A PROCESS FOR THE EXTRACTION OF ANTHOCYANINS FROM BLACK RICE AND COMPOSITION THEREOF

A process of extracting a composition comprising anthocyanins from black rice (Oryza sativa L.) comprises separating an outer layer from a starchy endosperm in de-hulled black rice; adding a solution of at least one organic solvent and an acid to the separated outer layer; filtering and removing the solvent and the acid from the separated outer layer to produce a pigment fraction; separating constituents of the pigment fraction; and collecting the anthocyanin composition therefrom. This composition is useful in enhancing and/or preserving the stability of HDL-C and the atherogenic lipoproteins such as LDL-C, VLDL-C, and IDL-C from oxidation, in preventing, reducing, eliminating or ameliorating injuries due to oxidative stress, and in preventing, reducing, eliminating or ameliorating the development of atherosclerotic lesions and inflammation associated therewith.
TITLE: A PROCESS FOR THE EXTRACTION OF ANTHOCYANINS FROM BLACK RICE AND COMPOSITION THEREOF

FIELD OF THE INVENTION
This present invention relates to methods of treating or preventing cardiovascular disease and its underlying conditions, including atherosclerosis, oxidative stress, and dyslipidemic conditions or disorders in animals, particularly humans.

BACKGROUND OF THE INVENTION

While recent advances in science and technology are helping to improve quality and add years to human life, the prevention of atherosclerosis, an underlying cause of cardiovascular disease ("CVD") has not been sufficiently addressed and it remains the leading cause of death in the United states, Europe and part of Asia (1). Atherosclerosis is a degenerative process resulting from an interplay of inherited (genetic) factors and environmental factors such as diet and lifestyle. Research to date suggest that cholesterol may play a role in atherosclerosis by forming atherosclerotic plaques in blood vessels, ultimately cutting off blood supply to the heart muscle or alternatively to the brain or limbs, depending on the location of the plaque in the arterial tree (2,3). Overviews have indicated that a 1% reduction in an individual's total serum cholesterol yields a 2% reduction in risk of a coronary artery event (4). Statistically, a 10% decrease in average serum cholesterol (e.g. from 6.0 mmol/L to 5.3 mmol/L) may result in the prevention of 100,000 deaths in the United States annually (5). Accordingly, hyperlipidemic conditions associated with elevated concentrations of total cholesterol and low density lipoprotein (LDL) cholesterol are significant risk factors.

Studies also show that a low plasma concentration of high density lipoprotein (HDL) cholesterol is a significant risk factor for the development of atherosclerosis (6) and that high levels are protective.

Lipoproteins are complexes of lipids and proteins held together by non-covalent bonds.
Each type of lipoprotein class has a characteristic mass, chemical composition, density and physiological role. Irrespective of density or particle size, circulating lipids consist of a core of cholesteryl esters and triglycerides, and an envelope of phospholipids, free cholesterol and apolipoproteins. The apolipoproteins are involved in the assembly and secretion of the lipoprotein, provide structural integrity, activate lipoprotein-modifying enzymes, and are the ligand for a large assortment of receptors and membrane proteins. Lipoprotein classes found in plasma include HDL, LDL, intermediate density lipoproteins (IDL) and very low density lipoproteins (VLDL).

Each type of lipoprotein has a characteristic apolipoprotein composition or ratio. The most prominent apolipoprotein in HDL is apolipoprotein-AI (apoA-I), which accounts for approximately 70% of the protein mass, with apoA-II accounting for another 20%. The ratio of apoA-I to apoA-II may determine HDL functional and anti-atherogenic properties. Circulating HDL particles consist of a heterogeneous mixture of discoidal and spherical particles with a mass of 200 to 400 kilo-daltons and a diameter of 7 to 10 nm.

HDL is one of the major classes of lipoproteins that function in the transport of lipids in plasma, and has multiple functions within the body, including reverse cholesterol transport, providing the cholesterol molecule substrate for bile acid synthesis, transport of clusterin, transport of paraoxanase, prevention of lipoprotein oxidation and selective uptake of cholesterol by adrenal cells. The major lipids associated with HDL include cholesterol, cholesteryl ester, triglycerides, phospholipids and fatty acids.

To better understand how HDL is anti-atherogenic, a brief explanation of the atherosclerotic process is necessary. The atherosclerotic process begins when LDL becomes trapped within the vascular wall. Oxidation of this LDL results in the binding of monocytes to the endothelial cells lining the vessel wall. These monocytes are activated and migrate into the endothelial space where they are transformed into macrophages,
leading to further oxidation of the LDL. The oxidized LDL is taken up through the scavenger receptor on the macrophage, leading to the formation of foam cells. A fibrous cap is generated through the proliferation and migration of arterial smooth muscle cells, thus creating an atherosclerotic plaque.

HDL is essential for the transport of cholesterol from extra-hepatic tissues to the liver, where it is excreted into bile as free cholesterol or as bile acids that are formed from cholesterol. The process requires several steps. The first is the formation of nascent or pre-beta HDL particles in the liver and intestine. Excess cholesterol moves across cell membranes into the nascent HDL through the action of the ABCA1 transporter. Lecithin cholesterol acyl transferase (LCAT) converts the cholesterol to cholesteryl ester and the subsequent conversion of nascent HDL to mature HDL. Esterified cholesterol is then transferred by cholesteryl ester transfer protein (CETP) from HDL to apolipoprotein-B containing lipoproteins, which are taken up by numerous receptors in the liver. Nascent HDL is regenerated via hepatic triglyceride lipase and phospholipid transfer protein and the cycle continues. In addition to the cholesterol removed from peripheral cells, HDL accepts cholesterol from LDL and erythrocyte membranes. Another mechanism of reverse cholesterol transport may involve passive diffusion of cholesterol between cholesterol-poor membranes and HDL or other acceptor molecules.

HDL protects against the development of atherosclerosis both through its role in reverse cholesterol transport and possibly by impeding LDL oxidation. Several HDL-associated enzymes are involved in the process. Paraoxonase (PON1), LCAT, and platelet activating factor acetylhydrolase (PAFAH) all participate by hydrolyzing phospholipid hydroperoxides generated during LDL oxidation and act in tandem to prevent the accumulation of oxidized lipid in LDL. These enzymes are responsible for the anti-oxidative and anti-inflammatory properties of HDL.

Although hypercholesterolemia is an important risk factor for patients with CVD, other
risk factors such as increased oxidative stress and serum homocysteine must be considered (7). Modification of these harmful components in the arteries would be beneficial in creating new avenues for management of those who have or are at risk of developing CVD but do not have hypercholesterolemia (8).

It is well documented that oxidative stress has an important role in the initiation and propagation of many chronic diseases, including atherosclerosis (9). Reactive oxygen species (ROS) are ubiquitous and occur naturally in all aerobic species, arising from both endogenous production of metabolism and as exogenous sources derived from environmental sources. ROS are largely indiscriminately reactive molecules which readily damage biological macromolecules including DNA, protein, carbohydrates and lipids (10). Excessive or uncontrolled production of ROS has been implicated in the development of atherosclerosis and CVD at different stages, including vascular endothelial cell damage, foam cell formation, vascular smooth muscle proliferation, gene expression, impaired vasomotor reactivity and plaque instability (11,12a).

In the absence of adequate endogenous antioxidant defenses, the propagation of free radicals events can lead to the co-oxidation of nucleophilic cellular constituents as well as the reaction of secondary lipid autooxidation products with nucleophilic macromolecules. Examples of oxidative stress indicators in target tissues and blood include 8-hydroxydeoxyguanosine, malondialdehyde, 4-hydroxynoneanal and lipid peroxides, which include not only fatty acid oxidation products but also cholesterol oxidation products. Management of oxidative stress is achieved by the collective removal and detoxification of ROS from specific enzymatic and nonenzymatic cellular antioxidant systems. For example, enzymatic antioxidants involved in the detoxification of lipid and oxygen radicals include the Cu/Zn superoxide dismutase (SOD; cytoplasm), catalase (peroxisomes) selenium-glutathione peroxidatse/reductase redox cycle enzymes (GSH-Px and GSHG-Red in both the cytoplasm and mitochondria and finally the non-selenium glutathione-S-transferases (cytoplasm). Non enzymatic cellular
antioxidants include α-tocopherol, ascorbic acid and β-carotene.

Evidence from in vitro and in vivo studies suggests that oxidative modification of LDL is involved in the onset of atherosclerosis and exacerbates its clinical manifestations. Specific products of cholesterol oxidation have for example been identified in atherosclerotic plaque (12b). Accordingly, antioxidants especially those with dietary sources which can impact on either enhancing enzymatic antioxidant activity or complement non-enzymatic antioxidant activity have received considerable attention as possible agents that can protect against generation of ROS and thus the development of atherosclerosis and CVD.

The treatment of CVD with rice diets was suggested several decades ago. More than fifty years ago, it was reported that the consumption of white rice decreased blood pressure and lowered hypercholesterolemia in humans (13). However, there are many different types of rice. By far the most common rice consumed by humans is white rice (over 85%), followed by red and black rice. The latter two are cultivated mainly in South Asia, Greece, Italy, and the United States. While Europeans eat more coloured rice than South Asians (14), coloured rice has long been consumed in China and is considered to be a health food which confers body-strength. Thus, it is commonly known as a "blood strengthening rice" or "drug rice".

Black rice, which has a pigment level of 1 mg per 100 g rice, has 3 mg vitamin C and 0.2 mg riboflavin per 100 g and has more iron, calcium and phosphorus than non-pigmented rice. In Kerala, India, the variety Navara is believed to have medicinal properties and is used to rejuvenate the nerves in paralytic conditions: oridine, an alkaloid present in rice, has some antineurotic properties when impure.

Prior research in the field of black rice has focused on the preparation and use of black rice pigment as a colouring agent (Chinese Patent No. 93109627.8), beverages and
foods supplemented with black rice pigment (Chinese Patent No. 93109627.8) and the effects of whole dietary black and red rice on serum lipids and aortic plaque (15)

It is an object of the present invention to obviate or mitigate the disadvantages and insufficiencies of compounds and compositions known in this field to treat and prevent CVD and all of its underlying conditions, including atherosclerosis, oxidative stress, and dyslipidemic conditions. It is a further object of the present invention to manipulate black rice so as to optimize its' various therapeutic effects.

SUMMARY OF THE INVENTION
The present invention provides, in one aspect, a process of extracting a composition comprising anthocyanins from black rice (Oryza sativa L) which comprises:

a) separating an outer layer from a starchy endosperm in de-hulled black rice;
b) adding a solution of at least one organic solvent and an acid to the separated outer layer;
c) filtering and removing the solvent and the acid from the separated outer layer to produce a pigment fraction;
d) separating constituents of the pigment fraction; and
e) collecting the anthocyanin composition therefrom.

The present invention further comprises compositions which comprise cyanidin-3-O-glucoside and peonidin-3-O-glucoside derived from black rice.

The present invention further provides a method of treating or preventing cardiovascular disease and its underlying conditions, including atherosclerosis, inflammation, hyperlipidemic conditions, hypoalphalipoproteinemia, hypercholesterolemia, and oxidative stress in an animal which comprises administering a composition prepared by the extraction process of the present invention.
The present invention further provides a method of treating or preventing cardiovascular disease and its underlying conditions, including atherosclerosis, inflammation, hyperlipidemic conditions, hypoalphalipoproteinemia, hypercholesterolemia, and oxidative stress in an animal which comprises administering a composition comprising cyanidin-3-O-glucoside and peonidin-3-O-glucoside derived from black rice.

One of the key aspects of the process of the present invention is that anthocyanins, the active therapeutic components black rice, have been found to be most advantageously extracted from the separated outer layer of the rice granule. Distribution of anthocyanins is clearly related to the depth of the granule, with the first outer layer containing the highest level of anthocyanin. Prior known processes for the extraction of anthocyanins from black rice have not appreciated this important advantage.

These effects and other significant advantages are described in more detail below.

BRIEF DESCRIPTION OF THE DRAWINGS
The present invention is illustrated by way the following non-limiting drawings in which:

Figure 1 is a diagram showing the components of a rice granule in cross-section;

Figure 2 is a chromatogram of Bio-Gel P-2 elution of black rice extract, with peaks 1 and 2 being subsequently identified by LC/MS as peonidin-3-glucoside and cyanidin-3-glucoside respectively;

Figure 3 is a chromatogram of Bio-Gel P-2 elution of black rice extract;

Figure 4 is a chromatogram of Bio-Gel P-2 elution of black rice extract, with a peak being subsequently identified by LC/MS as peonidin-3-glucoside;
Figure 5 is a chromatogram of Bio-Gel P-2 elution of black rice extract, with a peak being subsequently identified by LC/MS as cyanidin-3-glucoside;

Figure 6 is a graph showing the effect of black rice extract on inhibition of DPPH radical;

Figure 7 is a graph showing the effect of black rice extract on the formation of conjugated diene in peroxyl radical induced liposome peroxidation at 37° C;

Figure 8 shows the agarose gel electrophoresis results of the effect of black rice extract in suppressing the negative charge of oxidative modified LDL wherein lane 1 = native LDL, lane 2 = LDL plus cupric ion, lane 3-7 = LDL plus cupric ion with black rice extract, and lane 8 = LDL plus cupric ion and EDTA;

Figure 9 is a bar graph showing the suppression of LDL oxidative modification of human LDL in vitro (CD and TBARS);

Figure 10 shows the agarose gel electrophoresis results of the effect of black rice extract in preventing peroxyl radical induced super-coiled DNA scission wherein S = super-coiled DNA, lane 1 = DNA plus PBS, lane 2 = DNA plus AAPH, lane 3, 4, 5 and 6 = DNA plus AAPH + 1, 10, 25, 100 μg/ml black rice extract, respectively, lane 7, 8 = DNA plus AAPH + 1, 10 μg/ml Trolox™, respectively;

Figure 11 shows the agarose gel electrophoresis results of the effect of the combination of peonidin-3-glucoside and cyanidin-3-glucoside (10 μg/ml) in reducing the damage of peroxyl radical induced DNA scission wherein lane 1, 2 = native and oxidative DNA respectively, lanes 3, 4, 5, 6 and 7 = DNA plus AAPH with 9/1, 4/1, 1/1, 1/4 and 1/9 of cyanidin-3-glucoside and peonidin-3-glucoside respectively, lane 8 = DNA plus AAPH with 10 μg/ml Trolox™;
Figure 12 shows the agarose gel electrophoresis results of the effect of black rice extract in preventing hydroxyl radical (non-site specific) induced DNA nicking wherein lane 1= DNA plus PBS, lane 2=DNA plus hydroxyl radical initiator, lanes 3 and 4=DNA plus hydroxyl radical initiator + 1.7, 17 mg/ml black rice extract (first layer), respectively, lanes 5,6= DNA plus hydroxyl radical initiator + 1.7, 170mg/ml extract of whole granule of black rice, respectively, lanes 7,8 = DNA plus hydroxyl radical initiator +0.17, 1.7 mg/ml Trolox™ respectively;

Figure 13 shows the agarose gel electrophoresis results of the effect of black rice extract in preventing hydroxyl radical (site specific) induced DNA nicking wherein lane 1= DNA plus PBS, lane 2=DNA plus hydroxyl radical initiator, lanes 3 and 4=DNA plus hydroxyl radical initiator + 1.7, 17 mg/ml black rice extract (first layer), respectively, lanes 5,6= DNA plus hydroxyl radical initiator + 1.7, 170mg/ml extract of whole granule of black rice, respectively, lanes 7,8= DNA plus hydroxyl radical initiator +0.17, 1.7 mg/ml Trolox™ respectively;

Figure 14 is a bar graph showing cell viability test using black rice extract;

Figure 15 is a bar graph showing inhibition of hepatic lipase activity by black rice extracts.

Figure 16 is a graph representing the body weights of mice in different treatment groups over time;

Figure 17 is a graph representing the areas of atherosclerotic plaque in different groups. The extent of atherosclerosis was quantified after 16 wk. Atherosclerotic lesion area in the sinus was measures by staining lesions with oil-red O. Values are means± SD, n=15. Bars without common letters are significantly different, P<0.01;
Figure 18 is a graph showing effect of the black rice fraction of the present invention on the inhibition of nitric oxide stimulated by bacterial lipopolysacharide in mouse macrophage cell RAW264.7; and

Figure 19 shows the agarose gel electrophoresis results of the effect of the black rice extract of the present invention on the expression of inducible nitric oxide synthase.

PREFERRED EMBODIMENTS OF THE INVENTION
The following detailed description is provided to aid those skilled in the art in practising the present invention. However, this detailed description should not be construed so as to unduly limit the scope of the present invention. Modifications and variations to the embodiments discussed herein may be made by those with ordinary skill in the art without departing from the spirit or scope of the present invention.

Extraction Process
According to the present invention, there is provided a process of extracting a composition comprising anthocyanins from black rice (Oryza sativa L) which comprises separating an outer layer from a starchy endosperm in de-hulled black rice; adding a solution of at least one organic solvent and an acid to the separated outer layer; filtering and removing the solvent and the acid from the separated outer layer to produce a pigment fraction; separating constituents of the pigment fraction; and collecting the anthocyanin composition therefrom.

The starting material for the process of the present invention is de-hulled black rice. Dehulling of the rough rice may be carried out by a variety of ways known in the art. Generally, hulling is done either manually (hand pounding) or mechanically. Mechanical hullers are of three main types: Engelberg mills, stone dehullers and rubber dehullers.
Stone dehullers are still common in tropical Asia. Rubber rollers are common in Japan, where de-hulled rice is stored instead of rough rice, with a resultant space saving.

The first step of the process of the present invention is critical and comprises separating the outer layer from the starchy endosperm in de-hulled black rice. For greater understanding, Figure 1 shows the basic components of a rice granule in cross-section. After removal of the husk components, for example cellulose and hemi-cellulose in the de-hulling process, what generally remains are the pericarp, tegmen and aleurone layers which are positioned over the starchy endosperm. Within the scope of the present invention, it is desired to separate this outer "aleurone" layer and from this, extract an anthocyanin composition. Heretofore, the entire rice granule has been used in anthocyanin extraction processes but never just this outer layer.

It is preferred that the separation of the outer layer from the starch endosperm be achieved by physical separation, most preferably by milling. Various types of milling are already used in the manufacture and refinement of coloured rice and may be employed herein. In such prior known manufacture, the outer layers are generally discarded as it is the de-hulled, milled and then polished rice granules which are purchased by consumers. Within the scope of the present invention, it is the milled material which is retrieved and from which the anthocyanin composition is ultimately extracted.

There are many commonly used rice mills from a single-pass Engelberg mill to multipass systems. Within the scope of the present invention, manual technology involving hand pounding may be used as well as machine-milling using abrasion or friction. Most preferably, a mill using physical scratching is employed. Examples of suitable mills are provided by Satake USA Inc. and Buhler AG.

Slender grains require less pressure to mill than bold (i.e. thick) grains because of their thinner aleurone layer. Milling is undertaken until substantially all of the outer layer
(comprising the aleurone layer) is removed. Generally, this layer comprises 5 to 15% of the rice granule. In one preferred form, as an index to determine the end of the milling process, one may gauge approximately 10% of the rice granule. Alternatively, one may assess the granule colour change and end the milling process as the granule becomes light coloured relative to the starting material. Processing can be adjusted for the required depth of granule.

Innovations introduced in the Japanese rice industry which may be taken advantage of within the scope of the present invention include microcomputer control of milling based on the desired degree of milling and germ rice milling. A germ rice milling machine introduced in 1976 that uses gentle, abrasive roll milling under very low pressure leaves the germ intact for more than 80 percent of the grains. This way, greater economic efficiency is achieved as the outer layer is used to extract therapeutically useful anthocyanin compositions and the remainder of the granule may be sold as a commercial product.

Table I shows the proximate analysis of whole black rice and milled black rice outer layer fraction from a Shimzu Mill for comparison:

<table>
<thead>
<tr>
<th></th>
<th>Black Rice (outer layer)</th>
<th>Black Rice (whole granule)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein g/100mg</td>
<td>17</td>
<td>10.0</td>
</tr>
<tr>
<td>Fat g/100mg</td>
<td>9.8</td>
<td>2.0</td>
</tr>
<tr>
<td>Moisture g/100mg</td>
<td>8.3</td>
<td>11.0</td>
</tr>
<tr>
<td>Ash g/100mg</td>
<td>7.6</td>
<td>1.4</td>
</tr>
<tr>
<td>Carbohydrate g/100mg</td>
<td>57.0</td>
<td>76.0</td>
</tr>
<tr>
<td>Energy kjoules/100mg</td>
<td>1610</td>
<td>1510</td>
</tr>
</tbody>
</table>

Clearly, the outer layer fraction has a higher concentration of nutrients per 100mg than the whole granule.
Table 2 shows the comparative distribution of anthocyanin in black rice, both whole granule and fractions thereof.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole granule</td>
<td>1645±25</td>
</tr>
<tr>
<td>1st layer</td>
<td>13,567±802</td>
</tr>
<tr>
<td>2nd layer</td>
<td>3850±876</td>
</tr>
<tr>
<td>Inside kernel</td>
<td>70±9</td>
</tr>
</tbody>
</table>

It has been found that the distribution of anthocyanin (combination of both cyanidin-3-glucoside and peonidin-3-glucoside) is related to the depth of the granule, with the first layer separated from the starchy endosperm containing the highest amount of anthocyanin. This finding provides a unique opportunity to optimize anthocyanin recovery.

The second step of the process of the present invention comprises adding a solution of at least one organic solvent and an acid to the separated outer layer. Preferably, the organic solvent is selected from the group consisting of alcohols, ketones, hydrocarbons and water.

The ketones are selected from the group having the general structure RCOR^1 where R and R^1 are alkyl groups. Preferably the alkyl groups are C_1 - C_6 groups. Most preferably, the ketone is 2-propanone (acetone). The hydrocarbon may be selected from the group comprising all C_5 - C_{10} hydrocarbons. Most preferably, the hydrocarbon is hexane. The alcohol is selected from the group having the general structures R-CHOHR, R-CH_2OH, and RCOH where R is a C_1 - C_4 alkyl group. Most preferably, the alcohol is either methanol or ethanol.

The acid may be any non-toxic, edible acid sufficient to attain the desired pH range. Preferably, the acid is selected from the group consisting of hydrochloric acid, acetic acid,
citric acid, tartaric acid and low concentration sulphuric acid. It is preferred that sufficient acid is added to achieve a pH of between 1 and 4. It is preferred that the separated outer layer is soaked in the solvent for a period of between 1 and 10 hours and that the temperature be in the range of from 25-40° C. It is preferred that the ratio of solvent to rice be between 30/1 to 100/1 (volume/weight).

The third step of the process of the present invention comprises filtering and removing the solvent and the acid from the separated outer layer to produce a pigment fraction. Preferably, the solvent is removed by filtrate extraction and the acid removed by evaporation, such as by rotary evaporation although other methods are clearly within the purview of the present invention, for example, vacuum drying and freeze drying.

The fourth and fifth steps of the process of the present invention comprise separating and collecting the constituents of the pigment fraction. Preferably this is achieved via fractionation. Most preferably, procedures such as column chromatography, (for example, polyamide gel filtration) may be used. At this stage, after fractionation, two major fractions are collected, one comprising cyanidin-3-glucoside and the other comprising peonidin-3-glucoside.

Verification of the identity of the fractions after separation may be achieved by high pressure liquid chromatography (HPLC), liquid chromatography/mass spectrometry (LC/MS) as required. Figure 2 is a chromatogram of Bio-Gel P-2 elution of black rice extract, with peaks 1 and 2 being subsequently identified by LC/MS as peonidin-3-glucoside and cyanidin-3-glucoside respectively.

Preferred black rice varieties, which may be used in accordance with the present invention include, but are not limited to: Wu Gong, Shang-nong, Hei Xiang Geng Nuo, Zhen Xi Hei Mi, Dong Bei Nuo 149, Ao Yu Nuo 349, Hong 280, Hong 463 and Xiang Xue
Anthocyanin Compositions

The present invention comprises compositions which comprise cyanidin-3-O-glucoside and peonidin-3-O-glucoside derived from black rice. Such compositions include those compositions derived from the extraction and purification process described herein and also those compositions which are synthesized de novo (i.e. not necessarily derived from this process) having the ratios of the two constituents as described further hereinbelow.

The structures of cyanidin-3-O-glucoside and peonidin-3-O-glucoside are as follows:

![Cyanidin-3-Glucoside](image1)

![Peonidin-3-Glucoside](image2)

In a preferred embodiment, the composition of the present invention comprises cyanidin-3-glucoside and peonidin-3-glucoside in a ratio of from 10 to 1, more preferably from 6 to 1, more preferably from 3:5:1 to 6:5:1. Most preferably, this ratio is 4:1.

Within the scope of the present invention, cyanidin-3-glucoside and peonidin-3-glucoside, for use in preparing such compositions, may be extracted or obtained from a number of sources, including but not limited to black rice, black fruit sources such as blackberries and black currant, black beans, black seeds such as sesame, and black grains such as...
black wheat. In addition, other sources include red currant and red rice.

In one embodiment of the present invention, in order to achieve compositions having the preferred anthocyanin ratios as described above, a most preferred process to “blend” anthocyanins from varying sources. This way, one may take advantage of particularly high levels of one component from one while another source may be richer in the other required component.

Combination with Anti-Oxidants and/or Phytosterols
In a further embodiment, the compositions of the present invention may be combined, prior to administration, co-administered or administered separately over a time interval with one or more anti-oxidants. Suitable anti-oxidants include, but are not limited to: vitamin E, beta-caroten, enzymatic superoxide dismutase, catalase, glutathion peroxidase, glutathione reductase, tea catechins, chelating agents such as citric acid, EDTA, phenylalanine, phosphoric acid, tartaric acid and tryptophane; preferentially oxidized compounds such as ascorbic acid, sodium bisulfite and sodium sulfite; water soluble chain terminators such as thiols and lipid soluble chain terminators such as alkyl gallates, ascorbyl palmitate, t-butyl hydroquinone, butylated hydroxyanisole, butylated hydroxytoluene, hydroquinone, nordihydroguaiaretic acid and alpha-tocopherol. It is believed that the combination of these anti-oxidants and the anthocyanin compositions of the present invention initiates and perpetuates the beneficial anti-oxidant effects.

In a further embodiment, the compositions of the present invention may be combined, prior to administration, co-administered or administered separately over a time interval with one or more sterols or stanols.

As used herein, the term "sterol" includes all sterols without limitation, for example: sitosterol, campesterol, stigmasterol, brassicasterol (including dihydrobrassicasterol), desmosterol, chalinosterol, poriferasterol, clionasterol, ergosterol, coprosterol, codisterol, isofucosterol, fucosterol, clerosterol, nervisterol, lathosterol, stellasterol, spinasterol,
chondrillasterol, peposterol, avenasterol, isoavenasterol, fecosterol, pollinastasterol, cholesterol and all natural or synthesized forms and derivatives thereof, including isomers. The term "stanol" refers to saturated or hydrogenated sterols including all natural or synthesized forms and derivatives thereof, and isomers. It is to be understood that modifications to the sterols and stanols i.e. to include side chains also falls within the purview of this invention. For example, the purview of this invention clearly includes 24 beta-ethylchlostanol, 24-alpha-ethyl-22-dehydrocholstanol. It is also to be understood that, when in doubt throughout the specification, and unless otherwise specified, the term "sterol" encompasses both sterol and stanol. In a most preferred form, the sterol is in its saturated form and is sitostanol.

These sterols and stanols for use in accordance with this invention may be procured from a variety of natural sources. For example, they may be obtained from the processing of plant oils (including aquatic plants) such as corn oil and other vegetable oils, wheat germ oil, soy extract, rice extract, rice bran, rapeseed oil, sunflower oil, sesame oil and fish (and other marine-source) oils. They may also be derived from fungi, for example ergosterol, or animals, for example cholesterol. Accordingly, the present invention is not to be limited to any one source of sterols. US Patent Serial No. 4,420,427 teaches the preparation of sterols from vegetable oil sludge using solvents such as methanol. Alternatively, phytosterols and phytostanols may be obtained from tall oil pitch or soap, by-products of forestry practises as described in US Patent Serial No.5,770,749, incorporated herein by reference.

It is believed that the combination of these sterols and/or stanols in conjunction with the anthocyanin compositions of the present invention yields at least additive and perhaps synergistic and complementary therapeutic effects, particularly in lipid modulation. While not intending to be bound by any one theory regarding the mechanism of action, it is possibly due to the different lipid targets of each component. For example, sterols and stanols have been shown to be effective agents to lower serum LDL-C while they
show minimal efficacy in increasing serum HDL-C. Conversely, the anthocyanin and sterol/stanol compositons of the present invention have been found to effectively and quite substantially to stabilize both HDL-C and LDL-C from oxidation. In addition, there is a complementary effect between the anthocyanin components and the sterol/stanol components on the reduction and substantial arrest of atherosclerotic lesion development.

**Therapeutic Efficacy**

In a first embodiment of the present invention, there is provided a method of treating or preventing cardiovascular disease and its underlying conditions, including atherosclerosis and inflammation associated therewith, and oxidative stress in an animal, preferably a human, which comprises administering to the animal a therapeutically effective amount of a composition derived from black rice which comprises cyanidin-3-glucoside and peonidin-3-glucoside.

In another embodiment of the present invention, there is provided a method of treating or preventing cardiovascular disease and its underlying conditions, including atherosclerosis, inflammation, hyperlipidemic conditions, hypoalphalipoproteinemia, hypercholesterolemia, and oxidative stress in an animal, preferably a human, which comprises administering to the animal a therapeutically effective amount of a composition derived from black rice which comprises cyanidin-3-glucoside and peonidin-3-glucoside and optionally one or more phytosterols or phytostanols and/or one or more antioxidants.

Such compositions include those in which:

1) the anthocyanin components are derived from the extraction and purification process described herein;

2) the anthocyanin components are synthesized *de novo* and having the ratios of the two anthocyanin constituents as described herein; and
3) the anthocyanin components are extracted and purified from more than one source, for example, black rice, black bean, black seeds, blackberries, and the blended to achieve the desired ratio cyanidin-3-glucoside and peonidin-3-glucoside.

The term "therapeutically effective" is intended to qualify the amount of the composition administered in order to achieve one or more of the following goals:

a) enhancing and/or preserving the stability of HDL from oxidation;

b) enhancing and/or preserving the stability of LDL, VLDL or IDL from oxidation

c) enhancing and/or preserving the stability of triglyceride (TG) from oxidation;

d) preventing, reducing, eliminating or ameliorating a dyslipidemic condition or disorder;

e) preventing, reducing, eliminating or ameliorating hypercholesterolemia, hypoalphalipoproteinemia,

f) preventing, reducing, eliminating or ameliorating the development of atherosclerotic lesions;

g) preventing, reducing, eliminating or ameliorating the development of inflammation associated with the development of cardiovascular disease and coronary artery disease;

h) preventing, reducing, eliminating or ameliorating any condition, disease or disorder which has as its basis or which is exacerbated by a deficiency in plasma HDL, or excess of either LDL, VLDL, Lp(a), beta-VLDL, IDL or remnant lipoproteins; and

i) preventing, reducing, eliminating or ameliorating injuries due to oxidative stress.

As will become apparent from the examples provided below, the compositions of present invention exhibit strong anti-oxidant effects as evidenced by numerous testing protocols and are effective at decreasing atherosclerotic plaque formation.

It has been found that the anthocyanin compositions of present invention, when administered therapeutically:

1) enhances and/or preserves the stability of HDL-C and the atherogenic lipoproteins
such as LDL-C, VLDL-C, and IDL-C from oxidation;
2) prevents, reduces, eliminates or ameliorates injuries due to oxidative stress;
3) prevents, reduces, eliminates or ameliorates the development of atherosclerotic lesions; and
4) prevents, reduces, eliminates or ameliorates inflammation associated with atherosclerosis, coronary artery disease (CAD), and cardiovascular disease (CVD). This is critical as it has recently been found that CAD and CVD occur in apparently low-risk people due to inflammation. Recent evidence also suggests that inflammation of the arteries may be an important indicator of future heart attacks and strokes. Inflammation occurs when the body responds to injury or infection.

It has been found that the anthocyanin and phytosterol/phytostanol compositions of present invention, when administered therapeutically:
1) prevents, reduces, eliminates or ameliorates the development of atherosclerotic lesions;
2) modulates or controls plasma lipoproteins;
3) provides for the prevention, reduction, elimination or amelioration of a number of conditions and disorders, including, but not limited to: cardiovascular disease and its underlying conditions, including atherosclerosis, dyslipidemic conditions or disorders, hypercholesterolemia, and hypoalphalipoproteinemia, development of atherosclerotic lesions and toxic shock syndrome; and
4) provides for the prevention, reduction, elimination or amelioration of a number of conditions and disorders which have as their basis or which are exacerbated by a deficiency in plasma HDL, or excess of either LDL, VLDL, or IDL.

**Methods of Use**
The compositions of the present invention may be administered by any conventional means available for use in conjunction with pharmaceuticals, nutraceuticals, foods, beverages, and the like.
Without limiting the generality of the foregoing, the compositions of the present invention may be admixed with various carriers or adjuvants to assist in direct administration or to assist in the incorporation of the composition into foods, beverages, nutraceuticals or pharmaceuticals. In order to appreciate the various possible vehicles of the delivery of the compositions, the list below is provided. The doses of the composition will vary depending upon, among other factors, the mode of delivery, the patient size and condition, the result to be achieved, as well as other factors known to those skilled in the art of food additives and medicinal agents.

The desired effects described herein may be achieved in a number of different ways. These compositions may be administered by any conventional means available for use in conjunction with pharmaceuticals, nutraceuticals, foods, beverages, and the like.

The amount of the composition which is required to achieve the desired effects will, of course, depend on a number of factors such as the particular profile of the composition, the mode of administration and the condition of the patient.

1) Pharmaceutical Dosage Forms:
The compositions of the present invention can be administered to a patient either by themselves, or in pharmaceutical compositions where they are mixed with suitable carriers or excipients.

Use of pharmaceutically acceptable carriers to formulate the compositions herein disclosed for the practice of the invention into dosages suitable for systemic administration is within the scope of the invention. With proper choice of carrier and suitable manufacturing practice, the compositions of the present invention, in particular, those formulated as solutions, may be administered parenterally, such as by intravenous injection. The compositions can be formulated readily using
pharmaceutically acceptable carriers well known in the art into dosages suitable for oral administration. Such carriers enable the compositions of the invention to be formulated as tablets, pills, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated.

Pharmaceutical formulations, comprising one or more of the compositions of the present invention, include formulations wherein the active ingredients are contained in an effective amount to achieve their intended purpose. Determination of the effective amounts is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

In addition to the active ingredients these pharmaceutical compositions may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. The preparations formulated for oral administration may be in the form of tablets, dragees, capsules, or solutions.

The pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the
suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added.
Oral liquid preparations may be in the form of, for example, emulsions, syrups, or elixirs, or may be presented as a dry product for reconstitution with water or other suitable vehicle before use. Such liquid preparations may contain conventional additives such as suspending agents, for example sorbitol, syrup, methyl cellulose, gelatin, hydroxyethylcellulose, carboxymethylcellulose, aluminium stearate gel, hydrogenated edible fats; emulsifying agents, for example lecithin, sorbitan monooleate, or acacia; non-aqueous vehicles (which may include edible oils), for example almond oil, fractionated coconut oil, oily esters such as esters of glycerine, propylene glycol, or ethyl alcohol; preservatives, for example methyl or propyl p-hydroxybenzoate or sorbic acid; and if desired conventional flavouring or colouring agents.

It is contemplated within the scope of the present invention that the compositions of the present invention may be incorporated into various conventional pharmaceutical preparations and dosage forms such as tablets (plain and coated) for use orally, buccally or lingually, capsules (hard and soft, gelatin, with or without additional coatings) powders, granules (including effervescent granules), pellets, microparticulates, solutions (such as micellar, syrups, elixirs and drops), lozenges, pastilles, ampoules, emulsions, microemulsions, ointments, creams, suppositories, gels, transdermal patches and modified release dosage forms together with customary excipients and/or diluents and stabilizers.

The compositions of the present invention, adapted into the appropriate dosage form as described above may be administered to animals, including humans, orally, by injection (intravenously, subcutaneously, intra-peritoneally, intra-dermally or intra-muscularly), topically or in other ways.

The precise modes of delivery of the composition of the present invention in each case will depend upon the objectives of the administration protocol. In the case of existing conditions and disorders, it will depend upon the severity of the disorder, and as
discussed above, the age, size and gender of the individual. Determining appropriate dosages and administration schedules is well within the purview of one skilled in this field.

2) Foods/Beverages/Nutraceuticals:
In another form of the present invention, the compositions of the present invention may be incorporated into foods, beverages and nutraceuticals, for on-going prophylactic use, including, without limitation, into the following:

1) Dairy Products—such as cheeses, milk and other dairy beverages, spreads and dairy mixes, ice cream and yoghurt;
2) Cereal-Based Products—comprising grains (for example, bread, pastas, crackers and cereal bars) whether these goods are cooked, baked or otherwise processed;
3) Confectioneries—such as chocolate, candies, chewing gum, desserts, non-dairy toppings (for example Cool Whip™), sorbets, icings and other fillings;
4) Beverages—whether alcoholic or non-alcoholic and including colas and other soft drinks, juice drinks, dietary supplement and meal replacement drinks such as those sold under the trade-marks Boost™ and Ensure™; and
5) Miscellaneous Products—including processed foods such as soups, pre-prepared pasta sauces, pre-formed meals and the like.

The compositions of the present invention may be incorporated directly and without further modification into the food, nutraceutical or beverage by techniques such as mixing, infusion, injection, blending, dispersing, emulsifying, immersion, spraying and kneading. Alternatively, the compositions may be applied directly onto a food or into a beverage by the consumer prior to ingestion. These are simple and economical modes of delivery.

EXAMPLES
The present invention is described by the following non-limiting examples:
Example 1 Extraction and Separation of Anthocyanin

The outer layer was removed from Wu Gong black rice by Shimzu Mill in POS (Saskatchewan). Black rice fraction was soaked in 1% HCl in methanol overnight. Whatman filter paper no 4 was used to filter extraction. Methanol was removed by rotary evaporation under 40° C. Obtained pigment fraction was loaded into Bio-Gel P-2 column (2.5 x 45cm) packed with water acidified to pH 2.5 with acetic acid to separate anthocyanin.

The column was eluted with water acidified to pH 2.5 with acetic acid at a flow rate of 1ml/min and fractions were collected by fractionation collector (5ml/tube or 5 min/tube). The sample was monitored at 520nm. Two major fractions were collected and concentrated under vacuum for identification.

Example 2 Quantitation and Identification of Anthocyanin

The two major fractions from example 1 were further analyzed by HPLC and LC/MS using standard reference (cyanidin-3-glucoside and peonidin-3-glucoside). The HPLC was performed using a Waters Alliance 2690 system equipped with an autosampler and column heater. HPLC conditions were as follows: Waters Xterra MS C18 column (2.1x50mm, 2.5μm) was used at 40°C), 5μl injection, solvent A was 100% methanol and solvent B was 5% formic acid in water. Concentration of A was 20-4-% within 5 minutes. Flow rate was set as 0.4ml/min. Waters996 PDA detector was used and wavelength set at 200-600nm.

LC/MS analysis of black rice fractions was conducted using electrospray Mass spectrometry coupled with Waters 2690 separation module. PDA showed that using this Bio-Gel P-2 chromatography, two fragments were obtained which were distinguishable in both retention time and spectrum (Figures 3, 4 and 5). Figure 3 shows fraction I and fraction II after Bio-Gel P-2 separation.
Example 3 Anti-Oxidant Activity DPPH Radical Scavenging Test
The radical scavenging activity of the first layer, second layer and whole granule of black rice was evaluated using a stable free radical 1,1-diphenyl-2-picryl ("DPPH") in ethanol solution. DPPH will change into non-radical form when it reacts with other free radical scavengers, especially, primary anti-oxidants (hydrogen donors). A spectrometric method was applied to measure the disappearance of DPPH in an alcohol solution.

Figure 6 shows the effect of each layer and the whole granule on the inhibition of the DPPH radical. Clearly, at each concentration tested, the first layer is superior.

Example 4 Anti-Oxidant Activity Liposome Model
The free radical scavenging activity of the anthocyanin composition of the present invention was evaluated in a liposome model, where oxidation of a phospholipid was induced by thermolysis of 2,2-azobis(\(\alpha\)-amidinopropane) dihydrochloride ("AAPH"). This test was done by continuously monitoring the formation of conjugated diene hydroperoxide at 37°C.

Figure 7 shows the efficacy of the anthocyanin composition in preventing the formation of the conjugated diene hydroperoxide at various concentrations, with over 10μg/ml being most effective.

Example 5 Suppression of LDL Oxidation
The prevention of LDL oxidation by the anthocyanin composition of the present invention was evaluated. Oxidative modification of LDL is a critical step in the development of atherosclerosis due to the modification of down-regulation of ox-LDL. The modification of LDL particles results in the formation of foaming cells with the macrophage cell, and in turn the formation of laden and plaque on the endothelial of blood vessels. Accordingly, the reduction of LDL is considered as an important step in the prevention of cardiovascular disease. Transitional metal ion was removed by dialysis and LDL
oxidation was initiated by co-incubating with the Cu$^{2+}$ ion at 37°C for a period of time. LDL oxidation was monitored by different approaches, including agarose gel electrophoresis, formation of conjugated diene and thiobarbituric acid reactive substrate. The results in Figure 8 show that the addition of the anthocyanin composition of the present invention reduced the electrophoretic migration distance, indicating enhancement of negative charge of oxidatively modified LDL was suppressed.

Moreover, the formation of conjugated diene and thiobarbituric acid reactive substrates were also reduced (Figure 9), suggesting that the generation of primary and secondary lipid peroxidation products were also inhibited by the composition of the anthocyanin composition. In addition, HDL oxidation was evaluated. Peroxyl radical induced HDL modification was prevented by the addition of the anthocyanin composition as measured by agarose gel electrophoresis.

**Example 7 Suppression of DNA Oxidation**

The effects of the anthocyanin composition in preventing DNA oxidation induced by oxidative radicals such as the peroxyl radical and hydroxyl radicals (site specific and nonsite specific) were investigated. Figure 10 shows that the peroxyl radical led to the disappearance of the supercoiled DNA strand and the addition of either the anthocyanin composition or the standard anti-oxidant Trolox resulted in partial recovery of such damage. A similar effect was seen by the addition of cyanidin-3-glucoside and peonidin-3-glucoside in combination at various ratios Figure 11).

The black rice extract or composition of the present invention inhibited the DNA scission-induced by both non-site specific and site-specific hydroxyl radical (Figures 12 and 13). In this hydroxyl radical model, the hydroxyl radical was generated by ascorbic acid mediated Fenton reaction. The black rice extract was found to scavenge the hydroxyl radical generated in the site-specific model and was also demonstrated to chelate transitional metal ion in the site-specific hydroxyl radical model.
Example 8 Cell Viability Test
The purpose of this test was to determine whether the extract of the present invention provided protection against free-radical induced cytotoxicity in cell culture. In THP-1, a leukemia human cell line (ATCC), the addition of ferrous ion (as oxidative stimulus) resulted in cell death via oxidative stress attributed to the Fenton reaction mechanism (Figure 14). The addition of the black rice extract of the present invention recovered the cell death in a concentration dependent manner.

Example 9 Hepatic Lipase Suppression
Hepatic lipase activity was suppressed by the extract of the present invention (referred to as 1st layer in Figure 15). Hepatic lipase hydrolyzes HDL phospholipid and triglyceride, an after-heparin plasma hepatic lipase activity is inversely related to plasma HDL-C levels. Accordingly, the results show that the extract of the present invention, high in anthocyanins, would likely increase HDL in vivo.

Example 10 DNA nicking prevention
Both peroxyl radical and hydroxyl radical were applied in this test to evaluate the effect of black rice extract of the present invention in preventing DNA scission by 0.7% agarose gel electrophoresis with 0.5µg/ml ethidium bromide using TAE buffer (40mM Tris-acetate, 2mM EDTA, pH8.5). DNA bands were visualized by ultraviolet bench top transilluminator (UVP Inc., Upland, CA) and densities of bands were analyzed by LabWork software (UVP Inc., Upland, CA). The inhibition of DNA nicking was calculated as following equation:

\[
\% Inh = \frac{D_{\text{native}} - D_{\text{sample}}}{D_{\text{native}} - D_{\text{oxidative}}} \times 100
\]

\(D_{\text{native}}, D_{\text{oxidative}}, D_{\text{sample}}\) represented the density of DNA without AAPH, DNA with AAPH and DNA with AAPH and test sample, respectively.
DNA integrity is vital to cell division and survival. DNA has been shown to be vulnerable to oxidative damage in vivo, leading to disrupt transcription, translation and DNA replication and eventually mutation and cell death. For example, Schneider et al. [19] have demonstrated that hydroxyl radical implicated in DNA damage, suggesting that early hydroxyl radical is biologically associated with the mutagenic and carcinogenic processes. Transitional metal-ascorbic acid-hydrogen peroxide system produced sequence-dependent damage of DNA with preferential oxidation of guanines, suggesting that localized production of the reactive oxygen species as a result of metal binding to the special regions of the DNA. Previous reports indicated that DNA nicking test could be a useful tool for evaluating antioxidant activity against active oxygen species induced DNA deterioration including both hydroxyl radicals and peroxyl radicals [20](Kitts et al., 1999).

In the present study, we adapted the concept of both non-site specific and site-specific hydroxyl radical and applied these to the DNA scission assays (Figures 10 and 11). It is evident that hydroxyl radical, no matter site-specific or non-site specific broke the integrity of supercoiled DNA strand (lane 2 of Figure 10).

In this hydroxyl radical-induced DNA nicking test black rice extract showed higher affinity of prevention in the non-site specific case than that in the site-specific case (Table 3).

Table 3. Inhibition percentage of blackrice extract on the prevention of DNA nicking induced by hydroxyl radical

<table>
<thead>
<tr>
<th>µg/ml</th>
<th>Anthocyanin content (%)</th>
<th>Non-site specific</th>
<th>Site specific</th>
</tr>
</thead>
</table>

30
Whole rice  1.36±0.01
   100  65.7  51.4
   1000 83.9  74.1

Fraction  0.16±0.02
   10  54.4  49.0
   100 82.8  69.5

Trolox
   0.17  44.0  25.7
   1.7  80.8  61.0

*Measured by LC-MS using cyanidin-3-glucoside and peonidin-3-glucoside as external standards.

It is also noteworthy that the prevention of supercoiled DNA from hydroxyl radical induced nicking was also associated with the anthocyanin distribution in the black rice, i.e., blackrice outer layer contains higher amount of anthocyanin exhibits higher capacity than whole rice in inhibiting hydroxyl radical induced DNA damage (Table 3). Standard antioxidant Trolox also showed similar trend as higher inhibition of DNA damage induced by non-site specific hydroxyl radical (Table 3), confirming that sequence-specification was involved in the hydroxyl radical induced DNA strand breakage. The relevance of hydroxyl radical induced DNA nicking is that Fenton reaction components, such as hydrogen peroxide and transitional metal ion, could be found in cell nucleus and in turn inducing cell toxicity associated with DNA scission.

Table 4. Effects of blackrice and anthocyanin in preventing supercoiled DNA strand against peroxy radical induced scission

<table>
<thead>
<tr>
<th>Sample</th>
<th>%Inh</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blackrice fraction, 1µg/ml</td>
<td>1.0±0.7</td>
</tr>
<tr>
<td>10µg/ml</td>
<td>7.4±0.3</td>
</tr>
<tr>
<td>Concentration</td>
<td>IC50 (μg/ml)</td>
</tr>
<tr>
<td>---------------</td>
<td>-------------</td>
</tr>
<tr>
<td>25μg/ml</td>
<td>25.8±8.7</td>
</tr>
<tr>
<td>100μg/ml</td>
<td>82.2±7.8</td>
</tr>
</tbody>
</table>

Cyanidin-3-glucoside:peonidin-3-glucoside 9:1 63.2±1.6
4:1 50.4±2.1
1:1 38.7±0.3
1:4 50.2±0.6
9:1 40.7±0.0

In our present study, we also found that the black rice extract suppressed the supercoiled DNA scission at a concentration dependent manner (Table 4), of which the significant effect was as low as 10μg/ml. As pointed above, cyanidin-3-glucoside and peonidin-3-glucoside exist in the black rice as major pigments contributors. In our study, we found that cyanidin-3-glucoside and peonidin-3-glucoside exhibited 11.5 and 11.4% protection on the DNA strand scission at 1μg/ml respectively, whereas, protections increased to 45.3% and 44.1% at 5μg/ml level, respectively. The effect of combination of cyanidin-3-glucoside and peonidin-3-glucoside was also measured in this peroxyl radical induced DNA scission model. Results showed that the protection decreased with the decrease of percentage of cyanidin-3-glucoside (Figure 10 and Table 3). A concentration-dependent suppression of supercoiled DNA scission was also observed with black rice extract (Figure 11 and Table 4).

Example 11 Anti-Inflammation Testing

A) Effect of black rice extract on preventing nitric oxide

B) Western blotting for the iNOS
A) Mouse macrophage cell RAW 264.7 (ATCC) was cultured in DMEM medium supplemented with 10% fetal bovine serum and antibiotics (100U/ml of penicillin and 100U/ml of streptomycin) at 37°C under 5% CO₂. Cell was plated at a density of 2×10⁵ cells/well into 96-well plates. After overnight growth when cells adhered to the bottom of well, various amount of the black rice extract in PBS and 1μg/ml bacterial lipopolysaccharide (LPS, Escherichia coli, serotype 0111:B4) were added for another 24 hr. Medium was aliquot (100μl) to another 96-well plate where 100μl of Greiss reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrocholide in water, 1:1 v/v) was then added [18]. Absorbance at 540nm was determined with ELISA plate reader. The concentration of nitrite was measured in according to standard curve obtained from the same procedure. The inhibition of nitric oxide was calculated according to the following equation:

\[
\text{\% Inhibition} = \frac{Abs_{\text{positive}} - Abs_{\text{sample}}}{Abs_{\text{positive}} - Abs_{\text{negative}}} \times 100
\]

of which, \( Abs_{\text{positive}} \), \( Abs_{\text{negative}} \), \( Abs_{\text{sample}} \) represent absorbance of cultural media with LPS, without LPS and sample with LPS.

B) Cell was collected into 2×sample reducing buffer and incubated at boiling water for 5 minutes. 20μl of sample was loaded onto 8% SDS-PAGE, followed by electric transfer to nitrocellulose membrane (Bio-Rad Laboratory). Membrane was blocked 1h at room temperature with 3% skimmed milk powder in 50mM Tris buffer (pH7.5) containing 150mM NaCl and 0.05% Tween-20. Membrane was then incubated at 4°C overnight with mouse anti-iNOS antibody (Pharmingen Transduction Laboratories) and mouse anti-α-tubulin antibody (Sigma) in 50mM Tris buffer with 150mM NaCl (pH7.5). Membrane was then incubated with goat anti-mouse IgG conjugated with horse radish peroxidase (Pharmingen Transduction Laboratories) for 1 h at room temperature. 4-chloro-1-naphthol and hydrogen peroxide were used for the visualization of target protein [19] (Bollag et al, 1996).
In addition to reactive oxygen species, reactive oxygen species such as nitric oxide and its metabolite peroxynitrite are considered mutagenic [21](Keefer and Wink, 1996). There are two types of nitric oxide synthesis (NOS) in the mammalian cell, i.e., constitutive NOS (cNOS) and inducible NOS (iNOS), the latter being most likely to be activated by endotoxins such as lipopolysaccharide (LPS) and cytokines. The iNOS is not present in most cells under normal conditions, however, following the appropriate stimulus such as LPS and cytokines, it is rapidly induced and responsible for the large quantity of nitric oxide. The incubation of bacterial lipopolysaccharide with macrophage provided a quick way to evaluate the generation of nitric oxide from the inducible nitric oxide synthase. Leeuwenburgh et al [22](1997) has reported that LDL from aortic atherosclerotic intima contained significantly higher 3-nitrotyrosine than plasma LDL, indicating the implication of reactive nitric species in aortic LDL oxidation and atherosclerosis. Further, it has been shown that iNOS inhibitor N-iminoethyl-L-lysine limited the progression of preexisting atherosclerosis in hypercholesterolemic rabbits thus suggesting that inhibition of iNOS might be beneficial for the preventing the progression of atherosclerosis.

Large amount of nitric oxide expression was made possible by using mouse macrophage cell line RAW264.7 co-incubated with bacterial LPS. The measurement of nitric oxide was indirectly measured by its metabolite nitrite in the supernatant of cell cultural media by Griess reagent. Nitrite was found in the cultural media at 25μM which was higher compared to the macrophage cell without LPS treatment, indicating that nitric oxide formation was boosted when RAW264.7 cell was stimulated by bacterial lipopolysaccharide.

The addition of black rice extract significantly reduced the production of nitrite, indicating the suppression of nitric oxide in the activated macrophage cells (Figure 18). It is also notable that the inhibition of nitric oxide production was not due to any
cytotoxicity, since the cell viability was normal with the addition of black rice extract (Figure 18). The inhibition of nitric oxide was also found for cyanidin-3-glucoside and peonidin-3-glucoside. Under the same working condition, inhibition of nitric oxide in LPS-activated RAW264.7 cell by cyanidin-3-glucoside and peonidin-3-glucoside is shown in Table 5.

**Table 5.** Inhibition percentage of nitric oxide generated in the LPS-activated mouse macrophage cell RAW264.7 (mean±SD)

<table>
<thead>
<tr>
<th>Concentration (μM)</th>
<th>Cyanidin-3-glucoside</th>
<th>Peonidin-3-glucoside</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 μM</td>
<td>12.7±2.7</td>
<td>14.4±0.6</td>
</tr>
<tr>
<td>10 μM</td>
<td>23.1±0.9</td>
<td>22.3±3.7</td>
</tr>
<tr>
<td>100 μM</td>
<td>72.9±4.8</td>
<td>35.0±2.5</td>
</tr>
</tbody>
</table>

It was noteworthy that cyanidin-3-glucoside revealed a higher inhibition of nitric oxide formation than that of peonidin-3-glucoside at 100 μM.

In our present study, it is clear that the addition of LPS to the RAW264.7 cell significantly induces the expression of iNOS protein as demonstrated by the western blotting (Figure 19). The addition of black rice extract in this cell culture model indicated that the expression of iNOS protein (MW 130kDa) was suppressed at a concentration dependent manner (Figure 19). In addition, cell lysate blotted with anti-α-tubulin antibody showed that this housekeeping protein remained unchanged with or without LPS and samples treatments, thus suggesting that black rice, specifically suppresses the inducible nitric oxide synthase express in the macrophage, thus reduces the nitric oxide production under this condition.

**Example 12 Reduction in the Development of Atherosclerosis in Apo-E Mice**
ApoE-deficient mice on a C57BL/6J background were purchased from Jackson Laboratories (Bar Harbor, Maine U.S.A), and were bred and maintained under conventional housing conditions. C57BL/6J mice were from the Animal Center of Sun-Yat Sen University of Medical Sciences.

A total 45 male apoE – deficient mice of 4wk age were randomly divided into 3 groups of 15 each group and matched for body weight. The mice received a purified diet based on the AIN-93G formulation (summarized in Table 6) and each group consumed one of the following diet: AIN-93G purified diet (Positive Group); AIN-93G purified diet with 5g/100g of the extract of the present invention: black rice fraction (BRF Group); AIN-93G purified diet with 5g/100g white rice fraction (WRF Group). 15 male C57BL/6J mice aged 4wk old as control (Control Group) were fed AIN-93G purified diet.

The contents of protein, fat and energy in the different diets were adjusted to the same level by adding casein and soybean oil (Table 7). The components of the BRF and WRF were listed in Table 5. All the groups were housed in plastic cages with stainless steel grid tops. Food and distilled water given ad libitum. The experiment lasted 16 weeks. The average amount of dietary intake in each mouse was ≈2.8g/d in four groups. The mice were weighed twice every week during the experiment.

At the end of the experiment, all mice were deprived of food overnight and kindly killed by withdrawing blood from retro-orbital plexus under anaesthesia. Serum was prepared by low speed centrifugation at 2800×g for 20 min at 4°C and used to determine the levels of serum lipids.

Table 6
AIN-93 Purified Diets for Laboratory Rodents

36
<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/kg diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cornstarch</td>
<td>397.486</td>
</tr>
<tr>
<td>Casein</td>
<td>200</td>
</tr>
<tr>
<td>Dextrinized Cornstarch</td>
<td>132</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100</td>
</tr>
<tr>
<td>Soybean Oil(no additive)</td>
<td>70</td>
</tr>
<tr>
<td>Fiber</td>
<td>50</td>
</tr>
<tr>
<td>Mineral mix(AIN-93G-MX)(^1)</td>
<td>35</td>
</tr>
<tr>
<td>Vitamin mix(AIN-93-MX)(^2)</td>
<td>10</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>3</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2.5</td>
</tr>
<tr>
<td>(41.1% choline)(^3)</td>
<td></td>
</tr>
<tr>
<td>Tert-butylhydroquinone</td>
<td>0.014</td>
</tr>
</tbody>
</table>

\(^1\)ICN Company, U.S.A  
\(^2\)Based on the molecular weight of the free base.

Table 7
Major nutrients of different dietary groups

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Control Group</th>
<th>Positive Group</th>
<th>BRF Group</th>
<th>WRF Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kcal/kg diet)</td>
<td>3766.0</td>
<td>3766.0</td>
<td>3766.0</td>
<td>3766.0</td>
</tr>
<tr>
<td>Carbohydrate (g/kg diet)</td>
<td>629.486</td>
<td>629.486</td>
<td>629.486</td>
<td>629.486</td>
</tr>
<tr>
<td>Protein (g/kg diet)</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
</tbody>
</table>
Total fat (g/kg diet)  70  70  70  70  70

*Control group: C57BL/6J mice fed the AIN-93G purified diet
Positive Group: apoE-deficient mice fed the AIN-93G purified diet
BRF Group: apoE-deficient mice fed the AIN-93G purified diet with 5g/100g black rice fraction
WRF Group: apoE-deficient mice fed the AIN-93G purified diet with 5g/100g white rice fraction.

Table 8
Components of the black and white rice fraction

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Black rice fraction</th>
<th>White rice fraction</th>
</tr>
</thead>
</table>


<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Units/100g</th>
<th>Units/100g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein, g</td>
<td>13.90</td>
<td>12.20</td>
</tr>
<tr>
<td>Fat, g</td>
<td>13.20</td>
<td>14.10</td>
</tr>
<tr>
<td>Carbohydrate, g</td>
<td>47.36</td>
<td>50.95</td>
</tr>
<tr>
<td>Moisture, g</td>
<td>9.80</td>
<td>7.96</td>
</tr>
<tr>
<td>Fiber, g</td>
<td>8.32</td>
<td>7.04</td>
</tr>
<tr>
<td>Salt mixture, mg</td>
<td>7420</td>
<td>7750</td>
</tr>
<tr>
<td>P</td>
<td>1694.10</td>
<td>1542.50</td>
</tr>
<tr>
<td>Ca</td>
<td>60.20</td>
<td>45.30</td>
</tr>
<tr>
<td>K</td>
<td>673.70</td>
<td>624.60</td>
</tr>
<tr>
<td>Mg</td>
<td>79.40</td>
<td>80.40</td>
</tr>
<tr>
<td>Na</td>
<td>2.11</td>
<td>4.35</td>
</tr>
<tr>
<td>Fe</td>
<td>16.46</td>
<td>6.30</td>
</tr>
<tr>
<td>Zn</td>
<td>8.96</td>
<td>4.92</td>
</tr>
<tr>
<td>Mn</td>
<td>11.67</td>
<td>7.93</td>
</tr>
<tr>
<td>Mo</td>
<td>0.33</td>
<td>0.28</td>
</tr>
<tr>
<td>Cu</td>
<td>1.49</td>
<td>0.91</td>
</tr>
<tr>
<td>Se</td>
<td>0.15</td>
<td>0.06</td>
</tr>
<tr>
<td>Vitamin, mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin B1</td>
<td>2.30</td>
<td>1.20</td>
</tr>
<tr>
<td>Vitamin B2</td>
<td>0.40</td>
<td>0.14</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>0.60</td>
<td>0.03</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>21.00</td>
<td>13.00</td>
</tr>
<tr>
<td>Total flavonoids, mg</td>
<td>6.4</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Serum lipid profile

Serum total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C) were measured by using a Hitachi Automatic Analyzer (Tokyo, Japan). Serum TC was determined by using a cholesterol esterase and
cholesterol oxidase assay. Serum concentrations of HDL-C were assayed by the same method. Serum LDL concentrations were calculated according to Friedwald formula.

Assessment of Atherosclerosis
Quantification of atherosclerosis fatty streaks was done by calculating the lesion size in the aortic sinus as previously described (18). Briefly, the heart and upper section of the aorta were removed from the animals and the peripheral fat cleaned carefully. The upper section was embedded in O.C.T compound and frozen at −20°C. Every other section (10μm thick) throughout the aortic sinus (400μm) was taken for analysis. The distal portion of the aortic sinus was recognized by the three valve cusps which are the junctions of the aorta and the heart. Cryostat sections were stained with oil red O and counter-stained with hematoxylin. Each section of the aortic valve was evaluated for oil-red O staining area by capturing images directly from an RGB camera attached to an Olympus BX-50 light microscope and displaying them on a Trinitron™ RGB monitor. Image analysis was determined using Optimas™ 4.1 software (Image Processing Solutions). Results were expressed as the percent of the total cross-sectional vessel wall area (normal+diseased area/section, excluding the lumen) stained with oil red O.

Statistical analysis
Results are expressed as means ± SD, and the differences were determined by one-way ANOVA coupled with the Student-Newman-keuls (SNK) multiple comparison test. Differences with P < 0.05 were considered significant.

Results
Body Weights
Fifteen mice in each group were initiated onto the experimental protocol. Initial mice body weights for the experiment were 17±1 g (mean±SD). Final average body weights were 25 to 27g. No significant difference in body weights was observed during the experimental period (Table 9) (Figure 16).
Table 9
Body weights of mice in different groups fed AIN-93G diet or AIN-93G diet containing WRF,BRF during 16wk

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>4wk</th>
<th>8wk</th>
<th>12wk</th>
<th>16wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Group</td>
<td>15</td>
<td>17.07±1.08</td>
<td>22.41±2.13</td>
<td>25.67±2.34</td>
<td>26.45±2.63</td>
</tr>
<tr>
<td>Positive Group</td>
<td>15</td>
<td>17.16±1.04</td>
<td>21.13±3.14</td>
<td>23.77±2.75</td>
<td>24.47±2.85</td>
</tr>
<tr>
<td>BRF Group</td>
<td>15</td>
<td>17.11±1.05</td>
<td>21.35±3.31</td>
<td>24.73±3.24</td>
<td>25.90±3.13</td>
</tr>
<tr>
<td>WRF Group</td>
<td>15</td>
<td>17.18±1.07</td>
<td>21.83±2.72</td>
<td>24.38±3.07</td>
<td>26.18±3.00</td>
</tr>
</tbody>
</table>

Serum Cholesterol Levels
Serum TC,LDL-C,HDL-C and LDL/HDL in control group differed from the other three groups (P<0.05). The levels of TC,LDL-C and LDL/HDL in BRF group were lower compared with positive group and WRF group (P<0.05), and there were no differences between positive group and WRF group. Although both BRF and WRF groups had higher levels HDL-C compared with positive group, the BRF group had lower LDL/HDL (Table 10).

Table 10
Serum lipid concentrations in mice fed AIN-93G diet or AIN-93G diet containing WRF,BRF for 16wk*

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>TC (mmol/l)</th>
<th>LDL-C (mmol/l)</th>
<th>HDL-C (mmol/l)</th>
<th>LDL/HDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Group</td>
<td>10</td>
<td>2.881±0.758a</td>
<td>0.280±0.083a</td>
<td>1.776±0.439a</td>
<td>0.160±0.038a</td>
</tr>
<tr>
<td>Positive Group</td>
<td>10</td>
<td>15.682±2.644</td>
<td>1.427±0.420b</td>
<td>2.546±0.442b</td>
<td>0.570±0.188b</td>
</tr>
</tbody>
</table>

*
Development of Atherosclerosis in the Aortic Sinus

After 16 weeks intervention, there were no visible atherosclerotic plaques in the aortic sinus of the control group fed the AIN-93G diet. But atherosclerotic plaques of various degrees were visible in aortic sinus in other three groups. Plaque was much more severe in positive group and WRF group than in BRF group (Table 11)(Figure 17).

The average plaque area in mice of BRF group was lower than that of positive group and WRF group (P<0.01). The plaque area of aortic sinus in mice fed BRF was 48.42% and 46.08% less compared to positive group and WRF group respectively. There was no significant difference in plaque areas of mice in positive group and WRF group.

Table 11
Atherosclerotic plaque of aortic sinus in mice fed AIN-93G diet or AIN-93G diet containing WRF,BRF after 16wk*

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Atherosclerotic Area(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Group</td>
<td>15</td>
<td>0a</td>
</tr>
<tr>
<td>Positive Group</td>
<td>15</td>
<td>26.25±9.2b</td>
</tr>
<tr>
<td>BRF Group</td>
<td>15</td>
<td>13.54±4.1c</td>
</tr>
<tr>
<td>WRF Group</td>
<td>15</td>
<td>25.11±7.15b</td>
</tr>
</tbody>
</table>
*Values without common letters in column are significantly different, p<0.01.

REFERENCES


15. Ling et al. Biomedical and Molecular Action of Nutrients 2001


WE CLAIM:

1. A process of extracting a composition comprising anthocyanins from black rice (Oryza sativa L) comprises:
   a) separating an outer layer from a starchy endosperm in de-hulled black rice;
   b) adding a solution of at least one organic solvent and an acid to the separated outer layer;
   c) filtering and removing the solvent and the acid from the separated outer layer to produce a pigment fraction;
   d) separating constituents of the pigment fraction; and
   e) collecting the anthocyanin composition therefrom.

2. The process of claim 1 wherein the outer layer is physically separated from the starchy endosperm at step a).

3. The process of claim 1 wherein the outer layer is physically separated from the starchy endosperm at step a) by milling the de-hulled black rice.

4. The process of claim 1 wherein the organic solvent is selected from the group consisting of alcohols, ketones, hydrocarbons and water.

5. The process of claim 1 wherein the organic solvent is a ketone having the structure RCOR¹ where R and R¹ are C₁ - C₆ alkyl groups.

6. The process of claim 1 wherein the organic solvent is a C₅ - C₁₀ hydrocarbon.

7. The process of claim 1 wherein the organic solvent is an alcohol selected from the group consisting of the general structures R-CHOHR, R-CH₂OH, and RCOH where R is a C₁ - C₄ alkyl group.
8. The process of claim 1 wherein the acid is selected from the group consisting of hydrochloric acid, acetic acid, citric acid, low concentration sulphuric acid and tartaric acid.

9. The process of claim 1 wherein sufficient acid is added to achieve a pH of between 1 and 4.

10. The process of claim 1 wherein the separated outer layer is soaked in the solvent for a period of between 1 and 10 hours.

11. The process of claim 1 wherein the solvent and acid are removed at step c) by evaporation.

12. The process of claim 1 wherein the constituents of the pigment fraction are separated at step d) by fractionation.

13. A composition prepared according to the process of any of claims 1-9.

14. A composition which comprises cyanidin-3-O-glucoside and peonidin-3-O-glucoside.

15. The composition of claim 14 in which the ratio of cyanidin-3-O-glucoside to peonidin-3-O-glucoside is 10 to 1.

16. The composition of claim 14 in which the ratio of cyanidin-3-O-glucoside to peonidin-3-O-glucoside is 6 to 1.

17. The composition of claim 14 in which the ratio of cyanidin-3-O-glucoside to peonidin-3-O-glucoside is from 3.5:1 to 6.5:1.
18. The composition of claim 14 in which the ratio of cyanidin-3-O-glucoside to peonidin-3-O-glucoside is 4:1.

19. The composition of claim 11 additionally comprising one or more anti-oxidants.

20. The composition of claim 11 additionally comprising one or more sterols.

21. The composition of claim 11 additionally comprising one or more stanols.

22. The composition of claim 11 additionally comprising one or more anti-oxidants selected from the group consisting of vitamin E, beta-carotene, enzymatic superoxide dismutase, catalase, glutathion peroxidase, glutathione reductase, tea catechins, chelating agents such as citric acid, EDTA, phenylalanine, phosphoric acid, tartaric acid and tryptophane; preferentially oxidized compounds such as ascorbic acid, sodium bisulfite and sodium sulfite; water soluble chain terminators such as thiols and lipid soluble chain terminators such as alklyl gallates, ascorbyl palmitate, t-butyl hydroquinone, butylated hydroxyanisole, butylated hydroxytoluene, hydroquinone, nordihydroguaiaretic acid and alpha-tocopherol.

23. A method of treating or preventing cardiovascular disease and its underlying conditions, including atherosclerosis, inflammation, hyperlipidemic conditions, hypoalphalipoproteinemina, hypercholesterolemia, and oxidative stress in an animal which comprises administering to the animal the composition prepared according to the process of claim 1.

24. A method of treating or preventing cardiovascular disease and its underlying conditions, including atherosclerosis, inflammation, hyperlipidemic conditions, hypoalphalipoproteinemia, hypercholesterolemia, and oxidative stress in an animal
which comprises administering to the animal the composition of claim 11.

25. A method of treating or preventing cardiovascular disease and its underlying conditions, including atherosclerosis, inflammation, hyperlipidemic conditions, hypoalphalipoproteinemia, hypercholesterolemia, and oxidative stress in an animal which comprises administering to the animal the composition of claim 12
Figure 2

peonidin-3-glucoside
[M+H]^+ = 463, [M-glu]^+ = 301

cyanidin-3-glucoside
[M+H]^+ = 449, [M-glu]^+ = 287
Figure 4

<table>
<thead>
<tr>
<th>Peak Name</th>
<th>RT</th>
<th>Area</th>
<th>% Area</th>
<th>Height</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyanidin-3-glucoside</td>
<td>1.083</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>peonidin-3-glucoside</td>
<td>1.704</td>
<td>227249</td>
<td>100.00</td>
<td>18020</td>
</tr>
</tbody>
</table>
Figure 5

<table>
<thead>
<tr>
<th>Peak Name</th>
<th>RT</th>
<th>Area</th>
<th>% Area</th>
<th>Height</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyanidin-3-glucoside</td>
<td>1.073</td>
<td>386101</td>
<td>100.00</td>
<td>36332</td>
</tr>
<tr>
<td>peonidin-3-glucoside</td>
<td>1.744</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 7

7/19
Figure 11
Figure 15

Hepatic lipase 1st layer, 0.1mg/ml 1st layer, 1mg/ml whole, 1mg/ml whole, 10mg/ml

cpm/min/ml
Figure 19

LPS       ++ + + + -
BRE (mg/ml)  0.5 0.2 0.1 - -

--- iNOS, 130 kDa

--- α-tubulin

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INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K35/78 A61P9/10

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)

IPC 7 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 practical, search terms used)

FSTA, MEDLINE, BIOSIS, EPO-Internal, WPI Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category *</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tr>
<td>X</td>
<td>SU NOH RYU ET AL: &quot;High performance liquid chromatographic determination of anthocyanin pigments in some varieties of black rice.&quot; JOURNAL OF FOOD AND DRUG ANALYSIS 6 (4) 729-736 1998 CORRESPONDENCE (REPRINT) ADDRESS, CHI-TANG HO, DEP. OF FOOD SCI., COOK COLL., RUTGERS STATE UNIV., NEW BRUNSWICK, NJ 08901-8520, USA, XP001153058 abstract page 734; table 4 page 730, right-hand column, paragraph 2 page 730, left-hand column, paragraph 1</td>
<td>1-5,7, 9-25</td>
</tr>
</tbody>
</table>

Further documents are listed in the continuation of box C.

* 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 document but published on or after the international filing date

*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

*C* 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

**"** 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

**"** 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

- 30 June 2003

Date of mailing of the international search report

21/07/2003

Name and mailing address of the ISA

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Authorized officer

Escolar Blasco, P

Form PCT/ISA/210 (second sheet) (July 1992)
<table>
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<td>Y</td>
<td>DATABASE FSTA 'Online! INTERNATIONAL FOOD INFORMATION SERVICE (IFIS), FRANKFURT/MAIN, DE; HEE JONG KOH ET AL: &quot;Varietal variation of pigmentation and some nutritive characteristics of colored rices.&quot; Database accession no. 97-1-05-m0033 XP002245980 abstract &amp; KOREAN JOURNAL OF CROP SCIENCE 1996 COLL. OF AGRIC. &amp; LIFE SCI., SEOUL NAT. UNIV., SUWON 441-744, KOREA, vol. 41, no. 5, pages 600-607,</td>
<td>1-12</td>
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<td>A</td>
<td>ABDEL-AAL E S M ET AL: &quot;A rapid method for quantifying total anthocyanins in blue aleurone and purple pericarp wheats.&quot; CEREAL CHEMISTRY 76 (3) 350-354 1999 CROP DEV. CENT., DEP. OF PLANT SCI., UNIV. OF SASKATCHEWAN, SASKATOON, SASK. S7N 5A8, CANADA. FAX 306/966-5015. E-MAIL ABDELAAAL(A)SASK.USASK.CA, XP009013063 page 350, left-hand column, paragraph 2 - paragraph 3 page 353, right-hand column; table II</td>
<td>1-14</td>
</tr>
</tbody>
</table>
INTERNATIONAL SEARCH REPORT

Box I  Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
   - because they relate to subject matter not required to be searched by this Authority, namely:
     Claims 23–25: Rule 39.1(iv) PCT – Method for treatment of the human or animal body by therapy

2. ☐ Claims Nos.:
   - because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
   - because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II  Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple Inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.