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Activation of intracellular antioxidant master gene Nrf2 by the extracts of *Garcinia subelliptica, Ocimum gratissimum L.*, and *Plectranthus ornatus*

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Abstract
Body is always exposed to both endogenous and exogenous oxidative stress, and neutralizes them with antioxidant defense system. Imbalance between oxidant and antioxidant produces reactive oxygen and nitrogen species, causing oxidation of protein, lipid, and DNA damage that lead to aging and various types of diseases such as neurodegenerative, pulmonary, or cardiovascular disease. Therefore, antioxidative agents are important for prevention and treatment of these diseases. In this study, we screened Okinawan plant extract library of 80 species to find nuclear factor erythroid-derived two-like-2 (Nrf2)-dependent antioxidant activity by antioxidant response element (ARE)-lucifearase reporter assay. The three species extracts of Garcinia subelliptica, Ocimum gratissimum, and Plectranthus Ornatus dose-dependently induced mRNA and protein expression of HO1, a downstream antioxidant gene of Nrf2. Furthermore, these extracts induced nuclear translocation of Nrf2 and the reduction of reactive oxygen species (ROS). These results suggested that these plants are natural antioxidant sources of Nrf2 activators.

Keywords: Oxidative stress, Nrf2, herb extract, antioxidant

1. Introduction
Plants have been the important resources of medicines for humankind since thousands years and formed traditional medicine elements in medical systems of China and rest of the world [1, 2]. A number of medicines like penicillin were derived from natural plants and traditional medical plants are culturally acceptable nowadays. Research on plants and use of traditional medicinal information has received considerable interest as bioresources for a new medicine. Of all physiological activities of plants, antioxidant one is one of the most interesting and important activities for medical usage [3]. In this study, we have focused on the antioxidant activity of the plants in Okinawan Islands, a subtropical resion in Japan, because these vegetables grow under exposure to strong ultraviolet light and are thought to contain high antioxidant activities. In fact, the extracts of these vegetables contain strong radical scavenging activities and directly neutralize reactive oxygen species (ROS) in vitro and in vivo [4].
Antioxidants have a role in protecting plants against oxidative damage and are abundantly contained in plant and fruits [5]. Natural antioxidants gather attention for maintaining healthy life due to its low toxicity compared to synthetic antioxidants and a number of natural antioxidants have been intensely searched. Some substances such as sulforaphane in broccolis [6, 7], curcumin in turmeric [8, 9], and Carnosic acid in rosemary [10, 11] are reported to show antioxidant effect through activation of Nrf2.
Nuclear factor erythroid-derived two-like-2 (Nrf2) [12] is well known as a transcription factor to maintain cellular redox homeostasis [13]. Nrf2 is characterized as a member of the cap “n” collar (CNC) subfamily of basic region leucine zipper (bZip) transcription factor and bound in cytoplasm in basal state to Keap1 which is anchored to the actin cytoskeleton and an adopter protein for CULLI3-based ubiquitin E3 ligase that ubiquitinates Nrf2 for proteasomal degradation [14]. In addition to Keap1-CULLI3 pathway, degradation of Nrf2 is also regulated by β-TrCP -GSK3 pathway. Phosphorylation of Neh6 domain of Nrf2 by GSK3 leads to the binding β-TrCP in this domain and the formation of CULLI1-based ubiquitin E3 ligase [15, 16]. Under oxidative stress, reactive oxygen species oxidizes cysteine thiol in Keap1 causing the conformation change of Keap1 that lead to the disruption of keap1-Nrf2 association and Nrf2 is released from Keap1 and translocated to the nucleus. Thiol residues in KEAP1 act as electrophilic sensors [17]. Then Nrf2 binds to antioxidant response element (ARE) and induces ARE target genes coding antioxidant enzymes such as Heme oxigenase 1 (HO1) [18], NADPH

dehydrogenase quinone 1 (NQO1), Cystathionine gamma-synthase (CGS) [19]. Recently, Nrf2 and Keap1 system is the critical therapeutic target and the drugs to activate Nrf2 have been intensely investigated [20].

In this study, we screened a herb extract library of 80 species living in Okinawa Islands to find direct antioxidant Nrf2-dependent activity using ARE-lucifearase reporter assay system. Then the three candidates of plants, *Garcinia subelliptica*, *Ocimum gratissimum L., and Plectranthus ornatus*, were chosen for natural source of Nrf2 activators.

2. Materials and methods

2.1 Plant extracts

The library of 50 % aqueous ethanol extract of plants (OP Biofactory, Okinawa, Japan) was gifted by Kobayashi Pharamaceutical Co. Ltd. (Osaka, Japan). Lyophilized 50 % aqueous ethanol extract of *Garcinia subelliptica, Ocimum gratissimum L.*, and *Plectranthus ornatus* were purchaced from OP Biofactory and dissolved in DMSO.

2.2 Cells

HEK 293T cells or HEK293 cells (RIKEN cell bank) were cultured in 10% FBS-containing Dulbecco's Modified Eagle's medium (DMEM)-low glucose or 10% FBS-containing DMEM-high glucose at 37 ºC in a humidified 5% CO_2 incubator.

2.3 Nrf2 luciferase reporter assay

HEK 293T cells were seeded at 1.5 x 10^5 cells / well of 6-well plate and cultured 16 h then transfected with pGL4.37[luc2p/ARE/Hygro] vector (Promega, Madison WI) by using PEI-MAX (Polyscience, Warrington, PA) according to the instructions. Six hours after transfection, cells were collected and seeded at 1.5 x 10^4 cells /well in a 96-well plate with various concentrations of plant extracts in 10 % FBS containing-DMEM (High glucose) medium and cultured for 23h. Cells were lysed with 70 µl of lysis buffer and transferred in white 96-well plate. The luciferase activity was measured as the intensity of luminescence by using a micro plate reader (TriStar LB 941, BERTHOLD TECHNOLOGIES, Bad Wildbad, Germany).

2.4 MTT assay

HEK293 cells were seeded in 96-well plate at 0.75 x 10^4 cells /well and cultured 16 h. Cells were treated with plant extracts in 2% FBS containing-DMEM (high glucose) medium or 0.2% or 0.3% DMSO containing 2% FBS-DMEM (high glucose) mediun for 24h. Culture medium was replaced with 833 µg/ml of MTT in DMEM medium and cells were incubated at 37ºC for 2h in the absence of light. After the medium was removed, MTT-formazan crystals were dissolved in 150 µl of elution solution (4 mM HCl, 0.1% NP-40 in isopropanol) per wells by shaking. The absorbance was measured using a micro plate reader at 590 nm.

2.5 Gene expression analysis

HEK 293 cells were seeded at 5 x 10^4 cells / well of 24-well plate and cultured for 16 h. After treatment with several concentration of plant extracts in DMEM (high glucose) medium with 2% FBS for 6h, total RNA was extracted from each well by using RNeasy Mini kit (Qiagen, Hilden Germany). Complementary DNA was synthesized with ReverTra Ace (TOYOBO, Osaka Japan). Gene expression was quantified by using KOD SYBR qPCR (TOYOBO) with the 7500 First Real-Time PCR System (Applied Biosystems Foster City, CA). The relative expression levels of each samples were calculated by using the ddCt method with the expression level of vehicle control. The primer pairs used are listed below.

<HO-1>
Forward: 5'-AAGACTGCGTTCCTGTCAAC-3'
Reverse: 5'-AAAGCCCTACAGCAACTGTCG-3'
<NQO1>
Forward: 5'-CCGCAGACCTTGTGATATTCCA-3'
Reverse: 5'-AGAGTACATGGAGCCATGCCA-3'
<γGCS>
Forward: 5'-CATCATCAATGGGAAGGAAGGT-3'
Reverse: 5'-TTTGCGATAAACTCCCTCATCC-3'
<Nrf2>
Forward: 5'- TTCCCGGTCACATCGAGAG-3'
Reverse: 5'- TCCTGTTGCATACCGTCTAAATC-3'
< GAPDH>
Forward: 5'-ACCTGCCGTCTAGAAAAACCTGC -3'
Reverse: 5'-CTTGACAAAGTGGTCGTTGAGGG-3'
<β-actin >
Forward: 5'- CATGTACGTTGCTATCCAGGC-3'
Reverse: 5'- CTCCTTAATGTCACGCACGAT-3'

2.6 Protein preparation

HEK 293 cells were seeded at 7.5 x 10^4 cells / well of 12-well plate and cultured for 16 h. After treatment with several concentration of plant extracts in DMEM (high glucose) medium with 2% FBS for 24 h. Cells in each well were lysed with 100 µl of RIPA buffer for 40 min on ice. Cell lysates were centrifugated at 8000 x g for 10 min at 4 ºC and supernatants were collected as cell lysate. Protein concentrations were quantified using Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA).

2.7 Preparation of whole cell lysates and Nuclear fractions

HEK293 cells were seeded at 1.5 x 10^6 cells/10 cm dish and cultured for 40 h.

Culture medium was changed with 60 µg/ml of plant extracts in DMEM (high glucose) medium with 2% FBS and incubated for 3h. Cells were collected with trypsinization, and lysed with RIPA buffer as whole cell lysates. In the other experiments, cells were washed with PBS and suspended with 1.2 ml of cold TMK buffer (20 mM Tris-HCl pH7.4, 25 mM KCl, 5 mM $MgCl_2$), then homogenized and centrifuged at 600 x g for 10 min at 4 ºC. Pellets were collected as nuclear fractions and lysed with RIPA Buffer. Protein concentrationw were quantified using Pierce BCA Protein Assay kit (Thermo Fisher Scientific).

2.8 Antibodies

Anti-HO-1 antibody (sc-136960 Santa Cruz Biotechnology, Dallas TX) anti-Nrf2 antibody (#200-3 MBL, Nagoya, Japan) and Anti-Lamim B1 antibody (sc377000, Santa Cruz Biotechnology) were used. Horseradish peroxidase-conjugated goat anti-rabbit IgG (or horseradish peroxidase-conjugated goat anti-mouse IgG antibody) (CST Danvers, MA) was used as the secondary antibody.

2.9 Western blotting analysis

SDS-solved cell lysates were denatured at 95 ºC for 5 min, and resolved by 12% SDS-PAGE and transferred to a Hybond ECL nitrocellulose membrane (GE Healthcare, little Chalfont, UK) by using semi-dry transfer cell (Bio-Rad, Hercules, CA). For the detection of Nrf2 and Lamin B1, cell lysate was denatured at 95 ºC for 5 min resolved by 10% SDS-PAGE

and transferred to immobilion-P (Merck Millipore Ltd. Burlington, MA) by using Mini Trans-Blot Cell (Bio-Rad). The membrane was blocked with blocking buffer (5% skim milk in Tris-based buffer with 0.1% Tween 20) at 4 ºC for 16 h, then washed 3 times with TBST Buffer (Tris-based buffer with 0.1% Tween 20) for 5 min, and incubated with primary antibody diluted in Can Get Signal Solution A (TOYOBO, Osaka, Japan) by 500-fold at 4 ºC for 16 h. After washed 3 times with TBST buffer for 5 min the membrane was incubated with secondary antibody diluted in Can Get Signal Solution B (TOYOBO) by 1000-fold at room temperature for 1 h, followed by 3 times-wash with Tris-based buffer with 0.1% Tween 20 for 5 min. Detection of Chemiluminescence was performed by using enzyme-linked chemiluminescence (ECL) prime Western Blotting Detection Reagent (GE Healthcare, Little Chalfont, UK) and Image Quant LAS 4000 digital Image system (GE Healthcare).

2.10 Isolation of mouse primary hepatocytes

Primary hepatocytes were obtained from 8-10w-old female ICR mice as described [21]. Hepatic cells were isolated from the liver by perfusing 0.0125% collagenase type X (Wako), followed by passing through 70-µm opening mesh. Hepatocytes were purified by two times of centrifugation at 50 x g for 2 min. Nonparenchymal cells in the supernatant were discarded. Dead hepatocytes were removed by density gradient centrifugation in 45% Percoll (GE Healthcare) at 50 x g for 10 min. The obtained hepatocytes were then suspended in William's Medium E (Sigma) supplemented with 5% fetal bovine serum (FBS) (Japan Bio Serum) and seeded on collagen-coated 96-well plate at 8 x 10^3 cells / well seeding density in Williams medium E (Sigma) containing 5% FBS, 2 mM L-Glutamine (Sigma), 0.1 µM insulin (Sigma), 0.1 µM Dexamethasone（Nacalai）.

2.11 Statistical analysis

Multiple comparisons were performed with Bonferroni's multiple comparison tests depending on the combinations of comparisons, after one-way or two-way ANOV A. All analyses were done with GraphPad Prism 7 for Mac OS X software (GraphPad Software, Inc., La Jolla, CA).

3 Results

3.1 Screening of plant extracts as activators of Nrf2.

To evaluate the 80 plant extracts for activators of Nrf2, luciferase reporter assay with ARE elements was conducted. Extracts of 8 plants, *Garcinia subelliptica*, *Ocimum gratissimum L.*, *Plectranthus ornatus*, *Talinum crassifolium*, *Alpinia zerumbet*, *Lactuca sativa*, *Benincasa hispida*, *and Basella alba* significantly increased luciferase activity (data not shown). Dose dependency of Nrf2 activation by these 8 candidates was examined (Fig.1). Extracts of *Garcinia subelliptica*, *Ocimum gratissimum L.*, and *Plectranthus ornatus* showed dose-dependent luciferase activity. At high concentration (200 µg/mL), their effect on luciferase activity was comparable to that of a potent electrophilic Nrf2 activator, tertiary butylhydroquinone (tBHQ). These three plant extracts were chosen for further validation.

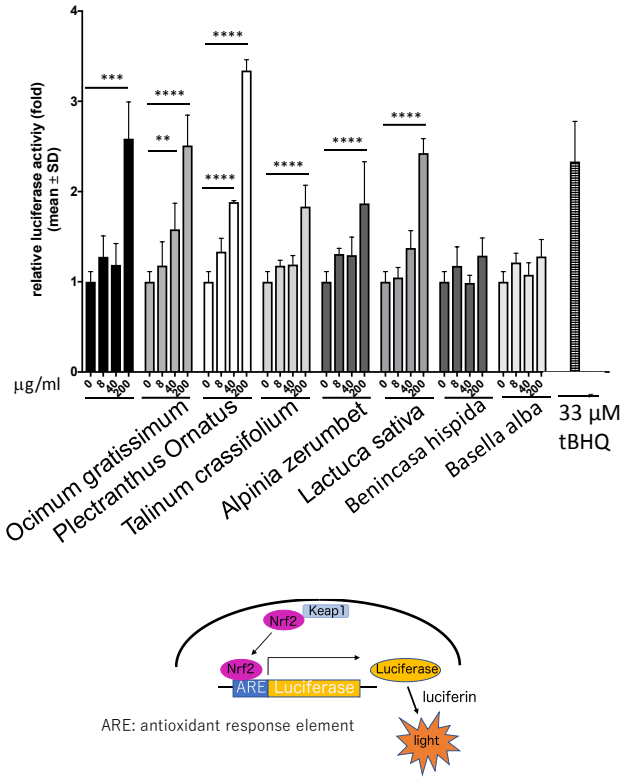

Fig 1: Screening of a plant extract library for Nrf2 activation

HEK293T cells were transfected with ARE pGL4.37[luc2p/ARE/Hygro] vector and treated by various concentration of plant extracts with the concentrations described for 24 hrs. Luciferase activity was measured as the Nrf2 activation. As a positive control, 33µM of tBHQ was used. mean±SD, n=3, Two-way ANOVA t- test *p<0.05, **p<0.01, ***p<0.001, ****p< 0.0001

3.2 Induction of ARE-dependent transcriptional activity by the plant extracts.

The induction of three antioxidant genes downstream of Nrf2, HO-1 (hemuoxygenase-1), NQO1 (NADPH dehydrogenase), and γGCS (γ glutamylcysteine synthetase) were analyzed. The mRNA expression of HO-1 was significantly induced by stimulations of *Garcinia subelliptica*, *Ocimum gratissimum L.*, and *Plectranthus ornatus* in dose-dependent manner (Fig.2a) while either NQO1 or gGCS was not (data not shown). The strong expression of HO-1 protein by these three plant extracts was also confirmed (Fig.2b). These results indicate that these plants extract practically activated intracellular Nrf2 molecules.

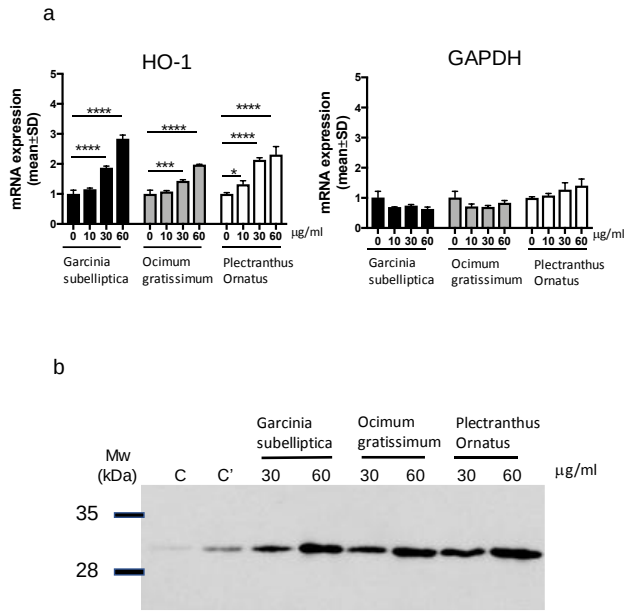

Fig 2: Extracts from *Garcinia subelliptica, Ocimum gratissimum L*., and *Plectranthus ornatus* induce the expression of Nrf2-dependent molecule, HO-1 at both mRNA and protein levels.

(a) Messenger RNA expression of HO-1.

HEK293 cells were treated with various concentrations of plant extracts and the mRNA samples collected 6 h after treatment was subjected to qRT-PCR. The relative expression levels of HO-1 and GAPDH as a control gene were shown. mean±SD, n=3, Two-way ANOVA t-test *p<0.05, ***p<0.001, ****p< 0.0001

(b) The induction of HO-1 protein.

HEK293 cells were treated with various concentrations of plant extracts. Protein samples collected 24h after treatment with various concentration of plant extracts were subjected to immunoblotting. C: control for treatment of *Garcinia subelliptica* or *Plectranthus ornatus*. Cells were treated with 0.2%DMSO/2% FBS containing DMEM medium, C': control for treatment of *Ocimum gratissimum*. Cells were treated with 0.3%DMSO/2% FBS containing DMEM medium.

3.3 Nuclear translocation of Nrf2 by treatment of *Garcinia subelliptica*

Activation of Nrf2 released Keap1 from Nrf2/Keap1 complex and released Nrf2 translocates into the nuclei. To confirm the translocation of Nrf2 by *Garcinia subelliptica*, nuclear Nrf2 protein was examined by western blotting analysis. Three hours after the addition of *Garcinia subelliptica* extracts into culture medium, the amount of nuclear Nrf2 protein was increased as compared to that of the control without any change in that of total Nrf2 protein (Fig. 3a). Figure 3b shows the protein ratio of Nrf2 to the control nuclear protein, Lamin B1 by scanning the images of Fig. 3a.

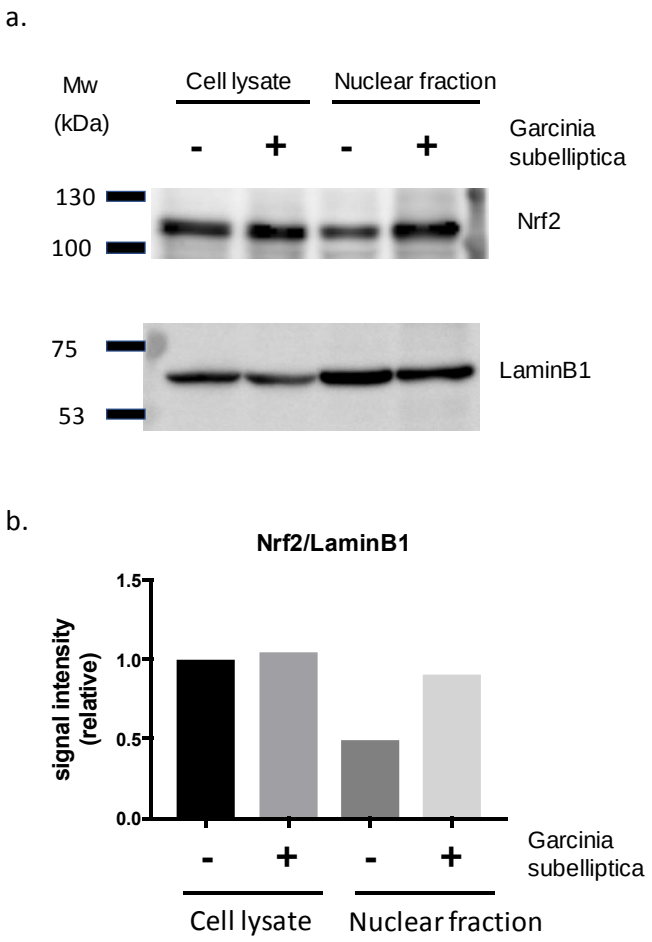

Fig 3: Extract of *Garcinia subelliptica* induces nuclear localization of Nrf2

(a) Nuclear protein and total cellular protein were collected 2 h after treatment with 60μg/mL of *Garcinia subelliptica* and subjected to immunoblotting with the indicated antibody. 20 μg protein samples were loaded per lane. (b) Signal intensities of bands detected in (a) were scanned and quantified.

3.4 The selected plant extracts do not show cell cytotoxicity.

MTT assay was performed to examine whether plant extracts induce cellular cytotoxicity. The extracts of either *Garcinia subelliptica* or *Ocimum gratissimum L*. did not exhibit cytotoxicity at even relatively high concentration (60 μg/mL), whereas the extract of Plectranthus Ornatus show slight cytotoxicity at the concentration of over 50 ug/ml (Fig.4).

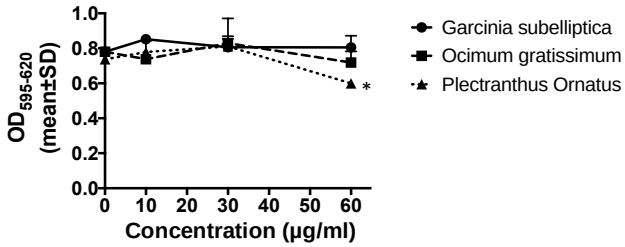

Fig 4: Evaluation of cytotoxicity of plant extracts.

HEK293 cells were treated with various concentration of plant extracts and incubated 24h. MTT assay was performed for evaluation

of cell toxicity. mean±SD, n=3, Two-way ANOVA t- test *p<0.05

3.5 Extract of Garcinia subelliptica prevented IFN-γ-induced cytotoxicity in primary hepatocytes.

We have previously reported that IFN-γ itself induced oxidative stress and promoted apoptosis in hepatocytes [22]. To evaluate the antioxidative effect of extract of Garcinia subelliptica in a pharmaceutical condition, the protective effect against IFN-γ-induced cytotoxicity in primary hepatocyte was examined by MTT assay. IFN-γ at 100 U/ml caused cytotoxicity in hepatocytes and extract of Garcinia subelliptica successfully prevented it in dose-dependent manner (Fig.5). It suggested that the extract of Garcinia subelliptica is effective for cytoprotection in liver damage.

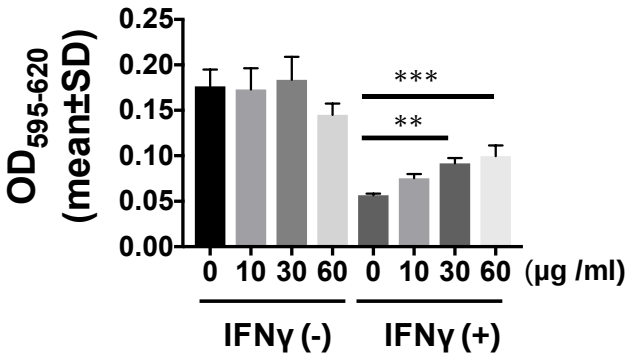

Fig 5: Extract of Garcinia subelliptica suppresses ROS-dependent cell death in primary hepatocytes

Primary hepatocytes were treated with several concentrations of Garcinia subelliptica. with / without 100 U/ml of IFN-γ. The effect of Garcinia subelliptica on IFN-induced cytotoxicity were evaluated by MTT assay.
mean±SD, n=4, Two-way ANOVA t-test **p<0.01, ***p<0.001,

4. Discussion

Antioxidant activity is divided in two major mechanisms. That is, (1) extracellularly trapping ROS and deleting the molecule as radical scavengers and (2) inducing antioxidant genes and intracellular quenching ROS [4]. The latter is mainly dependent on the activation of the master molecule Nrf2 [4]. In this study, we have screened plant library of Ryukyu (Okinawa) islands by the index of Nrf2 activity and identified several promising species, and examined the details in Garcinia subelliptica, Plectranthus Ornatus, and Ocimum gratissimum L.

Garcinia subelliptica Merr. (Fukugi) has been used for long time in Okinawa area as the traditional medical herb. This herb contains mainly benzophenones, xanthones, and biflavonoids and show various medicinal activities such as anti-inflammatory and anti-bacterial functions [23]. Plectranthus Ornatus is a member of plectranthus family, which contains various subspecies and has been employed as tranditional medicine herbs worldwide [24], however, the feature of Ornatus is little known except the herb contains antimicrobacteria components [25]. Ocimum gratissimum belongs to the family of Labiatae and is indigenous to tropical areas such as India and West Africa. This plant is worldwidely known by various names has been also commonly used as tranditional medicne for long time. The antimicrobial and antifungal activities of the herb are widely known function and some other medical activities such as anti-inflammatory function have been reported [26]. So far, to our knowledge, the function of Nrf2 activation in these three plants have not been reported. Our results may in part explain the mechanisms of these traditional medical activities.

Plant extracts contain miscellaneous components as direct radical scavengers, Nrf2 activators or bifunctional molecules. A large number of phytochemicals were identified as Nrf2 activators with diversified mechanisms [27, 28]. The fact that the different mechanisms by various phytochemicals control Nrf2 activation may explain that the three plant extracts in our present study induced HO-1 but not NQO-1 (NAD (P) H: qinone oxidoreductase-1) or GCS (g-glutamylcysteine synthetase) despite all are under Nrf2 regulation. The activated genes by Nrf2 are supposed to depend on the specific componets of plant extracts.

Because Nrf2 functions as a protective gene againt various diseases through the antioxidant activity, a large number of the drug candidates are under development [20]. Especially, phytochemicals are one of the most attractive candidate category for varoius diseases including chronic ones [29, 30].

Of the Nrf2 protective functions, it has been gathering attention that Nrf2 suppresses ferroptosis, a novel type of regulated cell death characterized by the iron-dependent accumulation of lipid peroxides to lethal levels, which is morphologically, biochemically, and genetically distinct from apoptosis, necroptosis, and autophagy [31]. Interestingly, the recent accumulating data show that ferroptosis is the central role in the pathogenesis of various diseases such as liver [32, 33], renal [34], and cardiovascular [35]. Ferroptosis is regulated by two distinct pathways, mitochondria-dependent and –independent/GPX inhibition-dependent pathway [36]. It is noteworthy that Nrf2 suppresses the both pathways via the expression of its downstream genes [37]. Accordingly, this function has Nrf2 becomes the one of the most important taraget molecules for the clinical usages.

Extracts of three natural plant, Garcinia subelliptica, Ocimum gratissimum L., and Plectranthus Ornatus, showed induction of Nrf2 activation dose-dependently. The induction efficacy of them were comparable to the classic Nrf2 activator, tBHQ. Furthermore, these three extracts caused potent HO-1 induction. To confirm the Nrf2 activation by Garcinia subelliptica, nuclear translocation of Nrf2 was examined after treatment of Garcinia subelliptica extract. Garcinia subelliptica extract increased the amount of nuclear Nrf2 protein, but interestingly there was almost no change in total amount of Nrf2 protein. It suggested that it had no effect on Nrf2 degradation itself. Nrf2 activators such as tBHQ or Sulforaphane modify cysteine residue in KEAP1 which leads to inhibition of proteasomal degradation of Nrf2. So, total protein of Nrf2 is increased by treatment of these Nrf2 activators. Garcinia subelliptica might activate Nrf2 activation through different mechanism from general Nrf2 activators [38, 39]. Further examinations are required to answer the question about the precise mechanism of Nrf2 activation by Garcinia subelliptica.

Interferon g, one of the major inflammatory cytokines, induces ROS-dependent cell death in primary hepatocytes [22]. It is pharmaceutically significant that extract of Garcinia subelliptica suppressed the cell death because the fact would partly support the various clinical effects of these herbal medicines.

Component identification for Nrf2 activation in these plant lead to discover the novel potent antioxidants. At the same time, it might lead to discover new mechanism of Nrf2 activation and inactivation. Results from this study showed

novel potent sources of antioxidant through Nrf2 activation.

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6. References

1. Kong J-M, Goh N-K, Chia L-S and Chia T-F Recent advances in traditional plant drugs and orchids. Acta Pharmacol. Sin 2003;24:7-21.
2. Karunamoorthi K, Phil M, Jegajeevanram K, Vijayalakshmi J and Mengistie E Traditional medicinal plants: A source of phytotherapeutic modality in recource-constrained health care settings. J. Evidence-Based Complementary & Alternative Medicine 2013;18: 67-74.
3. Dragland S, Senoo H, Wake K, Holte K and Blomhoff R Several culinary and medicinal herbs are important sources of dietary antioxidants. J. Nutr 2003;133:1286-1290.
4. Aniya Y Development of biorecources in Okinawa. J. Toxicol. Pathol 2018;31:241-253.
5. Lee MT, Lin WC, Yu B and Lee TT Antioxidant capacity of phytochemicals and their potential effects on oxidative status in animals - A review. Asian-Australas J Anim Sci 2017;30:299-308.
6. Zhang Y, Talalay P, Cho CG and Posner GH A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. Proc Natl Acad Sci USA 1992;89:2399-2403.
7. Kubo E, Chhunchha B, Singh P, Sasaki H and Singh DP Sulforaphane reactivates cellular antioxidant defense by inducing Nrf2/ARE/Prdx6 activity during aging and oxidative stress. Scientific Reports 2017;7:14130.
8. Balogun E, Hoque M, Gong P, Killeen E, Green CJ, Foresti R *et al*. Curcumin activates the haem oxygenase-1 gene via regulation of Nrf2 and the antioxidant-responsive element. Biochem J 2003;371:887-895.
9. Lin X, Bai D, Wei Z, Zhang Y, Huang Y, Deng H *et al*. Curcumin attenuates oxidative stress in RAW264.7 cells by increasing the activity of antioxidant enzymes and activating the Nrf2-Keap1 pathway. PLoS One 2019;14:e0216711.
10. Satoh T, Kosaka K, Itoh K, Kobayashi A, Yamamoto M, Shimojo Y *et al*. Carnosic acid, a catechol-type electrophilic compound, protects neurons both in vitro and in vivo through activation of the Keap1/Nrf2 pathway via S-alkylation of targeted cysteines on Keap1. J Neurochem 2008;104:1116-1131.
11. de Oliveira MR, Peres A, Ferreira GC, Schuck PF, Gama CS and Bosco SMD Carnosic Acid Protects Mitochondria of Human Neuroblastoma SH-SY5Y Cells Exposed to Paraquat Through Activation of the Nrf2/HO-1Axis. Mol Neurobiol 2017;54:5961-5972.
12. Moi P, Chan K, Asunis I, Cao A and Kan YW Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. Proc Natl Acad Sci USA 1994;91:9926-9930.
13. Hayes JD and Dinkova-Kostova AT The Nrf2 regulatory network provides an interface between redox and intermediary metabolism. Trends Biochem Sci 2014;39:199-218.
14. Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD *et al*. Keapl represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. Genes Dev 1999; 13:76-86.
15. Rada P, Rojo AI, Evrard-Todeschi N, Innamorato NG, Cotte A, Jaworski T *et al*. Structural and functional characterization of Nrf2 degradation by the glycogen synthase kinase 3/beta-TrCP axis. Mol Cell Biol 2012;32:3486-3499.
16. Chowdhry S, Zhang Y, McMahon M, Sutherland C, Cuadrado A and Hayes JD Nrf2 is controlled by two distinct beta-TrCP recognition motifs in its Neh6 domain, one of which can be modulated by GSK-3 activity. Oncogene 2013;32:3765-3781.
17. Kobayashi A, Kang MI, Watai Y, Tong KI, Shibata T, Uchida K *et al*. Oxidative and electrophilic stresses activate Nrf2 through inhibition of ubiquitination activity of Keap1. Mol Cell Biol 2006;26:221-229.
18. Lee BS, Heo J, Kim YM, Shim SM, Pae HO, Kim YM *et al*. Carbon monoxide mediates heme oxygenase 1 induction via Nrf2 activation in hepatoma cells. Biochem Biophys Res Commun 2006;343:965-972.
19. Rahal A, Kumar A, Singh V, Yadav B, Tiwari R, Chakraborty S *et al*. Oxidative stress, prooxidants, and antioxidants: the interplay. Biomed Res Int 2014, 761264.
20. Cuadrado A, Rojo AI, Wells G, Hayes JD, Cousin SP, L. RW *et al*. Therapeutic targeting of the NRF2 and KEAP1 partnership in chronic diseases. Nature Rev 2019;18:295-317.
21. Morita M, Watanabe Y and Akaike T Inflammatory cytokines up-regulate intercellular adhesion molecule-1 expression on primary cultured mouse hepatocytes and T-lymphocyte adhesion. Hepatology 1994;19:426-431.
22. Watanabe Y, Suzuki O, Haruyama T and Akaike T Interferon-g induces reactive oxygen species and endoplasmic reticulum stress at the hepatic apoptosis. J. Cell. Biochem. 2003;89:244-253.
23. Inoue T, Kainuma M, Baba K, Oshiro N, Kimura N and Chan EWC Garcinia subelliptica Merr. (Fukugi): A multipurpose coastal tree with promising medicinal properties. J. Intercult. Ethnopharmacol 2017;6:121-127.
24. Rice LJ, Brits GJ, Potgieter CJ and Staden JV Plectranthus: A plant for the future? South African J. Botany 2011;77:947-959.
25. Rijo P, Rodoriguez B, Duarte A and Simoes MF Antimicrobial properties of Plectranthus ornatus extracts, 11-acetoxy-halima-5,13-dien-15-oic acid metabolite and its derivatives. Natural Products J. 2011;1:57-64.
26. Prabhu KS, Lobo R, Shirwaikar AA and Shirwaikar A Ocimum gratissimum: A review of its chemical, pharmacological and ethnomedicinal properties. Open Complementary Medicine J 2009;1:1-15.
27. Qin S and Hou D-X Multiple regulations of Keap1/Nrf2 system by dietary phytochemicals. Mol. Nutr. Food Res 2016;60:1731-1755.
28. Stefanson AL and Bakovic M Dietary regulation of Keap1/Nrf2/ARE pathway: Focus on plant-derived compounds and trace minerals. Nutrients 2014;6:3777-3801.
29. Matzinger M, Fischhuber K and Heiss EH Activation of Nrf2 signaling by natural products-can it alleviate diabetes? Biotechnol. Advances 2018;36:1738-1767.
30. Jadeja RN, Upadhyay KK, Devkar RV and Khurana S

Naturally occurring Nrf2 activators: potential in treatment of liver injury. Oxidative medicine and cellular longevity 2016. Articlel ID 3453926
31. Dixon SJ, Lemberg KM, Lamprecht MR, Skouta R, Zaitsev EM, Gleason CE *et al*. Ferroptosis: an iron-dependent form of nonapoptotic cell death. Cell 2012;149:1060-1072.
32. Lorincz T, Jemnitz K, Kardon T, Mandl J and Szarka A Ferroptosis is Involved in Acetaminophen Induced Cell Death. Pathol Oncol Res 2015;21:1115-1121.
33. Tsurusaki S, Tsuchiya Y, Koumura T, Nakasone M, Sakamoto T, Matsuoka M *et al*. Hepatic ferroptosis plays an important role as the trigger for initiating inflammation in nonalcoholic steatohepatitis. Cell Death Dis 2019;10:449.
34. Friedmann Angeli JP, Schneider M, Proneth B, Tyurina YY, Tyurin VA, Hammond VJ *et al*. Inactivation of the ferroptosis regulator Gpx4 triggers acute renal failure in mice. Nat Cell Biol 2014;16:1180-1191.
35. Fang X, Wang H, Han D, Xie E, Yang X, Wei J *et al*. Ferroptosis as a target for protection against cardiomyopathy. Proc Natl Acad Sci USA 2019;116:2672-2680.
36. Gao M, Yi J, Zhu J, Minikes AM, Monian P, Thompson CB *et al*. Role of mitochondria in ferroptosis. Molecular Cell 2019;73:354-363.
37. Dodson M, Castro-Portuguez R and Zhang DD NRF2 plays a critical role in mitigating lipid peroxidation and ferroptosis. Redox Biol. 2019.
38. Jo GH, Kim GY, Kim WJ, Park KY and Choi YH Sulforaphane induces apoptosis in T24 human urinary bladder cancer cells through a reactive oxygen species-mediated mitochondrial pathway: the involvement of endoplasmic reticulum stress and the Nrf2 signaling pathway. Int J Oncol 2014;45:1497-1506.
39. Macari ER and Lowrey CH Induction of human fetal hemoglobin via the NRF2 antioxidant response signaling pathway. Blood 2011;117:5987-5997.