Title: COMPOSITIONS-OF-MATTER FOR LOWERING SERUM CHOLESTEROL AND/OR TRIGLYCERIDE LEVEL AND METHODS OF PRODUCING AND USING SAME

Abstract: A composition-of-matter for lowering serum cholesterol and/or triglyceride level including an isolated fraction of a Nigella sativa organic extract. The isolated fraction includes at least one substance which migrates with an Rf value of about 0.12 in a thin-layer-chromatography assay employing a 1:1:0.1 dichloromethane:hexane : methanol solvent system.
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COMPOSITIONS-OF-MATTER FOR LOWERING SERUM CHOLESTEROL AND/OR TRIGLYCERIDE LEVEL AND METHODS OF PRODUCING AND USING SAME

5 FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to compositions-of-matter and methods of lowering serum cholesterol and/or triglycerides in mammals and, more particularly, to isolated fractions of *Nigella sativa* organic extract selected capable of lowering serum cholesterol and triglycerides. In addition, the present invention relates to methods of producing the isolated fractions and their use in therapy.

Elevated level of serum cholesterol (hypercholesterolemia) and serum lipids (hyperlipidemia) are major contributing factors to the onset and progression of cardiovascular heart diseases, such as atherosclerosis (Erkkila Arja et al., 1999; Gotto, 1998; Brewer, 1999). Atherosclerosis afflicts over 6 million Americans and is the leading cause of death in the United States; direct and indirect medical costs associated with this disease exceed 60 billion dollars per year. Secondary physiological effects, which often accompany the onset of atherosclerosis, include cerebral strokes, sub-optimal liver function, renal artery blockage, senility, male impotence, arteriosclerotic aneurysms and limb gangrene.

Hypercholesterolemia and hyperlipidemia may be inherited or may be secondary to another disorder, such as systemic lupus erythematosus, hypothyroidism, nephrotic syndrome, cushing’s syndrome, diabetes mellitus, obesity, alcoholism, corticosteroid therapy or estrogen therapy. The maintenance of low level serum cholesterol and triglycerides effectively reduces the risk of developing atherosclerosis particularly by individuals having diabetes, hypertension, smoking habit, old age or physically inactive life style.

Maintenance of low level serum cholesterol and triglycerides may be controlled by proper dietary management, behavior modification, exercise and drug therapy. Typically, the first recommendation in treating or preventing hyperlipidemia or hypercholesterolemia is dietary intervention, whereby lipid intake is restricted, accompanied by physical exercise. Accordingly, a variety of dietary supplements or “health” foods (e.g. brans, psylliums, guar gum, lecithins, whey, red wines, fish oils and ginseng root extract) have been reported to be effective. However, while a
proper diet and exercise treatment can be effective in keeping serum lipids levels down, it is usually insufficient for substantially lowering levels of serum cholesterol and triglycerides in hypercholesterolemic or hyperlipidemic or subjects. Therefore, the use of drugs is often necessary.

Presently available pharmaceutical drugs for treating hypercholesterolemia and hyperlipidemia include lipids anti-absorption drugs (e.g. melinamide, thioesters); bile acid sequestrants (e.g. cholestipol and cholestyamine); and lipids biosynthesis inhibitors (e.g., lovastatin and its analogs, nicotinic acid and probucol). However, presently available drugs often fail to control severe cases of hypercholesterolemia and hyperlipidemia. In addition, a long term use of such drugs may result in undesired side effects.

Plant-derived fatty acids, such as oleic acid, linoleic acid and linolenic acid have been reported capable of lowering levels of serum lipids in animals (MacDonald, 2000) and human patients (Chan et al., 1991; Cho et al., 2002).

Silymarin, a mixture of flavonolignans obtained from Silybum marianum was reported active in lowering serum lipids in animals (Skottova et al., 1998; Krecman et al., 1998).

Scoparone (6,7-dimethoxycoumarin) obtained from Artemisia scoparia was reported capable of lowering total cholesterol and triglycerides in hypercholesterolaemic diabetic rabbits (Hoult et al., 1996).

Lycopene obtained from tomato inhibits an essential enzyme involved in cholesterol synthesis (Rao, 2002).

Astilbin, obtained from Engelhardtia chrysolepis was reported capable of lowering serum and liver lipid concentrations (Igarashi et al., 1996).

The present invention provides novel compositions-of-matter and articles-of-manufacturing which include an isolated fraction of Nigella sativa organic extract selected capable of substantially lowering serum cholesterol and triglyceride levels in hyperlipidemic animals. The isolated fraction of the present invention can be used, as an active ingredient, for treating or preventing hyperlipidemia, hypercholesterolemia and atherosclerosis.
SUMMARY OF THE INVENTION

According to one aspect of the present invention there is provided a composition-of-matter for lowering serum cholesterol and/or triglyceride level including an isolated fraction of a *Nigella sativa* organic extract. The isolated fraction includes at least one substance which migrates with an Rf value of about 0.12 in a thin-layer-chromatography assay employing a 1:1:0.1 dichloromethane: hexane : methanol solvent system.

According to another aspect of the present invention there is provided an article-of-manufacturing including packaging material and a pharmaceutical composition identified for use in lowering serum cholesterol and/or triglyceride level being contained within the packaging material. The pharmaceutical composition includes, as an active ingredient, an isolated fraction of a *Nigella sativa* organic extract comprising at least one substance which migrates with an Rf value of about 0.12 in a thin-layer-chromatography assay employing a 1:1:0.1 dichloromethane: hexane : methanol solvent system.

According to yet another aspect of the present invention there is provided a method of producing a composition-of-matter capable of lowering serum cholesterol and/or triglyceride level. The method includes fractionating a *Nigella sativa* organic extract to thereby obtain a fraction comprising at least one substance which migrates with an Rf value of about 0.12 in a thin-layer-chromatography assay employing a 1:1:0.1 dichloromethane: hexane : methanol solvent system.

According to still another aspect of the present invention there is provided a method of lowering serum cholesterol and/or triglyceride level. The method includes administering to a subject in need thereof an effective amount of an isolated fraction of a *Nigella sativa* organic extract. The isolated fraction includes at least one substance which migrates with an Rf value of about 0.12 in a thin-layer-chromatography assay employing a 1:1:0.1 dichloromethane: hexane : methanol solvent system.

According to an additional aspect of the present invention there is provided a method of treating or preventing hypercholesterolemia. The method includes administering to a subject in need thereof an effective amount of an isolated fraction of a *Nigella sativa* organic extract. The isolated fraction includes at least one
substance which migrates with an Rf value of about 0.12 in a thin-layer-chromatography assay employing a 1:1:0.1 dichloromethane: hexane : methanol solvent system.

According to yet an additional aspect of the present invention there is provided a method of treating or preventing hyperlipidemia. The method includes administrating to a subject in need thereof an effective amount of an isolated fraction of a *Nigella sativa* organic extract. The isolated fraction at least one substance which migrates with an Rf value of about 0.12 in a thin-layer-chromatography assay employing a 1:1:0.1 dichloromethane: hexane : methanol solvent system.

According to still an additional aspect of the present invention there is provided a method of treating or preventing atherosclerosis. The method includes administrating to a subject in need thereof an effective amount of an isolated fraction of a *Nigella sativa* organic extract. The isolated fraction includes at least one substance which migrates with an Rf value of about 0.12 in a thin-layer-chromatography assay employing a 1:1:0.1 dichloromethane: hexane : methanol solvent system.

According to further features in preferred embodiments of the invention described below, the isolated fraction includes at least one compound having a molecular weight ranging from 245 to 378.

According to still further features in the described preferred embodiments the isolated fraction is characterized by FAB/MS spectrum peaks at 355, 357, 447 and 463 m/z.

According to still further features in the described preferred embodiments the *Nigella sativa* organic extract is a *Nigella sativa* seed organic extract.

According to still further features in the described preferred embodiments the *Nigella sativa* seed organic extract is a *Nigella sativa* seed acetonitrile extract.

According to still further features in the described preferred embodiments the step of fractionating is effected by a liquid chromatography.

According to still further features in the described preferred embodiments the subject is a human.

According to still further features in the described preferred embodiments the administering is effected parenterally.
According to still further features in the described preferred embodiments the step of administering is effected orally.

According to still further features in the described preferred embodiments the effective amount is at a dosage ranging from 0.1 to 100 mg/Kg/day.

The present invention successfully addresses the shortcomings of the presently known configurations by providing a novel isolated fraction of *Nigella sativa* organic extract selected capable of substantially lowering serum cholesterol and triglyceride levels in a subject and methods of producing and using same in therapy.

**BRIEF DESCRIPTION OF THE DRAWINGS**

The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only, and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention, the description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

In the drawings:

FIGS. 1A-B illustrate the effect of *Nigella sativa* ethanol extract (NS4; 1 g/Kg), *N. sativa* chloroform extract (NS6; 1 g/Kg) and lipitor (positive control; 20 mg/Kg) on the level of serum cholesterol (Figure 1A) and serum triglyceride (Figure 1B) in model hyperlipidemic rats. The cholesterol and triglyceride levels are in mg/dL.

FIG. 2 illustrates the effect of *Nigella sativa* ethanol extract (NS4) and lipitor (positive control), administered at different doses, on the levels of serum cholesterol and serum triglyceride in model hyperlipidemic rats. The cholesterol and triglyceride levels are in mg/dL.
FIGS. 3A-B illustrate the effect of *Nigella sativa* petroleum ether extract (NS1), *N. sativa* ethyl acetate extract (NS2) and *N. sativa* ethanol extract (NS4), administered at 1 g/Kg to model hyperlipidemic rats, on the levels of serum cholesterol (Figure 3A) and serum triglyceride (Figure 3B). The cholesterol and triglyceride levels are in mg/dL.

FIG. 4 illustrates a TLC separation profile of *Nigella sativa* ethanol extract (Pal-10) and *N. sativa* acetonitrile extract (Pal-20) using a 1:1:0.1 dichloromethane: hexane : methanol solvent system.


FIG. 7A-B illustrate the effect of *Nigella sativa* ethanol extract fractions Pal-302, Pal-303, Pal-307, Pal-311 or Pal-312 (100 mg/Kg) on the levels of serum cholesterol (Figure 7A) and serum triglyceride (Figure 7B) in model hyperlipidemic rats. The cholesterol and triglyceride levels are in mg/dL.

FIGs. 8A-B illustrate the effect of *Nigella sativa* acetonitrile extract fractions Pal-403, Pal-404, Pal-405 and Pal-406 (50 mg/Kg) on the levels of serum cholesterol (Figure 8A) and serum triglyceride (Figure 8B) in model hyperlipidemic rats. The cholesterol and triglyceride levels are in mg/dL.

FIGs. 9A-B illustrate the effect of *Nigella sativa* acetonitrile extract fractions Pal-405 (200 mg/Kg), Pal-405-1 (100 mg/Kg), Pal-405-2 (100 mg/Kg) , Pal-405-3 (100 mg/Kg) and *Nigella sativa* ethanol extract volatile fraction Pal-101 (50 mg/Kg) on the level of serum cholesterol (Figure 9A) and serum triglyceride (Figure 9B) in model hyperlipidemic rats. The cholesterol and triglyceride levels are in mg/dL.

FIGs. 10A-B illustrate the effect of linoleic acid, oleic acid and stearic acid methyl-ester (100 mg/Kg) on the levels of serum cholesterol (Figure 10A) and serum
triglyceride (Figure 10B) in model hyperlipidemic rats. The cholesterol and triglyceride levels are in mg/dL.

FIGs. 11A-B illustrate the effect of linoleic acid ethyl ester, linoleic acid methyl ester, oleic acid ethyl ester, oleic acid methyl ester and palmatic acid ethyl ester (100 mg/Kg) on the levels of serum cholesterol (Figure 11A) and serum triglyceride (Figure 11B) in model hyperlipidemic rats. The cholesterol and triglyceride levels are in mg/dL.

FIGs. 12A-B illustrate a TLC separation profile of sub-fractions of *Nigella sativa* acetonitrile extract Pal-406, using dichloromethane : hexane : methanol, 45 : 45 : 10 solvent (Figure 12A) and petroleum ether : ether acetate, 1:1 solvent (Figure 12B).

FIGs. 13A-B illustrate the effect of fraction Pal-406 dissolved in either 100% propylene glycol (PG), or in a mixture of 66% propylene glycol and 33% methylene cellulose (PG/MS) and orally administered to model hyperlipidemic rats, on the level of serum cholesterol (Figure 13A) and serum triglyceride (Figure 13B). The cholesterol and triglyceride levels are in mg/dL.

FIGs. 14A-B illustrate the effect of sub-fraction Pal-406-6.2 (Pal 662) orally administered to model hyperlipidemic rats at a dosage of 50 or 100 mg/Kg (10 or 20 mg per 200 g animal), on the level of serum cholesterol (Figure 14A) and serum triglyceride (Figure 14B). The cholesterol and triglyceride levels are in mg/dL.

FIGs. 15A-B illustrate the effect of 3,6 dihydroxy falvone (J18), 3,7 dihydroxy flavone (J19), chrysin 5,7 dihydroxyflavone (J20), catechin hydrate (J22), Morin, 2',3,4',5,7-pentahydroxy flavone (J30) and Baicalein, 5,6,7-trihydroxyflavone (J31) IP administered at 50 mg/Kg, on the level of serum cholesterol (Figure 15A) and serum triglyceride (Figure 15B) in model hyperlipidemic rats. The cholesterol and triglyceride levels are in mg/dL.

FIG. 16 illustrates the molecular structure of chrysin 5,7 dihydroxyflavone.

FIG. 17 illustrates the molecular structure of 3,6 dihydroxy falvone.

FIG. 18 illustrates the molecular structure of 3,7 dihydroxy flavone.

FIGs. 20A-E illustrate spectra of GC/MS headspace analysis of the Pal-20 extract of the present invention.

FIGs. 21A-J illustrate spectra of GC/MS liquid injection analysis of the Pal-20 extract of the present invention.

FIG. 22 illustrates spectra of $^1$H NMR analysis of the Pal-20 extract of the present invention.

FIG. 23 illustrates spectra FAB/MS analysis of the Pal-20 extract of the present invention.

FIG. 24 illustrates spectra of $^1$H NMR analysis of the Pal-400 fraction of the present invention.

FIG. 25 illustrates spectra of FAB/MS analysis of the Pal-400 fraction of the present invention.

FIG. 26 illustrates spectra of $^1$H NMR analysis of the Pal-401 fraction of the present invention.

FIG. 27 illustrates spectra of FAB/MS analysis of the Pal-401 fraction of the present invention.

FIG. 28 illustrates spectra of $^1$H NMR analysis of the Pal-402 fraction of the present invention.

FIG. 29 illustrates spectra of FAB/MS analysis of the Pal-402 fraction of the present invention.

FIG. 30 illustrates spectra of $^1$H NMR analysis of the Pal-403 fraction of the present invention.

FIG. 31 illustrates spectra of FAB/MS analysis of the Pal-403 fraction of the present invention.

FIG. 32 illustrates spectra of $^1$H NMR analysis of the Pal-404 fraction of the present invention.

FIG. 33 illustrates spectra of FAB/MS analysis of the Pal-404 fraction of the present invention.

FIG. 34 illustrates spectra of $^1$H NMR analysis of the Pal-405 fraction of the present invention.

FIG. 35 illustrates spectra of FAB/MS analysis of the Pal-405 fraction of the present invention.
FIG. 36 illustrates spectra of $^1$H NMR analysis of the Pal-405-1 sub-fraction of the present invention.

FIG. 37 illustrates spectra of FAB/MS analysis of the Pal-405-1 sub-fraction of the present invention.

FIG. 38 illustrates spectra of $^1$H NMR analysis of the Pal-405-2 sub-fraction of the present invention.

FIG. 39 illustrates spectra of FAB/MS analysis of the Pal-405-2 sub-fraction of the present invention.

FIG. 40 illustrates spectra of $^1$H NMR analysis of the Pal-405-3 sub-fraction of the present invention.

FIG. 41 illustrates spectra of FAB/MS analysis of the Pal-405-3 sub-fraction of the present invention.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of a compositions-of-matter and article-of-manufacturing comprising isolated fractions of *Nigella sativa* organic extract selected capable of lowering serum cholesterol and triglycerides in mammals and methods of their production and use in therapy.

The principles and operation of the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details of construction and the arrangement of the components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

*Nigella sativa* Linn., commonly known as “black seed” or “black cumin”, has been used in folk medicine for thousands of years. *N. sativa* seed oil has been used worldwide for the treatment and prevention of asthma, hypertension, diabetes, inflammation, cough, bronchitis, headaches, eczema, fever, dizziness and influenza. Traditionally, there is a common Islamic belief that the “black seed” is a remedy for
all ailments.

*N. sativa* seed includes substances which have been shown to have diuretic activity (Nadkarni 1976), cholagogic and antispasmodic activities (Tennekoon, et al. 1991), carminative activity (Shayeb and Mabrouk, 1984), galactogogic activity (Vihan 1987), antibacterial activity (Hassan, et al. 1989), antifungal activity (Agarwal et al., 1979), anti-diabetic activity (al-Awadi et al.1985), anthelminthic activity (Akhtar, 1991) and emmenagogic activity (Siddiqui et al., 1988).

*N. sativa* fixed oil exhibits an anti-oxidant activity and inhibits non-enzymatic lipid peroxidation in liposomes (Houghton et al., 1995), antinociceptive activity (Khanna et al., 1993) and anti-inflammatory and analgesic activity (Al-Ghamdi, 2001, Mutabagani and El-Mahdi, 1997). Meral et al., (2001) reported that *N. sativa* fixed oil is capable of lowering glucose and lipid peroxides in diabetic rabbits. *N. sativa* volatile oil and its active constituent thymoquinone were reported capable of lowering arterial blood pressure and heart rate in a dose-dependent fashion (El-Tahir et al., 1993). The methanol soluble portion of *N. sativa* oil was reported to be capable of inhibiting blood coagulation (Enomoto et al., 2001). El- Dakhkhny et al. (2000) reported that *Nigella sativa* oil intake by rats for 4 weeks (800 mg/Kg) lowers the levels of low density lipoprotein and triglycerides in serum. More recently, Zaoui (2002a) reported that oral treatment of rats with *N. sativa* oil (1 mg/Kg/day/12 weeks) lowers the levels of cholesterol, triglycerides and glucose in serum of the treated animals.

U.S. Pat. Application Ser. No. 10/029885 describes a “lipid fraction” of *N. sativa* organic extract and its use in treating or preventing skin infections, wounds, bacterial infections, respiratory diseases and disorders, cellulite, cardiovascular disorders and septic infections. The lipid fraction comprises primarily fatty acids and fatty-acid glyceryl esters (96-99%) and the remaining 1-4% constitutes volatile oils (monoterpenes, carbonyl compounds, thymoquinone, dihydrothymoquinone, alcohols, esters and phenols) and sterols (sitosterol, β-amyrin, stigmasterol and campesterol). However, the lipid fraction described by this reference is not identified as being capable of lowering the level of cholesterol or triglycerides in serum.

While reducing the present invention to practice, the present inventors evaluated different organic extracts of *N. sativa* for their capacity to lower the level
of cholesterol and triglycerides in sera of hyperlipidemic animals (Examples 2-3). Selected active extracts were fractionated and the resulting fractions were selected based on their capacity to lower serum cholesterol and triglyceride levels in the hyperlipidemic animals (Examples 4-6). The biologically active fractions were isolated and chemically characterized (Example 9). The selected most active non-lipid fractions were chemically characterized by including one or more substances capable of migrating with an Rf value of about 0.12 in a thin-layer-chromatography (TLC) assay employing a 1:1:0.1 dichloromethane: hexane: methanol solvent system, which is indicative of being substantially devoid of lipids.

Thus, according to one aspect of the present invention there is provided a method of producing a composition-of-matter capable of lowering serum cholesterol and/or triglycerides level. The method includes fractionating a *Nigella sativa* organic extract to thereby obtain a fraction which is substantially devoid of lipids and capable of lowering serum cholesterol and/or triglycerides level in a subject.

As used herein, the phrase "*Nigella sativa* organic extract" refers to a mixture of substances extracted from *N. sativa*, preferably from *N. sativa* seed, using an organic solvent. The organic solvent of the present invention can be, but not limited to, ethanol, ethyl-acetate, hexane or acetonitrile. Preferably the organic solvent is ethanol, more preferably the organic solvent is acetonitrile.

The *N. sativa* organic extract of the present invention can be obtained by using standard extraction procedures known in the art. Preferably, *N. sativa* seeds are ground and mixed in the organic solvent to allow solvent extraction to take place for a period of 24-48 hours. The organic solvent is then removed from the crude solvent extract using standard techniques such as, for example, rotary evaporation.

Fractionation of the *N. sativa* organic extract can be effected by a number of chromatography and separation methods known in the art including, but not limited to, liquid chromatography, gas liquid chromatography, gas solid chromatography, high pressure or high performance liquid chromatography (HPLC; normal, reverse, or chiral), ion exchange chromatography, or size exclusion chromatography [see, for example, in Advances in Chromatography, Brown, Eds., Marcel Dekker, Pub. (1998); Basic Gas Chromatography, Harold et al., John Wiley & Sons, Pub. 1997]; Column Handbook for Size Exclusion Chromatography, Wu, Ed., Academic Press,

Preferably, the fractionation is effected using a silica gel column-chromatography procedure, such as that described in Examples 4-6 hereinbelow. Briefly, *N. sativa* extract or fraction is applied on top of a silica gel column and eluted successively with different solvents. The eluates are collected sequentially in fractions, followed by removal of the solvent by evaporation. The collected fractions are evaluated for biological activity using procedures such as those described in Examples 1-2 hereinbelow. Fractions exhibiting biological activity are selected for repeated, or modified, chromatography steps until sufficiently effective sub-fractions are obtained. The selected biologically active fractions are further analyzed for their chemical characteristics by TLC, GC/MS, LC/MS, FAB/MS, proton-NMR, or by other suitable analytical methods known in the art.

The *N. sativa* extract fraction isolated by the present invention includes one or more substances capable of migrating with an Rf value of about 0.12 in a thin-layer-chromatography assay employing a 1:1:0.1 dichloromethane: hexane : methanol solvent system. Preferably, the isolated fraction of the present invention is further characterized by FAB/MS spectrum peaks at 355, 357, 447 and 463 m/z. More preferably, the isolated fraction includes one or more compounds having molecular weights ranging from 245 to 378.

As is mentioned herein, one of the proposed uses for the isolated fraction of the present invention is in therapy of diseases or disorders characterized by high serum cholesterol and/or triglyceride levels.

Thus according to another aspect of the present invention there is provided a method of treating or preventing a disease or condition which is associated with high level of cholesterol and/or triglycerides in a subject in need thereof.

As used herein, the phrase "subject in need" refers to a mammal, preferably a human having an abnormal level of serum cholesterol and/or triglycerides. As per
the American Heart Association guidelines, the safe maximal levels of serum total cholesterol, low density lipoproteins (LDL)-cholesterol and triglycerides in adult human are 240, 35 and 150 mg/dL, respectively. Individuals having abnormal (higher) levels of cholesterol, LDL-cholesterol and/or triglycerides are typically placed on a restrictive diet and treated with drugs.

High levels of cholesterol, LDL-cholesterol and/or triglycerides may lead to hypercholesterolemia or hyperlipidemia.

Hypercholesterolemia denotes chronic elevated levels of serum total cholesterol, LDL-cholesterol and very low density lipoproteins (VLDL)-cholesterol while Hyperlipidemia denotes chronic elevated levels of serum lipids, including LDL, VLDL and triglycerides.

Hypercholesterolemia and Hyperlipidemia are major risk factors for diseases such as, atherosclerosis, coronary diseases, cerebral ischemia, intermittent claudication and gangrene.

The isolated fraction of the present invention can be administered to the subject in many different routes including, for example, oral, rectal, transmucosal, intestinal or parenteral delivery, including intramuscular, subcutaneous and intramedullary injections as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections. Preferably, the administration is effected parenterally, more preferably, orally.

Depending on the severity and responsiveness of the condition to be treated, dosing can be of a single or a plurality of administrations, with course of treatment lasting from several days to several weeks or diminution of the disease state is achieved. Preferably, the cholesterol and triglyceride levels in sera of treated subjects are frequently monitored during the course of treatment using standard AOAC methods routinely used in clinical labs.

The amount of a composition to be administered will be dependent on the subject being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, etc. Generally, an effective daily dosage of the isolated fraction of the present invention to an adult human will range from 0.01 to 1,000 mg/Kg, more preferably from 0.1 to 100 mg/Kg. Additional guidelines for
administration and formulation which can be utilized for administration are provided hereinbelow.

The isolated fraction of the present invention can be used in therapy *per se*, or as part (active ingredient) of a pharmaceutical composition.

As used herein a "pharmaceutical composition" refers to a preparation of one or more of the active ingredients described herein with other chemical components such as physiologically suitable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of a compound to an organism.

Hereinafter, the phrases "physiologically acceptable carrier" and "pharmaceutically acceptable carrier" which may be interchangeably used refer to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound. An adjuvant is included under these phrases.

Herein the term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

A pharmaceutical composition which includes the isolated fraction of the present invention may also include one or more compounds such as, for example, fibrates (e.g., chlorofibrate, benzaflibrate and gemfibrozil), nicotinic acid, its derivatives and analogues (e.g., acipimox and probucol), bile acid binding resins (e.g., cholestyramine and cholestypol), compounds inhibiting cholesterol absorption (e.g., sitosterol or neomycin) and compounds controlling the biosynthesis of cholesterol (e.g. HMG-CoA reductase inhibiting agents such as lovastatin, simvastatin and pravastatin).

Techniques for formulation and administration of drugs may be found in “Remington's Pharmaceutical Sciences,” Mack Publishing Co., Easton, PA, latest edition, which is incorporated herein by reference.

Pharmaceutical compositions of the present invention may be manufactured by processes well known in the art, e.g., by means of conventional mixing,
dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active ingredients into preparations which can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For injection, the active ingredients of the pharmaceutical composition may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank’s solution, Ringer’s solution, or physiological salt buffer.

For oral administration, the pharmaceutical composition can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the pharmaceutical composition to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for oral ingestion by a patient. Pharmacological preparations for oral use can be made using a solid excipient, optionally grinding the 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, hydroxypropylmethylcellulose, sodium carboxymethylcellulose; and/or physiologically acceptable polymers such as polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

As used herein, the term “oral administration” includes administration of the pharmaceutical compound to any oral surface, including the tongue, gums, palate, or other buccal surfaces. Addition methods of oral administration include provision of the pharmaceutical composition in a mist, spray or suspension compatible with tissues of the oral surface.

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, 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 compositions 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 may contain the active ingredients in admixture with filler such as lactose, binders such as starches, lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active ingredients may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for the chosen route of administration.

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

For administration by nasal inhalation, the active ingredients for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from a pressurized pack or a nebulizer with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane or carbon dioxide. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin for use in a dispenser may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The pharmaceutical composition described herein may be formulated for parenteral administration, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multidose containers with optionally, an added preservative. The compositions may be suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical compositions for parenteral administration include aqueous solutions of the active preparation in water-soluble form. Additionally, suspensions of the active ingredients may be prepared as appropriate oily or water based injection
suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acids esters such as ethyl oleate, 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 active ingredients to allow for the preparation of highly concentrated solutions.

Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water based solution, before use.

The pharmaceutical composition of the present invention may also be formulated in rectal compositions such as suppositories or retention enemas, using, e.g., conventional suppository bases such as cocoa butter or other glycerides.

Pharmaceutical compositions suitable for use in context of the present invention include compositions wherein the active ingredients are contained in an amount effective to achieve the intended purpose. More specifically, a therapeutically effective amount means an amount of active ingredients (e.g. antisense oligonucleotide) effective to prevent, alleviate or ameliorate symptoms of a disorder (e.g., mammary tumor progression) or prolong the survival of the subject being treated.

For any preparation used in the methods of the invention, the therapeutically effective amount or dose can be estimated initially from \textit{in vitro} and cell culture assays. For example, a dose can be formulated in a hyperlipidemia animal model such as described in Example 1 hereinbelow. Such information can be used to more accurately determine useful doses in humans.

Toxicity and therapeutic efficacy of the active ingredients described herein can be determined by standard pharmaceutical procedures \textit{in vitro}, in cell cultures or experimental animals. The data obtained from these \textit{in vitro} and cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage may vary depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g., Fingl, \textit{et al.}, 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1).
Compositions of the present invention may, if desired, be presented as an article-of-manufacturing in a pack or dispenser device, such as an FDA approved kit, which may contain one or more unit dosage forms containing the active ingredient. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser may also be accommodated by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert. Compositions comprising a preparation of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition, as if further detailed above.

As used herein, the term "about" denotes ± 10%.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

**EXAMPLES**

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below.

**EXAMPLE 1**
**Induction of hyperlipidemia in model rats**

**Materials and methods:**

*Animal maintenance:* Male Sprague Dawley rats, weighing about 200 g were randomly divided into six groups of 5-6 rats each, and housed in polycarbonate cages. The cages were bedded with wood shavings and fitted with stainless-steel wire mash tops. Animal rooms were maintained at 23°C under a 12/12 hr diurnal cycle with free access to tap water and standard commercial food (Cat No. 19510, Koffolk Ltd., Tel-Aviv, Israel).

*Hyperlipidemia induction:* Administering Triton WR-1339 (tyloxapol) to laboratory animals induces blockage of peripheral utilization of VLDL, inhibition of lipoprotein removal from the circulation and, consequently, a rapid rise in plasma lipids in laboratory animals and is a widely used animal model for evaluating serum lipids lowering substances (see, for example, Cayen *et al.*, 1982; Brunet *et al.*, 1984; Zeniya and Reuben, 1988; Nutting and Tso, 1989; Kasim *et al.*, 1992; Tanabe *et al.*, 1993; Pedrosa *et al.*, 2002; Kourounakis *et al.*, 2002; Ara *et al.*, 2002).

Accordingly, at the start of each experiment described hereinbelow, all rats were anaesthetized with diethyl-ether and blood samples were collected by ocular puncture. The animals were then administered intraperitoneally with 400 mg/Kg Tyloxapol [(1,1,3,3-tetramethylbutyl), phenol polymer with formaldehyde and oxirane; Sigma-Aldrich, Cat # T876] to induce hyperlipidemia. A second blood sample was drawn from the treated rats 24 post treatment. All blood samples were centrifuged at 300 rpm for 10 min then their sera were removed and kept at −20°C until being analyzed.

*Measurement levels of serum cholesterol and triglyceride:* Levels of serum cholesterol and total triglycerides in samples were determined using the Sigma Diagnostics Infinity Cholesterol Reagent (Procedure No. 402) and Triglycerides Reagent (Procedure No. 344), according to the manufacturer instructions. Briefly, one volume of rat serum was mixed with 100 volumes of supplied reagent followed by incubation at 37°C for 5 min. The optical absorbance values of mixtures were then measured using spectrophotometer at 500-550 nm and the levels of cholesterol and triglycerides in samples were determined based on standards solutions.

*Statistically analysis:* Data was analyzed by a one-way analysis of variance.
Results:

As can be seen in Table 1 below, the Tyloxapol treatment resulted in a significant 150% increase of serum cholesterol level just 24 hr post treatment.

<table>
<thead>
<tr>
<th>Time of sampling (hr after treatment)</th>
<th>Mean serum cholesterol level (mg/dL)</th>
<th>SD²</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>66.52 a</td>
<td>10.06</td>
</tr>
<tr>
<td>24</td>
<td>166.50 b</td>
<td>51.03</td>
</tr>
</tbody>
</table>

¹ Means followed by the same letter are not significantly different according to one way ANOVA test at p < 0.05.
² SD = Standard Deviation.

EXAMPLE 2

The effect of Nigella sativa organic extracts on lowering serum cholesterol and triglyceride in hyperlipidemic model rats

Materials and methods:

Animal maintenance: Male Sprague Dawley rats, weighing about 200 g, were randomly divided into six groups of 5-6 rats each, and maintained as described in Example 1 hereinafter.

Preparation of Nigella sativa extracts:

Ethanol extract (NS4): Two hundred gram of Nigella sativa seeds were air-dried, grounded and soaked in 95% ethanol (1 L) for 48 hours at room temperature. The solvent was then removed using a rotary evaporator yielding a 20 ml extract.

Chloroform extract (NS6): Two hundred gram of Nigella sativa seeds were air-dried, grounded and soaked in chloroform (1 L) for 48 hours extraction at room temperature. The solvent was then removed using a rotary evaporator yielding a 20 ml extract.

Petroleum ether extract (NS1): Two hundred gram of Nigella sativa seeds were air-dried, grounded and soaked in petroleum ether (1 L) for 48 hours extraction at room temperature. The solvent was then removed using a rotary evaporator yielding a 20 ml extract.

Ethyl acetate extract (NS2): Two hundred gram of Nigella sativa seeds were air-dried, grounded and soaked in ethyl acetate (1 L) for 48 hours extraction at room temperature. The solvent was then removed using a rotary evaporator yielding a 20
Acetonitrile extract (NS-AcN): Two hundred gram of Nigella sativa seeds were air-dried, grounded and soaked in acetonitrile (1 L) for 48 hours extraction at room temperature. The solvent was then removed using a rotary evaporator yielding a 20 ml extract.

Hexane extract (NS-Hexane): 200 Two hundred gram of Nigella sativa seeds were air-dried, grounded and soaked in hexane (1 L) for 48 hours extraction at room temperature. The solvent was then removed using a rotary evaporator yielding a 20 ml extract.

Dioxane extract (NS-Dioxane): Two hundred gram of Nigella sativa seeds were air-dried, grounded and soaked in dioxane (1 L) for 48 hours extraction at room temperature. The solvent was then removed using a rotary evaporator yielding a 20 ml extract.

Evaluation of serum lipids lowering activity of test substances: At the start of each experiment rats were anaesthetized with diethyl-ether and blood samples were collected by ocular puncture. The animals were then administered intraperitoneally with Tyloxaopil to induce hyperlipidemia, as described in Example 1 hereinabove. Test substances were concurrently administered intraperitoneally. Lepitor (Pfizer, USA) was used as a positive control. A second blood sample was drawn from the treated rats 24 post treatment. The blood samples were centrifuged at 300 rpm for 10 min then the sera were removed and kept at -20°C until analyzed for cholesterol and triglycerides as described in Example 1 hereinabove.

Statistically analysis: Data were analyzed by a one-way analysis of variance.

Results:

As can be seen in Figures 1A-B and in Tables 2-3 below, N. sativa ethanol extract (NS4) administered to model rats at 1 g/Kg, lowered the levels of serum cholesterol and triglyceride by 70.7 and 91.1%, respectively, as compared with the untreated control (statistically significant at p < 0.05). The administration of NS4 at different doses (0.1, 0.5 and 1 g/Kg) resulted in a dose response reduction of serum cholesterol and triglyceride (Figure 2).

In contrast, administering a chloroform extract (NS6) increased the levels of serum cholesterol and triglyceride in model rats by 33.8 and 7.4%, respectively, as
compared with the untreated control (see in 1A-B and in Tables 2-3 below).

### Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean serum cholesterol level (mg/dL)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (untreated)</td>
<td>270.00 a</td>
<td>124.39</td>
</tr>
<tr>
<td>Lepitor 20 mg/Kg</td>
<td>248.67 a</td>
<td>50.81</td>
</tr>
<tr>
<td>NS4 1 g/Kg</td>
<td>79.20 b</td>
<td>17.53</td>
</tr>
<tr>
<td>NS6 1 g/Kg</td>
<td>361.33 a</td>
<td>41.25</td>
</tr>
</tbody>
</table>

1 Means followed by the same letter are not significantly different according to one way ANOVA test at p < 0.05.
2 SD = Standard Deviation.

### Table 3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean serum triglyceride level (mg/dL)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (untreated)</td>
<td>1.547.3 a</td>
<td>932.2</td>
</tr>
<tr>
<td>Lepitor 20 mg/Kg</td>
<td>1.410.0 a</td>
<td>917.0</td>
</tr>
<tr>
<td>NS4 1 g/Kg</td>
<td>137.6 b</td>
<td>57.8</td>
</tr>
<tr>
<td>NS6 1 g/Kg</td>
<td>1.661.3 a</td>
<td>207.1</td>
</tr>
</tbody>
</table>

1 Means followed by the same letter are not significantly different according to one way ANOVA test at p < 0.05.
2 SD = Standard Deviation.

Administration of ethanol extract (NS4) and ethyl acetate extract (NS2) to model hyperlipidemic rats at 1 g/Kg, resulted in a significant reduction of serum cholesterols by 82.5 and 65.8%, respectively, and a significant reduction of serum triglyceride by 86.5 and 78.5%, respectively, as compared with the untreated control (Figures 3A-B and Tables 4-5). The petroleum ether extract (NS1) did not significantly affect the levels of serum cholesterols or triglyceride under the experimental conditions.

### Table 4

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean serum cholesterol level (mg/dL)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (untreated)</td>
<td>288.33 a</td>
<td>98.98</td>
</tr>
<tr>
<td>NS1 1 g/Kg</td>
<td>208.67 ab</td>
<td>144.51</td>
</tr>
<tr>
<td>NS2 1 g/Kg</td>
<td>98.67 bc</td>
<td>62.40</td>
</tr>
<tr>
<td>NS4 1 g/Kg</td>
<td>56.33 c</td>
<td>19.12</td>
</tr>
</tbody>
</table>

1 Means followed by the same letter are not significantly different according to one way ANOVA test at p < 0.05.
2 SD = Standard Deviation.
The effect of *Nigella sativa* extracts on the level of serum triglyceride in rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean serum triglyceride level (mg/dL)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (untreated)</td>
<td>1,713.30 a</td>
<td>814.70</td>
</tr>
<tr>
<td>NS1 1 g/Kg</td>
<td>1,262.00 ab</td>
<td>1,070.30</td>
</tr>
<tr>
<td>NS2 1 g/Kg</td>
<td>375.30 bc</td>
<td>409.50</td>
</tr>
<tr>
<td>NS4 1 g/Kg</td>
<td>144.00 c</td>
<td>106.60</td>
</tr>
</tbody>
</table>

Means followed by the same letter are not significantly different according to one way ANOVA test at p < 0.05.

Administration of acetonitrile extract (NS-AcN), hexane extract (NS-Hexane) and dioxane extract (NS-Dioxane) to model hyperlipidemic rats at 0.6 g/Kg, resulted in a significant reduction of serum cholesterol level by 72.9, 57.1 and 37.1%, respectively, and a significant reduction of serum triglyceride level by 80.2, 73.1 and 42.2%, respectively, as compared with the untreated control (Tables 6-7).

Table 6  
**The effect of *Nigella sativa* extracts on the level of serum cholesterol in rats**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean serum cholesterol level (mg/dL)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-1 (untreated)</td>
<td>245.4 a</td>
<td>145.69</td>
</tr>
<tr>
<td>Control-2 (untreated)</td>
<td>241.6 a</td>
<td>46.97</td>
</tr>
<tr>
<td>Control-3 (untreated)</td>
<td>223.0 ab</td>
<td>105.98</td>
</tr>
<tr>
<td>NS-Dioxane 0.6 g/Kg</td>
<td>148.8 ab</td>
<td>60.16</td>
</tr>
<tr>
<td>NS-Hexane 0.6 g/Kg</td>
<td>94.2 b</td>
<td>50.25</td>
</tr>
<tr>
<td>NS-AcN 0.6 g/Kg</td>
<td>101.6 b</td>
<td>48.17</td>
</tr>
</tbody>
</table>

Means followed by the same letter are not significantly different according to one way ANOVA test at p < 0.05.

Table 7  
**The effect of *Nigella sativa* extracts on the level of serum triglyceride in rats**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean serum triglyceride level (mg/dL)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-1 (untreated)</td>
<td>1,217.0 ab</td>
<td>882.3</td>
</tr>
<tr>
<td>Control-2 (untreated)</td>
<td>1,464.6 a</td>
<td>404.7</td>
</tr>
<tr>
<td>Control-3 (untreated)</td>
<td>995.8 ab</td>
<td>720.3</td>
</tr>
<tr>
<td>NS-Dioxane 0.6 g/Kg</td>
<td>708.8 ab</td>
<td>377.8</td>
</tr>
<tr>
<td>NS-Hexane 0.6 g/Kg</td>
<td>329.6 b</td>
<td>141.7</td>
</tr>
<tr>
<td>NS-AcN 0.6 g/Kg</td>
<td>242.4 b</td>
<td>307.8</td>
</tr>
</tbody>
</table>

Means followed by the same letter are not significantly different according to one way ANOVA test at p < 0.05.

Thus, the results clearly indicate that *Nigella sativa* ethanol extract (NS4), acetonitrile extract (NS-AcN), ethyl acetate extract (NS2) and hexane extract (NS-Hexane) are capable of substantially lowering serum cholesterol and triglycerides levels in hyperlipidemic model animals.
EXAMPLE 3

Chemical analysis of active extracts

Materials and methods:

Extracts: N. sativa ethanol extract (NS4) and acetonitrile extract (NS-AcN) were re-designated Pal 10 and Pal 20, respectively, for further evaluation and analysis.

Thin-layer chromatography (TLC): Active extracts were dissolved in dichloromethane: hexane : methanol (1:1:0.1, v/v/v) solvent and separated in a TLC (Merck) using a procedure essentially as described by Touchstone and Dobbins (1982).

GC/MS analysis: Headspace analyses were performed using Hewelett-Packard (HP-5972) mass spectrometer coupled with Hewelett-Packard (HP-6890) gas chromatograph. The Gas Chromatograph was equipped with a 20 m X 0.18 mm X 0.2 μm Quadrex 007-1 (100% dimethyl polysiloxane) capillary column. Helium was used as a carrier gas (about 0.6 ml/min).

Silylation analyses were performed in order to enhance the volatility of compounds which do not come out of the GC column (e.g., glycerol). The analyses were performed using BSTFA silylating agent (Fluka)

$^1$H-NMR analysis: Proton nuclear magnetic-resonance ($^1$H-NMR) analyses were performed using Varian model unity plus, 500 MHz and Brucker 400 MHz spectrometer. $^1$H-NMR Spectra were obtained in deuto-Chloroform (CDCl₃) (Merck). Chemical shifts (δ) are reported in parts per million (ppm) downfield from tetramethysilane.

FAB/MS analysis: Analyses were performed using Fast-Atom Bombardment (Micromass, VG Prospec) and Electrospray Mass Spectrometry (ES/MS; Finnigen MAT model LCQ).

LC/MS analysis: Mass spectrometry was conducted using (LC-MS/MS, Micromass Quattro Utima) in direct injection (ESI) mode. The source temperature of the mass spectrometer was set to 120°C, with a cone gas flow of 112 l/h and desolvation gas flow of 598 l/h. Peak spectra were monitored in scan mode ranging between 100 and 1000 m/z.

Results:
As can be seen in Figure 4, TLC analyses of Pal-10 (ethanol extract) and Pal-20 (acetonitrile extract) resulted in similar profiles comprising six distinct spots having Rf values of 0.81, 0.72, 0.47, 0.34, 0.22, and 0.12.

GC/MS analysis of extract Pal-20 identified putative glycerol; fatty acids 16:0, 18:2:Me, 18:2, 18:1, 18:0; Monopalmitin; glycerol trimethylsilyl ether; 1-Monolinoleoylglycerol trimethylsilyl ether; and 1-Monooleoylglycerol trimethylsilyl ether (Figure 12-13).

$^1$H NMR analysis of extract Pal-20 identified eight putative functional groups including fatty acids and glycerol esters (Figure 14, Table 8).

<table>
<thead>
<tr>
<th>Table 8: $^1$H NMR analysis of N. sativa extract Pal-20</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMR value (ppm)</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>0.88 (t)</td>
</tr>
<tr>
<td>1.2 (m)</td>
</tr>
<tr>
<td>1.6 (m)</td>
</tr>
<tr>
<td>2.0 (m)</td>
</tr>
<tr>
<td>2.3 (t)</td>
</tr>
<tr>
<td>2.7 (t)</td>
</tr>
<tr>
<td>4.1 (m)</td>
</tr>
<tr>
<td>5.3 (m)</td>
</tr>
</tbody>
</table>

FAB/MS analysis of Pal-20 uncovered peaks at 618, 602, 600, 578, 576, 522, 520, 496, 493, 387, 337, 313, 279 and 273 m/z (Figure 15).

**EXmkle 4**

The effect of Nigella sativa extract Pal-20 and fractions thereof on lowering serum cholesterol and triglyceride levels in hyperlipidemic model rats

Materials and methods:

Animal maintenance: Male Sprague Dawley rats were maintained as described in Example 1 hereinafter.

Fractionation of N. sativa acetonitrile extract (Pal-20):

A 1,000 ml silica gel column (230-400 mesh, Merck Cat# 109-385-2500) was eluted with one volume (1,000 ml) of hexane. A 100 ml sample of Pal-20 extract was then loaded into the column, allowed to penetrate into the silica gel then eluted sequentially with organic solvents to separate the extract sample different fractions.

The column was first eluted with one volume (1,000 ml) of dichloromethane
and hexane (500 ml each) yielding fraction Pal-400.

The column was then eluted with one volume (1,000 ml) of dichloromethane yielding fraction Pal-401.

The column was then eluted with one volume (1,000 ml) of dichloromethane yielding fraction Pal-402.

The column was then eluted with one volume (1,000 ml) of dichloromethane yielding fraction Pal-403.

The column was then eluted with one volume (1,000 ml) of a mixture of dichloromethane, hexane and methanol (495, 495 and 10 ml, respectively) yielding fraction Pal-404A.

The column was then eluted with one volume (1,000 ml) of a mixture of dichloromethane, hexane and methanol (495, 495 and 10 ml, respectively) yielding fraction Pal-404B.

The column was then eluted with one volume (1,000 ml) of a mixture of dichloromethane, hexane and methanol (490, 490 and 20 ml, respectively) yielding fraction Pal-404C.

The column was then eluted with one volume (1,000 ml) of a mixture of dichloromethane, hexane and methanol (490, 490 and 20 ml, respectively) yielding fraction Pal-404D.

The column was then eluted with one volume (1,000 ml) of a mixture of dichloromethane, hexane and methanol (485, 485 and 30 ml, respectively) yielding fraction Pal-405.

The column was then eluted with one volume (1,000 ml) of a mixture of dichloromethane, hexane and methanol (485, 485 and 30 ml, respectively) yielding fraction Pal-406.

Fractions Pal-404A-D were mixed together and designated as fraction Pal-400.

_Evaluation of serum lipids lowering capacity:_ The capacity of test substances (e.g., fractions and sub-fractions of _Nigella sativa_ organic extracts) to lower levels of serum cholesterol and/or triglyceride in hyperlipidemic model rats was determined as described in Example 2 hereinabove.
Results:

The administration of Pal-20 (acetonitrile extract) fractions Pal-404, Pal-405 and Pal-406 to model hyperlipidemic rats at 100 mg/Kg, resulted in a significant reduction of serum cholesterol level by 54.8, 74.5% and 72.9% %, respectively, and a significant reduction of serum triglyceride level by 61.0, 84.9 and 88.7%, respectively, as compared with the untreated control (Figures 8A-B, Tables 9-10).

<table>
<thead>
<tr>
<th>Treatment (100 mg/Kg)</th>
<th>Mean serum cholesterol level (mg/dL)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (untreated)</td>
<td>339.23 a</td>
<td>42.35</td>
</tr>
<tr>
<td>Pal-403</td>
<td>245.43 ab</td>
<td>98.36</td>
</tr>
<tr>
<td>Pal-404</td>
<td>153.53 bc</td>
<td>101.87</td>
</tr>
<tr>
<td>Pal-405</td>
<td>86.58 c</td>
<td>26.39</td>
</tr>
<tr>
<td>Pal-406</td>
<td>91.90 c</td>
<td>9.73</td>
</tr>
</tbody>
</table>

1 Means followed by the same letter are not significantly different according to one way ANOVA test at p < 0.05.
2 SD = Standard Deviation.

<table>
<thead>
<tr>
<th>Treatment (100 mg/Kg)</th>
<th>Mean serum triglyceride level (mg/dL)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (untreated)</td>
<td>1,151.60 a</td>
<td>165.40</td>
</tr>
<tr>
<td>Pal-403</td>
<td>614.30 ab</td>
<td>280.90</td>
</tr>
<tr>
<td>Pal-404</td>
<td>448.80 bc</td>
<td>442.80</td>
</tr>
<tr>
<td>Pal-405</td>
<td>173.70 c</td>
<td>51.50</td>
</tr>
<tr>
<td>Pal-406</td>
<td>130.1 c</td>
<td>43.50</td>
</tr>
</tbody>
</table>

1 Means followed by the same letter are not significantly different according to one way ANOVA test at p < 0.05.
2 SD = Standard Deviation.

The administration of fraction Pal-406 to model hyperlipidemic rats at 50 mg/Kg resulted in a significant reduction of serum cholesterol and triglyceride levels, by 54.4 and 64.8%, respectively, as compared with the untreated control (Tables 11-12).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean serum cholesterol level (mg/dL)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (untreated)</td>
<td>382.40 a</td>
<td>44.58</td>
</tr>
<tr>
<td>Pal-20 (400 mg/Kg)</td>
<td>151.00 b</td>
<td>68.49</td>
</tr>
<tr>
<td>Pal-403 (50 mg/Kg)</td>
<td>302.20 ab</td>
<td>37.22</td>
</tr>
<tr>
<td>Pal-404 (50 mg/Kg)</td>
<td>316.20 ab</td>
<td>65.47</td>
</tr>
<tr>
<td>Pal-405 (50 mg/Kg)</td>
<td>324.40 ab</td>
<td>135.41</td>
</tr>
</tbody>
</table>
Table 12

The effect of fractions of extract Pal-20 on the level of serum triglyceride in rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean serum triglyceride level (mg/dL)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (untreated)</td>
<td>2,290.40 a</td>
<td>360</td>
</tr>
<tr>
<td>Pal-20 (400 mg/Kg)</td>
<td>544.00 c</td>
<td>357.90</td>
</tr>
<tr>
<td>Pal-403 (50 mg/Kg)</td>
<td>1,839.60 ab</td>
<td>331.10</td>
</tr>
<tr>
<td>Pal-404 (50 mg/Kg)</td>
<td>1,799.40 ab</td>
<td>452.50</td>
</tr>
<tr>
<td>Pal-405 (50 mg/Kg)</td>
<td>1,908.40 ab</td>
<td>979.70</td>
</tr>
<tr>
<td>Pal-406 (50 mg/Kg)</td>
<td>805.20 bc</td>
<td>791.40</td>
</tr>
</tbody>
</table>

1 Means followed by the same letter are not significantly different according to one way ANOVA test at p < 0.05.
2 SD = Standard Deviation.

EXAMPLE 5

The effect of Nigella sativa extract fraction Pal-405 and sub-fractions thereof on lowering serum cholesterol and triglyceride levels in hyperlipidemic model rats

Materials and methods:

Animal maintenance: Male Sprague Dawley rats were maintained as described in Example 1 hereinabove.

Sub-fractionation of fraction Pal-405: Fraction Pal-405 was subjected to further fractionation using Sephadex LH20 (Pharmacia Biotech; prepared according to manufacturers instructions). A 0.5 gram sample of fraction Pal-405 was added to 30 cm column and eluted with dichloromethane. Three fractions (5 ml each) were collected sequentially and designated Pal-405-1, Pal-405-2 and Pal-405-3.

Evaluation of serum lipids lowering capacity: The capacity of test substances (e.g., fractions and sub-fractions of *Nigella sativa* organic extracts) to lower levels of serum cholesterol and/or triglyceride in hyperlipidemic model rats was determined as described in Example 2 hereinabove.

Results:

The administration of sub-fractions Pal-405-2 and Pal-405-3 (at 100 mg/Kg) and volatile fraction 101 (at 50 mg/Kg) to model hyperlipidemic rats, significantly reduced the level of serum cholesterol (by 29.9, 29.1 and 49.7% %, respectively) and significantly reduced the level of serum triglycerides (by 46.4, 54.4 and 72.1%,
respectively), as compared with the untreated control (Figures 8A-B, Tables 13-14). On the other hand, sub-fraction Pal-405-1 (administered at 100 mg/Kg) and fractions Pal-102, Pal-103 and Pal-104 (administered at 300 mg/Kg) did not significantly lower the level of serum cholesterol and triglycerides.

**Table 13**
The effect of different N. sativa extract-fractions and sub-fractions on the level of serum cholesterol in rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean serum cholesterol level (mg/dL)</th>
<th>SD ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (untreated)</td>
<td>269.75 a</td>
<td>21.32</td>
</tr>
<tr>
<td>Pal-101 (50 mg/Kg)</td>
<td>135.74 c</td>
<td>44.27</td>
</tr>
<tr>
<td>Pal-405 (200 mg/Kg)</td>
<td>200.10 abc</td>
<td>16.90</td>
</tr>
<tr>
<td>Pal-405-1 (100 mg/Kg)</td>
<td>244.00 ab</td>
<td>98.17</td>
</tr>
<tr>
<td>Pal-405-2 (100 mg/Kg)</td>
<td>189.18 bc</td>
<td>32.94</td>
</tr>
<tr>
<td>Pal-405-3 (100 mg/Kg)</td>
<td>191.13 bc</td>
<td>27.38</td>
</tr>
</tbody>
</table>

¹ Means followed by the same letter are not significantly different according to one way ANOVA test at p < 0.05.
² SD = Standard Deviation.

**Table 14**
The effect of different N. sativa extract-fractions and sub-fractions on the level of serum triglyceride in rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean serum triglyceride level (mg/dL)</th>
<th>SD ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (untreated)</td>
<td>1,062.80 a</td>
<td>233.50</td>
</tr>
<tr>
<td>Pal-101 (50 mg/Kg)</td>
<td>296.50 b</td>
<td>121.10</td>
</tr>
<tr>
<td>Pal-405 (200 mg/Kg)</td>
<td>495.60 b</td>
<td>85.0</td>
</tr>
<tr>
<td>Pal-405-1 (100 mg/Kg)</td>
<td>1,001.10 a</td>
<td>487.70</td>
</tr>
<tr>
<td>Pal-405-2 (100 mg/Kg)</td>
<td>569.50 ab</td>
<td>173.20</td>
</tr>
<tr>
<td>Pal-405-3 (100 mg/Kg)</td>
<td>484.90 b</td>
<td>83.20</td>
</tr>
</tbody>
</table>

¹ Means followed by the same letter are not significantly different according to one way ANOVA test at p < 0.05.
² SD = Standard Deviation.

**EXAMPLE 6**
The effect of Nigella sativa extract fraction Pal-406 and sub-fractions thereof on lowering serum cholesterol and triglyceride levels in hyperlipidemic model rats

**Animal maintenance:** Male Sprague Dawley rats were maintained as described in Example 1 hereinafter.

**Sub-fractionation of fraction Pal-406:** Fraction Pal-406 was generated as described in Example 4 above and was subjected to further fractionation using the following procedure.

A 1,000 ml silica gel column (230-400 mesh, Merck Cat# 109-385-2500) was
eluted with one volume (1,000 ml) of hexane. A 100 ml sample of fraction Pal-406 was then loaded into the column, allowed to penetrate into the silica gel then eluted sequentially with organic solvents to separate the extract sample different sub-fractions.

The column was first eluted with one volume (1,000 ml) of a mixture of hexane : dichloromethane : dichloromethane with 0.25% methanol : dichloromethane with 0.5% methanol (0.25 : 2 : 2 : 4, by volume), yielding sub-fraction Pal-406-F4.

The column was then eluted with one volume (1,000 ml) of dichloromethane yielding sub-fraction Pal-406-F2.

The column was first eluted with two volumes (2,000 ml) of a mixture of dichloromethane with 1% methanol, yielding sub-fraction Pal-406-F5.

The column was first eluted with two volumes (2,000 ml) of a mixture of dichloromethane with 1.5% methanol, yielding sub-fraction Pal-406-F6.

The column was first eluted with two volumes (2,000 ml) of a mixture of dichloromethane with 2% methanol, yielding sub-fraction Pal-406-F7.

The column was first eluted with two volumes (2,000 ml) of a mixture of dichloromethane with 2.5% methanol, yielding sub-fraction Pal-406-F8.

Sub-fractions Pal-406-F6 and Pal-606-F7 allowed to air dry and were then separated into gelatinous (liquid) and solid portions. The liquid and solid portions of sub-fraction Pal-406-F6 were designated Pal-406-F6.1 and Pal-605-F6.2, respectively. Similarly, the liquid and solid portions of sub-fraction Pal-406-F7 were designated Pal-406-F7.1 and Pal-605-F7.2, respectively.

**Evaluation of serum lipids lowering capacity:** The capacity of test substances (Pal-406 sub-fractions) to lower levels of serum cholesterol and/or triglyceride in hyperlipidemic model rats was determined either intraperitoneally as described in Example 2 hereinabove, or by oral administration. Oral administration was effected using rats weighing 200 g. Two hours following oral administration, 400 mg Tyloxapol was provided to each of animal by gavages. The levels of serum cholesterol and triglycerides were determined 24 hr post oral administration.

**Results:**

Administration of sub-fractions Pal-406-F6.2, Pal-406-F6.1 and Pal-406-F7.1 (IP at 50 mg/Kg) to model hyperlipidemic rats, significantly reduced the level of
serum cholesterol (by 51.3, 39.9 and 40.2%, respectively; Table 15) and significantly reduced the level of serum triglycerides (by 69.5, 71.2 and 57.4% respectively; Table 16), as compared with the untreated control. On the other hand, sub-fraction Pal-406-F7.2 and 406-F8 did not significantly lower the level of serum cholesterol and triglycerides.

| Table 15 |
|---|---|
| **The effect of different Pal-406 sub-fractions on the level of serum cholesterol in rats** |
| Treatment | Mean serum cholesterol level (mg/dL) | SD¹ |
| Control (untreated) | 279.41 a | 71.57 |
| Pal-406-5 | 171.12 b | 70.20 |
| Pal-406-F6.1 | 168.06 b | 66.20 |
| Pal-406-F6.2 | 136.08 c | 54.32 |
| Pal-406-F7.1 | 167.03 b | 83.19 |
| Pal-406-F7.2 | 228.66 ab | 23.81 |
| Pal-606-F8 | 266.12 ab | 30.51 |

¹ Means followed by the same letter are not significantly different according to one way ANOVA test at p < 0.05.

² SD = Standard Deviation.

| Table 16 |
|---|---|
| **The effect of different Pal-406 sub-fractions on the level of serum triglyceride in rats** |
| Treatment | Mean serum triglyceride level (mg/dL) | SD² |
| Control (untreated) | 1038.9 a | 369.3 |
| Pal-406-F5 | 597.2 ab | 340.4 |
| Pal-406-F6.1 | 299.2 b | 182.1 |
| Pal-406-F6.2 | 316.4 b | 170.3 |
| Pal-406-F7.1 | 443.0 b | 253.2 |
| Pal-406-F7.2 | 614.8 ab | 188.3 |
| Pal-606-F8 | 810.3 ab | 316.6 |

¹ Means followed by the same letter are not significantly different according to one way ANOVA test at p < 0.05.

² SD = Standard Deviation.

The effect of oral administration of Pal-406 on lowering serum levels of cholesterol and triglycerides was evaluated by dissolving the test substance in either 100% propylene glycol (PG), or in a mixture of 66% PG and 33% methylene cellulose (PG/MC) prior to oral administration. The dissolved test substance (fraction Pal-406) was administered to the animals at a dose of 125 mg/Kg (25 mg/200 g animal). As can be seen in Figures 13A-B, oral administration of fraction Pal-406 was substantially more effective on lowering serum levels of cholesterol and triglycerides when the substance was dissolved in PG only, as compared with the
PG/MC mixture.

Oral administration of sub-fraction Pal-406-6.2 dissolved in PG at 50 and 100 mg/Kg significantly reduced the level of serum cholesterol by 18.9 and 29.9%, respectively) and significantly reduced the level of serum triglycerides (by 37.2 and 42.2% respectively) in model hyperlipidemic rats, as compared with the untreated control. On the other hand, oral administration of sub-fraction Pal-406-F6.1 was only moderately effective on lowering the level of serum triglycerides and did not substantially lower the level serum cholesterol under similar experimental conditions.

Hence, the results show that fraction Pal-406 is capable of substantially lowering the level of serum cholesterol and triglycerides when administered to model hyperlipidemic animals either orally or intraperitoneally. The results further show that the most active sub-fraction of Pal-406 is sub-fraction Pal-406-F6.2.

**EXAMPLE 7**

*The effect of fatty acids and fatty acid esters on the levels of serum cholesterol and triglyceride in hyperlipidemic model rats*

Since *N. sativa* organic extracts and certain extract fractions (e.g., Pal-403, Pal-404) may contain lipid compounds, the activity of selected pure fatty acids and fatty acid esters on lowering the levels of serum cholesterol and triglyceride has been evaluated.

**Materials and methods:**

**Animal maintenance:** Male Sprague Dawley rats were maintained as described in Example 1 hereinafore.

**Fatty acids and fatty-acid esters:** Linoleic acid oleic acid, stearic acid, linoleic acid ethyl ester, linoleic acid methyl ester, oleic acid ethyl ester, oleic acid methyl ester and palmitic acid ethyl ester were obtained from Sigma-Aldrich.

**Evaluation of Serum lipids lowering activity:** The capacity of fatty acids and fatty acid esters to reduce serum cholesterol and triglyceride in hyperlipidemia model rats was determined using the procedure as described in Example 2 hereinafore.

**Results:**

Since fatty acids and fatty acid esters are present in active *N. sativa* extracts, the serum lipids lowering capacity of pure fatty acids and fatty-acid esters was
evaluated under similar experimental conditions as were used in evaluating *N. sativa* organic extracts and fractions thereof (Examples 2 and 4).

The administration of linoleic acid to model hyperlipidemic rats at 100 mg/Kg resulted in lowering serum cholesterol and triglyceride levels by 53 and 71%, respectively, as compared with the untreated control (Figures 10A-B, Tables 17-18). However, these differences were not statistically significant according to the experimental conditions.

All other substances tested (oleic acid, stearic acid, linoleic acid ethyl ester, linoleic acid methyl ester, oleic acid ethyl ester, oleic acid methyl ester and palmitic acid ethyl ester) failed to reduce the levels of serum cholesterol or triglyceride in the model rats under experimental conditions (Figures 10A-B and 11A-B; Tables 17-20).

### Table 17

**The effect of different fatty acids on the level of serum cholesterol in rats**

<table>
<thead>
<tr>
<th>Treatment (100 mg/Kg)</th>
<th>Mean serum cholesterol level (mg/dL)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (untreated)</td>
<td>284.20 a</td>
<td>124.50</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>132.80 a</td>
<td>92.60</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>240.60 a</td>
<td>50.70</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>207.60 a</td>
<td>125.30</td>
</tr>
</tbody>
</table>

1 Means followed by the same letter are not significantly different according to one way ANOVA test at *p* < 0.05.  
2 SD = Standard Deviation.

### Table 18

**The effect of different fatty acids on the level of serum triglyceride in rats**

<table>
<thead>
<tr>
<th>Treatment (100 mg/Kg)</th>
<th>Mean serum triglyceride level (mg/dL)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (untreated)</td>
<td>1,542.00 a</td>
<td>847.20</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>438.20 a</td>
<td>450.90</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>933.20 a</td>
<td>392.40</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>975.80 a</td>
<td>753.60</td>
</tr>
</tbody>
</table>

1 Means followed by the same letter are not significantly different according to one way ANOVA test at *p* < 0.05.  
2 SD = Standard Deviation.

### Table 19

**The effect of different fatty acid esters on the level of serum cholesterol in rats**

<table>
<thead>
<tr>
<th>Treatment (100 mg/Kg)</th>
<th>Mean serum cholesterol level (mg/dL)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (untreated)</td>
<td>175.00</td>
<td>94.0</td>
</tr>
<tr>
<td>Linoleic acid EE</td>
<td>228.60</td>
<td>120.5</td>
</tr>
<tr>
<td>Linoleic acid ME</td>
<td>249.20</td>
<td>33.20</td>
</tr>
<tr>
<td>Oleic acid EE</td>
<td>192.40</td>
<td>112.0</td>
</tr>
<tr>
<td>Oleic acid ME</td>
<td>154.60</td>
<td>98.0</td>
</tr>
</tbody>
</table>
Table 20
The effect of different fatty acid esters on the level of serum triglyceride in rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean serum triglyceride level (mg/dL)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (untreated)</td>
<td>898</td>
<td>n/a</td>
</tr>
<tr>
<td>Linoleic acid EE</td>
<td>1,286</td>
<td>n/a</td>
</tr>
<tr>
<td>Linoleic acid ME</td>
<td>1,468</td>
<td>n/a</td>
</tr>
<tr>
<td>Oleic acid EE</td>
<td>1,012</td>
<td>n/a</td>
</tr>
<tr>
<td>Oleic acid ME</td>
<td>816</td>
<td>n/a</td>
</tr>
<tr>
<td>Palmitic acid EE</td>
<td>1,374</td>
<td>n/a</td>
</tr>
</tbody>
</table>

1 SD = Standard Deviation.
2 EE = ethyl ester; ME = methyl ester.

EXAMPLE 8
The effect of flavonoids on the levels of serum cholesterol and triglyceride in hyperlipidemic model rats

Since N. sativa extracts contain a variety of flavonoids the activity of selected pure compounds on lowering the levels of serum cholesterol and triglyceride has been evaluated.

Materials and methods:
Animal maintenance: Male Sprague Dawley rats were maintained as described in Example 1 hereinabove.

Flavonoids: 3,6 dihydroxy falvone (J18), 3,7 dihydroxy flavone (J19), chrysin 5,7 dihydroxyflavone (J20), catechin hydrate (J22), Morin, 2',3,4',5,7-pentahydroxyflavone (J30) and Baicalein, 5,6,7-trihydroxyflavone were obtained from Sigma-Aldrich.

Evaluation of serum lipids lowering activity: The capacity of pure flavonoids to reduce serum cholesterol and triglyceride in hyperlipidemia model rats was determined using the procedure as described in Example 2 hereinabove.

Results:
The administration of chrysin 5,7 dihydroxyflavone (J20), 3,6 dihydroxy falvone (J18) and 3,7 dihydroxy Flavone (J19) to model hyperlipidemic rats at 30 mg/Kg resulted in lowering serum cholesterol levels by 31.2, 18.9 and 5.9%, respectively (Figure 15A) and in lowering serum triglycerides levels by 38, 20.7%
and 7.9%, respectively (Figure 15B), as compared with the untreated control. On the other hand, the administration of catechin hydrate (J22), Morin, 2',3,4',5,7-Pentahydroxyflavone (J30) and Baicalein, 5,6,7-trihydroxyflavone (J31) resulted in higher levels of serum cholesterol and triglycerides in the treated animals.

EXAMPLE 9

Chemical analysis of active fractions and sub-fractions

Materials and methods:

Chemical analyses: TLC, GC/MS, ¹H-NMR, FAB/MS and LC/MS analyses were performed as described in Example 3 hereinabove.

Results:

TLC analysis of Pal-10 (ethanol) extract fractions identified substances migrating to Rf values of 0.22 and 0.72 (spot Nos. 5 and 2) in active fraction Pal-307 and substance(s) migrating to Rf 0.22 (spot No. 5) in active fraction Pal-312 (Figure 5A).

TLC analysis of Pal-20 (acetonitrile) extract fractions identified substances migrating to Rf values of 0.47, 0.34, 0.22, and 0.12 (spot Nos. 3-6) in active fraction Pal-405 and substances migrating to Rf 0.12 (spot No. 6) in active fraction Pal-406 (Figure 5B).

TLC analysis of Pal-405 sub-fractions identified substance(s) migrating to Rf 0.12 (spot No. 6) in active sub-fractions Pal-405-2 and Pal-405-3. However, no substance migrating to Rf 0.12 was present in the non-active sub-fraction Pal-405-3 (Figure 7).

TLC analysis of Pal-406 sub-fractions identified substance(s) migrating to Rf 0.12 (spot No. 6) in all sub-fractions when dichloromethane with 2% methanol was used as mobile phase solvent (Figures 12A). On the other hand, when the mobile phase solvent was petroleum ether and ethyl acetate (1:1), sub-fractions F7 and F8 gave different spot profile (Figure 12B).

GC/MS analysis of active fraction Pal-405 identified putative fatty acids 16:0, 18:2, 20:2; Glycerol-ester (2OH) and Glycerol (2OH)-18:2 (Table 21).
Table 21: GC/MS analysis of fractions of N. sativa Pal-20 extract

<table>
<thead>
<tr>
<th>Test sample</th>
<th>Sample code</th>
<th>Putative compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pal-401</td>
<td>SL-196-1</td>
<td>Fatty acids 16:0, 18:1, 18:2, 18:0, 20:2; 18:2-ethyl ester,</td>
</tr>
<tr>
<td>Pal-402</td>
<td>SL-196-2</td>
<td>Fatty acids 16:0, 18:2, 18:1, 18:0, 20:2</td>
</tr>
<tr>
<td>Pal-403</td>
<td>SL-193-3</td>
<td>Fatty acids 16:0, 18:2, 18:1, 18:0, 20:2</td>
</tr>
<tr>
<td>Pal-404</td>
<td>SL-196-4</td>
<td>Fatty acids 16:0, 18:2, 18:0, 20:2</td>
</tr>
<tr>
<td>Pal-405</td>
<td>SL-197-5</td>
<td>Fatty acids 16:0, 18:2, 20:2; Gly-ester (2OH), Gly (2OH)-18:2</td>
</tr>
</tbody>
</table>

GC/MS analysis of active fraction 101 identified the following putative compounds: palmitic acid, linoleic acid, ethyl stearate, ethyl oleate, ethyl linoleate, ethyl laurate, ethyl myristate, ethyl palmitate, ethyl-9-hexadecanoate, thymoquininone, carvacrol, longifolene, elimicin and thymo-hydroquinone.


FAB/MS analyses of active fraction Pal-400; active sub-fractions Pal-405-2 and Pal-405-3; non-active fractions Pal-400, Pal-401, Pal-402, Pal-403 and Pal-404; and non-active sub-fraction Pal-405-1 are illustrated in Figures 25,27,29,31,33,35,37,39 and 41 and in Table 22. Comparative evaluation of the FAB/MS spectra uncovered peaks which are present in the active fractions/sub-fractions but absent, or present in substantially reduced amounts, in the non-active fractions/sub-fractions (Table 23). Accordingly, the active fraction Pal-405 and active sub-fractions Pal-405-2 and Pal-405-3 have been uniquely characterized by having FAB/MS spectrum peaks at 355, 357, 447 and 463 m/z and by including substantially increased amount of substance(s) having a FAB/MS spectrum peak of 263.

Table 22: FAB/MS peaks identified in fractions and sub-fractions of N. sativa extract Pal-20

<table>
<thead>
<tr>
<th>Sample</th>
<th>M/Z value 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>C Pal-20</td>
<td>618, 616, 602, 600, 578, 576, 339, 337, 313, 263, 239</td>
</tr>
<tr>
<td>Pal-400</td>
<td>882, 880, 856, 602, 600, 576, 339, 373, 337, 309, 307, 279, 265, 263, 239</td>
</tr>
<tr>
<td>Pal-402</td>
<td>946, 896, 666, 618, 616, 602, 573, 561, 559, 545, 279, 263</td>
</tr>
<tr>
<td>Pal-403</td>
<td>620, 618, 616, 602, 600, 578, 576, 339, 337, 313, 295, 279, 263, 239</td>
</tr>
<tr>
<td>Pal-405-1</td>
<td>618, 616, 602, 600, 578, 576, 491, 339, 337, 313, 279, 263, 239</td>
</tr>
</tbody>
</table>

1 M/Z values in bold indicate relatively high concentrations
Table 23

Summary of FAB/MS peaks uniquely identified in active fraction Pal-405, active sub-fractions Pal-405-2 and Pal-405-3 and non-active sub-fraction Pal-405-1

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>239</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>263</td>
<td>+</td>
<td>+</td>
<td>++(^{1})</td>
<td>++</td>
</tr>
<tr>
<td>265</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>279</td>
<td>+</td>
<td></td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>313</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>355</td>
<td></td>
<td></td>
<td></td>
<td>++</td>
</tr>
<tr>
<td>357</td>
<td></td>
<td></td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>445</td>
<td>+</td>
<td></td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>447</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>463</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
</tbody>
</table>

\(^{1}\) ++ indicates substantially higher concentration than +.

Comparative LC/MS analyses of active sub-fraction Pal-406-6.2 (Pal-606-662), moderately active sub-fraction Pal 406-F6.1 (Pal 406-661) and non-active sub-fraction Pal-406-5 are illustrated in Figure 19. The analyses indicate that the concentration of a compound or a group of compounds having a molecular weight ranging from 245-378 correlated with the biological activity of the sub-fractions.

Hence, the results show that *Nigella sativa* ethanol, acetonitrile, ethyl acetate and hexane extracts can be used effectively to lower serum cholesterol and/or triglyceride levels in model animals. Further fractionation and analyses of the active extracts uncovered fractions (Pal-405 and Pal-406) and sub-fractions (Pal-606-662, Pal-405-2 and Pal-405-3) being capable of substantially lowering serum cholesterol and/or triglycerides levels in the model animals upon IP administration. It was further uncovered that lowering of serum cholesterol and/or triglycerides levels can also be effected upon oral administration of fraction Pal-406 or sub-fraction 406-662.

Chemical analyses uncovered that biologically active fractions Pal-405 and Pal-406 are characterized by including substance or substances which migrate with an Rf value of 0.12 in a thin-layer-chromatography assay employing a 1:1:0.1 dichloromethane: hexane: methanol solvent system. It was further uncovered that active sub-fractions Pal-405-2 and Pal-405-3 are characterized by having FAB/MS spectrum peaks at 355, 357, 447 and 463 m/z. The most active sub-fraction Pal-406-F6.2 (Pal-406-662) is characterized by including increased concentrations of compounds having molecular weights ranging from 245 to 378.
It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable sub-combination.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims. All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent and patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention.
39
REFERENCES CITED
(Additional references are cited hereinafore)


21. Igarashi K, Uchida Y, Murakami N, Mizutani K, Masuda H. Effect of astilbin in tea processed from leaves of Engelhardtia chrysolepis on the serum and


WHAT IS CLAIMED IS:

1. A composition-of-matter for lowering serum cholesterol and/or triglyceride level, comprising an isolated fraction of a Nigella sativa organic extract said isolated fraction including at least one substance which migrates with an Rf value of about 0.12 in a thin-layer-chromatography assay employing a 1:1:0.1 dichloromethane:hexane:methanol solvent system.

2. The composition-of-matter of claim 1, wherein said isolated fraction includes at least one compound having a molecular weight ranging from 245 to 378.

3. The composition-of-matter of claim 1, wherein said isolated fraction is characterized by FAB/MS spectrum peaks at 355, 357, 447 and 463 m/z.

4. The composition-of-matter of claim 1, wherein said Nigella sativa organic extract is a Nigella sativa seed organic extract.

5. The composition-of-matter of claim 4, wherein said Nigella sativa seed organic extract is a Nigella sativa seed acetonitrile extract.

6. An article-of-manufacturing, comprising packaging material and a pharmaceutical composition identified for use in lowering serum cholesterol and/or triglyceride level being contained within the packaging material, said pharmaceutical composition including, as an active ingredient, an isolated fraction of a Nigella sativa organic extract including at least one substance which migrates with an Rf value of about 0.12 in a thin-layer-chromatography assay employing a 1:1:0.1 dichloromethane:hexane:methanol solvent system.

7. The article-of-manufacturing of claim 6, wherein said isolated fraction includes at least one compound having a molecular weight ranging from 245 to 378.

8. The article-of-manufacturing of claim 6, wherein said isolated fraction
being characterized by FAB/MS spectrum peaks at 355, 357, 447 and 463 m/z.

9. The article-of-manufacturing of claim 6, wherein said *Nigella sativa* organic extract is a *Nigella sativa* seed organic extract.

10. The article-of-manufacturing of claim 9, wherein said *Nigella sativa* seed organic extract is a *Nigella sativa* seed acetonitrile extract.

11. A method of producing a composition-of-matter capable of lowering serum cholesterol and/or triglyceride level, comprising fractionating a *Nigella sativa* organic extract to thereby obtain a fraction including at least one substance which migrates with an Rf value of about 0.12 in a thin-layer-chromatography assay employing a 1:1:0.1 dichloromethane: hexane : methanol solvent system, said fraction being capable of lowering serum cholesterol and/or triglyceride level.

12. The method of claim 11, wherein said fractionating is effected by a liquid chromatography.

13. The method of claim 11, wherein said *Nigella sativa* organic extract is a *Nigella sativa* seed organic extract.

14. The method of claim 13, wherein said *Nigella sativa* seed organic extract is a *Nigella sativa* seed acetonitrile extract.

15. The method of claim 13, wherein said isolated fraction includes at least one compound having a molecular weight ranging from 245 to 378.

16. The method of claim 13, wherein said isolated fraction being characterized by FAB/MS spectrum peaks at 355, 357, 447 and 463 m/z.

17. A method of lowering serum cholesterol and/or triglyceride level, comprising administering to a subject in need thereof an effective amount of an
isolated fraction of a *Nigella sativa* organic extract said isolated fraction including at least one substance which migrates with an Rf value of about 0.12 in a thin-layer-chromatography assay employing a 1:1:0.1 dichloromethane: hexane : methanol solvent system, thereby lowering serum cholesterol and/or triglyceride level in said subject.

18. The method of claim 17, wherein said isolated fraction includes at least one compound having a molecular weight ranging from 245 to 378.

19. The method of claim 17, wherein said isolated fraction being characterized by FAB/MS spectrum peaks at 355, 357, 447 and 463 m/z.

20. The method of claim 17, wherein said subject is a human.

21. The method of claim 17, wherein said administering is effected parenterally.

22. The method of claim 17, wherein said administering is effected orally.

23. The method of claim 17, wherein said effective amount is at a dosage ranging from 0.1 to 100 mg/Kg/day.

24. The method of claim 17, wherein said *Nigella sativa* organic extract is a *Nigella sativa* seed organic extract.

25. The method of claim 24, wherein said *Nigella sativa* seed organic extract is a *Nigella sativa* seed acetonitrile extract.

26. A method of treating or preventing hypercholesterolemia, comprising administrating to a subject in need thereof an effective amount of an isolated fraction of a *Nigella sativa* organic extract said isolated fraction including at least one substance which migrates with an Rf value of about 0.12 in a thin-layer-
chromatography assay employing a 1:1:0.1 dichloromethane: hexane : methanol solvent system, thereby treating or preventing hypercholesterolemia in said subject.

27. The method of claim 26, wherein said isolated fraction includes at least one compound having a molecular weight ranging from 245 to 378.

28. The method of claim 26, wherein said isolated fraction being characterized by FAB/MS spectrum peaks at 355, 357, 447 and 463 m/z.

29. The method of claim 26, wherein said subject is a human.

30. The method of claim 26, wherein said administering is effected parenterally.

31. The method of claim 26, wherein said administering is effected orally.

32. The method of claim 26, wherein said effective amount is at a dosage ranging from 0.1 to 100 mg/Kg/day.

33. The method of claim 26, wherein said *Nigella sativa* organic extract is a *Nigella sativa* seed organic extract.

34. The method of claim 33, wherein said *Nigella sativa* seed organic extract is a *Nigella sativa* seed acetonitrile extract.

35. A method of treating or preventing hyperlipidemia, comprising administering to a subject in need thereof an effective amount of an isolated fraction of a *Nigella sativa* organic extract said isolated fraction at least one substance which migrates with an Rf value of about 0.12 in a thin-layer-chromatography assay employing a 1:1:0.1 dichloromethane: hexane : methanol solvent system, thereby treating or preventing hyperlipidemia in said subject.
36. The method of claim 35, wherein said isolated fraction includes at least one compound having a molecular weight ranging from 245 to 378.

37. The method of claim 35, wherein said isolated fraction being characterized by FAB/MS spectrum peaks at 355, 357, 447 and 463 m/z.

38. The method of claim 35, wherein said subject is a human.

39. The method of claim 35, wherein said administering is effected parenterally.

40. The method of claim 35, wherein said administering is effected orally.

41. The method of claim 35, wherein said effective amount is at a dosage ranging from 0.1 to 100 mg/Kg/day.

42. The method of claim 35, wherein said Nigella sativa organic extract is a Nigella sativa seed organic extract.

43. The method of claim 42, wherein said Nigella sativa seed organic extract is a Nigella sativa seed acetonitrile extract.

44. A method of treating or preventing atherosclerosis, comprising administrating to a subject in need thereof an effective amount of an isolated fraction of a Nigella sativa organic extract said isolated fraction at least one substance which migrates with an Rf value of about 0.12 in a thin-layer-chromatography assay employing a 1:1:0.1 dichloromethane: hexane : methanol solvent system, thereby treating or preventing atherosclerosis in said subject.

45. The method of claim 44, wherein said isolated fraction includes at least one compound having a molecular weight ranging from 245 to 378.
46. The method of claim 44, wherein said isolated fraction being characterized by FAB/MS spectrum peaks at 355, 357, 447 and 463 m/z.

47. The method of claim 44, wherein said subject is a human.

48. The method of claim 44, wherein said administering is effected parenterally.

49. The method of claim 44, wherein said administering is effected orally.

50. The method of claim 44, wherein said effective amount is at a dosage ranging from 0.1 to 100 mg/Kg/day.

51. The method of claim 44, wherein said *Nigella sativa* organic extract is a *Nigella sativa* seed organic extract.

52. The method of claim 51, wherein said *Nigella sativa* seed organic extract is a *Nigella sativa* seed acetonitrile extract.
Effect of Organic Extracts of NS on Lipid levels in Rats

Fig. 1a

Effect of Organic Extracts of NS on Lipid levels in Rats

Fig. 1b
Dose-Dependent effect of NS 4 and Lipitor on Lipids (Tyloxapol - treatment rats)

% Inhibition

0.1 g/kg  0.5 g/kg  1 g/kg  10 mg/kg  30 mg/kg
NSP4 Lipitor

Cholesterol  Triglyceride

Fig. 2
Effect of Organic Extracts of NS on Lipid levels in Rats

Fig. 3a

Effect of Organic Extracts of NS on Lipid levels in Rats

Fig. 3b
Fig. 4

Fig. 5a
Effect of NS fractions (100 mg/Kg), on Lipid levels in Rats

Fig. 7a

Effect of NS fractions (100 mg/Kg), on Lipid levels in Rats

Fig. 7b
Effect of NS fractions (100 mg/Kg), on Lipid levels on Rats

Fig. 8a

Effect of NS fractions (100 mg/Kg), on Lipid levels on Rats

Fig. 8b
Effect of NS fractions on Lipid levels in Rats

**Fig. 9a**

Effect of NS fractions on Lipid levels in Rats

**Fig. 9b**
Effect of Fatty acids (100mg/Kg) on Lipid levels on Rats

Fig. 10a

Effect of Fatty acids (100mg/Kg) on Lipid levels on Rats

Fig. 10b
Effect of Fatty acids (100mg/Kg) on Lipid levels in Rats

Fig. 11a

Effect of Fatty acids (100mg/Kg) on Lipid levels in Rats

Fig. 11b
Fig. 13a

Fig. 13b
Fig. 20a
Fig. 21a
Average of 6.117 to 6.156 min.; GRMS793.D (∗)

#257819: Trimethylsilyl ether of glycerol

Fig. 21b
#279141: Hexadecanoic acid, trimethylsilyl ester

Average of 10.678 to 10.691 min.: GRMSZ93.D (-)
Fig. 21 I

Average of 13.784 to 13.797 min.: GRMS793.D (-)

#368148: 1-Monolinoleoylglycerol trimethylsilyl ether §§9, 1

Abundance

m/z: 73

129

103

41

395

446

483

3000

2000

1000

0

50

100

150

200

m/z: 9000

8000

7000

6000

5000

4000

3000

2000

1000

0

50

100

150

200

250

300

350

400

450

500

207

147

34

103

249

177

305

30327

355

395

429

483
Fig. 21j

Average of 13.797 to 13.797 min.: GRMS793.D (-)

#368537: 1-Monolinoeleyglycerol trimethylsilyl ether $$ Octa
Fig. 23
Fig. 25
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**ACQUISITION**

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**Fig. 38**

ppm

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**Note:** The image contains a chart and a spectrum analysis with various parameters and values. The text is a mixture of numerical data and some chemical analysis steps.