ORIGINAL ARTICLE

IN VITRO ANTIFUNGAL ACTIVITY OF RHAZYA *STRICTA*

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ABSTRACT
Rhazya stricta is a small glabrous shrub, widely distributed throughout Western Asia from Yemen to Arabia, to the North West Province of India and abundantly found in various regions of Pakistan.Larvicidal and antifungal studies of polar and non polar aerial parts extracts of Rhazya stricta were performed using brine shrimps larvae for larvicidal study and for antifungal study microorganisms, *Trichophyton longifusis, Aspergillus flavus, Candida albicans, Microsporum canis* and *Fusarium solani* were used respectively.The methanol fraction showed significant cytotoxicity with LC_{50} 17.809µg/ml, having mortality rate 73.33 % at highest dose. While

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pet-ether, chloroform and carbon tetrachloride possessed moderate to low cytotoxicity with their LC_{50} values 49.077 µg/ml, 95.859 µg/ml and 80.489µg/ml respectivelt, ethyl acetate fraction showed no cytotoxicity.Results of antifungal studies showed that fractionated samples of methanol and chloroform possessed significant antifungal activities against, *Trichophyton longifusis, Aspergillus flavus, Candida albicans* and *Fusarium solani* respectively. Due to these promising results, further *in vivo* studies over *R. stricta* must be conducted.

Keywords: *Rhazya stricta*; aerial parts; extracts/fractions; antifungal activity.

INTRODUCTION

Use of traditional medicine is the mainstay of primary health care, virtually in all developing countries. The use of herbal medicine in developed countries is also expanding rapidly. It seems that people are turning towards alternative medicine, which they wish to be less harmful and with fewer side effects than Western medicine. Medicinal plants are the most exclusive source of life saving drugs for the majority of the world's population. Bioactive compounds currently extracted from plants are used as food additives, dyes, insecticides, cosmetics, perfumes and fine chemicals. These compounds belong to a group collectively known as secondary metabolites.

About 80 % of world population is reliant on medicinal plants to maintain their health and to cure their ailment. With growing interest world wide in medicinal plant as a source of medicine, there is need to introduce new important plants of established therapeutic values used either in modern or traditional system of medicine which are sold widely in world market, for herbal, homeopathic, allopathic formulations (Ahmad *et al*., 2004).

Pakistan occupies a unique position among developing countries as it has good potential within the variety of medicinal plants due to its varied climatic and edaphic factors, which reflect diversity and valuable medicinal plant heritage. About 6000 taxa of flowering plants have been reported to occur in Pakistan, including Kashmir. It is interesting that over 50% of the population in Pakistan is being treated with traditional medicines by almost 50,000 traditional herbal practitioners *Hakims* (Zaidi, 2006). More than 350 regular items (as whole herbs or specific parts) have been enlisted, which are used in unani herbal preparations by various Herbal Drugs Manufacturing Laboratories (D*awakhanas*) in Pakistan). Pakistani flora offers great possibilities for the discovery of new compounds with antimicrobial activities (Haq, 1983).

There are various types of fungal pathogens that infect humans, animals and plants some causes sever type of acute inflammation and infection of hair, nails and skin. Such as *trychophylon Longifusis*, some organisms cause chronic infection of lungs, ear, and bones etc., such as *candida albicans*, some causes infection of joint, skin and central nervous system, such as *Aspergillus flavis*, while *Microsporum canis* causes ring worn infection of skin and hair in dogs and cats. Keeping in view there is need for investigation of new antifungal compounds (Khan *et al*., 2004).

The objectives of this article are to address the recent therapeutic value of *R. stricta* shrub for in vitro antifungal activity which could serve as good source for the development of new antifungal agents and standardized phytomedicines. In this paper we report the results of our study on antifungal activity of *Rhazya stricta* used as a traditional medicine since long. The plant was chosen on the bases of its reported uses in literature (Rahman and Qureshi, 1990).

***Rhazya stricta**:*

Genus:	Rhazya
Family:	Apocynaceae
Arabic name:	Raziyun
Urdu name:	Sanwar, Vena, sehar, sewar, gunders
English name:	*Rhazya stricta* Decne
Parts used:	All parts of the plant
Quality/temperament:	Cold and dry in second order

Rhazya stricta Decne is a stiff-growing plant with erect stems, 2-3 feet high and upright thickish smooth leaves placed close together on the stem (Nazimuddin and Qaiser, 1983). Its infusion is a good tonic with peculiar bitter taste. Phytochemical analysis has identified more than 100 alkaloids (Gilani *et al.,* 2006). These alkaloids have several pharmacological properties. It is also used for throat sour, in fever, general debility and as curative for chronic rheumatism and tumor. *Rhazya stricta* is used traditionally in Asia for the treatment of different types of diseases such as skin diseases, stomach diseases and antihypertensive (Mukhopadhyay *et al*., 1981. The leaves, flowers and fruit are also used in joint infections and for cancer (Rahman and Qureshi, 1990).

MATERIALS AND METHODS

Materials

Ethyl alcohol, methanol, chloroform, carbon tetrachloride, dimethyl sulfoxide (Merck, Germany); ethyl acetate (Beijing, China) and sea salt (Sigma Chemical Co., UK), Sabouraud dextrose agar (Difco), were used as received. Micro organisms, *Trichophyton longifusis, Aspergillus flavus, Candida albicans, Microsporum canis, Fusarium*

solani were obtained from HEJ International Research Institute of Chemistry Karachi, Pakistan.

Methods

Collection of shrub: The aerial part of *Rhazya stricta Decne* was collected from area of District Lakki Marwat (N.W.F.P) Pakistan. The taxonomic identification of the plant material was confirmed by plant taxonomist in the Department of Pharmacognosy, Faculty of pharmacy, Gomal University, Dera Ismail khan (N.W.F.P) Pakistan. The collected material were washed under running tap water to remove the adhering dust, dried under shed and ground in a grinder to a powder with a 2mm diameter mesh. The total weight of powder was 800gm.

*Extracts: 7*00gm of the said powder was extracted with ethanol (1× 96% and 2 ×80%) The ethanolic extracts were combined and filtered using Whatman filter paper No-4I (Sargent-Welch, USA), and then evaporated at 40°C to dryness, using vacuum evaporator, and freeze dried (yield 38.5gms). 36.5gms of the semisolid crude extract was dissolved in 250ml distilled water and subsequently partitioned between petroleum ether (P.E), chloroform ($CHCl_3$), carbon tetra chloride (CCl_4), ethyl acetate (EtOAC) and methanol (MeOH) (Rojas *et al*., 2003). The resulting fractions were concentrated by rotary vacuum evaporator (Eyela, NE-1R. Japan) and freeze dried to obtain fractions, P.E (6.00gm) $CHCl_3$ (10.00gm), CCl_4 (4.00gm) EtOAC (3.00gm), methanol (12.5gm). These fractions were stored in a freezer till further use. The above processes were performed in the Drug Delivery Research Laboratory of the Faculty of Pharmacy, Gomal University, Dera Ismail Khan, (N.W.F.P) Pakistan under sterile conditions.

In vitro cytotoxic bioassay (Cytotoxicity test)

Hatching of Brine shrimp eggs:

Brine shrimp (Artemia salina) larvae, called nauplii, have been used in toxicological studies for more than 45 years. A positive correlation has been demonstrated between brine shrimp nauplii lethality and human cytotoxicity (Krishnaraju *et al.*, 2006). Consequently, brine shrimp nauplii can be used to pre-screen compounds for potentiality of tumor activity. In addition, brine shrimp bioassays predict pesticidal activities and respond to a broad range of chemically and pharmacologically diverse compounds. A rectangular dish (22 x 32 x 10 cm) was half filled with filtered brine solution of sea water prepared by dissolving 38.0gm/L sea salt (Sigma Chemical Co., UK) in distilled water. A plastic sieve no 20 was clamped to the dish to form two unequal compartments). The Brine shrimp eggs (100 mg) were sprinkled into the larger portion of the dish and covered with aluminum foil, while the smaller one was remained open and exposed for light. The eggs were incubated to hatch for 48 hours at 37 °C (Khan *et al*., 2004).

Preparation of stock solutions (Crude extract /Fractions) for Brine Shrimps testing: Twenty milligrams of each extract was dissolved in 2ml methanol separately, packed in sterile glass vials of 5ml capacity and used as stock solutions when required.

From each stock solution transferred 5µl, 50µl and 500µl to the experimental vials (3vials/concentration) the final concentrations of the above dose were equivalent to10µg/ml, 100µg/ml and1000µg/ml respectively. All these vials were kept at room temperature for the evaporation of organic solvents.

Cytotoxicity study against brine shrimps

Transferred 2ml Brine Shrimps solution to all of these vials (three vials for each concentration and one vial each for positive and negative control). The negative control will act as blank without test compound, contained only brine shrimps solution (artificial sea salt solution), while positive control contained brine solution plus standard reference drug *Etoposide* (Lianyungang Guiyuan Chem Pharm Co, China). After 48h, the larvae (nauplii) were collected with a help of 150mm Pasteur pipette (John Poulten Ltd, England) from the lighted side of the rectangular dish and transferred 10 shrimps to each vial including positive and negative controls. To all these vials added more brine solution to make volume up to 5ml (Atalay, 2001). The vials were incubated at 27°C for 24 hours under illumination. After 24h survivors were counted, by using a dissection microscope, and percentage of mortality (% M) of each dose was calculated as compared with control.

% M = Percentage of survival in the control - Percentage of survival in the treatment.

Activities were considered significant if the LC_{50} value was less than 30µg/ml (= 0.03mg/ml).In this bioassay, the mortality of brine shrimps, incubated in the test solution, was recorded. Repeated each experiment two times.

STATISTICAL ANALYSES

LC_{50} was determined from the 24h counts using the probit analysis method with 95% confidence interval (Finney, 1971).

Antifungal activity

Stock solution for antifungal activity: For antifungal study each extract was resuspended in DMSO at a concentration of 100mg/ml and stored in a refrigerator till further used. Antifungal activities of the extracts were evaluated by means of agar well diffusion assay. The assay was carried out according to the method of (Hufford *et al*., 1975). Sabouraud dextrose agar (Difco) was used for the growth of fungus. Media with acidic pH (pH 5.5 to 5.6) containing relatively high concentration of glucose (40%) is prepared by mixing (SDA) Sabouraud dextrose

Table 1: Brine Shrimps Lethality Bioassay of *Rhazya stricta in* different solvents

Dose µg/ml	Total No of Shrimps	No of survivors in treatment group					Survivors in controls		
		P.E	$CHCL_3$	CCl_4	EtOAC	MeOH	Std. drug Etoposide µg/ml	Negative	Positive
10	30	29	28	29	30	16	7.4625	100%	0 %
100	30	25	26	26	30	13			
1000	30	20	24	22	30	08			

Methanol fraction showed significant activity.
P.E: pet ether., $CHCL_3$: Chloroform: CCl_4: Carbon tetrachloride.,EtoAC:Ethyl acetate MeOH:Methanol., Std: Standard.

Table 2: Brine shrimps lethality bioassay of *Rhazya stricta* (MeOH fraction)

Dose µg/ml	Total no. of Shrimps	No. of dead	Sample %age Mortality	Sample LC_{50} µg/ml	Lower toxic conc. µg/ml	Upper toxic conc. µg/ml	Std. drug Etoposide LC50.µg/ml	Std. drug %age Mortality
10	30	14	46.67	17.809	4.452	71.235	7.4625	100
100	30	17	56.67					
1000	30	22	73.33					

Sample showed significant cytotoxicity with LC50 17.809 µg/ml.

Table 3: *In vitro* antifungal activity of various solvent extracts of *Rhazya stricta*, compared to the reference standard amphotericin B 0.2mg/ml.

Plant extracts 100 mg/ml	Diameter of zones of inhibition in mm				
	T. long	*A. flav*	*C. alb*	*M. canis*	*F. sol*
P.E	12 ± 0.5	10 ± 1	-	-	-
$CHCL_3$	21 ± 0.53	19 ± 1.5	20 ± 0	-	16 ± 0.53
CCl_4	10 ± 0.5	7 ±1	-	11± 1.5	-
EtOAc	-	-	-	-	-
MeOH	25 ± 0.5	-	23 ± 1	-	18 ± 1.5
Std.drug	26 ± 1	28±1	31 ± 0.5	29±1	32 ± 0.5

Zone: mean ±SD for N=3 - : No zone of inhibition.
T.Long: Trichophyton longifusis., A.flav:Aspergillus flavus., C.alb:Candida albicans.,M.canis: Microsporum canis., F.sol: Fusarium solani.

and distilled water and autoclaved at 121°C for 15 minutes. Twenty ml of molten (45°C) SDA medium was aseptically transferred into each 100mm×15mm sterile Petri dish. All these dishes were inoculated with 4mm diameter piece of inoculums removed from a seven days old culture of fungus (Janaki *et al.*, 1998).

For counting of colonies another 4mm culture (fungi) were suspended in normal saline to make volume up to 1ml and then counted with help of heamocytometer (neubar chamber). Once the agar was hardened, 11mm wells were bored using a sterile cork borer. Then 0.1ml (100µl) from each stock solution of the extracts having final concentration of 100mg/ml was placed in each the well and the plates were incubated for 24 hour at 29°C.

Two wells in each petri dish were supplemented with DMSO and reference antifungal drug Amphotericin B (0.2mg/ml) dissolved in DMSO (sigma) serve as negative and positive control respectively. The tests were carried out in triplicate. The antifungal activity was measured as the diameter (mm) of clear zone of growth inhibition. The humidity in incubation room should be maintained from 40% to 50 % (Umadevi *et al.*, 2003).

RESULTS

During these studies we found that *methanol* fraction showed significant cytotoxicity, pet. ether, carbon tetrachloride and chloroform fraction showed negligible while ethylacetate showed no activity at all as shown in table 1. LC_{50} value of methanol extract was 17.809 µg/ml which is significant in nature and percentage mortalities at a dose of 10, 100, and 1000µg/ml were 46.67, 56.67 and 73.33% respectively, while lower and upper toxic limit was 4.452 and 71.235µg/ml respectively as shown in table 2.

In vitro antifungal study was performed which was our main object of this study, by subjecting different micro organisms to various fractions of *Rhazya stricta* in different solvents. Five fractions were used in our studies named *pet ether*, c*arbon tetra chloride*, *chloroform*, *ethyl acetate* and *methanol.* Among these, methanol and chloroform fractions showed significant antifungal activities. Diameter of zones of inhibition in mm of methanol extract against *Trichophyton longifusis*, *Candida albican* and *Fusarium solani* were 25mm, 23mm and 18mm respectively. While chloroform fraction showed activity against four organisms, *Trichophyton longifusis*, *Aspergillus flavus*, *Candida albican* and *Fusarium solani* with a zone of inhibitions 21mm, 19mm, 20mm and 16mm in diameter respectively. Pet. ether and carbon tetrachloride fractions showed low activities against the said organisms, while ethyl acetate fraction no activity at all as shown in table 3. From literature review we knew that all parts of *Rhazya stricta* have been used to cure different types of diseases which strongly support our study.

DISCUSSION

Rhazya stricta Decne (Apocynaceae) is widely distributed in Pakistan and it has been used for the treatment of various ailments such throat infection, fever, rheumatism and cancer. All parts contain alkaloids which are responsible for their pharmacological activities. Name of some important alkaloids are rhazicine,strictamine N—oxide, postsecamidine, dehydroaspidospermidine N, rhazimine, leepacine, rhazizine, Strictimine and isositsirikine acetate (Rahman and Qureshi, 1990). Many in vivo cytotoxicity studies on *Rhazya stricta* have been performed. Different studies showed that when *Rhazya stricta* is administered individually in different doses and dosage forms it is more toxic as compared to be used in combination/mixture with other herbal drugs (Tahira *et al*., 2000). *In vitro* cytotoxic tests of *Rhazya stricta* on different kinds of larvae have been performed, which showed acute cytotoxicity (El-Hag, *et al*., 1999). But very less worked has been done on cytotoxicity against brine shrimps (*Artemia salina*) larvae. We select Brine shrimps larvae to determine the cytotoxicity of *Rhazya stricta* extracts in different solvents. It has been investigated that some alkaloids of *Rhazya stricta* are cytotoxic in nature which support our study (Mukhopadhyay *et al*., 1981) Other researchers also studied the cytotoxic effect of *Rhazya stricta* crude extract/fractions on different micro organisms (Adam,1 998). They found that the said shrub was cytotoxic to different larvae.

R. stricta was also studied by Rahman *et al*. (1990) they investigated that *R. stricta* posses anticancer activity. These studies support our cytotoxic studies with special reference to brine shrimps lethality bioassay.

CONCLUSIONS

From the results of the present study it was concluded that extracts as well as fractionated samples from the aerial parts of *Rhazya stricta* have significant *antifungal* activity. In order to further exploit the *in vivo* antifungal activities of this indigenous medicinal plant and to come up with a potent, safe and economically affordable formulation, further investigations are to be required.

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REFERENCES

Adam SE I (1998). Combined toxicity of Rhazya stricta and Silene villosa in rats. *Fitoterapia*., **69**: 415-419.

Ahmad H, Ghulam RB and Latif A (2004). Medicinal flora of the Thar Desert- an overview of problems and their feasible solutions. *Zonas Áridas*., **8**: 1-11.

Atalay S (2001). Antiviral and cytotoxic activities of extracts from the cell cultures and respective parts of some Turkish medicinal plants. *Turk. J. Biol*., **25**: 343-350.

El-Hag AE, El-Nadi AH and Zaitoon AA (1999). Toxic and growth retarding effects of three plant extracts on Culex pipiens larvae (Diptera: Culicidae). *Phytotherapy Research.*, **13**: 388-392.

Finney, D.J., Probit Analysis, Cambridge University Press, Cambridge (U.R) (1971)

Gilani SA, Kikuchi A, Shinwari ZK, Khattak ZI and Watanab KN (2006). Phytochemical, pharmacological and ethnobotanical studies of *Rhazya stricta* Decne. *Phytotherapy Research.*, **21**: 301-307.

Haq I (1983). Economic and therapeutic importance of medicinal plants. Report of Ministry of Health, Govt. of Pakistan. *Hamdard Foundation Press, Pakistan*, pp.1-13.

Hufford CD, Funderburk JM, Morgan JM, Robertson LW (1975).Two antimicrobial alkaloids from heartwood of *Liriodendron tulipifera*. *I.J.pharm. Sci*., **64**:789-792.

Janaki S and Vijayasekaram V (1998). Antifungal activities of Aglaia roxbur ghiana. *Biomedicine*, **18**: 86-89.

Khan SU, Khan GM, Mehsud SUK, Rahman A and Khan MF (2004). Antifungal activities of *Tamarix dioica* - An *in vitro* study. *Gomal J. Med. Sci*., **2**: 40-42.

Krishnaraju AV, Rao TVN, Sundararaju D,Vanisree M, Tsay H S and Subbaraju GV(2006). Biological screening of medicinal plants collected from Eastern Ghats of India using *Artemia salina* (brine shrimp test). Intern.J.Appl.Sci.Eng., **4**: 115-125

Mukhopadhyay S, Handy GA, Funayama S and Cordell GA(1981).Anticancer indole alkaloids of *Rhazya stricta*. *J. Nat. Prod*., **44**: 696-700.

Nazimuddin S and Qaiser M (1983). Apocynaceae. *In*: Flora of Pakistan, Nisir E and Ali SI (Eds.), **148**: 26-27.

Rahman AU and Qureshi MM (1990). Applications of modern 2D-NMR techniques in structure elucidation of natural products. *Pure & Applied Chem*., **62**: 1385-1388.

Tanira MOM and Ali BH (2000).Mechanism of the hypotensive action of *Rhazya stricta* leaf extract in rats. *Pharmaco Research*., **41**: 369-378.

Umadevi S, Mohanta G P, Chelladurai V, Manna PK, Manavalan R(2003).Antibacterial and antifungal activity of Andrographis echiodes.*J. Nat. Remedies*., **3**:185-188

Zaidi SH (2006). Existing indigenous medical plants of Pakistan, [Online] http://www.telmedpak.com/agriculture, accessed on June **22**, 2006.