Improved Chromatographic Method for the Purification of Phenolic Constituents of the Lichen *Parmotrema tinctorum* (Nyl.) Hale

G.K. Jayaprakasha*, L. Jaganmohan Rao, R.P. Singh, and **K.K. Sakariah**
Central Food Technological Research Institute, Mysore-570 013, India

Abstract

A method for the separation and fractionation of the phenolic compounds atranorin, methyl orsenillate, orsenillic acid, and lecanoric acid from the lichen *Parmotrema tinctorum* (Nyl.) Hale (*P. tinctorum*) using thin-layer chromatography (TLC) and column chromatography, respectively, is described. TLC of lichen extract with an acid-free developing solvent on silica gel G containing 1% oxalic acid is carried out, and the compounds are visualized as yellow spots when sprayed with 10% sulfuric acid in methanol followed by heating at 110°C. For the fractionation of pure lichen compounds, column chromatography is carried out using silica gel impregnated with 1% oxalic acid. To our knowledge, it is also the first report on the isolation and characterization of methyl orsenillate and orsenillic acid from *P. tinctorum* (Nyl.) Hale.

Introduction

Thin-layer chromatography (TLC) is an important tool for the identification of various lichen compounds such as depsides, depsidones, and depsones. TLC methods using three different solvent systems (1,2) and two-dimensional TLC have been developed for the separation of lichen compounds (3). Diethyl ether was one of the components of the solvent systems described in these methods. Lichenologists found handling of these solvent systems difficult because of the fast evaporation of the diethyl ether component, consequent compositional changes in the system, and the hazardous nature of diethyl ether (4). A higher boiling ether, methyl *tert*-butyl ether (MTBE), was attempted to substitute diethyl ether for TLC separation (5). Although MTBE was safe for use, it is not a commonly available solvent; hence the use of MTBE is limited.

Parmotrema tinctorum is an abundantly growing foliose lichen in Southern India that is widely used in food products. In the present work, acid-free developing solvent systems for TLC of lichen compounds were developed that avoid the use of either diethyl ether or MTBE. For the fractionation of lichen compounds by column chromatography, silica gel impregnated with 1% oxalic acid was used, and the fractionated compounds were characterized by nuclear magnetic resonance (NMR) spectroscopy. To our knowledge, this is the first report on the isolation and characterization of methyl orsenillate and orsenillic acid from *P. tinctorum* (Nyl.) Hale.

Experimental

All the solvents used were of analytical reagent grade. Silica gel (60–120 mesh size) was obatined from BDH (Bombay, India). Silica gel G (particle size, 10–40 µm) and solvents were obatined from Merck (Bombay, India). ^{1}H NMR and ^{13}C NMR spectra were recorded at 400 and 100 MHz, respectively, on a Bruker AMX 400 FT instrument (Bruker, Rheinstetten, Germany). ^{13}C NMR spectral assignments were given on the basis of spin–echo Fourier transform (SEFT) spectra. Tetramethyl silane was used as the internal standard.

The TLC slurry was prepared by suspending 40 g of silica gel G (particle size, 10–40 µm) in 100 mL of water containing 0.4 g of oxalic acid and was applied on glass plates using the applicator. For column chromatography, 0.5 g of oxalic acid was dissolved in 100 mL of methanol and impregnated on 50 g of silica gel (60–120 mesh size). The gel was dried in an oven at 100°C for 30 min. Sulfuric acid (10%) in methanol was used as the spraying reagent.

The lichen sample was collected from a local market. The species was identified by the International Mycological Institute (Egham, Surrey, U.K.) as *Parmotrema tinctorum* (Nyl.) Hale. A voucher specimen was deposited in the reference collection center (IMI No. 367182). For the extraction of lichen compounds, air-dried lichen powder (40 g) was extracted with acetone for 36 h with occasional stirring. The extract was filtered and evaporated in vacuo to get dry powder (8.0 g).

TLC of lichen compounds

Glass plates (20 × 20 cm) were coated with the silica gel G slurry to a uniform thickness of 0.25 mm. The plates were acti-

* Author to whom correspondence should be addressed.

vated by heating in an oven at 110°C for 1 h and stored in a desiccator. A 5-µL amount of lichen extract was spotted on the plate. The plate was developed to a height of 15 cm in a TLC chamber previously saturated with the solvent systems given in Table I by lining the chamber with filter paper. The plates were removed from the chamber and air-dried. Compounds were detected by spraying the plates with 10% sulphuric acid in methanol followed by heating at 110°C for 10–15 min.

Table I. TLC Pattern of Lichen Compounds

Compound	R_f values: Hexane–ethylacetate (80:20)	Chloroform–methanol (98:02)	Benzene–ethylacetate (95:05)
Atranorin	0.92	0.95	0.94
Methyl orsenillate	0.58	0.69	0.66
Orsenillic acid	0.33	0.37	0.31
Lecanoric acid	0.30	0.23	0.27

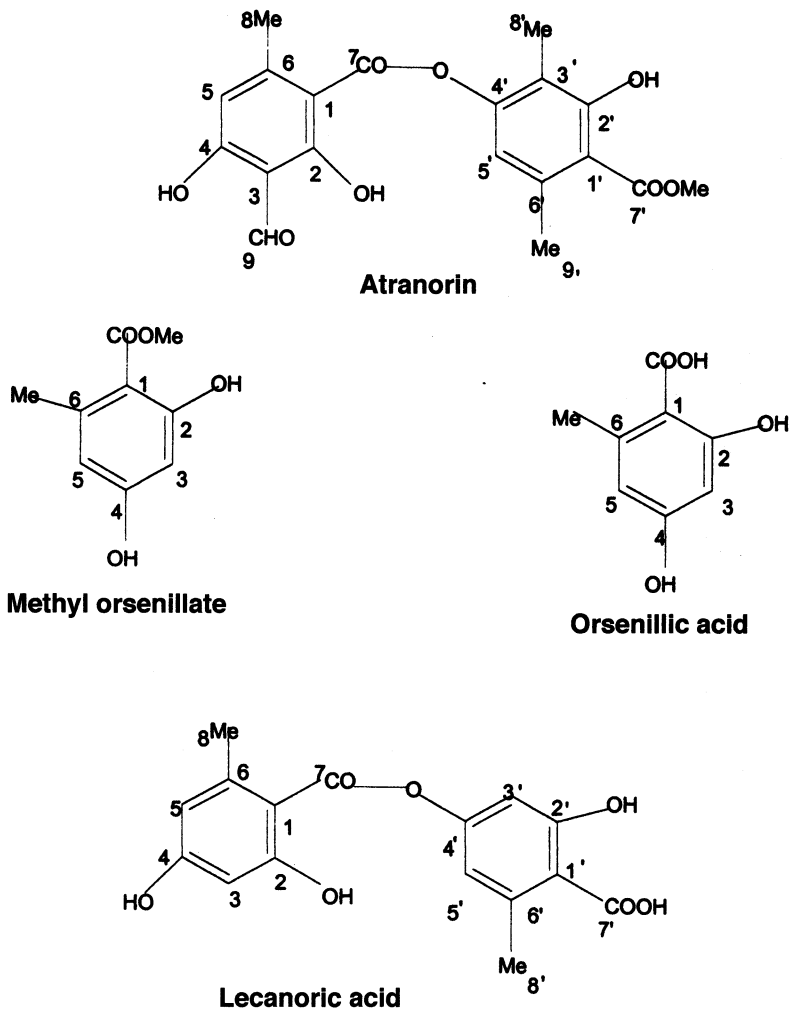

Figure 1. Structures of the four phenolic compounds of interest in this study.

Column chromatography of lichen compounds

Silica gel slurry (oxalic acid impregnated) was prepared in hexane and packed in a column (60 cm × 18 mm). Acetone-extracted lichen powder was dissolved in a minimum quantity of acetone, impregnated onto the silica gel, and loaded onto the column. The compounds were eluted regularly as the polarity of the eluent increased. Atranorin was eluted with hexane–benzene (3:1), whereas methyl orsenillate, orsenillic acid, and lecanoric acid were eluted with benzene and 2 and 5% ethyl acetate in benzene, respectively. The solvents from the eluates were evaporated in vacuo, and the compounds in the residue were recrystallized in dichloromethane (for atranorin), benzene (for methyl orsenillate), ether (for orsenillic acid), and 50% acetone in water (for lecanoric acid). Atranorin, orsenillic acid, and lecanoric acid crystallized as needles and yielded 450 mg, 640 mg, and 2.34 g, respectively; methyl orsenillate crystallized as polygonal crystals and yielded 880 mg.

Preparation of pure samples for TLC

Atranorin was dissolved in chloroform, whereas the other compounds were dissolved in acetone to make a solution of 1 mg/mL. Aliquots (5 µL) of each compound were spotted on the TLC plates. Retardation factors (R_f) were determined after development using the solvent systems given in Table I.

Identification of compounds

The melting points of atranorin, methyl orsenillate, orsenillic acid, and lecanoric acid were recorded as 187–88, 138–40, 172–75, and 172–74°C, respectively. The structures of the compounds (see Figure 1) were identified by ^1H NMR and ^{13}C NMR spectra.

Results and Discussion

The different solvent systems used for developing chromatograms and the R_f values of lichen compounds are shown in Table I. All lichen compounds appeared as stable yellow spots on developed TLC plates. The sensitivity of the method was 0.5 µg.

The compounds were characterized and identified as atranorin, methyl orsenillate, orsenillic acid, and lecanoric acid using ^1H NMR and ^{13}C NMR spectra. Chemical shifts of compounds were matched with reported values (6–9).

Often many TLC solvent systems contain a small amount of an acid, such as formic acid and acetic acid, the purpose of which is to suppress dissociation of the compounds being chromatographed and thereby prevent "tailing" of the spots. In earlier reports, the prepared silica gel plates and spotted plates were both exposed to an

acidic atmosphere of formic acid for better resolution (10). Similarly, in another method, acetic acid was used to prevent tailing of the phenolic lichen products (1,2). Also, in two-dimensional chromatography, unknown samples cannot be compared on the same chromatogram, and labile compounds may be decomposed before second-dimension chromatography (3). It was noticed in the present study that without using acidic medium, tailing of spots was observed on the TLC plates (Figure 2), and pure compounds could not be obtained using silica gel column chromatography. In the solvent systems containing acetic acid, phenolic compounds have the disadvantage that they often move as an irregular spot immediately ahead of a sharp secondary solvent front some distance below the true solvent front. This secondary front arises from the frontal analysis or demixing of the solvent mixture. Thus silica gel containing 1% oxalic acid was attempted, both for TLC and column chromatography. Several concentrations of oxalic acid were tried for the preparation of the plates. The range from 1 to 2% (w/w) in silica gel appeared to be satisfactory, although it was not critical. At higher concentrations of oxalic acid, the plates tended to be even less, whereas at low concentrations, the plates required more activation. Lichen extract after development on these plates showed clear spots with good separation from one another, and the compounds were separated on column chromatography.

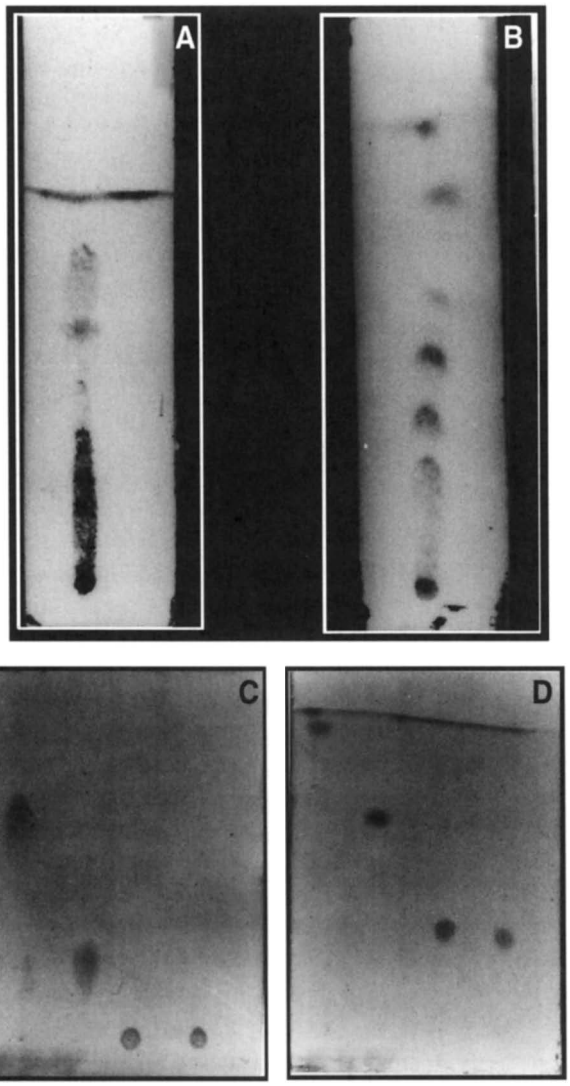

Figure 2. TLC of lichen products using the benzene–ethyl acetate (95:5) solvent system. (A) Crude extract on silica gel. (B) Crude extract on silica gel containing 1% oxalic acid. (C) Purified compounds on silica gel. (D) Purified compounds on silica gel containing 1% oxalic acid.

Conclusion

In the present study, solvent systems were developed for the separation of lichen compounds that could avoid the use of unstable and scarce solvents. Also, the use of oxalic acid in the preparation of column material facilitated the clear separation of the lichen compounds on column chromatography. The developed method was satisfactorily applied for the separation and fractionation of lichen compounds of another lichen species, *Parmotrema stuppeum*. To our knowledge, this is the first report for methyl orsenillate and orsenillic acid from *P. tinctorum*.

Acknowledgment

The authors thank Dr. B.C. Sutton, head of Biosystematics Services, International Mycological Institute, U.K., for identifying the lichen sample.

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Manuscript accepted September 9, 1997.