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# Black Garlic Antioxidant Activity Test (*Allium Sativum*) Compound Types with DPPH Method (1,1-*Difenil*-2-*Pikrilhidrazil*)

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## Abstract
Antioxidants are needed to neutralize Reactive Oxygen Species (ROS) in the body and prevent cardiovascular, cancer, and premature aging as degenerative diseases. Antioxidants will provide resistance to free radicals and prevent damage in normal cells, proteins, and fats, and are able to break chain reactions without disrupting their function. Black Garlic has higher antioxidant properties than regular garlic. S-allyl cysteine (SAC) is a compound from Black Garlic that functions as an antioxidant that can affect the cell cycle in apoptosis, which can provide cell protection from free radical damage. In a study, it was said that a single type of Black Garlic extract obtained IC50 52.055 ppm (µg/mL) which is included in the category of high antioxidant activity. While in the antioxidant activity test, this study used a compound type of black garlic on the grounds that the price is more affordable and easy to find in traditional markets. This study aims to evaluate the potential of compound-type black garlic extract against free radicals by testing antioxidant activity using the DPPH (1,1-diphenyl-2-picrylhydrazyl) method. The results of the compound type Black Garlic antioxidant test stated to have an IC50 value of 173 ppm (µg/mL) which is classified as moderate antioxidant activity. So researchers assume that the use of single type Black Garlic extract is more effective than compound type Black Garlic.

**Keywords:** Antioxidant, Black Garlic, *Allium Sativum*.

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## 1. INTRODUCTION

According to data from the World Health Organization (WHO), one worldwide problem in global health is infertility. The total number of ten percent women experience health issues related to infertility (World Health Organization, 2019). If a couple has not been pregnant in more than two years, they are considered to be infertile in Primary Infertility. However, infertility may also develop if a couple is unable to conceive for more than a year. In this instance, a married couple's attempt to conceive was unsuccessful due to their failure to take contraception. This is called Secondary Infertility (Rahmadiani, 2021). According to a survey, wives account for 64% of infertility and husbands for 36%. From WHO's perspective, women are responsible for 36% of Fallopian tube, 33% of ovulation, 6% of endometriosis, and 40% of other unexplainable or idiopathic conditions. Conversely, Oligozoospermia (16% multifactoral), endocrinologist (20%), and immunologic 2% are from the husband's side (World Health Organization, 2018), subfertility is the term used to describe the effects of lifestyle on exposure to environmental pollution, which often results in abnormal pregnancies. Smoke from cigarettes is one of the major contributing causes of ovulatory disorders.

Ovulatory disorders stand in the second position as infertility disorders owned by women (Irmawati, 2021). An imbalance in hormones caused by the suppression of FSH and LH hormone release is stated to be the cause of this ovulation issue. This hormone exhibits barriers as a result of hypothalamic and pituitary dysfunction. If problems with either hormone's release arise, the follicles' ability to develop will be hampered, which will impact the ovulation process. A variety of intricate chemicals included in cigarette smoke create huge impacts on various reproductive systems (De Angelis et al., 2020). Tobacco elements in smoke that contain nicotine have the potential to alter the body's hormone balance. This is contingent upon the quantity and duration of exposure to cigarette smoke. Reproductive function will be interfered with by any exposure to cigarette smoke. The substances included in cigarettes cause ovarian cell illness which affects estrogen levels. If this occurs, then genetic abnormalities will be more likely to affect female eggs or oocytes (Halimah & Winarni, 2018).

The smoke from cigarettes contains a lot of reactive oxygen species (ROS). Free radicals are defined as less stable molecules that are reactive and able to harm tissue because they can release electrons (Legowo, 2015). Oxidative stress will be impacted in this situation by an imbalance between pro-oxidants and antioxidants. Mitochondrial malfunction results in oxidative stress, which leads to defects in chromosomal segregation, as well as failures in maturation and conception (Ramdiana & Legiran, 2023). Cortisol from the adrenal glands is one of the glucocorticoid hormones produced by the body in response to stress. Gonadotropin-Releasing Hormone (GnRH) hormone released under stress inhibits the Hypothalamic-Pituitary-Gonadal pathway, which in turn impairs reproductive function. The influence of cortisol is a side effect of GnRH secretion inhibition at the pituitary level. It can take advantage of the ovarian level's lowered production of Follicle Stimulating Hormone (FSH) and cortisol, which can obstruct the creation of steroid hormones and cause apoptosis (Setiyono et al., 2015), it has been demonstrated that follicles contain cortisol and Cortisol-Binding Protein (CBP). This stimulates a variety of pathways including oocyte quality and direct steroid genesis effects. The number of follicles that mature from the primordial to the antral stage depends on how they respond to the hormone FSH. Follicles develop and grow in response to FSH stimulation until they reach the mature state. As a result, it may be said that cortisol may disrupt the GnRH pulse, lowering FSH hormone levels and inhibiting the growth of new follicles.

Atomic molecules without an electron pair in their atomic orbit are known as free radicals (Lobo et al., 2010). If free radicals in the body are unbalanced, it can cause a condition called oxidative stress which can eventually disrupt the function of organs in the body and cause disease (Nurkhasanah, 2023). These unbalanced pro-oxidants and antioxidants are said to lead to reproductive diseases such as endometriosis, polycystic ovary syndrome (PCOS), and unexplained infertility. Weight, lifestyle factors (smoking, alcohol use, drugs), and exposure to environmental pollutants have also been found to trigger the oxidative state and contribute to female infertility (Agarwal et al., 2012). The formation of free radicals or Reactive Oxygen Species (ROS) must be controlled by antioxidants because if not, it will turn into pro-oxidants (Hecht et al., 2016).

Antioxidant is defined as a substance that provides resistance to oxidation activities in the body (Kumar et al., 2012). The antioxidant work system provides one oxidant electron which aims to provide resistance to oxidant activity in cells (Winarsi, 2007). The body needs more antioxidants to balance oxidants and antioxidants. This is especially true if oxidants are present in higher amounts. The female reproductive system is one of the body processes affected by the provision of these antioxidants. Every day, the body will build up its defence system through physical exercise and consuming foods rich in nutrients (Ruder et al., 2008). Antioxidants inhibit free radicals and prevent damage to normal cells, proteins and fats, and can break chain reactions without disrupting their function (Halliwell & Gutteridge, 2007). Free radicals or Reactive Oxygen Species (ROS) trigger an increase in oxidative stress. When oxidative stress increases, there is an inhibition of GnRH (Gonadotropin-Releasing Hormone) secretion which will interfere with the production of FSH (Follicle Stimulate Hormone) and LH (Luteinizing Hormone) in the ovaries causing granulosa cell apoptosis and affecting nutrient supply and oocyte maturity. This affects the number and quality of oocytes and leads to ovulation disorders/infertility. The body is unable to naturally make antioxidants and secondary metabolic compounds so they must be obtained from foods such as spices, fruits, and vegetables. Antioxidants from plants consist of bioactive compounds such as flavonoids, phenolic compounds, tannins, phenolic diterpenes, and vitamins, and have a variety of benefits for the body (Ibroham et al., 2022).

Garlic has long been prescribed by traditional medicine to treat inflammatory and metabolic disorders, including diabetes mellitus, hypertension, cardiovascular diseases, and cancer (Falahatian et al., 2022). Garlic (Allium sativum) is part of a type of plant used for food and health. The development of technology makes the use of garlic not only used as a kitchen spice or eaten directly but can be processed by heating it to produce black garlic (Handayani et al., 2020). Black Garlic has higher antioxidant properties than ordinary garlic (Kimura et al., 2016). Black garlic extract itself is said to increase antioxidant activity and prevent cell damage. S-allyl cysteine (SAC) is a compound from Black Garlic that functions as an antioxidant that can affect the cell cycle in cell apoptosis. This can provide protection to cells from free radical damage (Handayani et al., 2020).

In a study of antioxidant activity tests on garlic compared to three types of garlic, namely compound type local garlic, single type local garlic, and single type imported garlic, it was found that the difference in antioxidant activity values in the three types of garlic varieties (Allium sativum) and the best antioxidant value was found in single type local garlic IC50 10.61 ppm (µg/mL) (Prasonto et al., 2017). While compared to the results of other studies that determine the levels of S-ally cysteine (SAC) by high-performance liquid chromatography (HPLC) on a single type of black garlic extract sample obtained IC50 52.055 ppm (µg/mL) which is included in the category of high antioxidant activity (Romsiah et al., 2022). The results of another study indicate that Black garlic or *Allium sativum*, when fermented in the dichloromethane extract fraction, has the highest total flavonoid content compared to water and n-hexane extracts, measuring at 55.68 mg QE/g. The $IC_{50}$ value of Black garlic in the dichloromethane extract is 361.07 µg/mL, classifying it as a compound with moderate antioxidant activity (Wardhani et al., 2020). The process of heating garlic at very high temperatures can result in black garlic. When processed, garlic turns black in color, has a sweet and slightly sour taste, and loses the fresh aroma of garlic. S-allyl cysteine is one of the bioactive substances found in black garlic. S-allyl cysteine is the main component of garlic derivatives formed by enzymatic reactions. The presence of heating carried out will form a change in γ-glutamylcysteine to S-allyl cysteine. This makes the S-allyl cysteine content show a higher ratio of five to six times compared to garlic (Handayani et al., 2020).

Previous research on female Wistar rats showed that black garlic extract contains flavonoids that can increase antioxidant activity which is said to reduce oxidative stress and thus inhibit the decline in the number of follicles (Amida et al., 2021). This study aims to evaluate the potential of compound-type black garlic extract against free radicals by testing antioxidant activity using the DPPH (1,1-diphenyl-2-picrylhydrazyl) method.

## 2. RESEARCH METHOD

The method used to test antioxidant activity in this study utilized the DPPH assay (*1,1-diphenyl-2-picrylhydrazyl*). The extract was dissolved in ethanol p.a and made in various concentrations of 500, 250, 125, 62, and 31 ppm as much as 50 ml each. Into each solution, 1 ml of 0.1 mM DPPH solution was added and incubated for 30 minutes and then measured at a wavelength of 516 nm. As a blank, ethanol p.a and 0.1 mM DPPH containing no test material were used as a positive control of quercetin with concentrations of 1,2,3,4, and 5 µg/mL. DPPH is a type of free radical that remains stable at room temperature and is commonly used to evaluate the antioxidant capability of various compounds or extracts from natural sources. When there is interaction between antioxidants and DPPH, either through electron transfer or hydrogen radical donation, the free radical property of DPPH is neutralized. Several advantages of using this approach include its quick, easy process and the requirement for relatively simple equipment. The lower the $IC_{50}$ value from the antioxidant activity testing using the DPPH method, the higher the antioxidant properties possessed by the substance or material.

This study is a laboratory experimental research with a quantitative experimental research design. The black garlic used in this study was obtained from a producer in Magelang Regency. Compound garlic was processed using a rice cooker at temperatures ranging from 34°C to 38°C for 15 days to produce black garlic. The selection of black garlic was due to its common consumption by the community and by cancer patients.

The inclusion criteria for subjects are: 1) Compound garlic. 2) Fermented black garlic. 3) The part of black garlic used is the bulb of black garlic (suing). The exclusion criteria for subjects are: 1) Rotten and wilted black garlic. The extraction process was conducted at the Herbal Materia Medika Laboratory in Batu Malang.

The materials used are compound black garlic, 96% p.a ethanol, DPPH (*1,1-diphenyl-2-picrylhydrazyl*), and quercetin standard (DPPH solution containing no test material). The equipment used in this experiment includes glassware, knives, rotary evaporator, blender, vortex mixer, magnetic stirrer, oven, analytical balance, filter cloth, and UV-VIS (*Ultraviolet-Visible*) spectrophotometer instrument Genesys.

Black garlic is prepared by peeling and cleaning to remove its skin. Next, the black garlic cloves are thinly sliced lengthwise. These slices are then arranged on a flat baking sheet in a thin layer, approximately 1 cm thick, and dried using an oven at 60°C. The drying process for black garlic is continued until it is completely dried, and then its moisture content is measured.

The black garlic simplisia is extracted using the maceration technique, where 1335 grams of simplisia is soaked in a mixture of 96% ethanol solvent with a total solvent volume of 8000 mL at a ratio of 1:7.5. After macerating for 1 day, the filtrate is filtered and collected in a bottle. The macerated filtrate is then evaporated using a rotary evaporator for 3 hours to remove any remaining solvent, resulting in a concentrated extract called a macerate. Weigh the extracted *Allium Sativum* or Black garlic and store it in the freezer.

The Black garlic extract is mixed with 2 mg of 0.1 mM DPPH powder in a reaction tube containing 96% ethanol solvent. The mixture is then homogenized and incubated. Subsequently, the absorbance of the solution is measured at a predetermined wavelength. The absorbance measurement data is used to determine the percentage of antioxidant activity using the established equation as follows:

$$\% \text{ Antioxidant Activity} = \frac{\text{Absorbance DPPH - Absorbance Sample}}{\text{Absorbance DPPH}} \times 100\%$$

Afterward, the IC50 value of each extract can be determined using a linear equation with the extract concentration on the x-axis and the percentage of antioxidant activity on the y-axis

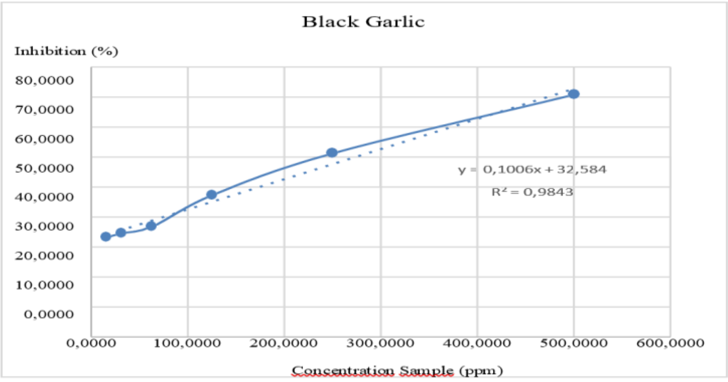
## 3. RESULTS AND DISCUSSION

This study utilized samples of compound garlic variety black garlic. Black garlic was extracted using the maceration method with 96% ethanol solvent. Following the extraction of the samples, antioxidant activity testing was conducted using the DPPH (*1,1-diphenyl-2-picrylhydrazyl*) method. The parameter used to evaluate antioxidant activity is the inhibition concentration value, commonly referred to as $IC_{50}$, which is the concentration of an antioxidant substance that causes a 50% loss of radical properties of DPPH or the concentration of an antioxidant substance that provides 50% inhibition. Substances with high antioxidant activity will have low $IC_{50}$ values. Below are the antioxidant test data for black garlic:
Equation: Y = AX + B; A = 0,1006; B = 32,584; R2 = 0,9843

**Table 1.** Inhibition Percentage Results

| Concentration Sample (ppm) | Absorbance Sample | Inhibition | % Inhibition |
|---|---|---|---|
| 500,0000 | 0,1283 | 0,8084 | 80,8448 |
| 250,0000 | 0,2591 | 0,6132 | 61,3163 |
| 125,0000 | 0,3524 | 0,4739 | 47,3867 |
| 62,5000 | 0,4228 | 0,3688 | 36,8759 |
| 31,2500 | 0,4374 | 0,3470 | 34,6961 |
| 15,6250 | 0,4463 | 0,3337 | 33,3674 |

In this study, an assessment of the antioxidant activity of black garlic was conducted. Graph 1 depicts the relationship between antioxidant concentration and inhibition percentage. The linear equation representing the concentration of antioxidants from the inhibition percentage is y = 0.1006x + 32.584, with a coefficient of correlation (R2) value of 0.9843. This indicates that the method used to assess antioxidant activity yields very good results, with a correlation coefficient value approaching 1 or -1, consistent with previous findings (Pramitha & Sundari, 2020).




**Graph 1.** The Relationship between Antioxidant Activity and Inhibition

Result: $IC_{50}$ = (50 – B) = 173,1213 ppm

UPT Herbal Laboratory Materia Medica Batu conducted the DPPH Black garlic compound type antioxidant test for this investigation, and the findings showed an $IC_{50}$ of 173 ppm. The three categories of antioxidant activity are determined by their $IC_{50}$ value: [1] Strong antioxidant activity, defined as <100 ppm (µg/ml); [2] Moderate antioxidant activity, defined as \100-500 ppm (µg/ml); and [3] Weak antioxidant activity, defined as \>500 ppm (µg/mL) (Wardhani et al., 2020). Through this result, the antioxidant activity of black garlic in this study was categorized as moderate antioxidant group.

A study of black garlic activity using the DPPH method said this method was chosen because it was better than the ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)) method in determining antioxidant activity. Where antioxidant activity is expressed in % inhibition to determine the IC50 value which describes the concentration of extracts that can capture free radicals by 50%. The smaller the IC50 value, the higher the antioxidant activity (Wardhani et al., 2020).

Black garlic undergoes a heating process that transforms a number of fresh chemical components into *Amadori/Heyns* compounds, the main substances in the Maillard reaction (Utama et al., 2024). Fresh garlic lacks many beneficial chemical compounds compared to black garlic. Black garlic contains *S-allyl cysteine* (SAC), which exhibits numerous benefits (Woo et al., 2022). According to several studies, black garlic's ability to combat diseases increases with age. Specifically, certain polyphenols, flavonoids, and Maillard reaction intermediates have been identified as potent antioxidants. Additionally, the biological activity of garlic varies geographically; nevertheless, black garlic has significantly higher biological activity, particularly in terms of antioxidant properties, compared to fresh garlic (Kimura et al., 2016). Flavonoids found in extract black garlic have the ability to boost antioxidant activity, which is thought to lower oxidative stress and prevent the loss of follicles (Amida et al., 2021). Consistent with recent research findings indicating that the extract of black garlic (Allium sativum) itself includes polyphenols that support the body's antioxidant defenses (Juniantari & Susanti, 2023).

From the results of this study, it can be concluded that the antioxidant activity of compound type Black Garlic is still less effective in inhibiting the adverse effects of free radicals because the compound type black garlic antioxidant used in this study is classified as a moderate antioxidant with an IC50 value of 173 ppm (µg/mL) where according to Wardhani (2020) the IC50 value range of 100-500 ppm (µg/mL) is categorized as a moderate antioxidant. So the researcher assumes that the use of single-type black garlic extract may be more effective than compound-type black garlic in inhibiting the adverse effects of free radicals.

A single type of black garlic was processed in a rice cooker at a temperature of 340-380 C and incubated for 15 days. The reason the researchers chose this single type of black garlic was because this product was sold and consumed by the public and CA patients, so the researchers applied it to experimental animals. The research literature states that heating black garlic is between 60-700 C because using temperatures above 700 C can damage the structure of reducing sugars, while temperatures below 600 C require a very long time in the process of making black garlic. The optimal heating time is 35 days to prevent free radicals, which produce many flavonoid compounds, tannins, sterols, and saponins (Agustina et al., 2020). Black garlic is generally processed at a temperature range of 30-900 C with relative humidity of 50-90% and an incubation period of 10-80 days. Several aspects can influence the properties and nutrition of Black garlic, including processing technology and processing variables such as time, temperature, humidity, pH, and type of pre-treatment (Ahmed & Wang, 2021).

The limitation of this study is that there is no comparative test between single and compound types of Black Galic extract, therefore it is hoped that further research will be carried out to analyze the content of compound-type black garlic and single-type black garlic in extract preparations and test their effectiveness against the adverse effects of free radicals.

## 4. CONCLUSION

The results concluded that the results of the DPPH test of antioxidant activity of compound-type Black Garlic were classified as moderate antioxidants with an IC50 value of 173 ppm (µg/mL) so the use of compound-type Black Garlic was less effective. It is hoped that further research will be conducted to analyze the content of compound-type black garlic and single-type black garlic in extract preparations and test their effectiveness against the adverse effects of free radicals..

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