Possible Fungistatic Implications of Betulin Presence in Betulaceae Plants and their Hymenochaetaceae Parasitic Fungi

Izabela Jasicka-Misiak*, Jacek Lipok, Izabela A. Świder, and Paweł Kafarski

Faculty of Chemistry, Opole University, Oleska 48, 45-052 Opole, Poland.
Fax: +4 87 74 52 71 15. E-mail: izajm@uni.opole.pl

* Author for correspondence and reprint requests

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Betulin and its derivatives (especially betulinic acid) are known to possess very interesting prospects for their application in medicine, cosmetics and as bioactive agents in pharmaceutical industry. Usually betulin is obtained by extraction from the outer layer of a birch bark. In this work we describe a simple method of betulin isolation from bark of various species of Betulaceae trees and parasitic Hymenochaetaceae fungi associated with these trees. The composition of the extracts was studied by GC-MS, whereas the structures of the isolated compounds were confirmed by FTIR and ^{1}H NMR. Additionally, the significant fungistatic activity of betulin towards some filamentous fungi was determined. This activity was found to be strongly dependent on the formulation of this triterpene. A betulin-trimyristin emulsion, in which nutmeg fat acts as emulsifier and lipophilic carrier, inhibited the fungal growth even in micromolar concentrations – its EC_{50} values were established in the range of 15 up to 50 μM depending on the sensitivity of the fungal strain. Considering the lack of fungistatic effect of betulin applied alone, the application of ultrasonic emulsification with the natural plant fat trimyristin appeared to be a new method of antifungal bioassay of water-insoluble substances, such as betulin.

Key words: Betulin, Triterpene, Trimyristin, Fungistatic Effect

Introduction

Betulin is a naturally occurring pentacyclic triterpene found in many plants. Generally, triterpenes are aliphatic polycyclic compounds with a skeleton of 30 carbon atoms. In most cases they are synthesized in plants from squalene (Jager *et al*., 2008; Pakdel *et al*., 2007). They are by far the most important secondary metabolites of birch bark and have been demonstrated to exhibit a variety of biological activities. The reported biological effects of triterpenoids include cytotoxic, antitumour, anticancer, anti-inflammatory, and antiviral activities (Drag *et al*. 2009; Fulda and Debatin, 2000; Gong *et al*., 2004; Mullauer *et al*., 2009; Recio *et al*., 1995; Ryu *et al*., 2000; Sun *et al*., 1998; Wang and Polya, 1996; Zuco *et al*., 2002). According to their structure, these triterpenes are subdivided into three classes: lanostanes, lupanes, and oleananes.

Although, the most widely reported source of betulin is the birch tree (*Betula* spp.), where it is present in high concentration (Yogeeswari and Sriram, 2005; Cichewicz and Kouzi, 2003), betulin has also been found in *Euclea divinorum* (Mebe *et al*., 1998), *Celtis philippinensis* (Hwang *et al*., 2006), *Acacia mellifera* (Mutai *et al*., 2004), *Byrsonima microphylla* (Aguiar *et al*., 2005). It is a valuable compound because it exhibits important biological properties and is also an important commodity, particularly in cosmetic and pharmaceutical industries (Dzubak *et al*., 2006). The anti-inflammatory activity of triterpenoids is connected with their interaction with several cellular and extracellular proteins. Betulin has been shown to be a potent phospholipase A2 inhibitor (Bernard *et al*., 2001).

The aim of the present study was to elaborate a simple method for extraction of betulin, determination of its antifungal properties, and identification of other triterpenes by means of GC-MS.

Results and Discussion

Choice of extractant

The rigid skeleton of betulin is composed of 30 carbon atoms substituted with only two hydroxy groups, and thus betulin is a lipophilic organic compound almost insoluble in water. Therefore, for its extraction various organic solvents are

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used, in which betulin is soluble at higher temperatures, and after concentrating the extract, it can crystallize directly from the extract.

To compare the extraction efficiency, several solvents were evaluated and extraction times were optimized. Thus the ground bark was extracted in a Soxhlet apparatus three times (using 5 probes each) with each studied solvent keeping the ratio of the material to the extractant (*n*-butanol, *n*-propanol and ethanol) at 1:20. The most effective solvent, in terms of both quantity and content of triterpenoic compounds, was *n*-butanol. In all three solvents betulin was found to be the most abundant component.

The qualitative analysis of the fractions obtained revealed also the presence of compounds other than betulin. Among the triterpenes, lupeol was the second major component in these fractions.

Content of betulin in various sources

Betulin and other triterpenic compounds were identified in *n*-butanol extracts of the outer bark of three different Betulaceae plants and their Hymenochaetaceae parasitic fungi by means of GC-MS.

Triterpenes are the dominant constituents in the extracts from plants of the genus Betulaceae. The *n*-butanolic extract of *Betula pendula* bark appeared to be the richest source of triterpenes providing 740 mg of a mixture of compounds per 1 g of powdered bark. Other species gave significantly lower amounts. These compounds constitute almost 95% in extracts from silver birch (*Betula pendula* L.) with betulin being the major component (over 60%), and lupeol and taraxasterol constituting together nearly 30% (Table I). A lower level of triterpenic compounds was found in extracts from common hazel (*Corylus avellana* L.) bark (about 70%), with lupeol being the major component and only a small fraction of betulin (Table I). On the contrary the extract from the bark of black alder (*Alnus glutinosa* L.) appeared to be very poor in the triterpenes fraction and lacks betulin.

Hymenochaetaceae also constitute a rich source of triterpenes and their amounts obtained from extracts were 620 mg, 600 mg, and 580 mg per 1 g of powdered tissue of *Inonotus obliquus*, *Piptoporus betulinus* and *Daedalea confragosa*, respectively. Triterpenes constitute the major fraction found in extracts from these fungi (Table II). Similarly as in the case of plants, the compositions of these extracts were variable with betulin being the major component of chaga mushroom (*I. obliquus*) extract. Chaga mushroom it is widespread parasitic fungus on birch and other trees. The content of betulin from this source was comparable with the content found in silver birch. The extracts from the remaining fungi were characterized by a small content of betulin with lupeol being the main compounds in birch bracket (*P.*

Table I. The average percentage content of the triterpenes in the *n*-butanolic extracts from Betulaceae.

Compound	*Betula pendula* L. (%)[a]	*Corylus avellana* L. (%)[a]	*Alnus glutinosa* L. (%)[a]
Betulin [lup-20(29)-en-3β,28-diol]	64.3	7.1	nd[b]
Taraxasterol	15.1	nd	nd
Lupeol [lup-20(29)-en-3-ol]	12.0	31.6	2.8
β-Sitosterol	0.1	11.9	15.6
β-Amyrin	nd	7.3	nd
Hop-22(29)-en-3β-ol	2.80	nd	nd
Lup-20(29)-en-3-one	0.30	nd	4.7
Cycloartenol (9,19-cyclolanost-24-en-3-ol)	0.30	nd	nd
Friedoolean-8-en-3-one	nd	4.7	nd
Urs-20-en-16-one	nd	3.6	nd
Stigmast-4-en-3-one	nd	2.3	9.4
Cholesterol	nd	nd	2.0
Vitamin E	nd	nd	1.8
Total	94.9	68.5	36.3

[a] % refers to the mean percentage of all samples analyzed against the total peak area.
[b] nd, not detected.

Table II. The average percentage content of the triterpenes in the *n*-butanolic extracts from Hymenochaetaceae parasitic fungi.

Compound	*Inonotus obliquus* (%)[a]	*Piptoporus betulinus* (%)[a]	*Daedalea confragosa* (%)[a]
Betulin	65.8	2.5	0.2
Lupeol	9.5	62.2	1.3
Cholesta-8,24-dien-3-*β*-ol	2.8	nd[b]	nd
Ergosta-7,22-dien-3-ol	1.6	7.8	nd
Ergosterol	1.0	nd	67.7
γ-Ergosterol	nd	nd	21.1
Taraxasterol	nd	5.7	nd
Hop-22(29)-en-3*β*-ol	nd	1.9	nd
Ergosta-7-en-3-ol	nd	nd	1.9
Total	80.7	80.1	92.2

[a] % refers to the mean percentage of all samples analyzed against the total peak area.
[b] nd, not detected.

betulinus) and ergosterol in blushing bracket (*D. confragosa*) (Table II).

Comparing the triterpene contents in Betulaceae plants with those of their pathogenic fungi has shown that the fungi are a richer source of these compounds. This seems to explain why Hymenochaetaceae are used in many ethnomedicines including these of eastern Poland, Russia and Belorussia (Johnson, 2006; Spiridonov *et al.*, 2005; Lindequist *et al.*, 2005).

Antifungal activity of betulin

Although, betulin is an abundant naturally occurring triterpene, the physiological implications of the presence of this compound in the bark is not known. Most of plant terpenes are well recognized as constituents of the antimicrobial defense systems in plants. To test the hypothesis that betulin is playing a similar role, we undertook studies on its influence on potentially pathogenic strains of fungi, namely *Alternaria alternata* (Fr.), *Fusarium dimerum* and *Fusarium oxysporum* (Schl.), and on the growth of the saprophytic strain of *Penicillium cyclopium* (Westling).

Betulin, when dispersed in water ultrasonically, had no influence on the fungal growth and development in solid or liquid Czapek media. We have speculated that this might result from the insolubility of this compound in water and consequently the lack of contact of it with the fungal mycelia. Thus, applied betulin in a form of dispersion in trimyristin, a common fat, which is extremely easily extracted from powdered nutmeg.

Trimyristin alone had no visible influence on fungal growth or development. The only differences observed concerned macroscopic features of the fungal mycelia, namely, with increasing trimyristin concentration the mycelia grew in more dispersed form. Independently on these macromorphological changes, the dry masses of fungi harvested after 14 days of experiments did not vary from appropriate controls.

When tested in microemulsions even in the smallest examined concentration (7.9 μM) betulin inhibited the growth of *F. dimerum* starting from the first day of experiment (Fig. 1), and from the third day in the case of *A. alternata*, *F. oxysporum* and *P. cyclopium*.

In time of intensive growth of the examined fungi, the suppression on fungal growth caused by betulin had grown day by day, reaching its maximum at the 7th day, what is shown in Fig. 2 considering the inhibition of growth versus betulin concentration. As can be seen the reduction of growth was fast up to 30 μM and then reached a steady state of fungal growth remaining in the range of 20–45%. Thus betulin seems to be a fungistatic rather than a toxic agent.

The betulin concentrations eliciting a 50% growth reduction over seven days (EC_{50}), which were determined from the exponential equation of correlation of inhibitory effect of betulin towards tested fungi in relation to its concentration, are given in Table III. These data indicate that *F. dimerum* appeared to be the most sensitive strain, whereas *P. cyclopium* was the most resistant.

It is worth to note that the natural content of betulin in birch bark and in phytopathogenic fungi studied in the present work is high enough to protect these organisms from the action of phytopathogenic and saprophytic lower fungi. Moreover, the phenomenon of effective inhibition of fungal growth by betulin incorporated in a betulin-trimyristin emulsion suggests that plant fats might act as carriers of betulin to lipophilic mycelia.

Table III. The 7th day EC_{50} values of betulin towards the examined fungal strains with confidence limit at 95%.

Test species	EC_{50} [μM]
Alternaria alternata	23.8 (21.2–26.4)
Penicillium cyclopium	46.8 (39.5–54.1)
Fusarium oxysporum	24.0 (19.4–28.6)
Fusarium dimerum	17.7 (15.3–20.1)

Experimental

General

All chemicals were purchased from POCh, Gliwice, Poland.

The ^{1}H NMR spectra were recorded at 400.13 MHz using a Bruker AVANCE DRX300 spectrometer with TMS (tetramethylsilane) as an internal standard. GC analyses were performed with a Hewlett Packard 6890 gas chromatograph equipped with an FID detector. Diethyl ether solution (1 μl) was applied onto an HP-5 capillary column (30 m × 0.32 mm, bonded-phase fused

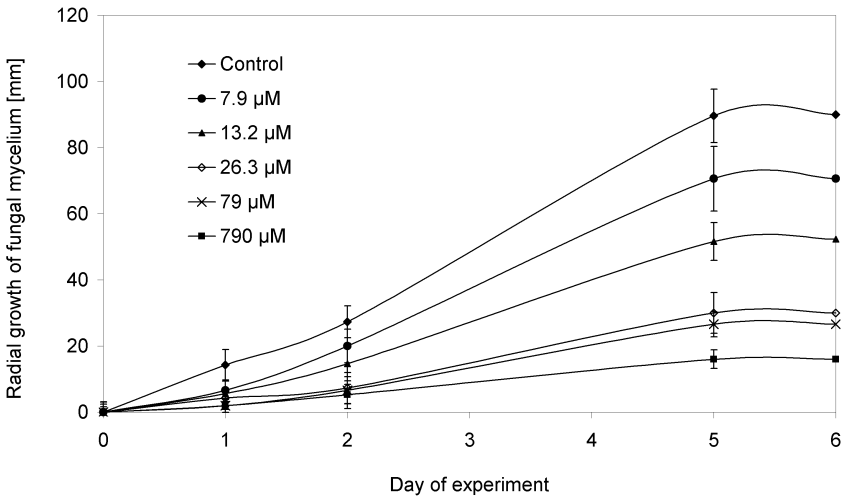

Fig. 1. The influence of betulin-trimyristin emulsion on the growth of *Fusarium dimerum* cultured in solid Czapek medium.

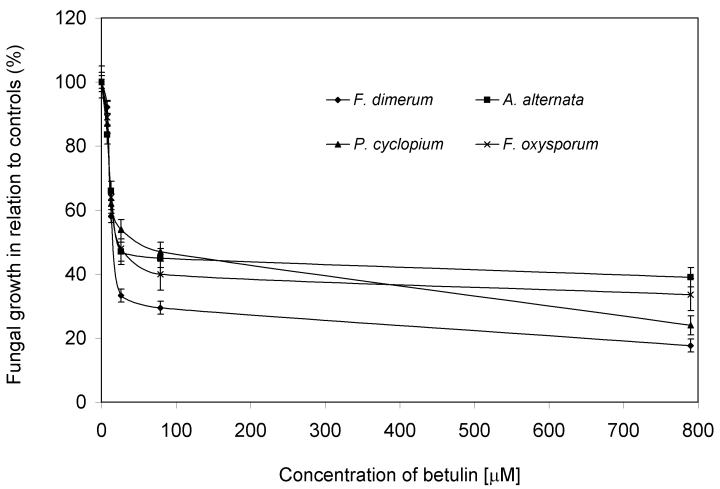

Fig. 2. The growth of the examined fungi dependent on the betulin dose in solid Czapek medium on the 7th day of the experiment.

silica). The initial oven temperature was maintained at 200 ºC for 2 min and then raised at 10 ºC min^{-1} to 280 ºC. Helium was used as a carrier gas. MS analyses were performed on a quadrupole Hewlett Packard 6897 instrument with ionization at 70 eV. The structure of the active compound was determined by peak matching library search of published standard mass spectra and by comparison with an authentic reference compound (Sigma-Aldrich Chemical Co.).

Plant material

The barks of *Betula pendula*, *Alnus glutinosa*, and *Corylus avellana* and the bracket fungi *Inonotus obliquus*, *Piptoporus betulinus*, and *Daedalea confragosa* were collected in October 2007 and May 2008 at Opole region, Poland.

Extraction and isolation

Betulin. Air-dried pulverized bark (10 g) was continuously extracted with 150 ml of the chosen solvent (ethanol, *n*-propanol, or *n*-butanol) in a Soxhlet apparatus for 5 h. The extract was concentrated under reduced pressure to a volume of 10 ml, and then it was mixed with 100 ml of distilled water and transferred into a glass separator where the aqueous phase was extracted 2 times with 30 ml of a mixture of benzene/diethyl ether (1:1, v/v). The organic phase was separated and washed with 25 ml water and 20% NaOH. Then the organic phase was dried over anhydrous $MgSO_4$ and evaporated *in vacuo*. Betulin was crystallized from methanol at room temperature. The purity of the white, crystalline product was examined by TLC (silica gel) using benzene/EtOAc (7:3) as eluent.

Trimyristin. Trimyristin was isolated from powdered nutmeg (*Myristica fragrans* Houtt.) according to a well known procedure described by Helmkamp and Johnson (1964).

The purities and molecular mass of the isolated compounds were determined by GC-MS, whereas their structure was confirmed by means of ^1H NMR spectroscopy.

Preparation of trimyristin and betulin-trimyristin microemulsions

Stable emulsions of pure trimyristin or a betulin-trimyristin mixture were obtained by placing 0.045 g of trimyristin or betulin solution in this fat into an 100-ml round-bottomed flask and adding 7.5 ml chloroform/methanol mixture (2:1, v/v). The organic solvents were gently removed on a rotary vacuum evaporator, heated up to 40 ºC, and rotated at 100 rpm. The obtained film was gently dried by passing cold air through the flask for 2 h. Then 39 ml of distilled water and 1 ml of physiological saline were added, and the flask was rotated at 60 rpm and heated up to 60 ºC for 15 min. After that the flask was closed, and its content was shaken out intensively for about 30 min. Finally the emulsion was stabilized ultrasonically – three times for 5 min with 3-min intervals – at 55 ºC. Emulsions of both trimyristin and betuline-trimyristin solution were stable for at least 28 d – neither their chemical composition was controlled by GC-MS nor microscopic picture changes were recorded during this time.

Bioassays on antifungal activity of betulin

Alternaria alternata (Fr.), *Fusarium dimerum* and *Fusarium oxysporum* (Schl.), and the saprophytic *Penicillium cyclopium* (Westling) originated from our fungal collection.

Stock suspension of the conidia of the examined fungi

The conidia of the examined strains were produced by appropriate fungus growing in standard Czapek medium at 26 ºC. The stock suspensions used for inoculation were prepared by washing the surface of 7- to 10-day-old cultures with 0.05% v/v sterilized suspension of Tween 80 in distilled water. Spore concentrations in the stock suspensions were determined using Thom's camera and then were diluted to reach maximally $1 \cdot 10^6$ conidia ml^{-1}.

Evaluation of the influence of trimyristin and betulin-trimyristin microemulsion on fungal growth

The bioassay was carried out in 9-cm Petri dishes using 25 ml of solidified Czapek medium supplemented with appropriate amounts of trimyristin (from 10 μM to 10 mM in the medium, added before sterilization) or betulin-trimyristin emulsion. The highest studied concentration of betulin in the medium, 790 μM, was chosen as this concentration is comparable with the natural content of this compound in *B. verrucosa* bark. Approximately 500-μl drops of spore stock suspension containing $5 \cdot 10^4$ spores of *A. alternata* and $5 \cdot 10^5$ spores of *F. oxysporum*, *F. dimerum*, and

P. cyclopium fungi were placed in the centre of the Petri dishes. These numbers of spores (established experimentally) guaranteed similar growth of the tested fungi in solid standard Czapek medium up to the 9th day of the experiment. Plates were incubated at 26 ºC, and the radial growth of each tested strain developing in the appropriate medium was recorded daily, by taking the mean diameter of fungal colony. The cultures of the examined fungi growing in standard Czapek medium were treated as the appropriate controls. Each experiment was carried out at least three times.

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