Ascorbic acid modulates pathogenecity markers of *Candida albicans*

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Abstract- *Candida albicans* is an opportunistic commensal of the human gastrointestinal tract and vaginal mucosa, causing opportunistic fungal infections in an immunocompromised patient. In the present study we have investigated the effect of ascorbic acid on growth and its several pathogenicity markers. Turbidometric measurement for growth; proteinase assay, WST-1 cell cytotoxicity assay, colony count method and inverted microscopy were performed to check pathogenecity markers of *C. albicans* ATCC 10261 strain. 150 mg/ml concentration of ascorbic acid arrests cell growth. It was observed that higher ascorbate level of 250 mg/ml reduces proteinase secretion (an important mechanism suggestive of virulence in *Candida*) exhibited by mean precipitation zone value of 2.375 which is remarkably less than that of Control cells (value 4.125). At higher concentration of ascorbic acid increases cell cytotoxcity (79.71 percent inhibition at 150 mg/ml) and decreases percent viability under oxidative stress (98 percent reduction at 250 mg/ml concentration). Transition studies showed cessation of germ tube induction and hyphae formation at lower concentrations (15 mg onwards) of ascorbic acid. Results indicate that higher ascorbic acid level somehow decreases pathogenic attribute of *Candida albicans,* while yeast to hyphal studies show an exception, were lower concentration was effective in inhibiting hyphae formation. Thus ascorbic acid exhibits its pro-oxidant nature in present *in-vitro* studies.
Key words: *Candida albicans,* ascorbic acid, proteinase, oxidative stress, cytotoxicity, hyphal transition

Introduction

Candida albicans is a normal inhabitant of the oral cavity and the gastrointestinal and genitourinary tracts, where it persists in equilibrium with the host's microflora. Alterations in the physiological or immunological status of the host such as infancy, pregnancy, diabetes, prolonged broad spectrum antibiotic administration, steroidal chemotherapy and AIDS can lead to opportunistic infections ranging from mild mucosal lesions to life-threatening systemic disease [1- 2]. The biochemical functions of Ascorbic acid include: stimulation of certain enzymes, collagen biosynthesis, hormonal activation, antioxidant, detoxification of histamine, phagocytic functions of leukocytes, formation of nitrosamine, and proline hydroxylation amongst others. However, many other functions of ascorbic acid as well as the precise mechanisms of its functions are still elusive. Yeast infections occur less frequently in patients treated with antibiotics if bowel tolerance doses of ascorbic acid are simultaneously used [3]. Till date there are no reports showing direct involvement of ascorbic acid in fighting candidal infection but research shows that vitamin C boosts immunity by keeping disease-fighting white blood cells increased, so that the body is better able to stave off infections, especially opportunistic ones such as *Candida* that take advantage of a weak immune system. Vitamin C also provides an acidic meliu for *Candida*-fighting *lactobacillus*, which grows in an acid environment. Treatment with antibiotics, poor oral hygiene, and vitamin C deficiency appeared as the most significant independent risk factors associated with topical candidiasis [4]. Neutrophils which offer first line of defense, against systemic candidiasis rapidly transport dehydroscorbic acid inside and reduce it to ascorbic acid. Ascorbic acid concentration may rise up to 10 mM when neutrophils are incubated with *Candida albicans* [5]. Functional role of this increased Ascorbate is still an enigma. Ascorbic acid is seen to have enhanced the lethal effects of amphotericin B on *Candida albicans*. It is assumed that ascorbic acid acting as a pro-oxidant augmented the oxidation-dependent killing of fungal cells induced by amphotericin B [6]. Locally applied ascorbic acid, while being not active or worsening the time course of acute vaginal candidiasis or other fungal infections, was very active in preventing fungal reinfection or super infection when applied after the completion of a successful standard antimycotic or antibiotic treatment to patients [7]. Studies on direct effect of Ascorbic acid on growth and pathogenecity markers of *Candida albicans* are lacking. In the present study we have explored the effect of Ascorbic acid on growth of *Candida albicans* ATCC 10261 and cytotoxicity and pathogenecity markers: Proteinase secretion, Dimorphism, Resistance to oxidative stress.

Materials and Methods

All biochemicals including media constituents and ascorbic acid were obtained from Sigma Chemical, USA, whereas all inorganic chemicals were of analytical grade and procured from E. Merck (India).

Growth conditions

Stock culture of *Candida albicans* (ATCC 10261) was maintained on slants of nutrient agar (yeast extract 1%, peptone 2%, D-glucose 2% and agar 2.5%) at 4ºC. To initiate growth for experimental purposes, one loop full of cells from an agar

culture was inoculated into 25ml of YEPD nutrient medium and incubated at 30ºC for 24 hr i.e. up to stationary phase (primary culture). When secondary culture was required, the cells from primary culture (10^8 cells ml^{-1}) were re-inoculated into 100 ml fresh YEPD medium and grown for 8-10 hrs i.e., upto mid-log phase (10^6 cells ml^{-1}).

Growth in presence of Ascorbic acid (Turbidometric measurement)
Cells from primary culture were inoculated to fresh YEPD media (50ml) containing various concentrations of Ascorbic acid varying from 50 to 250 mg in individual flasks. Cells were incubated at 30º C for 24 hours. For studying growth, the optical density of the cells was measured at 595nm at an interval of 2 hours up to 20 hours.

Proteinase Assay
Assay was performed as described by Rita et al [8]. Cell culture was transferred to flasks containing yeast peptone dextrose medium (YPD) with different concentrations of ascorbic acid and incubated at 37°C for 18 hours. Following incubation, 1.5ml of the yeast culture was transferred to an eppendorf tube and centrifuged at 3000 rpm, for 5 mins. The pellets obtained were washed twice by resuspension in saline and centrifugation under the same conditions to remove residual culture medium. After standardizing the suspension, volumes of 1µl were placed in duplicate, at equidistant points in proteinase agar medium containing BSA. The plates containing inoculates for the detection of proteinase were incubated at 37º C for 7 days. The presence of proteinase was determined by the formation of a transparent halo around the yeast colonies and enzyme activity (PZ) was measured by dividing the diameter of the colony plus precipitation zone by the diameter of the colony.

Yeast to Hyphal Transition
Transition studies were performed according to Padmashree et al [9]. Strain was routinely grown on a minimal (SD) medium (0.67% yeast nitrogen base without amino acids supplemented with 2%glucose) supplemented appropriately. To induce hyphal formation, cultures were grown up to stationary phase along with different concentrations of Ascorbic acid overnight at 30°C in SD medium, cells were then diluted the following day to an optical density of 0.4 at 600 nm in SD medium containing 20% bovine calf serum, and incubated at 37°C for varying times and examined under an inverted microscope (Motic AE31 -Germany).

Assay of resistance to oxidative stress
The susceptibility of the cells to H_2O_2 was measured as described by Huh et al. [10] with some modifications. Cells were grown in minimal defined medium along with different concentrations of Ascorbic acid (62.5, 125 and 250 mg) to mid-logarithmic phase harvested, and resuspended in 0.1 M potassium phosphate buffer, pH 7.0, to obtain an initial optical density of 0.1 at 600 nm To observe the sensitivity of the cells to oxidants, various concentrations of H_2O_2 (2.5, 5 and 7.5 mM) were added to the cell suspensions. After incubation for 1 h at 30°C, aliquots were taken from the cell suspensions, diluted appropriately in the same buffer, and plated onto solid minimal defined medium. Colonies were counted after incubation for 3 days at 28°C.

WST-1 Cell Cytotoxicity Assay
Cells were cultured in a micro-plate and then incubated with WST-1 and the assay was monitored with Biorad-680 Microplate Reader. For this varying concentrations of the ascorbic acid (10, 20, 30, 40, 50, 60, 100, 125 and 150 mg per ml) were added to culture cells and incubated for 24hrs. At the end of the treatment, 10 µl of WST-1/CEC assay dye solution was added to each well. After gentle shaking the plate was incubated for 30 mins. Plate was shaken again for 1 min. and reaction was stopped by adding 10µl of 1% SDS. WST-1 tetrazolium salt was reduced to formazan by cellular dehydrogenases, generating a deep yellow colored formazan that was measured at 420-480 nm in a Microplate reader (Bio rad-680) and is directly correlated to cell number.
% Cytotoxicity = (100 × (cell control – Experimental) / (Cell control)

Results and Discussion
C. albicans causes serious infection in immunocompromised hosts such as leukemic, diabetic, organ transplanted, and human immunodeficiency virus- infected patients. Ascorbic acid is required for the optimal activity of several important biosynthetic enzymes and is therefore essential for various metabolic pathways in the body. RDA (Recommended Dietary/Daily Allowances), for vitamin C has been suggested to be 120 mg/d [11]. The Institute of Medicine (National Academy of Sciences) issued a report in April 2000 which states that the highest level of daily intake that is likely to pose no risk of adverse health effects to the general adult population above 18 years is estimated at 2,000 mg. In the present study effect of varying concentrations of ascorbate (10-250 mg) on growth and pathogenicity markers of *Candida albicans* ATCC 10261 have been examined.

Growth: Growth was studied when *Candida* cells were exposed to varying concentrations of ascorbic acid (50 to 250mg). Control cells showed an initial lag, exponential log and final

stationary phase. At concentrations of 50 and 100mg/ml ascorbic acid, cells followed almost same pattern of growth as control cells. There was a delay in growth with increasing concentration of ascorbic acid. At 125mg/ml concentration cells remained in lag phase for 12 hours. This time period is more than double as compared to control and other lower concentrations of ascorbic acid. At this concentration log phase was very short and lasted for only 2-4 hours as compared to 8-10 hours in control and lower ascorbic acid concentrations. At higher concentrations of ascorbic acid i.e. at 150, 200 and 250 mg/ml the normal sigmoid growth curve could not be seen and almost flat line was observed indicating negligible growth above 150 mg/ml concentration, as depicted in figure 1.

Proteinase Assay: Proteinase assay was performed with varying concentrations of ascorbic acid and their respective mean of precipitation zone (PZ) values were determined. There was decrease in PZ values with increasing concentrations of ascorbic acid as compared to control (4.125cm). At concentration of 62.5 mg/ml ascorbic acid, PZ value was found to be 3.375 cm. At this concentration there is a reduction of around 18%. At 125mg/ml concentration, PZ value found to be was 3.0, which accounts to 27% reduction as compared to control. A PZ value of 2.375 cm was observed with the highest concentration of ascorbic acid (250mg/ml), where this value shows a drop by 42.43% which is well depicted in figure 2.

Yeast to Hyphal transition: Control *Candida* cells showed hyphal induction at 90 minutes post treatment of culture with 20% Bovine calf serum. After 2 hrs 80-100% cells were induced to form hyphae. Their hyphal length increased with increase in incubation period. Incubation for 2 hrs resulted in hyphal length of 20.3µm which reached up to the length of 117µm after 24 hours, with decreased population of hyphae. In case of ascorbic acid treated cells induction was negligible as compared to control. When treated with 5 mg of ascorbic acid, hyphal length was 8.1µm after 2 hrs incubation, increased up to 20.1µm after 6 hrs then reduced to 0.5µm and gradually hyphae disappeared from the population of cells. Post treatment of 10 mg Ascorbic acid, hyphae length increased from 9µm to 19.1µm after 6 hrs then length declined to 14.8µm and similarly gradual disappearance of hyphal population was observed. Results indicated that treatment with ascorbic acid at concentrations of 15 mg and onwards induction process was completely arrested and hyphae like extension were not observed at all. Post treatment with these concentrations of ascorbic acid, atypical structures were found which were neither round budded yeast cells nor elongated hyphae (illustrated in table 1).

Assay of Resistance to Oxidative stress: *Candida* culture was treated with varying concentrations of ascorbic acid to study its effect on resistance to oxidative stress. For this purpose, exponentially growing cells along with different concentrations of ascorbate were treated with varying concentrations of H_2O_2 and the viable cells were counted. As shown in figure 3 ascorbic acid treated cells were more sensitive to H_2O_2 than the control. With increasing concentration of ascorbic acid, the number of viable cell colonies decreased this implying that there is decreased resistance to oxidative stress imposed by H_2O_2 compared to the control. As indicated in table 2 at highest concentration of ascorbic acid (250mg/ml) along with 2.5 mM H_2O_2 percentage reduction in cfu was found to be 35.07 which significantly increased to 98% with 5mM H_2O_2.

Cell Cytotoxicity Assay: Cell cytotoxicity assay was performed with ascorbic acid treated cultures, concentrations ranging from 10 to 150 mg. Figure 4 indicates that at lower concentrations i.e. at 10 and 20 mg ascorbic acid, percent cytotoxicity was found to be 33.31% and 35.89% respectively. At 30 mg/ml concentration marked increase in percent cytotoxicity observed which is 65.39. At 40 and 50 mg/ml concentration cytotoxicity increased to 67.09 and 67.76 % respectively. Percent cytotoxicity remained almost same at 60 and 100 mg/ml concentrations, being 69.02 and 69.53 % respectively. At highest concentrations of 125 and 150 mg/ml of ascorbic acid percent cytotoxicity reached to 74.35 and 79.99 respectively.

Growth of *Candida albicans* when exposed to ascorbic acid concentration up to 100 mg, showed little deviation from the control cell growth pattern. With increasing concentration the curve is seem to become less sigmoid. The lag period shows an extension from 4 hours in control to 8 hours with 100 mg ascorbic acid. 125 mg ascorbic acid shows no growth till 12 hours. While above this concentration (150 mg, 200 mg and 250 mg) Candida cells showed complete arrest of growth. The results indicate that higher concentration of ascorbic acid above 125 mg completely inhibit this growth of pathogenic dimorphic fungus, and this concentration lies well below that tolerable upper intake level of vitamin C. There are various mechanisms that are suggestive of *Candida* virulence such as: capacity to take on various forms, called adaptive variations; ability to form hyphae and production of hydrolytic enzymes such as proteinases which hydrolyse peptides [12]. In the last decade, it has been demonstrated that secreted aspartyl

proteinases (Saps) are important virulence factors for several types of *Candida albicans* infections and that inhibition of these proteinases have a protective effect on the host [13-14]. With increasing concentrations of ascorbic acid precipitation zones (PZ) were declined. At highest concentration remarkable reduction in PZ value was observed indicating that *Candida albicans* ATCC 10261 is proteinase positive, moreover higher concentrations of ascorbic acid lowers proteinase activity and thus retards its pathogenic attribute. Proteinase production by *C. albicans* depends not only on strain type or type of infection, but also on phenotypic switch type and environmental conditions (13]. When yeast to hyphal transition studies were performed with ascorbic acid, surprisingly at lower concentrations length of hyphae was drastically reduced. Above 15 mg/ml concentration after serum induction blastoconidia didn't form hyphae, thus there was arrest of hyphal transition. Infact atypical (intermediate-neither budded nor filamentous) structures were observed in microscopic slide post ascorbate treatment. Arrest of hyphal growth is not because of antifungal activity which reduces germ tube formation, because at these concentrations there was no effect on growth, rather up to 100 mg very slight decline in growth curve observed in present study. It could be suggested that the inhibition of hyphal formation by ascorbic acid occurred by interruption of the hyphal formation signal of *C. albicans*. *Candida albicans* encounters high levels of oxidants following ingestion by professional phagocytes and through contact with hydrogen peroxide-producing bacteria. Antioxidant nutrients and enzyme defenses are fundamental protectors against all forms of stress. In the present study at higher concentration of ascorbic acid, the number of viable colonies was decreased. Ascorbic acid is a well known antioxidant whose precise role in protecting cells from oxidative challenge is uncertain. *In vitro* results have been confounded by pro-oxidant effects of ascorbic acid and an overlapping role of glutathione [15]. When cell cytotoxicity assay was performed with ascorbic acid; only a marginal decline in cytotoxicity observed at lower concentrations, while at higher concentrations i.e. at 125 and 150 mg percentage cytotoxicity reached to 74.85 and 79.71 respectively. Tetrazolium salts are widely used as an assay for bacterial, fungal and mammalian cell viability and are widely used as indicators of cellular proliferation and biomass for eukaryotic and prokaryotic cells [16-17]. These reductase assays have been used to assess the effectiveness of antifungal compounds by providing rapid, real-time and quantitative assay measurements of cell dependent activities. Live cells reduce the tetrazole ring and a colored formazan product is formed, which can be assessed visually and quantified spectrophotometrically. There are reports that such assays are used to test the efficiency of antifungal drugs in killing or inhibiting growth of *Candida albicans* and other fungi [18-20]. Assay result indicates that higher ascorbic acid concentration is inhibitory for cell proliferation and drastically reduces biomass of *Candida* culture. The intensive prophylactic use of anti-fungal drugs such as azoles [21] and allicin [22] leads to emergence of resistant strains of *C. albicans*. This causes a great concern for finding suitable new therapeutics and novel natural antifungal compounds and drugs. An important aspect of the present study is the observation that higher concentrations of ascorbic acid to some extent decreases virulence and pathogenecity of *Candida albicans.* One key issue is whether there is a relationship between higher ascorbate level in human and the clinical outcome in fungal infections. Because ascorbate a natural, harmless, dietary antioxidant has little toxicity and can be given by either enteral or parenteral administration to humans[23] and due to increasing antibiotic resistance[24], it may be worthwhile to explore the clinical potential of ascorbic acid in fungal infections.

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References

[1] Odds F.C. (1988) *Candida* and candidosis. 2nd ed. Bailliere-Tindall. London.

[2] Eggimann P., Garbino J. and Pittet D. (2003) *Lancet Infect. Dis,* 3, 685–702.

[3] Cathcart R.F., (1981 *Medical Hypotheses*, 7, 1359-1376.

[4] Paillaud E., Merlier I., Dupeyron C. Scherman E. Poupon J. and Bories P.N. (2004) *Br J Nut,* 92(5), 861-7.

[5] Yaohui W.,Thomas A., Oran K. Stephen C. Steven C. and Rumsey. (1997) *Proc. Natl. Acad. Sci. USA,* 94, 13816–13819.

[6] Brajtburg J., Elberg S., Kobayashi G.S. and Medoff G. (1989) *J Antimicrob Chemother*, 24(3), 333-7.

[7] Mailland, Federico. (Jan 2005). European Patent. EP1500394.

[8] Rita de C.M., Marlise I.K., Magda B.G. Janaina A.O.R. Reginaldo B.G. and Jose F.H (2006) *Brazilian Journal of Microbiology*, 37, 26-32.

[9] Padmashree C.G.R., Akiko N., Gena Y. Won and Neta D. (2006) *Molecular Biology of the Cell,* (17), 4364–4378.

[10] Huh W.K., Lee B.H., Kim S.T. Kim Y.R. Rhie G.E. and Baek Y.W. (1998) *Mol Microbiol*, 30, 895–903.
[11] Anitra C.C. and Balz F. (1999) *Am J Clin Nutr*, 69 (6), 1086-1107.
[12] Hube B. (1998) *Rev.Iberoam. Micol,* 15, 68-71.
[13] De Bernardis F., Sullivan P.A and Cassone A. (2001) *Med Mycol*, 39, 303–313.
[14] Hube B.and J. Naglik (2001) *Microbiology,* 147, 1997–2005.
[15] Victor H.G., Juan Carlos V.and David W.G. (2001) *J Biol Chem,* 276 (44), 40955–40961.
[16] Johnsen A.R., Bendixen K. and Karlson U. (2002) *Appl Environ Microbiol,* 68, 2683–2689.
[17] McCluskey C., Quinn J.P. and McGrath J.W. (2005) *Microb Ecol*, 49,379–387.
[18] Kuhn D.M., Balkis M., Chandra J. Mukherjee P.K. and Ghannoum M. (2003) *J Clin Microbiol,* 41, 506–508.
[19] Meletiadis J., Mouton J.W., Meis F., Bouman B.A. Donnelly J.P. and Verweij P.E. (2001) *J Clin Microbiol,* 39, 3402–3408.
[20] Wiederhold N.P., Kontoyiannis D.P., Prince R. and Lewis R.E (2005) *Antimicrob Agents Chemother*, 49, 5146–5148.
[21] Zhou W.C. and Zhou H.Y. (2006) *Chin J Pharm*, 37,125–133.
[22] Wen P., Chen L. and Guo Y.F. (2007) *J Microbiol*, 27:104–106.
[23] Levine M., Conry-Cantilena C., Wang Y. Welch R.W. Washko P.W. and Dhariwal K.R. (1996) *Proc.Natl. Acad.Sci. USA*,93, 3704-3709.
[24] Gold H.S. and Moellering R.C.J. (1996) *N.Engl.J.Med*, 335, 1445-1453.

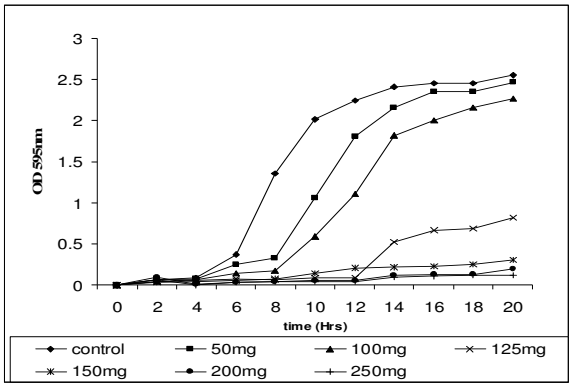

Figure 1: Growth curve in presence of varying concentrations of Ascorbic acid

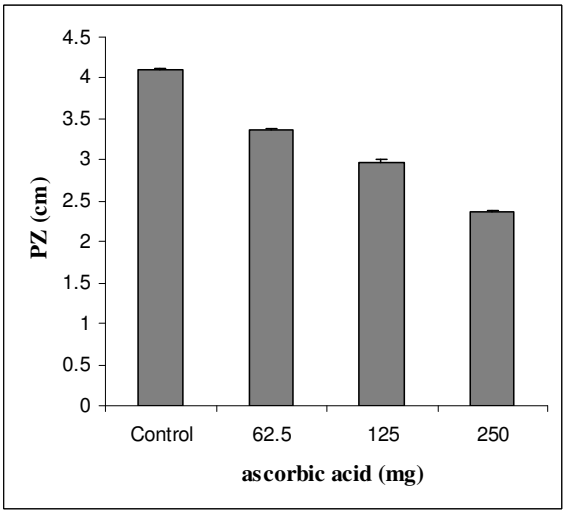

Figure 2: Effect of Ascorbic acid on Proteinase activity. The values are the means (indicated by the bars) ± standard deviations of three independent experiments

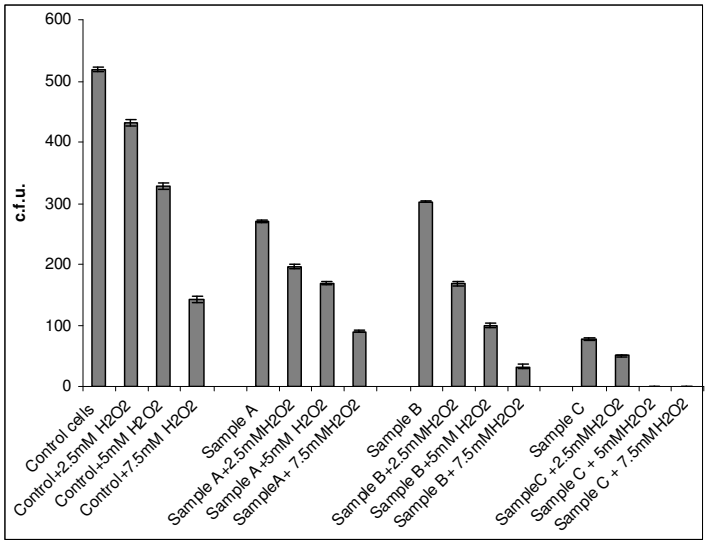

Figure 3: Effect of Ascorbic acid on Resistance to Oxidative stress. The values are the means (indicated by the bars) ± standard deviations of three independent experiments. Sample A: 62.5 mg ascorbic acid; Sample B: 125 mg ascorbic acid; Sample C: 250 mg ascorbic acid

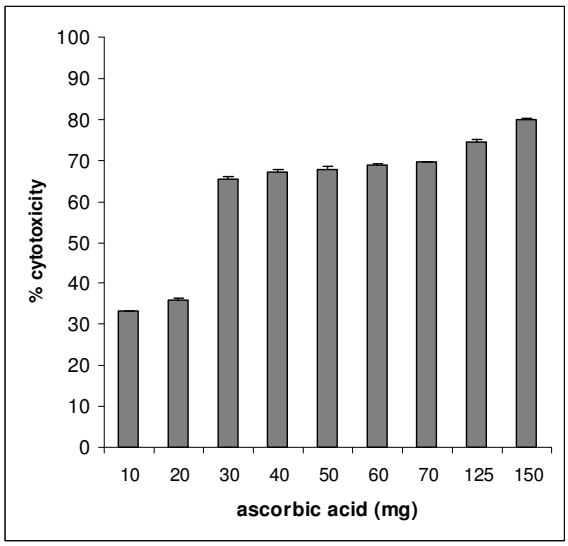

Figure 4: Effect of varying concentrations of Ascorbic acid on Cell Cytotoxicity. The values are the means (indicated by the bars) ± standard deviations of three independent experiments

Table 1: Hyphal induction in C abicans ATCC 10261 in the presence of Ascorbic acid

	Length of Hyphae (µm) with increasing Time				
Candida cells	2 hrs	4 hrs	6 hrs	8 hrs	24 hrs
Control	20.3	31.5	31.95	45	117.25
5 mg [a]	8.1	15.9	20.1	0.5	No hyphae
10 mg [a]	9.0	9.9	19.1	14.8	No hyphae
15 mg [a]	No hyphal structure found post treatment				

[a] *Ascorbic acid concentration.*

Table 2: Percentage reduction in Colony forming units of treated sample in Oxidative stress analysis

Ascorbic acid	2.5mM H_2O_2	5mMH_2O_2	7.5 mM H_2O_2
62.5 mg	27.41	37.41	66.67
125 mg	44.38	67.22	89.41
250 mg	35.07	98	-