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An overview of the current methodologies used for evaluation of aphrodisiac agents

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ABSTRACT

Discoveries in the past two decades have continued to improve our understanding of the pathophysiology of erectile dysfunction disease and animal models have played a significant role to define the basic mechanisms of erectile dysfunction treatment. Both *in vitro* and *in vivo* models have been developed in the past years to study the aphrodisiac agents. Methods that are used in aphrodisiac study can be categorized into physical methods including male sexual behavior (mount frequency, mount latency, intromission frequency, intromission latency, ejaculation frequency, post-ejaculatory interval, couplatory rate, index of libido, computed male sexual behavior parameter), pendiculation study, orientation behavior, determination of hesitation time & attraction towards female, test of potency, test for libido, penile microcirculation study, Intracavernous pressure study and biochemical methods, histopathology, sperm count, Fructose content in seminal vesicles, sperm preservation, organ weight, hormonal determination, assay of nitric oxide synthase, *In vitro* nitric oxide release & androgen receptor protein. This review aims to highlight some of the new and currently used experimental models that are used for the evaluation of aphrodisiac agents.

1. Introduction

1.1. Aphrodisiac

Aphrodisiac is the word derived from Aphrodite, the Greek goddess of sexual, love and beauty. An aphrodisiac is defined as an agent (food or drug) that arouses sexual desire. From time immemorial man's endeavour have been to increase his sexual powers. When man did not know metals and used only stones he exhibited his sexual powers by ritual dances accompanied by hunting. This lead early man was motivated by his quest for food, sex and self-preservation. The possibility of bioactive aphrodisiacs which may be derived from plants, animals or minerals, has been attractive throughout recorded history. Aphrodisiac are mentioned there as Vajikaranas, the word vaji meaning horse and karanta meaning making *i.e.* Measure to excite lust by charms *etc*[1-3].

Erectile dysfunction, sometimes, which also may imply to refer to "impotence," is the repeated inability to get or keep an erection firm enough for sexual intercourse. The causes of ED are varies from one individual to another. For whatever cause, since an erection requires a precise sequence of events, ED can occur when any of the events is disrupted. This sequence includes nerve impulses in the brain, spinal column, and area around the penis, and response in muscles, fibrous tissues, veins, and arteries in and near the *corpora cavernosa*. Thus, ED causes reported include, damage to nerves, arteries, smooth muscles, and fibrous tissues. These are often as a result of diseases, such as diabetes, kidney disease, chronic alcoholism, multiple sclerosis, atherosclerosis, vascular disease, and neurologic diseases that account for about 70 percent of ED cases. NIH reported that between 35 and 50 percent of men with diabetes experience ED. NIH further reported that the usage of many common medicines such as blood pressure drugs,

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antihistamines, antidepressants, tranquilizers, appetite suppressants, and cimetidine (an ulcer drug) can produce ED as a side effect. Nevertheless, psychological factors such as stress, anxiety, guilt, depression, low self-esteem, and fear of sexual failure cause 10 to 20 percent of ED cases. In addition, men with a physical cause for ED frequently experience the same sort of psychological reactions (stress, anxiety, guilt, and depression). Other possible causes are smoking, which affects blood flow in veins and arteries, and hormonal abnormalities, such as not enough testosterone. In modern medication of erectile dysfunction, the oral prescription medication of popular Viagra (Sildenafil) is effective, but in some men it is not compatible and Sildenafil works in less than 70% of men with various etiologies and has certain side effects[4]. The availability of Viagra has brought millions of couples to ED treatment. Oral testosterone can reduce ED in some men with low levels of natural testosterone, but it is often ineffective and may cause liver damage[7]. Other drugs such as Yohimbine, papaverine hydrochloride used under careful medical supervision, phentolamine, and alprostadil (marketed as Caverject) widen blood vessels[5].

Penile erection is a hemodynamic event in the penis. It is the end result of relaxation of the cavernous tissue that involves both the central nervous system and the local factors[5]. The basic concepts of penile erection were obtained from *in vivo* and *in vitro* animals models. The present review is an attempt to update the information on the aphrodisiac animal's models.

1.2 Guidelines follow during experiment

The following guidelines followed during experiment:

- Males were kept individually but females were kept in groups.
- Training of each male for 15 min at a time was performed until sexual behavior was elicited and when the behavior was noticed, males were exposed to receptive females (1 male with 5 females);
- Repeated training to overcome the lack of sexual response in the presence of observers;
- The study was conducted in a silent room under dim red light;
- Any jerking movement of the mating area was avoided to enable the rats to chase each other;
- Cleaning of the mating area was performed after each trial, since the urine trails left by one rat might alter the sexual behavior of the next rat[3].

1.3. Evaluation of aphrodisiac agents

The therapeutic value, efficacy and toxicity of drug may be evaluated in animals experimentally, followed by clinical trials. Both *in-vivo* and *in-vitro* animal models are employed to assess aphrodisiac activity in experimental animals (rat, mice & guinea pig etc.)

2. Parameters used in assessing aphrodisiac activity

For the study of aphrodisiac activity many *in-vitro* and *in-vivo* models have been used.

2.1. Physical methods: mating behavior test

The mating behavior tests would be carried out by the methods of Kolodny & Govier *et al*., and modified by Walsh *et al*[7-9]. Briefly, healthy and sexually experienced male albino rats that show brisk sexual activity should be selected for the study. After extract administration at various concentration to various groups of the animals depending on the experimental design and objective(s) of the study, the male animals should be brought to the laboratory and exposed to red dim light (in 1 w fluorescent tube in a laboratory of 14′ × 14′) at the stipulated time of testing daily for some days (3-6 d) before the experiment.

The female animals should be artificially brought into oestrus phase (heat) as the female rats allow mating only during the oestrus phase by administering either suspension of ethinyl oestradiol orally at the dose of 100 μg/animal 48 h prior to the pairing and subcutaneous administration of progesterone at the dose of 1 mg/animal 6 h before the experiment or alternatively by the sequential administration of estradiol benzoate (10 μg/100 g body weight) and progesterone (0.5 mg/100 g body weight) through subcutaneous injections, 48 h and 4 h respectively prior to pairing. The receptivity of the female animals should be confirmed before the test by exposing them to male animals, other than the control and test animals. The most receptive females should then be selected for the study. The experiment should be conducted at 20:00 h in the same laboratory and under light of same intensity. The receptive female animals should be introduced into the cages of male animals in the ratio 1 female to 1 male. The observation for mating behaviour should commence immediately and continued for first 2 mating series. The test should be terminated if the male failed to evince sexual interest. Any female animal that do not show receptivity should be replaced by another artificially 'warmed' female. The occurrence of events and phases of mating may be called out to be recorded on audio-cassette as soon as they appeared. Their disappearance should also be called out and recorded. Later, the frequencies and phases should be determined from cassette transcriptions. The parameters of male sexual behavior that should be monitored should include:

I. Mount frequency

Mounting is defined as the climbing of one animal by another usually from the posterior end with the intention of introducing one organ into another. Mount may also be

operationally defined as the male assuming the copulatory position but failing to achieve intromission. Mount Frequency (MF) is therefore defined as the number of mounts without intromission from the time of introduction of the female until ejaculation.

II. Intromission frequency

Intromission is the introduction of one organ or parts into another. *e.g.* the penis into the vagina. Intromission Frequency (IF) is therefore defined as the number of intromissions from the time of introduction of the female until ejaculation.

III. Mount latency

Mount latency (ML) is defined as the time interval between the introduction of the female and the first mount by the male.

IV. Intromission latency

Intromission latency (IL) is the time interval from the time of introduction of the female to the first intromission by the male. This is usually characterized by pelvic thrusting, and springing dismounts.

V. Ejaculatory latency

Ejaculation is the act of ejecting semen brought about by a reflex action that occurs as the result of sexual stimulation. Ejaculatory latency (EL) is defined as the time interval between the first intromission and ejaculation. This is usually characterized by longer, deeper pelvic thrusting and slow dismount followed by a period of inactivity or reduced activity.

VI. Post–ejaculatory interval

Post–ejaculatory interval (PEI) is the time interval between ejaculation and the first intromission of the following series[11].

VII. Copulatory rate

Copulatory rate was calculated by determining the number of mounts plus number of intromissions divided by the time from the first mount until ejaculation.

$$\text{Copulatory rate: } \frac{\text{Number of mounts + Number of intromissions}}{\text{Time from first mount till ejaculation}}$$

VIII. Index of libido

Index of Libido is defined as the ratio of number mated to number paired expressed in percentage. This can be expressed mathematically as:

$$\text{\% index of libido} = \frac{\text{Number mated}}{\text{Number paired}} \times 100$$

IX. Computed male sexual behaviour parameters

Using the above parameters of sexual behaviour, the following can thus be computed:

The parameters can be counted as:

$$\text{a) \% Mounted} = \frac{\text{Number mounted}}{\text{Number paired}} \times 100$$

$$\text{b) \% Intromitted} = \frac{\text{Number of intromissions}}{\text{Number paired}} \times 100$$

c) Intromission ratio =

$$\frac{\text{Number of Intromission}}{\text{Number of mounts + Number of intromissions}} \times 100$$

$$\text{d) \% Ejaculated} = \frac{\text{Number of ejaculations}}{\text{Number paired}} \times 100$$

$$\text{e) Copulatory efficiency} = \frac{\text{Number of intromissions}}{\text{Number of mounts}} \times 100$$

f) Intercopulatory efficiency = Average time between intromissions[10,11].

Any medicinal plant with aphrodisiac potential should produce statistically significant increase in the indices of sexual vigor of mount and intromission frequencies, significant decrease in mount and intromission latencies. These indices are indicators of stimulation of sexual arousability, motivation and vigour[12,13]. The significant decrease in mount and intromission latencies as well as significant increase in computed male sexual behavior parameters of %mounted, % intromitted, %ejaculated and the reduction in intercopulatory efficiency are indications of sustained increase in sexual activity and aphrodisiac property inherent in the plant extract[11].

2.2. Test for libido

Sexually experienced male albino rats should be kept singly in separate cages during the experiment. The female rats should be made receptive by hormonal treatment and all the animals should be accustomed to the testing condition as previously presented in mating behavior test. The animals should be observed for the mounting frequency (MF) on the evening of specific day according to the design of the experiment (likely 7th day) at 20:00 h. The penis should be exposed by retracting the sheath and apply 5% xylocaine ointment 30, 15 and 5 min before starting observations. Each animal should be placed individually in a cage with the receptive female rat in the same cage. The number of mountings should be noted. The animals should also be observed for intromission and ejaculation[9,14].

2.3. Test for potency

The male animals are kept singly. The Extracts are administered before half or one hour of the experiment. On the 8th day test for penile reflexes would be carried out by putting the animal on its back in a glass cylinder with partial restrain. The perpetual sheath would be pushed behind the glans for 15 min to stimulate genital reflexes. Total penile reflexes are obtained as sum of erections, quick flips and long flips.

From the above listed components of penile erection, the Total Penile Reflexes (TPR) which is the sum total of each of the components of penile erection *i.e.* E + QF + LF is obtained. Statistically significant increase in the frequency of penile reflexes suggests a medicinal plant with aphrodisiac potential[9,15,16].

2.4. Penile microcirculation study

Laser doppler flow meter may used for determine penile microcirculations. Intravenous anaesthesia of pentoborbitol sodium would given (dose 30 mg/kg body weight). Penile sheath would retracted manually, after 10 min of adaptation the Laser Doppler Flow detection probe would be positioned in a holder close (2-3 mm) to the dorsal side of penis. Average flow units (flux) within 10 min of the test would be calculated.

2.5. Intracavernous pressure (ICP) study

After 12 h of plant extracts administration animals would be given intraperitoneal anaesthesia of sodium pentobarbital (50 mg/kg body weight). This should be followed by the incision of the penile skin and degloving of the prepuce to expose the *corpora cavernosa*. A 26 gauge needle connected to a polyethylene tube (PE-50) filled with NSS with 100 IU/mL of heparin on one side of the *Corpora cavernosa* is inserted for ICP measurement. Another 22 gauge needle is placed into the right carotid artery connected to a PE-tube for the measurement of mean arterial pressure (MAP). Both tubes should be connected to blood pressure transducers which should also be connected to a data acquisition board via transducer amplifiers. Computers can be used to see real time display and recording of pressure measurements (mmHg). Similarly, the major pelvic ganglion, pelvic and cavernous nerves can be exposed by a midline abdominal incision. The cavernous nerve can then be stimulated by using a square pulse stimulator connected to a platinum bipolar electrode positioned on the cavernous nerve using five volts with a frequency of 50 Hertz and duration of 5 min as stimulus parameters. The stimulation may be done three times and the ICP can then be recorded. The ICP should be allowed to return to baseline before the next stimulation. Statistically significant increase in ICP may imply their role on nitric oxide (NO) and erectile function. Medicinal plants with aphrodisiac potential should be capable of stimulating cavernous nerve which normally should lead to increase in NO and cyclic Guanosine Phosphate (cGMP) signalling in corpus cavernosal smooth muscle relaxation. The subsequent arteriolar dilation leading to increased arterial inflow and impaired venous return (due to engorgement of the cavernosum) builds up a pressure system within the corpora that result in penile tumescence and rigidity[17].

2.6. Orientation behavior

Effects of extract of medicinal plant on behavior of male rats was gauged by evaluation of three different parameters viz., (1) behavior towards female which involved licking and sniffing of female anogential organ, (2) self exploratory behavior which included non-gential grooming and gential grooming, and (3) general behavior which comprised of exploration, rearing and climbing. The behavior of rats was digitally recorded by using camera. The number of episodes of each of the behavior parameters would be counted. Scoring is based on the episodes performed by the rats and every episode has certain points. The observations would be made daily and scores on day 0 (just before extract administration), and subsequent days of experimentation.

The scoring was made giving a value of 0 (no sexual activity), 1 (no interaction, rears and climbs on chamber), 2 (sniffs other rat), 3 self- exploratory behavior *i.e.*, grooming and sniffing of gentials, 4 (grooms female rat anywhere), 5 (rears and climbs sexually), 6 (pursues and sniffs other rat), 7 (tries to mount but easily discouraged), 8 (Mounts with an integrated deliberate manner, not easily discouraged), 9 (reflex and almost involuntary mount)[18,19].

2.7. Determination of hesitation time & attraction towards female

A female rat was placed in a cage which had a wooden barrier of 15 cm separating male and female compartments which could be passed by a motivated male rat. The hesitation time was recorded as the time (in sec) required by the male rat before making an attempt to cross the barrier. In the same way, a scoring for attraction towards female was recorded by a score between 0-5 during an observation period of 15 min. A complete cross of the partition by the male rat each time was given a score of 5 while an attempt to climb was given a score of 2 and disinterest to climb was rated as 0. The readings were recorded on Days 0, 7, 14, 21, and 28 of treatment. This test is useful in determining the willingness of a male rat to cross an aversive or obstructive position,

thus indicating the intent of sexual attraction. Male rats of all the groups were subjected to experimentation and their scores for attraction as well as hesitation time were recorded[18,19].

2.8. Pendiculation study

Pendiculations (act of yawing and stretching) were studied following the method of Baggio and Ferrari. The study was performed during the dark phase of the light-dark cycle (20:00-07:00 h) and in subdued red light. Treated male rats were given respective dose of test drugs while controls received water only. The rats were then placed individually in a wooden observation chamber with a glass and remained undisturbed during observation period. Yawing and stretching were counted from video recorded observation and not more than one rat was observed simultaneously. The occurrence of Pendiculations was recorded for animals responding during a 1h observation period. Each rat was used only once. The Pendiculations were recorded on day 0 and day 14 of treatment[20].

2.9. Biochemical studies

2.9.1. Determination of testicular and serum cholesterol (Chod PAP method)

Cholesterol is the precursor in the synthesis of many physiologically important steroids such as bile acids, steroid hormones and vitamin D and its requirement for normal testicular activity has been well established. Testicular and serum cholesterol concentrations may be determined by the Chod-PAP method as Briefly, 0.02 cm^3 of the sample is mixed with 2.00 cm^3 of the working reagent and the absorbance of the resulting mixture is read after five minutes at 546 nm.

A medicinal plant with potential for aphrodisiac should result in statistically significant increase in testicular and or serum cholesterol concentration. Such increase may imply stimulation in the steroid genesis, which may lead to increased testosterone concentration. Such increase in testosterone concentration should normally reflect a corresponding increase in libido[21-23].

2.9.2. Hormonal determination assay

The positive effects on the indices of male sexual behavior must have been brought about by the constituents of the medicinal plant on some reproductive hormones. These include testosterone, luteinizing hormone (LH), follicle stimulating hormone (FSH) and prolactin. Therefore, there is the need to evaluate the effect of administration of the extract of the plant associated with aphrodisiac potentials on the serum concentrations of these hormones. Testosterone supplementation has previously been shown to improve sexual function and libido, in addition to the intensity of orgasm and ejaculation which is likely to improve. Testosterone in the blood exists in three different forms namely: free, albumin-bound and sex-hormone binding globulin (SHBG). While it is generally considered that SHBG bound testosterone is not available for uptake by tissues, opinion is mixed as to whether the biologically active testosterone is restricted to the small quantity of the hormone that is free (2%) or includes the larger amount of albumin bound hormone (20%-80%). However, investigations suggest that both free and albumin-bound testosterone is biologically available. Generally, elevated testosterone level also enhances the sexual behavior in humans. Therefore, an increase in testicular and serum free testosterone concentration will confirm aphrodisiac potential inherent in the plant extract. Luteinizing hormones (LH) and Follicle Stimulating Hormone (FSH) produced by anterior pituitary lobe are necessary for maintaining testosterone levels such that as LH and FSH increases so do the testosterone. Therefore, a medicinal plant acclaimed to have aphrodisiac potential apart from being able to increase the concentration of bioavailable/free testosterone should cause increase in the concentrations of serum LH and FSH. An increase in the concentrations of LH and FSH should normally increase the testosterone concentration. Normally, prolactin is made by specialized pituitary cells called lactotrophs. Prolactin increases the production of breast milk and suppresses secretion of LH and FSH. The role of prolactin in men is not known. However, high levels of prolactin in men may cause hypogonadism, low blood testosterone levels and decrease in sex drive (libido) and sexual function. Therefore, any plant associated with aphrodisiac tendency should produce statistically significant reduction in the concentrations of prolactin in males which would enhance the levels of LH and FSH and by extension the testosterone concentration[24-26].

2.10. Assay for neuronic nitric oxide synthase (NOS) and androgen receptor protein

Activity of NOS can be estimated by use of western method. Nitric Oxide Synthase (NOS), a calcium/calmodulin dependent enzyme is responsible for the biosynthesis of nitric oxide (NO) from L-arginine. Since nitric oxide is responsible for the relaxation of smooth muscles of the cavernosum which eventually lead to in-flow of blood into the male organ, determination of the activity of NOS in the male copulatory organ and the testes is very imperative as this will lend credence to results that will be obtained from the ICP study. The activity of NOS can be estimated by the use of Western Blot. Similarly, analysis of Androgen Receptor (AR) protein will further give an idea of the receptors available for the binding of the androgens notably the free or

bioavailable testosterone[27].

2.11. In- vitro nitric oxide release

DS-1 cells (human corpus cavernosum cell line) were routinely cultured in a humidified 5% CO_2-95% air incubator in Dulbecco's modified eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 20 μM L-glutamine, 1 mM pyruvate, 200 U/mL penicillin G, 200 μg/mL streptomycin and 4.5 mg/mL of D-glucose at 37 ℃. Cells were allowed to attach to the bottom of a 96-well plate overnight and were then exposed to the extract (10 mg/mL PBS) or placebo PBS (control) for 24 h. The cell media was assayed for the concentrations of nitrate (NO_3^-) and nitrite (NO_2^-), the final *in-vivo* products of NO, using a commercial nitrate reductase preparation and Griess reagent kit (Cayman Chemical, USA) with the resultant absorbance being read at 550 nm using a plate reader[28].

2.12. Effect on sexual organ weight and histological studies

After 28 d of treatment, the body weights of animals were taken, after which all animals were killed by decapitation. Testis, seminal vesicles, epididymis and prostate glands were carefully removed and weighed. Testis of animals were cut into small pieces, fixed in Bovine's fixative and dehydrated with varying percentages of ethanol for histological studies. Sections were cut (6 m), stained with haemotoxylin and eosin and then analysed microscopically[29].

2.13. In vitro sperm preservation

The epididymides of the rats were taken into 5 mL of 1% sodium citrate solution and squashed thoroughly with the help of needle and forceps until a milky suspension was obtained. The solution was filtered through 80 m mesh and the volume was made up to 10 mL, inclusive of washings of the filter. A 1 mg/mL solution of extracts of *A. longifolia* (1 mg/mL) was prepared and added into the sperm specimens in the test sample in a ratio of 0.1:1 (100 μL sperm solution: 1 mL extract solution). The sperms were counted at 0 and 30 min after incubation at room temperature. The spermatozoa were counted in a five WBC counting chamber. The average number of sperm per chamber was reported[29].

2.14. In-vivo sperm count

In-vivo sperm count. Epididymis of rats of each group were homogenized and taken into 5 mL of 1% sodium citrate solution and squashed thoroughly with the help of needle and forceps until a milky suspension was obtained. The solution was filtered through 80 m mesh and the volume was made up to 10 mL with the same solution; the made up volume was inclusive of washings of the filter. The suspension was shaken thoroughly and the spermatozoa were counted in five WBC counting chambers of the haemocytometer. The average numbers of sperms per chamber are reported. The progressive sperm motility, spermcount, live/dead ratio (viability) and morphology were determined[30].

2.14.1. Fructose content in seminal vesicles

The seminal vesicles were macerated with 3 mL of distilled water and centrifuged at 4 000 r.p.m. for 12 min. To the supernatant, fluid collected after centrifugation add 0.5 mL of resoreinol and 1.5 mL of HCl. The mixture was kept at 80 ℃ for 12 min. The reaction with resorcinol developed a rosy colour, which was measured at 500 nm using spectrophotometer. A calibration curve was drawn using dilutions of fructose solution and measument of the colour developed with resorcinol and HCl[30].

3. Conclusion

Drug discovery and development consists of a series of processes starting with the demonstration of pharmacological effects in experimental animal models and cell lines and ending with drug safety and efficacy studies in patients. The main limitation is often the unacceptable level of toxicity with herbal drug. The study of aphrodisiac agents has been an important field of research. The article explains various types of animal models which are employed in the study of aphrodisiac agents.

Conflict of interest

We declare that we have no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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