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(*Z*)-3-Butylidenephthalide from *Ligusticum porteri*, an α-Glucosidase Inhibitor

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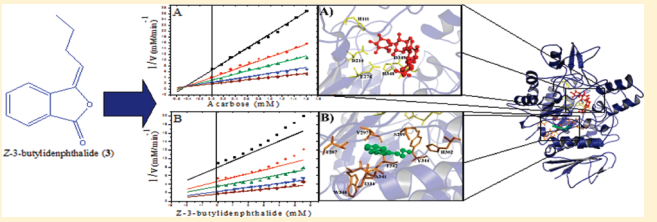
Supporting Information

ABSTRACT: An extract from the roots of *Ligusticum porteri*, orally administered to groups of normal and diabetic mice, showed significant hypoglycemic and antihyperglycemic effects. Experimental type-II DM was achieved by treating mice with streptozotocin 15 min after an injection of β-nicotinamide adenine dinucleotide. (*Z*)-6,6′,7,3′α-Diligustilide (**1**), (*Z*)-ligustilide (**2**), 3-(*Z*)-butylidenephthalide (**3**), myristicin (**4**), and ferulic acid (**5**) were isolated from the active extract. When tested In Vivo, compounds **1**−**3** showed antihyperglycemic activity, with **3** being the most active. Compound **3** (56.2 mg/kg) decreased blood glucose levels in NAD-STZ-diabetic mice after an oral sucrose load, suggesting that its antihyperglycemic effect is due to inhibition of α-glucosidase at the intestinal level. Furthermore, **3** inhibited the activity of yeast-α-glucosidase (IC_{50} 2.35 mM) in a noncompetitive fashion with a K_i of 4.86 mM. Docking analysis predicted that **3** binds to the enzyme in a pocket close to the catalytic site, but different from that for acarbose, with a K_i of 11.48 mM. Compounds **1** and **2** did not affect α-glucosidase In Vivo, but altered glucose absorption by a mechanism yet to be determined. The stimulatory effect of **5** on insulin secretion, present in high amounts in the extract, has been demonstrated in previous investigations. The present study provides scientific support of the use of *L. porteri* in Mexican folk medicine for the treatment of diabetes.



Type-II diabetes mellitus (DM) is a chronic disease associated with abnormally high blood glucose levels (hyperglycemia) due to defects in insulin secretion, or action, or both. Hyperglycemia provokes protein glycation, which cause tissue damage and devastating complications including heart and peripheral vascular complaints, retinopathy, nephropathy, and neuropathy.[1,2]

According to the World Health Organization, at least 2.8% of the world population suffered from type-II DM in 2000. The incidence of this illness is increasing rapidly, and it is estimated that by 2030 this figure will almost double. Type-II DM occurs throughout the world, but is more common in developed countries. The greatest increase in prevalence is, however, expected to occur in developing countries, where most patients will probably be found by 2030.[1]

There is currently no cure for diabetes, but the condition can be managed so that patients can live a relatively normal life. Treatment of diabetes focuses on two goals: keeping blood glucose within the normal range, thus preventing the development of long-term effects, and changes in diabetic patients' lifestyle. Oral medications are available to lower blood glucose in type-II diabetics, but most of these products possess side effects after prolonged use.[3] In consequence, the search for new therapeutic agents for treating type-II DM, including plants used in folk medicine, has increased notably in recent years.[4]

Mexico is rich in medicinal plants highly prized by the population for the treatment of diabetes, and according to a recent review, there are about 306 species from 235 genera and 93 families used as hypoglycemic agents. *Ligusticum porteri* Coult. & Rose (Apiaceae) is one of these.[4] Roots and leaves from this herb, commonly referred to as "osha", Porter's licorice-root, or "chuchupate", are employed to treat inflammatory, respiratory, gastrointestinal, and infectious complaints.[5−7] More recently, its use as an antidiabetic drug has been described.[4] The essential oil (100 μg/mL) prepared from the roots increased the antimicrobial activity of norfloxacin against a norfloxacin-resistant strain of *Staphylococcus aureus*.[8] Ethanolic and aqueous extracts did not affect the growth of human MCF-7/AZ breast or HCT8/E11 colon cancer cells.[9] A CH_2Cl_2−MeOH (1:1) extract of roots of *L. porteri* exhibited a significant antinociceptive effect, as detected by the writhing test in mice.[10] On the other hand, toxicity studies of the same extract according to the Lorke[11] and Ames[12] procedures revealed that the plant was devoid of genotoxic effects and allowed calculation of a medium lethal dose (LD_{50}) of 1 g/kg for mice.[13]

Chemical investigation of *L. porteri* led to the isolation and identification of secondary metabolites such as phenylpropanoids, terpenoids, and several phthalides including (*Z*)-6,6′,7,3′α-diligustilide (**1**), (*Z*)-ligustilide (**2**), and (*Z*)-3-butylidenephthalide (**3**).[14−19]

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Table 1. Effect of Compound 3 and Crude Extract of the Roots of *L. porteri* on Blood Glucose Levels in STZ-Diabetic Mice after an Oral Load of Sucrose (2 g/kg)[a]

		blood glucose concentration				
test sample	dose, mg/kg	0 h	0.5 h	1 h	1.5 h	2 h
vehicle		155 ± 4.7	236 ± 9.4(56)	169 ± 14.0(11.4)	163 ± 4.9(6.1)	145 ± 6.0(−4.3)
acarbose	5	153 ± 7.4	152 ± 8.3(−0.4)[b]	157 ± 7.9(0.5)[b]	159 ± 6.5(6.2)	158 ± 6.3(6.8)
extract	100	143 ± 3.7	194 ± 6.8(35)[b]	173 ± 2.1(21)	170 ± 6.0(19)	161 ± 3.9(12)
3	10	152 ± 8.7	214 ± 12.0(41)	180 ± 13.8(19)	173 ± 11.9(16)	163 ± 15.4(6.7)
3	56.2	149 ± 5.0	184 ± 7.3(23)[b]	161 ± 6.1(7.6)	153 ± 6.9(2.5)	159 ± 6.8(6.4)

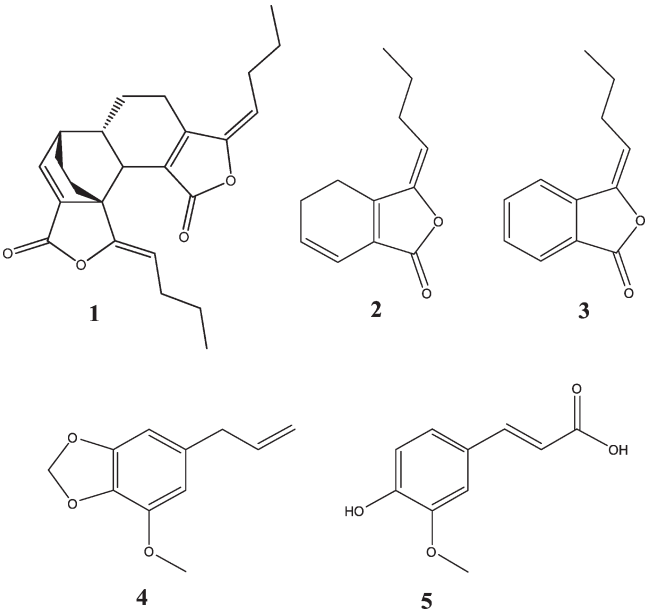
[a] Each value is the mean ± SEM for six mice in each group. Percent variations of glycemia are in parentheses. [b] $p < 0.05$ significantly different ANOVA followed by Dunnett's *t* test for comparison with respect to control group.

Since the pharmacological investigation of plants used in folk medicine could lead to the discovery of new antidiabetic agents and/or to the proposal of therapeutic alternatives for correcting impaired glucose homeostasis, the objectives of the present study were to ascertain if *L. porteri* had blood glucose lowering activity and to identify new α-glucosidase inhibitors that could effectively control postprandial glucose levels. Such control has been shown to be essential in preventing the progress of impaired glucose tolerance toward type-II DM by decreasing the incidence of hyperglycemia.

RESULTS AND DISCUSSION

The use of *L. porteri* in Mexican folk medicine prompted us to determine its efficacy as an antidiabetic agent using well-known animal models. First, an organic extract (56.2, 100, and 316 mg/kg) of the roots was tested using the oral glucose tolerance test (OGTT) in two sets of animals: normal and diabetic. Experimental type-II DM was achieved by treating mice with streptozotocin (STZ, 100 mg/kg) 15 min after an injection of β-nicotinamide adenine dinucleotide (NAD, 40 mg/kg).[20] This preliminary treatment with NAD provokes partial protection against the cytotoxic action of STZ by scavenging free radicals and causes only minor damage to pancreatic β-cell mass, creating a diabetic syndrome close to type-II DM. This model has recently proven to be a valuable tool for investigation of new antidiabetic agents.[20] In normal mice, the crude extract (Table S1, Supporting Information) did not affect the postprandial peak at all doses tested, but when assessed in NAD-STZ-diabetic mice, a significant decrease ($p < 0.05$) of the postprandial peak was observed (Figure S1 and Table S2, Supporting Information). The glucose-lowering effect (~45% at all doses tested) was noticed after 30 min and persisted throughout the experiment, suggesting that the extract was not only antihyperglycemic but also hypoglycemic. Next, in order to confirm the hypoglycemic action of the extract, its acute effect was evaluated in normal (Table S3, Supporting Information) and diabetic animals (Table S4, Supporting Information). The results revealed that the extract (56.2, 100, and 316 mg/kg) caused significant decreases in blood glucose levels only in NAD-STZ-diabetic mice when compared with vehicle-treated groups ($p < 0.05$). The effect of the extract was attained after 1.5 h and was maintained all through the experiment; in the case of glybenclamide (Gly), used as positive control, the initial effect was observed after 3 h of treatment and lasted throughout the experiment. Furthermore, the action of the lowest dose of extract tested was comparable to that of Gly. Finally, an oral sucrose tolerance test (OSTT), both in normal (Table S5, Supporting Information) and diabetic (Table 1) mice, was carried out. This test is usually performed to evaluate if the antihyperglycemic action of a drug involves inhibition of intestinal α-glucosidase; this enzyme reduces the rate of digestion of carbohydrates by preventing their immediate breakdown into monosaccharides, which then would be absorbed quickly into the bloodstream. Slowing the absorption of carbohydrates gives the β-cells in the pancreas more time to secrete adequate insulin to cover the meal.[21] The extract (31.6 and 56.2 mg/kg) lowered glucose levels in both diabetic and normal mice, revealing that inhibition of α-glucosidase was mediating the observed antihyperglicemic effect. The activity was compared with that of acarbose (5 mg/kg), a natural α-glucosidase inhibitor currently used to control blood glucose levels.

In order to determine the antidiabetic principle(s) of the plant, the active extract was subjected to chromatographic fractionation to yield compounds **1**−**5**. Phthalides **1**−**3** were identified by comparison of their spectroscopic and spectrometric data with those described previously,[16,22,23] and compounds **4** and **5** were identified by comparison with commercial authentic samples.



In the OGTT with normal mice, administration of compounds **1** (31.2 and 56.2 mg/kg) and **3** (10 and 20 mg/kg) displayed significant ($p < 0.05$) antihyperglycemic activity, provoking a decrease of the postprandial peak in comparison with the vehicle, whereas **2** (10 and 20 mg/kg), the most abundant

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Table 2. Effect of Compounds 1−3 from the Roots of *L. porteri* on Blood Glucose Levels in Normal Mice after an Oral Load of Glucose (1 g/kg)[a]

		blood glucose concentration				
test sample	dose mg/kg	0 h	0.5 h	1 h	1.5 h	2 h
vehicle		108 ± 5.3	172 ± 10.6(59)	109 ± 15.8(1.5)	110 ± 13.2(2.5)	94 ± 7.7(−12)
Gly	10	121 ± 7.0	71 ± 7.1(−42)[b]	43 ± 8.0(−65)[b]	37 ± 3.5(−69)[b]	39 ± 6.2(−68)[b]
1	31.2	93 ± 6.8	97 ± 8.3(3.8)[b]	66 ± 9.5(−28)[b]	61 ± 8.9(−35)[b]	46 ± 8.1(−50)[b]
1	56.2	95 ± 4.3	135 ± 8.7(41)[b]	92 ± 8.8(−3.8)	84 ± 13.1(−13)[b]	69 ± 9.0(−27)[b]
2	10	109 ± 2.9	151 ± 3.0(42)	130 ± 5.1(25)	113 ± 1.8(7)	96 ± 1.7(−8)
2	20	134 ± 4.9	187 ± 10.0(40)	150 ± 3.6(14)	126 ± 5.6(−3.5)	109 ± 6.9(−16)
3	10	122 ± 4.0	110 ± 10(−9)[b]	93 ± 6.9(−24)[b]	70 ± 10(−41)[b]	71 ± 9.4(−42)[b]
3	20	129 ± 8.6	153 ± 3.0(20)[b]	117 ± 3.7(−6.9)	103 ± 2.6(−18)[b]	98 ± 7.3(−23)[b]

[a] Each value is the mean ± SEM for six mice in each group. Percent variations of glycemia are in parentheses. [b] $p < 0.05$ significantly different ANOVA followed by Dunnett's *t* test for comparison with respect to control group.

Table 3. Effect of Compounds 2 and 3 from Roots of *L. porteri* on Blood Glucose Levels in STZ-Diabetic Mice after an Oral Load of Glucose (1 g/kg)[a]

		blood glucose concentration				
test sample	dose mg/kg	0 h	0.5 h	1 h	1.5 h	2 h
vehicle		218 ± 50.5	320 ± 59.3(51.9)	229 ± 39.0(10.3)	217 ± 49.5(5×10^{-3})	193 ± 49.4(−12.9)
Gly	10	163 ± 16.4	250 ± 31.1(42)	182 ± 27.7(3.8)	147 ± 17.6(−10)	130 ± 19.0(−21.5)
2	10	172 ± 6.0	192 ± 5.4(13)[b]	146 ± 6.0(−14)[b]	138 ± 6.7(−19)[b]	143 ± 8.6(−15.6)
2	31.2	185 ± 10.5	217 ± 14.2(17)[b]	170 ± 7.0(−6.3)[b]	160 ± 16.6(−12)[b]	165 ± 14.0(−10.0)
2	56.2	149 ± 1.6	186 ± 2.6(25)[b]	173 ± 2.1(17.1)	151 ± 2.2(2.1)	157 ± 1.0 (6.5)
3	10	225 ± 15.4	295.6 ± 17.4(35)	229 ± 16.6(3.5)	214 ± 12.4(1.5)	206 ± 15.8(−7.4)
3	31.2	284 ± 52.1	356 ± 63.7(27)[b]	292 ± 53.1(3.3)	290 ± 58.3(−0.3)	259 ± 48.4(−9.1)
3	56.2	429 ± 59.4	467 ± 52.8(12)[b]	413 ± 62.3(−5.2)	387 ± 62.4(−12)	352 ± 62.1(−21.7)

[a] Each value is the mean ± SEM for six mice in each group. Percent variations of glycemia are in parentheses. [b] $p < 0.05$ significantly different ANOVA followed by Dunnett's *t* test for comparison with respect to control group.

Table 4. Effect of 1 from the Roots of *L. porteri* on Blood Glucose Levels in STZ-Diabetic Mice after an Oral Load of Glucose (1 g/kg)[a]

		blood glucose concentration				
test sample	dose mg/kg	0 h	0.5 h	1 h	1.5 h	2 h
vehicle		218.5 ± 50.5	320 ± 59.3(51.9)	229.8 ± 39.0(10)	217 ± 49.5(0.005)	193 ± 49.4(−13)
Gly	10	163.5 ± 16.4	250.8 ± 31.1(42)	182 ± 27.7(3.8)	147.5 ± 17.6(−10)	130.6 ± 19.0(−21)
1	10	330.1 ± 77.0	391.6 ± 64.8(32)	331.1 ± 67.1(5.7)	334.6 ± 68.0(5.7)	304.1 ± 65.2(−5.5)
1	31.2	176.1 ± 11.5	248.1 ± 13.7(44)	218.5 ± 8.9(32)	205.3 ± 9.4(23)	179.1 ± 7.5(8.8)
1	56.2	208 ± 18.4	344 ± 50.2(57)	320 ± 38.1(52)	265 ± 31.7(25)	237 ± 28.2(8.9)

[a] Each value is the mean ± SEM for six mice in each group. Percent variations of glycemia are in parentheses. $p < 0.05$ significantly different ANOVA followed by Dunnett's *t* test for comparison with respect to control group.

compound in the organic extract, did not affect blood glucose levels after the glucose challenge (see Table 2). In NAD-STZ-diabetic mice, compound **2** (10, 31.2, and 56.2 mg/kg) induced a significant ($p < 0.05$) and dose-dependent decrease of the postprandial peak, suggesting that its mode of action did not involve inhibition of glucose absorption, but that it had a different mechanism yet to be demonstrated (Table 3). In contrast, compound **1** (10, 31.2, and 56.2 mg/kg), which affected the glucose level in normal mice, did not lower the postprandial peak in NAD-STZ-diabetic mice, indicating that its effect is mediated by an insulin-dependent mechanism. Finally, compound **3** (10, 31.2, and 56.2 mg/kg) inhibited (Table 4) the postprandial peak after the glucose load in NAD-STZ-diabetic mice at the two highest doses tested. The latter result was consistent with compound **3** being an inhibitor of glucose absorption. Since compound **3** was the only one lowering the postprandial peak in both normal and diabetic animals, its effect on glucose absorption In Vivo using the OSTT was investigated. The assay was performed only in NAD-STZ-diabetic mice due to scarcity of sample. Thus, administration of 3-(*Z*)-butylidenephthalide (**3**) (10 and 56.2 mg/kg) to NAD-STZ-diabetic mice provoked a decrement of 50% of the postprandial peak ($p < 0.05$) at a dose of 56.2 mg/kg (Figure 1).

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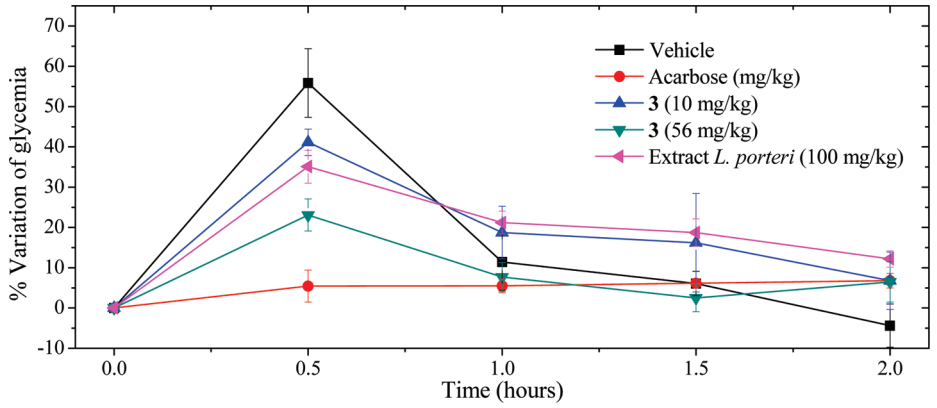

Figure 1. Effects of the crude extract of the roots of *L. porteri* and compound **3** on blood glucose levels in NAD-STZ-diabetic mice using the OST test. Each value is the mean ± SEM for 6 rats in each group. $^*p < 0.05$ significantly different ANOVA followed by Dunnett's *t*-test for comparison with respect to negative control values at the same time.

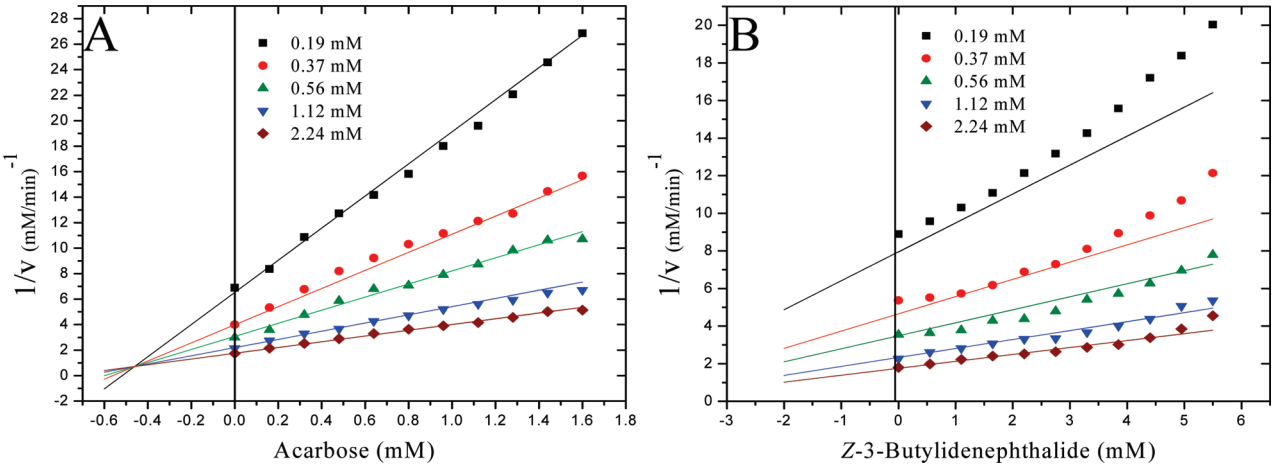

Figure 2. Plots of α-glucosidase inhibition at different concentrations of substrate: (A) acarbose; (B) (*Z*)-3-butylidenephthalide (**3**).

These results clearly revealed that compound **3** is able to inhibit α-glucosidase at the intestinal level with consequent decrease of the postprandial peak in NAD-STZ-diabetic mice.

Compounds **4** and **5** were not tested because their antidiabetic properties In Vivo and in vitro have been previously demonstrated. Ferulic acid (**5**) at a concentration of 25 μM causes a 2.98-fold increase in 2-deoxyglucose transport into the L6 myotubes; its performance was comparable to commercial oral hypoglycemic drugs.[24] Expression of GLUT4 and PI3K transcripts was also elevated in ferulic acid (**5**)-treated cells. In addition, **5** possesses stimulatory effects on insulin secretion in vitro in a concentration-dependent manner.[25] In other tests, ferulic acid (**5**) administered orally at low dosage (0.1% or 0.01%) suppressed hyperglycemia associated with induced diabetes in the model of KK-Ay mice.[26] Finally, ferulic acid (**5**) was the most potent insulin-secreting agent among several cinnamic acid derivatives, and its i.v. administration at a dose of 5 mg/kg significantly decreased plasma glucose and increased insulin concentration in normal rats.[27]

Myristicin (**4**) and some analogues from *Myristica fragrans* fruit are claimed as adiponectin production stimulators and health foods for prevention and treatment of type-II diabetes, hyperlipidemia, hypertension, obesity, and arteriosclerosis.[28]

The effects of compounds **1**−**5** on the activity of α-glucosidase was also tested in vitro according to a well-known procedure using *p*-nitrophenyl-α-D-glucopyranoside (*p*NPG) as substrate and yeast α-glucosidase.[29] The results were also compared with those of acarbose (Figure 2), revealing that **3** inhibited the activity of yeast α-glucosidase in a concentration-dependent manner. The IC_{50} calculated was 2.35 mM (acarbose = 0.42 mM). As expected from the In Vivo experiments, compounds **1** and **2** were not α-glucosidase inhibitors and neither were compounds **4** and **5.**

In order to obtain further evidence of the nature of inhibition exerted by compound **3**, a kinetic analysis of the enzyme inhibition activity was assessed using different amounts of compound **3** and acarbose. The results showed that **3** inhibited activity of the enzyme with a K_i of 4.86 mM vs K_i of 0.46 mM for acarbose. Dixon plots[30] for α-glucosidase from yeast in the presence of acarbose and 3-(*Z*)-butylidenephthalide (**3**) at different concentrations (Figure 2) revealed typical curves for competitive and mixed-type noncompetitive inhibitors, respectively. According to Figure 2, the increment in acarbose concentration resulted in lowering the slope of the lines, in agreement with a reversible inhibitor. On the other hand, compound **3** displayed an inhibition profile for a mixed-type inhibitor since the corresponding graphic showed a series of lines intersecting to the left of the vertical and above the horizontal axes.[28]

Altogether these results suggest that compound **3** does not bind to the same catalytic site as acarbose in α-glucosidase.

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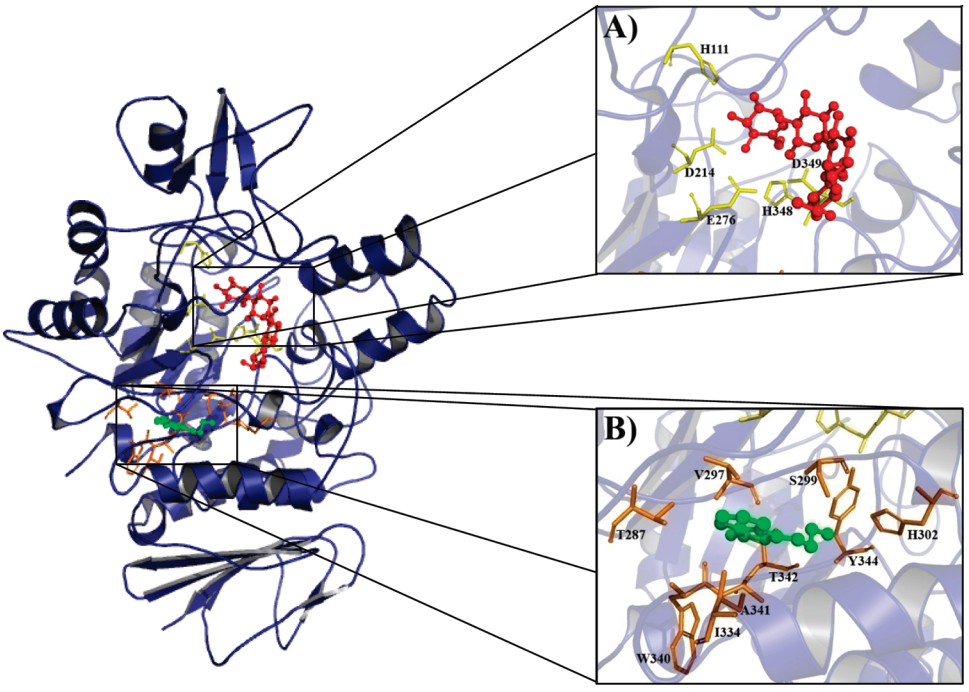

Figure 3. Docking results using the structural model of the α-glucosidase: (A) site of binding of acarbose, which comprises the catalytic site of the enzyme; (B) binding conformation of (*Z*)-3-butylidenephthalide (**3**).

Indeed, the docking analysis predicts that **3** binds to the enzyme in a different pocket than acarbose; the binding site for **3** is close to the catalytic site and is formed by residues Ser299, Thr287, Val297, Trp340, His302, Ala341, Thr342, Ile334, and Tyr344 (Figure 3), with a predicted K_i of 11.48 μM. As indicated in the Experimental Section, the docking was performed with a model built by homology with *Bacillus cereus* (1UOK.pdb) α-glucosidase, which possessed the highest sequence identity (38.9%) with α-glucosidase from yeast,[31] thus conserving the catalytic residues in most α-glucosidases (His111, Asp205, Glu276, His348, and Asp349). The docking study was validated with acarbose.

In conclusion, the information generated hitherto indicates that *L. porteri* extract and compounds have potent antihyperglycemic effect. One of its metabolites, (*Z*)-3-butilidenephthalide (**3**), displayed significant antihyperglycemic effect by inhibiting the activity of intestinal and yeast α-glucosidases In Vivo and in vitro. According to the in vitro studies, this compound acts as a noncompetitive, therefore allosteric, inhibitor of this important enzyme. This type of inhibitor has several advantages over competitive ones since their effects lasted for longer periods of time.[32] The related compounds, (*Z*)-6,6′,7,3′α-diligustilide (**1**) and (*Z*)-ligustilide (**2**), however, did not affect α-glucosidases In Vivo but affected glucose absorption by a mechanism yet to be determined. Finally, ferulic acid (**5**), present in high amounts in the extract, has demonstrated in previous investigations noted stimulatory effects on insulin secretion In Vivo and in vitro. Therefore, the extract of *L. porteri* represents a good phytotherapeutic agent with active principles having different modes of action. In addition, (*Z*)-3-butylidenephthalide (**3**) is a new α-glucosidase inhibitor potentially useful for the development of new antidiabetic, antiobesity, and antiviral agents. The present study provides scientific support of the use of *L. porteri* in Mexican folk medicine for the treatment of diabetes.

EXPERIMENTAL SECTION

Plant Material and Extract. The roots of *L. porteri* were collected in Basigochi (Chihuahua, Mexico) in October 2003. A voucher specimen (No. 31733) is deposited at the National Herbarium (MEXU), Mexico City. The air-dried and pulverized (250 g) roots were macerated with CH_2Cl_2−MeOH (1:1; 5 L × 2) at room temperature during 7 days, with subsequent evaporation of the solvent in vacuo to yield 87.5 g of a yellow residue.

Isolation of Compounds 1−5. The extract (87.5 g) was fractionated by column chromatography on silica gel (1 kg, 70−230 mesh, Merck) eluting with hexane, hexane with increasing amounts of CH_2Cl_2 (8:2 → 2:8), CH_2Cl_2, and CH_2Cl_2−MeOH (9:1) to yield 10 primary fractions. Fraction 3 (3 g), eluted with hexane, was further separated by TLC on silica gel (hexane−CH_2Cl_2, 8:2) to yield 400 mg of myristicin (**4**). Fraction 4 (3.5 g), eluted with hexane−CH_2Cl_2 (1:1), contained the major components (*Z*)-6,6′,7,3′α-diligustilide (**1**), (*Z*)-ligustilide (**2**), and (*Z*)-3-butylidenephthalide (**3**). Compounds **1**−**3** were separated by extensive TLC on silica gel (hexane−CH_2Cl_2, 1:1) to yield 5.5, 0.5, and 0.6 g, respectively. HPLC-UV analysis revealed the purity of the components [silica gel Hibar RT LiChrospher100 RP C-18, 5 μm, 4 × 250 mm, Merck; ACN−H_2O (0.5% AcOH) 20:80 → 100:0, 0−40 min; flow rate 1 mL/min; detecting at 260 and 280 nm]. The structures of compounds **1**−**3** were characterized as (*Z*)-6,6′,7,3′α-diligustilide, (*Z*)-ligustilide, and (*Z*)-3-butylidenephthalide by spectroscopic and spectrometric analyses and comparison with literature data.[16,22] Finally, 7.5 g of ferulic acid (**5**) precipitated from fraction 9 (20 g), eluted with CH_2Cl_2, which was identical to a reference sample.

Experimental Animals. Male ICR normoglycemic mice, weighing 20−25 g, 20−25 days old, were obtained from Centro UNAM-Harlan (Harlan México, SA de CV). Procedures involving animals and their care were conducted in conformity with the Mexican Official Norm for Animal Care and Handling (NOM-062-ZOO-1999) and in compliance with international rules on care and use of laboratory animals. Furthermore, clearance for conducting the studies was obtained from the Ethics Committee for the Use of Animals in Pharmacological and

Toxicological Testing, Facultad de Química, UNAM. For the pharmacological studies, groups of six animals were used. All doses are in mg/kg of body weight. The animals were housed in groups of eight under standard laboratory conditions (12 h light−dark cycle under controlled temperature, 22 ± 1 °C) and maintained on a standard pellet diet and water ad libitum.[33]

Preparation of Test Samples. All samples were suspended in 0.05% Tween 80 in saline solution and administered by intragastrical route. Gly (Sigma-Aldrich Corp., St. Louis, MO, USA) was used as a hypoglycemic model drug. Acarbose (Sigma-Aldrich) was used as an antihyperglycemic drug. Glucose and sucrose (Sigma-Aldrich) were used as carbohydrates to carry out the glucose and sucrose tolerance tests. Control group mice received only the vehicle (0.05% Tween 80 in saline solution) in the same volume (0.2 mL of vehicle/10 g of body weight) by the same route.[33]

Induction of Experimental Diabetes. Diabetes mellitus type-II was induced by a single intraperitoneal injection of freshly prepared STZ (100 mg/kg, Sigma-Aldrich) dissolved in 0.1 M citrate buffer, pH 4.5, in a volume of 10 mL/kg, 15 min after an injection of NAD (40 mg/kg; Sigma-Aldrich) dissolved in distilled water, in a volume of 10 mL/kg. After 7 days of STZ administration, blood glucose levels of each mouse were determined. Mice with blood glucose levels higher than 140 mg/dL were considered diabetic and included in the study.[33]

Collection of Blood Samples and Determination of Blood Glucose Levels. Blood samples were collected from the caudal vein by means of a small incision at the end of the tail. Blood glucose levels (mg/dL) were estimated by the enzymatic glucose oxidase method using a commercial glucometer (One Touch Ultra 2, Johnson & Johnson, CA, USA). The percentage variation of glycemia for each group was calculated with respect to the initial (0 h) level, according to the following equation, where G_i is initial glycemia value and G_t is the glycemia value after treatment administration:

$$\%\ \text{Variation of glycemia} = [(G_t - G_i)/G_i] \times 100$$

Acute Hypoglycemic Assay in Normal and Diabetic Animals. Normal or NAD-STZ-diabetic mice, placed in acrylic boxes in groups of six animals, were deprived of food for 12 h before experimentation, but allowed free access to tap water throughout. The crude extract of *L. porteri* was freshly prepared before experimentation and tested at doses of 56.2, 100, and 316.2 mg/kg. Blood samples were collected at 0, 1.5, 3, 5, 7, and 9 h after treatment administration. Gly was used as positive control.[33]

Oral Glucose Tolerance Test (OGTT). Normal or NAD-STZ-diabetic mice were placed in groups of six animals each. Mice were put on fast for 12 h before the experiment, with water ad libitum. In the normal and diabetic animals, the crude extract of *L. porteri* was tested at doses of 56.2, 100, and 316 mg/kg. Compound **1** was assayed at doses of 31.2 and 56.2 mg/kg in the case of normal mice, whereas diabetic animals were tested at doses of 10, 31.2, and 56.2 mg/kg. Compounds **2** and **3** were tested at doses of 10 and 20 mg/kg in normal animals, and in diabetic animals they were tested at doses of 10, 31.2, and 56.2 mg/kg. Gly (10 mg/kg) was suspended in the same vehicle. Time 0 min was set before treatment with the extract or compounds; 30 min later a glucose load (1 g/kg) was given to the animals. Blood samples were obtained 30, 60, 90, 120, and 180 min after the carbohydrate load.[33]

Oral Sucrose Tolerance Test (OSTT). The assay was performed exactly as described for the OGTT but using sucrose as the carbohydrate. In this experiment only the extract was tested in both normal and diabetic animals. In the case of normal mice the doses were 31.2, 56.2, and 100 mg/kg. For diabetic animals, however, the extract was tested at a single dose of 100 mg/kg. Finally, compound **3** was assayed at doses of 10 and 56.2 mg/kg, but only in diabetic animals. Acarbose was used as positive control.

Statistical Analysis. Data are expressed as the means ± SEM of the number (n = 6) of animals in each group. ANOVA was used to analyze the changes in blood glucose. Dunnett range posthoc comparisons were used to determine the source of significant differences where appropriate; $p \leq 0.05$ was considered statistically significant. Sigma Stat software was used for statistical analyses.

In Vitro α-Glucosidase Inhibition Study. The α-glucosidase inhibition assay was performed according to Oki et al., with modifications of our own.[29] α-Glucosidase (EC 3.2.1.20) from baker's yeast was purchased from Sigma-Aldrich. The inhibition was measured spectrophotometrically at pH 7.0 and 37 °C using 0.25 mM *p*-nitrophenyl-α-D-glucopyranoside (*p*NPG) as a substrate and 0.6 units/mL of enzyme, in 100 mM sodium phosphate buffer. Acarbose was dissolved in phosphate buffer, and serial dilutions from 0.1 to 1.0 mg/mL were made and used as positive control. The increments in absorption at 405 nm due to the hydrolysis of *p*NPG by α-glucosidase were determined spectrophotometrically on a microplate reader (Bio Instruments Incorporated, USA). A 10 μL amount of acarbose or 3-(*Z*)-butylidenephthalide (**3**) solution was incubated for 5 min with 20 μL of enzyme stock. After incubation, 10 μL of substrate (5 mM) was added and further incubated for 35 min at 30 °C. The concentration required to inhibit activity of the enzyme by 50% (IC_{50}) was calculated by regression analysis, using the following equation, where v is the percentage of inhibition, A_{100} is the maximum inhibition, I is the inhibitor concentration, IC_{50} is the concentration required to inhibit activity of the enzyme by 50%, and s is the cooperative degree:

$$v = \frac{A_{100}}{1 + (I/\text{IC}_{50})^S}$$

Kinetics of Enzyme Inhibition. Kinetic parameters were determined using the Dixon plot method at increasing concentrations of substrates and inhibitors, plotting slopes versus the reciprocal of the substrate concentration. All results are expressed as the mean of at least six experiments ± EM. Kinetic parameters such as V_m, K_m, and K_i were evaluated using the nonlinear regression method based on the following inhibition equation, where v is the initial velocity in the absence and presence of the inhibitor, s and I respectively are the concentration of substrate and inhibitor, V_m is the maximum velocity, K_m is the Michaelis−Menten constant, K_i is the competitive inhibition constant, and K'_i is the uncompetitive inhibition constant:

$$v = \frac{V_m S}{K_m(1 + {}^I/K_i) + s(1 + {}^I/K'_i)}$$

Homology Modeling of Yeast α-Glucosidase. Although the X-ray crystal structures of a few bacterial α-glucosidases have been reported, structural information is still unavailable for most eukaryotic α-glucosidases commonly used in biological assays, such as that from baker's yeast. Therefore, we carried out homology modeling of α-glucosidase from baker's yeast to obtain its structure. This homology modeling started with the retrieval of the amino acid sequence of α-glucosidase MAL12 (EC 3.2.1.20) from baker's yeast that comprises 584 amino acid residues from the UniProt (http://www.uniprot.org/uniprot/). Using the program CPH models (automated neural-network based protein modeling server; http://www.cbs.dtu.dk/services/CPHmodels/), the structural model was built based on the structure of oligo-1,6-glucosidase from *Bacillus cereus* (1UOK.pdb),[34] which revealed the highest sequence identity (38.9%) with the target. This model was then optimized geometrically with the program HyperChem 8 release, with the intention of correcting all the links and angles of the model and validated using the ProCheck (stereochemical quality analysis software),[31] Verify-3D (structure evaluation software),[35] and Whatcheck (protein verification tools software)[36] computer programs.

Docking of α-Glucosidase Inhibitor. Blind docking was carried out with AutoDock4 software (http://autodock.scripps.edu/)[37,38] using the default parameters, except for the number of GA runs (100) and the Lamarkian genetic algorithm with local search and 25 million energy evaluations (Long. Evals.) per run, on a parallel supercomputer of distributed memory; it contains 1368 processors AMD Opteron, around 3 terabytes of memory, and 160 terabytes of storage (http://www.super.unam.mx/). The target in each docking run was obtained from homology modeling of α-glucosidase. The starting conformation of the ligand was an energy-minimized form with geometric optimization using the program HyperChem 8 release. Acarbose and 3-(*Z*)-butylidenephthalide (**3**) molecules were first prepared by AutoDockTools 1.5.4 (http://mgltools.scripps.edu/), adding polar hydrogen atoms and Kollman charges to α-glucosidase and computing Gasteiger charges for acarbose and 3-(*Z*)-butylidenephthalide (**3**). The protein was held rigid during the docking process while the ligands were allowed to be flexible. The grid box size was 126 Å × 126 Å × 126 Å in the *x*, *y*, and *z* dimensions, with the center of the grid corresponding to the protein.

■ ASSOCIATED CONTENT

Supporting Information. Figure S1 and the Tables S1−S5 are available free of charge via the Internet at http://pubs.acs.org.

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Notes

[§]This paper is taken in part from the Ph.D. thesis of F.B.

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■ DEDICATION

This paper is dedicated to Dr. Koji Nakanishi from Columbia University for his pioneering work on bioactive natural products.

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