

EXTRACT FROM OIL PALM LEAVES COMPRISING PHENOLIC ACIDS

Technical Field of the Invention

This invention relates to extracts from oil palm leaves that have therapeutic activities for the reduction or prevention of oxidative stress in human and lower animals. In particular, this invention relates to a fraction of the extracts that are enriched with (-)-catechin gallate, ferulic acid, and phenolic acids such as gallic acid and protocatechuic acid that possess the said therapeutic activities.

Background of the Invention

The oil palms (*Elaeis*) comprise two species of the Arecaceae, or palm family. The African oil palm *Elaeis guineensis* is native to West Africa, while the American oil palm *Elaeis oleifera* is native to tropical Central America and South America. *Elaeis guineensis* is also widely cultivated in Malaysia and Indonesia for its oil producing fruits.

Mature oil palm trees are single-stemmed, and grow to 20 meter tall. The leaves or fronds are pinnate and can grow up to 3 – 5 meter long. A young tree produces about 30 leaves a year. The flowers are produced in clusters; each individual flower has three sepals and three petals.

The fruit is reddish and grows in large bunches which can weight between 10 to 40 kilogram each. The fruit comprises an oily and fleshy outer layer called pericarp, and with a single seed called palm kernel. Oil is extracted from both the pericarp and the palm kernel.

Oil palms are grown mainly for their fruits which are used mainly for the production of edible oil. Palm oil extracted from oil palm fruits also contains carotenes, tocopherols and tocotrienols. Some studies have been done on the beneficial effects of extracts derived from oil palm leaves.

Abeywardena M, et al (Asia Pac. J. Clin. Nutr., 11: S467 – S 472) discloses a polyphenol-enriched extract derived from leaves of oil palm (*Elaeis guineensis*). The said extract can be used to promote vascular relaxation via endothelium-dependent mechanisms. The presence of (-)-catechin gallate, ferulic acid, gallic acid, and protocatechuic acid were not studied or disclosed. The use of this extract as anti-oxidative stress agent was not studied or disclosed.

Suhaila Mohamed, et al (WHAT Medicine III Proceedings, 2007, PP 145 – 148) discloses the anti-hypercholesterolemic and anti-hypertensive effects of polyphenol rich oil palm leaf (*Elaeis guineensis*) extracts. However, the presence of (-)-catechin gallate, ferulic acid, gallic acid, and protocatechuic acid were not studied or disclosed. The use of this extract as anti-oxidative stress agent was not studied or disclosed.

Nagendran Balasundram, et al (Asia Pac. J. Clin. Nutr. 2005; 4 (4):319-324) discloses a phenolic-rich fraction isolated from oil palm fruits. This extract exhibits bioactive properties, in particular antioxidant effects. However, the chemical composition of the extract derived from oil palm leaves was not studied or disclosed. The use of the extract derived from oil palm leaves as anti-oxidative stress agent was also not studied or disclosed.

Tan Y. A., et al (The Royal Society of Chemistry 2001; 548-551) discloses the presence of phenolic compounds in palm oil that is derived from oil palm mesocarp and kernel, which is from the oil palm fruit. Such phenolic compounds include gallic, chlorogenic, protocatechuic, gentisic, coumaric, ferulic and caffeic acids, as well as catechins, hesperidine, narirutin, and 4-hydroxybenzoate. However, the chemical composition of the extract derived from oil palm leaves was not studied or disclosed. The use of the extract derived from oil palm leaves as anti-oxidative stress agent was also not studied or disclosed.

Run-Cang Sun, et al (J. Agric. Food Chem., 49 (11), 5122-5129, 2001) discloses a new method of quantitative determination of hydroxycinnamic acids in oil palm leaf fiber.

However, the presence of (-)-catechin gallate was not studied or disclosed. The use of this extract as anti-oxidative stress agent was not studied or disclosed.

Yew-Ai Tan, et al (European Journal of Lipid Sciences and Technology, Volume 109, Issue 4, pages 380-393, 2007) discloses the presence of phytochemicals such as sterols, vitamin E, carotenoids, phospholipids, squalene, and phenolics from the by-products of palm oil milling and refining, that is from the fruits of oil palm. However, the chemical composition of the extract derived from oil palm leaves was not studied or disclosed. The use of the extract derived from oil palm leaves as anti-oxidative stress agent was also not studied or disclosed.

Kinnoudo Celestin (WO2007129136) discloses extracts of *Elaeis guineensis* (oil palm) leaves that possess antimalarial properties. The chemical content of these extracts is not characterized. The presence of (-)-catechin gallate, ferulic acid, gallic acid, and protocatechuic acid in the extract derived from oil palm leaves was not studied or disclosed. The use of these extracts as anti-oxidative stress agent was also not studied or disclosed.

Oxidative stress

Over the past few decades, free and bound reactive radicals, highly reactive and thereby destructive molecules, have come to be appreciated increasingly for their importance to human health and disease. Many common and life-threatening human diseases, including atherosclerosis, cancer, and aging, have radical-based pathological reactions as an underlying mechanism of injury.

A radical, or a free radical, is generally understood as a molecule with one or more unpaired electrons in its outer orbital shell. Many molecular species with bound radicals are monoxides or other oxygen containing compounds, generally referred to as reactive oxygen species (ROS). These highly unstable molecules tend to react rapidly with adjacent molecules, donating, abstracting, or even sharing their outer orbital electron(s). This reaction not only changes the adjacent, target molecule, sometimes in profound and beneficial ways, but it can also damage it, or alternatively the unpaired

electron can be passed along to the target, i.e., as in a free radical, generating a second unwanted ROS, which can then go on to react positively or detrimentally with a new target. In fact, much of the high reactivity of ROS is due to their generation of such molecular chain reactions, effectively amplifying their effects many fold.

5 All living aerobic organisms use oxygen for the production of energy. However, there are many indications that the advantages of using oxygen are associated with the risk that the oxidative processes may also cause injury to the aerobic organisms. Various active oxygen metabolites participate in such oxidative processes and at the same time attack the various classes of biological materials. Almost all classes of chemical
10 compounds are subject to oxidative impairment by such oxygen compounds. For instance, the nucleic acids, proteins and free amino acids, lipids and hydrocarbon compounds are influenced by the oxygen toxicity. The human organism has a most refined, widespread and therefore obviously very important system to balance its oxidative and antioxidative processes (see H. Sies, Oxidative Stress, pages. 2-4, 1985).
15 However the naturally present antioxidant pool is not capable of counteracting an increase in generation of reactive oxygen species (ROS); in these cases, so-called "oxidative stress" occurs.

The shifting of this balance towards oxidative processes may have two reasons:

- i. overburden of the antioxidative system because of the production of an active
20 oxygen metabolite, and/or
- ii. insufficiency of the antioxidative system.

The results have been summarized by the expression "oxidative stress". It is known today that such a shifting of the balance may be caused by many different diseases, in particular the inflammatory diseases such as hepatitis, diseases of central nervous
25 system such as epilepsy or Parkinson's disease, diseases of the pulmonary organs such as asthma, psoriasis, side-effects of anticancer agents, chemicals such as paraquat and side-effects of radiation as well as diseases of the coronary circulation such as cardiac infarction.

While not wishing to be bound by any theory, oxidative stress has been implicated as a factor in various diseases and injury states in man and animals; while this does not mean that oxidative stress is the cause of these diseases, it does testify, as confirmed by a number of studies, that oxidative stress can have a negative influence on the progress of said diseases, causing further damage to the cells of an organism that is already sick.

A compelling body of scientific evidence now indicates that many dysfunctions and diseases in humans and animals are associated with oxidative stress.

These include,

Aging: Normal aging processes at a higher than normal rate.

Heart and Cardiovascular Disease: Atherosclerosis, Adriamycin cardiotoxicity.

Kidney: Autoimmune nephritic syndromes, Heavy metal nephrotoxicity.

Solar radiation: Skin wrinkling and pigmentation.

Eye: Cataractogenesis, Degenerative retinal damage, Macular degeneration.

Lung: Lung cancer (cigarette smoke), Emphysema, Oxidant pollutants (O_3 , NO_2),

Bronchopulmonary dysphasia, Asbestos carcinogenicity.

Nervous System disorders: Parkinson's disease, Neuronal ceroid lipofuscinoses, Alzheimer's disease, Muscular dystrophy, Multiple sclerosis.

Iron Overload: Idiopathic hemochromatosis, Dietary overload, Thalassemia.

Inflammatory-Immune Injury: Glomerulonephritis, Autoimmune disease, Rheumatoid arthritis.

Liver: Liver injuries induced by alcohol, halogenated hydrocarbons, and paracetamol.

Antioxidants afford protection because they can scavenge ROS and free radicals before they cause damage to the various biological molecules, or prevent oxidative damage from spreading, i.e., by interrupting the radical chain reaction of lipid peroxidation.

The reactivity of radicals in the body, and the burden on the body that results in eventual pathological conditions, is known as oxidative stress.

The effectiveness of an antioxidant (radical scavenger) thought to have the ability to eliminate the oxidative stress on the above diseases has studied from old times, and an

anti-inflammatory agent exhibiting a radical eliminating ability has been developed. Therefore, it can be said obvious that a substance having an antioxidative function is useful in decreasing oxidative stress.

5 Vitamins such as vitamin C and vitamin E, both of which are found in foods and available as supplements, help the body reduce effects of oxidative stress. A more powerful combatant against the free radicals and ROS, however, is the body's own self defense system of naturally produced chemicals called antioxidants. These antioxidants act to terminate the propagation of free and bound radicals on ROS either by giving an electron to the free radical or ROS or by hindering their formation.

10 The body's antioxidant defense system includes three important natural antioxidants: superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX). Studies conducted have indicated that these antioxidants can work synergistically as the reactions they catalyze are metabolically sequential, beginning first with SOD followed by the actions of CAT and GPX.

15 Despite the presence of SOD, CAT, and GPX, the body's antioxidant defense system is constantly subject to oxidative stress, and its ability to produce SOD, CAT, and GPX is compromised by the aging process and can further be impaired by inflammation, microbial or viral infections, the progression of cancer and neurological disorders, and other pathological conditions that produce, are caused by, or are exacerbated by
20 oxidative stress.

Recently there has been a growing interest in the search for natural antioxidants for three principal reasons (Dastmalchi, Dorman, Koşar, & Hiltunen, 2007):

- 25 (i) numerous clinical and epidemiological studies have demonstrated that consumption of fruits and vegetables is associated with reduced risks of developing chronic diseases such as cancer, cardiovascular disorders and diabetes;
- (ii) safety consideration regarding the potential harmful effects of the chronic consumption of synthetic antioxidants in foods and beverages; and

- (iii) the public's perception that natural and dietary antioxidants are safer than synthetic analogues. The result has been an increased interest in medicinal plants as sources of natural antioxidants.

5 The chemical compounds derived from extract of oil palm leaves that are responsible for the anti-oxidant and anti-oxidative stress activities have not been characterized.

The present invention seeks to provide a novel extract of oil palm leaves that is useful in reducing oxidative stress in mammals and poultry. The said extract which is enriched with (-)-catechin gallate, ferulic acid, and phenolic acids such as gallic acid and protocatechuic acid has anti-oxidative stress activity, and the combined effect of
10 (-)-catechin gallate, ferulic acid, and phenolic acids such as gallic acid and protocatechuic acid in the extracts of oil palm leaves have not been reported before.

To summarize, polyphenol enriched fractions of extracts derived from oil palm leaves have been disclosed and the said fractions can be used as anti-hypercholesterolemic and anti-hypertensive agents, but fraction derived from oil palm leaves containing (-)-
15 catechin gallate, ferulic acid, and phenolic acids such as gallic acid and protocatechuic acid was not studied or disclosed. The use of the said extract as anti-oxidative stress agent was not studied or disclosed.

Summary of the Invention

Accordingly, it is the primary objective of the present invention to provide a
20 composition from oil palm leaves that has therapeutic activities in mammals and poultry.

It is another object of the present invention to provide a composition comprising (-)-catechin gallate, ferulic acid, and phenolic acids such as gallic acid and protocatechuic acid.

25 It is yet another object of the present invention to provide a method for preparing an extract from oil palm leaves.

It is yet another object of the present invention to provide a pharmaceutical composition comprising the novel extract and at least one pharmaceutically acceptable carrier.

5 It is yet another object of the present invention to provide a pharmaceutical composition comprising the novel extract, stabilizers, carriers, excipients, extenders, and other suitable substances.

It is a further object of the present invention to provide a composition comprising the novel extract, in admixture and in association with pharmaceutical carriers, for use in reducing and preventing oxidative stress in mammals and poultry.

10 These and other objects of the present invention are accomplished by providing, a composition comprising an extract from oil palm leaves characterized in that; the said extract comprising (-)-catechin gallate, ferulic acid, and phenolic acids such as gallic acid and protocatechuic acid, and a method for producing an extract of oil palm leaves, containing (-)-catechin gallate, ferulic acid, and phenolic acids such as gallic acid and
15 protocatechuic acid using the steps of:

- a) extracting the herbal liquid from dried or fresh oil palm leaves, by mixing with water, ethanol, methanol, acetone, ethyl acetate, chloroform, isopropyl alcohol or mixture of these said solvents, or any other polar solvents, and heating at a temperature ranging from 25⁰C to 95⁰C for 0.5 to 96 hours;
- 20 b) filtering the herbal liquid extract obtained in (a);
- c) contacting the filtered herbal liquid extract obtained in (b) with an adsorptive chromatographic medium which adsorbs the fraction selectively, in which (-)-catechin gallate, ferulic acid, and phenolic acids such as gallic acid and protocatechuic acid are present; the adsorption medium may be column
25 adsorption medium or any other adsorption medium;
- d) eluting the said fraction obtained in (c) from the adsorptive chromatographic medium with water, ethanol, methanol, acetone, ethyl acetate, chloroform, isopropyl alcohol, or mixture of the said solvents, or any other polar solvents;

- e) drying the enriched herbal liquid extract obtained in (d) using a spray dryer, vacuum oven, conventional oven, microwave oven or freeze dryer.

It is yet another object of the present invention to use the said composition for reducing or preventing oxidative stress in mammals and poultry comprising administering to a subject in need of such treatment an effective amount of the composition.

Brief Description of the Figures

Figure 1 shows the HPLC identification of (-)-catechin gallate in extract of oil palm leaves.

Figure 2 shows the TLC identification of ferulic acid in extract of oil palm leaves.

Figure 3 shows the HPLC identification of gallic acid in extract of oil palm leaves.

Figure 4 shows the HPLC quantification of (-)-catechin gallate in extract of oil palm leaves.

Figure 5 shows the HPLC quantification of ferulic acid in extract of oil palm leaves.

Figure 6 shows the UV quantification of total phenolic acids in extract of oil palm leaves.

Detailed Description of the Invention

The invention relates to compositions comprising extracts from the oil palm leaves characterized by the said extracts comprising (-)-catechin gallate, ferulic acid, and phenolic acids such as gallic acid and protocatechuic acid.

The said oil palm leaves of this invention include leaves of plants selected from the group of plant genus *Elaeis* consisting of *Elaeis guineensis* or *Elaeis oleifera*.

The novel composition of the present invention comprises from about 0.1% to about 95% by weight of (-)-catechin gallate, from about 0.1% to about 95% by weight of ferulic acid, and from about 0.1% to about 95% by weight of total phenolic acids such as gallic acid and protocatechuic acid.

In a preferred embodiment of the present invention, the composition comprises from about 0.5% to about 10% by weight of (-)-catechin gallate, from about 1% to about 5% by weight of ferulic acid, and from about 5% to about 30% by weight of total phenolic acids such as gallic acid and protocatechuic acid.

- 5 In the most preferred embodiment of the present invention, the composition comprises about 0.5% by weight of (-)-catechin gallate, about 1% by weight of ferulic acid, and about 20% by weight of total phenolic acids such as gallic acid and protocatechuic acid.

10 The present invention further relates to a method for producing such an extract of oil palm leaves, comprising steps of:

- a) extracting the herbal liquid from dried or fresh oil palm leaves, by mixing with water, ethanol, methanol, acetone, ethyl acetate, chloroform, isopropyl alcohol, or mixture of these said solvents, or any other polar solvents, and heating at a temperature ranging from 25⁰C to 95⁰C for 0.5 to 96 hours;
- 15 b) filtering the herbal liquid extract obtained in (a);
- c) contacting the filtered herbal liquid extract obtained in (b) with an adsorptive chromatographic medium which selectively adsorbs the fraction in which (-)-catechin gallate, ferulic acid, and phenolic acids such as gallic acid and protocatechuic acid are present, such adsorption medium may be column
20 adsorption medium, or any other adsorption medium;
- d) eluting the said fraction obtained in (c) from the adsorptive chromatographic medium with water, ethanol, methanol, acetone, ethyl acetate, chloroform, isopropyl alcohol, or mixture of these said solvents, or any other polar solvents, at a temperature between about 10⁰C to about 80⁰C and at a pressure between
25 about 0.1 bar to about 10 bar to obtain an extract enriched in (-)-catechin gallate, ferulic acid, and phenolic acids such as gallic acid and protocatechuic acid; and
- e) drying the enriched herbal liquid extract obtained in (d) using a spray dryer, vacuum oven, conventional oven, freeze dry, or any other drying equipment.

In a preferred embodiment of the invention, 1 part of oil palm leaves (dry basis) is extracted with from about 5 to about 50 parts, preferably from about 10 parts to about 30 parts of solvent using an extraction apparatus where the solvent is mixed with the oil palm leaves for a period of time, preferably from about 1 hour to 96 hours, more preferably for 8 hours to 24 hours. The temperature of the solvent is maintained preferably from about 25°C to about 95°C, more preferably from about 50°C to 60°C.

In a further embodiment of the present invention, the preferred solvents for the extraction include water, ethanol, isopropyl alcohol, or mixture of these said solvents, and more preferably water, ethanol, or mixture of water and ethanol, and most preferably a mixture of 30% by weight of water and 70% by weight of ethanol.

In a preferred embodiment of the present invention, the adsorptive chromatographic medium includes styrene-divinylbenzene copolymer resin, phenol-formaldehyde resin, acrylic resin, methacrylate resin, and polyamide resin. Examples of such a resin are: Amberlite XAD-1, Amberlite XAD-2, Amberlite XAD-4, Amberlite XAD-7, Amberlite XAD-8, Amberlite XAD-11, Amberlite XAD-12, Amberlite XAD-1180, Amberlite 7HP, Amberlite 761, and Duolite S861 products of Rohm & Haas Co., Philadelphia, U.S.A.; Diaion HP-10, Diaion HP-20, Diaion HP-21, Diaion HP-30, Diaion HP-40, and Diaion HP-50, products of Mitsubishi Chemical Industries Ltd., Tokyo, Japan; and AB-8 Crosslinked polystyrene and H103 Crosslinked polystyrene resins, products of Tianjin Nankai Hecheng Science and Technology Co., Ltd, Tianjin, China.

In a more preferred embodiment of the present invention, the resins are styrene-divinylbenzene copolymer resins manufactured under trade names such as: AB-8 Crosslinked polystyrene (product of Tianjin Nankai Hecheng Science and Technology Co., Ltd, Tianjin, China), Amberlite XAD 1180, Amberlite 7HP, and Amberlite 761 (products of Rohm & Haas Co., Philadelphia, U.S.A).

In the most preferred embodiment of the present invention, the resin is styrene-divinylbenzene copolymer resins manufactured under trade name AB-8 Crosslinked

polystyrene (product of Tianjin Nankai Hecheng Science and Technology Co., Ltd, Tianjin, China).

Eluting solvents for eluting the enriched fraction from the adsorptive chromatographic medium may be water, ethanol, methanol, acetone, ethyl acetate, chloroform, isopropyl alcohol, or mixture of the said solvents, or any other polar solvents known to persons skilled in the art. The preferred solvent is mixture of water and ethanol, and most preferably is 30% aqueous ethanol.

In a preferred embodiment of the present invention, drying method includes the use of a spray dryer, vacuum oven, or conventional oven, and most preferably the use of a spray dryer.

Equipment for producing an extract of oil palm leaves of the present invention;

A system for producing an extract of *Elaeis guineensis* and *Elaeis oleifera* of the present invention comprises: herbal extraction tank, adsorptive chromatographic column, and dryer.

In accordance with one aspect of the invention, the herbal extraction tank is constructed with steam jacket and a speed agitator that stirs the tank's content. Steam is passed through the steam jacket to heat the content of the tank.

Oil palm leaves are mixed with water, ethanol, methanol, acetone, ethyl acetate, chloroform, isopropyl alcohol, or mixture of the said solvents, or any other polar solvents known to persons skilled in the art and heated in an herbal extraction tank at a temperature in the preferable range of 25⁰C to 95⁰C for 1 to 96 hours. The heating process produces an herbal extract liquid, which is then filtered to collect the filtrate.

In accordance with one aspect of the invention, the adsorptive chromatographic column is filled with adsorptive chromatographic resin, preferably comprises of polystyrene resin. The filtered herbal extract liquid is passed through the adsorptive chromatographic column whereby the fraction enriched in (-)-catechin gallate, ferulic acid, and phenolic acids such as gallic acid and protocatechuic acid is adsorbed in the

surface of the said resin. The said enriched fraction contained (-)-catechin gallate, ferulic acid, and phenolic acids such as gallic acid and protocatechuic acid is then eluted by eluting the adsorptive chromatographic column with water, methanol, ethanol, acetone, ethyl acetate, chloroform, isopropyl alcohol, or mixture of the said solvents, or any other polar solvents. The resultant enriched fraction of herbal liquid is finally dried by using spray dryer, vacuum oven, conventional oven, microwave oven, freeze dryer or other dryers which are apparent to those skilled in the art.

Qualitative tests to identify the presence of (-)-catechin gallate, ferulic acid, and gallic acid in an extract of oil palm leaves of the present invention.

A method of identifying the presence of (-)-catechin gallate in an extract of oil palm leaves by using HPLC:

- a) A reference standard solution of (-)-catechin gallate is prepared by dissolving 1 mg of (-)-catechin gallate reference standard in 1 ml of 0.1% phosphoric acid;
- b) A test solution is prepared by dissolving 200 mg of extract of the present invention in 10 ml of ethanol;
- c) These two solutions are injected separately into a HPLC system with the following conditions:

Equipment: Perkin Elmer series 200 LC

Column: Sinochrom ODS-BP column (250 mm x 4.6 mm ID, 5 μ m particle size)

Mobile phase: A mixture of (i) 2% (v/v) acetic acid and (ii) acetonitrile

The solvent mixture is started at 92% of solvent (i) and 8% of solvent (ii) and is increased to 31% of solvent (ii) in 50 minutes with linear gradient.

Flow rate: 1.0 ml / minute

Detection: UV 280 nm

The chromatograms obtained from reference standard solution and test solution showed a major peak at retention time about 28 minutes corresponding to (-)-catechin gallate. The presence of the peak confirmed the presence of (-)-catechin gallate in the composition of the present invention.

A method of identifying the presence of ferulic acid in an extract of oil palm leaves by using TLC Identification:

- a) The sample is prepared by dissolving the extract of the present invention in HPLC grade methanol, centrifuging at 4000 rpm for 15 minutes and collecting the
5 supernatant.
- b) The sample is first spotted on a pre-coated silica gel 60 TLC plate (E-Merck), along with known amount of ferulic acid reference standard, using a solvent phase consisting of a mixture of methanol / water (ratio 7:1).
- c) The plate is air dried and visualized under Ultra Violet (UV) lamp at wavelength
10 254 nm.

Light purple principal zones corresponding to ferulic acid were observed in both reference standard and sample solutions. The presence of the light purple principal zones confirmed the presence of ferulic acid in the composition of the present invention.

15 A method of identifying the presence of gallic acid in an extract of oil palm leaves by using HPLC:

- a) A reference standard solution of gallic acid is prepared by dissolving 10 mg of gallic acid reference standard in 10 ml of methanol;
- b) A test solution is prepared by dissolving 200 mg of extract of the present invention
20 in 10 ml of methanol;
- c) These two solutions are injected separately into a HPLC system with the following conditions:

Equipment: Perkin Elmer series 200 LC

Column: Hypersil BDS C₁₈ column (250 mm x 4.6 mm ID, 5 µm particle size)

25 Mobile phase: A mixture of methanol / water / orthophosphoric acid (20:79.9:0.1)

Flow rate: 1.0 ml / minute

Detection: UV 270 nm

The chromatograms obtained from reference standard solution and test solution showed a major peak at retention time about 6.6 minutes corresponding to gallic acid. The presence of the peak confirmed the presence of gallic acid in the composition of the present invention.

5 **Quantitative determination of (-)-catechin gallate and ferulic acid in an extract of oil palm leaves of the present invention by HPLC assay.**

A method of quantitative determination of the total amount of (-)-catechin gallate in an extract of oil palm leaves by using HPLC;

- 10 a) A reference standard solution of (-)-catechin gallate is prepared by dissolving 1 mg of (-)-catechin gallate reference standard in 1 ml of 0.1% phosphoric acid;
- b) A test solution is prepared by dissolving 200 mg of extract of the present invention in 10 ml of ethanol;
- c) These two solutions are injected separately into a HPLC system with the following conditions:

15 Equipment: Perkin Elmer series 200 LC

Column: Sinochrom ODS-BP column (250 mm x 4.6 mm ID, 5 μ m particle size)

Mobile phase: A mixture of (i) 2% (v/v) acetic acid and (ii) acetonitrile

The solvent mixture is started at 92% of solvent (i) and 8% of solvent (ii) in 50 minutes with linear gradient.

20 Flow rate: 1.0 ml / minute

Detection: UV 280 nm

Injection volume: 20 μ L

A peak area of (-)-catechin gallate was obtained at retention time about 28 minutes from the HPLC data.

25 The amount of (-)-catechin gallate in the test solution is obtained by comparing with the reference standard solution of (-)-catechin gallate.

Calculate the content of (-)-catechin gallate in the test solution in percentage (%) by using the following equation:

$$1000 C_{R_t} / W_{R_s}$$

where:

C = concentration of (-)-catechin gallate for test solution calculated from working standard curve (mg/ml)

W = weight (mg) of sample taken to prepare the test solution (mg)

5 r_t = peak area of (-)-catechin gallate obtained from the test solution

r_s = peak area of (-)-catechin gallate obtained from the reference standard solution

A method of quantitative determination of the total amount of ferulic acid in an extract of oil palm leaves by using HPLC;

10 a) Reference standard solutions of ferulic acid with four known concentrations of ferulic acid reference standard 0.15 mg/ml, 0.20 mg/ml, 0.25 mg/ml, and 0.5 mg/ml are prepared by dissolving ferulic acid reference standard in 70% ethanol;

b) A test solution is prepared by dissolving 10 mg of an extract of oil palm leaves of the present invention in 10 ml of 70% ethanol;

15 c) HPLC assays were carried out on these solutions by injecting separately into a HPLC system with the following conditions:

Equipment: Perkin Elmer series 200 LC

Column: Hypersil BDS C₁₈ column (250 mm x 4.6 mm ID, 5µm particle size)

Mobile phase: A mixture of methanol and 0.1% phosphoric acid aqueous solution (30:70)

20 Flow rate: 1.0 ml/minute

Detection: UV 325 nm

Injection volume: 20µL

A peak area of ferulic acid was obtained at retention time about 28.8 minutes from the HPLC data.

25 The amount of ferulic acid in the test solution is obtained by comparing with the reference standard solution of ferulic acid.

Calculate the content of ferulic acid in the test solution in percentage (%) by using the following equation:

$$1000 C_{r_t} / W_{r_s}$$

where:

C = concentration of ferulic acid for test solution calculated from working standard curve (mg/ml)

W = weight (mg) of sample taken to prepare the test solution (mg)

5 r_t = peak area of ferulic acid obtained from the test solution

r_s = peak area of ferulic acid obtained from the reference standard solution

Quantitative determination of total phenolic acids on an extract of oil palm leaves of the present invention by using UV spectrophotometry.

10 A method of quantitative determination of the total amount of phenolic acids in an extract of oil palm leaves of the present invention comprising steps of:

a) A reference standard solution is prepared by dissolving 10.0 mg of protocatechuic acid reference standard in 100 ml of HPLC grade methanol.

10 ml of the solution is diluted with HPLC grade methanol at a ratio of 1:9;

15 b) A test solution is prepared by dissolving 10 mg of the extract of oil palm leaves in 10 ml of 70% ethanol;

c) 0.9% potassium ferricyanide aqueous solution is prepared by dissolving 90 mg of Potassium ferricyanide in 10 ml of purified water;

d) 0.9% ferric chloride aqueous solution is prepared by dissolving 90 mg of ferric chloride in 10 ml of purified water;

20 e) Chromogenic reagent is prepared by mixing 9 ml of 0.9% potassium ferricyanide aqueous solution with 10 ml of 0.9% ferric chloride aqueous solution.

25 Leave all solutions (standard, test and blank solutions) in dark room for 5 minutes. Make up all solutions to volume with 0.1M HCL aqueous solution, mix well and leave in dark room for another 20 minutes. Measure absorbances of standard and test solutions at 679 nm against blank solution. Plot a standard calibration curve using concentration of standard solution as axis-x and absorbance as axis-y. Calculate the concentration of total phenolic acids from the standard calibration curve.

Calculate the content of total phenolic acids in the test solution in percentage (%) by using the following equation:

50,000 C / W

where:

C= concentration of total phenolic acids for test solution calculated from working standard curve (mg/ml)

5 W = weight (mg) of sample taken to prepare the test solution (mg)

The present invention encompasses a therapeutic composition comprising the extract prepared according to the present invention, wherein the composition is in the forms of tea, tablet, coated tablet, lozenge, chewable tablet, capsule, soft capsule, granule, coated granule, powder, coated powder, solution, syrup, emulsions, and suspension,
10 useful in humans or lower animals for the reduction or prevention of oxidative stress.

Furthermore, the present invention encompasses a pharmaceutical composition comprising an effective amount of the said extract, for example 1 mg to 800 mg, more preferably 300 mg to 500 mg, and a pharmaceutically acceptable carrier.

The present invention provides a pharmaceutical composition comprising the novel
15 extract formulated for oral, buccal, rectal or transdermal administration or in a form suitable for administration by inhalation or insufflation (either through the mouth or the nose).

For oral administration, the pharmaceutical compositions may take the forms of, for example, tea, tablet, coated tablet, lozenge, chewable tablet, capsule, softgel capsules,
20 granule, coated granule, powder, solution, syrup, emulsion or suspension prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g. pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g. lactose, microcrystalline cellulose, starch or tricalcium phosphate); lubricants (e.g. magnesium stearate, talc or silica); disintegrants (e.g. cross-linked polyvinylpyrrolidone, cross carmellose sodium, or sodium starch glycolate); or
25 wetting agents (e.g. sodium lauryl sulphate). The tablets may be coated by methods well known in the art.

Liquid preparations for oral administration may take the form of, for example, solutions, syrups, emulsions, or suspensions or they may be presented as a dry product for constitution with water or other suitable vehicles before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g. sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g. lecithin or acacia); non-aqueous vehicles (e.g. almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (e.g. methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavouring, colouring and sweetening agents as appropriate.

Preparations for oral administration may be suitably formulated to give controlled or extended release of the extract of the present invention.

For buccal administration the compositions may take the form of tablets or lozenges formulated in a conventional manner.

The pharmaceutical compositions according to the present invention may be formulated for administrations by nasal insufflation and oral inhalation. Examples of types of preparation for the said administrations include sprays and aerosols for use in an inhaler or insufflator. Transdermal preparation for external application may be formed with the aid of any suitable cream, ointment or lotion base known to those persons skilled in the art.

The pharmaceutical compositions according to the present invention may also be formulated in rectal compositions such as suppositories or retention enemas, e.g. containing conventional suppository bases such as cocoa butter or other fats and oils.

In a preferred embodiment of the present invention, the pharmaceutical compositions include capsule, soft capsule, or tablet comprising from about 1% to about 95% of the said extract, and from about 1% to about 95% of a pharmaceutical acceptable carrier.

The term 'pharmaceutically acceptable carrier' as used herein means one or more compatible solid or liquid filled diluents, or any pharmaceutical excipients known to persons skilled in the art.

Some examples of substances which can serve as pharmaceutically acceptable carriers are sugars such as lactose, glucose and sucrose, starch such as corn starch and potato starch, cellulose derivatives such as sodium carboxymethylcellulose, cellulose acetate, and microcrystalline cellulose.

A proposed dose of the composition according to the present invention for administration to a human (approximately 60 kg body weight) is 0.1 mg to 1 g, such as 1 mg to 500 mg dose of the active ingredient per unit dose, expressed as the weight of dry extract. The unit dose may be administered, for example, 1 to 4 times per day. The dose will depend on the route of administration. It will be appreciated that it may be necessary to make routine variations to the dosage depending on the age and weight of the patient as well as the severity of the condition to be treated. The dosage will also depend on the route of administration. The precise dose and route of administration will ultimately be at the discretion of the attendant physician or veterinarian.

The extract prepared according to the present invention demonstrates anti-oxidant effect and free radical scavenging effect in *in vitro* test models, and anti-oxidative stress effect in *in vivo* test model.

In accordance with another embodiment of the invention, the extract is used for reducing or preventing oxidative stress in mammals and poultry by administering to a subject in need of such treatment an effective amount of the composition.

Examples of compositions made according to the invention are set forth below.

Toxicity studies performed on the composition of the present invention has proven that the extract is non-toxic at an oral dose of 30 g of extract of oil palm leaves / kg of body weight in Sprague-Dawley rats.

According, oral dosage in humans of 0.03 g of extract of oil palm leaves per kg body weight per day is considered appropriate and safe.

EXAMPLE I

Manufacture of Extract of Oil Palm Leaves

5 About 50 g of dried oil palm leaves were extracted with about 500 mL of a mixture of water/ethanol (ratio 3:7 by volume), at about 60-65°C for approximately 60 minutes. The resulting slurry was filtered through two layers of muslin cloth and yielded about 502 g of crude extract liquid.

10 The resulting crude extract liquid was then subjected to column adsorptive chromatography.

Amberlite XAD 16HP (Manufactured by Rohm & Haas) was packed in a column (5.0 cm ID x 40 cm height) to give a column volume of about 780 mL. The column was washed with about 4-5 column volumes of deionized water. The crude extract liquid from the foregoing extraction step was pumped into the column. The column was first
15 eluted with 1500 ml of deionized water to remove salts, sugars, and other unwanted hydrophilic substances.

The column was then eluted with 1500 ml of a mixture of water/ isopropanol (ratio 3:7 by volume) with a constant flow rate of 25 ml / minute at a temperature of 60°C and a pressure of 0.5 to 1 bar to yield a purified eluant liquid enriched in (-)- catechin gallate, ferulic acid, and phenolic acids such as gallic acid and protocatechuic acid.
20

The resulting eluant liquid was further concentrated in a rotary evaporator to 50 ml and the concentrated liquid was dried in a vacuum oven at 60°C for 8 hours.

2.52 gm of yellow-beige powder with the following content was obtained:

(-)-Catechin gallate content – 1.1% (By HPLC assays)

25 Ferulic acid content – 1.5% (By HPLC assays)

Total phenolic acids content – 10.2% (By UV spectrophotometry method)

EXAMPLE 2

Manufacture of Extract of Oil Palm Leaves

100 kg of fresh oil palm leaves were cut into small pieces of about 2 cm in length and transferred to an extraction tank. 1000 litre of a mixture of water/isopropyl solution (ratio 3:7 by volume) were added and heated to 60°C for 8 hours.

The crude extract liquid was separated from the leaves by filtering through a cartridge filter with pore size of 10 µm. The filtered liquid was concentrated under a reduced pressure of 0.08-0.09 MPa at 70°C to form approximately 200 litre of concentrated fluid extract (Density = 1.10-1.20 g / cm.³).

The resulting concentrated fluid extract was subjected to adsorptive column chromatography by using a stainless steel column of 15 cm diameter and 150 cm length. The column was packed with polyamide resin with particle size ranging from 30-60 mesh.

The column was then washed with 200 litres of deionized water followed by elution with 200 litres of a mixture of water/isopropyl solution (ratio 3:7 by volume) with a constant flow rate of about 1.5 litre / minute at a temperature of about 20-30°C and at a pressure of about 0.5 to 1 bar to yield a purified eluant liquid enriched in (-)-catechin gallate, ferulic acid, and phenolic acids such as gallic acid and protocatechuic acid.

The resulting eluant liquid was then spray dried in a spray dryer. The inlet temperature in the spray dryer was set at about 170°C to about 180°C and the outlet temperature was set at about 100°C to about 105°C.

12.5 kg of yellow-beige powder with the following content was obtained:

(-)-Catechin gallate content – 1.15% (By HPLC assays)

Ferulic acid content – 1.6% (By HPLC assays)

Total phenolic acids content – 10.5% (By UV spectrophotometry method)

Characterization of the composition for the present invention by qualitative and quantitative analysis

Qualitative analyses to confirm presence of (-)-catechin gallate, ferulic acid and gallic acid in the composition for the present invention were carried out by Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC) methods as follows:

EXAMPLE 3

HPLC Identification of (-)-Catechin gallate in Extract of Oil Palm Leaves

1 mg of (-)-catechin gallate reference standard was dissolved in 1 ml of 0.1% phosphoric acid to form the reference standard solution.

In parallel, a test solution of the extract of the present invention obtained in Example 1 was prepared by dissolving 200 mg of extract to be analyzed in 10 ml of ethanol.

These two solutions were injected separately into a HPLC system with the following conditions:

Equipment: Perkin Elmer series 200 LC

Column: Sinochrom ODS-BP column (250 mm x 4.6 mm ID, 5 μ m particle size)

Mobile phase: A mixture of (i) 2% (v/v) acetic acid and (ii) acetonitrile

The solvent mixture is started at 92% of solvent (i) and 8% of solvent (ii) and is increased to 31% of solvent (ii) in 50 minutes with linear gradient.

Flow rate: 1.0 ml / minute

Detection: UV 280 nm

The chromatograms obtained from reference standard solution and test solution showed a major peak at retention time about 28 minutes corresponding to (-)-catechin gallate. The presence of the peak confirmed the presence of (-)-catechin gallate in the composition of the present invention (as shown in Figure 1).

EXAMPLE 4

TLC Identification of Ferulic acid in Extract of Oil Palm Leaves

The extract powder of oil palm leaves obtained in Example 1 was dissolved in HPLC grade methanol, centrifuged at 4000 rpm for 15 minutes and the supernatant was collected. The sample was first spotted on a pre-coated silica gel 60 TLC plate (E-Merck), along with known amount of ferulic acid reference standard, using a solvent phase consisting a mixture of methanol / water (ratio 7:1).

The plate was air dried and visualized under Ultra Violet (UV) lamp at wavelength 254 nm. Light purple principal zones corresponding to ferulic acid were observed in both reference standard solution and test solution (as shown in Figure 2).

EXAMPLE 5

HPLC Identification of Gallic acid in Extract of Oil Palm Leaves

10 mg of gallic acid reference standard was dissolved in 10 ml of methanol to form the reference standard solution.

In parallel, a test solution of the extract of the present invention obtained in Example 1 was prepared by dissolving 200 mg of extract to be analyzed in 10 ml of methanol.

These two solutions were injected separately into a HPLC system with the following conditions:

Equipment: Perkin Elmer series 200 LC

Column: Hypersil BDS C₁₈ column (250 mm x 4.6 mm ID, 5 µm particle size)

Mobile phase: A mixture of methanol / water / orthophosphoric acid (20:79.9:0.1)

Flow rate: 1.0 ml / minute

Detection: UV 270 nm

The chromatograms obtained from reference standard solution and test solution showed a major peak at retention time about 6.6 minutes corresponding to gallic acid. The presence of the peak confirmed the presence of gallic acid in the composition of the present invention (as shown in Figure 3).

Quantitative analysis of (-)-catechin gallate and ferulic acid present in the composition of the present invention were carried out by HPLC method as follows:

EXAMPLE 6

5 HPLC Quantification of (-)-Catechin gallate in Extract of Oil Palm Leaves

HPLC method was used to determine the amount of (-)-catechin gallate in the extract of oil palm leaves obtained in Example 1.

1 mg of (-)-catechin gallate reference standard was dissolved in 1 ml of 0.1% phosphoric acid to form the reference standard solution.

10 In parallel, a test solution of the extract of the present invention obtained in Example 1 was prepared by dissolving 200 mg of extract to be analyzed in 10 ml of ethanol.

HPLC assays were carried out on these solutions by injecting separately into a HPLC system with the following conditions:

Equipment: Perkin Elmer series 200 LC

15 Column: Sinochrom ODS-BP column (250 mm x 4.6 mm ID, 5 μ m particle size)

Mobile phase: A mixture of (i) 2% (v/v) acetic acid and (ii) acetonitrile

The solvent mixture is started at 92% of solvent (i) and 8% of solvent (ii) in 50 minutes with linear gradient.

Flow rate: 1.0 ml / minute

20 Detection: UV 280 nm

Injection volume: 20 μ L

A peak area of (-)-catechin gallate was obtained at retention time about 28 minutes from the HPLC data (as shown in Figure 4).

25 The amount of (-)-catechin gallate in the test solution was obtained by comparing with the reference standard solution of (-)-catechin gallate.

The content of (-)-catechin gallate in the test solution in percentage (%) was calculated by using the following equation:

$$1000 C_{r_t} / W_{r_s}$$

where

5 C = concentration of (-)-catechin gallate for test solution calculated from working standard curve (mg / ml)

W = weight (mg) of sample taken to prepare the test solution (mg)

r_t = peak area of (-)-catechin gallate obtained from the test solution

r_s = peak area of (-)-catechin gallate obtained from the reference standard solution

10 (-)-Catechin gallate content – 1.1% (By HPLC assays)

The HPLC assays showed that the amount of (-)-catechin gallate present in the composition obtained in Example 1 was 1.1%.

EXAMPLE 7

HPLC Quantification of Ferulic acid in Extract of Oil Palm Leaves

15 HPLC method was used to determine the amount of ferulic acid in the extract of oil palm leaves obtained in Example 1.

Reference standard solutions of ferulic acid with four known concentrations of ferulic acid reference standard 0.15 mg / ml, 0.20 mg / ml, 0.25 mg / ml, and 0.5 mg / ml were prepared by dissolving ferulic acid reference standard in 70% ethanol.

20 In parallel, a test solution of the extract of the present invention obtained in Example 1 was prepared by dissolving 10 mg of extract to be analyzed in 10 ml of 70% ethanol.

HPLC assays were carried out on these solutions by injecting separately into a HPLC system with the following conditions:

Equipment: Perkin Elmer series 200 LC

25 Column: Hypersil BDS C₁₈ column (250 mm x 4.6 mm ID, 5 µm particle size)

Mobile phase: A mixture of methanol and 0.1% phosphoric acid aqueous solution (30:70)

Flow rate: 1.0 ml / minute

Detection: UV 325 nm

Injection volume: 20 μ L

5 A peak area of ferulic acid was obtained at retention time about 28.8 minutes from the HPLC data (as shown in Figure 5).

The amount of ferulic acid in the test solution was obtained by comparing with the reference standard solution of ferulic acid.

The content of ferulic acid in the test solution in percentage (%) was calculated by using the following equation:

10
$$1000 C_{r_t} / W_{r_s}$$

where:

C = concentration of ferulic acid for test solution calculated from working standard curve (mg / ml)

W= weight (mg) of sample taken to prepare the test solution (mg)

15 r_t = peak area of ferulic acid obtained from the test solution

r_s = peak area of ferulic acid obtained from the reference standard solution

Ferulic acid content – 1.5% (By HPLC assays)

The HPLC assays showed that the amount of ferulic acid present in the composition obtained in Example 1 was 1.5%.

20

EXAMPLE 8

UV quantification of Total Phenolic Acids in Extract of Oil Palm Leaves

UV spectrophotometry method was used to determine the amount of total phenolic acids in extract of oil palm leaves obtained in Example 1.

25

10 mg of protocatechuic acid reference standard was dissolved in 100 ml of HPLC grade methanol to form the reference standard solution. 10 ml of the solution was diluted with HPLC grade methanol at a ratio of 1:9.

Different amounts of standard solutions and 1 ml of methanol (as blank solution) were pipette to separate 25 ml volumetric flasks respectively. To each flask, 5.0 ml of absolute ethanol, 2 ml of 3% sodium dodecyl sulfate solution, and 1.0 ml of chromogenic reagent [0.9% potassium ferricyanide solution: 0.9% ferric chloride solution (0.9:1)] were added.

The standard and blank solutions were left in dark room for 5 minutes. Each flask were made up to volume with 0.1M HCl and mixed well. The solutions were left in dark room for 20 minutes.

The absorbance of standard solutions were measured at 697 nm against the blank solution. A standard calibration curve was plotted by using concentration of standard solutions as axis-x and absorbance as axis-y.

The assay was performed according to the UV spectrophotometry method by dissolving the composition with 10 ml of 70% aqueous ethanol; pipetting 0.05 ml of the solution into a flask and the test solution was prepared by using the same method as above.

The absorbance of total phenolic acids in extract of oil palm leaves was obtained at wavelength of 679 nm (as shown in Figure 6).

According to the standard calibration curve, the concentration of total phenolic acids from the standard calibration curve was calculated.

The content of total phenolic acids in the test solution in percentage (%) was calculated by using the following equation:

$$500,000 C / W$$

where:

C= concentration of total phenolic acids for test solution calculated from working standard curve (mg / mL)

W = weight (mg) of sample taken to prepare the test solution (mg)

The UV spectrophotometry method showed that the amount of total phenolic acids present in the composition obtained in Example 1 was 10.2%.

EXAMPLE 9

Determination of antioxidant activity of composition for the present invention by *in vitro* and *in vivo* models.

Antioxidant activity of the composition for the present invention was determined by using *in vitro* and *in vivo* models as follows:

DPPH free radical scavenging activity of the composition of the present invention

DPPH free radical scavenging activity of the composition of the present invention obtained in Example 1 was determined by using 1,1-diphenyl-2-picryl-hydrazil (DPPH) colorimetry with detection at 517 nm. The activity was evaluated by the decrease in the absorbance as a result of DPPH color change from purple to yellow.

10 mg of sample powder obtained in Example 1 above was dissolved in 5 ml of absolute ethanol as sample solution and 0.2 mmol / L of DPPH solution in ethanol was prepared. 1 ml of the DPPH solution was added to 4 ml of sample solution. The sample solution was then left in dark room for 30 minutes. Absorbance of the sample solution was measured at 517 nm. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. Butylated hydroxinasole (BHA) was used as control. The higher the sample concentration used, the stronger was the free radical scavenging activity. The results obtained showed that the composition of the present invention exhibited a stronger antioxidant activity than that of BHA used.

Concentration (mg/ml)	Percentage (%) of DPPH free radical scavenging	
	BHA	Extract of oil palm leaves obtained from Example 1
0.125	58.15	92.52
0.25	67.79	95.78
0.5	79.76	97.18
1	86.84	96.84
2	94.12	94.34

Table 1: Percentage (%) of DPPH free radical scavenging activity of extract of oil palm leaves vs BHA

EXAMPLE 10**Reducing Power of the Composition for the present composition**

Reducing power of the composition of the present invention obtained in Example 1 was determined according to potassium ferricyanide reduction method. 10 mg of sample powder obtained in Example 1 above was dissolved in 10 ml of absolute ethanol as sample solution. 1 ml of the sample solution was dissolved in 1 ml of distilled water and mixed with 2.5 ml of 0.2 mol / L phosphate buffer and 2.5 ml of 1% potassium ferricyanide aqueous solution. The mixture was incubated at 50°C for 20 minutes. 2.5 ml of 10% trichloroacetic acid aqueous solution was then added to each sample solution. The sample solution was centrifuged at 3000 rpm for 10 minutes. 2.5 ml of the supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride aqueous solution. The yellow colour of the solution (potassium ferricyanide solution) changed to light yellow due to the reducing power of the composition. The presence of reducing agents (antioxidants) in the composition caused the reduction of Fe^{3+} (Ferricyanide complex) to ferrous form. Therefore the Fe^{2+} complex can be measured by the formation of Perl's Prussian blue at 700 nm (Absorbance was measured at 700 nm). Increased in absorbance indicated increased in reducing power and Butylated hydroxinasole (BHA) was used as control. The results showed that the composition exhibited reducing power activity as BHA.

Concentration (mg / ml)	Absorbance (measured at 700 nm)	
	BHA	Extract of oil palm leaves obtained from Example 1
1	0.8897	0.2296
0.5	0.7322	0.1603
0.25	0.5777	0.1177
0.0625	0.2769	0.0015
0.125	0.4054	0.0115

Table 2: Reducing power extract of oil palm leaves and BHA

EXAMPLE 11***In vivo* Reduction of Lipid Peroxidation by Extract of Oil Palm Leaves**

Anti-oxidative stress activity of extract of oil palm leaves can be demonstrated by determining the lipid peroxidation inhibitory property of extract of oil palm leaves in Malondialdehyde (MDA) test. One of the most devastating effects of oxidative stress by free radicals in the organism is the oxidation of lipids, resulting in the formation of Malondialdehyde (MDA). The level of lipid peroxidation can be assessed by measuring the level of thiobarbituric acid reactive substances (TBARS) in serum of lipid peroxidation-induced animals. MDA reacts with thiobarbituric acid to form a colored substance which can be measured calorimetrically.

Lipid peroxidation was induced by intraperitoneal injection of carbon tetrachloride

Group	No. of Rats (n=6)	Treatment (Days 1-5)	Administration of CCL ₄ (Day 6)
Control	6	-	-
Positive Control	6	Purified water	Single dose of 15 mg CCL ₄ / kg body weight
Treatment	6	1.1 g of composition obtained in Example 1 / kg body weight	Single dose of 15 mg CCL ₄ / kg body weight

As shown in Table 3 below, the serum MDA levels were higher in lipid peroxidation-induced rats (Positive Control group) compared to rats in the Control group. There was a decrease in the level of serum MDA in the rats treated with the composition obtained in Example 1 (Treatment group), indicating the lipid peroxidation inhibitory effect and anti-oxidative stress activity of oil palm leaf extracts.

Group	No. of Rats (n+6)	Mean Serum MDA Level (nmol / mL)
Control	6	1.913
Positive Control	6	2.851
Treatmen	6	1.947

5

EXAMPLE 12

Safety of Extract of Oil Palm Leaves

Determination of Maximum Tolerated Dose (MTD)

Animal studies have proven the safety of consuming extract of oil palm leaves. Toxicological studies performed on Sprague-Dawley rats administered with the extract of oil palm leaves obtained in Example 1 have shown that the composition is safe (without any occurrence of death) even at an oral dose of 2g of extract of oil palm leaves / kg of body weight in Sprague-Dawley rats.

Maximum tolerated dose was determined as follows:

15 The compositions were administered orally to groups of 3 rats weighing about 150 – 180 g each with doses of 2 g, 5 g, 7 g, 9 g, 11 g, 13 g, 16 g, 19 g, 25 g, 30 g, and 35 g / kg body weight. The compositions were dissolved in purified water before single oral dose was given to each animal. Purified water was orally administered to the same number of rats in the control group.

20 Animals were observed individually 30 minutes after dosing, periodically during the first 24 hours, with special attention given during the first 4 hours and daily thereafter, for a total of 14 days. Observations included gross behavioral changes and general motor activity, writhing, convulsion, response to tail pinching, gnawing, piloerection, pupil size, fecal output, feeding behavior, occurrence of death etc. Weight changes
25 were also recorded. The main observation was occurrence of death.

No dead or toxic effects occurred at 30 g of extract of oil palm leaves / kg body weight, hence the maximum tolerable dose was 30 g / kg body weight.

The normal recommended daily dose for human is about 0.03 g of extract of oil palm leaves / kg body weight.

By comparing the normal recommended daily dose for human with the maximum tolerable dose for rat,

5 Multiple of Maximum Tolerable Dose of rat:

$$= \frac{\text{Maximum Tolerable Dose for rat (g / kg body weight)}}{\text{Daily Dose for human (g / kg body weight)}}$$

$$= \frac{30.0}{0.03}$$

10 = 1,000 times

Conclusion: Maximum Tolerable Dose of extract of oil palm leaves obtained in Example 1 in rat is 30 g / kg body weight, equivalent to 1,000 times the recommended human oral dose. This shows that the acute toxicity of the composition of the present invention is very low and is very safe for human oral consumption.

15

EXAMPLE 13

Method of manufacture of a soft gelatin capsule

The following ingredients were mixed and formed into a homogenous oily suspension.

Soft gelatin capsule formula:

20

Ingredient	Amount / capsule	Function
Oil palm leaf extract powder as obtained in Example 1 (Standardized to contain 1.1% (-)-catechin gallate, 1.5% ferulic acid and, 10.2% total phenolic acids)	200.00 mg	Active ingredient
Beeswax	25.00 mg	Suspending agent
Medium-chain triglycerides	275.00 mg	Vehicle

The resulting mixture in oily suspension form is thereafter filled into a soft gelatin capsule.

EXAMPLE 14**Method of manufacture of a tablet**

A tablet with the following formulation was prepared as described below.

Direct compression method was used to prepare the tablet. All the ingredients were mixed homogenously and the resulting mixture was compressed into a round tablet (700 mg / tablet) in a tablet press.

Tablet formula:

Ingredient	Amount / tablet	Function
Oil palm leaf extract powder as obtained in Example 1 (Standardized to contain 1.1% (-)-catechin gallate, 1.5% ferulic acid, and 10.2% total phenolic acids)	350.00 mg	Active ingredient
Starch	77.00 mg	Diluent
Microcrystalline cellulose	175.00 mg	Diluent
Polyvinylpyrrolidone K-30	21.00 mg	Binding agent
Sodium starch glycolate	70.00 mg	Disintegrating agent
Magnesium stearate	7.00 mg	Lubricant

EXAMPLE 15**Method of manufacture of a hard gelatin capsule**

A composition of the following formulation was prepared in hard capsule by standard method known to those skilled in the art.

- 5 The following ingredients were mixed and formed into a homogenous powder mixture. The resulting mixture was then filled into a size #1 hard gelatin capsule.

Hard gelatin capsule formula:

10	Ingredient	Amount / capsule	Function
15	Oil palm leaf extract powder as obtained in Example 1 (Standardized to contain 1.1% (-)-catechin gallate, 1.5% ferulic acid and, 10.2% total phenolic acids)	200.00 mg	Active ingredient
	Starch	144.75 mg	Diluent
	Silicone dioxide	3.50 mg	Anti-caking agent
	Magnesium stearate	1.75 mg	Lubricant

EXAMPLE 16

Oral suspension

A liquid oral suspension with the following composition was prepared as follows:

Oil palm leaf extract powder of the present invention was then filled into an amber glass bottle.

Liquid oral suspension formula:

Ingredient	Amount / 100 ml	Function
Oil palm leaf extract powder as obtained in Example 1 (Standardized to contain 1.1% (-)-catechin gallate, 1.5% ferulic acid, and 10.2% total phenolic acids)	0.50 g	Active ingredient
Sodium carboxymethylcellulose	2.50 g	Suspending agent
Methyl paraben	0.09 g	Preservative
Propyl paraben	0.10 g	Preservative
Strawberry flavor oil	0.10 g	Flavoring agent
Polysorbate 80	0.10 g	Wetting agent
Purified water q.s.	100 ml	Vehicle

Claims:

1. A composition comprising an extract from oil palm leaves characterized in that;
the said extract comprises (-)-catechin gallate, ferulic acid, and phenolic
acids such as gallic acid and protocatechuic acid.
2. A composition of claim 1, wherein the oil palm leaves are selected from the
plant genera *Elaeis*, consisting of *Elaeis guineensis* and *Elaeis oleifera*.
3. A composition of claim 1 or 2, wherein the (-)-catechin gallate is present
from 0.1% to 95% by weight.
4. A composition of claim 1 or 2, wherein the ferulic acid is present from
0.1% to 95% by weight.
5. A composition of claim 1 or 2, wherein the phenolic acid is gallic acid.
6. A composition of any one of claims 1, 2 or 5, wherein the content of
phenolic acids is present from 0.1% to 95% by weight.
7. A method for producing an extract of oil palm leaves, containing (-)-
catechin gallate, ferulic acid, and phenolic acids such as gallic acid and
protocatechuic acid comprising the steps of:
 - a) extracting the herbal liquid from dried or fresh oil palm leaves, by
mixing with water, ethanol, methanol, acetone, ethyl acetate,
chloroform, isopropyl alcohol or mixture of these said solvents, or
any other polar solvents, and heating at a temperature ranging from
25°C to 95°C for 0.5 to 96 hours;
 - b) filtering the herbal liquid extract obtained in (a);

- 5 c) contacting the filtered herbal liquid extract obtained in (b) with an adsorptive chromatographic medium which adsorbs the fraction selectively, in which (-)-catechin gallate, ferulic acid, and phenolic acids such as gallic acid and protocatechuic acid are present; the adsorption medium may be column adsorption medium or any other adsorption medium;
- 10 d) eluting the said fraction obtained in (c) from the adsorptive chromatographic medium with water, ethanol, methanol, acetone, ethyl acetate, chloroform, isopropyl alcohol, or mixture of the said solvents, or any other polar solvents;
- e) drying the enriched herbal liquid extract obtained in (d) using a spray dryer, vacuum oven, conventional oven, microwave oven or freeze dryer.
- 15 8. An extract of oil palm leaves comprising (-)-catechin gallate, ferulic acid, and phenolic acids such as gallic acid and protocatechuic acid prepared by the method as claimed in claim 7.
9. A method according to claim 7, in which the extract is derived from leaves of *Elaeis guineensis*.
- 20 10. A method according to claim 7, wherein the solvent comprises at least one member selected from the group consisting of water, methanol, ethanol, acetone, ethyl acetate, methyl alcohol, chloroform, and mixtures thereof.
11. A method according to claim 7, wherein the separation medium comprises a polymeric chromatographic column.
- 25 12. A method according to claim 7, wherein the separation medium comprises a synthetic polymeric resin.
13. A method according to claim 7, wherein the polymeric resin is polystyrene.

14. A method according to claim 7, wherein the drying step comprises spray drying.
15. A method according to claim 7, wherein the eluent comprises at least one member selected from the group consisting of water, methanol, ethanol, acetone, ethyl acetate, methyl alcohol, chloroform, and mixtures thereof.
16. A therapeutic composition of any one of claims 1 to 6, wherein the composition is in the form of tea, tablet, coated tablet, lozenge, chewable tablet, capsule, softgel capsule, granule, coated granule, powder, solution, syrup, emulsion, and suspension.
17. A pharmaceutical composition comprising an effective amount of the composition as claimed in any one of claims 1 to 6 and a pharmaceutically acceptable carrier.
18. A pharmaceutical composition as claimed in claimed 17, wherein the pharmaceutically acceptable carrier may be stabilizers, carriers, extenders, and other suitable substances.
19. A pharmaceutical composition of any one of claims 1 to 6 and 16 to 18, wherein the said extract is present from 1 mg - 800 mg.
20. A pharmaceutical composition of any one of claims 1 to 6 and 16 to 18, wherein the said extract is preferably present from 300 mg - 500 mg.
21. Use of a composition of claims 1 to 6 and 16 to 20 for reducing or preventing oxidative stress in mammals and poultry comprising administering to a subject in need of such treatment an effective amount of the composition.

22. Use of a composition of claims 1 to 6 and 16 to ~~20~~, wherein the mammal is a human.
23. Use of a composition of claims 1 to 6 and 16 to 20, wherein the mammal is a cow, buffalo, goat, sheep, or any other mammal.
- 5 24. Use of a composition of claims 1 to 6 and 16 to 20, wherein the poultry is a goose, chicken, duck, turkey, pigeon, or any other poultry.