Rapid and Simple Biological Activity Screening of Some *Rumex* Species; Evaluation of Bioguided Fractions of *R. scutatus* and Pure Compounds

Ömür L. Demirezer and Ayşe Kuruüzüm

Hacettepe University, Faculty of Pharmacy, Department of Pharmacognosy, 06100 Ankara, Turkey

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Methanolic extracts of 11 *Rumex* L. species (Polygonaceae) were examined against brine shrimp and *R. scutatus* L. has shown significant brine shrimp lethality (LC_{50}=0.96 μg/ml). Methanolic extracts of the roots of *R. scutatus* were fractionated and the active fraction led to the isolation of 4 anthraquinone aglycones: emodin, chrysophanol, physcion and aloe emodin. Their toxic sequence was found as chrysophanol> aloe emodin> emodin> physcion. These anthraquinone aglycones showed a significant activity with LC_{50}=0.00, LC_{50}=0.01, LC_{50}=0.05, LC_{50}=0.15 μg/ml, respectively.

Introduction

Since ancient times, plants have been widely used in cancer treatment. Preparations some plants classified in the literature as cathartics were tested for their capacity to produce damage in Sarcoma 37. Among these plants, which produced the strongest effect in Sarcoma 37 were *Rhamnus cathartica, Rheum officinale* and *Rumex crispus* which contained anthraquinones as major substances (Belkin and Fitzgerald, 1952). *Rhamnus frangula* has been used in England and in the United States to treat cancer and other *Rhamnus* species have been used similarly in folk medicine since at least the time of Galen (Kupchan and Karim, 1976).

We have investigated whether other widespread anthraquinone-containing plants, *Rumex* L. species (Polygonaceae), have cytotoxicity or not.

In this paper, 11 *Rumex* species have been investigated with respect to cytotoxicity, using a brine shrimp bioassay. Among them *Rumex scutatus* L. has shown significant toxicity against brine shrimp. This paper reports on the identification of cytotoxic substances isolated from *R. scutatus* by bioguided fractionation.

Reprint requests to Prof. Demirezer.
Fax: 90/312/3114777.

Materials and Methods

Plant material

The roots of *Rumex* L. species were collected in different regions of Turkey. *R. acetosella* L., *R. angustifolius* Campd. *angustifolius*, *R. gracilescens* Rech., *R.tmoleus* L. were collected from Sivas (alt. 1600–2000 m). *R. alpinus* L. was collected from İzmir (alt. 1650 m); *R. conglomeratus* Murr., *R. dentatus* L. subsp. *halacsyi*, *R. sanguineus* L. were collected from Aydın (alt. 10 m); *R. scutatus* L. was collected from Yozgat (alt. 1200 m); *R. crispus* L. was collected from Ankara (alt. 1100 m) and *R. patientia* L. was collected from Niğde (alt. 1050 m). Voucher specimens are deposited in herbarium of the Faculty of Pharmacy, Hacettepe University, Ankara.

Completely dried material (root) was powdered with an electric grinder and stored in well-closed cellophane bags at room temperature.

Test for cytotoxic activity (Meyer *et al.*, 1982)

Extraction

The underground parts of the plants were dried in shadow, reduced to powder and 0.20 g of the powdered drug was heated with methanol (Merck, Darmstadt) (25 ml) for 15 min. under reflux. The extract was filtered from Schleicher-Schüll 2040a paper at room temperature and dried *in vacuo*.

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Sample preparation

The dried extract (20 mg) was dissolved in 2 ml methanol (Merck, Darmstadt) (Solution A: 1000 µg/ml). Solution B was prepared by diluting 0.2 ml of A to 2 ml with methanol (100 µg/ml). Solution C was prepared by diluting 0.2 ml of B to 2 ml with methanol (10 µg/ml), and Solution D was prepared by diluting 0.2 ml of C to 2 ml with methanol (1 µg/ml).

The dilutions of pure anthraquinone substances were prepared by using 5 mg substance in 2 ml methanol initially. 50, 5, 0.5 and 0.05 µg/ml concentrations were prepared by the same method above.

Three replicates were prepared for each dose level.

Hatching the shrimp

Artemia salina (Brine shrimp) eggs (Hobby, Auf der Kaiserführ 39, 53127 Bonn, Germany) were hatched in a dish filled with artificial sea water which was prepared with a commercial salt mixture (3.8%) (Artemia salz: Hobby Dohse Aquaristik, Bonn, Germany) by bidistilled water. After 48 h, the phototropic nauplii which were separated from their shells in the divided tank, were collected from the illuminated side, using a capillary.

Bioassay

Ten shrimps were transferred to each sample vial in a capillary and artificial sea water was added to make 5 ml. The nauplii which were in the body of the capillary were counted after 48 h of illumination with an overhead flourescent lamp (36 w) at a distance of 40 cm. The extract was added and the incubation was continued. After 24 h, percent deaths in controls and each dose of extract were determined.

LC_{50} determinations

The data were analyzed with the FINNEY (probit analysis method) computer program (DOS) to determine LC_{50} values and 95% confidence intervals. (The Finney computer program was obtained from Prof. McLaughlin, Purdue University, West Lafayette, IN 47907 USA).

Fractionation

Ground and dried roots of *R. scutatus* (20 g) were extracted (14 hours) with 140 ml methanol (Merck, Darmstadt) at 50 °C temperature in a Soxhlet apparatus. The extract was filtered and concentrated under reduced pressure. Yielded 2.69 g of residue. The methanol extract of *R. scutatus* roots was dissolved in aqueous methanol and the solution was chromatographed over a polyamide column eluted with methanol: water with increased polarity. Elution was initiated with 20% methanol in water. This process was continued through a number of steps, involving 40, 50, 60 and 80% aqueous methanol, finishing with 100% methanol. Each fraction (about 100 ml) was subsequently analyzed by TLC and HPLC. The fractions were evaluated for brine shrimp bioassay and the pure compounds were isolated only from the active fraction by column chromatography. Activity of these compounds against brine shrimp were performed by the procedure summarized in Fig. 1.

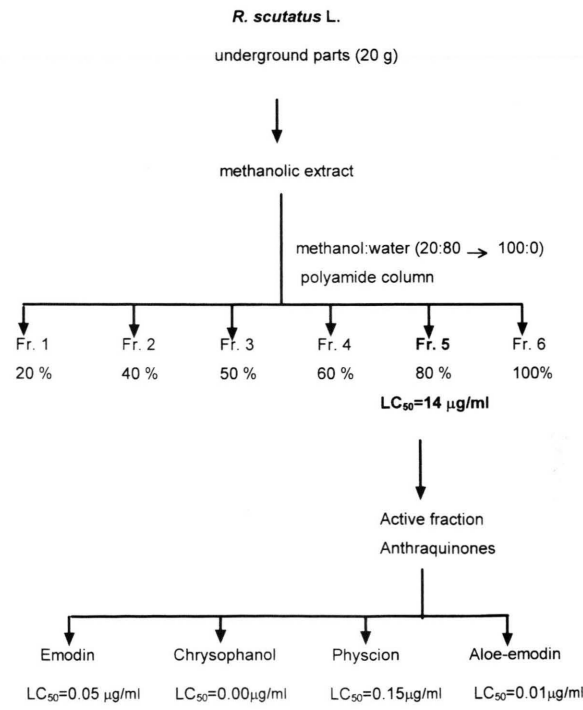

Fig. 1. Fractionation scheme for the isolation of active compounds from *R. scutatus*. The fractions and substances were monitored by TLC and HPLC.

Thin layer chromatography (TLC)

Thin layer, plates: Pre-coated TLC plates, silica gel 60 (Merck 5554)

Solvent system, and development:

Chloroform: methanol: water (80: 20: 2, v/v)

Cyclohexane: ethyl formiate: dichloromethane: formic acid (35: 30: 30: 5, v/v)

Petrol ether: ethyl formiate: formic acid (94: 25: 1,v/v)

Detection:

1. The spots were studied directly on the chromatogram in daylight and UV light (Camag)

2. Sprayed with 5% KOH in methanol (50% v/v) and heated for 15 min at 100 °C.

High performance liquid chromatography apparatus

The equipment consisted of a Waters 510 solvent delivery system (Waters, Milford, MA, USA) and a autosampler Waters WISP 710 B, Millipore. A Waters Modell 481, Lambda-Max, Millipore UV-detector was used. The detector was operated at 430 nm for anthraquinone aglycone. Separation was performed on an 0.8x10 cm, 10 µm Radial-Pak, C_{18} column at room temperature. The mobile phase (81.5:18.5:1, v/v) consisted of methanol:water:formic acid (Van Den Berg *et al.*, 1988). The flow rate was 1 ml/min. Each sample was chromatographed three times. The injection volume was 15 µl and the pressure was $82x10^5$ Pa

Sample Preparation: 3 mg of fraction was dissolved in exactly 10 ml methanol.

Standard Samples: Aloe-emodin, emodin, chrysophanol, physcion were isolated by us and comparison of these data with those previously reported (Demirezer, 1991; Labadie, 1971; Miething, 1984; Rauwald, 1983; Steglich and Lösel, 1969). A solution of 3 mg aloe-emodin, emodin, chrysophanol, physcion in 10 ml methanol was prepared as described in the sample preparation section.

Results and Discussion

Different concentrations of methanolic extracts of 11 *Rumex* species were examined for their effects on brine shrimp. The toxic potential of *Rumex* extracts in terms of LC_{50} values relating to the brine shrimp assay are given in Table I and Fig. 2. The crude methanolic extracts were toxic for the *Artemia salina* nauplii at a level ranging from 0.88 to 52 µg/ml.

The analyzed *Rumex* species were significantly active against brine shrimp except for three species. A weak lethal effect for *R. dentatus* (LC_{50}= 42 µg/ml), for *R. conglomeratus* (LC_{50}=36 µg/ml) and for *R. tmoleus* (LC_{50}=52 µg/ml) were found.

The brine shrimp assay revealed a significant lethal effect for *R. angustifolius* subsp. *angustifolius* (LC_{50}=0.88 µg/ml), *R. scutatus* (LC_{50}=0.96 µg/ml), *R. crispus* (LC_{50}=1.0 µg/ml) and *R. patientia* (LC_{50}=1.3 µg/ml).

R. angustifolius subsp. *angustifolius* showed strongest activity. But this plant is a very small herb and the root is too weak for industrial use. Therefore, the plant (*R. scutatus*) with the second

Table I. Cytotoxic activities of root extracts of some *Rumex* species.

Species	Percent deaths after 24 h				LC_{50}	95%
	1 µg/ml	10 µg/ml	100 µg/ml	1000 µg/ml	[µg/ml]	Confidence interval
R. acetosella	16.6	30	83.3	100	15	28.00–7.79
R. alpinus	43.3	56.6	93.3	100	2.6	5.84–0.76
R. angustifolius subsp. *angustifolius*	46.6	100	100	100	**0.88**	1.72–0.23
R. conglomeratus	16.6	50	56.6	73.3	36	132.15–10.44
R. crispus	43.3	100	100	100	**1.0**	1.90–0.30
R. dentatus subsp. *halacsyi*	13.3	20	66.6	100	42	71.97–23.47
R. gracilescens	23.3	46.6	100	100	10	17.27–4.36
R. patientia	36.6	100	100	100	**1.3**	2.30–0.52
R. sanguineus	20	33.3	86.6	100	9.3	16.81–4.88
R. scutatus	50	83.3	100	100	**0.96**	2.20–0.19
R. tmoleus	3.3	30	60	86.6	52	109.16–26.46

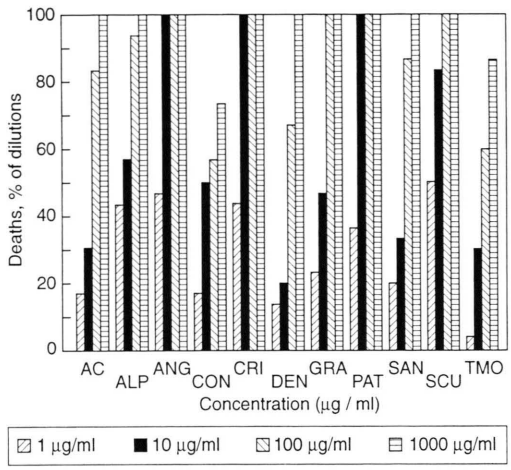

Fig. 2. Comparison of the cytotoxicity of eleven *Rumex* species.

strongest activity was investigated in detail. Methanolic extract of the roots of *R.scutatus* was fractionated over polyamide column with increasing polarity of methanol in water. The cytotoxicity of collected fractions were identified using brine shrimp method and the active fraction (LC_{50}= 14 µg/ml) was further investigated. The bioguided fractionation of the *R. scutatus* led to the isolation of 4 anthraquinone aglycones which were identified by TLC, HPLC (Fig. 3) and spectroscopic analysis and comparison of these data with those previously reported (Demirezer, 1991; Miething, 1984; Labadie, 1971; Steglich and Lösel, 1969; Rauwald, 1981; Van Den Berg *et al.*, 1988) and our

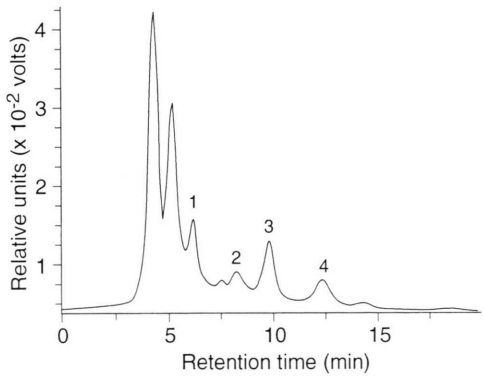

Fig. 3. The HPLC chromatogram of Fr. 5 obtained from *R. scutatus* (mobile phase: MeOH:H_2O:HCOOH (81.5:18.5:1, v/v). flow rate:1 ml/min., 430 nm). 1=Aloe emodin; 2=emodin; 3=chrysophanol; 4=physcion.

standard samples. These substances are emodin, aloe emodin, physcion and chrysophanol.

Aloe emodin has already been reported as antileukemic (Wei *et al.*, 1992), chrysophanol, physcion, emodin as cytotoxic against human hepatoma PLC/PRF/5 and KB cells. As far as we know our study is the first report for their toxicity against brine shrimp. The brine shrimp (*A. salina*) lethality assay is considered a useful tool for preliminary assessment of cytotoxicity. This method is a simple and inexpensive screening test for cytotoxic activity of compounds. It has the advantage of requiring only small amounts of compounds. The brine shrimp is a reliable detector of biological activity (McLaughlin *et al.*, 1991). Literatur data suggest a good correlation between the brine shrimp assay and some tumor cell lines (Solis *et al.*, 1993). The results are given in Table II. It is evident from the data that the strongest toxicity to brine shrimp was found in chrysophanol. Toxic potential sequence of anthraquinones were: chrysophanol> aloe emodin> emodin> physcion. Discussing the structure-activity relationship it can be said that; owing to the increase in substitutions at anthraquinones, activity decreases. There are disputed results of other studies with one another, and with our study. The comparison of our data with conflicted results is as follows:

1. *O*-Glycosidation or methylation of the C6-OH on emodin (physcion, emodin monoglucoside) enhances the cytotoxic effect against human hepatoma PLC/PRF/5 cells *in vitro* (Wei *et al.*, 1992).

These data are fully opposite when compared with our results which point out the decreasing of the activity by substitutions.

2. Emodin was the more active substance against HL-60 cells than physcion, chrysophanol and emodin-8-*O*-glucoside (Yeh *et al.*, 1988).

As shown above, these two data are completely different from each other. Using either varying cell lines or cytotoxicity methods may arise differences. While in first results, emodin has lower activity than physcion and emodin monoglucoside (Wei *et al.*, 1992), it was reported that emodin is the more active substance than physcion, chrysophanol and emodin-8-*O*-glucoside (Yeh *et al.*, 1988). In our study some different data were obtained. Physcion showed lower activity than emodin and chrysophanol. Chrysophanol was found to be that the most active substance among these

Table II. Cytotoxic activities of the pure anthraquinone substances.

Anthraquinone aglycones	Percent deaths after 24 h				LC_{50} [μg/ml]	95% Confidence interval
	0.05 μg/ml	0.5 μg/ml	5 μg/ml	50 μg/ml		
Aloe emodin	50	66.6	73.3	73.3	0.01	0.017–0.307
Emodin	43.3	96.6	100	100	0.05	0.015–0.10
Physcion	46.6	53.3	60	66.6	0.15	0.024–4.303
Chrysophanol	73.3	90	96.6	100	0.00	0.000-0.029

4 anthraquinone aglycones. It means that application methods may be important.

3. According to NCI (National Cancer Institute), aloe emodin (NSC-38628) was among the derivatives which were found to be inactive (Driscoll *et al.*, 1974).

4. But another author proved that aloe emodin showed significant inhibitory activity against the P-388 leucemia in mice and the author suggested a re-examination of other anthraquinones for potential antitumor activity with particular attention to possible vehicle dependence, may be rewarded by the discovery of new and useful structure activity relationship (Kupchan and Karim, 1976).

There are two opposite results on aloe emodin. In our study significant activity has also been found for aloe emodin.

In conclusion, it can be said that all of studied *Rumex* L. species have activities against *Artemia salina*. Active extracts determined by bioguided fraction was found to contain anthraquinone aglycones. These substances were obtained as pure forms and their cytotoxicity were established. All of the studied anthraquinone aglycones showed strong bioactivity.

Cytotoxic activity of *Rumex* species may well be dependent on the anthraquinone content.

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