



# The Effect of Mango Mistletoes (*Dendrophthoe pentandra*) Leaves Extract on Percentage of CD4<sup>+</sup>CD28<sup>+</sup>, CD8<sup>+</sup>CD28<sup>+</sup>, and interleukin-2 Levels of Aged Balb/c Mice

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## Abstract

**Edited by:** Slavica Hristomanova-Mitkovska  
**Citation:** Handono K, Pratama MZ, Sermoati IA, Yuniati MG, Haryati NPS, Norahmawati E, Endharti AT, Irwanto Y, Solikhin MB, Hidayat S. The Effect of Mango Mistletoes (*Dendrophthoe pentandra*) Leaves Extract on Percentage of CD4<sup>+</sup>CD28<sup>+</sup>, CD8<sup>+</sup>CD28<sup>+</sup>, and interleukin-2 Levels of Aged Balb/c Mice. Open Access Maced J Med Sci. 2021 May 23; 9(B):414-421.  
https://doi.org/10.3889/oamjms.2021.6182

**Keywords:** *Dendrophthoe pentandra*; Immunosenescence; Age; Aged Balb/C mice  
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**Received:** 12-Apr-2021

**Revised:** 29-Apr-2021

**Accepted:** 12-May-2021

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**Funding:** This article is supported by Faculty of Medicine, Universitas Brawijaya, Indonesia.

**Competing Interests:** The authors declared no conflict of interest

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**BACKGROUND:** Population aging is considered to be a global phenomenon today. Age-associated immune system dysfunction or “immunosenescence” is indicated by increased susceptibility to infections and chronic diseases, such as hypertension, diabetes mellitus, autoimmune diseases, heart disease, and atherosclerosis. One of the immunosenescence markers is a significant drop in CD28 and reduced proinflammatory cytokine interleukin-2 (IL-2). The mango mistletoes are deemed to have a better affinity for docking the CD28 and IL-2R receptors of  $\alpha$  and  $\beta$  subunits than other plants.

**AIM:** This study aims to determine the effect of ethanol extract of mango mistletoes on IL-2 levels, the percentage of CD4<sup>+</sup>CD28<sup>+</sup>, and the percentage of CD8<sup>+</sup>CD28<sup>+</sup> in aged female mice.

**METHODS:** The leaves of mango mistletoes were extracted using 96% ethanol solvent, and the extract was administered to aged female mice (18–20 months) orally with different doses for each group, namely 150, 300, and 600 mg/kg. Mango mistletoe leaves extract was administered once a day for 14 days. Then, the IL-2 levels of the mice were checked from their heart blood samples using Enzyme-Linked Immunosorbent Assay, while the percentages of CD4<sup>+</sup>CD28<sup>+</sup> and CD8<sup>+</sup>CD28<sup>+</sup> were examined from the spleen samples using flow cytometry.

**RESULTS:** The ethanol extract of mango mistletoe leaves was able to increase the percentage of CD4<sup>+</sup>CD28<sup>+</sup> significantly ( $p < 0.05$ ) at doses of 300 and 600 mg/kg and increase the percentage of CD8<sup>+</sup>CD28<sup>+</sup> significantly ( $p < 0.05$ ) at a dose of 600 mg/kg/BW, while other various doses had a strong enough correlation ( $r = 0.48$ ) to increase IL-2 levels.

**CONCLUSION:** The ethanol extract of mango mistletoe leaves has the good potential to inhibit the aging process in the immune system, as characterized by an increase in IL-2 levels and the percentage of CD4<sup>+</sup>CD28<sup>+</sup> and CD8<sup>+</sup>CD28<sup>+</sup>.

## Introduction

Population aging is a global phenomenon [1]. This fact is marked by the increase in the share of elderly population (60 years and over) worldwide. The global population of those aged 60 years or over totaled 962 million in 2017, and is expected to double to reach nearly 2.1 billion by 2050, with most of the increase in developing countries [2]. Age-associated immune system dysfunction or “immunosenescence” is indicated by increased susceptibility to infections and chronic diseases, such as hypertension, diabetes mellitus, autoimmune diseases, heart disease, and atherosclerosis [3]. Age-associated immune system dysfunction also includes changes

in immune tolerance, increases in autoantibodies, decreased function of NK cells, B lymphocytes, and especially T lymphocytes [4]. Another characteristic of immunosenescence is a chronic state of basal inflammatory activity that may result from increased production of pro-inflammatory cytokines, such as (interleukin-6) IL-6, tumor necrosis factor- $\alpha$ , and free radicals. In addition, molecules such as the cytokines IL-2 and interferon-gamma which are associated with T lymphocyte activation and proliferation also contribute to a high incidence of neoplastic disease and infection [3]. Immunosenescence or aging of immune cells is also correlated with several other changes, including decreased naive T cells, increased memory T cells, and loss of the CD28 molecule. CD28-T cells are considered to be replicating aging T cells in humans, and thus, the

frequency of CD28-T cells in peripheral blood increases with age [5].

Preliminary *in silico* study revealed that mango mistletoes leaves (*Dendrophthoe pentandra*) had better affinity for docking the CD28 receptors and IL-2R  $\alpha$  and  $\beta$  subunits than other plants. Moreover, our previous study found that the mango mistletoes leaves (*D. pentandra*) were able to inhibit the immune aging process in stimulated T cell cultures. The results of the study showed that administering the ethanol extract of mango mistletoe leaves with a dose of 1 mg/ml was able to reduce the percentage of aging cells, as indicated by an increase in CD8<sup>+</sup>CD28<sup>null</sup> T cells, a decrease in CD4<sup>+</sup>CD57<sup>+</sup>, and a significant decrease in CD8<sup>+</sup>CD57<sup>+</sup> compared to the control. In addition, the research also disclosed that the inhibition of the aging process due to the influence of mango mistletoes leaves was mediated by an increase in secretion of the proinflammatory cytokine IL-2, which was proven to be significantly higher after the provision of ethanol extract of mango mistletoe leaves at a dose of 1 mg/ml in cell culture.

Based on the results of the study of *in silico* and *in vitro*, this study aims to carry out further testing of mango mistletoe leaves *in vivo* on aged female Balb/c mice in inhibiting the immune aging process. It is expected that this research can develop a new drug derived from Indonesian herbs for an anti-immune aging therapy in the elderly.

## Methods

### Animals

Aged balb/c female mice about 18–20 months of age were used for the study. The animals were housed in standard cages at the animal house of Faculty of Medicine Universitas Brawijaya-Malang, Indonesia, and acclimatized to the new environment under optimum feeds with full access to drinking water for a period of 7 days before the commencement of the experiment. The mice had free access to water and were fed *ad libitum*. Mice that had been used for other studies, mice that were pregnant, and mice that died during the treatment process were excluded in this study. This research was approved by the Ethics Committee of Faculty of Medicine Universitas Brawijaya. Mice were housed at room temperatures of 26–7°C.

### Plant material extraction

The leaves of mango mistletoe (*D. pentandra*) were collected from UPT Materia Medica, Batu, Indonesia. The leaves of the mango mistletoe, an *arum manis* type that grow in Pasuruan, East Java, Indonesia, were dried for 5 days at room temperature and then made into a

powder. The leaves were macerated with 96% ethanol for 72 h and then filtered. The filtrate was evaporated under reduced pressure to obtain a solid extract.

### Administration of ethanol extract of mango mistletoe (*D. pentandra*) leaves

The diluted mango mistletoe leaves extract was administered orally to the mice using a feeding cannula with a total volume of 0.5 ml of the extract. The ethanol extract of mango mistletoe leaves was given in three different doses (150, 300, and 600 mg/kg, respectively) of the body weight and was administered once a day for 14 days.

### Flow cytometry analysis for marker measurement of aging T cells

The spleen samples were stained with CD4 anti-mouse PE (Biolegend), CD8 anti-mouse PerCP (Biolegend), and CD28 anti-mouse FITC (Biolegend). Samples were prepared according to the Biolegend protocol, and flow cytometry was performed with BD FACSMelody (BD Bioscience). The number of cells was analyzed by BD Cell Quest software. The analysis resulted in the percentage (%) of cells. Based on this, we measured the percentage of CD4<sup>+</sup>CD28<sup>+</sup> and CD8<sup>+</sup>CD28<sup>+</sup> cells.

### Enzyme-linked immunosorbent assay (ELISA) analysis for measurement of IL-2 levels

IL-2 levels were measured from cardiac blood serum by ELISA. IL-2 concentrations were measured with the ELISA IL-2 mouse (Legend Max) kit according to the manufacturer's instructions. The IL-2 concentration was obtained in the form of pg/ml.

### Statistical analysis

Normality test was conducted using the Shapiro–Wilk test. Homogeneity test was carried out using Levene's test. Data that were normally distributed and homogeneous were analyzed using the one-way ANOVA difference test, while the data with significant differences for each treatment were further tested with the *post hoc* test using the least significant difference (LSD) multiple comparison test with a confidence level of 95%. If the data were not normally distributed and/or not homogenous, a difference test analysis was performed with Kruskal–Wallis. However, when the results were significant, the process continued with the *post hoc* test using the Mann–Whitney test. Correlation test was performed using Pearson correlation test for the data with normal distribution, and Spearman correlation test was used for the data that were not normally distributed. The calculations were performed with the SPSS for Windows software.

## Results

### *The effect of D. pentandra administration on the percentage of CD4<sup>+</sup>CD28<sup>+</sup>*

The aging of CD4 and CD8 T cells is illustrated by the decrease in the percentage of CD4<sup>+</sup>CD28<sup>+</sup> cells. The results of the one-way ANNOVA test showed that the ethanol extract of mango mistletoe leaves had a significant effect ( $p < 0.05$ ) on the percentage of CD4<sup>+</sup>CD28<sup>+</sup> (Table 1). The results of the LSD test highlighted that the ethanol extract of mango mistletoe leaves had a significant effect on the percentage of CD4<sup>+</sup>CD28<sup>+</sup> at doses of 300 and 600 mg/kgBW. The correlation test using Pearson disclosed that the ethanol extract of mango mistletoe leaves had a strong correlation ( $r = 0.5$ ) to the percentage of CD4<sup>+</sup>CD28<sup>+</sup> (Table 1). This correlation is positive, which means that the higher the dose of ethanol extract of mango mistletoe leaves, the higher the percentage of CD4<sup>+</sup>CD28<sup>+</sup> (Figure 1).

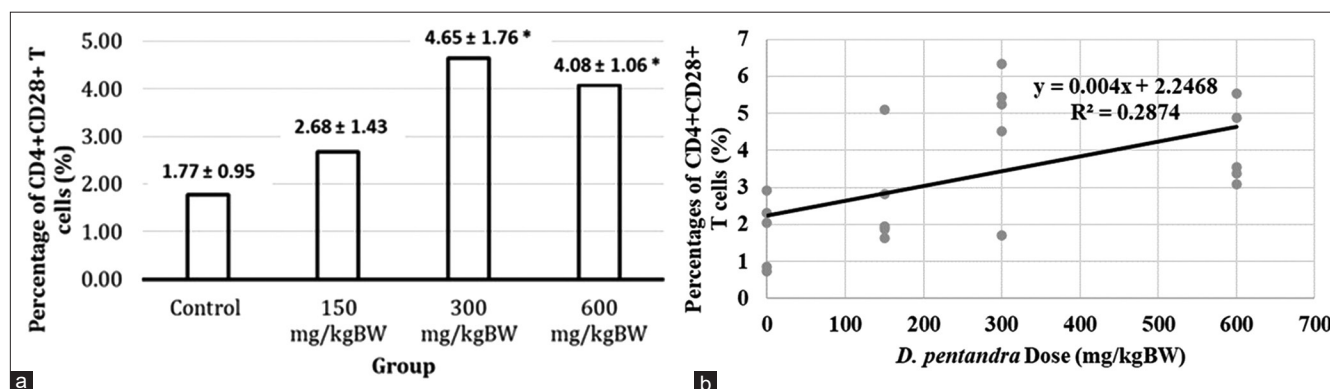
### *D. pentandra administration increases the percentage of CD8<sup>+</sup>CD28<sup>+</sup>*

A decrease in the percentage of CD8<sup>+</sup>CD28<sup>+</sup> is also an indicator of the aging immune system.

**Table 1: The effect of *Dendrophthoe pentandra* on immunosenescence marker**

Variables	Different test (p value)	Post-hoc (p value)	Correlation (r)
CD4+CD28+	0.015		0.536
Group:			
150 mg/kgBW		0.303	
300 mg/kgBW		0.004*	
600 mg/kgBW		0.015*	
CD8+CD28+	0.027		0.632
Group:			
150 mg/kgBW		0.675	
300 mg/kgBW		0.076	
600 mg/kgBW		0.016*	
IL-2	0.049		0.482
Group:			
150 mg/kgBW		0.206	
300 mg/kgBW		0.530	
600 mg/kgBW		0.117	

\*has a significant value versus control group.



**Figure 1: The effect of orally administered *Dendrophthoe pentandra* on the percentage of CD4<sup>+</sup>CD28<sup>+</sup> in mice; (a) the percentage of CD4+CD28<sup>+</sup> in mice with orally administered *D. pentandra* (\*has a significant p value); (b) correlation between dose of *D. pentandra* and percentage of CD4<sup>+</sup>CD28<sup>+</sup>**

The Kruskal–Wallis test revealed that the ethanol extract of mango mistletoe leaves had a significant effect ( $p < 0.05$ ) on the percentage of CD8<sup>+</sup>CD28<sup>+</sup> (Table 1). The results of multiple difference test analysis with LSD indicated that the ethanol extract of mango mistletoe leaves had a significant effect on the percentage of CD8<sup>+</sup>CD28<sup>+</sup> at a dose of 600 mg/kgBW. The correlation test using Spearman proved that the ethanol extract of mango mistletoe leaves had a strong correlation ( $r = 0.5$ ) to the percentage of CD8<sup>+</sup>CD28<sup>+</sup> (Table 1). The analysis presented that the higher dose of ethanol extract from mango mistletoe leaves means the higher percentage of CD8<sup>+</sup>CD28<sup>+</sup> (positive correlation) (Figure 2).

### *The ethanol extract of mango mistletoe leaves induces IL-2 levels*

In addition to changes in T cells, another indicator of immune system aging is decreased IL-2 levels. The different test analysis using Kruskal–Wallis highlighted that the ethanol extract of mango mistletoe leaves had a significant effect ( $p < 0.05$ ) on the percentage of IL-2 levels (Table 1). The analysis was continued with a multiple difference test with the LSD test which showed no significant difference at the dosage of 150, 300, or 600 mg/kgBW. Correlation analysis was performed using the Spearman correlation test, denoting that the ethanol extract of mango mistletoe had a strong enough correlation ( $r = 0.48$ ) to IL-2 levels (Table 1). This correlation generated a positive result, which means that the higher the dose of ethanol extract of mango mistletoe leaves is, the higher the IL-2 levels will be (Figure 3).

## Discussion

Mice are a widely used model for aging and senescence because of their similarity to humans. The

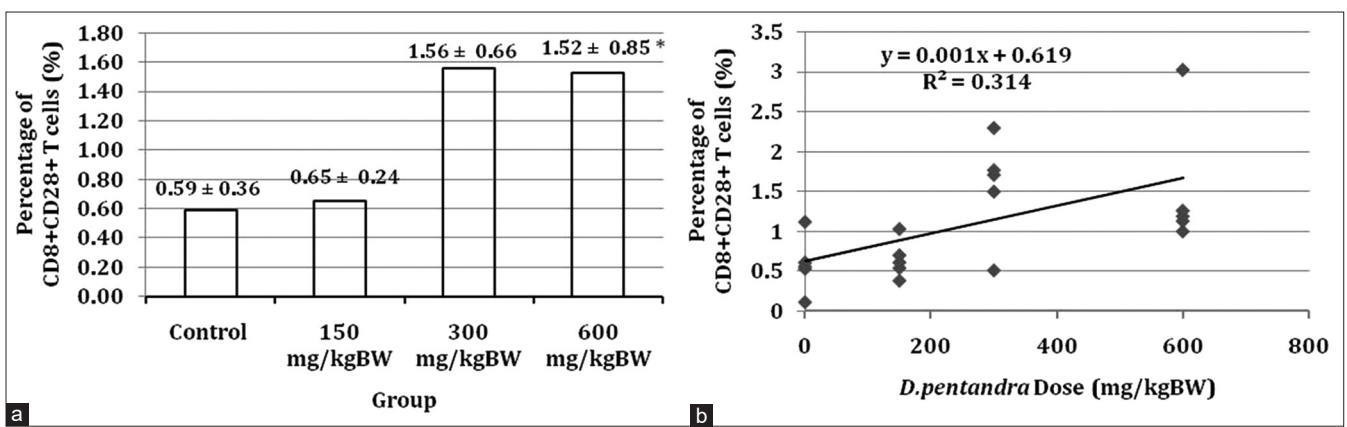


Figure 2: The effect of orally administered *Dendrophthoe pentandra* on the percentage of CD8<sup>+</sup>CD28<sup>+</sup> in mice; (a) the percentage of CD8<sup>+</sup>CD28<sup>+</sup> in mice with orally administered *D. pentandra* (\*has a significant p value); (b) correlation between dose of *D. pentandra* and percentage of CD8<sup>+</sup>CD28<sup>+</sup>

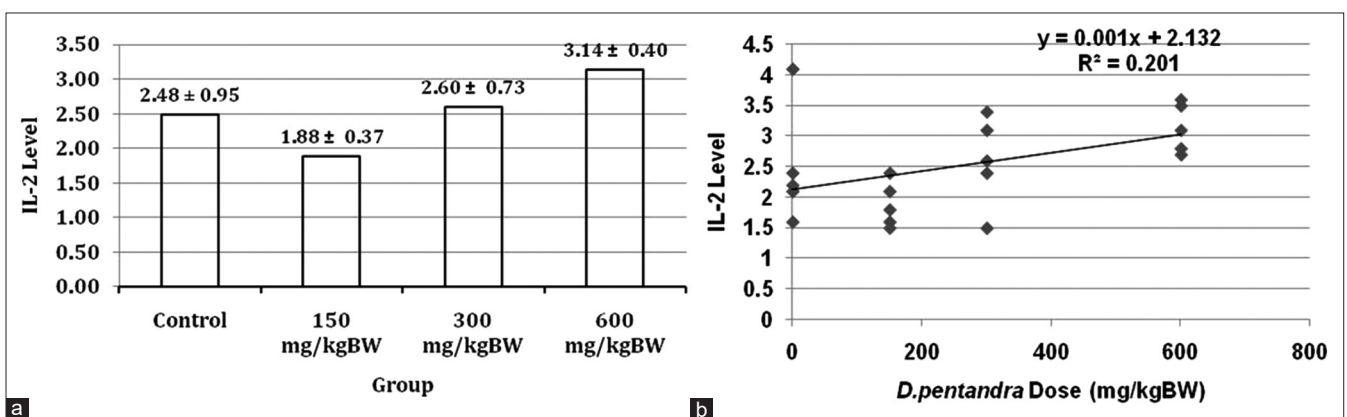


Figure 3: The effect of orally administered *Dendrophthoe pentandra* on interleukin-2 (IL-2) level in mice; (a) percentage IL-2 level in mice with orally administered *D. pentandra*; (b) correlation between dose of *D. pentandra* and IL-2 level

difference between the two terms is that aging refers to biological changes over time, while senescence refers to normal progressive dysfunction that occurs with age [6]. Balb/c is an albino congenital, immunodeficient strain of mice. Balb/c mice are characterized by easy reproduction and minimal weight variation between males and females. While the balb/c mouse serves as a general-purpose animal model, this strain is used extensively for hybridoma and monoclonal antibody production, for example, for the formation of anti-neuropilin-1 antibodies [7], antibody anti-tryptase [8], and very useful for cancer therapy and immunology study [9].

Aged balb/c mice become susceptible to infection with *Streptococcus pyogenes*, representing a possible analogy with decreased resistance to infection in elderly humans [10]. This study used mice aged 18–24 months, which correlates with human aged 56–69 years. This age range meets the definition of “old,” the change in old age in almost all biological markers in all animals, including immunosenescence [6]. In aged balb/c mice, a decrease in the proportion of T cells in the blood is associated with an increase in memory cells, a decrease in naive T cells and CD4/CD8

ratios [10], a decrease in T cells expressing CD28 [4] and regulatory T cells in the peritoneal cavity [10], and also a decrease of IL-2 levels [11]. The sign of immunosenescence that also appears in aged balb/c mice is the thymus involution which also occurs in elderly humans [4], [12]. Thymus involution results in changes in T cells, which implies a decreased immune response [4]. Aged balb/c mice showed a decreased response to CD8 T cells [13] as well as faster disease progression, earlier morbidity, and increased mortality compared to young balb/c mice [14].

In humans, with age, viral infections, exposure to antigens, thymus involution, and accumulation of ROS lead to aging of the immune system or known as immunosenescence [15]. These changes have an impact on disease progression, including cancer and autoimmune and cardiovascular diseases, worsening the severity of infection, and decreased response to vaccines, and increasing morbidity and mortality in the elderly [16].

There are two phenotypes of T lymphocytes, CD4 and CD8. The success of CD4 T cell activation is influenced by two signals, namely, TCR stimulation as the primary signal (signal 1) and antigen-independent costimulation as a secondary signal (signal 2), one of

which is CD28. CD28 is a major signaling receptor that transduces costimulatory 2 signals and is essential for activation, proliferation, and successful survival of T cells [17]. The CD28 molecule is expressed on CD4 and CD8 T cells. However, repeated antigenic stimulation over a lifetime results in CD28 progressively disappearing with age [18].

The administration of ethanol extract of mango mistletoe leaves to aged female mice showed an effect on the percentage of CD4<sup>+</sup>CD28<sup>+</sup>, as indicated by the fact that the doses of 300 and 600 mg/kgBW were able to increase the percentage of CD4<sup>+</sup>CD28<sup>+</sup> after administration for 14 days. The correlation test revealed that there was a strong positive correlation between the ethanol extract of mango mistletoe leaves and the percentage of CD4<sup>+</sup>CD28<sup>+</sup>. Similar results were also seen in the percentage of CD8<sup>+</sup>CD28<sup>+</sup>, in that the ethanol extract of mango mistletoe leaves was proven to have a significant effect at a dose of 600 mg/kgBW. This result is in line with that of the previous research that mango mistletoe leaves extract contains flavonoids, one of which is quercetin [19], which serves as an immunomodulator to improve immunosenescence conditions by suppressing mTOR activities [20]. The immunostimulatory effect of mTOR inhibition can be explained by increased activation of nuclear factor- $\kappa$ B [21], [22], [23] and inhibition of signal transducers and transcription activator 3 and glycogen synthase kinase 3 $\beta$  [24]. The effect of quercetin in modulating the immune system has been shown in previous studies that found a decrease in CD8 T cells thereby increasing the immune response [25].

Flavonoids are known to inhibit cancer cell proliferation, inhibit the cell cycle, induce apoptosis, and inhibit angiogenesis [26]. Flavonoids modulate the immune system, but its molecular mechanism remains unknown. Flavonoids suppress the PI3k/Akt/mTOR pathway which can decrease effector T cell differentiation and increase Treg cells [20]. This increase in apoptosis by flavonoids is thought to improve markers of aging which modulate CD28 expression characterized by increases in CD4<sup>+</sup>CD28<sup>+</sup> and CD8<sup>+</sup>CD28<sup>+</sup>.

Flavonoids are known to have antioxidant activity that can protect the body from oxidative damage caused by reactive oxygen species or reactive oxygen species (ROS). Antioxidants are able to donate one or more electrons they have to free radicals. The body is naturally able to produce antioxidants but sometimes the number of antioxidants produced is unable to neutralize free radicals. Therefore, the body requires exogenous antioxidants or intake of antioxidants from outside the body [27]. The flavonoids and tannins contained in the sample are phenolic compounds that are very reactive donating hydrogen atoms to free radicals so that these compounds are classified as primary antioxidants. Primary antioxidants are chain-breaking antioxidants that can react with free radicals to form stable radicals [28]. The flavonoid and triterpenoid

compounds contained in *D. pentandra* are able to act as ROS scavenger, inhibit enzymes in ROS formation, and prevent cellular and extracellular oxidation events. Flavonoids are able to inhibit cancer cell proliferation, inhibit the cell cycle, induce apoptosis, and inhibit angiogenesis [26].

Until now, it is not certain that the mechanism of the ethanol extract of mango parasite leaves in increasing CD4<sup>+</sup>CD28<sup>+</sup>, CD8<sup>+</sup>CD28<sup>+</sup>, and IL-2 levels; however, the active compounds of flavonoids in mango parasites have been shown to act as immunomodulators through several mechanisms. These mechanisms include *D. pentandra*'s potential to attenuate neutrophil migration and infiltration. Quercetin in *D. pentandra* extract is also able to increase regulatory T cells (CD4 + Foxp3 +), which increases IL-10 secretion and reduces Th17 cells. Th17 cells secrete IL-17, which is capable of acting as a neutrophil chemoattractant [29]. This increase in Treg has a good impact on aging conditions.

Other than that, the inhibitory effect of quercetin contained in *D. pentandra* on inflammatory responses can also occur due to the blocking of Kappa B (IKB) by phosphorylation inhibitors. This plant is also able to induce p53 expression, which is useful for overcoming excessive cell inflammation and preventing cancer cell proliferation by inhibiting it in the S phase. *D. pentandra* is able to reduce IL-22, which regresses the presence of breast cancer cells. A decrease in the amount of IL-22 causes a decrease in COX-2 and proinflammatory cytokines [19]. This reduction in proinflammatory cytokines improves the decreased immune response to immunosenescence, thereby reducing the risk of developing infectious diseases.

*D. pentandra* also has the ability to function as an immunomodulator by increasing the proliferation of splenocytes and thymocytes, which increases the potential for the treatment of cancer cells [30]. In addition, *D. pentandra* has great potential for cancer treatment since it does not induce cytotoxic activity in normal cell lines [26]. The increase in the percentage of CD4<sup>+</sup>CD28<sup>+</sup> and CD8<sup>+</sup>CD28<sup>+</sup> by ethanol extract of mango mistletoe leaves has positive implications for the provision of vaccination in the elderly. It is noteworthy that there has been a decreasing immune response of the elderly population to vaccination [31], [32] due to the suppressant effect of vaccines [33], especially a decrease in the cytolytic capacity of CD8<sup>+</sup> [32] T cells.

The administration of mango parasite extract was also proven to provide changes in the liver hepatology of codeine-induced rats [34]. The administration of mango parasite leaf extract orally at doses of 150, 300, and 600 mg/kg for 7 days in colitis-induced mice with 2,4,6-trinitrobenzene sulfonic acid per rectal (TNBS) gave the therapeutic effect of the extract depending on dosage; the extract provides strong protection against TNBS-induced colonic damage. Colon injury due to TNBS administration is also characterized by increased myeloperoxidase

activity (MPO) which indicates neutrophil infiltration in the inflamed tissue. TNBS-induced mice showed the highest MPO activity in the large intestine. MPO levels in the 300 and 600 mg/kgBW extract groups decreased significantly, whereas treatment with a dose of 150 mg/kgBW did not affect MPO levels in mice with TNBS-induced colitis. In addition, administration of mango parasite leaf extract at all doses can inhibit Th17 but increase the Treg response in TNBS-induced colitis [29]. Toxicity test of mango parasite leaf extract which was carried out on mice with various doses for 14 days showed the acute toxicity value (LD50) of male mice was 34.28 g/kgBW, while in female mice it was 22.41 g/kgBW [35].

Loss of CD28 with aging also results in decreased IL-2 levels [36], which results in inadequate immune response [37]. The results of this study indicated that the ethanol extract of mango mistletoe leaves was proven to increase IL-2 levels. Mango mistletoe leaves contain flavonoids [19]. Flavonoids have been shown to increase NFAT, which plays a role in the formation of IL-2. Increased NFAT activates the IL-2 promoter so that IL-2 transcription increases [38]; however, the mechanism of flavonoids to increase IL-2 definitely still needs further research.

Increased IL-2 has a positive effect on the elderly since it can improve response to vaccines [36]. Therefore, the results of this study indicate that the administration of ethanol extract of mango mistletoe leaves has a good potential to increase the immune response as indicated by an increase in CD4<sup>+</sup>CD28<sup>+</sup>, CD8<sup>+</sup>CD28<sup>+</sup>, and IL-2. Nevertheless, further research is needed before its application for immunosenescence therapy in humans.

## Conclusion

The results of this study indicate that the ethanol extract of mango mistletoe leaves has a good potential to inhibit the aging process in the immune system based on the observable changes in aging markers, including increased levels of IL-2 and an increase in the percentage of CD4<sup>+</sup>CD28<sup>+</sup> and CD8<sup>+</sup>CD28<sup>+</sup> of T cells.

## Acknowledgment

The authors would like to express their gratitude to Mr. Bambang for his technical assistance in flow cytometry analysis and Ms. Lilis for her technical assistance in ELISA analysis.

## Declarations

### **Ethic approval and consent to participate**

The study was approved by the Ethical Committee of Universitas Brawijaya (ethical number: 161/EC/KEPK/09/2020).

## Author Contribution

This study was carried out in collaboration between all authors. KH: Designing the study, supervising the study, editing manuscript. MZP: Designing the study, supervising the data analysis, editing manuscript. IAS: Extracting *D. pentandra*, administering *D. pentandra*, and analyzing data. MGY: Extracting *D. pentandra*, administering *D. pentandra*, and analyzing data. NPS: Extracting *D. pentandra*, administering *D. pentandra*, and analyzing data. MBS: Extracting *D. pentandra*, administering *D. pentandra*. SH: Extracting *D. pentandra*, administering *D. pentandra*. EN: Supervising the research. ATE: Supervising the research. YI: Supervising the research.

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