Title: DRUG FOR OSTEOFOROSIS COMPRISING KAMPFEROL AS THE ACTIVE INGREDIENT

Abstract: The present invention relates to a novel use of the phytoestrogen kaempferol. Kaempferol in accordance with the present invention can be usefully employed as a therapeutic agent for osteoblasts.
Description

DRUG FOR OSTEOPOROSIS COMPRISING KAMPFEROL AS THE ACTIVE INGREDIENT

Technical Field

The present invention relates to a therapeutic agent for osteoporosis, comprising a phytoestrogen kaempferol as an active ingredient. Specifically, the present invention relates to a therapeutic agent for osteoporosis, comprising, as an active ingredient, kaempferol represented by the following general formula (1), which has excellent promotion effects upon osteoblast proliferation:

[3]

Formula 1

Background Art

Bones are the physical supporters support structure of the body and serve to reserve necessary bone mass and maintain structural integrity. In addition, bones play an important role in maintaining blood calcium levels as they serve as the body's calcium (Ca²⁺) depository.

In order to perform such functions, bones always modulate decomposition and remodeling actions. Therefore, bones in normal persons actively progress undergo both bone absorption and bone formation, thereby resulting in a dynamic state to reach maximum in respect to metabolism.

However, osteoporosis is a disease characterized by the decrease of calcium in normal bone tissues leading to thinning of the compact bone structuresubstance of bones and the subsequent expansion of marrow cavities. Bones become brittle with the progress of the disease, and may be easily fractured by weak impacts from the external surroundings. Bone mass is affected by various factors such as genetic factors, nutritive condition, changes of hormone level, exercise and life style, and osteoporosis is known to be caused by aging, lack of exercise, low body weight, smoking, low calcium diet, menopause and ovariectomy. In the meantimeIn addition, it is said
known that bone mass is higher in the black due to a low bone resorption level, as compared to the white, even though there is a difference between individuals, and generally reaches the highest level around the age of 14 to 18 and then decreases by about 1% a per year at the old age thereafter. Particularly, in women, decrease of bone mass begins to continue from the age of 30, and around menopause, sharply progresses by due to changes in hormone secretion. That is, when reaching menopause, the concentration of estrogen rapidly decreases and vast amounts of B-lymphocytes are produced, and subsequent pre-B cell accumulation in bone marrow results in increased levels of IL-6 which in turn increases activity of osteoclasts, thus, bone mass becomes decreased decreasing bone mass.

As such, in aged people the elderly, especially in women of postmenopause postmenopausal women, osteoporosis is is not the avoidable disease unavoidable although the severity of the symptoms may vary, and therefore, a great deal of attention has been increasingly directed to osteoporosis and therapeutic agents thereof due to the aging of the general population in advanced countries. In addition, therapies associated with bone diseases are potentially to be worth about 130 billion dollars in terms of global market scale, and it is forecasted to gain that further increases in demand will occur. For these reasons, many research groups and pharmaceutical companies have made a great deal of exerted great efforts for development of therapeutic agents for bone diseases.

Therapeutic agents for osteoporosis now being used include estrogen preparations, androgenic anabolic steroid preparations, calcium preparations, phosphate preparations, fluoride preparations, ipriflavone, vitamin D3, etc. In addition, novel drugs for osteoporosis have been developed, which include aminobisphosphonate by Merck Co. (U.S.A.) in 1995 and Raloxifene which plays a role of acts as a selective estrogen receptor modulator (SERM) by Eli Lilly Co. (U.S.A.) in 1997.

Meanwhile, conventional therapeutic agents for osteoporosis are mostly estrogen substances which are known to cause adverse side effects such as cancer, cholelithiasis and thrombosis upon prolonged use. Long-term administration of drug is inevitable in the treatment of osteoporosis since it is not feasible to treat such a disease with short-term administration. Therefore, drug developers seek to develop novel effective agents which have no adverse side effects even when administered for a prolonged period of time and exhibit excellent efficacy enough sufficient to replace estrogen. Currently, a great deal of interest has been focused on a phytoestrogen as one of estrogen substitutes.

The phytoestrogen was first reported in 1946 by Bennetts et al. They revealed that the cause of clover disease, which was named for the high increase (over 30%) of infertility of the sheep fed with red clover (Trifolium subterraneum var. Dwalganup),
was an estrogen-like isoflavonoid contained in the plant, hence, the compound obtained from the plant has been named "phytoestrogen".

[11] As compounds known as phytoestrogens, mention may be made of isoflavone compounds such as daidzein and genistein, coumestan compounds such as coumestrol, lignan compounds such as enterolactone, and phenol compounds such as enterodiol.

[12] In general, phytoestrogens function similarly to the animal estrogens. That is, the phytoestrogens inhibit growth of breast cancer cells by binding to the estrogen receptor and have been used as an estrogen substitute in the treatment of cardiovascular diseases and other symptoms occurring in the postmenopausal women.

[13] Continued estrogen deficiency leads to increased incidence of cardiovascular diseases and osteoporosis in postmenopausal women. With the increase of in the postmenopausal women population, postmenopausal osteoporosis poses a variety of medical and socio-economical problems. Hormone replacement therapies have been practiced to prevent and treat cardiovascular diseases and osteoporosis in postmenopausal women, and it is well known that benefits obtained by prolonged hormone replacement therapies are much more greater than the risk due to associated with such therapies. However, resumption of menses that may occur upon implementing hormone replacement therapies and fear associated with possibly an increase in the risk of breast cancer upon long-term administration cause many women to be reluctant to receive this treatment or give it up early.

[14] For such reasons, there has been continued research to seek a promising estrogen that maintains the effects of estrogen on skeletons skeletal and cardiovascular systems while without affecting uterus and breast tissue and breast tissues. Clues for feasibility as such an ideal estrogen could be acquired from anti-estrogens that have been used to treat breast cancer. Anti-estrogens exhibit tissue-dependent different effects depending upon kinds of tissues. It was found that they block the action of estrogen on breast cancer cells, but have functions as a complete or partial estrogen agonist in other tissues. Estrogen agonists and antagonists exhibit their effects by binding to estrogen receptors, and thereby these substances are called selective estrogen receptor modulators (SERMs).

[15] The SERMs display tissue specificity that has different functions depending upon genes or cell types. The SERMs are known to play an active role by binding to the estrogen receptors and then affecting transcriptional processes, rather than by simply binding to the estrogen receptors in competition with estrogen thereby to inhibit the action of estrogen.

[16] Various actions of SERMs are determined by various kinds of SERMs, estrogen receptors (ERα and ERβ), hormone-responsive gene regulatory region (ERE, estrogen responsive element), various transcription factors and regulatory proteins. Estrogen
receptor (ER) functions as a transcription factor which is activated by a ligand. When SERMs bind to the estrogen receptors, inducing to result in conformational changes and subsequent activation of the receptors, the estrogen receptor-SERM conjugate binds to the gene of interest to initiate transcription. It is known that the mode of transcription varies depending upon types of cells and gene promoters.

When estrogen is deficient in postmenopausal women, a bone turnover rate increases and bone absorption exceeds bone formation, thereby decreasing bone mass. Upon administration of SERMs to postmenopausal women, bone density in the spine increases till for one year and then is maintained at the increased state level. Similar effects are also observed in hip joints. In contrast with postmenopausal women, when SERMs are administered to premenopausal women, decreases of bone density in the radii, spines and hip joints are observed. Therefore, SERMs have estrogen-like action on bones when blood estrogen level is low, while it they appears to function as an antagonist when blood estrogen level is high.

Meanwhile, it is known that estrogen, and both tamoxifen and raloxifene as SERMs, have effects of lowering cholesterol level. Administration of raloxifene to postmenopausal women results in significant decrease of total cholesterol and LDL cholesterol concentrations, but has no effects on concentrations of HDL cholesterol and triglyceride. In breast cancer patients who have received prolonged administration of tamoxifen, an about 50% reduction in cardiovascular diseases was observed.

Tamoxifen and raloxifene were developed as anti-estrogens for treating breast cancer. In the uterus, tamoxifen acts on the estrogen receptor as a partial agonist, while raloxifene acts as the antagonist.

In addition, recent research and study has reported that phytoestrogens like genistein affect increase of bone formation due to increased expression of estrogen receptors (ERα and ERβ)(Heim M. et al., 2003; Bonnellye et al., 2003).

**Disclosure of Invention**

**Technical Problem**

Therefore, the present invention has been made in view of the above problems, and it is an object of the present invention to provide a novel use of the phytoestrogen kaempferol, and a therapeutic agent for osteoporosis, comprising kaempferol having excellent cell proliferation promotion effects of osteoblasts, which is represented by a general formula (1), as an active ingredient.

**Technical Solution**

In accordance with the present invention, the above and other objects can be accomplished by the provision of a novel use of the phytoestrogen kaempferol that can be usefully used as a therapeutic agent for osteoblasts.
[23] Kaempferol of the above-general formula (1) is a polyphenol having one or more benzene rings containing more than one hydroxyl groups (-OH), in particular belonging to a flavonoid family. Structural characteristics of flavonoids consist in a structure of a flavonoid core in which two benzene rings are conjugated in the form of a heterocycle. Such kaempferol belonging to the flavonoid family has a molecular formula of C_{15}H_{10}O_{6} and a molecular weight of 286.2, and falls within the scope of phytoestrogens due to structural characteristics thereof and exhibits substitute effects for female estrogens against climacteric conditions. In addition, since kaempferol is known to have anticancer action, a variety of extensive and intensive study has been made and correlation with cardiovascular diseases has also been investigated. Further, kaempferol exerts anti-oxidative action, and thereby has anti-oxidative effects such as lowering incidence of arteriosclerosis and preventing cardiac diseases.

[24] Kaempferol is biosynthesized in nature as below:

\[ \text{2-oxoglutarate} + \text{dihydrokaempferol} \rightleftharpoons \text{kaempferol} \]

\[ \text{succinate} + \text{CO}_2 + \text{H}_2\text{O} \]

[26] Kaempferol utilized in the present invention may be obtained by a method, for example, described in Sigma-Aldrich, USA Biochemicals and Reagents (2003).

**Brief Description of the Drawings**

[27] The above and other objects, features and other advantages of the present invention will be more clearly understood from the following detailed description taken in conjunction with the accompanying drawings, in which:

[28] Fig. 1 shows the results of a pS2 gene expression assay, which determines pS2 that is expressed upon binding of estrogenic substance to an estrogen receptor. As can be
seen, expression (density) of pS2 mRNA induced by kaempferol was two-fold or more than that by quercetin, known to have therapeutic effects for osteoporosis, and was close to that of 17β-estradiol (E₂), thus suggesting that kaempferol acts as a potent estrogen analog by binding to the estrogen receptor and thereby it can be therapeutically effective for treating osteoporosis in the body.

**Best Mode for Carrying Out the Invention**

[29] EXAMPLES

[30] Now, the present invention will be described in more detail with reference to the following Examples. These examples are provided only for illustrating the present invention and should not be construed as limiting the scope and spirit of the present invention.

[31] Experimental Example 1: Osteoporosis therapeutic effects by estrogenic action of kaempferol

[32] In order to confirm whether kaempferol in accordance with the present invention exhibits estrogen-like effects and thereby can act as a therapeutic agent for osteoporosis, the following experiments were carried out. In order to confirm estrogenic effects of kaempferol, an MCF-7 cell line (Human breast cancer cell), available from KCLB (Korean Cell Line Bank), located at the Cancer Research Institute of the Seoul National University College of Medicine, Seoul, Korea, was used and osteoporosis therapeutic effects by estrogenic action were observed through experiments of cell proliferation effects by E-Screen assay and experiments of estrogen-responsive gene (pS2) expression.

[33] 1-1) Experiments on estrogenic action (osteoporosis therapeutic effects) by E-Screen assay

[34] The E-Screen assay is a method developed to assess the estrogenic effects of test substances. In accordance with this assay, estrogenic effects are evaluated by observing whether cell proliferation effects are exhibited by substances administered to MCF-7 cells. Such effects were confirmed by treating MCF-7 cells with kaempferol and determining the degree of cell proliferation of each cell exhibited after 6 days. As a result, as can be seen from Table 1, administration of kaempferol exhibited dose-responsive cell proliferation effects. In particular, the value of relative proliferative effect (RPE) which observes and compares cell proliferation effects was highest at a concentration of 10⁻⁵ M. In addition, in this test system, when the RPE value is more than 8, it can be said that the substance to be tested has estrogenic action as a partial agonist. Based on such criteria, kaempferol has a value higher than the above-mentioned range and thus it was confirmed that kaempferol may act as an estrogenic substance in the body. Additionally, upon comparing with quercetin, already known to
be effective for treating osteoporosis, it could be seen that kaempferol exerted significantly higher cell proliferation effects than quercetin, thereby having stronger action. From the above-mentioned results, it was confirmed that kaempferol acts as an estrogenic substance in the body and subsequently exhibits estrogen-supplying effects, and thereby can effectively treat osteoporosis.

The relative proliferative effect (RPE) is expressed by the following equation:

\[
RPE = \left[\frac{(S-1)}{(E-1)}\right] \times 100
\]

wherein S represents a proliferation rate of a sample and E represents a proliferation rate of a positive control (E). That is, RPE is the relative value expressed by taking a proliferation rate of \(10^{-10}\) M of \(E\) to be 100.

<table>
<thead>
<tr>
<th>Concentration (molar conc.: M)</th>
<th>Kaempferol</th>
<th>Quercetin</th>
</tr>
</thead>
<tbody>
<tr>
<td>(10^{-7}) M</td>
<td>5.8±2.5</td>
<td>0.9±1.2</td>
</tr>
<tr>
<td>(10^{-6}) M</td>
<td>14.5±2.4</td>
<td>4.1±2.3</td>
</tr>
<tr>
<td>(10^{-5}) M</td>
<td>74.4±1.3</td>
<td>8.5±9.7</td>
</tr>
</tbody>
</table>

**1-2) Experiment on estrogenic action (osteoporosis therapeutic effects) by measurement of estrogen-responsive gene (pS2) expression**

In order to confirm the estrogenic effects of kaempferol at the gene expression level, the pS2 gene expression assay, an assay to quantify expression of pS2, a gene that is expressed when estrogenic substances bind to estrogen receptors, was used to confirm the effects of kaempferol. Activity of this gene is determined by directly observing whether the treated agent acts as estrogen as changes in gene expression occurring when estrogen is introduced into cells from the outside and binds to the estrogen receptor. The pS2 gene expression assay was carried out by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). Kaempferol, and as positive controls, \(E_2\) (17β-estradiol) and quercetin were, respectively, administered to MCF-7 cells, at dose of \(10^{-5}\) M. As can be seen from Fig. 1, expression (density) of pS2 mRNA induced by kaempferol was two-fold or more than that induced by quercetin, known to be therapeutically effective for osteoporosis, and was near to that exhibited by \(E_2\), thus demonstrating that kaempferol has potent estrogenic effects by binding to the estrogen receptor. This results from effects of kaempferol on expression of mRNA of pS2 gene that is expressed upon binding of estrogenic substance to the estrogen receptor. Therefore, it can be seen that kaempferol acts as estrogen via the same estrogen-
regulated pathway as does E$_2$ (17β-estradiol) and thereby exerts estrogenic effects in the body, thus being capable of serving as a therapeutic agent for osteoporosis.

[41] **Experimental Example 2: Osteoporosis therapeutic effects by action of Selective Estrogen Receptor Modulators (SERMs)**

Estrogen receptors ERα and ERβ have different distributions in body tissues depending upon body organs, and selective estrogen receptor modulators (SERMs) selectively bind to each estrogen receptor (ERα and ERβ), thereby exerting different effects depending upon kinds of organs in the body. In addition, such drugs bind to estrogen receptors and thus act as estrogen agonists or antagonists depending upon estrogen concentration in the body.

[43] When estrogen is deficient in postmenopausal women, bone turnover rate increases and bone absorption surpasses bone formation, thereby resulting in decrease of bone mass leading to development of osteoporosis. Upon administration of SERMs such as currently developed raloxifene to postmenopausal women, bone density in the spine increases. Similar effects are also observed in hip joints. Therefore, in order to confirm whether, as the selective estrogen receptor modulator, kaempferol in accordance with the present invention is capable of acting as a therapeutic agent for osteoporosis, the following experiments were carried out.

[44] **2-1) Measurement of selective estrogen receptor binding properties:**

**Competitive binding assay**

In order to examine whether kaempferol acts as a selective estrogen receptor (ER) modulator, affinity of kaempferol for estrogen receptors ERα and ERβ was investigated. The experiment was performed by administering 2,4,6,7-[³H]E$_2$ and kaempferol to recombinant human ERα and ERβ, followed by incubation for 4 hours. As can be seen from Table 2, the results showed that binding capacity of kaempferol to ER increased in a concentration-dependent fashion, based on observation of a relative ratio (%) to a non-agent treated control, and kaempferol was observed to exhibit about 2-fold to 50-fold higher binding capacity, as compared to quercetin, known to be therapeutically effective for osteoporosis. In addition, it could be seen that kaempferol exhibits binding affinity for both ERα and ERβ and thus acts as an estrogen receptor modulator. Additionally, affinity of kaempferol for the estrogen receptors was greater for ERβ than for ERα. Therefore, it was confirmed that, as the estrogen receptor modulator, kaempferol is capable of acting as a therapeutic agent for osteoporosis by the same action mechanism as does the existing raloxifene.

[46] **Table 2**
The binding affinity of kaempferol for ERα and ERβ by competitive binding assay

<table>
<thead>
<tr>
<th>Concentration (molar conc.: M)</th>
<th>Kaempferol</th>
<th>Quercetin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ERα</td>
<td>ERβ</td>
</tr>
<tr>
<td>10⁻⁶ M</td>
<td>105.6</td>
<td>51.7</td>
</tr>
<tr>
<td>5x10⁻⁶ M</td>
<td>97.9</td>
<td>31.7</td>
</tr>
<tr>
<td>10⁻³ M</td>
<td>94.2</td>
<td>15.6</td>
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<tr>
<td>5x10⁻⁵ M</td>
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<td>2.1</td>
</tr>
<tr>
<td>10⁻⁴ M</td>
<td>31.9</td>
<td>1.9</td>
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[47] **Experimental Example 3: Osteoporosis therapeutic effects by osteoblast proliferation and differentiation**

In order to confirm whether kaempferol in accordance with the present invention has effects on cell proliferation of osteoblasts, Saos-2 cells, a human osteoblast-like cell line, available from KCLB (Korean Cell Line Bank), located at the Cancer Research Institute of the Seoul National University College of Medicine, Seoul, Korea, was used. Proliferation effects of osteoblasts were observed using, as an agent for comparison, genistein that is a kind of phytoestrogen and is known to have therapeutic action upon osteoporosis. Measurement of osteoblast proliferation was performed by a WST-1 assay that determines the degree to which tetrazolium salt substrate is cleaved to soluble formazan dye by enzymatic activation of a mitochondrial "succinate-tetrazolium reductase" system, which exists in the mitochondrial respiratory chain and is active only in metabolically intact cells, and a method that determines changes in activity of alkaline phosphatase (ALP) which increases during cellular differentiation processes. In addition, phytoestrogens genistein and quercetin were employed as comparative agents as they have been intensively studied as therapeutic agents for osteoporosis.

[49] **3-1) Cell proliferation experiment depending upon concentrations of the agents: WST-1 Assay**

For comparison of cell proliferation rate (%), using Saos-2 cell line which has similar properties to osteoblasts, the degree to which tetrazolium salt is cleaved by a mitochondrial "succinate-tetrazolium reductase" system in metabolically active cells was determined. Cell proliferation rate exerted by the agents was calculated as percentage of the OD of the agents administered group to the OD of a control group to which the agents were not administered. As can be seen from Table 3, the osteoblast proliferation effects by administration of kaempferol showed the maximum cell pro-
Proliferation rates of 101.1% at a concentration of 10^{-9} M, and thus were slightly higher than quercetin and slightly lower than genistein, known to be therapeutically effective for osteoporosis.

Table 3
Proliferation effects of kaempferol on Saos-2 cells using WST-1 assay

<table>
<thead>
<tr>
<th>Concentration (molar conc.: M)</th>
<th>Kaempferol (%)</th>
<th>Quercetin (%)</th>
<th>Genistein (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^{-9} M</td>
<td>101.1±10.3</td>
<td>98.1±0.1</td>
<td>105.0±11.7</td>
</tr>
<tr>
<td>10^{-8} M</td>
<td>99.6±10.9</td>
<td>93.9±0.8</td>
<td>106.2±12.2</td>
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<tr>
<td>10^{-7} M</td>
<td>97.6±10.0</td>
<td>98.6±1.0</td>
<td>104.4±13.7</td>
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</table>

3-2) Analysis of Alkaline Phosphatase (ALP) Activity

Osteoblasts exhibit specific alkaline phosphatase (ALP) activity upon differentiation thereof. In order to examine effects of kaempferol on osteoblasts, using hydrolysis of p-nitrophenylphosphate to p-nitrophenol and phosphate by ALP, the ALP activity was measured as the ratio of the OD at 405 nm of the groups to which respective substances were added relative to the OD of a control group at 405 nm.

As shown in Table 4, administration of kaempferol at a concentration of 10^{-8} M increased the ALP activity of Saos-2 cells up to 151.1% and exhibited higher ALP activity as compared to administration of quercetin or genistein. As a result, it was determined that kaempferol had therapeutic effects upon osteoporosis as evidenced by promotion of osteoblast differentiation and possessed stronger effects than quercetin or genistein.

Table 4
Differentiation effects of kaempferol on Saos-2 cells using ALP activity assay

<table>
<thead>
<tr>
<th>Concentration (molar conc.: M)</th>
<th>Kaempferol (%)</th>
<th>Quercetin (%)</th>
<th>Genistein (%)</th>
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<tbody>
<tr>
<td>10^{-9} M</td>
<td>138.2±10.5</td>
<td>98.1±3.4</td>
<td>117.7±6.8</td>
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<tr>
<td>10^{-8} M</td>
<td>151.1±10.0</td>
<td>104.4±3.9</td>
<td>123.6±8.1</td>
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<tr>
<td>10^{-7} M</td>
<td>142.0±19.9</td>
<td>101.2±3.1</td>
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<tr>
<td>10^{-6} M</td>
<td>127.4±9.3</td>
<td>127.2±3.5</td>
<td>121.9±7.1</td>
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In light of the experimental results as given above, it could be seen that kaempferol in accordance with the present invention exhibits remarkably superior therapeutic effects for treatment of osteoporosis, as compared to quercetin.

The kaempferol in accordance with the present invention may be mixed with phar-
maceutically acceptable carriers to prepare oral formulations such as tablets and capsules using conventional methods well known in the art. In order to obtain osteoporosis therapeutic effects, kaempferol is preferably administered in an amount of 40 mg to 200 mg, 2-3 times a day, that is, 80 mg to 600 mg a day.

**Industrial Applicability**

[58] The present invention relates to a novel use of the phytoestrogen kaempferol. Kaempferol in accordance with the present invention can be usefully employed as a therapeutic agent for treatment of osteoblasts.

[59] Although the preferred embodiments of the present invention have been disclosed for illustrative purposes, those skilled in the art will appreciate that various modifications, additions and substitutions are possible, without departing from the scope and spirit of the invention as disclosed in the accompanying claims.
Claims

[1] A therapeutic agent for osteoporosis, comprising kaempferol as an active ingredient.

[2] The therapeutic agent according to claim 1, wherein kaempferol is administered in an amount of 80 mg to 600 mg a day as an oral preparation.
Control  E₂  Quercetin  Kaempferol
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 A61K 31/352, A61P 19/10

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC as above

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean patents and applications for inventions since 1975

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKIPASS, Pubmed [kaempferol AND osteoporosis]

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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Further documents are listed in the continuation of Box C.

See patent family annex.

Date of the actual completion of the international search
02 MAY 2005 (02.05.2005)

Date of mailing of the international search report
09 MAY 2005 (09.05.2005)

Name and mailing address of the ISA/KR

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Form PCT/ISA/210 (second sheet) (January 2004)
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