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THE CYTOTOXIC POTENTIAL OF IVY (*Hedera helix* L.) LEAVES

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Abstract

Brine shrimp bioassay of *Hedera helix*L. showed that the leaves possess cytotoxicity (LC_{50}= 802.73µg). Further fractionation and subsequent brine shrimp bioassays of the fractions obtained, showed that the fraction F4 is the cytotoxic principle (LC_{50}= 161.84 µg). Infra red, Ultra violet spectroscopies and chemical tests showed that the fraction F4 is a phenolic compound.

Introduction

*Hedera helix*L. (Family Araliaceae). is found over a greater part of Europe, northern and central Asia (Grieve, 1974). In Pakistan it grows wild in Kurrum, Swat, Hazara, Murree hills and Kashmir from 5-9 thousand feet (Nasir & Ali, 1975). It is a common climber on *Pinus wallichiana* trees of Murree-Hazara hills, where it also grows as creeper over the ground in the woods. *Hedera helix* is an important medicinal plant, having antifungal (Timen-David *et al.*, 1980), antibacterial (Cioaca *et al.*, 1978), antitussive, antispasmodic, anti-inflammatory (Braudet, 1967), antiliposclerosis (Maffei *et al.*, 1990), antimutagenic (Elias *et al.*, 1990) and hypoglycemic (Ibrar and Ilahi, 2000) properties.

The anti-cancer property of *Hedera helix* was revealed some years back in a letter (unpublished, but in possession of the author) by Dr. Ilyas, an oncologist of Peshawar-Pakistan, when one of his patients, who was suffering from advance stages of Hodgkin lymphoma, recovered from the disease after using this plant. The present study was aimed to fractionate the leaf extract of *Hedera helix*, test the fractions for cytotoxicity, and to analyse the cytotoxic fraction in order to know its chemical nature.

Materials and Methods

Silicagel Kieselgel 60 for Column Chromatography.Silicagel Kieselgel 60HF254 for preparative TLC, ethanol, methanol, chloroform, n-hexane, n-butanol, pet. Spirit and ether.(all analytical grades.), Brine shrimp eggs, sea salt.

Leaves of *Hedera helix* L. were collected from Baragali, Abbottabad in the month of September. The leaves were washed to remove adhering dirt and were then dried in shade. After garbling and removing petioles the dried leaves were powdered using hand driven mincing machine. The powdered leaves were stored in a well closed ambered glass bottle at room temperature for further use.

Preparation of Methanolic Extract: Five hundred grams of the powdered leaves of *Hedera helix* were macerated in 1250 ml methanol for 7 days at room temperature and the extract was then filtered. The residue left was again soaked in methanol and

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extracted after 7 days. The two methanolic extracts were combined and after vacuum evaporation a semisolid extract was obtained (Harborne, 1973).Fourty grams of this extract were repeatedly washed with petroleum spirit to remove colouring and fatty materials. This methanolic extract was tested for cytotoxicity, using brine shrimp bioassay (Meyer *et al.*, 1982) and the data obtained was analysed using Finney computer programme (Finney, 1971).

Saponin and Non- Saponin Parts of the Leaves: Sixty grams of powder leaves of *Hedera helix* were first extracted with chloroform (300 ml) in a soxhlet apparatus for 24 hours. The residue left was next extracted with ethanol (300 ml) for 24 hours in soxhlet apparatus. The ethanolic extract was evaporated in vacuum to a rasidue, which was next dissolved in methanol (100 ml). To this methanolic solution ether solvent was added drop wise to precipitate the saponins (Bikram Singh *et al.*, 1987). The saponins were next tested for cytotoxicity, using the brine shrimp bioassay

The part left after the separation of saponin (non-saponin part) was retained; methanol and ether were evaporated from it and named as "Residue", which was also tested for cytotoxicity along with the methanolic extract and the saponins.

The residue was further fractionated through column chromatography using chloroform, methanol: chloroform (1:1) and methanol as mobile phases. The three fractions were evaporated and tested for cytotoxicity. The chloroform fraction showed significant cytotoxicity. In order to isolate the cytotoxic principle this chloroform fraction was further fractionated using preparative TLC. Various combinations of different solvents as mobile phase were tried in order to develop a suitable mobile phase. The n-Hexan: chloroform: methanol (1:1:2.5) system was adopted as it separated the sample in to six clear bands, F1 - F6. The Rf values of these bands were determined. The bands were then scrapped and collected separately. This process of preparative TLC was repeated many times to run 400 mg of the sample. The scratched fractions were freed from the silica gel, and after evaporating the solvents from these fractions, their weights were recorded.

These fractions were then tested for cytotoxicity using the same brine shrimp bioassay. Out of these six fractions, only the fraction F4 showed some significant cytotoxicity. In order to know the chemical nature of fraction F4, chemical test, Ultra Violet and Infra Red spectroscopies were performed.

Brine Shrimp Bioassay

Brine shrimp hatching: Sea salt solution was prepared by dissolving 38 gm sea salt in 1000 ml double distilled water and final solution thus prepared was filtered. The solution was taken in a small plastic tub, which was divided by a partition, having small holes in it. The eggs were sprinkled on one side of the partition, which was then covered with a black carbon paper. The other half of the tub was illuminated with an electric lamp. After 48 hours the shrimp hatched and matured as naupili. They actively swam and migrated through the holes in the partition wall to the illuminated side of the tub.

Bioassay Procedure: Twenty milligrams of each of the extracts/fractions was taken in small vial and dissolved in 2 ml of methanol to serve as stock solution.

From the stock solution, 500 µl, 50 µl and 5 µl (corresponding to 1000, 100, 10 µ g/ml, respectively) were transferred to vials with three replicates of each concentration.

The vials were placed uncovered for 24 hours for complete evaporation of the solvent, methanol, after which 2 ml sea salt solution was added to each of the vial. 10 brine shrimp were transferred to each vial (30 brine shrimps per dilution) with the help of long-tipped dropper and the volume was adjusted to 5 ml with the sea salt solution. After 24 hours, survived brine shrimps were counted for all the concentrations of the extracts/fractions.

In order to know the chemical nature of the cytotoxic principle, it was subjected to chemical test, UV and IR spectroscopies.

Results and discussion

The result of bioassays of the methanolic extract, saponin and the residue (Table 1) showed that the leaves contain cytotoxic principles, but the saponins are not cytotoxic and it is the "residue" part of the extract that is cytotoxic (LC_{50} =700.54μg ml^{-1}).

It can be concluded that though methonolic extract of *Hedera helix* leaf is cytotoxic ($LC50_{=802.73}$μg ml^{-1}), but the saponin isolated are not cytotoxic (LC_{50} > 1000 μg ml^{-1}). This fact is also confirmed by findings of Quetin *et al.* (1992) that the crude extract of *Hedera helix* exerted cytotoxic activity, both *in vitro* and *in vivo*, but the saponin isolated form this plant have no cytotoxic effect on cancer cells.

The bioassay result of the fraction left after separation of saponin, i.e., the *"RESIDUE"* (Table-1) is very significant ($LC_{50=}$ 700.54 μg ml^{-1}), indicating that this part of the extract of dried leaves contained the cytotoxic principle.

Bioassay data of the three fractions of the *"RESIDUE"* obtained through column chromatography (Table-2) further revealed that the cytotoxic principle is present in the first (chloroformic) fraction ($LC_{50=}$ 423.59 μg ml^{-1}), where as the other two fractions (2&3), are not cytotoxic, as their LC_{50} are greater than 1000 μg ml^{-1}.

For further separation this chloroform fraction was next subjected to preparative TLC, which resulted into six clear bands/ compounds, designated as F1, F2, F3, F4, F5 and F6,respectively.

Rf values and percent yield of these compounds are given in Table-3. These six fractions were next tested for their cytotoxic potentials. The bioassay data of these six compounds (Table-4) shows that only the compound F4, with Rf value 0.889,and a percent yield of 0.16 has a very significant cytotoxic activity ($LC_{50=}$ 161.84 μg ml^{-1}).

The λ_{max} (280 nm) of the compound F4 obtained after UV spectroscopy is closely resembling with phenol (λ_{max} 278nm) when run along with it in chloroform, suggesting phenolic nature of the compound F4. The phenolic nature of this compound is also established by Infrared spectroscopy (Fig-1), which shows a phenolic stretch at 3600 cm^{-1} . Ferric chloride colour test (Horborne, 1973) of F4 gave an intense green colour, which also confirmed the phenolic nature of the compound F4.

From early 1990s the biological activity, specially the anticancer activity of a number of phenolic compounds from the higher plants, marine algae, and fungi have been found to possess antitumor and cytotoxic activity; e.g. Phenolic compounds (kneglomeratanol, Kneglomeratanones A and B) isolated from *Knema*

glomerata possess cytotoxic activity when tested by brine shrimp bioassay and also showed moderate but significant toxicities against three human tumor cell lines, and inhibited the growth of crown gall tumors on discs of potato tubers (Zang *et al.*, 1994). Blunden *et al.* (1994) isolated polyphenols from marine algae and found them to possess cytotoxic activity. Similarly Takahashi *et al.* (1992 and 1993) have reported four phenolic compounds of fungal origin (Russuphelin A, B, C and D), to exhibit cytotoxic activity against 10 tumor cell lines including human derived carcinoma cells. These compounds are also tested *in vitro* against P388 leukemia cells and are found to be significantly active. Keeping in view the reported cytotoxicity of phenolic compounds and the cytotoxicity showed by the phenolic compound isolated from the *Hedera helix*, this plant can be used for further *in vitro* and *in vivo* cytotoxic and anti-cancer studies.

Table1. Data of bioassay of crude methanolic extract, saponin and the residue.

S.No	Dose (µg/ml)	No. of Brine shrimp taken	No. of Brine shrimp killed	LC_{50} (µg ml^{-1})
Methan				
ol	1000.0	30	15	
1	100.0	30	10	802.73
2	10.0	30	3	
3				
Saponin				
1	1000.0	30	6	
2	100.0	30	5	>1000
3	10.0	30	6	
Residue				
1	1000.0	30	16	
2	100.0	30	9	700.54
3	10.0	30	3	

Table 2. Data of bioassay of column chromatographic fractions of the "residue".

S.No	Dose (µg/ml)	No. of Brine shrimp taken	No. of Brine shrimp killed	LC_{50} (µg ml^{-1})
FRACTION No1				
1	1000.0	30	21	
2	100.0	30	5	423.6
3	10.0	30	2	
FRACTION No2				
1	1000.0	30	11	
2	100.0	30	2	>1000
3	10.0	30	2	
FRACTION No3				
1	1000.0	30	9	
2	100.0	30	4	>1000
3	10.0	30	1	

Fraction no1 = Chloroform Fraction, fraction no2 = Chloroform: Methanol (1:1) Fraction, fraction no3 = Methanol Fraction.

Table 3. Rf values and percent yield of compounds (F1-F6) obtained after TLC.

S.No	Compounds	Rf values	Percent yield
1	F1	0.375	0.24%
2	F2	0.565	0.18%
3	F3	0.813	0.14%
4	F4	0.889	0.16%
5	F5	0.924	0.12%
6	F6	0.965	0.32%

Table 4. Data of bioassay of compounds (F1-F6) obtained after the TLC of the chloroform fraction.

Compounds	Dose (µg/ml)	No. of Brine shrimp taken	No. of Brine shrimp killed	LC_{50} (µg ml^{-1})
F1				
1	1000.0	30	5	
2	100.0	30	3	>1000
3	10.0	30	2	
F2				
1	1000.0	30	5	
2	100.0	30	2	>1000
3	10.0	30	1	
F3				
1	1000.0	30	3	
2	100.0	30	3	>1000
3	10.0	30	1	
F4				
1	1000.0	30	22	
2	100.0	30	14	161.84
3	10.0	30	4	
F5				
1	1000.0	30	15	
2	100.0	30	8	>1000
3	10.0	30	10	
F6				
1	1000.0	30	10	
2	100.0	30	10	>1000
3	10.0	30	7	

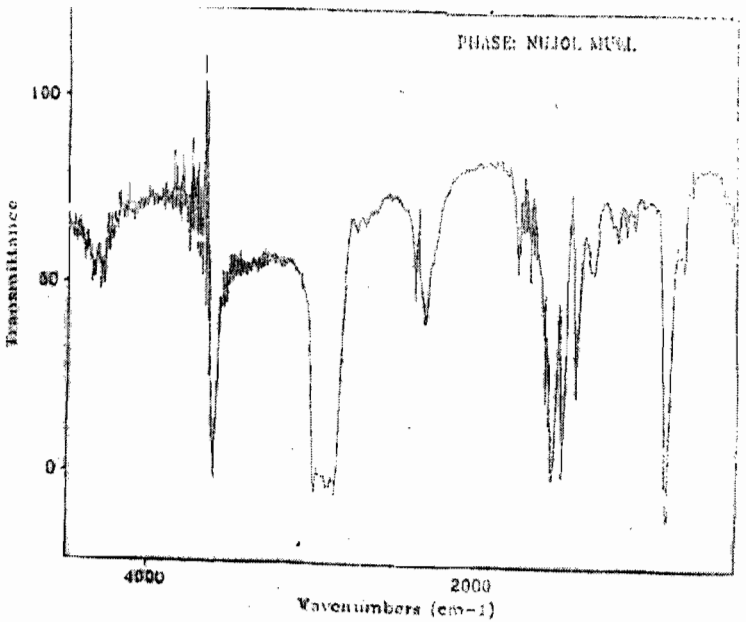

Fig1: Infrared Spectra of the Compound F4

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