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Antioxidant Activity of N-Hexane Extract from Tobacco Leaves (*Nicotiana tabacum* L.) Using the 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Method

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Abstract

Tobacco (*Nicotiana tabacum* L.) is a commercial plant that is often used for making cigarettes. In 2012, the government issued regulations for the diversification of tobacco products, in addition to cigarettes. Some of these diversified tobacco products include organic pesticides, anesthetics, cosmetic ingredients, and biochar as an alternative to coal. This is because tobacco also contains compounds such as phenol derivatives that have pharmacological activities. Therefore, to further diversify tobacco products, this study aimed to determine the antioxidant activity of n-hexane extracts from leaves using the Soxhlet extraction method. Antioxidant activity was assessed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) method, while the total phenolic content was determined by the Folin-Ciocalteu method, which allowed for the quantification of phenolic compounds in the extract, thereby confirming its antioxidant role. The IC_{50} value obtained from the antioxidant activity assay of the n-hexane extract of tobacco leaves was 426.042 µg/mL, indicating a very weak antioxidant activity. Meanwhile, the total phenolic content was 51.93 mg GAE/g. These results suggest that the high total phenol content may have other potential activities that can be tested, such as antibacterial activity.

Keywords: Antioxidant, total phenolic content, tobacco

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Introduction

A free radical is a very reactive molecule that consists of an unpaired electron in the outermost shell of electrons, also known as the valence shell. In the human body, the reactive form of free radicals is a byproduct of metabolic processes involved in cell respiration, phagocytosis, and prostaglandin synthesis[1]. The excessive amount of free radicals in the body causes stress oxidative, the imbalance between free radicals and endogenous antioxidant such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT), which can lead to several disease like the formation of atherosclerosis plaque, hyperlipidemia, dysfunctional kidney, and cancer[2].

Antioxidants are compounds that can neutralize free radicals within cells. Antioxidants protect against cell damage caused by free radicals[3]. Antioxidants can be classified into several categories. Based on their source, antioxidants are grouped into endogenous and exogenous antioxidants. Endogenous antioxidants are a group of enzymes produced by the body that act as antioxidants, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSHpx). Meanwhile, exogenous antioxidants are derived from the food we consume, such as vitamin C, vitamin E, and provitamin A. Based on their activity, antioxidants are categorized into two main types: enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants work by destroying and eliminating free radicals, while non-enzymatic antioxidants work by disrupting the reactions of free radicals[4].

Tobacco (*Nicotiana tabacum* L.) is a commercial plant that is often used for making cigarettes. The use of tobacco has been recognized for a long time, contributing to the increase in gross national income. In 2021, Indonesia ranked 4th among countries that produce tobacco, after China, India, and Brazil. The total production of tobacco in Indonesia reached 237,000 tons, accounting for around 5.03% of total tobacco production worldwide[5]. Similarly, antioxidant activities have been identified in various parts of tobacco, such as the leaves, roots, and stems[6]. Chemical analyses have shown that tobacco leaves are rich in sterols, diterpenoids, sesquiterpenes, alkaloids (including nicotine, a biomarker of tobacco), and phenolic compounds such as flavonoids, phenolic acids, and coumarins. Consequently, tobacco leaves may serve as neuroprotective, antioxidant, anti-inflammatory, and antiparasitic agents[7]. A previous study of the n-hexane extract of tobacco roots demonstrated excellent antioxidant activity, with an IC_{50} value of 2 µg/mL, comparable to that of Trolox, the standard[8].

This study aims to provide scientific support for the antioxidant properties of tobacco leaves by determining the IC_{50} value and total phenolic content (TPC). By investigating the antioxidant activity of the n-hexane extract of tobacco leaves, the research will offer valuable insights into its effectiveness. Additionally, the study will contribute information on the potential for diversifying tobacco products beyond cigarettes, highlighting opportunities for industry and research development.

Method

Preparation

The tobacco leaves were collected from Jember Regency, East Java, Indonesia. They were identified at the Bandungense Herbarium, Biology Study Program, School of Life Sciences and Technology, Bandung Institute of Technology. The sample was later cut into small pieces and dried for the extraction process.

Extraction of Tobacco Leaves

Approximately 200 grams of dried leaves were inserted into a Soxhlet extractor, which was connected to n-hexane as the solvent (ratio 1:10). The obtained extract was then concentrated using a rotary evaporator for 15 minutes, until half of the solvent had evaporated. The filtrate was concentrated using a water bath at 50°C until a thick extract was obtained[9].

Phytochemical Screening

Phytochemical screening was conducted on both the dry leaves and the n-hexane extract of tobacco leaves. Some of the compounds that were identified include:

Alkaloid Test

About 500 mg of sample was stirred with a few drops of 2N HCl and 9 mL of aquadest, then heated on the water for 2 minutes. The mixtures were then cooled and filtered. The filtrate was used to perform a test with various reagents (Table 1)[10].

Phenolic Test

Approximately 1-2 mL of sample was added to 2 mL of water, and 1 to 2 drops of diluted ferric chloride solution were added. A dark green or blue-green coloration indicates the presence of phenolic[11].

Flavonoid Test

About 0.5 g of sample was shaken with petroleum ether to remove the fatty materials (lipid layer). The defatted residue was dissolved in 20 ml of 80% ethanol and filtered. Approximately 3 mL of filtrate was treated with 1 mL of 10% NaOH solution. The formation of an intense yellow color indicated the presence of flavonoids[12].

Tanin Test

A small quantity of sample is dissolved in distilled water, and 2 ml of 1% solution of gelatin containing 10% sodium chloride is added to it. The appearance of white precipitate indicated the presence of tannins[11].

Anthraquinones Test

Approximately 1.0 g of sample was boiled in 6 mL of 1% HCl and then filtered. The filtrate was shaken with 5 mL of benzene, and the benzene layer was removed. 10% NH_4OH was added, and the colour in the alkaline phase was observed. Formation of pink/ violet or red colour indicated the presence of anthraquinone[13].

Saponin Test

A small quantity of sample is diluted with distilled water to 20mL. The suspension is shaken in a graduated cylinder for 15 minutes. Foam or froth that is stable for 10 minutes[10].

Monoterpenoids and Sesquiterpenoids Test

A small quantity of sample was ground with ether and then filtered. The filtrate was then placed in an evaporating dish and left to evaporate. Vanillin-sulfuric acid was added to the dish. Positive monoterpenoids and sesquiterpenoids were indicated by an array of colour changes[11].

Steroids and Triterpenoids test

The reaction of Liebermann-Burchard sought steroids and triterpenoids. The sample was dissolved in 2 mL of acetic anhydride and 1-2 drops of concentrated. H_2SO_4. The appearance of a ring of blue-green at the interphase showed a positive reaction[11].

Antioxidant Activity Using DPPH Method

The DPPH was dissolved in methanol to create a stock solution with a concentration of 60 µg/mL. This solution was later put in a dark place to protect it from the light due to its photosensitivity. Vitamin C was used as the reference, prepared in concentrations of 2, 4, 6, 8, and 10 µg/mL by diluting a 100 µg/mL

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stock solution. Simultaneously, the n-hexane extract of tobacco leaves was prepared in concentrations of 100, 200, 300, 400, and 500 µg/mL by diluting a 1000 µg/mL stock solution. In separate procedures, each of the vitamin C and n-hexane extracts of tobacco leaves (2 mL) was mixed with the DPPH solution (2 mL) in a dark vial and homogenized using a vortex mixer. The incubation time was 30 minutes. Later, the sample was measured at λ 515 nm using a UV-Vis spectrophotometer[14]. The minimum concentration required to achieve a 50% reduction of free radicals was determined using a linear curve equation. The result is shown in Table 2.

Total Phenolic Content

The total phenolic content (TPC) was determined using the Folin-Ciocalteu method. 1 mL of the extract was mixed with 5 mL of 7.5% Folin-Ciocalteu reagent. After 8 minutes, 4 mL of $NaHCO_3$ was added to the mixture. The mixture was then incubated for 1 hour. The absorbance of the solution was measured using a UV-Vis Spectrophotometer at λ = 748 nm. Later, the absorbance of the sample extract was substituted in the curve calibration of gallic acid (20-100 µg/mL). The outcome was expressed as mg GAE/g of dry extract[15].

Result and Discussion

Phytochemical Screening

Phytochemical screening is an initial step taken to determine the presence of secondary metabolites in a plant by adding a reagent that will produce a color reaction or precipitation[16]. The results of the phytochemical screening of both the dry leaves and the n-hexane extract of tobacco leaves can be seen in Table 2.

Based on the data in Table 2, the chemical compounds detected in the dry tobacco leaves include phenols, flavonoids, alkaloids, monoterpenes, and steroids. Meanwhile, the groups of compounds detected in the n-hexane extract obtained through the Soxhlet method are alkaloids, monoterpenes, and steroids. Compared to the research by Prommaban et al. (2022) on several varieties of Nicotiana tabacum, tannins were detected in all the tobacco varieties tested[6]. In contrast, the study by Kanmani et al. (2021) detected only saponins, steroids, and terpenoids in the phytochemical screening of tobacco leaves using n-hexane extract[17]. Another study, conducted by Shekins et al. (2016), showed positive results for polyphenols in methanol extracts of tobacco; however, the same group of compounds was not detected in water extracts[18]. According to Li et al. (2012), factors that can influence the compound content in a plant include genetics (variety), growth location, climate, and post-harvest storage conditions such as temperature. Meanwhile, factors such as the choice of extraction method, solvent selection, and reagents can impact the results of phytochemical screening[19].

Antioxidant Activity of Tobacco Leaves

Antioxidant activity testing using the DPPH method of n-hexane extract of tobacco leaves was carried out at a series of concentrations of 100 - 500 µg/mL with vitamin C as the standard at concentrations of 2 – 10 µg/mL at a wavelength of 515 nm. The results of the scavenging percentage for each concentration of vitamin C and n-hexane extract of tobacco leaves are presented in Table 3.

The test results revealed an IC_{50} value of 426.04 µg/mL (Table 3), indicating that the test extract requires a high concentration of 426.04 µg/mL to provide a 50% inhibitory effect on DPPH free radicals. These results are not as strong as those of the standard vitamin C, whose IC_{50} value is 7.1 µg/mL. According to Molyneux (2004), the result above 200 µg/mL provides very weak antioxidant activity[14].

The research conducted by Al-Lahham et al. (2019) demonstrated potent antioxidant activity in the n-hexane extract of tobacco roots, with an IC_{50} value of 2 µg/mL, which approaches the standar[8]. This difference may be due to the varying parts of the tobacco plant being tested, as well as differences in the growing locations of the tobacco.

Total Phenolic Content of Tobacco Leaves

To verify the results of antioxidant activity using the DPPH method, total phenol content was also measured to ensure and confirm the obtained results. Determination of total phenol content is a process used to calculate the amount of phenolic compounds in a sample. Phenolic compounds contained in the material can donate electrons to free radicals so that these compounds can act as antioxidants[20]. The determination of total phenol levels was carried out using gallic acid as a comparison, with concentrations of 40 - 120 µg/mL, and a test solution of the n-hexane extract of tobacco leaves at a concentration of 1000 µg/mL. The total phenolic content of tobacco leaves is presented in Table 4.

Based on these results, the total phenolic content measured was 51.93 mg GAE/g (Table 4), which is contrary to the phytochemical screening, which did not detect phenols during the screening process. This discrepancy could be due to the phenols in the extract existing in more complex forms, such as being conjugated with other molecules, making them difficult to detect using qualitative methods. However, they can be measured in total phenol content tests[21,22]. While this study's TPC was relatively high compared to the research by Zou et al (2021) which extracted components from tobacco leaves using methanol and showed vigorous antioxidant activity with a total phenol content of only 24.82 mg GAE/g, several factors may influence this disparity and contribute to the observed negative correlation[23,24]. A negative correlation indicates weak antioxidant activity despite a high total phenol content. Some phenolic compounds may exhibit more potent antioxidant effects than others. However, a high total phenol content does not necessarily mean that all these compounds are equally effective in providing antioxidant activity[24]. The high phenol content in the extract might be utilized for other activities, such as antibacterial activity[25].

Conclusion

Based on the results, it can be concluded that the IC_{50} of the n-hexane extract of tobacco leaves, obtained using the Soxhlet extraction method, was 426,024 µg/mL, which is classified as very weak. Meanwhile, the total phenolic content measured was 51,93 mg GAE/g. Due to the high total phenolic content, it is recommended that future research investigate other activities beyond antioxidant activity.

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Conflict of Interest

None declared.

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Table 1. Test with Various Alkaloid Reagents.

No.	Reagents	Observations
1	Dragendoff's test	A reddish-brown precipitate
2	Mayer's test	A creamy white/yellow precipitate

Table 2. The result of phytochemical screening of dry leaves and n-hexane extract of tobacco leaves.

Class of Compounds	Reagent	Dry Leave	Extract
Alkaloids	Dragendorff	+	+
	Mayer	+	+
Phenolics	Ferric chloride	+	-
Flavonoids	Alkaline	+	-
Tannins	Gelatin	-	-
Anthraquinones	Ammonium hydroxide	-	-
Monoterpenoids and Sesquiterpenoids	Vanilin-H2SO4	+	+
Steroids and Triterpenoids	Liebermann-Burchard	+	+

Note :
(+) Detected, (-) Not detected.

Table 3. %Inhibition and IC_{50} Value of Vitamin C and N-Hexane Extract

Sample	Concentration (µg/mL)	Inhibition (%)	IC_{50} (µg/mL)
Vitamin C	2	18.948	7.1
	4	30.420	
	6	44.070	
	8	55.300	
	10	67.585	
N-hexane extract of tobacco leaves	100	5.615	426.04
	200	25.669	
	300	34.670	
	400	44.786	
	500	60.116	

Table 4. Total Phenolic Content of Tobacco Leaves.

Sample	Concentration (µg/mL)	Total Phenolic Compound (mg GAE/g)
Gallic Acid	40	51.93
	60	
	80	
	100	
	120	
N-hexane extract of tobacco leaves	1000	