In vitro and in vivo anthelmintic efficacy of two pentacyclic triterpenoids, ursolic acid and betulinic acid against mice pinworm, *Syphacia obvelata*

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Received: 17 May 2017 / Accepted: 25 September 2017 / Published online: 9 October 2017
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Abstract Pinworm infections are one of the common problems in laboratory rodents and man. At present there are only few drugs against intestinal helminths, and new drugs are urgently needed to cope up any future risk of drug resistance. Interest in plant secondary metabolites (PSMs) has risen considerably in the recent years for the discovery and development of new drugs. In the present study, we explored the in vitro and in vivo anthelmintic potentials of two pentacyclic triterpenoids, ursolic acid (UA) and betulinic acid (BA), the important PSMs of many medicinal plants, against *Syphacia obvelata* (Nematoda: Oxyuridae), a common pinworm of mice. The results of this study indicated that in both, in vitro and in vivo assays, BA showed comparatively better anthelmintic effects than UA. In the in vitro assay, 1.00 mg/ml concentration of BA showed paralysis and mortality of worms in 1.20 ± 0.04 and 2.30 ± 0.03 h, respectively. In the in vivo assay, a single 10.00 mg/kg dose of BA, administered for 5 days, revealed 68.78% reduction in egg counts and 84.08% reduction in worm counts of infected mice. The present study suggests that BA holds a great promise to be pursued further for detailed testing against some other representative group of helminth parasites.

Keywords Anthelmintic · Betulinic acid · Ursolic acid · Triterpenoids · *Syphacia obvelata* · Pinworm · Scanning electron microscopy

Introduction

Pinworm infections are one of the common problems in laboratory rodents and man (Michels et al. 2006; Hotez and Gurwith 2011). At present, there exists only few drugs against intestinal helminths (e.g. mebendazole, pyrantel pamoate and albendazole) and there is a risk of drug-resistance against existing anthelmintics (Albonico et al. 2003). Though exhaustive work has been done to develop new anthelmintics, however, barring one, i.e. Emodepside (licensed for use only in cats), no new successful anthelmintic drug has been introduced in the market for almost last three decades (Panic et al. 2014). Therefore, new strategies are urgently needed to change this situation and there is a need to search and develop new classes of anthelmintics.

Of the various strategies attempted so far, some biologically active natural molecules—like plant secondary metabolites (PSM), which act as one of the main sources of templates for the synthesis of novel compounds, have been vigorously investigated for the development of new drugs (Li and Vederas 2009). Among various PSMs, terpenoids constitute as one of the most structurally diverse group of phytocompounds. Especially, the hydroxyl pentacyclic triterpenoic acids, such as ursolic acid (UA) and betulinic acid (BA), are considered as important PSMs which are commonly found in many traditionally used medicinal plants (Fontanay et al. 2008). In previous studies, both BA and UA have shown tremendous potentials for various beneficial biological effects, including antimicrobial, anti-inflammatory, anti-tumour, antiplasmodial activities, etc. (Liu 1995; Yogeeswari and Sriram 2005; Neto 2011; Innocente et al. 2012). The in vitro anthelmintic testing of UA and BA on *Hymenolepis diminuta* (Cestoda) has also revealed that both the compounds possess significant

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anthelmintic effects (Vijaya and Yadav 2016). The genotoxic testing of BA, using Ames and SOS chromotests, did not revealed any genotoxic or mutagenic effects (Frolova et al. 2015). Further, Mullauer et al. (2010) have also reported a complete absence of systemic toxicity by BA treatment in rodents up to a dose of 500 mg/kg. Similarly, toxicity studies on UA, involving clinical isolates MRC-5 and HaCaT cell lines, too have not indicated any apparent signs of toxicity on eukaryotic cells (Fontanay et al. 2008). But, in one study it was found that an intravenous treatment of UA in humans, through a liposome delivery system, may cause mild hepatotoxicity and minor adverse effects (Wang et al. 2013). However, minor adverse effects due to UA treatment were also found to be manageable in this study. The aim of the present study was to investigate the in vitro and in vivo anthelmintic potentials of these two pentacyclic triterpenoids, i.e. ursolic acid (also known as (3 β)-3-hydroxyurs-12-en-28-oic acid) and betulinic acid (also known as 3 β -3-hydroxylup-20(29)-en-28-oic acid) against *Syphacia obvelata* (Nematoda: Oxyuridae), a common pinworm of mice.

Materials and methods

Drugs and chemicals

The compounds UA and BA were of analytical grade and procured from Sigma-Aldrich, Bangalore, India. The reference drug, albendazole (ABZ) was manufactured by Ambalai Sarabhai Enterprises Ltd, Vadodara.

Experimental animals

Male and female Swiss mice, weighing 25–35 g and of 6–8 weeks age, were used. Animals were kept at standard room temperature with 12 h light and dark cycle and supplied with water ad libitum and commercially available mice feed. The experimental protocols involving laboratory mice are approved by the Institutional Animal Ethics Committee (Animal Models), North-Eastern Hill University, Shillong, vide letter no. nil dated 26 March, 2014. The natural infections of *S. obvelata* in experimental mice were identified by the use of anal tapes as suggested by Oge et al. (2000), taking a due account of sampling time (1600–1800 hours).

In vitro anthelmintic assay

In vitro testing of compounds was performed on adult *S. obvelata*, which was obtained from the intestine of freshly necropsied laboratory mice. The specimens were washed in 0.90% phosphate buffered saline (PBS) at 37 ± 1 °C. The

test worms ($n = 5$) were then maintained in petridishes inside an incubator at 37 ± 1 °C, containing 0.25, 0.50 and 1.00 mg/ml concentrations of each compound and 1.00 mg/ml concentration of ABZ. One set of worms ($n = 5$) were maintained in PBS alone to serve as controls. The anthelmintic efficacy of compounds was evaluated on the basis of physical motility of worms, as observed by their paralysis or mortality after exposure to different concentrations of compounds and ABZ (Vijaya and Yadav 2016).

Scanning electron microscopic studies

Immediately after the paralysis of worms in the extract treated group, a set of worms from the highest compound-treated group (1 mg/ml), ABZ-treated group and control group were picked up, washed in PBS and fixed in 3% formaldehyde for scanning electron microscopy (SEM). The specimens were processed for SEM by standard procedures and observed under the scanning electron microscope JSM-6360 (JEOL).

In vivo nematocidal assay

Animals were randomly divided into five groups ($n = 5$). Group I of animals constituted as untreated control. Groups II, III and IV of animals were administered orally, once daily, with 1, 5 and 10 mg/ml concentrations of each test compound, i.e. BA and UA. Group V of animals were given 10 mg/ml dose of a reference drug, ABZ. The anal stick tapes of breadth 1.5 cm were taken from each animal to confirm the pinworm infection. Egg counts via anal tape test from gravid *S. obvelata* females were taken on moistened slides (Grice and Prociw 1993). The anal tape EPG counts of animals were taken for 3 days (day 1–3, pre-treatment), during the treatment of test compounds for 5 days (day 4–8, treatment) and for another 3 days after treatments (day 9–11, post-treatment). All the animals were autopsied on day 12 and their intestines, along with full colon and caecum, were incised under a stereomicroscope to count the number of worms per animal. All calculations with regard to the efficacy of compounds were done as suggested by Oge et al. (2000).

Statistical analysis

The experimental data reported herein are represented as mean $\pm$ standard error of the mean (SEM). The data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey post hoc test. The p value < 0.05 were considered as statistically significant.

Results

In the in vitro testing both the compounds, BA and UA revealed dose-dependent anthelmintic effects (Fig. 1). However, BA showed a comparative better efficacy than UA. At its highest tested dose, i.e. 1.00 mg/ml, BA resulted into paralysis and mortality of worms in 1.20 ± 0.04 and 2.30 ± 0.03 h, respectively. On the other hand, exposure of worms to ABZ showed paralysis and mortality of worms in 5.40 ± 0.08 and 7.20 ± 0.26 h, respectively. Herein, the control worms showed physical activity up to 33.56 ± 0.44 h. On the other hand, at its highest tested concentration (1.00 mg/ml), UA caused paralysis and

mortality of worms in 3.10 ± 0.05 and 4.20 ± 0.06 h, respectively. On the other hand, ABZ showed paralysis and mortality of worms 5.45 ± 0.03 and 7.22 ± 0.12 h, respectively. In the control medium the worms showed physical movements till 32.56 ± 0.36 h (Fig. 1).

Scanning electron microscopy observations

SEM observations of *S. obvelata* from control group showed normal cuticular transverse striations (Fig. 2). On the other hand, exposure of worms to 1.00 mg/ml concentration of UA revealed significant damages to cuticle in the form of wrinkles (Fig. 2). In contrast, the worms exposed to 1.00 mg/ml concentration of BA did not revealed any noticeable adverse effects on parasite's body surface. But, the worms exposed to 1.00 mg/ml concentration of ABZ also showed an extensive distortion of cuticular transverse annulations (Fig. 2).

In vivo results

Both the compounds, BA and UA showed a dose-dependent effect on paralysis and mortality of parasites (Table 1). The mice treated with 10.00 mg/kg concentration of BA showed quite prominent effects on *S. obvelata* in terms of significant reduction ($p < 0.001$) in parasite's egg counts as well as total worm counts. A single 10.00 mg/kg dose of BA, administered for 5 days, revealed 68.78% reduction in parasite's egg counts and 84.08% ($p < 0.01$) reduction in worm counts of experimental mice as compared to the control. On the other hand, 10.00 mg/kg dose of reference drug, ABZ, administered for the same duration, showed a reduction in parasite's egg counts up to 89.01% and worm counts up to 84.83% (Table 1). Compared to BA, administration of UA to mice at its highest tested dose, i.e. 10.00 mg/kg resulted into a slightly less decrease in parasite's egg counts (54.59%) and worm counts (74.43%) at necropsy (Table 2).

Discussion

Many plant secondary metabolites (PSMs) and/or their derivatives have been adopted for their various therapeutic uses worldwide (Anthony et al. 2005; Olliaro et al. 2011; Wink 2012). The bioefficacies of different phytochemicals have been evaluated using different screening methods (Kaplan 2004; Keiser and Utzinger 2010; Vijaya and Yadav 2016). In most of the previous bioactivity studies on phytochemicals, the main focus of investigations have mainly been on purified compounds, isolated either from a medicinal plant or obtained commercially. In general, most previous studies also reveal that either

Fig. 1 In-vitro anthelmintic effects of **a** Betulinic acid, **b** Ursolic acid on *S. obvelata*. Survival of worms in control medium was as follows: control for betulinic acid = 33.56 ± 0.44 h; Control for ursolic acid = 32.56 ± 0.36 h. All data are expressed as mean $\pm$ SEM ($n = 5$). *Significant at $p < 0.05$ level; **significant at $p < 0.001$ level

Fig. 2 Scanning electron micrographs of *S. obvelata* exposed to betulinic acid (1 mg/ml), ursolic acid (1 mg/ml) and albendazole (1 mg/ml). **a** Control, **b** Betulinic acid, **c** Ursolic acid, **d** Albendazole. Scale bar = 5 μ m

in vitro (Wabo et al. 2013; Elo et al. 2015; Vijaya and Yadav 2016) or in vivo screenings (Caboni et al. 2015; Wang et al. 2015) are the major pre-clinical assays which may provide useful information about the efficacy of any antiparasitic agent. Therefore, in this study we employed a suitable in vitro and in vivo test to investigate the anthelmintic efficacy of two important pentacyclic triterpenoids, namely betulinic acid (BA) and ursolic acid (UA) on *S. obvelata*, a pinworm of mice.

In the present study, BA showed comparatively better in vitro anthelmintic effects than UA. A previous study, on in vitro testing of UA, BA and two other phytocompounds namely, betasitosterol and biochanin A, on *Hymenolepis diminuta* (Cestoda), also showed that, except betasitosterol, UA, BA and biochanin A possess similar levels of anthelmintic effects (Vijaya and Yadav 2016). During the past two decades, a number of studies have been undertaken on the anthelmintic effects of an isoflavonoid, genistein, which is the active principle of *Flemingia ves-tita*, a folk anthelmintic plant of Meghalaya. In one study, the genistein was tested to establish its credentials as an

anthelmintic against *Raillietina echinobothrida*, a poultry cestode (Tandon et al. 1997). In this study, in vitro exposure of parasites to genistein caused spontaneous loss of movement in cestode parasite, followed by several structural alterations in its tegumental architecture (Tandon et al. 1997). Likewise, the anthelmintic activity of *Croton zehntneri* and *Lippia sidoides* essential oils and their major constituents, anethole and thymol were determined by in vitro assays against the eggs and larvae of *Haemonchus contortus* (Camurca-Vasconcelos et al. 2007). In this study, anethole and thymol prevented about 98.00% of *H. contortus* egg hatching at 1.25 mg/ml concentration and larval development inhibition by more than 90.00% at a concentration of 10.00 mg/ml concentration. In the SEM studies, the worms exposed to 1.00 mg/ml concentration of BA did not revealed any major noticeable changes on body cuticle. However, the exposure of worms to 1.00 mg/ml concentration of UA caused significant damages to the cuticle, which were visible as wrinkles. Mode of some anthelmintics has also been explained on the basis of their selective effects on cuticle of nematodes (Williams et al.

Table 1 Anthelmintic activity of betulinic acid on *S. obvelata* infections in mice as assessed by reductions in egg counts and worm counts at necropsy

Groups	Egg counts (mean $\pm$ SEM) Pre-treatment (days 1–3)	Egg counts (mean $\pm$ SEM) Post-treatment (days 9–11)	Percentage difference in egg counts	No. of worms recovered/mice (mean $\pm$ SEM)	Percentage reduction in worm counts
Control	180 $\pm$ 3.25	184 $\pm$ 3.52	02.19	868 $\pm$ 62.00	00.00
Betulinic acid					
1 mg/kg	173 $\pm$ 3.84	161 $\pm$ 1.51 ^a	19.74	425 $\pm$ 19.74 ^d	51.03
5 mg/kg	172 $\pm$ 5.45	134 $\pm$ 1.94 ^b	21.94	307 $\pm$ 18.02 ^c	64.60
10 mg/kg	174 $\pm$ 4.82	54 $\pm$ 2.91 ^c	68.78	138 $\pm$ 29.42 ^c	84.08
Albendazole					
10 mg/kg	181 $\pm$ 3.40	19 $\pm$ 1.02 ^c	89.01	131 $\pm$ 13.22 ^c	84.83

Number of animals in each group, n = 5

^aSignificant at $p < 0.05$ versus pre-treatment

^bSignificant at $p < 0.01$ versus pre-treatment

^cSignificant at $p < 0.001$ versus pre-treatment

^dSignificant at $p < 0.05$ versus control

^eSignificant at $p < 0.01$ versus control

Table 2 Anthelmintic activity of ursolic acid on *S. obvelata* infections in mice as assessed by reductions in egg counts and worm counts at necropsy

Groups	Egg counts (mean $\pm$ SEM) Pre-treatment (days 1–3)	Egg counts (mean $\pm$ SEM) Post-treatment (days 9–11)	Percentage difference in egg counts	No. of worms recovered/mice (mean $\pm$ SEM)	Percentage reduction in worm counts
Control	178 $\pm$ 4.44	179 $\pm$ 4.87	00.62	937 $\pm$ 19.02	00.00
Ursolic acid					
1 mg/kg	177 $\pm$ 3.13	150 $\pm$ 1.23 ^a	15.57	419 $\pm$ 19.49 ^d	55.28
5 mg/kg	176 $\pm$ 4.30	128 $\pm$ 1.83 ^a	27.12	325 $\pm$ 13.59 ^d	65.26
10 mg/kg	171 $\pm$ 4.84	78 $\pm$ 5.72 ^b	54.59	239 $\pm$ 20.07 ^c	74.43
Albendazole					
10 mg/kg	167 $\pm$ 5.62	19 $\pm$ 1.31 ^c	88.37	104 $\pm$ 12.38 ^c	88.81

Number of animals in each group, n = 5

^aSignificant at $p < 0.05$ versus pre-treatment

^bSignificant at $p < 0.01$ versus pre-treatment

^cSignificant at $p < 0.001$ versus pre-treatment

^dSignificant at $p < 0.05$ versus control

^eSignificant at $p < 0.01$ versus control

2014a, b). For example, in a similar study on the effects of hazelnut (*Corylus avellana*) skin-extract on the motility and morphology of adult *Oesophagostomum dentatum*, the worms showed clear signs of paralysis and structural damage, including blebbing and a rough cuticle (Williams et al. 2014b). Similarly, Costa et al. (2010) tested the anthelmintic efficacy of liquid extracted from the bark of the green coconut, *Cocos nucifera*, a medicinal plant of Brazil, on intestinal nematodes of mice. Interestingly, in this study the aqueous extract did showed much efficacy, but its butanol extract, containing triterpens, saponnins and

condensed tannins, showed significant anthelmintic efficacy. Likewise, Villasenor et al. (2002) isolated another PSM, beta-sitosterol from *Mentha cordifolia* and reported its anthelmintic effects against *Ascaris suum*. Also, Deepak et al. (2002) noticed that same compound possesses significant anthelmintic effects against *Caenorhabditis elegans*, in vitro.

In conclusion, this study suggests that two pentacyclic triterpenoids, betulinic acid and ursolic acid possess significant nematicidal effects against mice pinworm *S. obvelata*. Further studies on these compounds, involving

other representative mammalian parasite groups, seems desirable from a therapeutic point of view.

Acknowledgements Vijaya thank North-Eastern Hill University, Shillong for providing her the University Grants Commission's, New Delhi non-net fellowship.

Authors contributions AKY conceived and supervised the study, developed the final draft; Vijaya performed the experiments, analysed the data and developed the first draft; SG wrote the supplementary information and participated in preparation of second manuscript draft.

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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