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Aphrodisiac activity of vanari gutika formulated using milk obtained from jersey cow (*Bos taurus)*, indigenous cow (*Bos indicus*) and buffalo (*Bubalus bubalis*)

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Erectile dysfunction (ED) is a condition characterized by the inability to develop or maintain erection of the penis. Ayurveda recommends vanari gutika, prepared from *Mucuna pruriens* as one of the products suggested for the management of sexual dysfunction. We were interested to validate and compare aphrodisiac potential of vanari gutika formulated, as per the principles of Ayurveda, using milk obtained from jersey cow (*Bos taurus)*, indigenous cow (*Bos indicus*) and buffalo (*Bubalus bubalis*). In order to assess the aphrodisiac activity, Swiss Albino mice were used as experimental subjects (n = 6 animals per group) with oral administration of aforesaid three formulations of vanari gutika with different doses i.e., 8.56 mg/mL, 17.14 mg/mL and compared with standard sildenafil citrate (5 mg/kg) and vehicle (milk+ghee) for 7 consecutive days. Animals were observed for mating behavior. Hormonal analysis, sperm count and histopathological studies were carried out to confirm physiological changes. The study exhibit marked changes in sexual behavior. Vanari gutika formulated using milk from indigenous cow at higher dose (17.14 mg/mL) showed significant increase in mounting frequency, hormone levels, sperm count and histopathology which revealed the ruptured seminiferous tubules. These investigations confirmed significant intensification of sexual activities in experimental animals.

Keywords: Aphrodisiac, Erectile dysfunction, Hormonal levels, Mounting frequency, *Mucuna pruriens*

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Erectile dysfunction (ED) is a common medical condition affecting millions of people worldwide. It is defined as the inability to achieve or maintain penile erection which leads to unsatisfactory sexual intercourse. Prevalence of study indicates 152 million men worldwide in 1995 were affected by ED, which can project to 320 million by 2025[1]. Obesity, diabetes, smoking, hypertension, dyslipidemia and metabolic syndrome are risk factors leading to ED.

Most cultures in ancient history like holy texts and sculpture in Hindu Temples has helped society to improve the sexual life[2]. In 1990s Viagra (Sildenafil) was introduced as first approved remedy for impotence[3]. An aphrodisiac is defined as any food or drug that arouses the sexual instinct, induces venereal desire and further increases pleasure, performance. This word is derived from 'Aphrodiate' the Greek Goddess of love and these substances are derived from plants, animals or minerals. In modern therapeutics, treatment of sexual dysfunction includes medications (tadalafil (Cialis®), vardenafil (Levitra®, Staxyn®) and avanafil (Stendra®) which increase blood flow to the penis. Treatment also includes use of mechanical aids (vacuum devices, penile implants), sex therapy, behavioral treatment and psychotherapy. These treatments have high cost, complications like infections, failure of devices and drug side effects like visual disturbances, dizziness, nasal congestions and flushing[2]. Therefore, we were interested to validate and compare aphrodisiac potential of vanari gutika formulated as per the principles of Ayurveda, using milk obtained from indigenous cow (*Bos indicus*), jersey cow (*Bos taurus)* and buffalo (*Bubalus bubalis*).

Vanari gutika is herbal ayurvedic tablet containing kaunch seeds as major constituent. *Mucuna pruriens (kaunch seeds)*, also known as velvet bean (Family Fabaceae, Sub family Papilionaceae) is wide spread fodder plant in tropical and sub-tropical regions of the world. The whole plant is fed as silage containing 1-23% protein, 35-40% fiber. The dried beans contain 20-35% crude protein. Due to high proteins

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concentration (23–35%) it is considered as source of dietary protein. *M. pruriens* plant has long, slender branches; alternate, lanceolate leaves and white flowers with a bluish-purple, butterfly-shaped corolla. The pods or legumes are hairy, thick, and leathery; are shaped like violin sound holes and contain four to six seeds. They are dark brown color and covered with stiff hairs[4]. The beans are used in the treatment of Parkinson's disease.

Methodology

Material

Procurement of raw material like dried kaunch seeds *(Mucuna pruriens)*, milk (jersey cow, indigenous cow and buffalo), Ghee (jersey cow, indigenous cow, buffalo) and honey from Go-Vigyan Anusandhan Kendra Deolapar. Authentication of seeds was done by Dr Dongarwar, Department of Botany, Nagpur University, Nagpur (voucher specimen number 10009).

Preparation of Vanarigutika

Three different formulations were prepared in-house. Approximately 1 kg of dried *Mucuna pruriens* seed were boiled in about 4 L of fresh milk of three breeds of cow independently viz., jersey cow (*Bos taurus)*, indigenous cow (*Bos indicus*), and buffalo (*Bubalus bubalis*), until milk gets viscous. The boiled seeds were triturated with concentrated viscous milk after decoating until a lump mass was obtained. Small sphere shaped gutikas were prepared from the lump mass, allowed to dry and fried in different fresh ghee until brownish red. Gutika were finally coated with sugar syrup and stored in honey[5].

Evaluation of gutika

Pharmaceutical parameters like weight variation, hardness (Rockwell hardness tester), diameter (Screw gauge) and pH (pH meter) were determined using standard instruments and procedures[6]. Physicochemical parameters i.e., ashes, extractive values, along with phytochemical screening for determining presence of phytoconstituents[7] were carried out. Quantitative estimations[8] of in-house vanari gutika formulations was done to ascertain total alkaloid, phenol, flavonoid, tannin, carbohydrates, steroids and protein content equivalent to standard.

Thin Layer chromatographic evaluation

Silica gel G was used as stationary phase for thin layer chromatography. Sample and standard stock solution were prepared in Methanol. The solvent system used was n-butanol –water –glacial acetic acid (2:1:4) to obtain optimum resolution[9].

High Performance Liquid Chromatography Quantification

A stock solution of 100 µg/mL was prepared by dissolving 10 mg of L-Dopa in 10 mL of 0.1 M HCL and diluted up to 100 mL with HPLC grade methanol. Accurately weighed one gram vanari gutika formulation was refluxed with a mixture of methanol and 0.1 M HCL (70:30) for 30.0 min and filtered. The extracts were evaporated to dryness and residue was again dissolved in methanol. Further filtered through 0.45 µm membrane filter and used for HPLC analysis.

Experimental Animals

Swiss Albino mice weighing 25-35 g of either sex were housed under standard laboratory conditions; temperature (24-28℃), relative humidity (60-70%), 12 h. light-dark cycle with solid pellet diet (gold mohar, lipton India) and water *ad libitum* throughout the study except during the experiment[10]. The experimental protocol was approved by Central Animal Ethical Committee of RTMNU Nagpur University (dated 14/08/2017) (Reg. No 92/1999/CPCSEA Dated - 28/04/1999) (Reg. No IAEC/UDPS/2017/37).

Preparation of samples

Vanari gutika formulation 17.14 mg/mL (high dose group) and 8.56 mg/mL (medium dose group) were triturated and suspended in distilled water using CMC (0.5%) for oral administration. The dose was calculated on basis of prescription dose. Similarly, sildenafil citrate was prepared and used as standard.

Experimental Protocol

Sexually active male mice were screened and selected by mating a male with receptive female for two consecutive weeks. Animals showing ejaculation latency shorter than 15 min were selected and divided into six group (n=6). Control (Group 1) received 0.5 mL/kg of distilled water, mice were administered with 5 mg/kg standard sildenafil citrate (group 2)[12].

Group 3,4 received medium and Group 5, 6 received high dose of formulation[6]. Animals were observed for 3 h. for mounting frequency. The numbers of mounts were recorded during a 15 min' observation period at the start of first hour. Then the female was separated for 105 min. Again the female was introduced and the number of mounts was observed for 15 min as before[13,4].

Test for libido

Male albino mice were divided into 8 groups (n=6) where Group 1 (control group) received 10 mL/kg of milk and ghee orally. Group 2 (standard 5 mg/kg), Group 3,5 and 7 received high dose and Group 4,6 and 8 received medium dose of specified vanari gutika formulations, once a day for 7 days. The female mice were made receptive by hormonal treatment, 10 µg of oestradiol benzoate orally 48 h prior to the experiment and 500 µg of progesterone subcutaneously 7 h prior to the experiment[10,11]. The animals were also observed for intromission and ejaculation. The standard animal reading was statistically analyzed by employing two-way analysis of variance (ANOVA) method.

Biochemical estimations

The male mice were sacrificed on day 7, collected blood samples were centrifuged and serum was used for the estimation of testosterone, LH and FSH using respective standard Electro Chemiluminescence Method[15]. The entire hormonal study was performed at SS Pathlab Dhantoli, Nagpur.

Histopathological examination

Histopathological study included fixing of testes in 4% paraformaldehyde in 0.1 M phosphate buffer. Tissues were removed and dehydrated through upgraded ethanol and cleared with xylene and finally embedded in paraffin[15]. Microtome of 7 µm thickness was used for sectioning. Tissues were counterstained with haematoxylin in eosin, then examined and photographed under a Leica DM 2500 microscope[8].

Sperm count

Sperm count is concentration of sperm measured in millions of sperm per cubic centimeter. Semen was diluted in 1:200 having a concentration of 100 mil/mL. The semen normal saline solution of 9.3 cc was taken in a beaker, added with 0.5 cc of semen and mixed thoroughly. Eosin solution (9 cc of 0.5 cc) was taken in another beaker and then transferred 1 cc of mixed semen into second beaker. The solution was placed on Neubaur' shaemocytometer and was observed under LEICA DM 2500 microscope for sperm count[16].

Statistical analysis:

Experimental results were expressed as mean±SEM, followed by one-way analysis of variance (ANOVA). Newman-Keuls test for multiple comparisons was used for determining the statistical significance between different groups. Mounting behavior was followed by two-way ANOVA Bonferroni multiple comparision test.

Results

Physicochemical properties

The results in Table 1 represent the pharmaceutical parameters of vanari gutika formulations prepared in-house using different types of milk. The formulation made up of indigenous cow milk showed greater hardness and pH. The parameters evaluated were found to be within the limits stated by Indian Pharmacopoeia 1996 for tablets. Also, the water soluble ash value, ether and water extractive value was found to be higher as compared to that of jersey cow and buffalo milk, as per Table 2.

Table 1 — Pharmaceutical parameters of Vanarigutika formulation

Sr. no.	Parameter	Jersey Cow	Indigenous Cow	Buffalo
1	Weight variation	Pass	Pass	Pass
2	Hardness (kg/cm)	2.7 ± 0.05	3.0 ± 0.05	2.7 ± 0.05
3	Diameter (mm)	8.5	8.5	8.3
4	pH	5.84	6.21	6.11

Table 2 — Physicochemical properties of Vanarigutika formulation

Sr. no.	Parameter	Jersey Cow	Indigenous Cow	Buffalo
1	Total Ash (% w/w)	0.81	0.90	0.66
2	Acid insoluble Ash (% w/w)	0.06	0.05	0.07
3	Water soluble Ash value (%w /w)	0.08	0.08	0.10
4	Alcohol extractive value (% w/v)	9.27	7.8	12
5	Aqueous soluble extractive (% w/v)	30.8	29.9	32.6
6	Ether extractive value (% w/v)	3.2	5.9	2.6

Phytochemical standardization

Preliminary phytochemical screening mainly revealed the presence of sterols, alkaloids, carbohydrates, proteins, phenolic, amino acids, flavonoids and triterpenoids. Table 3 depicted that the formulation made from indigenous cow milk quantitatively estimates higher percentage of total alkaloid, total phenolic content and steroid content.

Chromatographic evaluations

Table 4 shows the TLC studies of vanari gutika formulations (jersey cow, indigenous cow, buffalo) confirmed the presence of L-dopa, having Rf value 0.60 used as standard (Fig. 1). Rf values of indigenous cow, jersey cow, buffalo milk samples were found to be 0.58, 0.60 and 0.57 respectively. HPLC analysis Table 5 represented greater concentration of L-Dopa in formulation prepared by using indigenous cow milk (Fig. 1).

Aphrodisiac activity

Mounting and intromission frequency, intromission latency and ejaculation frequency was found to be highest in the groups treated with high dose formulation made from indigenous cow milk showing

Table 4 — Thin Layer Chromatography analysis

Sample	No. of Spots	Rf values
Standard (L-dopa)	1	0.60
Jersey Cow (J)	1	0.58
Indigenous Cow(C)	1	0.60
Bufffalo (B)	1	0.57

Table 3 — Quantitative estimations of Vanarigutika formulation

Sr.no	Parameters	Jersey cow	Indigenous cow	Buffalo
1	Total alkaloids (%w/w)	0.46	0.60	0.52
2	Total Phenol content (mg/g) Eq	5.10	2.83	2.15
3	Flavonoid content (mg/g) Eq	2.18	1.92	1.38
4	Tannin content (mg/g) Eq	0.90	1.21	1.08
5	Carbohydrate content (mg/g)	0.39	1.29	1.80
6	Steroids content (mg/g) Eq	16.70	16.83	15.60
7	Protein content (mg/g)	23.82	22.83	27.44

Table 5 — HPLC results

Sr. no	Sample	Concentration taken in µg/ml	Retention Time (min)	Peak area	Concentration µg/ml
1	Standard	10	2.936	1238237	-
2	Indigenous Cow	100	2.948	1845453	2.98
3	Jersey Cow	100	2.914	1551444	2.50
4	Buffalo	100	3.005	1669313	2.69

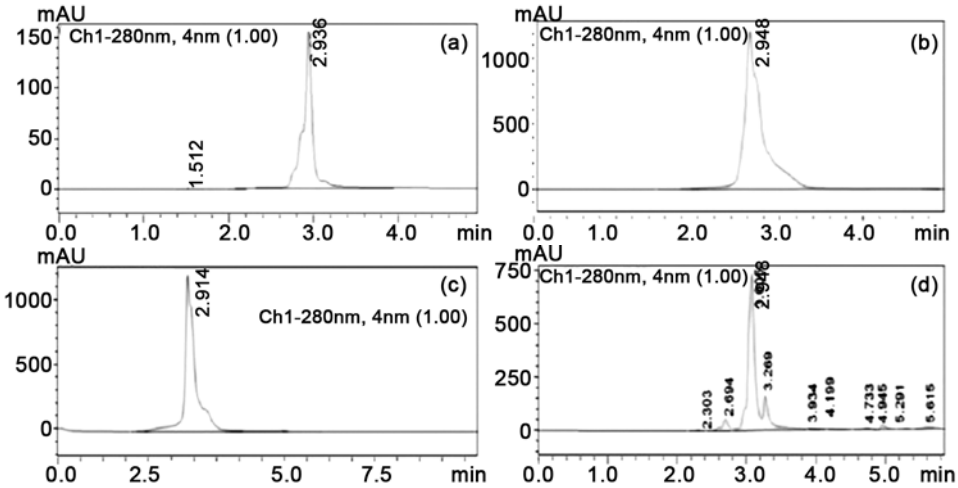

Fig. 1 — High performance Liquid Chromatographic analysis. Fig. A Chromatogram of Standard levodopa 10 mg Fig. B Vanarigutika formulation (ersey Cow) 100 mg Fig. C. vanarigutika formulation (Indigenous cow) 100 mg Fig. D Vanarigutika formulation (Buffalo) 100 mg

significant *($p<0.05$) results. Therefore, the comparatively higher sexual activity indicated the aphrodisiac potential of in-house formulation. However, those treated with jersey cow and buffalo milk formulation revealed moderate activity. The mounting latency was found decreased indicating reduction of time required from introduction to first mount (Fig. 2, 3 & Fig. 4).

Biochemical analysis

The biochemical analysis disclosed higher levels of hormones viz., testosterone, luteinizing, and follicle stimulating hormone in those treated with cow milk formulation (Fig. 5).

Histopathology

The histopathology studies revealed ruptured seminiferous tubules while in control, standard and low dose formulation groups it showed continuous seminiferous tubules intact in interstitial connective tissue and the Leydig cells (Fig. 6). The study revealed that vanari gutika formulated using indigenous cow milk in high dose shows higher aphrodisiac activity as compared to those formulated in jersey and buffalo milk.

Sperm count

The group treated with high dose formulation of indigenous cow milk disclosed high epididymal

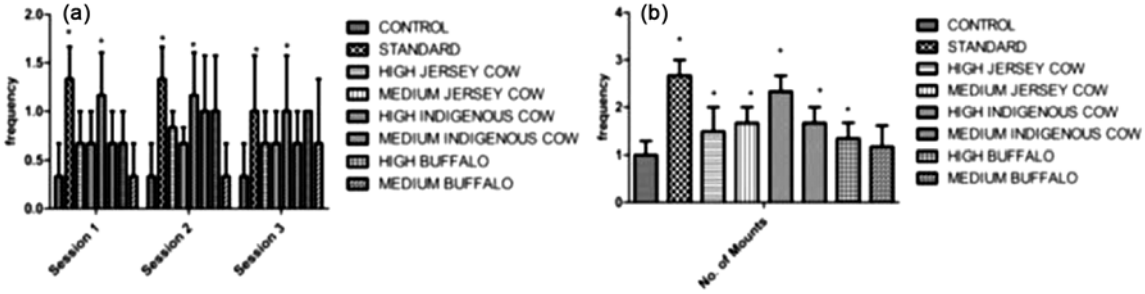

Fig. 2 — A. Mounting behavior sessions B. Mounting Frequency. Values are expressed as mean±S.E.M (n=3) *p<0.05 vs. Control; Two way Anova followed by Bonferroni multiple comparison test

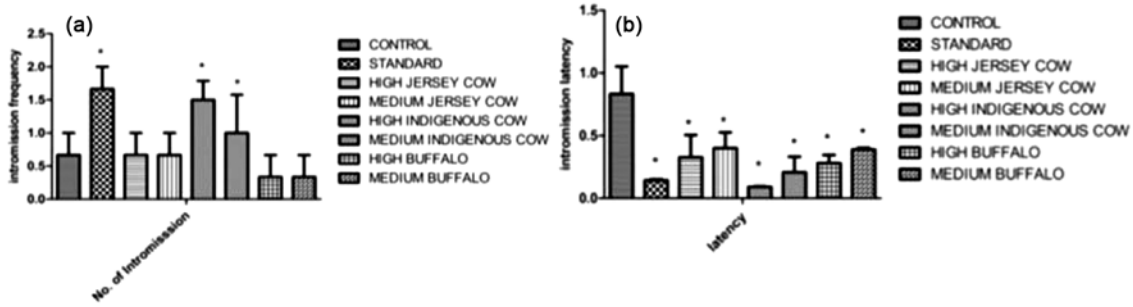

Fig. 3 — A. Intromission frequency B. Intromission latency Values are expressed as mean ±S.E. M. (n=3) *p<0.05 vs. Control; Two way Anova followed by Bonferroni multiple comparison tet

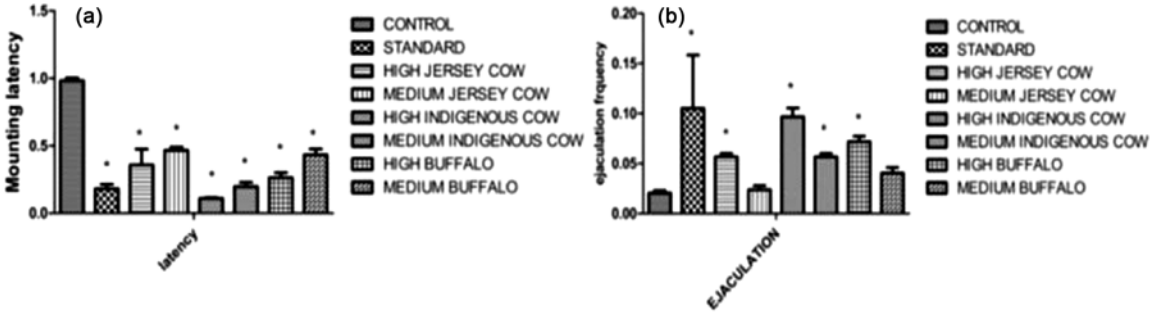

Fig. 4 — A. Mounting Latency B. Ejaculation. Values are expressed as mean ±S.E.M (n=3) *p<0.05 vs. Control; Two way Anova followed by Bonferroni multiple comparison test

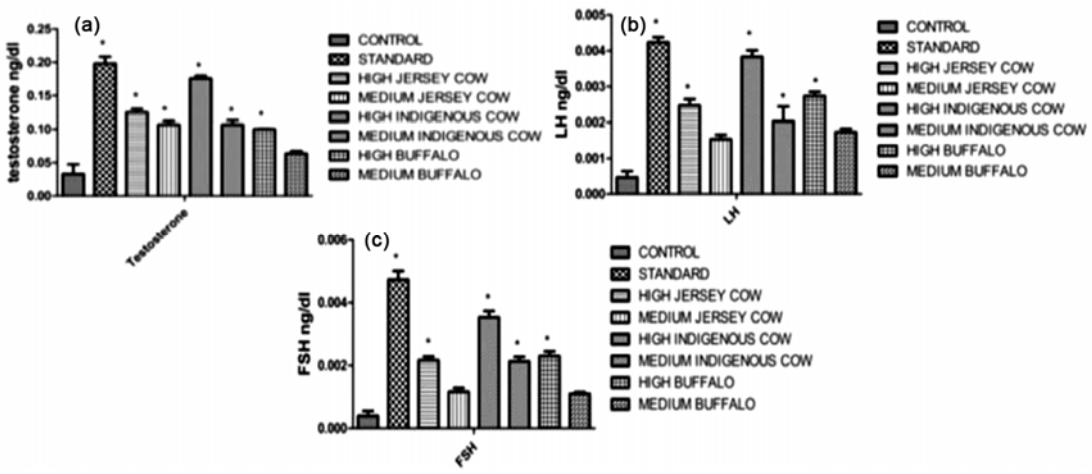

Fig. 5 — Biochemical analysis A. Testosterone level B. Luteinizing hormone C. Follicle stimulation hormone. Values are expressed as mean ± S.E.M (n=3) *p<0.05 vs. control; Two way Anova followed by Bonferroni multiple comparison test

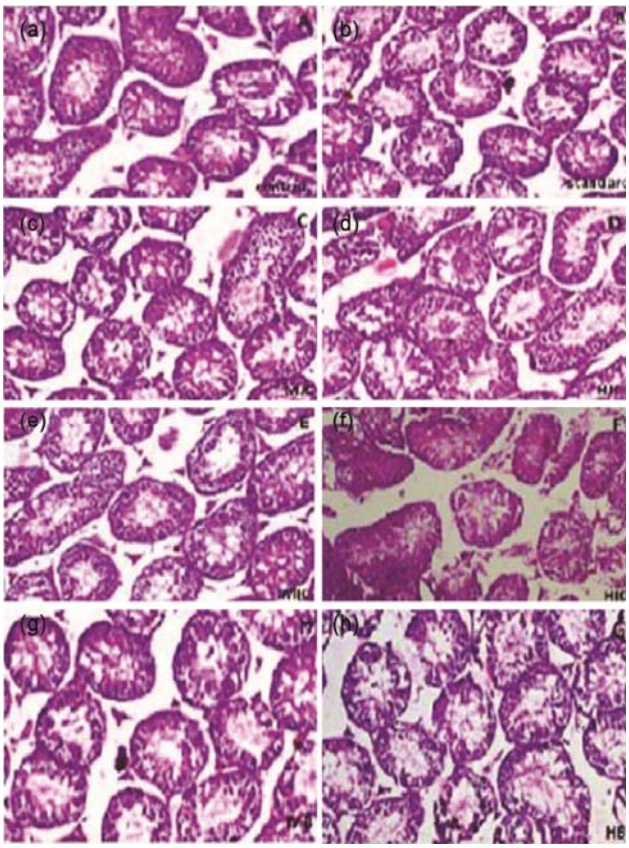

Fig. 6 — Histopathology of Testis (A): Control group, (B): Standard group, (C): High Jersey Cow Group, (D) Medium Jersey Cow group, (C): High Jersey Cow group, (D): Medium Jersey Cow group, (E): High Indigenous cow group, (F): Medium Indigenous cow group, (G): High Buffalo group, (H): Medium Buffalo group.

sperm count comparable to standard. The effect was found to be dose dependent**.** (Table 6)

Table 6 — EpididymalSperm count

Sr. no	Group	Sperm count ($\times 10^6$ Cu/mm)
1	Control	143.66±7.46
2	Standard	807.5±9.53**
3	Medium dose jersey cow	224.16±2.57
4	High dose jersey cow	584.66±50.50**
5	Medium dose indigenous cow	263.16±2.67
6	High dose indigenous cow	710.83±43.15**
7	Medium dose buffalo	248±4.34
8	High dose buffalo	498.72±3.82

Value are expressed as mean ± S.E.M.; n=6; *p<0.05 vs. Control. One-way ANOVA followed by Newman keuls test.

Discussion

Erectile dysfunction is common problem affecting people worldwide. Erection is the process which involve the psychogenic and reflex erection and erectile dysfunction is the condition in which person is unable to maintain erection. Sexual functions declines over ageing. Ten years of research, that has provided data regarding the prevalence of sexual dysfunctions has been reviewed. Global estimation stated that over 150 million people suffered in 1995 which would reach to 320 million by 2025. The synthetic drugs available in market are not only expensive but also showed adverse effects like abnormal vision, muscle pain, nausea, flushing etc. Instead of opting for expensive allopathic medicines, many people rely on economic herbal medicines for health care. In present research work vanari gutika formulation is made with the help of different ghee

and milk obtained from jersey, indigenous cow and buffalo. Kevanch seed is one of the major components of the vanari gutika and having a potent aphrodisiac potential. According to Ayurveda; vanari gutika is recommended for the management of sexual dysfunction. The aim of the comparative study was to determine formulation providing more potent activity along with its quality control profiling and evaluation of preclinical aphrodisiac potential. The present study also includes the pharmacognostic and pharmacological evaluation of seeds of *Mucuna pruriens* (Kevanch seed, family: Fabaceae). Estimation of various qualitative and quantitative parameters helped us to maintain quality and purity of formulation. The preparation procedure of gutika includes frying of gutika in ghee, which provide an oil/fat coating on the gutika and results in extended disintegration time; thus can be consumed in the form of chewable tablets. Due to negligible moisture content, formulation may not lead to early deterioration. Preliminary phytochemical screening helped in identification of the chemical nature of the active constituents. TLC was performed for the determination of different components present in the formulations. The aphrodisiac potential evaluated on the basis of mating behavior parameters exhibited marked changes in sexual behavior in male mice that were treated with standard drug or with 200 mg/kg high/medium dose of vanari gutika formulations made in indigenous cow milk. The study revealed that high dose of indigenous cow vanari gutika formulation shows increase in mounting frequency, hormone level, increased sperm count and histopathology revealed the ruptured seminiferous tubules.

This is the first study to report that vanari gutika prepared in indigenous cow milk enhances the sexual behaviors of male mice as compared to control and those prepared in milk of jersey cow and buffalo.

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