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Antifungal Activities of Different Extracts and Echimidine-N-oxide from *Symphytum sylvaticum* Boiss. subsp. *sepulcrale* (Boiss. & Bal.) Greuter & Burdet var. *sepulcrale*

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Abstract: Antifungal activity was determined by the tube dilution method on different extracts of *Symphytum sylvaticum* Boiss. subsp. *sepulcrale* (Boiss. & Bal.) Greuter & Burdet var. *sepulcrale*. The antifungal activity of the isolated compound from the root alkaloid fraction (Echimidine-N-Oxide) was also tested against ten fungal cultures. The activity was found to be mainly due to

Echimidine-N-Oxide (ENO). The quantitative determination of ENO was also carried out with capillary gas chromatography in the roots and the aerial parts of the plant.

Key Words: *Symphytum sylvaticum*, pyrrolizidine alkaloids, echimidine-N-oxide, antifungal activity, GC analysis

Introduction

There are 17 *Symphytum* species (*Boraginaceae*) growing in Turkey and eight of these are endemic (1-3). *Symphytum officinale* L. (Comfrey) has been applied topically in the treatment of inflammatory disorders, especially in Europe. It is considered to be useful in several skin complications such as chronic wounds, burns, sores, eczema and leg ulcers (4,5). There are reports of hepatotoxicity attributed to pyrrolizidine alkaloids present in comfrey preparations (6). They represent a serious health risk not only to livestock and animals, but also to human populations which may be exposed to them either through contamination of foodstuffs or in herbal teas or medicines (7). The external use of *Symphytum* species is still under discussion; additional research is necessary in order to determine its safety. In another study, which is to be published shortly, the isolation and structure elucidation of major pyrrolizidine alkaloids from *Symphytum sylvaticum* subsp. *sepulcrale* var. *sepulcrale* and *Symphytum aintabicum* are given in detail. HPLC and GC methods have been reported for the quantitation of pyrrolizidine alkaloids (8-11). Among these, GC was found to be the most useful and selective technique for the routine analysis of pyrrolizidine alkaloids. There are no reports in the literature about the antifungal activity of *Symphytum* species. The aim of this study was to investigate the antifungal activity of different extracts and the major pyrrolizidine alkaloid from *Symphytum*

sylvaticum subsp. *sepulcrale* var. *sepulcrale*. In addition to the antifungal activity, the quantity of the major alkaloid (Echimidine-N-oxide) in different parts of the plant was determined by gas chromatography.

Materials and Methods

Plant material

Symphytum sylvaticum Boiss. subsp. *Sepulcrale* (Boiss. & Bal.) Greuter & Burdet var. *sepulcrale* was collected from Rize-İkizdere, 1200-1400 m, during the flowering stage, in July 1993. The plant specimen has been deposited at the herbarium of the Faculty of Pharmacy, Ankara University, Ankara-TURKEY (AEF 17908).

Extraction procedure for antifungal activity

Dried powdered 400 g roots and 400 g aerial parts of the plant were each extracted with ethanol at room temperature by continually stirring and maceration. The mixture was filtered and evaporated in vacuo to a gummy residue. The extraction procedure was repeated for 7 days in the same manner and the combined extracts were evaporated under vacuum at 50°C. The details of the extraction procedure are given in Fig. 1. Then 780 mg and 375 mg of total crude alkaloid extracts were obtained from the roots (ARF) and the aerial parts (AAF) of the plant respectively.

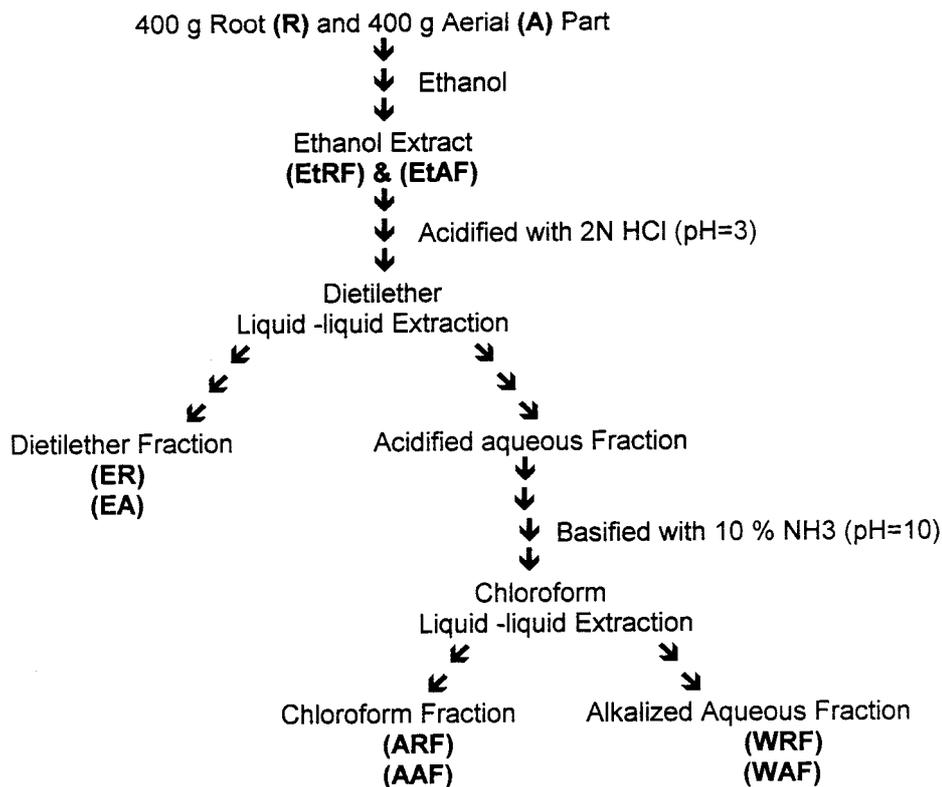


Figure 1. Extraction Procedure of *Symphytum sylvaticum* subsp. *sepulcrale* for Antifungal Activity.

Isolation of echimidine-N-oxide (ENO)

Crude alkaloid extract (9.39 g) from the roots of the plant (4.5 kg) was chromatographed on a silica gel column eluting with chloroform and chloroform:methanol and 100 ml fractions were collected and combined according to TLC. Fractions 95-105 eluted with chloroform:methanol (80:20) showed a strong alkaloid reaction. Evaporation of fractions 95-105 yielded 1.50 g. This fraction was loaded on another silica gel column and elution was started with chloroform; 50 ml fractions were collected and almost pure alkaloid (350 mg) was obtained between fractions 30 and 35 using 8% methanol in chloroform. Further purification of the alkaloid mixture was achieved by preparative TLC on silica gel plates (Merck-7748). ENO was obtained using $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{CH}_3\text{COCH}_3$, 10:7.5:2.5 (R_f 0.25) as eluent and was detected by Dragendorff reagent. The structure of ENO was elucidated based on IR, EIMS, ^1H , ^{13}C NMR analysis and also 2D NMR (COSY, HMBC, HMQC) experiments.

Gas chromatographic analysis

The gas chromatographic (GC) analysis was performed with a Varian instrument, model 3700, using a fused silica capillary column (0.32 mm x 30 m; SUPELCOWAX™10), carrier gas N_2 (1 kg/cm²). The injection temperature was 200°C (split was 1:10), flame-ionization detector (FID) temperature 250°C. The temperature programme of the column was 120°C-250°C at 5°C/min.

The calibration range for ENO was 2-20 mg/ml and 1 µl injections were made for each standard solution. The means of the peak areas at each concentration were plotted versus concentration (mg/ml), and a calibration curve was drawn. The least-square equation of the regression line and correlation coefficient of ENO was calculated.

Extraction for GC analysis

One hundred grams dried powdered roots and aerial parts of the plant were each extracted with methanol in a

soxhlet apparatus. The methanol was evaporated in vacuo at 50°C to a gummy residue. The crude alkaloid extracts were obtained with the same extraction procedure used for antifungal activity determination (Fig. 1). The extraction procedure was applied to duplicate roots and aerial parts samples. The total crude alkaloid extracts were obtained from the roots (190 mg and 208 mg) and the aerial parts (91 mg and 95 mg) of the plant. The solutions were prepared in methanol and 1 µl solutions were injected into the gas chromatograph as described above.

Medium

Mueller Hinton broth liquid medium was used for two-fold dilution of ENO for MIC determination. Sabouraud dextrose agar (Merck-5438) was used for anti-fungal activity assays of ENO and different extracts from the plant.

Test microorganisms

Anti-fungal studies were carried out against *Epidermophyton floccosum*, *Microsporum canis*, *Nigrospora oryzae*, *Allefsheria boydii*, *Pleuretus ostreatus*, *Stachbotrys atra*, *Curvularia lunata*, *Drechslera rostrata*, *Aspergillus niger* and *Candida albicans*. Pure fungal cultures were obtained from the Microbiology Department of H.E.J. Research Institute of Chemistry, University of Karachi.

Inoculation suspension

Fresh cultures of fungus suspensions at McFarland 0.5 density (10^8 CFU/ml) were used for inoculation.

Antifungal activity assay

For the antifungal activity test, 5 ml of medium (SDA) was added to each screw-capped test tube and they were autoclaved at 121°C 15 min. Tubes having 5 ml sterile SDA were inoculated with ENO (200µg/ml) and extracts (400µg/ml) in DMSO. Tubes were kept in the salutation position overnight for checking the sterility. The next day, the tubes were inoculated with fungal culture on the salutation position and all the test tubes were kept for ten days at 27-30°C incubation. Each compound or extract was tested against ten fungal cultures. After 10 days, the results were noted. The degree of activity was recorded in four grades according to the inhibition of growth: (-) inactive, (+1) less active, (+2) moderately active, and (+3) strongly active. One of the control tubes contained 5 ml SDA and 0.1 ml of fungal culture, the next control

tube contained 5 ml SDA and 0.1 ml DMSO, and the last two control tubes contained 5 ml SDA, griseofulvin in DMSO (100µg/ml) and fungal cultures of *Epidermophyton floccosum* and *Microsporum canis*, respectively as a positive control of the assay.

Results and Discussion

The results of the antifungal study are shown in Table 1. In each extract some activity was observed for certain fungi. However, evaluation of the extracts for antifungal property clearly indicated that the fungicidal activity was maximum in root alkaloid extract (ARF). This may lead to the conclusion that the alkaloids of this plant are responsible for this activity. Traditionally, the aqueous extract of *Heliotropium* (*Boraginaceae*) species are used topically for fungal infections of the feet in Turkey. This usage is generally attributed to pyrrolizidine alkaloids. There are reports in the literature on the antimicrobial activity of pyrrolizidine alkaloids from *Heliotropium* species (12,13). After isolation, the structure of the major alkaloid from the root was elucidated, based on IR, EIMS, ^1H , ^{13}C analysis and 2D NMR (COSY, HMBC, HMQC) experiments, to be Echimidine-N-oxide. As estimated from the results of the extracts, strong inhibitory activity for nine fungal cultures out of ten (except *C. albicans*) was demonstrated for ENO at 200µg/ml.

The quantitative determination of ENO was carried out with capillary gas chromatography, which yielded greatly improved resolution without derivatization of pyrrolizidine alkaloids. The linear regression equation, $y = 57700.05X - 65452.40$ (correlation coefficient $r^2 = 0.999$) was used to determine ENO in the range of 2-20 mg/ml. This method permits the quick and reliable determination of the pyrrolizidine alkaloids in *Symphytum* species. The percentage of crude alkaloid level, the amount of ENO and standard deviations of analysis for ENO in the duplicate samples of the roots and the aerial parts are shown in Table 2. Dried samples of plant gave a yield of total alkaloids of 0.199% in the roots and 0.093% in the aerial parts. The major alkaloid in the roots was Echimidine-N-oxide. The ENO content was higher in the roots (0.1078%) than in the aerial parts of the plant (0.0061%). The yield of ENO was 55.96-57.54% in the root (Fig. 2) and 6.66-6.86% in the aerial part (Fig. 3) crude alkaloid fraction. This

Table 1. Antifungal activity of extracts and ENO on fungal cultures.

Fungal Cultures	Different Extracts from <i>Symphytum sylvaticum</i> subsp. <i>sepulcrale</i> var. <i>sepulcrale</i>								Isolated Alkaloid ENO
	EtRF	EtAF	ARF	AAF	WRF	WAF	ER	EA	
<i>Epidermophyton floccosum</i>	++	+++	+++	+++	+++	++	+++	+++	+++
<i>Microsporum canis</i>	+++	+	+++	+	+++	-	++	-	+++
<i>Nigrospora oryzae</i>	+	++	++	+	-	-	+++	+++	+++
<i>Allefsheria boydii</i>	-	+	++	-	+	++	+++	+++	+++
<i>Pleuretus ostreatus</i>	+++	+++	++	+	+++	+++	-	+++	+++
<i>Stachbotrys atra</i>	-	++	++	-	-	+++	-	-	+++
<i>Curvularia lunata</i>	++	-	+++	+++	-	-	-	-	+++
<i>Drechslera rostrata</i>	-	+++	-	++	-	+	-	-	+++
<i>Aspergillus niger</i>	-	+	+++	-	-	-	-	-	++
<i>Candida albicans</i>	-	-	-	-	-	-	-	-	-

EtRF: Ethanol extract from root, EtAF: Ethanol extract from aerial part, ARF: Alkaloid fraction from root, AAF: Alkaloid fraction from aerial part, WRF: Water fraction from root, WAF: Water fraction from aerial part, ER: Diethyl ether fraction from root, EA: Diethyl ether fraction from aerial part, ENO: Echimidine-N-oxide
 The degree of activity: (-) inactive, (+1) less active, (+2) moderately active, and (+3) strongly active.
 Tubes having 5 ml sterile SDA were inoculated with the concentration of 200µg/ml ENO or 400µg/ml extracts in DMSO. The tests were done in triplicate.

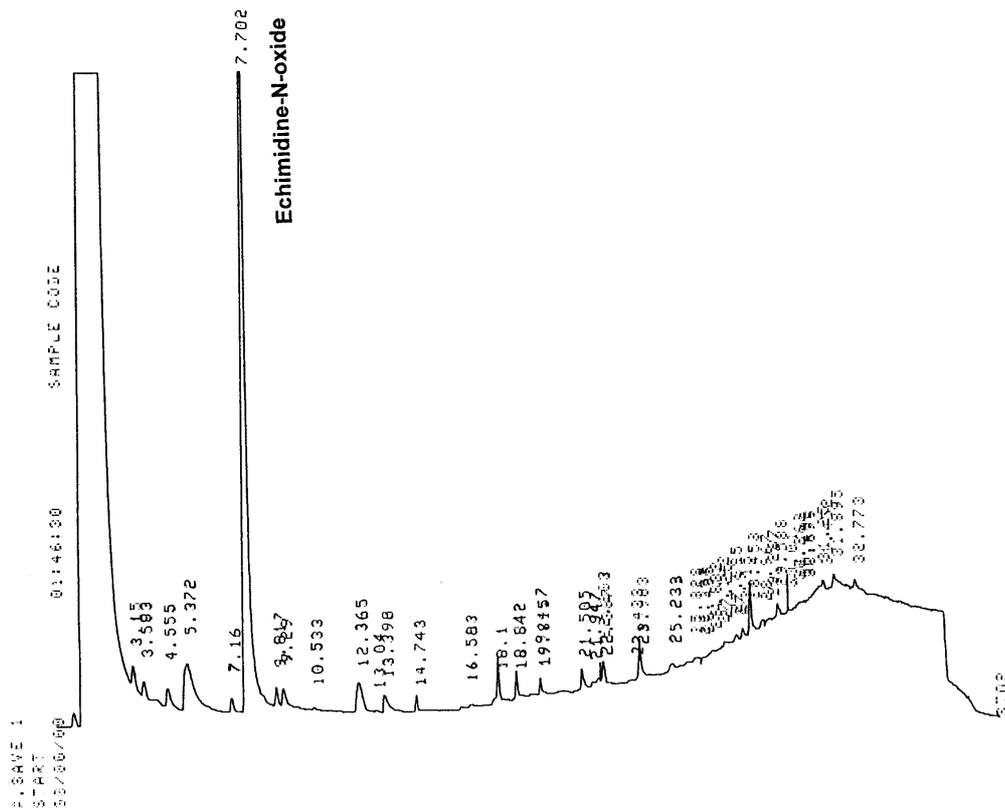


Figure 2. GC Chromatogram of root alkaloid fraction of *Symphytum sylvaticum* subsp. *sepulcrale* var. *sepulcrale*.

<i>Symphytum sylvaticum</i> subsp. <i>sepulcrale</i> var. <i>sepulcrale</i>	Crude Alkaloid %	ENO % \pm SD	RSD %
Root	0.199	0.1078 \pm 0.0021	1.97
Aerial Part	0.093	0.0061 \pm 0.0001	2.18

Table 2. The percentage of crude alkaloid level and ENO in the root and aerial part of *Symphytum sylvaticum* subsp. *sepulcrale* var. *sepulcrale*.

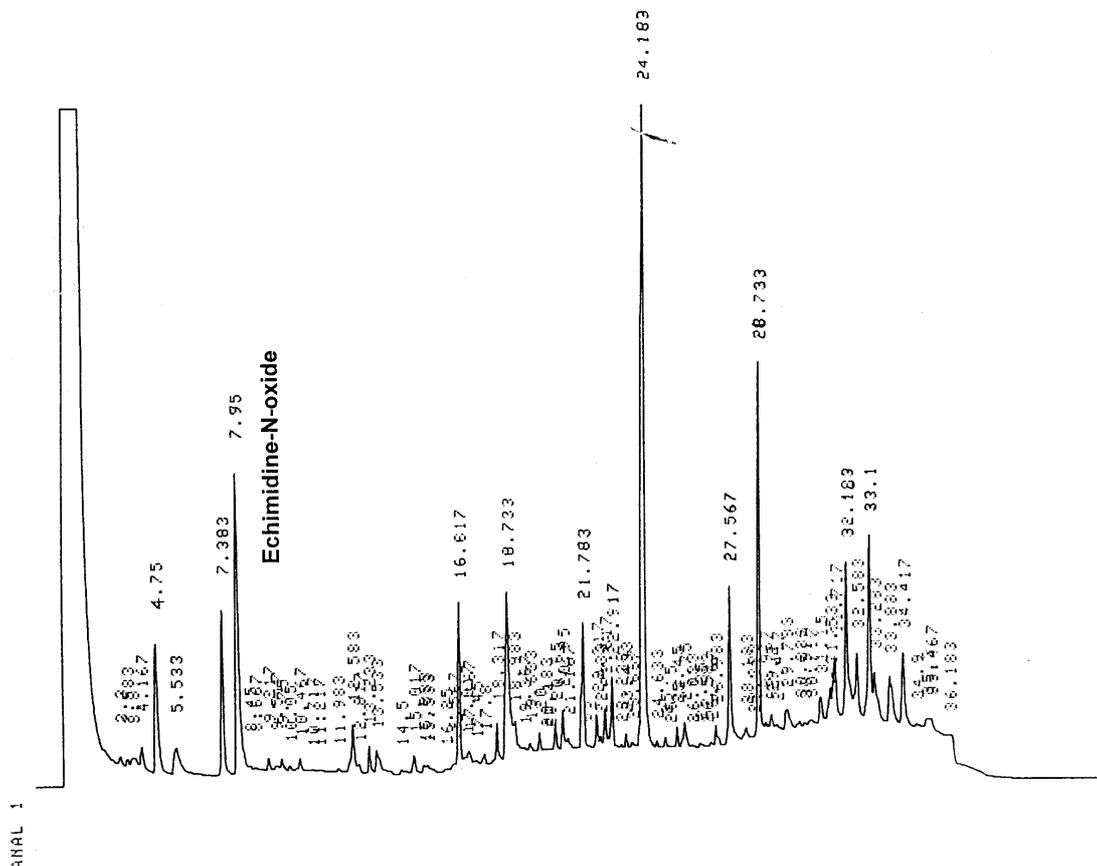


Figure 3. GC Chromatogram of aerial part alkaloid fraction of *Symphytum sylvaticum* subsp. *sepulcrale* var. *sepulcrale*.

explains why the alkaloid fraction from the root was more active than the alkaloid fraction from the aerial part compared with the fungal cultures used. Consequently, the activity was found to be mainly due to Echimidine-N-Oxide (ENO). This is the first antifungal study on *Symphytum* alkaloids.

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