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Molluscicides from Olive *Olea europaea* and Their Efficient Isolation by Countercurrent Chromatographies

Two iridoid glycosides possessing a molluscicidal property, oleuropein and ligstroside, were isolated from fresh fruits of olive *Olea europaea* by using two efficient countercurrent chromatographies, rotation locular countercurrent chromatography (RLCC) and droplet countercurrent chromatography (DCCC). This is the first isolation of ligstroside from olive.

In our preliminary screening for naturally occurring molluscicides for the control of schistosomiasis (Henderson et al., 1983; Kloos and McCullough, 1983; Kubo et al., 1983b), we found that the crude methanol extract of fresh fruits of bitter olive *Olea europaea* (Oleaceae) exhibited molluscicidal property against the South American snail *Biomphalaria glabratus*. Although the chemical constituents in this plant have been extensively studied (Schneider and Kleinert, 1972; Inoue et al., 1974), it was not known which of the constituents were responsible for this observed biological activity. Separation of the crude extract into ether, ethyl acetate, and water-soluble portions indicated the active components were in the ethyl acetate portion. Biological activity was monitored as previously described (Nakanishi and Kubo, 1977). Due to its polar nature, the bioactive ethyl acetate extract seemed ideally suited for further separation by countercurrent chromatography as has been previously applied to the resolution of many polar mixtures (Hostettmann, 1980; Kubo et al., 1983a).

This communication describes the efficient isolation of two molluscicidal iridoid glycosides by two countercurrent chromatographies, rotation locular countercurrent chromatography (RLCC) and droplet countercurrent chromatography (DCCC).

MATERIALS AND METHODS

Materials. A methanol extract (162.4 g) was obtained from fresh fruits of *O. europaea* (2.1 kg), which were collected in the University of California Berkeley campus in Sept 1982. Then, for the further separation, the methanol extract was partitioned into ether (6.8 g), ethyl acetate (24.6 g), and water-soluble (131.0 g) portions.

RLCC. A RLCC separation was performed on a Model RLCC—A (Tokyo Rikakikai Co., Tokyo, Japan). The RLCC solvent system of chloroform–methanol–water (13:7:4 v/v) was chosen by prescreening the components of the ethyl acetate extract on a TLC plate (Macherey, Wagel and Co., Duren, GFR, Polygram Sil G/UV 254). The upper phase was chosen as the mobile phase in our RLCC system. The crude methanol extract (1.0 g) was dissolved in a (1:1 v/v) mixture of the mobile and stationary phases and injected into the RLCC apparatus by using a 3-mL sample chamber. The eluents were collected in 1.4-mL fractions. Fractions were monitored by TLC (Sil G/UV 254) with the organic layer of this solvent system. Visualization of the compounds on the TLC plate was accomplished by UV spectroscopy (Chromato–UVE Cabinet, Model CC-60, Ultra Violet Products, Inc, CA) and using vanilin–sulfuric acid–ethanol 3 g:1.5 mL:100 mL) as a spray reagent.

DCCC. A Model DCC-300-G2 (Tokyo Rikakikai Co., Tokyo, Japan) with 300 glass columns (400 mm × 2 mm i.d.) was used for the DCCC separation. The same solvent system as for RLCC was chosen, and the ethyl acetate extract (3.8 g) was dissolved in the solvent of the mobile phase and injected into the DCCC apparatus by using a 10-mL sample chamber. The eluents were collected in 1.5-mL fractions. Fractions were monitored with the same way as on the RLCC separation.

Biological Assay. A molluscicidal activity was monitored as described previously (Nakanishi and Kubo, 1977).

RESULTS AND DISCUSSION

On a preliminary examination of the molluscicidal activity of the crude methanol extract of olive fruits, the extract killed the South American snail *B. glabratus* within 2 h at 2000 ppm. For further screening, the methanol extract was separated into ether, ethyl acetate, and water-soluble portions. The ethyl acetate extract was found to have the molluscicidal activity.

The R_f values of the main components in the active ethyl acetate extract were determined to be 0.2–0.5 on a TLC plate by using the organic layer of chloroform–methanol–water (13:7:4 v/v). In order to isolate such polar compounds, the very simple RLCC method was employed. In order to prevent any of loss of the active principles, the crude methanol extract was used for the RLCC separation. Figure 1 provides the weights of eluted compounds vs. the fraction number. Even though RLCC requires only small

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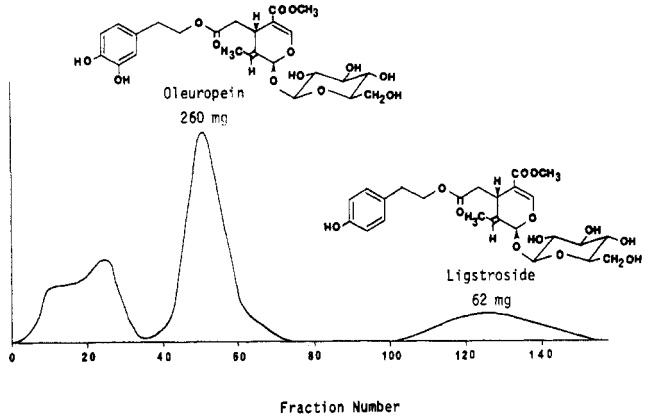

Figure 1. RLCC of the methanol extract of *O. europaea* (1.0 g) with $CHCl_3$–MeOH–H_2O (13:7:4 v/v) by the ascending method: 1.4 mL/fraction; 3 days.

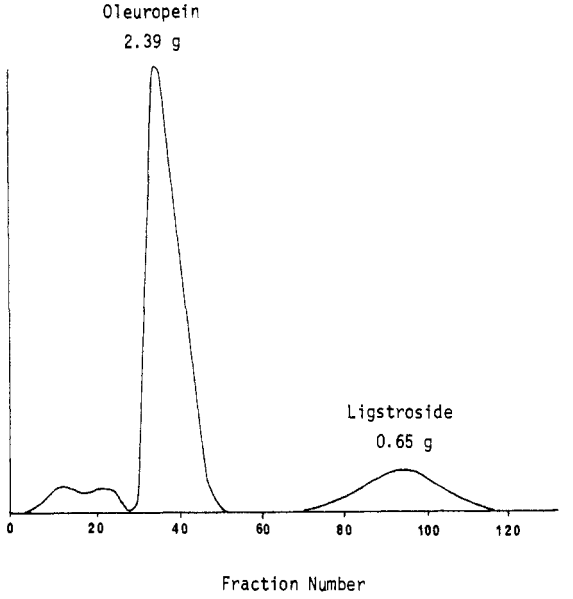

Figure 2. DCCC of the ethyl acetate extract of *O. europaea* (3.78 g) with $CHCl_3$–MeOH–H_2O (13:7:4 v/v) by the ascending method: 1.5 mL/fraction; 5 days.

volumes of solvents, two pure compounds were radpily and nondestructively isolated. It took about 3 days for this separation.

On the other hand, an alternative countercurrent chromatography, DCCC, method of separation was also examined. The ethyl acetate extract was used for this procedure. Figure 2 shows the weights of elutes vs. fraction number of this separation. This procedure took 5 days.

Spectral determination of the isolated compounds were made from various spectral techniques (UV, IR, SI-MS, ^1H NMR, and ^{13}C NMR) and showed them to be the bitter iridoid glycosides oleuropein (1) (Panizzi et al., 1960) and ligstroside (2) (Asaka et al., 1972). Spectral studies of the acetylated derivatives of these compounds confirmed the structures. This is the first time that RLCC has been applied to the separation of a plant constitution like iridoid glycosides and is also the first isolation of ligstroside from olive.

The molluscicidal activities of oleuropein and ligstroside were LD_{50} = 250 and 100 ppm, respectively, within 24 h against *B. glabratus*. When the olive is used for human food, these bitter iridoid glycosides are removed by extraction into water during the curing process. Perhaps some further commercial use could be made for these toxic glycosides, which at present are discarded.

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