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(54) Title: PLANT EXTRACT OF INULA RACEMOSA

(57) Abstract: The present invention is in the field of plant extracts suitable for the use in food, food products or additives for providing a vascular health benefit, and for oral and topical functional benefits. It is therefore object of the present invention to provide a highly active extract from the *Inula racemosa* (Pushkarmool) for providing a vasodilation and microcirculation benefit. It is a further object to provide active NO potentiating extracts of *Inula racemosa* with a low level of isoalantolactone, compared to known hexane extracts. It has now been found that an extract of plant material from *Inula Racemosa* with a solvent with a hydrogen bonding parameter of between 4 and 15, gives higher nitric oxide (NO) production and better vasodilation with less isoalantolactone in the extract.



PLANT EXTRACT OF INULA RACEMOSA

Field of the invention

The present invention is in the field of plant extracts suitable for the use in food,
5 food products or additives for providing a vascular health benefit, and for oral and topical functional benefits.

Background of the invention

Inula racemosa is a member of the Asteraceae family. It grows in the temperate
10 and alpine western Himalayas, and it is common in Kashmir. The roots are widely used locally in indigenous medicine as an expectorant and in veterinary medicine as a tonic. *Inula racemosa* is commonly known as Pushkarmool in India.

It is thought that one of the compounds present in the *Inula racemosa* root and
15 stem, isoallentolactone, is responsible for cholesterol lowering. Other lactones are known for cardiovascular benefits. Compounds in the same class as isoallentolactone have been tested for that in the past. This is disclosed amongst others in "Ginkgo biloba Extract Improves Coronary Blood Flow in Patients with Coronary Artery Disease: Role of Endothelium-Dependent Vasodilation", Wu,
20 Yuzhou; Li, Shuqin; Cui, Wei; Zu, Xiuguang; Wang, Fengfei; Du, Jun. *Planta Medica* vol. 73 issue 7 (2007) p. 624-628; and in "Toxic inhibition of smooth muscle contractility by plant-derived sesquiterpenes caused by their chemically reactive α -methylenebutyrolactone functions", Alastair J.B. Hay, Matthias
Hamburger, K. Hostettmann and J.R.S. Hoult, *Br. J. Pharmacol.* (1994), 112, 9-
25 12.

However, although *Inula racemosa* (Pushkarmool) root and stem contains high levels of isoallentolactone itself, known for the production of nitric oxide, which is being held responsible for vasodilation. The known methods of extraction of
30 isoallentolactone from Pushkarmool give dissatisfactory results, due to the lack of extraction of further compounds from the pushkarmool plant, that apparently contribute to the vasodilating properties.

Lactones, are skin sensitizers. Isoallantolactones are ROS (reactive oxygen species) inducers that may stimulate apoptosis in tumor cells, as is disclosed in literature in amongst others: Jean-luc Stampf et al Allergic contact dermatitis due to sesquiterpene lactones. British Journal of Dermatology (1978) 99, 163.; N. Alonso Blasi et al. A murine in vitro model of allergic contact dermatitis to sesquiterpene e-methylene-7-butyrolactones. Arch Dermatol Res (1992) 284:297-302; D. Belsito et al. A toxicological and dermatological assessment of macrocyclic lactone and lactide derivatives when used as fragrance ingredients. Food and Chemical Toxicology. 2011, Pages S219I Tox. Plant extracts containing reduced levels of isoallentolactone remain to be desired.

Improved extraction methods are proposed in “Two new eudesmanolides from *Inula racemosa* and their bioactivities”, Ting Zhang, Ting Gong, Yan Yang, Ruo-Yun Chen *, De-Quan Yu, Phytochemistry Letters 5 (2012) 229–232; and “Two New Sesquiterpenes from *Inula salsoloides* and Their Inhibitory Activities against NO Production”, Xiao-Jia Hua, Hui-Zi Jin, Xiao-Hua Liuc, and Wei-Dong Zhang, Helvetica 306 Chimica Acta – Vol. 94 (2011).

However, an improved extract, obtainable from the *Inula racemosa* plant, providing vasodilation, remains to be desired.

It is therefore object of the present invention to provide a highly active extract from the *Inula racemosa* (Pushkarmool) for providing a vasodilation and/or microcirculation benefit.

It is a further object to provide active NO potentiating extracts of *Inula racemosa* with a low level of isoalentolactone, compared to known hexane extracts.

It has been surprisingly found that an extract of plant material from *Inula Racemosa* with a solvent with a hydrogen bonding parameter of between 4 and 15, gives higher nitric oxide (NO) production and better vasodilation with less isoalantolactone in the extract.

Summary of the invention

Accordingly, the present invention provides a method for extraction of *Inula racemosa* stem, root or mixed stem and root powder comprising the steps of: extracting powdered stem or root or both stem and root of *Inula racemosa* with a solvent wherein the ratio of the polarity index to dielectric constant of the solvent is between 0.45 and 0.8; removing the stem and/or root powder; and evaporating the solvent from the extract.

These and other aspects, features and advantages will become apparent to those of ordinary skill in the art from a reading of the following detailed description and the appended claims. For the avoidance of doubt, any feature of one aspect of the present invention may be utilised in any other aspect of the invention. The word "comprising" is intended to mean "including" but not necessarily "consisting of" or "composed of." In other words, the listed steps or options need not be exhaustive. It is noted that the examples given in the description below are intended to clarify the invention and are not intended to limit the invention to those examples per se. Similarly, all percentages are weight/weight percentages unless otherwise indicated. Except in the operating and comparative examples, or where otherwise explicitly indicated, all numbers in this description indicating amounts of material or conditions of reaction, physical properties of materials and/or use are to be understood as modified by the word "about". Numerical ranges expressed in the format "from x to y" are understood to include x and y. When for a specific feature multiple preferred ranges are described in the format "from x to y", it is understood that all ranges combining the different endpoints are also contemplated.

Detailed description of the invention

Thus the invention provides for a method for extracting powdered *Inula racemosa* root and/or stem with a solvent, as well as a powdered material obtained from said extract, and a food and cosmetic products comprising the same.

Without being bound by a theory, for oral consumption the benefit of food products comprising *Inula racemosa* extract according to the invention, the effect is thought

to be increased NO production in endothelial cells, which provide better vascular function due to increase vasodilation.

Without being bound by a theory, for topic use, the effect of the increased NO
5 production by the extract is found to be enhanced micro circulation, improved skin health and improved detoxification of the skin as well as reduced skin aging and wrinkles.

Inula racemosa material

10 For the purpose of the invention, powdered material from the stem, root or stem and root of *Inula racemosa* is used. The powder may be obtained from commercial sources or prepared from plant material.

Inula racemosa powder may be prepared by powdering root, stem or both in a
15 pulveriser. For getting a discrete particle size range, a sieve may be used. It has been found that sieve fractions that pass through a 200 micron sieve (i.e. particles of less than 200 micrometer) perform the best.

Extraction

20 The powdered stem or root or both stem and root of *Inula racemosa* is preferably extracted with a solvent having a polarity index of between 3.5 and 4.8 and having a dielectric constant of at least 5.

Solvent

25 In commonly known pushkarmool extraction methods, the focus has been on providing extracts with a high isoalmentolactone content. However, the lactones have been found to have ROS related toxicity and are known to skin sensitizers and irritants.

30 We have found that extracts with a relatively low isoalmentolactone content still provide a good NO production, without the lactone related drawbacks.

The polarity index of a substance is a relative measure of the degree of interaction of the solvent with various and different polar test solutes. Polarity depends on the electro negativity difference between atoms in a bond, the greater the difference, the more polar the bond.

5

The polarity index of the solvent according to the invention is preferably between 4 and 4.5.

10 The polarity index is a measure of the relative polarity of a solvent. A numerical index is proposed that ranks solvents according to their different polarity. Polarity refers to the separation of electric charge leading to a molecule or its chemical groups having an electric dipole moment. Molecular polarity is dependent on the difference in electro-negativity between atoms in a compound and the asymmetry of the compound's structure. The polarity index value increases with increase in
15 polarity.

The dielectric constant (ϵ having the unit F m^{-1} , also known as permittivity) of a solvent is a measure of the solvent's ability to separate ions. The dielectric constant measures the solvent's ability to reduce the field strength of the electric
20 field surrounding a charged particle immersed in it. This reduction is compared to the field strength of the charged particle in a vacuum. The greater the dielectric constant, the greater the polarity.

25 The dielectric constant of the solvent according to the invention is preferably at least 5.5 and typically not more than 9.

The solvent has a ratio of the polarity index and the dielectric constant; wherein polarity index : dielectric constant is between 0.45 and 0.80.

30 The most preferred solvents are ethyl acetate and tetrahydrofuran. Other solvents are given in the table below.

Solvent	PI	D	PI/D
Tetrahydrofuran	4	7.5	0.53
diethyle ether	2.8	4.3	0.65
Ethyl Acetate	4.4	6	0.73
Carbontetrachloride	1.7	2.2	0.77
<i>n</i> -Butyl Acetate	4	5.1	0.78

Extraction

For the best results, the powder may be soaked in the solvent for at least 30 minutes, more preferably at least 1 hr, still more preferably at least 2 hours, even
5 more preferably at least 5 hrs, yet more preferably at least 10 hrs, or even at least 15 hrs.

In a preferred embodiment the solvent is refluxed for at least 30 minutes, more preferably at least 1 hr, still more preferably at least 2 hours, even more preferably
10 at least 5 hrs, or even at least 8 hrs. When refluxed the solvent is preferably left to cooled down to less than 30°C, or even to room temperature (21°C).

Removing stem and/or root powder

Then the extract is separated from the spent root powder to get a clarified solvent
15 solution of the extracted material. This may be done by any conventional way, including filtration, centrifugation, decanting, or otherwise. Filtration is the most preferred.

Evaporating the solvent from the extract.

20 The solvent may then evaporated to obtain a dry solid extract, preferably in powder form.

The dried extract may then be stored, preferably chilled, more preferably at a temperature of less than 10°C or even less than 5°C, but preferably more less
25 than -25°C, or even not less than -20°C.

Food product

The extract according to the invention may be used in a food product in a concentration of between 0.1 and 10% by weight.

Preferred food products include beverages, including tea and health drinks, ice-cream, bakery products, including biscuits, savoury products, such as noodles, spreads and soups.

The total dosage to a person by oral uptake is preferably between 0.1 and 6 mg per kg of body weight per day.

Topical compositions

The extract according to the invention may be used in a topical composition in a concentration of between 0.1 and 3% by weight.

Preferred topical compositions include skin creams, ointments, lotion, stearic acid bases, oil in water and water in oils, sprays, wet preparations, powders, shake lotions, creams, pastes, ointments, hydrocarbons, gels, and adherent dressings.

The total dosage to a person by topical use is preferably between 0.1 and 60 micro gram per square centimetre.

Other typical materials in topical compositions include polymers, including synthetic and natural polymers.

Synthetic polymers include, but are not limited to: polyalkylene oxides, polyethylene glycols, polyethylene oxides, partially or fully hydrolyzed polyvinylalcohols, poly(vinylpyrrolidone), poly(t-ethyloxazoline), poly(ethylene oxide)-co-poly(propylene oxide) block copolymers (poloxamers and merioxapols), polyols such as glycerol, polyglycerol (particularly highly branched polyglycerol), propylene glycol and trimethylene glycol substituted with one or more polyalkylene oxides, e.g., mono-, di- and tri-polyoxyethylated glycerol, mono- and di-polyoxyethylated propylene glycol, and mono- and di-polyoxyethylated trimethylene

glycol; polyoxyethylated sorbitol, polyoxyethylated glucose; acrylic acid polymers and analogs and copolymers thereof, such as polyacrylic acid, polymethacrylic acid, poly(hydroxyethylmethacrylate), poly(hydroxyethylacrylate), poly(methylalkylsulfoxide methacrylate), poly(methylalkylsulfoxide acrylate),
5 and/or with additional acrylate species such as aminoethyl acrylate and mono-2-(acryloxy)-ethyl succinate; polymaleic acid; polyacrylamides) such as polyacrylamide per se, poly(methacrylamide), poly(dimethylacrylamide), and poly(N-isopropyl-acrylamide); poly(olefinic alcohol)s such as poly(vinyl alcohol); poly(N-vinyl lactams) such as poly(vinyl pyrrolidone), poly(N-vinyl caprolactam),
10 and copolymers such as polyethylene glycol/poly(N-isopropylacrylamide) thereof; polyoxazolines, including poly(methyloxazoline) and poly(ethyloxazoline); polyvinylamines, polyacrylamide (PAA), poloxamines, carboxymethyl cellulose, and hydroxyalkylated celluloses such as hydroxyl-ethyl cellulose and methylhydroxypropyl celluloses. One may use various combinations, and further
15 various chemically modified forms or derivatives thereof.

Natural monomers may include glycosaminoglycans such as hyaluronic acid, chondroitin sulfate A, chondroitin sulfate C, dermatan sulfate, keratan sulfate, keratosulfate, chitin, chitosan, and derivatives thereof. Therefore, while not exhaustive, examples of natural monomers or polymers which may be used
20 include: polypeptides, polysaccharides or carbohydrates such as polysucrose, hyaluronic acid, dextran, heparin sulfate, chondroitin sulfate, heparin, or alginate, and proteins such as gelatin, collagen, albumin or ovalbumin or copolymers or blends thereof. Celluloses include cellulose and derivatives, dextrans include dextran and similar derivatives. Extracellular matrix proteins, such as collagens,
25 elastins, laminins, gelatins, and fibronectins include all the various types found naturally (e.g., Collagen I-IV) as well as those same collagens as produced by and purified from a recombinant source, fibrin, a naturally occurring peptide important for its a role in wound repair in the body, and alginate, a polysaccharide derived from seaweed containing repeating units of mannuronic and guluronic acid, may
30 also be used. One may use various combinations, and may include chemically modified forms, mimetics, or derivatives thereof. For proteins, one may use recombinant forms, analogs, forms containing amino acid mimetics, and other various protein or polypeptide-related compositions.

Examples

The invention will now be illustrated by means of the following non-limiting examples.

5 **Materials**

DAF FM-DA was purchased from Invitrogen (Eugene, Oregon). DMEM, Insulin, L-NAME were purchased from Sigma (St. Louis, MO, USA). Anti-eNOS Ser1177 antibody were purchased from Cell Signalling Technology (Beverly, MA). DAN assay kit (Cat # 780051) for estimation of Nitrites was purchased from Cayman
10 Chemicals (USA).

Preparation of EA.Hy926 cells

The cultured human endothelial cell line EA.Hy926 cells were procured from American Type Culture Collection (CRL-2922 - ATCC) and were cultured in
15 DMEM (Sigma) supplemented with 2mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin and 10% vol/vol FBS (Gibco, Invitrogen). Cells were cultured at 37°C in 95% humidified air with 5% CO₂. After attaining 70-80% confluence, the cells were sub-cultured by trypsinization (0.25% Tryp-EDTA, for 2-3 min). For experimental purpose, cells were seeded onto 24 well tissue culture
20 plates (CLS3524 Sigma). After adherence, cells were subjected to starvation in serum free low glucose DMEM for 12-14 hrs prior to any experiment, to maintain the cells in quiescent state and reduce the basal level of NO production.

Preparation of aqueous extract:

- 25 1. The aqueous extract was prepared from dry Inula root powder.
2. 100g dry *Inula Racemosa* root powder was soaked in 800ml water overnight and then boiled at 80°C for four hours.
3. After cooling down to 35 - 40°C, the extract was filtered to get a clear solution.
4. The solution was concentrated to dryness using rotary evaporator.
- 30 5. The dried aqueous extract was stored at 40°C.

Example 1: Quantification of Nitrites/Nitrate as a measure on NO release from the cell culture supernatant.

Experimental protocol:

- 5 1. EAHy926 (10^6) cells were allowed to adhere in 24 well cell culture plate.
2. After adherence, cells were starved in glucose deprived medium for 12 hr.
3. Cells were treated with or without L-NAME (10 mM; 30 min pre-incubation)
4. Cells were treated with Pushkarmool Aq. Extract for 30 min at 1, 10, 100 and 1000 $\mu\text{g/ml}$ concentrations.
- 10 5. Insulin (25 μM) was used as positive control.
6. After treatment period, the culture supernatant was collected and analyses for nitrite content using Di-amino naphthalene (DAN) assay kit as described in manufacturers protocol.
7. NaNO_3 was used as calibration standard.

15

Table 1. Estimation of Nitrites as a measure of NO released in endothelial culture supernatant.

Sample	Nitrite levels (μM)	SD	p
Untreated control	2.29	0.13	NS
Control +L-NAME	2.16	0.14	NS
Pushkarmool (1 $\mu\text{g/ml}$)	2.17	0.15	NS
Pushkarmool (1 $\mu\text{g/ml}$)+L-NAME	2.03	0.15	NS
Pushkarmool (10 $\mu\text{g/ml}$)	2.61	0.11	$p<0.01$.
Pushkarmool (10 $\mu\text{g/ml}$)+L-NAME	2.43	0.16	NS
Pushkarmool (100 $\mu\text{g/ml}$)	3.10	0.17	$p<0.01$.
Pushkarmool (100 $\mu\text{g/ml}$)+L-NAME	2.76	0.15	$p<0.01$.
Pushkarmool (1000 $\mu\text{g/ml}$)	2.69	0.19	$p<0.01$.
Pushkarmool (1000 $\mu\text{g/ml}$)+L-NAME	2.66	0.14	$p<0.01$.
Insulin 25 μM	2.94	0.15	$p<0.01$.
Insulin 25 μM +L-NAME	2.69	0.13	$p<0.01$.

EAHy926 (10^6) cells were allowed to adhere in 24 well cell culture plate. After
 20 adherence, cells were starved in glucose deprived medium for 12 hr.

Subsequently, cells were treated with or without L-NAME (10 mM; 30 min pre-incubation), followed by stimulation with Pushkarmool root extract for 30 min. After treatment period, the culture supernatant was collected and analyses for nitrite content using Di-amino naphthalene (DAN) assay kit as described in
5 manufacturers protocol. The measure of nitrites was expressed as nitrite equivalents to NaNO₃ standard. Insulin was used as positive control. L-NAME was used as negative control. Data are presented as mean $\pm$ SD (n = 6). Statistical comparisons were made by student t-test.

10 **Results:** Pushkarmool Aq extract increases nitrite levels in the culture supernatant, suggesting enhanced NO production. In presence of L-NAME, such increased response was not observed, suggesting that decrease in fluorescence was due to inhibition of NO production.

L-NAME is a substrate analogue to L-Arginine. It competitively binds to enzymes
15 active site and inhibit NO production.

Insulin (25 μ M) is a known NO inducer in endothelial cells, hence used as positive control.

20 **Conclusion:** Pushkarmool aquase extract enhance NO production in endothelial cells.

Example 2: Polarity guided fractionation of Pushkarmool:

Sequential solvent extraction procedure for *Inula racemosa*:

1kg of Inula root powder was soaked overnight in 2L of hexane at room
25 temperature and filtered. The process was repeated for three times. The filtrates were pooled and concentrated to dryness under vacuum using a rotary evaporator to obtain hexane extract.

The spent was air dried to remove the hexane completely. To the dried spent 2L
30 of ethyl acetate was added and soaked overnight at room temperature and filtered. The process was repeated for three times. The filtrates were pooled and

concentrated to dryness under vacuum using a rotary evaporator to obtain ethyl acetate extract.

The spent was air dried to remove the ethyl acetate completely. To the dried spent
 5 2L of 70% aqueous ethanol was added and soaked for 2hrs at room temperature and heated to 50°C for 2hrs. The process was repeated for three times. The filtrates were pooled and concentrated to dryness under vacuum using a rotary evaporator to obtain 70% EtOH extract.

10 ***Measurement of intracellular NO after flow-cytometry assisted segregation of activated and non-activated cells***

Experimental flow:

1. EAHy926 (5×10^6) cells were allowed to adhere in 24 well cell culture plate.
2. After adherence, cells were starved in glucose deprived medium for 12 hr.
- 15 3. Pre-starved cells (5×10^5) in 24 well tissue culture plates were loaded with DAF FM-DA ($1 \mu\text{M}$) for 30 min, washed twice with serum free medium.
4. Subsequently, cells were stimulated with agonist (Pushkarmool Aq. extracts or fractions) for 30 mins and washed twice with serum free medium.
5. The stimulated cells were trypsinized with 0.25% Trypsin-EDTA and fixed with
 20 2% PFA for 15 min.
6. The suspension of cells were acquired using flow-cytometry. A population of 10,000 cells were gated and segregated based on their relative fluorescence intensities using FACS Calibur (BD; San Diego). The mean yield of two distinct populations was measured and compared with the respective population in
 25 untreated cells.

Table 2. Estimation of intracellular NO produced in endothelial cell.

<i>Samples (10$\mu\text{g/ml}$)</i>	<i>NO levels (fold change)</i>	<i>SD</i>	<i>p</i>
Untreated control	1.00	0	NS
Control +L-NAME	0.69	0.09	NS
Ethanol fraction	1.24	0.11	$p < 0.01$.

Ethanol fraction+L-NAME	0.95	0.14	NS
Ethyl acetate fraction	1.87	0.16	p<0.01.
Ethyl acetate fraction+L-NAME	1.17	0.12	NS
Hexane fraction	1.59	0.20	NS
Hexane fraction+L-NAME	1.31	0.25	NS
Oil fraction	0.98	0.02	NS
Oil fraction+L-NAME	0.97	0.04	NS
Aqueous extract	1.57	0.15	NS
Aqueous extract+L-NAME	0.93	0.13	NS

Pushkarmool aqueous extract (Aq), fractions (Ethanol fraction [EF], ethyl acetate fraction [EAF] and Hexane fraction [HF]) were evaluated for its NO potentiating effect in endothelial cells. EA.Hy926 (1×10^6) cells were serum starved for 12 h, loaded with of DAF FM-DA (0.1 μ M, for 30 min). The cells were then stimulated with actives for 30 min in presence or absence of L-NAME. The cells were segregated using flow-cytometry, and fluorescent intensity of the eNOS activated cells was measured. Data represented as relative fold change with respect to untreated basal control. Data points are represented as mean $\pm$ SD of seven independent experiments. Statistical comparisons were made by student t-test. * values were statistically significant ($p < 0.01$, $n=7$) with respect to control. (Concentration of the entire active was 1 μ g/ml).

Results: Pushkarmool Ethyl acetate fraction showed significance enhancement in NO production in endothelial cells w.r.t Aq. Extract.

Conclusion: Pushkarmool Ethyl acetate fraction enhance NO production in endothelial cells, hence polarity index based extraction of whole herb should be used for further fractionation.

Example 3: Preparation of extract using different solvents.

1. 100g dry Inula root powder was soaked in 800ml different solvents (see table below) overnight and then refluxed for four hours.

2. After cooling down to 35 - 40°C, the extract was filtered to get a clear solution.
4. The solution was concentrated to dryness using rotary evaporator.
5. The dried extract was stored at 40°C.

5 **Table 3: Solvents and their PI and ϵ values**

Solvent	Polarity Index (PI)	Dielectric constant (ϵ)	Ratio PI/ ϵ
Hexane	0	1.89	0.00
Water	10.2	80	0.13
Ethanol	5.2	24.3	0.21
Acetone	5.1	20.7	0.25
Methylene dichloride (DCM)	3.1	9.1	0.34
Tetrahydrofuran (THF)	4	7.9	0.51
Diethyl ether	2.8	4.3	0.65
Ethyl acetate	4.4	6.0	0.73
n-butyl acetate	4	5.1	0.78
Chloroform	4.1	4.8	0.85
Toluene	2.4	2.4	1.00

Measurement of intracellular NO using flow-cytometry in endothelial cells

Experimental flow:

1. EAHy926 (5×10^6) cells were allowed to adhere in 24 well cell culture plate.
- 10 2. After adherence, cells were starved in glucose deprived medium for 12 hr.
3. Pre-starved cells (5×10^5) in 24 well tissue culture plates were loaded with DAF FM-DA (1 μ M) for 30 min, washed twice with serum free medium.
4. Subsequently, cells were stimulated with agonist (different solvent extracts) and 3 different concentrations (1, 10 and 100 μ g/ml) for 30 mins and washed
15 twice with serum free medium.
5. The stimulated cells were trypsinized with 0.25% Trypsin-EDTA and fixed with 2% PFA for 15 min.
6. The suspension of cells were acquired using flow-cytometry. A population of 10,000 cells were gated and segregated based on their relative fluorescence
20 intensities using FACS Calibur (BD; San Diego). The mean yield of two distinct

populations was measured and compared with the respective population in untreated cells.

Table 4: Evaluating the efficacy of different fractions of Pushkarmool.

Sample	NO levels (fold change)	SD	p
Untreated control	1.00	0	
Aqueous extract (1ug/ml)	1.19	0.08	NS
Aqueous extract (10ug/ml)	1.64	0.06	p<0.01.
Aqueous extract (100ug/ml)	2.58	0.12	p<0.01.
Ethanol extract (1ug/ml)	1.23	0.04	NS
Ethanol extract (10ug/ml)	1.54	0.05	NS
Ethanol extract (100ug/ml)	2.66	0.12	NS
Acetone extract (1ug/ml)	1.47	0.14	NS
Acetone extract (10ug/ml)	1.62	0.07	NS
Acetone extract (100ug/ml)	2.74	0.11	p<0.01.
CHCl ₃ (1ug/ml)	1.26	0.06	NS
CHCl ₃ (10ug/ml)	1.66	0.12	NS
CHCl ₃ (100ug/ml)	2.13	0.29	p<0.01.
Dichloromethane (1ug/ml)	1.26	0.16	NS
Dichloromethane (10ug/ml)	2.13	0.29	p<0.01.
Dichloromethane (100ug/ml)	2.52	0.16	p<0.01.
Tetrahydrofuran (1ug/ml)	3.24	0.03	p<0.01.
Tetrahydrofuran (10ug/ml)	3.97	0.26	p<0.01.
Tetrahydrofuran (100ug/ml)	5.23	0.04	p<0.01.
Diethyl ether (1ug/ml)	2.68	0.06	p<0.01.
Diethyl ether (10ug/ml)	3.40	0.17	p<0.01.
Diethyl ether (100ug/ml)	4.71	0.14	p<0.01.
EtOAc (1ug/ml)	2.41	0.30	p<0.01.
EtOAc (10ug/ml)	3.20	0.06	p<0.01.
EtOAc (100ug/ml)	3.89	0.33	p<0.01.
n-butyl acetate (1ug/ml)	2.16	0.07	p<0.01.
n-butyl acetate (10ug/ml)	2.86	0.12	p<0.01.
n-butyl acetate (100ug/ml)	3.59	0.18	p<0.01.

Toluene (1ug/ml)	1.32	0.05	NS
Toluene (10ug/ml)	1.49	0.11	NS
Toluene (100ug/ml)	2.41	0.30	p<0.01.

- 5 The extracts were prepared as defined in the protocol for extract preparation. The dried powders were tested for the efficacy on weight basis for enhanced NO production. Data represented as relative fold change with respect to untreated basal control. Data points are represented as mean $\pm$ SD of seven independent experiments. Statistical comparisons were made by student t-test.

10

Results: Pushkarmool Aq. Extract, Ethyl acetate fraction and THF fractions showed significant enhanced NO production in endothelial cells. Extract, Ethyl acetate fraction and THF fractions were significantly better at all the three concentrations tested. THF fraction had highest NO enhancing effect. The

15 Pushkarmool THF fraction shows enhanced NO production compared to other fractions. The activity lies according to the polarity index. The maximum activity lies between polarity index 4 to 4.5.

20

Chloroform has polarity index of 4.1, but do not show any NO potentiating effect, chloroform has lower dielectric constant, which also influences solubility during extraction. Hence the NO enhancing effect of different Pushkarmool solvent extracts fit better with the ratios of polarity index (PI) and dielectric constant of different solvent.

25

Conclusion:

The NO enhancing effect of Pushkarmool extracts using different solvents such as Ethanol, Acetone, Ethyl acetate, Tetrahydrofuran, Dichloromethane, Toluene, diethyl ether, n-butyl acetate and CHCl_3 is determined by the ratio of polarity index (PI) and dielectric constant of solvent used for extraction.

30

Thus the NO enhancing activity of Ayurvedic herb, Pushkarmool is enriched, if a solvent with polarity index of 3.5 and 4.8 and dielectric constant of above 5 is used for solvent extraction.

5 Example 4: Evaluating the efficacy of a) Aq and b) THF Extracts in CAM model.

CAM model is an alternate non-animal model for determining the vasodilation effect of any agonist.

Experimental setup:

- 10 1. Fertilized white leghorn chicken eggs incubated for four days were collected from the Poultry Research Centre.
2. The egg shell was cut open to expose the vitelline arteries.
3. A Nikon Cool Pix camera with an adapter was attached to the stereo microscope and a portion of the blood vessel was focused for video
15 recording.
4. The Aq. And THF fraction of Pushkarmool was prepared as defined in the protocol for extract preparation above.
5. Ethanol was used as vehicle control as it was used to dissolve THF fraction. Ethanol concentration in the extract was always kept below
20 0.01% as ethanol concentration above 0.05% is toxic.
6. The Aqueous Extract powder was dissolved in PBS.
7. A Whatman paper butt was soaked with the prepared Pushkarmool extract or vehicle control and placed on capillary vessel and the video was recorded for 20 min. Video was converted into JPEG images. 0,5,10,15,
25 20 min images were used to calculate the area of a fixed portion of blood vessel.
8. The change in area of the blood vessel after addition of extract was calculated with respected to 0 sec.

30 **Table 4a: Evaluating the efficacy of aqueous extract**

Time (mins)	% dilation control	SD	% dilation 50ug/ml	SD	p	% dilation 100ug/ml	SD	p
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	(PBS)							
0mins	100.00	0.00	100.00	0.00	NS	100.00	0.00	NS
5mins	88.51	0.71	101.80	0.58	NS	96.93	1.07	NS
10mins	86.85	1.74	99.80	1.04	p<0.01.	121.99	2.49	p<0.01.
15mins	95.01	1.59	100.75	2.09	p<0.01.	130.60	0.87	p<0.01.
20mins	94.72	0.82	102.54	2.04	p<0.01.	129.60	1.90	p<0.01.

Table 4b: Evaluating the efficacy THF extract

Time (mins)	% dilation control (PBS)	SD	% dilation 5ug/ml	SD	p	% dilation 10ug/ml	SD	p
0mins	100.00	0.00	100.00	0.00		100.00	0.00	NS
5mins	102.69	0.63	105.54	0.59	NS	110.42	1.78	p<0.01.
10mins	108.02	1.78	107.13	0.49	NS	115.84	2.09	p<0.01.
15mins	114.39	2.47	110.32	0.74	NS	119.70	2.02	p<0.01.
20mins	116.76	3.58	112.86	1.41	NS	126.42	4.11	p<0.01.

- 5 As demonstrated in the tables above, both Aq. And THF fraction of Puskarmool induced significant dilation in CAM model

Conclusion: THF fraction can be a potent lead for IPR and can be further evaluated for clinical benefit.

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Example 5: Isoallentolactone levels in different extracts.

Powdered stem and root from *Inula racemosa* was prepared as indicated above. The powder was directly extracted with hexane, ethyl acetate, THF or water as indicated above.

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The resulting extract was analysed for isoallentolactone content by HPLC.

Stationary phase: Phenomenex RP 18 column (reversed phase, 250 x 4.6 mm, particle size= 5 micrometer).

Mobile phase: Methanol(A): Acetonitrile (B)

- 5 Gradient run: 10 min(A:65, B:35), 30min((A:50, B:50), 45min((A:65, B:35), 50min((A:65, B:35); at a flow rate of 0.6ml/min.

A photo diode array detector was used for detection and the chromatogram was integrated with Shimadzu HPLC software to determine the peak area. The

- 10 wavelength at which the integration was done was 212nm.

Results

Solvent	Isoallentolactone (n=2)
Hexane	22.5%
Ethyl acetate	12.5%
THF	6%
Water	<1%

- 15 **Conclusion:** the results show that the isoallentolactone levels with solvents according to the invention are substantially lower than the levels in conventionally used hexane extracts.

Claims

1 A method for extraction of *Inula racemosa* stem, root or mixed stem and root powder comprising the steps of:

- 5 a Extracting powdered stem or root or both stem and root of *Inula racemosa* with a solvent wherein the ratio of the polarity index to dielectric constant of the solvent is between 0.45 and 0.8;
- b removing the stem and/or root powder; and
- c evaporating the solvent from the extract.

10

2 A method according to claim 1 wherein the solvent is selected from tetrahydrofuran, diethyl ether, ethyl acetate or n-butyl acetate.

3 A method according to any one of claims 1 or 2, the solvent having a polarity
15 index of between 3.5 and 4.8 and having a dielectric constant of at least 5.5.

4 A powdered extract of *Inula racemosa* obtainable by the method according to any one of claims 1 to 3.

20 5 A food product comprising 0.1-10% by weight of the extract according to claim 4.

6 A topical comprising between 0.1 and 3% by weight of the extract according to claim 4.

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/051590

A. CLASSIFICATION OF SUBJECT MATTER

INV. A23L1/30 A61K9/00 A61K36/28 A61Q19/00 A61K8/97
A61P9/08 A61P17/00

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)

A23L A61K A61Q 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, 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	LOHKANDE: "Antibacterial activity of isolated constituents and extract of roots of Inula racemosa", RESEARCH JOURNAL OF MEDICINAL PLANT, vol. 1, no. 1, 1 January 2007 (2007-01-01), page 7, XP055063125, ISSN: 1819-3455	1-6
Y	the whole document	4-6
Y	JP 2010 013400 A (ICHIMARU PHARCOS INC) 21 January 2010 (2010-01-21) paragraphs [0010] - [0019] paragraphs [0063] - [0085], [0089] - [0092], [0094], [0104]; claims 1-4 & JP 2010 013400 A (ICHIMARU PHARCOS INC) 21 January 2010 (2010-01-21) abstract	4-6
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Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2014/051590

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	OJHA SHREESH ET AL: "Effect of Inula racemosa root extract on cardiac function and oxidative stress against isoproterenol-induced myocardial infarction", INDIAN JOURNAL OF BIOCHEMISTRY AND BIOPHYSICS, vol. 48, no. 1, February 2011 (2011-02), pages 22-28, XP002697314, ISSN: 0301-1208 the whole document -----	1-5
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A	HUO Y ET AL: "Chemical constituents and pharmacological properties of Radix Inulae", PHARMAZIE, vol. 63, no. 10, October 2008 (2008-10), pages 699-703, XP002697315, ISSN: 0031-7144 the whole document -----	1-6
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2014/051590

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WO 2012090194	A2	05-07-2012	NONE	
CN 101380348	A	11-03-2009	NONE	