Title: METHOD FOR OBTAINING AN EXTRACT RICH IN TRITERPENIC ACIDS FROM EUCALYPTUS BARKS

Abstract: The present invention relates to a method of obtaining extracts having very high contents (up to 98%) of triterpenic acids, mainly oleanolic and ursolic acids, from eucalyptus barks. The method involves the solid-liquid extraction of the bark with organic solvents, preferably hexane, and the fractionation of the crude extract by means of a simple treatment with an alkaline solution, followed by separation of the aqueous phase, its filtration, acidification and finally isolation of the enriched triterpenic acids fraction by means of filtration, centrifugation or extraction with an organic solvent, preferably hexane, followed by solvent removal. The present invention has application in pharmaceuticals, nutraceuticals, cosmetics and functional foods industries.
TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.


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FIELD OF THE INVENTION

The present invention relates to a method of obtaining extracts having very high contents (up to 98%) of triterpenic acids, mainly oleanolic and ursolic acids, from eucalyptus barks. The method involves the solid-liquid extraction of the bark with organic solvents, preferably hexane, and the fractionation of the crude extract by means of a simple treatment with an alkaline solution, followed by separation of the aqueous phase, its filtration, acidification and finally isolation of the enriched triterpenic acids fraction by means of filtration, centrifugation or extraction with an organic solvent, preferably hexane, followed by solvent removal.

BACKGROUND OF THE INVENTION

Pentacyclic triterpenic acids with lupane, ursane and oleanane skeletons, like betulinic, ursolic, 3-acetyllursolic, oleanolic and 3-acetyloleanolic acids, have a wide range of unique and potentially usable biological effects and pharmacological activities (Yogeesswari and Sriram, 2005).

Betulinic acid is known to exhibit anti-HIV, antibacterial, antimalarial, anti-inflammatory and antioxidant properties (Tolstikova et al., 2006, Yogeesswari and Sriram, 2005). Oleanolic and ursolic acids are known also to have antimicrobial (Horiuchi et al., 2007, Kuete et al., 2007, Sattar et al., 1995) and significant anti-tumour activities (Li et al., 2002), as well as anti-inflammatory properties.
(Singh et al., 1992). Additionally, oleanolic acid revealed antiallergic and anti-HIV activities (Sultana and Ata, 2008) and ursolic acid is a useful suppressive compound for rheumatoid arthritis treatment with low risk of gastric problems (Kang et al., 2008).

The interest on bioactive natural compounds, for application in pharmaceuticals, nutraceuticals, cosmetics and functional foods, encouraged the development of several methods for obtaining vegetal extracts enriched in the above mentioned triterpenic acids. Several morphologic parts of plants have been described as potential sources of these natural compounds, as, for example, fir tree needles or bark (RU 2108803), persimmon leaves (CN 101538309), apple peel (EP 1250852, EP 1161879), olive leaves (WO 2005075614), Japanese loquat leaves (US 2009275778), and birch bark (WO 2005047304). The methodologies reported for obtaining extracts enriched in triterpenic acids involve the extraction of the vegetal substrates with an organic solvent system (e.g. ethyl alcohol, ethyl acetate, petroleum ether, acetone, hexane, chloroform, methyl-butyl ether, benzene, xylenes, among others) followed by complex fractionation procedures based on successive liquid-liquid extractions (US 6740778), column chromatography procedures (WO 03011891, CN 101538309, CN 101759756, US 2009275778), supercritical countercurrent column extraction (WO 2005075614), crystallization and recrystallization (WO 2006088385, RO 122244, WO 2005047304, CN 101759756, EP 1161879), or treatment of the organic extract with alkaline solutions (RU 2151139, RU 2108803, RU 2108107). The use of extremely hazardous organic solvents, namely chloroform, benzene and xylenes, and very complex fractionation procedures, when high purities are desired, constitute the major disadvantages of some of these technologies.
The outer bark of several Eucalyptus species contain high amounts of several triterpenic acids, namely betulonic, betulinic, 3-acetylbetulinic, ursolic, 3-acetylsalic, oleanolic and 3-acetylolanolic acids, and may be explored for obtaining such bioactive compounds (Domingues et al., 2010, Domingues et al., 2011, Freire et al., 2002). The novelty of the present invention resides in the use of Eucalyptus bark residues (and particularly Eucalyptus globulus) for obtaining extracts with high amounts (up to 98%) of triterpenic acids by means of solid-liquid extraction with an organic solvent, preferably n-hexane, followed by the fractionation through sequential alkaline/acid solutions treatment. The proposed method has the advantage of being highly efficient and easily industrialized when compared with some of the technologies available.

Summary of the invention

The object of the present invention is a method for obtaining extracts rich in triterpenic acids wherein it comprises the following steps:

i) drying and milling of Eucalyptus bark;
ii) extraction of bark with an organic solvent;
iii) fractionation of the crude extract by treatment with an aqueous alkaline solution;
iv) separation of the aqueous phase;
v) clarification by filtration;
vi) acidification with an inorganic or organic acid;
vii) isolation of the triterpenic acids precipitated fraction.

In a preferable embodiment the method further comprises a crystallization step.
In another preferable embodiment, the Eucalyptus bark is obtained from Eucalyptus species, namely *E. globulus*, *E. urograndis*, *E. grandis* × *globulus*, *E. maidenii*, *E. grandis* and *E. nitens* and the residual moisture and granulometry of the bark samples are below 15% and 60 mesh respectively.

In another preferable embodiment, the organic solvent used is dichloromethane, *n*-heptane, *n*-hexane or other non-polar solvent and the aqueous solution comprises at least one of: metal hydroxide (KOH or NaOH) or carbonate (Na$_2$CO$_3$) or K$_2$CO$_3$), as well as the corresponding hydrogenocarbonates, or phosphate (Na$_3$P$_4$O$_{10}$ or K$_3$PO$_4$), as well as the corresponding hydrogenophosphates and dihydrogenophosphates, or ammonium hydroxide (NH$_4$OH), being that the pH of the alkaline aqueous solution is between 8 and 14.

In another preferable embodiment, the separation is performed by decantation or centrifugation.

In another preferable embodiment, the acidification of the aqueous phase is done with inorganic acid solutions, such as sulphuric, hydrochloric and nitric acids, and organic acid solutions, such as acetic, and propionic acids and the pH of the acid solution is between 1 and 5.

Another object of the present invention is the extracts, obtained by the method described above, wherein the triterpenic acids content is in the range of 93-98%.

Another object of the present invention is the use of the extracts described above wherein they are used in pharmaceuticals, nutraceuticals, cosmetics and functional food industries.
Description of the drawings

Figure 1 - Steps of the method of obtaining extracts enriched in triterpenic acids, from Eucalyptus species barks.

Figure 2 - Typical GC-MS chromatogram of the extracts enriched in triterpenic acids obtained using the present methodology. (IS: internal standard)

Detailed Description of the invention

The present invention relates to a method of obtaining extracts enriched in triterpenic acids, from *Eucalyptus* species barks, outlined in Fig. 1.

The method comprises four main steps:

1. drying and milling the bark;
2. solid-liquid extraction of the bark residues with an organic solvent, preferably n-hexane;
3. fractionation of this crude extract by treatment with an alkaline aqueous solution, followed by separation of the aqueous phase, its filtration and acidification; and finally
4. isolation of the enriched solid triterpenic acids fraction by means of filtration, centrifugation or extraction with a non polar organic solvent, preferably hexane, followed by solvent removal.

Finally, this process can also comprise a crystallization step.

The result of this process is a whitish powder with a triterpenic acids content of about 93-98%.
The bark used as raw material can be obtained from different *Eucalyptus* species, namely *E. globulus*, *E. urograndis*, *E. grandis* x *globulus*, *E. maldenii*, *E. grandis*, and *E. nitens*, among others. Fresh or decayed biomass can be used but should be preferably dried and milled before the solvent extraction. The drying and milling steps include known methodologies such as lyophilization, air drying, hot air drying, and any mechanical milling such as, e.g., blade, knife or hammer milling, respectively. The residual moisture and granulometry of the bark used in this process are below 15% and 60 mesh, respectively.

The solvent used for extraction includes non-polar organic solvents, preferably n-hexane. This operation can be performed using conventional procedures, such as maceration, extraction under reflux, Soxtec, Soxhlet, accelerated solvent extraction, or any other solid-liquid extraction technique. The crude organic extract is then treated with an alkaline aqueous solution, until pH between 8 and 14, preferably NaOH or KOH, or carbonate (Na$_2$CO$_3$ or K$_2$CO$_3$), as well as the corresponding hydrogenocarbonates, or phosphate (Na$_3$PO$_4$ or K$_3$PO$_4$), as well as the corresponding hydrogenophosphates and dihydrogenophosphates, or ammonium hydroxide (NH$_4$OH), to promote the conversion of the triterpenic acids into the corresponding salts and its migration to the aqueous phase. The separation of the organic phase is performed by decantation or by any other liquid-liquid separation technique, such as centrifugation. The aqueous phase is filtrated, by means of conventional methods, and acidified until pH<3 with preferably an inorganic acid, such as sulphuric, hydrochloric and nitric
acids and organic acid solutions, such as acetic, and propionic acids. The precipitated triterpenic acids can be finally isolated by extraction with an organic solvent, filtration or by centrifugation.

Examples
Hereinafter, the present invention is described in more detail and specifically with reference to the Examples, which however are not intended to limit the present invention.

Solid-Liquid Extraction of outer bark
Approximately 100 g of Eucalyptus globulus outer bark were air dried, at room temperature, to produce approximately 70 g of dried bark with less than 10% by weight of moisture, and then milled to pass through a 2 mm screen. The extraction of the dried milled bark was performed in a Soxhlet extraction apparatus with 1 L of n-hexane as solvent, at reflux temperature. The extraction lasted 6 hours.

The evaporation, in a vacuum rotation evaporator, and drying of the above crude n-hexane extract yield 1.1 g (1.8 wt. % yield) of a green dry crude residue. The total triterpenoids content of this residue was 49.1% by weight, containing 14.4% of ursolic acid, 12.8% of 3-acetyltursolic, 5.7% of oleanolic acid, 5.3% of betulinic acid, 5.1% of betulonic acid and 3.5% of 3-acetyloleanolic acid, being the remaining 2.4% of other triterpenic compounds.

Fractionation of the outer bark extract
The 1 L n-hexane crude extract was extracted with 1 L 0.1 M sodium hydroxide (NaOH) aqueous solution. The aqueous phase was separated from the n-hexane phase by decantation, and
vacuum filtrated through 0.45 μm porosity nylon membranes disks, a clear solution being obtained. The said solution was acidified to pH < 3 with 2 M sulfuric acid (H₂SO₄) resulting in a whitish precipitate suspension. The precipitate was vacuum filtrated, washed with 1 L of distilled water up to neutral pH, and dried at 105 °C for 3 h.

407 mg of a whitish powder was obtained, consisting of a sum of triterpenic acids, with a minimum 97.8% by weight content composed of: 52.4% of ursolic acid, 17.3% of oleanolic acid, 13.2% of betulonic acid, 9.7% of betulinic acid, and 5.2% of other triterpenic acids (Fig. 2)

Qualitative and quantitative characterization of the crude n-hexane extract and of the triterpenic acids enriched fractions, produced according to the invention, was performed by application of Gas-chromatography coupled with mass spectrometry (GC-MS).

References:


Eucalyptus species cultivated in Brazil and in Portugal, Industrial Crops and Products 33, 158-164.

EHLKHA AOOT, 2000. Method of preparing biologically active sum of triterpenic acids. RU 2151139


Li, J., Guo, W.J., Yang, Q.Y., 2002. Effects of ursolic acid and oleanolic acid on human colon carcinoma cell line HCT15. World J. Gastroenterol. 8, 493-495.


The following claims set out a particular embodiment of the invention.
CLAIMS

1. Method for obtaining extracts rich in triterpenic acids, wherein it comprises the following steps:
   i) drying and milling the Eucalyptus bark;
   ii) extraction the bark with an organic solvent;
   iii) fractionation of the crude extract by treatment with an aqueous alkaline solution;
   iv) separation of the aqueous phase;
   v) clarification by filtration;
   vi) acidification with an inorganic or organic acid;
   vii) isolation of the triterpenic acids precipitated fraction.

2. Method according to claim 1, wherein it further comprises a crystallization step.

3. Method according to any of the previous claims, wherein the Eucalyptus bark is obtained from Eucalyptus species, namely E. globulus, E. urograndis, E. grandis x globulus, E. maidenii E. grandis and E. nltens.

4. Method according to any of previous claims, wherein in step i) the residual moisture and granulometry of the bark samples are below 15% and 60 mesh, respectively.

5. Method according to any of previous claims, wherein in step ii) the organic solvent is dichloromethane, n-heptane, n-hexane or other non-polar solvents.

6. Method according to any of the previous claims, wherein the alkaline solution of step iii) is an aqueous solution comprising at least one of: metal hydroxide (KOH or NaOH) or carbonate (\( \text{Na}_2\text{CO}_3 \) or \( \text{K}_2\text{CO}_3 \)), as well as the corresponding...
hydrogenocarbonates, or phosphate (Na\textsubscript{3}P\textsubscript{2}O\textsubscript{7} or K\textsubscript{3}PO\textsubscript{4}), as well as the corresponding hydrogenophosphates and dihydrogenophosphates, or ammonium hydroxide (NH\textsubscript{4}OH).

7. Method according to previous claim, wherein the pH of the alkaline aqueous solution in step iii) is between 8 and 14.

8. Method according to any of previous claims, wherein the separation of step iv) is performed by decantation or centrifugation.

9. Method according to any of the previous claims, wherein the acidification of the aqueous phase in step vi) is done with inorganic acid solutions, such as sulphuric, hydrochloric and nitric acids, and organic acid solutions, such as acetic, and propionic acids.

10. Method according to previous claim, wherein the pH of the acid solution is between 1 and 5.

11. Extracts obtained by the method described in previous claims, wherein the purity of the purified triterpenic acids fraction is in the range 93-98%.

12. Use of the extracts described in previous claim, wherein they are used in pharmaceuticals, nutraceuticals, cosmetics and functional foods industries.
Eucalyptus Bark

Bark milling

Non-polar organic solvent → Solid - liquid extraction → Extracted bark

Crude extract

Aqueous alkaline solution → Liquid - liquid extraction → Exhausted Organic fraction

Aqueous fraction with triterpenic acid salts

Filtration

Filtered aqueous fraction

Aqueous acid solution → Solid - liquid separation → Aqueous solution

Solid triterpenic acids fraction

Figure 1
Figure 2
**INTERNATIONAL SEARCH REPORT**

**A. CLASSIFICATION OF SUBJECT MATTER**

INV. A61K36/61 A23L1/30 C11B9/02

According to International Patent Classification (IPC) etc., both national classification and IPC.

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

A61K A23L C11B

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, FSTA, WPI Data

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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<td>CN 1 521 182 A (UNIV PLA 2ND MIRITARY MEDICAL [CN]) 18 August 2004 (2004-08-18) claims 1, 10; examples 1, 5, 6</td>
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<td>DOMINGUES R M A ET AL: &quot;Eucalyptus globulus biomass residues from pulping industry as a source of high value tri terpenic compounds&quot;, INDUSTRIAL CROPS AND PRODUCTS, ELSEVIER, NL, vol. 31, no. 1, 1 January 2010 (2010-01-01), pages 65-70, XP2677147, ISSN: 0926-6690, DOI: 10.1016/J.INDCROP.2009.09.002 [retrieved on 2009-10-07] cited in the application on abstract; figures 3, 4, 5; table 2</td>
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Further documents are listed in the continuation of Box C. See patent family annex.

Date of the actual completion of the international search: 9 September 2013

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Name and mailing address of the ISA:
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Authorized officer: DILLER, Reinhard
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