Trop J Nat Prod Res, April 2025; 9(4): 1487 – 1491 ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Toxicity and Aphrodisiac Activity of the Capsules Derived from the Cortex of *Uvaria rufa*

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ARTICLE INFO

Article history:
Received 03 January 2025
Revised 31 January 2025
Accepted 28 February 2025
Published online 01 May 2025

ABSTRACT

Sexual dysfunction is a condition characterized by disturbance in sexual function. About 12 to 51.6% of men aged 60 years and older experience decreased sexual desire and about 14.3 to 70% experience erectile dysfunction. The people of East Nusa Tenggara, Indonesia, traditionally use the cortex of *Uvaria rufa* as a treatment for male sexual dysfunction. This study aimed to determine the safety of 70% ethanol extract of *U. rufa* cortex (URCE) in zebrafish (*Danio rerio*) and evaluate the aphrodisiac activity of capsules derived from the 70% ethanol extract of *U. rufa* cortex (URCC). The acute toxicity was assessed by estimating the LD_{50} of URCE using the fixed dose method. Aphrodisiac activity was assessed by measuring Leydig cells, Sertoli cells, seminiferous tubule diameter, and testicular weight following the administration of URCC at doses of 1.34, 2.69, and 5.37 mg/20 g BW. Sildenafil at a dose of 0.13 mg/20 g BW was used as the positive control. The result shows that URCE showed low acute toxicity in zebrafish with an LD_{50} value of 1.582 µg/mL. Meanwhile, in the aphrodisiac activity evaluation, administration of URCC at a dose of 5.37 mg/20 g BW of mice/day was found to be the optimal dose resulting in significant increase in testicular weight, Leydig cells, Sertoli cells, and the diameter of the seminiferous tubules. These findings therefore revealed the potential of *Uvaria rufa* as a safer alternative to conventional drugs for the treatment of male sexual dysfunction.

Keywords: *Uvaria rufa*, Aphrodisiac Activity, Sexual Dysfunction, Toxicity.

Introduction

Sexual dysfunction is a condition characterized by a disturbance in the aspect of sexual function or response to sexual cycle.[1,2] According to a systematic review conducted on 76 articles globally, data showed that about 12 to 51.6% of men aged 60 years and above experience decreased sexual desire and about 14.3 to 70% experience erectile dysfunction.[2] There is no definitive data regarding the incidence and prevalence of male sexual dysfunction, both globally and regionally. This is due to the assumption that experiencing sexual dysfunction in men is something shameful. The large number of sexual dysfunction cases can be seen from the data on the sales of male sexual dysfunction drugs globally. Phosphodiesterase (PDE) Inhibitors was valued at USD 2.82 billion in the year 2022, and was forecasted at a compound annual growth rate of 6.1% from 2023 to 2030. The large market demand for PDE Inhibitors is thought to be due to increasing awareness among the population and the increasing prevalence of male sexual dysfunction.[3] The first line of treatment for sexual dysfunction in men is currently the phosphodiesterase-5 inhibitor (PDE5i) class of drugs. More than 30 million men worldwide are treated with sildenafil. Sildenafil works by inhibiting phosphodiesterase, increasing intracellular concentration of cyclic guanosine 3',5' monophosphate (cGMP), which causes amplification of the endogenous NO-cGMP signaling pathway.[3,]

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Citation: Ma'arif B, Muslikh FA, Boelan EG, Maulina N, Azzahra A, Sawjana OH, Aszari EH, Prameswari FA, Taek MM. Toxicity and Aphrodisiac Activity of the Capsules Derived from the Cortex of *Uvaria rufa*. Trop. J. Nat. Prod. Res. 2025; 9(4): 1487 – 1491. https://doi.org/10.26538/tjnpr/v9i4.16

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

Unfortunately, the use of sildenafil is associated with side effects such as headaches (9-39%) and visual disturbances (5-11%).[5] The use of sildenafil can also increase the risk of cardiovascular events, especially fatal arrhythmias, so it is considered unsafe for consumption by patients with coronary artery disease.[6] Therefore, there is a need for alternative treatments with minimal side effects, one of such alternative treatments is the use of herbal products derived from ethnomedicine.[7]

One of the Indonesian plants used in ethnomedicine as an aphrodisiac is *Uvaria rufa*. This plant is known by several names according to local languages in the Western Timor, Indonesia. In the Kupang dialect of Indonesian, it is called *lelak*, Dawan people call it *koknaba*, and Tetun people know it by the name *koke*. Traditionally, the stem bark (or cortex) of *U. rufa* has been widely used by people in Timor and Flores -islands, Nusa Tenggara Timur (NTT) Province to maintain physical fitness, prevent illness, and restore fitness and stamina after illness, including increasing stamina in men.[8,9] In traditional medical practice, a boiled water extract of the cortex of the plant is consumed for therapeutic purposes, maintain physical fitness, prevent illness, and restore freshness and stamina after illness, as well as to increase stamina in men.[7]

Metabolic profiling of the ethanol extract of *U. rufa* cortex using UPLC-QTOF MS/MS showed the presence of 46 compounds, with the major compound being 3',7-Dimethoxy-3-hydroxyflavone, with a percentage peak area of 21.92%. In addition, the compound 8-oxocoptisine was also identified with an activity probability of 84.09% to the target receptor Thromboxane A2, and a percentage peak area of 0.24%. Flavonoids are known to play a role in improving vascular health, including improving blood flow, which is particularly relevant in the context of erectile dysfunction, where healthy blood flow is essential for normal erectile function.[10] Flavonoids have protective effects on spermatogenesis and maturation defects in the testes through the mechanism of restoring normal glutathione (GSH) levels which play a role in hydrogen peroxide metabolism. Flavonoids also increase the regulation of kinases regulated through the phosphorylated extracellular

signaling pathway (ρ-ERK). As a result of this signaling, the mitogen activated protein kinase (MAPK) pathway is activated, resulting in the proliferation of sertoli cells.[11] Additionally, alkaloids with its high nitrogen composition, is suggested to be potential inhibitors of the enzyme phosphodiesterase type 5 (PDE5). Inhibition of PDE5 is the mechanism by which blood flow to the genitals is increased.[12] Alkaloids are thought to be able to modulate dopamine-2 (D2) receptors so that dopamine is released which has a stimulant effect and increases libido. In addition, alkaloids also bind to α-1 adrenergic receptors. As a result of this bond, the smooth muscle of the corpus cavernosum is constricted leading to increase in the sensitivity of the penis, and subsequently increases erection.[13] Furthermore, alkaloids increase steroidogenesis by increasing cholesterol import by the mitochondria so that there is an increase in the amount of steroid hormones that is converted into dehydroepiandrosterone (DHEA).[14] The flavonoid and alkaloid contents in *U. rufa* cortex is predicted to be responsible for its aphrodisiac activity.

The great potential of *U. rufa* cortex can be harnessed into readily acceptable medicinal product. However, active substances derived from natural ingredients usually have poor bioavailability due to differences in fat solubility and molecular size. This problem can limit the passage of the drug through biological membranes, thus disrupting systemic absorption.[15] Therefore, there is the need for further research on formulation of *U. rufa* cortex 70% ethanol extract that could improve its bioavailability. This study aimed to determine the safety of 70% ethanol extract of *U. rufa* cortex (URCE) in zebrafish (*Danio rerio*) and to evaluate the aphrodisiac activity of capsules (URCC) containing 70% ethanol extract of *U. rufa* cortex (URCE).

Materials and Methods

Reagents/Chemicals

The chemicals used in this study included 70% ethanol, Tween 80, 0.5% DMSO, distilled water, 80% ethanol, 96% ethanol, absolute alcohol, 10% formalin, ketamine, xylazine, hematoxylin, eosin, and xylol. All the chemicals were products of Merck (Darmstadt, Germany).

Collection and identification of plant material

The cortex of the *U. rufa* plant was collected from Kupang, NTT Province, Indonesia on the 13th of August 2024, and identified by a botanist of Widya Mandira Catholic University, Kupang, Indonesia. Herbarium specimen with voucher number 234/WM.H9/KET/XI/2024 was deposited.

Extraction

The cortex of *U. rufa* was finely ground into powder, and then extracted by maceration in 70% ethanol at a powder-to-solvent ratio of 1:15 at room temperature for 48 h. The extract was filtered and the resulting filtrate was concentrated using a rotary evaporator at 50°C, 175 psi, and 70 rpm. The residual solvent was evaporated in an oven at 50°C to obtain a thick ethanol extract of *U. rufa* cortex (URCE).

Formulation of U. rufa cortex capsule URCC

The URCC (*U. rufa* cortex capsule) was formulated and provided by PT. Agaricus Sido Makmur Sentosa, Malang, Indonesia. URCC was formulated using wet granulation method using 900 mg of 70% ethanol extract of *U. rufa* cortex (URCE). The excipients used include lactose, microcrystalline cellulose, sodium starch glycolate, hydrated silica, magnesium stearate, copovidone, and methylparaben.

Toxicity test

In vitro toxicity test was performed on zebrafish embryos in accordance with OECD Guideline No. 236 (2013) for acute toxicity testing on zebrafish embryos over a 96-hour period. Eight-hour-old embryos at the organogenesis stage were placed in well plates containing the test solution. The test solution concentrations included extract doses of 500 ppm, 1000 ppm, 1500 ppm, and 2000 ppm. Distilled water was used as the negative control (NC). Each well plate contained 20 embryos, and each concentration was tested in triplicate. The embryos were incubated with the test extract (URCE) at room temperature for up to 96 hours.[15] The mortality rate was calculated every 24 hours using probit analysis.

Evaluation of aphrodisiac activity

Animals

Thirty-five (35) male albino mice weighing between 15 – 25 g were obtained from were obtained from the CV. Satwa Sehat Sejahtera veterinary clinic, Malang, East Java, Indonesia. The mice were kept in well-ventilated cages and acclimatized to the laboratory conditions for 7 days. The mice were fed with standard rodent pellets and allowed access to drinking water *ad libitum*.

Ethical approval

The study was approved by the Research Ethics Commission (Animal Care and Use Committee) at Brawijaya University, Malang, East Java, Indonesia. Ethical approval with reference number 065-KEP-UB-2024 was issued.

Experimental design

Aphrodisiac activity was evaluated by Leydig cell count, Sertoli cell count, and measurement of the diameter of the seminiferous tubules, and testicular weight. The mice were divided into five groups of 7 mice per group. The groups include; Negative control (NC) which received 1% Tween 80 suspension in 0.5% DMSO, Positive control (PC) which received sildenafil citrate suspension at a dose of 0.13 mg/20 g BW mice/day, (A1) which received 1.34 mg URCC suspension/20 g BW mice/day, (A2) which received 2.69 mg URCC suspension/20 g BW mice/day, and (A3) which received 5.37 mg URCC suspension/20 g BW mice/day. The selection of the dose of URCE was based on the empirical dose or the dose of *U. rufa* herb used traditionally by the NTT community to treat sexual dysfunction in men. Each treatment was administered orally at 0.5 mL per dose for 28 days. After treatment, mice were euthanized, and testicular organs were removed. The testes were weighed. The weighed testes were then washed in 0.9% NaCl solution. Thereafter, the testes were prepared for histological examination after Hematoxylin-Eosin (HE) staining. The diameter of the seminiferous tubules was measured using a light microscope at 100x magnification (10x10) in five fields of view. The diameter of the seminiferous tubules was measured using the Image Ruster application. Measurements were made by drawing perpendiculars at the longest and shortest distances connecting the two outermost edges of the seminiferous tubules. Quantitative analysis of Leydig and Sertoli cells was performed using a light microscope with 400x magnification (40x10) in five fields of view.

Statistical analysis

Statistical analysis was performed using IBM statistical product and service solutions (SPSS) version 27.0 by applying analysis of variance (ANOVA). Furthermore, post hoc test was performed using the Least Significant Difference (LSD) at 5% confidence interval. Previously, the data were tested for normality using the Shapiro-Wilk test, and homogeneity of variance was tested using the Levene test.

Results and Discussion

Yield of Uvaria rufa ethanol extract

This extraction process yielded 93.8 grams of dry extract from 525 grams of crude powdered sample, which is equivalent to a percentage yield of 17.86%.

Toxicity of Uvaria rufa ethanol extract

The acute toxicity test showed that exposure of zebra fish embryos to *Uvaria rufa* ethanol extract (URCE) at 1000 - 2000 ppm concentrations resulted in the highest mortality rate in 24 hours, with a significant reduction in mortality rate from 48 to 96 hours (Figure 1). The LD_{50} value obtained was 1.582 ppm. The Fish and Wildlife Service Acute Toxicity Scale was used to classify the toxicity of URCE. This scale was chosen because it provides a reliable framework for assessing the environmental impact of toxic substances on aquatic organisms. Based on this scale, the acute toxicity of URCE is classified as relatively harmless (>1000 ppm).

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

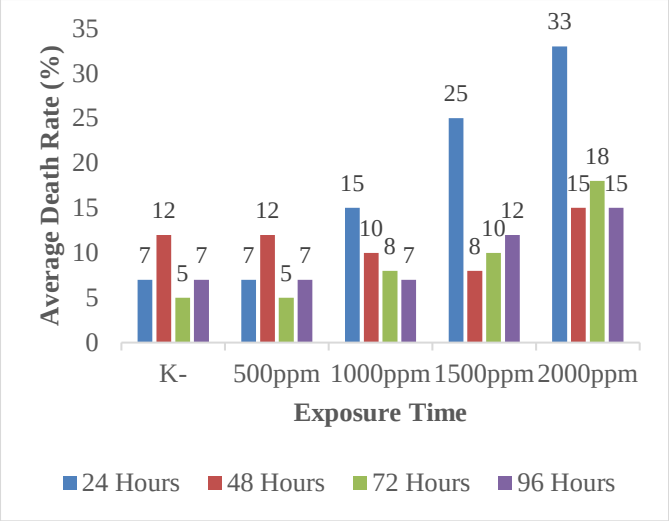

Figure 1: Total zebrafish embryo mortality after 96 hours of exposure to *Uvaria rufa* ethanol extract

Aphrodisiac activity of Uvaria rufa ethanol extract capsule

The aphrodisiac activity was evaluated by observing the weight of the testes, the number of Leydig cells, the number of Sertoli cells, and the diameter of the seminiferous tubules. The increase in testicular weight after the administration of *Uvaria rufa* ethanol extract capsule (URCC) was thought to be caused by anatomical modifications of the testicles (seminiferous tubule hypertrophy), thickening of seminiferous tubule epithelial cells, decreased degeneration of spermatogenic cells and Leydig cells, and significant development of the epididymal tissue structure with a large sperm load. The increase in the weight of the testicular organ is also caused by increased androgen hormones which play a role in the development, growth, and normal function of the testicles and male sex organs.[16] The average testicular weights following treatment with URCC are presented in Figure 2.

Post-hoc analysis using the Least Significant Difference (LSD) test showed that the testicular weight after administration of PC and the URCC treatment group (A1, A2, and A3) showed no significant difference compared to the negative control ($p > 0.05$). The highest testicular weight was observed in the PC and A3 groups (Figure 2). Observation of the number of Leydig cells is required to determine the normality of androgen synthesis, because Leydig cells are the main site of androgen synthesis in mammals.[13]

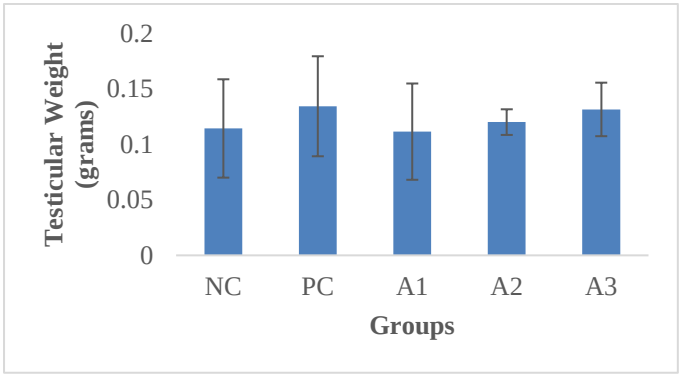

Figure 2: Effect of *Uvaria rufa* ethanol extract on testicular weight

Sertoli cells are cells that are stimulated by androgens through the expression of androgen binding protein (ABP), so that an increase in the number of sertoli cells correlates with an increase in ABP, where ABP binds free testosterone in the blood and as a transporter of androgen, it help maintain normal androgen level.[17] The average number of Leydig and Sertoli cells following treatment with URCC are presented in Figures 3 and 4, respectively.

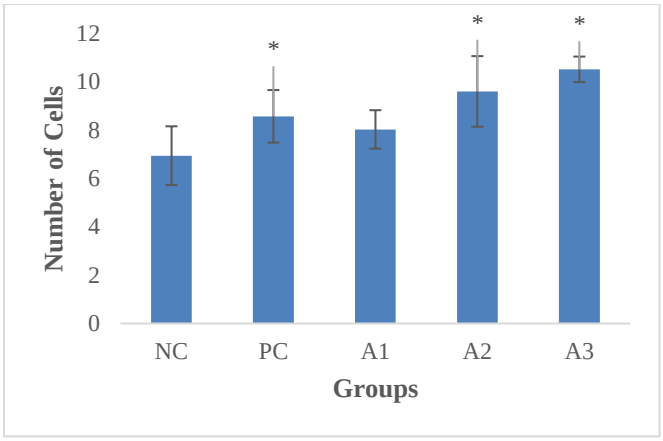

Figure 3: Effect of *Uvaria rufa* ethanol extract on Leydig cells. *Significant difference compared to negative control, $p < 0.05$.

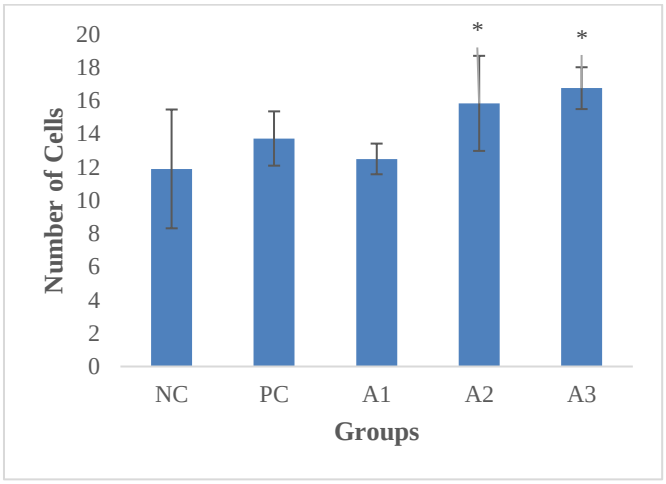

Figure 4: Effect of *Uvaria rufa* ethanol extract on Sertoli cells. *Significant difference compared to negative control, $p < 0.05$.

Post-hoc analysis using the Least Significant Difference (LSD) test showed that the number of Leydig and Sertoli cells after administration of PC and URCC (groups A2 and A3) was significantly increased compared to the NC group ($p < 0.05$), with the highest number of Leydig and Sertoli cells observed in A3 group.

Figure 5 shows the average diameter of the seminiferous tubules following the administration of URCC and PC. It was observed that the administration of URCC at 2.69 mg/20 g BW (A2) and 5.37 mg/20 g BW (A3), as well as the positive control (sildenafil 0.13 mg.20 g BW) resulted in increased diameter of the seminiferous tubules. This increase was significant compared to the negative control ($p < 0.05$). The observation of the diameter of the seminiferous tubules aims to determine if there are spermatogenesis disorders. The process of spermatogenesis is influenced by hormones, so that indirectly the presence of spermatogenesis disorders indicates sexual dysfunction.[18]

One of the risk factors for sexual dysfunction is age. As from age 30, testosterone production by Leydig cells decreased by 1% each year.[17] At age 80 years, testosterone level is expected to have decreased by 50%. Another study stated that the number of Leydig cells in the testes of elderly men (>50 years) decreased by 44% compared to young men with serum luteinizing hormone (LH) levels almost twice as high. At older age, the Leydig cells are less responsive, and there is decline in testosterone synthesis. In addition, cholesterol transport into the mitochondria of the testes is increasingly disrupted in older men. Testicular volume also decreases by 30% in men over 55 years.[19]

Aphrodisiacs are substances or drugs that stimulate sexual arousal or libido.[20] Herbal plants are known to affect the action of the hypothalamic-pituitary-testicular axis and increase libido.[14] The cortex of *U. rufa*, an ethnomedicinal plant used by the people of NTT to treat

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

sexual dysfunction in men, contains compounds with aphrodisiac activity, including flavonoids and alkaloids.[21] Based on preliminary study, it is possible that the major compounds with aphrodisiac activity are the flavonoid 3',7-Dimethoxy-3-hydroxyflavone and alkaloid 8-oxocoptisine (Figure 6A and 6B**)**.

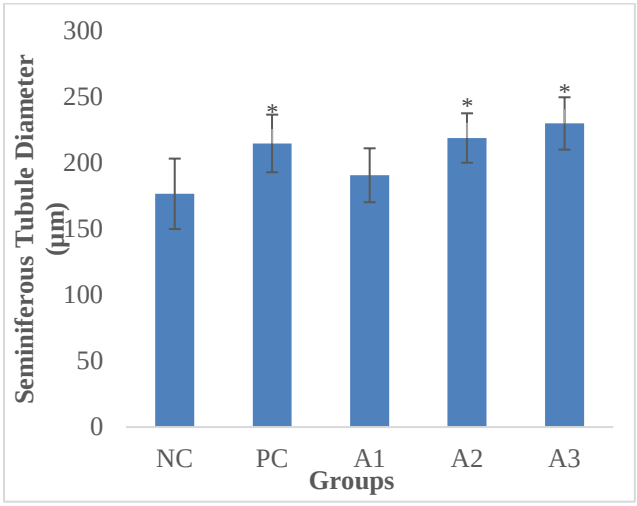

Figure 5: Effect of *Uvaria rufa* ethanol extract on seminiferous tubule diameter. *Significant difference compared to negative control, $p < 0.05$.

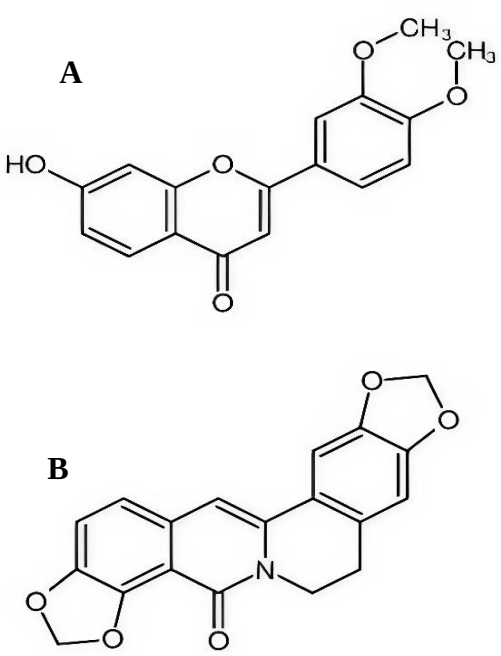

Figure 6: Molecular structures of possible aphrodisiac compounds in *Uvaria rufa* ethanol extract. **(A):** 3',7-Dimethoxy-3-hydroxyflavone, **(B)** 8-Oxocoptisine

Flavonoids and alkaloids have been shown to possess antiapoptotic activity, and thus inhibit the death of Leydig cells and Sertoli cells through the hypothalamic-pituitary-testicular mechanism. Normal levels of plasma gonadotropins (LH and FSH) in the testicles cause dynamic performance of Leydig cells as a site for testosterone synthesis and also Sertoli cells that play a role in the spermatogenesis process. Sertoli cells release hormonal factors and nutrients for the development of germ cells.[22]

Conclusion

The present study has shown that URCE is relatively safe as it was non-toxic to zebrafish with an LD_{50} value of 1.582 µg/mL. Findings from this study also showed that URCC has aphrodisiac activity, with the 5.37 mg/20 g BW of mice/day (A3) being the optimal dose as it significantly increased testicular weight, the number of Leydig and Sertoli cells, and seminiferous tubule diameter. Therefore, these findings offer valuable opportunities to expand the understanding of toxicology and pharmacology of herbal formulations while ensuring the safe and effective use of natural products.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

The authors would like to express their gratitude and highest appreciation to the Ministry of Higher Education, Science and Technology, Republic of Indonesia, for the research funding provided through the Bantuan Biaya Luaran Prototipe 2024 (Contract No. 136/E5/PG.02.00/PROTOTIPE/2024).

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