Antioxidant Evaluation of *Eysenhardtia* Species (Fabaceae): Relay Synthesis of 3-O-Acetyl-11α,12α-epoxy-oleanan-28,13β-olide Isolated from *E. platycarpa* and Its Protective Effect in Experimental Diabetes

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The antioxidant activities of plant extracts from *Eysenhardtia platycarpa*, *E. punctata*, and *E. subcoriacea* (Fabaceae) species, used in Mexican traditional medicine for the treatment of diabetes complications, were analyzed in a rat pancreas homogenate model. Methanolic extracts of *E. platycarpa*, *E. punctata*, and *E. subcoriacea* protected the pancreatic homogenate from 2,2-azo-bis(2-aminodipropyl)dihydrochloride (AAPH)-induced damage. The inhibitory effect was dose-dependent at concentrations of 10—1000 ppm and correlated with 1,1 diphenyl-2-picrylhydrazyl (DPPH) radical scavenger capacity. 3-O-Acetyl-11α,12α-epoxy-oleanan-28,13β-olide (1, EC50 = 21.2 ± 2.2 μM), (+)-catechin (2, EC50 = 7.4 ± 1.1 μM), and (+)-catechin 3-O-β-D-galactopyranoside (3, EC50 = 11.5 ± 1.5 μM), natural constituents isolated from the branches of *E. platycarpa*, displayed significant antioxidant activity. Compounds 1 and 2 significantly increased (p<0.001) the pancreatic glutathione (GSH) concentration alone and in combination with AAPH treatment. Compound 1 was obtained from oleanolic acid by relay synthesis via acetylation, bromo-lactonization, dehydrobromination, and oxidation, and its antioxidant effect was evaluated on streptozotocin (STZ)-induced diabetic rats. On its own, 1 at a dose of 100 mg/kg b. wt. (i.p.) for 5 d significantly increased the activities of glutathione peroxidase (GSHPs) and catalase (CAT). Simultaneous treatment of 1 (100 mg/kg b. wt.) and STZ significantly reduced the pancreatic triobarbituric acid reactive substances (TBARS) concentration together with a significant increase in the activities of GSHPs and CAT, preventing hyperglycemia induced by STZ after 5 d of treatment. The present study demonstrates the antioxidant and antihyperglycemic activities of compound 1 isolated from *Eysenhardtia* species used in traditional medicine.

**Key words** *Eysenhardtia platycarpa*; Fabaceae; 3-O-acetyl-11α,12α-epoxy-oleanan-28,13β-olide; glutathione; glutathione peroxidase; diabetes

Recently, attention has been focused on the relationship between reactive oxygen species (ROS) and several disorders including aging, various inflammatory diseases, carcinogenesis, neurodegenerative diseases, and diabetes.1) In fact, diabetes is usually accompanied by increased production of ROS2) and impaired antioxidant defense,3) indicating a central contribution of ROS to the onset, progression, and pathological consequences of diabetes. There is considerable evidence that chronic hyperglycemia is the proximate cause of retinopathy, kidney failure, neuropathies, and macrovascular diseases in diabetes.4) In addition, it has been demonstrated that β cells are particularly susceptible to oxidative damage.4) Therefore, as hyperglycemia worsens, β cells steadily deteriorate, secrete less insulin, and participate in a downward spiral of loss of pancreatic functions.

The current treatments of diabetes are based on a combination of diet, oral hypoglycemic agents, and insulin intake, which focus on decreasing the blood glucose level.5) Although some oral hypoglycemic agents have been associated with enhancement of the antioxidant system,6) they do not prevent the gradual loss of β-cell functions, which is known to account for gradual deterioration of glucose homeostasis. Therefore, the treatment of micro- and macrovascular complications has required the combined use with several herbal preparations and dietary supplements.7) Many natural products8)—9) and medicinal plants3) have been shown to significantly reduce oxidative stress and increase the antioxidant endogenous system, which represent a important property of plant medicines used for the treatment of several diseases including diabetes. In this sense, approximately 306 species from 235 genera and 93 families have been used empirically as hypoglycemic agents in Mexican traditional medicine.10)

*Eysenhardtia platycarpa*, *E. subcoriacea*, and *E. punctata*, known indiscriminately as ‘palo dulce’ (sweet wood), are traditionally used for the treatment of bladder infections and kidney diseases related to diabetes.11) *Eysenhardtia* is a small genus that comprises 14 species indigenous to North and Central Mexico.12) Previous studies on *Eysenhardtia* species have highlighted the hypoglycemic activity of *E. polyacantha*, the antihyperglycemic activity of *E. platycarpa*, as well as the isolation of cytotoxic flavonoids13,14) and 3-O-acetyloleanolic acid [which displayed antihyperglycemic activity in streptozotocin (STZ)-induced diabetic rats15)]

As part of our screening of plants for the isolation of substances with antioxidant and antihyperglycemic potential,14,17) this study was conducted to (A) assess the antioxidant effects of extracts from *E. platycarpa*, *E. punctata*, and *E. subcoriacea*, as well as of compounds 1—8, natural constituents isolated from *E. platycarpa* (Chart 1), in a rat pancreas model and in a 1,1 diphenyl-2-picrylhydrazyl (DPPH) radical scavenger assay; (B) synthesize 1, which displayed a protective effect against 2,2-azo-bis(2-aminodipropyl)dihydrochloride (AAPH)-induced damage in pancreatic homogenate; and (C) evaluate the protective effect of 1 in STZ-induced diabetic rats.

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MATERIAL AND METHODS

**Chemicals** AAPH, DPPH, sodium dihydrogen phosphate monohydrate, anhydrous sodium hydrogen phosphate, trichloroacetic acid (TCA), EDTA, α-tocopherol, xanthine, diethylenetriamine penta-acetic acid (DETAPAC), disodium salt of bathocuproine-disulphonic acid (BDA), bovine serum albumin, nitrotetrazolium blue (NTB), sodium carbonate, nitrate, sodium dihydrogen phosphate, and trichloroacetic acid (TCA) were purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.).

**Apparatus** Compound isolated from *E. platycarpa* and intermediates of the relay synthesis of compound 1, were purified by vacuum column chromatography performed on Merck Kiesel gel 60 (0.040—0.863 mm) and the eluent is specified in each experiment. Analytical and preparative TLC were conducted on precoated Alugram Sil G/UV 254 silica gel plates using both a 254-nm UV lamp and by ceric ammonium sulfate/H₂SO₄ spray reagent. Melting points were measured on a Fisher–John melting point apparatus. Infrared spectra were recorded with Perkin-Elmer 283B instruments.

**Plant Material** Plants were selected following the reported information and focusing on Fabaceae species used in Mexican traditional medicine, particularly those belonging to *Eysenhardtia*. *E. platycarpa* PELL & SAFFORD ex PENNELL & S Afford (1325), *E. punctata* PENNELL (88577) and *E. subcoriacea* PENNELL (11322) were collected in Tepic, Guerrero; Huatulca, Morelos; and Ixmiquilpan, Hidalgo, respectively. Voucher specimens (in parenthesis) are kept in the Herbarium of Facultad de Ciencias of the Universidad Nacional Autónoma de México.

**Extraction and Isolation** The plant material (quantities detailed in Table 1) was dried, ground to powder and extracted (3×51) successively with n-hexane, CH₂Cl₂ and MeOH at room temperature. The solvents were removed under reduced pressure to obtain the corresponding residues. The extracts were stored dry at 4°C in amber recipients.

General experimental procedures detailed for the extraction, isolation, purification and structure elucidation of 1, 4—8, isolated from the methanolic extract of branches of *E. platycarpa* (Chart 1) are described previously. The fractionation of methanolic extract of bark from *E. platycarpa*, was monitored following the antioxidative effect in the rat pancreas model. Methanolic extract (25.2 g, *EC*_5₀ = 21.6±2.9 ppm) of *E. platycarpa* was suspended in EtOAc to yield a soluble (4.9 g, *EC*_5₀ = 18.3±1.1 ppm) and insoluble (19.8 g) fractions. The soluble fraction was subjected to vacuum column chromatography (5×17 cm) with n-hexane/EtOAc/MeOH/H₂O gradient to give eight fractions: fr. 1 (n-hexane 100%, 175 mg), fr. 2 (EtOAc/n-hexane = 1:9, 241 mg), fr. 3 (EtOAc/n-hexane = 3:7, 206 mg), fr. 4 (EtOAc/
General Synthetic Procedure for 3-O-Acetyloleanolic Acid (6) Compound 5 (1 g, 2.19 mmol, previously obtained from other natural sources in our laboratory) was treated with acetic anhydride (10 ml) and pyridine (10 ml) at room temperature for 24 h. After this

Figure 1. Synthesis of 3-O-Acetyl-11α,12α-epoxy-oleanan-28,13β-olide (1)
time, 10 ml of water was added to the reaction mixture and was conventionally treated. The crystallization with CH₂Cl₂ afforded a solid (2.10 mmol, 98%) of 6 with mp 214—215 °C. IR (CHCl₃) cm⁻¹: 3514, 2950, 2877, 1722, 1695, 1638, 1371, 1027.²³

3-O-Acetyl-oleanan-12α-bromo-28,13β-olide (9) Compound 6 (1 g, 2.006 mmol) in CH₂Cl₂ was treated with a solution of bromine (1.20 g, 7.62 mmol) in MeOH (50 ml) at room temperature. After 15 min 50 ml of water was added. The mixture was extracted with CH₂Cl₂, and the organic phase was washed with NaHSO₃ (5%), brine and dried (Na₂SO₄). Elimination of the solvent yielded 9 (1.13 g, 1.96 mmol) as crystalline solid. mp 194—196 °C; yield 97%. IR (CHCl₃) cm⁻¹: 2958, 2937, 1762, 1721, 1468, 1389, 1369, 1301, 1257.²³

3-O-Acetyl-oleanan-11-en-28,13β-olide (10) Compound 9 (1 g, 1.72 mmol) was refluxed with DBU (7.6 ml, 0.05 mmol) in o-xylene (15 ml) for 18 h at 170 °C. The mixture of reaction was cooled and 20 ml of water was added. The mixture was extracted with CH₂Cl₂ and washed with HCl (5%), NaHCO₃ and brine. Elimination of solvent under reduced pressure afforded 10 (665 mg, 1.34 mmol, 78%) as colourless powder. mp 226—227 °C. IR (CHCl₃) cm⁻¹: 3053, 2953, 1754, 1720, 1639, 1390, 1368, 1320, 1299, 1257, 1139, 1084.²³

3-O-Acetyl-11α,12α-epoxy-oleanan-28,13β-olide (1) Compound 10 (1 g, 2.01 mmol, obtained from several repetitions of the above described reactions), was dissolved in CH₂Cl₂ (25 ml) and was cooled in ice bath. A mixture of sample following the protocol above described. 20) The reaction of a solution of STZ and NaHCO₃ was conventionally treated. The crystallization with CH₂Cl₂ afforded a solid (2.10 mmol, 98%) of 9 as colourless powder. mp 226—227 °C. IR (CHCl₃) cm⁻¹: 161 (36.2), 119 (50), 43 (57).
untreated group (0.02±0.01 nmol MDA/mg protein). The methanolic extracts of branches, leaves, and bark from *E. subcoriacea* and *E. platycarpa* showed higher antioxidant effects in pancreatic homogenate compared with the other extracts. For *E. punctata*, only the methanolic extracts from branches displayed antioxidant activity. In addition, CH$_2$Cl$_2$ extracts of branches and bark from *E. subcoriacea* and of branches from *E. punctata* and the n-hexane extract of bark from *E. subcoriacea* were found to inhibit lipid peroxidation. These extracts displayed DPPH radical scavenging effects in dose-dependent manners.

**Antioxidant and DPPH Radical Scavenger Activities and Effects on Pancreatic GSH Concentration of Compounds 1—8** The natural constituents 1—8 (Chart 1) were isolated from *E. platycarpa*. Compounds 4—8 did not display biological activity. Compounds 2 and 3 displayed potent antioxidant and DPPH radical scavenger effects, whereas 1 displayed antioxidant but not DPPH radical scavenger activity (Table 2). Quercetin and α-tocopherol (used as references) displayed both biological activities. Treatment of pancreatic homogenate with AAPH caused a marked decrease (p<0.001) in pancreatic GSH levels (0.32±0.06 μg GSH/mg protein) compared to the untreated group (0.89±0.15 μg GSH/mg protein). Compound 3 did not display an effect upon pancreatic GSH levels. Compounds 1 and 2 at 30 μM significantly increased the pancreatic GSH level alone (1.35±0.18 and 1.04±0.22 μg GSH/mg protein, respectively) and in combination with AAPH (1.13±0.17 and 1.01±0.14 μg GSH/mg protein, respectively) compared to the untreated group.

**Relay Synthesis of 1** Compound 1 was obtained from oleoanolic acid via a series of chemical transformations that are outlined in Chart 2. Oleoanolic acid (5) was acetylated to obtain 3-O-acetyl-oleoanolic acid (6, 98%). Bromination of 6 in CH$_2$Cl$_2$ afforded 3-O-acetyl-12α-bromo-oleanolate (9, 97%). Dehydrobromination of 9 afforded Δ$^{11}$-3-O-acetoxy-oleanolate (10, 78%). Physical and spectroscopic properties of 6, 9, and 10 were quite similar to those previously reported. Exposure of 10 to H$_2$O$_2$-glacial acetic acid (CH$_2$Cl$_2$, room temperature, for 4 h) gave 3-O-acetyl-11α,12α-epoxy-olean-28,13β-olide (1, 74%).

**Antioxidant Effect of 1 on STZ-Induced Diabetic Rats** The administration of 1 to normal rats for 5 d significantly decreased the serum glucose concentration (4.7±0.21 mM), compared to the untreated group (6.1±0.25 mM). STZ treatment produced a significant increase (18.6±0.89 mM) in serum glucose with respect to the untreated group. Treatment with 1 significantly reduced (7.9±0.41 mM) the increased serum glucose concentration induced by STZ after a period of 5 d (Fig. 1). Compound 1 alone did not induce any change in pancreatic TBARS levels (Fig. 2). STZ treatment significantly increased the TBARS levels. Immediately after diabetes induction with STZ, treatment with 1 for 5 d reduced
the production of pancreatic TBARS.

STZ treatment significantly increased SOD and GSHPx activities but decreased CAT activity (Figs. 3–5) compared to the untreated control. The administration of 1 alone to normal rats for 5 d did not vary the pancreatic activity of SOD enzyme in comparison to the untreated group, but GSHPx and CAT activities were significantly increased (*p < 0.001). Treatment with STZ and 1 for 5 d significantly decreased the pancreatic SOD activity with respect to diabetic rats treated with STZ alone. In contrast, treatment with STZ and 1 increased the GSHPx and CAT activities compared to treatment with STZ alone.

DISCUSSION

Free radicals and other reactive species are considered to be important causative factors in various diseases including diabetes.1) This relationship has led to considerable interest in the search for antioxidants to scavenge free radicals and boost defense systems. Therefore, natural antioxidants have attracted attention, and great efforts have been made to analyze the relationship between the antioxidant activity and the antihyperglycemic effect of plant extracts and plant products using several experimental models of diabetes.3) For example, Maritim et al.30) discussed how increased TBARS associated with diabetes is prevented by treatment with some compounds if the treatment is given before or immediately after the diabetogen. Also, several studies3) using in vivo models of diabetes have demonstrated that some plant extracts belonging to the Fabaceae family decreased oxidative stress and enhanced the activities of several components of the endogenous antioxidant system, in particular GSH, vitamins C and E, SOD, GSHPx, and CAT. However, chemical studies of these extracts have allowed the isolation of only a few bioactive constituents. The present study differs from others in that we specifically measured the lipid peroxidation inhibitory effects of extracts in a rat pancreas model induced by AAPH. With this model we could assess the antioxidant effects of extracts from three plants and of constituents isolated from E. platycarpa, and these activities could be corroborated using an in vivo model.

AAPH is a water-soluble azo compound that is used extensively as a free radical generator, often in the study of lipid peroxidation and in the characterization of antioxidants.31) Decomposition of AAPH produces molecular nitrogen and two carbon free radicals. The carbon radicals may combine to produce stable products or react with molecular oxygen to produce peroxyl radicals. After treatment of pancreatic homogenate with AAPH, the observed increased TBARS and decreased GSH levels suggested persistent oxidative stress. The low levels of GSH are probably due to increased utilization of GSH by cells in an attempt to counteract the increased formation of lipid peroxides. The decreased pancreatic GSH levels obtained upon treatment of pancreatic homogenate with AAPH, the observed increased TBARS and decreased GSH levels suggested persistent oxidative stress. The low levels of GSH are probably due to increased utilization of GSH by cells in an attempt to counteract the increased formation of lipid peroxides. The decreased pancreatic GSH levels obtained upon treatment of pancreatic homogenate with AAPH are consistent with previous reports and reflect the utilization of GSH by pancreatic cells for countering the damage induced by radicals formed by peroxidation.32,33)

Treatment with extracts of E. platycarpa, E. punctata, and E. subcoriacea displayed a protective effect against AAPH-induced damage, indicating a strong antioxidant activity that may be partially responsible for many of the biological properties manifested by these species. E. subcoriacea extracts were the most active as antioxidant agents, followed by E. platycarpa and E. punctata extracts. The methanolic extracts of bark from E. subcoriacea and from E. platycarpa were the
most active against DPPH radicals (Table 1). Piao et al.\textsuperscript{34} demonstrated that organic extracts and some flavonoids from \textit{Sophora flavescens} (Fabaceae), which displayed DPPH radical scavenger activity, recovered cell viability decreased by AAPH treatment. The antioxidant activity displayed by extracts could be due to the fact that the extracts reduced the peroxyl formed by AAPH during the initiation of lipid peroxidation, preventing lipid peroxidation from occurring. However, the methanolic extract of branches of \textit{E. platycarpa} displayed a relatively small radical scavenging activity but a potent antioxidant activity, suggesting additional mechanisms responsible for the antioxidant effect.

We have demonstrated previously that the methanolic extract of branches from \textit{E. platycarpa} displayed antihyperglycemic and hypoglycemic activities, and chemical analysis of this extract allowed the isolation of compounds 1 and 4—8.\textsuperscript{14} The present results showed antioxidant effects of 3-O-acetyl-11\textalpha,12\textalpha,\textbeta,13\textbeta-epoxy-oleanan-28,13\textbeta-olide (1), (\textplus/)-catechin (2), and (\textplus/)-catechin 3-O-\textbeta-D-galactopyranoside (3). In addition, 2 and 3 displayed both DPPH radical scavenger and antioxidant activities, indicating that the biological effect is based on the radical scavenger capacity. Compound 1 displayed a remarkable antioxidant activity but no radical scavenger activity. The observed increase in pancreatic GSH levels caused by 1 or 2 alone or in combination with AAPH suggested promotion of the GSH cycle, mainly the activity of GRd and/or GSHPx, which could be the mechanism responsible for the antioxidant activity. Quine and Raghu\textsuperscript{39} showed that intraperitoneal administration of (\textminus/)-epicatechin at doses of 15 and 30 mg/kg to STZ-induced diabetic rats for a period of 35 d resulted in significant decreases in blood glucose and TBARS and significant increases in the concentrations of GSH and antioxidant enzymes. In addition, Du and Ming\textsuperscript{35} observed increased serum GSH in rats induced by oleic acid (5).

To investigate the antioxidant activity of 1 in STZ-induced diabetic rats, this substance was synthesized from oleic acid (5) via a series of chemical transformations: acetylation, bromination, bromo-lactonization, and oxidation. Using CH\textsubscript{2}Cl\textsubscript{2} instead of MeOH in the bromo-lactonization significantly improved the yield (from 63 to 97%). Dehydrobromination of 9 afforded \textDelta\textsuperscript{11,13}-O-acetyl-oleanolide (10, 78%). The total yield of the reaction sequence 5→6→9→10→1 was 54% (compared to the 28% yield previously reported).\textsuperscript{22}

In this study, the main finding was that 1 protected against to STZ-induced damage and increased the pancreatic activities of antioxidant enzymes. Also, compound 1 displayed hypoglycemic activity in normal rats (Fig. 1). In this context, we previously demonstrated the antihyperglycemic activity of 3-O-acetyloleanolic acid (6, 31 mg/kg b. wt. oral) in STZ-induced diabetic rats.\textsuperscript{14} Likewise, Shaiq \textit{et al.}\textsuperscript{36} demonstrated that oleic acid and some of its derivatives inhibit \textalpha/-glucosidase; therefore, the hypoglycemic effect of 6 and 1 could be due to this mechanism.

Intraperitoneal administration of STZ to male rats increased food consumption and decreased body weight after 5 d of induction of diabetes due to excessive breakdown of tissue proteins. Also, treatment with STZ produced a sustained increase in serum glucose concentration together with an increase in pancreatic TBARS, suggesting that lipid peroxidation occurs well after induction of hyperglycemia. Kakkar \textit{et al.}\textsuperscript{25} showed a major increase in TBARS in the pancreas compared with the liver, suggesting a low capacity for the antioxidant system in the pancreas. It has been suggested that STZ causes cytotoxic effect probability increasing the production of H\textsubscript{2}O\textsubscript{2} and ·OH in \textbeta/-cells.\textsuperscript{37} As a result of STZ effects, \textbeta/ cells undergo destruction by necrosis and the production of insulin is decreased.

Simultaneous treatment with 1 (100 mg/kg) for 5 d significantly reduced the elevated serum glucose concentration (Fig. 1) and TBARS levels (Fig. 2) induced by STZ. The effects of 1 on serum glucose and pancreatic lipid peroxidation in STZ-induced rats may be correlated with the significant rise in pancreatic GSH induced by 1 in the \textit{in vitro} model (Table 2). Therefore, 1 may enhance the GSH/GSSG ratio to improve both the antioxidant system and serum glucose regulation, which is consistent with the observed antiadipic effect. Paolisso \textit{et al.}\textsuperscript{38} have proposed that the ratio of GSH/GSSG plays a critical role in the glucose homeostasis of diabetes. It has been suggested that thiol groups are important in the intracellular and membrane redox states of \textbeta/-pancreatic cells and thus influence their secretory function.

In addition, we found that the administration of STZ increased the activity of SOD and GSHPx and decreased the activity of CAT after 5 d of induction of diabetes, indicating persistent oxidative stress. The glutathione system, SOD, GSHPx, GRd, and CAT comprise the most important endogenous antioxidant defense against ROS-induced damage of the cell membrane. SOD protects tissues from oxygen free radicals by catalyzing the removal of O\textsuperscript{2-}. GSHPx and CAT were shown to be responsible for the detoxification of significant amounts of H\textsubscript{2}O\textsubscript{2}. Increased activities of antioxidant enzymes have been demonstrated in some organs of diabetic rats,\textsuperscript{9,25} possibly as a defense mechanism against the production of ROS induced by STZ. In addition, studies of Kakkar \textit{et al.}\textsuperscript{25} have shown decreased SOD, GSHPx, and CAT activities after 20 d of STZ treatment in rats, suggesting an impaired antioxidant defense. In the present study, the increased SOD activity (Fig. 3) could be due to its induction by increased production of O\textsuperscript{2-}. In addition, it has been proposed that O\textsuperscript{2-} is an inhibitor of CAT but not of GSHPx.\textsuperscript{39} The increase in GSHPx activity (Fig. 4) reflects the production of H\textsubscript{2}O\textsubscript{2} by SOD in diabetic tissues and suggests its induction by both organic and inorganic peroxides. On its own, 1 did not vary the pancreatic activity of the SOD enzyme during the period of study, but 1 significantly increased the GSHPx and CAT activities (Fig. 5). Simultaneous treatment with STZ and 1 also produced significant increases in GSHPx and CAT activities. The enhanced activities of the antioxidant enzymes promoted by 1 protect against STZ-induced damage; therefore, hyperglycemia does not develop. Our results show that GSHPx plays a primary role in minimizing oxidative damage. The protection against lipid peroxidation offered by GSHPx and the effect of 1 on this enzyme appear to be relevant responses to ROS-induced membrane damage.

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