Studies on the Physiological Functions of Kaki Fruit Extracts against Oxidative Stress in Skeletal Muscle

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（骨格筋の酸化ストレスに対するカキ果実エキスの生理機能に関する研究）

2020

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I. INTRODUCTION

Aging is thought to be a complex phenomenon since it involves many biological levels and signaling pathways in all living things, including humans. Even now, aging is a topic which attracts curiosity and generates unresolved questions, among researchers. There is no exact mechanism that would describe the emergence of aging in living things. López-otín et al. (2013) and Fedarko (2011) stated that aging is a progressive loss of physiological homeostasis which caused functional impairment and enhance the risk factor of pathological conditions. The onset and rate of aging of individuals are incredibly varied, dependent on a person's homeodynamic maintenance and ability to maintain optimal biological function. These mechanisms of maintenance, such as DNA repair, the detection and clearance of defective biological macromolecules, damaged organelles and cells, regenerative capacity, and defensive capacity against pathogens, keep individual homeostasis on track and ensure optimal biological function (Fedarko, 2011).

One popular mechanism that may explained the incident of aging is free radical theory of aging, which stated that accumulated free radical species in oxidative stress condition may impair macromolecule of the cell, leading to deleterious effect of aging (Harman, 1956). Oxidative stress is an imbalance state between oxidant and antioxidant levels in human body. The bioavailability of reactive oxygen species (ROS) as an oxidant become excessive while antioxidant defenses tend to remain the same or even decrease (Kregel & Zhang, 2007). High level of ROS can alter or damage macromolecule of the cell such as DNA, proteins, and lipids leading to cell death (Gomes et al., 2017). Together with reactive nitrogen species (RNS), ROS-RNS can induce nitration, nitrosylation, carbonylation, and glycation (Baumann et al., 2016).

On the other hand, frailty is a geriatric state which manifests by increased vulnerability to stressors and decreased physiological, defensive capacity to maintain homeostasis and to respond to internal and external stresses (Topinková, 2008). This geriatric syndrome is usually characterized by weakness, weight loss, low activity, and adverse health outcomes. There are at least five main criteria used in clinical screening for frailty: poor endurance, sudden weight loss of up to 10 lbs/year, slowed performance, weakness, and low physical activity (Fried et al., 2001). Recently, frailty has been recognized as one of the most problematic clinical conditions in an aged population, worldwide. As a consequence of multiple physiological impairments, related to aging, frail, elderly people have an increased risk of falls, disability, long-term hospitalization, and death. Clegg et al., (2013) stated that a quarter to a half of people over 85 years of age are estimated to be frail. Moreover, according to the WHO, the aged population worldwide is expected to rise rapidly from 600 million, in the year 2000, to around 2 billion by 2050. With this astonishing figure in mind, there is a growing interest to overcome the problem of frailty. Without proper management, people with frailty may became a burden, with high dependency, that affects not only individuals and family, but also the health care system and society (Buckinx et al., 2015).

Skeletal muscle is a strong predictor of frailty in elder population. Skeletal muscle, the most abundant tissue in the body, contributes not only to mobility and movement but also have an important role in metabolic activity. Therefore, muscle health can support human quality of life and functionality of locomotion (J. Liu et al., 2018). Unfortunately, since skeletal muscle consume large quantities of oxygen, this organ also tend to produce large quantities of ROS (Brioche & Lemoine-Morel, 2016). According to Meng & Yu (2010), the combination among oxidative stress, chronic inflammation, and mitochondrial

dysfunction have a significant role in accelerated skeletal muscle aging through the imbalances between muscle protein synthesis and muscle protein breakdown and apoptosis. These vicious cycles eventually leading to sarcopenia, an age-related syndrome which characterized by progressive loss of mass and strength of skeletal muscle (Seene & Kaasik, 2012). Unfortunately, until now there have been no effective clinical strategies to overcome sarcopenia. Physical therapists use a resistance training approach to deal with sarcopenic patients. Although this method is relatively safe and proves effective in maintaining muscle strength and function, the degree of muscle response may vary among individuals. Pharmacological intervention, such as testosterone treatment, growth hormone treatment, dehydroepiandrosterone supplementation, and myostatin regulation also give promising results but there are potential adverse effects to be considered (Jones et al., 2009).

The impact of food on human health has recently attracted an unprecedented scale of scientific research. Many scientists have focused their research on the functional nutrition, innovation and mass production of foods. The role of food in decreasing the risk of illness and improving health status has highlighted a new class of foods, known as functional foods (Lordan et al., 2011). Fruits and vegetable are now considered as functional foods that have the capability to deliver health benefits and to fulfil biological needs (Vinha et al., 2012). The health promoting potential of fruits, vegetables and other plant-based foods are associated with the presence of bioactive components. The synergistic combination of their phytochemical extracts can offer various health benefits, beyond the basic nutritional value of a food product (Yaqub et al., 2016). Some have strong antioxidant and antiproliferative activities, to prevent the development of chronic disease (Liu, 2004). Therefore, although most bioactive compounds (such as tannins, flavones,

triterpenoids, steroids, saponins, and alkaloids) are nonnutritive, they may have important roles in protection against various diseases (Zhang et al., 2015). Several studies relating botanical compounds to muscle health have shown promising results. Although few studies use humans as subjects to evaluate plant bioactive compounds on the welfare of skeletal muscle, recent *in vivo* and *in vitro* findings are worth consideration.

Persimmon (*Diospyros kaki*) is one of a well-known and highly distributed fruits around the world, especially among east asian country. Different studies reported therapeutic effects and phytoconstituents profile of various part of persimmon plant such as leaf (Kotani et al., (2000); Khan et al., (2017)), peel (Kawase et al., 2003), pulp (Forouzanfar et al., 2016), or even calyx (Park et al., 2017). Persimmon fruit is a good source of phenolic compound, especially polyphenols (Zillich et al., 2015). In particular, persimmon contains high levels of phenolics, carotenoids, and vitamin C; the latter is considered a powerful antioxidant. Antioxidants play an important role in protecting biomolecules from free radicals, which cause oxidative stress (Giordani et al., 2011). Different kind of strategies are presented to boost antioxidant level inside cells. Beside enzymatic antioxidant that naturally available inside human body, non-enzymatic antioxidant through food supplementation is expected to become additional defense system to counter free radical reaction.

The present study contributes toward the development of a novel delivery system of persimmon extract that has potential health benefits, including protective effects against oxidative stress. In this study, C2C12 cells were used as an *in vitro* model of skeletal muscle to analyze the effect of Japanese persimmon extract on H_2O_2-induced oxidative stress in myoblast cells. Considering that oxidative stress is one of the pathophysiological factors that contribute towards the development of sarcopenia, the protective effect of

persimmon extract against oxidative stress in C2C12 cells was evaluated. Furthermore, many of the recent *in vitro* studies on health claims of plant bioactive compounds have been relatively biased. Potential plant extracts or food compounds may not be able to target the skeletal muscle directly since they are processed by our digestive system and are influenced by various biological reactions before reaching the target tissue. Such processes may change the properties of the tested compound, not only physically, but also chemically (Rein et al., 2012). Therefore, to mimic physiological conditions, we constructed a co-culture system using Caco-2 human intestinal cells as a model of the intestinal epithelium.

II. LITERATURE STUDY

1. Skeletal muscle aging and pathophysiological mechanisms of sarcopenia

The term “sarcopenia” comes from Greek roots: sarx which means “flesh” and penia which means “loss”. Rosenberg proposed this term to describe the phenomenon of age-dependent loss of skeletal muscle mass and strength (Cruz-Jentoft et al., 2010; Rosenberg, 1997). The operational definition of sarcopenia is important, either for clinical practice or research studies, in determining parameters for diagnosis of sarcopenia. The European Working Group on Sarcopenia in Older People (EWGSOP) defines sarcopenia as a degenerative syndrome, characterized by the presence of low muscle mass and low muscle function, both in strength and performance (Cruz-Jentoft et al., 2010). Based on this definition, EWGSOP also established specific parameters to identify sarcopenia and conceptual stages to measure its severity. There are three main variables to use as parameters for the assessment of sarcopenia syndrome: muscle mass, muscle strength, and physical performance. Furthermore, the detailed measurements of these three variables are used as guidelines to categorize each subject’s case as ‘presarcopenia’, ‘sarcopenia’, or ‘severe sarcopenia’(Cruz-Jentoft et al., 2010; Santilli et al., 2014). The picture below is the general illustration of sarcopenia (Figure 1).

Until now, the exact mechanism associated with muscle aging has been poorly understood. The aetiology of sarcopenia is rather complex, since it involves diverse factors, including physiological impairment, changes in physical activity, and nutritional factors. A general illustration of the molecular mechanisms of sarcopenia can be seen on Figure 2. The complexity of factors and their effects on molecular mechanisms, are probable reasons why finding an effective treatment has proved illusive. Nevertheless,

several possible mechanisms have been proposed, to understand sarcopenia and to find the potential therapeutic avenues with the most promise.

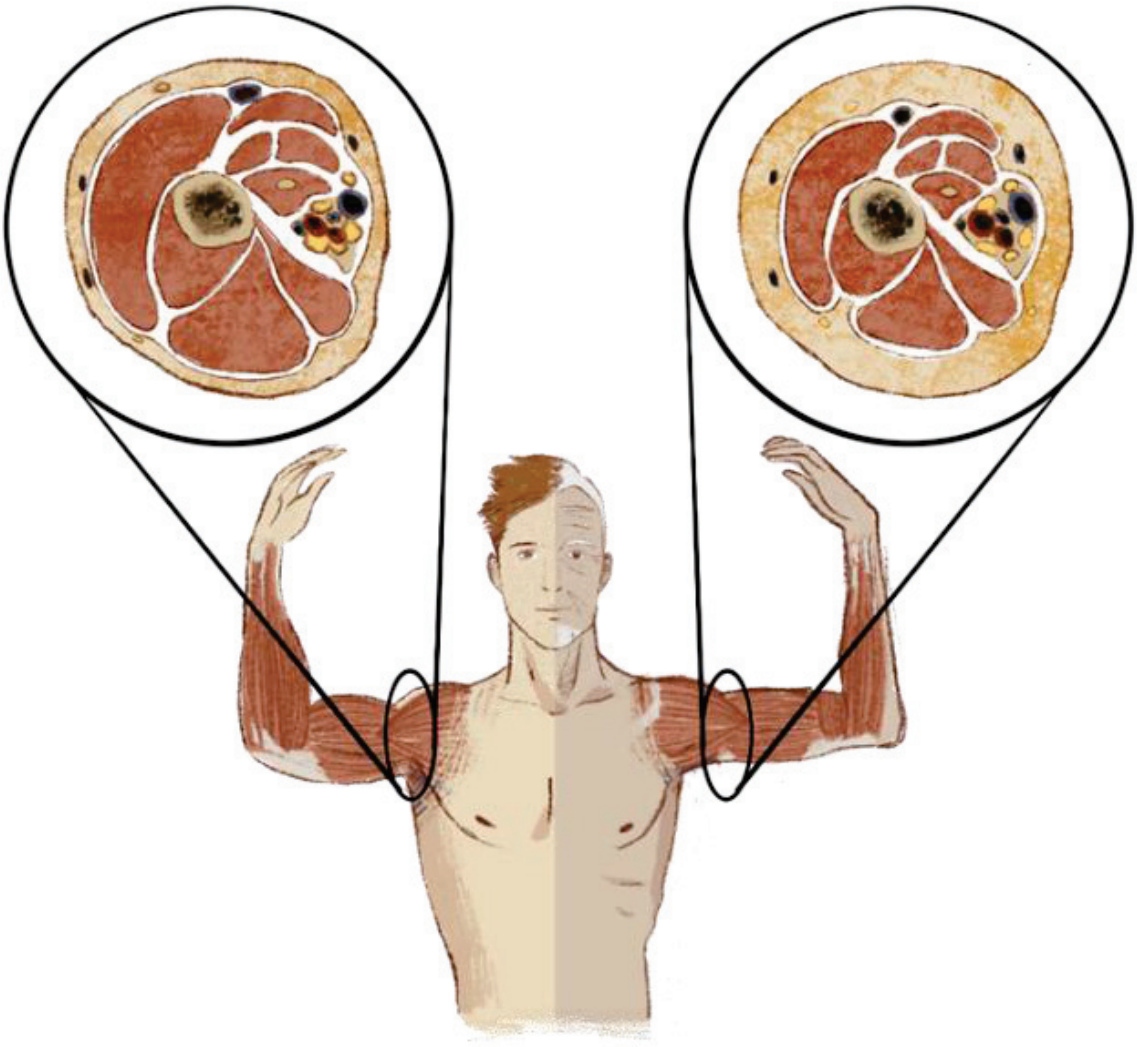

Figure 1. Sarcopenia and Skeletal Muscle Age

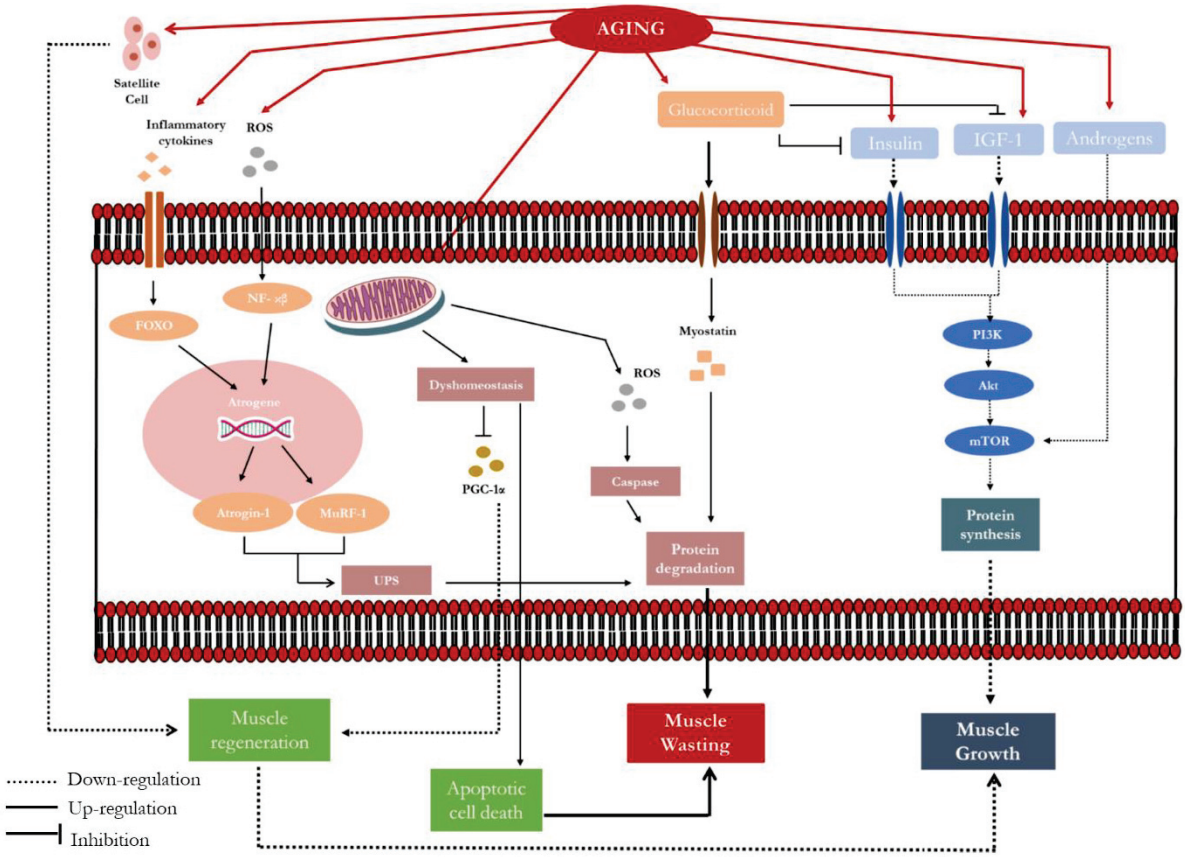

Figure 2. Molecular Mechanism of Sarcopenia (Alfarafisa et al., 2020)

1. Oxidative stress muscle aging

Oxidative stress is an imbalance state between oxidant and antioxidant leves in human body. The bioavailability of Reactive Oxygen Species (ROS) as an oxidant become excessive while antioxidant defenses tend to remain the same or even decrease (Kregel & Zhang, 2007). Normally, ROS play an essential role in activating or inhibiting enzymes that involve in cell survival. ROS can regulate signal transduction, gene expression, and enzyme regulation of the cells (Bouzid et al., 2015). ROS are mainly generated in mitochondria as a by-product of cell respiration (Joseph et al., 2016). ROS are continuously produced in small quantities, therefore could effectively controls by antioxidant mechanism such as superoxide dismutase, catalase, and glutathione peroxidase (Bouzid et al., 2015). Unfortunately in oxidative stress condition, ROS are exaggerated produce and antioxidant mechanism are no longer able to neutralize ROS effect (Brioche & Lemoine-Morel, 2016). High level of ROS can alter or damage macromolecule of the cell such as DNA, proteins, and lipids leading to cell death (Gomes et al., 2017). Together with Reactive Nitrogen Species (RNS), ROS-RNS can induce nitration, nitrosylation, carbonylation, and glycation (Baumann et al., 2016).

The first free radical theory of aging was proposed by Harman (1956). He stated that the damage of macromolecule of the cell caused by free radical species can accumulated within organism, causing deleterious effect. Organism lifespan is closely related with their ability in coping with such deleterious effect (Harman, 1956). Since skeletal muscle consume large quantities of oxygen, this organ also tend to produce large quantities of ROS (Brioche & Lemoine-Morel, 2016). Mitochondria are the main source of ROS production in the cell. Inappropriate electron leakage during aerobic respiration eventually leading to free radical deliverance (Joseph et al., 2016). In general, there are

three factors that believed to be the main contributor in ROS production within mitochondria: mitochondria membrane potential, intracellular Ca^{2+}, and NO. High mitochondrial membrane potential can lead to high production of ROS due to the extended period of electron transport and ubisemoquinone radical occupancy in complex III. On the other hand, Ca^{2+} can increase ROS production trough the stimulation of TCA cycle. While NO production that inhibit complex IV, and dissociation of cytochrome c from inner mitochondrial membrane (Bouzid et al., 2015).

ROS also can can be produced as a response to environmental stimuli such as growth factors, cytokine, radiation, UV, chemical oxidant, chemotherapy, hyperoxia, toxin, and transition metals (Bouzid et al., 2015). According to Meng & Yu (2010), the combination between oxidative stress, chronic inflammation, and mitochondrial dysfunction have a significant role in accelerated skeletal muscle aging through the imbalances between muscle protein synthesis and muscle protein breakdown and apoptosis. These molecular mechanism causing the atrophy and fiber loss in sarcopenic patient.

a. Sarcopenia and altered mitochondrial function

As organelles, mitochondria have a role in providing cellular ATP (McBride et al., 2006), maintaining redox stability (Marzetti et al., 2010), regulating cellular metabolism, organizing the cellular cycle (McBride et al., 2006), and integrating signaling pathways for cellular death (Wang & Youle, 2009). Notably, skeletal muscle and heart muscle contain two different mitochondrial subpopulations: subsarcolemmal mitochondria, which are located beneath the plasma membrane, and intermyofibrillar mitochondria, located between the myofibrils (Marzetti et al., 2010). These two subpopulations differ bioenergetically and structurally (Calvani et al., 2013; Ferreira et al., 2010). In addition, they might contribute differently to the pathogenesis of sarcopenia.

The aging of mitochondria has many consequences for cell function, especially in bioenergetic activity, to produce ATP. When mitochondria age, their capacity to synthesize ATP will decline, both in the resting state and at maximum respiration short (Short et al., 2005). Coen et al. (2013) found there is a strong correlation between reduced mitochondrial capacity to synthesize ATP and low walking speed in older participants, one of the defining criteria of sarcopenia. Beside their age-dependent decline, mitochondria are also major producers of oxidant as well as the major target of oxidative damage. The mtDNA, an important mitochondrial component, is reported to be more vulnerable to oxidative stress, compared with nuclear DNA. Their proximity to oxidants, their lack of histones and introns, and relatively weak repair mechanisms make them prone to defects and dysfunction (Yakes & Van Houten, 1997). The contribution of mtDNA damage to the progression of sarcopenia was demonstrated by Kujoth et al. (2005). They found that mice which express a defective version of the proofreading enzyme, mitochondrial polymerase-γ, accumulate a high load of mtDNA mutation. Mice also expressed a premature aging phenotype, including age-related loss of skeletal muscle. Regardless of their capability to generate macromolecular damage, oxidative stress from mitochondria also can regulate the expression of signaling molecules in catabolic pathways. Reactive oxygen species (ROS) can activate several proteolytic enzymes (Smuder et al., 2010) or modify the elements to make them targets for proteolysis (Grune et al., 2003). In common with sarcopenia, this condition is aggravated by the suppression of muscle protein synthesis (Wu et al., 2010).

In addition, any alteration in mitochondrial fusion and fission events may also contribute to mitochondrial dyshomeostasis, a major cause of muscle atrophy (Calvani et al., 2013). Yoon et al. (2006) stated that the highly interconnected networks found in aged

muscle, with aberrant morphology and enlarged mitochondria, may be caused by an enhancement of fusion activity and or a significant decline in fission activity, along with low autophagic rates. Fusion is an event in which mitochondria can join and their contents become mixed. This event leads to an equal redistribution of metabolites and other important macromolecules, to equilibrate nuclear-encoded proteins in each mitochondrion (Ono et al., 2001). Fission events allow mitochondria to segregate their unnecessary or damaged components, so that autophagic mechanisms can remove them from the system (Twig et al., 2008).

Another mitochondria-related mechanism that may contribute to the sarcopenic condition is the altered function of mitochondrial renewal. Mitochondrial turnover is regulated by the balance between autophagy and biogenesis (Marzetti et al., 2010). During autophagic events, dysfunctional and redundant mitochondria will be degraded, while replacement organelles will be synthesized through biogenesis events. If these coordinated processes are hampered, specifically in muscle cells, mitochondrial function and myocyte homeostasis will also be disturbed. The potential link between mitochondrial turnover and muscle wasting is through peroxisome proliferator activated receptor-γ coactivator1-α (PGC-1α). Koltai et al. (2012) found that the expression of PGC-1α in old muscle of Wistar rats can indicate a decrease in mitochondriogenesis. In 2006, Sandri et al. published that the disruption of PGC-1α signaling can escalate muscle protein degradation, via the ubiquitin-proteasome system, coordinated by FoxO3, causing severe muscular atrophy.

b. Elevated low-grade systemic inflammation

Chronic inflammation in the elderly has long been closely associated with aspects of frailty, including sarcopenia (Allen, 2017). The mechanism is clearly complex, since it

involves a number of biochemical reactions and physiological functions that interact with each other. In general, the immune system has an important role in muscle growth and regeneration, in acute or chronic conditions (Sun et al., 2009; Tidball, 2017). During aging, this important role tends to become aberrant and eventually drives the development of degenerative dysfunction of skeletal muscle (Jo et al., 2012). As a result, negative outcomes, such as skeletal muscle regenerative failure (Mourkioti & Rosenthal, 2005), muscle protein turnover impairment (Toth et al., 2005), and apoptosis activation (Dirks & Leeuwenburgh, 2006) are more likely to happen in the elderly. These three factors are also suggested to be the main mechanisms of inflammation-induced muscle wasting (Jo et al., 2012).

When a person experiences inflammaging, a chronic low-grade inflammation that develops with advanced age, then pro-inflammatory cytokines (IL-1β, IL-6), tissue necrosis factor (TNF-α), and C-reactive protein (CRP) will increase by 2-4 fold, while the serum levels of anti-inflammatory cytokines (IL-10, IL-1ra) are reduced (Wilson et al., 2017). Several studies have investigated the effects of excessive inflammaging. There is a strong correlation between high levels of IL-6 and CRP in serum and the loss of muscle strength (Schaap et al., 2006). IL-6 elevation can cause muscle degradation by activating lysosomal and non-lysosomal protease pathways in C2C12 cells (Ebisui et al., 1995), can induce ubiquitin expression in cachexia patients (Dejong et al., 2005), and promotes E3 ligase expression in a mouse cachexia model (White et al., 2012). Pro-inflammatory cytokines also upregulate the proteolytic pathway by activating the FoxO3a signaling pathway (Xia et al., 2017). In addition, chronically elevated IL-6 levels can influence glucose homeostasis, leading to insulin-stimulated glucose uptake impairment (Febbraio et al., 2004; Franckhauser et al., 2008). On the other hand, TNF-α can stimulate

muscle wasting by increasing the ubiquitin/proteasome pathway, via NF-κB activation. To activate NF-κB, the TNF-α signal will stimulate type 1 TNF-α receptors. Consequently, this step will increase ROS production, via mitochondrial electron transport. ROS then acts as a second messenger to activate NF-κB, directly or indirectly (Reid & Li, 2001). TNF-α, together with insulin-like growth factor I (IGF-1)/Akt, also controls the expression of two main factors in the proteolysis of skeletal muscle, Atrogin and MuRF1 (Clavel et al., 2006). The innate immune system is also found to contribute, indirectly, to the development of sarcopenia. This fact was considered after Leng et al. (2009) confirmed that high neutrophil and monocyte levels, in disabled older women, were associated with frailty status. Neutrophils, known for their chemotactic ability, lose their efficiency with aging. This may cause undesirable tissue defects and secondary inflammation. Neutrophil activity in injured muscle causes secondary damage to surrounding healthy muscle, such as myocyte apoptosis, muscle fiber degradation, and inflammatory enhancement (Wilson et al., 2017). Although it is still unclear how impairment of the innate immune system can directly affect skeletal muscle health, the mechanisms outlined above may provide an explanation of the contribution of leukocytes to sarcopenia.

c. Oxidative stress and sarcopenia

There are several mechanisms that may link oxidative stress and sarcopenia development. Several papers stated that oxidative stress can disturb satellite cell activity to repair damaged muscle fibers by creating unfavorable environment. As mentioned in recent paragraph, oxidative stress can cause lipid peroxidation that proofed to be linked with the reduction of myoblast fusion capacity (Beccafico et al., 2007; Fulle et al., 2004), deter the regenerative ability of skeletal muscle.

Other way, oxidative stress can depress the muscle protein synthesis while intensify muscle protein degradation. First, oxidative stress can change redox status environment and induce the expression of UPS inducer or UPS effector. Second, oxidative stress can escalate the activity of calpains, protease that degrade muscle protein. This event usually marked with the enhancement of H_2O_2 production. Third, oxidative stress also can overload the level of cytosolic calcium. The disturbance of calcium homeostasis usually mediated by the formation of reactive aldehydes, which eventually the activity of Ca^{2+} ATPase. As above mentioned, Ca^{2+} escalation may also enhance the production of ROS itself, creating the worsen cycle of oxidative damage and protein muscle degradation. In addition, ROS can also directly oxidize skeletal muscle protein as they also oxidize others important macromolecule of the cell (Brioche & Lemoine-Morel, 2016).

2. Non-enzyme Antioxidant from Plant Bioactive Compound

Antioxidant play an important role in protecting substantial biomolecules in human body from free radical that can cause oxidative stress (Giordani, et al., 2011). Nowadays, scientist believes that oxidative stress is a major cause of many degenerative disease like atherosclerosis, cancer, diabetics, rheumatoid arthritis, post-ischemic perfusion injury, myocardial infarction, cardiovascular diseases, chronic inflammation, stroke and septic shock, and aging (Fang et al., 2002). Synthetic antioxidants indeed present higher stability and performance, low cost, and high availability compared with natural antioxidants. However, the safety issues were gradually rise since several paper stated that long term intake may increase the incident of several health problem. Therefore, plant with potential bioactive component as antioxidative agent considered beneficial and had focusing research attention to satisfy the demands for exclusively natural origin antioxidant product (Lourenço et al., 2019).

Natural antioxidant mostly comes from fruit, vegetables, herbs, spices, and other plant materials. According to its bioactive compound, natural antioxidant from plants can be classified into three main groups: phenolic, vitamins, and carotenoids. Another classification of natural antioxidant can be seen on Figure 3 below. Though natural origin compounds relatively have lower risk compared with synthetic compound, toxicity studies are still needed to define its consumption condition. Natural compound must be met several requirements such as did not have negative effect on color, odor, and flavor; showed effective effect at relatively low concentration (0.001-0.01%); compatible with foods and convenient; stable; low cost; possess LD_{50} values lower than 1000mg/kg body weight; and showed no harmful effect in long-term consumption (Lourenço et al., 2019). The quality of natural antioxidant depends on the quality of source and extraction method. Extraction using organic solvents is the most common chosen method. Only with optimum solvent and extraction method, an optimum extract can be obtains: maximum amount of targeted bioactive compound with low degradation degree and minimum non antioxidant substance (Chandrasekhar et al., 2012).

Most of the natural antioxidants work as compound that trap free radical species. Non enzymatic antioxidant will trap the ROS, donating their electrons, turn the ROS into more stable compound. Non enzymatic antioxidant eventually will become free radical due to electron transfer, but this free radical is less reactive and easily to neutralize compare with the initial one (Sharma et al., 2012)fr. Natural based antioxidant also prevent lipid oxidation caused by oxidative stress, convert the oxidized lipid into more stable compound and breaking the harmful chemical chain (Anwar et al., 2018).

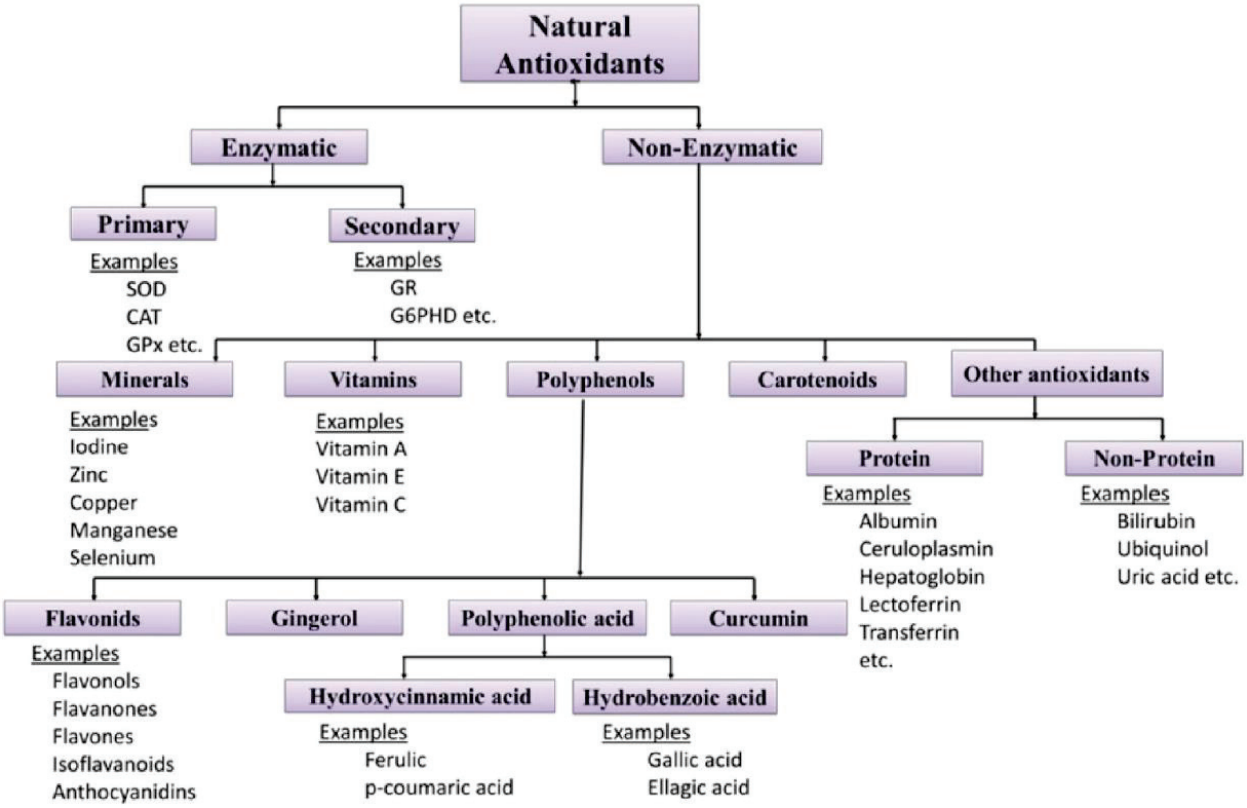

Figure 3. Natural Antioxidant Classification (Anwar et al., 2018)

3. Japanese persimmon antioxidant potential

Persimmon (*Diospyros kaki*) is one of a well-known and highly distributed fruits around the world, especially among east asian country. This fruit is a native crop from China. History showed that China society had start to cultivate this fruit even before Christ century. After 7th century, the cultivar began to spread to Japan and reached Korea at 11th century. In the middle ages, Persimmon had well spread to many other country, even to another continent (Heras et al., 2017) such as southern european country (Kashif et al., 2017). Taxonomically, *Diospyros kaki* or persimmon belongs to family Ebenaceae and genus Diospyros (Syn: Persimmon, ebony) (Xie et al., 2015). Genus *Diospyros* itself had more than 249 species and considered as the most important plant genus, both numerically and economically. Among these species, *Diospyros kaki* was reported as the best fruit yielding species (Mallavadhani et al., 1998).

Persimmon is a climacteric plant, so the ripening of its fruit was regulated by ethylene. During climacteric phase, rapid softening occurs and resulting a jelly-like flesh. The eating quality of persimmon is considered best at the end of pre-climacteric stage. At that stage, persimmon owing the maximum sugars content and desired color (Butt et al., 2015). Depending on the genotype, some fruits had an astringent flavor before reached a completely mature stage. The astringency of persimmon is associated with the amount of accumulated tannins, tannin characteristics, and the ability of seeds to produce volatile compound (Yaqub et al., 2016). In general, this astringent persimmon accumulate an amount of one type of tannins named proanthocyanidins (PAs) in their flesh during development, which causes the sensation of astringency due to coagulation of oral proteins (Denev & Yordanov, 2013).

Unique astringency characteristic and the ability of seeds to produce volatile compound usually became the basis for persimmon cultivar type division. According to Yaqub et al., (2016), persimmon can be classified into four main types based on their mode of astringency loss: Pollination Constant Astringent (PCA), Pollination Constant Non-Astringent (PCNA), Pollination Variant Astringent (PVA), and Pollination Variant Non-Astringent (PVNA). The fruit also classified into two main groups based on the formation of volatile compound (such as ethanol and its derivate), there are: Volatile Independent Group (VIG) and Volatile Dependent Group (VDG). VIG comprises PCNA cultivars, while VDG comprises PCA, PVA, and PVNA cultivars. VIG type proanthocyanidins are usually water insoluble with high molecular weights. The accumulation of soluble proanthocyanidins will finishes at early stage of fruit development, so that the fruit had non-astringent characteristics. Meanwhile, the VDG cultivars show a higher concentration of water soluble proanthocyanidins with low molecular weight, even in the final stage of maturation, causing the fruits had an astringent characteristic. In PCA and PVA cultivars, seeds only produce low amount of volatile compound that cannot completely insolubilises water soluble proanthocyanidins. Even after reached maturation stage, the level of fruit astringency still inedible. On the other hand, volatile compounds in PVNA cultivars is high enough to insolubilises water soluble proanthocyanidins after maturation stage, so the fruits are edible at rippening time (Denev & Yordanov, 2013).

Persimmon fruit is a good source of phenolic compound, especially polyphenols (Zillich, et al., 2015). Some particular components are also prevalent in persimmon fruit such as proanthocyanidins (Jung et al., 2005), flavonoid oligomers, tannins, phenolic acids, and cathecins (J. H. Lee et al., 2012). The other study categorized persimmon

phenols according to their absorption patterns in digestive tract, there are water soluble / extractable (EPP) phenols and water insoluble / non-extractable (NEPP) phenols (Butt et al., 2015). In general, among the phenolic compounds, the most prominent substances are caffeic acid, p-coumaric acid, ferulic acid, and gallic acid (Lee, Y. A. et al., 2008). The major phenolic acid founds in persimmon fruit pulp are ferulic acid, p-coumaric acid, and gallic acid (Yaqub, et al., 2016).

The chemical structure of persimmon's phenolic acids is a vital factor that affected the radical-scavenging and metal chelating activities (Tsao, 2010). The numbers and position of hydroxil groups with respect to the carboxyl functional group will determine antioxidant activity level of phenolic acids (Bei et al., 2005). Phenolic acids had a potential role in preventing oxidative stress damage and scavenging oxidative stressor, such as reactive oxygen species (ROS) (Zhou et al., 2016) or lipid peroxidase (Toschi et al., 2000). In a recent report, 32 low molecular weight phenolic acids have been found on persimmon fruit, there are: gallic acid, glycosides of p-coumaric, vanillic and cinnamic acids and different flavone di-C-hexosides, catechin, epicatechin, epigallocatechin, chlorogenic acid, caffeic acid (Chen et al., 2008), ferulic acid, tannic acid, protocatechuic acid, vanillic acid, epicatechin gallate, catechin gallate (Lee, J. H. et al., 2012). Research showed that gallic acid had the highest antioxidant activity compare to the other phenolic acids in persimmon fruits (Kashif et al., 2017).

Due to its diverse bioactive compound and antioxidant potential, persimmon has long been used for medicinal purposes in various way. Several experiments showed that persimmon may exert chemoprtotective activity, reduce the incident of stroke-induced haemorrhage, protective effect against genotoxic oxidative, and protective effect against epileptic episode (Giordani et al., 2011). It may also possess anti-diabetic affect, lowering

high blood pressure, and lowering LDL level (George & Redpath, 2008). It is worth to study another potential effect of persimmon and its efficacy in a way to develop a new functional food product.

4. Co-culture system for evaluating potential bioactive compound *In vitro*

Pharmacological, toxicological, and substance bioavailability analysis using *in vitro* methods are still frequently choose since for its simplicity and relatively low cost (Kämpfer et al., 2017). Science development had replaced the use of monoculture system with co-culture system which taught to be more represent the multicellularity of human composition. Co-culture system allow researcher to deeper the study of phytochemical compound effect and cellular communication (Gonzales et al., 2015). In general, co-culture is a cell cultivation set-up, in which two or more different populations of cells are grown with some degree of contact between them (Goers et al., 2014). Recently, the studies on natural bioactive compound and its interaction effect gain awareness due to the possible threats that may pose in human body. Pharmacokinetics researcher also consider the absorption mechanism, bioavailability, and possible metabolism of tested bioactive compound by mimicking the physiological condition of human body (Awortwe et al., 2014).

Absorption of plant-based supplement mainly occur at duodenum and jejenum segments of small intestine. Enterocyte of the epithelial cells of intestine are responsible for the absorption through passive diffusion or carrier mediated mechanism (Awortwe et al., 2014). Epithelial cell also responsible for the intrusion of harmful agent through tight junction mechanism (Kämpfer et al., 2017).

Caco-2 cells offer the transport and permeability properties of human intestinal tissue, therefore this cell line is extensively in cell-based model for bioactive compound

assessment. Caco-2 cell can spontaneously differentiated into human epithelial cell once they reached confluency, developing brush border anatomy, tight junctions, and uptake-efflux transporter mechanism (Awortwe et al., 2014). Below are several types of co-culture system in general that may use in testing bioactive compound *in vitro* (Figure 4).

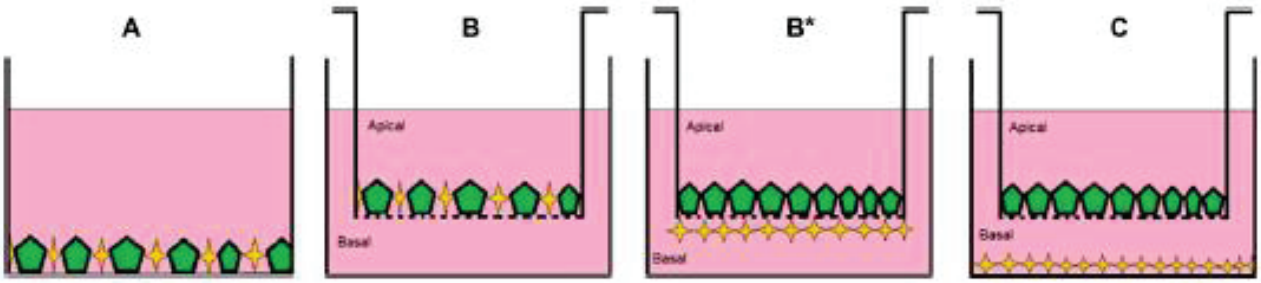

Figure 4. Several type of Co-culture Systems: Contact System (A); Transwell Contact System (B and B*); Non-contact System (C) (Gonzales et al., 2015).

III. MATERIALS AND METHODS

1. Sample preparation and Chemical Reagents

Diospyros kaki Nishimura cultivars were provided by Gifu Prefectural Agricultural Technology Center (Gifu, Japan). The samples were kept at -80°C until it was ready to process. The samples were washed and dried using lyophilization. After removing the calyx and seeds, the samples were weighed and their dry weight was recorded. The samples were powdered using a mortar to increase their surface area and improve extraction efficiency.

Trolox and (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Sigma-Aldrich, St. Louis, USA) were used as a standard for measuring antioxidant activity. Hydrogen peroxide was used to induced oxidative stress (Wako Pure Chemical Industries, Osaka, Japan). 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Wako Pure Chemical Industries, Osaka, Japan) was used as a stable free radical which turns yellow when scavenged. Catechin, fluorescein sodium salt (FL), 2,2-azobis-2-methyl-propanimidamide dihydrochloride (AAPH) was obtained from Wako Pure Chemical Industries (Osaka, Japan). Millicell ERS-2 chopstick Silver/SilverChloride electrodes for measuring TEER was purchased from Merck KGaA (Darmstadt, Germany). Cell Counting Kit-8 (CCK-8) were purchased from Dojindo Molecular Technologies Inc. (Kumamoto, Japan). DCFDA/H2DCFDA-Cellular ROS Assay Kit was purchased from Abcam (Cambridge, UK). Dulbecco's modified Eagle's medium (DMEM) powder, high glucose from Gibco (Thermo Fisher Scientific, Waltham, USA) was used for culturing Caco-2 cell, while autoclavable powdered DMEM from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan) was used for culturing C2C12. Fetal Bovine Serum (Biosera, Kansas City, USA), Nonessential Amino Acids solution (NEAA), L-glutamine, and Penicillin-streptomycin

from Nacalai Tesque Inc. (Kyoto, Japan) were used for making cell culture growth medium.

2. Extraction method

Japanese persimmon was extracted using the hot ethanol method (Oosthuizen et al., 2018). The powdered samples (42.5 g) were extracted in 420 mL of 99 % ethanol for 30 min under boiling conditions. After three cycles of extraction, the obtained product was mixed and filtered using a Buchner funnel. The solvents were vaporized using a rotary evaporator and dried by lyophilization. All crude extracts were stored at –30 ºC until further analysis.

3. Fractionation method

Japanese persimmon crude extract was fractionated using different concentrations of ethanol (25 %, 50 %, and 75 %). The extracts (5 g) were dissolved in 50 mL of each solvent and homogenized using a magnetic stirrer for 1 h at room temperature. When the crude extract was completely dissolved in ethanol solvent, the samples were fractionated again using butanol solvent (1:1) until two different phases were obtained. The solvent was vaporized using a rotary evaporator and dried by lyophilization. Each fraction product was weighed and recorded as the yield. Below are the flow charts that generally describe the extraction and fractionation method of Japanese persimmon in this study (Figure 4)

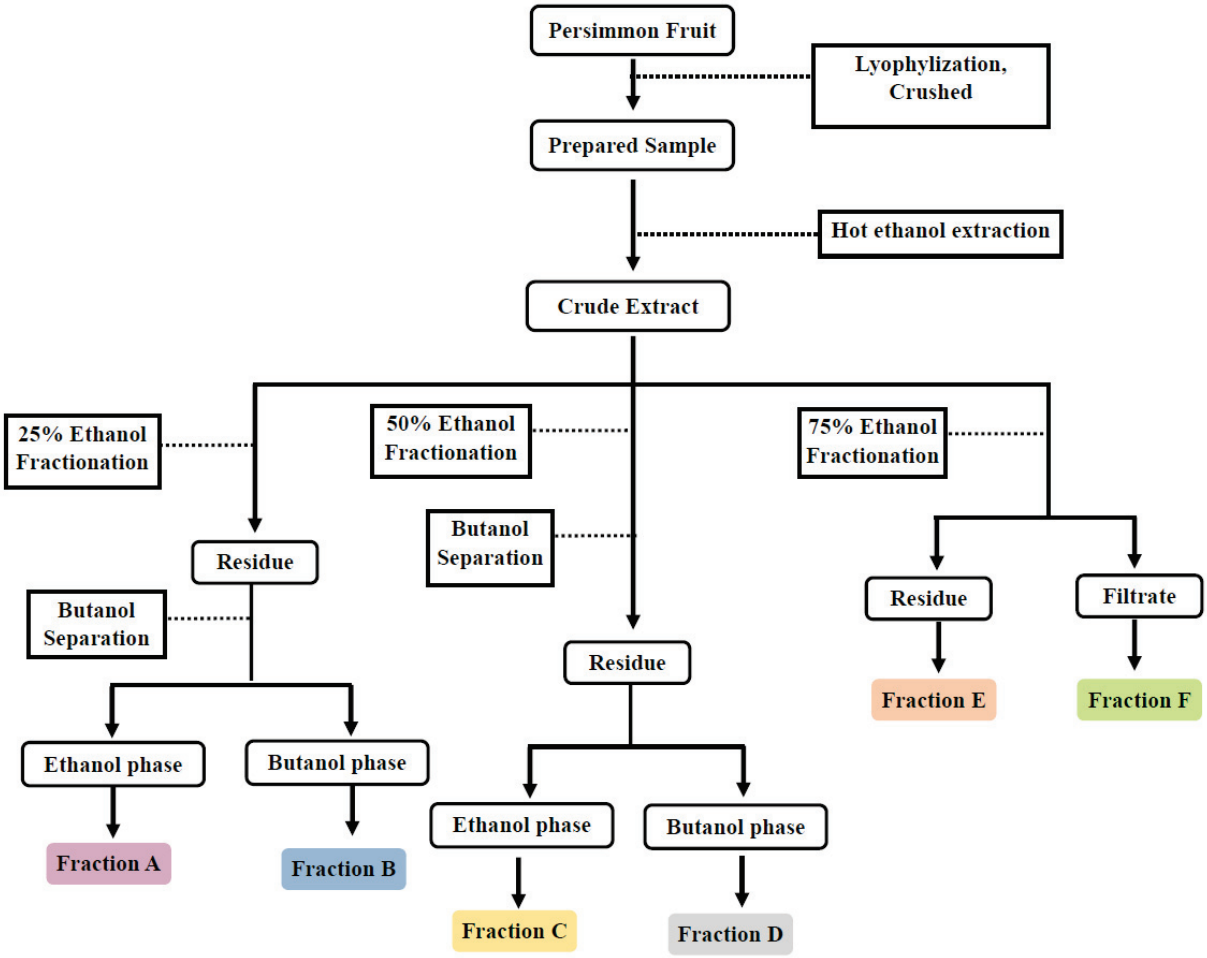

Figure 4. Persimmon Fruit Fraction-Extraction Flow Chart

4. Determination of antioxidant activity *in vitro*

a. DPPH (α, α-Diphenyl-β-picrylhydrazyl) free radical scavenging activity

The free radical scavenging activity of the extract was measured in terms of hydrogen donating or radical scavenging ability using the stable free radical DPPH as describe by (Akter et al., 2010) with some modifications. Antioxidant compound will donate its hydrogen atom to neutralize electron of nitrogen atom in DPPH. This event is marked by the decolorization of violet color which usually have an absorption at around 520nm in ethanol solution (Kedare & Singh, 2011). Gradient concentrations (3-11 mg/mL) of Japanese persimmon fraction solutions in water were prepared. On the other hand, Trolox solution was prepared by dissolving Trolox in ethanol. Gradient concentrations of Trolox were used as a standard. Trolox solutions and 30 µL of Japanese persimmon fraction were added to a 96-well plate, followed by 240 µL of methanol. Water was used as the blank. Solution of 1 mM DPPH in ethanol was prepared and added to each well of the samples. After 30 min of incubation in the dark, the absorbance was measured at 540 nm. The assay was performed in triplicate. The percentage of inhibition of free radical DPPH was calculated by using equation bellows. *Ab* was the absorbance of the blank and *As* was the absorbance of the sample. The amount of sample used to reduce the absorbance of DPPH by 50 % (IC_{50}) was also subtracted.

$$\text{DPPH Scavenging Activity (\%)} = \frac{(Ab - As)}{Ab} \text{ x } 100$$

b. ORAC (Oxygen Radical Absorbance Capacity) assay

The oxygen radical absorbance capacity (ORAC) assay was performed according to Ou et al. (2001) with some modifications. In principle, this method measured the antioxidant-mediated protection of the fluorescent protein (fluorescein) from free radical

damage (Sueishi et al., 2011). Different concentration of Japanese persimmon fraction solutions in water, blank, and trolox standard solutions (50 µL) were added to a 96-well plate. Then, 50 µL of fluorescein was added. The plate was incubated in the dark at 37 ^{o}C. After 15 min, 25 µL of AAPH was added to each well. The fluorescence intensity was monitored every 5 min for 90 min using a microplate reader at an excitation of 485 nm and an emission of 535 nm at an incubation temperature of 37 ºC. The assay was performed in triplicate. The ORAC value denotes the net area under the curve (AUC) of the fluorescence decay of the standard or sample minus the AUC of the blank

5. Total phenolic assay

The total phenolic content of the extracts was determined using the Folin–Denis method as describe by (Araujo et al., 2013). In general, Folin-Denis have the same principle with Folin-Ciocalteu method. The only difference is the addition of lithium sulfate in Folin-Ciocalteu reagent to reduce the formation of lithium salts. Phenolic compound will cause Folin reagent reduction and resulting in the production of blue color molybdenum-tungsten that can be detected at 760nm. The intensity of blue color is linearly correlate with the concentration of tested phenolic compound (Malta & Liu, 2014). Briefly, 1 mL of the diluted sample extract was transferred to reaction tubes containing 1 mL of Folin-Denis reagent. After 3 min, 1 mL of sodium carbonate solution was added to the mixture. The tubes were then allowed to stand at room temperature for 1 hour before measuring the absorbance at 760 nm. The total phenolic content of the extracts was calculated as catechin equivalents.

6. Biological antioxidant activity in C2C12 myoblast cell culture against oxidative stress

a. C2C12 mouse myoblast cell line

Mouse myoblast cell line C2C12 cells (Riken BRC Cell Bank, Tsukuba, Japan) was maintained in growth medium consisting of DMEM supplemented with 10 % fetal bovine serum (FBS), 1 % penicillin-streptomycin, and 2 % L-glutamine. 8 % $NaHCO_3$ were used as buffer for maintaining medium pH. The cells were grown on 100 mm cell culture dishes and kept at 37 °C in a 5 % CO_2 incubator. The cells were maintained at 70 % confluency. After exceeding 70 %, the cells were harvested with trypsin/EDTA and placed into new vessels.

b. Human enterocyte-like cell line Caco-2 cell

The human enterocyte-like cell line Caco-2 cells (American Type Culture Collection, Manassas, USA) were also maintained in DMEM supplemented with 10 % FBS, 1 % penicillin-streptomycin, and 1 % NEAA. The complete medium was sterilized using a membrane filter. Cells were maintained at 70 % confluency. After exceeding 70 %, the cells were harvested with trypsin/EDTA and placed into new vessels.

c. Co-culture system mimicking healthy human intestine

For the co-culture experiment, 0.3×10^6 Caco-2 cells were cultured on 0.4-μm semipermeable support membranes in 6-well cell culture inserts. After fully differentiating into integrated cell monolayers (21 days), samples were applied to the apical side and then the medium on the basolateral side was isolated. The medium was stored at -20°C for further analysis. Below are the schematics figure of co-culture system in this study (Figure 5)

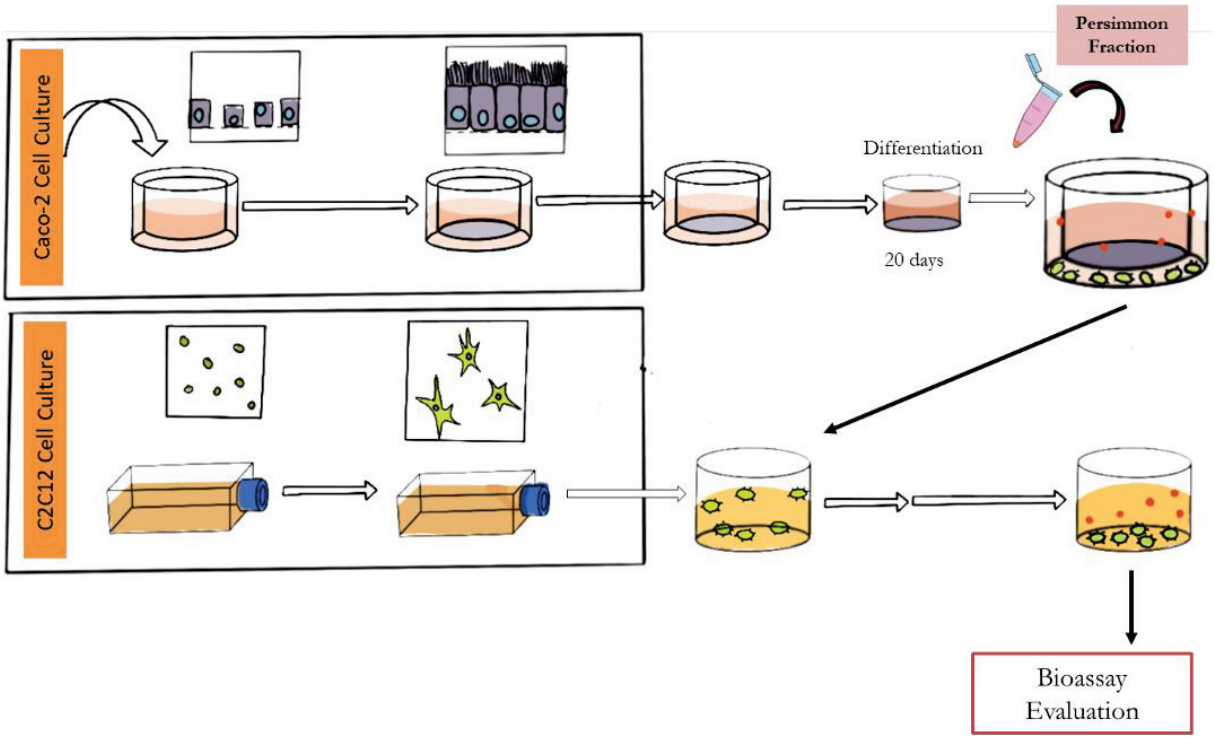

Figure 5. Co-culture system of C2C12 and Caco-2 cell culture

d. Monitoring barrier integrity by TEER

Trans-endothelial electrical resistance (TEER) was measured in Transwell inserts with Millicell ERS-2 chopstick Silver/SilverChloride (Ag/AgCl) electrodes (Ferraretto et al., 2018). After preparing and sterilizing a Millicell chopstick, the electrical resistance from each well of the co-culture was measured at three different locations. The average resistance was calculated, and the final TEER values (Ω) were by subtracting the read-out value from the cell and the baseline measurement in the absence of cells (Rblank) as shown below:

$$TEER = R - R_{blank}$$

e. Induction of oxidative stress

Oxidative stress was induced by 1mM H_2O_2 in DMEM without FBS. After 24 h pretreatment with basolateral medium from the co-culture system, 1×10^4 of C2C12 cells in a flat transparent 96-well plate for measuring C2C12 cell viability and 2.5×10^4 of C2C12 cells in a 96-well plate with a dark clear bottom for measuring intracellular oxidative stress were induced with H_2O_2 solution for another 6 h. Cell viability and intracellular ROS were quantified as a percentage against the control.

f. Measurement of C2C12 cell viability

Tetrazolium reagent WST-8, which was utilized in the CCK-8 kit (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt) (Dojindo), was used to evaluate the cell viability and cytotoxicity as describe by (Cai et al., 2018). WST-8 solution (5 %) in complete medium was added to the culture. After 2 h of incubation, the absorbance was measured at 450 nm.

g. Measurement of intracellular oxidative stress

The production of intracellular ROS was quantified using the oxidation-sensitive fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA). After treatment, the cells were washed with 1 × buffer, and then incubated with 10 μM H2DCFDA for 45 min under culture conditions in the dark. The fluorescence intensity was measured using a microplate reader at Ex/Em = 485/535.

7. Statistical analysis

All results are expressed as mean ± standard deviation (SD). Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test. IBM SPSS Statistics version 24.0 (IBM Corp., Armonk, USA) was used as software for statistical processing. Differences between means were considered significant at $P < 0.05$.

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IV. RESULTS

1. Japanese persimmon extraction and fractionation

Table 1 showed an extraction yield of hot ethanol extraction from Japanese persimmon. Using hot ethanol extraction, 27.8 g of the crude extract was obtained from 42.5 g of dry sample of Japanese persimmon with a yield of 65.3 %. Theoretically, the quantity of extractive yield is directly affected by the type of extraction methods/solvents and their extraction efficiency (Sharma & Cannoo, 2016). In this research, three cycles were conducted for each extraction step. After the extraction step, the crude extract of Japanese persimmon was fractionated.

Table 2, 3, and 4 showed the total yield of fractionation from 5 g of Japanese persimmon crude extract. Six different fractions were obtained from the Japanese persimmon crude extract: ethanol (A) and butanol (B) phase from 25 % ethanol fractionation, ethanol (C) and butanol (D) phase from 50 % ethanol fractionation, ethanol (E) and filtrate (F) phase from 75 % ethanol fractionation. Butanol separation was conducted using 25 % and 50 % ethanol solvent to completely dilute the Japanese persimmon extract. Therefore, an additional step was necessary to separate the Japanese persimmon bioactive compound based on its polarity. In addition, 75 % ethanol solvent was used to successfully separate the Japanese persimmon crude extract into two different phases: the ethanol phase or the diluted compound and the filtrate phase. Each fraction showed a different yield percentage from 5 g of Japanese persimmon crude extract sample. The physiological analysis of each fraction was conducted to evaluate the activity of the bioactive compounds. Fractions with robust physiological activity were selected for further biological analysis. The quantitative assay consisted of *in vitro* antioxidant evaluation and total phenolic content. According to Moharram & Youssef (2014), there

are two common types of *in vitro* antioxidant activity evaluation: (1) the reaction of redox type and (2) thermodynamic competition of peroxyl radicals between antioxidant compounds and substrates.

Table 1. Japanese persimmon extraction yield

Dry Sample (g)	Crude Extract (g)	Yield (%)
42.5	27.8	65.3

Table 2. Total yield from 25% ethanol fractionation and butanol separation

Crude Extract (g)	A phase (g)	Yield of A phase (%)	B Phase (g)	Yield of B phase (%)
5.00	1.75	35.00	2.85	57

Table 3. Total yield from 50% ethanol fractionation and butanol separation

Crude Extract (g)	C phase (g)	Yield of C phase (%)	D Phase (g)	Yield of D phase (%)
5.00	3.53	70.60	0.92	18.40

Table 4. Total yield from 75% ethanol fractionation

Crude Extract (g)	E phase (g)	Yield of E phase (%)	F Phase (g)	Yield of F phase (%)
5.00	4.30	86.00	0.64	12.80

2. DPPH IC_{50} values of Japanese persimmon fractions

Figure 6 showed IC_{50} values of each fraction of the Japanese persimmon. The DPPH radical scavenging assay was used to assess the antioxidant potential of each fraction. The degree of reduction was based on the discoloration of the DPPH compound and used to evaluate the antioxidant potential. Overall, the fractions exhibited DPPH radical scavenging activity to different degrees. The concentrations ranged from 3 to 19mg/mL. In this research, DPPH IC_{50} value was used to simplify the result of DPPH radical scavenging assay result (Figure 3.1). The DPPH IC_{50} value is the concentration at which an antioxidant reaches 50 % of DPPH free radical scavenging activity. Therefore, the fraction with the lowest IC_{50} concentration has the strongest antioxidant activity among others. In Figure 3.1, the F fraction possessed the strongest antioxidant activity (1.76 mg/mL), followed by D (3.83 mg/mL), then B (6.32 mg/mL) and E (5.96 mg/mL), and finally C (15.33 mg/mL). Meanwhile, the A fraction showed the lowest radical scavenging activity, with IC_{50} values greater than 19 mg/mL (26.21 mg/mL).

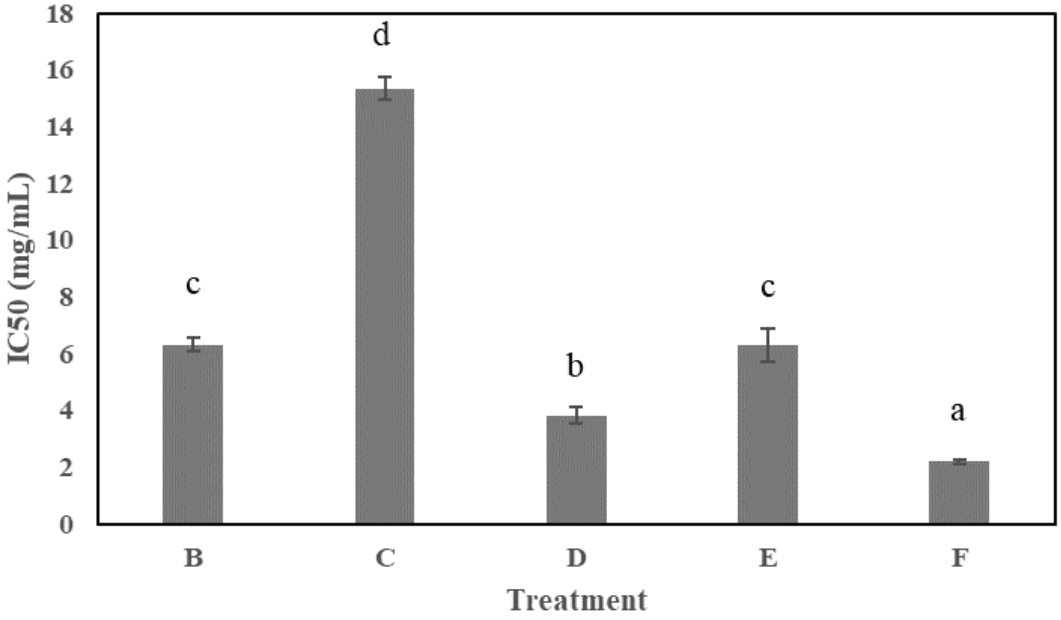

Figure 6. IC_{50} (mg/mL) values of the persimmon fractions for DPPH radical scavenging activity. Values denote the mean ± SD (n=7/group). Statistically significant differences between groups were determined by Tukey's test. Different letters indicate statistically significant differences between groups.

3. ORAC values of Japanese persimmon fractions

The ORAC values of the Japanese persimmon fractions are shown in Figure 3. 2. Compared with DPPH, ORAC measures the degree of inhibition of peroxy-radical-induced oxidation by the compounds of interest in a chemical milieu. Concentrations of 0.1-1 mg/mL for each fraction were used. The ORAC values were expressed relative to the Trolox Equivalent (TE), since Trolox is the conventional standard for the ORAC assay (Sueishi et al., 2011). ORAC value showed a slightly different pattern compared to the DPPH results (Figure 7). There were no significant differences between fractions B (6160 μmol TE/100 g), D (7150 μmol TE/100 g), E (7140 μmol TE/100 g), and F (8010 μmol TE/100 g). On the other hand, fractions A (2730 μmol TE/100 g) and C (3510 μmol TE/100 g) were significantly lower. Overall, the hydrophobic fraction had a better ORAC value than the hydrophilic fraction, except for the E fraction, which had a value comparable to the other hydrophobic fractions.

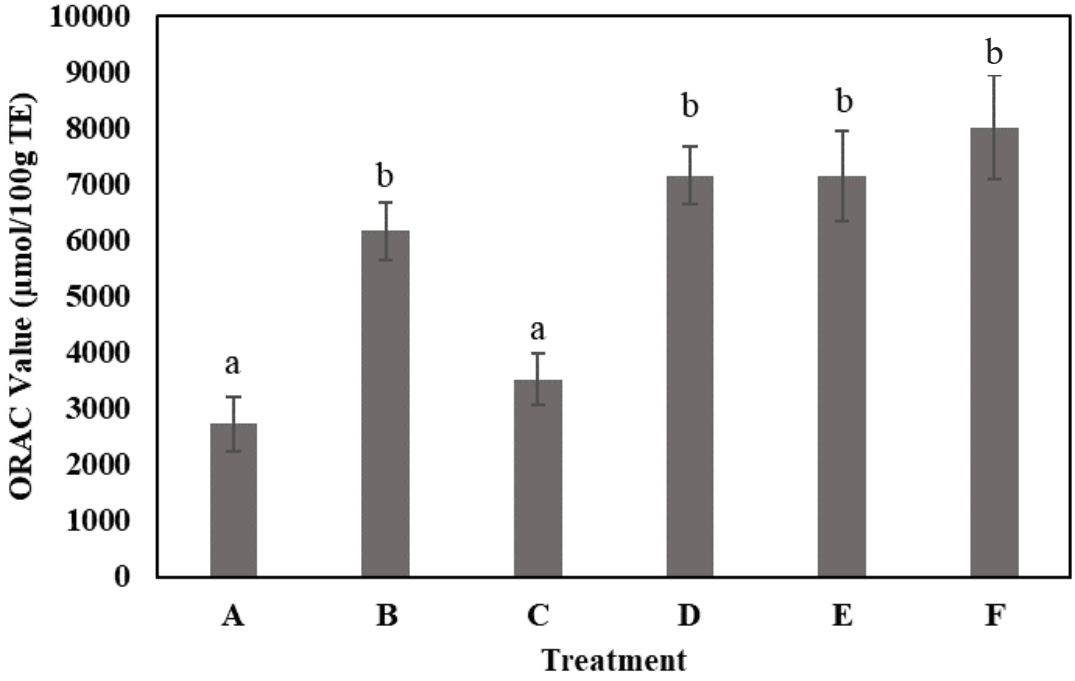

Figure 7. ORAC values (µmol/100 g TE) of persimmon fractions. Values denote the mean ± SD (n=4/group). Statistically significant differences between groups were determined by Tukey's test. Different letters indicate statistically significant differences between groups.

4. Total phenolic content of Japanese persimmon fractions

Phenolic compound believed to be the major components of plant compounds and act as primary antioxidants (Chen et al., 2008). The total phenolic content provides an insight into the antioxidant capability of plant extract compounds. The total phenolic content (TPC) of the persimmon fraction can be seen in Figure 8, and the results were expressed as the catechin equivalent (CE). Fraction F had the highest phenolic content (3.87 g CE/100g) followed by D (1.10 g CE/100g), B (0.75g CE/100g), and E (0.84 g CE/100g). Fractions A (0.22 g CE/100g) and C (0.39g CE/100g) had a relatively lower total phenolic content. However, the total phenolic content of fraction F was much higher than that of the other. According to ORAC analysis, the antioxidant activity of the F fraction was not significantly different from that of fractions B, D, and E.

In general, the hydrophobic fractions (B, D, F) from Japanese persimmon tended to have a stronger antioxidant activity than the hydrophilic fractions (A and C). On the other hand, fraction E always showed the highest antioxidant activity and phenolic content among the hydrophilic fractions, and was comparable to that of the hydrophobic fractions Some reports revealed a positive relationship between antioxidant activity and potential health of skeletal muscle cells *in vitro* (Goutzourelas et al., 2015; Han et al., 2019). We believed that compounds with high antioxidant activity tend to have a more positive effect on skeletal muscle health. This result indicates that compound with high antioxidant activity tend to have a more positive effect on skeletal muscle health. Therefore, fractions B, D, E, and F were selected for further analysis.

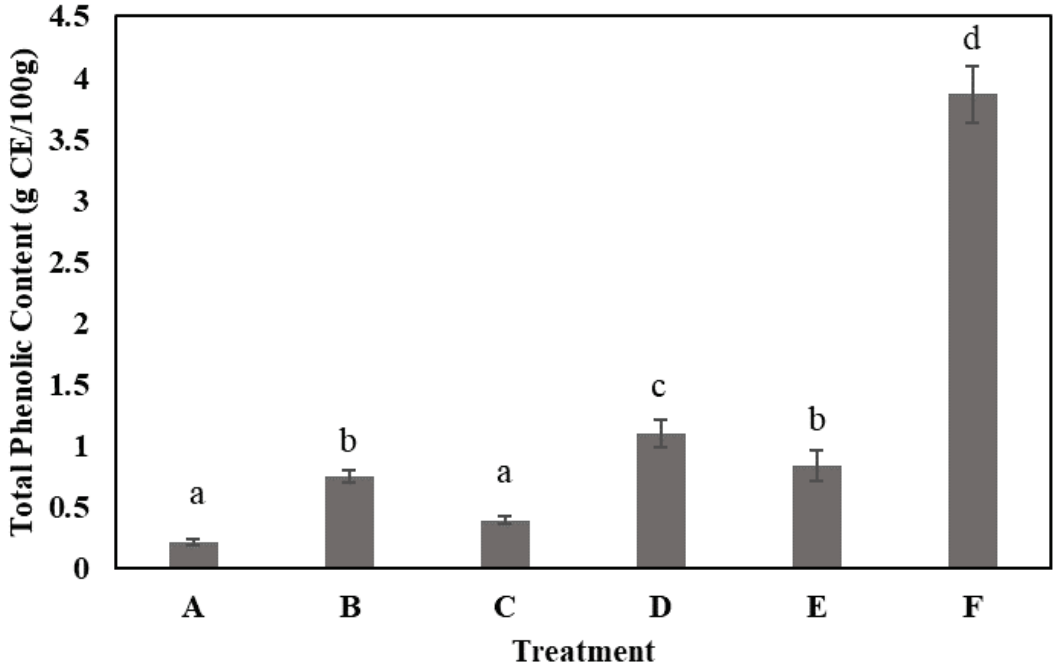

Figure 8 Total phenolic content (g/100 g CE) of persimmon fractions. Values denote the mean ± SD (n=6/group). Statistically significant differences between groups were determined by Tukey's test. Different letters indicate statistically significant differences between groups.

5. Direct cytotoxicity evaluation of selected Japanese persimmon fractions

Direct cytotoxicity tests were performed for B, D, E, and F fractions both in Caco-2 cells and C2C12 cells. Below are the results of selected Japanese persimmon fractions direct cytotoxicity analysis on Caco-2 (Figure 9) and C2C12 (Figure 10) cell cultures. The result showed that there was no toxicity in Caco-2 cell culture for all fractions from 0.1 until 1 mg/mL concentration (Figure 9). On the other hand, direct addition of Japanese persimmon fractions into C2C12 cell culture also did not showed no cytotoxicity in the range concentration from 0.1 until 1 mg/mL (Figure 10). All treatment showed above 50 % percent of cell viability in compared with control, indicated that Japanese persimmon fractions B, D, E, and F did not have toxicity effect for C2C12 cell culture. To deepen understanding of the effect of the Japanese persimmon fraction on Caco-2 and C2C12 cell cultures, two different concentrations from each fraction were choosed: 0.1 mg/mL and 0.5 mg/mL as low and high concentrations. Co-culture system was used to deepen the analysis for further analysis.

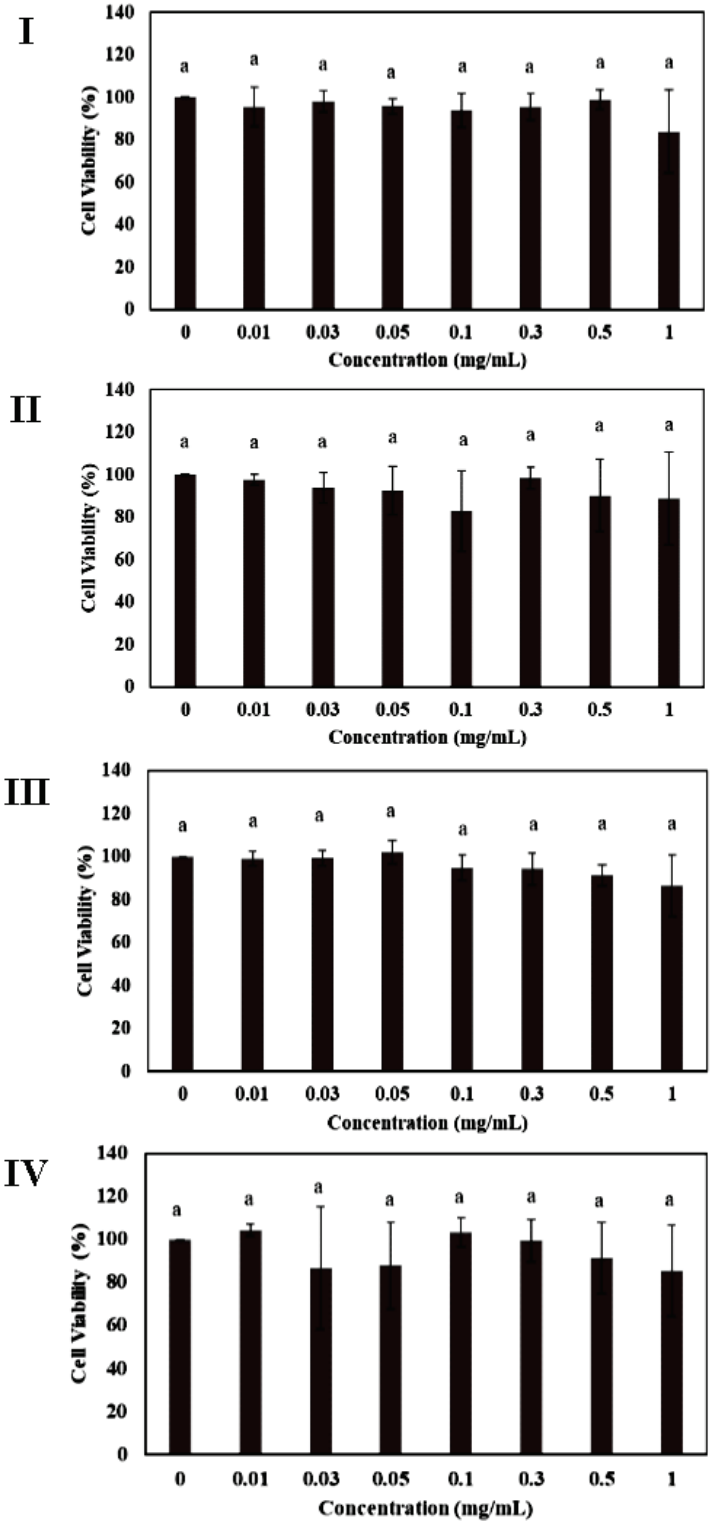

Figure 9. Caco-2 direct cytotoxicity analysis of the Japanese persimmon fractions B (I), fraction D (II), fraction E (III) and fraction F (IV) using different concentration. Values denote the mean ± SD (n=4/group). Statistically significant differences between groups were determined by Tukey's Test. Different letters indicate statistically significant differences between groups.

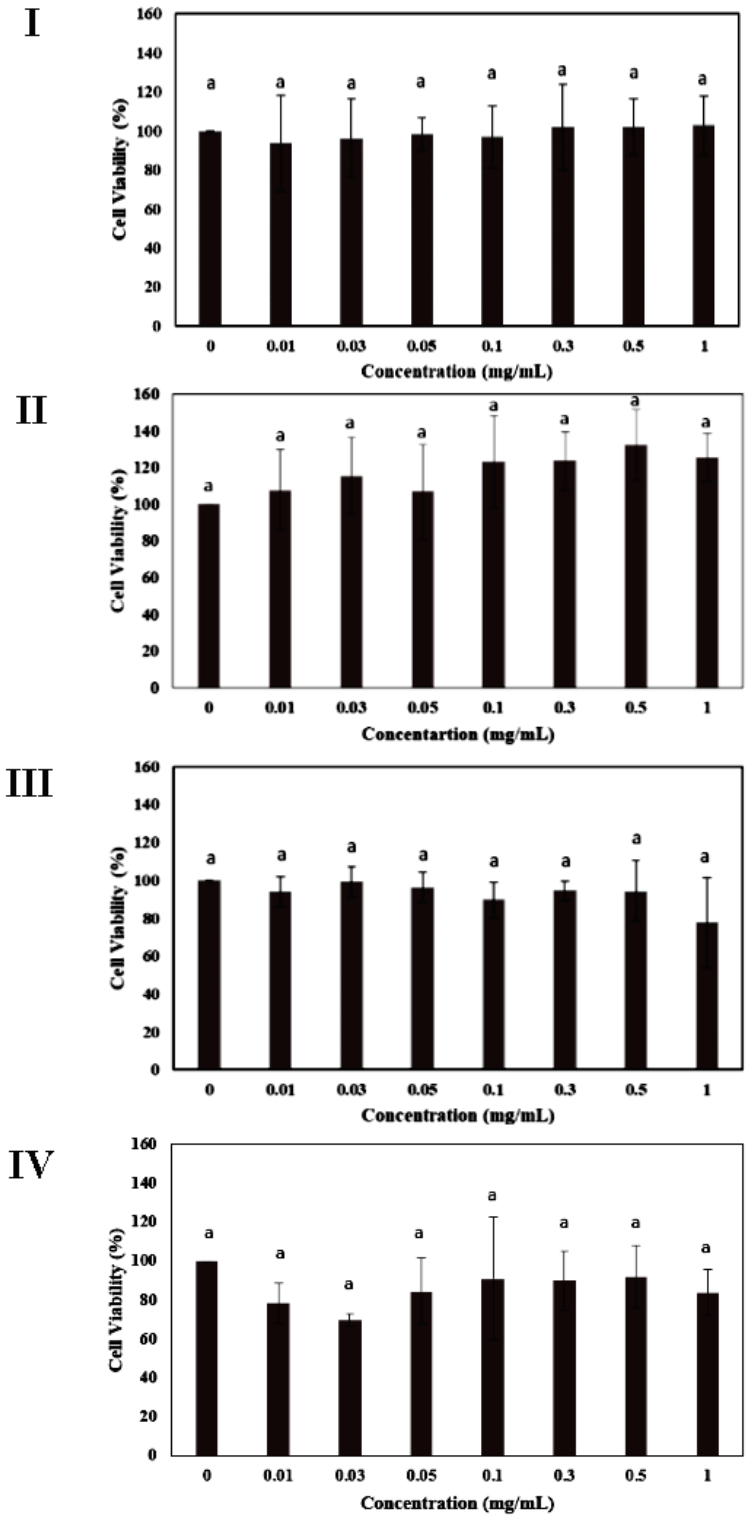

Figure 10. C2C12 direct cytotoxicity analysis of persimmon fractions B (I), fraction D (II), fraction E (III) and fraction F (IV) using different concentration. Values are means ± SD, n=4/group. Statistically significant difference of each group was determined by Tukey Test. Different letters indicate statistically significant difference between groups.

6. TEER value analysis

Below are the results of TEER value from Caco-2 cell culture on day 21 and 22 before and after 24 h of incubation of persimmon fractions (Figure 11). The graph showed that TEER value of Caco-2 cell culture is fluctuate from 5-21 days post seeding culture. The values are relatively increased although it suddenly fell off on day 13. In this study, the culture reached its peak steady state value after day 21 of culture. This result was in line with (Awortwe et al., 2014) who stated that 21 days were the minimal amount of culture days needed for Caco-2 cells to form well-developed tight junctions. From this graph, I concluded that after 21 days of culture, the Caco-2 cell in co-culture system had already reached the fully differentiated state. Therefore, day 21 was chosen to performed bioassay evaluation of persimmon fractions both in Caco-2 cell and C2C12 cell.

The addition of 0.1 mg/mL persimmon fractions to the Caco-2 cell culture caused changes in the TEER value from hour to hour. There was a dropped of TEER value right after the addition fraction B, D, E, and F from persimmon. However, observation for 24 h with measurements at 2 h-intervals showed that the TEER value increased and even recovered to its initial value after 24 h of post-fraction exposure (Figure 12). Same trend was also shown when 0.5 mg/mL of fractions B, D, E, and F were added to the co-culture system (Figure 13).

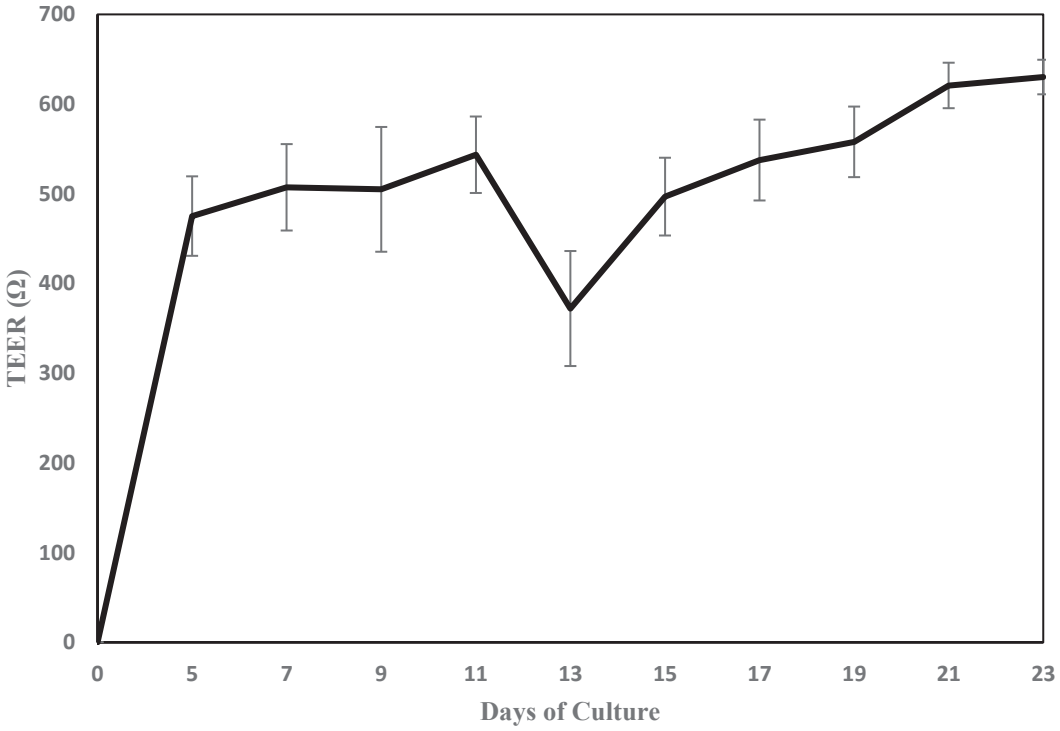

Figure 11. TEER values of Caco-2 cell grown on 24 mm Transwell inserts from day 0 to 23 post-seeding. Cells were seeded at a density of 3 x 10^5 cells/cm^2 and maintained in DMEM at 37 °C and 5 % CO_2 (mean ± SD, n=10)

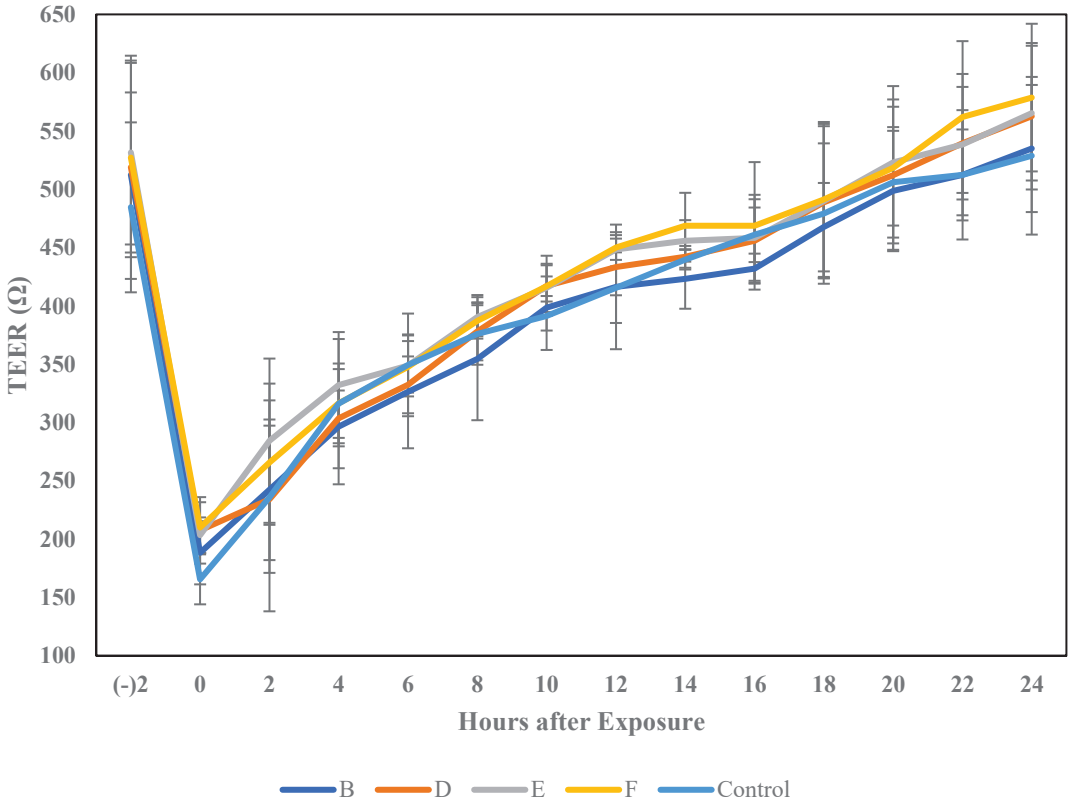

Figure 12. TEER values of Caco-2 cell grown on 24 mm Transwell inserts after exposure of 0.1 mg/mL of Japanese persimmon fractions. Cells were seeded at a density of 3 x 10^5 cells/cm^2 and maintained in DMEM at 37 °C and 5 % CO_2 (mean ± SD, n=3)

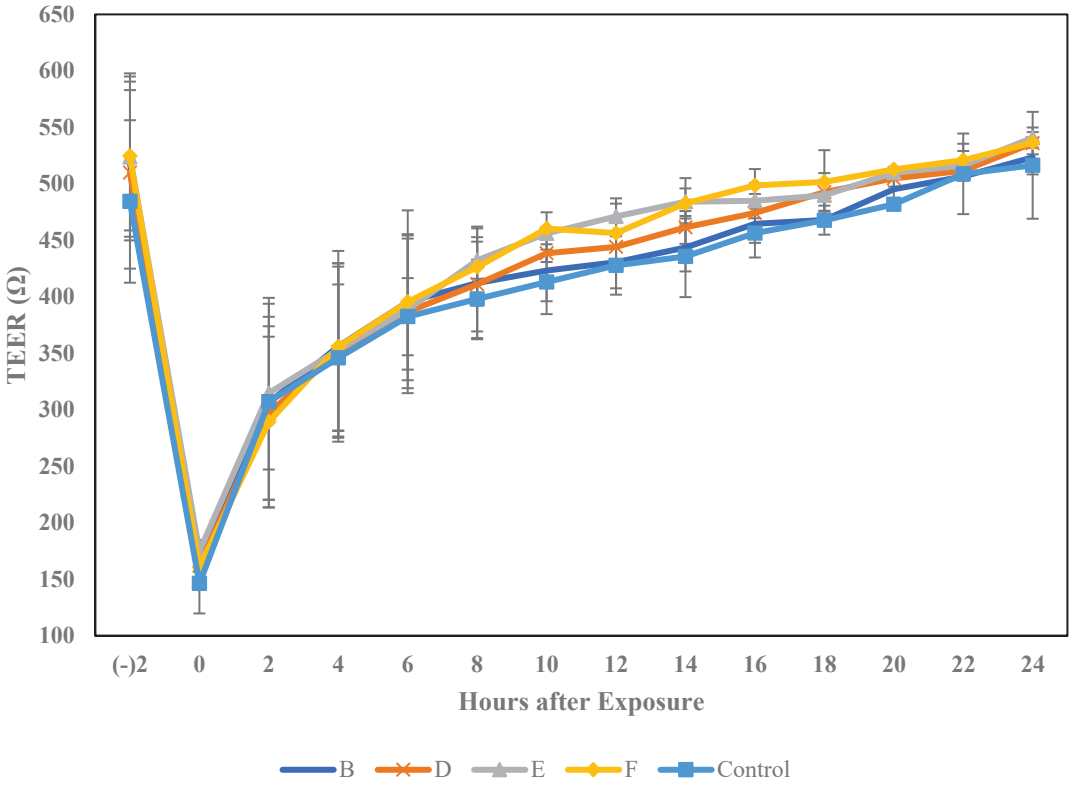

Figure 13. TEER values of Caco-2 cell grown on 24 mm Transwell inserts after exposure of 0.5 mg/mL of Japanese persimmon fractions. Cells were seeded at a density of 3 x 10^5 cells/cm^2 and maintained in DMEM at 37 °C and 5 % CO_2 (mean ± SD, n=3)

7. C2C12 cell growth evaluation

After 24 h of incubation, the basolateral medium (BM) of the co-culture systems was isolated and used for further analysis. Incubation of the C2C12 myoblast cells with BM treated with 0.1 and 0.5 mg/mL of the B, D, E, and F persimmon fraction for 3 days resulted in different effects on cell proliferation, both at low and high concentrations. Incubation of the C2C12 myoblast cells with BM treated with 0.1 and 0.5 mg/mL of the B, D, E, and F persimmon fraction for 3 days resulted in different effects on cell proliferation, both at low and high concentrations. Among the low concentration group on day 1 (Figure 14 I), treatment with fraction B alone resulted in a decrease in viability, although the difference was not statistically significant. There were no significant cells growth in both group on day 2 (Figure 14 II). The most prominent cell growth differences were observed after three days of culture (Figure 14 III). Fraction B showed the lowest cell viability, but with no statistically significant differences in cell viability among fractions D, E, and F. On the other hand, among the high concentration groups, none of the groups showed statistically significant cell viability compared with the control.

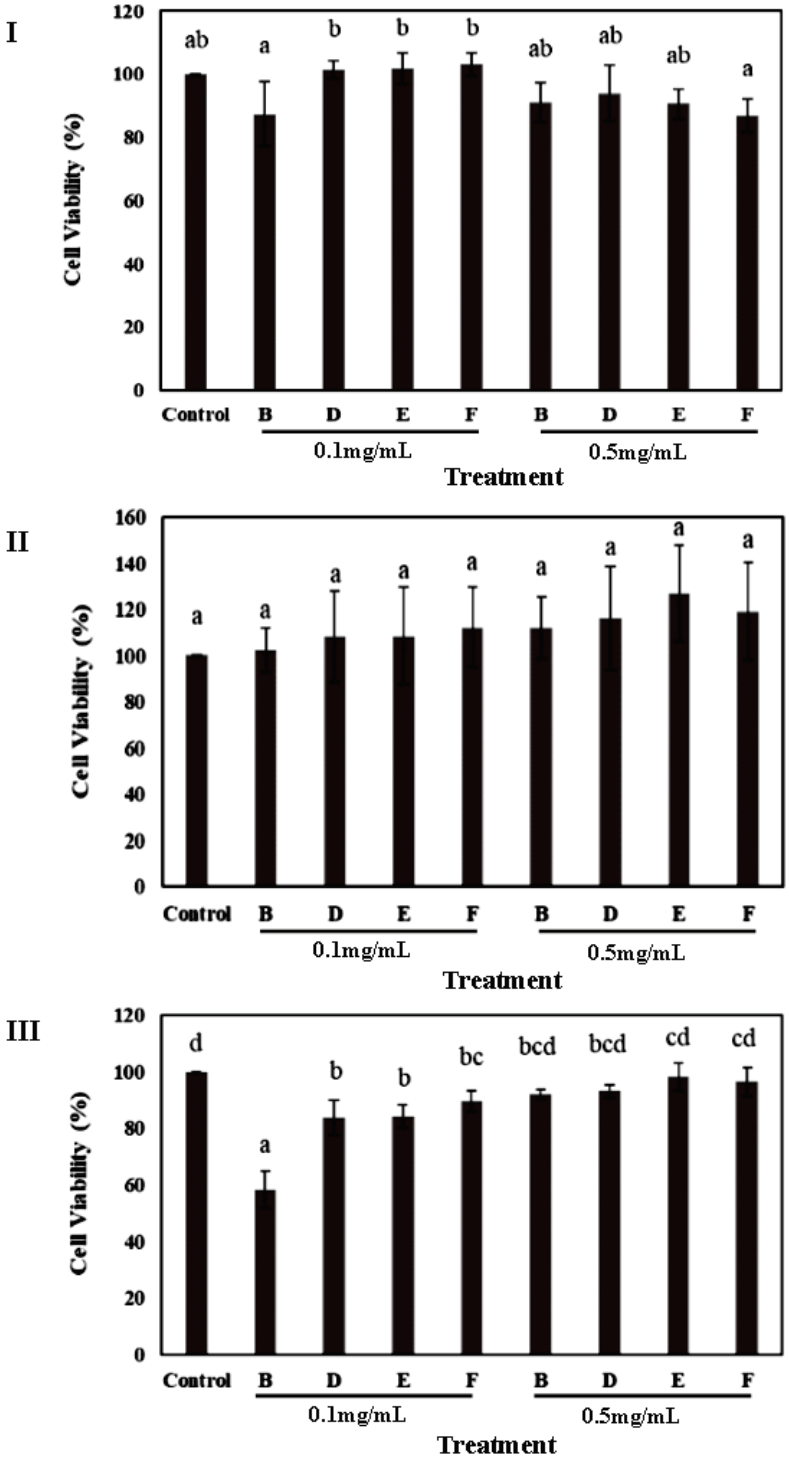

Figure 14. C2C12 cell growth after the addition of basolateral medium of Caco-2 cells treated with 0.1 and 0.5 mg/mL persimmon fractions. I, Culture for 1 day. II, Culture for 2 days. III, Culture for 3 days Values are the mean ± SD, n=4/group. Statistically significant differences between groups were determined by Tukey's Test. Different letters indicate statistically significant differences between groups.

8. Cytoprotective effect of Caco-2 cell basolateral medium against H_2O_2 induced oxidative stress in C2C12 myoblast cells

After concluded that basolateral fraction from co-culture systems was nontoxic, both through direct exposure (Caco-2 cell culture) or undirect exposure (C2C12 myoblast cell culture), its cytoprotective effect against hydrogen peroxide induced oxidative stress in C2C12 myoblast cell culture were evaluated. Treatment of 1 mM H_2O_2 for 6 h resulted in approximately 20 % loss of cell viability (Fig. 15). Pre-treatment of C2C12 myoblast cell culture using BM with low (0.1 mg/mL) and high (0.5 mg/mL) persimmon fraction concentration for 24 h have different effect on cell viability. In 0.1 mg/mL concentration, treatment with BM cannot restore cell viability percentage. On the other hand, pretreatment with BM of persimmon fraction in high concentration (0.5 mg/mL) showed more prominent cytoprotective effect against H_2O_2, especially in hydrophobic fraction (B, D and F). In fact, this result is comparable with the control group. In conclusion, 24 h pretreatment with BM added by 0.5mg/mL persimmon fraction B, D, and F could restore the cell viability of C2C12.

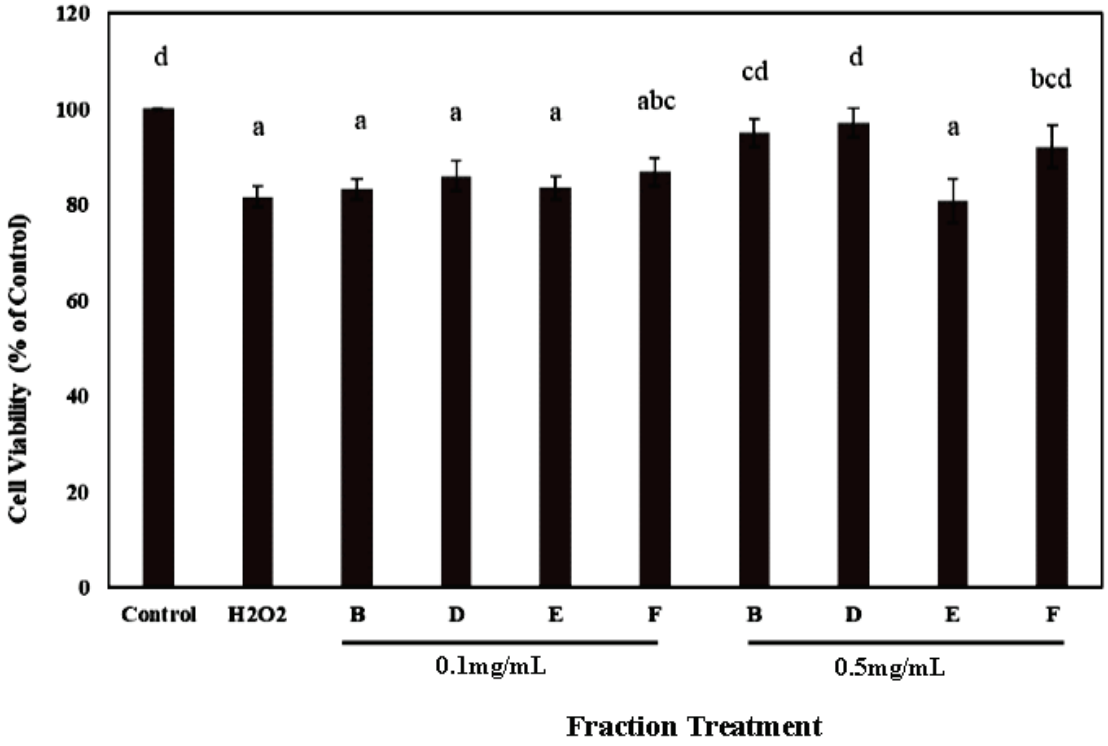

Fig. 15. C2C12 cell viability after 24 h treatment with persimmon fraction conditioned medium 0.1 mg/mL and 0.5 mg/mL, followed by 6 h treatment of 1 mM H_2O_2. Values are means ± SD, n=3/group. Statistically significant difference of each group was determined by Tukey Test. Different letters indicate statistically significant difference between groups.

9. Caco-2 cell basolateral medium attenuates intracellular ROS formation in H_2O_2-induced oxidative stress C2C12 myoblast cells

The levels of intracellular ROS were measured to evaluate the ability of persimmon extracts to quench ROS. The ROS levels in oxidized C2C12 myoblast cell cultures with and without BM pretreatment are shown in Fig. 16. Treatment with 1 mM H_2O_2 for 6 h resulted in an increase in the level for intracellular ROS. The ROS levels in the myoblast cells cultured in BM containing secretions of Caco-2 cells that were only exposed to fraction B of persimmon extract in high concentrations (0.5 mg/mL) significantly decreased (Fig. 16), suggesting that BM from pretreatment with persimmon extract fractions B had quenching effect on oxidative stress induced by H_2O_2. Therefore, the protective effect from oxidative stress-induced cytotoxicity was also most likely related to the inhibitory effect against oxidative stress-induced ROS generation.

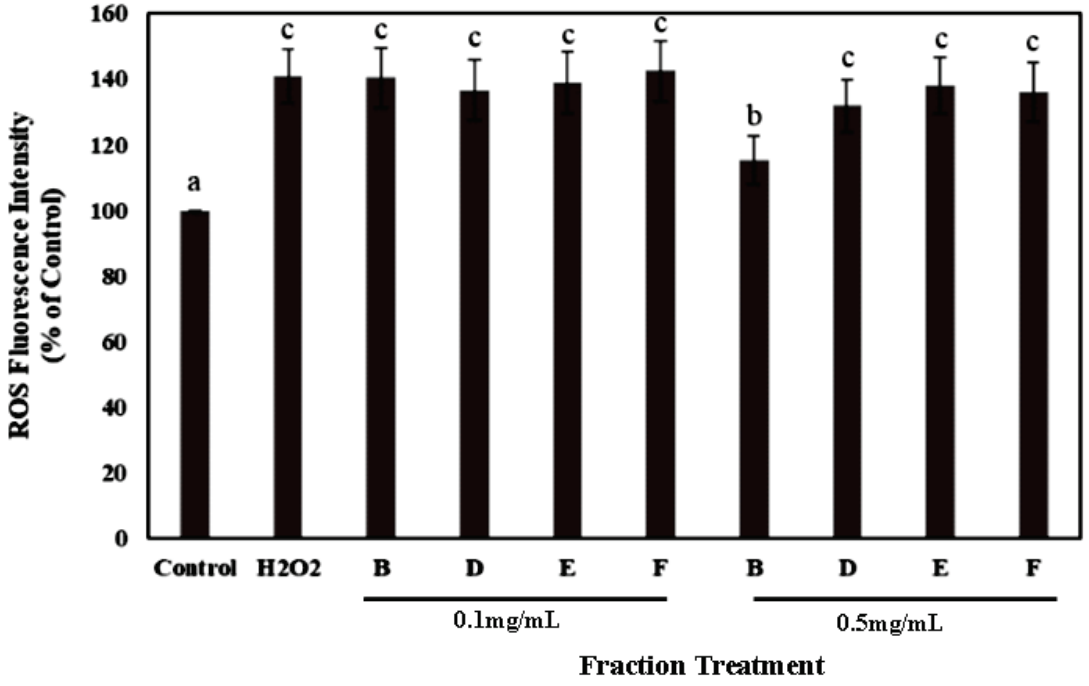

Fig. 16. Intracellular ROS level after 24 h treatment with persimmon fraction conditioned medium 0.1 mg/mL and 0.5 mg/mL followed by 6 h treatment of 1 mM H_2O_2. Values are means ± SD, n=3/group. Statistically significant difference of each group was determined by Tukey Test. Different letters indicate statistically significant difference between groups.

V. DISCUSSION

1. Japanese persimmon fractions quantitative analysis

In this study the physiological effects of Japanese persimmon extract on C2C12 myoblast cell culture, especially its protective effect against H_2O_2 induced oxidative stress were investigated. As a first step, Japanese persimmon fruit was extracted using hot ethanol method. Extraction is an important step in plant potent bioactive compound study. Suitable extraction method can isolate desirable soluble compound and leaving out undesirable component. In addition, it will obtain high yield extract with minimal changes to the required functional properties (Dhanani et al., 2017). On the other hand, the choice of solvent in the extraction step of plant bioactive compound is also very important. Menstruum or solvent properties can determine the successful of extraction itself. Das *et al*. (2010) stated that solvent should have low toxicity, not interfere the physiological action of bioactive compound, easy to evaporate, and promote rapid physiological absorption of the extract. Ethanol was selected as the main solvent because it has several advantageous characteristics. In addition to its polarity, ethanol has self-preservative and nontoxic properties and easily evaporates (Abubakar & Haque, 2020). Kim *et al*. (2017) reported that ethanol was a preferable solvent compared to methanol for extracting water-soluble phenol in astringent persimmon.

From hot ethanol extraction step, 27.8 g crude extract were obtained from 42.5 g of dry sample of Japanese persimmon fruit with a yield of 65.3%. According to Choe *et al*. (2014), plant extraction yield can be affected by various factor such as the method of extraction, selected solvent, extraction time, and extraction temperature. After the extraction step, the crude extract of Japanese persimmon was fractionated. In general, fractionation procedure can further separate bioactive compound of plant extract based

on their relative solubility and polarity. Fractionation and fraction evaluation step are necessary as standardize for preparing potential pharmaceutical drug (Seelinger *et al*., 2012).

For fractionation step, three different concentration of ethanol were used: 25 %, 50 %, and 75 % as solvent. Butanol separation was conducted since 25 % and 50 % ethanol solvent to completely dilute the Japanese persimmon crude extract. Therefore, an additional step was necessary to separate the Japanese persimmon bioactive compound based on its polarity. On the other hand, 75 % ethanol solvent was used to successfully separate the Japanese persimmon crude extract into ethanol phase and filtrate phase. Six fractions from fractionation step had different total yield (Table 2, 3, and 4) of fractionation relative to 5 g of Japanese persimmon crude extract. As antioxidant is one of the main targeted property, antioxidant activity and total phenolic content analysis were conducted for quantitative evaluation. Fraction with robust physiological activity will be selected accordingly for another biological analysis.

According to (Moharram & Youssef, 2014) there are two common types of *in vitro* antioxidant activity evaluation: (1) the reaction of redox type and (2) thermodynamic competition of peroxyl radicals between antioxidant compounds and substrates. In this research, DPPH method was selected to represent the antioxidant evaluation by reaction of redox type. DPPH was used to test the ability of compounds to act as free radical scavengers or hydrogen donors (Pisoschi *et al*., 2016). DPPH generally exhibits antioxidant activity when free radical inhibition reached 50 %. Therefore, the result was usually defined as IC_{50} value (Choe *et al*., 2014). The DPPH radical scavenging activity of Japanese persimmon fraction was ordered as follows: fraction F > fraction D > fractions B and E > fractions C > fraction A (Figure 6). Jang *et al*. (2011) find out that

IC_{50} of persimmon fruit were varied, depend on persimmon cultivar, solvent, and parts of persimmon. Calyx part of Nishimura-Wase persimmon (non-astringent) that extracted with acetone showed the highest DPPH radical scavenging activity compared with calyx, peel, and pulp part of astringent persimmon sample.

As mentioned by (Číž *et al*., 2010), it is important to use more than one antioxidant activity evaluation method for better understanding the main principles of antioxidant properties in the compound itself. ORAC method measured the antioxidant-mediated protection of the fluorescent protein (fluorescein) from free radical damage (Sueishi et al., 2011). By measuring the antioxidant scavenging activity against peroxyl-radical induced-oxidation induced by AAPH, ORAC can be performed both in aqueous and lipophilic solution (Masuda *et al*., 2015). Therefore, this method was choose for analyzing antioxidant activity evaluation of persimmon fraction since it is also considered as more physiologically relevant method (Denev & Yordanov, 2013).

The ORAC value of Japanese persimmon fraction was ordered as follows: fraction F, B, D, and E > fractions A and C (Figure 7). Denev & Yordanov (2013) found out that ORAC value of the persimmon is varied depend on cultivar and ripening stage. In general, ORAC value of plant material depend on several factor. Within same materials, environmental factors such as climate, location, and temperature can significantly affect the accumulation of antioxidant component and ORAC value (Číž *et al*., 2010). Genotype, origin, and cultivation techniques also have great effect on ORAC value (Li *et al.*, 2013). In addition, several papers also found that solvent composition can cause significantly different ORAC value (Yalcin & Sogut, 2014; Boeing *et al*., 2014).

Overall, antioxidant evaluation of persimmon exhibited different degrees of antioxidant activity. The hydrophobic fractions (B, D, and F) tended to show stronger

antioxidant capacity than the hydrophilic fractions (A and C), except for the E fraction, which had a value comparable to the other hydrophobic fractions. Solvent polarity indeed had a great effect on antioxidant properties of plant extracts. High polar solvent usually less effective in extracting antioxidant compound from plant extract (Iloki-Assanga *et al*., 2015).

Phenols are one of the major groups of non-essential dietary components present in fruits and vegetables with various health benefits. Several papers stated that phenol had redox properties which allowed them to act as radical scavengers, metal chelators, reducing agents, hydrogen donors, and singlet oxygen quenchers (Phang *et al*., 2011). Therefore, antioxidant activity evaluation normally accompanied by total phenolic content quantification.

Among all fractions, fraction F had the highest phenolic content. The total phenolic content was ordered as follow: fraction F > fraction D > fractions B and E > fractions A and C (Figure 8). Again, hydrophobic fraction (B, D, and F) tended to show higher level of total phenolic content compared with the hydrophilic fractions (A and C), except for the E fraction, which had a value comparable to the other hydrophobic fractions. According to previous report, total phenolic content of the plant extract can be affected by several factors: water content, method of extraction, and the chemical properties (Shon *et al*., 2014). Gao *et al*. (2014) append, sample varieties, genomics, plant part, and time collection may also affect the result. (Jang *et al*., 2010) find out that solvent have high impact on the total phenolic content of plant extract. Polar have better result in extracting phenolic content in compare with non-polar solvent. This may explain why F fraction showed higher result in compare with the other fractions. In addition, it was suggested

that the major antioxidant components in several fractions might not be phenolics compound. Further analysis is needed to explain this phenomenon in detailed.

2. Japanese persimmon cytotoxicity analysis

Beside evaluate its potential bioactivity, plant bioactive compound possible risks such as undesirable side effects, overdose and toxicity need to be standardized and evaluate (Ogbole *et al*., 2017). WST-8 that utilized in CCK-8 kit (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4 disulfophenyl)-2H tetrazolium, monosodium salt) was used to evaluate cell viability and cytotoxicity. In principle, water-soluble tetrazolium salts (WST) is reduced by NAD(P)H and produced WST formazan product. This formazan product had a strong orange color with maximum absorption at 450 nm. NADPH itself is an important biological molecule that acts as cofactor in various cellular metabolism. Therefore, the availability of NADPH indirectly indicated living cell (Chamchoy *et al.*, 2019). All selected fractions (B, D, E, and F) showed no sign of direct cytotoxicity both in Caco-2 (Figure 9) and C2C12 (Figure 10) cell culture. Japanese persimmon fraction B, D, E, and F in concentration range between 0.01-1 mg/mL did not showed any significant reduction of cell viability percentage after 24 h of culture.

An *in vitro* alternative to monoculture is coculture of multiple cell types including most commonly epithelial cells. This approach is being used with increasing frequency as a solution to bridge the gap between overly simplistic single lineage in vitro models and the dynamic biological processes that occur in vivo. Coculturing epithelial cells with other cell types in proportional levels approximated to known tissue constituency have been used to mimic the in- situ interactions of various body systems. Co-culture system is a new in vitro model for studying the toxicity and metabolism of drug, including plant bioactive compound. Caco-2 cells are used as a cell line that represents the human

intestine, which is the main absorption component of substances (Sambruy *et al*., 2001). Compared to other intestinal epithelial cell lines, the Caco-2 cell line is advantageous due to its spontaneous differentiation to an enterocyte-like phenotype, formation of tight junction network, and good functional correlation to human intestinal tissue. The fully differentiated Caco-2 cells have several properties that resemble colon crypt cell, such as electrical properties, permeability, and ion conductivity (Grasset et al., 1984). TEER is a simple and reliable method for measuring the electrical resistance of a cell. This method can be used to measure epithelial cell monolayer tightness and integrity. When Caco-2 cells establish confluent and functional tight junctions, the TEER values reach their maximum steady state values (Sambruy *et al*., 2001). This period marks the fully differentiated and fully functional process of Caco-2 cells in co-culture systems. In this study, the stable value was seen after 21 days of culture (Figure 11). Literature showed that Caco-2 will start to proliferate after 48 h of seeding with the most rapid proliferation occurred between day 3 until 9. After reached day 21, the proliferation activity will stop, metabolic rate relatively not high and Caco-2 cells were fully differentiated and functional (Tan *et al*., 2018).

Beside indicating the fully differentiated Caco-2 cell culture, TEER value also can indicate the sub-lethal toxicity of tested compound in intestinal cells. Toxic compound can cause cell death, leading to the tight junction alteration of the culture. Intestinal tight junctions are linked to the cytoskeleton, permeability, and paracellular barrier (Sambruy *et al*., 2001). According to Mukherjee *et al*. (2004), the recovered dropped of TEER value after the addition of tested compound is a sign of Caco-2 cell tight junction restoration. This also means that the tested compound did not possessed any toxic effect that can be harmful for intestinal cell. Although TEER value was dropped after the addition of

Japanese persimmon fractions, 24 h cultured could recovered the initial value of TEER, both in 0.1 mg/mL (Figure 12) and 0.5 mg/mL concentration (Figure 13). The intestinal absorption of drug or any potential plant compound is a critical step for their next fate in human body system. Before reached out targeted organ, the interaction between tested compound and intestinal organ is important to evaluate, either for analyzing their potential toxicity, or their effectivity after crossing intestinal barrier (Sambruy *et al*., 2001).

In conclusion, cell cytotoxicity evaluation using WST-8 assay and TEER value showed that all selected fraction of Japanese persimmon did not possessed any toxic effect on Caco-2 and C2C12 cell culture viability.

3. Basolateral medium added with Japanese persimmon fractions induce C2C12 cell growth

Interesting results were found in C2C12 cell growth evaluation which incubated with basolateral medium (BM) from co-culture system added to Caco-2 cells with persimmon fractions, both in low (0.1 mg/mL) and high (0.5 mg/mL) concentration. After 24 h incubation of C2C12 cells, low concentration group showed that treatment with fraction B alone resulted in a decrease in viability, although the difference was not statistically significant in compared with control (Figure 14 I). In addition, fraction B again showed the lowest cell viability compared to fractions D, E, and F (Figure 14 III). Another study using Jerusalem artichoke extract showed that phenolic content of the fraction may had effect on cell proliferation. Fraction with higher phenolic content showed higher growth activity of fibroblast cell in lower concentration, beside showing higher antioxidant activity (Nizioł-ŁUkaszewska *et al*., 2018). This result was in line with this study in which fraction F, fraction with highest phenolic content level, showed the highest growth activity level of C2C12 myoblast cell in low concentration (0.1 mg/mL). Until now, there

were no studies that can explained the cytotoxic phenomenon from basolateral medium added with 0.1 mg/mL fraction B. Plant bioactive compound evaluation in related with possible cellular interaction and bioavailability are still rarely done. Low concentration (0.1 mg/mL) of fraction B from Japanese persimmon extract may induced the secretion of metabolite substances from Caco-2 cell that caused relatively mild cytotoxic effect on C2C12 cell culture.

On the other hand, among the high concentration groups (0.5 mg/mL), none of the groups showed statistically significant cell viability compared with the control after three days of culture (Figure 14 III). This suggests that the basolateral medium from co-culture system added with 0.5mg/mL fractions B, D, E, and F had no cytotoxic effect and can maintain cell growth comparable with control even after three days of culture. The aforementioned phenomenon was not seen on direct cytotoxicity evaluation. There were no significantly different of C2C12 cell viability after the addition of Japanese persimmon fractions (Figure 10). It is suggested that Japanese persimmon fractions could induced metabolite secretions from Caco-2 cell that eventually had an effect on C2C12 myoblast cell culture. In addition to evaluating the bioavailability of the tested compound (Gonzales *et al*., 2015) a co-culture system can allow researchers to deepen the study of the effects of phytochemical compounds and cellular communication that may be involved in the metabolism of the tested compound. Nevertheless, further analysis is needed to explain all aforementioned phenomenon.

Antioxidant properties do not only have strong correlation with anti-tumor activity but also mitogenic activity, angiogenesis, collagen production, and DNA synthesis (Pitz *et al*., 2016) that may contributes to C2C12 cell proliferation and growth. In addition, another study in related with muscle and persimmon extract are also worth to find out

since compound with antioxidant properties proved to promotes skeletal muscle health (Nallamuthu *et al*., 2014; Receno *et al*., 2019).

4. Basolateral medium added with Japanese persimmon fractions protective effect against H_2O_2 oxidative stress

In this research, H_2O_2 was used to induced oxidative stress in C2C12 cell culture. H_2O_2 were naturally produced in cellular mitochondria together with superoxide anion radical through electron transport mechanism. This substance can cross the cell membrane easily. When it exposed to UV or interacted with transition metal ion inside human body, H_2O_2 will decomposed and become a highly reactive hydroxyl radical (Venkatesan *et al*., 2019).

Cytoprotective effect of BM added with Japanese persimmon fractions showed prominently in high concentration group (0.5 mg/mL) and hydrophobic fraction (B, D, and F). Cell viability of these three groups were comparable with control group (Figure 15), indicating that BM added with 0.5 mg/mL fraction B, D, and F of persimmon fraction have a cytoprotective effect against oxidative stress induced by H_2O_2. This result is in line with another study with same method (1 mM H_2O_2; 6 h induction; C2C12 cell culture) yet using different plant extract as tested compound such as garlic saponin (Kang *et al*., 2016b), morin extract (M. H. Lee et al., 2017), *Sargassum horneri* (Kang, S. S. *et al*., 2015), *Schisandrae semen* essential oil (Kang, J. S. *et al*., 2015), and *Prunus mume* (Kang, *et al*, 2016a).

To explained cytoprotective phenomenon, the levels of intracellular ROS were measured. The production of intracellular ROS was quantified using the oxidation-sensitive fluorescent probes 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA). H2DCFDA is cell-permeable, therefore it can diffuse into cells and deacetylated by cellular esterases to form 2',7'-dichlorofluorescein (H2DCF). During assay, H_2O_2 can oxidize H2DCF into DCF, a highly fluorescent compound (Yang *et al*., 2014). Among all group of treatment, only BM containing secretions of Caco-2 cells that were only exposed

to fraction B of persimmon extract in high concentrations (0.5 mg/mL) significantly decreased ROS levels in C2C12 induced with H_2O_2 oxidative stress. This finding is in line with another study that used persimmon extract as a tested compound and Caco-2 cells as a model in which persimmon ethanolic extract exhibited the scavenging activity of ROS induced by H_2O_2 (Kim *et al*., 2017).

Theoretically, natural antioxidants act as compounds that trap free radical species. Antioxidant will trap ROS, donate their electrons, and turn ROS into more stable compounds (Sharma et al., 2012). Although, all human cells have antioxidant mechanism to protect themselves against oxidative damage, these sometimes are not sufficient to prevent oxidative damage (Mandal *et al*., 2011). In this research, Japanese persimmon fraction showed several antioxidant properties and cytoprotective effect against oxidative stress. This result may indicate that Japanese persimmon fruit is a potential source of natural antioxidant and new functional food product. However, further analysis will be needed to improve our understanding of Japanese persimmon physiological mechanism in protecting muscle cell from oxidative stress *in vitro*.

Persimmon fruit contains several bioactive compounds that may contribute to its antioxidant activity properties. Dalvi *et al*. reported that the main mechanism by which the persimmon extract neutralized oxidative stress was radical sequestration (Dalvi et al., 2018). In this case, the persimmon compound reacted directly with hydroxyl radicals. This study suggested that the radical scavenging activity of the persimmon fraction was derived from non-phenolic compounds. Kondo et al. (2004) suggested that such persimmon scavenging activity was derived from catechins, ascorbic acid, or carotenoids. Although antioxidant activity may vary depending on persimmon cultivars and fruit development, they found that the major radical scavenging capacity of the pulp and peel

of non-astringent persimmons were mainly derived from ascorbic acid (Kondo et al., 2004). Ascorbic acid is reported as water soluble antioxidant with an effective scavenger against several radical species, especially O_2^{-}, H_2O_2, OH·, $^1O_2$, and NO·(Gulcin, 2020; Padayatty et al., 2003; Timoshnikov et al., 2020). To scavenge radical species, ascorbic acid is oxidized to dehydroascorbate, which is later recycled to ascorbic acid by the enzymatic activity of dehydroascorbate reductase (Gulcin, 2020). In addition, the ability of ascorbic acid to trap radical oxygen species generated by H_2O_2 was also reported by Cozzi *et al*. (Cozzi et al., 1997). They also found that additional ascorbic acid could supress ROS production in CHO cells.

Furthermore, Japanese persimmon fractions may also stimulate intestinal epithelium cells to secrete soluble factors that contribute to the reduction of oxidative stress in myoblasts *in vitro*. Besides being involved in absorption functions, Caco-2 cell also play a significant role in acting as food component sensors for signal transmission (Satsu, 2017; Zhao et al., 2008). In addition, the transwell co-culture technique makes it possible to study this paracrine signaling after addition of test compounds (Bogdanowicz & Lu, 2013; Jiang et al., 2008). Nevertheless, further studies are needed to improve our understanding of this mechanism.

VI. CONCLUSION

This study was conducted as a part of a screening program for potential natural antioxidant agents against oxidative stress in skeletal muscle cell, one of possible mechanism that can caused sarcopenia. Persimmon fruit (*Diospyros kaki*) is enriched with many bioactive compounds that considered as powerful antioxidants. Antioxidant play an important role in protecting substantial biomolecules in human body from free radical that can cause oxidative stress.

The physiological effect of Japanese persimmon extract on C2C12 myoblast cell culture, especially its protective effect against H_2O_2 induced oxidative stress was investigated. For mimicking physiological condition, co-culture system using human Caco-2 intestinal cells were constructed as a model of intestinal epithelium. As a first step, Japanese persimmon fruit was extracted and fractionated using graded concentration of ethanol. Six fractions from fractionation step had different total yield of fractionation relative to 5 g of Japanese persimmon crude extract.

As antioxidant is one of the main targeted property, antioxidant activity and total phenolic content analysis were conducted for quantitative evaluation. DPPH method was selected to represent the antioxidant evaluation by reaction of redox type while ORAC was used to evaluate antioxidant scavenging activity against peroxyl-radical induced-oxidation. Overall, antioxidant evaluation of persimmon exhibited different degrees of antioxidant activity. The hydrophobic fractions (B, D, and F) tended to show stronger antioxidant capacity than the hydrophilic fractions (A and C), except for the E fraction, which had a value comparable to the other hydrophobic fractions. In addition, hydrophobic fraction (B, D, and F) also tended to show higher level of total phenolic

content compared with the hydrophilic fractions (A and C), except for the E fraction, which had a value comparable to the other hydrophobic fractions.

Cell cytotoxicity evaluation using WST-8 assay and TEER value showed that all selected fraction of Japanese persimmon (B, D, E, and F) did not possessed any toxic effect both in Caco-2 and C2C12 cell culture viability. Basolateral medium from co-culture system added with 0.1 mg/mL of fraction B caused a decrease in viability of C2C12 after three days of incubation. On the other hand, three days incubation of basolateral medium from co-culture system added with 0.5 mg/mL of fraction B, D, E, and F have no toxic effect and can maintain cell growth comparable with control even after three days of culture.

A 20 % reduction in cell viability of C2C12 was observed after 6 h incubation with 1 mM H_2O_2. Nevertheless, the addition of basolateral medium from co-culture system of Caco-2 cells added with 0.5 mg/mL Japanese persimmon fraction B, D, and F could restored C2C12 cell viability comparable with control group. Furthermore, basolateral medium of Caco-2 cells added with 0.5 mg/mL fraction B could significantly restored ROS levels in C2C12 after H_2O_2 oxidative stress induction. This phenomenon indicated that persimmon fractions were found to stimulate intestinal epithelial cells to produce secretions that reduced oxidative stress in myoblasts *in vitro*. This feature is closely linked to the ability of persimmon extracts to stimulate epithelial cells to produce secretions with the ability to inhibit intracellular ROS production.

ACKNOWLEDGEMENTS

I would like to thank Gifu Agricultural Technology Center, Japan, for providing the persimmons used in this research.

I am most grateful to my main supervisors, Professor Tomio Yabe for his guidance throughout the thesis, for always had the door open for all questions and discussions concerning all aspects of the work. I am very grateful to Professor Kohji Kitaguchi for all support, kindly guidance, advices, and insightful comments. I express my special thanks to Dr. Yasunori Chiba of the National Institute of Advanced Industrial Science and Technology (AIST), Japan for his support and suggestions, and the constructive advice and criticism. In addition, I also wat to express my sincere gratitude to Professor Nakako Katsuno for accepting to review my PhD dissertation with her valuable suggestions.

I wish to express my sincere acknowledgment to all members of Functional Food Biochemistry Laboratory for their help during experiment, for accepting all my mistake, flaw, and misunderstanding. Without your friendly and happy working environment, I will never go through all the obstacle.

I also wish to extend my thanks to all my friend in Gifu: Aini, Randa, Arifin, Mas Zaki, Kak Ai, Kak Lia, Mbak Maya, Mas Viga,, Teh Ria and Nagaya Sensei for all the countless help. Finally, I would like to dedicate this work to my family: Bapak, Ibu, Nadiya without whom I would have never been able to reach this point in life. Your encouragement and unconditional support will always stay in my heart.

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