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ANTIOXIDANT AND *In-vitro* CYTOTOXIC ACTIVITY OF TYPICAL ACEHNESE PLANTS AGAINST HUMAN CERVICAL CANCER (HeLa) CELL LINE AND THEIR PHYTOCHEMICAL PROPERTIES

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ABSTRACT

Michelia Alba (*M. Alba*) plant is one of the typical Acehnese plants that has a lot of bioactivity and potential as a basic ingredient for drug manufacture. The aim of this study was to determine the phytochemical properties, antioxidant and in vitro cytotoxic activity of *M. Alba* leaves and flowers against the human cervical cancer (HeLa) cell line. This research was conducted by maceration method, where dry samples were soaked with ethanol solvents, and then crude ethanol extract was tested for antioxidant and in vitro cytotoxic activity against the HeLa cell line. The results of phytochemical properties showed leaves and flowers of *M. alba* contained secondary metabolites including alkaloids, terpenoids, flavonoids, tannins, and saponins. Antioxidant testing using DPPH solution of *M. alba* leaves showed IC50 value was 81.99 and for flowers was 30.18, this result indicates that *M. alba* flowers have very strong antioxidant activity, while the leaves have weak activity. The results of the cytotoxic test on the HeLa cell line from *M. alba* leaves and flowers showed very active where the CD_{50} values were 4 ppm and 2 ppm, respectively.

Keywords: *Michelia Alba,* Antioxidant, Cytotoxic Activity, Hela Cell, Medical Plant.

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INTRODUCTION

One of the biggest users of medicinal plants in the world is Indonesia along with other countries in Asia, such as India and China.[1] Aceh is one of the provinces in Indonesia which has very abundant biodiversity. Many of the plants were used as traditional medicine by the people of Aceh before.[2] The use of medicines has been going on for thousands of years and was used as a plant [3], one of them is Cempaka Putih (*Michelia alba*) this plant is known as "Jeumpa Gadeng" in the Aceh region. Previous studies reported that the *Michelia alba* (*M. alba*) plant has bioactivity as an antibacterial, antifungal, antioxidant, anti-gout, anti-depressant, anti-cytotoxic, and tyrosinase inhibition. Several studies have focused on bioactive compounds found in *Michelia alba* such as linalool (72.8% in flower oil and 80.1% in leaf oil), α-terpineol (6.04% in flower oil), phenylethyl alcohol, β-pinene (2.39 % in flower oil), and geraniol (1.23% flower oil). Specifically, linalool, several studies on linalool have reported anti-cancer, anti-inflammatory, anti-hypertensive, anti-ulcer, anti-hypertriglyceridemia, anti-psoriasis, antidepressant, anti-diarrhea, and antioxidant activities.[4] Antioxidants are substances that can slow down or prevent cell damage due to oxidation by free radicals. Compounds that act as antioxidants are flavonoids and phenolic compounds or polyphenols. Polyphenolic compounds are natural compounds found in plants that act as antioxidants. So far, many studies have shown the natural antioxidant activity of various plants. One of them is a typical Acehnese plant, namely *M. alba*, according to a report from Munira et al., secondary metabolites from the methanol extract of *M. alba* flowers detect the presence of alkaloids, terpenoids, steroids, saponins, phenols, and flavonoids.[5] From the studies that have been conducted, it can be concluded that the *M. alba* plant has potential as an antioxidant agent.

Antioxidant activity is closely related to anticancer or anti-cytotoxic activity. Where compounds that have cytotoxic activity include groups of alkaloid compounds, tannins, flavonoids [6], steroids [7], terpenoids [8] and polyphenols.[9] Cancer is the growth of cell tissue that is not normal and hurts cell function. There are two

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main cases of cancer in Indonesia that cause the highest number of deaths in women, namely breast cancer and cervical cancer. Several methods to cure patients suffering from cancer such as chemotherapy, taking drugs, or surgery. Chemotherapy can also kill normal cells and cause side effects such as hair loss, nausea and vomiting, diarrhea, and others.[10] Each of these methods still has its drawbacks which still require the development of several alternative methods such as herbal medicine.[8] The most common genital cancer and one of the leading causes of death among the female population is cervical cancer. It accounts for approximately 12% of all cancers in women and it is the second most common cancer in women worldwide especially in developing countries, including Indonesia.[11,12,13] The World Health Organization (WHO) reports that cervical cancer is the second most dominant cancer after breast cancer in Indonesian women, with prevalence in women aged 15 to 44 years. A comprehensive statistic estimated in 2012, reports that about 20,928 new cervical cancer cases are diagnosed annually in Indonesia. The incidence rate of cervical cancer is 17 per 100,000 women per year.[13] Based on the description above, this study aims to determine the phytochemical properties, antioxidant and cytotoxic activity in *M. alba* plants, so that can further highlight the potential of *M. alba* plants as candidates for drug discovery in the future and the result from this study can be used as additional references for further studies.

EXPERIMENTAL

Material and Methods

The apparatus used in this study were glassware, filter paper, rotary evaporator Hahnvapor, HS-2005S), incubator, and UV-visible spectrophotometer (Thermo Scientific). The materials used in this study were Roswell Park Memorial Institute 1640 media (RPMI 1640), ethanol solvent, methanol pro-analyst, distilled water, and 1,1-diphenyl-2-picryhydrazyl (DPPH) solution. The bioindicator used is the human cervical cancer (HeLa) cell line.

Extraction

The *M. alba* plant used in this study is the leaves and flowers. Sample preparation was carried out by cleaning and drying at room temperature, then the dry sample was mashed, and macerated using ethanol solvent for 2x24 hours. The macerated sample was filtered using filter paper to obtain a liquid ethanol extract. The liquid ethanol extract was evaporated using a rotary evaporator and crude ethanol extract was obtained. This extract was then tested for antioxidant and anti-cancer activities.

Phytochemical Screening

Alkaloid

M. alba dried crushed and an ammonia solution (3 ml) was added to it. They were allowed to stand for a few minutes. Then chloroform (10 ml) was added to the test tube samples which were shaken and then filtered to separate the residue. The filtrate was added 10 mL of sulfuric acid 2N, shaken vigorously, and left for a minute until the sulfuric acid solution and chloroform separated. The top layer (sulfuric acid layer) is divided into three tubes and each tube is tested by Mayer, Dragendorff's, and Wagner reagents to indicate the presence of alkaloids.

Steroid, Terpenoid and Saponin

The *M. alba* was finely ground and extracted with hot methanol. The filtrate was concentrated with a rotary evaporator to yield methanol extract. Then the methanol extract was partitioned with hexane, and hexane soluble extract was tested with Liebermann-Burchard reagent. The formation of blue or green color indicates the presence of the steroid and the formation of red color indicates terpenoids. The soluble residue in hexane was added to water and shaken vigorously. The formation of a stable form for 30 minutes showed the presence of saponins.

Flavonoid

The *M. alba* plant was extracted with methanol and concentrated with a rotary evaporator. The crude methanol extract was partitioned with n-hexane. The residue was extracted with 10 mL of 80% ethanol, then 0.5 g of magnesium and 0.5 M HCl were added, if a positive pink or purple color indicated the presence of flavonoids.

Phenolic
The *M. alba* extract was tested by a Ferric Chloride reagent. Then 3 – 4 drops of $FeCl_3$ solution were into an extract, and the formation of a bluish-black colour indicates the phenol compound.

Tannin
M. alba flowers and leaves were boiled in 10 ml of water in the test tube and then filtered. Then added a few drops of $FeCl_3$ 0.1%. The formation of a brownish-green or bluish-black color indicates a tannin compound.

Antioxidant Test Using DPPH
About 2.5 mg of *M. alba* crude ethanol extract was added with 5 mL of methanol to obtain a sample concentration of 500 ppm. The dissolved sample was diluted to concentrations of 5, 10, 25, 50, and 100 ppm. Then 0.6 mL of DPPH 0.4 mM solution was added to each tube, then methanol was added to the 3 mL mark in a test tube (which was covered with aluminum foil), then homogenized using a vortex mixer and incubated for 30 minutes at 37 ºC. Then the absorbance was measured using a UV-visible spectrophotometer at the maximum wavelength (517 nm).

MTT Assay
The cytotoxic activity in this study was treated against the breast cancer (MCF-7) cell line. The procedure of the test according to Amna et al.[25]

RESULTS AND DISCUSSION

The flowers and leaves from the *M. alba* plant are cleaned beforehand with the aim of removing dirt adhering to the sample and dried at room temperature to avoid decay and fungal growth in the sample which can change the chemical compound in it. Then the sample is mashed to make the surface area of the sample larger so that the solvent can easily extract the sample. The sample is macerated using ethanol solvent with the aim of extracting all the contents contained in the sample, both polar and non-polar compounds, the maceration process for 2x24 aims to maximize the process of extracting chemical compounds contained. After that, the sample was filtered which aims to separate the filtrate and residue, the filtrate was concentrated using a rotary evaporator to evaporate the solvent and obtain crude ethanol extract. The yield value (%) obtained from the ethanol extract of *M. alba* leaves was 7.021% from a 200 g dry sample, and the yield value of *M. alba* flowers was 29.71% from a dry sample as much as 44.48 grams.

Phytochemical Screening
Phytochemical screening was carried out as a preliminary test to determine the group of secondary metabolites contained in the sample. The phytochemical screening tests included tests for alkaloids, saponins, terpenoids, steroids, flavonoids, phenols, and tannins. The test results can be seen in Table-1.

Table-1: Phytochemical Screening of Leave and Flower *M. alba*

Secundary Metabolites		*M. alba* Leave	*M. alba* Flower
Alkaloid	Mayer	+	+
	Wagner	+	+
Terpenoid		+	+
Steroid		-	-
Flavonoid		+	+
Saponin		-	-
Tanin		+	+
Saponin		+	+
Fenol		-	-

Based on the results of phytochemical testing, it was shown that the leaves and flowers of *M. alba* contain compounds belonging to alkaloids, terpenoids, flavonoids, tannins, and saponins. Previous research also reported that the leaves and flowers of *M. alba* contain the same secondary metabolites. The results of phytochemicals from a plant can be a reference for the activity of the plant. The results of this study are also in line with the results of research from Munira *et al*.[6] which obtained the same phytochemical results. Flavonoids are thought to have antioxidant and anti-cytotoxic activities.[5] Terpenoids are secondary

metabolites essential for plant growth and development which contribute to the aroma, flavor, and color of the plant. They protect the plant from environmental stress, pests, and microorganisms.[24] Terpenoid group compounds show interesting pharmacological activities as antiviral, antibacterial, anti-inflammatory, inhibitors of cholesterol synthesis, and anticancer .[14]

Antioxidant Activity

The results of antioxidant testing on the ethanol extract of *M. alba* leaves and flowers can be seen in Table-3 and Fig.-3 below. Each sample was made with different concentration variations for the antioxidant activity test, namely 5, 10, 25, 50, and 100 ppm and DPPH solution with a concentration of 0.4 mM. Based on Table-2. It can be seen that only *M. alba* flowers have a strong antioxidant activity with an IC_{50} value is 30.18 ppm, while the leaves have a weak category of antioxidant activity with an IC_{50} value is 81.99 ppm. The results on *M. alba* flowers which are suspected of having potential as antioxidants are also proven by Suryani *et al*. [15] with IC_{50} values for methanol extracts is 11.04 ppm and 4.61 ppm for ethyl acetate extracts. Multiple studies have revealed the antioxidant activity of M. alba metabolites, including geraniol, α-terpineol, linalool, and (−)-N-formylanonaine, in addition to the antioxidant activity of M. alba extract. In contrast to ascorbic acid, which had a stronger scavenging ability (IC50 value: 52.1 μM), (−)-N-formylanonaine was shown to impede the detoxification of DPPH free radicals with an IC50 value: 121.4 μM.[16]

Table-2: Antioxidant Activity of Ethanol Extract of *M. alba* Leaves and Flower

Sample		Concentration (ppm)	Absorbance Average	% Inhibition	IC_{50} Value (ppm)
Michelia alba Plant	Flower	5	0.490	-3.1	30.18
		10	0.360	24.21	
		25	0.083	82.52	
		50	0.048	89.89	
		100	0.055	88.42	
	Leave	5	0.463	2.11	81.99
		10	0.455	3.8	
		25	0.367	22.41	
		50	0.361	25.28	
		100	0.179	62.15	

On the other hand, Chaudhari and colleagues reported the antioxidant activity of α-terpineol using ABTS and DPPH free radical scavenging assays. The study showed that α-terpineol has an IC50 value of 7.25 μL/mL for ABTS analysis and scavenges DPPH free radical with an IC50 value of 57.86 μL/mL. Encapsulation of α-terpineol with chitosan nanoemulsion enhanced DPPH free radical scavenging activity with an IC50 value of 39.57 μL/mL.[17] The bioactivity of a plant cannot be separated from the compounds contained in it, according to research Ueyama *et al.*[4] *M. alba* flowers contain linalool as a major component, and several minor components including α-terpinol, β-phenyl ethyl alcohol, β- pinene and geraniol. These compounds are thought to be antioxidant agents by slowing down or preventing the occurrence of free radicals by donating H atoms to free radicals.[18]

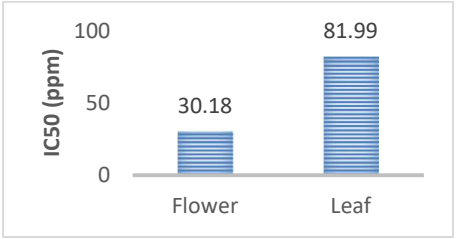

Fig.-1: IC50 Value of Extract Ethanol of *M. alba* Leaves and Flower

Oxidative stress has been recognized as one of the classical risk factors for human diseases such as cardiovascular diseases, cancers, and neurodegenerative diseases. In biological systems, macromolecules such as lipids, proteins, and nucleic acids are prone to oxidation upon exposure to free radicals. Excessive

production of free radicals and a low antioxidant level collectively contribute to oxidative stress leading to a negative impact on physiological function.[19]

Cytotoxic Activity

The cytotoxic activity of the ethanol extract in this study was tested on the leaves and flowers of *M. alba*. The ethanol extract of these two samples was evaluated for cytotoxic activity against the HeLa cell line. Vincristine is a conventional cancer drug used as a positive control against HeLa cells and shows a CD_{50} value of 0.6 µg/ml. The cytotoxicity of the extract was assayed at various concentrations of 1000, 500, 100, 50, 20, 10, 5, and 1 µg/ml under continuous exposure for 72 h, expressed in CD_{50} values and summarized in Table-3. Then, viability data is plotted with concentration doses to determine CD_{50} (Fig.-2). Results showed that CD_{50} represents the extract concentration doses that reduced the mean absorbance at 595 nm to 50% of those in the untreated control wells. The CD_{50} value was obtained from the plot of the concentrations of extract versus percent of cell viability. The value was used to describe the degree of cytotoxicity of the extract towards cell lines.

Table-3: Cytotoxic Activity of Ethanol Extract of *M. alba* Leaves and Flowers Against HeLa Cells

HeLa Cells (µg/ml)	*M. alba* Leave		*M. alba* Flower	
	Average	% Viability	Average	% Viability
1000	0.019	2.925	0.019	2.824
500	0.047	7.060	0.035	5.295
100	0.242	36.611	0.139	20.978
50	0.263	39.778	0.154	23.248
20	0.326	49.269	0.202	30.560
10	0.312	47.151	0.231	34.874
5	0.440	66.616	0.367	55.572
1	0.662	100.000	0.661	100.000

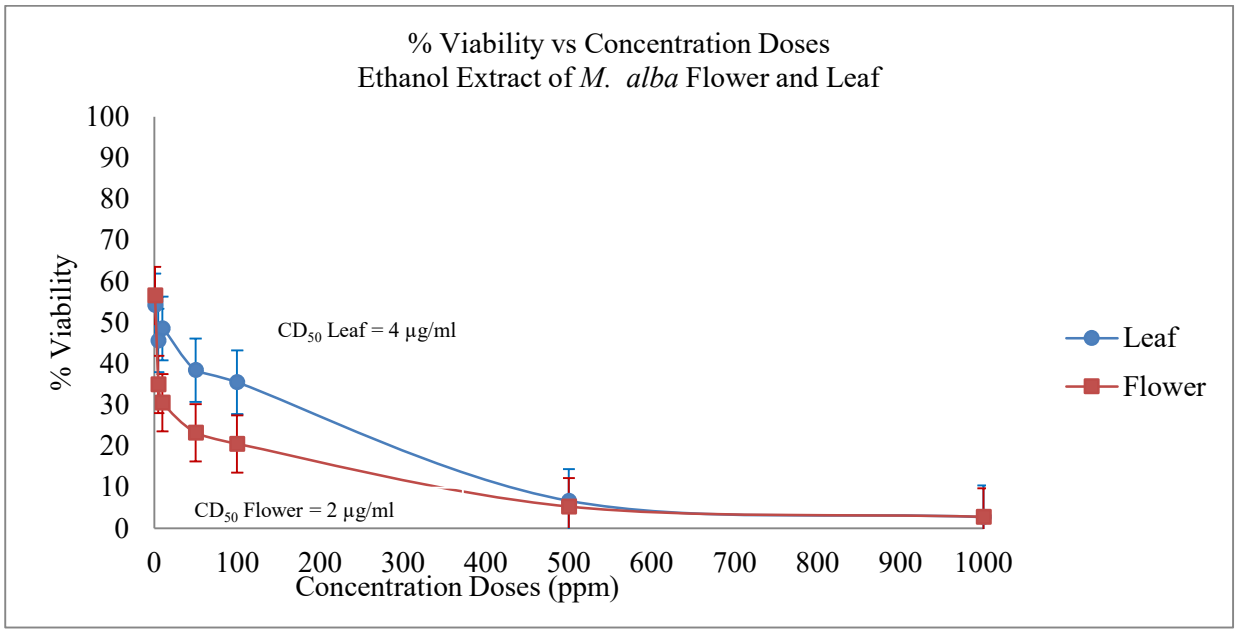

Fig.-2: Viability (%) vs Concentration Doses (µg/ml) of ethanol extract of *M. alba* Leave and Flower

Based on Table-3 and Fig.-2. It shows that the ethanol extract of *M. alba* leaves and flowers has very active cytotoxic activity. Compounds that have very active cytotoxic activity have a CD_{50} value <5.0 µg/mL, compounds with a CD_{50} value <10 µg/mL are categorized as having moderate activity, and compounds with a CD_{50} value between 10-25µg/mL are categorized as having weak cytotoxic activity.[25] The potential anti-cancer activities of *M. alba* may be attributed to two of its bioactive compounds, including linalool and (−)-anonaine. Linalool is the most abundant component in the flower oil (72.8%) and leaf oil (80.1%) of *M.*

alba.[4] It was reported to exhibit anticancer properties and induce apoptosis in the U937 leukemia cells and HeLa cervical cancer cells. The anticancer activity may be associated with the expression of cyclin-dependent kinase inhibitors (CDKIs) as well as the non-expression of cyclin-dependent kinases (CDK) activit.[21] All of these results point to the anti-cancer qualities of M. alba's main bioactive ingredients, linalool and (−)-anonaine, which could be turned into medicinal bioproducts. Particularly, Han *et al.* created linalool-incorporated nanoparticles that showed anti-cancer effects in patient-derived xenograft animal models of epithelial ovarian cancer as well as cell lines (including HeyA8, A2780, and SKOV3ip1).[22]

CONCLUSION

M. alba plant contains secondary metabolites which are thought to have potential as antioxidants and anti-cytotoxic. Antioxidant test results of *M. alba* flowers showed very strong activity, while the leaves had weak activity. Test results of anti-cytotoxic activity M. alba plants showed very active activity. Therefore *M. alba* plant has a potential as a natural medicine base ingredient.

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CONFLICT OF INTERESTS

We have no conflicts of interest to disclose. All authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

All the authors contributed significantly to this manuscript, participated in reviewing/editing, and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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