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Abstract : Extracts from respective parts and cell cultures of some Turkish medicinal plants have been assessed for their antiviral and cytotoxic properties. None of the extracts tested showed notable activity against herpes simplex viruses (HSV-I and II), but a slight antiretroviral activity against HIV-I was determined in an extract from *Hypericum capitatum* cell cultures. On the other hand, according to cytotoxic activity test results against brine shrimp (*Artemia salina*), an activity level of LD_{50} at 34.9 µg/ml was found on the extracts of *Ecbalium elaterium* cell cultures.

Key Words: Antiviral activity, antiretroviral activity, cytotoxicity, plant cell cultures, crude extracts, Turkish medicinal plants, *Artemia salina*

Bazı Türk Tıbbi Bitkilerinden ve Bu Bitkilerin Hücre Kültürlerinden Elde Edilen Özütlerin Antiviral ve Sitotoksik Aktiviteleri

Özet : Bazı Türk tıbbi bitkilerinden ve bu bitkilerden başlatılan hücre kültürlerinden elde edilen özütlerin antiviral ve sitotoksik özellikleri incelenmiştir. Test edilen özütlerin hiçbiri Herpes Simpleks Viruslarına (tip I ve Tip II) karşı kayda değer bir aktivite göstermemiş, ancak *Hypericum capitatum* hücre kültürlerinden elde edilen bir özütte HIV-I' e karşı zayıf bir antiretroviral aktivite saptanmıştır. Diğer taraftan, *Artemia salina* LEACH (Crustacea) (Brine shrimp) ile yapılan sitotoksik aktivite test sonuçlarına göre, *Ecbalium elaterium* hücre kültürlerinden elde edilen özütte LD_{50} 34.9 µg/ml' lik bir aktivite saptanmıştır.

Anahtar Sözcükler: Antiviral aktivite, antiretroviral aktivite, sitotoksisite, bitki hücre kültürleri, kaba özüt, Türk tıbbi bitkileri, *Artemia salina*

Introduction

The *in vitro* antibacterial activities of extracts from some Turkish medicinal plants have been reported recently (1). As a result of an attempt to establish a link between medicinal plants and plant biotechnology, callus and cell suspension cultures of some Turkish medicinal plants have been established (2) and their antimicrobial properties have also been examined *in vitro* (3).

The screening of plant extracts or pure compounds for exploring their antimicrobial properties can be more meaningful with cytotoxicity assays. For instance, the brine shrimp (*Artemia salina* LEACH) bioassay can be employed for this purpose as it appears to be a convenient, rapid and inexpensive assay for the determination of the cytotoxic properties of bioactive extracts (4, 5).

The objective of the present study was to assess the antiviral and cytotoxic properties of extracts from both cell cultures and respective parts of some Turkish medicinal plants against herpes simplex virus types I and II (HSV-I and II), human immunodeficiency virus type I strain MN, (HIV-I) and brine shrimp (*Artemia salina* LEACH), respectively.

Materials and Methods

Collection of plant material

Seeds of *Pimpinella anisum* (Apiaceae), *Linum usitatissimum* (Linaceae), *Nigella sativa* (Ranunculaceae) and *Cannabis sativa* var. *sativa* (Cannabinaceae) were purchased from a local market in Sivas. *Nine Allium* species, namely *Allium dicytoprosum, A. chrysanthemum, A. macrochaetum subsp. macrochaetum, A. macrochaetum subsp. tuncelianum, A. pustulosum, A. flavum* and *A. scorodoprasum,* (Liliaceae) and *Sideritis libanotica* var. *libanotica* (Lamiaceae) were collected, identified and kindly donated by Professor Mehmet Koyuncu at the Faculty of Pharmacy, the University of Ankara, Turkey. The remaining plant species, collected and identified by Professor Bayram Yıldız, at the Departmant of Biology, Faculty of Science and Literature, İnonü University, Malatya-Turkey, are as follows:

Rhus coriaria (Bursa, September-1993)(Anacardiaceae), *Bupleurum sulphureum* (Hafik-Sivas, August-1993)(Apiaceae), *Achillea biebersteinii* and *A. teretifolia* (Zara-Sivas, September-1993)(Asteraceae) and *Helianthemum ledifolium* var. *ledifolium* (Ankara, July-1993)(Cistaceae), *Ecbalium elaterium* (Ankara, September-1993)(Cucurbitaceae), *Hypericum capitatum* (Siverek-Urfa, June-1993) and *H. scabrum* (Sivas, August-1993)(Hypericaceae), *Phlomis kurdica* (Doğanşehir-Malatya, August-1993) and *Thymus fallax* (Malatya, August-1993)(Lamiaceae), *Urtica dioica* (Ankara, September-1993)(Urticaceae) and *Peganum harmala* (Ankara, August-1993)(Zygophyllaceae).

Callus and cell suspension culture conditions

The establishment and maintenance of callus and cell suspension cultures of plants used in this study were carried out using the procedures described before (2).

Extract preparation

For preliminary screening, the respective plant parts were powdered and material from callus and cell suspension cultures were lyophilised and extracted by a method described elsewhere (6). In short, samples weighing about 100 g were individually extracted in a Soxhlet with methanol (MeOH) at 60ºC for 6 hours.The extract was then filtered and concentrated *in*

vacuo at 45ºC.The resulting extract was resuspended in water and partitioned with chloroform ($CHCl_3$) to separate less polar, water insoluble compounds.

In addition to the above extraction procedure, ethyl acetate (EtOAc) extracts were employed for *Rhus coriaria*. Furthermore, the aerial parts and callus material of *Thymus fallax* were extracted with n-hexane followed by methanol. The diethyl ether (Et_2O) extracts of both the ground seeds and cell cultures of *Nigella sativa* were obtained using a method described elsewhere (7). All extracts prepared were lyophilised and then kept in the dark at 4ºC until tested.

Antiviral activity tests

For antiviral activity tests, the extracts were weighed and dissolved in phosphate buffer saline (PBS) (pH = 7.0-7.2) and dimethylsulphoxide (DMSO) at a 1:1 ratio to prepare extract solution at 2000 µg/ml. The following dilutions of extracts were then prepared: 1000, 100, 10 and 1 mg/ml.

Vero cells, obtained from Gibco Biocult Ltd., Paisley, Scotland, at passage level 145, were used for the preparation of stock virus and for antiviral activity tests against HSV-I and II as described previously (6). The percent reduction in the number of plaques produced by each virus in the presence of each concentration of the extracts was calculated as follows: % reduction = 100 – [(pfu at given extract dose / pfu in control) x 100].

From the above calculation, a graph was plotted for the percentage of plaque reduction against each extract concentration. The 50% inhibitory concentration (IC_{50}) was directly read from the graph. An acyclovir was used as a positive control.

Activity against Human Immunodeficiency virus type-I (HIV-I) strain MN was determined by the syncytia inhibition assay (6). In short, the MN strains of HIV-I was inoculated onto MT-2 cells in growth medium RPM-1 plus a concentration of 10% foetal calf serum (FCS), and 0.01% glutamine and antibiotics. The concentration of virus used was calculated to give a 50 syncytia per low power field after 72 h incubation. One hour following virus infection, cell cultures were inoculated with plant extracts that were previously diluted in growth medium in log condition. The extracts were also inoculated on non-virus infected cells to determine the exact toxicity. After 72 h incubation at 37ºC in 5% CO_2, aliquots ofvirus infected cells were removed, and the number of syncytia was counted. Anti-HIV activity was taken as the concentration of the extract which is non-toxic to normal cells, but which shows a significant reduction in syncytia number compared to normal cells infected with HIV-I in the absence of any extract.

Cytotoxicity test using Brine shrimp assay

Brine shrimp bioassay was carried out according to a method described elsewhere (8). Brine shrimp eggs were hatched in artificial sea water prepared from sea salt (Sigma Chemical Co., U.K.) 40 g/l supplemented with 6 mg/l dried yeast. After 48 h inoculation at room temperature,

nauplii were collected with a pasteur pipette after the organisms were attracted to one side of the vessel with a light source. Nauplii were separated from the eggs by pipetting into small beakers.

The above plant extracts (0.6 mg dry weight) for testing were made up of 1 mg/ml in artificial sea water except for water insoluble ones, which were dissolved in 50 µl dimetylsulphoxide (DMSO) prior to adding sea water.

Serial dilutions were made in the wells of 96-well microplates in triplicate in 100 ml sea water. A suspension of nauplii containing ca. 10 organisms was added to each well and the covered plate incubated at room temperature for 24 hours. After incubation plates were examined under binocular microscope and the dead nauplii in each well were counted. 100 ml methanol was then added to each well and after 15 minutes the total numbers of shrimps in each well was obtained. LC_{50} values were directly determined from the dose-response data, which were transformed into a straight line by means of a logit transformation (8).

Results and Discussion

Before evaluating antiviral and antiretroviral activity test results, it is important to indicate that all extracts studied had markedly toxic effects to the host (Vero and MT-2, respectively) cells, with concentrations ranging from 400 to 1000 µg/ml. As shown in Table 1, extracts of the respective parts of *R. coriaria, A. dicytoprosum, A. chrysanthemum, P. harmala, N. sativa, H. capitatum* and *H. scabrum* exhibited slight antiviral activity against HSV-I, and all had an IC_{50} at around 50 µg/ml, but against HSV-II and HIV-I.

In reports concerning the *in vitro* antiviral activity of many extracts or chemicals isolated from plants employed for this study or their closely related species, the aqueous extract of *P. harmala* seeds has been shown to be active against HSV-I with an IC_{50} dose at 35 µg/ml (9). The antiviral activities of β-carboline (harman) alkaloids isolated from *P. harmala* seeds have also been reported (10). Also, the aerial parts (11) as well as aromatic polycyclic diones (12) of *Hypericum species* and extracts and compounds from Allium sativum (garlic) (13) exhibit antiviral activity against several viruses. The *in vitro* antiviral activity of the extracts of N. sativa and *R. coriaria* has not been reported before.

None of the extracts of callus and/or cell cultures showed any activity against herpes simplex viruses (HSV-I and HSV-II) and HIV-I (strain MN) except for the methanolic extract (ethyl acetate part) of *H. capitatum* cell suspension cultures, which had an IC_{50} at 500 µg/ml against HIV-I (data not included in Table 1).

The results of the cytotoxicity test using the brine shrimp bioassay are presented in Table 2. Although fruit and/or callus culture extracts of *E. elaterium* did not show antiviral activity against HSV-1 and 2 or HIV-1, their toxic effects on Vero cells, even at concentrations lower than 100 µg/ml were interesting. This finding is further supported, as presented in Table 2, by

Table 1. Antiviral activity of extracts from some Turkish medicinal plants againstHSV-I.*

Botanical Name	Extraction/ Partition	Concentration of the test material (mg/ml)				
		1000	100	10	1	IC_{50} [1]
		MEAN[2]2±S.E. (% RED.)	MEAN±S.E.[3] (% RED.)	MEAN±S.E. (% RED.)	MEAN±S.E. (% RED.)	
Rhus coriaria	MeOH/ EtoAc	TOXIC[4]	No plaque (100)	139.67±3.84 (7.8)	144.67±4.84 (-)	49.0
Ecbalium elaterium	MeOH / $CHCl_3$	TOXIC	TOXIC	TOXIC	186.67±3.48 (-)	
Hypericum capitatum	MeOH/ $CHCl_3$	TOXIC	No plaque	194.33±7.84 (100.0)	194.00±7.02 (-)	50.0 (-)
Hypericum capitatum	Acetone	TOXIC	No plaque (100.0)	168.33±4.48 (5.7)	183.67±6.33 (-)	52.0
Hypericum scabrum	MeOH/ $CHCl_3$	TOXIC	No plaque (100.0)	144.00±3.46 (6.5)	158.67±2.40 (-)	50.0
Hypericum scabrum	Acetone	TOXIC	No plaque	144.33±3.48 (100.0)	145.67±4.63 (-)	50.0 (-)
Allium dictyoprosum	MeOH/ H_2O	TOXIC	No plaque (100)	188.33±10.14 (1.0)	190.67±10.35 (-)	52.0
Allium chrysanthemum	MeOH/ H_2O	TOXIC	No plaque (100)	165.33±6.36 (7.5)	182.33±7.88 (-)	51.0
Nigella sativa	MeOH / $CHCl_3$	TOXIC	74.00±3.60 (58.3)	174.00±3.60 (1.9)	179.00±4.93 (58.3)	59.0
Peganum harmala	MeOH/ $CHCl_3$	TOXIC	No plaque (100)	97.00±4.36 (31.6)	144.00±1.00 (-)	50.0

* Acyclovir was used as a positive control with a IC_{50} which is previously found at 0.05 µg/ml against HSV-I.

[1] The 50% inhibitory concentration of the particular extract is given as µg/ml.

[2] Mean is the average of three replicates.

[3] Standard error (n = 3).

[4] The dose chosen was lethal against host cells.

the brine shrimp bioassay results, which clearly indicate the toxic effects of extracts prepared from fruits and callus cultures of *E. elaterium*. In particular, extracts prepared from callus cultures were found to be more toxic than fruit extracts of the same plant. The former extracts had LD_{50} at 34.9 µg/ml, while the latter ones had LC_{50} at 102 µg/ml. The toxic effects of the remaining extracts used in brine shrimp bioassay were not strong against *Artemia salina* LEACH (Table 2).

Table 2 Brine shrimp bioassay results of the extracts from both respective parts and callus and/or cell suspension cultures of some Turkish medicinal plants.

Botanical Name	Part used	Extract	Percent death at 48 hours					
			10 µg/ml	30 µg/ml	100 µg/ml	300 µg/ml	100 µg/ml	LD_{50} [1] µg/ml
Thymus fallax	Aerial	n-hexane	0[2]	0	13.9	24.4	73.6	446.7
Thymus fallax	CC[3]	n-hexane	0	1.0	2.3	2.8	35.7	944
Ecbalium elaterium	Fruit	H_2O	3.7	8.2	63.5	75.9	95.7	102
Ecbalium elaterium	CC	H_2O	1.9	12.6	91.6	100	100	34.9
Pimpinella anisum	Seed	$CHCl_3$	3.8	6.3	29.4	35.5	58.3	595.6
Pimpinella anisum	CC	$CHCl_3$	0.1	1.0	3.7	9.9	44.30	≥1000
Achillea bibersteinii	Aerial	$CHCl_3$	0	0	43.4	43.1	63.4	251.2
Achillea bibersteinii	CC	$CHCl_3$	2.0	3.0	4.0	8.0	29.0	≥1000
Rhus coriaria	Fruit	EtOAc	2.0	10.8	32.1	41.6	70.0	400.0
Rhus coriaria	CC	EtOAc	0	2.0	4.7	6.9	22.4	≥1000
Allium dictyoprosum	Bulb	H_2O	0	0.5	5.7	25.4	61.7	635
Allium dictyoprosum	CC	H_2O	0	1.2	3.3	11.80	41.8	≥1000
A. chrysanthemum	Bulb	H_2O	0	1.6	9.9	41.4	69.7	598
A. chrysanthemum	CC	H_2O	0	0.5	2.0	9.7	36.0	≥1000
Nigella sativa	Seed	$CHCl_3$	0	4.4	3.4	26.2	52.2	530
Nigella sativa	CC	$CHCl_3$	0	1.0	9.7	17.8	48.9	780
Peganum harmala	Seed	$CHCl_3$	1.4	0.5	5.0	21.9	85.2	535
Peganum harmala	CC	$CHCl_3$	0	0	4.9	11.5	22.6	≥1000
Hypericum capitatum	Aerial	EtOAc	2.1	2.5	6.8	22.4	61.7	950
H. capitatum	CC	EtOAc	1.4	1.9	13.5	30.4	84.2	400
H. scabrum	Aerial	EtOAc	0.8	7.4	15.8	35.6	73.4	440
H. scabrum	CC	EtOAc	1.4	7.9	15.1	30.6	70.0	450

[1] LD_{50}'s were estimated using logit transformation. Therefore confidence intervals are not provided.
[2] An average of three replicates.
[3] Callus and/or cell suspension cultures.

Conclusion

The cytotoxic activity observed in *E. elaterium* callus cultures may provide useful data for the utilisation of cytotoxic principles produced by callus and/or cell cultures of this plant.

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