The Seed Oil of *Bernardia pulchella* (Euphorbiaceae) —A Rich Source of Vernolic Acid

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ABSTRACT: The fatty acids from the seed oil of *Bernardia pulchella* (Euphorbiaceae) have been analyzed by gas chromatography (GC) and GC–mass spectrometry (MS) analysis of their methyl esters. Vernolic acid is the main compound (91%), along with other usual fatty acids. In addition to the quantitation by GC analysis, ^{1}H-nuclear magnetic resonance (NMR) signals from the seed oil have been used to estimate the total epoxy fatty acid content. The structure of vernolic acid has been proven by spectroscopic methods (infrared, ^{1}H, and ^{13}C-NMR) and by GC–MS analysis of the corresponding silylated hydroxy–methoxy derivative. The 4,4-dimethyloxazoline derivatives of the fatty acid mixture have also been examined by GC–MS, and it was shown that this derivatization reaction is not suitable for the structure analysis of vernolic acid.

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KEY WORDS: *Bernardia pulchella,* epoxy fatty acids, Euphorbiaceae, GC–MS, silylated hydroxy–methoxy derivatives, vernolic acid.

The plant *Bernardia pulchella* belongs to the plant family Euphorbiaceae, which consists of approximately 300 genera with about 8000 species (1). The plant occurs as a 1- to 4-m height shrub in South Brazil, Argentina, and Paraguay (2) and yields about 5-mm long capsular fruits that contain three oily seeds.

The seed oils of the Euphorbiaceae show great variability in their fatty acid (FA) composition, and various unusual derivatives have been isolated (1,3). In the present study, as a part of our current research project about new crops from South Brazilian plants, the FA composition and nuclear magnetic resonance spectroscopic (NMR) data of the seed oil of *B. pulchella* have been investigated. This is the first chemical report about the plant *B. pulchella.*

MATERIALS AND METHODS

The fruits from *B. pulchella* (Baillon) Mueller Argoviensis were collected in June 1995 in the National Park of Turvo, Derrubadas, in the Federal State Rio Grande do Sul, Brazil. For extraction of the oil, the air-dried seeds were repeatedly crushed and homogenized with a pestle in a mortar under petroleum ether (40–60°C). After filtration, the solvent was removed by vacuum distillation at 30°C, and the extract was flushed with nitrogen and stored at −18°C until use.

Transesterification to the FA methyl esters (FAME) was carried out with 0.5 N sodium methoxide in anhydrous methanol as described by Christie (4). A part of the FAME mixture was allowed to react with BF_3/MeOH and was subsequently silylated with *bis*-(trimethylsilyl)-trifluoroacetamide (BSTFA) to achieve the silylated hydroxy–methoxy derivative of the epoxy acids (5,6). The 4,4-dimethyloxazoline derivatives (OXFA) were prepared by reaction of one part free FA mixture (obtained after hydrolysis of the oil with 1 N potassium hydroxide in 95% ethanol and subsequent acidification with HCl to pH 1) with five parts of 2-amino-2-methylpropanol for 1 h at 170°C (7).

A Hewlett-Packard (Palo Alto, CA) 5890 GC Series II chromatograph, a flame-ionization detector (FID, 240°C), and a split/splitless injector 240°C, split 1:100, with glass insert was used for separation and quantitation of the FAME. Hydrogen was the carrier gas at an inlet pressure of 150 kPa. For peak integration, an HP3392A integrator (Hewlett-Packard) was used. Separation of the compounds was achieved on an HP 20M (Hewlett-Packard) fused-silica capillary column (50 m, 0.2 mm i.d., 0.2 μm phase thickness) using an oven temperature of 195°C.

Gas chromatography–mass spectrometry (GC–MS) analyses were done with the Nermag Automass (Paris, France) operating at an ionization energy of 70 eV, a source temperature of 225°C, and an interface temperature of 220°C. The separation of the FAME and OXFA derivatives was carried out on a BPX70 (Axel Semrau GmbH, Sprockhövel, Germany) fused-silica capillary column (25 m, 0.22 mm i.d., 0.25 μm film thickness) with the following temperature program: 160–230°C, 4°C/min. Helium was used as carrier gas (0.7 bar).

Chemical ionization (CI) GC–MS analysis was carried out on a Hewlett-Packard instrument Model 5890 Series II/5989A with methane as reactant gas (1.2 torr). Separation of the FAME was carried out on a Permabond OV-1 fused-silica capillary column (25 m × 0.32 mm i.d., 0.23 μm film thickness; Macherey-Nagel, Düren, Germany) with the fol-

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lowing temperature program: the column was initially kept at 70°C for 5 min, then programmed from 70 to 240°C at 12°C/min, and the final temperature was held for 30 min. Other operating conditions were split/splitless injector temperature of 280°C, interface temperature of 280°C, and ion source temperature of 200°C. The carrier gas was helium at a flow rate of 1.5 mL/min.

The infrared (IR) spectrum of the oil was obtained with a Shimadzu (Tokyo, Japan) Fourier transform infrared (FTIR)-8101 in a liquid film. The ^{1}H-NMR (200 MHz) and ^{13}C-NMR-spectra [50 MHz, broad-band decoupled and attached proton test (ATP)] were run in $CDCl_3$ solution on a Varian VLX-200 (Palo Alto, CA) spectrometer with trimethylsilyl as internal standard.

RESULTS AND DISCUSSION

Extraction of the seeds of *B. pulchella* with petroleum ether yielded 26.4% of a light-yellow oil. The IR spectrum of this extract revealed two characteristic bands at 825 and 849 cm^{-1} (m), indicating the presence of an epoxy group (8). This finding was confirmed by the ^{1}H-NMR spectrum, which showed, besides the usual seed oil signals, a triplet at δ2.9 attributable to the two protons of a *cis*-epoxy group (9). Because the triplet at δ0.9 can be seen as representative for the terminal methyl group of all present FA, the concentration (mol%) of the total epoxy FA can be obtained by comparing the integration of the latter signal with the integral of the epoxy protons signal at δ2.9 using the following equation:

$$\text{epoxy acids (\% molar fraction)} = 100\,E/M \qquad [1]$$

where E = integral value of the two epoxy protons divided by 2, and M = integral value of the three terminal methyl protons divided by 3. The result of the estimation of the epoxy FA by ^{1}H-NMR analysis of the seed oil of *B. pulchella* is included in Table 1.

To obtain more detailed structure information, the ^{13}C-NMR spectrum of the oil was also recorded. The ^{13}C signals observed at δ27.4, 123.8, 132.4, 26.2 (two not resolved signals), 57.1, 56.5, 27.4, 26.1, 31.7 were almost identical with the values published for the carbon atoms C-8 to C-16 of *cis*-vernolic acid ester from the seed oil of *Vernonia galamensis* (10). Due to the predominance of the epoxy FA (see Table 1), the ^{13}C-NMR signals of the common FA were weak under the experimental conditions.

GC and GC–MS analyses of the FAME indicated the presence of some usual FA in low abundance (Table 1), identified by co-injection of standard compounds and by comparison of the mass spectra with the spectra of standards. The predominant GC peak (91%) was eluted after the common FAME, and its electron impact (EI) and CI mass spectra were identical with that of authentic vernolic acid methyl ester. Because the well-known tendency of double bonds to migrate along the aliphatic carbon chain of FAME during ionization prevents the exact identification of the original position of the double bond and of the epoxy ring in unsaturated epoxy FAME, the FAME mixture was allowed to react with BF_3/MeOH. FAME that contain an oxirane ring are thus transformed into isomeric methoxy–hydroxy derivatives, and after further trimethylsilanization of the OH-group, the mass spectra of these derivatives (Fig. 1) are less complicated and enable the correct structure assignments (5,9). Because in earlier works, separation of the two resulting positional isomers of the silylated methoxy–hydroxy derivatives was not possible on packed GC columns, the mass spectra of the isomer mixture

TABLE 1
The Fatty Acid Composition of the Seed Oil of *Bernardia pulchella*

Fatty acid	Weight percentage
16:0	1.05
18:0	1.05
18:1n-9	0.42
18:1n-7	0.31
18:2n-6	5.18
18:3n-3	0.14
20:0	trace
20:1n-9	0.14
?	trace
Vernolic acid	90.97
Unknown epoxy acid	0.66
Total epoxy acids by ^{1}H-nuclear magnetic resonance integral analysis (mol%)	90.8

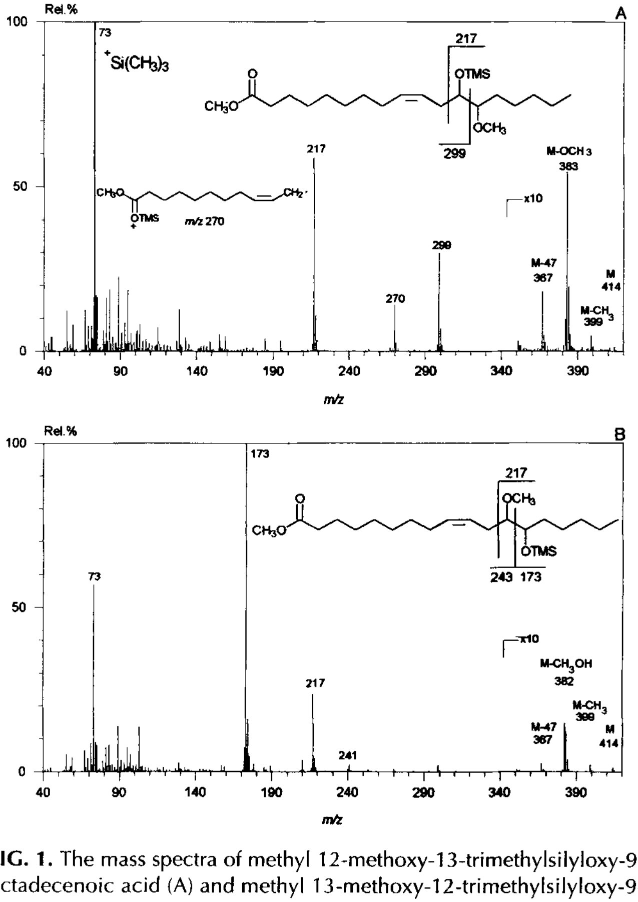

FIG. 1. The mass spectra of methyl 12-methoxy-13-trimethylsilyloxy-9-octadecenoic acid (A) and methyl 13-methoxy-12-trimethylsilyloxy-9-octadecenoic acid (B). TMS, trimethylsilyl.

were published (5,9). By using a capillary GC column, it was now possible to obtain the mass spectra (Fig. 1) of the individual isomers, and the compounds could be identified subsequently as methyl 12-methoxy-13-trimethylsilyloxy-9-octadecenoic acid and methyl 13-methoxy-12-trimethylsilyloxy-9-octadecenoic acid by analyzing their fragmentation patterns. The spectra confirmed the location of the original oxirane ring between C-12 and C-13, and the position of the double bond at C-9 was indicated by the abundance of the peak at *m/z* 217 (Fig. 1A) (5,9). Thus, in conjunction with the other spectroscopic data, the main epoxy FA was identified as *cis*-12,13-epoxy-octadec-*cis*-9-enoic acid (vernolic acid).

Another approach to overcome the problem of bond migration of unsaturated FAME during ionization is the remote-site derivatization to 2-substituted OXFA by condensation of the free FA with 2-amino-2-methylpropanol. Such derivatives show improved MS properties due to enhanced charge stabilization and the reduced tendency of bond migration as well as appropriate GLC properties (7). It was shown recently (11) that the mass spectra of synthetic oxazoline derivatives of unsaturated epoxy FA can be useful for their structure elucidation but that the formation of oxidation products can be a problem during the derivatization reaction. To evaluate this method in seed oil analysis, the oxazoline derivatives of the FA mixture from *B. pulchella* also have been examined. Under our experimental conditions, however, the expected oxazoline derivative of vernolic acid did not appear in the chromatogram after the oxazoline derivatives of the usual FA. Instead, some new minor peaks with similar peak areas were observable at higher retention times than predicted for the corresponding oxazoline derivative of vernolic acid. The mass spectra of these oxazoline derivatives indicated that oxidation must have occurred during derivatization. Thus, this derivatization method does not appear to be suitable for this type of analysis.

An unknown minor peak (0.66%), which eluted after vernolic acid, disappeared in the GC after reaction with BF_3/MeOH and subsequent silylation. Instead, a pair of two new minor peaks appeared after the pair of peaks from methyl 12-methoxy-13-trimethylsilyloxy-9-octadecenoic acid and methyl 13-methoxy-12-trimethylsilyloxy-9-octadecenoic acid. This observation shows that the unknown compound must have reacted with the used reagents. As the GC retention time shift of these two peaks in relation to the original minor peak was proportional to the shift from vernolic acid and its corresponding methoxy–trimethylsilyloxy derivatives, it is probable that the unknown compound also may contain an oxirane ring. Further structure analysis of this compound is in progress.

Seed oils that contain epoxy fatty acids are of potential interest as stabilizers in plastic formulations and attain a role in coatings and adhesive technology (12). By using nucleophilic agents, the oxirane ring can be opened to produce a series of trifunctional compounds. The technical applications of most of these products have not been fully investigated. Although epoxy FA can be obtained by epoxidation of unsaturated FA, natural sources of these compounds are of interest for the future (13). Important for commercial suitability of a crop as source for epoxy oils is its absolute oil content and the content of epoxy acid(s). As the seed oil of *B. pulchella* revealed more than 90% vernolic acid, it would be interesting to consider this wild plant for breeding experiments. To our knowledge, *B. pulchella* seed oil has the highest natural epoxy fatty acid content yet described for a seed oil.

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