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Deciphering molecular mechanism underlying antileishmanial activity of *Nyctanthes arbortristis*, an Indian medicinal plant

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ABSTRACT

Ethnopharmacological relevance: Nyctanthes arbortristis L. (Oleaceae) is widely used in the traditional medicine of India. The plant is shown to have antibacterial and antileishmanial activities.
Aim of the study: Evaluation of iridoid glucosides from the plant as inhibitor of trypanothione reductase (TryR), a validated drug target enzyme of the Leishmania parasite. The study contributes towards understanding mechanism of antileishmanial effect of the plant.
Materials and methods: TryR of Leishmania parasite is expressed and purified. Iridoid glucosides are isolated from the plant and tested as inhibitor of TryR enzyme of the parasite.
Results: Inhibitory constant (K_i) of various iridoid glucosides ranges from $3.24 \pm 0.05\,\mu M$ to $6.49 \pm 0.05\,\mu M$. Thus, the molecular mechanism underlying antileishmanial activity of these compounds is mediated through inhibition of TryR.
Conclusion: The current study also points out towards potential application of iridoid glucosides as novel drugs against the disease.

1. Introduction

Various parts of *Nyctanthes arbortristis* reported to have immunostimulant, hepatoprotective, antileishmanial, antiviral and antifungal activities (Puri et al., 1994). The leaves have been used in ayurvedic medicine to treat sciatica, arthritis, fevers and as laxative (Saxena et al., 2002). Flower of *Nyctanthes arbortristis* is shown to have antibacterial activity against many gram-positive and gram-negative microorganisms (Khatune et al., 2001). Three iridoid glucosides (arbortristosides A, B, C) and 6 β-hydroxyloganin have been isolated from the plant and tested as antilieshmanial agents (Tandon et al., 1991). However, the molecular mechanism underlying the antileishmanial activity was not reported.

Leishmaniasis is a widespread tropical disease caused by more than 20 species of protozoan *Leishmania* parasite. This disease is common in rural areas and is endemic in Central and South American countries (Tempone et al., 2005). Available drugs against the disease have limitations in terms of high cost and side effects (Shukla et al., 2010). Thus, search for a better antileishmanial drug is still on. Leishmania parasite has a unique thiol metabolism for the homeostasis to overcome the oxidative stress (Shukla et al., 2010). TryR is the key enzyme of thiol metabolism in Trypanosomatids. TryR helps in maintaining redox balance by detoxification of hydroperoxides, formation of DNA precursors by NADPH dependent reduction of trypanothione. Because neither trypanothione nor its reductase is found in mammalian host, so TryR is considered to be selective target for antiparasitic agents. In the current report, we have identified an inhibitor of TryR from *Nyctanthes arbortristis* which may be further developed as affordable drug.

2. Experimental

2.1. Plant

Nyctanthes arbortristis is also known as night-flowering jasmine and is very common in India. Stem, leaf and flowers of the plant were collected from IIT Guwahati campus and identified by taxonomist. The plant was authenticated in the herbarium of Botany Department, Gauhati University, India by Prof. S.K. Borthakur (Herbarium voucher specimen No. 69432). Plant material was dried at room temperature (without sunlight and heat) for 24–48 h and then crushed to fine powder form. TryR gene inserted in the NdeI-HindIII unique sites of pET28b Novagen, in frame with N-terminal His-tag was generously donated by Drs. Andrea Ilari and Gianni Colotti, Università "La Sapienza" Rome, Italy.

2.2. Preparation of plant extracts

Plant powder (50 g) was soaked in methanol (50 ml) and distilled water for 2–4 h to prepare methanolic and aqueous extracts,

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respectively. Then this solution was subjected to sonication to disrupt the cell wall. After sonication the extract was centrifuged (2500 rpm for 20 min) and supernatant was collected. The pellet was again dissolved in methanol/water for subsequent extraction. The supernatant was dried to get powder by lyophilization. Equal amount of the powder (1 mg/ml) was dissolved in distilled water and used for TryR activity assay.

2.3. Extraction of iridoid glucosides

Iridoid glucosides were isolated from *Nyctanthes* seeds (Rathore et al., 1989). 2 kg seed kernel of plant was dried at room temperature and extracted in 50% ethanol by incubating overnight and sonication. After sonication, sample was centrifuged and supernatant was dried in vacuum to get powder (400 g). Extract was fractionated using n-hexane, chloroform and n-butanol subsequently. n-Butanol fraction (20 g) was subjected to column chromatography using silica gel 60-120 (300 g) and eluted with ethyl acetate and ethyl acetate-MeOH (increasing MeOH content) which gave two fractions, fraction 1 (4 g) and fraction 2 (2.3 g). Fraction 1 was again subjected to column chromatography using 100 g Silica gel and eluted with $CHCl_3$–MeOH. 7% MeOH–$CHCl_3$ eluted compound a (white needles, m.p. 220–222 °C) and 10% MeOH–$CHCl_3$ eluted compound b (white amorphous powder, m.p. 200–202 °C). Fraction 2 was also further purified by column chromatography using 70 g silica gel and ethyl acetate-MeOH as eluents. 2% EtOAc–MeOH yielded compound c (colourless, m.p. 221–223 °C). Structures of these compounds are available in literature (Rathore et al., 1989).

2.4. Enzyme expression, purification and activity assay

Recombinant *Leishmania infantum* TryR was expressed in *Escherichia coli*, purified using Ni-affinity chromatography and His-tag was removed using method reported earlier (Baiocco et al., 2009). Enzyme was stored at 4 °C in phosphate buffer pH 7.5. Trypanothione was purchased from Bachem and DTNB from Sigma. Microtitre plate assay was carried out on Tecan spectrophotometer using DTNB as an indicator for reduced thiol groups using method of Hamilton et al. (2003). For microplate assays, the final assay mixture (250 μl) was containing Try R (1 m unit), 40 mM Hepes (pH 7.5), 15 mM NADPH, 1 mM EDTA, 25 μM DTNB and different concentrations of trypanothione $\{T(S)_2\}$ ranging from 0.5 to 200 μM and 2.5 μl of plant extract or 5, 10 and 20 μM of iridoid glucosides. Enzyme mixture was pre-incubated with NADPH for 5 min at 26 °C before starting the reaction by addition of substrate followed by plant extract. Enzyme activity was monitored at 412 nm due to conversion of DTNB into yellow colored TNB.

2.5. Calculation of IC_{50} values of compounds a, b and c against trypanothione reductase

The IC_{50} values (concentration required for 50% inhibition) of all the three compounds were calculated in 96-well plate using assay mixture containing TryR (1 mU), 40 mM HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) pH 7.5, 0.15 mM nicotinamide adenine dinucleotide phosphate-oxidase (NADPH), 1 mM ethylenediaminetetraacetic acid, 25 μM (5,5′-dithiobis-(2-nitrobenzoic acid), 5 μM trypanothione as substrate and different concentrations of inhibitor 1, 2, 4, 8, 10 and 20 μM. The resulting slope of the (dAbs/dt) of absorbance versus time plot is the measure of enzyme activity (Hamilton et al., 2003). The enzyme inhibition was determined by taking the ratio of activity with drug and that of without drug (Cota et al., 2008). Thus the percentage inhibition can be calculated as,

$$\text{Percent inhibition} = \left(1 - \frac{(\text{Activity})_{\text{exp}}}{(\text{Activity})_{\text{contr}}}\right) \times 100.$$

$(\text{Activity})_{\text{exp}}$ and $(\text{Activity})_{\text{contr}}$ represents activity in presence and absence of inhibitor.

Table 1
Inhibition of trypanothione reductase by iridoid glucosides. Structures of these compounds are available in literature (Rathore et al., 1989).

Sr. no.	Name of compound	Source	K_i (μM)	IC_{50} (μM)
1	Compound a	*Nyctanthes arbortristis*	3.24 ± 0.05	2.29 ± 0.03
2	Compound b	*Nyctanthes arbortristis*	3.34 ± 0.03	2.65 ± 0.05
3	Compound c	*Nyctanthes arbortristis*	6.49 ± 0.05	4.74 ± 0.05

3. Results and discussion

3.1. Effect of plant extracts on trypanothione metabolism

Both the methanolic and aqueous extracts obtained from leaf, stem and flower have shown positive inhibition of TryR with an increase of Michaelis constant (K_m value) from 5.44 ± 0.05 μM to 21.73 ± 0.15, 83.33 ± 1.0, 33.34 ± 1.0 μM for stem, leaf and flower in case of methanolic extract and 5.74 ± 0.05, 6.66 ± 0.08, 8.33 ± 0.05 μM for stem, leaf and flower in case of aqueous extract, respectively. Therefore some of the biological components present inside this medicinal plant have inhibition effect on trypanothione metabolism via inhibiting the TryR and can be targeted as naturally occurring chemotherapeutic agents against leishmaniasis. These results prompted us for further purification of compounds from the plant and assay their inhibitory effect against TryR.

3.2. Calculation of inhibitory constant (K_i) and IC_{50} values of iridoid glucosides

All the three isolated iridoid glucosides (compounds a, b and c) have shown inhibition of TryR with an increase of K_m value form 5.44 μM to 22.23 μM, 21.73 μM and 13.81 μM for compounds a, b and c, respectively (with 10 μM of inhibitor conc.). Thus, these three isolated glucosides have shown very high competitive inhibition of TryR with K_i values of 3.24 ± 0.05 μM and 3.34 ± 0.04 μM and 6.49 ± 0.05 μM, respectively. As V_{max} of the enzymatic reaction does not change, the compounds are likely to be competitive inhibitor (Fig. 1). Additionally, IC_{50} values are also calculated and given in Table 1. It is worth mentioning that TryR (from *Trypanosoma cruzi*) inhibitor of plant origin is reported in the literature (Cota et al., 2008; Oliveira et al., 2006). Iridoid glucosides as inhibitor of TryR shows several fold lower IC_{50} value.

Therefore these iridoid glucosides can be targeted as naturally occurring chemotherapeutic agents against leishmaniasis. This is the first report of iridoid glucosides inhibitor for this class of enzyme. It is interesting to note that the structure of these iridoid glucosides is completely distinct from the peptide backbone of the trypanothione substrate. As the TryR activity is required for parasite's survival, inhibition of the enzyme will results in parasitic death (Kannan et al., 2010). However, further studies of these compounds are required to confirm the druggability and safety. Moreover, we are also working on effects of these compounds on redox-homeostasis of the parasite, as TryR has key role in redox balance.

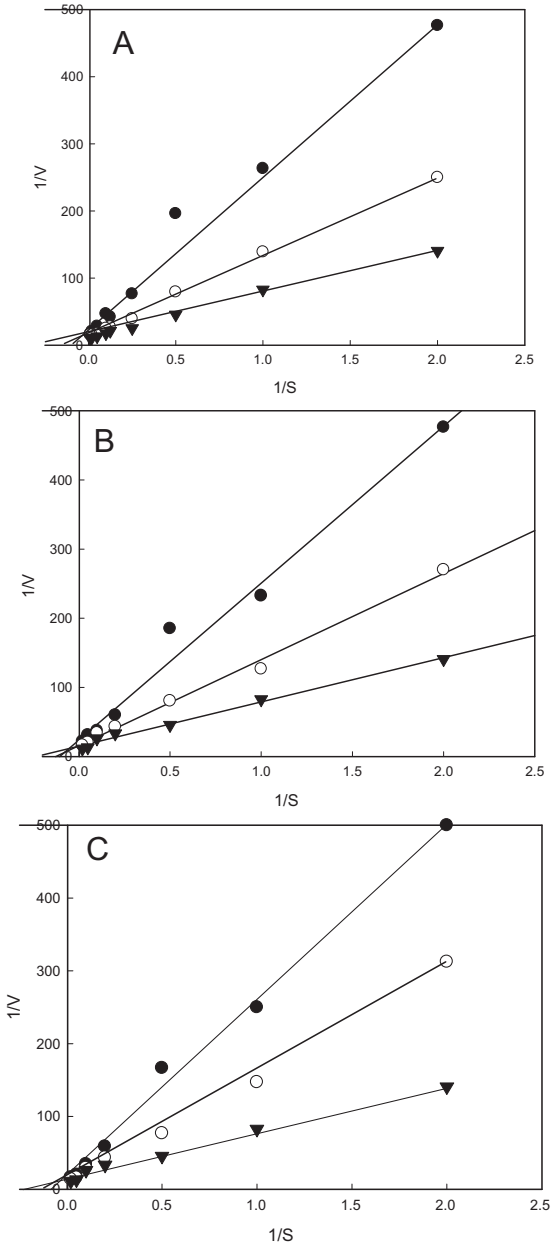

Fig. 1. Enzymatic assay of trypanothione reductase in presence of different iridoid glucosides. (A) In presence of compound a, (B) in presence of compound b and (C) in presence of compound c. Triangle, circle and dot represents data with 0 μM, 5 μM and 10 μM inhibitor concentration.

4. Conclusion

Natural products have played an important role in treating and preventing human diseases. Here, we have reported the molecular mechanism of antileishmanial activity of *Nyctanthes arbortristis*, an Indian medicinal plant. Additionally, we discovered a new class of inhibitor of TryR. As the plant is easily available, these compounds may be developed as an affordable drug. Finally, our results reinforce the potential of Indian medicinal plants as a useful source of chemical diversity for drug discovery.

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