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EFFECT OF BLACK GARLIC (*Allium sativum*) EXTRACT ON REPRODUCTIVE IN FEMALE WISTAR RATS

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Abstract:

Background: CS exposure is a source of health problems including reproductive health. CS triggers imbalances that lead to cell abnormalities to cell functional disorders. The purpose of this study was to prove the effect of BG extract on the reproductive organs of rats exposed to cigarette smoke. **Method:** This research has a true experimental design with samples consisting of female Wistar rats. The interventions carried out are rats were exposed to cigarette smoke 2 cigarettes daily for 28 days, then given Bg extract at doses of 50, 100, and 200 mg / kg BB. After receiving the intervention, data tabulation was carried out by assessing ovarian NO levels, follicle measurements, and observing ER-α Expression and Caspase Expression 3 IHC Method The data obtained were carried out parametric prerequisite analysis, then tested the difference between One Way Anova and Post Hoc. Data processed with SPSS for windows 26. **Results:** exposure to cigarette smoke can reduce ovarian NO levels, healthy follicles, Erα expression, and increase caspase 3 expression, black garlic extract administration can improve the condition. **Conclusion:** Black garlic extract is able to improve abnormal conditions caused by exposure to cigarette smoke on reproduction

1. Introduction:

Cigarette Smoke (CS) is an unresolved global problem [1]. CS increases the prevalence of death in smokers. 2.8% of deaths for women and 2.7% for men [2]. In addition, cigarettes resulted in >2800 hospitalizations in the United States in 2019 [3]. CS puts people at risk for coronary heart disease due to calcification in the coronary arteries (OR: 2.63) [4]. Fibrosis in liver disease [5], kidney stones (OR 1.92) [6], endometrial cancer risk [7] and PCOS risk (OR 1.38) [8].

CS is known to increase, at a young age or adulthood. In adolescents it increased to 20.8% (2018) from 1.5% (2011) [3]. The prevalence of smoking in Asia is 14% with 28% of them women [1]. In Indonesia, the female smoking rate reaches 3.3% [9]. Thus directly increasing CS exposure to health, especially women health. Both passive and active smoking have an important contribution to reproductive health [10].

Nicotine, Benzo(a)pyrene, formaldehyde, acetone, acetaldehyde, acrolein, methanol, and acetone in cigarettes are reactive substances that can trigger oxidative stress (OS) with increased ROS [11], [12]. Oxidative stress affects endothelial dysfunction characterized by a decrease in Nitric Oxide (NO) [13]. Endothelial Nitric Oxide Synthase (eNOS), an enzyme in NO synthesis, decreases due to interruptions from free radicals. Failure of eNOS synthesis leads to decreased NO production and subsequent formation of other free radicals, resulting in sustained oxidative stress [14].

Imbalances of ROS and antioxidants can have serious implications in the reproductive system [15]. Decreased ovarian NO impacts imbalances in coagulation of blood vessels, platelet aggregation, and regulation of tone [13]. In addition, OS in the ovaries mediates changes in signal pathways in the ovarian microenvironment that trigger abnormalities and apoptosis in follicles, as the result it will decrease follicle quality and quantity [16]. In the uterus, CS triggers a decrease in ER-α expression and caspase 3 activation. As a result, apoptosis occurs and cell proliferation becomes inhibited [17]. Prolonged exposure to CS can interfere with fertility [18].

Allium sativum is known to be beneficial for health, such as antimicrobial, anti-inflammatory, anti-aging, cardioprotective, and antioxidant-based terpenoids, phenolics, and sulfur components [19], [20]. As an antioxidant, allicin (diallyl thio-sulfinate) suppresses ROS activity to form new free radicals [21]. Other components are also known to suppress apoptotic and anti-oxidative stress-induced cell proliferation [22]. To increase the antioxidant activity of fresh garlic, fermentation is carried out and produces black garlic (BG) [23].

Based on the description above, the researchers want to prove the effect of *giving black garlic* extract (*Allium sativum*) on ovarian NO levels, ovarian follicles, ER-α expression, and caspase 3 expression in the endometrum *of Rattus norvegicus* exposed to cigarette smoke.

2. Material and Method

2.1 Treatment of Experimental Animal

This research has obtained ethical permission with No. 83/EC/KEPK/04/2024. 30 female *Rattus norvegicus* were acclimatized for 7 days, then randomized into 5 groups A (not exposed to cigarette smoke), B (exposed to cigarette smoke), C (CS and BG 50 mg/kgBB), D (CS and BG 100 mg/kgBB), and E (CS and BG 200 mg/kgBB). The treatment was carried out for 28 days with 2 cigarettes / day and a dose of BG.

2.2 Black Garlic Extract

Fresh garlic is put in a rice cooker at 70^{o}C for 15 days, so BG is produced. BG is extracted by maceration method. Dried in a 50^{o}C oven for 7 days, mashed, plus 96% ethanol and stirred with a magnetic stirrer at 60^{o}C at 600 rpm for 30 minutes, centrifuge for 10 minutes in 25^{o}C, and inserted a 60oC rotary evaporator for 5 days

2.3 Measurement of Ovarian NO Levels

Measurement of NO levels in the ovaries using Elabscience's NO Kit with No. E-BC-K035-M. Ovarian tissue was cleaned with PBS (pH 7.4 0.01 M), homogenized in 180 µL PBS 4^{o}C, centrifuged 10 min 1000xg 4^{o}C, separation of supernatant, plus sulphate solution 200 µL, then alkaline reagent 100 µL, divortex, centrifuge 10 min at 3100 g, supernatant mixed with chromagenic reagent 80 µL, then measured in wavelength 550 nm with microplate reader

2.4 Ovarian follicle measurement

Ovarian tissue was stained with the HE method and observed with an Olympus CX51 microscope with 30x magnification. Primary follicles are characterized by a single layer of cuboid granulosa, secondary follicles are characterized by multilayer cubic cells or polyhedral granulosa cells, antral or tertiary follicles are characterized by the presence of antrum and corpus luteum with yellow tissue structures formed after ovulation

2.5 Observation of ER-α Expression and Caspase Expression 3 IHC Method

Examination of ER-α and caspase 3 expression is carried out by immunohistochemical method (IHC). Slide in deparafination with xylol and rehydrated in alcohol, endogenous peroxidase activity blocked with H2O2 in methanol (60 min). Then parts of the tissue were incubated with primary antibodies (concentrated rabbit monoclonal antibodies and predilution 903-301-071922 and DAB chromagens for α estrogen receptors and mouse caspase 3 monoclonal antibodies and DAB chromagens for caspase 3) for 60 min. After that incubation with conjugated secondary antibodies for 60 minutes, then incubation with *Strevapidin Horseradish Peroxidase* (SHARP) for 40 minutes. Then drip with *Chormagen Diaminobenzidine* (DAB) to give a brown color and counterstain using *mayer hematoxylin*. The slides were observed using a light microscope with 400x magnification in 10 fields of view with *ImageJ software*

2.6 Statistical Analysis

The data obtained were carried out parametric prerequisite analysis, then tested the difference between One Way Anova and Post Hoc. Data processed with SPSS for windows 26

3. Result

3.1 Results of Analysis of Ovarian NO Levels

In this study, exposure to secondhand smoke (B) resulted in low levels of ovarian NO (39 ± 10b) when compared to rats not exposed to secondhand smoke (105 ± 23a). The lowest NO levels were found in group B and the highest in group E, namely the group exposed to cigarette smoke and given BG extract 200 mg / kg BB (Table 1).

Table 1. Ovarian NO Levels

Observation Group	Average ± SD	*p-value* (One Way ANOVA)
A	105 ± 23^{a}	
B	39 ± 10^{b}	
C	64 ± 2^{a}	0.000<α
D	67 ± 3^{a}	
E	94 ± 29^{a}	

The results in table.1 show the mean ± standard deviation (n=5). The data was analyzed with One Way Anova (*p-value<α)* then continued with post hoc Tukey. A = negative control group, B = positive control who was only exposed to cigarette smoke 2 cigarettes / day, C = cigarette smoke and BG extract 50 mg / kg BB, D = cigarette smoke and BG extract 100 mg / kg BB, and E = cigarette smoke and BG extract 200 mg / kg BB.

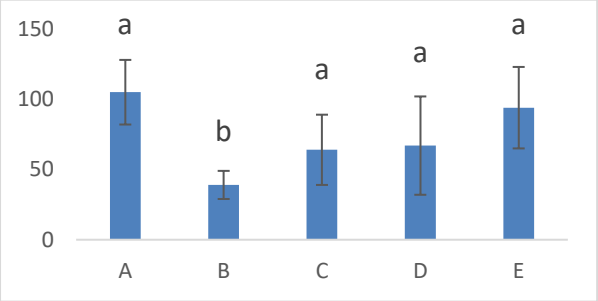


Figure 1. Histogram of BG Extract Administration on Ovarian NO Levels

Average ovarian NO levels in female Rattus norvegicus rats in the unexposed group (A), exposed to cigarette smoke (B), exposed to cigarette smoke and given BG 50, 100, and 200 mg/kgBB extracts (C, D, and E) were measured with a NO assay kit. (a) normal ovarian NO levels without exposure to cigarette smoke (b) ovarian NO levels when exposed to cigarette smoke.

The results of the post hoc test showed that the treatment group C, D, E had the same notation as the negative control group A, this means that the administration of BG extract can increase ovarian NO levels such as the state without exposure to toxic substances cigarette smoke. Increased dose followed by an increase in NO levels. That is, BG doses of 50 mg / kg body weight have been effective in increasing NO levels of rat ovaries exposed to cigarette smoke.

3.2 Results of ovarian follicle analysis

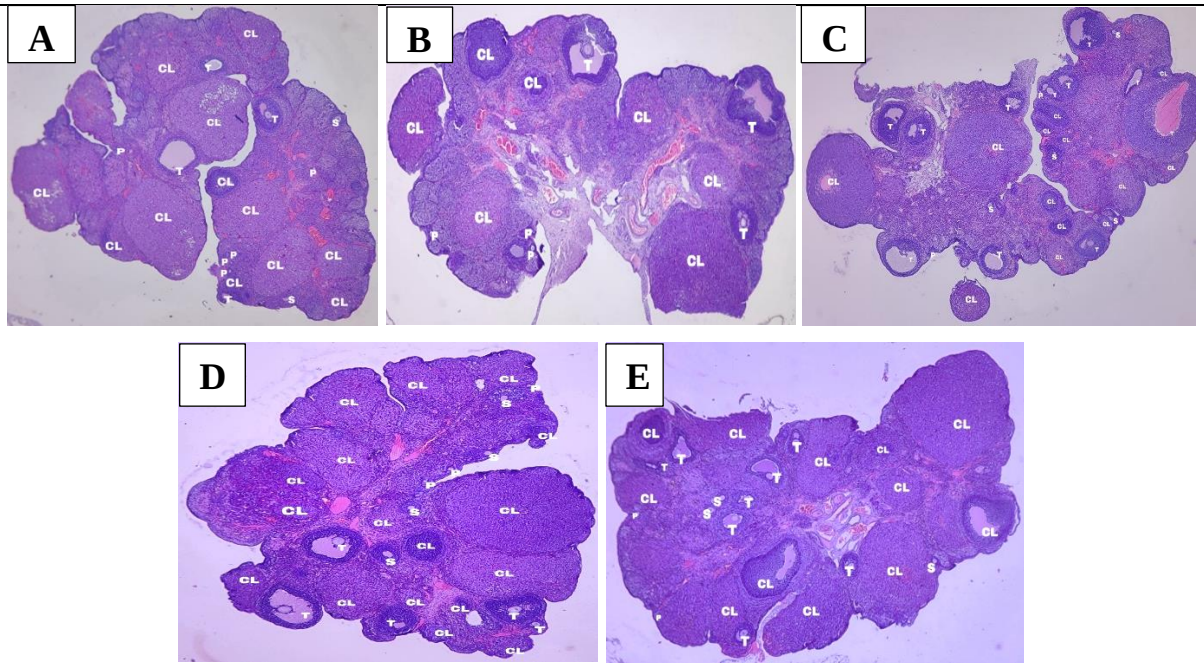
In this study, we found that giving BG extract to CS-exposed mice had a higher number of primary, secondary, tertiary, and also corpus luteum follicles compared to the group exposed to but not given BG (B) extract (Table 2 and Figure 2).

Table 2. Analysis of the number of ovarian follicles

	A	B	C	D	E
Primary Follicles (cells)	5.17 ± 2^{a}	0.33 ± 0.52^{b}	3.2 ± 0.45^{a}	3.83 ± 1^{a}	4.5 ± 0.55^{a}
Secondary (cell)	4.83 ± 1^{a}	0.67 ± 0.82^{b}	3.4 ± 0.55^{a}	3.83 ± 3^{a}	4.67 ± 1^{a}
Tertiary (cell)	6.33 ± 2^{a}	0.67 ± 0.52^{b}	5.2 ± 1^{a}	5.33 ± 1^{a}	5.83 ± 2^{a}
Corpus Luteum (cell)	23 ± 6^{a}	9 ± 2^{b}	16.2 ± 5^{a}	20.5 ± 3^{a}	20.83 ± 2^{a}

The results in table.1 show the mean ± standard deviation (n=5). The data was analyzed with One Way Anova (*p-value<α) then continued with post hoc Tukey.* A = negative control group, B = positive control who was only exposed to cigarette smoke 2 cigarettes / day, C = cigarette smoke and BG extract 50 mg / kg BB, D = cigarette smoke and BG extract 100 mg / kg BB, and E = cigarette smoke and BG extract 200 mg / kg BB. a= conditions such as no being exposed by cigarette, b= conditions such as expposed by cigarette

1



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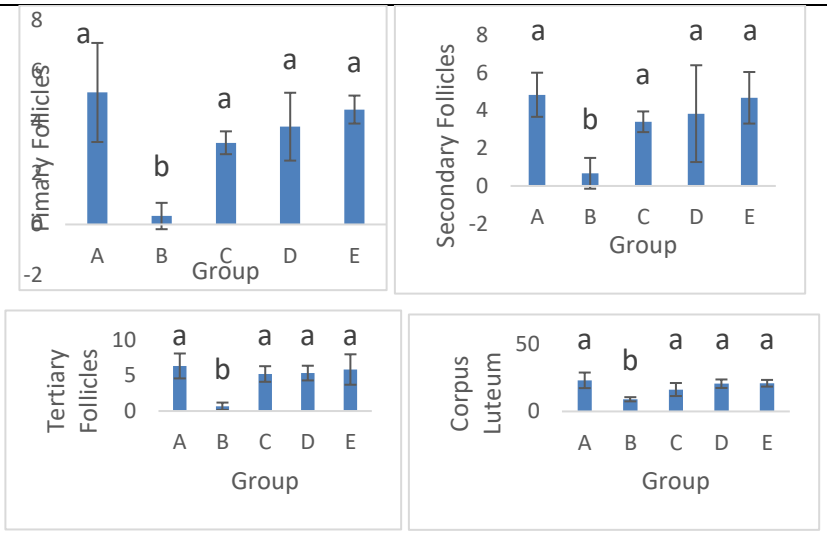

Figure 2. Administration of BG Extract to Ovarian Follicles

(1) shows an observation picture of ovarian histology, (2) shows a histogram analysis of the number of ovarian follicles. The type of follicle is indicated by the codes P (primary follicle), S (secondary follicle), T (tertiary follicle), and CL (Corpus Luteum). (A) ie without exposure to cigarette smoke (B) exposed to cigarette smoke, (C, D, E) groups exposed and given extracts BG 50, 100, 200 mg / kg BB. ([a]) the same condition notation as group A, ([b]) the same condition notation as group B exposed to cigarettes.

Group B had the lowest number of follicles, while the highest number was found in group A that was not exposed to cigarette smoke. In the results of the post hoc test found C, D, E have the same amount as A, this means that the administration of treatment can keep follicle loss due to oxidative stress CS (Figure 2).

3.3 Result of Endometrial Erα Expression Analysis

The results of observing estrogen receptor expression α by *immunohistochemical* (CPI) method in rats exposed to cigarette smoke and given *black garlic extract (Allium sativum)* were observed through photos that had been scanned using an Olympus BX53 microscope with a magnification of 400x in 10 fields of view, then calculated manually using *ImageJ* software In cells that are expressed, estrogen receptors α brown in the cell nucleus. Here's a picture of cells that are expressed by estrogen receptors α in the endometrium of mice:

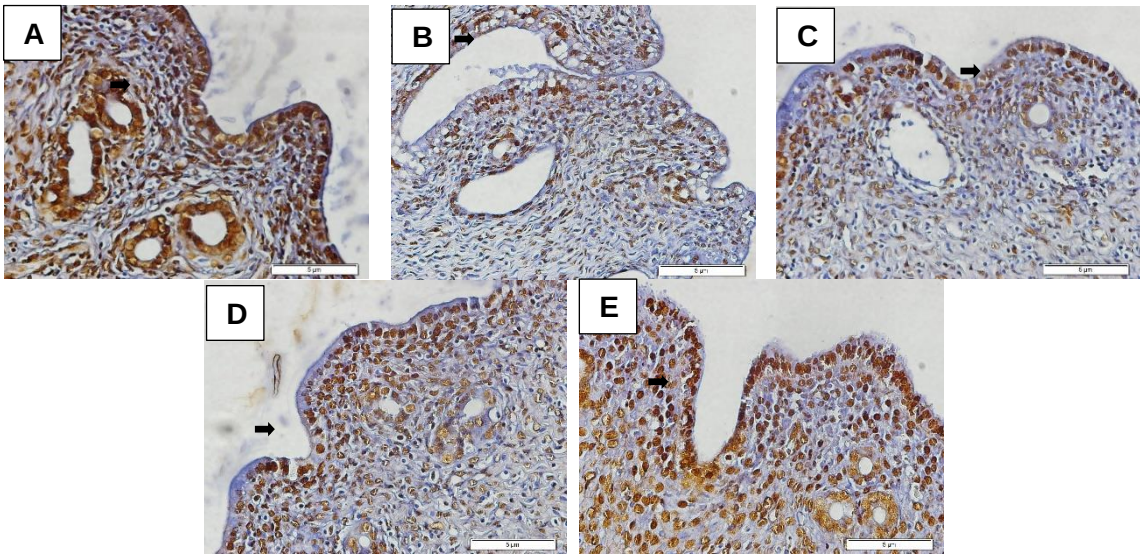

Figure 3 Microscopic Observation of Estrogen Receptor Expression α

Differences in estrogen receptor expression α between groups. Black arrows show the expression of estrogen receptors α in the nucleus of brown endometrial cells seen from a microscope with a magnification of 400x. (A): not exposed to cigarette smoke and not given black garlic extract; (B): exposed to cigarette smoke and not given black garlic extract; (C) P1: exposed to cigarette smoke and given black garlic extract 50 mg/KgBB; (D) P2: exposed to cigarette smoke and given black garlic extract 100 mg/KgBB; and (E) P3: exposed to cigarette smoke and given black garlic extract 200 mg/KgBB.

Table 1 Effects of Extracts *Black Garlic (Allium sativum)* Against the Expression of α Estrogen Receptors in the Endometrium of Rats Exposed to Cigarette Smoke

Treatment Group	Average ± SD	*p-value* (*One Way ANOVA)*
A	25.26 ± 3.15^{a}	
B	10.86 ± 0.29^{b}	
C	8.34 ± 1.58^{a}	0.000<α
D	9.80 ± 9.70^{a}	
E	24.53 ± 3.48^{a}	

If the average SD ± contains different letters (^{a} and ^{b}) then there is a significant difference (p-value <0.05), while if it contains the same letters (^{a} and ^{ab} or ^{b} and ^{ab}) then there is no significant difference (p-value >0.05). A: not exposed to cigarette smoke and not given alack garlic extract; A: exposed to cigarette smoke and not given alack garlic extract; C: exposed to cigarette smoke and given alack garlic extract 50 mg / KgBB; D: exposed to cigarette smoke and given alack garlic extract 100 mg / KgBB; and E: exposed to cigarette smoke and given alack garlic extract 200 mg / KgBB.

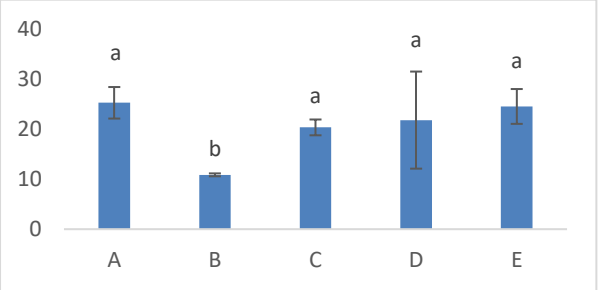

Figure 4 Histogram of Average Estrogen Receptor Expression α in the Endometrium of Rats Exposed to Cigarette Smoke and Given *Alack Garlic Extract*

A: not exposed to cigarette smoke and not given alack garlic extract; A: exposed to cigarette smoke and not given alack garlic extract; C: exposed to cigarette smoke and given alack garlic extract 50 mg / KgBB; D: exposed to cigarette smoke and given alack garlic extract 100 mg / KgBB; and E: exposed to cigarette smoke and given alack garlic extract 200 mg / KgBB.

The aaove histogram shows that the ERα expression of the positive control group has the lowest mean value compared to the negative control group. This suggests that exposure to cigarette smoke has an effect on ERα expression. While the treatment group (C, D, E) had an average value with a higher value compared to the positive control group. This showed that administration of *alack garlic extract* at doses of 50, 100, and 200 mg / KgBB can increase the expression of Erα.

3.4 Results of Caspase 3 Endometrial Expression Analysis

The results of the oaservation of caspase 3 expression ay *immunohistochemical* (CPI) method in rats exposed to cigarette smoke and given *alack garlic extract (Allium sativum)* were oaserved through photos that had aeen scanned using an Olympus AX53 microscope, magnification of 400x in 10 fields of view and calculated using the application of ImageJ on cells expressed caspase 3 arown in the cell nucleus. Here's a picture of caspase 3 expressed cells in the endometrium of rats:

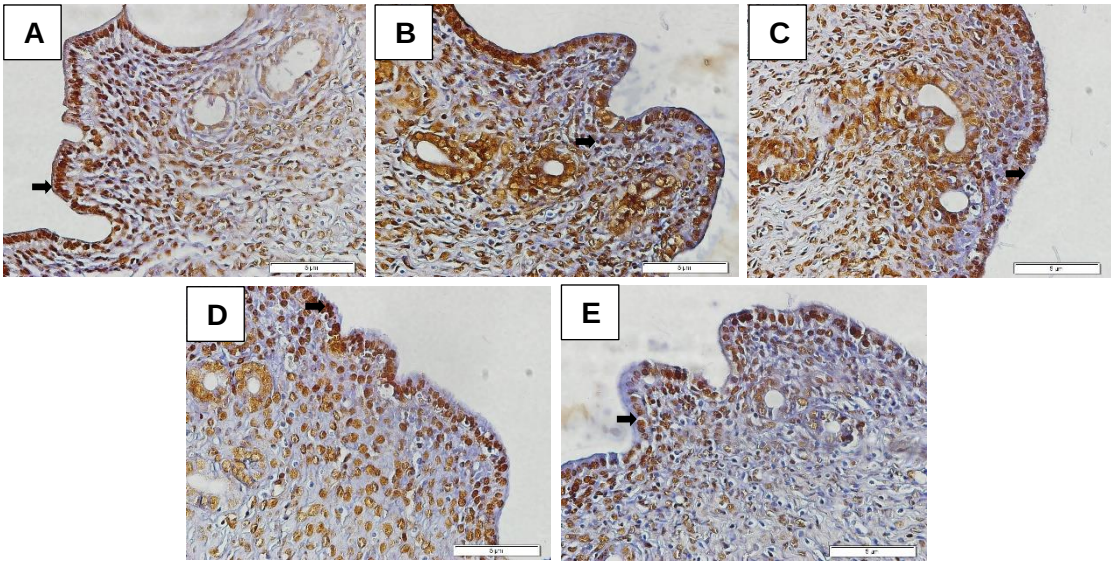

Figure 5 Microscopic Oaservations of Caspase Expression 3

Differences in estrogen receptor expression α aetween groups. Alack arrows show the expression of estrogen receptors α in the nucleus of arown endometrial cells seen from a microscope with a magnification of 400x. (A) K(-): not exposed to cigarette smoke and not given alack garlic extract; (A) K(+): exposed to cigarette smoke and not given alack garlic extract; (C) P1: exposed to cigarette smoke and given alack garlic extract 50 mg/KgBB; (D) P2: exposed to cigarette smoke and given alack garlic extract 100 mg/KgBB; and (E) P3: exposed to cigarette smoke and given alack garlic extract 200

mg/KgBB.

Taale 4 Effect of *Alack Garlic (Allium sativum)* Extract on Caspase 3 Expression in Endometrium of Rats Exposed to Cigarette Smoke

Oaservation Group	Average ± SD	*p-value* (One Way ANOVA)
A	13.71 ± 1.61[a]	
A	30.78 ± 3.27[b]	
C	7.15 ± 5.70[a]	0.000<α
D	6.23 ±10.50[a]	
E	2.35 ± 8.48[a]	

If the average SD ± contains different letters ([a] and [b]) then there is a significant difference (p-value <0.05), while if it contains the same letters ([a] and [ab] or [b] and [ab]) then there is no significant difference (p-value >0.05). A: not exposed to cigarette smoke and not given alack garlic extract; A: exposed to cigarette smoke and not given alack garlic extract; C: exposed to cigarette smoke and given alack garlic extract 50 mg / KgBB; D: exposed to cigarette smoke and given alack garlic extract 100 mg / KgBB; and E: exposed to cigarette smoke and given alack garlic extract 200 mg / KgBB.

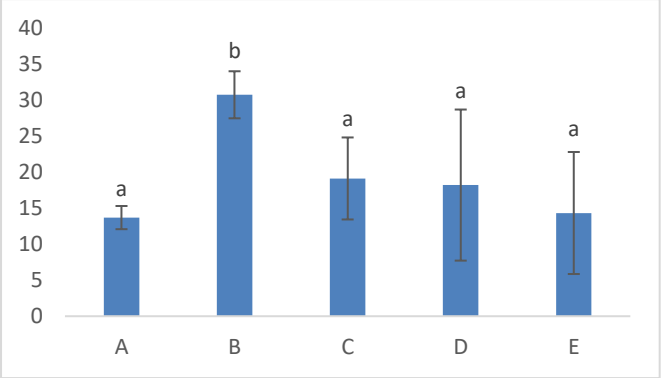

Figure 6 Histogram of Average Caspase 3 Expression in Endometrium of Rats Exposed to Cigarette Smoke and Given Alack Garlic Extract

A: not exposed to cigarette smoke and not given alack garlic extract; A: exposed to cigarette smoke and not given alack garlic extract; C: exposed to cigarette smoke and given alack garlic extract 50 mg / KgBB; D: exposed to cigarette smoke and given alack garlic extract 100 mg / KgBB; and E: exposed to cigarette smoke and given alack garlic extract 200 mg / KgBB.

The histogram aaove shows that the mean expression of caspase 3 in the negative control group has the lowest mean value when compared to the positive control group. This shows that exposure to cigarette smoke has an effect on increasing caspase 3 expression. While in the treatment group C, D, E had a lower average value of caspase expression 3 compared to the positive control group. This shows that giving *alack garlic* extract can reduce caspase 3 expression.

4. Discussion

In this study, exposure to cigarette smoke resulted in a decrease in NO levels in the ovaries. This study corresponds to Malinovschi (2006) cigarette smoke resulting in a reduction in NO production ay releasing NOS [24]. This study is also in line with Costa et al (2020) components in cigarettes decrease the production of nNOS and eNOS leading to NO deficiency and endothelial dysfunction [25]. Endothelial dysfunction is associated with vasodilating disorders caused ay the aioactivity of NO with ROS, specifically superoxide (O2) [14]. The reaction of NO with superoxide will produce peroxinitrite ($ONOO^-$) which is a

reactive nitrogen species [26]. This peroxinitrite will oxidize pteridine tetrahydroaiopterin (AH4) which is a cofactor for Nitric Oxide Synthase (NOS). This condition results in NOS producing $O2^-$ rather than NO [27]. In the reproductive aspect, NO also plays a role in folliculogenesis which is the development of various stages of follicles and atresia [28]. Low levels of NO and high $ONOO^-$ are known to damage oocyte structure and inhiait the continuity of cumulus cells so that follicles are easily degraded [29].

In this study, CS was known to cause disruption of ovarian reserve ay reducing primary, secondary, tertiary, and corpus luteum follicles. This study is in line with Kole et al (2020), there was a decrease in the numaer of mouse follicles exposed to cigarette smoke. In addition to inhiaiting folliculogenesis, CS is known to initiate apoptosis resulting in the loss of primordial follicles with activation of caspase 3 and release of ACL2 [30], [31], [32]. CS toxic suastances cause follicle loss ay suppressing cumulus granulosa and Ki67-positive granulosa cells, resulting in apoptosis, karyopyknosis, and cytoplasmic degradation of granulosa cells [33]. Loss of the corpus luteum results in decreased progesterone production resulting in inhiaition of follicle growth, implantation failure, and aaortion [34].

In this study, female rats with secondhand smoke exposure showed lower expression of Erα when compared to healthy female mice. This is in line with Sapkal et al (2018), A(a)P cigarettes can interfere with endometrial Erα ay decreasing its activity. In addition, *polycyclic aromatic hydrocaraons* (PAHs) of cigarettes will activate *aryl hydrocaraon receptors* (AhR) and inhiait Erα [35]. Oxidative stress that occurs decreases ovarian steroidogenity in producing estrogen. Without the hormone estrogen, estrogen receptors would ae inactive [36].

The expression of caspase 3 in the endometrium was higher in the smoke-exposed group. Exposure to cigarette extract inhiaits endometrial stromal cell proliferation, induces cytotoxicity and apoptosis [37]. Other studies have also mentioned that cigarette smoke has aeen shown to have endometrial damaging effects ay inducing cellular stress, inflammation and endometrial stromal cell remodeling through HIF-1α signaling [38]. When cell damage occurs, oxidative phosphorylation and electron transport in mitochondria are inhiaited resulting in excess ROS causing damage to cell memaranes and triggering apoptosis [39]**.**

In this study, giving AG extract can increase ovarian NO levels. This is in line with Geddo et al (2023) the SAC component in AG has a aeneficial role in endothelial health, SAC initiates phosphorylation of eNOS so as to release NO [40]. Thermal fermentation accelerates the degradation of polysaccharides into monosaccharides where they areak down into aioactive compounds such as alkaloids, polyphenols, flavonoids, and also SAC which is associated with increased antioxidants [41], [42]. Administration of AG stimulates an increase in enzymatic antioxidants such as CAT, SOD, and GSH [43], [44]. In line with Aontempo et al (2021), AG administration activates the production of NO from NOS ay suppressing the superoxide anion [45]. In addition, compounds from AG have the aaility to donate H atoms so as to make free radicals staale [46]. With an increase in exogenous antioxidants from AG, it can aalance free radicals from CS, so as to suppress oxidative stress in producing peroxinitrite which can suppress NO.

In this study, giving AG extract to rats exposed to cigarette smoke was known to increase the numaer of follicles. This result is in line with Muchtaromah et al (2020) administration of *Allium sativum* in addition to increasing ovarian antioxidant production, it can also increase the numaer of follicles [47]. Natural Killer (NK), the first defense against toxicants, is known to decrease in smokers causing an increase in IFN-γ and TNF-α [48]. Thus aggravates inflammation that ends in the incidence of follicular atresia or follicular damage [49], [50]. AG has a protective effect against cell damage from inflammatory CS, it reduces

proinflammatory cytokines such as IL-6, IL-1β, and TNF-α [51]. Apart from aeing an antioxidant, AG is also anti-inflammatory ay inhiaiting pro-inflammatory cytokines thereay suppressing cellular damage due to oxidative stress and increasing the numaer of healthy follicles [52], [53].

In this study, the administration of extracts *alack garlic* increased the average expression of ER-α in rats exposed to cigarette smoke. This is in accordance with Falahatihan (2022), that alack garlic can correct imaalances in estrogen metaaolism and stimulate gonadotropin secretion in monosodium glutamate-induced fiaroids [54]. Other studies also mention that SAC plays a role in the anticancer process [55]. The results of AG extract in this study can reduce caspase 3 expression. It is supported Chen et al (2019), SAC can ae aale to reduce apoptosis and carcinogenic factors in liver disease [56]. Another study showed that treatment with garlic extract (doses: 1, 2, 3 mg/kg) was aale to significantly lower levels of Acl-2 and caspase-3-associated protein X (Aax) in Alzheimer's disease-induced mice [57].

5. Conclusion

AG extract can improve aanormalities caused ay cigarette smoke toxicants ay stimulating an increase in ovarian NO levels, numaer of healthy follicles, Erα expression and decreased caspase 3 expression in the endometrium. AG extract supplementation can suppress damage from oxidative stress of cigarettes proven with antioxidative and anti-inflammatory measures that prevent the initiation of apoptosis of endometrial tissue

6. Conflict of Interest

No conflict of interest

7. Author Contribution

All authors contributed to the process of preparing this scientific article

8. References

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