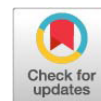


Research Article



Impact of *Ocimum basilicum* Essential Oils Supplementation in Semen Extenders on the Quality of Cryopreserved Canine Sperm

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Abstract | During cryopreservation, canine sperm faces a significant challenge: oxidative stress, which leads to reduced sperm quality. This study was conducted to evaluate the effects of *Ocimum basilicum* essential oils supplementation in the dilution medium on the quality of canine sperm frozen at -196°C . Sperm quality was assessed based on progressive motility, viability, membrane integrity, and oxidative resistance. The results showed that sperm quality improved at lower concentrations of essential oils (below $25\text{ }\mu\text{g/ml}$) and decreased at concentration of $25\text{ }\mu\text{g/ml}$. Notably, the essential oils concentration of $20\text{ }\mu\text{g/ml}$ yielded the best sperm quality, with significant differences compared to other levels ($P<0.05$). The study concludes that *Ocimum basilicum* essential oils affects canine sperm quality in a concentration-dependent manner, with $20\text{ }\mu\text{g/ml}$ being the optimal concentration to reduce oxidative stress and enhance sperm quality during cryopreservation.

Keywords | Canine sperm, Frozen-thawed, *Ocimum basilicum*, Essential oils, Antioxidants, Cryopreservation

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INTRODUCTION

Sperm cryopreservation and artificial insemination techniques are becoming increasingly important and essential in canine breeding, aiming to produce dog breeds with traits that align with human needs. Artificial insemination overcomes many drawbacks of natural mating and provides several benefits, including the ability to collect semen from male dogs at an optimal level without diminishing sperm quantity or quality, as well as the convenience of not having to transport the male dog or spend time allowing the female dog to adjust. However, a major challenge in sperm cryopreservation is the deterioration of sperm quality due to various factors, one of which is oxidative stress.

The sperm plasma membrane, rich in unsaturated fatty acids, is prone to oxidation when reactive oxygen species (ROS) are present (Vieira *et al.*, 2017). As a result, various synthetic antioxidants have been explored for preserving canine sperm to mitigate this problem (Thiangtum *et al.*, 2012). The most commonly employed antioxidants for sperm cryopreservation include vitamin E (α -tocopherol/Trolox), glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), ascorbic acid (vitamin C), quercetin, and melatonin (Moradi *et al.*, 2020). Moreover, since semen often contains bacteria, antioxidants combined with antibiotics have been utilized to inhibit microbial growth in the sperm storage medium (Bresciani *et al.*, 2014). Recently, plant extracts have attracted significant scientific interest

as a safe alternative for preserving and enhancing sperm quality. Certain plant species are potent antioxidants that neutralize free radicals, reducing the detrimental effects of oxidative stress on sperm. Additionally, these natural compounds possess antibacterial properties and promote the activity of various antioxidant enzymes (Nguyen *et al.*, 2023). This suggests that plant extracts are an ideal alternative for protecting sperm from oxidation and unwanted microorganisms.

Ocimum basilicum, a perennial herb found in Vietnam, grows throughout the year, reaching a height of 0.5-1.2 meters. It has multiple branches and a pleasant fragrance. The plant contains essential oils with concentrations ranging from 0.5-1.7% (Zheljazkov, 2008). These essential oils are rich in antioxidant compounds, including linalool, cineole, bergamotene, eugenol, estragole, γ -cadinene, muurolene, germacrene, tau-cadinol, and γ -gurjunene (Silva *et al.*, 2015). Although they show promise, as our knowledge no studies have been conducted to explore their effects on animal sperm quality. Therefore, adding *Ocimum basilicum* essential oils to semen extenders could improve the quality of frozen canine sperm by minimizing lipid peroxidation during the freezing process. This study aims to assess the impact of supplementing semen extenders with *Ocimum basilicum* essential oils on the quality of frozen dog sperm.

MATERIALS AND METHODS

EXTRACTION OF ESSENTIAL OILS

Ocimum basilicum essential oils was extracted using the steam distillation technique. After harvesting, the leaves were dried at 40°C for 60 hours using vegetable drying oven until their weight remained constant. The dried leaves were then finely ground into powder using grinder machine. A quantity of 150 g of *Ocimum basilicum* leaves powder was combined with 1300 ml of distilled water and placed in a 1000 ml flask of a Clevenger essential oils distillation apparatus. The mixture was heated with an electric stove at 70°C until the volume of essential oils remained constant, which took approximately 2 hours. The extracted *Ocimum basilicum* essential oils appeared as a pale-yellow liquid, lighter than water, and emitted a pleasant aroma. To remove any remaining moisture, the essential oils was treated with sodium sulfate (Na₂SO₄). The extracted essential oil was thoroughly mixed and covered with aluminum foil to prevent oxidation, then stored at 4°C. The essential oils composition was determined using gas chromatography-mass spectrometry (GC-MS) (Adams, 2007).

EVALUATION OF THE ESSENTIAL OIL'S ANTIOXIDANT ACTIVITY

The antioxidant activity was determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay by measuring the reduction in colour of the DPPH radical (Sharma and Bhat,

2009). The reaction involved 100 μ l of *Ocimum basilicum* essential oils dissolved in methanol at concentrations ranging from 100-800 μ g/ml, which was then mixed with 100 μ l of DPPH (0.2 mM) in a corresponding 96-well plate. Vitamin E was used as a positive control, while methanol served as a negative control. The reaction mixture was incubated in the dark for 30 minutes, and the absorbance was measured at 517 nm using a spectrophotometer. The IC₅₀ values, representing the concentration required to inhibit 50% of DPPH activity, were determined by graphing the inhibition percentage against the concentration and interpreting the resulting linear plot.

ANIMALS

The study utilized three healthy male Pug dogs, aged 2 to 5 years, all confirmed to be reproductively healthy. The dogs were housed at the same kennel and trained for semen collection. The research was conducted with the approval of the Institutional Animal Care and Use Committee of Tra Vinh University, Vietnam.

SEMEN COLLECTION AND EVALUATION

The semen collection technique outlined by Linde-Forsberg (1991) was used to gather semen samples from the dogs over a period of one week. After collection, the quality of sperm from each ejaculate was assessed. Sperm motility was observed directly under a microscope, while sperm morphology and viability were evaluated using eosin-nigrosin staining. In this study, canine semen was selected based on criteria of progressive sperm motility greater than 70%, sperm viability above 90%, sperm concentration exceeding 200 $\times$ 10⁶ sperm/ml, and abnormal sperm morphology below 5%.

PREPARATION OF EXTENDERS

All chemicals needed for preparing the semen diluent were provided by Sigma-Aldrich. The semen extender used in this study was a Tris buffer containing citric acid and fructose, enriched with 20% egg yolk. Various treatments were applied by adding essential oils extracted from *Ocimum basilicum* leaves at concentrations of 0, 15, 20, and 25 μ g/ml to the base extender. The essential oils were dissolved in dimethyl sulfoxide (DMSO), with each semen extender containing 0.8% DMSO. Glycerol was incorporated as a cryoprotectant. Two experimental groups were created: group 1 had 3% glycerol, while group 2 had 7% glycerol. After sperm dilution, the final glycerol concentration in each diluent was 5%. The detailed composition of the diluent is provided in Table 1.

SEMEN PROCESSING AND EXPERIMENTAL DESIGN

The cryopreservation process was carried out following the method of (Michael *et al.*, 2007). The collected semen was pooled and divided into four sterile tubes. The semen was then centrifuged to remove the seminal plasma at 720 $\times$ g

for 5 minutes. The sperm was diluted in the group 1 medium (3% glycerol) to a concentration of 100×10^6 sperm/ml. The sperm samples were placed in a styrofoam box containing water at 25°C and gradually cooled (0.3°C/minute) to 4°C by adding ice over the course of 1 hour. Next, the sperm samples were further diluted (1:1 v/v) with the group 2 medium (7% glycerol) at 4°C to achieve a concentration of 100×10^6 sperm/ml. After 30 minutes at 4°C, the diluted semen was loaded into 0.5ml straw straws, which were then sealed. The straws were frozen manually by placing them 7 cm above the surface of liquid nitrogen for 5 minutes (Bucci *et al.*, 2019). The straws were then immediately transferred to liquid nitrogen and stored in it for at least two weeks before being thawed for evaluation. The straws were thawed in a water bath at 70°C for 8 seconds (Peña and Linde-Forsberg, 2000) and then diluted in Tris buffer (38°C) at a 1:1 ratio before evaluation. The experimental design in this study was a completely randomized design with four replicates.

Table 1: The formulation of the four semen diluents utilized for canine sperm dilution.

Diluent ingredients	Semen extenders			
	Control	OB15	OB20	OB25
Tris (hydroxymethyl) aminomethane (mg)	3025	3025	3025	3025
Citric acid (mg)	1700	1700	1700	1700
Fructose (mg)	1250	1250	1250	1250
Egg yolk (ml)	20	20	20	20
Ocimum basilicum essential oils (mg)	0	1.5	2.0	2.5
Streptomycin (mg)	100	100	100	100
Penicillin (mg)	60	60	60	60
DMSO (ml)	0.8	0.8	0.8	0.8
Glycerol (ml)	5	5	5	5
Distilled water (ml)	To 100	To 100	To 100	To 100
pH	6.73	6.74	6.75	6.75
Osmolality (mOsmol/kg)	1425	1548	1554	1560

OB15, OB20, and OB25 represent 15, 20, and 25 µg/ml concentrations of *Ocimum basilicum* essential oils, respectively.

SPERM MOTILITY

The progressive motility of spermatozoa was analysed using a microscope set at 400x magnification. Before assessment, the sperm solution was incubated for 15 minutes at 38°C in a thermostatic bath. Observations were made across five randomly chosen fields, with approximately 200 spermatozoa evaluated in each field. The progressive motility was calculated as the proportion of spermatozoa displaying forward movement out of the total counted (Shah *et al.*, 2011).

SPERM VIABILITY

According to the method of Tamuli and Watson (1994), live and dead spermatozoa were identified by eosin-nigrosin staining. A 5µl drop of eosin-nigrosin dye solution was placed on a glass slide, followed by the addition of 5µl of sperm next to the dye drop. Using a wooden stick with the tip cut off, the semen was thoroughly mixed and stirred in a clockwise direction for about 30 seconds, then spread evenly on the slide. After spreading, the sample was left to air dry naturally and then observed under a microscope with 100x magnification. Dead spermatozoa have a cytoplasmic membrane that allows the eosin-nigrosin dye to penetrate, turning the sperm pink. In contrast, live spermatozoa have an intact cytoplasmic membrane that prevents dye penetration, maintaining their original white colour. A minimum of 200 spermatozoa were evaluated.

$$\text{Sperm viability} = \frac{\text{total number of live sperm}}{\text{total number of observed sperm}} \times 100$$

SPERM MEMBRANE INTEGRITY

The hypo-osmotic swelling test (HOST) was used to assess the integrity of the sperm membrane after freezing and thawing (Goericke-Pesch *et al.*, 2012). HOST relies on the sperm membrane’s ability to resist swelling in a hypotonic environment. The test was performed by adding 10 µl of sperm solution to 100 µl of hypo-osmotic solution (150mOsm). The mixture was incubated at 37°C for 30 minutes. After incubation, 0.2 ml of the mixture was placed on a glass slide and observed under a microscope with 400x magnification. A total of 200 spermatozoa (swollen and non-swollen tails) were evaluated. Swelling of the sperm indicated a healthy plasma membrane, whereas the lack of swelling suggested a damaged membrane.

SPERM LIPID PEROXIDATION

The thiobarbituric acid (TBA) assay was conducted following the procedure outlined by (Maia *et al.*, 2010). Lipid peroxidation in sperm was assessed by measuring the malondialdehyde (MDA) concentration. MDA levels in each sample were measured immediately after inducing lipid peroxidation in sperm with 0.24 mM FeSO4 at 37°C for 15 minutes. Next, 1ml of TBA reagent was added to 0.5ml of sperm sample. The mixture was incubated in a water bath for 15 minutes. After cooling, the solution was centrifuged at 1000×g for 10 minutes. The supernatant was separated, and absorbance was measured at 535nm using a microplate spectrophotometer (Multiskan SkyHigh, Thermo Fisher Scientific). The MDA concentration was calculated by comparing the sample’s absorbance at 535nm to the standard MDA curve. The results are reported as MDA concentration in nmol/50×10⁶ sperm.

STATISTICAL ANALYSIS

One-way analysis of variance (ANOVA) was used to evaluate the effect of semen extenders on sperm quality using

SPSS 22.0 software. Tukey's test was applied to compare the differences between the mean values of the treatments. Differences between the mean values were considered statistically significant when $P < 0.05$. The results are presented as mean $\pm$ standard deviation.

Table 2: The constituent profile of *Ocimum basilicum* essential oils.

Constituents	Retention time (min)	Relative percentage (%)
Eucalyptol	12.73	0.54
cis- β -Ocimene	13.89	0.39
β -Linalool	17.10	4.34
Fenchol	17.69	0.78
Camphor	19.35	0.85
Borneol	20.46	0.23
α -Terpineol	21.70	0.43
Estragole	22.24	75.2
Fenchyl acetate	22.97	0.38
Bornyl acetate	25.47	0.36
β -Elemene	28.95	1.11
Methyl eugenol	29.32	1.31
β -Caryophyllene	29.77	0.22
trans- α -Bergamotene	30.24	2.39
α -Guaiene	30.33	0.26
Humulene	30.76	0.5
cis-Muurola-4(15).5-diene	31.03	0.33
Germacrene D	31.52	0.41
Bicyclogermacrene	31.92	0.6
δ -Guaiene	32.13	0.58
γ -Cadinene	32.33	1.61
δ -Cadinene	32.52	0.32
Nerolidol	33.33	0.13
Spathulenol	33.69	0.17
Epicubenol	34.38	0.55
tau-Cadinol	34.83	3.84
β -Eudesmol	35.01	0.17
α -Cadinol	35.06	0.16
Total		98.16

RESULTS

EXTRACTION AND CHEMICAL COMPOSITION ANALYSIS OF ESSENTIAL OILS

Using the steam distillation method, 1.1 ml of essential oils was extracted from 150 g of dried *Ocimum basilicum* leaves powder, yielding 0.74%. The composition of the essential oil's biological compounds is presented in Table 2. GC-MS analysis identified 28 compounds, accounting for

98.16% of the total components. The primary constituents of *Ocimum basilicum* essential oils were estragole (75.2%), β -linalool (4.34%), tau-cadinol (3.84%), trans- α -bergamotene (2.39%), γ -cadinene (1.61%), β -elemene (1.11%), methyl eugenol (1.31%), camphor (0.85%), and eucalyptol (0.54%).

ANTIOXIDANT POTENTIAL OF ESSENTIAL OILS

The antioxidant activity evaluation revealed that the IC₅₀ value, which represents the concentration needed to neutralize 50% of DPPH radicals, was 1.38 mg/ml for *Ocimum basilicum* leaf essential oils and 0.047 mg/ml for vitamin E, as determined from the standard curve equation. These findings demonstrate that vitamin E exhibits significantly greater antioxidant potential compared to *Ocimum basilicum* essential oils at the same concentration. Nevertheless, the essential oils still displayed a considerable antioxidant capacity, despite being lower than the vitamin E standard.

Table 3: Impact of different *Ocimum basilicum* essential oils supplements in semen extenders on progressive motility, plasma membrane stability, and viability of frozen canine sperm.

Parameters	Control	OB15	OB20	OB25	P-value
Progressive motility (%)	47.98 $\pm$ 0.49 ^c	52.02 $\pm$ 0.65 ^b	55.43 $\pm$ 0.35 ^a	52.24 $\pm$ 0.35 ^b	<0.001
Plasma membrane integrity (%)	48.44 $\pm$ 0.22 ^c	52.26 $\pm$ 0.40 ^b	55.75 $\pm$ 0.24 ^a	52.63 $\pm$ 0.52 ^b	<0.001
Viability (%)	48.32 $\pm$ 0.24 ^c	52.31 $\pm$ 0.53 ^b	55.98 $\pm$ 0.22 ^a	52.63 $\pm$ 0.44 ^b	<0.001

OB15, OB20, and OB25 represent 15, 20, and 25 μ g/ml concentrations of *Ocimum basilicum* essential oils, respectively. Data are presented as mean $\pm$ SD based on four replicates, with each replicate consisting of a pooled sample from three ejaculates. Superscript letters (a, b, or c) within the same row denote significant differences among extenders ($P < 0.05$).

SPERM PROGRESSIVE MOTILITY, PLASMA MEMBRANE INTEGRITY AND VIABILITY

Table 3 presents the average proportions of sperm progressive motility, viability, and plasma membrane integrity. The findings indicate that sperm progressive motility, viability, and membrane integrity increased steadily from the control treatment to the treatments with essential oils concentrations of 15 μ g/ml and 20 μ g/ml, but declined at the highest concentration of 25 μ g/ml. Notably, the treatment with 20 μ g/ml had the highest average values for progressive motility, viability, and membrane integrity, showing significant differences compared to the control and the 15 μ g/ml and 25 μ g/ml treatments ($P < 0.05$). However, the treatment with the highest essential oils concentration (25 μ g/ml) had a similar average viability to the 15 μ g/ml treatment, both of which were significantly higher than

the control treatment (P<0.05).

SPERM LIPID PEROXIDATION

The malondialdehyde (MDA) levels produced by sperm after cryopreservation are shown in Table 4. The results of sperm oxidative susceptibility (MDA concentration) showed a gradual decrease from the control treatment to treatments with essential oils concentrations of 15 µg/ml and 20 µg/ml, followed by an increase at the highest concentration of 25 µg/ml. Notably, the treatment with 20 µg/ml had the lowest MDA concentration, which was significantly different from the other treatments (P<0.05). However, the treatment with the highest essential oils concentration of 25 µg/ml had an MDA concentration similar to that of the 15 µg/ml treatment, and both were significantly lower than the control treatment (P<0.05).

Table 4: Impact of different *Ocimum basilicum* essential oils supplements in semen extenders on malondialdehyde (MDA) production (nmol/50x10⁶sperm) in frozen canine sperm.

Parameters	Control	OB15	OB20	OB25	P-value
MDA	18.96 ±0.69 ^a	11.31 ±0.23 ^b	8.01 ±0.67 ^c	11.41 ±0.29 ^b	<0.001

OB15, OB20, and OB25 represent 15, 20, and 25 µg/ml concentrations of *Ocimum basilicum* essential oils, respectively. Data are presented as mean ± SD based on four replicates, with each replicate consisting of a pooled sample from three ejaculates. Superscript letters (a, b, or c) within the same row denote significant differences among extenders (P < 0.05).

DISCUSSION

This study highlighted a dose-dependent effect of *Ocimum basilicum* essential oils on the quality of frozen canine sperm. Concentrations below 20 µg/ml positively influenced sperm quality, whereas concentrations above this threshold proved detrimental. The optimal concentration for preserving frozen canine sperm during storage was identified as 20 µg/ml. The essential oils of *Ocimum basilicum* exhibited protective effects on canine sperm during cryopreservation, primarily due to its antioxidant components. These include linalool, cineole, bergamotene, eugenol, estragole, β-caryophyllene, γ-cadinene, muurolene, germacrene, tau-cadinol, and γ-gurjunene. These compounds readily inhibit excessive ROS by neutralizing harmful free radicals such as hydrogen peroxide and hydroxyl radicals. These results are consistent with previous research on the use of essential oils from rosemary, clove bud, and *Ocimum gratissimum* in extenders for ram, ovine, and canine sperm, respectively (Motlagh *et al.*, 2014; Baghshahi *et al.*, 2014; Nguyen *et al.*, 2023). However, differing outcomes were reported for thymol and *Thymus munbyanus* essential oils,

which were found to have adverse effects on human sperm (Chikhoun *et al.*, 2015). The study findings emphasize the potential use of *Ocimum basilicum* essential oil for preserving canine sperm and suggest that it could be applied to other livestock species as well. Earlier research has shown that linalool interacts directly with free radicals, including hydroxyl (OH⁻) and peroxy (ROO⁻) radicals, by donating a hydrogen atom or an electron to neutralize them. This process safeguards cellular structures like membranes, proteins, and DNA from oxidative harm, preserving the cells' structural integrity and functionality (Cheng *et al.*, 2022). Eugenol has been shown to boost sperm motility and mitigate oxidative damage by donating a hydrogen atom or an electron, thereby stabilizing free radicals and slowing oxidative processes (Barboza *et al.*, 2018; Mahmoud *et al.*, 2020). β-caryophyllene has demonstrated the ability to improve sperm quality in mice by enhancing motility, viability, and plasma membrane integrity through its capacity to combat oxidative stress and neutralize free radicals (OH⁻ and ROO⁻) during sperm oxidation (Francomano *et al.*, 2019). Furthermore, estragole acts as an antioxidant by neutralizing free radicals, such as hydroxyl (OH⁻) and superoxide (O²⁻), through electron or hydrogen atom donation, thereby minimizing their damaging effects on cellular components (Júniora *et al.*, 2020).

In addition to their antioxidant activity, the phenolic compounds in *Ocimum basilicum* essential oils also inhibit bacterial growth, such as eugenol and linalool, which alter membrane permeability, affect ion transport, ATP, and modify the fatty acid structure of bacteria. Due to their insolubility in water, these phenolic compounds can interact with phospholipids and proteins in sperm cell membranes. As a result, they influence membrane permeability and ion flow (Nazzaro *et al.*, 2013). The estragole component in *Ocimum basilicum* essential oils, when used at appropriate concentrations, exhibits antioxidant activity by donating electrons and hydrogen atoms to neutralize harmful hydroxyl (OH⁻) and superoxide (O²⁻) radicals, thus stabilizing and protecting the cell membrane and DNA, reducing adverse effects on sperm. However, at higher concentrations, it can be toxic to sperm cells, causing morphological changes, disrupting nuclear membrane integrity, enhancing chromatin condensation, and compromising the cell membrane's integrity (Lashkari *et al.*, 2020). This demonstrates that a concentration of 20 µg/ml is most effective for optimal sperm membrane protection, while concentrations above 25 µg/ml can inhibit or poison the sperm.

MDA concentration is an indicator of lipid peroxidation as well as a sign of oxidative stress; the higher MDA level associated with lower sperm quality (Vieira *et al.*, 2017). The addition of antioxidants to the sperm dilution medium can prevent sperm lipid peroxidation by inhibiting MDA production. The MDA concentrations in this study include

the lipid peroxidation process that occurs in both the sperm and the dilution medium. Therefore, lipid peroxidation in the sperm dilution medium can significantly influence the MDA concentration results. The MDA levels in treatments with added essential oils were lower than in the control, with MDA concentrations decreasing from low to high essential oils concentrations, due to the increasing levels of antioxidants in *Ocimum basilicum* essential oils, which help in sperm protection and reduce oxidation. Moreover, these bioactive compounds may directly contribute to or collaborate with extracellular antioxidant systems, including catalase, glutathione peroxidase, phospholipids, hydro-peroxide, glutathione, peroxidase, and superoxide dismutase, to counteract oxidative stress (Angrimani *et al.*, 2014). On the other hand, if antioxidant concentrations are too high, they may inhibit ROS, increasing the permeability of the plasma membrane, making sperm more sensitive (Takeshima *et al.*, 2004), leading to a loss of motility, reduced protein phosphorylation, and increased susceptibility to oxidation and cell death, ultimately raising MDA levels. Our study highlights the effect of *Ocimum basilicum* essential oil on canine sperm quality, specifically examining parameters like motility, plasma membrane integrity, viability, and lipid peroxidation. However, factors such as DNA integrity and fertility potential need to be explored further in future research to ensure the production of high-quality sperm for livestock breeding.

CONCLUSIONS AND RECOMMENDATIONS

Ocimum basilicum essential oils is rich in antioxidant compounds that offer beneficial effects on sperm when incorporated into the cryopreservation dilution medium, with the impact varying based on its concentration. The optimal concentration of 20 µg/ml enhances sperm quality by improving progressive motility, viability, membrane integrity, and oxidative resistance. However, higher concentrations may have adverse effects, reducing sperm quality during cold storage. Further experiments are suggested to assess the DNA integrity and fertilization potential of sperm following thawing.

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NOVELTY STATEMENT

This study introduces a novel approach by incorporating essential oil extracted from *Ocimum basilicum* leaves into frozen canine semen extenders. The findings highlight the positive effects of these essential oils on frozen dog

sperm, suggesting their potential utility in practical breeding practices.

AUTHOR'S CONTRIBUTIONS

Nguyen Van Vui conceptualized and designed the experiments. Luu Nguyen Trong Nghia and Huynh Thanh Tan carried out the experimental work. Nguyen Van Vui, Luu Nguyen Trong Nghia, and Huynh Thanh Tan analysed the data and drafted the manuscript. All authors reviewed and approved the final version of the manuscript.

CONFLICT OF INTEREST

Authors declared no conflict of interest.

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