

# Modulatory effects of *Crataeva nurvala* bark against testosterone and N-methyl-N-nitrosourea-induced oxidative damage in prostate of male albino rats

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## ABSTRACT

**Background:** Antioxidant properties of *Crataeva nurvala* bark contains a variety of the bioactive phytochemical constituents in medicinal plants which include flavonoids, phenolic compounds, tannins, anthracene derivatives, and essential oils. Components from *Crataeva nurvala* bark have been accounted to play an important role in scavenging free radicals generated by mutagens and carcinogens. Androgens are the key factors in either the initiation or progression of prostate cancer by inducing oxidative stress. In the present set of investigations, the antioxidative potential of *Crataeva nurvala* bark extract against androgen-mediated oxidative stress in male Wistar rats has been studied. **Materials and Methods:** Oxidative damage in prostate was induced in rats by the injection of testosterone (100 mg/kg body weight [bw]) for 3 days followed by injection of chemical carcinogen N-Methyl N-Nitroso Urea (50 mg/kg bw) for 1 week. The oxidative damage in prostate-induced rats were treated with the ethanolic extract of *Crataeva nurvala* bark (150 mg/kg bw) and testosterone injection (2 mg/kg bw) was also continued through the experimental period of 4 months. The prostate tissue was dissected out for biochemical analysis of lipid peroxidation and enzymic-antioxidants viz. catalase, superoxide dismutase, glutathione peroxidase, glutathione-S-transferase, and glutathione reductase; the non-enzymic antioxidants viz. reduced glutathione, and Vitamin C. **Results:** The results revealed that testosterone administration induced the oxidative stress in rat prostate; however, in drug (150 mg/kg bw) supplemented groups, a significant protective effect of *Crataeva nurvala* bark against testosterone-induced oxidative injury was recorded. **Conclusion:** Hence, the study reveals that constituents present in *Crataeva nurvala* bark impart protection against androgen-induced oxidative injury in prostate.

**Key words:** Reactive oxidative species, oxidative stress, testosterone, N-methyl-N-nitrosourea, antioxidant enzymes, *Crataeva nurvala* bark

## INTRODUCTION

Prostate cancer (PCA) continues to be the most frequently diagnosed neoplasm, and the second leading cause of cancer-related mortality in men.<sup>[1]</sup> The cause of this disease is not well understood; however, certain factors are commonly linked to its development. Non-modifiable risk factors include age, race, and genetic/family history; diet is a modifiable risk factor.<sup>[2]</sup> The etiological factors that initiate and enhance the

progression of this malignancy are emerging. The roles of prostate hormonal environment and diet/nutrition have emerged as two major direction of research focus.<sup>[3]</sup>

Studies in culture system have shown that black tea extract and theaflavins are capable of inhibiting the growth of several human carcinoma cells including androgen-sensitive human prostate carcinoma cells LNCaP.<sup>[4]</sup> The reactive oxygen species (ROS) are known to play a major role in either the initiation or progression of carcinogenesis by inducing oxidative stress.<sup>[5]</sup>

The source of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in tissues is mainly through superoxide dismutase (SOD)-mediated dismutation of O<sub>2</sub><sup>-</sup> generated in the tissues by endogenous

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enzyme system as well as by non-enzymatic pathways. In addition, the highly reactive hydroxyl radical ( $\cdot\text{OH}$ ), generated from  $\text{H}_2\text{O}_2$ , is known to damage DNA which produces the pathological alterations.<sup>[6]</sup> Cancer chemoprevention studies have shown that following administration of chemopreventive agents, levels of antioxidant enzymes are induced in various organs of the rodents.<sup>[7]</sup> Other studies have shown that activities of antioxidant, glutathione peroxidase (GPx) and catalase (CAT) and phase II detoxifying enzymes, glutathione-S-transferase (GST), and glutathione reductase (GR) could be analyzed for cancer chemopreventive effects observed with green tea.<sup>[8]</sup> ROS, such as superoxide radicals,  $\text{H}_2\text{O}_2$ , and  $\cdot\text{OH}$ , are shown to cause lipid peroxidation (LPO), thus altering the activity of sulfhydryl ( $-\text{SH}$ )-dependent enzymes and of damaging DNA and other critical cellular organelles. ROS-associated oxidative damage is well documented in human PCA<sup>[9-11]</sup> and down modulation of antioxidant enzymes in human prostate carcinoma cell lines viz. DU145 and LNCaP.<sup>[12]</sup>

United States as well as India has PCA as a leading cause of cancer-related deaths.<sup>[13,14]</sup> Steroid hormones, particularly testosterone and carcinogen MNU (N-Methyl N-Nitroso Urea), are suspected to play a role in human prostate carcinogenesis, but the precise mechanism by which androgens exert this process and the possible involvement of estrogenic hormones are not clear. Therefore, attempts are being made in present study to evaluate the role of testosterone in oxidative damage in prostate tissue and its modulation by supplementation of *Crataeva nurvala* bark (150 mg/kg bw) in male Wistar rats.

## MATERIALS AND METHODS

### Plant collection and extract preparation

The bark of *Crataeva nurvala* was collected from Pollachi, Tamil Nadu, India. It was authenticated by Dr. G.V.S. Murthy, Botanical Survey of India, Tamil Nadu Agricultural University Campus, Coimbatore, Tamil Nadu, India. A Voucher specimen was deposited in the laboratory for future reference (BSI/SC/5/23/8-9/Tech/766). The powdered bark of *Crataeva nurvala* (100 g) was extracted with 500 ml of 99% ethanol.

### Chemicals

Testosterone was purchased from SD Fine Chemicals, Mumbai; MNU was purchased from Sigma U.S.A.; and Propylene glycol was purchased from Qualigens Fine Chemicals, Mumbai.

### Preliminary phytochemical screening

The phytochemical screening of *Crataeva nurvala* was performed as per procedure.<sup>[15,16]</sup>

### Animals used

The Wistar strain of male albino rats weighing between 160 and 180 g were used in the present study. The animals were housed in large spacious cages and they were given food and water ad libitum during the course of the experiment. The study was approved by Institutional Animal Ethical Committee constituted for the purpose of CPCSEA, Government of India.

### Induction of oxidative damage in prostate using carcinogen and hormone

Step 1: The rats received daily intraperitoneal injection of 100 mg/kg body weight (bw) of testosterone in 0.3 ml propylene glycol for 3 days.

Step 2: One day after the last testosterone, all the rats received a single intravenous dose (50 mg/kg bw) of MNU dissolved in saline at (10 mg/ml) through the tail vein.

Step 3: One week after MNU administration, the rats received daily intraperitoneal injection of 2 mg/kg bw of testosterone for 60 days.

### Experimental design

The experimental animals were divided into five groups. Each group consisted of six rats.

**Group 1** served as control (propylene glycol)

**Group 2** rats were induced prostate cancer by daily intraperitoneal injection (100 mg/kg bw) of testosterone in 0.3 ml propylene glycol for 3 days. One day after the testosterone injection, all the rats received intravenous injection (50 mg/kg bw) of MNU (dissolved in saline at 10 mg/ml), through the tail vein for a period of one week. One week after MNU administration, rats received daily intraperitoneal injection of 2 mg/kg bw testosterone for 60 days

**Group 3** rats were treated as group 2 and simultaneously treated with weekly twice the dose of (150 mg/kg bw) of the crude ethanolic extract of *Crataeva nurvala* bark through oral gavage for 16 weeks.

**Group 4** rats received weekly twice the dose of (150 mg/kg bw) of crude, ethanolic extract of *Crataeva nurvala* bark through oral gavage for 16 weeks.

**Group 5** rats were treated as group 2 along with Finasteride (25 mg/kg bw) supplemented throughout the entire experimental period of 16 weeks.

After the experimental period, the animals were sacrificed under light chloroform anesthesia.

### Biochemical estimations

LPO was analyzed by the method of Okhawa.<sup>[17]</sup> The

reaction mixture in a final volume of 1.0 ml contained of 0.1M Phosphate buffer (pH 7.4). The degree of LPO was assayed by estimating the thiobarbituric acid reactive substances (TBARS) by using the standard methods with minor modifications. Different concentrations of the ethanolic extract (20, 40, 60, 80, and 100 µg/ml) were added to 0.1 ml of sample mixture. LPO was initiated by the addition of 100 µl of 15mM ferrous sulfate solution to the reaction mixture. After 30 minutes, 100 µl of this mixture was taken in a tube containing 1.5 ml of 10% TCA, mixed well, and kept for 10 minutes. The tubes were then centrifuged; supernatants were separated and mixed with 1.5 ml of 0.67% TBA in 50% acetic acid. Then, the mixture was heated for 30 minutes in boiling water. The intensity of the pink-colored complex formed was measured at 535 nm. The results were expressed as nanomoles of MDA formed/mg protein. The protein content of the tissue was determined by the method of Lowry<sup>[18]</sup> using bovine serum albumin as standard.

SOD was analyzed as per the protocol of Kakkar.<sup>[19]</sup> The assay mixture in a final volume of 3 ml contained 50mM phosphate buffer (pH 7.4). 1.4 ml aliquot of the reaction mixture was pipetted in a test tube. 100 µl of the sample was added followed by preincubation at 37°C for 5 minutes. 80 µl of riboflavin was added and the tubes were exposed for 10 minutes to 200 watt Philips fluorescent lamps. The control tube contained equal amount of buffer instead of sample. The sample and its respective control were run together. At the end of the exposure time, 0.1 ml of Griess reagent was added to each tube and the absorbance of the color formed was measured at 543 nm.

The activity of CAT was analyzed according to the method of Sinha<sup>[20]</sup> using H<sub>2</sub>O<sub>2</sub> as substrate. In brief, the assay mixture contained 0.5 ml of H<sub>2</sub>O<sub>2</sub>, 10 ml of 0.01 M Phosphate buffer (pH 7.0), and 0.4 ml water. 0.2 ml of the enzyme protein was added to initiate the reaction. 2.0 ml of the dichromate/acetic acid reagent was added after 0, 30, 60, and 90 seconds of incubation. The enzyme activity was measured following the disappearance of H<sub>2</sub>O<sub>2</sub> at 610 nm using a spectrophotometer and was expressed as mmole/min/mg protein.

The activity of GPx was estimated by the method of Rotruck<sup>[21]</sup> using sodium azide as substrate. In brief, 0.4 ml of phosphate buffer (pH 7.0), 0.1 ml of sodium azide, 0.2 ml of reduced glutathione (GSH), 0.1 ml of H<sub>2</sub>O<sub>2</sub>, 0.2 ml of enzyme, and 1.0 ml of water were added to a final incubation volume of 2.0 ml. The tubes were incubated for 0, 30, 60, and 90 seconds. The reaction was then terminated by the addition of 0.5 ml TCA. To determine the glutathione content, 2.0 ml of the supernatant was removed by centrifugation and added 3.0 ml disodium hydrogen phosphate solution and 1.0 ml of DTNB reagent. The

enzyme activity was measured at 340 nm. The activity was expressed in terms of µg of glutathione utilized/mg protein

GST was analyzed as per the protocol of Habig *et al.*<sup>[22]</sup> In brief, 1.0 ml of 0.5M phosphate buffer (pH 6.5), 0.1 ml of sample, 1.7 ml of water, and 0.1 ml of CDNB were added and incubated at 37°C for 5 minutes. After incubation, 0.1 ml of GSH was added. The increase in optical density of the enzyme was measured at 340 nm. The enzyme activity is calculated in terms of µmoles of CDNB conjugate formed/min/mg protein.

GR activity was measured by the protocol of Carlberg and Mannervik;<sup>[23]</sup> briefly, the assay mixture in a final volume of 3.0 ml contained 0.067M phosphate buffer (pH 6.6), NADPH, 7.5×10K3 GSSG (pH adjusted to 6.6 with 1N NaOH) enzyme, and water. The reaction was initiated with enzyme preparation. The difference in optical density per 30 s was measured for 3 minutes at 340 nm against a reference cuvette devoid of GSSG and NADPH.

GSH was estimated by the method of Moran *et al.*;<sup>[24]</sup> briefly, 1.0 ml of 10% tissue homogenate was mixed with 4.0 ml of metaphosphoric acid. The precipitate was removed by centrifugation. To 2.0 ml of the supernatant, 2.0 ml disodium hydrogen phosphate and 1.0 ml of DTNB (40 mg DTNB in 100 ml of 1% trisodium citrate) reagent were added. The absorbance was read within 2 minutes at 412 nm against a reagent blank. A set of standards was also treated in the above manner. The amount of glutathione was expressed as µg/mg protein.

Vitamin C was estimated by the method of Omaye *et al.*;<sup>[25]</sup> briefly, 1.0 ml of 10% homogenate was precipitated with 5% ice-cold TCA and centrifuged for 20 minutes at 3 500 g. 1.0 ml of the supernatant was mixed with 0.2 ml of DTCS (3 g of 2,4-dinitrophenyl hydrazine, 0.4 g thiourea, and 0.05 g copper sulfate were dissolved in 9N sulfuric acid and made up to 100 ml with the same) reagent and incubated for 3 hours at 37°C. Then, 1.5 ml of ice-cold 65% sulfuric acid was added, mixed well, and the solutions were allowed to stand at room temperature for an additional 30 minutes. Absorbance was determined at 520 nm. These results were expressed as µg/mg protein.

### Statistical analysis

The values were expressed as mean±SD. The statistical analysis was carried out by one-way analysis of variance using SPSS (version 10) statistical analysis program. Statistical significance was considered at  $P<0.05$ .

## RESULTS

The phytochemical screening of the plant extract revealed

**Table 1: Preliminary phytochemical screening of the ethanolic extract of *Crataeva nurvala***

Phytochemicals	Inference
Alkaloids	+
Amino acids	+
Proteins	+
Carbohydrates	+
Flavonoid	+
Steroids	+
Saponins	+
Tannins	+
Oils and fats	+

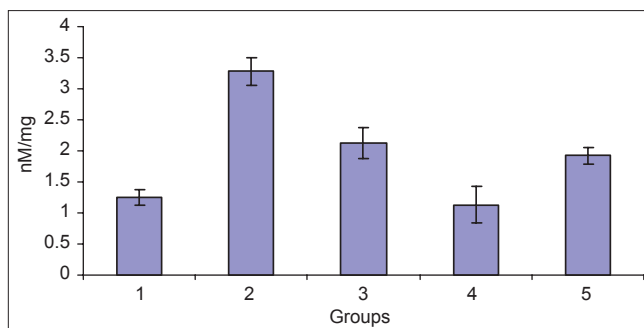
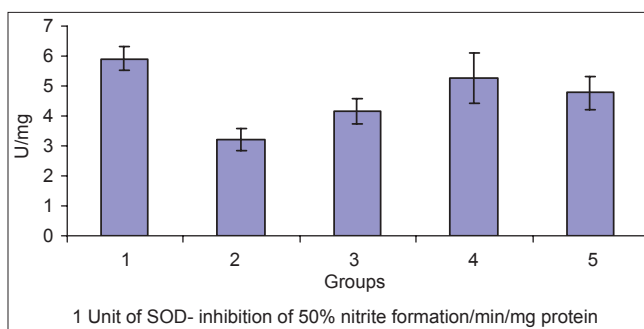
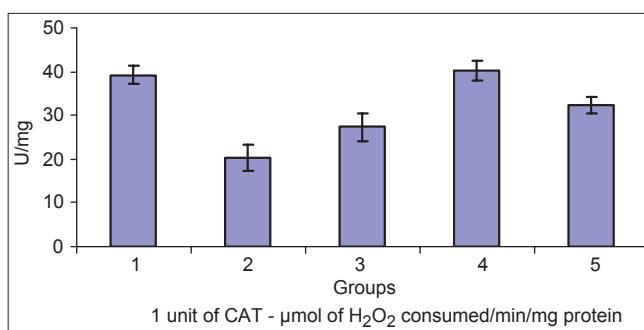
+ = Presence; - = Absence

the presence of major chemical constituents such as alkaloids, tannins, steroids, saponins, flavonoids, oils and fats, carbohydrates, amino acids, and proteins [Table 1].

Androgen is a known inducer of oxidative stress, and its intraperitoneal administration has been found to modulate the levels of antioxidant enzymes in male Wistar rats. The LPO level (TBARS) was found to be significantly elevated in group 2 testosterone- and MNU-induced rats when compared to group I control rats. Oral administration of the ethanolic extract of *Crataeva nurvala* bark to the group 3 rats significantly ( $P < 0.05$ ) reduced the testosterone- and MNU-induced prostate damage which is near to that of finasteride-treated group 5 rats. The LPO activity in group 2 and group 5 animals were close to that of normal group 1 animals. There is no significant ( $P < 0.05$ ) difference between group 1 control rats and the ethanolic extract of *Crataeva nurvala* bark alone treated group 4 rats [Figure 1].

The level of enzyme SOD in the prostate homogenate was found to be significantly ( $P < 0.05$ ) decreased following testosterone and MNU administration in group 2 rats when compared to control group I animals. *Crataeva nurvala* bark supplementation has been found to significantly ( $P < 0.05$ ) elevate the SOD activity in group 3 rats which is to the near levels of standard finasteride-treated group 5 animals. The SOD levels in group 2 and group 5 animals were close to that of normal group 1 animals. There is no significant ( $P < 0.05$ ) difference between group I control rats and the ethanolic extract of *Crataeva nurvala* bark alone treated group 4 rats [Figure 2].

The level of CAT activity was significantly ( $P < 0.05$ ) decreased in testosterone- and MNU-induced oxidative damaged group 2 rats when compared with group 1 control animals. The CAT activity was found to be significantly ( $P < 0.05$ ) increased in group 3 animals treated with the ethanolic extract of *Crataeva nurvala* bark and group 5 animals treated with finasteride [Figure 3]. No significant ( $P < 0.05$ ) difference was found between the group I control rats and the ethanolic

**Figure 1:** Effect of *Crataeva nurvala* bark extract on lipid peroxidation in prostate tissue**Figure 2:** Effect of *Crataeva nurvala* bark extract on superoxide dismutase in prostate tissue**Figure 3:** Effect of *Crataeva nurvala* bark extract on CAT in prostate tissue

extract of *Crataeva nurvala* bark alone treated group 4 rats.

GPx activity was found to be significantly ( $P < 0.05$ ) decreased in testosterone- and MNU-induced group 2 rats. Oral administration of *Crataeva nurvala* bark has been found to significantly ( $P < 0.05$ ) increase the GPx which is near to that of standard finasteride-treated group 5 animals. GPx activity in group 2 and group 5 animals were close to that of normal group 1 animals. No significant ( $P < 0.05$ ) difference was found between the group I control rats and the ethanolic extract of *Crataeva nurvala* bark alone treated group 4 rats [Figure 4].

Testosterone and MNU administration has been found to significantly ( $P < 0.05$ ) reduce the level of GST in group 2

rats when compared to that of control group 1 rats. The treatment of ethanolic extract of *Crataeva nurvala* bark significantly ( $P < 0.05$ ) induces GST activity as that of finasteride-treated group 5 rats. GST activity in group 2 and group 5 animals were close to that of normal group 1 animals as showed in Figure 5. No significant ( $P < 0.05$ ) difference was found between the group I control rats and the ethanolic extract alone treated group 4 rats.

A significant ( $P < 0.05$ ) decrease in the activity of enzyme GR has been recorded in the oxidative damage of rat prostate homogenate (group 2) following testosterone and MNU administration. A significant ( $P < 0.05$ ) increase in the level of GR to near normal level was seen in *Crataeva nurvala* bark supplemented group as comparable to standard group 5 and normal group 1 animals. No significant ( $P < 0.05$ ) difference was found between the group I control rats and the ethanolic extract alone treated group 4 rats [Figure 6].

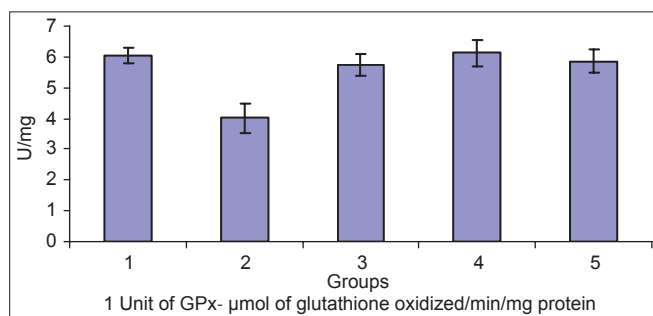
The levels of GSH and vitamin C were significantly ( $P < 0.05$ ) decreased in the testosterone- and MNU-induced group 2 as compared to the normal groups (group 1). On treatment with the ethanol extract of *Crataeva nurvala* bark (group 3), GSH and vitamin C levels were found to be enhanced significantly ( $P < 0.05$ ). There was no significant difference between the ethanol extract alone treated group 4 and normal group 1 rats [Figures 7 and 8]. No significant ( $P < 0.05$ ) difference was found between the group I control rats and the ethanolic extract alone treated group 4 rats.

## DISCUSSION

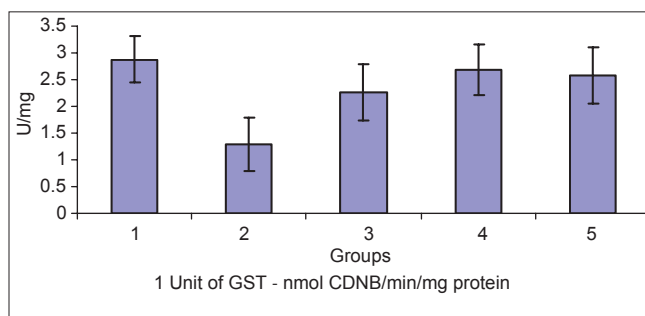
Herbal medicines prescribed by tribal healers are either preparation based on single plant part or a combination of several plant parts. They believe that combination of several plant parts cures diseases rapidly. Cancer chemoprevention by using antioxidant approaches has been suggested to offer a good potential in providing important fundamental benefits to public health, and is now considered by many clinicians and researchers as a key strategy for inhibiting, delaying, or even reversal of the process of carcinogenesis.<sup>[26]</sup>

The phytochemical screening of the plant extract revealed the presence of major chemical constituents such as alkaloids, tannins, steroids, saponins, flavonoids, oils and fats, carbohydrates, amino acids, and proteins. Some of their active principles including flavonoids are known to be used for the treatment of carcinogenic activity. Moreover, alkaloid-containing drug demonstrated anti-tumor activity.<sup>[27]</sup> on the basis of the above evidences, it is made clear that the presence of alkaloids, flavonoids, terpenoids, and tannins are responsible for the observed anti-tumor activity. Several such compounds are known to possess potent antioxidant activity.<sup>[28]</sup>

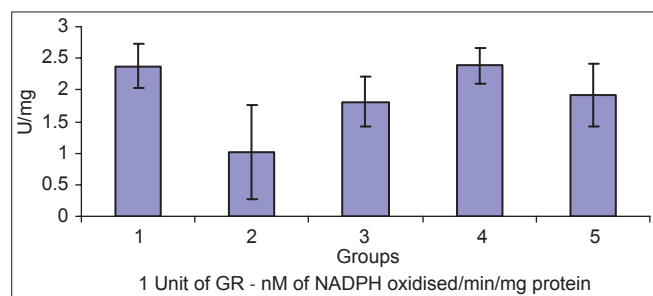
In prostate tissue, LPO levels were elevated significantly in testosterone- and MNU-induced prostate cancer rats as compared to control rats. The oral administration



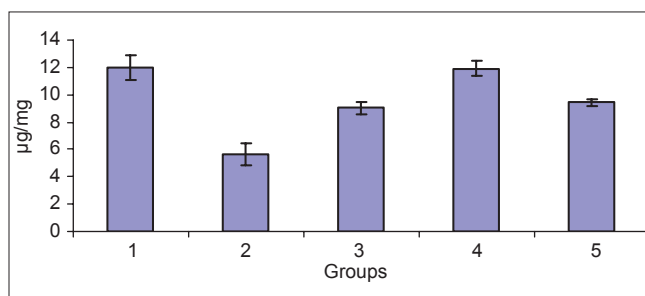
**Figure 4:** Effect of *Crataeva nurvala* bark extract on glutathione peroxidase in prostate tissue



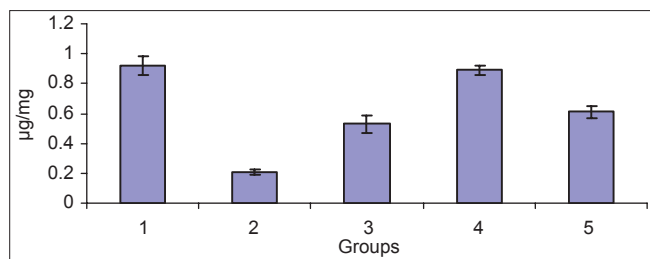
**Figure 5:** Effect of *Crataeva nurvala* bark extract on glutathione-S-transferase in prostate tissue



**Figure 6:** Effect of *Crataeva nurvala* bark extract on glutathione reductase in prostate tissue



**Figure 7:** Effect of *Crataeva nurvala* bark extract on glutathione in prostate tissue



**Figure 8:** Effect of *Crataeva nurvala* bark extract on Vit C in prostate tissue

of *Crataeva nurvala* bark extract for 16 weeks lowered the elevated values to near normal level significantly ( $P < 0.05$ ). *Crataeva nurvala* bark extract alone treated rats did not show any significant difference when compared to normal rats. The comparison was done with standard drug finasteride-treated rats. Previous studies reported that finasteride prevents or delays the appearance of prostate cancer. Androgens are involved in the development of prostate cancer. Finasteride, an inhibitor of  $5\alpha$ -reductase, inhibits the conversion of testosterone to dihydrotestosterone, the primary androgen in the prostate, and may reduce the risk of prostate cancer.<sup>[29]</sup> Finasteride is the only intervention that has been shown to reduce the incidence of prostate cancer in a long-term prospective clinical trial.<sup>[30]</sup>

LPO is one of the main causes of oxidative damage initiated by ROS and it has been linked with altered membrane structure and enzyme inactivation. It is initiated by the abstraction of a hydrogen atom from the side chain of PUFA in the membrane.<sup>[31]</sup> The increase observed in tissue MDA levels in the present study demonstrates LPO to have developed. The acetaminophen-induced elevation of the LPO in rats was significantly ( $P < 0.05$ ) decreased in the presence of *Uvaria chamae* root bark methanol extract.<sup>[32]</sup> Therefore, our results suggest that the agents in the extract may be acting by preventing the T+MNU-induced LPO.

A significant reduction in the enzymatic antioxidants like SOD, CAT, GPx, GST, and GR were observed in T+MNU-induced prostate cancer rats when compared with control rats. On oral administration of *Crataeva nurvala* bark and finasteride standard drug for 16 weeks showed significant elevation ( $P < 0.05$ ) in all the enzymatic antioxidant values and reached near normal values. There was no significant difference in *Crataeva nurvala* bark alone treated rats and their values were similar to control rats.

The major defense mechanism involves the antioxidant enzymes, including SOD, CAT, and GPx, which convert active oxygen molecules into non-toxic compounds.<sup>[33]</sup>

SOD is widely distributed in cells with high oxidative

metabolism and has been proposed to protect against the deleterious effect of superoxide anion. SOD catalytically scavenges the superoxide radicals and thus renders cytoprotection against the free radical damage.<sup>[34]</sup> CAT and GPx play a pivotal role in  $H_2O_2$  catabolism and the detoxification of endogenous metabolic peroxides and hydroperoxides which catalyses GSH. Antioxidant activity and the inhibition of free radical generation are important in terms of protecting the prostate after treatment.

The most extensively studied anti-oxidant enzyme in prostate cancer is GST. In particular, expression of GST has been extensively studied in human prostatic tissues. Two thirds of normal basal prostatic epithelial cells immune stain intensely for GST, whereas acinar epithelia stain weakly for GST.<sup>[35]</sup> Reduced activity of GST observed in the prostate cancer state may be due to the inactivation caused by ROS.

GR is the most important biomolecule against chemically induced toxicity which can participate in the elimination of reactive intermediates by reduction of hydroperoxides in the presence of GPx. It also functions as a free radical scavenger and in the repair of free radical caused biological damage.<sup>[36]</sup> The erythrocyte antioxidant enzyme, i.e., GR activity was found to be increased significantly in patients with prostate cancer.<sup>[37]</sup>

Glutathione being the most important biological molecule against chemically induced toxicity can participate in the elimination of reactive intermediates by reduction of hydro peroxides in the presence of GPx. Glutathione also function as a free radical scavenger and in the repair of free radical-induced biological damage.<sup>[38]</sup>

A high dose of vitamin C is reported to act as an oxidative agent. Membrane sulphhydryl groups play an important role in maintaining the structural integrity of membranes.<sup>[39]</sup> Vitamin C plays an important role in detoxification of reactive intermediates produced by cytochrome p 450, which detoxifies xenobiotics.

Thus, the present study suggests that *Crataeva nurvala* bark can be used against testosterone- and MNU-induced oxidative damage in prostate of male albino rats.

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