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Evaluation of Selected *in-vitro* Biological Activities of the Seed Extracts of *Ricinodendron heudelotii*

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ABSTRACT

Background and Objective: Plant crude extracts are analyzed to evaluate their potency as a starting material for preparation of eco-friendly and natural product-based drugs and substances for their potential therapeutic benefits. This study investigated the antileishmanial, anti-cancer, anti-inflammatory, cytotoxic and antibacterial activities of *Ricinodendron heudelotii* seed extracts. **Materials and Methods:** The seeds were collected, extracted and fractionated using hexane, DCM and ethyl acetate. The 96 microtitre plate bioassay, MTT HeLa cells, Brine shrimp assay and MABA assays were employed for antileishmanial, anti-cancer, anti-inflammatory and cytotoxicity activities of *Ricinodendron heudelotii* seed extract respectively. **Results:** The antileishmanial activity showed that all the extracts inhibited *in vitro* growth of *Leishmania tropica* promastigotes at the experimental dose with an IC_{50} of 3.16, 3.49 and 3.34 µg/mL for hexane, ethyl acetate and DCM fractions, respectively. Hexane fraction showed the highest inhibition (60%) of ROS with IC_{50} of 32.4 The cytotoxic and *in vitro* anticancer activity of the extracts against human HeLa cervical cancer cell lines at 30 µg/mL showed that hexane, ethyl acetate and DCM fractions had 64.5, 59.9 and 15.6% cell growth inhibition, respectively. The cytotoxic activity using Brine shrimp (*Artemia salina*) lethality bioassay showed that hexane fraction had cytotoxicity at the highest concentration with LC_{50} of 1548.82 antibacterial activity revealed that ethyl acetate fraction had a moderate inhibition against *Staphylococcus aureus*. **Conclusion:** This study revealed that *Ricinodendron heudelotii* seeds had *in vitro* antileishmanial, anti-cancer, anti-inflammatory, cytotoxic and antibacterial activities. The study thus provides evidence for the biological activities of *Ricinodendron heudelotii* seeds as the extracts showed varying potency levels for all investigated activities.

KEYWORDS

Antileishmanial, anti-inflammatory, anti-bacterial, Brine shrimp, heLa cells, *Ricinodendron heudelotii* seeds, reactive oxygen species (ROS)

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INTRODUCTION

Plant crude extracts are analyzed to either isolate biologically active compounds that can be directly used as drugs or to identify bioactive compounds that can be used as a starting material in the preparation of semi-synthetic drugs[1].

Phytotherapy has played a significant role in healthcare and therapeutics. Plant-sourced medications have been recognized and encouraged by WHO due to their less toxic, eco-friendliness, availability and economic advantages[2].

Leishmaniasis is a tropical disease that is caused by the protozoan parasites, which belong to the genus *Leishmania*, the parasites are transmitted by the bite of an insect vector, the phlebotomine sandfly. The need to develop drugs for the treatment of leishmaniasis is expedient because it's been reported that there are presently no vaccines for leishmaniasis, hence the fast spreading of the parasite[3].

Inflammation has been reported as a mechanism that develops collectively in response to pathogens, tissue damage, harmful chemicals and stimuli[4]. Formation of ROS in living organisms is essential for the production of the energy required for use in biological processes, however, their presence due to their imbalance status propagates inflammation via the release of cytokines which further stimulates the recruitment of neutrophils and macrophages[5]. These processes damage biological molecules such as DNA, lipids and proteins in organisms[6,7].

Brine shrimp lethality bioassay is a preliminary screening for further experiments on mammalian animal models for the evaluation of toxicity of natural products, xenobiotics, heavy metals and pesticides etc. It is based on the mortality of test samples on a simple zoological organism-Brine shrimp (*Artemia salina*)[8].

This study evaluated some biological activities of *Ricinodendron heudelotii* seed extracts, such as antileishmanial, anti-cancer, anti-inflammatory, cytotoxic and antibacterial activities for their possible potency as a starting material for the preparation of eco-friendly and natural product-based drugs and substances.

MATERIALS AND METHODS

Study duration and location: This study was carried out between March 2022 to August 2022 at the International Center for Chemical and Biological Science, University of Karachi, Pakistan.

Plant seed collection: The seeds of *Ricinodendron heudelotii* were collected, manually ground into powder and kept in a polyethylene bag at -5.5°C until needed.

Extraction: Powdered samples (1500 g) were extracted by maceration using 80% ethanol (2500 mL) for 72 hrs accompanied by occasional shaking and stirring. The extract was filtered through cotton wool, followed by Whatman filter paper No. 1. The filtrate was subjected to a rotary evaporator (BUCH rotavapor R-210) to allow evaporation of the solvent under pressure of 175 mbar and bath temperature of 50°C. The crude extract (48.67 g) was suspended in distilled water (350 mL). The mixture was transferred into a separatory funnel and extracted successively with equal volume of hexane, dichloromethane, ethylacetate and butanol, after which they were evaporated to dryness using rotary evaporator to yield 9.8, 5.1, 11.7 and 3.3 g crude fractions respectively. The fractions were kept in beakers until further analysis.

Antileishmanial activity (96 microtitre plate bioassay): The plant extracts (5 mg/mL) were dissolved and derived fractions in 1 mL of Dimethyl Sulfoxide (DMSO). This served as the anti-leishmanial assay stock solution. Serial dilutions using DMSO were made (2500, 1250, 625, 312.5, 156.3, 78.1, 39.1 and 19.5 µg/mL) to obtain a concentration of 333.3 µg/mL to 1.3 µg/mL in the wells. The samples were filtered using a 0.45 µm syringe filter.

The promastigotes form of the *Leishmania tropica* was grown in bulk early in liquid medium RPMI-1640 supplemented with 10% fetal bovine serum. Log phase promastigotes at 1×10^6/100 µL were used for the entire assay. About 90 µL of 199 media, 50 µL of *Leishmania tropica* 50129 (ATCC) log phase culture and 10 µL of each extract dilution were dispensed to different wells of microtiter plate. The DMSO was used as a negative control while amphotericin B and pentamidine were as a positive control. Afterwards, the microtiter plate was incubated at 24°C for 72 hrs. After incubation, about 15 µL of each dilution was pipetted on a Neubauer counting chamber and was counted under a light microscope. The 50% lethal dose (LC_{50}) by scheming concentration against percentage growth inhibition graphically was estimated. The anti-leishmanial activity was additionally determined by microscopic counting of the dead parasites for each well and the percentage growth inhibition for each concentration of extract was calculated in relation to negative control.

Reactive oxygen species (ROS) scavenging activity[5]: The RAW 264.7 cells were plated at 1×10^6 cells/well. After 4 hrs, the cells were treated with 10, 50 and 100 µg/mL *Ricinodendron heudelotii* seed extracts and LPS for 24 hrs. After incubation, the cells were washed with PBS and harvested. The cells were then incubated with Dichlorofluorescein Diacetate (DCF-DA) (25 µM) for 30 min at 37°C in the dark. After several washings with PBS, the fluorescence was captured using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA). The DCF fluorescence was measured at an excitation wavelength of 488 nm and an emission wavelength of 515-540 nm.

Anti-cancer activity

Cancer cell line: The human cervical cancer cell line (HeLa) was obtained from National Centre for Cell Science, Pune and grown in Eagles minimum essential medium containing 10% fetal bovine serum (FBS). All the cells were maintained at 37°C, 5% CO_2, 95% air and 100% relative humidity. Maintenance cultures were passed weekly and the culture medium was changed twice a week.

Cell treatment and 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay: The monolayer cells were detached with trypsin-ethylenediaminetetraacetic acid to make single cell suspensions and viable cells were counted using a hemocytometer and diluted with the FBS medium with 5% FBS to give a final density of 1×10^5 cells/mL. About 100 µL/well of cell suspension was seeded into 96-well plates at a plating density of 10,000 cells/well and incubated to allow for cell attachment at 37°C, 5% CO_2, 95% air and 100% relative humidity. After 24 hrs the cells were treated with serial concentrations of the extracts. They were initially dissolved in neat Dimethyl Sulfoxide (DMSO) and further diluted in a serum-free medium to produce five concentrations. About 100 µL per well of each concentration was added to plates to obtain final concentrations of 500, 250, 125, 62.5 and 31.25 µg/mL. The final volume in each well was 200 µL and the plates were incubated at 37°C, 5% CO_2, 95% air and 100% relative humidity for 48 hrs.

The medium containing no samples served as a control while doxorubicin was used as the standard drug. Triplicate was maintained for all concentrations. After 48 hrs of incubation, 15 µL of MTT (5 mg/mL) in phosphate-buffered saline was added to each well and incubated at 37°C for 4 hrs. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100 µL of DMSO and then the absorbance was measured at 570 nm using a microplate reader.

The cell inhibition (%) was determined using the formula[5]:

$$\text{Cell inhibition (\%)} = 100 - \frac{\text{Abs sample}}{\text{Abs control}} \times 100$$

Where,
Abs=Absorbance

Nonlinear regression graph was plotted between cell inhibition (%) and Log_{10} concentration and IC_{50} was determined using GraphPad Prism 6 software (GraphPad, San Diego, CA).

Brine shrimp (*Artemia salina*) lethality bioassay: The Brine shrimp (*Artemia salina*) lethality test was used to assess the cytotoxic activity of the crude extract and subsequent fractions of *Ricinodendron heudelotii* seeds.

For the experiment, (20 mg) of the samples were dissolved in 2 mL of methanol and from this solution, 5, 50 and 500 µL were transferred to vials (3 vials/ concentration) to make a concentration of 10, 100 and 1000 µg/mL respectively. The solvent is to evaporate overnight. After 2-days of hatching and maturation as nauplii, 10 larvae were placed in each vial using a Pasteur pipette, the volume was made up to 5 mL with seawater and Incubated at 25-27ºC for 24 hrs, under illumination. Other vials were supplemented with solvent and reference cytotoxic drug (Etoposide), which served as negative and positive controls, respectively. After 24 hrs, the vials were inspected using a magnifying glass and the number of survived nauplii in each vial was counted. The mortality endpoint of this bioassay was defined as the absence of controlled forward motion during 30s of observation[9]. From this data, the percent of the lethality of the Brine shrimp nauplii for each concentration and control was calculated. An approximate linear correlation was observed when logarithm of concentration versus percentage of mortality was plotted on the graph paper and the values of LC_{50} were calculated using Probit analysis and Microsoft Excel 2016.

Anti-bacterial activity

Microplate Alamar Blue Assay (MABA): The test organisms were grown in Mueller Hinton medium and inoculums were adjusted to 0.5 McFarland turbidity index. Stock solutions of the crude extract and fractions were prepared in DMSO (1:1 concentration) and the media were dispensed to all wells. All work was done in triplicate. The samples were added to the wells; the control wells did not contain any test samples. The 96 well plates were made up to 200 µL. The 5×10^6 cells were added in all wells including control and test. The plates were sealed with parafilm and incubated for 18-20 hrs. Alamar Blue dye was dispensed in each well and shaken at 80 RPM in a shaking incubator for 2-3 hrs. Plates were covered with foil in a shaking incubator. A change in color of Alamar Blue dye from blue to pink indicated the growth in bacterial strains. Absorbance was recorded at 570 and 600 nm by the ELISA reader-BR-580, BR Biochem Life Sciences Pvt., Ltd., India.

RESULTS AND DISCUSSION

The result on antileishmanial activity of *Ricinodendron heudelotii* seed extracts showed that all the extracts tested were potent for antileishmanial activity and inhibited *in vitro* growth of *L. tropica* promastigotes at the experimental dose with an IC_{50} of 3.16, 3.49 and 3.34 µg/mL for hexane, ethyl acetate and DCM fractions respectively as shown in Table 1.

Hexane fraction had the highest 60% inhibition of ROS with IC_{50} of 32.4 while DCM had the least 40.4% inhibition of ROS and was inactive against ROS. Ethyl acetate fraction was also inactive against ROS as shown in Table 2.

The cytotoxicity activity of the plant extracts was carried out against HeLa cells at 30 µg/mL to determine the IC_{50} by MTT assay. The result shows that hexane, ethyl acetate and DCM fractions had 64.5, 59.9 and 15.6% cell growth inhibition respectively as shown in Table 3. Hexane fraction had the highest inhibition percentage with IC_{50} value of 5.2 (unit) and DCM fraction had the least inhibition percentage and was inactive. The highest cytotoxicity of the extracts against HeLa cells was found with the hexane fraction with 64.5% of cell growth inhibition, while DCM fraction was inactive with 15.6% cell growth inhibition.

Table 1: Antileishmanial activity of *Ricinodendron heudelotii* seed extracts

Test samples	IC_{50} (µg/mL)±SD
R. heudelotii hexane fraction	3.16±0.00
R. heudelotii ethyl acetate fraction	3.49±0.2
R. heudelotii DCM fraction	3.34±0.1
Amphotericin B	3.14±0.02
Pentamidine	4.49±0.04

Table 2: ROS scavenging activities of *Ricinodendron heudelotii* seeds extracts

Sample	Concentration (µg/mL)	Inhibition (%)	IC_{50}±SD (µg/mL)
Hexane fraction	50	60.0	32.4±5.2
Ethyl acetate fraction	50	48.0	Inactive
DCM fraction	50	40.4	Inactive
Ibuprofen	25	73.2	11.2±1.9

Table 3: Cytotoxicity/anticancer activity of *Ricinodendron heudelotii* seed extracts against HeLa cell line by MTT assay

Sample	Concentration of sample	Inhibition (%)	IC_{50}±SD (µg/mL)
Hexane fraction	30 (µg/mL)	64.5	5.2±0.3
Ethyl acetate fraction	30 (µg/mL)	59.9	22.5±1.0
DCM fraction	30 (µg/mL)	15.6	Inactive
Doxorubicin	30 (µm)	100	0.9±0.14

Table 4: Cytotoxicity (Brine shrimp (*Artemia salina*) lethality bioassay

		Hexane fraction		DCM fraction		Etoposide	
Concentration (µg/mL)	Number of shrimps	No of survivors	Mortality (%)	No of survivors	Mortality (%)	Dose (µg/mL)	Mortality (%)
10	30	26	13.34	28	6.67	7.5	70
100	30	25	16.67	27	10		
1000	30	15	50	23	23.34		
		LC_{50} (µg/mL)	1548.82	LC_{50} (µg/mL)	144543.9		

Table 5: Antibacterial activity (Microplate Alamar Blue Assay-MABA) of *Ricinodendron heudelotii* seed extracts

Bacteria	Hexane (inhibition (%))	DCM (inhibition (%))	Ethylacetate (inhibition (%))	Standard drug (Ofloxacin)
Escherichia coli (ATCC 25922)	NI	NI	NI	89.1
Bacillus subtilis (ATCC 23857)	NI	NI	NI	89.15
Staphylococcus aureus (NCTC 6571)	28.19	7.93	47.53	88.72
Pseudomonas aeruginosa (ATCC 10145)	NI	NI	NI	88.78
Salmonella typhi (ATCC 14028)	NI	NI	NI	90.36

N/B: Concentration of sample = 3000 µg/mL, Amount of sample = 60 mg, concentration of standard drug = 100 µg/mL, Amount of drug = 10 mg and NI: No inhibition

Table 4 showed results on the cytotoxic activity of *Ricinodendron heudelotii* seeds using the Brine shrimp (*Artemia salina*) Lethality bioassay and showed that the hexane fraction had cytotoxicity at highest concentration, the DCM fraction showed no cytotoxicity at all concentration.

The result in Table 5 shows that the antibacterial activity of *Ricinodendron heudelotii* seed extracts was tested against 5 bacteria. All fractions tested had inhibition against just one bacterium; *Staphylococcus aureus*. Hexane fraction inhibited *Staphylococcus aureus* at 28.19%, while DCM and ethyl acetate fractions inhibited at 7.93 and 47.53%, respectively at 3000 µg/mL.

DISCUSSION

This study investigated the antileishmanial, anti-cancer, anti-inflammatory, antibacterial and cytotoxicity activities of *Ricinodendron heudelotii* seeds. An investigation on the crude extracts of plants has been reported to be useful in the identification of bioactive compounds that can be used as a starting material in the preparation of semi-synthetic drugs[10]. Access to conventional medications and health care in rural areas has been a challenge[11], hence the need and necessity of plant based sources for therapeutic remedies.

This result obtained for the antileishmanial activity as shown in Table 1 revealed that all extracts tested had potent antileishmanial activity on *L. tropica* promastigotes. The hexane, ethyl acetate and DCM extracts had IC_{50} of 3.16, 3.49 and 3.34 µg/mL, respectively and were toxic against the J774 macrphage cell line at the experimental concentration. Previous studies have reported the antileishmanial potency of various plant extracts. da Silva *et al.*[12] observed that extracts from *C. gilliesii*, *S. hortensis*, *C. copticum* heirm and *T. migricus* contained active compounds that could serve as alternative agents in the control of leishmaniasis. García *et al.*[13]; Grecco *et al.*[14] in their studies reported the flavonols constituent of Pluchea carolinensis as antileishmanial agent, also Gonzalez-Coloma *et al.*[15] reported the antileishmanial activity of alkaloids. Odinga *et al.*[16] reported the bioactive constituents and its anti-dengue and insecticidal potencies of *Ricinodendron heudelotii* seed oil, making the seeds potential therapeutic substances. This further supported the antileishmanial potency of the seed extracts of *Ricinodendron heudelotii*.

Table 2 shows the ROS scavenging activity of the seed extracts of *Ricinodendron heudelotii*. The ROS scavenging activity of the seed extracts of *Ricinodendron heudelotii* showed that the hexane fraction had the highest 60% inhibition of ROS with IC_{50} of 32.4 while DCM had the least 40.4% inhibition of ROS and was inactive against ROS. Ethyl acetate fraction was also inactive against ROS. The ROS has been identified as a regulatory factor in mitochondrial activities in humans, it has a role in inflammation processes, ROS scavenging has been reported to inhibit lipid oxidation and aging in humans[17]. Despite the roles of ROS in physiological cellular processes, their imbalance and excessive production give rise to oxidative stress. This further leads to damage to biomolecules such as lipids, proteins and DNA[18]. Zielinska-Blizniewska *et al.*[19] in their report observed natural plant supplements may provide increased health expectancy through ROS neutralization, this may be achieved through their antioxidant capacity and effects on the mitochondrial biogenesis, reduction of inflammation and regulation of the sympathetic nervous system. Odinga and Nwaokezi[20] also observed the potency of plant extract to inhibit oxidative stress in diabetic rats. The above literature was in agreement with the ROS-inhibiting potency of the hexane fraction of the seeds of *Ricinodendron heudelotii.* Park *et al.*[21] reported the ROS activity of white rose flower hexane fraction due to its polyphenol and volatile components properties.

The cytotoxic and *in vitro* anticancer activity of *Ricinodendron heudelotii* extracts against human HeLa cervical cancer cell lines using MTT colorimetric assay is seen in Table 3. The values for cytotoxicity measured by MTT assay were measured as concentration that causes a 50% decrease in cell viability (IC_{50}, µg/mL). The cytotoxicity activity of the plant extracts was carried out against HeLa cells at 30 µg/mL to determine the IC_{50} by MTT assay. The result as shown in Table 3 shows that hexane, ethyl acetate and DCM fractions had 64.5, 59.9 and 15.6% cell growth inhibition respectively. Hexane fraction had the highest inhibition percentage with an IC_{50} value of 5.2 (unit), DCM fraction had the lowest inhibition percentage and was inactive.

The highest cytotoxicity of the extracts against HeLa cells was found with the hexane fraction with 64.5% of cell growth inhibition, while DCM fraction was inactive with 15.6% of cell growth inhibition. Artun *et al.*[22] observed that novel anticancer drugs have been discovered from natural products and the discoveries are still ongoing. Cytotoxic natural products are significant in their anticancer activities selectively by their synergistic work with conventional chemotherapeutic drugs, thereby improving their efficacy or reducing their toxicity[23]. This study shows that the hexane fraction of *Ricinodendron heudelotii* seed has a potential *in vitro* anticancer activity and cytotoxicity activity against HeLa cell line. The potency of *Laurus nobilis* and *Rosmarinus officinalis* to inhibit the proliferation of HeLa cells has been reported[24].

The result on the cytotoxic activity of *Ricinodendron heudelotii* seeds using the Brine shrimp (*Artemia salina*) lethality bioassay showed that the hexane fraction had cytotoxicity at highest concentration, the DCM fraction showed no cytotoxicity at all concentrations. The LC_{50} values obtained from the Brine shrimp

lethality bioassay as shown in Table 4 were 1548.82 and 144543.9 µg/mL for hexane, EA, DCM fractions, respectively. Rieser *et al.*[25] reported that crude extracts resulting in LC_{50} values less than 250 µg/mL were considered significantly active with potentials for further investigation. The cytotoxic activity exhibited by the extracts of *Ricinodendron heudelotii* seeds from the above statement indicates its cytotoxic inactivity on (*Artemia salina*) for further investigations.

The antibacterial activity using Microplate Alamar Blue Assay (MABA) of *Ricinodendron heudelotii* seed extracts revealed that ethyl acetate fraction had a moderate inhibition against *Staphylococcus aureus* only. All other fractions had insignificant inhibition of only *Staphylococcus aureus*. Table 5 shows that the antibacterial activity of *Ricinodendron heudelotii* seed extracts was tested against 5 bacteria. All fractions tested had inhibition against just one bacterium; *Staphylococcus aureus*. Hexane fraction inhibited *Staphylococcus aureus* at 28.19%, while DCM and ethyl acetate fractions inhibited at 7.93 and 47.53%, respectively. In comparison to a standard antibacterial drug; ofloxacin which inhibited *Staphylococcus aureus* at 88.72%, hexane and DCM had non-significant antibacterial activity while ethyl acetate fraction had a moderate inhibition activity. The antibacterial activities of various plant extracts have been reported by various researchers[26-28]. The findings of this study further suggested the inhibitory potency of *Ricinodendron heudelotii* seed extract against *Staphylococcus aureus* at 3000 µg/mL.

The findings of this study imply that the crude extract of *Ricinodendron heudelotii* seeds possesses *in vitro* antileishmanial, anti-cancer, anti-inflammatory, cytotoxic and antibacterial activities, hence its ethnobotanical and medicinal use.

CONCLUSION

This study assessed the *in vitro* biological activities of *Ricinodendron heudelotii* seed extracts. All tested fractions had potent activity for antileishmanial on *L. tropica* promastigotes, hexane and DCM fractions had ROS scavenging activity while ethyl acetate fraction was also inactive. Hexane and ethyl acetate fraction had cytotoxicity against HeLa cells, while DCM fraction was inactive, hexane fraction also had cytotoxic activity *Artemia salina* and all extracts had inhibitory activity against *Staphylococcus aureus*. In conclusion, all data from the study showed that the seeds of *Ricinodendron heudelotii* had some biological activities, thus its potent therapeutic uses.

SIGNIFICANCE STATEMENT

The quest to exploit the potencies of the seeds of *Ricinodendron heudelotii* as it serves as a form of complementary and alternative medicine necessitated this study. This study revealed the *in vitro* antileishmanial, anti-cancer, anti-inflammatory, cytotoxic and antibacterial activities of the seeds of *Ricinodendron heudelotii* and also its therapeutic potency. These aforementioned activities of the seed extract will curb some of the challenges that are faced with the use of synthetic drugs in treatment of biochemical disorders and diseases.

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