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**SUPPLEMENTATION WITH WATERMELON RENDERS PROTECTION AGAINST TOXICITY INDUCED BY PARACETAMOL IN ALBINO RATS: THE MUTUAL AND FINE INTERACTION OF ANTIOXIDANTS PREVENTED THE CELLULAR DAMAGE**

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**ABSTRACT**

Vegetables and fruits are well known for their protective effects against oxidative stress because of their antioxidant properties. In the present studies locally available watermelon has been evaluated for its protective effects against paracetamol induced oxidative stress in albino rats. Three doses of fresh water melon extract (2.5ml, 5.0 ml and 7.5 ml per Kg body weight) along with paracetamol (125mg/Kg body weight) have been administered orally to three groups (5 each) of experimental rats for 30 days. Paracetamol control group was administered only paracetamol (125mg/Kg body weight) and normal control group was given equal volume of distilled water. At the end of the experimental period, after 30 days, blood samples were collected from all the experimental animals into heparin coated centrifugation tubes. Blood was centrifuged and plasma was separated to measure thiobarbituric acid reactive substances (TBARS), plasma alanine transaminase (ALT), plasma aspartate transaminase (AST) reduced glutathione (GSH), tocopherol, glutathione per oxidase (GPx) and super oxide dismutase (SOD). Results indicate that fresh watermelon extract inhibits lipid peroxidation induced by paracetamol, maintains the levels of glutathione peroxidase, glutathione and tocopherol. It also enhances the activity of super oxide dismutase and glutathione peroxidase. Carotenoids including lycopene, ascorbic acid and phenols of WMJ are responsible for the protection mechanisms. A mutual and fine interaction between antioxidants present in WMJ is proposed for prevention of oxidative stress and damage.

**Keywords:** Carotenoids, Thiobarbituric Acid Reactive Substance, Glutathione, Super Oxide Dismutase, Glutathione Peroxidase

**INTRODUCTION**

Paracetamol is usually recommended for mild pain and fever. With increasing status of physical and mental stress in developed as well as developing countries, consumption of paracetamol has increased beyond limit. Metabolism of paracetamol produces N-acetyl-P-benzoquinonimine which is metabolized by glutathione to a non toxic metabolite. When levels of glutathione are low, the toxic metabolite binds to sulphhydryl containing protein in the liver cells and causes lipid peroxidation because of oxidative stress (Knight *et al.*, 2003). Lipid peroxidation results diseases like arthritis, liver toxicity, cardiovascular disorders, kidney failure, diabetes and promotes aging. Oxidative stress induced by paracetamol usually results into liver toxicity either due to overdose or due to prolonged usage of paracetamol (James *et al.*, 2003). To curb this increasing problem of oxidative stress, supplementation of diet with natural vegetables and fruits which are rich in anti-oxidants is the best means to overcome this problem. Vegetables and fruits are rich sources of anti-oxidants and act as natural medicine because they prevent the oxidative stress by scavenging free radicals and bring the stressed physiological status of the body to normal (Charoensiri *et al.*, 2009).

Watermelon is very popular fruit throughout the world including Botswana. It forms the staple diet in village community. Watermelon (*Citrullus lanatus*) belongs to family *Cucurbitaceae*. It is a vine-like climber herb. The fruit of this plant is pepo with exocarp and fleshy mesocarp and endocarp. The endocarp is juicy, sweet and usually red in colour. It is an excellent source of beta carotene (616

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ug/100g), another carotenoid lycopene (6693ug/100g) (Charoensiri *et al.*, 2009; Perkins- Veazie *et al.*, 2006) and vitamin C (0.7 to 7mg/100g) (Sevcan *et al.*, 2011). It is also rich source of phenolic and flavonoids. According to Sevcan *et al.*, 2011, the total phenol content is 2490 ugGAE/ml juice and total flavonoid content is  $9.84 \pm 1.54$  ug QUE/ml juice in red water melon. Watermelon is a rich source of citrulline that is metabolized to arginine, an essential amino acid used to synthesize nitric oxide and thus plays an essential role in cardiovascular and immune functions (Collins *et al.*, 2007). Watermelon pomace juice acts as a functional food and increases the availability of arginine, reduces concentrations of cardiovascular risk factors, improves glycemic control, and ameliorates vascular dysfunction in obese animals with type-II diabetes (Wu *et al.*, 2007). Vitamin C, carotenoids, phenols and flavonoids are well known for their anti-oxidant and free radical scavenging activities and water melon is rich in these anti-oxidants (Bosku, 2006). Hence it is proposed that water melon could be very beneficial in preventing diseases caused of oxidative stress. Protective effects of Diyarbakir water melon (red variety) against carbon tetra chloride induced toxicity has been reported by Sevcan *et al.*, 2011. Our previous findings also indicate the hepatoprotective properties of water melon against paracetamol induced hepatotoxicity (Chaturvedi 2012). No work has yet been reported regarding the role of water melon juice (WMJ) indigenous to Botswana on anti-oxidant system in preventing paracetamol induced hepatotoxicity. Therefore our present study is mainly focused at evaluating the role of WMJ in preventing the oxidative toxicity induced by daily administration of paracetamol to albino rats.

## **MATERIALS AND METHODS**

### ***Water Melon Fruit and Preparation of Extract***

Fresh water melon was obtained from local farmers and its authenticity was confirmed by the Botany section of the Biological Sciences department, University of Botswana. About 5 Kg of endocarp of the ripe fruit was chopped into thin slices and crushed to pulp into a blender. The crushed pulp was filtered through fine mesh cloth to get the fresh water melon fruit juice (WMJ). The juice was allowed to stand for half an hour that allowed the clear juice to settle at the bottom with frothy topping which was carefully removed. The total volume of the juice was 500 ml. The juice was stored at  $-70^{\circ}\text{C}$  until use.

### ***Chemicals***

Thiobarbituric acid, reduced glutathione (GSH), ascorbic acid, trichloroacetic acid (TCA), 5-dithiobis 2-nitrobenzoic acid (DTNB), NADPH, L-methionine, Nitroblue tetrazolium (NBT), EDTA, Riboflavin, Glutathione reductase bought from Sigma-Aldrich (St Louis-MO). Other chemicals were bought from local suppliers and were of analytical grade.

### ***Animals***

Male albino rats of Wistar strain of approximately 200-250 g were used for all the experiment. They were housed in an air conditioned room at 23 to  $25^{\circ}\text{C}$  with 12 hours dark and 12 hours light photoperiod. Animals had free access to water and were fed on standard laboratory rat diet.

### ***Experimental Design***

#### ***In vivo Experiment***

Twenty five rats were used for this experiment and were divided into five groups of five each. Group NC was normal control group, administered distilled water; Group PC was paracetamol control group administered liquid paracetamol (125mg/kg body weight) Group EX1, EX2 and EX3 were the experimental groups that received plant extract at the dose of 2.5, 5 and 7.5ml /kg body weight respectively for 30 days. All the experimental groups were also administered liquid paracetamol (125mg/kg body weight) along with the fruit extract.. All the administrations were done orally with the help of a syringe and a rubber tubing. At the end of the experiment, animals were anaesthetized using ether Blood was removed from brachial artery into heparin coated tubes. Plasma was separated from it and kept at  $-70^{\circ}\text{C}$  to measure biochemical parameters.

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### Statistical Analysis

Programme used for data analysis was Sigma Stat (3.1 version). Data was subjected to descriptive statistics after that differences among the groups were analyzed using two way ANOVA followed by Student-Newman-Keuls Method and Tukeys test for comparisons.

### Biochemical Measurements

#### Thiobarbituric Acid Reactive Substances

Lipid peroxidation in plasma was estimated in terms of thiobarbutaric acid reactive substance by the method described by Tripathi *et al.*, 2001 with little modification (Chaturvedi, 2007). 0.1ml of plasma was treated with 2 ml of TCA-TBA-HCL reagent (1:1:1) and incubated in boiling water bath for 10 minutes. After that, the mixture was cooled, mixed with 2ml of freshly prepared 1N NaOH. The absorbance was measured at 535 nm.

ALT and AST were estimated using the kit bought from Agape Diagnostics, India and the manufacturer guidelines were followed.

#### Reduced Glutathione (GSH)

Reduced glutathione was measured by the method described by Ellman (1959). Red blood cell lysate precipitated with metaphosphoric acid (0.25 ml of plasma) was mixed with 0.5ml of precipitating buffer (1.67 g Metaphosphoric acid, 0.2g EDTA, 30 g sodium chloride dissolved in 100 ml of double distilled water) and centrifuged at 3000 rpm. The supernatant was collected and mixed with 2.5ml of 0.3 M phosphate buffer. Colour was developed by adding 100 $\mu$ l of 0.01% 5, 5-dithiobis 2-nitrobenzoic acid (DTNB) and read at 412 nm.

#### Superoxide Dismutase

Super oxide dismutase was assayed by the method described by Tripathi *et al.*, 2001. Lysate was treated with a mixture of chloroform and ethanol. The reaction mixture consisted of 150 $\mu$ l EDTA (0.5 mmol), 600 $\mu$ l L-methionine (130 mmol) and 300 $\mu$ l NBT (750  $\mu$ mol). The volume of reaction mixture was made up 2.8 $\mu$ l with SOD buffer. Then 200 $\mu$ l of sample was added except in the control. Finally 200 $\mu$ l of riboflavin was added to start the reaction. All the test tubes were placed under fluorescent lamp except the blank. Absorbance was recorded at 540nm after for 4 minutes. One unit of enzyme activity was calculated as the activity that was required to inhibit the reduction of NBT by 50%.

#### Tocopherol (Vitamin-E)

Tocopherol was estimated using the method of Martinek, 1964. The sample (0.5 ml), distilled water as blank (0.5ml) and standard solution (0.5ml) were taken in three centrifuge tubes. To all three tubes 0.5ml xylene was added. The tubes were stoppered, mixed and centrifuged. The xylene layer (containing the precipitated tocopherol) was then carefully pipetted into clean tubes and mixed with 0.35 ml  $\alpha$ - $\alpha'$  Dipyrindyl reagent. The extinction of the test and standard against blank were read at 460nm.

In turn, beginning with the blank tube, 0.33ml of ferric chloride solution was added to all the tubes. Optical density of the test and the standard against blank were read at 520nm after 1.5minutes.

Amount of tocopherol present in mg/100 ml was calculated using the formula: mg/100 ml = (Reading unknown at 520nm- Reading unknown at 460nm)\* 0.29/ Reading of standard at 520nm.

#### Glutathione Peroxidase

Activity of glutathione peroxidase was measured by the method described by Paglia and Valentine, 1967. Into 1 ml of phosphate buffer were added : 200 $\mu$ l EDTA, 200 $\mu$ l GSH, 200 $\mu$ l of sodium azide, 200 $\mu$ l of H<sub>2</sub>O<sub>2</sub>, 200 $\mu$ l of NADPH, one unit of glutathione reductase and an appropriate amount of the sample (100 $\mu$ l). The decrease in absorbance due to NADPH oxidation was monitored at 340nm for 3 minutes. Glutathione Peroxidase activity was calculated using the extinction coefficient of NADPH (6.22 \* 10<sup>3</sup> M<sup>-1</sup>cm<sup>-1</sup>) and the results were expressed as U/L (equivalent to micro mol of NADPH oxidized /min/L).

## RESULTS

### WMJ Reduced the Lipid Peroxidation Induced by Paracetamol

Results of WMJ on paracetamol induced lipid peroxidation are presented in Figure 1. The results showed that there was a significant increase in the levels of TBARS in PC group on day 31 as compared to levels

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in NC, EX2 and EX3. The levels in EX2 and EX3 were significantly low as compared to PC ( $p < 0.001$ ). Levels in EX2 and EX3 differed significantly with the levels in PC. There was no significant difference found in these groups as compared to NC but levels of MDA were still higher by 50% in EX2 and by 40% in EX3. Levels in EX1 differed significantly from PC and NC ( $p < 0.001$ )

### WMJ Reduced the Activity of ALT and AST

Effects of WMJ on ALT and AST in treated rats are presented in Figure 2 and 3 respectively. The result showed similar trend in both cases. There was a marked increase in the activity of these two enzymes in PC on day 31 as compared to NC. The levels of ALT in two experimental groups EX2 and EX3 were lower than PC ( $p < 0.05$ ). Similar was the case with AST. The levels of AST were 20% higher in EX2 and 15% higher in EX3 as compared to the levels in NC. No significant difference was found after statistical comparisons with NC but their levels still had not achieved the value parallel to NC.

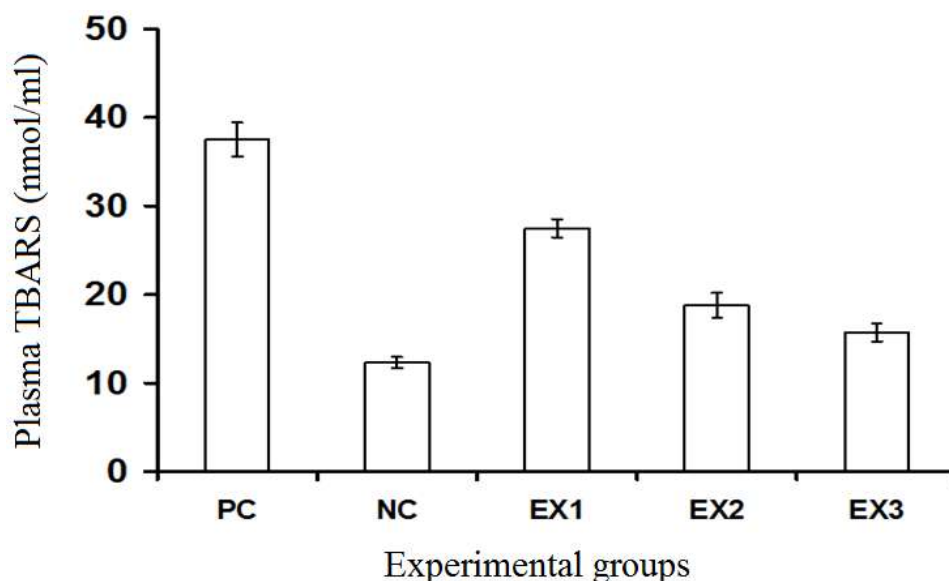


Figure 1: Effects of WMJ on plasma TBARS

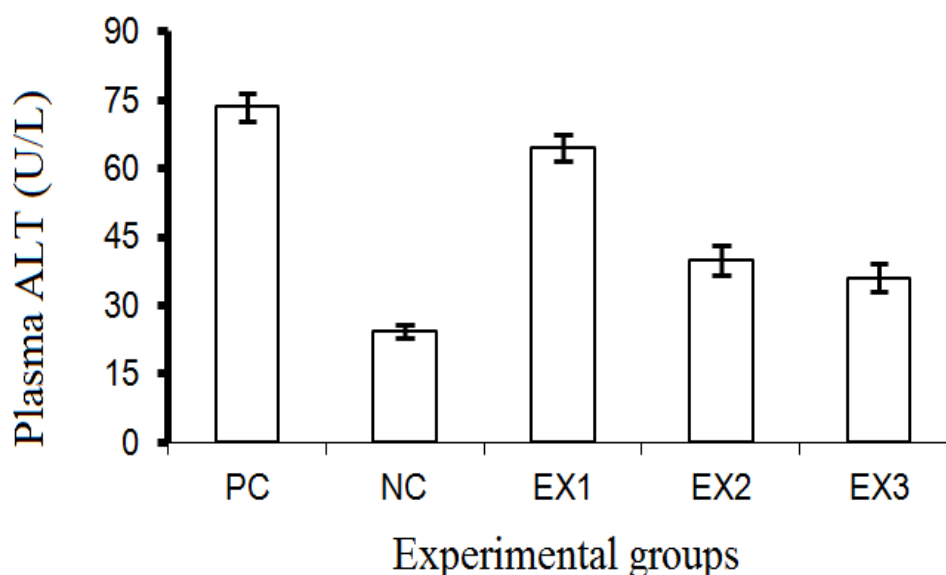
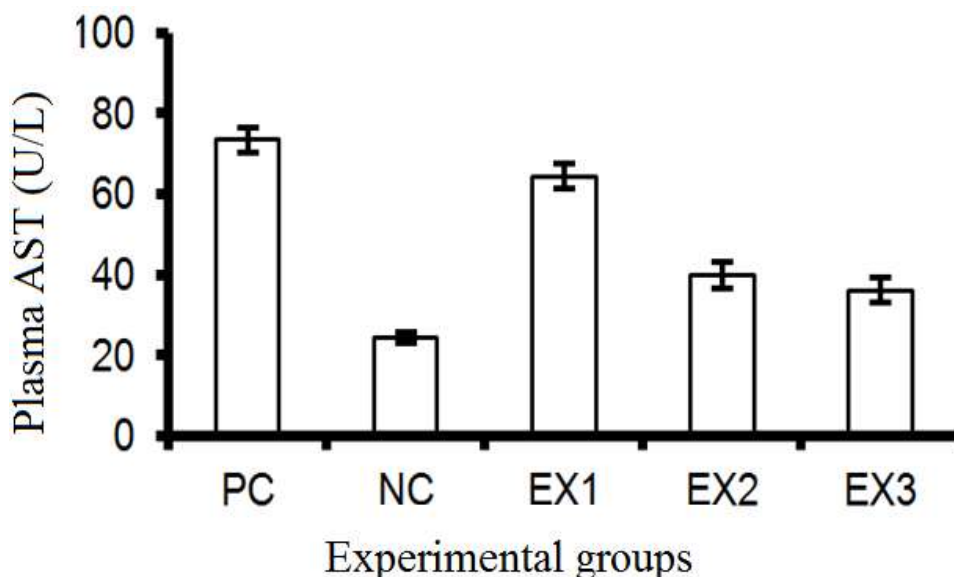


Figure 2: Effects of WMJ on plasma ALT

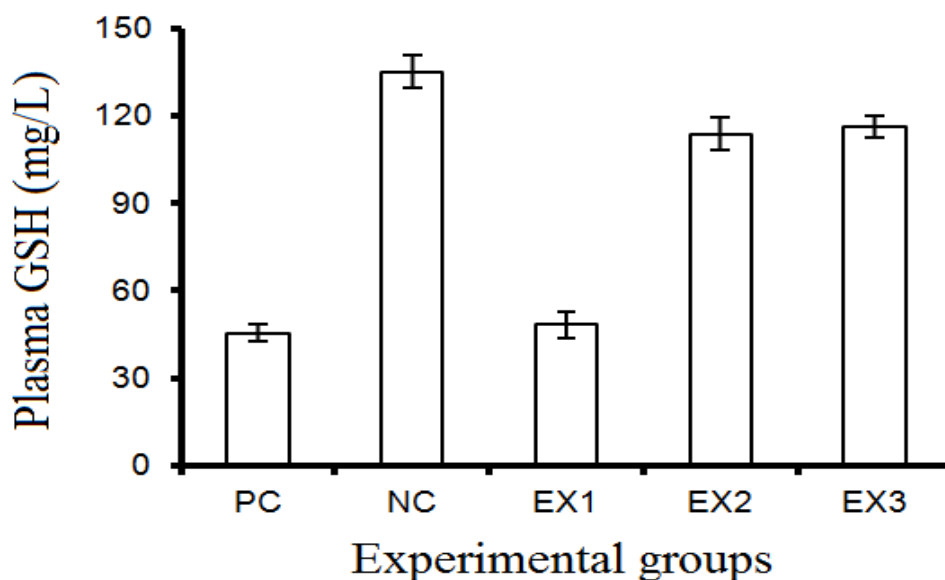
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**Figure 3: Effect of WMJ on plasma AST**

## WMJ Maintained the Levels of GSH, GPx and Vitamin-E

Results of effects of WMJ on GSH, GPx and Vitamin E are presented in Figure 4, 5 and 6 respectively. The results showed that there was a significant decrease in the levels of reduced glutathione content in plasma in PC and as compared to NC and all experimental groups... In EX2 and EX3 the levels of GSH were elevated and differed significantly with PC ( $p < 0.001$ ). The differences between NC and EX2 were not significant.



**Figure 4: Effect of WMJ on plasma AST Reduced Glutathione (GSH)**

Similar case was observed with GPx. Activities of GPx were significantly low in PC as compared to NC ( $p < 0.001$ ) and this activity was being picked up slowly in all experimental groups. The levels of GPx were elevated in all experimental groups at the end of experiment and differed significantly with PC ( $p < 0.001$ ) In EX3, the level went up but did not reach the normal activity level like NC.

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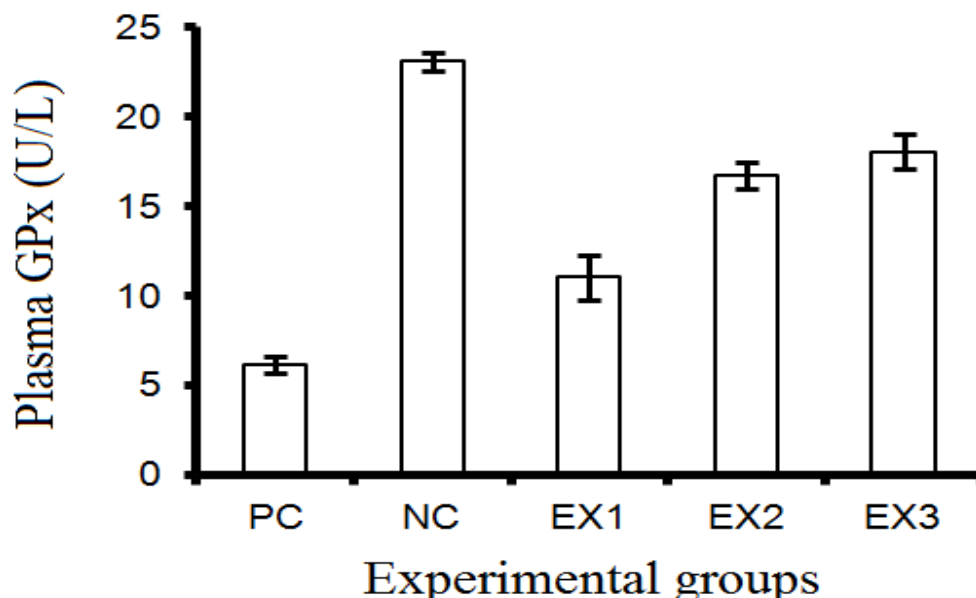


Figure 5: Effect of WMJ on plasma Glutathione Peroxidase

Vitamin E also showed the similar trends (Figure 6) Treatment groups also showed high levels of tocopherol in all groups as compared to the levels in PC. The group EX3 showed most significant difference from PC ( $p < 0.05$ ). Actually the levels in this group were higher than the levels in NC.

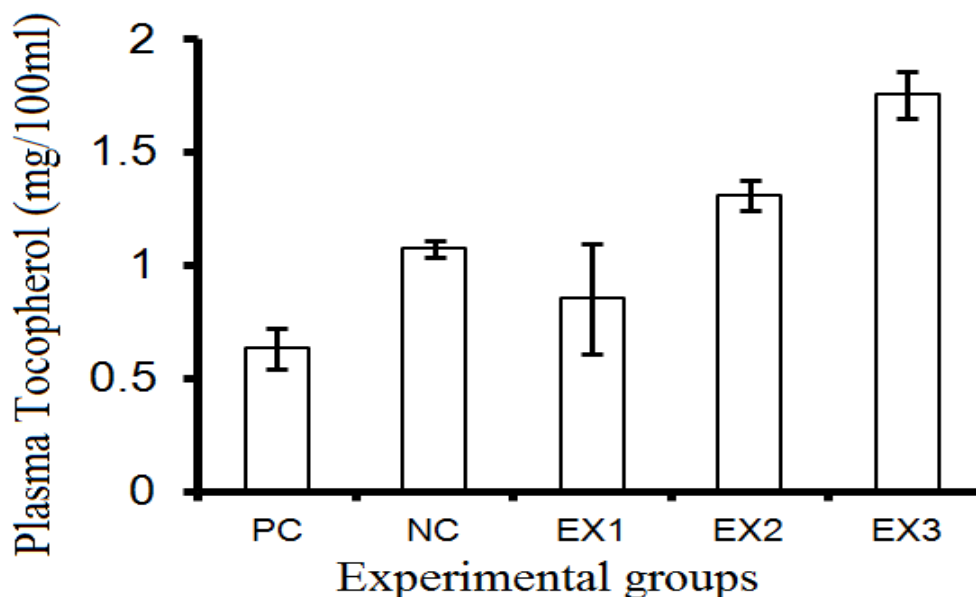


Figure 5: Effect of WMJ on plasma Tocopherol

## WMJ Enhanced the Activities of Superoxide Dismutase

Results of effects of WMJ on SOD are presented in Figure 7. The results showed that there was a significant decline in the activities of SOD in PC as compared to NC ( $p < 0.001$ ). This decline was gradually compensated in experimental groups and the results were significantly different in all experimental groups as compared to PC ( $p < 0.001$ ). The differences were no significant in EX2 as compared to NC. In EX3, the activities was much higher than NC ( $p < 0.05$ )



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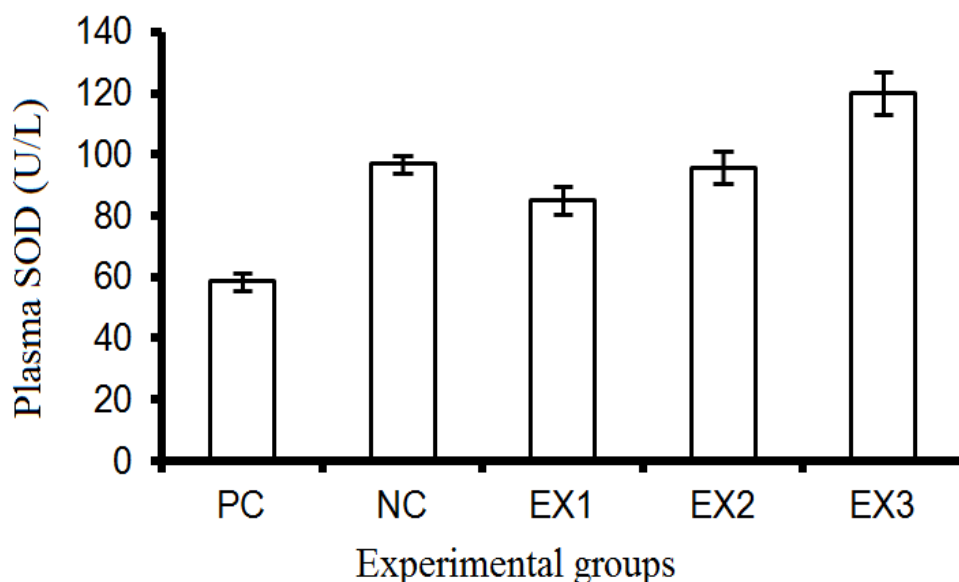


Figure 5: Effect of WMJ on plasma Super Oxide Dismutase

## DISCUSSION

Paracetamol undergoes metabolism in the liver through cytochrome P450 pathway and free radical N-acetyl-P-benzoquinoneimine is produced in the process. This metabolite binds to sulphhydryl – containing proteins in the liver cell and causes lipid peroxidation<sup>2</sup> and produces thiobarbituric acid reactive substance like malonyldialdehyde (MDA)

MDA is one of the marker indices of lipid peroxidation caused by oxidative stress and hence the increase in the levels of TBARS in plasma is the direct reflection of oxidative injury of the liver tissue (Pandey *et al.*, 2012). The present study reveals, the significant increase in the levels of plasma TBARS in PC which is the indication of high levels of oxidative stress and lipid peroxidation leading to membrane damage. This was further supported by the high activities of AST (aspartate amino transferase) and ALT (alanine amino transferase) and low levels of total proteins. These two enzymes are marker parameters of hepatic cell damage. They are located in hepatic cells and are released after cell damage (Himmerich *et al.*, 2001). Because of liver cell damage, rate of protein synthesis is reduced that resulted into low levels of plasma proteins in PC.

Administration of paracetamol together with the WMJ had significantly reduced the oxidative stress generated by paracetamol metabolism in EX2 and EX3. The levels of plasma MDA were significantly low in these experimental groups as compared to PC. Continuous decrease in the levels of TBA-RS in all experimental groups clearly indicates the reduction in the rate of the lipid peroxidation induced by reactive metabolites of paracetamol metabolism by WMJ and attenuation of hepatic cells to normal,

Glutathione (GSH) is the abundant thiol compound present in mammalian cells and plays an important role to scavenge free radicals in the first line of anti-oxidant defense system. Glutathione is a natural antioxidant which donates one electron to hydrogen peroxide and in the process oxidized glutathione is formed. Reaction is catalyzed by glutathione peroxidase. Subsequently, the oxidized glutathione (GSSG) is reduced to GSH via NADPH-dependent reduction by glutathione reductase. Depletion of glutathione results in the inhibition of glutathione peroxidase activity and thus enhancement in the lipid peroxidation activities (Ehrhart, 2003). The antioxidant role of glutathione is both direct and indirect in that it stimulates other endogenous antioxidants.

Results of present experiments demonstrate that the level of GSH was significantly reduced in PC and EX1 as compared to NC. Depletion of glutathione could be attributed to its overusage to neutralize the toxic metabolite N-acetyl-P-benzoquinonimine of paracetamol. GSH level is maintained in both experimental group as compared to the levels in PC and EX1. It has reached almost the normal level.

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Depletion of glutathione results in the inefficiency of glutathione peroxidase. In PC both the levels of GSH and GPX are low while the activities of glutathione peroxidase has been increased in all experimental groups.

Ascorbic acid and beta carotene maintain the normal levels of GSH (Winkler *et al.*, 1994; Takeda *et al.*, 2008). Both beta carotene and ascorbic acid are present in WMJ (Wee and Wai, 2012) and its consumption elevates the levels of both beta carotene and lycopene (Edwards *et al.*, 2003). Vitamin C and beta carotene are very strong anti-oxidants (Polidori *et al.*, 2004; Maritim *et al.*, 2002). Vitamin C is an important dietary anti-oxidant. It protects lipids, DNA and proteins from oxidative damage (Niki *et al.*, 1982). It reduces the reactive oxygen species before they oxidize lipoprotein (Doba *et al.*, 1985; Kalayanraman *et al.*, 1992). Once vitamin C is depleted, the remaining antioxidants provide only partial protection from ROS that initiate lipid peroxidation reaction (Ganesh *et al.*, 2003). But ascorbic acid is water soluble antioxidant and scavenge aqueous radicals efficiently but cannot clear lipophilic radicals in the membranes. Beta carotene and alpha tocopherol work synergistically and can scavenge free radicals generated at in lipoproteins and membranes (Palozza *et al.*, 1992). Alpha-tocopherol acts at the surface while beta carotene in the interior. Beta carotene forms peroxy radical after scavenging free radical which is scavenged by alpha tocopherol (Palozza, 1991). Alpha tocopherol also generates tocopheroxyl radical when it reacts with a free radical. Alpha-tocopheroxyl radicals are further reduced by Vitamin C to alpha-tocopherol (Lieber *et al.*, 1986). Thus it inhibits alpha-tocopheroxyl radical-mediated propagation of lipid peroxidation by converting it to tocopherol (Niki *et al.*, 1982). In the present study, level of alpha tocopherol is significantly elevated in EX3 as compared to PC and in fact the level is higher than the level in NC. High levels of alpha tocopherol in experimental group could be due to the interaction of three antioxidants and ultimate transformation of alpha- tocopheroxyl radical to tocopherol by ascorbic acid. Apart from this, WMJ is also rich in alpha tocopherol (Charoensiri *et al.*, 2009) and this might also have contributed to the high levels of this vitamin in experimental groups. Since water melon consumption also increases lycopene content (Edwards *et al.*, 2003) and the significant role of lycopene in rendering protection in the present study can also not be denied. Lycopene is very efficient in quenching singlet oxygen because of its conjugated diene content (DiMascio *et al.*, 1989). It inactivates hydrogen peroxide and nitric oxide and is the most efficient carotenoid to scavenge reactive oxygen species (Lou *et al.*, 1995; Bohm *et al.*, 1995). WMJ is also very rich in phenol and flavonoid content (Sevcan *et al.*, 2011) which are claimed to be strong anti-oxidants. Thus there is a possibility that these anti-oxidants might have also played a mutual role in protecting liver damage.

During formation of NAPQI in the metabolism of paracetamol, superoxide radicals are also formed which are ultimately converted to hydrogen peroxide (James *et al.*, 2003). SOD converts superoxide radical to  $H_2O_2$  and prevents the formation of hydroxyl radical through Fenton reaction.  $H_2O_2$  is finally removed by glutathione peroxidase. In this study, activities of both SOD and glutathione peroxidase are significantly low in PC as compared to the activity in NC ( $p < 0.001$ ). High paracetamol toxicity could be the reason for the low activity. The endogenous enzymatic anti-oxidant system might not be coping with the high production of superoxide radicals. The activities of glutathione peroxidase and SOD in all experimental groups have been enhanced significantly by WMJ and they differ significantly with PC and there exist non significant difference with NC. In EX3, the activities of SOD have been enormously enhanced and in fact elevated more than NC ( $p < 0.004$ ). Ascorbic acid in WMJ could be the reason for this recovery as vitamin C enhances the activities of SOD during toxicity Wee and Wai, 2012). Beta carotene suppresses the activities of superoxide dismutase and glutathione peroxidase in healthy subjects (Kalayanraman *et al.*, 1992). Water melon juice has also been reported to suppress the activity of SOD in normal rats (Edwards *et al.*, 2003). Chemiluminescence and Electron Spin Resonance study has shown that carotenoid scavenge hydroxyl and superoxide radicals (DiMascio *et al.*, 1989) and this could be the reason that SOD activities were suppressed by beta carotene and water melon juice in previous studies. In the present investigation, the experimental rats were under oxidative stress induced by paracetamol and carotenoids present in WMJ (Bohm *et al.*, 1995) might not be able to scavenge the super oxide radicals efficiently which has lead to the activation of SOD and GPx. Ascorbic acid enhances the activities of



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superoxide dismutase and glutathione peroxidase. As WMJ is rich in vitamin C and animals were under oxidative stress, there is possibility that vitamin C might have enhanced the activities of these enzymes.

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