Antileishmanial, insecticidal and phytotoxic potential of leaves and stems of *Rumex nervosus* Vahl.

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Abstract: Biological assays including; phytotoxicity, brine shrimp lethality, antileishmanial and insecticidal activities were carried out on crude methanolic extracts of stems and leaves of *R. nervosus* and their fraction namely; *n*-Hexane, $CHCl_3$, EtOAc, and MeOH fractions. The highest significant phytotoxicty activity showed by chloroform, *n*-hexane fractions and crude extract of leaves, the growth regulation were (95%, 90% and 90%) respectively against *Lemna minor*, while ethyl acetate fraction and *n*-hexane fractions of stems displayed significant phytotoxicty (100% and 90%) respectively against *Lemna minor* at high dose (1000µg/ml). The results obtained from cytotoxicity assays revealed that none of the fractions are cytotoxic. The chloroform fraction of stems was showed good antileishmanial activity against *L. major* with LC_{50}±S.D: 70.3±1.2 at µg/ml. The crude methanolic extracts of leaves, chloroform fraction and ethyl acetate fraction exhibited low mortality against tested insect *Rhyzopertha dominica* while, the rest of extracts were found almost inactive against insects species.

Keywords: *Rumex nervosus*, extract, antileishmanial, insecticidal, phytotoxic, cytotoxic.

INTRODUCTION

Leishmaniasis is diseases caused by more than a 20 species of protozoan parasite. It is still one of the world's most neglected diseases affecting largely the poorest of the poor, particularly in the developing countries (García *et al.*, 2011) Documented literature report that about fifty thousand death occurs each year due to leishmania as well as 12 million get infected. The infectious leishmaniasis can be categorized into three types: (a) mucocutaneous leishmaniasis, (b) visceral leishmaniasis, (c) cutaneous leishmaniasis. Reported study has revealed that about 0.5 and 1.5 million cases of visceral and cutaneous leishmaniasis respectively (Shah *et al*., 2014; Ejazi and Ali, 2013; Abdel-Sattar *et al*., 2010). Therefore, the Leishmaniasis is considered one of the life threatening disease, particularly which affects mostly to people of developing countries, living below the poverty line (Abdel-Sattar *et al*., 2010). Synthetic drugs available for treating have been associated with greater hazards which made the search for alternatives in an urgent need, while, emphasis the important of natural products as greatest sours of novel drugs with low side effects. Some of plants have potential agents cannot be ignoring it. Species of Genus Rumex have been used vastly in traditional medicine. Both roots and aerial parts of genus *Rumex* have been used for curing of serval diseases such as; constipation, depurative, purgative, inflammation, blood purification, rheumatism, gout, burns, topically applied in scabies, eczema (Kahraman and Yanardag, 2011; Tynybekov, 2013). *Rumex nervosus* is medicinal plant has been used traditionally for treating of several diseases such as; stomach-ache, wounds, ophthalmic, dysentery, diarrhea, pharyngitis (Al-Dubai and Al-Khulaidi, 1997). Recently studies reported that the potential *R. nervosus* is an effective source of urease inhibitory agents, analgesic earthworms and influenza A virus (Khan *et al*., 2014; Alwashli *et al*., 2012; Raju and Yesuf, 2010; Getie *et al*., 2003). The current study was performed on the crude methanolic extracts of both stems and leaves of *R. nervosus* as well as their sub-fractions namely; *n*-hexane fraction, $CHCL_3$ fraction, EtOAc fraction, and MeOH fraction, for antileishmanial, insecticidal, phytotoxicity and brine shrimp lethality.

MATERIAL AND METHODS

Plant materials

Rumex nervosus was collected in August and September (2013) from Taiz city, Yemen and identified in Department of Botany, University of Peshawar, Pakistan by Ghulam Jelani. A voucher specimen (UPESH-Bot: 20040O (pup)) was deposited in the Herbarium of the said department.

Preparation and fractionation of extracts

Stems (1150g) and leaves (3kg) of *R. nervosus* were grinded and extracted discretely with hot methanol by using soxhlet extractor. The rotary evaporator was using for removing methanol, the final extracts obtained were (350g) and (875g) for stems and leave respectively. Both crude extracts of stems and leaves were discretely fractionated with organic solvents namely: *n*- hexane, chloroform, ethyl acetate and methanol. Solvents were

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evaporated under reduced pressure by using rotary evaporator, accordingly their sub-fractions obtained from both stems and leaves were; *n*-hexane (29g), (75g), chloroform (21g), (112g), ethyl acetate (15g), (105g), and methanol (58g), (49g), respectively.

Biological activity

Antileishmanial activity

Screening of antileishmanial activity was carried out on the pre-established cultures of *L. major* (DESTO) according to standard protocol (Khan *et al*., 2011). (1mg) of each test extracts were dissolved in (1ml) of solvent (methanol, ethanol, DMSO and water) depending on solubility). (1mg) of Pentamidine was dissolved in (1mL) DMSO as reference drug. Parasite at log phase were centrifuged at (3000rpm) for (3 min) and were diluted in fresh culture medium to a final density of 2×106 cells/mL. In different wells, medium (180µL) was added. Extract/ fraction (20µL) was added to medium than serially diluted. Parasite (100µL) culture was added in all the wells. Rows (4) left for positive and negative controls: (water, MeOH, EtOH, and DMSO), respectively, serially diluted in medium whereas positive control contained varying concentrations of standard antileishmanial compound, Pentamidine. The dishs were incubated for (72 hour) at 24°C. Results of antileishmanial screening were analyzed through dose versus response using nonlinear regression curve fit with Graphad Prims 5.

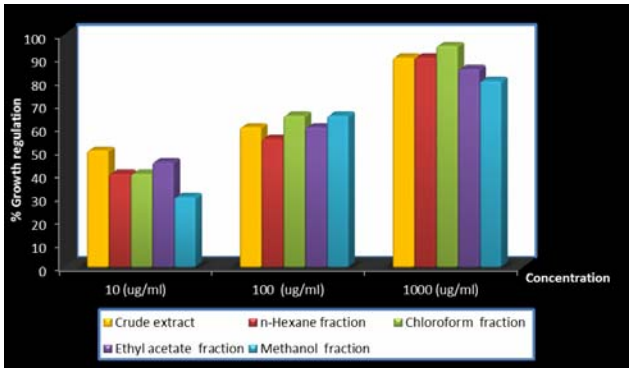

Fig. 1: Phytotoxicity of Methanolic Crude Extract and their Sub-fractions of Leaves of *R. nervosus*.

Insecticidal activity

Crude methanolic extracts of both leaves and stems of *R. nervosus* as well as their sub-fraction were evaluated against two insects including; Rhyzopertha dominica and Tribolium castaneum according to standard protocol (Nisar *et al*., 2010). The test sample was prepared by dissolving (200mg) of extracts in (3ml) acetone and loaded in a Petri plate wrapped with the filter papers. The plate was left (24h) to ensure complete evaporate the solvent. Then for each plate (10) test insects were added and incubated at (27°C) for (24h) with 50% relative humidity in growth chamber. The results were analyzed as percentage mortality and calculated with reference to the negative and positive controls. For standard drug the (Permethrin) was used, while Permethrin, acetone and test insects were used as positive and negative controls. The percentage mortality (%) was calculated by the formula:

Mortality (%)= (Number of insects alive in test / Number of insects alive in control) × 100

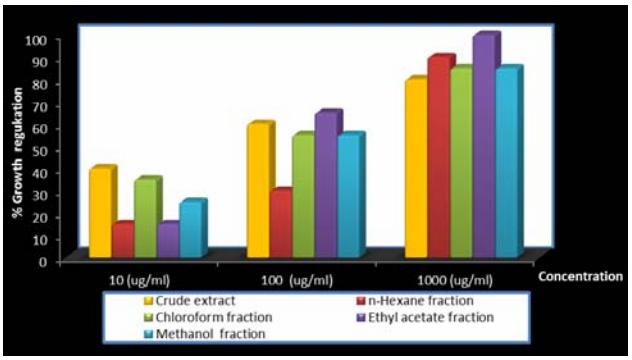

Fig. 2: Phytotoxicity of Methanolic Crude Extract and their Sub-fractions of Stems of *R. nervosus*.

Phytotoxicity activity

Phytotoxic activity was determined by using standard protocol of *Lemna minor* (Ali *et al*., 2009). The medium for this screening was prepared by employing diverse constituents in (100ml) of distal water with adjust the range of PH from 5.5 to 6.5 and the autoclaved for (15 min) at (121°C). The extracts/fractions were dissolved in ethanol to get stack solution. (3) flasks for each concentration were incubated with (10µL), (100µL) and (1000µL) of the stock solution for (5ppm), (50ppm) and (500ppm) and then left overnight to evaporate the solvent. (20ml) medium, (10) plants and (3) fronds of *Lamna minor* was added to each flask along with (1) flask which comprise (Paraquat) as standard. All flask was clogged by cotton and kept for (7) days. After (7) days the number of fronds in each flask was calculated and % activity was calculated by following formula:

$$\text{Growth regulation}\,(\%) = 100\,\frac{\text{- Number of sample test in fronds}}{\text{Number of fronds in negative control}} \times 100$$

Shrimp lethality assay

Cytotoxicity of crude extracts of both stems and leaves of *R.* nervosus as well as their fractions were carried out using brine shrimp cytotoxic assay on extracts (Nisar *et al*., 2010). Concisely, (3.8g) of sea salt in (1L) distilled was dissolved and then filtered to obtained sea water, brine shrimps eggs (1mg) were then added into sea water in a small tank. The tank was covered with aluminum foil. And placed in dark for (24 h) at (25°C) which supplied a large number of larvae. (20mg) of concentrated sample was dissolved in (2ml) of chloroform (20mg/2ml) and transferred to (500µl), (50) and (5µl) vials corresponding to (1000µg), (100µg) and (10µg) per (ml) respectively. For each concentration (3) replicates were prepared for making a total of (9) vials. The vials including material was concentrated, dissolved in (50µl) of DMSO and (5ml) of "sea water" was added to each. Follow that, (10) shrimps were added to each vial, then left for (24h), then

Table 1: Antileishmanial activity of leaves and stems of *R. nervosus* extracts and its fractions

Extractions	IC_{50}(µg/ml) ± S.D.	
	Leaves	Stem
Crude extracts	>100	91.7 ± 0.5
n-hexane	>100	>100
Chloroform	>100	70.3 ± 1.2
Ethyl acetate	>100	92.35 ± 0.3
Methanol	>100	92.3 ± 0.35
Standard (Pentamidine)	5.09 ±0.09	5.09 ±0.09

IC_{50}: values indicated the effective concentration of a extract in µg/mL necessary to achieve 50% inhibition. S.D: Standard deviation.

Table 2: Insecticidal activity of leaves and stems of *R. nervosus* extracts and its fractions

Insect species	% Mortality										
			Extractions								
			leaves					Stems			
	+ve control	-ve control	Crude extract	*n*-hexane	Chloroform	Ethyl acetate	Methanol	Crude extract	*n*-hexane	Ethyl acetate	Methanol
Tribolium castaneum	100	0	0	0	0	0	0	0	0	0	0
Rhyzopertha dominica	100	0	20	0	20	20	0	0	0	0	0

Table 3: Phytotoxic activity of crude methanolic extracts of leaves and stems and their sub-fractions of *R. nervosus*

Extracts	concentration (µg/ml)	% Growth Regulation	
		Leaves	Stems
Crude extract	10	50	40
	100	60	60
	1000	90	80
n-Hexane fraction	10	40	15
	100	55	30
	1000	90	90
Chloroform Fraction	10	40	35
	100	65	55
	1000	95	85
Ethyl acetate fraction	10	45	15
	100	60	65
	1000	85	100
Methanol fraction	10	30	25
	100	65	55
	1000	80	85

Standard drug = Paraqua (0.015)

recorded and counted the number of remain alive of shrimps. Etoposide was used as positive control. The Finney computer program was used to analyze and determined the LD_{50} values

RESULTS

The crude methanol extracts of both stems and leaves of *R. nervosus* and their fractions including; *n*-hexane, $CHCL_3$ fraction, EtOAc fraction and MeOH fraction were tested for their potential biological activities namely; antileishmanial, insecticidal, phytotoxic and brine shrimp lethality. Results obtained are presented in tables (1, 2, 3 and 4). The crude extracts of both leaves and stems of *R. nervosus* and their fractions showed variable effect degree against biological studies examined.

DISCUSSION

Antileishmanial activity

Nowadays, the parasite leishmaniasis is considered one of the important problems in the World and there has to be an urgent need to discover new drugs for potential use in

Table 4: Cytotoxicity activity of methanolic extracts of leaves and stems and their sub fractions *R. nervosus* from

Extractions	Dose	Leaves			Stems		
		No. of shrimps Survived	No. of Death	LD_{50}	No. of shrimps Survived	No. of Death	LD_{50}
Crude extracts	1000	26	04	-	26	04	-
	100	28	02	-	30	0.0	-
	10	30	0.0	-	30	0.0	-
n-hexane	1000	29	01	-	27	03	-
	100	30	0.0	-	28	02	-
	10	30	0.0	-	30	0.0	-
Chloroform	1000	29	01	-	26	04	-
	100	30	0.0	-	27	03	-
	10	30	0.0	-	30	0.0	-
Ethyl acetate	1000	27	03	-	26	04	-
	100	29	01	-	28	02	-
	10	30	0.0	-	30	0.0	-
Methanol	1000	26	04	-	27	03	-
	100	28	02	-	29	01	-
	10	30		-	30	0.0	-

Standard drug = Etoposide LD_{50} (7.4625 ppm). No of Shrimps used: 30 (for each extract).

therapy of leishmaniasis (Shah *et al*., 2014). Here in our investigation the crude extracts of both stems and leaves of *R. nervosus* and their fractions subjected as leishmancidal agent. Antileishmanial profile revealed that the stems of the *R. nervosus* have more antileishmanial potential then the leaves table 1. The crude methanolic extracts of stems as well as their fractions were found to be good and low activity. Chloroform fraction showed good activity with IC_{50} (µg/ml) ± S.D: 70.3±1.2; while the crude extracts, methanol fraction and ethyl acetate fraction showed low activity with IC_{50} (µg/ml) ± S. D:(91.7±0.5, 92.3±0.35 and 92.35±0.3) respectively. The *n*-hexane fraction showed inactive with (IC50 (µg/ml) ± S.D; >100). The crude methanolic extracts of leaves and their fractions exhibited inactive with (IC_{50} (µg/ml) ± S.D; >100). Activities observed exactly in the chloroform fraction of stems maybe due to the phenolic and flavonoids compounds that reported as antileishmanial agents (Shah *et al*., 2014), which requires further analysis that maybe lead to explore new medicine against leishmaniasis diseases.

Insecticidal activity

The crude methanolic extracts of stems and leaves as well as various fractions namely *n*-Hexane, Chloroform, Ethyl acetate and Methanol of *R. nervosus* were evaluated against two insects species as; Rhyzopertha dominica and Tribolium castaneum. The results obtained are shown in table 2. Only crude methanolic extracts of leaves as well as Chloroform fraction and Ethyl acetate fraction exhibited very low mortality against tested insect *Rhyzopertha dominica*, while *n*-hexane fraction and methanol fraction showed inactive. Meanwhile crude methanolic extracts of stems and their fractions exhibited inactive against insect species selected.

Phytotoxicity activity

Phytotoxicity assays involve the use of plants to determine toxicity of chemicals. It helps in selecting plant species that are able to withstand high levels of contaminants and screening out those that are not able to establish themselves in such conditions as present in contaminated sites (Njoku *et al*., 2011). Synthetic weedicides (herbicides) are often toxic, non-specific and expensive, natural products on of the sources of weedicides and having improved characteristics could therefore have a promising future (Atta-ur-Rahman *et al*., 2005). In current study; the crude extracts of both stems and leaves additional to their fractions of *R. nervosus* were subjected to phytotoxic assays. Various concentration including (1000µg/ml, 100µg/ml and 10µg/ml) were tested for their toxicity against *lemna minor* and (0,015µg/ml) of Parquet was used as standard. Facilitate interpretation impressible against *lemna minor* by crude methanolic extracts and their fractions of both leaves and stems of *R. nervosus were* recorded in table 3. At heights dose (1000µg/ml), The $CHCL_3$ and *n*-hexane fractions as well as the crude extract of leaves showed significant phytotoxic against *Lemna minor* respectively, while, EtOAc and MeOH fractions exhibited very good phytotoxic against *Lemna minor*. Also at dose (100.10) µg/ml the crude extract of leaves and their sub-fraction exhibited moderate and good phytotoxic agent. Meanwhile the EtOAc fraction and *n*-hexane fraction of stems showed significant phytotoxic at heights dose (1000ug/ml) against *Lemna minor* respectively; while, MeOH fraction, $CHCl_3$ fraction, and crude extract of stems exhibited very good phytotoxic against *Lemna minor* respectively (figs.1, 2). Activities observed may be due to the flavonoids compounds which reported as the Phytotoxic agent (Castro *et al*., 2010).

Cytotoxic activity

Cytotoxicity was evaluated using brine shrimp lethality assay. The crude extracts of both-stems and leaves as well as their fractions namely; *n*-Hexane, $CHCL_3$, EtOAc and MeOH fractions were found to be relatively non-toxic as compared to standard toxic drug (table 4).

CONCLUSION

It is concluded from current work that methanolic extracts and sub-fractions of stems and leaves of the *R. nervosus* plant have significant phytotoxic potential. Crude methanolic extract of stems and their sub-fractions were found to be more active against Leishmaniasis then leaves and their sub-fractions. Both stems and leaves of *R. nervosus* were found to be relatively non-toxic. The biological activity of the *R. nervosus* maybe due to the presence of bioactive compounds in various solvent fractions. This finding directed the natural product chemist to isolated these bioactive compounds from various fractions of *R. nervosus*.

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