DESCRIPTION

EXTERNAL PREPARATION FOR SKIN CONTAINING FLAVANONE DERIVATIVE

Technical Field

[0001] The present invention relates to an external preparation for skin, and more specifically, to an external preparation for skin containing a flavanone derivative. The present invention also relates to a method of enhancing the collagen production ability of fibroblast. Further, the present invention relates to a method of screening a substance having a wound-healing effect.

Background Art

[0002] The skin is formed of the stratum corneum, epidermis, basal stratum, and dermis. It is said that the structure of the dermis has a large effect on the properties of the "skin" which can be commonly recognized. It is also said that one of the important constituents of the dermis is collagens that have a large effect on elastic properties and so on. The collagens of the dermis include collagen I, collagen III, collagen IV, collagen V, collagen VII, and the like and have their own important functions. These collagens are derived from procollagen produced by fibroblasts (see, for example, Non-patent Document 1, Non-patent Document 2, and Non-patent Document 3). A decrease in collagens (including

procollagen) production ability of fibroblast is also known to be due to aging and damage caused by UV rays and is responsible for deterioration of skin function. In other words, an important factor to recover the skin function is to restore a collagens production ability of fibroblast from the deterioration.

[0003] A person changes in appearance with age and shows aging signs. One of such signs is the formation of wrinkles on the skin, and the major theme of cosmetics is to prevent the formation of such a sign. It has been considered that one of developmental mechanisms of the wrinkle formation may be the collapse of the collagen fiber bundle structure of the dermis or the disorder of such a structure. Sometimes, the collapse or disorder of the collagen fiber bundle structure of the dermis leads to a partial lack of the dermis collagen fiber bundle itself. The skin portion in this condition may have a structure similar to that of wounded skin and is popularly termed a "large wrinkle". It has been known that terpene such as monoterpene or triterpene, or a derivative thereof is used for restoring the dermis collagen fiber bundle to improve the collapse or disorder of the collagen fiber bundle structure of the dermis (see, for example, Patent Document 1). In addition, it has been also known that some of plant extracts have the remodeling action on the dermis collagen fiber bundle (see, for example, Patent Document 2 and Patent Document 3). Among the plant extracts, a Hypericum extract has been known to be excellent in remodeling action of the

dermis collagen fiber bundle (see, for example, Patent Document 4). These terpenes and extracts have an action to enhance the collagen production ability of fibroblast and, if the collagen fiber bundle is located around, they can build a collagen fiber bundle along such a fiber bundle. However, these substances cannot construct a collagen fiber bundle sufficiently in the case where the dermis collagen fiber bundle itself is lacked and absent around the fibroblasts. Therefore, the above-mentioned terpenes and extracts cannot promote sufficient remodeling of a collagen fiber bundle on a portion lacking collagen fiber bundle, such as a large wrinkle. Therefore, there is a problem in that a sufficient improvement effect on the above-mentioned symptom cannot be exerted.

[0004] In addition, the skin is an important tissue for protecting a living body as well as a respiratory organ, so any defect of the skin will threaten both the defense mechanism and the respiratory function of the living body. Thus, there is an established theory that the loss of one-third of the epidermis by suffering burns or the like leads to a danger of life. In this context, the development of a technology for quickly regenerating a defected skin portion has been demanded for many years. Substances with wound-healing action which have been known in the art include, for example, a Curcuma extract and lactoferrin (see, for example, Patent Document 5 and Patent Document 6). However, any of these substances has an insufficient effect of regenerating the dermal tissue because

of no remodeling action on the collagen fiber bundle structure of the dermis. In such a circumstance, as substances for wound healing to promote the growth of cells and enhance the collagen productivities thereof, inulins (see, for example, Patent Document 7), collagen hydrolysates (see, for example, Patent Document 8), lactoferrins (see, for example, Patent Document 6), and so on have been developed. The wound-healing action of these substances have been confirmed by means of the regeneration activity of the dermal tissue or the RNA expression of enzymes or the like involved in the proliferative activities of fibroblasts. However, any phenomenon evidently leading to actual skin regeneration by these substances is not observed at all. Further, as for these substances, any influence on the reconstruction of a defective portion of the living body, such a lack of a collagen fiber bundle structure, caused by wound has not been observed at all.

[0005] In this way, any substance having an excellent effect in tissue regeneration, wrinkle improvement, wound healing and so on in the living body tissue with a defective portion due to the lack of the collagen fiber bundle structure or the like has not been found at all.

[0006] On the other hand, farrerol is a compound having a flavanone structure and known to be included in various plants, and also the synthetic method thereof is already known (see, for example, Patent Document 9, Patent Document 10, and Patent Document

11). However, any technology for incorporating it into an external preparation for skin has not been known at all. Besides, any remodeling action on the dermis collagen fiber bundle and any wound-healing promoting effect have not been known at all.

[0007] Further, the co-cultivation study has revealed that the action of keratinocytes may influence an improvement in collagen production ability of fibroblast (see, for example, Non-patent Document 4). However, any substance influencing keratinocytes to enhance the collagen production ability of fibroblast has not been known.

[0008] On the other hand, with reference to the collagen production ability of fibroblast, any of the above-mentioned substances for wound healing such as inulins, collagen hydrolysates, and lactoferrins has been screened as one for enhancing the collagen production ability of fibroblast. However, an improvement in collagen production ability of fibroblast is a necessary but not a sufficient condition for promoting wound healing. The present situation is that there is a substance without any effect of promoting wound healing even having an ability of improving collagen productivity.

[0009] In addition, a screening method for an effect of promoting wound healing has been also conceived with reference to the migration activity of fibroblasts (see, for example, Non-patent Document 5). In this method, a collagen gel is prepared using

fibroblasts and a defective portion is then formed therein, followed by counting the fibroblasts migrating into the defective portion. However, an improvement in migration ability of fibroblasts is a necessary but not a sufficient condition for promoting wound healing. Therefore, there is an ingredient without any effect of promoting wound healing even if it is screened with respect to the migration ability of fibroblasts.

[0010] In other words, these screening methods could not screen a substance having both an effect of improving the collagen production ability of fibroblast and an effect of improving the migration ability of fibroblasts, which are required for promoting wound healing. Under such a circumstance, the development of a technology for obtaining a substance having an excellent wound-healing promoting effect has been demanded.

[0011] [Patent Document 1] JP 2002-104921 A

[Patent Document 2] JP 2002-29988 A

[Patent Document 3] JP 2002-29987 A

[Patent Document 4] JP 2002-29986 A

[Patent Document 5] JP 11-199461 A

[Patent Document 6] JP 07-196529 A

[Patent Document 7] JP 2004-501176 A

[Patent Document 8] JP 2003-137807 A

[Patent Document 9] WO 02/092110

[Patent Document 10] EP 122053 A

[Patent Document 11] JP 58-21678 A

[Non-patent Document 1] Keene et al., J. Cell Biol., 104, 611-621 (1987)

[Non-patent Document 2] Shimizu et al., Lab. Invest., Jun., 76(6), 753-63 (1997)

[Non-patent Document 3] Vazquez F. et al, Maturitas, 25, 209-15 (1996)

[Non-patent Document 4] Eming S.A. et al., Hum. Gene Ther., 9(4), 529-39 (1998)

[Non-patent Document 5] Roman O'Leary, Mark Rerek, and Edward John Wood, Biol. Pharm. Bull., 27(2), 266-270 (2004)

Disclosure of the Invention

[0012] The present invention intends to provide an external preparation for skin having an excellent remodeling action of disordered collagen fiber bundle structure of the dermis (hereinafter, simply also referred to as a "collagen fiber bundle"). In particular, the present invention intends to provide a technology for promoting wound healing on a skin defect area typified by a large wrinkle or wound by enhancing a tissue regeneration ability of a living body which is typified by a collagen production ability of fibroblast or the like in the dermis.

Further, the present invention intends to provide a technology for regenerating the dermal tissue. Specifically, the present

invention intends to provide a technology for enhancing a collagen production ability of fibroblast by acting on keratinocytes.

Further, the present invention intends to provide a method of screening a substance having an excellent effect on promoting wound healing.

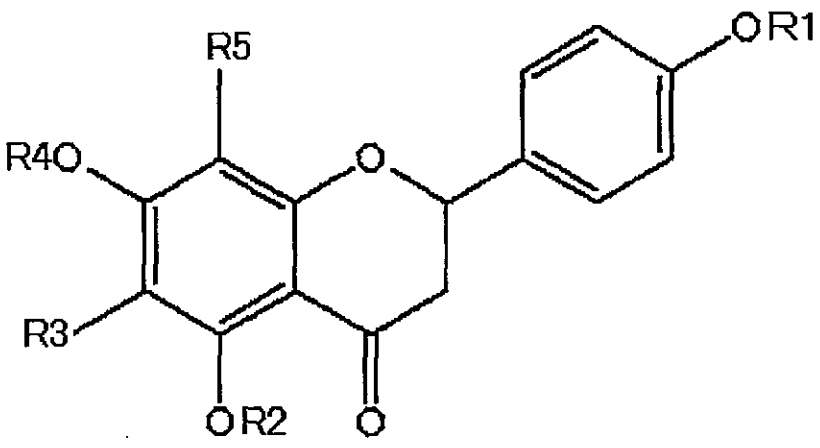
[0013] The present inventors have searched a substance that can be contained in an external preparation for skin, having effects of enhancing a collagen production ability of fibroblast and promoting wound healing, and finally found out that flavanone derivatives such as farrerol can be excellent in such effects. Besides, the present inventors have also found that the flavanone derivatives can effect on keratinocytes to enhance a collagen production ability of fibroblast. Further, the present inventors have completed the present invention by finding out that cosmetics containing flavanone derivatives have excellent effects of improving wrinkles and dermal medicines for external application containing flavanone derivatives have excellent effects of promoting wound healing.

[0014] In addition, the present inventors have completed the present invention by finding out that a substance having an effect on promoting wound healing can be efficiently screened by testing the remodeling action of collagen fiber bundle with a method using a skin wound model in the process of searching a substance having a wound-healing promoting activity.

That is, the present invention is as follows.

[0015] [1] A first invention relates to an external preparation for skin (hereinafter, also referred to as "an external preparation for skin of the present invention"), containing a flavanone derivative represented by the following general formula (1) and/or a salt thereof.

[0016]

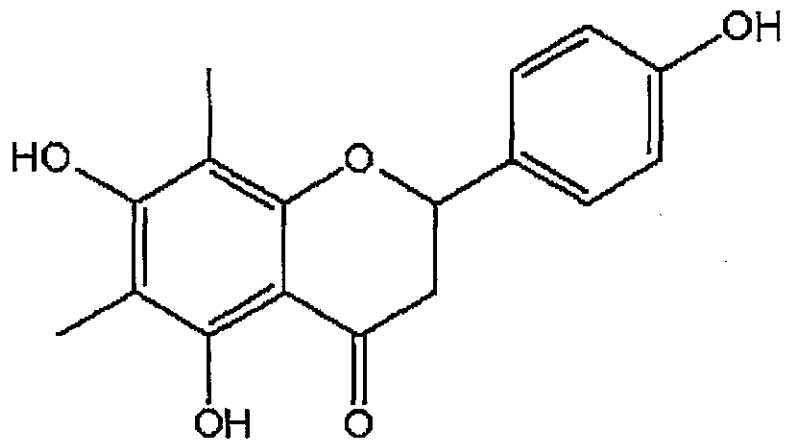


general formula (1)

[0017] In the general formula (1), R1, R2, R3, R4, and R5 each independently represent a hydrogen atom or an alkyl group with 1 to 4 carbon atoms.

The flavanone derivative is preferably farrerol.

[0018]



farrerol

[0019] Further, in a preferred embodiment, the external preparation for skin of the present invention contains an extract of *Hypericum erectum*, *Guttiferae* containing 10^{-6} to 0.1% by mass of the flavanone derivative and/or the salt thereof.

[0020] In addition, the external preparation for skin of the present invention can be used for normalizing a skin structure. In particular, it is preferably used for remodeling a disordered dermis collagen fiber bundle. Further, it is preferably used for promoting the regeneration of a skin defect area and the occlusion of the defect area with a regenerated tissue and curing a wound. Besides, it is also preferably used for enhancing the activity of keratinocytes to increase the collagen production ability of fibroblast.

[0021] [2] A second invention is the use of a flavanone

derivative represented by the general formula (1) and/or a salt thereof in the production of an external preparation for skin.

[0022] [3] A third invention relates to a method of normalizing a skin structure, including administrating to the skin an external preparation for skin containing a flavanone derivative represented by the general formula (1) and/or a salt thereof.

In the above-mentioned second and third inventions, preferable aspects of the flavanone derivative and preferable use of the external preparation for skin are the same as those of the first invention.

[0023] [4] A fourth invention relates to a method of enhancing an effect of keratinocytes to increase a collagen production ability of fibroblast, including administering a flavanone derivative represented by the general formula (1) and/or a salt thereof under a coexistence of keratinocytes with fibroblasts.

A preferable aspect of the flavanone derivative is the same as that of the first invention.

[0024] The administration may be transdermal administration. Specifically, the flavanone derivative and/or a salt thereof is preferably contained in an external preparation for skin and then applied to the skin.

[0025] [5] A fifth invention relates to a method of screening a substance having a wound-healing effect, including: culturing normal animal fibroblasts in the presence of a test substance; preparing a collagen gel by adding a collagen medium solution to

an obtained culture; culturing the fibroblasts in the collagen gel in the presence of the test substance; forming a defect area in the collagen gel; culturing the fibroblasts under a coexistence of the collagen gel having the defect area with the test substance; and observing the degree of filling or reduction of the defect area during the culture (hereinafter, also referred to as a "screening method of the present invention").

[0026] Further, in the screening method of the present invention, a test substance having a wound-healing promoting effect can be screened by making a comparison between the degree of filling or reduction of the defect area in the presence of the test substance and the degree of filling or reduction of the defect area in the absence of the test substance.

Specifically, when the degree of filling or reduction of the defect area in the presence of the test substance is larger than the degree of filling or reduction of the defect area in the absence of the test substance, it can be determined that the test substance has a wound-healing promoting effect.

Brief Description of the Drawings

[0027] FIG. 1 is a diagram illustrating the results obtained in Example 1, where graph 1 represents the relation between the number of incubation days and the number of cells in the absence of heparin treatment and graph 2 represents the relation between

the number of incubation days and the number of cells in the presence of 0.01%-heparin treatment.

FIG. 2 is a photographic representation of the behavior of fibroblasts observed in a wound-healing model of Example 2.

FIG. 3 is a photographic representation of the behavior of constructing a collagen fiber bundle observed in Example 3.

FIG. 4 is a photographic representation of the state of a collagen-gel-defect area observed in Example 6.

FIG. 5 is a diagram illustrating the results obtained in Experiment 1 of Example 9, where OD values (450 nm) are represented in response to the addition of the respective concentrations of a farrerol solution.

FIG. 6 is a diagram illustrating the results obtained in Experiment 2 of Example 9, where the growth rates of keratinocytes are represented in response to the addition of the respective concentrations of a farrerol solution.

FIG. 7 is a photographic representation of the shape of keratinocytes observed in Experiment 2 of Example 9.

FIG. 8 is a diagram illustrating the results obtained in Experiment 3 of Example 9, where the amount of procollagen produced is represented in response to the addition of the respective concentrations of a farrerol solution.

FIG. 9 is a photographic representation of the state of a collagen-gel-defect area observed in Example 11.

Best Mode for Carrying out the Invention

[0028] (A) Flavanone derivative and salt thereof used in the present invention

The flavanone derivative which can be used in the present invention is any of compounds represented by the general formula (1).

In the general formula (1), R1, R2, R3, R4, and R5 each independently represent a hydrogen atom or an alkyl group with 1 to 4 carbon atoms.

At least one of R1, R2, and R4 is preferably a hydrogen atom, and in particular all of the three are preferably hydrogen atoms. Examples of the alkyl group include a methyl group, an ethyl group, a propyl group, and a butyl group. Of those, in particular, the methyl group is preferable.

[0029] Further, at least one of R3 and R5 is preferably an alkyl group with 1 to 4 carbon atoms, and in particular both of them are preferably alkyl groups with 1 to 4 carbon atoms. Examples of the alkyl group include a methyl group, an ethyl group, a propyl group, and a butyl group. Of those, the methyl group is more preferable. In particular, both R3 and R5 are preferably methyl groups.

[0030] Examples of such a flavanone derivative include farrerol, methyl farrerol, and dimethyl farrerol.

[0031] The flavanone derivatives can be synthesized from known

compounds according to JP 58-21678 A. In addition, they are also present in plants, so they can be also isolated and purified from plant extracts. Farrerol, one of the compounds represented by the general formula (1), can be isolated and purified from an extract of plant, *Hypericum erectum,* Guttiferae. An example of the production process thereof will be described below. Needless to say, when the plant-origin flavanone derivative and/or a salt thereof is contained in an external preparation for skin as described later, an extract containing a given concentration of the flavanone derivative and the salt thereof enough to exert a desired effect may be also used.

[0032] <Production Example 1>

10 l of an aqueous solution of 80% ethanol was added to 1 kg of a dried aerial part of *Hypericum erectum Thunberg* (Guttiferae) and then refluxed for 4 hours while stirring. After cooling, an insoluble matter was removed by filtration and then concentrated under reduced pressure, followed by freeze-drying. Consequently, 51 g of amorphous was obtained. This product was dispersed in water, charged on DIAION HP20, washed with the flows of 5 l of water and 5 l of an aqueous solution of 50% ethanol in this order, and eluted with 99% ethanol. The resulting eluate was fractionated. Subsequently, fractions thus obtained are further subjected to thin-layer chromatography and HPLC to checkout the contents thereof to combine the fractions including the same single substance together.

On the other hand, the fractions including two or more ingredients are further purified by subjecting them to the chromatography again. The obtained fraction containing the single ingredient is investigated with respect to the action thereof to remodel the collagen fiber bundle. Then, the structure of the single ingredient in the fraction showing such an action strongly was identified using proton NMR, elemental analysis, mass analysis, and ^{13}C-NMR. As a result, the ingredient was farrerol with a yield of 3.2 mg. In addition, about 0.006% by mass of the farrerol was included in a solvent-eliminated product which was obtained from the material extracted with an aqueous solution of 80% ethanol.

[0033] Preferable examples of the salt of the flavanone derivative include, but not limited thereto so long as they are physiologically acceptable, alkali metal salts such as sodium salt and potassium salt; alkali earth metal salts such as calcium salt and magnesium salt; organic amine salts such as ammonium salt, triethyl amine salt, triethanol amine salt, and monoethanol amine salt; and basic amino acid salts such as lysine salt and arginate.

[0034] The above-mentioned flavanone derivative and the salt thereof have an effect of promoting the ability of fibroblasts to remodel the collagen fiber bundle structure of the dermis. Specifically, the flavanone derivative allows fibroblasts to migrate to anywhere even an area where no collagen fiber is present and simultaneously increase the collagen production ability of

fibroblast itself, thereby remodeling a collagen fiber bundle on the tissue defect area. Further, wound healing can be promoted by promoting the regeneration of the dermal tissue on the skin defect area and enhancing the ability of the regenerated tissue to occlude the defect area.

[0035] (B) External preparation for skin of the present invention

A characteristic feature of the external preparation for skin of the present invention is to contain the flavanone derivative and/or the salt thereof. For example, it may contain the flavanone derivative and/or the salt thereof as a plant extract. The flavanone derivative may be preferably farrerol. Preferably, the external preparation for skin of the present invention may include a *Hypericum erectum* extract containing farrerol.

[0036] As described above, the flavanone derivative and the salt thereof have an effect of remodeling the collagen fiber bundle structure of the dermis. Thus, the external preparation for skin of the present invention can be used for normalizing the skin structure. Here, the phrase "normalizing the skin structure" means to lower the degree of destructing the structures of the epidermis, the dermis, and the subcutis which constitute the skin. Specifically, for example, it includes the remodeling of a disordered collagen fiber bundle structure of the dermis. Here, the phrase "the remodeling of a disordered collagen fiber bundle structure of the dermis" means

to build the collagen fiber bundle structure of the dermis at an area where the dermis collagen fiber bundle structure is disordered. The phrase "the dermis collagen fiber bundle structure is disordered" means that the collagen fiber bundle structure cannot be kept in normal structure. For example, it includes the collagen fiver bundle becomes thin and weak, the amount of the collagen fibers becomes small, the collagen fibers are severed, or the collagen fiber bundle is lacked. Further, the phrase "normalizing the skin structure" also includes the regeneration of skin defect area. It also includes recovering the defect area and promoting the occlusion of the defect area with the regenerated tissue enable to prevent infection from occurring by the defect area and recover the defense mechanism and respiratory function of the skin. Here, the phrase "defect of the skin" means a destroyed state of the skin structure, where, for example, skin roughness, wrinkles, and wound are formed. The external preparation for skin of the present invention is particularly suitable for wound healing. Further, the phrase "normalizing the skin structure" also includes that the activity of keratinocytes to increase the collagen production ability of fibroblast is enhanced.

[0037] The external preparation for skin of the present invention can be applied without any specific limitation as long as it is externally applicable to the skin. Examples of the external preparation include cosmetics containing quasi-drugs, dermal

medicines for external application, and externally applicable dermatological sundries. Of those, it is particularly preferable to be applied to the cosmetics and the dermal medicines for external application. For example, the cosmetics are preferable for improving wrinkles and mild skin roughness. Specifically, it is preferably used for recovering the dermis collagen fiber bundle from disorder due to photo-aging and for regenerating the collagen fiber bundle structure of the dermis being defected with wound or severe optical damage. In contrast, the dermal medicines for external application are preferable for improving severe skin roughness and for wound healing.

[0038] The dosage form of the external preparation for skin of the present invention is not limited in particular and may be any of dosage forms that the conventional external preparations for skin can be taken depending on the intended use. Preferable examples of the dosage form of the cosmetics include the dosage form to be commonly used for basic skin care such as lotion, milky liquid, essence, cream, and pack. In addition, Preferable examples of the dosage form of the external medicines for skin include ointment, lotion, cream, milk, and essence.

[0039] The content of the flavanone derivative and/or the salt thereof in the external preparation for skin of the present invention, which can be adjusted depending on the application, target, and administration method of the external preparation for skin, is

preferably 1×10^{-6}% by mass and more preferably 1×10^{-4}% by mass as a lower limit. The upper limit is preferably 1% by mass and more preferably 0.1% by mass.

Further, particularly in the case of the dermal medicines for external application to be used for wound healing, the content of the flavanone derivative and/or the salt thereof may be preferably 1×10^{-6} M and more preferably 1×10^{-4} M as a lower limit and preferably 1 M and more preferably 0.1 M as an upper limit.

[0040] Further, when a plant extract containing the flavanone derivative and/or the salt thereof is contained in the external preparation for skin for the present invention, it is preferable to use one with an increased concentration of the flavanone derivative and/or the salt thereof by purifying the flavanone derivative and/or the salt. The external preparation for skin of the present invention does not include the external preparation for skin to be used for remodeling of the dermis collagen fiber bundle containing a crude *Hypericum erectum* extract and containing no additional flavanone derivative and/or a salt thereof other than one originally included in the extract. The plant extract which can be used in the external preparation for skin of the present invention may be preferably an extract with an increased concentration of farrerol obtained by purification of a plant extract of *Hypericum erectum*. The concentration of the flavanone derivative and/or the salt thereof in the plant extract is 10^{-6} to 0.1% by mass and more preferably

10^{-5} to 0.01% by mass. In the case of using such a plant extract, the content of the plant extract in the external preparation for skin is preferably 0.001 to 10% by mass and more preferably 0.01 to 1% by mass. Here, the content of the plant extract is synonymous with the content thereof in a solvent-removed product.

[0041] The external preparation for skin of the present invention can contain optional ingredients used commonly in an external preparation for skin as well as those essential ingredients. Preferred examples of such an optional ingredient include: oils/waxes such as macadamia nut oil, avocado oil, corn oil, olive oil, rapeseed oil, sesame oil, castor oil, safflower oil, cottonseed oil, jojoba oil, coconut oil, palm oil, liquid lanolin, cured coconut oil, cured oil, Japan wax, cured castor oil, beeswax, candelilla wax, carnauba wax, ibota wax, lanolin, reduced lanolin, hard lanolin, and jojoba wax; hydrocarbons such as liquid paraffin, squalane, pristane, ozokerite, paraffin, ceresin, vaseline, and microcrystalline wax; higher fatty acids such as oleic acid, isostearic acid, lauric acid, myristic acid, palmitic acid, stearic acid, behenic acid, and undecylenic acid; higher alcohols such as cetyl alcohol, stearyl alcohol, isostearyl alcohol, behenyl alcohol, octyldodecanol, myristyl alcohol, and cetostearyl alcohol; synthetic ester oils such as cetyl isooctanoate, isopropyl myristate, hexyldecyl isostearate, diisopropyl adipate, di-2-ethylhexyl sebacate, cetyl lactate, diisostearyl malate, ethylene glycol

di-2-ethyl hexanoate, neopentylglycol dicaprate, glyceryl di-2-heptylundecanoate, glyceryl tri-2-ethylhexanoate, trimethylolpropane tri-2-ethylhexanoate, trimethylolpropane triisostearate, and pentaerythritol tetra-2-ethylhexonate; silicone oil, such as chain polysiloxanes such as dimethylpolysiloxane, methylphenylpolysiloxane, and diphenylpolysiloxane; cyclic polysiloxanes such as octamethylcyclotetrasiloxane, decamethylcyclopentasiloxane, and dodecamethylcyclohexanesiloxane; modified polysiloxanes such as amino-modified polysiloxane, polyether-modified polysiloxane, alkyl-modified polysiloxane, and fluorine-modified polysiloxane; anionic surfactants such as fatty acid soaps (such as sodium laurate and sodium palmitate), potassium laurylsulfate, and triethanolamine alkylsulfate ether; cationic surfactants such as trimethyl ammonium stearyl chloride, benzalkonium chloride, and laurylamine oxide; amphoteric surfactants such as imidazoline-based amphoteric surfactants (such as a 2-cocoyl-2-imidazolinium hydroxide-1-carboxyethyloxy disodium salt), betaine-based surfactants (such as alkyl betaine, amide betaine, and sulfo betaine), and acylmethyl taurine; nonionic surfactants such as sorbitan fatty acid esters (such as sorbitan monostearate and sorbitan sesquioleate), glycerin fatty acid esters (such as glycerin monostearate), propyleneglycol fatty acid esters (such as propyleneglycol monostearate), cured castor oil derivatives,

glycerol alkyl ether, POE sorbitan fatty acid esters (such as POE sorbitan monooleate and polyoxyethylene sorbitan monostearate), POE sorbitol fatty acid esters (such as POE-sorbitol monolaurate), POE glycerol fatty acid esters (such as POE-glyceryl monoisostearate), POE fatty acid esters (such as polyethyleneglycol monooleate and POE distearate), POE alkyl ethers (such as POE2-octyldodecyl ether), POE alkylphenyl ethers (such as POE nonylphenyl ether), pluronic types, POE/POP alkyl ethers (such as POE/POP2-decyltetradecyl ether), tetronic types, POE castor oil/cured castor oil derivatives (such as POE castor oil and POE cured castor oil), sucrose fatty acid ester, and alkyl glycoside; polyvalent alcohols such as polyethylene glycol, glycerin, 1,3-butylene glycol, erythritol, sorbitol, xylitol, maltitol, propylene glycol, dipropylene glycol, diglycerin, isoprene glycol, 1,2-pentanediol, 2,4-hexanediol, 1,2-hexanediol, and 1,2-octanediol; moisture components such as sodium pyrrolidone carboxylate, lactate, and sodium lactate; fine particles such as mica, talc, kaolin, synthetic mica, calcium carbonate, magnesium carbonate, silicic anhydride (silica), aluminum oxide, and barium sulfate, whose surfaces may be treated; inorganic pigments such as red iron oxide, yellow iron oxide, black iron oxide, cobalt oxide, ultramarine blue, iron blue, titanium oxide, and zinc oxide, whose surfaces may be treated; pearl agents such as mica titanium, fish scale foil, and bismuth oxychloride, whose surfaces may be treated;

organic dyes such as Red No. 202, Red No. 228, Red No. 226, Yellow No. 4, Blue No. 404, Yellow No. 5, Red No. 505, Red No. 230, Red No. 223, Orange No. 201, Red No. 213, Yellow No. 204, Yellow No. 203, Blue No. 1, Green No. 201, Purple No. 201, and Red No. 204, which may be laked; organic fine particles such as polyethylene powder, polymethyl methacrylate, nylon powder, and organopolysiloxane elastomer; p-aminobenzoate-based ultraviolet absorbent; an anthranilate-based ultraviolet absorbent; a salicylate-based ultraviolet absorbent; a cinnamate-based ultraviolet absorbent; a benzophenone-based ultraviolet absorbent; a sugar-based ultraviolet absorbent; ultraviolet absorbents such as 2-(2'-hydroxy-5'-t-octylphenyl)benzotriazole, and 4-methoxy-4'-t-butyldibenzoylmethane; lower alcohols such as ethanol and isopropanol; vitamins such as vitamin A or derivatives thereof; vitamin B types such as vitamin B_6 hydrochloride, vitamin B_6 tripalmitate, vitamin B_6 dioctanoate, vitamin B_2 or derivatives thereof, vitamin B_{12}, and vitamin B_{15} or derivatives thereof; vitamin E types such as α-tocopherol, β-tocopherol, γ-tocopherol, and vitamin E acetate, vitamin D types, vitamin H, pantothenic acid, pantethine, and pyrroloquinoline quinone; and antibacterial agents such as phenoxyethanol.

[0042] Of those, the optional ingredient to be contained in the dermal medicines for external application for promoting wound healing is particularly preferably any of ingredients effective

to the regeneration of the dermal tissue defected with wound. Specifically, optical ingredient may be particularly preferably, an antibiotic substance for preventing microbial infection such as penicillin, fradiomycin, tetracycline, or a salt thereof, a sterilizer such as acrinol or isodine, or the like. Alternatively, a cicatrization depressant such as tranilast is also preferable. The content of any of these ingredients effective to the regeneration of the dermal tissue is preferably 0.1 to 10% by mass.

[0043] The external preparation for skin of the present invention can be produced by processing the flavanone derivative and/or the salt thereof and the optical ingredient by a conventional method.

In addition, the usage of the external preparation for skin of the present invention can be modified depending on the use, dosage form, application site, symptom, and so on.

[0044] (C) Method of enhancing action of keratinocytes to increase collagen production ability of fibroblast

A characteristic feature of the method of enhancing the action of keratinocytes to increase the collagen production ability of fibroblasts of the present invention (hereinafter, also referred to as the "method of the present invention") is to administer the flavanone derivative represented by the general formula (1) and/or a salt thereof in the presence of both keratinocytes and fibroblasts.

The flavanone derivative and/or the salt thereof used in the

method of the present invention follows the above description of (A). The flavanone derivative and/or the salt thereof have an activity to increase a collagen production ability of fibroblast through an effect on keratinocytes.

The meaning of "collagens" conceptually includes both collagen, and procollagen which is precursor of collagen.

[0045] The phrase "in the presence of both keratinocytes and fibroblasts" means a state in which these cells are coexistent with each other in vitro or in vivo. For example, in vitro, such a state can be produced by co-culture of these cells. In contrast, in vivo, such a state can be found in the skin containing the epidermis and the dermis.

The administration of the flavanone derivative and/or the salt thereof can be performed, for example, by allowing the flavanone derivative and/or the salt thereof to be mixed with a culture medium for co-culture of keratinocytes and fibroblasts. In this case, the amount (dosage) of the mixture is preferably 10^{-8} M and more preferably 10^{-7} M as a lower limit and preferably 10^{-3} M and more preferably 10^{-5} M as an upper limit in terms of the concentration thereof in the medium.

Further, the in vivo administration method may be transdermal administration. The method of transdermal administration is not particularly limited to as long as it is generally used, but preferably a method of applying an external preparation for skin containing

the flavanone derivative and/or the salt thereof to the skin. The external preparation for skin which can be used in the method of the present invention is the external preparation for skin of the present invention as described above and a preferred aspect thereof is also formulated as described above.

[0046] (D) Screening method of the present invention

A characteristic feature of the screening method of the present invention is that normal fibroblasts from an animal are cultured in the presence of a test substance, a collagen medium solution is added to the obtained culture to prepare a collagen gel, the fibroblasts in the collagen gel are cultured in the presence of the test substance and a defect area is then formed in the collagen gel, the fibroblasts are cultured such that the collagen gel with the defect area is coexistent with the test substance, and the degree of filling or reduction of the defect area is observed during the culture.

[0047] The normal fibroblast of the animal used in the screening method of the present invention is not particularly limited as long as it is generally used in an assay. Preferable examples of the animal include a mouse, a rat, a rabbit, and a human. Of those, the human is particularly preferable because of slight differences among the animal species. The normal fibroblasts are noncancerous cells having ability to form organized collagen gel. Such normal fibroblasts can be obtained from the intraoral mucosa, the skin,

or the like or the primary culture thereof may be prepared and used. Alternatively, an established culture cell line is commercially available and such a commercial product may be used. The commercial product is preferably, for example, one obtained as "Normal Human Skin Fibroblast (Primary Culture)" manufactured by Sanko Junyaku Co., Ltd., and "Normal Human Fibroblast" manufactured by Kurabo Co., Ltd.

Note that, hereinafter, the normal fibroblasts of these animals are also simply referred to as fibroblasts.

[0048] It is preferable that fibroblasts are precultured in advance to an appropriate number of the cells and then subjected to a subsequent culture. The preculture may be performed under the general culture conditions for culture cells, for example, where a culture medium such as DMEM (manufactured by GIBCO) added with 10% FBS, is used and the cells are then incubated at 37°C under the flow of 5% carbon dioxide gas until the cells grow to 60 to 90% confluency. The cell cluster obtained by the preculture is preferably broken into individual cells in the presence of 0.01 to 0.1% of trypsin, preparing a cell suspension. In this case, the presence of ethylenediaminetetraacetate with trypsin is more preferable.

[0049] For the culture of fibroblasts in the presence of a test substance, a method of adding the test substance is not particularly limited. The test substance may be previously added to the medium,

added simultaneously with seeding of cells, or added after the cells being seeded and then cultured for an appropriate time. Preferably, the method may be one in which cells are seeded on a plate at first and then cultured in a medium free of a test substance, followed by replacing the medium with a flesh medium containing the test substance. The amount of the test substance added may be of any concentration. Preferably, the culture is performed using media containing various concentrations of the test substance for testing an effective amount of the test substance. In addition, the number of cells of fibroblasts used in the culture is not particularly limited and may be adjusted to the size of a culture plate. For example, the number of the cells may be preferably adjusted to $10^3/cm^2$ to $10^5/cm^2$. The culture may be performed under normal conditions in a manner similar to the preculture. The culture time is not particularly limited as long as the number of cells can be increased to an appropriate one, so it may be, for example, about 3 to 5 days as a standard. During the period, the medium may be replaced with a flesh medium if required.

Specifically, for example, the suspension of cells obtained by breaking the precultured fibroblasts into the individual cells is seeded on plates and then cultured for about 24 hours, followed by addition of a solution of the test substance at various concentrations. The culture is preferably performed for 3 to 5 days.

[0050] Subsequently, a collagen gel is prepared by adding a

collagen medium solution to the fibroblast culture obtained as described above. The amount of the collagen medium solution added and the concentration of collagen in the collagen medium solution are not particularly limited as long as they are within the range allowable to the dispersion of fibroblasts in the collagen gel. The collagen medium solution used may be, for example, one prepared by mixing 0.3 to 0.8% by mass of a type-I collagen solution, a DMEM medium, 100 to 300 mM of HEPES, 2 to 4% by mass of a sodium hydrogen carbonate solution, an aqueous solution of 0.05 to 0.15 N sodium hydroxide, FBS, and water at a ratio of about 4 : 4 : 2 : 2 : 1 : 2 : 3. The amount of the collagen medium solution added can be defined so that, for example, the ratio of the collagen medium solution to the fibroblast culture will be 8 to 10 parts by mass to 1 to 2 parts by mass.

[0051] Next, the fibroblasts in the prepared collagen gel are cultured in the presence of the test substance. In other words, the collagen gel prepared as described above is placed in a medium containing the test substance, thereby performing the suspension culture of the fibroblasts in the collagen gel.

The concentration of the test substance in the medium to be used in the culture may correspond to the concentration of the medium used in the culture of fibroblasts in the presence of the test substance. The culture time can be controlled depending on the reduction state of the collagen gel. Specifically, the culture is

preferably performed until about 1/5 to 1/10 reduction of the gel size is attained with reference to the size thereof at a time of preparing the gel with addition of the collagen solution. The culture time may be 3 to 5 days as a standard.

[0052] Next, the defect area is formed in the reduced collagen gel. The defect area is a portion where part of the collagen is pierced and kept in a hollow shape. The shape and size of the defect area is not particularly limited as long as it is suitable for the observation of a change in shape. For example, the center portion of the reduced collagen gel can be punched out in a circular form, providing a donut-shaped gal. The punching procedure may use, for example, a disposable biopsy punch with a diameter of 1 to 5 mm.

A method of preparing such a model of damaged collagen gel is disclosed in Roman O'Leary, Mark Rerek, and Edward John Wood, Biol. Pharm. Bull., 27(2), 266-270 (2004).

[0053] Next, the collagen gel provided with the defect area is placed in the presence of a test substance and fibroblasts in the collagen gel are then cultured. A plate used for the culture may be selected depending on the size of the prepared collagen gel. For example, if the collagen gel has a diameter of about 2 to 7 mm, a 6-well plate may be selected. In addition, the collagen gel is preferably fixed on the bottom of the well. The fixation of collagen gel can be performed by, for example, dropping a collagen solution on the center portion of the well and incubating the collagen

gel thereon for 15 minutes at 37°C. The concentration of the test substance in the medium may correspond to one in the medium used in the culture of the above-mentioned fibroblasts in the presence of the test substance. The culture conditions used may be also general conditions just as in the case with the above-mentioned culture conditions. The culture period (i.e., the observation period) is, but not particularly limited to, 5 to 20 days as a standard.

[0054] During the culture as described above, the degree of filling or reduction of the collagen-gel-defect area is observed. By recording the results of the observation, the action of the test substance to remodel the collagen can be determined. For example, the shape of the defect area can be objectively recorded using photographs. Optionally, these photographs can be compared and determined by converting them into digital image data. Specifically, when the degree of filling or reduction of the above-mentioned defect area in the presence of the test substance is larger than one in the absence of the test substance, it can be determined that the above-mentioned test substance has a wound-healing promoting effect. In addition, the degree of filling or reduction can be evaluated such that a score or the like is used to replace the degree of filling or reduction with a numerical value. For example, when the volume of the above-mentioned defect area in the presence of the test substance is 85% or less and preferably 50% or less of one in the absence of the test substance, it can be determined that the

above-mentioned test substance has a wound-healing promoting effect.

As the wound-healing promoting effect of the test substance can be evaluated by such a method, it becomes possible to remove any substance that only promotes the migration of fibroblasts and does not promote the collagen production of the fibroblasts or any substance that only promote the collagen production of fibroblasts and does not promote the migration (movement) of fibroblasts. Therefore, any substance effective to wound-healing can be more efficiently screened.

Example 1

[0055] The remodeling action of farrerol on a dermis collagen fiber bundle was confirmed by an in vitro experiment. In other words, utilizing the fact that, when fibroblasts were cultured in a collagen gel in a heparin supplemented medium, collagen fibers produced from cells were damaged and a strong collagen fiber bundle cannot be formed, the degree of a reduction in damage of the collagen fiber was investigated by allowing farrerol to coexist with the heparin supplemented system. The procedures will be described below.

[0056] <Cells and reagents used>

- Normal human fibroblasts (Kurabo)
- DMEM with 10% FBS (GIBCO)
- WST-8/1-Methyl PMS (Cell Counting Kit-8: DOJINDO LABORATORIES)

Farrerol was dissolved in dimethylsulfoxide (DMSO) and used.

[0057] The toxicity of farrerol was examined by the MTT assay and the dose of farrerol added to the heparin supplemented system was then obtained.

<Protocol>

1. Fibroblasts grown to 80% confluency were dispersed with 0.05% Trypsin/EDTA. The dispersant was seeded on 96 wells/plate at a concentration of 3×10^3 cells/well.

2. After 24 hours, farrerol with adjusted concentration was added to the respective wells in a volume of 10 ml/well (n = 8).

3. WST-8/1-Methyl PMS was added to the respective wells in a volume of 10 ml/well after 24 hours, 48 hours, and 72 hours from the addition of farrerol and then incubated at 37°C for 4 hours.

4. Absorbance was measured at a measurement wavelength of 450 nm and a reference wavelength of 650 nm.

<Results>

Significant cytotoxicity could not be confirmed at any of the concentrations being considered. In contrast, it was confirmed that the addition of farrerol could promote the cell proliferation. In particular, the cell proliferation was promoted when farrerol was at concentrations of 10^{-6} ml/ml-DMEM, 5×10^{-7} ml/ml-DMEM, and 10^{-7} ml/ml-DMEM, so the following collagen production test was carried out using these three concentrations.

[0058] On the basis of the above-mentioned results, the action

of farrerol to promote the production of collagen was examined using a heparin supplemented system.

<Cells and reagents used>

- Normal human fibroblasts (Kurabo)
- HEPARIN (SIGMA: H-3149)
- DMEM (GIBCO)
- FBS

<Protocol>

1. Fibroblasts were grown to confluency in a medium with 10% FBS and then dispersed by trypsin treatment, followed by centrifugation.
2. Supernatant was removed and the fibroblasts were then resuspended in DMEM with 10% FBS, followed by seeding the fibroblasts on a 12-well plate (4.5 cm^2/well) at 3 × 10^3 cells/cm^2.
3. After 4 hours from seeding the cells, the supernatant DMEM was removed and replaced with DMEM with 0.4% FBS, followed by 72-hour incubation under the conditions for preventing cell proliferation (37°C, 5% CO_2).
4. DMEM with 0.4% FBS was removed and then replaced with DMEM with 10% FBS to release the prevention of cell proliferation. In this procedure, the culture conditions were 10% FBS-DMEM, 0.1% heparin/10% FBS-DMEM, and 1.0% heparin/10% FBS-DMEM (each n = 3) for investigating the influence of heparin. Further, those added with farrerol at concentrations of 10^{-6}ml/ml-DMEM, 5×10^{-7}ml/ml-DMEM, and 10^{-7} ml/ml-DMEM were prepared, respectively.

<Results>

The results were represented in the graphic diagram of FIG. 1. Graph 1 represents a cell proliferation effect when each concentration of the farrerol solution was added to the medium under the heparin nonsupplemented condition and Graph 2 represents one under the heparin supplemented condition. Any of the conditions indicated that the farrerol-added group tended to promote cell proliferation, compared with the control, DMSO-added group. Consequently, it is found that farrerol has an effect to promote the growth of fibroblasts.

Example 2

[0059] The above-mentioned experiment confirmed that farrerol had an effect to promote cell proliferation. In addition, for confirming the influence of farrerol on the movement ability of fibroblasts, a "wound-healing model" prepared by punching out a three-dimensional collagen gel by a disposable biopsy punch was used in a comparison between the farrerol-added group and the farrerol-free group with respect to the behavior of fibroblasts derived from the wound area.

<Cells and reagents used>

· Normal human fibroblasts (Kurabo)

· DMEM with 10% FBS (GIBCO)

· Collagen medium solution (0.5% type-I collagen : 5 × DMEM : 200 mM HEPES : 2.2% $NaHCO_3$: 0.1 N NaOH : FBS : water were mixed

at a ratio of 4 : 4 : 2 : 2 : 1 : 2 : 3)

· Disposable biopsy punch (3.0 mm in tip diameter)

<Protocol>

1) The culture of fibroblasts obtained by Protocol 4 of Example 1 and the collagen medium solution were mixed together at a ratio of 1 : 9 (volume) and then suspension-cultured in a 10%-FBS medium for 1 week.

2) When the size of a collagen gel was reduced from one at the time of being prepared by the addition of the collagen medium solution to a size of about 1/5 to 1/10, the center portion of the gel was punched out by a disposable biopsy punch with a diameter of 3 mm.

3) A 6-well plate was prepared and 15 μl of the collagen solution was then dropped on the center of the well. Subsequently, the punched three-dimensional collagen gel was mounted thereon and incubated for 10 minutes (37°C, 5% CO_2).

4) When the dropped collagen was solidified and the three-dimensional collagen gel was attached to the bottom of the well, 10% FBS-DMEM, 0.1% heparin/10% FBS-DMEM, and 1.0% heparin/10% FBS-DMEM were added in a volume of 3 ml/well.

5) Further, a group where 10^{-6} ml/ml-DMEM of farrerol was added to each culture condition of (4) was prepared.

<Results>

The behavior of fibroblasts after 24 hours from the preparation

of the "wound-healing model" was observed. Photographs of fibroblasts derived from the wound area punched out by the disposable biopsy punch were represented in FIG. 2. With respect to the unsupplemented 10% FBS-DMEM, the tendencies of a longer movement distance of fibroblasts and a more active cellular movement were observed in the farrerol-added group, compared with the farrerol-free group. In contrast, with respect to 0.1% heparin/10% FBS-DMEM and 1.0% heparin/10% FBS-DMEM, there was no difference in cell movement between the farrerol-added group and the farrerol-free group and the movement of each of them was prevented by the action of heparin.

Consequently, the results suggest that farrerol does not inhibit the influence of heparin on the movement ability of fibroblasts but directly act on the fibroblasts to enhance the movement ability of the cells.

Example 3

[0060] The following procedures were employed to investigate whether farrerol had the action of preventing an inhibitory effect of heparin added on the building of a collagen fiber bundle. The results are represented in FIG. 3. It is found that farrerol prevents an inhibitory effect of heparin on the building of a collagen fiber bundle and exerts a remodeling action of the collagen fiber bundle of the dermis.

[0061] <Protocol>

A collagen solution was prepared by mixture of 0.5% type-I collagen : 5 × DMEM : 200 mM HEPES : 2.2% $NaHCO_3$: 0.1 N NaOH : FBS : water at a ratio of 4 : 4 : 2 : 2 : 1 : 2 : 3 (performed while cooling).

↓

A lower-layer collagen solution was prepared by mixing collagen solution with water at a ratio of 9 to 1.

↓

The lower-layer collagen solution was dispensed into the respective wells of a 48-well plate in a volume of 100 µl/well.

↓

Solidification was carried out in a CO_2 incubator (about 15 minutes).

↓

NHDF was recovered according to the routine procedure.

↓

(Cells were separated from one another by a cell strainer).

↓

The number of the cells was counted (Trypan blue) and then adjusted to 1×10^5 cells/ml.

↓

The collagen solution was mixed with the cell suspension at a ratio of 9 to 1 (volume) and then dispensed into the respective wells in a volume of 300 µl/well.

(For preventing the cells from uneven dispersion, the operation

proceeded while intermittently stirring (1×10^4 cells/ml at final).

↓

The cells were left standing for 4 hours in the CO_2 incubator.

↓

The gel was separated from the inner wall of the well by an injection needle not to prevent the gel from reduction.

↓

The addition of 1.8% Heparin/10% FBS-DMEM was performed in a volume of 500 µl/well (1% Heparin/well at final).

↓

Farrerol (dissolved in DMSO) was added in a volume of 0.9 µl/well (1,000-fold dilution).

↓

Incubation was carried out in the CO_2 incubator (5 days).

↓

A sample for SEM observation was obtained.

Example 4

[0062] According to the formulation described below, cosmetics (essences) which are the external preparation for skin of the present invention were prepared. A, B, and C were heated to 80°C, B was gradually added to A while stirring to emulsify, and C was then added for neutralization, followed by stirring under cooling. As a result, the essence 1 was obtained. Similar operation was carried out to prepare Comparative Example 1 in which farrerol of essence

1 was substituted with ethanol.

[0063]

Table 1

Ingredients	% by mass
A	
Squalane	5
POE (20) behenyl ether	1
Behenyl alcohol	2
Sorbitan sesquistearate	1.5
Behenic acid	0.5
B	
1,3-butanediol	8
1,2-hexanediol	4
Phenoxyethanol	0.5
"Carbopol 1382" (Alkyl-modified carboxyvinyl polymer: manufactured by Goodrich Corporation)	0.1
"Pemulen TR-1" (Alkyl-modified carboxyvinyl polymer: manufactured by Goodrich Corporation)	0.1
Water	57.1
0.1% farrerol solution in ethanol	0.1
C	
Potassium hydroxide	0.1
Water	20
Total	100

[0064] <Test Example 1>

Using two groups of 5 individuals, 10 panelists (40 to 50 year old) in total, essence 1 prepared as described above and cosmetics of Comparative Example 1 were subjected to an application test for 8 weeks. These cosmetics were used twice every morning and night on consecutive days and the replicas of the outer corners of the eyes were obtained before and after the application test. Under light irradiation at an oblique angle of 45°, the shadows formed by wrinkles were taken in pictures from a microscope and then binarized

to obtain the area ratios of the shadows in the visual field, followed by obtaining the average value thereof in the group. The results are listed in Table 2. Consequently, the cosmetics (essences) which are the external preparation for skin of the present invention are found to have an excellent improvement effect on wrinkles.

[0065]

Sample	Average shadowed area ratio (%)	
	Before test	After test
Essence 1	6.8±2.3	3.3±0.9
Comparative Example 1	7.1±2.8	5.7±1.6

Example 5

[0066] <Test Example 2>

Essence 2 which is the external preparation for skin of the present invention was prepared according to the following formulation in a manner similar to essence 1. It was evaluated by the method described in Test Example 1. Consequently, an effect was confirmed such that the wrinkle area ratio was changed from 8.2 ± 3.2% before the test to 3.9 ± 1.1% after the test.

[0067]

Table 3

Ingredients	% by mass
A	
Squalane	5
POE (20) behenyl ether	1
Behenyl alcohol	2
Sorbitan sesquistearate	1.5
Behenic acid	0.5
B	
1,3-butanediol	8
1,2-hexanediol	4
Phenoxyethanol	0.5
"Carbopol 1382" (Alkyl-modified carboxyvinyl polymer: manufactured by Goodrich Corporation)	0.1
"Pemulen TR-1" (Alkyl-modified carboxyvinyl polymer: manufactured by Goodrich Corporation)	0.1
Water	57.1
0.001% farrerol solution in ethanol	0.1
C	
Potassium hydroxide	0.1
Water	20
Total	100

Example 6

[0068] Using a "model of defective dermal tissue with wound" in which a defect area was prepared by punching out a collagen gel, the effect of farrerol to promote the tissue regeneration of a defect area was investigated. The procedures will be described below. The results are represented in FIG. 4. From the figure, it is found that the reduction of a punched hole size is promoted clearly at the final concentration of farrerol of 10^{-5} M or 10^{-6} M but the promotion of the reduction is unclear at the concentration of 10^{-7} M.

Consequently, it is found that a lower limit of farrerol is preferably 10^{-6} M in the external preparation for skin of the present invention.

1) Normal human fibroblasts grown to 80% confluency were dispersed with 0.05% Trypsin/EDTA. The fibroblasts were seeded at a cell density of 3×10^5 cells/25 cm^2.

2) After 24 hours, the medium was replaced with one added with farrerol (10^{-5} M, 10^{-6} M, or 10^{-7} M in final concentration) and DMSO, followed by culturing 4 days.

3) A collagen gel was prepared by adding a collagen medium solution to the culture obtained by culturing under each of the conditions in a manner similar to Example 2. The prepared collagen gel was then suspension-cultured for 1 week in the same medium as that of the culture condition of (2). The fibroblasts cultured in the medium with 10^{-5} M, 10^{-6} M, and 10^{-7} M farrerol in (2) were cultured in a medium with 10^{-5} M, 10^{-6} M, and 10^{-7} M farrerol after preparing the collagen gel, respectively.

4) When the size of a collagen gel was reduced from one at the time of being prepared by the addition of the collagen medium solution to a size of about 1/5 to 1/10, the center portion of the gel was punched out in a donut form by a disposable biopsy punch with a diameter of 3 mm. As a result, a "wound model" was prepared.

5) A 6-well plate was prepared and 5 µl of collagen was then dropped on the center portion of the well. Subsequently, the collagen gel of the wound model was mounted thereon and incubated at 37°C

for 15 minutes.

6) When the dropped collagen was solidified and the gel was attached to the bottom of the well, 3 ml of the medium with the same condition as that of (3) was added, followed by culturing as a "model of defective dermal tissue with wound". Subsequently, the process of closing up the wound area of the collagen gel was visually observed.

Example 7

[0069] According to the formulation described below, cosmetics (essences) which are the external preparation for skin of the present invention were prepared. A, B, and C were heated to 80°C, B was gradually added to A while stirring to emulsify, and C was then added for neutralization, followed by stirring under cooling. As a result, essence 3 was obtained. Similar operation was carried out to prepare Comparative Example 2 in which farrerol of essence 3 was substituted with ethanol.

[0070]

Table 4

Ingredients	% by mass
A	
Squalane	5
POE (20) behenyl ether	1
Behenyl alcohol	2
Sorbitan sesquistearate	1.5
Behenic acid	0.5
B	
1,3-butanediol	8
1,2-hexanediol	4
Phenoxyethanol	0.5
"Carbopol 1382" (Alkyl-modified carboxyvinyl polymer: manufactured by Goodrich Corporation)	0.1
"Pemulen TR-1" (Alkyl-modified carboxyvinyl polymer: manufactured by Goodrich Corporation)	0.1
Water	57.1
Material extracted with an aqueous solution of 80% ethanol of Production Example 1	0.1
C	
Potassium hydrate	0.1
Water	20
Total	100

[0071] <Test Example 3>

Using essence 3 and Comparative Example 2, the effect of promoting the tissue regeneration of a defect area with wound was studied on panelists. A trimming die was put on the medial part of the upper arm of a panelist and the epidermis was stripped off with a scalpel to obtain three parts of 1 mm × 1 mm. The essence 3 was applied to one part once a day for 5 days from the day of removing the epidermis. Comparative Example 2 was applied to another one part once a day for 5 days from the day of removing the epidermis,

and the remaining one part was not subjected to any treatment. The observation was performed after 1 week and 2 weeks from the application to determine the degree of tissue regeneration on the defect area. The results are represented in Table 5. Consequently, essence 3, the external preparation for skin of the present invention, is found to be excellent in effect of promoting the tissue regeneration of a defect area. Besides, the formation of scar was not observed at all.

[0072]

Table 5

Specimen	Observation result after 1 week	Observation result after 2 weeks
Essence 1	Most of defect areas are recovered	No difference from normal areas
Comparative Example 1	Scab is present and partial defect remains	Most of defect areas are recovered
Untreated	Scab is present and partial defect remains	Most of defect areas are recovered

Example 8

[0073] <Test Example 4>

According to the formulation described below, a dermal medicine 1 for external application, the external preparation for skin of the present invention, was prepared in a manner similar to Example 7. The same evaluation as that of Test Example 3 was performed. As a result, the medicine showed an excellent effect of promoting the regeneration of a defect dermal tissue even it coexists with an antibiotic substance.

[0074]

Table 6

Ingredient	% by mass
A	
Squalane	5
POE (20) behenyl ether	1
Behenyl alcohol	2
Sorbitan sesquistearate	1.5
Behenic acid	0.5
B	
1,3-butanediol	8
1,2-hexanediol	4
Phenoxyethanol	0.5
"Carbopol 1382" (Alkyl-modified carboxyvinyl polymer: manufactured by Goodrich Corporation)	0.1
"Pemulen TR-1" (Alkyl-modified carboxyvinyl polymer: manufactured by Goodrich Corporation)	0.1
Water	56.1
Fradiomycin sulfate	1
Extracted with an aqueous solution of 80% ethanol of Production Example 1	0.1
C	
Potassium hydrate	0.1
Water	20
Total	100

Example 9

[0075] The effect of farrerol on keratinocytes was studied according the following procedures.

[0076] <Experiment 1: Cytotoxicity evaluation of farrerol on keratinocytes>

[Cells and reagents used]

· Normal human epidermal keratinocytes (manufactured by Kurabo Co., Ltd.)

· Humedia KG2 (manufactured by GIBCO Co., Ltd.)

· WST-8/1-Methyl PMS (Cell Counting Kit-8: DOJINDO LABORATORIES)

[Sample preparation]

A farrerol sample was prepared by diluting 10^{-2} M of farrerol dissolved in DMSO with DMSO to adjust the concentration thereof.

[Protocol]

1) Normal human epidermal keratinocytes grown to 80% confluency were dispersed with 0.05% Trypsin/EDTA. The dispersant was seeded on 96 wells/plate at a concentration of 3×10^3 cells/well and 6×10^3 cells/well.

2) After 24 hours, the farrerol sample with adjusted concentration was added to the respective wells in a volume of 100 µl/well (n = 8).

3) WST-8/1-Methyl PMS was added to the respective wells in a volume of 10 µl /well after 24 hours and 48 hours from the addition of the farrerol sample, and then incubated at 37°C for 4 hours.

4) Absorbance (OD value) was measured at a measurement wavelength of 450 nm and a reference wavelength of 650 nm.

<Results>

The results of the evaluation with MTT are represented in a graphic diagram of FIG. 5. It was indicated that the OD value increased depending on the concentration of farrerol. For the OD value of 3×10^3 cells/well at 24 hours, 10^{-5}M farrerol showed a

value two or more times higher than that of the control, DMSO. At 48 hours, it also showed a value 1.7 times higher than that of DMSO. However, the number of the cells was small and the OD value thereof intended to low, so that the reproducibility of the result was confirmed using a doubled number of seeded cells, 6×10^3 cells/well. As a result, almost the similar result could be obtained.

On the other hand, the visual evaluation did not give the impression that the cell proliferation of the farrerol-added group was promoted compared with that of DMSO.

[0077] <Experiment 2: Cell proliferation effect of farrerol on keratinocytes (direct evaluation)>

[Cells and reagents used]

· Normal human epidermal keratinocytes (manufactured by Kurabo Co., Ltd.)

· Humedia KG2 (manufactured by GIBCO Co., Ltd.)

[Protocol]

1) Normal human epidermal keratinocytes were prepared at a cell density of 6×10^3 cells/ml and 1-ml aliquots thereof were then seeded on 96 wells/plate, respectively.

2) After 24 hours, the supernatant was removed from the respective wells and 1 ml of a medium added with a farrerol sample with a concentration adjusted with DMSO was then added to each of the wells.

3) After 24 hours, 48 hours, and 72 hours from the sample addition,

the cells were dispersed with 0.05% Trypsin/EDTA and the number thereof was then counted by a blood cell counter. By the way, in Experiment 1, the evaluation for WST-8 was used as an indicator of the number of cells. Since such a method is provided for indirect evaluation of the number of cells by acting on dehydrase in mitochondria, the present study directly count the number of cells.

[Results]

The results of the cell count are represented in FIG. 6. The graph represents the rate of cell proliferation when the number of cells in the control (DMSO-added group) is defined as 100%. Comparing with the control, the cell proliferation rate tended to be inhibited in a concentration-dependent manner. The cell proliferation rate of the 10^{-5} M farrerol-added group was inhibited to 75.1 ± 5.2% after 24 hours. In addition, with respect to the other concentrations, the cell proliferation rates were held at 75.5 ± 5.8% with 10^{-6} M and 77.1 ± 8.6% with 10^{-7} M. After 48 hours, the 10^{-5} M farrerol-added group showed a proliferation rate of about 71.9 ± 16.6%. However, at any concentration lower than the above-mentioned concentration showed almost the same proliferation rate as that of the control group. In addition, there was no change in cell shape (FIG. 7). That is, the results of the direct evaluation were not coincident with those of the indirect evaluation on the number of cells with WST-8. As a result, it is implicated that farrerol may promote the reaction of the citric acid cycle in the

mitochondria per keratinocyte. Thus, it is expected that the effect of NADH generated during the reaction of the citric acid cycle may also promote a subsequent reaction step, the oxidative phosphorylation reaction. As a result, it is indicated that the generation of ATP provided as an energy source in the cell, or the energy metabolic capacity may be accelerated.

[0078] <Experiment 3: Study of influence on fibroblasts through epidermal keratinocytes>

[Outline]

To find out whether or not any interaction between epidermal keratinocytes and fibroblasts or any effect of farrerol on fibroblasts in coculture could be occurred, an evaluation was performed with reference to the amount of procollagen produced. The method is based on one described in Eming SA et al., Hum. Gene. Ther., 9 (4), 529-39 (1998).

[Cells and reagents used]

· Normal human epidermal keratinocytes (manufactured by Kurabo Co., Ltd.)

· Humedia KG2 (manufactured by Kurabo Co., Ltd.)

· Normal human fibroblasts (manufactured by Kurabo Co., Ltd.)

· DMEM (manufactured by GIBCO Co., Ltd.)

· PIP EIA Kit (manufactured by TAKARA BIO INC.)

[Protocol]

1) Epidermal keratinocytes

DAY 1: The cells are seeded on a 24-well plate at a cell density of 3×10^4 cells/well.

DAY 6: The plate is washed with PBS and the medium is then replaced with DMEM + 2% FBS, followed by addition of farrerol (10^{-6} M, 10^{-7} M, 10^{-8} M, DMSO).

DAY 7: The supernatant of the culture is collected.

2) Fibroblasts

DAY 3: The cells are seeded on a 24-well plate at a cell density of 2.5×10^4 cells/well.

DAY 7: The plate is washed with PBS and the culture supernatant (900 µl) of the epidermal keratinocytes is then added, followed by culturing for 48 hours.

DAY 9: The plate is washed with PBS and serum-free DMEM and the medium is then replaced with 500 µl of serum-free DMEM, followed by culturing for 2 hours. The supernatant of the culture is collected and the amount of procollagen in the centrifuged supernatant (15000 rpm, 5 sec.) was then measured depending on the method described below.

[Quantitative determination of amount of procollagen produced]

1) After adding 100 µl of a standard antibody solution to each of wells of a plate, a previously-prepared PIP standard solution at each of concentrations (320, 160, 80, 40, 20, and 10 µl/ml; the test substance is diluted with DMEM) and 20 µl of the test sample were added to each well of two rows by means of a micropipette,

followed by reacting at 37°C for 3 hours (first reaction).

2) The reaction solution was removed and the wells were then washed with PBS four times. Then, 100 µl of a substrate solution (TMBZ) was added to each well using an eight-tip pipette, followed by reacting at room temperature (20°C to 30°C) for 15 minutes (second reaction).

3) Subsequently, 100 µl of a stop solution was added to each well in the order of the addition of the substrate solution to terminate the reaction and then mixed well.

4) A standard curve was formed by plotting absorbance values at a wavelength of 450 mm while the absorbance of the medium was set to zero as a reference. Then, the PIP concentration was read out from the corresponding absorbance of the test substance.

[Results]

The results are represented in FIG. 8. The amount of procollagen increased significantly depending on the concentration of farrerol. On the other hand, another system in which an evaluation was performed by a method of adding farrerol at the time of culturing fibroblasts and then adding the culture supernatant of epidermal keratinocytes did not show a large change in amount of procollagen produced. From this fact, the effect of farrerol on fibroblasts to directly promote an increase in procollagen production may be small. Consequently, it can be determined that farrerol acts on epidermal keratinocytes and the epidermal keratinocytes promote

the effect of fibroblasts to enhance the production of collagens. In this way, the collagen production ability of fibroblast can be changed by using keratinocytes in the coculture system. Thus, for example, with respect to the three-dimensional culture skin or the like, it is applicable to change the properties of three-dimensional skin into desired properties.

Example 10

[0079] A farrerol-containing external preparation for skin (cosmetics: lotion) was prepared according to the formulation described below. The formulated ingredients were mixed and dissolved at room temperature to prepare lotion. The amount of procollagen produced in the dermis of the skin can be increased by transdermally administering the lotion to the skin of a mouse or a human, leading to an increase in amount of collagen in the dermis. This is because keratinocytes are coexistent with fibroblasts in the skin.

[0080]

Table 7

Ingredients	% by mass
1,3-butanediol	5
1,2-pentanediol	2
1,2-hexanediol	3
Glycerine	5
Phenoxyethanol	0.5
Hypericum erectum extract of Production Example 1	0.001
Water	89.499
Total	100

Example 11

[0081] <Preparation of test substance>

As a test substance for screening, a *Hypericum erectum* extract was prepared. 10 l of an aqueous solution of 80% ethanol was added to 1 kg of a dried aerial part of *Hypericum erectum Thunberg* (Guttiferae) and then refluxed for 4 hours while stirring. After cooling, an insoluble matter was removed by filtration and then condensed under reduced pressure, followed by freeze-drying. Consequently, 51 g of amorphous was obtained. This product was dispersed in water, charged on DIAION HP20, washed with the flows of 5 l of water and 5 l of an aqueous solution of 50% ethanol in this order, and eluted with 99% ethanol. The resulting eluted fraction was fractionated. Subsequently, fractions thus obtained are further subjected to thin-layer chromatography and HPLC to checkout the contents thereof to combine the fractions including the same single substance together. On the other hand, the fractions including two or more ingredients are further purified by subjecting them to the chromatography again. consequently, seven fractions containing the single ingredient were obtained.

[0082] <Example of screening>

The fractions containing the single ingredients obtained as described above are provided as test substances and the effect of each test substance to construct collagen is then evaluated to screen the substance with a wound-healing effect. The specific procedures

will be described later.

Using a "wound model" provided with a defect area formed by punching a collagen gel containing animal fibroblasts, the degree of filling or reduction of the defect area in the presence of a test substance will be observed. The procedures are as follows. The single-ingredient structure of the test substance with the highest degree of filling or reduction of the defect area, compared with the rest of the test substances, was subjected to proton NMR, mass analysis, and ^{13}C-NMR and identified as farrerol. By the way, the yield of farrerol from the above extract was 3.2 mg. The content of farrerol in a solvent-removed product of an extract with an aqueous solution of 80% ethanol was about 0.006% by mass.

Further, the final concentration of farrerol was adjusted to each of 10^{-5} M, 10^{-6} M, and 10^{-7} M and the degree of reduction of the defect area at each concentration was then observed. The results are represented in FIG. 9. From the figure, it is found that the reduction of the punch hole (defect area) is distinctly promoted when the final concentration of farrerol is 10^{-5}M or 10^{-6}M but the promotion of reduction at 10^{-7}M is obscure. Consequently, the effect of farrerol to promote the collagen construction can exert a wound-healing promoting effect when at least 10^{-6} M of farrerol is used. In this way, according to the screening method of the present invention, the effective concentration of a test substance with a wound-healing promoting effect can be also obtained.

[0083] <Procedures>

1) Normal human fibroblasts grown to 80% confluency in preculture were dispersed with 0.05% Trypsin/EDTA. The fibroblasts were then seeded on a plate at a cell density of 3×10^5 cells/25 cm^2 (1×10^4 cells/cm^2).

2) After culturing for 24 hours, the medium was replaced with one added with farrerol (10^{-5}M, 10^{-6}M, or 10^{-7}M in final concentration) and DMSO, followed by culturing for 4 days.

3) A collagen gel was prepared by adding a collagen medium solution to the obtained culture in a manner similar to Example 3. The fibroblasts in the collagen gel were cultured using the medium used in (2). The concentration of the test substance in the medium corresponded to the concentration of the medium of (2). That is, the fibroblasts cultured in the medium with farrerol with final concentrations of 10^{-5} M, 10^{-6} M, and 10^{-7} M in (2) were cultured in a medium with farrerol with final concentrations of 10^{-5} M, 10^{-6} M, and 10^{-7} M even after preparing the collagen gel, respectively.

4) After culturing for 1 week, the size of a collagen gel was reduced from one at the time of being prepared by the addition of the collagen medium solution to a size of about 1/5 to 1/10. Subsequently, the center portion of the collagen gel was punched out in a donut form by a disposable biopsy punch with a diameter of 3 mm. As a result, a "wound model" was prepared.

5) A 6-well plate was prepared and 5 µl of the collagen solution

was then dropped on the center portion of the well. Subsequently, the collagen gel of the wound model was mounted thereon and incubated at 37°C for 15 minutes, allowing the collagen gel to be attached on the bottom of the well.

6) Subsequently, 3 ml of the same medium as that of (2) was added to each of the wells and the fibroblasts in the above-mentioned collagen gel was then cultured for 7 days. During the culture, the process of closing up the wound area of the collagen gel was visually observed.

Industrial Applicability

[0084] The external preparation for skin of the present invention is applicable to cosmetics, dermal medicines for external application, and so on. In addition, wound healing can be promoted using the external preparation for skin of the present invention.

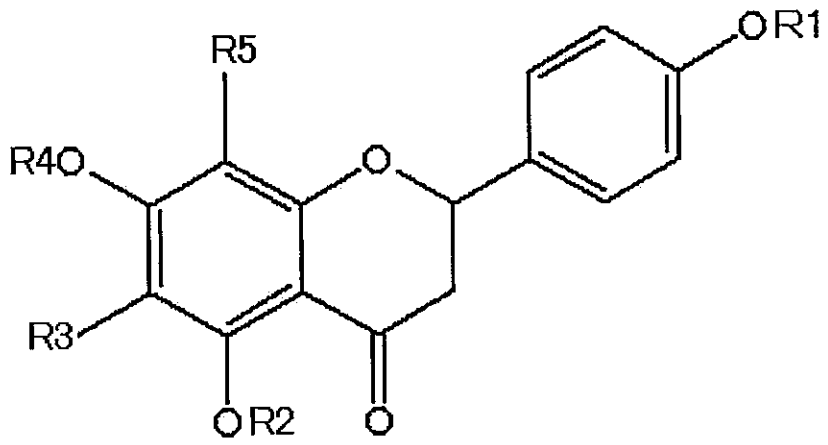
Further, with respect to three-dimensional culture skin or the like, the method of enhancing a collagen production ability of fibroblast may be used in the production of artificial skin by changing the properties of three-dimensional skin into desired properties.

A substance with a wound-healing promoting effect suitable for dermal medicines for external application can be screened using the screening method of the present invention.

Claims

1. An external preparation for skin, comprising:

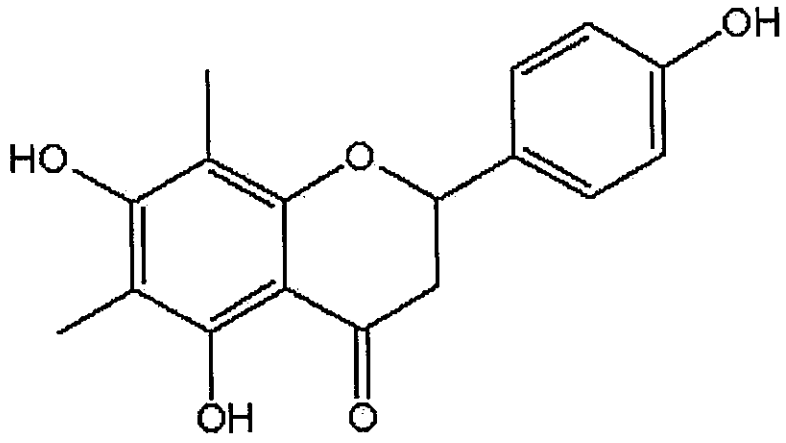
a flavanone derivative represented by the following general formula (1) and/or a salt thereof:



general formula (1)

wherein R1, R2, R3, R4, and R5 each independently represent a hydrogen atom or an alkyl group with 1 to 4 carbon atoms.

2. The external preparation for skin according to claim 1, wherein the flavanone derivative is farrerol.



farrerol

3. The external preparation for skin according to claim 1 or 2, comprising:

an extract of *Hypericum erectum, Guttiferae* which contains 10^{-6} to 0.1% by mass of the flavanone derivative and/or the salt thereof.

4. The external preparation for skin according to any one of claims 1 to 3, which is used for normalizing a skin structure.

5. The external preparation for skin according to claim 4, wherein the normalization of the skin structure is to remodel a disordered collagen fiber bundle structure of the dermis.

6. The external preparation for skin according to claim 4, wherein the normalization of the skin structure is to regenerate a skin defect area.

7. The external preparation for skin according to claim 4, wherein the normalization of the skin structure is to regenerate the skin defect area and to promote occlusion of the defect area by a regenerated tissue.

8. The external preparation for skin according to claim 6 or 7, wherein the skin defect is due to wound.

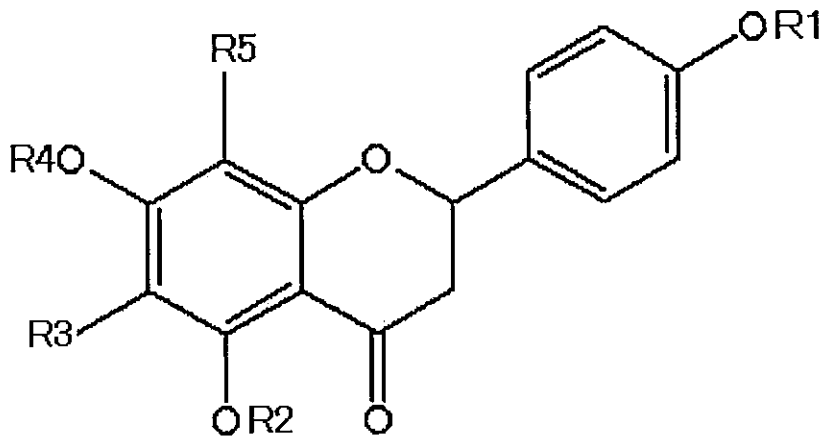
9. The external preparation for skin according to any one of claims 1 to 8, wherein the content of the flavanone derivative and/or the salt thereof is 1×10^{-6} to 1% by mass.

10. The external preparation for skin according to claim 4, wherein the normalization of the skin structure is to enhance an effect of keratinocytes to increase a collagen production ability of fibroblast.

11. A method of enhancing an effect of keratinocytes to increase a collagen production ability of fibroblast in vitro,

comprising:

administering a flavanone derivative represented by the general formula (1) and/or a salt thereof under a coexistence of keratinocytes with fibroblasts in vitro:



general formula (1)

wherein R1, R2, R3, R4, and R5 each independently represent a hydrogen atom or an alkyl group with 1 to 4 carbon atoms.

12. The method according to claim 11, wherein the flavanone derivative is farrerol.

13. The method according to claim 11 or 12, wherein the dosage of the flavanone derivative and/or the salt thereof is 10^{-8} M or more.

14. The method according to any one of claims 11 to 13, comprising:

administering an extract of *Hypericum erectum* containing the flavanone derivative and/or the salt thereof.

15. The method according to any one of claims 11 to 14, wherein the collagens are procollagen.

16. A method of screening a substance with a wound-healing effect, comprising:

culturing normal animal fibroblasts in the presence of a test substance;

preparing a collagen gel by adding a collagen medium solution to an obtained culture;

culturing the fibroblasts in the collagen gel in the presence of the test substance;

forming a defect area in the collagen gel;

culturing the fibroblasts under a coexistence of the collagen gel having the defect area with the test substance; and

observing the degree of filling or reduction of the defect area during the culture.

17. The screening method according to claim 16, further comprising:

culturing normal animal fibroblasts in the absence of a test substance;

preparing a collagen gel by adding a collagen medium solution to an obtained culture;

culturing the fibroblasts in the collagen gel in the absence of the test substance;

forming a defect area in the collagen gel;

culturing the fibroblasts while placing the collagen gel having the defect area in the absence of the test substance; and

observing the degree of filling or reduction of the defect area during the culture, wherein when the degree of filling or reduction of the defect area in the presence of the test substance is larger than the degree of filling or reduction of the defect area in the absence of the test substance, it is determined that the test substance has a wound-healing promoting effect.

18. The screening method according to claim 17, wherein when the volume of the defect area in the presence of the test substance is 85% or less of the volume of the defect area in the absence of the test substance, it is determined that the test substance has

a wound-healing promoting effect.

19. The screening method according to any one of claims 16 to 18, wherein the normal fibroblasts are of human origin.

20. The screening method according to any one of claims 17 to 19, comprising the following steps:

(1) culturing normal animal fibroblasts separated into individual cells in a medium containing a test substance and a medium free of the test substance;

(2) preparing a collagen gel by adding a collagen medium solution to a culture obtained in (1);

(3) suspension-culturing the normal fibroblasts in the collagen gel of (2) using the same medium as that of (1);

(4) forming a defect area by punching out the center portion of the collagen gel by a punch when the size of the collagen gel is reduced to about 1/5 to 1/10 of the size at the time of preparing the collagen by the addition of the collagen medium solution;

(5) dropping a collagen solution on a well of a plate , and mounting and culturing the collagen gel prepared in (4) on the well to allow the collagen gel to attach on the bottom of the well; and

(6) culturing the collagen gel using the same medium as that of (1) and observing the degree of filling or reduction of the defect area during the culture.