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Original Article

The Effect Ethanol Extract of Phyllanthus niruri l on Malondialdehyde (MDA) Expression and Extracellular Signal-Regulated Protein Kinase-1 (ERK-1) on Vaginal Epithelial Cell Thickness in Menopausal Mice

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ABSTRACT

Introduction: Menopause is the condition of the ovaries stops produce estrogen so that resulting in vaginal bleeding atrophy that is become dry cause pain moment relate sexual so that lower quality life. The ROS pathway with MAPK regulates proliferation, differentiation, motility, and survival cell life. Research purposes is know influence giving extract Phyllanthus niruri l against Malondialdehyde (MDA) expression and Extracellular Signal-Regulated Protein Kinase-1 (ERK-1) expression against thickness cell vaginal epithelium of menopausal model mice. Material from Phillantus niruri l processed become extract. Treatment animal try mice (mus muscullus) first acclimatized during one next week done ovariectomy of both ovaries, after two weeks checked vaginal examination to be sure phase diestrus (menopause). Stage treatment given extract for 21 days with dose different 14 mg, 28 mg and 56 mg/20gBW/ day. **Methods:** True Experimental research method with Post Test only with control group design. Data analysis used one way ANOVA. **Results:** The research group that produced the highest average expression of Malondialdehyde (MDA) was the control group. The highest expression of Extracellular Signal-Regulated Protein Kinase-1 (ERK-1) was in the P3 treatment group (dose 56 mg/20gBW/day). The results of statistical analysis showed that there was a significant effect of Phyllanthus niruri l extract on decreasing MDA expression with a sig value of 0.000 < p = 21 0.001 and increasing ERK-1 with a sig value of 0.000 < 0.001, but there was no effect on increasing the thickness of the vaginal wall epithelial cells in menopausal model mice. with a sig value of 0.220 > 0.05. **Conclusion:** The three doses of phillantus niruri decreased MDA and increased ERK-1. The Folin-Ciocalteau.

Keywords: Phyllanthus niruri l, MDA, ERK-1, Vaginal epithelium, Menopausal Mice.

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INTRODUCTION

Menopause is a stage of bodily changes experienced by women, both physically and psychologically, resulting from the cessation of reproductive hormone production by the ovaries [1,2]. This phase is preceded by a transition period known as perimenopause, characterized by a gradual loss of oocytes, changes in response to gonadal steroid feedback, significant hormonal fluctuations, and irregular menstrual patterns [1,3]. Women experiencing menopause often complain of vaginal dryness (atrophy), which can lead to pain during sexual intercourse. Vaginal atrophy involves the thinning of the vaginal epithelium and decreased cervical secretion, closely associated with reduced estrogen levels [4]. These complaints can significantly affect women's sexual quality of life [5,6].

This study utilized experimental animals that underwent ovariectomy (OVX), a process involving sterilization of female animals by surgically removing the ovaries (ovariectomy). OVX is employed to create a model of menopause. Studies have shown a decrease in estradiol levels in the blood of ovariectomized rats [7]. Bilateral ovariectomy eliminates testosterone secretion by the ovaries, leading to a state resembling menopause. Ovariectomy increases the expression of Fas, a marker of apoptosis protein in the endometrium of Rattus norvegicus [8]. The surgical removal of ovaries has become a valuable tool for understanding estrogen deficiency through experiments on animals. Animal models that mimic menopausal phases are crucial for better understanding the biological processes involved during this life stage [9,10]. Numerous physiological, metabolic, and immunological changes occur in the body during menopause. After menopause or post-ovariectomy, the ovaries cease estrogen production, resulting in decreased estrogen levels [10]. Women in menopause experience clinical symptoms ranging from vaginal dryness (10%), vaginal discharge (6%), to dyspareunia (20%). From our clinical observations, we found that 44% of women suffer from atrophic vaginitis based on vaginal secretions, 42% based on macroscopic surface/integrity of vaginal epithelium, 54% based on rugae and vaginal elasticity, and 42% based on vaginal color. However, 80% of all participants exhibited a vaginal pH greater than 7, most of whom were categorized as suffering from atrophic vaginitis [11].

Atrophy is associated with MDA and ERK-1, which are pathways of oxidative stress, characterized by an imbalance between free radicals and antioxidants

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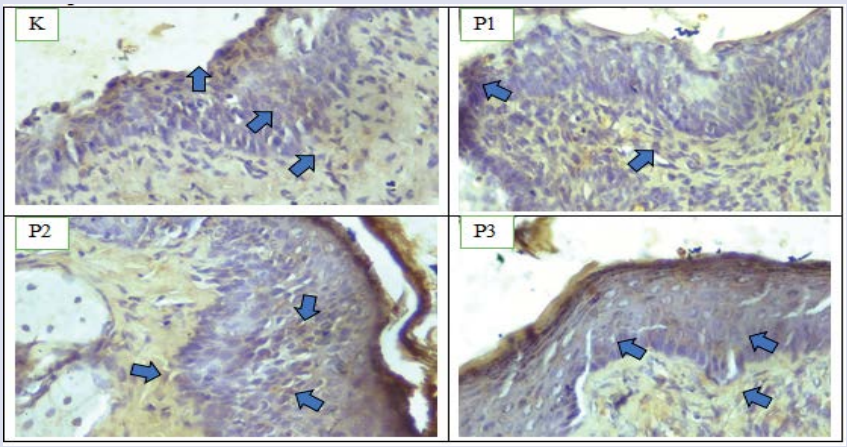

Figure 1. Expression of MDA in Epithelium and Lamina Propria in each Control and Treatment group with ethanol extract of Phyllanthus niruri l administration in a mouse model of menopause.

Primary Data: Comparison of MDA Expression in Epithelium and Lamina Propria of each treatment group. Red arrows indicate the expression of MDA in vaginal tissue, marked by brown chromogen color (arrow sign). IHC. 400x

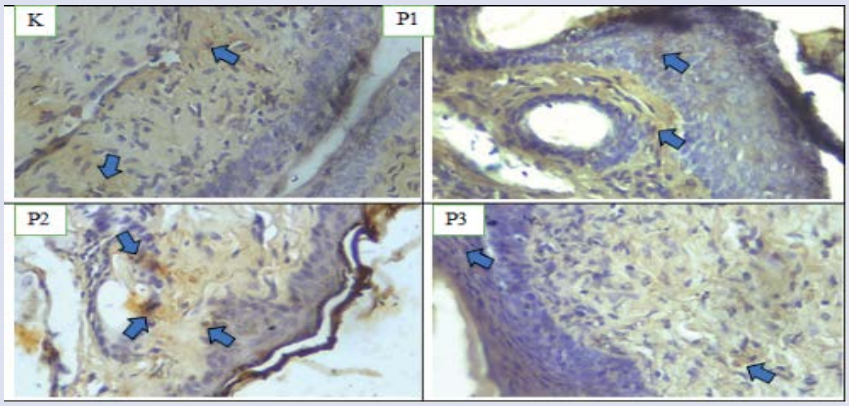

Figure 2. Expression of ERK-1 in Epithelium and Lamina Propria in each Control and Treatment group with administration of ethanol extract of Phyllanthus niruri l in a mouse model of menopause.

Primary Data: Comparison of ERK-1 Expression in Epithelium and Lamina Propria of each treatment group. Red arrows indicate ERK-1 expression in vaginal tissue, marked by brown chromogen (arrow sign). IHC. 400x

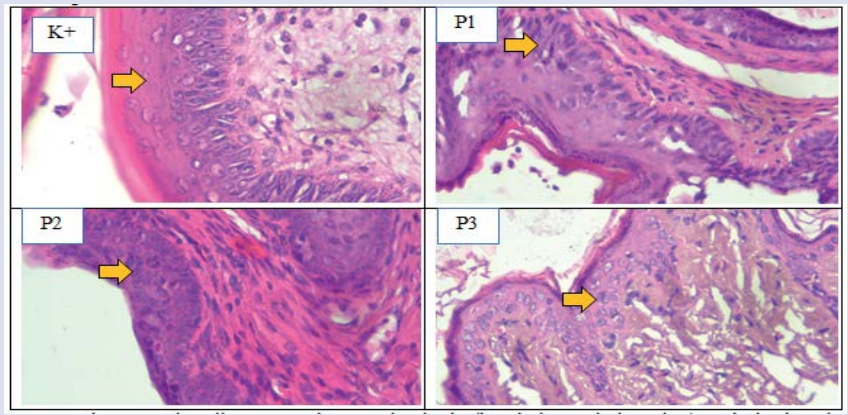

Figure 3. Histological depiction of vaginal epithelial thickness in each Control and Treatment group administered with extract of Phyllanthus niruri l. in a mouse model of menopause.

Primary Data: Comparison of Histological Images (vaginal epithelial thickness) in various groups (K+, P.1, P.2, P.3). H.E.. 400x

Graph 1. Expression of MDA and ERK in each Control and Treatment group administered with ethanol extract of Phyllanthus niruri l in a menopause mouse model.

Primary Data: Graph Description of MDA and ERK-1 Variables

Graph 2. Thickness of Vaginal Epithelium in each Control and Treatment group administered with ethanol extract of Phyllanthus niruri l in a menopause mouse model.

Primary Data: Graph Description of Vaginal Epithelium Thickness Variable

in the body. This imbalance can result from excessive production of reactive oxygen species (ROS) or reactive nitrogen species (RNS), which can damage biomolecules such as lipids, proteins, and nucleic acids. Malondialdehyde (MDA) serves as a stable and accurate biomarker of oxidative stress, reflecting the oxidation processes within cell membranes [12].

Atrophy is associated with MDA and ERK-1, which are pathways of oxidative stress, characterized by an imbalance between free radicals and antioxidants in the body. This imbalance can result from excessive production of reactive oxygen species (ROS) or reactive nitrogen species (RNS), which can damage biomolecules such as lipids, proteins, and nucleic acids. Malondialdehyde (MDA) serves as a stable and accurate biomarker of oxidative stress, reflecting the oxidation processes within cell membranes [12].

METHODS

Design, subjects, and research variables

This study employed a True Experimental design with a Post Test Only with Control Group design using female mice (Mus musculus) as the research subjects. The mice were aged 5-6 months and weighed between 20-40 grams, selected based on their healthy condition characterized by smooth, glossy fur and absence of defects. Immunohistochemical examinations were conducted by a pathology expert at the Faculty of Veterinary Medicine, Airlangga University.

The experimental procedure involved the following steps, Animal Preparation (Acclimatization): Mice were individually placed in cages. During acclimatization, attention was given to cleanliness, nutrition, and overall health. Specific conditions such as humidity, light, and temperature were controlled to ensure homogeneity and consistency. Mice had ad libitum access to pellet food and water. Cage cleanliness and comfort were maintained throughout the research process. After one week of acclimatization, mice underwent ovariectomy by surgical removal of both ovaries. Prior to ovariectomy, vaginal smears were taken to ensure the mice were not pregnant [13.] Post-ovariectomy, mice were administered antibiotics at a dose of 60 mg/kg body weight per day for three days to prevent infection and facilitate recovery. Mice were kept in separate cages during the wound healing process for up to two weeks. One week post-ovariectomy, vaginal cytology was performed to confirm entry into the diestrus phase [14]. The sample size calculation determined that a minimum of 8 mice per group was required. An additional 20% was added to account for any potential failures or deaths of mice within each group. The research protocol was approved by the ethics committee of the Faculty of Veterinary Medicine, Airlangga University, with approval number No: 2.KEH.104.06.2023.

The Extract of Phyllanthus niruri l

The ethanol extract of Phyllanthus niruri L was prepared at the UPT Lab Herbal Materia Medica in Batu, East Java. The extraction process of Phyllanthus niruri l (stonebreaker) was conducted using the maceration method with 96% ethanol solvent. First, Phyllanthus niruri L was ground into a powder form, which was then placed into an Erlenmeyer flask. Next, 96% ethanol solvent was added, and the mixture was stirred for 2-3 hours. Subsequently, the flask was sealed with aluminum foil and allowed to soak. The extraction process continued by maceration

for 24 hours. Afterward, the mixture was filtered to obtain the filtrate, which resulted in a concentrated extract free from solvents.

Methods for MDA Expression Analysis

The histopathological examination aimed to determine MDA expression in the uterus. Each sample's data was assessed semi-quantitatively using a modified Remmele method [15]. The Remmele scoring index (Immuno Reactive Score/IRS) was calculated by multiplying the percentage score of immunoreactive cells with the intensity score of color in these cells (Table 1). Each sample's data represented the average IRS observed across ten different Fields Of View (FOVs) under 100x and 400x magnifications.

Assessment of MDA expression was conducted using immunohistochemistry cell density scoring. MDA, a metabolite of reactive oxygen species (ROS), appears brown when positive, indicating oxidative stress. Understanding oxidative stress mechanisms and measuring biomarkers like MDA provide crucial insights into its role in various pathological conditions, paving the way for the development of more effective prevention and treatment strategies [12].

Examination of ERK-1 Expression

The histopathological examination aimed to determine ERK-1 expression in the vagina. Each sample's data was assessed semi-quantitatively using a modified Remmele method [15]. The Remmele scoring index (Immuno Reactive Score/IRS) was calculated by multiplying the percentage score of immunoreactive cells with the intensity score of color in these cells (Table 1). Each sample's data represented the average IRS observed across ten different Fields Of View (FOVs) under 100x and 400x magnifications.

Evaluation of ERK expression experiments using inhibitors confirms that ERK activity is necessary for various pathological responses, including epithelial repair after injury, inflammation, and formation of cancer metastatic niches [16]. In conclusion, biosensors for ERK will be powerful and valuable tools for investigating the role of ERK in the field.

Examination of Vaginal Epithelial Thickness

The histopathological examination aimed to determine the thickness of the vaginal epithelium. Epithelial thickness measurements were

Table 1. Expression of MDA and ERK in each Control and Treatment group administered with ethanol extract of Phyllanthus niruri l in a menopause mouse model

Variable	Treatment	N	Mean	Std. Deviation	Minimum	Maximum
Malondialdehyde Expression (MDA)	K	8	6.0250	1.06066	4.60	7.80
	P1	8	4.0625	1.43819	1.90	6.00
	P2	8	3.8625	0.65174	2.20	6.00
	P3	8	3.1143	0.65174	2.30	4.20
	Total	32	4.3032	1.56429	1.90	7.80
Extracellular Signal-Regulated Protein Kinase-1 Expression (ERK-1)	K	8	2.0250	0.72457	1.10	3.00
	P1	8	4.0286	1.26322	2.20	6.20
	P2	8	3.9375	1.37107	2.20	6.00
	P3	8	4.2375	1.64311	2.60	7.00
	Total	31	3.5419	1.53292	1.10	7.00

Primary Data: Analysis of MDA and ERK-1 expression description

Table 2. Results of normality testing and data transformation on the variables MDA, ERK-1, and Vaginal Wall Thickness in the menopausal mouse model treated with *Phyllanthus niruri* l. Extract

Variable	Shapiro-Wilk			Description
	Statistic	df	Sig.	
Malondialdehide (MDA)	0.939	32	0.079	Normal
Extracellular Signal-Regulated Protein Kinase-1 (ERK-1)	0.962	32	0.322	Normal

Table 3. Results of Homogeneity Test on MDA, ERK-1, and Vaginal Epithelial Thickness Data in Menopausal Mice given *Phyllanthus niruri* l. Extract

Variable	Levene Statistic	Sig.	Description
Malondialdehide (MDA)	1.810	0.169	Homogeneous
Extracellular Signal-Regulated Protein Kinase-1 (ERK-1)	1.429	0.256	Homogeneous
Vaginal epithelial cell thickness	0.272	0.845	Homogeneous

Primary data: Homogeneity test of MDA, ERK-1, and Vaginal Epithelial Thickness variable

Table 4. Results of *One Way* ANOVA Analysis on the variables MDA, ERK-1, and Vaginal Epithelial Thickness in the mouse model of menopause treated with ethanol extract of Phyllanthus niruri l.

Variable	Statistic	Sig.	Description
Malondialdehide (MDA)	8.487	0.000	Significant
Protein Kinase-1 (ERK-1)	5.004	0.007	Significant
Vaginal epithelial cell thickness	1.568	0.220	Not Significant

Primary data: One Way ANOVA Analysis on the variables MDA, ERK-1, and Vaginal Epithelial Thickness

Table 5. Tukey's Test Results for the variables MDA, ERK-1, and Vaginal Epithelial Thickness in the mouse model of menopause treated with ethanol extract of Phyllanthus niruri l.

Variable	Treatment	N	Subset for alpha = 0.05	
			1	2
MDA	P1	8	3.1143	
	P2	8	3.8625	
	P3	8	4.0625	
	K	8		6.0250
	Sig		0.409	1.000
ERK-1	K	8	2.0250	
	P2	8		3.9375
	P1	8		4.0286
	P3	8		4.2375
	Sig		1.000	0.968

Primary data: Tukey's Test for the variables MDA, ERK-1

conducted using calibrated Image Raster 3 software. Measurements were taken in ten fields of view at 400x magnification, with ten measurements drawn per field of view. The thickness of the vaginal epithelial cells has implications for menopausal women, potentially reducing menopausal symptoms and complaints. Vaginal thickness was examined using Hematoxylin and Eosin (H&E) staining[17].

Data analysis

Statistical analysis was performed using One-Way ANOVA. First, the normality of the data distribution was assessed using the Shapiro-Wilk test. If the data did not follow a normal distribution, logarithmic transformation was applied. Subsequently, homogeneity of variance was tested to determine if the data were homogenous across groups using Levene's test based on the mean. The criteria for testing stated that if the significance value was > 0.05, the data were considered homogenous. Data were tested using the Shapiro-Wilk test to assess normality because the sample size was < 50. Data were presented with ± standard deviation. One-Way ANOVA was used to compare parameters between control and treatment groups under the condition of normal data distribution [18].

RESULTS

Characteristics of Subjects

Results of One Way ANOVA with the significance value less than alpha (5% or 0.05):

1. The statistical analysis of Malondialdehyde (MDA) variable yielded a significance value of 0.000, indicating a significant effect of ethanol extract of Phyllanthus niruri L on reducing Malondialdehyde (MDA) expression. Tukey's test results indicate that group P1 tends to have the highest reduction in Malondialdehyde (MDA) compared to the other groups. However, its effectiveness is similar to groups P2 and P3. Group P3 tends to have the highest increase in expression.
2. The statistical analysis of Extracellular Signal-Regulated Protein Kinase-1 (ERK-1) variable yielded a significance value of 0.000, indicating a significant effect of ethanol extract of Phyllanthus niruri L on increasing Extracellular Signal-Regulated Protein Kinase-1 (ERK-1) expression. Tukey's test results indicate that group P3 tends to have the highest increase in ERK-1 expression compared to the other groups, although its effectiveness is similar to groups P1 and P2.
3. The statistical analysis of vaginal epithelial cell thickness variable yielded a significance value of 0.220, indicating that there is no significant effect of ethanol extract of Phyllanthus niruri L on increasing vaginal epithelial cell thickness.

DISCUSSION

This study explores oxidative stress pathways related to vaginal atrophy experienced in menopausal women. The study demonstrates that Phyllanthus niruri L. extract acts as an antioxidant, reducing Malondialdehyde (MDA) expression and increasing Extracellular Signal-Regulated Protein Kinase-1 (ERK-1) expression in menopause model mice (Mus musculus). Active compounds such as saponins, phenols, flavonoids, and tannins were identified through phytochemical screening. Quercetin, a flavonoid, has significant potential as a natural antioxidant source [19]. Antioxidants play a crucial role in preventing and repairing cell damage caused by free radical exposure within the body. Phytochemicals from plant extracts, particularly polyphenols, act as antioxidants in the body. Water extracts of P. niruri leaves have shown antioxidant activity in vitro [20]. Flavonoids protect against hypoxia, which induces changes at all levels of red blood cell organization, affecting the functional properties of hemoglobin oxygen transport and eventually leading to total red blood cell degradation [21].

The thickness of epithelial cells in the vagina did not show significant thickening. This is due to the physiological decrease in estrogen during menopause, leading to thinning or atrophy. Vaginal atrophy is a common symptom of postmenopausal estrogen deficiency and can manifest as dryness, irritation, bleeding, infections, dyspareunia, and may affect sexual function and quality of life [22,23].

Dryness in the vagina during menopause can also be caused by fluid deficiency or dehydration. Elderly women are at higher risk of dehydration because they may replenish fluids more slowly. Estrogen therapy increases osmotic sensitivity to mechanisms that maintain body water, helping menopausal women regulate body fluids and avoid dehydration [24].

The research results can be summarized as follows: extract of Phyllanthus niruri l. had the highest effect in reducing MDA and increasing ERK-1 expression, particularly in treatment group P-1 (14 mg/20gBW/day for 21 days). Phyllanthus niruri extract has been proven to decrease MDA expression and increase ERK-1 expression. Although not significant in thickening vaginal epithelial cells during menopause, it has been shown to act as an antioxidant essential for the body. Other studies indicate that Phyllanthus niruri L. can increase endometrial thickness in cases of endometriosis [25]. Additionally, research mentions that Phyllanthus niruri L. has potential hepatoprotective activity, improving liver conditions by protecting liver tissue from oxidative damage and stimulating repair mechanisms in the liver [26]. From these findings, it can be concluded that Phyllanthus niruri provides significant impacts on tissues that have not undergone atrophy as a natural antioxidant [25].

CONCLUSION

Phyllanthus niruri L. extract at doses of 14 mg, 28 mg, and 56 mg/20gBW/day for 21 days can reduce MDA expression and increase ERK-1 as antioxidants to reduce oxidative stress. However, it does not increase the thickness of the vaginal epithelial cells.

CONFLICTS OF INTEREST

There are no conflicts of interest in this research.

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