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(54) **Title:** IMPROVED METHOD FOR EXTRACTING LICORICE AND SUCH LICORICE EXTRACT

(57) **Abstract:** The present disclosure provides a method of extracting Glabridin (Gla) from Glycyrrhiza roots, the method comprises (a) providing licorice particulates, and (b) maintaining the licorice particulates within an extractant comprising a volatile C_2-C_6 alkyl ether for a time sufficient to allow extraction of Gla from said licorice particulates into its surrounding medium and to thereby form an extract comprising a liquid medium with dissolved Gla. Also provided by the present disclosure is a Glabridin-rich extract compositions comprising the extract and uses thereof. The Glabridin rich extract is of Glycyrrhiza roots and comprises Glabridin (Gla) in an amount that provides a Gla to Glycyrrhizic acid (GA) weight/weight ratio in the extract, if GA is present in the Gla- rich extract, of at least 2:1.

IMPROVED METHOD FOR EXTRACTING LICORICE AND SUCH LICORICE EXTRACT

TECHNOLOGICAL FIELD

The present disclosure provides an improved method

BACKGROUND ART

References considered to be relevant as background to the presently disclosed subject matter are listed below:

- US Patent No. 7,572,470 to Cohen et al.
- Petr G. Gorovoy et al. Natural Product ResearchVolume 31 (Issue 4): Pages 445-452, 2017 - *Phenolic compounds from Glycyrrhiza pallidiflora Maxim. and their cytotoxic activity*
- European Patent No. 1 925 312

Acknowledgement of the above references herein is not to be inferred as meaning that these are in any way relevant to the patentability of the presently disclosed subject matter.

BACKGROUND

Ethanolic extraction of licorice has been described. For example, US Patent No. 7,572,470 to Cohen et al. reports a preparation containing a licorice extract that is water insoluble. The extract is prepared by grinding and drying licorice root and then steeping the ground root in absolute ethanol at room temperature, stirring for several hours and filtering. The ethanol was then evaporated to obtain the dried extracted mass. According to Cohen et al. ethanol is the preferred solvent for extraction of licorice root.

Petr G. Gorovoy et al. describe twenty-one phenolic compounds isolated from the roots of *Glycyrrhiza pallidiflora Maxim*. and their evaluation for their cytotoxic activity with respect to model cancer cell lines.

European Patent No. 1 925 312 describes the use of a composition comprising flavonoids obtained by licorice root fermentation for treating diabetes mellitus complications, such as diabetic nephropathy, peripheral neuritis, cataract and retinopathy. The composition is described to be effective by inhibiting aldose reductase enzyme and thereby preventing transformation of glucose to sorbitol and its accumulation in tissues which is the cause of these diabetes mellitus complications.

GENERAL DESCRIPTION

The present disclosure provides, in accordance with a first of its aspects, a method of extracting Glabridin (Gla), from *Glycyrrhiza* roots, the method comprises:

- maintaining *Glycyrrhiza Glabra* root particulates (also referred to herein by the term *licorice* particulates) within an extractant comprising a volatile C_2-C_6 alkyl ether for a time sufficient to provide extraction of Gla from said licorice particulates into its surrounding medium and to thereby form a liquid medium comprising extracted Gla.

As subsequent steps, the method can involve:

- separating between the particulates and the liquid medium; and
- removing at least a portion of the extractant from the liquid medium to obtain an extract comprising Gla.

The liquid medium, preferably after removal of the particulates and extractant is referred to herein as a Gla-rich extract.

Generally, *Glycyrrhiza* is a perennial plant belonging to the family *Fabaceae*, and grows naturally or is cultivated. In accordance with some embodiments, the licorice extract is one obtainable or obtained from *Glycyrrhiza Glabra* root. In a preferred embodiment, the licorice extract is a *Glycyrrhiza Glabra* root extract.

In accordance with a second disclosed aspect, there is provided by the present disclosure a Gla-rich extract of *Glycyrrhiza Glabra* roots comprising Gla in an amount that provides a Gla to Glycyrrhizic acid (GA) weight/weight ratio (if GA is present in the Gla-rich extract) of at least 2:1.

In accordance with yet a further aspect, there is disclosed herein the Gla-rich extract or a composition comprising the Gla-rich extract for use as a therapeutic agent. In some embodiments, the use is as an anti-oxidation agent, as further discussed below.

Finally, disclosed herein is a method of treatment comprising administering to a subject in need of treatment an amount of the Gla-rich extract disclosed herein. In this context, treatment also encompasses prophylactic treatment.

BRIEF DESCRIPTION OF THE DRAWINGS

In order to better understand the subject matter that is disclosed herein and to exemplify how it may be carried out in practice, embodiments will now be described, by way of non-limiting example only, with reference to the accompanying drawings, in which:

Figure 1 is a photographic image of *Glycyrrhiza Glabra* root extracted with MtBE (Left image) or EtOH (right image)

Figure 2 is a bar graph showing the dry weight of different *Glycyrrhiza Glabra* root extracts.

Figure 3 is a bar graph showing total phenols (mg/g root) as determined compared to the reference Gallic Acid ("gallic acid equivalent")

Figure 4 provide total amount (mg/g root) of Glabridin (Gla) or Glycerhizic acid (GA) in each extract, as determined spectrophotometrically by absorbance at 230 nm (for Gla) or 252nm (for GA).

Figures 5A-5B are HPLC graphs showing Gla peak (at 230nm), Figure 5A providing Gla peaks from the EtOH extract and MtBE extract while Figure 5B provides the standard peaks of Gla (230nm) and GA (252nm)

Figure 6 is a bar graph showing antioxidant activity of each extract as compared to Trolox and Vitamin E.

Figure 7 is a graph showing the protective/anti-oxidative effect of MtBE-based extract on Omega-3.

DETAILED DESCRIPTION OF EMBODIMENTS

The present disclosure is based on the unexpected finding the extraction of *Glycyrrhiza Glabra* root (also referred to herein by the term "*licorice*") with an ether-based volatile and poorly water soluble organic solvent, specifically, methyl tert-butyl ether (MtBE), provides an extract that is richer in Glabridin content as compared to the conventional ethanol based extraction, this being exhibited, *inter alia*, by the Gla/GA w/w ratio in the extract.

The MtBE-based *Glycyrrhiza Glabra* root extract disclosed herein has also an improved anti-oxidative activity as compared to that of the EtOH-based extract, as exemplified hereinbelow with respect to some non-limiting *in vitro* examples.

Specifically, it has been found that the disclosed MtBE-based extract has at least a 2 fold increase in *in vitro* anti-oxidative activity as compared to a corresponding EtOH-based extract and at least a 4 or even 5 fold increase as compared to an equivalent recommended dose of Vitamin E.

Based on the current finding, an improved method for extracting Gla from *Glycyrrhiza* roots (licorice) is disclosed, the method comprising (a) providing licorice particulates; and (b) holding the licorice particulates within an extractant comprising a volatile C_2-C_6 alkyl ether for a time sufficient to cause extraction of Gla from the licorice particulates into its surrounding medium, which as a result, a liquid medium comprising the Gla is formed (this stage (b) being regarded herein, at times, as the extraction stage).

This liquid medium can then be further processed by separating between the particulates and the liquid medium; and then removing at least a portion of the extractant from the liquid medium to obtain a clear or essentially clear (root-free) extract comprising Gla.

In the context of the present disclosure, an *extractant* is regarded as the liquid used to cause extraction of at least Gla from the particulated roots. In some embodiments, the extractant comprises or is an organic volatile, poorly water soluble or water insoluble solvent. In some embodiments, the extractant comprises or is a branched C_3-C_6 alkyl ether.

In a preferred embodiment, the extractant comprises or is MtBE.

In yet a specifically preferred embodiment, the extractant is MtBE. In some embodiments, the MtBE is MtBE 100%.

The *Glycyrrhiza* roots (licorice) is to be provided in particulate form, i.e. processed into smaller particles. The particulating can be done by any means available in the art, from chopping, grinding, powdering or otherwise reducing size. The particulates can be a priori prepared, provided commercially, of prepared just before the extraction step.

In some embodiments, the licorice is particulated into powder. In the context of the present disclosure, when referring to licorice powder, it is to be understood as forming root particles having an average size of between 0.5 centimeter to 3 centimeter.

In some embodiments, the root particles or root powder formed by the said particulating are essentially uniform in size.

The particulated licorice/licorice powder is then introduced into the extractant for the actual extraction stage.

In some embodiments, the extractant containing the particulated licorice, the combination of which is referred to herein as the liquid medium surrounding the licorice particulate, is agitated or shaken to cause suspension of the particualated matter within the medium. The agitation may be continuous, during the entire extraction period, and may be intermittent.

In some embodiments, the extraction stage takes place at any temperature between 10°C -50°C. In some embodiments, the extraction is at ambient temperature; this being defined as a temperature of 22°C±5°.

The extraction stage can take from several minutes to several hours. In some embodiments, the extraction stage is for a period of at least 10 minutes, at times between 10 minutes and 10 hours or for any period of time between this defined range. The end of the extraction period can be determined empirically, e.g. spectrophotometrically based on the level or intensity of absorbance at 230nm (absorbance wavelength of Gla).

Once the extraction stage is completed or even arbitrarily terminated, the method may further involves the separation between the particulate roots and the liquid medium that at this stage holds the extracted Gla. The separation between the

particulated matter and the liquid medium can be by any means known in the art, including filtration, centrifugation, press, extrusion.

In some embodiments, the separation is by centrifugation, and collecting the supernatant (separation between the supernatant and the pellet).

The removed liquid medium may then be further processed by, for example, partially or completely drying. Drying can be achieved by evaporation. Since the extractant is volatile, evaporation can be achieved by simply placing the liquid medium within a ventilated device, such as a fume hood. Other evaporation techniques, as known in the art, may be employed at this stage.

The result of evaporation may be a concentrated extract or a dry or semi-dried Gla-rich powder.

In some embodiments, the drying of the liquid medium is until all extractant liquid is removed. In some embodiments, the drying provides a powder (art times referred to herein as the extract powder or Gla-rich powder). Drying can be by any known method in the art, such as, without being limited thereto, spray drying, drum drying, vacuum belt drying etc.

The extract powder may then be further processed, e.g. for storage, into commercial products, re-dissolved within a desired medium, etc.

The extract, be it in a form of a liquid, semi liquid or powder, is considered unique due to its Gla content, and can be characterized as a Gla-rich extract of *Glycyrrhiza* roots comprising a Gla to Glycyrrhizic acid (GA) weight/weight ratio, if any GA is present in the Gla-rich extract, of at least 2:1, at times, at least 2.1:1, or at least 2.2:1, or at least 2.3:1, or at least 2.5:1 or at least 2.6:1 or at least 2.7:1 or at least 2.8:1 or at least 2.9:1 or at least 3:1.

In addition or alternatively, the extract may be characterized by a chromatography profile that is substantially similar to **Figure 5A**. Without being bound by theory, it is believed that the absence of a peak as compared to the profile obtained from an EtOH extract and/or the apparent difference in physical properties of the two extracts may be indicative of the absence or low amount of carbohydrates (sugars) in the MtBE derived extract. The HPLC profile can be obtained, as described hereinbelow, with respect to some non-limiting examples (See for example **Figure 5A**), using a diode

array detector (DAD) at 280 nm and 370 nm [Natalia Martins, et al. Characterization of phenolic compounds and antioxidant properties of *Glycyrrhiza glabra* L. rhizomes and roots, RSC Adv., 5:26991-26997 (2015)]. It is noted that the absence of sugars or low amount of sugars in the extract is of advantage when turning to process the extract into solid dosage forms. For example, sugars are known to impose difficulties in spray drying and their absence from the extract would thus overcome such difficulties.

In addition or alternatively, the extract may be characterized by its anti-oxidative activity that is statistically significantly greater that the activity of an extract similarly obtained, yet with ethanol as the extractant. The superior activity is exhibited in the non-limited example of Figure 6.

In addition or alternatively, the licorice extract can be characterized by its Gla content that is at least 5% w/w greater than the amount obtained from the same licorice source, subjected to the same extraction method steps yet with EtOH (96%) instead of MtBE.

In accordance with some embodiments, the Gla-rich extract (before or after the further processing) can be characterized by a total phenolic content of at least 10%w/w, at times, at least 15%w/w or at least 16%w/w, or at least 17%w/w or even at least 18%, or 19% or 20%w/w out of the total weight of the extract; the total phenolic content being determined, for example, by a commonly used Folin–Ciocalteu method.

In some embodiments, phenolic compounds can be determined by HPLC, carried out in the diode array detector (DAD) using 280 nm and 370 nm as wavelengths and in a mass spectrometer (MS) connected to the HPLC system via the DAD cell outlet. Peaks can be identified based on their UV-vis and mass spectra and comparison with data reported in the literature. Quantification can be performed from the areas of the peaks recorded at 280 and 370 nm using calibration curves (1–100 mg mL1) obtained with phenolic standards of the same group [Natalia Martins, et al. Characterization of phenolic compounds and antioxidant properties of Glycyrrhiza glabra L. rhizomes and roots, RSC Adv., 5:26991-26997 (2015)]. The results will then be expressed in mg per g of extract.

In some embodiments, when referring to phenol compounds it is to be understood as encompassing any one and at least one of flavones, flavanones and

chalcones, as well as possible isoflavones. Some non-limiting phenol compounds found in the Gla-rich extract are selected from the group consisting of Hispaglabridin A, HispaglabridinB, 4'-O-Methylglabridin, Glabridin, Isoprenylchalcone, Isoliquiritigenin, Formononetin.

A preferred phenol in accordance with the present disclosure that is present in the extract is Glabridin (Gla).

In some embodiments the licorice extract can be characterized by low glycyrrhizic acid (GA) content. In some embodiments, the GA content is of less than 2%w/w, at times, less than 1.5%w/w, less than 1%w/w or even less than 0.9% w/w, 0.8% w/w, 0.7% w/w, 0.6% w/w, 0.5% w/w; this being determined, for example, by dissolving Gla-rich extract in ethanol, filtering to remove aggregates, if any, and running the filtered matter on HPLC with a phenyl-hezyl column (mobile phase: A- 1% Acetic Acid in water, B- 1% Acetic Acid in acetonitrile. Gradient conditions: 0%-90% B in 50 min and 90%-100% B in 10 min, temperature kept constant at 25°C). The samples can be tested at 230nm, 252nm and 280nm may be analyzed versus Glycyrrhizic acid standard.

The low content of GA is of importance, *inter alia*, due to its undesired effect on elevating blood pressure.

In some embodiments, the Gla-rich extract is characterized by its Gla/GA ratio. The Gla/GA ratio can be determined by re-dissolving the extract within an organic solvent in which the powder is soluble. The selection of the solvent would typically be determined based on the analytical method used for determination of the ratio. In some embodiments, the analytical method requires a water soluble solvent, and thus, ethanol could be used. The dissolved extract may then be subjected to various analyses, for example, HPLC, ultraviolet-visible spectroscopy or ultraviolet-visible spectrophotometry (UV-Vis or UV/Vis).

The Gla-rich extract was found to be superior in various therapeutically beneficial aspects.

Specifically, the Gla-rich extract was found to have superior anti-oxidative effect as compared to the effect of licorice extract from EtOH.

In some embodiments, the Gla-rich extract exhibits an anti-oxidative activity that is at least 2.2 fold greater than the activity of a licorice extract produced from the same licorice source, subjected to the same extraction method steps, albeit with EtOH (100 or 96%) instead of MtBE as the extractant.

A similar anti-oxidative effect was shown with respect to Omega-3 where the presence of the MtBE based extract (i.e. Gla-rich extract) reduced level of oxidation by ~ 95% (see non-limiting example).

In some embodiments, at the recommended dose of Gla-rich extract exhibits an anti-oxidative activity that is at least 5 fold greater than the activity of an equivalent recommended dose of vitamin E.

The improved anti-oxidative properties of the Gla-rich extract as compared to the extract received from the commonly used extractant – EtOH, was unexpected since the common understanding was that ethanol is the best solvent for extraction of licorice. This is described, for example, by Cohen et al. (*ibid.*) stating that ethanol gives the highest yield and activity as compared to extracts with acetone, ethyl acetate or hexane.

The Gla-rich extract disclosed herein can have various applications.

In some embodiments, the Gla-rich extract is for use as an antioxidant. As such, the Gla-rich extract can be used, for example, for treating or preventing a disease or disorder associated with oxidative damage.

When referring to *treatment* in the context of the present disclosure it is to be understood as meaning the administering of an amount of the Gla-rich composition disclosed herein which is effective to ameliorate undesired symptoms associated with a disease or disorder, to prevent the manifestation of such symptoms before they occur, to slow down the progression of the disease or disorder, slow down the deterioration of symptoms, slow down the irreversible damage caused in the progressive chronic stage of the disease, to delay the onset of any such progressive stage, to lessen the severity or cure the disease, to improve survival rate or more rapid recovery. Any of the above may be collectively regarded as improving the condition of the subject being treated with the extract, the improvement being determined by a change of at least 10% in one or more measured parameters as compared to the level thereof before said treatment. At times, the improvement is by more than 10%, at times by at least 20%, or by at least 30%, or

by at least 40%, or by at least 50%, or by at least 60%, or by at least 70%, or by at least 80%, or by at least 90%, or even by at least about 100% as compared to the level thereof before said treatment.

When referring to *prevention* (prophylactic treatment), in the context of the present disclosure, it is to be understood as preventing disease or disorder form occurring. This may be, for example, applicable a subject is susceptible to get the disease, further, for example, being exposed to a toxin, being exposed to a pathogen, being exposed to oxidative stress etc. further, as an example, a subject having diabetes or receiving anti-cancer treatment may thus be in predisposition of developing neuropathy and will be selected for receiving the Gla-rich extract as disclosed herein. In addition, or alternatively, the prevention is also applicable to a subject suffering from ongoing/chronic diabetic neuropathy and has a yet non-diagnosed/identified foot ulcer e.g. the subject is at early states of developing ulcer, which could develop into ulcer or even to a gangrene. The effectiveness of the prophylactic treatment can be determined by the lack of appearance of one or more of the parameters indicative of neuropathy, or level of such parameters of no more than 10% as compared to a standard level (e.g. the expected level in a healthy subject).

Generally, the Gla-rich extract disclosed herein has beneficial use in treating of any one or any combination of two or more of the following (each constituting a separate embodiment of the present disclosure): atherosclerosis, hyperlipidemia, high triglyceride levels, fatty liver, neuropathy, retinopathy, claudication, menopausal symptoms, Peripheral artery disease (PAD), chronic obstructive pulmonary disease COPD.

In some embodiments, the Gla-rich extract is for use in the treatment or prevention of conditions associated with oxidative damage.

When referring to *conditions associated with oxidative damage* it is to be understood as encompassing any condition or symptom known in the art to result from oxidative damage. Thus, the Gla-rich extract can be regarded and used as an anti-oxidant.

In some embodiments, the Gla-rich extract is for use in inhibiting LDL oxidation.

In some embodiments, the Gla-rich extract is for use in reducing or controlling blood pressure.

In some embodiments, the Gla-rich extract is for use in reducing or controlling blood glucose concentration.

In some embodiments, the Gla-rich extract is for use in reducing or controlling blood triglyceride concentration.

In some embodiments, the Gla-rich extract is for use in treating retinopathy.

In some embodiments, the Gla-rich extract is for use in treating neuropathy.

In the context of the present disclose, when referring to *neuropathy*, it is to be understood as encompassing any condition manifested by one or more parameter *a priori* known to be associated with neuropathy, i.e. parameter indicative of neuropathy.

In some embodiments, the neuropathy is peripheral neuropathy, namely, various degrees of pain in feet or hands and/or numbness, prickling or tingling in the feet or hands, which can spread upward into legs and arms and/or sharp, jabbing, throbbing, freezing or burning pain and/or extreme sensitivity to touch and/or lack of coordination and falling and/or muscle weakness or paralysis if motor nerves are affected, as well as a range of other symptoms all caused by one or more damaged peripheral nerves (that connect the brain and spinal cord to the muscles, eyes, lungs, skin, and internal organs).

In some embodiments, neuropathy is secondary to another condition. For example, certain complications of cancer or cancer treatments can cause or worsen neuropathy.

When being secondary to another condition, and in accordance with some embodiments, the neuropathy is selected from diabetic neuropathy, vitamin deficiency derived neuropathy, autoimmune neuropathy (e.g. AIDS, rheumatoid arthritis), hypothyroidism, carpel tunnel syndrome, post-herpetic neuralgia, alcoholic neuropathy, toxin or drug induced neuropathy, ischemic neuropathy, tumor associated neuropathy, and idiopathic neuropathy, each representing a separate embodiment.

The Gla-rich extract can be formulated into a composition, when combined with a physiologically acceptable carrier. The carrier will be selected in accordance with the desired use.

In some embodiments, the carrier is one suitable for oral delivery. For example, and without being limited thereto, the carrier is suitable for formulating the Gla-rich extract in a dosage form selected from tablets, pills, powders, granules, capsules, etc.

In some embodiments, the carrier is one suitable for topical delivery. For example, and without being limited thereto, the carrier is suitable for formulating the Gla-rich extract as a cream, lotion, ointment, gel etc.

In some embodiments, the composition can be a pharmaceutical composition, to which end, the carrier is a pharmaceutically acceptable carrier.

In some embodiments, the composition is a food supplement or food ingredient, to which end, the carrier is one acceptable in the food industry.

In some embodiments, the composition is a cosmetic product, to which end, the carrier is a cosmetically acceptable carrier.

The daily dose of the Gla-rich extract can be formulated into a single dosage form or in two or more dosage forms to be taken on same day (e.g. in intervals of several hours).

The daily dose can be administered alone or in combination with other therapeutic agents, and may be administered sequentially or simultaneously with conventional therapeutic agents.

The daily dose can be administered at different schedules and the schedule of treatment can depend on the type of condition to be treated as well as other factors, such as age, sex, severity of neuropathy etc. as discussed above.

For example, when the condition is neuropathy that is induced by chemotherapy, the schedule of treatment may comprise administration of the Gla-rich extract in conjugation with the chemotherapeutic treatment and/or for a defined period after said chemotherapeutic treatment terminates. In some other embodiments, when the condition is neuropathy that is associated with alcohol consumption, treatment may take place for

several weeks or months after cessation of the alcohol consumption (e.g. until the liver returns to normal function and there is no more pain associated therewith).

In some embodiments, the Gla-rich extract is effective in treatment when administered as a sole, herb extracted, active ingredient in the same pharmaceutical dosage form (e.g. tablet, capsule). This is unique in view of the fact that medical herbs are typically administered as a cocktail of extracts.

When referring to "*sole herb extract*" it is to be understood that the Gla-rich extract may be combined with other herb extracts but such that do not have a detectable effect on the treated condition or any one of the parameters being indicative of severity of the condition being treated with the Gla-rich extract. In other words, if and when another herb extract is used with the Gla-rich extract it is considers as an additive and not an active ingredient.

In some embodiments, the Gla-rich extract is the sole herb extract, namely, the composition consists, as active, herb-derived, ingredient, essentially only or only the Gla-rich extract.

The above uses of the Gla-rich extract are provided as examples only and should not construed as limiting the scope of the present disclosure.

As used herein, the forms "***a***", "***an***" and "***the***" include singular as well as plural references unless the context clearly dictates otherwise.

Further, as used herein, the term "***comprising***" is intended to mean that the Gla-rich extract but not excluding other elements that do not originate from the plant. The term "***consisting essentially of***" is used to define the Gla-rich extract comprising, respectively, the extract, and excludes other agents that may have an essential significance on treatment of the condition, e.g. neuropathy; "***Consisting of***" shall thus mean excluding more than trace elements of other elements, e.g. GA. Embodiments defined by each of these transition terms are within the scope of this invention.

Further, all numerical values are approximations which are varied (+) or (-) by up to 20%, at times by up to 10% of from the stated values. It is to be understood, even if not always explicitly stated that all numerical designations are preceded by the term "***about***".

The disclosure will now be exemplified in the following description of experiments that were carried out in accordance with the disclosure. It is to be understood that these examples are intended to be in the nature of illustration rather than of limitation. Obviously, many modifications and variations of these examples are possible in light of the above teaching. It is therefore, to be understood that within the scope of the appended claims, the invention may be practiced otherwise, in a myriad of possible ways, than as specifically described hereinbelow.

NON LIMITING EXAMPLES

Example 1 – Extract preparation and characterization

Materials

- Glabridin and Glycyrrhizic acid were purchased from Sigma Aldrich.
- Ground Licorice root was obtained from "Derech Hatavlinim (also known as "Havat Hatavlinim" from Bethlehem of the Galilee's Specialties, Israel).
- Ethanol was purchased from Bio-lab Yoram. Unless otherwise states, EtOH is ethanol 96%.
- Methyl tert-butyl ether (MtBE) was purchased from Bio-lab

Methods

Licorice root was grinded in a coffee grinder, to powder and 1 gram of the powder was subjected to EtOH or MTBE extraction according to the following procedures (in triplicates):

- ***EtOH***- 10ml 100% EtOH, 75 minutes shaking,
- ***EtOH+S*** - 10ml 100% ethanol, 60 minutes in sonication bath and 15 minutes shaking,
- ***EtOH 96%*** -4 ml 96% ethanol, shaking for 4h
- ***MtBE*** – 10ml 100% MtBE, 75 minutes shaking
- ***MtBE+S*** – 10ml 100% MtBE, 60 minutes in sonication bath and 15 minutes shaking.

At the end of the extraction samples were centrifuged 4000g, 10 min, 25°C and supernatant (7 ml) was collected, dried in a chemical hood (evaporating the solvent) and weighted.

The dried pellet (from either method of extraction) was then dissolved in 100% ethanol (in correlation to the type of EtOH used in the extraction) to a concentration of 20mg/ml for further analysis.

Specifically, a sample of 5µl from each dissolved extract was subjected to HPLC-DAD (An Agilent 1200 HPLC equipped with a Phenyl-Hexyl column) using Mobile phase: A- 1% Acetic Acid in water, B- 1% Acetic Acid in acetonitrile; Gradient conditions: 0%-90% B in 50 min and 90%-100% B in 10 min with the Temperature being kept constant at 25°C. The samples were tested at 230nm and 252nm and as reference standard Glycyrrhizic acid and Glabridin standards from Sigma were used.

Further, a sample of 0.5µl from each dissolved extract was subjected to antioxidant (ORAC) test, using a commercial kit (BioVision: Total Antioxidant Capacity (TAC) Colorimetric Assay Kit No.#K274). This test determines the anti-oxidative effect of the extract(ability to protect Cu ions from oxidation). As reference, Torolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) a Vitamin E analog was use. Synthetic vitamin E (96%; Sigma) was also used for comparison.

Results:

Extract properties

Figure 1 provides photographic images of transparent containers containing EtOH extract and MtBE extract after extraction with 100% MtBE or Ethanol allowing the biomass to sediment, showing that MtBE extract is transparent while the EtOH is cloudy and darker, probably due to the presence of humic acids within the material.

The dry weight of each extract as compared to the weight of extract obtained with 100% EtOH (representing the baseline) and its ratio from the original root weight is presented in **Table 1** below and in **Figure 2**.

Table 1: Dry weight of licorice root extract (MtBE or EtOH) out of the total original root weight.

	Avg. yield (dry mg/g root)	**SD**	**Compared to EtOH**
MTBE	70	1.5	33%
MTBE+Sonication	80	12	53%

96% EtOH	110	1.5	100%
96% EtOH+Sonication	250	10	126%

The results in **Table 2** and **Figure 3** show that MtBE resulted in 12% more total phenols compared to EtOH. Extraction with EtOH and sonication resulted in a highest total Phenols (including a wide range of different chemicals).

Table 2: Total phenols (TP) extracted from licorice root (MtBE or EtOH)

Extraction method	**Avg.**	**SD**	**Compared to EtOH**
MTBE	102.8	7.87	112%
MTBE + Sonication	88.4	8.55	97%
Ethanol (EtOH)	91.5	7.02	100%
EtOH + Sonication	178.2	21.70	195%

Further, the relative amount of Glabridin (detected spectrophotometrically at 230nm) and Glycyrrhizic acid (GA) (detected spectrophotometrically at 252nm) were determined, as shown in **Table 3** and **Figure 4**.

Table 3 – relative amounts of Glabridin (Gla) and Glycyrrhizic acid (GA)

All numbers mg/g root	**Gla (230nm)**		**GA (252nm)**		**% Gla relative to EtOH**	**% GA relative to EtOH**	**% Glabridin out of total extract**
	Avg	**SD**	**Avg**	**SD**			
MtBE	2.0	0.1	1.0	0.4	105%	55%	**8.0%**
MtBE+Sonication	2.0	0.1	1.0	0.1	105%	55%	**5.0%**
EtOH	1.9	0.5	1.8	0.6	100%	100%	2.5%
EtOH+Sonication	1.8	0.1	1.1	0.1	95%	61%	1.9%

The results show that MtBE extraction provides 5% higher amount of Gla and 45% less GA. The percentage of Gla out of the total weight of the final dry extract was the highest when using MtBE (8%w/w without sonication, 5% with sonication, that verses only 2.5% of Ethanol and 1.9% of Ethanol with sonication). MtBE is thus over 3 times more effective in extracting Gla and over 2 times more effective in reducing GA presence in the extract.

The results also show that there is no advantage in applying sonication during the extraction.

HPLC Analysis

The MtBE extract and EtOH extract samples were also subjected to HPLC analysis as shown in **Figure 5A**, and the peaks observed were compared to Gla standard peak and GA standard peak as shown in **Figure 5B.**

Antioxidants (ORAC)

To determine anti-oxidative activity, the different dried extracts were dissolved in 100% ethanol, at a concentration of 20 mg solid/ml. Total *Antioxidant Capacity* was determined based on the ability to reduce Cu^{+} ions, using Trolox® as standard reference. The different samples were diluted and from each sample a volume equivalents to 10 µg extract was taken. Results are presented in **Figure 6** (each data point is an average of 3 extraction replicates).

Specifically, the results show that the highest anti-oxidative activity was exhibited with the extract from MtBE, this being 2.3 fold higher than the activity observed with the extract from EtOH (defined as 96%). The MtBE extract activity was about 70% of equivalent weight of vitamin E and EtOH activity was about 30% and the activity of MtBE extract was 5.3 fold higher than that of an equivalent dose of commercial vitamin E (the Reference, i.e. 100mg/day vs. 15mg/day, See Vitamin E, fact sheet at the National Institute of Health, NIH)) and the activity of EtOH was only 2.2 fold higher than the reference.

Further observed is that sonication reduced the anti-oxidative activity.

Example 2 - Anti-oxidative effect of MtBE extract on Omega 3 fish oils oxidation

Materials

Omega 3 fish oil capsules 600 mg/capsule from Omega Galil (Active Ingredient: 600 mg\capsule consisting of: 400 mg EPA : 200 mg DHA) was used and maintained prior to analysis according to manufacturer's instructions (storage at 4˚C in the dark).

Methods

Samples of Omega 3 fish oil with or without MtBE extract were placed in glass beakers at an extract to oil ratio of 10mg MtBE extract/ml oil. Each beaker remained uncovered and thus exposed to air, for a period of 24 hours during which the samples were shaken. The assay was conducted in triplicates.

Peroxide value (PV) was determined according to the ferric thiocyanate protocol, based on Fe^{+2} oxidation into Fe^{+3} by the peroxide. [Ueda, S., Hayashi, T., Namiki, M. (1986). Effect of ascorbic acid on lipid autoxidation in a model food system. Agric. Biol. Chem., 50 (1), 1-7.]

Results

Oxidation levels of Omega 3 fish oil at starting point (t0), after 24 exposure to air (oxygen) are presented in **Table 4** and **Figure 7**.

Table 4: micro-equivalent peroxide O_2/kg levels

Sample	µEq peroxide O_2/kg			
	Average	SD	Increase	% Increase
Omega 3 before exposure ("*O_3 at t0*")	0.09	0.005		
Omega 3 after 24 h exposure ("*O_3 after O.N.*")	0.73	0.037	0.64	714.4%
Omega 3 + MtBE extract after 24 h exposure ("*O_3+MtBE after O.N.*")	0.121	00008	0.03	34.4%

As evident both from **Table 4** and **Figure 7**, MtBE extract significantly prevented oxidation of Omega 3 fish oil. Specifically, without the extract, Omega 3 fish oil was oxidized by 714% within 24 hours exposure to air, as compared to the level of peroxides at time point 0, i.e. before exposure to oxygen. When the extract was added, the Omega 3 fish oil was protected from oxidation with only 34% increase in level of peroxide as compared to the level before exposure. In order words, the presence of the MtBE extract reduced level of oxidation by ~ 95%.

Example 3 – Clinical efficacy of MtBE extract

Diabetic peripheral neuropathy (DPN) is a common complication of diabetes mellitus (DM), affecting up to 50% of patients with types 1 and 2 Diabetes mellitus (DM).

The purpose of the following clinical study is to determine effectiveness and safety of MtBE extract in the treatment of painful diabetic neuropathy (PDN) and other symptoms related to DPN as compared to usual care and to evaluate the feasibility of large-scale clinical research.

Method

60-80 participants with a ≥ six month history of PDN and/or sensory loss and/or diabetic wounds and a mean weekly pain score of ≥ 4 on the 11-point Pain Intensity Numerical Rating Scale (PI-NRS) will be assigned to the MtBE group or placebo (≥20 placebo and ≥40 MTBE).

The participants assigned to the MtBE group will receive 200-400 mg MtBE extract in capsule form once (or twice 100-200mg each) daily for 16 weeks. Inclusion criteria patients who score≥ 7 in the Michigan Neuropathy Screening Instrument and/or sensory loss and/or diabetic wounds. The follow-up will be twice weekly for 16 weeks after random allocation. The Short-Form McGill Pain Questionnaire (SF-MPQ) score assessed at the 16-24 weeks will be the primary outcome measurement used in this study. Additionally, EMG/NCV tests will be conducted at the beginning and end of the study. For patients who have observable DPN wounds and photos will be taken on a bi-weekly basis to record the progress . Safety will be assessed at every visit to include physical exam, EKG, and laboratory test (e.g. CMP, CBC, UA).

In addition, every 1-2 month, the following tests will be done:

- Lipid Panel, LDL oxidation
- Hg A1C
- C-reactive protein (CRP)
- Homocysteine
- Vitals exams (heart, lungs, Abd, extremities).

Eye Exam will be conducted at the beginning and the end of the clinical trial to ascertain effect of MtBE extract consumption on diabetic retinopathy.

Discussion

The MtBE is expected to reduce PDN symptoms, reverse numbness and/or improve sensation, heal diabetic wounds in legs, feet or hands and possibly slow down progress of retinopathy. It is also expected to show improvements in diabetes profile and lipid profile, reduced inflammation, as well as reduce LDL oxidation levels. MtBE extract to cause no significant side effects.

CLAIMS:

1. A method of extracting Glabridin (Gla) from *Glycyrrhiza* roots, the method comprises:

(a) providing licorice particulates,

(b) maintaining the licorice particulates within an extractant comprising a volatile C_2-C_6 alkyl ether for a time sufficient to allow extraction of Gla from said licorice particulates into its surrounding medium and to thereby form an extract comprising a liquid medium with dissolved Gla.

2. The method of claim 1, comprising separating between particulate matter and the liquid medium and removing at least a portion of the extractant from the liquid medium to obtain an extract comprising Gla.

3. The method of claim 1 or 2, wherein said C_2-C_6 alkyl ether is Methyl tert-butyl ether (MtBE).

4. The method of claim 2 or 3, wherein said separating comprise removing of particulate matter from the medium and evaporation of the ether solvent.

5. The method of any one of claims 2 to 4, wherein said separating comprises drying the extract comprising Gla.

6. A Glabridin-rich extract of *Glycyrrhiza* roots comprising Glabridin (Gla) in an amount that provides a Gla to Glycyrrhizic acid (GA) weight/weight ratio in the extract, if GA is present in the Gla-rich extract, of at least 2:1.

7. The Gla-rich extract of claim 6, having a chromatography profile that is substantially similar to Figure 5A.

8. The Gla-rich extract of claim 6 or 7, which when subjected to high-performance liquid chromatography is substantially absent a peak corresponding to sugars.

9. The Gla-rich extract of any one of claims 6 to 8, for use as an anti-oxidant.

10. The Gla-rich extract of claim 9, for use in protecting oxidation of oil.

11. The Gla-rich extract of any one of claims 6 to 9, for use in the treatment of conditions associated with oxidative damage.

12. The Gla-rich extract of any one of claims 6 to 9, for use in inhibiting LDL oxidation.

13. The Gla-rich extract of any one of claims 6 to 8, for use in reducing or controlling blood pressure.

14. The Gla-rich extract of any one of claims 6 to 8, for use in reducing or controlling blood glucose concentration.

15. The Gla-rich extract of any one of claims 6 to 8, for use in reducing or controlling blood triglyceride concentration.

16. The Gla-rich extract of any one of claims 6 to 8, for use in treating neuropathy.

17. The Gla-rich extract of claim 16, for use in treating diabetic neuropathy.

18. A composition comprising Gla-rich extract as defined in any one of claims 6 to 8, and a physiologically acceptable carrier.

19. A method of treatment comprising administering to a subject in need of treatment an amount of a Gla-rich extract as defined in any one of claims 6 to 8.

20. The method of claim 19, for treating a condition associated with oxidative damage.

21. The method of claim 20, comprising administering to the subject in need of said treatment an amount sufficient to ameliorate said condition.

22. A composition comprising a oxidation sensitive oil and Gla-rich extract as defined in any one of claims 6 to 9, wherein level of peroxides in said composition is statistically significantly lower than the level of peroxide in said oxidation sensitive oil in the absence of said Gla-rich extract.

23. The composition of claim 22, wherein said oil is Omega 3.

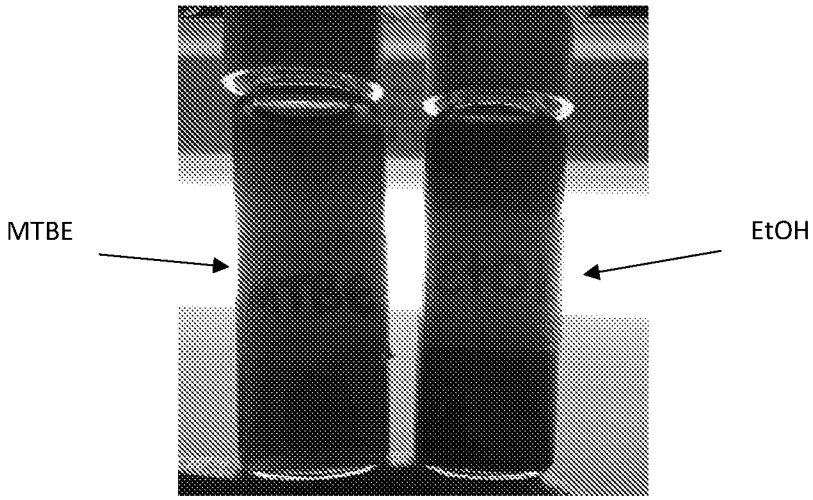


Figure 1

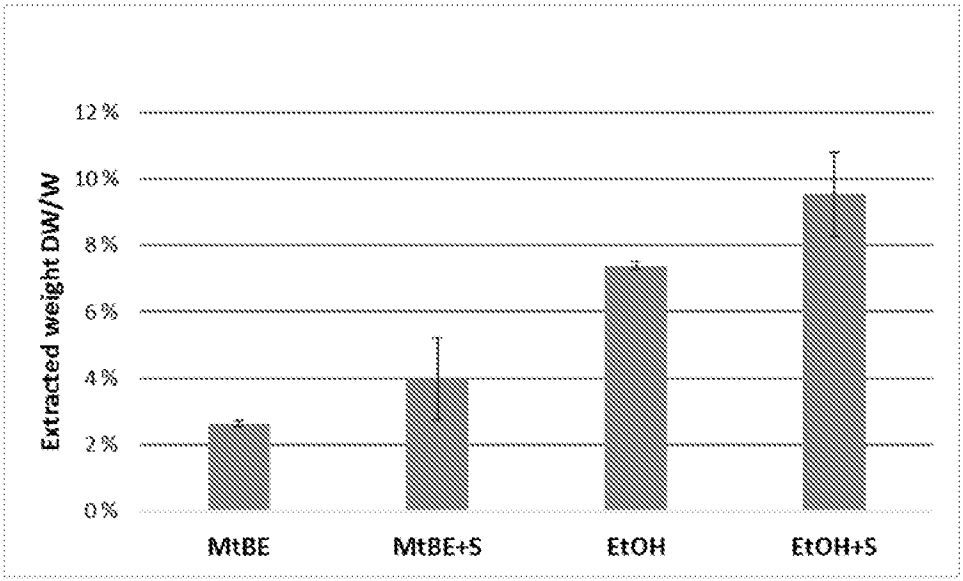


Figure 2

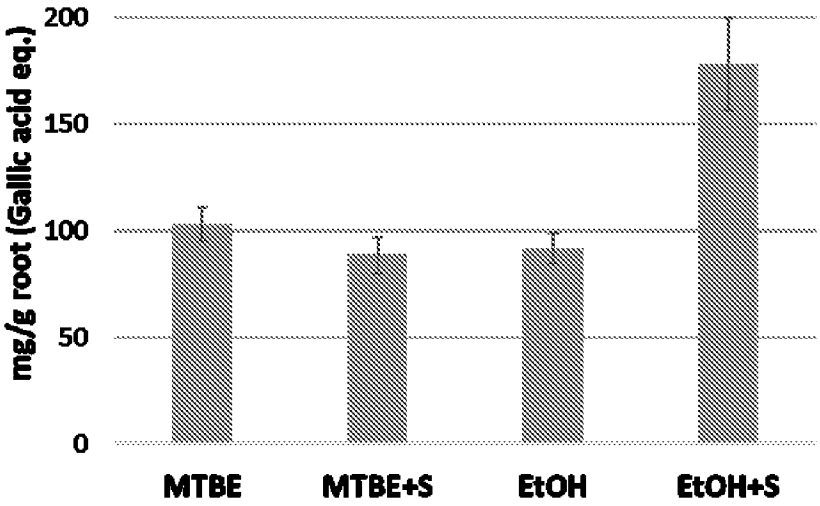


Figure 3

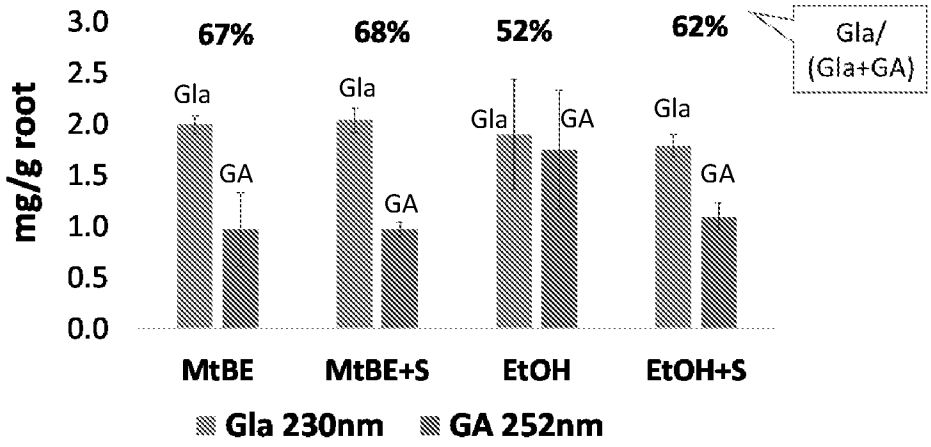
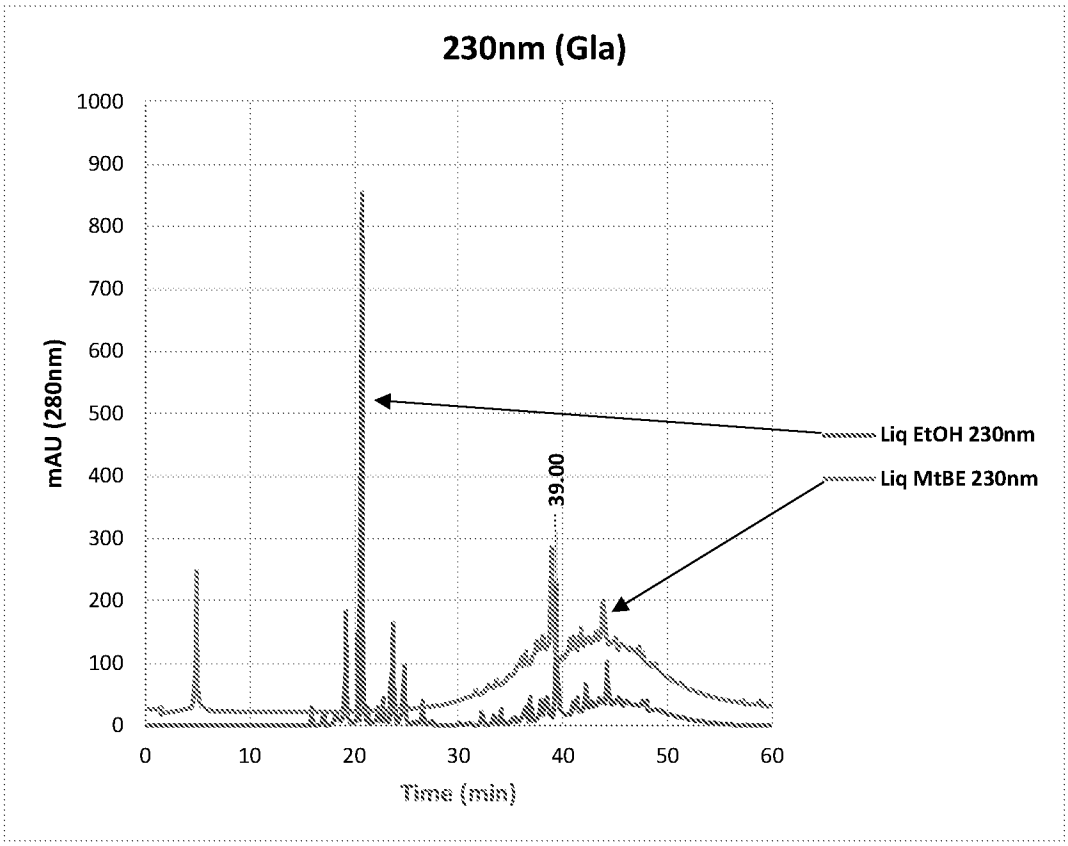


Figure 4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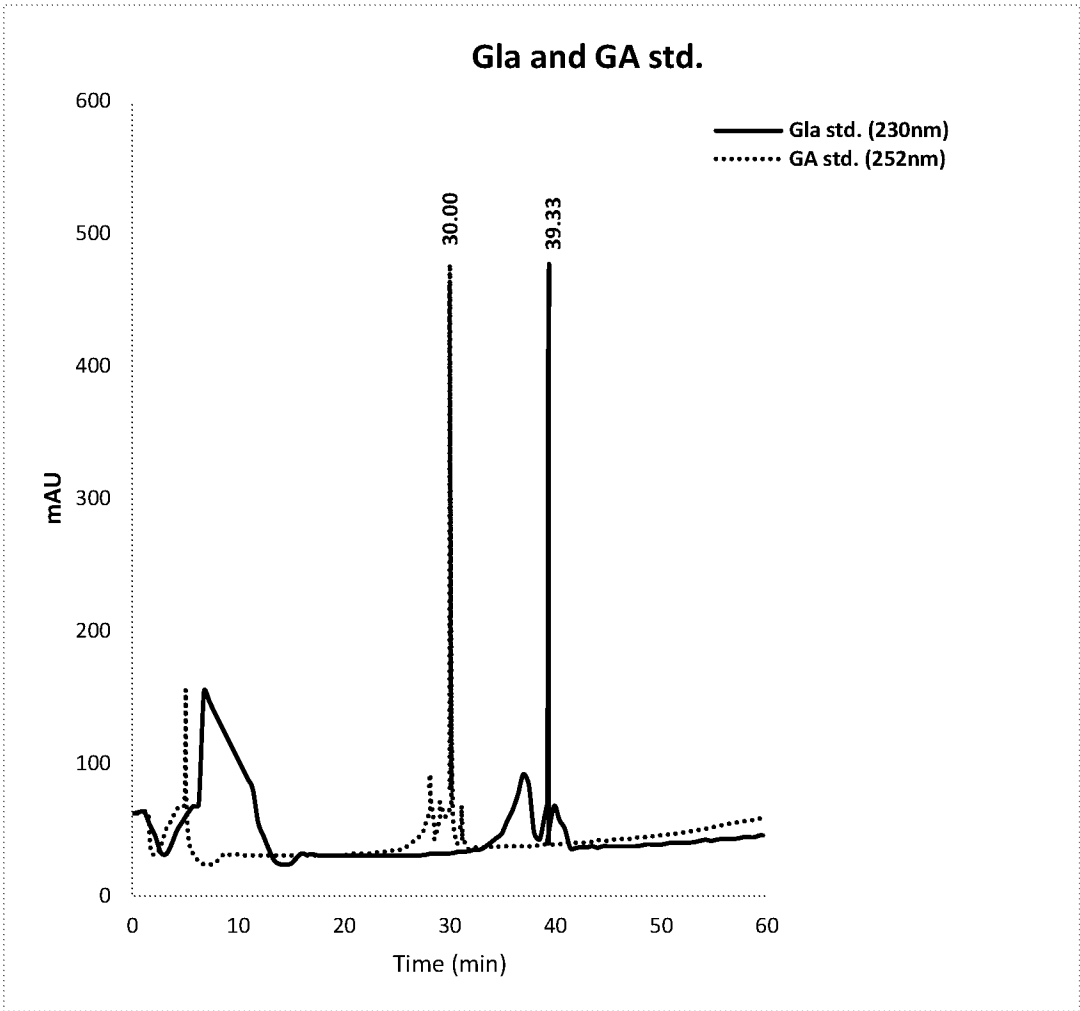
mAU (280nm)
1000
900
800
700
600
500
400
300
200
100
0
0
10
20
30
40
50
60
Time (min)
39.00
Liq EtOH 230nm
Liq MtBE 230nm

Figure 5A


Gla and GA std.
Gla std. (230nm)
GA std. (252nm)
30.00
39.33
mAU
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500
400
300
200
100
0
0
10
20
30
40
50
60
Time (min)

Figure 5B

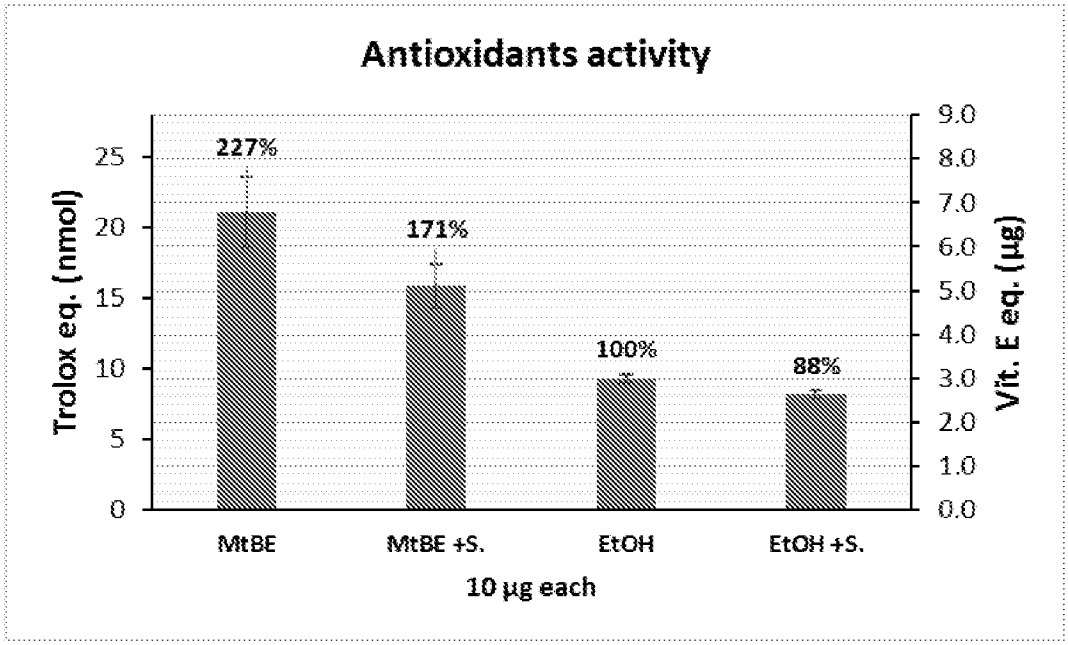

Figure 6

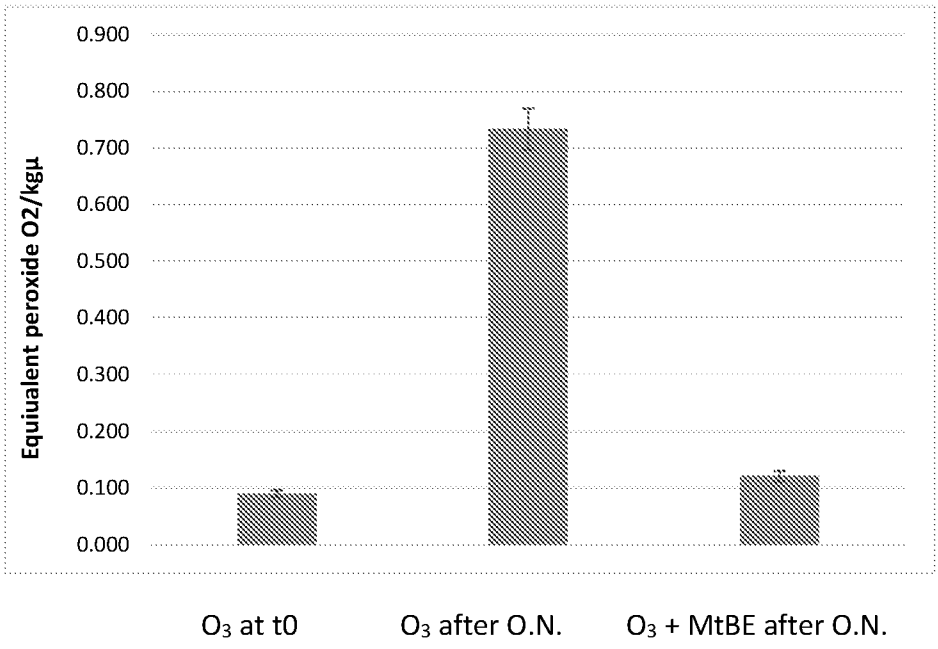

Figure 7

INTERNATIONAL SEARCH REPORT

International application No
PCT/IL2019/050592

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K36/484 A61K31/353 A61P3/06 A61P9/12 A61P3/10
ADD.

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

B. FIELDS SEARCHED

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

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ELVIRA E. SHULTS ET AL: "Phenolic compounds from Glycyrrhiza pallidiflora Maxim. and their cytotoxic activity", NATURAL PRODUCT RESEARCH, vol. 31, no. 4, 16 February 2017 (2017-02-16), pages 445-452, XP055384624, GB ISSN: 1478-6419, DOI: 10.1080/14786419.2016.1188094 cited in the application page 446, last paragraph - page 448, paragraph f ----- -/--	1-5

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents :

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

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

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 July 2019	02/08/2019

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

1

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SIMMLER CHARLOTTE ET AL: "Phytochemistry and biological properties of glabridin", FITOTERAPIA, IDB HOLDING, MILAN, IT, vol. 90, 10 July 2013 (2013-07-10), pages 160-184, XP028739855, ISSN: 0367-326X, DOI: 10.1016/J.FITOTE.2013.07.003 the whole document -----	1-23
X	N.N.: "Scientific Opinion on the safety of 'Glavonoid ', an extract derived from the roots or rootstock of Glycyrrhiza glabra L., as a Novel Food ingredient", THE EFSA JOURNAL, vol. 9, no. 7, 1 July 2011 (2011-07-01), XP055608000, Parma, IT ISSN: 1831-4732, DOI: 10.2903/j.efsa.2011.2287 pages 2-3; tables 1-4 -----	6-23
T	N.N.: "Glabridin (Licorice Root Extract)", , 23 July 2019 (2019-07-23), XP055607997, Retrieved from the Internet: URL:https://www.in-cosmetics.com/__novadocuments/66627?v [retrieved on 2019-07-23] the whole document -----	