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Research Article

IN VITRO ANTITRYPANOSOMAL EVALUATION OF *PICRORHIZA KURROA* RHIZOMES

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ABSTRACT
In this study, *Picrorrhiza kurroa* rihzomes were cold extracted with methanolic solvent at concentrations (250-1000 µg ml-1). The methanolic plant extract (MPE) obtained was tested against *Trypanosoma evansi* for antitrypanosomal activity. This was carried out on Vero cells grown in Dulbecco's Modified Eagle Medium (DMEM) and supplemented with foetal calf serum (FCS) 20-40% at appropriate conditions. *In vitro* cytotoxicity test of *P. kurroa* rhizomes extract at concentrations (1.56-100 µg ml-1) was done on Vero cells but without FCS. *In vivo* infectivity test was done in mice. Results of *in vitro* antitrypanosomal activity varied from immobilization, reduction and to the killing of trypanosomes in corresponding ELISA plate wells. At 750 µg ml-1of MPE, there was marked reduction of average mean trypanosomes count in the corresponding ELISA plate wells as observed (39.00±0.0to 19.00±0.0). But at 1000 µg ml-1 of the test extract of *P. kurroa,* there was complete killing of trypanosomes at 8 h of incubation, which was comparable to diminazine aceturate (50 µg ml-1) at 4 h. Trypanosomes counts decreased in concentration and time –dependent manner with significant difference ($P<0.05$). MPE of *P. kurroa* and diminazine aceturate, standard drug, were cytotoxic to Vero cells except at concentrations of 1.56-6. 25 µg ml-1. Both MPE and diminazine aceturate had the same cytotoxic levels. Group of mice inoculated with contents of ELISA plate wells with apparently killed trypanosomes survived for more than 30 days. While, the other group of mice inoculated with contents of ELISA plate wells with reduced trypanosomes died of parasitaemia. Moderate antitrypanosmal activity of MPE of *P. kurroa was* observed.
KEY WORDS: *Picrorrhiza kurroa R*hizomes, Antitrypanosomal Activity, *In Vitro C*ytotoxicity Test, *In Vivo* Infectivity Test

INTRODUCTION

Trypansomosis is an important blood protozoan parasite disease. It is a zoonotic in nature. It is caused by different *Trypanosoma* species (e.g. *T. evansi, T. brucei rhodesiense*).[1,2]. Trypanosomosis play a major role as a hindrance to livestock production where the disease thrives in different parts of Africa.[2,3]

Reports of resistance to limited classes of available trypanocides on the fields and otherwise are on the increase in different parts of the world.[3,4]. Reports of the resurgence of the disease in recent years in the endemic regions, Africa and Latin America, where millions of population and cattle are affected with considerable morbidity and mortality are on the increase.[3,5,6,7]. Estimated losses in agricultural production as a result of the disease amounted to 3 billion pounds annually[7].

Chemotherapy and chemoprophylaxis are the only available means of combating the disease. But the chemotherapy of trypanosomosis is faced with problems such as limited choice of trypanocides in the market, high cost, toxicity, and emergence of drug-resistant trypanosome strains that have been reported[8-11]

Rhizomes of *Picrorhiza kurroa*, "an important herb in the Indian [medical] system of Ayurveda," has been used traditionally for asthma, bronchitis, malaria, chronic dysentery, viral hepatitis, upset stomach, scorpion sting, as a bitter tonic (stimulating the appetite and improving digestion), and as a liver protectant (hepato-protectant).[1213] Also, it has been used in the treatment of skin conditions, peptic ulcer and neuralgia, vitiligo, and rheumatic arthritis.[14.]

Ethno pharmacology and ethno medicine research revealed that several medicinal plants possess trypanocidal compounds, which may hold the key for future potential trypanocides.[3,15-19,3,31] More so, several semi-synthetic and synthetic drug derivatives were originally isolated from natural compounds.[21, 22]

Biological activity of *P. kurroa,* such as antibacterial and anti-inflammatory, has been documented.[14.]

Chemical components such as glucoside (simple sugar plus alcohol), a bitter principle called kutkin, a non-bitter compound called kurrin, and other components, including vanillic acid, kutkiol, and kutki-sterol have been isolated It was later discovered that kutkin is a mixed crystal of two glucosides -- glucoside-A and kutkoside., androsin, and apocynin..[23]

As a result of existing problems bedeviling limited classes of available trypanocides, *Picrorhiza kurroa* rhizomes were evaluated for antitrypanosomal activity.

MATERIALS AND METHODS

Chemicals

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

Plant materials

Picrorrhiza kurroai rhizomes at matured stages were collected in September, 2006 and identified at Institute of Himalayan Biosource and Technology, Palampur, India.

Preparation of extract

The extraction was carried out according to the method of Stahl.[24] 20 g of *P. 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 37oC and stored at 4oC until used.

Solvent systems

The following 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

Aliquot (0.2 ml) of extract 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 extract. This was also done following the method of Stahl.[24]

Animals

Swiss albino mice (20-30 g) of either sex were obtained from Animal Research Laboratory Section of Indian Veterinary Research Institute (IVRI) Izatnagar. The mice were 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 of committee on Ethics and Cruelty to Animals of the institute.

Test organism

T. evansi were obtained from the Division of Parasitology, Indian Veterinary Research Institute (IVRI), Izatnagar. Trypanosomes were maintained in the laboratory by serial sub-passages in Swiss albino mice. The strain was routinely tested for virulence following the method of Williamson.[25]

Trypanosomes count

Counting of trypanosomes was carried out following the method of Lumsden.[26] A number of fields (10-15) of each drop of blood or incubated media and trypanosomes 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 with modified method of Oliveira.[6] A Vero cell line (SIGMA) was grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20-40% foetal calf serum (FCS), GIBCO USA and antibiotics (100 iu penicillin, 100 µg streptomycin and 40 µg gentamycin) in 96-wells flat bottom microculture plates (NUNC, Denmark). Each well received 100 µl of DMEM containing 5x105 cells ml-1. Plates were incubated at 37oC under 5% CO2 for 12h. After the formation of confluent monolayer, the medium was discarded and replaced with a fresh one. Finally, a high parasitaemic blood from mouse was diluted with DMEM to obtain 1x106 parasites ml-1. Suspension (100 ml of medium with trypanosomes) was added at the rate of 1:1 to test MPE of *P. kurroa* and the plates were incubated under the same conditions mentioned above. The test was repeated at least thrice.

Stock of test MPE of *P. kurroa* rhizomes was solubilized in 1% dimethylsuphoxide (DMSO). The concentration in the experiment had no deleterious effect by itself on host cells or parasites. 1% DMSO in distilled water was used as control.[27].

***In vivo* infectivity assessment**

After incubation for antitrypanosomal activity was completed, contents of ELISA plates wells with reduced and apparently killed trypanosomes by MPE of *P. kurroa* rhizomes were inoculated (0.1ml mouse-1) into two groups of mice (six group-1) intra-peritoneal, and observed for more than 30 days for parasitaemia.[28]

***In vitro* cytotoxicity test**

It was done according to the method of Sidwell and Hoffman. [29] Vero cell line (SIGMA) was grown in DMEM in 96-wells microculture plates without FCS. Each well was seeded with 500,000 cells ml-1 and plates were incubated at 37oC with 5% CO2 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-1) of MPE of *P kurroa* rhizomes in triplicate and incubated for 72 h consecutively under the same conditions described previously. After 24 h interval, ELISA plates were 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. Plate was then incubated for 24 h at 37oC in ordinary incubator. Plates were later observed under inverted microscope for cytotoxic effects.

STATISTICAL ANALYSIS

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

RESULTS

Extraction

During the extraction process of *P. kurroa*, methanolic solvent was suitable in extraction of bioactive constituents as observed on TLC plates (plates not shown). Presence of bioactive constituents from MPE of *P. kurroa* rhizomes was detected on TLC plates.

Thin layer chromatography plates analysis

In the analysis of thin layer chromatography (TLC), combinations of solvent systems were tested. Solvent system, methanol/chloroform (20:80), was more suitable than other solvent systems tested in the analysis of thin layer chromatography (TLC) plates with applied aliquots of plant extract. TLC plates (plates not shown) showed different patterns of bioactive constituents of *P. kurroa* that were subsequently responsible for antitrypanosomal activity.

***In vitro t*rypanocidal activity**

Results of *in vitro* antitrypanosomal activity of *P. kurroa* rhizomes are presented in Table 1. Antitrypanosomal activity varied from immobilization, reduction and to the killing of trypanosomes at different concentrations used. At concentration of 250 µg ml-1 of MPE (*P. kurroa*), there was reduction of trypanosomes (40.00±0.0 to 8.33±0.33). But at 750 µg ml-1 of MPE (*P. kurroa*) trypanosomes were not detectable in the corresponding ELISA plate wells, which is statistically comparable to 4 h of diminazine aceturate (Berenil, a standard drug at 50 µg ml-1).

***In vivo* infectivity test**

Group of mice inoculated with contents of ELISA plate wells (medium, MPE of *P. kurroa* and completely killed trypanosomes) after completion of i*n vitro* antitrypanosomal test survived for more than 30 days. While, the other group of mice inoculated with contents of ELISA plate wells (medium, MPE of *P. kurroa* and immobilized trypanosomes) died of parasitaemia.

***In vitro* cytotoxicity test**

In vitro cytotoxic effects of MPE of *P. kurroa* rhizomes and diminazine aceturate at the same concentrations on Vero cells depicted different effects such as distortion, swelling, sloughing and death of Vero cells compared to negative normal cells in control wells (Table 2). MPE of *P. kurroa* and diminazine aceturate were cytotoxic to Vero cells at all concentrations except at 1.56-6.25 µg ml-1, respectively. Both the test extract and dimiazine aceturate had the same levels of cytotoxic effects.

DISCUSSION

In this current report, methanollic solvent used in the extraction of *P. kurroa* rhizomes and the obtained MPE that was applied on TLC is comparable to extraction of MPES of

Camellia sinensis leaves and *Piper nigrum* buds (fruits) in which similar solvent was used.[18,15]

The method used in the TLC analysis of *P. kurroa* rhizomes in a suitable solvent system is comparable to that used by Freiburghaus *et al* in bioassay-guided isolation of a diasterolisomer of kolavenol from *Entada Abyssinia* active on *T. brucei. rhodesiense* and (Shaba *et al.,* 2012b) in TLC analysis of *Zanthoxylum alatum* leaves and *Eugenia caryophyllatum* buds (fruits) that depicted the presence of bioactive constituents, and detected by vanillin-sulfuric spray and iodine vapour in different chambers[11,6]

Antitrypanosomal activity of *P. kurroa* rhizomes is comparable to *in vitro* trypanocidal activity of MPES of medicinal plants used in the treatment of trypanosomosis in northern Nigeria at an effective concentration of 8.3 mg ml-1, *in vitro* trypanocidal activity of methanolic extracts of *Khaya senegalensis* root bark with complete killing of trypanosomes at 250 µg /ml and therapeutic effects of *Zanthoxylum alatum* leaves and *Eugenia caryophyllatum* buds (fruits) against trypanosomes where trypanosomes were not detected in the corresponding ELISA plate wells at 750 and 1000 µg /ml of the test extracts at 8 and 9 h of incubation.[2,16,18] An average mean trypanosomes count of 37.67±0.58 is statistically critical value. Average mean trypanosomes count from 37.67±0.58 and below was significant between the treatment groups and negative control ($p \leq 0.05$). Kurrin, vanillic acid, kutkiol, and kutki-sterol identified from *P. kurroa* may be responsible for the antitrypanosomal activity observed.

Validation of *in vitro* trypanocidal activity via *in vivo* infectivity assessment of antitrypanosomal activity is comparable to antitrypanosomal effects of the aqueous extract of *Brassica oleracea* buds (fruits), MPES of *Ageratum houstonionum* flowers and *Terminalia chebula* dried fruits where inoculated mice with contents of ELISA plate wells with apparently killed trypanosomes survived.[31, 33]

Result of *in vitro* cytotoxicity of MPE of *P. kurroa* rhizomes is comparable to *in vitro* cytotoxicity tests of extraction of *Ageratum houstonianum* flowers and methanolic extract of *Khaya senegalensis* root bark in which similar cytotoxic effects were observed.[30-32]

CONCLUSION

In conclusion, MPE of *P. kurroa* rhizomes at different concentrations demonstrated moderate degree of antitrypanosomal activity. *In vitro* cytotoxicity effects of MPE of *P. kurroa* and diminazine aceturate were the same levels. It seems further purification of *P. kurroa* rhizomes will make it safer to use than diminazine aceturate if its antitrypanosomal activity is fully studied. Further research such as bioassay-guided isolation of active constituents and i*n vivo* test in mice are required to fully understand its trypanocidal potential.

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Table 1. *In vitro* trypanocidal activity of methanlic extract of *Picrorhiza. Kurroa* against *Trypanosma evansi* on Vero cell line

Concentration of plant extract in µg ml-1	1 h	2 h	3 h	4 h	5 h	6 h	7 h	8 h	9 h
250	39.00±0.0	38.00±0.33	36.67±0.33	35.33±0.58	33.00±0.58	29.00±0.58	25.00±0.33	21.33±0.58	19.00±0.0
500	38.33±0.33	36.67±0.33	34.33±0.33	31.67±0.33	29.00±0.58	24.00±0.67	20.33±0.33	17.33±0.33	15.67±0.33
750	31.00±0.58	27.00±0.58	21.67±0.33	15.00±0.58	10.67±0.33	5.000±0.58	0.33±0.33	0.0±0.0	0.0±0.0
1000	33.00±0.58	26.33±0.67	20.00±0.58	12.67±0.67	5.667±0.67	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Diminazine aceturate (50) Positive control	22.33±0.33	9.333±0.67	1.000±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	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	40.00±0.0	40.00±0.0	40.00±0.0	40.00±0.0

Bioassay status: there was significant reduction of trypanosomes counts from concentration of 250 µg ml-1 and complete killing of trypanosomes at 750 µg ml-1at 8th h of incubation as observed. Average mean trypanosomes counts of 37.67± 0.58 are statistically critical value. Average mean from 37.67± 0.58 and below is significant between the treatment groups and negative control. ($P \leq 0.05$ to 0.01).

Table 2. Cytotoxic effect of methanolic extract of *Picrorhiza korroa* rhizomes on Vero cell line compared to diminazine aceturate (Berenil)

Concentration of test material in µg ml-1	Effects of test extract at various periods of incubation (24 h, 48 h, 72 h)						
	Picrorhiza korrea	Berenil	*Picrorhiza korrea*	Bernil	*Picrorhiza korrea*	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

Picrorhiza korrea and diminazine aceturate were toxic to Vero cell line in all concentrations used except at of 1.56-6.25 µg ml-1

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