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BIOASSAYS ON *PERIPLOCA GRAECA* L.(SILK VINE)

PERIPLOCA GRAECA L. (İPEK FIDANI) İLE BİYOLOJİK AKTİVİTE ÇALIŞMALARI

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Silk vine, Periploca graeca L. (Asclepiadaceae) which is a poisonous plant, was collected in July 1997 from Eskişehir (Aşağı Küplü-Mayıslar Village). The methanolic extracts of the air dried leaves and twigs were separately subjected to a series of simple benchtop biological assays. Brine-shrimp in vivo lethality bioassay, Lemna bioassay for plant growth promoters or inhibitors, antifungal and antibacterial bioassays, insecticidal bioassay, nematocidal bioassay and antileishmanial assay were performed using the crude methanolic extracts of the plant. Promising results were obtained especially on the antileishmanial assay. The bioassay results are reported here.

İpek fidanı olarak bilinen ve zehirli bir bitki olan Periploca graeca L. (Asclepiadaceae) Temmuz 1997'de Eskişehir'in Aşağı Küplü-Mayıslar Köyünden toplanmıştır. Kurutulmuş toprak üstü yaprak ve dalların metanollü ekstreleri ile bir seri basit ve kolay uygulanabilen biyolojik aktivite çalışmaları (biyoassay) yapılmıştır. Bitkinin metanollü ekstreleri ile Artemia salina toksisite testi, bitki büyümesi/inhibisyonu için Lemna deneyi, antifungal ve antibakteriyel etki, insektisidal etki, nematosidal etki ve antileishmanial etkileri incelenmiştir. Bu makalede, Periploca graeca L. ile yapılan biyolojik aktivite çalışmaları ve sonuçları verilmiştir. Özellikle antileishmanial etki açısından olumlu sonuçlar elde edilmiştir.

Keywords: *Periploca graeca L.; Asclepiadaceae; Bioassay; Antileishmanial activity*

Anahtar Kelimeler: *Periploca graeca L.; Asclepiadaceae; Biyolojik aktivite çalışmaları (biyoassay); Antileishmanial etki*

Introduction

200 *Periploca* species and around 50 genera are found in tropical and temperate regions of the world; in tropical Africa, Western Mediterrenean and Eastern Asia including Turkey. It has a wide distribution from sea level upto 1200 m in Turkey, and is known as "ipek fidanı". *Periploca graeca* is used in medicine since ancient times. It is also an ornamental plant. It is used for the treatment of heart diseases and as a tonic. The aerial parts, the seeds and the cortex are also used in different forms. The plant contains a poisonous latex, sometimes used as an arrow poison. The latex turns black when dry. The leaves when applied to the skin can produce a burning effect (1-6). Besides *Periploca graeca* many other *Periploca* species have found medical uses. In Chinese medicine some of them are used for the treatment of tumors and cancer, especially *P. sepium* (Chin.=Wujapi), *P. calophylla*, *P. nigrescens* and *P. aphylla*. Other uses of these plants like purgative and local analgesic, also against rheumatism, lumbago, swellings and impotency are also reported (7, 8). Recently CNS activities of Chinese drugs including *Periploca* species have been reported by Zhu and co-workers (9).

Ethnopharmacological uses as cardiotonic and breath-reliever were reported in Iran for the whole plant but due to the toxic effects this practice has been left (10).

The plant is known to contain flavonoids, saponins and tannins through simple identification tests (11). Other compounds isolated include chlorogenic acids, coumaric acid, p-coumaroyl- and ferulocylquinic acids, gentisic acid, ursolic acid, glucose esters, polyphenolic substances, cardenolides, triterpenes, cardiac glycoside periplocin, periplocymarin, poenidin and other glycosides; aesculetin, isoquercetin, rutin, astraglin and nicotiflorin (12-18). Calocinin, locin, plocin, plocinin (19), and more than 16 other pregnanes and cardenolides with antitumor activity were also isolated by

Indian and Japanese researchers from *Periploca* species (20).

Despite immense medicinal importance of the plant, no bioassay-guided isolation investigation has been conducted so far. Recent interest on natural products as medicine is mainly due to their supposedly nontoxic effects on the living organism (21-23). In this perspective phytochemical and biological assays were conducted on the crude extracts of *Periploca graeca* L. Active fractions were identified.

Materials and Methods

Plant Material: Leaves and twigs of the plant were collected when flowering from Aşağı Küplü-Mayıslar Village at a altitude of 500 m, Eskişehir, Turkey (June 1997). Voucher specimen was deposited at the Faculty of Pharmacy Eskişehir, Turkey (ESSE 12364).

Extraction: Air dried leaves (200 g) and twigs (200 g) were macerated separately with methanol exhaustively for three days (1:20, w/v). Then filtered, and concentrated at reduced pressure to yield crude leaf (LE, 23 g) and twig (TE, 14 g) extracts, respectively.

These extracts were subjected to various bioassays as follow;

-Brine-shrimp in vivo Lethality Bioassay

Brine Shrimp hatching: *Artemia salina* Leach (Brine-shrimp) eggs were hatched in a shallow dish filled with artificial sea water which was prepared with a commercial sea salt mixture and distilled water. A plastic divider with several 2 mm holes was mounted in the dish to divide two unequal compartments. The eggs were sprinkled into the bigger part which was darkened, while the smaller part was illuminated at 27°C for 48 h. After this period phototropic nauplii were collected with the pipette.

Sample preparation: 20 mg crude extracts of leaves (LE) and twigs (TE) were dissolved separately in 2 ml methanol stock solution each. 0.5 ml, 0.05 ml and 0.005 ml volumes of extract were transferred into vials. After evaporation of the solvent 3 different concentrations have been prepared and replicated 3 times; 1000 µg/ml, 100 µg/ml and 10 µg/ml, respectively for both extracts separately. After hatching procedure 10 *Artemia salina* nauplii were transferred in each vial with sea water having the volume of 5 ml. After 24 h mortality of *Artemia salina* was checked and LD_{50} values and 95% confidence intervals for the extracts were determined by Finney Computer Program.

Reference Drug: Cu_2SO_4

-Lemna Bioassay for Plant Growth Promoters or Inhibitors

Plant Material: *Lemna aequinoctialis* Welv. - (*Lemnaceae*) were collected under aseptic conditions. The plant material was cultured in E-medium and grown until they achieve standard size and shape in a Fisons Fi-Totron 600 H growth cabinet at 30°C, 56±10° relative humidity, 9000 Lux light intensity and 12 hours day length for seven days.

E-medium: KH_2PO_4 0.68, KNO_3 1.515, $Ca(NO_2)_2.4H_2O$ 1.18, $MgSO_4.7H_2O$ 0.492, H_3BO_3 0.00286, $MnCl_2.4H_2O$ 0.00362, $FeCl_3.6H_2O$ 0.0054, $ZnSO_4.5H_2O$ 0.00022, $Na_2MnO_4.2H_2O$ 0.00012, EDTA 0.01120 g/L.

Sample preparation: 15 mg crude extracts of leaves (LE) and twigs (TE) were dissolved separately in 1.5 ml methanol stock solution each. 1000 µl, 100 µl and 10 µl solutions were transferred into 30 ml vials in triplicate respectively for both extracts separately. The vials were left overnight for evaporation of the solvent. After complete evaporation, 20 ml of E-media and 10 healthy *Lemna* plants were placed in the vial, sealed and left for incubation in the growth cabinet for 7 days. Effects of the extracts to individual *Lemna* plants were observed and reported in percentage (Table-1).

Reference Drug: Paraquat (inh.), Gibberellic Acid (Grw.)

-Antifungal Bioassay

Sample preparation: Stock solutions of crude extracts of *Periploca graeca* leaves (LE) and twigs (TE) were freshly prepared with 24 mg in 1 ml sterile dimethylsulfoxide (DMSO). Dilutions were added into test tubes having sterile Sabouraud dextrose agar (SDA) media to reach a final concentration of 400 µg/ml. SDA with the dilution of samples were kept in slanting position at room temperature for solidification. Fungal cultures obtained from clinical/pathogenic isolates were inoculated onto the slant and growth inhibition was observed after an incubation period of 7-10 days at 27-29°C.

Growth in the compound containing media was determined by measuring linear growth in mm, and growth inhibition calculated with reference to the negative control (Table-2).

Reference Drugs: Miconazole, Ketoconazole; Amphotericin-B for *Aspergillus niger*; Benlate for the plant pathogens

-Antibacterial Bioassay

Sample preparation: Stock solutions of crude extracts of *Periploca graeca* leaves (LE) and twigs (TE) were freshly prepared. 3 mg of each extract was dissolved separately in 1.5 ml dimethylsulfoxide (DMSO). Each solution was diluted to 200 µg/100 µl final concentration. One loop of 24 h old culture obtained from clinical/pathogenic isolates containing approx. 10^4-10^6 CFU (Colony Forming Unit) was spread on the surface of Mueller-Hinton agar plates. 10 mm diameter wells were dug in the medium with a sterile metalic borer and each dilution was added into the respective wells. Zones of inhibition were measured after an incubation period of 24 h at 37°C (Table 3.).

Reference Drugs: Amoxicillin, Ampicillin, Cephalexin-Na

-Insecticidal Bioassay

Insect: Common store grain insect *Tribolium castaneum* (Red flour beetle) was reared in the laboratory under controlled conditions (31°C and 60-65% humidity). Wheat bran was used as food and breeding media. After emergence the new insects were transferred into fresh media.

Sample preparation and application: 1 gr of crude branch extract was dissolved in 5 ml methanol as stock solution and 1 ml was applied on a 4.5 cm diameter filter paper

to obtain 10% concentration (w/v) dose 785.85 μg/cm^2. Permethrin in same concentration and methanol was applied on different filterpapers as control. Methanol was evaporated. The filter papers were placed in petri dishes with ten insects. After 24 h of treatment, mortality percentage was noted. Mortality here is defined as inability to move when disturbed.

Reference Drug: Permethrin (Coopex)

-Nematocidal Bioassay

Nematod hatching: Egg masses were obtained from *Meliodogyne* spp. (Root-knot nematode) and culture was maintained on tomato and egg plants in the experimental plot of National Nematological Research Center, University of Karachi. Egg masses were separated from the soil and left overnight in distilled water for hatching. After 24 hours, hatched 2nd stage juvenile nematodes were collected and larval suspention was prepared. Number of larvae counted per ml.

Sample preparation: 1000 ppm concentrated solution of leaf (LE) and twig (TE) crude extracts were prepared separately in methanol. 2 ml of each extract was taken into a small watch glass and left for the complete evaporation of the solvent. After removal of the solvent, 2 ml of the larval suspension was poured in each watch glass. Watch glasses without extract were used as control and each extract was replicated 3 times. Observations were recorded after 24, 48 and 72 hours and the percentage of mortality was calculated (Table-4).

-Antileishmanial Assay

Sample preparation: Dried extracts were dissolved in 5% methanol/PBS (pH 7.4) so the final concentration was 1.0 mg/ml in each. The toxicity of methanol was checked by adding different amounts of solvent (0.1 to 5%) to the suspension of promastigotes. After 3 days the value of non-toxic dilutions was found to be 0.62% and growth inhibition (upto 10%) was observed between 0.62 and 1.25%, therefore higher concentrations of methanol were never used. Pakistani isolates of *Leishmania, Leishmania major* (MHOM/PK/88/DESTO) promastigotes were used for checking the leishmanicidal activity. Parasites were grown in medium RPMI-1640 buffered with 25 mM TES and supplemented with 5% HIFCS + 1.5 % human urine at 23-25°C in dark. To study the effect, 4 x 10^6 promastigotes were taken into 96 well microtiter plate, leaf and twig extracts were added separately and diluted serially starting from 100 μg/ml upto 0.75 μg/ml. Only methanol/PBS was added in control. The culture was left for 3-5 days at 23-25°C in dark after which growth was assessed by counting the parasites microscopically on an improved Neubauer chamber (Table-5).

Reference Drugs: Pentamidine

Results

Previous studies on *Periploca graeca* extracts have shown that it has a negative chronotropic effect. The water extract was found to be active on the heart. Also found active on an animal trial as an intra venal infusion. It also has hypertensive activity and positive inotropic effect was shown on the water extract (24).

Our studies showed that the plant has only slight toxicity in brine-shrimp *in vivo* lethality assay with the value of LD_{50} 705.47 μg/ml (389.24- 1743.18) for both leaf and twig extract. But further work on different cell-lines are worthwile to screen; as many active compounds were reported previously from *Periploca* species (7,8,19,20).

Lemna bioassay (25) for plant growth assay on the plant has shown inhibition of plant growth with significant effects at high concentrations. Leaf extract of the plant was found to be more active. Table-1 summerizes the phytotoxicity effects at different concentrations.

Agar tube dilution protocol (26) was used to determine the fungicidal activity of the crude extracts against 10 different human, animal and plant pathogenic fungi isolated from clinical or plant pathogenic sources. Only weak activity was observed at the concentration of 400 μg/ml. The results are shown in Table-2.

Agar well diffusion protocol (27) was employed to screen antibacterial activity of the extracts. The twig extract especially showed good activity against *Bacillus cereus* which is responsible for food poisonings in humans. Twig extract inhibited *B. cereus* at a concentration of 200 μg/100 μl. This showed that the plant may yield promissing active compounds after further purification. Some minor inhibitory effects on *Klebsiella pneumoniae* and *Salmonella typhi* were observed. All other bacteria showed strong resistance towards the extracts (Table-3).

Filter paper exposure method for insecticidal activity was applied on both extracts. No effect on *Tribolium castaneum.* was observed. The bioassay showed that the insects are resistant to the extracts in 20% concentration (w/v).

Nematocidal activity (28) was observed at a concentration of 1000 ppm in leaf extract significantly (Table-4).

Methanolic extract of *Periploca graeca* twigs showed a concentration dependent potent antileishmanial activity according the bioassay (29). The IC_{90} and IC_{50} values were found to be 12.5 μg/ml and 2.31 μg/ml respectively on 3rd day after inoculation. Whereas methanolic extract of the leaves showed no activity.

The comparative results are shown in Table-5 below.

As the final conclusion, it is worthwhile toconduct bioassay guided fractionations towards antileishmanial activity, nematocidal activity and plant growth inhibition. Also bactericidal activity against *Bacillus cereus* of further fractions of the leaf extract can be performed to yield useful compounds.

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Table1. Inhibition in percentage of Lemna after treatment with extracts

SAMPLE	Inhibition (%)		
Concentration	500 ppm	50 ppm	5 ppm
Control	100	100	100
Leaf extract (LE)	100	20	12
Twig extract (TE)	52	24	12

Table2. Fungicidal activities of the extractract against the controls in mm inhibition

Fungus	LE (mm)	Ref.D. (mm)	INH. %	TE (mm)	Ref.D. (mm)	INH. %
Trichophyton schoenleinii	60	65	7.6	55	65	15.3
Trichophtyon longifusus	55	60	8.3	60	60	-
Pseudallescheria boydii	50	60	16.5	40	50	20.0
Candida albicans	55	55	-	55	55	-
Aspergillus niger	72	85	15.2	45	60	25.0
Microsporum canis	68	78	12.8	40	50	20.0
Trichophyton mentagrophytes	40	50	20.0	40	50	20.0
Fusarium oxysporum var. *lycopersici*	75	75	-	75	75	-
Fusarium solani var. *lycopersici*	51	60	15.0	55	65	15.3
Rhizoctonia solani	68	78	12.8	60	78	23.0

LE: Leaf Extract ,TE: Twig Extract , INH: Inhibition, Ref.D.: Reference Drug

Table3. Bactericidal activity measured in inhibition zones (mm) of extracts compared with the controls

Bacteria	LE INH. (mm)	Ref.D. (mm)	TE INH. (mm)	Ref.D. (mm)
Bacillus cereus	-	-	7	7.75
Cornynebacterium diptheriae	-	-	-	-
Escherichia coli	-	-	-	-
Klebsiella pneumoniae	7	21.0	6.5	30
Proteus mirabilis	-	-	-	-
Pseudomonas aeroginosa	-	-	-	-
Salmonella typhi	5.5	23.3	-	-
Shigella boydii	-	-	-	-
Staphylococcus aureus	-	-	-	-
Strephtococcus pyogenes	-	-	-	-

Table4. Nematocidal activity and mortality percentage of two extracts after different timings

Nematocidal activity (1000 ppm)	Mortality (%) after		
	24 h	48 h	72 h
Control	0	0	2
Leaf extract (LE)	75	83	86
Twig extract (TE)	0	0	0

Table5. Percent inhibition of *Leishmania* promastigotes

Parasite/ compound (µg/ml)	100	50	25	12.5	6.25	3.125	1.5	0.75
Control	-	-	-	-	-	-	-	-
LE	-	-	-	-	-	-	-	-
TE	100	100	92	90	70	60	40	30
Pentamidine	100	65	42	30	20	8	-	-

The values are (mean + 5%) of triplicate determinations

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