Effects of *Kaempferia parviflora* extracts on reproductive parameters and spermatic blood flow in male rats

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Abstract

Krachaidum (KD, *Kaempferia parviflora* Wall. Ex. Baker), a native plant of Southeast Asia, is traditionally used to enhance male sexual function. However, only few scientific data in support of this anecdote have been reported. The present study investigated the effects of feeding three different extracts of KD (alcohol, hexane, and water extracts) for 3–5 weeks on the reproductive organs, the aphrodisiac activity, fertility, sperm motility, and blood flow to the testis of male rats. Sexual performances (mount latency, mount frequency, ejaculatory latency, post-ejaculatory latency) and sperm motility were assessed by a video camera and computer-assisted sperm analysis respectively, while blood flow to the testis was measured by a directional pulsed Doppler flowmeter. The results showed that all extracts of KD had virtually no effect on the reproductive organ weights even after 5 weeks. However, administration of the alcohol extract at a dose of 70 mg/kg body weight (BW)/day for 4 weeks significantly decreased mount and ejaculatory latencies when compared with the control. By contrast, hexane and water extracts had no influence on any sexual behavior parameters. All types of extracts of KD had no effect on fertility or sperm motility. On the other hand, alcohol extract produced a significant increase in blood flow to the testis without affecting the heart rate and mean arterial blood pressure. In a separate study, an acute effect of alcohol extract of KD on blood flow to the testis was investigated. Intravenous injection of KD at doses of 10, 20, and 40 mg/kg BW caused dose-dependent increases in blood flow to the testis. The results indicate that alcohol extract of KD had an aphrodisiac activity probably via a marked increase in blood flow to the testis.

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Introduction

For several hundred years, people around the world have used locally grown plants as supplements to energize, vitalize, and eventually to improve male sexual functions. Up to now aphrodisiac activity of plant extracts including *Eurycoma longifolia* Jack (Ang *et al.* 2000), *Terminalia catappa* (Ratnasooriya & Dharmasiri 2000), *Lepidium meyenii* (Maca; Zheng *et al.* 2000, Cicero *et al.* 2001), *Tribulus terrestris* (protodioscin; Gauthaman *et al.* 2002, 2003), *Montanoa tomentosa* (Carro- Juárez *et al.* 2004), and *Fadogia agrestis* (Yakubu *et al.* 2005) have been reported. The active components of these plants have not yet been identified. However, most investigators used aqueous or alcohol extracts that contain largely phenols, alkaloids, and saponins. Of these plants, the flavonoids, which are normally extracted by less polar or non-polar solvents, are not subjected to many studies, in spite of the fact that flavones and isoflavones are known to have estrogenic (Hiremath *et al.* 2000, Haddad & Faqua 2001, Das *et al.* 2004) or androgenic activity (Qin *et al.* 2000, Yousef *et al.* 2005).

Krachaidum (KD, *Kaempferia parviflora* Wall. Ex. Baker), a native plant of Thailand, belongs to Zingiberaceae family. This plant has been used in Thai traditional

medicine to treat various ailments such as inflammation, diarrhea, vertigo, and heart diseases. As a herbal product, KD has been used in different preparations such as fresh or dried rhizomes, dried powder in tea bags, and wine. It is usually claimed that KD increases male sexual activity and reproductive functions. However, the claim is based on consumer opinions. Scientific data in support of the aphrodisiac activity and reproductive functions are still controversial. Thus, Sudwan *et al.* (2006, 2007) have shown that alcohol extracts of KD and *Boesenbergia rotunda* (Krachai) had no effects on aphrodisiac activity and reproductive organs of male rats. Similarly, KD powder suspended in water exerted no androgen-like effects on reproductive organs of the castrated immature male rats (Trisomboon *et al.* 2007). On the other hand, Somphol *et al.* (2003) have shown that ground rhizome of *Boesenbergia pandurata* (former name of KD), increased ejaculation volume and had a tendency to increase total number, viability, and progressive motility of spermatozoa in rabbit semen. The positive effects of tea from KD on the seminal vesicle and spermatogenesis in rats have also been reported (Jitjaingam *et al.* 2005). Such discrepancy is partly due to differences in the preparations and doses because chemical analyses of different fractions of KD

extracts showed that hexane and alcohol extracts yielded phenols and flavonoids but at different percentages. Phenols are the major component of the alcohol extract while flavonoids are present mainly in the hexane extract. The water extract contains only small amounts of phenols and flavonoids (Sutthanut *et al.* 2007).

Although the effects of extracts of KD and its related plant on male reproductive organs and function have been reported, the mechanism by which these extracts mediate changes in reproductive function has not been elucidated. Since plants in the Zingiberaceae family such as curcuma and ginger have been shown to relax smooth muscles (Itthipanichpong *et al.* 2003) especially smooth muscle of blood vessels (Sasaki *et al.* 2003, Goto *et al.* 2005), it is possible that KD might relax vascular smooth muscle leading to an increase in blood flow to the reproductive organs and finally improvement of male reproductive functions. Therefore, the present study was designed to examine the effects of KD on aphrodisiac activity, reproductive functions, and spermatic blood flow in male rats.

Results

To investigate the long-term effect of KD on androgen-dependent and -independent organs, rats were treated with three different extracts for 5 weeks. We found that the relative weights of testes, epididymides, seminal vesicles, prostate glands, liver, kidneys, and levator ani muscle of KD-treated animals were not significantly different from the control group (Table 1).

To investigate the effect of KD on sexual behaviors, rats were treated with three extracts of KD as indicated in Experiment 1. Interestingly, the alcohol extract of KD significantly reduced both mounting and ejaculation latencies of the treated animals compared with those of the control group ($P < 0.05$) whereas similar effects were not observed with hexane or water extracts (Table 2). However, none of the alcohol, hexane, and water extracts of KD altered the percentage of fertility or sperm motility (Table 3).

The reduction in mounting and ejaculation latencies observed in the animals treated with the alcohol extract suggests an increase in sexual motivation that might be due to the favorable pharmacological effect of the extract on blood flow to male reproductive organs. To investigate this

possibility, we measured spermatic blood flow after the sexual parameters were assessed. We found that chronic treatment of the alcohol extract of KD did not alter mean arterial blood pressure (mABP) or (resting) heart rate (HR). However, a significant increase ($P < 0.05$) in blood flow through the spermatic artery from 0.59 ± 0.01 ml/min in control animals to 0.96 ± 0.08 ml/min in KD-treated animals was observed (Fig. 1).

It was of interest whether KD would produce an immediate effect on mABP and spermatic blood flow following an i.v. injection. Therefore, mABP and spermatic blood flow were measured after i.v. injections of the alcohol extract of KD at doses of 10, 20, and 40 mg/kg body weight (BW). Acetylcholine at a dose of 10 µg/kg BW was also administered as a positive control. As shown in Figs 2 and 3, all doses of alcohol extract, immediately produced dose-dependent decreases in mABP. Meanwhile, dose-dependent increases in spermatic blood flow were observed after KD administration. Notably, acetylcholine significantly reduced mABP and increased spermatic blood flow in the same range as that of alcoholic extract at a dose of 20 mg/kg BW.

Discussion

In the present study, we have shown that among the three different fractions of KD extracts, only the alcohol extract has an effect on sexual behavior by shortening mount latency (ML) and ejaculation latency (EL), which are considered to be the indicators of an increase in sexual motivation. However, there were no changes in other sexual parameters including mount frequency (MF), post-ejaculation latency (PEL), and MF₃₀. Concurrent with the enhancement of sexual behaviors, spermatic blood flow was significantly augmented after treatment with the alcohol extract. All extracts had virtually no effect on androgen-dependent and -independent organs. Previous work has revealed flavones in the hexane extract of KD, all of which are methoxyflavones (Sutthanut *et al.* 2007). The finding in this study that hexane extract, which contains primarily flavones, had virtually no effect on sexual performance and reproductive organs of male rats suggests that methoxyflavones at the dose 70 mg/kg have neither androgenic nor estrogenic activity. Since flavones and isoflavones are phytoestrogens and hence have estrogenic or

Table 1 Effects of Krachaidum (KD) at a concentration of 70 mg/kg BW/day for 5 weeks on % organ weight/BW.

Group	Testis	Epididymis	Liver	Kidney	Seminal	Prostate	Levator Ani muscle
AC	0.98 ± 0.02	0.14 ± 0.00	3.86 ± 0.18	0.75 ± 0.02	0.17 ± 0.01	0.11 ± 0.01	0.26 ± 0.01
AKD	1.01 ± 0.03	0.15 ± 0.01	3.78 ± 0.09	0.76 ± 0.02	0.16 ± 0.02	0.11 ± 0.01	0.25 ± 0.01
HC	0.95 ± 0.05	0.14 ± 0.01	3.59 ± 0.11	0.74 ± 0.02	0.19 ± 0.01	0.09 ± 0.00	0.25 ± 0.01
HKD	0.95 ± 0.01	0.14 ± 0.00	4.04 ± 0.21	0.78 ± 0.02	0.18 ± 0.01	0.09 ± 0.01	0.25 ± 0.01
WC	0.98 ± 0.03	0.14 ± 0.01	3.80 ± 0.06	0.74 ± 0.02	0.18 ± 0.01	0.11 ± 0.00	0.24 ± 0.01
WKD	0.93 ± 0.01	0.14 ± 0.00	4.10 ± 0.08	0.79 ± 0.01	0.17 ± 0.01	0.09 ± 0.11	0.25 ± 0.00

AC, alcohol control group; AKD, alcohol extract of KD treatment group; HC, hexane control group; HKD, hexane extract of KD treatment group; WC, water control group; WKD, water extract of KD treatment group. All values are shown in mean $\pm$ S.E.M. ($n = 8$ rats).

Table 2 Effects of alcohol, hexane, and water extracts of Krachaidum (KD) at a dose of 70 mg/kg BW/day for 4 weeks on sexual behaviors.

Group	ML (min)	MF	EL (min)	PEL (min)	MF ₃₀
AC	0.78±0.38	44.75±7.75	25.53±3.27	7.78±0.77	55.13±7.10
AKD	0.18±0.12*	25.63±5.20	8.95±2.80*	6.42±0.23	53.50±6.13
HC	0.61±0.19	42.25±7.16	17.00±2.69	6.74±0.44	62.50±5.47
HKD	0.21±0.12	41.00±8.59	11.51±1.87	6.96±0.41	65.50±6.01
WC	0.27±0.13	28.38±5.32	13.54±2.41	8.19±0.73	45.13±5.93
WKD	0.13±0.15	31.50±4.65	12.21±2.22	6.48±0.44	51.38±4.99

AC, alcohol control group; AKD, alcohol extract of KD treatment group; HC, hexane control group; HKD, hexane extract of KD treatment group; WC, water control group; WKD, water extract of KD treatment group. ML, mount latency; MF, mount frequency; EL, ejaculation latency; PEL, post-ejaculation latency; MF₃₀, number of mounts within 30 min. All values are shown in mean ± s.e.m. (*n*=8 rats). **P*<0.05 significantly different from AC.

anti-estrogenic activity (Haddad & Fuqua 2001), it appears that methylation of the phenolic groups abolishes this activity. Although hexane and alcohol extracts yield similar flavones, the major components in each fraction are different. Thus, the three major flavones in the hexane extract are in the following order: 5,7-dimethoxyflavone > 5-hydroxy-7,4'-dimethoxy-flavone > 5,7,4'-trimethoxyflavone. By contrast, those in the alcohol extract are 5,7,4'-trimethoxyflavone > 5,7-dimethoxyflavone > 3,5,7,3',4'-pentamethoxyflavone. Furthermore, alcoholic extract contains a substantial amount of phenols while only a trace of these compounds is present in the hexane extract. On the other hand, aqueous extract has only small amount of flavones and no phenols (Sripanidkulchai *et al.* 2006). The differences in the composition of various extracts could explain the disparity of the results in this study.

As a Thai tonic herb for men, KD is usually consumed as tea or, more popularly, alcoholic drinks. However, most investigations of KD used aqueous extract. Tea prepared from KD powder at a dose of 120 mg/kg increased seminal vesicle weight, sperm density in the cauda epididymidis, and the diameter of seminiferous tubules of rats (Jitjaingam *et al.* 2005). On the other hand, aqueous suspension of KD powder at a dose of 1000 mg/kg failed to have any effect on the reproductive organs of the castrated immature rats although serum testosterone was elevated (Trisomboon *et al.* 2007). To our knowledge, only one study reported the effect of alcohol extract of KD on sexual behavior of male rats

(Sudwan *et al.* 2006). However, these authors showed that KD (50% alcohol extract) had virtually no effect on all sexual behavior parameters even though the dose was as high as 240 mg/kg and the duration was 60 days. The discrepancy between this and our study may be due to the active constituents in the extract and the animal model. In the present study, we used sexually experienced male rats. Our data, therefore, showed higher sexual activity compared with those of Sudwan *et al.* (2006). Indeed, their data indicated that the time of courtship significantly decreased over 30-min period even in the control group. In addition, we also used sexually experienced females for testing the mating behavior of the males. This might partly explain the relatively high values of ML, MF, and EL in the alcohol control group compared with other control groups, since the same females were used in testing the alcohol control and subsequently other groups. Taken together, our animal study provides some scientific support to the anecdotal belief that KD extract served as an alcoholic drink enhances male sexual libido. Other preparations such as tea fail to have such an effect. However, more studies in support of this notion are still required.

In order to investigate the mechanism by which KD enhanced sexual behavior, we measured blood flow to the testis via spermatic artery. Indeed, spermatic blood flow was markedly enhanced after treatment with alcohol extract of KD. The increase in blood flow to the testis would stimulate testosterone production and secretion which, in turn, acts on the central nervous system (CNS) and gonadal tissues to modulate male sexual behavior. There is evidence that testosterone production and secretion are closely related with the testicular blood flow (Damber & Janson 1978). It has been shown that exposure of the rat testes to γ -irradiation caused a reduction in testis weight and disruption of spermatogenesis. Testicular blood flow per testis was decreased in proportion to the reduction in testicular weight and, as a result, the production of testosterone was markedly reduced (Wang *et al.* 1983). Furthermore, the measurement of testicular capillary blood flow in adult foxes by the radioactive inert gas clearance technique demonstrated that, during the mating period, at which time spermatozoa were observed in the testes and the testicular weight and

Table 3 Effects of alcohol, hexane, and water extracts of Krachaidum (KD) at a dose of 70 mg/kg BW/day for 3 and 5 weeks on fertility and sperm motility.

Group	% Fertility ^a	% Motility ^b	% Progressive motility ^b
AC	72.5±7.6	83.4±2.3	37.4±2.6
AKD	80.4±4.9	81.2±2.2	34.3±2.7
HC	71.2±4.8	81.1±3.1	33.5±2.2
HKD	73.6±6.3	82.9±4.6	34.3±2.3
WC	74.9±5.4	86.9±2.4	37.0±2.1
WKD	76.1±4.8	89.5±1.5	41.1±1.6

AC, alcohol control group; AKD, alcohol extract of KD treatment group; HC, hexane control group; HKD, hexane extract of KD treatment group; WC, water control group; WKD, water extract of KD treatment group. All values are shown in mean ± s.e.m., *n*=8 rats.

^aAfter 3 weeks KD treatment. ^bAfter 5 weeks KD treatment.

Figure 1 Effects of alcohol extract of Krachaidum (KD) treatment for 4 weeks on (A) mean arterial blood pressure, (B) heart rate, (C) spermatoc blood flow. Values are mean $\pm$ S.E.M. from eight rats. AC, alcohol control group; AKD, alcohol extract of KD treatment group; * $P < 0.05$ significantly different from AC group.

circulating testosterone level were increased, testicular capillary blood flow increased (Joffe 1977). In contrast, during the period of testicular regression, testicular capillary blood flow and also androgenic activity decreased (Joffe 1977). However, the data in the present study do not support the notion that enhancement of sexual behaviors is due to the increase in testosterone production or secretion as a result of increased spermatoc

Figure 2 Representative tracings of the blood pressure (BP) and spermatoc blood flow (SBF) in response to i.v. injections of (A) vehicle (PVP) and alcohol extract of Krachaidum (KD) at doses of (B) 10, (C) 20, and (D) 40 mg/kg BW.

blood flow for several reasons. First, KD did not affect the weights of reproductive organs, such as epididymis, seminal vesicles and prostate glands, or the levator ani muscle, all of which are androgen-dependent tissues. Secondly, it has been reported that KD has no testosterone-like effect on the reproductive organs including seminal vesicles plus coagulating glands and ventral prostate and also the non-reproductive tissue such as levator ani muscle in the castrated immature rats treated with KD (Trisomboon *et al.* 2007). Thirdly, there is evidence that the aphrodisiac and fertility-enhancing properties of *L. meyenii* in healthy men have no correlation with serum reproductive hormone including testosterone (Gonzales *et al.* 2001, 2003). However, in view of the recent findings that in the castrated immature rats orally treated with dry KD powder in water for 5 days significantly increase serum testosterone levels were observed (Trisomboon *et al.* 2007), the possibility that KD enhances sexual behaviors via an increase in the testosterone cannot be ruled out.

Blood flow to the testis measured in the present study was in the same range as previously reported in which the flow was measured by various techniques in a variety of animal species during resting conditions,

Figure 3 Effects of i.v. injections of alcohol extract of Krachaidum (KD) at doses of 10, 20, and 40 mg/kg BW on (A) mABP and (B) blood flow compared with acetylcholine (ACH) at a dose of 10 µg/kg BW in male rats. Values are mean $\pm$ S.E.M. from eight rats. * P < 0.05 significantly different from baseline value.

and when stimulated by vasodilators such as tropic hormone (Setchell *et al.* 1988) and human chorionic gonadotropins (Wang *et al.* 1984). The findings in the present study that an oral administration of alcohol extract of KD at a dose of 70 mg/kg BW/day for 4 weeks significantly increased spermatid blood flow without changing the HR and the mean arterial blood pressure suggest that KD induces a local vasodilating effect. This result is consistent with the previous reports in humans and animals that plant extracts including those from garlic and flavonoid-rich cocoa induced vasodilation without altering the mean arterial blood pressure and HR (Naomi *et al.* 2003, Anim-Nyame *et al.* 2004). In order to confirm the vasodilating effect of KD, we performed an acute experiment in which alcohol extract of KD was intravenously injected at various doses. The results showed that KD caused a dose-dependent increase in blood flow to the testis, which was maintained for 30–100 s. This effect resembled that after 10 µg/kg acetylcholine. The vasodilating response to KD could be a result of pharmacological effects of flavonoids, derivatives of methoxyflavones isolated from rhizome of KD (Sutthanut *et al.* 2007) on endothelial function. Studies in cultured human umbilical vein endothelial cells showed that KD produced a dose-dependent increase in nitrite concentration. Furthermore,

expression of NOS mRNAs, the indicators of NO-cGMP induced vasodilation, was also enhanced (Wattanapitayakul *et al.* 2007). Studies in laboratory animals have shown that ginseng saponin enhanced libido and copulatory performance by directly acting on the CNS and gonadal tissues (Murphy & Lee 2002). There is evidence that ginseng saponin facilitated penile erection by directly inducing the vasodilation and relaxation of penile corpus cavernosum via nitric oxide mechanism (Chen & Lee 1995). Moreover, alteration of the activity of hypothalamic catecholamines was involved in the facilitation of copulatory behavior (Murphy *et al.* 1998). Whether *K. parviflora* promotes sexual behavior via acting centrally or peripherally needs further investigation.

In the present study, the reproductive functions, which are male fertility and sperm motility, were also investigated following administration of KD. The results showed that feeding the intact rats with a dose of 70 mg/kg BW/day of alcohol, hexane, and water extracts for 3 and 5 weeks had no effect on fertility nor sperm motility respectively. The failure to demonstrate the enhancing effect of KD may be due to the fact that these parameters are already high in the control animals. It has been shown that *L. meyenii* (1500 and 300 mg/kg per day for 4 months) improved sperm production and sperm motility in adult normal men (Gonzales *et al.* 2001). Whether sperm production is enhanced by KD is not known. By contrast, an administration of 500 mg/kg dose of *Artocarpus heterophyllus* seed suspension in 1% methylcellulose in rats, failed to alter male fertility (Ratnasooriya & Jayakody 2002). The results obtained from the previous and present studies indicate that plant extracts produce a wide range of effect on sexual behavior parameters and reproductive functions. This, perhaps, might be explained by the differences in the active constituents of the plant, doses, and duration of treatment, and also experimental animal models used in the studies.

Materials and Methods

Animals

Male and female Sprague–Dawley rats, 5 weeks old and weighing 140–150 g, were obtained from the National Laboratory Animal Center, Mahidol University. They were housed separately in stainless steel hanging cages at 25 °C and under a 12-h darkness:12-h light regimen with free access to food and water. At the time of experiment, the male (320–400 g) and female (200–250 g) rats were sexually mature. All experiments were carried out according to the International Guiding Principles for Biomedical Research Involving Animals of CIOMS and approved by the Faculty of Science, Mahidol University Animal Care and Use Committee.

Plant extracts

KD rhizomes were collected from Loei province, Thailand. The alcohol, hexane, and water extracts of KD were provided by

Assoc. Prof. Bungorn Sripanidkulchai, Director of Center for Research and Development of Herbal Health Product, Khon Kaen University, Thailand. The dried plant rhizome powder was prepared by the continuous Soxhlet extraction with solvents of different polarity in the following order: hexane, 95% ethanol, and water. After complete evaporation of the solvents, the dried extracts were used for gavage. Suspension of alcohol, hexane, and water extracts were prepared in 6.5% polyvinylpyrrolidone (PVP) in water, 5% PVP dissolved in corn oil, and water respectively.

Chemicals

Estrogen (β -estradiol 3-benzoate), progesterone (4-pregnene-3,20-dione), and other reagent grade chemicals were purchased from Sigma. Sodium pentobarbital (Nembutal) was obtained from Sanofi (Sanofi Sante Animale SA, Paris, France).

Sexual behavior study

The sexual behaviors of male rats with proven fertility were tested with sexually experienced, ovariectomized female rats during the night (from 0630 to 1000 h) in a quiet dark room at 22 °C. Male rats were introduced into a rectangular aluminum observation cage (28×58×17 cm) with a wire mesh front and allowed to acclimatization for about 10 min. Thereafter, a stimulus-receptive female rat was introduced and sexual behavior was recorded by a digital video camera recorder (DCR-TRV40E) for 30 min. Female receptivity was induced by the sequential s.c. administration of 10 mg/100 g BW estradiol benzoate (Sigma Chemical Company) and 500 mg/100 g BW progesterone (Sigma Chemical Company) for 48 and 4 h before the sexual behavior study respectively. Only receptive females exhibiting good lordosis in response to mounting when screened with non-experimental mature males were chosen in the study. The following sexual behavior parameters were registered according to the standard procedure (Gauthaman *et al.* 2002).

- 1) ML: the time interval (min) between the introduction of female into the cage until the first mount.
- 2) MF: the number of mounts from the first mount until ejaculation.
- 3) EL: the time (min) taken from the introduction of female into the cage until ejaculation.
- 4) PEL: the time (min) taken from the first ejaculation until the next mount.
- 5) MF within 30 min (MF₃₀): the number of mounts within 30 min.

For ethical reasons, a set of sexually experienced females was used throughout. However, the females were randomly assigned to mate with the males.

Male fertility

The fertility of male rats was assessed by natural mating. Each mature male rat was individually housed in a large cage with two virgin female rats for 5 days. Successful mating was verified by the presence of spermatozoa in the vaginal smears taken

every morning. The female rats showing thick clumps of spermatozoa in the vaginal smear were separated for 8–10 days, and then euthanized with overdose of diethyl ether (Sigma) for confirmation of pregnancy. The numbers of fetuses and corpora lutea were determined by the use of a dissecting microscope. The fertility of the male rat was expressed as the percentage fertility, calculated from the ratio of the number of fetuses to the number of corpora lutea×100. When both female rats were impregnated by the same male, the average value was used.

Sperm motility

After completion of 4-week KD feeding, animals were euthanized by overdose of i.p. sodium pentobarbital. The epididymis was removed and trimmed of fat. Spermatozoa were obtained by puncturing the distal cauda epididymidis and the epididymal fluid was collected into a hematocrit tube (id 1.1–1.2 mm; Sherwood Medical, St Louis, MO, USA) for 0.25–0.3 cm (2.65 μ l) and rapidly transferred to 1 ml modified Tyrode's solution. After 10 min incubation, the solution containing spermatozoa was dropped into an analysis chamber (depth 100 μ M, Art. No. SC 100-01-c, The Netherlands). The sperm motility was then assessed by Hamilton Thorne Integrated Visual Optical System at 35 °C and the parameters measured were the percentage of motile sperm and the percentage of progressively motile sperm (Goyal *et al.* 2001).

Measurement of blood flow

The male rat was anesthetized with an i.p. injection of 60 mg/kg BW of pentobarbital sodium. The level of surgical anesthesia was maintained during the experiment by supplementary doses of pentobarbital sodium (10–20% of the initial dose) given intravenously when necessary. A tracheostomy was performed by inserting a short polyethylene cannula (Clay-Adam, PE-240) to prevent airway obstruction. The left femoral artery was exposed and cannulated with a polyethylene catheter (Clay-Adam, PE-50) containing heparinized saline (50 IU heparin/ml). mABP and HR were measured by connecting the polyethylene catheter to a Statham P23 AC pressure transducer and a Grass polygraph recorder. The left femoral vein was also cannulated with a polyethylene catheter (Clay-Adam, PE-50) containing normal saline for the administration of KD and other substances at a volume of 0.1 ml each time. Body temperature of the rat was monitored by a rectal thermister probe and maintained at ~37 °C throughout the experimental period with an overhead lamp. Spermatic blood flow was measured using a directional pulse Doppler flowmeter (545C-4, the University of Iowa). After the abdominal wall over the spermatic artery was open, the vessel was isolated from the surrounding tissues. The piezoelectric crystal probe was gently placed around the vessel at the point where the spermatic artery enters the testis. The maximal blood flow was obtained by adjusting the range control, while listening to the audio from the speaker and turning the control for the best sound and monitoring the highest

peaks of the output, which is a fine adjustment for the center of the flow stream.

Instantaneous volume flow was calculated as follow:

$$Q = ED^2/0.2712 \cos A$$

where Q is instantaneous volume flow (ml/min), E is mean output (volt), D is lumen diameter of probe embed tube, and A is angle between sound beam and blood velocity vector which was constantly set at 45° .

Experimental protocol

Experiment 1: effects of extracts of KD on male reproductive parameters

The animals were divided into six groups, eight rats per group. The first group (AC) received 6.5% PVP dissolved in water serving as control for alcohol extract. The second group (alcohol extract of KD treatment group) received alcohol extract of KD at a dose of 70 mg/kg BW/day. The third group (hexane control group) received 5% PVP dissolved in corn oil serving as control for hexane extract. The fourth group (hexane extract of KD treatment group) received hexane extract of KD at a dose of 70 mg/kg BW/day. The fifth group (water control group) received distilled water serving as control for water extract. The sixth group (water extract of KD treatment group) received water extract of KD at a dose of 70 mg/kg BW/day. Animals were fed by gavage daily with KD extracts or their vehicles at 2 ml/kg BW/day. After 3 weeks of treatment, they were allowed to have sexual experience by mating with virgin females, so as to test for their fertility. These male rats were further treated until the fourth and fifth weeks at which time sexual behaviors and sperm motility study were performed.

Experiment 2: effect of KD on spermatoc blood flow

Experiment 2.1: chronic effects of alcohol extract of KD on blood pressure, HR, and spermatoc blood flow. The rats were divided into two groups, eight rats each. The first group (AC) received 6.5% PVP in water serving as control. The second group received alcohol extract of KD dissolved in 6.5% PVP as in Experiment 1 at a dose of 70 mg/kg BW/day for 4 weeks. After completion of KD treatment, the rats were prepared for the measurements of blood pressure, HR, and spermatoc blood flow as described above.

Experiment 2.2: acute effects of alcohol extract of KD on blood pressure and spermatoc blood flow. This part of study was carried out in eight intact rats receiving no treatment. After a steady spermatoc blood flow recording was established for about 10 min, alcohol extract of KD dissolved in 6.5% PVP as in Experiment 1 at a dose of 10 mg/kg BW was administered through the femoral vein and spermatoc blood flow was recorded until the effect of KD subsided. This protocol was repeated using 20 and 40 mg/kg doses to obtain a dose-response curve. Finally, 10 µg/kg acetylcholine was administered as a positive control.

Statistical analysis

All results were expressed as mean $\pm$ S.E.M. Significant differences between groups were analyzed by using one-way ANOVA followed by the *post hoc* Tukey's test for % fertility, % organs weight/BW, and blood flow data. The sexual behavior parameters were analyzed by Kruskal–Wallis ANOVA followed by Mann–Whitney U test. The differences between two groups were considered to be statistically significant, if the statistical probability (P value) was less than 0.05.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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