



EVALUATION OF *NEPETA LEAVIGATA*, *NEPETA KURRAMENSIS* AND *RHYNCHOSIA RENIFORMIS* ON ANTIMALARIAL AND ANTILEISHMANIAL ACTIVITIES

Memoona A¹, Amir MK¹, Sultan A², Razia P³, Sumera P¹, Lal M⁴, Nisar Ahmed^{1*}

¹Department of Botany, Kohat University of Science and Technology, Pakistan

²Department of Zoology, Kohat University of Science and Technology, Pakistan

³Department of Biotechnology, Kohat University of Science and Technology, Pakistan

⁴Kohat Institute of Medical Sciences, Kohat, Pakistan

*Corresponding Author: Dr. Nisar Ahmed, Chairman, Department of Botany, Kohat University of Science and Technology, Pakistan

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Abstract: The pharmacological investigation of methanolic extracts and different fractions of the *Nepeta leavigata*, *Nepeta kurramensis* and *Rhynchosia reniformis* were designed to assess the antimalarial and antileishmanial screening of medicinal plants for development of drugs. Materials and Methods: The shade-dried whole plant material of *Nepeta leavigata* was soaked in methanol for 10 days. The powdered drug was extracted with 80 % methanol three times and filtered at room temperature. The filtrate was evaporated in rotary to get a dark-greenish residue (extract), which was further suspended in water and partitioned successively with *n*-hexane, chloroform, ethyl acetate and *n*-butanol to obtain *n*-hexane-soluble, chloroform-soluble, ethyl acetate-soluble, *n*-butanol-soluble and aqueous fractions, respectively (Khan, et al., 2010a). Same extraction and fractionation procedure was adopted for *Nepeta kurramensis* and *Rhynchosia reniformis*. The antimalarial / antileishmanial activity of crude extracts/fractions was determined through standard protocol (Kerharo & Adam, 1974/). Results: The crude extract, chloroform fraction of *Nepeta leavigata* and fractions chloroform and ethyl acetate of *Nepeta kurramensis* showed promising antileishmanial and antimalarial activity against parasites of leishmania and malaria (promistogetes and schizonts) by observing 1 or 0 number of parasites on slide field where ≤ 1 level is significant, while all fractions of *Rhynchosia reniformis* did not showed any action against promistigotes and schizonts. Conclusions: The bioassays guided isolation and phytochemical screening of active fractions against these activities will further enhance research in the field of pharmacology.

Keywords: Antileishmanial, Antimalarial, *Nepeta leavigata*, *Nepeta kurramensis*, *Rhynchosia reniformis*

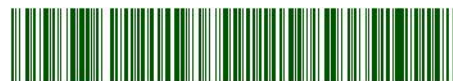
INTRODUCTION

Malaria and leishmaniasis are two of the most common parasitic diseases and infect a large human population over five continents. Malaria is caused by intraerythrocytic protozoan parasites of the genus *Plasmodium*. It is responsible for more than 300 million clinical cases and over 2 million deaths annually (Reisen and Boreham., 1982). *Plasmodium falciparum*, the organism that causes the most lethal form of the disease, is becoming increasingly resistant to almost all available drugs in the antimalarial armamentarium. Malaria is disease caused by an infectious parasite known as, *Plasmodium*, which cause infection in red blood cells and is one of the most well known parasites ever known to world. It affects at least 91 different countries and some 300 million people and it is one of the most common tropical diseases in the world (Khan, et al., 2005).

Plasmodium falciparum, *P. malariae*, *P. vivax* and *P. ovale* are the species which causes Malaria in Humans. The primary mode of transmission of malarial parasite

by the bite of an infected female anopheles mosquito, but infected blood products and congenital transmission can also cause infection. Malaria parasites are transmitted by the female anopheles mosquito. There are about 380 species of this type of mosquito, but only about 60 species can transmit disease. Leishmaniasis is a disease caused by any of the flagellate protozoan of the genus *Leishmania*, transmitted to humans and animals by parasitical sand flies. It is an infection, such as kala-azar or moreover of two clinically diverse ulcerative skin diseases (Menedez, et al., 2000).

Nepeta genus has about 250 species of flowering plants belonging to family Lamiaceae. The genus is native to Europe, Asia, and Africa and now also common in North America. Various chemical compounds and ingredients isolated from *Nepeta* genus control calcineurin *in vitro* (Prescott et al., 2011). *Nepeta* acts as an insect repellent source in many countries (Zhu et al., 2009; Birkett et al., 2011).



Rhynchosia reniformis belongs to family Fabaceae. Its flowering season is from May to September. This small species can be found mainly in the southern half of Alabama in counties which contain sandy soil, sandhills, and dry wood.

MATERIALS AND METHODS

Plant Material for Biological Activities:

Fresh plants or parts of *Nepeta leavigata* and *Nepeta kurramensis* were collected randomly from Swat and Kurram Agency Pakistan respectively, while *Rhynchosia reniformis* was collected from district Karak. The taxonomic identity of these plants was determined by qualified plant taxonomist Mr. Nisar Ahmad, Chairman, Botany Department, Kohat University of Science & Technology (KUST), Kohat, Khyber Pakhtunkhwa, Pakistan. Fresh plant materials were washed under running tap water; air dried and then was homogenized to fine powder and stored in airtight bottles. The Biological activities of these plants were carried out in the laboratory of Zoology Department, KUST, Kohat.

Extraction and Fractionation:

The shade-dried whole plant material of *Nepeta leavigata* was soaked in methanol for 10 days. The powdered drug was extracted with 80 % methanol three times and filtered at room temperature. The filtrate was evaporated in rotary to get a dark-greenish residue (extract), which was further suspended in water and partitioned successively with *n*-hexane, chloroform, ethyl acetate and *n*-butanol to obtain *n*-hexane-soluble, chloroform-soluble, ethyl acetate-soluble, *n*-butanol-soluble and aqueous fractions, respectively (Khan, et al., 2010a). Same extraction and fractionation procedure was adopted for *Nepeta kurramensis* and *Rhynchosia reniformis*.

Preparation of stock solution from different fraction of plant materials:

The stock solution was prepared from each 6 fractions i.e. methanol, *n*-hexane, chloroform, ethyl acetate, *n*-butanol and aqueous fraction. Each fraction was dissolved in the distilled water at the dose rate of 0.025µg/ml and 0.050µg/ml respectively to conduct the antileishmanial and antimalarial activities.

Biological Activities:

Application of valuable and negative consequences of a medicine in living organisms can be brought about by phytochemical evaluation/screening of plants. The antileishmanial and antimalarial bioassays were carried out, which helped in the selection of appropriate plants fraction for bioassay guided isolation of the dynamic compound.

Antileishmanial Activity:

Antileishmanial activity was conducted by following procedure.

Sample collection:

The collection of infection (Leishmaniasis) sample from the lesion of a Leishmaniasis patient with suspected Cutaneous Leishmaniasis was brought about from *leishmania centre Kuwait hospital Peshawar, Khyber Pakhtunkhwa province*. Some Samples were collected from patients infected of cutaneous leishmaniasis from district Karak. The skin of the CL patient where lesion of leishmaniasis appeared was scraped with the help of blade (scalpel) at one point. The scraping was continued till oozes out of the blood from the injury and the cut/scratch were often given at the swollen and irritating area of wound. Some of the scraped infection was mixed with 1% Formalin and stored at 4°C for staining and the remaining scraped infection was mixed with buffer at pH 7.2 and stored in sterile eppendorf for more investigation. The study was carried out from April, 2011 to March, 2012 in the departmental laboratory of Zoology, KUST, Pakistan.

Media preparation and culturing of Leishmanial sample:

For the growth and culture of Leishmanial parasite 0.3g / 30ml of medium RPMI-1640 was dissolved in distilled water (10.43g / 1000ml of distilled water). This culture medium was dropped in the 10 vials of bijou bottle, each bottle having 3ml of the dissolved media with supplemented 10% fetal bovine serum (FBS). The antibiotic consisting of penicillin G and kanamycine were mixed in the culture medium to avoid bacterial contamination.

The bijou bottles were placed in ice jar and were taken to hospital where sample were collected from the recognized Leishmaniasis suspected person. Infection scraping obtained from CL patient were directly mixed with culture medium already present in each bottle in ice jar and then were brought back direct to the laboratory of Zoology Department of Kohat University of Science and Technology Kohat. The bottles containing CL infection scraping mixed with culture medium were kept in the incubator (memmert type Inb 500, Germany) for incubation at 26°C to avoid contamination. After 24 hours of incubation the very small amount of culture was dropped on the slide and then stained with Giemsa stain to check the promastigotes of *Leishmania* parasite and watched at 10x, 40x and 100x intensification (magnification) of microscope.

Application of test sample on leishmania culture:

For the determination of antileishmanial activity, 100µl culture sample of leishmaniasis patient was taken in 8 wells of micro titer plate and added equal quantity

of culture media RPMI-1640. 200 unit penicillin and 10 μ l kanamycine was also added in that media to protect bacterial contamination. After this micro titer plate was shaken vigorously to get homogenous and standardized suspension in each well. 20 μ l of drug concentration both level of dose i.e. 0.025 and 0.050 μ g/ml was poured into the concerned well like A1= well 1, A2 = well 2, B1= well 3, B2 = well 4, C1 = well 5, C2 = well 6 and sodium gluconate (Sodium gluconate) kept as positive control of drug concentration of known chemo factor in well 7. Similarly negative control in well 8 was maintained till the end of experiment. The micro titer plate was kept in the incubator at 37.0°C for 5 days.

Microscopic study and results:

During the duration of incubation, daily slides were prepared to check the parasite growth in wells. Glass slide were prepared from each well of micro titer plate and all slides were dully marked for identification. Slides were air dried and fixed with methanol. After drying, each slide was stained with Giemsa solution, and examined under microscope at 10x, 40x and 100x specification and the growth of amastigotes and promistogetes observed in the slide field was recorded.

Antimalarial Activity:

Antimalarial activity was tested in the laboratory by following steps:

Collection of malarial samples:

Blood samples were collected from clinical suspected patients in KDA Hospital Kohat, confirmed through microscopy and were used for further process.

Culturing of malaria sample:

For the growth of malarial parasites, the malaria blood samples were cultured in microtiter plate well using RPMI-1640 media. 100 μ l blood was taken from malarial sample blood in 8 wells of micro titer plate and added equal quantity of culture media RPMI-1640. 200unit penicillin and 10 μ l kanamycine was also added in micro titer plate well to protect bacterial contamination. Giemsa-stained blood films on glass slide were prepared from blood sample of each microtiter plate well. Their number of parasites was observed microscopically which showed the early growth of trophozoits and schizont.

Application of test samples on malarial culture:

The Antimalarial activity of the medicinal plants extracts was determined through standard protocol (Kerharo & Adam, 1974). 100 μ l blood was taken from malarial sample blood in 8 wells of micro titer plate and added equal quantity of culture media RPMI-1640. 200unit penicillin and 10 μ l kanamycine was also added in microtitre plate well to protect bacterial

contamination. After this, micro titer plate was shaken vigorously to get homogenous and standardized suspension in each well. 20 μ l of drug concentration of both dose level i.e. 0.025 and 0.050 μ g/ml was poured into the concerned well like A1= well 1, A2 = well 2, B1= well 3, B2 = well 4, C1 = well 5, C2 = well 6 and chloroquine kept as positive control of drug concentration of known chemo factor in well 7. Similarly negative control in well 8 was maintained till the end of experiment. The micro titer plate was kept in the incubator at 37.0°C for 5 days.

Microscopic study and results:

During the duration of incubation, daily slides were prepared to check the parasite growth in wells. Glass slide were prepared from each well of micro titer plate and all slides were dully marked for identification. Slides were air dried and fixed with methanol. After drying, each slide was stained with Giemsa solution, and examined under microscope at 10x, 40x and 100x specification and the growth of Trophozoits and Schizonts observed in the slide field was recorded.

RESULTS AND DISCUSSION

Antileishmanial Activity:

The Plants fractions, at two dose level concentrations, 0.025 μ g/ml and 0.050 μ g/ml were tested against *Leishmania tropica* amastigotes and promastigote cultures. Result revealed that out of investigated 6 fractions, methanol fraction and chloroform fraction of *Nepeta leavigata* and fractions chloroform and ethyle acetate of *Nepeta kurramensis* are active against Leishmaniasis as the number of promistigotes isolated were ≤ 1 i.e. equal or less than 1 (Table. 1 & 2), while no one fraction of *Rhynchosia reniformis* is active against *Leishmania* as the number of isolated parasites (promistigotes) was more than significant level (Table.3).

The sodium gluconate (Sodium gluconate) was kept as positive control of drug concentration of known chemofactor which inhibits the growth of amastigotes and promistigotes. Similarly negative control i.e. media with distilled water was maintained till the end of experiment which showed the complete growth of leishmanial parasites. A number of publications revealed that a large number of plants extracts are enriched of ingredients against leishmanial activity (Singh *et al.*, 2011; Ghosh *et al.*, 2011). It is important to investigate the plants ingredients against Leishmaniasis because the existing and available medicines which are in use of people are so costly and has more or less side effects for patients. In the current research work, the 2 plants showed significant efficacy against *L. tropica* promastigotes.

Table.1: Antileishmanial activity of *Nepeta leavigata*

S.No	Test Sample	Dose Level	Fraction Concentration (µg/ml)	Number of Parasite isolated in slide field of each fraction (Promistogotes)					
				Methanol	n-Hexane	Chloroform	E.acetate	n-Butanol	Aqueous
1	A	A ₁	0.025	01	02	01	03	02	04
		A ₂	0.050	01	03	01	02	02	03
2	B	B ₁	0.025	01	04	01	04	02	03
		B ₂	0.050	0	03	0	04	02	03
3	C	C ₁	0.025	0	02	01	02	03	02
		C ₂	0.050	01	02	01	03	02	04
4	D	D	Sodium gluconate	Growth inhibited					
5	E	E	Media with distilled water	100 % growth					

Note: ≤ 1 level is significant

Table.2: Antileishmanial activity of *Nepeta kurramensis*

S.No	Test Sample	Dose Level	Fraction Concentration (µg/ml)	Number of Parasite isolated in slide field of each fraction (Promistogotes)					
				Methanol	n-Hexane	Chloroform	E.Acetate	n-Butanol	Aqueous
1	A	A ₁	0.025	02	06	01	01	04	06
		A ₂	0.050	02	03	01	0	03	04
2	B	B ₁	0.025	02	05	01	01	04	03
		B ₂	0.050	04	04	0	01	02	05
3	C	C ₁	0.025	02	04	01	01	03	04
		C ₂	0.050	03	04	01	0	02	03
4	D	D	Sodium gluconate	Growth inhibited					
5	E	E	Media with distilled water	100 % growth					

Note: ≤ 1 level is significant

Table.3: Antileishmanial activity of *Rhynchosia reniformis*

S.No	Test Sample	Dose Level	Fraction Concentration (µg/ml)	Number of Parasite isolated in slide field of each fraction (Promistogotes)					
				Methanol	n-Hexane	Chloroform	E.Acetate	n-Butanol	Aqueous
1	A	A ₁	0.025	04	05	04	04	03	04
		A ₂	0.050	04	03	06	06	05	06
		B ₁	0.025	04	04	06	03	04	04
2	B	B ₂	0.050	03	04	04	04	04	03
		C ₁	0.025	04	04	05	04	03	05
3	C	C ₂	0.050	04	02	06	06	03	04
4	D	D	Sodium gluconate	Growth inhibited					
5	E	E	Media with distilled water	100 % growth					

Note: ≤ 1 level is significant

Molecular characterization of *Leishmania* species which cause cutaneous leishmaniasis in Kohat Khyber Pakhtunkhwa was reported by Sharma, et al., 2005. Sporadic of CL are reported in other parts of Pakistan, Afghanistan, India and Iran where both *L. tropica* and *L. donovani* were found that cause leishmaniasis in particular areas (Sharma et al., 2005). CL is prevalent in Pakistan and has been reported from all the provinces (Kassi et al., 2008), where the causative agent is *Leishmania tropica*, a protozoan parasite (Amtul and Shaheen, 2001).

For the vitro culture and direct microscopically diagnosis high number of amastigotes of CL is required which is very low in skin lesions (kassi et al., 2008; Kochar et al., 2000).

Antimalarial Activity:

The investigational study was conducted to evaluate the active ingredients of *Nepeta leavigata*, *Nepeta kurramensis* and *Rhynchosia reniformis* extracts fractions against Malaria disease. Results of this research work on antimalarial activity are shown in tables (4, 5 & 6). Out of all fractions, the methanol and chloroform fraction of *Nepeta leavigata* and chloroform and ethyl acetate fraction of *Nepeta kurramensis* are most remarkable fractions obtained against malarial activity (significant level is equal or less than 1).

Table.4: Antimalarial activity of *Nepeta leavigata*

S.No	Drug	Drug dose level	Drug Concentration (µg/ml)	Number of Parasite isolated in slide field of each fraction (Schizont)					
				Methanol	n-Hexane	Chloroform	E. Acetate	n-Butanol	Aqueous
1	A	A ₁	0.025	01	02	01	02	03	05
		A ₂	0.050	01	02	0	02	02	03
2	B	B ₁	0.025	0	04	01	04	02	03
		B ₂	0.050	0	02	0	03	03	02
3	C	C ₁	0.025	0	04	01	03	03	04
		C ₂	0.050	01	03	0	02	02	04
4	D	D	Chloroquine	Growth inhibited					
5	E	E	Media with distilled water	100 % growth					

Note: ≤ 1 level is significant

Table.5: Antimalarial activity of *Rhynchosia reniformis*

S.No	Drug	Drug dose level	Drug Concentration (µg/ml)	Number of Parasite isolated in slide field of each fraction (Schizont)					
				Methanol	n-Hexane	Chloroform	E.Acetate	n-Butanol	Aqueous
1	A	A ₁	0.025	03	03	0	01	03	04
		A ₂	0.050	03	02	01	0	03	02
2	B	B ₁	0.025	03	03	0	0	04	05
		B ₂	0.050	02	04	0	01	02	05
3	C	C ₁	0.025	02	04	01	01	03	04
		C ₂	0.050	04	02	0	0	04	04
4	D	D	Chloroquine	Growth inhibited					
5	E	E	Media with distilled water	100 % growth					

Note: ≤ 1 level is significant

Table.6: Antimalarial activity of *Nepeta kurramensis*

S.No	Drug	Drug dose level	Drug Concentration (µg/ml)	Number of Parasite isolated in slide field of each fraction (Schizont)					
				Methanol	n-Hexane	Chloroform	E.Acetate	n-Butanol	Aqueous
1	A	A ₁	0.025	04	04	04	03	04	06
		A ₂	0.050	03	03	04	03	03	05
2	B	B ₁	0.025	05	05	04	04	05	05
		B ₂	0.050	03	05	04	02	02	05
3	C	C ₁	0.025	03	04	05	05	04	03
		C ₂	0.050	04	03	04	03	04	04
4	D	D	Chloroquine	Growth inhibited					
5	E	E	Media with distilled water	100 % growth					

Note: ≤ 1 level is significant

The microscopic study of the slides of these significant fractions stained with Giemsa-stain showed less development or absence of mature schizonts in the field area of slides at both dose level concentrations i.e. 0.025µg/ml and 0.050µg/ml (the result is shown in Table. 4 & 5). While out of all fractions of *Rhynchosia reniformis*, no fraction showed any activity against malaria (Table. 6). Chloroquine was kept as positive control of drug concentration of known chemofactor which did not show the development of schizonts. Similarly negative control (Media with distilled water) was maintained till the end of experiment which did not affect the growth of schizonts. A survey reported that some species of *Nepeta* are used as mosquito repellent. It is used to cure fever as it promotes the sweating (Virendra, *et al.*, 2011).

Nepeta is also used as expectorant and helpful in the treatment of jaundice. It is highly effective against insect stings and bite of snake (Hedgewitchery, 2010). Thus the result reveals that 2 plants of *Nepeta* genus selected for antimalarial and antileishmanial activities are active. Because of this these properties, *Nepeta leavigata* and *Nepeta kurramensis* needs to be further investigated through isolation to find out pure and active compounds and molecules against Leishmania and Malaria.

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