USE OF A SEEDS EXTRACT OF MYRISTICA FRAGRANS OR ACTIVE COMPOUNDS ISOLATED THEREFROM FOR PREVENTING OR TREATING OSTEOPOROSIS

FIG. 1

Myristica fragrans (1.8kg)

5L MeOH / 7 days 3times

MeOH extract (300g)

2L H₂O / 2L ethylacetate 3times

Ethylacetate fraction (255g)

n-Butanol fraction (6g)

2L n-butanol 3times

Water fraction (39g)

(57) Abstract: The present invention relates to a use of a compound selected from the group consisting of machilin A, macelignan, machilin F, nectandrin B, safrole, licarin A, licarin B, myristagenol A, meso-dihydroguaiaretic acid and a mixture thereof, or a seeds extract of Myristica fragrans comprising at least one of the compounds for preventing or treating osteoporosis.
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USE OF A SEEDS EXTRACT OF MYRISTICA FRAGRANS OR ACTIVE COMPOUNDS ISOLATED THEREFROM FOR PREVENTING OR TREATING OSTEOPOROSIS

Field of the Invention

The present invention relates to a use of a seeds extract of *Myristica fragrans* or compounds isolated therefrom for preventing or treating osteoporosis.

Background of the Invention

Bone homeostasis is controlled by bone remodeling which maintains a balance between the actions of bone resorption by osteoclast and bone formation by osteoblast. However, over-activation of the osteoclast or reduced activation of the osteoblast induces bone diseases such as osteoporosis. In order to correct such an imbalance, a variety of methods to inhibit the over-activation of the osteoclast and/or to stimulate the osteoblast activation have been used.

Osteoblast cells are derived from mesenchymal stem cells, and mineralization such as calcium formation by the osteoblast differentiation maintains the bone integrity, which has an important role in calcium and hormone homeostasis of the body. The calcium formation by the osteoblast differentiation is controlled by vitamin D, parathyroid hormone, and others, and the bone formation by the osteoblast differentiation is accomplished by synthesizing alkaline phosphatase (ALP) associated with the osteoblast differentiation in the early stage by cross-talking between various signal transducers such as bone morphogenetic protein (BMP), Wnt, MAP kinase, calcineurin-calmodulin kinase, NF-κB and AP-I in the cell, followed by synthesizing mineralization-related elements such as osteopontin, osteocalcin and type I collagen.
Accordingly, an ideal drug for stimulating the osteoblast activation should have a low toxicity and be administered orally for a long period. Therefore, there has been a need to develop a non-toxic drug and functional food as a stimulator of the osteoblast activation.

*Myristica fragrans* is an evergreen tree that belongs to the *Myristicaceae* family, and its extract can be prepared by harvesting the fruit, isolating the seed therefrom, drying the seed, removing black shell therefrom, soaking in a lime water overnight, and drying at room temperature.

There have been reported that the seeds extract of *Myristica fragrans* is: cytotoxic against cancer cells [Lee et al., *Kor. J. Pharmacogn.* 37(3), 206-211, 2006]; antibacterial and antioxidant [Singh et al., *Journal of Food Science*, 70(2), 141-148, 2005]; antibacterial [Orabi et al., *Journal of Natural Products*, 54(3), 856-859, 1991; Narasimhan et al., *Journal of Medicinal Food*, 9(3), 395-399, 2006; Miyazawa et al., *Natural Product Letters*, 8(4), 271-273, 1996]; effective in detoxication of the liver [Singh et al., *Food and Chemical Toxicology*, 31(7), 517-521, 1993]; capable of inhibiting cerebral cholinesterase which is related to dementia [Dhingra et al., *Journal of Medicinal Food*, 9(2), 281-283, 2006]](1); and active in suppressing anxiety [Sonavane et al., *Biochemistry and Behavior*, 71(1-2), 239-244, 2002]. However, it has not yet been reported that the seeds extract of *Myristica fragrans* stimulates the osteoblast activation.

The present inventors have unexpectively found that a seeds extract of *Myristica fragrans* can be used for preventing or treating osteoporosis and bone diseases caused thereby.

**Summary of the Invention**

Accordingly, it is an object of the present invention to provide a pharmaceutically active substance for preventing or treating osteoporosis.

It is another object of the present invention to provide a food for preventing osteoporosis.

It is still another object of the present invention to provide a method for
preventing or treating osteoporosis.

In accordance with one aspect of the present invention, there is provided a use of a compound selected from the group consisting of machilin A of formula (I), macelignan of formula (II), machilin F of formula (III), nectandrin B of formula (IV), safrole of formula (V), licarin A of formula (VI), licarin B of formula (VII), myristagenol A of formula (VIII), meso-dihydroguaiaretic acid of formula (IX) and a mixture thereof, or a seeds extract of *Myristica fragrans* comprising at least one of said compounds for preventing or treating osteoporosis:

![Chemical structures](image-url)
In accordance with another aspect of the present invention, there is provided a health care food for preventing osteoporosis, which comprises at least one of the compounds of formula (I) to (IX), a mixture thereof, or a seeds extract of *Myristica fragrans* comprising the same.

In accordance with still another aspect of the present invention, there is provided a method for preventing or treating osteoporosis in mammals, which comprises administering thereto an effective amount of at least one of the compounds of formula (I) to (IX), a mixture thereof, or a seeds extract of *Myristica fragrans* comprising the same.

**Brief Description of Drawings**

The above and other objects and features of the present invention will become apparent from the following description of the invention taken in conjunction with the following accompanying drawings, which respectively
show:

Fig. 1: a diagram showing the process of fractionating a methanol extract of *Myristica fragrans* according to solvents;

Fig. 2: a diagram showing the process of isolating and purifying active ingredients of the ethyl acetate fraction of the seeds extract of *Myristica fragrans*;

Fig. 3A: the result of staining the calcium formed by the action of the seeds extract of *Myristica fragrans* by using alizarin red S staining method;

Fig. 3B: the amount of the calcium formed by the action of the seeds extract of *Myristica fragrans*;

Fig. 4: the amount of the calcium formed by the action of the compound isolated from the seeds extract of *Myristica fragrans*;

Fig. 5: a graph showing the increased number of osteoblast after treating with machilin A;

Fig. 6A: the effect of machilin A on the activity of alkaline phosphatase (ALP) as a differentiation marker of the osteoblast;

Fig. 6B: the result of staining the ALP to measure the ALP expression after treating the osteoblast with machilin A;

Fig. 7A: the amount of the calcium formed by the action of machilin A;

Fig. 7B: the calcium formed by the action of machilin A by using alizarin red S staining method;

Fig. 8: the result of staining the osteoblast by using von Kossa staining method to confirm the osteoblast mineralization induced by machilin A;

Fig. 9A: a graph showing the expression patterns of genes related to the osteoblast differentiation induced by machilin A;

Fig. 9B: a graph showing the expression patterns of genes related to the osteoblast mineralization induced by machilin A; and

Fig. 10: the result of measuring the activation of mitogen activated protein (MAP) kinase induced by machilin A.

**Detailed Description of the Invention**
The seeds extract of *Myristica fragrans* of the present invention may be prepared by extracting the seeds of *Myristica fragrans* with a solvent after drying. Specifically, the seeds of *Myristica fragrans* may be dried in the shade, sliced and extracted with an organic solvent at 10 to 30°C for 1 to 20 days, preferably 5 to 10 days, the volume of the solvent being 2 to 200, preferably 10 to 30 based on one unit volume of the seeds of *Myristica fragrans*. The extract solution is then filtered and concentrated under a reduced pressure to obtain the seeds extract of *Myristica fragrans*.

The organic solvent used in this procedure may be selected from the group consisting of C1-C4 alcohol and aqueous solutions thereof such as methanol, aqueous methanol, ethanol, aqueous ethanol, butanol, dichloromethane, ethyl acetate, and a mixture thereof, preferably methanol or aqueous methanol.

Further, the seeds extract of *Myristica fragrans* thus obtained may be further subjected to fractionation by extracting with butanol or ethylacetate, preferably ethyl acetate.

In a preferred embodiment of the present invention, *Myristica fragrans* or dried *Myristica fragrans* is soaked in 100% cold methanol over a period of 1 week, and the resulting solution was filtered and concentrated under a reduced pressure to obtain a methanol extract. Then, the methanol extract is suspended in distilled water, and the resulting suspension is fractionated by extraction with ethylacetate, followed by concentrating the fraction under a reduced pressure to obtain various components of the methanol extract of *Myristica fragrans*.

Further, in the present invention, chromatography is performed to isolate or purify the active compounds of formula (I) to (IX) from the seeds extract of *Myristica fragrans*. The chromatographic procedure preferably involves 1 or 2 rounds of reversed-phase or silica gel column chromatography, which is preferably conducted according to a gradient elution method using n-hexane/ethyl acetate (100:1-0:1) or methanol/water (50-100% MeOH) as the mobile phase. The fractions thus collected were tested for their osteoblast-stimulating activities, leading to the identification of the active compounds of formula (I) to (IX), which may be chemically synthesized, if needed.

In order to prevent or treat osteoporosis, the seeds extract of *Myristica fragrans* or one of the compounds of formula (I) to (IX) isolated therefrom can
be administered to a mammal in the form of a composition containing, e.g., a pharmaceutical composition or a health care food composition.

The pharmaceutical composition having the above effect may be prepared by admixing the seeds extract of *Myristica fragrans* or the compounds of formula (I) to (IX) isolated therefrom as an active ingredient with a pharmaceutically acceptable carrier or excipient; or diluting with a diluent in accordance with any of the conventional methods. Examples of suitable carrier, excipient and diluent are lactose, dextrose, sucrose, sorbitol, mannitol, xylitol, erythritol, malditol, starch, acacia gum, alginate, gelatin, calcium phosphate, calcium silicate, cellulose, methylcellulose, microcrystalline cellulose, polyvinylpyrrolidone, water, methylhydroxybenzoate, propylhydroxybenzoate, talc, magnesium stearate and mineral oil. The pharmaceutical composition may additionally include fillers, anti-agglutinating agents, lubricants, wetting agents, flavoring agents, emulsifiers, preservatives and the like.

The pharmaceutical compositions of the present invention may also be formulated so as to provide quick, sustained or delayed release of an active ingredient after their administration to a mammal by employing any of the methods well known in the art, and the formulation may be in the form of a tablet, pill, powder, sachet, elixir, suspension, emulsion, solution, syrup, aerosol, soft and hard gelatin capsule, sterile injectable solution, sterile packaged powder and the like.

The pharmaceutical composition of the present invention can be administered via various routes including oral, transdermal, subcutaneous, intravenous and intramuscular introduction. The inventive seeds extract of *Myristica fragrans* may be administered in an effective amount ranging from about 10 to 100 mg/kg body weight, preferably 10 to 30 mg/kg body weight per day in a single dose or in divided doses. And, the compounds of formula (I) to (IX) isolated therefrom may be administered in an effective amount ranging from about 1 to 30 mg/kg body weight, preferably 1 to 10 mg/kg body weight per day in a single dose or in divided doses. However, it should be understood that the amount of the active ingredient actually administered ought to be determined in light of various relevant factors including the condition to be
treated, the chosen route of administration, the age, sex and body weight of the individual patient, and the severity of the patient's symptom; and, therefore, the above dose should not be intended to limit the scope of the invention in any way.

Further, the present invention provides a health care food composition for preventing osteoporosis comprising any one of the compounds of formula (I) to (IX), a mixture thereof, or the seeds extract of *Myristica fragrans* comprising thereof.

The health care food may be, but not limited to, various foods, beverages, snacks, cookies, gums, ice creams, tea bag-teas, instant teas, granules, flavoring agents, vitamin complexes, and other health supplement foods.

Moreover, the seeds extract of *Myristica fragrans* or the compounds isolated therefrom can be incorporated in prepared health care foods or raw materials thereof. In this case, the content of any one of the compounds of formula (I) to (IX), a mixture thereof, or the seeds extract of *Myristica fragrans* comprising thereof in the health care food may range from 0.01 to 30 wt% based on the total weight of the health care food.

The inventive pharmaceutical composition or health care food may additionally include other pharmaceutically acceptable medicinal herbs or the extract thereof for enhancing or complementing the desired effect. Examples of the other medicinal herb is *Siegeseckiae herba, Alpiniae katusumadaii semen, Nelumbo semen, Eugenia caryophyllata* and the like. The other medicinal herb may be used in the amount of 0.01 to 50 wt% based on the total weight of the composition.

The present invention also provides a method for preventing or treating osteoporosis in mammals, which comprises administering thereto an effective amount of the seeds extract of *Myristica fragrans* or the compounds isolated therefrom optionally together with an additional herbal extract.

The following Examples are intended to further illustrate the present invention without limiting its scope.
Example 1: Preparation of a seeds extract of *Myristica fragrans*

1.8 kg of dried seeds of *Myristica fragrans* purchased at Gyeongdong market (Korea) was extracted by soaking in 5.6 of cold methanol over a period of 1 week, and the resulting solution was filtered and concentrated under a reduced pressure. The above procedure was repeated three times to obtain 300 g of methanol extract. Then, the methanol extract was suspended in 2.6 of distilled water, and the suspension was extracted three times with 2.6 of ethylacetate. The combined organic layer was concentrated under a reduced pressure to obtain 255 g of an ethylacetate fraction. Then, the aqueous layer after ethylacetate extraction was further extracted three times each with 2 E of n-butanol, the organic layers were combined, and the solvent was removed under a reduced pressure to obtain 6 g of n-butanol fraction. The water layer recovered after the n-butanol extraction process was lyophilized.

Example 2: Separation of machilin A from the seeds extract of *Myristica fragrans* and identification thereof

60 g of the ethylacetate fraction from the seeds extract of *Myristica fragrans* obtained in Example 1 was fractionated by silica gel chromatography (silica gel: 70-230 mesh, 2 kg; column size: Φ=10.0 x 28 cm) using a gradient elution of n-hexane/ethyl acetate (10:1 to 0.1) at a flow rate of 3 ml/min to collect five 3 I fractions which were respectively subjected to solvents removal to obtain Fractions 1 to 5. Fraction 1 (7.9 g) was further fractionated by silica gel chromatography (silica gel: 70-230 mesh, 1 kg; column size: Φ=7.0 x 28 cm) using n-hexane/ethyl acetate (100:1) as the mobile phase at a flow rate of 3 ml/min to collect two 3 E fractions which were treated as above to obtain Fractions 11 and 12. Then, Fraction 12 was purified by reversed-phase chromatography (mobile phase: 80% methanol) to isolate 930 mg of machilin A as a yellow oil.

EI-MS m/z: 326 [M]+;

¹H-NMR (300 MHz, CDCl₃): δ 0.75 (6H, d, J = 6.7 Hz, H-9, 9'), δ 1.67 (2H, m, H-8, 8'), δ 2.29 (2H, dd, J = 13.4, 9.3 Hz, H-7a, 7'a), δ 2.65 (2H, dd, J = 13.4, 4.8 Hz, H-7b, 7'b), δ 5.82 (4H, s, -OCH₂O- x 2), δ 6.52 (2H, dd, J-7.8, 1.6 Hz, H-6, 6'); and
\[ ^{13} \text{C-NMR (125 MHz CDCl}_3): \delta \ 135.6 \text{ (C-I, 1')}, 107.9 \text{ (C-2, 2')}, 145.4 \text{ (C-3, 3')}, 147.4 \text{ (C-4, 4')}, 109.3 \text{ (C-5, 5')}, 121.7 \text{ (C-6, 6')}, 39.4 \text{ (C-7, 7')}, 39.0 \text{ (C-8, 8')}, 16.1 \text{ (C-9, 9')}, 100.6 (-OCH}_2\text{O-).} \]

**Example 3: Separation of maceligiian from the seeds extract of Myristica fragrans and identification thereof**

Fraction 3 (13.6 g) of Example 2 was further fractionated into 2 fractions, Fractions 31 and 32, by a procedure similar to that of Example 2 using silica gel chromatography (silica gel: 70-230 mesh, 1.2 kg; column size: \( \Phi = 7.0 \times 32 \text{ cm} \)) with n-hexane/ethyl acetate (5:1) as a mobile phase at a flow rate of 3 ml/min. Fraction 31 obtained in an amount of 3.9 g was identified to be macelignan, a colorless crystal.

m.p.: 70-72°C;
[\( \alpha \rceil \text{D}^{20} = +5.28^\circ \text{ (c = 1.8, CHCl}_3 \)];

EI-MS m/z (rel. int.): 328 [M]+ (I1), 137 [C\textsubscript{8}H\textsubscript{9}O\textsubscript{2}]\textsuperscript{+} (100) and 135 [C\textsubscript{8}H\textsubscript{7}O\textsubscript{2}]\textsuperscript{+} (68);

\[^1\text{H-NMR (300 MHz, CDCl}_3): \delta 6.87 \text{ (IH, d, J = 7.8 Hz, H-5)}, \delta 6.76 \text{ (IH, d, J = 1.8 Hz, H-2)}, \delta 6.63 \text{ (IH, d, J = 1.8 Hz, H-6)}, \delta 6.70 \text{ (IH, dd, J = 7.8, 1.8 Hz, H-6)}, \delta 6.64 \text{ (IH, d, J = 7.8, 1.8 Hz, H-2)}, \delta 5.95 \text{ (2H, s, -OH)}, \delta 5.32 \text{ (IH, s, -OCH}_3\text{)}, \delta 2.76 \text{ and 2.30 (each 2H, m, H-7, T)}, \delta 1.78 \text{ (2H, m, H-8, 8')}, \delta 0.88 \text{ (6H, m, H-9, 9')}; \]

\[^{13}\text{C-NMR (125 MHz, CDCl}_3): \delta 135.6 \text{ (C-I, 1')}, 107.8 \text{ (C-2, 2')}, 147.4 \text{ (C-3, 3')}, 109.3 \text{ (C-5, 5')}, 121.7 \text{ (C-6, 6')}, 39.0 \text{ (C-7, 7')}, 39.4 \text{ (C-8, 8')}, 16.1 \text{ (C-9, 9')}, 100.6 (-OCH}_2\text{O-).} \]

**Example 4: Separation of machilin F from the seeds extract of Myristica fragrans and identification thereof**

Fraction 4 (6.7 g) of Example 2 was further fractionated into 4 fractions, Fractions 41 to 44, by a procedure similar to that of Example 2 using silica gel chromatography (silica gel: 70-230 mesh, 420 g; column size: \( \Phi = 4.0 \times 35 \text{ cm} \)) with n-hexane/ethyl acetate (5:1) as a mobile phase at a flow rate of 3 ml/min.
Fraction 43 (670 mg) thus obtained was further fractionated into 5 fractions, Fractions 431 to 435, by reversed-phase chromatography (Φ=2.0 x 25 cm) with 80% methanol as a mobile phase. Fraction 432 obtained in an amount of 60 mg was identified to be machilin F, a colorless crystal.

\[ \delta \text{H-NMR (CDCl}_3, \text{300 MHz)}: 1.37 (3H, d, } J = 6.8 \text{ Hz, CH}_3-8 \), 1.86 (3H, d, } J = 6.5 \text{ Hz, CH}_3-9 \), 3.44 (IH, m, H-8), 3.88 (6H, s, -OCH}_3\times2\), 5.09 (IH, d, } J = 9.4 \text{ Hz, H-7}, 5.65 (IH, s, OH), 6.06 - 6.16 (IH, m, H-8 \)), 6.33 (IH, br-d, } J = 15.7 \text{ Hz, H-7 \}}, 6.76 - 7.00 (5H, m, H-aromatic); and

\[ \delta \text{C-NMR (CDCl}_3, \text{125 MHz)}: 108.9 (C-2), 146.7 (C-3), 145.7 (C-4), 114.1 (C-5), 119.9 (C-6), 93.8 (C-7), 45.6 (C-8), 132.2 (C-1 \)), 113.3 (C-2 \)), 133.3 (C-3 \)), 146.6 (C-4 \)), 144.2 (C-5 \)), 109.2 (C-6 \)), 130.9 (C-7 \)), 123.5 (C-8 \)), 18.4 (C-9 \)), 17.6 (\text{-CH}_3 \), 55.9 (OCH}_3 \), 56.0 (OCH}_3 \).

**Example 5: Separation of nectandrin B from the seeds extract of Myristica fragrans and identification thereof**

Fraction 5 (11.4 g) of Example 2 was subjected to reversed-phase chromatography (250 x 40 mm i.d Merck, 40-63 μm using preparative HPLC and 60-100% methanol as a mobile phase at a flow rate of 3 ml/min to isolate 820 mg of nectandrin B as a colorless oil.

\[ \delta \text{H-NMR (CDCl}_3, \text{300 MHz)}: 1.03 (6H, d, } J = 6.6 \text{ Hz, CH}_3-3, 4 \), 2.32 (2H, m, H-3, 4), 3.88 (6H, s, OCH}_3\times3, 3 \)), 4.49 (2H, d, } J = 6.4 \text{ Hz, H-2, 5}, 5.57 (2H, s, HO-4 \)), 6.80 - 6.95 (6H, m, H-2 \)), 5 \)), 6 \)), 2 \)), 5 \)), 6 \)); and

\[ \delta \text{C-NMR (CDCl}_3, \text{125 MHz)}: 87.5 (C-2, 5), 44.5 (C-3, 4), 134.3 (C-1 \)), 109.2 (C-2 \), 146.6 (C-3 \)), 145.2 (C-4 \)), 114.1 (C-5 \)), 119.2 (C-6 \)), 13.1 (CH}_3-3, 4 \), 55.8 (OCH}_3 \).

**Example 6: Separation of safrole from the seeds extract of Myristica fragrans and identification thereof**
The procedure of Example 2 was repeated except for using Fraction 11 to obtain 3.2 g of safrole as a cream yellow oil.

EI-MS m/z: 162 [M]+;

$^1$H-NMR (300 MHz, CDCl$_3$): $\delta$ 3.22 (2H, d, $J = 6.6$ Hz, H-7), $\delta$ 5.00 (IH, m, H-9a), $\delta$ 5.02 (IH, m, H-9b), $\delta$ 5.85 (IH, m, H-8), $\delta$ 5.83 (2H, s, -OCH$_2$O-), $\delta$ 6.58 (IH, $J = 8.1$ Hz, H-6), $\delta$ 6.63 (IH, m, H-2), $\delta$ 6.67 (IH, d, $J = 8.1$ Hz, H-5); and

$^{13}$C-NMR (125 MHz, CDCl$_3$): $\delta$ 133.4 (C-I), 108.0 (C-2), 147.3 (C-3), 145.5 (C-4), 108.8 (C-5), 121.1 (C-6), 40.0 (C-7), 137.8 (C-8), 115.4 (C-9),

100.7 (-OCH$_2$O-)

**Example 7:** Separation of licarin A from the seeds extract of *Myristica fragrans* and identification thereof

The procedure of Example 4 was repeated except for using Fraction 434 to obtain 150 mg of licarin A as a colorless oil.

$[\alpha]_D$: $0^\circ$ (c = 0.1, CHCl$_3$);

EI-MS m/z: 342.15 [M]+;

$^1$H-NMR (CDCl$_3$, 300 MHz): $\delta$ 1.00 (6H, d, $J = 6.6$ Hz, Me-3, 4), 2.26 (2H, m, H-3, 4), 3.88 (3H, s, CH$_3$O-3”), 4.45 (2H, d, $J = 5.1$ Hz, H-2, 5), 5.66 (IH, s, HO-4”), 5.95 (2H, s, -OCH$_2$O-), 6.77 - 6.97 (6H, m, H-Ar); and

$^{13}$C-NMR (CDCl$_3$, 125 MHz): 136.2 (C-I”), 146.9 (C-4”), 146.9 (C-4”), 109.0 (C-5”), 119.6 (C-6”), 87.3 (C-2), 44.5 (C-3), 44.6 (C-4), 87.4 (C-5), 134.0 (C-I”), 108.0 (C-2”), 146.5 (C-3”), 145.0 (C-4”), 114.2 (C-5”), 119.9 (C-6”), 12.8 (Me-3, 4), 55.9 (-OCH$_3$), 100.9 (-OCH$_2$O).

**Example 8:** Separation of licarin B from the seeds extract of *Myristica fragrans* and identification thereof

Fraction 2 (19.0 g) of Example 2 was subjected to reversed-phase chromatography (Φ=5.O x 30 cm) using 60% methanol as a mobile phase at a flow rate of 3 ml/min to isolate 140 mg of licarin B as a yellow oil.

$[\alpha]_D$: $-45.4^\circ$ (c = 0.1, CHCl$_3$);

EI-MS m/z: 324 [M]+;

$^1$H-NMR (300 MHz, CDCl$_3$): $\delta$ 1.31 (3H, d, $J = 6.8$ Hz, H-9), $\delta$ 1.79...
\( \text{(3H}_2 \text{, J} = 6.7 \text{ Hz, H-9}^1 \), \( \delta 3.3 \) (IH, m, H-8), \( \delta 3.82 \) (3H, s, -OCH\(_3\)), \( \delta 5.02 \) (IH, d, \( J = 9.0 \text{ Hz, H-7} \), \( \delta 5.88 \) (2H, s, -OCH\(_2\)O-), \( \delta 6.03 \) (IH, dq, \( J = 15.7, 6.7 \text{ Hz}, \text{H-8}^1 \), \( \delta 6.28 \) (IH, \( \text{d}, J = 15.7 \text{ Hz}_5 \text{H-7}^1 \), \( \delta 6.70 - 6.86 \) (5H, m, H-2, 4, 5, 2', 6'); and

\( ^{13} \text{C-NMR (125 MHz, CDCl}_3\): \( \delta 134.3 \) (C-I), 106.7 (C-2), 147.8 (C-3), 147.5 (C-4), 108.1 (C-5), 121.1 (C-6), 93.3 (C-7), 45.7 (C-8), 17.8 (C-9), 132.2 (C-I'), 113.3 (C-2'), 133.0 (C-3'), 146.5 (C-4'), 144.1(C-5'), 109.4 (C-6'), 130.9 (C-7'), 123.3 (C-8'), 18.3 (C-9'), 56.0 (-OCH\(_3\)), 101.0 (-OCH\(_2\)O-).

**Example 9**: Separation of myristagenol A from the seeds extract of *Myristica fragrans* and identification thereof

Fraction 32 (5.0 g) of Example 3 was subjected to silica gel (70-230 mesh, 400 g) column (Φ=4.0 x 32 cm) chromatography using n-hexane/ethyl acetate (5:1) as a mobile phase at a flow rate of 3 ml/min to isolate 570 mg of myristagenol A as a white powder.

\( ^1 \text{H-NMR (300 MHz, acetone-d}\_6\): \( \delta 0.62 \) (3H, d, \( J = 7.0 \text{ Hz, H-9} \), 0.85 (3H, d, \( J = 7.0 \text{ Hz, H-9} \), \( \delta 1.2 \) (IH, m, H-8), \( \delta 2.38 \) (IH, m, H-8'), \( \delta 2.13 \) (IH, dd, \( J = 12.9, 2.0 \text{ Hz, H-7a} \), \( \delta 2.95 \) (IH, dd, \( J = 12.9, 3.4 \text{ Hz, H-7b} \), \( \delta 4.45 \) (IH, dd, \( J = 9.2, 1.0 \text{ Hz, H-7} \), \( \delta 6.80 \) (IH, dd, \( J = 9.0, 1.3 \text{ Hz, H-6} \), \( \delta 6.80 \) (IH, dd, \( J = 8.1, 2.0 \text{ Hz, H-6} \), \( \delta 7.01 \) (IH, d, \( J = 1.3 \text{ Hz, H-2} \), \( \delta 3.84 \) (3H, s, -OCH\(_3\)), \( \delta 5.94 \) (2H, s, U-CH\(_2\)-O) \( \delta 5.62 \) (s, Ar-OH); and

\( ^{13} \text{C-NMR (125 MHz, acetone-\text{d}_6\): \( \delta 138.6 \) (C-I), 111.5 (C-2), 148.6 (C-3), 148.5 (C-4), 115.6 (C-5), 121.0 (C-6), 77.4 (C-7), 46.4 (C-8), 12.3 (C-9), 137.8 (C-I'), 109.0 (C-2'), 148.9 (C-3'), 146.8 (C-4'), 110.6 (C-5'), 123.2 (C-6'), 38.2 (C-7'), 36.6 (C-8'), 18.4 (C-9'), 56.7 (-OCH\(_3\)), 102.0 (0-CH\(_2\)-O).

**Example 10**: Separation of meso-dihydroguaiaretic acid from the seeds extract of *Myristica fragrans* and identification thereof

The procedure of Example 9 also yielded 1.2 g of meso-dihydroguaiaretic acid as a colorless crystal,

m.p.: 102-104 °C;

\([\alpha]_D +52.0^\circ \ (c = 0.79, \text{MeOH})\);
EI-MS m/z: 326 [M]+;

$^1$H-NMR (CDCl$_3$, 300 MHz): $\delta$ 1.37 (3H, d, $J = 6.8$ Hz, CH$_3$-S), 1.86
(3H, d, $J = 6.5$ Hz, CH$_3$-9), 3.44 (IH, m, H-8), 3.88 (6H, s, -OCH$_3^2$), 5.09 (IH, d, $J = 9.4$ Hz, U-T), 5.65 (IH, s, OH), 6.06 - 6.16 (IH, m, H-8'), 6.33 (IH, br-d,
$J = 15.7$ Hz, H-7'), 6.76 - 7.00 (5H, m, H-aromatic); and

$^{13}$C-NMR (CDCl$_3$, 125 MHz): $\delta$ 108.9 (C-2), 146.7 (C-3), 145.7 (C-4),
114.1 (C-5), 119.9 (C-6), 93.8 (C-7), 45.6 (C-8), 132.2 (C-1'), 113.3 (C-2'),
133.3 (C-3'), 146.6 (C-4'), 144.2 (C-5'), 109.2 (C-6'), 130.9 (C-7'), 123.5 (C-8'),
18.4 (C-9'), 17.6 (-CH$_3$), 55.9 (OCH$_3$), 56.0 (OCH$_3$).

**Test Example 1: The influence of the seeds extract of *Myristica fragrans* on the calcium formation in the osteoblast**

(1-1) Culture and differentiation induction of osteoblast

MC3T3-E1 subclone 4 (ATCC CRL-2593) was used as osteoblast cells, and medium and other materials used in cell culture were purchased from HyClone Laboratories Inc.. The osteoblast cells were cultured in an $\alpha$-MEM medium supplemented with 10% FBS, and the medium was replaced with a fresh medium every 3 days. At 70% confluency, the cultured cells were removed from the culture plate by using trypsin, and 1 x $10^4$ cells were transferred to each well of a 48-well plate. At a confluency of 95% and more, the medium was changed to a differentiation-inducing medium ($\alpha$-MEM containing 10% FBS, 50 $\mu$g/ml ascorbic acid and 10 mM $\beta$-glycerophosphate). Cells were then cultured while replacing the medium with a fresh medium every 3 days. 0.1, 1 and 10 $\beta$glml of the seeds extract of *Myristica fragrans* of Example 1, the ethylacetate fraction, n-butanol fraction and water fraction were each added thereto (treatment).

(1-2) Staining and quantitation of calcium formed in the osteoblast

After 21 days of the treatment, the cells were washed with PBS, reacted with an alizarin red S staining solution (0.1368 g in 10 ml water, pH 4.2), and washed with water to detect the presence of accumulated calcium which was stained (Fig. 3A). The accumulated calcium was extracted with 1N HCl, and
quantified using Calcium C kit (Wako, Japan) (Fig. 3B).

As shown in Fig. 3A, when 10 μg/mL of the methanol extract of *Myristica fragrans*, ethylacetate fraction and water fraction, respectively, were used to treat cells, increased amounts of stained calcium were observed as compared with the non-treated control group. Further, as shown in Fig. 3B, the treated groups formed more calcium as compared with the non-treated control group in the view of statistical significance: the statistical significance was tested by t-test (* p<0.01, ** p<0.001) based on the mean value of the control groups of 2.75 mg/dL.

**Test Example 2: Analysis of calcium formation in the osteoblast by the compounds isolated from the seeds extract of *Myristica fragrans***

The effects of the compounds of Examples 2 to 9 isolated from the seeds extract of *Myristica fragrans* on the calcium formation in cases of osteoblast were tested as described in Test Example 1.

As shown in Fig. 4, the treatment with 10 μM machilin A showed the highest amount of accumulated calcium, while the treatments with 5 μM macelignan, machilin F, nectandrin B, safrole, licarin A, licarin B, myristagenol A and meso-dihydroguaiaretic acid were each most effective in obtaining the highest amount of accumulated calcium in the osteoblast cases under test. Consequently, the amounts of calcium formation in the osteoblast case induced by the compounds of Examples 2 to 9 were 15 to 100 times higher than that of the non-treated control group.

**Test Example 3: Analysis of the osteoblast proliferation by machilin A, and the increase of the activity and expression of alkaline phosphatase (ALP) as an early differentiation marker of the osteoblast**

(3-1) Analysis of the osteoblast proliferation

1 x 10^3 cells/well of the MC3T3-E1 subclone 4 used in Test Example 1 were seeded into a 96-well plate, and 0, 0.625, 1.25, 2.5 and 10 μM machilin A were added, respectively, to each well after 12 hours. After 1 and 3 days of
the treatment, the osteoblast proliferation was measured using CCK-8 kit (Dojindo, Japan). The results are shown in Fig. 5. Before the 12-hour measurement, the plate with serially diluted cells \((1 \times 2, 4, 8 \times 10^3 \text{cells/well})\) was prepared in order to determine the cell number of each test group, wherein the measured absorbance was used to determine the cell number.

(3-2) Analysis of the activity and expression of ALP

In order to examine the effect of machilin A on the activity and expression of ALP, the following experiment was performed.

Osteoblasts cells were treated with machilin A and cultured as described in (3-1). After 5 days of the treatment, the cells were lysed with lysis solution \((10 \text{ mM Tris-HCl, pH 7.5, 0.5 mM MgCl}_2, 0.1\% \text{ Triton X-100})\) at room temperature for 30 min, and centrifuged at 12,000 rpm at 4°C for 20 min. Protein concentration in the supernatant was measured with BCA kit (Bio-Rad). The supernatant thus obtained was used for measurement of the activity of ALP with LabAssay ALP kit (Wako, Japan). The results are shown in Fig. 6A. The measured activity was expressed as unit/mg protein, and the statistical significance was tested by t-test (ALP activity in general medium versus in differentiation-inducing medium, * p<0.05; ALP activity in differentiation-inducing medium versus in differentiation-inducing medium containing machilin A, ## p<0.01).

Further, in order to examine the effect of machilin A on the expression of ALP in cases of osteoblast, the following ALP staining was performed.

The cells were washed three times with PBS, fixed with 10% formalin solution for 30 sec, and washed again with water. The cells were stained in the dark for 1 hour by treating with an alkaline solution, which was prepared by dissolving Fast Blue JAR 1 capsule (Sigma, included in a kit (cat. no. 85LI-I)) in 48 mL water and adding 2 mL Naphthol AS-MX phosphate (Sigma, included in a kit (cat. no. 85LI-I)) thereto. Then, the cells were washed with water. The stained ALP was purple, and the results are shown in Fig. 6B.

(3-3) Result and analysis
As shown in Fig. 5, the increased cell number after treating with 10 μM of machilin A was observed as compared with the control group. Further, the activity and expression of ALP were significantly increased when compared with the control group (Figs. 6A and 6B). Therefore, these results demonstrate that machilin A stimulates the osteoblast differentiation.

**Test Example 4: Analysis of the calcium formation and mineralization in the osteoblast by machilin A**

(4-1) Staining and quantitation of calcium formed in the osteoblast

In order to examine the effect of machilin A on the calcium formation in the osteoblast, the cells were treated with machilin A as described in Test Example 3, and, after the 12- and 21-day treatment, calcium staining and quantitation were performed as described in (1-2) of Test Example 1. The results are shown in Fig. 7A (calcium concentration in general medium versus in differentiation-inducing medium, ** p<0.01; *** p<0.001; calcium concentration in differentiation-inducing medium versus in differentiation-inducing medium containing machilin A, ### p<0.001) and Fig. 7B.

As shown in Figs. 7A and 7B, after the 14-day treatment of 5 to 10 μM of machilin A, an amount of accumulated calcium in the treated groups was at least 2.5-fold higher than that of the control group. Further, after the 21-day treatment, greatly increased amount of accumulated calcium was observed, and, even in the group treated with 0.625 μM of machilin A, the calcium was significantly accumulated.

(4-2) Analysis of the mineralization of the osteoblast

The effect of machilin A on the mineralization in osteoblast cells was examined using von Kossa staining method. First, osteoblast cells were washed with PBS, fixed with 2.5% glutaraldehyde/PBS solution for 30 min, and washed twice with distilled water. The cells were treated with 5% silver nitrate solution and subjected to UV radiation at room temperature to observe the mineralized region (black). The results are shown in Fig. 8.

As shown in Fig. 8, the mineralization in osteoblast cells was increased
with increasing an amount of machilin A. Therefore, the result reveals that machilin A stimulates the calcium formation and the mineralization by stimulating the osteoblast differentiation.

5 **Test Example 5: Analysis of the expressions of genes related to the osteoblast differentiation and mineralization induced by machilin A**

The biomarker expressed at the time of the osteoblast differentiation is changes according to the time point. It is reported that the ALP is expressed at the early differentiation, and certain genes such as osteopontin (OP), osteocalcin (OC) and type I collagen (Col) are highly expressed at the stage of the mineralization induced by osteoblast cells. Accordingly, in order to examine how the expression levels of the above four genes related to the osteoblast differentiation are changed after the 5- and 14-day machilin A treatments, the osteoblast cells were treated with machilin A as described in Test Example 3, and after the 5- and 14-day treatment, the following real time RT-PCR was performed.

(5-1) Real time RT-PCR

Total RNA from the cultured osteoblast cells was prepared with Trizol reagent (Life Technologies), and cDNA was synthesized by using 1 µg of RNA, 1 µM oligo-dT15 primer and 1 µM Omniscript Reverse Transcriptase (Qiagen). The synthesized cDNA was diluted with the culture medium (1/50), and PCR was performed by using Brilliant SYBR Green Master Mix (Stratagene), 20 pmole primers and Mx3000P (Stratagene). The following primers were used for PCR amplification:

(1) ALP
5'-atgggccgtctccacagtaac-3' (Forward; SEQ ID NO: 1)
5'-tcacccgagtgtgtcaca-3' (Reverse; SEQ ID NO: 2)

(2) OP
5'-cccggtgaaagtgactgatt-3' (Forward; SEQ ID NO: 3)
5'-tctcctggctctctttggaa-3' (Reverse; SEQ ID NO: 4)

(3) OC
PCR reaction was carried out as follows: an initial melt at 94°C for 3 min; 40 cycles of φ melting at 94°C for 40 sec, (2) annealing at 60°C for 40 sec and (S) extension at 72°C for 1 min; and a final extension at 72°C for 5 min. The amplified levels of products were quantified by normalizing to that of GAPDH (glyceraldehyde -3-phosphate dehydrogenase) using 2-^[ΔΔCT] method (Livak et al., Methods, 25(4), 402-408, 2001), and expressed relative to the control group. The statistical significance was tested by t-test, and the results are shown in Figs. 9A and 9B (* p<0.05, ** p<0.01, *** p<0.001).

As shown in Fig. 9A, the tested four genes (ALP, OP, OC and Col) were significantly increased in the cell after the 5-day machilin A treatment. Further, as shown in Fig. 9B, among the tested genes, three genes (OP, OC, Col) related to the mineralization were significantly increased in the cell after the 14-day machilin A treatment.

**Test Example 6: Analysis of the activation of mitogen-activated protein (MAP) induced by machilin A**

It is reported that the increase of the MAP kinase activity can stimulate the osteoblast differentiation. Therefore, in order to examine what is the effect of machilin A on the MAP kinase activity in osteoblasts cells, the following experiment was performed.

(6-1) Western blot

The osteoblast cells were cultured in the differentiation-inducing
medium for 7 days while treating machilin A every 3 days as described in Test Example 1, and the cells were lysed with RIPA solution to obtain cellular proteins. The proteins thus obtained were quantified with BCA kit (Bio-Rad). The proteins were separated on 12% SDS-gel (loading amounts of the proteins: 10 /ug/lane), followed by transferring to a nitrocellulose (NC) membrane. The membrane was blocked with a 10% skimmed milk, and reacted with antibodies (SantaCruz, USA) against several members of MAP kinase family, i.e., p-JNK (phosphorylated jun N-terminal kinase), JNK (jun N-terminal kinase), p-ERK (phosphorylated externally regulated kinases), ERK (externally regulated kinases), p-p38 and p38. The results are shown in Fig. 10.

As shown in Fig. 10, elevated phosphorylation of JNK was observed after 7 days of the differentiation with increasing concentration of machilin A. Therefore, such results indicate that machilin A stimulates the osteoblast differentiation through the activation of JNK that is one member of MAP kinase family.

Exemplary pharmaceutical formulations or foods were prepared by using the seeds extract of Myristica fragrans or one of the active components obtained in the above Examples as follows.

**Formulation Example 1: Preparation of powder**

2 g of dried seeds extract of *Myristica fragrans* or one of the active components thereof and 1 g of lactose were mixed, and the resulting mixture was filled in a sealed package according to a conventional method.

**Formulation Example 2: Preparation of tablet**

100 mg of dried seeds extract of *Myristica fragrans* or one of the active components thereof, 100 mg of corn starch, 100 mg of lactose and 2 mg of magnesium stearate were mixed, and the resulting mixture was tabletted according to a conventional method.

**Formulation Example 3: Preparation of capsule**

100 mg of dried seeds extract of *Myristica fragrans* or one of the active...
components thereof, 100 mg of corn starch, 100 mg of lactose and 2 mg of magnesium stearate were mixed, and the resulting mixture was filled in a gelatin capsule according to a conventional method.

5  **Formulation Example 4: Preparation of injection solution**

100 mg of dried seeds extract of *Myristica fragrans* or one of the active components thereof was dissolved in quantum satis of distilled water for injection, and adjusted to pH approximately 7.5 using quantum satis of pH adjuster. The resulting solution was filled in 2 ml of ample with distilled water for injection and sterilized according to a conventional method.

10  **Formulation Example 5: Roast grain powder**

Brown rice, barley, lutinous rice and coix were gelatinized and dried according to a conventional method, and the resulting mixture was distributed and pulverized to obtain 60-mesh size grain powder. Black bean, black sesame and perilla were steamed and dried according to a conventional method, and the resulting mixture was distributed and pulverized to obtain 60-mesh size seed powder.

The seeds extract of *Myristica fragrans* or the active compound of the present invention was concentrated under a reduced pressure, and spray-dried with a hot-air dryer. The dried material thus obtained was pulverized to obtain 60-mesh sized, dried powder.

The dried powders obtained from the grains, seeds and the seeds extract of *Myristica fragrans* or the active compound were all mixed in the following ratio, and the resulting mixture was formed to a granule according to a conventional method:

Grain powder (30 wt% of brown rice, 15 wt% of coix, 20 wt% of barley and 9 wt% of lutinous rice),

Seed powder (7 wt% of perilla, 8 wt% of black bean and 7 wt% of black sesame),

3 wt% of the seeds extract powder of *Myristica fragrans*,

0.5 wt% of *Ganoderma lucidum*, and

0.5 wt% of *Rehmannia glutinosa*. 


Formulation Example 6: Preparation of chewing gum

0.1 wt% of the seeds extract of Myristica fragrans or one of the active components thereof was mixed with 20 wt% of gum base, 76.9 wt% of sugar, 1 wt% of flavor and 2 wt% of water, and the resulting mixture was formed to a chewing gum according to a conventional method.

Formulation Example 7: Preparation of candy

0.1 wt% of the seeds extract of Myristica fragrans or one of the active components thereof was mixed with 60 wt% of sugar, 39.8 wt% of dextrose syrup and 0.1 wt% of flavor, and the resulting mixture was formed to a candy according to a conventional method.

Formulation Example 8: Preparation of biscuit

1 wt% of the seeds extract of Myristica fragrans or one of the active components thereof was mixed with 25.59 wt% of weak flour (I), 22.22 wt% of medium flour (I), 4.80 wt% of sucrose, 0.73 wt% of edible salt, 0.78 wt% of glucose, 11.78 wt% of palm shortening, 1.54 wt% of ammonium, 0.17 wt% of baking soda, 0.16 wt% of sodium metabisulfite, 1.45 wt% of rice flour, 0.001 wt% of vitamin B₁, 0.0001 wt% of vitamin B₂, 0.04 wt% of milk flavor, 20.6998 wt% of water, 1.16 wt% of whole milk powder, 0.29 wt% of imitation milk powder, 0.03 wt% of calcium phosphate monobasic, 0.29 wt% of spray salt and 7.27 wt% of spray-dried milk, and the resulting mixture was formed to a biscuit according to a conventional method.

Formulation Example 9: Preparation of health care beverage

1 wt% of the seeds extract of Myristica fragrans or one of the active components thereof was mixed with 0.26 wt% of honey, 0.0002 wt% of thioctamide, 0.0004 wt% of nicotinamide, 0.0001 wt% of riboflavin sodium hydrochloride, 0.0001 wt% of pyridoxine hydrochloride, 0.001 wt% of inositol, 0.002 wt% of orotic acid and 98.7362 wt% of water, to obtain a health care beverage according to a conventional method.
Formulation Example 10: Preparation of health supplement food

10 wt% of the seeds extract of *Myristica fragrans* or one of the active components thereof was mixed with 55 wt% of spirulina, 10 wt% of enzymatic hydrolysate of guar gum, 0.01 wt% of vitamin B₁ hydrochloride, 0.01 wt% of vitamin B₆ hydrochloride, 0.23 wt% of DL-methionine, 0.7 wt% of magnesium stearate, 22.2 wt% of lactose and 1.85 wt% of corn starch, and the resulting mixture was tabletted to obtain tablets of a health supplement food according to a conventional method.

While the invention has been described with respect to the above specific embodiments, it should be recognized that various modifications and changes may be made and also fall within the scope of the invention as defined by the claims that follow.
What is claimed is:

1. A use of a compound selected from the group consisting of machilin A of formula (I), macelignan of formula (II), machilin F of formula (III), nectandrin B of formula (IV), safrole of formula (V), licarin A of formula (VI), licarin B of formula (VII), myristagenol A of formula (VIII), meso-dihydroguaiaretic acid of formula (IX) and a mixture thereof, or a seeds extract of *Myristica fragrans* comprising at least one of said compounds for preventing or treating osteoporosis:
2. The use of claim 1, wherein the seeds extract of Myristica fragrans is obtained by extracting the seeds of Myristica fragrans with a solvent selected from the group consisting of C$_{1-4}$ alcohols, aqueous solutions thereof, dichloromethane, ethyl acetate, and a mixture thereof.

3. A health care food for preventing osteoporosis, which comprises a compound selected from the group consisting of machilin A of formula (I), macelignan of formula (II), machilin F of formula (III), nectandrin B of formula (IV), safrole of formula (V), licarin A of formula (VI), licarin B of formula (VII), myristagenol A of formula (VIII), meso-dihydroguaiaretic acid of formula (IX) and a mixture thereof, or a seeds extract of Myristica fragrans comprising at least one of said compounds:
4. The health care food of claim 3, wherein the amount of any of the
compounds of formula (I) to (IX), a mixture thereof, or the seeds extract of *Myristica fragrans* in the food is 0.01 to 30 wt% based on the total weight of the food.

5. The health care food of claim 3, wherein the extract of *Myristica fragrans* is obtained by extracting the seeds of *Myristica fragrans* with a solvent selected from the group consisting of C$_{1-4}$ alcohols, aqueous solutions thereof, dichloromethane, ethyl acetate, and a mixture thereof.

6. The health care food of claim 3, wherein the food is a beverage.

7. A method for preventing or treating osteoporosis in mammals, which comprises administering thereto an effective amount of a compound selected from the group consisting of machilin A of formula (I), macelignan of formula (II), machilin F of formula (III), nectandrin B of formula (IV), safrole of formula (V), licarin A of formula (VI), licarin B of formula (VII), myristagenol A of formula (VIII), meso-dihydroguaiaretic acid of formula (IX) and a mixture thereof, or a seeds extract of *Myristica fragrans* comprising at least one of said compounds:

![Chemical structures](image)
FIG. 1

Myristica fragrans (1.8kg)

5L MeOH / 7 days
3times

MeOH extract (300g)

2L H₂O / 2L ethylacetate
3times

Ethylacetate fraction (255g)

2L n-butanol
3times

n-Butanol fraction (6g)

Water fraction (39g)
FIG. 2

Ethylacetate

Fraction (60g)

1 (17.9g)

2 (19.0g)

3 (13.6g)

4 (6.7g)

5 (11.4g)

Silica gel c.c.

(Hex:EA = 10:1-0:1)

Silica c.c.

(Hex:EA = 100:1)

Silica c.c.

(Hex:EA = 5:1)

Silica c.c.

(Hex:EA = 5:1)

RP-18 c.c.

(60% MeOH)

RP-18 c.c.

(60% MeOH)

RP-18 c.c.

(60% MeOH)

Safronine A

(3.2g)

Nectandrin B

Methylglyoxal A

Licarin A

Myristigenol A

Interference of 
Meso-Dihydroguaiaretic 
Acid (0.5g)

(930mg)

(150mg)

(570mg)

(140mg)

(3.9g)

(20mg)

(1.2g)

(60mg)
FIG. 3A

Methanol extract of Myristica fragrans

Ethylacetate fraction

Butanol fraction

Water fraction

0
0.1
1
10 (ug/ml)
FIG. 3B

Calcium concentration (mg/dL)

Methanol extract of Myristica fragrans
Butanol fraction
Ethylacetate fraction
Water fraction

0 0.1 1 10 0 0.1 1 10 0 0.1 1 10

*  **

6 5 4 3 2 1 0
FIG. 4

Calcium Concentration (mg/dL)

Machilin A  Macelignan  Machilin F  Nectandrin B  Safrole  Licarin A  Licarin B  Myristagenol A  meso-Dihydro guaiaretic acid
FIG. 6A

ALP activity (unit/mg)

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<th>General medium</th>
<th>0</th>
<th>0.625</th>
<th>1.25</th>
<th>2.5</th>
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<th>10 (μM) Machilin A</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</table>

FIG. 6B

General medium | 0 | 1.25 | 10 (μM) Machilin A |
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FIG. 8

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<td>20 days</td>
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<td>10 (μM) Machilin A</td>
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</table>

Differentiation-inducing medium
FIG. 9A

mRNA expression level (relative to GAPDH)

FIG. 9B

mRNA expression level (relative to GAPDH)
FIG. 10

0 0.625 1.25 2.5 5 10 (µg/ml) Machilin A on day 7

- p-JNK
- JNK
- p-ERK
- ERK
- p-p38
- p38
INTERNATIONAL SEARCH REPORT

International application No
PCT/KR2008/000676

A. CLASSIFICATION OF SUBJECT MATTER

A61K 31/357(2006.01)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 8 A61K

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 practicable, search terms used)

eKIPASS, STN (Caplus), Pubmed

* Key words: myristica fragrans, machuh, machegnian, nectandΠ, safrole, licaΠ, myΠstagenol, osteoporosis, etc

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
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<tr>
<td>Y</td>
<td>WO 2007/001 150 A2 (HWANG, JAE-KWAN) 04 January 2007 See the claim 1 and 2, pages 3-6 and Fig 1</td>
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<tr>
<td>A</td>
<td>US 6,261,565 B1 (ARCHER DANIELS MIDLAND COMPANY) 17 July 2001 See the whole document</td>
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Further documents are listed in the continuation of Box C

See patent family annex

* Special documents are cited documents
'A' document defining the general state of the art which is not considered to be of particular relevance
'E' earlier application or patent 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 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
13 MAY 2008 (13 05 2008)

Date of mailing of the international search report
13 MAY 2008 (13.05.2008)

Name and mailing address of the ISA/KR

Korean Intellectual Property Office
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gu, Daejeon 302-701, Republic of Korea

Facsimile No 82-42-472-7140

Authorized officer

LEE, SUN HWA

Telephone No 82-42-481-5606

Form PCT/ISA/210 (second sheet) (April 2007)
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. 7** because they relate to subject matter not required to be searched by this Authority, namely:

   Although claim 7 is directed to a treatment method of the human body by therapy and thus relates to a subject matter under Rule 39 of PCT, a search has been carried out based on the treatment effect of osteoporosis of the active ingredients (formulae I - IX) from the extract of Myristica fragrans.

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 64(a).

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 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.
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