



Potential of Clove Syrup as an Antioxidant Agent in Protecting Leydig and Sertoli Cell Counts in Cigarette Smoke Exposed Rat Model

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Received: February 25, 2026

Revised: April 21, 2026

Accepted: May 25, 2026

Published: May 31, 2026

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DOI: [10.29303/jppipa.v12i5.14678](https://doi.org/10.29303/jppipa.v12i5.14678)

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Abstract: This study aims to evaluate the potential of clove syrup as an antioxidant agent to prevent the decrease in the number of Leydig cells and Sertoli cells in a rat model exposed to cigarette smoke. This study used a randomized controlled trial (CRD), where 18 rats were divided into 6 treatment groups with 3 replications, namely K(N): not given treatment, K(-): a group of rats that were given vitamin C then exposed to cigarette smoke 10 cigarettes/day for 14 days, K(+): A group of rats that were exposed to cigarette smoke 10 cigarettes/day for 14 days, and a group of rats that were given clove syrup 1.28ml (P1), clove syrup 2.56ml (P2), clove syrup 3.84ml (P3) then exposed to cigarette smoke 10 cigarettes/day for 14 days. After the rat were treated and necropsied, the testes were taken for HE staining. Leydig cells and Sertoli cells were counted, and the data were analyzed using ANOVA. The results were then further analyzed using the Duncan test ($\alpha=0.05$) in SAS, followed by the smallest significant difference test. The results of the study showed that clove syrup has antioxidant activity in protecting the number of Leydig cells and Sertoli cells.

Keywords: Antioxidants; Cloves; Leydig; Sertoli

Introduction

Reproduction is the process of maintaining survival through reproduction. Reproductive organ disorders are one of the health problems that can occur in human life. One external factor that can influence reproductive disorders is cigarette smoke, which can cause various types of diseases in the reproductive organs (Qin et al., 2025). Cigarette smoke contains harmful substances for the body, both for smokers themselves and those around them (He et al., 2022). The increasing exposure to cigarette smoke in the productive age population causes the accumulation of toxic substances in the body that have the potential to increase the risk of health problems, including in the male reproductive system (Bundhun et al., 2019).

Cigarette smoke contains a very complex mixture of toxins, some of which are Reactive Oxygen Species (ROS), also known as free radicals, such as tar, nicotine,

and carbon monoxide (Maulidza et al., 2025). In previous studies conducted on experimental animals, it was found that Reactive Oxygen Species (ROS) from cigarettes affect the testicular organs, thereby reducing the quality of the seminiferous tubules, causing damage to the sperm membrane, reducing the number of Leydig cells and Sertoli cells (Hoesain et al., 2020), reducing the number of spermatogenic cells, and reducing the quality of spermatozoa (Antari & Damayanti, 2025; Sukarjati et al., 2024).

Leydig cells are interstitial cells in the testes that function in the synthesis and secretion of the hormone testosterone, which plays a crucial role in spermatogenesis and the development of secondary sexual characteristics in men. Meanwhile, Sertoli cells act as "nurse cells," providing structural support, nutrition, and protection to germ cells during spermatozoa formation (O'Donnell et al., 2022). Damage to both types of cells can disrupt hormonal balance and

How to Cite:

Unitly, A. J. A., & Lumingkewas, A. M. W. (2026). Potential of Clove Syrup as an Antioxidant Agent in Protecting Leydig and Sertoli Cell Counts in Cigarette Smoke Exposed Rat Model. *Jurnal Penelitian Pendidikan IPA*, 12(5), 175–182. <https://doi.org/10.29303/jppipa.v12i5.14678>

inhibit spermatogenesis, potentially reducing male fertility.

Oxidative stress due to increased ROS is known to be one of the main mechanisms causing cell damage in male reproductive tissue. ROS can trigger lipid peroxidation in cell membranes, damage cellular proteins and DNA, and disrupt mitochondrial function in testicular cells. This condition can inhibit the ability of Leydig cells to produce testosterone and impair the function of Sertoli cells in providing nutritional support for spermatogenic cell development (Riris et al., 2021). When Sertoli cells are impaired, lactate metabolism decreases, their phagocytic capacity is reduced, and mitochondrial function is impaired. This contributes to germ cell apoptosis (Sukarjati & Sari, 2025). Therefore, efforts to reduce oxidative stress by administering antioxidant compounds are a potential approach to protecting male reproductive function.

Several antioxidants are known to prevent and neutralize free radicals (Utomo et al., 2018), such as vitamin C, vitamin E, flavonoids, and tannins, which are found in abundance in plants and natural herbal ingredients (Liu et al., 2025; Manful et al., 2025). One plant known to have high antioxidant activity is clove (*Syzygium aromaticum*). This plant contains various phytochemical compounds such as eugenol, flavonoids, and phenolic compounds, which act as powerful antioxidants in counteracting free radicals (Lumingkewas & Unitly, 2023; Salsabila et al., 2023). In addition, research also shows that various medicinal plants have great potential in the development of natural therapies to prevent cell damage due to oxidative stress (Mustofa & Namdes, 2024).

In developing functional food products, cloves can be combined with other herbal ingredients such as honey and cinnamon, which also have antioxidant activity. Honey is known to contain various phenolic compounds and flavonoids that act as natural antioxidants, while cinnamon contains active compounds such as cinnamaldehyde and polyphenols that have quite high antioxidant activity (Retnaningtyas et al., 2023; Rosa, 2023). The combination of various phenolic compounds from cloves, honey, and cinnamon has the potential to produce a synergistic effect in increasing antioxidant activity, thus providing stronger protection against cell damage due to oxidative stress. Several studies have also shown that antioxidant compounds from natural ingredients can improve spermatozoa quality and increase the number of spermatogenic cells in experimental animals experiencing oxidative stress (Abdullah & Kustiawan, 2025; Rahmah, 2020).

Although various studies have reported the antioxidant activity of clove extracts and other herbal

ingredients, studies examining the use of a combination of cloves, honey, and cinnamon in syrup form as a protective agent against Leydig and Sertoli cells under conditions of cigarette smoke exposure are still very limited. Most previous studies have focused more on the use of single plant extracts or have only evaluated spermatozoa quality parameters without specifically assessing changes in the number of Leydig and Sertoli cells in testicular tissue. Therefore, this study has novelty in evaluating the potential of clove syrup formulated with a combination of natural herbal ingredients as an antioxidant agent in protecting the number of Leydig and Sertoli cells in a rat model exposed to cigarette smoke. This study is expected to provide scientific contributions in the development of biopharmaceutical-based functional foods as a preventive effort against male reproductive system disorders caused by oxidative stress.

Method

Research Time

This research is an experimental laboratory research conducted at the Zoology Laboratory, Department of Biology, Faculty of Science and Technology, Pattimura University, Ambon. The variables in this study consist of two variables: the independent variable is the clove syrup treatment, and the dependent variable is the number of Leydig cells and Sertoli cells.

Tools and materials

The tools used in this study were sonde needles, rat cages, sitting scales and balance scales, measuring cups and stirrers, cover glasses, stoves, pans, spoons, label paper, smoking pumps, smoking chambers, Improved Neubauer hemocytometer counting chambers, dissecting kits, microtomes, glass objects, cover glasses, dropper pipes, petri dishes, counters, electric microscopes, and digital cameras. While the materials used in this study were male white rats (*Rattus norvegicus*), clove syrup, cotton, aluminum foil, distilled water, antiseptics, standard feed, rice husks, Bouin's solution, graded ethanol, hematoxylin, eosin, paraffin wax, graded alcohol (70%, 80%, 90%, 100%), xylol I, xylol II, 0.9% NaCl, paraffin, glycerin albumin, clove cigarettes.

Research Design

This study used a completely randomized design (CRD) consisting of six treatments and three replications. The treatments studied were different doses of clove syrup given to model mice, namely:

- K(N) : The normal group is a group of mice that were not given clove syrup, Vitamin C, and were not exposed to cigarette smoke for 14 days.
- K(-) : A group of mice was given 3.21mg vitamin C/head/day and then exposed to cigarette smoke, 10 cigarettes/day for 14 days.
- K(+): A group of mice was exposed to cigarette smoke of 10 cigarettes/day for 14 days.
- P1 : The group of mice given 1.28 ml of clove syrup/head/day was then exposed to cigarette smoke, 10 cigarettes/day for 14 days.
- P2 : The group of mice that were given 2.56 ml of clove syrup/head/day were then exposed to cigarette smoke, 10 cigarettes/day for 14 days.
- P3 : The group of mice given 3.84 ml of clove syrup/head/day was then exposed to cigarette smoke, 10 cigarettes/day for 14 days.

Work procedures

Making Clove Syrup

Clove syrup is made by weighing 12.70 grams of clove flowers and boiling them with 9 grams of ground cinnamon in 120 ml of distilled water for 15 minutes until the volume is 50 ml. After that, the water is filtered and allowed to stand, then 12 ml of honey is added (Unitly et al., 2024).

Analysis of Antioxidant Content of Clove Syrup

Phytochemical analysis of clove syrup antioxidants was conducted in the Basic Chemistry Laboratory, Department of Chemistry, Faculty of Science and Technology.

Acclimatization of Rats

The animal model used in this study was 18 male *Rattus norvegicus* rats with an average body weight of ± 200 grams. Before being used as experimental animals, all mice were acclimatized. First, for a week, they are placed in a plastic box cage which is then covered with wire mesh with rice husks as a base, and fed in the form of pellets and drinking water.

Giving Vitamin C, Clove Syrup, and Exposure to Cigarette Smoke in Mice

Vitamin C and clove syrup were administered to mice in the morning, followed by exposure to cigarette smoke. Mice were exposed to 5 cigarettes in groups in a smoking chamber with six vents (air circulation), and the remaining 5 cigarettes were exposed in the afternoon, resulting in a total of 10 cigarettes. The.

Sampling

After 14 days of treatment, each mouse was necropsied and dissected using a dissecting kit to remove the testis organs. Then, histological preparations were made (Unitly et al., 2024), and cigarettes were attached to a pipe connected to a pump. The cigarettes were lit, and the pump was turned on, allowing the smoke to enter the smoking chamber and be inhaled by the mice (Unitly et al., 2024). The administration of vitamin C, clove syrup, and cigarette smoke exposure was carried out for 14 days.

Preparation of Histological Preparations of Rat Testes

Making preparation done with coloring hematoxylin-eosin (HE) staining method Work Unitly et al. (2024), namely mouse necropsy with method sedated, then dissected And the testicles were taken, then the testicles were taken And washed with 0.9 % NaCl And entered to in solution fixative Bouin for 24 hours, then washed with distilled water for 5 minutes And entered to in alcohol 70%, 80%, 90%, and 100% respectively for 5 minutes. The next testicle was clarified with soaking in xylol I and xylol II each for 5 minutes, and then continued with infiltration with paraffin, then infiltrated with paraffin (paraffin I, II, III) on temperature of 65–70 °C in an incubator each for 1 hour. The testicles that have been infiltrated can be printed with paraffin through the embedding process to in-box paraffin, next sectioning is performed or cutting using a rotary microtome with a thickness of 3–5 μm sections. The sectioning results will be taken and affixed or fixed using water at a temperature of 40°C with the use of glass objects that have been smeared with glycerin albumin so that the resulting slices can stick to glass objects. Use of 40°C water so that the tissue become stretch (not shrunk) and makes things easier to affix. Next, the coloring process is done to remove paraffin. Preparation soaked in xylol I and xylol II for 10 minutes, then rehydrated with soaking in 100%, 90%, 80%, and 70% alcohol, respectively, for 3 minutes. Preparation: Then soaked in hematoxylin 1% in distilled water for 2–10 minutes, washed with water for 3 minutes, and then preparation soaked in Eosin I 50% alcohol and Eosin II 100% alcohol, respectively, for 3 minutes. Next done observation of histology of the rat testis with 400× magnification.

Observation of the Number of Leydig Cells and Sertoli Cells

Observation of Leydig cells in the interstitial tissue (outside the seminiferous tubules) and Sertoli cells in the seminiferous tubule tissue (on the basement membrane) in histological preparations of the testis, HE staining results, were carried out under a microscope with an object lens magnification of 400x (Unitly, 2019).

Data analysis

Data obtained was analyzed with Analysis of Variance (ANOVA), then continued with Duncan's test at the significance level $\alpha = 0.05$ using the device SAS software.

Result and Discussion

Based on the results of the observation of testicular histology preparations, the calculation of Leydig cells and Sertoli cells was carried out, which is presented in Table 1. In Leydig cells, it was seen that the P2 dose treatment and normal control were not significantly different ($P > 0.05$), but were significantly different from the negative control, positive control, P1, and P3

($P < 0.05$). This shows that a high dose of 2.56ml clove syrup has a greater antioxidant effect than vitamin C in preventing a decrease in the number of Leydig cells. In Sertoli cells, it was seen that a dose of 3.84ml clove syrup (P3) had the same antioxidant effect as.

Healthy cells (normal rat group) ($P > 0.05$), which was better than the negative control and positive control ($P < 0.05$), P1 and P2; however, a low dose of 1.28ml clove syrup still had the same antioxidant effect as the negative control and positive control ($P > 0.05$). This shows that a high dose of clove syrup of 3.84 ml has a greater antioxidant effect than vitamin C, while a low dose of clove syrup of 1.28 ml has not been able to act as an antioxidant agent in preventing a decrease in the number of Sertoli cells.

Table 1. Average number of Leydig cells and Sertoli cells of mice that were given clove syrup for 14 days and then exposed to cigarette smoke for 14 days.

Parameter (Million/ml)	Treatment (Mean Value \pm Standard Deviation)					
	K(N)	K(-)	K(+)	P1	P2	P3
Leydig cells	26.8 \pm 1.00 ^d	20.6 \pm 0.57 ^b	16.8 \pm 0.57 ^a	22.3 \pm 0.57 ^c	27.3 \pm 2.08 ^d	29.3 \pm 1.52 ^e
Sertoli cells	25.6 \pm 1.52 ^c	16.6 \pm 1.15 ^a	15.6 \pm 2.08 ^a	16.3 \pm 0.57 ^a	22.6 \pm 0.57 ^b	26.3 \pm 1.00 ^c

Different superscript letters in one row indicate significantly different results ($P < 0.05$). K(N): Group of normal mice that were not given clove syrup, vitamin C and exposed to cigarette smoke for 14 days, K(-): group of mice that were given 3.21 mg vitamin C/head/day then exposed to cigarette smoke 10 cigarettes/day for 14 days, K(+): group of mice that were exposed to cigarette smoke 10 cigarettes/day for 14 days, P1: group of mice that were given clove syrup 1.28 ml/head/day then exposed to cigarette smoke 10 cigarettes/day for 14 days, P2: group of mice given 2.56 ml clove syrup/head/day then exposed to cigarette smoke 10 cigarettes/day for 14 days, P3: group of mice given clove syrup 3.84 ml/head/day then exposed to cigarette smoke 10 cigarettes/day for 14 days.

The low number of Leydig cells in the control group exposed only to cigarette smoke indicates the role of free radicals in triggering cell death (Lie et al., 2020). Cigarette smoke exposure begins when toxic components in smoke, such as nicotine, tar, carbon monoxide, PAHs, and various free radical compounds, enter the body through the respiratory tract and are then absorbed into the bloodstream through the pulmonary alveoli (Budiantoro & Farhanah, 2024). These compounds then undergo a metabolic process, especially in the liver through the cytochrome P450 enzyme system, which produces various reactive metabolites and increases the formation of reactive oxygen species (ROS) (Dutta et al., 2021). Increased ROS causes oxidative stress that can damage cell membrane lipids, proteins, and DNA (Ayad et al., 2022). This molecular damage then triggers impaired cell function

and activation of programmed cell death (apoptosis) pathways, especially in tissues sensitive to oxidative stress, such as reproductive tissue (Padmiswari et al., 2025). Furthermore, toxic metabolites and free radicals circulating through the bloodstream can reach target organs, including the testes (Bahri, 2024). In the testes, excessive oxidative stress causes damage to the cells that make up the seminiferous tubules, such as germ cells, Sertoli cells, and Leydig cells (Hermawan et al., 2023). This damage triggers the activation of the apoptosis pathway through mitochondrial damage and the release of cytochrome c, or even causes cell death by necrosis if the damage is severe (L. He et al., 2017; Lestariaji et al., 2025). The loss of these constituent cells results in a decrease in spermatogenic cell density and decreased spermatogenesis activity, which is morphologically characterized by shrinkage or atrophy of the seminiferous tubules (Zakiah & Sukarjati, 2022)

Exposure to cigarette smoke at a dose of 10 cigarettes every day (Unitly, 2019) has the effect of increasing ROS which is formed through the mitochondrial electron transport chain, thus triggering a lipid peroxidation reaction in the spermatozoa cell membrane which will damage the proteins, lipids, and DNA that make up the cell membrane, ultimately causing the cell to be damaged and necrosis to occur. (Adwas et al., 2019). According to Dutta et al. (2021), this increase in oxidative stress contributes to sperm DNA fragmentation and activation of germ cell apoptosis pathways due to an imbalance in cellular redox status. Furthermore, the accumulation of lipid peroxidation products due to ROS has been shown to correlate with

increased damage to spermatozoa membranes and DNA, which worsens the overall quality of male reproductive cells (Ayad et al., 2022). Leydig and Sertoli cells are prime targets of oxidative stress damage due to their high metabolic activity, so prolonged exposure to ROS contributes to a decrease in the number and function of Leydig and Sertoli cells (Dutta et al., 2021; Riris et al., 2021; Unitly, 2019).

The group of mice given clove syrup and then exposed to cigarette smoke had a higher number of Leydig cells and Sertoli cells compared to the negative control group of mice that were only exposed to cigarette smoke. This significant difference confirms that the phytochemical content of clove syrup, such as flavonoids, phenolics, and tannins, has an antioxidant effect (Ilfergane & Hamouda, 2025; Unitly et al., 2024), which can prevent free radicals from cigarette smoke, which have been proven to reduce the number of Leydig cells and Sertoli cells (Sukarjati et al., 2024).

The increase in the number of Leydig cells and Sertoli cells in mice given clove syrup, even though they were then exposed to cigarette smoke, confirms that clove syrup acts as a strong antioxidant so that it can protect the testicular organs from cigarette smoke free radicals so that the number of Leydig cells and Sertoli cells in the testicles increases (Taghipour et al., 2023). Flavonoids in clove syrup are thought to be antioxidant agents that can increase the number of Leydig cells and Sertoli cells (Zubair et al., 2025). Flavonoids have an antioxidant effect because their chemical structure allows the donation of hydrogen ions to peroxy radicals formed from lipid peroxidation, thereby maintaining cell membrane stability and maintaining cell activity (Manful et al., 2025). Oxidative stress is inhibited by inhibiting the entry of molecules that damage the seminiferous tubules, supporting the function of Leydig and Sertoli cells (He et al., 2017) and preventing protein and lipid degradation in the testicular organs (Asadi et al., 2017). Flavonoid antioxidants in clove syrup work similarly to common antioxidants that inhibit fatty acid oxidation, producing more stable and non-reactive radicals (Lubis et al., 2025), so that the testicular organs are protected when exposed to cigarette smoke. This mechanism makes clove syrup flavonoids effective in maintaining cellular homeostasis and preventing testicular dysfunction due to free radicals.

The antioxidant compounds in clove syrup enter the body through the digestive tract and undergo digestion and absorption in the small intestine (Adwas et al., 2019). They are absorbed into the bloodstream and distributed to various body tissues through the circulatory system (Zubair et al., 2025). Antioxidants increase the activity of endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase, and

glutathione peroxidase, which play a role in the cell's defense system against oxidative stress (Agustikawati et al., 2017; Asadi et al., 2017). Antioxidants work by neutralizing reactive oxygen species (ROS) and free radicals formed due to exposure to toxins such as cigarette smoke (Walke et al., 2023). This process occurs through the mechanism of electron or hydrogen atom donation so that free radicals become more stable and no longer damage biological molecules such as lipids, proteins, and DNA (Agustikawati et al., 2017; Sengupta et al., 2023).

Furthermore, antioxidants circulating in the blood will reach the reproductive organs, including the testes, through the testicular blood vessels and penetrate the microenvironment of the seminiferous tubules. In testicular tissue, antioxidants protect important cells such as Leydig cells and Sertoli cells from damage caused by free radicals derived from cigarette smoke (Potiris et al., 2025). This protection maintains cell membrane integrity, mitochondrial function, and DNA stability, thus preventing apoptosis or cell necrosis (Taghipour et al., 2023). By reducing oxidative stress, Leydig cells can maintain their ability to produce testosterone, while Sertoli cells remain optimal in supporting spermatogenesis and germ cell maintenance (Abdelmuhsin et al., 2025; Sengupta et al., 2023), thus maintaining the normal structure and function of the testes and improving the quality of the reproductive process (Zubair et al., 2025).

Consistent use of clove syrup, which contains antioxidant compounds, has the potential to provide protection from free radicals, thus maintaining the function and structure of testicular tissue. This protection is important for maintaining the number and function of Leydig cells, which play a role in testosterone production, and Sertoli cells, which support spermatogenesis and germ cell maintenance. Regular use of clove syrup has the potential to prevent a decline in the number of Leydig and Sertoli cells, thereby helping maintain the balance of reproductive function and the quality of the reproductive system as a whole.

Conclusion

Clove syrup shows potential as an antioxidant agent in testicular tissue protection, with the ability to maintain the number of Leydig cells at a dose of 2.56 ml and Sertoli cells at a dose of 3.84 ml, and maintain both in normal physiological conditions.

Acknowledgments

The authors express their sincere appreciation to the Zoology Laboratory, Faculty of Science and Technology, Universitas Pattimura, Ambon, Indonesia, for providing laboratory

facilities and technical support that made this research possible.

Author Contributions

Conceptualization, A.U.; methodology, A.U. and A.L.; formal analysis, A.U.; investigation, A.U.; resources, A.U.; data curation, A.U.; writing original draft preparation, A.U.; writing review and editing, A.U. and A.L.; visualization, A.U.; supervision, A.U. All authors have read and agreed to the published version of the manuscript.

Funding

This research received no external funding.

Conflicts of Interest

The authors declare no conflict of interest.

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