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Hepatoprotective Properties in the Rat of *Mitracarpus scaber* (Rubiaceae)

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Abstract

The effect of *Mitracarpus scaber* on carbon tetrachloride-induced acute liver damage in the rat has been evaluated.

Results showed that treatment with *Mitracarpus scaber* decoction resulted in significant hepatoprotection against CCl_4-induced liver injury both in-vivo and in-vitro. In-vivo, *Mitracarpus scaber* pretreatment reduced levels of serum glutamate-oxalate-transaminase ($P < 0{\cdot}01$ for 250, 500 and 1000 mg kg^{-1}) and serum glutamate-pyruvate-transaminase ($P < 0{\cdot}05$ for 250 mg kg^{-1} and $P < 0{\cdot}01$ for 1000 mg kg^{-1}) previously increased by administration of CCl_4. In-vitro results indicated that addition to the culture medium of *Mitracarpus scaber* extracts significantly reduced glutamate-oxalate-transaminase ($P < 0{\cdot}05$ for 100 μg mL^{-1} and $P < 0{\cdot}01$ for 10 and 1000 μg mL^{-1}) and lactate dehydrogenase activity ($P < 0{\cdot}05$ for 10 μg mL^{-1}). *Mitracarpus* treatment also resulted in a good (> 93%) survival rate for the CCl_4-intoxicated hepatocytes as demonstrated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Moreover, as in the in-vitro assay, *Mitracarpus scaber* had radical-scavenging properties, shown by its reaction with the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (EC50, the extract concentration resulting in a 50% reduction in the absorbance of DPPH blank solution, $= 41{\cdot}64 \pm 1{\cdot}5$ μg mL^{-1}).

The results of this study showed that *Mitracarpus scaber* had antihepatotoxic potential, a finding which supports the validity of traditional usage of this drug in Mali for the treatment of liver diseases.

Liver disease is a world-wide problem. Conventional drugs used in the treatment of liver diseases are sometimes inadequate and can have serious adverse effects. It is therefore necessary to search for alternative drugs for the treatment of liver disease to replace currently used drugs of doubtful efficacy and safety.

Many natural products of vegetable origin are in use in the traditional medicine of Mali for the treatment of liver diseases. *Mitracarpus scaber* Zucc. (Rubiaceae) aerial parts are used as a decoction for the treatment of many diseases including hepatic syndromes, jaundice, hepatitis and other hepatic disorders (Kerharo & Adam 1974). The same plant is also used for the treatment of skin diseases (infectious dermatitis, eczema, scabies) (Adjanohoun et al 1986, 1989).

Previous studies have shown that *Mitracarpus scaber* has pronounced antimicrobial, antifungal (Irobi & Daramola 1993, 1994; Sanogo et al 1996) and anti-inflammatory properties (Ekpendu et al 1994). More recently, phytochemical studies on *Mitracarpus scaber* methanolic extract have identified the presence of gallic acid, 4-methoxyacetophenone, 3,4,5-trimethoxyacetophenone, 3,4,5-trimethoxybenzoic acid, kaempferol-3- *O*-rutinoside, rutin, and psoralen (Bisignano et al 1998).

In this publication we report the hepatoprotective effects of *Mitracarpus scaber* decoction on CCl_4-induced hepatotoxicity in-vivo and in-vitro using hepatocytes isolated from rats. CCl_4 is frequently

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used as a chemical inducer of experimental liver cirrhosis. It is metabolized to the $\cdot CCl_3$ radical in the microsomes of hepatocytes and this radical then reacts with unsaturated fatty acids to form lipid peroxides in the hepatocyte plasma membranes. Microsomes, mitochondria and the nuclei of the hepatocytes are damaged by the lipid peroxides and the hepatocytes are destroyed (Recknagel & Glende 1973; Comporti 1985).

The hepatoprotective effect of *Mitracarpus scaber* was evaluated in-vivo by measuring serum glutamate-oxalate-transaminase (sGOT) and serum glutamate-pyruvate-transaminase (sGPT) activity. The indices used to evaluate cytotoxicity were plasma membrane integrity as assessed by the leakage of GOT and lactate dehydrogenase (LDH) into the culture medium, and mitochondrial reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) as a general measure of mitochondrial dehydrogenase activity and cell viability.

The in-vitro radical scavenging activity of *Mitracarpus scaber* decoction was also measured by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) reduction assay.

Materials and Methods

Plant material

Mitracarpus scaber aerial parts were collected in the belt of Bamako (Mali) and authenticated by the botanists of the Division of Traditional Medicine (DMT) of Bamako. Representative specimens of the drug were preserved in the DMT herbarium.

Preparation of extracts

The plant material was air-dried and powdered. A 10% decoction was prepared and after filtration the liquid was lyophilized (yield 14·96%). Just before use the lyophilized decoction was again dissolved in water.

Chemicals

Bovine serum albumin Fr. V, foetal bovine serum, collagenase type IA (449 int. units mg^{-1}), dexamethasone, (*N*-[2-hydroxyethyl]piperazine- *N*′-[2-ethanesulphonic acid]) (HEPES), insulin, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), RPMI-1640 medium, and sodium bicarbonate were from Sigma; $CCl_4 > 99{\cdot}5\%$ purity, dimethylsulphoxide (DMSO), 1,1-diphenyl-2-picrylhydrazyl stable radical (DPPH), and ethylene glycol-*O*,*O*′-bis (2-aminoethyl)- *N*, *N*, *N*′*N*′-tetra-acetic acid (EGTA) were from Fluka; Hank's Ca^{2+}- and Mg^{2+}-free balanced salt solution was from Euro-Clone, isopropanol from Lab-Scan, Triton X-100 from Bio-Rad, and Trypan Blue from Flow Laboratories.

Animals

Male Charles River rats, 242–325 g, were used for all experiments. Rats were kept under standard conditions, with free access to food and tap water, for two weeks before treatment.

CCl_4-induced hepatocyte injury in-vivo

The animals were fasted for 24 h with water freely available. Liver damage was induced in rats by means of a single intraperitoneal injection of CCl_4, 0·3 mL kg^{-1}, in olive oil (5 mL kg^{-1}) (Fleurentin et al 1986).

Animals were divided into five groups of five rats each. Group 1 received water (5 mL kg^{-1}, orally) as normal control; Group 2 received water and 1 h later was injected with CCl_4 (CCl_4-treated group); Groups 3, 4 and 5 were treated orally with *Mitracarpus scaber* decoction, at doses equivalent to 250, 500 and 1000 mg kg^{-1}, respectively, of the dry drug, 1 h before CCl_4 intoxication.

Assessment of hepatotoxic activity in-vivo

Rats were killed by ether anaesthesia 24 h after administration of the hepatotoxic agent. After blood collection, the blood samples were left to coagulate at room temperature for 1 h. Serum was separated by centrifugation at 3000 rev min^{-1} and 4°C for 20 min. sGOT and sGPT activity were measured by use of AST/GOT UV-Autom and ALT/GPT UV-Autom test kits (Sentinel CH, Milan, Italy) with a ARCO PC bioanalyser. The data are reported in int. units L^{-1}.

CCl_4-induced hepatocyte injury in-vitro

Isolation of hepatocytes. Liver cells were isolated by the procedure of Seglen (1973, 1976) with modifications. The abdomen of the rat was opened under ether anaesthesia. A midline incision was made and the portal vein was cannulated with a needle fitted with a teflon catheter (0·7 mm × 19 mm). After the teflon catheter had been tied in place and the needle removed, the lower abdominal vena cava was cannulated (1·1 mm × 32 mm). Perfusion of the liver was started with Ca^{2+}- and Mg^{2+}-free Hank's buffer containing 0·5 mM EGTA

and 0·05 M HEPES (pH 7·3) at 37°C. The flow rate was 30 mL min^{-1}. After this first perfusion for 8–10 min, a second perfusion of 100 mL RPMI-1640 medium containing collagenase (0·061 mg mL^{-1}) and 0·05 M HEPES (pH 7·4) at 37°C was started at a flow rate of 30 mL min^{-1}. After perfusion, the liver was moved to a Petri dish containing 10 mL cell-wash medium (RPMI-1640 medium containing 0·05 M HEPES, pH 7·3), cooled on ice and gently dispersed with forceps. The crude cell suspension was filtered and then centrifuged at 50 *g* for 1 min. The viability of the cells to exclude trypan blue was determined by incubating the cell suspension (100 μL) with 0·4% trypan blue (25 μL) and phosphate buffered saline (PBS, 375 μL) and then counting the number of cells which excluded the dye and the number of cells the nuclei of which stained blue. The culture medium was RPMI-1640 medium supplemented with 10% inactivated (56°C for 30 min) foetal bovine serum, penicillin (100 int. units mL^{-1}), streptomycin (100 μg mL^{-1}), dexamethasone (10 mM) and insulin (1 mM). The cells were seeded into Falcon 24-well multiplates at a density of 1×10^6 cells mL^{-1} and pre-incubated in a humidified incubator at 37°C under 5% CO_2 in air before treatment.

After preculture (30 min), the cells were exposed to fresh medium (1·0 mL) containing CCl_4 (10 mM) in ethanol (0·01 mL) with or without test samples in DMSO (0·01 mL) (Kiso et al 1984). Concentrations of the tested extracts were 10, 100 and 1000 μg mL^{-1}. Control cultures contained the same amount of DMSO.

Assessment of hepatotoxic activity in-vitro

GOT and LDH activity in the medium were measured 60 min after CCl_4 challenge as indicators of plasma membrane integrity. After incubation, the media which contained detached cells were collected and centrifuged at 50 *g* and 4°C for 2 min. The supernatant was used to assay GOT and LDH activity by means of the AST/GOT UV-Autom and LDH UV Liquid test kits (Sentinel) with a ARCO PC bioanalyser. The data are reported in int. units L^{-1}. It is generally accepted that experiments are valid only if the background activity of control cells is $\leq$25% of total available intracellular activity. So, for control cells, the total intracellular enzyme content was determined after treatment of cells with Triton X-100 detergent to induce 100% lysis (Tyson et al 1983).

Measurement of hepatocyte survival rate (MTT reduction assay)

The cell survival rate was assessed 60 min after CCl_4 intoxication by the MTT reduction assay according to Mosmann (1983), with modifications. Briefly, MTT (0·3 mg mL^{-1}) was dissolved in PBS (pH 7·4) and MTT solution (200 μL mL^{-1}) was added to each well and incubated for 2 h at 37°C with 5% CO_2. After incubation, cell suspension with formazan crystals was transferred into Eppendorf tubes and centrifuged at 5000 *g* for 3 min. Supernatant was discarded and lysis-solubilization solution (10% Triton X-100 in isopropanol, pH 2; 1 mL) was added to each tube. The pellet was dissolved by sonication and cell debris was collected by brief centrifugation. The absorbance of the purple solution was measured at 565 nm with a spectrophotometer (Shimadzu UV-1601).

The cell survival rate was expressed as percentage formazan production by treated samples compared with control samples.

Assay of antioxidative activity (DPPH method)

The antiradical activity of *Mitracarpus scaber* was assessed by the method of Ohnishi et al (1994). The extract was added at different concentrations (10–1000 μg mL^{-1}) to an equal volume of DPPH solution (0·1 mM in 95% EtOH). After letting the mixture stand at room temperature for 20 min the absorbance was recorded at 517 nm. Radical-scavenging activity was expressed as a 50% effective concentration (EC50; the extract concentration required to reduce the absorbance of DPPH blank solution by 50%). Ascorbic acid was used as reference substance.

Statistical analysis

Values are given as arithmetic means $\pm$ standard deviation. Student's *t*-test was used to compare unpaired means of two data sets.

Results

Effect on CCl_4-induced liver injury in-vivo

The hepatoprotective effect of *Mitracarpus scaber* decoction is shown in Table 1. The CCl_4-treated group developed significant hepatic damage ($P < 0·01$) as indicated by elevated serum levels of GOT and GPT compared with the normal group. A significant decrease in sGOT ($P < 0·01$) and sGPT levels ($P < 0·05$ with 250 mg kg^{-1} and $P < 0·01$ with 1000 mg kg^{-1}) was observed for all the groups treated with *Mitracarpus* compared with the group treated with CCl_4 alone. The hepatoprotective activity of *Mitracarpus* did not seem to be dose-dependent.

Table 1. Hepatoprotective activity of *Mitracarpus scaber* extracts on CCl_4-induced hepatic injury in-vivo.

Treatment	Serum glutamate-oxalate-transaminase	Serum glutamate-pyruvate-transaminase
Control	61·12 ± 8·50	38·14 ± 6·51
CCl_4–olive oil (0·3 mL kg^{-1})	477·00 ± 59·17†	323·40 ± 49·78†
CCl_4 + 250 mg kg^{-1} *Mitracarpus scaber*	191·60 ± 24·66**	184·40 ± 20·34*
CCl_4 + 500 mg kg^{-1} *Mitracarpus scaber*	169·40 ± 48·88**	271·20 ± 20·81
CCl_4 + 1000 mg kg^{-1} *Mitracarpus scaber*	194·00 ± 28·55**	124·20 ± 10·87**

Doses are expressed as mg of dry drug. Each value (units L^{-1}) is the mean ± s.d. of results from five rats. †$P < 0·01$ compared with control. *$P < 0·05$, **$P < 0·01$ compared with CCl_4-treated rats.

Effect on CCl_4-induced liver injury in-vitro

Results obtained for the hepatoprotective activity of *Mitracarpus scaber* extracts are shown in Table 2. The extent of liver cell injury was expressed in terms of GOT and LDH levels released into the medium. GOT and LDH release of CCl_4 control groups was significantly increased ($P < 0·01$ compared with control) 1 h after CCl_4 exposure. *Mitracarpus scaber* had significant hepatoprotective activity as demonstrated by the reduction of GOT levels in the group treated with the extract ($P < 0·05$ with 100 μg mL^{-1} and $P < 0·01$ with 10 and 1000 μg mL^{-1}). The results indicate that *Mitracarpus scaber* also reduces the leakage of LDH even if significance ($P < 0·05$) is achieved only at the lower dose.

Table 2. Hepatoprotective activity of *Mitracarpus scaber* extracts on CCl_4-induced hepatic injury in-vitro.

Treatment	Glutamate-oxalate-transaminase	Lactate dehydrogenase
Control	29·20 ± 9·10	97·20 ± 20·10
CCl_4	109·10 ± 9·52†	336·50 ± 11·18†
CCl_4 + 10 μg mL^{-1} *Mitracarpus scaber*	79·45 ± 6·13**	283·97 ± 13·15*
CCl_4 + 100 μg mL^{-1} *Mitracarpus scaber*	86·38 ± 6·62*	322·22 ± 40·90
CCl_4 + 1000 μg mL^{-1} *Mitracarpus scaber*	54·45 ± 14·75**	296·48 ± 36·60

Doses are expressed as mg of lyophilized decoction. Each value (units L^{-1}) is the mean ± s.d. of results from two experiments in triplicate. †$P < 0·01$ compared with control. *$P < 0·05$, **$P < 0·01$ compared with CCl_4-treated cells.

The survival rate of hepatocytes, expressed as a percentage of MTT reduction relative to control, was measured 60 min after CCl_4 exposure in samples pretreated with *Mitracarpus scaber* and in untreated samples. As shown in Table 3, the survival rate of the cells, reduced to 88% in the CCl_4 control group, remained at more than 93% in all the groups treated with *Mitracarpus scaber* extracts after CCl_4 challenge.

Radical-scavenging properties

Mitracarpus scaber extract inhibited DPPH activity with an EC50 of 41·64 ± 1·5 μg mL^{-1}; the EC50 for ascorbic acid was 2·37 ± 0·12 μg mL^{-1}.

Discussion

Pretreatment with different doses of *Mitracarpus* decoction resulted in a significant reduction in hepatic injury as a result of the ability of the extract either to stimulate or to protect hepatic drug-metabolizing enzymes. In CCl_4-induced acute liver damage, elevated levels of GOT and GPT in serum are indicative of cellular leakage and loss of functional integrity of cell membranes in liver (Drotman & Lawhorn 1978). The reduction in the levels of sGOT and sGPT after administration of the *Mitracarpus* decoction could be an indication of the

Table 3. Effect of *Mitracarpus scaber* extracts on the survival rate of hepatocytes after CCl_4 exposure.

	Control	CCl_4	*Mitracarpus scaber* (μg mL^{-1})		
			10	100	1000
MTT reduction	100·00 ± 0·00	88·00 ± 2·26	97·10 ± 1·69	94·30 ± 1·41	93·80 ± 3·39

Doses are expressed as mg of lyophilized decoction. Each value (% of cells surviving) is the mean ± s.d. of results from two experiments in triplicate.

stabilization of the plasma membrane and repair of hepatic tissue damage caused by CCl_4.

This is in agreement with the commonly accepted view that serum levels of transaminases return to normal with healing of hepatic parenchyma and the regeneration of the hepatocytes (Thabrew et al 1987).

Hepatoprotective activity against CCl_4-intoxication was also confirmed in-vitro on isolated rat hepatocytes. *Mitracarpus scaber* extracts significantly reduced GOT and LDH release into the medium. The results of MTT assay confirm the hepatoprotective activity, although they did not reach statistical significance. The short time of CCl_4 exposure (60 min) is most probably responsible for the slight reduction in cell viability (only 12% less than control) observed in CCl_4-treated cells.

Detoxification of xenobiotic and toxic substances is an important hepatic function in the course of which large amounts of reactive oxygen intermediates are produced. Acute doses of toxic agents or chronic exposure to such substances can overpower the antioxidant defence system and cause hepatic damage.

CCl_4 is metabolized by the drug metabolizing enzymes of cytochrome P450 to a highly reactive trichloromethyl free radical ($\cdot CCl_3$) and trichloromethyl peroxy radical ($\cdot OOCCl_3$). Free radicals attack membrane lipid and protein and thus cause destruction of microsomes and liver cells (Recknagel 1983). Any compound, natural or synthetic, with antioxidant properties, would contribute towards the partial or total alleviation of this damage.

The scavenger activity of *Mitracarpus scaber* is indicative of its antioxidant properties and the protective effect of *Mitracarpus* extracts against CCl_4-induced liver damage both in-vivo and in-vitro could be explained in terms of beneficial action against the pathological alterations caused by the presence of free radicals which occur in certain hepatic disorders.

Phytochemical analyses on *Mitracarpus scaber* methanolic extract have identified the presence of gallic acid, 4-methoxyacetophenone, 3,4,5-trimethoxyacetophenone, 3,4,5-trimethoxybenzoic acid, kaempferol-3- *O*-rutinoside, rutin, and psoralen (Bisignano et al 1998). 3,4,5-Trimethoxy-benzoic acid is a known antioxidant added to different pharmaceutical preparations and foodstuffs to inhibit their oxidation (Boussenadji et al 1993). The antioxidant activity of gallic acid, a naturally occurring plant phenol, is well known (Masaki et al 1995; Salah et al 1995). Rutin, another flavonoid widely occurring in the plant kingdom, has been reported to have strong anti-lipoperoxidant activity (Saija et al 1995).

Although the hepatoprotective effects of these single substances should be further confirmed, their involvement in the mechanism of hepatoprotection could be hypothesized.

The results of this study are in accord with the traditional use of the drug in Mali for the treatment of liver diseases.

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