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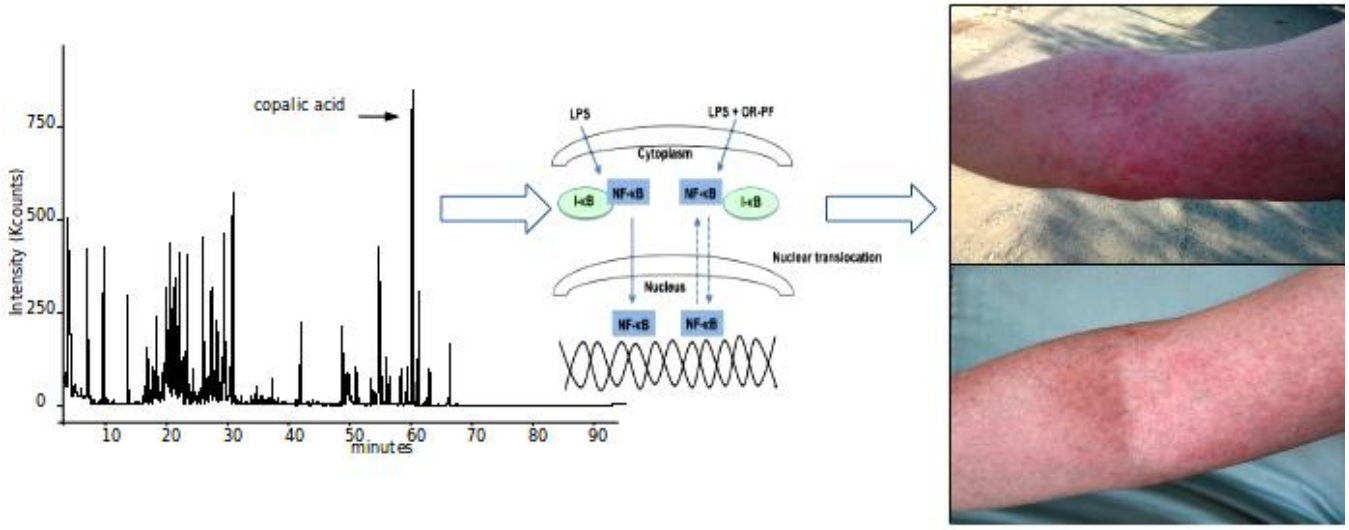
Graphical abstract



GC-MS profiling of the phytochemical constituents of the oleoresin from *Copaifera langsdorffii* Desf. and a preliminary *in vivo* evaluation of its antipsoriatic effect

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Abstract

Copaiba is the oleoresin (OR) obtained from *Copaifera* (*Fabaceae*), a neotropical tree which grows in Amazon regions. The balsam, constituted by an essential oil and a resinous fraction is used as folkloristic remedy in the treatment of several inflammatory diseases and for its antioxidant and antibacterial properties. Aim of this work was (a) to carry out a characterization by GC-MS of the volatile and nonvolatile constituents of *Copaifera langsdorffii* Desf. oleoresin (OR); (b) to investigate the mechanism of its anti-inflammatory activity; (c) to evaluate its antipsoriatic effect after oral intake/topical application. The volatile fraction (yield: 22.51% w/w) shows: α-bergamotene (48.38%), α-himachalene (11.17%), β-selinene (5.00%) and β-caryophyllene (5.47%). The OR residue (77,49% w/w), after derivatization, showed as main constituents the following compounds: copalic, abietic, daniellic, lambertinic, labd-7-en-15-oic, pimaric, isopimaric acids and kaur16-en18-oic acid.

Preincubation of LPS-stimulated human THP-1 monocytes with increasing concentrations of the OR purified fraction (OR-PF), containing diterpene acids, diterpenes and sesquiterpenes, reduced the release of pro-inflammatory cytokines (IL-1β, IL-6, TNFα) in a dose-range of 0.1-10 μM.

In addition, in cell culture system of human THP-1 monocytes, 1μM OR-PF counteracts LPS-driven NF-kB nuclear translocation.

In a preliminary clinical trial three patients affected by chronic psoriasis, treated with oral intake or topical application of the OR, exhibited a significant improvement of the typical signs of this disease, *i.e.* erythema, skin thickness, and scaliness.

In conclusion, the results of this work, beside an extensive analytical characterization of the OR chemical composition, provide strong evidences that its anti-inflammatory activity is related to the inhibition of the NF-κB nuclear translocation, and consequently of proinflammatory cytokines secretion.

Keywords: *Copaifera langsdorffii* Desf., chemical composition, GC-MS analysis, diterpenes and diterpene acids, cytokines secretion, Nf-κB translocation, psoriasis.

1. Introduction

Copaiba is the oleoresin (OR) obtained from Copaifera (*Fabaceae*), a family of neotropical trees which grows throughout the Amazon regions. Copaiba balsam is obtained from more than twenty arboreal species, among which the most studied are *C. officinalis, C. reticulata, C. duckei, C. multijuga, C. langsdorffii, C. hayn, C. epunctata, C. guyanensis* and *C. panamensis*.

The balsam, constituted by an essential oil and a xyloglucanic resinous fraction (Stupp *et al.*, 2008), accumulates in cavities within the tree trunk, and it is used in traditional medicine in the treatment of several inflammatory diseases involving the respiratory airways (such as asthma, sore throat, bronchitis), the genital-urinary apparatus an the skin diseases (healing of scurs skin ailments: wounds, eczema and herpes).

It constitutes one of the most important renewable source of natural remedy for the populations of the amazon region.

In United States it was an official drug reported in the U.S.P. from 1820 to 1910, and in 1940 was admitted in the National Formulary. In 1972 the OR has been approved as dietary supplement by the FDA, after assessment of its safety, and more recently in Italy as food supplement by the Italian Ministry of Health (http://www.salute.gov.it; 2011).

Notwithstanding the clinical evidences that the OR from *C. langsdorffii* Desf. can ameliorate the outcome of inflammatory-mediated gastrointestinal, genital, urinary and pulmonary diseases (Santos *et al.*, 2008), few studies have been focused on its molecular mechanisms through which this OR exerts its action. This is due to the poor analytical background on its chemical constituents: while several informations are reported in literature about the composition of the essential oil (Veiga *et al.*, 2001), few are those available (and some of them still in progress) relative to the profile of its nonvolatile components, such as dipertenes, diterpenoic acids and sesquiterpenes (Leandro *et al.*, 2012; do Nascimento *et al.*, 2012).

Hence, despite these recently published papers, the relationship between the *C. langsdorffii* Desf. OR chemical composition and its pharmacological activity remains undefined. In the light of this gap, and of the re-emerging interest for this popular and traditional remedy, aim of this study was: a) to perform an exhaustive study on the composition of its constituents; b) to isolate the most active fraction of the OR; c) to evaluate in an immunocompetent cell culture system, THP-1 human monocytes, their inhibitory activity on the LPS-stimulated secretion of the pro-inflammatory cytokines, and of the nuclear translocation of NF-κB.

Finally we have carried out a preliminary testing on the potential antipsoriatic activity of the OR in a restricted number of subjects affected by recalcitrant localized psoriasis.

2. Materials and methods

2.1 Copaiba oil

The OR was collected from the trunks of *C. langsdorffii* Desf. trees, growing wild in the Amazon region in Northern Brazil during October 2011. Authentication of the species was carried out by the examination of its seeds, fruits and leaves by Prof. Gelsomina Fico, Department of Biology, Faculty of Pharmacy, University of Milan. Two voucher specimens were deposited in the herbarium of the Department of Biology of the same University.

2.2 Chemicals

Thiobarbituric acid, *n*-hexane, trolox, butanol, methanol, ethanol, gallic acid, ascorbic acid, Ethylenediaminetetraacetic acid sodium salt, trichloroacetic acid, hydrogen peroxide 30%, potassium hydroxide, gaseous hydrochloric acid, Bradford reagent, phenol, bovine serum albumin (BSA), abietic acid (AA) and lipopolysaccharide (LPS) were all from Sigma–Aldrich (Milan, Italy). 1,1-diphenyl-2-picrylhydrazyl (DPPH), and Folin–Ciocalteau reagent were from Fluka (Buchs,

Switzerland). Glucose, $FeCl_3$ were from Carlo Erba (Milan, Italy). Human IL-1β, IL-6 and TNF-α enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D Systems (Minneapolis, MN, USA).

2.3 Physical parameters

The physical constants were analyzed (20 ± 0.2°C) according to the standard methods of the Association Française de Normalisation (AFNOR). The refractive index of the essential oil (EO) was determined at 25 °C with an Abbe optical refractometer (Ivymen System). The optical activity of essential oil was measured in ethanol at 25 °C with a D7 optical polarimeter (Bellingham & Stanley Ltd., Tunbridge Wells, Kent U.K.) working at λ 589 nm. The density of the OR was determined gravimetrically as m/vol ratio (g/mL). The saponification and acidic indexes were determined according to the methods reported in the European Pharmacopeia 7^{th} edition.

2.4 Free sugar determination

The analysis of free monosaccharide fraction was performed according to Dubois (Dubois *et al.*, 1956) and expressed as glucose equivalent/mg_{OR}.

2.5 Polysaccharide analysis

The average molecular weight (MW) of the xyloglucan fraction was determined dissolving the matrix in ethanol, since its poor solubility in water. Detection was carried out in off line mode using a light scattering multiangle DAWN-DSP-F apparatus (Wyatt Technology model Corp., Santa Barbara, CA) equipped with a 5-mW He-Ne laser source (632.8 nm). The average MW was between $5x10^3$ - $1x10^4$ da.

2.6 Total reducing substances

The total content of reducing substances was determined using two different methods:

(a) Folin–Ciocalteau assay with minor modifications (Vinson and Bose, 2001), and expressing the results as mg of gallic acid equivalent (mg_{GAE}/mg of samples).

Briefly, 300 μl of the OR properly diluted in ethanol (30 μl/ml) were added to 2.7 mL of Folin–Ciocalteau reagent previously diluted (1:10) with milliQ water. The mixture was vortexed for 2 min, and the spectrophotometric absorbance was measured at 750 nm, after 20 min incubation at room temperature against a blank (reagents and ethanol). Gallic acid was used to calibrate the concentration as a function of absorbance. The gallic acid equivalent content (mg_{GAE} /$mL_{samples}$ ± SD) was calculated by comparison with a calibration curve plotted with a diluted stock solution (1 mg/mL) of gallic acid in EtOH/H_2O (1:1). The calibration line (y = 0.01162x + 0.04261) was linear (R^2 = 0.9997) between 0.01 and 0.50 mg/mL.

(b) Prussian blue method (Price and Butler, 1977), with minor modifications: 100 μL of ethanol containing different amounts of OR (from 1 to 50 μL) were added to a solution composed by 3 mL of 0.1 M $FeCl_3$ in 0.1 N HCl and 3.0 mL of 8.0 mM of $K_3[Fe(CN)_6]$ and vigorously vortexed. The absorbance was determined spectrophotometrically at λ_{max} 720 nm, after 10 min incubation at room temperature. All analyses were done in triplicate. The results were expressed as mg_{GAE}/mL_{EO} ± S.D. The GAE was calculated as described above, and the calibration curve (y=0.03690x+0.06004) was linear (R^2=0.9995) between 0.01 and 0.50 mg/mL.

2.6.1 Free radical scavenging activity of C. langsdorffii Desf. OR

The H/e^- transferring ability of the components of the OR *in toto,* of its resinous fraction, and of the essential oil were evaluated by the conventional DPPH assay. The extent of the DPPH radical quenching, expressed as: (a) *RSC%* according to the formula: RSC% = [(ACTR − AEO)/ACTR] × 100; (b) IC_{50} value was calculated as previously described (Beretta *et al.*, 2011).

154 *2.6.2 Scavenging effect of C. langsdorffii* Desf. OR *of hydroxyl radicals*

155 The hydroxyl radical scavenging activity was evaluated according to the conventional method of
156 Halliwell *et al*. (1987). The OR was diluted 1:1000 v/v with ethanol and 10-40 μL, corresponding to
157 10-40 nL of OR, added to the assay mixture under vigorous vortexing at 37°C for 1 hour.
158 Measurements were carried out against a blank in which pure ethanol was added.

159

160 *2.6.3 Scavenging effect of the OR on hydrogen peroxide*

161 The ability of the OR to scavenge H_2O_2 was determined according to the method of Yen and Duh
162 (1994) with minor modifications. A solution (2 mM) of H_2O_2 was prepared in phosphate-buffered
163 saline (PBS) pH 7.41 at 20 °C. H_2O_2 concentration was determined spectrophotometrically at λ 230
164 nm using a molar extinction coefficient for hydrogen peroxide of 81 M^{-1} cm^{-1} (Beers and Sizer,
165 1952). Different amounts of OR (1, 5, 10, 15 μL) were dissolved in EtOH (1 mL): 10 μL of each
166 solution (containing respectively 10, 50, 100, and 150 nL of OR) were added to 990 μL of
167 H_2O_2/PBS mixture at 20 °C. The absorbance of H_2O_2 was determined spectrophotometrically at 230
168 nm after 10 min of incubation (CARY 50, Varian) against a blank solution containing the OR
169 dissolved in the same conditions and diluted in PBS without H_2O_2. All tests and analyses were run
170 in three replicates and averaged.

171

172 *2.7 EO and resinous complex isolation*

173 The EO from *C. langsdorffii* Desf. was isolated by conventional steam distillation (10 g of OR;
174 yield 22,50% w/w) and then submitted to GC-MS analysis for the characterization of its
175 components. The OR resinous fraction was prepared from the same amount of OR, after
176 evaporation of the EO under reduced pressure (2.8 mbar, 100 °C, chilling temperature -5 °C), until
177 constant weight of the residue.

178

2.8 Sample derivatization

The resinous complex was submitted to GC-MS analysis after derivatization. Briefly, 50 μL of samples were treated with 450 μL of MeOH saturated with gaseous HCl, and left to react for 1 hour at 40 °C. The mixture was then exhaustively extracted with 1 mL of *n*-hexane, and 1 μl of the extract submitted to GC-MS analysis. This procedure allows to characterize the diterpenoic acids and derivatives, and the sesquiterpenes embedded into the polymeric matrix of the resinous complex, as well as the residual traces of paraffins, not eliminated during the evaporation of the essential oil.

2.9 Isolation of the sesquiterpenes and of diterpenoic acids from the polymeric matrix

The isolation of the purified fraction of the OR (OR-PF), containing diterpenes acids, diterpenes and sesquiterpenes was performed according to protocol shown in **Scheme 1**.

2.10 GC-MS conditions

The essential oil profile of Copaiba OR was performed by GC-MS analysis using a Bruker Scion SQ instrument (Bruker Daltonics, Macerata, Italy), equipped with a Factor Four capillary column (VF-5 ms, 30 m; 0.25 mm i.d., film thickness 0.25 μm) coupled with a SQ (single quadrupole) detector. The oven temperature was initially set at 60 °C (hold time 3min), with a gradient from 60 to 120 °C (3.0 °C/min, hold 1 min), from 120 to 280 °C (2 °C/min, hold 1 min), and from 280 to 300 °C (10 °C/min, hold 2 min); injector temperature 290 °C, hold 95 min. Column flow 1.00 mL/min. Carrier gas helium 5.5; ionization energy 70 eV; the split/splitless ratio was set to 1:30 after 45 s. Peaks were identified by matching their mass spectra with those of the commercial library NIST mass spectral database (vers. 2.0, 2011) and with those of commercial standards when necessary. The percentage composition of the constituents was obtained by normalization of the peak areas.

204 *2.11 Biological activity: in vitro studies*

205

206 *2.11.1 Cell culture*

207 Human THP-1 monocyte cell line was used (American Type Culture Collection, ATCC). Cells
208 were routinely cultured as follows: they were diluted to 10^6 cells/ml in RPMI 1640 containing 2
209 mM L-glutamine, 0.1 mg/ml streptomycin, 100 IU/ml penicillin, 0.05 mM 2-mercaptoethanol,
210 supplemented with 10% heated-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY) and
211 cultured in 37°C in 5% CO_2 incubator. For IL-1β, IL-6 and TNF-α release experiments, THP-1 cells
212 were seeded in 24-well plates, while for Western blot analysis $4x10^6$ cells were cultured in 15 ml
213 polypropylene tubes. Cells were incubated with or without LPS in the presence or absence of
214 increasing concentrations of OR-PF (0.1-100 μM). THP-1 cell viability rate was assessed by trypan
215 blue exclusion test. AA (abietic acid), at the dose of $4x10^{-5}$ M, was used as a positive control of OR-
216 PF (Kim 2010). Dimethyl sulphoxide and ethanol (0.1% final concentration) were used as vehicle
217 controls.

218

219 *2.11.2 IL-1β, IL-6 and TNF-α ELISA*

220 The most effective dose of LPS was 1 μg/mL, in accordance with previous studies on THP-1
221 (Corsini, 2011; Esafi-Benkhadir, 2012). Cells were pretreated or not for 1 h with OR-PF (0.1-100
222 μM) and $4x10^{-5}$ M AA; then exposed to 1 μg/mL LPS for further 24 h. Culture medium was then
223 removed, centrifuged for 10 min at 1.200 rpm at 4 °C and stored at -80 °C until measurement. The
224 lowest limits of sensitivity were 3.9 pg/mL for human IL-1β; 0.70 pg/mL for human IL-6 and 0.5-
225 5.1 pg/mL for human TNF-α. The serum in the culture media did not interfere with the assay.
226 Cytochine levels were normalized to the cell number.

227

228

2.11.3 Western blot analysis (WB)

THP-1 cells were cultured in 15 ml polypropylene tubes at the density of $4x10^6$ cells. They were pretreated or not for 1 h with 1μM OR-PF then exposed to 1 μg/mL LPS for another 30 minutes. NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific, Rockford, IL, USA) were used according to the manufacturer's instructions to extract nuclear and cytoplasmic proteins. Protein concentrations were assessed by bicinchoninic acid assay (BCA) Thermo Scientific, Rockford, IL, USA. Proteins were denatured and fractionated on a 4-20% gradient gel by SDS-PAGE then transferred electrophoretically to Hybond C-extra nitrocellulose membranes. Non-specific binding sites were blocked by treating the membranes with Tris-buffered saline-Tween (TBS-T) containing 5% non-fat dried milk for 1 h at 22°C.

To assess the presence of NF-κB in nuclear and cytoplasmic fractions, membranes were incubated for 16 h at 4°C with a 1:100 dilution of anti-NF-κB p65 polyclonal antibody (Santa Cruz Biotechnology, CA) and incubated for 1 h at 22 °C with a 1:4000 dilution of horseradish peroxidase-linked anti-rabbit IgG. To normalize total NF-κB to nuclear and cytoplasmic fractions, membranes were immunoblotted with 1:1000 anti-histone H3 (Cell Signaling Technology, MA) and 1:2000 anti-tubulin (Sigma-Aldrich, St. Louis, MO), respectively. Membranes were then washed with TBS-T, immersed in the chemiluminescence detection solution. The luminescence was then quantified in a Chemidoc system and the bands were quantified by Image Lab (both from Bio-Rad Laboratories, Milan, Italy).

2.12 In vivo studies: antipsoriatic activity

2.12.1 Oral intake

Two patients (36 and 45 years old, woman and man respectively) with an 8 year history of chronic psoriasis from mild to moderate (grade 1 and 2 of the Psoriasis Area Scale Index, PASI scale: 0-

none-, 1-slight-, 2-mild-, 3-moderate-, 4-severe; Feldman and Krueger, 2005), localized in man on the legs, and in the woman on the elbows, were considered.

The patients, recalcitrant to topical treatment with conventional anti-psoriatic drugs (corticosteroids and vitamin D analogues) and not affected by considerable comorbidities, gave their informed consent to the oral treatment with the *C. langsdorffii* Desf. OR.

Before the treatment, patients carried out an extensive therapeutic wash out (four weeks) and then started the intake of the OR, with an initial dose of one drop *t.i.d.* (*ter in die*), daily increasing the dose from one to seven drops t.i.d., equivalent to 1 mL_{OR}/*die*.

2.12.2 Topical treatment

A 36 year-old-man affected by severe psoriasis localized at both the elbows, graded from 2 to 3 according to the PASI scale, was treated on the left with an ointment containing 0.005% of calcipotriol, and on the right with an ointment constituted by 5% of *C. langsdorffii* Desf. OR dissolved in karitè shea butter, containing the 0.1% of tea tree oil as skin penetration enhancer. The treatment was carried out twice daily on symmetrical lesions for 6 weeks. The patient gave informed consent to the treatments. The effect was assessed by clinical visual examination of erythema, of scaling, and finger palpation of the lesions.

2.13. Statistical analysis

Statistical analyses were conducted with the R-commander GUI for R (v. 1.5–6) (Fox, 2005). Results are expressed as the mean (S.D.) of at least three independent experiments. Student's *t*-test was used; *P*-values <0.05 were considered significant.

3.1 Results and discussion

The results relative to the organoleptic characteristics and to the physical-chemical descriptors of the OR and of the EO (22% w/w) are reported in **Table 1**. Color, odor, boiling point, refractive index, optical rotation, and specific gravity, match those already reported in literature (Remington and Wood, 1918: the US Dispensatory). All this data indicate the authenticity of the OR *in toto* and of its EO.

The total free acids value of OR was 102.450 ± 5.044 (mg_{KOH}/g_{OR}), and the saponification index 107.450 ± 2.040 (mg_{KOH}/g_{OR}): the overlapping of these two values indicates the presence of acids in free form only. From a stoichiometric calculation based on these data, and considering the MW of the two prototype diterpene acids (kaurenoic and copalic acids, $C_{20}H_{32}O_2$, MW=304.24), we can estimate a content in acid fraction in the OR of 56,89 ± 1,22%.

The free sugar content expressed as mg glucose/ mL_{OR} was 0.69 ± 0.03 % (w/w). The low amount of free sugars indicates that these may be considered more as contaminants of the OR than as a hydrolytic products from its xyloglucan fraction. This last is known to be insensitive to acidic or basic hydrolysis and susceptible only in part to degradation by enzymatic treatment with cellulase and β-galactosidase (Silva Tiné *et al.*, 2003). The sticky structure of the hydrocolloid confirms its protective function inside and outside the trunk against wounds and attack of bacteria, insect and fungi.

3.2 Antioxidant and antiradical activities

3.2.1 Total reducing substances

The total content of reducing substances determined by both the Folin-Ciocalteau and $FeCl_3/Fe(CN)_6^-$ in OR *in toto* were 0.318 ± 0.04 mg_{GAE}/g_{OR} and 0.212 ± 0.01 mg_{GAE}/g_{OR}, respectively. The content of reducing substances determined on the essential oil, and on the resinous

complex, gave a value below the limit of detection.

The DPPH assay was first performed on the OR *in toto* (IC_{50} 2.30 mg/mL), and then on its constitutive components, *i.e.* the essential oil (IC_{50} 8.71 mg/mL) and on the xyloglucanic resin which contains entrapped diterpenes and diterpenoic acids (IC_{50} 9.23 mg/mL). From these results, it is clear a synergistic effect of the components, commonly observed in several phytocomplexes containing H/e- tranferring structures (Beretta *et al.*, 2011).

3.2.2 ·OH scavenging activity

The ·OH scavenging activity evaluated according to Halliwell *et al.* (Hall, 1987) demonstrated a dose dependent inhibition of the TBA-MDA chromogen of 35.54%, 56.39%, 70.31%, and 75.10% after addition of 10 nL, 20 nL, 30 nL, and 40 nL respectively of the OR suitably diluted in the minimal amount of ethanol , indicating that the balsam, at least in part, is able to quench the flux of highly reactive ·OH radicals generated by the Fe^{3+}/H_2O_2/ascorbate redox system. The observed effect may be in part due also to the direct quenching activity of the OR towards H_2O_2.

3.2.3 Effect on H_2O_2

A dose dependent effect of the OR on H_2O_2 (2 mM) was observed: 10, 50, 100, and 150 nl_{OR}/mL induce a progressive decrease in the absorbance at λ 230 nm of 2 mM H_2O_2 (from 1.98 mM to 0.30 mM), to indicate that the components of OR are able to quench directly H_2O_2.

Interestingly, 1 mL of EtOH containing 2 μL of OR shows in the UV spectrum a sharp peak at 272 nm typical of endocyclic conjugated dienes, which, when added with 2 μL of concentrated H_2O_2 (30% v/v), undergoes complete disappereance (**Fig. 1**). This behavior may suggest the presence in the OR of a set of α-β unsaturated diterpenic compounds. Not surprisingly the antiradical activity (DPPH assay) of the OR when saturated with H_2O_2, undergoes a dramatic drop. The IC_{50} value rises from 2.30 mg/mL to 13.52 mg/mL. These findings, although preliminary, indicate that in the

diterpene structure of the compounds the active groups responsible for the antiradical activity, and probably for the other scavenging properties, specifically involves this kind of electron rich structures. Finally, the positive correlation observed between the antioxidant response, measured by the different methods used and the concentration of the OR, provides a strong demonstration of its antioxidant activity.

In the light of these findings, we thought a) to characterize the low MW components present in the resinous fraction, and b) to evaluate the anti-inflammatory activity of OR-PF using a cell system model of inflammation; c) to evaluate its anti-psoriatic activity.

3.4 GC-MS profile of the EO and of the resinous fraction from C. langsdorffii Desf. OR

As evidenced in **Fig. 2** and **Tables 2** and **3**, copaiba contains (a) volatile compounds (essential oil) and (b) several classes of compounds which become volatiles after derivatization. All the compounds were identified and characterized by GC-MS: the essential oil was constituted by a set of forty terpenes and paraffins, constituted by sesquiterpenes (96.36 %), the main of which were α-trans-bergamotene (48.38%), α-himachalene (11.17%), β-caryophyllene (5.47%), β-elemene (5.06%), cyclosativene (5.02%), and β-selinene (5.00%), paraffins (2.21%), sesquiterpenols (0.85%), and diterpenes methyl esthers (0.47%).

Among the nonvolatiles compounds present in the resinous fraction (after derivatization) we found some interesting labdanic structures, diterpenoic acids, and diterpenes bearings α-β conjugated dienes, to the best of our knowledge never identified in the *C. langsdorffii* Desf. OR before 2012 (Leandro *et al.*, 2012; do Nascimento *et al.*, 2012), *i.e.* copalic, pimaric, isopimaric, abietic, daniellic, lambertinic, giberellic acids and a group of labdenoic acid (see **Table 3**).

In particular, the GC-MS analysis of this last fraction evidenced the presence of a 44.73% of diterpenic and labdenoic acids, 31.74% of sesquiterpenes, 1.21% of phytormones and pheromones, 3.85% of fatty acids, and 5.58% of diterpenes, among which the 35.68% were unsaturated

conjugated structures, confirming the results obtained by the UV experiments with H_2O_2 (see above).

Among, the bulk of diterpene acids found by us in *C. langsdorffii* Desf. OR, only kauran-19-oic acid has been unequivocally characterized by Costa-Lotufo (Costa-Lotufo *et al*., 2002). In our samples, kaurenoic acid is present in a very small percentage (approximately 1%), less than the value reported by the same author. Conversely, in *C. langsdorffii* Desf. OR, we have found an array of structurally different diterpene acids, some of which reported in the OR from other *Copaifera* species, *i.e.* pimaric acid, and palustric acid (Imaizumi *et al*., 2002; Velikova *et al*., 2000).

Since we have no doubt on the botanical characterization of *Copaifera langsdorffii* Desf., and on the reliability of physical and chemical characterization performed by us (refractive index, optical rotation), we believe that the great variability of these active structures may stem from a plant metabolic process of interconversion of these intermediates along the time. Hence, these compounds can be considered as metabolic intermediates of the plant metabolism which leads to the biosynthesis of giberellins (Graebe, 1987).

To gain an insight into the role of this diterpenoic acids into the anti-inflammatory activity of *C. langsdorffii* Desf. OR, we investigated this effect using a purified fraction (PF) of the resinous complex free of the xiloglucanic fraction and containing only the bulk of diterpene acids, diterpenes and sesquiterpenes. To evaluate its inhibitory effect on IL-1β, IL-6 and TNF-α cytokines secretion, and on the nuclear translocation of NF-κB, we used a model of human THP-1 monocytes, a well-known and largely used model of macrophages precursor (Chanput et al., 2010; Essafi-Benkhadir et al., 2012).

3.5 Effects of Copaiba on cytokine secretion by human THP-1 monocytes

A 24-h exposure of THP-1 cells to 1 μg/mL LPS induced the maximal secretion of IL-1β (50-fold,

377 p<0.001 *vs* control; **Fig. 3a**), IL-6 (44-fold, p<0.001 *vs* control; **Fig. 3b**) and TNF-α (400-fold,
378 p<0.001 *vs* control; **Fig. 3c**), which are released in small amounts in basal conditions. Preincubation
379 (1 h) with $4x10^{-5}$ M AA, used as positive control of OR-PF, reduced LPS-stimulated IL-1β, IL-6
380 and TNF-α secretion respectively by 34% (p<0.05), by 22% (p<0.05), and non-significantly by
381 18%, *vs.* LPS-stimulated cells (**Fig. 3a-c**). After 1-h incubation, OR-PF significantly reduced the
382 secretion of IL-1β (by 44-36%; dose range 0.1 – 10 μM, all p<0.05; **Fig. 3a**), IL-6 (by 28% and
383 37%, at the doses of 0.1 and 1 μM; all p<0.05, **Fig. 3b**) and TNF-α (by 37% and 31% a the doses of
384 0.1 and 1 μM respectively; both p<0.05). Interestingly, there was no inhibition of cytokine secretion
385 when the cells were exposed to the highest concentration of OR-PF (100 μM). None of the OR-PF
386 concentrations had any effect on the unstimulated secretion of IL-1β, IL-6 and TNF-α, nor elicited
387 any sign of cell toxicity, as indicated by the Trypan blue exclusion test (data not shown).
388
389 *3.5.1 Effects of OR-PF on NF-κB nuclear translocation in human THP-1 monocytes*
390 We then examined the effect of OR-PF in counteracting LPS-induced nuclear translocation of NF-
391 κB in THP-1 cells. WB analysis of cytoplasmic and nuclear extracts showed that LPS alone
392 promoted translocation of the 52-kDa NF-κB subunit of the IκB/NF-κB complex from the cytosol
393 to the nucleus, as expected, and 1 μM OR-PF reduced the amount of this protein in the nucleus. The
394 efficiency of the separation of the nuclear and cytoplasmic fractions was confirmed by the detection
395 of histone 3 and tubulin, specific markers for the nucleus and the cytoplasm (**Fig. 4**).
396 For what concern the mechanism of inhibition of cytokines secretion, we believe that the most
397 likely candidates moieties are the group of α-β unsaturated diterpene acids. If this is true, we can
398 speculate that due to their highly lipophilic character they are able to permeate the THP-1 cells
399 membrane and establish a covalent binding to the specific target(s) within the cells with the
400 electrophilic groups of the α-β unsaturated moieties.

3. 6 Anti-psoriatic activity: oral administration

After four weeks of treatment, only a slight decrease of scaling and infiltration, compared to the basal condition, was observed on the legs of the patient 1. For patient 2 lesions on the extensor surfaces of the elbows appeared not significantly improved (data not shown). After six weeks, the 45-years-old man (patient 1) showed a further decrease of the scaliness of the legs lesions and of the skin infiltration, as well as an initial attenuation of the erythema redness (**Fig. 5A-B**). Also for the 36-years-old woman (patient 2) there was an appreciable attenuation of redness of the target plaques, and a more prominent skin scaling decrease (**Fig. 6A-C**).

At the end of this period both patients asked to continue the OR oral assumption, and for this reason the treatment was extended to three months (keeping 1 mL/*die* as dose). After this additional time period a fairly complete remission, with almost disappearance of the lesions, was observed (95% PASI score reduction) for both the subjects and in the follow up period, the therapeutic response was maintained (one year, data not shown). No allergic reactions or dermatitis were observed. In addition, blood parameters and liver- and renal- function tests remained in the normal range, further confirming the safety of *C. langsdorffii* Desf. OR.

3.6.1 Topical treatment

At baseline, the extension of the lesions on the right elbow of patient 3 (36 year-old-man) appeared not larger than that present on the left elbow, but erythema was evident, and as well as the scaling, and the infiltration nearly severe for both of them. At 2 weeks, no significant improvement was assessed; at 4 weeks, erythema appeared only a little bit improved, not yet to decrease from 3 to 2 of the PASI scale, but both infiltration and scaling achieved a point degree of improvement, from severe to moderate. At 6 weeks, erythema improved by two points, from moderate to mild; the scaling and infiltration follow the same trend from moderate to mild (**Fig. 7**). Tolerability for *C.*

langsdorffii Desf. OR ointment was declared to be very good by the patient.

The results of these pilot and preliminary oral and topical treatments demonstrate the efficacy of the OR from *C. langsdorffii* Desf., in ameliorating the typical clinical signs of psoriasis.

4. Conclusions

In conclusion, the results of this study demonstrate for the first time that the oleoresin (OR) from *C. langsdorffii* Desf. possesses different well established biological properties, which range from a strong antioxidant capacity to an anti-inflammatory activity and to a potential anti-psoriatic effect. These properties are not due to the presence of phenolic/polyphenolic species but to several volatile sesquiterpenes, and to the concomitant presence of unsaturated diterpene acids components (of which copalic acid is the most abundant in *C. langsdorffii* Desf. OR, followed by abietic acid, polialtic acid, kaurenoic acid etc.) and of diterpene and sesquiterpene species, all embedded within the polymeric xiloglucan matrix.

We also have demonstrated that these components exert a significant anti-inflammatory action based on the inhibition of cytokine secretion, consequent to an interaction with the NF-κB signalling pathway, thus explaining the health benefits deriving from the topical or oral administration *in vivo* of the oleoresin.

In this context, the significant clinical improvement, without any apparent systemic adverse effect, observed in psoriatic patients recalcitrant to conventional pharmacological therapy (corticosteroids and vitamin D analogues), is a clear indication of the anti-inflammatory and potentially anti-proliferative action of the same.

These results open the way to further in depth pharmaceutical studies on the use of this potent natural phytocomplex in the treatment of inflammatory diseases, as well as to investigations aimed to maximize its bioavailability and improve its delivery to the target sites (inclusion in cyclodextrins, microencapsulation, etc.) and its safety after topical and/or systemic administration.

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Figure Captions

Fig.1. Conjugated dienes disappearance (λ 272) in OR from *C. langsdorffii* Desf. induced by the addition of 2 mM H_2O_2.

Fig. 2. GC-MS profile of: (A) EO and (B) OR-PF isolated in OR from *C. langsdorffii* Desf. Peaks identification is reported in Tables 2 and 3.

Fig. 3. Effect of *C. langsdorffii* Desf. OR-PF (Oleoresin-Purified Fraction) on IL-1β, IL-6 and TNF-α secretion in cultured THP-1 monocytes. Cells were pretreated or not for 1 h with different OR-PF concentrations (ranging from 0.1 to 100 μM) and then exposed (or not) to 1 μg/mL LPS for another 24 h with the same OR-PF concentrations. Abietic acid (AA, $5x10^{-4}$ M) was used as positive OR-PF control. Data are expressed as mean ± SEM, n = 3; °$p<0.001$ *vs* control, *$p<0.05$ *vs* LPS (ANOVA).

Fig. 4. Effect of OR-PF on the nuclear translocation of NF-κB in cultured THP-1 monocytes. The cells, pretreated or not, for 1 h with 1 μM OR-PF, were stimulated (or not) for 30 min with 1 μg/mL LPS, again with or without 1 μM OR-PF. The subcellular localization of the NF-κB p65 subunit (52 kDa) was examined by Western blotting analysis. The quality of the separation of the nuclear and cytoplasmic fractions was confirmed by detection of the respective specific proteins, histone 3 (17 kDa) and tubulin (50 kDa). (C): control (RPMI).

Fig. 5. Patient 1, legs of a 45 -years-old man with an 8-year history of recalcitrant chronic psoriasis. (A) baseline and (B) after 6 weeks of oral intake of OR from *C. langsdorffii* Desf.

Fig. 6. Patient 2, right elbow of a 36-years-old woman with an 8-year history of recalcitrant chronic

555 psoriasis. (A) at the baseline; (B) after 4 weeks of oral intake of OR from *C. langsdorffii* Desf.; (C)
556 after 6 weeks of oral intake of OR from *C. langsdorffii* Desf..
557
558 **Fig. 7.** Patient 3, elbows of a 36-years-old man with an 8-year history of recalcitrant chronic
559 psoriasis. (A) right elbow at the baseline; (B) right elbow after 6 weeks of calcipotriol topical
560 treatment; (C) left elbow at the baseline; (D) left elbow after 6 weeks of topical treatment with OR
561 from *C. langsdorffii* Desf.

Table 1

Physical descriptors of the *C. langsdorffii* Desf. OR.

Descriptors	Experimental evaluation	U.S. Dispensatory Reference
Color OR	pale yellow clear oily	colorless or pale yellow
Odor OR	sweet, linalolic odor, woody	odor and taste of Copaiba
Boiling point	255.00 ± 0.01	252.00-256.00 °C
Specific gravity OR (g/mL)	0.902 ± 0.001	0.896 to 0.910
Refractive index EO(t= 25 °C)	1.4967 ± 0.004	1.4940 to 1.5000
Optical rotation OR [α]20/D	- 12 °	-7° to -35°

Table 2

GC-MS analysis of *C. langsdorffii* Desf. essential oil: peak identification, and area percentage.

Compounds	**R.T. (min)**	**%**	**Compounds**	**R.T. (min)**	**%**
3-octene, (*Z*)-	3.050	0.32	isolongifolene, 4,5-dehydro	20.312	0.13
3-octene, (*E*)-	3.168	1.05	β-guaiene	20.397	0.24
unidentified paraffin	3.259	0.15	cuparene	20.667	0.48
4-octene, (*Z*)-	3.296	0.68	isocaryophyllene	20.842	1.63
(+)-cyclosativene	16.365	5.02	β-selinene	21.145	5.01
α-copaene	16.560	0.57	α-selinene	21.422	2.57
τ-gurjunene	16.753	0.14	α-himachalene	21.901	11.17
α-farnesene	16.879	0.58	isolongifolene, 4,5,9,10-dehydro-	22.532	0.21
β-elemene	17.027	5.06	β-chamigrene	22.987	0.14
di-*epi*-α-cedrene	17.426	0.16	caryophyllene oxide	23.264	0.52
δ-selinene	17.664	1.42	diepicedrene-1-oxide	24.915	0.17
α-longipinene	17.906	1.22	aromadendrene oxide-(2)	25.254	1.09
α-santalene	18.151	0.27	α-elemene	25.848	0.39
β-caryophyllene	18.270	5.47	longipinocarveol, *trans*-	26.555	0.14
α-bergamotene	18.771	48.38	ledene oxide-(II)	27.841	0.12
cedrene	18.976	0.37	τ-himachalene	28.267	0.13
bergamotol, *Z*-α-*trans*-	19.111	0.60	*unidentified sesquiterpene*	28.721	0.20
epi-β-santalene	19.277	0.76	(-)-spathulenol	29.449	0.11
β-farnesene	19.386	1.01	dehydroabietic acid methyl ester	59.429	0.13
α-humulene	19.704	1.67	abietic acid, methyl ester	61.398	0.34

Table 3
GC-MS analysis of *C. langsdorffii* Desf. OR-PF*:* peak identification, and area percentage

Compounds	**R.T. (min)**	**%**	**Compounds**	**R.T. (min)**	**%**
1-hexene, 4-methyl-	3.196	0.29	β-humulene	29.572	0.79
heptane, 2,4-dimethyl-	3.602	5.03	sclaral	30.729	4.58
β-clavene	16.662	0.39	androst-5-en-4-one,	40.977	0.67
δ-selinene	17.427	0.30	androstan-17-one, 3-ethyl-3-hydroxy-,(5a)-	41.177	0.15
longifolene-(V4)	17.649	0.44	gibberellic acid	41.468	0.40
alloaromadendrene	17.834	0.26	isopimaric acid	41.734	1.48
caryophyleine-(I3)	18.096	1.08	pentadecanoic acid, 13-methyl-	41.777	1.51
n.i. sesquiterpene	19.349	0.79	pimarinal	43.313	0.20
cyperene	19.616	0.93	abietic acid	43.744	0.58
β-guajene	20.110	1.07	13-Isopimaradiene	44.562	0.21
τ-selinene	20.365	2.14	kaur-16-ene	44.677	1.57
τ-muurulene	20.478	1.97	sclarene	45.880	0.54
cuparene	20.622	1.04	biformene	46.566	0.39
cadinene	20.947	2.65	ent kaur-16-ene	46.833	0.46
β-selinene	21.350	1.72	cembrene	47.370	0.16
elixene	21.454	1.24	8,11-Octadecadienoic acid	49.382	1.54
germacrene B	21.901	3.38	elaidic acid	49.727	0.55
α-himachalene	22.631	0.88	sclareol	50.685	0.46
α-gurjunene	22.943	0.67	labd-7-en-15-oic acid	54.757	7.15
β-chamigrene	23.232	2.31	*n.i. labdane*	55.328	0.25
cubenol	24.148	0.46	labd-7-en-15-oic acid	55.995	0.47
n.i. sesquiterpenol	25.723	2.32	copalic acid isomer A	58.249	0.81
(-)-caryophyllene-(I1)	26.152	0.71	copalic acid	59.441	22.15
isoaromadendrene epoxide	26.297	0.31	valencene	60.111	3.36
β-eudesmene	27.026	0.65	pimaric acid	60.531	1.80
eudesma-4(14),11-diene	27.975	1.53	daniellic acid	61.313	5.38
τ-eudesmol	28.645	0.21	lambertinic acid	62.496	2.15
calarene epoxide	28.952	0.57	kauran-19-oic acid	63.348	1.08
n.i. sesquiterpene	29.346	2.14	labd-8(20)-ene-15,18-dioic acid, dimethyl ester	65.802	1.67

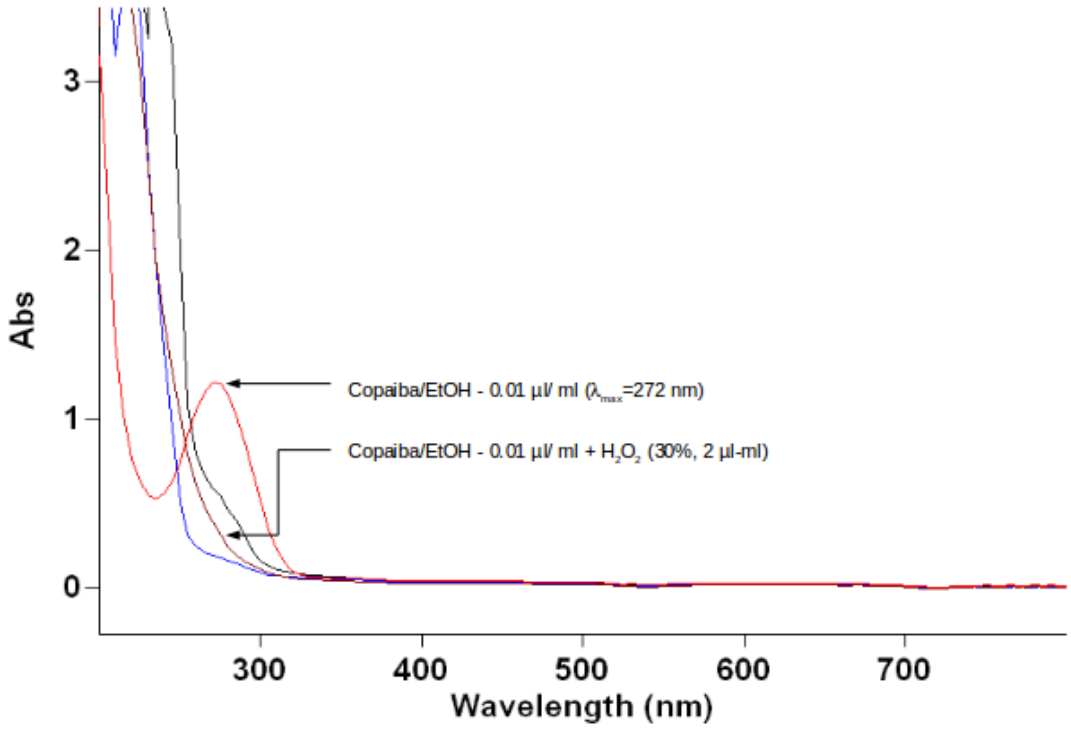


Figure 1

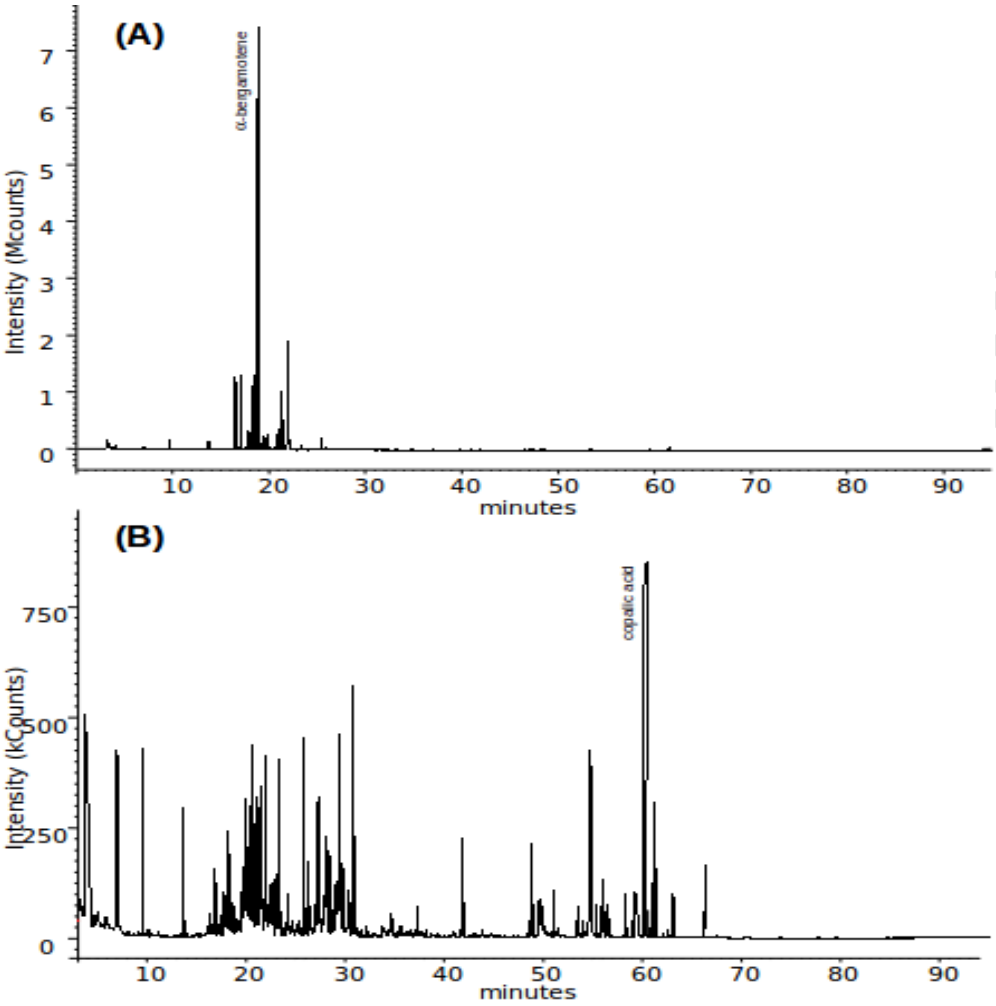


Figure 2

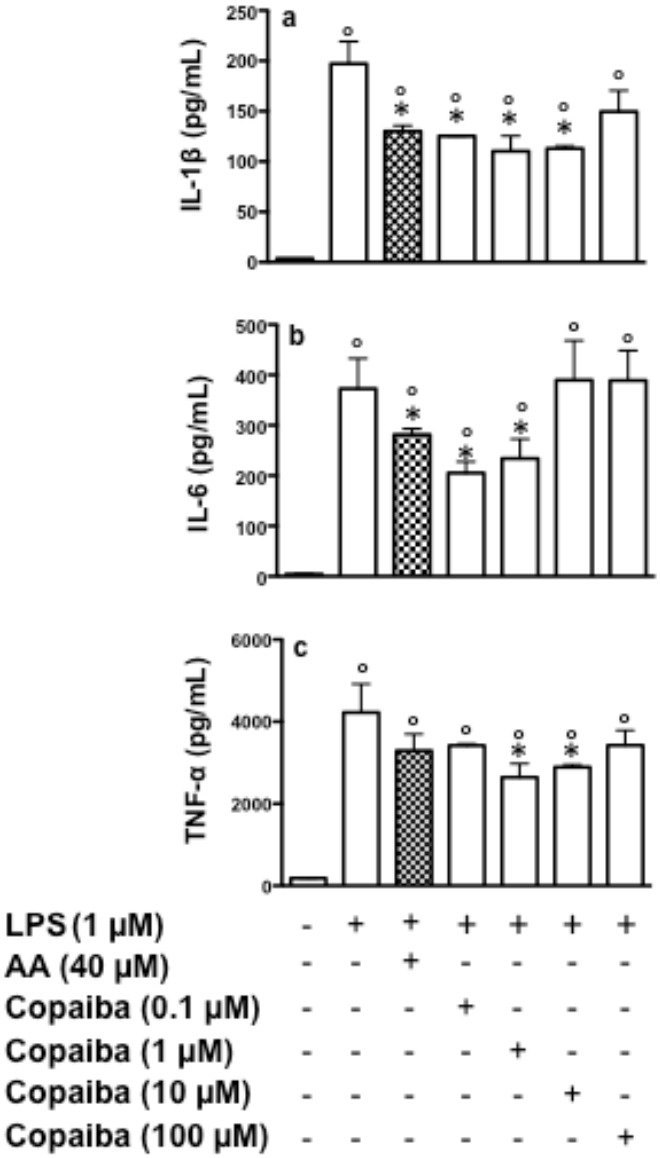


Figure 3

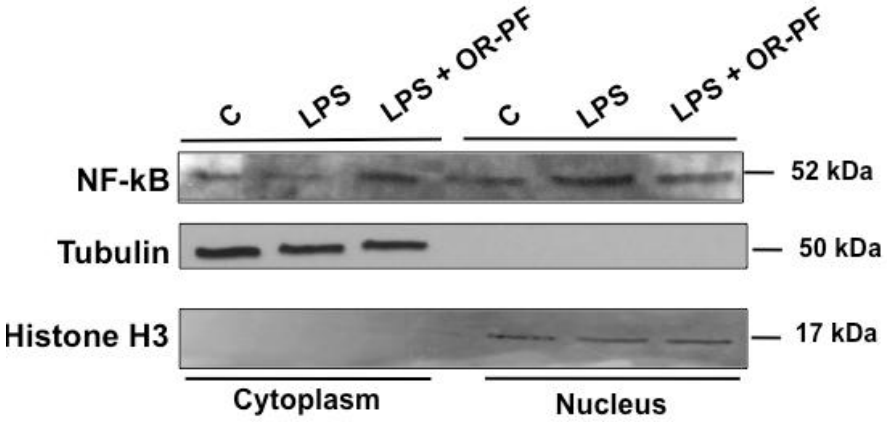


Figure 4

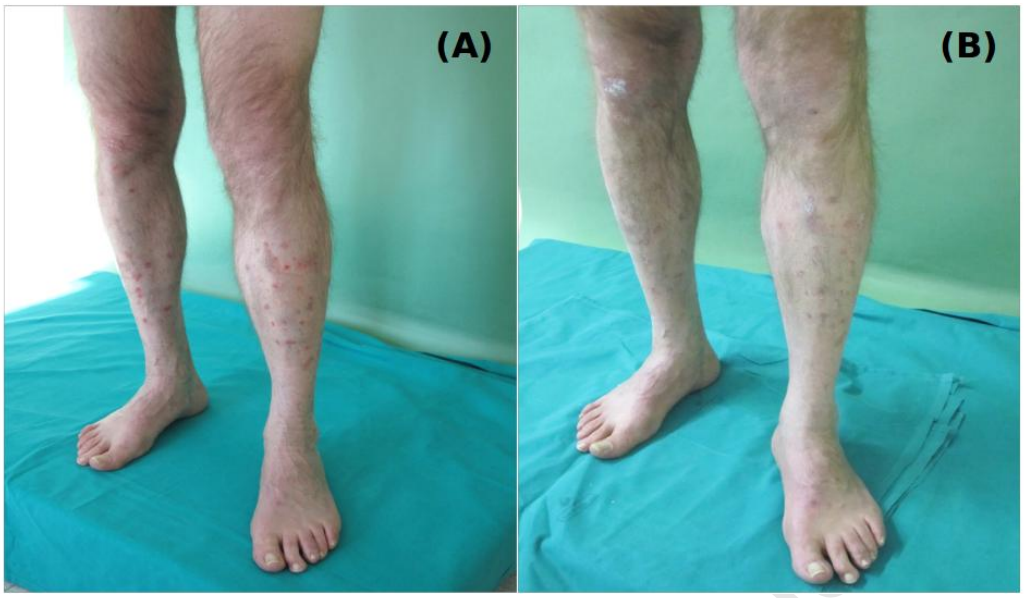


Figure 5

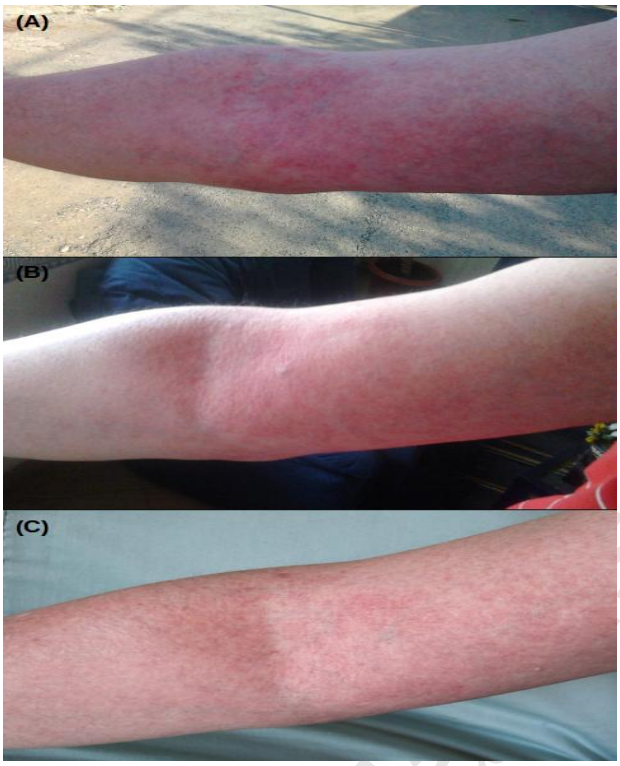


Figure 6

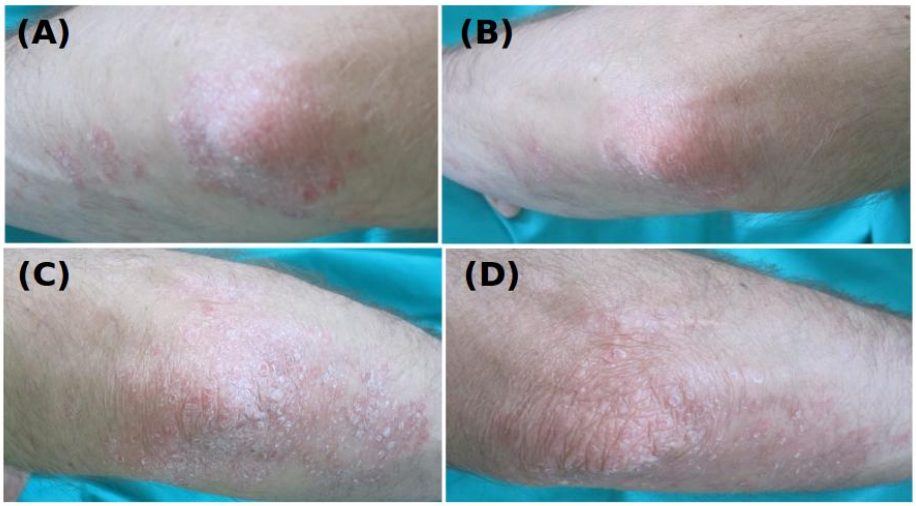
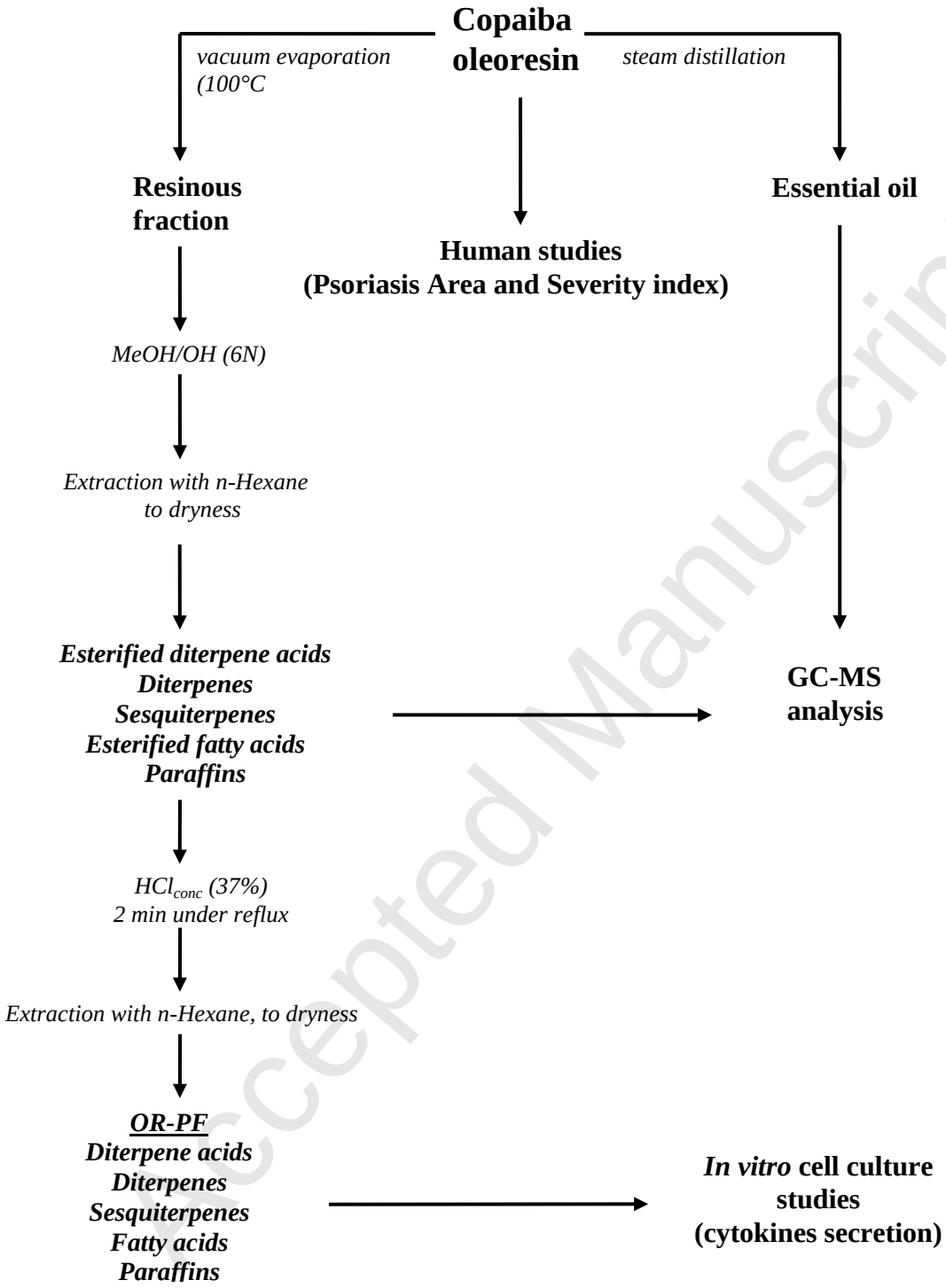


Figure 7



Scheme 1