(RESEARCH ARTICLE)

# The effect of *Abelmoschus esculentus* pods ethanol extract on mice (*Mus musculus*) preantral follicle, antral follicle, and corpus luteum numbers exposed to carbon black

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## Abstract

**Introduction:** This study aims to determine the preventive effect of okra (*Abelmoschus esculentus*) pods ethanol extract on the mice (*Mus musculus*) preantral follicle, antral follicle, and corpus luteum numbers exposed to carbon black.

**Material and Methods:** This study used 25 mice which were divided into 5 groups. The negative control group (C-) and the positive control group (C+) were given Na-CMC 0.5% solution orally, while the treatments groups T1, T2, and T3 were pretreated with okra pods ethanol extract orally with a dose of 4 mg/20gBW, 8mg/20gBW and 16 mg/20gBW. Later the C+, T1, T2, and T3 were exposed to 1064 mg/m$^3$ carbon black for 6 hours/day for 30 days.

**Results:** The results of the study showed that there was a significant difference between control group C- (18.20$^c$±2.28; 11.20$^d$±0.83), C+ (8.60$^a$±1.14; 5.00$^a$±1.00), treatment group T1 (12.60$^b$±2.70; 8.00$^b$±1.22), T2 (15.20$^{bc}$±4.76; 8.80$^{bc}$±2.04) and T3 (17.80$^c$±2.77; 9.80$^{cd}$±0.83) (p<0.05) in ovarian preantral and antral follicle numbers, while there was not shown any significant difference between the corpus luteum numbers (p>0.05) in all treatment groups.

**Conclusion:** It can be concluded that okra pods (*Abelmoschus esculentus*) ethanol extract can maintain the preantral and antral follicle numbers in mice exposed to carbon black.

**Keywords**: Carbon Black; Okra; Ovarian Follicles; Reproductive Health

## 1. Introduction

One of the health problems that occur around us is air pollution. Hendrawan et al. [10] stated that sources of air pollution possibly come from various activities, including industry, transportation, and housing, as well as various natural disasters, such as forest fires, volcanic eruptions, and toxic natural gas. Air pollution that comes from various sources can be in the form of particles of dust, gas, lead, and combustion products called Particulate Matter (PM). One of the main particles of PM is in the form of carbonaceous particles, which is known as carbon black.

Carbon black is one of the aerodynamic particles measuring 0.1 μm (100 nm) or less that enters through the respiratory tract and can penetrate the alveolar-capillary, thus being distributed throughout the body through the circulatory system

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[14]. Niranjan and Thakur [19] mentioned that carbon black particles can induce an increase in inflammatory cytokines in the body. The amount of carbon black deposited in the lungs can increase Reactive Oxygen Species (ROS) production [10]. Niwa et al. [20] and Li et al. [16] explained carbon black in the lungs causes mitochondrial abnormalities in alveolar macrophages and alveolar epithelial cells, leading to increase ROS production. Proinflammatory cytokines and increased intracellular ROS are released into the bloodstream and cause systemic disturbances.

Agarwal et al. [2] explained that an increase in ROS production may result in oxidative stress in the body. Oxidative stress can cause various health problems and is known to harm women's reproductive health. Further PM exposure also affects women's reproductive health and is closely related to the process of folliculogenesis. The research of Luderer et al. [18] concluded that exposure to PM resulted in a reduction in the number of developing follicles, inducing cell death due to DNA damage and increased cell apoptosis. Previous studies on PM exposure to ovarian function and folliculogenesis in mice have shown a decrease in fertility, an increase in the incidence of irregular estrous cycles, and a reduction in the number of follicles [26].

*Abelmoschus esculentus* is known to have many health benefits. The *A. esculentus* plant belongs to the Malvaceae family originating from Africa but has been widely planted in other areas such as Asia, Southern Europe, and America with tropical, subtropical, and warm climates. All parts of this plant have various uses, with the main part often used being the pods [5]. *Abelmoschus esculentus* pods and seeds contain flavonoid active ingredients such as procyanidin B2, procyanidin B1, rutin, quercetin, catechin, and epicatechin which function as antioxidants [12]. Shui and Peng [25] reported about 70% of the antioxidant effect of *A. esculentus* in the form of quercetin. Quercetin can reduce oxidative stress conditions and induce antioxidant enzymes in the body [13].

The antioxidant effect in *A. esculentus* is expected to prevent the production of ROS in the body exposed to carbon black. Therefore, the study was conducted to observe the effect of *A. esculentus* in preventing oxidative stress caused by exposure to carbon black and maintaining the number of preantral-antral follicles in the ovaries.

## 2. Material and methods

### 2.1. Sample and materials

The study used 25 female mice (*Mus musculus*), aged 8 weeks with 20-25 grams bodyweight and obtained from a Rat-mice breeder. The Materials for daily mice maintenance are water given ad libitum and feed in the form of Hi-Pro-Vite Medicated pellets 594. The materials for treatment are *A. esculentus* pods ethanol extract and carbon black. The *A. esculentus* pods were obtained from farmers in Ngemplak Village, Sambikerep District, Surabaya City, East Java, Indonesia. The *A. esculentus* pods were extracted by maceration method using 96% ethanol solution with three levels of preventive doses. The carbon black exposure used the Vulcan N330 and Carbon Black Inhalation Chamber as the inductor.

### 2.2. Experimental design

This study used an experimental principle, by applying the Completely Random Design (CRD) method. Mice were divided into 5 treatment groups, where each group consisted of 5 mice and then acclimatized for 7 days in a homogeneous state. Then, a vaginal smear of the mice was conducted to determine the estrous cycle of the mice [1]. The negative control (C-) group was given Na-CMC 0.5% solution (Aquadest added with Na-CMC 0.5% powder) orally without being exposed to carbon black and the positive control (C+) group was given Na-CMC 0.5% solution (Aquadest added with Na-CMC 0.5% powder) orally and exposed to carbon black. Based on Shintaningrum [24], the dose of *A. esculentus* pods ethanol extract was divided into Treatment 1 (T1), Treatment 2 (T2), and Treatment 3 (T3). Each group was given *A. esculentus* pods ethanol extract at doses of 4mg/20gBW, 8mg/20gBW, and 16mg/20gBW orally. The C+, T1, T2, and T3 groups were then exposed to carbon black at a dose of 1064mg/m$^3$ [11] and inhaled 6 hours/day for 30 days.

### 2.3. Histopathological examination

On the 31st day, the mice were then euthanized, and the ovarium samples were collected to be made for histopathological preparation using Hematoxylin-Eosin (HE) staining. The histopathological preparations were observed using Nikon Eclipse E-100 using a 100x magnification microscope equipped with an optical camera connected to a computer. The counting is manually done with the Image Raster Application on the computer. Five different fields of view were taken by image using

the computer and were calculated. The calculation was done by identifying the number of preantral follicles, antral follicles, and corpus luteum.

Preantral follicles consist of primary and secondary follicles. The primary follicle is an oocyte surrounded by a layer of cuboid granulosa cells. The secondary follicle is an oocyte covered by a few layers of cuboid granulosa cells. Antral follicles consist of tertiary and de Graaf follicles. The tertiary follicle is an oocyte covered by granulosa cells and begins to form an antrum filled with follicular fluid which are called Call-Exner bodies. Graafian follicle is an oocyte that has begun to pull over to one side, surrounded by cumulus oophorus, heca interna and theca externa cells, and corona radiata, and has a wider antrum compared to the tertiary follicle. The Corpus luteum is a structure composed of theca lutein cells and lutein cells, originating from ovulated Graafian follicles [9, 7].

## 2.4. Statistical analysis

The data was analyzed using Statistical Product and Service Solution (SPSS). Data on the number of follicles from the five treatment groups were analyzed using descriptive statistical tests, different tests using one-way ANOVA, and Duncan's test with a significance of 0.05.

## 3. Results and discussion

The data was gathered by examining the histopathological preparations of the mice ovaries (*Mus musculus*). The variables observed were preantral follicles (primary and secondary follicles), antral follicles (tertiary and Graafian follicles), and corpus luteum (Figure 1). Histopathological assessment was performed on five different visual fields for each variable in one preparation. The average value and Standard Deviation (SD) are shown in Table 1 and Figure 2.

The results showed the calculation of preantral follicle, antral follicle, and corpus luteum numbers. One-way ANOVA analysis of the number of preantral and antral follicles showed a significant difference (p<0.05). Table 1 showed Duncan's analysis, where it was shown that all treatments have significantly different results (p<0.05) between groups C- (18.20 ± 2.28 and 11.20 ± 0.83), C+ (8.60 ± 1.14 and 5.00 ± 1.00), T1 (12.60 ± 2.70 and 8.00 ± 1.22), and T2 (15.20 ± 4.76 and 8.80 ± 2.04) on the number of preantral and antral follicles. The C- (18.20 ± 2.28 and 11.20 ± 0.83) and C+ group (8.60 ± 1.14 and 5.00 ± 1.00) showed significant differences between each other (p<0.05) indicating there is an effect of carbon black exposure to the number of the preantral and antral follicle, but showed no significant difference (p>0.05) between groups T3 (17.80 ± 2.77 and 9.80 ± 0.83) and C- (18.20 ± 2.28 and 11.20 ± 0.83). For the number of corpus luteum, it was found that there was no significant difference (p>0.05) between all treatment groups, but the C+ (2.80 ± 0.83) group has the lowest average number compared to the other treatment groups.
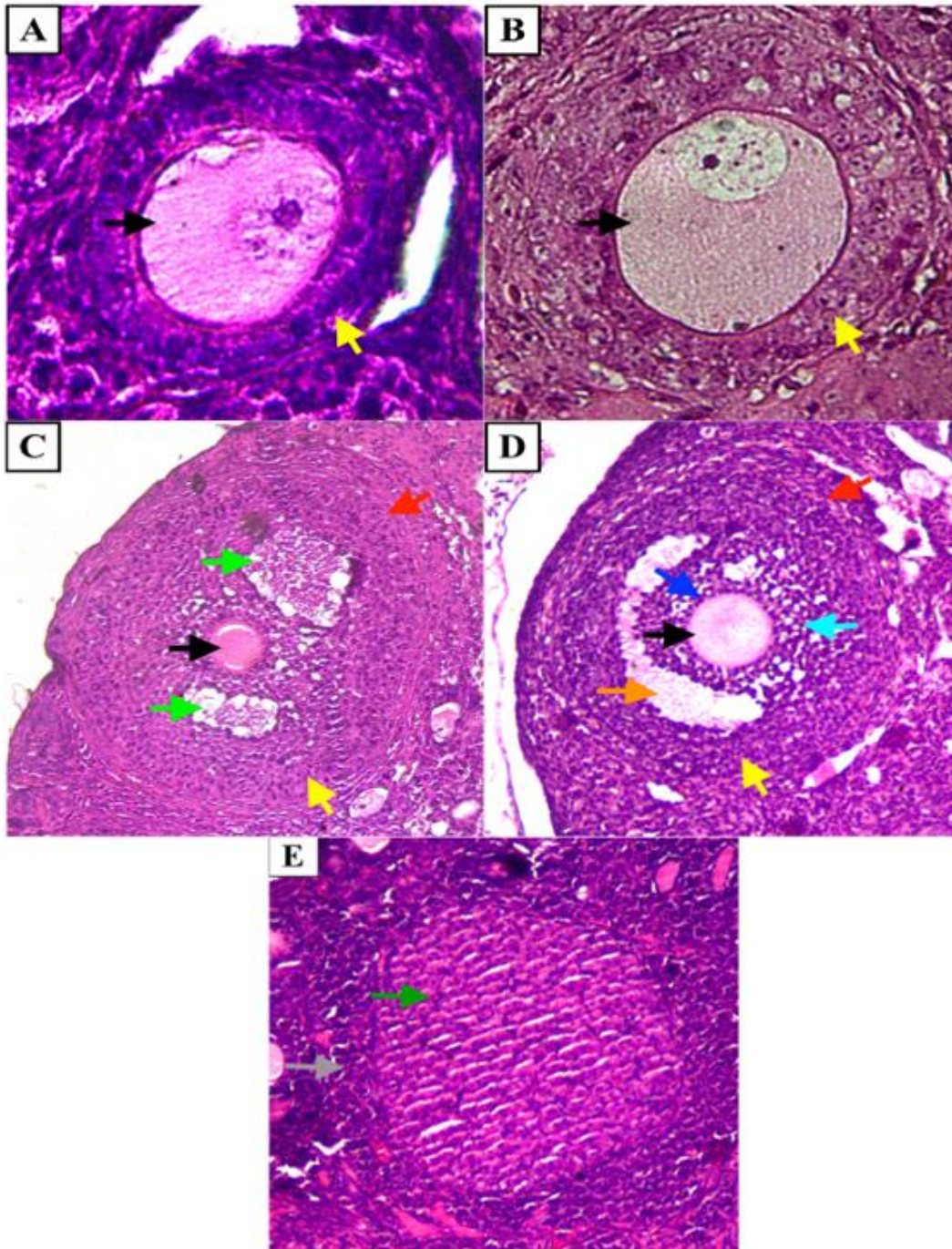
**Figure 1** Follicle identification ovarian histopathology of mice (*Mus musculus*) which was given *A. esculentus* pods ethanol extract as preventive medicine against exposure to carbon black with HE staining. Preantral follicle (A: primary follicle; B: secondary follicle; 400x magnification), Antral follicle (C: tertiary follicle; D: Graafian follicle; 100x magnification), and corpus luteum (E; 100x magnification). Black arrows show oocytes, yellow arrows show granulosa cell membrane, light green arrows show Call-Exner bodies, red arrows show corona radiata, orange arrows show follicular antrum, dark green arrows show lutein cells and grey arrows show theca lutein cells.

**Table 1** The average preantral follicle, antral follicle, and corpus luteum numbers in each group (Mean ± SD).

| Group | Preantral Follicle Number (Mean ± SD) | Antral Follicle Number (Mean ± SD) | Corpus Luteum Number (Mean ± SD) |
|---|---|---|---|
| C- | 18.20[c] ± 2.28 | 11.20[d] ± 0.83 | 7.40[a] ± 3.64 |
| C+ | 8.60[a] ± 1.14 | 5.00[a] ± 1.00 | 2.80[a] ± 0.83 |
| T1 | 12.60[b] ± 2.70 | 8.00[b] ± 1.22 | 7.20[a] ± 3.96 |
| T2 | 15.20[bc] ± 4.76 | 8.80[bc] ± 2.04 | 5.60[a] ± 4.61 |
| T3 | 17.80[c] ± 2.77 | 9.80[cd] ± 0.83 | 6.20[a] ± 4.08 |

Notes: different superscripts in one column showed significant differences ($p<0.05$).
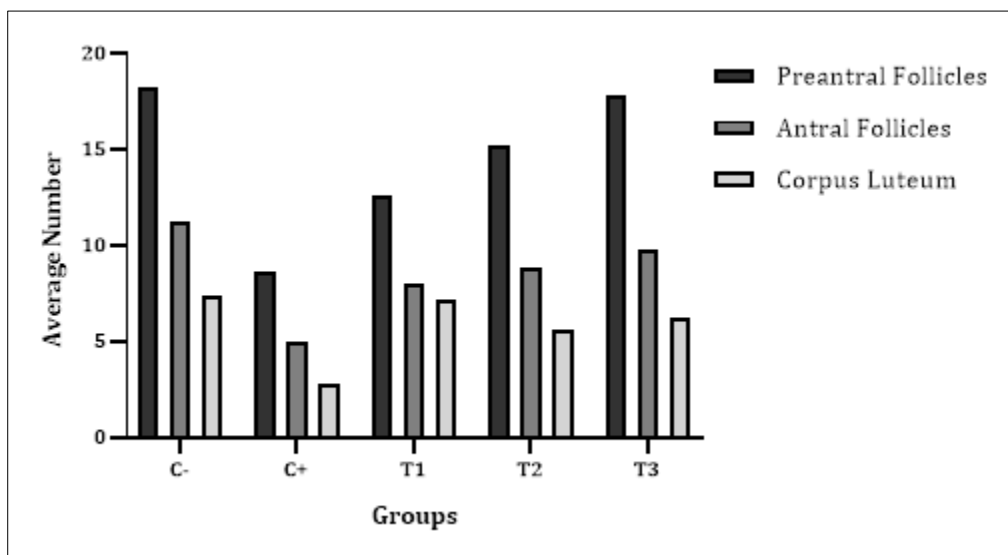


**Figure 2** Bar chart of the mean number of preantral follicles, antral follicles, and corpus luteum in 5 groups of mice (*Mus musculus*).

From Figure 2, the graphic of preantral and antral follicle numbers shows a decrease between the C- and C+ groups, while showing an increase after the treatment of *A. esculentus* pods ethanol extract with three levels of doses (T1, T2, and T3). The graphic of the corpus luteum numbers showed that the C+ group was also having the lowest graphic average number (2.80 ± 0.83), followed by the T2 group (5.60 ± 4.61), T3 group (6.20 ± 4.08), T1 group (7.20 ± 3.96), and C- group (7.40 ± 3.64) as the highest corpus luteum average number.

The preantral follicles are known to be gonadotropin independent, in which the growth and the differentiation of the follicles are affected by growth factors such as Transforming Growth Factor-β (TGF-β) superfamily and Insulin-like Growth Factor 1 (IGF-1). These growth factors stimulate the recruitment of primordial follicles to differentiate into preantral follicles and also stimulates the proliferation of granulosa cells. While undergoing the transition to antral follicles, many of the growing follicles died and formed atretic follicles.

The antral follicles are gonadotropin dependent, marked by the rapid growth of their size affected by the Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH), and other growth factors which would affect the gonadotropin activities. Estrogen will mainly be produced inside the transitioning antral follicles, and forming the antral cavities which would increase the size of antral follicles. This explained that in the result, only the preantral (Primary and secondary) follicles undergo a significant increase in number, while the antral (tertiary and de Graaf) follicles undergo an increase in size [1, 27]. Increased LH production will eventually make the ovulation happen inside the ovary, thus leaving behind a structure called corpus luteum [7].

The corpus luteum numbers in all groups are shown to be insignificantly different from each other's but showed a decrease in the average number of C+ group if compared to other groups. The decrease happened because the preantral and antral follicles in the C+ group showed a significant decrease following the carbon black exposure, thus having a lower follicle number to be ovulated if compared to the C- group (without the exposure of carbon black) and the treatment groups (T1, T2, and T3).

Based on research by Niwa et al. [20] and Li et al. [16], The interaction between carbon black and cells in the lungs will trigger an inflammatory response in the body. The occurring inflammatory response is the secretion of pro-inflammatory cytokines IL-1β and IL-8 in the bronchiolar epithelium. Continued exposure might affect the secretion of other inflammatory marker proteins such as MPC-1, IL-6, and CRP, which will also increase circulation. This inflammatory response will increase the production of TNF-α, which induces the release of ROS in mitochondrial cells. Lu et al. [17] stated that excessive ROS production causes an imbalance between oxidation and antioxidants, causing oxidative stress in the body. As the carbon black exposure continued, the inflammation responses and the production of ROS will also continue. The decrease in the follicle number has been stated in the study of Luderer et al. [18], in which ROS adversely affects folliculogenesis and increases the risk of infertility.

Granulosa cells strongly influence the development of follicles in the ovary. The granulosa cells are known to be the receptor of FSH and LH from the bloodstream, regulating the release of estrogen hormone, the formation of the corpus luteum, and nurse cells that provide nutrition to oocytes inside the ovarium [7]. The release of the inflammatory responses and ROS is known to affect the integrity of cell membranes, reducing cell viability, and eventually leading to cell death [19, 4]. Luderer et al. [18] also stated that oxidative stress will eventually cause the death of granulosa cells, leading to oocyte death caused by apoptosis and the follicle will degenerate into an atretic follicle. Cell death is caused by the activation of caspase-3 in the ovary. Caspase-3 is known to be a major caspase in the regulation of cell apoptosis [27]. The decrease in follicular number in all treatments exposed to carbon black may also cause a decrease in the formation of the corpus luteum, following the decrease in the antral follicle, and thus having lesser mature oocytes to be ovulated.

*Abelmoschus esculentus* pods are known to have many benefits, including as an anti-inflammatory and antioxidant). About 70% of the antioxidant activity of *A. esculentus* comes from quercetin (a type of flavonoid) [25]. Granado-Serrano et al. [8] explained that quercetin can interact with cellular defense systems and induce antioxidant enzymes and Glutathione (GSH). Induction of the release of antioxidant enzymes and glutathione in the body can counter the increase in ROS production in the body and keep levels low. In addition, Quercetin can bind directly to ROS [23]. Li et al. [15] explained that quercetin, as a major active substance in the *A. esculentus* pods, inhibits the release of pro-inflammatory cytokines by inhibiting the lipopolysaccharide-Induced TNF-α production in the macrophage.

Flavonoid content in *A. esculentus* can also give an antioxidant effect by inhibiting the excess production of ROS, directly scavenging ROS, or indirectly increasing endogenous antioxidant enzymes [3]. *Abelmoschus esculentus* extract contains various antioxidant compounds such as flavonoids, quercetin, and phenolic acids that can donate $H^+$ from antioxidant compounds to ROS compounds [6]. Yang et al. [28] mentioned that quercetin may inhibit the activation of caspase-9 and caspase-3 from endoplasmic reticulum stress of buffalo ovarian granulosa cells and reduce ROS activities. Rashidi et al. [22] explained that quercetin could reduce apoptosis of granulosa cells through Nuclear Factor (erythroid-derived 2)-like 2 (Nrf2)/Antioxidant Response Element (ARE) pathway and Thioredoxin (Trx) system. Nrf2 is a key regulator of phase II antioxidant enzyme expression through binding to the ARE. In the normal states, Nrf2 is disabled in the cytosol by binding to Kelch-like ECH-Associated Protein 1 (Keap1). But the Keap1–Nrf2 complex is separated under oxidative stress and Nrf2 is transferred into the nucleus where it connects to the ARE region. It was reported before that quercetin stimulates Nrf2-mediated ARE activity and cytoprotective genes.

## 4. Conclusion

The studies above can explain that the bar chart of the T1, T2, and T3 treatment group seems to have an increase in both preantral follicle, antral follicle, and corpus luteum numbers and maintain their healthy count when compared to the C+ group. The Conclusion showed that *Abelmoschus esculentus* pods ethanol extract administration can maintain the number of preantral follicles, antral follicles, and corpus luteum of mice (*Mus musculus*) exposed to carbon black. Further studies are needed to explain more about the adverse effect of *Abelmoschus esculentus* pods ethanol extract's active components as the exact mechanism are still poorly understood.

## Compliance with ethical standards

*Disclosure of conflict of interest*

All authors declare no conflict of interest in the manuscript.

*Statement of ethical approval*

The research was conducted appropriately following the ethics in using experimental animals and has been approved by the ethics committee of the Faculty of Veterinary Medicine, Universitas Airlangga, No. 1.KE.138.12.2021 on 12th December 2021.

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