Pharmacological activities of different species and varieties of turmeric (*Curcuma spp.*)

(*Curcuma* spp.の薬理作用の研究)

A dissertation submitted to The United Graduate School of Agricultural Sciences, Kagoshima University, Kagoshima, Japan in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Turmeric (*Curcuma* spp.) is a rhizomatous perennial herbs with broad spectrum of pharmacological actions including antioxidant, anticancer, antibacterial, antifungal and insecticidal properties. There are more than 80 species of turmeric and 70 varieties/strains of *Curcuma longa*, which may possess different chemical properties and biological activities. Hence, we compared the major active components, antifungal, antioxidant, vasodilatory and phytotoxic effects of three *Curcuma longa* varieties (Ryudai gold; RD, Okinawa ukon, and BK2), *C. xanthorrhiza*, *C. aromatica*, *C. amada*, and *C. zedoaria*. The content of curcuminoides (curcumin, demethoxycurcumin and bisdemethoxycurcumin) were compared by HPLC and the active compounds were isolated and purified by using silica gel, TOYOPEARL® HW-40F, and high-performance liquid chromatography. The structural identification of the compounds was conducted using ^{1}H NMR, ^{13}C NMR, and liquid chromatography-tandem mass spectrometry.

The chapter I of this thesis compared curcuminoids content and antifungal activity of different species and varieties of turmeric against *Fusarium solani sensu lato* (FSSL). The antifungal activity was measured by the diameter of colonies grown on Petri dish, microscopic observation, and CLSI microdilution methods. The BK2 variety of *C. longa* contained highest concentration of curcumin, demethoxycurcumin, and bisdemethoxycurcumin followed by RD, *C. xanthorrhiza*, Okinawa ukon, and *C. aromatica*. These compounds were not present in *C. amada* and *C. zedoaria*. Among the different turmeric, RD showed strongest antifungal activity against FSSL. The order of IC_{50} against FSSL was RD (78 to 92 µg/mL) > BK2 (89 to 101 µg/mL) > *C. xanthorrhiza* (98 to 114 µg/mL) > *C. aromatica* (183 to 204 µg/mL) > *C. amada* (183 to 206 µg/mL) > Okinawa ukon (191 to 216 µg/mL) > *C. zedoaria* (354 to 385 µg/mL). Our results found a correlation

between the antifungal activity and curcuminiods contents of turmeric. However, RD contained lower curcuminoid than BK2 but showed stronger antifungal activity. Similarly, *C. amada* and *C. zedoaria* had no curcuminoids, but showed antifungal effects which indicated that other compounds present in RD, *C. amada* and *C. zedoaria* could also inhibit the growth of FSSL. Therefore, RD (contain curcuminoids) and *C. amada* (contain no curcuminoids) were chosen for the isolation and purification of antifungal compounds. The purified compounds were turmeronol B, turmeronol A, (*E)*-α-atlantone, dihydrobisdemethoxycurcumin, demethoxycurcumin, and curcumin from RD and zederone and furanodienone from *C. amada*. The IC_{50} of the isolated compounds against the four isolates of FSSL ranged from 116 to172, 127 to 185, 88 to 109, 90 to 112, 74 to 80, 63 to 68, 115 to 129, and 82 to 91 μM for turmeronol B, turmeronol A, (*E)*-α-atlantone, dihydrobisdemethoxycurcumin, demethoxycurcumin, curcumin, zederone and furanodienone, respectively.

The chapter II of this thesis compared antioxidant activity, total phenolic and flavonoid content of different species and varieties of turmeric. The antioxidant activity was determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity, oxygen radical absorbance capacity (ORAC), reducing power and 2-deoxyribose (2-DR) oxidation assay. The results suggested that RD contained significantly higher concentrations of total phenolic (157.4 mg gallic acid equivalent/g extract) and flavonoids (1089.5 mg rutin equivalent/g extract). It also showed significantly higher DPPH radical-scavenging activity (IC_{50}: 26.4 μg/mL), ORAC (14090 μmol Trolox equivalent/g extract), reducing power absorbance (0.33) and hydroxyl radical scavenging activity (IC_{50}: 7.4 μg/mL). Therefore, I purified 9 antioxidant compounds from RD namely, bisabolone-9-one (**1**), 4-methyllene-5-hydroxybisabola-2,10-diene-9-one (**2**), turmeronol B (**3**), 5-hydroxy-1,7-bis(4-hydroxy-3-

methoxyphenyl)-1-hepten-3-one (**4**), 3-hydroxy-1,7-bis(4-hydroxyphenyl)-6-hepten-1,5-dione (**5**), cyclobisdemethoxycurcumin (**6**), bisdemethoxycurcumin (**7**), demethoxycurcumin (**8**) and curcumin (**9**). The IC_{50} for DPPH radical-scavenging activity were 474, 621, 234, 29, 39, 257, 198, 47 and 18 μM and hydroxyl radical-scavenging activity were 25.1, 24.4, 20.2, 2.1, 5.1, 17.2, 7.2, 3.3 and 1.5 μM for compound **1**, **2**, **3**, **4**, **5**, **6**, **7**, **8** and **9**, respectively.

The chapter III clarify the vasodilatory mechanism of turmeric that traditionally used for the treatment of cardiovascular disorders, such as hypertension, and palpitations. A tissue-organ-bath system was used to investigate the vasoactive effects of methanol extracts of turmeric on the isolated porcine basilar artery. All turmeric extracts (20–800 μg/mL) induced concentration-dependent relaxation of the isolated porcine basilar artery without significant difference. No significant differences were observed between the endothelium-intact and denuded arteries. In depolarized, Ca^{2+}-free medium, the turmeric extracts inhibited $CaCl_2$-induced contractions. In addition, propranolol (a non-specific β-adrenoceptor antagonist) slightly inhibited the relaxation induced by turmeric. In contrast, Nω-nitro-L-arginine, indomethacin, tetraethylammonium, glibenclamide and 4-aminopyridine did not affect turmeric-induced relaxation. These results demonstrated that turmeric induced endothelium-independent relaxation of the porcine basilar artery, which may be due to the inhibition of extracellular and intracellular Ca^{2+} and the partial inhibition of β-adrenergic receptors.

The chapter IV evaluated the plant growth inhibitory activities of two cultivars of *Curcuma longa* (*C. longa*; Ryudai gold and Okinawa ukon) against radish, cress, lettuce and *Bidens pilosa* (*B. pilosa*). The methanol extracts of RD had a significantly higher inhibitory effect on the seed germination and root and shoot growth of the plants than Okinawa ukon. The growth inhibitors from RD were identified as dihydrobisdemethoxycurcumin, bisdemethoxycurcumin, demethoxycurcumin, and curcumin. The IC_{50} of the curcuminoids

against the root and shoot growth of *B. pilosa* ranged from 8.7±1.7 to 12.9±1.8 and 15.5±1.8 to 38.9±2.8 µM, respectively.

This thesis concluded that different species and varieties of turmeric contained different levels of curcuminoides, phenolic, flavonoids and other components, and showed different degree of antifungal, antioxidant, relaxation and plant growth inhibitory effects. Some other compounds except curcuminoids present in different turmeric species and strains had antifungal and antioxidant properties. All the nine compounds isolated from the turmeric RD showed antioxidant activities in different levels. The turmeric variety ‘Ryudai gold’ developed by the University of the Ryukyus showed stronger antifungal, antioxidant, relaxation and phytotoxic effects than other turmeric, which could be a potential source of natural compounds with various pharmacological actions.

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GENERAL INTRODUCTION

Plant products have been used throughout human history for numerous purposes. Medicinal plants have provided a reliable source for the preparation of new drugs as well as combating diseases, from the dawn of civilization. Although modern medicine has been routinely used in treatment of various diseases, it is less than 100 years old. However, traditional medicine, in comparison, has served mankind for thousands of years, is quite safe and effective. Even with the marked advancement in modern medicine, plants and their derivatives with diverse biological activities are still an important source for the development of new drug. Although many synthetic drugs are produced through combinatorial chemistry, plant-based drugs are more suitable, at least in biochemical terms, for human use. Nonetheless, modern medicine has neither held in very high esteem nor encouraged the medicinal use of natural products. The Indian system of holistic medicine known as "Ayurveda" and traditional Chinese medicine use mainly plant-based drugs or formulations to treat various ailments, including cancer. The mechanism or the scientific basis of traditional medicine, however, is less well understood. In addition, thousands of plant products are produced as a secondary metabolites by higher plants as a natural defense mechanism against disease and infection. Many of these have pharmacological or biological activity that can be exploited in pharmaceutical drug discovery and drug design. They are found to be more effective with least side effects as compared to commercial/synthetic products. Therefore, there is an increasing interest in the study of medicinal plants to validate their consumption as an alternative/functional food. Among spices, turmeric has been extensively studied and more number of edible *Curcuma* species is yet to be explored.

Turmeric (*Curcuma spp*.) is an important dietary spice belonging to the family Zingiberaceae, an economically important genus having both medicinal and food values. The generic name Curcuma originated from the Arabic word kurkum, meaning "yellow", and

refers to the deep yellow color of the rhizome. It is a perennial, erect and leafy plant with very large, lily like leave up to 1.2 m long. It has oblong, pointed leaves and funnel-shaped yellow flowers. Turmeric is a plant distributed throughout the tropical and subtropical regions of the world. It is widely cultivated in Asian countries, mainly in China, India, Pakistan and Bangladesh. The processed underground rhizome is commonly used in Asian cuisine as an important constituent of curry powder, dye for food and textile, and as cosmetics additive. Native and tribal people in most Asian countries use many more Curcuma species as medicinal plants, spices, food (as vegetable and source of starch) and in religious rituals. It is highly regarded as a universal panacea in the herbal medicine with a wide spectrum of pharmacological activities. As a result, plants belonging to the genus Curcuma are gaining importance all over the world and are being the subject for many investigation and exploration in recent years. The plants have been shown to contain bioactive molecules that possess pharmacological properties like antioxidant, anti-inflammatory, antimicrobial, anti-fertility, anti-venom, hypocholestraemic, hypolipidemic, antirheumatic, antiviral, antifibrotic, antivenomous, antihepatotoxic, antidiabetic, antinociceptive, anticancerous, and gastroprotective properties, antialzheimer anticoagulant, digestive stimulant. (Padalia *et al*., 2013, Policegoudra *et al*., 2010, Jeon *et al*., 2015, Srivastava *et al*., 2006). The plant has also shown to possess anti HIV activity to combat AIDS. Some species have been reported for their antifungal, antioxidant, vasodilatory and phytotoxic properties.

The genus *Curcuma* comprises approximately 80 perennial rhizomatous species (Xia *et al*., 2005 Sasikumar, 2005) and some species has multiple cultivars/varieties, for example *C. longa* has about 70 varieties in India (Sasikumar, 2005). They could be different in their chemical properties and biological activities. Therefore, the present thesis compared some

selected biological activities of different species and varieties of turmeric namely; *C. longa* (var: BK2, Ryudai gold, Okinawa ukon), *C. xanthorrhiza, C. aromatica, C. amada and C. zedoaria. Curcuma longa*, is a common turmeric species cultivated throughout the tropics and perhaps the mostly studied species for biological activities (Sharma *et al*., 2011). *Curcuma xanthorrhiza*, characterized by its deeply colored rhizome, is native to Indonesia and was used as a dye earlier but now; it is often used as a substitute for *C. aromatica* in cosmetics (Ravindran *et al*., 2007; Sharma, 2012; Liu and Wu, 1999). *Curcuma aromatica*, also known as aromatic turmeric, is a wild turmeric and the most widely used Curcuma species next to the common turmeric (*Curcuma longa*) (Bown, 1995). *C. aromatica*, are also used medicinally for the treatment of snakebites, contraceptive purposes, and as blood purifier (Reddy *et al*., 2009; Watt, 1908; Perry, 1980; Parthasarathy and Sasikumar, 2006; Chopra *et al*., 1956; Bhosle *et al*., 2009). *Curcuma amada*, popularly known as mango ginger, are morphologically similar to *Zingiber officinale* and *Curcuma longa* but lacks pungency and has a raw mango flavor due to the presence of car-3-ene and cis-ocimene (Rao *et al*., 1989; Ravindran *et al*., 2007; Sharma, 2012). *C. amada* have been reported to be used as an appetizer, alexteric, antipyretic, aphrodisiac, laxative, for mood disorders, biliousness, and bronchitis (Warrier *et al*., 1994). *Curcuma zedoaria* also known as white turmeric or zedoary, is indigenous to Bangladesh, Sri Lanka, and India (Burkill, 1966). The rhizomes and roots of *C. zedoaria* are also being utilized as a rubefacient, carminative, expectorant, demulcent, diuretic, and stimulant (Sharma *et al*., 2011; Wilson *et al*., 2005). Apart from the usage of *C. longa* in cosmetics and food industry, the other edible species of Curcuma find application as ornamental plants due to their exotic fragrance and are also used as functional foods with high nutritive value. Therefore, research on pharmacological studies of new uninvestigated compounds from turmeric is desirable and will provide immense opportunities for the

development of new plant-based food and pharmaceutical products.

The rhizome of turmeric species is a rich source of beneficial phenolic compounds known as curcuminoids (Srinivasan, 1953; Lechtenberg *et al.*, 2004). Curcumin, demethoxycurcumin, and bisdemethoxycurcumin are major chemical constituent in turmeric, having a wide range of pharmacological properties. However, different species and varieties have been reported to contain different concentration of these major curcuminoids. Some of the turmeric species even do not contain these curcuminoids but showed pharmacological activities. Therefore, beside these major curcuminoids, many other active compound with diverse pharmacological action could be explored. The amounts of the active principles vary with variations in different agro-climatic zones, from one season to another, with different plant organs, with different plant clones, even with diurnal rhythms. Co-occurrence of undesirable principles may cause synergistic, antagonistic, or other undesirable, and possibly unpredictable, modulations of the bioactivity. Therefore, isolation of pure bioactive principles, and proper physicochemical characterization of bioactive principles could help chemists to synthesize new compounds within the laboratory which may also lead to the cost effective production of these natural drugs. This thesis primarily deals with the following specific objective.

1. Comparison of the major curcuminoids (curcumin, demethoxycurcumin and bisdemethoxycurcumin) content of different species and varieties of turmeric.
2. Comparison of antifungal activity of different species and varieties of turmeric and isolation of active antifungal compounds.
3. Comparison of antioxidant activity of different species and varieties of turmeric and isolation of active compounds.

4. Comparison of the vasomotor effect of different species and varieties of turmeric on isolated porcine cerebral artery.
5. Comparison of plant growth inhibitory activity of different species and varieties of turmeric and isolation of active compounds.

Chapter 1

Study 1

Antifungal Activity of Various Species and Varieties of Turmeric (*Curcuma* spp.) against *Fusarium solani sensu lato*

1. ABSTRACT

Turmeric (*Curcuma* spp.) are rhizomatous perennial herbs with broad spectrum of pharmacological actions. There are more than 80 species of turmeric and 70 varieties/strains of *Curcuma longa*, which may have different chemical properties and biological activities. Hence, we compared the major active components (curcuminoides) and antifungal activity of three *Curcuma longa* strains (Ryudai gold (RD), Okinawa ukon, and BK2), *C. xanthorrhiza*, *C. aromatica, C. amada,* and *C. zedoaria* against *Fusarium solani sensu lato* (FSSL). The content of curcuminoides was determined by HPLC and the antifungal activity was measured by the diameter of colonies grown on Petri dish, microscopic observation, and CLSI microdilution methods. The BK2 turmeric contained highest concentration of curcumin, demethoxycurcumin, and bisdemethoxycurcumin followed by RD, *C. xanthorrhiza*, Okinawa ukon, and *C. aromatica*. These compounds were not detected in *C. amada* and *C. zedoaria*. All turmeric species inhibited fungal growth in a concentration-dependent manner. The order of IC_{50} against FSSL was RD (78 to 92 µg/ml) > BK2 (89 to 101 µg/ml) > *C. xanthorrhiza* (98 to 114 µg/ml) > *C. aromatica* (183 to 204 µg/ml) > *C. amada* (183 to 206 µg/ml) > Okinawa ukon (191 to 216 µg/ml) > *C. zedoaria* (354 to 385 µg/ml). The results showed a correlation between the antifungal activity and curcuminiods contents of turmeric. Curcumin itself showed marked antifungal activity against FSSL (IC_{50} = 23 to 25 µg/ml) followed by demethoxycurcumin (IC_{50} = 25 to 27 µg/ml), while the antifungal activity of bisdemethoxycurcumin was extremely low (IC_{50} = 216 to 238 µg/ml). However, *C. amada* and *C. zedoaria* had no curcuminoids but showed antifungal effects which indicated that other compounds could also inhibit the growth of FSSL. The obtained results demonstrated that turmeric species *C. longa* (strains Ryudai gold and BK2) and *C. xanthorrhiza* had higher content of curcuminoids and showed excellent antifungal activity against FSSL.

Keywords: Turmeric species/strains; curcuminoids; antifungal activity; *Fusarium* spp.

2. INTRODUCTION

Turmeric (Zingiberaceae family, *Curcuma* genus) species grow predominantly in the tropical and subtropical regions of Asia and Africa. The *Curcuma* genus comprises over 80 species and some of these species have multiple strains/varieties, for example, *C. longa* has about 50 cultivars and 20 improved varieties (Sasikumar, 2005). The rhizome of turmeric species is a rich source of beneficial phenolic compounds known as curcuminoids (Srinivasan, 1953; Lechtenberg *et al*., 2004). The main curcuminoids are curcumin, demethoxycurcumin, and bisdemethoxycurcumin. Curcumin from *C. longa* was reported to exhibit antifungal activity, for example, it was effective against human pathogenic fungi such as *Candida* spp. and *Paracoccidioides brasiliensis* (Garcia-Gomes *et al*., 2012; Martins *et al*., 2009). Curcumin also exhibited strong to moderate fungicidal activity against plant diseases such as rice blast, rice sheath blight, cucumber gray mold, tomato late blight, wheat leaf rust and barley powdery mildew caused *in vivo* by *Botrytis cineria, Erysiphe graminis, Phytophthora infestans, Puccinia recondita,* and *Rhizoctonia solani* (Kim *et al*., 2003). The volatile oils of *C. longa, C. amada, C. zedoaria* and *C. aromatica* were effective against various fungi such as *Curvularia pallescens, Aspergillus niger,* and *Aspergillus terreus* (Singh *et al*., 2002). Methanol extract of *C. longa* inhibited the growth of *Candida albicans* in stored foods such as potato, chicken, cooking oil, masala and tomato, which suggested that turmeric extracts can provide potential alternatives to the currently used toxic fungicides in controlling post-harvest fungal deterioration of crops (Gul *et al*., 2015).

Fusarium solani sensu lato (FSSL) are well-known pathogens of plants, insects, and humans (Majumdar *et al*., 2008). There are at least 111 plant species from 87 genera are commonly infected by FSSL (Kolattukudy and Gamble, 1995). It causes crown and root rots on a wide range of plants such as pea, bean tomato, and passion fruit. Many agriculturally

important *Fusarium* fungi produce toxins (mycotoxins) which are harmful to animals and humans (Nelson *et al*., 1994). FSSL is most commonly (~50%) associated with human infections among *Fusarium* spp. It is the common cause of keratitis, onychomycosis, endophthalmitis, and skin and musculoskeletal infections (Mansoory *et al*., 2003). It is well known that FSSL species are resistant to commercially available antifungal agents.

In this study, we hypothesize that various turmeric species and strains might contain different types and concentrations of active compounds producing pharmacological effects. Comparative studies of curcuminoids contained in *Curcuma* spp. and antifungal activities of various species and strains of turmeric on FSSL isolates have never been thoroughly conducted. The use of synthetic fungicides is gradually restricted due to undesirable impacts on the environment and human health. Therefore, we compared the content of curcuminoids and antifungal activity of *C. longa* (Ryudai gold, Okinawa ukon and BK2), *C. xanthorrhiza*, *C. aromatica, C. amada,* and *C. zedoaria* turmeric species.

3. MATERIALS AND METHODS

3.1 Plant material preparation. Five turmeric species, namely *C. longa* (strains: Ryudai gold, Okinawa ukon, and BK2), *C. xanthorrhiza*, *C. aromatica*, *C. amada* and *C. zedoaria* were cultivated under the same condition at the Subtropical Field Science Center, University of the Ryukyus, Okinawa, Japan, from April 2015 to March 2016. Fresh rhizomes of turmeric harvested from the field were visibly different in morphology and color (Figure 1-1). The rhizomes were washed, sliced, and oven dried at 60˚C for 72 h. Then turmeric powder was prepared and studied.

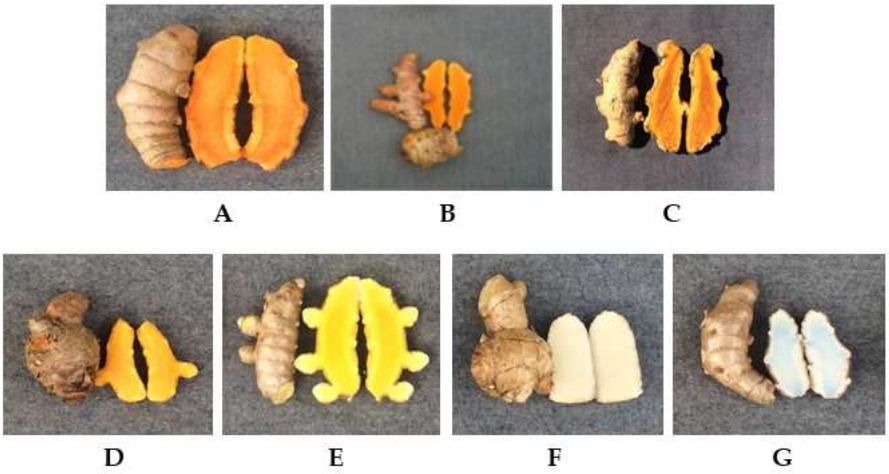


Figure 1-1. Various turmeric species and strains: *Curcuma longa* [Ryudai gold (A), BK2 (B), Okinawa ukon (C)], *Curcuma xanthorrhiza* (D), *Curcuma aromatica* (E), *Curcuma amada* (F), and *Curcuma zedoaria* (G).

3.2 Chemicals and Reagents. Potato Dextrose Agar (PDA), Sabouraud Dextrose Agar (SDA) (Becton, Dickinson and Company, France), RPMI-1640 (Life Technologies, USA), methanol (MeOH) (Nacalai Tesque, Kyoto, Japan) and pure curcuminoids (Nacalai Tesque, Kyoto, Japan) were purchased.

3.3 Microorganisms. Four isolates of FSSL (F1, F3, F10, and F17) were derived from skin

lesions of American manatees (*Trichechus manatus*) with three different genotypes (Sano *et al*., 2013). These were maintained in PDA slants and stored at 24-26˚C. These fungi were sub-cultured for one week before using in the experiments.

3.4 Extraction of samples. The extractions were carried out by dissolving different turmeric powders in MeOH at room temperature (27˚C) and atmospheric pressure, and kept for 2 days with shaking. The soluble compounds were filtered using filter paper (Advantec no. 5A). Fresh solvents were added to the used material and the process was repeated three times. The filtered solutions containing plant compounds were dried on a rotary evaporator. The deposits of all extracts were kept in refrigerator at 4˚C for experimental analyses.

3.5 HPLC analysis of curcumin, demethoxycurcumin and bisdemethoxycurcumin. For the analysis of curcuminoids, 0.1g of turmeric powder was dissolved in 40 mL ethyl alcohol (99.5%) at room temperature (27˚C) and atmospheric pressure using vortex mixer followed by ultrasonic water bath at 50˚C for 10 min. The soluble compounds were filtered using filter paper (Advantec no. 5A). Fresh solvents were added into the residues on filter paper and the process was repeated three times. Then, ethyl alcohol was added up to 250 mL solution. Curcumin, demethoxycurcumin, and bisdemethoxycurcumin were determined by HPLC. Shimadzu Co. Ltd. Column (Imtack Cadenza CD-C18 100 × 3.0 mm, 3 µm) was run at 40˚C with acetonitrile and 1% phosphoric acid mixture at a 43:57 ratio. A 5 µL solution sample was supplied at 0.5 mL/min rate and analyzed for 11 min. All tests were performed in triplicate.

3.6 Mycelial growth inhibition. To evaluate the activity of different turmeric extracts against FSSL (isolates F1, F3, F10, F17) mycelial growth, extracts were incorporated into the fungal growth medium (SDA) at various concentrations (32, 64, and 128 µg/mL). After

mixing, the amended SDA was dispensed into 9 cm diameter Petri dishes and allowed to cool. Mycelia from 7 day old FSSL culture were placed at the centers of Petri dishes. The plates were incubated at 25˚C and the radial growth of mycelium after 7 days was measured. The plates without extract were used as control. All the tests were performed in triplicate. The relative mycelial growth inhibition (MGI) by test turmeric compared to the control was calculated using the following formula:

$$\%MGI = (dc - dt)/dc \times 100\ [\%],$$

Where *dc* and *dt* represent mycelia growth diameters in control and treated Petri plates, respectively.

3.7 Microscopic observation. The morphological aspects of FSSL mycelia treated by various turmeric extracts at different concentrations (32, 64, and 128 µg/mL) were microscopically examined. Photomicrographs of the mycelia of FSSL were taken after 48 h of treatment with different turmeric extracts and the effect of turmeric extract on hyphal growth was monitored and compared to that in the control.

3.8 Antifungal susceptibility test. Susceptibility tests were performed in 96-well flat-bottom microplates using a standard two-fold broth micro dilution method, based on the Clinical and Laboratory Standards Institute (CLSI) recommendations (CLSI, 2008). Liquid RPMI-1640 medium buffered to pH 7.0 with 1.0 M NaOH, was filter sterilized. Isolates were cultured on PDA slants at 25˚C for 7 days. The inoculum was prepared by rubbing the surface of fungal colonies with 3 mL of sterile liquid RPMI-1640 from PDA slant using a loop. The suspension was then filtered to remove the hyphae and adjusted to a final inoculum ranging from 2×10^3 to 10×10^3 colony-forming units (CFU)/mL by the use of hemocytometer (Neubauer chamber). A stock solution of turmeric was prepared in 100%

MeOH. The medium with the fungal isolates, without any extract, was used as a growth control. Microplates were prepared with serial dilutions of each samples ranging from 0.25 to 256 µg/mL. Aliquots (50 µL) of fungal suspensions with different concentration of samples were incubated at 25˚C. All tests were performed in triplicate. After 24, 48 and 72 h, hyphal growth was monitored to determine the minimum effective concentration (MEC). The MEC was defined as the lowest concentration of an agent that led to abnormal growth leading to small, rounded, compact hyphal forms of the test fungus.

3.9 Statistical analysis. Results were expressed as the mean ± standard error of mean (SEM). Multiple comparisons were performed using one-way analysis of the variance (ANOVA) followed by the Tukey's HSD test. All experiments were carried out three times in triplicate. Differences were considered significant at $P<0.05$.

4. RESULTS

4.1 Curcumin, Demethoxycurcumin and Bisdemethoxycurcumin Content of Various Turmeric Samples

Experimental results showed that BK2 contained the highest concentration of curcumin, demethoxycurcumin and bisdemethoxycurcumin, followed by RD (*C. longa*), *C. xanthorrhiza*, Okinawa ukon (*C. longa*), and *C. aromatic*, while *C. amada* and *C. zedoaria* did not contain any of these three active compounds (Figure 1-2).

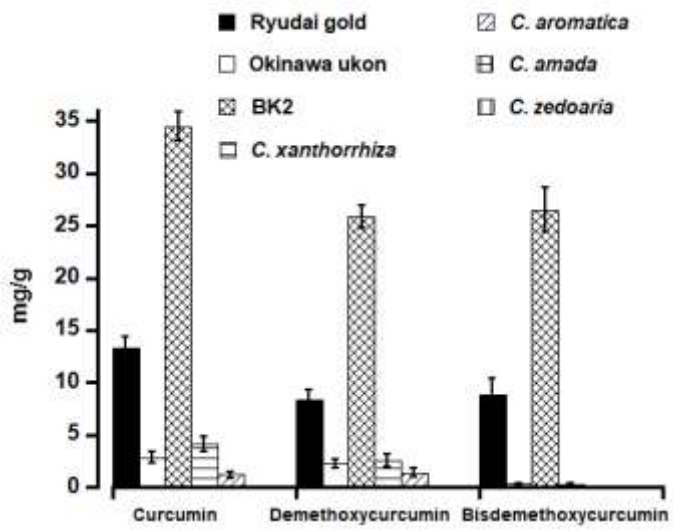


Figure 1-2. Comparison of the content of active components in different turmeric species and strains. Each bar represents the mean ± SEM for three independent experiments.

4.2 Mycelial Growth Inhibition

Experimental results showed that pure curcuminoids and methanolic extracts of various turmeric samples exhibited dose-dependent antifungal activity. Curcumin and demethoxycurcumin showed strong antifungal activity with IC_{50} ranging from 23 to 25 and 25 to 27 µg/mL, respectively, which were significantly lower than that of total

turmeric. There were no significant differences between the IC_{50} values of RD, BK2 and *C. xanthorrhiza* (78 to 92, 89 to 101, and 98 to 114 μg/mL, respectively) which were significantly lower than the values for *C. aromatica* and *C. amada*, Okinawa ukon, and bisdemethoxycurcumin (183 to 204, 183 to 206, and 191 to 216 μg/mL, respectively); *C. zedoaria* showed the lowest antifungal effect with IC_{50} value ranging from 354 to 385 μg/mL in case of total fungal isolates (Table 1-1). The order of antifungal activity of the pure curcuminoids and 7 different turmeric extracts was as follows: curcumin > demethoxycurcumin > RD > BK2 > *C. xanthorrhiza* > *C. aromatica* > *C. amada* > Okinawa ukon > bisdemethoxycurcumin > *C. zedoaria* (Figure 1-3).

Table 1-1. IC_{50} Values of Curcuminoids Isolated from Various Turmeric Species and Strains

	IC_{50} (μg/mL)			
	F. solani 1	*F. solani* 3	*F. solani* 10	*F. solani* 17
Curcumin	25.6±0.6[a]	23.6±0.8[a]	23.9±0.6[a]	24.4±0.8[a]
Demethoxycurcumin	26.1±0.2[a]	25.2±0.6[a]	25.7±0.5[a]	27.3±0.3[a]
Bisdemthoxycurcumin	216.2±2.8[c]	238.0±1.5[c]	225.4±1.0[c]	219.9±1.0[c]
C. longa (Ryudai gold)	88.6±4.6[b]	78.4±0.6[b]	88.9±3.1[b]	91.9±2.3[b]
C. longa (Okinawa ukon)	191.1±4.8[c]	215.6±6.7[c]	201.7±11.5[c]	188.1±1.2[c]
C. longa (BK2)	89.5±1.5[b]	92.3±1.7[b]	101.2±3.9[b]	101.3±2.7[b]
C. xanthorrhiza	105.5±4.9[b]	104.9±4.4[b]	97.7±0.9[b]	113.8±4.1[b]
C. aromatica	204.2±10.9[c]	182.7±1.2[c]	185.2±4.2[c]	193.1±2.3[c]
C. amada	190.3±2.9[c]	182.6±27.1[c]	188.2±2.5[c]	206.7±5.9[c]
C. zedoaria	353.8±10.5[d]	357.1±11.5[d]	367.8±8.2[d]	384.7±8.8[d]

Mean values with various indices a-d within a column are significantly different for Tukey's HSD, $p < 0.05$.

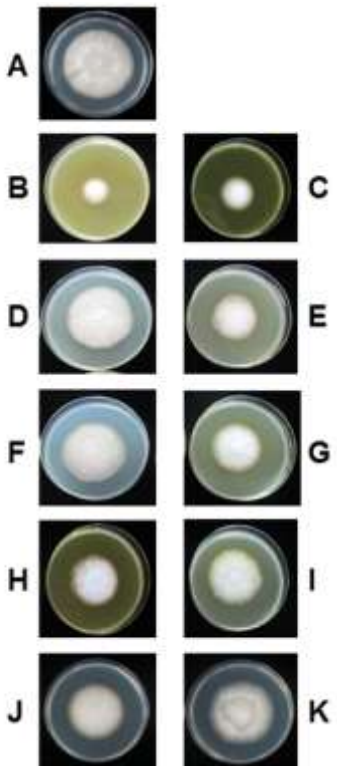


Figure 1-3. Effects of curcuminoids and extracts of various turmeric species and strains on mycelial growth of *F. solani* (F10) at 64 µg/mL after 7-day incubation: (A) control; (B) curcumin; (C) demethoxycurcumin; (D) bisdemethoxycurcumin; (E) RD; (F) Okinawa ukon; (G) BK2; (H) *C. xanthorrhiza*; (I) *C. aromatica*; (J) *C. amada*; (K) *C. zedoaria*.

4.3 Fluorescent Microscopic Observation of Hyphal Growth

Curcumin and demethoxycurcumin produced concentration-dependent decrease in hyphal growth rates compared to control. Similarly, RD, BK2 and *C. xanthorrhiza* exhibited higher antifungal effects against *F. solani* as compared to that of *C. amada*, *C. aromatica,* and *C. zedoaria* at all tested concentrations (32, 64 and 128 µg/mL). Similar to the mycelial growth inhibition test, the order of the turmeric effect on hyphal growth was as follows: curcumin > demethoxycurcumin > RD > BK2 > *C. xanthorrhiza* > *C. aromatica* > *C. amada* > Okinawa ukon > bisdemethoxycurcumin > *C. zedoaria* (Figure 1-4).

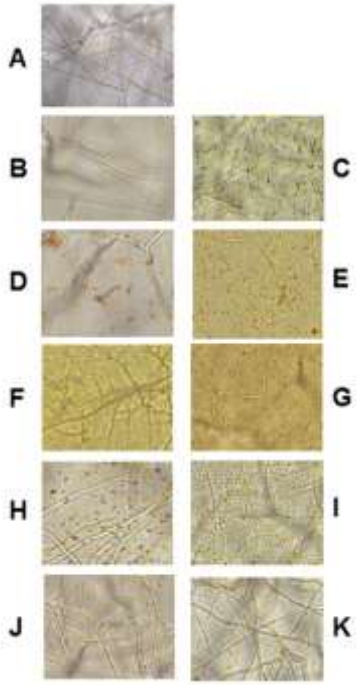


Figure 1-4. Microscopic examination of the hyphal growth of *F solani* (F10) after 48-h treatment with curcuminoids and extracts of various turmeric species and strains at 64 µg/mL: (A) control; (B) curcumin; (C) demethoxycurcumin; (D) bisdemethoxycurcumin; (E) RD; (F) Okinawa ukon; (G) BK2; (H) *C. xanthorrhiza*; (I) *C. aromatica*; (J) *C. amada*; (K) *C. zedoaria*.

4.4 Antifungal Susceptibility Test

The MEC values obtained for four FSSL isolates at 24, 48 and 72 h are shown in Table 1-2. The MEC moderately increased in all cases at 48 and 72 h in comparison to 24 h. Curcumin had lower MEC followed by demethoxycurcumin at all observation times for all fungal isolates. Among the seven turmeric extracts studied, RD showed the lowest MEC followed by BK2 and *C. xanthorrhiza*. The MEC values of *C. aromatica*, *C. amada* and *C. zedoaria* were two- to four-fold higher than those of RD, BK2, and *C. xanthorrhiza* (Table 1-2).

Table 1-2. Minimum effective concentration (MEC) of MeOH extract of various turmeric species and strains against four isolates of *F. solani* at various treatment times

Sample	MEC (µg/mL)											
	Isolate F1			Isolate F3			Isolate F10			Isolate F17		
	Time (h)			Time (h)			Time (h)			Time (h)		
	24	48	72	24	48	72	24	48	72	24	48	72
Curcumin	2	4	4	2	8	16	1	4	4	4	8	16
Demethoxycurcumin	4	8	8	2	16	16	2	8	16	4	16	32
Bisdemethoxycurcumin	64	64	128	64	128	256	32	128	128	64	256	256
C. longa (Ryudai gold)	4	8	16	2	16	32	2	16	32	8	16	64
C.longa(Okinawa ukon)	32	64	128	64	128	128	16	128	128	64	128	256
C. longa (BK2)	4	16	16	4	16	32	4	32	64	8	32	64
C. xanthorrhiza	4	16	32	8	32	64	8	64	64	16	32	64
C. aromatica	8	32	32	16	64	64	8	64	16	32	64	128
C. amada	16	64	64	32	64	16	16	64	16	64	64	256
C. zedoaria	64	16	16	64	256	256	64	128	256	128	256	256

5. DISCUSSION

In this study, we hypothesize that various turmeric species and strains might contain different type/concentration sets of active compounds responsible for pharmacological effects. Our study demonstrated that there are significant variations in the content of curcuminoids and antifungal activity against FSSL of different turmeric species and strains. Particularly, *C. longa* strain BK2 contained the highest amount of curcumin, demethoxycurcumin and bisdemethoxycurcumin followed by RD (*C. longa), C. xanthorrhiza*, Okinawa ukon (*C. longa*) and *C. aromatica*. These results are concordant with a previous study which compared curcumin, demethoxycurcumin and bisdemethoxycurcumin content among *C. longa, C. aromatica* and *C. zedoaria* and reported that *C. longa* contained highest amount of all three curcuminoids (Tohda *et al*., 2006). Minami *et al*. (Minami *et al*., 2009) reported that *C. xanthorrhiza* contained 1.12% dry weight of curcumin, whereas our result showed that *C. xanthorrhiza* contained 0.41% dry weight of curcumin. The difference might be due to variations in the soil factors, climatic factors, management factors, etc. It has been previously suggested that curcumin content of turmeric depends on environmental factors such as minimum relative humidity, altitude, soil nitrogen content and soil pH (Akbar *et al*., 2016). Earlier study suggested that *C. aromatica* contained trace amount of curcumin (Tohda *et al*., 2006; Minami *et al*., 2009) but not demethoxycurcumin and bisdemethoxycurcumin (Tohda *et al*., 2006), which is very close to our result that demonstrated *C. aromatica* containing trace amount of curcumin and demethoxycurcumin but not bisdemethoxycurcumin. Similar to Tohda *et al*. (Tohda *et al*., 2006) and Minami *et al*. (Minami *et al*., 2009), we found that C. *zedoaria* did not contain curcuminoids. A very small amount of curcumin, demethoxycurcumin and bisdemethoxycurcumin were present in acetone extract of *C. amada* (Gupta *et al*., 1999). However, we found that MeOH extract of *C. amada* did not

contain any of these three curcumins. There are no reports about the content of curcuminoids in Okinawa ukon and BK2 (two strains of *C. longa*).

In this study, curcumin and demethoxycurcumin showed strong antifungal activity with the IC_{50} ranging from 23 to 25 µg/mL and 25 to 27 µg/mL, respectively, for all fungal isolates. The antifungal activity of curcumin against two phytophagous fungi, FSSL and *Helminthosporium oryzae,* has been reported in (Chowdhury *et al*., 2008). Demethoxycurcumin and bisdemethoxycurcumin also exhibited antifungal activity against *Phomopsis obscurans* and *Phomopsis viticola*, respectively (Radwan *et al*., 2014). However, in our study, bisdemethoxycurcumin showed weaker antifungal activity against FSSL.

According to the obtained results, the antifungal activity was correlated with the curcumin and demethoxycurcumin content of turmeric species. For example, curcuminoid fractions isolated from the turmeric of BK2, RD and *C. xanthorrhiza* were greater than those of Okinawa ukon and *C. aromatica* and showed higher antifungal activity accordingly. Similarly, the MEC of curcumin was lowest among all fungal isolates. Among the seven turmeric extracts studied, RD showed the lowest MEC followed by *C. longa* strain BK2 and *C. xanthorrhiza*. However, the calculated IC_{50} for RD was minimum (78 to 92 µg/mL) among the turmeric species and strains studied, which implies its highest antifungal potential. This result suggested that some other active compounds beside curcuminoids existed with synergistic/additive effects probably responsible for stronger antifungal activity of *C. longa* strain RD than those of BK2. However, *C. xanthorrhiza* contained comparatively lower amount of curcuminoids but showed stronger antifungal activity against FSSL. This result could be explained by the fact that it contains a compound called xanthorrhizol that has been reported for antifungal

activity (Rukayadi and Hwang, 2007). Xanthorrhizol decreased the germination of conidia of *A. flavus*, *A. fumigatus*, *A. niger*, *F. oxysporum*, *R. oryzae* and *T. mentagrophytes* (Rukayadi and Hwang, 2007). Xanthorrhizol has also been reported for anticandidal activity against *C. albicans, C. glabrata, C. guilliermondii, C. krusei, C. parapsilosis* and *C. tropicalis* (Rukayadi *et al*., 2006). In addition, *C. aromatica* and Okinawa ukon showed moderate antifungal activity against FSSL in this study. Considering these results, it appears that turmeric species and strains containing curcuminoids exhibit higher antifungal activity.

On the other hand, *C. amada* and *C. zedoaria* did not contain curcuminoids but showed antifungal activity, which suggested that *C. amada* and *C. zedoaria* contained some other active antifungal compounds. It was reported that myrcene and pinene were the major components of volatile oils in *C. amada* responsible for antifungal activity against *Curvularia pallescens, Aspergillus niger, A. terreus, F. moniliforme* and *F. oxysporium* (Singh *et al*., 2002). Although *C. amada* (Udayakumar *et al*., 2015) and *C. zedoaria* (Wilson *et al*., 2005) were found effective against *Candida albicans, Candida glabrata, Cryptococcus* sp. and *Microsporum canis* (Udayakumar *et al*., 2015;Wilson *et al*., 2005), there was no specific information regarding their active compounds. Information concerning chemical composition of *C. amada* and *C. zedoaria* are very restricted. Therefore, active antifungal compounds of *C. amada* and *C. zedoaria* will be identified in future research.

Chapter 1

Study 2

Isolation and Structural Elucidation of Antifungal Compounds from Ryudai Gold (*Curcuma longa*) Against *Fusarium solani sensu lato*

1. ABSTRACT

In a previous study, we reported that *Curcuma longa* strain Ryudai gold (RD) showed antifungal activity against *Fusarium solani sensu lato* (FSSL) among the different species and strains of turmeric. The present study focused on isolation, identification and structural elucidation of antifungal compounds in RD. The ethyl acetate (EtOAc) fraction was eluted with *n*-hexane and EtOAc with gradually increasing the concentration of EtOAc (*n*-hexane: EtOAc; 100:0; 80:20; 60:40, 40:60, 20:80 and 0:100). The antifungal compounds were isolated from the most effective fraction by using silica gel, TOYOPEARL® HW-40F column, and high-performance liquid chromatography. Structural identification of the antifungal compounds was conducted using ^{1}H NMR, ^{13}C NMR, and liquid chromatography-tandem mass spectrometry. The MeOH extract of the rhizome of RD inhibited the growth of FSSL in a concentration-dependent manner. The EtOAc fraction of the MeOH extract of RD demonstrated the highest antifungal activity against FSSL. The purified antifungal compounds were turmeronol B (**1**), turmeronol A (**2**), (*E)*-α-atlantone (**3**), dihydrobisdemethoxycurcumin (**4**), demethoxycurcumin (**5**) and curcumin (**6**). These six compounds showed concentration-dependent antifungal activity against FSSL. The concentration required for 50% growth inhibition (IC_{50}) of the four isolates of FSSL ranged from 116 to172, 127 to 185, 88 to 109, 90 to 112, 74 to 80 and 63 to 68 μM/L for turmeronol B, turmeronol A, (*E)*-α-atlantone, dihydrobisdemethoxycurcumin, demethoxycurcumin and curcumin, respectively. These results suggested that RD contained potential antifungal compounds that could be useful to control FSSL. The RD strain of *C. longa* can be a good source of natural antifungal agent.

Key words: Turmeric, Ryudai gold, *Fusarium solani sensu lato*, Antifungal compounds, ^{1}H NMR, ^{13}C NMR.

2. INTRODUCTION

Turmeric (*Curcuma longa* L.), (family: Zingiberaceae) is native to Southeast Asia and extensively used as a spice, food preservative, and coloring material. It has a variety of therapeutic uses such as antimicrobial, antifungal, insecticidal, anti-inflammatory, vasodilatory and antioxidant properties (Apisariyakul *et al*., 1995; Khattak *et al*., 2005; Akter *et al*., 2018a, 2018b). The main bioactive constituent of turmeric is curcumin which is widely used in traditional Indian medicine for the treatment of anorexia, hepatic diseases, cold, cough, and other disorders (Chin *et al*., 2013; Huminiecki *et al*., 2017). *Curcuma longa* (*C. longa)* mediated biosynthesized silver nanoparticles showed potent antibiofilm activity against gram-positive bacteria such as *S. aureus* and *S. pneumonia* (Kamble and Shinde, 2018). The EtOAc extract of *C. longa* showed potent fungicidal activities against phytopathogenic fungi under greenhouse (Kim *et al*., 2003). Curcumin, demethoxycurcumin and bisdemethoxycurcumin derived from *C. longa* were found effective against plant pathogenic fungi *Phomopsis obscurans* and *Phomopsis viticola* (Radwan *et al*., 2014). A recent study isolated curdione, isocurcumenol, curcumenol, curzerene, β-elemene, curcumin, germacrone and curcumol from *C. longa* and found effective against *Fusarium graminearum* (Chen *et al*., 2018). Turmeric has been found effective for controlling the mycelial growth of *Fusarium oxysporum* (Singh *et al*., 2002). Lean and Mohamed (1999) reported that turmeric was more antimycotic and antioxidative than lemon-grass, betel leaves, clove, black pepper leaves and *Garcinia atroviridis* (Lean and Mohamed, 1999). Moreover, the increasing resistance to antifungal compounds and the reduced number of available drugs led us to search for therapeutic alternatives among plants. Plants are the promising source for new natural antifungal drugs, even though they have relatively mild effects against human pathogenic fungi compared with commercial synthetic antifungal drugs (Devkatte *et al*.,

2005; Cavelario *et al*., 2006; Pauli, 2006).

Ryudai gold (RD) is a strain of *C. longa* developed by the University of the Ryukyus, Okinawa and registered (Registration No. 21485) by the Ministry of Agriculture, Forestry and Fisheries, Japan. This is a dwarf and short duration turmeric variety, which provides higher yield (30-40 ton/ha, fresh rhizome) with about three times bigger rhizome, deep orange color, and higher curcumin content, compared to Okinawa indigenous turmeric variety (Okinawa ukon) (Akter *et al*., 2018a). The cultivation period is about 45 days shorter and plant height is about 40 cm shorter than that of Okinawa turmeric strain. The MeOH extract of RD has shown stronger antifungal activity against FSSL than BK2 (*C. longa*), Okinawa ukon (*C. longa*), *C. xanthorrihiza*, *C. aromatica*, *C. zedoaria* and *C. amada*.[15] However, no antifungal compound has been identified from the RD rhizomes.

Fusarium solani sensu lato (FSSL) causes several diseases in human, animals, and plants (Nelson *et al*., 1994; Majumdar *et al*., 2008). According to our previous research, curcumin and demethoxycurcumin which are the major active constituents of turmeric showed the strong inhibitory effect on FSSL. Though RD contained a comparatively lower concentration of curcumin and demethoxycurcumin, however, showed the strongest antifungal effect on FSSL (Akter *et al*., 2018a). Therefore, it was thought that there are some other compounds in the RD which are responsible for the strong antifungal effect on FSSL. Therefore, the aim of our present study was to isolate and characterize the antifungal compounds from the RD rhizomes.

3. MATERIALS AND METHODS

3.1 Chemicals and materials

Methanol (MeOH), ethyl acetate (EtOAc) and *n*-hexane were purchased from Nacalai Tesque (Kyoto, Japan). Silica gel (63-200 μm (Kanto Chemical CO. Tokyo, Japan)), MeOH-d_4 (CD_3OD, Merck KGaA, Germany), Potato Dextrose Agar (PDA), Sabouraud Dextrose Agar (SDA) (Becton, Dickinson and Company, France) were purchased. TOYOPEARL® HW-40F was obtained from Tosoh Corporation, Japan. RPMI-1640 (Life Technologies, USA), antifungal drug coated plate (Eiken Chemical Co., Ltd. Tochigi, Japan) and miconazole (Wako Pure Chemical Industries, Ltd. Osaka, Japan) were purchased.

3.2 Microorganism

Four isolates of *Fusarium solani sensu lato* (FSSL; 1, 3, 10, and 17) were derived from skin lesions of American manatees (*Trichechus manatus*) with 3 different genotypes (Sano *et al*., 2013). FSSL 1, 3 and 10 were isolated from the cutaneous lesion of the face (Isolate 1: genotype A, GenBank accession number AB775568) and body skin (Isolate 3: genotype B, GenBank accession number AB775569; Isolate 10: genotype C, GenBank accession number AB775570) of a male manatee-1. FSSL 17 was isolated from palm skin of a male manatee-2 (genotype A, GenBank accession number; AB775568). The genotypes based on internal transcribed spacer 1 – 5.8s – ITS 2 regions of ribosomal RNA gene sequence. The isolates 1 and 17 were genetically identical. However, the source of the manatee was different. Thus we used the above 4 isolates. All the isolates are stored in the Department of Animal Sciences, Faculty of Agriculture, University of the Ryukyus, Japan. These were maintained in PDA slants and stored at 24-26℃. These fungi were subcultured for one week before using in the experiments.

3.3 Plant material

C. longa strain Ryudai gold (RD) was cultivated in the field of Subtropical Field Science Center, University of the Ryukyus. It has been cultivating and depositing at the Subtropical Field Science Center, Faculty of Agriculture, University of the Ryukyus since registration in 2012. We have evaluated cluster dendrogram based on fragment length pattern of different turmeric lines and species and found some dissimilarities among them (data unpublished). We also found some morphological differences between RD and other *C. longa* strains. Three kilograms of fresh rhizomes were harvested, washed, sliced and dried in a hot air oven at 40℃ for 96 h. The dried rhizomes were ground finely and a total of 300g powder was taken for the experiment.

3.4 Extraction of samples

The extraction was carried out by dissolving 300g turmeric powder in MeOH at room temperature (25℃) and at atmospheric pressure and kept for 2 days with shaking. Then it was filtered by using filter paper (No. 2; Advantec, Tokyo Roshi Kaisha Ltd., Tokyo, Japan). Fresh solvents were added into the used plant material and the process was repeated three times. The filtered solutions containing plant compounds were dried on a rotary evaporator to afford 46 g of yield. The extract yield was kept in the refrigerator at 4℃ temperature for experimental analyses.

3.5 ***Antifungal assays***

Mycelial growth inhibition

According to our previous study, we used two-fold dilution of the RD extract from 2-256 μg/mL based on the Clinical and Laboratory Standards Institute (CLSI) recommendations where the effective concentrations were 32, 64 and 128 μg/mL. Different concentrations

of RD extracts (32, 64 and 128 µg/mL) were incorporated into the fungal growth medium (SDA). After mixing, the amended SDA was dispensed into 9 cm diameter Petri dishes and allowed to cool. Mycelia from 7 days old culture of FSSL were placed in the center of the Petri dishes. The plates were incubated at 25℃ and the radial growth of mycelium was measured after 7 days. The plates without extract were used as the control. All tests were performed in triplicate. The relative growth inhibition of the treatment compared to the control was calculated as a percentage, using the following formula:

MGI (%) = (dc−dt)/dc × 100

Where dc and dt represent mycelia growth diameter in control and treated Petri plates, respectively.

Hyphal morphology of FSSL

The morphological aspects of FSSL mycelia treated by different concentration (32, 64 and 128 µg/mL) of RD extracts were examined by an automated inverted fluorescent microscope (Leica, Model No. 4000B, Germany). Photomicrographs of the mycelia of FSSL were taken after 48 h of treatment with turmeric RD extracts and the effect of turmeric extract on hyphal growth was compared with that of control.

3.6 Antifungal susceptibilities to commercial compounds

Susceptibility test was performed in a commercially available antifungal drug-coated plate, based on the CLSI standard M38-A2 broth microdilution method (CLSI, 2008). Liquid RPMI-1640 medium buffered to pH 7.0 with 1.0M NaOH, was filter sterilized. Four isolates were cultured on PDA slants at 25℃ for 7 days. The inoculum was prepared by rubbing the surface of fungal colonies with 3 mL of sterile liquid RPMI-1640 from PDA slant using a loop. The suspension was then filtered to remove the hyphae and adjusted to a final inoculum of 10^3 colony-forming units/mL using hemocytometer

(Neubauer chamber). Seven antifungal drugs including; micafungin (MCFG), amphotericin B (AMPB), flucytosine (5-FC), fluconazole (FLCZ), itraconazol (ITCZ), voriconazol (VRCZ), miconazole (MCZ) were used in this study. Hundred μL fungal suspensions was added into 96 wells of antifungal drug plates and incubated at 25℃. The medium without fungal strain was used as negative control and fungal strain without drugs was used as positive control. After 48 h, hyphal growth was monitored and compared with control. All tests were performed in triplicate. The susceptibility of FSSL isolates was graded inhibitory concentration of 100% (IC_{100}), IC_{50} and minimum effective concentration (MEC). The IC_{100} was defined as the lowest concentration of an agent that completely inhibited the growth of FSSL. IC_{50} was defined as the lowest concentration of an agent that inhibited approximately 50% of the drug-free growth control. The MEC was defined as the lowest concentration of an agent that led to abnormal growth, such as leading to small, rounded, compact hyphal forms of the fungus. Susceptibilities to amphotericin B, itraconazole and voriconazole were evaluated by IC_{100}. Susceptibility to micafungin was evaluated by the MEC. Susceptibility to flucytosine, fluconazole, and miconazole were evaluated by IC_{50} (CLSI, 2008).

3.7 Isolation of bioactive compounds from crude extract of Ryudai gold

To identify the antifungal compounds, the flowchart of bioactivity-guided purification from the crude extract of RD was described in Figure 2-1. The crude extract was diluted with 200 mLof distilled water and extracted with an equal volume of *n*-hexane and then with EtOAc. All fractions were concentrated to dryness on a rotary evaporator at 40℃. The extract yield of EtOAc, *n*- hexane and water fraction were 24, 9 and 7 g, respectively. Antifungal activity of these three fractions was determined using mycelial growth inhibition assay according to the aforementioned procedure. As EtOAc fraction showed

the strongest antifungal activity, it was selected for the isolation and purification of the bioactive compounds. The dried extract from the EtOAc fraction was subjected to chromatography on a silica gel (70g) column (30 x 3 cm). Elution was carried out using *n*-hexane and EtOAc with increasing amount of EtOAc [100:0 (F1), 80:20 (F2), 60:40 (F3), 40:60 (F4), 20:80 (F5) and 0:100 (F6)]. Antifungal activity was carried out on all six fractions and fraction 6 (F6) was determined to have the strongest antifungal activity. Thereby, the F6 was further subjected to TOYOPEARL® HW-40F column chromatography using 60% MeOH (F6-1), 80% MeOH (F6-2), and 100% MeOH (F6-3). As the fraction F6-1 and F6-3 showed strong antifungal activity, they were tested for their purity using C_{18} reversed-phase HPLC (COSMOSIL 5C_{18}-AR-II; Nacalai Tesque, Inc., Kyoto, Japan) eluted with 50% acetonitrile in water (v/v) at a flow rate of 2 mL/min, monitored at 280 nm. Three peaks from F6-1 eluted at 14.11, 15.86 and 24.47 min (Figure 2-7A) as colorless substances and three peaks from F6-3 eluted at 8.96, 10.9 and 12.04 min (Figure 2-7B) as yellow substances showed antifungal activity. The isolated compounds were dissolved in MeOH-d_4 and then subjected to spectral analysis. Nuclear magnetic resonance (NMR) spectra were recorded on BRUKER NMR spectrometers (500 MHz for ^{1}H and 125 MHz for ^{13}C) at room temperature. Chemical shifts (δ) were recorded as parts per million (ppm) relative to tetramethylsilane (TMS) as the internal standard. Mass Spectrometry (MS) experiments were carried out on Waters mass spectrometer (Quattro Micro, USA) with an electrospray ionization probe under the following instrumental conditions: Column: COSMOSIL5C18ARII, 2×150 mm. Solvent A: Water (0.1% Formic acid), Solvent B: Acetonitrile, Flow rate: 0.2 mL/min, Injection Volume: 10 μL, Run Time: 30 min, Pump mode: Binary gradient. Time program: 10% B (0 min) →100% B (20 min) → 100% B (25 min) →10% B (25.1 min) →10% B (30 min).

Oven details: CTO-20AC, temperature 40℃.

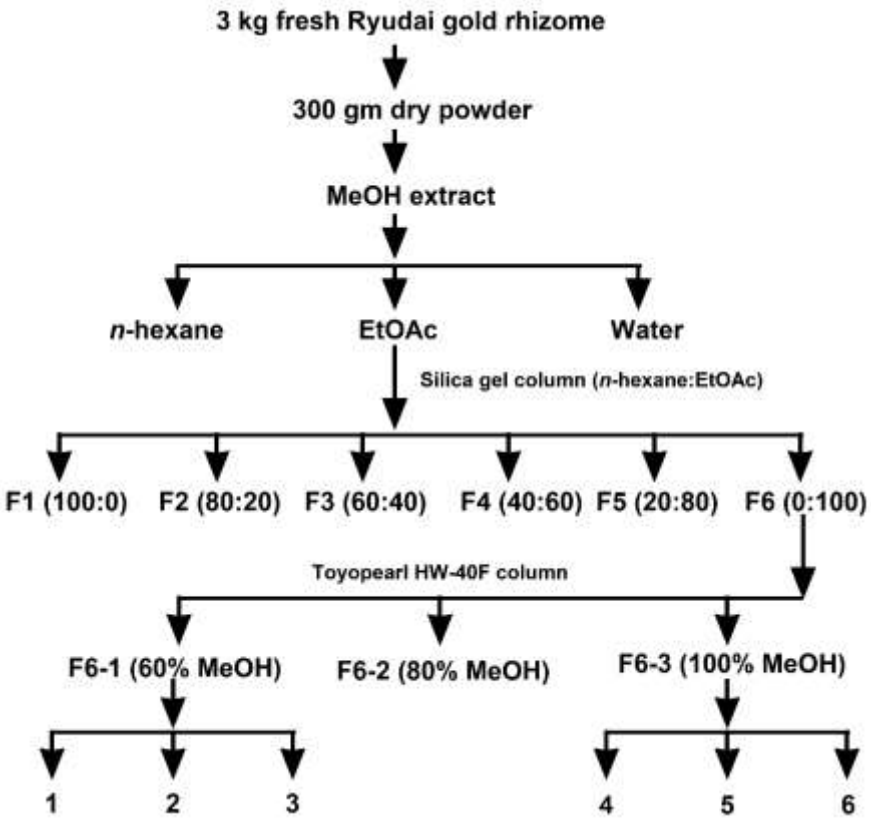


Figure 2-1. Flowchart for the isolation of active antifungal compounds from Ryudai gold.

3.8 Bioassay of the isolated compounds

Isolated compounds were dissolved in MeOH to make the stock solution of 10, 20, 50, 100 and 500 μM for each compound. Different concentrations of the isolated compounds were incorporated into the fungal growth medium (SDA). After mixing, the amended SDA was dispensed into 9 cm diameter Petri dishes and allowed to cool. Mycelia from 7 days old culture of FSSL were placed in the center of the Petri dishes. The plates were incubated at 25℃ and the radial growth of mycelium was measured after 7 days. The plates without compounds were used as a negative control. Miconazole was used as a positive control. All the tests were performed in triplicate. The relative growth inhibition of the treatment compared to the control was calculated as a percentage, using the following formula:

$$MGI\ (\%) = (dc - dt)/dc \times 100$$

Where dc and dt represent mycelia growth diameter in control and treated Petri plates, respectively.

3.9 Statistical analysis

Results are expressed as the mean ± SEM. Multiple comparisons were performed using one-way analysis of variance followed by Duncan's multiple range tests. Differences were considered significant at $p < 0.05$. The concentration required for 50% inhibition of fungal growth (IC_{50}) and isolated compounds in the assay was determined from the regression equation of the concentration-response curves. All the experiments were carried out three times.

4. RESULTS

4.1 Mycelial growth inhibition

MeOH extract of Ryudai gold (RD) inhibited the growth of all the strains of FSSL in a concentration-dependent manner (Figure 2-2). At 128 μg/mL RD inhibited about 80% growth of all the strains of FSSL (Figure 2-2). There were no significant differences in the antifungal activity of RD against the four isolates of FSSL.

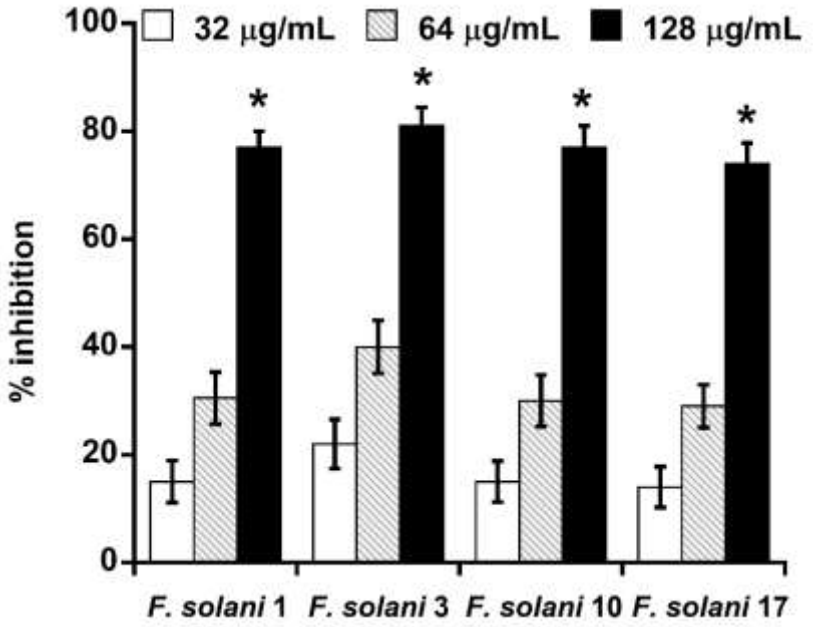


Figure 2-2. Effect of different concentrations of Ryudai gold against the four isolates of FSSL. Results are expressed as the mean ± SEM of three independent experiments. $^{*}p<0.05$ vs 64 and 32 μg/mL.

4.2 Fluorescent microscopic observation of hyphal growth

Our result demonstrated that RD had the concentration-dependent inhibitory effect on the hyphal growth of FSSL (Figure 2-3).

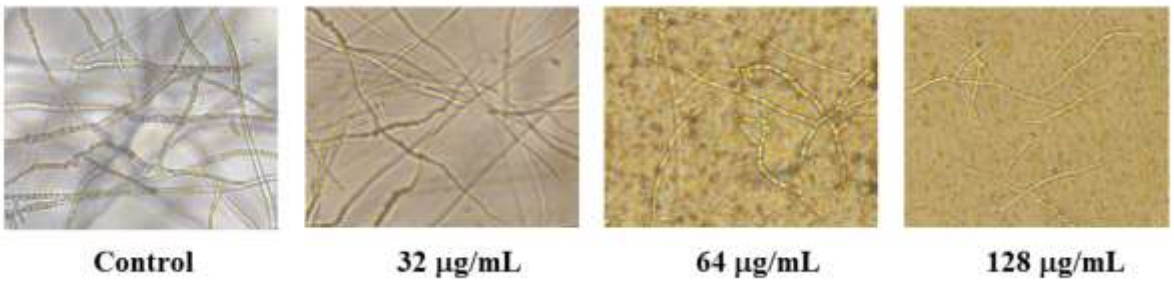


Figure 2-3. Microscopic examination of the hyphal growth of FSSL (isolate 3) after 48 h of treatment with different concentrations of Ryudai gold.

4.3 Antifungal susceptibilities to commercial compounds

Among the 7 commercial antifungal drugs, FSSL were susceptible to AMPB and MCZ. However, FSSL isolate 3 was resistant to AMPB. 5-FC, FLCZ, ITCZ, and VCZ were not effective against FSSL (Table 2-1).

Table 2-1. Antifungal susceptibilities to commercial compounds evaluated by CLSI method (CLSI, 2008).

	Antifungal agent (μg/mL)						
Isolate	MCFG (MEC)	AMPB (IC_{100})	5-FC (IC_{50})	FLCZ (IC_{50})	ITCZ (IC_{100})	VCZ (IC_{100})	MCZ (IC_{50})
1	>16	4	>16	>16	>8	>8	4
3	16	>16	>16	>16	>8	>8	4
10	>16	2	>16	>16	>8	>8	16
17	16	2	>16	>16	>8	>8	8

MCFG; micafungin, AMPB; amphotericin-B, F-FC; flucytocin, FLCZ; fluconazole, ITCZ; itraconazole, VCZ, voriconazole, and MCZ; miconazole, MEC; minimum effective concentration.

4.4 Identification of antifungal compounds

Antifungal activity of water, n-hexane and EtOAc fractions of MeOH extract of RD

EtOAc fraction obtained from MeOH extract of RD resulted in 85% inhibitory activity, whereas *n*-hexane and water fraction of RD resulted in 34% and 4% inhibitory activity, respectively (Figure 2-4).

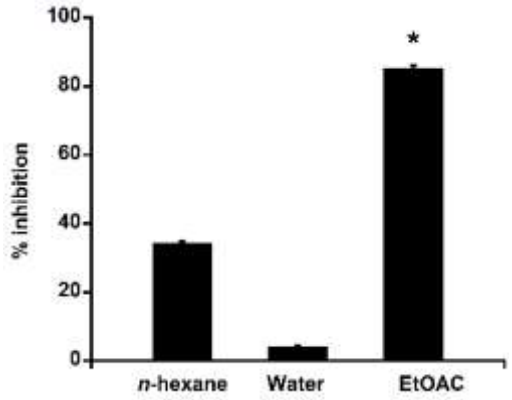


Figure 2-4. Effect of *n*-hexane, water and EtOAc extract of Ryudai gold (128 μg/mL on FSSL (isolate 3). Results are expressed as the mean ± SEM of three independent experiments. $^{*}p<0.05$ vs. water and *n*-hexane.

4.5 Antifungal activity of different fractions of EtOAc part of RD

The antifungal activity of the six fractions [EtOAc: *n*-hexane 0:100 (F1), 20:80 (F2), 40:60 (F3), 60:40 (F4), 80:20 (F5) and 100:0 (F6)] differed significantly. Among these, the F6 showed significantly stronger inhibitory activity than other fractions (Figure 2-5).

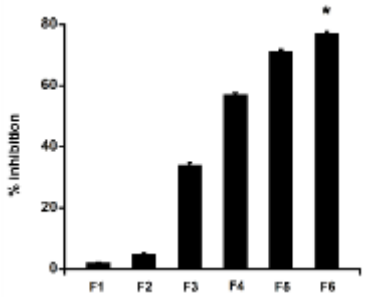


Figure 2-5. Effect of six fractions of EtOAc part of Ryudai gold against FSSL (isolate 3). Results are expressed as the mean ± SEM of three independent experiments. $^{*}p<0.05$ vs. other fractions.

4.6 Antifungal activity of three fractions obtained from F6 part of RD

Further three fractions were prepared from the purification of the F6 (F6-1: 60% MeOH, F6-2: 80% MeOH and F6-3: 100% MeOH), wherein the first fraction (F6-1) and third fraction (F6-3) resulted in 79% and 77% inhibitory activity, while F6-2 resulted in 29% inhibitory activity, respectively (Figure 2-6).

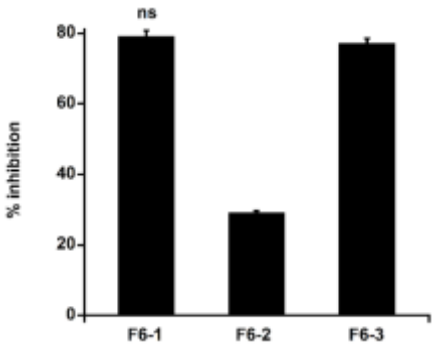


Figure 2-6. Effect of three fractions of F6 part of Ryudai gold against FSSL (isolate 3). Results are expressed as the mean ± SEM of three independent experiments. ns: not significant vs F6-3.

The antifungal compounds from the first fraction (F6-1) showed three (1-3) peaks at 280 nm at a retention time of 14.11, 15.86 and 24.47 min in the HPLC Chromatogram (Figure 2-7). The antifungal compounds from the third fraction (F6-3) showed three (4-6) peaks at 280 nm at a retention time of 8.96, 10.9 and 12.04 min in the HPLC Chromatogram (Figure 2-7).

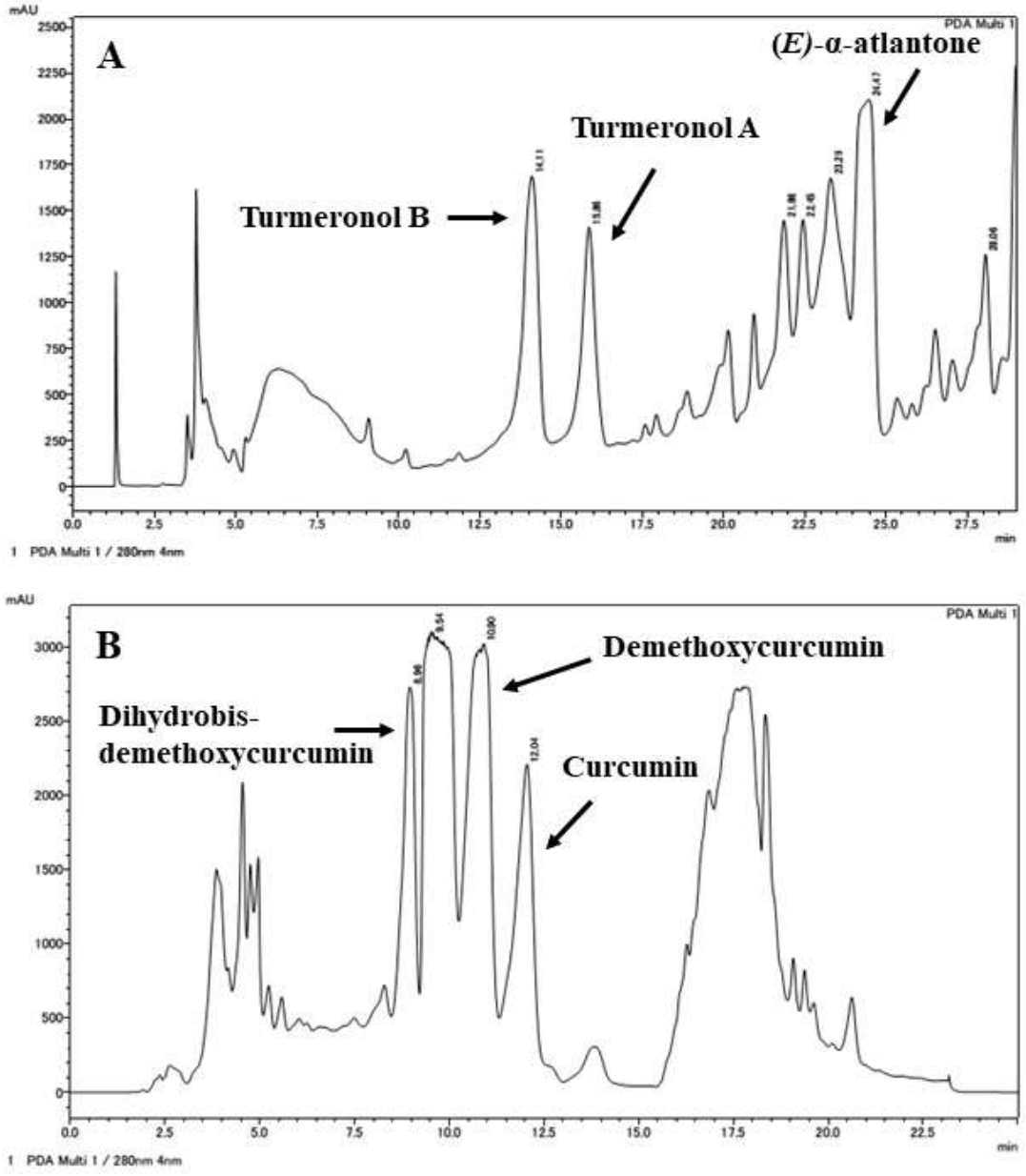


Figure 2-7. HPLC chromatograms of MeOH extract of Ryudai gold (detected at 280 nm; A: F6-1; B: F6-3).

4.7 Chemical structures of the isolated compounds

The chemical structures of the six antifungal compounds isolated from the first fraction (F6-1) and third fraction (F6-3) were identified according to their ^{1}H NMR and ^{13}C NMR spectra. Peak data were as follows:

Compound 1: Colorless oil, UV λ_{max} nm: 241. ESI-MS (+): *m/z* 233.3 $[M+H]^{+}$. ^{1}H-NMR (CD_3OD) δ: 6.89 (1H, s, H-3), 6.82 (1H, dd, *J*=8, 2 Hz, H-6), 6.62 (1H, d, *J*=8 Hz, H-5), 6.10, (1H, s, H-10), 3.12 (1H, m, H-7), 2.65 (1H, dd, *J*=15, 7 Hz, H-8a), 2.57 (1H, dd, *J*=15, 7 Hz, H-8b), 2.13 (3H, s, H-15), 2.03 (3H, s, H-13), 1.85 (3H, s, H-12), 1.18 (3H, d, *J*=7 Hz, H-14). ^{13}C-NMR (CD_3OD) δ: 203.0 (C-9), 156.4 (C-11), 154.6 (C-2), 138.5 (C-4), 130.2 (C-1), 125.8 (C-6), 125.33 (C-10), 125.26 (C-5)115.5 (C-3), 53.9 (C-8), 36.8 (C-7), 27.6 (C-12), 22.7 (C-14), 20.8 (C-13), 16.3 (C-15). From the comparison of these data with those reported in the literature by Imai *et al*. (1990) and Sharma and Tek Chand (1996), the substance was identified as turmeronol B (Figure 2-8) (Imai *et al*., 1990; Sharma and Tek Chand, 1996).

Compound 2: Colorless oil, UV λ_{max} nm: 240, 280 (sh). ESI-MS (+): *m/z* 233.3 $[M+H]^{+}$. ^{1}H-NMR (CD_3OD) δ: 6.93 (1H, d, *J*=8 Hz, H-5), 6.58 (1H, s, H-2), 6.56 (1H, dd, *J*=8, 2 Hz, H-5), 3.12 (1H, m, H-7), 2.67 (1H, dd, *J*=15, 7 Hz, H-8a), 2.58 (1H, dd, *J*=15, 7 Hz, H-8b), 2.11 (3H, s, H-15), 2.04 (3H, s, H-13), 1.85, (3H, s, H-12), 1.18 (3H, d, *J*=7 Hz, H-14). ^{13}C-NMR (CD_3OD) δ: 202.7 (C-9), 157.0 (C-11), 156.4 (C-3), 146.4 (C-1), 131.6 (C-5), 125.2 (C-10), 123.2 (C-4), 118.8 (C-6), 114.1 (C-2), 53.6 (C-8), 37.2 (C-7), 27.6 (C-12), 22.5 (C-14), 20.8 (C13), 15.8 (C-15). From the comparison of these data with those reported in the literature by Kitahara *et al*., (1993) and Sharma and Tek Chand (1996), the substance was identified as turmeronol A (Figure 2-8) (Sharma and Tek Chand, 1996; Kitahara *et al*., 1993).

Compound 3: Colorless oil, UV λ_{max} nm: 266. ESI-MS (+): *m/z* 219.4 $[M+H]^+$. ^{1}H-NMR (CD_3OD) δ: 6.14 (1H, m, H-10), 6.12 (1H, m, H-8), 5.41 (1H, brs, H-5), 2.22 (1H, m, H-1), 2.12 (3H, s, H-13), 2.11 (3H, s, H-14), 2.07 (1H, m, H-3a), 1.99 (1H, m, H-3b), 1.90 (3H, s, H-12), 1.78 (1H, m, H-2a), 1.64 (3H, brs, H-15), 1.59 (1H, m, H-2b), 1.32 (1H, m, H-6a), 1.28 (1H, m, H-6b). ^{13}C-NMR (CD_3OD) δ: 194.3 (C-9), 163.5 (C-7), 156.1 (C-11), 134.8 (C-4), 127.4 (C-10), 125.4 (C-8), 121.1 (C-5), 45.8 (C-1), 31.4 (C-3), 31.2 (C-6), 28.6 (C-2), 27.7 (C-12), 23.6 (C-15), 20.7 (C-13), 17.8 (C-14). From the comparison of these data with those reported in the literature by Manville *et al*., (1989), the substance was identified as (*E*)-α-atlantone (Figure 2-8) (Manville *et al*., 1989).

Compound 4: Yellow powder, UV λ_{max} nm: 243, 362. ESI-MS (+): *m/z* 311.2 $[M+H]^+$. ^{1}H-NMR (CD_3OD) δ: 7.50 (1H, d, *J*=16 Hz, H-4'), 7.44 (2H, d, *J*=8 Hz, H-6', 10'), 7.01 (1H, d, *J*=8 Hz, H-6, 10), 6.79 (2H, d, *J*=8 Hz, H-7', 9'), 6.68 (2H, d, *J*=8 Hz, H-7, 9), 6.43 (1H, d, *J*=16 Hz, H-3'), 2.84 (2H, t, *J*=10 Hz, H-4), 2.62 (2H, t, *J*=10 Hz, H-3). ^{13}C-NMR (CD_3OD) δ: 199.2 (C-2), 178.1 (C-2'), 159.5 (C-8'), 155.3 (C-8), 139.9 (C-4'), 131.7 (C-5), 128.9 (C-6, 10), 126.6 (C-5'), 119.1 (C-3'), 115.4 (C-7', 9'), 114.8 (C-7, 9), 41.7 (C-3), 30.4 (C-4). From the comparison of these data with those reported in the literature by Li *et al*., (2009), the substance was identified as 5-hydroxy-1,7-bis(4-hydroxyphenyl)-4,6-heptadien-3-one (dihydrobisdemethoxycurcumin) (Figure 2-8) (Li *et al*., 2009).

Compound 5: Yellow powder, UV λ_{max} nm: 247, 414. ESI-MS (+): *m/z* 339.3 $[M+H]^+$. ^{1}H-NMR (CD_3OD) δ: 7.56 (2H, d, *J*=16 Hz, H-4, 4'), 7.48 (2H, d, *J*=8 Hz, H-6, 10), 7.20 (1H, s, H-6'), 7.10 (1H, d, *J*=8 Hz, H-10'), 6.80 (3H, d, *J*=8 Hz, H-7, 10, 9'), 6.60 (2H, br, H-3, 3'), 3.90 (3H, s, 7'-OCH_3). ^{13}C-NMR (CD_3OD) δ: 184.7 (C-2, 2'), 161.1 (C-8, 8'), 150.5 (C-8'), 149.4 (C-7'), 142.1 (C-4'), 141.9 (C-4), 131.1 (C-6, 10), 128.6 (C-5'), 128.0 (C-5), 124.1 (C-10'), 122.3 (C-3'), 122.0 (C-3), 116.9 (C-10), 116.6 (C-9'), 111.8 (C-6'), 56.5 (7'-

OCH_3). From the comparison of these data with those reported in the literature by Kiuchi *et al*., (1993) and Li *et al*., (2009), the substance was identified as 1-(4-hydroxy-3-methoxyphenyl)-7-(4-hydroxyphenyl)hepta-1,6-diene-3,5-dione (demethoxycurcumin) (Figure 2-8) (Li *et al*., 2009; Kiuchi *et al*., 1993).

Compound 6: Yellow powder, UV λ_{max} nm: 262, 419. ESI-MS (+): *m/z* 369.2 $[M+H]^+$. ^{1}H-NMR (CD_3OD) δ: 7.57 (2H, d, *J*=16 Hz, H-4, 4'), 7.21 (2H, s, H-6, 6'), 7.10 (2H, bd, *J*=8 Hz, H-10, 10'), 6.82 (2H, d, *J*=8 Hz, C-9, 9'), 6.62 (2H, bd, *J*=16 Hz, H-3, 3'), 3.90 (6H, s, 7, 7'-OCH_3). ^{13}C-NMR (CD_3OD) δ: 184.8 (C-2, 2'), 150.6 (C-8, 8'), 149.5 (C-7, 7'), 142.1 (C-4, 4'), 128.6 (C-5, 5'), 124.1 (C-10, 10'), 122.3 (C-3, 3'), 116.6 (C-9, 9'), 111.8 (C-6, 6'), 56.5 (7, 7'-OCH_3). From the comparison of these data with those reported in the literature by Kiuchi *et al*., (1993), Li *et al*., (2009) and Payton *et al*., (2007), the substance was identified as 1,7-bis(4-hydroxy-3-ethoxyphenyl)-1,6-heptadiene-3,5-dione (curcumin) (Figure 2-8) (Li *et al*., 2009; Kiuchi *et al*.,1993; Payton *et al*., 2007).

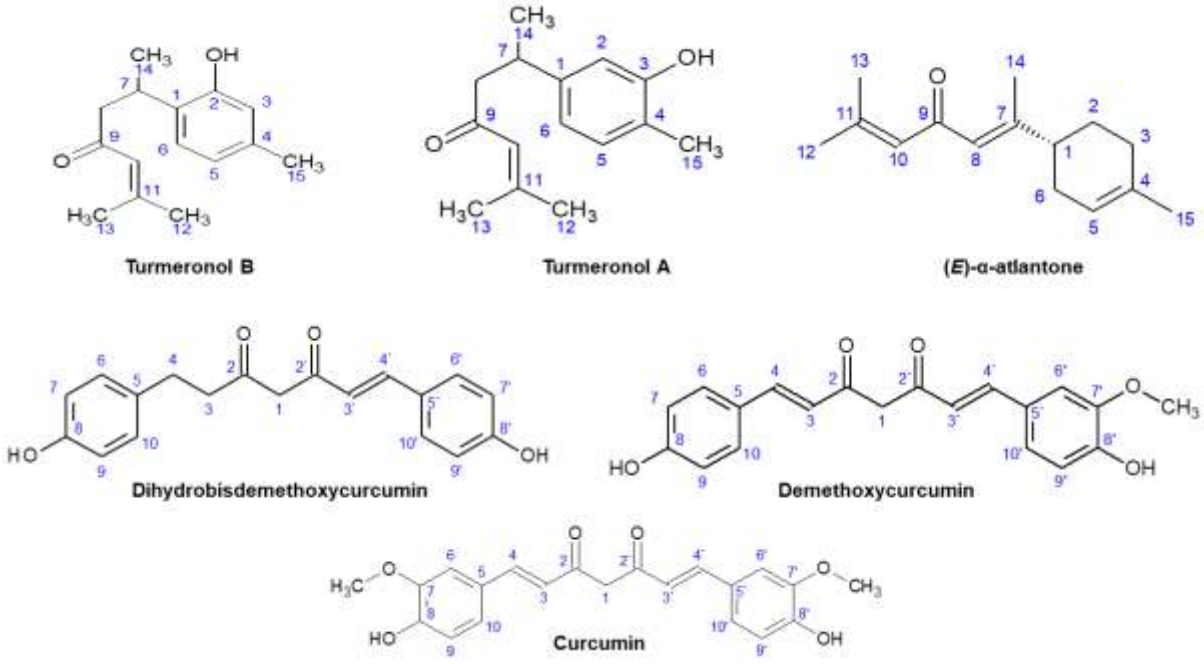


Figure 2-8. Chemical structures of the six antifungal compounds isolated from Ryudai gold were identified according to their ^{1}H NMR and ^{13}C NMR spectra; Turmeronol B, Turmeronol A, (*E*)-α-atlanton, dihydrobisdemethoxycurcumin, demethoxycurcumin and curcumin.

4.8 Antifungal activity of isolated compounds

All the isolated compounds from RD (turmeronol B, turmeronol A, (*E)*-α-atlantone, dihydrobisdemethoxycurcumin, demethoxycurcumin and curcumin) and the standard commercial drug miconazole (positive control) inhibited fungal growth in a concentration-dependent manner (Figure 2-9). Among the six compounds, demethoxycurcumin and curcumin showed lower IC_{50} than others.

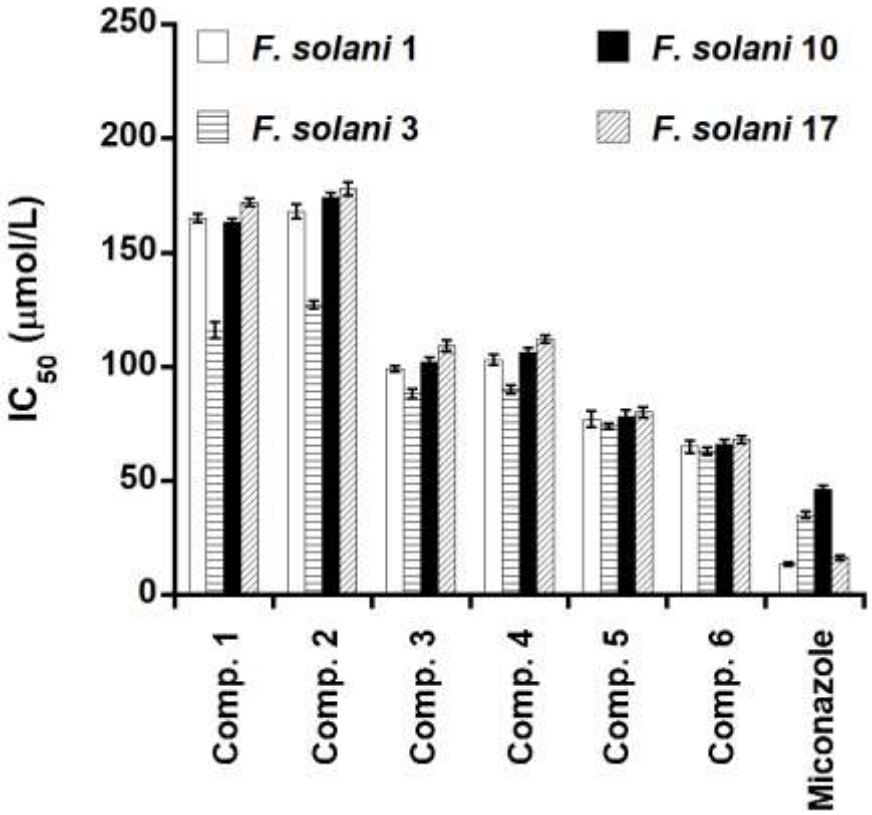


Figure 2-9. IC_{50} of turmeronol B (**1**), turmeronol A (**2**), (*E)*-α-atlantone (**3**), dihydrobisdemethoxycurcumin (**4**), demethoxycurcumin (**5**) and curcumin (**6**).

5. DISCUSSION

In our previous study, MeOH extract of RD showed a stronger antifungal effect than the other species and strains of turmeric (Akter *et al*., 2018a). Turmeric RD contained lower curcumin but showed a stronger antifungal effect, which indicated that some other compounds possess antifungal properties (Devkatte *et al*., 2005). In the present study, we isolated 6 antifungal compounds from RD by using repeated column chromatography of EtOAc fraction of MeOH extract. Although two previous studies isolated some antifungal compounds from *C. longa* (Radwan *et al*., 2014; Chen *et al*., 2018). However, this is the first study that discovered turmeronol B, turmeronol A, (*E)*-α-atlantone, dihydrobisdemethoxycurcumin as antifungal compounds isolated from *C. longa* strain RD. Although these were not newly discovered molecules, however, their antifungal effect had never been addressed.

Imai *et al*., (1990), isolated turmeronol B (**1**) and turmeronol A (**2**), phenolic sesquiterpene kitones from *C. longa* and developed their structures (Imai *et al*., 1990). These two compounds inhibited soybean lipoxygenase and catalyze the oxidation of certain useful unsaturated fatty acids during food processing (Imai *et al*., 1990). (*E)*-α-atlantone (**3**), a bisabolane sesquiterpenes were previously isolated and purified form Alpine fir and *C. longa* (Manville *et al*., 1989; Braga *et al*., 2003). However, no study reported its biological activity.

Li *et al*., (2009) isolated dihydrobisdemethoxycurcumin (**4**), demethoxycurcumin (**5**) and curcumin (**6**) from *C. longa* and developed their structure (Li *et al*., 2009). Dihydrobisdemethoxycurcumin has been reported to show antiplatelet, anti-inflammatory, antibacterial, wound healing and antioxidant activities (Dong and chen, 1998; Sudsai *et al*., 2014; Sudsai *et al*., 2017; Sudsai *et al*., 2016). Demethoxycurcumin and curcumin are the major chemical constituents in turmeric, having a wide range of pharmacological properties

including antioxidant, anti-cancer, antimalarial, and nematocidal activities (Kiuchi *et al*., 1993; Sonkaew *et al*., 2012; Adams *et al*., 2004; Ji and Shen, 2009). It also has potential neuroprotective properties including antioxidant, anti-neuroinflammatory, antiproliferative, anti-amyloidogenic, anti-dementia, and neuro-regulative effects (Chin *et al*., 2013). The concentrations of turmeronol B, turmeronol A, (*E)*-α-atlantone, dihydrobisdemethoxycurcumin, demethoxycurcumin and curcumin were 0.019, 0.038, 0.018, 0.12, 24.4 and 36 μmol/g of dry RD, respectively. The range of calculated IC_{50} values of turmeronol B, turmeronol A, (*E)*-α-atlantone, dihydrobisdemethoxycurcumin, demethoxycurcumin and curcumin were 116 to172, 127 to 185, 88 to 109, 90 to 112, 74 to 80 and 63 to 68 μM/L, respectively. The order of antifungal activity against FSSL was curcumin> demethoxycurcumin> (*E)*-α-atlantone> dihydrobisdemethoxycurcumin> turmeronol B> turmeronol A. Our previous study reported that curcumin and demethoxycurcumin had a strong effect against FSSL (Akter *et al*., 2018a). The effectiveness of (*E)*-α-atlantone was 1 to 1.7 fold higher than turmeronol B, turmeronol A and dihydrobisdemethoxycurcumin and 1.1 to 1.6 fold lower than curcumin and demethoxycurcumin. However, the concentration of (*E)*-α-atlantone was 1 to 2000 fold lower than other compounds. Antifungal susceptibility test showed that FSSL was resistant to most of the commercially available antifungal agents. Amphotericin B and miconazole were found effective against FSSL. However, FSSL isolate 3 was resistant to amphotericin B. In contrast, our present study suggested that the crude extract and all the isolated compounds from RD had the strongest effect on FSSL isolate 3. Our result suggested that not only curcumin and demethoxycurcumin but also turmeronol B, turmeronol A, (*E)*-α-atlantone and dihydrobisdemethoxycurcumin had a strong antifungal effect against FSSL. However, further investigation is necessary to know the mode and site of action of these isolated compounds.

Chapter 1

Study 3

Isolation and structural elucidation of antifungal compounds from *Curcuma amada* against *Fusarium solani sensu lato*

1. ABSTRACT

Curcumin, demethoxycurcumin and bisdemethoxycurcumin are the major active components of turmeric (*Curcuma spp*.) and they have a strong antifungal effect. However, in a previous study we found that *Curcuma amada* (*C. amada*) does not contain any of these major curcuminoids but showed strong antifungal activity against *Fusarium solani senso lato* (FSSL). Therefore, the aim of the present study was to isolation and identification of the antifungal compounds from *C. amada*. The antifungal activity was measured by the diameter of colonies grown on Petri dish, microscopic observation, and CLSI microdilution methods. Methanol extract of the rhizome of *C. amada* inhibited fungal growth in a concentration dependent manner. The ethyl acetate (EtOAc) fraction from methanol extract of *C. amada* demonstrated the highest antifungal activity against FSSL. Then the EtOAc fraction was further eluded with *n*-hexane and EtOAc with gradually increasing the amount of EtOAc (*n*-hexane:EtOAc; 100:0; 80:20; 60:40, 40:60, 20:80 and 0:100). The antifungal compounds were isolated from the most effective fraction (60:40) of *C. amada* using silica gel column and high performance liquid chromatography. Structural identification of the antifungal compounds was conducted using ^{1}H NMR, ^{13}C NMR, and liquid chromatography-tandem mass spectrometry. The purified antifungal compounds were zederone and furanodienone. These two compounds showed dose-dependent antifungal activity against FSSL. The concentration required for 50% growth inhibition (IC_{50}) of FSSL ranged from 115 to 129 µM and 82 to 91 µM for zederone and furanodienone, respectively. This study suggested that the isolated compound from *C. amada* could be a promising natural antifungal agent to control the diseases caused by FSSL.

Key word: *Curcuma amada*, *Fusarium solani sensu lato*, antifungal compounds, zederone, furanodienone, NMR.

2. INTRODUCTION

Curcuma amada Roxb. is a perennial, rhizomatous herb belongs to the family Zingiberaceae (Policegoudra *et al*., 2007a; Tamta *et al*., 2016). Morphologically it resemble to ginger (*Zingiber officinale*), however the rhizome posses mango (*Mangifera indica*) like aroma. For this it is known as mango ginger (Tamta *et al*., 2016). Among the 68 volatile components in the essential oil of rhizome of *C. amada*, car-3-ene and cis-ocimene are responsible for this mango flavor (Achut and Bandyopadhyaya 1984; Srinivas Rao *et al*., 1989; Singh *et al*., 2002, 2003). It is found in various parts of the world. In India, it is cultivated in West Bengal, Gujarat, Uttar Pradesh, Tamil Nadu, Karnataka, Konkan and in the hills of Western coast (Ghani, 1998). The height of this plant is about 1 m. The leaves are long, lanceolate, sheathed, radical and petiolate. The rhizome of *C. amada* is 5-10 cm long and 2-5 cm in diameter. The rhizomes are use in the manufacture of pickles, oleoresin, essential oil, candies, preserves, sauces, salads and curres (Gupta, 2001; Verghese 1990). The rhizome of *C. amada* has been extensively used as alexteric, appetizer, aphrodisiac, antipyretic and laxative. Moreover, it showed anti-inflammatory, antifungal, analgesic, anticancer and antihyperglyceridemic properties (Chowdhury *et al*., 2015; Ghosh *et al*., 1980; Mujumdar *et al*., 2000; Gupta, 2003; Kumari Bai and Shannukanada, 2015). Currently, some compounds from the rhizome of *C. amada* have been identified. Particularly, difurocumeninol, amadannulen and amadaldehyde were demonstrated as antibacterial, platelet aggregation inhibition, cytotoxicity and antioxidant activities (Policegoudra *et al*., 2007a, 2007b, and 2010). However, no antifungal compound has been identified from *C. amada* rhizomes.

Fusarium solani sensu lato (FSSL) causes several diseases in human, animals, and plants (Nelson *et al*., 1994 and Majumdar *et al*., 2008). Our previous research demonstrated

that curcumin and demethoxycurcumin which are the major active constituent of turmeric showed strong inhibitory effect on FSSL. On the other hand, without having curcuminoids *C. amada* exhibited strong inhibitory effect against FSSL. Therefore, the aim of our present study was to isolate and characterize the antifungal compounds from *C. amada* rhizomes.

3. MATERIALS AND METHODS

3.1 Chemicals and materials

Methanol (MeOH), ethyl acetate (EtOAc) and *n*-hexane were purchased from nacalai Tesque (Kyoto, Japan). Silica gel (63-200 μm, Kanto Chemical Co. Tokyo, Japan), MeOH-d_4 (CD_3OD, Merck KGaA, Germany), Potato Dextrose Agar (PDA), Sabouraud Dextrose Agar (SDA) (Becton, Dickinson and Company, France), RPMI-1640 (Life Technologies, USA), antifungal drug coated plate (Eiken Chemical Co., Ltd. Tochigi, Japan) and miconazole (Wako Pure Chemical Industries, Ltd. Osaka, Japan) were purchased.

3.2 Microorganism

Four isolates of FSSL (1, 3, 10, and 17) were derived from skin lesions of American manatees (*Trichechus manatus*) with 3 different genotypes (Sano *et al*., 2013). FSSL 1, 3 and 10 were isolated from the cutaneous lesion of the face (Isolate 1: genotype A, GenBank accession number AB775568) and body skin (Isolate 3: genotype B, GenBank accession number AB775569; Isolate 10: genotype C, GenBank accession number AB775570) of a male manatee-1. FSSL 17 was isolated from palm skin of a male manatee-2 (genotype A, GenBank accession number; AB775568). The genotypes based on internal transcribed spacer 1 – 5.8s – ITS 2 regions of ribosomal RNA gene sequence.

The isolates 1 and 17 were genetically identical. However, the source of the manatee was different. Thus we used the above 4 isolates. All the isolates are stored in the Department of Animal Sciences, Faculty of Agriculture, University of the Ryukyus, Japan. These were maintained in PDA slants and stored at 24–26^0 C. These fungi were subcultured one week before using for experiments.

3.3 Plant material

C. amada was cultivated in a field of gray soil (coarse sand 3.6%, fine sand 30.9%, silt 24.3%, clay 32.8%, pH 7.4, NO_3-N 0.07%, NH_4-N 0.08%, P 4.6 ng/g, K 42.9 ng/g; Hossain *et al*., 2005a) at the Subtropical Field Science Center, University of the Ryukyus, Okinawa, Japan. The average monthly temperature, humidity, and precipitation during the cultivation period were 17–29℃, 61–83% and 22–369 mm, respectively. Rhizomes were harvested on February 10, 2016, when all the shoots of the species withered completely. One and half kilogram of fresh rhizomes were harvested, washed, sliced and dried in a hot air oven at 50^0 C for 72 h (Policegoudra *et al*., 2007b). A total of 300 g dried powder was obtained from that 1.5 kg of fresh rhizomes.

3.4 Extraction of Samples

The extraction was carried out by dissolving 300 g turmeric powder into MeOH at room temperature (25℃) with continuous magnetic stirring to prevent oxidation by air and shielding from sunlight. The solvent soluble compounds were filtered using filter paper (No. 2; Advantec, Tokyo Roshi Kaisha Ltd., Tokyo, Japan). Fresh solvents were added into the used plant material and the process was repeated three times. The filtered solutions containing plant compounds were dried on rotary evaporator under reduced pressure at 40℃. The yield of extract (26 g) was kept in refrigerator at 4℃ for

experimental analyses.

3.5 Antifungal assays

a. *Mycelial growth inhibition*: To study the effect of different concentrations (32, 64 and 128 µg/mL) of *C. amada* extracts against FSSL (1, 3, 10 and 17 isolates) mycelia growth, sample was incorporated into the fungal growth medium (SDA). After mixing, the amended SDA was dispensed into 9 cm diameter Petri dishes and allowed to cool. Mycelia from 7 day old culture of FSSL were placed in the center of the Petri dishes. The plates were incubated at 25℃ and the radial growth of mycelium was measured after 7 days. The plates without extract were used as control. All tests were performed in triplicate. The relative growth inhibition of the treatment compared to the control as calculated as a percentage, using the following formula:

$$MGI\ (\%) = (dc - dt)/dc \times 100$$

dc and dt represent mycelia growth diameter in control and treated Petri plates, respectively.

b. *Hyphal morphology of FSSL:* The morphological aspects of *FSSL* mycelia treated by different concentration (32, 64 and 128 µg/mL) of *C. amada* extracts were examined by an automated inverted fluorescent microscope (Leica, Model No. 4000B, Germany). Photomicrographs of the mycelia of *FSSL* were taken after 48 hours of treatment with *C. amada* extracts and the effect of the extract on hyphal growth was compared with that of control.

c. *Antifungal susceptibilities to commercial compounds:* Susceptibility test was performed in a commercially available antifungal drug-coated plate, based on the CLSI standard M38-A2 broth microdilution method (CLSI, 2008). Liquid RPMI-1640 medium

buffered to pH 7.0 with 1.0M NaOH, was filter sterilized. Four isolates were cultured on PDA slants at 25℃ for 7 days. The inoculum was prepared by rubbing the surface of fungal colonies with 3 mL of sterile liquid RPMI-1640 from PDA slant using a loop. The suspension was then filtered to remove the hyphae and adjusted to a final inoculum of 10^3 colony-forming units/mL using hemocytometer (Neubauer chamber). Seven antifungal drugs including; micafungin (MCFG), amphotericin B (AMPB), flucytosine (5-FC), fluconazole (FLCZ), itraconazol (ITCZ), voriconazol (VRCZ), miconazole (MCZ) were used in this study. Hundred μL fungal suspensions was added into 96 wells of antifungal drug plates and incubated at 25℃. The medium without fungal strain was used as negative control and fungal strain without drugs was used as positive control. After 48 h, hyphal growth was monitored and compared with control. All tests were performed in triplicate. The susceptibility of FSSL isolates was graded inhibitory concentration of 100% (IC_{100}), IC_{50} and minimum effective concentration (MEC). The IC_{100} was defined as the lowest concentration of an agent that completely inhibited the growth of FSSL. IC_{50} was defined as the lowest concentration of an agent that inhibited approximately 50% of the drug-free growth control. The MEC was defined as the lowest concentration of an agent that led to abnormal growth, such as leading to small, rounded, compact hyphal forms of the fungus. Susceptibilities to amphotericin B, itraconazole and voriconazole were evaluated by IC_{100}. Susceptibility to micafungin was evaluated by the MEC. Susceptibility to flucytosine, fluconazole, and miconazole were evaluated by IC_{50} (CLSI, 2008).

d. *Isolation of bioactive compounds from crude extract of C. amada:* To isolate the antifungal compounds from *C. amada*, the bio-assay guided fractionation was described in figure 3-1.

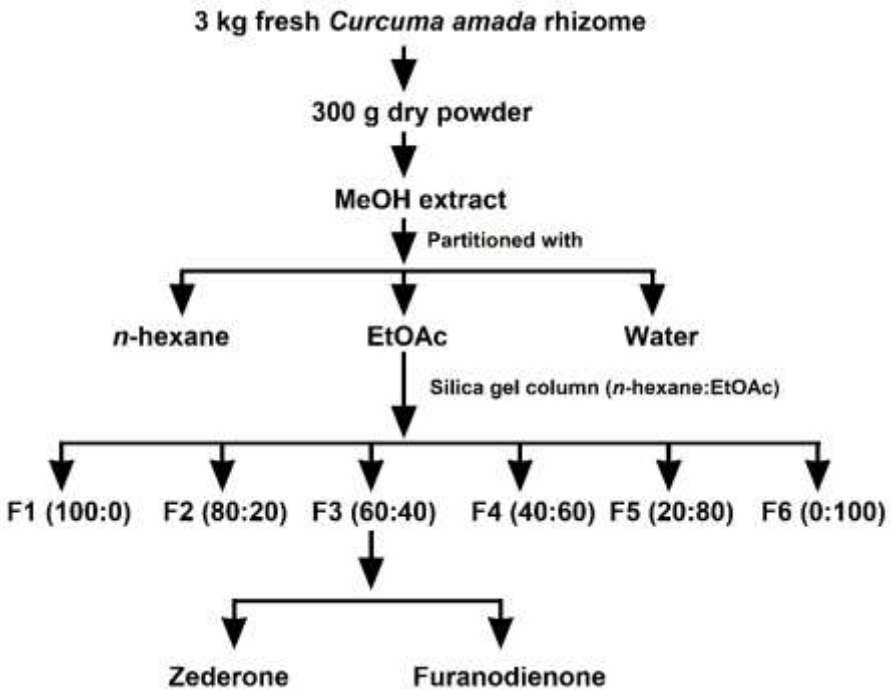


Figure 3-1. Flow chart for isolation of antifungal compounds from *C. amada*.

The crude extract was diluted with 200 mL distilled water and extracted with an equal volume of *n*-hexane and then with EtOAc. All fractions were concentrated to dryness on rotary evaporator at 40℃. Antifungal activity of these three fractions was determined using mycelial growth inhibition assay according to the aforementioned procedure. As EtOAc fraction showed the strongest antifungal activity, it was selected for the isolation and purification of the bioactive compounds. The dried extract from EtOAc fraction was subjected to chromatography on a silica gel (75 g) column (30 x 3 cm). Elution was carried out using *n*-hexane and EtOAc with increasing portion of EtOAc [100:0 (F1), 80:20 (F2), 60:40 (F3), 40:60 (F4), 20:80 (F5) and 0:100 (F6) (v/v)]. Antifungal activity was carried out on all six fractions and fraction 3 (F3) showed the strongest antifungal activity. For this, F3 was purified by C_{18} reversed-phase HPLC (COSMOSIL 5C18-AR-II; nacalai Tesque, Inc., Kyoto, Japan) eluted with 50% acetonitrile in water (v/v) (50%)

at a flow rate of 2.5 mL min^{-1}, monitored at 280 nm. Antifungal activity was detected in two peaks fraction eluted at 10 and 17.5 min as white substances (Figure 3-2). The isolated compounds (approximately 10 mg each) were dissolved in MeOH-d_4 and then subjected to spectroscopic analyses. Nuclear magnetic resonance (NMR) spectra were recorded on BRUKER NMR spectrometers (500 MHz for ^{1}H and 125 MHz for ^{13}C) at room temperature. Chemical shifts (δ) were recorded as parts per million (ppm) relative to tetramethylsilane (TMS) as the internal standard. Mass Spectrometry experiments were carried out on Waters mass spectrometer (Quattro Micro, USA) with an electrospray ionization probe under the following instrumental conditions: Column: COSMOSIL 5C18-AR-II, 2×150 mm. Solvent A: Water (0.1% Formic acid), Solvent B: Acetonitrile, Flow rate: 4 mL/min, Injection Volume: 100 μL, Run Time: 35 min, Pump mode: Binary gradient. Time program: 75% B (0 min) →75% B (20 min) → 100% B (20.1 min) →100% B (27 min) →75% B (27.1 min) →75% B (35 min). Oven details: CTO-20AC, temperature 40℃. MS ionization mode: ES (+), Capillary voltage: 4.0 kV, Cone voltage: 20 V, Source Temp.: 120℃, Desolvation Temp.: 350℃, Cone Gas flow: 100 L/h, Desolvation Gas flow: 800 L/h.

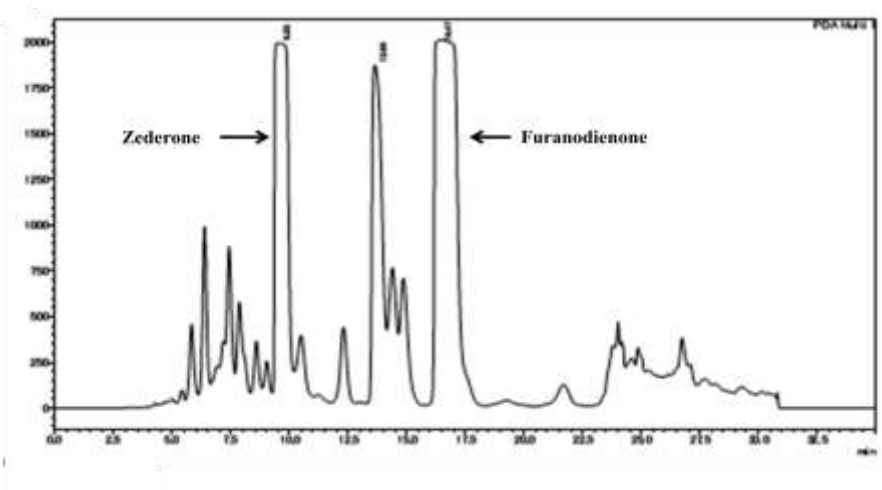


Figure 3-2. HPLC chromatogram of F3, detected at 280 nm.

3.6 Antifungal activity of the isolated compounds.

Isolated compounds were dissolved in MeOH for making the concentrations of 10, 20, 50, 100 and 500 μM for each compound. Different concentrations of the isolated compounds were incorporated into the fungal growth medium (SDA). After mixing, the amended SDA was dispensed into 9 cm diameter Petri dishes and allowed to cool. Mycelia from 7 days old culture of FSSL were placed in the center of the Petri dishes. The plates were incubated at 25℃ and the radial growth of mycelium was measured after 7 days. The plates without compounds were used as a negative control. Miconazole was used as a positive control. All the tests were performed in triplicate. The relative growth inhibition of the treatment compared to the control was calculated as a percentage, using the following formula:

$$MGI\ (\%) = (dc - dt)/dc \times 100$$

Where dc and dt represent mycelia growth diameter in control and treated Petri plates, respectively.

3.6 Statistical Analysis

The experiments were carried out three times in triplicate. Means and standard errors (SE) of the sample were calculated using Duncan's new multiple-range test at a level of $p \leq 0.05$. All the statistical analyses were performed using Cropstat version 7.0. The concentration required for 50% inhibition of fungal growth (IC_{50}) of the turmeric extract and isolated compounds in the assay was determined from the regression equation of the concentration response curves.

4 RESULT

4.1 Mycelial growth inhibition

MeOH extract of *C. amada* inhibited the growth of all the strains of FSSL in a concentration dependent manner (Figure 3-3). At 128 μg/mL concentration, *C. amada* inhibited about 35% growth of all the strains of FSSL. There were no significant differences in the antifungal activity of *C. amada* against the four isolates of FSSL.

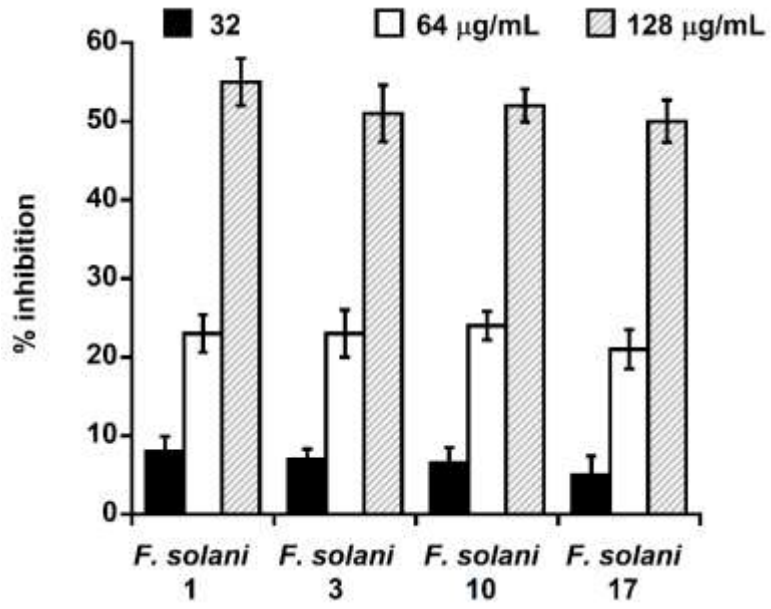


Figure 3-3. Effect of different concentrations of *C. amada* on four isolates of FSSL. Results are expressed as the mean ± SEM of three independent experiments in triplicate. $^{*}p<0.05$ vs 64 and 32 μg/mL.

4.2 Fluorescent microscopic observation of hyphal growth

Our result suggested that different concentrations (32, 64 and 128 µg/mL) of *C. amada* had strong effect on hyphal growth of FSSL (Figure 3-4).

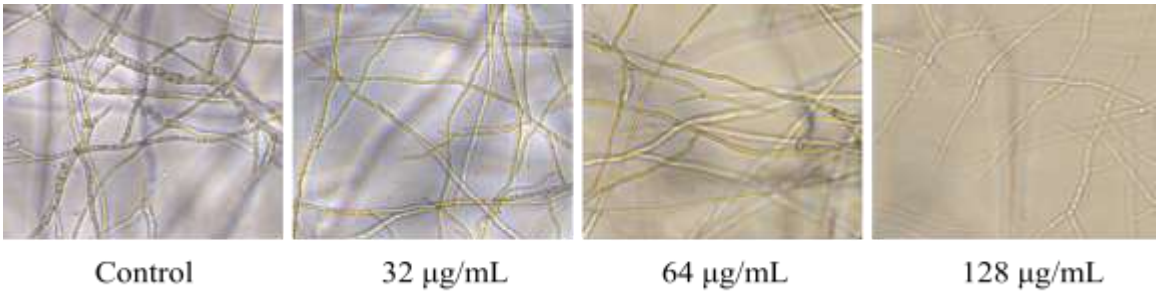


Figure 3-4. Microscopic examination of the hyphal growth of FSSL (F3) after 48 h of treatment with different concentrations of *C. amada.*

4.3 Antifungal susceptibilities to commercial compounds

Among the 7 commercial antifungal drugs, FSSL were susceptible to AMPB and MCZ. However, FSSL isolate 3 was resistant to AMPB. 5-FC, FLCZ, ITCZ, and VCZ were not effective against FSSL (Table 3-1).

Table 3-1. Antifungal susceptibilities to commercial compounds evaluated by CLSI method (CLSI, 2008).

	Antifungal agent (μg/mL)						
Isolate	MCFG (MEC)	AMPB (IC_{100})	5-FC (IC_{50})	FLCZ (IC_{50})	ITCZ (IC_{100})	VCZ (IC_{100})	MCZ (IC_{50})
1	>16	4	>16	>16	>8	>8	4
3	16	>16	>16	>16	>8	>8	4
10	>16	2	>16	>16	>8	>8	16
17	16	2	>16	>16	>8	>8	8

MCFG; micafungin, AMPB; amphotericin-B, F-FC; flucytocin, FLCZ; fluconazole, ITCZ; itraconazole, VCZ, voriconazole, and MCZ; miconazole, MEC; minimum effective concentration.

4.4 Identification of antifungal compounds

Antifungal activity of water, n-hexane and EtOAc fraction of MeOH extract of C. amada

EtOAc fraction obtained from *C. amada* resulted in 74% inhibitory activity, whereas *n*-hexane and water fraction resulted in 49% and 17% inhibitory activity, respectively (Figure 3-5).

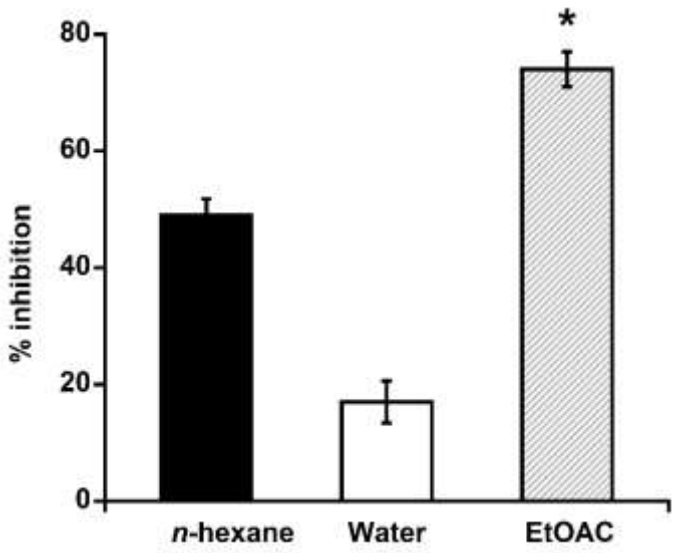


Figure 3-5. Effect of different solvents extract of *C. amada* on FSSL (F3). Results are expressed as the mean ± SEM of three independent experiments in triplicate. $^{*}p<0.05$ vs. water and *n*-hexane.

4.5 Antifungal activity of different fractions of EtOAc part of C. amada

The antifungal activity of the six fractions [*n*-hexane: EtOAc; 100:0 (F1), 80:20 (F2), 60:40 (F3), 40:60 (F4), 20:80 (F5) and 0:100 (F6) (v/v)] differed significantly. Among these, the F3 showed 79% inhibitory activity, whereas the remaining fractions, F1, F2, F4, F5 and F6 resulted in 0, 2.2, 63, 38 and 22% inhibitory activity, respectively (Figure 3-6). The antifungal compounds from F3 showed two peaks at 280 nm at a retention time of 10 and 17.5 min in the HPLC Chromatogram.

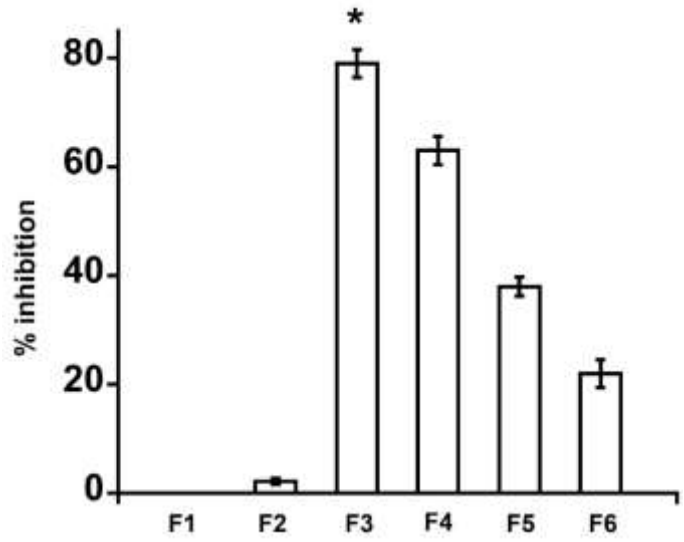


Figure 3-6. Effect of six fractions of EtOAc part of *C. amada* on FSSL (F3). Results are expressed as the mean ± SEM of three independent experiments in triplicate. $^{*}p<0.05$ vs. other fractions.

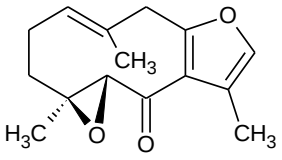
4.6 Chemical structures of the isolated compounds

The chemical structures of the two antifungal compounds isolated from F3 were identified according to their ^{1}H NMR and ^{13}C NMR spectra. Peak data were as follows:

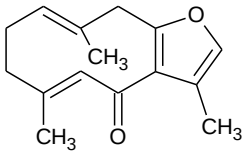
Compound -1: Colourless needle shape crystal, UV λ_{max}: nm 234, 285. ESI-MS (+) *m/z*: 247.3 $[M+H]^{+}$, 229.4 $[M+H-H_2O]^{+}$. ^{1}H-NMR (CD_3OD): δ 7.22 (1H, s, H-12), 5.59 (1H, br d,

J = 12 Hz, H-1), 3.99 (1H, s, H-5), 3.85 (1H, d, *J* = 16 Hz, H-9a), 3.69 (1H, d, *J* = 16 Hz, H-9b), 2.57 (1H, dddd, *J* = 13, 13, 12, 4 Hz, H-2a), 2.26 (1H, m, H-3a), 2.20 (1H, m, H-2b), 2.09 (3H, s, H-13), 1.57 (3H, s, H-15), 1.32 (1H, ddd, *J* = 13, 13, 4 Hz, H-3b), 1.28 (3H, s, H-14). ^{13}C-NMR (CD_3OD): δ 194.2(C-6) 160.2(C-8), 139.7(C-12), 132.8 (C-1), 132.0(C-10), 124.3(C-11), 123.0(C-7), 67.8(C-5), 65.2(C-4), 42.5(C-9), 39.0(C-3), 25.4(C-2), 15.7(C-15), 15.4(C-14), 10.7(C-13). From the comparison of these data with those reported in the literature by Giang *et al*., (2000) and Asem *et al*., (2012), the substance was identified as zederone (Figure 3-7).

Compound -2: Colourless oil, UV λ_{max}: nm 243, 280. ESI-MS (+) *m/z*: 231.0 $[M+H]^+$, 223.3 $[M+H-H_2O]^+$. ^{1}H-NMR (CD_3OD): δ 7.16 (1H, s H-12), 5.83 (1H, s, H-5), 5.21 (1H, dd, *J* = 12 Hz, 5 Hz, H-1), 3.73 (1H, d, *J* = 16 Hz, H-9a), 3.63 (1H, d, *J* = 16 Hz, H-9b), 2.45 (1H, ddd, *J* = 15 Hz, 11 Hz, 4 Hz, H-3a), 2.31 (1H, m, H-2a), 2.20 (1H, dddd, *J* = 12 Hz, 12 Hz, 12 Hz, 4 Hz, H-2b), 2.06 (3H, s, H-13), 1.92 (3H, s, H-14), 1.89 (1H, m, H-3b), 1.25 (3H, s, H-15). 13 C-NMR (CD_3OD): δ 191.8 (C-6), 158.6 (C-8), 147.6 (C-4), 140.0 (C-12), 136.1 (C-10), 133.3 (C-5), 132.0 (C-1), 124.6 (C-11), 123.1(C-7), 42.4 (C-9), 41.4 (C-3), 27.3 (C-2), 19.3 (C-14), 15.9 (C-2), 9.9 (C-13). From the comparison of these data with those reported in the literature by Dekebo *et al*., (2002), the substance was identified as furanodienone (Figure 3-7).



Zederone



Furanodienone

Figure 3-7. Chemical structures of the two antifungal compounds isolated from *C. amada* were identified according to their ^{1} H NMR and ^{13}C NMR spectra; zederone and furanodienone.

4.7 Antifungal activity of the isolated compounds

Two compounds namely zederone and furanodienone were isolated from the fraction F3. The isolated compounds and the standard commercial drug miconazole (positive control) inhibited fungal growth in a concentration-dependent manner. The IC_{50} of furanodienone was lower than that of zederone (Figure 3-8).

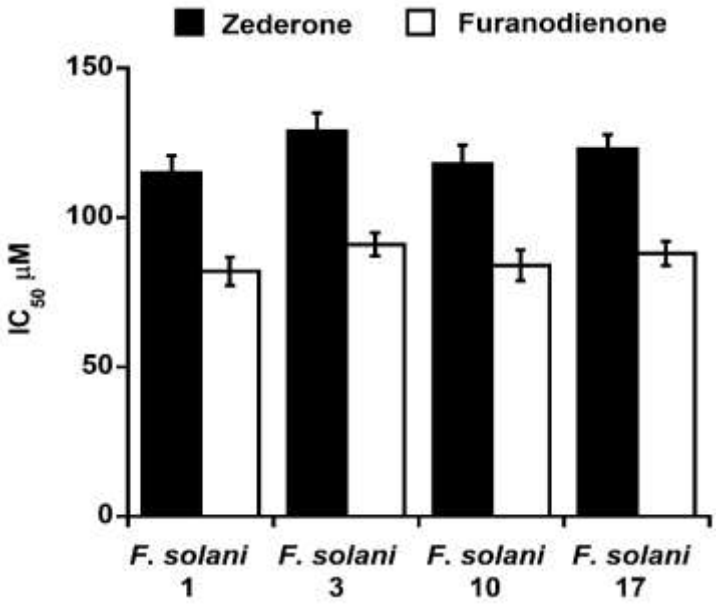


Figure 3-8. IC_{50} of zederone and furanodienone on FSSL. Results are expressed as the mean ± SEM of three independent experiments in triplicate. $^{*}p<0.05$ vs. zederone.

5 DISCUSSION

Our previous research suggested that without having curcuminoids (curcumin, demethoxycurcumin and bisdemethoxycurcumin), the major active constituent of turmeric, *C. amada* showed strong inhibitory effect on FSSL. The present study demonstrated that *C. amada* had a dose-dependent inhibitory effect on hyphal growth of FSSL. For this, we analyzed antifungal compounds from *C. amada* by using silica gel column and high performance liquid chromatography of EtOAc fraction of *C. amada* extract. In our study, we identified two antifungal compounds from *C. amada* namely, zederone and furanodienone. It has been reported that zederone was isolated from *C. amada* and *C. zedoaria* (Faiz Hossain *et al*., 2015; Ahmed Hamdi *et al*., 2014; Makabe *et al*., 2006). Zederone is a sesquiterpene, had analgesic principle (Faiz Hossain *et al*., 2015), showed anti-inflammatory (Makabe *et al*., 2006) and cytotoxic activity (Ahmed Hamdi *et al*., 2014). Previous study suggested that furanodienone was isolated from *Lindera pulcherrima* (Nees.) Benth. ex Hook. f (Joshi and Mathela, 2012), *Curcuma zedoaria* (Makabe *et al*., 2006) and *Curcuma wenyujin* (Yang *et al*., 2007). Furanodienone is a furanosesquiterpenoids, exhibited anti-inflammatory (Makabe *et al*., 2006), anti cancer (Li *et al*., 2011), antibacterial and antioxidant activity (Joshi and Mathela, 2012). To the best of our knowledge, this is the first time furanodienone was discovered from *C. amada*. Though zederone and furanodienone are not new compounds, however their antifungal activity has never been reported before. The concentration of zederone and furanodienone was 1.4 and 1.7 µmol g^{-1} of dry *C. amada*, respectively. The calculated IC_{50} value of zederone and furanodienone was 115 ± 5.7 to 129 ± 6 and 82 ± 4.8 to 91 ± 3.9 µM, respectively. The effectiveness and concentration of furanodienone was 1.4 and 1.2 fold higher than that of zederone. Antifungal susceptibility test showed that FSSL was resistant to most of the commercially available antifungal agents. Amphotericin B and

miconazole were found effective against FSSL. However, FSSL isolate 3 was resistant to amphotericin B. In contrast, our present study suggested that the crude extract and two isolated compounds from *C. amada* had strong effect on FSSL isolate 3.

In conclusion, we strongly suggested that zederone and furanodienone isolated from *C. amada* can play an important role for controlling the soil borne diseases caused by FSSL. Moreover, further research is necessary to know their mode and site of action.

Chapter 2

Antioxidant activity of different species and varieties of turmeric (*Curcuma* spp): Isolation of active compounds

1. ABSTRACT

There are more than 80 species of turmeric (*Curcuma spp.*) and some species have multiple varieties, for example, *Curcuma longa* (*C. longa*) has 70 varieties. They could be different in their chemical properties and biological activities. Therefore, we compared antioxidant activity, total phenolic and flavonoid content of different species and varieties of turmeric namely *C. longa* [variety: Ryudai gold (RD) and Okinawa ukon], *C. xanthorrhiza*, *C. aromatica*, *C. amada*, and *C. zedoaria.* The antioxidant activity was determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity, oxygen radical absorbance capacity (ORAC), reducing power and 2-deoxyribose (2-DR) oxidation assay. Our results suggested that RD contained significantly higher concentrations of total phenolic (157.4 mg gallic acid equivalent/g extract) and flavonoids (1089.5 mg rutin equivalent/g extract). RD also showed significantly higher DPPH radical-scavenging activity (IC_{50}: 26.4 µg/mL), ORAC (14090 µmol Trolox equivalent/g extract), reducing power absorbance (0.33) and hydroxyl radical scavenging activity (IC_{50}: 7.4 µg/mL). Therefore, RD was chosen for the isolation of antioxidant compounds using silica gel column, Toyopearl HW-40F column, and high-performance liquid chromatography. Structural identification of the compounds was conducted using ^{1}H NMR, ^{13}C NMR, and liquid chromatography-tandem mass spectrometry. The purified antioxidant compounds were bisabolone-9-one (**1**), 4-methyllene-5-hydroxybisabola-2,10-diene-9-one (**2**), turmeronol B (**3**), 5-hydroxy-1,7-bis(4-hydroxy-3-methoxyphenyl)-1-hepten-3-one (**4**), 3-hydroxy-1,7-bis(4-hydroxyphenyl)-6-hepten-1,5-dione (**5**), cyclobisdemethoxycurcumin (**6**), bisdemethoxycurcumin (**7**), demethoxycurcumin (**8**) and curcumin (**9**). The IC_{50} for DPPH radical-scavenging activity were 474, 621, 234, 29, 39, 257, 198, 47 and 18 µM and hydroxyl radical-scavenging activity were 25.1, 24.4, 20.2, 2.1, 5.1, 17.2, 7.2, 3.3 and 1.5 µM for compound **1**, **2**, **3**, **4**, **5**, **6**, **7**, **8** and **9**, respectively. Our

findings suggested that the RD variety of *C. longa*, developed by the University of the Ryukyus, Okinawa, Japan, is a promising source of natural antioxidants.

Key words: Turmeric; Ryudai gold; antioxidant; phenolic; flavonoid; active compound.

2. INTRODUCTION

Antioxidants are an important part of our regular diet that prevents oxidative cell damage by acting as free radical scavengers (Godic *et al*., 2014). Free radicals (superoxide radicals [$O_2^{\bullet-}$], hydroxyl radicals [OH•], and singlet oxygen [^{1}O2]) are producing continuously in the human body by complex redox reactions and play a crucial role in the development of many chronic diseases such as cancer, cardiovascular diseases, arteriosclerosis, diabetes, aging, and neurodegenerative diseases (Pham-Huy *et al*., 2008). In the normal physiology, specific enzymes in the body such as superoxide dismutase, catalase and glutathione peroxidase usually control the levels of free radicals (Gülçin *et al*., 2002). However, when the levels of free radicals are higher than those of the endogenous enzymes responsible for clearing them leads to oxidative stress.

To counter the effects of oxidative stress, many people take antioxidants in the form of commercial food additives that are produced synthetically and contain high amounts of preservatives (Shasha, 2014). Synthetic antioxidants are also used as food additives to prevent the oxidative deterioration of fats and oils in processed foods (Nanditha and Prabhasankar, 2009). However, synthetic antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene or tertiary butyl hydroquinone have been reported to produce toxins or act as carcinogens (Grice 1986; Shasha, 2014). In contrary, spices and herbs are rich in antioxidant compounds with no toxic effects (Yanishlieva *et al*., 2006). Hence, the need for health-promoting natural antioxidants has increased due to limitations on the use of synthetic antioxidants and enhanced public awareness of health issues. Therefore, identifying antioxidant constituents in plant material can be an alternative source of natural antioxidant to ensure sound health.

Turmeric (genus: *Curcuma*) is an herb belonging to the family Zingiberaceae, a

yellow spice of high economic importance due to its wide medicinal values. In Indian cooking, it is used in both vegetarian and non-vegetarian preparations as a major spice for the preparation of different types of 'curries'. Numerous studies have been reported the pharmacological activity of turmeric including antioxidant, anti-inflammatory, anti-angiogenic, antibacterial, antifungal, analgesic, immunomodulatory, vasodilatory, antidiabetes, anti-Alzheimer's disease (Ringman *et al*., 2005; Ramadan *et al*., 2011; Boaz *et al*., 2011; Akter *et al*., 2018a, 2018b). However, the genus *Curcuma* comprises over 80 species and some of the species have multiple varieties, for example, *Curcuma longa* (*C. longa*) has about 70 varieties in India (Sasikumar, 2005). Although some turmeric species have been reported for the antioxidant activity there is a lack of comparative information among the different species and varieties of turmeric. Furthermore, purification of potential antioxidant compounds from *C. longa* has not yet been done. Additionally, RD is a high yielding variety of *C. longa* developed by the University of the Ryukyus, Okinawa and registered by the ministry of agriculture, Japan (registration no. 21485). This is a dwarf and short duration turmeric variety, which provides higher yield (30-40 ton/ha, fresh rhizome) with higher curcumin content, compared to Okinawa indigenous turmeric variety (Okinawa ukon) (Akter *et al*., 2018a). The rhizome is 5 to 10 cm long, ovoid, cylindrical, branched and deep orange in color. We previously reported that MeOH extract of Ryudai gold (RD) exhibited significantly stronger antifungal activity against *F. solani* than other species and varieties of turmeric (Akter *et al*., 2018a). Its other pharmacological activities are yet to be explored. Therefore, the aim of this research was to evaluate the antioxidant activity along with the selected phytochemicals (total phenolic and flavonoid) content of different species and varieties of turmeric such as *C. longa* [variety: RD, Okinawa ukon], *C. xanthorrhiza*, *C. aromatica*, *C. amada* and *C. zedoaria* and purify the active compounds.

3. MATERIALS AND METHODS

3.1 Chemicals

Rutin, $AlCl_3$, gallic acid, potassium ferricyanide, $FeCl_3$, 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH), hydrogen peroxide (H_2O_2) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox®) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Folin-Ciocalteu reagent, sodium carbonate, trichloroacetic acid, methanol (MeOH), ethyl acetate (EtOAc), ascorbic acid, 2-deoxy-D-ribose and *n*-hexane were obtained from nacalai tesque (Kyoto, Japan). Silica gel (63-200 μm) was from Kanto Chemical Co. (Tokyo, Japan). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2- Thiobarbituric acid (TBA) and sodium fluorescein were purchased from Sigma-aldrich (Germany). Toyopearl HW-40F was obtained from Tosoh Corporation, Japan. Ethylenediaminetetraacetic acid (EDTA) was obtained from Dojindo Laboratories, Japan.

3.2 Plant Material Preparation

Different species and varieties of turmeric namely *C. longa* (variety: RD and Okinawa ukon), *C. xanthorrhiza*, *C. aromatica*, *C. amada* and *C. zedoaria* were cultivated in a field of gray soil (coarse sand 3.6%, fine sand 30.9%, silt 24.3%, clay 32.8%, pH 7.4, NO_3-N 0.07%, NH_4-N 0.08%, P 4.6 ng/g, K 42.9 ng/g; Hossain *et al*., 2005a) at the Subtropical Field Science Center, University of the Ryukyus, Okinawa, Japan. The average monthly temperature, humidity, and precipitation during the cultivation period were 17-29℃, 61-83% and 22-369 mm, respectively. Sixty seed rhizomes of each turmeric species were planted on April 15, 2015, to obtained sufficient sample. Common agronomic practices including fertilizer and irrigation were provided according to Ishimine *et al*., 2004 and

Hossain *et al*., 2005b. Rhizomes were harvested on February 10, 2016, when all the shoots of the species withered completely. The rhizomes were washed, sliced and dried in a hot air oven at 50℃ for 72 h. We got 10, 10.8, 13.1, 9.3, 20 and 9.1% dry powder from fresh rhizomes of RD, Okinawa ukon, *C. xanthorrhiza, C. aromatica, C. amada* and *C. zedoaria*, respectively.

3.3 Extraction and Yield of Samples

The equal amount of different turmeric powder (20 g) was extracted with MeOH (200 mL) for 2 days at room temperature (25℃) with continuous magnetic stirring to prevent oxidation by air and shielding from sunlight. The extraction was carried out so that the components were completely extracted and not oxidized. Then the solutions were filtered through double filter paper (Whatman™ No. 1). Fresh MeOH was added into the used plant material and the process was repeated three times. The filtered solutions were dried on a rotary evaporator under reduced pressure at 40℃. The yield of all extracts was recorded and kept in the refrigerator at 4℃ for experimental analyses.

3.4 Estimation of Total Phenolic Contents

The amount of total phenolic contents (TPC) of the test samples were performed by using a method of Kahkonen *et al*., 1999. Briefly, 500 µL of Folin-Ciocalteu and 500 µL of distilled water were added to 200 µL of the test samples (1000 µg/mL). After 1 min, 800 µL of sodium carbonate solution (7.5 %) was added to the mixture and incubated at room temperature for 30 min. Absorbance was measured at 760 nm by using Biotek Powerwave XS2 Spectrophotometer (Winooski, USA). The TPC was expressed as gallic acid equivalents (GAE) in mg per g extract. The experiment was carried out in triplicate.

3.5 Estimation of Total Flavonoid Contents

Total flavonoid contents (TFC) were estimated according to the method describe by Djeridane *et al*., 2006. Rutin was used to make a calibration curve. A hundred microliters of samples (1000 µg/mL) were put in the microplate and 100 µL of 2% $AlCl_3$ was added. The reaction was mixed and stand at room temperature for 15 min. The absorbance of the mixture was measured at 430 nm by using a Biotek Powerwave XS2 Spectrophotometer (Winooski, USA). The TFC was expressed as rutin equivalents in mg per g extract. The experiment was carried out in triplicate.

3.6 Determination of Antioxidant Activities

3.6.1 DPPH Radical Scavenging Assay

The DPPH radical scavenging assay was estimated according to the procedure of Boskou *et al*., 2006. Forty µL of DPPH and 80 µL of sodium acetate buffer (0.1M, pH =5.5) were added into 80 µL samples at different concentrations (10, 25 and 50 µg/mL). The mixture solution was incubated at room temperature in the dark for 30 min. The absorbance was measured at 517 nm by using a Biotek Powerwave XS2 Spectrophotometer (Winooski, USA). Trolox was used as positive control in this assay.

The % DPPH radical scavenging activity was calculated using the formula:

$$\% \text{ scavenging capacity} = (A_{control} - A_{sample})/A_{control} \times 100$$

Where control is the absorbance of control without test sample and sample is the absorbance of the sample. The experiment was carried out in triplicate. The % radical scavenging activity was plotted against the corresponding extract concentration to obtain the IC_{50} value.

3.6.2 Oxygen Radical Absorption Capacity (*ORAC)*

The ORAC assay measures the ability of antioxidant compounds in test materials to

protect against oxidation induced by the peroxyl radical generator AAPH. ORAC of the samples was measured according to the method of Huang *et al*., 2002, with the excitation and emission wavelengths of 485 and 528 nm, respectively. Trolox (5, 10, 15, 25, 40 and 60 µM) was used as a standard. The results were expressed as Trolox equivalents (TE) in micromole per g extract (µmol TE/g). The experiment was carried out in triplicate.

3.6.3 Reducing Power Assay (RPA)

Reducing power of the extract was determined according to the method of Murrant *et al*., 2001. Fifty microliters (500 µg/mL) of the test samples were mixed with 125 µL phosphate buffer (0.2 M, pH 6.6) and 125 µL potassium ferricyanide [$K_3Fe(CN)_6$] (1%). The mixture was incubated at 50℃ for 20 min. Trichloroacetic acid (10 % w/v) of 125 µL was added to the mixture, then centrifuged at 1000 g for 10 min. The upper layer of the solution (100 µL) was mixed with distilled water (100 µL) and $FeCl_3$ (20 µL, 0.1 % w/v). The absorbance was measured at 700 nm by using a Biotek Powerwave XS2 Spectrophotometer (Winooski, USA). Trolox was used as positive control. Increased absorbance of the reaction mixture indicates greater reducing power capacity. The experiment was carried out in triplicate.

3.6.4 The 2-deoxyribose degradation assay (2-DR)

The assay was performed by using a method of Halliwell *et al*., 1987 with slight modification. In brief, 100 µL of different concentrations (5, 10, 20 and 50 µg/mL) of turmeric extracts solution was brought to 400 µL phosphate buffer (2 mM, pH 7.4). Then, 50 µL of 2-deoxy-D-ribose (50 mM), 50 µL of EDTA (104 µM), 50 µL of $FeCl_3$ (100 µM), 50 µL of H_2O_2 (1 mM) and 50 µL of ascorbic acid (100 µM) were added. The total volume of the reaction mixture was adjusted to 800 µL with buffer. After incubation at

30℃ for 1h, the reaction was terminated by 250 µL of trichloroacetic acid (10%, w/w). The color was then developed by addition of 150 µL of TBA (1%, in 50 mM NaOH solution) and heating in an oven at 100℃ for 20 min. The mixture was cooled and absorbance was measured at 532 nm (Biotek Powerwave XS2 Spectrophotometer, Winooski, USA) against the buffer (as blank). The hydroxyl radical-scavenging activity was expressed as:

$$\text{Inhibition\%} = (A_{control} - A_{sample})/A_{control} \times 100$$

Where control is the absorbance of control without test sample and sample is the absorbance of the sample. The experiment was carried out in triplicate. The IC_{50} value represented the concentration of the extracts that caused 50% inhibition. Trolox was used as positive control.

3.7 Isolation of Bioactive Compounds from the Crude Extract of RD

Among the different turmeric extracts, RD showed the highest TPC, TFC and antioxidant activity. Therefore bioactive compounds were isolated from the crude extract of RD. For this, 300 g dried RD powder obtained from 3 kg fresh rhizome was used. The flow chart for the bioactivity guided purification of the antioxidant compounds from the crude extract of RD was described in figure 4-1. After repeated fractionation of RD with different solvents, eight active fractions were purified by C18 reversed-phase HPLC (Inter Sustain C18 column) equipped with water and MeOH as the mobile phase with a flow rate of 4.0 mL/min and detected at 280 nm. Antioxidant activity was detected in 9 peaks fraction. The isolated peaks were dissolved in MeOH-d_4 and then subjected to spectral analysis. Nuclear magnetic resonance (NMR) spectra were recorded on BRUKER NMR spectrometers (500 MHz for ^{1}H and 125 MHz for ^{13}C) at room

temperature. Mass Spectrometry experiments were carried out on a Waters Mass Spectrometer (Quattro Micro, USA) using an electrospray ionization (ESI) probe under the following instrumental conditions: Column: COSMOSIL 5C18 AR-II, 2×150 mm. Solvent A: water (0.1% formic acid), Solvent B: acetonitrile, Flow rate: 0.2 mL/min, Injection volume: 5 μL, Run time: 30 min, Pump mode: Binary gradient, Time program: 10% B (0 min) - 100% B (20 min) - 100% B (25 min) - 10% B (25.1 min) - 10% B (30 min), MS ionization mode: ES (+), Capillary voltage: 4.0 kV, Cone voltage: 20 V, Source Temp.: 120℃, Desolvation Temp.: 350℃, Cone Gas flow: 100 L/h, Desolvation Gas flow: 800 L/h.

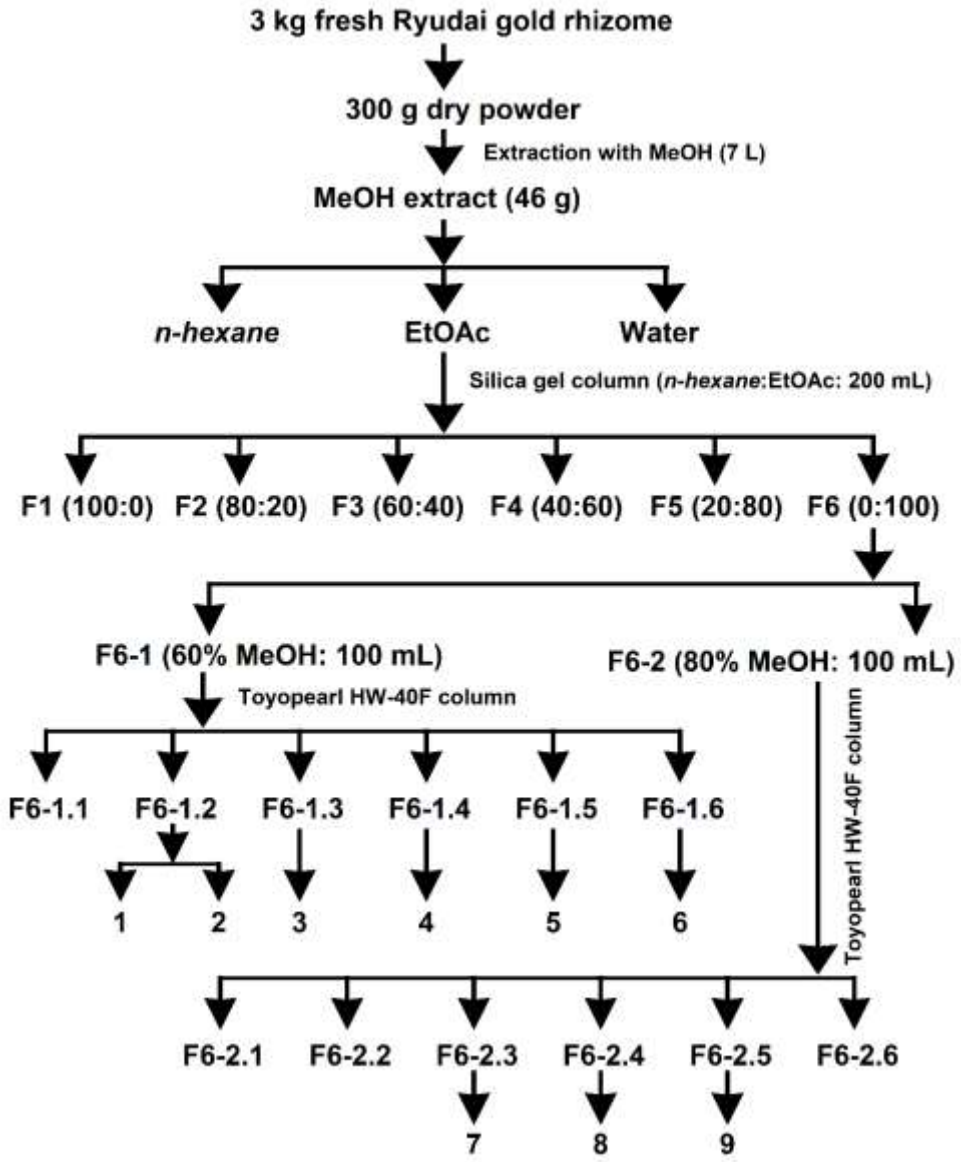


Figure 4-1. Flow chart for the bioactivity-guided purification of antioxidant compounds from the fresh rhizome of turmeric Ryudai gold. Structural identification of the compounds was conducted using ^{1}H NMR, ^{13}C NMR, and liquid chromatography-tandem mass spectrometry.

3.8 Antioxidant Activity of the Isolated Compounds

Isolated compounds were dissolved in MeOH for making the concentrations of 5, 10, 30, 50, 100, 200, 500 and 1000 μM for each compound. Antioxidant activity was determined using DPPH radical scavenging and 2-DR oxidation assay according to the aforementioned procedure.

3.9 *Statistical Analysis*

Results are expressed as the mean ± SEM of three independent experiments in triplicate. Multiple comparisons were performed using one-way analysis of variance followed by Duncan's multiple range tests. Differences were considered significant at $p<0.05$.

4. Results

4.1. Estimation of Yield of Extract

The MeOH extract of dry RD powder resulted the highest extract yield (15%) followed by *C. xanthorrhiza* (14%), *C. aromatica* (10.5%), *Okinawa ukon* (10%), *C. zedoaria* (7.5%) and *C. amada* (6.5%).

4.2. Estimation of Total Phenolic and Flavonoid Contents:

TPC and TFC of different species and varieties of turmeric extracts are shown in Table 4-1. Their content was significantly different among the various species and variety of turmeric. RD contained a significantly higher amount of total phenolic (157.4 ± 1.0 mg GAE/g extract) and TFC (1089.5 ± 0.9 mg rutin/g extract) content than those of others. Whereas *C. zedoaria* contained the lowest amount of this two phytochemicals (Table 4-1).

Table 4-1. Total phenolic and flavonoid content of different species and varieties of turmeric.

Sample	Total phenolic content (mg GAE/g extract)	Total flavonoid content (mg Rutin/g extract)
Ryudai gold	157.4 ± 1.0^{a}	1089.5 ± 0.9^{a}
Okinawa ukon	59.2 ± 0.7^{b}	620.7 ± 0.9^{b}
C. xanthorrhiza	38.5 ± 0.9^{c}	797.5 ± 0.9^{c}
C. aromatica	37.9 ± 1.0^{c}	310.7 ± 0.9^{d}
C. amada	48.7 ± 0.9^{d}	89.3 ± 0.9^{e}
C. zedoaria	43.7 ± 0.9^{e}	15.3 ± 0.8^{f}

Data are expressed as the mean ± SEM of three independent experiments in triplicate. Different letters (a, b, c, d, e and f) in each column indicate a significant difference ($p < 0.05$) according to Duncan's multiple range tests.

4.3. Antioxidant Activity of Different Species and Varieties of Turmeric

The DPPH radical scavenging activity, total reducing power, ORAC and 2-DR oxidation assay are shown in table 4-2. Among the six different turmeric extracts, RD evidenced the significantly lower IC_{50} for the DPPH radicals (26.4 µg/mL), 2-DR oxidation (7.4 µg/mL), stronger reducing power (0.33) and higher ORAC value (14090 µmol TE/g extract) than those of others. There was no significant difference in the calculated IC_{50} values of DPPH activity between RD and the Trolox (a potent commercial antioxidant).

The correlations (R) between the antioxidant activity revealed by the four assays [DPPH ($1/IC_{50}$ values), RPA, ORAC and 2-DR ($1/IC_{50}$ values) assays] and TPC and TFC are represented in Table 4-3. The best correlations were found for RPA *vs.* ORAC (R = 0.95) followed by ORAC *vs.* DPPH (R = 0.94) while the lowest correlation was found for TFC *vs.* 2-DR (R = 0.27) (Table 4-3).

Table 4-2. Antioxidant activity of different species and varieties of turmeric.

Sample	DPPH (IC_{50} μg/mL)	RPA (Abs)	ORAC μmol TE/g extract	2-DR oxidation assay (IC_{50} μg/mL)
Ryudai gold	26.4 ± 0.2^{a}	0.3 ± 0.006^{a}	14090 ± 0.9^{a}	7.4 ± 1.3^{a}
Okinawa ukon	291.3 ± 3.1^{f}	0.1 ± 0.002^{bc}	4119.7 ± 0.7^{b}	28.3 ± 2.3^{d}
C. xanthorrhiza	80.4 ± 0.7^{b}	0.2 ± 0.001^{b}	6490.3 ± 0.7^{c}	19.0 ± 1.7^{c}
C. aromatica	130.7 ± 2.0^{d}	0.1 ± 0.053^{c}	1680 ± 0.9^{d}	20.4 ± 2.0^{c}
C. amada	92.8 ± 3.4^{c}	0.2 ± 0.004^{b}	6600 ± 0.8^{c}	12.8 ± 0.7^{b}
C. zedoaria	228.4 ± 3.4^{e}	0.1 ± 0.062^{c}	1790.3 ± 0.7^{d}	20.2 ± 2.0^{c}
Trolox	23.2 ± 0.6^{a}	1.4 ± 0.023^{d}		2.4 ± 0.8^{e}

Data are expressed as the mean ± SEM of three independent experiments in triplicate. Different letters (a, b, c, d and e) in each column indicate a significant difference ($p < 0.05$) according to Duncan's multiple range tests.

Table 4-3. Correlations (R) between antioxidant capacities [DPPH ($1/IC_{50}$ values), RPA, ORAC and 2-DR ($1/IC_{50}$ values) assays] of different turmeric extracts, TPC and TFC.

	TPC	TFC	DPPH	RPA	ORAC	2-DR
TPC		0.70	0.92	0.92	0.90	0.78
TFC			0.73	0.60	0.80	0.27
DPPH				0.90	0.94	0.91
RPA					0.95	0.83
ORAC						0.82

R: correlation coefficient

4.4. Antioxidant Activity of Water, n-hexane and EtOAc Fraction of MeOH Extract of RD

The MeOH extract of RD was partitioned with water, *n*-hexane, and EtOAc. The antioxidant activity of these three fractions was determined by using DPPH radical scavenging assay according to the aforementioned procedure. Among them, the EtOAc part showed significantly stronger effect than that of water and *n*-hexane (Figure 4-2A).

4.5. Antioxidant Activity of Different Fractions of EtOAc Part of RD

The EtOAc fraction was subjected to chromatography on a silica gel (75 g) column (30 x 3 cm). Elution was carried out using *n*-hexane and EtOAc with increasing amount of EtOAc [*n*-hexane:EtOAc; 100:0 (F1), 80:20 (F2), 60:40 (F3), 40:60 (F4), 20:80 (F5) and 0:100 (F6)]. Among the six fractions of EtOAc part of RD, F6 showed significantly higher antioxidant activity than others (Figure 4-2B).

4.6. Antioxidant Activity of Two Fractions of F6 Part of RD

The F6 part of RD was passed through Sep-Pak C18 and obtained two fractions by using 60% (F6-1) and 80% (F6-2) MeOH. There was no significant difference in the antioxidant activity of F6-1 (74%) and F6-2 (68%) (Figure 4-2C).

4.7. Antioxidant Activity of Twelve Fractions from F6-1and F6-2 part of RD

As both fractions (F6-1 and F6-2) exhibited strong antioxidant activity (Figure 4-2C), they were divided into 12 fractions (F6-1.1 to F6-1.6 and F6-2.1 to F6-2.6) using Toyopearl HW-40F column (50 x 1.5 cm) chromatography, eluted with 40%, 50%, 60%, 70% and 90% MeOH. Among these 12 fractions, 8 fractions (F6-1.2 to F6-1.6 and F6-2.3 to F6-2.5) exhibited high antioxidant activity (Figures **4-**2D, 4-2E).

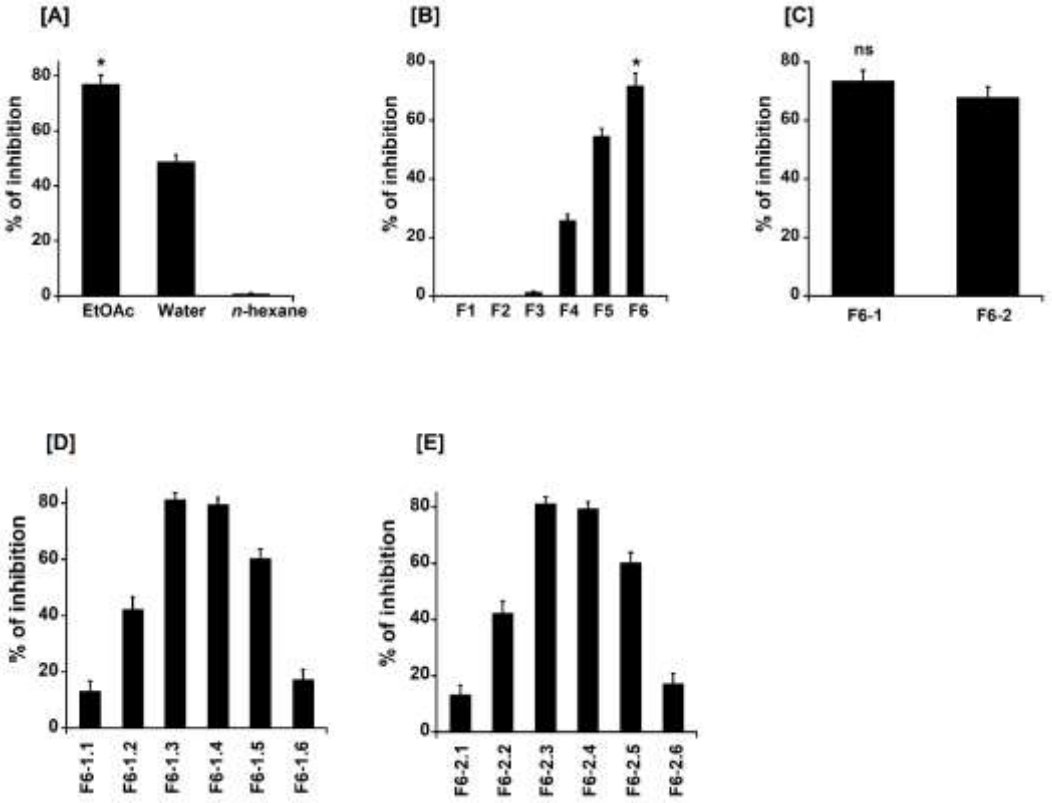


Figure 4-2. Antioxidant activity of different solvent extract [A], fractions of EtOAc part [B], fractions of F6 of EtOAc [C], fractions of F6-1 [D] and F6-2 [E] of Ryudai gold according to DPPH radical scavenging assay. Results are expressed as the mean ± SEM of three independent experiments in triplicate. $^{*}p<0.05$ vs. others; ns= not significant.

4.8. Chemical Structures of the Isolated Compounds

The chemical structures of the isolated compounds were identified according to their ^{1}H NMR and ^{13}C NMR spectra. Peak data were as follows:

Compound 1: Colorless oil**,** UV λ_{max} nm: 239. ESI-MS (+): *m/z* 235.2 $[M+H]^{+}$, 257.2 $[M+Na]^{+}$. ^{1}H-NMR (CD_3OD) δ:6.18 (1H, s, H-10), 5.80 (1H, s, H-5), 2.77 (1H, m, H-7), 2.46 (1H, dd, *J*=16, 7 Hz, H-8a), 2.37 (1H, dd, *J*=10, 5 Hz, H-8b), 2.18 (1H, dt, *J*=12, 4 Hz, H-1), 2.10 (3H, s, H-13), 1.99 (1H, m, H-2a), 1.96 (3H, s, H-15), 1.90 (3H, s, C-12), 1.80 (1H, m,

H-2b), 0.81 (3H, d, J=7 Hz, H-14). ^{13}C-NMR (CD_3OD) δ:203.3 (C-9), 202.9 (C-6), 165.5 (C-4), 157.4(C-11), 127.1 (C-5), 124.9 (C-10), 50.8 (C-1), 49.8 (C-8), 31.6 (C-3), 28.9 (C-7), 27.7 (C-12), 24.2 (C-2), 24.1 (C-15), 20.9 (C-13), 16.5 (C-14). After comparing these data with previously reported data in the literature Zeng *et al*., (2007), the compound was identified as bisabolone-9-one (Figure 4-3).

Compound 2: Colorless oil**,** UV λ_{max} nm: 236. ESI-MS (+): *m/z* 217.3 $[M+H-H_2O]^+$, 235.2 $[M+H]^+$, 257.3 $[M+Na]^+$. ^{1}H-NMR (CD_3OD) δ: 6.18 (1H, s, H-10), 6.13 (1H, d, *J*= 10 Hz, H-3), 5.72 (1H, d, *J*= 10 Hz, H-2), 5.04 (1H, s, H-15a), 4.93 (1H, s, H-15b), 4.35 (1H, s, H-5), 2.52 (1H, dd, *J*= 15, 5, H-8a), 2.45 (1H, m, H-1), 2.26 (1H, m, H-8b), 2.16 (1H, m, H-7), 2.11 (3H, s, H-13), 1.90 (3H, s, H-12), 1.82 (1H, m, H-6a), 1.53 (1H, m, H-6b), 0.88 (3H, d, *J*= 7 Hz, H-14). ^{13}C-NMR (CD_3OD) δ: 203.4 (C-9), 157.3 (C- 11), 146.2 (C-4), 134.1 (C-2), 128.7 (C-3), 125.0 (C-10), 113.7 (C-15), 69.6 (C-5), 50.0 (C-8), 37.5 (C-1), 34.4 (C-7), 33.2 (C-6), 27.7 (C-12), 20.9 (C-13), 17.0 (C14). After comparing these data with previously reported data in the literature Li *et al*., (2008), the compound was identified as 4-methyllene-5-hydroxybisabola-2,10-diene-9-one (Figure 4-3).

Compound 3: Colorless oil, UV λ_{max} nm: 241. ESI-MS (+): *m/z* 233.3 $[M+H]^+$. ^{1}H-NMR (CD_3OD) δ: 6.89 (1H, s, H-3), 6.82 (1H, dd, *J*=8, 2 Hz, H-6), 6.62 (1H, d, *J*=8 Hz, H-5), 6.10, (1H, s, H-10), 3.12 (1H, m, H-7), 2.65 (1H, dd, *J*=15, 7 Hz, H-8a), 2.57 (1H, dd, J=15, 7 Hz, H-8b), 2.13 (3H, s, H-15), 2.03 (3H, s, H-13), 1.85 (3H, s, H-12), 1.18 (3H, d, *J*=7 Hz, H-14). ^{13}C-NMR (CD_3OD) δ: 203.0 (C-9), 156.4 (C-11), 154.6 (C-2), 138.5 (C-4), 130.2 (C-1), 125.8 (C-6), 125.33 (C-10), 125.26 (C-5)115.5 (C-3), 53.9 (C-8), 36.8 (C-7), 27.6 (C-12), 22.7 (C-14), 20.8 (C-13), 16.3 (C-15). Comparison of these spectroscopic features with previously reported literatures by Imai *et al*., (1990) and Sharma and Tek Chand, (1996), the compound was identified as turmeronol B (Figure 4-3).

Compound 4: Yellow powder, UV λ_{max} nm: 340. ESI-MS (+): *m/z* 373.2 $[M+H]^+$. ^{1}H-NMR (CD_3OD) δ: 7.56 d (1H, d, *J*=16 Hz, H-4'), 7.21 (1H, s, H-6'), 7.11 (1H, d, *J*=8 Hz, H-10'), 6.81 (1H, d, *J*=8Hz, H-9'), 6.78 (1H, s, H-6), 6.69 (d, *J*=16 Hz, H-3'), 6.68 (1H, d, *J*=8Hz, H-9), 6.64 (1H, d, *J*=8Hz, H-10), 4.11 (1H, m, H-2), 3.89 (3H, s, H-11), 3.81 (3H, s, H-11'), 2.86 (1H, dd, *J*=16, 8 Hz, H-3a), 2.79 (1H, dd, J=16, 5Hz, H-3b), 2.71 (1H, m, H-4a), 2.60 (1H, m, H-4b), 1.78 (2H, m, H-1). Comparing these data with previously reported data in the literature by Li *et al*., (2009), the compound was identified as 5-hydroxy-1,7-bis(4-hydroxy-3-methoxyphenyl)-1-hepten-3-one (Figure 4-3).

Compound 5: Orange powder, UV λ_{max} nm: 288, 325. ESI-MS (+): *m/z* 327.1 $[M+H]^+$, 349.1 $[M+Na]^+$. ^{1}H-NMR (CD_3OD) δ 7.91 (2H, d, *J*=8 Hz, H-6, 10), 7.61 (1H, d, *J*=16 Hz, H-4'), 7.50 (2H, d, *J*= 8 Hz, H-6', 10'), 6.84 (2H, d, *J*= 9 Hz, H-7, 9). 6.81 (2H, d, *J*= 9 Hz, H-7', 9'), 6.69 (1H, d = 16 Hz, H-3'), 4.71 (1H. m, H-2), 3.15 (2H, dd, *J* =10, 8 Hz, H-3), 2.93 (2H, dd, *J*= 7, 4 Hz, H-1). ^{13}C-NMR (CD_3OD) δ: 201.4 (C-2'), 199.4 (C-4), 164.0 (C-8), 161.6 (C-8'), 145.7 (C-4'), 132.0 (C-6, 10), 131.6 (C-4), 130.3 (C-5), 127.2 (C-5'), 124.4 (C-3'), 116.9 (C-7', 9'), 116.3 (C-7, 9), 66.5 (C-2), 48.3 (C-1i), 46.2 (C-3). Comparing these data with previously reported data in the literature by Li *et al*., (2009), the compound was identified as 3-hydroxy-1,7-bis(4-hydroxyphenyl)-6-hepten-1,5-dione (Figure 4-3).

Compound 6: Yellow powder, UVλ_{max} nm: 358. ESI-MS (+): *m/z* 309.2 $[M+H]^+$. ^{1}H-NMR (CD_3OD) δ: 7.39 (2H, d, *J*= 8Hz, H-6, 10), 7.32 (2H, d, *J*=8 Hz, H-6', 10'), 7.29 (1H, d, *J*=16 Hz, H-4), 6.81 (2H, d, *J*=8 Hz, H-7', 9'), 6.73 (2H, d, *J*=8 Hz, H-7, 9), 6.57 (1H, d, *J*=16 Hz, H-3), 5.55 (1H, s, H-1), 5.40 (1H, dd, *J*=14, 4 Hz, H-4'), 2.93 (1H, dd, *J*=17, 14Hz, H-3'a), 2.53 (1H, dd, *J*= 17, 3 Hz, H-3'b). ^{13}C-NMR (CD_3OD) δ: 171.5 (C-2), 160.8 (C-8), 139.3 (C-4), 130.9 (C-6, 10), 130.5 (C-5'), 129.3 (C-6', 10'), 127.7 (C-5), 119.1 (C-3), 116.8 (C-7, 9), 116.4 (C-7', 9'), 105.3 (C-1), 82.1 (C-4'), 43.2 (C-3'). From the comparison of these

data with those reported in the literature by Jiang *et al*., (2012) and Jiang *et al*., (2013), the compound was identified as cyclobisdemethoxycurcumin (Figure 4-3).

Compound 7: Yellow powder**,** UV λ_{max} nm: 247, 414. ESI-MS (+): *m/z* 309.1 $[M+H]^+$. ^{1}H-NMR (CD_3OD) δ: 7.56 (2H, d, *J*=16 Hz, H-4, 4'), 7.51 (4H, d, *J*=8 Hz, H-6, 10, 6', 10'), 6.84 (4H, d, *J*=8 Hz, H-7, 10, 7', 10'), 6.62 (2H, d, *J*=16 Hz, H-3, 3'). ^{13}C-NMR (CD_3OD) δ: 184.8 (C-2, 2'), 161.1 (C-8, 8'), 141.8 (C-4, 4'), 131.1 (C-6, 10, 6', 10'), 128.0 (C-5, 5'), 122.0 (C-3, 3'), 116.9 (C-7, 9, 7', 9'). From the comparison of these data with those reported in the literature by Kiuchi *et al*., (1993) and Li *et al*., (2009), the compound was identified as 1,7-bis(4-hydroxyphenyl)hepta-1,6-diene-3,5-dione (bisdemethoxycurcumin) (Figure 4-3).

Compound 8: Yellow powder**,** UV λ_{max} nm: 247, 414. ESI-MS (+): *m/z* 339.3 $[M+H]^+$. ^{1}H-NMR (CD_3OD) δ: 7.56 (2H, d, *J*=16 Hz, H-4, 4'), 7.48 (2H, d, *J*=8 Hz, H-6, 10), 7.20 (1H, s, H-6'), 7.10 (1H, d, *J*=8 Hz, H-10'), 6.80 (3H, d, *J*=8 Hz, H-7, 10, 9'), 6.60 (2H, br, H-3, 3'), 3.90 (3H, s, 7'-OCH_3). ^{13}C-NMR (CD_3OD) δ: 184.7 (C-2, 2'), 161.1 (C-8, 8'), 150.5 (C-8'), 149.4 (C-7'), 142.1 (C-4'), 141.9 (C-4), 131.1 (C-6, 10), 128.6 (C-5'), 128.0 (C-5), 124.1 (C-10'), 122.3 (C-3'), 122.0 (C-3), 116.9 (C-10), 116.6 (C-9'), 111.8 (C-6'), 56.5 (7'- OCH_3). From the comparison of these data with those reported in the literature by Kiuchi *et al*., (1993) and Li *et al*., (2009), the compound was identified as 1-(4-hydroxy-3-methoxyphenyl)-7-(4-hydroxyphenyl)hepta-1,6-diene-3,5-dione (demethoxycurcumin) (Figure 4-3).

Compound 9: Yellow powder, UV λ_{max} nm: 262, 419. ESI-MS (+): *m/z* 369.2 $[M+H]^+$. ^{1}H-NMR (CD_3OD) δ: 7.57 (2H, d, *J*=16 Hz, H-4, 4'), 7.21 (2H, s, H-6, 6'), 7.10 (2H, bd, *J*=8 Hz, H-10, 10'), 6.82 (2H, d, *J*=8 Hz, C-9, 9'), 6.62 (2H, bd, *J*=16 Hz, H-3, 3'), 3.90 (6H, s, 7, 7'-OCH_3). ^{13}C-NMR (CD_3OD) δ: 184.8 (C-2, 2'), 150.6 (C-8, 8'), 149.5 (C-7,

7'), 142.1 (C-4, 4'), 128.6 (C-5, 5'), 124.1 (C-10, 10'), 122.3 (C-3, 3'), 116.6 (C-9, 9'), 111.8 (C-6, 6'), 56.5 (7, 7'-OCH_3). From the comparison of these data with those reported in the literature by Kiuchi *et al*., (1993), Li *et al*., (2009), and Payton *et al*., (2007), the compound was identified as 1,7-bis(4-hydroxy-3-ethoxyphenyl)-1,6-heptadiene-3,5-dione (curcumin) (Figure 4-3).

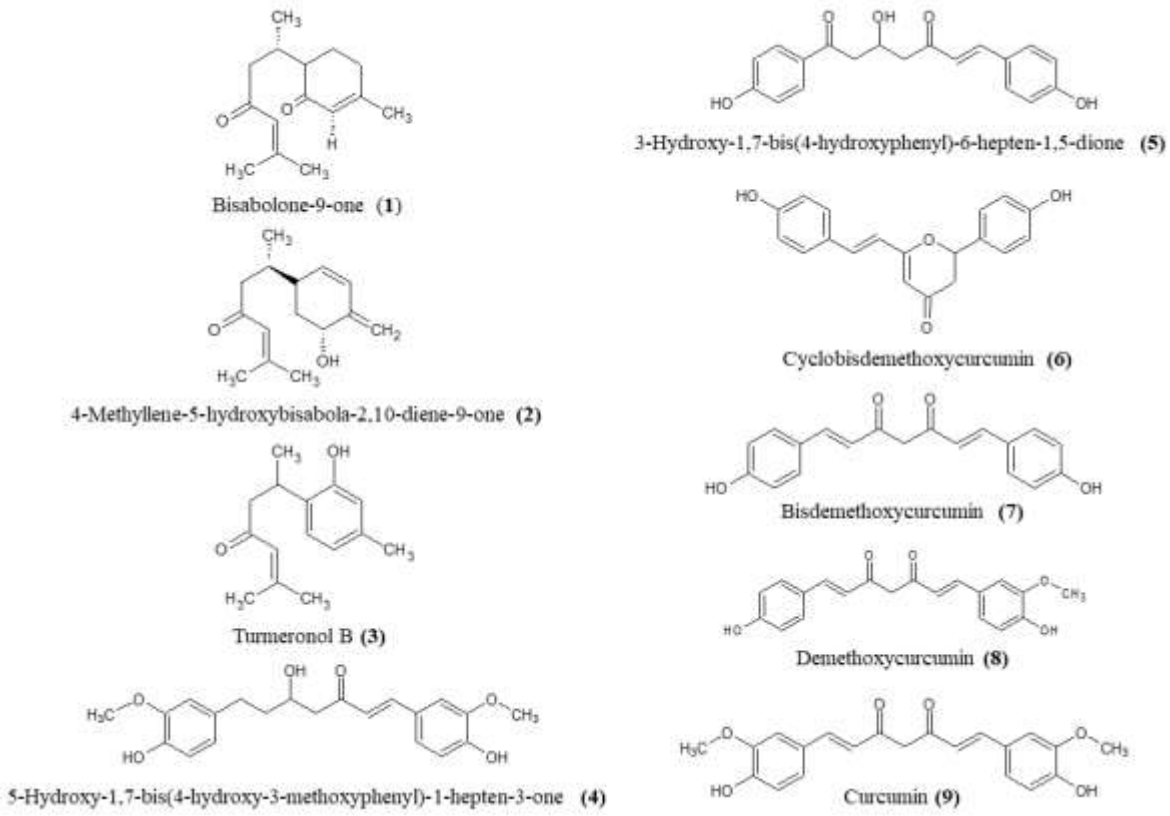


Figure 4-3. Chemical structures of the nine compounds isolated from Ryudai gold were identified according to their ^{1}H NMR and ^{13}C NMR spectra; bisabolone-9-one (**1**), 4-methyllene-5-hydroxybisabola-2,10-diene-9-one (**2**), turmeronol B (**3**), 5-hydroxy-1,7-bis(4-hydroxy-3-methoxyphenyl)-1-hepten-3-one (**4**), 3-hydroxy-1,7-bis(4-hydroxyphenyl)-6-hepten-1,5-dione (**5**), cyclobisdemethoxycurcumin (**6**), bisdemethoxycurcumin (**7**), demethoxycurcumin (**8**) and curcumin (**9**).

4.9 Antioxidant Activity of the Isolated Compounds

Nine compounds namely bisabolone-9-one (**1**), 4-methyllene-5-hydroxybisabola-2,10-diene-9-one (**2**), turmeronol B (**3**), 5-hydroxy-1,7-bis(4-hydroxy-3-methoxyphenyl)-1-hepten-3-one (**4**), 3-hydroxy-1,7-bis(4-hydroxyphenyl)-6-hepten-1,5-dione (**5**), cyclobisdemethoxycurcumin (**6**), bisdemethoxycurcumin (**7**), demethoxycurcumin (**8**) and curcumin (**9**) were isolated from RD. All the compounds exhibited antioxidant activity in a concentration-dependent manner. The IC_{50} values for DPPH and 2-DR oxidation assay are shown in Figure 4-4[A] and 4-4[B]. Among the nine compounds, compound **4, 5, 8** and **9** showed significantly stronger antioxidant activity than Trolox.

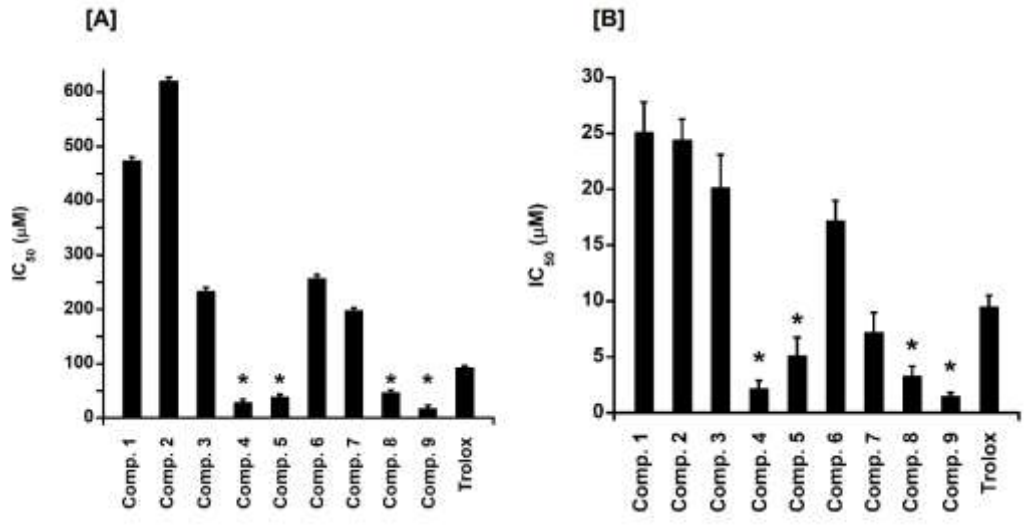


Figure 4-4. IC_{50} value for the antioxidant activity of bisabolone-9-one (**1**) , 4-methyllene-5-hydroxybisabola-2,10-diene-9-one (**2**), turmeronol B (**3**), 5-hydroxy-1,7-bis(4-hydroxy-3-methoxyphenyl)-1-hepten-3-one (**4**), 3-hydroxy-1,7-bis(4-hydroxyphenyl)-6-hepten-1,5-dione (**5**), cyclobisdemethoxycurcumin (**6**), bisdemethoxycurcumin (**7**), demethoxycurcumin (**8**) and curcumin (**9**). DPPH radical scavenging assay [A] and 2-DR oxidation assay [B] were performed to measure antioxidant activity. Values are expressed as the mean ± SEM of three independent experiments. $^{*}p<0.05$ vs. Trolox.

5. DISCUSSION

The present study demonstrated that there are significant variations in the antioxidant activity, TPC and TFC among different species and varieties of turmeric. Among the turmeric, RD showed significantly stronger antioxidant activity and contained a higher amount of TPC and TFC than those of others. Therefore, we have purified 9 active antioxidant compounds from RD.

Phenolics including flavonoid are ubiquitously present in vegetables as well as in medicinal plants. These are the secondary metabolite that contribute to color and flavor, and have significant roles as free radical scavengers, hydrogen donors, reducing agent and singlet oxygen quenchers. Therefore to counter the potential hazards of oxidative damage, the dietary consumption of antioxidant phenolics and flavonoids may be regarded as the first line of defense against highly reactive toxicants (Denre, 2014). Our result showed that RD had 3-4 fold higher concentration of phenolic content (157.4 mg GAE/g extract) than that of other turmeric (Table 4-1). These results are in consistent with a previous study (Nahak and Sahu, 2011) that compare phenolic content of several turmeric and reported that *C. longa* contained the highest concentration of TPC followed by *C. zedoaria*, *C. aromatica* and *C. amada*, respectively. From the present study, we got 1 g extract from 6.6 g and 10 g dry weight of RD and Okinawa ukon, respectively. From 1 g of extract, we obtained 157.4 mg GAE TPC from RD. According to the previous report, TPC of *C. longa* from West Bengal was 13 mg GAE/ g dry turmeric (Denre, 2014) which was higher than Okinawa ukon, but lower than RD. The results suggested that there is a variation in the TPC content in different varieties of the *C. longa*. The difference in TPC might be due to the amount of active substances, solvent, extraction time, temperature and geographic area of cultivation of turmeric. Like TPC, RD contained significantly higher flavonoids than others (Table 4-1) that are partially consistent

with Alafiatayo *et al*., 2014 who reported *C. longa* contain higher flavonoid than *C. xanthorrhiza* and *C. amada*. Tanvir *et al*., 2017 reported significant variation in TFC of different varieties (mura and chora) of *C. longa*.

To determine the antioxidant capacity, we measured the DPPH, ORAC, RPA and 2-DR oxidation assay. The DPPH free-radical scavenging activity assay is one of the most common methods for investigating the free-radical scavenging activities of plant products. The antioxidants scavenge DPPH radical by donating hydrogen atoms leading to a non-radical state with yellow color. In our study, RD showed the highest scavenging activity with the lowest IC_{50} (26.5 µg/mL), which was not significantly different with the IC_{50} value of the standard antioxidant Trolox (IC_{50}: 23.2 µg/mL). On the other hand, Okinawa ukon showed the highest IC_{50} value (291.3 µg/mL). Our results are similar to Alafiatayo *et al*., 2014 that compared the antioxidant activity of several turmerics and reported that *C. longa* showed the highest scavenging activity followed by *C. xanthorrhiza* and *C. amada*. These results are also partially consistent with Nahak and Sahu 2011, who compared antioxidant activity of ethanolic extract of *C. longa*, *C. aromatica, C. zedoaria* and *C. amada* and reported stronger antioxidant activity of *C. longa*. In RPA, the reducing capacity of antioxidant is evaluated by the transformation of Fe^{3+} to Fe^{2+} form. Table 4-2 showed the reductive capability of turmeric compared with Trolox. Among the six turmerics, the highest reducing power was observed in RD followed by *C. amada* and Okinawa ukon. ORAC assay is considered to be a ‘standard’ method for comparing the antioxidant activity of food materials and supplements as it measures the hydrogen-atom transfer reactions and initiates *in vivo* antioxidant action (Haytowitz and Bhagwat, 2010). Similar to DPPH and RPA, RD observed significantly higher ORAC value (14090 µmol TE/ g extract) whereas *C. aromatica* had the lowest value (1680 µmol TE/ g extract). So far, there is no report on ORAC value of different species and

varieties of turmeric except *C. longa*. The ORAC value of *C. longa* from Thailand was 815.4 µmol TE/ g dry weight (Kettawan *et al*., 2012) which was much lower than RD, but higher than Okinawa ukon. Similar to DPPH, RPA and ORAC, RD resulted the highest hydroxyl radical scavenging activity with the lowest IC_{50} (7.4 µg/mL), while the highest IC_{50} value was observed in Okinawa ukon (IC_{50}; 28.3 µg/mL). Kim *et al*., 2011 compared the 2-DR oxidation activity of several spices and reported that hydroxyl radical scavenging activity of the turmeric (*C. longa*) extract was higher than other spices extract. Another report suggested that the IC_{50} values for 2-DR oxidation assay of different turmeric (*C. longa*) samples collected from different region of India varied from 39.8 to 72.9 µg/mL (Arya *et al*., 2015) which were higher than our findings. The variations in the antioxidant activity of different turmeric in the present study and others might be due to the genetic variations, chemical composition of turmeric, different geographical area including soil types and climatic conditions of turmeric cultivation. The significantly higher antioxidant activity of RD may be attributed to the higher TPC and TFC as we found a very strong correlation between TPC and TFC of turmeric with their antioxidant activity. High correlations were found between the TPC and TFC, and DPPH radical scavenging activity in various spices including turmeric (Kim *et al*., 2011).

Though antioxidant capacity of some species and varieties of turmeric have been reported previously (Kim *et al*., 2011; Tanvir *et al*., 2017) and many compounds have been isolated from turmeric for various biological effects. However, to the best of our knowledge active antioxidant compound from *C. longa* has never been purified and studied. Moreover, RD is a high yielding variety of *C. longa* that is rich in curcumin (major active compound of turmeric) content. So it is necessary to study its chemical constituents and biological activities. Therefore, we purified nine antioxidant compounds from RD, namely, bisabolone-

9-one (**1**), 4-methyllene-5-hydroxybisabola-2,10-diene-9-one (**2**), turmeronol B (**3**), 5-hydroxy-1,7-bis(4-hydroxy-3-methoxyphenyl)-1-hepten-3-one (**4**), 3-hydroxy-1,7-bis(4-hydroxyphenyl)-6-hepten-1,5-dione (**5**), cyclobisdemethoxycurcumin (**6**), bisdemethoxycurcumin (**7**), demethoxycurcumin (**8**) and curcumin (**9**).

Zeng *et al*., (2007), isolated and identified bisabolone-9-one (**1**), a sesquiterpene from *C. longa.* Li *et al*., (2008) isolated 4-methyllene-5-hydroxybisabola-2, 10-diene-9-one (**2**), a natural bisabolane sesquiterpenoid from the aerial parts of *Schisandra propinqua* var. *intermedia.* However, this is the first report of 4-methyllene-5-hydroxybisabola-2,10-diene-9-one to isolate from turmeric. Turmeronol B (**3),** a phenolic sesquiterpene kitone isolated from *C. longa* (Imai *et al*., 1990). It inhibited soybean lipoxygenase enzyme which is present in soybean seed and catalyze the oxidation of certain useful unsaturated fatty acids during food processing (Imai *et al*., 1990). Li *et al*., (2009) isolated 5-hydroxy-1,7-bis(4-hydroxy-3-methoxyphenyl)-1-hepten-3-one (**4**), 3-hydroxy-1,7-bis(4-hydroxyphenyl)-6-hepten-1,5-dione (**5**), bisdemethoxycurcumin (**7**), demethoxycurcumin (**8**) and curcumin (**9**) from *C. longa* rhizomes and developed their structure. Cyclobisdemethoxycurcumin (**6**), a diarylheptanoid was isolated from *C. longa* (Jiang *et al*., 2012). Although these compounds were previously isolated from turmeric and other plant, there is no information about their biological activity except demethoxycurcumin (**8**) and curcumin (**9**).

Curcuminoids (curcumin and its related compounds) are major chemical constituent in turmeric, having a wide range of pharmacological properties including antioxidant, anti-hypertensive (Nakmareong *et al*., 2011), neuroprotective and anti-ischemic (de Alcântara *et al*., 2016), anti-cancer (Jiang *et al*., 2012), antiplasmodial, antimalarial (Kettawan *et al*., 2012) and nematocidal activities (Kiuchi *et al*., 1993). In a different study, we found that RD contained a significantly higher amount of curcumin, demethoxycurcumin and

bisdemethoxycurcumin (Akter *et al*., 2018a). These could be the partial reason for the highest antioxidant activity of RD among the six different turmerics. It has been reported earlier that curcumin, demethoxycurcumin are the good scavenger of DPPH radical (Somparn *et al*., 2007), which is consistent with our results. The concentrations of compound **1, 2, 3, 4, 5, 6, 7, 8** and **9** were 0.017, 0.046, 0.02, 0.012, 0.30, 0.12, 28.4, 24.4, and 36 µmol/g of dry RD, respectively. The range of calculated IC_{50} values of the nine isolated compounds was 18 to 621 µM and 1.5 to 25.1 µM for DPPH and 2-DR oxidation assay, respectively. The order of scavenging activity of these compounds was **9**> **4**> **5**> **8**> Trolox> **7**> **3**> **6**> **1**> **2** (DPPH) and **9**> **4**> **8**> **5**> 7> Trolox> 6> **3**> **2**> **1** (2-DR oxidation assay). The calculated IC_{50} values of compound **4**, **5**, **8** and **9** were significantly lower than that of Trolox (a potent commercial antioxidant) indicating that these compounds could be used for potential natural antioxidant.

Structure-activity relationship studies suggest that the activity of these compounds depends significantly on the introduction of electron donating groups (methoxy) in the *ortho* position of the 4-hydroxyphenyl group. Therefore, compound **9**, **8**, and **4** with two electron donating groups (two phenoxyl groups, and two methoxyl groups for **9** and **4**, one methoxyl group for **8**) exhibited the most activity. Although many of works about the antioxidant mechanism of curcumin has been investigated, its redox behaviour is more complex and has led to conflicting views on the contribution of heptadione linker in the curcumin. More recently, H- atom transfer from CH_2 group at the centre of the heptadione link also plays an important role in the antioxidant properties of curcumin along with that of its hydroxy phenol group (Jha *et al*., 2015).

The role of free radical has been established in different diseases such as atherosclerosis, cancer, diabetes, cirrhosis, aging etc (Halliwell *et al*., 1992). Therefore, compounds that can scavenge these free radicals have great potentials to prevent or inhibit

these degenerative diseases. As our isolated compounds showed strong antioxidant activity, it would be good to study their efficacy against oxidative stress related diseases and to prevent oxidative deterioration of processed food. Besides this, further study could be conducted to explore other biological activities. Also, *in vivo* trial is necessary to confirm the health-promoting potential of these compounds.

Chapter 3

Endothelium-independent and calcium channel-dependent relaxation of the porcine cerebral artery by different species and strains of turmeric

1. ABSTRACT:

The present study were undertaken to clarify the underlying mechanism of turmeric, which is traditionally used as a medicinal plant for the treatment of cardiovascular disorders, such as hypertension, and palpitations. Methanol extracts of different turmeric were used. A tissue-organ-bath system was used to investigate the vasoactive effects of methanol extracts from 5 kinds of turmeric on isolated porcine basilar arteries. The arterial rings were suspended in physiological solution that was maintained at 37℃ temperature with a continuous supply of 95% O_2 and 5% CO_2. All turmeric extracts (20–800 μg/mL) induced concentration-dependent relaxation of the isolated porcine basilar artery pre-contracted with U46619 (1-5×10^{-9} M) in arterial rings with or without endothelium. There were no significant differences in the relaxation induced by different turmeric or between the endothelium-intact and denuded arteries. In depolarized, Ca^{2+}-free medium, the turmeric extracts inhibited $CaCl_2$-induced contractions and caused a concentration-dependent rightward shift of the response curves. In addition, propranolol (a non-specific β-adrenoceptor antagonist) slightly inhibited the relaxation induced by turmeric. In contrast, Nω-nitro-L-arginine, indomethacin, tetraethylammonium, glibenclamide and 4-aminopyridine did not affect turmeric-induced relaxation. These results demonstrated that turmeric induced endothelium-independent relaxation of the porcine basilar artery, which may be due to the inhibition of extracellular and intracellular Ca^{2+} receptors and the partial inhibition of β-adrenergic receptors in vascular smooth muscle cells.

Key words: Turmeric, cerebral artery, vasorelaxation, Ca^{2+}-channel, β-adrenergic receptor.

2. INTRODUCTION

Currently, vascular diseases, such as hypertension, atherosclerosis, subarachnoid hemorrhage, stroke, and Alzheimer's disease, have become public health challenges; therefore, it is necessary to develop modulators that control vascular tone to treat these diseases (Miller *et al*., 2005; Kearney *et al*., 2005). Vascular reactivity is an important factor for the treatment of cardiovascular diseases because it affects blood flow and pressure. The efficacy of synthetic antihypertensive drugs increases in a dose-dependent manner, which leads to various adverse effects (Handler, 2005). In addition to synthetic drugs, the use of herbs or herbal extracts is increasing in China, Japan, and Korea (Alaerts *et al*., 2010). Many plants used in traditional medicine have been investigated for treating cardiovascular disease (Ibarra-Alvarado *et al*., 2010). The effect of curcumin and its parent plant *Curcuma* spp. (Family: Zingiberaceae) on cardiovascular systems has recently received much attention.

Turmeric, especially, *Curcuma longa*, has been extensively studied for its various biological activities both *in vivo* and *in vitro*, including anti-inflammatory (de Alcantara *et al*., 2016; Verma *et al*., 2016; Salahshoor *et al*., 2015), anti-diabetic (Dehghan *et al*., 2016), wound healing (Sidhu *et al*., 1998), anti-hemolytic (Mathuria *et al*., 2007), antioxidant (Salahshoor *et al*., 2015; Nakmareong *et al*., 2011), and anti-carcinogenic (Aqqarwal *et al*., 2003) effects, as well as protection against gastrointestinal and respiratory disorders (Gilani *et al*., 2005). Curcuma drugs have been traditionally used for "Oketsu" (syndromes caused by the obstruction of blood circulation such as psychataxia, arthralgia and dysmenorrhea) in the Chinese medicine system (Sasaki *et al*., 2003). It has been reported that *C. longa* lowers arterial blood pressure and heart rate in rats (Adaramoye *et al*., 2008; Adaramoye *et al*., 2009) and induces endothelium-independent vasorelaxation in isolated rat aorta (Sasaki *et al*., 2003) and superior mesenteric arteries (Adaramoye *et al*., 2008; Adaramoye *et al*., 2009).

The genus *Curcuma* comprises approximately 80 species (Larsen, 2005), and some species have multiple cultivars/varieties, for example, *C. longa* comprises approximately 70 varieties in India (Sasikumar *et al*., 2005). The most important component of turmeric responsible for its biological activities is curcumin, which has been reported to have anti-hypertensive (Nakmareong *et al*., 2011) neuroprotective and anti-ischemic (de Alcantara *et al*., 2016), anti-angiogenic (Dehghan *et al*., 2016), hepatoprotective (Salahshoor *et al*., 2015) and vasorelaxation effects in isolated rabbit basilar (Ahn *et al*., 2009) and porcine coronary arteries (Xu *et al*., 2007). Curcumin also reverses the pathogenesis of cerebral ischemia and stroke (de Alcantara *et al*., 2016). Tetrahydrocurcumin and hexahydrocurcumin, biotransformed products of curcumin, have hypotensive effects and induce vasorelaxation of isolated rat thoracic aorta, respectively (Nakmareong *et al*., 2011; Moohammadaree *et al*., 2015). We found that there are significant differences in the curcumin, demethoxycurcumin and bisdemethoxycurcumin content of different species and strains of turmeric (Akter *et al*., 2018a). Therefore, we hypothesized that different species and strains of turmeric might vary in their vasomotional effects in porcine basilar arteries. This study was designed to compare the vascular reactivity and underlying mechanisms of different species and strains of turmeric in isolated porcine basilar arteries.

3. MATERIALS AND METHODS

3.1. Reagents

Bradykinin (BK) acetate salt, N′-tetraacetic acid (EGTA), nifedipine, Nω-nitro-L-arginine (L-NA), sodium nitroprusside (SNP), tetraethylammonium (TEA; Sigma-Aldrich, St, Louis, MO, USA) and U46619 (Cayman Chemical Co., Ann Arbor, MI, USA) were used. All Krebs salts and other chemicals were general-purpose or analytical grade and

purchased from Nakalai Tesque (Kyoto, Japan) or Wako (Osaka, Japan). Stock solutions were dissolved in distilled water, except nifedipine, which was dissolved in ethanol. The solutions were prepared fresh on the day of the experiment.

3.2. Plant material and extraction

Seven different species and strains of turmeric, including *C. longa* (strain: Ryudai gold, Okinawa ukon and BK2), *C. xanthorrhiza*, *C. aromatica*, *C. amada* and *C. zedoaria*, were studied in this experiment. Fresh, healthy rhizomes were harvested from the field of the University of the Ryukyus. Botanical identification and authentication of the turmeric samples were confirmed by qualified taxonomists of the Subtropical Field Science Center, Faculty of Agriculture, University of the Ryukyus, Japan. The rhizomes were washed, sliced and oven-dried at 60℃ for 72 h. Then, turmeric powder was prepared and passed through a sieve with a nominal mesh size of 2 mm. The extractions were carried out by dissolving the different turmeric powders in methanol at room temperature (27℃) and atmospheric pressure for 2 days with shaking. The solvent-soluble compounds were filtered using double filter paper (Whatman™). Fresh solvent was added into the used plant material, and the process was repeated three times. The filtered solutions containing plant compounds were dried by rotary evaporator. All extracts were kept in a refrigerator at 4℃ for experimental analyses. Prior to the experiments, the methanol extracts were dissolved in DMSO (1%) and further diluted in distilled water to give the desired concentration. The final concentration of DMSO in the organ-bath was less than 0.1%.

3.3. Tissue preparation

Basilar arteries were obtained from freshly slaughtered pigs (both sexes, approximately 6–7 months old, LWD cross-breed) at a local slaughter house and transferred to our

laboratory in ice-cold physiological saline solution (119 mM NaCl, 4.7 mM KCl, 1.6 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4 and 10 mM glucose, pH 7.4) aerated with carbogen (95% (v/v) O_2, 5% (v/v) CO_2). After the adherent tissues had been carefully removed, several rings approximately 4 mm long were cut from each artery. When required, the endothelium was removed by gently rubbing the intimal space with a stainless-steel rod with a diameter equivalent to the lumen of the artery. Arterial rings were mounted vertically between two L-shaped stainless steel holders, with the upper part fixed to an isometric force transducer (TB-611T, Nihon Kohden Kogyo, Tokyo, Japan), and immersed in a 5-ml water-jacketed organ bath containing oxygenated salt solution at 37℃ (pH 7.4). Each suspended ring was left to equilibrate under a resting tension of 0.75 g, which allowed us to induce maximum contractions in the artery. KCl (60 mM) was applied to stimulate the artery. After the contraction reached the maximum, the artery was washed, re-equilibrated and again stimulated with 60 mM KCl. This process was continued until the contraction amplitude reached a constant value. The isometric tension was recorded with an amplifier (AP-621 g, Nihon Kohden Kogyo, Tokyo, Japan), digitized with an analog-digital converter (PowerLab/8SP, ADInstruments Co., Castle Hill, NSW, Australia) and stored on the hard disc of a personal computer. The presence of endothelial cells was confirmed pharmacologically by testing the relaxant response to BK under pre-contracted conditions with U46619 (this response was abolished by endothelial denudation) (Miyamoto *et al*., 1999).

3.4. Turmeric-induced relaxation of basilar arteries pre-contracted with U46619

A thromboxane analog (U46619; 1-5×10^{-9} M) was used to induce stable pre-contraction of arterial rings with the endothelium intact or denuded, and turmeric extract was added cumulatively (20-800 μg/mL) to obtain the concentration response curve (CRC). At the

end of the experiments, 10^{-4} M SNP (sodium nitroprusside) was added, and the resulting relaxation was taken as 100%. Turmeric-induced relaxation was calculated as a percentage relative to the response elicited by 10^{-4} M SNP. The test for the Ca^{2+} influx-inhibitive effect of turmeric was modified from Khan and Gilani (2009) (Khan and Gilani, 2009). Endothelium-denuded arterial segments were allowed to stabilize in normal physiological saline (PSS), which was then replaced with Ca^{2+}-free PSS containing EGTA (2 mM) for 30 min to remove extracellular Ca^{2+} from the tissues. This solution was finally replaced with Ca^{2+}-free and K^{+}-rich (60 mM) PSS. After an incubation period of 30 min, the CRC was obtained by addition of extracellular $CaCl_2$ (extracellular Ca^{2+} CRC) to the bath fluid. After the extracellular Ca^{2+} CRCs were found to be superimposable (after 2 cycles), the arterial segment was pre-treated with different concentrations of turmeric or nifedipine for 30 min, and the next extracellular Ca^{2+} CRC was constructed in the presence of these agents. The concentration-dependent inhibitory effect of turmeric was tested. The change in extracellular Ca^{2+} CRC induced by turmeric or nifedipine was used to estimate the Ca^{2+} influx-inhibitory effect.

3.5. Statistical analysis

The contraction response was expressed as a percentage of the response obtained with SNP (10^{-4} M). The relaxation response was expressed as a percentage of the response obtained with 10^{-4} M SNP. The results are expressed as the means ± standard error mean (SEM). Statistical analyses were performed by paired t test or the Bonferroni test after one-way analysis of variance (one-way ANOVA). Significance was established when the probability level was equal to or less than 5%.

4. RESULTS

4.1. Effect of different species and strains of turmeric on isolated porcine basilar arteries pre-contracted with U46619

Methanol extracts of different species and strains of turmeric (20-800 μg/mL) induced relaxation of the porcine basilar artery rings pre-contracted with U46619 in a concentration-dependent manner. There were no significant differences observed among the different species and strains of turmeric-induced relaxation of the isolated porcine basilar artery (Figure 5-1).

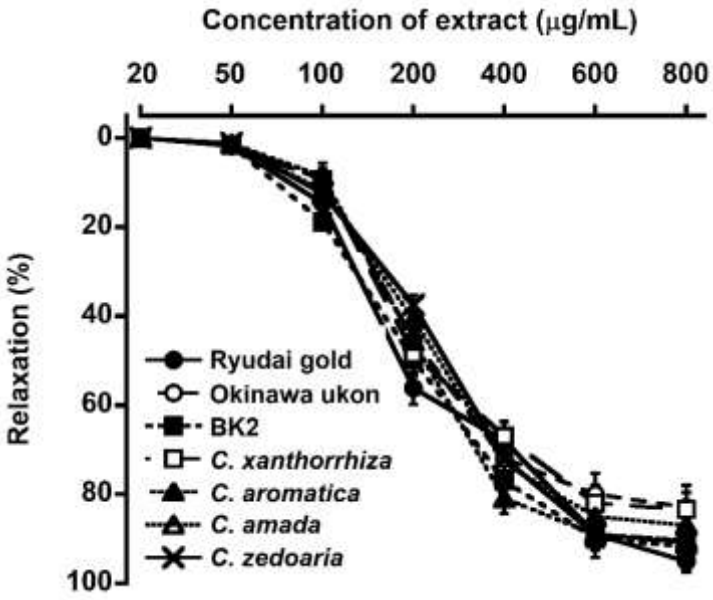


Figure 5-1. Effect of methanol extracts from different species and strains of turmeric on isolated porcine basilar arteries. The results are expressed as the mean ± SEM (n = 7-9 pigs).

4.2. Effect of endothelium denudation, L-NA and indomethacin on turmeric-induced relaxation in porcine basilar arteries pre-contracted with U46619

Because a similar pattern of vascular reactivity for the different species and strains of turmeric was observed, we took Ryudai gold (containing curcumin, demethoxycurcumin and bisdemethoxycurcumin) and *C. amada* (does not contain curcumin, demethoxycurcumin or bisdemethoxycurcumin) for the graphical presentation of the subsequent experiments. Endothelium denudation, pretreatment of endothelium-intact arteries with L-NA (a nitric oxide synthase inhibitor) and indomethacin (a non-selective cyclooxygenase inhibitor) for 30 min had no significant effect on the turmeric-induced relaxation of the pre-contracted porcine basilar artery (Figure 5-2).

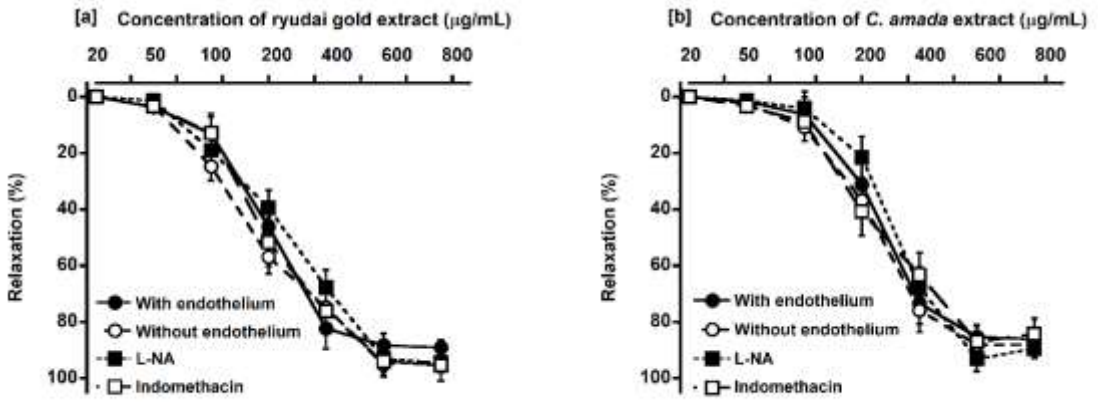


Figure 5-2. Effects of endothelial denudation and pretreatment of endothelium-intact arterial rings for 30 min with L-NA (a nitric oxide synthase inhibitor) and indomethacin (a non-specific cyclooxygenase inhibitor) on vascular relaxation induced by Ryudai gold [a] and *C. amada* [b] in isolated porcine basilar arteries. The results are expressed as the mean ± SEM (n = 5-7 pigs).

4.3. Role of K^+-channel on turmeric-induced relaxation of the pre-contracted arterial rings

To test the involvement of K^+-channels in turmeric-induced relaxation of endothelium-denuded arterial rings, the arteries were pre-treated with K^+-channel antagonists, such as tetraethylammonium (TEA; a non-selective K^+-channel inhibitor, 10 mmol/L)[26], glibenclamide (a non-specific ATP-sensitive K^+-channel antagonist, 10 μmol/L)[27] and 4-aminopyridine (4-AP; a voltage-dependent K^+-channel antagonist, 1 mmol/L)[28] for 30 min before pre-contraction with U46619 ($1\text{-}5\times10^{-9}$ M). As shown in figure 5-3, pretreatment with K^+-channel antagonists had no significant effect on turmeric-induced relaxation.

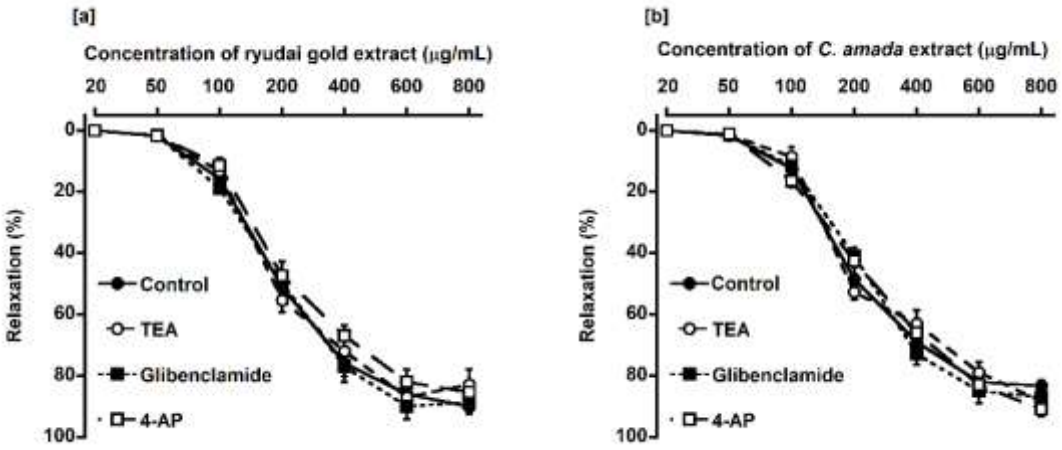


Figure 5-3. Effect of K^+-channel antagonists on vascular relaxation induced by Ryudai gold [a] and *C. amada* [b] in endothelium-denuded porcine basilar arterial rings pre-contracted with U46619. The arterial rings were pre-incubated with tetraethylammonium (TEA; 10 mM), glibenclamide (10 μM) and 4-aminopyridine (4-AP; 1 mM) for 30 min. The results are expressed as the mean ± SEM (n = 6-8 pigs).

4.4. Effect of propranolol (a non-selective β-adrenergic receptor antagonist) on turmeric-induced relaxation in pre-contracted rings

To test the involvement of the β-adrenergic receptor in turmeric-induced relaxation in endothelium-denuded porcine basilar arteries, we pre-treated arterial rings with propranolol (1 μM), a non-selective β-adrenergic receptor antagonist, for 30 min. Propranolol significantly decreased the maximal relaxation from 93.1 ± 2.7% and 91.0 ± 3.4 to 81.3 ± 3.9 and 80.3 ±3.6 for Ryudai gold and *C. amada*, respectively. Propranolol significantly inhibited the relaxation when the turmeric concentration was 600 μg/mL and 800 μg/mL (Figure 5-4).

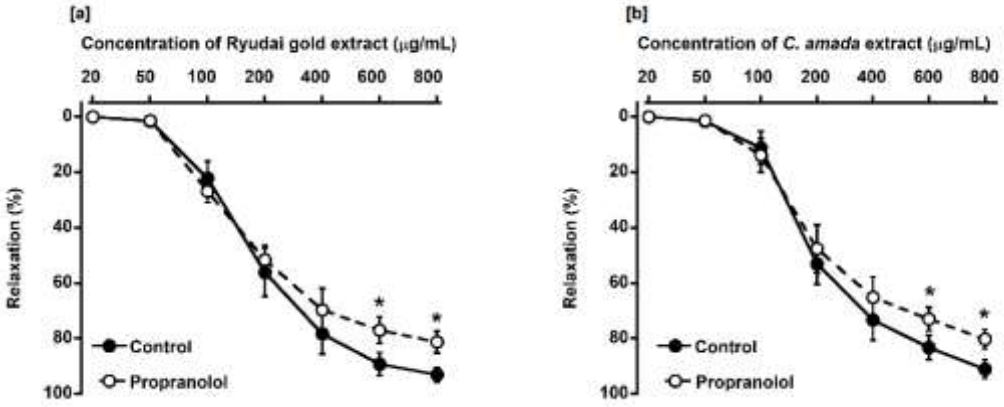


Figure 5-4. Effect of propranolol (a nonselective β-adrenergic receptor antagonist, 1 μM) on vasorelaxation induced by Ryudai gold [a] and *C. amada* [b] in endothelium-denuded porcine basilar artery rings. The arteries were pre-contracted with U46619 (10^{-9} M). The results are expressed as the mean ± SEM (n = 6 pigs). $^{*}p<0.05$ vs. control.

4.5. Effect of turmeric extracts on $CaCl_2$-induced contraction in isolated porcine basilar arteries

Pre-treatment with turmeric extract at 100, 200 and 400 μg/mL and nifedipine (10^{-4} M) for 30 min significantly attenuated the $CaCl_2$-induced contraction of the endothelium-denuded arterial rings exposed to Ca^{2+}-free medium containing high K^+ in a concentration-dependent manner (Figure 5-5). Pre-treatment with turmeric extracts and nifedipine shifted the concentration response curve for $CaCl_2$ to the right.

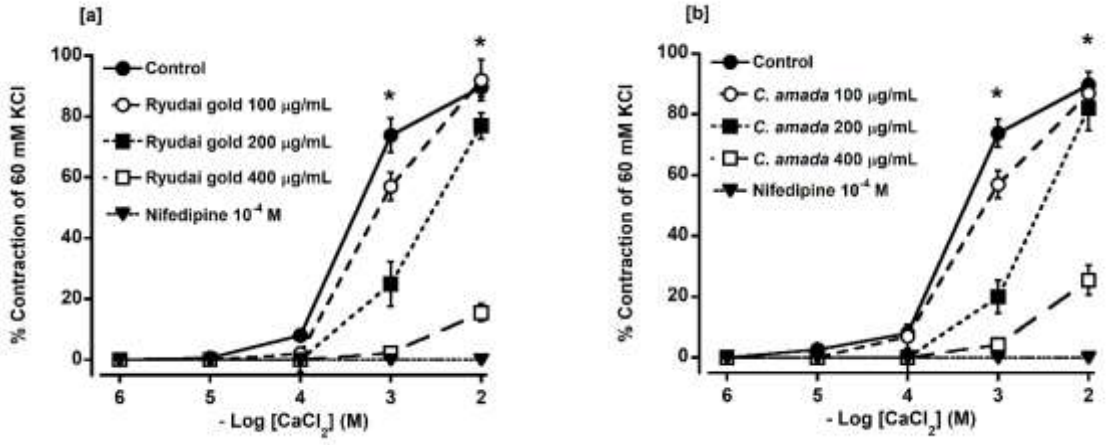


Figure 5-5. Effect of Ryudai gold [a], *C. amada* [b] extracts and nifedipine on $CaCl_2$-induced contraction of endothelium- denuded porcine basilar arteries. Concentration–response curves were determined in Ca^{2+}-free solution after the depletion of extracellular Ca^{2+}, where the $CaCl_2$-contractile effect was dependent on Ca^{2+}-influx through voltage-operated Ca^{2+}-channels activated by KCl (60 mM). The curves were constructed in the absence of added substance (control) or after 30 min incubation in the presence of Ryudai gold, *C. amada* (100, 200 and 400 μg/mL) and nifedipine (10^{-4} M) or turmeric extract prior to the cumulative addition of $CaCl_2$. The results are expressed as the mean ± SEM (n = 7-9 pigs). $^{*}p<0.05$ vs. control.

4.6. Effect of the main active compounds of turmeric (curcumin, demethoxycurcumin and bisdemethoxycurcumin) on isolated porcine basilar arteries pre-contracted with U46619

We studied the effect of curcumin, demethoxycurcumin and bisdemethoxycurcumin on isolated porcine basilar arteries pre-contracted with U46619. All three compounds induced relaxation of the isolated porcine basilar artery. There were no significant differences at lower concentrations (10^{-9}–10^{-6} M) of curcuminoids. However, at higher concentrations (10^{-5} and 10^{-4} M), demthoxycurcumin induced significantly higher relaxation, followed by curcumin and bisdemethoxycurcumin (Figure 5-6).

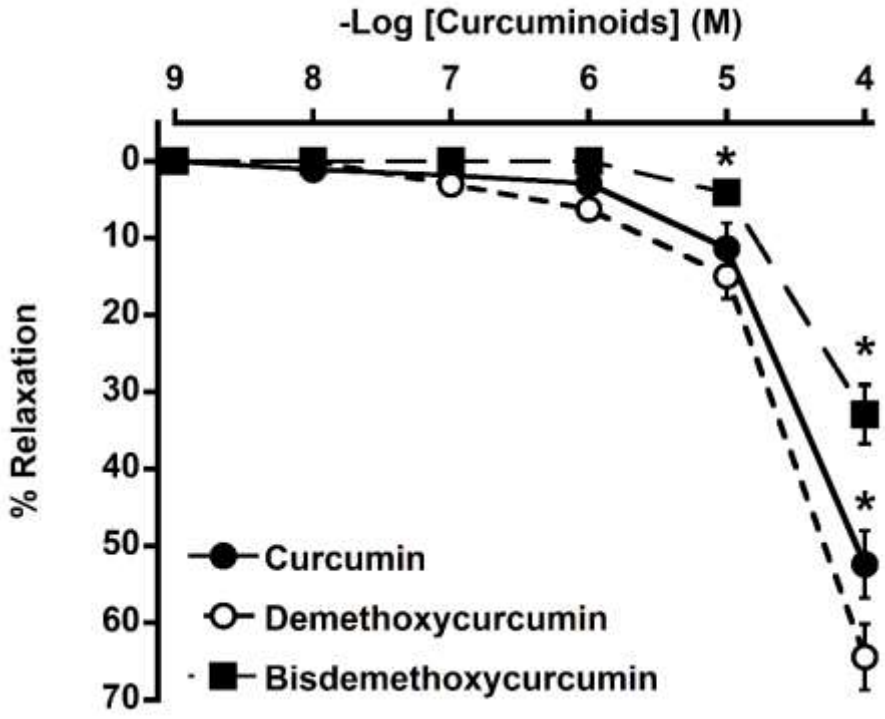


Figure 5-6. Effect of curcumin, demethoxycurcumin and bisdemethoxycurcumin on porcine basilar artery rings. The arteries were pre-contracted with U46619 (10^{-9} M). The results are expressed as the mean ± SEM (n = 6 pigs). $^{*}p<0.05$ vs. demethoxycurcumin.

5. DISCUSSION

Our results showed that, despite the variation in curcuminoid content, all turmeric induced strong relaxation of isolated porcine basilar arteries. There were no significant differences in relaxation effects among the different species and strains of turmeric. It is well known that, in addition to nitric oxide, the relaxation of vascular smooth muscles is induced by prostaglandin I_2, Ca^{2+} antagonists, and β-adrenoceptor agonists. Therefore, these pathways were evaluated to reveal the mechanism of vasorelaxation induced by turmeric. In this study, pretreatment of endothelium-intact arterial rings with L-NA (a nitric oxide synthase inhibitor) did not affect turmeric-induced relaxation. Mechanical removal of the endothelium also had no significant effect on turmeric-induced relaxation. These results suggested that turmeric induces NO and endothelium-independent relaxation and directly acts through smooth muscle cells of the porcine basilar artery. These results are in accordance with the findings of Sasaki (Sasaki *et al*., 2003), who reported the effectiveness of curcuma drugs as vasorelaxant agents in the vascular smooth muscle of rat aortas. It has also been reported that methanol extracts of *C. longa* induce endothelium-independent relaxation in isolated rat aorta (Sasaki *et al*., 2003) and superior mesenteric arteries (Adaramoye *et al*., 2008; Adaramoye *et al*., 2009). Similarly, the major active compounds of turmeric such as curcumin, demethoxycurcmin and bisdemethoxycurmin induce endothelium-independent relaxation of isolated rat pulmonary arteries (Kruangtip *et al*., 2015). However, one study reported that curcumin induces partial endothelium and NO-dependent relaxation of porcine coronary arteries (Xu *et al*., 2007). This difference might be due to the difference in the vascular bed studied. Indomethacin (a non-selective cyclooxygenase inhibitor) did not affect turmeric-induced relaxation in endothelium-intact porcine basilar arteries, which indicates that the release of vasodilator prostanoids is not involved in turmeric-induced relaxation of the porcine basilar artery.

Similarly, vasodilator prostanoids are not involved in the curcumin-induced relaxation of porcine coronary arteries (Xu *et al*., 2007).

It is well known that K^+-channels play a substantial role in the regulation of vascular contractility and tone (Jackson, 2005). Direct activation of K^+-channels in arterial smooth muscle cells leads to membrane hyperpolarization, inhibits Ca^{2+} influx through voltage operated Ca^{2+}-channel and suppresses smooth muscle contraction and subsequent vasodilation (Jackson, 2005). Therefore, we evaluated the influence of K^+-channel inhibitors on the vasorelaxant response induced by turmeric. However, TEA (a Ca^{2+}-activated K^+-channel antagonist), glibenclamide (a non-specific ATP-sensitive K^+-channel antagonist) and 4-AP (a voltage-dependent K^+-channel antagonist) had no significant effect on turmeric-induced relaxation. These results suggested that opening of the K^+-channels was not involved in the mechanism of the vasomotional action of turmeric. Similar results were observed in *C. longa*-induced vasorelaxation in rat thoracic aorta (Adaramoye *et al*., 2008).

KCl induces smooth muscle contraction via the activation of voltage-dependent Ca^{2+}-channels and subsequent release of Ca^{2+} from the sarcoplasmic reticulum, causing a significant increase in the intracellular Ca^{2+} concentration (Ratz *et al*., 2005). Turmeric antagonism of the Ca^{2+}-channel was confirmed by our experiment in which extracellular Ca^{2+} removal (using 2 mM EGTA) suppressed KCl-induced contraction, and pretreatment with turmeric significantly inhibited Ca^{2+}-induced contraction of 60 mM KCl-depolarized arteries. In addition, the magnitude of the inhibitory effect of turmeric and nifedipine (an L-type Ca^{2+}-channel antagonist) was similar, which suggested the involvement of the L-type voltage-operated Ca^{2+}-channel. This observation is consistent with a study by Gilani *et al* (Gilani *et al*., 2005), who concluded that Ca^{2+}-channel antagonism is the main mechanism of vasorelaxation elicited by curcuma drugs and forms the basis of traditional uses in

hyperactive states of the gut, airway inflammation disorders, palpitation and hypertension. Similarly, curcumin analogs and synthesized curcumin mimics dilate rabbit basilar arteries via antagonism of the L-type Ca^{2+}-channel (Kruangtip *et al*., 2015; Park *et al*., 2015).

Stimulation of vascular smooth muscle by β-adrenoreceptors results in vasorelaxation of the porcine basilar artery (Miyamoto, *et al*., 1993). Our results showed that propranolol (a non-selective β-adrenergic receptor antagonist) inhibited the turmeric-induced relaxation when the concentrations were 600 μg/mL and 800 μg/mL, indicating that the high degree of turmeric-induced relaxation might be attributed to β-adrenoceptor activation. Our results consistent with the results of Xu (Xu *et al*., 2007) and Moohammadaree (Moohammadaree *et al*., 2015), who demonstrated the involvement of β-adrenoceptors in curcumin and hexahydrocurcuminin-induced relaxation of porcine coronary arteries and rat thoracic aorta, respectively. It has been reported that noradrenaline-induced relaxation of the porcine basilar artery occurs via the stimulation of β-adrenoceptors on vascular smooth muscle cells, which is inhibited by propranolol (Miyamoto *et al*., 1993). We performed HPLC to detect the presence of noradrenaline in the turmeric extracts. However, noradrenaline was not detected in any of the tested samples (data not shown).

Like the effect of the turmeric extract, pure curcuminoids (curcumin, demethoxycurcumin and bisdemethoxycurcumin) induced relaxation of the isolated porcine basilar artery. A previous study also reported that these three curcuminods induce endothelial-independent and Ca^{2+}-channel-dependent relaxation of rat pulmonary arteries (Kruangtip *et al*., 2015). Demethoxycurcumin induced significantly more relaxation, followed by curcumin and bisdemethoxycurcumin at higher concentrations. However, the relaxation induced by the turmeric extract showed no significant differences. This might be due to the difference in the concentration and ratio of the active compounds present in the turmeric extract. Moreover, we

reported previously that among the different species and strains of turmeric, *C. amada* and *C. zedoaria* do not contain curcumin, demethoxycurcumin and bisdemethoxycurcumin (Akter *et al*., 2018a) but still induce relaxation. Therefore, vascular relaxation induced by *C. amada* and *C. zedoaria* indicated that there are other vasoactive compounds besides curcuminoids present in turmeric, which is consistent with the results of Sasaki (Sasaki *et al*., 2003), who isolated curcumin and eight sesquiterpenes that induced NO-independent relaxation with almost the same intensities.

In conclusion, our results suggested that different species and strains of turmeric induce nitric oxide- and endothelium-independent relaxation of the porcine basilar artery. Possible mechanisms of vasorelaxation include partial activation of β-adrenoceptors and inhibition of Ca^{2+} from both extracellular and intracellular sources. Turmeric, its active compounds and/or synthetic curcuminoids may be used as novel vasodilation agents for the treatment of cardiovascular diseases such as atherosclerosis, essential hypertension, pulmonary artery hypertension, coronary artery disease, stroke, and diabetic complications of vasculature. However, additional work is required to investigate the vasoactive compounds and molecular mechanisms of the vasorelaxation induced by turmeric.

Chapter 4

Plant growth inhibitors in turmeric (*Curcuma longa*) and their effects on *Bidens pilosa*

1. ABSTRACT

Turmeric (*Curcuma* spp.) has numerous biological activities including anticancer, antibacterial, antifungal and insecticidal properties. Here, we evaluated the plant growth inhibitory activities of two cultivars of *Curcuma longa* (*C. longa*; Ryudai gold and Okinawa ukon) against radish, cress, lettuce and *Bidens pilosa* (*B. pilosa*). The methanol extracts of both turmeric varieties inhibited the seed germination and seedling growth of all the tested plants. Ryudai gold had a significantly higher inhibitory effect on the seed germination and root and shoot growth of the plants than Okinawa ukon. Therefore, Ryudai gold was chosen for the isolation of plant growth inhibitory compounds using a silica gel column and high-performance liquid chromatography. The structural identification of the compounds was conducted using ^{1}H NMR, ^{13}C NMR, and liquid chromatography-tandem mass spectrometry. The growth inhibitors were identified as four curcuminoids; dihydrobisdemethoxycurcumin (**1**), bisdemethoxycurcumin (**2**), demethoxycurcumin (**3**) and curcumin (**4**). The IC_{50} of the curcuminoids against the root and shoot growth of *B. pilosa* ranged from 8.7±1.7 to 12.9±1.8 and 15.5±1.8 to 38.9±2.8 μmol L^{-1}, respectively. Compound **1** showed the lowest IC_{50} against the root and shoot growth of *B. pilosa*. These results suggested that Ryudai gold has a potential growth inhibitory effect against *B. pilosa*.

Key words: *Curcuma longa*, plant growth inhibitors, *Bidens pilosa*

2. INTRODUCTION

Weeds, which are a large group of undesirable plants, are the key problem in agriculture and reduce 12% of the world's crop production (Anaya 1999; Kordali *et al*., 2016). Weeds show great adaptability in different habitats and compete for nutrients, space, water and light, which cause massive economic loss (Wu *et al*., 2017). *Bidens pilosa* (*B. pilosa*) is a cosmopolitan weed that is distributed widely all over the world, particularly in the tropical and subtropical regions (Ballard, 1986). Although it originated from tropical America, it is found in the Pacific region and parts of Asia, including Okinawa, Japan (Ishimine *et al*., 1986; Nakama *et al*., 2011). Arthur *et al*., (2012) reported that *B. pilosa* is a weed of 31 crops in more than 40 countries. It is a major weed in crops, pastures, gardens, wastelands and cultivated areas and on roadsides. It has been reported that *B. pilosa* is resistant to imazaquin in Brazil (Heap, 1997) and is a host and vector of harmful parasites such as root knot nematodes (*Meloidogyne* species).

Traditionally, weeds are controlled by using synthetic herbicides. However, the widespread use of herbicides not only creates negative impacts on agricultural products, human health and the environment but also develops weed resistance (Anaya 1999; Kordali *et al*., 2009). Moreover, synthetic herbicides may affect nontarget plants and crops grown in rotation as their residues may persist in the soil (Burke and Bell 2014). Therefore, there is an urgent need to develop new, efficient plant growth inhibitors that are safe to human health and the environment.

Turmeric (*Curcuma* spp.) is a rhizomatous perennial herb belonging to the family Zingiberaceae. It has a broad spectrum of pharmacological functions such as antioxidant, anticoagulant, anticarcinogenic, antimutagenic, antifertility, antidiabetic, antibacterial and antifungal activities (Chattopadhyay *et al*., 2004). Among the different species and varieties

of turmeric, *Curcuma longa* (*C. longa*) has been reported to have repellent (Jilani and Saxena 1990; Su *et al*., 1982), insecticidal (Chander *et al*., 1992) and antifeeding activities (Jilani and Saxena 1990) against some stored-product insects such as *Dysdercus koenigiin* (Walk) and *Schistocerca gregaria* (Forsk) (Chowdhury *et al*., 2000). It has been reported that the essential oil *C. zedoaria* has allelopathic effects on seed germination and seedling growth of lettuce achenes and tomato (De Melo *et al.*, 2017). However, there is no report regarding the growth inhibitory effect of turmeric on *B. pilosa*, an important weed that causes the loss of agricultural crops. Moreover, no phytotoxic compounds have been isolated from turmeric. Therefore, we evaluated the growth inhibitory potential of two strains of *C. longa* (Ryudai gold and Okinawa ukon) against several plant species, including *B. pilosa*, and isolated the effective compounds.

3. MATERIALS AND METHODS

3.1 Plant materials collection

There are many strains/varieties of *C. longa*; the turmeric variety 'Ryudai gold' provides a higher yield and quality, and the turmeric variety 'Okinawa ukon' is an indigenous variety of Okinawa, Japan. Therefore, Ryudai gold and Okinawa ukon were evaluated in this experiment. These two turmeric varieties were cultivated under the same conditions at the Subtropical Field Science Center, University of the Ryukyus, Okinawa, Japan. Three kilograms of fresh rhizomes were harvested, washed, sliced and dried in a hot air oven at 60℃ for 72 h. An experiment conducted at the Faculty of Agriculture, University of the Ryukyus, showed that the turmeric rhizome slices dried at 50, 60 and 70℃ had almost similar values of curcuminoids (Hossain, data not published). Therefore, the turmeric rhizome slices were dried at 60℃. A total of 300 g and 450 g dried powder was obtained from those 3 kg of fresh rhizomes of Ryudai gold and Okinawa ukon, respectively.

Radish (*Raphanus sativus* L.), garden cress (*Lepidum sativum* L.) and lettuce (*Lactuca sativa* L.) usually used as model plants for bioassays were purchased from Green Field Project (Kumamoto, Japan). The seeds of *B. pilosa* were collected from the field of the University of the Ryukyus, Okinawa, Japan. The seeds were screened visually on the basis of size and color for the germination test. The screened seeds were dried at room temperature (25-28℃) for three days and stored at room temperature for 30 days.

3.2 Chemicals

Methanol (MeOH), ethyl acetate (EtOAc), ethanol (EtOH; 99.5%) and *n*-hexane were purchased from Nacalai Tesque (Kyoto, Japan). Silica gel (63-200 μm, Kanto Chemical Co. Tokyo, Japan) and MeOH-d_4 (CD_3OD, Merck KGaA, Germany) were purchased.

3.3 Extraction of samples

The extraction was carried out by dissolving 300 g turmeric powder into 3 L of MeOH at room temperature (27℃) and at atmospheric pressure and kept for 2 days with shaking. After filtration (No. 2; Advantec, Toyo Roshi Kaisha Ltd., Tokyo, Japan), the residue was extracted with 2 L of MeOH. This process was repeated, and the filtrates were combined. The solvents were evaporated under reduced pressure to obtain 46 g and 30 g of extracts from Ryudai gold and Okinawa ukon, respectively. The extracts were kept in the refrigerator at 4℃ for experimental analyses.

3.4 Turmeric extract bioassay

A sheet of sterilized filter paper (No. 2; Advantec, Toyo Roshi Kaisha Ltd., Tokyo, Japan) was placed in a sterilized Petri dish (i.d. 3 cm) and treated with 0.2 mL of MeOH containing no (control) or 0.5, 1, 2 mg mL^{-1} turmeric extract. After evaporation of the solvent, the filter paper was wetted with 0.4 mL of distilled water.

For the germination bioassay, 10 seeds of radish, cress, lettuce and *B. pilosa* were sown separately on the filter paper in the Petri dishes with three replications and then incubated in a dark chamber at 25℃ for 48 h (cress, radish and lettuce) and 4 days (*B. pilosa*). The bioassay was repeated three times. The percentage of inhibition of germination compared to control (without extracts) was determined using following formula:

% of inhibition of germination= ((C- S)/C × 100))

where C = germination in the control and S = germination in the sample.

For the growth bioassay, 10 uniform germinated seedlings of lettuce, cress, radish and *B. pilosa* were sown separately on the treated filter paper in the Petri dishes with three replications. The lengths of their shoots and roots were measured after 48 h of incubation

at 25℃ in the dark. The percentage length of the seedlings was then calculated and compared to the length of the control (without extracts) seedlings. The bioassay was repeated three times using a completely randomized design. The percentage inhibition of root and shoot length was calculated by using the following formula:

% of inhibition of growth= ((C- S)/ C × 100))

where S = Root or shoot length in Sample and C = Root or shoot length in Control.

3.5 Isolation of bioactive compounds from crude extracts of turmeric variety Ryudai gold

The crude extract (46 g) of Ryudai gold was diluted with 200 mL distilled water and extracted with an equal volume of *n*-hexane and then with EtOAc. All fractions were concentrated to dryness on a rotary evaporator at 40℃ to obtain 24, 9 and 7 g of EtOAc, *n*- hexane and water fractions, respectively. The inhibitory activity of these three fractions was determined using the *B. pilosa* growth bioassay according to the aforementioned procedure.

As activity was found in the EtOAc fraction, this fraction was selected for the isolation and purification of bioactive compounds. The most active EtOAc fraction was evaporated to dryness and subjected to silica gel (75 g, 63-200 µm) column (30 x 3 cm) chromatography. Elution was carried out using *n*-hexane and EtOAc with increasing portions of EtOAc (100:0 (F1), 80:20 (F2), 60:40 (F3), 40:60 (F4), 20:80 (F5) and 0:100 (F6) (v/v)). These fractions were examined for their phytotoxicity in *B. pilosa* as described before. The most active fraction, F4, was further purified by C_{18} reversed-phase HPLC (COSMOSIL 5C_{18}-AR-II; Nacalai Tesque, Inc., Kyoto, Japan) eluted with 50% acetonitrile in water (v/v) (50%) at a flow rate of 2.5 mL min^{-1} and monitored at 280 nm.

A total of four peaks were eluted at 8.96, 9.54, 10.9 and 12.04 min as yellow substances,

and their inhibitory activity was detected. The isolated compounds (approximately 10 mg each) were dissolved in MeOH-d_4 and then subjected to spectroscopic analyses. Nuclear magnetic resonance (NMR) spectra were recorded on BRUKER NMR spectrometers (500 MHz for ^{1}H and 125 MHz for ^{13}C) at room temperature. Mass Spectrometry (MS) experiments were carried out on a Waters mass spectrometer (Quattro Micro, USA) with an electrospray ionization probe.

3.6 Plant growth inhibitory activity of the isolated compounds

The isolated compounds were dissolved in MeOH for making the concentrations of 1, 3, 5, 10, 30, 50, 100, 200 and 300 μmol L^{-1} for each compound. Growth inhibitory activity was determined using the *B. pilosa* growth bioassay according to the above procedure.

3.7 HPLC for the determination of the four curcuminoids in turmeric

For curcuminoids analysis, 0.1 g powder of turmeric was dissolved into 40 mL EtOH (99.5%) at room temperature (27℃) and atmospheric pressure using vortex mixing followed by mixing in an ultrasonic water bath at 50℃ for 10 min. The solvent soluble compounds were filtered using filter paper (Advantec no. 5A). Fresh solvents were added to the residues on filter paper, and the process was repeated three times. Then, EtOH was added to make a 250 mL solution. Curcumin (**4**), demethoxycurcumin (**3**), bisdemethoxycurcumin (**2**) and dihydrobisdemethoxycurcumin (**1**) were determined by HPLC (Shimadzu Co. Ltd.). The column (Imtakt Cadenza CD-C18 100 x 3.0 mm, 3 μm) was run at 40℃, and the acetonitrile and 1% phosphoric acid solvents were used at the ratio of 43:57. Each 5 μL solution was supplied at 0.5 mL min^{-1} and analyzed for 11 min. All tests were performed in triplicate.

3.8 Statistical analysis

The results are expressed as the mean ± standard error. Statistical differences between two means were evaluated by Student's t-test. Multiple comparisons were performed using one-way analysis of variance followed by Bonferroni test. Differences were considered significant at $p < 0.05$.

4. RESULTS

4.1 Germination bioassay

We compared the effects of the MeOH extracts of Ryudai gold and Okinawa ukon (0.5, 1 and 2 mg mL^{-1}, respectively) on the germination of radish, cress, lettuce and *B. pilosa* (Figure 6-1). Our results demonstrated that Ryudai gold and Okinawa ukon inhibited the germination of radish, cress, lettuce and *B. pilosa* in a concentration-dependent manner. The inhibitory effects of Ryudai gold on germination were significantly stronger than those of Okinawa ukon for all tested plants at all concentrations examined (Figure 6-1).

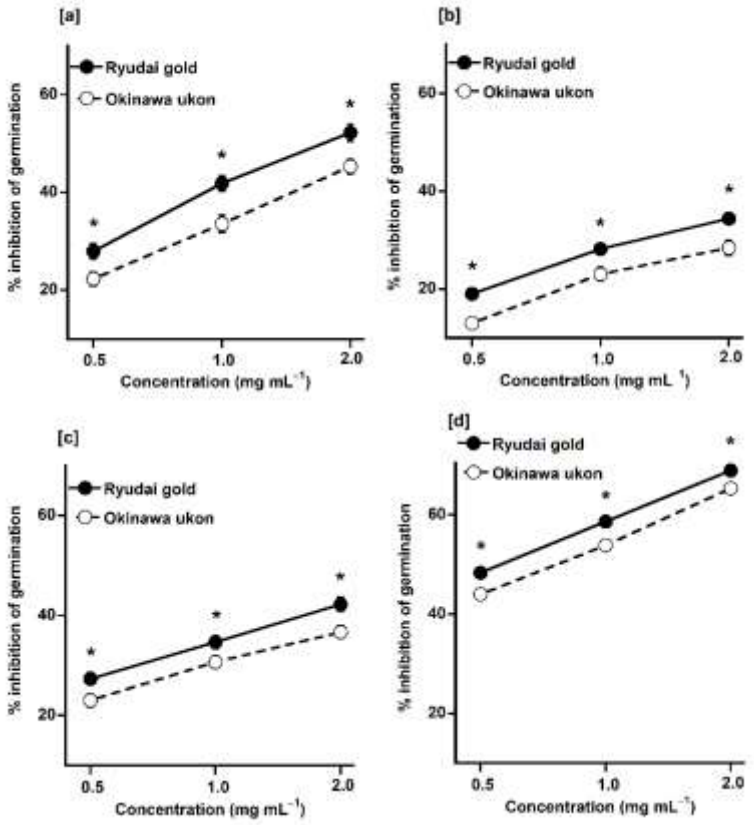


Figure 6-1. Effect of the MeOH extract of turmeric Ryudai gold and Okinawa ukon on the germination of radish [a], cress [b], lettuce [c], and *Bidens pilosa* [d]. Results are expressed as the mean ± standard error of three independent experiments in triplicate. $^{*}p<0.05$ vs. Okinawa ukon.

4.2 Shoot and root growth

The effects of the MeOH extracts of Ryudai gold and Okinawa ukon on the root and shoot growth of radish, cress, lettuce and *B. pilosa* at all three concentrations of 0.5, 1 and 2 mg mL^{-1} are shown in figures 6-2 and 6-3. The results demonstrated that Ryudai gold and Okinawa ukon inhibited the root (Figure 6-2) and shoot (Figure 6-3) growth of radish, cress, lettuce and *B. pilosa* in a concentration-dependent manner. The inhibitory effects of Ryudai gold on root and shoot growth were significantly stronger than those of Okinawa ukon for all the tested plants at all the concentrations examined (Figures 6-2 and 6-3).

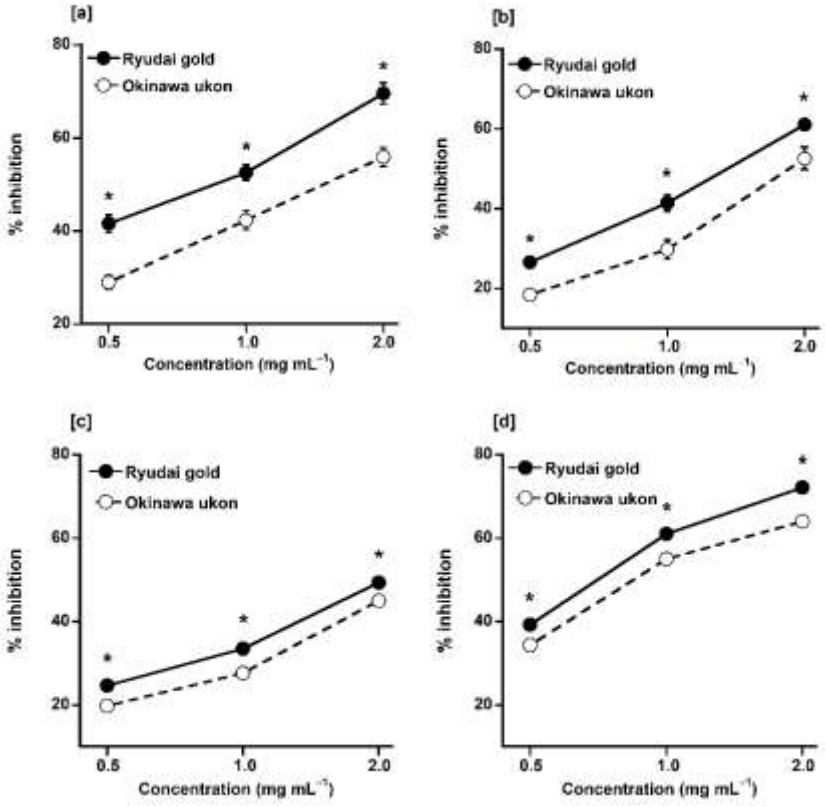


Figure 6-2. Effect of the MeOH extract of turmeric Ryudai gold and Okinawa ukon on the root growth of radish [a], cress [b], lettuce [c], and *Bidens pilosa* [d]. Results are expressed as the mean ± standard error of three independent experiments in triplicate. $^{*}p<0.05$ vs. Okinawa ukon.

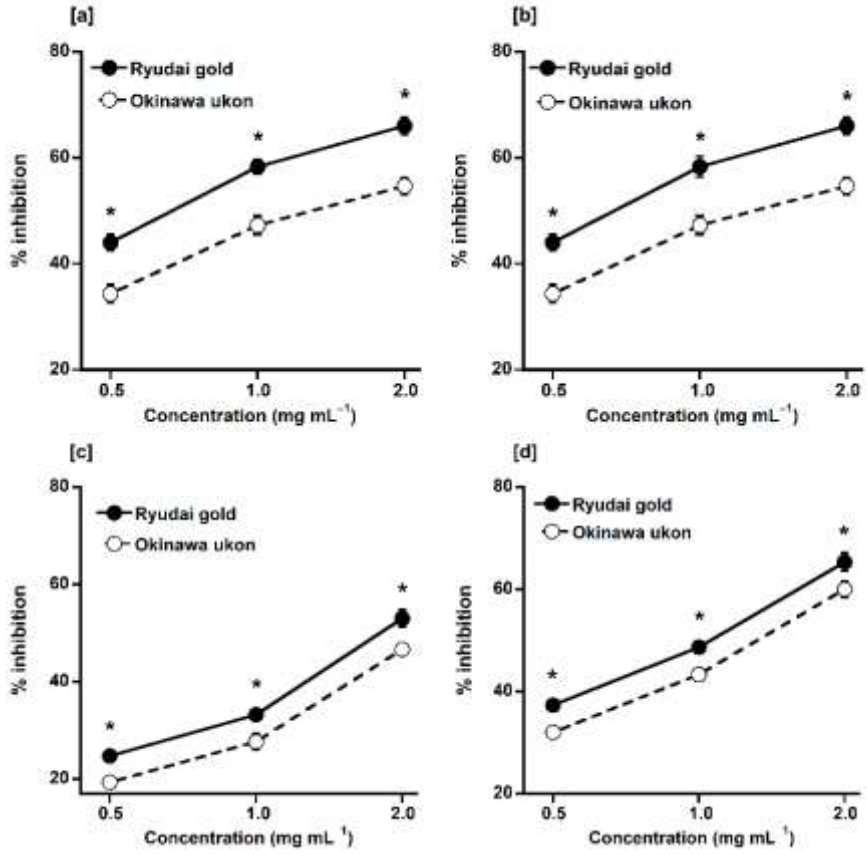


Figure 6-3. Effect of the MeOH extract of turmeric Ryudai gold and Okinawa ukon on the shoot growth of radish [a], cress [b], lettuce [c], and *Bidens pilosa* [d]. Results are expressed as the mean ± standard error of three independent experiments in triplicate. $^{*}p<0.05$ vs. Okinawa ukon.

4.3 Effects of aqueous, n- hexane and EtOAc fraction

MeOH extracts of Ryudai gold showed significantly stronger growth inhibitory effect than those of Okinawa ukon in all the tested plants. Therefore, Ryudai gold extract was subjected to solvent partitioning with water, *n*-hexane and EtOAc. Among them, EtOAc extracts showed strongest inhibitory effect (Figure 6-4). Therefore, the EtOAc extracts were taken for further fractionation.

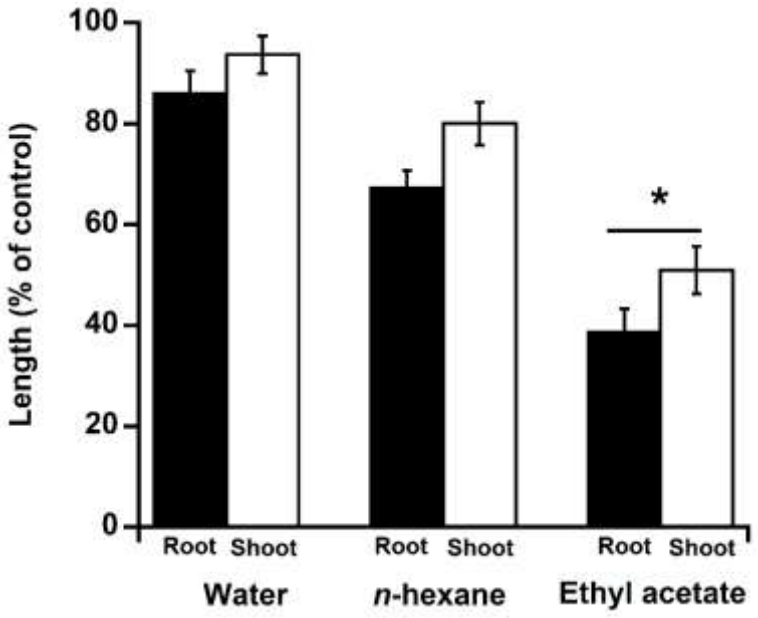


Figure 6-4. Effect of the different solvent extract of Ryudai gold on the root and shoot growth of *Bidens pilosa*. Results are expressed as the mean ± standard error of three independent experiments in triplicate. $^{*}p<0.05$ vs. water and *n*-hexane.

4.4 Effects of different fractions of EtOAc extracts of Ryudai gold on the root and shoot growth of B. pilosa

Six fractions were obtained from the EtOAc extracts of Ryudai gold by silica gel chromatography using *n*-hexane-EtOAc [100:0 (F1), 80:20 (F2), 60:40 (F3), 40:60 (F4), 20:80 (F5) and 0:100 (F6)]. Among the six fractions, fraction 4 (F4) showed the highest inhibitory activity on the root and shoot growth of *B. pilosa* (Figure 6-5).

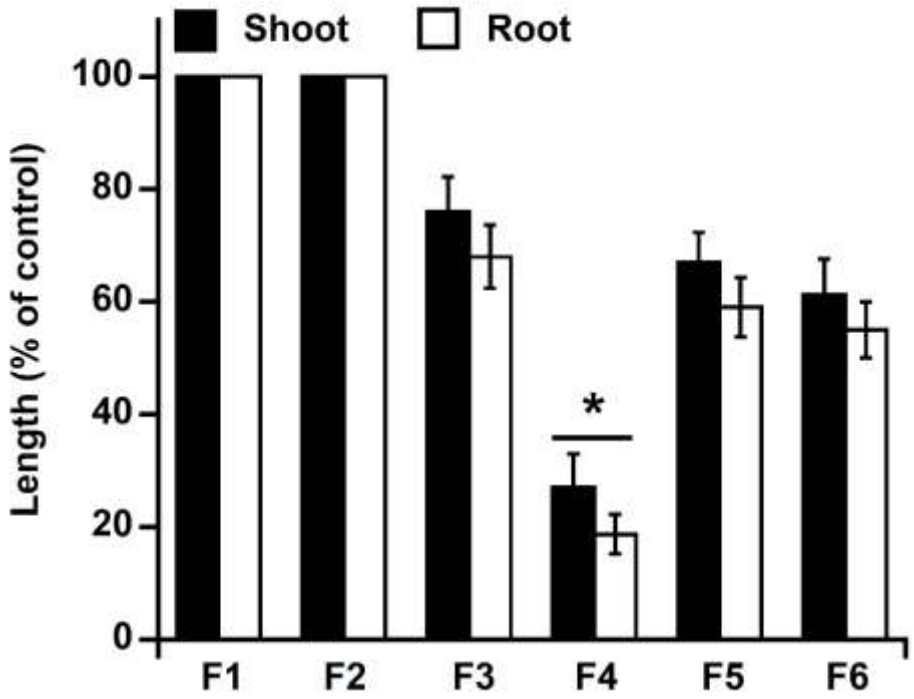


Figure 6-5. Effect of the six fractions [*n*-hexane: EtOAc (v/v) (100:0 (F1), 80:20 (F2), 60:40 (F3), 40:60 (F4), 20:80 (F5) and 0:100 (F6))] of EtOAc part of Ryudai gold on the root and shoot growth of *Bidens pilosa*. Results are expressed as the mean ± standard error of three independent experiments in triplicate. $^{*}p<0.05$ vs. other fractions.

4.5 Chemical structures of the isolated compounds

The chemical structures of the four compounds from fraction F4 were identified according to their ^{1}H NMR and ^{13}C NMR spectra. The peak data were as follows:

Compound 1: Yellow powder**,** UV λ_{max} nm: 243, 362. ESI-MS (+): *m/z* 311.2 $[M+H]^{+}$. ^{1}H-NMR (CD_3OD) δ: 7.50 (1H, d, *J*=16 Hz, H-4'), 7.44 (2H, d, *J*=8 Hz, H-6', 10'), 7.01 (1H, d, *J*=8 Hz, H-6, 10), 6.79 (2H, d, *J*=8 Hz, H-7', 9'), 6.68 (2H, d, *J*=8 Hz, H-7, 9), 6.43 (1H, d, *J*=16 Hz, H-3'), 2.84 (2H, t, *J*=10 Hz, H-4), 2.62 (2H, t, *J*=10 Hz, H-3). ^{13}C-NMR (CD_3OD) δ: 199.2 (C-2), 178.1 (C-2'), 159.5 (C-8'), 155.3 (C-8), 139.9 (C-4'), 131.7 (C-5), 128.9 (C-6, 10), 126.6 (C-5'), 119.1 (C-3'), 115.4 (C-7', 9'), 114.8 (C-7, 9), 41.7 (C-3), 30.4 (C-4). From the comparison of these data with those reported in the literature by Li *et al*., (2009), the substance was identified as 5-hydroxy-1,7-bis(4-hydroxyphenyl)-4,6-heptadien-3-one (dihydrobisdemethoxycurcumin) (Figure 6-6).

Compound 2: Yellow powder**,** UV λ_{max} nm: 247, 414. ESI-MS (+): *m/z* 309.1 $[M+H]^{+}$. ^{1}H-NMR (CD_3OD) δ: 7.56 (2H, d, *J*=16 Hz, H-4, 4'), 7.51 (4H, d, *J*=8 Hz, H-6, 10, 6', 10'), 6.84 (4H, d, *J*=8 Hz, H-7, 10, 7', 10'), 6.62 (2H, d, *J*=16 Hz, H-3, 3'). ^{13}C-NMR (CD_3OD) δ: 184.8 (C-2, 2'), 161.1 (C-8, 8'), 141.8 (C-4, 4'), 131.1 (C-6, 10, 6', 10'), 128.0 (C-5, 5'), 122.0 (C-3, 3'), 116.9 (C-7, 9, 7', 9'). From the comparison of these data with those reported in the literature by Kiuchi *et al*., (1993) and Li *et al*., (2009), the substance was identified as 1,7-bis(4-hydroxyphenyl)hepta-1,6-diene-3,5-dione (bisdemethoxycurcumin) (Figure 6-6).

Compound 3: Yellow powder**,** UV λ_{max} nm: 247, 414. ESI-MS (+): *m/z* 339.3 $[M+H]^{+}$. ^{1}H-NMR (CD_3OD) δ: 7.56 (2H, d, *J*=16 Hz, H-4, 4'), 7.48 (2H, d, *J*=8 Hz, H-6, 10), 7.20 (1H, s, H-6'), 7.10 (1H, d, *J*=8 Hz, H-10'), 6.80 (3H, d, *J*=8 Hz, H-7, 10, 9'), 6.60 (2H, Br, H-3, 3'), 3.90 (3H, s, 7'-OCH_3). ^{13}C-NMR (CD_3OD) δ: 184.7 (C-2, 2'), 161.1 (C-8, 8'),

150.5 (C-8'), 149.4 (C-7'), 142.1 (C-4'), 141.9 (C-4), 131.1 (C-6, 10), 128.6 (C-5'), 128.0 (C-5), 124.1 (C-10'), 122.3 (C-3'), 122.0 (C-3), 116.9 (C-10), 116.6 (C-9'), 111.8 (C-6'), 56.5 (7'-OCH_3). From the comparison of these data with those reported in the literature by Kiuchi *et al*., (1993) and Li *et al*., (2009), the substance was identified as 1-(4-hydroxy-3-methoxyphenyl)-7-(4-hydroxyphenyl)hepta-1,6-diene-3,5-dione (demethoxycurcumin) (Figure 6-6).

Compound 4: Yellow powder, UV λ_{max} nm: 262, 419. ESI-MS (+): *m/z* 369.2 $[M+H]^+$. ^{1}H-NMR (CD_3OD) δ: 7.57 (2H, d, *J*=16 Hz, H-4, 4'), 7.21 (2H, s, H-6, 6'), 7.10 (2H, bd, *J*=8 Hz, H-10, 10'), 6.82 (2H, d, *J*=8 Hz, C-9, 9'), 6.62 (2H, bd, *J*=16 Hz, H-3, 3'), 3.90 (6H, s, 7, 7'-OCH_3). ^{13}C-NMR (CD_3OD) δ: 184.8 (C-2, 2'), 150.6 (C-8, 8'), 149.5 (C-7, 7'), 142.1 (C-4, 4'), 128.6 (C-5, 5'), 124.1 (C-10, 10'), 122.3 (C-3, 3'), 116.6 (C-9, 9'), 111.8 (C-6, 6'), 56.5 (7, 7'-OCH_3). From the comparison of these data with those reported in the literature by Kiuchi *et al*., (1993), Li *et al*., (2009), and Payton *et al*., (2007), the substance was identified as 1,7-bis(4-hydroxy-3-ethoxyphenyl)-1,6-heptadiene-3,5-dione (curcumin) (Figure 6-6).

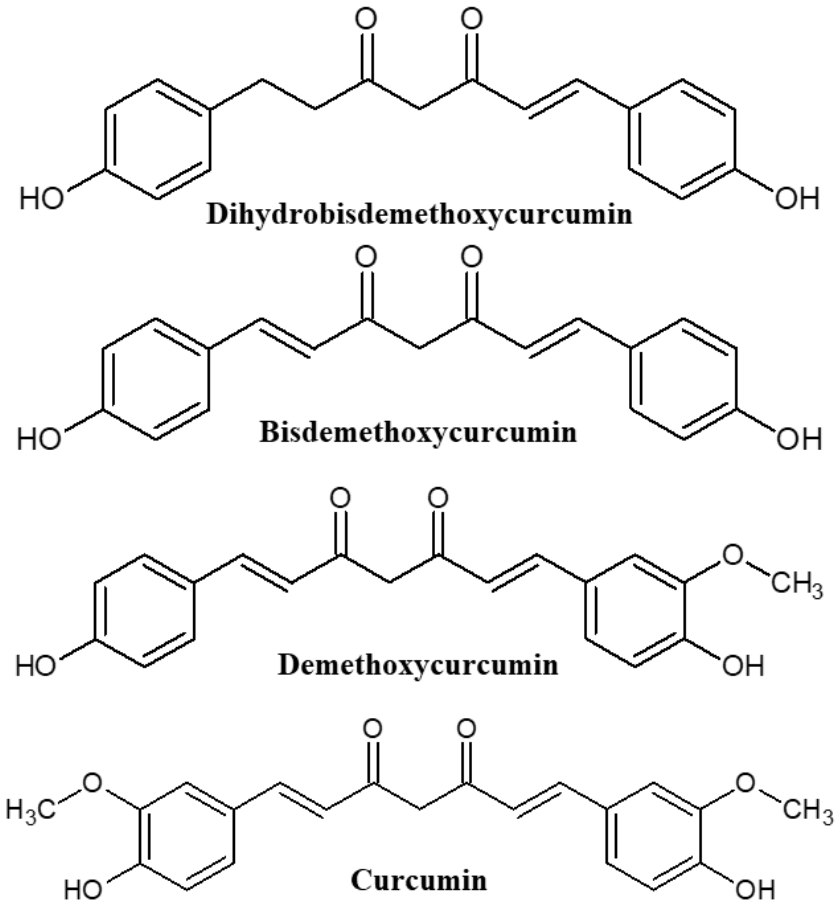


Figure 6-6. Chemical structures of the curcuminoids isolated from Ryudai gold.

4.6 Effects of isolated compounds from Ryudai gold on the root and shoot growth of B. pilosa

Four compounds, namely, dihydrobisdemethoxycurcumin (**1**), bisdemethoxycurcumin (**2**), demethoxycurcumin (**3**) and curcumin (**4**), were isolated from fraction 4 of EtOAc extracts. All the isolated compounds inhibited the root and shoot growth of *B. pilosa* in a concentration-dependent manner (Figure 6-7).

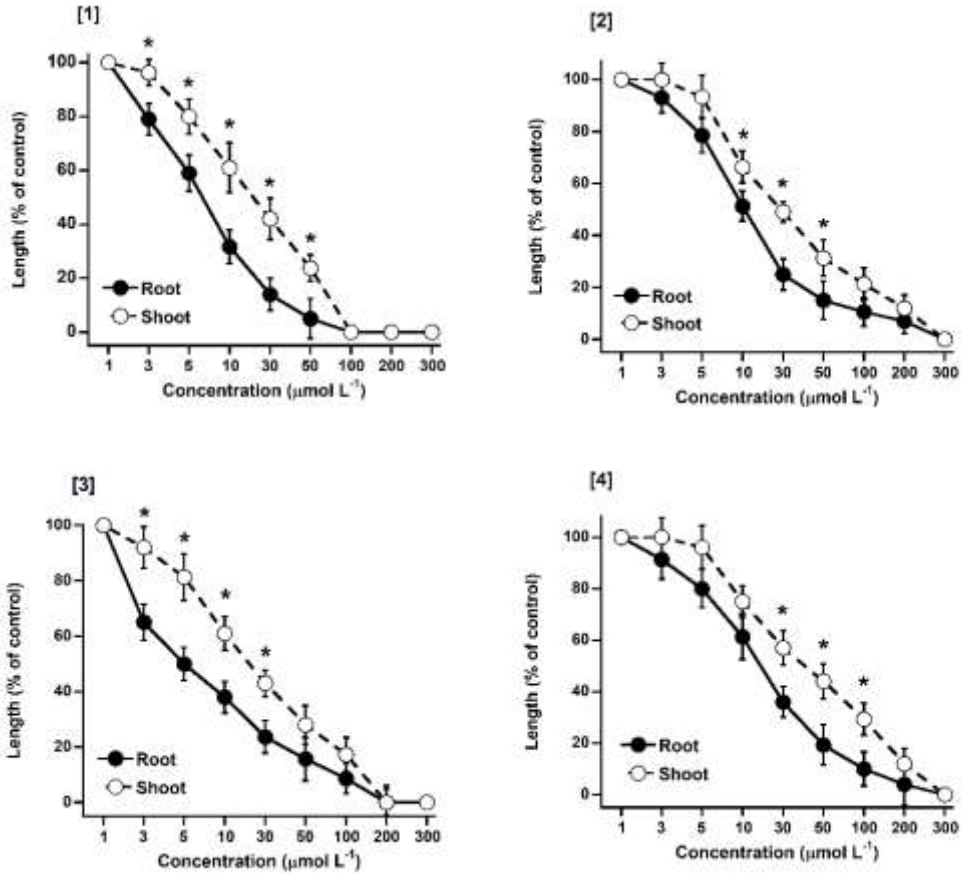


Figure 6-7. Effect of the dihydrobisdemethoxycurcumin (**1**), bisdemethoxycurcumin (**2**), demethoxycurcumin (**3**) and curcumin (**4**) on the root and shoot growth of *Bidens pilosa*. Results are expressed as the mean ± standard error of three independent experiments in triplicate. $^{*}p<0.05$ vs. root.

4.7 Curcuminoids content in two cultivars

The results showed that Okinawa ukon contained lower contents of curcumin (**4**), demethoxycurcumin (**3**) and bisdemethoxycurcumin (**2**) than Ryudai gold. Moreover, Okinawa ukon did not contain dihydrobisdemethoxycurcumin (**1**) (Table 5-1).

Table 5-1. Four curcuminoids content in two cultivars (Ryudai gold and Okinawa ukon) of *C. longa*. Results are expressed as the mean ± standard error of three independent experiments in triplicate. $^{*}p<0.05$ vs. Okinawa ukon.

Sample	Compound **1** (mg/g)	Compound **2** (mg/g)	Compound **3** (mg/g)	Compound **4** (mg/g)
Ryudai gold	0.04±0.003*	8.85±0.002*	8.37±0.007*	13.33±0.022*
Okinawa ukon	0±0	0.32±0.005	2.29±0.032	2.82±0.032

5. DISCUSSION

To the best of our knowledge, this is the first report that demonstrates the plant growth inhibitory potential of turmeric. Germination and seedling growth are principal implements for checking phytotoxic activity (Inderjit and Dakshini, 1995). In our study, Ryudai gold and Okinawa ukon significantly reduced the seed germination as well as the root and shoot growth of all the tested plants in a concentration-dependent manner. Our results also demonstrated that Ryudai gold has a significantly stronger inhibitory effect than Okinawa ukon on seed germination and seedling growth against radish, cress, lettuce and *B. pilosa*. Here, we isolated four compounds, namely, curcumin (**4**), demethoxycurcumin (**3**), bisdemethoxycurcumin (**2**) and dihydrobisdemethoxycurcumin (**1**). We found that Ryudai gold contains significantly higher concentrations of curcumin (**4**), demethoxycurcumin (**3**), and bisdemethoxycurcumin (**2**) than Okinawa ukon. Moreover, dihydrobisdemethoxycurcumin (**1**) was not detected in Okinawa ukon. These might be the reasons for the significant differences in the growth inhibitory effects between Ryudai gold and Okinawa ukon. Similarly, Masum *et al*., (2018) reported that the inhibition of weed seed germination differed between rice varieties due to the differences in the active compound levels. Our results suggested that root growth is more susceptible than shoot growth, which is in agreement with the study of Javaid *et al*., (2008). This result might be because the roots were in direct contact with the extracts and subsequently absorb more active compounds from the filter paper, resulting in reduced length. As root tissue is more permeable than shoot tissue (Nishida *et al*., 2005), root length is the best indicator of the phytotoxic effect of plant extracts (Chon *et al*., 2000). The germination of *B. pilosa* was most strongly inhibited by the turmeric extracts, followed by radish, lettuce and cress. In addition, *B. pilosa* is a seriously problematic weed in various crops that can be controlled by using alternative measures.

Therefore, *B. pilosa* was used for the phytotoxic bioassay of the isolated compounds. The inhibitory effects of Ryudai gold and Okinawa ukon were specific against particular seeds, which might be due to the thickness of the seed coat, the presence of sensitive enzymes in the seeds, the permeability of the cell membrane, the pH of the extracts, etc.

Due to higher phytotoxic effects of Ryudai gold, we analyzed its phytochemicals capable of affecting germination and seedling growth of the tested plants. In our study, we identified four compounds from Ryudai gold, namely dihydrobisdemethoxycurcumin (**1**), bisdemethoxycurcumin (**2**), demethoxycurcumin (**3**) and curcumin (**4**), which were yellow in color. These four compounds were the first plant growth inhibitory compounds isolated from turmeric varieties such as Ryudai gold. Although these compounds were not newly discovered molecules, their phytotoxic effects had never been reported.

Li *et al*., (2009) isolated dihydrobisdemethoxycurcumin (**1**), bisdemethoxycurcumin (**2**), demethoxycurcumin (**3**) and curcumin (**4**) from *Curcuma longa* and determined their structures. Curcuminoids (curcumin and its related compounds) are the major chemical constituents in turmeric, having a wide range of pharmacological properties including antioxidant (Sonkaew *et al*., 2012; Sudsai *et al*., 2016), anticancer (Adams *et al*., 2004), antimalarial (Ji and Shen, 2009), antiplatelet (Dong and Chen, 1998), anti-inflammatory (Sudsai *et al*., 2014), wound healing (Sudsai *et al*., 2016) and nematocidal activities (Kiuchi *et al*., 1993). The contents of dihydrobisdemethoxycurcumin (**1**), bisdemethoxycurcumin (**2**), demethoxycurcumin (**3**) and curcumin (**4**) were 0.128, 28.46, 24.46, and 36 µmol g^{-1} or 0.04, 8.8, 8.3 and 13.3 mg g^{-1} of dry powder of Ryudai gold. The minimum concentrations of the isolated four compounds that inhibited the root and shoot growth of *B. pilosa* were 3 and 5 µmol L^{-1}, respectively (Figure 6-6). The calculated IC_{50} values for *B. pilosa* root and shoot growth from the assay were 8.7±1.7 to 12.9±1.8 and 15.5±1.8 to 38.9±2.8 µmol L^{-1},

respectively. Comparing IC_{50} values, dihydrobisdemethoxycurcumin (**1**) had the strongest inhibitory effect on both the root and shoot growth of *B. pilosa*, followed by demethoxycurcumin (**3**), bisdemethoxycurcumin (**2**) and curcumin (**4**). The effectiveness of dihydrobisdemethoxycurcumin (**1**) on the root and shoot growth of *B. pilosa* was approximately 1.2, 1.1, 1.4-fold and 1.9, 1.7, 2.5-fold higher than that of bisdemethoxycurcumin (**2**), demethoxycurcumin (**3**) and curcumin (**4**), respectively. However, the contents of dihydrobisdemethoxycurcumin (**1**) in the crude extract were approximately 220, 207.5 and 332.5-fold lower than those of bisdemethoxycurcumin (**2**), demethoxycurcumin (**3**) and curcumin (**4**), respectively. The plant growth inhibitory activity of each compound might be dependent on the number of methoxyl groups. Both dihydrobisdemethoxycurcumin (**1**) and bisdemethoxycurcumin (**2**) have no methoxyl groups. Therefore, methoxyl group may have lower allelopathic activities on *B. pilosa* in curcuminoids compounds.

In conclusion, this study assessed that four curcuminoids present in Ryudai gold inhibited the germination and growth of *B. pilosa*. Moreover, dihydrobisdemethoxycurcumin (**1**) and demethoxycurcumin (**3**) exhibited strong inhibitory activity against *B. pilosa*. These results suggested that these molecules, when released by the plant, can prevent the germination and seedling growth of surrounding species. This can be confirmed by further studies aimed to understand the structure-activity relationship of these molecules, their method of release and their persistence in the environment.

General discussion

The genus Curcuma comprises numerous species and varieties with diverse pharmacological action. We hypothesize that various turmeric species and varieties might contain different type/concentration of active compounds and thereby could be different in their pharmacological action.

The chapter I of this thesis studied antifungal effect of different species and varieties of turmeric against FSSL. Our study demonstrated that there are significant variations in the curcuminoids content and antifungal activity of different turmeric species and varieties. *C. longa* strain BK2 contained the highest amount of curcumin, demethoxycurcumin and bisdemethoxycurcumin followed by RD (*C. longa), C. xanthorrhiza*, Okinawa ukon (*C. longa*) and *C. aromatica*. However, RD showed strongest antifungal activity. On the other hand, *C. amada* did not contain any of these three curcumins but showed good antifungal activity. This result suggested that some other active compounds also responsible for antifungal activity of *C. longa* strain RD and *C. amada*. Although *C. longa* and few others species have been reported for their antifungal effect in some other types of fungus. But there is no report about their effect on FSSL, an important pathogenic fungi for human, animal and plants. Moreover, most of the studies evaluabted the effect of the crude extract of turmeric. Therefore, very little is known about their active compounds. So, in the subsequent study, 6 antifungal compound namely curcumin, demethoxycurcumin, turmeronol B, turmeronol A, (*E)*-α-atlantone, dihydrobisdemethoxycurcumin from *C. longa* strain RD and zederone and furanodienone from *C. amada* were purified. Although these were not newly discovered molecules and some have been reported for various pharmacological activities including antioxidant, anti-inflammatory, anti-cancer, antimalarial, and nematocidal activities however, their antifungal effect had never been addressed. Furthermore, antifungal susceptibility test

showed that FSSL was resistant to most of the commercially available antifungal agents. In contrast, our present study suggested that all the isolated compounds showed strong effect on the FSSL. Therefore, the isolated compound can play an important role to control *Fusarium solani sanso lato* infection of human, animal, and plants. However, further investigation is necessary to know their effect on other fungal species, *in vivo* efficacy, mode and site of action of these isolated compounds.

Chapter II of this thesis demonstrated that there are significant variations in the antioxidant activity, TPC and TFC contnent among the different species and varieties of turmeric. The results showed that RD had 3-4 fold higher concentration of phenolic and flavonoid content than that of other turmerics. Phenolics and flavonoides are secondary metabolite that have significant roles as free radical scavengers, hydrogen donors, reducing agent and singlet oxygen quenchers. Hence, we compared the antioxidant activity by using DPPH, ORAC, RPA and 2-DR oxidation assay. RD showed the highest scavenging activity with the lowest IC_{50} (26.5 µg/mL), which was not significantly different with the IC_{50} value of the standard antioxidant Trolox (IC_{50}: 23.2 µg/mL). Similar to DPPH, RD resulted the highes RPA, ORAC, and 2-DR activity with the lowest IC_{50}, while the highest IC_{50} value was observed in Okinawa ukon. The significantly higher antioxidant activity of RD may be attributed to the higher TPC and TFC as we found a very strong correlation between TPC and TFC of turmeric with their antioxidant activity. Though antioxidant capacity of some species and varieties of turmeric have been reported previously (Kim *et al*., 2011; Tanvir *et al*., 2017). However, to the best of our knowledge, active antioxidant compound from *C. longa* has never been purified. Moreover, RD is a high yielding variety of *C. longa* that is rich in curcumin (major active compound of turmeric) content. Therefore, we purified nine antioxidant compounds from RD, namely, bisabolone-9-one (**1**), 4-methyllene-5-hydroxybisabola-2,10-

diene-9-one (**2**), turmeronol B (**3**), 5-hydroxy-1,7-bis(4-hydroxy-3-methoxyphenyl)-1-hepten-3-one (**4**), 3-hydroxy-1,7-bis(4-hydroxyphenyl)-6-hepten-1,5-dione (**5**), cyclobisdemethoxycurcumin (**6**), bisdemethoxycurcumin (**7**), demethoxycurcumin (**8**) and curcumin (**9**). Although these compounds were previously isolated from turmeric and other plant, there is no information about their biological activity except demethoxycurcumin (**8**) and curcumin (**9**). The calculated IC_{50} values of compound **4**, **5**, **8** and **9** were significantly lower than that of Trolox (a potent commercial antioxidant) indicating that these compounds could be used for potential natural antioxidant. Structure-activity relationship studies suggest that the activity of these compounds depends significantly on the introduction of electron donating groups (methoxy) in the *ortho* position of the 4-hydroxyphenyl group. Therefore, compound **9**, **8**, and **4** with two electron donating groups (two phenoxyl groups, and two methoxyl groups for **9** and **4**, one methoxyl group for **8**) exhibited the most activity. Free radical has been implicated in different diseases such as atherosclerosis, cancer, diabetes, cirrhosis, aging etc (Halliwell *et al*., 1992). Therefore, compounds that can scavenge these free radicals have great potentials to prevent or inhibit these degenerative diseases. Future research is to study the efficacy of the isolated compounds against oxidative stress related diseases in vitro and in vivo, and to prevent oxidative deterioration of processed food. Besides this, further study could be conducted to explore other biological activities.

Chapter III examine the vascular effect of turmeric on isolated porcine basilar artery. The results showed that, despite the variation in active compound content found in chapter I, all turmeric induced strong relaxation of isolated porcine basilar arteries. There were no significant differences in relaxation effects among the different species and strains of turmeric. To evaluate the mechanism of turmeric induced relaxation, we examine the role of endothelium, nitric oxide, prostaglandin, K+-channel, Ca^{2+} antagonists and β-adrenoceptor

agonists. Mechanical removal of the endothelium, pretreatment of L-NA and indomethacin did not affect the turmeric induced relaxation. These results suggested that turmeric induces NO, prostaglandins and endothelium-independent relaxation and directly acts through smooth muscle cells of the porcine basilar artery. It is well known that K^+-channels play a substantial role in the regulation of vascular contractility and tone (Jackson, 2005). However, TEA (a Ca^{2+}-activated K^+-channel antagonist), glibenclamide (a non-specific ATP-sensitive K^+-channel antagonist) and 4-AP (a voltage-dependent K^+-channel antagonist) had no significant effect on turmeric-induced relaxation. These results suggested that opening of the K^+-channels was not involved in the mechanism of the vasomotional action of turmeric. Extracellular Ca^{2+} removal (using 2 mM EGTA) suppressed KCl-induced contraction, and pretreatment with turmeric significantly inhibited Ca^{2+}-induced contraction of 60 mM KCl-depolarized arteries. This observation is consistent with a study by Gilani *et al*., 2005, who concluded that Ca^{2+}-channel antagonism is the main mechanism of vasorelaxation elicited by curcuma drugs and forms the basis of traditional uses in hyperactive states of the gut, airway inflammation disorders, palpitation and hypertension. Similarly, curcumin analogs and synthesized curcumin mimics dilate rabbit basilar arteries via antagonism of the L-type Ca^{2+}-channel (Kruangtip *et al*., 2015; Park *et al*., 2015). Stimulation of vascular smooth muscle by β-adrenoreceptors results in vasorelaxation of the porcine basilar artery (Miyamoto, *et al*., 1993). Our results showed that propranolol (a non-selective β-adrenergic receptor antagonist) inhibited the turmeric-induced relaxation when the concentrations were 600 μg/mL and 800 μg/mL, indicating that the high degree of turmeric-induced relaxation might be attributed to β-adrenoceptor activation. Like the effect of the turmeric extract, pure curcuminoids (curcumin, demethoxycurcumin and bisdemethoxycurcumin) induced relaxation of the isolated porcine basilar artery. Moreover, we reported previously that among the different

species and strains of turmeric, *C. amada* and *C. zedoaria* do not contain curcumin, demethoxycurcumin and bisdemethoxycurcumin (Akter *et al*., 2018a) but still induce relaxation. Therefore, vascular relaxation induced by *C. amada* and *C. zedoaria* indicated that there are other vasoactive compounds besides curcuminoids present in turmeric. Turmeric, its active compounds and/or synthetic curcuminoids may be used as novel vasodilatory agents for the treatment of cardiovascular diseases such as atherosclerosis, essential hypertension, pulmonary artery hypertension, coronary artery disease, stroke, and diabetic complications of vasculature. However, additional work is required to investigate the vasoactive compounds and molecular mechanisms of the vasorelaxation induced by turmeric.

Chapter IV demonstrates the plant growth inhibitory potential of turmeric. Ryudai gold has a significantly stronger inhibitory effect than Okinawa ukon on seed germination and seedling growth against radish, cress, lettuce and *B. pilosa*. Hence, we isolated four compounds, namely, curcumin (**4**), demethoxycurcumin (**3**), bisdemethoxycurcumin (**2**) and dihydrobisdemethoxycurcumin (**1**) from RD. We found that Ryudai gold contains significantly higher concentrations of curcumin (**4**), demethoxycurcumin (**3**), and bisdemethoxycurcumin (**2**) than Okinawa ukon. Moreover, dihydrobisdemethoxycurcumin (**1**) was not detected in Okinawa ukon. These might be the reasons for the significant differences in the growth inhibitory effects between Ryudai gold and Okinawa ukon. These four compounds were the first plant growth inhibitory compounds isolated from turmeric varieties Ryudai gold. Although these compounds were not newly discovered molecules, their phytotoxic effects had never been reported. The calculated IC_{50} values for *B. pilosa* root and shoot growth from the assay were 8.7±1.7 to 12.9±1.8 and 15.5±1.8 to 38.9±2.8 μmol L^{-1}, respectively. Comparing IC_{50} values, dihydrobisdemethoxycurcumin (**1**) had the strongest inhibitory effect on both the root and shoot growth of *B. pilosa*, followed by

demethoxycurcumin (**3**), bisdemethoxycurcumin (**2**) and curcumin (**4**). The plant growth inhibitory activity of each compound might be dependent on the number of methoxyl groups. Both dihydrobisdemethoxycurcumin (**1**) and bisdemethoxycurcumin (**2**) have no methoxyl groups. Therefore, methoxyl group may have lower allelopathic activities on *B. pilosa* in curcuminoids compounds. This can be confirmed by further studies aimed to understand their method of release and their persistence in the environment.

The present thesis compared antifungal, antioxidant, vasodilatory and plant growth inhibitory effects of different species and varieties of turmeric and purified the active compounds. Further research is necessary to understand the structure-activity relationship of these compounds, molecular mechanism of action, and their in *vivo* effects.

General Conclusions

C. longa is the mostly studied turmeric for pharmacological activities. Howevere, there are many species and even the single species *C. longa* has multiple varieties for example, Okinawa ukon is an indigeneous variety of *C. longa* cultivated in the Okinawa island, Japan. On the other hand, RD is new variety of *C. longa* develop by the University of the Ryukyus and registered by the ministry of agriculture, Japan (registration no. 21485). This two varieties of *C. longa* and other studied species were cultivated in a common place with common agronomic practices were different in their active component content and pharmacological actions. Therefore, future research on more turmeric species with genetic variations, different geographical area including soil types and climatic conditions of turmeric cultivation could be done to explore more active compounds with deverse pharmacological actions. In vivo experiment is also required to validate the safety and efficacy of the isolated compounds.

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