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Short Communication

Antiprotozoal activity of Betulinic acid derivatives

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ABSTRACT

Betulinic acid (**1**), isolated from the crude extract of the leaves of *Pentalinon andrieuxii* (Apocynaceae), together with betulinic acid acetate (**2**), betulonic acid (**3**), betulinic acid methyl ester (**4**), and betulin (**5**) were evaluated for their antiprotozoal activity. The results showed that modifying the C-3 position increases leishmanicidal activity while modification of the C-3 and C-28 positions decreases trypanocidal activity.

Introduction

Protozoal diseases continue to be considered an important group of illnesses because of their significant number of cases and mortalities every year (WHO 2004). The chemotherapy against diseases such as leishmaniosis and trypanosomiosis is limited by the existence of only a few drugs in the market, most of which are of low efficacy, require a long and supervised treatment, show toxic side effects, and frequently lead to the appearance of resistant strains (Kayser et al. 2002; Croft et al. 2006; Ashutosh et al. 2007); this reflects the need to continue searching for new and better antiprotozoals. Triterpenes are a group of natural products that have shown a wide range of biological activities, including leishmanicidal, trypanocidal and antiplasmodial (Chan-Bacab and Peña-Rodríquez 2001; Kayser et al. 2003; Tolstikova et al. 2006). Recently, the chemical transformation of natural triterpenoids has been used to prepare derivatives with improved biological activity (Tolstikov et al. 1997; Baltina 2003). Betulinic acid (**1**) is a lupane-type triterpene found in many plant species, which has been reported to exhibit anti-HIV-1, antibacterial, antifungal, antiplasmodial, and anti-inflammatory activities (Yogeeswari and Sriram 2005). Betulinic acid (**1**) has also been reported to inhibit growth of cancer cells, without affecting normal cells (Einzhammer and Xu 2004), and its lack of cytotoxic activity has been demonstrated in human astrocytes (Wick et al. 1999), human dermal fibroblasts, peripheral blood lymphoblasts (Einzhammer and Xu 2004; Zuco et al. 2002), and animal studies (Zuco et al. 2002; Pisha et al. 1995). Additionally, a number of betulinic acid derivatives have been prepared to enhance anti-HIV activity (Kashiwada et al. 1996; Baglin et al. 2003), to reduce the organotoxic effect of antitumor drugs, and to be evaluated as new anticancer agents (Rajendran et al. 2008). However, and although **1** has been shown to suppress the activity of DNA topoisomerase I (Chowdhury et al. 2002), and metabolites able to interact with human DNA are reported to be antiparasitic (Nenortas et al. 1998; Rowe et al. 2001; Baraldi et al. 2004), little is known about the antiprotozoal activity of this triterpene. We wish to report here on the leishmanicidal, trypanocidal and antiplasmodial activities of betulinic acid (**1**) and a number of its derivatives (**2**-**5**).

Materials and Methods

Plant material

Leaves of *Pentalinon andrieuxii* Muell. Arg. (Apocynaceae) were collected 3.5 km northwest of Campeche, México, on the road to Chiná. A voucher specimen (P. Simá 2503) has been deposited in the Herbarium of Centro de Investigación Científica de Yucatán.

*Isolation of betulinic acid (**1**)*

Dry, ground leaves of *P. andrieuxii* (365 g) were extracted three times with ethanol (4 l) at room temperature. Evaporation of the solvent yielded the corresponding crude extract (54.5 g, 14.9%), which was fractionated by successive liquid-liquid partition with hexane, ethyl acetate and butanol, to produce the corresponding low, medium and high polarity fractions. Purification of the low polarity fraction using silica gel (70-230 mesh, E.M. Merck) column chromatography and a gradient elution [$CHCl_3$/MeOH

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(99:1-90:10) mixtures] produced 62.7 mg (0.11%) of pure betulinic acid (**1**), identified by comparing its spectroscopic data with those reported in the literature (Mahato and Kundu 1994; Macias et al. 1994).

Preparation of betulinic acid derivatives

The structures of all derivatives were established by comparing their corresponding spectroscopic data with those reported in the literature (Macias et al. 1994). Betulin (**5**), amphotericin B and chloroquine were purchased from Sigma.

Betulinic acid acetate (**2**). A mixture of betulinic acid (10 mg), acetic anhydride (1 ml) and pyridine (0.5 ml) was allowed to stir at room temperature for 72 h. The reaction mixture was poured over distilled water (20 ml) and the resulting suspension was extracted twice with ethylacetate (2:1 v/v). The organic layer was washed successively with equal volumes of HCl (5%), NaOH (3%), H_2O, and NaCl satd., and then dried over anhydrous Mg_2SO_4. Filtration and evaporation of the solvent yielded 10.5 mg (96.2%) of crude acetylated product, identified as betulinic acid acetate (**2**).

Betulonic acid (**3**). A solution of 23.5 mg of betulinic acid in dichloromethane (2 mL) was treated with an excess of Corey's reagent (PCC) (Corey and Suggs 1975). The reaction mixture was allowed to stir at room temperature for 24 h and then filtered through a plug of silica gel (70-230 mesh), eluting with 50 ml of a 99:1 mixture of dichloromethane-methanol to produce 16.1 mg (68.6%) of crude oxidized product identified as betulonic acid (**3**).

Betulinic acid methyl ester (**4**). A mixture of betulinic acid (13.5 mg), K_2CO_3 (200 mg), CH_3I (1 ml) and acetone (1 ml) was stirred for 72 h at room temperature. The reaction mixture was poured over distiller water (13 ml) and the resulting suspension was extracted twice with EtOAc (4:1, v/v); the organic phase was dried with anhydrous Na_2SO_4, filtered and the solvent evaporated to produce 12 mg (86.2%) of crude esterified product identified as betulinic acid methyl esther (**4**).

Leishmanicidal assay

This assay was carried out following the procedure reported by Muñoz et al. (1994) and Inchausti et al. (1997). Briefly, strains of *L. amazonensis* (IFLA/BR/75/PH8), *L. braziliensis* (MHOM/BR/75/M2903) and *L. donovani* (MHOM/74/PP75) were maintained in Schneider culture medium with 10% fetal bovine serum (FBS); parasites in the log phase of their growth cycle were transferred to a microplate (96 wells; 1×10^6 parasites/well). Stock solutions of DMSO (blank), amphotericin B (positive control), betulinic acid, and the various betulinic acid derivatives were diluted in Schneider medium at $<100\,\mu g/ml$, added to the plate, and incubated at 27 °C for 72 h. The IC_{50} (μg/ml) values were obtained using a GraphPad Software Inc. ver. 4.00. All the assays were carried out in triplicate.

Trypanocidal assay

Trypanosoma cruzi strain Tulahuen parasites were maintained in liver infusion tryptose (LIT) medium supplemented with 5% FBS, following the procedure modified by Chataing et al. (1998). Briefly, parasites in the log phase of their growth cycle were transferred to a microplate (96 wells; 1×10^6 parasites/well). Stock solutions of benznidazole, DMSO (positive control and blank respectively), betulinic acid, and the various betulinic acid derivatives were diluted in LIT medium and added to a plate (100 μl) at concentrations $<100\,\mu g/ml$, and incubated at 27 °C for 72 h. The IC_{50} (μg/ml) value was obtained using a GraphPad Software Inc. ver. 4.00.

Antiplasmodial assay

Plasmodium falciparum strain F32 was cultivated at 37 °C in RPMI medium with 10% of human serum and 4% of hematocrite (O, Rh+), under anaerobic conditions, according to the method of Trager and Jensen (1976). Cultures with 1% parasitemic and 2% hematocrite (100 μl) were transferred to a 96 wells plate. Stock solutions of chloroquine (control), betulinic acid, and the various betulinic acid derivatives were diluted in RPMI medium to a concentration of $<10\,\mu g/ml$ and added to each well. The plate was then incubated at 37 °C for 48 h. The IC_{50} values were calculated using the program a GraphPad Software Inc. ver. 4.00.

Results and Discussion

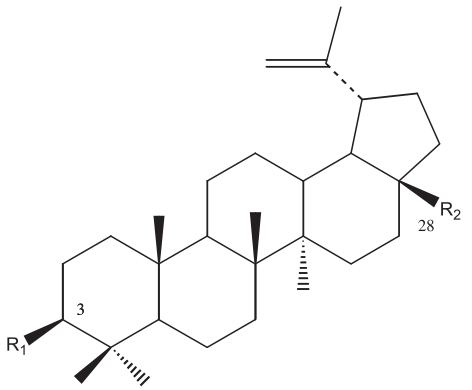
Betulinic acid (**1**) was obtained from the purification of the low polarity fraction of the leaf extract of *P. andrieuxii*, a plant whose extracts have been shown to possess leishmanicidal activity (Chan-Bacab et al. 2003). Although the low polarity fraction had shown moderate leishmanicidal activity, the lack of activity of pure betulinic acid (**1**) indicated that the triterpene was not responsible for the weak leishmanicidal activity originally detected. However, testing of the pure metabolite against *T. cruzi* and *P. falciparum* showed that **1** has a moderate trypanocidal activity and a good antiplasmodial activity (Table 1). In order to explore the possibility of improving the antiprotozoal activity of **1**, a number of simple semisynthetic derivatives were prepared and/or evaluated; these derivatives included betulinic acid acetate (**2**), betulonic acid (**3**), betulinic acid methyl esther (**4**) and betulin (**5**) (Fig. 1).

The results showed that modification of the C-3 hydroxyl group, either by esterification (to produce **2**) or oxidation (to

Table 1
IC_{50} (μM) values of antiprotozoal activity of betulinic acid and derivates.

Sample	Leishmanicidal		Trypanocidal	Antiplasmodial
	LA	M2903	TULA	F32
Betulinic acid (1)	>200	>200	50.0	22.5
Betulinic acid acetate (2)	44.9	>200	154.4	11.8
Betulonic acid (3)	51.2	>200	161.4	>20
Betulinic acid methyl esther (4)	>200	69.9	93.3	>20
Betulin (5)	>200	>200	173.0	>20
Amphotericin B	0.6	0.2	0.2	
Benznidazol			384.4	
Chloroquine				0.2

LA: *Leishmania amazonensis*; M2903: *L. braziliensis*; TULA: *Trypanosoma cruzi tulahuen*; F32: *Plasmodium falciparum*.



	R_1 α	R_1 β	R_2
(**1**)	H	OH	COOH
(**2**)	H	$OCOCH_3$	COOH
(**3**)	O		COOH
(**4**)	H	OH	$COOCH_3$
(**5**)	H	OH	CH_2OH

Fig. 1. Betulinic acid and derivatives.

produce **3**), resulted in a significant increase of leishmanicidal activity against *L. amazonensis* (Table 1). Alternatively, the modifications on the C-28 carboxyl group did not show a clear effect since while betulinic acid (**1**) and betulin (**5**) lack leishmanicidal activity, betulinic acid methyl ester (**4**) showed an improved activity against *L. braziliensis* (Table 1). On the other hand, the evaluation of the trypanocidal activity of the various terpenoids showed that any modification on the betulinic acid skeleton resulted in a lower activity (Table 1). Finally, and although our results showing betulinic acid (**1**) as an adequate antiplasmodial agent are in agreement with those reported in the literature (Ziegler et al. 2004; Duker-Eshun et al. 2004), testing of the various derivatives showed that only the esterification of the C-3 hydroxyl group (to produce **2**) results in an improved activity (Table 1).

The increase in leishmanicidal activity shown by **2**, **3**, and **4** suggests that preparation of other acyl derivatives at the C-28 position, maintaining a ketone or an ester group in C-3, can produce new derivatives with improved leishmanicidal and antiplasmodial activities, since an ester at the C-3 β position and a free carboxylic acid, a high hydrophilic group, or a hydrogen donating group at C-28 are required for cytotoxic and anti-HIV activities (Baglin et al. 2003). Interestingly enough, betulinaldehyde has been reported to be active against amastigotes of *L. amazonensis* (Sauvain et al. 1996), while the C-28 aldehyde derivative of betulonic acid has been shown to have a higher antitumoral activity than that of the parent metabolite (Kim et al. 1998; Jeong et al. 1999; Hata et al. 2003; Dzubak et al. 2006). Furthermore, betulonic acid (**3**) has been reported to be able to reduce the organotoxic effect of antitumor drugs (Sorokina et al. 2004), while its C-28 amine derivatives are reported as potent inhibitors of tumor cell growth (Shintyapina et al. 2007).

To date, there exist a number of reports in the literature describing the chemical modifications of betulinic acid to produce semisynthetic derivatives with enhanced anti-HIV and cytotoxic activities (Baglin et al. 2003); however, little is known about the importance of the various functional groups in the betulinic acid skeleton, for the expression of antiprotozoal activity. This is the first report of the evaluation of the antiprotozoal activity of betulinic acid and a number of its derivatives and the first report of the betulinic acid derivative **2** with improved antiplasmodial activity.

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