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**Acknowledgements and
funding:** We thank MVZ Mario
Soriano, Biol. Dora Salazar and
Biol. Isabel Antunez (Bioterium
of the Faculty of Sciences, UNAM,
México) for technical assistance
in providing and maintaining the
hamsters used in this study. This
work was supported by Dirección
General de Estudios de Posgrado,
UNAM and CONACYT through a
fellowship to Silvia Arteaga and
by DGAPA, UNAM (PAPIIT No.
IN204703-3).

Effect of *Larrea tridentata* (creosote bush) on cholesterol gallstones and bile secretion in hamsters

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Abstract

Larrea tridentata (Sesse and Moc. ex DC.) Coville is used for the treatment of gallstones in traditional Mexican medicine. The possible prevention or elimination of gallstones by ethanolic and aqueous extracts of the leaves and twigs of *L. tridentata* was tested in hamsters fed a rich carbohydrate, fat-free diet. In addition, the effects of the ethanolic extract and its main metabolite, nordihydroguaiaretic acid, on bile secretion in the perfused liver were tested. In the experiment on prevention of gallstones, the dry ethanolic extract at a level of 0.5% of diet completely inhibited gallstone formation, lowered biliary moles percent cholesterol and increased the proportion of chenodeoxycholic acid of hepatic bile. The dry aqueous extract at a level of 1% of diet did not affect gallstone frequency or biliary parameters. In the experiment on elimination of gallstones, the ethanolic extract significantly reduced gallstone frequency, gallbladder bile cholesterol concentration and moles percent cholesterol. Both the ethanolic extract and nordihydroguaiaretic acid had cholestatic effects in the perfused liver, with an EC₅₀ of 34 and 28 mg dL⁻¹, respectively, when perfused for 10 min. This effect was reversible with concentrations up to 40 mg dL⁻¹. The results indicate that *L. tridentata* could be useful in the treatment of gallstone disease, however care must be taken due to its hepatotoxicity.

Introduction

Cholesterol gallstone disease is common in the Western world, with the greatest incidence in North American Indians (64.1% in women aged 47 years and older; Everhart et al 2002). The Mexican Ministry of Health reported that in 2002 it was the third cause of hospitalization (33 474 cases, 2.05% of all cases; SSA 2002). The aetiology of cholesterol gallstones is multifactorial, being affected by age, gender and diet, among other factors, and involves supersaturation of bile with cholesterol either by increased cholesterol or reduced bile salt secretion, followed by precipitation of excess cholesterol in the gallbladder and favoured by abnormalities in gallbladder emptying (Méndez et al 1996).

Patients with type 2 diabetes mellitus have a greater incidence of gallstones (Pazzi et al 2000). Between 40% and 50% of North American Indians over 35 years of age are diabetic (Knowler et al 1978). Diabetics tend to be obese and to have hypertriglyceridaemia, and both of these conditions are associated with an increased risk of gallstones. Although some reports do not agree, several studies have found that patients with diabetes present cholesterol supersaturated bile as well as inadequate gallbladder emptying (Pazzi et al 2000).

Larrea tridentata (Sessé & Moc. ex DC.) Coville (Zygophyllaceae; creosote bush) has been used in the treatment of diabetes (Winkelman 1989), and nordihydroguaiaretic acid (NDGA), extracted from *L. tridentata*, has been shown to be effective in lowering hyperglycaemia and hypertriglyceridaemia in diabetic animals (Reed et al 1999). Thus, it is possible that *L. tridentata* extracts would also affect cholesterol gallstone formation and/or elimination. Factors that reduce biliary cholesterol secretion or that increase cholesterol 'solubility' in bile, where it is transported in mixed micelles and vesicles, are known to prevent or eliminate gallstones (e.g. chenodeoxycholate and ursodeoxycholate; Méndez et al 1996). In traditional Mexican medicine, one of the uses reported for *L. tridentata* (known as Gobernadora) is in the treatment of biliary stones (Diaz 1976). However, abuse of *L. tridentata* has been associated with nephropathy and hepatotoxicity, including cholestatic

hepatitis (Brent 1999), and so care must be taken when using this plant in alternative or complementary medicine.

L. tridentata is a well-branched shrub with no defined trunk, reaching heights of up to 1–1.5 m. The stems are grey/black, leaves are opposite, bifoliately compound, each leaflet joined at the base, divergent, but the tips often point toward each other. Leaflets are generally olive green in colour, up to 1 cm in length, 3–4 mm wide, asymmetrical, oblong to obovate in shape, and broader away from the base (USDA Forest Service). It is widely found in the desert southwest of the USA and north of Mexico. It has many secondary metabolites, with the lignan NDGA representing 5–10% of the dry weight of leaves (Mabry & Bohnstedt 1981). Among several other properties, NDGA is an antioxidant and an inhibitor of lipoxigenase and vesicular transport (Fujiwara et al 1998). The resin that covers the leaves contains 19 flavonoid aglycones and several lignans (Konno et al 1990). Some glycosylated flavonoids, saponinins, essential oils, halogenic alkaloids and waxes have also been isolated from *L. tridentata* (Romo de Vivar 1985; Argueta 1994). *L. tridentata* contains almost 0.1% volatile oils and 67 compounds have been identified, which constitute more than 90% of *L. tridentata* oils. The remaining 10% is a mixture of more than 300 constituents, mainly hydrocarbon monoterpenes, oxygenated monoterpenes and aromatic sesquiterpenes (Mabry & Bohnstedt 1981; Xue et al 1988). Products from the mevalonic, shikimic and fatty acid pathways are predominant. Vinyl and methyl ketones contribute significantly to the typical odour of *L. tridentata*. Three sterols have been identified (campesterol, stigmaterol and sitosterol) as well as saponins of the C₃₀ type, representing less than 1% of the dry weight, the main component being ursolic acid (Mabry & Bohnstedt 1981). Alkaloids have been isolated from the bark and roots, but not from leaves and flowers (Lara & Márquez 1996). Two reviews of the uses and pharmacology of *L. tridentata* and NDGA have been published (Lambert et al 2004; Arteaga et al 2005).

In the present study, the possible prevention and/or elimination of cholesterol gallstones by *L. tridentata* extracts was tested, as well as their effects on bile secretion and composition. Hamsters fed a lithogenic diet (Dam 1969) were used in the study. Serum lipids were also monitored since changes in serum lipids can be associated with biliary lipids; for example, hypocholesterolaemic agents, such as clofibrate and legume, produce an increase in biliary cholesterol (Rigotti et al 1989). In addition, the effect of *L. tridentata* ethanolic extract on bile secretion as an indicator of hepatotoxicity was assessed in perfused hamster liver.

Materials and Methods

Plant material and extracts

The *L. tridentata* plants used to prepare the extracts were collected at San Luis Potosí State, Mexico. Voucher specimens were deposited at the National Herbarium, Institute of Biology, UNAM (MEXU) (no. 534807), and at the

Herbarium of Medicinal Plants, S. XXI Medical Center (IMSSN) (nos 11 319–11 321).

The ethanolic extract was prepared from three batches of 100 g of coarsely fragmented leaves and twigs. Each batch was extracted in 1 L absolute ethanol, mixed for 30 min at room temperature, filtered with Whatman No. 1 paper and reduced to approximately 40 mL in a rotary evaporator under reduced pressure. This extract was dried in an oven at 40°C overnight. Between 9 and 10 g of resin were obtained per batch of plant extracted. The ratio of the herbal drug to the native herbal drug preparation (DER native) was 10:1 (Gaedcke & Steinhoff 2003).

In order to obtain an aqueous extract, 100 g of coarsely fragmented leaves and twigs were placed in a 2-L flask with 1 L of boiling distilled water and left to boil for 10 min. The extract was filtered through a sieve and a fine nylon mesh, and then lyophilized. Between 15 and 20 g of powder were obtained per batch of plant extracted. DER native mean was 6:1.

Animals

The animals used in this study were housed, cared for and used in accordance with the Official Mexican Regulations on Technical Specifications in the Production, Maintenance and Use of Laboratory Animals (NOM-062-ZOO-1999). Sixty-day-old male golden hamsters (*Mesocricetus auratus*) were used. The animals were obtained from the stock colony kept at the animal house from the Faculty of Sciences, UNAM, Mexico. They were maintained in stainless steel cages (wire mesh floor and front), three animals per cage, at room temperature and humidity, under a 14-h light/10-h dark cycle.

Gallstone prevention assay

Four groups of 12 hamsters each were formed, and fed for 62 days on the following diets and tap water *ad libitum*: Group 1 was fed powdered Purina Nutricubes for small rodents (Purina Mexico, Cuautitlan Izcalli, Mexico); Group 2 was fed the lithogenic diet; Group 3 was fed the lithogenic diet + 0.5% *L. tridentata* ethanolic extract; Group 4 was fed the lithogenic diet + 1.0% *L. tridentata* aqueous extract. The extracts were added instead of an equivalent amount of glucose. The ethanolic extract was first dissolved in a small volume of ethanol and thoroughly mixed with a small amount of glucose before being incorporated into the rest of the glucose, giving a homogeneous light green colour. This glucose was placed in an oven at 40°C overnight before mixing with the other components of the diet.

The lithogenic diet (Dam 1969) consisted of 20% casein, 74.3% glucose, 5% mineral mix (4% of mineral mix No 2 USP XIII and 1% of supplement for mineral mix No. 2 USP XIII), 0.5% vitamin mix (vitamin diet fortification mixture) and 0.2% choline chloride (all from ICN Biomedicals, Mexico City, Mexico).

Food consumption was measured on Day 55: 50 g of each diet per cage was given to the animals at 12:00 hours; 24 h later, the food remaining was weighed.

At the end of the experimental period, the animals were fasted overnight, anaesthetized with pentobarbital (50 mg kg⁻¹ i.p.), the cystic duct was ligated and a polyethylene cannula PE-10 (Clay Adams, Parsipany, NJ, USA) was placed in the common bile duct. Bile was collected for 1 h in 2-mL siliconized polypropylene tubes kept on ice; the volume was determined gravimetrically. Once bile was collected, the hamsters, still under anaesthesia, were bled by cardiac puncture, and the serum was separated by centrifugation. Post-mortems were immediately performed and the livers were excised and weighed.

Gallstone elimination assay

Four groups of nine hamsters were formed: Group A was fed powdered Purina Nutricubes for small rodents; Groups B, C and D were fed the lithogenic diet *ad libitum*. After 6 weeks, the animals in Group B were killed by excess of pentobarbital. Post-mortems were performed and the gallbladder bile aspirated, diluted with 9 vols of isopropyl alcohol and kept at -20°C until analysis the following week. Groups A and C continued on the same diets, while Group D received the lithogenic diet + 0.5% *L. tridentata* ethanolic extract for another 6 weeks before gallbladder bile was collected as for Group B.

Liver perfusion

Liver perfusion was performed in-situ at 37°C based on Seglen (1976). Briefly, hamsters were anaesthetized with pentobarbital (50 mg kg⁻¹ i.p.) and the common bile duct was cannulated. The portal vein (inlet) and the suprahepatic cava vein (outlet) were cannulated. The perfusion medium was Krebs-Henseleit-bicarbonate buffer fortified with 2 g L⁻¹ glucose, 10 g L⁻¹ bovine serum albumin and 50 μM sodium taurocholate, pH 7.4. It was oxygenated with a mixture of oxygen and carbon dioxide (95:5). The perfusion medium was delivered at a flow rate of ~3–4 mL min⁻¹ (g liver weight)⁻¹ in a re-circulated manner. Samples of bile were collected in 10-min periods. After the first two samples of bile were collected, the medium was substituted with medium containing different amounts of the ethanolic extract of *L. tridentata* or NDGA (Sigma Aldrich, Toluca, Mexico), in a volume to be

perfused for 10 min by single pass (~100–110 mL), followed by 60 min perfusion of medium without additions. Both compounds were previously dissolved in 100 μL of ethanol, mixed with 50 μL of glycerol and 5 mL of perfusion medium. Control livers were perfused with vehicle.

Bile and serum analysis

The bile volume was determined gravimetrically. Bile was analysed for cholesterol by an enzymatic method with a Merck reagent kit (Trinder; Merck-Mexico, Naucalpan de Juarez, Mexico), bile salts with 3-α-hydroxysteroid dehydrogenase (Turley & Dietschi 1978), and phospholipids as organic phosphorus (Bartlett 1959). Biliary bile acid profiles were determined by HPLC (Rossi et al 1987), using a Nucleosil 100-5 C18 column (250 × 4.6 mm; Machery-Nagel, Mexico City, Mexico) and an isocratic elution with methanol/phosphate buffer (75:25), pH 5.35, in a Merck-Hitachi HPLC system. Serum was analysed for cholesterol by an enzymatic method with a Sigma kit (Sigma Aldrich), HDL cholesterol with the same kit after precipitation of other lipoproteins with phosphotungstic acid, and triglycerides by an enzymatic method with a triglycerides GPO-PAP kit (Boheringer Mannheim, Mexico City, Mexico).

Statistical analysis

Data were analysed by the χ²-test and one-way analysis of variance followed by Bonferroni's multiple comparison test, with the level of significance set at *P* < 0.05.

Results

Gallstone prevention assay

The lithogenic diet (Dam 1969) is a fatty acid deficient diet and so it was not surprising that the animals on this diet (Groups 2, 3 and 4) did not gain weight (Table 1). Animals that were fed the lithogenic diet had similar food consumption; their food consumption was lower than that of animals fed the Purina diet (Group 1), however the difference was statistically significant only for Groups 2 and 3.

Table 1 Bodyweight, liver weight and food consumption in hamsters fed a lithogenic diet with or without *Larrea tridentata* extracts

Group	Diet	Final body weight (g)	Change in bodyweight (g)	Liver weight (g/100g bodyweight)	Food consumption (g/animal per day)
1	Purina	118 ± 8 ^a (n = 12)	27 ± 6 ^a (n = 12)	3.4 ± 0.2 ^a (n = 12)	10.0 ± 0.9 ^a (n = 12)
2	Lithogenic	94 ± 19 ^b (n = 12)	4 ± 19 ^b (n = 12)	4.2 ± 0.5 ^b (n = 12)	7.6 ± 1.2 ^b (n = 12)
3	Lithogenic + 0.5% ethanolic extract	87 ± 14 ^b (n = 9)	-4 ± 13 ^b (n = 9)	4.2 ± 0.4 ^b (n = 9)	7.8 ± 1.7 ^b (n = 9)
4	Lithogenic + 1% aqueous extract	95 ± 9 ^b (n = 12)	5 ± 6 ^b (n = 12)	3.6 ± 0.4 ^a (n = 12)	8.9 ± 0.8 ^{a,b} (n = 12)

Values are given as mean ± s.d. Different letters in the same column indicate a statistical difference at *P* < 0.05.

In relation to liver weight (expressed as a percentage of bodyweight; Table 1), it was observed that the livers of animals fed the lithogenic diet (Group 2) or the lithogenic diet and the ethanolic extract (Group 3) were significantly larger compared with the control group (Group 1). The liver weight of animals fed the lithogenic diet and the aqueous extract (Group 4) was similar to the control group. Three animals on the lithogenic diet and ethanolic extract (Group 3) died during the first 2 weeks of the experiment.

Gallstone frequency is shown in Table 2. Group 2 exhibited a high frequency of cholesterol gallstones (75%). Similarly, the group that received the aqueous extract (Group 4) presented high gallstone incidence (67%), and two animals without gallstones had cholesterol crystals. In animals that received the ethanolic extract (Group 3), cholesterol gallstone formation was completely inhibited; only cholesterol crystals were found in two animals.

In relation to bile parameters (Table 2), there were no differences in bile flow among the four groups. The biliary cholesterol concentration was greater in the groups that received the lithogenic diet (Groups 2, 3 and 4), being approximately double the concentration in the control group. Although the animals that received the ethanolic

extract (Group 3) had a lower cholesterol concentration than those fed with only the lithogenic diet (Group 2), the difference was not statistically significant. Bile salt concentrations were slightly greater in the groups that received the *L. tridentata* extracts, especially the ethanolic extract (Group 3), however the differences were not significant compared with Groups 1 and 2. Similarly, the phospholipid concentration was greater in groups fed the lithogenic diet; the animals that received the ethanolic extract showed the highest value, being the only parameter statistically significant when compared with the control group (Group 1).

Due to the increase in biliary cholesterol, the moles percent cholesterol (i.e. $[\text{cholesterol}] \times 100 / [\text{bile acids}] + [\text{phospholipids}] + [\text{cholesterol}]$; Carey 1978) showed a considerable increase in all groups fed the lithogenic diet (Groups 2, 3 and 4), the differences among them not being significant (Table 2). Animals that received the ethanolic extract had a moles percent cholesterol intermediate between that of the group fed only the lithogenic diet (Group 2) and the control (Group 1), the differences among them not being significant.

The profile of bile acids in hepatic bile (Table 3) showed a reduction in cholate and an increase in

Table 2 Gallstone frequency and biliary parameters in hepatic bile from hamsters fed a lithogenic diet with or without *Larrea tridentata* extracts

Group	Diet	Gallstone frequency	Bile flow (mg min ⁻¹ /100 g bodyweight)	Cholesterol (mM)	Bile salts (mM)	Phospholipids (mM)	Moles percent cholesterol
1	Purina	0/12 ^a	5.89 ± 0.44 ^a (n = 12)	0.18 ± 0.06 ^a (n = 12)	9.2 ± 3.4 ^a (n = 12)	2.23 ± 0.66 ^a (n = 12)	1.61 ± 0.45 ^a (n = 12)
2	Lithogenic	9/12 ^b	6.05 ± 1.50 ^a (n = 9)	0.40 ± 0.14 ^b (n = 9)	9.9 ± 3.0 ^a (n = 9)	2.90 ± 1.00 ^{a,b} (n = 9)	3.15 ± 1.09 ^b (n = 9)
3	Lithogenic + 0.5% ethanolic extract	0/9 ^a	6.14 ± 1.41 ^a (n = 7)	0.37 ± 0.13 ^b (n = 7)	11.3 ± 4.1 ^a (n = 7)	3.82 ± 1.15 ^b (n = 7)	2.43 ± 0.60 ^{a,b} (n = 7)
4	Lithogenic + 1% aqueous extract	8/12 ^b	5.94 ± 1.14 ^a (n = 10)	0.41 ± 0.11 ^b (n = 10)	10.9 ± 2.9 ^a (n = 10)	3.18 ± 0.85 ^{a,b} (n = 10)	2.98 ± 1.18 ^b (n = 10)

Values are given as mean ± s.d. Moles percent cholesterol: $[\text{cholesterol}] \times 100 / [\text{bile salts}] + [\text{phospholipids}] + [\text{cholesterol}]$. Different letters in the same column indicate a statistical difference at $P < 0.05$.

Table 3 Proportion of glycine to taurine conjugates and percentage of main bile acids in bile of hamsters that received the ethanolic extract of *Larrea tridentata*

Group	Diet	Glycine/taurine conjugation	Cholate (%)	Chenodeoxycholate (%)	Deoxycholate (%)
1	Purina	1.2 ± 0.8 ^a (n = 4)	74.6 ± 3.3 ^a (n = 4)	21.5 ± 4.1 ^a (n = 4)	3.9 ± 1.0 ^a (n = 4)
2	Lithogenic	2.3 ± 0.6 ^a (n = 4)	59.2 ± 6.6 ^b (n = 4)	28.4 ± 5.0 ^a (n = 4)	12.5 ± 3.0 ^b (n = 4)
3	Lithogenic + 0.5% ethanolic extract	2.0 ± 0.5 ^a (n = 4)	44.1 ± 10.3 ^b (n = 4)	46.0 ± 9.8 ^b (n = 4)	10.0 ± 4.7 ^{a,b} (n = 4)

Values are given as mean ± s.d. Different letters in the same column indicate a statistical difference at $P < 0.05$.

deoxycholate in the animals fed the lithogenic diet (Group 2). The addition of the ethanolic extract increased the proportion of chenodeoxycholate ($P < 0.05$) and reduced that of cholate ($P < 0.05$) and deoxycholate (not significant). As expected, the proportion of glycine conjugates was greater in the animals on the semi-purified diet.

Table 4 shows the results with respect to serum lipids. The animals fed the lithogenic diet (Group 2) had the highest cholesterol concentrations. Animals that received the ethanolic extract or the aqueous extract (Groups 3 and 4) had significantly lower serum cholesterol concentrations compared with Group 2; these concentrations were not significantly different compared with the control group (Group 1).

The lithogenic diet induced the highest concentrations of HDL cholesterol; the addition of *L. tridentata* extracts produced values intermediate between the lithogenic diet and the control diet (differences were not significant). The proportion of HDL cholesterol to total cholesterol was increased 10% by the lithogenic diet compared with

the control group and was not modified by the addition of extracts.

The serum triglyceride concentration was slightly higher in Group 2, however it was not significantly different compared with the control group. The addition of extracts induced triglyceride values similar to that in the control group.

Gallstone elimination assay

After 6 weeks on the lithogenic diet, the animals developed a high incidence of cholesterol gallstones (Group B). Feeding the ethanolic extract to hamsters previously on the lithogenic diet for 6 weeks (Group D) caused a significantly lower incidence of gallstones. In these assays, gallbladder bile was analysed instead of hepatic bile. The results show that the reduced incidence of gallstones is associated with a decrease in moles percent cholesterol (mainly due to a lower biliary cholesterol concentration) and a slight increase in total bile acids (Table 5).

Table 4 Serum lipids in hamsters fed a lithogenic diet with or without *Larrea tridentata* extracts

Group	Diet	Cholesterol total (mg dL ⁻¹)	Cholesterol HDL (mg dL ⁻¹)	Triglycerides (mg dL ⁻¹)
1	Purina	105 ± 4 ^a (n = 12)	68 ± 2 ^a (n = 12)	100 ± 8 ^a (n = 12)
2	Lithogenic	179 ± 13 ^b (n = 12)	131 ± 6 ^b (n = 12)	133 ± 16 ^a (n = 12)
3	Lithogenic + 0.5% ethanolic extract	138 ± 23 ^{a, c} (n = 7)	108 ± 7 ^{a, b} (n = 7)	102 ± 27 ^a (n = 7)
4	Lithogenic + 1% aqueous extract	139 ± 8 ^c (n = 12)	106 ± 4 ^{a, b} (n = 12)	106 ± 38 ^a (n = 10)

Values are given as mean ± s.d. Different letters in the same column indicate a statistical difference at $P < 0.05$.

Table 5 Effect of the ethanolic extract of *Larrea tridentata* on gallstone frequency and gallbladder bile composition in hamsters previously fed a lithogenic diet

Group	Diet		Gallstone frequency	Cholesterol (mM)	Bile salts (mM)	Phospholipids (mM)	Moles percent cholesterol
	0–6 weeks	6–12 weeks					
A	Purina	Purina	0/9 ^a	0.66 ± 0.22 ^a (n = 9)	19.9 ± 4.0 ^a (n = 9)	4.40 ± 1.06 ^a (n = 9)	2.69 ± 0.90 ^a (n = 9)
B	Lithogenic	–	9/9 ^b	1.96 ± 0.75 ^b (n = 6)	24.3 ± 15.5 ^{a, b} (n = 6)	3.36 ± 0.88 ^a (n = 6)	8.44 ± 5.21 ^b (n = 6)
C	Lithogenic	Lithogenic	8/9 ^b	2.08 ± 0.33 ^b (n = 7)	27.7 ± 8.7 ^{a, b} (n = 7)	3.35 ± 0.67 ^a (n = 7)	6.71 ± 1.77 ^b (n = 7)
D	Lithogenic	Lithogenic + 0.5% ethanolic extract	2/9 ^a	1.11 ± 0.41 ^a (n = 9)	32.8 ± 8.6 ^b (n = 9)	3.37 ± 0.69 ^a (n = 9)	2.98 ± 0.92 ^a (n = 9)

Values are given as mean ± s.d. Different letters in the same column indicate a statistical difference at $P < 0.05$.

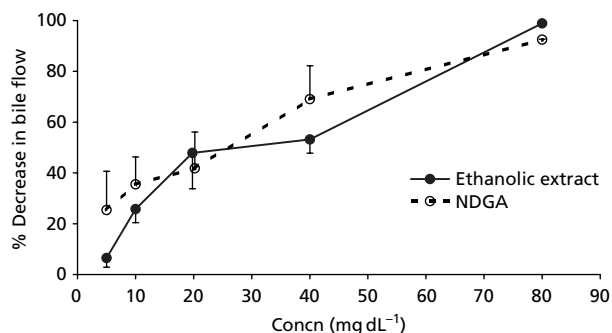


Figure 1 Cholestatic effect of an ethanolic extract of *Larrea tridentata* and nordihydroguaiaretic acid (NDGA) on perfused liver in hamsters. After a 20-min stabilizing period, liver was perfused with medium containing the ethanolic extract or NDGA for 10 min and the effect on bile secretion was determined gravimetrically. Data are the mean \pm s.e., $n = 4$.

Bile secretion on perfused liver

Both the ethanolic extract and NDGA showed a dose-dependent cholestatic effect when perfused in the hamster liver for 10 min (Figure 1), with an EC₅₀ of 34 and 28 mg dL⁻¹, respectively. This effect was reversible with concentrations up to 40 mg dL⁻¹.

Discussion

The prevention and elimination assays showed that the ethanolic extract of *L. tridentata* is effective in reducing the incidence of cholesterol gallstone in hamsters fed a lithogenic diet. In traditional medicine, only the aqueous extract has been used in the treatment of cholesterol gallstone disease. In this study, the aqueous extract had no effect on gallstone formation in hamsters. This may be due to differences in species sensibility and/or the strength of the gallstone inducer. It is possible that humans are more sensitive to the *L. tridentata* extract than hamsters. It is also possible that the lithogenic diet used in the present study is a very strong gallstone inducer and that the aqueous extract, with an expected much lower concentration of NDGA and flavonoids, was ineffective in the animal model used.

The ethanolic extract was added to the lithogenic diet at the 0.5% level, whereas the aqueous extract was added at the 1% level; therefore the animals ingested approximately 400 and 800 mg kg⁻¹ per day, respectively. For the aqueous extract, this dose is 8 times greater than a 70-kg person would ingest drinking 1 L of tea made with 40 g L⁻¹ of leaves and twigs. Despite the high dose, the aqueous extract was well tolerated by the animals and even showed a hepatoprotective effect (mean liver weight in this group was similar to that of controls) and improved plasma lipid profile. The ethanolic extract was less well tolerated and three hamsters died within the first 2 weeks of the experiment.

The effect of the ethanolic extract on gallstone formation and dissolution may be due to the lignan NDGA. NDGA is the most abundant metabolite present in the resin of

L. tridentata and is quite soluble in alcohol and slightly soluble in hot water. The ethanolic extract of this plant has been reported to contain up to 26% NDGA. However, the resin also contains a great number of secondary metabolites, including, among others, six other lignans and 19 flavonoid aglycones (Mabry & Bohnstedt 1981; Abou-Gazar et al 2004), and any of these could be the active compound. It is also possible that more than one compound acting synergistically or through potentiation could be responsible for the effects found.

The effect of the ethanolic extract was associated with a reduction of the moles percent cholesterol, which favours cholesterol 'solubility' in bile. This was mainly due to increases in biliary phospholipids and bile salts, and a decrease in cholesterol, although the differences were not significant compared with the lithogenic control. In the elimination assay, gallbladder bile was analysed, which is more concentrated and where gallstones are formed. In this bile, more pronounced reductions in the cholesterol concentration and moles percent cholesterol were found.

Hamsters that received the ethanolic extract showed an increase in chenodeoxycholate and a reduction of deoxycholate; both changes are known to favour a less cholesterol-saturated bile, indicated by a decrease in the moles percent cholesterol. The first of these bile acids has been used for the dissolution of gallstones, and the latter is known to increase cholesterol secretion and gallstone formation (Hofmann 1999). It is proposed that the modification of the bile acid profile by the ethanolic extract is the reason for the reduction in the moles percent cholesterol, and this, in turn, is the reason for the gallstone prevention and elimination.

The mechanism by which bile acid profiles in bile are affected by *L. tridentata* remains to be established. Deoxycholate is produced in the colon and caecum from cholate by bacterial dehydroxylation. The alteration of intestinal flora by the known antibiotic activity of *L. tridentata* (Mabry & Bohnstedt 1981) could explain the decrease in deoxycholate concentration. The altered proportions of the primary bile acids, chenodeoxycholate and cholate, must be an effect on the liver where they are synthesized. In relation to serum lipids, it is noteworthy that both extracts exhibited a hypocholesterolaemic effect. This suggests that the action exerted by the extracts on bile is independent from that exerted on plasma.

The cholestatic effect exhibited by the ethanolic extract of *L. tridentata* and NDGA in the perfused liver confirms their hepatotoxicity (Sheik et al 1997). However, it was shown that the effect is reversible at low levels (5–40 mg dL⁻¹). Since the dose–effect curves are very similar, it seems that the cholestatic effect of *L. tridentata* is due to NDGA. However, when orally administered, NDGA is oxidized by catalase to an ortho-quinone form in the ileum and caecum before being absorbed (Evan & Gardner 1979). Although this form is nephrotoxic, it remains to be established whether this oxidation modifies the cholestatic effect of NDGA found in the perfused liver. The determination of blood concentrations of NDGA or its metabolite in hamsters fed a lithogenic diet and in humans drinking chaparral tea could help to establish therapeutic and toxic doses.

Conclusions

The ethanolic extract of *L. tridentata* prevents cholesterol gallstone formation and eliminates cholesterol gallstones in hamsters fed a high carbohydrate, fat-free lithogenic diet. The prevention and elimination of cholesterol gallstones by the ethanolic extract was associated with a significant reduction in the moles percent cholesterol, which favours cholesterol 'solubility' in bile. The extract also alters the bile acid composition of bile, with an increase in chenodeoxycholate and a reduction of deoxycholate. A cholestatic effect, reversible at low doses, of both the ethanolic extract and NDGA was found, confirming their hepatotoxicity. The results suggest that *L. tridentata* could be useful in cholesterol gallstone treatment, however care must be taken in its use.

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