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The Effects of Water Clover (*Marsilea Crenata*) Extract against Estrogen, Progesterone And Uterine Histology on Rat (*Rattus Norvegicus*)

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Abstract : Isoflavones in Water clover (Marsilea crenata) shown to mimic the role of the female hormone estrogen. It is expected to replace the function of the synthetic estrogen hormone. However, it still needs further research to determine the most effective dose. The purpose of this study was to determine the effects of water clover extract to estrogen and progesterone levels and how it can affect the histology of uterine. This study used 30 female Wistar Rats (Ratus norvegicus) divided into six groups. Group I (P1) as a negative control group, group II (P2) as a positive control group. Group III, Group IV, Group V and Group VI (P3, P4, P5, P6) were given extract water clover concentrations are given as follow (6.25%, 12.5%, 25%, 50%). The results were analyzed with one-way ANOVA showed not significant ($p > 0.05$) between groups, even though group P3, P4, P5 and P6 produce estrogen that is higher than the negative control (P1) and a positive control (P2). The P1 group produces the highest progesterone level but after the ANOVA test showed no significant different ($p>0,05$) between-group. Histological features of the uterus in the P2 group showed endometrial lining thicker than the P1 group. Histology of the uterus in the treatment group P3, P4, P5, and P6 have also seen endometrium thickening. This research has proved that water clover extract consumption showed a promising replacement estrogen hormone therapy in the future. However, there was needed a much further research in order to found a proper dose for human consumption.
Keywords : Water Clover (Marsilia crenata), Estrogen, Progesterone, Uterine Histology.

Introduction

Reproduction is an essential factor in life. Reproduction in mammals closely related to the estrous cycle. Regulation of the estrous cycle involves reciprocal interaction between reproductive hormones from hypothalamus, the anterior pituitary, and ovary. A steroid hormone involved in the estrous cycle produced by the ovaries are estrogen and progesterone[1]. This hormone can also induce endometrial histological changes that followed the ovarian cycle per month[2].

Menopause, ovariectomy, and reproductive system disorders (amenorrhea) can affect target cells resulting in decreased levels of estrogen in the body. Declining level of estrogen can cause interference with the urogenital system, cardiovascular disease, and osteoporosis[3]. In women, hormone replacement therapy is often conducted. Hormone replacement therapy, among others, can be done with a synthetic homone estrogen. However, the granting of these hormone in the long term have a variety of side effects, such as breast pain, vaginal bleeding, and trigger breast cancer[4] .

Marsilea crenata or water clover has a round shape of leaves and consists of four strands small leaves. This plant is a plant group of salviniales, live wild in aquatic environments such as ponds, paddy fields, lakes, and marshes[5]. Freshwater clover plant content phytochemical such as sugar, steroid, carbohydrate, and flavonoids. Flavonoids also have a function as an antibacterial, anti-inflammatory, antitumor, allergenic, and prevent osteoporosis[6].

The main content of the water clover isoflavones is a genistein and daidzein. Water clover contains more genistein than daidzein. Isoflavones are phytoestrogens that are part of this has important functions in the defense mechanisms of plants. Isoflavones are an active substance that contains estrogen hormone from plant material[7].

Water clover has shown to mimic the role of the female hormone estrogen. The Estrogenic feature is contained in the active substances of flavonoid compound, when administered in high doses it can lead to an increase levels of estrogen in the blood[8]. The estrogenic activity of isoflavones has been linked with a chemical structure similar to stilbesterol[9], which is used as an isoflavones estrogenic drug that can activated estrogen receptors in mammals, so it is often called isoflavone phytoestrogens[10].

In humans isoflavone compounds are widely used as medical action in postmenopausal women without any side effects. If the body consume isoflavones, there will be an effect of binding isoflavones to estrogen receptors that produce beneficial effects, thereby reducing the symptoms of menopause, water clover will reduce the clinical symptoms that appear before and while entering the stage of menopause and to improve the quality of bone to avoid osteoporosis[7].

Isoflavones that are contained in water clover is expected to replace the function of the synthetic estrogen hormone. But it still needs further research to determine the most effective dose. The purpose of this study was to determine the effects of water clover extract to estrogen and progesterone levels and how it can affect the histology of uterine.

Materials and Methods

Materials

Water clover (Marsilea crenata) purchased from the local market in Surabaya, East Java, Indonesia. Rats supplied by the laboratory of molecular biology, faculty of mathematics and natural sciences, University of Brawijaya. Phosphate buffered saline (PBS), Mycroginon (synthetic estrogen), ELISA Kit estradiol, Sephadex Gel for separated of the isoflavone derivatives, histological coloring agent for soft tissue, hormones PGF2α as estrus synchronization experimental animals.

Preparation Marsilea crenata

Water clover (Marsilea crenata) were analyzed by an independent institution with a certificate of determination by Materia Medica. Water clover is dried by the sun, dried using sunlight to avoid damage to the composition. Then mashed into simplicia use dish meal, in order to obtain water clover leaf meal (WCLM) amount of 1 kg. A stock solution of WCLM (100%) the extraction of N Hexane of 1 kg of crude drugs. Stock dilution solution (100%) into a solution treatment (P3, P4, P5, P6) WCLM solution with a concentration of 6.25%, 12.5%, 25%, 50%.

Animal experiments

This study used 30 female Wistar Rats (Ratus norvegicus) 2 months old, 100-150 grams, divided into six groups each group consisting of 5 rats. Group I (P1) as a control group, rats given Phosphate buffered saline (PBS) orally. Group II (P2) as a positive control group, rats given Mycrogynon orally. Group III, Group IV, Group V and Group VI (P3, P4, P5, P6) is the treatment group was given water clover with different concentrations which are 6.25%, 12.5%, 25%, 50%. All groups were injected PGF2 α for estrus synchronization before experimental. The treatment group, water clover leaf extract according to the concentration is given orally 2 mL per rats, every day for 21 days. the last period of research, the animals were sacrificed in compliance with Standard Research Ethics Guidelines. Necropsy performed to take a sample of uterine organs

and blood samples. The whole animal has been in compliance with the guidelines declared by institutional animal care and application committee.

Data analysis

Blood estrogen and progesterone hormone profile were observed quantitatively by using SPSS 16.0 for Windows with statistical analysis one way ANOVA.If there is a significant difference ($p<0.05$) then followed by Tukey test with $\alpha = 0.05$. Results of histological preparations uterine were observed using a microscope with a comparison between control and treatment groups.

Results

Hormone estrogen level

Levels of the hormone estrogen on group I (P1) as negative control group, mean levels of estrogen was the lowest among the five groups other treatments (231.67 ± 34.06 pg/mL), Group II (P2) showed the average levels of the hormone estrogen amounted to 396.00±76.71 pg/mL. The P2 group is a positive control group was given microgynon orally. Estrogen levels in the treatment group given water clover that are Group III, Group IV, Group V, and Group VI (P3, P4, P5 and P6) sequentially which is 468.67 ± 34.18 pg/mL, 441.67 ± 108.26 pg/mL, 519.33 ± 93.41 pg/mL, 494.33 ± 47.16 pg/mL. Table 1. showed that the administration of water clover extract with various doses produces estrogen that was higher than the negative control (P1) and a positive control (P2). The test results in estrogen levels then performed statistical analysis using one way ANOVA and obtain measurement results were not significant ($p>0.05$) between the treatment groups.

Table 1. The result of hormone estrogen level (pg/mL)

Group	Estrogen (pg/mL) Mean±SD
P1 (Control)	231.67[a] ± 34.06
P2 (microgynon)	396.00[a] ± 76.71
P3 (6.25% extract)	468.67[a] ± 34.18
P4(12.5% extract)	441.67[a] ± 108.26
P5(25% extract)	519.33[a] ± 93.41
P6 (50% extract)	494.33[a] ± 47.16

Hormone progesterone level

Table 2. showed progesterone level in each groups. Differ with estrogen level, in progesterone the higher mean of progesterone level was group I (P1) as negative control group(33.30 ± 4.24 pg/mL) among the five other treatments groups. The lowest level of progesteron was group P3 (16.21±6.08 pg/mL). Group P2 as a positive control showed progesteron level (19.95 ± 3.47 pg/mL) that was not much differ with all treatment group (P3, P4, P5, P6). Progesterone levels in the Group IV, Group V, and Group VI (P4, P5 and P6) sequentially which is 19.84 ± 9.21 pg/mL, 18.75 ± 6.08 pg/mL, 20.84 ± 3.49 pg/mL. The test results in progesterone levels then performed statistical analysis using one way ANOVA and obtain measurement results were not significant ($p>0.05$) between groups.

Table 2. The result of hormone Progesterone level (pg/mL)

Group	Progesterone (pg/mL) Mean±SD
P1 (Control)	33.30[b] ± 4.24
P2 (microgynon)	19.95[a] ± 3.47
P3 (6.25% extract)	16.21[a] ± 6.08
P4 (12.5% extract)	19.84[a] ± 9.21
P5 (25% extract)	18.75[a] ± 6.08
P6 (50% extract)	20.84[a] ± 3.49

Uterine histology

Histology of the uterus in the negative control group showed endometrial lining inactive and compact containing blood vessels and connective tissue cells such as fibroblasts, macrophages and mast cells (Figure 1.A). Histological features of the uterus in the positive control group showed endometrial lining thicker than the negative control group. Histological features of treatment group showed superficial layers form at the functional zones consisting of loose connective tissue. During estrus would appear a large cavities were irregular were lack of fluid between cells in the functional zone called endometrial edema (Figure 1.B). Histology of the uterus in the treatment group P4, and P5 have also seen endometrium thickening as in group P3.

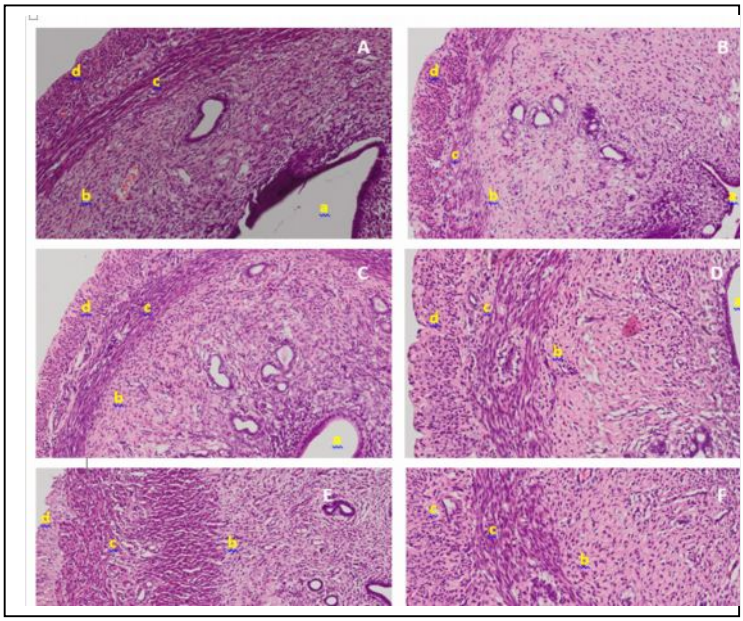

Figure 1. Photomicrograph uterine of rat (A) normal-control negative group; (B) microgynone-control positive group; (C) 6,25% extract-treatment group; (D) 12,5% extract-treatment group; (E) 25% extract-treatment group; (F) 50% extract-treatment group. (a) uterine lumen, (b) uterine glands, (c) longitudinal muscle layers (d) tunica serosa (H & E staining, 400x).

Disscusion

Hormone Estrogen and Progesteron on Rats After Therapy of Water Clover Extract

Estrogen levels in normal rats is between 132-140 pg/mL of blood[11]. In this research, all rats were in estrus phase that has been confirmed by the vaginal swap. During fertile period, the estrogen hormone reached a maximum level then declined rapidly[12]. This may cause a high amount of estrogen levels in the study above normal. When compared with the blood serum levels of a rat model of menopause which showed the average 40,73 pg/mL[13], then the estrogen levels in this study were much higher up to 10 times ranged at 396.00 to 519,33 pg/mL. This means the water clover extract can be a promising estrogen hormone replacement. Other study showed that the clover extract 0,8 g plus 10 mg once daily of vitamin B1 can significantly increase the concentration of estrogen in postmenopausal women and delay the increase in the imbalance of bone remodeling in postmenopausal women[14].

The content of phytoestrogens in water clover plant leaves that has been investigated showed that the estrogen content of the juice of fresh clover was 538,0 pg/g, while the dried clover increased to 1068,0 pg/g[15]. As phytoestrogens, isoflavones in the plant has two important effects. First, when estrogen levels are high, phytoestrogens can stop a more potent form of estrogen produced by the body (by blocking the estrogen receptor) and can help prevent diseases driven by hormones, such as breast cancer. Secondly, when estrogen levels are low, such as in a state after menopause, phytoestrogens can replace the body's own estrogen, which can reduce hot flashes and protect bones[16].

Isoflavones in the water clover shown to emulate the role of the female hormone estrogen[7]. Estrogen binds to estrogen receptors as part of a hormonal activity, causing a series of reactions that benefit the body. Phytoestrogens role in stabilizing the hormonal functions, namely by inhibiting the excessive estrogen activity that can induce cancer and also can substitute when the estrogen levels in the body are low.

The negative control group showed progesterone levels are highest compared to other treatments even though the mice just get distilled water treatment. It is suspected because it has to do with the lowest levels of estrogen produced compared to the other treatment groups. While on the treatment given clover extract water have lower levels of progesterone compared to negative controls. It is suspected due to the possibility of phytoestrogens that mimic estrogen so that progesterone produced is lower.

Progesterone is a steroid hormone involved in the estrous cycle and pregnancy. Progesterone is produced by the corpus luteum after ovulation in the ovaries and in the adrenal glands that is located near the kidneys, as well as in the placenta during pregnancy. Progesterone has a dominant role in regulating the estrous cycle[17].

After ovulation, estrogen levels begin to be replaced by increased levels of progesterone. Increased progesterone levels indicating ovulation has occurred and progesterone levels will peak at midluteal phase of the cycle. Fluctuations in hormone levels of these hormones in response to the operation of pituitary hormones in ovarian organ[18].

If fertilization in the ovum result of ovulation, the progesterone levels maintained until pregnancy occurs. On the contrary, if the ovum is not fertilized the results of ovulation, subsequently the progesterone levels will decrease gradually, and continued to decline until the end of the cycle[19]. Progesterone levels remain low at the beginning of the following cycle until ovulation occurs again[20].

Uterine Histology

Phytoestrogen affected organs include the ovaries, uterus, testis, prostate, and several other organs[21]. Uterus divided into 3 parts, which is the outermost layer (perimetrium), the middle layer (myometrium), and the mucosa lining (endometrium). The mucosal lining of the uterus (endometrium) in the inactive state, especially on the endometrial tissue, merely consists of tubular glands, fibroblastic stroma dense and thin blood vessels normally occur during anestrus or before puberty. After menopause at the age of non-productive animals are found a lining mucosa of the uterus (endometrium) also experienced a state of inactive proved to be a process of proliferation and secretion, thinner form, and often accompanied by a cyst in the abdominal cavity with flattened or cuboidal cells, as well as the presence of fibrotic stroma[22].

The estrus phase was also called follicular phase, will have an enlarged uterus and swollen due to the accumulation of fluid under the influence of estrogen. While progesterone only able to influence the endometrium after endometrial gain influence by estrogen[23]. Progesterone works in the endometrium that has been prepared by estrogen to turn it into a layer that is tolerant and contains many nutrients for the fertilized ovum. Progeterone also ready to prepare the endometrium for embryo implanted and accommodating by stimulating endometrial glands that secrete and store glycogen in large quantities and also in stimulating an increase in vascularization[24].

Uterine glands on histology also look wider, an increase in the cotnent of estrogen in the blood can stimulate the growth of uterine glands (25). The Histological uterus is changed according to reproductive status, because of the influence of reproductive hormones such as progesterone and estrogen. Visible wall thickening of the endometrium or endometrial dilution according to estrus phase (4, 26). In the estrus phase which occurred in the treatment group showed an increase of endometrium and myometrium mass in the form of hyperplasia and hypertrophy Thus increased levels of estrogen in the estrus phase will also affect the weight of the uterus and ovaries. This shows that the water clover can increase the uterine weight that seen from the changes in histology and thibeans ts is also confirmed by examination of blood serum estrogen. Research using soy bean also has a uterothrophic activity of phytoestrogens that was seen an increase in the uterus mass (27).

Estrogen has two types of receptors that are estrogen receptor alpha (ERα) and beta (ERβ). α receptors present in organs ovary, breast, uterus, testis, pituitary, kidneys, epididymis, and adrenal whereas β receptors are found in organ ovary[28]. Phytoestrogen although not a hormone, but because its structure similar to estradiol

may also occupy estrogen receptors and capable of causing the withdrawal effects of endogenous estrogen itself[29]. Phytoestrogens have a chemical structure similar to 17βestradiol, that can bind to the estrogen receptor that is ERα and ERβ. Phytoestrogens binding affinity to both receptors is not the same, phytoestrogens greater affinity for ERβ compared to Erα[21].

In the ovary, estrogen will occupy estrogen receptors α and β, while in the uterus will occupy the estrogen receptor α so that the ovaries and uterus will occur a proliferation. This proliferation would lead to organ becomes heavier weights. The hormone estrogen causes development and the maintain secondary sex signs in women, such as breast, and are also involved in the thickening of the endometrium as well as in regulating the menstrual cycle[30]. Estrogen causes a significant change in endometrial glands and consequently the size of the uterus increase two to three times more than before puberty[31].

Conclusion

This research has proved that water clover extract consumption shows a promising replacement estrogen hormone therapy in the future. However, there was needed a much further research in order to found a proper dose for human consumption.

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Reference

1. Khanum SA, M Hussain, R Kausar. 2008. Progesterone and estradiol profiles during estrous cycle and gestation in dwarf goats (Capra hircus). Pakistan Vet J, 28:1-4.
2. Junqueira, L.C. and J Carneiro . 2007. Basic Histology: Text and Atlas Edisi 10. EGC. Jakarta
3. Lindberg, Z. Weihva, Andersson, Moverare. 2002. Endocrinology Disorder. Journal of Applied Sciences 174:167-178.
4. Wang H, B Masironi, H Eriksson and L Sahlin . 1999. A comparative study of estrogen receptors alpha and beta in the rat uterus. Journal of Biology of Reproduction 61 955–964.
5. Afriastini, JJ. 2003. Marsilea crenata C.Presl . in : de Winter WP, Amoroso VB, editor. Cryptograms: Ferns and fern allies. Bogor : LIPI
6. Yacoeb AM, Nurjanah, M Arifin, W Sulistiono, SS Kristiono. 2010. Description histological and changes in the chemical composition of the leaves and stalks of clover (Marsilea crenata Presl., Marsileaceae) by boiling. Journal of Fishery Products Processing Indonesia XII (2): 81-95.
7. Kumar A, R Ilavarasan , T Jayachanran, M Decaraman, P Arivindha, N Padmanabhan , MVR Krishman. 2009. Phytochemical investigation on a tropical plan, Syzgium cumini from Kattuppalayam, Erode District, Tamil Nadu, South India. Journal of Nutrition Pakistan 8(1) : 83-85
8. Zabri H, C Kodjo, A Benie, JM Bekro, YA Bekro. 2008. Phytochemical sceening and determination of flavonoids in Secamone afzelii (Asclepiadaccae) axtracts, Journal of Pure Applied Chemistry 2(8):80-82.
9. Ausbel,K. 2000. Tempest in a Tonic Bottle : A Bunche of Weeds?Herbal Gram. Journal American Botanical Council 49:32-43
10. Knight DC, PL Wall, JA Eden. 1999. A Review Of Phytoestrogen And Theirs Effect In Relations To Menopausal Symptoms. Centre for Management of the Menopause. Royal Houspital for Women, Sidney
11. Suarsana, Nyoman, I Dharmawan, I Gorda,B Pontjo Priosoeryanto. 2011. Tepung Tempe Kaya Isoflavon MeningkatkanKadar Kalsium, Posfor dan Estrogen Plasma Tikus Betina Normal. Jurnal Veteriner Vol. 12 No. 3: 229-234, September 2011
12. Sophia, R.A. 2003. Uji Efek Diuretic Suspensi Simplisia Daun Sambiloto (Andrographis Paniculata ness) terhadap Tikus Putih (Rattus norvegicus L.) Betina Galur Sprague-Dawley. Skripsi. Fakultas Matematika dan Ilmu Pengetahuan Alam Universitas Indonesia. Jakarta.

13. Widjayanti, Y. 2014. Effect of Tomato Juice (Lycopersicum Commune) Against Estradiol levels, proliferation of fibroblasts and Wall Thickness Vagina Menopause Rat Model. Thesis. Post Graduate Airlangga University Surabaya.
14. Putra, HL. 2011. Green Clover Potentiates Delaying The Increment Of Imbalance Bone Remodeling Process In Postmenopausal Women. Folia Medica Indonesiana Vol. 47 No. 2 April - June 2011 : 112-117
15. Mahaputra L anda Putra H.L. 2011. Phytoestrogen in several fruits and leaves. Indonesian Journal of Clinical Pathology and Medical Laboratory. Vol 18/No1/ Published:2011-01.
16. Andria, Y. 2012. Pengaruh Pemberian Ekstrak Daun Peganggan terhadap Kadar Hormon Estradiol dan Kadar hormon Progesteron Tikus Putih (Rattus norvegicus) Betina. Program Studi Ilmu Biomedik.
17. Hadley, M.E. 2000. Endrocrinology. Ed. Ke-5. Prentice-Hall, Inc., New Jersey : xxii.
18. Champbell, A.N., J.B. Reece, and L.G. Mitchell. 2004. Biology. W. Manalu (Penterjemah). Edisi ke-5. Erlangga, Jakarta
19. Nadjamudin, Rusdin, Sriyanto, Amrozi, S. Agungpriyono, dan T.L. Yusuf. 2010. Penentuan siklus estrus pada kancil (Tragulus javanicus) berdasarkan perubahan sitologi vagina. Jurnal Veteriner 11:81-86. javanicus) berdasarkan perubahan sitologi vagina. Jurnal Veteriner 11:81-86
20. Ward, J.P.T., R.W. Clarke and R.W.A. Linden. 2005. Physiology at a Glance. Blackwell Publishing, USA.
21. Tsourounis, C. 2004. Clinnical Effect of Fitoestrogens. Clinical Obstretict and Gynecology: 44(4): 836-42
22. Dellman, H. D. 2010.Text Book of Veterinary Histology. 5th. Ed. William and Wilkins a Wavery Company.
23. Turner CD and Bagnara JJ. 1976. General Endocrinology. Interpreter : Harjoso. Surabaya: Airlangga University Press
24. Sherwood, L.2001. Fisiologi Manusia dari sel ke Sistem. Brahm, Penerjemah. Jakarta (ID): EGC. Terjemahan dari Human Physiology From Cells to Systems
25. Dellman HD, and EM Brown. 1992. Buku Teks Histologi Veteriner. Ed ke-3. R. Hartono, penerjemah. Jakarta: Universitas Indonesia Press.
26. Ismudiono. 2009. Reproduction in Animal Physiology. Edition II. Faculty of Veterinary Medicine, University of Airlangga.
27. Ford JA Jr, SG Clark, EM Walters, MB Wheeler and WL Hurley. 2006. Estrogenic effects of genistein on reproductive tissues of ovariectomized gilts. J Anim Sci. 84:834-842
28. Ganong, W.F. 2003. Fisiologi Kedokteran. Penerbit Buku Kedokteran EGC, Jakarta.
29. Harrison RM, PP Phillippi, KF Swan, and MC Henson. 1999. Effect Of Genistein On Steroid Hormon Production In The Pregnant Rhesus Monkey Society For Experimental Biology and Medicine vol 222.
30. Alam M.N., A Ahmad, FA Al-Abbasi. Female ovarian steroids in epilepsy: a cause or remedy.Pharmacol Rep. 2013;65:802–812
31. Guyton, A.C. and J.E. Hall. 1997. Textbook of Medical Physiology. 9th edition. Book Medical Publishers EGC, Jakarta.

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