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Pharmacological Effect of *Muntingia calabura* Leaves on the Expression of *sod1* and *sod2* in *Drosophila*

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Abstract: The lifespan of *Drosophila melanogaster* can last longer when consuming ethanol extract of *Muntingia calabura* leaves (EMCL) before starvation. Lifespan extension can be affected by several factors, including the increased expression of endogenous antioxidants. This study aimed to assess the level of endogenous antioxidants *sod1* and *sod2* in *D. melanogaster* upon ingestion of ethanol extract of *M. calabura* leaves (EMCL) before starvation. Fifty *D. melanogaster* were divided into five groups: healthy control group, starvation control group, and three groups of EMCL-treated: 0.2% group, 1% group, and 5% group. Each group was fed with fly food in the presence or absence of EMCL for seven days, and all groups, except the healthy control group, were subsequently subjected to a starvation procedure for seven hours. RNA extraction was performed on each group before *sod1* and *sod2* measurements using the reverse-transcriptase quantitative PCR method. Our results revealed that the expression of *sod2* was increased in response to starvation. On the contrary, reduced expression of *sod1* was observed in the starvation-treated group. Treatment of flies with EMCL at 1% and 5% increased the mRNA level of *sod1*, but did not affect the mRNA level of *sod2*. Taken together, our results demonstrated the potential effect of *Muntingia calabura* leaves in the modulation of *Drosophila sod1* and *sod2* gene expression in *Drosophila*. Similar endogenous antioxidants are present in humans and have been associated with aging, implying that prospective pharmacological investigations are feasible to pursue in future studies.

Keywords: *Muntingia calabura*; *sod1*; *sod2;* antioxidant; *Drosophila melanogaster*.

1. Introduction

Free radicals consist of one or more unpaired electrons as metabolic products of metabolism in the mitochondria [1, 2]. Production of free radicals in low concentrations has beneficial effects on the body. However, it can cause oxidative stress if they are overproduced [3-5]. Oxidative stress has been suggested to play an essential role in the induction of apoptosis, a programmed cell death [5, 6]. Apoptosis is essential for the normal function and survival of multi-cellular organisms, including host defense against pathogenic microbes [6-8]. However, excessive apoptosis can induce harmful host responses that may impair tissue functions and may lead to physiological abnormalities [9].

In living things, cell death can be triggered by several factors: continuous starvation [10, 11]. Cells primarily respond to starvation via the autophagy pathway, but in the event of prolonged starvation, cells may undergo autophagic cell death in addition to apoptosis [11].

Calorie restriction (CR) by controlled starvation has been shown to be associated with a prolonged duration of life, implying the anti-aging effect of such an approach [12]. However, at certain conditions, starvation may promote detrimental injuries to tissues [10]. Starvation-induced modifications in physiological responses, for example, extended lifespan, have been demonstrated using different types of model animals [12, 13], including in the fruit fly *Drosophila melanogaster* [14].

Drosophila melanogaster has been widely applied as a model system to investigate various biological phenomena in metazoan species, including the supportive role of the virus in the defense against cancerous cells [15], the mechanistic basis of cell death [16], and aseptic inflammation [17], the impeccable role of innate immunity in the fight against bacterial [18], viral [19, 20], and fungal [21] infections, and even in the continuous global efforts to discover new drug entities from medicinal plants [22-26] and/or to repurpose old drugs for new pharmacological indications [27]. In fact, just recently, *D. melanogaster* has been proposed as a potential model organism to study SARS-CoV-2 virulence genes and host factors responsible for the pathogenesis of COVID-19 [28].

Extended lifespan is affected by several factors, including antioxidants both exogenous and endogenous that can inhibit free radicals [3, 4]. Normally, the human body produces intrinsic or endogenous antioxidants; one of them is superoxide dismutases (SODs), the first class and one of the most important enzymatic antioxidants to inhibit reactive oxygen species (ROS), especially superoxide anion radicals [3, 29, 30]. Two SODs, CuZn-SOD (*SOD1*) and Mn-SOD (*SOD2*) are important in humans [29]. In *Drosophila* itself, these two SODs have been described to be important in life extension [31]. Some studies using *D. melanogaster* as a model organism demonstrated the involvement of endogenous antioxidants *SOD1* and/or *SOD2* in regulating *D. melanogaster* lifespan [31, 32] and its improvement in the presence of a certain drug or plant extracts [3, 33, 34].

One of the natural resources proven to have antioxidant activity *in vitro* and *in vivo* is *Muntingia calabura* L. [35, 36]. Previous experiments using the mice model revealed that *M. calabura* leaves extract possessed anti-aging activity, possibly reducing the level of oxidative stress [35]. Indeed, we also found that *Drosophila melanogaster* experienced an extended lifespan upon ingestion of ethanol extracts of *Muntingia calabura* leaves (EMCL) before the starvation process was initiated [Unpublished data]. Based on the currently available data, there is an indication that EMCL can extend the lifespan of *D. melanogaster* in stress conditions. Here we reported a study to examine the effect of EMCL on the expression of endogenous antioxidants CuZn-SOD (*sod1*) and Mn-SOD (*sod2*) using the starved *Drosophila* model.

2. Materials and Methods

2.1. Plant extract preparation.

Ethanol extract of *M. calabura* leaves (EMCL) samples was obtained from Makassar, South Sulawesi, Indonesia. This sample was prepared by maceration method with a ratio 1:10 of simplistic and 70% ethanol for three days in a place protected from direct sunlight with occasional stirring was performed during the process. After that, the macerated mixture was filtered then the residue was re-macerated again for three more days. Later, the filtrate was evaporated using a rotary evaporator until a thicken extract was obtained.

2.2. Fly stocks.

The *w*^{1118} *Drosophila melanogaster* line used in this study was generously provided by the Host and Response Laboratory of the University of Kanazawa, Japan. Flies' maintenance were carried out in standard conditions (25°C, 12 hours light and 12 hours dark cycles, and standard corn-meal food). In this study, adult male flies were anesthetized in a refrigerator at -20°C for ±5 minutes before RNA extraction and gene expression analysis.

2.3. Fly treatment and starvation induction.

Adult male flies at the age of 10 days were prepared, assigned into five different groups, treated according to their groups, and then induced into the starvation condition (10 flies per vial, 3 vials in each group). Flies were maintained in an incubator with a steady temperature at 25°C. Fly feeding was carried out for seven days. A group fed with standard fly food was used as the healthy control group in this experiment. Flies in the starvation control group were given standard fly food for seven days and then transferred in an empty vial for seven hours. Other groups were similarly treated as the negative control group but fed with standard fly food containing EMCL at a concentration of either 0.2 %, 1%, or 5%.

2.4. Gene Expression analysis.

Isolation of total RNA was conducted on all treatment groups. Ten live flies from each group were transferred into a Treff tube then anesthetized in a freezer at -20°C for ±5 minutes prior to crushing using a micropestle. Total RNA was extracted using the RNA Isolation System (Promega) reagent. The level of *sod1* and *sod2* were quantitatively examined by using the reverse transcriptase quantitative PCR (RT-qPCR) method. A set of *sod1* primer (sequence of *sod1* forward primer: 5'–AGGTCAACATCACCGACTCC–3' and sequence of *sod1* reverse primer: 5'–GTTGACTTGCTC AGCTCGTG–3') and a set of *sod2* primer (sequence of *sod2* forward primer: 5'–TGGCCACATCAACCA CAC–3'and sequence of *sod2* reverse primer: 5'–TTCCACTGCGACTCGATG–3') in a total volume of 20 µl using GoTaq® 1-Step RT-qPCR System (Promega) were used, as per manufacturer's instruction. Verification of whether the expected product had been amplified was carried out based on the assigned samples' standard melt curve profile. As an internal control in the RTqPCR assay, the level of *rp49,* a host gene responsible for the expression of ribosomal protein, was examined by using a set of *rp49* primer (*rp49* forward primer: 5'–AGATCGTGAAGAAGCGCACCAAG–3' and *rp49* reverse primer: 5'–CACCAGGA ACTTCTTGAATCCGG–3'). A real time thermal cycler (Rotor Gene Q, Qiagen, Germany) was used with a running profile: 37°C for 15 minutes, 95°C for 10 minutes, and a set of 95°C for 10 seconds, 60°C for 30 seconds, and 72°C for 30 seconds for 40 cycle repeat following by melt curve analysis from 60°C to 95°C. Data were analyzed using the relative quantification method.

2.5. Data processing and statistical analysis.

Data obtained in all experiments were processed and statistically analyzed by a One-Way Anova approach using GraphPad Prism® 8. For all statistical analyses, data were presented as mean ± S.D and p values of less than 0.05 were considered significant.

3. Results and Discussion

3.1. Expression of sod1 and sod2 in response to starvation.

Starvation is one of the approaches that can be used to increase the number of free radicals in cells [37, 38]. The excessive free radicals can further promote the occurrence of oxidative stress, which will eventually affect the quality of life [3, 39]. To encounter such stress conditions, cells are programmed to produce endogenous antioxidants, for example, the *SOD1* and *SOD2* that are available from an invertebrate *D. melanogaster* to a more complex eukaryote such as a human.

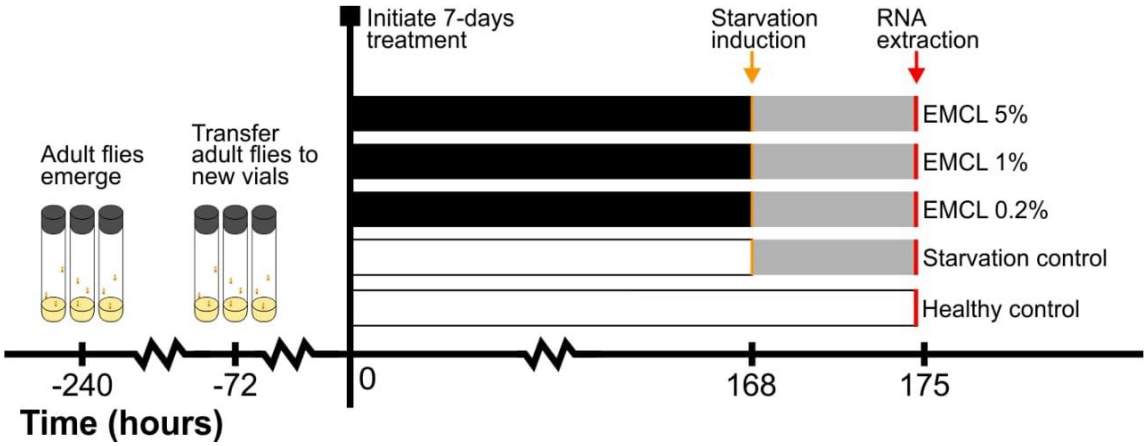

Figure 1. Timeline of the experimental design. Three groups of adult flies were subjected to treatment with food containing ethanol extract of *M. calabura* leaves (EMCL) at three different concentrations: 0.2, 1, or 5% for seven days prior to starvation for seven hours. For control groups, flies were given standard fly food in the presence (negative control) or absence (healthy control) of starvation at length, similar to treatment groups. RNA extraction was carried out at 175 hours after treatment initiation, followed by an amplification process using RT-qPCR method.

In this study, we designed an experimental approach (Fig. 1) to assess the effect of ethanol extract of *M. calabura* leaves (EMCL) on the expression of *sod1* and *sod2*, two important endogenous antioxidants in *Drosophila* that are also available in humans.

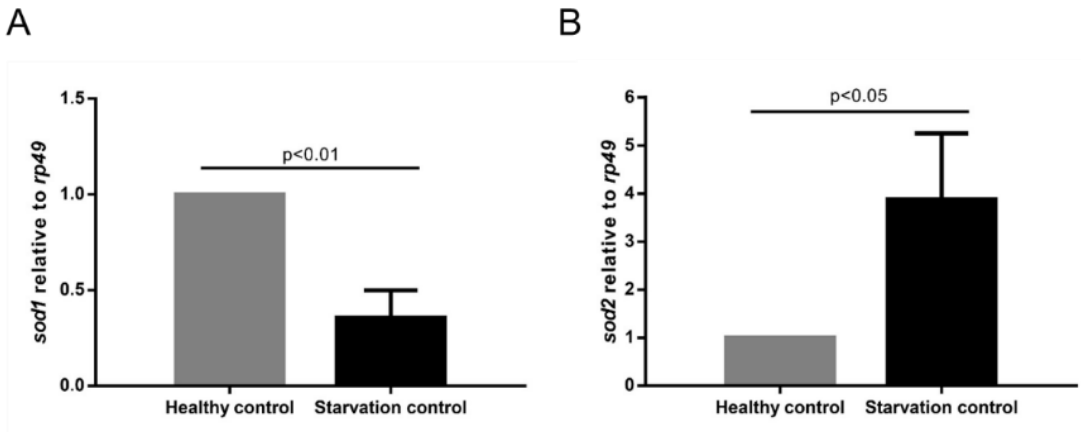

Figure 2. Levels of *sod1* and *sod2* gene expression in the healthy control and the starvation control groups. Adult flies were maintained and fed with standard fly food for seven days prior to starvation. After seven days, flies were transferred to either vial containing standard fly food (healthy control group) or to an empty vial (starvation control group) and incubated at 25°C for seven hours, followed by RNA isolation. The levels of *sod1* (A) and *sod2* (B) were calculated compared to *rp49* after amplification by RT-qPCR method.

As can be seen in Fig. 2A, it appears that the starvation control group showed reduced expression of *sod1* compared to the healthy control group. Surprisingly, a different result was

obtained in the expression of *sod2* (Fig. 2B). Starved *Drosophila* expressed a higher level of *sod2* than its healthy counterpart. This might be caused by the autophagy process that can increase energy production in the mitochondria.

However, such a process also forms free radicals that can intensify *sod2* production as feedback response of free radicals due to starvation condition, as has been shown previously in freshwater shrimp *Neocaridina davidi* [40]. Nevertheless, increased expression of *sod2* gene upon starvation induction would be a beneficial response to the host because *sod2* has been shown to be important to counteract the free radicals that first formed in the mitochondria [41].

3.2. Effect of Muntingia calabura L. on the expression of sod1 and sod2.

Lifespan extension is affected by several factors, including the use of endogenous antioxidants to inhibit the excessive formation of free radicals [3]. Humans and *Drosophila* express some endogenous antioxidants, including superoxide dismutase 1 (CuZn-*SOD*, *SOD1*) and superoxide dismutase 2 (Mn-*SOD*, *SOD2*). Based on the results displayed in Fig. 3, it seems that the ingestion of EMCL by *D. melanogaster* before starvation-induced condition showed contrast effects on the level of expression of *sod1* gene (Fig. 3A) and *sod2* gene (Fig. 3B). While the expression of *sod1* was increased in response to EMCL treatment, the expression of *sod2* remains unaltered.

Increased expression of *sod1* in response to EMCL treatment at concentrations of 1% and 5% but not at 0.2% under starvation condition may occur due to the activity of unidentified EMCL compound(s) that can activate the production of *sod1* in *D. melanogaster* in a dose-dependent manner that was optimally reached at 1%. Naturally, *SOD1* can be found in the cytoplasm of human and *D. melanogaster*. *T*his enzyme is very stable because it has non-covalent bonds formed by disulfide in each subunit [42, 43]. Also, there is a possibility of this high expression related to the amount of *SOD1* as the dominant enzymatic antioxidant in some cells and tissues, which is an account for 70-80% of the total cellular activity of SODs, as has been shown in mice and human cells [44-46].

Surprisingly, while the induction of *sod2* expression was observed in response to starvation (Fig. 2B), EMCL treatment demonstrated no stimulatory effect on the expression level of *sod2* in starved *D. melanogaster* (Fig. 3B).

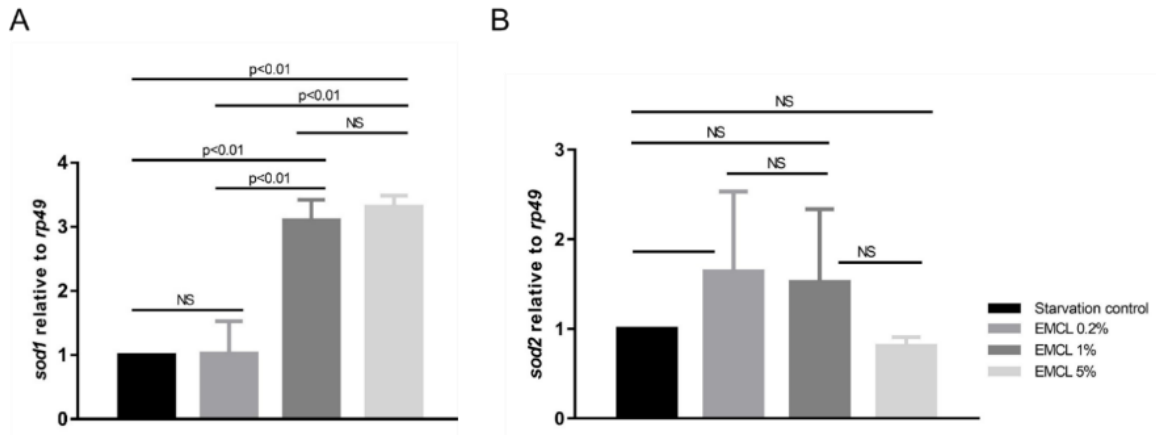

Figure 3. Levels of *sod1* and *sod2* gene expression in the starvation control and ethanol extract of *Muntingia calabura* (EMCL)-treated groups. Adult flies were maintained and fed with standard fly food for seven days before starvation. After seven days, flies were transferred to empty vials and incubated at 25°C for seven hours, followed by RNA isolation. The levels of *sod1* (A) and *sod2* (B) were calculated compared to *rp49* after amplification by RT-qPCR method.

The reason(s) why these unexpected results have occurred remains unknown. Nevertheless, taken together, our results implied that starvation could induce the expression of *sod2* but not *sod1*, and this expression profile was reversed under EMCL treatment at certain concentrations (1% and 5%).

In addition to the *sod* genes, a thorough investigation on the effect of EMCL on the expression of some genes known to express endogenous antioxidants (for example, cat gene that expresses catalase) will provide additional information on the prospective use of EMCL in the modulation of endogenous antioxidants. In the long run, follow-up research is urgently required to elucidate the responsible compound(s) in the modulation of *sod1* and *sod2* expression and also to unveil the detailed mechanisms on how such compound(s) can yield the demonstrated effects.

4. Conclusions

In the current study, we provided initial evidence that the expression of endogenous antioxidants *sod1* and *sod2* in *D. melanogaster* was modulated by starvation. The administration of ethanol extract of *Muntingia calabura* L. leaves (EMCL) was able to induce the expression of *sod1* but not *sod2* in starved *D. melanogaster*. Here, we reported the potential in vivo anti-aging effect of EMCL in a *Drosophila* platform system that shall warrant future research in higher model animals or even human subjects.

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Conflicts of Interest

The authors declare no conflict of interest.

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