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(54) Title: TUNICATE EXTRACTS AND USES THEREOF FOR TREATING METABOLIC DISORDERS

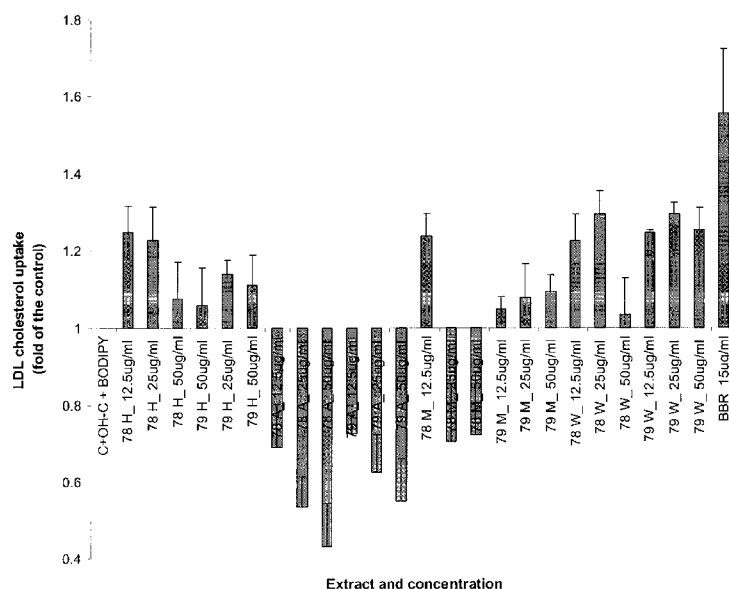


FIG. 3A

(57) Abstract: The present invention relates water extracts of tunicate. The water extracts may be obtained by extracting a tunicate sample selected from *Styela clava*, *Ciona intestinalis*, *Botrylloides violaceus*, *Botryllus schlosseri*, and any combination thereof with hot water; and collecting the tunicate extracts. Also provided are acetone extracts of tunicate, for example *Botrylloides violaceus*, *Botryllus schlosseri*, and any combination thereof. Methods of treating or preventing metabolic disorders by administration of the extracts of the present invention are provided. Specifically, a method of enhancing LDL-cholesterol uptake by administering the tunicate water extract is provided; also provided is a method of inhibiting adipogenesis by administering the acetone tunicate extract.



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TUNICATE EXTRACTS AND USES THEREOF FOR TREATING METABOLIC DISORDERSFIELD OF THE INVENTION

The present invention relates to tunicate extracts and uses thereof for treating metabolic disorders. More specifically, the invention relates to tunicate extracts and uses thereof for treating or preventing obesity and/or hyperlipidemia.

BACKGROUND OF THE INVENTION

Obesity, hyperlipidemia, and diabetes are three major disorders related to metabolic syndrome.

It is well-known that elevated circulating cholesterol levels, in particular low-density lipoprotein cholesterol (LDL-c), is one of the major risk factors for developing and accelerating the progression of cardiovascular disease (CVD) [1, 2], including coronary heart disease, disease of blood vessels, and stroke. Reducing blood cholesterol has long been a primary care approach for cardiovascular health and disease prevention. This approach aims to minimize the development of atherosclerosis, which is mainly caused by elevated blood cholesterol levels, especially LDL-C. Low-density lipoproteins are highly enriched with cholesterol and are easier than other lipoproteins to be taken up by the epithelial cells of blood vessels and macrophages; this leads to atherosclerotic plaque formation and development, which progresses to atherosclerosis, and possible vessel blockage and damage. The maintenance of circulating blood cholesterol levels is a result of the balance of cholesterol input from dietary source in addition to that synthesized within the body (*de novo* synthesis) *versus* the output or elimination of cholesterol via uptake, catabolism and clearance by the liver, bile secretion and fecal excretion. Enhancement of liver clearance LDL-C results in increased clearance of cholesterol from the body, which consequently lowers the circulating blood cholesterol levels [3].

Hyperlipidemia, which refers to high circulating levels of lipids including cholesterol and cholesterol esters, is mainly treated using statins – a family of drugs that inhibit cholesterol synthesis. While these drugs have been widely used and have made a significant contribution to reduction of blood lipids, side effects of varying severity, including death, have been reported [4, 5]; moreover, a sub-group of the population does not respond well to treatment with statins [6]. Another cholesterol-lowering drug is ezetimibe, which lowers blood cholesterol

by inhibiting cholesterol absorption. However, its efficacy is low; for example, the cholesterol-lowering efficacy of ezetimibe is similar or even lower than the popular natural products of the sterol/stanol family [7].

Compounds that can increase the expression or activity of LDL receptor in liver cells would enhance the uptake and clearance of LDL-C by the liver. Currently, no effective drug treatments or nutraceutical products are available to reduce blood cholesterol through up-regulation of LDL receptor expression. This is mainly due to limitations in methods for identifying such compounds.

Obesity, another metabolic disorder, has become a world-wide epidemic [8-12]. Obesity results from a positive balance of energy intake *versus* expenditure; this leads to adipogenesis, the formation and storage of fat and increased mass of fatty tissue. Balance of this metabolic process would be beneficial to the control of body weight in those that are at risk for obesity, and reduction or reversal of this process (negative energy balance) will result in weight loss. The socio-economic impact of obesity extends well beyond its direct effects on health; obesity can also cause other health problems or complications such as diabetes, dyslipidemia, hypertension, and cardiovascular problems [13-15]. This problem has been increasing in North America and other countries.

A number of programs and products are available to the treatment of obesity. Generally, obesity drugs work by regulating appetite, reducing the absorption of calories, or increasing energy expenditure [16]. However, side effects of the currently available weight loss drugs are of significant concern. Some anti-obesity drugs have severe and often life-threatening side effects (for example, fen-phen), which are often associated with the drug's mechanism of action [16]. For example, stimulants carry a risk of high blood pressure, faster heart rate, palpitations, closed-angle glaucoma, drug addiction, restlessness, agitation, and insomnia. Another drug, orlistat, blocks absorption of dietary fats, and as a result causes oily spotting bowel movements (steatorrhea), oily stools, stomach pain, and flatulence. The neural basis of appetite is not fully understood – nor is a method to modulate it. Drugs that abolish appetite seem to carry a higher risk of mortality and are thus unsuitable for clinical use.

In parallel with the increasing prevalence of obesity, diabetes has also achieved epidemic status [17-21]. More than 90% of diabetes patients suffer from non-insulin-dependent diabetes mellitus (NIDDM, type 2 diabetes). Type 2 Diabetes is associated with two principal physiological defects: resistance to the action of insulin resistance and a deficiency of insulin

secretion [22, 23]. The incidence of type 2 diabetes, which highly correlates with diet and lifestyle [22, 24], has particularly increased. Hyperglycemia can be managed in various ways. Some approaches include: 1) protection or regeneration of pancreatic beta cell mass and improvement of insulin secretion is one approach [25, 26]; 2) inhibition of the intestinal
5 breakdown of carbohydrates to decrease or slow glucose absorption [27]; and 3) increase insulin sensitivity. Alternatively, hyperglycemia may be managed via an increase of glucose clearance (uptake) in the peripheral tissues such as muscle and adipose tissues and/or an inhibition of glucose production by the liver [26, 28, 29]. When diabetes becomes severe or involves significant insulin deficiency (later stage of type 2 diabetes or type 1 diabetes), insulin
10 supplementation is essential.

Insulin-sensitizing drug such as rosiglitazone have been used for the treatment of diabetes; these compounds mainly target PPAR-gamma, which affects fat cell differentiation and insulin signalling [26]. The main disadvantage with this type of anti-diabetic drug is the increased risk of heart attack; this is of particular concern, since diabetics are already several times more
15 likely to suffer heart attacks [30]. Another significant side effect of this class of compounds (rosiglitazone) is weight gain, which in turn worsens insulin resistance. If used long-term, rosiglitazone results in loss of insulin efficiency, and patients eventually must utilize commercially-available insulin [30]. Another medication targeted to patients with type 2 diabetes is Acarbose, which partially blocks absorption of carbohydrates in the small intestine,
20 and produces side effects including stomach pain, and flatulence [31].

To prevent or reduce the impact of hyperlipidemia, obesity and diabetes on the quality of life and socioeconomic development, approaches relating to lifestyle changes to minimize the incidence and improve control of these diseases have met with limited success. People prefer solutions that can be easily incorporated in their daily life and health management.
25 Additionally, factors such as food culture, lifestyle, and substantial changes in the nature of jobs (reduction in manual labour) and increased sedentary behaviour associated with increase of leisure time all appear to be compounding factors.

Development of effective and safe natural health products that can be conveniently used as alternative treatment options or co-solutions to resolve or minimize the impact of
30 hyperlipidemia, obesity and diabetes has become increasingly important. Because most metabolic diseases (non-genetic) are lifestyle-related, they are generally thought to be preventable. Dietary changes have been employed but compliance appears to limit the effectiveness of this intervention, and the development of products such as dietary

supplements, nutraceuticals, and/or functional foods are being considered as prevention and treatment options prior to using an anti-obesity drug. Such products could have a modulatory role on metabolic processes that may prevent or delay the onset and progression of obesity, type-2 diabetes, or hyperlipidemia. In many cases, the safety profile of natural products are better than pharmaceuticals, and these are presently used widely by the general population as home care or self-management products to maintain their health or prevent potential health problems, in particular metabolic disorders.

SUMMARY OF THE INVENTION

The present invention relates to tunicate extracts and uses thereof for treating metabolic disorders. More specifically, the invention relates to tunicate extracts and uses thereof for treating or preventing obesity and/or hyperlipidemia.

There is increasing interest in developing effective and safe natural health products that can be used as alternative treatment options or co-solutions in treating, preventing, or delay the onset of metabolic disorders such as hyperlipidemia and/or obesity. The present invention provides methods and products as a means to attain this goal.

The present invention provides a method of preparing tunicate water extract comprising:

- a) extracting a *Styela clava*, *Ciona intestinalis*, *Botrylloides violaceus*, *Botryllus schlosseri* tunicate sample or any combination thereof with hot water; and
- b) collecting the tunicate water extract obtained in step a).

In the method as just described, the tunicate sample may be dried or lyophilized prior to step a); optionally, the dried or lyophilized sample may be milled prior to step a). Additionally, the method as described herein may comprise sequentially extracting the tunicate sample with hexane, acetone, methanol, and hot water in step a).

In the method as described herein, the water extract may be further processed by ethanol precipitation, chromatography, ultrafiltration, desalting, drying by rotatory evaporator, centrifugal vacuum evaporator and/or spray dryer, or any combination thereof. Additionally, the water extract may be further fractionated by ion exchange chromatography and/or size exclusion chromatography, and/or papain hydrolysis followed by precipitation at basic pH, yielding sub-fractions; optionally, the sub-fractions may be further processed by ethanol

precipitation, chromatography, ultrafiltration, desalting, drying by rotatory evaporator, centrifugal vacuum evaporator and/or spray dryer, or any combination thereof.

The present invention also provides a tunicate water extract, fraction, or sub-fraction thereof obtained from *Styela clava*, *Ciona intestinalis*, *Botrylloides violaceus*, *Botryllus schlosseri*, or
5 any combination thereof. In one non-limiting example, the tunicate water extract, fraction or sub-fraction thereof is obtained from *Styela clava*; in another non-limiting example, the extract, fraction or sub-fraction thereof is obtained from *Ciona intestinalis*; in yet another non-limiting example, the extract, fraction or sub-fraction thereof is obtained from a sample comprised of a combination of *Botrylloides violaceus* and *Botryllus schlosseri*. The tunicate water extract,
10 fraction or sub-fraction thereof may be obtained by the method as described herein.

The tunicate water extract or sub-fraction thereof of the present invention may be characterized by any one of the proton NMR spectrum of Figure 1D, 6, or 7. Additionally, the tunicate water extract or sub-fraction thereof of the present invention may comprise acetamide-substituted polysaccharides or protein glycans as the main components. The polysaccharides
15 and/or protein glycans in the water extract, fraction, or sub-fraction thereof may comprise a molecular weight in the range of about 50 KDa to 10 MDa. Furthermore, the polysaccharides in the main component of the water extract, fraction, or sub-fraction thereof may comprise galactose and glucose as the main sugar units.

The present invention additionally provides a tunicate acetone extract, fraction or sub-fraction thereof obtained from *Botrylloides violaceus*, *Botryllus schlosseri*, or any combination thereof.
20 The tunicate acetone extract, fraction or sub-fraction thereof may be obtained by the method as described herein. The tunicate acetone extract, fraction, or sub-fraction thereof of the present invention may be obtained by a method comprising:

- a) extracting a *Botrylloides violaceus*, *Botryllus schlosseri* tunicate sample or any
25 combination thereof with acetone; and
- b) collecting the tunicate acetone extract obtained in step a).

In the method as described, the tunicate sample may be dried or lyophilized prior to step a); optionally, the dried or lyophilized sample may be milled prior to step a). Additionally, the method as described herein may comprise sequentially extracting the tunicate sample with
30 hexane and acetone in step a).

In the method as described herein, the acetone extract may be further processed by chromatography, drying by rotatory evaporator, centrifugal vacuum evaporator, or any combination thereof. Additionally, the acetone extract may be further fractionated by normal or reverse phase chromatography, silica gel chromatography, or thin layer chromatography. The tunicate acetone extract of the present invention may be characterized by the proton NMR spectrum of Figure 1B (1).

The present invention further provides a method of treating, preventing, or delaying the onset of metabolic disorders by administering one or more than one extract of the present invention. The metabolic disorders may include hyperlipidemia and/or obesity. The treatment, prevention, or delay of onset of hyperlipidemia may be addressed by enhancing LDL-cholesterol uptake by the liver; treatment prevention, or delay of onset of obesity may be addressed by inhibiting adipogenesis.

Thus, the present invention provides a method of enhancing LDL-cholesterol uptake by the liver comprising administering one or more than one of the tunicate water extract or sub-fraction thereof of any one of the present invention to a subject in need thereof.

Also, the present invention provides a method of inhibiting adipogenesis comprising administering one or more than one of the water and/or acetone tunicate extract, fraction, or sub-fraction thereof obtained from *Botrylloides violaceus*, *Botryllus schlosseri* or combination thereof; the water extract, fraction, or sub-fraction thereof obtained from *Styela clava*; any mix of compounds or single compounds obtained therefrom; or any combination thereof to a subject in need thereof.

It is presently shown that water extracts of various types of tunicates (including *Styela clava*, *Ciona intestinalis*, *Botrylloides violaceus*, *Botryllus schlosseri*) can increase LDL cholesterol (LDL-C) uptake by liver cells. The increase or enhanced LDL-cholesterol uptake results in a reduction of plasma total and LDL cholesterol [3], which are both closely related to the development of atherosclerosis and cardiovascular disease. The water extracts of the present invention may decrease blood cholesterol via these or other pathways or mechanisms, in addition to enhancing LDL cholesterol uptake/clearance in the liver. For example, and without wishing to be bound by theory or limiting in any manner, the water extracts may decrease cholesterol absorption by epithelial cells and macrophages, inhibit cholesterol biosynthesis, increase bile acid synthesis and/or bile secretion. Additionally, it is presently shown that water and acetone extracts of *Botrylloides violaceus* and *Botryllus schlosseri* and the water extract

of *Styela clava* have a potential to prevent weight gain by suppressing fat accumulation in adipose tissue.

Additional aspects and advantages of the present invention will be apparent in view of the following description. The detailed description and examples, while indicating preferred
5 embodiments of the invention, are given by way of illustration only, as various changes and modifications within the scope of the invention will become apparent to those skilled in the art in light of the teachings of this invention.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features of the invention will now be described by way of example, with
10 reference to the appended drawings, wherein:

FIGURE 1 shows proton NMR spectra for various extracts of the tunicate samples. FIGURE 1A shows hexane extracts (1- SH PTC-3100; 2- BR PTC-2100; 3- MB PTC-1100; 4- INH-OS-79H; 5- INH-OS-78H); FIGURE 1B shows acetone extracts (1- SH PTC-3200; 2- BR PTC-2200; 3- MB PTC-1200; 4- INH-OS-79A; 5- INH-OS-78A); FIGURE 1C shows methanol
15 extracts (1- SH PTC-3300; 2- BR PTC-2300; 3- MB PTC-1300; 4- INH-OS-79M; 5- INH-OS-78M); and FIGURE 1D shows hot water extracts (1- SH PTC-3400; 2- BR PTC-2400; 3- MB PTC-1400; 4- INH-OS-79W; 5- INH-OS-78W).

FIGURE 2 shows bar graphs showing the effect of tunicate extracts on the low-density lipoprotein cholesterol uptake by hepatoma cells (HepG2). FIGURE 2A shows tunicate
20 extracts PTC1100, PTC1200, PTC1300 and PTC1400 at concentrations of 12.5, 25.0 and 50.0 µg/ml. FIGURE 2B shows tunicate extracts PTC2100, PTC2200, PTC2300 and PTC2400 at concentrations of 12.5, 25.0 and 50.0 µg/ml. FIGURE 2C shows tunicate extracts PTC3100, PTC3200, PTC3300 and PTC3400 at concentrations of 12.5, 25.0 and 50.0 µg/ml. A statin drug was used as a positive control.

FIGURE 3A shows the effect of tunicate extracts from INH-OS-78 and INH-OS-79 on LDL-cholesterol uptake in liver cells (HepG2) in the presence of cholesterol and hydroxyl-cholesterol (mean ±SD, n = 5). Berberine was used as a positive control. FIGURE 3B shows the effect of tunicate extracts on LDL-cholesterol uptake in liver cells (HepG2) in the absence of cholesterol and hydroxyl-cholesterol (mean ±SD, n = 5). Statin was used as a positive
30 control.

FIGURE 4 is a bar graph showing the accumulation of triacylglycerol in 3T3-L1 adipocytes treated with tunicate extracts PTC1100, PTC1200, PTC1300, PTC1400, PTC2100, PTC2200, PTC2300, PTC2400, PTC3100, PTC3200, PTC3300, or PTC3400.

FIGURE 5 shows bar graph showing the accumulation of triacylglycerol in 3T3-L1 adipocytes treated with tunicate extract INH-OS-78W. At 50µg/ml, the extracted reduced lipid level to 74.5 ± 1.5 of the control value. Results are mean $\pm$ s.e.m. of 3 separate experiments.

FIGURE 6 is a comparison of proton NMR spectra from water extracts and fractions YW-T-6 (1), YW-T-4 (2), MB-PTC-1400 (3), INH-OS-78W-2 (4), INH-OS-78W-1 (5), and INH-OS-78W (6), in D₂O, 600 MHz.

FIGURE 7 is a comparison of proton NMR spectra of fractions/sub-fractions from water extracts 78W-52-5 (1), 78W-52-4 (2), 78W-52-3 (3), 78W-52-2 (4), 78W-52-1 (5), 78W-54-1 (6), 78W-49-1 (7), 78W-48-1 (8), INH-OS-78W (9), in D₂O, 600 MHz.

FIGURE 8 is a GC chromatogram of INH-OS-78W derivatives for sugar composition analysis, where (1) is D-xylose, (2) is D-mannose, (3) is D-glucose; (4) is L-galactose; and (5) is the reference peak.

FIGURE 9 shows the effect of sub-fractions on LDL cholesterol uptake in liver cells (HepG2) in the presence of cholesterol and hydroxyl cholesterol (mean $\pm$ SD, n = 3). FIGURES 9A, 9B, and 9C show results of assays performed on different days. Extracts labelled 79W-1N and 79W-2N are the same extracts as 79W1 and 79W2, but aliquoted at a different time from the same stock. Results are expressed as fold of the negative control, with berberine (BBR) at a concentration of 15 µg/ml being used as the positive control.

FIGURE 10 shows the effect of water extract T2 and sub-fractions T4 and T6 on LDL-cholesterol uptake in liver cells (HepG2) in the presence (FIGURE 10A; n=3-6) and absence (FIGURE 10B; n=5-6) of cholesterol and hydroxyl cholesterol (mean $\pm$ SD). Results are expressed as fold of the negative control, with berberine (BBR) at a concentration of 15 µg/ml being used as the positive control in Figure 10A and Statin (1µM) used for Figure 10B, respectively.

FIGURE 11 shows the effect of further subfractions of INH-OS-78W on LDL-cholesterol uptake in liver cells (HepG2) (mean $\pm$ SD, n=4). Berberine was used as a positive control.

FIGURE 12 shows size exclusion chromatograms of tunicate extracts and selected sub-fractions (MALS trace vs. eluting time). FIGURE 12A shows all samples; FIGURE 12B shows INH-OS-78W and INH-OS-79W; FIGURE 12C shows INH-OS-78W and its sub-fractions.

DETAILED DESCRIPTION OF THE INVENTION

- 5 The present invention relates to tunicate extracts and uses thereof for treating metabolic disorders. More specifically, the invention relates to tunicate extracts and uses thereof for treating or preventing obesity and/or hyperlipidemia.

The present invention provides a method of preparing tunicate extract comprising:

- 10 a) extracting a *Styela clava*, *Ciona intestinalis*, *Botrylloides violaceus*, *Botryllus schlosseri*, tunicate sample or any combination thereof, with hot water; and
b) collecting the tunicate water extract obtained in step a).

The tunicate sample may be obtained or harvested from any suitable tunicate species. Tunicates, also referred to as "urochordates", are marine organisms belonging to a group of underwater filter feeders with incurrent and excurrent siphons. Of particular interest in the
15 present invention are tunicates of species *Styela clava*, *Ciona intestinalis*, *Botrylloides violaceus*, *Botryllus schlosseri*, or any combination thereof. In one non-limiting example, the tunicate sample may be *Styela clava*; in another non-limiting example, the tunicate sample may be *Ciona intestinalis*; in yet another non-limiting example, the tunicate sample may be comprised of a combination of *Botrylloides violaceus* and *Botryllus schlosseri*. Tunicate
20 samples may be harvested from coastal areas globally, including but not limited to the coastal areas of North America, Europe, Asia, and South America. For example and without wishing to be limiting in any manner, the tunicate sample may be obtained from coastal areas in eastern Canada or the north-eastern USA. The tunicate sample may be collected at any suitable time of year. For example, and without wishing to be limiting in any manner, the
25 tunicate sample may be collected between about September and May. Alternatively, the tunicate samples may be obtained from cultured tunicates. The tunicates may be cultured in an appropriate environment; methods for cultivating tunicates would be known to those of skill in the art. Optionally, the cultivated tunicates may be bioengineered (via hybridization or genetic engineering) to produce extracts exhibiting increased bioactivity characteristics.

- 30 The tunicate sample is extracted with hot water. As would be understood by one of skill in the art, the tunicate sample may be extracted directly with hot water to obtain a water extract;

alternatively, the tunicate sample may be sequentially extracted with various solvents, including hot water. For example, and without wishing to be limiting in any manner, the tunicate sample may be sequentially extracted with hexane, acetone, methanol, and hot water. By the term "sequentially extracted", and according to this example, it is meant that the tunicate sample is first extracted with hexane, and the resulting solid residue is then extracted with acetone; the solid residue resulting from acetone extraction is then extracted using methanol; the resulting solid residue is subsequently extracted with hot water. The tunicate sample may be extracted with additional or different solvents, not limited to those included herein. As would also be understood by a person of skill in the art, the tunicate sample may be extracted in a different order than that listed herein.

For water extraction, the solid residue resulting from the methanol extraction may be mixed with hot water. The water may be at a temperature between about 30°C and 100°C; for example, and without wishing to be limiting, the water may be at about 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100°C, or any temperature therebetween or any range of temperatures defined by these values. In a specific, non-limiting example, the water may be at about 80 to 90°C. The water is mixed with the solid residue in an a ratio of about 1:20 original sample weight:water, or in a ratio in the range of about 1:5 to 1:100; in a non-limiting example, the ratio may be about 1:5, 1:10, 1:15, 1:20, 1:25, 1:30, 1:35, 1:40, 1:45, 1:50, 1:55, 1:60, 1:65, 1:70, 1:75, 1:80, 1:85, 1:90, 1:95, 1:100, or any ratio therebetween. In a specific, non-limiting example, 1 g of solid residue may be mixed with 20 mL water. The water extraction may proceed for any suitable time; for example and without wishing to be limiting, the water extraction may proceed for a time in the range of about 30 min up to several hrs; for example, and without wishing to be limiting in any manner, the extraction time may be about 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 135, 150, 165, 180, 195, 210, 225, 240, 255, 270, 285, or 300 minutes or more, or any time therebetween. In a specific, non-limiting example, the extraction time may be about 300 minutes (5 hrs). As would be understood by one of skill in the art, the extraction time may vary based on the extraction temperature. The water extraction may also be done with the assistance of microwave, sonication, stirring, or other approaches facilitating extraction that are known in the art. Following extraction, the sample may be cooled then filtered using any suitable filtration method known in the art, such as paper filtration or vacuum filtration. Alternatively, centrifugation may also be used for separation of water extract from solid residue.

For optional hexane, acetone, and methanol extractions, the tunicate sample or the solid residue may be mixed with a suitable amount of the respective solvent. For example, and

without wishing to be limiting in any manner, the amount of solvent may be in a ratio of about 1:20 original sample weight:solvent, or in a ratio in the range of about 1:5 to 1:100; in a non-limiting example, the ratio may be about 1:5, 1:10, 1:15, 1:20, 1:25, 1:30, 1:35, 1:40, 1:45, 1:50, 1:55, 1:60, 1:65, 1:70, 1:75, 1:80, 1:85, 1:90, 1:95, 1:100, or any ratio therebetween. In a specific, non-limiting example, 1 g of sample or solid residue may be mixed with 20 mL solvent. The extraction may proceed for any suitable time; for example, and without wishing to be limiting, the extraction time may be about 30 minutes, or for a time in the range of 5min to 2hrs; in a non-limiting example, the extraction time may be about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 70, 80, 90, 100, 110, 120 minutes, or any time therebetween. The extraction may proceed at any suitable temperature; for example and without wishing to be limiting, the temperature may be about room temperature, or between about room temperature and the boiling point of the solvents used. As would be known to a person of skill in the art, the extraction temperature will vary based on other conditions and solvents used; it would be within the capabilities of the skilled person to adjust the temperature appropriately. The extraction may additionally incorporate using any acceptable physical or mechanical method known in the art; for example, and without wishing to be limiting, the extraction may incorporate stirring and/or sonication. Additionally, the extraction may also include immersing and/or refluxing.

Following each extraction step, the sample may be filtered using any suitable filtration method known in the art, such as paper filtration or vacuum filtration. Alternatively, separation of liquid extract and solid residue may be done by centrifugation. Following filtration or centrifugation, the flow-through or supernatant fraction is collected and is labelled as the respective extract, while the solid filtrate or precipitate (also referred to herein as the solid residue) is subjected to further extraction.

Prior to extraction, the fresh tunicate may be dried, lyophilized and/or homogenized, by any suitable method known in the art. For example, and without wishing to be limiting in any manner, the tunicate sample may be homogenized using a commercially available homogenizer, grinder, blender, etc. The tunicate sample may be dried or lyophilized, also referred to herein as "freeze-dried", using any suitable method of freeze-drying or other drying methods known in the art, such as but not limited to oven drying, drum dryer, or conveyor dryer.

The dry tunicate tissue may optionally be milled by any suitable method known in the art. For example, and without wishing to be limiting in any manner, the tunicate sample may be milled

using a commercially available grinder, or manually ground (for example, using a mortar and pestle). Subsequent to milling, the sample may be immediately subjected to extraction, or may be stored. The sample may be stored under any suitable conditions, for example and without wishing to be limiting in any manner, the sample may be stored at -80°C to room temperature.

- 5 In a specific, non-limiting example, the sample may be stored at -80°C, -20°C, 4°C, 10°C, 25°C, or room temperature.

The water extract resulting from the extraction method described herein may optionally be processed or refined further using any suitable method in the art; for example, and without wishing to be limiting in any manner, the water extract may be subjected to ethanol
10 precipitation, ultrafiltration, chromatography, desalting, drying by using rotary evaporator or centrifugal vacuum evaporator and/or spray dryer, or any combination thereof. The water extract may optionally also be further fractionated. Such further fractionation may be accomplished using any suitable method known in the art, for example, but not limited to a method described herein, or the method as follows. In a non-limiting example, the water
15 extract may be submitted to ion exchange and/or size exclusion chromatography and/or papain hydrolysis followed by precipitation at basic pH or with EtOH, yielding (first stage) sub-fractions of the water extract. Methods for ion exchange or size exclusion chromatography, papain hydrolysis, basic and/or EtOH precipitation would be known to those of skill in the art. The sub-fractions may also optionally be further processed by any suitable method known in
20 the art, for example, but not limited to ethanol precipitation, chromatography, ultrafiltration, desalting, drying by rotatory evaporator, centrifugal vacuum evaporator and/or spray dryer, or any combination thereof.

Similarly, hexane, acetone, and/or methanol extracts may be further processed by chromatography, drying by rotatory evaporator, centrifugal vacuum evaporator, or any
25 combination thereof. Additionally, these extracts may be further fractionated by normal or reverse phase chromatography, silica gel chromatography, or thin layer chromatography.

The present invention also provides a tunicate water extract or fraction/sub-fraction thereof obtained from *Styela clava*, *Ciona intestinalis*, *Botrylloides violaceus*, *Botryllus schlosseri*, or any combination thereof. In one non-limiting example, the tunicate water extract, fraction or
30 sub-fraction thereof is obtained from *Styela clava*; in another non-limiting example, the extract, fraction or sub-fraction thereof is obtained from *Ciona intestinalis*; in yet another non-limiting example, the extract, fraction or sub-fraction thereof is obtained from a sample comprised of a

combination of *Botrylloides violaceus* and *Botryllus schlosseri*. The tunicate water extract, fraction or sub-fraction thereof may be obtained using the methods as described herein.

The tunicate water extract, fraction or sub-fraction thereof of the present invention may be characterized by any one of the proton NMR spectrum as shown in Figure 1D, 6, or 7. The tunicate water extract may further be described as comprising acetamide-substituted polysaccharides and protein glycans as the main components. The polysaccharides and/or protein glycans may comprise a molecular weight in the range of 50 KDa to 10 MDa; for example, and without wishing to be limiting in any manner, the average molecular weight of the polysaccharides and/or protein glycans may be between about 0.4 and 4.6 MDa, or any value therebetween. In a non-limiting example, the average molecular weight of the polysaccharides and/or protein glycans may be about 2-3 MDa. The polysaccharides and/or glycans in the main component of the water extract may comprise galactose and glucose as the main sugar units.

The present invention additionally provides a tunicate acetone extract, fraction, or sub-fraction thereof obtained from *Botrylloides violaceus*, *Botryllus schlosseri*, or any combination thereof. The tunicate acetone extract, fraction, or sub-fraction thereof of the present invention may be obtained by a method comprising:

- a) extracting a *Botrylloides violaceus*, *Botryllus schlosseri* tunicate sample or any combination thereof with acetone; and
- b) collecting the tunicate acetone extract obtained in step a).

In the method as just described, the tunicate sample may be dried or lyophilized prior to step a); optionally, the dried or lyophilized sample may be milled prior to step a). Additionally, the method as described herein may comprise sequentially extracting the tunicate sample with hexane and acetone in step a), or with other/additional solvents, as described above. The tunicate acetone extract, fraction, or sub-fraction thereof may be obtained using methods as described herein. The acetone extract may be further processed by chromatography, drying by rotatory evaporator, centrifugal vacuum evaporator, or any combination thereof; additionally, the extract may be further fractionated by normal or reverse phase chromatography, silica gel chromatography, or thin layer chromatography. The tunicate acetone extract, fraction, or sub-fraction thereof may be characterized by the proton NMR spectrum of Figure 1B (1).

The present invention further provides a method of treating, preventing, or delaying the onset of metabolic disorders such as hyperlipidemia and obesity by administering one or more than

one extract of the present invention (as described above). The treatment, prevention, or delay of onset of hyperlipidemia may be addressed by enhancing LDL-cholesterol uptake by the liver; treatment, prevention, or delay of onset of obesity may be addressed by inhibiting adipogenesis.

- 5 As such, the present invention provides a method of enhancing LDL-cholesterol uptake by the liver comprising administering one or more than one of the tunicate water extract, fraction or sub-fraction thereof described herein, or any mix of compounds or single compounds obtained therefrom, to a subject in need thereof. The tunicate water extract, mix of compounds or single compounds obtained therefrom, may optionally be administered in combination with
10 other natural compounds/extracts or drugs, for example, but not limited to compounds from the sterol/stanol family. Such natural compounds/extracts or drugs may be anti-diabetic or anti-obesity compounds/extracts or drugs.

The present invention further provides a method of inhibiting adipogenesis, comprising administering one or more than one of the acetone and/or water tunicate extract, fraction or
15 sub-fraction thereof obtained from *Botrylloides violaceus*, *Botryllus schlosseri*, or any combination thereof as described herein; the *Styela clava* water extract, fraction, or sub-fraction as described herein; any mix of compounds or single compounds obtained therefrom; or any combination thereof, to a subject in need thereof. The phrase "inhibiting adipogenesis", as used herein may include inhibition of pre-adipocyte differentiation and fat storage or
20 accumulation in fat cells, decreasing fatty acid and triglyceride synthesis, and/or increasing fat (fatty acids) oxidation.

The extract may be administered in an effective amount to obtain the desired effect. As would be known to those of skill in the art, a specific dosage will vary based on several factors such as age and body weight. For example, and without wishing to be limiting in any manner, an
25 effective dosage may be approximately 0.7 -2.1 grams, or any amount in the range described; in a specific, non-limiting example, the dosage may be 1-2 g/day.

It is presently shown that water extracts of various types of tunicates (including *Styela clava*, *Ciona intestinalis*, *Botrylloides violaceus*, *Botryllus schlosseri*) can increase LDL cholesterol (LDL-C) uptake by liver cells. The increase or enhanced LDL-cholesterol uptake results in a
30 reduction of plasma total and LDL cholesterol [3], which are both closely related to the development of atherosclerosis and cardiovascular disease. The water extracts of the present invention may decrease blood cholesterol via these or other pathways or mechanisms, in

addition to enhancing LDL cholesterol uptake/clearance in the liver. For example, and without wishing to be bound by theory or limiting in any manner, the water extracts may decrease cholesterol absorption by epithelial cells and macrophages, inhibit cholesterol biosynthesis, increase bile acid synthesis and/or bile secretion. Additionally, it is presently shown that water and acetone extracts of *Botrylloides violaceus* and *Botryllus schlosseri*, and water extracts of *Styela clava* have a potential to prevent weight gain by suppressing fat accumulation in adipose tissue.

The present invention will be further illustrated in the following examples. However, it is to be understood that these examples are for illustrative purposes only and should not be used to limit the scope of the present invention in any manner.

Example 1: Preparation of tunicate extracts

Tunicate samples can be collected from coastal areas in eastern Canada (for example Nova-Scotia, New-Brunswick, Prince Edward Island, Newfoundland) or the northeastern USA (for example Maine, Massachusetts, Connecticut, Rhode Island, New York, New Jersey).

Three tunicate samples were obtained from PEI Aquaculture and Fisheries Research Initiative, Inc from different sites in PEI in 2008:

- 081006MB: primarily *Styela clava* (Clubbed tunicate) collected on October 6, 2008 at Malpeque Bay;
- 081112BR: a mix of *Ciona intestinalis* (Vase tunicate) and *Styela clava* (Clubbed tunicate) collected on November 12, 2008 at Brudenell River; and
- 081203SH: a mix of *Botrylloides violaceus* (Violet tunicate) and *Botryllus schlosseri* (Golden star tunicate) collected on December 3, 2008 at Savage Harbour.

Two pure species of tunicate samples were obtained from PEI Aquaculture, Fisheries and Research Initiative Inc in PEI in 2010:

- INH-OS-78: *Styela clava*, collected in January/February; and
- INH-OS-79: *Ciona intestinalis*, collected in April/May.

These samples were extracted with hexane, acetone, methanol and water, sequentially as described below. All samples were subject to global profiling of bioactive compounds using NMR (see Example 2).

The samples were processed, freeze-dried, milled, and kept at -80°C. The dry powder of each tunicate sample was extracted sequentially using four different solvents: hexane, acetone, MeOH, and hot water. The tunicate sample was mixed 1:20 in hexane (1g in 20mL solvent), stirred for 30min at room temperature and then sonicated for 30min. For this example, the mixture was then filtered using filtration paper and the flowthrough was collected. The extraction was repeated once and the solvent in the combined liquid extract was removed by rotary evaporator then centrifugal evaporator (Genevap) to yield the hexane extract (noted by an "H" suffix). The solid filtrate was then mixed 1:20 (original sample weight) in acetone and stirred and then sonicated for 30min; the mixture was filtered and the flow-through was collected. Similarly, the extraction was repeated once and the solvent in the combined liquid extract evaporated (acetone extract, noted by "A" suffix). The solid filtrate was then re-suspended in MeOH (1:20), followed by 30min stirring and sonication; the mixture was then filtered and the flow-through was collected. The extraction was repeated once and the solvent in the combined liquid extract evaporated (MeOH extract, noted by "M" suffix). Finally, the solid filtrate was mixed with hot water (1:20) and stirred at 80-90°C for 5hrs. After cooling to room temperature and filtration the flow-through was mixed with 3 volumes of 95% EtOH and put in ice bath for 2 hrs. The precipitate was then filtered, washed with EtOH, and collected by centrifugation. The solvent was removed from each extract by centrifugal evaporator (Genevap) and freeze-dryer to yield water extracts (noted by "W" suffix).

Results of the extractions are shown in Table 1.

Table 1. Tunicate species and extracts

Sample	Sample Mass	Extract	Extract Name	Extract Mass	% by mass
081006MB	40.204	Hexane	PTC-1100 or MB-H	0.475	1.181
		Acetone	PTC-1200 or MB-A	0.203	0.505
		Methanol	PTC-1300 or MB-M	8.509	21.165
		Water	PTC-1400 or MB-W	3.373	8.39
081112BR	39.995	Hexane	PTC-2100 or BR-H	0.726	1.815
		Acetone	PTC-2200 or BR-A	0.454	1.135
		Methanol	PTC-2300 or BR-M	11.582	28.959
		Water	PTC-2400 or BR-W	1.411	3.528

081203SH	40.015	Hexane	PTC-3100 or SH-H	0.417	1.042
		Acetone	PTC-3200 or SH-A	0.257	0.642
		Methanol	PTC-3300 or SH-M	11.835	29.576
		Water	PTC-3400 or SH-W	1.72	4.298
INH-OS-78	40.007	Hexane	INH-OS-78-H	0.424	1.06
		Acetone	INH-OS-78-A	0.224	0.56
		Methanol	INH-OS-78-M	8.46	21.146
		Water	INH-OS-78-W	2.688	6.719
INH-OS-79	40.003	Hexane	INH-OS-79-H	0.151	0.377
		Acetone	INH-OS-79-A	0.127	0.317
		Methanol	INH-OS-79-M	19.601	48.999
		Water	INH-OS-79-W	2.078	5.195
INH-OS-78	195.18	Hexane	INH-OS-78-H	1.15	0.59
		Acetone	INH-OS-78-A	0.549	0.28
		Methanol	INH-OS-78-M	48.83	25.02
		Water	INH-OS-78-W	17.61	9.02
INH-OS-79	94.54	Hexane	INH-OS-79-H	0.363	0.38
		Acetone	INH-OS-79-A	0.64	0.55
		Methanol	INH-OS-79-M	56.69	59.97
		Water	INH-OS-79-W	7.484	7.92

Example 2: Proton NMR characterization of tunicate extracts

The extracts obtained in Example 1 were submitted to Proton NMR profiling.

Briefly, 2 mg of the hexane, acetone, MeOH extracts were separately dissolved in 100 μ L of DMSO-d₆, while 2 mg of water extracts were dissolved in 100 μ L D₂O. Sample solutions were transferred to 1.7 mm NMR tubes and proton NMR spectra were acquired on Bruker Bruker Avance III 600 MHz NMR spectrometer (Bruker Corporation, East Milton, ON) operating at 600.28 MHz ¹H observation frequency and a temperature of 25 $\pm$ 0.2°C. The signals were acquired, processed and analyzed using TopSpin[®] NMR data acquisition and processing Software (Bruker Biospin Ltd, East Milton, ON) integrated with the spectrometer.

Results in the form of NMR spectra are shown in Figure 1. General proton NMR profiling indicated that there is certain level of similarity in the main components of extracts prepared from different tunicate species. Hexane and acetone extracts comprised fatty acids (including polyunsaturated FAs), while the water extracts showed the presence of polysaccharides or protein glycans as the main components.

Example 3: Effect of tunicate extracts on LDL-cholesterol uptake assays

Heptoma cell line was used to test the effect of tunicate extracts of Example 1 on LDL-cholesterol (LDL-C) uptake. Although the circulating cholesterol levels are regulated by several cholesterol metabolic pathways, LDL receptor-mediated LDL cholesterol uptake in liver
5 is an important process. An increase of LDL cholesterol uptake in liver leads to a reduction of blood cholesterol. There is a strong relationship between *in vitro* and *in vivo* effects [3].

Hepatoma cells (HepG2) were maintained in Eagle's Minimum Essential Medium (EMEM) with 10% fetal bovine serum (FBS) in T-75 culture flasks. When they reached ~80% confluence, cells were detached by incubation with 0.25% trypsin. Cells were seeded on 6, 12, 24, or up
10 to 96-well plates in EMEM containing 10% FBS and incubated overnight. Following incubation, cells were treated with EMEM medium containing 0.5% lipoprotein-deficient serum (LPDS) and the extracts of Example 1 were added at 12.5, 25, or 50 µg/ml for 18 hours or overnight; cells were incubated in the presence or absence of cholesterol (10 µg/ml) and OH-cholesterol (1 µg/ml) in EMEM medium containing 0.5% LPDS. 1 µM simvastatin in dimethyl sulfoxide
15 (DMSO) or 15 µg/ml berberine was used as a positive control; cells cultured under the same conditions but without extract or simvastatin were used as baseline or background. On day 3, the medium was aspirated and 0.5 ml of EMEM with 0.5%LPDS and 5 µg/ml of LDL-BODIPY (final concentration) was added to each well. Following 4-hour incubation at 37°C and 5% CO₂, medium was aspirated and cells were washed twice with PBS. Cells were then
20 detached/dissociated with but not limited to the use of trypsin, re-suspended in PBS, transferred to micro-centrifuge tubes, and centrifuged for 5 minutes at 2,800 RPM in a Eppendorf centrifuge 5810R. Supernatant was removed and cells was re-suspended in 300 µl PBS then transferred into flow-cytometry tubes or onto 96-well-plates at 100-250 µl/well. The fluorescence density was measured using a cell flow cytometry or fluorescence plate reader.
25 For cells seeded in 96-well plates (including all INH-OS-78 and -79 samples), after treatment and washing, the plate was read directly fluorescent intensity on a Varioskan Plate Reader, without detaching or dissociation of cells. The fluorescence density in the cells correlates with the amount of the labelled LDL-C taken up by the cells, and illustrates the ability of the liver cells to clear LDL-C from the peripheral tissues thus reducing blood cholesterol levels.
30 The results presented in Figure 2 are the average of 2-3 repeats, while Figure 3 is an average of 5 repeats, all conducted separately on different days.

It was consistently shown that the extracts PTC1100 and PTC1200 were ineffective at increasing LDL-C uptake by liver cells (HepG2); rather, they inhibited uptake in a dose-dependent manner. The magnitude of inhibition of these extracts was very similar. PTC1300 had a neutral effect on LDL-C uptake. Interestingly, PTC1400 showed a significant increase in LDL-C uptake, which appeared to be dosage-dependent in the tested range; the magnitude of the increase in uptake is larger than that of the positive control, a statin drug that inhibits cholesterol synthesis and up-regulates the expression of LDL receptor.

PTC2100 and PTC2200 also showed a dosage-dependent inhibitory effect on LDL-C uptake by liver cells (Fig. 2B). PTC2300 showed a mild up-regulating effect. PTC2400 showed the strongest effect, though no dose-response was observed; it is plausible that the maximum effect was reached at a concentration of 12.5 µg/ml and decreased when at a concentration of 50 µg/ml.

PTC3100 showed a mild enhancing effect on LDL-C uptake at a concentration of 12.5 µg/ml, though the response was smaller than the positive control. PTC3200 and PTC3300 showed a dosage-dependent inhibitory effect on LDL-C uptake. PTC3400 increased LDL-C uptake, with a pattern similar to that observed for PTC2400.

Similarly, and as shown in Figure 3, water extracts of both the pure *S. clava* and *C. intestinalis* species (INH-OS-78W and INH-OS-79W) and methanol extract of *C. intestinalis* (INH-OS-79M) increased LDL-cholesterol uptake. However, the methanol extract activity was less potent and less consistent than water extracts.

Based on these results, it appears that water extracts upregulate LDL-C uptake in liver cells. This function is closely associated with cholesterol clearance from blood and peripheral tissues, and thus leads to the reduction of circulating cholesterol levels in general [3]. Clearance of LDL-cholesterol is an important pathway through which the body removes extra LDL cholesterol from the peripheral tissues or reduces LDL cholesterol levels in the circulating system. This process is mediated by LDL-receptor on the liver cell surface. Thus the expression of LDL receptor regulates human blood LDL cholesterol homeostasis [32, 33]. Increased hepatic LDL receptor expression results in improved clearance of plasma LDL-cholesterol through receptor mediated endocytosis, and consequently lower plasma LDL and total cholesterol levels.

Example 4: Effect of tunicate extracts on adipogenesis

The tunicate extracts of Example 1 were assessed for their ability to inhibit fat cell differentiation (adipogenesis) and triacylglycerol deposition.

3T3-L1 pre-adipocytes were grown to confluence in DMEM (10% calf serum; CS) in 24-well
5 plates. Two days post-confluence, differentiation was initiated by adding DMEM, 10% fetal
bovine serum (FBS), insulin (10µg/ml), dexamethasone (0.3µg/ml), and 3-Isobutyl-1-
methylxanthine (500µM). Test extracts were added at a concentration of 25 and 50 µg/ml
(extracts of INH-OS-78 and INH-OS-79), or 20 µg/ml (all others). Control wells were treated
10 with differentiation medium alone (differentiated Control), DMEM (10% CS; non-differentiated
Control), or rosiglitazone (10 µM; adipogenesis stimulator). After 2 days, the media were
refreshed with DMEM (10% FBS) plus insulin (10 µg/ml); test extracts from Example 1 and
rosiglitazone were present at the same concentrations as for the first two days. On day 5 or 6,
cells were washed with Dulbecco's phosphate-buffered saline (DPBS) and then assessed for
triacylglycerol content using the AdipoRed reagent (Lonza Walkerville, Inc., Walkerville, MD) or
15 Oil red O. The amount of triacylglycerol (in relative fluorescent units) was corrected for amount
of cell protein determined by the BCA method. Duplicate determinations for each extract were
made and the results were averaged. Results are presented in Figures 4 and 5 as a
percentage of the value for differentiated control cells.

Results in Figure 4 show inhibition of adipogenesis in the presence of hexane and acetone
20 extracts (PTC-2100, PTC-3100, PTC-2200 & PTC-3200), as well as with water extract PTC-
3400. There was no observed stimulatory effect on adipogenesis (rosiglitazone-like activity)
for any of the 2008 samples. Treatment of 3T3-L1 cells with PTC-2100, PTC-3100, PTC-3200,
and PTC-3400 resulted in a reduction in triacylglycerol accumulation (71%, 64%, 63%, and
55% of Control, respectively). None of the extracts exhibited rosiglitazone-like activity (i.e. they
25 do not stimulate PPAR-γ).

Figure 5 shows that INH-OS-78W inhibited adipogenesis by over 30%, with the other extracts
showing no effect. With the exception of 78-W, none of the extracts showed consistent
inhibitory or stimulatory effects on adipogenesis (results not shown). At 50µg/ml, the extracted
reduced lipid level to 74.5 ± 1.5 of the control value. Results are mean $\pm$ s.e.m. of 3 separate
30 experiments.

Example 5: Further fractionation and characterization of tunicate extracts

Based on the results of Example 3, the methanol extract INH-OS-79M and water extracts INH-OS-78W and INH-OS-79W were selected for further fractionation.

For INH-OS-79M, 2 g of methanol extract INH-OS-79M was fractionated on a C18 column
5 (Octadecyl-functionalized silica gel, 200-400 mesh, Sigma-Aldrich), eluted with aqueous MeOH (5, 25, 50, and 100%), and MeOH-CH₂Cl₂ to yield INH-OS-79M-1 (1.8 g), 79M-2 (18.8 mg), 79M-3 (16.1 mg), 79M-4 (118.1 mg), and 79M-5 (207.0 mg).

For INH-OS-78W and INH-OS-79W, 250 mg of extract was dissolved in 5 ml 5% sodium acetate buffer with 5 mM EDTA and 5 mM cysteine. 25 mg papain was added, vortexed for 20
10 sec and then sonicated for 10 min before being incubated at 60°C for 24h. The hydrolysis was stopped by boiling in water bath for 10 min. The mixture was centrifuged at 1590 RPM for 15 min. 95% ethanol (12 ml) was added to the supernatant (4 mL), vortexed for 20 sec and then sonicated for 20 min; 2 g sodium acetate was added and stored at 4°C for 24 hr, then centrifuged at 1500 rpm for 15min to collect precipitate. Solvent of supernatant was removed
15 using a rotary evaporator under reduced pressure, and then freeze-dried. The papain-treated products, the precipitate (INH-OS-78W-1 and INH-OS-79W-1) and the supernatant (INH-OS-78W-2 and INH-OS-79W-2) were further processed to remove salt. 200 mg each of these freeze-dried products were desalted by filtering the water solution through a 3 KDa centrifuging membrane filter, and freeze-dried, to obtain INH-OS-78W-1 (0.7 mg), INH-OS-78W-2 (1.2 mg),
20 INH-OS-79W-1 (1.0 mg), and INH-OS-79W-2 (0.9 mg).

Water extract INH-OS-78W consistently showed potent bioactivity (Example 3), and was thus chosen for further fractionation. INH-OS-78W was subjected to further fractionation via papain hydrolysis or ion-exchange chromatography. In preparation, 3.2 g of INH-OS-78W was desalted by washing with 60%, 80%, and 95% EtOH then dried (78W-48-1).

For fractionation through papain hydrolysis, 2 g of 78W-48-1 was subjected to papain
25 hydrolysis in NaOAc, pH 6.8 for 18 hr. After deactivation at 95°C for 0.5 hr and cooling down, the pH was adjusted to 1-2 and the precipitate was removed by centrifugation. The pH of the supernatant was then adjusted to 11-12, and the precipitate was collected by centrifugation; the precipitate was rinsed with EtOH and dried in a centrifugal evaporator (Genevap) followed
30 by freeze-drying, yielding 105.3 mg of extract 78W-49-1. The supernatant was mixed with 4 volumes of 95% EtOH, stored at 4°C, and the resulting precipitate was collected; the precipitate was rinsed with EtOH and further purified on a Q-Sepharose Fast Flow ion-

exchange column (QFF, GE Healthcare), where it was eluted with 1.28 M NaCl, desalted by dialysis (1K MWCO), and freeze-dried. 9.8 mg of extract 78W-54-1 was obtained.

The MB-W extract (PTC-1400 or YW-T-2) was similarly fractionated, except that the extract was desalted by washing with 70% EtOH and dried. The dried extract was then submitted to
5 papain hydrolysis and ethanol precipitation as described above, except that 70% EtOH was used for precipitation. This yielded two fractions: the soluble YW-T-4 (not desalted) and the precipitate YW-T-6 (not desalted).

For fractionation with ion-exchange column, 1 g of 78W-48-1 was loaded on a Q Sepharose – fast flow ion-exchange column (5 x 2.6 cm), eluted with a linear gradient of 2 M NaCl in water
10 (0, 0.15, 0.25, 0.40, 0.60, 0.80 M NaCl), followed by 2 M NaCl. The fractions collected were dialyzed and freeze-dried, yielding fractions 78W-52-1 (11.4 mg), 78W-52-2 (6.3 mg), 78W-52-3 (11.0 mg), 78W-52-4 (16.4 mg), and 78W-52-5 (29.5 mg).

As water extract INH-OS-78W and its fractions and sub-fractions consistently showed potent bioactivity (see Example 6), focus was placed upon these in chemical profiling and
15 characterization. For proton NMR characterization, samples were dissolved in D₂O and analyzed on a Bruker Avance III 600 MHz NMR spectrometer, as described in Example 2.

Figure 6 shows proton NMR spectra of initial fractions of INH-OS-78W and other similar fractions (YW-T-4 and YW-T-6). The proton NMR spectra for additional set of fractions/sub-fractions prepared from INH-OS-78W are shown in Figure 7. The NMR analysis revealed that
20 the water extracts INH-OS-78W and INH-OS-79W contain polysaccharides (peaks at 5.5-3.0 and 2.5-1.5 ppm) comprising an acetamide group (with NMR peak for the methyl protons at around 2.5-1.5 ppm) and protein (7.5-6.5 and 1.0-0.5 ppm) components. Papain hydrolysis, intended to remove protein, appears to be without significant effect on bioactivity (INH-OS-78W-1 and INH-OS-78W-2). However, the acetamide on the sugar backbone may be
25 important for bioactivity, as no activity was observed for the polysaccharide fraction 78W-49-1 (see Example 6).

Sugar composition analysis was also performed for extract INH-OS-78W. The samples were derivatized as acetylated aldononitriles for GC-MS analysis. INH-OS-78W was completely hydrolyzed with 2 M TFA at 110°C for 6 hr. The hydrolyzed sample was mixed with
30 5 mg hydroxylamine hydrochloride, and acetylated with 500 µl pyridine at 90°C for 30 min; 500 µl Ac₂O was then added to the mixture and allowed to react at 90°C for 30 min. Inositol was derivatized at the same condition. The sugar sample was spiked with inositol derivatives as

reference. 1 µl of each derivatized solution was injected on a GC (Agilent HP6890, USA) system using a fused silica capillary column HP-INNOWax, 0.25mm * 30m * 0.15µm at 220°C and monitored with using a flame-ionization detector at 245°C. Figure 8 shows the GC chromatogram of INH-OS-78W derivatives. The results show that the polysaccharide components of the water extract comprises sugars such as galactose and glucose.

Example 6: Effect of tunicate extracts on LDL-cholesterol uptake assays

The activity of the sub-fractions of water extract from Example 5 on LDL-cholesterol uptake in liver cells was tested.

First, extracts INH-OS-78W1, INH-OS-78W2, INH-OS-79W1, INH-OS-79W2, INH-OS-79M1, INH-OS-79M2, INH-OS-79M3, INH-OS-79M4, and INH-OS-79M5 were tested for their effect on LDL-cholesterol uptake. The methods utilized are essentially as described in Example 3. Results are shown in Figure 9. INH-OS-78W1 and -78W2 enhanced LDL cholesterol uptake with a comparable or higher efficacy than the positive control. INH-OS-78W-1 showed the strongest and most consistent effect, while INH-OS-78W-2 and INH-OS-79W-2 were promising, but less effective and less consistent. None of the five fractions of INH-OS-79M showed a promising effect.

Sub-fractions of water extract MB-W (081006MB) were also tested for effect on LDL-cholesterol uptake. The methods utilized are essentially as described in Example 3. Results are shown in Figure 10. All fractions showed a potent effect on LDL cholesterol uptake in liver cells relative to the positive control of berberine, with fraction T4 showing the best potency. The water extract T2 and sub-fraction T4 showed dose-dependent effects to increase LDL-cholesterol uptake in HepG2 cells in the presence of cholesterol and hydroxyl-cholesterol. Fraction T6 increased LDL uptake in the present of cholesterol and OH-cholesterol in medium but decreased LDL uptake in the absence of cholesterol and OH-cholesterol. These results are in line, to certain degree, with the observed effect of INH-OS-78W, the sub-fractions INH-OS-78W-1, and INH-OS-78W-2.

Finally, further sub-fractions of INH-OS-78W were further tested for their effect on LDL-cholesterol uptake. The methods utilized are essentially as described in Example 3. Results are shown in Figure 11. Fractions INH-OS-78W-52-1, INH-OS-78W-52-2, INH-OS-78W-52-3, INH-OS-78W-52-4, INH-OS-78W-52-5, and INH-OS-78W-54-1 were consistently positive in three independent experiments. The efficacies are similar or slightly lower than the positive

control, berberine. INH-OS-78W-49-1, however, showed little to no bioactivity, indicating that the acetamide on the sugar backbone may be important for bioactivity.

Example 7: Molecular weight analysis of tunicate water extracts and some fractions

Water extracts, fractions and sub-fractions thereof were submitted to molecular weight
5 analysis.

Briefly, tunicate water extracts (INH-OS-78W and INH-OS-79W) and sub-fractions of INH-OS-78W (78W-48-1, 78W-49-1, 78W-52-1, 78W-52-2, 78W-52-3, 78W-52-4, 78W-52-5, and 78W-54-1) were analyzed to obtain molecular weights on an Agilent 1100 HPLC system using a size exclusion chromatography (SEC) column TSK gel G5000PWxl (Tosoh Bioscience LLC),
10 coupled with a Dawn Heleos II multi-angle light scattering detector (MALS, Wyatt Technologies) and an OptiLab T-rEx refractive index detector (RI, Wyatt Technologies). Samples were dissolved in 0.1 M NaCl at a concentration of 1 mg/mL. The mobile phase used was 0.1 M NaCl, with a flow rate at 0.6 mL/min. Samples were run twice to obtain average values of molecular weight. Dextran (MW 50 KDa, Fluka) was used as a reference to validate
15 the system. Astra software (Wyatt Technologies) was used to process data and obtain weight average molecular weight.

Results are shown in Table 2 and size exclusion chromatograms of Figure 12A-C. The results show that the tunicate extracts and sub-fractions contain polysaccharides or protein glycans with a molecular size ranging from about 50 KDa up to 10 MDa, as shown in Figures 12A-C.
20 The average molecular weight of each sample is listed in Table 2, where the average molecular weight of the main components of the water extract is shown to be between 0.4 and 4.58 MDa.

Table 2. Weight average molecular weight of tunicate water extracts and selected sub-fractions by using SEC-MALS-RI method.

Sample	Average MW (MDa)		
	Run 1	Run 2	Average
INH-OS-78W	3.05	3.29	3.17
INH-OS-79W	Component 1: 3.78	3.04	3.41
	Component 2: 1.85	1.67	1.76
78W-48-1	3.84	3.29	3.57
78W-49-1	Component 1: 0.95	1.10	1.03
	Component 2: 0.35	0.44	0.40
78W-52-1	4.28	4.87	4.58
78W-52-2	3.75	3.37	3.56

78W-52-3	3.25	3.78	3.52
78W-52-4	3.38	3.10	3.24
78W-52-5	2.64	3.37	3.00
78W-54-1	2.37	2.42	2.40

The embodiments and examples described herein are illustrative and are not meant to limit the scope of the invention as claimed. Variations of the foregoing embodiments, including alternatives, modifications and equivalents, are intended by the inventors to be encompassed by the claims. Furthermore, the discussed combination of features might not be necessary for the inventive solution.

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All patents, patent applications and publications referred to herein are hereby incorporated by reference in their entirety, where permitted.

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CLAIMS:

1. A method of preparing tunicate water extract comprising:
 - a) extracting a *Styela clava*, *Ciona intestinalis*, *Botrylloides violaceus*, *Botryllus schlosseri* tunicate sample or any combination thereof with hot water; and
 - 5 b) collecting the tunicate water extract obtained in step a).
2. The method of claim 1, wherein the tunicate sample is a *Styela clava* tunicate sample.
3. The method of claim 1, wherein the tunicate sample is a *Ciona intestinalis* tunicate sample.
4. The method of claim 1, wherein the tunicate sample is a sample comprised of *Botrylloides violaceus*, *Botryllus schlosseri*, or a combination thereof.
- 10 5. The method of any one of claims 1 to 4, wherein the tunicate sample is dried or lyophilized prior to step a).
6. The method of claim 5, wherein the dried or lyophilized sample is milled prior to step a).
7. The method of any one of claims 1 to 6, wherein the step a) comprises sequentially extracting the tunicate sample with hexane, acetone, methanol, and hot water.
- 15 8. The method of any one of claims 1 to 7, wherein the water extract is further processed by ethanol precipitation, chromatography, ultrafiltration, desalting, drying by rotatory evaporator, centrifugal vacuum evaporator and/or spray dryer, or any combination thereof.
9. The method of any one of claims 1 to 8, wherein the water extract is further fractionated by ion exchange or size exclusion chromatography and/or papain hydrolysis followed by
- 20 precipitation at basic pH, yielding sub-fractions.
10. The method of claim 9, wherein the fractions are further processed by ethanol precipitation, chromatography, ultrafiltration, desalting, drying by rotatory evaporator, centrifugal vacuum evaporator and/or spray dryer, or any combination thereof.
11. A tunicate water extract, fraction or sub-fraction thereof obtained from *Styela clava*, *Ciona*
- 25 *intestinalis*, *Botrylloides violaceus*, *Botryllus schlosseri*, or any combination thereof.

12. The water extract of claim 11, wherein the extract, fraction or sub-fraction thereof is obtained from *Styela clava*.
13. The water extract of claim 11, wherein the extract, fraction or sub-fraction thereof is obtained from *Ciona intestinalis*.
- 5 14. The water extract of claim 11, wherein the extract, fraction or sub-fraction thereof is obtained from a combination of *Botrylloides violaceus* and *Botryllus schlosseri*.
15. A tunicate water extract, fraction or sub-fraction thereof obtained by the method of any one of claims 1 to 10.
16. The tunicate water extract or sub-fraction thereof of claim 11 to 14, characterized by any
10 one of the proton NMR spectrum of Figure 1D, 6, or 7.
17. The tunicate water extract or sub-fraction thereof of any one of claims 11 to 16, comprising acetamide-substituted polysaccharides or protein glycans as the main components.
18. The tunicate water extract or sub-fraction thereof of any one of claims 11 to 17, wherein the polysaccharides and/or protein glycans comprise a molecular weight in the range of about
15 50 KDa to 10 MDa.
19. The tunicate water extract or sub-fraction thereof of any one of claims 11 to 18, wherein the polysaccharides in the main component of the water extract may comprise galactose and glucose as the main sugar units.
20. A tunicate acetone extract, fraction, or sub-fraction thereof obtained from *Botrylloides violaceus*, *Botryllus schlosseri*, or any combination thereof.
21. A tunicate acetone extract, fraction, or sub-fraction thereof of claim 20, obtained by the method comprising:
- a) extracting a *Botrylloides violaceus*, *Botryllus schlosseri* tunicate sample or any combination thereof with acetone; and
25 b) collecting the tunicate acetone extract obtained in step a).
22. The tunicate acetone extract, fraction, or sub-fraction thereof of claim 21, wherein step a) of the method comprises sequentially extracting the tunicate sample with hexane and acetone.

23. The tunicate acetone extract, fraction, or sub-fraction thereof of any one of claims 20 to 22, characterized by the proton NMR spectrum of Figure 1B (1).

24. A method of treating, preventing, or delaying the onset of metabolic disorders by administering one or more than one extract, fraction, or sub-fraction thereof of any one of
5 claims 11 to 23 to a subject in need thereof.

25. The method of claim 24, wherein the metabolic disorder is hyperlipidemia and one or more than one of the extract, fraction, or sub-fraction thereof is selected from any one of claims 11 to 19.

26. The method of claim 24, wherein the metabolic disorder is obesity and the one or more
10 than one of the extract, fraction, or sub-fraction thereof is selected from any one of claims 12 or 20 to 23.

27. A method of enhancing LDL-cholesterol uptake comprising administering one or more than one of the tunicate water extract, fraction, or sub-fraction thereof of any one of claim 11 to 19 to a subject in need thereof.

15 28. A method of inhibiting adipogenesis comprising administering one or more than one of the water and/or acetone tunicate extract of any one of claims 12 or 20 to 23 to a subject in need thereof.

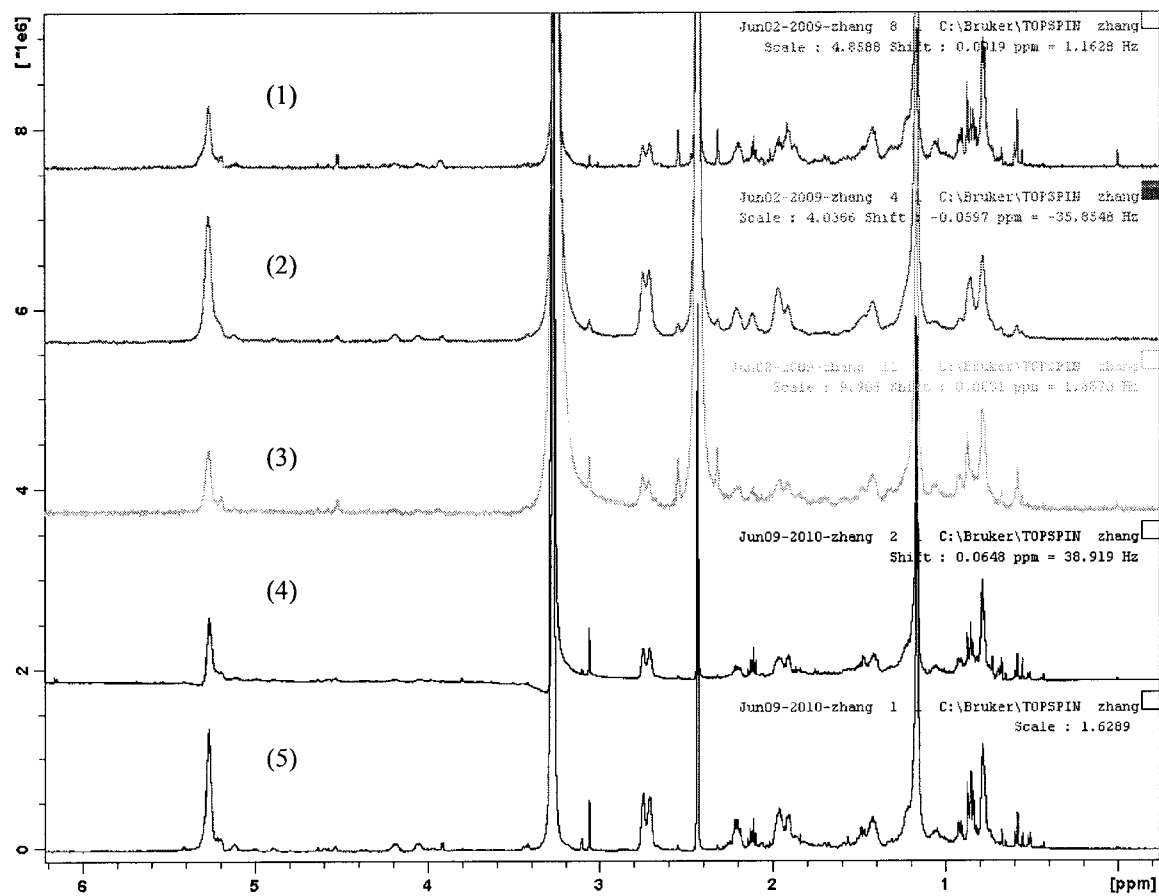


FIG. 1A

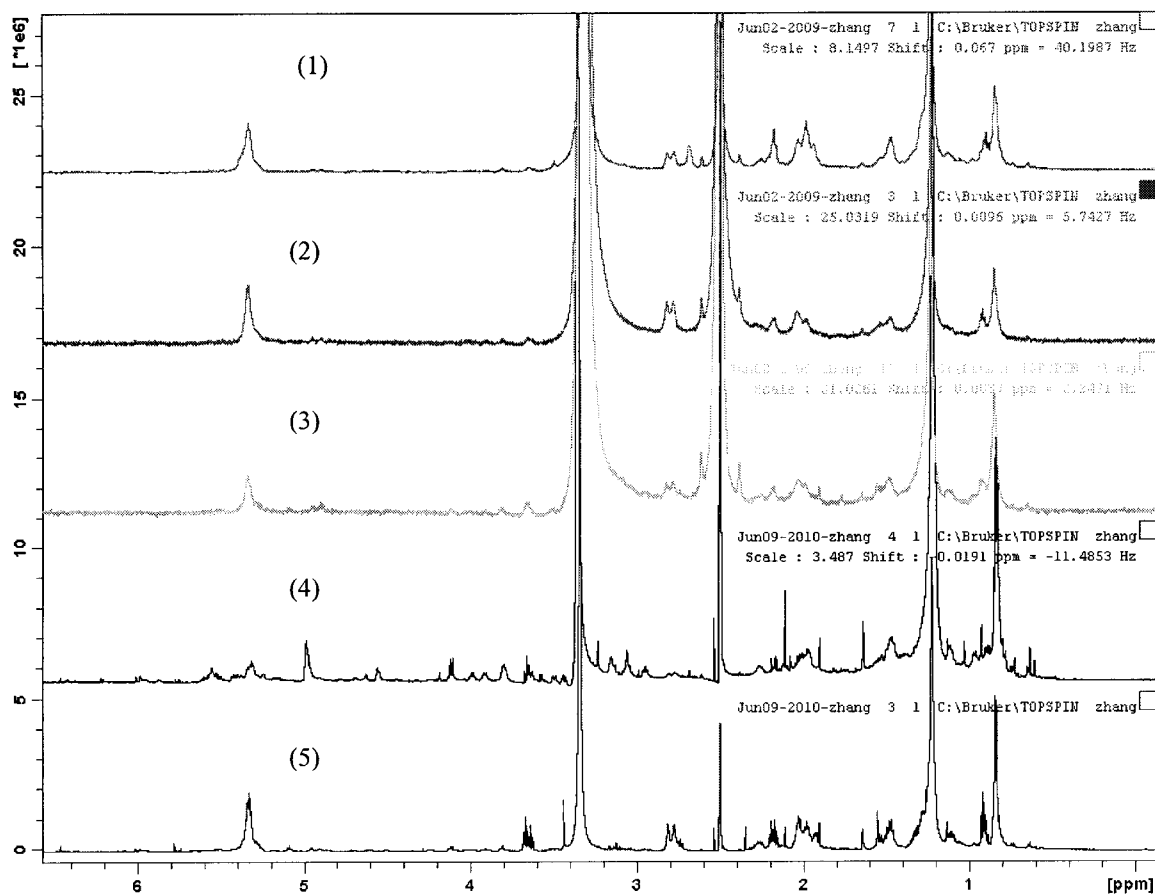


FIG. 1B

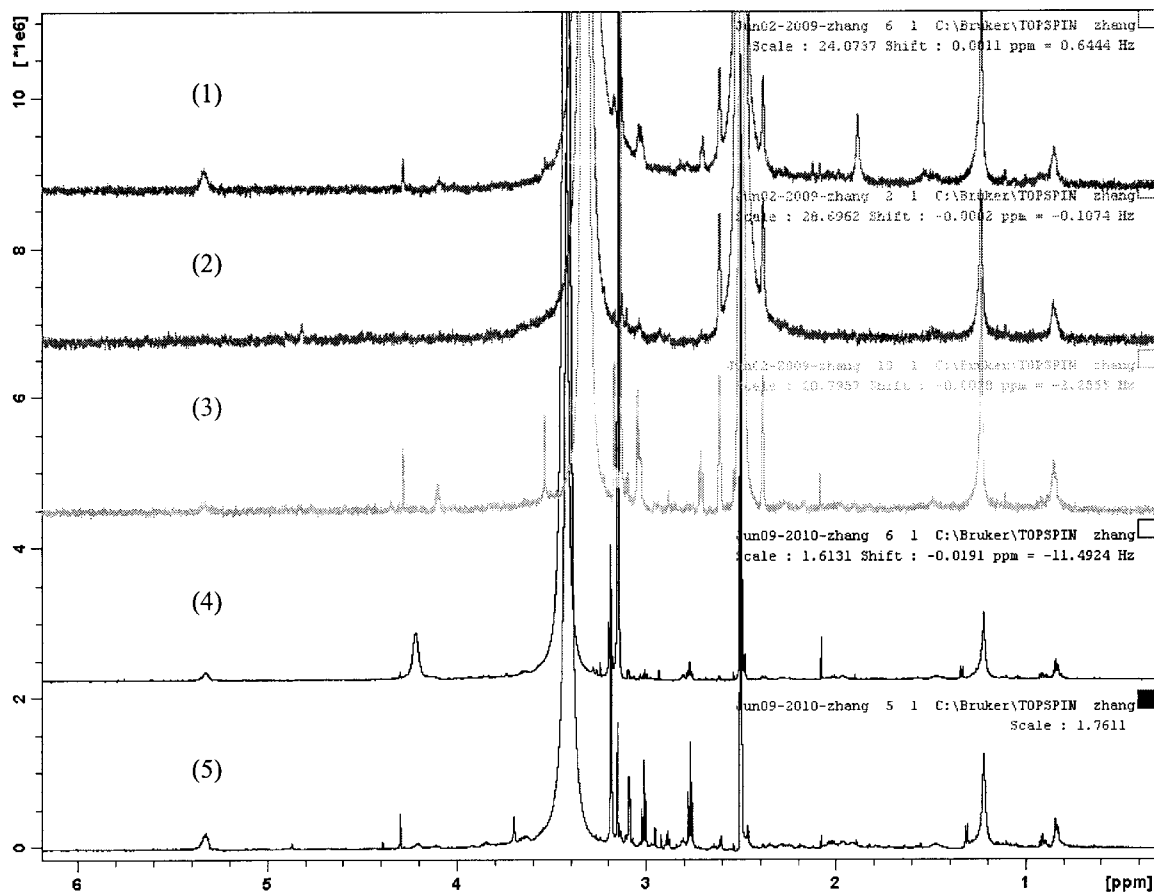


FIG. 1C

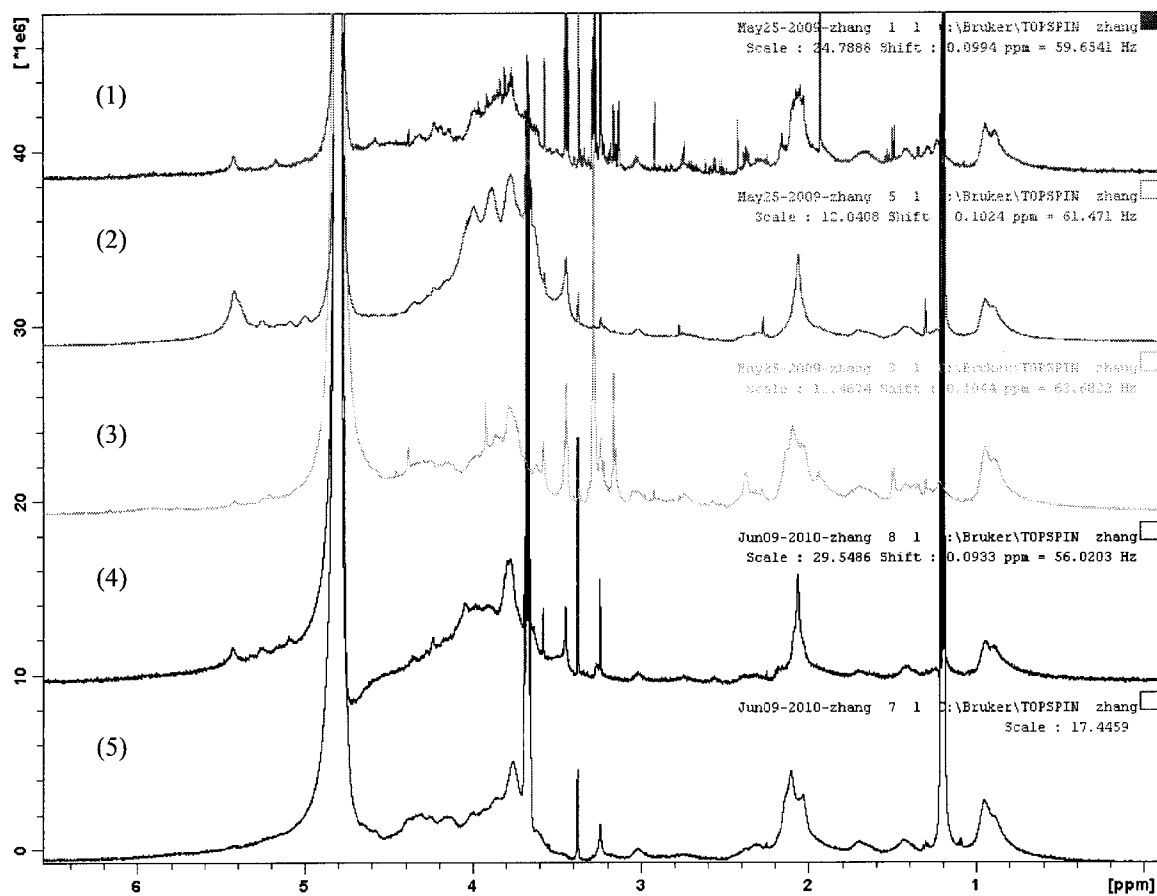


FIG. 1D

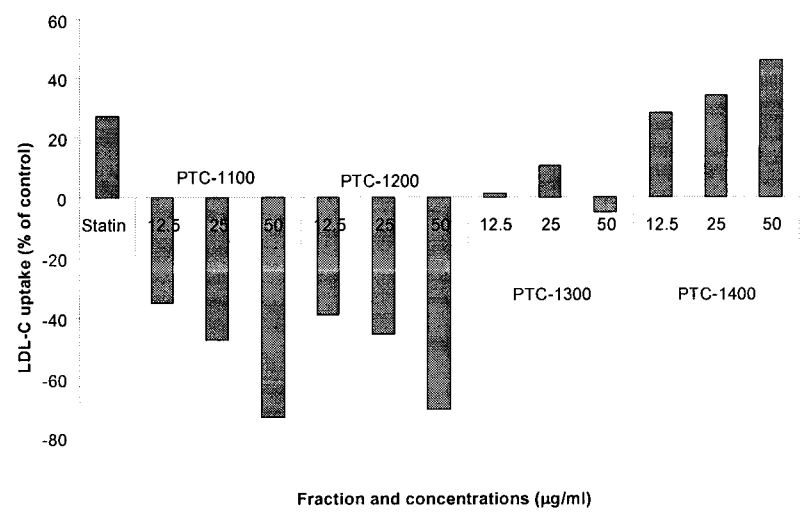


FIG. 2A

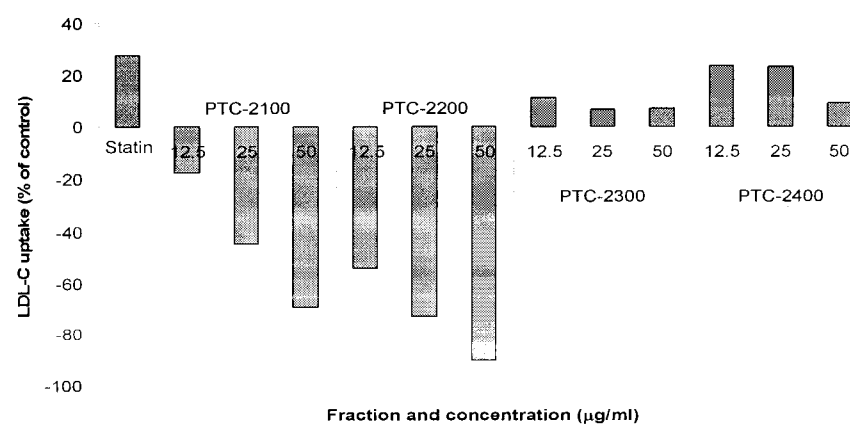


FIG. 2B

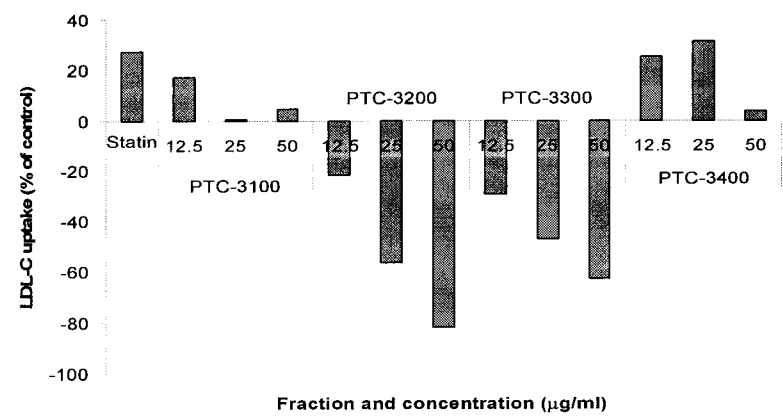


FIG. 2C

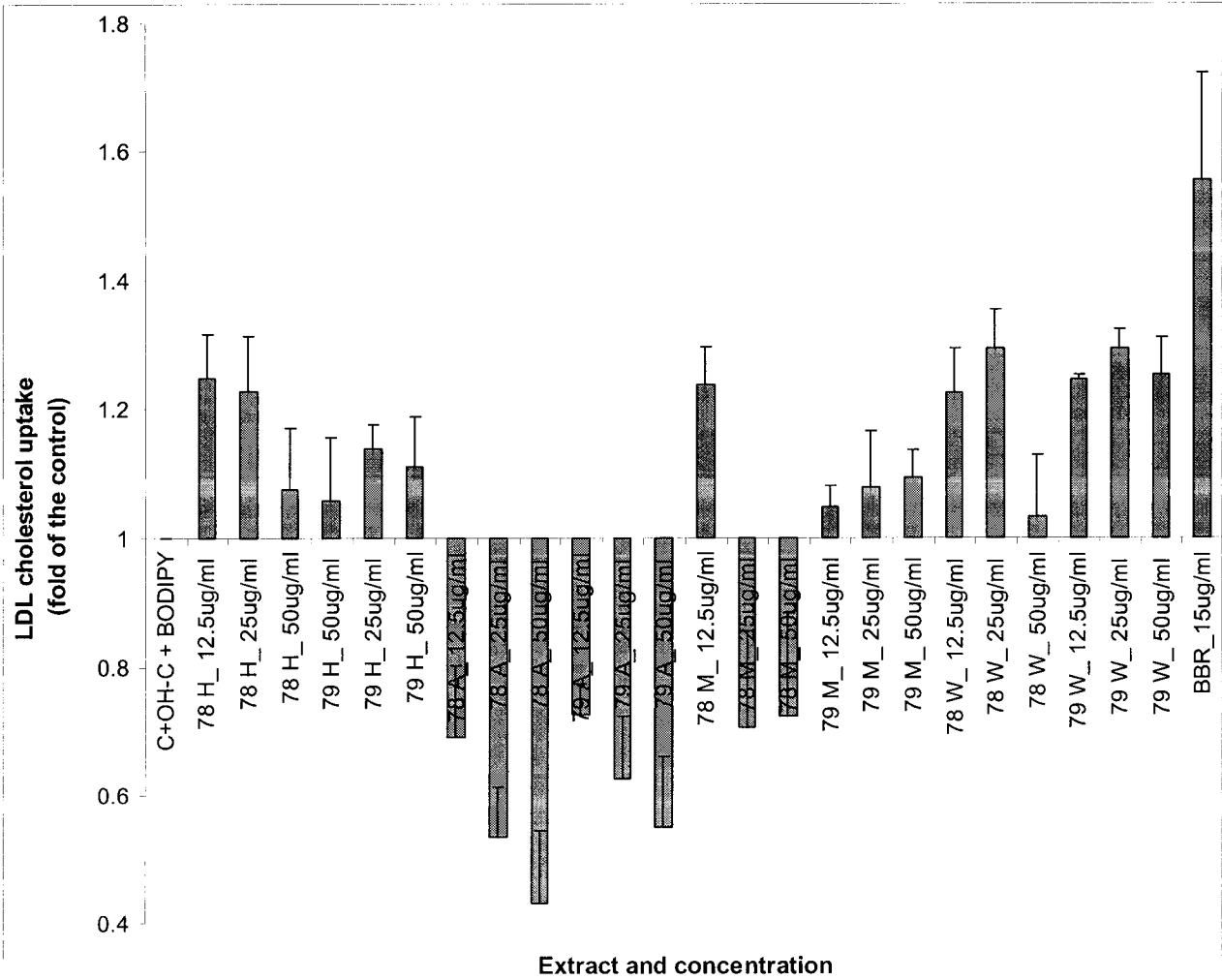


FIG. 3A

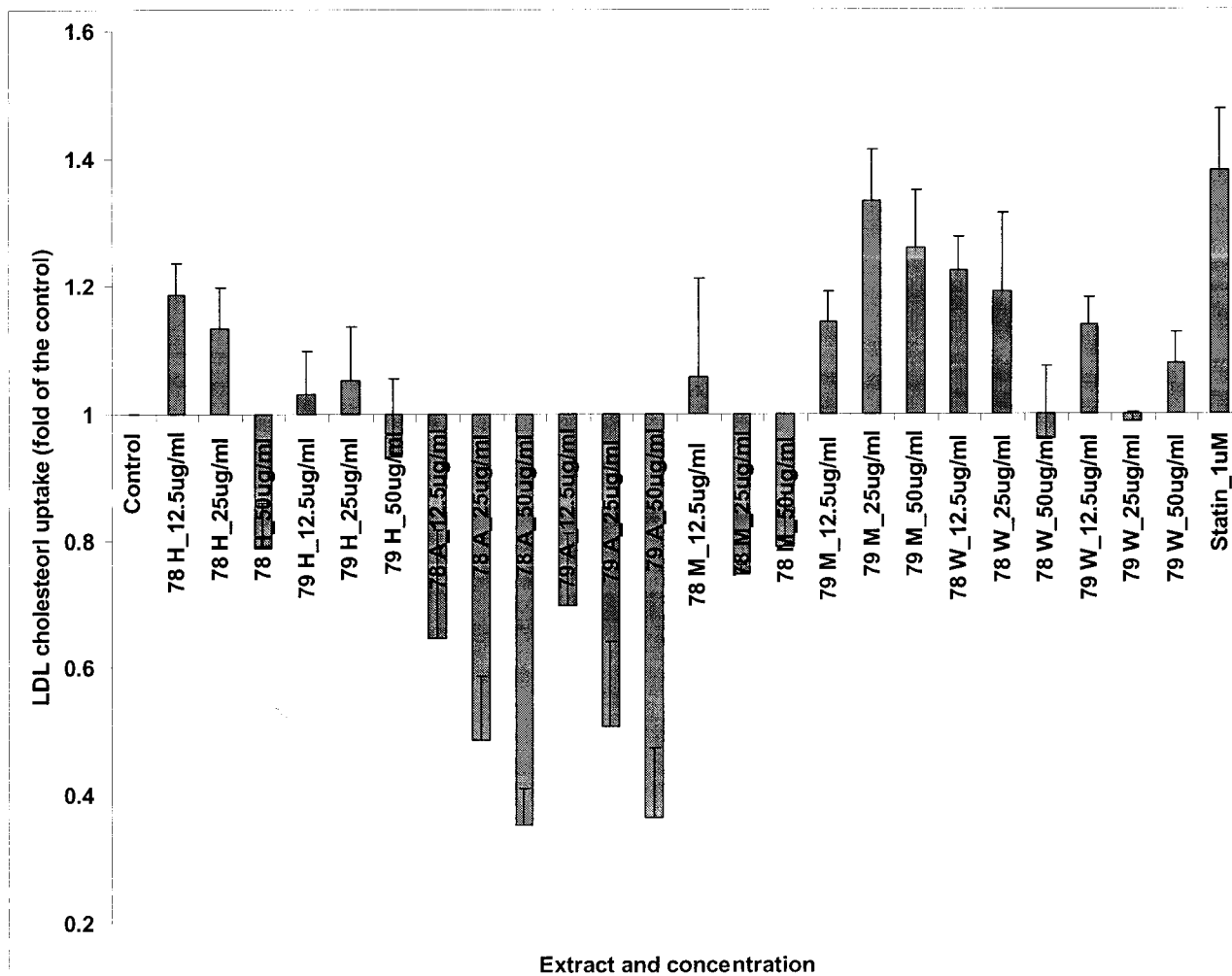


FIG. 3B

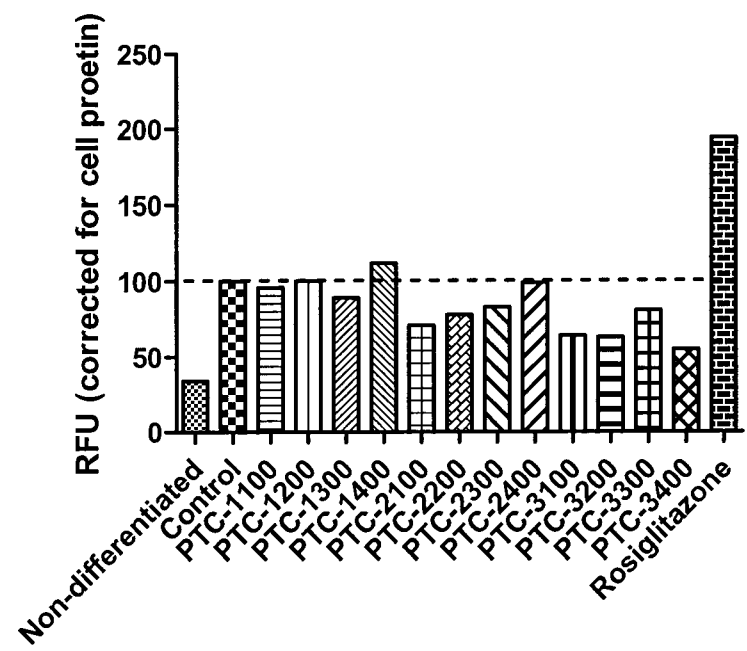


FIG. 4

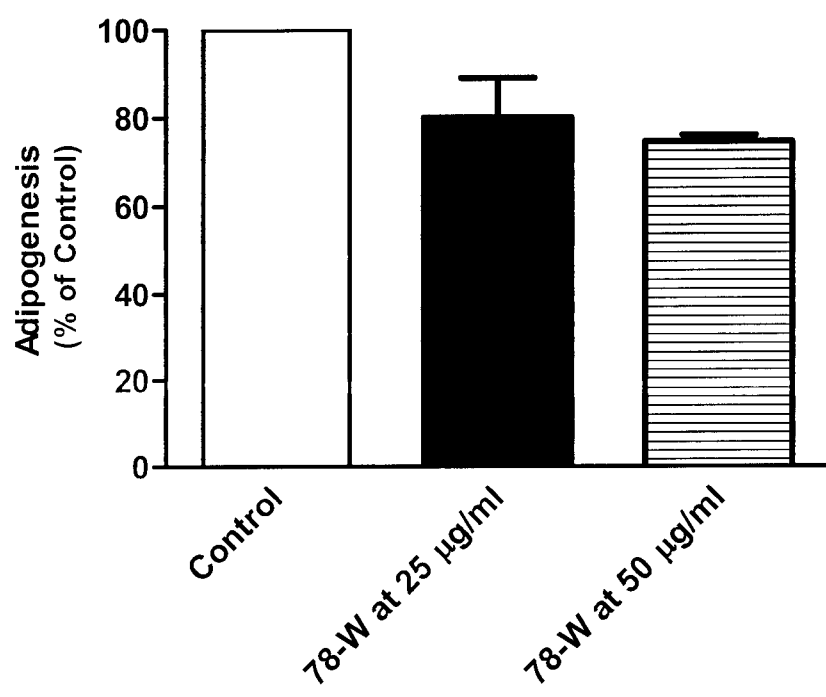


Fig. 5

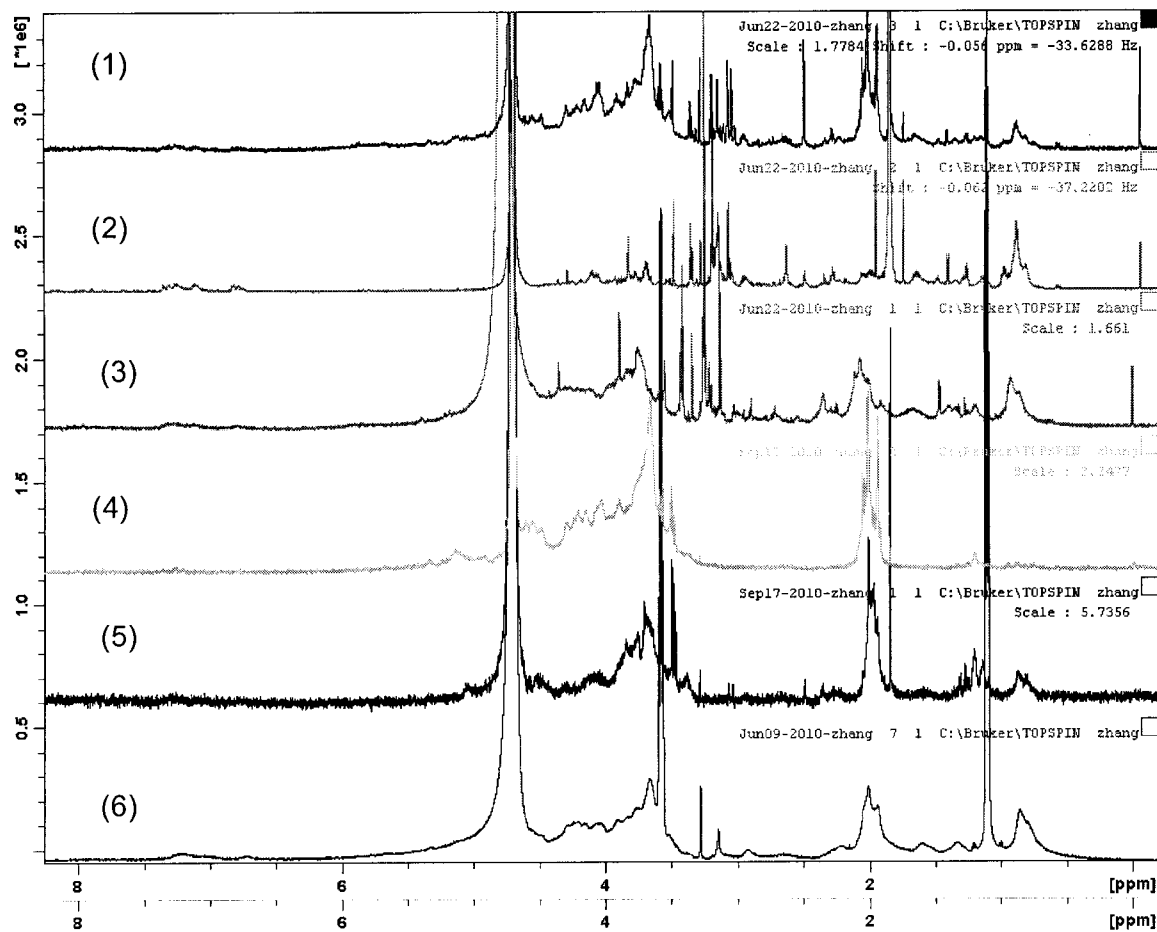


FIG. 6

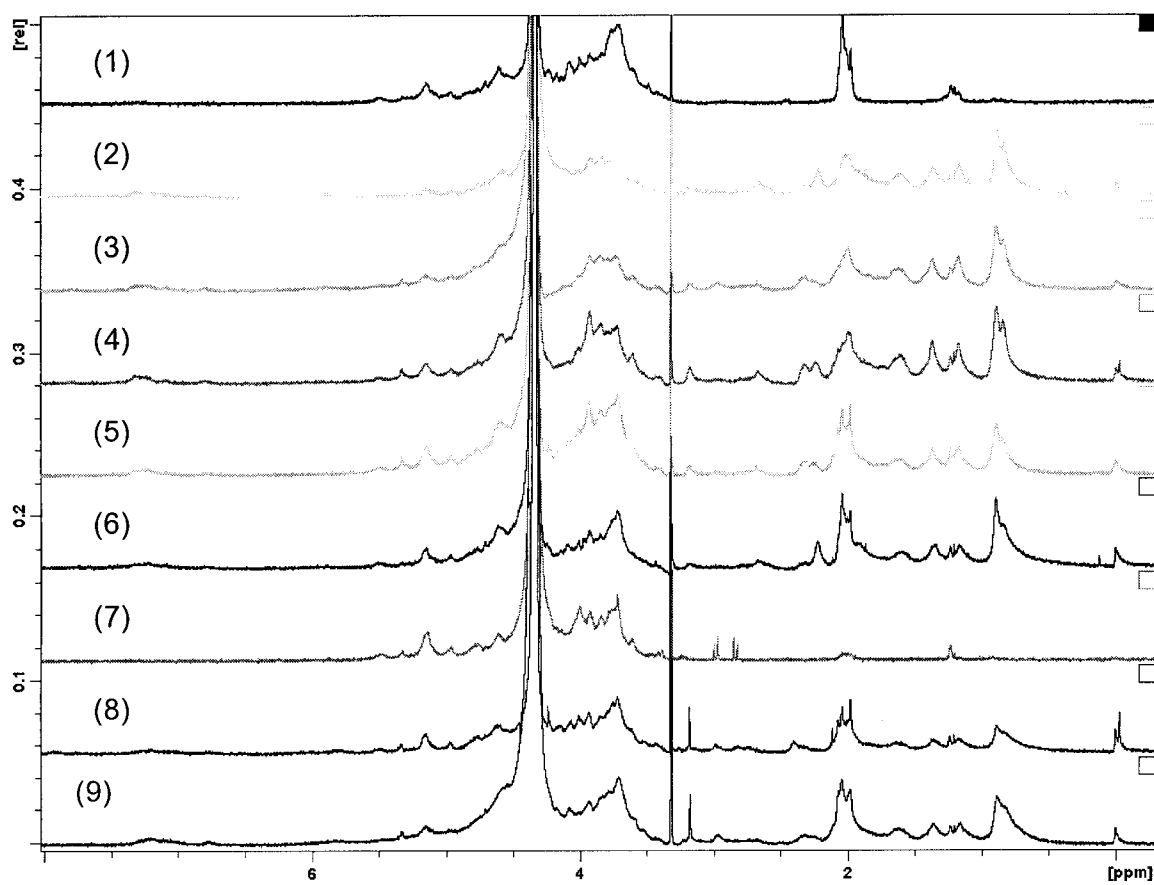


FIG. 7

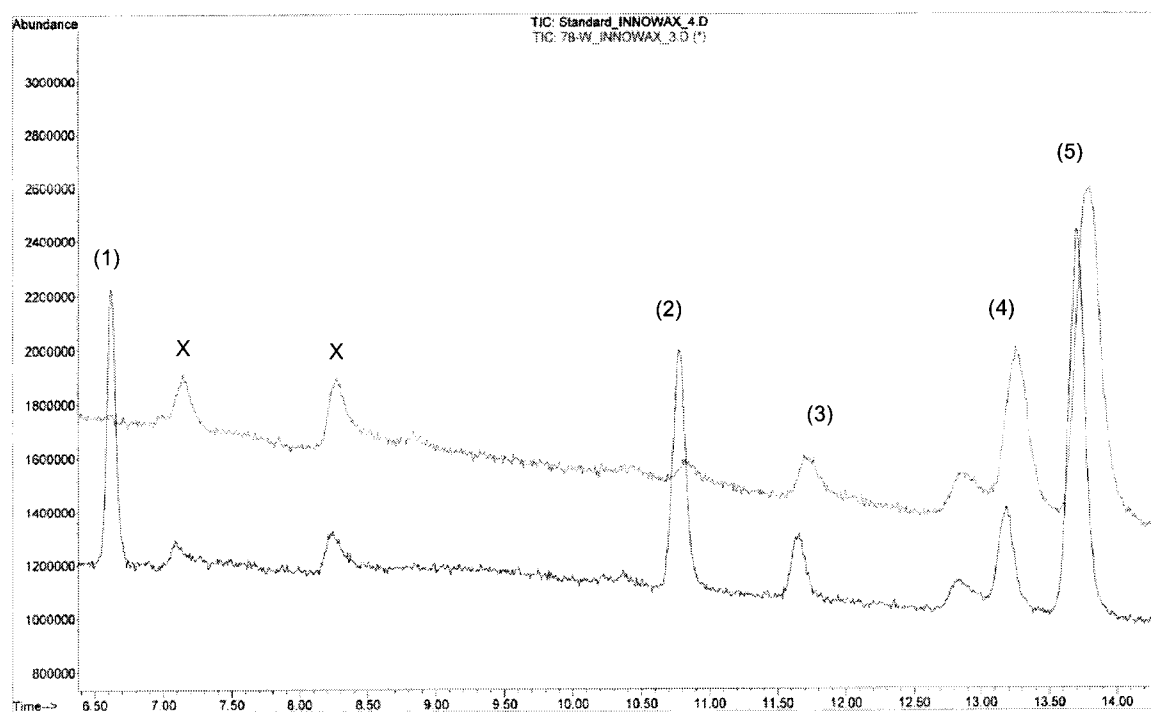


FIG. 8

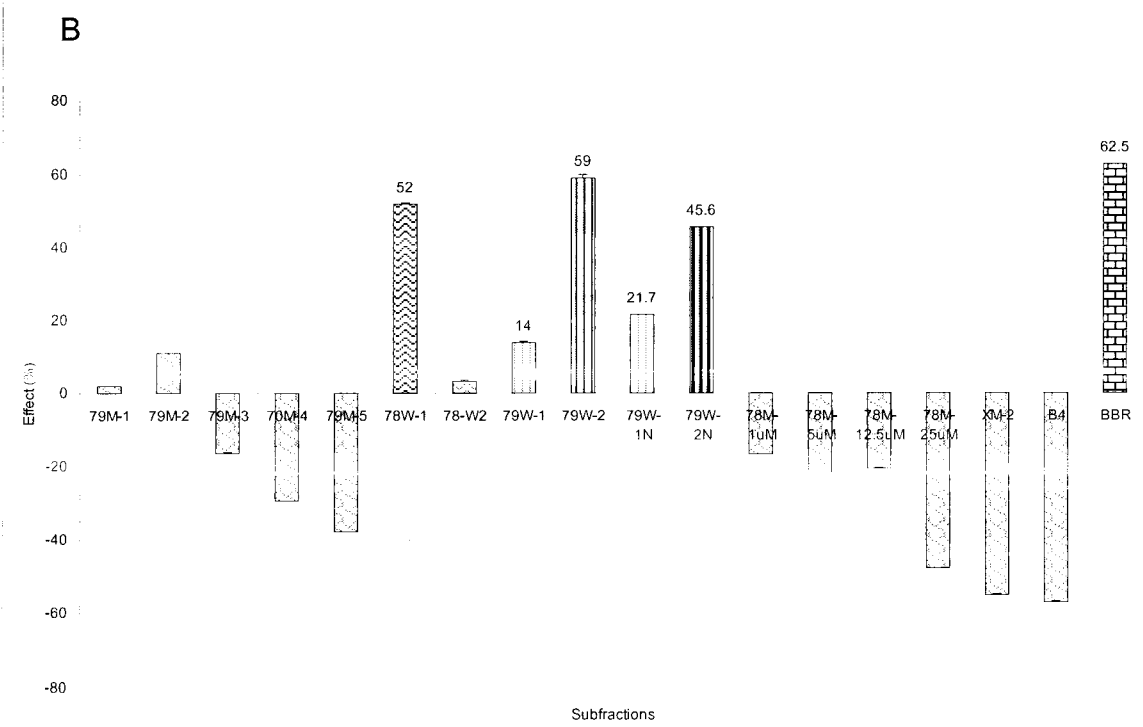
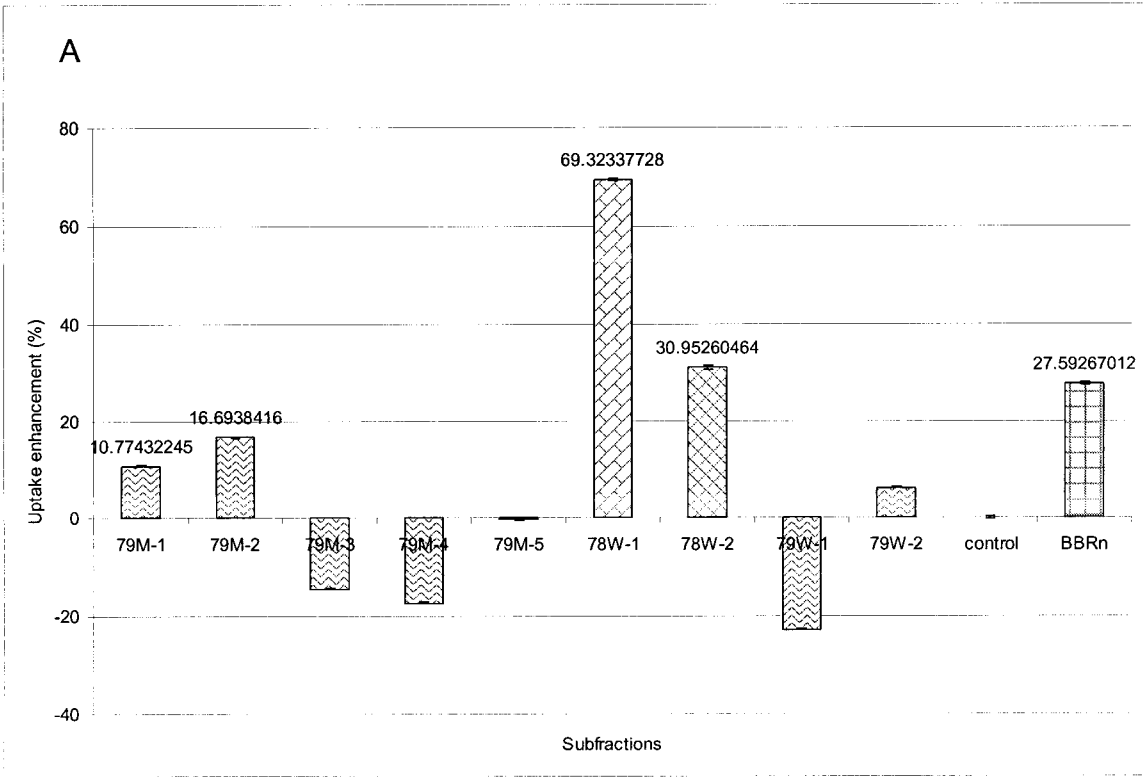


FIG. 9

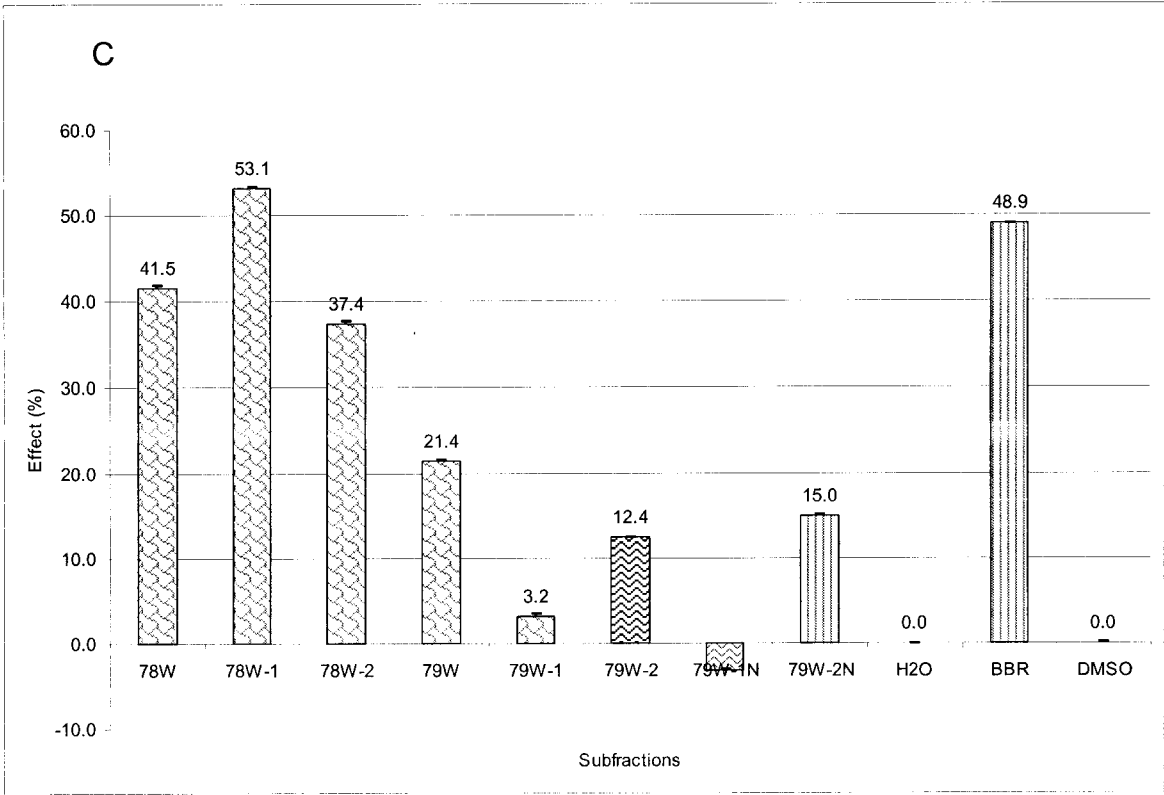


FIG. 9C

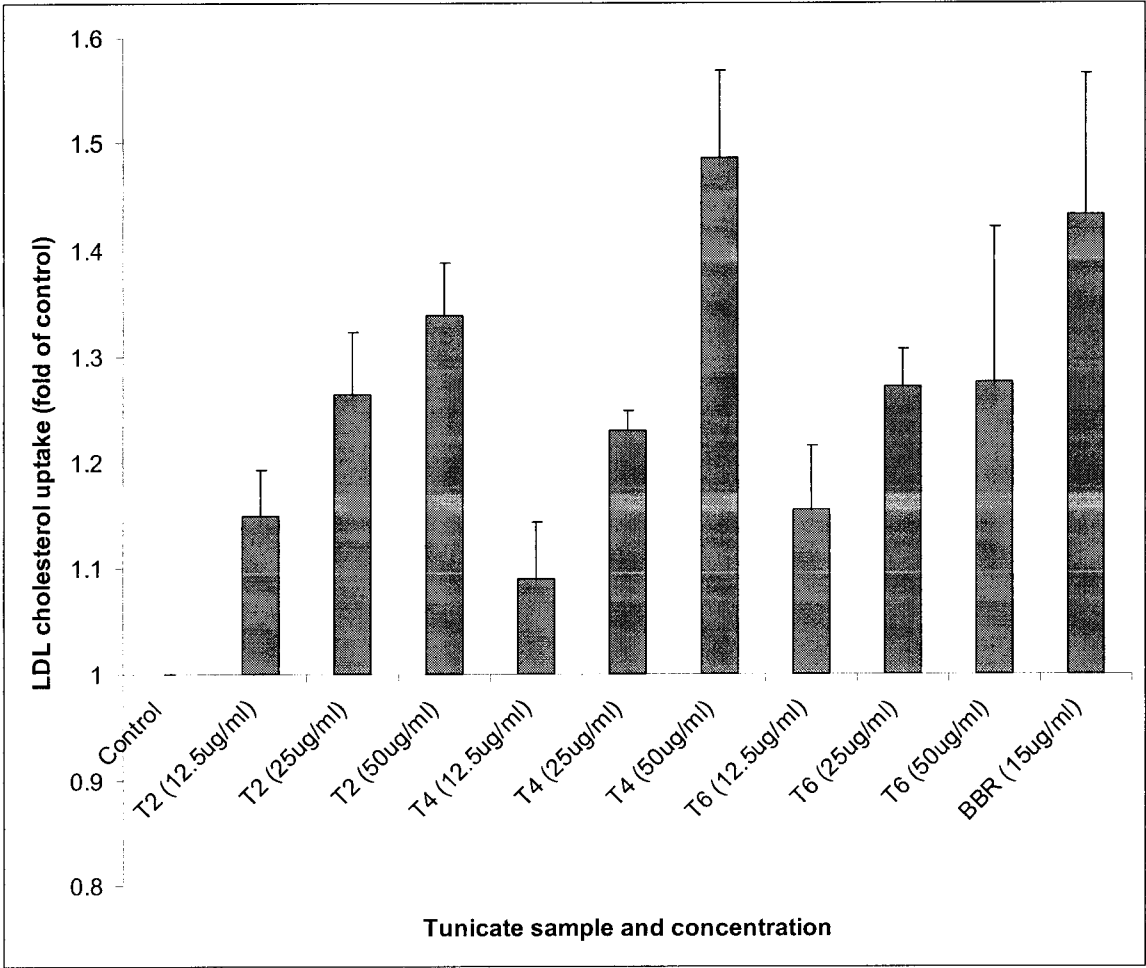


FIG. 10A

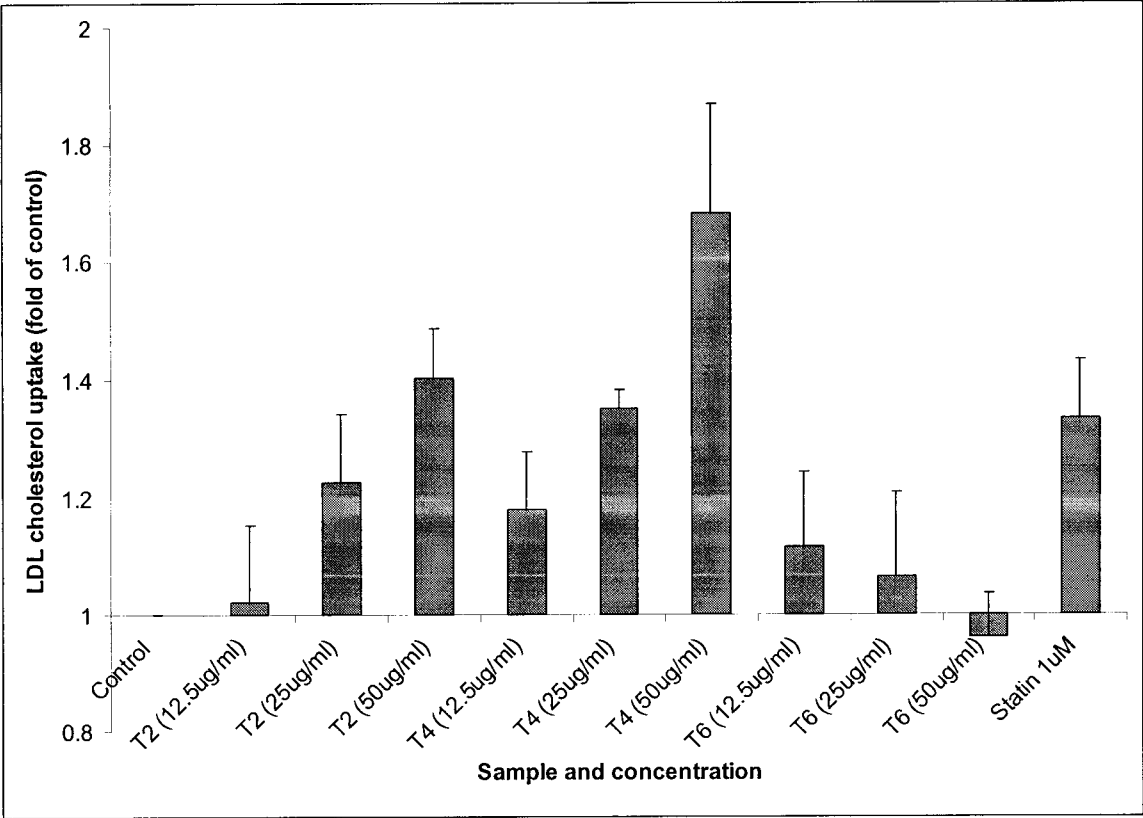


FIG. 10B

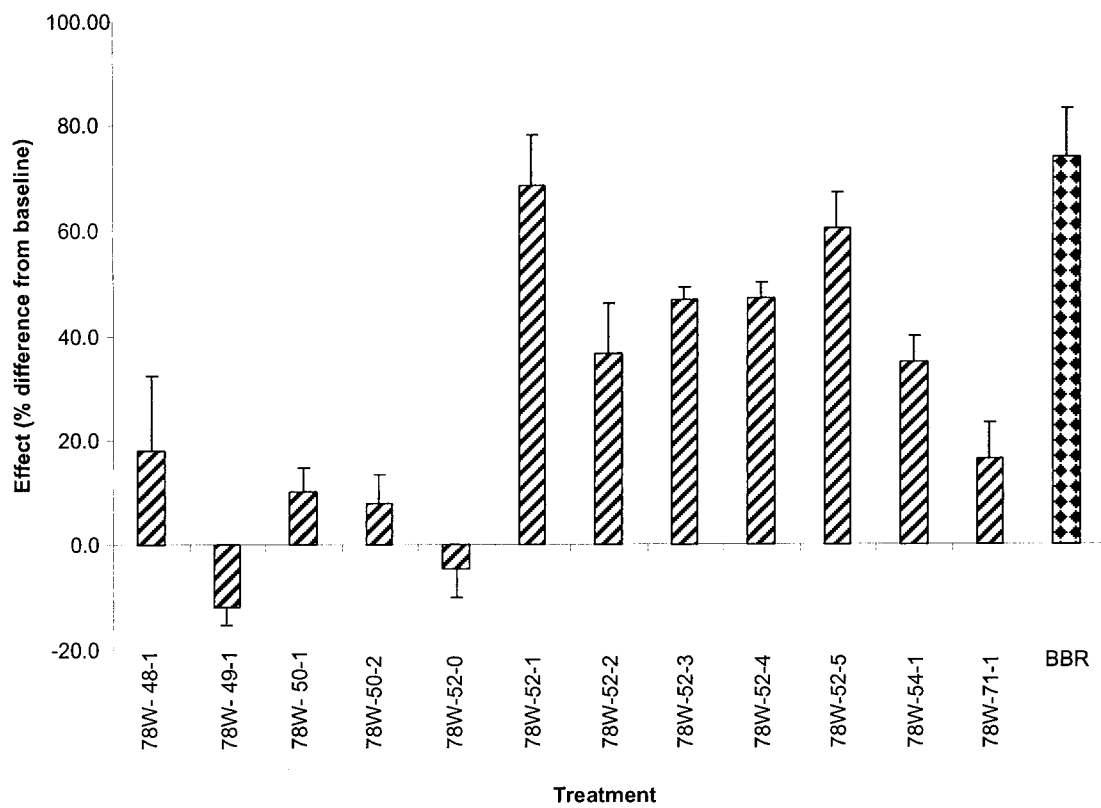


FIG. 11

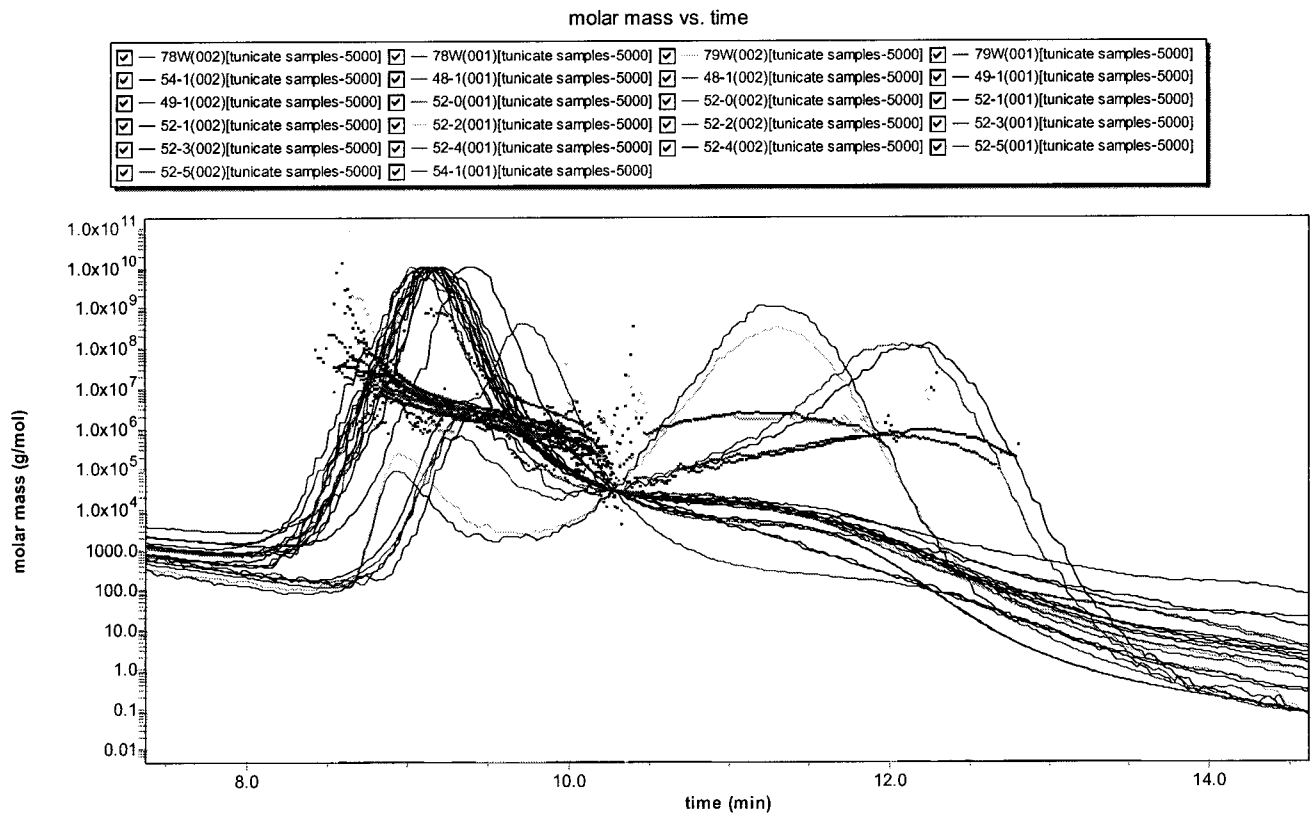


FIG. 12A

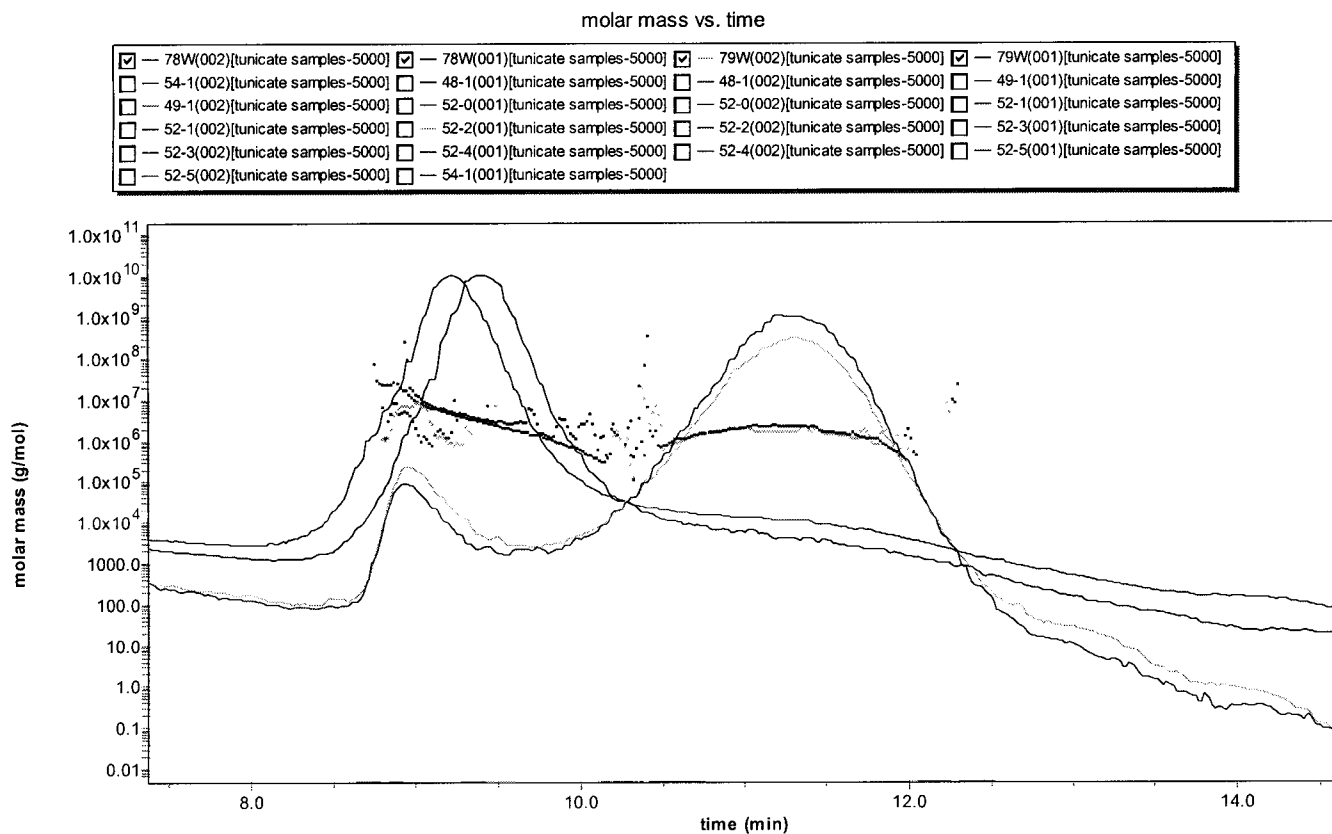


FIG. 12B

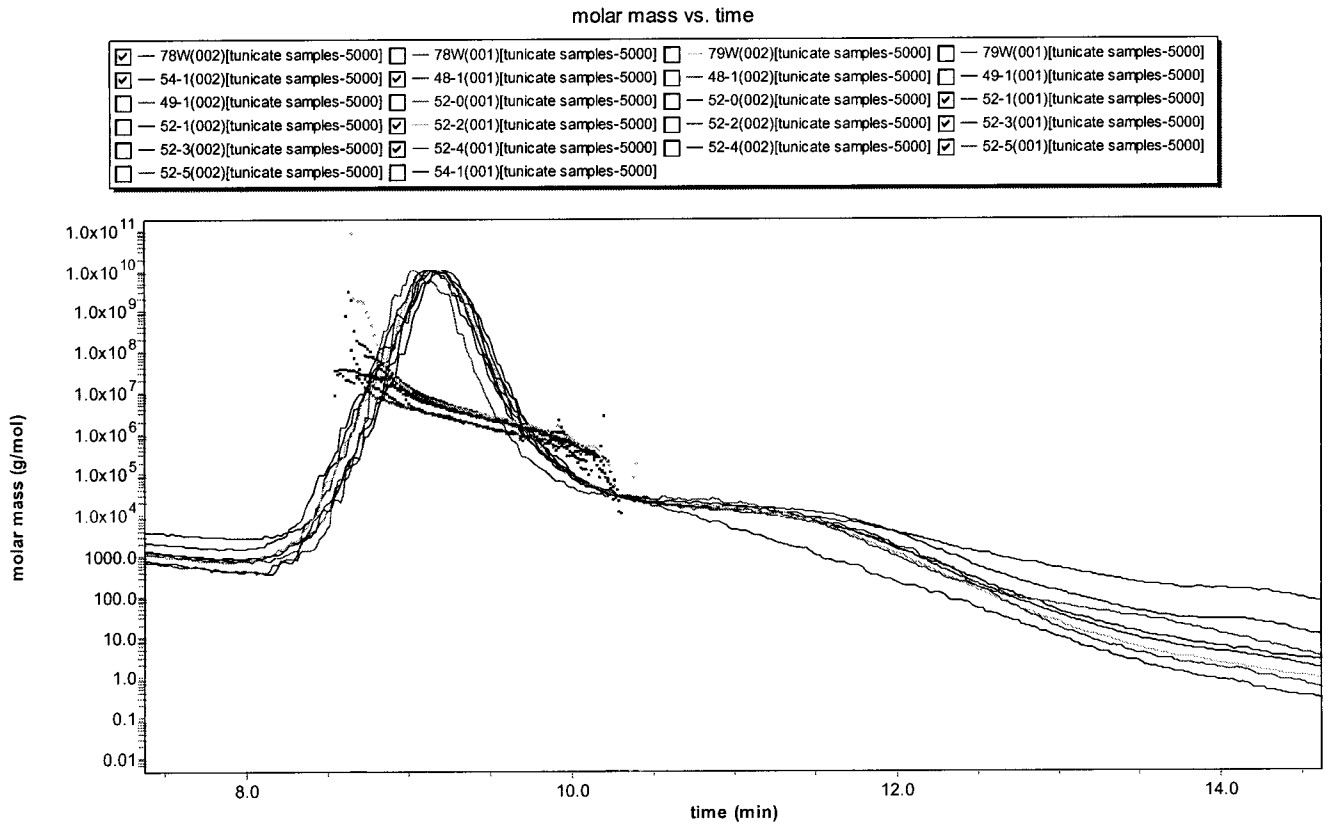


FIG. 12C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2011/000204

A. CLASSIFICATION OF SUBJECT MATTER

IPC: **A61K 35/56** (2006.01) , **A61P 3/06** (2006.01) , **B01D 11/00** (2006.01)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: **A61K 35/56** (2006.01) , **A61P 3/06** (2006.01) , **B01D 11/00** (2006.01)

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

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
Canadian Patents Database, Total Patent, Medline, Scopus (keywords: tunicate, Styela clava, Ciona intestinalis, Botrylloides violaceus, water, extract, hot, acetone, metabolic, disorder, hyperlipidemia, obesity, LDL, cholesterol, adipogenesis)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KR100743569B1 (CHANGWON NATIONAL UNIVERSITY INDUSTRY ACADEMY COOPERATION CORPS) 23 July, 2007 (23-07-2007). Abstract.	1, 2, 5, 6, 8-12, 15-19
Y		4, 7, 14
X	KR1020090030831A (AQUA GREEN TECHNOLOGY CO. LTD.) 25 March, 2009 (25-03-2009). Abstract.	1, 2, 5, 6, 8-12, 15-19
Y		4, 7, 14
X	KR1020080018590A (INDUSTRY-ACADEMIC COOPERATION FOUNDATION OF KYUNGNAM UNIVERSITY) 28 February, 2008 (28-02-2008). Abstract.	11, 12, 15-19
Y		7, 20-23

☒ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents :	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

10 June 2011 (10-06-2011)

Date of mailing of the international search report

8 July 2011 (08-07-2011)

Name and mailing address of the ISA/CA
Canadian Intellectual Property Office
Place du Portage I, C114 - 1st Floor, Box PCT
50 Victoria Street
Gatineau, Quebec K1A 0C9
Facsimile No.: 001-819-953-2476

Authorized officer

Seema Bissoon-Haqqani (819) 934-7932

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons :

1. ☒ Claim Nos. : 24-28

because they relate to subject matter not required to be searched by this Authority, namely :

Although claims 24-28 are directed to a method of treatment of the human/animal body, which this authority is not obliged to search under Rule 39.1(iv) of the PCT, the search has been carried out based on the alleged effects of the compositions referred to therein.

2. ☐ Claim Nos. :

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically :

3. ☐ Claim Nos. :

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

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

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

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

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

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

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2011/000204

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JIN-JU, K. ET AL. Antioxidant and anticancer activities of extracts from <i>Styela clava</i> according to the processing methods and solvents. Journal of the Korean Society of Food Science and Nutrition. 8 May, 2006 (08-05-2006). Vol. 35, no. 3, pgs 278-283. ISSN:1226-3311, Abstract.	11, 12, 15-19
Y		20-23
X	THORNDYKE, M. ET AL. Identification and localization of material with gastrin-like immunoreactivity in the neural ganglion of a protochordate, <i>Ciona intestinalis</i> . Regul. Pept. 30 Dec, 1986 (30-12-1986). Vol. 16, no. 3-4, pgs 269-79. ISSN: 0167-0115, Abstract.	1, 3, 13, 15-19
Y		4, 7, 14
X	SATAKE, H. ET AL. Tachykinin and tachykinin receptor of an Ascidian, <i>Ciona intestinalis</i> . J. Biol. Chem. 17 Dec, 2004 (17-12-2004) [published on-line 12 October, 2004 (12-10-2004)]. Vol. 279, no. 51, pgs 53798-53805. ISSN: 0021-9258 (print) and 1083-351X (electronic).	1, 3, 13, 15-19
Y		4, 7, 14
Y	HIROSE, E. et al. A new type of the manifestation of colony specificity in the compound Ascidian, <i>Botrylloides violaceus</i> Oka. Biol. Bull. October, 1988 (10-1988). Vol. 175, no. 2, pgs 240-245. ISSN: 1062-3590, Abstract.	4, 14, 20-23
Y	BALLARIN, L. ET AL. Cytochemical properties of <i>Botryllus schlosseri</i> haemocytes: indications for morpho-functional characterisation. Eur. J. Histochem. 2005. Vol. 49, no. 3, pgs 255-264. ISSN: 1121-760X.	4, 14, 20-23
X	KR1020060087765A (KANG, H. J.) 3 August, 2006 (03-08-2006). Abstract.	24, 25, 27
X	CN1431188A (WANG, C.) 23 July, 2003 (23-07-2003). Abstract.	24, 26, 28

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CA2011/000204

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
KR100743569B1	23 July 2007 (23-07-2007)	None	
KR1020090030831A	25 March 2009 (25-03-2009)	None	
KR1020080018590A	28 Feb 2008 (28-02-2008)	KR100836569B1	2008-06-10
KR1020060087765A	3 August 2006 (03-08-2006)	None	
CN1431188A	23 July 2003 (23-07-2003)	None	