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DEFENSIVE ACTION OF LIMONENE ON TE INDUCED BPH IN MALE WISTAR RATS

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Abstract: *The Primary goal of the present investigation is to assess the defensive impact of Limonene on testosterone enanthate (TE) incited benign prostatic hyperplasia (BPH) in male Wistar rats. Wistar rats were segregated into six exploratory groups (n=6); Group 1 (Normal control), Group 2 (Disease control), Group 3 (Standard treated), Group 4 and 5 (Disease treated) and Group 6 (Normal treated) for imparting experimental treatment for a period of twenty one days. Testosterone Enanthate (TE) 25mg per day was used to induce benign prostatic hyperplasia in Wistar rats belonging to Groups from 2-5. At the end of the study Prostate gland was disengaged from all groups of lab animals after cervical dislocation for the biochemical estimation of MDA, Nitrite, Zinc, SOD and GSH and for the histopathological examination. Variation in the body weights were also recorded during the tenure of exploration.*

TE induced BPH rat groups , after three weeks of treatment with Limonene has shown significant ($p<0.05$) downturn in the levels of prostatic weight, prostatic index, MDA, nitrite and zinc but expansion in the levels of SOD and GSH in a dosage reliant way, alike Finasteride drug , a therapeutic agent for the treatment of benign prostatic hyperplasia. At the end, 21 days of treatment has emphasized the defensive action of Limonene against TE induced BPH in Wistar rats in comparison to Finasteride, a commissary therapeutic agent that treats BPH.

Keywords: *Benign prostatic hyperplasia (BPH), Limonene, Finasteride and Testosterone Enanthate (TE).*

INTRODUCTION

Benign prostatic hyperplasia (BPH) is the most common neoplasm in aging men. BPH is nonmalignant uncontrolled enlargement of the prostate gland involving the proliferation of epithelium and fibromusclar tissue leading to urethral obstruction lower urinary tract symptoms (LUTS)[1].

Expansion of the stromal and epithelial cells in the prostate organ increases progressively with ageing in all the ethnic groups. The frequency of BPH arises pointedly after the age of 40 and no less than half of the men more than 50 years of age experience the ill effects of it. Further 90% of the men who are in eighties of their life are said to have amassed with histopathological incidence of BPH[2]. It is a multi-factorial disease characterized by various LUTS like bladder outlet obstruction, increased urinary frequency, urgency and nocturia. It is associated with intermittent weak urinary stream, incomplete bladder emptying, acute and chronic urinary retention resulting in complications like urinary infections, urosepsis, bladder stones, uropathy and haematuria[3].

The development of BPH is associated with ageing and hormonal changes of men. Some studies have demonstrated that many factors such as obesity, hypertension and diabetes that are associated with cardiovascular diseases can be considered as risk factors for BPH[4]. Androgens have been identified as the major cause of the disease process. The proliferation, growth and development of prostatic tissue depends upon the action of testosterone and Dihydrotestosterone(DHT). Therefore, inhibition of DHT causes reduction in size of prostate tissue. In the prostate gland, testosterone which is produced by testicular interstitial cells is converted to Dihydrotestoterone (DHT) by the enzyme 5α-reductase, which on continuous production and accumulation causes hyperplasia. This is commonly seen in elderly men with the age of 50 years and above. The 5-alpha reductase enzymes (5αR1 and 5αR2) which trigger the production of testosterone are seen in enough amounts from the ninth week of gestation. The recent studies have shown that the main enzyme involved in the hyperplasia is 5αR2[5].

DHT increases the mitochondrial activity in the prostatic cells resulting in an excessive free radical induced oxidative damage. Conventionally in the management of BPH, the first choice of drugs are 5α Reductase inhibitors like Finasteride and Dutasteride followed by α1

adrenergic blockers like Alfuzocin, Doxazocin, Tamsulosin and Terazocin have been used[6]. But these drugs possess side effects like impotence, ejaculatory disorders like ejaculatory dysfunction from painful ejaculation to no ejaculation, decreased ejaculatory volume, decreased libido, gynaecomastia (rare), dizziness, headache fatigue, upper respiratory infections. Furthermore, post marketing investigations reported occurrence of rashes, tachycardia and chest pain. The complications and side effects with surgical treatments like prostatectomy made the choice for the screening of new drugs.

Various studies implicate the role of oxidative stress in either initiation or progression of BPH. Augmentative functioning of antioxidants and redox systems against OS in male reproductive tissues has been reported. It is suggested that OS do significant role in prostate cancer. Some reports suggest the association of OS in BPH. An extract from cactus flower inhibits the 5 alpha reductase enzyme and showed significant antioxidant effect in rat prostate homogenates. Anthocyanin from black soya bean, lupeol and mango extract[7],Diallyl sulfide[8],black tea extract[9], Vitamin E[10] and D were augmented to play a significant modulator effect in testosterone induced oxidative stress by virtue of their antioxidant property. It gives an idea that the use of exogenous antioxidant therapy may be protective effect on BPH.

Limonene (1-methyl-4-(1-methylethenyl)-cyclohexene) is the predominant flavonone (natural cyclic monoterpene) found in several citrus fruit peel oils and juices like orange, lemon, mandarin, lime, and grapefruit [11]. Limonene is a natural flavonoid that has been proved to have no side effects when used as medicine. It has been listed and recognized as safe in food supplement by the Food and Drug Administration, USA. Limonene is used to promote weight loss, treat cancer, and treat bronchitis. It also possess antiulcer, antimicrobial, anticancer, anti-diabetic and antioxidant effects[12].

These striking features of Limonene has made us curious to investigate its protective action against testosterone induced benign prostatic hyperplasia in rats. However till date no study has been reported on the effect of Limonene on testosterone induced benign prostatic hyperplasia in rats.

MATERIALS AND METHODS

Chemicals

Chemicals and reagents were procured from Sigma Chemical Company (USA) - Limonene, Cadila healthcare limited, Goa-Testosterone Enanthate, and Dr. Reddy's laboratories, Hyderabad- Finasteride.

Experimental Animals

Male wistar rats weighing (180-230g) were purchased from national institute of nutrition (NIN), Hyderabad. They were placed individually in clean, transparent polypropylene cages with free access to food and water with 12: 12 hr dark/light cycle is followed. They were acclimatized for a period of one week and grouped into experimental groups.

Experimental Design

Male Wistar rats (187-238 g) were divided into six groups (n=6) and received the following treatment for 21days.

Table 1. Experimental design of the study

Group	*Nature of group*	*Treatment/kg B.W*
Group 1	**Normal control**	**Distilled water**
Group 2	**Disease control**	**TE(25mg)**
Group 3	**Diseased treated with Standard drug**	**Finastride(5mg)**
Group 4	**Diseased treated with low dose of test drug**	**Limonene(40mg)**
Group 5	**Diseased treated with high dose of test drug**	**Limonene(40mg)**
Group 6	**Normal treated with test drug**	**Limonene(40mg)**

Parameters monitored

Body weight was measured on day1 and day 22. Drug treatment from day1 to 21 days was given. On 22nd day all the animals were sacrificed by cervical dislocation. The prostate gland was isolated and weighed immediately. Finally prostatic tissue was used for biochemical estimations (MDA, Nitrite, Zinc, GSH& SOD) and histological examination.

A. Body weight (BW)

Animals were weighed in the beginning and at the end of the experiment.

B.Prostate weight. (PW)

Animals were sacrificed by cervical dislocation and prostates were removed and weighed immediately.

C.PW and BW Ratio

PW to BW ratio was calculated.

D. Percentage of inhibition: a) Prostate weight.

b) PW/BW ratios.

$$\text{Percentage of inhibition} = 100 - [(T\text{-}NC)/(PC\text{-}NC) \times 100]$$

Where NC, PC, and T were the values of the Negative control, Positive control and treatment group, respectively.

BIOCHEMICAL CONSTITUENTS

Preparation of 2% tissue homogenate

100 mg of tissue was weighed appropriately and homogenized in 5ml of 0.15mol/L Kcl with remi motor at a speed of 2500 rpm for 2 minutes in ice cold surrounding environment. The homogenate is centrifuged at 200 rpm for 2 minutes.

1. Determination of Malondialdehyde (MDA) Concentration

The level of Malondialdehyde was determined by the procedure described by Ohkawa*et al.,* 1979[13].

Principle

The test is based on the reaction of MDA with Thiobarbituric acid (TBA); forming MDA-TBA2 product that absorbs strongly at 532nm as in the following.

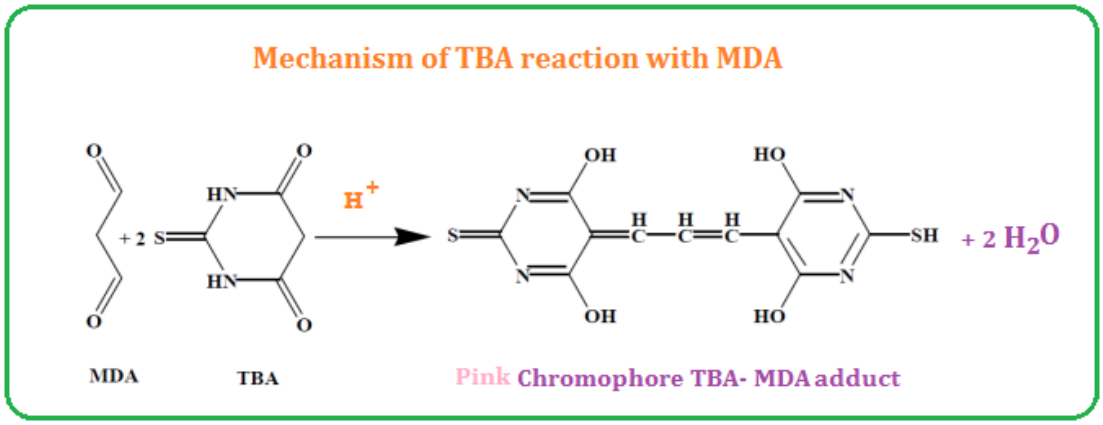

Figure 1. Reaction of TBA with Malondialdehyde to yield pink colored compound

Procedure

To 500µl of 2% tissue homogenate, 0.15mol/L Kcl, 200µl of 8.1% SDS are mixed and incubated for 5 minutes at room temperature, then it is added with1.5ml of 20% acetic acid (pH 3.5) & 1.5ml 0.8% thiobarbituric acid. The reaction mixture is heated to 95ºc for 90

minutes, the mixture is cooled and 1ml of distilled water is added along with 5ml butanol/pyridine (15:1) solution by agitation using a vortex. Solution is centrifuged at 1000xg for 15min & the resultant colouredsupernant layer is separated and its absorbance is measured at 532nm using spectrophotometer. Finally the concentration of MDA is expressed as nmol of MDA/ mg protein.

2. Determination of Super Oxide Dismutase (SOD)

The SOD level was determined by the procedure described by Mishra *et al.,* 1972[14].

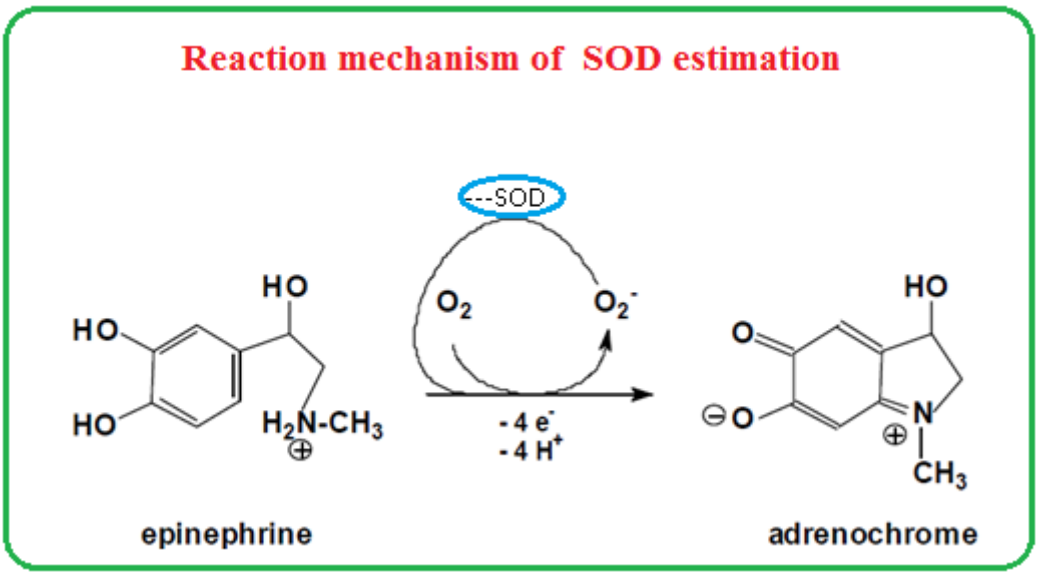

Figure 2. Oxidation of epinephrine and inhibition by SOD

Procedure

To0.5ml of tissue homogenate 1.5ml of carbonate buffer (pH 10.2),0.5ml of 0.1mM EDTA,0.4ml of epinephrine was added ,optical density is measured at 480 nm and SOD is expressed as units / min/ mg protein.

3. Determination of Reduced Glutathione (GSH) Level (Ellman)

Glutathione GSH was analyzed according to Ellman's method, 1959[15].

Principle

5, 5-dithiobis (2-nitrobenzoic acid) (DTNB) is a disulfide chromogen that is readily reduced by sulfhydryl group of GSH to an intensely yellow compound. The absorbance of the reduced chromogen is measured at 412 nm, directly proportional to the GSH concentration as in the following.

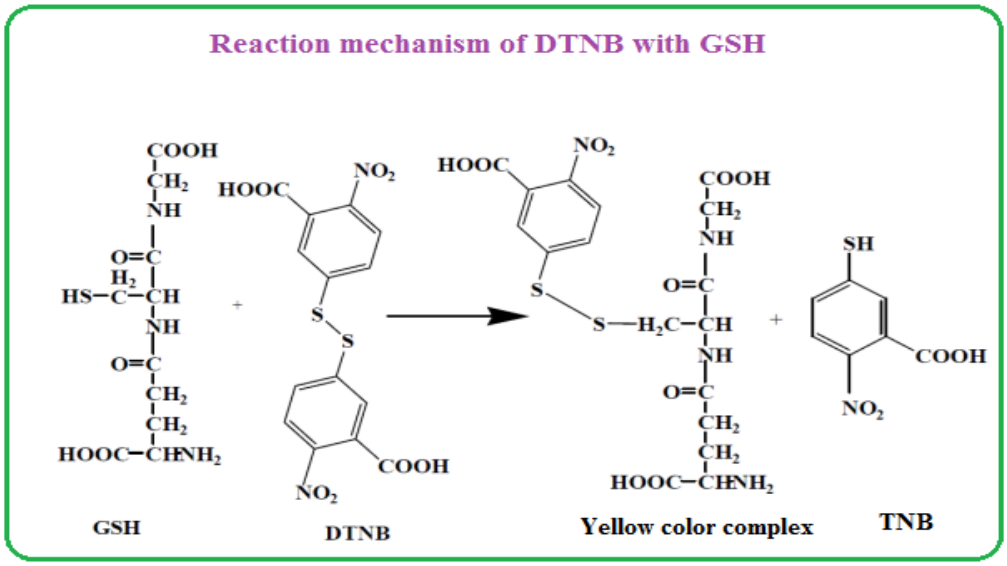

Figure 3. Principle of GSH estimation byEllman's method

Procedure

Tissue was taken and homogenized in 0.1M phosphate buffer pH 7.4.The homogenate was added with equal volume of 20% trichloro acetic acid containing 1mM EDTA, the mixture is allowed to stand for 5 minutes and centrifuged at 200 rpm for 10 minutes. 1.8ml of the Ellman's reagent is added to 200 µl of the supernatant and its volume is made up to 2ml. Absorbance is measured at 412 nm against blank and GSH is expressed as µmol / gm tissue.

Estimation of total nitrite content (Griess reaction)

Nitrite estimation was done in accordance to Griessreaction [16, 17]

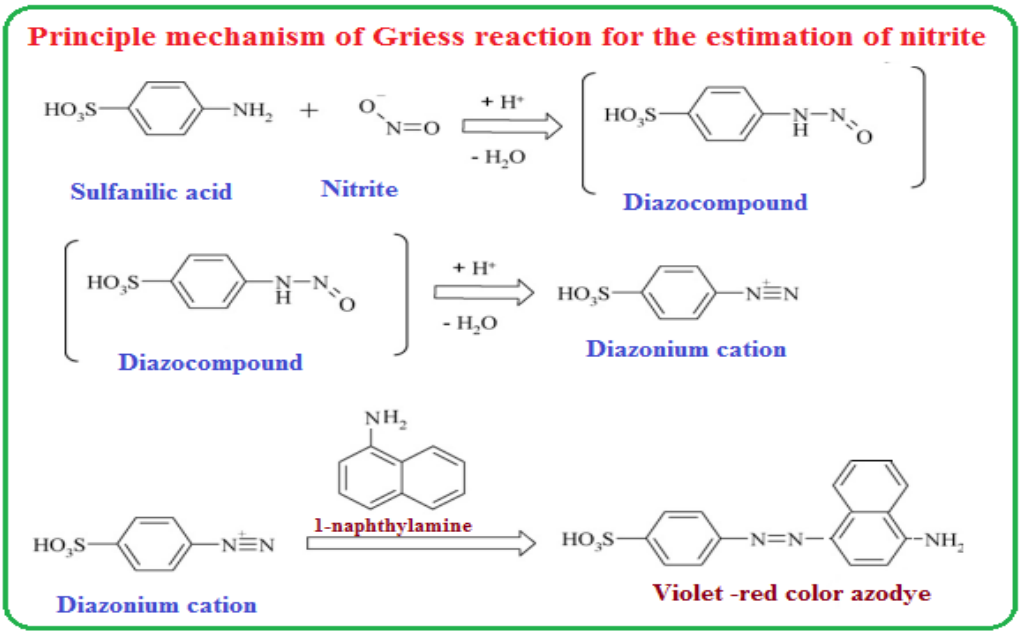

Figure 4. Principle of Griess Reaction

Procedure

To100µl of 2% tissue homogenate 100µl $FeCl_3$ solution and 100µl of Griess reagent are added, mixed well and incubated at 37ºc for 3 minutes.Absorbance is read at 540 nm. Nitrite content is expressed in µmoles.

Determination of zinc levels in prostate

Prostates were isolated and separated, homogenized in 5 ml of normal saline (NaCl 0.9%) and centrifuged at 3500g for 10 min. The supernatant was obtained for zinc determination by colorimetric assay[18].

Histopathological Examination

To assess morphological changes in prostate, tissues were embedded in paraffin and cut into sections of 4µm thickness and stained with H&E solution (hematoxylin and eosin). Tissues were subsequently mounted and cover slipped using mounting medium and then examined microscopically at 40X.

Statistical analysis

Data were expressed as means ± standard error of mean (SEM). Statistical significancewas determined using analysis of variance (ANOVA). Tests that showed a significantdifference among groups were analyzed by a multiple comparison procedure usingBonferroni's multiple comparison test. The level for significance was set at $P < 0.05$[24].

RESULTS

Body weights

There were no significant differences in the body weights of rats before and after testosterone treatment among the groups (Table 2).

Prostate weight

Administration of testosterone significantly increases prostate weight when compared with normal control rats. Treatment with Limonene (40mg/kg and 80mg/kg, p.o) dose dependently decreased prostate weights when compared to disease control. Similar effect was observed in Finasteride (5mg/kg p.o) treated group. Whereas, treatment with Limonene(80mg/kg) in normal rats did not show significant change in prostate weight when compared to normal control rats.Percentage inhibition for Finasteride, Limonene 40mg/kg and 80mg/kg, were found to be 92%, 60.0%, and 78.6% respectively (Table2).

Prostatic index (PI)

Induction of BPH significantly increases PI in testosterone treated group when compared with normal control rats. Treatment with Limonene (40mg/kg and 80mg/kg, p.o) and Finasteride (5mg/kg, p.o) significantly decreases testosterone induced prostatic index when compared with testosterone treated group. There was no change prostatic index in normal animal treated with Limonene (80mg/kg) when compared to normal control rats. Percentage inhibition of PI was found to be 93%, 73.7%, and 89% for Finasteride, Limonene 40mg/kg and 80mg/kg respectively (Table 2).

Prostatic MDA, Nitrite and zinc levels

Induction of BPH significantly increased the prostatic levels of MDA, nitrite and zinc when compared to normal control rats. Treatment with (Limonene 40mg/kg and 80mg/kg) significantly decreased the elevated levels of MDA, Nitrite and zinc in a dose dependent manner and these changes were restored to normal by treatment with high dose of Limonene except prostatic zinc levels. Treatment with Finasteride significantly restored testosterone induced MDA, nitrite and zinc levels. There were no changes observed in Limonene treated normal rat when compared to normal control (Table 3& Fig 5).

Prostatic GSH and SOD levels

Induction of BPH significantly decreased the prostatic levels of GSH and SOD were observed when compared with normal control rats. Treatment with Limonene (40& 80mg/kg) significantly increased GSH and SOD levels when compared to disease control rats. Whereas, treatment with Finasteride (5mg/kg p.o) restored prostatic GSH and SOD levels. There were no changes observed in Limonene treated normal rats when compared to normal control rats (Table 3& Fig6).

Histoarchitectural changes

Histopathological examination showed that oral administration of Limonene attenuated TE induced prostatic hyperplasia. Prostatic enlargement is used as one of important marker of BPH. In the present study, induction of BPH significantly increased in relative prostatic weight and development of stromal proliferation, hypertrophy, irregular acinar folding, hemorrhage, was observed (Fig.7B) compared with the normal control group (Fig.7A). Treatment with Limonene (40 mg/ kg) showed mild glandular hyperplasia, fatty changes, acinar folding (Fig.7D). Whereas, treatment with Limonene (80mg/kg) restored the

histoarchitecture of the prostate (Fig.7E).Similar changes was observed by treatment with Finasteride (Fig.7C). There were no changes in in normal rats treated with Limonene (80mg/kg) when compared to normal control (Fig.7F).

Table 2: Body weight, Prostate weight and Prostatic index in normal & Testosterone Enanthate(25mg/day,i.m) induced BPH rats concurrently treatedwith Vehicle, Limonene(40 and 80mg/kg, p.o) or finasteride (5mg/kg, p.o) for 21 days.

S. No.	Treatment	MDA (nmol/mg tissue)	Nitrite (mMol/mg tissue)	SOD (µmol/mg tissue)	GSH (µmol/mg tissue)	Zinc (mg/L)
I	Normal control	39.52 ± 3.84	14.57 ± 0.19	12.75 ± 0.54	5.10 ± 0.07	0.43 ± 0.02
II	Disease control TE + Vehicle	175.2 ± 4.27^{a}	30.45 ± 0.75^{a}	3.15 ± 0.22^{a}	0.87 ± 0.055^{a}	3.43 ± 0.15^{a}
III	Standard treated TE+Finasteride	65.15 ± 3.85$^{b\beta}$	14.56 ± 0.57$^{\beta}$	11.05 ± 0.32$^{\beta}$	4.87 ± 0.10$^{\beta}$	0.67 ± 0.02$^{\beta}$
IV	Disease treated TE+NR(40mg/kg)	94.01 ± 5.40$^{a\beta}$	18.89 ± 0.14$^{a\beta}$	7.6 ± 20.48$^{a\beta}$	3.2 ± 0.11$^{a\beta}$	1.18 ± 0.06$^{a\beta}$
V	Disease treated TE+NR(80mg/kg)	56.74 ± 3.8$^{\beta}$	15.26 ± 0.28$^{\beta}$	10.77 ± 0.44$^{\beta}$	4.08 ± 0.08$^{\beta}$	0.83 ± 0.18$^{\beta c}$
VI	Normal treated NR(80mg/kg)	36.32 ± 4.87	12.69 ± 0.50	15.43 ± 0.57	6.0 ± 0.11	0.48 ± 0.09

NR (Limonene); Values are expressed as mean ± SEM. (n=6).

$^{a}p < 0.0001$, $^{b}p<0.001$ when compared with normal control; $^{\beta}$ $p < 0.0001$ when compared with disease control.

Table 3. Prostatic levels of MDA, Nitrite, SOD, GSH and zinc in Normal and Testosterone Enanthate(25mg/day i.m 1,7,14) induced BPH rats concurrently treated with Vehicle, Limonene (40, 80mg/kg, p.o) or Finasteride (5mg/kg,p.o) for 21 days.

Groups	Treatment	Initial Body Weight (gms)	Final Body Weight (gms)	Prostate Weight (mgs)	%inhibition (P.W)	Prostate Index* 10^{-3}	%inhibition (P.I)
I	Normal control	203.5±4.0	209.2±4.54	470.7±4.08	-----	2.220±0.5	------
II	Disease control TE + Vehicle	206.8±1.57	213.7±2.63	875.5±3.59^{a}	-----	4.12±0.04^{a}	------
III	Standard treated TE + Finasteride	207.7±3.66	222.8±5.7	503.2±3.16$^{a\beta}$	92	2.37±0.03$^{\beta}$	93.0
IV	Disease treated TE + NR (40mg/kg)	229.2±9.45	233.7±4.36	636.8±5.96$^{a\beta}$	60	2.72±0.03$^{a\beta}$	73.7
V	D isease treated TE + NR(80mg/kg)	218.2±15.08	229.2±6.26	557.5±4.79$^{a\beta}$	78.6	2.43±0.04$^{b\beta}$	89
VI	Normal treated NR (80mg/kg)	209.5±3.91	218.5±3.73	480.8±3.94	-----	2.19±0.02	------

NR (Limonene); Values are expressed as mean ± SEM (n=6):

$^{a}p,< 0.0001$, $^{b}p<0.001$, $^{c}p<0.01$ when compared with normal control.

$^{\beta}p< 0.0001$ when compared with disease control.

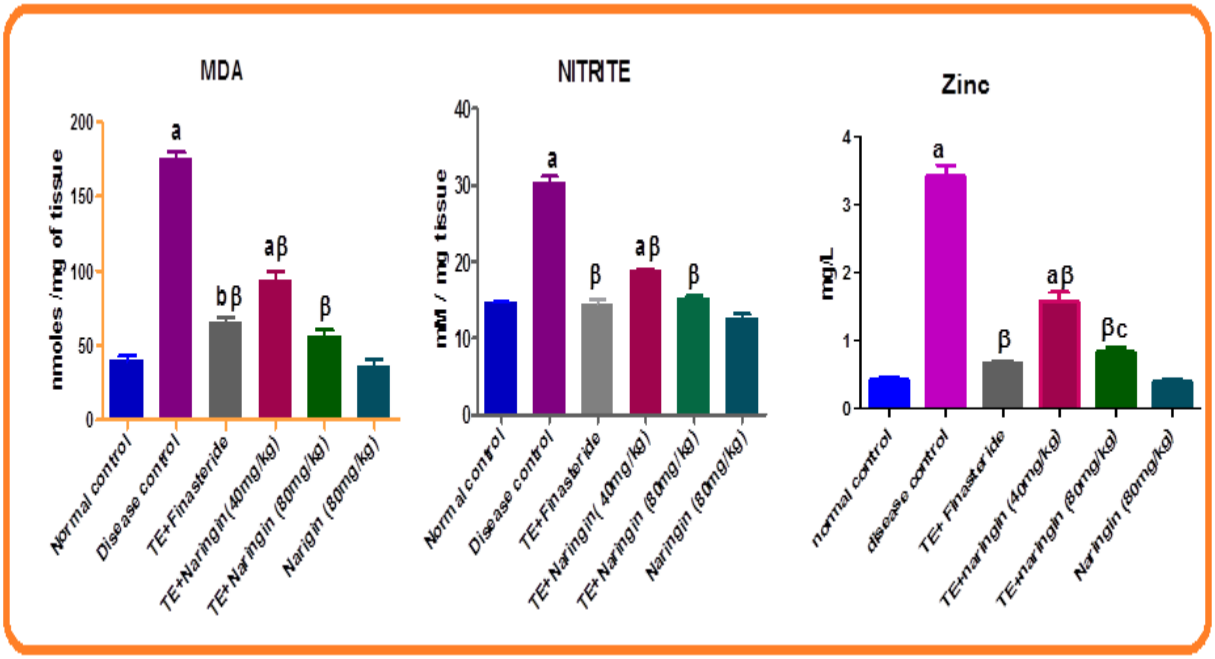

Figure 5. Prostatic MDA, Nitrite and Zinc levels in normal & Testosterone Enanthate (25mg/day,i.m) induced BPH rats concurrently treatedwith Vehicle, Limonene(40 and 80mg/kg, p.o) or finasteride (5mg/kg, p.o) for 21 days. Values are expressed as mean ± SEM (n=6). [a]p< 0.0001, [b]p<0.001, [c]p<0.01 when compared with normal control. [β] p < 0.0001 when compared with disease control.

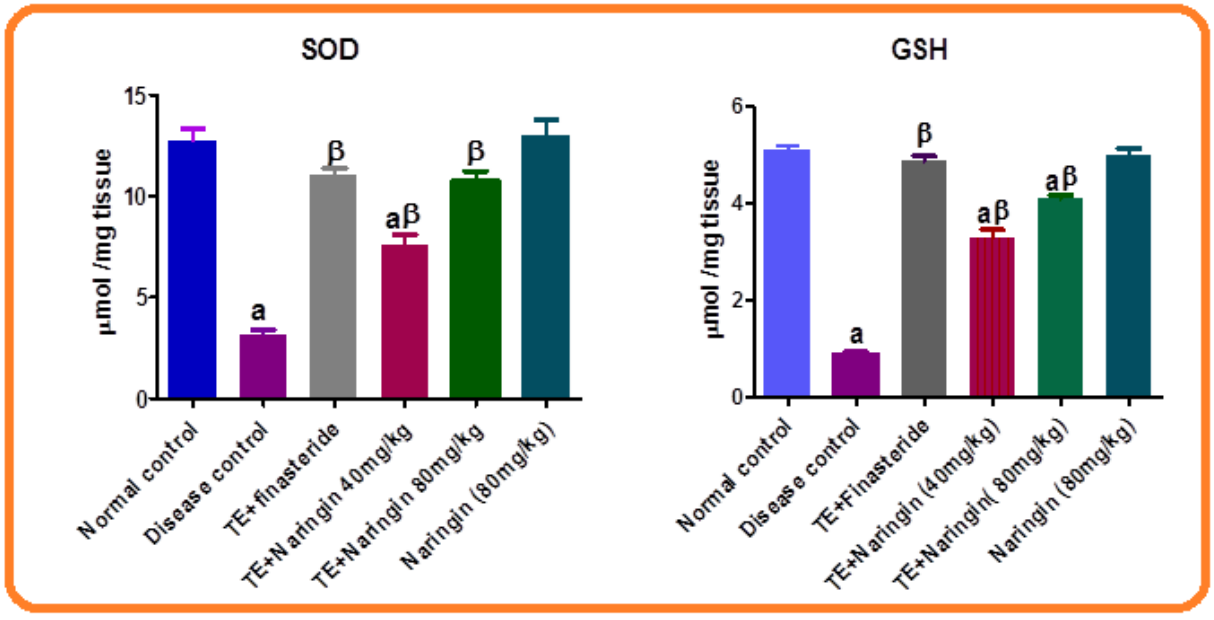

Figure 6. Prostatic SOD and GSH levels in normal & Testosterone Enanthate(25mg/day,i.m) induced BPH rats concurrently treatedwith Vehicle, Limonene(40 and 80mg/kg, p.o) or finasteride (5mg/kg, p.o) for 21 days. Values are expressed as mean ± SEM.[a]p<0.0001 when compared with Normal control.[β] p < 0.0001 when compared with Disease control.

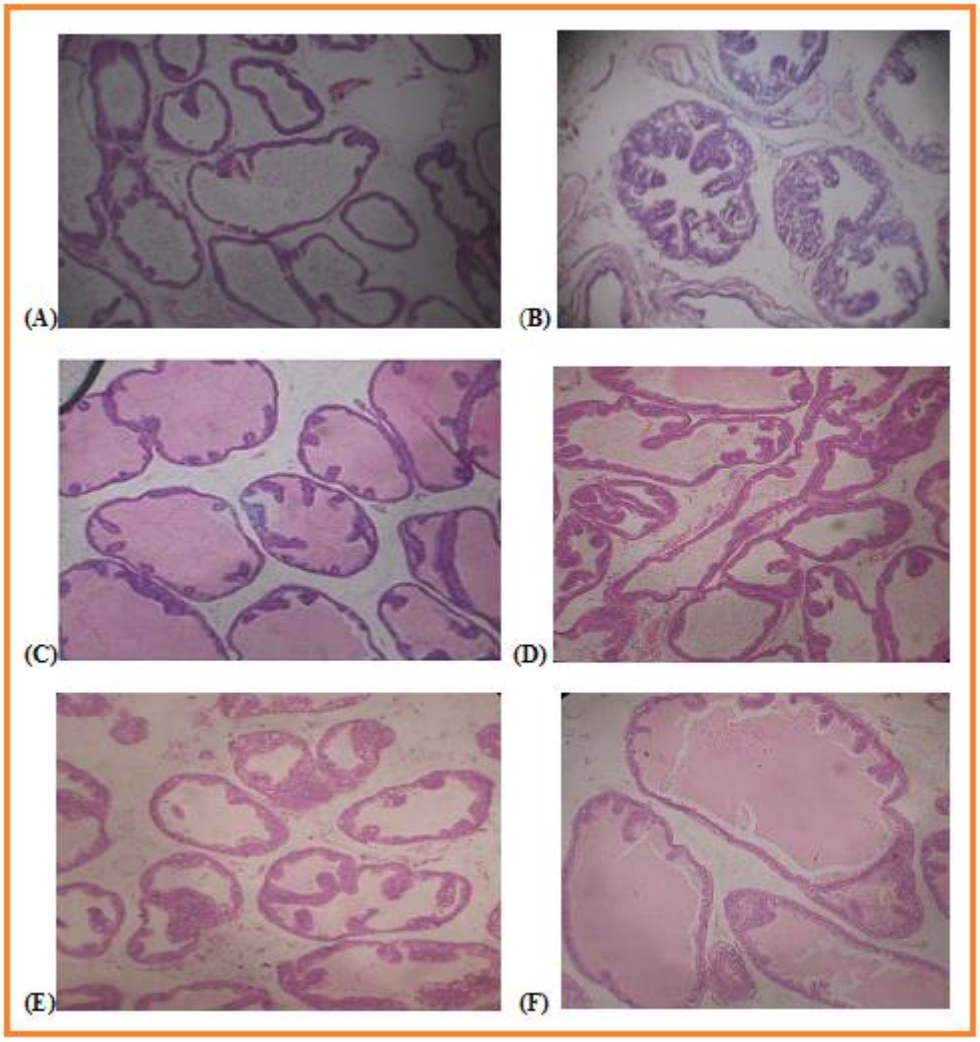

Fig 7. Effect of Limonene and Finasteride on testosterone induced histoarchitechture of rat (40x) prostate. A: Normal control; B: Disease control (Testosterone 25mg/day, i.m);C: Testosterone + Finasteride (5mg/kg, p.o); D Testosterone + Limonene (40mg/kg, p.o);E: Testosterone + Limonene (80mg/kg, p.o); F: Limonene (80mg/kg, p.o).

DISCUSSION

In the present study treatment with Limonene prevented testosterone induced benign prostatic hyperplasia which was affirmed by decrease in prostate weight and prostatic index. In consistent with previous reports, in the present study, treatment with testosterone significantly developed BPH in rats attributed by increased prostatic weight and prostatic index[5].Apart from 5-alpha reductase various clinical and preclinical studies have demonstrated that involvement of oxidative stress plays a major role in prostate cancer and BPH[7]**.** Similarly in the present study development of BPH was mediated by oxidative stress in testosterone treated rats, evidenced by increased levels of MDA, nitrite radicals and

decreased levels of GSH and SOD were observed[7]. Oxidative stress is mainly due to imbalance between the antioxidant enzymes and ROS[7]. Most of the reactive oxygen species act either by increasing the number or by inhibiting the antioxidant defense system[19].

It is well known that exogenous administration of testosterone is converted into DHT by the enzyme 5-alpha reductase in stromal cells of prostate gland.DHT which is more potent than testosterone sensitizes the androgen receptor in epithelial cells and leads to transcriptional changes in the DNA which leads to mitochondrial leakage and production of free radicals.Lipid peroxidation is an important marker for oxidative stress. Peroxide production enhances cancer progression by damaging the cell integrity and cellular enzymes[20].Glutathione plays a key role by detoxifying the toxic metabolites and lipid peroxides in the body. Increased levels of reduced glutathione significantly protect the cells[20]. SOD which is considered to be a primary antioxidant enzyme eliminates the free radicals from the body. Inhibition or deficiency of SOD in the body leads to increased production of free radicals which leads to cellular damage.Similarly various clinical studies demonstrated an increased level of nitrite radicals in BPH patients.With this observation various clinical and preclinical studies have observed beneficial effect of antioxidants in BPH[21].

In the present study treatment with Limonene had significantly elevated the antioxidant enzymes levels in prostate gland which may be due to its antioxidant property. Similar effect was observed by treatment with Limonene restores pancreatic function in streptozotocin induced diabetic rats by virtue of its antioxidant property. Similarly in the present study treatment with finasteride, a 5-alpha reductase inhibitor significantly restores antioxidant status by inhibiting the DHT mediated oxidative stress.Prostate zinc levels were increased by TE administration in animal models to induce BPH. The outcomes from the present study revealed that the treatment with Limonene reduced zinc levels in TE treated rats. In constituent to previous studies, zinc avoids citrate oxidation to regulate the proliferation of prostatic cells in BPH and also various studies support that altered androgen metabolism and higher zinc levels in patients with BPH[22].

In the present study, a positive co-relation was found between zinc and prostate weight. In consistent with earlier reports, in the present study, induction of BPH significantly altered

histoarchitecture of prostate gland[23].BPH animals experienced stromal proliferation and glandular hyperplasia in the prostate. Treatment with Limonene (40 mg/ kg) showed mild glandular hyperplasia, whereas animals treated with Limonene (80mg/kg) restored the histoarchitecture of the prostate. Similar changes were observed with treatment of Finasteride.

CONCLUSION

Exogenous administration of TE induces BPH mediated by oxidative stress, evidenced by increase in prostate weight, prostatic index, MDA and nitrite levels and decreased levels of GSH, SOD and altered histoarchitecture. Treatment with Limonene (40 & 80mg/kg) significantly inhibited TE induced BPH and oxidative stress in a dose dependent manner.

In conclusion, the present study suggests that treatment with Limonene prevents the testosterone induced BPH and this was mediated by antioxidant mechanism. Thus these findings may open novel prospective in cancer chemoprevention.However we cannot exclude other possible mechanism of action of Limonene in cancer prevention. Further experimental studies are required to confirm the present findings.

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REFERENCES

1. Arruzabala M.L, Molina V, Rosa M.C, Marrero D &Gonazalez D, Effect of coconut oil on Testosterone Induced Hyperplasia in Sprague Daweley Rat, *J of Pharm Pharmacol*, 59 (2007) 995.
2. Oesterling JE, Benign Prostatic Hyperplasia. Medical and minimally invasive treatment options, *N Engl J Med*, 332, (1995) 99.
3. Barry M.J, Evaluation of symptoms and quality of life in men with benign prostatic hyperplasia, *Urol,* 58 (2001) 20
4. Lepor H, Pathophysiology, Epidemiology and Natural History of Benign Prostatic Hyperplasi, *Urology*, 6(9) (2004) 3.
5. Veeresh Babu S, Veeresh B, Anup A.P &Warke Y, Lauric acid and Myristic acid Prevent Testosterone Induced Prostatic Hyperplasia in rats, *European journal of pharmacology*, 626 (2010) 262.

6. Anand K Patel & Christopher R C, Benign prostatic hyperplasia: treatment in primary care, *British Medical Journal*, 333(7567) (2006) 535.
7. Sahdeo P, Neetu K, Singh M & Shukla Y, Protective effect of Lupeol and Mango extract on Androgen induced oxidative stress in Swiss albino Mice. *Asian JouAndrol*. 10(2) (2008) 313.
8. Sahdeo P, Neetu K, Singh M & Shukla Y, Modulatory effect of Diallylsulhide against Testosterone induced oxidative stress in Swiss albino Mice. *Asian J. Androl*. 8(6) (2006) 719.
9. Siddiquia I.A, Raisuddind S & Shukla Y, Protective effect of Black Tea extract on Testosterone induced oxidative damage in prostat, *Cancer Letters,* 227 125-132.
10. Zhou D.X, quiu SD, Zhang J, Tian. H & Wang H.X, The Protective effect of vit. E against oxidative damage caused by Formaldehyde in the testes of Adult Rats, *J of Androl,* 8 (2006) 584.
11. Sun J.D-Limonene: safety and clinical applications.*Altern Med Rev*. 2007 Sep; 12(3):259-64.
12. Leelavinothan Pari&SelvarajuSuman, Antihyperglycemic and antilipidperoxidative effects of flavanoidnaringin in streptozotocin-nicotinamide induced diabetic rats, *Int J Biol Med Res,* 1 (2004) 206.
13. Ohkawa H, Ohishi N & Yagi K, Assay for lipid peroxides in animal tissues by Thiobarbituric acid reaction, Analytical biochemistry, 95 (1979) 351.
14. Mishra H.P &Fridovich I, The role of Superoxide anion in the Autoxidation of Epinephrine and a simple assay for Superoxide Dismutase, *J of Biological Chem*, 247 (1972) 3170.
15. Ellman G.L, Tissue Sulfhydryl groups, *Archives of biochemistry and biophysics*, 82 (1959) 70.
16. Green L. C, Wagner D.A, Glogowski J, Skipper P.L, Wishnok J.S &Tannenbaum S.R, Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids, Analytical Biochemistry,12 (1982) 131.
17. Tsikas D, Analysis of nitrite and nitrate in biological fluids by assays based on the Griess reaction: appraisal of the Griess reaction in the L-arginine/nitric oxide area of Research, J of Chromatography, 851 (2007) 51.

18. Gonzales C, Leiva-Revilla J, Rubio J, Gasco M & Gonzales GF, Effect of Red Maca (Lepidiummeyenii) on Prostate Zinc levels in rats with Testosterone-induced Prostatic Hyperplasia, *J of Androl,* 44 (2011) 362.
19. Ott M, Gogvadze V, Orrenius S & Boris Z**,** Mitochondria, oxidative stress and cell death, *Apoptosis,* 12 (2007) 913.
20. Manal K & Abdel-Rahman,Effect of Pumpkin seed (Cucurbitapepo L.) Diets on Benign Prostatic hyperplasia (BPH):Chemical and Morphometric Evaluation in rats, *World J Chem,* 1 (2006) 33.
21. Zorica A S, Ayse E, Nadics M &Onur. Increase oxidative/ nitrosative stress and decreased antioxidant enzymes in prostate cancer, *Clinical Biochemistry*, 42 (2009) 1228.
22. Duncan JR, Aspartate transcarbamylase from regenerating rat liver – a zinc activated enzymes, Nutr Res. 4 (1984) 93.
23. Babsinki MA, Chagas MA & Costa WS, Santaio.FJ, Prostatic epithelial and luminal area in the transition zone in acini: morphometric analysis in normal and hyperplastic human prostate, *BJU. Int,* 92 (2003) 592.
24. Bonferroni, C. E., Teoriastatisticadelleclassi e calcolodelleprobabilità, Pubblicazioni del R IstitutoSuperiore di ScienzeEconomiche e Commerciali di Firenze 1936.