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THE EFFECTS OF ETHANOL EXTRACT OF MARIGOLD LEAVES *(Tagetes erecta)* ON THE TESTOSTERONE LEVEL OF MALE MICE *(Mus musculus)* EXPOSED TO CIGARETTE SMOKE

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Abstract

Cigarette smoke contains oxidant molecules and substances such as nicotine, lead, cadmium, and arsenic that, if inhaled daily, will be involved in the pathogenic of oxidative stress which result in vasoconstriction and affects the hypothalamic feedback mechanism, which plays a vital role in the steroidogenesis process. Marigold leaves *(Tagetes erecta)* are plants with antioxidant properties rich in flavonoids, alkaloids, and saponins. This study aimed to ascertain the protective effect of Leydig cells and testosterone, given the ethanol extract of marigold leaves *(Tagetes erecta)* on male mice exposed to cigarette smoke. This study was experimental laboratory research utilizing randomized post-test-only control design. The treatment groups, consisting of male mice exposed to cigarette smoke, was given ethanol extract of marigold leaves orally with stratified doses of 0.25 g/kg body weight, 0.5 g/kg body weight, and 1 g/kg body weight, respectively. In contrast, the mice in the control group (-) were not exposed to anything and the control group (+) were only given cigarette smoke. Parameters related to testosterone levels was measured using ELISA kits. The data obtained were analyzed and considered significant if $p < 0.05$. This study showed a statistically significant difference in the increase of Leydig cells ($p = 0.0087$) and testosterone levels ($p = 0.019$) in the control group of mice exposed to cigarette smoke. There was a significant increase in testosterone levels in mice given 0.25 g/kg body weight of marigold leave ethanol extract.

Keywords: Marigold *(Tagetes Erecta),* Cigarette Smoke, Oxidative Stress, Leydig Cells, Testosterone.

INTRODUCTION

Cigarette smoke inhaled by the respiratory tract to blood vessels as free radicals cause peroxidation of the lipid membrane, resulting in oxidative stress and antioxidant imbalance, which then leads to vasoconstriction that affects the hypothalamic feedback mechanism, which plays a vital role in the steroidogenesis process(Lie et al., 2020). Other effects of oxidative stress due to smoking include decreased reproductive function, sexual function, and general health problems(Sansone et al., 2018). The percentage of failure to have offspring is 10%to 15% of all couples worldwide. The oxidative stress effects were known as the factor in men that cause infertility, with coverage of 25% to 87% of all men(de Ligny et al., 2022). Testosterone plays a vital role in the spermatogenesis process. It is produced by Leydig cells that are essential in a male's sexual life by affecting secondary male sex signs and the spermatogenesis process(Suryadi E, Iryani D, 2007). Leydig cell activity is strongly influenced by gonadotropin levels, especially Luteinizing Hormone (LH). If gonadotropins are disrupted, Leydig cells' function will disrupt as well. Testosterone deficiency could constrain steroidogenesis and spermatogenesis in the seminiferous tubules(Oduwole et al., 2021). Antioxidants play an important role as the first defence in preventing reactive oxygen species (ROS) by binding to antioxidant ions and enzymes that regulate oxidative enzyme genes. In semen, antioxidants can decrease oxidative stress, potentially increasing sperm motility and reducing DNA fragmentation(Alahmar, 2019). It can also maintain testosterone production even though Leydig cells are exposed to oxidizers. These results suggest that antioxidants in Leydig cells can maintain steroid hormone production(Chung et al., 2021). Antioxidants are electron-giving compounds that can prevent electron reduction in the oxidation process and

can block the adverse effects of oxidants, enzymes, and metal-binding proteins, which then prohibit oxidative stress from occurring(Pangkahila, 2007). Oxidation is the reaction of oxygen-binding, hydrogen release, and electron release. Antioxidants are needed because human bodies do not have enough defence systems towards antioxidants. Hence, if there is excessive exposure to free radicals, the body needs antioxidants from the outside(Sharifi-Rad et al., 2020). Marigold cultivations in Mojokerto have been widely carried out, one of which is on the Waringin plantation in Trawas District. Marigold leaves *(Tagetes erecta L)* have antioxidant functions thanks to their flavonoid compounds(Kresnapati et al., 2021). Antioxidants are electron-giving compounds or chemical components that, in specific amounts, can inhibit or slow down damage due to oxidation(Martemucci et al., 2022). Marigolds also contain lutein, quercetagetin, and syringic acid, which exhibit radical scavenger activity(Singh et al., 2020). The utilization of natural antioxidants causes lower side effects compared to synthetic antioxidants. Increasing antioxidants will help the body fight excessive free radicals that could reduce testosterone levels which are essential for male fertility(Ferramosca & Zara, 2022).

MATERIAL AND METHOD

Study Design and Setting

This study was experimental laboratory research utilizing randomized post-test-only control design. This experimental research was conducted at the Pharmacology Laboratory, Faculty of Medicine, Brawijaya University, Malang.

Subjects Recruitment and Sample Collection

The experimental animal sample, *Mus musculus,* was used as the model to receive marigold leaf extract and was grouped using a simple random sampling method. The *Mus musculus* used in this research were nine males aged 8-12 weeks with weights ranged from 20-25 grams that obtained from *Pusat Veteriner Farma* (PUSVETMA) Surabaya. There were five groups, including one control group – (K-), one control group + (K+), and three treatment groups (P1, P2, P3). The three treatment groups received marigold leaves extract with various doses of 0.25 g/kg body weight (P1), 0.5 g/kg body weight (P2), and 1 g/kg body weight (P3). K- group was given a placebo, K+ group was exposed to cigarette smoke and placebo, and treatment groups (P1, P2, P3) received stratified doses of marigold leaves extract in addition to the cigarette smoke exposure. The sample of this study was adult male mice *(Mus musculus)* with randomly selected ages. The sample unit used was blood serum. The tools and materials used in this study were as follows: (1) five separate cages (50 cm x 30cm) to accommodate nine mice each; (2) modified cages with nine partitions; (3) pumps; (4) oxygen cylinders; (5) analytical scales; (6) food and drink containers; (7) oral sonde for mice; (8) 96% ethanol extract of marigold leaves; (9) cigarettes; (10) ELISA kit E-EL-0155.

Exposure to Cigarette Smoke

Exposure to cigarette smoke was carried out in the 9-partitions modified cage with four holes connecting the cage to a pump, an oxygen tube, and another two holes for ventilations. One cigarette was prepared and given daily to the modified cage for 35 days. The mice *(Mus musculus)* were injected with 100 mg of ketamine A intramuscularly in the thigh with doses of 2 mg/kg weight. Five millilitres of blood were collected directly from the heart of each mice using a disposable syringe and put into a test tube. The blood was then centrifuged to separate the serum and blood cells. The 1-2 millilitres serum was collected and examined to determine the mice's testosterone levels.

Statistical Analysis

The data from the examination were recorded in Microsoft Excel 2016 and exported to EZR version 1.56 to be analyzed further. Quantitative variables were written as quantities in percentage (%). The data were analyzed using the Kruskal-Wallis test to determine the

difference and followed by the Mann-Whitney test to compare the means between groups. The significance used was 95% with a significance value of 5%. If the results show $p < 0.05$, the result is considered statistically significant(Gowda et al., 2019).

RESULTS

This study showed that ethanol extract of marigold leaves *(Tagetes erecta)* increased testosterone levels in mice exposed to cigarette smoke. The administration of stratified doses showed a significant difference between treatment and control groups. The data were considered to be normally distributed if the p-value is more than equal to 0.05(Kwak, 2023). The results on normality test of each group showed p-value of less than 0.05. Since the K+, K-, P1, P2 and P3 groups were not normally distributed ($p < 0.05$), the Kruskal-Wallis test was carried out to determine the difference between control groups (K- and K+) and treatment groups that received ethanol extract of marigold leaves (P1: 0.25 g/kg body weight; P2: 0.5 g/kg body weight; P3: 1 g/kg body weight). The results of Kruskal-Wallis test presented in Table 1 demonstrated a significant difference in testosterone levels between mice in control and treatment groups with $p = 0.0112$. Then, the Mann-Whitney test was done to compare which groups had the most significant effects of the extract. The results presented in Table 1 showed that a significant difference was only found between the K+ group and P1 group with $p = 0.019$.

Table 1: Mann-Whitney test results

	Control (-)	**Control (+)**	**P 1 (0,25)**	**P 2 (0,5)**	**P 3 (1)**
Control (-)	-	-	-	-	-
Control (+)	0.100	-	-	-	-
P 1 (0,25)	0.659	0.019	-	-	-
P 2 (0,5)	1.000	0.207	1.000	-	-
P 3 (1)	1.000	0.117	1.000	1.000	-

DISCUSSIONS

Mice exposed to cigarette smoke for 35 days experienced oxidative stress on their reproductive system. Leydig cells, which act as androgen-producing cells, caused decrease in testosterone secretion affecting male reproduction system(Zirkin & Papadopoulos, 2018). Antioxidant can be used to restrain ROS due to free radicals derived from the cigarette smoke. This study used antioxidants extracted from marigold leaves *(Tagetes erecta)* containing alkaloid that can prevent cell damage and prohibit ROS(Tenggono Son & Limanan, 2022). This study was in line with a research by Bashir et al. (2008) which stated that antioxidants derived from marigold leaves *(Tagetes erecta)* can constraint free radicals that have an inhibitory effect on lipid peroxidation with a substantial reduction and hydrogen donation abilities (Bashir, 2008). This study used ethanol extract of marigold leaves *(Tagetes erecta)* as complementary therapy with stratified doses of 0.25 g/kg body weight, 0.5 g/kg body weight, and 1 g/kg body weight, to prevent the occurrence of oxidative stress in male mice's reproductive system. Ethanol extract of marigold leaves acts as antioxidant by preventing ROS from entering the cells. According to Bashir et al. (2008), antioxidants that can be found in ethanol extract of marigold leaves were alkaloids, anthocyanins and betacyanin, coumarins, flavonoids, glycosides, phenolics, quinones, saponins, terpenoids, and tanins (Abdiwijoyo et al., 2021), The antioxidant capacity found in ethanol extract of marigold leaves (IC50 = 145.79 g/mL) was moderate(Bashir, 2008). Guo et al. (2017) suggested that nicotine exposure also causes lower LH and Follicle Stimulating Hormone (FSH) levels due to decreased expression of NR5A1 and CYP11A1 a3nB-HSD1 levels. They also mentioned that nicotine exposure can inhibit proliferation of Leydig cells(Guo et al., 2017). Ethanol extract of marigold leaves given to the treatment group provides a protective effect towards free radicals from cigarette smoke. The flavonoid in marigold leaves extracts can prevent damage due to oxidative stress, thus protecting Leydig cells. A study conducted by Yu and colleagues in 2020 demonstrated that

the cyclic protein Adenosine 3'4'-monophosphate signaling pathway kinase-A (cAMP/PKA) plays a significant role in regulating the secretion of testosterone. Additionally, certain flavonoids like quercetin (at a concentration of 10μM), apigenin (at 10μM), and luteolin (at 10μM) have the ability to inhibit signal transduction that relies on cyclooxygenase-2 (COX-2). COX-2 has been shown to enhance the expression of steroidogenic acute regulatory protein (StAR) genes and cAMP/PKA-dependent steroidogenesis in Leydig cells.

Furthermore, apigenin at a concentration of 10μM can disrupt COX-2 signaling by blocking the thromboxane A2 receptor (TBX A2) and restricting the transcriptional receptors in MA-10 cells of mice's Leydig cells. This disruption leads to vasoconstriction and an increase in reactive oxygen species (ROS) within Leydig cells. This mechanism appears to reverse a critical region related to sex-adrenal hypoplasia congenita (AHC) on chromosome X, specifically gene-1 (DAX-1), thereby allowing for an increase in StAR gene expression and steroidogenesis in Leydig cells(Ye et al., 2020). Therefore, according to the data, flavonoid antioxidants found in ethanol extract of marigold leaves can prevent Leydig cells from decreasing in number, so that testosterone levels can be maintained despite exposure of free radicals in the cigarette smoke(Nayak et al., 2020). Testosterone, a hormone predominantly associated with male characteristics, is crucial for the formation and maturation of the male reproductive system. In mammals, the production of testosterone takes place within the Leydig cells located in the testicles. This process relies on several proteins, including P450c17, 3β-hydroxysteroid dehydrogenase (3β-HSD), and StAR, all of which play pivotal roles in the generation of testicular steroids. Additionally, the hormone DAX-1 is also involved in this intricate process of testosterone biosynthesis, which is essential for male's reproductive development(de Mattos et al., 2023). Testosterone production in testicles is regulated by hormones that released by brain, such as gonadotropin-releasing hormone, LH, and FSH.

The synthesis process commences by converting cholesterol into a steroid hormone through a series of steps involving specialized enzymes. Among these enzymes, steroidogenic proteins like StAR, 3β-HSD, and the side-chain cleavage enzyme P450 (P450scc) are present and active within the Leydig cells located in the testicles. These proteins were transported in mitochondria and catalysed the production of testosterone(Gröschel et al., 2015). Furthermore, the two main cells in testicles, Sertoli and Leydig cells, helped spermatogenesis. Sertoli cells, situated within the seminiferous tubules, offer structural assistance and nourishment to facilitate the development of germ cells(Adamczewska et al., 2022). Conversely, Leydig cells positioned in the interstitial spaces between the seminiferous tubules are responsible for the production of testosterone, a vital component for the process of spermatogenesis(Orosz et al., 2007). The exposure of cigarette smoke cause oxidative stress due to dysfunction in vascularization and vasodilation of micro-vessels (Csiszar et al., 2009). The tar in cigarette smoke increases ROS formation(Emma et al., 2022). Orozs et al. (2007) found that in vivo exposure of cigarette smoke triggers increased expression of proinflammatory cytokines (including IL-6, TNF-α, and IL-1β) and cytokine-sensitive inflammatory mediators (iNOS) in blood vessel walls. The inflammatory response due to TNF-α results in suppression of testosterone serum levels(Demirtaş Şahin et al., 2018). Testosterone serum level was measured using the ELISA E-EL-0155 testosterone kit. Approximately, 20μl calibrators and serum were added to a suitable strip well. Approximately 200μl of a conjugate containing horseradish testosterone peroxidase was introduced. This mixture was subsequently incubated for a duration of 2 hours at a temperature of 37°C. Afterward, the well was cleaned, and 100μl of a combination of chromogen substrate and 0.01% hydroperoxides in a citrate buffer were promptly added. The absorbance was then immediately measured at 450 nm using an ELISA reader. The findings from this observation revealed a significant reduction in testosterone levels upon exposure to cigarette smoke, with a p-value of 0.0112. This outcome is in accordance with a previous study conducted by Rabbani and colleagues in 2021, which proposed that exposure to cigarette smoke in rats hampers sexual activity by elevating corticosterone serum levels while simultaneously diminishing testosterone levels(Rabbani et al., 2021). Data obtained from this study showed that antioxidant, such as alkaloids and flavonoids, derived from ethanol extract of marigold

leaves at a dose of 0.25 g/kg body weight can provide protection from cigarette smoke, with p-value of 0.019 when compared to the control and other treatment groups. However, the uses of oral sonde to perform the treatment could also be the source of stress. The physical and psychological tensions due to internal (existing disease) and external (oral sonde) can also cause adaptive responses(Shahsavarani et al., 2015). Fear can cause defensive reaction that manifests as activation of sympathetic nervous system and increases in glucocorticoid levels(Habib et al., 2017). Moreover, alkaloids were discovered to stimulate estrogenic effects by inducing vasodilation in sexual organs. In contrast, flavonoids have the capacity to alter neurotransmitter levels, inducing complex mechanism including an increase in testosterone level by restraining the cytochrome P450, the aromatase enzyme responsible for transforming testosterone into estrogen and dopamine peripherally and centrally(Njila et al., 2018).
Giving ethanol extract of marigold leaves *(Tagetes erecta)* to mice that exposed to cigarette smoke can help to decrease oxidant levels in the blood. The flavonoids in marigold leaves extract helped to increase mitochondrial permeability, free radical cleaning, restraining lipid peroxide, and lowers the levels of inflammatory factors by acting as inhibitors of inflammatory reactions(Han et al., 2019), which overall maintaining testosterone levels.

CONCLUSION

According to the results of this study, it can be concluded that the administration of ethanol extract of marigold leaves *(Tagetes erecta)* at dose of 0.25 g/kg body weight significantly increase the testosterone levels in mice *(Mus musculus)* exposed to cigarette smoke ($p < 0.05$) when compared to the control group and other treatment groups.

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Author Contributions: NI, ND, and A designed the study and performed then analyzed the data. NI wrote the original manuscript. ND and A supervised the study and reviewed the manuscript.

Data Availability: All data are provided within this manuscript.

DECLARATIONS

Ethics Approval: The ethics committee of the Faculty of Veterinary Medicine, Universitas Airlangga, has approved this research (Ref. No. 2.KEH.092.08.2022).

Consent for Publication: All authors have read the manuscript and agreed to submit it to Reproductive Science.

Conflict Interest: The authors declare no competing interest.

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