Chapter 8

Glucose Uptake and Oxidative Stress Studies of Polyphenols from Faba Beans on 3T3-L1 Cell Line

8.1. Introduction

Excessive production of reactive oxygen species (ROS) is the result of oxidative stress. Oxidative stress is also one of the factors for the development of insulin resistance, β-cell dysfunction, impaired glucose tolerance and *type 2 diabetes mellitus* (*T2DM*) (Ceriello and Motz, 2004; Newsholme et al., 2007). Free radical production is balanced by the antioxidative defense system, however, polyphenols (gallic-acid, ellagic-acid, catechin, epicatechin) acquire propensity to neutralize excessive free radical generation due to antioxidant property.

Recently, growing interest has emerged on the beneficial effects of dietary polyphenols for the prevention of chronic diseases including obesity, diabetes, and cardiovascular disease. Highly rich in phenolic compounds, flavonoids, terpenoids and other constituents are responsible for the reduction in blood glucose levels. (Goel et al., 2012; Patel et al., 2012). Among phytochemicals, phenolic compounds are gaining increasing interest for their health-promoting properties(Hemingway and Laks, 2012). Polyphenols in Faba beans are located in several parts of the plant (e.g. leaves, roots, and seeds) (Baginsky et al., 2013)

Faba beans **(***Vicia faba***)** possess diverse bioactive constituents such as ellagic-acid, gallic-acid, ellagitannins and gallotannins etc. and widely known for pharmacological effects like antioxidant, antidiabetic and antibacterial (Turco et al.,

2016). Recently, the anti-diabetic potential of polyphenols had been linked to reducing oxidative stress in experimental diabetes by M. Sabu.et al.2002 (Sabu et al., 2002). Therefore, the present study focused on the effect of polyphenols from faba bean on the oxidative study and glucose uptake in 3T3-L1 cell line.

8.2. Experimental

8.2.1. Seed material

Detail descriptions of faba beans and its extraction, purification, and characterization have been described in Chapter 3.

8.2.2. Chemicals and reagents

2-NBDG (Sigma Aldrich), 2′,7′ dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma-Aldrich, USA), propidium iodide (PI, Himedia, India), DAPI (Himedia, India) dexamethasone (Himedia, India), oil red, 4′, 6′- diamidino-2 phenylindole dye (DAPI, Himedia, India), DMEM (Himedia, India), fetal bovine serum (FBS, Himedia, India), 3-(4,5-dimethylthiazol- 2-yl) - 2,5-diphenyltetrazolium bromide (MTT, Himedia, India). Penicillin and streptomycin antibiotics and other biochemical materials were purchased from Himedia, India. All the other reagents were of high purity and of analytical grade.

8.2.3. Cell culture and differential assay

The 3T3-L1 cell line of mouse muscle fibroblasts was bought from NCCS, Pune, India. 3T3-L1 preadipocytes culture and differentiation protocol were described previously(Nakashima et al., 2000). Cells were cultured and maintained in DMEM containing 10% FBS, and 100 µl/ml streptomycin, 100 U/ml penicillin in a 5% CO_2 at 37°C constant temperature in $C0_2$ incubator. After 80% confluence, the cells were transferred to the differential medium, 0.5mM methylisobutylxanthine, 0.25 µM

dexamethasone, DMEM containing 10% FBS and 1 µg/ml insulin, for 2 days. After that cells were incubated in DMEM containing 10% FBS and insulin (1 µg/ml) for 2 days. Later, the cells were subcultured in 10% FBS-DMEM.

8.2.4. Oil red o staining

3 T3-L1 confluent cells (80%) were washed two times with phosphate buffer solution (PBS) and then fixed for 1 h at room temperature with 10% formalin, prepared in PBS. At that point, cells were washed two times with PBS. Then, cells were washed with PBS and stained with a filtered Oil Red solution (0.5% in 60% isopropanol) for 1 h. At last, cells were washed twice with distilled water.

8.2.5. MTT assay

Cell viability of 3T3-L1 preadipocytes was followed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. First of all, preadipocytes cells (1× 10^4 cells/well) was seeded out in 96- well-tissue plates properly and cultured for 3 days. Then the cells were washed once in PBS and treated with 1.0 mg/mL concentrations of different seed extract (0.5 mg/ml) and incubated for 24 h. All the seed extract samples were removed and 10µl of MTT solution (0.5 mg/mL) was added to each well. Cells were treated with different faba bean seed extract and incubated at 37 °C for 1 h in 96-well plates. Finally, 150µl DMSO (0.1-0.5%) was added to dissolve the formazan crystals. Absorbance was evaluated by spectrophotometer at 540 nm.

8.2.6. Oxidative stress study through different approaches

8.2.6.1. ROS evaluation by DCF-DA (2',7'-dichlorofluorescein diacetate) assay

This method is extensively applied for the profile the oxidative status of living cells (Keston and Brandt, 1965). 3T3-L1 cells (1× 10^4 cells) were treated with H_20_2 or insulin and extract and incubated at 37°C in CO_2 for 1 hrs with All the samples were washed with PBS and added with 20 µM H2DCFDA. Air drying was utilized for 1 hrs at

room temperature. Cells were analyzed using the LSM510 confocal microscope (Zeiss, Jena, Germany)

8.2.6.2. Oxidative stress study through flow cytometry approach

Trypsinization method was utilized to harvest treated and untreated cells and wash with phosphate buffer saline and resuspended the pellet in PBS. 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.

8.2.6.3. DAPI staining

DAPI staining protocol was followed by the modified procedure (Rashmi et al., 2003). 3% paraformaldehyde was applied for fixation of control and treated cells. Cells were permeabilized with 0.2% Triton X-100 in phosphate buffered saline (PBS).20 µl of 5 mg/mL of DAPI was added into each sample and air dried it. Then samples were visualized by ZEISS LSM780 confocal microscopy.

8.2.6.4. Propidium iodide staining

PI staining protocol was used to differentiate apoptotic cells from normal cells, which displays nuclear changes during apoptosis (Sarker et al., 2000). 3T3-L1 cells (1×10^4 cells) were treated with H_20_2 and extract for 1 hr at 37°C. It was permeabilized with a combination of acetone: methanol (1:1) at 37°C for 10 minutes. Then, PI (10 µL) was

added to each sample and fixation was done by air. It was observed under ZEISS LSM780 confocal microscopy.

8.2.6.5. Atomic force microscopy

The primary point of utilizing, this technique was to know the impact of hydrogen peroxide and seed extract on yeast cells in terms of cell roughness. Hydrogen peroxide (H_2O_2) is regularly utilized used as a source of oxygen-derived free radicals. Control (1×10^4 cells) and treated *cells (*1×10^4 cells) were visualized under AFM model-Nt-Mdt, Russia for the surface morphological changes. The cell samples had been scanned in semi contact modes and experiments were accomplished in the air at room temperature (Adya et al., 2005). The air-drying was executed to immobilize to cells on the slide.

8.2.6.6. Oxidative stress through scanning electron microscopy

Scanning electron microscopy (SEM) is extensively used to study structural details on the surface of biological samples. The conventional sample preparation process for SEM includes fixation, dehydration, drying, and optionally, conductive coating. Cells were cultured at 37°C and 5% CO_2 in DMEM supplemented with 10% FBS. Sterile coverslip per well was inserted in 6 cell-well plates. 1×10^4 cell were then treated with extract and H_20_2 for 60 minutes at 37 ºC. Then Primary fixation of cells was performed with freshly made 3.0% glutaraldehyde or and 0.1% glutaraldehyde in PBS for 20 min at room temperature. Post-fixation was done by 1% OsO_4 in osmium tetroxide (OsO_4). The samples were then dehydrated by a graded series of ethanol (50%, 60%, 70%, 80%, 90%, 95% and three times 100%) at 20-minute intervals. Following dehydration, the solvent was removed by either air drying with HMDS (hexamethyldisilazane). the dehydrated specimens were immersed with HMDS for 20 minutes for HMDS drying, Then HMDS was decanted, and the samples were left under a hood to air-dry at room temperature.

Optionally, the conductive coating was performed. SEM microscope model was EVO - Scanning Electron Microscope MA15 / 18 (CARL ZEISS microscopy).

8.2.7. Glucose uptake study in 3 T3-L1 cell line

8.2.7.1. Estimation of glucose uptake study in 3T3-L1 cells by confocal microscopy

Cultures were grown until 80% confluent where the medium was removed, and culture plates were rinsed three times in phosphate buffer. Coverslips were inserted in 6 wells microtiter well plate. Cells ($1\times10^{4)}$ were then incubated at 37°C with 200 μM 2-NBDG and extract in the presence of phosphate buffer. After 1 h, buffers were removed. 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.

8.2.7.2. Glucose uptake assay by flow cytometry

The glucose uptake assay was performed as previously described with slight modification(Chen et al., 2010). Briefly, cells were seeded in 6-well plates at a density of 1×10^4 cells/well and incubated overnight. The medium was then replaced with the glucose- and serum-free DMEM containing insulin (100 nM), 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). After incubation for 24 h, the medium was removed and cells 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 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 $F_{Ibackground}$ is the fluorescence intensity in the absence of 2-NBDG. Relative fluorescence intensity (FI) was calculated image J software.

8.3. Results and Discussion

8.3.1. Cell differentiation study

3T3-L1 preadipocytes appeared to have a fusiform and flattened morphology and some cell converted into a 'rounded-up' morphology after initial 24 hrs observations(**Figure 8.1)**.

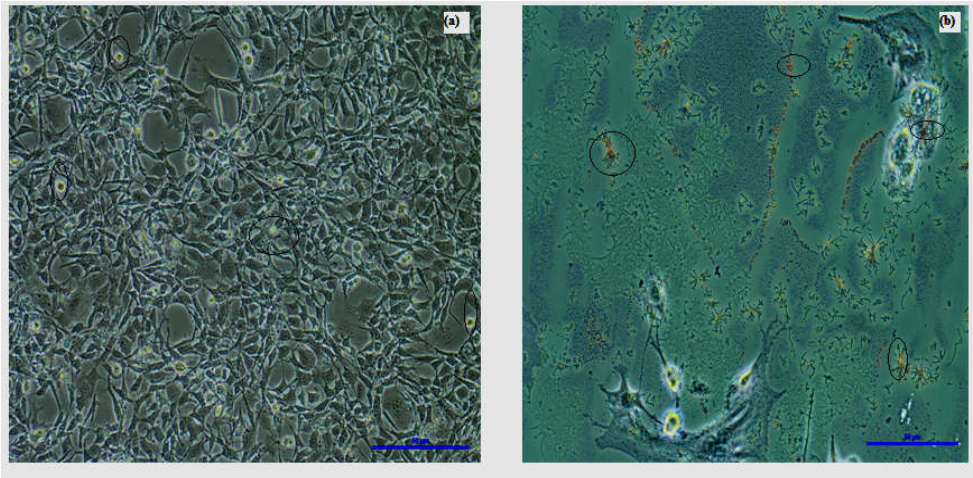

Figure 8.1. Phase contrast microscopy observation (a) circle represents lipid droplet after differentiation assay (b) spherical red color shows the presence of oil or lipid droplet after 24 hrs

The fat droplets in 3T3-L1 cells were observed by phase contrast microscopy(**Figure 8.1**). Lipid droplets were visualized by Oil Red O staining under

phase contrast microscopy. Their size and numbers were gradually increased in a differentiating time-dependent manner. Cells were differentiated into 3T3-L1 adipocytes which revealed features of adipose cells, counting the expression of lipid metabolic genes and the production of lipid droplets(Yagi et al., 2004).

8.3.2. Cell viability study

Maximum survival rate was found in acetone seed extract (87.067 %, $P<0.05$) as compared to control (97.36%, $P<0.05$) and chloroform seed extract had least survival potential (75.48%, $P<0.05$) (**Figure 8.2**). Literature also authenticated that acetone extract is effective for cell survival. (Cheng et al., 2003).

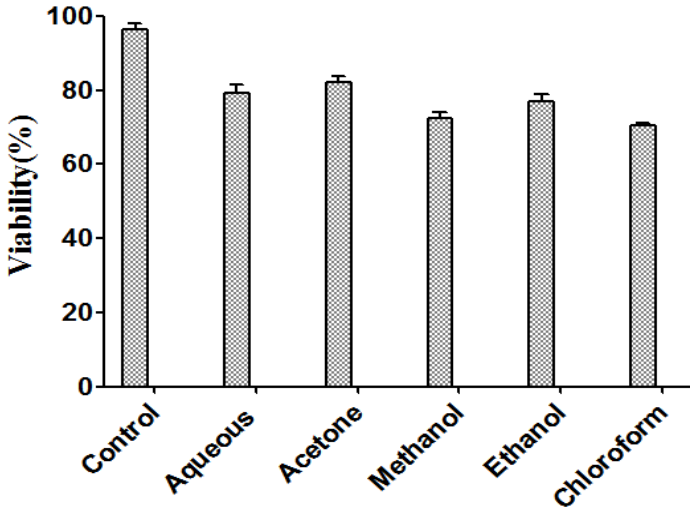

Figure 8.2. MTT assay represents the effect of different seed extract (0.1mg/ml) on cell viability with respect to control

8.3.3.Oxidative stress analysis through different techniques.

8.3.3.1. Investigation of oxidative stress study by flow cytometry approach

Anti-oxidative properties of polyphenols as suggested in literature may contribute to the protection of 3T3-L1 cells from H_2O_2 induced oxidative stress. H_2O_2 exposure

increased the apoptosis rate of 3T3-L1 Cells from 9.72% to 41.66% compared to the control, which was declined to 17.55% to 34.53 % respectively by extract and extracts with H_2O_2 treatment (**Figure 8.3**). The result suggested that seed extract pre-treatment markedly protected the cell death of 3T3-L1 cells. The result was consistent with previous studies, which have also affirmed the protective effects of quercetin against oxidative stress (Nabavi et al., 2012).

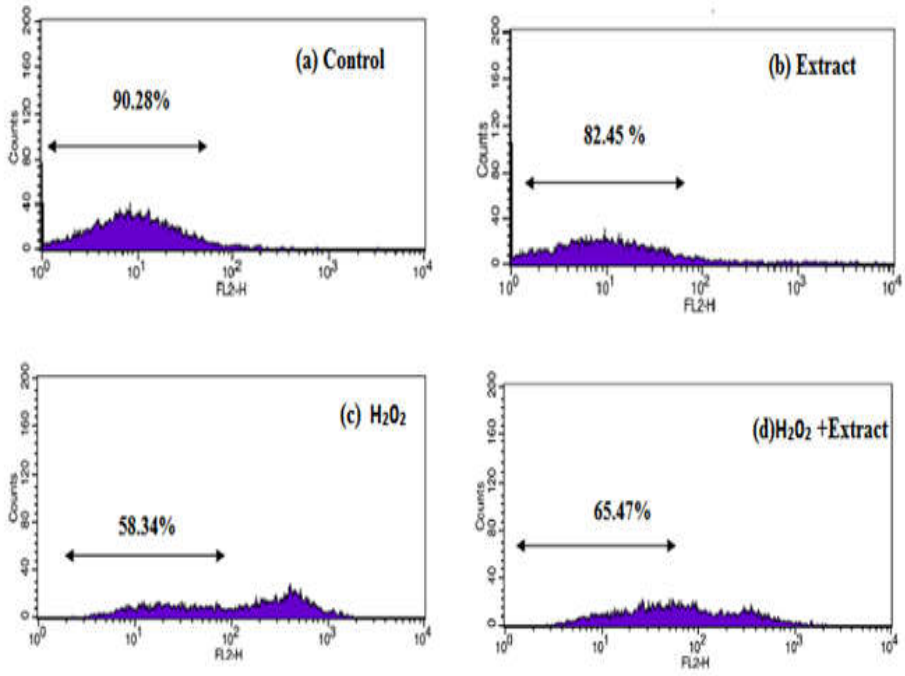

Figure 8.3. (a) Control (b) extract (C) H_20_2 (d) extract+H_2O_2 left side in the image represent lives cell and right side dead cells (Flow cytometry**)**

8.3.3.2. Evaluation of ROS measurement in 3T3-L1 cells by confocal microscopy

3T3-L1 cells incubated with H_2O_2 showed a significant increase in ROS intensity (172±4.29, $P<0.05$) as compared to control (137±2.64, $P<0.05$). A cell in the presence of acetone extract showed lesser ROS intensity (143± 6.6, $P<0.05$) than H_2O_2 treated cells (**Figure 8.4**). 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 either insulin stimulation or H_2O_2 treatment in 3T3-L1 cell line as evident from the **Figure 8.4**. These results demonstrated that H_2O_2, similar to insulin treatment, could increase intracellular ROS generation and change the redox balance in 3T3-L1 cells. 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)

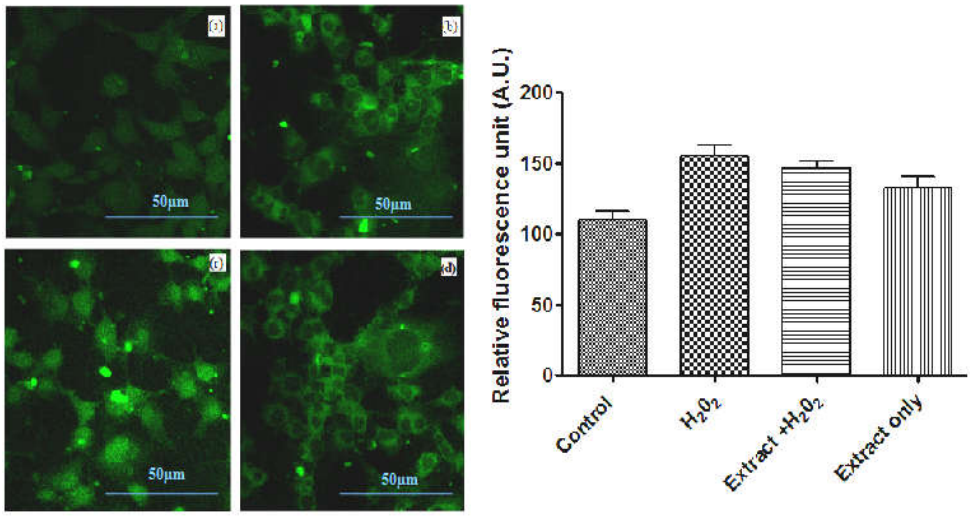

Figure 8.4. Measurement of ROS level by confocal microscopy (a) control **(**b) H_20_2 **(**C**)** extract **(**d) **e**xtract+H_2O_2 (right side of image relative fluorescence intensity measured by Image J software)

8.3.3.3. Qualitative and quantitative analysis of 3T3-L1 cells like apoptotic nuclei, apoptotic cell population by confocal microscopy

Some cells in population on PI, DAPI, and DCF staining was shown apoptotic cells based on their characteristic morphology that includes chromatin condensation, nuclear fragmentation, plasma membrane blebbing, contained condensed cytoplasm, dense bright green fluorescence as observed in **Figure 8.5, Figure 8.6, Figure 8.7, Figure 8.8.** Induction of apoptosis on 3T3-L1 cells was investigated by microscopic analysis of PI and DAPI. We observed numerous apoptotic cells with nuclear condensation and fragmentation **(Figure 8.7 and Figure 8.8).** Table 3 indicated the effect of H_2O_2 on 3T3-L1 cells as it becomes permeable to PI and their % PI positive (dead cell) populations were found to be 35 ± 2.62% ($P<0.05$) with respect to control (**Table 8.1**). Control having % PI positive was 0.08± 0.04 ($P<0.05$). 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 7 ± 0.042 %, 9 ±0.065% and 30± 2.54 ($P<0.05$) respectively (Table 4). It was evident from (**Table 8.1 and Table 8.2**) extract was efficient for reducing oxidative stress in terms of % apoptotic nuclei and % cell death. The reason for using PI and DAPI in tandem was to measure the apoptosis and necrosis as DAPI enters all cells while PI only enters in necrotic cells or those undergoing late apoptosis (or secondary necrosis/necrosis) when membrane integrity is lost (**Figure 8.7, Figure 8.8**). Literature also supported the findings that regardless of the type of cell death, loss of membrane integrity is correlated to nuclear morphology in the same cell (Darzynkiewicz et al., 1992). Researchers have supported that H_2O_2 treatment was responsible for inducing a proliferation arrest associated with an increase in mitochondrial content in 3T3-L1 preadipocytes,

preconditioning with some major dietary polyphenols totally or partially protects the cells against oxidative stress consequences.

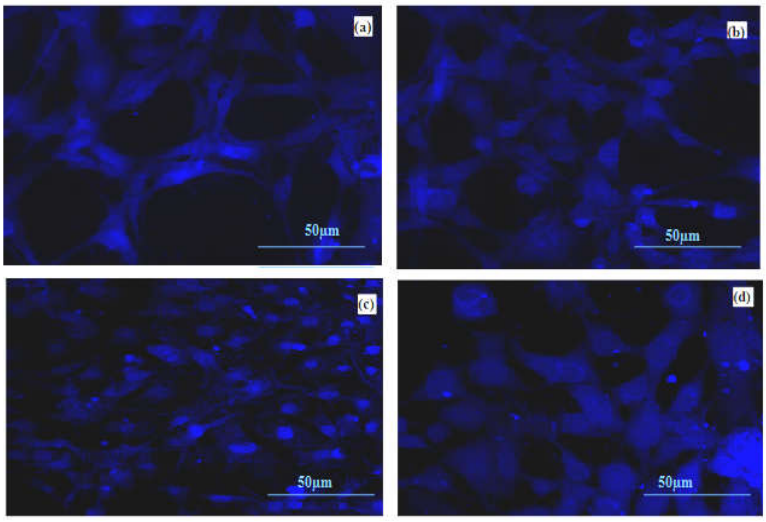

Figure 8.5. DAPI staining (Confocal microscopy) showing oxidative stress effect of seed extract and H_2O_2 on 3T3-L1 cells nuclei. (a) Control (b) extract (c) H_2O_2 (d) H_2O_2 +extract.

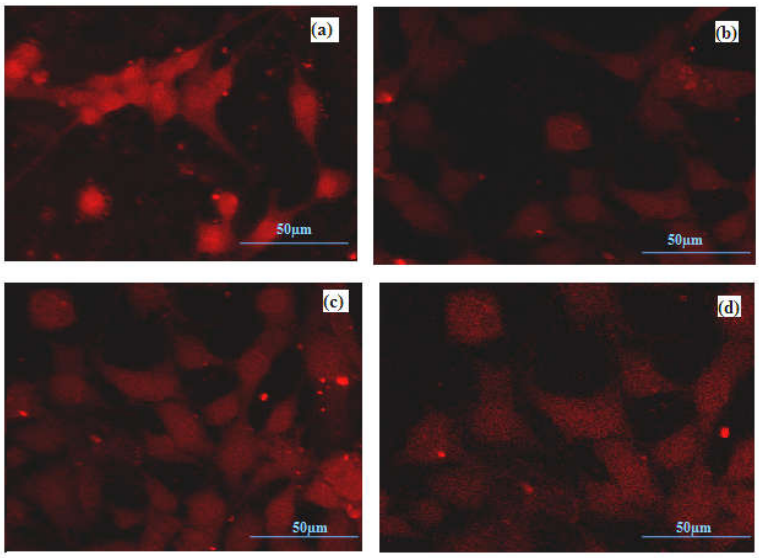

Figure 8.6. Propidium iodide staining (Confocal microscopy) showing oxidative stress effect of seed extract and H_2O_2 on 3T3-L1 cell population (a) Control (b) extract (c) H_2O_2 (d) H_2O_2 +extract

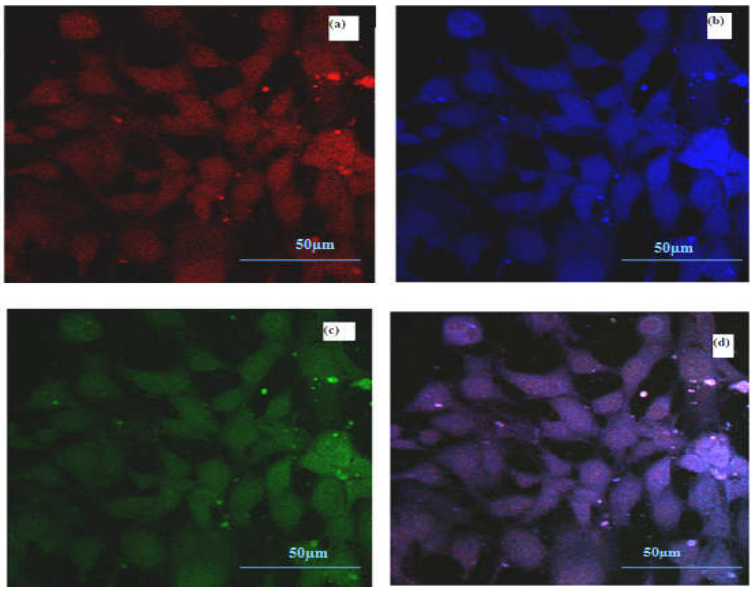

Figure 8.7. Apoptotic cell population study under a combination of different dye (Confocal microscopy) (a) Propidium iodide staining (B) DAPI staining (b) DCF staining (c) Merged image of PI, DAPI, and DCF

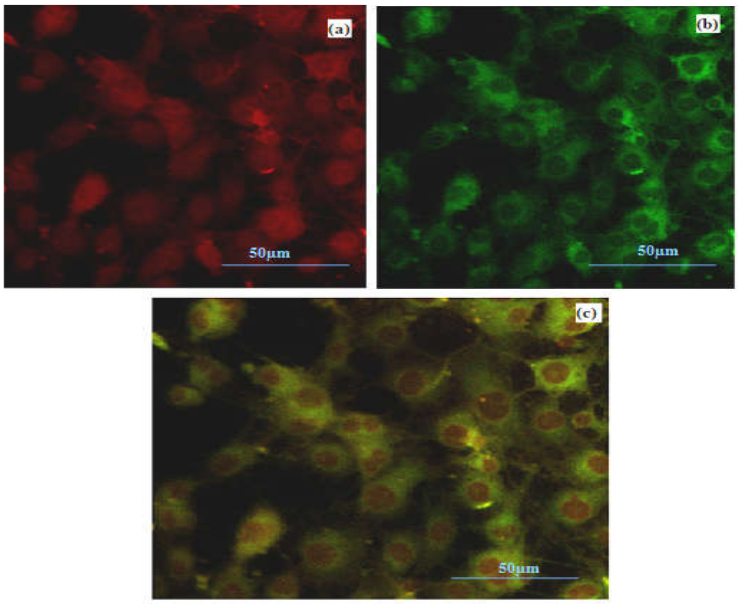

Figure 8.8. Apoptotic cell population study under a combination of different dye (Confocal microscopy) (a) Propidium iodide staining (b) DCF staining (c) Merged image of PI and DCF.

Table 8.1: Quantitative analysis of 3T3-L1 cell population subjected to *oxidative* stress by propidium iodide staining

Samples(mg/ml)	Number of apoptotic cells per 100 cells	Apoptotic ratio
Control	7 ± 0.042	0.07
Extract	9 ±0.065	0.09
H_20_2	30± 2.54	0.30
Extract + H_20_2	22±1.64	0.22

Table 8.2: Quantitative analysis of nuclear change in 3T3-L1 cell population subjected to *oxidative* stress by DAPI staining

Samples(mg/ml)	Number of apoptotic cells per 100 cells	Apoptotic ratio
Control	8 ± 0.04	0.08
Extract	10 ±0.05	0.10
H_20_2	35± 2.62	0.35
Extract + H_20_2	26±1.72	0.26

8.3.3.4. Roughness study in 3T3-L1 cell population due to oxidative stress

Cells on hydrogen peroxide treatment roughness were maximum (182.5±17.3 nm) and cells on treatment with H_2O_2 in combination with extract roughness decreased to be 208±12.2. Oxidative stress resulted in a decrease in the mean cell volumes of the 3T3-L1 cell **(Table 8.3**) whereas when the cells were exposed to H_2O_2 concentrations for 1 hr, it raised the cell roughness but on treatment with extract with H_2O_2 the effect was reversed (**Figure 8.9**).

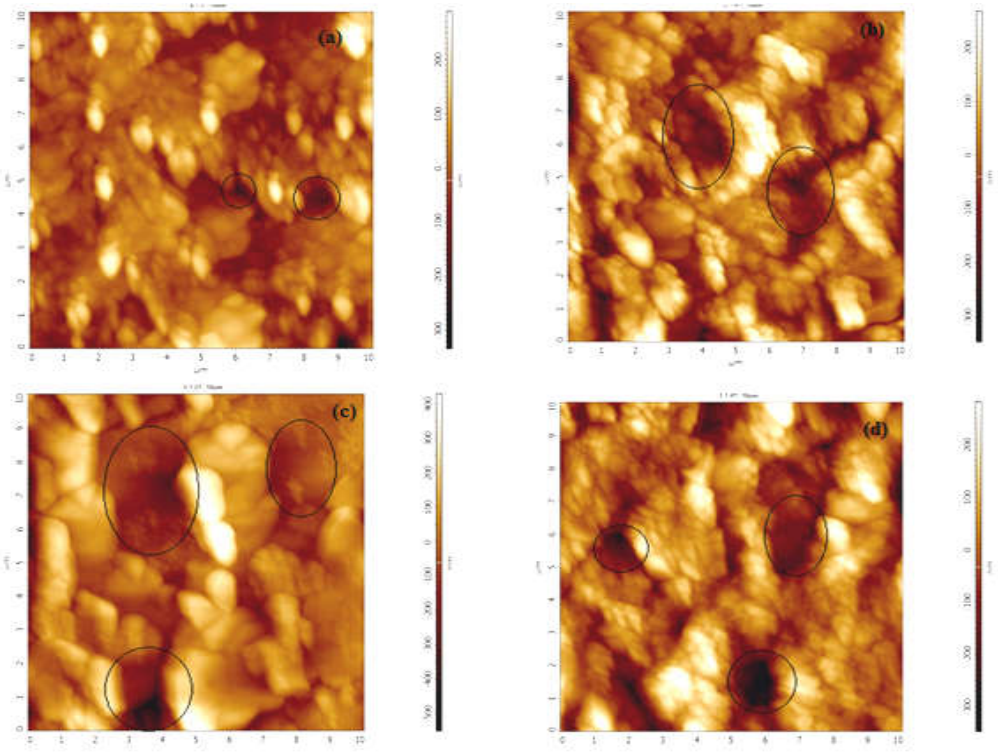

Figure 8.9. Atomic force microscopy shows cell roughness((nm) and mean cell volume (μm^3) Control **(**b) extract (0.1mg/ml) **(**C) H_20_2 **(**d) **e**xtract+H_2O_2 (circle represents cavity)

Figure 8.9 clearly indicated the formation of "cavities" on the cell surfaces as the H_2O_2 concentration was increased. 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, and initial compression of cell walls followed by the formation of cavities on cell surfaces, changes in cell morphologies and increase in surface roughness in the 3T3-L1 cell. *C*ell roughness was diminished might be due to the presence of polyphenols (phenolics, flavonoids or synergistic effect) in seed extract. Finding by other researchers also authenticated the observations made in the present study (Daw et al., 2013).

Table 8.3: Analysis of cell roughness (nm) and mean cell volume (μm^3) of Control **(**b) extract **(**C) H_20_2 and (d) **e**xtract+H_2O_2 by atomic force microscopy.

Sample	**Cell roughness (nm)**	**Mean cell volume (μm^3)**
Control	120.8±12.8	266±16.4
Extract	134.8±16.4	245±15.6
H_2O_2	182.5±17.3	208±12.2
Extract + H_2O_2	162.32 ±14.5	235±14.8

8.3.3.5. Oxidative stress analysis by SEM

Surface morphology (spanning topographic features) of the cell image of treated and untreated was observed by SEM. Control showed fibroblast-like morphology (**Figure 8.10**). In addition, H_20_2 treated cells exhibited an altered morphology, as evident from **Figure 8.10,** a larger and more irregular, three-dimensional (3D)-cuboidal or polygonal morphology was seen. Literature also authenticated that H_2O_2 treatment induces a proliferation arrest associated with an increase in mitochondrial content in 3T3-L1 preadipocytes (Baret et al., 2013).

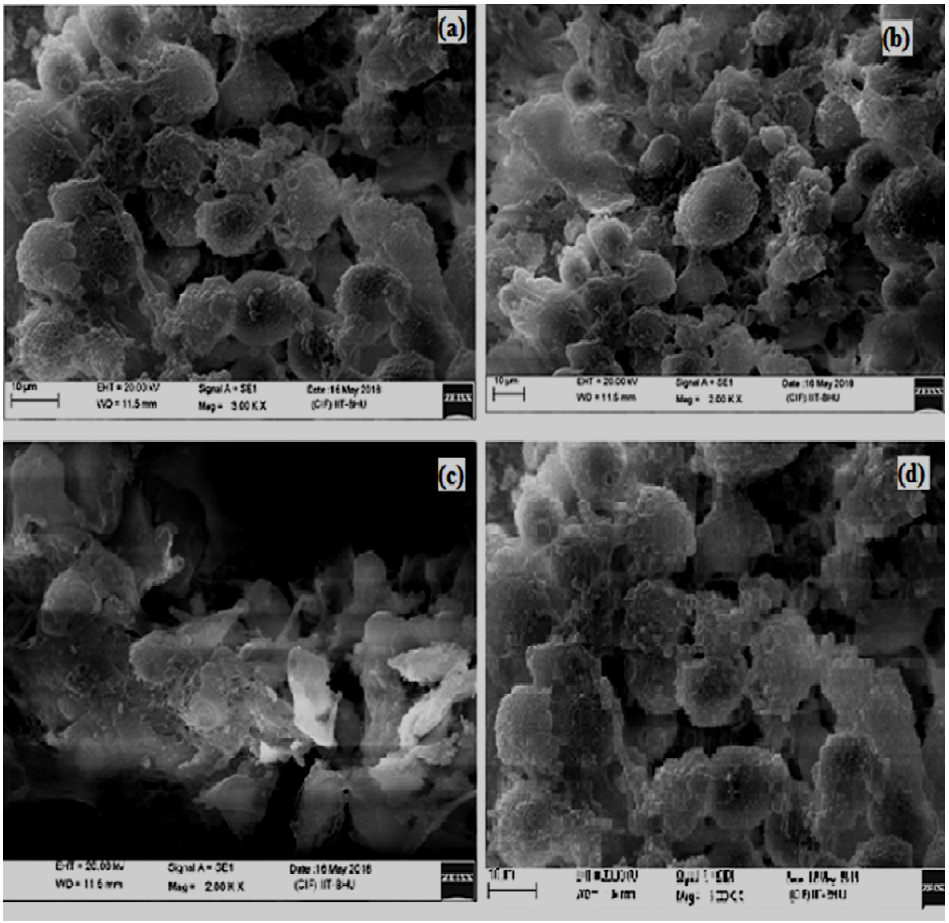

Figure 8.10. SEM Showing surface morphology of 3T3-L1 cell (oxidative stress study) (a) Control (b) Extract (c) H_20_2 (d) H_20_2 +extract.

8.3.4. Evaluation of glucose uptake study in 3T3-L1 cell line by confocal microscopy and flow cytometry

We examined changes in glucose uptake ability in 3T3-L1 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 acetone extract had better fluorescence intensity (0.86±0.031) than NBDG (0.45+0.016) (**Figure 8.11**).

Flow cytometry approach also revealed that 2- NBDG (2.08 ±0.31) showed relative fluorescence intensity lesser than acetone (4.98 ±0.62) and aqueous (3.52 ±0.52) seed extract **(Figure 8.12**).

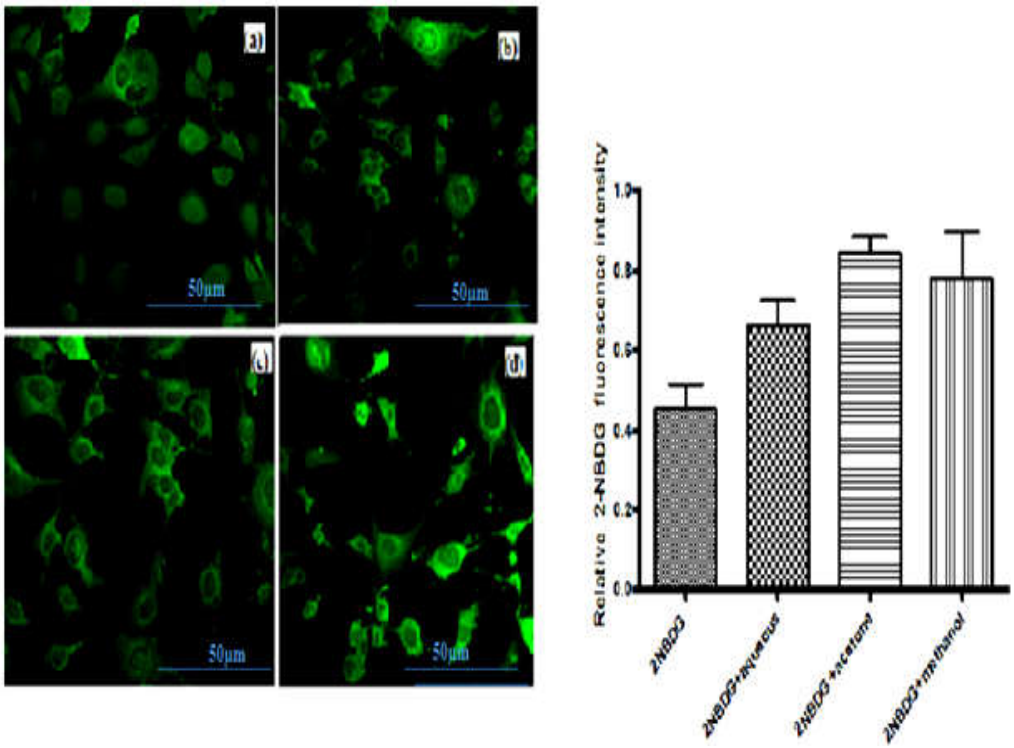

Figure 8.11. Measurement of glucose uptake by confocal microscopy in 3T3-L1. Cells were seeded in 6-well plates and treated with 100 nM insulin and 10 µM 2-NBDG in the presence (0.1mg/ml) or absence. (A) 2-NBDG (B) aqueous seed extract (C) acetone seed extract (d) methanol seed extract (right side of image relative fluorescence intensity measured by Image J software).

Higher relative fluorescence intensity is indicative of more glucose uptake by 3T3 -L1 cells. PPARγ agonists are responsible for glucose transport by modulating the expression of several genes involved in glucose uptake (Picard and Auwerx, 2002).

Literature also indicated that ethanol extract may promote glucose uptake in 3T3-L1 preadipocytes via the insulin signaling pathway (Takahashi et al., 2008).

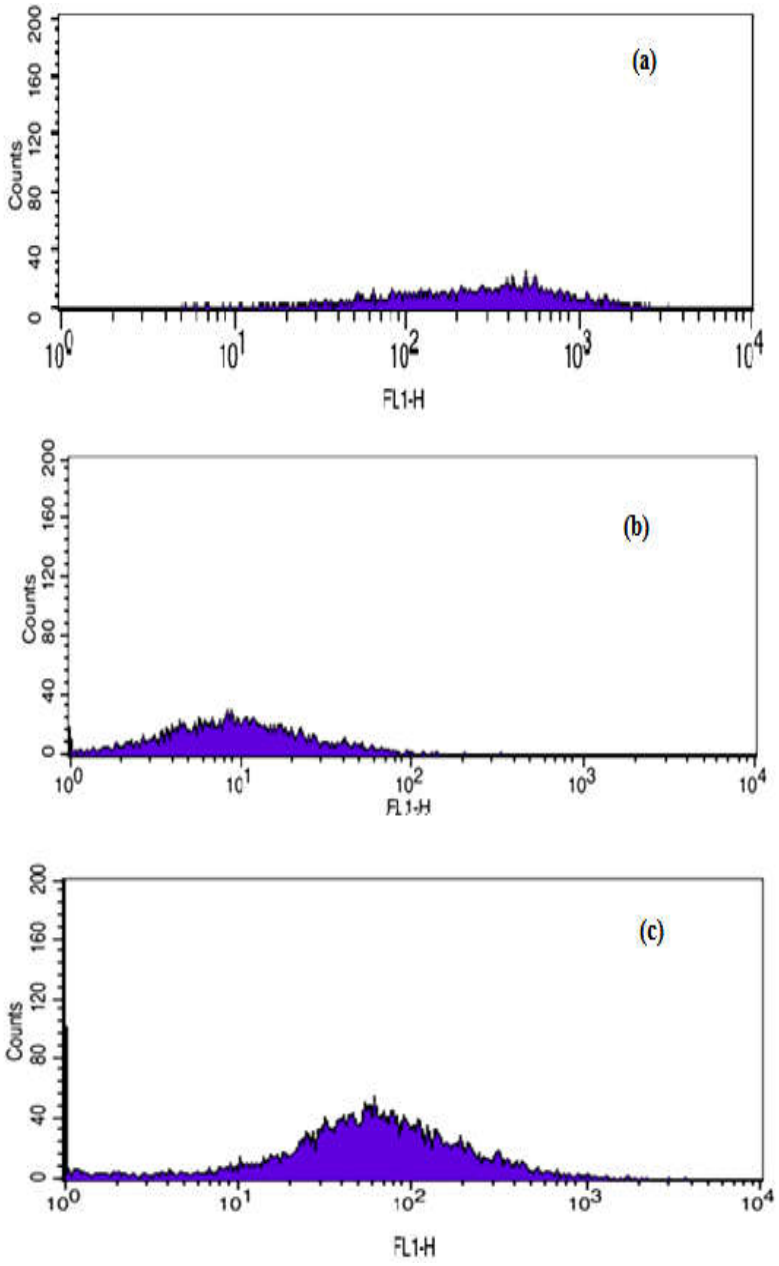

Figure 8.12. Evaluation of glucose uptake by flow cytometry in 3T3-L1. Cells were seeded in 6-well plates and treated with 100 nM insulin and 10 μM 2-NBDG in the presence or absence of samples (50 μM) for 24 h. After incubation, cells were washed with PBS and 2-NBDG uptake was calculated by a flow cytometer. (A) Control, (B) aqueous seed extract (C) acetone seed extract.

8.4. Conclusion

Dietary polyphenols may control type 2 diabetes (T2D) through diverse mechanisms, such as promoting the uptake of glucose in tissues, and as a result of improving insulin sensitivity. Hypoglycemic and anti-oxidative stress potential of faba bean may be due to the synergistic effect of polyphenols present in seed extract or acting independently. Dietary faba bean polyphenols may be substitutes for a drug for future prospect. Findings were proposed in future drugs which might be useful for oxidative stress treatment and its consequences might be useful for diabetic disorders. Still, we require more experimental trials to find out the effects of polyphenols- rich foods, their efficient dose, and mechanisms of their effects in managing diabetes.