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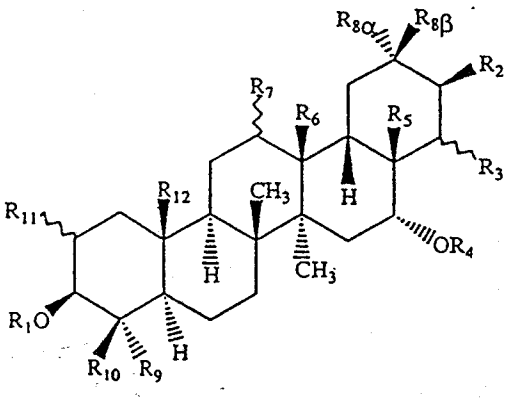
(54) **Titre :** Antiprotozoal saponins.

(57) **Abrégé :** Saponins of formula (I), a stereoisomeric form thereof or a pharmaceutically acceptable addition salt thereof, wherein R_1 to R_{12} have the meaning given in the description, can be isolated from plants of the family *Myrsinaceae* and used to decrease the infectiousness of and reduce the mortality associated with protozoan parasites of the genus *Leishmania* which are responsible for a group of conditions known as leishmaniases.



ANTIPROTOZOAL SAPONINS

The present invention is concerned with a process for the isolation of antiprotozoal saponins from plants belonging to the family *Myrsinaceae* and the use of said saponins for preparing a medicament for treating hosts, both men and animals, infected by protozoan parasites of the genus *Leishmania*, and for alleviating clinical manifestations of, and curing disorders known as leishmaniases in said hosts.

Leishmaniases present a large variety of disease manifestations differing markedly in their severity and health impact. Primarily, leishmaniases are debilitating conditions caused by any of several species of *Leishmania* and are transmitted by several *Phlebotomine* sandflies. The leishmaniases appear to be far more abundant and of greater public health importance than has been previously recognized. Control of leishmanial infections is complicated because many species of sandfly are potential vectors, because many animal species can act as reservoir hosts and because diagnostic procedures (clinical, serological, parasitological) are not always applicable or have limited acceptable diagnostic value.

The manifestations may be visceral, mucocutaneous and/or cutaneous and the strain of the infecting organism and the immunologic status of the host can influence the clinical manifestations and outcome of the parasitic disease. Treatment of leishmaniases is complex and prolonged systemic treatment is imperative. The objectives of treatment are to cure the human or animal patient of an intracellular parasitic infection, to prevent relapse, to avoid development of unresponsiveness and to keep hospitalisation and overall treatment costs to a minimum. To achieve these objectives, appropriate drugs must be given at adequate dose levels and frequency for a suitable period of time. Despite the extensive research in the search of effective and well tolerated antileishmanial agents, only few agents have been discovered and are available to the patient. Currently, two pentavalent antimony compounds that have to be administered by deep intramuscular injection are commonly used as first-line drugs: meglumine antimonate (Glucantim™, Farmitalia) and sodium stibogluconate (Pentostam™, Wellcome). Second-line drugs are amphothericin-B (in particular the liposomal formulations), pentamidine and allopurinol. The currently available therapies are not sufficiently effective and cause toxic side effects in the patient. In addition, their spectrum of activity is not sufficiently broad. For these reasons, the need for new medications remains very high. The present identification of new active principles will

have use in the treatment of disorders caused by protozoan parasites belonging to the genus *Leishmania*.

Unexpectedly, triterpene saponins having very potent prophylactic as well as therapeutical activity against *Leishmania* have been isolated from the plants *Maesa balansae and M. lanceolata*, two species from the family *Myrsinaceae*, genus *Maesa*.

The invention is directed to a process for the isolation of triterpene saponins from plants belonging to the family *Myrsinaceae*, characterized in that said process comprises the steps of

(a) removing apolar material from the dried plant parts with an apolar solvent,
(b) extracting said plant parts with an alcohol, and
(c) further purifying the saponins in the alcohol extract by liquid-liquid extraction, filtration and chromatography.

In particular, the apolar solvent is a halogenated hydrocarbon, e.g. dichloromethane or chloroform ; and the alcohol is methanol, ethanol, isopropanol, butanol, each optionally admixed with water. Particularly useful is a mixture of methanol : water (90 : 10) or a mixture of ethanol : water (70 : 30) for isolating the fraction containing the saponins.

The saponins of the alcohol extract are further purified by
(c1) evaporating the extract to dryness,
(c2) partitioning the residue between butanol and water,
(c3) evaporating the organic layer to dryness,
(c4) washing the residue in a ketone and
(c5) filtering off the crude saponin mixture.
In step (c2), the water layer is preferably extracted several times with n-butanol.

The invention is also directed to an alternative process for the isolation of triterpene saponins from plants belonging to the family *Myrsinaceae*, characterized in that said process comprises the steps of

(a) extracting the dried plant parts with an alcohol and concentrating the extract,
(b) removing the apolar fraction from the extract by liquid-liquid extraction with an apolar solvent, and
(c) further purifying the saponins in the alcohol extract by liquid-liquid extraction, filtration and chromatography.

In particular, the alcohol is methanol, ethanol, isopropanol, butanol, each optionally

admixed with water, preferably a mixture of ethanol : water (70 : 30); the apolar solvent is a hydrocarbon, e.g. hexane.

The saponins of the alcohol extract are further purified by
(c6) extracting the aqueous fraction with butanol satured with water,
(c7) evaporating the organic layer to dryness,
(c8) washing the residue in a ketone, and
(c9) filtering off the crude saponin mixture,
In step (c6), the water layer is preferably extracted several times with n-butanol.

When the saponins are isolated from the plant genus *Maesa*, the chromatography can comprise reversed-phase liquid chromatography with gradient eluent system using

A : 0.5 % ammonium acetate in water
B : methanol
C : acetonitrile

wherein at t = 0, (A:B:C) = (60:20:20) and t = end, (A:B:C) = (0:50:50), or straight-phase liquid chromatography on silicagel.

These processes yield a mixture that consists essentially of saponins. In many pharmacological experiments described in the experimental part, this mixture of saponins was used. For the purpose of structure elucidation, this mixture was separated into the individual constituents by HPLC as described in the experimental part. .

The present invention thus also relates to one or more triterpene saponins obtainable by the processes described herein, whether as a mixture or as isolated products.

In particular, the invention concerns triterpene saponins obtainable from the plant genus *Maesa*, by chromatography comprising reversed-phase liquid chromatography with gradient eluent system using

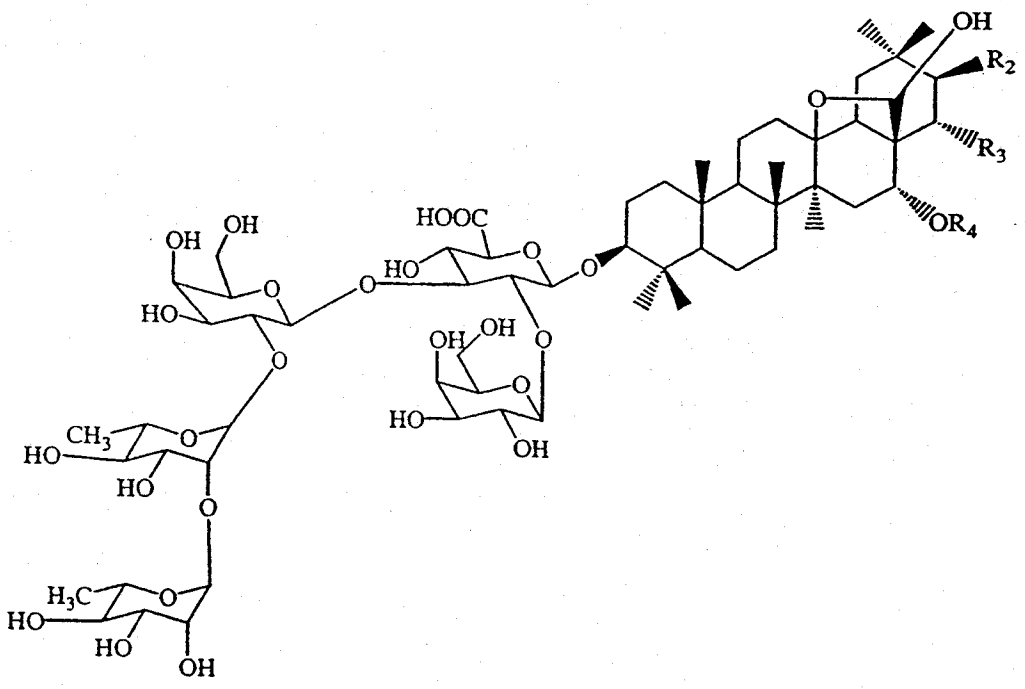
A : 0.5 % ammonium acetate in water
B : methanol
C : acetonitrile

wherein at t = 0, (A:B:C) = (60:20:20) and t = end, (A:B:C) = (0:50:50), and wherein said saponin has the following characteristics :

Compound 1 : MW = 1532, λ_{max} = 228.6 nm, λ_{max2} = 273.3 nm ; t_R = 8.97
Compound 2 : MW = 1510, λ_{max} = 223.9 nm, λ_{max2} = 274.5 nm ; t_R = 9.39
Compound 3 : MW = 1532, λ_{max} = 279.2 nm, λ_{max2} = 223.9 nm ; t_R = 9.68
Compound 4 : MW = 1510, λ_{max} = 280.4 nm, λ_{max2} = 222.7 nm ; t_R = 10.09

Compound 5 : MW = 1574, λ_{max} = 276.8 nm, λ_{max2} = 225.0 nm ; t_R = 10.87 ; and
Compound 6 : MW = 1552, λ_{max} = 279.2 nm, λ_{max2} = 223.9 nm ; t_R = 11.37.
The relative retention time t_R is the mean value of 10 measurements versus the retention time of uracil on a column Hypersil BDS C-18, 3 µm, 100 x 4 mm.

Specifically, the present invention concerns triterpene saponins having the formula

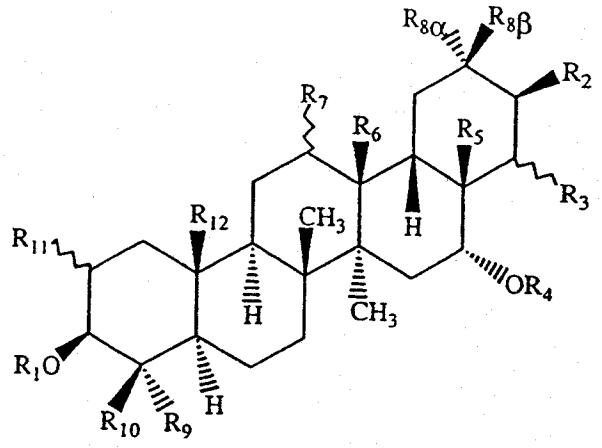


wherein R_2 is $-O(C=O)C_6H_5$ or $-O(C=O)C(CH_3)=CHCH_3$,
R_3 is (E) or (Z) $-O(C=O)CH=CH-C_6H_5$, and
R_4 is hydrogen or $-(C=O)CH_3$;
more in particular,
in compound 1, R_2 is $-O(C=O)C_6H_5$,
R_3 is (Z) $-O(C=O)CH=CH-C_6H_5$,
R_4 is hydrogen;
in compound 2, R_2 is $-O(C=O)C(CH_3)=CHCH_3$,
R_3 is (Z) $-O(C=O)CH=CH-C_6H_5$,
R_4 is hydrogen;
in compound 3, R_2 is $-O(C=O)C_6H_5$,
R_3 is (E) $-O(C=O)CH=CH-C_6H_5$,
R_4 is hydrogen;
in compound 4, R_2 is $-O(C=O)C(CH_3)=CHCH_3$,
R_3 is (E) $-O(C=O)CH=CH-C_6H_5$,
R_4 is hydrogen;

in compound 5, R_2 is $-O(C=O)C_6H_5$,
R_3 is (E) $-O(C=O)CH=CH-C_6H_5$,
R_4 is $-(C=O)CH_3$;
in compound 6, R_2 is $-O(C=O)C(CH_3)=CHCH_3$,
R_3 is (E) $-O(C=O)CH=CH-C_6H_5$,
R_4 is $-(C=O)CH_3$;

Preferred compounds for use in the pharmaceutical compositions and methods of treatment of the present invention are compounds 3 and 4, in particular compound 3.

Specifically, the present invention concerns the use of one or more triterpene saponins for the preparation of a pharmaceutical composition for treating leishmaniases in hosts infected by *Leishmania* species, characterized in that the saponin has the formula (I)



a stereoisomeric form thereof or a pharmaceutically acceptable addition salt thereof, wherein

R_1 is hydrogen, $-(C=O)C_{1-5}$alkyl, $-(C=O)C_{2-5}$alkenyl, $-(C=O)C_{2-5}$alkenyl substituted with phenyl, a monosaccharide group or an oligosaccharide group ;

R_2 is hydrogen, hydroxy, $-O(C=O)C_{1-5}$alkyl, $-O(C=O)C_{2-5}$alkenyl, $-O(C=O)C_6H_5$, or $-O(C=O)C_{2-5}$alkenyl substituted with phenyl ;

R_3 is hydrogen, hydroxy, $-O(C=O)C_{1-5}$alkyl, $-O(C=O)C_{2-5}$alkenyl, $-O(C=O)C_6H_5$, or $-O(C=O)C_{2-5}$alkenyl substituted with phenyl ;

R_4 is hydrogen, C_{1-6}alkyl, $-(C=O)C_{1-5}$alkyl, $-(C=O)C_{2-5}$alkenyl, $-(C=O)C_6H_5$, or $-(C=O)C_{2-5}$alkenyl substituted with phenyl ;

R_5 is CH_3, CH_2OH, CH_2OCH_3, CH_2O-$C(=O)CH_3$, CHO, COOH ; or

R_5 and R_2 form a divalent radical of formula $-C(=O)-O-$;

R_6 and R_7 are hydrogen; or taken together they form a bond; or

R_5 and R_6 form a divalent radical of formula

$-CH_2-O-$ (a),

$-CH(OR_{13})-O-$ (b),

$-C(=O)-O-$ (c),

wherein R_{13} is hydrogen, C_{1-6}alkyl or $-(C=O)C_{1-5}$alkyl ;

$R_{8\alpha}$ and $R_{8\beta}$ each independently represent CH_3, CH_2OH, CH_2OCH_3, $CH_2O-C(=O)$ C_{1-5}alkyl, CHO, $CH(OCH_3)_2$, CH=NOH, COOH ; or

$R_{8\beta}$ and R_3 form a divalent radical of formula $-C(=O)-O-$; or

$R_{8\beta}$ and R_5 form a divalent radical of formula $-CH_2O-CHOH-$;

R_9 is CH_3, CH_2OH, CH_2OCH_3, $CH_2O-C(=O)C_{1-5}$alkyl, CHO, COOH ;

R_{10} is CH_3, CH_2OH, CH_2OCH_3, $CH_2O-C(=O)C_{1-5}$alkyl, CHO, COOH ;

R_{11} is hydrogen, hydroxy or $O-C(=O)C_{1-5}$alkyl ; or R_{10} and R_{11} form a divalent radical of formula - CH_2O- ; and

R_{12} is CH_3, CH_2OH, CH_2OCH_3, $CH_2O-C(=O)CH_3$, CHO, CH=NOH or COOH.

Preferred are the compounds of formula (I) wherein

R_1 is hydrogen, $-(C=O)C_{1-5}$alkyl, or an oligosaccharide group ;

R_3 is hydrogen, hydroxy, $-O(C=O)C_{1-5}$alkyl, $-O(C=O)C_{2-5}$alkenyl, $-O(C=O)C_{2-5}$alkenyl substituted with phenyl ;

R_4 is hydrogen, C_{1-6}alkyl, $-(C=O)C_{1-5}$alkyl, $-(C=O)C_{2-5}$alkenyl ;

R_5 is CH_2OH, $CH_2O-C(=O)CH_3$, CHO ; and

R_6 and R_7 taken together form a bond; or

R_5 and R_6 form a divalent radical of formula

$-CH_2-O-$ (a),

$-CH(OR_{13})-O-$ (b),

$-C(=O)-O-$ (c),

wherein R_{13} is hydrogen, C_{1-6}alkyl or $-(C=O)C_{1-5}$alkyl ; and

R_7 is hydrogen ;

$R_{8\alpha}$ represents CH_3 ;

$R_{8\beta}$ represents CH_3, CH_2OH, CHO, $CH(OCH_3)_2$, CH=NOH, COOH ; or

$R_{8\beta}$ and R_3 form a divalent radical of formula $-C(=O)-O-$; or

$R_{8\beta}$ and R_5 form a divalent radical of formula $-CH_2O-CHOH-$;

R_{10} is CH_3, CH_2OH ;

R_{11} is hydrogen, hydroxy or $O-C(=O)C_{1-5}$alkyl ; or

R_{10} and R_{11} form a divalent radical of formula $-CH_2O-$; and

R_{12} is CH_3, CH_2OH, $CH_2O-C(=O)CH_3$, CHO, CH=NOH.

Especially preferred compounds are those of formula (I) wherein

R_1 is hydrogen or an oligosaccharide group ;

R_2 is hydrogen, hydroxy, -O(C=O)C_{1-5}alkyl, -O(C=O)C_{2-5}alkenyl, -O(C=O)C_6H_5 or -O(C=O)C_{2-5}alkenyl substituted with phenyl ;

R_3 is hydrogen, hydroxy, -O(C=O)C_{1-5}alkyl, -O(C=O)C_{2-5}alkenyl, -O(C=O)C_{2-5}alkenyl substituted with phenyl ;

R_4 is hydrogen, C_{1-6}alkyl, -(C=O)C_{1-5}alkyl, -(C=O)C_{2-5}alkenyl, -(C=O)C_{2-5}alkenyl substituted with phenyl ;

R_5 is CH_2OH, CH_2OCH_3, CH_2O-C(=O)CH_3, CHO, COOH ; and

R_6 and R_7 taken together form a bond; or

R_5 and R_6 form a divalent radical of formula

-CH_2-O- (a),

-$CH(OR_{13})$-O- (b),

-C(=O)-O- (c),

wherein R_{13} is hydrogen ; and

R_7 is hydrogen ;

$R_{8\alpha}$ and $R_{8\beta}$ both represent CH_3 ;

R_9 is CH_3 ;

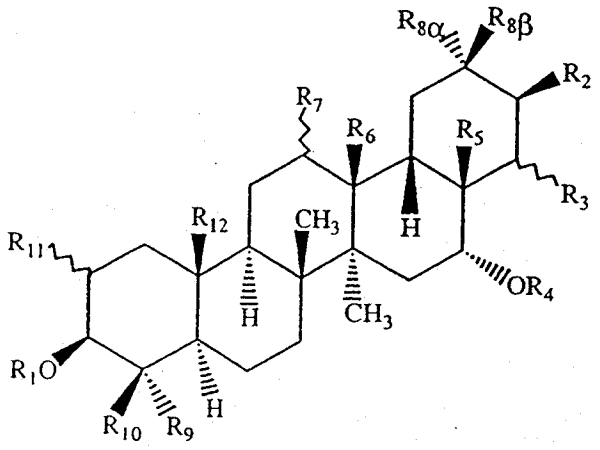
R_{10} is CH_3 ;

R_{11} is hydrogen ; and

R_{12} is CH_3.

The compounds of formula (I) may be converted into each other through art-known processes. Particularly interesting processes are saponification in basic media, trans-esterification in acidic media, and enzymatic degradation of the oligosaccharide moiety so as to produce aglycones, *i.e.* compounds of formula (I) wherein R_1 is hydrogen.

The present invention also concerns a method of alleviating clinical manifestations of, and curing disorders known as leishmaniases attributable to infection by protozoan parasites of the genus *Leishmania* in both men and animals, comprising administering to an infected host a therapeutically effective amount of a compound of formula (I)



a stereoisomeric form thereof or a pharmaceutically acceptable addition salt thereof, wherein

R_1 is hydrogen, -(C=O)C_{1-5}alkyl, -(C=O)C_{2-5}alkenyl, -(C=O)C_{2-5}alkenyl substituted with phenyl, a monosaccharide group or an oligosaccharide group ;

R_2 is hydrogen, hydroxy, -O(C=O)C_{1-5}alkyl, -O(C=O)C_{2-5}alkenyl, -O(C=O)C_6H_5, or -O(C=O)C_{2-5}alkenyl substituted with phenyl ;

R_3 is hydrogen, hydroxy, -O(C=O)C_{1-5}alkyl, -O(C=O)C_{2-5}alkenyl, -O(C=O)C_6H_5, or -O(C=O)C_{2-5}alkenyl substituted with phenyl ;

R_4 is hydrogen, C_{1-6}alkyl, -(C=O)C_{1-5}alkyl, -(C=O)C_{2-5}alkenyl, -(C=O)C_6H_5, or -(C=O)C_{2-5}alkenyl substituted with phenyl ;

R_5 is CH_3, CH_2OH, CH_2OCH_3, CH_2O-C(=O)CH_3, CHO, COOH ; or

R_5 and R_2 form a divalent radical of formula -C(=O)-O- ;

R_6 and R_7 are hydrogen; or taken together they form a bond; or

R_5 and R_6 form a divalent radical of formula

-CH_2-O- (a),

-$CH(OR_{13})$-O- (b),

-C(=O)-O- (c),

wherein R_{13} is hydrogen, C_{1-6}alkyl or -(C=O)C_{1-5}alkyl ;

$R_{8\alpha}$ and $R_{8\beta}$ each independently represent CH_3, CH_2OH, CH_2OCH_3, CH_2O-C(=O) C_{1-5}alkyl, CHO, $CH(OCH_3)_2$, CH=NOH, COOH ;

$R_{8\beta}$ and R_3 form a divalent radical of formula -C(=O)-O- ;

$R_{8\beta}$ and R_5 form a divalent radical of formula -CH_2O-CHOH- ;

R_9 is CH_3, CH_2OH, CH_2OCH_3, CH_2O-C(=O)C_{1-5}alkyl, CHO, COOH ;

R_{10} is CH_3, CH_2OH, CH_2OCH_3, CH_2O-C(=O)C_{1-5}alkyl, CHO, COOH ;

R_{11} is hydrogen, hydroxy or O-C(=O)C_{1-5}alkyl ; or R_{10} and R_{11} form a divalent radical of formula -CH_2O- ; and

R_{12} is CH_3, CH_2OH, CH_2OCH_3, CH_2O-C(=O)CH_3, CHO, CH=NOH, or COOH.

For the purposes of treating leishmaniases, the compounds of formula (I) may be administered orally, topically, parenterally, by inhalation spray or rectally in dosage unit formulations containing conventional nontoxic pharmaceutically acceptable carriers, adjuvants and vehicles. The term parenteral as used herein includes subcutaneous injection, intravenous, intramuscular, intrasternal injection, intraarticular injection, or infusion techniques in subjects susceptible to leishmania organism infection.

The pharmaceutical compositions containing the active ingredient may be in a form suitable for oral use, for example, as tablets, troches, lozenges, aqueous or oily solutions or suspensions, dispersible powders or granules, emulsions, hard or soft capsules, or syrups or elixirs. Compositions intended for oral use may be prepared according to any method known to the art for the manufacture of pharmaceutical compositions and such compositions may contain one or more agents selected from the group consisting of sweetening agents, flavoring agents, coloring agents and preserving agents in order to provide a pharmaceutically elegant and palatable preparation. Tablets contain the active ingredient in admixture with non-toxic pharmaceutically acceptable excipients which are suitable for the manufacture of tablets including but not limited to inert diluents, granulating and disintegrating agents, and lubricating agents. Tablets may be uncoated or they may be coated by known techniques to delay disintegration and adsorption in the gastrointestinal tract thereby providing a sustained action over a longer period ; to mask an unpleasant taste or mouthfeel; or to improve appearance and recognizability.

Aqueous suspensions contain the active materials in admixture with excipients suitable for the manufacture of aqueous suspensions. Such excipients are suspending agents, dispersing or wetting agents. The said aqueous suspensions may also contain one or more preservatives, and oily suspensions may be formulated by suspending the active ingredient in a suitable vegetable oil or in a mineral oil such as liquid paraffin. The oily suspensions may contain thickening agents, sweetening agents, and flavoring agents to provide a palatable oral preparation. These compositions may be preserved by the addition of an acceptable antioxidant.

Dispersible powders and granules suitable for preparation of aqueous suspensions by the addition of water provide the active ingredient in admixture with a dispersing or wetting agent, suspending agent and one or more preservatives.

The pharmaceutical compositions of the present invention may also be in the form of

oil-in-water (o/w) or water-in-oil (w/o) emulsions. The oily phase may be a pharmaceutically suitable vegetable oil, arachis oils, or a mineral oil, containing suitable emulsifying agents and antioxidants. The aqueous phase may contain emulsifying agents, thickening agents and preservatives. The emulsions may also contain sweetening, coloring and flavoring agents.

Syrups and elixirs may be formulated with sweetening agents. Such formulations may also contain a demulcent, a preservative and flavoring and coloring agents.

The pharmaceutical compositions may be in the form of a sterile injectable preparation, for example as a sterile injectable aqueous or oleaginous solution or suspension in a non-toxic parenterally acceptable diluent or solvent. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono- or diglycerides, fatty acids and other suitable additives of injectables. Suspensions may be formulated according to the known art using suitable dispersing or wetting agents and suspending agents.

The compounds of formula (I) may also be administered in the form of suppositories or other formulations such as solutions or suspensions for rectal administration of the drug. Suppositories can be prepared by mixing the drug with a suitable non-irritating excipient which is solid at ordinary temperatures but liquid at the rectal temperature to release the drug.

The daily dosage of the compounds of formula (I) may be varied over a wide range, *e.g.* from 1.0 to 2,000 mg. Preferably, the compound of formula (I) with a carrier in a pharmaceutical composition, is administered in subdivided doses containing 5, 10, 25, 50, 100, 150, 250 or 500 mg of the active ingredient for the appropriate dosage to the patient to be treated. An effective amount of the drug is ordinarily supplied at a dosage level of from about 0.01 mg to about 50 mg/kg of body weight. Preferably, the range is from about 0.1 mg to about 7 mg/kg of body weight.

It will be understood, however, that the specific dose level for any particular patient will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, sex, diet, time of administration, route of administration, rate of excretion, drug combination and the severity and organ systems affected and in need of therapy.

EXPERIMENTAL PART

ISOLATION OF TRITERPENE SAPONINS FROM *MAESA BALANSAE*

The air-dried powdered leaves (3 kg) of *Maesa balansae* were extracted with chloroform to remove apolar material, and then with methanol : water (9:1). The alcoholic extract was evaporated under reduced pressure and the residue was partitioned between n-butanol (saturated with water) and water. The organic layer was evaporated to dryness and the residue was washed with acetone and filtered. The acetone insoluble part containing the saponins (10 g) was purified by reversed-phase HPLC (stationary phase RP-18 HS BDS Hyperprep 100 Å, 8 µm; 200g, ø column 5 cm) with a gradient eluent system using :

A : 0,5% ammonium acetate in water,

B : methanol and

C : acetonitrile

at a flow rate of 80 ml/min with UV-detection at 235 nm. Using the gradient eluent system (t = 0 min) A:B:C (60:20:20) to (t = 50 min) A:B:C (0:50:50) a pure saponin mixture (5 g) is obtained comprising six compounds.

Isolation of each of the six saponins was performed on the same column under the same conditions using the above described gradient solvent system. In order of elution, the following compounds were obtained :

Compound 1 : MW = 1532, λ_{max} = 223.3 nm ; was further purified using isocratic solvent system A:B:C (33:64:03) ; yield 230 mg.

Compound 2 : MW = 1510, λ_{max} = 209.2 nm ; gradient elution system : (t = 0 min) A:B:C (42:29:29) to (t = end) A:B:C (24:38:38) ; yield 110 mg.

Compound 3 : MW = 1532, λ_{max} = 222.1 nm ; isocratic solvent system : A:B:C (40:30:30) ; yield 1,000 mg.

Compound 4 : MW = 1510, λ_{max} = 202.2 nm ;isocratic solvent system : A:B:C (59:00:41) ; yield 1,000 mg.

Compound 5 : MW = 1574, λ_{max} = 203.4 nm ; and isocratic solvent system : A:B:C (32:34:34) ; yield 220 mg.

Compound 6 : MW = 1552, λ_{max} = 216.3 nm ; isocratic solvent system : A:B:C (32:34:34) with recycling (4 times) ; yield 230 mg.

EVALUATION OF ANTILEISHMANIA ACTIVITY

The test drug PX used in the following examples comprises the mixture of saponins isolated from *Maesa balansae*.

1. *In vitro* antileishmanial activity

Methods for the *in vitro* growth of *Leishmania* organisms and screening methodology are well documented in the international literature. Testing protocols are flexible and may be adapted according to the specific objectives and the characteristics of the test compounds. Briefly, the following *in vitro* methodology has been used:

Primary peritoneal macrophages derived from laboratory rodents or macrophage cell lines were seeded in multiwell tissue culture vessels and allowed to attach for about 24 hours. Amastigotes of the *Leishmania* species (obtained from target tissues of an infected donor animal or from amastigote-infected tissue cultures) or promastigotes of the *Leishmania* species were added at an appropriate infection ratio together with varying serially diluted concentrations of the drug or test compound. The test drug was solubilized in an appropriate solvent which was tolerated in the *in vitro* test system (DMSO ; water, alcohols, and the like work equally well) and added to the tissue culture medium. The cultures were incubated at 37°C in 5% CO_2 for 5-15 days. Treatment of uninfected control cultures was also included in order to determine a selectivity index. Reference drug treated cultures were included as well so as to determine the relative potency of the test drug. Drug activity was determined in stained preparations as the percentage reduction of the total parasite load or the number of infected macrophages compared to the untreated control cultures. Reading was performed microscopically and EC_{50}-values (effective concentration for 50% inhibition) were determined. The percentage reduction and the EC_{50}-value serve as an indication for antileishmanial activity *in vitro* and provide significant leads for clinically useful agents.

Table I: *In vitro* antileishmanial activity in primary mouse macrophages

Leishmania species		EC_{50} (microgram/ml)				
		PX	meglumine	pentostam	ampho-B	itraconazole
Visceral	*L.donovani*	0.05	12	6	0.1	>12.5
	L.infantum	0.05	12	6	0.1	>12.5
Cutaneous	*L.mexicana*	1	>50	25	0.1	nd
	L.major	5	>50	>50	0.2	nd
Mucocutaneous	*L.panamensis*	nd	nd	nd	nd	nd
	L.major	nd	nd	nd	nd	nd

nd: not done

2. *In vivo* antileishmanial activity

Methods for *in vivo* maintenance of *Leishmania* organisms and animals models are well documented in the international literature. Balb-C mice and golden hamsters are the preferred laboratory animal species for primary isolation, maintenance and use in artificial infection models. Testing protocols are flexible and may be adapted according to the specific objectives and characteristics of the test compounds. Briefly, the following *in vivo* methodologies have been used:

For visceral *Leishmania* species: Balb-C mice or young hamsters were intravenously infected with about 10^6 to 10^7 amastigotes derived from the target organs (generally spleen) of an infected donor animal or from an *in vitro* culture of parasite forms. The animals were treated with the test compound at different dose levels (dose range : 0.1 to 80 mg/kg in 100% DMSO or any other acceptable vehicle), using different routes of administration and treatment schedules. Initiation of treatment was either at different times after infection (curatively), concomitant with infection (prophylactically) or before infection (residual activity). In the prophylactic study design, the first administration of the test drug is given immediately before or together with the artificial infection with the *Leishmania* species. In the curative study design, the first administration of the test drug is given several weeks after the artificial infection with the *Leishmania* species (early curative = when the first clinical signs appear ; late curative = when the clinical symptoms are well established or become chronic). Drug activity was evaluated by determination of the total parasite burdens in the liver or any other relevant target tissue/organ, compared to the tissue/organ burdens in untreated control animals. The mean number of amastigotes is enumerated quantitatively or semi-quantitatively on stained impression smears or slides. The percentage reduction serves as an indication for antileishmanial activity and provides significant leads for clinically useful agents. The lowest active dose (LAD) is defined as the lowest dose which reduces the parasite burden in the primary target organ/tissue by at least 80%.

Table II: *In vivo* antileishmanial activity in mice and hamsters against visceral *Leishmania* species

Animal	dosing regimen		% reduction of parasite load in target organ after parenteral dosing at (mg/kg)							
species	freq.	timing	40	20	10	5	2.5	1.25	0.63	0.32
Mouse	5x	proph.	100	100	100	100	100	100	100	98
		cur.	100	100	100	100	100			
	1x	proph.	100	100	100	100	100			
		cur.				98	90	80		
Hamster	5x	proph.	100	100	100					
		cur.				100	100			
	1x	proph.				100	100	100		
		cur.								

Table III: *In vitro* and *in vivo* activity against visceral *Leishmania infantum*

Product	Activity against L.infantum		Result
	in vitro IC50 (ng/ml)	*in vivo* LAD (mg/kg 1x)	Activity score
PX	50	0.4	+++
compound-1	70	0.8	++
compound-2	50	>0.8	++
compound-3	20	0.2	+++
compound-4	20	0.4	+++
compound-5	3400	>0.8	+
compound-6	700	>0.8	+
Tri-OH derivative	10,000	>40	not active
Aglycon derivative	>50,000	>40	not active

For cutaneous and mucocutaneous *Leishmania* species: Balb-C mice or young hamsters were infected intradermally or subcutaneously with about 10^6 to 10^7 amastigotes derived from the target organs (generally skin lesion) of an infected donor animal or from an *in vitro* culture of parasite forms. The animals were treated with the test compound at different dose levels (mg/kg in 100% DMSO or any other acceptable vehicle), using different routes of administration and treatment schedules. Initiation of

treatment was either before infection (prophylactically) or at different times after infection (curatively). In the prophylactic study design, the first administration of the test drug is given immediately before or together with the artificial infection with the *Leishmania* species. In the curative study design, the first administration of the test drug is given several weeks after the artificial infection with the *Leishmania* species (early curative = when the first dermal lesions appear ; late curative = when dermal lesions are well established or become chronic). Drug activity was evaluated either by determination of the severity of the lesion in the relevant target tissue/organ (primary parameter) or of the parasite burdens in the relevant target tissue/organ (secondary parameter), compared to untreated control animals. The lesion size was assessed quantitatively using the method of J. El-On. and A.D. Hamburger [Trans. Roy. Soc. Trop. Med. Hyg., 81, 734-737 (1987)]. The percentage reduction serves as an indication for antileishmanial activity and provides significant leads for clinically useful agents. The lowest active dose (LAD) is defined as the lowest dose which prevents lesions to develop, stops further evolution of the lesions or induces a clinical cure of the lesions in the primary target organ or tissue..

Table IV: *In vivo* antileishmanial activity in mice and hamsters against cutaneous and mucocutaneous *Leishmania* species

A. Prophylactic treatment

In the prophylactic study design, the first administration of the test drug is given immediately before the artificial infection with the *Leishmania* species

L.mexicana (proph.)	skin lesion size* at weeks post infection												
Group	0	1	2	3	4	5	6	7	8	9	10	11	12
Untreated control	0	0	0	0	0	1	1	10	33	59	86	108	98
Ampho-B 10 mg/kg	0	0	0	0	0	1	2	7	13	34	85	101	112
Pentostam 250 mg/kg	0	0	0	0	0	0	1	9	13	22	37	50	51
PX 10 mg/kg	0	0	0	0	0	0	0	0	0	0	0	0	0
PX 5 mg/kg	0	0	0	0	0	0	0	0	0	0	0	0	0
PX 2.5 mg/kg	0	0	0	0	0	0	0	0	0	0	0	0	0

* using the formula: [vertical diameter x horizontal diameter]/2 (mm^2)

L.major (proph.) Group	skin lesion size* at weeks post infection								
	0	1	2	3	4	5	6	7	8
Untreated control	0	0	1	1	17	20	27	72	113
Ampho-B 10 mg/kg	0	0	1	1	12	31	41	57	95
Pentostam 250 mg/kg	0	0	1	1	7	16	20	32	60
PX 10 mg/kg	0	0	0	0	0	0	0	0	0
PX 5 mg/kg	0	0	0	0	0	0	0	0	0
PX 2.5 mg/kg	0	0	0	0	0	0	0	0	0

* using the formula: [vertical diameter x horizontal diameter]/2 (mm^2)

L.panamensis (proph.) Group	skin lesion size* at weeks post infection												
	0	1	2	3	4	5	6	7	8	9	10	11	12
Untreated control	0	0	0	0	1	6	30	42	49	58	55	67	56
Ampho-B 10 mg/kg	0	0	0	0	1	9	15	30	30	42	42	43	39
Pentostam 250 mg/kg	0	0	0	0	0	0	0	0	0	0	0	0	0
PX 10 mg/kg	0	0	0	0	0	0	0	0	0	0	0	0	0
PX 5 mg/kg	0	0	0	0	0	0	0	0	0	0	0	0	0
PX 2.5 mg/kg	0	0	0	0	0	0	0	0	0	0	0	0	0

* using the formula: [vertical diameter x horizontal diameter]/2 (mm^2)

B. Curative treatment

In the curative study design, the first administration of the test drug is given several weeks after the artificial infection with the *Leishmania* species (generally when the first clinical signs appear).

L.mexicana (cur) Group	Dose mg/kg	freq./ week	skin lesion size* at weeks post infection 5**	6	7	8	9
Untreated control			1.7	3.6	10.7	17.7	32.8
Pentostam	250	2x	2.5	4.9	8.5	13.3	19.2
PX	1	1x	1.8	3.6	4.2	3.1	2.2
PX	1	2x	2.2	3.7	3.6	2.4	2.3
PX	2	1x	1.6	2.6	1.3	1.3	1.4
PX	2	2x	1.3	1.1	1.8	0.7	0.6

* using the formula: [vertical diameter x horizontal diameter]/2 (mm^2)
** initiation of treatment

L.major (cur) Group	Dose mg/kg	freq./ week	skin lesion size* at weeks post infection 2**	3	4	5	6	7	8	9
Untreated control			1	14	32	42	50	59	64	148
Pentostam	250	2x	1	13	24	33	36	42	48	123
PX	1	1x	1	14	27	34	40	46	59	64
PX	1	2x	1	12	19	27	32	33	32	33
PX	2	1x	1	16	25	30	33	38	42	42
PX	2	2x	1	9	23	29	30	28	32	35

* using the formula: [vertical diameter x horizontal diameter]/2 (mm^2)
** initiation of treatment

L.panamensis (cur) Group	Dose mg/kg	freq./ week	skin lesion size* at weeks post infection 5**	6	7	8	9
Untreated control			2.8	8.5	20.8	30.5	
Pentostam	250	2x	2.3	1.2	0.5	0.1	
PX	1	1x	2.2	2.1	3.5	3.7	
PX	1	2x	1.2	1.8	2.8	1.2	
PX	2	1x	1.9	4.0	4.8	2.6	
PX	2	2x	2.9	1.6	2.3	1.7	

* using the formula: [vertical diameter x horizontal diameter]/2 (mm^2)
** initiation of treatment

Table V : *In vivo* antileishmanial activity of the PX mixture against different *Leishmania* species

Model	Lowest active dose (LAD) regimen in Balb-C mice*		
	PX	**Sodium stibogluconate** Pentostam®	**Amphothericin-B** Fungizone®
L.donovani			
prophylactic	0.4 mg/kg, 1x	250 mg/kg, 1x	not effective
curative early	1.6 mg/kg, 1x	250 mg/kg, 1x	not effective
curative late	nd	nd	nd
residual activity	5 days after single 2.5 mg/kg dose	nd	not effective
L.mexicana			
prophylactic	< 0.5 mg/kg, 6x (alternate days)	>> 250 mg/kg, 6x (alternate days)	not effective
curative early	< 1 mg/kg, 4x (in 4 weeks)	>> 250 mg/kg, 8x (in 4 weeks)	nd
curative late	< 1 mg/kg, 2x/w for 4 weeks	nd	nd
L.panamensis			
prophylactic	< 0.5 mg/kg, 6x (alternate days)	< 250 mg/kg, 6x (alternate days)	not effective
curative early	1 mg/kg, 4x (in 4 weeks)	< 250 mg/kg, 8x (in 4 weeks)	nd
curative late	< 1 mg/kg, 2x/w for 4 weeks	nd	nd
L.major			
prophylactic	2 mg/kg, 6x (alternate days)	>> 250 mg/kg, 6x (alternate days)	not effective
curative early	1 mg/kg, 4x (in 4 weeks)	>> 250 mg/kg, 8x (in 4 weeks)	nd
curative late	1 mg/kg, 22x (in 11 weeks)	nd	nd

*: based on amastigote burdens in the liver for visceral forms and on lesion size for cutaneous forms

Claims

1. A process for the isolation of triterpene saponins from plants belonging to the family *Myrsinaceae*, characterized in that said process comprises the steps of
   (a) exracting the dried plant parts with an alcohol and concentrating the extract,
   (b) removing the apolar fraction from the extract by liquid-liquid extraction with an apolar solvent, and
   (c) further purifying the saponins in the alcohol extract by liquid -liquid extraction, filtration and chromatography.

2. A process according to claim 1 wherein the alcohol is methanol, ethanol, isopropanol, butanol, each optionally admixed with water.

3. A process according to claim 1 wherein the saponins of the alcohol extract are further purified by
   (c6) extracting the aqueous fraction with butanol satured with water,
   (c7) evaporating the organic layer to dryness,
   (c8) washing the residue in a ketone, and
   (c9) filtering off the crude saponin mixture.

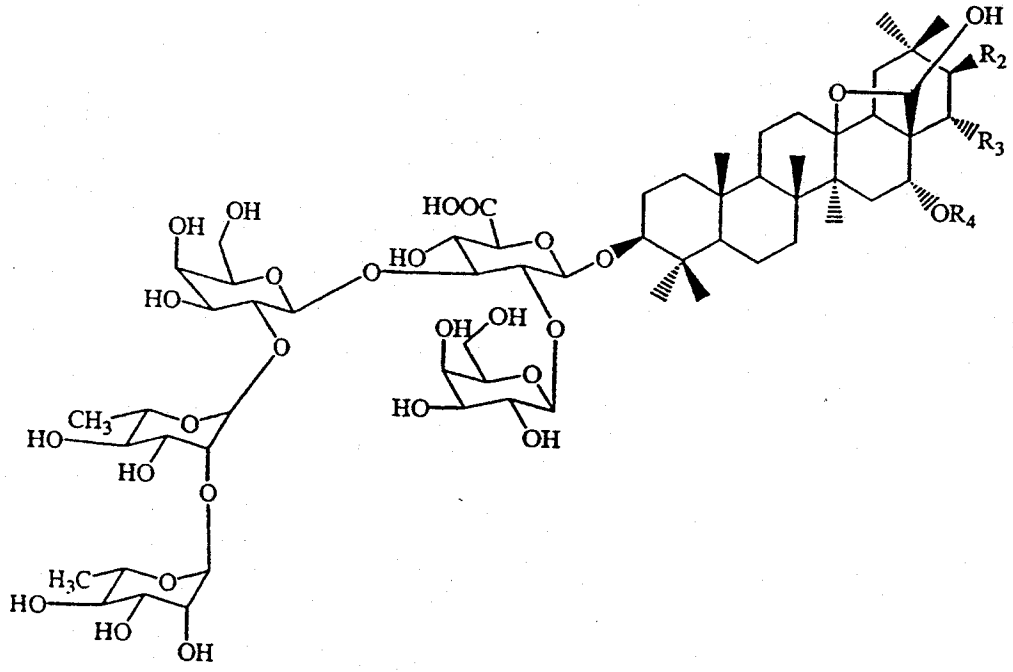
4. A process according to claim 1 wherein the saponins are isolated from the plant species *Maesa balansae*, and the chromatography comprises straight phase chromatographyliquid chromatography on silicagel or reversed-phase liquid chromatography with gradient eluent system using
   A : 0.5 % ammonium acetate in water
   B : methanol
   C : acetonitrile
   wherein at t = 0, (A:B:C) = (60:20:20) and t = end, (A:B:C) = (0:50:50).

5. A triterpene saponin obtainable by a process according to anyone of claims 1 to 4.

6. A triterpene saponin according to claim 5 wherein said saponin is isolated from the plant species *Maesa* balansae, and the chromatography comprises reversed-phase liquid chromatography with gradient eluent system using
   A : 0.5 % ammonium acetate in water
   B : methanol
   C : acetonitrile
   wherein at t = 0, (A:B:C) = (60:20:20) and t = end, (A:B:C) = (0:50:50), and wherein said saponin has the following characteristics :

Compound 1 : MW = 1532, λ_{max} = 228.6 nm, λ_{max2} = 273.3 nm ;
Compound 2 : MW = 1510, λ_{max} = 223.9 nm, λ_{max2} = 274.5 nm ;
Compound 3 : MW = 1532, λ_{max} = 279.2 nm, λ_{max2} = 223.9 nm ;
Compound 4 : MW = 1510, λ_{max} = 280.4 nm, λ_{max2} = 222.7 nm ;
Compound 5 : MW = 1574, λ_{max} = 276.8 nm, λ_{max2} = 225.0 nm ; or
Compound 6 : MW = 1552, λ_{max} = 279.2 nm, λ_{max2} = 223.9 nm.

7. A triterpene saponin having the formula



wherein R_2 is $-O(C=O)C_6H_5$ or $-O(C=O)C(CH_3)=CHCH_3$,
R_3 is (E) or (Z) $-O(C=O)CH=CH-C_6H_5$, and
R_4 is hydrogen or $-(C=O)CH_3$.

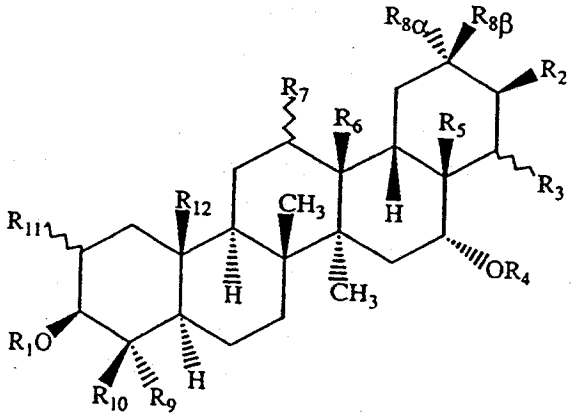
8. A compound according to claim 7 wherein
in compound 1, R_2 is $-O(C=O)C_6H_5$,
R_3 is (Z) $-O(C=O)CH=CH-C_6H_5$,
R_4 is hydrogen;
in compound 2, R_2 is $-O(C=O)C(CH_3)=CHCH_3$,
R_3 is (Z) $-O(C=O)CH=CH-C_6H_5$,
R_4 is hydrogen;
in compound 3, R_2 is $-O(C=O)C_6H_5$,
R_3 is (E) $-O(C=O)CH=CH-C_6H_5$,
R_4 is hydrogen;

in compound 4, R_2 is $-O(C=O)C(CH_3)=CHCH_3$,
R_3 is (E) $-O(C=O)CH=CH-C_6H_5$,
R_4 is hydrogen;
in compound 5, R_2 is $-O(C=O)C_6H_5$,
R_3 is (E) $-O(C=O)CH=CH-C_6H_5$,
R_4 is $-(C=O)CH_3$;
in compound 6, R_2 is $-O(C=O)C(CH_3)=CHCH_3$,
R_3 is (E) $-O(C=O)CH=CH-C_6H_5$,
R_4 is $-(C=O)CH_3$.

9. A pharmaceutical composition comprising a pharmaceutically acceptable excipient and as an active ingredient a triterpene saponin as defined in claim 5, 6, 7 or 8.

10. A composition according to claim 7 adapted for parenteral administration.

11. Use of one or more triterpene saponins for the preparation of a pharmaceutical composition for treating leishmaniases in hosts infected by *Leishmania* species, characterized in that the saponin has the formula



a stereoisomeric form thereof or a pharmaceutically acceptable addition salt thereof, wherein

R_1 is hydrogen, $-(C=O)C_{1-5}$alkyl, $-(C=O)C_{2-5}$alkenyl, $-(C=O)C_{2-5}$alkenyl substituted with phenyl, a monosaccharide group or an oligosaccharide group ;

R_2 is hydrogen, hydroxy, $-O(C=O)C_{1-5}$alkyl, $-O(C=O)C_{2-5}$alkenyl, $-O(C=O)C_6H_5$, or $-O(C=O)C_{2-5}$alkenyl substituted with phenyl ;

R_3 is hydrogen, hydroxy, $-O(C=O)C_{1-5}$alkyl, $-O(C=O)C_{2-5}$alkenyl, $-O(C=O)C_6H_5$, or $-O(C=O)C_{2-5}$alkenyl substituted with phenyl ;

R_4 is hydrogen, C_{1-6}alkyl, $-(C=O)C_{1-5}$alkyl, $-(C=O)C_{2-5}$alkenyl, $-(C=O)C_6H_5$, or $-(C=O)C_{2-5}$alkenyl substituted with phenyl ;

R_5 is CH_3, CH_2OH, CH_2OCH_3, CH_2O-C(=O)CH_3, CHO, COOH ; or
R_5 and R_2 form a divalent radical of formula -C(=O)-O- ;
R_6 and R_7 are hydrogen; or taken together they form a bond; or
R_5 and R_6 form a divalent radical of formula

-CH_2-O- (a),
-CH(OR_{13})-O- (b),
-C(=O)-O- (c),

wherein R_{13} is hydrogen, C_{1-6}alkyl or -(C=O)C_{1-5}alkyl ;
$R_{8\alpha}$ and $R_{8\beta}$ each independently represent CH_3, CH_2OH, CH_2OCH_3, CH_2O-C(=O) C_{1-5}alkyl, CHO, $CH(OCH_3)_2$, CH=NOH, COOH ;
$R_{8\beta}$ and R_3 form a divalent radical of formula -C(=O)-O- ;
$R_{8\beta}$ and R_5 form a divalent radical of formula -CH_2O-CHOH- ;
R_9 is CH_3, CH_2OH, CH_2OCH_3, CH_2O-C(=O)C_{1-5}alkyl, CHO, COOH ;
R_{10} is CH_3, CH_2OH, CH_2OCH_3, CH_2O-C(=O)C_{1-5}alkyl, CHO, COOH ;
R_{11} is hydrogen, hydroxy or O-C(=O)C_{1-5}alkyl ; or R_{10} and R_{11} form a divalent radical of formula -CH_2O- ; and
R_{12} is CH_3, CH_2OH, CH_2OCH_3, CH_2O-C(=O)CH_3, CHO, CH=NOH, or COOH.

12. Use according to claim 11 wherein
R_1 is hydrogen, -(C=O)C_{1-5}alkyl, or an oligosaccharide group ;
R_3 is hydrogen, hydroxy, -O(C=O)C_{1-5}alkyl, -O(C=O)C_{2-5}alkenyl, -O(C=O)C_{2-5}alkenyl substituted with phenyl ;
R_4 is hydrogen, C_{1-6}alkyl, -(C=O)C_{1-5}alkyl, -(C=O)C_{2-5}alkenyl ;
R_5 is CH_2OH, CH_2O-C(=O)CH_3, CHO ; and
R_6 and R_7 taken together form a bond; or
R_5 and R_6 form a divalent radical of formula

-CH_2-O- (a),
-CH(OR_{13})-O- (b),
-C(=O)-O- (c),

wherein R_{13} is hydrogen, C_{1-6}alkyl or -(C=O)C_{1-5}alkyl, ; and
R_7 is hydrogen ;
$R_{8\beta}$ represents CH_3, CH_2OH, CHO, $CH(OCH_3)_2$, CH=NOH, COOH ;
$R_{8\alpha}$ represents CH_3 ;
$R_{8\beta}$ and R_3 form a divalent radical of formula -C(=O)-O- ; or
$R_{8\beta}$ and R_5 form a divalent radical of formula -CH_2O-CHOH- ;
R_{10} is CH_3, CH_2OH ;
R_{11} is hydrogen, hydroxy or O-C(=O)C_{1-5}alkyl ; or

R_{10} and R_{11} form a divalent radical of formula $-CH_2O-$; and
R_{12} is CH_3, CH_2OH, $CH_2O-C(=O)CH_3$, CHO, or CH=NOH.

13. Use according to claim 12 wherein
R_1 is hydrogen or an oligosaccharide group ;
R_2 is hydrogen, hydroxy, $-O(C=O)C_{1-5}$alkyl, $-O(C=O)C_{2-5}$alkenyl, $-O(C=O)C_6H_5$, or $-O(C=O)C_{2-5}$alkenyl substituted with phenyl ;
R_3 is hydrogen, hydroxy, $-O(C=O)C_{1-5}$alkyl, $-O(C=O)C_{2-5}$alkenyl, $-O(C=O)C_{2-5}$alkenyl substituted with phenyl ;
R_4 is hydrogen, C_{1-6}alkyl, $-(C=O)C_{1-5}$alkyl, $-(C=O)C_{2-5}$alkenyl, $-(C=O)C_{2-5}$alkenyl substituted with phenyl ;
R_5 is CH_2OH, CH_2OCH_3, $CH_2O-C(=O)CH_3$, CHO, COOH ; and
R_6 and R_7 taken together form a bond; or
R_5 and R_6 form a divalent radical of formula
$-CH_2-O-$ (a),
$-CH(OR_{13})-O-$ (b),
$-C(=O)-O-$ (c),
wherein R_{13} is hydrogen ; and
R_7 is hydrogen ;
$R_{8\alpha}$ and $R_{8\beta}$ both represent CH_3 ;
R_9 is CH_3 ;
R_{10} is CH_3 ;
R_{11} is hydrogen ; and
R_{12} is CH_3.