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Trypanocidal Activity of Purified Precocene I by Reverse-Phase High-Performance Liquid Chromatography from Essential Oil of *Ageratum houstonianum* aerial Parts

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Abstract

Freshly harvested leaves sample of *Ageratum houstonionum* were dried under shade and powdered. Leaf sample of *A. houstonionum* was extracted by process of hydrodistillation using a Clevenger-type apparatus for the preparation of essential oil. Extract from *A. houstonianum* was prepared by dissolving 5 µL of the essential oil in 10 mL methanol. All the sample was filtered through a Whatman (Maidstone, England) stainless steel syringe assembly using a 0.22 µm Durapore (Millipore: Milford, USA) membrane filter. Purification processes via column chromatography, thin layer chromatography and preparative thin layer chromatography were done. Reverse phase HPLC analysis was carried out via a Waters HPLC system consisting of model 510 and 515 pumps, a Rheodyne injector, a Novapak C_{18} column (250 × 4.6 mm i.d.; 4 µm), a model 490 E multi-channel detector and Millennium 2010 sata manager. The mobile phase constituents were filtered using a Durapore 0.22 µm membrane filter. The elution was carried out with a linear gradient of acetonitrile: water (40:60) to pure acetonitrile in 60 min at a flow rate of 1 mL/min. detection was at 210, 240, 280 and 320 nm. The precocene was eluted within 25min, the peak areas showed good reproducibility (average relative standard deviation were 0.78%), and the calibration curves (i.e., mass of precocene standard injected vs. peak area detected at 210 nm) were linear over the range of 0.05-10 µg (for precocene I, y= 6654454 $\times$ + 176626, r^2=0.99 and for precocene II, y=4618457 $\times$ + 133472, r^2 =0.99). Standard sample containing precocene I (1 mg/mL) and precoceneII (1 mg/mL) obtained from Sigma (St Louis, MO, USA) were prepared in methanol. Identified precocene I was screened against *Trypanosoma evansi* for trypanocidal activity 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 precocene I at concentrations (1.56-100 µg ml^{-1}) was done on Vero cells but without FCS. *In vitro* trypanocidal activity varied from immobilization, reduction and to the killing of trypanosomes in corresponding ELISA plate wells. At 250 µg ml^{-1} of purified precocene I, there was drastic reduction of average mean trypanosomes count to complete killing of trypanosomes (40.00 ± 0.0 to 0.00 ± 0.00) at 9h of incubation, which was statistically the same as diminazine ceturate (50 µg ml^{-1}) at 4 h. Trypanosomes counts decreased in concentration and time–dependent manner with significant difference (P ≤ 0.05 to 0.01). During *in vitro* cytotoxicity test, Purified precocene I and diminazine aceturate standard drug, were cytotoxic to Vero cells at all concentrations except at concentrations of 6.25-1.56 µg ml^{-1} and 1.56 µg ml^{-1} respectively. Precocene I was responsible for higher anti-Trypanosoma l activity. Precocene I could be near future trypanocidal compound for a new trypanocide.

Keywords: *Ageratum houstonionum* leaves; Reverse phase HPLC purified precocene I; *In-vitro* trypanocidal activity; *In-vitro* cytotoxicity test

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Introduction

Trypansomosis, is a blood protozoan parasite disease of zoonotic importance, affect both animals and humans, which poses a huge hindrance to livestock production in some parts of Africa where the disease thrives at distinct levels of occurrence [1-3]. Reports of its resistance to available classes of trypanocides have been documented [4-7]. Trypanosomosis being a neglected disease has resurged in certain parts of Africa and Latin America, where millions of population and cattle are affected with considerable morbidity and mortality [8-10]. Different species of trypanosomes (e.g. *Trypanosoma evansi* in animals as to *Trypanosoma brucei rhodesiense* in humans in Africa) have been incriminated in the cause of trypanosomosis [3,9,10]. Estimated losses in agricultural production due to trypanosomosis are 3 billion pounds annually [11].

There are documents of herbal preparations being used in the treatment of many diseases with trypanosomosis inclusive in both animals and humans [3]. The only available mean of combating this menace is via effective and efficient usage of trypanocides as chemotherapy and chemoprophylaxis. But the chemotherapy of trypanosomosis is confronted 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 [3,9,10,12].

Ethno pharmacology and ethno medicine showed that several medicinal plants possess trypanocidal compounds, which may hold lead to discovery of a future potential trypanocide(s) [4,6,7,13-18]. More so, several semi-synthetic and synthetic drug derivatives were originally isolated from natural compounds [19].

Previously, we reported the anti-trypanosmal activity of *Ageratum houstonianum* flowers (Asteraceace) also known as *Ageratum mexicanum* (blue big Billy goat weed) with interesting findings [14]. *Ageratum houstonianum* leaves are a common poisonous weeds found on the vast valley of Kangra in Palampur, Himachal Pradesh State.

Previously, precocene I and II were identified and isolated as the bioactive chromenes in *Ageratum spp*. [20,21]. Both of these chromenes have insecticidal activity [20,22]. In addition to this, precocenes act as anti-juvenile hormones and cause genotoxicity [23,24].

Both, precocene I and II may be extracted by hydro distillation into an essential oil fraction [25-29]. Most of the studies reported so far have been carried out using GC systems which are limited in application to volatile compounds or to components which have been previously derivatised. HPLC analysis, however, permits the possible separation and quantification of natural products with a wide range of polarity. In search of new trypanocidal compound(s), aerial part of *A. houstonianum* was screened for its anti-trypanosmal activity.

Materials and Methods

Chemicals

The chemicals and solvents used in HPLC analysis were of HPLC grade. They were purchased from Qualigens Fine Chemicals (Mumbai, India). Precocenes I and II were obtained from Sigma (St. Louis, MO, USA).

Plant materials

The aerial parts of *A. houstonianum* were collected from Palampur in the Kangra Valley (Himachal Pradesh State, India). The plant material was identified by Institute of Himalaya Biosource Technology, Palampur, Himachal Pradesh, India.

Extraction

The samples were extracted by hydro-distillation using a Clevenger-type apparatus for the preparation of essential oil. Standard samples containing precocene I (1 mg/mL) and precocene II (1 mg/mL) were prepared in methanol. Extract from *A. houstonianum* was prepared by dissolving 5 µL of the essential oil in 10 mL methanol. The sample was filtered through a Whatman (Maidstone, England) stainless steel syringe assembly using a 0.22 µm Durapore (Millipore: Milford, USA) membrane filter.

Thin Layer Chromatography (TLC) plates

Aliquots (0.2 ml) of methanolic plant extract (MPE) and purified precocene I were applied on TLC plates, which were dried under room temperature and immersed inside the solvent systems in glass jar listed in the next subsection. This was done to detect, if any, the presence of bioactive constituents in applied MPE. The same was applied to fractions from column chromatographic purified precocene from reverse phase HPLC. After full development of plates in solvent systems, plates were dried at room temperature. Then, one set of TLC plates were immersed in iodine vapours in a glass jar. Second set of TLC plates were sprayed with Vanillin-sulphuric acid spray. Both media used facilitated the detection of bioactive constituents. This was carried out according to the method of Stahl [30].

Solvent systems applied

The following solvent systems were tested to develop the TLC plates to obtain a more suitable system according to the method of Stahl [30].

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

Column Chromatography (CC)

The extracted essential oil of *A. houstonianum* leaves underwent the process of column chromatograph as a first stage of purification with appropriate solvents ratios. This was carried out as per the methods of Stahl [30]; Sharma and Sharma [21].

Preparative Thin Layer Chromatography (PTLC)

Aliquots of fractions of *A. houstonianum* from column chromatography were applied on PTLC for identification of its purity state. This was done as per the methods of Stahl [30]; Sharma and Sharma [21].

Reverse Phase Higher Performance Liquid Chromatography (RHPLC) Analysis of HPLC purified precocene I from *A. houstonianum* leaves

High performance liquid chromatography (HPLC) analysis was done in reverse phase according to Sharma and Sharma [21]. HPLC (Waters) analysis by injecting 20 µl of HPLC purified precocenes dissolved in HPLC graded methanol via 18 columns. Gradient of methanol: water (40:60) to methanol (100%) for 30 min was used. (250 × 4.6 mm i.d; 4 µm), a model 490E multi-channel detector and Millennium 2010 sata manager. The mobile phase constituents were filtered using a Durapore 0.22 µm membrane filter. The elution was carried out with a linear gradient of acetonitrile: water (40:60) to pure acetonitrile in 60 min at a flow rate of 1 mL/min. detection was at 210, 240, 280 and 320 nm. The precocenes were eluted within 25 min, the peak areas showed good reproducibility (average relative standard deviation were 0.78%), and the calibration curves (i.e., mass of precocene standard injected vs. peak area detected at 210 nm) were linear over the range of 0.05-10 µg (for precocene I, y = 6654454 × + 176626, r^2=0.99; for precocene II, y=4618457 × + 133472, r^2=0.99).

Test organisms

Trypanosoma evansi was obtained from the Division of Parasitology, Indian Veterinary Research Institute (IVRI), Izzatnagar and was maintained in the laboratory by serial sub-passage in Swiss albino mice. The strain was routinely tested for virulence following the method Williamson et al. [31].

In vitro tryponocidal activity

In vitro trypanocidal activity was carried out on modified method of Oliveira et al. [8]. In this method, a Vero cell line was grown in Dulbecco's Modified Eagle Medium (DMEM) (Sigma) in 96-well flat bottom micro culture plates (Nunc, Denmark). Each well received 100 µ of DMEM containing 5 × 10^5 cells/ml. The plates were incubated at 37°C under 5% CO_2 for 48 h to complete development of monolayer. After the formation of confluent monolayer, the medium (DMEM) was discarded and replaced with a fresh DMEM. And the medium was supplemented with 20-40% fetal calf serum (FCS), Gibco USA and antibiotics (100 units penicillin, 100 µg streptomycin and 40 µg gentamycin). A high parasitemic blood from mouse was diluted with DMEM to obtain a final parasite of 1x106 parasites/ml. The suspension (100 ml of medium with trypanosomes was added at rate of 1:1 to MPE and precocene I of *A. houstonianum* leaves at concentrations (250-1000 µg/ml). The suspension (100 ml of medium with trypanosomes) was added at rate of 1:1 to test sample and the plates were incubated at 37°C under 5% CO_2. The mixture was incubated for 9h. The test was repeated at least thrice and the plate was incubated under the same conditions mentioned above.

Stock of test MPE was solubilized in 1% dimethylsuphoxide (DMSO).

The concentration of DMSO in the experiment had no deleterious effect by itself on host cells or parasites.1% DMSO in distilled water was used as control [32].

In vivo infectivity assessment

In vivo infectivity of reverse phase HPLC purified precocene I was carried out after successful completion of anti-Trypanosoma l activity. Contents of microculture plate wells that contained reduced and apparently killed trypanosomes with HPLC purified precocene I were inoculated (0.1 ml mouse^{-1}) into two groups of mice (six group^{-1}) via intra-peritoneal, and observed for more than 60 days for parasitaemia [33,34].

In vitro cytotoxicity test

Cytotoxic effects of the reverse phase HPLC purified precocene I was determined according to the method described by Sidwell and Hoffman [35]. Vero cell line was grown in Dulbecco's Modified Eagle Medium (DMEM) (Sigma) Gibco, USA antibiotics (100 units penicillin, 100 µg streptomycin and 40 µg gentamycin) in 96-well flat bottom microculture plates (Nunc, Denmark). Each well received 100 µl of DMEM containing 5 × 10^5 cells/ml. The plates were incubated at 37°C under 5% CO_2 for 48 h. After the formation of confluent monolayer, the medium was discarded and replaced with a fresh one. A high parasitaemic blood from mouse was diluted with DMEM to obtain a final parasite of 1 × 10^6 parasites/m. Confluent monolayer of Vero cell was treated with serial dilutions of reverse phase HPLC purified precocene I of *A. houstonianum* (1.56-100 µg/ml) in triplicate and incubated under the same conditions described previously. After 24 h of incubation, the culture plate was observed for evidence of cytotoxic effects. The plate was incubated for 72 h and observed daily. It was repeated thrice. In each case, after the 72 h of incubation, the culture media of the incubated Vero cells were discarded. The adhered cells were stained with a drop of crystal violet in phosphate buffered solution. The plate was incubated for 24 hours at 37°C in an ordinary incubator. After 24 h of incubation, the culture plate was observed for evidence of cytotoxic effects.

Institute Committee on Welfare and Cruelty to Animals

Indian Veterinary Research Institute Committee on Welfare and Cruelty to Animals received and approved application for the usage of mice in this research.

Statistical Analysis

Results of trypanocidal activity were expressed as mean ± SEM. Statistical significance was determined by Sigma Stat (Jandel), USA.

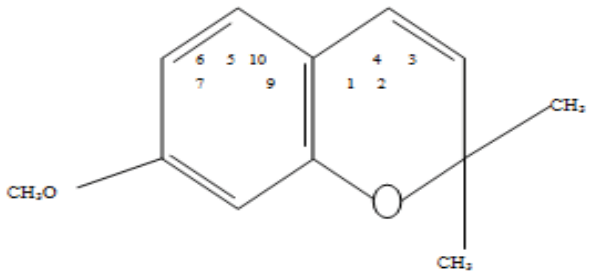
Results

As shown in the **Table 1,** as a result of purification of *A. houstonianum* leaves, quantification of precocenes I and II in the essential oil of *A. houstonianum* was quantified accordingly.

Figure 1 showed the elucidated structure of a precocene I. In the **Table 2,** *in vitro* trypanocidal activity of purified precocene

Table 1 Quantification of precocenes in the essentially oil of *Ageratum houstonianum.*

Amount (µmol/mL essential oil)	Amount (µmol/kg fresh weight of plant sample)
Ageratum houstonianum	*Ageratum houstonianum*
Precocene I 1421 ± 98	Precocene I 1706 ± 116
Precocene II 2133 ± 98	Precocene II 1421 ± 98



Precocene I

Figure 1 An elucidated structure of Precocene I from *A. houstonianum* leaves.

Table 2 *In vitro* trypanocidal activity of precocene I against *Trypanosmae vansi* on V*ero* cell lines.

Concentration of plant extract in µg/ml	1 h	2 h	3 h	4 h	5h	6 h	7 h	8 h	9 h
250	35.33 ± 0.33	31.00 ± 0.0	26.67 ± 0.33	22.00 ± 0.58	17.33 ± 0.33	12.67 ± 0.33	8.667 ± 0.33	4.333 ± 0.33	0.0 ± 0.0
500	33.33 ± 0.67	30.00 ± 0.0	24.33 ± 0.33	19.67 ± 0.33	13.67 ± 0.33	8.667 ± 0.33	2.333 ± 0.33	0.0 ± 0.0	0.0 ± 0.0
750	25.67 ± 0.33	27.00 ± 0.58	23.33 ± 0.33	17.00 ± 0.58	11.33 ± 0.33	4.667 ± 0.33	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
1000	30.33 ± 0.67	25.67 ± 0.67	19.67 ± 0.33	12.67 ± 0.33	5.667 ± 0.33	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Berenil (50)	22.00 ± 0.0	9.333 ± 0.33	1.333 ± 0.33	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

at varied hours. At 250 µg/ml, there was drastic reduction of the trypanosomes count, and none was detected at 9thof incubation, which was statically equivalent to diminazine (50 µg/ml) the standard drug at4th of incubation. An average mean trypanosomes count of 37.67 ± 0.58 is statistically critical value. Average mean trypanosomes counts from 37.67 ± 0.58 and below is significant between the treatment groups and negative control ($P \leq 0.05$ to 0.01).

In vitro cytotoxicity test was as contained in the **Table 3.** Precocene I and dimainazineaceturate were toxic to Vero cell line except at concentrations of 1.56 and 6.25-1.56 µg/ml. The same concentrations were used for both precocenel and diminazine aceturate (Berenil)

Figure 2 showed the reverse phase HPLC purified precocene I and II as indicated by different picks detected at wavelength (210 nm).

Discussion

During the tedious and cumbersome processes of purification of *A. houstonianum,* a lot of solvents and distinct solvent combinations were used to arrive at it final stage using HPLC to detect the purity status of the precocenes. The HPLC protocol reported here provides a facile method for the separation and quantification of these compounds and will, therefore, be useful in monitoring the precocenes as biocides and for studies concerning their further metabolism in animal systems

In the process of purification as shown above, **Figure 2** shows the HPLC separation of precocenes I and II in the essential oil of *A. houstonianum*. The acetonitrile: water gradient allowed baseline separation with sharp peak and narrow retention windows. Large differences in the absorbance of precocenes at different detection wavelengths were observed (data not shown), and detection at 210 nm provided sharper peaks and higher sensitivities than previously reported by Hsia et al. [36], Haplin et al. [37], Siebertz et al. [38] and Sharma and Sharma [21]. The usage of HPLC in purification of plant metabolites (Preccocenes) is comparable to HPLC partially purified *Emblica officinalis* dried fruits [18].

In vitro trypanocidal activity of HPLC purified precocene I could be compared to *in vitro*try panocidal activity of HPLC partially purified fractions of *Emblicaofficinalis* dried fruits [18].

In vivo infectivity test

Group of mice inoculated with contents of ELISA wells with completely killed trypanosomes and precocne I survived for more than 30 days. While other group of mice inoculated with contents of wells with reduced trypanosomes died of parasitaemia. *In vivo* Infectivity assessment of anti-Trypanosoma I activity is

Table 3 Cytotoxic effect of precocene I on Vero cell lines compared to diminazine aceturate (Berenil).

Concentration of test material in µg/ml	Effects of Proconsul I at various periods of incubation (24 h, 48 h, 72 h)						
	Proconsul I.	Berenil	Proconsul I.	Berenil	Proconsul I.	Berenil	Control
100	100%	66.6%	100%	100%	100%	100%	0
50	100%	33.3%	100%	100%	100%	100%	0
25	0	0	100%	100%	100%	100%	0
12.5	0	0	100%	0	100%	33.3%	0
6.25	0	0	0	0	66.6%	0	0
3.13	0	0	0	0	33.3%	0	0
1.56	0	0	0	0	0	0	0

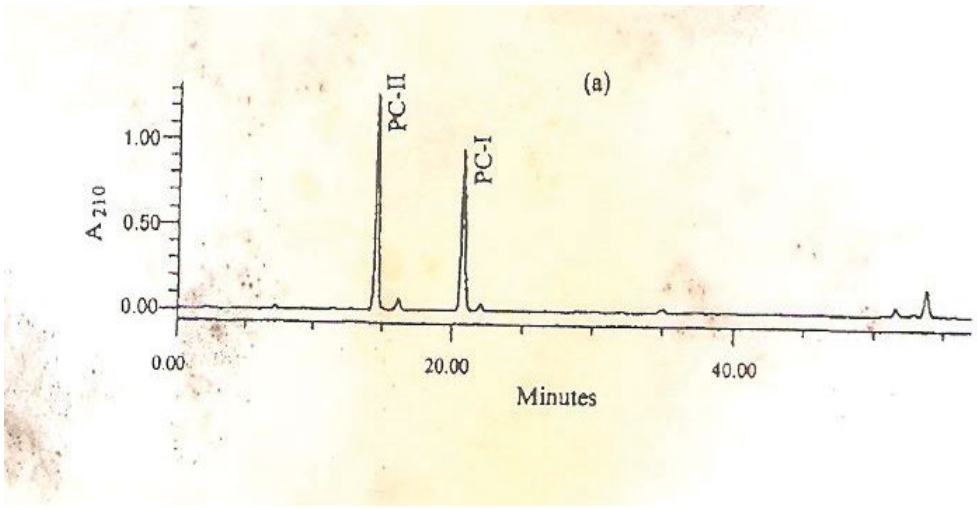


Figure 2 HPLC purified precocene I from *Ageratum houstonianum* leaves. HPLC chromatograms (detected at 210 nm) of the purified precocenes from essential oil extracts of (a) *Ageratum houstonianum* Key to peak identities: PC-I, precocene I; PC-II, precocene II.

comparable to anti-Trypanosoma l effects of the aqueous extract of *Brassica oleracea*buds (fruits) and methanolic plant extract (MPE) of *Terminalia chebula* dried fruits where inoculated mice with contents of ELISA wells with apparently killed trypanosomes survived [6-7,34].

Similarly, *in vitro* cytotoxicity of HPLC purified precocenel 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. These cytotoxic effects are in line with *in vitro* cytotoxicity tests of methanolic extract of *Khaya senegalensis* root bark and effects of *Terminalia arjuna* bark extract on opoptosis of human hepatoma Cell line (HEPG2) in which similar cytotoxic effects were observed [16,39].

Conclusion and Recommendations

In this current report, it shows that HPLC provides a vital tool in the purification and isolation of precocenes (I and II) from *A. houstonianum* leaves. From the results of *in vitro* tryoanicidal activity of precocene Ichomene, it possesstry panocidal property. To attest its full and firm trypanocidal activity potential, *in vivo* test need to be conducted alongside the *in vitro* method. Also, purified precocene I was just one concentration more toxic than diminazine aceturate, the standard drug used, and it's the same drug being used in the treatment of clinical cases of trypanosomosis both in the fields and veterinary hospitals/clinics. Further research is being carried out to determine precocene I full trypanocidal potential.

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