Research Article **Vol 1/Issue 1/Oct-Dec 2011**

International Journal of Life science & Pharma Research

VANCOMYCIN RESISTANT *STAPHYLOCOCCUS AUREUS* INDUCED OXIDATIVE STRESS IN LIVER AND KIDNEY: PROTECTIVE ROLE OF NANOCONJUGATED VANCOMYCIN

SUBHANKARI PRASAD CHAKRABORTY[1], SABYASACHI DAS[1], PANCHANAN PRAMANIK[2] AND SOMENATH ROY[1*]

[1]Immunology and Microbiology Laboratory, Department of Human Physiology with Community Health, Vidyasagar University, Midnapore-721 102, West Bengal, INDIA.
[2]Nanomaterials Laboratory, Department of Chemistry, Indian Institute of Technology, Kharagpur, Pin-721 302, West Bengal, INDIA.

ABSTRACT

Staphylococcus aureus is a most common pathogen in hospital and community acquired disease that causes a wide range of infection such as skin and soft tissue infection to life threatening disease like respiratory tract infection, musculoskeletal infection, endocarditis and urinary tract infection. The aim of the present study was to evaluate the possible protective effects of nanoconjugated vancomycin against VRSA infection on selective makers of oxidative damage and antioxidant status in liver and kidney. A coagulase positive VRSA strain was used for this study. VRSA infection was developed in Swiss mice by intraperitoneal injection of 5 X 10^6 CFU/ml bacterial solutions. Nanoconjugated vancomycin was treated to VRSA infected mice at its effective dose for 10 days. After decapitation, liver and kidney were excised from control and experimental groups, homogenized and used for different biochemical estimation. Nitrate level, myeloperoxidse activity, lipid peroxidation, protein oxidation, oxidized glutathione, DNA fragmentation level were increased significantly ($p<0.05$) in liver and kidney of VRSA infected group as compared to control group, and reduced glutathione level, activity of antioxidant enzymes (SOD and CAT), glutathione dependent enzymes (GPx, GR and GST) were decreased significantly ($p<0.05$); which were increased or decreased significantly ($p<0.05$) near to normal in nanoconjugated vancomycin treated group. These finding suggests the potential use and beneficial role of nanoconjugated vancomycin against VRSA infection induced oxidative stress and DNA damage in liver and kidney.

Key-words: VRSA, free radical, lipid peroxidation, antioxidant enzyme, DNA fragmentation.

1. INTRODUCTION

Staphylococcus aureus is facultitatively anaerobic, gram positive coccus and is the most common cause of staph infections (Kluytmans J et al. 1997). *S. aureus* has developed resistance to most classes of antimicrobial agents. Penicillin was the first choice of antibiotics to treat staphylococcal infection. In 1944, by destroying the penicillin by *penicillinase*, *S. aureus* become resistant (Kirby W. 1944). More than 90% *S. aureus* strains are resistant to penicillin (Neu H. 1992). Methicillin, a semi synthetic penicillin was used to treat Penicillin Resistant *Staphylococcus aureus* but resistance finally emerge in 1962

(Livermore D. 2001; Lowry O et al. 1951). Vancomycin, a glycopeptide antibiotic continues to be an important antimicrobial agent to treat MRSA but resistance finally emerges. In June 2002, the World's first reported clinical infection due to *S. aureus* with high resistance to vancomycin (VRSA) was diagnosed in a patient in the USA (Sievert D et al. 2002). Recently, we have isolated thirty pathogenic *S. aureus* from post operative pus sample by standard biochemical test and detection of *S. aureus* specific *nuc* gene; out of them twenty two were vancomycin sensitive and rests were vancomycin resistant (Chakraborty S et al. 2011c).

S. aureus causes chronic/relapsing diseases and reported to persist as an opportunistic intracellular organism both in vitro and in vivo (Brouillette E et al. 2003). *S. aureus* were able to survive within phagocytic cells both in polymorphonuclear leukocytes (PMN) and monocytes (Steigbigel R et al. 1974). In vitro studies demonstrated that pathogenic strains of *S. aureus* could survive for long periods of time inside both PMN and monocytes isolated from different animals and humans. To induce an infection in host the pathogenic must cope with their changing environment and have to attack continuously to the host to weak the immune system (Dryla A et al. 2003).

Chitin is the major structural component of invertebrates like crab, shrimp, shells and the cell walls of fungi. Chitosan (CS), the deacetylated form of chitin, is a linear polysaccharide, composed of glucosamine and N-acetyl glucosamine linked in a β linkage (Khor E and Lim LY, 2003). CS has been reported to possess immune stimulating properties such as increasing accumulation and activation of macrophages and polymorphonucleus, augmenting antibody responses and inducing production of cytokines (Koide S. 1998). Carboxymethyl chitosan (CMC) is a linear polysaccharide composed of β (1, 4) glycosidic linkages between 6-carboxymethyl-D-glucosamine monomers. CMC is synthesized from CS by carboxylation of the hydroxyl and amine groups (Liu X et al. 2001). In our previous laboratory report, we synthesized CMC-EDBE-FA nanoparticle based on carboxy methyl chitosan tagged with folic acid by covalently linkage through 2, 2' (ethylenedioxy) bis-(ethylamine), vancomycin was loaded onto it, complex is called "nanoconjugated vancomycin" and observe it's bactericidal activity against *S. aureus* (Chakraborty S et al. 2010); and reported that CMC-EDBE-FA nanoparticle is non toxic (Chakraborty S et al. 2011a). We also reported that in vivo challenge of VSSA and VRSA for 5 days can produce the highest degree of damage in lymphocyte through the increased production of nitric oxide, TNF-α that leads to decreased antioxidant status in cell and ten days successive treatment of nanoconjugated vancomycin also eliminate in vivo VSSA and VRSA infection (Chakraborty S et al. 2011b). Recently we reported the possible antioxidant effects of nanoconjugated vancomycin against VRSA infection on select makers of oxidative damage and antioxidant status in spleen (Chakraborty S et al. 2011d). In light of these findings, the present study was conducted to obtain information on the possible protective effects of nanoconjugated vancomycin against VRSA infection on select makers of oxidative damage and antioxidant status in liver and kidney.

2 MATERIALS AND METHODS

2.1 Chemicals and reagents

Sodium dodecyl sulfate (SDS), 5', 5'-dithio (bis)-2-nitrobenzoic acid (DTNB), standard reduced glutathione (GSH), glutathione reductase (GR), NADPH,Na_4, NADPH, oxidized glutathione (GSSG) were purchased from Sigma Chemical Co., USA. Sodium chloride (Nacl), sodium dodecyl sulfate, sucrose, ethylene diamine tetra acetate (EDTA), tryptic soy broth, mannitol salt agar, agarose was purchased from Himedia, India. Tris-Hcl, KH_2PO_4 and K_2HPO_4, alcohol, diphenylamine (DPA), O-phenylenediamine and other chemicals were procured from Merck Ltd., SRL Pvt. Ltd., Mumbai, India. All other chemicals were from Merck Ltd., SRL Pvt., Ltd., Mumbai and were of the highest grade available.

2.2 Animals

Experiments were performed using eighteen (18) Swiss male mice 6–8 weeks old, weighing 20–25 g. The animals were fed standard pellet diet and water

were given *ad libitum* and housed in polypropylene cage (Tarson) in the departmental animal house with 12 h light:dark cycle, and the temperature of 25±2 ºC. The animals were allowed to acclimatize for one week. The animals used did not show any sign of malignancy or other pathological processes. Animals were maintained in accordance with the guidelines of the National Institute of Nutrition, Indian Council of Medical Research, Hyderabad, India, and approved by the ethical committee of Vidyasagar University.

2.3 Bacterial strain

We used a coagulase positive Vancomycin resistant *Staphylococcus aureus* (MMC-17) strain that was isolated from human post operative pus sample and was grown at 37°C for overnight in tryptic soy broth (Chakraborty S et al. 2011c). The bacterial culture was centrifuged at 15,000 rpm for 15 minutes. The pellet was resuspended and washed with sterile phosphate buffer saline (PBS). Using a UV-spectrophotometer (Schimadzu, USA) at an absorbance of 620 nm, we adjusted the viable bacterial count to approximately 1.0 X 10^9 colony-forming units (CFU)/ml, which corresponded to an optical density of 1.6. The bacterial suspension was adjusted by serial dilution in PBS to give a final concentration of approximately 5 X 10^6 in 100µl of bacterial suspension (Hattie D et al. 2000).

2.4 Preparation of CMC-EDBE-FA nanoparticle and loading of vancomycin

CMC-EDBE-FA nanoparticle was prepared and vancomycin was loaded onto it according to our previous laboratory report (Chakraborty S et al. 2010).

2.5 Development of VRSA infection in Swiss mice

VRSA infection was developed in male Swiss mice by intraperitoneal (i.p.) injection of 100µl of bacterial suspension containing 5 X 10^6 CFU/ml according to our previous laboratory report (Chakraborty S et al. 2011b).

2.6 Experimental design

VRSA infected mice were treated with nanoconjugated vancomycin for successive 10 days at a dose of 500 mg/kg bw/day. The dose and duration of nanoconjugated vancomycin was selected from our previous laboratory report (Chakraborty S et al. 2011b). The following groups were considered for the experiment: **Group I**: Control, **Group II**: VRSA infection, **Group III**: VRSA infection + 500 mg/kg bw/day nanoconjugated vancomycin. After the termination of experiment, animals were sacrificed by an intraperitoneal injection of sodium pentobarbital (60–70 mg/kg body weight) (Chandran K and Venugopal PM, 2004).

2.7 Separation and homogenization of liver and kidney

After decapitation, liver and kidney were excised from experimental mice of different experimental groups and washed with cold normal saline and homogenized in the ice-cold buffer containing 0.25 M sucrose, 1 mM EDTA, and 1 mM Tris-Hcl, pH 7.4. The homogenate was first centrifuge at 600×g for 10 min at 4°C, and the supernatant was stored at -80ºC for the biochemical estimation of different parameters.

2.8 Biochemical estimation

2.8.1 Nitrite (NO) level

After treatment schedule, 100 µl of Griess reagent (containing 1 part of 1% sulfanilamide in 5% phosphoric acid, and 1 part of 0.1% of N-C-1 napthyl ethylene diamine dihydrochloride) was added to 100 µl of sample, incubated at room temperature for 10 minutes, readings were taken in a UV spectrophotometer at 550nm and compared to a sodium nitrite standard curve (values ranging between 0.5 and 25µM). The levels of NO were expressed as µ M/mg protein (Chakraborty S et al. 2011b).

2.8.2 Determination of myeloperoxidase (MPO) activity

200 µl of sample was reacted with 200 µl substrate (containing H_2O_2 and OPD) in dark for 30 min. The blank was prepared with citrate phosphate buffer (pH 5.2) and substrate, in absence of cell free supernatant. The reaction was stopped with addition of 100 µl 2(N) sulfuric acid and reading was taken at 492 nm in a spectrophotometer (Chakraborty S et al. 2011b). The MPO activity was expressed in terms of µ M/mg protein.

2.8.3 Determination of Lipid Peroxidation (MDA) level

Lipid peroxidation of liver and kidney homogenate was estimated by the method of KarMahapatra et al., 2009 (Kar Mahapatra S et al. 2009). Briefly, the reaction mixture contained Tris-HCl buffer (50 mM, pH 7.4), tetra-butyl hydroperoxide (BHP) (500 µM in ethanol) and 1 mM FeSO4. After incubating the samples at 37°C for 90 min, the reaction was stopped by adding 0.2 ml of 8% sodium dodecyl sulfate (SDS) followed by 1.5 ml of 20% acetic acid (pH 3.5). The amount of malondialdehyde (MDA) formed during incubation was estimated by adding 1.5 ml of 0.8% TBA and further heating the mixture at 95°C for 45 min. After cooling, samples were centrifuged, and the TBA reactive substances (TBARS) were measured in supernatants at 532 nm by using 1.53 x 10^5 M^{-1} cm^{-1} as extinction coefficient. The levels of lipid peroxidation were expressed in terms of n mol/mg protein.

2.8.4 Determination of protein carbonyls (PC) contents

Protein oxidation was monitored by measuring protein carbonyl contents by derivatization with 2, 4-dinitrophenyl hydrazine (DNPH) (Kar Mahapatra S et al. 2009). In general, liver and kidney proteins in 50mM potassium phosphate buffer, pH 7.4, were derivatized with DNPH (21% in 2N HCl). Blank samples were mixed with 2N HCl incubated at 1h in the dark; protein was precipitated with 20% trichloro acetic acid (TCA). Underivatized proteins were washed with an ethanol: ethyl acetate mixture (1:1). Final pellets of protein were dissolved in 6N guanidine hydrochloride and absorbance was measured at 370nm. Protein carbonyls content was expressed in terms of n mol/mg protein.

2.8.5 Determination of reduced glutathione (GSH) level

Reduced glutathione estimation in liver and kidney homogenate was performed by the method of KarMahapatra et al., 2009 (Kar Mahapatra S et al. 2009). The required amount of sample was mixed with 25% of TCA and centrifuged at 2,000×g for 15 min to settle the precipitated proteins. The supernatant was aspirated and diluted to 1 ml with 0.2 M sodium phosphate buffer (pH 8.0). Later, 2 ml of 0.6 mM DTNB was added. After 10 minutes the optical density of the yellow-colored complex formed by the reaction of GSH and DTNB (Ellman's reagent) was measured at 405 nm. A standard curve was obtained with standard reduced glutathione. The levels of GSH were expressed as µg of GSH/mg protein.

2.8.6 Determination of oxidized glutathione (GSSG) level

The oxidized glutathione level in liver and kidney homogenate was measured after derevatization of GSH with 2-vinylpyidine according to the method of KarMahapatra et al., 2009 (Kar Mahapatra S et al. 2009). In brief, with 0.5 ml sample, 2 µl of 2-vinylpyidine was added and incubates for 1 hr at 37°C. Then the mixture was deprotenized with 4% sulfosalicylic acid and centrifuged at 1,000×g for 10 min to settle the precipitated proteins. The supernatant was aspirated and GSSG level was estimated with the reaction of DTNB at 412 nm in spectrophotometer and calculated with standard GSSG curve. The levels of GSSG were expressed as µg of GSSG/mg protein.

2.8.7 Determination of super oxide dismutase (SOD) activity

SOD activity of liver and kidney homogenate was determined from its ability to inhibit the auto-oxidation of pyrogalol according to KarMahapatra et al., 2009 (Kar Mahapatra S et al. 2009). The reaction mixture considered of 50 mM Tris (hydroxymethyl) amino methane (pH 8.2), 1 mM diethylenetriamine penta acetic acid, and 20–50 µl of sample. The reaction was initiated by addition of 0.2 mM pyrogalol, and the absorbance measured kinetically at 420 nm at 25°C for 3 min. SOD activity was expressed as unit/ mg protein.

2.8.8 Determination of catalase (CAT) activity

Catalase activity of liver and kidney homogenate was measured by the method of KarMahapatra et al., 2009 (Kar Mahapatra S et al. 2009). The final reaction volume of 3 ml contained 0.05 M Tris-buffer, 5 mM EDTA (pH 7.0), and 10 mM H_2O_2 (in 0.1 M

potassium phosphate buffer, pH 7.0). About 50 µl of sample was added to the above mixture. The rate of change of absorbance per min at 240 nm was recorded. Catalase activity was calculated by using the molar extinction coefficient of 43.6 M^{-1} cm^{-1} for H_2O_2. The level of CAT was expressed as unit/ mg protein.

2.8.9 Determination of glutathione peroxidase (GPx) activity

The GPx activity of liver and kidney homogenate was measured by the method of KarMahapatra et al., 2009 (Kar Mahapatra S et al. 2009). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM sodium azide, 0.2 mM NADPH, 1 U glutathione reductase and 1 mM reduced glutathione. The sample, after its addition, was allowed to equilibrate for 5 min at 25°C. The reaction was initiated by adding 0.1 ml of 2.5 mM H_2O_2. Absorbance at 340 nm was recorded for 5 min. Values were expressed as n mol of NADPH oxidized to NADP by using the extinction coefficient of 6.2 x 10^3 M^{-1} cm^{-1} at 340 nm. The activity of GPx was expressed in terms of n mol NADPH consumed/min/mg protein.

2.8.10 Determination of glutathione reductase (GR) activity

The GR activity liver and kidney homogenate was measured by the method of KarMahapatra et al., 2009 (Kar Mahapatra S et al. 2009). The tubes for enzyme assay were incubated at 37°C and contained 2.0 ml of 9 mM GSSG, 0.02 ml of 12 mM NADPH, Na4, 2.68 ml of 1/15 M phosphate buffer (pH 6.6) and 0.1 ml of sample. The activity of this enzyme was determined by monitoring the decrease in absorbance at 340 nm. The activity of GR was expressed in terms of n mol NADPH consumed/min/mg protein.

2.8.11 Determination of glutathione-s-transferase (GST) activity

The GST activity of liver and kidney homogenate was measured by the method of KarMahapatra et al., 2009 (Kar Mahapatra S et al. 2009). The tubes of enzyme assay were incubated at 25°C and contained 2.85 ml of 0.1 M potassium phosphate (pH 6.5) containing 1 mM of GSH, 0.05 ml of 60 mM 1-chloro-2, 4-dinitrobengene and 0.1 ml of sample. The activity of this enzyme was determined by monitoring the increase in absorbance at 340 nm. The activity of GST was expressed in terms of n mol NADPH consumed/min/mg protein.

4.8.12 DNA fragmentation assay by diphenylamine (DPA) assay

The diphenylamine (DPA) reaction of liver and kidney was performed by the method of Paradones et al., 1993 (Paradones C et al. 1993). Perchloric acid (0.5 M) was added to the sample containing uncut DNA (resuspended in 200 µl of hypotonic lysis buffer) and to the other half of the supernatant containing DNA fragments. Then two volumes of a solution consisting of 0.088 M DPA, 98% (v/v) glacial acetic acid, 1.5% (v/v) sulphuric acid, and a 0.5% (v/v) concentration of 1.6% acetaldehyde solution were added. The samples were stored at 4ºC for 48 h. The reaction was quantified spectrophotometrically at 575 nm. The percentage of fragmentation was calculated as the ratio of DNA in the supernatants to the total DNA.

2.8.13 Protein estimation

Protein was determined using bovine serum albumin as standard according to Lowry et al. 1951 (Lowry O et al. 1951).

2.9 Statistical analysis

The data were expressed as mean ± SEM, n=6. Comparisons between the means of control and VRSA treated group were made by two-way ANOVA test (using a statistical package, Origin 6.1, Northampton, MA 01060 USA) with multiple comparison t-tests, $p < 0.05$ as a limit of significance.

3. RESULTS

3.1 Nitrite (NO) level and Myeloperoxidase (MPO) activity

Nitrate (NO) is an indicator of free radical generation. Myeloperoxidase (MPO) is an important enzyme to produce hypochlorus acid (HOCl) in cellular system that leads to oxidative damage. NO level and MPO activity were significantly ($P < 0.05$) increased by 251.05%, 139.68% and 156.45%, 122.18% in VRSA

infected liver and kidney, respectively, as compared to control group, which were significantly ($P < 0.05$) decreased by 49.56%, 49.14% and 45.52%, 45.95%, respectively, due to treatment of nanoconjugated vancomycin (Figure 1-2).

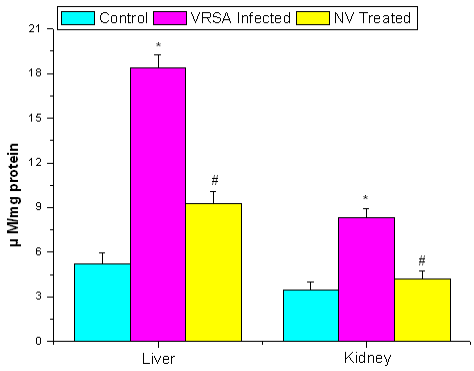

Figure 1: ***Nitrite (NO) generation in liver and kidney of control, VRSA-infected and nanoconjugated vancomycin treated group. Values are expressed as mean±SEM, n=6. * indicates significant difference (P<0.05) compared to control group. # indicates significant difference (P<0.05) compared to VRSA-infected group.***

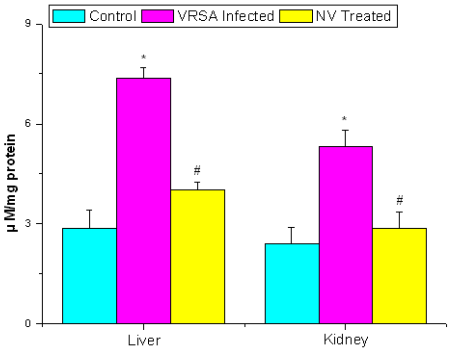

Figure 2: ***Myeloperoxidase (MPO) activity in liver and kidney of control, VRSA-infected and nanoconjugated vancomycin treated group. Values are expressed as mean±SEM, n=6. * indicates significant difference (P<0.05) compared to control group. # indicates significant difference (P<0.05) compared to VRSA-infected group.***

3.2 Lipid peroxidation (MDA) and protein oxidation (PC) level

Lipid peroxidation and protein oxidation are two important determinants to access the cellular damage. Lipid peroxidation in terms of malondialdehyde level and protein oxidation in terms of protein carbonyl level were significantly ($P < 0.05$) increased by 270.20%, 125.55% and 222.39%, 110.09% in VRSA infected liver and kidney, respectively, as compared to control group, which were significantly ($P < 0.05$) decreased by 61.19%, 42.07% and 55.86%, 40.01%, respectively, due to treatment of nanoconjugated vancomycin (Figure 3-4).

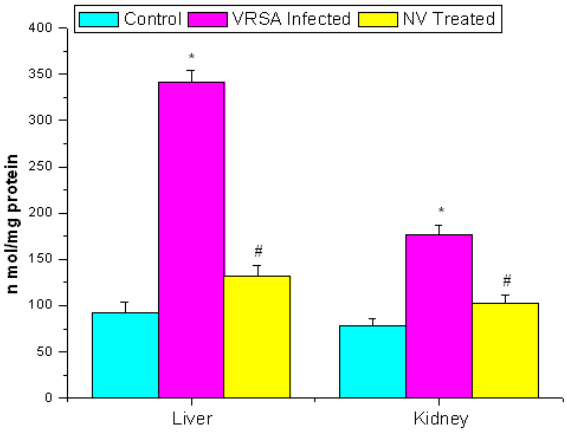

Figure 3: ***Lipid peroxidation (MDA) level in liver and kidney of control, VRSA-infected and nanoconjugated vancomycin treated group. Values are expressed as mean±SEM, n=6. * indicates significant difference (P<0.05) compared to control group. # indicates significant difference (P<0.05) compared to VRSA-infected group.***

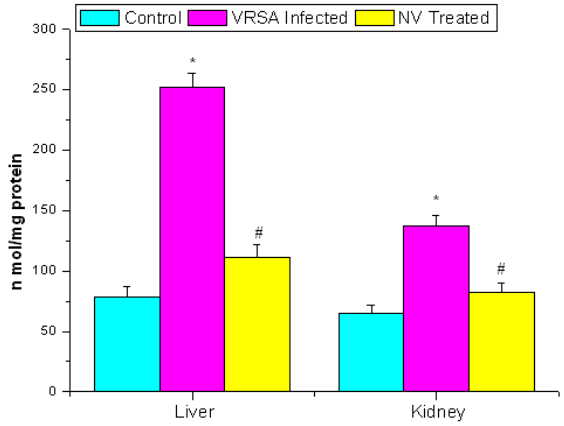

Figure 4: ***Protein carbonyls (PC) contents in liver and kidney of control, VRSA-infected and nanoconjugated vancomycin treated group. Values are expressed as mean±SEM, n=6. * indicates significant difference (P<0.05) compared to control group. # indicates significant difference (P<0.05) compared to VRSA-infected group***.

3.3 Reduced glutathione (GSH) and oxidized glutathione (GSSG)

Glutathione is an important antioxidant in cellular system. So, to understand glutathione level, we have measured both reduced and oxidized form of glutathione. The reduced glutathione level was decreased significantly ($P < 0.05$) by 59.26% and 54.65% in liver and kidney of VRSA infected group, respectively, as compared to control; where as the oxidized glutathione level was increases significantly ($P < 0.05$) by 109.41% and 106.97%, respectively, as compared to control. Treatment of nanoconjugated vancomycin significantly ($P < 0.05$) increased GSH level by 116.16% and 99.55%; and decreased GSSG level significantly ($P < 0.05$) by 30.48% and 42.06%, respectively, in liver and kidney (Fig. 5-6).

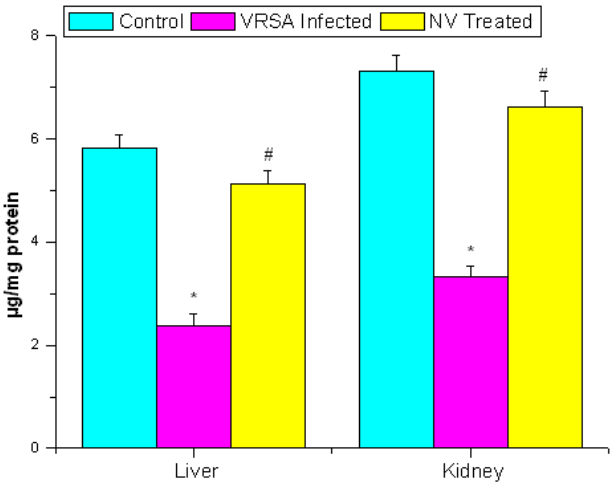

Figure 5: ***Reduced glutathione (GSH) level in liver and kidney of control, VRSA-infected and nanoconjugated vancomycin treated group. Values are expressed as mean±SEM, n=6. * indicates significant difference (P<0.05) compared to control group. # indicates significant difference (P<0.05) compared to VRSA-infected group.***

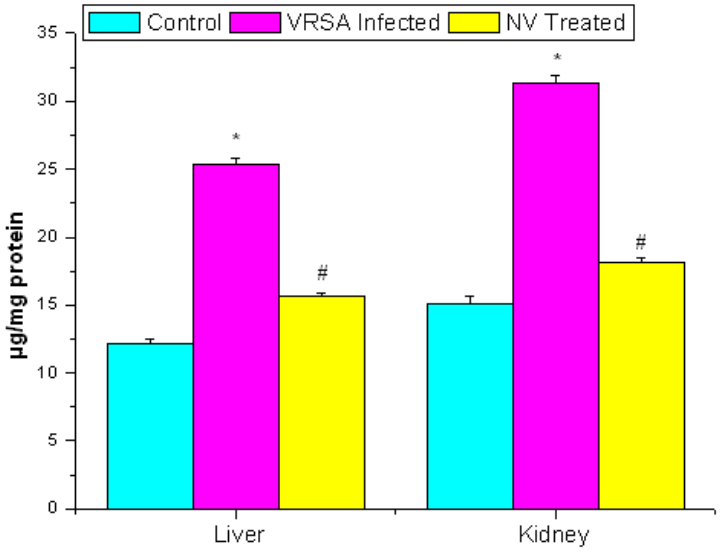

Figure 6: ***Oxidized glutathione (GSSG) level in liver and kidney of control, VRSA-infected and nanoconjugated vancomycin treated group. Values are expressed as mean±SEM, n=6. * indicates significant difference (P<0.05) compared to control group. # indicates significant difference (P<0.05) compared to VRSA-infected group.***

3.4 Superoxide dismutase (SOD) and catalase (CAT) activity

The super oxide dismutase (SOD) and catalase (CAT) activity were measured to understand the antioxidant enzymes status in liver and kidney of VRSA infected group. SOD and CAT activity were decreased significantly (P<0.05) by 44.71%, 61.48% and 51.40%, 61.99% in VRSA infected liver and kidney, respectively, as compared to control group, which were significantly ($P < 0.05$) increased by 55.71%, 129.08% and 78.80%, 133.42%, respectively, due to treatment of nanoconjugated vancomycin (Figure 7-8).

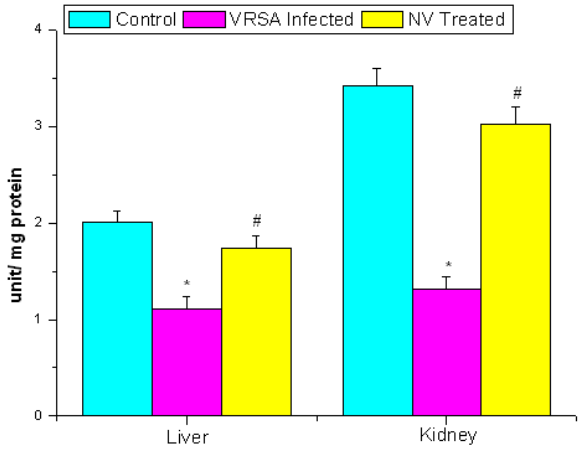

Figure 7: ***Superoxide dismutase (SOD) activity in liver and kidney of control, VRSA-infected and nanoconjugated vancomycin treated group. Values are expressed as mean±SEM, n=6. * indicates significant difference (P<0.05) compared to control group. # indicates significant difference (P<0.05) compared to VRSA-infected group.***

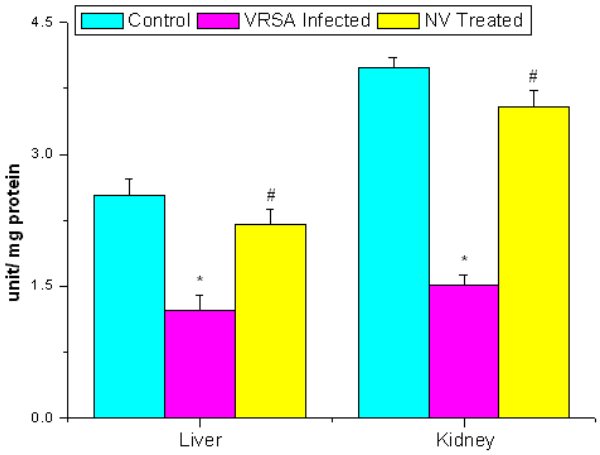

Figure 8: ***Catalase (CAT) activity in liver and kidney of control, VRSA-infected and nanoconjugated vancomycin treated group. Values are expressed as mean±SEM, n=6. * indicates significant difference (P<0.05) compared to control group. # indicates significant difference (P<0.05) compared to VRSA-infected group.***

3.5 Glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-s-transferase (GST) activity The activity of glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione-s-transferase (GST) were measured to understand the antioxidant enzymes status in liver and kidney of VRSA infected group. GPx, GR and GST activity were decreased significantly (P<0.05) by 75.47%, 76.33%; 57.30%, 61.73% and 59.35%, 66.13% in VRSA infected liver and kidney, respectively, as compared to control group, which were significantly ($P < 0.05$) increased by 267.93%, 286.54%; 123.08%, 140.39% and 120.94%, 163.61%, respectively, due to treatment of nanoconjugated vancomycin (Figure 9-11).

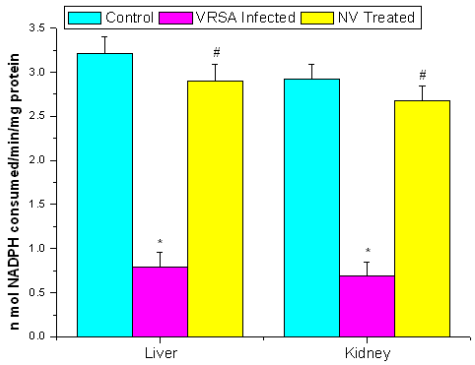

Figure 9: ***Glutathione peroxidase (GPx) activity in liver and kidney of control, VRSA-infected and nanoconjugated vancomycin treated group. Values are expressed as mean±SEM, n=6. * indicates significant difference (P<0.05) compared to control group. # indicates significant difference (P<0.05) compared to VRSA-infected group.***

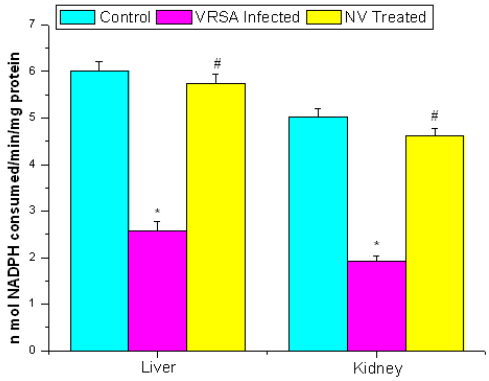

Figure 10: ***Glutathione reductase (GR) activity in liver and kidney of control, VRSA-infected and nanoconjugated vancomycin treated group. Values are expressed as mean±SEM, n=6. * indicates significant difference (P<0.05) compared to control group. # indicates significant difference (P<0.05) compared to VRSA-infected group.***

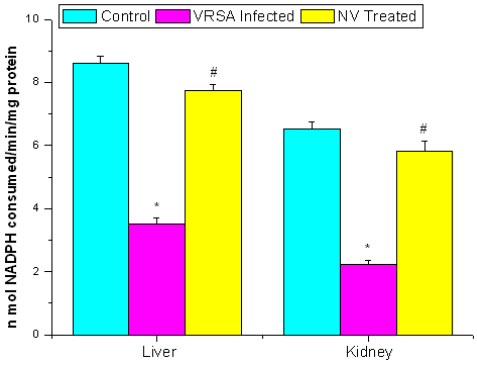

Figure 11: ***Glutathione-s-Transferase (GST) activity in liver and kidney of control, VRSA-infected and nanoconjugated vancomycin treated group. Values are expressed as mean±SEM, n=6. * indicates significant difference (P<0.05) compared to control group. # indicates significant***

3.6 DNA fragmentation

DNA fragmentation was significantly ($P < 0.05$) increased in liver and kidney by 50.66% and 62.98% as compared to control which was significantly ($P < 0.05$) decreased by 63.70% and 72.40% due to treatment of nanoconjugated vancomycin (Figure 12).

difference (P<0.05) compared to VRSA-infected group.

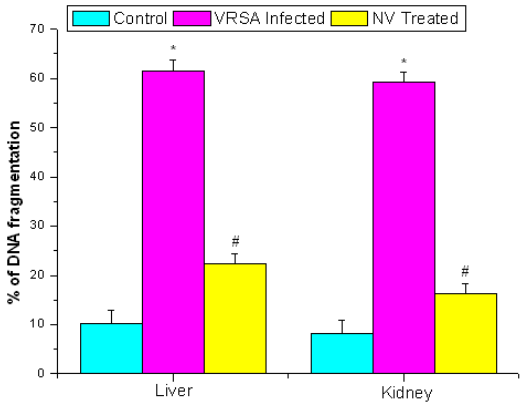

Figure 12: ***Quantitative estimation of DNA fragmentation assay by diphenylamine (DPA) assay in liver and kidney of control, VRSA-infected, and nanoconjugated vancomycin treated group. Values are expressed as mean ± SEM, n = 6. * indicates significant difference (P < 0.05) compared to control group. # indicates significant difference (P < 0.05) compared to VRSA-infected group.***

4. DISCUSSION

Staphylococcal infections caused by VRSA are increasing and their reduced therapeutic responsiveness to vancomycin represents an emerging threat to public health. Infection is a major complication of implanted devices. Patients frequently do not respond to high doses of antimicrobial agents even if administered for prolonged periods, and foreign bodies usually must be removed to achieve cure. It is known, susceptibility to antibiotics may be profoundly affected by growth of microorganisms near biomaterials. In vivo models are characterized by an artificial environment in which bacteria grow for a short time in the absence of host factors under selected, not necessarily physiologic, conditions.

The result of our study demonstrate that, VRSA infection in mice is associated with enhanced nitrate level, MPO activity, MDA level, PC level, GSSG level and decreased GSH level and as well as decreased enzymatic antioxidant (SOD, CAT, GPx, GR and GST) activity in liver and kidney, which are ameliorated by treatment of nanoconjugated vancomycin (Figure 1-11). More over DNA damage assessed by DPA assay due to VRSA infection was also observed in liver and kidney, which is protected by treatment of nanoconjugated vancomycin (Figure 12).

In this study, significant elevation of nitrate level and MPO activity in liver and kidney was observed in VRSA infected mice; which were decreased in nanoconjugated vancomycin treated group. Treatment of nanoconjugated vancomycin to VRSA infected mice decreased NO level and MPO activity significantly in liver and kidney (Figure 1-2). Nitric oxide (NO) is a free radical synthesized by nitric oxide synthase (NOS). NOS is composed of two identical monomers with molecular weights ranging from 130 to 160 kDa (Nikki PYL and Cheng CY, 2008). Our previous study shown that, nitric oxide synthesis in lymphocytes and as well as release in serum is high during VSSA and VRSA infection, which can be related to an alteration in oxidant-

antioxidant potential (Chakraborty S et al. 2011b). Thus, higher level of nitrite by VRSA infection may be due to high production of free radicals. Nanoconjugated vancomycin play the role of antioxidant to prevent the nitrate generation may be through the inhibition of inducible nitric oxide synthase (iNOS) expression (Li W et al. 2006). Hypochlorous acid (HOCl) is generated in the presence of myeloperoxidase and initiates the deactivation of antiproteases and the activation of latent proteases and leads to the cellular damage (Sullivan G et al. 2000). In this study, nanoconjugated vancomycin inhibited the myeloperoxidase activity which was increased due to VRSA infection; suggesting protective role of nanoconjugated vancomycin (Figure 2). These results suggest that either the cellular antioxidants level reached in a higher concentration to exert antioxidant effects or scavenged the free radical produced by the myeloperoxidase (Ogata M et al. 2000). Thus, in addition to the antioxidant system, nanoconjugated vancomycin may indirectly protect liver and kidney from VRSA infection induced oxidative damage. Thus, free radical depletion by the antioxidant agents seems to be beneficial for preventing the damage of lipid and protein.

In this study, significant elevation of malondialdehyde (MDA) and protein carbonyl level was observed in liver and kidney of VRSA infected mice; where as treatment of nanoconjugated vancomycin to VRSA infected mice decreased lipid peroxidation and protein oxidation significantly (Figure 3-4). It may be due to the generation of free radicals (mainly NO) which may react with protein in addition to lipids. Lipid peroxidation is known to disturb the integrity of cellular membranes; leading to the leakage of cytoplasmic enzymes (Bagchi M et al. 1995). Protein carbonyls formation has been indicated to be an earlier marker of protein oxidation. Oxidation of protein may be due to either excessive oxidation of proteins or decreased capacity to clean up oxidative damaged proteins. Oxidative modification of proteins may lead to the structural alteration and functional inactivation of many enzyme proteins, (Reznick AZ and Packer L, 1994) as evidenced by the decreased activity of different antioxidant enzymes like SOD, CAT, GPx, GR, and GST.

Imbalance between the generation of reactive oxygen species (ROS) and the antioxidant system causes oxidative stress. Glutathione, an important cellular reductant, is involved in protection against free radicals, peroxides, and toxic compounds in cellular systems (Gerster H. 1995). In the present study, the reduced glutathione level was significantly decreased in liver and kidney of VRSA infected mice; where as treatment of nanoconjugated vancomycin to VRSA infected mice increased the GSH level (Figure 5). In this study, it was observed that oxidized glutathione level was increased in liver and kidney of VRSA infected mice, which was ameliorated due to nanoconjugated vancomycin treatment (Figure 6). The decreased GSH levels represent its increased utilization due to VRSA infection. On the other hand, decreasing GSH level may be due to increasing level of lipid oxidation products which may be associated with less availability of NADPH required for the activity of glutathione reductase (GR) to transform GSSG to GSH due to the increasing production of ROS in form of NO (Sarkar S et al. 1995). In our present study, the increasing levels of GSSG and decreasing GR activity (Figure 10) due to VRSA infection may support the explanation.

Antioxidant enzymes are considered to be a primary defense that prevents biological macromolecules from oxidative damage. SOD rapidly dismutates superoxide anion ($O_2^{\cdot-}$) to less dangerous H_2O_2, which is further degraded by CAT and GPx to water and oxygen (Wetscher G et al. 1995). The results of the present study showed a significant fall in SOD and CAT activities in liver and kidney of VRSA infected group; where as treatment of nanoconjugated vancomycin to VRSA infected mice significantly increased the SOD and CAT activity (Figure 7-8). SOD, dismutate $O_2^{\cdot-}$ and the same in turn is a potent inhibitor of CAT (Ashakumari L and Vijayammal PL, 1996). The depletion in SOD activity was may be due to dispose off the free radicals, produced due to VRSA infection. Beside this, during infection, H_2O_2 produced by dismutation of superoxide anion, may have been efficiently converted to O_2 by CAT and the

enzyme activities showed a marked reduction. The depletion of antioxidant enzyme activity was may be due to inactivation of the enzyme proteins by VRSA infection induced NO generation, depletion of the enzyme substrates, and/or down-regulation of transcription and translation processes.

GPx works non-specifically to scavenge and decompose excess hydroperoxides including H_2O_2, which may be prevalent under oxidative stress (Somani S. 1996). Glutathione-S-transferase (GST) mainly detoxifies electrophilic compounds (Hemachand T et al. 2002) and has a well-established role in protecting cells from mutagens and carcinogens as a free radical scavenger along with glutathione. In the present study, the significant decreasing of GSH level and GSH-dependent enzymes, i.e. GPx, GR, and GST (Figure 9-11) in VRSA infection may be due to increased utilization to scavenge the free radical generation. The results of the present study showed a significant fall of GPx, GR and GST activities in liver and kidney of VRSA infected group; where as treatment of nanoconjugated vancomycin to VRSA infected mice significantly increased the GPx, GR and GST activity in liver and kidney (Figure 9-11). In the present study, it was observed that MDA level (Figure 3) and DNA fragmentation (Figure 12) were significantly elevated in liver and kidney due to VRSA infection. This elevated MDA level decreases GSH level (Figure 5) and SOD activity (Figure 7), which may be associated with DNA fragmentation. In this study, it was observed that DNA fragmentation increased in VRSA infected liver and kidney, which was brought back near to control due to nanoconjugated vancomycin treatment.

5. CONCLUSION

In conclusion, the study described here, liver and kidney is susceptible to *S. aureus* infection through the increased production of nitric oxide which leads to decreased antioxidant status; and nanoconjugated vancomycin protects the liver and kidney from such infection by decreasing free radical generation, lipid and protein damage, and also by increasing the antioxidant status. Hence, the nanoconjugated vancomycin can be used as a potent free radical scavenger antioxidative product and can be used as a potential therapeutic agent against staphylococcal infection.

Abbreviations:

CAT	: Catalase
CFU	: Colony formation unit
CMC	: Carboxymethyl chitosan
CMC-EDBE-FA	:Carboxymethyl chitosan-2, 2' ethylenedioxy bis ethylamine-Folate
CS	: Chitosan
DNA	: Deoxyribonucleic acid
DPA	: Diphenylamine
DTNB	: 5', 5'-dithio (bis)-2-nitrobenzoic acid
EDTA	: Ethylene diamine tetra acetate
GPx	: Glutathione peroxidase
GR	: Glutathione reductase
GSSG	: Oxidized glutathione
GSH	: Reduced glutathione
GST	: Glutathione-s-transferase
H_2O_2	: Hydrogen peroxide
MDA	: Malondialdehyde
MPO	: Myeloperoxidase
MRSA	: Methicillin resistant *Staphylococcus aureus*
NADPH	: Nicotinamide adenine dinucleotide phosphate
NO	: Nitric oxide
PC	: Protein carbonyls
ROS	: Reactive oxygen species
SDS	: Sodium dodecyl sulfate

SOD : Superoxide dismutase
SSA : Sulfosalicylic acid
TBA : Thiobutiric acid
TBARS : Thiobutiric acid reactive substance
TCA : Trichloro acetic acid
VRSA : Vancomycin resistant *Staphylococcus aureus*

ACKNOWLEDGEMENT

The authors express gratefulness to the Department of Biotechnology, Government of India for funding. The authors also express gratefulness Indian Institute of Technology, Kharagpur and Vidyasagar University, Midnapore for providing the facilities to execute these studies.

Declaration of interest
Authors declares that there are no conflict of interests.

REFERENCE

1. Ashakumari L, Vijayammal L. Addictive effect of alcohol and nicotine on lipid peroxidation and antioxidant defense mechanism in rats. J. Applied Toxicol. 1996; 16: 305-308.
2. Bagchi M, Bagchi D, Adickes E, Stohs SJ. Chronic effects of smokeless tobacco extract on rat liver histopathology and protection of HSP-90. J. Environ. Pathol. Toxicol. Oncol. 1995; 14 (2): 61-68.
3. Brouillette E, Grondin G, Shkreta L, Lacasse P, Talbot G. In vivo and in vitro demonstration of that *Staphylococcus aureus* is an intracellular pathogen in the presence or absence of fibronectin binding proteins. Microb Pathog. 2003; 35: 159-168.
4. Chakraborty Prasad S, KarMahapatra S, Sahu Kumar S, Pramanik P, Roy S. Amelioratory Effect of Nanoconjugated Vancomycin on Spleen during VRSA-Induced Oxidative Stress. Pathology Research International. 2011d; (Accepted), doi:10.4061/2011/420198
5. Chakraborty Prasad S, KarMahapatra S, Bal M, Roy S. Isolation and identification of Vancomycin Resistant *Staphylococcus aureus* from post operative pus sample. Al Ameen J of Med Sci. 2011c; 4(2): 52-68.
6. Chakraborty Prasad S, KarMahapatra S, Sahu Kumar S, Chattopadhyay S, Pramanik P, Roy S.. Nitric oxide mediated *Staphylococcus aureus* pathogenesis and protective role of nanoconjugated vancomycin. Asian Pacific J of Tropical Biomedicine. 2011b; 1(2): 105-112.
7. Chakraborty Prasad S, KarMahapatra S, Sahu Kumar S, Pramanik P, Roy S. Antioxidative effect of folate-modified chitosan nanoparticles. Asian Pacific J of Tropical Biomedicine. 2011a; 1(1): 29-38.
8. Chakraborty Prasad S, Sahu Kumar S, KarMahapatra S, Santra S, Bal M, Roy S, Pramanik P. Nanoconjugated vancomycin: new opportunities for the development of anti-VRSA agents. Nanotechnology, 2010; 21: 105103.
9. Chandran K, Venugopal M. Modulatory effects of curcumin on lipid peroxidation and antioxidant status during nicotine induced toxicity. Pol. J. Pharmacol. 2004; 56: 581-586.
10. Dryla A, Gelbmann D, Gabain A, Nagy E. Identification of a noveliron regulated Staphylococcal surface protein with haptogloinhemoglobinbinding activity. Mol Microbiol, 2003; 49: 37-53.
11. Gerster H. β-Carotene, vitamin E and vitamin C in different stages of experimental carcinogenesis. Eur J Clin Nutr. 1995; 49: 155-168.
12. Hattie G, Jon L, Tony C, Bridget W, Ambrose C, Frederik L. Survival of *Staphylococcus aureus* inside neutrophils contributes to infection. J Immunol, 2000; 164: 3713-3722.
13. Hemachand T, Gopalakrishnan B, Salunke M, Totey M, Shaha C. Sperm plasmamembrane-

associated glutathione S-transferases as gamete recognition molecules. J Cell Sci. 2002; 115: 2053-2065.
14. KarMahapatra S, Chakraborty Prasad S, Das S, Roy S. Methanol extract of *Ocimum gratissimum* protects murine peritoneal macrophages from nicotine toxicity by decreasing free radical generation, lipid and protein damage and enhances antioxidant protection. Oxidative Medicine and Cellular Longevity. 2009; 2(4): 1-9.
15. Khor E, Lim Y. Implantable applications of chitin and chitosan. Biomaterials. 2003; 24: 2339-2349
16. Kirby M. Extraction of a highly potent penicillin inactivator from penicillin resistant staphylococci. Science. 1944; 99: 452-453.
17. Kluytmans J, Belkum A, Verbrugh H. Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks. Clin. Microbiol. Rev. 1997; 10 (3): 505-520.
18. Koide S. Chitin-chitosan: Properties, benefits and risks. Nutrition Research. 1998; 18: 1091-1101.
19. Li W, Tsubouchi R, Qiao S, Haneda M, Murakami K, Yoshino M. Inhibitory action of eugenol compounds on the production of nitric oxide in RAW 264.7 macrophages. Biomed. Res. 2006; 27 (2): 69-74.
20. Liu F, Guan L, Yang Z, Li Z, Yao D. Antibacterial action of chitosan and carboxymethylated chitosan. J. Appl. Polym. Sci. 2001; 79 (7): 1324-1335.
21. Livermore M. Antibiotic resistance in staphylococci. Int J Antimicrob Agents. 2001; 16: 3-10.
22. Lowry H, Rosenbrough J, Farr L, Randall J. Protein measurement with the Folin Phenol Reagent. J Biol Chem. 1951; 193: 255-275.
23. Neu C. The crisis in antibiotic resistance. Science. 1992; 257: 1064-1073.
24. Nikki Lee, Cheng Y. Nitric oxide and cyclic nucleotides: Their roles in junction dynamics and spermatogenesis. Oxid Med Cell Longev. 2008; 1(1): 25-32.
25. Ogata M, Hoshi M, Urano S, Endo T. Antioxidant activity of eugenol and related monomeric and dimeric compounds. Chem. Pharm. Bull. 2000; 48 (10): 1467-1469.
26. Paradones E, Illera A, Peckham D, Stunz L, Ashman F. Regulation of apoptosis in vitro in mature spleen T cell. J Immunol. 1993; 151: 3521-3529.
27. Reznick Z, Packer L. Oxidative damage to proteins: Spectrophotometric methods for carbonyl assay. Methods Enzymol. 1994; 233: 357-363.
28. Sarkar S, Yadav P, Trivedi R, Bansal K, Bhatnagar D. Cadmium-induced lipid peroxidation and the status of the antioxidant system in rat tissues. J. Elem. Med. 1995; 9: 144-147.
29. Sievert M, Boulton L, Stoltman G, Johnson D, Stobierski G, Downes P. *Staphylococcus aureus* resistant to vancomycin—United States, 2002. Morb Mortal Wkly Rep, 51, pp. 565-567.
30. Somani M. Exercise, drugs and tissue specific antioxidant system. In S. M. Somani (Ed.), Pharmacology in exercise and sports. Boca Raton, FL: 1996; CRC Press, pp. 57- 95.
31. Steigbigel T, Lambert H, Remington S. Phagocytic and antibacterialproperties of normal human monocytes. J Clin Invest. 1974;.53: 131-142.
32. Sullivan W, Sarembock J, Linden J. The role of inflammation in vascular diseases. J Leuk Biol. 2000; 67: 591-602.
33. Wetscher J, Bagchi G, Perdikis G, Hinder R, Glaser K, Hinder A. Free radical production in nicotine treated pancreatic tissue. Free Radic. Biol. Med. 1995; 18: 877-882.