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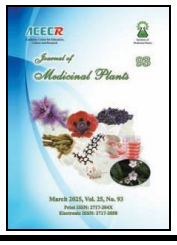

Research Article

Effectiveness of *Morinda citrifolia* L. leaves extract to improve semen quality and reproductive hormone concentrations in Wistar rats exposed to cigarette smoke

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ABSTRACT

Background: *Morinda citrifolia* L. possesses antioxidant activity that can ameliorate the decline in semen quality of male rats due to exposure to cigarette smoke. **Objectives**: This study intend to assess the effectiveness of *M._citrifolia* leaves extract in ameliorating male infertility associated with oxidative dysregulation induced by exposure to tobacco smoke. **Methods**: The animals used in the study were evenly and randomly divided into five groups, each containing five rats. Group X1 served as the normal control without any treatment, whereas group X2 comprised rats that were exclusively subjected to cigarette smoke exposure. Groups X3, X4, and X5 were exposed to cigarette smoke and subsequently administered *M. citrifolia* leaves extract orally via a nasogastric tube at doses of 100, 200, and 300 mg/kg BW, respectively, for a period of 52 days. Twenty-four hours after the final treatment, blood samples were collected to examine FSH, LH, and testosterone levels using ELISA technique. Semen was collected from the cauda epididymis to analyze the quality of spermatozoa. **Results**: The administration of *M. citrifolia* leaves extract improved sperm concentration, progressive motility, and viability, while sperm morphological abnormalities were not affected by the extract (P = 0.618). FSH concentration decreased following *M. citrifolia* leaves extract administration, particularly at dose of 100, and 200 mg/kg BW. LH concentration increased significantly after treatment with 100_mg/kg BWof *M. citrifolia* leaves extract and testosterone levels improved after treated with leaves extract of *M. citrifolia* (P <0.001). **Conclusions**: Methanol extract of *M. citrifolia* leaves enhance sperm quality and testosterone levels but does not affect FSH and LH concentrations in male rats exposed to cigarette smoke.

Abbreviations: RD, Ratna Dewi; SW, Sri Wahyuni; AS, Amalia Sutriana; TNS, Tongku Nizwan Siregar; TA, Teuku Armansyah; *M. citrifolia* L, *Moringa citrifolia* L; FSH, follicle stimulating hormone; LH, luteinizing hormone; ELISA, enzymelinked immunosorbent assay; BW, body weight; WHO, World Health Organization; ROS, reactive oxygen species; RNS, reactive nitrogen species; ANOVA, analysis of variance; DNA, deoxyribonucleic acid; GnRH, gonadotropin releasing hormone;

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1. Introduction

In 2020, the WHO estimated that about 48 million couples and individuals experience infertility problems, with 50% of cases involving men [1]. Infertility in this population may be associated with various factors, including genetic variations, hormonal disorders, environmental influences, psychological issues, and lifestyle habits such as diet, alcohol intake, and drug abuse [2]. Although recent epidemiological studies have shown no direct association between smoking and male infertility [3, 4], smoking is a common route of exposure to toxic substances that can disrupt oxidative stress balance [5]. Extensive analyses of spermatogenic profiles indicated that smoking contributes to a significant reduction in semen volume as well as sperm concentration [2]. Additionally, infertile men exhibiting clinical oxidative stress have a unique proteomic profile in their seminal plasma, as indicated by the presence of 23 specific proteins [6].

The male reproductive system is vulnerable to free radicals, including reactive oxygen species (ROS) and reactive nitrogen species (RNS). Exposure to exogenous oxidants significantly increases ROS and RNS levels, causing excessive oxidative stress. This condition promotes the oxidative degradation of lipids, carbohydrates, proteins, and nucleotides, which negatively impacts spermatozoa and sperm quality. Testosterone, synthesized by Leydig cells within the testes in response to LH stimulation, is pivotal for the development and continuing function of male reproductive tissues. In the process of spermatogenesis, follicle-stimulating hormone (FSH) promotes nutrient provision by activating Sertoli cells. Oxidative stress has been found to reduce testosterone secretion, potentially leading to endothelial dysfunction [7]. Moreover, LH and FSH have been reported to inversely correlate with ROS concentration [8, 9].

Recently, researchers are interested in phytomedicine to develop infertility treatments related to oxidative stress. *Morinda citrifolia* L. is one of such traditional medicinal plant that has attracted significant attention due to its therapeutic properties. This plant believed to originate from Southeast Asia and has been distributed in various countries, such as Polynesia, South Asia, Southeast Asia, Australia, Hawaii and Caribia [10]. *M. citrifolia* is a member of the Rubiaceae family, comprising over 80 genera that are distributed across tropical and subtropical regions [11-13]. These plants can grow in a variety of different environments, such as coastal areas, plains, infertile soils, grasslands and humid forest areas with low luminosity [14,15], and can also be found up to an altitude of 1300 feet above sea level [16] with rainfall of 1500-3500 mm/year, soil pH 5-7, temperature 22-30°C and humidity 50-70% [17].

M. citrifolia possess several therapeutic properties, including antioxidant, anticancer, antimicrobial, antidyslipidemia, and anti-inflammatory activities [18]. The ethanol extract of *M. citrifolia* fruits has been quantified to exhibit a total phenolic concentration of 11.69 ± 0.04 mg GAE/g DW [19]. *M. citrifolia* has been demonstrated to attenuate indicators of oxidative damage, including reactive_oxygen_species (ROS) and ‘malondialdehyde, while significantly enhancing serum levels of glutathione (GSH which capable in mitigating ROS formation [20]. Previous research also indicated that *M. citrifolia* extract could produce protective effects on spermatozoa, increasing sperm viability and motility [21, 22]. Additionally, the fruit extract can counteract paclitaxel-induced toxicity in testicular parenchyma [23]. Despite its promising as an antioxidant, the potential of *M. citrifolia* leaves in enhancing male fertility has not been extensively documented. This study represented

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the first effort to explore the therapeutic potential of *M. citrifolia* L. leaves extract in addressing male infertility using animal models. Infertility in experimental animals was induced by exposure to cigarette smoke, following recommendations from previous research [24].

This study aimed to evaluate the therapeutic potential of *M._citrifolia* leaves extract in mitigating male infertility, particularly focusing on the oxidative stress-induced damage to reproductive health caused by ‘exposure to ‘cigarette ‘smoke.

2. Materials and methods

2.1. Sample Preparation and Extraction Process

M. citrifolia leaves were collected from KecamatanMeraxa, Banda Aceh, Indonesia. The plant specimens were brought to the Biology Laboratory of Universitas Syiah Kuala for taxonomic identification with no. 269/UN11. 18.4/ TA 0003/2023. Fifteen kilograms of fresh *M. citrifolia* leaves were carefully rinsed with distilled water and then dried at, room temperature for a period of 15,0days. The dried leaves were then’ pulverized into fine `powder. Maceration was carried out on the *M. citrifolia* leaves powder using hexane, ethyl acetate, and methanol solvents. The powder was soaked in a closed container filled with n-hexane and stored at room temperature without exposure to direct sunlight. During maceration, the mixture was gently stirred twice every 24 hours. Macerate was collected after 72 hours, and the leftover residue was then re-macerated with ethyl acetate, followed by methanol, using the same extraction procedure. The macerate was evaporated using vacuum evaporator, at 50°C’ until a thick powder was obtained. The methanol extract’ of *M. citrifolia* leaves were then used in this study.

2.2. Phytochemical analysis

Phytochemical analysis was carried out at the Natural_Pharmaceutical_Laboratory, Faculty of Mathematics and Natural Sciences Universitas` Syiah` Kuala. The procedure followed the protocols established in prior research [25].

2.3. Experimental animals

Thirty healthy male Wistar rats (*Rattus norvegicus*), aged 2-2.5 months and weighing 150-200 g, were acclimatized for 7 days under a 12-hour light/dark cycle in standard cages (36 × 30 × 12 cm) with five rats per cage and free access to food and water. The rats were randomly assigned into five groups (n = 5) using an online randomization tool (https://www.random.org/). Sample size determination followed the Federer formula: $(t-1)(r-1) \geq 15$, where t is number of groups and r is number of replications.

2.4. Cigarette smoke exposure and treatment with M. citrifolia leaves extract

The Wistar rats were randomly assigned into five equal groups (n = 5 per group): a control group (X1); a group exposed solely to cigarette smoke (X2); and three groups subjected to cigarette smoke followed by oral administration of *M. citrifolia* leaves extract at doses of 100 mg/kg BW (X3), 200 mg/kg BW (X4), and 300 mg/kg BW (X5), respectively. The dosages administered to the rats were adapted from previous studies using *Azadirachta indica* leaves extract with doses of 50, 100 and 200 mg/kg BB [26]. *M. citrifolia* leaves extract was orally administered through a nasogastric tube for duration of 52 days. Each day, 4 cigarettes were used to create exposure to cigarette smoke, which was conducted for 21 consecutive days. The exposures took place in a smoking chamber where the rats inhaled the smoke delivered through an inhaler for 15 minutes at one-hour intervals. A detailed design of the

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smoking chamber and the mechanism has been described previously [27].

2.5. Sample preparation and hormonal determination

Twenty-four hours post-treatment, blood samples were obtained from the heart to measure LH, FSH, and testosterone levels. Blood was collected in a 3 ml EDTA vacuum container and centrifuged for 15 minutes at 3500 rpm. The levels of FSH, LH, and testosterone were measured employing ELISA kit manufactured by DRG Instruments GmbH, Germany. Subsequently, the rats were euthanized with Zoletil® 100 at a dosage of 40 mg/kg BW following an overnight fasting period. Semen was collected from the cauda epididymis to analyze the quality of spermatozoa. The cauda epididymis was placed in 2 mL of phosphate-buffered saline (PBS) and meticulously dissected with a scalpel. The resulting suspension was gently mixed and allowed to incubate for 5 minutes to promote the release of sperm. This suspension was subsequently utilized for sperm quality assessment [28].

2.6. Data analysis

Phytochemical screening results were presented descriptively, while sperm quality and hormone concentrations were analyzed using one-way ANOVA followed by Tukey's post hoc test, with significance set at $P < 0.05$. Analyses were performed using SPSS version 20.0 (SPSS Inc., Chicago, USA).

3. Result

3.1. Phytochemical profile of M. citrifolia

Phytochemical tests showed that M. citrifolia leaves extract contains alkaloids, flavonoids, tannins, steroids, terpenoids, phenolics, and saponins (Table 1).

Table 1. Results of phytochemical screening of methanol extract of *M. citrifolia* leaves

Secondary metabolites	Observation
Flavonoids	+
Terpenoids	+
Steroids	+
Tannins	+
Phenolic	+
Saponins	+
Alkaloids	
Dragendroff	+
Meyer	+
Wagner	+

3.2. Sperm quality after Cigarette smoke exposure and M. citrifolia leaves extract therapy

The sperm quality observation was presented in Table 2. The results showed that cigarette smoke exposure did not significantly affect viability ($P = 0.131$) and abnormalities of sperm ($P = 0.994$). However, sperm concentration and motility experienced a significant decrease following smoke exposure ($P = 0.024$). The treatment with *M. citrifolia* leaves extract improved sperm motility ($P = 0.005$), concentration ($P = 0.040$), and viability ($P = 0.038$). No significant differences in sperm abnormalities were found among the groups ($P = 0.618$).

The effects of *M. citrifolia* leaves extract on FSH, LH, and testosterone concentrations were provided in Table 3. The cigarette smoke exposure did not significantly affect serum levels of FSH ($P = 0.089$), LH ($P = 0.066$) and testosterone ($P = 0.995$). The FSH levels showed a significant decrease following the administration of 100 and 200 mg/kg BW doses of *M. citrifolia* leaves extract, with concentrations of 1.99 ± 0.74 and 1.31 ± 0.48 mIU/mL, respectively ($P = 0.001$). The testosterone concentration significantly increased after treated with 100 and 200 mg/kg BW extracts ($P = 0.001$).

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Table 2. Average spermatozoa quality of Wistar rats treated with *M. citrifolia* leaves extract for 52 days after exposure to cigarette smoke for 21 days

Treatments	Variables of spermatozoa quality			
	Concentration (x106/mL)	Motility (%)	Viability (%)	Abnormality (%)
X1 (normal control)	99.4±31.36[b]	84.28±9.25[b]	67.81±6.04[a]	19.98±11.89
X2 (cigarette smoke exposure + 0 mg/kg BW extract)	60.0±21.74[a]	42.04±33.42[a]	41.35±17.20[a]	12.32±17.37
X3 (cigarette smoke exposure + 100 mg/kg BW extract)	82.0±8.89[b]	83.77±6.27[b]	79.54±3.69[b]	17.95±3.99
X4 (cigarette smoke exposure + 200 mg/kg BW extract)	96.3±52.00[b]	48.94±27.15[b]	44.11±22.39[a]	10.18±8.25
X5 (cigarette smoke exposure + 300 mg/kg BW extract)	122.33±52.32[b]	78.61±2.84[b]	73.23±2.05[ab]	16.21±8.66
Anova p-value	0.040	0.005	0.038	0.618

[a,ab,b]Different superscripts in the same column indicate significant differences (P < 0.05).

Table 3. Mean concentrations of testosterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) in Wistar rats treated with *M. citrifolia* leaves extract for 52 days after exposed to cigarette smoke for 21 days

Treatments	Concentration		
	Testosterone (ng/mL)	FSH (mIU/mL)	LH (mIU/mL)
X1 (normal control)	0.49 ± 30.29[a]	2.20 ± 0.53[b]	0.85 ± 0.11[a]
X2 (cigarette smoke exposure + 0 mg/kg BW extract)	0.74 ± 0.54[a]	3.38 ± 1.09[b]	2.41 ± 1.23[ab]
X3 (cigarette smoke exposure + 100 mg/kg BW extract)	2.35 ± 1.20[c]	1.99 ± 0.74[a]	1.34 ± 0.18[b]
X4 (cigarette smoke exposure + 200 mg/kg BW extract)	1.38 ± 0.27[bc]	1.31 ± 0.48[a]	1.74 ± 0.81[ab]
X5 (cigarette smoke exposure + 300 mg/kg BW extract)	1.02 ± 0.7[ab]	2.12 ± 0.69[b]	2.43 ± 1.22[ab]
Anovap-value	0.001	0.001	0.036

[a,ab,b]Different superscripts in the same column indicate significant differences (P < 0.05).

4. Discussion

Phytochemical screening of methanolic extract of *M. citrifolia* leaves in this study detected several bioactive constituents such as terpenoids, flavonoids, steroid, tannins, phenolic, saponins, and tannins. Previous study also reported similar finding. Erina *et al*. [29] stated that *M. citrifolia* plant contains various compounds, contributing to its antioxidant activities. It includes alkaloids, saponins, tannins, steroids, flavonoids, polyphenols, triterpenoids, and quinones. Sogandi and Rabima [30] also reported that this plant has numerous secondary metabolites, which potentially act as antioxidants depending on the solvents used. Ethanol extract of *M. citrifolia* contains anthraquinones, alkaloids, tannins, flavonoids, steroids, saponins, and phenolics. The hexane fraction holds anthraquinones, alkaloids, flavonoids, triterpenoids, saponins, and phenolic compounds. The chloroform fraction contains anthraquinones, alkaloids, flavonoids, steroids, triterpenoids, saponins, and phenolics. The ethyl acetate fraction includes anthraquinones, alkaloids, flavonoids, triterpenoids, and saponins. Finally, the water

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fraction contains anthraquinones, alkaloids, and triterpenoids.

Statistically, all spermatozoa quality variables, except for abnormality, in the group without administration of *M. citrifolia* leaves extract had lower quality ($P < 0.05$) than those in the control group and the group that received *M. citrifolia* leaves extract, as presented in Table 1. Based on the data, increasing doses of *M. citrifolia* leaves extract significantly improved sperm viability ($P < 0.05$) but did not affect concentration, motility, or sperm abnormality ($P > 0.05$). The *M. citrifolia* leaves extract showed potential to improve concentration, motility, and sperm viability in rats exposed to cigarette smoke ($P < 0.05$). These findings agree with previous reports that cigarette smoke can decrease sperm quality [31-35] and vice versa [36].

The decline in sperm quality observed in group C1 following smoke exposure is likely attributed to an increase in reactive molecules, including free radicals and reactive oxygen species (ROS), which subsequently induce oxidative stress [32, 37, 38]. Free radicals such as quinone, semiquinone, and hydroquinone result from incomplete combustion [39]. Quinone polymer and hydroquinone are highly reactive and can damage cell membranes, proteins, and deoxyribonucleic acid (DNA) [40-42]. The presence of free radicals may cause spermatozoa disorders, accounting for 30-80% of infertility cases in men [34, 43]. Elevated ROS levels and decreased antioxidant activity in sperm due to cigarette smoke can disrupt spermatogenesis. Cigarette smoke-derived free radicals have the potential to induce DNA damage and trigger apoptosis in spermatozoa, resulting in diminished concentration and motility of sperm, as well as alterations in sperm morphology [44].

The mechanism behind the decrease in semen quality due to high ROS is likely related to reduced antioxidant capacity, testicular endocrine changes, and hypothalamic-pituitary axis disturbance. Oxidative stress may disrupt sperm production by affecting the release of LH and FSH [37, 38]. Nicotine in cigarettes impacts the central nervous system by stimulating the hypothalamus, which subsequently reduces the secretion of GnRH. This reduction leads to a decrease in LH secretion, thereby impairing the activation of Leydig cells, the principal source of testosterone production [45].

The administration of *M. citrifolia* leaves extract in rats subjected to cigarette smoke exposure resulted in notable improvements in sperm quality, particularly in terms of concentration and motility as shown in Table 1. These improvements in sperm parameters are likely attributed to the potent antioxidant properties present in the *M. citrifolia* leaves extract. Phytochemical test results indicated that the extract in methanol solvent contains chemical compounds from the flavonoid, phenolic, tannin, and terpenoid groups (Table 1). Afif and Amilah [46] reported similar findings that ethanol extract of *M. citrifolia* leaves contains saponins, flavonoids, polyphenols, tannins, and triterpenes. Flavonoid compounds [47] and phenolics [48] can reduce peroxide and hydroxyl radicals related to cigarette smoke exposure, thereby helping regulate semen quality in the epididymis [49/40]. The antioxidant activity assessments of *M. citrifolia* leaves extract revealed an average value of 7.17 mg trolox equivalent per gram of extract [50]. The antioxidant activity of the ethanol extract from *M. citrifolia* leaves was comparable to that of ascorbic acid, exhibiting an IC_{50} value of 275.0792 ± 1.929 μg/mL [51]. The *M. citrifolia* leaves extract is assumed to

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have the capacity to neutralize peroxide free radicals that damage Sertoli and Leydig cells, thereby supporting their ability to secrete testosterone and facilitate effective sperm production. Increased testosterone levels stimulate spermatogenesis in the testes, resulting in more sperm cell production.

Exposure to cigarette smoke reduces testosterone and FSH concentrations, although LH levels do not show significant differences, as presented in Table 3. These results align with previous research, which described that adult rats exposed to smoke have reduced testosterone concentrations, even though FSH and LH levels remain unchanged [52]. Treatment with *M. citrifolia* leaves extract increased testosterone and LH concentration but did not significantly increase FSH concentrations. Other studies have demonstrated that rats treated with 100 mg/kg BW of lead acetate and extracts from *Eruca sativa*, *Apium graveolens* L., and *Nigella sativa* exhibited elevated levels of testosterone, FSH, and LH [54]. These different results are likely related to different infertility inductions. Lead exposure significantly decreases the concentrations of all three hormones (testosterone, FSH, and LH), while this study indicated that cigarette smoke exposure mainly reduces testosterone concentrations. Moreover, the effects of different plant treatments may also influence the outcomes.

5. Conclusion

In conclusion, *M. citrifolia* leaves extract improves sperm quality and increases testosterone levels in cigarette smoke-exposed male rats, without affecting FSH and LH concentrations. Further study is needed to identify the active antioxidant compounds in *M. citrifolia* leaves extract using GC-MS and antioxidant analysis.

Author contribution

RD: Conceptualization, methodology, investigation, resources, data curation, writing—original draft preparation, project administration. TNS: Conceptualization, software, validation, formal analysis, writing—original draft preparation, writing—review and editing, supervision, SM: writing—original draft preparation, visualization, supervision. AS: Conceptualization, methodology, formal analysis, data curation, writing—original draft preparation, writing—review and editing, supervision, project administration. TA: writing—original draft preparation, visualization, supervision. All authors have read and agreed to the published version of the manuscript.

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Ethics approval

Ethical approval for this study was granted by the Faculty of Veterinary Medicine's Ethics Board Universitas Syiah Kuala, with certificate No: 224/KEPH/VII/2023.

Conflict of interest

The author declares no conflict of interest in the publication of this manuscript.

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