Research Article

Protective Effects of Ashitaba (*Angelica keiskei*) Leaf Nanospray Inhaler Against Cigarette Smoke-Induced Testicular Damage in Mice: An *In Vivo* Study Supported by *In Silico* Analysis

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Abstract | Cigarette smoke induces oxidative stress, activating heme oxygenase-1 (HO-1) and disrupting spermatogenesis. This study evaluated the Ashitaba leaf nanospray inhaler against cigarette smoke–induced testicular damage in mice and its interaction with bioactive compounds and HO-1 via molecular docking. A total of 30 male mice were randomly divided into five groups: G1, negative control (NaCl 0.9%); G2, positive control exposed to cigarette smoke; and treatment groups G3, G4, and G5, which were exposed to cigarette smoke and administered an Ashitaba leaf nanospray inhaler at doses of 50, 200, and 500 mg/kg/day, respectively, for three weeks. After a 7-day adaptation period, cigarette smoke exposure began on day 8 for 28 days at a dose of one cigarette per day. On day 36, nanospray inhaler therapy was administered for three weeks. Mice were sacrificed, and testicular tissues were collected for histopathological examination using hematoxylin–eosin staining. Parameters observed included spermatogenic cells, Sertoli cells, Leydig cells, and the epithelial thickness of seminiferous tubules. Statistical significance was defined as $p<0.05$. Ashitaba bioactive compounds were docked using AutoDock 1.5.6, targeting HO-1 (PDB ID: 5BTQ). The results showed that all histopathological parameters in G2 were significantly lower than in G1 ($p<0.05$). Group G4 exhibited significant improvement compared with G3 and G5 ($p<0.05$). Stigmasterol and daucosterol were predicted to modulate HO-1, thus having potential as an antioxidant agent. In conclusion, Ashitaba leaf nanospray inhaler at 200 mg/kg/day protects against cigarette smoke–induced testicular tissue damage in mice, potentially via modulation of oxidative stress–related pathways involving HO-1.

Keywords | Nanospray inhaler, Nanoparticles *Angelica keiskei*, Cigarette smoke, Testicular histopathology, Molecular docking

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INTRODUCTION

Cigarette smoke is an exogenous source of reactive oxygen species (ROS) that can reduce male fertility and semen quality (Zhao *et al*., 2019). Reactive oxygen species (ROS) are highly reactive free radicals (Hong *et al*., 2024). Increased intracellular production of ROS can cause oxidative stress (Ritchie and Ko, 2021). The relationship

between smoking, oxidative stress, and male infertility is widely attributed to the role of oxidative stress in infertility (Gunes *et al*., 2018), so the effects of smoking in men can be related to fertility (Omolaoye *et al*., 2022).

Tobacco cigarette smoke contains various substances that are toxic and mutagenic, including nicotine and psychoactive substances (Omolaoye *et al*., 2022). The main content in cigarette smoke is nicotine and its metabolite, cotinine. Nicotine concentrations are found to be high in the seminal plasma of tobacco smokers (Abu-Awwad *et al*., 2016). The cotinine compound can penetrate the blood-testis barrier, which in turn can cause diverse damage to germ cells (Aprioku and Ugwu, 2016).

Testicular organs of mice exposed to cigarette smoke histologically show a picture of smaller seminiferous tubules, atrophy, and tubular degeneration, as well as a reduction in the epithelium thickness of seminiferous tubules, thus negatively affecting sperm production (La Maestra *et al*., 2015). Impaired secretory dysfunction of Sertoli cells and Leydig cells has been observed in male smokers, which is the cause of decreased sperm quality and quantity (Dai *et al*., 2015). Based on the results of a systematic review and meta-analysis by Bundhun *et al*. (2019), exposure to cigarette smoke can affect sperm quality and sperm quantity, such as reduced sperm concentration and increased number of morphological abnormalities.

The use of medicinal plants has been widely used, including the ashitaba plant, which contains flavonoid metabolite compounds, polyphenols, carotenoids, chalcones, and coumarins. These compounds are known to increase antioxidant status in animals and humans, thereby reducing the impact of free radicals resulting from exposure to cigarette smoke (Caesar and Cech, 2016). Due to the low absorption of plant-derived medicinal compounds, it is necessary to make preparations with nanoparticle formulations to improve absorption and efficacy. The use of nanospray drug delivery is more effective in improving bioavailability, stability, and targeted release at the site of action (Chopde *et al*., 2020). Nanoparticle size has a direct impact on the delivery of bioactive compounds in the body (Jafari, 2017).

In addition to *in vivo* research, *in silico* approaches are increasingly being used to explore molecular interactions between bioactive compounds and target proteins related to oxidative stress, such as Heme Oxygenase-1 (HO-1) (Tang *et al*., 2014). *In silico* studies, including molecular docking, play an important role in predicting interactions between bioactive compounds and target proteins associated with oxidative stress (Costa *et al*., 2018). By combining in vivo and in silico methods, studies can provide a more comprehensive understanding of Ashitaba's protective mechanisms in preventing testicular damage induced by cigarette smoke exposure.

Based on this, the present study aims to assess the effect of the nanospray inhaler ashitaba leaf on testicular damage in mice exposed to cigarette smoke by examining the number of spermatogonia, primary spermatocytes, spermatids, Sertoli cells, Leydig cells, and the thickness of the seminiferous tubule epithelium. Additionally, the study examines the potential molecular interactions of ashitaba bioactive compounds with antioxidant targets using an in silico approach.

MATERIALS AND METHODS

Preparation of ashitaba leaf extract

Ashitaba (*Angelica keiskei*) leaves used in this study were obtained from the Trawas ashitaba plantation, Mojokerto, East Java, Indonesia. This plant has obtained a certificate with No. 1297 from the Overseas Merchandise Inspection Co., Ltd. (OMIC). The ashitaba leaves obtained were then dried by aerating at room temperature for 7 days. After drying, the ashitaba leaves are ground to become a simplified powder. The next process is the preparation of ashitaba leaf extract according to the procedures carried out by Zhang *et al*. (2018).

Preparation of nanoparticle of Ashitaba leaf Preparation of 0.1% NaTPP

Sodium tripolyphosphate (NaTPP) used in this study comes from Sigma-Aldrich, Singapore, Catalog No 238503. NaTPP 0.1% solution was made by weighing 0.1 g, which was dissolved in 100 ml of distilled water.

Preparation of 0.2% chitosan

A 0.2% chitosan solution was prepared by weighing 0.2 g, which was dissolved in 100 ml of 2% acetic acid. Chitosan material was obtained from Sigma-Aldrich, Singapore, Catalog No. 448877.

Nanoparticle of Ashitaba leaf

Preparation of ashitaba leaf nanoparticles using the ratio of Ashitaba leaf extract: NaTPP: Chitosan is 1:1:6. 10 ml of 5% ashitaba leaf extract was mixed with 10 ml of 0.1% NaTPP and then mixed with 60 ml of 0.2% chitosan. The materials were mixed and sonicated with a sonicator machine for 60 minutes at a frequency of 20 kHz; the results were freeze-dried (Sawtarie *et al*., 2017).

Animal model

The experimental animals used in this study were 30 male mice (*Mus musculus*) with an average body weight of 25-30 g. The place where the animals were kept was at the Laboratory of Animal Experiments, Faculty of

Veterinary Medicine, Universitas Airlangga. The animals were adapted for 7 days, after which they were divided into 5 treatment groups, namely G1: Control group (-) not exposed to cigarette smoke and given NaCl 0.9%. G2: The control group (+) was exposed to cigarette smoke and not given a nanospray inhaler. Treatment groups G3, G4, and G5: Exposed to cigarette smoke and given nanospray inhaler ashitaba leaf at a dose of 50 mg/kg/day, 200 mg/kg/day, and 500 mg/kg/day, respectively, for 3 weeks (Al-Anshori *et al*., 2019). Cigarette smoke exposure was given after 7 days of adaptation. On day 8, the animals were exposed to Marlboro® cigarette smoke (13 mg tar and 1.0 mg nicotine) for 28 days at a dose of 1 cigarette/day in a smoking box (He *et al*., 2015). On day 36, the nanospray inhaler ashitaba leaf was started for 3 weeks according to the dose, after which the animals were sacrificed by injecting ketamine (40-100 mg/kg) and xylazine (5-16 mg/kg) intraperitoneally (Tobar *et al*., 2021). The surgical procedure is performed legeartis, starting with an incision of the abdominal wall or laparotomy surgical technique, after which the abdominal and pelvic cavities are opened to take the testicular organs. Schematic illustration of the experimental design and treatment timeline. Mice underwent a 1-week adaptation period (Days 1–7), followed by cigarette smoke exposure for 4 weeks (Days 8–35). Subsequently, mice received Ashitaba leaf nanospray inhaler treatment for 3 weeks (Days 36–56). Euthanasia was performed on Day 57 for testicular tissue collection (Figure 1).

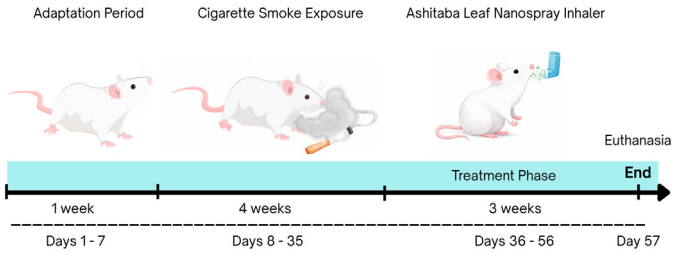

Figure 1: Schematic illustration of the experimental design and treatment timeline. Mice underwent a 1-week adaptation period (Days 1–7), followed by cigarette smoke exposure for 4 weeks (Days 8–35). Subsequently, mice received ashitaba leaf nanospray inhaler treatment for 3 weeks (Days 36–56). Euthanasia was performed on Day 57 for testicular tissue collection.

Histological examination

Testicular organs that have been taken are then placed in 10% neutral buffered formalin for 24 hours at room temperature for the fixation process. The next stage of histopathology slide-making is by the standard histopathology technique of Hematoxylin-eosin (HE) staining performed by Su *et al*. (2023). Histopathology slides of testes with HE staining have been made, then photographed using a light microscope (Nikon E200), magnification of 400x, equipped with Optilab Viewer Software Version 2.2. Calculation of the number of spermatogonium cells, primary spermatocytes, spermatids, Sertoli cells, Leydig cells, and the epithelium thickness of seminiferous tubules using Image Raster Software Version 3.0. The number of spermatogonium cells, primary spermatocytes, spermatids, and Sertoli cells was counted in the seminiferous tubules. The number of Leydig cells was counted in the inter-seminiferous tubule space. The epithelium thickness of the seminiferous tubules was measured from the spermatogonium near the basement membrane of the seminiferous tubules to the spermatids (Caldeira *et al*., 2010). Calculations and observations of all these parameters were carried out randomly with 5 replications to obtain an average value.

Statistical analysis

The data obtained were tested for normality first (Shapiro-Wilk test) to analyze the relationship between each variable. The results of the normality test in this study showed normal distribution, so it was analyzed using Analysis of Variance (ANOVA) and continued with Duncan's test to determine differences between groups ($p<0.05$). Data were analyzed using the SPSS® 23.0 software program (IBM Corp., NY, USA).

Molecular docking

The validation of the docking method is needed to minimize the inaccuracy during the docking process by redocking the natural ligand (biliverdin IX alpha) in the heme Oxygenase-1 (PDB ID 5BTQ) crystallographic structure to its binding site using AutoDockTools 1.5.6.

The same software is used for ligand and receptor preparation. The ligand, the chemical structure of isolated compounds from *Angelica keiskei* leaf, was obtained from previous research, then drawn using ChemDraw 20.0 and performed with MMFF94 minimization using Chem3D 20.0 (Caesar and Cech, 2016; Kil *et al*., 2017). The ligand was computed by Gasteiger charges with merged nonpolar. Meanwhile, the receptor was computed by Kollman charges, deleting its water, and adding polar hydrogens. Both were saved in pdbqt (Mardianingrum *et al*., 2024).

The detail of the grid specification is needed to conform to Autodock and Autogrid. The dimension box for heme Oxygenase-1 receptor (PDB ID 5BTQ) was 40 x 40 x 40 points, 0.375Å of spacing, and x = 68.566, y = 20.983, z = 72.88 of the center grid box. Lamarckian Genetic Algorithm (LGA) was used to calculate during 100 runs of the docking process, and the result was analyzed using Discovery studio 2021.

Table 1: Count data of spermatogonium cells, primary spermatocytes, spermatids, Sertoli cells, Leydig cells, and the epithelium thickness of seminiferous tubules in control and treatment groups (X̄±SD).

Group	Spermatogonium cells	Primary spermatocytes cells	Spermatid	Sertoli cells	Epithelium thickness (µm)	Leydig cells
G1	36.20^{a}±3.03	70.60^{a}±4.44	98.20^{a}±7.75	18.40^{a}±1.67	57.70^{a}±6.94	15.80^{a}±2.94
G2	26.20^{b}±2.86	40.80^{b}±5.49	56.20^{c}±10.61	9.80^{c}±1.92	41.58^{b}±3.26	9.20^{b}±2.58
G3	31.00^{ab}±5.38	50.80^{b}±12.83	73.60^{b}±17.61	13.80^{b}±2.38	44.60^{b}± 2.06	10.40^{b}±1.14
G4	35.80^{a}±7.49	75.40^{a}±4.77	94.60^{a}±8.96	16.60^{a}±2.40	52.89^{a}±2.82	13.40^{a}±1.51
G5	28.20^{b}±3.19	65.40^{a}±12.09	79.00^{b}± 8.15	9.60^{c}±1.51	40.44^{b}±2.61	9.60^{b}±1.14

Note: different superscripts (a,b,c) in the same column indicate significant differences between treatments ($p<0.05$). G1: Control group (-) not exposed to cigarette smoke and given NaCl 0.9%, G2: control group (+) exposed to cigarette smoke and not given nanospray inhaler. Treatment groups G3, G4, and G5: exposed to cigarette smoke and given nanospray inhaler ashitaba leaf at doses of 50 mg/kg/day, 200 mg/kg/day, and 500 mg/kg/day respectively.

RESULTS

The results of the calculation of the average number of spermatogonium cells, primary spermatocytes, spermatids, Sertoli cells, Leydig cells, and the epithelium thickness of seminiferous tubules in five different groups are presented in Table 1. Histology images of spermatogonium cells, primary spermatocytes, spermatids, Sertoli cells, and the epithelium thickness of seminiferous tubules in five different groups can be seen in Figure 2. Histology images of Leydig cells in five different groups can be seen in Figure 3.

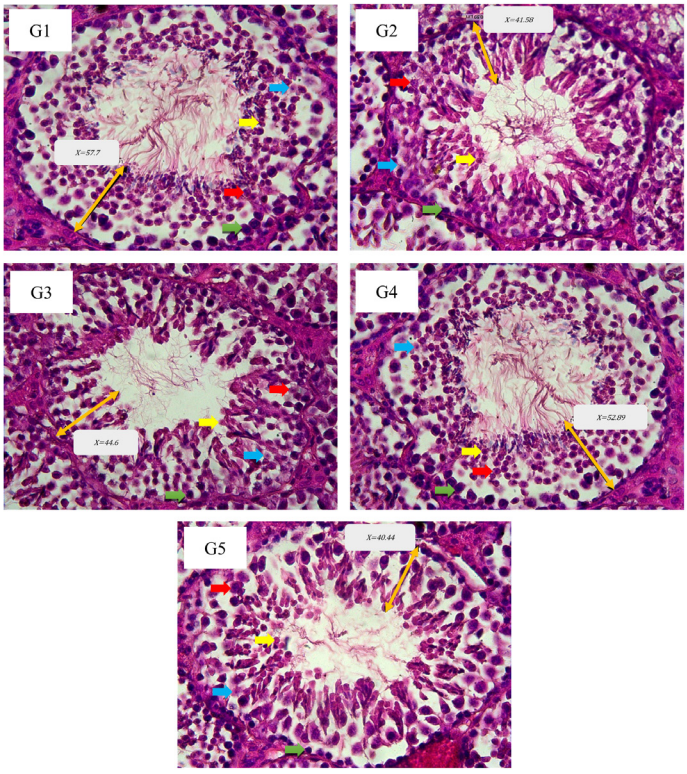

Figure 2: Representative H&E-stained microscopic images of seminiferous tubules from mice in the control and treatment groups at 400x magnification. Green arrow: spermatogonium cell, red arrow: primary spermatocyte, yellow arrow: spermatid, blue arrow: Sertoli cell, orange arrow: the epithelium thickness of seminiferous tubules.

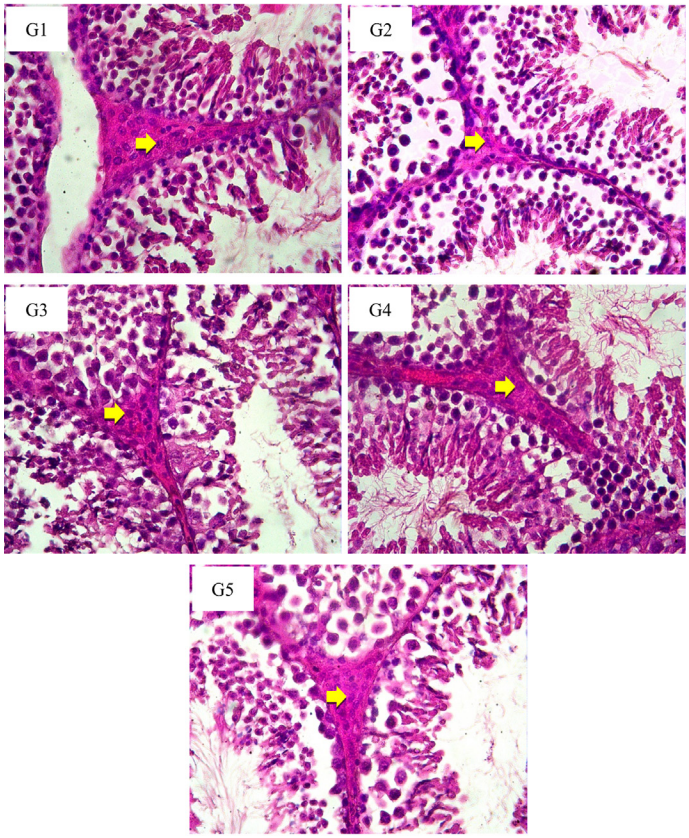

Figure 3: Representative H&E-stained microscopic images of Leydig cells from mice in the control and treatment groups at 400x magnification. Leydig cells were quantified within the interstitial area of the seminiferous tubules. Yellow arrows indicate Leydig cells.

The average number of spermatogonium cells in the negative control group (G1) showed a significant difference when compared to the positive control group (G2) and treatment group G5 ($p<0.05$) and showed no significant difference with groups G3 and G4 ($p>0.05$).

The average number of primary spermatocytes in the negative control group (G1) showed a significant difference with the positive control group (G2) and treatment group G3 ($p<0.05$), but did not show a significant difference

with treatment groups G4 and G5 ($p>0.05$). The average number of spermatids in the negative control group (G1) was significantly different from that in the positive control group (G2), as well as in groups G3 and G5 ($p<0.05$), whereas no significant difference was observed with group G4 ($p>0.05$). The average number of Sertoli cells in the negative control group (G1) was significantly different from that in the positive control group (G2), and in groups G3 and G5 ($p<0.05$), while no significant difference was found with group G4 ($p>0.05$). The average number of Leydig cells and the epithelium thickness of seminiferous tubules have the same pattern. The negative control group (G1) showed no significant difference from group G4 ($p>0.05$) and showed significant differences in the positive control group (G2), G3, and G5 ($p<0.05$).

The validation of the docking method was examined by Root Mean Square Deviation (RMSD). The method could be said to be valid if the RMSD value is less than or equal to 22Å. According to the result, the RMSD value of c was 0.70 Å. The native ligand structure from the co-crystal and after re-docking (Figure 4) shows that there are no significant changes during the process. Therefore, the method was valid and could be used for the docking process.

The most stable ligand-receptor interaction was predicted based on binding affinity (ΔG), inhibition constant (Ki), and percentage similarity to the native ligand using molecular docking. The molecular docking result was compared to the biliverdin as the native ligand from heme oxygenase-1 receptor (PDB ID 5BTQ) can be seen in Table 2.

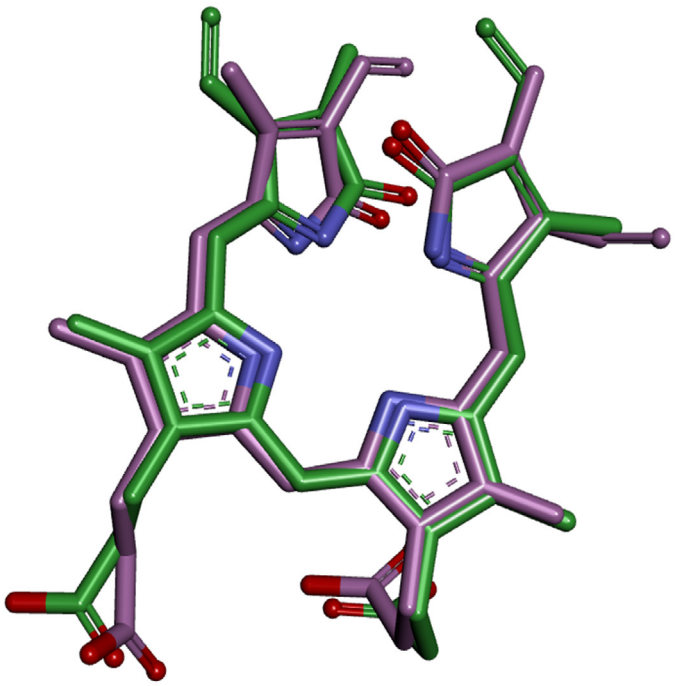

Figure 4: The overlay structure between biliverdine (native ligand) co-crystal (Green) and after redocking (Purple) on the heme Oxygenase-1 receptor (PDB ID 5BTQ).

According to the result (Table 2), biliverdin, as a native ligand, has a binding affinity value lower than all of the test ligands. However, three compounds that have lower binding affinity values than other compounds were stigmasterol, daucosterol, and xanthoangelol (-9.27 kcal/mol, -8.66 kcal/mol, and -7.87 kcal/mol, respectively). Therefore, it can be predicted that stigmasterol, daucosterol, and xanthoangelol have potential against heme Oxygenase-1.

Table 2: Molecular docking result against heme oxygenase-1 receptor (5BTQ).

No	Compound	Binding affinity (ΔG) kcal/mol	Inhibition constant (μM)
1	Biliverdine (Native Ligand)	-12.08	1.40×10^{-3}
2	Stigmasterol	-9.27	161.24×10^{-3}
3	Daucosterol	-8.66	447.27×10^{-3}
4	Xanthoangelol	-7.87	1.69
5	Xanthoangelol B	-7.79	1.94
6	Steviol-13-O-β-D-glucopyranoside 19-O-β-D-glucopyranosyl ester	-7.78	2.00
7	4,2',4'-Trihydroxy-3'-[(6E)-2-hydroxy-7- methyl-3-methylene-6-octenyl]-chalcone	-7.67	2.38
8	4-hydroxyderricin	-7.55	2.94
9	Xanthokeistal A	-7.40	3.75
10	Xanthoangelol E	-7.31	4.42
11	Luteolin	-6.08	34.91
12	1-Cerotol	-4.46	541.89
13	Quercitrin	-4.10	994.71

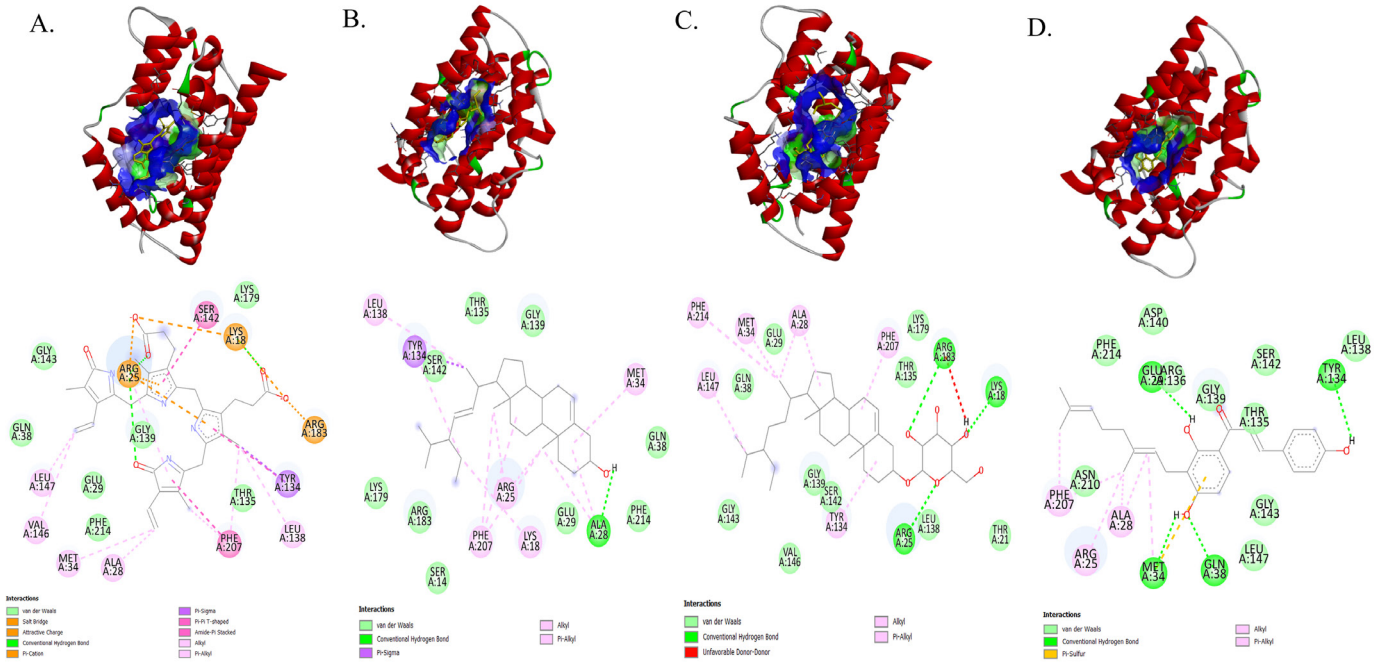

Figure 5: The 3D and 2D visualizations of biliverdine IXα (A), stigmasterol (B), daucosterol (C), and xanthoangelol (D) interactions with heme oxygenase-1 (5BTQ).

The binding energy and the inhibitory constant (Ki) value are directly correlated. The more effectively the ligand inhibits the receptor, the lower the inhibition constant (Ki) value. Furthermore, ligand-receptor bonds were used to analyze the percentage similarity to the native ligand. The more comparable the amino acid residues, the greater the possibility of similar activity with the native ligand. A hydrogen bond can affect the compounds' affinity to the protein. Moreover, hydrophobic bonds contribute to biological activity. It can improve the stability of ligand-receptor interaction by minimizing the interaction between non-polar residues and water. Based on the result in Figure 5 shows that stigmasterol and daucosterol have the same percentage similarity, reaching up to 56%. Meanwhile, the percentage similarity of xanthoangelol is up to 42%. It's in line with the free binding energy result.

DISCUSSION

The results showed that the average number of spermatogonium cells, primary spermatocytes, spermatids, Sertoli cells, Leydig cells, and the epithelium thickness of seminiferous tubules in the positive control group (G2) exposed to cigarette smoke was lower when compared to the negative control group (G1). This is because the G2 group inhaled cigarette smoke, which became an exogenous source of reactive oxygen species (ROS) production in the cell (Darbandi *et al*., 2018). Reactive oxygen species (ROS) produced from exogenous sources in excess can cause oxidative stress, impair reproductive function, reduce gonadal hormone production, and disrupt cross-talk between the hypothalamic-pituitary-gonadal (HPG) axis and endocrine axes (Rijal *et al*., 2022). The hypothalamic-pituitary-gonadal (HPG) axis has a major role in controlling reproductive function (Kaprara and Huhtaniemi, 2018). The hypothalamus regulates gonadotropin-releasing hormone (GnRH) and signals the anterior pituitary (Kanda, 2019). Gonadotropin-releasing hormone (GnRH) neurons play a role in the central axis to produce GnRH, which can bind to pituitary gonadotroph receptors and secrete gonadotropin hormones such as luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Stamatiades and Kaiser, 2018).

Spermatogenesis is a sequential biological event that favors the maturation of germ cells in the seminiferous tubules of the testes (Nishimura and L'Hernault, 2017). The development and maintenance of both the quality and quantity of spermatogenesis depend on the production of LH and FSH from the anterior pituitary gland, secreted by GnRH (Oduwole *et al*., 2021). LH controls testosterone production by Leydig cells, which are endocrine cells located in the interstitium of the seminiferous tubules of the testes (Li *et al*., 2024). FSH regulates germ cell proliferation and maturation independently, and its action is combined with LH exerted on Sertoli cells (Ramaswamy and Weinbauer, 2015). Sertoli cells are one of the important somatic cells in mammalian testes because they have the role of providing nutrients and supporting germ cell development (Su *et al*., 2023). The results of research by Al-Anshori *et al*. (2022) show that increasing the number of Sertoli cells has a good correlation with the epithelium thickness of the seminiferous tubules, with the presence of a large number of Sertoli cells, so that the nutritional needs of germ cells

are fulfilled and do not experience death.

There was a significant decrease in epithelium thickness of seminiferous tubules in the G2 group exposed to cigarette smoke and not given the nanospray inhaler ashitaba leaf due to a reduced number of Sertoli cells and spermatogenic cells or inhibition of spermatogenesis and germ cell apoptosis. The results of this study are consistent with the research report of En *et al.* (2020) that exposure to nicotine contained in cigarette smoke inhibits spermatogenesis as evidenced by a reduction in the diameter and epithelium thickness of the testicular seminiferous tubules. Based on the research report of Jana *et al.* (2010), nicotine administration also causes disturbances in seminiferous tubules and spermatogenic cells.

Reduction in the number of spermatogonium cells, primary spermatocytes, spermatids, Sertoli cells, and Leydig cells in the G2 group exposed to cigarette smoke and not given a nanospray inhaler ashitaba leaf is due to disruption of the spermatogenesis process, which is influenced by exposure to cigarette smoke, so that it can cause damage to the epithelium of seminiferous tubules and thinning. Inhaled cigarette smoke can act as an endocrine disruptor on the male hormone profile, especially on testosterone and FSH (Ramaswamy and Weinbauer, 2015). Testosterone, when associated with FSH, both hormones work in the seminiferous tubules to initiate and maintain spermatogenesis (Galdon *et al.*, 2016).

Inhaled cigarette smoke is also known to play a role in redox reactions and produce reactive oxygen species (ROS) (Darbandi *et al.*, 2018). Excessive levels of ROS can cause oxidative stress and cell death (Sies and Jones, 2020). The germ cell membrane of testicular seminiferous tubules contains a lot of polyunsaturated fatty acids (PUFAs) and undergoes division at a high rate, making spermatogenic cells very vulnerable to oxidative disorders that cause damage (Sansone *et al.*, 2018). Oxidative stress is a cause of intracellular molecular damage and cell death; the process is defined as an imbalance between reactive oxygen species (ROS) and antioxidants in the cell (Saygin *et al.*, 2023). Oxidative stress can cause Leydig cell dysfunction, thus disrupting the process of steroidogenesis and spermatogenesis (Monageng *et al.*, 2023). Oxidative stress-induced Leydig cell dysfunction leads to apoptosis, decreased Leydig cell population, and testosterone production (Ma *et al.*, 2020).

The treatment group exposed to cigarette smoke by administering a nanospray inhaler ashitaba leaf at a dose of 200 mg/kg/day (G4) experienced a good recovery when compared to other groups. This shows that the dose of nanospray inhaler ashitaba leaf can increase the number of spermatogonium cells, primary spermatocyte cells, spermatids, Sertoli cells, Leydig cells, and the epithelium thickness of the seminiferous tubule, so it can be said that the dose of administration is very effective.

Nanospray inhaler ashitaba leaf (*Angelica keiskei*) contains coumarins, flavanones, chalcones, and phenolic acids (Kim *et al.*, 2014). The phytochemical content of ashitaba leaves, such as flavonoids and phenolic acids, has been shown to have the capacity as a natural antioxidant with a strong free radical scavenging mechanism of action (Li *et al.*, 2009). Ashitaba is one of the medicinal plants originating from Japan and has been cultivated in several regions in Indonesia. The antioxidant activity of phytochemicals from medicinal plants has been shown to have a protective effect on Leydig cells against damage induced by ROS (Martin and Touaibia, 2020). Nanospray inhaler ashitaba leaf given to the treatment group can increase antioxidant activity and expression of steroidogenic enzymes to synthesize testosterone, which can reduce cell death, DNA damage, and lipid peroxidation (Monageng *et al.*, 2023). This is because the antioxidant activity of the nanospray inhaler ashitaba leaf has a mechanism of action as a strong free radical scavenger, thus helping to prevent oxidative stress, which has the effect of restoring Leydig cell function, steroidogenesis, and spermatogenesis.

The administration of nanospray inhaler ashitaba leaf at a dose of 200 mg/kg/day can protect mice from testicular tissue damage due to exposure to cigarette smoke through the ability to maintain the number of spermatogonium cells, primary spermatocytes, spermatids, Sertoli cells, Leydig cells, and the epithelium thickness of the seminiferous tubule.

Heme oxygenase-1 (HO-1) is a crucial enzyme in cellular antioxidant defense that decomposes heme into biliverdin, CO, and Fe^{2+}, and exerts cytoprotective effects through its derivative products (Gozzelino *et al.*, 2010). Biliverdin, as an endogenous ligand, exhibits the highest binding affinity to HO-1, reflecting a possible physiological feedback regulation between the product and the enzyme. Several test compounds, particularly stigmasterol and daucosterol, exhibited relatively high binding affinities (ΔG –9.27 and –8.66 kcal/mol), indicating a significant interaction with the active site of HO-1. These findings align with previous reports that phytosterols can induce HO-1 expression and exert antioxidant and anti-inflammatory effects (Bakrim *et al.*, 2022). Other compounds, such as xanthoangelol and B, exhibited lower affinities (ΔG –7.87 to –7.79 kcal/mol), possibly related to suboptimal spatial fit and chemical properties within the enzyme binding pocket. In addition, steviol glucoside ester displayed a low binding affinity (-7.78 kcal/mol) which indicates potential relevance to induce HO-1. Nevertheless, these moderate affinities still indicate potential interactions worthy of further evaluation

through molecular dynamics simulations or biological assays.

Overall, the docking results confirm biliverdin as a reference ligand with high affinity for HO-1 and identify several natural compounds with potential as enzyme modulators. These findings provide an important foundation for further research on HO-1 modulation and the development of bioactive compound-based antioxidant agents.

CONCLUSIONS

Cigarette smoke exposure causes significant testicular structural damage through oxidative stress–mediated mechanisms that impair spermatogenesis and hormonal regulation. Administration of an ashitaba leaf nanospray inhaler at a dose of 200 mg/kg/day provides a protective effect by restoring key testicular cell populations and improving seminiferous tubule epithelial integrity, likely through its antioxidant activity. In silico molecular docking analysis further supports these findings, demonstrating that ashitaba bioactive compounds, particularly stigmasterol and daucosterol, exhibit favorable interactions with heme oxygenase-1 (HO-1), a critical enzyme in cellular antioxidant defense. Overall, these results indicate that ashitaba may mitigate cigarette smoke–induced testicular damage by modulating oxidative stress–related pathways involving HO-1, highlighting its potential as a bioactive antioxidant agent for protecting male reproductive health.

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NOVELTY STATEMENT

The pulmonary delivery route has been extensively investigated not only for local lung therapy but also as an effective strategy for achieving systemic drug absorption. Building on this concept, this study introduces a novel Ashitaba (Angelica keiskei) leaf-based nanospray inhaler as a systemic delivery approach to mitigate cigarette smoke-induced testicular damage. Unlike previous studies that primarily utilize oral administration or intraperitoneal injection, as well as conventional extract forms, this research explores an inhalation-based nanoformulation targeting male reproductive health, offering a potentially less invasive and more efficient route of systemic delivery. Furthermore, the integration of in vivo and in silico analyses provides a comprehensive evaluation of both biological efficacy and underlying molecular mechanisms, which has not been widely reported.

AUTHOR'S CONTRIBUTION

Conceptualization: Al-Anshori AA, Maslachah L; Data curation: Al-Anshori AA; Formal analysis: Al-Anshori AA, Susilawati D; Investigation: Al-Anshori AA, Maslachah L; Methodology: Maslachah L, Al-Anshori AA; Project administration: Al-Anshori AA, Maslachah L; Resources: Maslachah L, Al-Anshori AA; Software: Susilawati D; Supervision: Maslachah L; Validation: Rajanagara AS; Visualization: Al-Anshori AA, Susilawati D; Writing - original draft: Al-Anshori AA, Susilawati D, Rajanagara AS; Writing - review & editing: Maslachah L.

Ethical approval

This study complies with institutional regulations on the handling and use of experimental animals. This study has obtained official approval with certificate No. 1.KE.102.06.2018 to conduct research protocols from the Animal Care and Use Committee (ACUC), Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia.

Generative AI and AI assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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