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Dendrophthoe pentandra methanolic leaf extract increases progesterone levels in female rats

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ABSTRACT

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BACKGROUND
Human infertiliy cases in Indonesia have tended to increase at about 2-5% annually since 2000. Many tropical plants in Indonesia are potential sources of novel anti-infertility compounds, e.g. *Dendrophthoe pentandra* L. Miq. (*benalu duku*), a parasitic plant growing on *Lansium domesticum*. The objective of this study was to identify the effect of crude methanolic *Dendrophthoe pentandra* leaf extract on follicle stimulating hormone (FSH) and progesterone levels in female rats.

METHODS
Fourteen *Rattus norvegicus* Wistar strain female rats were divided into an intervention group and a control group, and synchronized to estrus via the pheromone synchronizing method. The intervention group was given daily single intramuscular injections of crude methanolic *Dendrophthoe pentandra* leaf extract at 100 mg/kgBW for 4 days, while the control group was given daily single intramuscular injections of 1 mL of distilled water. Determination of FSH and progesterone levels in whole blood was done using the Randox Evidence Investigator analyzer. The data were subjected to the t test for two independent samples.

RESULTS
Mean FSH in the intervention group was 9.28 ± 6.72 mIU/mL, which was lower than mean FSH of 24.80 ±16.35 mIU/mL in controls ($p<0.05$). Mean progesterone level in the intervention group was 33.55±13.96 nmol/L, twice as high as that in the control group, which was 18.47± 06.47 nmol/L ($p<0.05$).

CONCLUSIONS
Crude methanolic *Dendrophthoe pentandra* leaf extract reduces FSH and increases progesterone levels in female rats, but cannot yet be recommended for use as fertility hormone inhibitor or stimulator in rats.

Key words: *Dendrophthoe pentandra***,** estrus synchronization, cuboidal cells, progesterone, female rats

Ekstrak methanol daun Dendrophthoe pentandra meningkatkan kadar progesteron pada tikus betina

ABSTRAK

LATAR BELAKANG

Kasus ketidaksuburan pada manusia di Indonesia cenderung naik 2-5% setiap tahun sejak tahun 2000. Disisi lain diketahui banyak tumbuhan tropis di Indonesia yang berpotensi sebagai sumber komponen baru anti infertilitas (contoh benalu duku atau Dendrophthoe pentandra L. Miq., tumbuh di Lancium demesticum). Tujuan penelitian ini adalah mengidentifikasikan kinerja ekstrak kasar metanol daun Benalu duku pada tikus betina terhadap induksi siklus oestrous dan pengaruhnya terhadap kinerja follicle stimulating hormone *(FSH) dan progesteron.*

METODE

Sebanyak empat belas tikus betina (Rattus norvegicus Wistar strain) dibagi dua kelompok (kelompok perlakuan dan kelompok kontrol) dan diatur menjadi oestrous melalui metode sinkronisasi feromon. Kelompok perlakuan selanjutnya diberi ekstrak kasar daun benalu duku 100 mg/kg berat badan (satu kali sehari) selama 4 hari melalui intra muskular. Kelompok kontrol diberi 1 mL aqua secara intra muskular (satu kali sehari) selama 4 hari. Pengukuran kadar FSH dan progesteron, sampel darah diproses menggunakan metode analisis Evidence Investigator *dari Randox. Data yang diperoleh dilakukan uji t dua sampel saling tak bergantung dengan tingkat siknifikansi sebesar 0,05.*

HASIL

Rata-rata kadar FSH pada kelompok perlakuan besarnya 9,28 ± 06,72 mIU/mL lebih rendah secara bermakna dari kelompok kontrol sebesar 24,80 ± 16,35 mIU/mL, (p<0,05). Pada kelompok perlakuan, rata-rata kadar progesteron besanya 33,55 ± 13,96 nmol/L dua kali lebih tinggi secara bermakna dari kelompok kontrol sebesar 18,47 ± 6,47 nmol/L (p<0,05).

KESIMPULAN

Ekstrak kasar metanol daun benalu duku mampu menurunkan kadar FSH dan meningkatkan kadar progesteron pada tikus betina. Pada penelitian ini masih belum dianjurkan untuk menggunakan hormone fertilitas lainnya sebagai penghambat atau peningkat hormon pada tikus betina.

Kata kunci: *Benalu duku, penyeragaman birahi, sel kubus, progesteron, tikus betina*

INTRODUCTION

The follicle stimulating hormone (FSH) and progesterone are fertility hormones in the human female and essential for control of the menstrual cycle. Fertility hormones are not only used for pregnancy testing in women but are also important in the diagnosis of cases of early onset of menopause and gonadal abnormalities. In males, fertility hormones can also be used as accurate indicators of cases of liver cirrhosis and testicular cancer.[1] Fertility hormones can be produced by medicinal plants such as *Jatropha curcas,* which produces testosterone,[2] and *Dioscorea macrostachya,* which produces diosgenine (intermediate product of cortisone), and compounds with anti-estrogenic activity.[3]

Dendrophthoe pentandra (L.) Miq (local name: *benalu duku*), a parasitic plant of the *Loranthaceae* family and growing on *Lansium domesticum,* has been known to possess

Table 1. Determination of sample size

Analyte availability on administration of dendrophthoe pentandra leaf extract (%)										Analyte availability without administration of dendrophthoe pentandra leaf extract (%)
10	20	30	40	50	60	70	80	90	100	
53	28	17	12	9	7	-	-	-	-	0
-	270	83	42	26	18	13	9	7	-	10
-	-	402	111	53	31	20	14	9	-	20
-	-	-	294	128	58	32	20	13	-	30
-	-	-	-	539	134	58	31	18	7	40
-	-	-	-	-	539	128	53	26	9	50
-	-	-	-	-	-	494	111	42	12	60
-	-	-	-	-	-	-	402	83	17	70
-	-	-	-	-	-	-	-	270	26	80
-	-	-	-	-	-	-	-	-	53	90

medicinal properties since 1990 and is still being investigated for its content of local and systemic anticancer compounds.[4] The plant is also known to contain compounds with anti-myeloma cell and antibiotic activity.[5,6] Several investigators have reported that *Dendrophthoe pentandra* contains components that are useful for preventing disease, such as compounds with antiproliferative activity. Among its known organic compounds are essential amino acids, alkaloids, flavonoids, polyphenols, terpenoids, and free steroids.[4] A more recent report states that members of the genus *Dendrophthoe* may contain immune hormones that prevent the formation of internal free radicals as a result of stimulation by external substances.[7] However, the use of *Dendrophthoe pentandra* L. Miq for treatment of menstrual cycle disorders has not yet been reported.

On the basis of the above mentioned background, we attempted to determine the levels of the steroid hormones follicle stimulating hormone (FSH) and progesterone in adult female rats receiving a methanolic extract of *Dendrophthoe pentandra* leaves. The objective of this study was to evaluate the influence of a methanolic *Dendrophthoe pentandra* leaf extract on FSH and progesterone levels in adult estrous female rats.

METHODS

Research design

This study was of experimental design with posttest only control group using experimental animals as research subjects. This study was conducted from July to October 2013 in the laboratory of the Veterinary Pharmacy Subdivision, Department of Veterinary Basic Science, Faculty of Veterinary Medicine, Airlangga University.

Sample size

The sample size was calculated according to Rumke's table with the assumption of a 100% success rate after administration of *Dendrophthoe pentandra* leaf extract and 40% failure rate without administration of *Dendrophthoe pentandra* leaf extract (Table 1).[4,8] Therefore the sample size required was 7 female rats for each treatment group, giving a total of 14 female rats (7 in the intervention group and 7 in the control group).

Plant material

The leaves of *Dendrophthoe pentandra* L., Miq growing on *Lansium domesticum* were collected from the plant's natural habitats in the Palembang District, South Sumatera, and

authenticated by Mrs. Yayah, Biological Research Center – Indonesian Science Institute (*Lembaga Ilmu Pengetahuan Indonesia,* LIPI), Bogor.

Extraction

The fresh *Dendrophthoe pentandra* leaves were cleaned, air dried, and powdered using an electric homogenizer. Then 450 g of the powdered sample was extracted with 2 L of analytical grade methanol for 72 h by using the rotating percolation method. The crude methanolic extract was dried by flushing with nitrogen gas to reduce the methanol level. From the crude methanolic extract subsequently a injectable preparation was made that was pyrogen-free, sterile, stable, isotonic, iso-ionic, isohydric, with a pH of 7.2-7.5. The last step in the preparation of the extract was filtration at 0.20 μm and dispensing to disposable sterile vials at 4^0C.

Experimental animals

Fourteen healthy adult female rats 3-4 months of age *(Rattus norwegicus* Wistar strain*)* were obtained from Rachmad Priyadi DVM in Trosobo, Sidoarjo. The rats were assigned by simple randomization to one control group (n=7) and one intervention group (n=7). All animals were ear-marked according to the following code: O : no ear markings; KA : markings on right ear, KI : markings on left ear, KK : markings on both ears. Ka3: code for cage No. 3; code for cage No. 4. Ear markings were notches on the ear lobes. In this study, cages Nos. 1 (Ka1) and 2 (Ka2) were used by male rats for estrus synchronization purposes only.

Synchronization of estrus

All rats were synchronized for estrus by the Whitten effect [pheromonally induced estrus synchronization in female rats by the presence of a male] or pheromone effect technique for two cycles at intervals of 10 days as follows: all fourteen female rats of the two groups were kept in cages no. 3 (8 females) and no. 4 (6 females). Cages no. 1 and 2 contained the male rats.[9,10] Giemsa stained vaginal smears of the female rats were examined at 1000 x magnification for cuboidal and cornified cells during the study period.[10]

Experimental procedures

The treatments in this study were applied after all rats were in estrus. During the four-day treatment period, the rats in the intervention group were given once daily single intramuscular injections of *Dendrophthoe pentandra* leaf extract diluted with aqua pro injection (w/v) at a dose of 100 mg/kg body weight. Control rats were given a single daily intramuscular injection of 1 mL aqua pro injection during the four days. At the end of the treatment period, the rats were sacrificed and cardiac blood samples were collected, ranging from 1.5 to 2 ml. Plasma was separated from the blood samples by centrifugation at 8000 g for 15 minutes and stored at 2-8^0C until required for determination of FSH and progesterone concentrations.

Measurement of FSH and progesterone

The levels of FSH and progesterone were measured using the Evidence Investigator™ semi-automated analyzer based on a proprietary biochip array technology. The complete Evidence Investigator package includes the biochip imaging module, nine-biochip carrier, biochip carrier handling tray, thermoshaker, as well as a personal computer, imaging software, and barcode scanner.

The core technology is the Randox Biochip, which is a 9-mm^2 solid substrate containing an array of discrete test regions for different markers. The biochip allows the simultaneous quantitative detection of multiple analytes from a single subject sample.

For chemiluminescent immunoassay determination of FSH and progesterone concentrations, the present study used the Randox fertility hormone array package (Cat No. EV3610) consisting of assay diluent, conjugate, biochip, calibrator, luminance or PX, and wash

buffer. The light signal generated from each of the test regions on the biochip was detected using a CCD camera and digital imaging technology, then compared to that from a stored calibration curve, from which the concentration was calculated.[11]

Procedural details

After adding 150 μL of assay diluent into the appropriate biochip wells and a volume of 75 μL each of calibrators, samples and controls into the respective wells, the reagents were mixed by gently tapping all edges of the handling tray. Then the handling tray containing the biochip carriers was fixed to the base plate of the thermoshaker, and incubated for 30 minutes at +37 ^{0}C and 350 rpm.

After removal of the handling tray from the thermoshaker, volumes of 75 μL of conjugate were added to the biochip wells and handled using the same sequence of tapping/mixing, fixing to thermoshaker and incubating under the same conditions as described above. At the end of the procedure, the reagents were discarded using a sharp flicking action of the handling tray, and immediately subjected to 2 quick wash cycles by adding approximately 350 μL wash buffer to each well, using the wash bottle containing diluted wash buffer. The edges of the handling tray were then tapped to release any reagents trapped below the biochip, then the reagents were flicked to waste with a sharp action. To reduce potential well-to-well contamination, care was taken not to overfill the wells.

After a further 4 wash cycles, in which the edges of the handling tray were tapped for approximately 10 to 15 seconds, the tray was then left to stand for 2 minutes to allow the biochips to soak in wash buffer. After the final wash, the wells were filled with wash buffer and left to soak until directly prior to imaging.

For imaging, the carrier was removed from the handling tray and 250 μL of working signal reagent added to each of the well, which were then covered to protect from light. Directly before addition of signal, the wash buffer was removed by flicking and tapping the carrier onto lint free tissue.

After exactly 2 minutes (±10 seconds) the carrier was then placed in the Evidence Investigator machine. Image capture was automatically initiated by the dedicated software. All blood samples of the intervention and control groups were assayed in triplicate, the results being coded n1, n2, and n3.

Statistical analysis

Data analysis was performed using the Minitab statistical software version 17.0. Independent-t test was used to test the differences between control and intervention groups at a significance level of 0.05.

Animal ethical clearance

The rats were handled according to the principles for animal experiments under control of the Commission on Animal Research Ethics from the Faculty of Veterinary Medicine, Airlangga University, and the minimum standard requirements for animal handling for experiments from the Experimental Animal Ethics Unit of the Indonesian Veterinary Pharmacy and Pharmacology Association (www.affaveti.org).

RESULTS

From the study results it is apparent that estrus synchronization was achieved in all animals in around 10 days. For a view of cuboidal cells at 1000 x magnification in vaginal smears prepared by the Giemsa staining method, see Figures 1 and 2 (at 1000x magnification).

The coefficient of variation interval for the FSH assays from 36 replicates was 10.01% to 16.68%, while for the progesterone assay it was 10.00% to 16.66%. The assay results for FSH and progesterone in both intervention and control groups performed in triplicate are presented in Table 2.

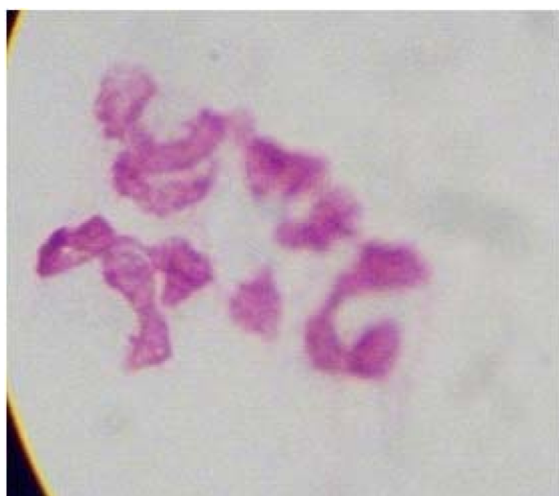

Figure 1. Cuboidal cells in vaginal smear (Giemsa stain, 1000x)

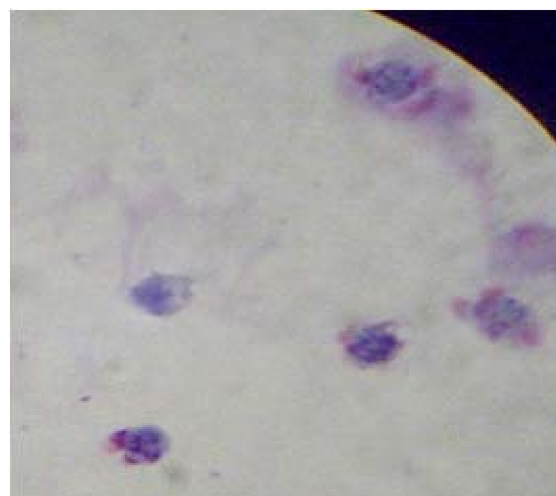

Figure 2. Cornified cells in vaginal smear (Giemsa stain, 1000x)

DISCUSSION

The present study achieved estrus in rats more rapidly than is possible by the Bruce effect [pheromonally induced pregnancy block and return to estrus by exposing recently inseminated females with an unfamiliar male], as described by other investigators in mice.(12,13) Thus our method is applicable for laboratory studies using rats for assays of short-acting hormones, such as luteinizing hormone.

This study demonstrated that the FSH concentrations in the intervention group was threefold lower than those in the control group, whereas the progesterone concentrations in the intervention group were twice those in the

Table 2. Effect of *Dendrophthoe pentandra* L. on FSH and progesterone levels in female rats

	Treatment group		p value
	Dendrophthoe pentandra	**Control**	
FSH (mIU/mL)			
Ka3KA	16.98 ± 27.85	15.24 ± 9.16	<0.05
Ka3KI	4.74 ± 6.26	17.55 ± 14.02	<0.05
Ka3KK	14.54 ± 12.30	5.74 ± 4.72	<0.05
Ka3O	16.15 ± 13.80	10.80 ± 0.00	<0.05
Ka4O	2.04 ± 0.33	46.92 ± 37.54	<0.05
Ka4KA	9.27 ± 10.47	35.14 ± 10.68	<0.05
Ka4KI	1.27 ± 1.05	42.23 ± 4.98	<0.05
Overall	9.28 ± 6.72	24.80 ± 16.35	<0.05
Progesterone (nmol/L)			
Ka3KA	46.04 ± 30.85	13.45 ± 5.76	<0.05
Ka3KI	38.56 ± 37.87	15.40 ± 9.58	<0.05
Ka3KK	16.30 ± 18.86	27.36 ± 23.61	<0.05
Ka3O	12.03 ± 8.36	28.67 ± 28.10	<0.05
Ka4O	35.66 ± 3.97	16.91 ± 13.34	<0.05
Ka4KA	38.77 ± 0.00	12.02 ± 8.76	<0.05
Ka4KI	47.52 ± 25.33	---	---
Overall	33.55 ± 13.96	18.47 ± 6.47	<0.05

Each pair of FSH and progesterone values is from a single blood sample —- Lyzed blood sample; Ka3: cage No. 3, Ka4: cage No. 4; O: no ear markings; KA: markings on right ear, KI : markings on left ear, KK: markings on both ears, Significance level at p <0.05 using independent t test

control group. These facts indicate that the analytes contain some unknown substance(s) with two pharmacodynamic actions, viz. as an FSH suppressor and progesterone stimulator. These unknown substances from the crude methanolic extract of *Dendrophthoe pentandra* leaves have been identified as having "suppressor FSH-like effects" and "stimulant progesterone-like effects". Several other substances with "suppressor FSH-like effects" are human seminal plasma components with 92-amino acid polypeptides and alpha-inhibin-92 (alpha-IB-92).[14] Synthetic compounds with "progesterone-like effects" that have been known as early as 2000 are dydrogesterone, 17-α-hydroxyprogesterone caproate, medroxy progesterone acetate, and 17-megestrol acetate.

The FSH suppression phenomenon is analogous to the concept of "receptor down-regulation of gonadotrophin releasing hormone (GnRH)". As down-regulation occurs, production of gonadotrophins by the pituitary i.e FSH ceases, effectively shutting down control hormones for cyclic ovarian functioning in the female. This effect was well known as early as 1989 and has been suggested as a potential estrus suppressor hormone.[15]

The analytes from the crude methanolic extract of *Dendrophthoe pentandra* leaves may act by occupying GnRH receptors in the pituitary and after a short period of stimulation cause the cells to reduce or stop the synthesis of receptor proteins, making the cells insensitive to GnRH.[15] The progesterone level increases after the cells receive a "calling signal" from the anterior pituitary, impacting on the corpus luteum in the form of inhibition of Graafian follicle development in the ovary. The relationship between FSH inhibition and increased progesterone is not a direct one, but has an influence on other fertility hormones, such as luteinizing hormone (LH), while estrogen may have an important role in strengthening the relationship between FSH inhibition and progesterone stimulation.[16]

Hormones are generally administered to patients for one of three purposes. First, when patients fail to produce sufficient quantities of hormones, therapy is directed at correcting the deficiency. Second, when no permanent hormonal deficiency exists, hormones are used to obtain more physiological effects. For example, synthetic progesterone, used as an estrus control agent, may be administered in cases of normal estrus. Third, when hormone production is excessive, therapy is directed to the target by administration of antagonist hormones to inhibit excessive production.

Our study results probably indicate that the analytes act by stimulating the corpus luteum so as to produce a larger than two-fold increase in progesterone as compared with the normal production. This will activate a feedback mechanism for decreasing FSH production by GnRH signaling to inhibit FSH, as referred to by Konishi et al.[17] Our study cannot clarify the mechanism whereby the analytes induce low FSH levels and excessive production of progesterone after administration of the crude methanolic extract of *Dendrophthoe pentandra* leaves. There is a possibility that through further exploration of the "progesteron-like" effects of the extract, the mechanism of abovementioned analyte action may be explained.

A limitation of our study is that we did not study the expression of enzymes encoded by base-paired microsomal DNA subunits acting as "control messenger" to inhibit FSH and stimulate progesterone, which may provide the answer to the analyte mechanism of action.

In women with fertility disorders, the unique phenomena of FSH inhibition and progesterone stimulation by crude methanolic extract of *Dendrophthoe pentandra* leaves may be used to treat pregnant woman with low progesterone levels, particularly in early pregnancy with a risk of miscarriage. In early pregnancy, abdominal pain and vaginal bleeding may be signs of a miscarriage, but consumption of crude

methanolic extract of *Dendrophthoe pentandra* leaves probably will be of benefit in increasing progesterone levels.

Several natural products from medicinal or other plants used to stimulate progesterone in early pregnancy are oils in yam and soy plants, compounds from *Curcuma comosa* especially its diaryl-heptanoid compounds, plants frequently growing in waste water (wwTPs) in Beijing, China, and alkaloids from *Digitalis lanata* leaves known as 5-â-cardenolides.[17-19] As explained earlier, some alkaloids of the *Dendrophthoe* family are very beneficial for the treatment of steroid disorders in experimental animals.[20]

Other researchers even mention that *Dendrophthoe* species are potential muscle relaxants. [21] However, through future studies to explore the leaf alkaloids of *Dendrophthoe pentandra* as a member of the *Dendrophthoe* family, we may presumably find new compounds for the treatment of cases of low progesterone levels in early pregnancy or for management of infertile uterine conditions.

CONCLUSIONS

The crude methanolic extract of *Dendrophthoe pentandra* leaves is capable of increasing progesterone concentrations and of decreasing FSH production in healthy female rats. Further studies are required on the components in crude methanolic extract of *Dendrophthoe pentandra* leaves that have progesterone stimulating properties. Other fertility hormones such as prolactin, testosterone, estrogen, and luteinizing hormone are not recommended yet to be used in the treatment of steroid disorders in healthy female rats.

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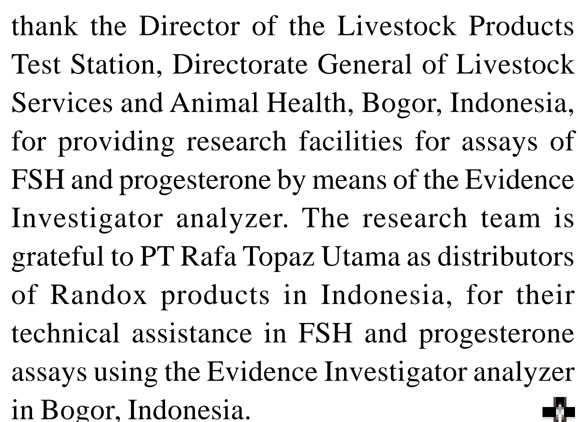
The investigations were financed by the Directorate General of Higher Education, Ministry of Education and Culture by contract No. 8714/UN3/KR/2013. The authors wish to thank the Director of the Livestock Products Test Station, Directorate General of Livestock Services and Animal Health, Bogor, Indonesia, for providing research facilities for assays of FSH and progesterone by means of the Evidence Investigator analyzer. The research team is grateful to PT Rafa Topaz Utama as distributors of Randox products in Indonesia, for their technical assistance in FSH and progesterone assays using the Evidence Investigator analyzer in Bogor, Indonesia.

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