4.1 INTRODUCTION

Reactive oxygen species (ROS) generated as a result of stress gain biological and toxicological importance because of its potent oxidative potential and indiscriminate reactivity with cellular components, such as, lipids of biological membranes, enzymes, proteins and DNA molecules (Richter, 1987; Stadtman and Levine, 2000; Jackson and Loeb, 2001). Interestingly, aquatic organisms can be considered as test species because of their sensitivity to oxidative damage by chronic exposure or sublethal concentrations of toxic chemicals. They can provide model systems for investigation of different mechanisms of ROS mediated damage to cellular components, how cells respond, how repair mechanisms ameliorate this damage and how oxidative stress can lead to disease (Di Giulio et al., 1989; Livingstone et al., 1994). These organisms are more sensitive to exposure and toxicity compared to terrestrial organisms including mammals and in this respect they may provide experimental data for evaluation of subtle effects of oxidative stress (Lackner, 1998).

Cyanobacteria are one of the dominant microorganisms in aquatic and semiaquatic ecosystems. Compared to algae and higher plants, cyanobacteria undergo a high degree of O₂ reduction by consuming 50% of the photosynthetic electrons instead of only 15% for plants (Badger et al., 2000). Hence, the chances of ROS production in N₂-fixing systems are much greater as high rates of respiration are required to support N₂ fixation (Becana et al., 2010). Additionally, compared to heterotrophic bacteria, cyanobacteria contain at least an order of magnitude more iron within their cells (Shcolnick et al., 2009) in order to supply the components of
the photosynthetic and respiratory electron transfer chains, which reside in the same internal membrane system (Cooley and Vermaas, 2001). Therefore, increase in iron concentration will eventually enhance the vulnerability of cyanobacteria via iron-mediated Fenton reactions or ROS.

Among the various defense mechanisms, the main antioxidant defense system is the enzymatic anti-oxidant system, which involves the sequential and simultaneous action of a number of enzymes including superoxide dismutase (SOD), peroxidase (POD) and Catalase (CAT) to remove excess active oxygen species. In fact, activities of antioxidant enzymes are inducible by oxidative stress due to exposure to abiotic or biotic stresses (Baisak et al., 1994; Foyer et al., 1994). But the direction and size of the response varies with organism and the kind and intensity of stress treatment (Schutzendubel and Polle, 2002).

DNA is another key cellular component that is particularly susceptible to oxidative damage by ROS (Cerutti, 1985). The polyanionic nature of DNA provides a useful substrate for infiltration through membranes and adherence of metal cations, thus facilitating the formation of HO\(^*\) adjacent to these critical biological targets (Halliwell and Aruoma, 1991). Additionally, the heterogeneity of DNA molecules allows for HO\(^*\) attacks, including the nucleo bases and the sugar–phosphate backbone (Buxton et al., 1988) resulting in fragmentation of deoxyribose with single-strand breaks and oxidation of the sugar moiety leading to programmed cell death (PCD) (Dizdaroglu et al., 1975; Breen and Murphy, 1995). However, DNA fragmentation has been used to diagnose apoptosis in animals and plants (Ameisen 2002; Williams & Dickman 2008). Interestingly,
several studies has reported DNA fragmentation in cyanobacteria under stress conditions. In a study, DNA fragmentation was observed in the unicellular cyanobacteria, *Microcystis aeruginosa* when it is placed in darkness or is exposed to elevated temperatures by Bouchard & Purdie (2011). In another study, it was reported that *Microcystis aeruginosa* exposed to H$_2$O$_2$ lead to DNA fragmentation (Ding *et al*., 2012). Similar results are reported for *Anabaena* sp. (Ning *et al*. 2002), *Dunaliella viridis* Teodoresco (Jimenez *et al*. 2009) and *Skeletonema costatum* (Greville) Cleve (Chung *et al*. 2005).

Program Cell Death (PCD) is an irreversible, genetically controlled form of cell suicide that is essential for the proper development, function and ultimate survival of multicellular organisms (Ameisen 2002). PCD-like cell death is well established in all eukaryotes ranging from unicellular organisms such as amoebae, fungi and yeast (Matsuyama *et al*., 1999; Frohlich and Madeo, 2000), invertebrates (Bergmann *et al*., 1998), to higher animals and plants. The physiological cell death processes occurring in many prokaryotic microorganisms including bacteria (Ameisen. 1996; Yarmolinsky, 1995; Chaloupka and Vinter 1996; Samuilov *et al*., 2000) and the phytoplankton cyanophyceae (Lee and Rhee, 1997) are also considered types of PCD (Ameisen, 1996; Lee and Rhee, 1997; Samuilov *et al*., 2000). A well-known form of PCD is apoptosis, which is accompanied by plasma membrane blebbing, chromatin condensation, involvement of cysteine proteases (caspases) and fragmentation of DNA (Kerr *et al*. 1972).
Caspases (cysteinyl aspartate-specific proteases) are one of the most important and widely researched apoptotic proteins in mammalian PCD. Caspases have only been isolated from multicellular animals (sponges to humans) but their activity has also been reported for vascular plants, yeast and bacteria (De-Jong et al. 2002; Madeo et al. 2002; Herker et al. 2004; Jimenez et al. 2008, 2009; Chen & He 2009). Uren (2000) and his colleagues identified two ancient families of caspase-like proteins, paracaspases and metacaspases in silico and it has been reported that cyanobacteria maintain a rich metacaspase pool and many of these genes have been identified in silico in some sequenced cyanobacteria strains, including Gloeobacter violaceus PCC 7421, Thermosynechococcus elongatus BP-1, Synechocystis sp. PCC 6803, Trichodesmium erythraeum ISM 101, Nostoc punctiforme PCC 73102, Nostoc sp. PCC 7120, and Anabaena variabilis ATCC 29413 (Bidle and Falkowski, 2004). However, within the caspase family, caspase-3 is believed to be the final executor of apoptotic cell death in animal cell; it elicits organized degradation of many cellular proteins, and it changes the nuclear morphology (Fischer et al. 2003; Rice & Bayles 2008). Interestingly, it was found by Ding et al (2012) that caspase-3–like activity increased over time after exposure to 250 and 325 µM H$_2$O$_2$ in Microcystis aeruginosa. Similar results have also been reported during PCD of the green algae Micrasterias denticulata (Darehshouri et al. 2008), Dunaliella tertiolecta Butcher (Segovia et al. 2003) and Chlorella saccharophila (Kru¨ ger) Migula (Zuppini et al. 2007). In addition, under diverse environmental stresses, the filamentous marine cyanobacterium Trichodesmium sp. IMS101 were also found to initiates PCD with an increase in
caspase activity (Berman-Frank et al. 2004). ROS can also act indirectly by modifying the cellular redox potential, which modulates key regulatory proteins involved in PCD (Mignotte and Vayssiere, 1998). During cell death induced by high concentrations of H$_2$O$_2$ or ROS, there is an increase in cytoplasmic vacuolation, a feature regarded as a cytological hallmark of paraptosis. Further, cytoplasm vacuolation was also observed during PCD in *Anabaena* sp. (Ning et al. 2002), *Micrasterias denticulate* (Darehshouri et al. 2008) and *Amphidinium carterae* Hulbert (Franklin & Berges 2004).

Proteins are another important target of ROS. Metal-catalyzed damage to proteins involves oxidative scission, loss of histidine residues, bityrosine crosslinks, the introduction of carbonyl groups, and the formation of protein-centered alkyl, R·, alkoxy, RO·, and alkylperoxyl, ROO•, radicals. (Davis, 1987; Stadtman and Levine, 2000) oxidative modifications of amino acid side chains, reactive oxygen-species-mediated peptide cleavage, reactions of peptides with lipids and carbohydrate oxidation products. Alterations in the expression and activities of proteins act as the most important molecular biomarker of environmental stress. To face stress, the functioning of some proteins is inhibited or lost and that of others are enhanced or induced.

In the previous chapter, *N. muscorum* growth was found inhibited by different classes of compounds (curcumin, quercetin, gallic acid, hydroquinone, cypermethrin, saponin and bromelain) and IC$_{50}$ for each compound against *N. muscorum* was successfully calculated using standard MTT and INT assays. Based on these observations of the previous chapter, the study presented in this
Chapter 4

This chapter explores the comprehensive and comparative study on the dose-dependent effect of these compounds on the enzymatic defense systems. Further to decipher the molecular mechanism of effect of these compounds on *N. muscorum*, this chapter, also focused on DNA fragmentation studies with protein analysis by SDS-PAGE.
4.2 MATERIAL AND METHODS

4.2.1 Free radical generation

4.2.1.1 Superoxide

Superoxide radical was estimated in compound treated and untreated cells of *N. muscorum* as per the method of Elstner and Heupel (1976)

*Reagents*

(a) Hydroxylamine 6.94 mg in 10 ml
(b) Sulphanilamide 129.27 mg in 15 ml
(c) NEDD 108.57 mg in 15 ml

*Procedure*

15 ml treated and untreated cells of *N. muscorum* was homogenized in 2 ml phosphate buffer and centrifuged at 8000 rpm. To 1 ml of supernatant, 0.9 ml phosphate buffer and 0.1 ml hydroxylamine was added and incubated at 25 °C. After incubation 1 ml sulphanilamide and 1 ml NEDD was added and optical density was measured at 530 nm incubated after 20 min of incubation.

4.2.1.2 Peroxide

The level of peroxide in the cells was determined by following the method of Sagisaka (1976)

*Reagents*

(a) Ferrous ammonium sulphate 39.2 mg
(b) KSCN 1.21 gm
For total peroxide estimation, 10 ml culture were extracted in 3.5 ml of 5% trichloroacetic acid and after centrifugation at 8000 rpm for 20 min level of total peroxide in the supernatant was measured by taking absorption at 480 nm.

4.2.2 Lipid peroxidation

Lipid peroxidation in cyanobacterial sample under compound stress conditions was measured by estimating the end product malondialdehyde (MDA) in the compound treated and untreated cells of *N.muscorum* after each treatment as per the method of Heath and Packer (1968).

Reagents

(a)Thiobarbituric acid (TBA)       0.5% (W/V) in 0.2 N HCL.
(b)Trichloro acetic acid (TCA)     20% (W/V) in TBA Solution

Procedure

Cells from different test samples were collected by centrifugation and washed twice in 5 mM phosphate buffer (pH 7.0). The cellular pellets were homogenised in 50 mM phosphate buffer. The resulting homogenate was centrifuged at 8000 rpm for 20 min. To 0.5 ml aliquot of the supernatant, 2 ml of 20% TCA containing 0.5% TBA was added. The mixture was heated at 90 °C for 20 min and then quickly cooled in ice bath. After centrifugation at 8000 rpm for 10 min, the...
4.2.3 Antioxidant enzyme activity

4.2.3.1 SOD

SOD activity was measured spectrophotometrically by the method of Giannopolitis and Ries (1977).

Reagents

(a) Riboflavin - 1.3 µM
(b) Methionine - 13 µM
(c) NBT - 63 µM
(d) Sodium carbonate - 0.05 M
(e) EDTA - 100 mM
(f) K₂HPO₄ - 100 mM

Procedure

Cyanobacterial cells after four days of treatment were collected by centrifugation and washed twice with 100 mM EDTA-phosphate buffer, pH 7.8. The cellular pellet was grounded in an ice cold mortar with 100mM EDTA-phosphate buffer, pH 7.8. The homogenate was centrifuged for 20 min at 8000 rpm. The supernatant fraction was used as the enzyme source. The reaction mixture 3 ml contained 1.3
µM riboflavin, 13 mM L-methionine, 0.05 M Na₂CO₃, (pH 10.2) 63 µM-p-nitroblue tetrazolium chloride and 0.1 ml of crude extract. Reaction was carried out in similar test tubes under illumination from fluorescent lamp at 25°C. The initial rate of reaction as measured by the difference in increase in absorbance at 560 nm in the presence and absence of extract was proportional to the amount of enzyme. The unit of superoxide dismutase activity was defined as the amount of enzyme which caused a 50% inhibition of the reaction observed in the absence of enzyme. For the blank the reaction was run in darkness.

4.2.3.2 POD

For the estimation of POD the method of Gahagen et al. (1968) was followed

Reagents

(a) 25 mM H₂O₂ in 100 mM phosphate buffer (pH 7.0)

(b) 100 mM pyrogallol

(c) 100 mM phosphate buffer (pH 7.0)

Procedure

Cyanobacterial cells after treatment were collected and homogenised in 2 ml, 100 mM phosphate buffer (pH 7.0) at 5°C. The homogenate was centrifuged at 8000 rpm for 30 min. The supernatant was taken as the enzyme extract. 3 ml reaction mixture consist of 1 ml 25 mM H₂O₂, 1 ml 100 mM pyrogallol prepared in distilled water and 1.0 ml enzyme extract. After thorough mixing of the reaction mixture, change in optical density was monitored at 430 nm for 2-3 minutes.
4.2.4 Yield and quality of DNA extracted from *Nostoc muscorum* using different methods of DNA isolation for optimization of best method.

**DNA extraction and analysis**

The following extraction procedures were repeated 3 times to assess the reproducibility of the methods. For each DNA extraction cells were collected by centrifugation of 50 ml from a stock culture (10 min, 3500 rpm) before being treated accordingly to each protocol.

**4.2.4.1 DNA isolation by Miniprep of bacterial genomic DNA (Ausubel et al. 1995).**

**Reagents**

1M Tris HCl (pH 7.2)

0.5M EDTA (pH 8.0)

5M NaCl

SDS 10%

Proteinase K 20 mg/ml

CTAB – NaCl solution CTAB-10% and NaCl- 0.7M.

Chloroform: Isoamyl alcohol (24:1)

Isopropanol

Ethanol 70%
**Procedure**

A 50-mL of logarithmic phase culture of untreated and compound treated (curcumin and quercetin for 24 hrs) *N.muscorum* was taken and DNA was extracted by miniprep method of bacterial genomic DNA (Ausubel et al. 1995). Cells were resuspended in 567 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) by repeated pipetting. Lysis of the cells was performed using 30 µl of 10 % SDS and 3 µl of 20 mg/ml proteinase K. The mix was subsequently incubated for 1 h at 37 °C. Selective precipitation of proteins and polysaccharides was performed using 80 µl of CTAB/NaCl (10 % CTAB, 0.7 M NaCl) in presence of 100 µl of 5 M NaCl. The samples were gently mixed by inversion and incubated for 10 min at 65 °C. Nucleic acids were thereafter isolated by a chloroform:isoamyl alcohol (24:1) separation. DNA was finally recovered by precipitation using 0.6 volume of isopropanol and centrifugation (5 min, 4 °C, 15000 rpm). The DNA pellets were washed with 1 ml of cold 70 % ethanol. Finally, the tubes were centrifuged one last time 5 min (4 °C, 15000 rpm), the supernatant was discarded, and each pellet dried before being resuspended in 100 µl TE Buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

**4.2.4.2 DNA Isolation by Himedia® Hipura™ DNA Xpress Kit.**

The extraction was performed according to the instruction of the manufacturer using DNA Xpress reagent.
4.2.4.3 DNA extraction protocol for animal cell lines. (Sam brook & Russell, 2001)

Reagents

1M Tris HCl (pH 7.2)
0.5M EDTA (pH 8.0)
5M NaCl
SDS 20%
Proteinase K 100 µg/ml
Chloroform: Isoamyl alcohol (24:1)
Isopropanol
Ethanol 70%

Cells were resuspended in 620 µl TE buffer (150 mM Tris, 20 mM EDTA, pH 8.0) by repeated pipetting. Lysis of the cells was performed using 30 µl of 20% SDS and 15 µl of 100µg/ml proteinase K. The mix was subsequently incubated for 12 - 24h at 55 °C. Added 375 µl of 5 M NaCl to this solution and incubated at 37°C for 30 min. The tubes were centrifuged for 30 min (4 °C, 14000 rpm). Nucleic acids were thereafter isolated by a chloroform:isoamyl alcohol (24:1) separation. DNA was finally recovered by precipitation using 0.6 volume of isopropanol (Kept at -20°C for 30 minutes) and centrifugation (5 min, 4 °C, 15000 rpm). The DNA pellets were washed with 1 ml of cold 70 % ethanol. Finally, the tubes were centrifuged one last time for 5 min (4 °C, 15000 rpm), the
supernatant was discarded, and each pellet dried before being resuspended in 100 µl TE Buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

**4.2.4.4 Optimized DNA extraction protocol**

Cells were resuspended in 620 µl TE buffer (150 mM Tris, 20 mM EDTA, pH 8.0) by repeated pipetting. Lysis of the cells was performed using 30 µl of 20 % SDS and 20µl of 100µg/ml proteinase K. The mix was subsequently incubated for 2 hrs at 55 °C. Added 375 µl of 5 M NaCl to this solution and incubated at 37°C for 30 min. The tubes were centrifuged for 30 min (4 °C, 14000 rpm). Nucleic acids were thereafter isolated by adding chloroform separation. DNA was finally recovered by precipitation using 0.6 volume of isopropanol (Kept at 4°C for overnight) and centrifugation (5 min, 4 °C, 15000 rpm). The DNA pellets were washed with 1 ml of cold 70 % ethanol. Finally, the tubes were centrifuged one last time 5 min (4 °C, 15000 rpm), the supernatant was discarded, and each pellet dried before being resuspended in 100 µl TE Buffer (10 mM Tris, 1 mM EDTA, pH 8.0). This method is further used for the DNA fragmentation study for all the compounds after 24 hrs. of treatment.

**DNA Concentration, yield and purity**

Tris -10mM

EDTA- 1mM

Spectrophotometric analysis and agarose gel electrophoresis will reveal the concentration and the purity of the genomic DNA. The samples were diluted with
TE buffer and the absorbance was taken at 260 nm and 280 nm using a quartz cuvette. An absorbance of 1.0 at 260 nm corresponds to 50 µg/ml of DNA. Purity of DNA is determined by measuring the ratio of absorbance at 260 nm and 280 nm.

Concentration of DNA sample (µg/ml) = 50 * A$_{260}$ * dilution factor

**Gel preparation**

Agarose 1% in TAE buffer (40mM Tris-HCL, pH 8.0 containing 2 mM EDTA) was dissolved by heating. The solution was cooled to about 60°C, ethidium bromide (0.5 µg/ml) was added and then poured into the gel tray and allowed to solidify at room temperature.

**Agarose gel electrophoresis**

Both untreated and treated DNA were mixed with the loading buffer (0.125% bromophenol blue, 30% glycerol) in 3:1 ratio and loaded in the wells of 1% agarose gels. Electrophoresis was done for 2 hr at 50V and the gels were viewed under UV light.

**4.2.5 Analysis of protein by SDS-PAGE**

**Preparation of crude extract**

The total crude protein was extracted from cyanobacterial biomass using modified method of Ivleva and Golden, 2008
**Chapter 4**

**Reagents**

a) Glass beads: diameter 0.2–0.3 mm (Sigma), acid-washed.

b) Phosphate-buffered saline (PBS) buffer (pH to 7.4)

136 mM NaCl,

2.6 mM KCl,

10 mM Na$_2$HPO$_4$,

1.76 mM KH$_2$PO$_4$;

c) PMSF 0.01M

0.01M stock solution of PMSF was prepared by dissolving 17.42 mg in 10 ml Isopropanol and stored at -20°C

**Procedure**

A 100-mL of logarithmic phase culture of *N. muscorum* with optical density 0.5 at 750 nm was taken and the cells were spin down for 10 min at 1500g. The pellets were washed by resuspending in 2 mL of PBS buffer and repeating the spin. The pellets were again resuspended in 200 µL of PBS. The cells were frozen at –80°C and then quickly thawed at 37°C to allow partial cell breakage. For the remainder of the procedure, the samples were kept on ice and in the presence of protease inhibitors. 100 µL of PBS buffer was added and suspension was spin at 1000g. The supernatant fraction was collected and again spin at 1000g for 10s to pellet
residual glass beads. The resulting green supernatant fraction was a crude cell extract.

The protein concentration was measured using Lowry method using BSA as standard. The cell extract was used for SDS-PAGE analysis.

Estimation of protein

Protein was measured by the method developed by Lowry et al. (1951) and modified by Herbert et al. (1971).

Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of the Crude protein extract

SDS-PAGE of cyanobacterial protein was performed on 12% polyacrylamide resolving gel after seven days of curcumin and quercetin treatment and carried out in a vertical system (Bangalore Geni) according to the method of Laemmli (1970)

SDS-PAGE is used for analyzing mixtures of proteins. In this technique, proteins are made to react with an anionic reagent, Sodium Dodecyl-sulfate (SDS), to form negatively charged complexes. The amount of SDS bound by protein, and so the charge on the complex, is proportional to its size. The proteins are denatured and solubilized due to their binding with SDS. Thus, proteins of either acidic or basic pI form negatively charged complexes that can be separated on the basis of differences in charge and size by electrophoresis through a sieve like matrix of polyacrylamide gel.
Reagents for SDS Gel Electrophoresis

Gel Preparation

Reagents

Resolving gel (5ml)

\[
\begin{align*}
\text{H}_2\text{O} & \quad 1.6 \text{ ml} \\
\text{Acrylamide mix} & \quad 0.33 \text{ ml (30\% W/V)} \\
\text{Tris (pH 8.8)} & \quad 1.3 \text{ ml (1.5 M)} \\
\text{SDS} & \quad 50 \mu\text{l (10\% W/V)} \\
\text{Ammonium persulphate} & \quad 50 \mu\text{l (10\% W/V)} \\
\text{TEMED} & \quad 3 \mu\text{l} \\
\end{align*}
\]

(N, N, N, N –Tetramethylethylene diamine)

Stacking gel

\[
\begin{align*}
\text{H}_2\text{O} & \quad 1.4 \text{ ml} \\
\text{Acrylamide mix} & \quad 500 \mu\text{l (30\% W/V)} \\
\text{Tris (pH 6.8)} & \quad 250 \mu\text{l (1 M)} \\
\text{SDS} & \quad 20 \mu\text{l (10\%)} \\
\text{(Sodium dodecyl sulphate)} & \\
\text{Ammonium persulphate} & \quad 20 \mu\text{l (10 \%)} \\
\end{align*}
\]
TEMED — 2 µl

(N, N, N, N, Tetramethyl ethylene diamine)

Procedure

Thoroughly cleaned, dried glass plates and three spacers were taken and assembled properly. The spacers were set with 1 mm distance from the edges of the glass plates. The construction was held together with bull-dog clips. The chamber was clamped in an upright, level position. A sufficient volume of separating gel mixture (5ml) was prepared. The freshly mixed solution was carefully pipetted or poured into the chamber without generating air bubbles. It was poured to a level about 1 cm below, where the bottom of the well-forming comb would rest when it would be in position. A thin layer of water or methanol was poured to prevent oxidation of resolving gel. The layer was discarded when resolving gel was polymerized. The mixture was left to stand until it was set.

Stacking gel (2 ml) was prepared properly. The solution was pipetted into the chamber. The comb was inserted and the gel was allowed to stand until set. When the stacking gel was set, the comb was removed without distorting the shape of the wells. The clips, holding the plates together, were removed and gel was installed in the apparatus. The apparatus was filled with reservoir buffer (5 X Tris glycine electrophoresis buffer: (stock solution) (500 ml) is prepared by adding 7.55g Tris Base and 47g Glycine (pH 8.3 ) in 400 ml of dH₂O. Then 25 ml of a 10 % (w/v) stock solution of SDS was added and adjusted the volume to 500 ml)
Samples were mixed with loading dye (100 mM DTT (Dithiothreitol), 50 mM Tris-HCl, pH 6.8, 2% SDS, 10% (v/v) glycerol, 0.1% bromphenol blue dye) while the gel was setting/polymerizing. The sample solution was heated in boiling water for 2 min and cooled before loading. The gel was finally loaded. Required volume of sample solution was taken up in a pipette and carefully injected into a sample well through the reservoir buffer.

After loading all the samples, electrophoresis was started by turning on power (DC) on a gel of about 1mm thickness and about 14 cm length, with an applied voltage of about 80 V when samples are in stacking gel then on 120 V through running gel. At the end of electrophoresis (when the dye front reached the bottom of the gel), protein bands in the gel are visualized by staining.

The gel was removed from the glass plates and immersed in the protein stain immediately. The gel was left there with gentle agitation until the dye had penetrated the gel. Dye that was not bound to the protein was removed by transferring the gel to a destaining solution. After about 24 hr, with gentle agitation and several changes of destaining solution, the gel background became colorless and protein bands of blue, purple color is visible.

Finally, the whole cell protein profiles of the samples were visualized under Trans white light and captured using Gel Doc (BIO-RAD). All experiments were performed in three independent replicates and only those bands present in at least two gels of the independent set were taken for analysis.
4.2.6 Statistical analysis

All the data are given as the mean ± S.E. of three measurements. Statistical analysis was carried out using the SPSS software (SPSS Inc., version 10.).

4.3 RESULTS

The results in this chapter show the compounds induced alterations in the level of active oxygen species generation (AOS), antioxidative enzymes and MDA content. The generation of reactive oxygen species in *N. muscorum* might occur due to compound’s induced stress and therefore, the status of reactive oxygen species in the cells treated with different classes of compounds (curcumin, quercetin, gallic acid, hydroquinone, cypermethrin, saponin and bromelain) were investigated along with the defense mechanisms.

4.3.1 Total Superoxide

It is clear from the results that superoxide radical content in *N. muscorum* was increased by 65%, 40%, 12%, 63% and 10% following the exposure of curcumin, quercetin, gallic acid, cypermethrin and hydroquinone at 50 µM, respectively. While, 23% and 10% increase in superoxide content were observed in case of (100 µM) saponin and (1mAnson U) bromelain, respectively (Figure 4.1-4.7). Further increase in superoxide content was noticed on raising the concentration of compounds, indicating the dose dependent effects of the compounds on superoxide generation.
The increase in the superoxide was found by 65% - 130%, 40% - 140%, 12% - 40%, 63% - 146% and 10% - 85% in the *N. muscorum* when treated with 50-400 µM concentration of curcumin, quercetin, gallic acid, cypermethrin and hydroquinone, respectively (Figure 4.1 - 4.5).

**Figure 4.1:** Effect of different concentrations (0-400 µM) of curcumin on the superoxide radical level. Values are means ± SE of three replicates. The superoxide content in untreated control was 1240 ± 20 µM (g dry wt)^{-1} x10^{-3}.
Figure 4.2: Effect of different concentrations (0-400 µM) of quercetin on the superoxide radical level. Values are means ± SE of three replicates. The superoxide content in untreated control was 1240 ± 20 µM (g dry wt)$^{-1}$ x10$^{-3}$.

Figure 4.3: Effect of different concentrations (0-400 µM) of gallic acid on the superoxide radical level. Values are means ± SE of three replicates. The superoxide content in untreated control was 1240 ± 20 µM (g dry wt)$^{-1}$ x10$^{-3}$.
Figure 4.4: Effect of different concentrations (0-400 µM) of cypermethrin on the superoxide radical level. Values are means ± SE of three replicates. The superoxide content in untreated control was $1240 \pm 20 \, \mu M \, (g \, dry \, wt)^{-1} \times 10^{-3}$.

Figure 4.5: Effect of different concentrations (0-400 µM) of hydroquinone on the superoxide radical level. Values are means ± SE of three replicates. The superoxide content in untreated control was $1240 \pm 20 \, \mu M \, (g \, dry \, wt)^{-1} \times 10^{-3}$.
Increase in superoxide content was observed from 23% to 58% % in the *N. muscorum* when saponin concentration increased from 100 to 800 µM (Figure 4.6). However, bromelain also showed a dose dependent (1mAnson U to 100 mAnson U) increase in superoxide content from 10% to 65% (Figure 4.7).

**Figure 4.6:** Effect of different concentrations (0-800 µM) of saponin on the superoxide radical level. Values are means ± SE of three replicates. The superoxide content in untreated control was 1240 ± 20 µM (g dry wt)⁻¹ x10⁻³.
Figure 4.7: Effect of different concentrations (0-100 mAnson U) of bromelain on the superoxide radical level. Values are means ± SE of three replicates. The superoxide content in untreated control was $1240 \pm 20 \mu M (g \text{ dry wt})^{-1} \times 10^{-3}$.

4.3.2 Total Peroxides

All the test compounds induced dose dependent formation of total peroxide (Figure 4.8-4.14). At the low concentration (50 µM), compared to all other compounds, curcumin and quercetin showed the minimal increase in peroxide content i.e., 10% and 15%, respectively. However, on increasing the concentration from 50 µM to 400 µM a dose dependent sharp increase in peroxide content can be observed for both curcumin and quercetin. At 400 µM concentration, increases in peroxide content by 60% and 80% were observed in case of curcumin and quercetin, respectively (Figure 4.8-4.9).
Figure 4.8: Effect of different concentrations (0-400 µM) of curcumin on total peroxide. Values are means ± SE of three replicates. The total peroxide content in untreated control was 540 ± 11 µM (g dry wt)\(^{-1}\) x10\(^2\)

Figure 4.9: Effect of different concentrations (0-400 µM) of quercetin on total peroxide. Values are means ± SE of three replicates. The total peroxide content in untreated control was 540 ± 11 µM (g dry wt)\(^{-1}\) x10\(^2\)
Gallic acid, cypermethrin and hydroquinone showed dose dependent increase in the level of peroxide by 55% to 85%, 38% to 65% and 25% to 60%, respectively in *N. muscorum* on increasing their concentration from 50µM to 400µM (Figure 4.10-4.12).

![Figure 4.10: Effect of different concentrations (0-400 µM) of gallic acid on total peroxide. Values are means ± SE of three replicates. The total peroxide content in untreated control was 540 ± 11 µM (g dry wt)^{-1} \times 10^2](image1)

![Figure 4.11: Effect of different concentrations (0-400 µM) of cypermethrin on total peroxide. Values are means ± SE of three replicates. The total peroxide content in untreated control was 540 ± 11 µM (g dry wt)^{-1} \times 10^2](image2)
Figure 4.12: Effect of different concentrations (0-400 µM) of hydroquinone on total peroxide. Values are means ± SE of three replicates. The total peroxide content in untreated control was 540 ± 11 µM (g dry wt)\(^{-1}\) x10\(^2\).

Increase in peroxide content from 26% to 138% was observed on increasing the concentration (100µM- 800µM) of saponin, while, increasing concentration (1mAnson U to 100 mAnson U) of bromelain showed increase in peroxide content from 34% to 65% (Figure 4.13 –Figure 4.14).
Figure 4.13: Effect of different concentrations (0-800 µM) of saponin on total peroxide. Values are means ± SE of three replicates. The total peroxide content in untreated control was 540 ± 11 µM (g dry wt)$^{-1} \times 10^2$

Figure 4.14: Effect of different concentrations (0-100 mAnson U) of bromelain on total peroxide. Values are means ± SE of three replicates. The total peroxide content in untreated control was 540 ± 11 µM (g dry wt)$^{-1} \times 10^2$
4.3.3 Lipid peroxidation

The data pertaining to membrane damage measured in terms of lipid peroxidation are summarized in Figure 4.15 - Figure 4.21. Lipid peroxidation is probably the most extensively investigated biomarker of oxidative stress. The level of MDA in the cells is considered a measure of lipid peroxidation status. Treatment with quercetin (50-400 µM) showed an enhancement in MDA content by 30% - 228% (Figure 4.16). In contrast, curcumin, gallic acid, cypermethrin and hydroquinone showed an enhancement by 25% - 200%, 20% - 168%, 10% -128%, 1% -177%, respectively, on increasing the concentration from 50-400 µM (Figure 4.15, Figure 4.17- 4.19). Whereas, saponin also showed a dose dependent (100 -800 µM) increase in the MDA content i.e., 1% - 92%, while, bromelain showed increase in MDA content from 1% to 56% on increasing concentration from 1 mAnson U to 100 mAnson U (Figure 4.20-4.21).
Figure 4.15: Effect of different concentrations (0-400 µM) of curcumin on the level of MDA content. The MDA content in untreated control was 705 ± 9 nmol MDA (g dry wt)⁻¹. Values are means ± SE of three replicates.

Figure 4.16: Effect of different concentrations (0-400 µM) of quercetin on the level of MDA content. The MDA content in untreated control was 705 ± 9 nmol MDA (g dry wt)⁻¹. Values are means ± SE of three replicates.
Figure 4.17: Effect of different concentrations (0-400 µM) of gallic acid on the level of MDA content. The MDA content in untreated control was 705 ± 9 nmol MDA (g dry wt)$^{-1}$. Values are means ± SE of three replicates.

Figure 4.18: Effect of different concentrations (0-400 µM) of cypermethrin on the level of MDA content. The MDA content in untreated control was 705 ± 9 nmol MDA (g dry wt)$^{-1}$. Values are means ± SE of three replicates.
Figure 4.19: Effect of different concentrations (0-400 µM) of hydroquinone on the level of MDA content. The MDA content in untreated control was 705 ± 9 nmol MDA (g dry wt)^{-1}. Values are means ± SE of three replicates.

Figure 4.20: Effect of different concentrations (0-800 µM) of saponin on the level of MDA content. The MDA content in untreated control was 705 ± 9 nmol MDA (g dry wt)^{-1}. Values are means ± SE of three replicates.
Figure 4.21: Effect of different concentrations (0-100 mAson U) of bromelain on the level of MDA content. The MDA content in untreated control was 705 ± 9 nmol MDA (g dry wt)^{-1}. Values are means ± SE of three replicates.

4.3.4 Antioxidant enzyme system

The activity of antioxidant enzymes such as superoxide dismutase and peroxidase, which scavenge superoxide O$_2^-$ and H$_2$O$_2$, respectively, produced in large quantity under compound stress, were estimated after 4 days of treatment.

4.3.4.1 SOD activity

Data related to SOD activity in *N. muscorum* under curcumin, quercetin, gallic acid, cypermethrin, hydroquinone, saponin and bromelain treatment are presented in Figure 4.22 - 4.28. The activity of the SOD in untreated *N. muscorum* was 6.5 ± 0.25 Unit (mg protein)$^{-1}$. The treatment of *N. muscorum* with curcumin at 50, 100 and 200 µM concentration resulted in 20%, 56 %, 144 % increase, respectively, in SOD activity whereas 400 µM of curcumin showed only 11%
increase indicating the loss of enzymatic activity or synthesis (Figure 4.22). *N. muscorum* treated with quercetin at low concentrations (50 - 200 µM) stimulated the SOD activity by 66% - 245%, whereas, at high concentration (400 µM), increase in the SOD activity was only 145% (Figure 4.23). In case of gallic acid treated cells the dose dependent increase i.e., 2% to 129% in SOD activity was noticed on increasing the concentration from 50 to 400 µM (Figure 4.24). Increasing concentration of the cypermethrin from 50 to 400 µM enhanced the SOD activity by 1 % to 173 %. However, in contrast, increased SOD activity was from 2% to 133% was noticed in hydroquinone (50 to 400 µM) (Figure 4.25-4.26).

![Figure 4.22](image)

**Figure 4.22:** Effect of different concentrations (0-400 µM) of curcumin on SOD activity. The activity of the SOD in untreated *N. muscorum* was 6.5 ± 0.25 Unit (mg protein)^{-1}. Values are means ± SE of three replicates.
Figure 4.23: Effect of different concentrations (0-400 µM) of quercetin on SOD activity. The activity of the SOD in untreated *N. muscorum* was 6.5 ± 0.25 Unit (mg protein)⁻¹. Values are means ± SE of three replicates.

Figure 4.24: Effect of different concentrations (0-400 µM) of gallic acid on SOD activity. The activity of the SOD in untreated *N. muscorum* was 6.5 ± 0.25 Unit (mg protein)⁻¹. Values are means ± SE of three replicates.
Figure 4.25: Effect of different concentrations (0-400 µM) of cypermethrin on SOD activity. The activity of the SOD in untreated *N. muscorum* was 6.5 ± 0.25 Unit (mg protein)^{-1}. Values are means ± SE of three replicates.

Figure 4.26: Effect of different concentrations (0-400 µM) of hydroquinone on SOD activity. The activity of the SOD in untreated *N. muscorum* was 6.5 ± 0.25 Unit (mg protein)^{-1}. Values are means ± SE of three replicates.
Figure 4.27 and 4.28 shows the effect of saponin and bromelain on the SOD activity of *N. muscorum*. SOD activity increased by 12% to 109% when the concentration of saponin was increased up to 400 µM, whereas, further increase in concentration to 800 µM showed a decrease as SOD activity increase was found to be only 1%. In contrast, bromelain showed a dose dependent (1 mAnson U to 100 mAnson U) effect with an increase of 1% to 50% in SOD activity.

![Figure 4.27](image)

**Figure 4.27**: Effect of different concentrations (0-800 µM) of saponin on SOD activity. The activity of the SOD in untreated *N. muscorum* was 6.5 ± 0.25 Unit (mg protein)^{-1}. Values are means ± SE of three replicates.
Figure 4.28: Effect of different concentrations (0-100 mAnson U) of bromelain on SOD activity. The activity of the SOD in untreated *N. muscorum* was 6.5 ± 0.25 Unit (mg protein)^{-1}. Values are means ± SE of three replicates.

### 4.3.4.2 POD activity

The activity of POD in *N. muscorum* treated with curcumin, quercetin, gallic acid, cypermethrin, hydroquinone, saponin and bromelain after 4 days of treatment is depicted in Figure 4.29-4.35. The activity of POD in the cells of *N. muscorum* following the treatment with 50, 100, 200 and 400 µM curcumin increased by 54, 60, 64 and 80 %, respectively (Figure 4.29). In contrast, when *N. muscorum* was treated with 50, 100, 200 and 400 µM of quercetin, POD activity exhibited an enhancement of 60, 78, 76 and 82%, respectively (Figure 4.30). In case of gallic acid treated cells the POD activity showed an enhancement of 6% to 69% on
increasing doses from 50 µM to 400 µM (Figure 4.31). Cypermethrin showed a progressive increase in the enhancement of POD activity by 38% to 72% on increasing the concentration (50-400 µM) (Figure 4.32). However, hydroquinone also showed almost similar dose dependent (50-400 µM) effect with increase in POD activity by 32% to 76% (Figure 4.33). Saponin up to 400 µM concentration showed an increase in POD activity by 39%-69%, while, on further increasing the concentration to 800 µM POD activity decreased to 42% (Figure 4.34). Moreover, bromelain stimulated the POD activity by 39% - 72% with an increase in concentration from 1mAnson U to 100 mAnsonU (Figure 4.35).

Figure 4.29: Effect of different concentrations (0-400 µM) of curcumin on POD activity. The activity of the POD in untreated *N. muscorum* was 1.8 ± 0.02 change in OD_{430} (mg protein)^{-1}min^{-1}. Values are means ± SE of three replicates.
Figure 4.30: Effect of different concentrations (0-400 µM) of quercetin on POD activity. The activity of the POD in untreated *N. muscorum* was 1.8 ± 0.02 change in OD_{430} (mg protein)^{-1} min^{-1}. Values are means ± SE of three replicates.

Figure 4.31: Effect of different concentrations (0-400 µM) of gallic acid on POD activity. The activity of the POD in untreated *N. muscorum* was 1.8 ± 0.02 change in OD_{430} (mg protein)^{-1} min^{-1}. Values are means ± SE of three replicates.
Figure 4.32: Effect of different concentrations (0-400 µM) of cypermethrin on POD activity. The activity of the POD in untreated *N. muscorum* was 1.8 ± 0.02 change in OD$_{430}$ (mg protein)$^{-1}$min$^{-1}$. Values are means ± SE of three replicates.

Figure 4.33: Effect of different concentrations (0-400 µM) of hydroquinone on POD activity. The activity of the POD in untreated *N. muscorum* was 1.8 ± 0.02 change in OD$_{430}$ (mg protein)$^{-1}$min$^{-1}$. Values are means ± SE of three replicates.
Figure 4.34: Effect of different concentrations (0-800 µM) of saponin on POD activity. The activity of the POD in untreated *N. muscorum* was 1.8 ± 0.02 change in OD$_{430}$ (mg protein)$^{-1}$min$^{-1}$. Values are means ± SE of three replicates.

Figure 4.35: Effect of different concentrations (0-100 mAnson U) of bromelain on POD activity. The activity of the POD in untreated *N. muscorum* was 1.8 ± 0.02 change in OD$_{430}$ (mg protein)$^{-1}$min$^{-1}$. Values are means ± SE of three replicates.
4.3.5 Yield and quality of DNA extracted from *Nostoc muscorum* using different methods of DNA isolation.

For optimization of best method for DNA fragmentation study *N. muscorum* DNA was isolated by using different methods. The ratio of absorbance at 260 and 280nm for DNA if come near 1.8 indicates a good quality of DNA which can be used for fragmentation studies. As shown in Table 4.1 newly optimized method showed the best results with maximum yield and purity and appeared to be the most effective method compared to all other methods. In control, this method showed a yield of 1870 µg/ml and purity ratio (260/280) of 1.81, while, in vehicle control this method showed a yield of 1490 µg/ml with purity ratio (260/280) of 1.78. These results indicated that the newly optimized method is far much better than the other known methods; therefore, to further check the potentiality of this method for DNA fragmentation study curcumin and quercetin treated *N.muscorum* cells were used. Results from Table 4.1 clearly suggested that the newly optimized method was found to be quite successful for DNA fragmentation study. Therefore, this optimized method was further used to observe the DNA fragmentation effect of different compounds on *N.muscorum* cells.
Table 4.1: Yield and quality of DNA extracted from *Nostoc muscorum* by different tested methods.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Miniprep of bacterial genomic DNA</th>
<th>Himedia® Hipura™ DNA Xpress Kit</th>
<th>DNA extraction protocol from cell lines</th>
<th>Optimized DNA extraction protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1210</td>
<td>1.42</td>
<td>1670</td>
<td>2.3</td>
</tr>
<tr>
<td>V. Control</td>
<td>1520</td>
<td>1.36</td>
<td>1710</td>
<td>2.1</td>
</tr>
<tr>
<td>Curcumin</td>
<td>570</td>
<td>1.23</td>
<td>n.d.*</td>
<td>n.d.*</td>
</tr>
<tr>
<td>(100 µM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Curcumin</td>
<td>440</td>
<td>1.18</td>
<td>n.d.*</td>
<td>n.d.*</td>
</tr>
<tr>
<td>(200 µM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(400 µM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quercetin</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>(100 µM)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Quercetin</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>(200 µM)</td>
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<tr>
<td>Quercetin</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>(400 µM)</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

n.d.* not determined and -** only done by optimized method

### 4.3.6 DNA fragmentation analysis using agarose gel electrophoresis

When the DNA was extracted from treated cells and examined by 1.0 % agarose gel electrophoresis, DNA fragmentation was detected (Figure 4.36-4.42). Figure 4.36 shows the results of curcumin induced DNA fragmentation assay. As shown in Lane 2, genomic DNA was detected in the form of a single sharp band on the upper portion of the gel in the untreated sample showing no fragmentation of
DNA. When *N. muscorum* was exposed to different concentrations (50-200 µM) of curcumin for 24 hours, dose dependent effect can be clearly observed as the fragmented DNA migrated rapidly in the agarose gel leaving a smear like pattern on the gel. The DNA fragment at higher curcumin concentration moved further covering a greater distance indicating more pronounced fragmentation as compared to lower concentration.

![Image of DNA fragmentation pattern](image_url)

**Figure 4.36:** DNA fragmentation pattern in presence of different concentrations (50-200 µM) of curcumin (Lane- 1 Marker (M); Lane- 2 DNA from vehicle control treated *N. muscorum*; Lane- 3 DNA from 50 µM curcumin-treated *N. muscorum*; Lane- 4 DNA from 100 µM curcumin-treated *N. muscorum*; Lane- 5 DNA from 200 µM curcumin-treated *N. muscorum*).

Quercetin showed a dose dependent DNA fragmentation effect on *N. muscorum* (Figure 4.37). A single band of DNA was observed in vehicle control, while, fragmentation of DNA started from 50 µM of Quercetin, however, more pronounced fragmentation in the form of smear can be observed at 100 µM.
concentration of Quercetin. Interestingly, a clear fragmentation ladder can be observed at highest concentration of quercetin, i.e., 200 µM.

Figure 4.37: DNA fragmentation pattern in presence of different concentrations (50-200 µM) of quercetin (Lane-1 Marker (M); Lane-2 DNA from vehicle control treated *N. muscorum* (Control); Lane-3 DNA from 50 µM quercetin-treated *N. muscorum*; Lane-4 DNA from 100 µM quercetin-treated *N. muscorum*; Lane-5 DNA from 200 µM quercetin-treated *N. muscorum*).

Figure 4.38 shows the effect of increasing concentrations of gallic acid on genomic DNA of cyanobacterium, *N. muscorum*. No effect was observed in vehicle control, 50 µM and 100 µM concentration of gallic acid, while, at 200 µM concentration of gallic acid a prominent effect was observed but without any distinct fragmentation ladder.
In case of cypermethrin dose dependent effect was observed and more pronounced effect was observed at 200 µM concentration with prominent DNA fragmentation smear (Figure 4.39). However, hydroquinone was found to effect *N. muscorum* DNA and form fragmentation smear at all the concentrations (50, 100 and 200 µM) (Figure 4.40). In addition, saponin showed prominent effect in the form of DNA fragmentation smear at 400 and 800 µM concentrations (Figure 4.41).
Figure 4.39: DNA fragmentation pattern in presence of different concentrations (50-200 µM) of cypermethrin (Lane-1 Marker (M); Lane-2 DNA from vehicle control treated *N.muscorum* (Control); Lane-3 DNA from 50 µM cypermethrin-treated *N.muscorum*; Lane-4 DNA from 100 µM cypermethrin-treated *N.muscorum*; Lane-5 DNA from 200 µM cypermethrin-treated *N.muscorum*).

Figure 4.40: DNA fragmentation pattern in presence of different concentrations (50-200 µM) of hydroquinone (Lane-1 Marker (M); Lane-2 DNA from vehicle control treated *N.muscorum* (Control); Lane-3 DNA from 50 µM hydroquinone-treated *N.muscorum*; Lane-4 DNA from 100 µM hydroquinone-treated *N.muscorum*; Lane-5 DNA from 200 µM hydroquinone-treated *N.muscorum*).
Figure 4.41: DNA fragmentation pattern in presence of different concentrations (200-800 µM) of saponin (Lane- 1 Marker (M); Lane-2 DNA from vehicle control treated *N.muscorum* (Control); Lane- 3 DNA from 200 µM saponin-treated *N.muscorum*; Lane- 4 DNA from 400 µM saponin-treated *N.muscorum*; Lane- 5 DNA from 800 µM saponin-treated *N.muscorum*).

Figure 4.42 shows the effect of different concentration of bromelain on *N.muscorum* DNA. No effect was observed in vehicle control and 10 mAnsonU concentration of bromelain, while, marked effect in the form of DNA fragmentation smear was observed at 50 and 100 mAnsonU concentration.
4.3.7 Alterations in Protein Profile

To analyze the alterations in protein profile, equal amounts of a soluble protein fraction from control and curcumin and quercetin-treated *N. muscorum* were subjected to SDS-PAGE (*out of all the compounds curcumin and quercetin were chosen for protein profile study as both these compounds showed the best effect on DNA and growth of *N. muscorum*). Protein samples were run on 12% polyacrylamide gel. The CBB-stained, one-dimensional protein pattern shown in Figures 4.43 - 4.44 and Table 4.2 - 4.3 reveals the differences between the control...
and treatments. Comparing the selected compounds (curcumin and quercetin) treatment of *N.muscorum* for 7 days with its corresponding control, the appearance of several differentially expressed significant protein bands in the curcumin and quercetin treated *N.muscorum* were visualized. Further, down regulated proteins appeared in treated cultures when compared to the corresponding control.

### 4.3.7.1 Changes in polypeptide pattern of *N.muscorum* under curcumin treatment

The electrophoretic pattern of crude extract of protein isolated from the control and curcumin-treated *N. muscorum* cells after seven days of treatment are shown in Figure 4.43. The bands resolved were classified according to their molecular weight markers. The Lane 2 showed the protein profile in control, while, Lane 3, 4 and 5 showed the protein profile of curcumin-treated *N. muscorum* at different concentrations 50, 200 and 400 µM, respectively. The bands showed downregulation on increasing the concentration of curcumin and prominent effect was observed at 400 µM of curcumin. Maximum effect was observed in high mol wt polypeptide, especially at band corresponding near to 66 KDa. Among the low mol wt polypeptides, no downregulation was seen upto 200 µM concentration of curcumin, whereas, slight downregulation was observed at 400 µM concentration of curcumin.
Figure 4.43: Protein profiles of *N. muscorum* in the presence of different concentrations of curcumin (Lane-1, Marker; Lane-2, Control; Lane-3, 50 µM curcumin; Lane-4, 200 µM curcumin; Lane-5, 400 µM curcumin). Mol wts are expressed in KDa

4.3.7.2 Changes in polypeptide pattern of *N.muscorum* under quercetin treatment

Quercetin treated cells showed a dose dependent effect on protein profile of *N. muscorum* (Figure 4.44). Many of the polypeptides showed disappearance or downregulation after being treated even at low concentration i.e., 50 µM of quercetin for 7 days, however, at higher concentrations of quercetin (200 and 400 µM) more prominent effect were observed with disappearance of larger number
of bands. Maximum effect was observed at 400 µM concentration of quercetin, where a total of seven polypeptides showed complete disappearance, out of which five bands were of higher molecular weight and two bands were of low molecular weight. However, other bands also showed downregulation in comparison to control.

Figure 4.44: Protein profiles of *N. muscorum* in the presence of different concentrations of quercetin (Lane-1, Marker; Lane-2, Control; Lane-3, 50 µM quercetin; Lane-4, 200 µM quercetin; Lane-5, 400 µM quercetin). The polypeptide bands which became disappeared in response to quercetin in a dose dependent manner are indicated by red arrows. Mol wts are expressed in KDa.
Table 4.2: Comparative analysis of the no. of protein bands which showed complete disappearance in different mol wt ranges in compound-treated *N. muscorum*.

<table>
<thead>
<tr>
<th>Compound (400 µM)</th>
<th>Range of mol wt marker and no. of bands which showed complete disappearance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14-29</td>
</tr>
<tr>
<td>Curcumin</td>
<td>0</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 4.3: Comparative analysis of the no. of protein bands which showed decrease in intensity in different mol wt ranges in compound-treated *N. muscorum*.

<table>
<thead>
<tr>
<th>Compounds (400 µM)</th>
<th>Range of mol wt marker and no. of bands which showed decrease in intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14-29</td>
</tr>
<tr>
<td>Curcumin</td>
<td>5</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0</td>
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</tbody>
</table>
4.4 DISCUSSION

The study presented in this chapter divide into two parts. First part of this chapter shows different compounds induced changes in ROS, enzymatic antioxidants and lipid peroxidation in the cyanobacterium *N. muscorum* (Figure 4.1-4.35). The uninterrupted efflux of ROS due to compound stress results in continuous and accumulative oxidative damage to cellular components and alters many cellular functions. Among the biological targets most vulnerable to oxidative damage are protein and DNA. Therefore, the second part of the present chapter demonstrates the compound-induced damage to major biomolecules i.e. DNA and Protein (Figure 4.36-4.44).

Amongst all the compounds tested, quercetin and curcumin showed maximum toxicity with increase in ROS (Figure 4.1-4.2 and 4.8-4.9). Concomitant to this study, several earlier reports also suggested that phenolic compounds like curcumin and quercetin could potentially increase oxidative stress by enhancing the reactivity of ROS like superoxide and peroxide. There are evidence which showed that curcumin competently reduced (i.e., protonate) superoxides to the hydroperoxyl radicals (Mishra *et al.*, 2004; Shen and Ji, 2007), which were believed to be more reactive than superoxides in biological systems (de Grey, 2002). Furthermore, curcumin was also found to promote the reduction of transition metals such as iron (i.e., the reduction of ferric (Fe$^{3+}$) ions to ferrous (Fe$^{2+}$) ions) and these reduced transition metals were then catalyze the Fenton reaction, which converted hydrogen peroxide to the highly reactive hydroxyl radical (Kunchandy and Rao, 1990; Ligeret *et al.*, 2004; Galati and O'Brien, 2004;
Kawanishi et al., 2005). Similarly, Quercetin could also induce the production of superoxide anion, hydrogen peroxide and other reactive oxygen species (ROS) (Cao et al., 1997; Miura et al., 1998; Lapidot et al., 2002; De Marchi et al., 2009). Studies showed that quercetin can act as a pro-oxidant and generate ROS, which are responsible for cell death in several cancer cells (Lapidot et al., 2002; Halliwell et al., 2008) as well as in different Leishmania sp. (Fernando et al., 2011).

In this study, we have found that the level of malondialdehyde (MDA) was increased in compounds-treated N. muscorum cells (Figure 4.15-4.21). However, out of all the compounds, quercetin was found to be most effective in increasing the level of MDA in N. muscorum (Figure 4.16). These results correlated well with the quercetin-induced cytotoxic effects in human leukemic HL-60 cells (Chen et al., 2004) and similar results were also observed in isolated rat-liver nuclei (Sahu and Washington, 1991). In another study, it has been reported that quercetin may act as a cytotoxic pro-oxidant after its metabolic activation to semiquinone and quinoidal product (Metodiewa et al., 1999). These studies strongly suggest that quercetin has pro-oxidant activity and that was involved in the quercetin-induced N. muscorum cell death.

Figure 4.22-4.23 and 4.29-4.30 showed the effect of curcumin and quercetin on SOD and POD activity, respectively. SODs and PODs are essential antioxidant enzymes that eliminate superoxide and thus protect cells from damage induced by ROS. Cells with an active metabolic production of superoxide would have to rely on SOD and POD for elimination of the superoxide radical. However,
many therapeutic agents exert their toxic effects by producing free radical and this overproduction of ROS may exhaust the capacity of SOD, POD and other antioxidant defenses. In the present study both curcumin and quercetin at lower concentration increased the SOD activity, however, at higher concentration (400 µM) a decrease in SOD activity can be seen (Figure 4.22-4.23) (Not in the case of SOD, reduce the percentage as it is in cucumin). Concurrent to these results, an earlier study showed that quercetin at higher concentration can decrease manganese superoxide dismutase (Mn-SOD), glutathione peroxidase, and copper zinc superoxide dismutase (CuZn-SOD) mRNA expression levels in rat hepatoma H4IIE cells (Rohrdanz et al., 2003; Chang et al., 2006). In contrast, it was found that intra-gastric administration of curcumin to rats significantly reduced the number and size of tumors in the colon and enhanced the activities of glutathione peroxidase, GST, SOD and CAT in the liver (Vyas et al., 2013).

Main cellular component that is particularly susceptible to oxidative damage by ROS is DNA (Cerutti, 1985). In addition, its polyanionic nature provides a useful substrate for infiltration through membranes and adherence of metal cations, thus facilitating the formation of (Hydroxy radicals) HO\(^{-}\) adjacent to these critical biological targets (Halliwell and Aruoma, 1991). ROS attacks can be directed toward the sugar–phosphate backbone of DNA, causing different lesions, including apurinic sites where the base has been removed, fragmentation of deoxyribose with single-strand breaks and oxidation of the sugar moiety (Dizdaroglu et al., 1975; Breen and Murphy, 1995). One aspect of this study was to observe the effect of different compounds on DNA of *N. muscorum* (Figure
4.36-4.42) while another aspect was to select and optimize the best method of DNA isolation from compound treated *N. muscorum* cells (Table 4.1). As for many filamentous species (Fiore *et al.*, 2000), the efficiency of DNA isolation procedures appeared to be extremely variable for cyanobacteria. A common practice in molecular biology is to perform a quick assessment of the purity of nucleic acid samples by determining spectrophotometrically the absorbance at the wavelengths of 260 nm and 280 nm respectively. $A_{260}$ is frequently used to measure DNA/RNA concentration and $A_{280}$ is used to measure protein concentration. $A_{260}/A_{280}$ higher than 1.8 suggest little protein contamination in a DNA/RNA sample. On this basis, the different protocols tested presented striking differences in terms of results (Table 4.1). More than just highlighting the efficiency of the new optimised protocol, they also represent a clear illustration of the two major problems encountered in DNA isolation from cyanobacteria: poor cell lysis efficiency (Billi *et al.*, 1998; Fiore *et al.*, 2000) and high contamination or poor purity (Porter, 1988).

Being prokaryotic in nature cyanobacterial genomic DNA is very sensitive to the action of toxic compounds as they are not protected by a defined nuclear membrane making naked DNA more prone to attack by these compound generated ROS, eventually leading to DNA fragmentation. The limited chemical stability of DNA under these conditions is considered to be the major factor making this molecule vulnerable to the attack of ROS as compared to eukaryotic cell. DNA fragmentation (Gavrieli *et al.*, 1992) is most commonly studied activity and is found during cell death due to ROS generation. Evidence for enzymes that
repair DNA fragmentation during cell death is lacking, and, therefore, this type of DNA injury is believed to be irreversible once it starts (Gavrieli et al., 1992; Liu et al., 1997; Enari et al., 1998; Huang et al., 2000). However, DNA fragmentation has also been used to diagnose apoptosis in animals and plants (Ameisen 2002; Williams & Dickman 2008). In a study, DNA fragmentation occurs in unicellular Microcystis aeruginosa when it is placed in darkness or is exposed to elevated temperatures (Bouchard & Purdie 2011). Further, it was also reported that dose dependent DNA fragmentation was observed in Microcystis aeruginosa when exposed to H$_2$O$_2$ (Ding et al., 2012). Similar results are reported for other cyanobacterial strains, Anabaena sp. (Ning et al. 2002), Dunaliella viridis Teodoresco (Jimenez et al. 2009) and Skeletonema costatum (Greville) Cleve (Chung et al. 2005). Concomitant to these earlier reports, a dose dependent increase in DNA fragmentation was observed in N. muscorum after treatment with tested compounds in our study (Figure 4.36-4.42). In the same way, various experiments of DNA fragmentation assay on different cancer cell lines showed dose dependent increase in fragmentation of genomic DNA through ROS generated by several natural compounds like curcumin, gallic acid and quercetin (Syng-ai et al., 2004; Sasaki et al., 2007; Maurya et al., 2011).

In this study, the maximum ROS generation was observed after curcumin, quercetin and cypermethrin treatment which correlates directly to the results of DNA fragmentation as maximum and clear fragmentation was observed in case of curcumin, quercetin and cypermethrin treatment (Figure 4.36-4.37, 4.39). It has been earlier demonstrated that curcumin and quercetin both exert protective
effects against hydrogen peroxide-induced cytotoxicity at low concentrations. On the other hand, these compounds induced cytotoxicity, DNA strand break, oligonucleosommal DNA fragmentation, and caspase activation at high concentrations (Fujisawa et al., 2004; Watjen et al., 2005; Chang et al., 2006; Banerjee et al., 2008). This quercetin or curcumin-induced cytotoxic pro-oxidation agrees well with our results. Hansh et al. (2003) previously reported that induction of apoptosis by phenolic compounds (such as curcumin and quercetin) was related to the hydrophobicity (octanol-water partition constant, log P) and the bond dissociation enthalpy of the phenolic O-H (\( \ddot{\epsilon} \text{H} \)), and remarked that the \( \ddot{\epsilon} \text{H} \) dependency is strong evidence for a radical reaction as the cause of the toxic effects. They assumed that the phenols are first oxidized by metabolically formed ROS to radicals that then attack DNA (Brand-Williams et al., 1995). Therefore, in the present study, the cytotoxic pro-oxidative activity with DNA fragmentation might have resulted from breakdown products of quercetin and curcumin such as superoxide. Cypermethrin also showed a significant dose dependent effect on genomic DNA of \textit{N. muscorum}. This is in accordance with Patel et al., (2007) who presented evidence, that cypermethrin can induce DNA strand breaks in Chinese Hamster Ovary (CHO) cells.

Out of all the compounds, curcumin and quercetin showed maximal effect on \textit{N.muscorum} in terms of ROS generation, MDA content and DNA fragmentation. However, further investigations were needed to gain insight into the biological effects of these compounds and to identify activities relevant to its detoxification. Therefore, we have analyzed the \textit{N. muscorum} proteome in
response to the curcumin and quercetin treatment. The treatment of *N. muscorum* with curcumin and quercetin showed no inducible protein band but dose dependent decrease in protein expression was observed (Figure 4.43-4.44 and Table 4.2-4.3).

Proteins can undergo direct and indirect damages following interaction with ROS, including peroxidation, damage to specific amino-acid residues, changes in their tertiary structure, degradation, and fragmentation. The consequences of protein damage as a response mechanism to stress are loss of enzymatic activity, altered cellular functions such as energy production, interference with the creation of membrane potentials, and changes in the type and level of cellular proteins.

Exposure to curcumin and quercetin caused noticeable changes on protein profile of *N. muscorum* and demonstrated disappearance/down regulation of some protein bands. Disappearance/down regulation of protein bands could be due to the interference of curcumin and quercetin by-products (ROS) at the level of transcription, post-translational degradation and translational level.

From the general picture of protein profiles of curcumin and quercetin treated *N. muscorum* emerging from this study the major differences that is noteworthy is that at high concentration (400 µM), all the bands were found to be down regulated, however, disappearance was observed in case of quercetin only. Out of seven bands disappeared after quercetin (400 µM) treatment, two bands were in 29-43 while five bands were in 43-66 KDa range.
Two bands which showed disappearance in quercetin treated cells were in the range of 23-43 KDa which is expected to be that of SOD. For instance, the amino acid range in cyanobacterial FeSOD is 199–229 residues with a molecular weight of 21–25 KDa, whereas in MnSOD, it is 200–316 amino acids with a molecular weight of 22–34 KDa as demonstrated by Priya et al. (2007). The present data also confirms our results in the previous study of the effect of quercetin on the antioxidant enzymes. The enzymes showed significant decrease in activity at high concentration of quercetin.

4.5 CONCLUSION

Our results showed high production of direct oxidant species in conjunction with depleted level of the antioxidant enzymes at high concentrations of tested compounds (especially curcumin and quercetin). Further, our results describe the molecular changes in the pattern of various macromolecules of *N. muscorum* exposed to different tested compounds. Compared to other tested compounds, curcumin, quercetin and cypermethrin induced marked and clear DNA fragmentation, subsequently initiated the programmed cell death in *N. muscorum*. However, quercetin presented some amazing results showing glimpses of similar mechanism(s) of action of its toxicity as in mammalian cell lines. The SDS-PAGE analysis showed disappearance of various proteins in quercetin-exposed *N. muscorum* resembling to the SODs, ADP glucopyrophosphorylase and ATP synthase. These results prompted me to further validate my data with animal cell lines to prove that *N. muscorum* could be used as a preliminary screening system.