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(54) Title: COMPOSITION WITH PREVENTIVE OR IMPROVEMENT EFFECT ON SYMPTOMS OR DISEASES ASSOCIATED WITH STRESS-INDUCED BEHAVIOR DISORDERS

(57) Abstract: A composition with a preventive or improvement effect on symptoms or diseases associated with stress-induced behavior disorders, comprising arachidonic acid and/or a compound comprising arachidonic acid as a constituent fatty acid.

## DESCRIPTION

5 COMPOSITION WITH PREVENTIVE OR IMPROVEMENT EFFECT ON  
SYMPTOMS OR DISEASES ASSOCIATED WITH STRESS-INDUCED  
BEHAVIOR DISORDERS

Technical Field of the Invention

10 The present invention relates to a preventive or improvement agent for symptoms or diseases associated with stress-induced behavior disorders, comprising as an active ingredient arachidonic acid and/or a compound comprising arachidonic acid as a constituent fatty acid, as well as to a composition with a preventive or improvement effect on symptoms or diseases associated with stress-induced behavior disorders, and a method for its production. More specifically, the invention relates to a preventive or improvement agent for stress-induced habits such as finger sucking and onychophagy, adjustment disorder, attention deficit hyperactivity disorder, acute 15 stress disorder or posttraumatic stress disorder, comprising as an active ingredient at least one selected from the group consisting of arachidonic acid, arachidonic acid alcohol esters, and triglycerides, phospholipids or glycolipids wherein all or a portion of the constituent fatty acid is arachidonic acid, as well 20 as to a food or beverage with such a preventive or improvement effect and a method for its production.

25

Background Art

30 Stress is recognized as a response which can lead to behavior disorders, and the symptoms associated with stress-induced behavior disorders are known as stress-related disorders; these are classified into several types of symptoms based on two factors: the nature of the stress and the disposition of the individual experiencing 35 the stress. The symptoms span a wide range from simple habits such as finger sucking or onychophagy seen primarily during the infant period and resulting from

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light stress factors, to adjustment disorder and attention deficit hyperactivity disorder which are considered to be highly influenced by individual disposition, and further to acute stress disorder or 5 posttraumatic stress disorder, which are associated with extremely intense stress beyond individual disposition (Shindan to Chiryo 91, 1333, 2003).

Recently, increased blood IL-1 $\beta$  has been reported in post-traumatic stress disorder patients (Biol. Psychiatry 10 42, 345, 1997), and research has focused on the relationship between IL-1 $\beta$  and neuronopathy.

Drugs used for stress-related disorders include benzodiazepine-based drugs used for insomnia and anxiety, serotonin reuptake inhibitors and tricyclic 15 antidepressant drugs used to alleviate symptoms of flashbacks, adrenaline antagonists and anticonvulsant drugs used for symptoms of hypervigilance, and antipsychotic agents used for exaggerated startle response or increased irritability. However, all such 20 agents are symptomatic treatment for improvement of superficial symptoms such as depression, insomnia and excitement, and unfortunately no agents for causal treatment exist at this time.

One possible treatment for stress-related disorders 25 is to suppress their progression by removing the cause of stress. However, given the modern environment it is very difficult to eliminate the causative factors of stress.

Thus, to date no drug has existed which is effective 30 as a pharmaceutical agent having a preventive or improvement effect on symptoms or diseases associated with stress-induced behavior disorders. Furthermore, applications to food products have been hampered by the limitation to components which produce no side effects.

The brain consists of a lipid mass-like tissue, with 35 phospholipids constituting about 1/3 of the white matter and about 1/4 of the gray matter. The polyunsaturated fatty acids in phospholipids of the various cell

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membranes in the brain consist primarily of arachidonic acid and docosahexaenoic acid. However, arachidonic acid and docosahexaenoic acid (DHA) cannot be synthesized *de novo* in animal bodies and must be directly or indirectly obtained through diet (for example, as the arachidonic acid and docosahexaenoic acid precursors, linoleic acid and  $\alpha$ -linolenic acid).

Burgess et al. have demonstrated that arachidonic acid and DHA contents of plasma phospholipids are significantly lower in posttraumatic stress disorder patients (Am J Clin Nutr 71, 327S, 2000). It has also been reported that liver microsome  $\Delta$ 5-desaturase and  $\Delta$ 6-desaturase activity is reduced in separately bred stress model rats (Proc Soc Exp Biol Med. 205, 56, 1994), and the reduction in activity of these desaturases is believed to be responsible for a lack of brain levels of arachidonic acid and DHA, polyunsaturated fatty acids with a high degree of unsaturation.

On the other hand, several experiments have been reported using administration of arachidonic acid to animal stress models. Song et al. reported that administration of free arachidonic acid was not effective for IL-1 $\beta$  induced stress anxiety behavior models (J Lipid Res. 44, 1984, 2003). Also, Clements et al. describe giving attention deficit hyperactivity disorder (ADHD) models (spontaneously hypertensive rats, SHR) feed containing 0.5% arachidonic acid and 0.9% DHA for 8 weeks, and reported increased DHA in the brain phospholipids but no observable effect (Dev Psychobiol. 43, 57, 2003). No other reports indicate that administration of arachidonic acid improves stress-induced behavior disorders.

Thus, while it has been reported that arachidonic acid levels in the body are lowered by stress, it has not been clearly demonstrated whether arachidonic acid or compounds including arachidonic acid as a constituent

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fatty acid according to the invention are effective for the prevention or improvement of symptoms or diseases associated with stress-induced behavior disorders, and in fact the experiments conducted to date have been 5 definitively negative.

Non-patent document 1: Shindan to Chiryo 91, 1333, 2003

Non-patent document 2: Biol. Psychiatry 42, 345, 1997

10 Non-patent document 3: Am J Clin Nutr 71, 327S, 2000

Non-patent document 4: Proc Soc Exp Biol Med. 205, 56, 1994

Non-patent document 5: J Lipid Res. 44, 1984, 2003

15 Non-patent document 6: Dev Psychobiol. 43, 57, 2003

#### DISCLOSURE OF THE INVENTION

Thus, a strong demand exists for development of pharmaceuticals which prevent and improve symptoms or 20 diseases associated with stress-induced behavior disorders, as well as such compounds which are highly suitable for consumption and lacking notable side effects.

As a result of much diligent research conducted with 25 the purpose of elucidating the preventive or improvement effects on symptoms or diseases associated with stress-induced behavior disorders by agents comprising as active ingredients arachidonic acid and/or compounds comprising arachidonic acid as a constituent fatty acid, the present 30 inventors found that the active ingredients of the invention exhibit apparent behavioral pharmacologic effects in a behavioral observation test using mice subjected to restraint stress, which is considered to approximate human emotional stress.

35 We also succeeded in realizing industrial production of a triglyceride containing at least 10% microbially generated arachidonic acid, and supplied the triglyceride

for testing in order to elucidate the effect of the invention.

Specifically, the present invention provides a preventive or improvement agent for symptoms or diseases associated with stress-induced behavior disorders and a composition with a preventive or improvement effect on symptoms or diseases associated with stress-induced behavior disorders, comprising as an active ingredient arachidonic acid and/or a compound comprising arachidonic acid as a constituent fatty acid, as well as a method for their production. More specifically, the invention provides a preventive or improvement agent for stress-induced habits such as finger sucking and fingernail biting, adjustment disorder, attention deficit hyperactivity disorder, acute stress disorder or posttraumatic stress disorder, comprising as an active ingredient at least one selected from the group consisting of arachidonic acid, arachidonic acid alcohol esters, and triglycerides, phospholipids or glycolipids wherein all or a portion of the constituent fatty acid is arachidonic acid, as well as to a composition with such a preventive or improvement effect and a method for its production.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graph showing the results for Example 3, indicating the effect of arachidonic acid on the rearing behavior of stressed mice.

Fig. 2 is a graph showing the results for Example 3, indicating the effect of arachidonic acid on the ultromotivity of stressed mice.

#### Best Mode for Carrying Out the Invention

The present invention relates to a preventive or improvement agent for symptoms or diseases associated with stress-induced behavior disorders and a composition with a preventive or improvement effect on symptoms or diseases associated with stress-induced behavior disorders, comprising as an active ingredient arachidonic acid

acid and/or a compound comprising arachidonic acid as a constituent fatty acid, as well as a method for their production.

As "symptoms or diseases associated with stress-induced behavior disorders" there may be mentioned habits such as finger sucking and onychophagy, adjustment disorder, attention deficit hyperactivity disorder, acute stress disorder or posttraumatic stress disorder, but the symptoms and diseases are not limited to these and include all symptoms and diseases associated with stress-induced behavior disorders.

The active ingredient of the invention is arachidonic acid, but any compound comprising arachidonic acid as a constituent fatty acid may be used. As compounds comprising arachidonic acid as a constituent fatty acid there may be mentioned arachidonic acid salts, such as calcium or sodium salts. There may also be mentioned arachidonic acid lower alcohol esters such as arachidonic acid methyl ester and arachidonic acid ethyl ester. There may also be used triglycerides, phospholipids or glycolipids wherein all or a portion of the constituent fatty acid is arachidonic acid. However, the invention is not limited to the compounds mentioned above, and includes any compound comprising arachidonic acid as a constituent fatty acid.

For application to food products, the arachidonic acid is preferably in the form of a triglyceride or phospholipid, and most preferably in the form of a triglyceride. While virtually no natural sources of arachidonic acid-containing triglycerides (i.e., triglycerides including a triglyceride wherein all or a portion of the constituent fatty acid is arachidonic acid) exist, the present inventors have been the first to clearly demonstrate that it is possible to industrially utilize triglycerides comprising arachidonic acid as a constituent fatty acid, that the active ingredients of the invention exhibit apparent behavioral pharmacologic

effects in mice subjected to restraint stress and evaluated by a behavioral observation test and have preventive or improvement effects for symptoms or diseases associated with stress-induced behavior 5 disorders, and that the effects are attributable to arachidonic acid.

According to the invention, therefore, triglycerides including a triglyceride wherein all or a portion of the constituent fatty acid is arachidonic acid (arachidonic acid-containing triglycerides) may be used as the active 10 ingredients of the invention. For application in foods, the arachidonic acid-containing triglycerides are preferably oils or fats (triglycerides) in a form wherein the arachidonic acid content of the total constituent 15 fatty acid of the triglycerides is at least 10 wt% (w/w), more preferably at least 20 wt%, even more preferably at least 30 wt%, and most preferably at least 40 wt%. Thus, the present invention may employ any such compounds which are obtained by culturing microorganisms capable of 20 producing arachidonic acid-containing oils or fats (triglycerides).

As microorganisms capable of producing oils or fats (triglycerides) containing arachidonic acid, there may be mentioned microorganisms belonging to the genera 25 *Mortierella*, *Conidiobolus*, *Pythium*, *Phytophthora*, *Penicillium*, *Cladosporium*, *Mucor*, *Fusarium*, *Aspergillus*, *Rhodotorula*, *Entomophthora*, *Echinoporangium* and *Saprolegnia*.

As examples of microorganisms belonging to the genus 30 *Mortierella*, subgenus *Mortierella*, there may be mentioned *Mortierella elongata*, *Mortierella exigua*, *Mortierella hygrophila* and *Mortierella alpina*. More specifically, there may be mentioned the strains *Mortierella elongata* IFO8570, *Mortierella exigua* IFO8571, *Mortierella hygrophila* IFO5941, and *Mortierella alpina* IFO8568, 35 ATCC16266, ATCC32221, ATCC42430, CBS219.35, CBS224.37, CBS250.53, CBS343.66, CBS527.72, CBS529.72, CBS608.70,

CBS754.68, etc.

All of these strains may be acquired without any special restrictions from the Institute for Fermentation, Osaka (IFO), American Type Culture Collection (ATCC) or Centralbureau voor Schimmelcultures (CBS). There may also be used the strain *Mortierella elongata* SAM0219 (FERM-P 8703) (deposited under the provisions of the Budapest Treaty on March 19, 1986 with the Patent Microorganism Depository of National Institute of Industrial Science and Technology at Chuo 6, 1-1, Higashi 1-chome, Tsukuba city, Ibaraki pref., Japan, as FERM BP-1239), isolated from soil by the research group for the present invention.

For culturing of a strain to be used for the invention, spores, hypha or a pre-culture solution obtained by pre-culturing the strain may be seeded in a liquid medium or solid medium for culturing. In the case of liquid culturing, the carbon source used may be a common one such as glucose, fructose, xylose, saccharose, maltose, soluble starch, molasses, glycerol or mannitol, although there is no limitation to these.

As nitrogen sources there may be used organic nitrogen sources including urea, and natural nitrogen sources such as peptone, yeast extract, malt extract, meat extract, casamino acid, corn steep liquor, soybean protein, defatted soybean and cotton seed meal, or inorganic nitrogen sources such as sodium nitrate, ammonium nitrate and ammonium sulfate. Trace nutrient sources including inorganic salts such as phosphoric acid salts, magnesium sulfate, iron sulfate and copper sulfate, or vitamins, may also be used if necessary. The medium components are not particularly restricted so long as they are in concentrations which do not prevent growth of the microorganisms. For most practical applications the carbon source may be used at a concentration of 0.1-40 wt% and preferably 1-25 wt%. The initial nitrogen source addition may be at 0.1-10 wt% and preferably 0.1-6

wt%, with further feeding of the nitrogen source during culturing.

By controlling the carbon source concentration of the medium it is possible to obtain oils or fats (triglyceride) containing at least 45 wt% arachidonic acid as the active ingredient of the invention. The cell growth phase is the culturing period up to the 2nd-4th day of culturing, while the fat/oil accumulation phase is from the 2nd-4th day of culturing. The initial carbon source concentration is 1-8 wt% and preferably 1-4 wt%, with successive supplemental addition of the carbon source only between the cell growth phase and the early fat/oil accumulation phase, for a total supplemental carbon source addition of 2-20 wt% and preferably 5-15 wt%. The amount of carbon source added between the cell growth phase and the early fat/oil accumulation phase will depend on the initial nitrogen source concentration, and if the carbon source concentration in the medium is 0 from the 7th day of culturing, preferably from the 6th day of culturing and more preferably from the 4th day of culturing, it will be possible to obtain oils or fats (triglyceride) containing at least 45 wt% arachidonic acid, as the active ingredient of the invention.

The culturing temperature for the arachidonic acid-producing cells will differ depending on the microorganism used, but is 5-40°C, preferably 20-30°C, while culturing at 20-30°C for proliferation of the cells may also be followed by continued culturing at 5-20°C to produce unsaturated fatty acids. Such temperature control can also be utilized to increase the proportion of polyunsaturated fatty acids among the produced fatty acids. The pH of the medium may be 4-10 and preferably 5-9, for jar fermentor culturing, shake culturing or stationary culturing. The culturing is normally carried out for 2-30 days, preferably 5-20 days and more preferably 5-15 days.

In addition to controlling the carbon source concentration of the medium as a strategy for increasing the proportion of arachidonic acid in the arachidonic acid-containing oils or fats (triglyceride), arachidonic acid-rich oils or fats may also be obtained by selective hydrolysis of the arachidonic acid-containing oils or fats. Since lipases used for such selective hydrolysis do not have regiospecificity for triglycerides and the hydrolytic activity decreases in proportion to the number of double bonds, the ester bonds of the fatty acids other than the polyunsaturated fatty acids are preferentially hydrolyzed. Furthermore, ester-exchange reaction between the produced PUFA glycerides may be used to produce triglycerides with an increased polyunsaturated fatty acid content ("Enhancement of Arachidonic Acid: Selective Hydrolysis of a Single-Cell Oil from *Mortierella* with *Candida cylindracea* Lipase": J. Am. Oil Chem. Soc., 72, 1323-1327, 1998).

Thus, oils or fats (triglyceride) with a high content of arachidonic acid obtained by selective hydrolysis of arachidonic acid-containing oils or fats can be prepared as the active ingredient of the invention. The proportion of arachidonic acid with respect to the total fatty acid content of the arachidonic acid-containing oils or fats (triglyceride) of the invention is preferably higher from the standpoint of eliminating the effect of other fatty acids, but it does not necessarily have to be a high proportion, and in fact the absolute amount of arachidonic acid can pose a problem for application to some foods. Oils or fats (triglycerides) containing arachidonic acid at 10 wt% or greater can be suitably used in most cases.

As triglycerides wherein all or a portion of the constituent fatty acid is arachidonic acid according to the invention, there may be used triglycerides having medium chain fatty acids bonded at the 1,3-positions and arachidonic acid bonded at the 2-position. The oils or

fats (triglycerides) used may also comprise at least 5 mole percent, preferably at least 10 mole percent, more preferably at least 20 mole percent and most preferably at least 30 mole percent, of triglycerides having medium 5 chain fatty acids bonded at the 1,3-positions and arachidonic acid bonded at the 2-position. The medium chain fatty acids bonded at the 1,3-positions of the triglyceride may be selected from among C6-12 fatty acids. As examples of C6-12 fatty acids there may be 10 mentioned caprylic acid or capric acid, with 1,3-capryloyl-2-arachidonoyl-glycerol (hereinafter, "8A8") being particularly preferred.

Such triglycerides having medium chain fatty acids bonded at the 1,3-positions and arachidonic acid bonded 15 at the 2-position are optimum oils or fats (triglycerides) for elderly persons. Generally speaking, ingested oils or fats (triglycerides) are hydrolyzed by pancreatic lipases upon entering the small intestine, but since pancreatic lipases are 1,3-specific, the 1,3- 20 positions of the triglycerides are cleaved to form two free fatty acids while simultaneously producing a single 2-monoacylglycerol (MG). As 2-MG has extremely high bile solubility and is highly absorbable, the 2-position fatty acid is generally considered to be better absorbed. In 25 addition, 2-MG dissolved in bile acid acts as a surfactant and thus increases the absorption of the free fatty acids.

The free fatty acids and 2-MG then form bile acid 30 complex micelles together with cholesterol, phospholipids and the like and are incorporated into the intestinal epithelial cells where triacylglycerols are resynthesized, being finally released into the lymph as chylomicrons. However, the fatty acid specificity of pancreatic lipases is higher for saturated fatty acids, 35 whereas arachidonic acid is not as easily cleaved. Another problem is that pancreatic lipase activity declines with age, and therefore triglycerides having

medium chain fatty acids bonded at the 1,3-positions and arachidonic acid bonded at the 2-position are more optimal oils or fats (triglycerides) for the elderly.

One specific production method for triglycerides 5 having medium chain fatty acids bonded at the 1,3-positions and arachidonic acid bonded at the 2-position is a method using a lipase which acts only on the 1,3-position ester bonds of triglycerides, in the presence of arachidonic acid-containing oils or fats (triglyceride) 10 and a medium chain fatty acid.

The oils or fats (triglyceride) starting material are a triglyceride comprising arachidonic acid as a constituent fatty acid, but in the case of a high proportion of arachidonic acid with respect to the total 15 constituent fatty acid of the triglycerides, reduced reaction yield due to excess unreacted oils or fats (the triglyceride starting material and triglycerides wherein only one of the 1,3-position fatty acids has been converted to a medium chain fatty acid) can be prevented 20 if the temperature is above the normal enzyme reaction temperature of 20-30°C, such as 30-50°C and preferably 40-50°C.

As examples of lipases which act specifically on the 1,3-position ester bonds of triglycerides there may be 25 mentioned lipases produced by microorganisms such as *Rhizopus*, *Rhizomucor* and *Aspergillus*, as well as porcine pancreatic lipases. Any such commercially available lipases may be used. For example, there may be mentioned 30 *Rhizopus delemar* lipase (Talipase, Tanabe Pharmaceutical Co., Ltd.), *Rhizomucor miehei* lipase (Ribozyme IM, Novo Nordisk Co., Ltd.) and *Aspergillus niger* lipase (Lipase A, Amano Pharmaceutical Co., Ltd.), although there is no limitation to these enzymes and any 1,3-specific lipases 35 may be used.

The form of the lipase used is preferably an immobilized form on an immobilizing support in order to impart heat resistance to the enzyme, since the reaction

temperature is 30°C or above and preferably 40°C or above for increased reaction efficiency. The immobilizing support may be a porous (highly porous) resin, for example, an ion-exchange resin with pores of 5 approximately 100 Å or greater such as Dowex MARATHON WBA. However, this condition is not restrictive on the immobilizing support, and any immobilizing support capable of imparting heat resistance may be used.

The immobilizing support may be suspended in an 10 aqueous solution of a 1,3-specific lipase at a weight proportion of 0.5-20 of the latter with respect to the former, and a 2- to 5-fold amount of cold acetone (for example, -80°C) may be slowly added to the suspension while stirring to form a precipitate. The precipitate 15 may then be dried under reduced pressure to prepare the immobilized enzyme. As a simpler method, a 1,3-specific lipase in a proportion of 0.05-0.4 with respect to the immobilizing support may be dissolved in a minimal amount of water and mixed with the immobilizing support while stirring and dried under reduced pressure to prepare the 20 immobilized enzyme. This procedure can immobilize approximately 90% lipase on the support, but since absolutely no ester exchange activity will be exhibited in that state, pretreatment may be carried out in a 25 substrate containing 1-10 wt% (w/v) water and preferably a substrate containing 1-3 wt% water, in order to activate the immobilized enzyme to maximum efficiency before it is provided for production.

The amount of water added to the reaction system is 30 extremely important depending on the type of enzyme, because a lack of water will impede ester exchange while an excess of water will cause hydrolysis and a reduced glyceride yield (since hydrolysis will produce diglycerides and monoglycerides). However, if the 35 immobilized enzyme used has been activated by pretreatment the amount of water added to the reaction system is no longer crucial, and an efficient ester

exchange reaction can be carried out even in a completely water-free system. Also, selection of the type of enzyme agent may allow the pretreatment step to be omitted.

Thus, by using a heat-resistant immobilized enzyme and raising the enzyme reaction temperature, it is possible to efficiently produce triglycerides having medium chain fatty acids bonded at the 1,3-positions and arachidonic acid bonded at the 2-position (8A8), without lowering the reaction efficiency even for arachidonic acid-containing oils or fats (triglycerides) with low reactivity for 1,3-specific lipases.

A method for production of a dietary product having a preventive or improvement effect on symptoms or diseases associated with stress-induced behavior disorders, involves adding arachidonic acid and/or a compound including arachidonic acid as a constituent fatty acid alone, or in combination with a dietary material containing substantially no arachidonic acid or only a slight amount thereof. Here, a "slight amount" means that even if arachidonic acid is present in the dietary product material and a food composition containing it is ingested by a human, the amount does not reach the daily amount of arachidonic acid consumption according to the invention, as described hereunder.

An unlimited number of uses exist for oils or fats (triglycerides) wherein all or a portion of the constituent fatty acid is arachidonic acid: for example, they may be used as starting materials and additives for foods, beverages, cosmetics and pharmaceuticals. The purposes of use and amounts of use are also completely unrestricted.

As examples of food compositions there may be mentioned ordinary foods, as well as functional foods, nutritional supplements, food for specified health uses, preterm infant formula, term infant formula, infant foods, maternal foods or geriatric foods. As examples of fat/oil-containing foods there may be mentioned natural

fat/oil-containing foods such as meat, fish and nuts, foods to which oils or fats are added during preparation, such as soups, foods employing oils or fats as heating media, such as donuts, oils or fats foods such as butter, 5 processed foods to which oils or fats are added during processing, such as cookies, or foods which are sprayed or coated with oils or fats upon finishing, such as hard biscuits. Such compositions may also be added to agricultural foods, fermented foods, livestock feeds, 10 marine foods and beverages which contain no oils or fats. They may also be in the form of functional foods or pharmaceuticals, and for example, in processed form such as enteral nutrients, powders, granules, lozenges, oral solutions, suspensions, emulsions, syrups and the like.

15 A composition of the invention may also contain various carriers or additives ordinarily used in foods and beverages, pharmaceuticals or quasi drugs, in addition to the active ingredient of the invention. Antioxidants are particularly preferred as additives to 20 prevent oxidation of the active ingredient of the invention. As examples of antioxidants there may be mentioned natural antioxidants such as tocopherols, flavone derivatives, phyllodulcins, kojic acid, gallic acid derivatives, catechins, fukiic acid, gossypol, 25 pyrazine derivatives, sesamol, guaiaol, guaiac acid, p-coumaric acid, nordihydroguaiaretic acid, sterols, terpenes, nucleotide bases, carotenoids, lignans and the like, and synthetic antioxidants including ascorbic palmitic acid esters, ascorbic stearic acid esters, 30 butylhydroxyanisole (BHA), butylhydroxytoluene (BHT), mono-t-butylhydroquinone (TBHQ) and 4-hydroxymethyl-2,6-di-t-butylphenol (HMBP).

As tocopherols there may be mentioned  $\alpha$ -tocopherol,  $\beta$ -tocopherol,  $\gamma$ -tocopherol,  $\delta$ -tocopherol,  $\epsilon$ -tocopherol,  $\zeta$ -tocopherol,  $\eta$ -tocopherol and tocopherol esters (tocopherol acetate and the like), as well as tocopherol

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analogs. As examples of carotenoids there may be mentioned  $\beta$ -carotene, canthaxanthine, astaxanthine and the like.

The composition of the invention may also contain, 5 in addition to the active ingredient of the invention, supports such as carrier supports, extenders, diluents, bulking agents, dispersing agents, excipients, binder solvents (for example, water, ethanol and vegetable oils), dissolving aids, buffering agents, dissolving 10 accelerators, gelling agents, suspending agents, wheat flour, rice flour, starch, corn starch, polysaccharides, milk protein, collagen, rice oil, lecithin and the like. As examples of additives there may be mentioned vitamins, 15 sweeteners, organic acids, coloring agents, aromatic agents, moisture-preventing agents, fibers, electrolytes, minerals, nutrients, antioxidants, preservatives, fragrances, humectants, natural food extracts, vegetable extracts and the like, although there is no limitation to these.

20 Arachidonic acid is the main active ingredient of the compound which is either arachidonic acid or comprises arachidonic acid as a constituent fatty acid. The daily intake of arachidonic acid from dietary sources has been reported to be 0.14 g in the Kanto region and 25 0.19-0.20 g in the Kansai region of Japan (Shishitsu Eiyougaku 4, 73, 1995), and in consideration of reduced oils or fats intake and reduced pancreatic lipase function in the elderly, a correspondingly greater amount of arachidonic acid must be ingested. Thus, the daily 30 intake of the arachidonic acid or the compound comprising arachidonic acid as a constituent fatty acid according to the invention for an adult (for example, 60 kg body weight) is 0.001-20 g, preferably 0.01-10 g, more preferably 0.05-5 g and most preferably 0.1-2 g, based on 35 the arachidonic acid content.

When the active ingredient of the invention is to be actually applied for a food or beverage product, the

absolute amount of arachidonic acid in the product is an important factor. However, since the absolute amount added to foods and beverages will differ depending on the amount of consumption of those foods or beverages,

5       triglycerides including a triglyceride wherein all or a portion of the constituent fatty acid is arachidonic acid may be added to food products in amounts of at least 0.001 wt%, preferably at least 0.01 wt% and more preferably at least 0.1 wt% in terms of arachidonic acid.

10      For addition to food and beverage products of triglycerides having medium chain fatty acids bonded at the 1,3-positions and arachidonic acid bonded at the 2-position, the amount may be at least 0.0003 wt%, preferably at least 0.003 wt% and more preferably at least 0.03 wt%.

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When the composition of the invention is to be used as a pharmaceutical, it may be produced according to a common method in the field of pharmaceutical preparation techniques, such as according to a method described in

20      the Japanese Pharmacopeia or a similar method.

When the composition of the invention is to be used as a pharmaceutical, the content of the active ingredient in the composition is not particularly restricted so long as the object of the invention is achieved, and any appropriate content may be employed.

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When the composition of the invention is to be used as a pharmaceutical, it is preferably administered in the form of an administrable unit, and especially in oral form. The dosage of the composition of the invention will differ depending on age, body weight, symptoms and frequency of administration, but for example, the arachidonic acid and/or compound including arachidonic acid as a constituent fatty acid according to the invention may be administered at about 0.001-20 g, preferably 0.01-10 g, more preferably 0.05-5 g and most preferably 0.1-2 g (as arachidonic acid) per day for adults (approximately 60 kg), either once a day or

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divided among multiple doses, such as three separate doses.

The major fatty acid components of phospholipid membranes in the brain are arachidonic acid and docosahexaenoic acid, and therefore from the standpoint of balance, a combination with docosahexaenoic acid is preferred. Also, since the proportion of eicosapentaenoic acid in brain phospholipid membranes is very small, a combination of arachidonic acid and docosahexaenoic acid containing virtually no eicosapentaenoic acid is especially preferred. Furthermore, the arachidonic acid/docosahexaenoic acid ratio in the combination of the arachidonic acid and docosahexaenoic acid is preferably in the range of 0.1-15, and more preferably in the range of 0.25-10. Also, the amount of eicosapentaenoic acid in the food or beverage preferably does not exceed 1/5 of the arachidonic acid (weight ratio).

## 20 EXAMPLES

The present invention will now be explained in greater detail by the following examples, with the understanding that the invention is not limited to these examples.

### 25 Example 1 Method for production of arachidonic acid-containing triglycerides

*Mortierella alpina* CBS754.68 was used as the arachidonic acid-producing strain. After preparing 6 kL of medium containing 1.8% glucose, 3.1% defatted soybean powder, 0.1% soybean oil, 0.3%  $\text{KH}_2\text{PO}_4$ , 0.1%  $\text{Na}_2\text{SO}_4$ , 0.05%  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  and 0.05%  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  in a 10 kL culturing tank, the initial pH was adjusted to 6.0.

30 A 30 L portion of the preculturing solution was transferred for 8 days of jar fermentor culturing under conditions with a temperature of 26°C, an airflow of 360  $\text{m}^3/\text{h}$  and an internal pressure of 200 kPa. The stirring rate was adjusted to maintain a dissolved oxygen

concentration of 10-15 ppm. Also, the glucose concentration was adjusted by the feeding culture method for a glucose concentration in the range of 1-2.5% in the medium up to the 4th day, with 0.5-1% maintained thereafter (where the percentage values are weight (W/V) %).

After completion of the culturing, the cells containing triglycerides having arachidonic acid as a constituent fatty acid were collected by filtration and drying, and the fat and oil portion was extracted from the collected cells by hexane extraction and subjected to dietary oils or fats purification steps (degumming, deoxidation, deodorization, decolorizing) to obtain 150 kg of arachidonic acid-containing triglycerides (triglycerides including a triglyceride wherein all or a portion of the constituent fatty acid is arachidonic acid). The obtained oils or fats (triglycerides) were methylesterified, and the obtained fatty acid methyl ester mixture was analyzed by gas chromatography and found to have an arachidonic acid proportion of 40.84 wt% of the total fatty acid.

The contents of palmitic acid, stearic acid, oleic acid, linoleic acid,  $\gamma$ -linolenic acid and dihomo- $\gamma$ -linolenic acid were 11.63%, 7.45%, 7.73%, 9.14%, 2.23% and 3.27% by weight, respectively. The arachidonic acid-containing oils or fats (triglycerides) (TGA40S) were also ethylesterified, and the fatty acid ethyl ester mixture including 40 wt% arachidonic acid ethyl ester was separated and purified by an established high-performance liquid chromatography method to obtain 99 wt% arachidonic acid ethyl ester.

Example 2 Production of triglycerides including at least 5 mole percent 8A8

After suspending 100 g of an ion-exchange resin carrier (Dowex MARATHON WBA: Dow Chemical) in 80 ml of *Rhizopus delemar* lipase aqueous solution (12.5% Talipase powder, Tanabe Pharmaceutical Co., Ltd.), 240 ml of cold

acetone (-80°C) was stirred therewith and the mixture was dried under reduced pressure to obtain the immobilized lipase.

5 Next, 80 g of the triglycerides containing 40 wt% arachidonic acid (TGA40S) obtained in Example 1, 160 g of caprylic acid, 12 g of the aforementioned immobilized lipase and 4.8 ml of water were reacted for 48 hours at 30°C while stirring (130 rpm). Upon completion of the reaction, the reaction solution was removed to obtain the 10 activated immobilized enzyme.

A 10 g portion of immobilized lipase (*Rhizopus delemar* lipase, carrier: Dowex MARATHON WBA) was then packed into a jacketed glass column (1.8 x 12.5 cm, 31.8 ml volume), and the reaction oils or fats comprising a 15 mixture of the TGA40S obtained in Example 1 and caprylic acid (TGA40S:caprylic acid = 1:2) was flowed through the column at a fixed speed (4 ml/h) for continuous reaction, to obtain 400 g of reaction oils or fats. The column 20 temperature was 40-41°C. The unreacted caprylic acid and free fatty acids were removed from the obtained reaction oils or fats by molecular distillation, and then subjected to dietary oils or fats purification steps (degumming, deoxidation, deodorization, decolorizing) to obtain 8A8-containing oils or fats (triglycerides).

25 The 8A8 proportion of the obtained 8A8-containing oils or fats (triglycerides) was determined by gas chromatography and high-performance liquid chromatography to be 31.6 mole percent. (Incidentally, the proportions of 8P8, 8O8, 8L8, 8G8 and 8D8 were 0.6, 7.9, 15.1, 5.2 and 4.8 mole percent, respectively. The fatty acids P, 30 O, L, G and D bonded at the triglyceride 2-position represent palmitic acid, oleic acid, linoleic acid,  $\gamma$ -linolenic acid and dihomo- $\gamma$ -linolenic acid, respectively, and therefore 8P8 represents 1,3-capryloyl-2-palmitolein-glycerol, 8O8 represents 1,3-capryloyl-2-oleoyl-glycerol, 35 8L8 represents 1,3-capryloyl-2-linoleoyl-glycerol, 8G8

represents 1,3-capryloyl-2- $\gamma$ -linolenoyl-glycerol and 8D8 represents 1,3-capryloyl-2-dihomo- $\gamma$ -linolenoyl-glycerol). Separation and purification from the obtained 8A8-containing oils or fats (triglycerides) by an established high-performance liquid chromatography method yielded 96 mole percent 8A8.

Example 3 Evaluation of effect on behavior disorder of TGA40S by behavioral observation test

The experimental groups consisted of 51 two- to three-month-old male ICR mice, divided into a control diet group (27 mice) and a TGA40S-containing diet group (24 mice), with the control diet or TGA40S-containing diet shown in Table 1 being given to each group. Each group was further divided into non-restrained groups (non-restrained control diet group (9), non-restrained arachidonic acid (ARA) diet group (12)) and restrained groups (restrained control diet group (18), restrained ARA diet group (12)). The restraining was accomplished using a wire mesh restraining tube, once for a 6 hour period three weeks after the start of feeding. The control diet or TGA40S-containing diet shown in Table 1 continued to be fed to each group for the remaining experiment period. The TGA40S used for the TGA40S-containing diet was the product obtained in Example 1.

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Table 1 Experimental diet

	Control diet	TGA40S-added diet
Casein (g/kg)	200	200
DL-methionine	3	3
Corn starch	150	150
Sucrose	500	500
Cellulose powder	50	50
Corn oil	50	45
Mineral AIN-76	35	35
Vitamin AIN-76	10	10
Choline bitartrate	2	2
Vitamin E	0.05	0.05
TGA40S	0	5

Since the daily ingestion was approximately 5 g per mouse, the daily intake of TGA40S was 25 mg per mouse. Also, since the total fatty acids bonded to the arachidonic acid-containing oils or fats (triglycerides) prepared in Example 1 included 40 wt% arachidonic acid, the daily intake of arachidonic acid was 10 mg per mouse.

The 6-hour restraint with a wire mesh restraining tube was immediately followed by a behavioral observation test. The behavioral observation test was conducted by transferring the mouse into a new cage and then after 30 minutes observing and recording the behavioral indices according to the Irwin neurobehavioral test method (sniffing, circling, rearing, jumping, digging). No difference was observed between the control diet mice and ARA diet mice in the absence of restraint stress. However, the mice of the control diet group which had experienced restraint stress clearly exhibited increased rearing, indicating cautious reaction, and reduced spontaneous movements such as sniffing and circling, compared to the non-restrained mice, whereas the mice given TAG40S (arachidonic acid) recovered to the same level of behavior as the mice without restraint stress (Figs. 1, 2).

Thus, for the first time it has been clearly demonstrated that administration of TGA40S improves behavioral patterns which have altered as a result of stress, and that arachidonic acid exhibits an improving effect against behavioral disorders.

Example 4 Preparation of capsules comprising arachidonic acid-containing oils or fats (triglycerides)

Water was added to 100 parts by weight of gelatin and 35 parts by weight of food additive grade glycerin for dissolution at 50-60°C, to prepare a gelatin coating with a viscosity of 2000 cp. Next, 0.05 wt% of vitamin E oil was combined with the arachidonic acid-containing oils or fats (triglycerides) obtained in Example 1 to prepare filling 1. Vitamin E oil was also combined at

0.05 wt% with oils or fats (triglycerides) containing 32 mole percent of the 8A8 obtained in Example 2 to prepare filling 2. Also, 50 wt% of the arachidonic acid-containing oils or fats (triglycerides) obtained in  
5 Example 1 was combined with 50 wt% fish oil (tuna oil: the eicosapentaenoic acid and docosahexaenoic acid proportions of the total fatty acids were 5.1% and 26.5%, respectively) and then 0.05 wt% vitamin E oil was added to prepare filling 3.

10 Also, 80 wt% of arachidonic acid-containing oils or fats (triglycerides) was combined with 20 wt% fish oil (tuna oil: the eicosapentaenoic acid and docosahexaenoic acid proportions of the total fatty acids were 5.1% and 26.5%, respectively) and then 0.05 wt% vitamin E oil was  
15 added to prepare filling 4. Separately, 0.05 wt% of vitamin E oil was combined with the 99% arachidonic acid ethyl ester obtained in Example 1 to prepare filling 5. These fillings 1 to 5 were used for production of soft capsules containing 180 mg of filling per capsule,  
20 obtained by capsule molding and drying by ordinary methods.

Example 5 Use for oil infusion

After combining 400 g of the oils or fats (triglycerides) containing 96 mole percent 8A8 obtained in Example 2, 48 g of purified egg yolk lecithin, 20 g of oleic acid, 100 g of glycerin and 40 ml of 0.1 N caustic soda and dispersing the mixture with a homogenizer, distilled water for injection was added to make 4 liters. This was emulsified with a high-pressure spray emulsifier to prepare a lipid emulsion. The lipid emulsion was dispensed into plastic bags at 200 ml per bag and then subjected to high-pressure steam sterilization treatment at 121°C for 20 minutes to prepare an oil infusion.

Example 6 Use for juice

35 A 2 g portion of  $\beta$ -cyclodextrin was added to 20 ml of 20% aqueous ethanol, and then 100 mg of the arachidonic acid-containing triglycerides obtained in

- 24 -

Example 1 (containing 0.05% vitamin E) were added thereto while stirring with a stirrer, and the mixture was incubated for 2 hours at 50°C. After room temperature cooling (approximately 1 hour), stirring was continued 5 while incubating for 10 hours at 4°C. The resulting precipitate was recovered by centrifugal separation and then washed with n-hexane and lyophilized to obtain 1.8 g of a cyclodextrin clathrate compound comprising arachidonic acid-containing triglycerides. A 1 g portion 10 of this powder was uniformly mixed into 10 L of juice to prepare a juice comprising arachidonic acid-containing triglycerides.

CLAIMS

1. A composition with a preventive or improvement effect on symptoms or diseases associated with stress-induced behavior disorders, comprising arachidonic acid and/or a compound comprising arachidonic acid as a constituent fatty acid.

2. A composition according to claim 1, wherein said compound comprising arachidonic acid as a constituent fatty acid is an arachidonic acid alcohol ester, or a triglyceride, phospholipid or glycolipid wherein all or a portion of the constituent fatty acid is arachidonic acid.

3. A composition according to claim 2, wherein the triglyceride in which all or a portion of the constituent fatty acid is arachidonic acid is a triglyceride having medium chain fatty acids bonded at the 1,3-positions and arachidonic acid bonded at the 2-position.

4. A composition according to claim 3, wherein said medium chain fatty acids are selected from among C6-12 fatty acids.

5. A composition with a preventive or improvement effect on symptoms or diseases associated with stress-induced behavior disorders, comprising triglycerides which include a triglyceride in which all or a portion of the constituent fatty acid is arachidonic acid.

6. A composition according to claim 5, characterized in that the arachidonic acid content of said triglycerides which include a triglyceride in which all or a portion of the constituent fatty acid is arachidonic acid, is at least 10 wt% of the total fatty acids of the triglycerides.

7. A composition according to claim 5 or 6, wherein said triglycerides which include a triglyceride in which all or a portion of the constituent fatty acid is arachidonic acid, are extracted from a microorganism belonging to the genus *Mortierella*, *Conidiobolus*, *Pythium*, *Phytophthora*, *Penicillium*, *Cladosporium*, *Mucor*,

*Fusarium, Aspergillus, Rhodotorula, Entomophthora, Echinosporangium or Saprolegnia.*

8. A composition according to any one of claims 5 to 7, wherein said triglycerides which include a triglyceride in which all or a portion of the constituent fatty acid is arachidonic acid, are triglycerides containing virtually no eicosapentaenoic acid.

9. A composition with a preventive or improvement effect on symptoms or diseases associated with stress-induced behavior disorders, comprising triglycerides of which at least 5 mole percent consists of a triglyceride having medium chain fatty acids bonded at the 1,3-positions and arachidonic acid bonded at the 2-position.

10. A composition according to claim 9, wherein said medium chain fatty acids are selected from among C6-12 fatty acids.

11. A composition according to any one of claims 1 to 10, wherein said symptoms associated with stress-induced behavior disorders include habits such as finger sucking and onychophagy.

12. A composition according to any one of claims 1 to 10, wherein said diseases associated with stress-induced behavior disorders include adjustment disorder and attention deficit hyperactivity disorder.

13. A composition according to any one of claims 1 to 10, wherein said diseases associated with stress-induced behavior disorders include acute stress disorder and posttraumatic stress disorder.

14. A composition according to any one of claims 1 to 13, wherein said composition is a food composition or pharmaceutical composition.

15. A composition according to claim 14, characterized in that said food composition is a common food (food and drink), functional food, nutritional supplement, food for specified health uses, preterm infant formula, term infant formula, infant food, maternal food or geriatric food.

16. A composition according to any one of claims 1 to 15, which comprises docosahexaenoic acid and/or a compound comprising docosahexaenoic acid as a constituent fatty acid.

5 17. A composition according to claim 16, wherein said compound comprising docosahexaenoic acid as a constituent fatty acid is a docosahexaenoic acid alcohol ester, or a triglyceride, phospholipid or glycolipid wherein all or a portion of the constituent fatty acid is  
10 docosahexaenoic acid.

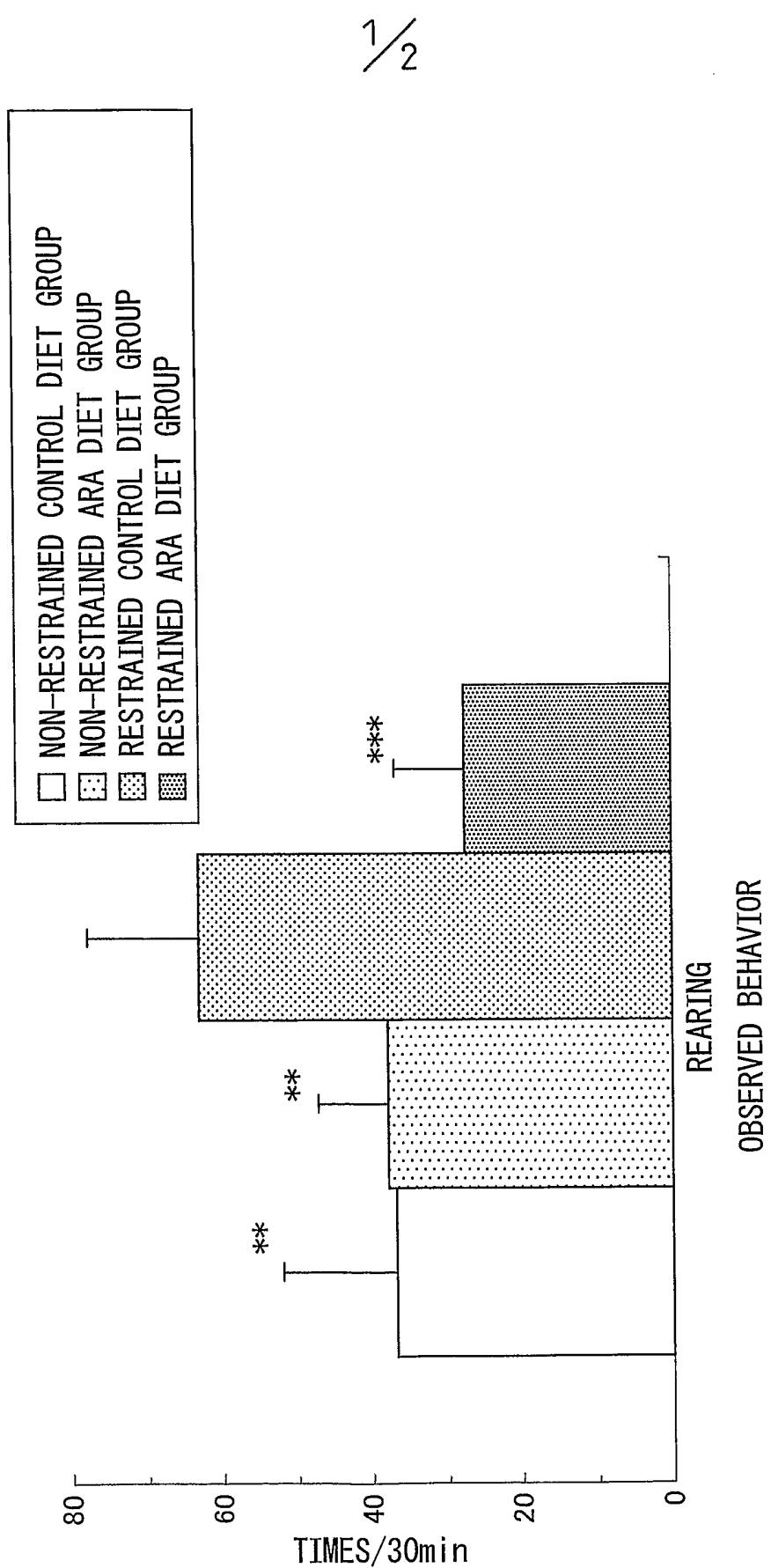
15 18. A composition according to claim 16 or 17, characterized in that the arachidonic acid/docosahexaenoic acid ratio (by weight) in the combination of said arachidonic acid and docosahexaenoic acid is in the range of 0.1-15.

19. A composition according to any one of claims 1 to 18, characterized in that the amount of eicosapentaenoic acid in the composition does not exceed 1/5 of the arachidonic acid in the composition.

20 20. A method for production of a dietary product having a preventive or improvement effect on symptoms or diseases associated with stress-induced behavior disorders, the method being characterized by adding arachidonic acid and/or a compound comprising arachidonic acid as a constituent fatty acid alone, or in combination with a dietary material containing substantially no arachidonic acid or only a slight amount thereof.

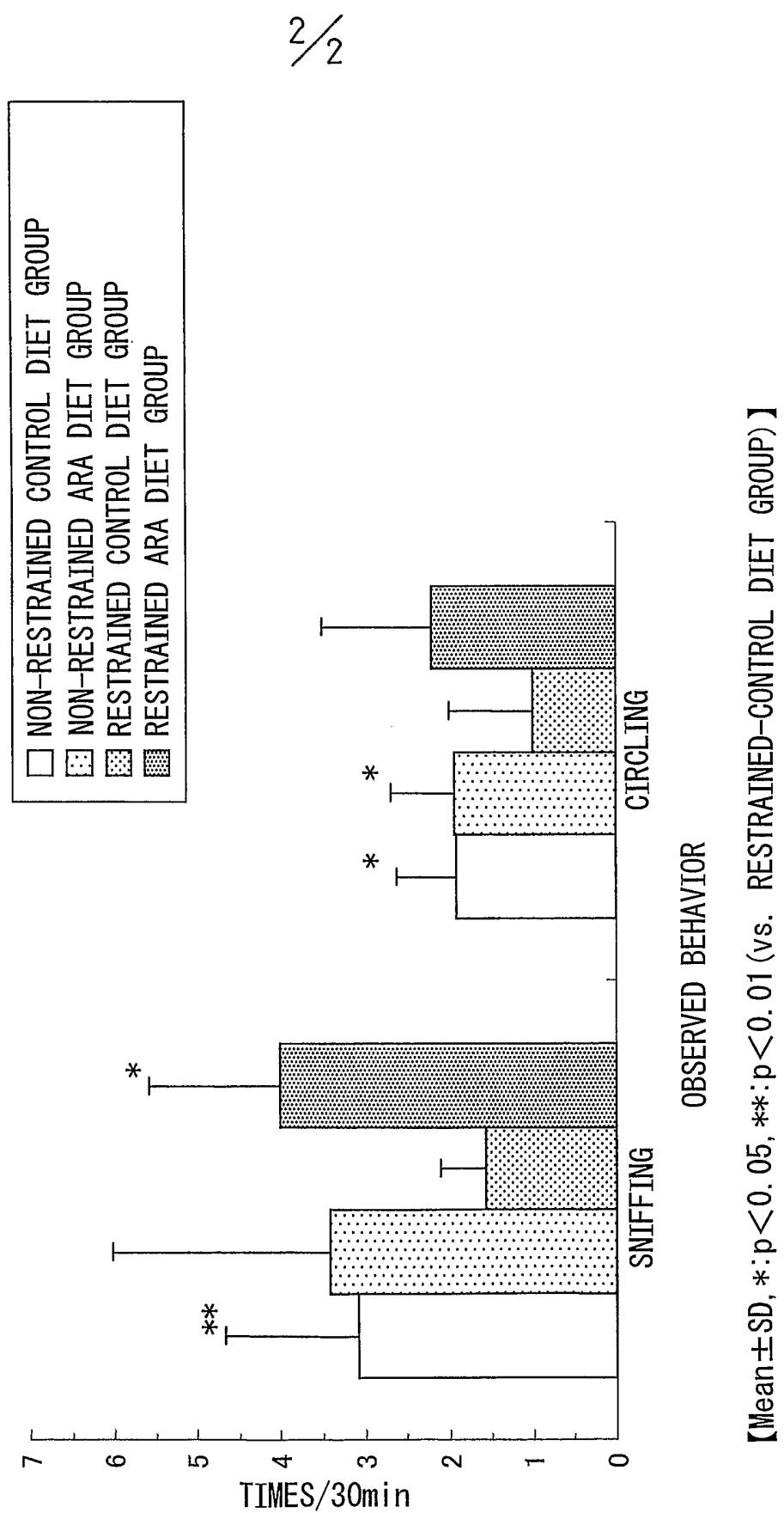
25 30 21. A method for prevention or medical treatment of symptoms or diseases associated with stress-induced behavior disorders, which comprises administering arachidonic acid and/or a compound comprising arachidonic acid as a constituent fatty acid, to a patient in need of its administration.

Fig. 1



[Mean $\pm$ SD, \*\*:p<0.01, \*\*\*:p<0.001 (vs. RESTRAINED-CONTROL DIET GROUP)]

Fig. 2



# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/JP2005/005623

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 7 A61K31/202 A61K31/232 A23L1/30 A61P25/00

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

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 A61K A61P A23L

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data, PAJ

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2004/028529 A (SUNTORY LIMITED; AKIMOTO, KENGO; KOGA, YOSHIHIKO) 8 April 2004 (2004-04-08) claims 1-35 examples 1-8 ----- EP 1 419 768 A (SUNTORY LIMITED) 19 May 2004 (2004-05-19) page 9, lines 25-32 examples 1-8 ----- -/-	1-21
X		1-21

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

° Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

29 June 2005

Date of mailing of the international search report

11/07/2005

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## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/JP2005/005623

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BURGESS J R ET AL: "LONG-CHAIN POLYUNSATURATED FATTY ACIDS IN CHILDREN WITH ATTENTION-DEFICIT HYPERACTIVITY DISORDER" AMERICAN JOURNAL OF CLINICAL NUTRITION, BETHESDA, MD, US, vol. 71, no. 1, SUPPL, January 2000 (2000-01), pages 327S-330S, XP008000462 ISSN: 0002-9165 cited in the application abstract -----	1-21
Y	WO 02/19839 A (UNIVERSITY OF MARYLAND BIOTECHNOLOGY INSTITUTE) 14 March 2002 (2002-03-14) claims 1,2 -----	1-21
Y	AUGUSTE L-J ET AL: "PREVENTION OF STRESS-INDUCED EROSION GASTRITIS BY PARENTERAL ADMINISTRATION OF ARACHIDONIC ACID" JOURNAL OF PARENTERAL AND ENTERAL NUTRITION, vol. 14, no. 6, 1990, pages 615-617, XP009049858 ISSN: 0148-6071 abstract -----	1-21

**INTERNATIONAL SEARCH REPORT**International application No.  
PCT/JP2005/005623**Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

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

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

Although claim 21 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2.  Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3.  Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box 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 an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest** The additional search fees were accompanied by the applicant's protest. No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International Application No  
PCT/JP2005/005623

Patent document cited in search report		Publication date		Patent family member(s)		Publication date
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WO 0219839	A	14-03-2002	AU CA EP WO US	9258601 A 2424570 A1 1324671 A1 0219839 A1 2002110582 A1		22-03-2002 14-03-2002 09-07-2003 14-03-2002 15-08-2002