

## ORIGINAL ARTICLE

**Improvement of penile erection, sperm count and seminal fructose levels *in vivo* and nitric oxide release *in vitro* by ayurvedic herbs**M. Thakur<sup>1,2,3</sup>, D. Thompson<sup>1</sup>, P. Connellan<sup>1</sup>, M. A. Deseo<sup>1</sup>, C. Morris<sup>1</sup> & V. K. Dixit<sup>2</sup><sup>1</sup> Centre for Phytochemistry and Pharmacology, Southern Cross University, Lismore, NSW, Australia;<sup>2</sup> Department of Pharmaceutical Sciences, Dr. H. S. Gour University, Sagar (M.P.), India;<sup>3</sup> Zentral Institut für Labor medizin und Pathobiochemie, Charité University of Medicine, Campus Benjamin Franklin, Berlin, Germany**Keywords**

Aphrodisiac—nitric oxide—penile erection index—seminal fructose concentration—sperm count—Vajikaran-rasayana

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Accepted: January 22, 2010

doi: 10.1111/j.1439-0272.2010.01068.x

**Summary**

In the present study, the effect of four Vajikaran Rasayana herbs on penile erection, sperm count, seminal fructose content *in vivo* and nitric oxide (NO) release *in vitro* was assessed. Lyophilised aqueous extracts of *Asparagus racemosus* Willd. (AR), *Chlorophytum borivilianum* Sant. F. (CB), *Curculigo orchoides* Gaertn. (CO), and *Dactylorhiza hatagirea* (D. Don) Soo (DH) were orally administered at 100 mg/kg body weight to Wistar strain male albino rats. Penile erection index and sperm count were determined by visual observation; the seminal fructose concentration was measured spectrophotometrically using resorcinol reagent; and NO release was assessed in a mouse macrophage cell line (RAW264) spectrophotometrically using a commercial Griess reagent kit. Penile erection index, sperm count, seminal fructose concentration and *in vitro* NO release were the parameters measured. A significant effect on the sperm count, seminal fructose content and penile erection index was observed upon treatment with the extracts. The effect of extracts on inducible NO release *in vitro* directly correlated with the enhanced erectile function *in vivo*. The aphrodisiac claims attributed to the four Vajikaran Rasayana herbs were tested and a distinctive effect of all extracts tested was observed, with *C. borivilianum* showing a highly significant response for all parameters measured *in vivo* and *in vitro*. The present study also provides a good correlation between the *in vivo* improvement of penile erection and *in vitro* NO releasing activity of the extracts. Increase in seminal fructose levels and sperm count further validates the role of these herbs in improving reproductive function.

**Introduction**

Aphrodisiacs have most often nestled in the arms of medicine, in whatever form medicine was practised at a given time and place. Kamasutra of Vatsyayan, a treatise on the art and science of love making, discusses the use of numerous herbs for enhancing virility (Hooper, 2002). Ayurveda, the ancient Indian system of medicine, holds a special position and is the basis of drugs recommended in Kamasutra (Puri, 1977). *Vajikaran* is a specialty in Ayurvedic system of medicine in India dealing with herbs promoting fertility, virility and strength. In the present study

*Asparagus racemosus* Willd., *Chlorophytum borivilianum* Sant. F., *Curculigo orchoides* Gaertn. and *Dactylorhiza hatagirea* (D. Don) Soo were evaluated for their folkloric claims of being used as aphrodisiacs and sexual tonic (Puri, 2003). All the investigated herbs are also mentioned in Ayurvedic treatise like Sushruta Samhita and Charak Samhita, thus leading to the present investigations (Triveni, 1976). Use of these herbs for improving the sexual dynamics and increasing the overall potency is an age old practice but lack of scientific evidence in support of these drugs thwarts their recognition as useful remedies for sexual dysfunction.

Previous studies on the herbs have been successful in validating their role in improving sexual behaviour and ameliorating sexual dysfunction in rats (Thakur *et al.*, 2009). In the present study, we investigated the effect of administration of lyophilised aqueous extract of four herbs on *in vivo* sperm count, seminal fructose level, penile erection index (PI) and nitric oxide (NO) release activity *in vitro*. A complete improvement of reproductive parameters along with an improved sexual behaviour and enhanced erectile function are a guiding principle behind the use of aphrodisiacs, therefore testing of herbal extracts for penile erection and NO release could provide lucid evidence for their vestige as potent aphrodisiacs (Argiola *et al.*, 1988). Similarly, aphrodisiac herbs have also been attributed with their ability to assist in semen production and preservation of the sperm count *in vivo* and *in vitro* (Sharma *et al.*, 2009). With these aspects as background, the aqueous extracts of all these herbs were subjected to screening.

## Materials and methods

### Plant materials

All the plant materials were purchased from the local market and identified at the Department of Pharmaceutical Sciences, Dr H.S. Gour University Sagar (M.P.), India. Voucher specimens of *Asparagus racemosus* Willd. (AR), *Chlorophytum borivilianum* Sant. F. (CB), *Curculigo orchoides* Gaertn. (CO), and *Dactylorhiza hatagirea* (D. Don) Soo (DH) have been submitted and assigned voucher specimen codes MT-2007-21 to 24 respectively. Roots of all the four plants were coarsely powdered, defatted by extraction with petroleum ether and the defatted material was extracted with water as per the methodology previously reported [8]. The water extracts were used in the succeeding *in vivo* and *in vitro* assays.

### Test animals

Wistar strain male albino rats weighing 170–190 g were fed on a standard pellet diet and water *ad libitum*. The animals were housed at room temperature ( $24 \pm 2^\circ\text{C}$ ) on a reversed day-night cycle (06:00 to 18:00 hours). Determination of PI was carried out under dim red light and the episodes of penile erection were video recorded (Olympus EX120, New Delhi, India). Animal experimentation were performed with permission from the institutional ethical committee of Dr H.S. Gour University, Sagar (M.P.), India, and the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India were adhered to during the experimentation.

Male rats were divided into six groups with each group comprising of six animals. One group was administered with water only and served as the control (Group I). Four groups were treated with an oral dose of aqueous extracts (100 mg/kg body weight) once daily for 14 days, and the sixth group served as the positive control as outlined below:

Group I – administered water only and served as control;

Group II – 100 mg AR extract per kg body weight (b.w.);

Group III – 100 mg CB extract per kg b.w.;

Group IV – 100 mg CO extract per kg b.w.;

Group V – 100 mg DH extract per kg b.w.;

Group VI – testosterone in arachis oil (0.5 mg/kg b. w.) twice weekly by intramuscular route [9].

The animals were treated for 14 days and observations were recorded on days 0 and 14 for PI. On day 14, following documentation of PI, rats were killed by cervical decapitation and their seminal vesicles and epididymes were removed, freed from adhering material and subjected to the determination of sperm count as well as seminal fructose content.

### Penile erection index

Determination of penile erection in noncontact position was determined by the methodology reported by Matsumoto *et al.* (1997). In brief, each male rat was placed in a transparent plexiglass cabin ( $60 \times 40 \times 40$  cm) that was divided in half by two sheets of plastic fibre mesh preventing contact but allowing auditory, visual, and olfactory stimuli. Ventral as well as lateral viewing and recording of the whole experimentation was facilitated by appropriately placing a mirror. After a 5-min adaptation period, the test was started by placing an estrous female on the other side of the cage. A recording of the behavioural parameter was carried out for 15 min after the first erection. Cages were cleaned before shifting the animals of different groups. The number of erections were recorded and tabulated. Erection in rats was marked by the visibility of the penis out of its sheath. Grooming of penis is another indicator of penile erection in rats and was recorded as well. PI was calculated as per the methodology reported by Islam *et al.* (1991).

### *In vivo* sperm count

After the PI determination and cervical decapitation, left and right epididymes of all the rats were placed into 1 mL of 1% sodium citrate solution (pH 7.2) and squashed thoroughly with the help of needle and forceps until a milky suspension was obtained. The solution was

filtered through 80  $\mu\text{m}$  mesh and the filtrate was stained with 1% aqueous eosin Y. The liquid was collected in a leucocyte pipette and subjected to counting of mature spermatozoa as per the standard procedure (Hadley *et al.*, 1981).

#### Corrected seminal fructose content

The determination of corrected seminal fructose content was performed using the standard methodology (Gonzales & Villena, 2001) with slight modification. In brief, the seminal vesicles of decapitated rats were weighed, minced and homogenised using a tissue homogeniser. The fructose content was measured spectrophotometrically using resorcinol reagent (Erb *et al.*, 1956). Corrected seminal fructose content, which correlates with sperm motility, was calculated as follows (Gonzales *et al.*, 1988):

$$\text{Corrected seminal fructose content} = (\log_{10} \text{sperm count}) \times (\text{seminal fructose concentration}).$$

#### Statistical analysis

The *in vivo* data are expressed as the mean  $\pm$  standard error of the mean. The results were analysed by comparison of the treatments (groups II–VI) versus control (group I) using Dunnet's test. A *P*-value of  $<0.05$  (\*) is considered significant and *P*  $< 0.01$  (\*\*) is highly significant.

#### *In vitro* nitric oxide activity

RAW264 cells (mouse macrophage) were routinely cultured in a humidified 5% CO<sub>2</sub>–95% air incubator in Dulbecco's modified eagles medium (DMEM) supple-

mented with 10% foetal bovine serum (FBS), 20  $\mu\text{M}$  L-glutamine, 1 mM pyruvate, 200 U/mL penicillin G, 200  $\mu\text{g}/\text{mL}$  streptomycin and 4.5 mg/mL of D-glucose at 37 °C. Cells were allowed to attach to the bottom of a 96-well plate overnight and were then exposed to the extracts (10 mg/mL concentration) for 24 h. The cell media were assayed for nitrate and nitrite concentrations using a commercial Griess reagent kit (Cayman Chemicals, Michigan, USA) with the resultant absorbance being read at 550 nm using a VictorX™ plate reader (Perkin Elmer, Salem, MA, USA).

#### Results

The effect of various extracts and control on sperm count, seminal fructose levels and PI are shown in Table 1. Results showed that all the four plant extracts produced a significant increase in the mean sperm count when compared with the controls (*P*  $< 0.05$ ). The percentage increase in sperm count was 25.23% for group II (AR-treated), 28.04% for group III (CB-treated), 23.87% for group IV (CO-treated), and 28.22% for group V (DH-treated) as compared with control group animals (group I). All the groups treated with the plant extract showed higher percentage increase in sperm count than the group that was treated with testosterone (group VI), which showed only a 9.80% increase compared to the control group.

The seminal fructose concentration was measured and the resulting data were used to calculate for the true corrected seminal fructose concentration, which is a better marker of the function of seminal vesicles (Gonzales & Villena, 2001). The corrected seminal fructose concentration in mg/g of seminal vesicle was found to be 5.63  $\pm$  0.15 in control group animals. For the test extract-treated

**Table 1** Effect of treatment with various 'Vajikaran Rasayan' herb aqueous extracts on sperm count, seminal fructose level and penile erection index

Group <sup>a</sup>	Sperm count ( $\times 10^6$ )	Log <sub>10</sub> sperm count	Seminal fructose concentration (mg/g of seminal vesicle)	Corrected seminal fructose concentration (mg/g of seminal vesicle)	Penile erection index
I	110.2 $\pm$ 12.3	8.04	0.70 $\pm$ 0.02	5.63 $\pm$ 0.15	24.6 $\pm$ 1.4
II	138.0* $\pm$ 11.2	8.14	0.80* $\pm$ 0.01	6.51* $\pm$ 0.17	48.7* $\pm$ 1.8
III	141.1** $\pm$ 10.4	8.15	0.90** $\pm$ 0.02	7.33** $\pm$ 0.19	59.1** $\pm$ 1.4
IV	136.5* $\pm$ 11.4	8.14	0.76 $\pm$ 0.03	6.18* $\pm$ 0.10	51.6** $\pm$ 1.6
V	141.3** $\pm$ 9.6	8.15	0.71 $\pm$ 0.05	5.79* $\pm$ 0.25	49.8* $\pm$ 1.1
VI	121.0* $\pm$ 11.2	8.08	0.58* $\pm$ 0.03	4.69 $\pm$ 0.92	51.4** $\pm$ 1.1

Statistical significance within the column by Dunnet's test: \**P*  $< 0.05$ , \*\**P*  $< 0.01$ . No asterisk means there is no significant difference between the treatment and the control (group I).

<sup>a</sup>Treatments: group I – control (treated with vehicle only); group II – *A. racemosus* aqueous extract (100 mg/kg b.w.); group III – *C. borivilianum* aqueous extract (100 mg/kg b.w.); group IV – *C. orchiooides* aqueous extract (100 mg/kg b.w.); group V – *D. hatagirea* aqueous extract (100 mg/kg b.w.); group VI – testosterone in arachis oil (0.5 mg/kg b.w.).

groups, AR recorded  $6.51 \pm 0.17$ ; CB,  $7.33 \pm 0.19$ ; CO,  $6.18 \pm 0.10$ ; and DH,  $5.79 \pm 0.25$ . Interestingly, the value was lower for testosterone treated group ( $4.69 \pm 0.92$ ) than the control. The corrected seminal fructose levels demonstrated a 15.63% increase in the case of AR, 30.20% in CB, 9.77% in CO, and 2.84% in case of DH treated animals. In contrast, the corrected seminal fructose concentration of the testosterone-treated animals was found to be 16.70% less than the control.

The data for PI in Table 1 showed an enhanced erectile function promoting activity in all the groups treated with the extracts and in the group treated with testosterone compared with the control. The PI for groups II to VI showed at least twofold increase compared with the control, with CB-treated group (PI = 59.1) > CO-treated (51.6); and testosterone-treated (51.4) > DH-treated (49.8) > AR-treated (48.7).

Inducible NO release was evaluated using a macrophage cell line and the relative % enhancement in NO release compared with the control ( $4.93 \pm 0.4 \mu\text{M}$ ) is shown in Fig. 1. The relative increase in NO release was highest in case of CB ( $49.5 \pm 1.6 \mu\text{M}$ ) ( $P < 0.005$ ). The next to follow was CO ( $24.1 \pm 1.3 \mu\text{M}$ ) ( $P < 0.005$ ); DH showed a  $12.9 \pm 1.7 \mu\text{M}$  ( $P < 0.01$ ) concentration of NO closely followed by AR with a  $9.2 \pm 1.3 \mu\text{M}$  NO release.

## Discussion

The increased sperm count in extract-treated groups suggests that the herbal drugs under test provide a conducive environment for the survival of spermatozoa, which may in major part be contributed by the carbohydrate rich composition of the extracts (Wiwanitkit, 2005; Thakur & Dixit, 2008). The presence of steroid compounds and progenitors of testosterone biosynthesis has already been validated in all the four herbs under investigation (Uniyal

*et al.*, 2002; Thakur & Dixit, 2006; Chauhan *et al.*, 2007). Therefore, the overall composition of the extract supports sperm production as well as preservation.

The sperm count of the testosterone treated group was significantly higher than the control but the seminal fructose concentration was significantly lower. This resulted in a lower corrected seminal fructose concentration, although the value is not significantly different than the control. This thus provides evidence that treatment with hormones alone is not an appropriate methodology for the treatment of oligozoospermia and the overall reproductive mechanism is dependent on proper nutrition to the spermatozoa.

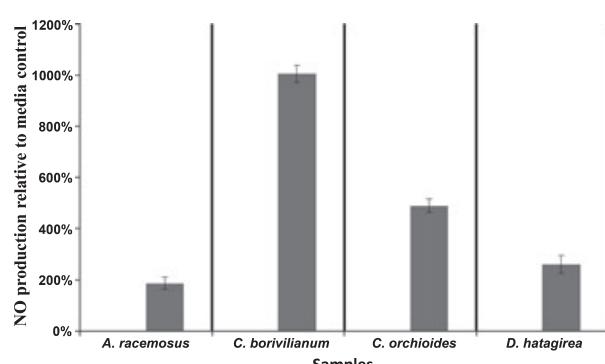
Corrected seminal fructose level further validated the role of extracts under investigation in improving the seminal parameters, which hold a strong correlation with an improved fertility. In general, a coherence from all the parameters evaluated *in vivo* suggests a composite role of extract in improving the sperm count on one hand and maintaining it by providing enough nutritive replenishment. An improvement in sperm count as well as corrected seminal fructose distinctively correlates with the purported ethno-pharmacological usage of these herbs in oligozoospermia and infertility (Anonymous, 2002).

Enhanced erectile function is directly correlated to enhanced sexual performance in most cases (Fink *et al.*, 2002). Penile erection is under direct control of NO release via neuronal, endothelial and inducible NO synthase activity (Burnett *et al.*, 1992). The enhanced PI activity *in vivo* of the extracts directly correlated with the inducible NO activity *in vitro* by the extracts of all the drugs, and is suggestive of improved erectile response and enhanced sexual pleasure, which are desirable attributes of any aphrodisiac agent.

## Conclusion

The results of the present study give support to the aphrodisiac claims attributed to the four Vajikaran Rasayana herbs tested. Of the four herbs tested, CB consistently showed highly significant response for all the parameters tested *in vivo* (sperm count, corrected seminal fructose concentration and PI) and *in vitro* (NO release activity). The present study also provides a good correlation between the *in vivo* improvement of penile erection and NO releasing activity of the extracts. Increase in seminal fructose levels and sperm count further validates the role of these herbs in improving reproductive function.

Impairment of sexual functions remains a major cause of psychological distress and associated personality related disorders if remained untreated (Laumann *et al.*, 1999). Herbal drugs and remedies have been a part of the lexicon of traditional system of medicines throughout the world



**Fig. 1** Effect of aqueous extracts of various Vajikaran Rasayana herbs at 10 mg/mL concentration on *in vitro* NO production by RAW264 cells in the absence of lipid polysaccharides.

and numerous herbs find usage as aphrodisiacs (Puri, 2003). Vajikaran herbs are proclaimed to enhance sexual pleasure and performance and the results of the present study along with previously reported data provide support for aphrodisiac claims attributed to all the four herbs investigated (Thakur & Dixit, 2007).

### Acknowledgement

One of the authors MT would like to thank DEEWR, Australia, for providing Endeavour Research fellowship.

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