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In-vitro evaluation of hepatoprotective activity of *Pergularia daemia* Forsk.

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ABSTRACT

Studies on the hepatoprotective effect of acetone and ethanol sub fractions of ethanolic fraction obtained from total ethanol extract was carried out using carbon tetrachloride-induced toxicity in primary cultured rat hepatocytes. In vitro activity was assessed by determining the change in hepatocyte viability and other biochemical parameters such as glutamic oxaloacetic transaminase, glutamic pyruvic transaminase and total protein. Acetone and ethanol sub fractions showed significant ($P<0.05$) protective effect by restoring altered parameters in the selected in vitro model. The flavonoids present in acetone and ethanol sub fractions of total alcohol extract from *Pergularia daemia* may be responsible for significant hepatoprotective properties. The results justify the claims of *Pergularia daemia* in folk medicine as a hepatoprotective agent.

KEY WORDS: Carbon tetrachloride; Ethanol extract; Hepatocytes; In-vitro; *Pergularia daemia* Forsk; Silymarin.

INTRODUCTION

Pergularia daemia Forsk. Syn. *Daemia extensa* R Br. (Asclepiadaceae) commonly known as "Uttaravaruni" in Sanskrit and "Utranajutuka" in Hindi is a perennial twining herb, grows wildly along the road sides throughout Andhra Pradesh state. The plant is used by the people of Chittoor district of Andhra Pradesh state in India to treat jaundice. The plant is known for its anthelmintic, laxative, antipyretic and expectorant properties, also used to treat infantile diarrhoea and malarial intermittent fevers (1). Sathish et al. (2) reported the presence of triterpenes and saponins cardenolides and alkaloids. Presence of various triterpenes and steroidal compounds was reported by Aanjaneyulu et al (3). Sathish et al investigated the anti inflammatory, anti pyretic and analgesic activities of the plant (2). The plant exhibited anti diabetic activity also (4). Our preliminary investigations on the extracts from the *Pergularia daemia* have shown significant hepatoprotective activity. (5). Samia et al reported the presence of various flavonoidal compounds in the plant (6).

The present study was undertaken to standardise an in vitro test system using primary cultured rat hepatocytes to detect the protective effect of extracts against carbon tetrachloride-induced cellular damage, as in vitro models can offer a more detailed approach to understanding the mechanism of toxic actions (7), thus permitting a better analysis of the hepatoprotective action of drugs being developed. In present study the effect of acetone and ethanol sub fractions of ethanolic fraction from total ethanol extract was examined against the hepatotoxin, in vitro using primary cultured rat hepatocytes. Silymarin was used as a positive control.

MATERIALS AND METHODS

Plant material

The aerial parts of *Pergularia daemia* were procured from the foot hills of Tirumala, Andhra Pradesh, India. The identity of the plant was conformed at The Botanical Survey of India, Southern circle, Coimbatore, India. The voucher specimen (BSI/SC/5/21/05-06/Tech: 1512) was deposited at the Madras herbarium, The Botanical Survey of India, Coimbatore.

Preparation of extracts

About 43 g of the ethanolic fraction (EFTE) obtained by the fractionation of 60 g of total alcohol extract (TE) was adsorbed on to the 250 g of silica gel of 60-120 mesh size and fractionated with chloroform, acetone and 95% ethyl alcohol, resulting fractions concentrated in vacuum yielded 2.32 g, 11.57 g and 20.26 g solid mass respectively. Preliminary TLC studies of EFTE revealed the presence of flavonoids and cardenolides. The chloroform sub fraction (CFEFTE) showed cardenolides, acetone sub fraction (AFEFTE) showed flavonoids and cardenolides while ethanol sub fraction (EFEFTE) showed flavonoids (8). The AFEFTE and EFEFTE were used for hepatoprotective activity in vitro.

Animals - Wistar albino rats weighing 175-225 g of either sex, maintained under standard husbandry

conditions were used for all studies. Animals were allowed to take standard laboratory feed and tap water. The experiments were performed after the experimental protocols approved by the institutional animal ethics committee, M.S.University of Baroda, Vadodara, Gujarat, India.

In-vitro hepatoprotective activity

Hepatotoxins and test substances

For in vitro studies carbon tetrachloride (CCl_4) (0.1N), was used to produce submaximal toxicity in isolated rat hepatocytes. The test solutions are tested at dose levels of 100, 500 and 1000 µg/ml. Silymarin was used as a positive control at a dose level of 100 µg/ml. All the substances are dissolved in a vehicle 30% DMSO (9).

Isolation of rat hepatocytes

The method developed by Sarkar and Sil, (10) was used for the isolation of hepatocytes with slight modifications. The livers were isolated under aseptic conditions and placed in HEPES (*N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid*) buffer I containing HEPES (0.01 M), NaCl (0.142 M), and KCl (0.0067 M), pH 7.4. The livers were cut into small pieces and then incubated with a second buffer containing HEPES (0.1 M), NaCl (0.0667 M), KCl (0.0067 M), and 0.5% Collagenase type IV, pH 7.6 for about 45 min at 37°C in an incubator with constant shaking. Hepatocytes were obtained after filtration and cold centrifugation (4°C, 200 rpm/min for 2 min, three times) and suspended in HEPES buffer I. The viability of the hepatocytes was assessed by trypan blue (0.2%) exclusion method.

Primary cultures of rat hepatocytes

The method of Tingstrom and Obrink (11) with slight modifications was used for the primary culturing of rat hepatocytes. The freshly isolated viable hepatocytes were suspended in culture medium RPMI-1640 supplemented with calf serum (10%), HEPES and gentamicin (1 µg/ml). These cells (approximately 1-1.2 X 10^6/ml) were then seeded into culture bottles and incubated at 37°C in atmosphere of 5% CO_2 in carbon dioxide incubator. Upon incubation for 24 h the hepatocytes formed a mono layer. The newly formed cells were round and most appeared as individual cells. These cells were 95-96% viable as confirmed by trypan blue exclusion test.

Hepatic cytotoxicity testing

The sub fractions of the total ethanolic extract i.e AFEFTE and EFEFTE were tested for their hepatic cytotoxicity at 500, 1000 and 1500 µg/ml on isolated rat hepatocytes. After 24 h of incubation at 37°C in CO_2 incubator, the percentage viability of hepatocytes was tested using trypan blue exclusion (12) and by the estimation of total protein content.

Hepatoprotective activity

Twenty four hours after the establishment of the monolayers of hepatocytes, the medium was decanted and the culture was washed with HEPES buffer I and finally the hepatocytes were suspended in Buffer I. The hepatic cytotoxicity was induced with carbon tetrachloride (0.1N). Triplicate hepatocyte suspensions (0.1 ml) from different cultures were distributed into various culture tubes labelled as control, toxicant, standard (silymarin + toxicant) and test (test sample + toxicant). The control group received 0.1 ml of vehicle (30% DMSO) and toxicant groups received 0.1 ml of carbon tetrachloride, while the test groups received 0.1 ml of respective test solutions (100, 500 and 1000 µg/ml) followed by 0.1 ml (0.1N) of hepatotoxin. The standard groups received 0.1 ml of silymarin solution (100 µg/ml) followed by hepatotoxin. The content of the all culture tubes were made up to 1 ml with HEPES buffer I. The contents of all the tubes were mixed well and incubated in a CO_2 incubator for 24 h at 37°C. In test and standard groups the hepatocytes were pre incubated with respective solutions for 30 min and then exposed to hepatotoxin. After incubation hepatocyte suspensions were collected to assess cell damage. Cell viability was evaluated by trypan blue exclusion method (13). Hepatocytes suspensions were centrifuged at 200 rpm. The leakage of the enzymes GOT, GPT and total proteins secreted outside the cells were determined from the supernatant.

Assessment of hepatoprotective activity

The effect of different extracts in liver protection was determined by measuring an increase in the percentage of viable cells in that group of cells incubated with extracts, compared with the control and toxicant groups. Reversal of toxin-induced elevations in the levels of enzymes and toxin-induced reductions in levels of proteins were also considered to be an important criterion of hepatoprotective activity. The UV-Kinetic method based on the reference method of International Federation of Clinical Chemistry (14) in which both SGOT and SGPT were assayed based on enzyme-coupled system; where keto acid formed by the aminotransferase reacts in a system using NADH. The coenzyme is oxidised to NAD and the decrease in absorbance at 340 nm is measured. For SGOT malate dehydrogenase is used to reduce oxaloacetate to malate where as for SGPT the pyruvate formed in the reaction is converted to lactate by lactate dehydrogenase, was followed for the assessment of

activity of glutamic oxaloacetic transaminase and glutamic pyruvic transaminase in the cells. Total protein (TPTN) was estimated by Biuret method (15) where proteins produce a violet colour complex with copper ions in an alkali solution. The absorbance of the colour complex is directly proportional to the protein in the sample.

Statistical analysis

The mean values±SEM are calculated for each parameter. Percentage reduction against the hepatotoxin by the test samples was calculated by considering the difference in enzyme level between the hepatotoxin treated group and the control group as 100% reduction. For determining the significant inter group difference each parameter was analysed separately and one-way analysis of variance (16) was carried out and the individual comparisons of the group mean values were done using Dunnet's test (17).

RESULTS

Hepatic cytotoxicity testing

When normal hepatocytes were treated with the extracts under test, there are no alterations in the values of % viable cells and TPTN content as compared to the control at the dose level up to 1500 µg/ml indicating that the extracts were not toxic to the cells.

Effect against paracetamol induced toxicity

Incubation of hepatocytes with carbon tetrachloride (0.1N) resulted in 64.78% and 54.59% depletion in viability and TPTN content of hepatocytes respectively. Similarly an elevation about 268.45% and 267.06% of GOT and GPT levels were observed respectively upon intoxication with carbon tetrachloride. Hepatocytes treated with AFEFTE showed a concentration dependant (100-1000 µg/ml) protective effect by restoring the viability of hepatocytes (40.36-79.20%), TPTN content (24.08-63.63%), GOT (28.16-88.48%) and GPT (36.05-83.87%). EFEFTE also showed concentration dependant protective effect by altering the viability of cells (27.63-59.29%), TPTN content (10.05-51.35%), GOT (09.02-44.25%) and GPT (23.07-65.16%), while the positive control silymarin showed good protective effect by restoring viability (86.77%), TPTN (72.72%), GOT (97.59%) and GPT (92.85%). The Maximum protection was seen with AFEFTE 1000 µg/ml. Results were represented in Table 1.

DISCUSSION

The present studies were performed to assess the hepatoprotective activity of *Pergularia daemia* in hepatocytes against carbon tetrachloride as hepatotoxin in an attempt to prove its claims in folklore practice against liver disorders and to develop an in vitro test system using primary cultured rat hepatocytes. These results were compared with our previous investigations (5).

Isolated hepatocytes have become a useful model for pharmacological, toxicological, metabolic and transport studies of xenobiotics since the development of techniques for high yield isolation of rat hepatocytes (18). Freshly isolated rat hepatocytes are also very useful and common tool for study of cytotoxicity and metabolic studies in this area, as they keep enzymatic activity similar to in vivo for several hours (19). Various hepatotoxins viz. carbon tetrachloride, thioacetamide and paracetamol have been shown to result in the reduction of viability of hepatocytes, and leakage of enzymes which are considered to be the markers of cellular injury (20) Similar changes in the present study confirm these changes and also indicate satisfactory standardisation of our isolation and culture procedures.

CCl_4 is metabolised by the microsomal cytochrome P450 to a highly reactive trichloromethyl free radical and trichloromethyl peroxy radical. These free radicals start a chain of free radical formation which attack membrane lipids and proteins there by causing the destruction of microsomes and liver cells causing cell lysis. Leakage of cytosolic enzymes out of the cells thus occurs due to increase in cell permeability, membrane damage and cell necrosis (21, 22). In the present study, the hepatotoxin employed reduced cell viability possibly due to injury of plasma membrane of hepatocytes resulting in the leakage of cellular enzymes. Incubation of hepatocytes with extracts significantly restored their viability as well as altered biochemical parameters induced by hepatotoxin (Table 1). In our study we have observed significant but sub maximal toxicity after 24 h of incubation with hepatotoxin. Though both the extracts were able to restore the changes caused by hepatotoxin significantly, the restoration exhibited by AFEFTE at dose levels of 1000 µg/kg was maximum.

The results of the present study also support our earlier published preliminary in vivo work (5) of hepatoprotective activity of extracts from pergularia daemia. It will also support our studies (23) regarding the protective effect of AFEFTE and EFEFTE against carbon tetrachloride toxicity in vivo. It was observed that in our previous in vivo studies of these extracts against carbon tetrachloride toxicity, EFEFTE was not able to produce significant protective effect at the dose levels (up to 150 mg/kg) tested; reason may be

Table 1: Effect of Pergularia daemia on carbon tetrachloride-induced toxicity in rat hepatocytes.

Group	Viable cells (%)	GOT (IU/L)	GPT (IU/L)	TPTN (g/dl)
Control	93.88 ± 0.81	18.70 ± 0.46	21.53 ± 0.63	4.03 ± 0.09
CCl_4	33.06 ± 1.14	50.20 ± 0.61	57.50 ± 0.94	1.83 ± 0.14
Silymarin 100 µg/ml	85.84 ± 1.19 (86.77)*	19.46 ± 0.35 (97.59)*	24.10 ± 0.37 (92.85)*	3.43 ± 0.14 (72.72)**
AFEFTE 100 µg/ml	57.63 ± 1.58 (40.39)*	41.33 ± 0.62 (28.16)*	44.53 ± 1.25 (36.05)*	2.36 ± 0.21 (24.08)**
AFEFTE 500 µg/ml	77.60 ± 0.57 (73.72)*	38.50 ± 0.45 (37.14)*	32.80 ± 0.65 (68.66)*	2.86 ± 0.12 (46.81)**
AFEFTE 1000 µg/ml	81.24 ± 1.67 (79.20)*	22.33 ± 0.50 (88.48)*	27.33 ± 0.69 (83.87)*	3.23 ± 0.18 (63.63)**
EFEFTE 100µg/ml	33.67 ± 1.82 (01.01)	47.36 ± 0.75 (09.02)*	49.20 ± 0.63 (23.07)*	1.53 ± 0.08 (-)
EFEFTE 500 µg/ml	49.87 ± 2.46 (27.63)*	40.70 ± 0.71 (30.16)*	41.90 ± 0.81 (43.36)*	2.06 ± 0.11(10.45)
EFEFTE 1000 µg/ml	69.13 ± 0.44 (59.29)*	36.26 ± 0.49 (44.25)*	34.06 ± 1.10 (65.16)*	2.96 ± 0.20 (51.35)**

Data represents the mean±SEM of three values, AFEFTE: Acetone sub fraction of total ethanol extract, EFEFTE: Ethanol sub fraction of total ethanol extract. Values in parenthesis indicate percentage protection against toxicant.

** Significant reduction compared to hepatotoxin (P<0.05). ** Significant increase compared to hepatotoxin (P<0.05).*

due to that still high concentration is required to exhibit the effect. The invivo studies required a large number of animals (six per group), and needed up to 3-5 days of drug administration for a significant effect to be produced and thus required large quantities of drugs. The present in vitro models on the other hand is more rapid and requires small quantities of test substances and fewer animals, which may be particularly useful in studies of natural products. In such studies a large number of plant fractions need to be examined which are generally only available in small quantities.

In literature many authors reported the hepatoprotective activity of flavonoid compounds. Galisteo et al., (24) reported the hepatoprotective activity of flavonoids of *Rosmarinus tomentosus*. The hepatoprotective effect of quercetin was reported by Janbaz et al., (25). Protective effect of rutin was reported by Janbaz et al., (26). Silymarin obtained from *Silybum marianum* is a good hepatoprotective agent (27). Samia et al (6) reported various flavonoids like quercetin, kaempferol and isorhamnetin glycosides in the *Pergularia daemia*. In accordance with these results, it may be hypothesized that flavonoids, which are present in AFEFTE and EFEFTE, could be considered responsible for the hepatoprotective activity. Further work is in progress to isolate the bio active principle the plant. In conclusion this study underlines the therapeutic potential of *Pergularia daemia*.

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