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Inhibition by the Bioflavonoid Ternatin of Aflatoxin B_1-induced Lipid Peroxidation in Rat Liver

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Abstract

Aflatoxin B_1, a metabolite of *Aspergillus flavus* is a potent hepatotoxic and hepatocarcinogenic mycotoxin. Lipid peroxidation and oxidative DNA damage are the principal manifestations of aflatoxin B_1-induced toxicity which could be mitigated by antioxidants. Many plant constituents, e.g. flavonoids, lignans and spice principles (capsaicin, curcumin, eugenol, etc.) have been reported to prevent liver damage associated with lipid peroxidation. In this study we investigated ternatin, a tetramethoxyflavone isolated from *Egletes viscosa*, for possible protection against liver injury induced by aflatoxin B_1 in rats.

Seventy two hours after a single intraperitoneal dose of aflatoxin B_1 ($1\,mg\,kg^{-1}$), the concentration of malondialdehyde, the product of lipid peroxidation in liver homogenates, and serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were significantly elevated ($P < 0{\cdot}01$). Subcutaneous ternatin ($25\,mg\,kg^{-1}$) pretreatment greatly reduced aflatoxin B_1-induced increases in the levels of serum enzymes (ALT from 5071 ± 763 to 293 ± 66 international units L^{-1} and AST from 4241 ± 471 to 449 ± 108 international units L^{-1}) and elevated malondialdehyde levels (from $11{\cdot}37 \pm 1{\cdot}27$ to $0{\cdot}79 \pm 0{\cdot}22$ nmol (mg wet tissue)$^{-1}$) in a manner similar to oral vitamin E ($300\,mg\,kg^{-1}$), a standard antioxidant. Further, histological changes induced by aflatoxin B_1 such as hepatocellular necrosis and bile-duct proliferation were markedly inhibited in animals pretreated with ternatin or vitamin E.

These data provide evidence that ternatin inhibits lipid peroxidation and affords protection against liver damage induced by aflatoxin B_1. Ternatin might, therefore, be a suitable candidate for the chemoprevention of aflatoxicosis associated liver cancer.

Mycotoxicosis as a result of aflatoxins is a health hazard to man. In acute and subacute poisoning the liver is the main target organ and the information available indicates a higher risk of liver cancer in populations consuming aflatoxin-contaminated foods (Burfening 1973). Among the aflatoxins, aflatoxin B_1, a metabolite of *Aspergillus flavus*, induces hepatocellular necrosis and carcinoma in man and in animals (Newberne & Butler 1969; Peers & Linsell 1977). The biochemical basis of aflatoxin B_1-induced cellular damage and carcinogenecity has not been well established. Its activation by the cellular cytochrome P450 enzyme system to form the reactive intermediate, aflatoxin B_1-8,9-epoxide has been described (Baertschi et al 1988). This epoxide can subsequently be trapped as a DNA-adduct which is generally considered as the critical step in tumour initiation (Choy 1993). Aflatoxin B_1 has recently been shown to induce lipid peroxidation-associated liver damage in rats (Shen et al 1994).

Many plant substances, e.g. flavonoids (Cholbi et al 1991; Galvez et al 1995; Keli et al 1996); lignans and neolignans (Haraguchi et al 1997) and spice principles such as capsaicin, eugenol and curcumin have antioxidant properties and can inhibit lipid peroxidation (Pulla Reddy & Lokesh 1992). These substances often serve as hepatoprotective agents in the prevention of toxicity caused by certain drugs and environmental chemicals wherein pathogenic oxidative and lipid peroxidative reactions are believed to play an essential role (Hara et al 1991; Kwan et al 1995), but their potential to inhibit

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aflatoxin B_1-induced peroxidation has not been evaluated.

Egletes viscosa L. (Asteraceae), popularly known as Macela da terra, is a small medicinal herb that grows abundantly in the northeast of Brazil. Infusions prepared from its flower heads are commonly employed in folk medicine for the treatment of gastritis, intestinal colic and liver disorders (Braga 1960). The bioflavonoid ternatin (4′5-dihydroxy-3,3′,7,8 tetramethoxyflavone) has been isolated from the dried flower heads (Lima et al 1996) and studied for its biological activity in laboratory animals. Ternatin was found to have anti-inflammatory, anti-anaphylactic, antithrombotic and hepatoprotective properties in animal models (Souza et al 1992; Rao et al 1994; Souza et al 1994). It has also been shown to have antiviral activity and to moderate the cytotoxicity of ternatin against tumour cells in man (Beutler et al 1998). More recently, we have demonstrated that ternatin has antiperoxidative action in the rat model of hepatotoxicity induced by carbon tetrachloride (Souza et al 1997) and paracetamol (Souza et al 1998). In this study we investigated whether ternatin prevents hepatic damage induced by aflatoxin B_1 in rats.

Materials and Methods

Animals

Male Wistar rats, 140–160 g, were used for the experiments. They were housed in polypropylene cages and maintained at 22 ± 2°C with a 12-h light–dark cycle. They were allowed free access to water and feed (purina chow).

Drugs and treatments

Aflatoxin B_1 and thiobarbituric acid (TBA) were obtained from Sigma (St Louis, MO). Ternatin was isolated from dried flower buds of *E. viscosa* L. by procedures and methods described elsewhere (Lima et al 1996). Aflatoxin B_1 and ternatin were dissolved in dimethylsulphoxide (DMSO; Reagen, Brazil) and then diluted with distilled water to the required concentration. The final concentration of DMSO in these solutions was 3%. Vitamin E (Ephymal) was purchased from Roche, Brazil, and all other chemicals used were of analytical grade. Aflatoxin B_1 was administered intraperitoneally (i.p.) and ternatin subcutaneously (s.c.), in volumes of 5 mL kg^{-1}. Vitamin E was suspended in corn oil at the desired concentration and was given orally (p.o.; 10 mL kg^{-1}). Control animals received the same volume of vehicle (3% DMSO, s.c., or corn oil, p.o.).

Aflatoxin B_1-induced liver damage

Liver damage was induced in overnight-fasted male rats by a single intraperitoneal injection of aflatoxin B_1 (1 mg kg^{-1}). Five groups of animals (n = 8) were pretreated before injection of aflatoxin B_1. The first group served as vehicle-treated control and received the vehicles 3% DMSO (5 mL kg^{-1}, s.c.) + corn oil (10 mL kg^{-1}, p.o.). The second group served as ternatin control and received ternatin in 3% DMSO (25 mg kg^{-1}, s.c.). The third group served as aflatoxin B_1 control and received vehicles only (as for the first group). The fourth and fifth groups of animals were treated with ternatin (25 mg kg^{-1}, s.c.) and vitamin E (300 mg kg^{-1}, p.o.), respectively. All pretreatments were given once daily for four consecutive days. At the end of this period, the third, fourth and the fifth groups of animals were given aflatoxin B_1 (1 mg kg^{-1}, i.p.) injection. Blood samples were collected 72 h (3 days) later, under light ether anaesthesia from the sino-orbital plexus. The blood was centrifuged at 2000 *g* for 5 min and the serum was separated and kept at 4°C to assay for alanine aminotransferase (ALT) and aspartate aminotransferase (AST). The rats were then killed by cervical dislocation and decapitation, and liver tissues were collected, washed with cold saline, blotted dry, and a 200-mg sample from each liver was homogenized in ice-cold 0·15 M potassium chloride solution to give a 10% homogenate.

The doses of 25 mg kg^{-1} ternatin and 300 mg kg^{-1} vitamin E were selected on the basis of previous work and on literature findings (Shen et al 1994; Souza et al 1997).

Measurement of lipid peroxidation

Lipid peroxidation was assayed by measuring malondialdehyde as thiobarbituric acid (TBA)-reactive material (Uchiyama & Mihara 1977). In brief, H_3PO_4 (1%, 3 mL) and aqueous TBA solution (0·6%, 1 mL) were added to the 10% homogenate (0·5 mL). The mixture was well shaken and heated on a boiling-water bath for 45 min. After cooling *n*-butanol (4 mL) was added and the mixture shaken. After separation of the butanol layer by centrifugation at 1200 *g* for 15 min its optical density at 535 and 520 nm was determined in a spectrophotometer (Beckman DU 640B). The difference between the results of the two optical density determinations was taken as the TBA value and the amount of malondialdehyde in the liver

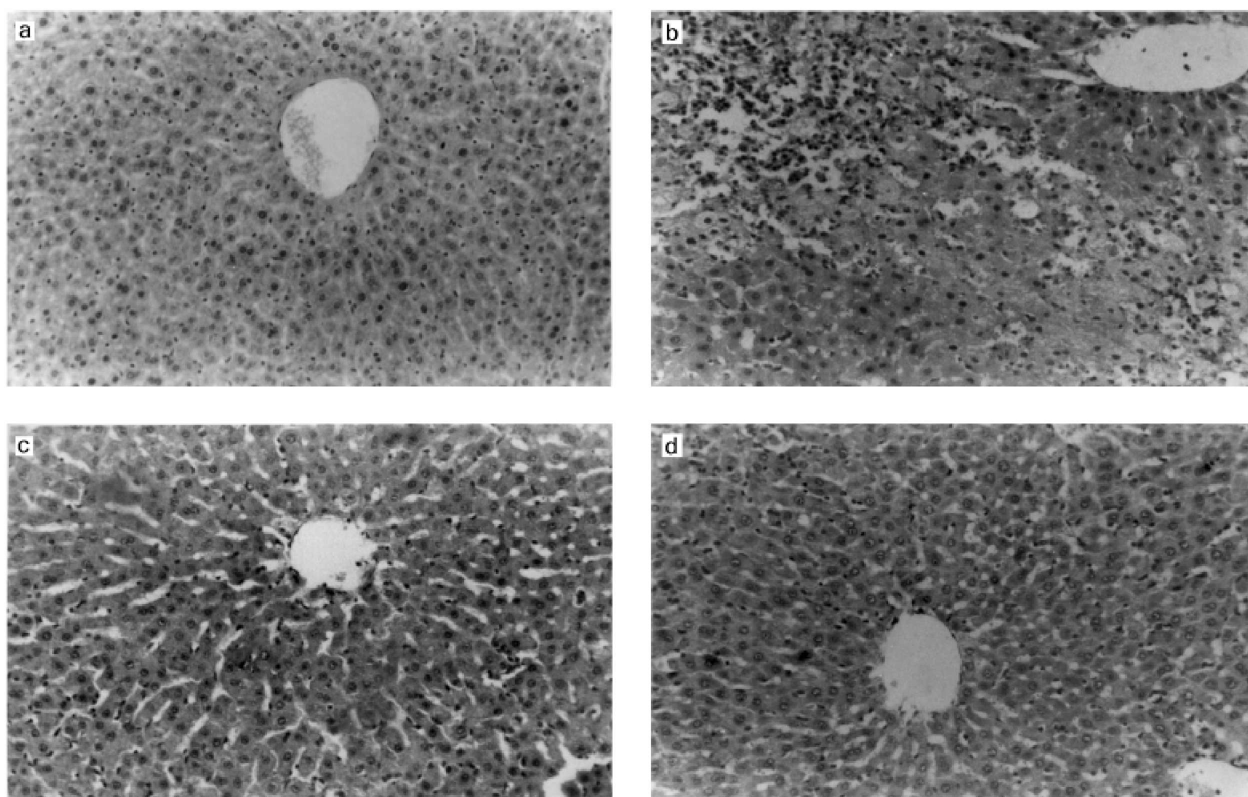

Figure 1. Photomicrograph of vehicle-treated rat liver section showing normal architecture (a); liver section from rat treated intraperitoneally with aflatoxin B_1 (1 mg kg^{-1}), showing cellular necrosis, intense hepatocellular vacuolization, cellular exudation and bile duct proliferation (b); liver section from rat treated subcutaneously with ternatin (25 mg kg^{-1}) showing almost complete protection against aflatoxin B_1 toxicity (c); cellular exudation is minimal and the liver architecture is well preserved; and liver section from rat treated orally with vitamin E (300 mg kg^{-1}) showing protection against aflatoxin B_1 toxicity (d). Necrosis and centrilobular changes are not remarkable. All sections were stained with haematoxylin and eosin dye; magnification ×100.

homogenate was calculated on the basis of a molar extinction coefficient of 13·700 $\text{M}^{-1}\,\text{cm}^{-1}$ (Esterbauer et al 1984).

Determination of serum enzymes

Levels of ALT and AST in rat serum were analysed by use of the RA-XT biochemical analyser with the respective test kits (Technicon, Bayer Diagnostic, Brazil) and the values were expressed in international units L^{-1}.

Histological changes in the liver

Liver tissue from each rat was fixed in 10% neutral formalin and processed for paraffin embedding. Sections (6 μm) were prepared and stained with haematoxylin and eosin dye. Histological changes

Table 1. Effects of pretreatment with ternatin and vitamin E on aflatoxin B_1-induced changes in serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), and on malondialdehyde concentration in rat liver homogenates.

Group	Dose (mg kg^{-1}) and route	Alanine aminotransferase (international units L^{-1})	Aspartate aminotransferase (international units L^{-1})	Malondialdehyde (nmol mg^{-1} wet tissue)
Vehicle control	–	129·3 ± 17·6	162·5 ± 12·7	2·48 ± 0·57
Ternatin control	25, intraperitoneal	106·9 ± 22·4	164·9 ± 26·4	1·78 ± 0·38
Aflatoxin B_1control	1, intraperitoneal	5071·4 ± 763·0*	4240·9 ± 471·1*	11·37 ± 1·27*
Aflatoxin B_1 +ternatin	1, intraperitoneal +25, subcutaneous	293·1 ± 66·1†	449·2 ± 108·0*†	0·79 ± 0·22†
Aflatoxin B_1 +vitamin E	1, intraperitoneal +300, oral	218·5 ± 58·4†	203·9 ± 46·2†	4·81 ± 0·52†

Results are expressed as means ± standard error of the mean (n = 8). Malondialdehyde concentrations are given in terms of thiobarbituric acid-derived conjugates. *$P < 0·001$, significantly different from vehicle control value. †$P < 0·001$, significantly different from aflatoxin B1 control value.

were studied under light microscopy by a person unaware of the treatment groups.

Statistical analysis

The results are expressed as the mean ± the standard error of the mean (s.e.m.) and statistical significance between groups was analysed by one-way analysis of variance and then the Student–Newman–Keuls multiple comparison test. Values of $P < 0{\cdot}05$ were considered to be indicative of significance.

Results and Discussion

The thiobarbituratic acid reaction of liver homogenates and the levels of serum enzymes in rats from vehicle and test groups are presented in Table 1. Significant (fivefold) elevation of malondialdehyde concentration was observed 72 h after a single intraperitoneal injection of aflatoxin B_1, indicating the occurrence of lipid peroxidation. This study confirms the earlier report of the lipid peroxidation-inducing effect of aflatoxin B_1. Shen et al (1994) observed an increase in malondialdehyde concentration as early as 1 day after administration of aflatoxin B_1 (1 mg kg^{-1}), with a peak level after 3 days. Therefore this study examined the effect of ternatin, a natural flavonoid from *Egletes viscosa*, on the aflatoxin B_1-induced peak increase in the levels of malondialdehyde and the serum enzymes ALT and AST. Although ternatin per se had no significant effect on these levels, the aflatoxin B_1-induced elevations of the concentration of malondialdehyde in the liver homogenates and the levels of the serum enzymes were significantly ($P < 0{\cdot}05$) inhibited by ternatin pretreatment in a manner similar to that observed after treatment with vitamin E, a known antioxidant (Table 1). These results indicate that ternatin and vitamin E are effective inhibitors of aflatoxin B_1-induced lipid peroxidation.

Aflatoxin B_1 treatment markedly enhanced serum enzyme activity—39- and 26-fold for ALT and AST, respectively, compared with the vehicle-treated control group. This elevation of serum enzyme activity reflects the extent of cell damage; this was further confirmed by histopathological examination—the appearance of liver sections from vehicle-treated control animals was normal (Figure 1a) whereas intense hepatocellular microvacuolization, necrosis and focal areas of parenchymal collapse were apparent in those from aflatoxin B_1-treated rats. These lesions were mostly perilobular. Inflammatory exudate and bile duct proliferation were apparent throughout the lobule (Figure 1b). In contrast, for ternatin and vitamin E-treated rats lobular structure was well preserved, and less exudation and hyperplasia of bile ducts were apparent (Figures 1c, d).

Although several plant substances such as flavonoids (rutin, quercetin, catechin, sylimarin, gardinin D), spice principles (curcumin, eugenol, capsaicin), lignans and neolignans (honokiol, magnolol) have been reported to afford protection in other models of lipid peroxidation, they have not been tested against aflatoxin B_1-induced lipid peroxidation. Most of these substances have been reported to be potent antioxidants and it is probable that they not only inhibit hepatocellular damage but also the carcinogenecity of aflatoxin B_1. In this study ternatin was effective in mitigating the severity of histopathological changes and the elevation of serum transaminases (ALT, AST). Aflatoxin B_1 toxicity is believed to be mediated by the generation of toxic metabolites (Baertschi et al 1988) that initiate lipid peroxidation (Shen et al 1994). Indeed, the report that ternatin protects against CCl_4-induced lipid peroxidation (Souza et al 1997) suggests that protective action is at the lipid peroxidation step rather than on aflatoxin B_1 metabolism. Studies indicate that polymethoxyflavonoids are more potent inhibitors of lipid peroxidation in-vivo than are highly polar hydroxylated compounds (Cholbi et al 1991), which are extensively metabolized and become less active in-vivo. Ternatin, being a tetramethoxyflavone, can act as a free-radical scavenger and prevent the lipid peroxidation induced by toxic metabolites of aflatoxin B_1.

In conclusion, the results of this study are in-vivo evidence of the protective effect of ternatin against aflatoxin B_1-induced hepatotoxicity. Inhibition of lipid peroxidation might constitute the principal mechanism of its hepatoprotective function. Further studies are required to establish its interaction with the cytochrome P-450 system in the formation of toxic metabolites. We suggest that ternatin might be a suitable candidate for the chemoprevention of aflatoxicosis-associated liver damage and cancer.

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References

Baertschi, S. W., Raney, K. D., Stone, M. P., Harris, T. M. (1988) Preparation of 8,9-epoxide of the mycotoxin afla-

toxin B_1, the ultimate carcinogen species. J. Am. Chem. Soc. 110: 7929–7931

Beutler, J. A., Hamel, E., Vlietinck, A. J., Haemers, A., Rajan, P., Roitman, J. N., Cardellina II, J. H., Boyd, M. R. (1998) Structure-activity requirements for flavone cytotoxicity and binding to tubulin. J. Med. Chem. 41: 2333–2338

Braga, R. (1960) Plantas do Nordeste, especialmente do Ceará. Centro de divulgação Universitária, Fortaleza

Burfening, P. J. (1973) Ergotism. J. Am. Vet. Med. Assoc. 163: 1288–1290

Cholbi, M. R., Paya, M., Alcaraz, M. J. (1991) Inhibitory effects of phenolic compounds on CCl_4-induced microsomal lipid peroxidation. Experientia 47: 195–199

Choy, W. N. (1993) A review of the dose-response induction of DNA adducts by aflatoxin B_1 and its implications to quantitative cancer-risk assessment. Mutat. Res. 296: 181–198

Esterbauer, H., Lang, J., Zadravec, S., Slater, T. F. (1984) Detection of malonaldehyde by high-performance liquid chromatography. Methods Enzymol. 105: 319–328

Galvez, J., De La Cruz, J. P., Zarzuelo, A., De La Cuesta, F. S. (1995) Flavonoid inhibition of enzymic and nonenzymic lipid peroxidation in rat liver differs from its influence on the glutathione-related enzymes. Pharmacology 51: 127–133

Hara, S., Endo, T., Kuriiwa, F., Kano, S. (1991) Mechanism of paraquat-stimulated lipid peroxidation in mouse brain and pulmonary microsomes. J. Pharm. Pharmacol. 43: 731–733

Haraguchi, H., Ishikawa, H., Shirataki, N., Fukuda, A. (1997) Antiperoxidative activity of neolignans from *Magnolia obovata*. J. Pharm. Pharmacol. 49: 209–221

Keli, S. O., Hertog, M. G. L., Feskens, E. J. M., Krohout, D. (1996) Dietary flavonoids, antioxidant vitamins, and incidence of stroke: the Zutphen study. Arch. Intern. Med. 156: 637–642

Kwan, D., Bartle, W. R., Walker, S. E. (1995) Abnormal serum transaminases following therapeutic doses of acetaminophen in the absence of known risk factors. Dig. Dis. Sci. 40: 1951–1955

Lima, M. A. S., Silveira, E. R., Marques, M. S. L., Santos, R. H., Gambardela, M. T. P. (1996) Biologically active flavonoids and terpenoids from *Egletes viscosa*. Phytochemistry 41: 217–223

Newberne, P. M., Butler, W. H. (1969) Acute and chronic effects of aflatoxin on the liver of domestic and laboratory animals. A review. Cancer Res. 29: 236–250

Peers, F. G., Linsell, C. A. (1977) Dietary aflatoxins and human primary liver cancer. Ann. Nutr. Alim. 31: 1005–1018

Pulla Reddy, A. Ch., Lokesh, B. R. (1992) Studies on spice principles as antioxidants in the inhibition of lipid peroxidation of rat liver microsomes. Mol. Cell. Biochem. 111: 117–124

Rao, V. S. N., Figuerido, E. G., Melo, C. L., Viana, G. S. B., Menezes, D. B., Matos, M. S. F., Silveira, E. R. (1994) Protective effect of ternatin, a flavonoid isolated from *Egletes viscosa* Less. in experimental liver injury. Pharmacology 48: 392–397

Shen, H. M., Shi, C. Y., Lee, H. P., Ong, C. N. (1994) Aflatoxin B_1-induced lipid peroxidation in rat liver. Toxicol. Appl. Pharmacol. 127: 145–150

Souza, M. F., Rao, V. S. N., Silveira, E. R. (1992) Antianaphylactic and anti-inflammatory effects of ternatin, a flavonoid isolated from *Egletes viscosa* Less. Brazilian J. Med. Biol. Res. 25: 1029–1032

Souza, M. F., Cunha, G. M. A., Fontenele, J. B., Rao, V. S. N., Silveira, E. R. (1994) Antithrombotic activity of ternatin, a tetramethoxyflavone from *Egletes viscosa* Less. Phytother. Res. 8: 478–481

Souza, M. F., Rao, V. S. N., Silveira, E. R. (1997) Inhibition of lipid peroxidation by ternatin, a tetramethoxyflavone from *Egletes viscosa* L. Phytomedicine 4: 25–29

Souza, M. F., Rao, V. S. N., Silveira, E. R. (1998) Prevention of acetaminophen-induced hepatotoxicity by ternatin, a bioflavonoid from *Egletes viscosa* Less. Phytother. Res. 12: 1–5

Uchiyama, M., Mihara, M. (1977) Determination of malondialdehyde precursor in tissues by thiobarbituric acid test. Anal. Biochem. 86: 271–278