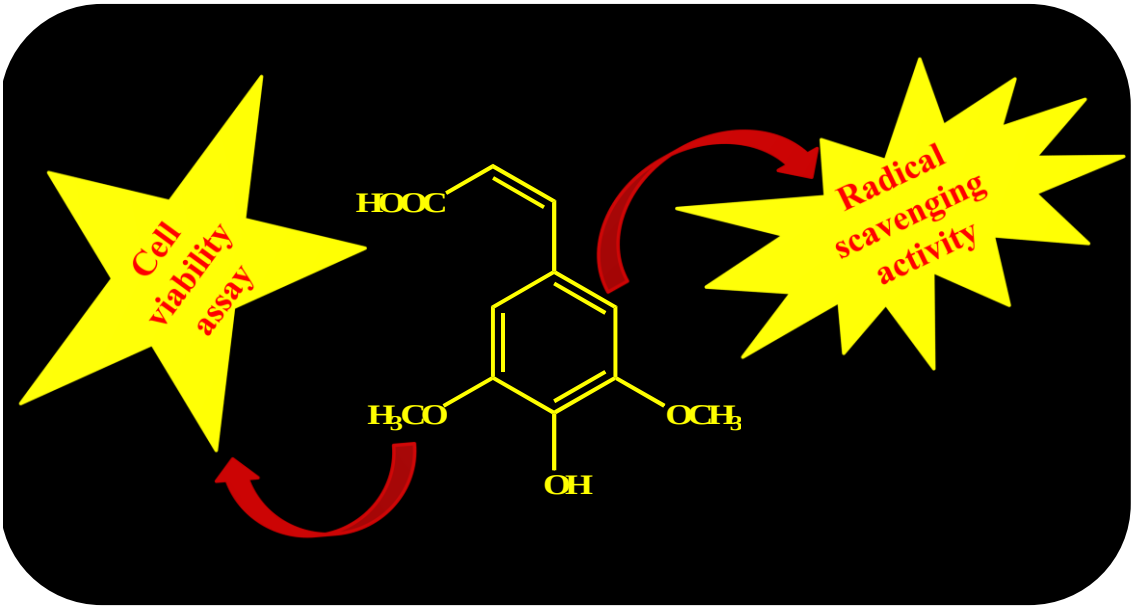
Chapter 3B

In vitro application of sinapic acid



3B.1. Introduction

Ultraviolet radiation is a potent environmental carcinogen, induces oxidative and inflammatory skin damage and eventually cancer. Naturally occurring polyphenols are potent antioxidants and anti-inflammatory agents which can potentially reduce the damaging effects of ultraviolet (UV) rays on the human body. Reduction in the ozone layer in the atmosphere promotes considerable UV radiation to enter into the earth surface which may cause damages to the biological systems. Although both UVA and UVB are potentially harmful, the effect of UVB is more severe as it causes burn to the absorbing tissues. UV ray can penetrate the skin and contributes its damaging effects mostly on the keratinocyte and fibroblast. Most of the UVB is absorbed in the epidermis layer of the skin leaving little to enter into the dermis layer. Exposure to UVB causes formation of reactive oxygen species (ROS), cell cycle arrest, activation of various genes and cell markers [Khalil et al., 2017]. Among all three types of UV rays, UVA and UVB rays can penetrate in human body. The UVA rays possessing the longest wavelength can reach deeper into the human skin, beneath the dermis layer, while, comparatively little UVB rays can penetrate into the dermis layer. UVC rays are fully absorbed by ozone layer and cannot reach the Earth surface. UVB is potentially the more dangerous than UVA rays and it causes burning of the cells and responsible for producing skin cancer. The carcinogenicity of UVB is well established and has been understood as a potential damaging agent to DNA from which gene mutation arises. On the other hand, UVA is generally far more abundant on earth surface, and goes deeper into the skin surface, and therefore contribute equally to the carcinogenicity of sunlight. In contrast to UVB, UVA is hardly absorbed by DNA and therefore less harmful from UVB regarding carcinogenic potential [de Gruijl, 2000]. Therefore, whenever the UVB rays hit any soft tissue it causes cell death or alteration in cell cycle. The intensity of the UV exposure depends on the geographical position of a particular area. In tropics, the solar UV is more intense than in higher latitudes and the intensity of exposure decreases in arctic and polar region. That's why people in equatorial and tropical region are more vulnerable to UV exposure than any higher latitudes. But the question is not only limited to solar exposure of UV rays. People are exposed to UVB rays nowadays through occupational, medical and cosmetic exposure from UV. Sun beds are extensively used in tanning parlous to tan skin and it uses UVB. Regular uses of Sun beds may contribute significantly to person's annual cumulative UVB exposure. UV sources are used in various diagnostic and therapeutic purposes, and intensity may vary according to the type of treatment. Arc welding, UV light manufacturing industry, use of UV

in research are also the potential contributing factors for UV exposure. All these route of exposure either alone or in combination give a high cumulative exposure to people working in these fields. Therefore, occupational exposure and environmental exposure can lead to a considerable risk from UV exposure on human body. Polyphenols possess diverse functions including antioxidant, anti-inflammatory, antimutagenic and anticancer activities. To explore the role of polyphenol in amelioration of UVB induced cell damage we planned to work out the effect of sinapic acid (SA) on UVB exposed peripheral blood mononuclear cells (PBMC). The polyphenols, with over 8000 currently known compounds, represent one of the largest chemical groups in the plant kingdom, classified into 4 major classes (phenolic acids, flavonoids, stilbenes, and lignans) [Pandey et al., 2009].

SA is one of the four most common hydroxycinnamic acids and is widespread in the plant kingdom. It has been proposed as a potent antioxidant by many researchers, [Natella et al., 1999; Pekkarinen et al., 1999; Sengupta et al., 2018; Zou et al., 2002]. In recent years SA and some of its derivatives (sinapine, 4-vinylsyringol, sinapoyl esters, syringaldehyde) have gained tremendous attention because of their various biological activities as an antimicrobial, anti-inflammatory, anti-cancer, and anti-anxiety activity. It has also a potent anticholinesterase activity and thereby can be used in diseases like Myasthenia Gravis and other neurological disorders like Alzheimer's disease, Parkinson's disease, etc. [Niciforovic et al., 2014]. SA, like other phenolic compounds, can be found in fruits, grains, and vegetables as well as in some medicinal plants and species (for instance borage, sage, mace, or rosemary) **[**Zych et al., 2018**]**.

The protective properties of exogenous antioxidants have been documented in a number of epidemiological, interventional and *in vitro* studies [Kampa et al., 2004]. Therefore we hypothesized that SA could work to ameliorate radiation induced cellular toxicity. To evaluate the role of SA for combating UV radiation-induced damage, we have studied its effect on UVB exposed cells. Further, in this study, the solvent for SA has been studied to evaluate the best solvent for its delivery to get the maximum effectiveness.

The effect of drugs and its efficacy depends on the density of bioavailable molecules in the system and its half-life [Larsen et al., 2016]. An increase in bioavailable molecules in the blood increases the concentration of the said drug at the target site. Therapeutic drugs are generally low-molecular-weight molecules that result in non-specific distribution within the body. This helps it to excrete itself through renal filtration resulting in rapid renal clearance and concomitant short plasma circulatory time [Markovsky et al., 2012; Sleep et al., 2013]. Although polyphenols are a good choice for combating diseases, dietary supplementation of

flavonoids cannot reach the therapeutic concentration at the target site because of their low absorption rate from the gastrointestinal tract and low bioavailability [Wang et al., 2013]. The usual and best possible way of any drug administration is the oral route [Wang et al., 2013]. Polyphenols are poorly absorbed from the gut and therefore the alternative approach of administration may be required to raise the therapeutic concentration at the target site [Wang et al., 2013; Yhee et al., 2015; Srinivasan et al., 2015]. Therefore, in this study we have also sought the alternative route of flavonoid delivery and selective organic solvent for SA delivery.

Small nonprotein molecules with electrophilic properties are transported through the blood to their target site by binding with albumin. It is efficient for its relatively long half-life and abundance [Yhee et al. 2015; Vachali et al., 2016]. Regarding its binding and transporting activity, it is important to find out it's mode of interaction with a drug molecule. Polyphenols are small electrophilic molecule and non-protein in nature and therefore transported inside the plasma with the help of albumin through binding. We are therefore interested to study the interaction of SA with albumin to compare the bioavailability of SA and its biological activity in different solvent systems.

We have chosen three solvents: SA dissolved in alcohol, SA dissolved in dimethyl sulfoxide (DMSO) and SA dissolved in 13% PEG-4000 as stock solutions for solvents. The binding constant of SA in these three solvents with albumin has been judged crucially to follow the extent of SA delivery to the biological system and carrying of SA in the system by binding with albumin. The stock solution for PEG, alcohol and DMSO were 20%. 14 µl of SA (0.00224 gm SA in 2 ml of stock solution, i.e, 400 µl alcohol in 1600 µl PBS buffer) in 20% alcohol was dissolved in 1986 µl of PBS to meet the final concentration as 0.01%, which is then used to incubate cells. Similarly, the final concentration of DMSO is 0.005% and PEG is 0.01% in working solution in which the peripheral blood mononuclear cells were cultured and incubated for further study.

To evaluate the biological activity and efficacy of candidate solvent for transporting SA into the blood and its bioavailability at the blood and target site we have done *in vitro* study on human PBMC. Here we studied the effects of UVB on PBMC to show the cytotoxicity which may also true for keratinocytes. We used cultured PBMC as study model because it is easy to culture and maintain. In the later stage of our study we studied the effect of UVB exposure with and without SA on immortalized keratinocytes (HaCat).

3B.2. Subjects and reagents

3B.2.1. Subject selection

For in-vitro experiments 5ml of EDTA anticoagulated whole blood were taken from 6 healthy male volunteers. The average age was 34 years with 65 kg average weight. All the subjects were nonsmoker and neither has any known disease or nor under any medication. Their average blood pressure and heart rate were 124/86 mmHg and 80 beats/min. Written consent were taken from each volunteers before enlisting their name in the study. From each participant cells were separated and studied in triplicate for each experiment. Therefore, in each experiment like, MTT assay, LDH assay, Nitric oxide assay, plasma free sialic assay the number of observation were 18 for each concentration and solvent of SA.

3B.2.2. Reagents

SA (≥98%, powder), and phosphate buffer solution (PBS, pH 7.4) were purchased from Sigma-Aldrich, USA and used without further purification. PEG-4000, ethyl alcohol, and DMSO were purchased from Merck Millipore. All the solutions were prepared using 0.1 M of PBS buffer at pH 7.4. LDH - cell cytotoxicity assay kit was purchased from HIMEDIA and kept at -20^{0}C until work. MTT was purchased from SRL, dissolved in 0.1 M PBS freshly and kept in dark until work. Ficoll-Histopaque and phytohaemoagglutinin were purchased from Sigma. Immortalized keratinocyte cell line 'HaCat' was obtained from Thermo Fisher Scientific.

3B.3. Instruments

All the UV-Vis spectra were recorded on Hitachi U-4100 spectrophotometer at 298 K. The measurements at the respective wavelengths were taken using photometry option in the instrument.

The emission spectra were measured on F-7000 Hitachi Spectrofluorimeter equipped with a 1.0 cm path-length quartz cell and a circulating water bath, using an excitation wavelength of 280 nm. All the measurements were done at the micromolar range to avoid the aggregation and inner filter effect.

3B.4. Sample collection and preparation

On the day of experiment peripheral blood was collected from healthy volunteers by venipuncture with EDTA-anticoagulated autoclaved polypropylene tube. From whole blood

plasma cell compartments were separated using centrifugation. PBMCs were separated from the whole blood by the usual method of ficol density gradient centrifugation. Written informed consent was taken from every volunteer before taking their blood. Ethical principles followed by the institute are guided by rules as formulated by the Indian Council of Medical Research and these are in agreement with Helsinki rules.

3B.4.1. Isolation and irradiation of peripheral blood mononuclear cells

Whole blood was collected from healthy human volunteers by phlebotomy. EDTA anti-coagulated whole blood was then subjected to ficol density gradient for isolation of PBMCs. Buffy coat was isolated by centrifugation and PBMCs were then washed in cold PBS and incubated at 37 ºC for 1 hour with 5% CO_2 in a CO_2 incubator. Cells were then subjected to UVB after 15 min incubation under 37 ºC with different concentration of SA dissolved (DMSO, alcohol, and PEG) in different solvents according to the design of the experiments in a CO_2 incubator. The viability of cells was evaluated by MTT assay before and after SA administration with UVB exposure.

The UVB source consisted of a bank of four Philips TL-12 lamps (Philips, Eindhoven, Netherland). These lamps emit UV rays over the range of 250-400 nm, primarily in the UVB region (280-320 nm), with a peak at 315 nm. PBMC suspended in PBS in Petri dishes (2 x 10^6 cells in 2 ml per dish), were placed under the TL- 12 lamps after removal of the lid, and exposed to single doses of UVB (selected range of radiation was 10 mJ/cm^2) (Becton Dickinson) [Teunissbn et al., 1993]. Before use, the culture media of the petri dishes (35 mm; Greiner) were treated with a graded concentration of SA (1 µM, 5 µM, 10 µM, 20 µM, 35 µM, 40 µM, 50 µM) in three different solvent medium. For each medium and each concentration of SA, MTT assay was performed to evaluate the percentage of viable cells present with and without administration of SA to UVB exposure.

3B.4.2. MTT assay

From each subjects three aliquots of PBMC prepared were subjected to MTT assay. MTT assay was carried out on UVB-exposed PBMCs to evaluate the effect of SA in ameliorating UV radiation-induced cell death. SA was administered through three different solvent systems to compare its efficacy in the solvent systems. In brief, the single-cell suspension was made from fresh human blood by ficol. Cells were then washed in cold PBS and seeded in 96 well plates to incubate first at 37 ºC for 1 hour with 5% CO_2 in a CO_2 incubator with SA dissolved in different solvent media (in alcohol, in PEG, and in DMSO). Briefly, 1 x10^4 to 5

$x10^4$ human PBMCs were treated with SA in three particular solvent systems prior to incubating under UVB rays ($10mJ/cm^2$). The graded concentration of SA was also used in a similar design but a different set of experiment was performed to select the best effective concentration of SA against radiation-induced cellular toxicity. Cells were then incubated with 50 µl of MTT solution for 2.5 hrs at 37 °C. Formazan crystals formed, was then dissolved in 150 µl MTT solvent (4 mM HCl, 0.1% NP40 in isopropanol) and the absorbance was measured at 590 nm. The effect of SA on radiation-induced cellular toxicity after UVB was compared to the cytotoxicity developed (if any) in control untreated cells, assigned here as 100%. Negative control was prepared by addition of MTT in cell-free PBS. Samples were prepared for each concentration and each solvent of SA in triplicate. Controls were prepared in same fashion without the exposure of SA and UVB.

Cytotoxicity was determined by corrected absorbance: % cytotoxicity = 100 x (control-sample)

3B.4.3. Cell cycle analysis

SA treated (with 5 µM, 10 µM, 20 µM, 30 µM, 50 µM of SA) and untreated PBMC cells after washing with PBS were plated in a 24-well plate at a density of $1x10^6$ cells/well and exposed to UV B irradiation at 10 mJ/cm^2. Following incubation for 2 h, cells were harvested and re-suspended in PBS and fixed in 70% ethanol at 4˚C overnight. They were then washed twice in cold PBS and incubated with propidium iodide staining solution (Beyotime Institute of Biotechnology, Haimen, China) for 30 min at room temperature. The percentage of cells at various phases of the cell cycle, namely the G0/G1, S and G2/M phases, were determined by flow-cytometric analysis of $1x10^6$ cells. Same experiment was conducted with cultured immortalized keratinocytes, HaCat. In case of keratinocytes the dose of UVB were graded in one experiment to determine the dose dependent inhibition of cell cycle and in other the graded doses of SA were prior to UVB radiation and culture.

3B.4.4. Assay of Cell Necrosis

Cytoplasmic leakage of LDH in extracellular space and measurement of this LDH is an indirect way to access the extent of cellular necrosis. Therefore to study the extent of necrosis PBMCs were separated from whole blood by ficol and then harvested in RPMI 1640 medium (HiMedia) using Dulbecco's modified Eagle medium (HiMedia) and 5% fetal bovine serum and 5% CO_2 in a 96 well microtiter plate at a density of $1x10^4$ - $5x10^4$ cells per well. The

cells were incubated overnight at 37 ºC equilibrated with 5% CO_2. After completion of the culture, lysis solution was added to each well and incubated further for another 45 minutes at the same condition. The contents were then centrifuged at 2000 rpm for 5 minutes and the supernatant was restored in a new vial. To the supernatant, LDH assay buffer was added and subjected to incubation in dark at 37 ºC for about 25 min and the absorbance was taken at 520 nm. Percentage of cytotoxic cells was calculated by the following formula according to Chan et al. with minimum modification [Chan et al., 2013]. Samples were prepared for each concentration and each solvent of SA in triplicate. Controls or untreated cells were prepared neither with SA nor with UVB. Negative controls are prepared in same way without addition of cells according to Chan et al. [Chan et al., 2013].

% Cytotoxicity = 100 x (Corrected reading from test well - Corrected reading from untreated well)/(Corrected reading from untreated well- corrected maximum LDH release negative control)

3B.4.5. Sialic acid assay

Plsama free sialic acid was estimated from every sample (whole blood) exposed to UVB with and without SA administration by the method of Jourdian et al. [Jourdian et al., 1971].

3B.4.6. Nitric oxide synthase activity assay

Nitric oxide synthase (NOS) activity was measured by Nitric oxide assay kit (colorimetric) from Abcam (ab211083). Briefly, PBMCs were cultured with and without SA prior to UVB exposure for 15 min. Then, they were washed twice with ice-cold PBS and then homogenized in homogenization buffer (250 mM Tris EDTA, 10 mM EDTA, 10 mM EGTA). Cellular debris and unbroken cells were precipitated by centrifugation at 12000 rpm for 15 min and the supernatant was used for NOS activity study. The supernatant was subjected to react with Griess reagent 1 and 2 (provided with the kit) and the colour generated was studied for its intensity colorimetrically at 540 nm.

3B.4.7. Serum Amyloid A assay

The PBMCs were cultured on Poly-D Lysine/Laminin coated plates. The cells were then irradiated under UVB source with and without graded concentration of SA. PBMCs were then harvested and total protein extracted for ELISA assay to detect the serum amyloid-A level using commercially available α- amyloid kit (BioAssay Systems, Hayward, CA).

3B.4.8. Assay of ROS scavenging activity assay

3B.4.8.1. DPPH assay

The DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging activity was determined according to the method of Xie et al [Xie et al., 2014]. The initial stock solution of SA was prepared in both alcohol-water and DMSO medium. The required concentration for the assay (1, 5, 10, 20, 30, and 50 µM) was then achieved by further dilution of the stock solutions with double distilled water. According to the method, 0.5 mL of the sample solution with different concentration of SA was added to 3 mL of DPPH (0.1 mM) in methanol and mixed well. The solutions were kept in dark for 20 minutes and then absorbance was taken at 517 nm. The below-mentioned equation was utilized to calculate the percentage of scavenging activity:

$$\text{Radical Scavenging activity (\%)} = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100 \qquad \text{(3B.1)}$$

3B.4.8.2. ABTS assay

ABTS assay measure the relative ability of an antioxidant to scavenge the ABTS generated in the aqueous phase, as compared with Trolox (water-soluble vitamin E analog) standard. The ABTS is generated by reacting with a strong oxidizing agent with the ABTS salt. It is a decolouration assay applicable to both lipophilic and hydrophilic antioxidants. The assay was performed according to the protocol of Re et al., with different concentration of SA (1, 5,10, 20, 30 and 50 µM), in which radical monocation of $ABTS^{\cdot+}$ (2,2' azinobis-3 ethylbenzothiazoline-6-sulfonic acid) is generated by oxidation of ABTS with potassium persulfate and is reduced in the presence of such hydrogen donating antioxidants [Re et al., 1999]. This assay was also performed in two different solvent system of SA, alcohol water and DMSO water.

3B.4.9. Statistical Analysis

Significance of differences between the mean of different parameters has been studied by paired two-tail t-test and one way ANOVA to find out the difference between group means. Significance of difference was studied against a particular concentration of SA treated UVB exposed group and SA untreated UVB exposed group. The computed F score for ANOVA was compared with critical F to find out the level of significance [Das et al., 2008].

3B.5. Results

3B.5.1. Steady-state fluorescence studies

The characteristics of the interaction of plasma protein with SA were studied initially in three different solvent media (DMSO/buffer, alcohol/buffer, PEG/buffer) using steady-state fluorescence. The plasma shows intrinsic fluorescence due to the presence of different plasma proteins mainly serum albumin. Serum albumin contains various fluorophores such as tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) residues, among them Trp residue shows the major while Tyr shows minor contribution for intrinsic fluorescence of serum albumin [Abou-Zied etal., 2008; Paul et al., 2014]. Hence, we have monitored the fluorescence spectra of the blood plasma by exciting at 280 nm where both the Trp and Tyr residues contribute to the emission spectra (vide Fig. 3B.1.(A and B)).

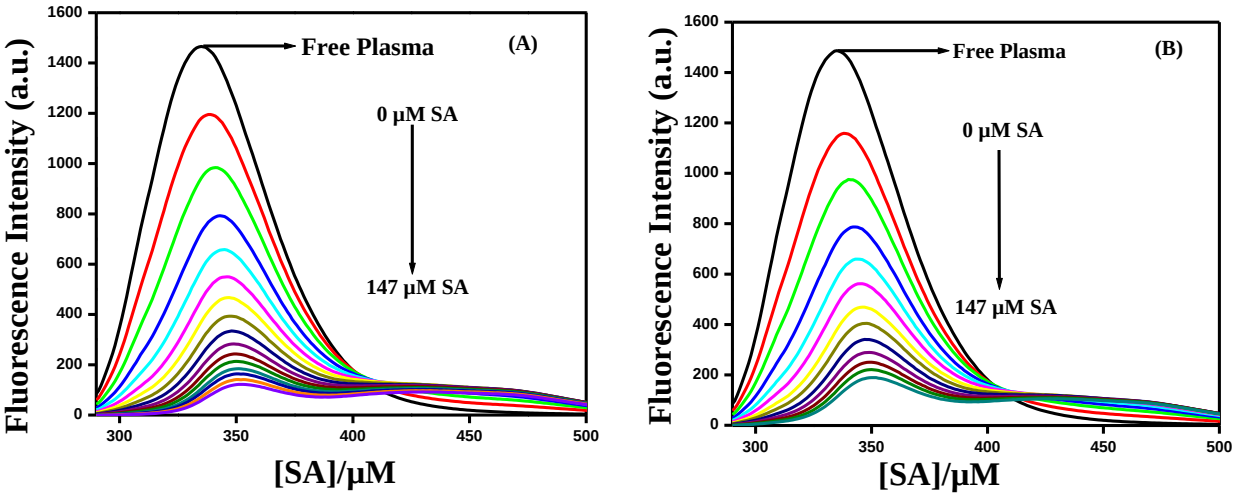


Figure 3B.1. Fluorescence quenching spectra of plasma protein (albumin) in the presence of varying concentrations of SA at 298 K; λ_{ex} = 280 nm, pH = 7.4 (0.1M PBS). Excitation band pass = 5 nm and Emission band pass = 5 nm. (A) alcohol/buffer medium; (B) DMSO/buffer medium

3B.5.2. Evaluation of Binding Constant

To understand the interaction of SA in different solvent medium with human plasma, we have exploited the fluorescence titration data using the following equation (equation 3B.2) [Lakowicz, 1983; Bi et al., 2004; Naveenraj et al., 2013; Sengupta et al., 2018]:

$$\log\left(\frac{F_0 - F}{F}\right) = \log k_b + n \log[Q] \qquad (3B.2)$$

where, F_0 and F are the fluorescence intensities in the absence and presence of quencher

molecule (Q, here SA), respectively, k_b is the binding constant, [Q] is the concentration of the quencher and n is a number of binding sites.

The binding constant of SA with human plasma decreases 10 times in PEG-buffer medium (k_b = 2.344x10^4 M^{-1}) in comparison to DMSO and alcohol, while that of DMSO/buffer and alcohol/buffer remained almost comparable (vide Fig. 3B.2, Table 3B.1).

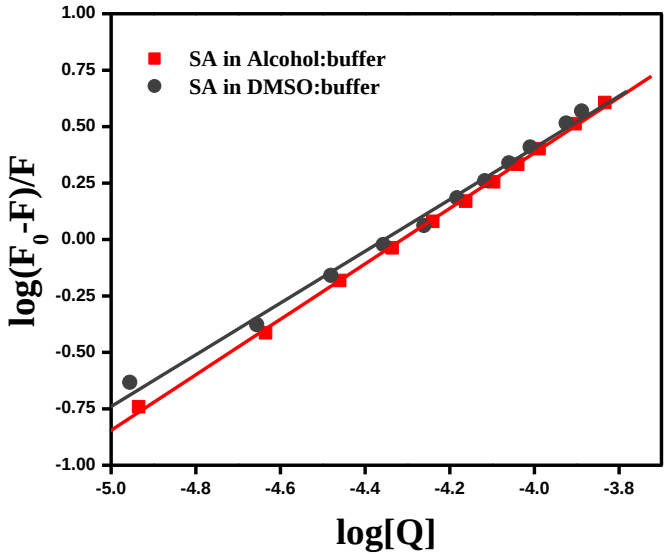


Figure 3B.2. The double-logarithm plot of log[(F_0-F)/F] vs. log[Q] for the interaction between human blood plasma with SA in two different media

Figure 3B.1.(A and B) depict the effect of gradual addition of SA in alcohol/buffer and DMSO/buffer respectively to human blood plasma. The percentage of alcohol and DMSO and PEG in working conditions was less than 0.01 %. The intrinsic fluorescence of the blood plasma decreases with the gradual addition of SA in both the medium, which indirectly indicated that, the increase in the binding of SA with plasma protein and formation of a non-fluorescent complex. As evident from the plot, with increasing SA concentration, gradual quenching of intrinsic fluorescence of plasma protein was observed in both alcohol/buffer and DMSO/buffer media.

Based on the above results we have performed the biological assays by dissolving SA in all of the above-mentioned media.

Table 3B.1. List of binding constant (k_b) and binding sites (n) for the interaction of SA in different media with human blood plasma at 298K

	Medium	**k_b (M^{-1})**	**n**	**R^2**
Human Blood Plasma	Alcohol-buffer	$2x10^5$	1.22	0.99
	DMSO-buffer	$1x10^5$	1.14	0.99

3B.5.3. MTT assay

The efficacy of SA to ameliorate radiation-induced cellular damage has been studied by evaluation of percentage of cytotoxicity and viable cell on UVB exposed PBMCs in three different solvent systems (DMSO, Alcohol, PEG). Percentage of viable cells and cytotoxicity were evaluated by MTT assay and LDH-cytotoxicity cell necrosis assay respectively. Cell death by tissue necrosis has been studied by nitric oxide synthase activity. UVB and SA exposed cultured PBMCs extract has been studied and compared for Serum amyloid A assay in cells before and after radiation exposure. MTT assay on PBMCs after radiation exposure have been studied and showed that the prior administration of SA to PBMCs before UVB exposure is beneficial for cells as they survive more after treatment with SA. A significantly higher percentage of viable cells ($p<0.05$) were observed in aliquots incubated with SA, when compared between without exposure of SA and with graded concentration of SA in UVB exposed cells dissolved in both DMSO and alcohol. No dose-response pattern has been seen in our study considering all the concentration of SA together. However, up to 10 µM of SA concentration the percentage of viable cell increases in a dose response pattern. The percentage of the viable cell after higher dose (above 20µM) of SA administration with UVB rather showed a non-dose responsive status. We have studied each concentration of SA in triplicate with a minimum concentration of 1 µM and found a dose response up to 10 µM of SA. The number of viable cell in case of alcohol/buffer and DMSO /buffer system was highest at a concentration of 10µM (61.74%) and lowest at 1µM (41.36%) which shows a dose response relationship. At the concentration 35, 20 and 5µM the percentage of cell viability was 52, 48 and 49 respectively. Similar results were also obtained in DMSO and PEG buffer system (vide Fig. 3B.3).

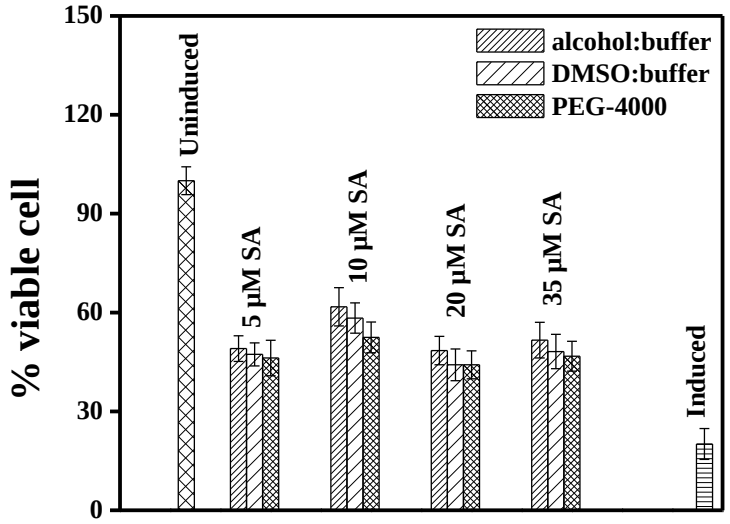


Figure 3B.3. Comparative analysis of percent viable cells (analyzed by MTT assay) after administration of different concentrations of SA in three different solvent systems, on UVB exposed human peripheral blood mononuclear cells

3B.5.4. Assay of cell cycle analysis

Result indicates a fall in cell growth and division as it exposed to UVB rays in both normal PBMC and in HaCat cells. Co-administrations of SA reduce the percentage of cell death and restore some cell division and growth which is maximum in 30µM of SA administration in both PBMC and HaCat cells. The percentage of apoptotic cell death and necrotic cell death are highest at UVB exposure without any co-administration of SA. Apoptotic and necrotic cell death was reduced efficiently after SA administration in the culture medium before exposed to UVB radiation. However, graded concentration of SA can successfully increase the percentage of live cell after UVB exposure and this showed a dose response curve up to 30 µM concentration. Beyond this level the percentage of live cell reduced again instead of higher concentration of SA application. Again, the percentage of apoptotic and necrotic cells showed higher concentration when the culture was maintained without SA or very low concentration of SA. Both in PBMC and in HaCat the percentage of necrotic cell is highest in UVB exposure with only 10µM of SA. Whereas, the percentage of apoptotic cell is almost similar in UVB exposure without SA and with 5µM of SA when maintained in PBMC. The cultured HaCat shows slightly different concentration of apoptotic cell when maintained with 5 µM of SA with UVB in comparison to only UVB (vide Fig. 3B.4).

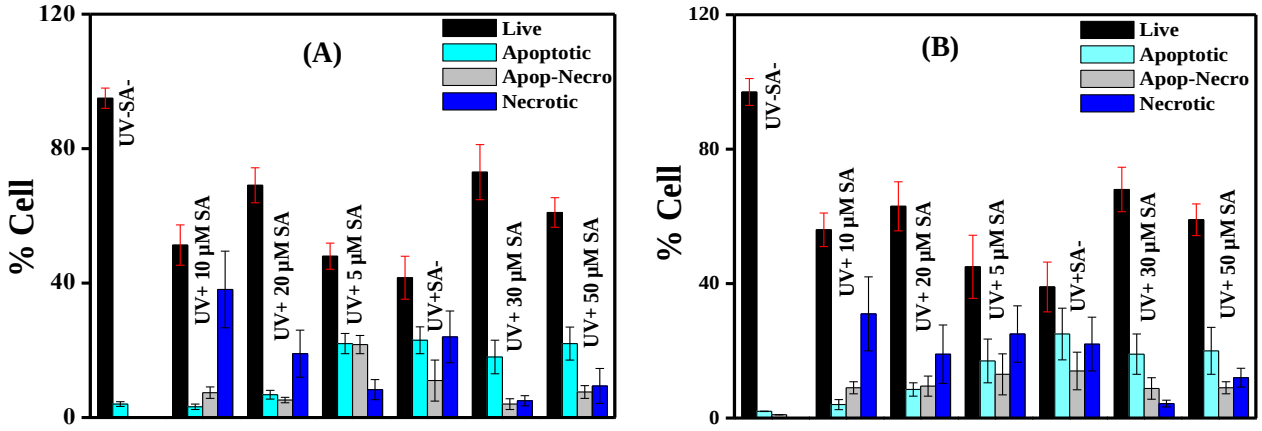


Figure 3B.4. Assay of Cell growth and cell death by flow cytometric analysis using human blood peripheral momonuclear cells and immortalized keratinocytes (HaCat) after UVB exposure with and without SA administration, (A) PBMC cell culture with UVB and SA (B) HaCat culture with UVB and SA

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3B.5.5. LDH assay

LDH level is an indicative of cellular death. The percentage of cytotoxicity induced by UVB and its amelioration by co-administration of SA showed interesting results. The percentage of cytotoxicity in case of SA administered through alcohol/buffer system was in the order of 5µM (38.49%)>20µM (37.92%)>10µM (35.78%)>35µM (20.68%) and that for DMSO/buffer media was 20µM (53.15%)>5µM (45.10%)>10µM (38.18%)>35µM (20.15%) and in case of SA in PEG medium that result was 5µM (58.03%)>10µM (55.69%)>20µM (52.33%)>35µM (43.00%). The observation primarily indicated that a concentration of 35 µM of SA was able best to prevent cell damage against UVB. Interestingly, the optimum concentration for alcohol/buffer, DMSO/buffer and SA in PEG/buffer medium was 35 µM. The percent cytotoxicity for alcohol/buffer, DMSO/buffer and PEG/buffer media was also comparable to each other (vide Fig. 3B.5). We have also studied the experiment with 1 µM SA which showed a poor amelioration from UVB induced damage. The level of LDH before and after administration of SA in UVB exposed PBMC was significantly different ($p<0.01$) between 5 and 35 µM of SA ($p<0.05$).

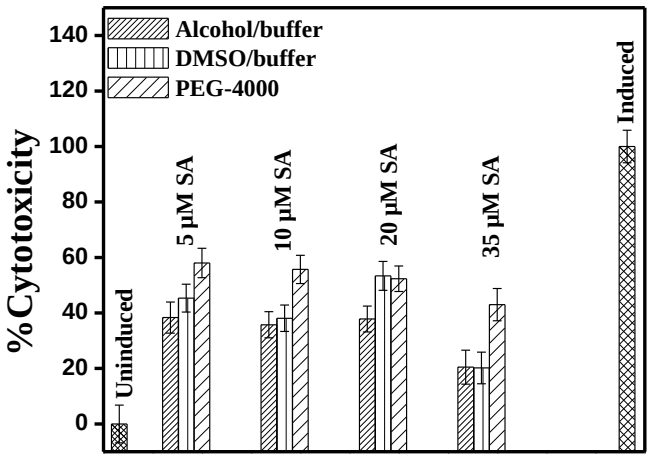

Figure 3B.5. LDH Assay (mean ± SD) for % cytotoxicity of human PBMC exposed to UVB after prior administration of SA in DMSO/buffer, alcohol/buffer media and PEG 4000 in buffer medium

3B.5.6. Sialic acid assay

From sialic acid assay it has been shown that plasma free sialic acid concentration increases in UVB irradiated blood cells in comparison to blood cells irradiated under UVB with SA. Plasma-free sialic acid level in irradiated blood cells is significantly higher ($p<0.01$) in comparison to un-irradiated samples. When human blood samples were subjected to UVB irradiation with a graded concentration of SA it showed a decrease in plasma-free sialic acid concentration. A concentration of 10 µM of SA has shown maximum effectiveness against UVB irradiation in this assay. The difference in sialic acid concentration between UVB irradiated 10 µM SA added cells and without SA added cells was also significant ($p<0.05$).

The analysis indicated that alcohol/buffer medium was the suitable one and the order of effectiveness was 10µM<5µM<1µM<35µM<20µM. In the case of the DMSO/buffer solvent system and SA-PEG, the order followed a similar trend as 10µM<5µM<1µM<35µM<20µM. In all three cases, the absorbance value was lowest at a 10 µM concentration of SA, which suggested a lesser release of sialic acid from irradiated blood cell at that particular concentration (vide Fig. 3B.6).

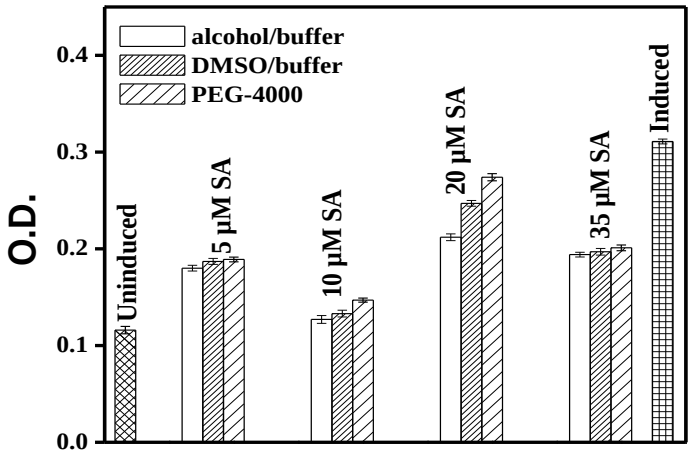

Figure 3B.6. Sialic Acid Assay for evaluation of cell death ($O.D_{630nm}$) of human PBMC exposed to UVB with prior administration of SA in DMSO/buffer and alcohol/buffer media and SA in PEG/buffer medium

3B.5.7. Radical scavenging assay (DPPH and ABTS assay)

Free radical scavenging activity of SA has been studied by DPPH and ABTS assay. It is seen from the result of DPPH activity that gradual increase in SA concentration increases the free radical scavenging activity of SA in a dose-dependent manner ($p<0.05$) (Fig. 3B.7A). A similar result has also been observed when the ABTS assay performed to evaluate the Trolox equivalent (Fig. 3B.7B). This study also showed a dose-response pattern when SA concentration was increased gradually.

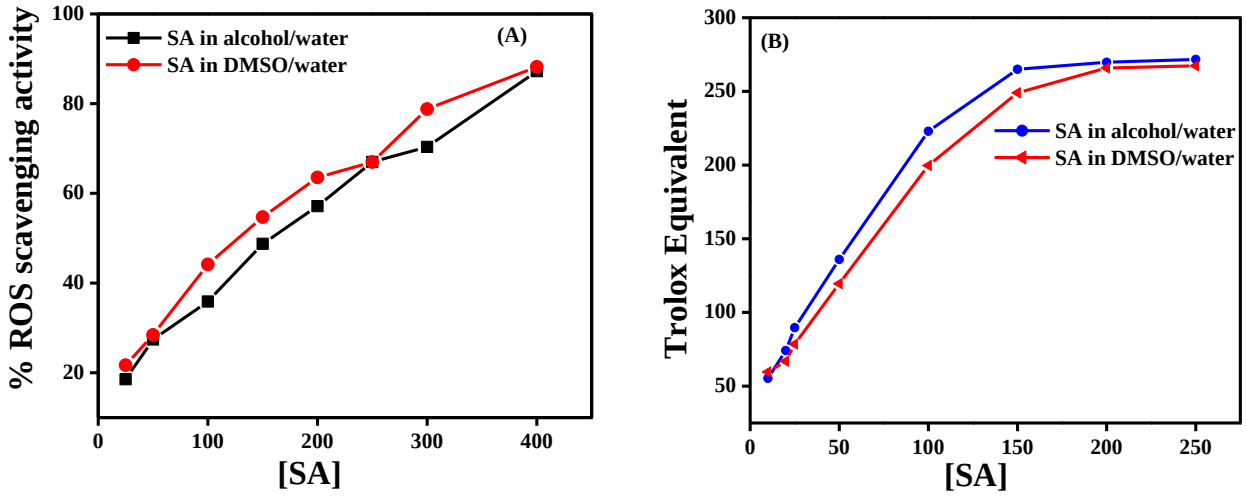

Figure 3B.7. (A) ROS scavenging activity of SA before and after exposure of UVB measured by DPPH activity. (B) ROS scavenging activity of SA measured by Trolox equivalent through ABTS activity after UVB exposure

3B.5.8. Serum amyloid A assay

Serum amyloid A (SAA) is an apolipoprotein and a major acute phase reactant which has a central role in the inflammatory response. SAA is expressed primarily in the liver, although it is also found in extrahepatic sources such as adipocytes and macrophages. SAA is involved in cholesterol sequestering and lipid metabolism and has been shown to induce extracellular-matrix-degrading enzymes, pro-inflammatory cytokines and to recruit immune cells to sites of inflammation by chemotaxis [Sproull et al., 2015]. Radiation can induce SAA in rodents and other primates [Sproull et al., 2015]. Here we have shown that UVB induces SAA at a significantly high level ($p<0.05$) in comparison to normal unexposed PBMCs. Radiation-induced SAA expression is increased to show a dose-response relationship (data not shown here, when gradually the time of exposure has been increased from 5 min to 25 min). 15 min UVB exposure with graded concentrations of SA can reduce the SAA concentration in comparison to UVB exposure without SA, although did not attain the normal unexposed level (Fig. 3B.8).

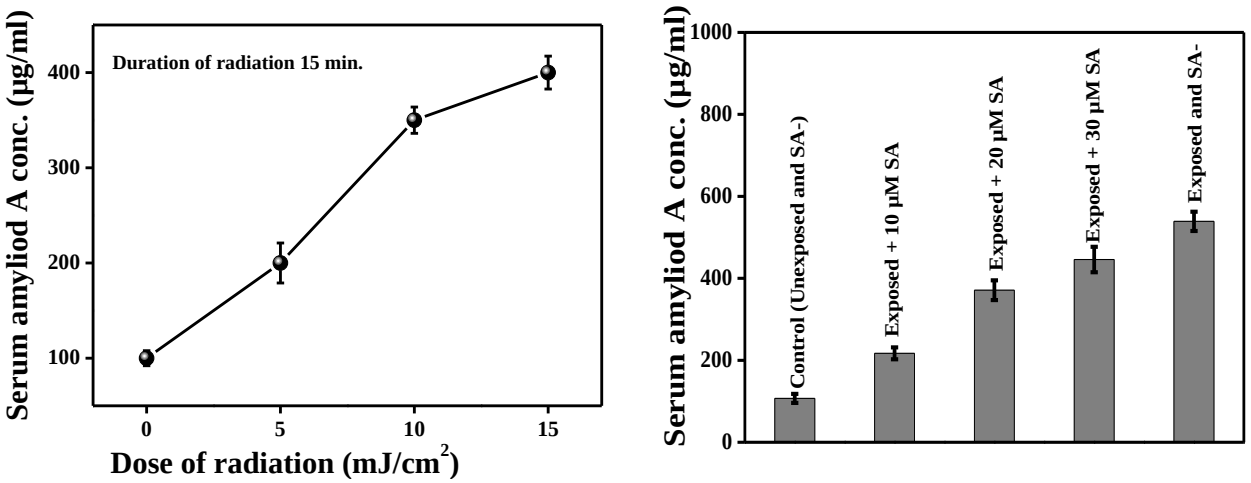

Figure 3B.8. Serum amyloid A (SAA) level in UVB exposed human peripheral blood before and after administration of sinapic acid. The bar diagram shows the mean ±SD value of SAA before and after SA administration on UVB exposed blood cells

3B.5.9. Nitric oxide synthase

Our experiment on nitric oxide synthase (NOS) activity after UVB exposure to PBMC revealed that the activity of NOS is increased dramatically when exposed to radiation and decreased when the cells are cultured with different concentration of SA. The nitrate concentration (due to NOS activity) is increased 4 fold after UVB exposure without administration of SA. A significant decrease ($p<0.05$) in nitrate concentration has been noted

after graded concentrations (1µM, 5µM, 10µM, 20µM, 35µM, and 50µM) of SA administration prior to UVB exposure in culture medium of PBMC cells. The lowest concentration of nitrate has been noted in cells treated with 35 µM of SA (vide Fig. 3B.9).

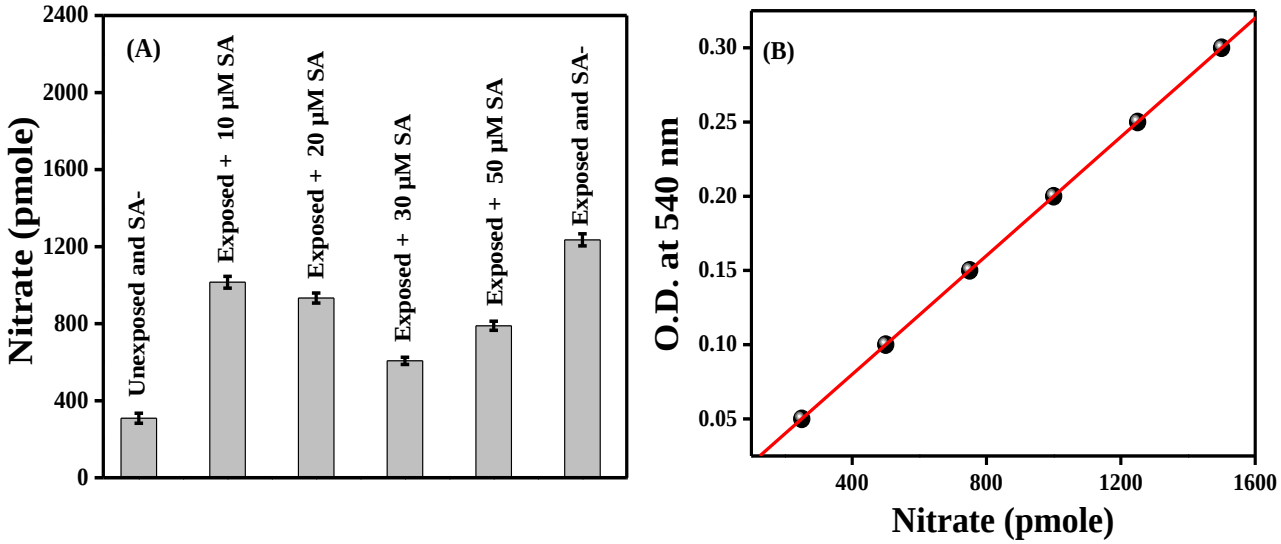

Figure 3B.9. (A) represents the pmole concentration of nitrate evolved against OD level at 540nm. (B) represents the concentration of nitrate evolved in cells after UVB exposure with and without SA administration

3B.6. Conclusion

UV radiation induces apoptosis through two biochemically and morphologically distinct processes, apoptosis and necrosis. Intricate cell biology shows that various cell lines undergo apoptosis following low doses of UVB irradiation, whereas prolonged exposure induces necrosis [Xie et al., 2016]. UVA and UVB both are potentially harmful and both can induce squamus cell carcinoma (SCC) in nude mice but the ability to produce SCC of UVA alone is about 5000 to 10000 times lower than UVB alone [De Laat et al., 1997; Griffiths et al., 1998; Ley, 2001]. Both in vitro and in vivo experiments and epidemiological studies indicate that long lasting chronic exposure to UVB is the main cause of SCC of the skin [De Laat et al., 1997]. Before 1990, for this reason, only UVB was considered as carcinogenic, not UVA. It was revealed experimentally, that the UVA is less toxic than UVB for induction of melanocyte tumors in Monodelphis domestica [Robinson et al., 2000]. Experiments with UVA on new born rodents and on isolated human skin cell couldn't reproduce the results obtained with UVB [Berking et al., 2004; Kulms et al., 2000]. All these experimental

evidence suggest that similar dose of UVB is more dangerous than UVA to produce melanoma and SCC. Besides skin, our lips, conjunctiva and iris muscles can be exposed to UV radiation. Therefore, although UVB cannot penetrate deep beneath the skin, these soft tissues are under the threat of being exposed and damaged. This is one of the major reasons for choosing UVB to explore the cellular effects on it and the ameliorating action of SA on UVB induced cellular damage.

UV radiation affects the biological system through disruption of the cellular homeostasis by damaging the membrane and macromolecules inside the cell including DNA, RNA, and protein. ROS are generated by UVA and UVB which are highly reactive and cause alteration of the molecular structure of DNA, lipids, and proteins through inhibiting DNA repair pathway [Denning et al., 2002; Assefa et al., 2003; Lee et al., 2013]. In our study, post UV exposure cellular toxicity profile and cellular damage was assessed by MTT, NOS and LDH assay which shows differential damage to the cell and causes cellular death. We have shown that SA effectively reduces generation of ROS in post UVB exposed peripheral blood mononuclear cells. May be, due to its hydrogen or electron donation property it can act as a free radical scavenger and antioxidant molecule. In this chapter, we have tried to evaluate not only the cyto-protective effect of SA but also the effective dose of SA in isolated in-vitro cellular system at which, it most effectively scavenges the ROS generated by UVB and prevents cellular death from UVB exposure. The study also indicated the best suitable solvent for SA delivery.

The pharmacokinetic study revealed that serum albumin is responsible for the transportation of SA in the blood, due to its ability to combine with serum albumin. It is also evident from our study that SA after administered to the ex-vivo human plasma, combine with plasma protein to transport to the cellular system. Study with HSA and BSA revealed that SA bind with albumin and thus reduces the fluorescence. The maximum reduction of fluorescence intensity has been observed with 147 µM of SA, when it is gradually added to the human plasma (Figure 3B.1). Reduction in fluorescence is indicative of the quenching of SA with plasma protein. But it is not known what amount of these transported SA is released to the tissue space or target site. It is rather more complicated to work out. Actually, the biological activity is the reflection of the internalized SA into the cell which ameliorates radiation-induced cellular death.

Administration of graded concentration of SA on the PBMCs subjected to UVB causes a reduction in radiation-induced cellular damage significantly. Significant reduction in cellular death and an increase in viable cells in SA administered PBMCs subjected to UVB is actually

the indicative of beneficial role of SA against UVB induced cellular death. Reduction in LDH and NOS activity reflected the capability of SA to protect cells from UVB induced necrosis and radiation induced damage. We have done all the experiments of SA with 1 to 50 µM of concentration. The reason for choosing this range of SA is that we have found a significant beneficial effect in this range. Beyond 50 µM concentration the cells undergoes shrinkage which may indicate the early signs of cell death. At a concentration of 100 µM of Sinapic acid about 50% of the cells undergoes membrane corrugation and death. Therefore we have planned to hold the concentration of SA up to 50 µM when administered prior to UVB. Radiation causes generation of different types of ROS in the biological system. Disruption of balance between its production and scavenging can cause cell death and break of macromolecules like DNA, RNA and proteins which in turn damage the cells. SA is a class of phenolic molecules which efficiently scavenge generated ROS in the body and thereby protect the system. It can scavenge $O^{\cdot}$, $OH^{\cdot}$, OOH, $ONOO^{-}$ and other ROS generated in the body as a result of radiation [Kim et al., 2018]. Although we have significant number of literature in respect to the beneficial role of SA, still, the dose dependence of SA and solvent system for its delivery has not been studied in radiation induced cytotoxicity. To our knowledge, we are the first to propose the effective solvent for SA delivery and its effective dose at which it can best ameliorate the radiation-induced cellular damage at least in vitro system. By this preliminary work we think, we are inviting lots of works in this field to evaluate the role of SA with its effective dose and solvent in radiation induced toxicity.

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