

**Adaptation of marine derived fungi, *Chaetomium globosum* (NIOCC 36), under alkaline stress using antioxidant properties**

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**Abstract:**

The fungi are an exceptionally useful model in elucidating the cell functions under extreme conditions (temperature, salinity, pH values) and mechanisms underlying adaptation to those extreme environmental conditions. Here, *Chaetomium globosum* was examined for its adaptation mechanism under alkaline stress using antioxidant properties. The aqueous extracts of *C. globosum* exhibited different levels of antioxidant activity in all the *in vitro* tests such as  $\alpha,\alpha$ -diphenyl- $\beta$ -picrylhydrazyl (DPPH), Hydrogen peroxide, Hydroxyl Radical Scavenging Assay (HRSA), Ferric reducing/antioxidant power (FRAP) assay, Metal chelating assay and  $\beta$  – carotene – linolic acid model system. The antioxidant capacity of marine fungi showed an increase in activity with increase in stress. In addition, the production of intra and extracellular antioxidant enzymes of the fungus at various pH stresses were analyzed and discussed for their possible role in the stress mechanism. The present study elucidates that the scavenging activity is one of the protective mechanisms developed to avoid the deleterious effect of stress. Furthermore, the *in vitro* assays also clearly indicate that fungal extracts are significant source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stresses.

**Key words:** antioxidants; free radical scavenging; marine fungi; pH stress;

## 1. Introduction

A variety of physiological signals and stresses may cause translation level controls to direct competent tissues of microorganisms to undertake specific differentiation processes [1]. The general theory of microbial eukaryotic cell differentiation postulates that this phenomenon is triggered by a hyperoxidant state, which induces the cell to isolate itself from molecular oxygen [1, 2]. A hyperoxidant state is an unstable pro-oxidant (oxidative) state in which the amount of oxygen-free radicals inside the cell exceeds the cell's capacity to neutralize them. Although not all of them are radicals, the term "oxygen-free radicals" has been used to refer to all species of oxygen (reactive oxygen species, ROS) that are more reactive than  $O_2$  in its ground state or triplet state ( $^3O_2$ ). These are dioxygen in its excited state singlet forms ( $^1O_2$ ) and the partially reduced forms of oxygen, that is superoxide radical ion and its protonated form ( $O_2^-$  and  $HO_2^-$ , respectively), hydroxyl radical ( $HO^\cdot$ ) and hydrogen peroxide ( $H_2O_2$ ) [1].

It has been known that ROS influence molecular and biochemical processes and signal transduction pathways, which affect proliferation, differentiation, and death in fungi, and in a variety of other organisms [3]. The fungal cell differentiation is related to different growth strategies, various forms of resistance to adverse environmental conditions, different ways of reproduction and differentiation. The physical factors that mostly influence the marine fungi are salinity and pH, low water potential, high concentrations of sodium ions, low temperature, oligotrophic nutrient conditions and high hydrostatic pressure (the last three parameters being unique to the deep-sea environment). The research on abiotic stress still emphasizes NaCl as main subject and it is deeply developing towards various aspects such as  $Na^+$  metabolism, molecular biology of salt-resistance genes, and salt stress signal transduction. There are only a few reports about stress by alkali. There have been studies about alkaline soil [4] and alkaline salt stress [5], and also it has been clearly demonstrated that the existence of alkali stress is more severe than salt stress [6]. Ambient pH is thus one component that plays a major role in influencing growth, physiology and differentiation of many microorganisms. The diverse niches that fungi occupy vary greatly with respect to their pH, for example, in the marine water, mangrove wood litters alkaline soils etc. This pH adaptation presumably addresses the impact of pH variation upon protein activities, nutrient availability and the proton gradient across the plasma membrane [7]. One of the central problems associated with growth in alkaline medium is to maintain the cytoplasmic pH at a level compatible with metabolic functions, i.e., more acidic than that of the external medium. To solve this problem, the cells have to evolve efficient mechanisms to sustain the pH gradient across the cytoplasmic plasma membrane [8].

Fungus *Aspergillus nidulans* is the first eukaryotic organism whose adaptation to ambient pH was characterized. This fungus can grow in a wide pH range from 2.5 to 9.0 and apparently disposes of a regulatory mechanism of controlling gene expression according to the circumstances [9; 10], and the genes involved in the pH regulatory system of *A. nidulans* was found homologues with other major groups of fungi [11]. Further, in the last few years the alkaline response of the eukaryotic model organism such as *Saccharomyces cerevisiae* has been studied quite thoroughly, mainly through analysis of the transcriptional remodeling or the identification of mutants that grow poorly under such conditions [12]. In addition, it was also reported that the phenotypic effects including part of the transcriptional response derived from exposure to high pH must be result of an oxidative stress situation [13].

Fungi were reported for using both the enzymatic and non-enzymatic defense systems against oxidative injury caused by ROS due to external factors [14]. But there was no study on the eukaryotic organism for its adaptation to ambient pH using antioxidant properties. Thus, in the present study, specific adaptations of the filamentous marine derived fungus, *C. globosum*, which grows in a wide range of pH from 4 to 12 [15] was investigated for its adaptive mechanisms under different pH conditions using antioxidant properties. *C. globosum*, the type species of the genus, *Chaetomium* (Phylum *Ascomycota*, Class *Sordariomycetes*) can be isolated easily from decaying plant material, seeds and other cellulosic substrates. It is the most frequently isolated and cosmopolitan genus of over 150 species [16]. It has also been reported that some isolates of *C. globosum* produce antibiotics that can suppress plant diseases like damping-off of sugar beet [17], leaf spot disease of corn, rice blast, sheath blight of rice and tomato wilt [18]. *C. globosum* has also been proven to be an important source of novel bioactive compounds (like ergosterol, ergosteryl palmitate, chrysophanol, chaetoglobosin C, alternariol monomethyl ether, echinuline, iso chaetoglobosin D), further chaetomanone and echinulin has been shown to have activity towards *Mycobacterium tuberculosis* [19] and chaetopyranin, shown to exhibit moderate to weak cytotoxic activity toward several tumor cell lines [20]. Some of the above compounds are also noted for their antioxidant properties for example chaetopyranin for its DPPH radical scavenging property [20]. In addition, the whole genome of *Chaetomium globosum* sequence project is in progress with part of the Broad Institute of Harvard and MIT Fungal Genome Initiative (<http://www.broadinstitute.org/>). Therefore, the present study was conducted using marine derived fungus, *C. globosum*, as the candidate species with the objective to assess the pH responses of the fungus under alkaline stress using antioxidant defense components.

## **2. Materials and methods**

### **2.1. Chemicals**

Sodium bicarbonate, Folin–Ciocalteu reagent, Gallic acid, Sodium nitrite (NaNO<sub>2</sub>), Aluminium chloride (AlCl<sub>3</sub>), Sodium hydroxide (NaOH), Quercetin, 1, Ethanol, xylenol orange, Butylated Hydroxy Anisole (BHA), Butylated Hydroxy Toluene (BHT) were obtained from Merck, Mumbai, India.  $\alpha,\alpha$ -diphenyl- $\beta$ -picrylhydrazyl (DPPH) were purchased from Sigma Aldrich (Steinheim Germany). H<sub>2</sub>O<sub>2</sub>, HPLC grade methanol, Ammonium ferrous sulphate, deoxy ribose, Ferric chloride, Ethylenediaminetetraacetic acid (EDTA), Ascorbic acid, Thiobarbituric acid (TBA), Trichloroacetic acid (TCA), Potassium hexacyanoferrate, Ferric chloride, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), ferrous sulphate, Ferrozine, Chloroform, Tween 80 were obtained from SD fine chemicals, India.  $\beta$ -carotene and Linoleic acid were purchased from HiMedia, Mumbai, India. All other reagents were of analytical grade.

### **2.2. Isolation of alkaline stress tolerant fungi**

The isolation of stress tolerant fungi was done as described earlier [15]. Briefly, the wood pieces were collected from mangrove swamps (Sal, Chorao and Zuari in Goa, India), washed with sterile sea water and were incubated ( $25 \pm 2^\circ\text{C}$ ) in sterile polythene bags. The wood pieces were screened under a stereomicroscope for the fungal structures at different intervals of incubation (2, 8, 16 and 32 weeks) and isolated using a sterile needle and transferred to Malt extract agar (MEA) medium (Himedia, Mumbai, India) containing 10,000 units of sodium benzyl penicillin and 0.05 g of streptomycin sulfate per 100 ml of medium to inhibit bacterial growth. The isolated fungus was inoculated at the centers of MEA medium plates of different pH viz., 4, 6, 8, 10, and 12. The plates were made up in triplicates and incubated at room temperature. The growth of the mycelium was noted on each day and the time course of growth was plotted.

### **2.3. Identification of fungi**

The fungus was identified based on the colony morphology and microscopical features using standard taxonomic keys [16] as described earlier [15]. In addition, the sequence data derived from the fungal strain using an automated multicapillary DNA sequencer (ABI Prism 3130xl Genetic analyzer, Applied Biosystems, Foster City, CA, USA), has been submitted and deposited at GenBank under accession number GQ412081.

## **2.4. Total antioxidant and antioxidant activity assay**

### **2.4.1. Sample preparation**

The fungus grown in the MEA broth for 6-7 days at different pH conditions was separated into mycelial mat and culture filtrate by filtration with Whatman No 1 filter paper (9 cm) under vacuum. Culture filtrate thus obtained was further centrifuged at 4000 rpm for 10-15 minutes to separate the remaining mycelial mass from the filtrates. The clear final culture filtrate sample was stored at 4°C until use within 24 h. The mycelial mat was extracted with ethyl acetate to get the crude extract.

### **2.4.2. Determination of total phenolic content (TPC)**

The total soluble phenolic content was estimated for each extract using a modified version of the Folin assay of Vatter and Shetty [21], and gallic acid was used as the phenolic standard. Briefly, 100 µl of sample and 2 ml sodium bicarbonate mix were incubated at room temperature for 2 min followed by addition of 100 µl of Folin-Ciocalteu reagent and incubated in dark for 30 min. The samples were vortexed and assayed for absorbance at  $\lambda = 725$  nm using a spectrophotometer. Gallic acid 1mg/ml was used as standard and standard curve was obtained using various concentrations of gallic acid.

### **2.4.3. Determination of total flavonoid content**

The total flavonoid content of the extract was estimated by colorimetric method described by Zhishen et al. [22] with some modifications. The extract (100µl) was mixed with 300 µl distilled water and 30µl of 5% NaNO<sub>2</sub>. After 5 min 30 µl of 10% AlCl<sub>3</sub> was added and mixed well. The mixture was incubated for 5 min and 0.2 ml of 1 mM NaOH was added. Finally, the volume was made to 1 ml using distilled water and mixed well. The absorbance was measured at 510 nm. Quercetin was used to calculate the standard curve. The concentration versus absorbance was plotted and the slope value was determined.

### **2.4.4. DPPH scavenging assay**

The fungal extracts containing antioxidants was aliquoted into different concentrations (50 – 200 µg/ml) to determine its ability to scavenge 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals using the method of Yildirim et al. [23]. DPPH solution (1 mM DPPH radical solution in 95% ethanol) of 800 µl was mixed with 200 µl of sample extract, vortexed well, and then incubated for 30 min at

room temperature in dark. After 30 min incubation, the samples were poured into microcentrifuge tubes and centrifuged for 5 min at 13,500 rpm (at room temperature). Then, the absorbance of each sample at  $\lambda = 517$  nm was measured and 1 ml of 95% EtOH was used as a control. Butylated Hydroxy Anisole (BHA) and Butylated Hydroxy Toluene (BHT) were used as reference compounds. The antioxidant activity is given as percent (%) DPPH scavenging, calculated using the formula:  $[(\text{control absorbance} - \text{extract absorbance}) / (\text{control absorbance}) \times 100]$ .

#### **2.4.5. Hydrogen peroxide scavenging assay**

The hydrogen peroxide scavenging activity was determined using Long et al. [24] method with minor changes. An aliquot of different concentration of sample (50 – 200  $\mu\text{g/ml}$ ) and 50 mM  $\text{H}_2\text{O}_2$  was mixed in the ratio of 1:1 (v/v) and incubated for 30 min at room temperature. After 30 min, 90  $\mu\text{l}$  of  $\text{H}_2\text{O}_2$  – sample solution was mixed with 10  $\mu\text{l}$  HPLC grade methanol and 0.9 ml of previously prepared FOX reagent (Ferrous Oxidation Xylenol (FOX) reagent was prepared by mixing 9 volumes of 4.4 mM BHT dissolved in HPLC grade methanol mixed with 1 volume of 1 mM xylenol orange and 2.56 mM ammonium ferrous sulphate in 0.25 M  $\text{H}_2\text{SO}_4$ . It was prepared before the assay) was added. The reaction mixture was mixed well and incubated at room temperature for 30 min. The reaction mixture without the sample was used as blank. The absorbance of ferric xylenol orange complex was measured at 560 nm. The scavenging activity leads to the inhibition of ferric xylenol orange complex formation and the scavenging activity was calculated based on the formula: % Hydrogen peroxide scavenging =  $[(\text{control absorbance} - \text{extract absorbance}) / (\text{control absorbance}) \times 100]$ .

#### **2.4.6. Hydroxyl Radical Scavenging Assay (HRSA)**

In this assay the hydroxyl radical was produced by the  $\text{Fe}^{3+}$  ascorbate EDTA  $\text{H}_2\text{O}_2$  system i.e. fenton reaction [25]. The reaction was performed in phosphate buffer (20 mM, pH 7.4) containing 2-deoxyribose (2.8 mM),  $\text{FeCl}_3$  (100  $\mu\text{M}$ ),  $\text{H}_2\text{O}_2$  (1 mM), EDTA (100  $\mu\text{M}$ ) and various concentrations (50–200  $\mu\text{g/ml}$ ) of sample of the test sample and reference compound. The ascorbic acid (100  $\mu\text{M}$ ) was added at last and incubated at 37  $^\circ\text{C}$  for 1 hr. The reaction volume of the above said mixture was 1 ml. After incubation 1 ml of 2.8 % TCA and 1 ml of 1 % aqueous TBA was added and the mixture was incubated at 90  $^\circ\text{C}$  for 15 min to develop color. The absorbance was measured at 532 nm after cooling. The mannitol was used as reference compound. The percentage of scavenging was determined as, % scavenging =  $[1 - (\text{sample absorbance} / \text{blank absorbance}) \times 100]$

#### **2.4.7. Ferric reducing/antioxidant power (FRAP) assay**

The FRAP assay was performed for the samples and reference compound of various concentrations (50 – 200 µg/m) based on Benzie and Strain [26]. The FRAP reagent (2.5 ml of 20 mmol/l 2,4,6-tri(2-pyridyl)-1,3,5-triazine TPTZ solution in 40 mmol/l HCl plus 2.5 ml of 25 mmol/l FeCl<sub>3</sub>.6H<sub>2</sub>O and 25 ml of 0.3 mol/l acetate buffer (pH 3.6)) was prepared freshly and incubated at 37 °C. To the 900 µl of FRAP reagent 90 µl of distilled water was added and 30 µl of the test sample was added. The mixture was incubated at 37 °C for 30 min. in water bath. After incubation, the absorbance was measured at 593 nm using spectrophotometer. The methanolic solution of known Fe (II) (FeSO<sub>4</sub>) was prepared for calibration curve. BHT was used as the reference compound. The reducing power was determined based on the amount of reduced product of Fe (II) formed and it is determined from the calibration curve.

#### **2.4.8. Metal chelating assay**

The metal chelating activity was estimated by the method of Dinis et al. [27] with slight modification. The reaction was performed in HEPES buffer (20 mM, pH 7.2). Various concentrations (50–200 µg/ml) of samples were mixed with solution of 12.5 µM ferrous sulphate solution. The reaction was initiated by the addition of 75 µM ferrozine and the mixture was shaken vigorously and incubated for 20 min at room temperature. After incubation the absorbance was measured at 562 nm. EDTA was used as the reference compound and the percentage chelating capacity was calculated as; % Chelating activity =  $[(A_0 - A_1)/A_0] \times 100$  where, A<sub>0</sub> = Absorbance of the blank; A<sub>1</sub> = Absorbance of the sample.

#### **2.4.9. Antioxidant assay using 1 β – carotene – linoleate model system**

The antioxidant activity of the extract was evaluated by β-carotene–linoleate model system [28] for which, β-carotene–linoleate emulsion was prepared freshly. Aliquots of 2.8 ml of emulsion were transferred into assay tubes containing various concentration of extract (0.2 ml). As soon as the emulsion was added the initial absorbance was measured at 470 nm for each reaction and the tubes were incubated at 50 °C in a water bath for 2 hr. The absorbance after 2 hr incubation was also measured. A blank devoid of β-carotene was prepared for the background subtraction. The same procedure was used for synthetic antioxidant BHT (reference compound) at various concentrations (50–200 µg/ml). The antioxidant activity was calculated using the following equation: Antioxidant activity =  $(\beta\text{-carotene content after 2 hr} / \beta\text{-carotene content at initial stage}) \times 100$ .

## **2.5. Antioxidant enzyme assay**

Antioxidant enzyme was assayed in culture liquid filtrates (extracellular) and in the mycelium (intracellular). The mycelium was separated from the medium on filter paper. Samples of mycelium were washed with distilled water and dissolved in 50 mM phosphate buffer (pH 7.0). The cells were destroyed by treatment with quartz sand in a porcelain mortar for 4–5 min and suspended in the buffer. The homogenate was centrifuged at 4000 rpm for 30 min.

### **2.5.1. Catalase assay**

The catalase assay was performed based on the protocol described earlier [29]. The enzymatic activity of catalase was assayed based on the principle that the catalase enzyme degrades hydrogen peroxide to water and oxygen and hence the absorbance is reduced. The rate of change of absorbance was measured at 240 nm. The assay mixture was prepared by mixing 2.9 ml of the substrate solution i.e. 0.036% H<sub>2</sub>O<sub>2</sub> and 100 μl of the sample solution. The blank was prepared using phosphate buffer instead of the sample. The decomposition of the H<sub>2</sub>O<sub>2</sub> (substrate) was measured as the reduction in absorbance at 240 nm. One unit is defined as the decomposition of 1 μM of H<sub>2</sub>O<sub>2</sub> per minute per milligram of protein at pH 7.0 at 25°C. The rate of disappearance of H<sub>2</sub>O<sub>2</sub> is followed by observing the rate of decrease in the absorbance at 240 nm.

### **2.5.2. Guaiacol peroxidase assay**

Based on the procedure described earlier [30], the rate of decomposition of hydrogen peroxide by peroxidase, with guaiacol as hydrogen donor, was determined by measuring the rate of colour development spectrophotometrically at 436 nm. The assay mixture for peroxidase assay comprised of 2.8 ml of 0.1 M phosphate buffer (pH 7), 50 μl of 0.018 M guaiacol and 50 μl of substrate (H<sub>2</sub>O<sub>2</sub>, Dilute 0.1 ml 30% hydrogen peroxide with distilled water to 120 ml. Store the solution on ice and prepare fresh). The assay mixture was pipetted directly into the cuvette and 100 μl of sample was added. The rate of increase in absorbance per minute was observed at 436 nm. A blank was prepared without the sample and used for background subtraction. One unit is defined as the amount of enzyme which catalyses the conversion of one micromole of hydrogen peroxide per minute.



### **2.5.3. Glutathione S Transferase assay:**

The Glutathione S Transferase activity was measured by observing the conjugation of 1-Chloro, 2, 4-Dinitrobenzene (CDNB) with reduced glutathione (GSH). This is done by measuring an increase in absorbance at 340 nm. One unit of enzyme will conjugate 10 nmol of CDNB with reduced glutathione per minute at 25°C. This assay was performed based on the procedure described by Mannervik [31]. The assay cocktail was prepared prior to the assay. For each reaction the assay cocktail composed of 980 µl of Phosphate buffer saline (PBS) pH 6.5, 10 µl of 100 mM CDNB (dissolved in ethanol), and 10 µl of 100 mM glutathione. For each sample and a blank, 900 µl of assay cocktail was added directly into the cuvette and 100 µl of PBS buffer was added in case of blank to auto zero the instrument. For test reaction sample was added instead of PBS and the rate of increase in absorbance was measured at 340 nm.

### **2.6. Statistical analysis:**

All data are given as the mean  $\pm$  SD of triplicates (n = 3). Analysis of variance ( $p < 0.05$ ) was performed using Microsoft excel 2007. The  $EC_{50}$  values were calculated from the linear regression equation determined by XY scatter plot in Excel. Analysis of variance was performed for all the assays with different pH as groups to calculate the significance of variance. The ANOVA test was performed using Microsoft excel 2007. Statistical differences at  $P < 0.05$  were considered to be significant difference. The coefficient of determination ( $r^2$ ) to determine the relationship between the scavenging activity and increase in phenol concentration were calculated using MS Excel for FRAP assay. The correlation coefficient ( $r$ ) for the increase in scavenging activity with increase in the pH was calculated for all the assays using MS Excel.

## **3. Results and Discussion**

### **3.1. Species identification of genus *Chaetomium***

In our earlier study we had identified the isolate number NIOCC 36 as *Chaetomium* sp., using morphological and microscopical characters [15]. The special adaptation of the fungus under different pH prompted us to identify this fungal species employing molecular characters. We sequenced a portion of the nuclear large ribosomal subunit rRNA gene (28S) from the extracted DNA of the fungal culture isolated from marine source. The BLAST search of the obtained sequence of the *Chaetomium* sp (NIOCC 36) (GenBank accession number GQ412081) showed that the sequence was most similar (100%) to that of the 28S large subunit ribosomal RNA gene sequences

of *Chaetomium globosum* (compared with gb AB449672 to AB449688, AB292591, AY545729.1, AF286403.1). Thus, *Chaetomium globosum* was confirmed for its identity of species using molecular analysis. *C. globosum* has also been isolated from several marine sources such as an endophytic fungus derived from the marine green alga *Ulva pertusa* [32], marine red alga *Polysiphonia urceolata* [20], marine fish *Mugil cephalus* [33], and sea shore soil [34] and mangrove wood litters [15].

### **3.1.1 Alkaline tolerant fungi**

We had optimized the media for *C. globosum* using plate assay and broth with different commonly used media and found fungus with maximum growth in MEA [15]. Thus, MEA was used in testing the marine derived fungus, *C. globosum* for its ability to grow in alkaline pH. *C. globosum* was found capable of growing in wide range of pH from 4 – 12 (Fig. 1). There are several other reports with respect to fungi obtained from natural habitats [35; 36], and *Chaetomium* sp., as soil fungi, for their alkali tolerance and alkalophilic nature [37]. *C. globosum* obtained from American Type Culture Collection 16021 (ATCC, Manassas, Virginia) was also reported for its capability to grow in pH ranging from 4.3 to 9.4, and the optimal growth of this isolate was found to occur at a neutral pH [38]. In addition, their results show that sporulation by *C. globosum* is favored in an acidic environment. Our marine derived isolate too showed the optimal growth in neutral pH and showed more sporulation in acidic environment (data not shown), which is in agreement with the above study of *C. globosum* isolate (ATCC 16021).

## **3.2. Antioxidants and Antioxidant activity of extracts of *C. globosum***

### **3.2.1. Total antioxidant content from extracts of *C. globosum***

The lifespan of a fungal organism is tightly connected with its stability to the environmental factors, which is determined by the state of the cell antioxidant defense components e.g. phenolic compounds [3]. Phenolic and flavonoid compounds seem to have important role in stabilizing lipid oxidation and to be associated with antioxidant activity, which is emphasized in several reports. The mechanism of action of flavonoids is through scavenging or chelation [39] and phenolic compounds are also very important plant and fungal constituents because their hydroxyl groups confer scavenging ability [40]. Therefore, in this study we determined the total phenolics and flavonoid contents of *C. globosum* fungal extracts obtained from mycelia and broth (Table 1). The total phenols and flavonoids of the *C. globosum* found increased with the increased level of pH stress from 4 to 12 (Table 1). Many phenolic compounds have been found to be effective antioxidants in

many strains of moulds belonging to species of *Aspergillus*, *Penicillium*, *Rhizopus*, *Mortierella* [41; 42], *Chaetomium*, *Cladosporium*, *Torula*, *Phoma* [43]. Mushrooms [44], and marine derived fungus, *Chaetomium globosum* [20], *Acremonium* sp. [45] and *Wardomyces anomalus* [46].

### 3.2.2. DPPH scavenging activity

The free radical-scavenging activities of fungus, *C. globosum* along with reference standard BHA were determined by the DPPH assay. DPPH is usually used as a substrate to evaluate antioxidative activity of antioxidants [47]. The fungal extracts of the present study showed a concentration dependant antiradical activity by inhibiting DPPH radical (Table 2). The decrease in absorbance of the DPPH radical caused by antioxidant was due to the scavenging of the radical by hydrogen donation. It is visually noticeable as a colour change from purple to yellow. Also, a lower value of EC<sub>50</sub> (concentration at which the 50% scavenging activity is obtained) indicates a higher antioxidant activity at lower concentration. The fungal extract was able to reduce the stable radical DPPH to the yellow-coloured diphenylpicrylhydrazine. It has been found that cysteine, glutathione, ascorbic acid, tocopherol, polyhydroxy aromatic compounds (e.g., hydroquinone, pyrogallol, gallic acid), and aromatic amines (e.g., p-phenylene diamine, p-aminophenol), reduce and decolorise  $\alpha,\alpha$ -diphenyl- $\beta$ -picrylhydrazyl by their hydrogen donating ability [48]. The results showed that the fungal extracts had hydrogen donating capabilities and acts as an antioxidant. The scavenging effects were found increased with increasing concentration of the extract in all the pH tested, and the EC<sub>50</sub> values decreased with increased pH when compared with the standard. The correlation coefficient (r) for the pH versus scavenging activity was found to be 0.511. The positive value of r shows that there is positive responses i.e. increase in scavenging activity for the increase in pH. The Analysis of variance (ANOVA) test was found to be significant with P=0.0019 which is less than 0.05 (Fig. 4). However, scavenging activity of BHA, a known antioxidant used as positive control, was relatively more pronounced than that of fungal extracts (Table 2). Several antioxidant compounds such as 3,3-di-OH terphenyllin, 3-OH terphenyllin, and candidusin B obtained from the extracts of *Aspergillus candidus* has been reported for their scavenging effects on DPPH radicals [42]. About ten compounds have been isolated from marine derived *C. globosum*, among which four compounds including chaetopyranin are identified for their DPPH scavenging activity [20]. The extracts from *C. globosum* was observed to have the DPPH scavenging activity, which is in agreement with previous study of marine derived algicolous fungus, *C. globosum* [20] and *Xylaria* sp. [49].

### 3.2.3. Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging was assayed by the FOX reagent method. Table 2 shows that the fungal extracts are very poor scavengers of H<sub>2</sub>O<sub>2</sub> compared to standard sodium pyruvate. Hydrogen peroxide is a weak oxidizing agent that inactivates few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Inside the cell, H<sub>2</sub>O<sub>2</sub> can cross cell membranes rapidly and probably react with Fe<sup>2+</sup> and Cu<sup>2+</sup> ions to form a hydroxyl radical which produces pronounced toxic effects [50]. In the present study, H<sub>2</sub>O<sub>2</sub> scavenging activity of the fungal extracts at different pH are negligible compared to that of the standard sodium pyruvate. But interestingly, scavenging activity of the fungal extract showed an increase with increased concentration of extracts in all the pH tested, and EC<sub>50</sub> values decreased with increased pH (Table 2). The correlation coefficient value  $r = 0.44$  and ANOVA test value  $P = 0.35$  showed that is lesser degree of significance for the hydrogen peroxide scavenging activity (Fig. 4). It has previously been proven that dietary phenols protect mammalian and bacterial cells from cytotoxicity induced by hydrogen peroxide [51], indicating that the observed H<sub>2</sub>O<sub>2</sub> scavenging activity of *C. globosum* could be due to the presence of antioxidant compounds.

### 3.2.4. Hydroxyl radical scavenging activity

Hydroxyl radical scavenging assay showed the abilities of the extract and standard mannitol in inhibiting hydroxyl radical-mediated deoxyribose degradation in a Fe<sup>3+</sup> EDTA ascorbic acid and H<sub>2</sub>O<sub>2</sub> reaction mixture (Table 2). The EC<sub>50</sub> values of the extract were found distinctly decreased with increased pH when compared to the standard (Table 2). Hydroxyl radicals are the major active oxygen species causing enormous biological damage by lipid peroxidation in cells [52]. When a hydroxyl radical reacts with aromatic compounds, hydroxycyclohexadienyl radical is formed, which will undergo further reaction with oxygen, to give peroxy radical, or decompose to phenoxy-type radicals by water elimination [53]. Hydroxyl radical were produced in this study by incubating ferric-EDTA with ascorbic acid and H<sub>2</sub>O<sub>2</sub> at pH 7.4 and reacted with 2-deoxy-2-ribose to generate a malondialdehyde (MDA)-like product. This compound forms a pink chromogen upon heating with TBA under low pH [54]. When fungal extracts of the samples was added to the reaction mixture, it will remove the hydroxyl radicals from the sugar and prevent the reaction. The EC<sub>50</sub> value indicates that the scavenging activity of the extracts was more in fungus grown in pH 12 than the other pHs tested (Table 2). The correlation coefficient ( $r$ ) value (0.84) showed that there is a strong positive correlation between the pH and the scavenging activity. The ANOVA test also showed that there exists a greater significance level in the scavenging activity under stress by having  $P$  values less than 0.0001 (Fig. 4). Several other fungi have been reported for scavenging activity against metal ion

dependant hydroxyl radical [55]. One among which possessed Graphislactone A, an antioxidant compound of fungus *Cephalosporium* sp. that shows a pronounced activity in eliminating hydroxyl radicals in a dose-dependent manner [56]. Thus, Graphislactone A like antioxidants may attenuate the process of metal ion-dependent hydroxyl radical formation and thus reduce hydroxyl radical-induced cell injury, in the present study.

### 3.2.5. $\beta$ -carotene–linoleate model system antioxidant activity

In this model system  $\beta$ -Carotene undergoes rapid discoloration in the absence of an antioxidant. This is because of the coupled oxidation of  $\beta$ -carotene and linoleic acid, which generates free radicals. The linoleic acid free radical, formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups, attacks the highly unsaturated  $\beta$ -carotene molecules. As a result,  $\beta$ -carotene will be oxidized and broken down in part; subsequently, the system loses its chromophore and the characteristic orange colour, which can be monitored spectrophotometrically. The presence of different antioxidants can hinder the extent of  $\beta$ -carotene bleaching by neutralizing the linoleate-free radical and other free radicals formed in the system [57]. The antioxidant activities of the extracts from *C. globosum* and of reference (BHT), measured by the bleaching of  $\beta$ -carotene, are shown in Table 2. It can be seen that the inhibition values of both the fungal extract and the standards increased with concentration. At a low concentration of 50  $\mu\text{g/ml}$ , the fungal extract exhibited the lowest activity. At the concentration of 100, and 150  $\mu\text{g/ml}$  the extract exhibited weaker activity than BHT, whereas 200  $\mu\text{g/ml}$  concentrations, the activity of the extract from *C. globosum* increased rapidly, showing the highest inhibition of  $\text{EC}_{50}$  values ( $39.6 \pm 5.6 \mu\text{g/ml}$ ) than BHT ( $60.28 \pm 1.5 \mu\text{g/ml}$ ) (Table 2). The correlation coefficient value of  $r=0.83$  and the ANOVA test result with  $P=0.0013$  showed a significant increase in the scavenging activity with increased level of stress (Fig. 4). Further, the antioxidants isopestacin isolated from culture fluid of the fungus *Pestalotiopsis microspora* [58] and Graphislactone A from *Cephalosporium* sp. (endophytic fungus) were demonstrated to show antioxidant activity against linoleic acid free radical [56]. Thus, the ability of the above fungal antioxidants to scavenge free radical may be comparable to that of the present fungal extracts.

### 3.2.6. Metal chelating activity

In this assay, both fungal extract and EDTA, interfered with the formation of ferrous and ferrozine complex, indicating the presence of chelating activity which captures ferrous ion before ferrozine, ferrozine can quantitatively form complexes with  $\text{Fe}^{2+}$ . In the presence of other chelating

agents, the complex formation is disrupted which is determined by the decrease in red colour. Measurement of the rate of colour reduction is used in the estimation of chelating activity of the coexisting chelator [40]. It was reported that chelating agents that form r-bonds with a metal, are effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion [59]. In the present study, the absorbance of  $\text{Fe}^{2+}$ -ferrozine complex was decreased dose-dependently; otherwise the activity increased with the increasing concentration from 50 to 200  $\mu\text{g/ml}$  and the  $\text{EC}_{50}$  values decreased with increased pH compared to the standard, EDTA (Table 2). The correlation coefficient value  $r=0.74$  and the ANOVA test value  $P=0.0001$  shows there was a significant level of increase in the chelating activity to suppress the generation of free radicals with increased stress condition (Fig. 4). Though  $\text{Cu}^{+2}$  and  $\text{Fe}^{+2}$  are required by the organisms for transport, protection against oxidative stress, cell growth, and development they can catalyze hydroxyl radical formation by the Fenton reaction [60]. Therefore, the levels of  $\text{Fe}^{+2}$  and  $\text{Cu}^{+2}$  in cells should be carefully controlled. Recent data suggest that yeast protein frataxin controls the iron needed for the living body biosynthesis of iron-sulfur clusters [61; 62] and of haem [63]. In fungus *P. brasiliensis*, a gene coding for frataxin (PbAEST 858), in relation to the frataxin homolog (Yfh1p; NP-010163.1) of *S. cerevisiae* was identified [60]. In addition, the chelating activity of several fungi of earlier studies for ferrous ion was assayed by the inhibition of formation of red colored ferrozine and ferrous complex as in present study using fungal extracts of *Trametes hirsuta*, which includes secondary antioxidants like transferrin and ceruloplasmin [64; 65] and *Aspergillus* sp. [66].

### 3.2.7. Ferric reducing antioxidant power assay (Frap assay)

FRAP assay measures the antioxidant effect of any substance in the reaction medium that has reducing ability. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity [67]. Further, the reducing properties are generally associated with the presence of reductones [68], which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom [59]. Reductones are also reported to react with certain precursors of peroxide, thus preventing the peroxide formation. Antioxidant potential of the fungal extracts of different pH was estimated from their ability to reduce TPTZ-Fe (III) complex to TPTZ-Fe (II) complex. In an earlier study, extracts of two *Aspergillus* sp. have been observed for a direct correlation between antioxidant activities and reducing power [66]. Thus, our data on the reducing power of fungal extract suggest that it is likely to contribute significantly towards the observed antioxidant effect. Moreover, there was a noticeable correlation between extractable total phenolics

concentration and FRAP values (pH 4,  $r^2 = 0.9803$ ; pH 6,  $r^2 = 0.9928$ ; pH 8,  $r^2 = 0.974$ ; pH 10,  $r^2 = 0.996$ ; pH 12,  $r^2 = 0.985$ ) (Fig. 2), in all the pH of the fungal extracts. The ANOVA test value of P lesser than 0.05 ( $P=0.0001$ ) emphasizes the result was significant. The correlation coefficient value between the stress level and the scavenging activity was 0.85 (Fig. 4). This shows that there is a strong positive correlation.

#### 4. Antioxidant enzyme assay

##### 4.1 Catalase assay

The antioxidant enzymes catalase and peroxidase serve as the primary line of defense in destroying free radicals. Thus, we assayed the above enzymes production in *C. globosum* under different pH conditions. Catalase (EC 1.11.1.6) catalyzes the degradation of hydrogen peroxide with release of molecular oxygen. Here, we examined the extra and intracellular conditions for antioxidant enzyme synthesis of the fungus at different pHs (Fig. 3). The catalase enzyme activity was maximal at pH 12 in both the intra and extracellular conditions (Fig. 3), but the intracellular catalases were less when compared to that of the extracellular catalase production (Fig. 3), which is in agreement with the previous study of microscopic fungi from various taxonomic groups [69]. In addition, the increased production of catalase in *C. globosum* (MO96) for its defense reaction was observed with increase of hydrogen peroxide after fungus cell interference of stress made with the petroleum ferrofluid [70]. This supports the present study that the increased amounts of catalase produced with increased pH stress (Fig. 3) in considerable defense reaction of the fungus against hydrogen peroxide.

It was reported that several genes of the antioxidant enzyme catalase are expressed at different metamorphic states of *Aspergillus nidulans*, *A. fumigatus* and *Neurospora crassa* [1]. *A. fumigatus* expresses three active catalases, one that is present in the conidia and two that are present in the mycelium, which are encoded by three separate structural genes CATA, CAT1, and CAT2 [71]. CatAp is unglycosylated catalase present only in resting conidia and absent in hyphae. These conidial and mycelial catalases were investigated for the protection of fungus against hydrogen peroxide *in vitro*. However, it was found that the conidial catalase, CatAp, is not a virulence factor, but both mycelial catalases, Cat1p and Cat2p, are involved in the degradation of hydrogen peroxide *in vitro* and transiently protected the fungus against the oxidative burst [71]. Further, *Aspergillus nidulans* have different localizations of expressing catalases, that is enzyme CatA activity was associated with spores [72] and CatB in the hyphal cell wall and cytosol [73] and catalase 3 of *N.*

*crassa* accumulates in conidia, like CatA [74]. Thus, the production of catalase by marine isolate *C. globosum* in the present study is comparable to the above kinds of active catalase genes in protecting against the oxidatative burst caused due to various environmental stresses.

#### 4.2. Peroxidase assay

It was also reported that catalase activity in fungi would be high if the selected strains exhibited no pronounced activity of peroxidase (1.11.17), which catalyzes the oxidation of organic compounds by using hydrogen peroxide [69]. Further, catalase/peroxidases (KatGs) are known for their unique bifunctional oxidoreductases accomplishing efficiently both peroxidatic and catalatic activity within a single active site [75]. Thus, we examined the peroxidase activity in *C. globosum* and its production was very less compared to that of the catalase production (Fig. 3). But, we could observe the peroxidase levels high in alkaline pH stress (Fig. 3). Moreover, phytopathogenic fungi such as the rice blast fungus *Magnaporthe grisea* are reported for their uniqueness in having two catalase/peroxidase (KatG) paralogues located either intracellularly (KatG1) or extracellularly (KatG2) [76]. The *C. globosum* of the present study also showed the presence of intra and extracellular catalases/peroxidases (Fig. 3).

Catalase/peroxidases (KatGs) are known for their sequence similarities and close phylogenetic relationship with ascorbate peroxidases and cytochrome-c peroxidases (CCP). CCP, the haem protein which was found localized in the mitochondrial intermembrane space of *Saccharomyces cerevisiae* have been demonstrated for their protection of the organisms from damage caused by high concentrations of H<sub>2</sub>O<sub>2</sub> [60]. This CCP enzyme was also reported in fungus *Paracoccidioides brasiliensis* [60], which indicates that the fungus *C. globosum* may have a conserved extra H<sub>2</sub>O<sub>2</sub> detoxification mechanism.

#### 4.3. Glutathione S Transferase assay

Catalase (CAT) reduces H<sub>2</sub>O<sub>2</sub> to water and molecular oxygen, and Glutathione peroxidase (GPX) catalyzes the reduction of H<sub>2</sub>O<sub>2</sub> to water and organic peroxide to alcohols at the expense of reduced glutathione (GSH). Enzyme Glutathione-S-transferase (GST) (EC 2.5.1.18) conjugates xenobiotics with GSH for excretion. Thus, we assayed the GST activity levels in *C. globosum* under different pH stress conditions. The enzyme activity was maximal with pH 12 in both the intra and extracellular conditions (Fig. 3). The results suggest that GST activity of the fungus differs according to their various pH stress tolerance. The GST activity has been identified in several fungal species [77] with divergent classes of GST dependent on the species such as the  $\omega$  class from *S. cerevisiae*



(Gtt2p, Gto1p, Gto2p, Gto3p),  $\theta$  class from *Issatchenkia orientalis* (GST Y-1 and Y-2),  $\gamma$  class from *C. elegans* (GST1-1, GST2-2), GstA from *A. nidulans* and GstA, GstB, EF1B $\gamma$  from *A. fumigatus* [78]. Among the above classes of GSTs  $\omega$  class, EF1B $\gamma$  class, and MAK16 class GST are widely distributed in fungi in Ascomycetes, Basidiomycetes, and Zygomycetes, whereas Ure2p class GST were found only in Ascomycetes. Some class of GST like GstB orthologs belong exclusively to Ascomycetes and especially to the *Aspergillus* and *Fusarium* genera which indicates that GstB-like GSTs are distributed in restricted fungal species.  $\gamma$  class GSTs are another example of GSTs found only in the Zygomycetes *Cunninghamella elegans* and *Rhizopus oryzae* [78]. Thus by the above classes of GST distribution in different groups of fungi *C. globosum* could be of with  $\omega$  class, EF1B $\gamma$  class, and MAK16 classes of GST.

## 5. Conclusion

In conclusion, the stress tolerance mechanism of marine derived fungi for its amount of antioxidant components and scavenging activity at different level of stress confirms that at an increased level of stress at pH 12, higher amount of ROS is generated. The fungus that can tolerate the stress produced increased amount of antioxidants to scavenge ROS. Thus, the present study elucidates that the scavenging capability is one of the phenomenon that help the fungus to survive in extreme stress conditions. In addition, the study also helps in understanding how the pathogenic fungus tackles the oxidative burst i.e. hypersensitivity reaction performed by host to kill the pathogens. Furthermore, the *in vitro* assays also indicate that fungal extracts are significant source of natural antioxidant, which may be helpful in preventing the progress of various oxidative stresses.

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## Figure Legend

**Figure 1:** Growth of *Chaetomium globosum* on different pH in malt extract agar (MEA) media. *C. globosum* showed a wide range of growth with pH 4 to pH 12 conditions, and growth was maximal at under pH 6. Values are means  $\pm$  SD (n=3).

**Figure 2:** Correlation of total phenolic content and Ferric reducing antioxidant power (FRAP) Assay. The extracts of *C. globosum* showed positive correlation of total phenolic content and FRAP of extracts with all the pH tested. The correlation values of **pH 4** (0  $\mu\text{g}/\text{ml}$  = 0; 50  $\mu\text{g}/\text{ml}$  = 22.10046, 100  $\mu\text{g}/\text{ml}$  = 36.57534, 150  $\mu\text{g}/\text{ml}$  = 50.68493, 200  $\mu\text{g}/\text{ml}$  = 63.78995),  $r^2 = 0.9803$ ; **pH 6**, (0  $\mu\text{g}/\text{ml}$  = 0; 50  $\mu\text{g}/\text{ml}$  = 35.25114, 100  $\mu\text{g}/\text{ml}$  = 60.86758, 150  $\mu\text{g}/\text{ml}$  = 83.74429, 200  $\mu\text{g}/\text{ml}$  = 116.8037),  $r^2 = 0.9928$ ; **pH 10**, (0  $\mu\text{g}/\text{ml}$  = 0; 50  $\mu\text{g}/\text{ml}$  = 55.25114, 100  $\mu\text{g}/\text{ml}$  = 91.91781, 150  $\mu\text{g}/\text{ml}$  = 138.0822, 200  $\mu\text{g}/\text{ml}$  = 190.1826),  $r^2 = 0.996$ ; **pH 12**, (0  $\mu\text{g}/\text{ml}$  = 0; 50  $\mu\text{g}/\text{ml}$  = 39.77169, 100  $\mu\text{g}/\text{ml}$  = 89.26941, 150  $\mu\text{g}/\text{ml}$  = 114.4292, 200  $\mu\text{g}/\text{ml}$  = 146.6667),  $r^2 = 0.985$ .  $r^2$  = coefficient of determination.

**Figure 3:** Production of antioxidant enzymes by *Chaetomium globosum* at different pH conditions. Extracellular and intracellular catalase levels are maximal at pH 12, but extracellular catalase levels are significantly higher than that of intracellular catalase. Extracellular and intracellular peroxidase production was less comparative to that of the extra and intra cellular catalase and GST, but a significant maximal production was observed at higher pH. Extra and intracellular GST production was maximal with other antioxidant enzymes. Values are means  $\pm$  SD (n=3).

**Figure 4:** Group Means with 95% Confidence Intervals. The values of P obtained after ANOVA test are DPPH scavenging 0.0019,  $\text{H}_2\text{O}_2$  scavenging – 0.35,  $\text{OH}^-$  scavenging – 0.0001,  $\beta$  Carotene Antioxidant activity – 0.0013, Iron Chelation – 0.0001, FRAP assay – 0.0001.

**Table 1 Total antioxidant content of alkaline tolerant *C. globosum* at different pH**

Fungus	Stress	Total phenols (mycelia) (mg/g)	Total phenols (Broth) (mg/g)	Total flavonoids (Broth) (mg/g)
<i>C. globosum</i>	pH 4	10.64±0.02	29.60±0.01	7.78±0.01
	pH 6	12.88±0.01	31.59±0.03	88.53±0.04
	pH 10	34.83±0.03	73.53±0.04	165.78±0.04
	pH 12	83.65±0.01	110.10±0.01	216.30±0.04



**Table 2** Extracts of alkaline tolerant *C. globosum* scavenging activity and EC<sub>50</sub> values

Sample Conc (µg/ml)	DPPH <sup>a</sup> Scavenging (%)	EC <sub>50</sub> (µg/ml)	H <sub>2</sub> O <sub>2</sub> <sup>b</sup> Scavenging (%)	EC <sub>50</sub> (µg/ml)	·OH <sup>c</sup> Scavenging (%)	EC <sub>50</sub> (µg/ml)	β Carotene <sup>d</sup> antioxidant activity (%)	EC <sub>50</sub> (µg/ml)	Iron chelation <sup>e</sup> (%)	EC <sub>50</sub> (µg/ml)
<b>pH 4</b>										
50	12.19±0.51	403.9±4.24	-	4514.2±28.0	-	-	47.75±1.00		132.1±4.4	12.45±0.10
653.02±1										
100	14.97±0.81	-			-		49.70±1.69		13.57±0.03	
150	22.31±0.28	-			-				55.67±0.66	15.19±0.04
200	24.00±0.35	2.80±0.32		-		57.44±0.94		16.05±0.08		
<b>pH 6</b>										
50	18.87±1.01	302.2±5.6	-	1249.0±22.8	-	1282.1±16.7	59.00±3.67	67.5±2.9	12.22±0.27	768.4±2.7
100	24.21±0.99	-			-				79.56±0.43	13.56±0.23
150	27.16±0.25	3.60±0.10		0.99±0.88		87.78±1.00		13.80±0.12		
200	32.31±1.04	8.56±1.78		9.64±0.49		94.06±0.00		13.92±0.00		
<b>pH 10</b>										
50	16.48±0.40	304.2±1.9	-	680.7±08.4	-	962.5±18.4	68.28±3.70	62.9±4.4	12.30±0.13	637.5±2.6
100	19.36±0.23	-				01.13±1.00	75.01±1.40	13.49±0.13		
150	27.10±0.30	04.87±1.00		06.80±1.27		89.82±2.00		14.81±0.37		
200	32.52±0.26	17.18 ±0.32		10.06±0.64		95.60±0.20		16.66±0.10		
<b>pH 12</b>										
50	14.70±0.90	305.3±5.4	-	635.2±17.5	09.0±0.64	522.9±8.9	88.60±1.40	39.6±5.6	27.00±2.40	315.1±6.9
100	22.59±0.30	-		14.6±0.24			91.04±1.20		27.35±0.50	
150	27.43±0.30	03.75±2.1		17.0±1.27			91.71±2.90		29.20±0.10	
200	31.15±1.70	19.33±0.8		18.5±1.07			93.97±2.90		30.46±0.40	

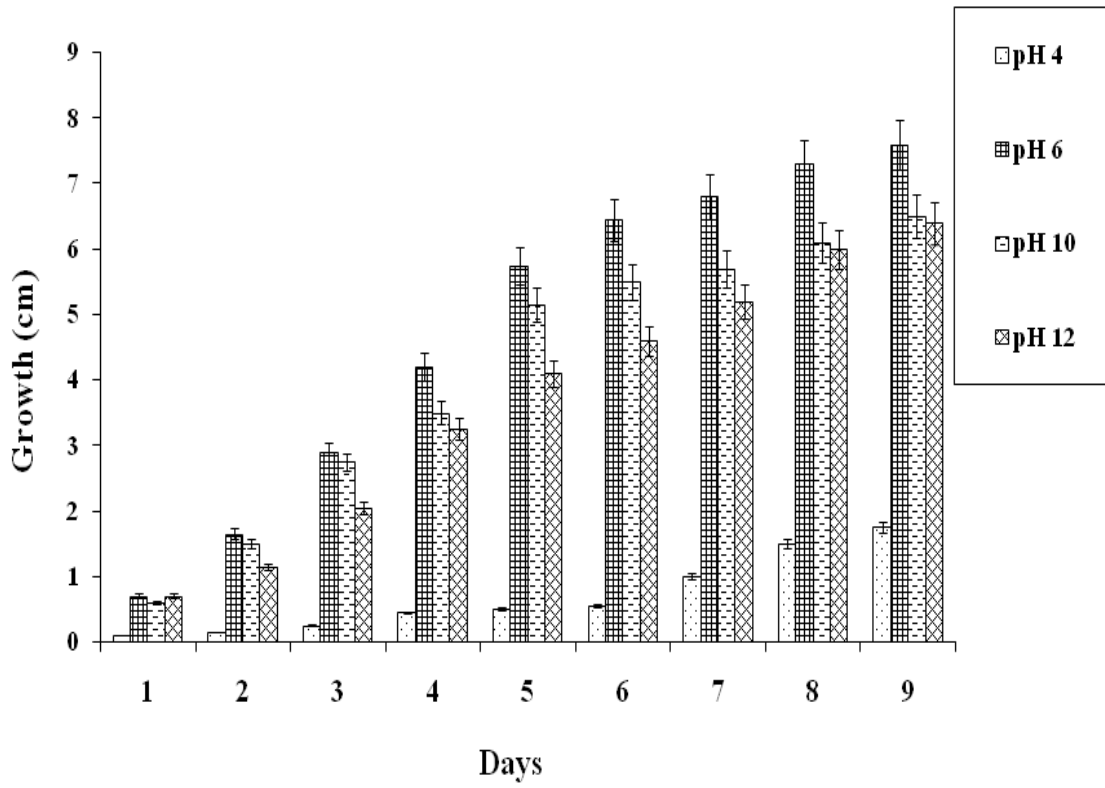
**Artificial  
Standard**

50	26.15±0.05	123.5±1.15	-	424.33±11.3	12.80±1.00	346.48±8.25	67.53±0.01	60.2±1.5	90.45±0.00	35.59±0.63
100	54.23±0.41		80.53±2.02		20.50±0.52		83.50±1.27		95.05±0.00	
150	63.22±0.37		13.13±0.57		21.84±1.50		87.28±0.00		97.70±0.74	
200	66.46±0.73		24.80±1.70		29.11±1.30		94.94±1.25		99.09±0.27	

**Standards used for various assays:** <sup>a</sup>DPPH scavenging assay – Butylated Hydroxy Anisole (BHA); <sup>b</sup>H<sub>2</sub>O<sub>2</sub> scavenging assay – Sodium pyruvate; <sup>c</sup>OH scavenging- Mannitol; <sup>d</sup>Antioxidant activity using β -carotene linoleate emulsion system – Butylated Hydroxy Toluene (BHT); <sup>e</sup>Metal chelating assay – EDTA.

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Figure 1



**Figure 2**

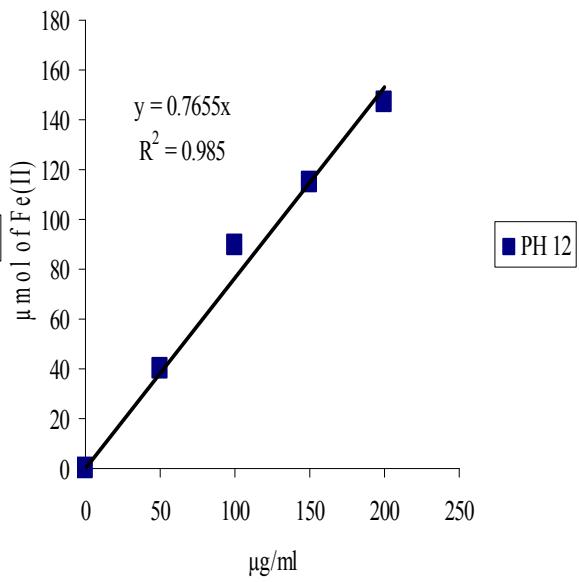
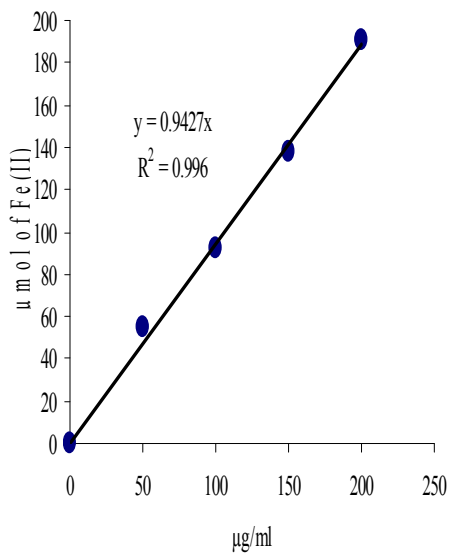
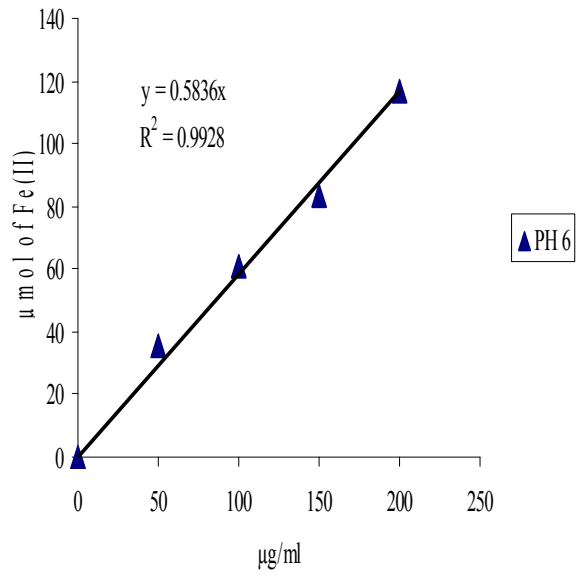
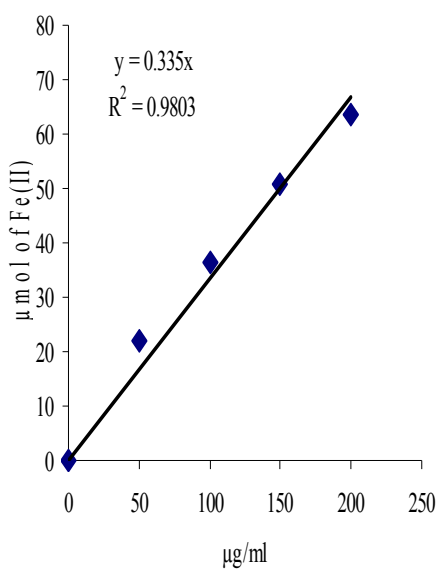


Figure 3

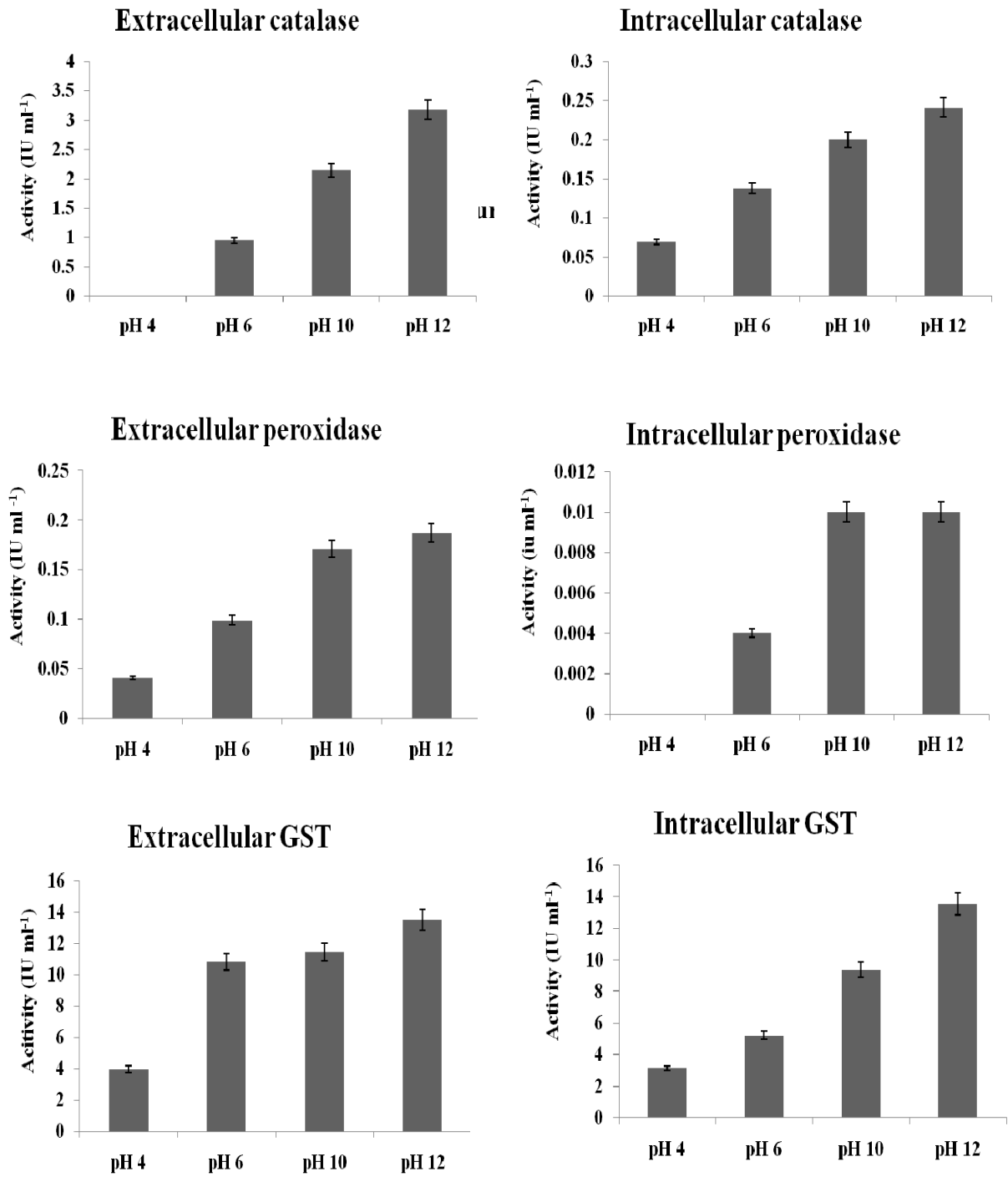


Figure 4

