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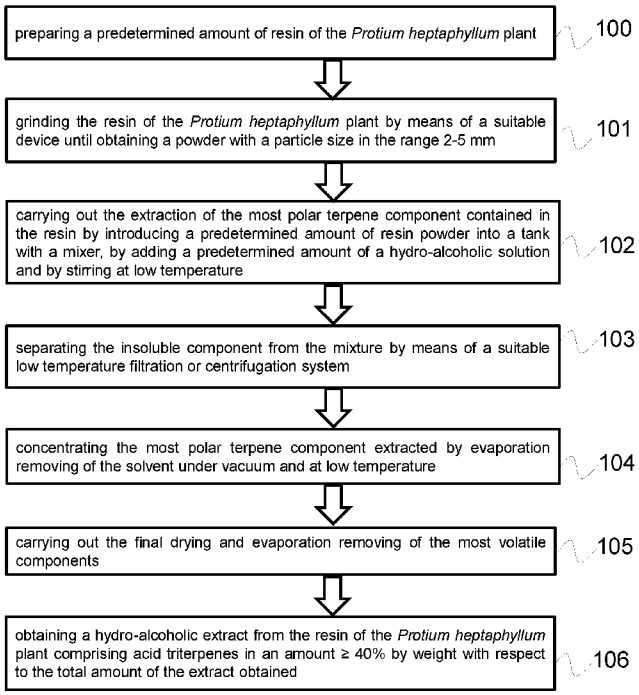

Fig. 1

(57) Abstract: The invention relates to a hydroalcoholic extract from the resin of the *Protium heptaphyllum* plant enriched for the acid triterpene component. The invention also relates to pharmaceutical formulations comprising the aforesaid extract. The invention also relates to a hydroalcoholic extraction process at controlled pressure and temperature of the aforesaid extract. The present invention finds advantageous applications in the inhibition of the gene expression of the HMG-CoA reductase enzyme, in the alteration of the gene expression in liver cells, in the alteration of the gene expression of key factors involved in the lipid metabolism and in the treatment of hypercholesterolemia. Furthermore, the hydroalcoholic extraction process according to the present invention is economically and qualitatively more advantageous than the known solutions thanks to the use of simple and inexpensive equipment as well as of solvents

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(water and ethanol) non-toxic or anyway widely used at the food level and more environmentally friendly.

"Extract from the resin of the *Protium heptaphyllum* plant, formulations comprising such extract and hydroalcoholic extraction process at controlled pressure and temperature"

DESCRIPTION

TECHNICAL FIELD

The present invention relates to the nutraceutical, pharmaceutical and cosmetic fields.

Specifically, the present invention relates to a hydroalcoholic extract from the resin of the *Protium heptaphyllum* plant enriched for the acid triterpene component.

The invention also relates to pharmaceutical formulations comprising the aforesaid extract.

The invention also relates to a hydroalcoholic extraction process at controlled pressure and temperature of the aforesaid extract.

The present invention finds advantageous applications in the inhibition of the gene expression of the HMG-CoA reductase enzyme, in the alteration of the gene expression in liver cells, in the alteration of the gene expression of key factors involved in the lipid metabolism and in the treatment of hypercholesterolemia.

Furthermore, the hydroalcoholic extraction process according to the present invention is economically and qualitatively more advantageous than the known solutions thanks to the use of simple and inexpensive equipment as well as of solvents (water and ethanol) non-toxic or anyway widely used at the food level and more environmentally friendly.

STATE OF THE ART

The risk of atherosclerosis and coronary heart diseases is increased in patients with high serum concentrations of cholesterol low-density lipoprotein (LDL), total cholesterol (TC) and triglycerides (TG).

When LDLs are present in quantities greater than the physiological ones, they tend to deposit on the artery wall, causing its progressive thickening and hardening (atherosclerosis).

This process can lead, over time, to the formation of real plaques that can make difficult the correct blood flow and that, in the most serious cases, can lead to obstructions.

For this reason, the need is felt to have available a technical solution able to exert an effect on the regulation of the gene expression of the most important enzymes involved in the metabolism of cholesterol.

Statins are organic molecules able to inhibit the action of hydroxymethylglutaryl-CoA reductase (or HMG-CoA reductase), a key enzyme of the cholesterol metabolism since it converts the 3-hydroxy-3-methylglutaryl-CoA molecule into mevalonic acid. There are different kinds of statins both of the natural and the synthetic type; given their action, they are used in the pharmaceutical field for the treatment of hypercholesterolemia-related disorders since they are able to directly limit the cholesterol biosynthesis; some molecules of this class are also able to directly interact with the LDLs receptor favouring its biosynthesis and thus preventing any damage caused by atherosclerosis. In this pharmaceutical class there are several molecules that are currently used as active principles; among the most important molecules there are simvastatin, atorvastatin, lovastatin, pravastatin, rosuvastatin and fluvastatin.

In particular, monacolin K is one of the molecules that can be obtained from the fermentation of red rice carried out with the *Monascus purpureus* fungus, it has a similar structure to that of lovastatin and, as such, it is used in supplements intended to prevent damages resulting from hypercholesterolemia.

The molecule efficacy has been recognized to such an extent that, in 2013, EFSA awarded the 1924/2006 claim of "maintaining cholesterol normal levels in blood" to monacolin K of fermented red rice in doses of 10 mg/day.

In addition to statins, there are other drugs on the market able to regulate hypercholesterolemia and the biosynthesis of fatty acids, such as ezetimibe (it selectively inhibits the absorption of exogenous cholesterol), resins that sequester bile acids (they induce a reduction of the blood levels of LDL), acipimox (it reduces the blood levels of triglycerides and cholesterol) and fibrates drugs (they regulate the blood levels of cholesterol and triglycerides).

Like all drugs, even the abovementioned ones for the treatment of hypercholesterolemia have various adverse effects if regularly taken; among the most frequent disorders possible allergic reactions, disorders of the gastrointestinal tract, weakness and muscle aches, headache, altered taste and skin disorders were detected.

In the most severe cases it was possible to notice problems to the liver system.

From the Italian phytosurveillance system (EpiCentro) 52 notifications of adverse reactions due to the intake of fermented red rice recorded between 2002 and 2015 emerged, which include myalgia and/or increase in creatine phosphokinase (CPK), liver damage, increase of liver enzymes and hepatitis, damages to the gastrointestinal tract, skin reactions including urticaria or skin rash and other reactions, among which the symptoms: INR increase for interaction with warfarin, tachycardia, tingling in the extremities, dizziness, blurred vision.

These results led to the conclusion that the safety profile of fermented red rice is similar to that of statins (Mazzanti *et al.*, 2017).

Furthermore, the fermentation process mediated by the *Monascus purpureus* fungus can generate, as a secondary metabolite, the mycotoxin known as citrinin, which has a nephrotoxic, hepatotoxic and probably carcinogenic action (source EFSA); for this reason, the EU Regulation 2019/1901 was issued on November 7, 2019, which obliges that, from April 1, 2020, the maximum amount of citrinin present in food supplements based on rice fermented with *Monascus purpureus* red yeast must be 100 µg/kg.

Given the high number of adverse reactions caused by drugs used to treat this pathology, in recent years the research for alternative bioactive molecules of natural origin that do not show particular complications is increasingly of study interest; in particular, the need is felt to have available an extract deriving from a plant resin rich in secondary metabolites that give it different pharmacologically interesting properties, with different potential effects on the organism, high tolerability and poor toxicity and also absent of adverse reactions.

The *Protium heptaphyllum* resin is rich in secondary metabolites that give it different pharmacologically interesting properties; it is rich in molecules of natural origin with therapeutic effects of which the exact nature is not known, but which could be used in order to generate supplements with possibly preventive function that have high tolerability and low toxicity or adverse reactions.

The best known and studied metabolites of this resin are the molecules of α-amyrin and β-amyrin: they are among the most widespread pentacyclic triterpenes in plant matrices and they are particularly abundant in the resin exudates and in the oils of

various plants, including in particular those of the *Burseraceae* family as the same *Protium heptaphyllum*; in these resins the concentration of the two isomers can even reach 50%. In addition to this category of triterpenes and of their oxidized derivatives, in the *Protium heptaphyllum* resin there are also molecules defined acid triterpenes, in particular tetra- and penta- cyclic acid triterpenes (molecular weight 450 - 500 Da) that are distinguished from the class of triterpenes described above (amyrins and oxidized derivatives) for the presence of one or more carboxylic substituents that give them different chemical-physical characteristics.

Historically, the *Protium heptaphyllum* resin has been studied for the presence of amyrins, which belong to the class of oleanic pentacyclic triterpenes and differ from each other due to a different arrangement of two methyl groups positioned on the fifth ring.

The amyrins have aroused a lot of interest in the research field since they turned out to be pharmacologically active molecules; several studies have already been carried out on *Protium heptaphyllum* extracts and on purified amyrins but, to date, the Inventors are not aware that the pharmacological activity of the acid triterpene fraction as well as the method for their enrichment starting from the resin of *Protium heptaphyllum* have ever been investigated.

The amyrins and the identified related studies, in summary, focused on investigating what is listed hereinbelow:

- the analysed resin extracts concerned only some selected parts of the *Protium* resin separated and purified to enrich the content in α-amyrin and β-amyrin;
- the biological activity of the pentacyclic triterpene components (amyrins) was tested *in vivo* in relation to anti-nociceptive activity, anti-inflammatory activity, activity on the Central Nervous System, their hypoglycemic action, on the metabolism and on the digestive tract;
- the few studies present in relation to the cholesterol metabolism were carried out on a purified fraction of α-amyrin and β-amyrin and they were realized only considering the variation of the blood levels of LDL and HDL;
- no pharmacological study is known to date regarding the acid triterpene component of the *Protium heptaphyllum* resin as well as an economically and

ecologically sustainable industrial method for the extraction and enrichment of this component with respect to the amyrinic component.

Examples of known solutions are disclosed in EP 2812005 B1, EP 3068412 B1, BR 102015012884-3 A2 and US 9233066 B2.

EP 2812005 B1 presents an in-depth analysis of the disorders that respond to the reduction of the activity of the HMG-CoA reductase enzyme in mammals; the invention consists of an extract of the *Amelanchier alnifolia* plant usable in the prophylaxis and/or therapy for the treatment of hypercholesterolemia, hyperlipidemia, HDL/LDL ratio.

The composition is generated by the set of natural molecules with an inhibitory action against the HMG-CoA reductase enzyme; the polyhydroxylated pentacyclic triterpenes, which include euscaphic acid, tormentic acid, myriantic acid, corosolic acid, oleanic acid, ursolic acid, are the protagonists.

EP 3068412 B1 analyses the problems relating to the metabolism of fatty acids and sugars; in particular the role of the HDL is highlighted and how it can be considered a potential target for coronary heart diseases; the invention consists of a medicinal composition obtained from the extract of *Emblica officinalis* seeds, with nutraceutical and pharmaceutical applications for the reduction of hypercholesterolemia, blood triglyceride level, blood glucose level, LDL reduction and HDL increase.

The treatment of dyslipidemia and hypercholesterolemia is carried out with triterpenoids (β-amyrin, β-sitosterol, lupeol) and hydroxycinnamic acids (ferulic acid, p-coumaric acid).

BR 102015012884-3 A2 deals with diseases associated with dysfunctions of the metabolism of fatty acids and sugars, such as type II diabetes and obesity; the invention consists of a phytopharmaceutical product of natural origin able to inhibit digestive enzymes and to exert hypoglycemic, hypolipidemic and antiobesity action.

The natural source is the resin of the *Protium paniculatum* plant var. "Nova", which is subjected to extraction with mixtures of hexane and ethyl acetate, in different proportions, with increasing polarity. This kind of processing allows to obtain a rich extract of α/β amyrins pentacyclic triterpenes, which are then oxidized with the aim of obtaining α/β amyrones; the latter are the claimed bioactive components and they

form up to 60% of the final product.

US 9233066 B2 faces the difficulty related to the search for products with antimicrobial action and biofilm removal properties; the invention consists of an essential oil obtained from at least one plant belonging to the *Protium*, *Guatteria* and *Cyperus* genera. This essential oil can be used for realizing a phytocosmetic and/or phytotherapic product for personal hygiene, medicine and veterinary. The choice of introducing the *Protium* genus in the invention is given to its antimicrobial properties and to its ability to speed up wound healing.

A hydroalcoholic extract from the resin of the *Protium heptaphyllum* plant purified for the acid triterpene component, the corresponding pharmaceutical formulations comprising the aforesaid extract and the relevant hydroalcoholic extraction process at controlled pressure and temperature of the aforesaid extract, would satisfy the need to have available a solution able to have an effect on the regulation of the gene expression of the most important enzymes linked to the metabolism of cholesterol and lipids.

The present invention aims at answering the aforesaid need.

In particular, the present invention intends to solve the technical problem of intervening on the modulation of the metabolism of cholesterol and lipids through the regulation of the transcription of enzymes genes and factors involved in these metabolic pathways and not on the same, as it occurs today in most commercially available drugs and supplements that in some cases have potential adverse effects.

Moreover, the present invention intends to solve the technical problem of identifying an economically, ethically and ecologically sustainable method of extraction and purification of organic substances or mixtures having a pharmacological action on the metabolism of cholesterol and lipids. The substances that generally interact on these metabolic pathways are, by their nature, poorly soluble in polar solvents (water) and, therefore, their extraction and purification require strongly apolar solvents (for example hexane and ethyl acetate), with a high degree of toxicity, that therefore require subsequent very expensive solvent removal processes and whose use presents a high environmental risk.

Moreover, the present invention intends to solve the technical problem of producing

drugs or supplements containing, as active principle, substances or molecules alternative to those obtained through synthesis, semi-synthesis or obtained through microbial fermentation processes, obtained from the intensive cultivation of plant organisms at the expense of forest soil or of soil used for the cultivation of plants intended for basic food.

Finally, the present invention intends to solve the technical problem of identifying a process of extraction and purification of natural substances or mixtures for food and pharmaceutical use that is optimized, linear, inexpensive and easily implementable even in technologically disadvantaged geographical areas and in which it is difficult to find highly advanced manpower and tools, without reducing quality and stability of the product and also obtaining an extract easily usable and integrable into the formulations of pharmaceutical products and supplements.

In summary, therefore, up to the present time, as far as the Inventors know, there are no known solutions allowing to achieve - through a hydroalcoholic extract from the resin of the *Protium heptaphyllum* plant enriched for the acid triterpene component, the corresponding pharmaceutical formulations comprising the aforesaid extract and the relevant hydroalcoholic extraction process at controlled pressure and temperature of the aforesaid extract - an effect on the regulation of gene expression of the most important factors linked to the metabolism of cholesterol and lipids.

Therefore the Applicant, with the hydroalcoholic extract from the resin of the *Protium heptaphyllum* plant enriched for the acid triterpene component, the corresponding pharmaceutical formulations comprising the aforesaid extract and the relevant hydroalcoholic extraction process at controlled pressure and temperature of the aforesaid extract according to the present invention, intends to remedy this lack.

OBJECTS AND SUMMARY OF THE INVENTION

It is the object of the present invention to overcome the drawbacks of the known art related to the impossibility of obtaining a direct effect on the regulation of the gene expression of the most important enzymes involved in the cellular homeostasis of cholesterol.

These objects are achieved with the hydroalcoholic extract from the resin of the *Protium heptaphyllum* plant, the corresponding pharmaceutical formulations comprising the

aforesaid extract and the relevant hydroalcoholic extraction process at controlled pressure and temperature of the aforesaid extract according to the present invention that, advantageously and thanks to the enrichment for the acid triterpene component, allow to have an effect on the regulation of the gene expression of the most important enzymes involved in the cholesterol metabolism.

Specifically, the abovementioned and other objects and advantages of the invention, which will become apparent from the following description, are achieved with a hydroalcoholic extract from the resin of the *Protium heptaphyllum* plant according to claim 1.

Another independent aspect of the present invention relates to a pharmaceutical formulation comprising the extract and it forms the subject of claim 5.

Another independent aspect of the present invention relates to a hydroalcoholic extraction process at controlled pressure and temperature of the extract and it forms the subject of claim 15.

Preferred embodiments and variants of the present invention form the subject of the dependent claims.

It is understood that all the appended claims form an integral part of the present description and that each of the technical features claimed therein is possibly independent and autonomously usable with respect to the other aspects of the invention.

It will be immediately evident that innumerable changes (for example related to shape, sizes, arrangements and parts with equivalent functionality) could be brought to what described, without departing from the scope of the invention as claimed in the appended claims.

Advantageously, the technical solution according to the present invention, which provides a hydroalcoholic extract from the resin of the *Protium heptaphyllum* plant enriched for the acid triterpene component, the corresponding pharmaceutical formulations comprising the aforesaid extract and the relevant hydroalcoholic extraction process at controlled pressure and temperature of the aforesaid extract, allows to obtain a plant extract able to modulate the main enzymes and factors involved in the metabolism of cholesterol and lipids directly at level of their gene

transcription.

This particular hydroalcoholic extract from the resin of the *Protium heptaphyllum* plant is obtained by using solvents (water and ethanol) compatible with both food and environment and whose use during the process does not involve the adoption of further steps for their removal below the admissible limits. The particular combination of these solvents and the control of definite temperature and pressure values during the process steps allows to selectively extract from the resin of the *Protium heptaphyllum* plant the class of molecules of interest for the modulating action on the metabolism of cholesterol and lipids.

The process of extraction and purification of the identified bioactive components is economically advantageous because it uses inexpensive solvents, simple equipment, widely spread and at low cost that can be used also by unskilled manpower. Despite this, it guarantees at the same time speed, scalability, component stability and food safety of the extract during all the different process steps.

In addition, the collection of the raw material (resin) takes place from spontaneous and easily available trees within the whole area of the Amazon rainforest, thus not requiring the use of new areas for the cultivation or felling of the same producing trees for the exploitation of this resource.

The final extract obtained from this innovative process consists of an easily crushable solid material, which, once powdered, can be easily combined with the excipients necessary for the final formulations.

The extract can be easily conveyed in its powdered form (POWDERED EXTRACT OF PROTIUM) for the realization of products intended for oral intake (capsules / tablets / sachets). Possible problems related to the intake of the abovementioned products can be prevented through a liquid formulation (FLUID EXTRACT OF PROTIUM), for making syrups; in fact the extract, due to its lipophilic character, can be easily solubilized in vegetable oils widely used for food purposes (e.g. rice oil).

To overcome the problems related to the bioavailability of the bioactive molecules, the extract is suitable for realizing liposomal formulations (LIPOSOMAL EXTRACT OF PROTIUM) that exploit the creation of phospholipid micelles with an amphiphilic character containing the mixture or substance to be conveyed by increasing the

assimilable amount in the intestine.

To overcome the problems related to the crossing of the gastric tract and the possible degradation or alteration of the biomolecules, the extract is suitable for realizing formulations in microcapsules (MICROENCAPSULATED EXTRACT OF PROTIUM) obtained by gelling with alginate, whose composition gives the necessary protection to the extract during the gastric transit and the subsequent dissolution in the intestinal environment.

Further advantageous features will appear more evident from the following description of preferred but not exclusive embodiments, given by mere way of non-limiting example.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will be described hereinafter by means of some preferred embodiments, given by way of non-limiting example, with reference to the attached drawings. These drawings illustrate different aspects and examples of the present invention and, where appropriate, similar structures, components, materials and/or elements in different figures are denoted by similar reference numbers.

FIG. 1 is a flow chart of the extraction process according to the present invention;

FIG. 2 is a bar graph illustrating the expression of key enzymes in the cellular cholesterol biosynthesis using the extract according to the present invention;

FIG. 3 is a bar graph illustrating the expression of regulating factors of the cholesterol homeostasis in the liver using the extract according to the present invention;

FIG. 4 is a bar graph illustrating the expression of regulating factors of the cholesterol homeostasis in the blood using the extract according to the present invention;

FIG. 5 is a bar graph illustrating the expression of factors involved in lipid metabolism, in particular the ACC1 gene, using the extract according to the present invention;

FIG. 6 is a bar graph illustrating the expression of factors involved in lipid metabolism, in particular the FASN gene, using the extract according to the present invention;

FIG. 7 is a bar graph illustrating the expression of factors involved in lipid metabolism, in particular the PPARα gene, using the extract according to the present invention;

FIG. 8 is a bar graph illustrating the expression of factors involved in suppressing the conversion of cholesterol into bile salts using the extract according to the present

invention.

DETAILED DESCRIPTION OF THE INVENTION

While the invention is susceptible to various modifications and alternative constructions, some preferred embodiments are shown in the drawings and will be described in detail hereinbelow.

It should be understood, however, that there is no intention to limit the invention to the specific embodiments illustrated, but, on the contrary, the invention is intended to cover all modifications, alternative constructions, and equivalents falling within the scope of the invention as defined in the claims.

In the following description, therefore, the use of "for example", "etc.", "or" denotes non-exclusive alternatives without any limitation, unless otherwise indicated; the use of "also" means "including, but not limited to" unless otherwise indicated; the use of "includes / comprises" means "includes / comprises, but not limited to" unless otherwise indicated.

The hydroalcoholic extract from the resin of the *Protium heptaphyllum* plant, the corresponding pharmaceutical formulations comprising the aforesaid extract and the relevant hydroalcoholic extraction process at controlled pressure and temperature of the aforesaid extract according to the present invention are based on the innovative concept of carrying out an enrichment for the acid triterpene component.

The present invention finds advantageous applications in the inhibition of the gene expression of the HMG-CoA reductase enzyme, in the alteration of the gene expression in liver cells, in the alteration of the gene expression of key enzymes involved in the lipid metabolism and in the treatment of hypercholesterolemia.

In the present description, the term "extract" means the mixture of organic molecules obtained at the end of the invented process starting from the *Protium heptaphyllum* resin.

In the present description, the term "hydroalcoholic extract" means the mixture of organic molecules obtained at the end of the process in which a mixture of water and ethanol is exclusively used as a solvent.

In the present description, the term "hydroalcoholic extraction" means the selective solubilization of organic molecules in a fluid exclusively consisting of a mixture of

water and ethanol.

In the present description, the term "resin" means the organic material obtained from the incision of the *Protium heptaphyllum* trunk.

In the present description, the term "acid triterpene component / acid triterpenes" means the set of organic molecules present in the extract characterized by 30 carbon atoms, by the presence in the structure of 4 or 5 cycles and in particular by one or more carboxylic substituents that give the molecule an acid dissociation constant lower than organic molecules with the same basic triterpene structure with 4 or 5 cycles, but having possible non-carboxylic substituents. These acid triterpenes have a molecular weight ranging between 450 Da and 500 Da; in the present description the terms "acid triterpene component" and "acid triterpenes" will be used indistinctively, as synonyms.

In the present description, the term "pharmaceutical formulation" means the form in which each type of pharmaceutical product is presented, whether medicinal or non-medicinal. Pharmaceutical products are not in themselves exclusively medicines or active principles, therefore also cosmetic, food and herbal products classified as pharmaceutical products or even having exclusively pharmaceutical quality or grade fall into this classification.

In the present description, the term "inhibition of the gene expression of the HMG-CoA reductase enzyme" means the reduction of the amount of intracellular messenger RNA (mRNA) subsequently involved in the translation processes for obtaining the HMG-CoA reductase protein (EC number - 1.1.1.88) .

In the present description, the term "alteration of the gene expression in liver cells" means the ability to modify the amount of different messenger RNA (mRNA) sequences, present inside the cellular lines of human hepatocytes, transcribed starting from DNA sequences of certain genes.

In the present description, the term "alteration of the gene expression of key enzymes involved in lipid metabolism" means the ability to modify the amount of different messenger RNA (mRNA) sequences, transcribed starting from DNA sequences of certain genes involved in lipid metabolism.

In the present description, the term "hypercholesterolemia" means the condition in an

organism in which LDL levels higher than the normal physiological levels are present in the plasma.

In this description, the term "controlled pressure and temperature" means the possibility of monitoring and adjusting the temperature and pressure parameters during the process steps, keeping them within definite ranges of values.

In the present description, the term "polarity" means a chemical-physical property for which a molecule (called polar) has one or more positive partial charges on one part of the molecule and one or more negative partial charges on the opposite part of it. Molecules that do not exhibit the polarity phenomenon are called apolar or non-polar.

The main innovation that characterizes the extract of the present application is based on the combination between the method of obtaining the extract from the starting resin, by means of a technology allowing the selective solubilization and the following enrichment of a certain class of molecules present in the starting resin (the fraction of acid triterpenes), and the significant action that this fraction has, even at low doses, in the regulation of the cholesterol metabolism through the regulation of the gene expression of certain key genes in the cholesterol metabolism.

The effect that is noticed on the gene expression is found out to be also innovative compared to those commonly studied (blood measurements of LDL and HDL levels) since it is based on the investigation of the molecular mechanisms upstream the cholesterol metabolism and on the regulation thereof, and it is not based on effects resulting from the change in metabolism.

In addition to the above, the extract complies with the hygiene and quality requirements required for the production of pharmaceutical formulations.

The identified extraction process falls within the green chemistry technologies, safeguarding the environment and avoiding the use of highly toxic or fossil-based solvents.

This extraction method has been developed to be particularly optimized and economical: it consists of few steps; it provides the use of instruments that are simple and easily available on the market. The extraction is maximized in order to obtain the highest extraction yield of the fraction of interest depending on the costs necessary for its obtaining.

The use of this extract derived from the resin of a spontaneous plant present in the Amazon rainforest area, in view of an environmentally friendly, supportive and circular economy, favours the responsible use of the natural resources by shifting the interest of the local populations from different exploitations of the resources of the Amazon rainforest providing, for example, deforestation works and proposes, at the same time, an economic return for the local populations in line with their traditional lifestyle. Finally, the by-products obtained as a secondary result from the extraction activity find application in the agricultural field as natural biostimulants in replacement, for example, of pesticides.

It is an aspect independent and usable autonomously with respect to the other aspects of the invention a hydroalcoholic extract from the resin of the *Protium heptaphyllum* plant comprising acid triterpenes in an amount ≥ 40% by weight with respect to the total amount of the extract obtained.

Preferably the extract comprises acid triterpenes molecules having a molecular weight in the range of 450 Da - 500 Da.

Preferably the extract further comprises other triterpenes, sequiterpenes and monoterpenes (with polarity lower than the acid triterpenes) characteristic of the resin of *Protium heptaphyllum* in amounts lower than the concentrations present in the resin as such.

Preferably the ratio by weight of acid triterpenes with respect to α and β amyrins triterpenes ranges between 1.4 and 8, more preferably it is equal to 4.

It is an aspect independent and usable autonomously with respect to the other aspects of the invention a pharmaceutical formulation comprising the extract as described above.

Preferably the pharmaceutical formulation further comprises one or more additional ingredients selected from:

- a carrier of the extract selected from:
    - rice starch and tribasic calcium phosphate
    - rice oil
- amphiphilic molecules such as phospholipids and derivatives
- sodium alginate, calcium chloride and microcrystalline cellulose

- further botanical extracts such as, for example:
    - extract from the *Commiphora mukul* plant
    - extract from the *Berberis vulgaris* plant
    - extract from the *Allium sativum* plant
- one or more flavouring and sweetening agents
- one or more bulking agents, excipients and diluents of pharmaceutical, food or cosmetic grade.

The extract as described above and the pharmaceutical formulation comprising such extract are intended for use in the inhibition of the gene expression of the HMG-CoA reductase enzyme.

The extract as described above and the pharmaceutical formulation comprising such extract are intended for use in the alteration of the gene expression in liver cells.

The extract as described above and the pharmaceutical formulation comprising such extract are intended for use in the alteration of the gene expression of key enzymes involved in the lipid metabolism.

The extract as described above and the pharmaceutical formulation comprising such extract are intended for use in the treatment of hypercholesterolemia.

With reference to FIG. 1, which illustrates the preferred embodiment of the present invention, it is noted that the hydroalcoholic extraction process at controlled pressure and temperature comprises the following steps:

- step 100: preparing a predetermined amount of resin of the *Protium heptaphyllum* plant;
- step 101: grinding the resin of the *Protium heptaphyllum* plant by means of a suitable device until obtaining a powder with a particle size in the range 2-5 mm;
- step 102: carrying out the extraction of the most polar terpene component contained in the resin by introducing a predetermined amount of powdered resin into a tank with a mixer, by adding a predetermined amount of a hydroalcoholic solution and by stirring at low temperature;
- step 103: separating the insoluble component from the mixture by means of a suitable low temperature filtration or centrifugation system;

- step 104: concentrating the most polar terpene component extracted by evaporation removing of the solvent under vacuum and at low temperature;
- step 105: carrying out the final drying and evaporation removing of the most volatile components;
- step 106: obtaining a hydroalcoholic extract from the resin of the *Protium heptaphyllum* plant comprising acid triterpenes in an amount ≥ 40% by weight with respect to the total amount of the extract obtained.

Preferably the process provides

- step 100: the predetermined amount of resin of the *Protium heptaphyllum* plant ranges between 80 kg and 160 kg, preferably it is equal to 110 kg;
- step 101: the grinding of the resin of the *Protium heptaphyllum* plant takes place by means of a refrigerated hammer mill, with a temperature maintained between -20 °C and 15 °C;
- step 102: the predetermined amount of powdered resin ranges between 75 kg and 150 kg, preferably it is equal to 100 kg, the predetermined amount of hydroalcoholic solution ranges between 350 litres and 1,000 litres, preferably it is equal to 350 litres and the extraction of the most polar terpene component contained in the resin takes place at a temperature ranging between -20 °C and 15 °C;
- step 103: separating the insoluble component, i.e. the most apolar terpene component mainly consisting of α and β amyrins, from the mixture by filtration with Buchner filter, press filter or cellulosic passive filters having porosity of 25 µm at a temperature ranging between -20 °C and 15 °C;
- step 104: concentrating the most polar terpene component extracted by evaporation removal of the solvent under vacuum and at a temperature ranging between -20 °C and 15 °C and at a relative pressure value lower than -950 mBar with respect to the normal atmospheric pressure value;
- step 105: carrying out the final drying of the concentrated material together with the evaporation removal under conditions of forced ventilation in a nitrogen atmosphere at a temperature ranging between 40 °C and 130 °C for a time ranging between 1 and 12 hours of the most volatile components, i.e. of the

residual content, still present in traces, of water and ethanol and of most of the mono and sesquiterpene component still present in the extract.

Preferably the hydroalcoholic solution is selected from a mixture containing water and a water-miscible alcohol, more preferably it is a mixture consisting of water and ethyl alcohol.

Preferably 100 kg of powdered resin are introduced into a tank with mixer, into which a hydroalcoholic solution is added (450 l, weight/volume ratio ranging between 1:4.5 and 1:5) having an ethanol concentration ranging from 50% to 96% by volume.

Although the resin is formed by almost completely liposoluble elements (only a small fraction thereof, in fact, is solubilized in water) these particular process conditions allow the extraction of a specific fraction of molecules from the resin, a more "polar" fraction, avoiding to solubilize all the remaining material.

The hydroalcoholic extraction process at controlled pressure and temperature is further specified hereinbelow.

The *Protium heptaphyllum* resin (raw material) is crushed by means of a refrigerated hammer mill until obtaining a powder with a particle size of 2-5 mm. During this step the temperature is maintained between -20 °C and 15 °C.

100 kg of powdered resin are introduced into a tank with mixer, into which a hydroalcoholic solution (450 l, weight/volume ratio ranging between 1:4.5 and 1:5) is added, which can have an ethanol concentration ranging from 50% to 96% by volume. The extraction process takes place under agitation, at a temperature between -20 °C and 15 °C.

The insoluble fraction is separated from the mixture by filtration (Buchner or press filter) with cellulosic passive filters having porosity of 25 µm or by centrifugation, at a temperature between -20 °C and 15 °C.

The filtered mixture is concentrated in a low temperature vacuum solvent evaporation system (pressure = -950 mBar, evaporation chamber temperature = 15 °C).

The concentrated mixture is finally subjected to a process of removal of the residual solvent and of the volatile compounds still present (monoterpenes and sesquiterpenes) by introducing it into a forced ventilation cabinet at a controlled temperature and atmosphere (ambient pressure, temperature between 40 °C and 130 °C, in a nitrogen

atmosphere).

The obtained final product consists of an extract in a solid-crystalline form. The extraction yield by weight is usually between 40% and 60% of the starting material.

After obtaining the solid extract (denomination: "Concentrated extract of Protium"), this can be finally processed in different ways in order to provide an ingredient intended for the food, pharmaceutical and cosmetic industries that is technologically valid for the production of finished products.

The main forms in which the extract is supplied to the companies that will carry out the different finished products are as follows.

POWDERED EXTRACT OF PROTIUM

The extract and the excipients are crushed and homogenized by means of a refrigerated hammer mill (temperature between 10 °C and 15 °C) until a homogeneous powder with an average particle size of 200 µm is obtained.

Composition example:

1- CONCENTRATED EXTRACT OF PROTIUM = 40-60%

2- Rice native starch = 20-40%

3- Tribasic Calcium Phosphate = 10-25%

FLUID EXTRACT OF PROTIUM

The extract is mixed with a low viscosity vegetable oil (for example rice oil) in a melter with mixer (temperature between 50 and 80 °C, controlled atmosphere with nitrogen) until the complete solubilization and homogenization of the extract is obtained.

Composition example:

1- CONCENTRATED EXTRACT OF PROTIUM = 40-60%

2- Rice oil = 40-60%

Other possible (secondary) forms of marketing of the ingredient are:

LIPOSOMAL EXTRACT OF PROTIUM

Composition example:

1- CONCENTRATED EXTRACT OF PROTIUM = 40-60%

2- Liposomal mixture containing Phospholipids = 40-60%

MICROENCAPSULATED EXTRACT OF PROTIUM

Composition example:

1- CONCENTRATED EXTRACT OF PROTIUM = 25-80%

2- Microcrystalline cellulose = 15-50%

3- Sodium alginate = 5-15%

4- Calcium Chloride = 1-5%

Some examples of the use of the different forms of ingredient in the final formulations (finished products) intended for consumption for the food supplements, pharmaceutical and cosmetic markets are listed hereinbelow:

A. Example of capsule formulation (quantity per capsule):

1. MICROENCAPSULATED EXTRACT OF PROTIUM: 50-200 mg
2. Microencapsulated extract titrated in guggulsterones from *Commiphora mukul*: 50-200 mg
3. Pullulan-based vegetable capsule

B. Example of tablet formulation (quantity per tablet):

1. POWDERED EXTRACT OF PROTIUM: 50-200 mg
2. Extract titrated in berberine from *Berberis vulgaris*: 50-200 mg
3. Microcrystalline cellulose: 0.1-50 mg
4. Magnesium stearate: 0.1-50 mg
5. Hydroxypropylmethylcellulose: 0.1-50 mg

C. Example of formulation of effervescent granules in sachets (quantity per sachet):

1. POWDERED EXTRACT OF PROTIUM: 50-200 mg
2. Tartaric acid: 100-200 mg
3. Sodium bicarbonate: 1-1.5 g
4. Citric acid: 500-800 mg
5. Sweeteners: 50-200 mg
6. Natural flavours: 10-50 mg

D. Example of formulation in syrups (quantity per 150 ml bottle):

1. LIPOSOMAL EXTRACT OF PROTIUM: 1,000 mg
2. Liposomal extract titrated in guggulsterones by *Commiphora mukul*: 1,000 mg
3. Extract titrated in berberine from *Berberis vulgaris*: 500 mg
4. Glycerol: 20 g
5. Natural flavours: 0.1-200 mg

6. Steviol glycosides (sweeteners): 100-200 mg
7. Sodium benzoate: 100 mg
8. Water: just enough up to volume

E. Example of soft-gel formulation:

1. FLUID EXTRACT OF PROTIUM: 50-200 mg
2. Fluid extract titrated in guggulsterones from *Commiphora mukul*: 50-200 mg
3. Vegetable oil as bulking agent: 10-200 mg
4. Hydroxypropylmethylcellulose-based vegetable capsule

The hydroalcoholic extract from the resin of the *Protium heptaphyllum* plant enriched for the acid triterpene component, the corresponding pharmaceutical formulations comprising the aforesaid extract and the relevant hydroalcoholic extraction process at controlled pressure and temperature of the aforesaid extract according to the present invention are hereinafter described more in detail with reference to the following experimental data, which are to be construed as illustrative but not limitative of the present invention.

To determine the effects of the extraction process, the resin matrix and the extract were quantified with the aim of characterizing the bioactive molecules present therein.

Comparing the initial composition present in the raw material with the extract obtained it is possible to observe how the extraction method used and the following heat treatment allow to concentrate the triterpene component with carboxylic substituents (with acid function), while the quantities of α and β amyrins (during the low temperature extraction phase) and the volatile fraction (during the final heat treatment in an inert atmosphere) are reduced. In the extract there is however present a minimal oxidized triterpene component (amyrones, ≤ 6%) exclusively due to the presence of these components already in the starting material and not due to oxidative phenomena during the process.

The acid triterpene component, which is the target of the invented extraction process and of which the extract is enriched, consists of a group of molecules that, each and all, have at least one carboxyl function, characterized by having 30 carbon atoms and by the presence in the structure of 4 or 5 cycles and by having a molecular weight ranging between 450 Da and 500 Da.

Through an HPLC-HRMS qualitative analysis, the acid triterpene fraction was characterized, which turns out to be mainly composed of acid triterpenes having molecular weight comprised, in particular, in the ranges 452 - 457 Da, 468 - 473 Da and 494 - 498 Da.

The residual volatile component after the process mainly consists of monoterpenes and sesquiterpenes in which the most representative molecules are carene, limonene, terpineol, cymene and terpinene.

Comparison of the typical composition of the extract obtained with the starting resin

	RESIN	EXTRACT
Acid triterpene fraction	**25%**	**60%**
α amyrins	25%	12%
β amyrins	20%	9%
α amyrone	2.5%	3%
β amyrone	2%	3%
breine	2.5%	1.5%
maniladiol	3%	2.5%
volatile component	20%	9%

Concentration ranges (maximum and minimum) obtainable for the different components of the extract

	Minimum value	Maximum value
Acid triterpene fraction	**40%**	**80%**
α amyrins	5%	15%
β amyrins	5%	15%
α amyrone	0.5%	4%
β amyrone	0.5%	4%
breina	0.5%	3%
maniladiol	0.5%	3%
volatile component	4%	10%

Characterization of the acid triterpene fraction in the extract

m/z [M-H] -*	MS/MS (m/z)**	Abundance in the acid fraction
455.3492	437.3390; 373.2721; 341.2824	**5-30%**
453.3374	435.2563; 407.2618	**5-30%**
471.3439	453.3336; 425.3389; 359.2714	**1-15%**
467.3124	449.3026; 423.3231; 353.2455	**1-15%**

469.3283	451.3181; 425.3383; 355.2611	**1-15%**

* Molecular ion in ESI ionization with negative polarity

* * Characteristic fragmentation pattern

Maximum residual volatile component in the extract:

- 2.5% Carene
- 1% Limonene
- 1% Terpineol
- 4.5% Cymene
- 1% Terpinene

PHARMACOLOGICAL ACTIVITY

The extract obtained as reported above (hydroalcoholic extraction) was tested on cells of the HepG2 liver tumour line characterized by the expression of a set of expressed genes typical of several specialized liver tissues.

The used extract concentrations were calculated depending on the dilution that the pharmaceutical formulation undergoes in the volume of fluids present in the digestive tract and depending on the absorption at the intestinal level.

The cells in the control and treatment culture medium at different concentrations of extract were incubated and used for subsequent gene expression analysis and cytotoxicity assessment.

The transcripts whose activity was monitored after administration of the resin extract are relevant to some of the genes that code for the main direct and indirect key mechanisms linked to cholesterol metabolism and in particular:

- HMG-CoAR (HMG-CoA Reductase)
- PPARα (Peroxisome Proliferator-Activated Receptors)
- FXR (Farnesoid X Receptor)
- PCSK9 (Proprotein Convertase Subtilisin/Kexin Type 9)
- IDOL/MYLIP (Inducible Degrader of the LDL receptor)
- FASN (Fatty Acid Synthase)
- ACC1 (Acetyl-CoA carboxylase)

For the qRT-PCR analyses (*semi-quantitative PCR*), the expression of the target genes in

proliferating cells in media at different concentrations of the extract, in the range between 1 and 50 µg/ml, was taken into account.

As negative control, the transcripts in cells treated with the same volume of solvent without extract have been analysed.

The measurement, in addition to these transcripts, of a constitutively expressed reference gene (*housekeeping*) such as beta-actin, whose activity is not altered by substances such as those under study, was monitored in order to normalize the expression data of the other genes among all the treatments carried out excluding variables linked to the repeatability of the tests.

The qRT-PCR analysis of the target genes led to the identification of regulation events of the gene expression correlated in a dose-dependent manner to the treatment received by the cells.

The relevant graphs are reported hereinbelow, in which the dose-dependent effects (at concentrations of 1, 10, 25 and 50 mg/L) of the extract on the transcriptional activity of the genes involved in the cholesterol metabolism are highlighted, comparing them to the normal levels present in the control (reference value on the ordinate axis equal to 1):

<u>Expression of key enzymes in the cellular cholesterol biosynthesis</u>

The HMG-CoAR enzyme, mostly present in the hepatocytes (liver cells), appears to be the limiting, and therefore regulating, stage of cholesterol synthesis. The down-regulation of the gene that codes for this enzyme is correlated to a minor biosynthesis of the intracellular cholesterol.

With reference to FIG. 2 it is noted that the messenger RNA of the HMG-CoA reductase gene is strongly down-regulated after treatment with the extract, already at amounts equal to 10 mg/L.

<u>Expression of regulating factors of the cholesterol homeostasis in the liver and in the blood</u>

The factors IDOL and PCSK9 both contribute to reduce the levels of LDLR, membrane receptor involved in the LDL uptake into the bloodstream, so the down-regulation of their expression results in a larger quantity of receptors able to internalize LDLs.

With reference to FIG. 3 it is noted that the messenger RNA of the IDOL/MYLIP gene

is down-regulated in a way that is directly proportional to the increase in the amount of extract for the treatment.

With reference to FIG. 4 it is noted that the messenger RNA of the PCSK9 gene is significantly down-regulated after treatment with the extract, starting from concentration values of the extract equal to 10 mg/L.

Expression of factors involved in lipid metabolism

The lipid metabolism is regulated by a class of enzymes called Acetyl-CoA carboxylase, among whom there is the ACC1 isoform that is responsible, when present and active, for the formation of malonyl-CoA, a key precursor in the biosynthesis of fatty acids. This is followed by the activity of FASN, a multi-enzyme complex that uses malonyl-CoA and acetyl-CoA in the presence of NADPH for the synthesis of palmitic acid.

Inversely to ACC1 and FASN, the class of receptors activated by alpha-type peroxisome proliferators (PPARα) is involved, in particular in the liver, in the processes linked to the catabolism of fatty acids. The down-regulation of ACC1 and FASN and the up-regulation of the PPARα expression indicates a reduction in the biosynthesis of intracellular fatty acids versus an increase in their catabolism.

With reference to FIG. 5 it is noted that the messenger RNA of the ACC1 gene is strongly down-regulated after treatment with the extract even at very low concentrations (1 mg/L).

With reference to FIG. 6 it is noted that the messenger RNA of the FASN gene is significantly down-regulated after treatment with the extract, starting from concentration values of the extract equal to 10 mg/L.

With reference to FIG. 7 it is noted that the messenger RNA of the PPARα gene is significantly up-regulated after treatment with the extract even at very low concentrations (1 mg/L).

Expression of factors involved in the inhibition of the conversion of cholesterol into bile salts

FXR is a receptor present in the intestine and liver that, once bound to the bile acids, migrates as a heterodimer into the nucleus, cascade inhibiting the activity of the CYP7A1 enzyme responsible for one of the key steps in the conversion of cholesterol

into bile acids. The down-regulation of the FXR gene consequently decreases the amount of receptor present, then allowing to reduce the inhibition of CYP7A1.

With reference to FIG. 8 it is noted that the messenger RNA of the FXR gene is down-regulated after treatment with the extract even at very low concentrations (1 mg/L).

ASSESSMENT OF THE EXTRACT CYTOTOXICITY

The assessment of the potential cytotoxicity on HepG2 cells depending on the administered concentration of extract allowed to exclude a potential cytotoxic effect; in fact for all the tested concentrations of extract (1, 10, 25, 50 µg/ml) the cells have shown to maintain a viability equal to 100%.

As can be deduced from the above, the innovative technical solution herein described has the following advantageous characteristics:

- the extract of the invention does not exert any cytotoxic effect on the cellular line of hepatocytes, thus proving to be safe at all tested concentrations, which comprise the concentration of extract intended for the production of pharmaceutical formulations;
- the bioactive molecules contained in the extract exert a significant modulatory action on the transcription of the most important genes correlated to the metabolism of cholesterol and fatty acids favouring, through a careful administration and an appropriate monitoring during treatment, the maintenance of normal physiological values of cholesterol and intracellular lipids.

From the description reported hereinabove it is evident, therefore, how with the hydroalcoholic extract from the resin of the *Protium heptaphyllum* plant enriched for the acid triterpene component, the corresponding pharmaceutical formulations comprising the aforesaid extract and the relevant hydroalcoholic extraction process at controlled pressure and temperature of the aforesaid extract according to the present invention allow to achieve the proposed aims.

It is likewise evident, to a person skilled in the art, that it is possible to make modifications and further variations to the solution described with reference to the attached figures, without thereby departing from the teaching of the present invention and from the scope as defined by the attached claims.

CLAIMS

1. A hydroalcoholic extract from the resin of the *Protium heptaphyllum* plant comprising acid triterpenes in an amount ≥ 40% by weight with respect to the total amount of the extract obtained.
2. The extract according to claim 1, comprising acid triterpenes molecules having molecular weight in the range 450 Da - 500 Da.
3. The extract according to claim 1 or 2, further comprising other triterpenes, sequiterpenes and monoterpenes with polarity lower than that of the acid triterpenes and characteristic of the resin of *Protium heptaphyllum* in amounts lower than the concentrations present in the resin as such.
4. The extract according to claim 3, wherein the ratio by weight of acid triterpenes with respect to α and β amyrins ranges between 1.4 and 8, preferably it is equal to 4.
5. A pharmaceutical formulation comprising the extract according to any claims 1 to 4.
6. The pharmaceutical formulation according to claim 5, further comprising one or more additional ingredients selected from:

- a carrier of the extract selected from:
    - rice starch and tribasic calcium phosphate
    - rice oil
- amphiphilic molecules such as phospholipids and derivatives
- sodium alginate, calcium chloride and microcrystalline cellulose
- further botanical extracts selected from:
    - extract from the *Commiphora mukul* plant
    - extract from the *Berberis vulgaris* plant
    - extract from the *Allium sativum* plant
- one or more flavouring and sweetening agents
- one or more bulking agents, excipients and diluents of pharmaceutical, food or cosmetic grade.

7. The extract according to any claim 1 to 4 for use in the inhibition of the gene expression of the HMG-CoA reductase enzyme.

8. The pharmaceutical formulation according to claims 5 or 6 for use in the inhibition of the gene expression of the HMG-CoA reductase enzyme.
9. The extract according to any claim 1 to 4 for use in the alteration of the gene expression in liver cells.
10. The pharmaceutical formulation according to claims 5 or 6 for use in the alteration of the gene expression in liver cells.
11. The extract according to any claim 1 to 4 for use in the alteration of the gene expression of key enzymes involved in the lipid metabolism.
12. The pharmaceutical formulation according to claims 5 or 6 for use in the alteration of the gene expression of key enzymes involved in the lipid metabolism.
13. The extract according to any claim 1 to 4 for use in the treatment of hypercholesterolemia.
14. The pharmaceutical formulation according to claim 5 or 6 for use in the treatment of hypercholesterolemia.
15. A hydroalcoholic extraction process at controlled pressure and temperature comprising the following steps:
    - step 100: preparing a predetermined amount of resin of the *Protium heptaphyllum* plant;
    - step 101: grinding the resin of the *Protium heptaphyllum* plant by means of a suitable device until obtaining a powder with a particle size in the range 2-5 mm;
    - step 102: carrying out the extraction of the most polar terpene component contained in the resin by introducing a predetermined amount of powdered resin into a tank with a mixer, by adding a predetermined amount of a hydroalcoholic solution and by stirring at low temperature;
    - step 103: separating the insoluble component from the mixture by means of a suitable low temperature filtration or centrifugation system;
    - step 104: concentrating the most polar terpene component extracted by evaporation removing of the solvent under vacuum and at low temperature;
    - step 105: carrying out the final drying and evaporation removing of the most volatile components;

- step 106: obtaining a hydroalcoholic extract from the resin of the *Protium heptaphyllum* plant comprising acid triterpenes in an amount ≥ 40% by weight with respect to the total amount of the extract obtained.

16. The process according to claim 15, wherein

- step 100: the predetermined amount of resin of the *Protium heptaphyllum* plant ranges between 80 kg and 160 kg, preferably it is equal to 110 kg;
- step 101: the grinding of the resin of the *Protium heptaphyllum* plant takes place by means of a refrigerated hammer mill, with a temperature maintained between -20 °C and 15 °C;
- step 102: the predetermined amount of powdered resin ranges between 75 kg and 150 kg, preferably it is equal to 100 kg, the predetermined amount of hydroalcoholic solution ranges between 350 litres and 1,000 litres, preferably it is equal to 350 litres and the extraction of the most polar terpene component contained in the resin takes place at a temperature ranging between -20 °C and 15 °C;
- step 103: separating the insoluble component, i.e. the most apolar terpene component mainly consisting of α and β amyrins, from the mixture by filtration with Buchner filter, press filter or cellulosic passive filters having porosity of 25 µm at a temperature ranging between -20 °C and 15 °C;
- step 104: concentrating the most polar terpene component extracted by evaporation removal of the solvent under vacuum and at a temperature ranging between -20 °C and 15 °C and at a relative pressure value lower than -950 mBar with respect to the normal atmospheric pressure value;
- step 105: carrying out the final drying of the concentrated material together with the evaporation removal under conditions of forced ventilation in a nitrogen atmosphere at a temperature ranging between 40 °C and 130 °C for a time ranging between 1 and 12 hours of the most volatile components, i.e. of the residual content, still present in traces, of water and ethanol and of most of the mono and sesquiterpene component still present in the extract.

17. The process according to claim 15 or 16, wherein the hydroalcoholic solution is selected from a mixture containing water and a water-miscible alcohol, preferably

it is a mixture consisting of water and ethyl alcohol.

18. The process according to any claim 15 to 17, wherein 100 kg of powdered resin are introduced into a tank with a mixer, into which a hydroalcoholic solution (450 l, weight/volume ratio ranging between 1:4.5 and 1:5) is added, having an ethanol concentration ranging from 50% to 96% by volume.

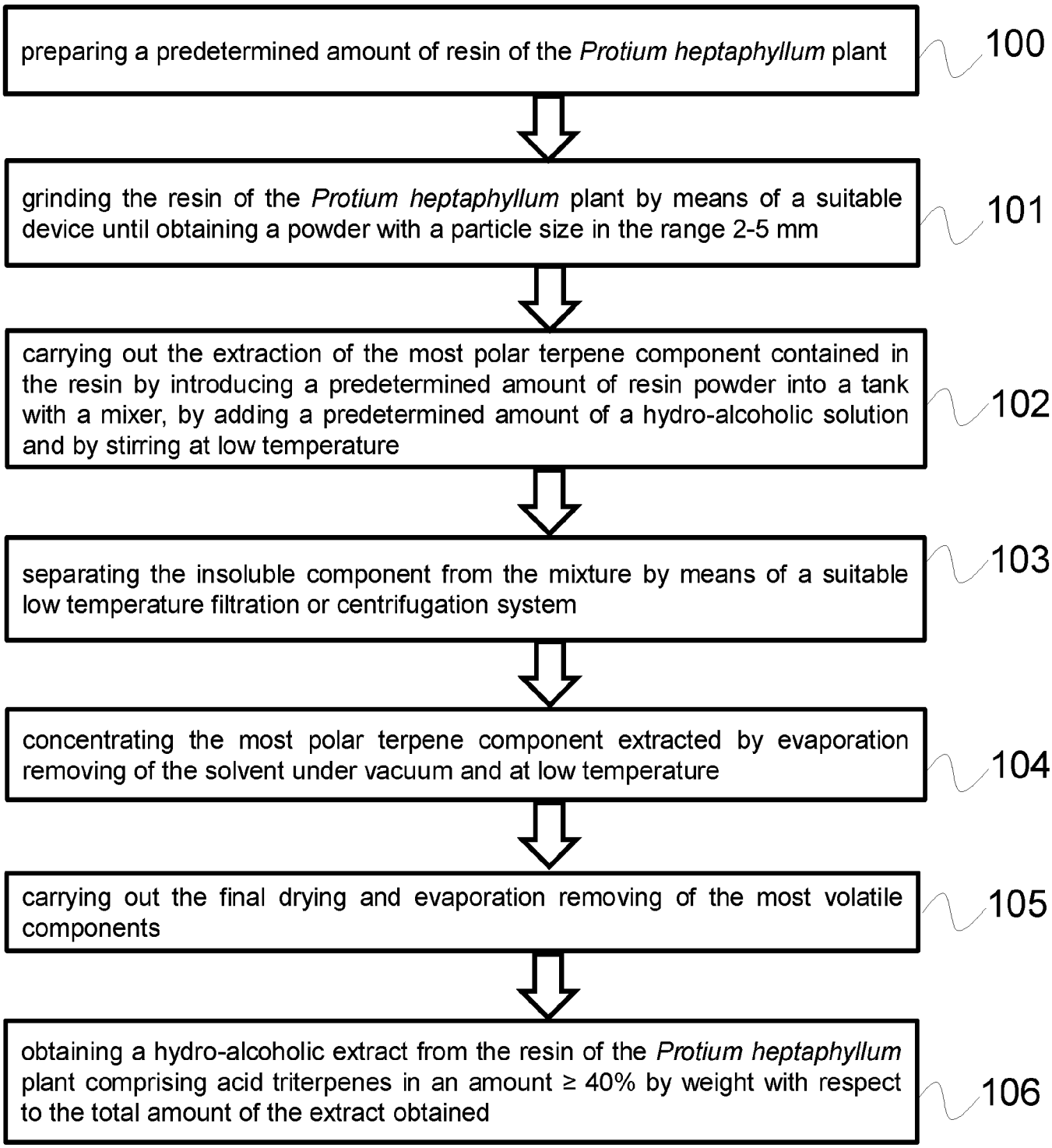
preparing a predetermined amount of resin of the *Protium heptaphyllum* plant
100
grinding the resin of the *Protium heptaphyllum* plant by means of a suitable device until obtaining a powder with a particle size in the range 2-5 mm
101
carrying out the extraction of the most polar terpene component contained in the resin by introducing a predetermined amount of resin powder into a tank with a mixer, by adding a predetermined amount of a hydro-alcoholic solution and by stirring at low temperature
102
separating the insoluble component from the mixture by means of a suitable low temperature filtration or centrifugation system
103
concentrating the most polar terpene component extracted by evaporation removing of the solvent under vacuum and at low temperature
104
carrying out the final drying and evaporation removing of the most volatile components
105
obtaining a hydro-alcoholic extract from the resin of the *Protium heptaphyllum* plant comprising acid triterpenes in an amount ≥ 40% by weight with respect to the total amount of the extract obtained
106

Fig. 1

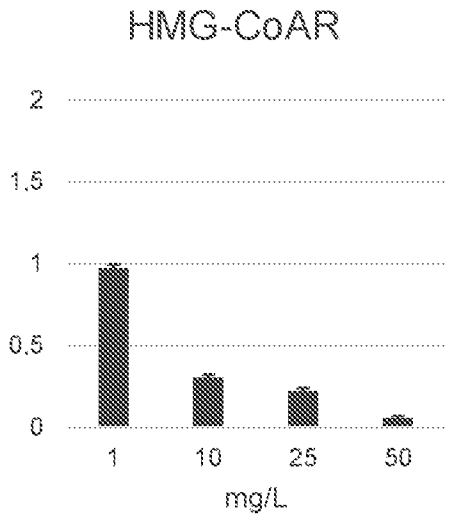

Fig. 2

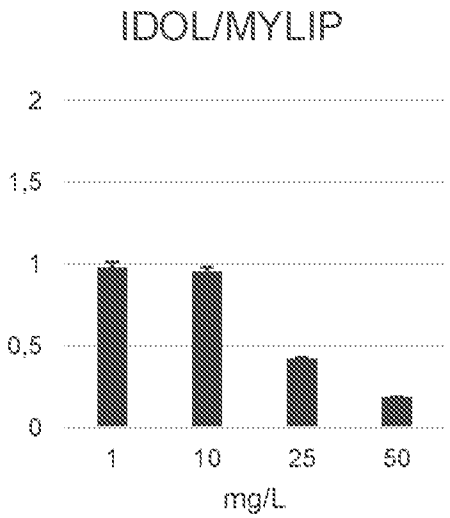

Fig. 3

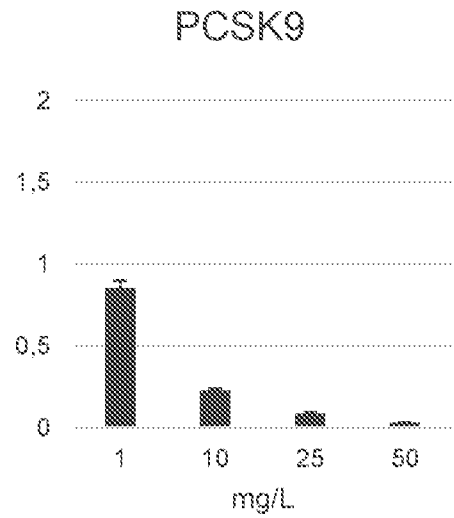

Fig. 4

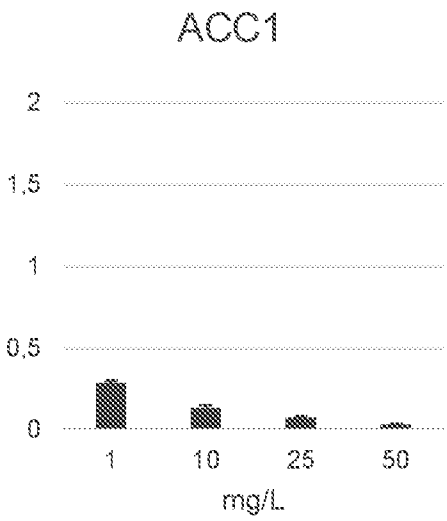

Fig. 5

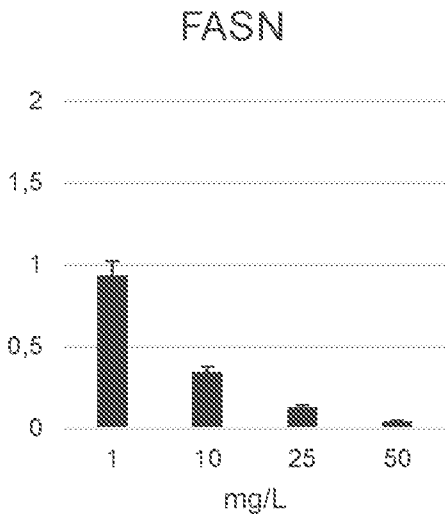

Fig. 6

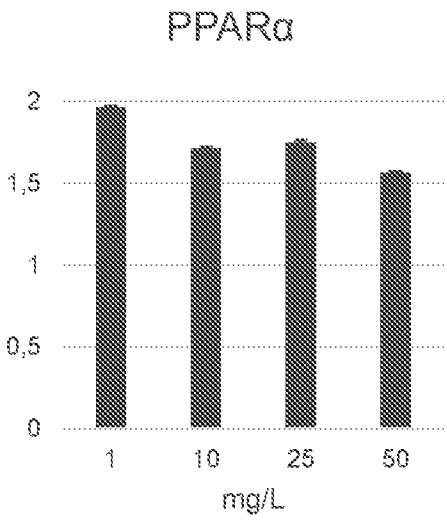

Fig. 7

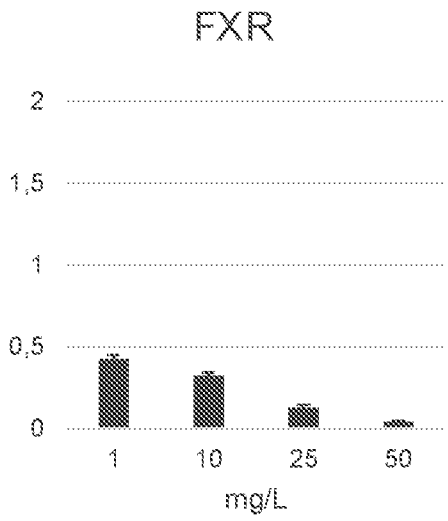

Fig. 8

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2021/053411

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K36/32 A61P3/00 A61P3/06
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, EMBASE, FSTA, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FLÃ VIA ALMEIDA SANTOS ET AL: "Antihyperglycemic and hypolipidemic effects of Î , Î2-amyrin, a triterpenoid mixture from Protium heptaphyllum in mice", LIPIDS IN HEALTH AND DISEASE, BIOMED CENTRAL, LONDON, GB, vol. 11, no. 1, 6 August 2012 (2012-08-06) , page 98, XP021107471, ISSN: 1476-511X, DOI: 10.1186/1476-511X-11-98	1,4,5, 13,14
Y	table 1	1-18
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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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Date of the actual completion of the international search	Date of mailing of the international search report
14 June 2021	24/06/2021

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Thalmair, Michaela

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2021/053411

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	OLIVEIRA F A ET AL: "Protective effect of @a- and @b-amyrin, a triterpene mixture from Protium heptaphyllum (Aubl.) March. trunk wood resin, against acetaminophen-induced liver injury in mice", JOURNAL OF ETHNOPHARMACOLOGY, ELSEVIER IRELAND LTD, IE, vol. 98, no. 1-2, 8 April 2005 (2005-04-08), pages 103-108, XP027757229, ISSN: 0378-8741 [retrieved on 2005-04-08]	1,4,5, 13,14
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Y	XU GUO-BO ET AL: "Hepatoprotective natural triterpenoids", EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY, ELSEVIER, AMSTERDAM, NL, vol. 145, 8 January 2018 (2018-01-08), pages 691-716, XP085414807, ISSN: 0223-5234, DOI: 10.1016/J.EJMECH.2018.01.011 page 702, right-hand column	1-18

Y	RÜDIGER A L ET AL: "The Chemistry and Pharmacology of the South America genus Protium Burm.f. (Burseraceae)", PHARMACOGNOSY REVIEWS,, vol. 1, no. 1, 1 January 2007 (2007-01-01) , pages 93-104, XP008136454, page 95 - page 96; table 1	1-18

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		US 2016021906 A1	28-01-2016
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