

Original Article

## **Effect of aqueous extracts of khaini on fertility of adult male albino rats**

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### **Abstract**

The use of tobacco without burning is referred as smokeless tobacco use. Smokeless tobacco use has been increased rapidly throughout the world including India. In India khaini is one of the popular form of smokeless tobacco. Smokeless tobacco produces oxidative tissue damage and apoptosis. Sperm is highly susceptible to oxidative damage. This study was undertaken in order to assess the effect of chronic treatment of khaini on fertility of male albino rats. In our present investigation treatment of rat with extract of khaini significantly reduced concentration, motility and viability of epididymal sperm. Fertility and fecundity of male albino rat was significantly less in khaini treated group than untreated counterpart. Results of biochemical analysis showed significant decrease of antioxidant, ascorbic acid level in testis and decrease activity of enzyme super oxide dismutase both in testis and sperm. Khaini extract induced decrease of male fertility may be due to alteration of sperm indices from khaini-induced decrease potentiality of testicular antioxidant system. The obtained result conclude that smokeless tobacco

khaini is detrimental to the reproductive potentials of male rats.

**Key words** Khaini, Smokeless tobacco, fertility, Superoxide dismutase , vitamin-C.

### **Introduction**

The use of tobacco without burning is known as smokeless tobacco ( SLT ) use. SLT use has been increased rapidly throughout the world in recent years especially among adolescent boys and young men (1). In India male, female and teenagers take SLT. In India tobacco is used in a smokeless manner in a wide variety of ways with multitudes of products such as betel quid, tobacco with lime, manufactured SLT products like gutkha, khaini, mawa and many others (2).

Khaini is one of the popular SLT in India. It is made from sun-dried or fermented tobacco leaves (*Nicotina rustica* and/or *Nicotina tabacum* ), slaked lime paste and sometimes areca nut (3). This SLT is prepared by rubbing tobacco leaves and slake lime with the thumb by users at the time of use. It is also now available commercially. It is kept in the mouth for 10 to 20 minute and sucked from time to time.

SLT adversely affects the various physiological system including reproductive system.

A number of study have shown higher incidence of abnormal morphology of sperm (4), decrease sperm motility, decrease sperm density and damage DNA (5,6) in men who smoked. Exposure of spermatozoa to seminal plasma from smokers resulted in a significant reduction in sperm viability and possibly their fertilizing ability (7,8). Like cigarette main ingredient of SLT is tobacco and tobacco contains along with other harmful materials- nicotine. Nicotine exposure due to SLT use is much more than smoking because of prolonged absorption (9). Smokeless tobacco extract produces oxidative tissue damage and apoptosis which can be attenuated by antioxidants including vitamin-C (10).

Antioxidant enzymes viz. catalase, superoxide dismutase and glucose 6-phosphate dehydrogenase levels were lower in SLT users than nonusers (11). An increase in the seminal ROS level has been reported in 40% of infertile male (12). In our previous study we found that crude extract of khaini significantly decreased sperm count and adversely changed sperm morphology. Thus the present study was under taken to assess the effect of one SLT, khaini on male fertility and its correlation with gonadal antioxidant level system.

## **Materials and Methods**

### ***Preparation of extract of khaini***

Aqueous extract of khaini (AEST) was prepared by modifying the method originally described by Avti et al. (13). Commercially available khaini was finely powdered and 20g was dissolved in 100ml distilled water and incubated at 37°C for 1hr with through shaking. The dissolved contains were filtered

twice through Whatman number1 filter paper and kept in -20°C in screw-capped bottle before lyophilization. The dried yield of AEST was found to be around 1mg per 15mg khaini.

### ***Animals and grouping***

Pure wister strain male albino rat weighing between 160- 200 gm were used for the experiments. The study was approved by animal ethical committee of the Institution. The animals were housed in standard laboratory condition in a photoperiod cycle of 12 hr: 12 hr ( light and dark ) and were supplied with standard laboratory diet and drinking water *ad libitum*.

Animals were randomly divided into three groups- one control and two experimental group. Each group consisted 6 animals. Animals of 1<sup>st</sup> experimental group were treated with khaini extract ( 25 mg /kg body weight /day ) orally for 60 days. Animals of 2<sup>nd</sup> experimental group were orally treated with khaini extract for 90 days with same dose. Dose were selected according to Bagchi et. al. (14). Animals of control group were orally given distilled water every day for as placebo treatment.

### ***Separation of epididymal sperm***

Epididymal spermatozoa were separated by modification of method of Brooks (15). Caudal portion of epididymis was cut out. It was then cut into small pieces by sharp blade. Spermatozoa from epididymal pieces were removed by vortexing gently in Krebs Ringer phosphate buffer ( pH 7.4 ) for 10 min. Suspension was used for sperm count.

### ***Sperm concentration :***

Spermatozoa were counted as per the method of Zaneveld and Polakoski (16). Sperm suspension was placed on both side of Neubaure's hemocytometer and allow to settle for 15 min . The number of spermatozoa in the

appropriate squares of the hemocytometer was counted under the microscope at 40x magnification. Sperm concentration was expressed as number of sperm/cauda of epididymis as well as number of sperm / gm weight of epididymis.

**Sperm viability:**

Assessment of viability of sperm was done by hypo-osmotic swelling test (17). 0.1ml epididymal washed in physiological saline was mixed with 1.0ml 150m mol/kg hypo osmotic solution ( prepared by 7.35 gm sodium citrate and 13.5 gm fructose in 1000ml distilled water). The mixture was incubated 60 min at 37°C. Then 0.2ml of the mixture was placed on a slide and mounted with a cover slip and immediately examined at a magnification of 400. The percentage of reacted sperm (curled tail) and un-reacted sperm (uncurled tail) were assessed by counting 100 sperm.

**Sperm motility:**

For the study of sperm motility spermatozoa were expressed out by cutting the distal end of cauda epididymal tubule (18). It was then diluted with physiological saline and placed on a thin glass slide. Sperm motility was studied according to Aboua et.al. (19). 10 random fields were manually scored for the number of motile and non-motile sperm. Motility was expressed as a percentage of motile sperm compared to total sperm counted.

**In vivo fertility assessment:**

In different set of experiment, each male was allowed to undergo mating with two proestrus females (3-4 months old ) during the last 10 days of treatment. The vaginal smear was examined for the presence of sperm as a criterion of successful insemination. Each sperm positive female was caged separately and observed after 21-

24 days for parturition (20). The number of rats that became pregnant and litter size ( number of pups delivered) were noted to assess the fertility and fecundity of male rats respectively (18).

**Biochemical estimation of testicular SOD and vitamin-C:**

Testes were removed and immediately placed in cold 0.9% NaCl and washed in the same (21) and homogenized in 0.9% sodium chloride solution (1 ml per 50 mg tissue). Suspension was allow to settle for 15 min at - 20° C. Supernatant was centrifuged at 800 x g for 15 min (18) and was used for estimation of SOD and vitamin-C following the method of Martin et. al. (22) and Srikrishna and Suresh (23) respectively. The result was expressed on the basis of tissue weight.

**Biochemical estimation of antioxidant enzymes in epididymal sperm:**

2 cm part from caudal portion of epididymis was cut out . It was then rinsed with 10 ml Krebs Ringer phosphate buffer ( pH 7.4 ). Suspension was allowed to settle for 5 min. It was then centrifuged at 800 x g for 15 min. Supernatant was used for biochemical estimation SOD. SOD activity was expressed on the basis of number of sperm.

**Statistical analysis :** Results were expressed as mean  $\pm$  standard error of mean. Significance was determined by students' t test. Differences were considered significant when  $p < 0.05$ .

**Results**

Oral administration of khaini extract decreased sperm concentration in epididymis. The result was more significant in 90 days treatment than 60 days ( table-1 ).

Table 1: Effect of chronic treatment of khaini on epididymal sperm concentration

Animal group	Sperm count ( million/cauda )	Sperm count ( million/gm)
Control	5.735 ± 0.225	569.57 ± 20.27
60 Days treatment	3.555 ± 0.368 [p<0.001]	361.31 ± 32.56 [p<0.001]
90 Days treatment	2.173 ± 0.042 [p<0.001]	233.45 ± 15.15 [p<0.001]

Data represent mean ± SEM; n=6 in each group  
Treatment of male albino rat with extract of Khaini significantly affect sperm viability. Number of viable sperm in experimental group of rats was significantly lower than control group (fig.1). There was positive correlation between khaini treatment and decrease sperm viability. Similar result was obtained for sperm motility ( fig.2).

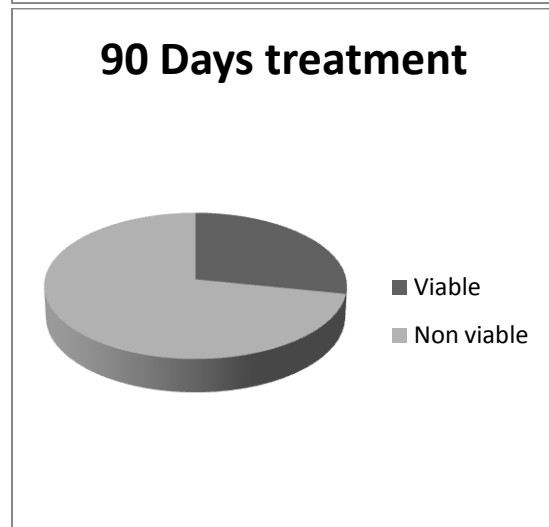
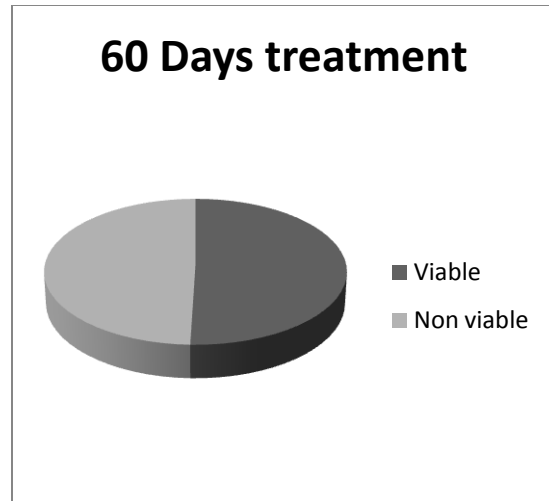
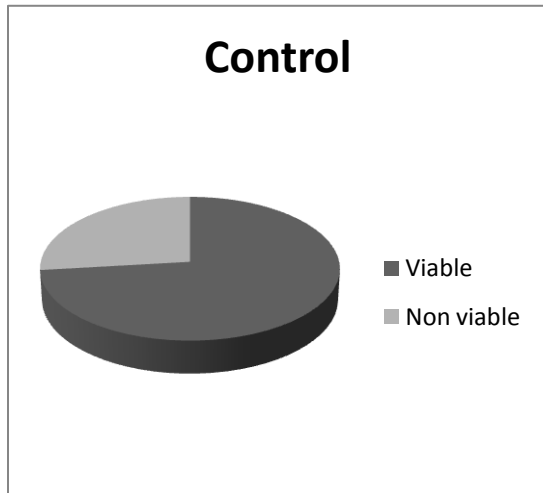


Fig. 1: Pie diagram showing the effect of chronic treatment of khaini on viability of epididymal sperm (n=6; p<0.001 in all cases)

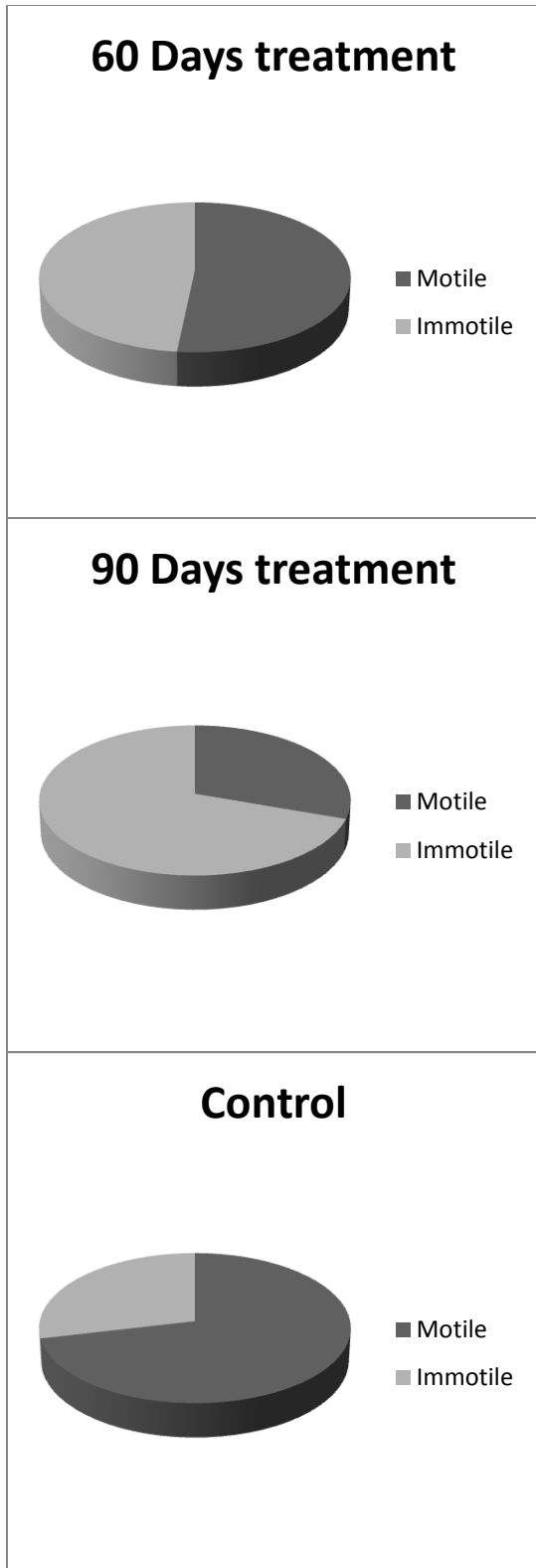


Fig. 2: Pie diagram showing the effect of chronic treatment of khaini on motility of epididymal sperm (n=6; p<0.001 in all cases)

Chronic khaini treatment significantly decreased male fertility and fecundity (table-2). The effect was more significant in 90 days treated group than 60 days treated.

Table.2: Effect of chronic treatment of khaini on the fertility and fecundity of male albino rats

Parameter	Control	60 Days treatment	90 Days treatment
% Insemination	100	83.33	58.33
Fertility index	66.66	50.00	28.57
Fecundity	6.87	4.60	3.50

Data represent mean ± SEM; n=6 in each group

SOD, the principal antioxidant enzyme of testis was estimated in control and khaini treated albino rats. Chronic khaini treatment significantly decreased activity of SOD in testis (table-3) as well as in epididymal sperms (table-4).

Table. 3: Effect of chronic treatment of khaini on the activity of super oxide dismutase of testicular tissue

Animal group	Super oxide dismutase (U/mg tissue)	p-value
Control	0.528 ± 0.031	
60 Days treatment	0.385 ± 0.027	0.001
90 Days treatment	0.243 ± 0.035	0.001

Data represent mean ± SEM; n=6 in each group

*Table. 4: Effect of chronic treatment of khaini on the activity of super oxide dismutase of epididymal sperms*

Animal group	Super oxide dismutase ( U/million sperm )	p-value
Control	0.216 ± 0.021	
60 Days treatment	0.144 ± 0.019	0.001
90 Days treatment	0.087 ± 0.013	0.001

Data represent mean ± SEM; n=6 in each group

Vitamin -C , the one of the nonenzymic antioxidant of testis was estimated in control and khaini treated albino rats. Chronic khaini treatment significantly decreased vitamin-C level in testis. The effect was more significant in 90 days treatment than 60 days treatment ( table-5).

*Table. 5: Effect of chronic treatment of khaini on the level of ascorbic acid in testicular tissue*

Animal group	Ascorbic acid ( µg/ mg tissues )	p-value
Control	11.40 ± 1.428	
60 Days treatment	7.74 ± 0.810	0.001
90 Days treatment	5.49 ± 0.506	0.001

Data represent mean ± SEM; n=6 in each group

### Discussion

Chronic treatment of rat with khaini extract decreased sperm count, sperm viability and sperm motility. These results were coincide with khaini-induced decrease of fertility and fecundity.

Oxidative stress has been established as one of the cause of male infertility (24). Oxidative stress is detrimental to cell function and survival (25). SLT extract produces oxidative tissue damage and apoptosis (26) (Kilinc et.al. , 2004). In mammals the epididymis plays an important role in the maturation and storage of sperm. During epididymal transit, sperm metabolism increases, accompanied by the threat of oxidative stress (26). Oxidative stress is a cellular condition associated with an imbalance between the production of ROS and their scavenging capacity by antioxidants. When the production of ROS exceeds the available antioxidant defense, significant oxidative damage occurs to many cellular organelles due to damage of lipids, proteins, carbohydrates and DNA. These processes can ultimately lead to cell death. Sperm is susceptible to oxidative damage as it contains high poly-unsaturated fatty acid in its plasma membrane (27-29). Though antioxidant defense system is active in the semen its activity is limited as the amount of cytoplasm of the sperm is low (12). Leukocytes and spermatozoa have been shown to be the main sources of ROS (30). I studied antioxidant level in testis and sperm to correlate it with khaini-induced decrease of male fertility.

Dietary antioxidants, endogenous antioxidants and metal binding proteins play important role in reducing oxidative stress in male (31,32). Endogenous antioxidants comprise antioxidants present in seminal plasma and spermatozoa. Seminal plasma contains three main enzymatic antioxidant: SOD, catalase and glutathione peroxidase / glutathione reductase. In addition there is wide range of nonenzymic antioxidants like ascorbate, urate, vitamin -E and many others. We

studied activity of SOD, principal enzymatic antioxidant of seminal plasma and ascorbate level, a nonenzymic antioxidant.

Activity of SOD significantly low in khaini treated rats than control rats. Similar result was obtained for ascorbic acid. These result indicate that khaini treatment induces oxidative stress. The result was coincide with previous observation of Kilinc et al. (11) Khaini-induced decrease of sperm motility may be due to decrease ROS scavenging capacity. Previous studies have shown a correlation between high level of ROS and sperm motility (33,34). The ROS particularly H<sub>2</sub>O<sub>2</sub> might diffuse across the membrane into the cells and inhibit the activity of glucose -6-phosphate dehydrogenase which lead to a decrease in the availability of NADPH. As a result there is decrease formation of ATP essential for sperm motility (19).

The sperm plasma membrane is very sensitive to the effect of ROS since it contains abundant poly unsaturated fatty acid ( PUFA ). These abundant PUFA create fluidity which is necessary for sperm motility and acrosomal reaction. However, the unsaturated nature of these molecules predisposes them to ROS attack and ongoing lipid peroxidation throughout the sperm plasma membrane (19). This lead to damage with subsequent sperm dysfunction or cell death. Thus khaini-induced decrease of sperm count and sperm viability may be due to increase oxidative stress.

Insemination is decreased by 25% in khaini treated rat. Penile erection is dependent on vascular smooth muscle relaxation of erectile tissues and arteries by nitric oxide. Super oxide anion and other ROS inactivate nitric oxide results in impaired male libido (35). Smoking causes erectile dysfunction by

increasing ROS generation (36). Thus SLT, Khaini-induced decrease of male libido may be due to decrease of ROS scavenging capacity .

### Conclusion

In conclusion it was suggested that chronic khaini treatment significantly decreases male fertility by reducing sperm density, sperm motility and sperm viability. Adverse effect on male fertility may be due to decrease ROS scavenging capacity of khaini treated rats.

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### References

1. A. G. Christen, J. L. McDonald, B. L. Olson J. A. Christen. *Pediatrician*. 1989, 16, 170.
2. P. C. Gupta. *Curr. Sci*. 2001, 81, 475.
3. M. R. Piano, N. L. Benowitz, G. A. FitzGerald, S. Carbridge, J. Heath, E. Hahn, T. F. Peachacek, G. Howard. *Circulation*. 2010, 122, 1520.
4. A. Lewin, O. Gonen, R. Orvieto, J. G. Schenker. *Arch. Androl*. 1991, 275 , 51.
5. R. Kunzle, M. D. Mueller, W. Hanggi, M. H. Brikhauser, H. Drescher, N. A. Bersinger. *Fertil. Steril*. 2003, 79 , 287.
6. M. F. Vine, C. K. Tse, P. Hu, K. Y. Truong. *Fertil. Steril*. 1996, 65, 835.
7. P. M. Zovos, J. R. Correa, S. Antypas, P. N. Zarmakoupis-zovas, C. N. Zarmakoupis. *Fertil. Steril*. 1998a, 69, 425.
8. P. M. Zovos, J. R. Correa, C. S. Karagounis, A. Ahparaki, C. Phoroglou, C. L. Hicks, P. N. Zarmakoupis-Zovos. *Fertil. Steril*. 1998b, 69 , 430.



9. N. L. Benowitz, H. Porchet, L. Shiener, P-III. Jacob. *Clin. Pharmacol. Therap.* 1988, 44, 23.
10. M. Bagchi, J. Balmoori, D. Bagchi, S. D. Roy, L. Kuszynski, S. J. Stohs. *Free Radical Res.* 1999, 26, 992.
11. M. Kilinc, E. Okur, E. B. Kurutas, E. I. Gular, I. Vildirih. *Cell Biol. Funct.* 2004, 22(4): 233.
12. S. E. Lewis, E. S. Sterling, I. S. Young, W. *Fertil. Steril.* 1997, 67, 142.
13. P. K. Avti, S. Kumar, C. M. Pathak, K. Vaiphei, K. L. Khanduja. *Toxicol. Sci.* 2006, 89(2), 547.
14. M. Bagchi, D. Bagchi, E. A. Hassowan, S. J. Stohs. *Toxicology.* 1998, 127, 29.
15. D. E. Brooks. *Biochem J.* 1976, 156 ;527.
16. L. J. D. Zaneveld, K. L. Polakoski. *Techniques of Human Andrology.* ed. Hafez E. S. E, North Holand Biomedical Press , Amsterdam. 1977
17. M. Buckell. *Asian J. Androl.* 2003, 5 , 209.
18. V. Srikanth, T. Malini, J. Arunakaran, P. Govindarajulu, K. Balasubramanian. *J. Pharmacol. Expt. Therap.* 1999, 288, 509.
19. Y. G. Aboua, S. S. duPlessis, N. Brooks. *African J. Biotech.* 2009, 8(22), 6416.
20. F. Nagoula, P. Watcho, M. C. Dongmo, A. Kenfack, P. Kamtchouing, J. Tchoumboue. *African Health Sci.* 2007, 7(1), 3.
21. R. Sisodia, R. K. Yadav, K. V. Sharma, A. L. Vatia. *Ind. J. Pharmaceut. Sci.* 2008, 70, 320.
22. J. P. Martin, M. Daily, E. Sugarmann. *Arch. Biochem Biophys.* 1987, 255, 329.
23. R. Srikshna, D. R. Suresh. *J. Med. Pharmacol.* 2009, 2 ( 1 ), 35.
24. K. Makker, A. Agarwal A, R. Sharma. *Indian J. Med. Res.* 2009, 29 , 357.
25. E. De Lamirande C. Gagnon. *Human Reprod.* 1995, 10 ( suppl-1 ), 15
26. J. L. Dacheux, J. L. Gatti JL, F. Dacheux. *Microscopy Res. Tech.* 2003, 61, 7.
27. R. Garg, Y. Kumbkarni, A. Aljada, P. Mohanty, H. Ghanim, W. Hamouda, P. Dandona. *Hypertension,* 2000, 36, 430.
28. A. Lenzi, L. Gandini, V. Maresca, R. Rago, F. Dondero, M. Picardo. *Mol. Human Reprod.* 2000, 6, 206.
29. D. Sanocke, M. Kurpisz. *Reprod. Biol. Endocrinol.* 2004, 2, 12.
30. N. Garrido, M. Meseguer, C. Simon, A. Pellicer, J. Remohi. *Asian J. Androl.* 2004, 6, 59.
31. A. Agarwal, K. P. Nallella, S. S. Allamaneni, T. M. Said. *Reprod. Biol. Med. Online,* 2004, 8, 616.
32. E. Greco, M. Iacobelli M, L. Rienzi, F. Ubaldi, S. Ferrero, J. Tesarik. *J. Androl.* 2005, 26, 349.
33. A. Agarwal, R. A. Saleh, M. A. Bedaiwy. *Fertil. Steril.* 2003, 79, 829.
34. J. S. Armstrong, M. Rajasekaran, W. Chamulitrat, P. Gatti, W. J. Hellstrom, S. C. Sikka. *Free Radical Biol. Med.* 1999, 26, 869.
35. R. W. Johes, R. W. Rees, S. Minhas, D. Ralph, R. A. *Expart Opin. Pharmacother.* 2002, 3(7), 889.
36. R. S. Tostes, F. S. Cameiro, A. J. Lee, F. R. Giachini, R. Liete, Y. Osawa, R. C. Webb. *J. Sex Med.* 2008, 10, 1284.