Studies on the Chemical Characteristics of Flavonoid Glycosides Isolated from Subtropical Plants in Okinawa and their Biological Activities

（沖縄産亜熱帯植物から分離したフラボノイド配糖体の化学特性とそれらの生理活性に関する研究）

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List of Abbreviations and Acronyms

AGEs	Advanced glycation end products
ANOVA	Analysis of variance
BSA	Bovine serum albumin
COSY	Correlated spectroscopy
DPPH	1,1-Diphenyl-2-picrylhydrazyl
DMSO-d_6	Dimethylsulfoxide-d_6
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
Hb	Hemoglobin
HbA	Hemoglobin A
HMBC	Heteronuclear multiple bond correlation
HMQC	Heteronuclear multiple quantum coherence
HPLC	High performance liquid chromatography
HSA	Human serum albumin
IC	Inhibitory activity
MES	2-Morpholinoethanesulfonic acid
MPLC	Medium pressure liquid chromatography
NBT	Nitro blue tetrazolium
NMR	Nuclear magnetic resonance
ODS	Octadecylsilane
ROS	Reactive oxygen species
SC	Scavenging activity
SDM	Standard deviation of means
SPE	Solid phase extraction
TMS	Tetramethylsilane
XOD	Xanthine oxidase

Abstract of dissertation
(in English)

Free radicals and reactive oxygen species do not only cause denaturation in foods but also cause oxidative damage to the human body. The development of diabetic complications was shown to be related with the excessive oxidation by these molecular species and hyperglycemia, which produced reactive carbonyl species (RCS) and glycation of protein. Natural antioxidants from plants such as flavonoids and polyphenols were found to reduce oxidative damage to food during storage and protecting the human body cells from oxidative damage.

Okinawa plants are reported to contain richly in antioxidants, which might be contributed to the development of good health to the Okinawa people. Two Okinawa plants, Ooitabi (*Ficus pumila* L.) and Taumu (*Colocasia esculenta* S.) were selected in this research. The antioxidant components in these plants were investigated and their structural-activity relationship was further evaluated. One of the isolated antioxidant compounds from Ooitabi, rutin, was also investigated for its potential in inhibiting the formation of RCS from glucose autoxidation and glycation of an amine under high glucose concentration.

The separation of antioxidants from Ooitabi and Taumu were based on their DPPH radical scavenging activities. Ooitabi and Taumu were extracted with aqueous ethanol and methanol, respectively. The extracts of these plants were fractionated using C18 solid phase extraction followed by a series of separation using reversed phase MPLC and HPLC. All isolated compounds were identified with MS and NMR spectroscopies. Four flavonoid glycosides had been isolated and identified from Ooitabi, which were apigenin 6-neohesperidosyl, kaempferol 3-robinobioside, kaempferol 3-rutinoside and rutin. As for Taumu, seven flavonoid glycosides were also isolated and identified as isoorientin, isoschaftoside, isovitexin, luteolin 7-sophoroside, orientin, schaftoside and vitexin.

The structural-activity relationship of these flavonoid glycosides were evaluated with comparison to apigenin, isoquercitrin, kaempferol, luteolin, quercetin and quercitrin using DPPH radical scavenging, superoxide radical inhibitory and β-carotene bleaching assays. It was found that not only the presence of hydroxyl groups in position 3 and 3' of the aglycone had an effect on the antioxidant activities but also the glycosylation characteristics such as the glycosidic bonding position, bonding type, the number of glycosides and the type of glycosides. Analysis on the ranking of the antioxidant activities of these compounds in these three assays showed that they have different mechanisms of actions.

Hyperglycemic is an important characteristic of diabetes mellitus. The formation of RCS was found to increase under high sugar concentration and high temperature. The presence of air and metal ions such as Cu^{2+} and Fe^{2+} were found to promote its formation. RCS was found to react readily with lysine to form intermediate advanced glycation end products (AGEs), one of the contributing factors of diabetic complications. Rutin as one of the strong flavonoid antioxidant was shown to inhibit the formation of RCS as well as the glycation of lysine under high glucose concentration. The results of this study suggested that the flavonoid glycosides in Ooitabi and Taumu as antioxidants have potential in inhibiting the formation of intermediate AGEs. Hence, these Okinawa plants can be used as a part of dietary management for diabetes patients and health promotion.

Abstract of dissertation

(in Japanese)

フリーラジカルや活性酸素種は食品の変性を引き起こすのみならず、人体にも酸化障害を起こすことが知られている。これらの分子種による生体内での過剰な酸化反応は、高血糖下における活性カルボニルの形成や生体タンパク質の糖化を通して、2 型糖尿病の病態形成にも関与していることが指摘されている。植物性天然抗酸化成分であるフラボノイドやポリフェノールは、食品を長期保存する際に起こりうる酸化防止効果を持つだけでなく、生体においても細胞を酸化損傷から保護する効果を示すことが報告されている。沖縄産植物は抗酸化成分を豊富に含んでおり、それが沖縄県民の健康に貢献していると推論している報告もある。そこで本研究ではオオイタビ（Ficus pumila L.)とタウム（Colocasia esculenta S.)の 2 種類の沖縄産植物を実験材料として選び、これらの材料から抗酸化成分を分離・同定した。同定化合物については、抗酸化活性と分子構造との相関を調べた。さらに、オオイタビから得られた化合物のひとつ rutin について、本化合物がタンパク質糖化モデル反応系でアミノ基の糖化や活性カルボニル種(RCS)の形成を抑制できるか否かを研究した。概要は以下の通りである。

オオイタビ及びタウムに含まれている抗酸化成分を、DPPH ラジカル補足活性を指標として分離・精製した。すなわち、乾燥したオオイタビ葉とタウムの葉・茎それぞれをエタノール水及びメタノールで抽出した。この抽出液を C18 固相抽出法で分画し、得られた画分を逆相カラムを用いて MPLC、HPLC で精製した。得られた精製化合物を LC/MS と NMR で同定した結果、オオイタビからは Apigenin 6-neohesperidosyl、kaempferol3-robinobioside、kaempferol3-rutinoside と rutin の 4 つフラボノイド配糖体を、タウムからは isoorientin、isoschaftoside、isovitexin、luteolin 7-sophoroside、orientin、schaftoside と vitexin の 7 つのフラボノイド配糖体を得た。

DPPH ラジカル捕捉法、スーパーオキシドラジカル抑制法と β ーカロテン退色法を用いて、単離したフラボノイド配糖体、apigenin、isoquercitrin、kaempferol、luteolin、quercetin と quercitrin の抗酸化活性を測定し、分子構造と抗酸化活性の相関を解析した。その結果、フラボノイド配糖体の抗酸化活性は、アグリコンの B 環の 3 と 3´の OH 基の存在、アグリコンに対する配糖体の結合部位、結合の種類、糖の数及び種類などにより強く影響を受けることが分かった。これら化合物の抗酸化活性は、測定法によって強さの順位が異なることより、化合物間で抗酸化メカニズムが異なることが推測された。

2 型糖尿病の特徴は血糖値が高くなるところにある。試験管テストで、RCS の形成が高糖濃度と高温度の状況下で顕著に増加することを明らかにした。RCS の形成は、金属と空気の存在下でさらに促進されることを示した。この RCS はリジンと反応し、容易に中間 AGEs（終末糖化産物）を生成することが分かった。AGEs は糖尿病の合併症を引き起こす要因の 1 つであるが、rutin は高糖濃度下において RCS の形成及びリジンの糖化を抑制することが分かった。本研究で用いたオオイタビやタウムには rutin をはじめとするフラボノイド配糖体が豊富に含まれており、これら化合物の作用を通して、これらの植物が健康増進あるいは糖尿病を改善するための食品として刊用できる可能性を示唆している。

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Chapter 1

Introduction

1.1 Oxidative stress and complications of diabetes mellitus

Oxidative reactions by free radicals and reactive oxygen species (ROS) could cause denaturation in foods in terms of nutritional value and sensory quality (Yasaei et al., 1996; Pokorný, Yanishlieva and Gordon, 2001). These reactions have been reported to cause oxidative damages to the cellular constituents in our body such as DNA, lipids and proteins (Aniya, 2002; Sies, 1991). External factors such as radiation exposure, ozone, pollutants and chemicals were reported to produce free radicals and ROS. These species were also found to be generated from the normal essential metabolic processes in our body (Bagchi and Puri, 1998).

The harmful effects ofthese radical species cause biological damages, which termed by researchers as oxidative stress. Oxidative stress have been recognized to be involved in the pathogenesis of various life threatening chronic diseases like atherosclerosis, cancer, diabetes mellitus and reperfusion disorder (Benzie, 2002; Dalle-Donne et al., 2005; Kunimoto, 2007).

Diabetes mellitus (Type 2 diabetes) accounts for around 90% of all diabetes worldwide (WHO, 2009). In a report by Roglic et al. (2005), diabetes accounted for 5.2% of the global death in the year 2000. An important characteristic of diabetes mellitus is high blood sugar due to insulin resistance and impaired insulin secretion (Porte, 2001). Brownlee and Cerami (1981) suggested that because of high concentration of sugar in blood, the decreased uptake of glucose into muscle and adipose tissue leads to chronic extracellular hyperglycemia. This would result in tissue damage and pathophysiological complications, involving heart disease, atherosclerosis, cataract formation, peripheral nerve damage, retinopathy and other diabetic complications.

The involvement of oxidative stress had been experimentally shown as one of the causes of hyperglycemia-induced trigger of diabetic complications (Baynes, 1991; Kaneto et al., 1999; Lipinsky, 2001). Since then, researchers have discovered several pathways to show the relationship of oxidative stress and diabetic complications (Evans et al., 2002; Robertson and Harmon, 2006). Among them is the formation of advanced glycation end products (AGEs) due to chronic hyperglycemia (Ahmed and Thornalley, 2007; Goh and Cooper, 2008).

1.2 Advanced glycation end products (AGEs)

AGEs formation is a complex non-enzymatic reaction of reducing sugars, α-oxoaldehydes and other sugar derivatives with proteins. The reaction was first proposed by Maillard in 1912 in the chemistry of food processing.

Investigation on the mechanisms of the formation of AGEs to-date by the researchers concluded with three pathways leading to the formation of AGEs in physiological systems as summarized in Figure 1.1. Hodge proposed the first AGEs formation pathway (Hodge pathway) in 1953. Reducing sugars reacted non-enzymatically with amines to form Schiff base products. These unstable products underwent Amadori rearrangement to form Amadori products such as fructosamine. The products continue to react within themselves as well as neighboring proteins that end up as AGEs.

The second pathway (Namiki pathway) of the formation of AGEs was without the need for Amadori rearrangement, i.e. through the fragmentation of Schiff's base (Namiki and Hayashi, 1983). In their report, Schiff base intermediates reacted with free radicals to form AGEs without proceeding to Amadori rearrangement. The third pathway (Wolff pathway) was reported by Wolff and Dean (1987) where the formation of AGEs was also possible through autoxidation of reducing sugars unlike the pathways described byHodge and Namiki (1983).

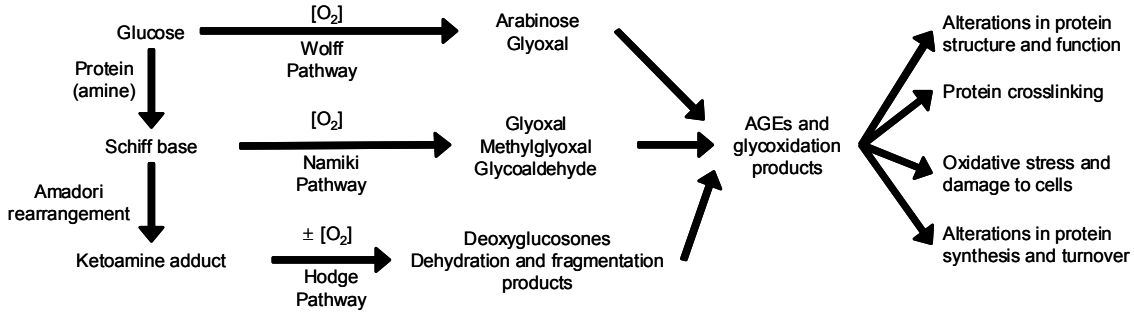


Figure 1.1 The pathways of the formation of AGEs. AGEs are formed via the Wolff pathway by autoxidative glycosylation, the Namiki pathway by autoxidation of Schiff base adducts or the Hodge pathway by reactions of the Amadori adduct on protein (Requena and Baynes, 2000).

Further researches supported Wolff and Dean's finding that monosaccaharides could autoxidize under physiological conditions generating reactive α-oxoaldehydes, which were precursors of AGEs (Thornalley et al., 1984; Argirov et al., 2003). Autoxidation of glucose generated glyoxal, glucosone, 3-deoxyglucosone, methylglyoxal and arabinose (Wells-Knecht et al., 1995; Usui et al., 2007).

In adults, 97% of hemoglobin (Hb) is formed from hemoglobin A (HbA) (Lapolla, Traldi and Fedele, 2005). It has three minor components called as HbA1a, HbA1b and HbA1c (Allen, Schroeder and Balog, 1958). Subsequent studies found that HbA1c was derived from the non-enzymatic reaction between glucose and the amino groups of valine and lysine of β-globulin (John, 1997). HbA1c was discovered by Rahbar (1968) to occur widely in diabetic patients. Structural studies in the later years established that the diabetic hemoglobin was identical to HbA1c, which was elevated two- to three-fold in diabetic patients (Rahbar, Blumenfeld and Ranney, 1969).

The *in vitro* exposure of protein to glucose resulted in the non-enzymatic covalent attachment of glucose to lysine side chains in a manner that resembled that observed *in vivo* (Coussons et al., 1997). This process is normal in individuals with normal control of blood glucose concentration. However, human serum albumin (HSA) was reported to be two to three times more glycated in hyperglycemic condition especially during periods of poor control of

plasma glucose concentration (Guthrow et al., 1979). The extent of glycation of proteins in physiological systems was typically 0.01-1% of lysine and arginine residues (Ahmed et al., 2002). For HSA in blood plasma, the concentration ranges (mol/mol of albumin) of Schiff's base adduct, fructosamine and AGEs are about 1-5, 6-15 and 0.01-7%, respectively (Day, Thorpe and Baynes, 1979; Thornalley, Langborg andMinhas, 1999).

Most AGEs were formed by the modification of lysine and arginine residues in the proteins (Usui, Watanabe and Hayase, 2006). The same authors also stated that α-oxoaldehyde compounds attack the lysine and arginine residues of proteins and the following glycation process was accelerated.

Examples of AGEs that have been found in the physiological system are shown in Figure 1.2: Pyrraline (Hayase et al., 1989), CML (N^{ε}-(carboxymethyl)lysine) (Ahmed et al., 1997), GA-pyridine (glycoaldehyde-pyridine) (Nagaiet al., 2002), GLAP (3-hydroxy-5-hydroxymethyl-pyridinium) (Usui and Hayase, 2003) and OP-lysine (2-aminio-6-(3-oxidopyridinium-1-yl)hexanoate) (Argiov, Lin and Ortwerth, 2004). They were formed as a result of α-oxoaldehyde derived lysine dimer.

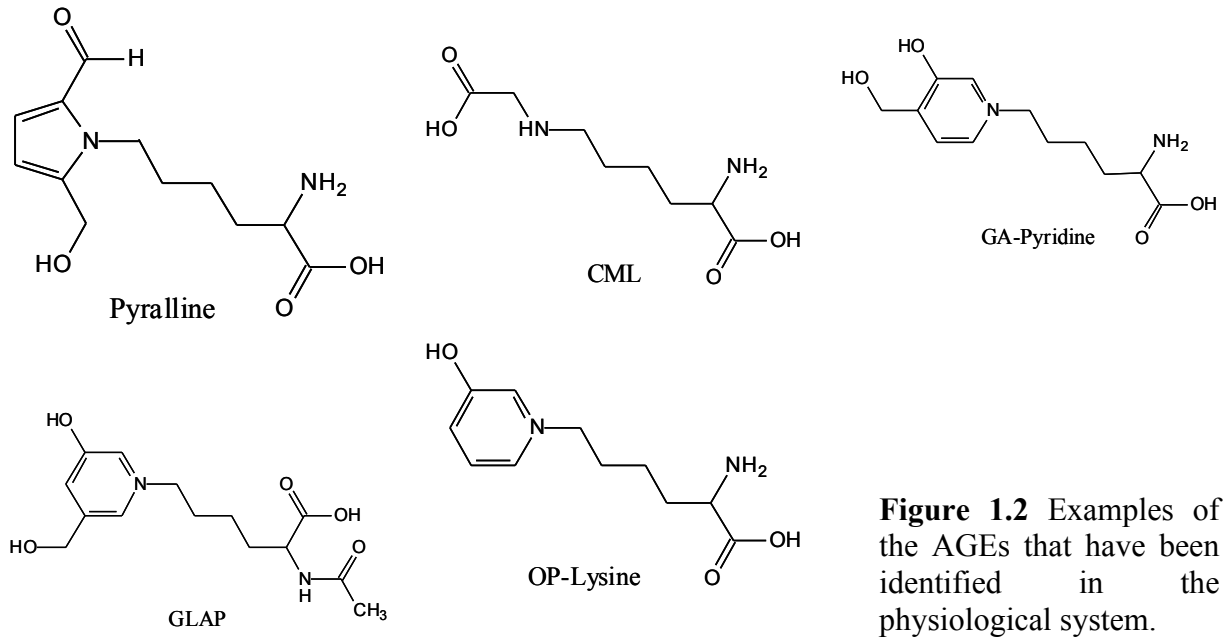


Figure 1.2 Examples of the AGEs that have been identified in the physiological system.

1..3 Natural antioxidants from Okinawan plants

Our body has unique enzymatic antioxidants such as superoxide dismutase, catalase and glutathione peroxidase that protect the cells from oxidative damage (Rahman, Biswas and Kode, 2005). However, the imbalance of oxidative stress and endogenous antioxidant in the body leads to deleterious effect of oxidative stress (Schafer and Buettner, 2001). Therefore, our body requires "reinforcement", which is antioxidant from food to defend against oxidative stress. Natural antioxidants from plants are widely studied nowadays not just as preferred food preservatives (Pokorný, Yanishlieva and Gordon, 2001) but also their potential in preventing oxidative damages in the body (Wiseman, 1996). Epidemiological studies showed that antioxidants from plants such as polyphenols were effective in the protection against cancer and cardiovascular diseases (Arts and Hollman, 2005; Manach, Mazur and Scalbert, 2005).

The subtropical climate of Okinawa has blessed the islands with various plants that have important roles in the daily lives of Okinawans. The utilization of these plants in the Okinawan food culture as well as medicinal purpose has attracted different communities from around the world because Okinawans have the longest life expectancies (Okinawa Centenarian Study, 2009). Sho (2001) expected that these plants might contain important components that contributed to their excellent health.

Okinawan plants have been shown by researchers to contain rich polyphenols that have antioxidant activities (Nakatani, 2003; Suda et al., 2005). The antioxidant activities of these plants were shown to protect liver from inflammation and preventing diabetes complications (Aniya, Itokazu and Shimoji, 2002; Aniya et al., 2002). Nakatani (1992) suggested that the antioxidant compounds contained in the Okinawan plants contributed to the good health of the Okinawans. Table 1.1 shows some of the natural antioxidants that have been identified in the Okinawan plants.

In consideration of the significance of the involvement of oxidative stress in diabetes mellitus, a supplement of antioxidants is required in response to the inhibition of the formation of AGEs as theoretical strategy for the prevention of diabetic complications. Many researchers had shown that antioxidants were capable in inhibiting the formation of AGEs (Morimitsu et al., 1995; Kim and Kim, 2003) but they failed to explain how these antioxidants worked, i.e. the pathway that the antioxidants could inhibit. The background study about this research had shown that there were three pathways to the formation of AGEs from reducing sugars. Therefore, it was necessary to identify the pathway the antioxidant could inhibit for the sake of future application in preventing the development of diabetic complications.

Table 1.1 Natural antioxidants that have been identified in the Okinawan plants.

Plant name	Antioxidant compounds	References
Botanbofu *(Peucedanum japonicum)*	Isoquercitrin, rutin, chlorogenic acid, caffeoylquinic acids	Nakatani, 2003
Ryukyu Yomogi (*Artemisia campestris*)	Dicaffeoylquinic acids, methyl 3,5-dicaffeoylquinate acid	Nakatani, 2003
Getto (*Alpinia speciosa*)	(+)-epicatechin, ethyl 4-feruloyl-glucoside, 4-hydroxy-3-methoxyphenyl 4-feruloyl-glucoside	Masuda et al., 2000
Hosobawadan (*Crepidiastrum lanceolatum*)	Caffeic acid, chlorogenic acid, chicoric acid, luteolin 7-glucuronide	Maeda, 2009

The reactive dicarbonyls were shown to increase in diabetes and they were a potential precursor of AGEs as a post-translational modification of protein. Hence, the pathway proposed by Wolff and Dean (1987) was further investigated in this study. Lysine as one of the amino acid in the albumin that was susceptible to glycation (Garlick and Mazer, 1983; Iberg and Flückiger, 1986) was used as a model of study in this research.

As mentioned above, Okinawan plants were found to have antioxidants that might contribute to the good health of the Okinawan people. In this study, two Okinawan plants

were selected namely Ooitabi (*Ficus pumila* L.) and Taumu (*Colocasia esculenta* S.). Ooitabi was used traditionally by the elderly folks of Okinawa as a leisure herbal tea or medication to treat diabetes, dizziness, high blood pressure and neuralgia (Okada, 1988; Tobinaga, 1989). Taumu was an important source of vegetable in the Okinawan diet mainly during the summer (Shimono, 1980). Although the tuber of Taumu is mainly consumed, the stem is also used as food. Taumu leaf is not consumed and it was interesting to study whether there are any possibilities of utilizing it as food. Therefore, it was necessary to investigate the active antioxidant compounds in these plants as there were no reports about them.

With respect to these hypotheses, the objectives of this study were:

1. Investigation of the antioxidants in Ooitabi and Taumu.
2. Identification of the pathway for the formation of intermediate AGEs.
3. Investigation of the inhibitory activity of the antioxidant on the formation of AGEs.

Chapter 2

Antioxidants in Ooitabi

2.1 Introduction of Ooitabi

Ooitabi (*Ficus pumila* L.) of the Moraceae family is also known as Chita or Ishibaaki in Okinawa. The plant is a scandent shrub with evergreen coriaceous leaves that is naturally grown between the trees as well as on fragmented surface. It also can be found mostly in the southern part of China, Taiwan and southern region of the mainland Japan.

The leaves of Ooitabi can be harvested throughout the year. The dried leaves are infused either in hot water or in liquor for consumption (Okada et al., 1988). Figure 2.1 shows the photographs of Ooitabi taken at the Okinawa Churaumi Aquarium at Motobu-cho of Okinawa in April 2007. In this study, the antioxidants in the leaves of Ooitabi were investigated using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay as the marker for antioxidant activity.

Figure 2.1 Photographs of *Ficus pumila* L. taken at the Okinawa Churaumi Aquarium at Motobu-cho in Okinawa. Coriaceous growth of the leaves (left) and a close-up view of the leaves (right). The photographs were taken in April 2007.

2.2 Materials and methods

2.2.1 Chemicals and reagents

All chemicals and reagents used were of analytical grade. 2-Morpholinoethanesulfonic acid (MES), bovine serum albumin (BSA), DPPH, ethanol, methanol, nitro blue tetrazolium (NBT), α-tocopherol and xanthine were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Copper (II) chloride, ethylenediaminetetraacetic acid (EDTA), dimethylsulfoxide-d_6 (DMSO-d_6), formic acid, methanol-d_4, rutin and xanthine oxidase (XOD) from buttermilk were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). L(+)-Ascorbic acid was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Tetramethylsilane (TMS) for NMR was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Water (18.3 Ω) was purified using a MilliQ-Labo purification system by Millipore Corp. (Osaka, Japan).

2.2.2 Instruments for chromatography

MPLC by Yamazen Corp. (Osaka, Japan) consisted of a GR-200 gradient mixer, a Pump 600A pump, and a Prep. UV254 UV detector. HPLC system by Shimadzu Corp. (Kyoto, Japan) consisted of a SCL-6B system controller, a DGU-12A degasser, two units of LC-6A pump, a CTO-6A column oven with a 1 ml sample loop injector (Rheodyne LLC Co., Rohnert Park, CA, U.S.A.), a SPD-6A UV spectrophotometric detector, and a C-R4A chromatopac.

2.2.3 Plant material and extraction

The leaves of Ooitabi were collected from Motobu-cho in Okinawa in April 2006. The leaves were washed with tap water and air-dried at 60°C for an overnight. The dried leaves were pulverized using a household food processor. Then, 2 L of 50% aqueous ethanol was used to extract about 600 g of the pulverized dried leaves at room temperature for 24 hr. The extract was filtered and the residue was extracted for another twice with the same solvent giving a total volume of 6 L of crude extract. These crude extracts were pooled and concentrated using rotary evaporator.

2.2.4 Separation by chromatography

The separation scheme of the dried leaves of Ooitabi extract is shown in Figure 2.2. The crude extract was chemically filtered using Sep-Pak C18 SPE cartridge (35 cc. reservoir) by Waters Corp. (Milford, MA, U.S.A.). The filtrated extract was evaporated into syrup and then dissolved in water. Then, this extract was fractionated using the same cartridge with a stepwise elution to yield five eluates: water, 25, 50, 75% aqueous ethanol and ethanol.

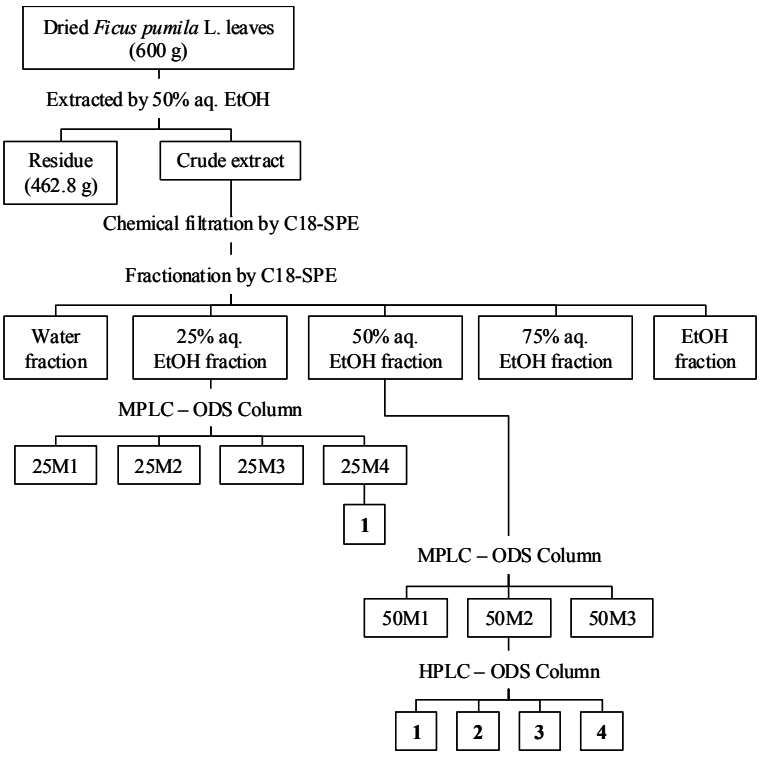


Figure 2.2 The separation scheme of the dried leaves of *Ficus pumila* L.

The 25% aqueous ethanol eluate was separated by MPLC using Ultra Pack ODS-S-50B (300×26 mm i.d. column, 50 μm) column by Yamazen Corp. (Osaka, Japan) with monitoring at 254 nm. A gradient elution from 10 to 60% aqueous methanol in 35 minutes at a flow rate of 9 ml/min was applied to the column. The MPLC chromatogram of the separation is shown in Figure 2.3. Compound **1** was precipitated from fraction 25M4 after concentrated under vacuum evaporation.

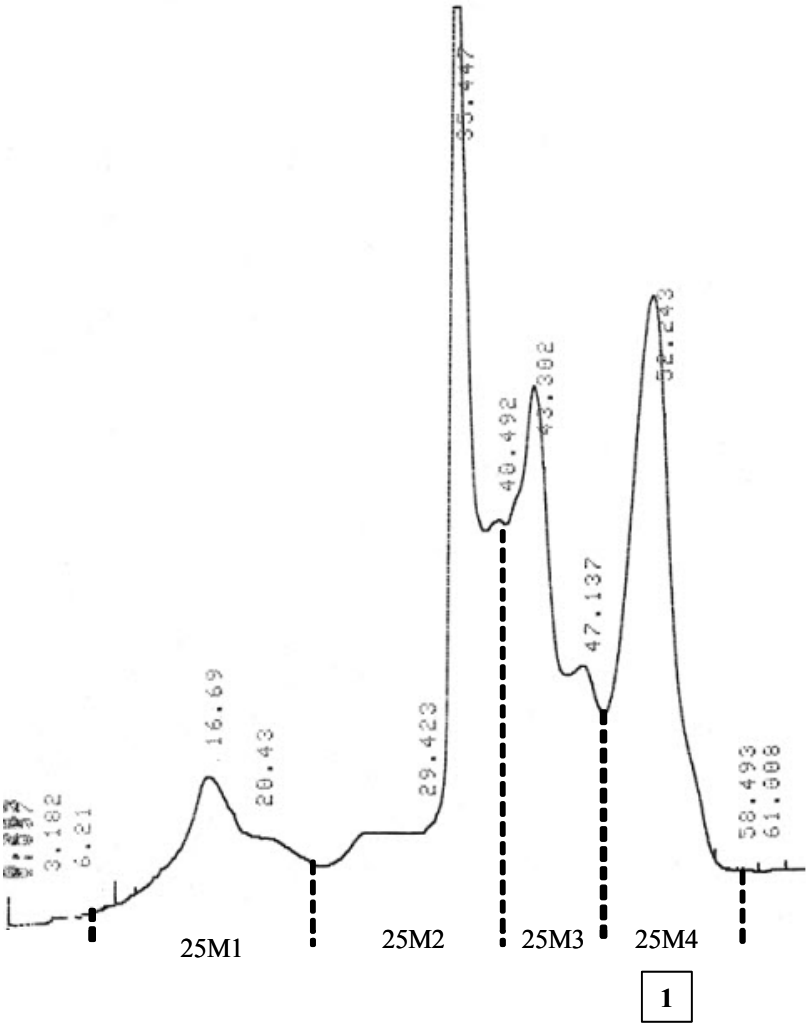


Figure 2.3 MPLC chromatogram of the separation of 25% aqueous ethanol eluate.

The 50% aqueous ethanol eluate was also separated by MPLC using the same column. An isocratic elution of 80% aqueous methanol was applied to the column. Figure 2.4 shows the chromatogram of the separation. The fraction 50M2 showed DPPH radical scavenging activity

and was further separated by HPLC using Cosmosil C18-AR-II Waters (250×10 mm i.d. column, 5 μm) column by Nacalai Tesque, Inc. (Kyoto, Japan). A binary mobile phase was used in which mobile phase A was water and mobile phase B was methanol. The elution was started with 2 min of 30% of B, ascended to 50% of B in 28 min and to 60% of B in 5 min. The separation was monitored at 254 nm. The separation afforded compounds **1**, **2**, **3** and **4**. The HPLC chromatogram of the separation is shown in Figure 2.5.

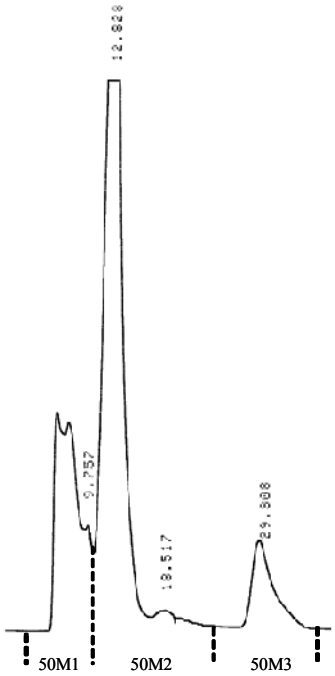


Figure 2.4 MPLC chromatogram of the separation of 50% aqueous ethanol eluate.

The isolated compounds were purified by crystallization. Compounds **1**, **3** and **4** were dissolved in DMSO-d_6 while compound **2** was dissolved in methanol-d_4. TMS was added as an internal reference prior to analysis by NMR spectrometer. NMR spectra (^{1}H, 500 MHz; ^{13}C, 125.65 MHz) were recorded at ambient temperature by JNM-α500 (JEOL, Ltd., Tokyo, Japan).

2.2.5 DPPH radical scavenging assay

DPPH radical scavenging assay by Suda (2000) was used. A reaction mixture of 0.3 ml of 0.4 mM DPPH in methanol, 0.3 ml of 200 mM MES buffer (pH 6.0) and 0.3 ml of 50% aqueous methanol was prepared. Then, 0.3 ml of sample in 50% aqueous methanol in different concentrations up to 0.4 mM was mixed with the reaction mixture (total volume: 1.2 ml). The assay was allowed to stand at room temperature for 1 hr before measuring its absorbance at 520 nm.

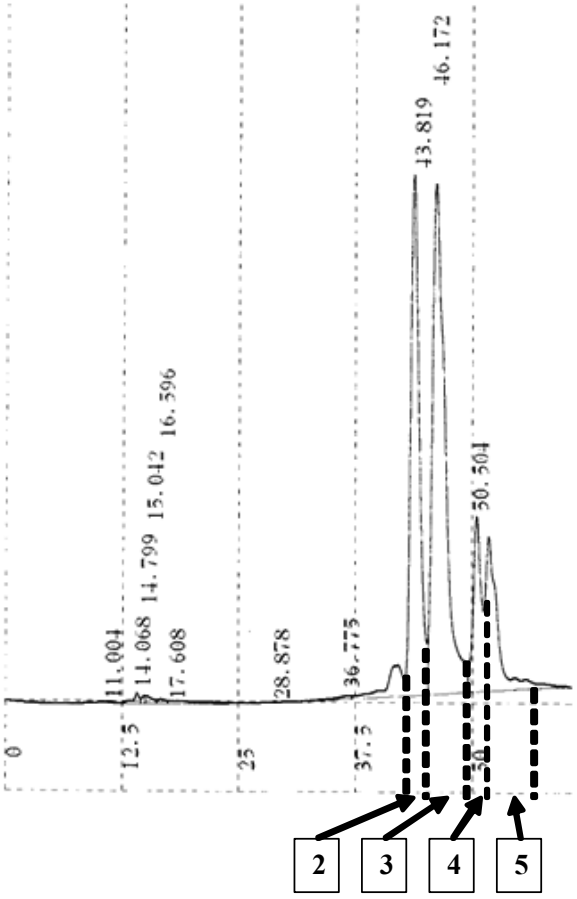


Figure 2.5 HPLC chromatogram of the semi-preparative separation of 50M2 fraction.

The DPPH radical scavenging activity (SC) of the sample was calculated using the following formula: $SC(\%)=[1-(A_s/A_0)]\times100$ where A_s was the absorbance of the sample and A_0 was the absorbance of the blank. The SC_{50} (the concentration required to scavenge 50% of DPPH radicals) value was determined from the linear regression of curve. α-Tocopherol was used as a positive control while 50% aqueous methanol was used as a blank.

2.2.6 Superoxide radical inhibitory assay

The improved assay method for superoxide dismutase described by Imanari et al. (1977) was used. Briefly, 1 mM EDTA, 0.05% (w/v) BSA, 0.25 mM NBT and test sample in different concentrations up to 0.5 mM were prepared in water while 1 mM xanthine was prepared in 0.05 M sodium carbonate buffer (pH 10.2). A reaction mixture was made by adding 0.1 ml of each solution into 2.4 ml of 0.05 M sodium carbonate buffer (pH 10.2). The reaction mixture was pre-incubated at room temperature for 10 min. Then, 0.1 ml of XOD (0.1 U/ml) in water was added to initiate the reaction. After 30 min of reaction, 0.1 ml of 6 mM copper (II) chloride was added to stop the reaction. The formation of formazan was measured from the absorbance at 560 nm.

The superoxide radical inhibitory activity (IC) of the sample was calculated using the following formula: IC (%)=$[1-A_s/A_0]\times100$ where A_s was the absorbance of the sample and A_0 was the absorbance of the blank. The IC_{50} (the concentration required to inhibit 50% of superoxide radicals) value was determined from the linear regression of curve. Due to the difficulty of dissolving the test samples in water, a maximum concentration of 0.5 mM was used. L(+)-Ascorbic acid in different concentrations up to 2.0 mM was used as a reference compound while water was used as a blank.

2.2.7 Quantification

The extraction capacities of water, 50% aqueous ethanol and ethanol on the dried Ooitabi leaves were investigated. Glass weighing bottles (40×40 mm) were weighed and dried in an oven at 90°C for an hour. The bottles were transferred into a desiccator contained with silica gel and

left for 1 hr to cool down. The weight of each bottle was measured and these procedures were repeated until a constant weight was attained on each bottle.

A hundred milliliter of water, 50% aqueous ethanol and ethanol was used to extract 1 g of dried Ooitabi leaves. The extracts were filtered and filled up to 100 ml. Then, 5 ml of each extract was transferred into the dried weighing bottles. These bottles with their contents were dried in the oven and placed into the desiccator to cool down. The weight of each bottle was measured and the procedures were repeated until constant weight was attained on each bottle.

The amount of the isolated compounds contained in these extracts of the dried Ooitabi leaves was analyzed with HPLC. Each extract in a concentration of 1 mg/ml was prepared and 50 μl was injected into HPLC separately. Cosmosil 5C18-AR-II Waters (4.6×150 mm, 5 μm) column by Nacalai Tesque, Inc. (Kyoto, Japan) at 40°C was used in the HPLC analyses. Mobile phase A was 1% (v/v) formic acid in water while mobile phase B was 1% (v/v) formic acid in methanol. The column was developed with a flow rate of 0.8 ml/min using a gradient elution from 30% of B (0-1 min) to 60% of B (1-34 min). The column was reconditioned in 30% of B for 15 minutes before every sample injection. Detection wavelength was set at 254 nm.

Apigenin 6-neohesperidosyl, kaempferol 3-robinobioside and kaempferol 3-rutinoside isolated from Ooitabi (>95% purity by HPLC) as well as commercially obtained rutin were used as the standard compounds for the quantification. Each standard compounds in concentrations of 0.0625, 0.125, 0.25, 0.5 and 1 mg/ml were prepared and 5 μl was subjected to HPLC separately. The column was developed with the same HPLC parameters as described above.

A calibration curve was plotted as peak area (arbitrary units) obtained from the UV absorbance at 254 nm against the known amount of standard compound (mg). The data points were fitted into a line of best fit by the linear regression method. The amount of the isolated compounds extracted by these three solvents was calculated based on the peak area of the chromatogram of the extract with the calibration curve of the standard compounds.

2.3 Results and discussion

DPPH assay was used in this research to detect antioxidant activity as it was considered a valid and easy assay (Sanchez-Moreno, 2002). The result of the DPPH radical scavenging assay for all eluates from the SPE cartridge is shown in Figure 2.6. The 25 and 50% aqueous ethanol eluates reduced the absorbance of the purple color of DPPH radicals at 520 nm in concentration dependent manner. The result showed that these two eluates contained antioxidants that have DPPH radical scavenging activity. The other eluates did not show scavenging activity.

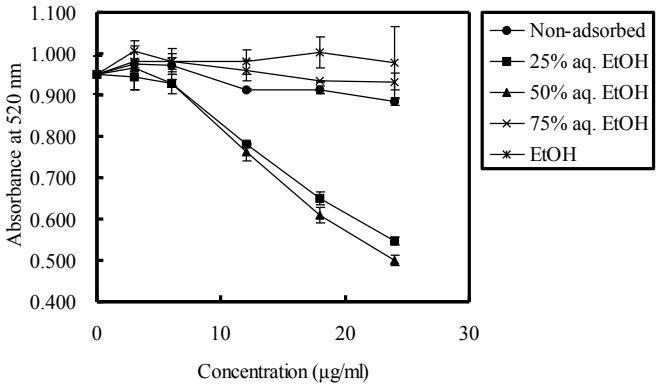


Figure 2.6 DPPH radical scavenging activity of the eluates from C18 SPE cartridge. Error bars shown were standard deviation of mean of three independent experiments.

Crystallization of all isolated compounds gave yellow amorphous solids. The ^{1}H- and ^{13}C-NMR spectral data are shown in Table 2.1 and Table 2.2, respectively. Compound **1** was identified as rutin and confirmed with comparison made with authentic compound. Compound **2** was identified as apigenin 6-neohesperidosyl (apigenin 6-*C*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside). The NMR spectral data of compound **2** corresponded to the results by Rayyan, Fossen and Andersen (2005).

The ^{1}H-NMR spectral data of both compounds **3** and **4** were similar as shown in Table 2.1. However, the ^{13}C-NMR spectra of these compounds were different. Comparison of these ^{13}C-NMR spectral data with the reported data by Brasseur and Angenot (1989) and Agrawal and

Bansal (1989) found that compound **3** was identified as kaempferol 3-robinobioside (kaempferol 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside) while compound **4** was identified as kaempferol 3-rutinoside (kaempferol 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside). Hence, the isolated compounds were found to be flavonoid glycosides. The chemical structures of each compound are shown in Figure 2.7.

Table 2.1 ^{1}H-NMR spectral data for compounds **1**, **2**, **3** and **4**.

Position	**1**[a]	**2**[b]	**3**[a]	**4**[a]
H-5	12.60 (OH, s)	-	12.56 (OH, s)	12.57 (OH, s)
H-6	6.18 (1H, d, *J*=2.0 Hz)	-	6.20 (1H, d, *J*=2.0 Hz)	6.18 (1H, d, *J*=1.3 Hz)
H-8	6.39 (1H, dd, *J*=7.3, 1.5 Hz)	6.51 (1H, s)	6.41 (1H, d, *J*=2.0 Hz)	6.41 (1H, d, *J*=1.3 Hz)
H-2'	7.65 (1H, dd, *J*=8.5, 1.9 Hz)	7.83 (2H, d, *J*=8.8 Hz)	7.98 (2H, d, *J*=8.8 Hz)	8.05 (2H, d, *J*=8.9 Hz)
H-3'	-	6.92 (2H, d, *J*=8.8 Hz)	6.88 (2H, d, *J*=8.8 Hz)	6.86 (2H, d, *J*=8.9 Hz)
H-5'	6.83 (2H, d, *J*=8.8 Hz)	6.92 (2H, d, *J*=8.8 Hz)	6.88 (2H, d, *J*=8.8 Hz)	6.86 (2H, d, *J*=8.9 Hz)
H-6'	7.54 (2H, d, *J*=8.7 Hz)	7.83 (2H, d, *J*=8.8 Hz)	7.98 (2H, d, *J*=8.8 Hz)	8.05 (2H, d, *J*=8.9 Hz)
H-1''	5.33 (1H, d, *J*=7.4 Hz)	3.11 (1H, t, *J*=9.3 Hz)	5.31 (1H, d, *J*=7.8 Hz)	5.31 (1H, d, *J*=7.6 Hz)

[a]Solvent: DMSO-d_6. δ values in ppm from TMS.

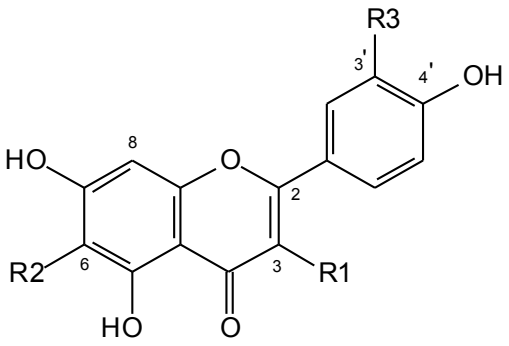
[b]Solvent: Methanol-d_4. δ values in ppm from TMS

Table 2.2 ^{13}C-NMR spectral data for compounds **1**, **2**, **3** and **4**.

Position	**1**[a]	**2**[b]	**3**[a]	**4**[a]
C-2	156.4	166.2	156.4	156.4
C-3	133.3	104.0	133.2	133.1
C-4	177.4	184.2	177.2	177.3
C-5	156.6	n.d.	161.0	161.1
C-6	98.7	109.5	98.7	98.6
C-7	164.0	164.9	164.0	164.0
C-8	93.6	96.0 94.9	93.7	93.6
C-4a	104.0	105.4	103.6	103.9
C-8a	161.2	158.8	156.4	156.7
C-1'	121.2	123.1	120.7	120.8
C-2'	115.2	129.5	130.8	130.8
C-3'	144.7	118.0	114.9	115.0
C-4'	148.4	162.9	159.8	159.8
C-5'	116.2	117.1	114.9	115.0
C-6'	121.6	129.5	130.8	130.8
C-1"	101.2	73.4	101.9	101.2
C-2"	74.1	77.8	71.0	74.1
C-3"	76.4	81.6	72.8	76.2
C-4"	70.0	72.1	67.9	69.8
C-5"	75.9	82.6	73.4	75.6
C-6"	67.0	62.8	65.1	66.8
C-1"'	100.7	102.5	99.9	100.7
C-2"'	70.4	72.4	70.5	70.2
C-3"'	70.5	72.2	70.3	70.5
C-4"'	71.8	73.7	71.8	71.7
C-5"'	68.2	69.9	68.2	68.1
C-6"'	17.7	18.1	17.8	17.6

[a]Solvent: DMSO-d_6. δ values in ppm from TMS.

[b]Solvent: Methanol-d_4. δ values in ppm from TMS



Compound	R1	R2	R3
Rutin	*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside	H	OH
Apigenin 6-neohesperidosyl	H	*C*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside	H
Kaempferol 3-robinobioside	*O*-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside	H	H
Kaempferol 3-rutinoside	*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside	H	H

Figure 2.7 Chemical structures of the flavonoid glycosides isolated from the dried leaves of *Ficus pumila* L.

Table 2.3 shows the values of SC_{50} and IC_{50} of the isolated flavonoid glycosides from Ooitabi determined by DPPH radical scavenging and superoxide radical inhibitory assays, respectively. Rutin had the highest DPPH radical scavenging activity with the SC_{50} value of 0.04 mM followed by the reference compound, α-tocopherol (0.10 mM). The rest of the flavonoid glycosides did not scavenge DPPH radicals as efficient as rutin.

Rutin also had the highest inhibitory activity on the formation of NBT formazan caused by the superoxide radicals generated by xanthine oxidase with the IC_{50} value of 0.14 mM. L(+)-Ascorbic acid and the other flavonoid glycosides did not show inhibitory activity up to 2.0 and 0.4 mM, respectively.

Based on the chemical structures of each compound, the antioxidant strength were shown to be affected by the number of hydroxyl group at the B-ring of the aglycone (Matsuda et al.,

2003; van Acker et al., 1996b). This complied with rutin as it has two hydroxyl groups at the B-ring compared with the other three flavonoid glycosides, which has only one hydroxyl group at the B-ring.

Table 2.3 Antioxidant activities of the flavonoid glycosides isolated from the leaves of *Ficus pumila* L.

Compound	SC_{50} of DPPH radical (mM)	IC_{50} of superoxide radical (mM)
α-Tocopherol	0.10	n.t.
L(+)-Ascorbic acid	n.t.	>2.0
Rutin	0.04	0.14
Apigenin 6-neohesperidosyl	>0.4	>0.5
Kaempferol 3-robinobioside	>0.4	>0.5
Kaempferol 3-rutinoside	>0.4	>0.5

n.t.: not tested

Table 2.4 shows the results of the dry weight matter of the extracts of dried Ooitabi leaves and the content of the flavonoid glycosides in these extracts. Water extract gave the highest yield of dry weight matter followed by 50% aqueous ethanol and ethanol. Among these three solvents, 50% aqueous ethanol extracted the most quantity of apigenin 6-neohesperidosyl.

Table 2.4 The dry weight matter of the extracts of the dried leaves of *Ficus pumila* L. and the content of the flavonoid glycosides in these extracts.

Extract	mg/g of dry weight of *Ficus pumila* L. leaves				
	dry weight matter	Rutin	Apigenin 6-neohesperidosyl	Kaempferol 3-robinobioside	Kaempferol 3-rutinoside
Water	12.6	1.69	0.90	trace	0.21
50% aq. EtOH	10.4	2.38	3.15	0.16	0.01
EtOH	1.5	trace	0.01	trace	trace

Values are means of triplicate analyses.

The most amount of flavonoid glycoside extracted by water was rutin followed by apigenin 6-neohesperidosyl, kaempferol 3-rutinoside and kaempferol 3-robinobioside. Most flavonoid glycosides extracted by ethanol could only be traced. The quantification by HPLC as described in Section 2.2.7 showed that the retention time of apigenin 6-neohesperidosyl, rutin kaempferol 3-robinobioside and kaempferol 3-rutinoside was 11.2, 12.8, 14.7 and 16.7 min, respectively.

The strongest antioxidant, rutin, could be extracted from the leaves of Ooitabi using the above solvents with 50% aqueous ethanol extracted the most. Although this experimental result showed that 50% aqueous ethanol solvent was the best extraction solvent, considerable amount of rutin could still be obtained using water. In conclusion, the antioxidants contained in the leaves of Ooitabi were of flavonoid glycosides. Among these compounds, rutin was found to have the highest antioxidant activity.

Chapter 3

Antioxidants in Taumu

3.1 Introduction of Taumu

Taumu or *Colocasia esculenta* Schott var. aquatilis Kitamura is one of the traditional plant foods in Okinawa. Taumu at one time was cultivated in various places in Okinawa. Nowadays, the only main cultivation area is in Kin town and Ginowan due to the decrease of paddy field (Kin Town Hall, 1994). The stem of Taumu can grow throughout the year although the tuber of Taumu is harvested in between the month of December and April.

The stem of Taumu is eaten either as salad, stew or as soup (Kinjo, Higashimori and Tahara, 2003). The leaf of Taumu is not being used as food. Hence, it was an interest to investigate the antioxidants in the leaf and its potential as food. In this study, the antioxidants in the shoots of Taumu, which included both the stems and the leaves (Figure 3.1), were investigated by using DPPH radical scavenging assay as the marker for antioxidant activity.

Figure 3.1 Photographs of the leaves (left) (Satoimo, 2009) and the stem (right) (Hamada Ichiro Shouten Manzokuya, 2009) of *Colocasia esculenta* S.

3.2 Materials and methods

3.2.1 Chemicals and reagents

All chemicals and reagents used were the same as described in Chapter 2.

3.2.2 Instruments for chromatography

The MPLC and HPLC systems used are the same as described in Chapter 2.

3.2.3 Plant material and extraction

The shoots of Taumu were collected from Kin town in Okinawa in April 2008. They were washed with tap water and freeze-dried. The dried shoots were pulverized using a household food processor. About 750 g of pulverized shoots were extracted with methanol at room temperature for 8 hr. The methanol extract was filtered and the residue was extracted twice again under the same conditions giving a total volume of 8 L of crude extract.

These crude extracts were pooled and vacuum evaporated using rotary evaporator to remove methanol. The dried extract was dissolved in 2 L of water. Hexane (1:1, v/v) was added to remove low polarity compounds from the water phase solution. The liquid-liquid partition was conducted five times.

3.2.4 Separation by chromatography

The separation scheme of the dried Taumu shoots extract is summarized in Figure 3.2. Fractionation of the water phase extract by Waters Sep-Pak C18 SPE cartridge (35 cc. reservoir) with a stepwise elution from water to methanol yielded five fractions. The MPLC and HPLC systems used for the separation of these eluates are the same as in Chapter 2.

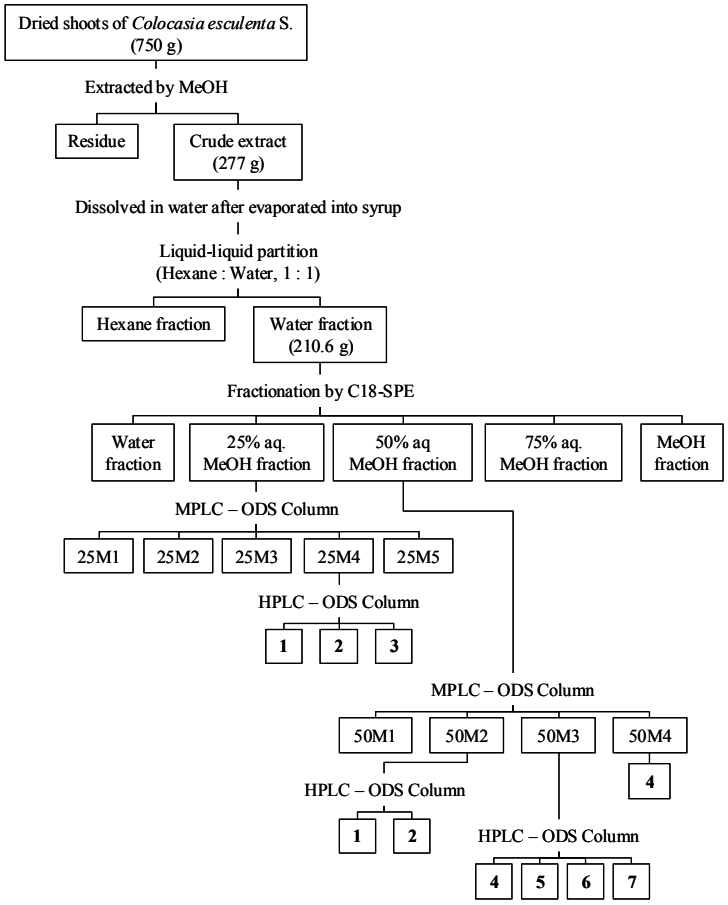


Figure 3.2 The separation scheme of the dried shoots of *Colocasia esculenta* S.

The 25% aqueous methanol eluate was separated by MPLC using Ultra Pack ODS-S-50B (300×26 mm i.d. column, 50 μm) (Yamazen Corp., Osaka, Japan) with monitoring at 254 nm. The following gradient profile at a flow rate of 7.5 ml/min was applied: 5 min of 40% aqueous methanol, increased to 70% aqueous methanol in 15 min and held for 10 min. Five fractions were obtained in this separation.

Fraction 25M4 was further separated by HPLC using a Cosmosil 5C18-AR-II (20×250 mm, 5 μm) (Nacalai Tesque, Inc., Kyoto, Japan) semi-preparative column at 40°C. The flow rate of the mobile phase was 4.5 ml/min with monitoring at 254 nm. Separation of this fraction with isocratic elution of 1% (v/v) formic acid in 30% aqueous methanol afforded compounds **1**, **2** and **3**. The HPLC chromatogram of the separation of fraction 25M4 is shown in Figure 3.3.

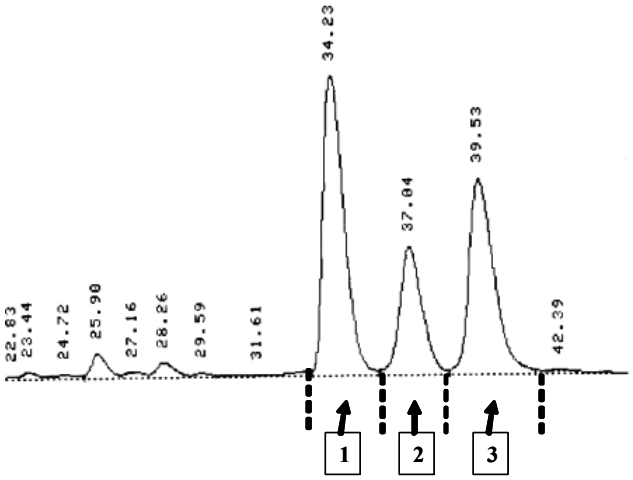


Figure 3.3 Semi-preparative HPLC chromatogram of the separation of fraction 25M4.

The 50% aqueous methanol eluate was also separated by MPLC. Separation was achieved using the following gradient elution: 10 min of 50% aqueous methanol increased to 80% aqueous methanol in 20 min and held for 10 min. Four fractions were obtained. Compound **4** was precipitated from fraction 50M4 when concentrated under vacuum evaporation.

The same semi-preparative column as described above was used for the separation of fractions 50M2 and 50M3 by HPLC. Separation of fraction 50M2 with isocratic elution of 1% (v/v) formic acid in 30% aqueous methanol afforded compounds **1** and **2.** The HPLC chromatogram is shown in Figure 3.4. Separation of fraction 50M3 with isocratic elution of 1% (v/v) formic acid in 35% aqueous methanol afforded compounds **4**, **5**, **6** and **7**. The HPLC chromatogram is shown in Figure 3.5.

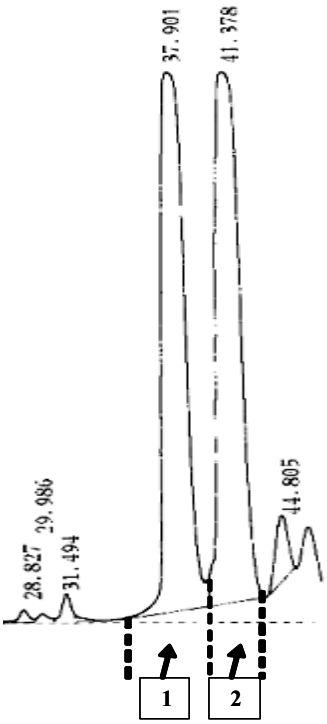


Figure 3.4 Semi-preparative HPLC chromatogram of the separation of fraction 50M2.

All isolated compounds were purified by crystallization. These purified compounds were dissolved in methanol for analysis by an Esquire 3000 plus ion-trap mass spectrometer in electro-spray ionization mode (Bruker Daltronics, Billerica, MA, USA).

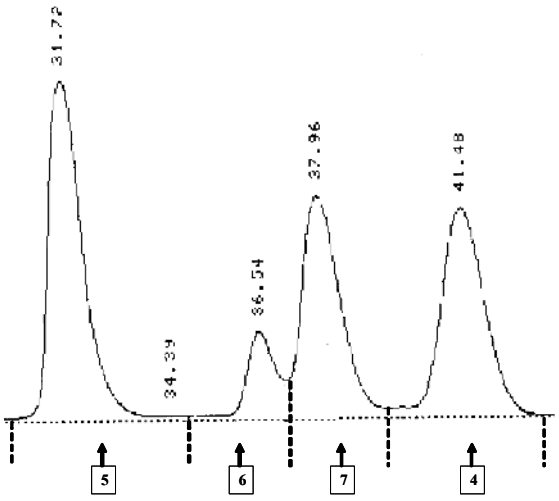


Figure 3.5 Semi-preparative HPLC chromatogram of the separation of fraction 50M3.

For analysis by NMR spectrometer, the purified compounds were dissolved in DMSO-d_6. Both 1D (^{1}H, 500 MHz; ^{13}C, 125.65 MHz) and 2D (^{1}H-^{1}H COSY, HMBC and HMQC) NMR spectra were recorded at ambient temperature by JNM-α500 (JEOL, Ltd., Tokyo, Japan).

3.2.5 DPPH radical scavenging assay

The DPPH radical scavenging assay as described in Chapter 2 was used.

3.2.6 Superoxide radical inhibitory assay

The superoxide radical inhibitory assay as described in Chapter 2 was used.

3.2.7 Quantification

The method as described by Leong et al. (2008) was slightly modified to quantify the extraction capacities of different solvents on the dried Taumu leaves and stems as well as the content of the isolated compounds in these extracts. Glass weighing bottles (40×40 mm) were weighed and dried in an oven at 90°C for an hour. The bottles were transferred into a desiccator contained with silica gel and left for 1 hr to cool down. The weight of each bottle was measured and these procedures were repeated until a constant weight was attained on each bottle.

A hundred milliliter of water and methanol was used to extract 2 g of pulverized dried Taumu leaves and stems. The extracts were filtered and filled up to 100 ml. Each extracts (5 ml) were withdrawn and placed into individual weighing bottles. The bottles with their contents were dried in the oven and transferred into the desiccator. The weight of each bottle was measured and the procedure was repeated until a constant weight was attained.

The amount of the isolated compounds contained in these extracts of dried Taumu leaves and stems was analyzed with HPLC. Each extract in a concentration of 20 mg/ml was prepared and 100 μl was injected into HPLC separately. Cosmosil 5C18-AR-II Waters (4.6×150 mm, 5 μm) column by Nacalai Tesque, Inc. (Kyoto, Japan) at 40°C was used for in the HPLC analyses. Mobile phase A was 1% (v/v) formic acid in water while mobile phase B was 1% (v/v) formic acid in methanol. The column was developed at a flow rate of 0.8 ml/min using a gradient elution from 5% of B (0-1 min) to 100% of B (1-55 min). The column was washed with 100% of B for 5 min before reconditioning in 5% of B for 20 min. Detection wavelength was set at 254 nm.

The isolated compounds from the shoots of Taumu were used as standard compounds (>95% purity by HPLC). Each standard compound was prepared in concentrations of 0.06, 0.1, 0.18, 0.3, 0.52 mg/ml. Five microlitres of the compounds were subjected to HPLC separately. The column was developed with the same HPLC parameters as described above.

A calibration curve was plotted as peak area (arbitrary units) obtained from the UV absorbance at 254 nm against the known amount of standard compound (mg). The data points were fitted into a line of best fit by the linear regression method. The amount of the isolated compounds extracted by these solvents was calculated based on the peak area of the chromatogram of the extract with the calibration curve of the standard compounds.

3.2.8 Solvent extraction capacity

The extraction capacity of water and methanol based on DPPH radical scavenging assay was investigated. Two grams of each leaf and stem of Taumu were extracted separately with 100 ml of water and methanol for 3 hours. Extraction was conducted once. Ekuseru-Toukei 2006 by Social Survey Research Information Co., Ltd. (Tokyo, Japan) was used for statistical analysis.

One-way ANOVA and Scheffé's method of multiple comparison were applied with statistical significance at $p<0.05$.

3.3 Results and discussion

Figure 3.6 shows the DPPH radical scavenging activity of the eluates from SPE at two different concentrations. At the concentration of 1.25 mg/ml, the 25% aqueous methanol eluate showed the highest DPPH radical scavenging activity followed by 50% aqueous methanol eluate. These two fractions were selected for further separation.

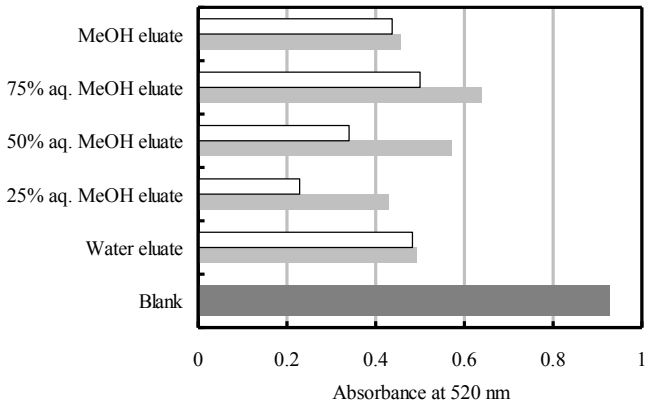


Figure 3.6 DPPH radical scavenging activity of the eluates from C18 SPE cartridge in different concentrations.

All isolated compounds gave yellow amorphous solids after crystallization. Both mass and ^{1}H-NMR spectral data of the compounds are summarized in Table 3.1 while the ^{13}C-NMR spectral data are listed in Table 3.2. Compounds **1** and **2** were identified as schaftoside (apigenin 6-*C*-β-D-glucopyranosyl-8-*C*-α-L-arabinopyranosyl) and isoschaftoside (apigenin 6-*C*-α-L-arabinopyranosyl-8-*C*-β-D-glucopyranosyl), respectively. Their ^{13}C-NMR spectral data was confirmed by comparison with the report by Agrawal and Bansal (1989).

Table 3.1 Mass and ^{1}H-NMR spectral data for compounds **1**, **2**, **3**, **4**, **5**, **6** and **7.**

	1	**2**	**3**	**4**	**5**	**6**	**7**
m/z	$[M-H]^-$ 563.1	$[M-H]^-$ 563.2	$[M-H]^-$ 447.3	$[M-H]^-$ 431.1	$[M-H]^-$ 447.1	$[M+H]^+$ 611.1	$[M-H]^-$ 431.1
H-3	6.74 (1H, s)	6.81 (1H, s)	6.58 (1H, s)	6.82 (1H, s)	6.66 (1H, s)	6.66 (1H, s)	6.8. (1H, s)
H-5	13.68 (OH, s)	13.67 (OH, s)	13.11 (OH, s)	13.60 (OH, s)	13.54 (OH, s)	12.89 (OH, s)	13.22 (OH, s)
H-6	-	-	-	-	6.47 (1H, s)	6.74 (1H, d, *J*=2.0 Hz)	6.31 (1H, s)
H-8	-	-	6.20 (1H, s)	6.53 (1H, s)	-	6.41 (1H, d, *J*=2.0 Hz)	-
H-2'	7.89 (2H, d, *J*=8.4 Hz)	8.02 (2H, d, *J*=8.8 Hz)	7.41 (1H, d, *J*=1.6 Hz)	7.96 (2H, d, *J*=8.8 Hz)	7.33 (1H, d, *J*=2.0 Hz)	7.32 (1H, d, *J*=2.0 Hz)	8.06 (2H, d, *J*=8.5 Hz)
H-3'	6.88 (2H, d, *J*=8.4 Hz)	6.88 (2H, d, *J*=8.5 Hz)	-	6.95 (2H, d, *J*=8.8 Hz)	-	-	6.92 (2H, d, *J*=8.5 Hz)
H-5'	6.88 (2H, d, *J*=8.4 Hz)	6.88 (2H, d, *J*=8.5 Hz)	6.79 (1H, d, *J*=8.3 Hz)	6.95 (2H, d, *J*=8.8 Hz)	6.88 (1H, d, *J*=8.5 Hz)	6.81 (1H, d, *J*=8.1 Hz)	6.92 (2H, d, *J*=8.5 Hz)
H-6'	7.89 (2H, d, *J*=8.4 Hz)	8.02 (2H, d, *J*=8.8 Hz)	7.47 (1H, dd, *J*=8.0, 2.0 Hz)	7.96 (2H, d, *J*=8.8 Hz)	7.36 (1H, dd, *J*=8.0, 2.2 Hz)	7.35 (1H, dd, *J*=8.3, 1.8 Hz)	8.06 (2H, d, *J*=8.5 Hz)
H-1''	4.49 (1H, d, *J*=9.9 Hz)	4.71 (1H, d, *J*=9.6 Hz)	4.61 (1H, d, *J*=9.9 Hz)	4.60 (1H, d, *J*=9.9 Hz)	4.56 (1H, d, *J*=9.6 Hz)	5.13 (1H, d, *J*=7.3 Hz)	4.71 (1H, d, *J*=9.9 Hz)
H-1'''	4.69 (1H, d, *J*=9.9 Hz)	4.75 (1H, d, *J*=9.9 Hz)	-	-	-	4.39 (1H, d, *J*=7.9 Hz)	-

δ values are in ppm.
DMSO-d_6 was used as the internal reference standard.

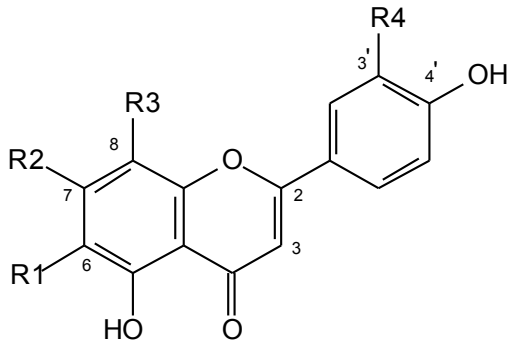
Table 3.2 ^{13}C-NMR spectral data for compounds **1**, **2**, **3**, **4**, **5**, **6** and **7**.

Position	**1**	**2**	**3**	**4**	**5**	**6**	**7**
C-2	164.12	164.14	164.05	163.39	163.78	164.45	163.95
C-3	102.64	102.64	102.39	102.73	102.91	103.11	102.47
C-4	182.45	182.35	182.00	181.87	182.01	181.92	182.14
C-5	159.16	160.97	160.35	103.18	160.79	161.08	160.41
C-6	108.04	108.14	98.09	160.70	108.95	99.65	98.14
C-7	160.83	158.22	162.51	108.91	163.38	162.75	162.60
C-8	104.06	105.14	104.50	-	93.62	94.74	104.61
C-4a	103.32	103.76	104.01	93.68	103.51	105.33	104.04
C-8a	153.77	155.12	155.98	156.27	156.32	156.85	156.01
C-1'	121.45	121.57	122.01	121.09	121.52	121.33	121.62
C-2'	129.14	129.09	114.06	128.47	113.36	113.52	129.01
C-3'	116.05	115.86	145.79	115.94	145.86	145.79	115.82
C-4'	161.28	161.24	149.57	160.70	149.83	149.98	161.16
C-5'	116.05	115.86	115.60	115.94	116.17	115.97	115.82
C-6'	129.14	129.09	119.36	128.47	119.11	119.15	129.01
C-1''	73.64	73.83	73.37	73.08	73.13	104.65	73.39
C-2''	70.97	68.44	70.74	70.64	70.72	82.71	70.82
C-3''	78.56	74.19	78.73	78.97	79.04	75.65[a]	78.65
C-4''	69.98	69.63	70.66	70.15	70.27	69.15[b]	70.51
C-5''	81.14	70.91	81.98	81.62	81.68	76.94[c]	81.88
C-6''	61.27	-	61.61	61.49	61.60	60.55[d]	61.27
C-1'''	74.96	73.29	-	-	-	98.35	-
C-2'''	68.86	70.56	-	-	-	74.69	-
C-3'''	75.21	78.89	-	-	-	76.19[a]	-
C-4'''	69.60	70.14	-	-	-	69.56[b]	-
C-5'''	70.97	81.94	-	-	-	76.94[c]	-
C-6'''	-	61.22	-	-	-	60.46[d]	-

[a-d]Assignments with the same superscripts may be interchanged.
δ values are in ppm.
DMSO-d_6 was used as the internal reference standard.

Compound **3** was identified as orientin (luteolin 8-*C*-β-D-glucopyranosyl) while compound **4** was identified as isovitexin (apigenin 6-*C*-β-D-glucopyranosyl). NMR spectral data of both compounds **3** and **4** corresponded to the results by Mun'im, Negishi and Ozawa (2003) and Peng et al. (2005), respectively. Compound **5** was identified as isoorientin (luteolin 6-*C*-β-D-glucopyranosyl) and was confirmed upon comparison with Mun'im et al. (2003).

Analysis of the NMR spectral of compound **6** found it was luteolin with a diglucoside at C7. The data were compared with the report by Imperato and Nazzaro (1996) and identified the compound as luteolin 7-sophoroside (luteolin 7-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside). Compound **7** was identified as vitexin (apigenin 8-*C*-β-D-glucopyranosyl), corresponded to the results by Zhou et al. (2005). Hence, the isolated compounds were flavonoid glycosides. Their chemical structures are shown in Figure 3.7.



Compound	R1	R2	R3	R4
Schaftoside	*C*-β-D-glucopyranosyl	OH	*C*-β-D-arabinopyranosyl	H
Isoschaftoside	*C*-α-L-arabinopyranosyl	OH	*C*-β-D-glucopyranosyl	H
Orientin	H	OH	*C*-β-D-glucopyranosyl	OH
Isovitexin	*C*-β-D-glucopyranosyl	OH	H	H
Isoorientin	*C*-β-D-glucopyranosyl	OH	H	OH
Luteolin 7-sophoroside	H	*O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside	H	OH
Vitexin	H	OH	*C*-β-D-glucopyranosyl	H

Figure 3.7 Chemical structures of the flavonoid glycosides isolated from the dried shoots of *Colocasia esculenta* S.

The SC_{50} and IC_{50} values of the isolated flavonoid glycosides determined by DPPH radical scavenging and superoxide radical inhibitory assays are shown in Table 3.3. Isoorientin had the highest DPPH radical scavenging activity (SC_{50}=0.031 mM) followed by luteolin 7-sophoroside and orientin when compared to α-tocopherol. Isovitexin and vitexin also showed radical scavenging activity but they had higher SC_{50} values than α-tocopherol. The SC_{50} values of schaftoside and isoschaftoside could not be determined, as 0.4 mM was the maximum concentration used in this assay.

Table 3.3 Antioxidant activities of the flavonoid glycosides isolated from the shoots of *Colocasia esculenta* S.

Compound	SC_{50} of DPPH radical (mM)	IC_{50} of superoxide radical (mM)
α-Tocopherol	0.095	n.t.
L(+)-Ascorbic acid	n.t.	>2.0
Schaftoside	>0.4	>0.5
Isoschaftoside	>0.4	>0.5
Orientin	0.038	0.171
Isovitexin	0.325	>0.5
Isoorientin	0.031	0.067
Luteolin 7-sophoroside	0.032	0.037
Vitexin	0.129	>0.5

n.t.: not tested.

In the superoxide radical inhibitory assay, luteolin 7-sophoroside showed the highest inhibitory activity with the IC_{50} value of 0.037 mM followed by isoorientin and orientin. L(+)-Ascorbic acid in various concentrations up to 2.0 mM did not show any inhibitory activity. The IC_{50} values of the rest of the flavonoid glycosides could not be determined, as 0.5 mM was the maximum concentration used in this assay.

Based on the chemical structures of each compound, the number of the hydroxyl groups at the B-ring of the aglycone affected the antioxidant activities of these flavonoid glycosides. Luteolin 7-sophoroside, isoorientin and orientin have two hydroxyl groups, which explained their

higher antioxidant activities than the other flavonoid glycosides that have only one hydroxyl group at the B-ring. This observation was similar as in Chapter 2 and the report by Matsuda et al. (2003).

Table 3.4 shows quantification results of the dry weight matter of the extracts of dried Taumu leaf and stem as well as the flavonoid glycosides content in these extracts. Water has a higher extraction capacity than methanol in which 28.8 mg/g of dry weight of the leaf and 33.5 mg/g of dry weight of the stem of Taumu had been extracted.

The linear equations and the R^2 values of the individual flavonoid glycosides used as the standard compounds for the quantification were as follow: schaftoside (y=112766x+4672.8, R^2=0.9995), isoschaftoside (y=73284x+1978.2, R^2=0.9994), orientin (y=115035x–955.35, R^2=0.9989), isovitexin (y=91286x+11320, R^2=0.9955), isoorientin (y=136877x–3987.1, R^2=0.9998), luteolin 7-sophoroside (y=77199x+2392.9, R^2=0.9998), and vitexin (y=109338x+3327, R^2=0.9990). The y-axis was the peak area (arbitrary units) of the HPLC chromatogram while the x-axis was the known amount of standard compound (mg).

Quantification on the content of the flavonoid glycosides in these extracts by HPLC found isovitexin was the main compound in both water (0.28 mg/g of dry weight extract) and methanol (0.51 mg/g of dry weight extract) extracts of the leaf of Taumu. Only a trace amount of isovitexin was detected in the water and methanol extracts of the stem. Schaftoside (0.06 mg/g of dry weight extract) was the main compound in the water extract of the stem. The retention times for schaftoside, isoschaftoside, orientin, isoorientin, luteolin 7-sophoroside, vitexin and isovitexin determined by HPLC as described in Section 3.2.7 were 24.50, 24.87, 25.20, 25.59, 26.76, 26.77 and 28.02 min, respectively.

Luteolin 7-sophoroside and vitexin could not be quantified individually because of the closeness of their retention times (0.01 min). The two compounds were quantified together based on the calibration curve of vitexin. The overall quantification results showed that the flavonoid

Table 3.4 The extraction capacity of different solvents upon the dried leaves and stems of *Colocasia esculenta* S. and the content of the flavonoid glycosides in these extracts.

Plant part	Extracted by	Dry weight matter	mg/g of dry weight of extract					
			Schaftoside	Isoschaftoside	Orientin	Isovitexin	Isoorientin	Luteolin 7-sophoroside and Vitexin
Leaf	Water	28.8	0.14	0.06	0.12	0.28	0.19	0.06
	MeOH	21.7	0.21	0.09	0.22	0.51	0.37	0.09
Stem	Water	33.5	0.06	0.02	0.01	trace	0.01	trace
	MeOH	24.1	trace	0.01	0.02	trace	0.02	trace

Values are means of triplicate analyses.

glycosides content in the leaf was higher than in the stem. As shown in Table 3.4, considerable amount of flavonoid glycosides can be extracted with water for both the leaf and stem of Taumu.

The DPPH radical scavenging activities of the water and methanol extracts of the Taumu leaves and stems are shown in Table 3.5. The methanol extract of the leaf had higher DPPH radical scavenging activity than the water extract. However, the DPPH radical scavenging activity of the water extract of the stem was higher than the methanol extract. This might due to the flavonoid glycosides content in these plant parts, which has been shown in Table 3.4.

Table 3.5 DPPH radical scavenging activity of the water and methanol extracts of the leaves and stems of *Colocasia esculenta* S. against blank.

Plant part	Dry weight (mg)	DPPH radical scavenging activity (%)	
		Water extract	MeOH extract
Leaves	0.03	12.1±0.6 [a]	15.8±2.9 [a]
	0.3	65.0±2.5 [c]	83.9±3.2 [b]
Stems	0.03	4.1±1.1 [d]	4.7±0.8 [d]
	0.3	17.8±1.4 [f]	14.4±1.1 [e]

Values are means±standard deviation of triplicate analyses. Values with different letters are significantly different at $p<0.05$.

In conclusion, the antioxidants in the shoot of Taumu are flavonoid glycosides. Luteolin 7-sophororide had the highest overall antioxidant activity among these compounds. In addition to the stem, the Taumu leaf is proposed to be used as food as it contained high amount of antioxidants.

3.4 Acknowledgement

I thank Reiko Kina for providing the shoots of Taumu in this research.

Chapter 4

Structural-activity relationship of flavonoid glycosides

4.1 Introduction of flavonoid glycosides study

The background study in Chapter 1 mentioned that long-term consumption of plant foods rich in antioxidants were effective in the protection against chronic diseases. Flavonoids are attributed to their protective effects on the biological systems due to their capacity to transfer electrons free radicals, chelate metal catalysts (Ferrali et al., 1997), reduce α-tocopherol radicals (Hirano et al., 2001) and inhibit oxidases (Cos et al., 1998). These observations suggested on the possible role of flavonoids in plant foods as radical scavenging nutrients.

Flavonoids are benzo-γ-pyrone derivatives consisting of phenolic and pyrone rings as shown in Figure 4.1. They are classified in accordance to the substitutions as described in the references (Heim, Tagliaferro and Bobilya, 2002; Andersen and Markham, 2006). The antioxidant activity of flavonoids depends upon the arrangement of functional groups about their molecular structures.

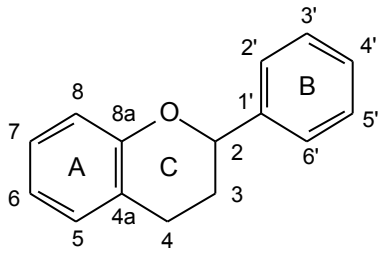


Figure 4.1 Basic chemical structure of a flavonoid.

The presence of an ortho-hydroxylation on the B-ring of the flavonoid molecule was generally pointed as the condition of antioxidant activities as found in the previous studies in

Chapter 2 and 3. Other conditions included the number of hydroxyl groups, the presence of a double bond in between C2-C3 and the presence of a hydroxyl group at C3 (van Acker et al., 1996b; Matsuda et al., 2003).

It was generally accepted that the antioxidant activities of the aglycones are higher than their corresponding glycosides (Ioku et al. 1995; Gao et al., 1999). However, dietary flavonoids occur in food primarily as glycosides (Hammerstone, Lazarus and Schmitz, 2000; Williams, 2006). The most common glycosidic unit is glucose but other examples include glucorhamnose, galactose, arabinose and rhamnose (Cook and Samman, 1996).

The position and the type of glycosides play an important role in the antioxidant activity of a flavonoid besides their mere presence and numbers. Ioku et al. (1995) reported that glucosidic bonding at C4' had higher antioxidant activity than at C3 or C7 of quercetin. In their same report, *O*-glycosylation at C7 instead of at C3, weakens the antioxidant effect of flavonoids in rat mitochondria. Although glycosides are considered as weaker antioxidants than aglycones, the bioavailability of a flavonoid is sometimes enhanced by a glycosidic moiety (Hollman et al., 1999).

Therefore, it was necessary to know the effect of glycosidic moiety on the antioxidant activity of the flavonoids. In this study, eleven flavonoid glycosides have been isolated from Ooitabi and Taumu as reported in Chapter 2 and 3, respectively. In addition to some known flavonoids, the objective of this part of study was to elucidate the relationship between the chemical structures of the isolated flavonoid glycosides with their antioxidant activities.

4.2 Materials and methods

4.2.1 Chemicals and reagents

Analytical grade chemicals and reagents were used for the DPPH radical scavenging and superoxide radical inhibitory assays were as described in Chapter 2. β-Carotene, linoleic acid, polysorbate 20 (Tween 20), α-tocopherol, quercetin and quercitrin were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Chloroform was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Apigenin 6-neohesperidosyl, kaempferol 3-robinobioside, kaempferol 3-rutinoside and rutin used were isolated from Ooitabi as described in Chapter 2. Luteolin 7-sophoroside, isoorientin, isoschaftoside, isovitexin, orientin, schaftoside and vitexin used were isolated from Taumu as described in Chapter 3. Apigenin, luteolin, isoquercitrin and kaempferol were purchased from Funakoshi Co., Ltd. (Tokyo, Japan).

4.2.2 DPPH radical scavenging assay

The DPPH radical scavenging assay as described in Chapter 2 was used.

4.2.3 β-Carotene bleaching assay

The assay by Kumaran and Karunakaran (2006) was used with modifications. A mixture containing 0.5 ml of β-carotene (1 mg/ml), 0.2 ml of linoleic acid (0.1 g/ml) and 1 ml of Tween 20 (0.2 g/ml) was prepared in chloroform. Chloroform solvent was removed under a stream of nitrogen. Then, 90 ml of water and 8 ml of sodium phosphate buffer (0.2 M, pH 6.8) were added into the dried mixture. The solution mixture was thoroughly mixed and 4.8 ml of the mixture were withdrawn into test tubes. After adding 0.2 ml of 1 mM samples prepared in methanol, the zero time absorbance of the resulting mixture (t=0 min) was measured at 470 nm. These tubes

were incubated at 50°C for 2 hours. After that, the absorbance of the solution mixtures was measured again.

The inhibitory activity was calculated using the following formula: Inhibitory activity (%)=[1-(A_s^0-A_s^{120})/(A_c^0-A_c^{120})]×100 in which A_s^0 was the absorbance of the sample at 0 min, A_s^{120} was the absorbance of the sample at 120 min, A_c^0 was the absorbance of the control at 0 min and A_c^{120} was the absorbance of the control at 120 min. α-Tocopherol was used as a positive control while methanol was used as a blank.

4.2.4 Superoxide radical inhibitory assay

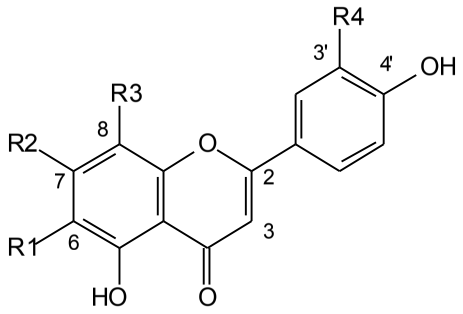
The superoxide radical scavenging assay as described in Chapter 2 was used.

4.3 Results and discussion

The chemical structures of the flavones and their glycosides are shown in Figure 4.2 while the chemical structures of the flavonols and their glycosides are shown in Figure 4.3.

Table 4.1 shows the antioxidant activities of the flavones and their glycosides. Luteolin showed the highest DPPH radical scavenging activity among all the flavones with the SC_{50} value of 0.023 mM. This was followed by isoorientin, luteolin 7-sophoroside, orientin, α-tocopherol, vitexin and isovitexin. These results suggested that the antioxidant mechanism of flavones were reduction of DPPH radicals through electron transfer (Prior, Wu and Schaich, 2005).

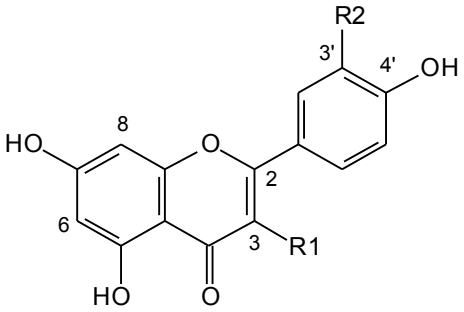
In the β-carotene bleaching assay, α-tocopherol showed the highest inhibitory activity (81.8%) followed by luteolin and luteolin 7-sophoroside with more than 50% of inhibitory activity. The rest of the flavones and their glycosides also showed inhibitory activity but with lower effect. The results suggested that these compounds could donate hydrogen atoms to quench



Compound	R1	R2	R3	R4
Apigenin	H	OH	H	H
Apigenin 6-neohesperidosyl	*C*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside	OH	H	H
Vitexin	H	OH	*C*-β-D-glucopyranosyl	H
Isovitexin	*C*-β-D-glucopyranosyl	OH	H	H
Schaftoside	*C*-β-D-glucopyranosyl	OH	*C*-α-L-arabinopyranosyl	H
Isoschaftoside	*C*-α-L-arabinopyranosyl	OH	*C*-β-D-glucopyranosyl	H
Luteolin	H	OH	H	OH
Luteolin 7-sophoroside	H	*O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside	H	OH
Orientin	H	OH	*C*-β-D-glucopyranosyl	OH
Isoorientin	*C*-β-D-glucopyranosyl	OH	OH	OH

Figure 4.2 Chemical structures of flavones and their glycosides.

the peroxyl radicals induced by heat that was necessary to oxidize β-carotene (Prior et al., 2005). For the result of superoxide radical inhibition assay, luteolin 7-sophoroside showed the highest inhibitory activity (IC_{50}=0.037 mM) followed by isoorientin and orientin.



Compound	R1	R2
Kaempferol	OH	H
Kaempferol 3-robinobioside	*O*-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside	H
Kaempferol 3-rutinoside	*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside	H
Quercetin	OH	OH
Quercitrin	*O*-α-L-rhamnopyranoside	OH
Isoquercitrin	*O*-β-D-glucopyranoside	OH
Rutin	*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside	OH

Figure 4.3 Chemical structures of flavonols and their glycosides.

Table 4.2 shows the antioxidant activities of flavonols and their glycosides. Quercetin showed the highest DPPH radical scavenging activity (SC_{50}=0.016 mM) followed by quercitrin, rutin, α-tocopherol and isoquercitrin. It also showed the highest superoxide radical inhibitory activity followed by quercitrin and rutin. In the β-carotene bleaching assay, the compounds that showed more than 50% of inhibitory activity were α-tocopherol (81.8%), quercetin (80.6%), kaempferol (72.5%) and kaempferol 3-robinobioside (52.8%).

As reported by van Acker et al. (1996b) and Matsuda et al. (2003), the presence of the catechol moiety (C3′, C4′-dihydroxyl group) in the B-ring increased the antioxidant activities of flavonoids as observed for apigenin with luteolin and kaempferol with quercetin. The additional

hydroxyl group at C3 also enhanced the antioxidant activity of kaempferol and quercetin when compared to apigenin and luteolin, respectively. In these results, the aglycone had more potent antioxidant activities than their corresponding glycosides. Glycosylation of flavonols at C3 reduced the antioxidant activities as observed on quercitrin, isoquercitrin, rutin, kaempferol 3-robinobioside and kaempferol 3-rutinoside.

Table 4.1 Antioxidant activities of the flavones and their glycosides.

Compound	SC_{50} of DPPH radical (nM)	β-Carotene bleaching inhibitory activity at 1 mM (%)	IC_{50} of superoxide radical (nM)
L(+)-Ascorbic acid	n.t.	n.t.	>2000
α-Tocopherol	95	81.8	n.t.
Apigenin	>400	-14.1	>500
Apigenin 6-neohesperidosyl	>400	28.9	>500
Vitexin	130	2.3	>500
Isovitexin	333	3.3	>500
Schaftoside	>400	18.4	>500
Isoschaftoside	>400	11.1	>500
Luteolin	23	63.1	>500
Luteolin 7-sophoroside	32	52.0	37
Orientin	38	17.6	171
Isoorientin	31	17.0	67

n.t.: not tested

Although it was reported that the aglycone had higher antioxidant activities than the glycosides, an exception was found for luteolin and luteolin 7-sophoroside. Glycosidic conjugation of luteolin at C7 had a significant effect on the inhibitory activity than luteolin against the formation of formazan caused by superoxide radical generated by xanthine oxidase. This could be due to the effect of the sugar moiety on the antioxidant activity of the luteolin against superoxide radicals, which requires further research.

Table 4.2 Antioxidant activities of the flavonols and their glycosides.

Compound	SC_{50} of DPPH radical (nM)	β-Carotene bleaching inhibitory activity at 1 mM (%)	IC_{50} of superoxide radical (nM)
L(+)-Ascorbic acid	n.t.	n.t.	>2000
α-Tocopherol	95	81.8	n.t.
Kaempferol	>400	72.5	>500
Kaempferol 3-robinobioside	>400	52.8	>500
Kaempferol 3-rutinoside	>400	21.4	>500
Quercetin	16	80.6	63
Quercitrin	29	42.5	102
Isoquercitrin	374	29.6	>500
Rutin	40	47.4	138

n.t.: not tested

Isoorientin and orientin showed higher DPPH radical scavenging and superoxide radical inhibitory activities among all the flavone glycosides yet they did not scavenge peroxyl radical as effective as compared to luteolin 7-sophoroside. Luteolin 7-sophoroside has an *O*-glycosylation bond to 7 of the aglycone while the other compounds have *C*-glycosyl bonded to 6 and 8. The result suggested that *C*-glycosylation in the A-ring could have a negative effect on the antioxidant activity of the flavone towards peroxyl radical inhibition compared to *O*-glycosylation. Similar result was also reported by Mora et al. (1990) that *C*-glycosylation in the A-ring decreases the antioxidant activity of a flavone in the lipid peroxidation assay.

The glycosidic bonding site also affected the antioxidant activities of orientin, isoorientin, vitexin and isovitexin as shown in Table 4.1. Vitexin has a *C*-β-D-glucopyranose bonded at C8 while isovitexin has a *C*-β-D-glucopyranose bonded at C6. DPPH radical scavenging activity of these two compounds differed about 2.5 times with such bonding site difference. The same was observed for orientin and isoorientin in which their superoxide radical inhibitory activity differed about 2.5 times. Isoorientin has a *C*-β-D-glucopyranose bonded at C6 while orientin has a *C*-β-D-glucopyranose bonded at C8.

The type of glycoside also played an important role in the antioxidant strength of the flavonol as observed for quercitrin and isoquercitrin. Quercitrin has an α-L-rhamnopyranoside while isoquercitrin has a β-D-glucopyranoside bonded at C3. The three assays used in this study showed that quercitrin had a higher antioxidant activity than isoquercitrin. The same observation was upon kaempferol 3-robinobioside and kaempferol 3-rutinoside in which the different glycoside moeity gave the latter weaker antioxidant activity when evaluated by β-carotene bleaching assay.

The conjugation of quercetin with two glycosides also reduced the antioxidant activities compared with the aglycone alone. Based on the results of the three assays used, the antioxidant activities of rutin were almost halved than quercetin. Although there was a difference in the chain length of the glycoside when compared to quercitrin and isoquercitrin, the overall results suggested that the type of glycoside played more significant role than the chain length of the glycoside in the antioxidant activity of a flavonol glycoside.

Glycosylation at C7 of luteolin 7-sophoroside showed better antioxidant activities than at C3 of rutin. This was observed especially in the superoxide radical inhibitory activity in which the IC_{50} values of luteolin 7-sophoroside was 0.037 mM while rutin was 0.138 mM. This result suggested that the glycosylated position at C3 and C7 interfered with the coplanarity of the B-ring with the rest of the flavonoids and the ability to delocalize the electrons (van Acker et al., 1996a).

Flavonoid glycosides are usually weaker antioxidants than aglycones as shown in this study. Hollman and Arts (2000) reported that an aglycone was easily absorbed into the body than a flavonoid glycoside. However, in their same report, a flavonoid glycoside could be absorbed and metabolized in the body. Hattori et al. (1988) reported that *C*-glycosylflavones could be metabolized in our body. Hence, it was possible that the flavonoid glycosides in Ooitabi and in the shoots of Taumu could be absorbed into the body.

This study showed that luteolin 7-sophoroside had the highest antioxidant activities among the flavone glycosides while rutin had the highest antioxidant activities among the flavonol glycosides isolated from Ooitabi and Taumu. In conclusion, besides the flavonoid glycosides isolated from Ooitabi and Taumu had different antioxidant mechanism of actions, the glycosyls moieties in the aglycones had an important effect towards their antioxidant activities.

Chapter 5

Rutin as antioxidant inhibiting AGEs formation

5.1 Introduction

An important characteristic of diabetes mellitus is high sugar level in the blood. In Chapter 1, background study found that autoxidation of glucose produced α-oxoaldehydes (α-dicarbonyl compounds such as glyoxal, methylglycoxal and 3-deoxyglucosone) as well as reactive oxygen species as described by Wolff and Dean (1987). α-Oxoaldehydes are potential precursors of AGEs as a post-translational modification of protein (Niwa et al., 1997).

Most AGEs were formed by the modification of lysine and arginine residues in the protein (Usui, Watanabe and Hayase, 2006). Amino acid analysis on human serum albumin after glycation with glucose showed significant losses of lysine (Coussons et al., 1997). Usui et al. (2006) reported that lysine and arginine residues of proteins were susceptible to attack by α-oxoaldehyde compounds.

In the previous study, luteolin 7-sophoroside had the highest antioxidant activities among the flavone glycosides while rutin had the highest antioxidant activities among the flavonol glycosides isolated from Ooitabi and Taumu. However, quantification study showed that rutin was more abundant (Leong et al., 2008) than luteolin 7-sophoroside (Leong et al., 2010). Also, rutin is a common flavonoids in fruits, vegetables and plant-derived beverages such as tea and wine (Łata, Trampczynska and Paczesna, 2009; Shou, Lu and Huang, 2007).

With respect to these background studies, the formation of α-oxoaldehydes were characterized using glucose as no study was made before. Rutin as antioxidant was investigated

for its inhibitory activities on the formation of α-oxoaldehydes and the formation of intermediate AGEs by using lysine as the model of glycation.

5.2 Materials and methods

5.2.1 Chemicals and reagents

All chemicals and reagents used were of analytical grade. Aminoguanidine hydrochloride, copper (II) chloride, D(+)-glucose and ethylenediaminetetraacetic acid (EDTA) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). L(+)-Ascorbic acid and Girard-T reagent were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan) while 40% of glyoxal solution, L-lysine monohydrochloride and nitro blue tetrazolium (NBT) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Water (18.3 Ω) was purified using a MilliQ-Labo purification system by Millipore Corp. (Osaka, Japan). Rutin used was from the separation of Ooitabi (*Ficus pumila* L.) as described in Chapter 2.

5.2.2 Generation of α-oxoaldehydes

The effect of temperature on the formation of α-oxoaldehydes was investigated with the following conditions: temperatures at 37, 60 and 80°C was used to incubate 500 mM of glucose, which has been dissolved with 0.75 ml of sodium phosphate buffer (0.1 M, pH 7.4) for 49 hr period. Glucose in various concentrations (5, 25, 100 and 500 mM) dissolved in 0.1 M of sodium phosphate buffer (pH 7.4) was incubated at 80°C for 2 hr to investigate the effect of glucose concentration on the formation of α-oxoaldehydes.

For the investigation of the effect of air, a final concentration of 0.5 M of glucose in 0.75 ml of sodium phosphate buffer (0.1 M, pH 7.4) was aerated with air for 15 min while another same concentration of glucose was aerated with nitrogen gas for 15 min to purge all the oxygen dissolved in the solution. The samples were incubated at 80°C for 2 hr.

The effect of metal ions on the formation of dicarbonyls was also studied. A solution mixture with the final concentration of 0.1 M of copper (II) chloride and 0.5 M glucose in 0.75 ml of sodium phosphate buffer (0.1 M, pH 7.4) was incubated at 80°C for 6 hr. EDTA at a final concentration of 1 mM was used as a negative control.

All samples were cooled with running water after incubation prior to Girard-T assay. The samples studied for the metal effect were diluted with 6.7 ml of 0.05 M of sodium phosphate buffer (pH 7.4) before conducting Girard-T assay. The α-oxoaldehydes generated from glucose was determined as glyoxal equivalent from the glyoxal standard curve. Sodium phosphate buffer (0.1 M, pH 7.4) was used as a blank.

5.2.3 Girard-T assay

The utilization of Girard-T assay to determine the dicarbonyls in glucose was adapted from Mitchel and Birnboim (1977) with slight modification. A reaction mixture contained 0.3 ml of sample, 0.1 ml of 0.5 M sodium formate buffer (pH 2.9) and 0.1 ml of 0.1 M Girard-T solution was incubated at 30°C for 10 min. Then, 0.5 ml of 0.1 M sodium formate buffer (pH 2.9) was added into the sample for dilution. The UV absorbance of the diluted sample was measured at 295 nm.

A glyoxal standard curve was prepared by diluting 40% of glyoxal solution in sodium phosphate buffer (0.1 M, pH 7.4) to make a series of concentrations of 0.005, 0.02, 0.05, 0.1 and 0.2 mM. For the blank, the sample in the reaction mixture was substituted with sodium phosphate

buffer (0.1 M, pH 7.4). A regression of curve of y=11.443x-0.0086 with R^2=0.9994 was obtained. The y-axis was the UV absorbance of glyoxal at 295 nm determined by the Girard-T assay while the x-axis was the amount of glyoxal standard (nmol) used in the Girard-T assay.

5.2.4 Generation of intermediate AGEs

Reaction mixtures containing final concentrations of 0.3 M lysine and various concentrations of glyoxal (1, 2, 5 and 25 mM) in 0.75 ml of sodium phosphate buffer (0.1 M, pH 7.4) were incubated at 80°C for 30 min. The samples were cooled with running water after incubation. Each samples were diluted with 2 ml of 0.05 M sodium phosphate buffer (pH 7.4). The samples were spot on TLC silica gel 60 F_{254} (Merck, Darmstadt, Germany) using a 2 μl glass capillary. These spots were observed under black light at 365 nm.

5.2.5 α-Oxoaldehydes inhibitory assay

Reaction mixtures containing final concentrations of 0.5 M glucose and rutin in two different concentrations was prepared in 0.75 ml of sodium phosphate buffer (0.1 M, pH 7.4). The reaction mixture was incubated at 80°C for one hour. After incubation, they were cooled with running water. Each samples were diluted with 0.05 M sodium phosphate buffer (pH 7.4) prior to Girard-T assay. α-Oxoaldehydes generated from glucose was determined as glyoxal equivalent from a glyoxal standard curve. L(+)-Ascorbic acid was used as a reference compound while sodium phosphate buffer (0.1 M, pH 7.4) was used as a blank. Inhibitory activities of rutin and L(+)-ascorbic acid on the formation of dicarbonyls were determined as follows: Inhibitory activity (%) = $[1-(A_{sample}/A_{blank})]\times100$, where A_{sample} was the UV absorbance of the test sample at 295 and A_{blank} was the UV absorbance of the blank at 295 nm.

5.2.6 Amadori products inhibitory assay

Reaction mixtures containing final concentrations of 0.5 M glucose, 0.3 M lysine and rutin in two different concentrations was prepared in 0.1 M sodium phosphate buffer (pH 7.4). The reaction mixture was incubated at 80°C for one hour. The samples were cooled with running water after incubation. Each samples were diluted with 0.05 M sodium phosphate buffer (pH 7.4) prior to NBT reduction assay. Aminoguanidine was used as a control while sodium phosphate buffer (0.1 M, pH 7.4) was used as a blank. Inhibitory activities of rutin and aminoguanidine on the formation of Amadori compounds were determined as follows: Inhibitory activity (%) = $[1-(A_{sample}/A_{blank})]\times100$, where A_{sample} was the absorbance of the test sample at 530 nm and A_{blank} was the absorbance of the blank at 530 nm.

5.2.7 NBT reduction assay

NBT reduction assay by Johnson, Metcalf and Baker (1982) was used to determine Amadori products produced by the glycation of glucose and lysine. Briefly, 0.1 ml sample was diluted with 0.8 ml of phosphate buffer saline. Then 0.2 ml of 0.25 M NBT solution in 0.1 M sodium carbonate buffer (pH 10.8) was added into the diluted sample. The mixture was incubated at 37°C for 15 min. Absorbance at 530 nm was measured.

5.2.8 Intermediate AGEs inhibitory assay

Reaction mixtures containing final concentrations of 0.3 M lysine, 5 mM glyoxal and rutin in two different concentrations was prepared in 0.75 ml of sodium phosphate buffer (0.1 M,

pH 7.4). The reaction mixture was incubated at 80°C for 30 min. Samples were cooled with running water after incubation. Each samples were diluted with 2 ml of sodium phosphate buffer (0.05 M, pH 7.4). The samples were spot on TLC silica gel 60 F_{254} using a 2 µl glass capillary. After dried, the spots were observed under black light at 365 nm. Aminoguanidine was used as a reference compound while sodium phosphate buffer (0.1 M, pH 7.4) was used as a blank.

5.2.9 Statistical analysis

All values and the error bars were shown as the standard deviation of the means (SDM) of triplicate independent analysis. SPSS ver. 16 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. One-way ANOVA and Scheffe's method for multiple comparison were applied with statistical significance at $p<0.05$.

5.3 Results and discussion

Figure 5.1 shows the formation of α-oxoaldehydes as glyoxal equivalent when 0.5 M glucose was incubated at different temperatures for a period of time. Glyoxal was chosen as a reference as it was more stable and cheaper than methylglyoxal and 3-deoxyglucosone. After 24 hours of incubation, 55 nmol of glyoxal were formed at 80°C while 10 nmol and 3 nmol of glyoxal was formed at 60 and 37°C, respectively. At 37°C, the formation of dicarbonyls was increasing slowly with time. However, it was impractical to such temperature to generate dicarbonyls in this study as it required a long time for the autoxidation of glucose. Instead of using 37°C of reaction temperature, this study used 80°C of reaction temperature to accelerate the formation of dicarbonyls.

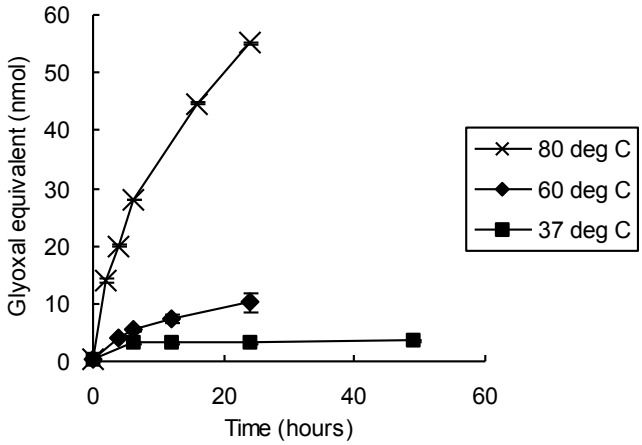


Figure 5.1 The formation of glyoxal from glucose under different incubation temperatures and time.

In Figure 5.2, the formation of glyoxal increased as concentration-dependent manner after 2 hr of incubation at 80°C. As shown in the figure, glucose at the concentration of 500 mM produced the highest amount of glyoxal (14 nmol). Diabetic concentration (25 mM) produced dicarbonyls more than physiological glucose concentration (5 mM) (Coussons et al., 1997). This result suggested that under prolonged exposure of high concentrations of glucose, more dicarbonyls are formed.

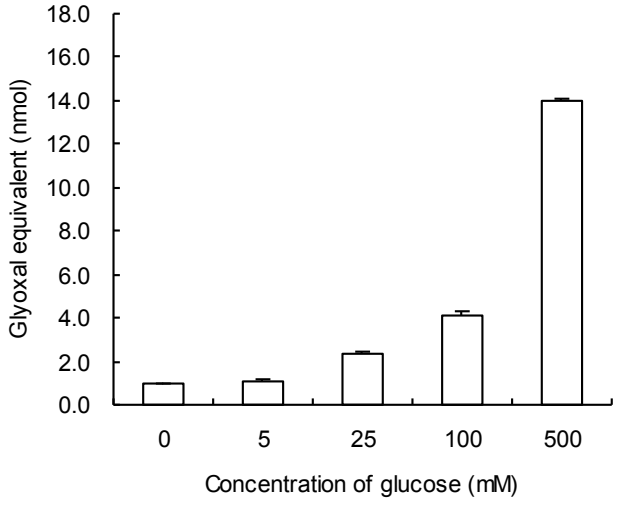


Figure 5.2 The formation of glyoxal from different concentrations of glucose. Different alphabetical letters showed significant differences at $p<0.05$.

The effect of excessive air on the formation of glyoxal was shown in Figure 5.3. Glucose solution saturated with air was found to contain more glyoxal (1.5 nmol) than nitrogen-treated (0.1 nmol) and non-treated (0.9 nmol) glucose solutions before incubation. After incubation at 80°C for 2 hr, glyoxal content in the air-saturated glucose solution increased to 8.3 nmol. Glyoxal content in nitrogen-saturated and non-treated glucose solution increased to 3.6 and 5.6 nmol, respectively.

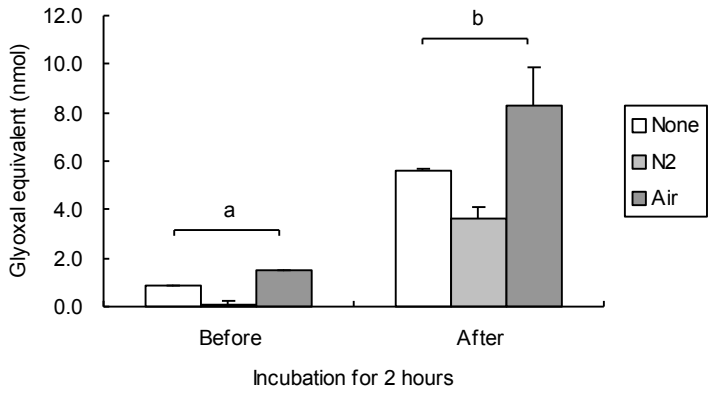


Figure 5.3 The formation of glyoxal from glucose when treated with nitrogen and air. Different alphabetical letters showed significant differences at $p<0.05$.

The result showed that the presence of air promoted the formation of α-oxoaldehydes. Oxygen was considered to play an important role in this process (Thornalley, Langborg and Minhas, 1999; Usui et al., 2007). However, even without the presence of excess air, the formation was still possible as observed for the nitrogen-treated sample. It could be due to the degradation of glucose under high temperature or the presence of phosphate anions because of the acid/base catalysis of aldehyde dehydration/enolization (Thornalley et al., 1984).

Transition metals such as copper, iron and manganese are essential in most biological systems but their accumulation in an excessive capacity in the cellular compartment is cytotoxic due to their involvement in the promotion of free radicals production (Sayre, Perry and Smith, 1999). The cause of cardiovascular disease as one of the complications in diabetes, was strongly related with the Fenton reaction generated physiologically (Sayre et al., 2005; Stadler, Lindner

and Davies, 2009). In Fenton reaction, the hydrogen peroxides are converted into hydroxyl radicals by a metal-catalyzed reaction (Brewer, 2007). This highly reactive hydroxyl radicals increased oxidative stress, which were found to be strongly related to atherosclerosis (Duffy et al., 2001).

In this part of study, the formation of glyoxal was promoted by the presence of metal as shown in Figure 5.4. At a concentration of 0.1 mM of copper (II) chloride, 111.7 nmol of glyoxal was generated from glucose compared with control, which produced 41.9 nmol of glyoxal. When a metal chelator agent, EDTA, was added, the amount of glyoxal was reduced to less than 15 nmol. The result suggested that autoxidation of glucose is a metal-catalyzed process. In overall, oxygen and traces of metal ions were required to form α-oxoaldehydes from glucose.

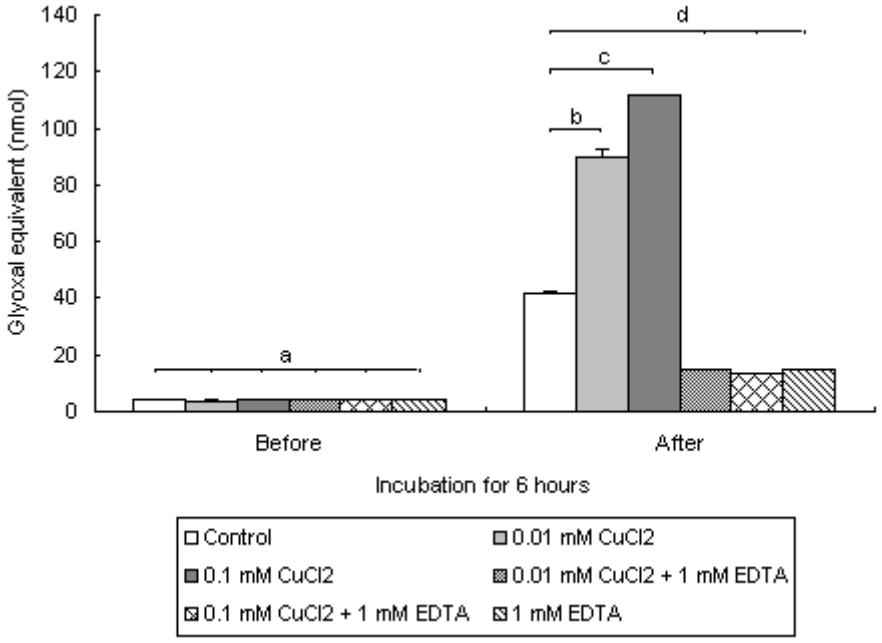


Figure 5.4 The formation of glyoxal from glucose with the presence of copper ion. Different alphabetical letters showed significant differences at $p<0.05$.

Cooper et al. (2005) reported that copper metabolism in a diabetic person was shown to be abnormal and the basal urinary output of Cu^{2+} was higher than a normal person. The increase was suggested to be originated from the fragmentation of the copper and zinc-superoxide

dismutase enzyme due to glycation (Ookawara et al., 1992). Another suggestion was the fragmentation of ceruloplasmin by hydroxyl radicals under hyperglycemia (Islam et al., 1995). With these reports, transition metals were the promoter of oxidative stress and glucose autoxidation. However, the origins of the transition metals *in vivo* level and the precise pathways in the promotion of oxidative stress and glucose autoxidation remained unclear due to the complexity of diabetic complications (Monnier, 2001).

When lysine was reacted with glyoxal, the solution mixture became yellowish-brown in color. Figure 5.5 shows these spots on the TLC silica gel 60 F_{254} fluorescence-like when observed under the black light. The fluorescent-like substance of these spots might be intermediate AGEs as suggested by Coussons et al. (1997). As the amount of glyoxal increased, the amount of intermediate AGEs also increased as shown by the area of the spot. The reacted solution of lysine with glyoxal did not show any NBT reduction activity (data not shown), which suggested that the formation of intermediate AGEs is possible without having to undergo Amadori rearrangement when radical compounds react with protein.

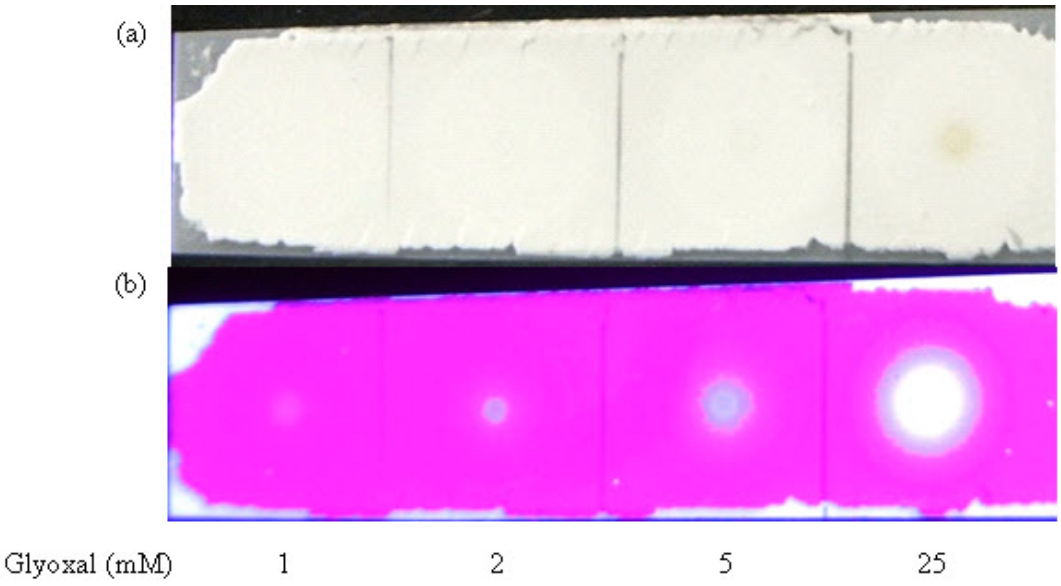


Figure 5.5 Fluorescence-like effects after different concentrations of glyoxal were incubated with lysine. Illumination with normal fluorescence light, (a), and with black light at 365, (b).

Figure 5.6 summarizes the pathway of the formation of intermediate AGEs in this part of study. As shown in the results above, glucose produced α-oxoaldehyde compounds after undergone autoxidation, which in turn reacted with lysine to produce intermediate AGEs. Denoted by the broken line as shown in the figure, such pathway was cofirmed with the studies conducted by Wolff et al. (1987) and Wells-Knecht et al. (1995). Another pathway to the formation of intermediate AGEs was through glycation with lysine to form Schiff base products and proceeded to produce Amadori products that lead to the formation of intermediate AGEs denoted by the solid lines. Thornalley et al. (1999) and Gopalkrishnapillai et al. (2003) also reported such pathway.

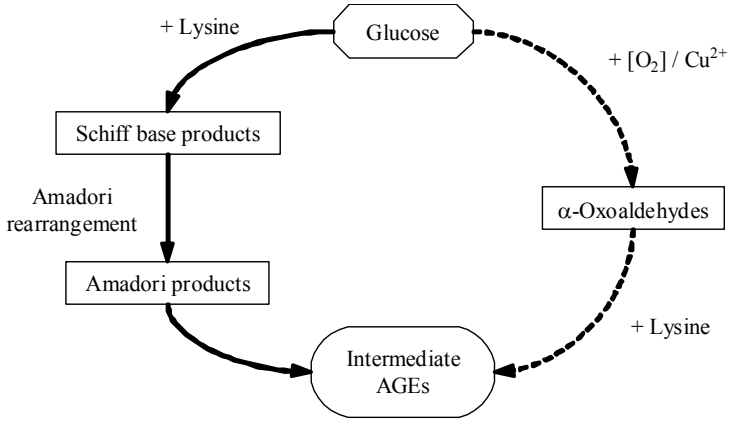


Figure 5.6 The pathways of the formation of intermediate AGEs by glucose autoxidation (dotted line) determined *in vitro* compared with Hodge pathway (solid line).

The inhibitory activities of rutin, L(+)-ascorbic acid and aminoguanidine on the formation of α-oxoaldehydes from glucose and Amadori products from the glycation of lysine are shown in Table 5.1. The formation of glyoxal was inhibited by rutin as dose-dependent. Rutin at 0.2 mM inhibited 42.9% of glyoxal compared to 0.2 mM L(+)-ascorbic acid, which inhibited 8.4% of glyoxal formation. The results showed that rutin effectively suppressed α-oxoaldehydes formation. Rutin also inhibited the formation of Amadori products in which 0.2 mM rutin inhibited 37.7% of Amadori products formation compared to 0.2 mM aminoguanidine.

Table 5.1 Inhibitory activities of rutin, L(+)-ascorbic acid and aminoguanidine on the formations of α-oxoaldehydes and Amadori products.

Sample	Inhibitory activity (%)	
	α-Oxoaldehydes	Amadori products
0.1 mM L(+)-Ascorbic acid	-6.9±1.2	n.t.
0.2 mM L(+)-Ascorbic acid	8.4±0.8	n.t.
0.1 mM Aminoguanidine	n.t.	3.3±0.3
0.2 mM Aminoguanidine	n.t.	23.2±0.2
0.1 mM Rutin	14.2±0.5	13.6±1.3
0.2 mM Rutin	42.9±0.8	37.7±1.4

Values are means±SD of triplicate analyses.
n.t.: not tested.

The inhibitory activities of rutin and aminoguanidine towards the formation of intermediate AGEs are shown in Figure 5.7. After incubation, all samples produced fluorescent-like spots when observed on TLC silica gel 60 F_{254} under the black light. However, the intensities of the spots decreased as the concentrations of rutin and aminoguanidine increased. This result suggested that rutin and aminoguanidine were effective for inhibiting the formation of intermediate AGEs. Although such method was easy to implement qualitatively, it was insufficient to quantify the inhibitory activities of these two compounds.

The beneficial effects of flavonoids on cardiovascular disease and cancer risk are well-documented. In diabetes mellitus, controlling the blood sugar level is important to mitigate the illnesses and prevent hyperglycemic and diabetes complications (Mertes, 2001). Flavonoids were reported to be able to inhibit α-glucosidase activity to control blood sugar level (Lee and Lee, 2001; Kawaguchi, Tanabe and Nagamine, 2004; Li et al., 2009). This enzyme is located at the brush-border surface membrane of the intestinal cells that is important for carbohydrate ingestion (Li et al., 2009).

A research by Odetti et al. (1990) showed that rutin has positive effect on the prevention of the formation of fluorescence compound in streptozotocin-induced diabetic rats. The prevention of the later stage of AGEs by rutin and its metabolites in collagen was also possible (Cervantes-Laurean et al., 2006; Urios, Grigorova-Borsos and Sternberg, 2007). Although these reports claimed the efficacy of rutin in inhibiting the formation of AGEs, they did not mention in which pathway rutin inhibited. In this study, it was found that rutin could inhibit the formation of intermediate AGEs by inhibiting the formations of α-oxoaldehydes and Amadori products as shown in Figure 5.8.

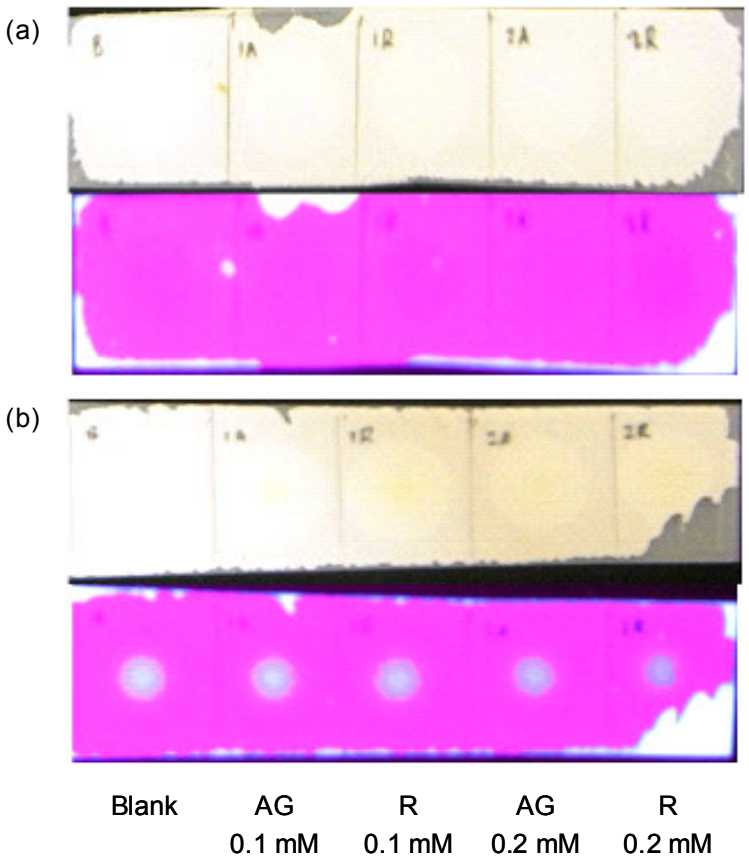


Figure 5.7 Effect of different concentrations of aminoguanidine and rutin on the intensities of fluorescent-like spots before, (a), and after, (b), incubation. AG=Aminoguanidine and R=Rutin.

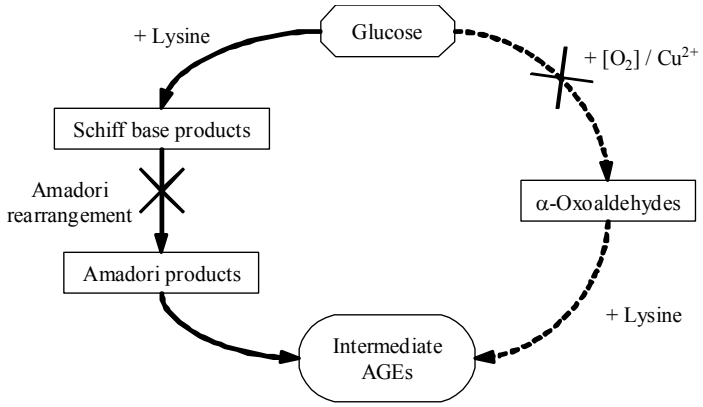


Figure 5.8 Inhibitory activities of rutin on the pathways of the formation of intermediate AGEs determined *in vitro*.

The prevention of the formation of AGEs through inhibition of these pathways are important in reducing oxidative stress that could cause complications in diabetes. The experimental results showed that rutin was capable in inhibiting glucose autoxidation and protein glycation. However, further experiments especially *in vivo* are needed to investigate whether rutin could be used in the prevention of the formation of AGEs.

This experiment showed the potential of antioxidant compound with rutin as an example in the prevention of intermediate AGEs. Both Okinawan plants, Ooitabi and Taumu, contained antioxidants as shown in this study. It is possible that the antioxidants of these plants could inhibit the formation of intermediate AGEs. In conclusion, Ooitabi and Taumu could be used as a functional food in the prevention and management of diabetes mellitus.

Chapter 6

Conclusion of the studies

Oxidative stress was associated with the development of diabetic complications under hyperglycemic through the formation of advanced glycation end products (AGEs). Natural antioxidants were found to have potential in preventing the deleterious effects of oxidative stress.

The antioxidants contained in the leaves of Ooitabi (*Ficus pumila* L.) and Taumu (*Colocasia esculentai* S.) were flavonoid glycosides. The flavonoid glycosides contained in the leaves of Ooitabi were identified as apigenin 6-neohesperidosyl, kaempferol 3-robinobioside, kaempferol 3-rutinoside and rutin. In Taumu, the antioxidants contained were identified as isoorientin, isoschaftoside, isovitexin, luteolin 7-sophoroside, orientin, schaftoside and vitexin. These flavonoid glycosides have antioxidant capabilities that could scavenge DPPH radicals by electron transfer, inhibiting superoxide radical by inhibiting xanthine oxidase and inhibiting peroxyl radicals through hydrogen donation.

Luteolin 7-sophoroside had the highest antioxidant activities among the flavone glycosides while rutin had the highest antioxidant activities among the flavonol glycosides isolated from these plants. Rutin was the major flavonoid glycoside in the water extract of the leaves of Ooitabi. In Taumu, isovitexin was the main flavonoid glycoside in the leaves while schaftoside was the main flavonoid glycoside in the water extract of the stem. The leaves of Taumu contained more flavonoid glycosides than the stems. Hence, the leaves of Taumu could also be used as food.

The formation of intermediate AGEs *in vitro* level was established through autoxidation of glucose. Besides possessing antioxidant activities, rutin was found to inhibit the early

formation of intermediate AGEs through inhibitions of glucose autoxidation and Amadori products formation.

Both Ooitabi and Taumu contained flavonoid glycoxides that not only have antioxidant activities but also inhibitory activity on the formation of intermediate AGEs. Hence, these Okinawan plants could be used as functional foods in the prevention and management of diabetes mellitus.

Chapter 7

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