Effect of the Basidiomycete *Poria cocos* on Experimental Dermatitis and Other Inflammatory Conditions

María Jesús CUELLAR, Rosa María GINER, María del Carmen RECIO, María José JUST, Salvador MAÑEZ,* and José Luis RIOS

Departament de Farmacologia, Facultat de Farmàcia, Universitat de València, Avda. Vicent Andrés Estellés s/n, E-46100 Burjassot, València, Spain. Received July 29, 1996; accepted November 19, 1996

The hydroalcoholic extract from *P. cocos* was examined for oral and topical anti-inflammatory activities. It proved to be active against carrageenan, arachidonic acid, tetradecanoyl phorbol acetate (TPA) acute edemas, TPA chronic inflammation and oxazolone delayed hypersensitivity in mice. Two lanostane-type triterpenes were isolated and identified by spectroscopic methods as dehydrotumulosic and pachymic acids. Their ID_{50} on acute TPA edema was 4.7×10^{-3} and 6.8×10^{-4} μmol/ear, respectively.

Key words *Poria cocos*; Polyporaceae; triterpene; lanostane; *in vivo* anti-inflammatory activity

Poria cocos (SCHW.) WOLF (Polyporaceae) is a saprophytic fungus growing on diverse *Pinus* species such as *P. densiflora* y *P. mansoniana*. Its sclerotium, properly called "Hoelen," is a crude drug used because of its diuretic, sedative and tonic effects.[1] Although it is prescribed as a constituent of many preparations in Oriental medicine, there is no stated chemical rationale for its use. In a screening of crude drugs, Hoelen was found to have a remarkable inhibitory effect on the secretion of the cytokines IL-1β, IL-6, TNF-α and GM-CSF from human peripheral blood monocytes.[2] A protective effect on stress-induced ulcers and an inhibitory effect on picryl chloride-induced contact dermatitis, when administered topically, have been described.[3]

It has been reported to contain lanostane type triterpenes, some of them revealing anti-emetic activity.[4] Previous studies demonstrated these compounds to be cytotoxic against certain tumours, as were the polysaccharides pachyman and pachymaran from the same source.[3]

In this work, we have dealt with the effect of this fungus on certain acute and chronic inflammatory processes. For that purpose, the hydroalcoholic extract was assayed against different experimental models and later fractionated in order to isolate the principles responsible for the activity.

The hydroalcoholic extract of *P. cocos* was first tested against acute inflammation models. Topical treatment with 0.5 mg/ear of the extract exhibited a high degree of activity in the tetradecanoyl phorbol acetate (TPA)-induced edema, and resulted in an 80% decrease in ear thickness. A more moderate effect, *i.e.* 40% reduction, was observed when arachidonic acid (AA) was used as the inducer. This value is quite relevant when the dose applied is compared with nordihydroguaiaretic acid (NDGA) (40% at 0.5 mg/ear for the extract *versus* 50% at 2 mg/ear for the standard), and because of the fact that this test is very selective and many extracts or products fail to evidence appreciable activity. The very slight effect of the extract against the carrageenan paw edema was not statistically significant (Table 1, Fig. 1).

Secondly, the extract was assayed against chronic inflammation induced by repeated applications of TPA. It caused a 53% reduction in ear thickness. This inhibition

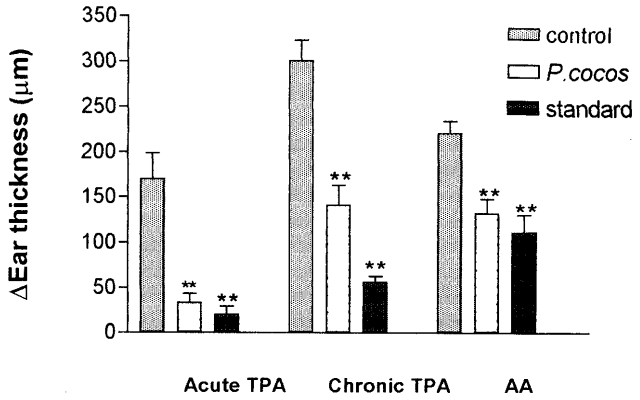

Fig. 1. ΔEar Thickness in Acute and Chronic Inflammation
** $p < 0.01$, $n = 6$.

Table 1. Percentages of Inhibition of Hydroalcoholic Extract on the Different Models of Inflammation in Comparison with Reference Drugs

	TPA[a] (Single)	TPA[a] (Repeated)	AA[a]	Oxazolone[a]					Carrageenan[b]		
				24 h	48 h	72 h	96 h	102 h	1 h	3 h	5 h
EtOH extract	80	53	40	15	14	41	29	32	7	22	9
Indomethacin	86	—	—	—	—	—	—	—	—	—	—
Dexamethasone	—	81	—	76	89	93	81	84	—	—	—
NDGA	—	—	50	—	—	—	—	—	—	—	—
Phenylbutazone	—	—	—	—	—	—	—	—	20	64	34

a) Topical administration. *b*) Subcutaneous paw administration. EtOH extract: 100 mg/kg, *p.o.* (carrageenan test); 0.5 mg/ear[a] (single TPA and AA tests); 1 mg/ear[a] (repeated TPA and oxazolone). Indomethacin: 0.5 mg/ear.[a] Dexamethasone: 0.05 mg/ear.[a] NDGA = nordihydroguaiaretic acid: 2 mg/ear.[a] Phenylbutazone: 100 mg/kg, *p.o.*

* To whom correspondence should be addressed.

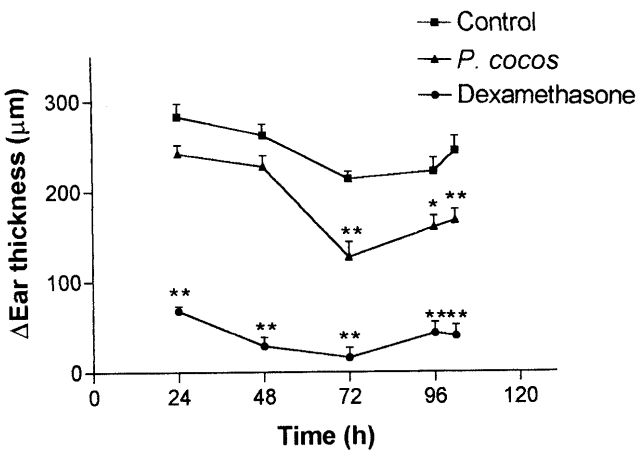

Fig. 2. ΔEar Thickness in the Oxazolone Test

$*p<0.05$, $**p<0.01$, $n=6$.

was concomitant with a 73% decrease in myeloperoxidase activity with respect to the TPA-treated controls, which indicates that leukocyte recruitment into the damaged tissue could be prevented at a dose of 1.0 mg/ear. With respect to the oxazolone-induced delayed hypersensitivity, the extract reduced the ear edema and had little effect on the myeloperoxidase activity. The reduction in the edema increases progressively along the daily treatment with the extract, reaching the highest inhibition rate at 72 h (Fig. 2). This means that either a latency period or a repetitive-dose administration schedule is needed prior to the extract to produce its effect. The myeloperoxidase assay is an end-point analysis for both chronic TPA inflammation and oxazolone delayed hypersensitivity. The extract has shown quite different behavior in these two models, which can only be justified by distinct abilities to face polymorphonuclear recruitment into skin. In spite of the data reported in the literature,[2] it seems that *P. cocos* better counteracts the infiltration caused by TPA, which is thought to be remarkably mediated by lipoxygenase products[5] like leukotriene B_4, than that of oxazolone, mediated by several interleukines secreted by activated T-lymphocytes.[6]

Two active compounds were isolated from the extract, by precipitating with cold MeOH, then with EtOAc and further purification by column chromatography. They were identified by spectroscopic methods as pachymic acid (3β-acetyloxy-16α-hydroxylanosta-8,24(31)-dien-21-oic acid) and dehydrotumulosic acid (3β,16α-dihydroxylanosta-7,9(11),24(31)-trien-21-oic acid). ^{1}H-NMR revealed signals for eight methyl and two methylene protons (2 singlets at 4.56 and 4.59 ppm) consistent with a lanostane triterpene structure, which was confirmed by the ^{13}C-NMR spectrum. It showed resonances attributable to alcoholic carbons at 3β-OH (substituted, 81.1 ppm) and 16α-OH (free, 76.7 ppm) and to carboxyls of an acetyl (171.5 ppm) esterifying 3-OH and one free at C-21 (178.7). Two unsaturated quaternary carbons (133.9 and 134.0 ppm) indicate the presence of a double bond at 8(9). The second isolate showed resonances indicative of two hydroxylated carbons, 3β-OH (79.0 ppm) and 16α-OH (76.2 ppm), and absence of esterification. Three double bonds at 24(31) (107.0 and 155.8 ppm), 7(8) (121.4 and 141.5 ppm) and 9(11) (146.0 and 116.4 ppm) were also observed. Both compounds have been described previously in this species.[1,7]

Table 2. Inhibitory Effect of Pachymic Acid, Dehydrotumulosic Acid and aq. EtOH Extract of *Poria cocos* on Acute TPA-Induced Inflammation in Mice

Sample	ID_{50} μmol/ear	ΔEar thickness (mm × 10^{-3}) ± S.E.M.	I.R.
TPA (control)	—	170.8 ± 27.9	—
EtOH extract	—	34.2 ± 9.4**	80
Pachymic acid	4.7×10^{-3} a) (1.9×10^{-3}—1.9×10^{-2})	58.1 ± 8.6**	66
Dehydrotumulosic acid	6.8×10^{-4} b)	44.4 ± 6.9**	74
Indomethacin	3.5×10^{-1} c) (4.3×10^{-1}—1.5×10^{-1})	20.8 ± 8.4**	88

ID_{50} = 50% inhibitory dose. 95% confidence limits given in parentheses. *a)* r (correlation coefficient) = 0.9993. $P=0.0241$ (Anova test, significant). *b)* $r=0.9950$, $P=0.0639$ (not quite significant). *c)* $r=0.9993$, $P=0.0231$ (significant). I.R. = inhibition ratio at 0.5 mg/ear. $**p<0.01$ by Dunnet's t-test compared with the control group.

Topical administration of these lanostane derivatives showed comparable and marked inhibition of edema, which reached values of more than 80%, and ID_{50} values were calculated (Table 2). They exhibited ID_{50} of 4.7×10^{-3} and 6.8×10^{-4} μmol/ear, respectively, in the TPA induced edema test, and both, but particularly dehydrotumulosic acid, were more potent than indomethacin. They are the major constituents of this fungus and could be the principles responsible for its anti-inflammatory activity. Further extraction of crude drug would make it possible to obtain the amounts necessary to assess their efficacy and potency in other tests.

These results support the use of this species in the so-called natural therapeutics. Its effectiveness in the models assayed makes it an interesting agent for the treatment of certain inflammatory diseases, and adds other indications to those provided by Chinese medicinal traditions. It has a low toxicity since it is used *p.o.* at a dose of 6—18 g of sclerotia/day without any trouble.[3] Interestingly, it should be noted that the rate of the active principle extraction is in fact low, only 1.4% extract *versus* the dry sclerotia and approx. 1% of each compound *versus* the extract.

Many studies have reported pentacyclic triterpenoids as active principles of various anti-inflammatory species, but few have described the structural requirements for this activity.[8] Basic skeleton has no influence on the activity, but an increase in oxygenated functions enhances the effect. On the other hand, glycosylation reduces it, as occurs with the triterpenoid derivatives from *Tetrapanax papyriferum*.[9]

Dehydrotumulosic and pachymic acids contain hydroxyl groups at 3 and 16, and a carboxyl group at 21, located on the tetracyclic lanostane nucleus and on the side chain. The lanostane derivatives are much less frequent than the well-known oleanane and ursane nuclei, and no studies are available on the structure–activity relationship. The higher potency of dehydrotumulosic acid could be attributed to the presence of a free 3-OH that is blocked by acetylation in pachymic acid. Moreover, dehydrotumulosic acid presents an heteroannular 7—8, 9—11-dien

group, which is thought to increase the planarity of the molecule and could be one reason for its greater activity.

Experimental

General Experimental Procedures Identification was carried out by ^1H (400 MHz) and ^{13}C (75 MHz) on a Bruker AMX-400 spectrometer using $CDCl_3$ solutions. High resolution FAB-MS was recorded on a VG Analytica Fisons spectrometer. Compounds were visualized on TLC plates by sulphuric anisaldehyde. Silica gel 60 (Merck) was used for CC and Silica gel 60G (Merck) on TLC plates.

Animals Groups of six female Swiss mice weighing 25—30 g were used. All animals were fed a standard diet *ad libitum* and maintained in suitable environmental conditions throughout the experiments.

Vegetal Material Sclerotia of *Poria cocos* (SCHW.) WOLF (Polyporaceae) were supplied by Asia Natural Products S.L. (Amposta, Spain).

Chemicals Carrageenan, 12-tetradecanoyl phorbol acetate (TPA), arachidonic acid, oxazolone, hydrogen peroxide, phosphate buffer saline (PBS), *N*,*N*-dimethylformamide, tetramethylbenzidine (TMB), hexadecyltrimethylammonium bromide (HTAB), the reference drugs phenylbutazone, indomethacin, nordihydroguaiaretic acid and dexamethasone were purchased from Sigma Chemical Co., St. Louis and sodium acetate from Panreac, Barcelona.

Extraction and Isolation of the Compounds Dried sclerotia of *Poria cocos* (298.8 g) were treated with hot 50% aq. EtOH. By concentration under reduced pressure the hydroalcoholic extract was obtained (3.4 g) and lyophilized. It was purified by precipitation with cold MeOH (5 °C), centrifuged and reprecipitated with EtOAc. The last supernatant (826.6 mg) was chromatographed over a silica gel column with $CHCl_3$/EtOAc mixtures to yield two compounds, **1** (43 mg) and **2** (40 mg), which were identified by ^1H- and ^{13}C-NMR spectral analysis.

Carrageenan-Induced Mouse Paw Edema[10] An edema was induced on the right hind foot of mice by subplantar injection of 0.05 ml of a solution of 3% carrageenan in 0.9% saline (w/v). The volumes of the injected and contralateral paws were measured 1, 3 and 5 h after induction of inflammation using a plethysmometer (Ugo Basile), and the edema was expressed as an increase in paw volume due to carrageenan injection. A reference group was treated with phenylbutazone (100 mg/kg, *p.o.*). Extract and pure compounds, dissolved in EtOH/Tween 80/H_2O (2 : 2 : 20, v/v), were administered orally at 100 mg/kg (0.50 ml) 1 h before carrageenan injection. A control group received the vehicle only.

12-*O*-Tetradecanoyl Phorbol-13-Acetate (TPA)-Induced Mouse Ear Edema[11] An edema was induced on the right ear by topical application of 2.5 μg/ear of TPA in acetone. The left ear (control) received the vehicle (acetone or 70% aq. EtOH). Extract and pure compounds, dissolved in 70% aq. EtOH and acetone, respectively, were applied topically (0.5 mg/ear) simultaneously with TPA. The thickness of the ears was measured 4 h after induction of inflammation using a micrometer (Mitutoyo Serie 293). The edema was expressed as an increase in the ear thickness due to TPA application. A reference group was treated with indomethacin (0.5 mg/ear). ID_{50} values of the isolated compounds were calculated by linear regression analysis of the relationship dose/response for at least three doses between 1×10^{-2} and 1×10^{-4} mg/ear.

Arachidonic Acid (AA)-Induced Mouse Ear Edema[12] AA was dissolved in acetone at a concentration of 100 mg/ml. An edema was induced on the right ear by topical application of 2 mg/ear of AA in acetone. The left ear (control) received the vehicle (acetone or 70% aq. EtOH). Extract and pure compounds, dissolved in 70% aq. EtOH and acetone, respectively, were applied topically (0.5 mg/ear), 30 min before the application of AA. The thickness of the ears was measured 1 h after induction of inflammation using a micrometer. The edema was expressed as an increase in the ear thickness due to AA application. A reference group was treated with nordihydroguaiaretic acid (2 mg/ear).

Mouse Ear Edema Induced by Multiple Topical Applications of TPA[13] Chronic inflammation was induced by topical application of 10 μl of TPA (2.5 μg/ear) to the inner and outer surface of both ears of each mouse with a micropipette on alternate days. The MeOH extract was dissolved in 70% aq. EtOH and applied topically (1 mg/ear) twice daily for 4 d, in the morning immediately after TPA application and 6 h later. Dexamethasone was used as the reference drug (0.05 mg/ear). The thickness of each ear was measured using a micrometer, before and after treatment; therefore each ear served as its own control. The swelling was assessed in terms of the means of the increase in the thickness of each ear.

Oxazolone-Induced Contact-Delayed Hypersensitivity Mouse Ear Edema[12] Female mice were sensitized by topical application on the shaven ventral abdomen of 50 μl of a 2% (w/v) solution of oxazolone in acetone on two consecutive days (days 1 and 2). Challenge was performed on day 6 by application of 30 μl of 2% oxazolone to both ears. Hydroalcoholic extract and dexamethasone were applied (30 μl) to right ears 6 h after challenge (single application) and 24, 48, 72 and 96 h after challenge (repeated dosage). Ear thickness measurements of treated and control groups were made with a micrometer 24, 48, 72, 96 and 102 h after challenge and just before drug application. The final measurement was performed immediately before sacrifice. The thickness of each ear was measured as described in the previous test.

Myeloperoxidase Assay[14] Each biopsy was placed in an Eppendorf tube containing 0.75 ml of 0.5% HTAB in 80 mM sodium phosphate buffer (pH = 5.4). The tissue was then homogenized (45 s). The supernatant (30 μl) was assayed by mixing it with 20 μl of TMB 18.4 mM and 15 μl of H_2O_2 0.017% in a 96-well microtiter plate. The mixture was incubated for 3 min at 37 °C. Enzyme activity was determined colorimetrically using a Labsystems Multiskan MCC/340 plate reader set to measure absorbance at 620 nm.

Statistics Percentages of edema reduction are expressed by the mean with S.E.M. Dunnet's *t*-test for unpaired data was used for statistical evaluation.

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