REPUBLIC OF SOUTH AFRICA

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PATENTS ACT, 1978

CERTIFICATE

n accordance with section 44 (1) of the Patents Act, No. 57 of 1978, it is hereby certified that:

ZHEJIANG UNIVERSITY

Has been granted a patent in respect of an invention described and claimed in complete specification deposited at the Patent Office under the number

2024/05212

A copy of the complete specification is annexed, together with the relevant Form P2.

In [illegible]mony thereof, the seal of the Patent Office has been affixed at Pretoria with effect from the **29th** day of **January 2025**

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Registrar of Patents

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REGISTER OF PATENTS

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71	Assignee(s):				Date registrered	
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APPLICATION OF TANGERETIN AND NOBILETIN MIXTURE IN PREPARING FUNCTIONAL PRODUCTS TO ACTIVATE ABILITY OF CELLS TO RESIST OXIDATIVE STRESS						
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FORM P7

REPUBLIC OF SOUTH AFRICA
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COMPLETE SPECIFICATION
[Section 30(1) – Regulation 28]

OFFICIAL APPLICATION NO.			LODGING DATE	
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FULL NAME(S) OF APPLICANT(S)

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TITLE OF INVENTION

54	APPLICATION OF TANGERETIN AND NOBILETIN MIXTURE IN PREPARING FUNCTIONAL PRODUCTS TO ACTIVATE ABILITY OF CELLS TO RESIST OXIDATIVE STRESS

APPLICATION OF TANGERETIN AND NOBILETIN MIXTURE IN PREPARING FUNCTIONAL PRODUCTS TO ACTIVATE ABILITY OF CELLS TO RESIST OXIDATIVE STRESS

TECHNICAL FIELD

[01] The present invention belongs to the field of foods and drugs, and relates to an application of a tangeretin and nobiletin mixture in preparing functional products to activate ability of cells to resist oxidative stress.

BACKGROUND ART

[02] Oxidation and reduction are common chemical reactions. Oxidative stress injury occurs when the accumulation rate of oxides in organisms exceeds the rates of reduction reaction and oxide removal of organisms. Natural products can help protect the body from oxidative stress injury, can enhance the antioxidant ability of cells, and even make cells maintain the ability to resist oxidative stress injury for a period of time. There are no reports on natural active products.

[03] Polymethoxylated Flavonoids (PMFs), mainly derived from citrus plants of rutaceae, especially rich in the peel of citrus fruits, are flavonoids compounds with 4 or more methoxyl substituents. PMFs ligands, such as nobiletin and tangeretin, attract more attention because of their stronger physiological activity than ordinary flavonoids. Tangeretin (TAN) has good activities in anti-tumor, anti-inflammation, anti-mutagenesis and cardiovascular protection. Nobiletin, served as a carbohydrate metabolism accelerator, has the functions of anti-hemagglutination, anti-thrombosis, anti-cancer, anti-fungus, anti-inflammation, anti-allergy, anti-cholinesterase and anti-epilepsy. However, there are no reports about studies of TAN and/or nobiletin on enhancing and maintaining the ability of cells to resist oxidative stress injury, and especially on enhancing the ability of L02 cell to resist oxidative stress by activating oxidative stress pathways.

SUMMARY

[04] The objective of the present invention is to provide an application of a TAN and nobiletin mixture in preparing functional products to activate ability of cells to resist oxidative stress. The products refer to functional foods, health care products or preventive drugs, the cells are human liver L20 cells, and a mass ratio of the TAN to the nobiletin in the mixture is 0.5-1.5:0.5-1.5.

[05] Preferably, to activate ability of cells to resist oxidative stress is significant increase of Nrf2 gene and protein expression in the human liver L02 cells, or significant inhibition of Keap1 gene and protein expression in the human liver L02 cells, or significant inhibition of Cul3 gene and protein expression in the human liver L02 cells, or significant inhibition of ubiquitination degradation of Nrf2 proteins in the human liver L02 cells, or significant inhibition of reactive oxygen species (ROS) accumulation induced by hydrogen peroxide in the human liver L02 cells, or is that the TAN can significantly increase activities of quinone oxidoreductase (NQO1) and catalase (CAT) in the human liver L02 cells.

[06] Preferably, the functional foods or the health care products are made from the TAN and nobiletin mixture as well as auxiliary materials acceptable for foods or health care products.

[07] Preferably, the drugs are made from the TAN and nobiletin mixture as well as pharmaceutically acceptable auxiliary materials.

[08] The TAN and nobiletin mixture provided herein can significantly increase the ability of the human liver L02 cells to resist hydrogen peroxide-induced oxidative stress injury, and enhance the expression of antioxidase NQO1 and CAT by regulating the oxidative stress-related Nrf2-Keap1-Cul3 pathways, and inhibit the ubiquitination degradation of Nrf2 by inhibiting the expression of Cul3, so as to maintain the ability of L02 cells to resist oxidative stress. Functional foods, health care products or preventive drugs can be prepared to prevent and treat diseases related to liver oxidative stress injury.

BRIEF DESCRIPTION OF THE DRAWINGS

[09] FIG. 1 shows relative effects of the TAN and nobiletin mixture on intracellular

ROS during oxidative injury from hydrogen peroxide, with human liver L02 cells as a model; wherein DMSO is used as a negative control, cells without hydrogen peroxide treatment are used as a blank, and a final concentration of the pretreated mixture retained or removed is 2 μmol/L; the pictures are cell photos taken by a fluorescence microscope.

[10] FIG. 2 shows relative effects of the TAN and nobiletin mixture on intracellular ROS during oxidative injury from hydrogen peroxide, with human liver L02 cells as a model; wherein the legends are shown in FIG. 1; the figure shows analysis of optical density values of fluorescence photos by ImageJ.

[11] FIG. 3 shows effects of the TAN and nobiletin mixture on activities of NQO1 and CAT, with human liver L02 cells as a model; wherein DMSO is used as a negative control, three concentration gradients are set for the pretreated TAN and nobiletin mixture retained or removed, i.e., 0.5 μmol/L, 1 μmol/L and 2 μmol/L, respectively; the result is expressed as a relative content of the treatment group and the control group, the significance of difference is analyzed by SPSS software, a *t* test is conducted, $*p < 0.05$, comparison is made to the control group.

[12] FIG. 4 shows effects of the TAN and nobiletin mixture on expression of NQO1 and CAT proteins, with human liver L02 cells as a model; wherein each legend is shown in FIG. 1.

[13] FIG. 5 shows effects of the TAN and nobiletin mixture on Nrf2, Keap1 and Cul3 genes, with human liver L02 cells as a model; wherein the legends are shown in FIG. 3.

[14] FIG. 6 shows effects of the TAN and nobiletin mixture on expression of Nrf2, Keap1 and Cul3 proteins, with human liver L02 cells as a model; wherein the legends are shown in FIG. 1.

[15] FIG. 7 shows effects of the TAN and nobiletin mixture on ubiquitination degradation of Nrf2 proteins, with human liver L02 cells as a model; wherein IgG is used as a blank, DMSO is used as a solvent control, MG-132 is used as a positive control, and a final concentration of the pretreated TAN and nobiletin mixture retained or removed is 2 μmol/L.

[16] FIG. 8 shows time effects of the TAN and nobiletin mixture on inhibition of ubiquitination degradation of Nrf2 proteins, with human liver L02 cells as a model; wherein the legends are shown in FIG. 7.

DETAILED DESCRIPTION OF THE EMBODIMENTS

[17] The present invention will be further described in combination with accompanying drawings and embodiments.

[18] **Example 1 Effects of the TAN and nobiletin mixture on intracellular ROS when human liver L02 cells suffered from hydrogen peroxide-induced oxidative stress injury**

[19] The human liver L02 cells were incubated with a RPMI-1640 medium containing 10% fetal calf serum in a 5% CO_2 cell incubator at 37℃, a fresh culture solution was used every other day, and the culture passed once every 2-3 days. During the experiment, the L02 cells were inoculated on a 6-well plate. When the cells grew to 70%-80% fusion, the original medium was discarded and a chemical reagent was added by group. A blank group (without hydrogen peroxide treatment), a DMSO solvent control group and a mixture (a mass ratio of the TAN to the nobiletin being 1:1) treatment group (a final concentration of 2 μmol/L) were set. Upon action for 6 h, the medium containing the mixture was retained (or removed) and a serum-free medium containing 0.78 mmol/L hydrogen peroxide was added. Upon incubation for 30 min, the original medium was removed, and washed twice with PBS, and a serum-free medium containing a DCFH-DA probe at a final concentration of 10 μmol/L was added. Upon incubation for 20 min, the original medium was removed, and washed with a serum-free medium 3 times, and the fluorescence of cells in the 6-well plate was observed by a fluorescence microscope. 3 repeated wells were set for each group, and the experiment was repeated in parallel three times.

[20] The data were expressed as mean ± standard error ($\bar{x} \pm$ SE), statistics on optical density values of fluorescence were conducted by ImageJ software, data were analyzed by SPSS software, and a *t* test was conducted. The specific effects of the TAN

and nobiletin mixture on intracellular ROS when human liver L02 cells suffered from a hydrogen peroxide-induced oxidative stress injury are shown in FIGS. 1-2.

[21] Example 2 Effects of the TAN and nobiletin mixture on intracellular autioxidant enzyme activities of human liver L02 cells

[22] The human liver L02 cells were incubated with a RPMI-1640 medium containing 10% fetal calf serum in a 5% CO_2 cell incubator at 37℃, a fresh culture solution was used every other day, and the culture passed once every 2-3 days. During the experiment, the L02 cells were inoculated on a 96-well plate. When the cells grew to 70%-80% fusion, the original medium was discarded and a chemical reagent was added by group. A DMSO solvent control group and a treatment group of mixtures (a mass ratio of the TAN to the nobiletin being 1:1) of different concentrations (a final concentrations being 0.5 μmol/L, 1 μmol/L and 2 μmol/L respectively) were set. Upon action for 6 h, the medium containing the mixture was retained (or removed). A CAT activity was detected by a CAT assay kit (S0051) produced by Beyotime. A NQO1 activity was detected by a NQO1 assay kit (ab184867) produced by Abcam.

[23] Detection was conducted by the method of example 1. The specific effects of the TAN and nobiletin mixture on intracellular antioxidant enzyme activities of human liver L02 cells, human stomach GES cells, human umbilical vein epithelial HUVEC cells and human fibroblast WI-38 cells are shown in FIGS. 3-4.

[24] Example 3 Effects of the TAN and nobiletin mixture on expression of intracellular CAT and NQO1 proteins of human liver L02 cells

[25] The human liver L02 cells were incubated with a RPMI-1640 medium containing 10% fetal calf serum in a 5% CO_2 cell incubator at 37℃, a fresh culture solution was used every other day, and the culture passed once every 2-3 days. During the experiment, the L02 cells were inoculated on a 6-well plate. When the cells grew to 70%-80% fusion, the original medium was discarded and a chemical reagent was added by group. A DMSO solvent control group and a mixture (a mass ratio of the TAN to the nobiletin being 1:1) treatment group (a final concentration being 2 μmol/L) were set. Upon action for 6 h, the cells were lysed by a NP40 lysate containing a protease inhibitor and a protein phosphatase inhibitor and a total protein (TP) was collected, a

protein concentration was determined by a BCA method, the proteins were isolated by a SDS-PAGE gel and a PVDF membrane was transferred, β-ACTIN was used as a reference, and an antibody dilution concentration was (CAT:1:1,000; NQO1:1:1,000; β-ACTIN:1:5,000).

[26] Detection was conducted by the method of example 1. The specific effects of the TAN and nobiletin mixture on expression of CAT and NQO1 proteins of human liver L02 cells are shown in FIG. 5.

[27] Example 4 Effects of the TAN and nobiletin mixture on Nrf2, Keap1 and Cul3 genes of human liver L02 cells

[28] The human liver L02 cells were incubated with a RPMI-1640 medium containing 10% fetal calf serum in a 5% CO_2 cell incubator at 37℃, a fresh culture solution was used every other day, and the culture passed once every 2-3 days. During the experiment, the L02 cells were inoculated on a 96-well plate. When the cells grew to 70%-80% fusion, the original medium was discarded and a chemical reagent was added by group. A DMSO solvent control group and a treatment group of mixtures (a mass ratio of the TAN to the nobiletin being 1:1) of different concentrations (a final concentrations being 0.5 μmol/L, 1 μmol/L and 2 μmol/L respectively) were set. Upon action for 6 h, a total RNA was extracted by a Trizol method; after the total RNA was reverse-transcribed into cDNA, a RT-PCR reaction was performed by a Bio-rad SsoFast™ EvaGreen® Supermix kit, wherein Nrf2 QPCR primer pairs were SEQ ID NO:1 (forward primer) and SEQ ID NO:2 (reverse primer), Keap1 QPCR primer pairs were SEQ ID NO:3 (forward primer) and SEQ ID NO:4 (reverse primer), and Cul3 primer pairs were SEQ ID NO:5 (forward primer) and SEQ ID NO:6 (reverse primer), the primer reference sequence was taking β-actin as an internal reference, the primer pairs were SEQ ID NO:7 (forward primer) and SEQ ID NO:8 (reverse primer), and the gene expression was calculated by a 2-ΔΔCt method.

[29] Detection was conducted by the method of example 1. The specific effects of the TAN and nobiletin mixture on expression of Nrf2 and Keap1 genes of human liver L02 cells are shown in FIG. 5.

Table 1 Primers Used in Example 4

Gene	Forward primers (5' to 3')	Reverse primers (3' to 5')
Nrf2	CACATCCAGTCAGAAACCAGTGG (SEQ ID NO: 1)	GGAATGTCTGCGCCAAAAGCTG (SEQ ID NO: 2)
Keap1	GGGAGGTGGCCAAGCAAGAGG (SEQ ID NO: 3)	TCACCTGCGTGGGCTTGTGCAG (SEQ ID NO: 4)
Cul3	AGAGCGGAAAGGAGAAGTCGTAGA (SEQ ID NO: 5)	CTCAAAGTCACCCGCAATAGTT (SEQ ID NO: 6)
β-actin	GGCTGTATTCCCCTCCATCG (SEQ ID NO: 7)	CCAGTTGGTAACAATGCCATGT (SEQ ID NO: 8)

[30] Example 5 Effects of the TAN and nobiletin mixture on Nrf2, Keap1 and Cul3 proteins of human liver L02 cells

[31] The human liver L02 cells were incubated with a RPMI-1640 medium containing 10% fetal calf serum in a 5% CO_2 cell incubator at 37℃, a fresh culture solution was used every other day, and the culture passed once every 2-3 days. During the experiment, the L02 cells were inoculated on a 6-well plate. When the cells grew to 70%-80% fusion, the original medium was discarded and a chemical reagent was added by group. A DMSO solvent control group and a TAN and nobiletin mixture (a mass ratio of the TAN to the nobiletin being 1:1) treatment group (a final concentration being 2 μmol/L) were set. Upon action for 6 h, the cells were lysed by a NP40 lysate containing a protease inhibitor and a protein phosphatase inhibitor and a TP was collected, a protein concentration was determined by a BCA method, the proteins were isolated by a SDS-PAGE gel and a PVDF membrane was transferred, β-ACTIN was used as a reference, and an antibody dilution concentration was (Nrf2:1:1,000; Keap1:1:1,000; Cul3:1:1,000; β-ACTIN:1:5,000).

[32] Detection was conducted by the method of example 1. The specific effects of the TAN and/or nobiletin on expression of Nrf2, Keap1 and Cul3 proteins of human liver L02 cells are shown in FIG. 6.

[33] Example 6 Effects of the TAN and nobiletin mixture on ubiquitination of Nrf2 proteins of human liver L02 cells

[34] The human liver L02 cells were incubated with a RPMI-1640 medium containing 10% fetal calf serum in a 5% CO_2 cell incubator at 37℃, a fresh culture solution was used every other day, and the culture passed once every 2-3 days. During the experiment, the L02 cells were inoculated on a 6-well plate. When the cells grew to 70%-80% fusion, the original medium was discarded and a chemical reagent was added by group. A DMSO solvent control group and a mixture (a mass ratio of the TAN to the nobiletin was 1:1) treatment group (a final concentration being 2 μmol/L) were set. Upon action for 6 h, the cells were lysed by a NP40 lysate containing a protease inhibitor and a protein phosphatase inhibitor and a TP was collected, a protein concentration was determined by a BCA method, the protein lysates were incubated with G protein magnetic beads (Bio-rad, 1614023) and the Nrf2 antibody or an IgG control of the same species, and the resulting solution was mixed-spun overnight at 4℃ on a mixer. Magnetic beads bound with immunoprecipitate were collected, washed with a PBS-T solution 5 times, boiled at 100℃, and supernatant was collected. The proteins were isolated by a SDS-PAGE gel and a PVDF membrane was transferred, β-ACTIN was used as a reference, MG-132 was used as a positive control, and an antibody dilution concentration was (Nrf2:1:1,000; Ubi:1:1,000; β-ACTIN:1:5,000).

[35] Detection was conducted by the method of example 1. The specific effects of the TAN and nobiletin mixture on ubiquitination degradation of Nrf2 proteins of human liver L02 cells are shown in FIG. 7.

[36] Example 7 Time effects of the TAN and/or nobiletin on ubiquitination inhibition of Nrf2 proteins of human liver L02 cells

[37] The human liver L02 cells were incubated with a RPMI-1640 medium containing 10% fetal calf serum in a 5% CO_2 cell incubator at 37℃, a fresh culture solution was used every other day, and the culture passed once every 2-3 days. During the experiment, the L02 cells were inoculated on a 6-well plate. When the cells grew to 70%-80% fusion, the original medium was discarded and a chemical reagent was added by group. A DMSO solvent control group and a mixture (a mass ratio of the TAN to the nobiletin being 1:1) treatment group (a final concentration being 2 μmol/L) were set. Upon action for 6 h, the medium was replaced with a medium without the mixture, the

cells were collected at 0.5 h, 1 h, 2 h and 4 h respectively after mixture removal and treatment, the cells were lysed by a NP40 lysate containing a protease inhibitor and a protein phosphatase inhibitor and a TP was collected, a protein concentration was determined by a BCA method, the protein lysates were incubated with G protein magnetic beads (Bio-rad, 1614023) and the Nrf2 antibody or an IgG control of the same species, and the resulting solution was mixed-spun overnight at 4℃ on a mixer. Magnetic beads bound with immunoprecipitate were collected, washed with a PBS-T solution 5 times, boiled at 100℃, and supernatant was collected. The proteins were isolated by a SDS-PAGE gel and a PVDF membrane was transferred, β-ACTIN was used as a reference, MG-132 was used as a positive control, and an antibody dilution concentration was (Nrf2:1:1,000; Ubi:1:1,000; β-ACTIN:1:5,000).

[38] Detection was conducted by the method of example 1. The specific effects of the TAN and nobiletin mixture on ubiquitination degradation of Nrf2 proteins of human liver L02 cells are shown in FIG. 8.

[39] Although the present invention has been described in detail with the above-mentioned embodiments, such embodiments are only a part of, rather than all of, the embodiments of the present invention. Based on the embodiments, other embodiments may be obtained without making creativity, and these embodiments should fall into the protection scope of the present invention.

WHAT IS CLAIMED IS:

1. An application of a tangeretin (TAN) and nobiletin mixture in preparing functional products to activate ability of cells to resist oxidative stress, wherein the products refer to functional foods, health care products or preventive drugs, the cells are human liver L20 cells, and a mass ratio of the TAN to the nobiletin in the mixture is 0.5-1.5:0.5-1.5.

2. The application of claim 1, wherein to activate ability of cells to resist oxidative stress is significant increase of Nrf2 gene and protein expression in the human liver L02 cells, or significant inhibition of Keap1 gene and protein expression in the human liver L02 cells, or significant inhibition of Cul3 gene and protein expression in the human liver L02 cells, or significant inhibition of ubiquitination degradation of Nrf2 proteins in the human liver L02 cells, or significant inhibition of reactive oxygen species (ROS) accumulation induced by hydrogen peroxide in the human liver L02 cells, or is that the TAN significantly increases activities of quinone oxidoreductase (NQO1) and catalase (CAT) in the human liver L02 cells.

3. The application of claim 1, wherein the functional foods or the health care products are made from the TAN and nobiletin mixture as well as auxiliary materials acceptable for foods or health care products.

4. The application of claim 1, wherein the drugs are made from the TAN and nobiletin mixture as well as pharmaceutically acceptable auxiliary materials.

Sibanda & Zantwijk Patent Attorneys

ABSTRACT OF THE DISCLOSURE

The present invention provides an application of a tangeretin (TAN) and nobiletin mixture in preparing functional products to activate ability of cells to resist oxidative stress. The products include functional foods, health care products or drugs. The TAN and nobiletin mixture can significantly increase the ability of human liver L02 cells to resist hydrogen peroxide-induced oxidative stress injury, and enhance the expression of antioxidase quinone oxidoreductase NQO1 and catalase CAT by regulating the oxidative stress-related Nrf2-Keap1-Cul3 pathways, and inhibit the ubiquitination degradation of Nrf2 by inhibiting the expression of Cul3, so as to maintain the ability of L02 cells to resist oxidative stress.

ABSTRACT DRAWING

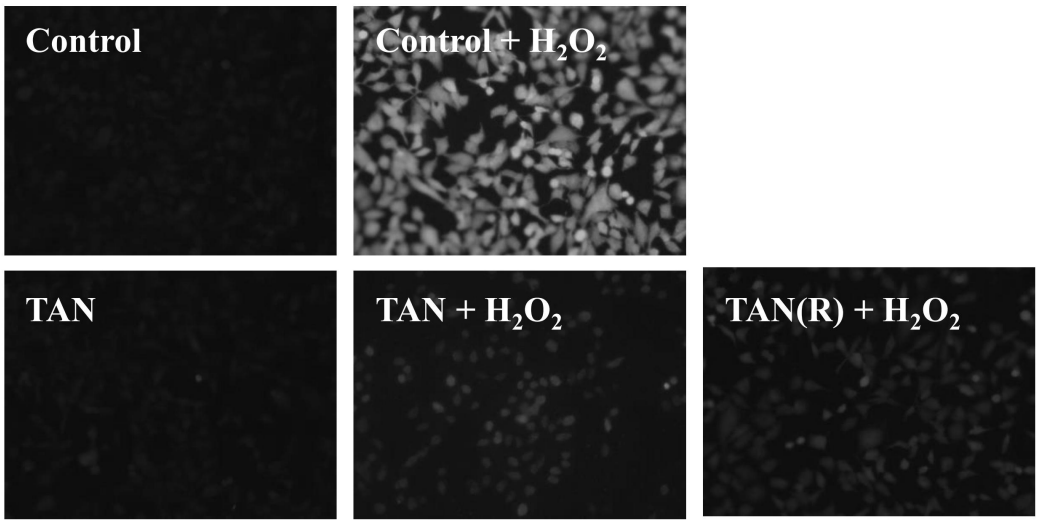

FIG. 1

Drawings

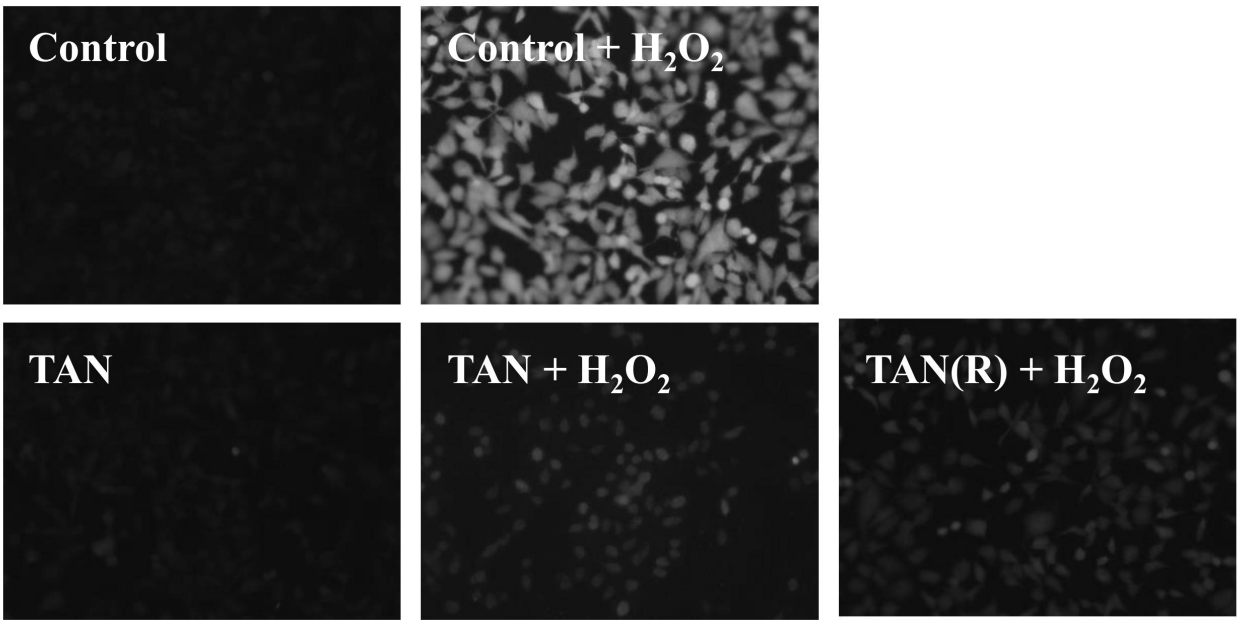

FIG. 1

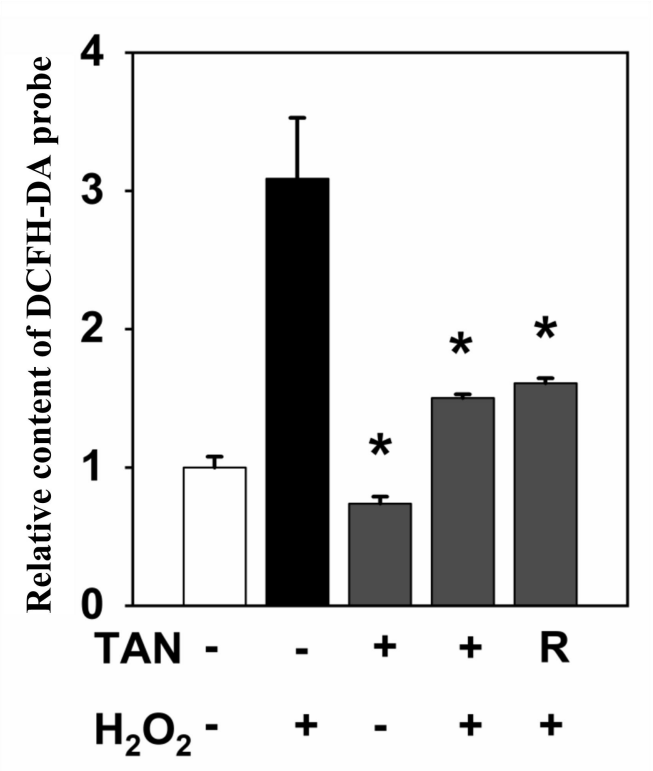

FIG. 2

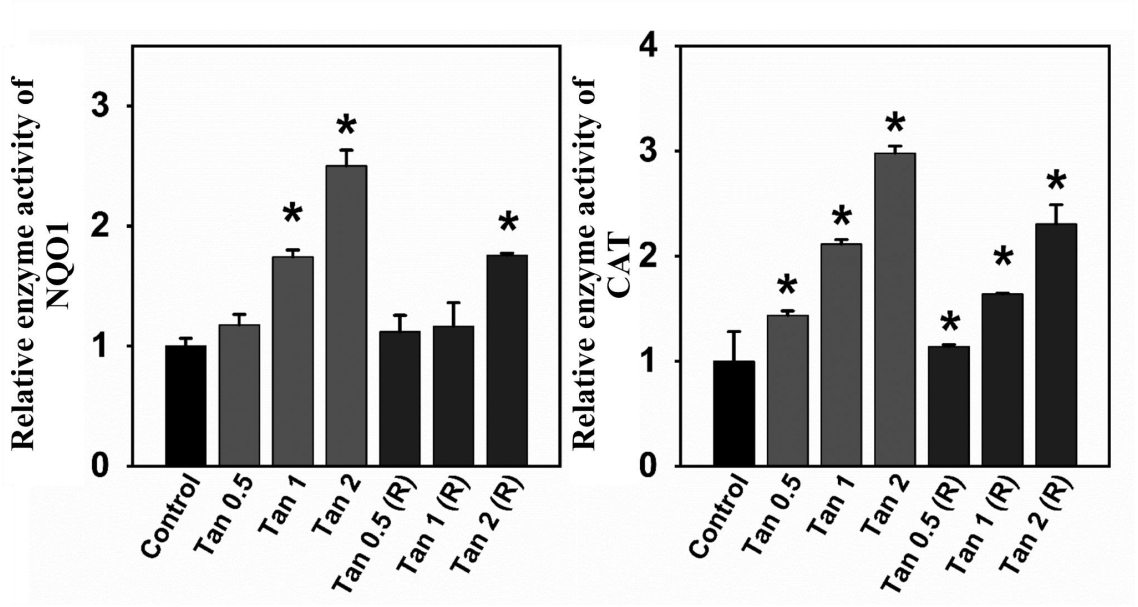

FIG. 3

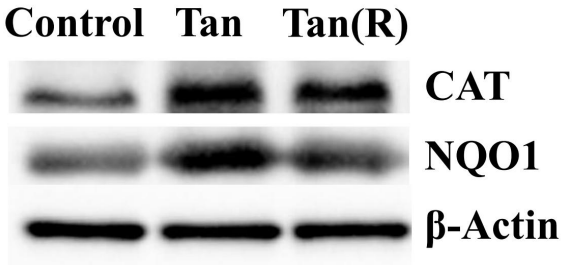

FIG. 4

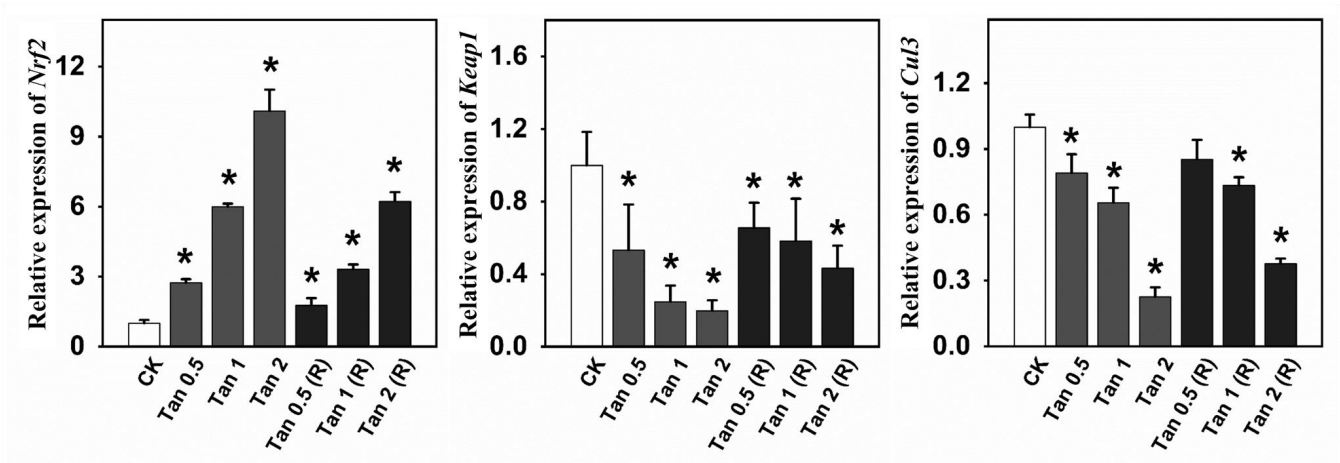

FIG. 5

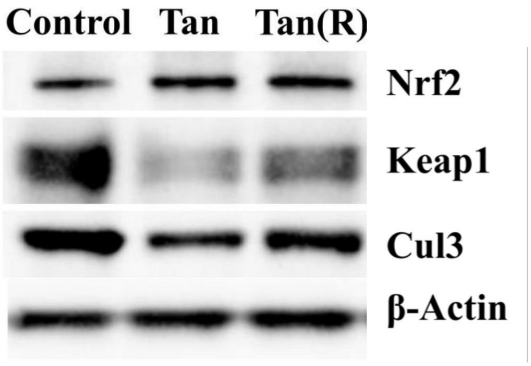

FIG. 6

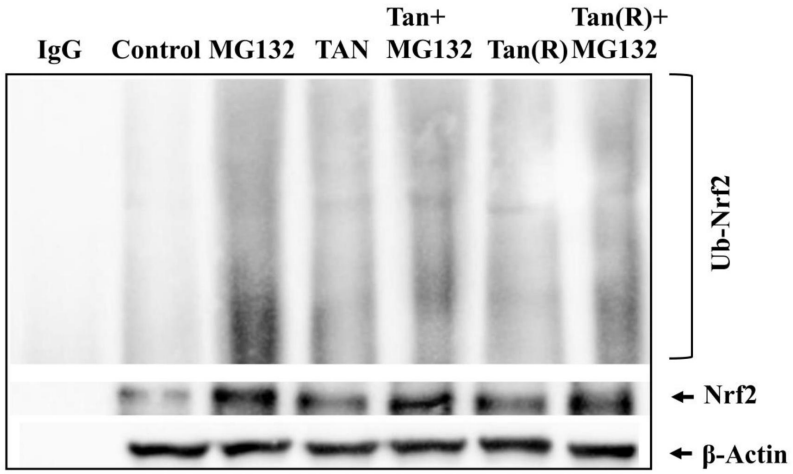

FIG. 7

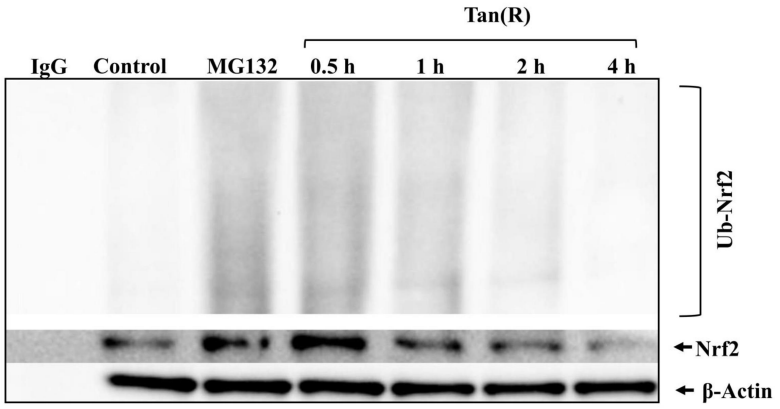

FIG. 8