COMPARATIVE EVALUATION OF DIFFERENT EXTRACTS OF LEAVES OF *PSIDIUM GUAJAVA* LINN. FOR HEPATOPROTECTIVE ACTIVITY

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ABSTRACT

The study was designed to evaluate the hepatoprotective activity of different extracts (petroleum ether, chloroform, ethyl acetate, methanol and aqueous) of *P. guajava* in acute experimental liver injury induced by carbon tetrachloride and paracetamol. The effects observed were compared with a known hepatoprotective agent, silymarin (100 mg/kg p.o.). In the acute liver damage induced by different hepatotoxins, *P. guajava* methanolic leaf extract (200 mg/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. *P. guajava* ethyl acetate leaf extract (200 mg/kg, p.o.) significantly reduced the elevated serum levels of aspartate aminotransferase, alanine aminotransferase and bilirubin in carbon tetrachloride induced hepatotoxicity whereas *P. guajava* aqueous leaf extract (200 mg/kg, p.o.) significantly reduced the elevated serum levels of alkaline phosphatase, alanine aminotransferase and bilirubin in carbon tetrachloride induced hepatotoxicity. *P. guajava* ethyl acetate and aqueous leaf extracts (200 mg/kg, p.o.) significantly reduced the elevated serum levels of aspartate aminotransferase in paracetamol induced hepatotoxicity. Histological examination of the liver tissues supported the hepatoprotection. It is concluded that the methanolic extract of leaves of *Psidium guajava* plant possesses better hepatoprotective activity compared to other extracts.

Keywords: Carbon tetrachloride, hepatoprotection, paracetamol, *Psidium guajava* Linn.

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 (Caceres *et al*., 1993), antidiarrheoal (Lutterodt, 1992), antimycobacterial (Malcolm and Sofowora, 1969), antihyperglycemic (Maruyama *et al*., 1985), antimalarial (Gessler *et al*., 1995), cytotoxic (Villareal *et al*., 1992) and antioxidant activities (Qian and Nihorimbere, 2004). 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 (Ross, 1999).

No concrete scientific study has been reported to prove the folklore claim in the utility of *Psidium guajava* leaf in the treatment of liver diseases and hence one of the objectives of the present study was to correlate the ethnobotanical evidence with scientific study. Further, the study also attempted to investigate relative protection afforded by different fractions of the leaf extracts from *Psidium guajava* using solvents of various polarities in chemically induced hepatotoxicity models in rats.

MATERIALS AND METHODS

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).

Extraction of Psidium guajava leaves - 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 of different extracts was calculated. The powder extracts obtained were then subjected to phytochemical analysis to detect the chemical constituents present in each extracts. The powdery leaf petroleum ether (EA), chloroform

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(CH), ethyl acetate (EA), methanol (MT) and aqueous (AQ) extracts of *P. guajava* leaves was suspended in water without adding any suspending agent for oral administration.

Chemicals – Carbon tetrachloride was purchased from S D Fine Chemicals Ltd., Mumbai, India and paracetamol was obtained from Lupin Ltd. Silymarin was obtained as a gift sample from Micro Labs, Hosur, India. All the other chemicals used were of analytical grade.

Acute toxicity study - The acute oral toxicity study was performed according to the OPPTS (Office of Prevention, Pesticide and Toxic Substance) Up and Down procedure (OPPTS, 2004).

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 paraffin (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: distilled water (1 ml/kg, p.o.)
Group 2: distilled water for 9 days + CCl_4 (1ml/kg, p.o.) on ninth day
Group 3: Silymarin (100mg/kg/day, p.o.) for 9 days + CCl_4 (1ml/kg, p.o.) on ninth day
Group 4: PE (500mg/kg/day, p.o.) for 9 days + CCl_4 (1ml/kg, p.o.) on ninth day
Group 5: CH (500mg/kg/day, p.o.) for 9 days + CCl_4 (1ml/kg, p.o.) on ninth day
Group 6: EA (200mg/kg/day, p.o.) for 9 days + CCl_4 (1ml/kg, p.o.) on ninth day
Group 7: MT (200mg/kg/day, p.o.) for 9 days + CCl_4 (1ml/kg, p.o.) on ninth day
Group 8: AQ (200mg/kg/day, p.o.) for 9 days + CCl_4 (1ml/kg, p.o.) on ninth day

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 and 8. 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) (Reitman and Frankel, 1957), alanine aminotransferase (ALT) (Reitman and Frankel, 1957), alkaline phosphatase (ALP) (Kind and King, 1954) and serum bilirubin (Mallay and Evelyn, 1937). The liver was immediately isolated and washed with normal saline, blotted with filter paper and weighed. The liver was then subjected to histopathological examination (Matsuda *et al*., 1991).

(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 (Yoshigurki *et al*., 1992).

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

Yields

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.

Preliminary phytochemical investigation- The preliminary phytochemical investigation of the different extracts of the *Psidium. Guajava* has revealed the presence of flavanoids, carbohydrates and saponins (table 1).

Acute oral toxicity study- The acute oral toxicity study was carried out and the results obtained are shown (table 2).

Carbon tetrachloride induced acute toxicity- A significant difference in biochemical markers was observed between normal and CCl_4 control groups. Comparative analysis of the effect of various extracts on ALT, AST and ALP levels revealed that methanol extract (200mg/kg body weight) has almost similar activity compared to the standard silymarin (100mg/kg body weight). The aqueous extract (200mg/kg body weight) and ethyl acetate extract (200mg/kg body weight) also showed protection against hepatoxin. Comparative analysis of the effect on serum bilirubin and between the extracts revealed that Methanol and aqueous extract (200mg/kg and 200mg/kg body weight) and silymarin have similar activity but ethyl acetate extract (200mg/kg body weight) showed very moderate activity. Whereas the petroleum ether extract and chloroform extract (500mg/kg body weight and 500mg/kg body weight) did not show any hepatoprotection (table 3).

Petroleum ether (500mg/kg body weight), ethyl acetate (200mg/kg body weight) and aqueous extract (200mg/kg body weight) showed no significant effect on liver weight. Silymarin (100mg/kg body weight), chloroform (500mg/kg body weight) and methanol extract (200mg/kg body weight) cause significant decrease in liver weight

Table 1: The qualitative chemical analysis is as follows:

Extracts/Test	Petroleum Ether	Chloroform	Ethyl Acetate	Methanol	Water
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	-	-	-	-	-

+ Presence of chemical constituent, - Absence of chemical constituent

Table 2: The acute oral toxicity study was carried out and the results obtained are as follows:

Extracts	Petroleum Ether	Chloroform	Ethyl Acetate	Methanol	Water
LD_{50}/kg body weight	5000mg/kg	5000mg/kg	2000mg/kg	2000mg/kg	2000mg/kg

Table 3: Effect of Silymarin, PE, CH, EA, MT and AQ extracts on serum ALT, AST, ALP, bilirubin levels and liver weights in CCl_4 induced acute hepatitis in rats.

Treatment	Dose (p.o.)	ALT (U/L)	AST (U/L)	ALP (U/L)	Serum Bilirubin (mg/dl)	Liver weight (g/100gm b.w)
Vehicle control	-	53.65 ± 1.35	149.18 ± 15.21	516.48 ± 94.10	0.21 ± 0.03	3.14 ± 0.07
CCl_4 control	-	386.88 ± 20.60^{a}	630.68 ± 27.91^{a}	752.15 ± 29.72^{b}	1.58 ± 0.18^{a}	3.86 ± 0.26^{c}
CCl_4 + Silymarin	100mg/Kg	56.03 ± 2.58***	204.33 ± 5.12***	405.96 ± 4.99***	0.25 ± 0.04***	3.07 ± 0.06*
CCl_4 + PE	500mg/Kg	393.11 ± 11.64*	726.48 ± 18.78^{ns}	793.38 ± 14.29^{ns}	1.58 ± 0.18^{ns}	3.64 ± 0.15 ^{ns}
CCl_4 + CH	500mg/Kg	368.55 ± 17.65^{ns}	652.18 ± 29.08^{ns}	766.41 ± 16.30^{ns}	1.36 ± 0.07^{ns}	2.93 ± 0.12**
CCl_4 + EA	200mg/Kg	309.42 ± 15.79**	471.83 ± 19.47*	753.70 ± 28.62^{ns}	1.01 ± 0.07*	4.18 ± 0.25^{ns}
CCl_4 + MT	200mg/Kg	86.23 ± 15.49*	179.06 ± 6.50***	341.53 ± 32.21***	0.55 ± 0.03***	2.88 ± 0.02**
CCl_4 + AQ	200mg/Kg	60.11± 6.29 ***	626.78 ± 64.84^{ns}	518.26 ± 68.48*	0.28 ± 0.09***	3.29 ± 0.12^{ns}

N = 6; 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.

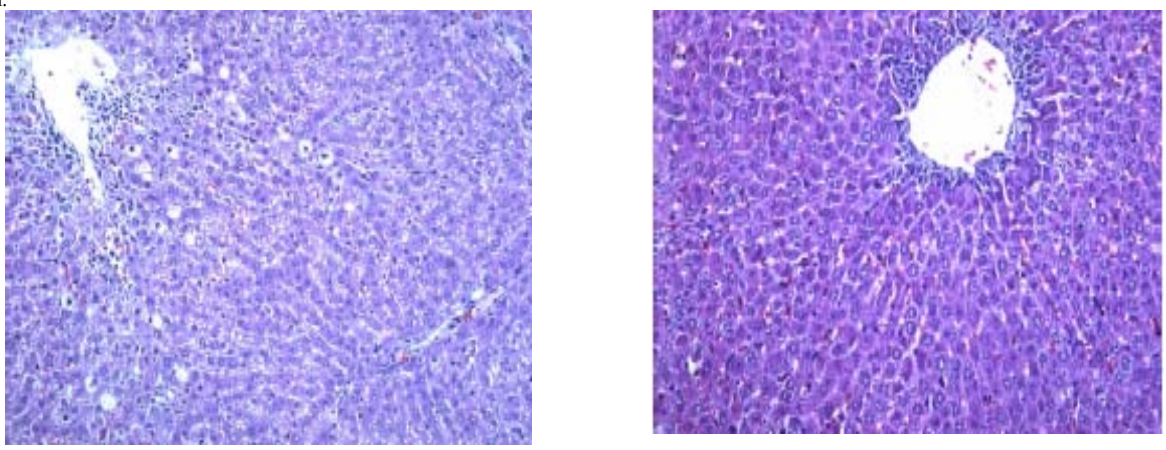
when compared to CCl_4 treated control (table 3). Histological examination of the liver tissue from CCl_4 treated animals revealed that CCl_4 had produced profound inflammation and congestion especially in the sinusoids. Hydropic degeneration and steatosis in the periportal region was also observed (fig1a). In animals pretreated with silymarin, and *Psidium guajava* methanolic extract reduced the inflammation, degenerative changes and steatosis (fig. 1b).

Paracetamol induced liver toxicity– Forty eight hours after treatment with paracetamol, the parameters, ALT, AST, ALP and bilirubin levels in the serum increased remarkably. In the experimental groups pretreated with chloroform, methanol (500mg/kg and 200mg/kg body weight respectively) and silymarin the ALT levels were significantly lowered than the paracetamol treated group whereas in animals pretreated with petroleum ether, ethyl acetate and aqueous extract (500g/kg, 200mg/kg and 200mg/kg body weight respectively) did not show significant effect on ALT level when compared with the paracetamol (PCM) control. In the experimental groups pretreated with ethyl acetate, methanol, aqueous extract (200mg/kg, 200mg/kg and 200mg/kg body weight respectively) and silymarin, the AST levels were significantly lowered than the paracetamol treated group whereas in animals pretreated with petroleum ether and chloroform (500g/kg and 500mg/kg body weight respectively) did not show significant effect when compared with the paracetamol (PCM) control. In the experimental groups pretreated with methanol (200mg/kg body weight) and silymarin the ALP levels were significantly lowered than the paracetamol treated group. Pretreatment with petroleum ether, chloroform, ethyl acetate and aqueous extract (500g/kg, 500mg/kg, 200mg/kg, 200mg/kg and 200mg/kg body weight respectively) did not show significant effect on the ALP levels when compared with the paracetamol (PCM) control. In the experimental groups pretreated with methanol (200mg/Kg body weight) and silymarin, the

Table 4: Effect of Silymarin, PE, CH, EA, MT and AQ extracts on serum ALT, AST, ALP, bilirubin levels and liver weights in paracetamol induced acute hepatitis in rats.

Treatment	Dose (p.o.)	ALT (U/L)	AST (U/L)	ALP (U/L)	Serum Bilirubin (mg/dl)	Liver weight (g/100gm b.w)
Vehicle control	-	53.65 ± 1.35	149.18 ± 15.21	516.48 ± 94.10	0.21 ± 0.03	3.14 ± 0.07
Para control	-	378.25 ± 28.45^{a}	645.61 ± 25.38^{a}	758.66 ± 30.58^{a}	1.68 ± 0.20^{b}	4.09 ± 0.33^{c}
Para + Silymarin	100mg/Kg	56.03 ± 2.58***	204.33 ± 5.12***	405.96 ± 4.99***	0.25 ± 0.04 ***	3.06 ± 0.06*
Para + PE	500mg/Kg	342.41 ± 29.37^{ns}	568.80 ± 13.80*	813.85 ± 14.71^{ns}	0.91 ± 0.34^{ns}	3.32 ± 0.09 ^{ns}
Para + CH	500mg/Kg	163.30 ± 11.18***	688.86 ± 12.73^{ns}	788.58 ± 10.96^{ns}	1.55 ± 0.19^{ns}	3.64 ± 0.15^{ns}
Para + EA	200mg/Kg	391.00 ± 8.06 ^{ns}	456.60 ± 15.71**	733.85 ± 19.24^{ns}	1.38 ± 0.29^{ns}	2.96 ± 0.12***
Para + MT	200mg/Kg	132.25 ± 7.73 ***	237.48 ± 15.39***	479.46 ± 7.52***	0.35 ± 0.09**	3.13 ± 0.07**
Para + AQ	200mg/Kg	332.78± 12.01^{ns}	405.96 ± 4.99***	755.56 ± 13.71^{ns}	1.5 ± 0.34^{ns}	3.59 ± 0.08^{ns}

N = 6; 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. paracetamol treated control.



(a) (b)

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

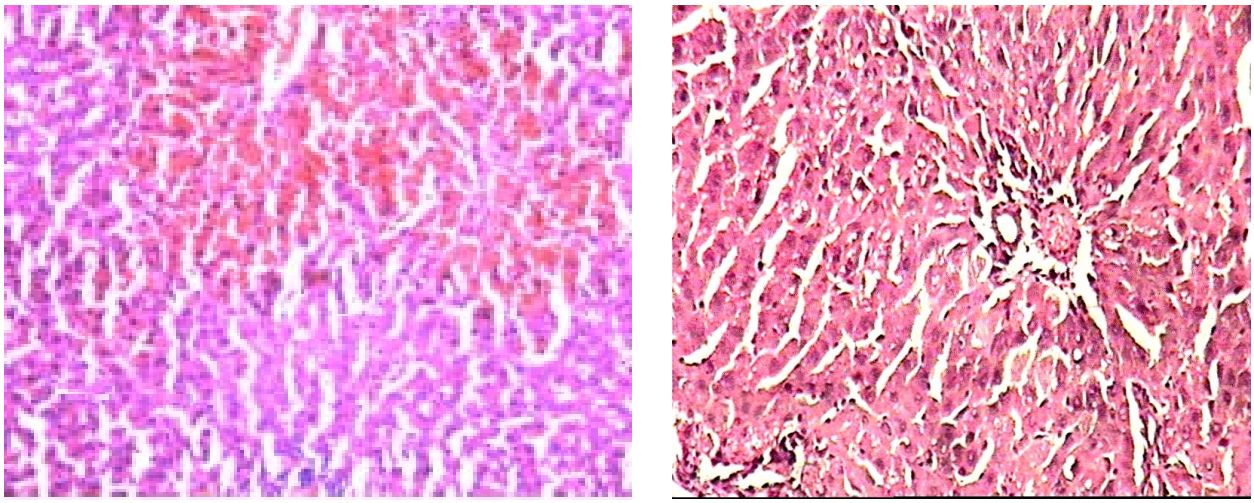
bilirubin levels were significantly lowered than the paracetamol treated group but pretreatment with petroleum ether, chloroform, ethyl acetate and aqueous extract (500g/kg, 500mg/kg, 200mg/kg and 200mg/kg body weight respectively) did not show significant effect when compared with the paracetamol (PCM) control (table 4).

Pretreatment with petroleum ether, ethyl acetate, methanol (500mg/kg, 200mg/kg and 200mg/kg body weight respectively) and silymarin significantly reduced the increase in the liver weight after intoxication with paracetamol whereas other extracts have not significant effect (table 4). 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 (fig 2a). 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 (fig. 2b).

DISCUSSION

The methanolic extract of *Psidium guajava* leaves showed significant hepatoprotective activity when administered at doses of 200 mg/kg orally. 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.

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 (Shenoy *et al*., 2001). 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 liver weight, which is due to blocking of secretion of hepatic triglycerides into the plasma (Yoko *et al*., 2005). Silymarin and methanolic extract of *Psidium guajava* (200mg/kg, p.o.) prevented the increase of liver weight in rats.



(a) (b)

Fig. 2: Effect of *P. guajava* leaf extract on PCM induced acute liver injury (a: PCM treated control, b: PCM + MT extract [H & E X200])

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 (Udem *et al*., 1997). Damage induced in the liver is accompanied by the increase in the activity of some serum enzymes. The anti-hepatotoxic action of the methanolic extract (200mg/kg, p.o.) 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). Methanolic extract of *Psidium guajava* prevented the increase in liver weight of rats pretreated with PCM.

In conclusion, the methanolic extract of leaves of *Psidium guajava* Linn. showed better hepatoprotective activity in CCl_4 induced acute liver damage and PCM induced liver damage compared to petroleum ether, chloroform, ethyl acetate and water extract. The hepatoprotective activity may be due to the anti-oxidant effect of the plant. Further studies to characterize the active principles and to elucidate the mechanism are in progress.

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