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CYTOTXICITY OF FOUR MEDICINAL PLANTS OF PAKISTAN

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Abstract

The extracts of the four biologically active medicinal plants *Grewia erythraea*, *Hymenocrater sessilifolius, Vincetoxicum stocksii* and *Zygophyllum fabago* were evaluated for their cytotoxicity. These plants are used in folk medicine but their scientific toxicity is not well established. The cytotoxicity of the six fractions of plant extracts was determined by flow cytometry on 2×10^6 CFU/ml of *C. albicans* at different concentrations of extracts for a period of 1, 2, and 24 h at 25ºC. The 1 h exposure showed that 1.0 g/ml extract of *Z. fabago* killed 98% of cells; *V. stocksii* killed 60%; *H. sessilifolius* 29% and *G. erythraea* only 2%. Similar results were obtained when the cytotoxicity was also checked through brine shrimps (*Artemia salina*) cytotoxicity assay. The chloroform fractions of all the extracts were most effective.

Introduction

In Balochistan, a province of Pakistan, a large number of medicinal plants have historically been used to treat a wide range of diseases (Atta-ur Rehman & Ahmad, 1986). In the rural areas, where health services are not adequate, people depend on locally available medicinal plants for curing various illnesses. Four such indigenous medicinal plants of Balochistan namely *Grewia erythraea* Schwein f. (Tiliaceae) , *Hymenocrater sessilifolius* Fisch. C.A. Mey (Lamiaceae)**,** *Vincetoxicum stocksii* Ali & Khatoon (Asclepiadaceae) and *Zygophyllum fabago* L. (Zygophyllaceae).

These plants are used locally for the treatment of a number of diseases. Native people use these plants by boiling 15–20 g of leaves and shoots in water for a few minutes and consume it as a hot drink twice a day. For instance, *Grewia erythraea* is used for cough, common cold and other respiratory tract illnesses whereas *Hymenocrater sessilifolius* is used for the cure of fever, headache, giddiness, cardiac diseases and wounds. In our previous publications we have reported their remarkable antibacterial and antifungal properties; presence of fatty acids, keto-alcohols and other effects (Mansoor *et al*., 1998; Zaidi & Crow, 2005). Flavonoids and essential oils are important constituents of *Hymenocrater sessilifolius* (Zaidi & Crow, 2005).

Vincetoxicum stocksii is known for its poisonous as well as its medicinal properties. Traditionally, it is used to treat external cancers, wounds and injuries in man and animals (M-Simándi, 1996). Leaves and shoots are ground and used externally as poultice. Ten glycosides and cytotoxic alkaloids from another species *Cynanchum vincetoxicum* have been reported (Stockel *et al*., 1969; Staerk *et al*., 2000). It was also found to be active against mammary carcinoma cells (Tanner &Wienheim, 1993).

Zygophyllum fabago is used as part of a drug for rheumatism and gout. It is also used externally for the cure of skin diseases, external wounds and injuries and is known to have some antiseptic properties. Some work on the isolation of natural compounds and their biological activities has been done on other species of these plants, but not on these four species. Only two triterpenoidal saponins were reported from the aerial parts of the same species of *Z. fabago* (Attia, 1999). Another species *Z. eichwaldii* has been described for its use as an antiseptic, antieczemic and antidiabetic medicine for stomach and liver diseases (Samsokov *et al*., 2001). Toxicity of yet another species of *Zygophyllum gaetulum* has also been established (Skim *et al*., 1999). Therefore, this study was conducted to investigate the cytotoxic effect of these plants.

Materials and Methods

Whole plants of *G. erythraea, H. sessilifolius, V. stocksii* and *Z. fabago* were dried in shade, ground and soaked in methanol for 10 days. Thick dark crude extracts were obtained after evaporating the solvent through rotary evaporator. Fractionation of the methanolic extracts was carried out to determine the active fraction responsible for these activities. Fifty grams of the crude extract of each plant was dissolved in distilled water, filtered and extracted with hexane in three steps. This procedure was repeated with ethyl acetate, chloroform, methanol and butanol. Fractions were dried through rotary evaporator in all experiments.

Cytotoxicity assay through flow cytometery: Cytotoxicity of crude extracts of four plants was determined through Flow Cytometery (FACScalibur, Becton Dickinson). 24 h old cultures of *Candida albicans* CA-30 were grown on Sabourad Dextrose Agar. After harvesting and washing twice at 8000 rpm for 10 minutes in saline, they were exposed to different concentrations of plant extracts 1.0g /ml, 0.1g /ml, and 0.01g/ml. A concentration of 2×10^6 CFU/ml of *Candida albicans* was used in all cases, and incubated for 1, 2 and 24h. After harvesting and washing twice they were stained with propidium iodide. The fluorescent emitted by the cells was read and the percentage of live vs. dead was calculated. The experiment was repeated three times and results are expressed in mean.

Brine shrimp Cytotoxicity assay: Cytotoxicity of crude extracts of these plant extracts was also checked through brine shrimp (*Artemia salina*) assay (Meyer *et al*., 1982). Larvae were produced from 50mg of eggs when incubated in artificial seawater at 25ºC for 48h. Extracts of all the plants were dissolved separately in 20mg/ml-distilled water. From this solution, different concentrations 1000µl, 100µl and 10µl were taken and 3 vials for each

concentration were made along with the controls. 30 shrimp were shifted to each concentration and control vial, after 24h the number of survivors was calculated. The experiment was repeated five times and results are expressed in mean and standard errors of mean.

Results and Discussion

Crude extracts of four medicinal plants were checked individually for their cytotoxicity. Among the plants tested *Z. fabago* and *V. stocksii* are considered toxic and therefore it is not surprising that sheep fed with the leaves and roots of these plants are reported to die or fall seriously ill. *Z. fabago* showed highest cytotoxicity, 1.0 g/ml/h of the extract killed 98% cells of *C. albicans* when checked through flow cytometry (Table 1). The extract also showed maximum toxicity against brine shrimps as 100% of the shrimps were killed at high concentration in 24h.

Vincetoxicum stocksii also exhibited very high cytotoxicity against brine shrimps as it killed 100% of the shrimps at high concentrations. It exhibited very strong activity against *C. albicans,* as it was found to be very toxic for *C. albicans,* killing 60% of the cells at 1.0g/ml/h when checked through flow cytometer (Table 1). It must also be noted that the effect of ethyl acetate and chloroform fractions was more profound when compared with other fractions. It is directly correlated with increasing concentrations as well.

Grewia erythraea cytotoxicity tests proved it to be non-toxic against brine shrimps and *C. albicans,* as no shrimp was killed even at highest concentration (Table 2). Results of flow cytometry showed 1.0g/ml/h of the extract killed only 2% cells of *C. albicans* (Table 1).

Table 1. Cytotoxicity of plant extracts through flow cytometry.

Plant extracts fractions	Concentrations in g/ml; % Dead Cells (mean)								
	1h			2h			24h		
	1.0g/ml	0.1g/ml	0.01g/ml	1.0g/ml	0.1g/ml	0.01g/ml	1.0g/ml	0.1g/ml	0.01g/ml
G. erythraea									
Hexane	2	0	0	2	0	0	3	0	0
Ethyl acetate	3	2	0	5	0	0	6	0	0
Chloroform	2	0	0	3	0	0	6	2	1
Methanol	1	0	0	0	0	0	1	1	1
Butanol	1	0	0	2	0	0	1	0	0
Water	2	1	0	0	0	0	0	0	0
H. sessilifolius									
Hexane	29	16	8	32	15	6	40	25	15
Ethyl acetate	27	15	12	28	16	5	30	23	10
Chloroform	15	5	7	8	10	0	16	12	0
Methanol	16	14	2	27	12	5	21	12	11
Butanol	8	10	6	13	5	0	6	10	0
Water	9	5	3	10	4	0	7	8	2
V. stocksii									
Hexane	60	30	20	72	45	30	100	72	46
Ethyl acetate	55	26	16	68	42	26	100	65	44
Chloroform	18	23	10	17	14	18	30	19	13
Methanol	20	16	9	15	12	5	55	24	21
Butanol	8	9	5	10	7	12	14	14	8
Water	12	15	11	10	13	14	20	12	7
Z. *fabago*									
Hexane	98	54	36	100	85	35	100	62	25
Ethyl acetate	90	43	24	100	80	32	100	79	50
Chloroform	40	29	25	67	44	20	36	24	20
Methanol	15	13	15	23	18	14	30	17	15
Butanol	10	5	8	11	6	5	12	9	8
Water	12	7	12	15	10	10	24	16	14

(*Candida albicans* 2×10^6 CFU/ml); Each value is an average of 3

The extract of *Hymenocrater sessilifolius* was mildly toxic at very high concentrations, as only 5 brine shrimp were killed at 1000µg/ml/24h (Table 2), 1.0g/ml/h of the crude extract killed 29% cells of *C. albicans* in 24h, but its cytotoxicity was not significant (Table 1).

The four medicinal plants of Balochistan were found to have biologically active compounds, which explain their use for the treatment of cancer, infectious diseases, injuries, wounds and boils, by locals and in folk medicine. The cytotoxicity of *Vincetoxicum stocksii* and *Zygophyllum fabago* is very important as these are extremely active against a common human pathogen *C. albicans* which causes a broad range of serious illnesses. The mortality rate reported for patients with *C. albicans* is the highest among all fungal infections (Crump & Collington, 2000). Our previous studies showed the extract of *Zygophyllum fabago* was highly effective against *Candida albicans* and *Escherichia coli*. The extract of *Vincetoxicum stocksii* was also found to be significantly active against *Candida albicans, Bacillus subtilis* and *Bacillus cereus* (Zaidi & Crow, 2005). Therefore, we propose that these two plants can be employed for the development of relatively inexpensive and effective antifungal drugs. High toxicity was found in *Z. fabago* and similar results were reported by Skim *et al*., (1999). The cytotoxicity of these plants is also very

important since they are being used for the treatment of external cancers. Previous studies have demonstrated the presence of cytotoxic phenanthroindolizidine alkaloids from another species of same genus *Cynanchum vincetoxicum* and from other members of family Asclepediaceae. These alkaloids exhibit pronounced cytotoxicity against memory carcinoma cells (Stockel *et al*., 1969; Staerk *et al*., 2000; Tanner & Wiegrebe, 1993). The hexane and Bioassay guided isolation, purification and identification of these active compounds is in progress. Its mode of action should also be further studied for drug development. On the other hand, extracts of *Hymenocrater sessilifolius* showed non-significant activity against *C. albicans* and was not toxic for brine shrimps. *Grewia erythraea* was also relatively non-toxic for *C. albicans* and brine shrimps. It may therefore be a potential candidate for antibacterial drugs.

Table 2. Brine shrimp cytotoxicity bioassay.

Dose conc. (µg/ml)	Extract fraction	No. of shrimp	No. of survivors (Mean)				
			G. erythraea	*H. sessilifolius*	*V. stocksii*	*Z. fabago*	Control
1000	Hexane	30	30	25	0	0	30
1000	Ethyl acetate	30	29	22	1	0	30
1000	Chloroform	30	28	22	0	0	30
1000	Methanol	30	30	24	0	0	30
1000	Butanol	30	30	26	0	0	30
1000	Water	30	30	27	2	0	30
100	Hexane	30	30	28	2	1	30
100	Ethyl acetate	30	30	26	2	1	30
100	Chloroform	30	30	25	4	4	30
100	Methanol	30	30	28	5	6	30
100	Butanol	30	30	28	5	7	30
100	Water	30	30	30	6	7	30
10	Hexane	30	30	29	6	2	30
10	Ethyl acetate	30	30	28	8	7	30
10	Chloroform	30	30	26	7	9	30
10	Methanol	30	30	29	14	12	30
10	Butanol	30	30	28	10	13	30
10	Water	30	30	29	10	10	30
LD_{50}			74.625	66.292	47.2711	32.738	74.625

Abbreviations: G=*G. erythraea*; H=*H. sessilifolius;* V=*V. stocksii*; Z= *Z. fabago*; each value is an average of 3. Standard drug Etoposide

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