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Antioxidant and Antiproliferative Activities of Methanol Extract from *Melaleuca cajuputi* subsp. Cumingiana [Turcz.] Fruit

Aktivitas Antioksidan dan Antiproliferatif Ekstrak Metanol Buah Melaleuca cajuputi subsp. Cumingiana [Turcz.]

Isnaini[1], Rosinta Dewi Achmadiyah[2], Gelvia Awaeh[2], Husnul Khatimah[3], Alfi Yasmina[1]

[1]Departement of Pharmacology and Therapeutics, Faculty of Medicine, Lambung Mangkurat University, Banjarmasin, Indonesia
[2]Medical Study Program, Faculty of Medicine, Lambung Mangkurat University, Banjarmasin, Indonesia
[3]Departement of Biomedic, Faculty of Medicine, Lambung Mangkurat University, Banjarmasin, Indonesia

Email: isnaini@ulm.ac.id

ABSTRACT

Melaleuca cajuputi subsp. Cumingiana [Turcz.] Barlow (*M. cajuputi*) is widely available in Banjarmasin. *M. cajuputi* contains phytochemical compounds in the form of polyphenols, including flavonoids, quinones, saponins, and alkaloids, that have antioxidant and antiproliferation activities. This research aimed to analyze the antioxidant and antiproliferation activity of *M. cajuputi* fruit methanol extract. The antioxidant activity was tested employing the DPPH method. The activities were observed in IC_{50} and measured using the UV-VIS spectrophotometer at 517 nm. The antiproliferation was conducted using true experimental with a post-test. This study used 30 mature zebrafish (length >2.5 cm), which were grouped into four, namely the negative control group (DMSO 0.05%), the methanol extract group of *M. cajuputi* with concentrations of 18.5 ppm, 37 ppm, and 74 ppm. The bound variable in this study was antiproliferation activity in the tail of an amputated fish. Data analysis was measured by one-way ANOVA and post-hoc Tukey HSD tests. The phytochemical test indicates the presence of phenol compounds, quinones, flavonoids, alkaloids, saponins, tannins, steroids, and terpenoids. The IC_{50} of the methanol extract of *M. cajuputi* fruit was 15.50 ppm (95% CI 8.31- 32.72). The antiproliferation activity of zebrafish tails increased in the administration of 74 ppm ($p < 0.05$) *M. cajuputi* fruit extract on day four and day eight of measurement compared to negative controls. It can be concluded that the methanol extract of *M. cajuputi* fruit has antiproliferative activity against the growth of amputated zebrafish tails.

Keywords: antioxidant, antiproliverative, *Melaleuca cajuputi,* zebrafish, amputated tail.

ABSTRAK

Melaleuca cajuputi subsp. Cumingiana [Turcz.] Barlow (*M. cajuputi*) merupakan tanaman yang mudah ditemukan di Banjarmasin, Kalimantan Selatan. *M. cajuputi* mengandung senyawa fitokimia polifenol antara lain flavonoid, kuinon, saponin, dan alkaloid yang diduga memiliki aktivitas antioksidan dan antiproliferasi. Penelitian ini bertujuan untuk mengetahui aktivitas antioksidan dan antiproliferasi ekstrak metanol buah *M. cajuputi*. Uji aktivitas antioksidan menggunakan metode DPPH. Aktivitas diamati di IC_{50} dan diukur

menggunakan spektrofotometer UV-VIS pada panjang gelombang 517 nm. Uji antiproliferasi menggunakan true eksperimental dengan post-test. Hewan yang digunakan dalam penelitian ini adalah 30 ekor zebra fish dewasa (panjang > 2,5 cm) yang dikelompokkan menjadi 4 yaitu kelompok kontrol negatif (DMSO 0,05%), kelompok ekstrak metanol *M. cajuputi* dengan konsentrasi 18,5 ppm, 37 ppm dan 74 ppm. Analisis data diukur dengan uji one-way ANOVA dan Post-Hoc Tukey HSD. Hasil uji fitokimia menunjukkan adanya senyawa fenol, kuinon, flavonoid, alkaloid, saponin, tanin, steroid dan terpenoid. Ekstrak metanol buah *M. cajuputi* berada pada IC_{50} sebesar 15,50 ppm (95% CI 8,31- 32,72). Aktivitas antiproliferasi ekor ikan zebra meningkat pada pemberian ekstrak buah *M. cajuputi* dalam konsentrasi 74 ppm ($p < 0,05$), baik pada hari ke-4 dan hari ke-8 pengukuran bila dibandingkan dengan kontrol negatif. Dapat disimpulkan bahwa ekstrak metanol buah *M. cajuputi* memiliki aktivitas antiproliferatif terhadap pertumbuhan ekor ikan zebra yang diamputasi.

Kata kunci: antioksidan, antiproliveratif, Malaleuca cajuputi, ikan zebra, ekor yang diamputasi.

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1. INTRODUCTION

Indonesia is the second largest biodiversity country in the world, with various plants containing various natural active ingredients beneficial to the human body. One of the most common plant genera found in almost every area of South Kalimantan is the genus Melaleuca. There are about 300 species in this genus. One of the species found in South Kalimantan is *M. cajuputi* (Al-Abd *et al.*, 2015; Takao *et al.*, 2015).

This plant contains phytochemicals such as flavonoids, quinones, saponins, and alkaloids in polyphenols (Wardhani *et al.*, 2018a, Wardhani *et al.*, 2018b). These four compounds are found in cancer because they induce electrogenic compounds, protective enzymes with conjugated activity, autophagy, increase apoptosis rates, inhibit cell proliferation, and metabolize carcinogens and tumorigenesis. It is believed to have great prophylactic and therapeutic effects-regulates expression, inhibits lipid peroxidation, inhibits angiogenesis, and inhibit DNA oxidation (Ren *et al.*, 2003). In addition, the four compounds also serve as antioxidants.

Antioxidant compounds have a significant influence on the prevention and treatment of cancer. Antioxidants can suppress oxidative stress to reduce the activation of cancer cells' prosurvival factors, such as NFkB and AP-1, and increase the activation of the p53 tumor suppressor gene. Small levels of oxidative stress can induce the proliferation of cancer cells. Therefore, antioxidants are vital for cancer patients. Epidemiological studies have also shown that a diet rich in polyphenols affects cancer risk. In particular, these four polyphenolic compounds can exert anticancer effects through mechanisms that include antioxidant and antiproliferative activities as well as their effects on subcellular signaling pathways, apoptosis, and induction of cell cycle shortening (Zakaria *et al.*, 2011).

One of the animal models used for preliminary study on anticancer drugs is zebrafish (*Brachydanio rerio*). The early stages of regeneration in amputated zebrafish tail fins have similarities to the development of cancer cells. Hence, the growth of the amputated zebrafish tail can be used as a model to study the antiproliferative effect of the fruit extract of *M. cajuputi* (Muñoz *et al.*, 2009). This study was conducted to prove the antiproliferative activity of the methanol extract of the fruit of *M. cajuputi* in inhibiting the growth of the amputated zebrafish tail. A close relationship between cancer and antioxidants has also encouraged researchers to examine the antioxidant activity of the

methanol extract of *M. cajuputi* using the DPPH method.

2. MATERIALS AND METHODS

2.1. Materials

The material used in this study was 1 kg of the young fruit of *M. cajuputi* (Fig. 1), methanol 50% technical grade, methanol grade p.a, DMSO, ketamine injection, sterile distilled water, Mayer reagent, Dragendorff reagent, Pb acetic acid 10%, NaOH, Gelatin 1%, FeCl3 3%, HCl benzene, chloroform, anhydrous acetic acid, and concentrated H_2SO_4 and DPPH.

2.2. Material collection and extraction

The young fruit of *M. cajuputi* was obtained from Jalan Kayutangi, Banjarmasin city, South Kalimantan (3°17'50.1"S 114°35'07.8"E). Verification of the *M. cajuputi*'s fruit was conducted by the Faculty of Mathematics and Natural Sciences, Lambung Mangkurat University, with certificate number 0.65a/LB.LABDASAR/III/2020. The young fruit of *M. cajuputi* was air-dried on paper at room temperature until dry. After drying, the fruit was crushed and sieved to become homogeneous simplicia. The simplicia was soaked in 50% methanol and 0.5% acetic acid. The immersion was performed three times. The thick methanol extract obtained was evaporated with a rotary evaporator at 60ºC, then re-evaporated with a water bath at 60ºC. It was then freeze-dried to obtain a concentrated extract (Dahlan, 2014).

2.3. Antioxidant Activity

Two mL of each methanol extract of *M. cajuputi* fruit (concentrations: 1, 5, 10, 15, 25, and 50 ppm) was added with 2 mL of 50 ppm DPPH solution. The mixture was then incubated for 30 minutes in a dark room. The absorbance was measured using a UV-VIS spectrophotometer at 517 nm. The formula used to calculate % damping is as follows: (Tiwari *et al.*, 2011).

$$DPPH_{radical\ scavenging\ act.}(\%) = \frac{A_{ctrl} - A_{sample}}{A_{ctrl}} \times 100\%$$

The percentage of free radical attenuation in the fruit extract of *M. cajuputi* was used to determine the IC_{50} value using probit analysis. The classification of IC_{50} values was determined based on Table 1.

Table 1 Classification of Antioxidant Activity

No.	Value of IC_{50}	Antioxidant activity
1	<50	Very strong
2	50-100	Strong
	100-150	Moderate
3	151-200	Weak

Figure 1. Young fruit of *M. cajuputi*

2.4. Antiproliferation Test

The sample was divided into four groups: the DMSO negative control group (0.05%, DMSO), the *M. cajuputi* group with concentrations of 18.5 ppm (EMFM 18.5), 37 ppm (EMFM 37), and 81 ppm (EMFM 81). The *M. cajuputi* group was added with 0.05% DMSO. This study obtained an ethical clearance letter from the Ethics Commission of the Faculty of Medicine, Lambung Mangkurat University No. 457/KEPK-FKUNLAM/EC/XI/2020.

Zebrafish were reared for one week at room temperature before receiving treatment to provide the same physical and psychological conditions. During maintenance, fish were fed 2-3 times a day. Before being treated with tail amputation, zebrafish were anesthetized with the anesthetic solution of ketamine at a dose of 2 mL/L for two minutes to reduce movement and stress. After being anesthetized, the fishtail was amputated by placing it on a petri dish with a little puddle of water and cut straight with a scalpel from top to bottom. Each of the amputated fish was put into four different aquariums containing 1 L of treatment solution. Each aquarium contains six zebrafish. The aquarium water was changed every two days. The length of the zebrafish's tail was measured at four and eight days after the amputation. Measurements were made using a caliper (millimeter scale).

2.5. Data statistical analysis

Data measurements of fishtail length were evaluated statistically. Normality and homogeneity analysis were performed. The normality of the data was based on the Shapiro-Wilk normality test (sample <50), and the homogeneity was based on the Rubin variance test. Since the study data was normal and evenly distributed, the parametric analysis was performed using a one-way ANOVA and a post-Tukey HSD test with a 95% confidence level ($\alpha = 0.05$) (Dahlan, 2014).

3. RESULTS AND DISCUSSION

3.1. Results

Based on the phytochemical tests (Table 2), it is known that the methanol extract of the fruit of *M. cajuputi* contains alkaloid compounds, flavonoids, polyphenols, saponins, quinones, steroids, terpenoids, and tannins. These results differed from Wardhani *et al.* (2018). The difference might be caused by the difference in each plant's growth site and soil nutrients. In this research, *M. cajuputi* was obtained from the Banjarmasin area, while Wardhani's research was obtained from the Palangkaraya area (Wardhani *et al.*, 2018a; Zafrial & Amalia, 2018).

After a phytochemical screening test, methanol extract was made into various concentrations and reacted with DPPH. Based on the probit analysis, the IC_{50} value of the methanol extract of *M. cajuputi* subsp Cumingiana [Turcz.] Barlow fruit was 15.50 ppm (95% CI 8.31 - 32.72).

Table 2. The Phytochemical Screening Tests of *Melaleuca cajuputi*

No	Compound Type	Reagen	Results	Wardhani *et al.* 2018
1	Polyphenol	$FeCl_3$ 3%	+	+
2	Flavonoids	Pb acetate	+	+
		Alkaline Reagent	+	+
3	Alkaloids	Mayer Reagent	+	-
		Reagen Dragendorft	+	-
4	Quinone	Benzene + ammonia	+	+
5	Saponins	Aquadest	+	+
6	Steroids	*Libermann Burchard's test*	+	-
7	Terpenoids	*Salkowski's test*	+	-
8	Tannin	Gelatine Solution 1%	+	-

Description:
(+) = contains secondary metabolites
(-) = does not contains secondary metabolites

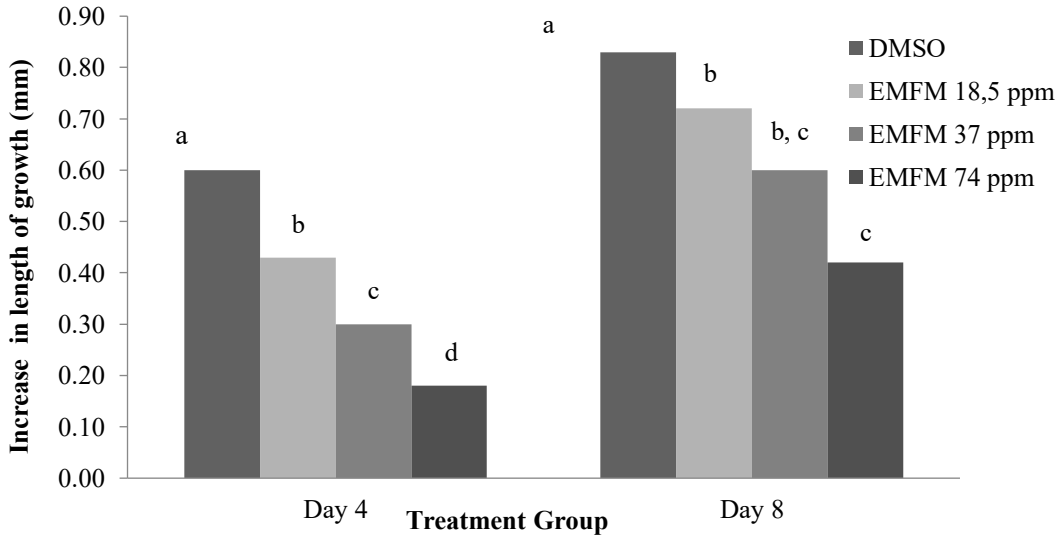

Figure 2. The average growth length of the amputated zebrafish tail in each treatment group (significant difference $p<0.05$)

Fig. 2 shows that on the fourth day of measurement, all treatments significantly differed in the length of zebrafish tail growth compared with the negative control. Furthermore, only EMFM 74 ppm had a significant difference with the negative control on the eighth day of measurement.

3.2. Discussion

EMFM inhibits the proliferation activity of zebrafish tails. There are three phases of regeneration in the growth of zebrafish tails. The first phase occurred 0-18 hours after amputation in the form of wound healing. Epithelial cells cover the wound by forming an epidermis on the wound. The second phase occurs through blastema formation (18-48

hours after amputation). In the last phase, regenerative growth occurred from 48 hours to 20 days after amputation. During this phase, patterns and differentiation occurred to restore the tissue architecture and function of the tail fin (Khoirunnisa & Sumiwi, 2019; Ren *et al.*, 2003). In this study, observations on the eighth day after amputation showed that the tissue architecture had not fully recovered in the negative control group and EMFM administration. The regeneration process after the zebrafish tail's amputation will return complete in 20 days after amputation (Khoirunnisa & Sumiwi, 2019).

The phytochemical screening of the methanol extract of *Melaleuca cajuputi* indicates the presence of antioxidant compounds, namely steroids, flavonoids, saponins, tannins, phenols, quinones, and terpenoids. In general, the antioxidant mechanism in steroids is not yet clearly known, but some steroids can have natural antioxidant activity, including estriol and 17β-estradiol (Mooradian, 1993). Flavonoids, saponins, and tannins have antioxidant activity by indirect mechanisms (Singh & Chaudhuri, 2018). Phenols, pheninones, tannins, terpenoids, alkaloids, and flavonoids are compounds that have antioxidant activity by the direct mechanism of donating hydrogen to free radicals (Grassmann, 2005; Nweze *et al.*, 2019).

In this study, DPPH acted as free radicals. The higher levels of hydrogen donor compounds, the higher the free radical damping activity, and the lower the IC_{50} value (Kusuma, 2019; Shekhar & Anju, 2014; Widyastuti, 2010). In addition, flavonoid compounds, saponins, quinones, steroids, terpenoids, and tannins are believed to have antiproliferative effects. Flavonoids inhibit ornithine decarboxylase, which plays a role in polyamine biosynthesis, DNA synthesis, and protein synthesis. Flavonoid compounds can also inhibit the cell cycle, either in G1/S or G2/M, by inhibiting cyclin-dependent kinases (CDKs), which are key regulators of cell cycle development. In addition, the flavonoid compounds block growth factor receptors inhibit mitogen-activated protein kinase (MAPK) through tyrosine kinase (RTK) receptor signaling pathways, inhibition of DNA activity of topoisomerase I/II, decrease ROS, modulation of apoptosis signaling pathways, activation of caspase-9 and caspase-3, activation of endonuclease, and decrease in Mcl-1 protein (Achmad *et al.*, 2014; Khoirunnisa & Sumiwi, 2019; Man *et al.*, 2010; Zhao *et al.*, 2018).

Furthermore, the methanol extract of Melaleuca cajuputi contains quinones, steroids, terpenoids, saponins, and tannins. Quinones have pharmacological activity as an anticancer. The mechanism of quinones as chemopreventive contributes to inducing apoptosis by stopping cell cycles, regulating carcinogen metabolism, and the expression of ontogenesis (Achmad *et al.*, 2014). Saponins can inhibit cell cycles, trigger autophagy, inhibit angiogenesis, dis-sync the cytoskeleton, inhibit metastasis intrinsically and extrinsically, and activate apoptosis pathways (Man *et al.*, 2010). Both quinones and saponins potentially become antiproliferative through the inhibition mechanism of their cell cycles. Steroids and terpenoids can inhibit the primary mechanisms in cell proliferation and trigger apoptosis and autophagy of cancer cells (Man *et al.*, 2010; Zhao *et al.*, 2018). Tannin compounds have an effect in inhibiting the cancer proliferation process, while in the process, protein kinase will be activated. The protein kinase inhibits the signal transmission pathway from the membrane to the cell nucleus. Tannins inhibit the activity of tyrosine kinase receptors that play a role in the growth of cancer cell malignancies (Firdaus, 2016).

After amputation, the three main regeneration phases are activated in the growth of the zebrafish tail. The first phase occurs 0–18 hours after amputation in the form of wound healing. The epithelial cells will cover the wound by forming the wound epidermis and secreting factors such as Fgf20a and Activin-βA to induce the next phase of the regeneration process. Then, this process is followed by blastema formations (18-48 hours after amputation). In the last phase, regenerative growth occurs from 48 hours to 20 days after amputation. During this phase, patterns and differentiation occur to restore the tissue architecture and tail fin function (Chahar MK *et al.*, 2011; Abidin IZZ *et al.*, 2020). In this research, observations on the eighth day after amputation showed that the tissue architecture had not fully recovered in the negative control group and the extract treatment group with concentrations of 18.5 ppm, 37 ppm, and 74 ppm. It is in accordance with research by Abidin IZZ *et al.* (2020), which states that the regeneration process after the amputation of the zebrafish's tail will be completed 20 days after the amputation (Abidin IZZ *et al.*, 2020).

In this study, the exact amount of anti-proliferative active compounds in *M. cajuputi* is unknown. Therefore, further research is suggested to carry out quantitative tests for the phytochemical content of *M. cajuputi* and trials on human cancer cells.

4. CONCLUSION

M. cajuputi has an antioxidant and anti-proliferation activity based on the phytochemical test conducted (phenol compounds, quinones, flavonoids, alkaloids, saponins, tannins, steroids, and terpenoids).

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