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### Original Article

## Anti-trypanosomal Activity of *Picrorrhiza kurroa* rhizomes against *Trypanosoma evansi*

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### ABSTRACT

In our quest for more effective and efficient means of obtaining trypanocidal extracts/compounds from medicinal plants, for leads in developing new trypanocide against trypanosomes, *Picrorrhiza kurroa* rhizomes were extracted with solvent, methanol, at different concentrations (250-1000 µg/ml) and tested against *Trypanosoma evansi* on Alsever's medium. Trypanosomes were suspended in Alsever's solution with inactivated bovine serum at 58°C for 1 h at appropriate conditions for antitrypanosomal activity. Trypanosomes concentration was  $1 \times 10^6$  parasites/ml. *In vitro* cytotoxicity test of methanolic plant extract (MPE) of *P. kurroa* at concentrations (1.56-100 µg/ml) was done on Vero cells grown in Dulbecco's Modified Eagle Medium (DMEM). At 250 µg/ml<sup>-1</sup> of MPE of *P. kurroa*, there was significant anti-trypanosomal activity with drastic reduction of trypanosomes count ( $40.00 \pm 0.0$  to  $4.667 \pm 0.67$ ) was observed after 5 h of incubation. But at 500 µg/ml of MPE of *P. kurroa*, trypanosomes were completely killed in corresponding ELISA plate wells ( $40.00 \pm 0.0$  to  $0.0 \pm 0.0$ ) after 5 h of incubation, which was statistically equivalent to diminazineaceturate (50 µg/ml) standard drug at 4 h. Both MPE of *P. kurroa* rhizomes and diminazineaceturate were cytotoxic to Vero cells in all concentrations except at 1.56-6.25 µg/ml. MPE of *P. kurroa* demonstrated marked anti-trypanosomal activity with significant difference ( $P = 0.05$  to  $0.01$ ). MPE of *P. kurroa* rhizomes and diminazineaceturate had the same level of *in vitro* cytotoxic effects on Vero cells. In this current report, Alsever's medium supported trypanosomes with observed anti-trypanosomal activity. The outcome of this research could be further strengthened by (e.g. bioassay-guided purification) of *P. kurroa* for isolation of trypanocidal compound(s) that may lead to development of new trypanocide.

**Keywords:** Anti-trypanosomal activity, Cytotoxicity test, *Picrorrhiza kurroa* rhizomes, *Trypanosoma evansi*.

## INTRODUCTION

Trypanosomosis, a blood protozoan parasitic disease of both animals and humans had resurged in endemic area where such disease thrives around the world particularly, in Africa and Latin America and pocket areas in USA being brought in by immigrants from Latin America (WHO, 2010). Trypanosomosis is caused by parasites under genus *Trypanosoma* with variant species in both animals and humans with devastating havoc (WHO, 2012). Direct losses due to trypanosomosis are estimated to amount to between US\$1-1.2 billion each year whereas the indirect impact of AAT on agriculture in sub-Saharan Africa exceeds this amount. A pondered evaluation extrapolated for the total tsetse-infested lands values the total losses, in terms of agricultural Gross Domestic Product, at US\$ 4.75 billion per year (FAO, 2004). Drawbacks (e.g. toxicity, resistance and emerged resistant strains of trypanosomes had emerged) had beset the limited class of available trypanocides (Nok and Nock, 2002)

From medicinal plant point of view, lots of research works had been documented on anti-trypanosomal activity potential with leads for further research and development of new trypanocide (Freiburghouse *et al.*, 1996; Nok and Nock, 2002; Kubuta *et al.*, 2005; Shaba *et al.*, 2006; 2007; 2011(ab); Wube *et al.*, 2012; Obbo *et al.*, 2013; Shaba *et al.*, 2014).

Pharmacological active compounds such as Kutkin that contained mixture of picroside I and picroside II (Kutkoside) and Apocynin have been isolated and structures elucidated (Lee *et al.*, 2006).

*P. kurroa* is hepatoprotective, anti-inflammatory, enhances cardiovascular functions, anti-asthmatic and antiviral. (Keche *et al.*, 2010).

Previously, were ported *in vitro* trypanocidal evaluation of *P. kurroa* rhizomes using different medium (Shaba *et al.*, 2012a). Current report was focused on assessing different medium and corresponding anti-trypanosomal activity.

As a result of problems affecting limited classes of trypanocides, *Picrorrhiza kurroa* rhizomes were extracted with methanol and obtained MPE of *P. kurroa* were tested against *Trypanosoma evansi*.

## MATERIALS AND MEMTHODS

### Chemicals

Silica gel-G for thin layer chromatography (TLC), solvents (hexane, chloroform, methanol, acetic acid and ethyl acetate) for extraction of plant material and development/analysis of TLC plates, vanillin for spray, and iodine for detection of bioactive constituents were used which were purchased from E. Merck, India.

### Plant Material

Fresh *Picrorrhiza kurroa* rhizomes (Family: scrophulariaceae) which is known locally in Ayurveda medicine as Kutki or Katuki were obtained from Palampur, Himachal Pradesh, India. The plant material was subsequently identified at Institute of Himalayan Biosource and Technology, Palampur, Himachal Pradesh, India.

### Preparation of Extract

The extraction was done based on the method of Stahl (1969). 20 g of dried *Picrorrhiza kurroa* rhizomes were powdered using laboratory pestle and mortar, and cold extracted with 200 ml of methanol (Analytical grade). Residues obtained were extracted twice in the same medium. The filtrates were combined, dried at 37°C and stored at 4°C until used.

### Solvent Systems

In order to obtain the most suitable solvent system, the under listed solvent systems were tested to develop the TLC plates according to the method of Stahl (1969).

- Chloroform/hexane/acetic acid (50:50:1)
- Chloroform/ethyl acetate/acetic acid (50:50:1)
- Methanol and chloroform (20: 80)

### Thin Layer Chromatography (TLC) Plates

Aliquots (0.2 ml) of methanolic plant extract (MPE) of *P. kurroa* was applied on TLC plates, dried under room temperature and immersed inside the appropriate solvent systems in a glass jar. It was done to detect the presence of bioactive constituents in applied extracts. Method of Stahl (1969) was applied in the development of the TLC plates.

## Animals

Swiss albino mice (20-30 g) of either sex were obtained from Animal Research Laboratory Section of Indian Veterinary Research Institute (IVRI) Izatnagar, maintained in standard environmental conditions and fed on a standard diet prepared by the institute with water *ad libitum*. Usage of mice in the experiment was strictly guided by laid down rules and regulations of committee on Ethics and Cruelty to Animals of the institute.

## Test Organism

*T. evansi* was obtained from the Division of Parasitology, Indian Veterinary Research Institute (IVRI), Izatnagar and was maintained in the laboratory by serial sub-passages in Swiss albino mice. The strain was routinely tested for virulence as per the method of Williamson *et al.*, (1982).

## Trypanosomes Count

Counting of trypanosomes was carried out following the method of Lumsden *et al.*, (1973). A number of fields (10-15) of each drop of blood or incubated media and parasites in triplicate were counted using glass slides under inverted microscope (400X). An average mean trypanosomes count was taken as number of trypanosomes per field.

## In Vitro Trypanocidal Activity

*In vitro* trypanocidal activity was carried out according to the method of Talakal *et al.*, (1995). A high parasitemic blood of a mouse was diluted with Alsever's solution to obtain final trypanosomes concentration of  $1 \times 10^6$  parasites/ml. The medium consist of Alsever's solution and inactivated bovine serum at 58°C for 1 h. Suspension (180 µl of medium with trypanosomes) was added to 20 µl of the test MPE of *P. kurroa* rhizomes and the plates were incubated at 37°C under 5% CO<sub>2</sub>. The test was repeated at least thrice.

Stock of MPE of *P. kurroa* was solubilized in 1% dimethylsulphoxide (DMSO). The concentration in the experiment had no deleterious effect by itself on host cells or trypanosomes. 1% DMSO in distilled water was used as control (Young *et al.*, 2000).

## In Vivo Infectivity Assessment

*In vivo* infectivity assessment of MPE of *P. kurroa* was carried out after completion of

incubation for anti-trypanosomal activity. Contents of ELISA plate wells with reduced or immobilized and apparently killed trypanosomes from MPE of *P. kurroa* rhizomes were inoculated (0.1ml mouse<sup>-1</sup>) into two groups of mice (six group<sup>-1</sup>) via intra-peritoneum, and observed for more than 60 days for parasitemia (Woo, 1970; Igweh *et al.*, 2002).

## In Vitro Cytotoxicity Test

It was done according to the method of Sidwell and Hoffman. (1997). Vero cell line (SIGMA) was grown in DMEM in 96 wells micro culture plates. Each well was seeded with 500,000 cells ml<sup>-1</sup> and plates were incubated at 37°C with 5% CO<sub>2</sub> for 48 h. After the formation of confluent monolayer, the supernatant was discarded and replaced with fresh medium. Confluent monolayer of Vero cell lines was treated with serial dilutions (1.56-100 µg ml<sup>-1</sup>) of MPE of *P. kurroa* in triplicate and incubated for 72 h consecutively under the same conditions described previously. At 24 h interval, ELISA plate was observed under inverted microscope for cytotoxic effects as compared to untreated normal cells that served as control. In each case, after 72 h of incubation, the culture media of the incubated Vero cells was discarded. Adhered cells were stained with a drop of crystal violet in phosphate buffered solution. ELISA plates were then incubated for 24 h at 37°C in ordinary incubator. Plates were later observed under inverted microscope for cytotoxic effects. It was repeated thrice.

## Statistical Analysis

Results of trypanocidal activity were expressed as mean  $\pm$  SEM. Statistical analysis was done using Sigma stat (Jandel, USA).

## RESULTS AND DISCUSSION

The results of current study were as presented in Tables (1 and 2).

## Extraction

Methanol was used in the cold extraction of *P. kurroa* rhizomes with resultant exhibition of bioactive constituents depicted on the TLC plates. This is in line with extraction of some medicinal plants from Nigerian ethno medicine (Abiodun *et al.*, 2012) and *Piper nigrum* (Black Pepper) (Shaba *et al.*, 2012).

### Solvent System

After concerted efforts in trial of solvent systems for a more suitable one, methanol/chloroform (20:80) appeared to be more effective in the development of TLCs plates than other solvents tested with applied aliquots of *P. kurroa* accordingly. Distinct patterns of bioactive constituents from extract of

*P. kurroa* rhizomes were detected, which were subsequently responsible for anti-trypanosomal activity. This is comparable to earlier report of development of TLC plates of MPE of *Picrorrhiza kurroa* rhizomes (2007) and *Khayasene galensis* bark (Shaba et al., 2011).

**Table 1: *In vitro* trypanocidal activity of 100% methanolic extract of *Picrorrhiza. Kurroa* against *Trypanosoma evansi* on Vero cell line**

Concentration of plant extract in µg/ml	1 h	2 h	3 h	4 h	5 h
250	40.00±0.0	35.00±0.58	17.67±0.67	10.33±0.33	4.667±0.67
500	40.00±0.0	13.33±0.33	4.667±0.33	0.0±0.0	0.0±0.0
750	38.33±0.33	10.67±0.67	0.0±0.0	0.0±0.0	0.0±0.0
1000	19.67±0.33	1.333±0.33	0.0±0.0	0.0±0.0	0.0±0.0
Diminazineaceturate (50) Positive control	22.33±0.33	9.000±0.58	1.333±0.33	0.0±0.0	0.0±0.0
Control (Negative control)	40.00±0.0	40.00±0.0	40.00±0.0	40.00±0.0	40.00±0.0

Bioassay status: significant reduction of parasites counts from concentration of 750 µg/ml and complete killing of parasites at 1000 µg/ml at 7<sup>th</sup> hour of observation. An average mean parasites count of 37.67±0.58 is statistically critical value. Average mean from 37.67±0.58 and below is significant between the treatment groups and negative control. (P = 0.05 to 0.01).

**Table 2: Cytotoxic effect of methanolic extract of *Picrorrhiza korrea* rhizomes on Vero cell line compared to diminazineaceturate (Berenil)**

Concentration of test material in µg/ml	Effects of test extract at various periods of incubation (24 h, 48 h, 72 h)						
	<i>Picrorrhiza korrea</i>	Berenil	<i>Picrorrhiza korrea</i>	Berenil	<i>Picrorrhiza korrea</i>	Berenil	Control
100	100%	66.6%	100%	100%	100%	100%	0
50	100%	33.3%	100%	100%	100%	100%	0
25	33.3%	0	100%	100%	100%	100%	0
12.5	0	0	0	0	33.3%	33.3%	0
6.25	0	0	0	0	0	0	0
3.13	0	0	0	0	0	0	0
1.56	0	0	0	0	0	0	0

*Picrorrhiza korrea* and diminazineaceturate were toxic to Vero cell line except at concentrations range of 6.25-1.56 µg/ml.

### *In vivo* Infectivity Test

Group of mice inoculated with contents of ELISA plate wells with completely killed trypanosomes by MPE of *P. kurroa* rhizomes survived for more than 60 days, while other group of mice inoculated with contents of ELISA plate wells with reduced trypanosomes count by MPE of *P. kurroa* rhizomes died of parasitemia. This is in line with *in vivo* infectivity test of *Terminalia belirica* dried fruits (Shaba et al., 2009) and aqueous extract of *Brassica oleracea* (Igweh et al., 2002) in which similar pattern of *in vivo* infectivity were observed.

### *In Vitro* Cytotoxicity Test

*In vitro* cytotoxicity test of MPE of *P. kurroa* rhizomes and diminazineaceturate at the same concentrations on Vero cells depicted different cytotoxic effects such as distortion, swelling,

sloughing and death of Vero cells compared to negative normal cells in control wells, which were observed in all concentrations range except at 6.25-1.56 µg/ml. *P. kurroa* had the same level of *in vitro* cytotoxicity as diminazine, standard reference drug used. These findings are comparable to *T. belirica* dried fruits and methanolic *Plumbago zeylanica* root bark with similar cytotoxic effects though at much higher toxic concentrations than the later.

### *In Vitro* Trypanocidal Activity

At t 250 µg/ml of MPE of *P. kurroa*, there was drastic reduction of trypanosomes counts but no complete killing of trypanosomes in the corresponding ELISA plate wells after 5 h of incubation. However, at 500 µg/ml of MPE of *P. kurroa*, trypanosomes were not detected in the corresponding ELISA plate wells at 5 h of incubation, which was statistically equivalent to

diminazineaceturate (50µg/ml) standard drug at 4 h. High activity of trypanocidal activity was observed with significant difference ((P 0.05 to 0. 01). Already isolated compounds with elucidated structures (Picroside I and Picroside II (Kutkoside) and Apocynin) could be responsible for its trypanocidal activity. This result is comparable to antitrypanosomal activity of methanolic extract of *Khayasene galensis* root bark (Shaba *et al.*, 2011), antitrypanosomal activity of some medicinal plants from Nigerian ethnomedicine (Abiodun *et al.*, 2012) and evaluation of anti-trypanosomal activity of methanolic extracts of *Moringa oleifera* lam) tree bark and seed pods (Shaba *et al.*, 2014) where different levels of anti-trypanosomal activity were observed irrespective of trypanosomes species used against methanolic extracts of different medicinal plants.

Also, ability of the extracts/purified compounds of medicinal plants to chelate trypanosomes DNA led to death of the trypanosomes had been documented (Sepúlveda-Boza, 1996).

## CONCLUSION

In this report, it could be concluded that MPE of *P. kurroa* possesses anti-trypanosomal compound(s), as previously documented, at distinct concentrations with maximum activity at the highest concentration. Also, Alsever's medium tested was suitable and supported trypanosomes for the period of incubation, and corresponding trypanocidal activity observed. MPE of *P. kurroa* rhizomes and diminazineaceturate had the same level of *in vitro* cytotoxic effects on Vero cells. This further allows flexibility in using alternate medium to carry out this type of bioassay. More research work is needed such as purification of MPE of *P. kurroa* rhizomes for possible isolation of trypanocidal compound(s), a key ingredient to new drug development against trypanosomes.

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