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Protective effect of crude extract from *Wedelia paludosa* (Asteraceae) on the hepatotoxicity induced by paracetamol in mice

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Abstract

Several in-vitro and in-vivo ethnopharmacological studies carried out with plants of the genus *Wedelia* have already demonstrated hepatoprotective effects in chemically-induced liver injury, including those induced by paracetamol. Here, the effects of the crude extract from *Wedelia paludosa* on paracetamol-induced hepatotoxicity in mice was investigated. Intraperitoneal injection of paracetamol (1000 mg kg^{-1}) caused 80% death after 24 h in mice, which was significantly reduced by oral pretreatment with *W. paludosa* (500 mg kg^{-1}). Hepatotoxicity was observed 24 h after an intraperitoneal injection of paracetamol (600 mg kg^{-1}), as evidenced by an increase in plasma activity of aspartate and alanine aminotransferases. That hepatotoxicity was significantly attenuated by *W. paludosa* pretreatment (100–500 mg kg^{-1}) in a dose–response manner. Paracetamol (1000 mg kg^{-1}) drastically depleted total glutathione levels and decreased glutathione peroxidase and δ-aminolevulinate dehydratase activity in the liver, such effects not being prevented by pretreatment with *W. paludosa*. Neither paracetamol treatment alone nor pretreatment with *W. paludosa* altered glutathione reductase and glutathione S-transferase activity or the levels of end-products of lipid peroxidation. In conclusion, we found that *W. paludosa* protected against paracetamol-induced hepatotoxicity, an effect not observed over oxidative stress-related parameters. Hepatoprotection is likely mediated by some terpenes present in *W. paludosa* extract. However, further studies will be required to explain the mechanisms involved in the hepatoprotection afforded by *W. paludosa*.

Introduction

Wedelia paludosa D.C. (Asteraceae), popularly known as margarida, margaridão, pseudo-arnica, mal-me-quer-do-brejo, wedelia and vedelia, is a small herb almost ubiquitous in Brazil. Infusions of leaf or stem are largely used in the treatment of diseases of the respiratory system, as an expectorant or antitussive. Additional ethnopharmacological uses of *W. paludosa* are: analgesic, anti-inflammatory, anti-rheumatic, antipyretic and anti-anaemic (Michalak 1997). It was found in preclinical studies that *W. paludosa* produces an antinociceptive effect in mice (Block et al 1998). Furthermore, toxicological assays have not revealed acute or sub-acute toxicity for *W. paludosa*, corroborating the pharmacological use of this plant (Bürger et al 2005).

Several ethnopharmacological studies carried out with plants of the genus *Wedelia* have already demonstrated hepatoprotection. Among them, *Wedelia calendulacea* and *Wedelia chinensis* provided protection from chemically-induced liver injury, both in-vitro (hepatocyte culture) and in-vivo (mice and rats) (Yang et al 1987; Lin et al 1994; Emmanuel et al 2001). In addition, the main chemical constituents of aerial parts from *W. paludosa* are diterpenes and triterpenes (Block et al 1998; Batista et al 1999; Bresciani et al 2004), which have been reported to produce hepatoprotective actions in clinical trials and in experimental models, including paracetamol-induced toxicity (Liu 1995).

Paracetamol (acetaminophen) is one of the most popular analgesic and antipyretic drugs worldwide, and overdose or idiopathic reaction are among the major causes of morbidity and mortality in its victims (Mirochnitchenko et al 1999). Paracetamol is

considered a safe and effective analgesic when used at therapeutic levels. However, at supra-therapeutic doses it may cause centrilobular hepatic necrosis, being able to induce mortality in man and in experimental animals (Mitchell et al 1973). In spite of massive efforts undertaken in the search for new drugs to counteract paracetamol toxicity, no effective treatments are available that completely neutralize its toxic effects.

In view of the absence of effective treatments for acute paracetamol intoxication, and considering that plants of the genus *Wedelia*, and their main constituents, terpenes, have been shown to have beneficial hepatotoxicity protection effects, the aim of this study was to determine the potential in-vivo hepatoprotective effects of *W. paludosa* crude extract assessed by markers of liver damage and oxidative stress-related parameters, as well as by the analysis of lethality in mice.

Materials and Methods

Plant material

Wedelia paludosa D.C. was collected in the botanic garden of the Universidade Federal de Santa Catarina, Florianópolis, Brazil, in March 2003, and classified by Dr Ademir Reis (Department of Botany, Federal University of Santa Catarina, UFSC). A voucher specimen was deposited in Barbosa Rodrigues Herbarium, Itajaí, Brazil. Dried aerial parts (400 g) were cut into small pieces and macerated with ethanol at room temperature for approximately 15 days. The extract was then concentrated (reduced pressure) to the desired volume, as previously described (Block et al 1998).

Chemical studies carried out with *W. paludosa* aerial parts have demonstrated the presence of many classes of constituents, such as diterpenes and triterpenes (identified as ent-kaur-16-en-19-oic acid (kaurenoic acid), ent-kaur-16 alpha-ol-19-oic acid, ent-kaur-9(11),16(17)-dien-19-oic acid, 3 alpha-angeloiloxy-ent-kaur-16-en-19-oic acid, 3 beta-*O*-hexa-decanoylolean-12-en-28-oic acid, 3 beta-*O*-pentadecanoylolean-12-en-28-oic acid, 3 beta-*O*-tetradecanoylolean-12-en-28-oic acid, 3 alpha-tigloyloxykaur-16-en-19-oic acid, 3 alpha-cinamoyloxykaur-16-en-19-oic acid and beta-amyrin acetate), flavones (luteolin), steroids (stigmasterol, 3 beta-*O*-beta-D-glycopyranosyl sitosterol and 3 beta-*O*-beta-D-glycopyranosylstigmasterol), olenoic acids and lactones (paludolactone) (Batista et al 1999; Carvalho et al 2001; Vieira et al 2001; Cechinel-Filho et al 2004).

Chemicals

The following chemicals were used: nicotinamide adenine dinucleotide phosphate (reduced form; NADPH), paracetamol, *tert*-butylhydroperoxide, oxidized (GSSG) and reduced glutathione; 1-chloro-2,4-dinitrobenzene (CDNB); glutathione reductase; bovine serum albumin; 5,5-dithio bis (2-nitrobenzoic acid); malondialdehyde; 2-thiobarbituric acid and δ-aminolevulinic acid from Sigma Chemical Co. (USA).

Animals

Male adult Swiss mice, 25–35 g, were kept in a controlled-temperature room (23 ± 2°C) under a 12-h light–dark cycle (lights on at 0600 h). Food and water were freely available. The experiments were carried out in accordance with the current guidelines for the care of laboratory animals, and approved by the local Ethics Committee. Every effort was made to minimize animal suffering.

Treatments

Mice were treated as follows: the vehicle/vehicle group received *W. paludosa*-vehicle (saline) by the oral route and, one hour later, paracetamol-vehicle (saline with 5% Tween 80) by the intraperitoneal route; the paracetamol group received an intraperitoneal injection of paracetamol one hour after the *W. paludosa*-vehicle; and the mice in the *W. paludosa* group received *W. paludosa* crude extract orally one hour before paracetamol intraperitoneal injection.

To investigate the possible hepatoprotective effects of *W. paludosa*, mice were pretreated with *W. paludosa* crude extract (100, 250 or 500 mg kg^{-1}, p.o.) one hour before the paracetamol (600 mg kg^{-1}, i.p.) treatment. After 24 h, mice were slightly anaesthetized with ether for blood collection. The hepatic function was analysed using plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity as markers of paracetamol toxicity and liver was removed for total glutathione (GSH-t) measurement.

In another treatment regimen, mice were pretreated with *W. paludosa* (500 mg kg^{-1}, p.o.) one hour before paracetamol (1000 mg kg^{-1}, i.p.). The samples were obtained 4 h after paracetamol administration for the measurement of the activity of the hepatic enzymes glutathione reductase (GR), glutathione peroxidase (GPx), glutathione S-transferase (GST), catalase (CAT), δ-aminolevulinate dehydratase (δ-ALA-D), and of GSH-t and thiobarbituric acid reducing substances (TBARS) content.

Survival assessment

Mortality was induced by paracetamol (1000 mg kg^{-1}, i.p.) and mice were observed for up to 24 h after administration, as previously described (Janbaz et al 2002). Groups were treated as outlined above.

Plasmatic transaminase activity

The activity of ALT and AST was measured according to protocols available in the commercial kit (Roche, SP/ Brazil).

Total glutathione levels

GSH-t measurement was carried out in the acid extract of liver (1:10 w/v) with 0.5 M perchloric acid. The homogenate was centrifuged at 15000 *g*, for 2 min at 4°C, and the resultant supernatant was used after neutralization to pH 7.0. The assay was performed using a standard curve with known GSSG concentration, following the enzymatic method of Tietze (1969), as modified by Akerboom & Sies (1981).

Antioxidant enzyme activity

Liver was homogenized (1:10 w/v) in 20 mM Hepes buffer, pH 7.4. The homogenate was centrifuged at 15000 *g* for 30 min at 4°C, and the resultant supernatant was used. GPx activity was measured by the Wendel (1981) method, using *tert*-butylhydroperoxide as a substrate. NADPH disappearance was monitored spectrophotometrically at 340 nm. GST activity was assayed by the Habig & Jakoby (1981) procedure, using CDNB as a substrate. The assay was conducted by monitoring the appearance of the conjugated complex of CDNB and glutathione at 340 nm. GR activity was determined according to the method described by Carlberg & Mannervik (1985). The reduction of oxidized glutathione (GSSG) in the presence of NADPH was measured spectrophotometrically at 340 nm. Catalase activity was assayed by monitoring the H_2O_2 decay at 240 nm, using relatively high H_2O_2 concentration, 10 mM (Aebi 1984).

Thiobarbituric acid reactive substances (TBARS)

The mice were killed four hours after paracetamol administration and the livers removed and homogenized (1:10 w/v) in a medium containing 150 mM NaCl. The homogenates were centrifuged at 4000 *g* for 10 min at 4°C and the TBARS were assayed. TBARS were evaluated as described by Ohkawa et al (1979). Briefly, samples were incubated at 100°C for 120 min in a medium containing 0.45% sodium dodecyl sulfate, 100 mM hydrochloric acid, 1.4 M acetic acid, pH 3.4, and 0.3% thiobarbituric acid. After centrifugation the reaction product was determined at 532 nm. The results were expressed in terms of malondialdehyde production.

δ-Aminolevulinate dehydratase activity (δ-ALA-D)

The livers of treated mice were quickly removed, placed on ice and homogenized in 150 mM NaCl (1:10 w/v). The homogenate was centrifuged at 4000 *g* for 10 min at 4°C to yield a low-speed supernatant fraction that was used for enzyme assay. Enzyme assay was carried out as described by Sassa (1982), except for the use of 200 mM potassium phosphate buffer, pH 6.4, and 2.5 mM δ-aminolevulinic acid, by measuring the rate of product (porphobilinogen) formation. Reaction was started 10 min after the addition of the liver homogenate by adding the substrate and carried out over 60 min at 39°C. The reaction product was determined using modified Ehrlich's reagent at 555 nm, with a molar absorption coefficient of $6.1 \times 10^4\ M^{-1}\ cm^{-1}$ for the Ehrlich-porphobilinogen salt.

Protein determination

The protein content in homogenates was quantified by the method of Bradford (1976), using bovine serum albumin as a standard.

Statistical analysis

All data are expressed as mean ± s.e.m. by one-way analysis of variance, followed by Duncan's test, when appropriate. Differences between groups were considered significant when $P < 0.05$.

Results

Lethal response

Paracetamol administration caused 80% death in mice 24 h after treatment. The pretreatment with *W. paludosa* crude extract significantly (25%) reduced paracetamol-induced death in mice.

Plasmatic transaminase activity

The treatment with paracetamol increased blood plasma AST and ALT activity about 100-fold (Figure 1). Pretreatment with *W. paludosa* crude extract (100–500 mg kg^{-1}, p.o.) to a significant extent, in a dose-dependent manner, prevented the paracetamol-induced increase in AST activity (Figure 1A).

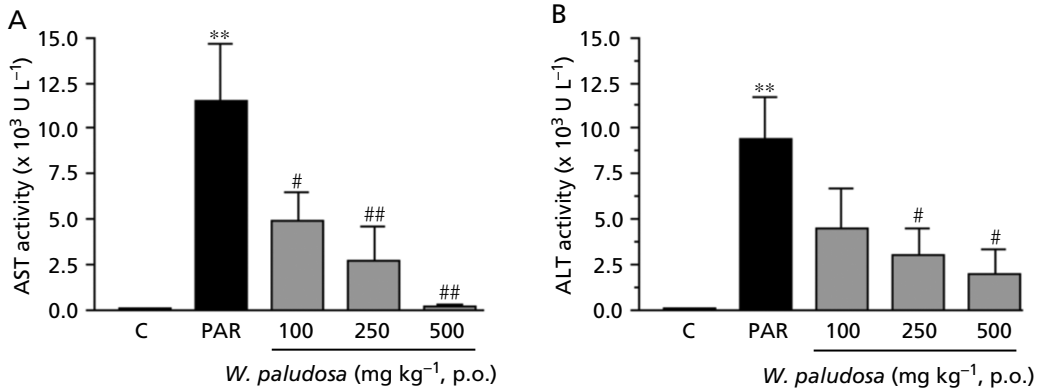

Figure 1 Effect of *W. paludosa* crude extract upon blood plasma AST and ALT activity. The blood plasma transaminases AST (aspartate aminotransferase, A) and ALT (alanine aminotransferase, B) were measured in blood plasma collected 24 h after paracetamol (PAR) treatment of mice. Mice were pretreated with *W. paludosa* at indicated doses 1 h before paracetamol (600 mg kg^{-1}, i.p.). Data are reported as mean + s.e.m. of 6 mice per dose. $^{**}P < 0.01$ compared with control (C) group; $\#P < 0.05$, $\#\#P < 0.01$ and $\#\#\#P < 0.001$ compared with PAR group.

Paracetamol-induced AST release was completely abolished by the highest dose of *W. paludosa* (500 mg kg^{-1}, Figure 1A). Also, *W. paludosa* treatment prevented paracetamol toxicity on ALT activity at the two higher doses employed (250 and 500 mg kg^{-1}) (Figure 1B).

Total glutathione (GSH-t) levels

It is known that paracetamol toxicity is associated with a decrease in glutathione (Gerber et al 1977). To determine whether *W. paludosa* has hepatoprotective effects with regard to glutathione, we evaluated GSH-t levels in the livers of treated mice. Neither paracetamol (600 mg kg^{-1}) nor *W. paludosa* crude extract (100, 250 and 500 mg kg^{-1}) plus paracetamol (600 mg kg^{-1}) treatment altered GSH-t levels after 24 h (data not shown). Nevertheless, paracetamol treatment (1000 mg kg^{-1}) almost completely depleted GSH-t levels 4 h after paracetamol administration. The *W. paludosa* crude extract (500 mg kg^{-1}) was unable to prevent such GSH-t decrease (Table 1).

Enzymatic activity

Within 4 h of paracetamol injection (1000 mg kg^{-1}, i.p.) a significant reduction in hepatic GPx activity was observed in the mice, which was not prevented by the pretreatment with *W. paludosa* crude extract (500 mg kg^{-1}) (Table 1).

The activity of CAT, GR and GST was not altered by paracetamol (1000 mg kg^{-1}, i.p.) treatment or by the paracetamol treatment in combination with *W. paludosa* crude extract as compared with the activity of the enzymes observed in vehicle-treated mice (Table 1).

Although CAT activity was not changed by paracetamol (1000 mg kg^{-1}, i.p.) treatment, pretreatment with *W. paludosa* crude extract increased CAT activity when compared with the paracetamol group ($P < 0.05$) (Table 1).

Hepatic δ-ALA-D activity was significantly reduced after exposure to paracetamol (1000 mg kg^{-1}, i.p.; Table 1). However, *W. paludosa* (500 mg kg^{-1}, p.o.) pretreatment did not prevent the paracetamol-induced reduction in the δ-ALA-D activity.

Thiobarbituric acid reactive substance (TBARS) measurements

The TBARS levels were not altered by either paracetamol (1000 mg kg^{-1}, i.p.) or paracetamol plus *W. paludosa* crude extract (500 mg kg^{-1}, p.o.) treatment. The TBARS levels were 9.27 ± 1.14; 7.77 ± 0.79 and 11.0 ± 1.94 (mean ± s.e.m.; n = 6) for the vehicle, paracetamol and *W. paludosa* plus paracetamol groups, respectively.

Discussion

A considerable number of studies suggest that extracts or isolated compounds obtained from *Wedelia paludosa* show a broad spectrum of biological activity, including trypanosomicidal, antifungal, bactericidal, antinociceptive, anti-inflammatory, antispasmodic, vasorelaxant and hypoglycaemic effects, which are mostly due to kaurenoic acid (Block et al 1998; Batista et al 1999; Bresciani et al 2004). Furthermore, ethnopharmacological studies with plants of the genus *Wedelia* have shown hepatoprotective effects in chemically induced liver damage (Yang et al 1987; Emmanuel et al 2001). We show here, for the first time, a protective effect of *W. paludosa* against paracetamol-induced hepatotoxicity, reflected in a better survival rate, and in the prevention of the increase in the activity of plasmatic AST and ALT in mice.

In fact, paracetamol treatment was lethal in 80% of mice and produced an approximately 100-fold increase in plasmatic AST and ALT activity. As an indication of *W. paludosa*'s protective effect, pretreatment with *W. paludosa* extract significantly (25%) reduced the lethality induced by paracetamol. In addition, *W. paludosa* pretreatment completely prevented the increase in AST activity and 80% of the increase in ALT activity in blood plasma.

A possible explanation for the protective effect of *W. paludosa* is that its main constituents are diterpenes and triterpenes, which have been shown to have beneficial effects on the liver in clinical trials and experimental models (Qu 1981; Liu 1995). Some terpene compounds are known for their hepatoprotective action against chemical-induced injury, including damage induced by paracetamol (Liu 1995).

Paracetamol toxicity is generally accepted to be primed by the formation of the alkylating intermediate *N*-acetyl-*para*-benzo-quinoneimine (NAPQI), a metabolite formed during cytochrome P450-catalysed oxidation of paracetamol (Mitchell et al 1973; Van de Straat et al 1988). NAPQI is a potent electrophilic substance, which causes glutathione depletion and binds covalently to a variety of hepatic cell proteins (Gerber et al 1977). The oxidation of protein thiols is thought to be an important step in determining paracetamol hepatotoxicity. In addition, the decrease in the levels of glutathione, which is one of the most important natural antioxidants of the

Table 1 Effect of paracetamol and *W. paludosa* crude extract on hepatic GSH-t levels and enzyme activity in mice

Treatment	GSH-t	GPx	GR	GST	CAT	δ-ALA-D
Vehicle	8.0 ± 0.6	14.8 ± 1.1	33.5 ± 1.5	27.9 ± 1.8	107.0 ± 6.0	25.1 ± 2.7
Paracetamol	0.4 ± 0.1**	8.1 ± 1.2**	33.2 ± 2.5	25.3 ± 2.3	70.4 ± 10.5	12.4 ± 3.9*
W. paludosa + paracetamol	1.0 ± 0.7**	5.1 ± 0.7**	45.1 ± 9.8	25.3 ± 4.5	165.9 ± 30.0#	14.1 ± 3.3*

Total glutathione (GSH-t) in μmol (g tissue)$^{-1}$; enzymatic activity in nmol min^{-1} (mg protein)$^{-1}$ for GPx (glutathione peroxidase), GR (glutathione reductase), GST (glutathione S-transferase) and CAT (catalase) and μmol PBG h^{-1}(mg protein)$^{-1}$ for δ-ALA-D (δ-aminolevulinate dehydratase). *$P < 0.05$, **$P < 0.001$ compared with vehicle; #$P < 0.05$ compared with paracetamol group. Data are mean ± s.e.m., n = 6.

hepatocyte, renders the cell remarkably susceptible to oxidative stress (Arnaiz et al 1995).

Several hypotheses have been put forward regarding the crucial early steps in the development of hepatic damage taking place directly after ingestion of paracetamol, once NAPQI is released in quantities that exhaust cellular GSH-t. Actually, depletion of GSH-t seems to be a cellular event that comes before paracetamol toxicity, and the reduction of liver thiol groups is a common finding after paracetamol intoxication (Kaushal et al 1999).

In this study, paracetamol treatment (1000 mg kg^{-1}) completely depleted hepatic GSH-t levels after 4 h, but not after 24 h (paracetamol 600 mg kg^{-1}). In line with this finding, previous studies have shown that hepatic GSH-t levels are decreased after paracetamol injection, returning to normal levels 8 h after (Mirochnitchenko et al 1999). The results reported here indicate that oral administration of *W. paludosa* crude extract failed to protect against GSH-t depletion induced by paracetamol. Based on these results, we may postulate that the hepatoprotective action of *W. paludosa* is not dependent on an antioxidant pathway. Accordingly, some antioxidant compounds, like flavones (luteolin), are moderately found in aerial parts of *W. paludosa* (Block et al 1998).

Glutathione peroxidase (GPx) is a critical antioxidant enzyme in the detoxification of peroxides. Its high affinity and relatively low substrate specificity for peroxides renders GPx more effective than CAT in the removal of peroxides (Toussaint et al 1993). In this study, GPx activity was markedly reduced by paracetamol treatment, and not prevented by *W. paludosa*. Lower GPx levels would increase steady-state hydroperoxide levels due to reduced capacity of peroxide elimination, which can lead to liver damage (Arnaiz et al 1995). Interestingly, CAT activity was not altered by paracetamol treatment. In fact, the administration of *W. paludosa* before paracetamol treatment caused an increase in CAT activity as compared with paracetamol treatment alone. Therefore, it may be suggested that lower GPx activity would be, to some extent, remedied by the increase in catalase activity, re-establishing, at least partially, the capacity to eliminate H_2O_2 when animals treated with paracetamol were pretreated with *W. paludosa*.

Considering that the P450 system can release reactive oxygen species, besides NAPQI-induced GSH-t depletion, Wendel et al (1979) postulated that paracetamol metabolism triggers massive lipid peroxidation, believed to be responsible for liver injury. However, no alteration in hepatic lipid peroxidation as measured by TBARS levels was observed in this study. Likewise, Wendel et al (1979, 1982) did not observe quantitative lipid peroxidation alterations in paracetamol-treated mice. For these authors, lipid peroxidation, followed by cell death and liver failure, was present only in mice fed with a diet low in vitamin E and high in polyunsaturated fatty acids, or in mice that received P-450 inducers (Wendel et al 1979, 1982). Conversely, an increase in lipid peroxidation was observed 4 h after paracetamol treatment, and a return to normal levels after an additional 4 h (Mirochnitchenko et al 1999). A possible discrepancy between our results and those obtained by Mirochnitchenko's group might be due to the difference between the mice strains employed.

It is known that δ-ALA-D activity is susceptible to a variety of treatments that are associated with oxidative stress (Folmer et al 2002; Soares et al 2003). In line with this, paracetamol treatment inhibits δ-ALA-D activity (Noriega et al 2000), a finding reproduced in our study. Furthermore, the inhibitory effect of paracetamol on δ-ALA-D activity was neutralized by an antioxidant (Rocha et al 2005). Here, we infer that the decrease in δ-ALA-D activity elicited by paracetamol treatment is causally linked to non-protein thiol group depletion, which may favour the development of an oxidizing environment in the liver. One possibility is that the reactive metabolite of paracetamol (NAPQI) may react directly with the essential thiol groups of δ-ALA-D, inactivating the enzyme. In this study, we observed a decrease in hepatic δ-ALA-D activity in paracetamol-treated mice, although no protection was observed with *W. paludosa* treatment. This emphasizes the idea that *W. paludosa* hepatoprotection is independent of an antioxidant route.

As mentioned above, the main constituents of *W. paludosa* are diterpenes and triterpenes, which are hepatoprotective (Qu 1981; Liu 1995). The terpenoids' defences against paracetamol toxicity are attributed to multiple mechanisms, but not antioxidant action, in line with the lack of effect on hepatic GPx and superoxide dismutase activity (Liu 1995). The main defence afforded by terpenoids is a reduction in the metabolic activation of liver cytochrome P-450 enzymes. This effect prevents liver lesions from developing fibrosis and cirrhosis, in parallel with an improved repair capacity in parenchymal cells, which allows better liver regeneration (Liu 1995).

Conclusion

Treatment with *W. paludosa* crude extract was able to reduce paracetamol-induced hepatotoxicity and mortality in mice. This protection may be attributed, at least in part, to effects of some terpenes present in *W. paludosa*, because these compounds reduce the metabolic activation of xenobiotics, including paracetamol, and improve liver regeneration. Furthermore, the protection is not directly related to any antioxidant action of the extract. However, further study will be required to explain the mechanisms involved in the hepatoprotection afforded by *W. paludosa*.

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