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Antiviral Activity of a «Rosmarinus officinalis L.» Extract

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Introduction

Various extracts were produced by different methods during research on the extraction of fractions, from Rosmarinus officinalis L., with antioxidant activity (1, 2). Previous studies also include preliminary research on the antimutagenic effect of some extracts (3, 4).

Several substances derived from plant extracts are known to have antiviral activity in vitro. Moreover, screenings have been reported describing plant extracts as a source of antiviral activity (5, 6). Some substances have been studied for antiviral activity in more detailed reports: flavonoids (7 – 9), alkaloids (10) triterpenoids (11).

Since 1958, about 25 antiviral screening studies concerning 900 plant species from about 150 different plant families have been published, but the responsible antiviral principles of only 37 species have been partially characterized, as referred by *Vanden Berghe* in a recent review (12).

It is not possible within the scope of this paper to review all antiviral extracts from natural sources.

Till today data referring to the Rosemary extracts with antiviral activity have not been published with the exception of the results concerning only one plant-derived molecule named rosmarinic acid (12).

In the present work we attempt to evaluate the activity of some Rosemary extracts against herpes simplex virus replication by plaque reduction assay. The extracts were also assayed for toxicity in uninfected cell cultures by the Trypan blue exclusion method.

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Experimental

This section deals with:
(i) the method of production of the extracts to be examined from the point of view of their antiviral activity
(ii) the results of the antiviral activity evaluation.

(i) Extract production

Complex extracts were first examined. Identification and isolation of active components with possible antiviral activity will be the subject of the second step of our research. Figure 1 shows the method of production of the extracts examined in this report.

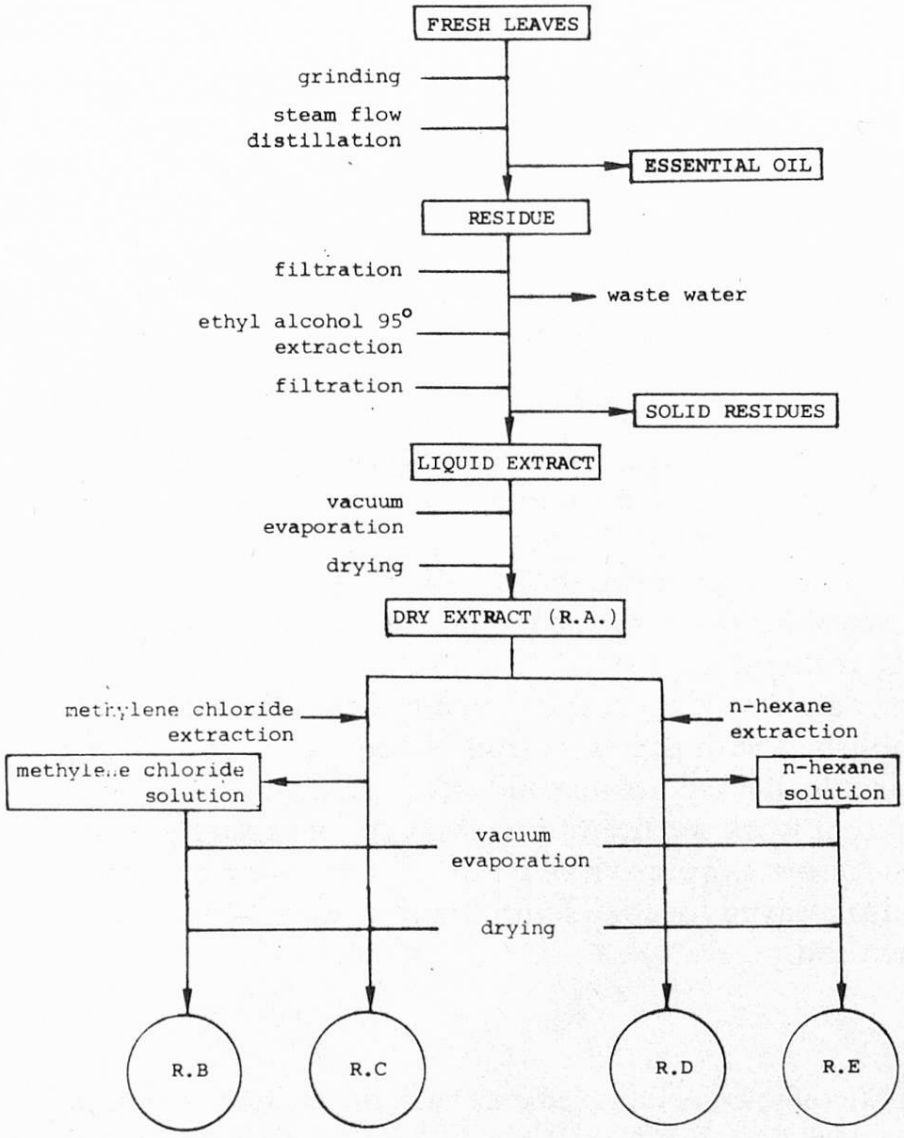

Fig. 1. Flow-sheet of various extracts from «Rosmarinus officinalis L.» produced for the evaluation of antiviral activity

Figure 1 shows the flow-sheet of the most simple production procedures of a dry extract (R. A.), which represent the first step of a more complete process described in a previous paper (4).

In this research, however, the dry R. A. extract undergoes two different kinds of purification: one with n-hexane, the other with methylene chloride as solvent. Both solvents have different molecular dipole values (0 for n-hexance and 1.5 for methylene chloride respectively, according to Debye) and enable the obtention of the four fractions (R. B., R. C., R. D., R. E.) whose antiviral activity is evaluated.

(ii) Antiviral activity evaluation

Materials and methods

Cells and virus

Human embryo lung fibroblast cell cultures (HELF) were grown in Eagle's Minimum Essential Medium (MEM), supplemented with 10% heat-inactivated fetal bovine serum, 50 U penicillin and 50 mg streptomycin per ml. Maintenance medium consisted of MEM with 2% fetal bovine serum and antibiotics.

Herpes simplex virus type 2 (HSV-2) ATCC, strain MS, was propagated in HELF cell culture monolayers. The culture fluids of HSV-infected cells were titrated and stored at –80 °C until use.

Plaque reduction assay

Confluent HELF cells grown in 96-well tissue culture microplates were infected with 50 µl/well virus suspension containing a 10 PFU/ml HSV-2. After 60 min adsorption at 37 °C in a 5% CO_2 atmosphere incubator, the viral inoculum was removed and 150 µl test compound (diluted in 2% MEM) was added to the appropriate well. The compound was assayed at concentrations ranging from 100 to 0.5 µg/ml.

After 24 h incubation at 37 °C under CO_2, the cells were fixed with absolute ethanol and the number of infection plaques was revealed by the immunoperoxidase staining technique (IP).

Immunoperoxidase technique

Fixed monolayers were washed with saline phosphate buffer (SPB) and incubated with an approximate dilution of peroxidase-conjugated rabbit immunoglobulines to herpes simplex virus type 2 (DAYKO). After 60 min incubation at 37 °C under CO_2, the cells were rewashed with SPB. We used a solution contain-

ing 2 mg 3-amino-9-ethyl-carbazole dissolved in 1 ml dimethylsulfoxide, 9 ml acetate buffer (pH 5.5) and 0.1% H_2O_2 as substrate. After 15 min of incubation at room temperature the virus-plaques were examined under the microscope and counted. The antiviral activity of the compound is expressed as ID50 that is the minimal extract concentration reducing plaque formation observed in the control cells by 50%.

Trypan blue exclusion test

The test was performed as described previously (11). Briefly, confluent HELF cells grown in 24-well tissue culture microplates were treated with extract at various concentrations (ranging from 100 to 0.5 μg/ml). After 24 h the monolayers were washed and removed with trypsin. Cell suspensions were subjected to low speed centrifugation and pellets were returned to the appropriate cell suspension: then 0.1 ml was mixed with 0.1 ml of a stock solution of trypan blue (0.5 %). After 10 min the viable cells were counted with a haemocytometer.

Results

The effect of Rosemary and four fractions derived from Rosemary on the replication of HSV-2 was studied by the plaque reduction assay. The compounds were added to cell cultures 60 min after virus inoculation and infection plaques were revealed after 24 h by the immunoperoxydase staining technique.

Table 1. Percentage of virus plaque formation of Rosemary evaluated by plaque reduction assay

Drug	conc.	% inhibition of plaque formation
Control	0	0
Rosemary extract (R. A.)	100 μg/ml	100
	50 μg/ml	70
	10 μg/ml	50
	2 μg/ml	0

Table 1 shows 50% inhibition of virus plaque formation of Rosemary at the concentration of 10 μg/ml. Rosemary fractions were tested by the same methods; ID50 are reported in table 2.

When tested by the same standard plaque reduction assay, the known antiviral molecule ß-glycerrhizic acid (13) used as a positive control, causes 50% plaque

Table 2. I. $D._{50}$ of four fractions obtained from Rosmarinus extract evaluated by plaque reduction assay

Rosemary fractions	I. $D._{50}$
R. B.	50 μg/ml
R. C.	90 μg/ml
R. D.	13 μg/ml
R. E.	11 μg/ml

I. $D._{50}$ = 50% infection plaques inhibiting dose

formation reduction at a concentration of 1.5 μg/ml. These results indicate that two fractions (D and E) possess a similar antiviral activity when added to monolayers 60 min after virus infection. In order to separate the cytotoxic properties from antiviral activity, HELF cells were examined for toxicity when treated with these compounds. Results obtained from the trypan blue exclusion method indicate alteration of cell viability in the presence of fraction D. Evidence for toxicity was not observed in fraction E.

Discussion

The results obtained in this study indicate the presence of a potential antiviral activity of extract against HSV-2.

When tested by a standard plaque reduction assay, Rosemary extract and two fractions derived from Rosemary (D and E) at concentrations of 10, 13 and 11 μg/ml respectively cause 50% plaque formation reduction. Furthermore, we found that fraction D, at the antiviral concentration, when tested by the trypan blue exclusion method, showed a toxic activity in HELF cells. These data seem to suggest that only fraction E contains a potential antiviral activity. However, it is important to emphasize that antiviral and toxic activities were studied in extracts containing several substances and further studies should be performed to make a chemical characterization and isolation of the possible active substances. Furthermore, antiviral activity must be studied in order to evaluate the inhibiting mechanism presumably involved.

Summary

Various extracts were produced by different methods during a number of experiments for the extraction of fractions, having antioxidant activity, from «Rosmarinus officinalis L.». The purpose of previous studies by the authors was the detection of active fractions.

Experiments on the antiviral activity of a particularly interesting rosemary extract were performed.

The results obtained indicate that one fraction has antiviral activity without significant signs of cytotoxicity in tissue cultures.

Zusammenfassung

Verschiedene Extrakte wurden mittels unterschiedlichen Technologien aus «Rosmarinus officinalis L.» gewonnen. So waren Experimente zur Auffindung von aktiven Fraktionen das Ziel von früheren Studien. Experimente auf antiviraler Wirkung interessanter Rosmarinextrakte wurden ausgeführt.

Die erhaltenen Ergebnisse zeigen, dass eine Fraktion antivirale Wirkung hat, ohne wesentliche Anzeichen von Zellgiftigkeit in Gewebekulturen aufzuweisen.

Résumé

Des essais ont été effectués pour évaluer l'activité antivirale d'extraits de «Rosmarinus officinalis L.», particulièrement intéressants. Le travail a été réalisé dans le cadre d'une étude sur les techniques de production d'extraits ayant une activité antioxydante.

Une des fractions examinées présente une bonne activité antivirale sans signes notables de cytotoxicité dans les cultures de tissus.

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