Chapter 7

Glucose Uptake and Oxidative Stress Studies of Polyphenols from Faba Beans in *Saccharomyces cerevisiae* 2376

7.1 Introduction

Oxidative stress related to the mechanism of superoxide production, is the common pathogenic factor leading to insulin resistance, impaired glucose tolerance (IGT) and ultimately to *type 2 DM* (*T2DM*)) (Wright et al., 2006). It has been reported that the imbalance between oxidative stress and antioxidant levels in diabetes may be because of the generation of ROS during glycation, and glucose and lipid oxidation (Rösen et al., 1998; Wolff, 1993).

Saccharomyces cerevisiae is one of the most ideal experimental models for the exploration of oxidative stress and its effects, from the perspective of programmed cell death (PCD) and aging. PCD and aging depend upon the ROS levels in the living system. ROS typically arise because of electron leakage from the electron transport chain onto dioxygen during aerobic respiration (Boveris et al., 1972; Halliwell, 2007; Turrens, 1997; Turrens and Boveris, 1980). Aerobic organisms have integrated antioxidant systems, which include enzymatic and nonenzymatic antioxidants that are usually effective in blocking the harmful effects of ROS (Birben et al., 2012).

Generally, the development of pharmaceutical agents depends on increasing the body's resistance to the influence of stress and hypoxic factors (Kamzolova et al., 2018). A similar strategy was exploited in yeast cells as an *in vitro* model system. Yeast as a model has several other industrial and therapeutic applications for the production of secondary metabolites (Kamzolova et al., 2014)**.** Yeast cells were used for glucose uptake

activity and oxidative stress potential as described by Gonzalez (González-Siso et al., 2009). It is accepted as an exceptional legume because of its rich nutrient content, mainly, being an enriched source of protein, carbohydrates, dietary fibre, minerals and secondary metabolites (phenolics and Levo dihydroxyphenylalanine (l DOPA) (Rabey et al., 1992) and also contain many functional phenolic and flavonoid compounds such as tannin, proanthocyanidins, L-3,4-dihydroxyphenylalanine (L-dopa), flavonols and flavones which possess antioxidant activity(Kwon et al., 2018) .

Therefore, present investigation focussed on yeast as an experimental potential for glucose uptake and oxidative stress potential.

7.2 Experimental

7.2.1. Seed Material

Details about faba bean and their extraction, purification, and characterization were already discussed in Chapter 3.

7.2.2. Chemicals

Dialysis bags (12000 MW cut-off) and extra pure Glucose Assay Kit were procured from Himedia laboratories, India. All the reagents used were propidium iodide (PI; Sigma, St. Louis, MO, USA); 4,6-diamino-2-phenylindole dihydrochloride (DAPI; Sigma); 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT; Sigma); sulforhodamine B (SRB; Sigma); paraformaldehyde (Hi-media) and also others reagents were of extra pure analytical grade used in this investigations.

7.2.3. Culture collection and maintenance

Saccharomyces cerevisiae (NCIM: 3187) were obtained from National Collection of Industrial Microorganism National Chemical Laboratory, Pune India. The yeast cells were grown in the YPD broth at 28°C and preserved on YPD agar slants at 4°C. Cells

were first grown in glucose-free (5% glycerol + 2% ethanol) medium to log phase ($O.D_{600nm}$ = 1.2–1.5).

7.2.4. Estimation of glucose uptake study in yeast cell

Glucose uptake study was based according to this modified method of Cirillo (Cirillo, 1962). Cells were first cultured in glucose-free (5% glycerol + 2% ethanol) medium to mid-log phase ($O.D_{600nm}$ = 1.2–1.5). Yeast cell culture broth was kept for centrifugation (4000 r/minutes, 10 minutes) in distilled water until the supernatant fluids cleared and a 10% (v/v) suspension was made in sterile distilled water ($O.D_{600}$=0.3-0.5). Dialysis bag containing 1 mL of glucose solution (5-25 mmol/L) and different concentrations of seed extracts (1-5 mg) were mixed properly and incubated together for 10 minutes at 37°C. 200 µL of yeast suspension was added and vortexed, then further incubated at 37°C for 60 minutes. After 60 minutes, the tubes were centrifuged (4000 r/minutes, 10 minutes) and glucose was estimated in the supernatant. The percent increase in glucose uptake by yeast cells was calculated by using the following formula:

$$\textbf{\% increase in glucose uptake} = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100$$

where, Abs control is the absorbance of the control reaction (containing all reagents except the test sample), and Abs sample is the absorbance of the test sample.

7.2.5. Determination of glucose adsorption capacity

The glucose adsorption capacity of the samples was evaluated according to this modified method Ou *et al*. (Ou et al., 2001). Seed extracts (1%) of 0.5 gm were mixed to 50 ml of increasing concentration of glucose solution (5, 10, 20, 50, and 100 mmol/L). The mixture was filled in a dialysis bag and kept in a shaking water bath at 37°C for 6 hrs, centrifuged at 8000 rpm for 10 minutes and the adsorbed glucose content in the samples was determined in the supernatant. It was calculated by using this equation:-

$$\text{Glucose Bound} = \left\{ \frac{G_1 - G_6}{Weight \text{ of the sample}} \times volume \text{ of the solution} \right\}$$

G_1 is the glucose concentration of the original solution.

G_6 is the glucose concentration after 6 h.

7.2.6. FRAP (Ferric Reducing Antioxidant Power) assay.

This assay was based upon the methodology (Benzie and Strain, 1996). The FRAP reagent was made up of TPTZ in 40 mM HCl, $FeCl_3$ and sodium acetate buffer (pH 3.6) and it was always freshly prepared. A 100 µl of extract solution containing 0.1 mg of different extracts (ethanol, acetone, methanol, chloroform and aqueous) were mixed with 900 µl of FRAP reagent. The mixture was allowed to stand at 37 °C for 4 min and the absorbance was determined at 593 nm against blank. BHT was used as a calibration standard. FRAP values were evaluated as mg of BHT equivalents/g extract.

7.2.7. Oxidative stress study condition

Yeast cells were treated with hydrogen peroxide (H_2O_2:30% w/v stock solution) (resuspending them in 25 ml of 0.5%, 1%, 2%, and 3% w/v H_2O_2 a final concentration of ~1×10^6 cells/ml) for *S. cerevisiae* for 60 minutes. Cells in the exponential growth phase (O.D at 1.2-1.4) were harvested. Cells pellets were resuspended in distilled water. The unstressed (controls) and H_2O_2-stressed on yeast suspensions were kept in a pre-equilibrated water bath at 30^oC and vortexed once for proper mixing. Seed extract (1mg/ml) in DMSO was prepared and treated with cells and H_2O_2 treated cells separately. Same study condition was maintained in all the experimental process

7.2.8. Cell survival study by MTT and SRB assay

Approximately 1×10^6 cells were seeded into 96-well plates and incubated for 1 hour to the H_2O_2-seed extract mixture. The cytotoxicity of the drugs was calculated by the

MTT assay, based on the protocol of Igarashi and Miyazawa(Igarashi and Miyazawa, 2001). MTT (50 µL) was added to the treated wells. It was incubated at 37ºC for 3 hours with mild shaking. After that, the cells were resuspended in 200 µL of 2-propanol containing 0.04N HCl overnight in the dark. The absorbance was measured at 650 nm in a synergy multimode biotech microtiter plate reader. Similarly, other protocols like SRB assay were performed to validate MTT assay data (Kiruthika and Padma, 2013). The cell survival was measured as the absorbance at 492 nm (Synergy H1 Hybrid Multi-Mode Reader). compared to the control (i.e., untreated (Flores, 1978)

7.2.9. Oxidative stress analysis by different approaches

7.2.9.1. ROS measurement by DCF-DA (2',7'-dichlorofluorescein diacetate) assay

The is a technique that is widely used to profile the oxidative status of living cells. It was initially developed by (Keston and Brandt, 1965). Cells were treated with extract and H_20_2 Control was made without extract. After that, all samples were incubated at 30^0C. 20 µl of DCF (5 mg/ml) was added in each sample. Fluorescence was measured at 450 nm wavelength by Synergy H1 Hybrid Multi-Mode Reader**.**

7.2.9.2. DNA fragmentation assay

DNA fragmentation was analyzed by agarose gel electrophoresis as described by (Moongkarndi et al., 2004) with slight modifications. Cells (1×10^6 cells) were incubated with extract and H_2O_2 for 60 minutes. It was scraped and harvested by centrifugation. The cell pellets were incubated for 60 min 50 ◦C in 100 µl lysis buffer (100 mM Tris–HCl pH 8, 100 mM NaCl and 10 mM EDTA). Proteinase K (10 µl of 20 mg/ml) was added and further incubated for 30 min at 50 ˚C. RNase (3µl of 10 mg/ml) was then added and incubated for 2 h at 50 ˚C. The DNA was extracted with phenol-chloroform-isoamyl

alcohol, subjected to 2.0% of agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light transilluminator

7.2.9.3. Atomic force microscopy

The main aim of using this technique was to know the effect of hydrogen peroxide and seed extract on yeast cells in terms of cell roughness. Hydrogen peroxide (H_2O_2) is often used as an experimental source of oxygen-derived free radicals. Control and treated *S. cerevisiae* yeasts were visualized by using an AFM model-Nt-Mdt, Russia for the surface morphological changes in terms of cell roughness and mean cell volume parameters. The yeast samples were scanned in semi contact modes and scanning speed was 0.5 Hz. Experiments were performed in air at room temperature (Adya et al., 2005). The air-drying was done to immobilize yeasts on hydrophilic glass slides. The AFM samples were prepared by spreading aliquots of 100 µl of yeast suspensions onto the surfaces of glass slides.

7.2.9.4. Propidium iodide staining

PI staining protocol was followed by (Sarker et al., 2000) and it was used to distinguish apoptotic cells from normal cells, which reflects nuclear changes during apoptosis. Control, cell treated with extract, H_20_2 treated cells were kept on the slide and permeabilized with a mixture of acetone: methanol (1:1) at 20ºC for 10 minutes. Then, 10 µL of 5 mg/ml PI was added to each slide, spread with a coverslip and air dried it. It was visualized under ZEISS LSM780 confocal microscopy.

7.2.9.5. DAPI staining

The apoptotic ratio of the H_20_2-treated cells (with or without the seed extract) and the untreated cells were evaluated by DAPI staining (Rashmi et al., 2003). The cells were then transferred to slides and were immediately fixed with 3% paraformaldehyde and

permeabilized with 0.2% Triton X-100 in phosphate buffered saline (PBS) for 10 minutes at room temperature. They were then incubated with 20 µl of 5 mg/mL of DAPI. The apoptotic nuclei were observed under a Zeiss LSM780 confocal microscopy.

7.2.9.6. Oxidative stress study via flow cytometry approach

1 ml of yeast cell culture (1×10^6 cells) were incubated with extract and H_20_2 for 1 hrs. Centrifugation method was utilized to harvest treated and untreated cells and wash with phosphate buffer saline and resuspended the pellet in PBS buffer. 10-20 µL of PI staining solution was added to each sample. The same volume was maintained for all samples. Mixed gently and incubated 1 minute in dark environment. PI Fluorescence was determined with FACS scan ^{TM} using FL-2 or FL-3 channel. Data were acquired for unstained and single positive control. Stop count was fixed on the viable cells from dot plot of forward scatter and red fluorescence FL-2 channel with 585/42 nm band-pass filter was observed. The compensations and the settings had been adjusted according to the protocol. Data were analyzed with the use of BD Cell Quest Pro software.

7.2.10. Glucose uptake assay through different approaches

7.2.10.1. Glucose uptake assay by flow cytometry

The glucose uptake assay was performed as previously described with slight modification(Chen et al., 2010). Briefly, 1 ml of yeast cell culture (1×10^6 cells) were incubated with different extract and the fluorescent glucose analog 2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-D-glucose (2-NBDG, 10 µM) and samples (50 µM) FOR 1 HRS. Equal volumes are maintained in all test tubes. After incubation Centrifugation was performed at 5000 rpm for 20 minutes. Then, pellets were washed with phosphate-buffered saline (PBS) twice. Cells were scraped out in 1 mL of PBS and transferred into 5 mL polystyrene round-bottom tubes (BD Falcon) and kept at 4 ºC. The

amount of 2-NBDG taken up by the cells was measured in the FL1 channel using a FACS Calibur flow cytometer (BD Bioscience, San Jose, CA). Data from 10,000 single cell events were collected. Values of relative fluorescence intensity (FI) were calculated as. Values of relative fluorescence intensity (FI) were calculated as $FI = F_{I2\text{-}NBDG} - F_{Ibackground}$ where $F_{I2\text{-}NBDG\ is}$ the fluorescence intensity of a single cell treated with or without a sample in the presence of 2-NBDG and $FI_{background}$ is the fluorescence intensity in the absence of 2-NBDG. Relative fluorescence intensity (FI) was calculated image J software.

7.2.10.2. Estimation of glucose uptake study in yeast cells by confocal microscopy

1 ml of yeast cell culture (1×10^6 cells) were incubated with different extract and the fluorescent glucose analog 2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-D-glucose (2-NBDG, 10 μM) and samples (50 μM) for 1 hrs. Equal volumes are maintained in all test tubes. After incubation centrifugation was performed at 5000 rpm for 20 minutes. Then, pellets were washed with phosphate-buffered saline (PBS) twice. Cells on slides were rinsed in Dulbecco's phosphate-buffered saline and then fixed for 10 min in 4% paraformaldehyde. Cells were examined with ZEISS LSM-780 confocal microscopy microscope. Image J software was used for fluorescence intensity measurement.

7.2.11. Statistical analysis

Experimental analysis was done in triplicate and the results were expressed as n=3, means ± standard deviation. The analysis of variance (ANOVA) was calculated using Graph Pad Prism 5.0 to compare the means. Differences were considered statistically significant at $p<0.05$.

7.3. Results and Discussion

7.3.1. Evaluation of Binding Capacity of Glucose with Seed Extract

Seed extracts were effective for glucose binding and their binding capacity was directly proportional to the molar concentration of glucose. The samples were effective in adsorbing glucose at both lower and higher concentrations (5 and 100 mmol/L) **(Figure 7.1).** Out of all the seed extracts, acetone extracts had higher binding activity(8.48±1.23 mg) this might be due to their both insoluble and soluble constituents and fibers from different sources. The hypoglycaemic action of the extract of *faba bean seed* may be due to blocking of glucose absorption (Kannur et al., 2006) Therefore, *in vitro* experimental outcome was in conformity with *in vivo* system reported by (Chau et al., 2004) that means that extract might reduce the amount of glucose available for transport across the intestinal lumen, thus blunting the postprandial hyperglycemia.

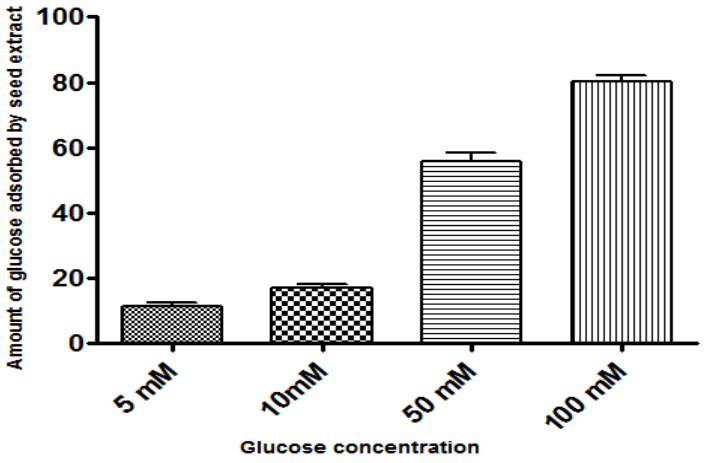


Figure 7.1. The glucose adsorption capacity of acetone extract at different glucose concentration(mM), (n=3,mean±S.D)

7.3.2. Effect of seed extract on glucose uptake in yeast cells

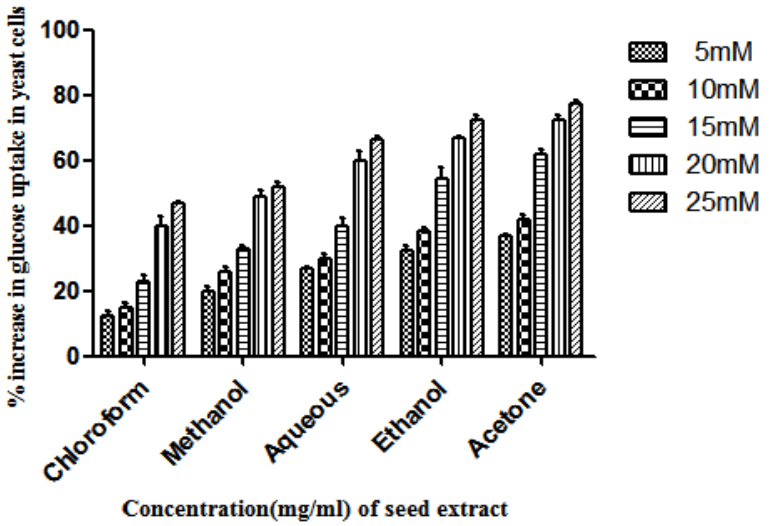
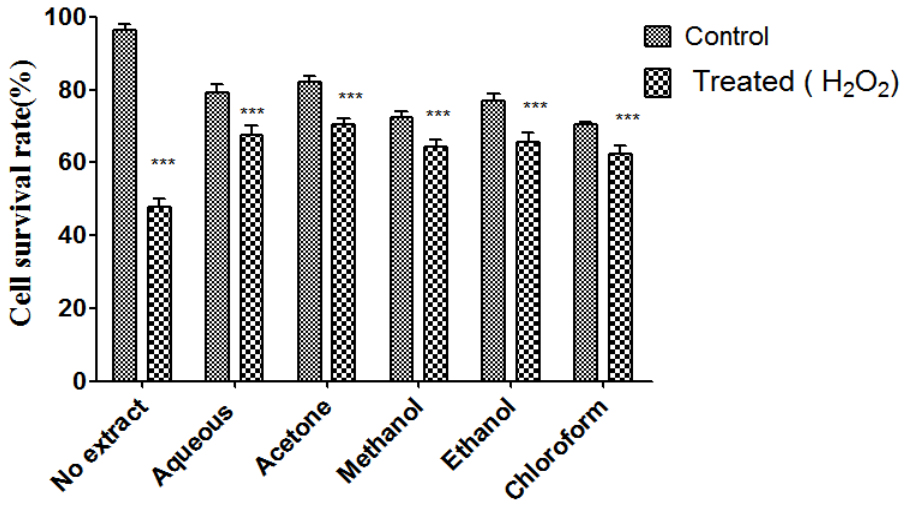


Figure 7.2. Glucose uptake by yeast cells by different seed extracts(0.1 (n=3,mean±S.D)

As from the **figure 7.2**, we got maximum 77.28±2.42% (n=3) glucose uptake in yeast cells at 25mM glucose concentration by acetone seed extract and minimum glucose uptake rate was found to be 52.36± 2.06% (n=3) by chloroform seed extract **(Figure 7. 2)**. Seed extracts increased glucose transport across the yeast cells. It was reported that in yeast cells (*Saccharomyces cerevisiae*) glucose transport is complex, and glucose transport in a yeast cell is done by a facilitated diffusion process (Gupta et al., 2013). Glucose transport in yeast cell may be due to at least six members of the glucose transporter family (Hxt1, 2, 3, 4, 6 and 7) (Boles and Hollenberg, 1997; Reifenberger et al., 1997).

7.3.3. Effect of seed extract and hydrogen peroxide on yeast cell survival rate

It was evident from **Figure 7.3** that maximum survival rate was found in acetone extract(82.067 %, $P<0.05$) as compared with control (96.36%., $P<0.05$) but least survival rate was found to be chloroform extract (70.48%, $P<0.05$) (Figure 3).



.**Figure 7.3.** MTT assay of different extract (0.1mg/ml) with H_20_2 as compared to control(n=3,mean±S.D, ANOVA at $p<0.05$,*** Control vs. treated (statistically extremely significant value).

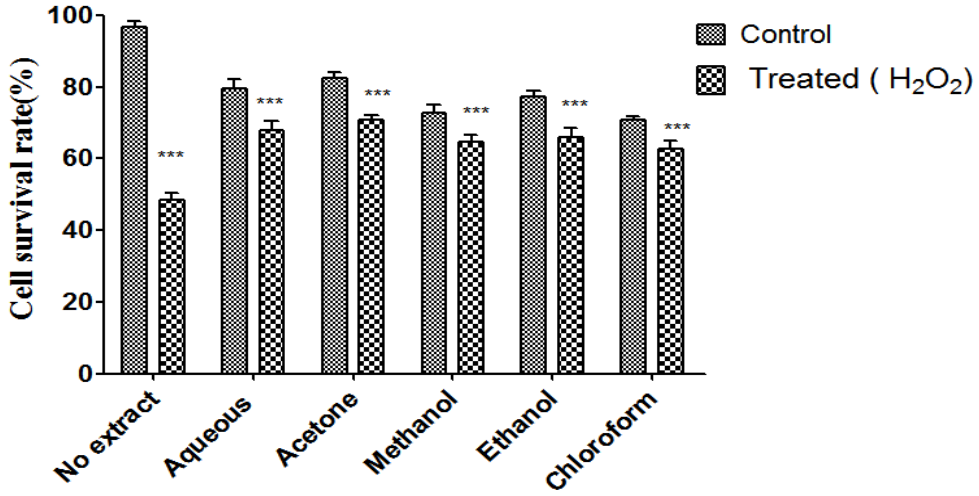


Figure 7.4. SRB assay of different extract (0.1mg/ml) with H_20_2 as compared to control (n=3, mean±S.D, ANOVA at $p<0.05$),*** Control vs. treated (statistically extremely significant value)

H_20_2 treated cell only showed the least survival (47.97%, P<0.05).SRB assay also gave approximately similar results **(Figure 7.4).** Results showed the anti-apoptotic effect of different *Vicia faba* seed extract on the survival of H_2O_2-injured yeast cells. When extracts were added to the cells the number of surviving cells was increased in comparison to the apoptotic population. The results clearly indicated that seed extracts were reducing oxidative stress. Literature also supported that it may be results of ROS that include upregulation of antioxidants mediated by complex transcriptional changes, activation of pro-survival pathways such as mitophagy, and programmed cell death (PCD) which, apart from apoptosis, includes pathways such as autophagy and necrosis, a form of cell death long considered accidental and uncoordinated (Farrugia and Balzan, 2012) .

7.3.4. Estimation of antioxidant by ferric reducing antioxidant power (FRAP) assay

This method was based on the reduction of the ferric tripyridyl-s-triazine complex to a colored ferrous complex compound form in the presence of antioxidants. Antioxidants in the samples decreased ferric TPTZ complex to form a blue colored complex which was measured at a wavelength of 593 nm. The intensity of the color is related to the number of reductants in the samples. The activity was obtained to be highest in the case of acetone extract (20.03 μg ± 0.73, P<0.05) BHT equivalents (BE)/mg of the sample and the least was found in chloroform extract (12.25±0.31 BHT equivalents (BE), P<0.05)/mg of the sample **(Table 7.1).** The previous study confirmed that rich phenolic content of *V. faba* pods suggesting to be an interesting novel source for antioxidant activity animal nutrition, promoting product quality and consumers' health (Valente et al., 2018).

Table 7.1: The FRAP (Ferric reducing antioxidant power) assay.

Seed extract(mg/ml)	Antioxidant potential (µg BE/mg of sample)
Ethanol	20.02 ±0.73
Acetone	18.56±0.65
Aqueous	15.45±0.45
Methanol	13.34±0.54
Chloroform	12.25±0.31

These data are represented in means ± S.D (n=3).One way ANOVA (P<0.05) * Ethanol vs. Methanol (P<0.05) and **Ethanol vs. Chloroform are significant value

7.3.5. Oxidative stress analysis by different techniques

7.3.5.1. Cell Roughness study in yeast cell due to oxidative stress

The damaging effects of different concentrations of H_2O_2 (oxidative stress) on the morphology (roughness and dimensions of cavities appearing on cell surfaces) and physiology (cell viability, mean cell volume) were examined by AFM.

When treated with 3% hydrogen peroxide concentration roughness was maximum (441±6.7 nm) and along with extract treatment roughness decreased to be 251`±6.2 nm. Oxidative stress resulted in a decrease in the mean cell volumes of *S. cerevisiae* **(Figure 7.5**) and raised the cell roughness as they were exposed to increasing H_2O_2 concentrations for 1 h but on treatment with extract with H_2O_2, the effect was reversed (**Figure 7.5**). This rapid decrease in the mean cell volumes of stressed yeasts could be due to compression of the cells caused by elevated H_2O_2 concentrations. *S. cerevisiae* (**Figure 7.5**) clearly disclosed the formation of "cavities" on the cell surfaces as the H_2O_2 concentration was increased. No genuine cavities could be found on unstressed (Control)

yeast cell surfaces. The change in cell morphologies was due to the oxidative damages of cell membranes by oxidation of proteins and lipids that are well known to modify the membrane permeability and fluidity (Rueda-Jasso et al., 2004). The findings indicated that oxidative stress was responsible for the rapid decline of cell viabilities, a decrease of mean cell volumes, initial compression of cell walls followed by the formation of cavities on cell surfaces, changes in cell morphologies, an increase in surface roughnesses in *S. Cerevisiae. C*ell roughness was diminished due to antioxidant property of seed extract or polyphenols (phenolics, flavonoids or synergistic effect).

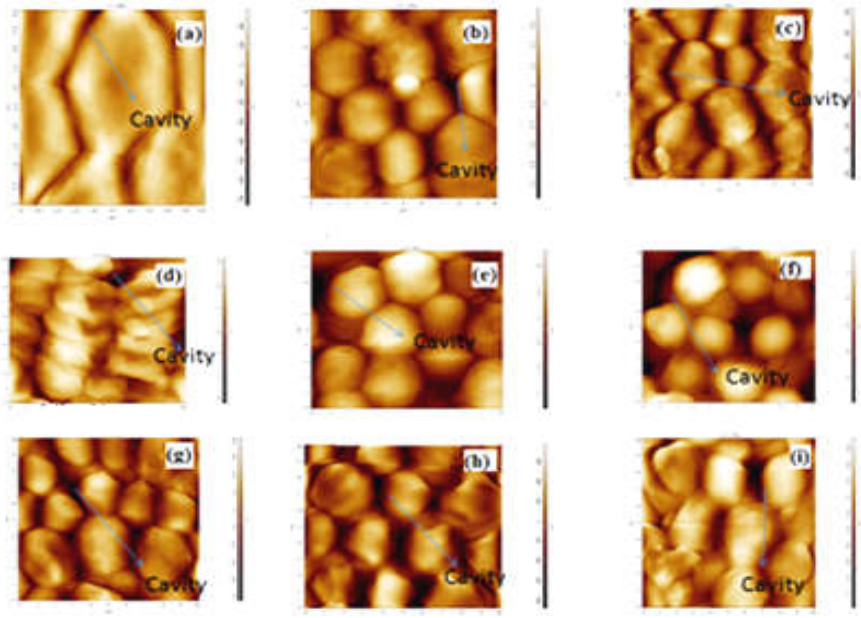


Figure 7.5. Morphological analysis of yeast cells by atomic force microscopy at different concentration of hydrogen peroxide **(a)** 0% H_2O_2 **(b)** extract **(c)** 0.5 % H_2O_2 **(d)** 1 % H_2O_2 **(e)** 2 % H_2O_2 **(f)** 3 % H_2O_2 **(g)** 1 % H_2O_2 +extract **(h)** 2 % H_2O_2 +extract **(i)** 3% H_2O_2+ extract (n=3,mean±S.D).

7.3.5.2. Effect of seed extract on ROS measurement in yeast cells

Yeast cells incubated with H_2O_2 showed a significant increase in ROS intensity (174±5.29, $P<0.05$) as compared to only control (142±2.64, $P<0.05$) and extract treated cell showed lesser ROS intensity (151±7.6, $P<0.05$) than H_2O_2 treated cells **(Figure 7.6)**.

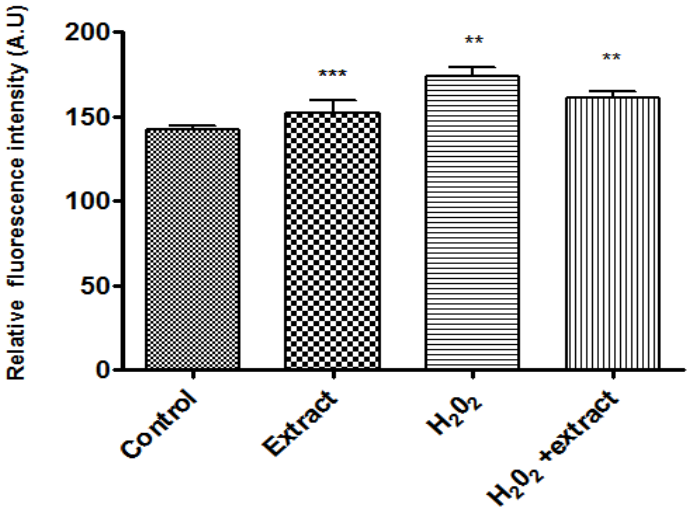


Figure 7.6. ROS measurement by DCF-DA (2',7'-dichlorofluorescein diacetate) assay with respect to control and extract (0.1 mg/ml)

(n=3, mean±S.D, One way ANOVA at $P<0.05$),*** Control vs. H_2O_2 (extremely significant value) , ** Control vs. H_2O_2 +extract and **Extract vs. H_2O_2 (significant value).

Phenolics, especially flavonoids and phenylpropanoids, are oxidized by peroxidase and act in H_2O_2 - scavenging, phenolic/ASC/POX system (Michalak, 2006). Similar another results demonstrated that retrieved intracellular ROS generation induced by HG (Shoaib et al., 2018). The study made by Han et al. (2014) also validated the ROS generation.

7.3.5.3. Detection of DNA fragmentation due to oxidative stress

Different concentration of hydrogen peroxide(1%,2%) and extract treated cells (T1, T2) showed more DNA fragmentation and smear formation than control (C), H_2O_2 and extract only **(Figure 7.7)**.

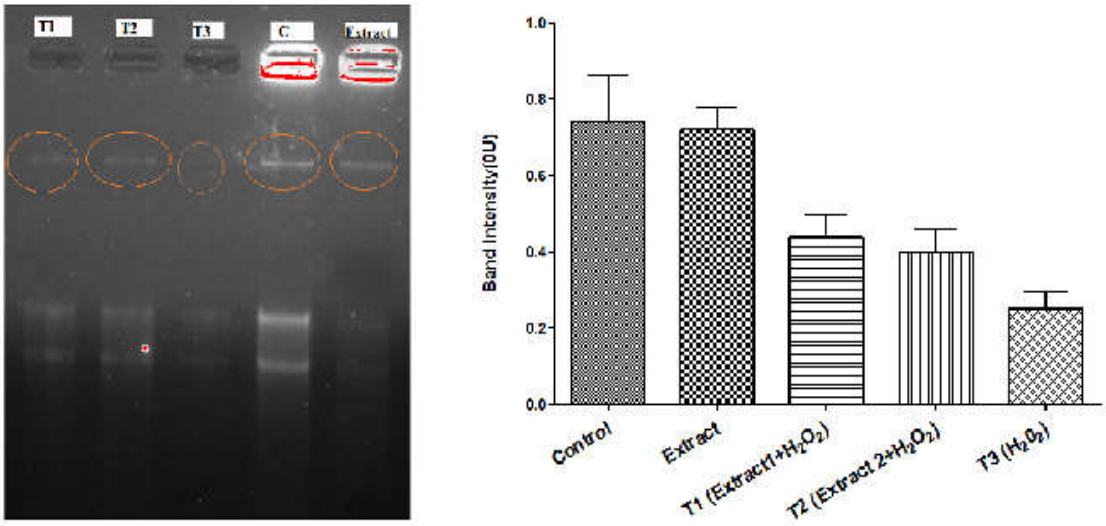


Figure 7.7. (a) Treated cell showing band fragmentation and smear formation with respect to control (b) Fig band intensity quantification (n=3,mean±S.D ,One way ANOVA at P<0.05 ⁎Control vs. T1 (Extract +1 % H_2O_2) and ⁎Control vs. T2 (Extract +2%H_2O_2) are significant values , ⁎⁎⁎Control vs. T3 (H_20_2) is extremely significant value.

Control showed more band intensity (0.74±0.21, P<0.05) than H_2O_2 treated cells (0.11±0.02,P<0.05) (Figure 8).These observations were consistent with a process that showed mammalian apoptosis, is enzyme dependent, degrades chromosomal DNA, and is activated only at low intensity of death stimuli (Ribeiro et al., 2006). Literature also supported that oxidized DNA also interferes with the normal response to environmental oxidative stress in yeast(Salmon et al., 2004) causing ROS accumulation and PCD in both *S. cerevisiae* and *S. pombe* (Burhans et al., 2003). Oxidative DNA damage and chromosome fragmentation are observed in ROS-mediated killing of yeast exposed to acetic acid (Ludovico et al., 2001) H_2O_2 (Madeo et al., 1999).

7.3.5.4. Qualitative and quantitative analysis of yeast cells like apoptotic nuclei, apoptotic cell population by confocal microscopy

The morphology of the cell nucleus was visualized by various DNA staining (PI and DAPI) (**Figure 7.8, Figure 7.9**).

Table 7.2: Quantitative analysis of yeast cell population subjected to *oxidative* stress by DAPI staining

Samples(mg/ml)	**Number of apoptotic cells per 100 cells**	**Apoptotic ratio**
Control	6 ± 0.05	0.06
Extract	9 ±0.06	0.09
H_20_2	40± 1.08	0.4
Extract + H_20_2	25±1.02	0.25

Table 7.3: Quantitative analysis of yeast cell population subjected to *oxidative* stress by propidium iodide staining

Samples(mg/ml)	**Number of apoptotic cells per 100 cells**	**Apoptotic ratio**
Control	6 ± 0.05	0.06
Treated (Extract)	9 ±0.06	0.09
H_20_2	40± 1.08	0.4
Extract + H_20_2	25±1.02	0.25

Induction of apoptosis on yeast cells was investigated by microscopic analysis of PI and DAPI. It was observed that numerous apoptotic cells with nuclear condensation and fragmentation(**Figure 7.8 Figure 7.9, Figure 7.10 Figure 7.11**). As the values in Table 3 indicated that the treatment of H_2O_2 caused a very high number of yeast cells to become permeable to PI and their % PI positive (dead cell) populations were found to be

40 ± 1.18% (P<0.05) with respect to control **(Table 7.2)**.Control having % PI positive was 0.06± 0.05 (P<0.05). DAPI was used to visualize the nuclear DNA in both living and fixed cells. It also forms nonfluorescent intercalative complexes with double-stranded nucleic acids (Bringezu et al., 2011).DAPI staining also confirmed that H_20_2 treated cell increased the apoptotic nuclei with respect to control.DAPI staining showed apoptotic nuclei of control, extract and H_2O_2 were found to be 8 ± 0.052 %, 12 ±0.065%, and 42± 1.16 (P<0.05) respectively **(Table 7.3**).

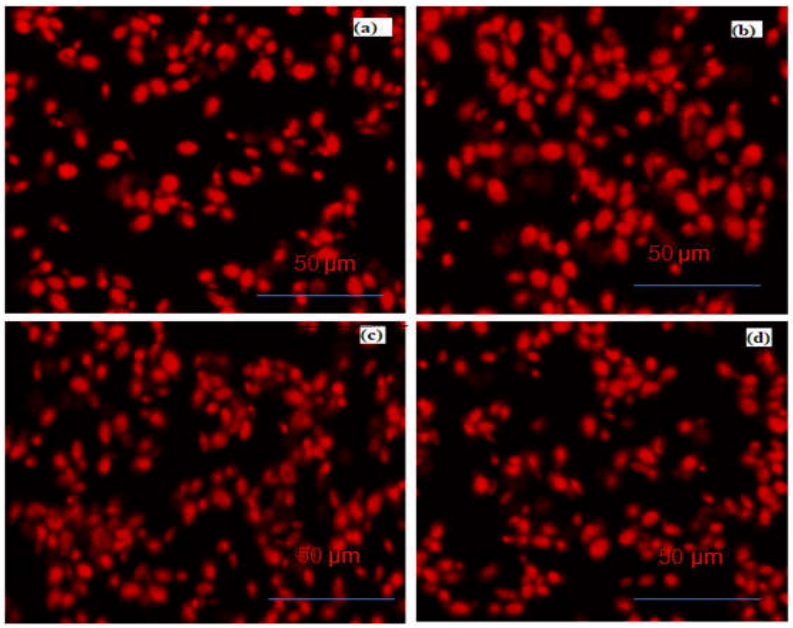


Figure 7.8. PI staining (Confocal microscopy) showing oxidative stress effect of seed extract and H_2O_2 on yeast cell population (a) Control (b) extract (c) H_2O_2 (d) H_2O_2 +extract

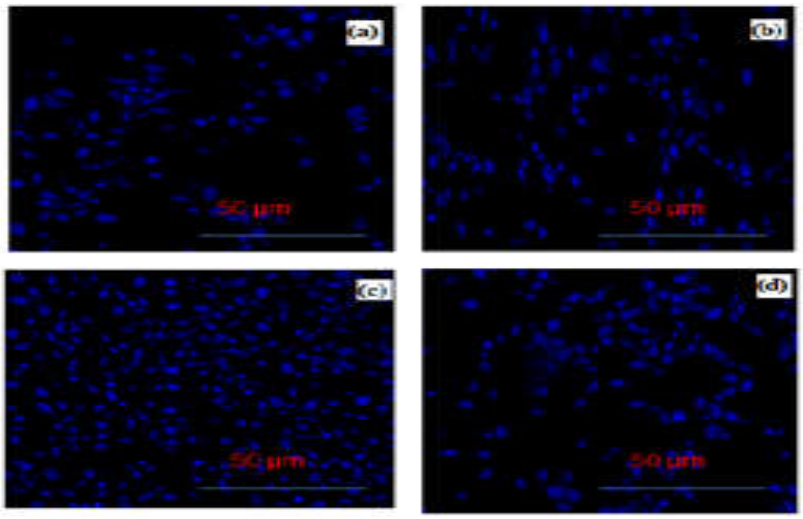


Figure 7.9. DAPI staining (Confocal microscopy) showing oxidative stress effect of seed extract and H_2O_2 on yeast cells nuclei. (a) Control (b) extract (c) H_2O_2 (d) H_2O_2 +extract

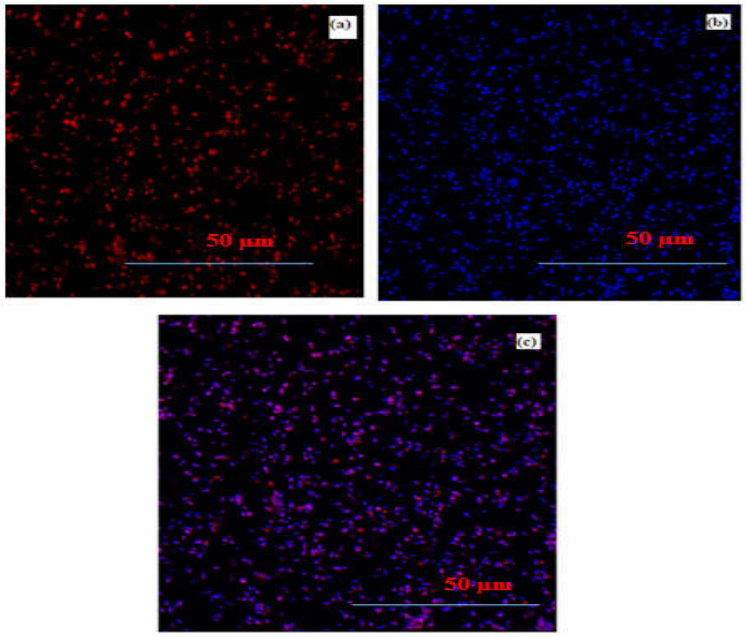


Figure 7.10. Apoptotic cell population study under a combination of different dye (Confocal microscopy) (a) Propidium iodide staining (b) DAPI staining (c) Merged image of PI and DAPI

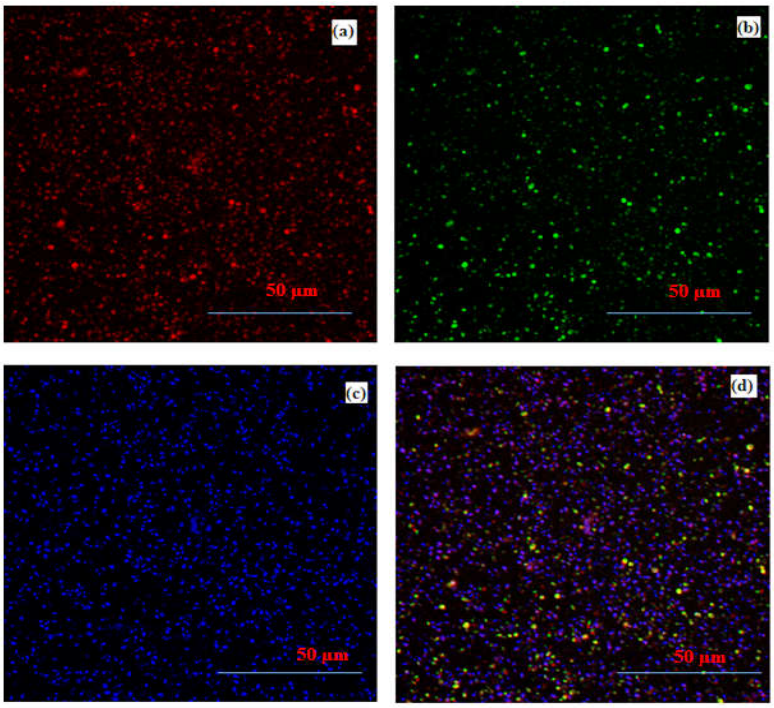


Figure 7.10. Apoptotic cell population study under a combination of different dye (Confocal microscopy) (a) Propidium iodide staining (B) DAPI staining (b) DCF staining (c) Merged image of PI, DAPI, and DCF.

7.3.5.5. Investigation of oxidative stress study by flow cytometry approach

Anti-oxidative properties of polyphenols as suggested in literature may contribute to the protection of yeast cells from H_2O_2 induced oxidative stress. H_2O_2 exposure increased the apoptosis rate of yeast cells from 8.20 % to 64.80% compared to the control, which was declined to 18.75% to 36.05 % respectively by extract and extracts with H_2O_2 treatment (**Figure 7.11**). The result suggested that seed extract pre-treatment markedly protected the cell death of yeast cells. The result was consistent with previous studies, which have also affirmed the protective effects of quercetin against oxidative stress (Nabavi et al., 2012)

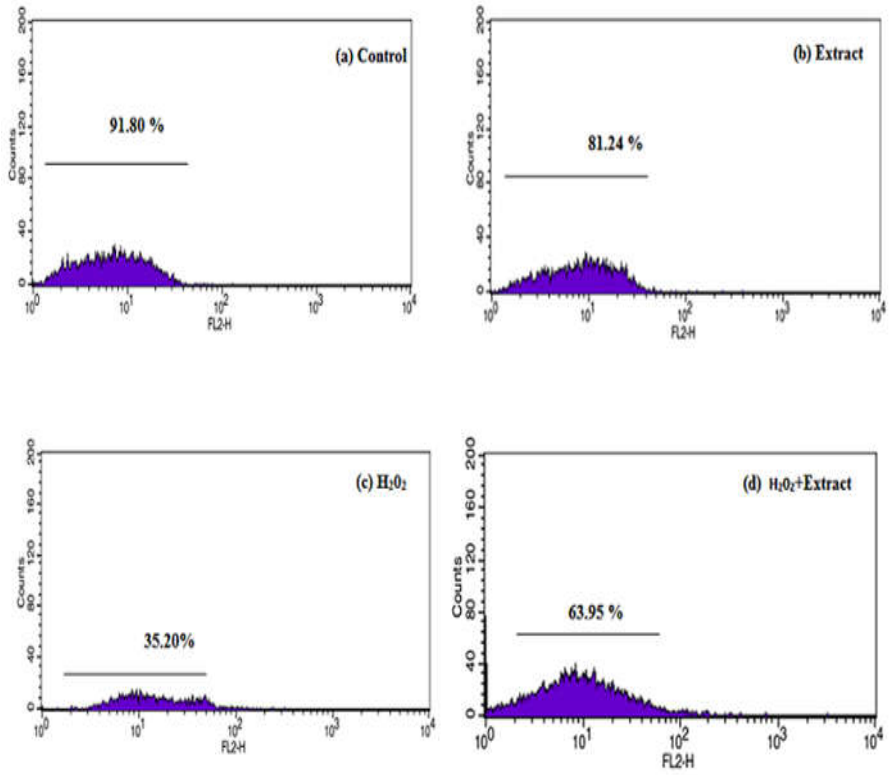


Figure 7.11. (a) Control (b) extract (C) H_20_2 (d) extract+H_2O_2 left side in the image represent lives cell and right side dead cells (Flow cytometry

7.3.5.6. Evaluation of ROS measurement in yeast cells by confocal microscopy

Yeast cells incubated with H_2O_2 showed a significant increase in ROS intensity (162±4.32, $P<0.05$) as compared to control (118±2.52, $P<0.05$). A cell in the presence of acetone extract showed lesser ROS intensity (138± 6.9, $P<0.05$) than H_2O_2 treated cells (**Figure 7.12**). Phenolics, especially flavonoids and phenylpropanoids, are oxidized by peroxidase and act in H_2O_2-scavenging, phenolic/ASC/POX system (Michalak, 2006). Normal cells show a weak cytosolic and a somewhat stronger mitochondrial-type DCF fluorescence pattern, while apoptotic cells demonstrate strong diffuse fluorescence (Shoaib et al., 2018). Furthermore, confocal microscopy results showed that fluorescence intensity was significantly increased after H_2O_2 treatment in yeast cell as evident from the

Figure 7.12. Reactive oxygen species (ROS) contribute to the progression of various human diseases, including type 2 diabetes mellitus (T2DM). ROS can suppress the insulin response and contribute to the development of insulin resistance, a key pathological feature of T2DM (Tiganis, 2011)

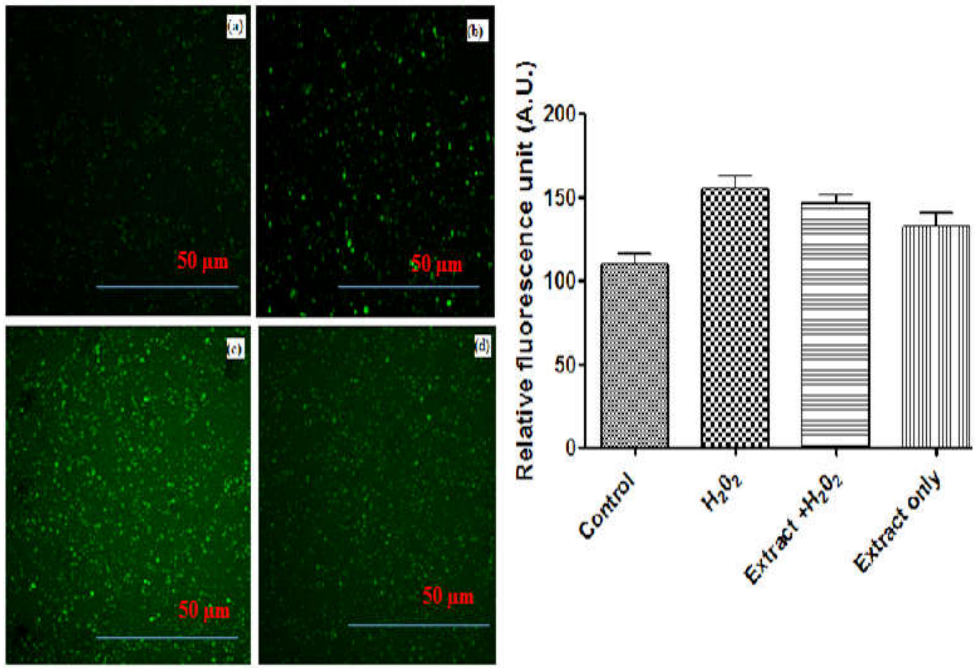


Figure 7.12. Measurement of ROS level by confocal microscopy (a) control **(**b)) H_20_2 **(**C**)** extract **(**d**)** extract+H_2O_2 (right side of image relative fluorescence intensity measured by Image J software)

7.3.6. Evaluation of glucose uptake study in yeast cells by confocal microscopy and flow cytometry

We examined changes in glucose uptake ability in yeast cells by different types of seed extract such as aqueous, methanol and ethanol., When comparison was made with 2-NBDG, it was found that with methanol extract had better fluorescence intensity (0.74±0.034) than NBDG(0.35+0.012) (**Figure 7.14**).

Flow cytometry approach also revealed that 2- NBDG (1.98 ±0.37) showed relative fluorescence intensity lesser than methanol (5.98 ±0.67) and acetone (4.43 ±0.55) seed extract**(Figure 7.13)**. Higher relative fluorescence intensity is an indication of more glucose uptake by yeast cells. Previous researchers have demonstrated that 2-NBDG is a fluorescent probe for glucose uptake in the yeast cells and Hxt1 is responsible for glucose uptake in yeast cells (Roy et al., 2015).

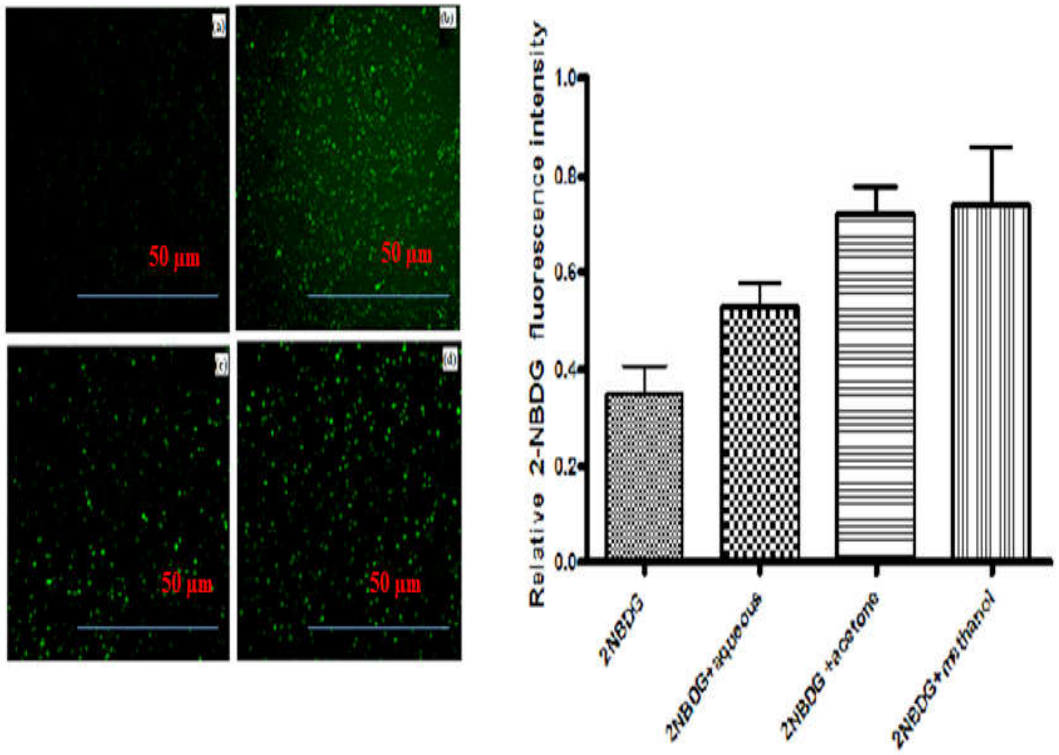


Figure. 7.13 Measurement of glucose uptake by confocal microscopy in 3T3-L1. Cells were seeded in 6-well plates and treated with 100 nM insulin and 10 µM 2-NBDG in the presence (0.1mg/ml) or absence of sample (0.1 (A) 2-NBDG (B) Aqueous seed extract (C) acetone seed extract (d) methanol seed extract (right side of image relative fluorescence intensity measured by Image J software).

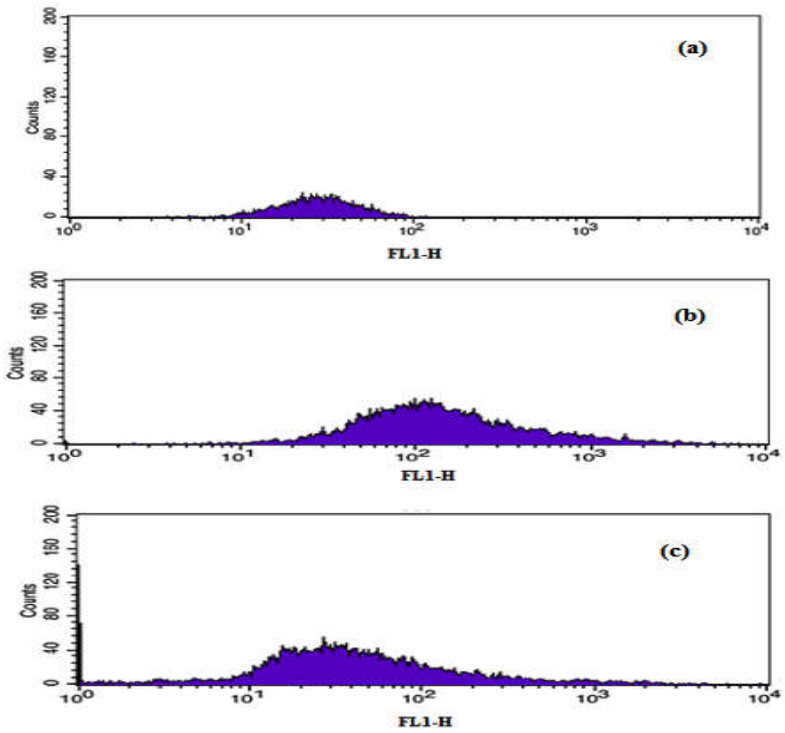


Figure 7.14. Evaluation of glucose uptake by flow cytometry in 3T3-L1. Cells were incubated within the presence or absence of samples (50 μM) for 1 hrs. After incubation, cells were washed with PBS and 2-NBDG uptake was calculated by a flow cytometer. (A) Control, (B) acetone extract (C) methanol seed extract.

7.4. Conclusion

The hypoglycaemic effect exhibited by the extracts of seed was mediated by increasing glucose adsorption, decreasing glucose diffusion rate and at the cellular level by promoting glucose transport across the cell membrane as revealed by utilizing simple *in vitro* model of yeast cells. A significant benefit of phytochemicals (polyphenols such as phenols and flavonoids) is thought to be capable of scavenging free radicals, lowering the incidence of chronic diseases. Role of oxidative stress in diabetes in their relationship with the antioxidant property of faba bean as an edible pulse might be an effective strategy for a person suffering from diabetes mellitus. Seed extract had a significant impact on oxidative stress in yeast cells. It might be due to results of phenolics

contents, flavonoids present in faba bean seed extract that shows the antioxidant oxidant properties or synergistic effects of all phytochemicals.