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Original article

Effect of methanol extract of cloves (*Syzigium aromaticum*) on Protein Gene Product 9.5 in the testes of rats (*Rattus norvegicus*) with induced cryptorchidism

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ABSTRACT

This study aimed to know the effect of cloves (*Syzigium aromaticum*) methanol extracts as an antioxidant on the germ cells of white rats (*Rattus norvegicus*) with induced cryptorchidism. The subjects of this study were 24 male white rats aged 21 days, who were divided into six groups. Rats were adapted for 5 days, after which cryptorchidism were induced. Cloves extract was given for 18 days and 36 days. On day-19 and day-37, rats were sacrificed and testicles were taken for Protein Gene Product (PGP) 9.5 detection by immunohistochemistry. These groups consisted of negative control group (given distilled water and sham surgery), positive control group (induction of cryptorchidism and given distilled water), and T treatment group (induction of cryptorchidism and given 70mg/kg bw cloves extract. Kruskal-Wallis test indicated a significant difference ($p < 0.05$). The differences between treatments were shown based on the outcome of the Mann-Whitney test. Cloves acted as an antioxidant for cryptorchid testicular germ cells. It could be concluded that administering methanol extract of clove flowers could ameliorate the expression of PGP 9.5 in the testicular germ cells of white rats in a model of unilateral cryptorchidism.

Keywords: antioxidant, immunohistochemical, methanol extract, Remmele scale index, testicle

INTRODUCTION

Cryptorchidism (retained testicles) is one of the most common disorders in mammals and humans. Cryptorchidism or undescensus testicles by definition is a developmental disorder characterized by failure to descend one or both testicles, which are not in their proper position in the scrotum at birth and cannot be moved manually to their correct position (Leslie *et al*., 2024).

Cryptorchid is one of the congenital defects found in dogs (Khan *et al*., 2018) and cats (Villalobos-Gomez *et al*., 2023). Apart from dogs and cats, cryptorchidism is also one of the most common congenital defects in humans (Soto-Heras *et al*., 2024). Cryptorchidism was caused by various factors that prevent the testicles from descending into the scrotum. The main cause of cryptorchidism was a defect in androgen secretion in the prenatal phase, either secondary to gonadotropin stimulation or low

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placental gonadotropin production (Rodprasert *et al*., 2020).

Cryptorchidism caused high intra-abdominal temperatures (Leslie *et al*., 2024) and affected spermatogenesis and induced hyperproduction of reactive oxygen species (ROS) so that it could increase oxidative stress (Wang *et al*, 2023a). Increased oxidative stress ultimately led to spermatogenic cells death, a mechanism that linked azoospermia to cryptorchidism (Gao *et al*., 2022). Long-term complications of untreated oxidative stress were malignant transformation of the testicles due to chronic inflammation outside the scrotum in the form of seminoma (Dutta *et al*., 2021).

Since the rate of neoplasia in the testicles was increasing, many studies have been carried out to diagnose it. One of the methods that had been developed was the immunohistochemical technique (Boccellino *et al*., 2017). In particular, many immunohistochemical markers had been introduced to accurately determine the histological diagnosis and pathogenesis of tumors, one of which was PGP 9.5. Protein Gene Product (PGP 9.5) is a 27-kDa protein originally isolated from the brain (Zhou *et al*., 2024).

Unilateral cryptorchidism was treated using an orchiectomy surgical approach. Orchiectomy is the only medical procedure recommended in cases of cryptorchidism in dogs aged 6 -18 months and could reduce fertility (Leslie *et al*, 2024). Treatment of unilateral cryptorchidism with orchiopexy in dogs before 6 months of age successfully restored spermatogenic function and spermatozoa quality (Mahiddine and Kim, 2021). More worrying for the treatment of cryptorchidism during puberty was that the spermatozoa in the testicles might never function properly even after treatment (Oremosu *et al*., 2017). Sometimes the owner also did not want castration, therefore natural treatment was carried out to prevent free radicals from becoming oxidative stress which could cause tumors to develop (Hernández-Jardón *et al*., 2022).

Body had an enzymatic and non-enzymatic defense system to neutralize the toxic influence of ROS compounds so that only small amounts of ROS were formed which were needed by the body to maintain normal cell function (Jena *et al*., 2023). Oxidative stress could be prevented by using antioxidants. Antioxidant therapy was effective in the neutralization of ROS and in the management of oxidative stress in testicular infertility (Walke *et al*., 2023). One plant that had antioxidant compounds is clove flowers (Lumingkewas and Unitly, 2023). Clove (*Syzigium aromaticum*) is a spice plant originating from Indonesia. Clove flowers have various uses. Historically, cloves were used as medicines, then as spices, and are currently used for various purposes (Batiha *et al*., 2020). Several studies utilized clove flowers, because of their phenolic content which functioned as a natural antioxidant. One of the antioxidants contained in clove flowers is the compound eugenol (Lumingkewas and Unitly, 2023). To our knowledge, no study has been found on the effect of methanol extract of clove flowers (*Syzigium aromaticum*) as a natural antioxidant on oxidative stress in the testes of white rats (*Rattus norvegicus*) in a unilateral cryptorchidism model.

MATERIALS AND METHODS

Experimental animals used in this study were 24 male white rats (*Rattus norvegicus*) of the Sprague Dawley strain, aged 21 days. Negative control C–1 and C–2: four rats each underwent sham surgery and were given distilled water orally for 18 and 36 days. Positive control C+1 and C+2: four rats each had cryptorchidism induced surgically and were given distilled water orally for 18 and 36 days. Treatments T+1 and T+2: four rats each had cryptorchidism induced surgically and treated with methanol extract of clove flower (*Syzigium aromaticum*) at a dose of 70 mg/kg bw orally for 18 and 36 days.

Unilateral cryptorchidism induction surgical procedure

This study has received ethical approval (1.KEH.011.01.2022) and all procedures were carried out according to the direction of the ethics committee. Surgical preparation began

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with anesthesia of rats using a combination of 2mg/kg bw of Xylazine and 10mg/kg bw of Ketamine administered intraperitoneally (Ritschl *et al*., 2015). The right inguinal region was incised, and the testicle gubernaculum was separated. The right testicle was pushed into the abdominal cavity through the internal inguinal ring. After the testicle was pushed, the external inguinal ring was closed with sutures to prevent descent of the testicle. Sham surgery in the control groups were performed by manipulating the gubernaculum by pushing it into the abdominal cavity as if cryptorchidism induction surgery was being performed. After manipulation, the gubernaculum was returned to its original position and closed with sutures (Okoye and Saikali, 2023).

Clove methanol extract

Fifty gram of cloves powder was wrapped in filter paper and then put into a Soxhlet, and 500 mL of 90% methanol solvent was put into a 1000 mL Soxhlet flask at a temperature of 65°C for 3 hours. The liquid extract obtained was then concentrated using a rotary vacuum evaporator to remove the solvent.

Histopathological evaluation

Animals from each group were sacrificed by cervical dislocation on day-19 and day-37 after cryptorchidism induction surgery. PGP 9.5 expression was observed using a microscope (Nikon Eclipse Ei at a 400x magnification). The semiquantitative assessment of PGP 9.5 assessment of PGP 9.5 expression used the modified Remmele scale index (Immunoreactive Scale, IRS) method. IRS was the result of multiplying the percentage score of positive cells (A) with the color reaction intensity score (B). Assessment of PGP 9.5 expression was carried out in different fields (Muwaffaq and Supriyo, 2021).

Table 1 Semi-quantitative Remmele scale index (Meyerholz and Beck, 2018)

A (percentage score of positive cells)	B (color reaction intensity score)
score 0: no positive cells	score 0: no color reaction (not stained)
score 1: less than 10% positive cells	score 1: low color intensity (unevenly stained)
score 2: positive cells between 11%-50%	score 2: medium color intensity (incomplete stained)
score 3: positive cells between 51%-80%	score 3: strong color intensity (completely stained)
score 4: positive cells of more than 80%	

RESULTS

Table 2 shows that cryptorchidism induction led to an increase in the mean of PGP 9.5 expression 18 and 36 days after surgery. The highest expression was found in groups C+1 and C+2, which were 3.62 and 3.93. The negative control group had the lowest mean expression which was 0.36 at C–1 and 0.44 at C–2. The results of immunohistochemical staining with PGP 9.5 are showed in Figure 1.

DISCUSSION

The negative control group still expressed a brownish color in the cytoplasm of the germ cells because there was an ubiquitin C-terminal hydrolase 1 (UCHL-1) which played an active role in the formation of new germ cells (Yang *et al*., 2023). Meanwhile, in the positive control, there were testicular germ cells that were stained with a brownish color. The presence of testicular germ cells staining brown indicates that PGP 9.5 in testicular germ cells plays an important role in the degradation of abnormal proteins (Choi *et al*., 2020).

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Table 2 Expression (Mean ± Standard Deviation) of PGP 9.5 in testicle germ cell

days after surgery	experimental group	PGP 9.5 expression
18		
	C–1	0.36 ± 0.24 [a]
	C+1	3.62 ± 1.44 [c]
	T+1	1.68 ± 0.39 [b]
36		
	C–2	0.44 ± 0.21 [a]
	C+2	3.93 ± 0.36 [c]
	T+2	1.88 ± 0.54 [b]

Different superscripts at each column indicate significant difference ($p < 0.05$); C–1 and C–2: rats underwent sham surgery and were orally given distilled water for 18 and 36 days, respectively; C+1 and C+2: rats were cryptorchidism induced surgically and were orally given distilled water for 18 and 36 days, respectively; T+1 and T+2: rats were cryptorchidism induced surgically and were orally treated with methanol extract of clove flower (*Syzigium aromaticum*) at a dose of 70 mg/kg bw for 18 and 36 days, respectively.

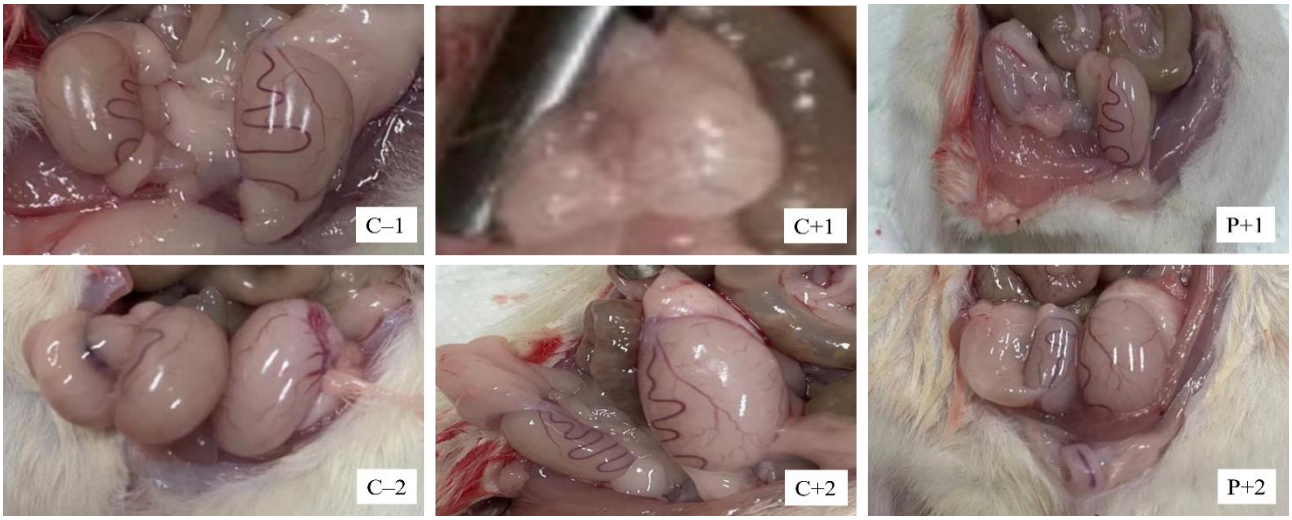

Figure 1 Gross anatomy of rat (*Rattus norvegicus*) testicles; C–1 and C–2: rats underwent sham surgery and were orally given distilled water for 18 and 36 days, respectively; C+1 and C+2: rats were cryptorchidism induced surgically and were orally given distilled water for 18 and 36 days, respectively; T+1 and T+2: rats were cryptorchidism induced surgically and were orally treated with methanol extract of clove flower (*Syzigium aromaticum*) at a dose of 70 mg/kg bw for 18 and 36 days, respectively.

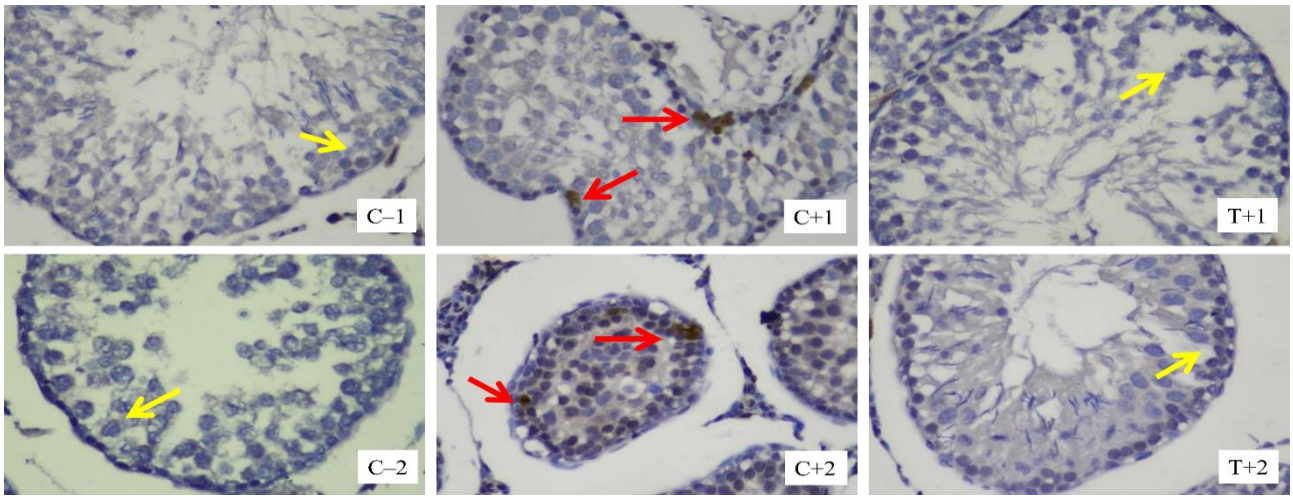

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Figure 2 Expression of PGP 9.5 in male white rats (*Rattus norvegicus*) testicles; (yellow arrow indicates low color intensity; red arrow indicates high color intensity, Nikon Eclipse Ei, 400x magnification); C–1 and C–2: rats underwent sham surgery and were orally given distilled water for 18 and 36 days, respectively; C+1 and C+2: rats were cryptorchidism induced surgically and were orally given distilled water for 18 and 36 days, respectively; T+1 and T+2: rats were cryptorchidism induced surgically and were orally treated with methanol extract of clove flower (*Syzigium aromaticum*) at a dose of 70 mg/kg bw for 18 and 36 days, respectively.

UCHL-1 was concentrated in germ cells in response to heat stress caused by cryptorchidism. The ubiquitin protein pathway in germ cells minimized DNA damage when germ cells are exposed to stress. However, if the ubiquitin pathway was overloaded or deactivated in spermatogonia with UCHL-1 dysfunction, spermatogonial stem cell proliferation would progressively decrease (Li *et al*., 2023).

Surgical induction of cryptorchidism in experimental animals caused degeneration of testicular germ cells (de Souza *et al*., 2019). This was because cryptorchidism could cause heat stress which caused reproductive toxicity with excessive ROS production and can cause damage to testicular tissue and sperm cell membranes (Aly and Hassan, 2018). Cryptorchidism also caused oxidative stress and could cause DNA strand breaks (Tsounapi *et al*., 2015). Spermatogenesis usually occured in the scrotum at a temperature below body temperature. If the testicles were in the abdomen (cryptorchidism), the testicles must adjust physiological activities, especially energy metabolism, to maintain normal testicular levels such as temperature in the scrotum (Jorban *et al*., 2024). ROS were produced in excess to induce oxidative stress which could inhibit the normal function of cellular lipids, proteins, DNA, and RNA (Chianese *et al*, 2021). ROS were free radicals that played an important role in several physiological processes in the body's organs. The formation of ROS could induce lipid peroxidation which was cytotoxic due to the initiation of a migration reaction into the membrane, followed by a propagation reaction which overall results in cell damage (Juan *et al*., 2021). The interaction of ROS with DNA bases could change the chemical structure of DNA, if not repaired it would experience mutations that could be inherited, especially if they occurred in germ cell DNA in both the testicles and ovaries. Meanwhile, DNA damage in somatic cells could lead to the initiation of malignancy (Wang *et al*., 2023b).

The degeneration of germ cell death was indicated by the morphology and characterization of DNA fragmentation. The temperature in the abdomen was higher than the temperature of the scrotum, which made the cryptorchid testicles in an abnormal physiological condition (Thorup *et al*., 2024). Hyperthermia had been shown to induce necrosis in various types of normal cells and tumor cells (Gao *et al*., 2022).

Clove flower methanol extract was an antioxidant that could increase the endogenous defense system of cells, inhibited ROS production, and could inhibit lipid peroxidation (Lumingkewas *et al*., 2023). Oxidative stress had been defined as an imbalance between ROS production and antioxidant defenses that caused disturbances in the balance of prooxidant-antioxidant reactions. The inhibitory mechanism of antioxidants usually occurred during the initiation or propagation reaction of the oxidation reaction of fats or other molecules in the body by absorbing and neutralizing free radicals or decomposing peroxides (Chaudhary *et al*., 2023).

Administration of antioxidants increased the possibility of preventing at least some germ cell death in cryptorchid testicles by interfering with necrosis (Aitken and Roman Soder, 2013). This showed that methanol extract of clove flowers could reduce the expression of PGP 9.5 in the testicular germ cells of white rats induced by cryptorchidism, although when compared with the negative control group the average was still

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higher than the negative control. This was thought to be caused by the pro-oxidant properties of some antioxidants which depended on concentration and environment (Sotler *et al*., 2019).

CONCLUSION

Therapy with methanol extract of clove (*Syzigium aromaticum*) flowers for 18 days was quite effective in reducing the expression of PGP 9.5.

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Faizah Zakiyyatun Nufus (FZN), Bambang Sektiari Lukiswanto (BSL), Nove Hidajati (NH), Eduardus Bimo Aksono Herupradoto (EBAH), Ira Sari Yudaniyanti (ISY), Budi Utomo (BU). FZN, BSL, and NH: conceived the idea, FZN: acquisition, analysis, and interpretation of data, manuscript drafting, EBAH, ISY, and BU: critically read and revised the manuscript for intellectual content. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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