

Triterpenes from *Agarista mexicana* as Potential Antidiabetic Agents

R. M. Perez G.^{1*} and R. Vargas S.²

¹Laboratorio de Investigacion de Productos Naturales, Escuela Superior de Ingeniería Química e Industrias Extractivas IPN, Punto Fijo 16, Col. Torres Lindavista C.P. 07708, México D.F. México

²Universidad Autónoma Metropolitana-Xochimilco A.P., 23-181 Mexico D.F.

Hypoglycaemic activity-guided fractionation together with chemical analysis led to the isolation of 12-ursene and a novel triterpene 23,24 dimethyl-24-ethyl-stigmast-25-ene from the chloroform extract of the dried stem of *A. mexicana*. Identification was based on spectroscopic methods. The isolated triterpenes were tested for hypoglycaemic activity in normal and alloxan-diabetic CD1 mice 25–30 g at a dose of 50 mg/kg body weight. The blood glucose levels were determined before and 1.5, 3, 4.5 and 24 h after intraperitoneal drug administration. The results showed that the triterpenes produced a significant hypoglycaemic effect in normal as well as in diabetic mice. Comparison was made between the action of the triterpenes and a known hypoglycaemic drug, tolbutamide (50 mg/kg). The 12-ursene was found to be slow and less effective than tolbutamide, and the 23,24 dimethyl-24-ethyl-stigmast-25-ene was shown to be more effective than tolbutamide. Copyright © 2002 John Wiley & Sons, Ltd.

Keywords: *Agarista mexicana*; hypoglycaemic; triterpenes; 23, 24 dimethyl-24-ethyl-stigmast-25-ene; 12-ursene.

INTRODUCTION

The plant *Agarista mexicana* (Hemsl) Judd (Ericaceae), is commonly known as ‘palo santo’. It is a common herb that grows wild and abundantly in the fields of Mexico. A water extract of the leaves has long been used in folk medicine for treating diabetes mellitus and it is generally believed to produce beneficial effects. In a previous communication we reported the hypoglycaemic activity of *Agarista mexicana* (Perez *et al.*, 1996). In this paper, we report the chemical analysis of the active fractions and the antihyperglycaemic activity of the isolated constituents in normal and alloxan-induced diabetic mice. Activity was compared with that of tolbutamide.

EXPERIMENTAL

General experimental procedures. IR: KBr, MS: 70eV, silica gel 60 (0.2–0.5 mm). NMR spectra were measured in CDCl₃. Chemical shifts were referenced to residual hydrogen (3.31 ppm) or carbon (49.15 ppm); coupling constants are given in Hz. ¹H and ¹³C NMR spectra were run on a DPX-300 MHz. Mass spectra were recorded on a Jeol CC-Mate.

Animals used. Healthy adult (male and female) mice of the CD1 strain weighing 25–30 g were used in these experiments. The animals were kept in an air-conditioned animal room (22°±2°C with a 12 h light–12 h dark cycle) in the Biotery of the University. The animals were given a commercial feed prepared by Purina and allowed tap

water *ad libitum*. The effects of extracts and triterpenoids were studied on the blood glucose levels of normal and alloxan-diabetic mice.

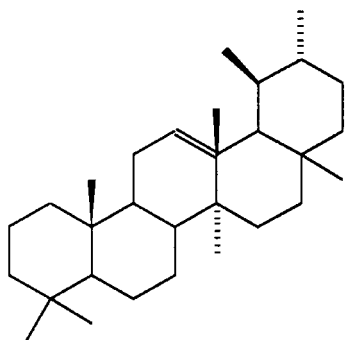
Studies of hyperglycaemic mice. A freshly prepared solution of alloxan in normal saline was injected into the caudal vein (70 mg/kg body wt), three times every third day (Rodriguez *et al.*, 1975). Seven days after administration blood samples were drawn from the caudal vein by the pinch clip method. Animals with a blood sugar of 250 mg/dL were considered to be diabetic and used in this study. The animals were divided into several groups and ten animals were maintained in each cage under uniform husbandry conditions. The triterpenes were then administered i.p. and, at the same time, a control test was carried out using only saline solution. The dosage administered was 50 mg/kg. The percentage decrease in glycaemia was determined as a function of time (Dulin, 1964).

Studies on normoglycaemic mice. To evaluate hypoglycaemic activity, a study was carried out on the variation of blood glucose levels after the administration of triterpenes (i.p.) to albino CD1 mice. The animals were fasted 12 h prior to each experiment, but water was allowed *ad libitum*. The room temperature was kept constant at 22°C. Blood glucose concentration was determined and noted as the initial glycaemia (G₀) and blood samples were drawn from the caudal vein at 1.5, 3, 4.5 and 24 h (G_x). The percentage of triterpene induced glycaemia was calculated as a time function by the following formula:

$$\% \text{ induced glycaemia} = \frac{G_x - G_0}{G_0} \times 100$$

where G₀ is the initial blood glucose level and G_x the

* Correspondence to: Dr R. M. Perez Gutierrez, Punto Fijo No. 16, Col. Torres de Lindavista C.P. 07708 Mexico, D.F. Mexico.
Email: dmex@df1.telnet.net.mx
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Figure 1

blood glucose level at 1.5, 3, 4.5 and 24 h (Gupta, *et al.*, 1984).

Preparation and administration of drug suspension.

The amount of triterpenes required for each mouse was calculated on a body weight basis. The drug was weighed, triturated with water and administered i.p. to each animal. The control group received 0.5 mL saline (i.p.), tolbutamide was administered i.p. as an aqueous solution.

Collection of blood. Before administration of the drug, 0.1 mL of blood was extracted for glucose estimation collected from the tail vein. Similar blood samples were also collected at 1.5, 3, 4.5 and 24 h after drug administration. After collection of the blood, samples for the glucose assay were to put into micro-test tubes containing 3.8% freshly prepared sodium citrate and then analysed using the glucose oxidase method on a glucose analyser (Beckman Instruments, Anaheim, USA). The pricked site of the tail was rubbed with cotton soaked with 70% alcohol to protect the mice against infection.

Comparison of triterpenes with a standard drug.

Fifteen white CD1 mice (25–30 g) were divided into three groups of five mice per group. The control group received 0.5 mL saline (i.p.), one group received 50 mg/kg i.p. tolbutamide and the other group received the triterpenoids (50 mg/kg, i.p.). The blood glucose levels in all groups were monitored 1.5, 3, 4.5 and 24 h after drug administration.

Statistical analysis. Blood glucose levels in each group were expressed as the percentage of the initial blood glucose (mean $\pm$ SEM) and the data were statistically analysed by the Student's *t*-test, using the program 'Microcal Origin'. Values of $P \leq 0.05$ were taken as significant.

Extraction and separation of compounds. *Agarista mexicana* stem was collected in the region of Taxco state of Guerrero. The material was identified in the Department of Botany of ENEP-Iztacala UNAM, and a voucher specimen of the plant (4645) in stored in the herbarium of this Department for reference. The material was dried at room temperature, 5 kg of the powder was successively extracted with hexane and chloroform. The yields

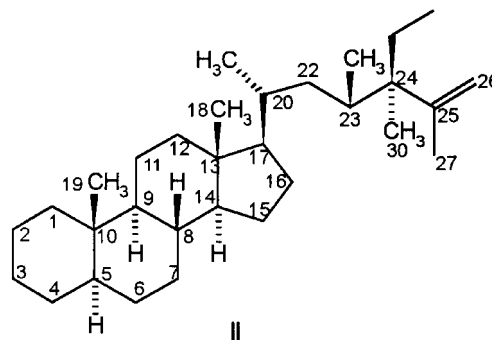


Figure 2

obtained for the hexane and chloroform extracts were 3% and 5%, respectively. The chloroform extract was concentrated under reduced pressure to produce a dark green viscous mass which was subjected to silica gel chromatography. The column was developed with benzene to give nine fractions. Of all the fractions tested the most pharmacologically active were fractions 2 and 5. Fraction 2 was separated by silica gel by eluting with CCl_4 – CH_2Cl_2 (7:1) to give a crude extract (I) which was then purified by preparative TLC over silica gel eluting with CHCl_3 – EtOAc (4:1) to give (I) (200 mg). Fraction 5 was then chromatographed on silica gel CCl_4 – CH_2 (5:1) to give seven subfractions. Another silica gel column for subfraction 3 (300 mg) eluted with CCl_4 –benzene– CHCl_3 (1:1:9) gave six fractions of which fraction 4 yielded compound (II) (100 mg). Compounds (I) and (II) were determined to purity by thin-layer chromatography.

RESULTS AND DISCUSSION

Structure determination

12-ursene (I). EIMS (m/z) 410.3893 (M^+ , calcd for $\text{C}_{30}\text{H}_{50}$); IR ν_{max} 2900–2800 (C–H) and 1650 (C=C); ^1H NMR δ 0.80 (3H, s), 0.84 (3H, s), 0.92 (3H, s), 0.95 (3H, s), 0.97 (3H, s), and 1.0 (3H, s) 0.86 or 0.88 (d, $J = 6.0$ Hz H-29 or H-30), and 5.28 (1H, t, $J_{12,11\alpha} = J_{12,11\beta} = 3.40$ Hz, H-12); ^{13}C NMR: C-1 (39.8), C-2 (19.2), C-3 (42.2), C-4 (33.1), C-5 (56.4), C-6 (19.4), C-7 (33.0), C-8 (39.6), C-9 (47.5), C-10 (37.0), C-11 (17.0), C-12 (126.7), C-13 (138.1), C-14 (42.1), C-15 (28.2), C-16 (26.2), C-17 (34.1), C-18 (57.8), C-19 (39.1), C-20 (38.8), C-21 (30.7), C-22 (39.8), C-23 (34.2), C-24 (20.5), C-25 (16.7), C-26 (16.9), C-27 (24.3), C-28 (28.1), C-29 (22.5), C-30 (21.2). EIMS 70 eV m/z : 356 ($\text{C}_{26}\text{H}_{44}$), 342 ($\text{C}_{25}\text{H}_{42}$), 328 ($\text{C}_{24}\text{H}_{40}$), 288 ($\text{C}_{21}\text{H}_{36}$), 274 ($\text{C}_{20}\text{H}_{34}$), 260 ($\text{C}_{19}\text{H}_{32}$), 217 ($\text{C}_{16}\text{H}_{25}$), 205 ($\text{C}_{15}\text{H}_{25}$), 203 ($\text{C}_{15}\text{H}_{23}$), 192 ($\text{C}_{14}\text{H}_{24}$), 138 ($\text{C}_{10}\text{H}_{18}$), 124 (C_9H_{16}), 84 (C_6H_{12}), 56 (C_4H_8). Compound (I) was identified as urs-12-ene by comparison of physical data (IR, M.S., PMR, CNM) with published values (Siddiqui *et al.*, 1988; Ukpabio *et al.*, 1994; Hazai *et al.*, 1992; Nakatani *et al.*, 1989; Budzikiewics *et al.*, 1963; Sea *et al.*, 1975).

23,24 dimethyl-24-ethyl-stigmast-25-ene (II). Colourless needles from hexane–MeOH, M^+ at m/z 426.537385 (calc. for $\text{C}_{31}\text{H}_{54}$); IR ν_{max} 2910, 2848, 1650, 1462; EIMS 70 eV m/z : 426 (4), 383 (2), 302 (3) 271 (4), 258 (3), 257

Table 1. Effect of 12-ursene (I) and 23,24-dimethyl-24-ethyl-stigmast-25-ene (II) on alloxan-induced hyperglycaemic mice

Sample (mg/kg)	Mean % deviation from initial glucose concentration (± SEM)			
	Hours after administration			
	1.5	3	4.5	24
Control	+0.7 ± 2.3	+0.9 ± 1.4	1.4 ± 1.1	2.6 ± 1.8
(I) 50	−12.3 ± 6.3 ^b	−29.6 ± 2.5 ^b	−22.5 ± 4.8 ^a	+5.1 ± 3.5 ^a
(II) 50	−19.3 ± 3.6	−38.3 ± 5.1 ^b	−65.8 ± 4.8 ^a	−9.2 ± 2.7
Tolbutamide 50	−17.8 ± 4.2 ^b	−35.1 ± 3.3 ^a	−61.3 ± 2.7 ^a	−8.42 ± 1.9 ^b

Significance ^a $p < 0.05$ or ^b $p < 0.01$; 10 mice per experimental group.

(6), 245 (4), 218 (100), 215 (3), 207 (10), 204 (18), 203 (41), 191 (11), 190 (13), 189 (26), 179 (2), 175 (15), 167 (10), 161 (12), 150 (5), 149 (26), 145 (11), 139 (5), 136 (14), 134 (13), 127 (12), 126 (5), 122 (13), 113 (5), 111 (15), 109 (22), 107 (23), 105 (21), 97 (22), 96 (9), 95 (27), 93 (17), 91 (13), 85 (12), 83 (23), 81 (21), 79 (15). ¹H NMR: δ 5.01 (bd, 5.10, bd, $J = 2.23$ Hz, H-26), 2.18 (m, H-11), 1.90 (m, H-12), 1.85 (m, H-23), 1.65 (s, H-27), 1.07 (d, $J = 6.6$ Hz, H-31), 0.92 (d, $J = 6.0$ Hz, H-21), 0.90 (s, H-30), 0.83 (s, H-19), 0.804 (t, $J = 7.5$ Hz, H-29), 0.79 (s, H-18), 0.781 (dd, $J = 7.4$ Hz, 6.8, H-28). ¹³C NMR: δ 37.10 (C-1), 23.51 (C-2), 26.11 (C-3), 29.69 (C-4), 47.18 (C-5), 28.07 (C-6), 32.46 (C-7), 36.90 (C-8), 48.20 (C-9), 38.54 (C-10), 20.50 (C-11), 39.74 (C-12), 41.67 (C-13), 55.12 (C-14), 26.89 (C-15), 32.60 (C-16), 47.50 (C-17), 14.48 (C-18), 18.34 (C-19), 31.65 (C-20), 18.96 (C-21), 27.02 (C-22), 29.14 (C-23), 38.94 (C-24), 145.16 (C-25), 116.84 (C-26), 19.6 (C-27), 23.61 (C-28), 20.60 (C-29), 27.50 (C-30), 19.42 (C-31).

The ¹H NMR spectrum of compound (II) displayed four tertiary (δ 0.79, 0.83, 0.90 and 1.65) and two secondary methyl groups (δ 0.92 and 1.07). The ¹³C NMR and DEPT spectra revealed 31 atoms consisting of 7 methyl carbons, 13 methylene 7 methine, the remaining four signals in the broad-band spectrum were attributed to the quaternary carbons, two olefinic carbon (δ 116.84 C-26, 145.16 C-25) were further identified. Moreover, the four methine carbons at δ 47.18 (C-5), 48.20 (C-9), 36.90 (C-8) and 55.12 (C-14) and four quaternary carbons at δ 14.48 (C-18), 18.34 (C-19), 19.6 (C-27) and 27.50 (C-30) were also indicative of a tetracyclic triterpene containing a side-chain. Hence, all spectral data could only be accommodated with a stigmastane skeleton which was also established by comparison of its spectral properties with the data given in the literature for stigmastanol derivatives (Sarwar *et al.*, 1996; Kawagishi *et al.*, 1997; Dos, *et al.*, 1990).

The olefinic methylene protons (H₂-26) were obtained

as a broad singlet at δ 5.01 and δ 5.1. The 27-methyl protons appeared as a singlet at δ 1.65 indicating that the group is on an olefinic double bond, which was shown to be coupled to one of two vinyl protons (H-26), thus indicating the presence of an isopropenyl group. The ¹³C NMR spectrum (Table 1) was in complete agreement with the existence of this group, in particular the characteristic vinyl carbon atom resonances (C-25, δ 145.16 and C-26, δ 116.85). The presence of the isopropylidene group was also confirmed by a [M-43]⁺ fragment corresponding to a hydrogen migration to side chain and loss of an isopropyl group (Kojima and Ogura, 1986). Allylic cleavage of the C-22–C-23 and C-23–C-24 was not observed. The mass spectrum of (II) showed [M]⁺ at m/z 426 corresponding to C₃₁H₅₄. Rupture of the C-17–C-20 bond gave a peak at m/z 257 for the tetracyclic part of compound as a result of the loss of the side chain plus two hydrogens, a process typical of sterols with one degree of unsaturation in the side chain (Wyllie and Djerassi, 1968). Analysis of the mass spectrum of compound (II) revealed the nature of the tetracyclic skeleton cleavage of ring C gave a peak m/z 150 which limited the choice of carbon skeleton to the stigmastane group. The mass spectrum strongly suggested a saturated sterol nucleus. The base peak is found at m/z 218 as a result of the cleavage of ring D. The presence of fragment ions at m/z 218 [M- side chain-C₃H₆ (ring D)] and 204 [218-CH₂] suggested also the absence of one additional nuclear methyl group. Two methyl groups must be in the side chain, assuming the presence of the usual C-26 and C-27 methyl groups, only positions 20, 22 or 23 are available for the remaining two secondary methyl substituents (Lin *et al.*, 1993). The spectrum showed the presence of the ion at m/z 301 [M-part of side chain by C-20, C-22 cleavage] suggesting the absence of methyl group at C-22. The presence of the C₈H₁₅ side chain was evident from the appearance of the ion M-111 in the spectra indicating that the C-24 is

Table 2. Effect of 12-ursene (I) and 23,24-dimethyl-24-ethyl-stigmast-25-ene (II) on normal mice

Sample (mg/kg)	Mean % deviation from initial glucose concentration (± SEM)			
	Hours after administration			
	1.5	3	4.5	24
Control	+0.2 ± 2.9	+1.3 ± 3.7	2.4 ± 4.8	1.8 ± 3.1
(I) 50	−5.7 ± 5.1 ^b	−14.3 ± 4.7	−25.9 ± 6.1 ^a	−3.9 ± 2.8
(II) 50	−9.6 ± 6.8 ^b	−26.7 ± 5.8 ^b	−38.4 ± 5.5	−10.7 ± 4.1 ^a
Tolbutamide 50	−8.1 ± 3.2	−24.1 ± 2.7 ^b	−31.1 ± 3.4	−8.72 ± 3.9

Significance ^a $p < 0.05$ or ^b $p < 0.01$; 10 mice per experimental group.

substituted by a methyl and an ethyl group and a 25(26) double bond (Sarwar *et al.*, 1996). The absence of *m/z* 314 peak in the spectrum suggested the presence of a quaternary centre at C-24, blocking McLafferty rearrangement of Δ^{25} double bond¹². ¹H NMR spectral features a quartet at δ 0.781 (dd, *J* = 7.4, 6.8 Hz) for the C-28 methylene proton, as well as the C-29 methyl proton at δ 0.804 (t, 7.5 Hz) these signals are known to characterize the ethyl group.

In order to determine the location of the groups, a ¹H-¹H COSY experiment was carried out. The observed correlations led to the sequences H-2/H-3, H-3/H-4, H-5/H-3, H-5/H-4, H-5/H-6, H-5/H-7, H-7/H-14, H-8/H-11, H-8/H-18, H-8/H-19, H-14/H-15, H-17/H-21, H-18/H-19, H-30/H-31, H-27/31, H-28/H-26. On this basis compound (II) was identified as 23,24 dimethyl-24-ethyl-stigmast-25-ene.

The hypoglycaemic effect of the triperpenes was determined at a dose of 50 mg/kg by i.p. administration, comparing the levels of blood glucose reduction with the activity produced with tolbutamide at the same dose. This dose was chosen as it produced a 61.3% reduction at 4.5 h after tolbutamide by i.p. administration (Chucla *et al.*, 1988).

The effects of 23,24 dimethyl-24-ethyl-stigmast-25-ene (II) and 12-ursene and tolbutamide on blood glucose levels of alloxan induced diabetic mice are shown in Table 1. With the 12-ursene the maximum hypoglycaemic activity (29.6% reduction) was observed 3 h after the

administration but the blood glucose concentration increased within 24 h which further indicates that the effect of the 12-ursene did not last for more than 24 h. In contrast, with 23-methyl-24-methyl-24-ethyl-stigmast-25-ene the maximum effect (–65.8% reduction) was observed 4.5 h after the administration of the compound. This effect persisted for a period longer than 24 h.

The administration of 50 mg/kg of 12-ursene and 23,24 dimethyl-24-ethyl-stigmast-25-ene to normoglycaemic mice (Table 2) produced a statistically significant lowering of blood glucose. The maximum reduction of blood glucose level obtained by using identical amounts of 12-ursene and tolbutamide (50 mg/kg of body wt) was –25.9% at 4.5 h while tolbutamide caused a –31.1% lowering of blood sugar. The group treated with 50 mg/kg of 23,24 dimethyl-24-ethyl-stigmast-25-ene showed only a 9.6% reduction in blood glucose after 1 h. The greatest effect on glucose levels (38.4%) was shown at 4.5 h.

The results obtained demonstrate that *A. mexicana* produced hypoglycaemic effects, which play a role in traditional local folk medicine, and may be due to the presence of 12-ursene and 23,24 dimethyl-24-ethyl-stigmast-25-ene.

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