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Original Article

Antitrypanosomal Effects of Brassica Oleracea (Cabbage) Fruits and Leaves

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Abstract

In our continuation of search for antitrypanosomal agents from medicinal plants and other sources, *Brassica oleracea* (cabbage) fruits and leaves were screened for their antitrypanosomal and cytotoxicity effects .Methanolic plant extracts (MPES) of *B. Oleracea* fruits and leaves at different concentrations (250-1000 µg/ml) were tested against *Trypanosoma evansi* on Vero cell line grown in Dulbecco's Modified Eagle Medium (DMEM) with foetal calf serum (FCS) (20-40%) at appropriate conditions. *In vivo* infectivity test of incubated MPES of *B. Oleracea* fruits, leaves and medium with trypanosomes were done in mice. *In vitro* cytotoxicity of the test extracts at concentrations (1.56-100 µg/ml) were performed on Vero cells but without FCS. Both MPES of *B. oleracea* fruits and leaves demonstrated trypanocidal activity, which ranged from immobilization, reduction and to the killing of trypanosomes. At 500 µg/ml of MPES of *B. oleracea* fruits and leaves *wi*th trypanosomes undergoing incubation, there were marked reductions of trypanosomes (19.33±0.33) (15.33±0.33) in the corresponding ELISA plate wells. But at 1000 µg/ml of MPE of *B. oleracea* fruits, there was no complete killing of the trypanosomes (5.33±0.33) as to that of MPE of leaves where trypanosomes could not be detected at 7 h of incubation, which was statistical comparable to the standard drug, diminazine aceturate at concentration of 50 µg/ml at 4 h of incubation. Trypanosomes counts decreased in concentration and time – dependent manner with significant difference ($P<0.05$). In *in vivo* infectivity test, 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 in the incubated medium died of parasitaemia. MPES of *B. oleracea* fruits, leaves and diminazine aceturate (Berenil) were cytotoxic to Vero cells at all concentrations except at 1.56, 1.56 and 6.25-1.56 µg/ml, respectively.

Keywords: *Brassica oleracea (*cabbage) fruits and leaves, antitrypanosomal effects, *in vivo* infectivity test, *in vitro* cytotoxicity test

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Introduction

Trypanosoma evansi is one of the species of the genus *Trypanosoma* that causes trypanosomosis in animals (Soulsby, 1982; Seed, 2001).Trypanosomosis being a zoonotic disease in nature has great impacts on both animals and humans especially where the disease thrives (WHO, 2004).

Trypanosomosis act as a hindrance to livestock production in endemic areas of Africa where the disease is rampart. (Freiburghaus *et al*., 1998; WHO, 2002)

Chemoprophylaxis and chemotherapy are the two major means of tackling the menace of the disease. But these means are faced with lots of challenges such as high cost of the available trypanocides, limited classes of trypanocides and emerged resistant strains of trypanosomes .Also, reports of resistance to the available trypanocides are on the increase (WHO, 2004; Dou and Yapo, 2001; Gutteridge, 1985).

Natural products are valuable sources for new drug formulation. Important classes of antimalarial drugs such as quinoline and endoperoxide atermisinin deravatives were originally identified from traditional medicine (El-Sayed *et al.,* 2001).

Traditional uses of *B. oleracea* fruits and leaves include condiment and consumed as vegetables (de Pascual-Teresa *et al.,* 2010).

Biological activity such as anti-inflammatory, enzyme inhibition, and antioxidant, ant allergic and vascular and cytotoxic antitumor activity has been reported (Chu *et al.,* 2000; Podsedek *et al.,* 2006).

Isolated compound such as tannins, antthocynins, hydroxycinnamic acids, flavonoids – flavones, flavan-3-ols, isoflavones have been reported (de Pascual-Teresa *et al.,* 2010; Cushnie *et al.,* 2005).

Prospects of introducing new trypanocidal drugs into the market are also not encouraging. Therefore, there is an urgent need for development of newer anti-trypanosomal drug. On this note, *Brassica oleracea* (cabbage) fruits and leaves were screened for their trypanocidal activity.

Material 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 material

Brassica oleracea (Brassicaceae) fruits and leaves at matured stage were collected in September, 2006 and identified at Institute of Himalayan Biosource and Technology, Palampur, India.

Preparation of extracts

The extraction was carried out according to the method of Stahl, (1969). 20 g each of *B. oleracea* fruits and leaves 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

Aliquots (0.2 ml) of extract were 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, (1969)

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 *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 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 *et al.,* (2004). 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 MPES of *B. oleracea* fruits, leaves and the plates were incubated under the same conditions mentioned above. The test was repeated at least thrice.

Stock of test MPES of *B. oleracea* fruits and leaves were 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 (Young *et al.,* 2000).

***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 *B. oleracea* leaves were inoculated (0.1ml mouse-1) into two groups of mice (six group-1) intra-peritoneally, and observed for more than 30 days for parasitaemia (Woo, 1970)

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 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 MPES of *B oleracea* fruits and leaves 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 were discarded. Adhered cells were stained with a drop of crystal violet in phosphate buffered solution. Plates were 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 and Discussion

Extraction

During the extraction processes of *B. oleracea* fruits and leaves, methanolic solvent was suitable in

extraction of bioactive constituents as observed on TLC plates (plates not shown). Presence of bioactive constituents from MPES of *B. oleracea* fruits and leaves were 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 extracts of *B. oleracea* fruits and leaves. TLC plates (plates not shown) showed different patterns of bioactive constituents of *B. oleracea* of fruits and leaves that were subsequently responsible for antitrypanosomal activity.

In vitro* trypanocidal *activity

Result of *in vitro* antitrypanosomal activity of *B. oleracea* fruits and leaves are presented in Tables 1 and 2. Antitrypanosomal activity varied from immobilization, reduction and to the killing of trypanosomes at different concentrations used. At concentration of 500 µg ml-1 of MPES *B. oleracea* fruits and leaves, there were marked reductions of trypanosomes (19.33±0.33) (15.33±0.33). But at 1000 µg ml-1 of MPE of *B. oleracea* leaves, live trypanosomes were not detectable in the corresponding ELISA plate wells, which is statistically comparable to diminazine aceturate (Berenil, a standard drug at 50 µg ml-1) after 4 h of incubation. There was no complete killing of trypanosomes of *B. oleracea* fruits in the corresponding ELISA plate wells at all concentrations used.

In vivo infectivity test

Group of mice inoculated with contents of ELISA plate wells (medium, MPE of *B. oleracea* leaves and killed trypanosomes) after completion of *in 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 *B. oleracea* and immobilized trypanosomes) died of parasitaemia.

In vitro cytotoxicity test

In vitro cytotoxic effects of MPES of *B oleracea* fruits, leaves 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 normal cells in the negative control wells (Tables 3 and 4). MPES of *B. oleracea* fruits leaves and diminazine aceturate were cytotoxic to Vero cells at all concentrations except at 1.56, 1.56 and 1.56-6.25 µg ml-1, respectively.

In the process of extraction, methanol solvent used in the extraction of *B. oleracea* fruits leaves and the obtained MPES that were applied on TLC plates were comparable to extraction of MPES of *Terminalia belirica* dried fruits, (Shaba *et al.,* 2009) *Plumbago zeylanica* (Shaba *et al.,* 2006) and *Piper nigrum* buds (fruits) (Shaba *et al.,* 2012a) in which similar solvent was used.

MPES of *B. oleracea* fruits and leaves were applied on TLC plates and the plates were subjected to TLC analysis of most suitable solvent system. This TLC analysis is comparable to that used by Freiburghaus *et al.,* (1998) in the 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.

Antitrypanosomal activity of *B oleracea* fruits and leaves are 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 extract 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 h and 9 h of incubation (Shaba *et al.,* 2012). An average mean trypanosomes count of 37.67±0.58 is statistically critical value.

Table 1: *In vitro* trypanocidal activity of methanlic extract of *Brassica oleracea* (Cabbage) fruits against *Trypanosma evansi on* Vero cell line

Concentration of plant extract in µg/ml	1 h	2 h	3 h	4 h	5 h	6 h	7 h	8 h	9 h
250	38.33± 0.33	38.00± 0.0	36.67± 0.33	36.67± 0.33	36.00± 0.0	34.00± 0.0	32.33± 0.33	30.33± 0.33	30.00± 0.58
500	38.67± 0.33	37.33± 0.33	36.00± 0.0	34.00± 0.0	32.33± 0.33	30.33± 0.33	26.00± 0.0	21.67± 0.33	19.33± 0.33
750	37.33± 0.33	36.00± 0.0	33.33± 0.33	30.00± 0.0	26.67± 0.33	25.00± 0.0	22.33± 0.33	17.67± 0.33	14.67± .67
1000	36.33± 0.33	31.33± 0.33	27.33± 0.33	23.33± 0.33	20.67± 0.33	17.33± 0.67	13.00± 0.58	10.00± 0.58	5.33±0 .33
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: significant reduction of trypanosomes counts from concentration of 500 µg /ml but not complete killing of parasites at hours of observation. An average mean parasites count of 37.67± 0.58 is statistically critical value. Average mean parasites counts 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: *In vitro* trypanocidal activity of methanlic extract of *Brassica oleracea* (Cabbage) leaves against *Trypanosma evansi on* Vero cell line

Concentration of plant extract in µg/ml	1 h	2 h	3 h	4 h	5 h	6 h	7 h	8 h	9 h
250	38.33 ±0.33	38.00 ±0.0	36.67 ±0.33	36.67 ±0.33	36.00 ±0.0	34.00 ±0.0	32.33 ±0.33	30.33 ±0.33	30.00±0. 58
500	37.67 ±0.33	36.33 ±0.33	35.00 ±0.0	34.00 ±0.0	30.33 ±0.33	26.33 ±0.33	22.00 ±0.0	18.67 ±0.33	15.33±0. 33
750	36.33 ±0.33	33.00 ±0.0	30.33 ±0.33	26.00 ±0.0	23.67 ±0.33	19.00 ±0.0	16.33 ±0.33	13.67 ±0.33	10.67±.6 7
1000	34.33 ±0.33	30.33 ±0.33	25.33 ±0.33	17.33 ±0.33	12.67 ±0.33	6.33± 0.67	0.0.0 0±0.0	0.00± 0.0	0.00±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: significant reduction of trypanosomes counts from concentration of 500 µg /ml and complete killing of trypanosomes at 7 h of incubation. An average mean parasites count of 37.67± 0.58 is statistically critical value. Average mean parasites counts from 37.67± 0.58 and below is significant between the treatment groups and negative control. ($P \leq 0.05$ to 0.01).

Table 3: Cytotoxic effects of methanolic extract of *Brassica oleracea* (cabbage) fruits on Vero cell line compared to diminazine aceturate (Berenil).

Concentration of test material in µg/ml	Cytotoxic effects of extract and DA at various time intervals of incubation						
	24 h		48 h		72 h		Control
	B. oleracea	DA	*B.oleracea. Ocinalis*	DA	*B. oleracea*	DA	
100	100%	66.6%	100%	100%	100%	100%	0
50	100%	33.3%	100%	100%	100%	100%	0
25	33.3%	0	100%	33.3	100%	66.6	0
12.5	33.3%	0	66.6%	0	100%	33.3%	0
6.25	0	0	33.3%	0	66.6%	0	0
3.13	0	0	0	0	33.3%	0	0
1.56	0	0	0	0	0	0	0

The same concentrations were used for both MPE of *Brassica oleracea (*cabbage) fruits and DA.
Cytotoxicity effects increased with period of incubation depending on the concentrations of the extract and DA.
DA (Diminazine aceturate)

Table 4: Cytotoxic effects of methanolic extract of *Brassica oleracea* (cabbage) leaves on Vero cell line compared to diminazine aceturate (Berenil).

Concentration of test material in µg/ml	Cytotoxic effects of extract and DA at various time intervals of incubation						
	24 h		48 h		72 h		Control
	B. oleracea	DA	*B.oleracea. Ocinalis*	DA	*B. oleracea*	DA	
100	100%	66.6%	100%	100%	100%	100%	0
50	100%	33.3%	100%	100%	100%	100%	0
25	33.3%	0	100%	33.3	100%	66.6	0
12.5	33.3%	0	66.6%	0	100%	33.3%	0
6.25	0	0	33.3%	0	66.6%	0	0
3.13	0	0	0	0	33.3%	0	0
1.56	0	0	0	0	0	0	0

The same concentrations were used for both MPE of *Brassica oleracea* (cabbage) leaves and DA.
Cytotoxicity effects increased with period of incubation depending on the concentrations of the extract and DA.
DA (Diminazine aceturate)

Average mean trypanosomes count from 37.67±0.58 and below was significant between the treatment groups and negative control ($p \leq 0.05$). Tannins, antthocynins, hydroxycinnamic acids, flavonoids –flavones, flavan-3-ols, isoflavones isolated from *B. oleracea may* be responsible for the antitrypanosomal activity observed. But in this investigation, the antitrypanosomal activity of MPE of *B. oleracea* fruits was lesser in activity as earlier reported by Igweh *et al.*, (2002). This may be due to differences in genetic code of the trypanosome specie and its susceptibility to the fruits extract of *B. oleracea*. This is in addition to differences in soil profiles of different continents and contents of compound(s) responsible for antitrypanosomal activity. Even though this is the preliminary study of *B. oleracea* fruits and leaves antitrypanosomal effects against *T. evansi*, anthocynins and flavonoids have been reported and isolated as antiparasitic and anttrypanosomal. The MPE of the leaf extract of *B. oleracea* exhibited more antitrypanosomal effects than the fruits extract. But the levels of *in vitro* cytotoxicity test effects of MPES of *B. oleracea* fruits and leaves were the same.

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 belirica* dried fruits where inoculated mice with contents of ELISA plate wells with apparently killed trypanosomes survived (lgweh *et al.*, 2002; Shaba *et al.*, 2011; Shaba *et al.*, 2009).

Conclusion

In conclusion, MPES of *B. oleracea* fruits and leaves at different concentrations exhibited moderate degree of antitrypanosomal activity. But in this investigation, the antitrypanosomal activity of MPE of *B. oleracea* fruits was lesser in activity as earlier reported.

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