



**COMPARATIVE STUDY ON ANTILEISHMANIAL AND CYTOTOXIC ACTIVITY OF
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Faculty of Pharmacy and Health Sciences, University of Balochistan, Quetta, Pakistan;****Tel.: +92 3356951284; E Mail: kashifiqbal321@gmail.com****ABSTRACT**

This work describes an invitro, invivo antileishmanial and cytotoxic activity of *Lawsonia Inermis* bark and *Aloe Vera* leaves extract. Methanolic extract of the plant material at concentrations from 100 - 500 µg/ml was tested invitro to get % inhibition activity on *L. tropica* KWH23 promastigotes in comparison with negative control and Amphotericin-Bat 12-24 hours, whereas invivo antileishmanial activity was checked against *L. tropica* infected Albino mice and cytotoxicity was analyzed via mammalian cell line. For *Lawsonia Inermis* bark, mean % inhibition in extracellular promastigotes at four different concentrations (100 µg/ml, 125 µg/ml, 250 µg/ml, and 500 µg/ml) at 24th hour were 98.02 ± 0.06, 98.70 ± 1.09, 99.41 ± 0.00 and 100.00 ± 0.00 respectively, whereas after 8 weeks, mean lesion size decreased from 0.81 ± 0.20mm to 0.10 ± 0.11mm (p < 0.01) and % cure rate against intracellular amastigotes at dose 75mg/kg was 98.022 (95% C.I = 96.13-98.09) in Albino mice. IC₅₀ value calculated for *Lawsonia Inermis* bark to estimate the cytotoxic activity was 25.105 µg/ml (95% C.I = 15.55-33.83) against lymphocytes. The study proved to be *Lawsonia Inermis* bark as safe and potent inhibitor against *Leishmania tropica* parasites.

Keywords: Lawsonia inermis, Aloe Vera, Leishmania tropica

INTRODUCTION

Leishmaniasis is a major public health problem in tropical and sub-tropical regions, and the disease is caused by parasites belonging to the genus *Leishmania* (Family Trypanosomatidae). WHO have reported that people from 98 countries - covering 5 continents - are at high risk of Leishmaniasis, and it is estimated that approximately 12 million people are currently infected. In Pakistan, *L. tropica* and *L. major* are the main cause of cutaneous leishmaniasis. First line therapy for cutaneous leishmaniasis in Europe, Asia and Africa is pentavalent antimonials, *i.e.*, sodium stibogluconate. However, antimonials have severe side effects like myalgia, pancreatitis, cardiac arrhythmia, hepatitis, and accumulation of the drug in liver and spleen. Thus, there is an urgent need for new chemical entities for non-toxic and effective treatment of leishmaniasis [1, 2].

Lawsonia inermis (Family Lythraceae) is native to Northern Africa and South-western Asia - is cultivated in many tropical and sub-tropical regions. Different parts of plant specially leaves including flowers, stem, bark and root are used as traditional medicine for years - have large number of identified compounds, flavonoids, coumarins, alkylphenones, terpenes (volatile terpenes,

non-volatile terpenes), aliphatic hydrocarbons, and alkaloids which possess a variety of pharmacological activities, including antioxidant, antiviral and antiparasitic [3, 4]. *Aloe Vera* belong to family Liliaceae; found in dry regions of Africa, Asia and Southern Europe – used as therapeutic agent and have diverse action against skin diseases - phytochemically includes Anthraquinones and Phenolic constituents which possessed antioxidant, antimicrobial and anticancer properties [5 - 7].

In the current work, comparison between antileishmanial activities of *Lawsonia inermis* and *Aloe Vera* were examined on *Leishmania tropica* promastigotes and amastigotes whereas cytotoxic effect of plant extract was checked on lymphocyte cell line.

MATERIALS & METHODS**Chemicals**

Fetal bovine serum (FBS), dimethyl sulfoxide (DMSO), RPMI-1640 medium, Amphotericin B, penicillin, streptomycin, formic acid, analytical grade solvents (Ethanol, Methanol and Acetone) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Water used for analysis was purified by deionization and 0.22 µm

membrane filtration (Millipore, Billerica, MA, USA).

Collection of plant material and preparation of crude extract

Plant material of *Lawsonia Inermis* bark and *Aloe Vera* Leaves were collected from the territory of Dera Ismail Khan, Khyber Pakhtunkhwa (KPK), Pakistan in August and September 2015. Identification was performed by Dr. Siraj – ud-Din, Department of Botany, and a voucher specimen (accession number: Bot, 200110 (pup) and 200111 (pup) was deposited at Department of Botany, University of Peshawar (UOP), KPK. The plant material was washed with distilled water before drying them in shade at temperatures below 35 °C. The dried material was stored in a cool dark place until use. Crude plant extract was obtained by macerating 1 kg of powdered leaves in 2 liter of methanol for 1 week with regular stirring in the early morning. The extract was filtered through muslin cloth, and subsequently through Whatman filter paper No. 41. The filtrate was concentrated under reduced

pressure at 46 °C using a rotary evaporator, which afforded 500 g of crude extract.

In vitro antileishmanial activity

In vitro antileishmanial activity of *Lawsonia inermis* bark and *Aloe Vera* Leaves were performed with clinically isolated *Leishmania tropica* promastigotes (KWH23). The *in vitro* antileishmanial growth inhibition assay was adopted from Ozbilgin *et al*, 2014. Promastigotes of *Leishmania tropica* were cultured in RPMI-1640 medium containing 10% fetal bovine serum, 200 U/mL of penicillin, and 0.2 mg/mL of streptomycin. The parasites were cultured at 26 °C for 4 days in BOD incubator (Gallenkamp, Size 1, UK), where after promastigotes were harvested in sterile tubes. The number of promastigotes were measured by transferring 5-10 µl to a haemocytometer (REICHERT, N.Y, U.S.A), and counting the number of promastigotes under upright microscope (CX31, OLYMPUS, Tokyo, Japan). The number of promastigotes were calculated using the formula:

$$\text{Viable cell Count (live cells/ml)} = \frac{\text{Number of live cells counted}}{\text{Number of large corner squares counted}} \times \text{Dilution} \times 10,000$$

The harvested promastigotes were subsequently centrifuge at 4°C at 2000 rpm for 10 min, the supernatant removed, and the pellet reconstituted in fresh RPMI-1640 medium with 10% FBS to obtain a

concentration of 2.1×10^6 promastigotes/mL in the required volume (10ml). Distribute 10 ml having 2.1×10^6 promastigotes/mL in 96 wells of culture plate through multipipette and fraction mix well. Left the culture plate

in BOD incubator at 26°C for two days. Haemocytometer was used to count the promastigotes under upright microscope (lens 40x) at 12 and 24 hours. Performed the in vitro promastigotes test in triplicate and in last the mean percentage inhibition of parasite were taken in record.

In vivo test

Animal Model and Administration of Extract

To study the pathogenesis of leishmania strain, the BALB/c mouse was used by applying very low amount of drug agent. The BALB/c mouse having weight (20-32 gm) sex male, age 6-8 week, cardiac route for drug input and 4 groups were made for in vivo screening.

Leishmania Tropica KWH23 promastigotes were cultured in RPMI-1640 medium along with 10% Fetal bovine Serum, penicillin 200 U/ml and Streptomycin was 0.2 mg/ml. The parasite was cultured and growth multiplied at 26 °C for 4 days in BOD incubator and harvested parasite was used. The harvested promastigotes were taken in sterile tube and was checked and counted by haemocytometer under upright microscope. Calculate the number of promastigotes and centrifuge at 4 °C at 2000 rpm for 10 min, Supernatant liquid was removed and discarded while pellet was left in tube. Fresh

RPMI-1640 medium with 10% FBS was added to get the required volume (10ml). Add required volume (10µl) of promastigotes (2.1×10^6 promastigotes/ml) into cardiac cavity (Intraperitoneally) of BALB/c mice. The development of lesion was measured weekly with dial micrometer during infection period. The infection was well established and cleared visible lesions were seen with naked eye.

4 groups (n=6) of BALB/c were made in which 2 groups comprised of drug control, 1 group have positive control and 1 group was negative control. After 36 days, the lesion was established, so treatment process was started. Dose of plant extract material to Group I and II for mice treatment were 15mg/kg for 5 days (Total dose 75mg/kg) in DMSO solvent up to final volume of 3 ml and 10 ml respectively. Amphotericin was used as Standard drug at a dose of 15mg/kg. No drug agent was used in IV group (Negative Control). Injection dose 10 µl, five times with 3 day intervals were administered to each mice and its result was recorded regularly. Dial micrometer was used to note the difference between size of the lesion in infected and uninfected mice weekly. Before and after treatment, needle aspirations samples were taken from infected lesion [8].

To detect amastigotes under light microscope, Giemsa stained was used in samples, under oil immersions. On the 48th and 60th day of infection, 60 mg of tissue sample was taken from the lesion for biopsy. To search amastigote in leishmania strain, sample was smeared on the slides stained with Giemsa and light microscope was used for examination.

Ethics Statement: BALB/c mice were supplied by Department of Pharmacology (Animal center), University of Peshawar, KPK, Pakistan and this study was approved by Animal and Ethics Committee [9], Faculty of Pharmacy and Health Sciences, University of Balochistan, Quetta. Standard diet along with water and *Lawsonia inermis* Barkitum was given to BALB/c mice during experiments.

Cytotoxicity test

Fresh blood (10ml) was taken in BD Vacutainer K2E (EDTA) obtained from healthy volunteer to get mammalian cell (lymphocyte) for cytotoxic analysis of plant material. PBS (Phosphate Buffered Saline) through 0.2 µm filter under laminar flow hood (kept sterile conditions) and then add equal quantity of PBS and blood in sterile tube. Put Ficoll solution (ratio 1:2) carefully at 165° angle in already mixed PBS and Blood tube. Centrifuge the 30ml mixture at

2000 rpm at 4°C for 30 minutes. Puncture the lower portion of transparent layer in tube with syringe, take liquid carefully, add 5 ml of RPMI-1640 medium and count the number of lymphocytes through haemocytometer under light microscope. Normally 10,000 cells (Lymphocytes) are present in each milliliter. Put 100 µl of lymphocyte media into 96 wells of culture plate [10].

Take the dose of plant material for mammalian cell test were 500, 250, 125, and 100 µg/ml in DMSO solvent up to final volume of 3 ml and 10 ml respectively. Amphotericin having concentration 25 µg/ml (positive control) was taken as reference drug while negative control was *Leishmania tropica* KWH23 promastigotes. Add required volume of promastigotes (2.1×10^6 promastigotes/ml) in 12 wells of culture plate through multipipette. Left the culture plate in incubator at 26 °C for two days (at least otherwise three days). Haemocytometer was used to count the viable lymphocytes and promastigotes under light microscope (lens 40x) at 12 and 24 hours. Performed the cytotoxic test of mammalian cell in triplicate and in last the IC₅₀ were taken in record.

Statistical analysis

In this study, invitro assay of plant extract was expressed as the % Inhibition of parasite

growth, and calculated as the mean of three replicate measurements with standard

deviation using the equation:

$$\text{Percentage inhibition} = \frac{\text{count of control promastigotes} - \text{count of treated promastigotes}}{\text{count of control promastigotes}} \times 100 \%$$

Result record of cytotoxic activity was expressed as % inhibitory concentration (IC₅₀) and was determined by non-linear regression analysis whereas for invivo assays, mean lesion size (mm) and percentage cure rate with 95% confidence level was taken in record, using GraphPad Prism 5 software (GraphPad software, San Diego, CA). P <0.05 was the level of significance.

RESULTS

Invitro Antileishmanial activity

The current work describes the extract of *Lawsonia inermis* bark and *Aloe Vera* Leaves- in combination with invitro antileishmanial profiling. Crude methanol extract of all plant material was tested for its ability to inhibit extracellular promastigotes (2.1 x 10⁶ cells) of *L. tropica* KWH23 at concentrations of 500, 250, 125, and 100 µg/ml, after 12-24 hours gave promising result as shown in **Table 1**, whereas Amphotericin-B was used as Standard drug.

At 12th hour analysis, the plant extract percentage inhibition ranging between 40.10 and 59.01 % at 100µg/ml concentration while at 500 µg/ml concentration, %age inhibition

ranging between 70 and 93%. At 24th hour, parasite %age inhibition ranging between 65 and 98 at 100µg/ml and ranging between 81 and 100% when extract was checked at 500 µg/ml concentration. *Lawsonia inermis* bark showed promising inhibitory activity, reached 98% at 100 µg/ml at 24th hours (p < 0.01). In comparison with Amphotericin-B, having parasitic inhibition ranging between 50.12 and 78.78% at 100 and 500 µg/ml concentration respectively, whereas at 24th hour showed parasite inhibition between 80.09 and 90.55% at 100 and 500 µg/ml concentration against *L. tropica*, *Lawsonia inermis* showed the significant results, ranging between 98.02 and 100% antileishmanial activity at 24th hour at 100 and 500 µg/ml concentration respectively.

Invivo Antileishmanial activity

Invivo activity of *Lawsonia inermis* bark and *Aloe Vera* Leaves were analyzed on Albino mice infected with 0.02 ml clinically isolated *L. Tropica* KWH23 having 2.1 x 10⁶ promastigotes via intraperitoneal route, after 36-120 days gave promising result as shown in **Table 2**. Four groups consisting of Six Albino mice each were made, in which Group I and II were used for sample analysis.

Group III was used as positive control and Group IV as negative control. *Leishmania* lesion of mice was checked by measuring its size before and after treatment of sample and difference in size of lesion was recorded and its mean value was taken in record. All the experimental procedure were go through in triplicate. In two (2) sample groups, the mean lesion size of mice decreased significantly from 0.51 ± 0.20 mm to 0.10 ± 0.10 mm after treatment with plant extract but negative group reached 1.5 ± 0.50 mm ($p > 0.05$), whereas Amphotericin-B decreased from 0.85 ± 0.60 mm to 0.15 ± 0.60 mm, at the end of 8th week. The mice receiving methanolic extract of *Lawsonia inermis* bark and *Aloe Vera* Leaves have percentage cure rate was 98.02% and 75.27% respectively after 8th week treatment. It is evident that

from the resultsthat *Lawsonia Inermis*bark exhibited significant invivo antileishmanial activity against *L. tropica* ($p < 0.01$) infected mice whereas rest of the plant material have promising activity as compared to negative control group.

Cytotoxic effect

Cytotoxic activity of *Lawsonia inermis* bark and *Aloe Vera* Leaves were analyzed invitro against Lymphocytes having count 1.8×10^4 , after 12-24 hours showed result as summarized in **Table 3**.

The methanolic extract of *Aloe Vera* Leaves showed highest cytotoxic activity at a concentration of more than 100 $\mu\text{g/ml}$. Lowest cytotoxic activity was observed when *Lawsonia Inermis* bark extract was exposed to lymphocyte cells having IC_{50} values of 25.105 $\mu\text{g/ml}$.

Table 1: In vitro Antileishmanial activity of Plant extract; Data represent mean percent inhibition \pm S.D of three replicates

Sample	Sample concentrations ($\mu\text{g/ml}$)	Percent inhibition at Time (h)	
		12	24
<i>Lawsonia inermis</i> bark	100	59.01 \pm 0.02	98.02 \pm 0.06
	125	70.68 \pm 0.03	98.70 \pm 1.09
	250	85.35 \pm 0.10	99.41 \pm 0.00
	500	93.00 \pm 0.34	100.00 \pm 0.00
<i>Aloe vera</i> Leaves	100	40.10 \pm 0.70	65.11 \pm 0.00
	125	53.00 \pm 0.20	70.03 \pm 0.07
	250	61.07 \pm 0.40	78.01 \pm 1.00
	500	70.00 \pm 0.20	81.96 \pm 0.00
Amphotericin-B	100	50.12 \pm 0.16	80.09 \pm 0.06
	125	63.34 \pm 0.09	84.67 \pm 1.09
	250	71.45 \pm 0.10	87.62 \pm 0.00
	500	78.78 \pm 0.07	90.55 \pm 0.57
Negative Control	100	0.00 \pm 0.00	0.00 \pm 0.00
	125	0.00 \pm 0.00	0.00 \pm 0.00
	250	0.00 \pm 0.00	0.00 \pm 0.00
	500	0.00 \pm 0.00	0.00 \pm 0.00

Table 2: In vivo Antileishmanial activity of Plant extract; Data represent mean lesion size (mm) with percentage cure rate with 95% confidence intervals

Sample	Dosing Regimen ^{a,b} (For 5 Days)	Mean Lesion (mm) Pre-treatment	Mean Lesion (mm) After Treatment (After 8 Weeks)	%age Cure Rate (with 95% Confidence intervals)	No: of mice cured/No: of mice Infected	Mean survival time (Days)
<i>Lawsonia inermis</i> bark	15mg/kg	0.81±0.20	0.10±0.10	98.02 (96.13-98.09)	6/6	≥60
<i>Aloe vera</i> Leaves	15mg/kg	0.72±0.30	0.51±0.20	75.27 (74.20-76.80)	5/6	≥60
Amphotericin-B	15mg/kg	0.85±0.60	0.15±0.60	96.00 (94.28-96.01)	6/6	≥60
Negative Control	15mg/kg	0.76±0.50	1.5±0.50	0.000	0/6	≥30

a: Total dose 75 mg/kg; b: Route of administration: Intraperitoneally (i.p) Injection

Table 3: Cytotoxic Effect of Plant extract; Data represent IC₅₀(µg/ml) with 95% confidence intervals

Sample	IC ₅₀ (µg/ml) 95% confidence intervals
<i>Lawsonia inermis</i> bark	25.105 (15.55-33.83)
<i>Aloe vera</i> Leaves	>100

DISCUSSION

In Algeria, *L. major* promastigotes were successfully treated with 10µl hydroalcoholic extracts of *Juglan regia*, *Lawsonia inermis* and *Salvia officinalis* [11]. *Heliectta apiculata* was reported to have significant invitro antileishmanial activity on *L. amazonensis*, *Leishmania Infantum* and *Leishmania Braziliensis* whereas invivo antileishmanial activity against *L. amazonensis* [12].

In literature, there are few studies having antileishmanial activity of plant extracts and there isolated natural compounds and secondary metabolites against *L. tropica*.

In a study conducted in Pakistan on antileishmanial activities of 5 plant species *Stellaria media*, *Sida cordata*, *Asparagus gracilis*, *Jurinea dolomiaea* and *Opuntia ficus-indica* in which *Jurinea dolomiaea*

showed significant antileishmanial activity against *L. tropica* [13]. Crude methanolic extracts of four Sudanese plants (*Azadirachta indica*, *Allium Sativum*, *Acacia nilotica* and *Balanites aegyotiaca*) were analyzed against *L. major* promastigotes in which *Balanites aegyotiaca* showed moderate antileishmanial activity [14]. The leishmanicidal activity of *Aloe vera* leaf exudate was reported to have promising invivo antileishmanial effect when checked against *L. donovani* strains [15].

In this work, methanol was selected as a solvent for extraction due to its polar nature in preparation of plant extract. Due to methanolic extract, secondary metabolites play crucial role in antileishmanial and cytotoxic activity. Bark of *Lawsonia Inermis* showed invitro % inhibition 100 ± 0.00 at 500 µg/ml concentration ($p < 0.01$), after 24

hours which confirmed antileishmanial activity from previous findings[11]. *Aloe Vera* leaves possessed 81.96 % at 500 µg/ml ($p < 0.04$), after 24 hours which is an agreement with the results reported for the antileishmanial activity as described earlier in literature [15]. Thus, it was confirmed that plant material possessed secondary metabolites such as Flavonoids, Tannins, Coumarins, Terpenes, Saponins and Alkaloids [4, 12] which exhibited antileishmanial and cytotoxic activity against *L. Tropica*.

Extract of *Lawsonia inermis* possessed very interesting and significant ($p < 0.01$) invitro and invivo activity against *L. tropica* promastigotes than *Aloe Vera* and Amphotericin-B. All Albino mice were cured at or after 60 day of infection, when extract of *Lawsonia iermis* was given whereas after 8th week, mean lesion size was decreased upto 0.10 ± 0.10 mm in mice. That effect may be due to killing of promastigotes/amstigotes or by inhibiting extra/intracellular metaboic pathways of *L. tropica* strains. Cytotoxic effect of *Lawsonia inermis* leaves was moderate (IC_{50} value: $25.105 \mu\text{g/ml}$) were known to results of test extract [16].

CONCLUSION

Antileishmanial and cytotoxic evaluation stated that bark of *Lawsonia Inermisis* safe and promising antileishmanial source against *L. tropica* parasites as compared to *Aloe Vera* leaves.

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