

Effect of Purwoceng Extracts (*Pimpinella Alpina* Molk) on Erectile Function Biomarker Enhancement

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ABSTRACT

Purwoceng (*Pimpinella alpina* Molk) extract could improve the testosterone levels, LH, and FSH in *Sprague Dawley* male rats. We hypothesized that Purwoceng extract can increase erectile-function biomarker in male rats *Sprague Dawley* through increased expression level of eNOS endothelial, nNOS nerve ending expression levels and the levels of cGMP in penile *corpus cavernosum*. We used Post test only controlled group design and used 30 male rats *Sprague Dawley* which randomized into two groups: castrated and noncastrated, consist of control group, 4 hours and 30-days treatment. Purwoceng extract supplementation dose of 50 mg increased significantly the number of eNOS, nNOS expression and cGMP levels of *corpus cavernosum* penile tissue compare to control group. The highest enhancement of cGMP level along with eNOS and nNOS expression were obtained after 30-days treatment. Enhancement of eNOS expression for endothelium was positively correlated to increment levels of cGMP and nNOS nerve ending smooth muscle *corpus cavernosum*. Purwoceng (*Pimpinella alpina* Molk) extracts could enhance erectile function biomarker.

Keywords: Purwoceng, *Pimpinella alpina* Molk, erectile function, cGMP, eNOS, nNOS

INTRODUCTION

The use of synthetic oral medication for the erectile dysfunction (ED) treatment of sildenafil (Viagra), vardenafil (Levitra) and tadalafil (Cialis) type has been widely used since the '90s. Synthesis of sildenafil derived compounds was done through a series of reactions which quite complicated and selective, starting with diketoster condensation with hydrazine, followed by six other reaction stage¹. This situation is thought to be the main cause of the high cost of synthetic drugs. In addition, the side effects such as headache, nausea and the decreasing of selectivity to against other PDE isoenzymes, especially PDE-1 and PDE-6, led the researchers interested to develop drugs from natural products. This situation also is supported by the existence of public confidence that natural ingredients are safer for health².

Systematic research of traditional medicine for erectile dysfunction drugs was generally done through the development of aphrodisiac claims³. Aphrodisiac is a stimulant substance which can

increase sexual desire by stimulating the excretion of testosterone and accelerate blood circulation to the brain that stimulates nerve function. Some drugs from natural ingredients that had been proven to have an aphrodisiac properties can increase the erection, for example *Tribulus terrestris* and *Panax ginseng*. *Tribulus terrestris* L., contains phytochemicals, flavonoids, alkaloids, glucosides, steroid and saponins. Protodiosin (saponin) was the most dominant compound in this plant, which is a phytochemical agent that can increase sexual arousal and erection through the conversion of testosterone to DHEA, thus can improve erectile dysfunction⁴. In addition, the proerectile has a relaxant effect which is claimed to be an aphrodisiac⁵. *Panax ginseng* root extract contains triterpene saponins called ginsenosides compound which has been reported to improve erectile function by increasing the production of NO. Clinical tests of this plant material also had been reported to have a good effect compared to the placebo⁶. The influence of ginsenosides in raising the nitric oxide (NO) production was done by relaxing the penile smooth muscle through the NO-cGMP pathway^{7,8}.

Purwoceng (*Pimpinella alpina* Molk), a typical plant thrives in Indonesia on the Dieng Mountains, Wonosobo, Central Java, have been proven to increase levels of testosterone. This plant has been used for generations to enhance vitality and sexual desire⁹. Purwoceng extract with 50mg dose in male *Sprague Dawley* rats have been proven to increase levels of testosterone, Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH)¹⁰. Phytochemistry

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screening of this plant contained steroid, triterpenoid, alkaloid and flavonoid compounds. Analysis of chemical component by gas chromatographic/mass spectroscopy technique of Purwoceng root had showed contained steroid substance as stigmasterol¹¹. These compounds from *Pimpinella alpina* Molk were the most playing role in increasing the blood serum's testosterone level and vitality in male rats *Sprague Dawley*.

Study of Purwoceng extract effect on increasing the erectile function biomarkers (eNOS endothelial, nNOS nerve ending and cGMP) has not been studied. Therefore, this study was purposed to prove the effect of Purwoceng extract which can increase the erectile-function biomarker of male rats *Sprague Dawley* through increased expression level of eNOS endothelial, nNOS nerve ending expression levels and the levels of cGMP in penile *corpus cavernosum*.

MATERIALS AND METHODS

Materials: male *Sprague Dawley* rats, materials for making tissue and immunohistochemically examination: formalin solution 10%, Natrium phosphat buffer, xylol, alcohol absolute, alcohol 90% and 70%, paraffin, object glass, antibody monoclonal IgG1 anti-eNOS (1:500) and anti-nNOS (1:500) (oncogene reseach Products, USA), phosphate buffered solution (PBS), avidin-biotin, biotinylated horseradich peroksidase, mouse monoklonal Ig anti eNOS, anti rat Ig-HRP dan anti-goat Ig-HRP (1:2000), cGMP kit. (Kat#K372-100).

Plant material and extract: Root of Purwoceng (*Pimpinella alpina* Molk) were collected in April 2015 from Dieng Mountains, Wonosobo, Central Java, were dried in an oven with circulating air at 40°C. Samples have been deposited in the Herbarium of the "University of Diponegoro" Semarang, Indonesia. Crude extracts were prepared by maceration of 500 g dried Purwoceng roots in room temperature with ethanol 96% (v/v) solvent during 3 x 24 h. The obtained extract was filtered and concentrated in a rotary evaporator at 45°C under reduced pressure (15 mmHg), yielding 5.75 g.

Animals and Ethics: *Sprague Dawley* male rat (weight 200-250g) were obtained from Integrated

Research and Testing Laboratory (LPPT) Unit IV, Gadjah Mada University. All the animals were maintained under standard condition, with temperature at 25±2 °C, 12 h dark-light cycle and relative humidity. The rat received food and water ad libitum. This study was approved by the Ethics Committee for Health Research and Medical University School of Medicine and Kariadi Hospital Semarang, Indonesia (Medical Letter No: 016/EC/FK/RSDK).

Experimental Design: After acclimatization period, 30 male *Sprague Dawley* rats were randomly allocated into two groups castrated (K) and noncastrated (NK). Each group was divided into a control group, treatment group of 4 hours and 30-days. Purwoceng extract was given orally to the treatment groups by a dose of 50 mg. The treatment was given daily at 8-9 am. The control group only had distilled water. After 4 hours and 30-days all rats were terminated and then the penile tissue was taken for analysis. Expression of eNOS endothelium cell and nNOS nerve ending smooth muscle corpus cavernosum were measured by IHC staining with monoclonal antibody eNOS and nNOS. Measurement of cGMP levels performed by the method of immuno assay on penis tissue supernatant. This procedure was conducted according to Park research¹².

Statistical analysis: The data are expressed as mean ± S.D. Results were statistically evaluated by using one-way analysis of variance (ANOVA) and Turkey's test were carried out to statistically compare the data among the groups. The values were considered significant when p values ≤0.05. Regression correlation analysis was used to analyze of data, will be significant if p values ≤0.05 with 95% CI.

RESULTS

The results of assessment of the average amount of eNOS, nNOS and cGMP expression both in castrated and non castrated mice which is performed with period for 4 hours and 30 days shown in Table 1.

Table 1. The average amount of eNOS, nNOS expression and cGMP level in various variables of study

Variables	Treatment group Mean and SD						p
	NK ₍₋₎	NK ₄	NK ₃₀	K ₍₋₎	K ₄	K ₃₀	
eNOS	6.52±0.60	15.80±0.83	24.80±1.30	5.68±1.17	11.80±0.44	17.84±0.16	0.000 ^a
nNOS	1.08±1.41	4.96±0.16	12.20±0.40	1.84±0.51	4.64±0.29	10.00±0.37	0.000 ^a
Level of cGMP	0.11±0.02	0.15±0.006	3.12±0.38	0.009±0.03	0.13±0.01	0.86±0.34	0.000 ^a

^aKruskal Wallis Value p=0.000.

Table 2: Summary of correlation test analysis results

No	Variables	Level of cGMP	
		Correlation Coefficient	p
1	eNOS on endothelium cell CC	0.885	0.000
2	nNOS in smooth muscle CC nerve ending	0.858	0.000

Spearman correlation test $p < 0.005$

The Mann Whitney test was performed to see whether the difference in eNOS, nNOS expression amount is significant or not on non-castrated rats group compared to controls of treatment for 4 hours and 30 days, the comparison treatment for 4 hours and 30 days showed a high significant difference ($p < 0.001$). Figure 1 and 2 showed the boxplot diagram of eNOS and nNOS expressions.

mean levels of cGMP in the non-castrated groups showed that administration of the purwoceng extract for 30 days showed highest levels, the difference of each treatment group was analysed by Mann Whitney test, it showed a highly significant difference ($p < 0.001$), whereas the castrated group treated for 4 hours with the controls did not show significant difference ($p > 0.001$).

Fig. 1: Boxplot diagram the number of eNOS expression on endothelium

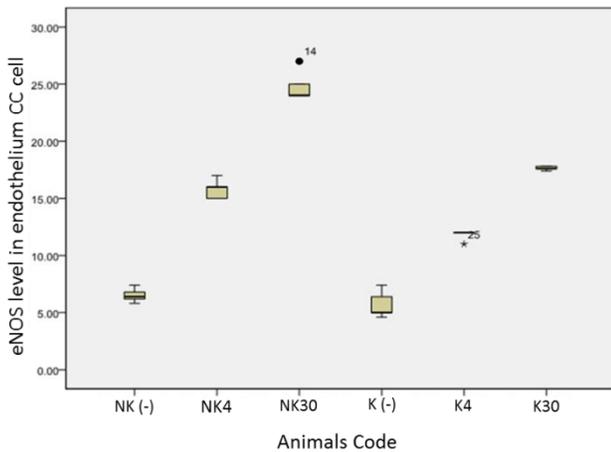
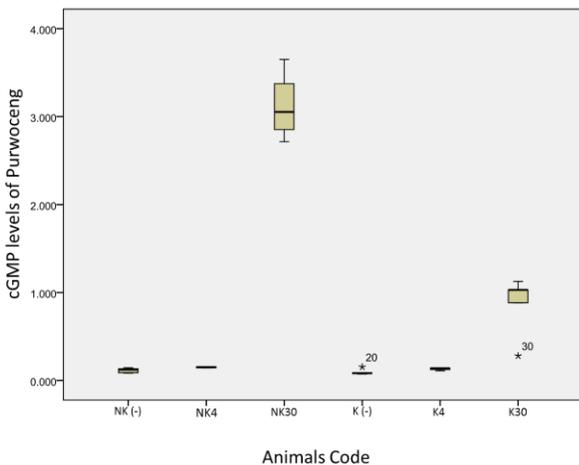
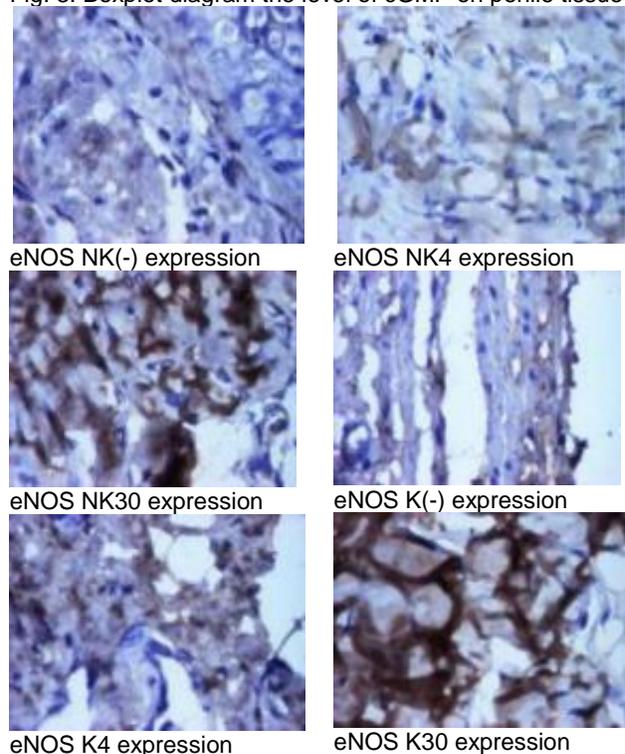


Fig. 2. Boxplot diagram the number of nNOS nerve ending corpus cavernosum



The statistics tests showed that the purwoceng extract was able to increase the amount of eNOS and nNOS expression in both groups of non-castrated as well as castrated rats. The measurements of the the

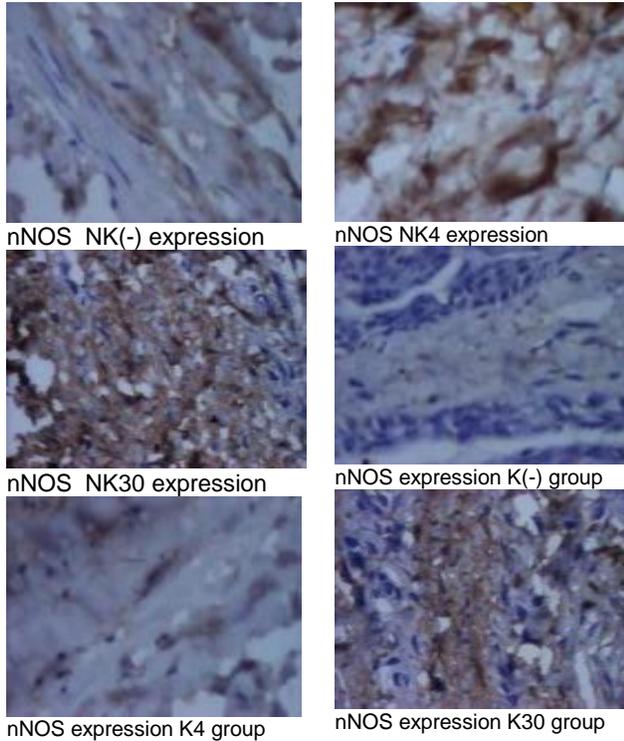
Fig. 3: Boxplot diagram the level of cGMP on penile tissue



Histopathologic features of eNOS and nNOS expressions with IHC method using a monoclonal antibody staining anti-eNOS and nNOS on mouse tissue for treatment 4 hours and 30 days were shown in Figures 4.

Figure 4 eNOS expression for non-castrated animal group with immunohistochemistry method using antibody monoclonal anti-eNOS coloration (*Mouse monoclonal IgG1 anti eNOS*) on rat penile tissue with 4 hours and 30 days treatment

Fig. 4: eNOS expression for non-castrated animal group with immunohistochemistry method using antibody monoclonal anti-eNOS coloration (*Mouse monoclonal IgG1 anti eNOS*) on rat penile tissue with 4 hours and 30 days treatment



The results showed that the treatment group for 4 hours and 30 days showed the increasing of expression number compared to the control group. The 30-day treatment group showed the highest expression of both treatments. Pathogenesis overview of Puwoceng extract role in improving the erectile function biomarker done through modeling using the line equation, firstly the analysis of correlation tests was performed. The analysis results obtained was a summary as in Table 2.

Based on the correlation test results show that the two variables play role in increasing the levels of cGMP.

Multivariate analysis with Spearman test obtained the regression line of the effects of purwoceng extract on the levels of cGMP are follows: $0.923 + 0.109$ (nNOS nerve endings) + 0.76 (eNOS endothelium). This equation indicates that the Purwoceng extract treatment contribute to the changes in the levels of cGMP along with the increasing number of eNOS and nNOS expression.

DISCUSSION

Research variables are the expression of eNOS, nNOS and cGMP levels in penile tissue of rats. The

three dependant variable is directly related to NO, because NO is produced through a reaction catalyzed by specific enzyme NOS¹³. Nitric Oxide is released from nerve endings nitregik produced by oxidation of L-arginine catalyzed by nNOS, whereas NO released from endothelial cells is catalyzed by eNOS. As a major neurotransmitter, NO is responsible for the occurrence of smooth muscle relaxation in the corpus cavernosum and diffuses directly into the corpus cavernosum smooth muscle guanylyl cyclase to activate enzymes to accelerate the formation of cGMP that causes relaxation of corpus cavernosum smooth muscle. The cGMP equilibrium is regulated by PDE-5 enzyme to change cGMP into 5-GMP. Thus increased levels of eNOS and nNOS would lead to an increase in cGMP levels, so these three variables is very important in the process of relaxation in the corpus cavernosum smooth muscle of the penis. Therefore, eNOS, nNOS and cGMP are biomarkers of erectile function.

Endothelial NOS is an enzyme the identified attached to the membrane of blood vessels and serve as material for vasodilation. NO produced by eNOS is responsible for keeping blood vessels functioning normally and as a mediator of the erectile mechanism^{14,15,16}. The results showed increament of the number of eNOS expression in Purwoceng extract for 4 hours and 30 days were significant compared to the control group rats castrated and non-castrated. Thus, increasing the amount of eNOS expression is also assumed to increase NO production. Increasing the amount of expression is highly influenced by the hormone testosterone, because testosterone is a central mechanism to regulate the erection process. In this study, enhancement of the amount eNOS expression allegedly caused by the presence of elevated levels of serum testosterone. Evidence showed that the effect of extract Purwoceng increase serum levels of testosterone through LH simulation and through the conversion of stigmaterol to testosterone in peripheral tissues¹⁰. The presence of stigmaterol compound in the extract Purwoceng also been shown¹¹.

This study uses the castrated rats, are intended to describe the state of andropouse because castrated can cause loss of testosterone-producing Leydig cells. While the use of non-castrated rat meant to portray a normal state. The results showed that the effect of administration Purwoceng extract in the castrated groups of rats for 4 hours and 30 days compared to normal rat groups showed differences in the amount of eNOS expression is significantly ($p < 0.001$). This is due to an increase in the amount of eNOS expression in castrated rats, because there is

the adrenal cortex to produce testosterone, although in very small amounts^{17,18,19,20}.

Provision for 30 days showed the highest expression of eNOS compared to 4 hours of treatment and control groups. This suggests that the longer time of administration, the higher expression of eNOS. These results reinforce earlier theory that medicinal plants takes time to reach and influence the target organ.

Increasing the amount of eNOS expression is suspected by the presence of bioactive flavonoid compounds in extracts Purwoceng. Flavonoid shown induce eNOS, increasing cytosolic calcium that works as a cofactor of eNOS activate and eliminate free radicals in the interstitial fluid that NO production can be maintained. In addition, flavonoids work as vasorelaxan by inhibiting PDE-5 and lower Ca²⁺ in smooth muscle cells²¹.

Flavonoid compounds have been found in the plant genus *Pimpinella* is illunginin A f B²². Effects of flavonoids have also been shown to activate eNOS in the provision of epigallocatechin gallate in the reperfusion of ischemic hearts. Another researcher, reported that injection of resveratrol which is a flavonoid compound intraperitoneally in rat showed increased expression of eNOS and nNOS²³.

Neuronal NOS is an enzyme located in the nerve tissue and striated muscle, which serves as a communication cell. nNOS in the penis expressed as neurons innervating the corpus cavernosum penis. Neuronal NOS to synthesize NO are dominant in the brain and released from the nerve terminal. Mechanism of action of nNOS in the relaxation process is not yet fully understood. Allegedly NO derived from L-arginine stimulates the activity of guanylyl cyclase to convert GTP to cGMP in the corpus cavernosum smooth muscle of the penis^{24,25}.

The results showed that there is a significant increase in the number of nNOS expression in the non-castrated and castrated rats after administration of Purwoceng extract for 4 hours and 30 days compared to the negative control group (p<0.001). The number of nNOS expression is proven to increase due to administration of a dose of 50 mg extract Purwoceng. Increasing the number of nNOS expression thus also increases the ability of an erection, because nNOS is a biomarker that erectile function can produce NO. An increase of the number of eNOS and nNOS expression, is expected to increase levels of NO resulting in vasodilation and smooth muscle relaxation in the corpus cavernosum penis of male *Sprague Dawley* rats.

Nitric oxide (NO) the activates the enzyme guanylate cyclase and induces smooth muscle relaxation by increasing cGMP levels. Cyclic GMP will stimulate protein kinase G (PKG) which is

inactive to active. Then the active PKG lowers calcium levels, causing dilatation and relaxation of corpus cavernosum smooth muscle, which in turn causes an erection. Cyclic GMP are second messengers resulting from changes in the GTP activated by NO via activation of guanylyl cyclase enzyme²⁶. NO neurotransmitters and second messengers cGMP are both relaxing vascular smooth muscle. Purwoceng extract in castrated and non-castrated for 30 days in this study increase cGMP levels significantly compared to the control group (p<0.001). These results prove that an increase in cGMP levels due to administration of the extract Purwoceng. The increasing of cGMP levels through increased expression of eNOS and nNOS.

Based on the Spearman correlation test of the biomarker increased erectile function it is obtained that the increasing role Purwoceng extract cGMP accompanied by increased expression levels of nNOS and eNOS. The increase of all biomarker eNOS, nNOS and cGMP caused by the presence of elevated levels of serum testosterone.

CONCLUSION

Purwoceng extract supplementation dose of 50 mg on male *Sprague Dawley* rats among castrated and non-castrated has been proven increased the biomarker erectile function. It has been proven from increase of the number of eNOS expression in endothelial cells, nNOS in the nerve endings smooth muscle of corpus cavernosum penile tissue, and cGMP levels. The highest enhancement were obtained after 30-days treatment. Enhancement of cGMP level was positively correlated to increment eNOS endothelium and nNOS nerve ending smooth muscle corpus cavernosum.

Conflict of interest: We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

REFERENCE

1. Terret, N.K., Bell, A.K., Brown, D., & Ellis, P., 1996. *Bioorg. Med. Chem. Lett.*, 6, pp.1819–1824.
2. Gratz, S.R., Flurer, C.L. & Wolnik, K.A., 2004. *N. J.Pharm. Biomed. Anal.*, 36, pp.525–533.
3. Traish, A.M., Goldstein, I. & Kim, N.N., 2007. Testosterone and Erectile Function: From Basic Research to a New Clinical Paradigm for Managing Men with Androgen Insufficiency and Erectile Dysfunction. *European Urology*, 52(1), pp.54–70.
4. Adimoelja, A., 2000. Phytochemicals and the breakthrough of traditional herbs in the management of sexual dysfunctions. *International Journal of Andrology*, 23(S2), pp.82–84.
5. Andaikan, P.G., Gauthaman, K. & Prasad, R.N., 2000. Pro-erectile Pharmacological Effects of Tribulus Terrestris Extract on The Rabbit Corpus Cavernosum. *Ann. Acad. Med. Singapore*, 29,

- pp.22–26.
6. Choi, H., Seong, D. & Rha, K., 1995. Clinical Efficacy of Korean Red Ginseng for Erectile Dysfunction. *Int J Impot Res*, 7, pp.181–186.
 7. Kim, Ji H., Hahm, D. H., Yang, D. C., Kim, Jang H., Lee, H. J., & Shim, Insop, 2005. Effect of Crude Saponin of Korean Red Ginseng on High-Fat Diet- Induced Obesity in the Rat. *Journal of Pharmacological Sciences*, 97, pp.124–131.
 8. Young-Mi, K., Seung, N., Yun, Y. G., Hong, H. D., Lee, Y. C., Ha, K. S., Lee, H., Kwon, H. J., Kwon, Y. G., & Kim, Y. M., 2007. Water Extract of Korean Red Ginseng Stimulates Angiogenesis by Activating the PI3K/Akt-Dependent ERK1/2 and eNOS Pathways in Human Umbilical Vein Endothelial Cells. *Biol. Pharm. Bull*, 30(9), pp.1674–1679.
 9. Caropeboka, A.M. & Lubis, I., 1975. Pemeriksaan Pendahuluan Kandungan Kimia akar Pimpinella alpina dalam Simposium Tanaman Obat I., pp.Bogor, 8–9 Desember.
 10. Taufiqurrachman & Wibowo, S., 1999. Pengaruh Ekstrak Pimpinella alpina Molk dan Eurycoma Longifolia Jack terhadap Peningkatan Kadar Testosteron LH dan FSH pada Tikus Jantan Sprague Dawley. *Media Medika Indonesia*, 35(2).
 11. Suzery, M., Ngadiwiyono, Nurhasnawati, E., & Cahyono, B., 2004. Senyawa Stigmasterol dari Pimpinella alpina Molk. *Media Medika Indosiana*, 29 Suppl.(1), pp.37–39.
 12. Park, C.S., Ryu, S.D. & Hwang, S.Y., 2004. Herbal Formula Enhances Erectile Responses in Aged, Diabetic, and Normal Rats via the Activation of NO/cGMP Signaling Pathways. *Journal of Ethnopharmacology*, 94, pp.85–92.
 13. Moncada, S. & A, H., 1993. The L-arginine-nitric Oxide Pathway. *N. Engl. J. Med.*, 329(27), pp.2002–2012.
 14. Moncada, S. & Higgs, A., 1997. The Arginine-nitric Oxide Pathway. *N. Engl. J. Med.*, 329, pp.2002–2012.
 15. Ghalayini, I., 2004. Nitric Oxide-cyclic GMP Pathway with Some Emphasis on Cavernosal Contractility. *International Journal of Impotence Research*, 16, pp.459–469.
 16. Moncada, S. & Higgs, A., 2006. The Discovery of Nitric Oxide and Its Role in Vascular Biology. *Br. J. Pharmacol*, 147(Suppl. I), pp.S193–201.
 17. Cooper, T.G. & Yeung, C.H., 1977. Physiology of Sperm Maturation and Fertilization in: Neischlag E, Behre AM, editors Andrology, Male Reproduction Health and Dysfunction. *Berlin Heldeberg: Springer-Verlag*, pp.178–181.
 18. Van, A.H. & Hertle, R., 1997. Erectile Dysfunction, Disorder of Sperm Deposition in: Neischlag E, Behre HM, (Editions), Andrology, Male Reproductive Health and Dysfunction. *Berlin Heldeberg: Springer-Verlag*, pp.178–181.
 19. Howel, S. & Shalet, S., 2001. Testosterone Deficiency and Replacement. *Horm. Res.*, 56(1), pp.82–92.
 20. Thijssen, J.H.H., 2002. Laboratory Test in Endocrine Evaluation of Aging Male (Appendix) in: Lunenfeld B, Gooren L (editors), Textbook of Men's Health, London: Yhe Pathenon Publishing Groups., pp.44–50.
 21. Akhlaghi, M. & B., B., 2009. Mechanisms of Flavonoid Protection Against Myocardial Ischemia-reperfusion Injury. *Journal of Molecular and Cellular Cardiology*, 46, pp.309–317.
 22. Qiao, B.-L., Wang, C. D., Shi, H., Mi, C. F., & Li, F., 1996. Studies on Chemicals Constituent of the Root of Pimpinella Thellungiana. *Zhongcaoyao*, 27(3), pp.136–138.
 23. Hung, L.M., Su, M.J. & Chen, J.K., 2004. Resveratrol Protects Myocardial Ischemia-reperfusion Injury Through Both NO-dependant and NO-independant Mechanisms. *Free Radic. Biol. Med.*, 36, pp.774–781.
 24. Magee, T.R., Ferrini, M. G., Davila, H. H., Zeller, C. B., Vernet, D., Sun, J., Lalani, R., Burnett, A. L., Rajfer, J., & Gonza, N. F., 2003. Protein Inhibitor of Nitric Oxide Synthase (NOS) and the N-Methyl-D-Aspartate Receptor Are Expressed in the Rat and Mouse Penile Nerves and Colocalize with Penile Neuronal NOS. *BIOLOGY OF REPRODUCTION*, 68, pp.478–488.
 25. Toda, N., Ayajiki, K. & Okamura, T., 2005. Nitric oxide and penile erectile function. *Pharmacology and Therapeutics*, 106(2), pp.233–266.
 26. ucas, K., Pitari, G.M., Kazerounian, S., Ruiz-Stewart, I., Park, J., & Schulz, S., 2000. Guanylyl Cyclases and Signaling by Cyclic GMP. *Pharmacol Rev.*, 52, pp.375–414.