

Full Length Research Paper

Biological activities of aerial parts of *Tylophora hirsuta* Wall

Ahmad Bashir^{1*}, Niaz Ali², Shumaila Bashir² and Muhammad Iqbal Choudhary³

¹Center of Biotechnology and Microbiology, University of Peshawar. N.W.F.P Pakistan.

²Department of Pharmacy, University of Peshawar. N.W.F.P Pakistan.

³International Center for Chemical and Biological Sciences, University of Karachi, Karachi, Pakistan.

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The methanolic extract from the aerial parts of *Tylophora hirsuta* was screened for various *in vitro* biological activities including antileishmanial, insecticidal, phytotoxicity, antibacterial and antifungal. General toxicity (brine shrimp lethality assay) of the methanolic extract was also performed. The extract was found to have significant antileishmanial activity against *Leishmania major*; reasonable insecticidal activity against *L. minor* L., low and non-significant antibacterial activity against *Shigella flexneri* and *Bacillus subtilis*, respectively, and moderate antifungal activity against *Fusarium solani*. No significant general toxicity was observed with the extract at the tested concentrations.

Key words: *Tylophora hirsuta* Wall, biological activities, phytotoxicity, antileishmanial, insecticidal, IC₅₀ values, α-amyrin acetate.

INTRODUCTION

Northern regions of Pakistan are rich with medicinal plants that are yet to be explored on scientific grounds. In folk medicine, the plants are used as decoctions, teas and juice preparations to treat different infections. They may be also used as poultice and applied directly on the infected wounds or burns for relief. World Health Organization is giving more emphasis on promoting traditional medicine particularly in the Third World Countries. More than 8,000 plants species in South Asia have medicinal values; out of which 2000 medicinal species exist in Pakistan (Olayiwola, 1984). Stating further, more than 80% of the population of South Asia cannot afford the primary health care cost and they only rely on traditional uses of herbs even in the therapeutic antenatal and postnatal cases. Global market is estimated over US\$ 60.0 billion and is expected to grow to US\$ 5.0 trillion by the year 2050 (Madhav, 2002).

In Pakistan, during 1999, the bill on the import of medicinal plants was worth of US\$ 31.0 million, whereas the export of medicinal plants could hardly reached US\$ 6.0 million (Khan et al., 1979). Interestingly, plants contain natural compounds that can work synergistically for

therapeutic purposes with fewer incidences of side effects (Kaufman et al., 1999). Only about 6% plants have been screened for biological activities, and a reported 15% have been evaluated phytochemically (Schultes, 1972). In the world, 30% of the pharmaceutical preparations are manufactured from plants. We selected *Tylophora hirsuta* for further work.

Traditionally tylophora is used in the treatment of asthma, high blood pressure, diarrhea, rheumatism and other allergic conditions. "Tylophora" is present in the list of herbal medicine in the *Bengal Pharmacopoeia* since 1884 and other species of the genus include *Tylophora indica*, *Tylophora asthmatica* and *Tylophora vomitoria*. Traditionally they have laxative, expectorant, diaphoretic, and purgative properties. The plants have been used for the treatment of various disorders besides asthma (Srivipuri, 1972; Thiruvengadam et al., 1978; Udupa et al., 1991). Other reported activities are anti-allergic and anti-arthritic (Gopalakrishnan et al., 1980; Wagner, 1989). Cyto-toxicity of *T. asthmatica* has been established in male rats (Dikshith et al., 1990). Similarly anticancer activity of derivatives of Tylophora genus has been established (Wenli and Wing, 2004).

Previously, compounds like tylophora alkaloids (Bhutani et al., 1984; Bhutani et al., 1985; Ali and Bhutani, 1987) have been reported from tylophora spe-

*Corresponding author. E-mail: bashirdr2001@yahoo.com.

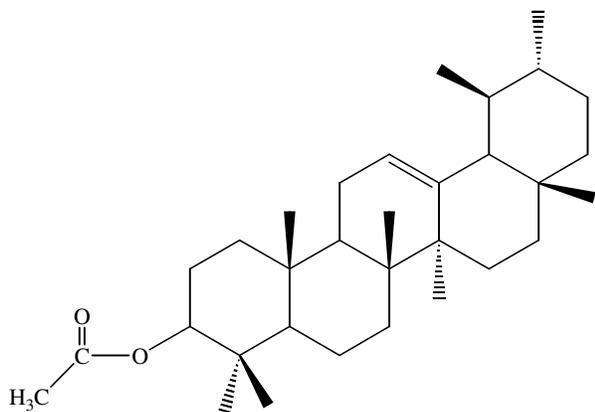


Figure 1. α -Amyrin acetate. (3 β -Acetoxyurs-12-ene). MS data of m/z (M^+ 468, 3.83%), 257 (3%), 218 (100%), 203 (22.53%).

cies with antiamoebic activities (Bhutani et al., 1987). Other constituents reported from the aerial parts of the *T. hirsuta* are Gymnorhizol and β -sitosterol (Ali and Bhutani, 1991). *Tylophora* is mentioned as herbal medicine in Chinese Materia Medica (Shiu-Ying Hu, 1980). Keeping in view the traditional uses and previous work done on the plant, we carried out our current work that is based on biological screenings of methanolic extract of aerial parts of *T. hirsuta* Wall for any further beneficial effect(s) of medicinal and/or agriculture importance.

MATERIALS AND METHODS

Plant material

The dried aerial parts of *T. hirsuta* Wall. (Asclepiadaceae) were collected in April – May 2005 from the nearby hills of the campus-I of University of Malakand N.W.F.P Pakistan. The plant was identified by Professor Dr. Jehandar Shah; a voucher has been deposited in the herbarium of University of Malakand.

Extraction

The shade dried aerial plant material was crushed into small pieces and finally pulverized into fine powder. The powdered plant material (7.5 kg) was soaked in methanol with occasional shaking, at room temperature. After 15 days, the methanol (commercial grade) soluble materials were filtered off. The filtrates were concentrated under vacuum at low temperature (below 40°C) using rotary evaporator. A blackish crude extract (1100 g) was obtained.

Fractionation and isolation

The crude methanolic extract (1000 g) was suspended in distilled water (500 mL) and sequentially partitioned with *n*-hexane (3 x 450 mL), chloroform (3 x 450 mL), ethyl acetate (3 x 450 mL) and *n*-butanol (3 x 450 mL) to yield *n*-hexane (500 g), chloroform (45 g), ethyl acetate (20 g), *n*-butanol (5 g) and aqueous (250 g) fractions, respectively. About 100 g crude methanolic was reserved for other pharmacological screenings.

Table 1. Antileishmanial activities of crude methanolic extract and fractions of *Tylophora hirsuta* against the susceptible strains of *Leishmania major* promastigotes (Pakistani isolates).

Drugs	IC ₅₀ values ($\mu\text{g/ml} \pm \text{S.D}$)	Mean relative index ($\text{IC}_{50}^{\text{Sample}} / \text{IC}_{50}^{\text{Standard}}$)
Pentamidine	2.5 \pm 0.09 (n=3)	1
Crude extract	34.44 \pm 0.06 (n=3)	13.78
<i>n</i> -hexane	38.93 \pm 0.65 (n=3)	15.57
Chloroform	92.26 \pm 0.13 (n=3)	36.90
Ethyl acetate	81.52 \pm 0.98 (n=3)	32.60
<i>n</i> -butanol	54.63 \pm 0.17 (n=3)	21.85
Water	69.17 \pm 0.57 (n=3)	27.67

Mean IC₅₀ values in $\mu\text{g/ml} \pm \text{S.D}$ obtained from three separate experiments are shown. Pentamidine was used as standard drug.

Isolation of pure compounds is under process. So far, apart from the previously reported compounds, upon gradient elution of the chloroform and *n*-hexane fractions with ethyl acetate : *n*-hexane 3:7, we have isolated α -amyrin acetate (Figure 1) for the first time from *T. hirsuta*. The α -amyrin acetate is a very potent and therapeutically active compound (Otuki et al., 2004) and our work continues on it.

Antileishmanial activity

Leishmania major Promastigotes were grown in bulk, early in modified NNN biphasic medium, using normal physiological saline. Then the promastigotes were cultured with RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS). The parasites (*Leishmania major*), at log phase, were centrifuged at 2000 rpm for 10 min and washed three times with saline at same speed and time. Finally the parasites were diluted with fresh culture medium to give a final density of 10^6 cells/ml.

In a 96-well micro titer plate, 180 μl of medium was added in different wells. 20 μl of the experimental compound was added in medium and serially diluted. 100 μl of parasite culture (final density of 10^6 cells/ml) was added in all wells. Two rows were left, one for negative and other for positive control. Negative controls received the medium while the positive control received Pentamidine as standard antileishmanial compound. The plate was incubated between 21-22°C for 72 h. The culture was examined microscopically for cell viability by counting the number of motile cells on an improved Neubauer counting chamber and IC₅₀ values of compounds possessing antileishmanial activity were calculated by Software Ezfit 5.03 Perella Scientific. IC₅₀ values of different fractions against the test pathogen are mentioned in Table 1.

All the experiments were performed in triplicate. IC₅₀ values of compounds possessing antileishmanial activity were calculated by Software Ezfit 5.03 Perella Scientific. The mean and standard deviation of at least three experiments were determined by using Microsoft XL sheet. P values of 0.05 or less was considered significant using student's "t" test.

Insecticidal activity

The insecticidal activity of the extract was determined by direct contact application using filter paper (Ahn et al., 1995). 3 ml of the extract (1 mg/ml) was applied to filter papers (90 mm diameter).

After drying, each filter paper was placed in the separate Petri dish along with 10 adults of each *Tribolium castaneum*, *Stiphilus oryzae*, *Rhyzopertha dominica* and *Callosbruchus analis*. Permethrin ($235.71 \mu\text{g}/\text{cm}^2$) was used as reference insecticide. All these were kept without food for 24 h after which mortality count was done.

Phytotoxic activity

Phytotoxic activity of the crude extract was tested against the *Lemna minor* L. (McLaughlin et al., 1991). Three flasks for each 1000, 100 and 10 $\mu\text{g}/\text{ml}$ were inoculated with stock solution of the extract (20 mg/ml). To each flask, 20 ml medium and 10 plants each containing a rosette of three fronds, was added. Paraquat was used as reference growth inhibitor. All flasks were incubated in the growth cabinet for seven days after which the growth regulation in percentage was calculated with reference to the negative control. IC_{50} was calculated with a Finney computer program with 95% confidence interval.

Antibacterial activity

The crude extract along with fractions was screened against various human pathogens including *Escherichia coli*, *Bacillus subtilis*, *Shigella flexenari*, *Staphylococcus aureus*, and *Salmonella typhi* by agar well diffusion method (Atta-ur-Rahman et al., 1999). In this method, 10 ml aliquots of nutrients broth (Sigma-Aldrich, Germany) was inoculated with the test organism and incubated at 37°C for 24 h. Using a sterile pipette, 0.6 ml of the broth culture of the test organism was added to 60 ml of molten agar, which had been cooled to 45°C , mixed well and poured into a sterile Petri dish (for the 9 cm Petri dish, 0.2 ml of the culture was added to 20 ml of agar). Duplicate plates of each organism were prepared. The agar was allowed to set and harden and the required numbers of wells were dug in the medium with the help of a sterile metallic cork borer ensuring proper distribution of the wells in the periphery and one in the center. Agar plugs were removed. Stock solutions of the test samples in the concentration of 1 mg/ml were prepared in the sterile dimethyl sulfoxide (DMSO) and 100 and 200 μl of each dilution was added in their respective wells. The control well received only 100 and 200 μl of DMSO. Imipinem was used as standard drug. The plates were left at room temperature for 2 h to allow diffusion of the samples then incubated face upwards at 37°C for 24 h. The diameter of the zones of inhibition was measured (mm).

Antifungal activity

Similarly antifungal activity was evaluated by agar tube dilution method (Atta-ur-Rahman et al., 1999). The samples at a concentration of 24-mg/ml were dissolved in the sterile (autoclaved) dimethyl sulfoxide (DMSO, Merck), which served as stock solution. Sabouraud dextrose agar (SDA, Sigma-Aldrich, Germany) was prepared by mixing 32.5 g sabouraud, 4% glucose agar and 4.0 g of agar-agar in 500 ml distilled water and the mixture was mixed thoroughly with magnetic stirrer. Then 4 ml amount was dispensed into screw cap tubes, which were autoclaved at 120°C for 15 min and then cooled to 15°C . The non-solidified SDA media was mixed with stock solution (66.6 μl) giving the final concentration of 400 μg of the extract per ml of SDA. Tubes were then allowed to solidify in the slanted position at room temperature. Each tube was inoculated with a piece (4 mm diameter) of inoculums removed from a seven days old culture of fungi for non-mycelial growth; an agar surface streak was employed. Other media supplemented with dimethyl sulfoxide (DMSO) and reference anti-fungal drugs served as negative and positive control respectively. Inhibition of fungal

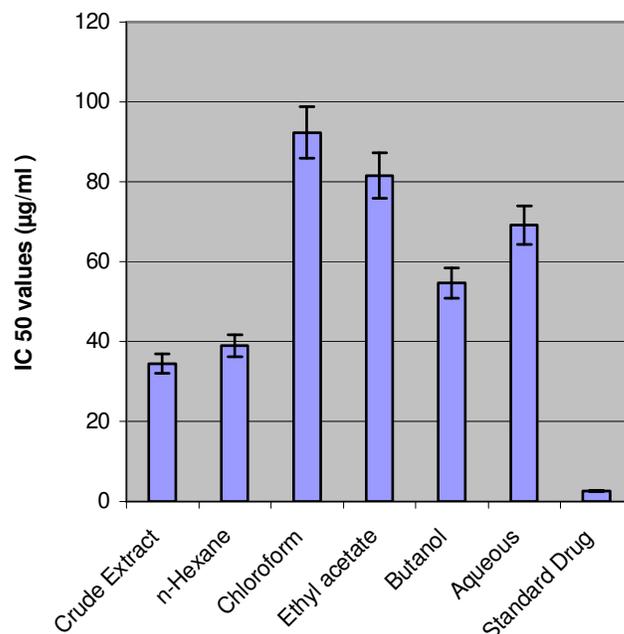


Figure 2. Antileishmanial activity of crude extract and fractions of *Tylophora hirsuta* against promastigotes of *Leishmania major* (Pakistani isolates). Mean IC_{50} values in $\mu\text{g}/\text{ml}$ \pm S.D obtained from three separate experiments are shown in bar charts to show their relative potency.

growth was observed visually after 7-days of incubation at $28 \pm 1^\circ\text{C}$. Humidity (40-50 %) was controlled by placing an open pan of water in the incubator. The percent growth inhibition was calculated with reference to the negative control.

Brine shrimp cytotoxicity

Artemia salina (brine-shrimp eggs) was used to determine the cytotoxicity of the extract (Meyer et al., 1982). Ten shrimps, 5 ml seawater and different concentrations of extract (1000, 100 and 10 $\mu\text{g}/\text{ml}$) were added to separate vials. Etoposide ($\text{LD}_{50} = 7.465 \mu\text{g}/\text{ml}$) was used as reference cytotoxic drug. All the vials were incubated at $26 \pm 1^\circ\text{C}$ for 24 h and the brine shrimps that survived were counted. The data were analyzed with a Finney computer program to determine LD_{50} values with 95% confidence interval.

RESULTS AND DISCUSSION

Rural population of Pakistan still mainly depends on the indigenous system of medicine for their health related matters (Khattak et al., 1985). Keeping in view the traditional uses of the plant and the past work done, we carried out the *in vitro* biological screening of the aerial parts of the *T. hirsuta*. The results of the antileishmanial activity of crude methanolic and various fractions are shown in Figure 2. The crude methanolic extract and n-hexane fraction showed significant antileishmanial activities. The lower the MIC, the greater will be the potency and vice versa. The order of potency of extract and various fractions with respect to standard drug, based on

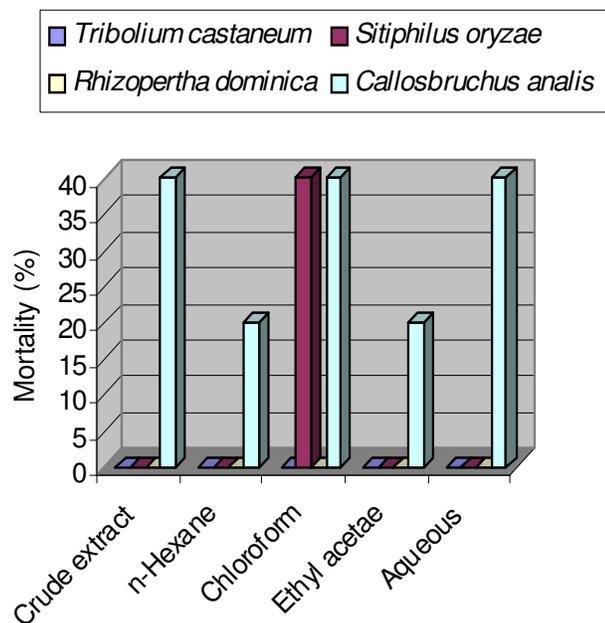


Figure 3. Insecticidal activity of crude extract and fractions of *Tylophora hirsuta*

their IC_{50} values is: pentamidine > crude methanolic extract > n-hexane fraction > n-butanol fraction > aqueous fraction > ethyl acetate fraction > chloroform fraction (Table 1). The plant can be a potential target for the isolation of the bioactive constituents having antileishmanial activity.

Similarly interests in environment friendly insecticides have been increased as the synthetic insecticides are more toxic to environment (Pavela, 2004). The insecticidal activity of crude methanolic and fractions were tested against *T. castaneum*, *Sitiphilus oryzae*, *R. dominica* and *Callosbruchus analis* pests. The results are showed in Figure 3. Crude extract, chloroform and aqueous fraction showed moderate (40%) and n-hexane and ethyl acetate fraction showed weak (20%) insecticidal activity against *C. analis*, whereas moderate (40%) was observed against *S. oryzae* by chloroform fraction. The rest of the test insects were not affected by the test samples.

As the existing herbicides are becoming less and less effective against the weed biotypes (Bhowmilk and Inderjit, 2003); and plants' origin herbicides are environment friendly, therefore, more attention is given to search for environment friendly herbicides. The results of the phytotoxic activity are shown in Figure 4. Against the *L. minor*, the methanolic extract exhibited only 25 and 18.75% growth inhibition at concentration 1000 and 100 $\mu\text{g}/\text{m}$ respectively. No phytotoxic activity was displayed at concentration 10 $\mu\text{g}/\text{m}$.

Against the tested pathogens, Chloroform, ethyl acetate and n-butanol fractions showed non-significant to low antibacterial properties. Crude methanolic extract, n-

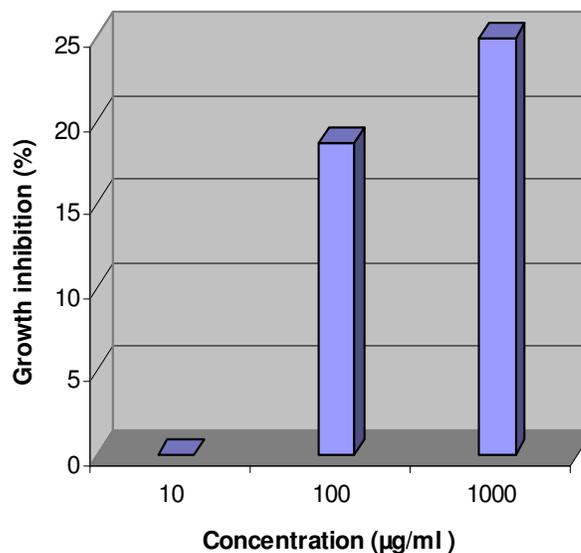


Figure 4. Phytotoxic activity of methanolic extract of *Tylophora hirsuta* against *L. minor L.*

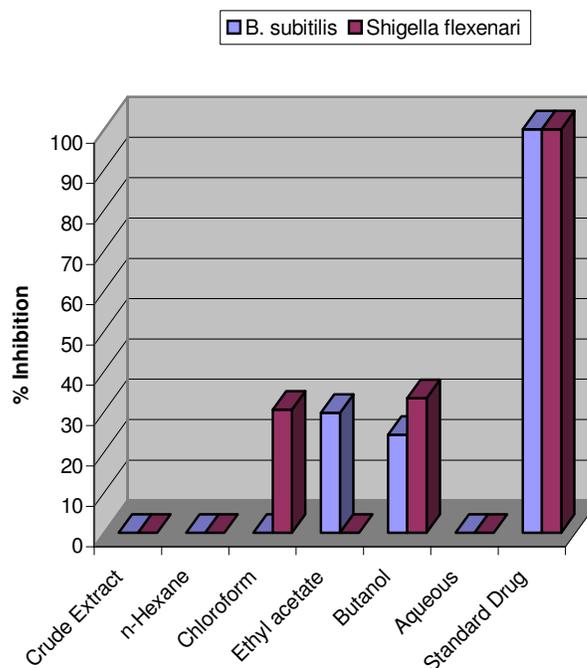


Figure 5. Antibacterial activity of crude methanolic and fractions of *Tylophora hirsuta*.

hexane and water fractions do not have the antibacterial property. As mentioned in Figure 5, ethyl acetate and n-butanol fractions displayed non-significant antibacterial activity (29.72 and 24.32%, respectively) against *B. subtilis*. Against the *S. flexenari*, the chloroform and n-butanol fractions also displayed non-significant antibacterial activities (30.55 and 33.33%) respectively. Similarly,

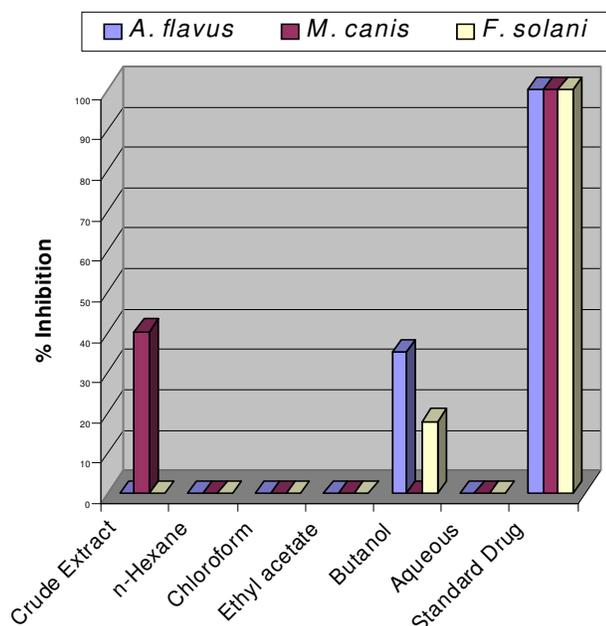


Figure 6. Antifungal activity of crude methanolic and fractions of *Tylophora hirsuta*.

as mentioned in Figure 6, crude methanolic extract displayed moderate antifungal activity against *Microsporum canis* (40%). Non-significant to low antifungal activity was displayed by the n-butanol fraction against *Aspergillus flavus* (35%) and against *F. solani* (8%), respectively.

Thus the plant specie could be potential target for activity-guided isolation of bio-active leishmanicidal, insecticidal and phytotoxic constituents that may be used either directly or used as starting material for searching more potent and novel derivatives to treat the infections or control the pests.

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