CHAPTER 2

CHAPTER 2

Anti-proliferative and Cytotoxicity Evaluation of Plant Extracts

2.1 Introduction

When evaluating herbal remedies for their potential in combating cancer, it is crucial to assess their cytotoxic effects and ability to inhibit cell proliferation. This process ensures the quality and safety of these products by confirming their efficacy and identifying any associated risks. Studies on cytotoxicity and anti-proliferative properties also contribute to the development of personalized cancer treatment strategies, as they help identify plant extracts that show effectiveness against specific types of cancer cells or tumours. Because of this reason, natural plant products must undergo standardization and initial investigations to assess potential hazards, including adverse effects, overconsumption, and toxicity. (Hemaiswarya & Doble, 2006). So it is important to analyse the preliminary studies of anti-proliferative activity of plant extracts on breast cancer cell lines and cytotoxicity on normal cell lines as well for preliminary screening of plant extracts for further studies.

This chapter explains the anti-proliferative activity of Bm, Sa, Ma and Sv plant parts on breast cancer cell lines (MCF-7 and MDA-MB-231). On the basis of the anti-proliferative assay, the selected plant extract was subjected to cytotoxicity evaluation on normal non-cancerous cell line (HEK-293). The study was performed at different doses and time points.

2..2 Materials and method

2.2.1 Chemicals

All cell lines were purchased from National Centre for Cell Science (NCCS) Pune, India. Trypan blue (cat# TC193), dimethyl thiazolyl tetrazolium bromide (MTT) (cat# TC191), Dulbecco's Modified Eagle Medium (DMEM) (cat# AL066A), Leibovitz's L-15 Medium (L-15) (cat# AL011S), 0.25% (w/v) Trypsin- Ethylene diamine tetra acetate (EDTA) (cat#

TCL047), Penicillin-Streptomycin (Peni-Strep) (cat# A018), and Fetal Bovine Serum (FBS) (cat# RM9955) were purchased from HiMedia Laboratories Pvt. Ltd., India.

2.2.2 Cell line maintenance

The MCF-7(ER+/PR+) cell line was harvested in high-glucose DMEM, while the HEK-293 cell line was cultured in MEM, and the MDA-MB-231 (ER-/PR-) cell lines were cultured in L-15 medium and maintained at 37°C with 5% CO_2 in a CO_2 incubator (Remi, India) with 95% humidity. All media were supplemented with 10% fetal bovine serum and 1% peni-strep (penicillin, 100 U/mL; streptomycin, 100 µg/mL). The medium was changed every 2–3 days, and the cells were passaged at a confluency of 80-85%.

2.2.3 Preparation of extractions and compounds for treatment

All methanolic extracts were poorly soluble in water so considered as hydrophobic. These extracts were prepared in 0.2% DMSO (0.2 mL DMSO solution in 99.8 mL autoclaved dH_2O). The 100 mg extract was dissolved in 1 mL of 0.2% DMSO. The aqueous extracts were easily soluble in water so the stock were prepared by dissolving 100 mg extracts in autoclaved dH_2O. Further dilutions were prepared by using respective solvents to reach the concentration 1 mg/mL for assays. The working solutions of various concentration range 6.25,12.5,25,50,100 ,200 µg/mL were prepared by dissolving stock solution in complete growth medium.

2.2.4 Cell Viability assay: IC_{50} determination using 3-(4, 5-dimethylthiazol2-yl)− 2, 5-diphenyltetrazolium bromide (MTT) assay (Corry et al.,1991 and Patel et al.,2023)

The cytotoxic effect of all extracts was evaluated using the tetrazolium dye MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)) assay. The MTT assay was performed in a dose- and time-dependent manner.

2.2.4.1 Principle

The MTT assay working on the principle of reduction of the tetrazolium dye MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)) in to an insoluble, coloured (purple)

formazan crystals by the mitochondrial succinate dehydrogenase. This reduction of MTT occurs only in metabolically active cells. The cells are further solubilized with organic solvent (e.g. DMSO) and the released solubilized formazan product was measured using a UV-Visible spectrophotometer. The reduction of MTT in formazan crystals were occurred only in metabolically active cells, more absorbance indicates more live cells which helps in the assessment of cell viability for treated group vs control untreated groups.

2.2.4.2 Experimental design

The MCF-7, MDA-MB-231, and HEK-293 were seeded in 96-well plates at a density of 5×10^3 cells/well in 200 µL culture medium. Following 24 hr of incubation and attachment, the cells were treated with different concentrations of extracts (6.25µg/mL, 12.5µg/mL, 25µg/mL, 50µg/mL, 100µg/mL,200µg/mL) and similar concentrations of diluents (0.2% DMSO and only media) for further 24 hr, 48 hr and 72 hr. After treatment, the medium was replaced with MTT solution (50 µL of 0.5 mg/mL per well) prepared in 1X PBS and incubated for three hours at 37°C in a humidified incubator with 5% CO_2. The yellow MTT dye was reduced to purple formazan crystals by succinate dehydrogenase in the mitochondria of viable cells. To solubilize the formazan, 100 µL of DMSO was added to each well. After 30 min of incubation, the plates were gently shaken for 1 min, and the absorbance was measured at 492 nm using a microtiter plate reader (Analytical Technologies Ltd., India). The percentage of cell cytotoxicity was calculated as {(Y-X)/(Y)} × 100, where Y is the mean optical density of the control (0.2% DMSO-treated cells for alcoholic extracts or only media containing cells for aqueous extracts) and X is the mean optical density of cells treated with plant extracts. The concentration-effect curves predicted the IC_{50} of the extracts in the cell lines. Doxorubicin was used as the positive control. Among all IC_{50} concentrations of plant extracts, the extracts were subjected to further assays, which showed anti-proliferative activity at lower concentrations.

2.2.5 Statistical Analysis

All experiments were performed in triplicates with similar results. Data shown are expressed as mean ± standard error mean (SEM) of three different measurements per extract.

2.3 Result

Bm, *Sa*, *Ma* and *Sv* extracts were subjected to breast cancer cell line (MCF-7 and MDA-MB-231) to check their efficacy as an anti-proliferative agent. Figure 2.3.1 and 2.3.2 has shown the comparative IC_{50} (µg/mL) for *Bm*, *Sa*, *Ma* bark and leaf extracts and *Sv* unripe fruit, ripe fruit and leaf extracts for MCF-7 cell line while figure 2.3.3 and 2.3.4 has shown the comparative IC_{50} (µg/mL) for MDA-MB-231 cell lines. In case of all extracts, the IC_{50} concentrations were getting decreased at time dependent manner (24 hr,48 hr,72 hr). In case of both breast cancerous cell lines, amongst all extracts, *Sv* plant extracts were showing significantly higher cytotoxicity towards cells as it was observed that the half minimal cell death was occurs at very lower concentrations in all *Sv* extracts compare to *Bm*, *Sa* and *Ma* (Table 2.3.1A,2.3.1B).

Figure 2.3.5 and 2.3.6 represents the dose response curve for *Sv* extracts for MCF-7 and MDA-MB-231 which indicates that *Sv* leaf extracts were showing promising anti-proliferative activity in comparison with fruit extracts. In case of MCF-7, there is no significance difference between aqueous and methanolic activities of *Sv* leaf. Previously it has been studied that aqueous extract is considered as non-toxic solvent for in-vivo studies during safety evaluations (Akanji et al,2013; Awounfack et al.,2016). That is way for further studies, *Sv* leaf aqueous extract were considered for MCF-7.

In anti-proliferative assay on MCF-7 and MDA-MB-231 cell lines, amongst all extracts, methanolic and aqueous extracts of *Sv* leaf shown highest anti-proliferation activity on these cell lines at lower concentration which is 10 µg /mL and 12 µg /mL accordingly. The cytotoxicity of *Sv* leaf aqueous and methanolic extracts were performed on HEK-293 cell line

which is non-cancerous normal cell line (Figure 2.3.7A,2.3.7B). The highest cell cytotoxicity at the lower dose was observed for *Sv* aqueous leaf extract on MCF-7 cell line at 24 hr incubation (IC_{50} concentration 10 ± 1.13 µg/mL), whereas IC_{50} for aqueous leaf extract for HEK-293 was 58.44±3.40 µg/mL at 24 hr. In same way, *Sv* methanolic leaf extract were showing 94.55±3.40 µg/mL at 24 hr for HEK-293 cells (Figure 2.3.7B). These findings suggest that *Sv* leaf extracts is non-toxic to normal cells and can be consider for further evaluation.

Hence, for further analysis, 10 µg/mL concentration (IC_{50}) was considered, and 5 µg/mL (sub IC_{50}) dose of *Sv* aqueous leaf extract for MCF-7 and 12 µg/mL concentration (IC_{50}) was considered, and 6 µg/mL (sub IC_{50}) dose of *Sv* methanolic extract for MDA-MB-231 cell lines to evaluate its synergy with melatonin (Chapter 5) and migration inhibition and tumour formation inhibition activities (Chapter 6).

The overall MTT results showed that *Sv* UNRF and *Sv* RF extracts showed cytotoxicity normal cells HEK-293, so only *Sv* leaf extract IC_{50} and sub-IC_{50} were considered for further analysis.

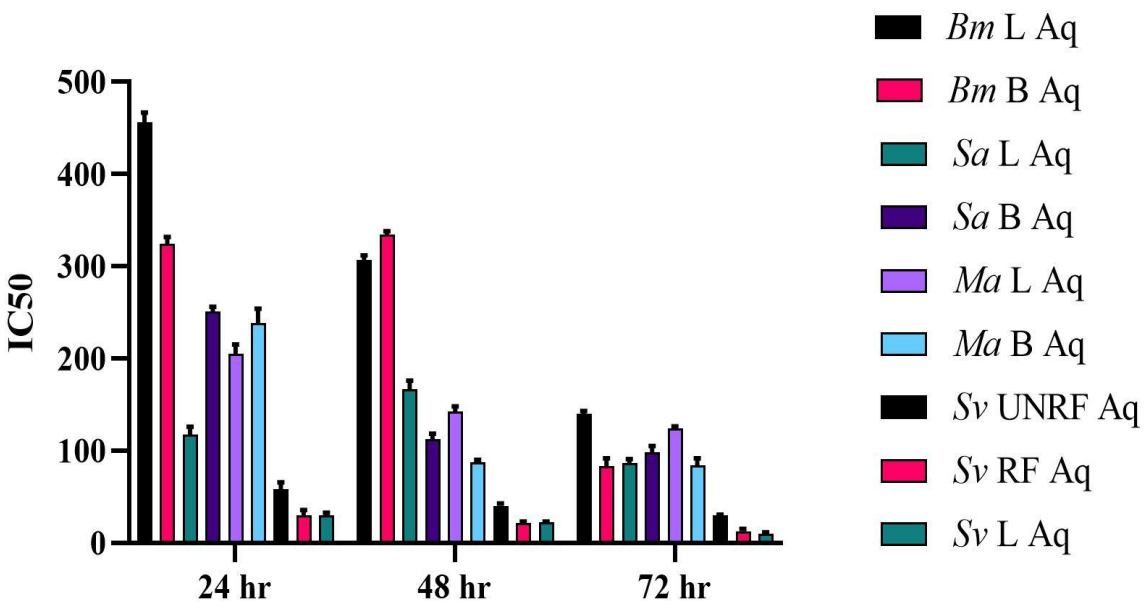

Figure 2.3.1: Anti-proliferative activity: IC_{50} (µg/mL) of *Bm*, *Sa*, *Ma* and *Sv* plant parts' aqueous extracts on MCF-7 for the treatment of 24 hr,48 hr and 72 hr.

Table 2.3.1(A): Anti-proliferative activity of aqueous extracts on MCF-7. IC_{50} was calculated values represent mean± SEM

	Butea monosperma (Bm)		***Saraca asoca (Sa)***		***Melia azedarach (Ma)***		***Solanum virginianum (Sv)***		
hr	**Leaf**	**Bark**	**Leaf**	**Bark**	**Leaf**	**Bark**	**Unripe fruit**	**Ripe fruit**	**Leaf**
24	456.6 ± 10.2	324.4±7.3	117.5±8.4	250.8±5.1	204.7±10.3	238.2±15.6	58.30 ±7.48	29.74 ± 5.78	19.66 ±3.0
48	307.4 ±4.5	334.1 ±4.1	166.5±9.4	112.1±6.3	142.4±5.6	87.27±2.8	40.05 ± 2.45	21.76 ± 1.29	12.18 ± 0.74
72	140.1 ± 3.2	83.13±8.9	86.41±4.5	97.92±7.4	124.0±2.3	83.86±7.9	29.99±0.58	12.91± 2.28	10.4 ± 1.13

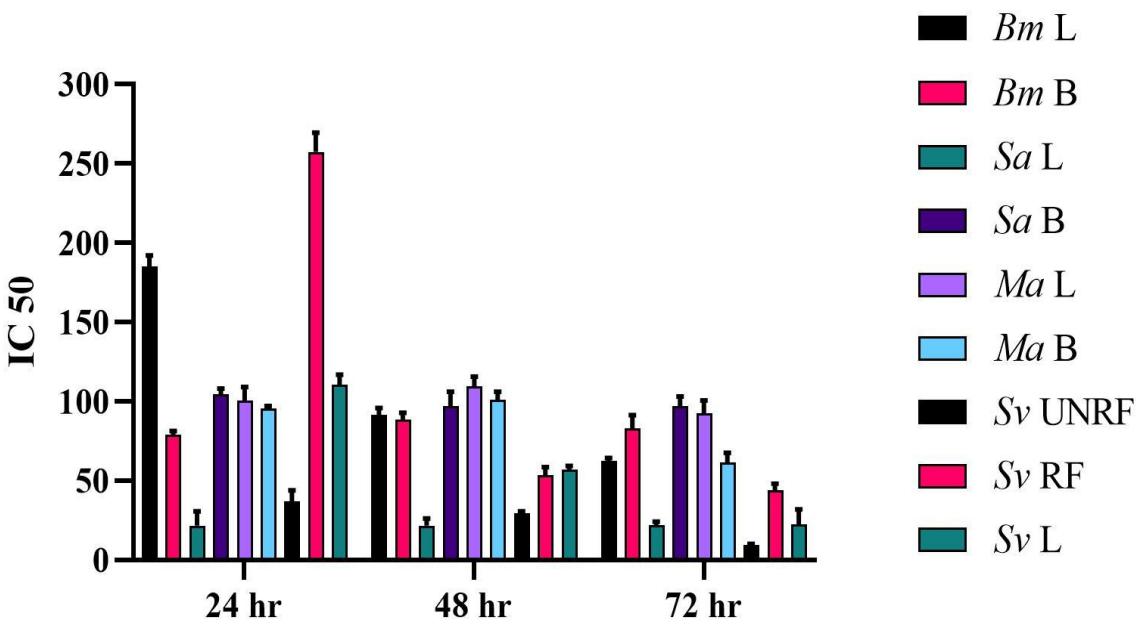

Figure 2.3.2: Anti-proliferative activity determination: IC_{50} (µg/mL) of *Bm*, *Sa*, *Ma* and *Sv* plant parts' aqueous extracts on MDA-MB-231 for the treatment of 24 hr,48 hr and 72 hr.

Table 2.3.2: Anti-proliferative activity of aqueous extracts on MDA-MB-231. IC_{50} was calculated values represent mean± SEM

	Butea monosperma (Bm)		*Saraca asoca (Sa)*		*Melia azedarach (Ma)*		*Solanum virginianum (Sv)*		
hr	**Leaf**	**Bark**	**Leaf**	**Bark**	**Leaf**	**Bark**	**Unripe fruit**	**Ripe fruit**	**Leaf**
24	185.2±6.8	78.78±2.5	21.56±9.1	104.6±3.5	100.5±8.5	95.43±1.8	36.98±6.98	257.3±12.3	110.6±6.3
48	91.70±4.1	88.39±4.6	21.59±4.6	96.75±9.4	109.5±6.2	101.2±4.9	29.37±1.34	53.04±5.2	56.92±2.5
72	62.55±1.9	83.15±8.2	21.90±2.3	97.08±6.1	92.68±7.9	61.29±6.3	9.37± 0.81	43.87±4.3	22.44±9.4

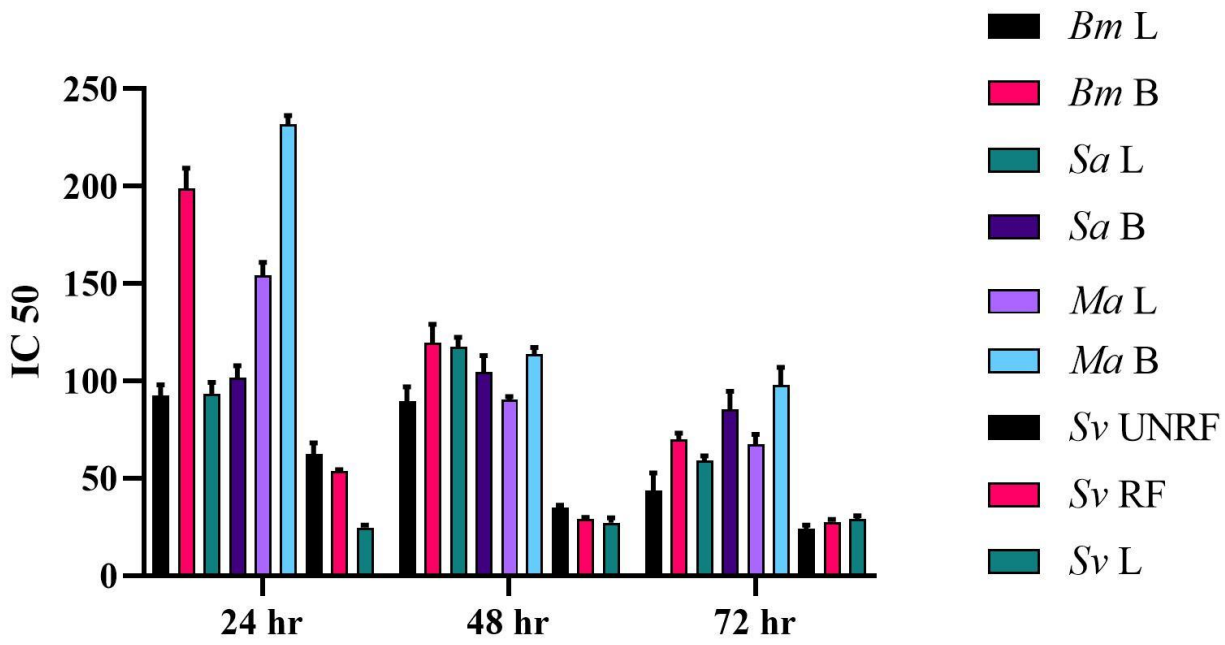

Figure 2.3.3: Anti-proliferative activity: IC_{50} (µg/mL) of *Bm*, *Sa*, *Ma* and *Sv* plant parts' methanolic extracts on MCF-7 for the treatment of 24 hr,48 hr and 72 hr..

Table 2.3.3: Anti-proliferative activity of methanolic extracts on MCF-7. IC_{50} was calculated values represent mean± SEM

	Butea monosperma (Bm)		***Saraca asoca (Sa)***		***Melia azedarach (Ma)***		***Solanum virginianum (Sv)***		
hr	**Leaf**	**Bark**	**Leaf**	**Bark**	**Leaf**	**Bark**	**Unripe fruit**	**Ripe fruit**	**Leaf**
24	92.42 ±5.6	198.9±10.4	93.16±6.1	101.5±6.4	154.2±6.7	231.7±4.6	62.53 ± 5.78	53.60 ±0.95	24.62 ± 1.25
48	89.54 ±7.4	119.4±9.7	117.7±4.6	104.6±8.4	90.48±1.6	113.7±3.5	34.96 ± 1.14	29.05 ± 0.79	27.10 ± 2.5
72	43.63±9.1	69.80±3.4	59.25±2.3	85.56±9.2	67.38±5.3	97.99±8.9	24.05 ± 1.94	27.69 ± 1.23	29.05 ± 1.78

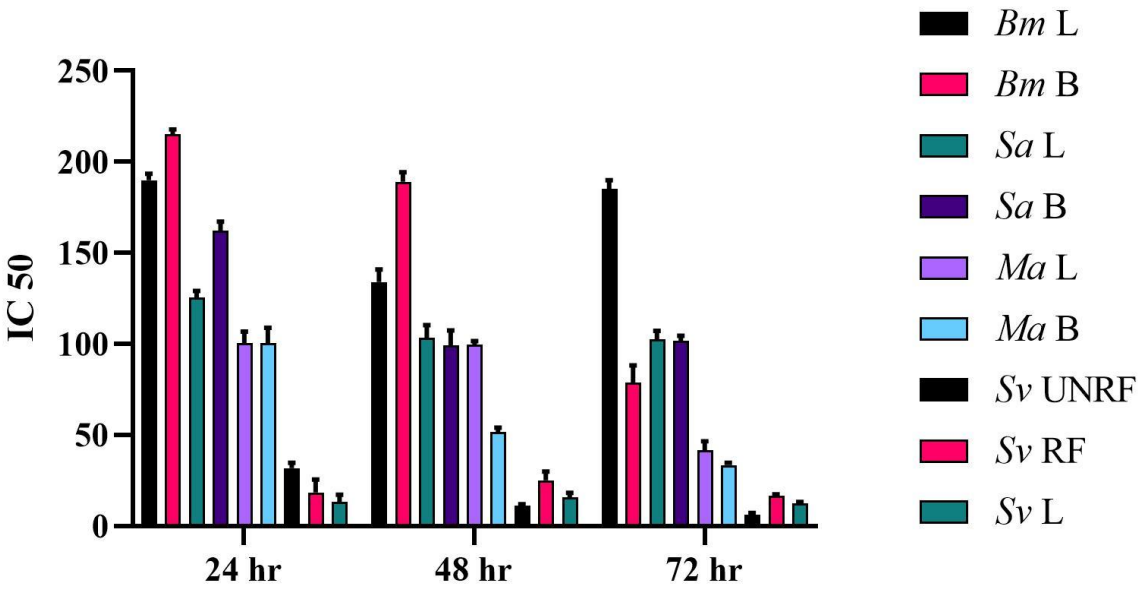

Figure 2.3.4: Anti-proliferative activity: IC_{50} (µg/mL) of *Bm*, *Sa*, *Ma* and *Sv* plant parts' methanolic extracts on MDA-MB-231 for the treatment of 24 hr,48 hr and 72 hr.

Table 2.3.4: Anti-proliferative activity of methanolic extracts on MDA-MB-231. IC_{50} was calculated values represent mean± SEM

	Butea monosperma (Bm)		***Saraca asoca (Sa)***		***Melia azedarach (Ma)***		***Solanum virginianum (Sv)***		
hr	**Leaf**	**Bark**	**Leaf**	**Bark**	**Leaf**	**Bark**	**Unripe fruit**	**Ripe fruit**	**Leaf**
24	189.9±3.6	215.1±2.8	125.5±3.6	162.2±4.9	100.4±6.3	100.4 ±8.4	31.67±3.11	18.34±7.20	13.29±3.81
48	134.0±6.9	189.1±5.1	103.2±7.2	99.0±8.3	99.4±2.1	51.53±2.5	11.06±1.01	25.055 ±4.94	15.8±2.39
72	185.2±4.7	78.79±9.4	102.6±4.6	101.7±2.8	41.56±4.9	33.45±1.2	6.14±1.0	16.55±0.9	12.47±0.67

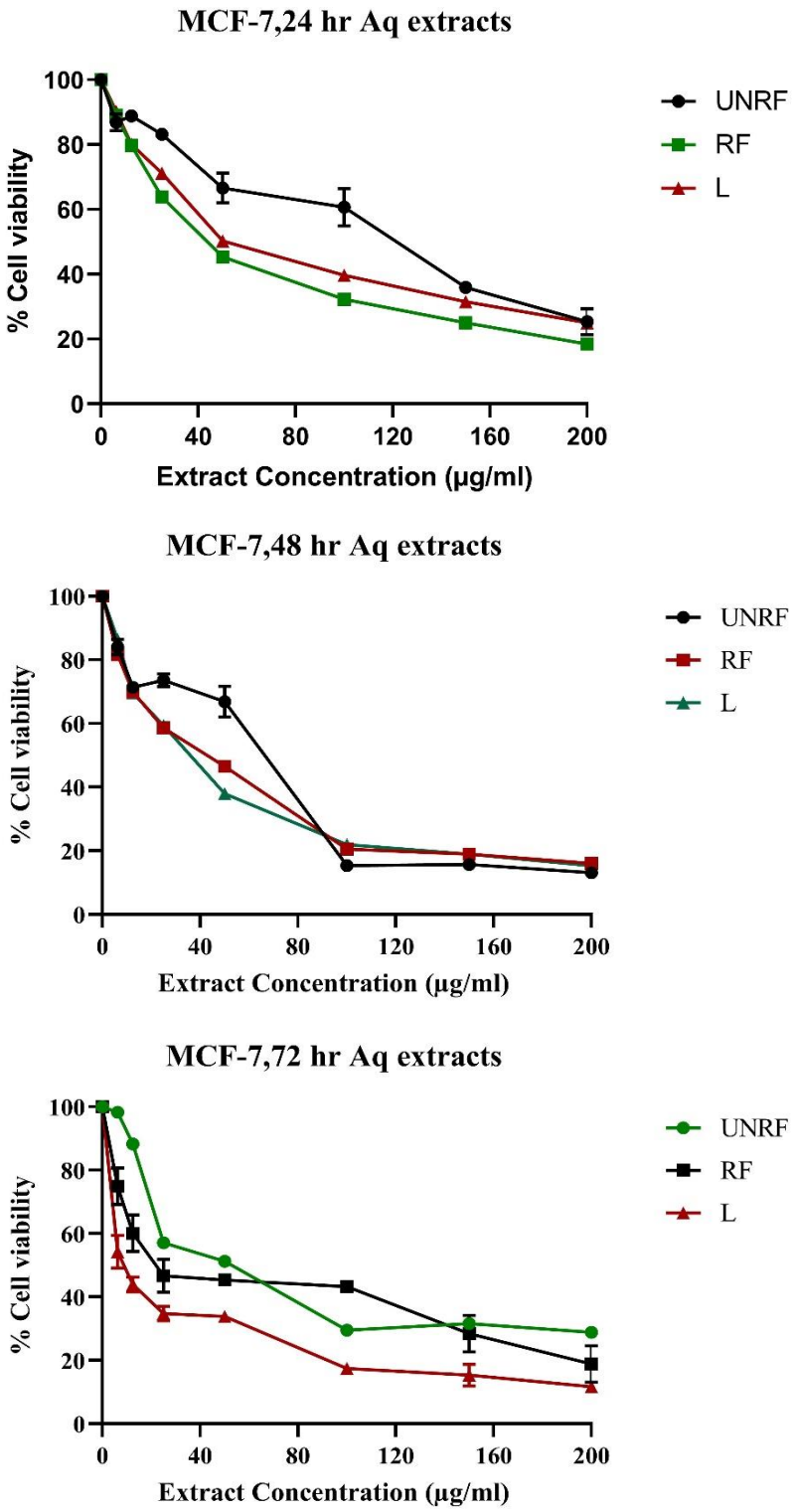

Figure 2.3.5 (A): Anti-proliferative activity of *Sv* plant extracts on Human breast cancer cell line MCF-7. The dose response curve of plant extracts (6.25-200 µg/ml) on MCF-7 cell line were plotted to evaluate IC_{50} concentration. Present graph shows the cell viability at 24 hr,48 hr and 72 hr respectively for **aqueous extract** treatment.

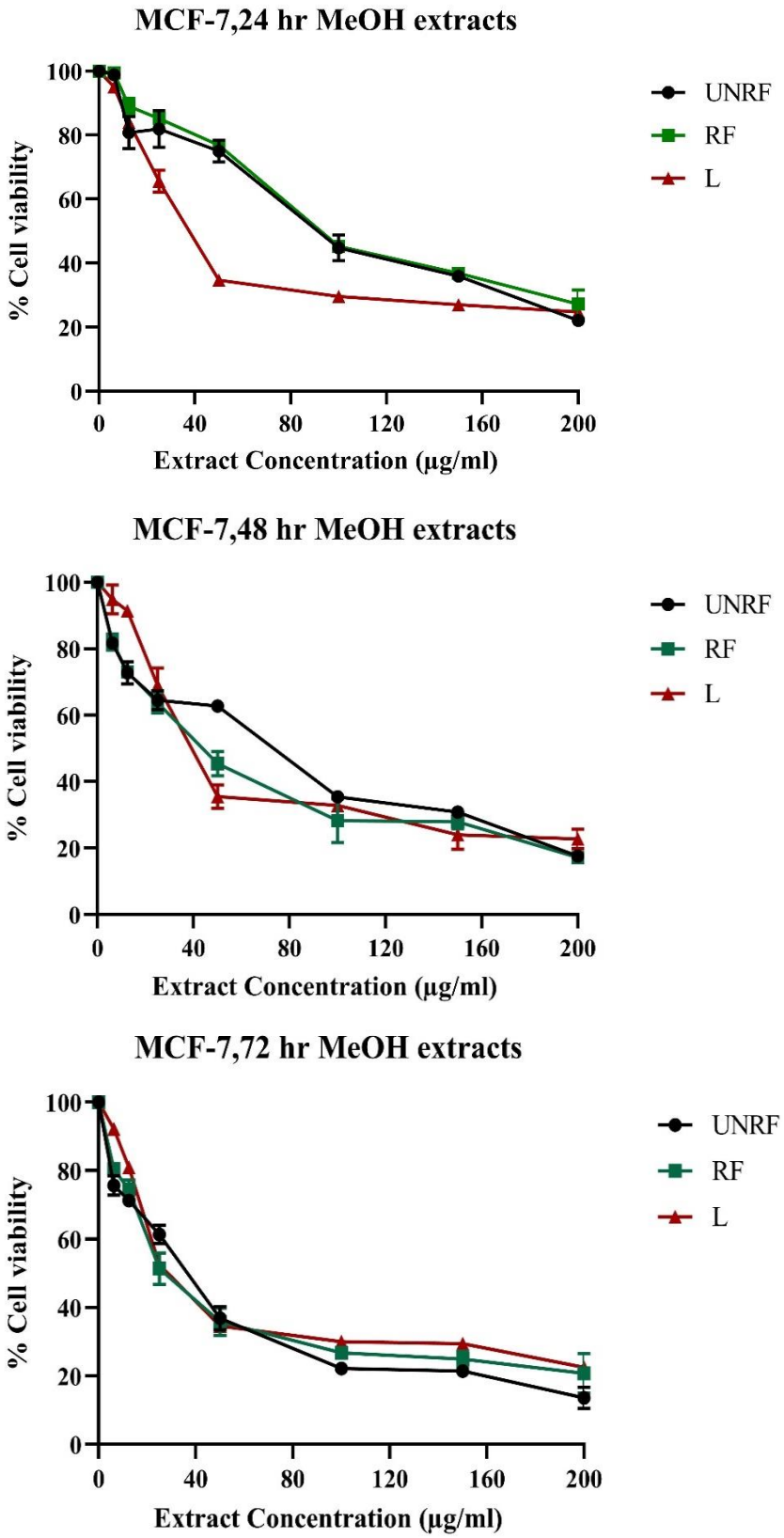

Figure 2.3.5 (B): Anti-proliferative activity of *Sv* plant extracts on Human breast cancer cell line MCF-7. The dose response curve of plant extracts (6.25-200 µg/ml) on MCF-7 cell line were plotted to evaluate IC_{50} concentration. Present graph shows the cell viability at 24 hr,48 hr and 72 hr respectively for **methanolic** extract treatment.

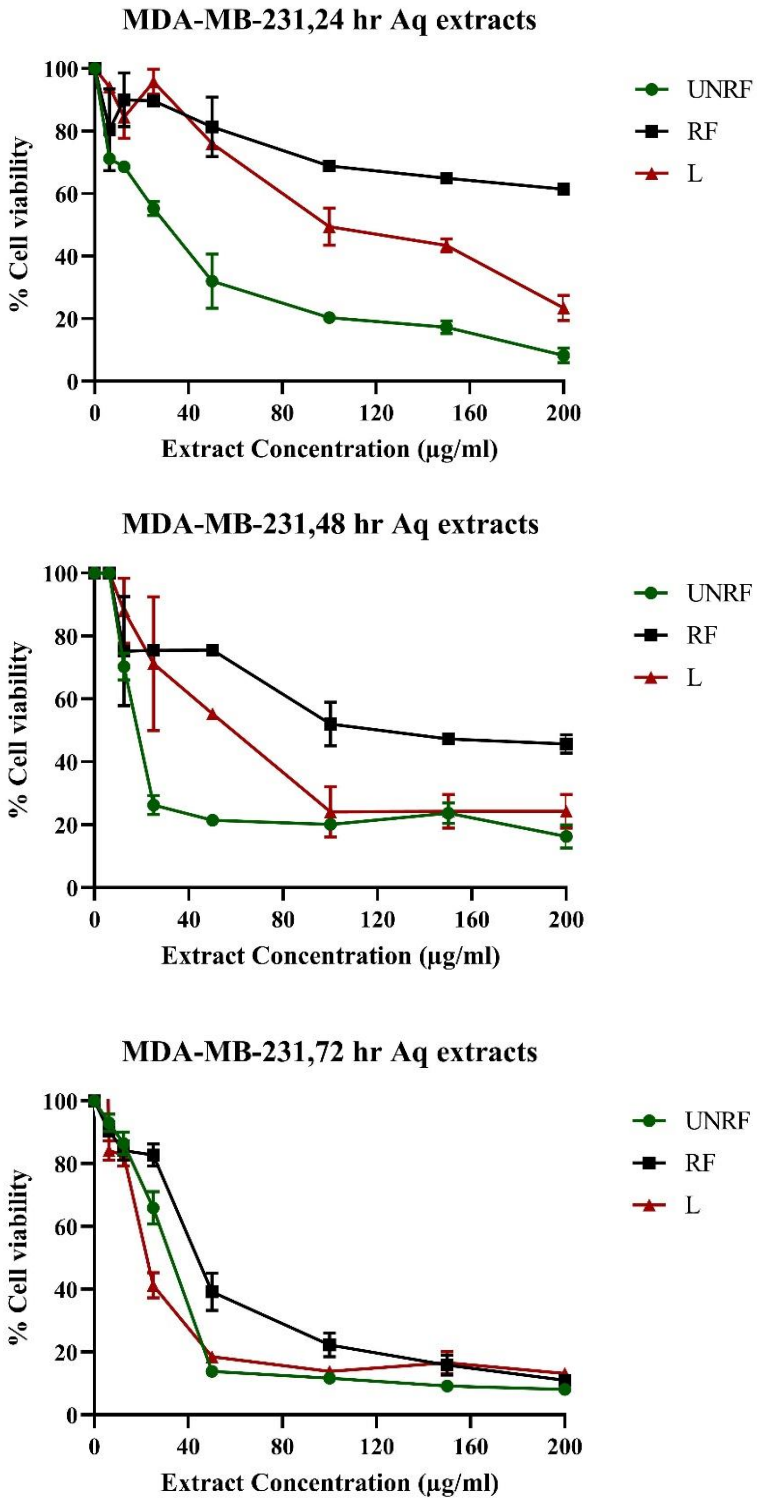

Figure 2.3.6(A): Anti-proliferative activity of *Sv* plant extracts on Human breast cancer cell line MDA-MB-231. The dose response curve of plant extracts (6.25-200 µg/ml) on MDA-MB-231 cell line were plotted to evaluate IC_{50} concentration. Present graph shows the cell viability at 24 hr,48hr and 72 hr respectively for **aqueous extract** treatment

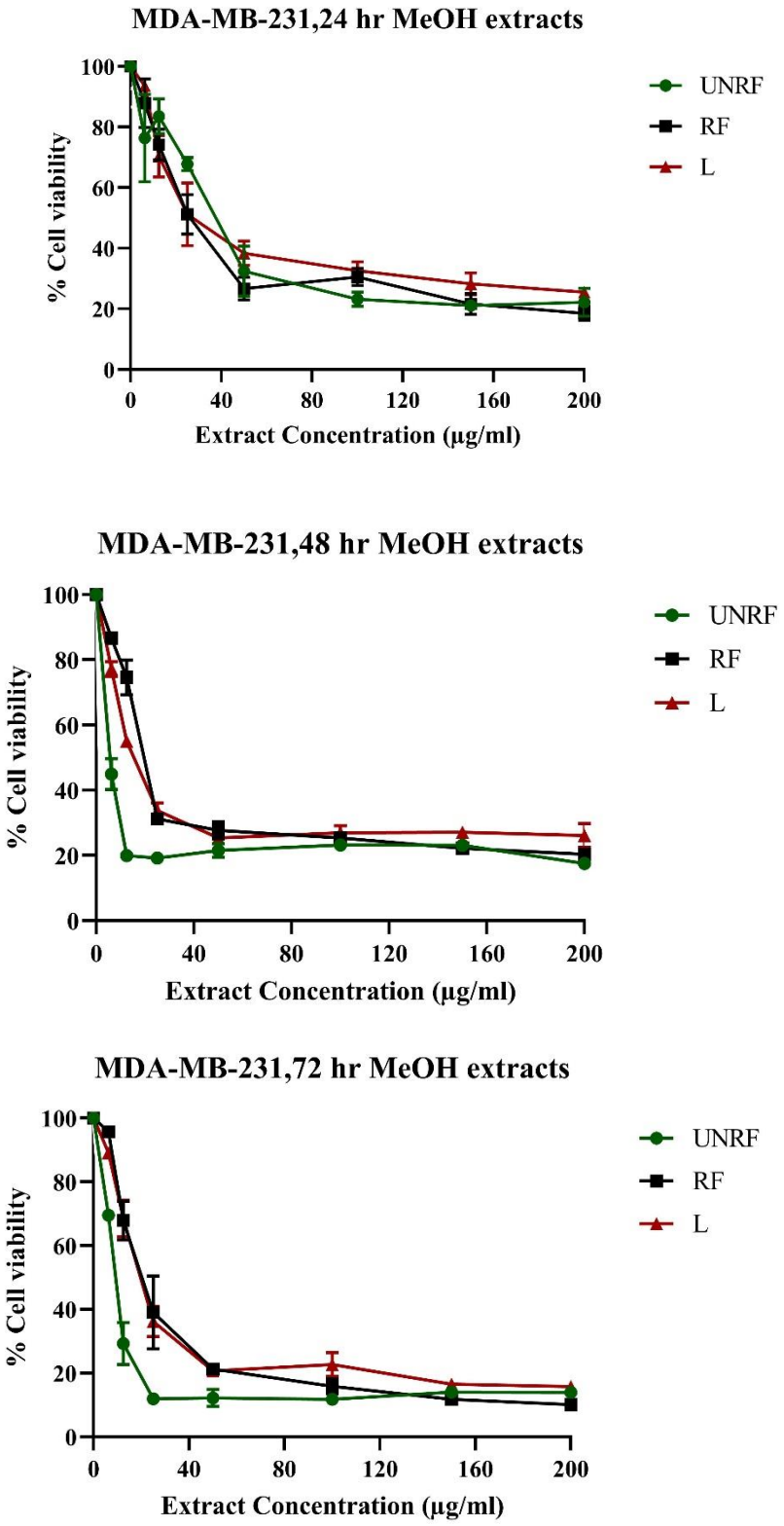

Figure 2.3.6(A): Anti-proliferative activity of *Sv* plant extracts on Human breast cancer cell line MDA-MB-231. The dose response curve of plant extracts (6.25-200 µg/ml) on MDA-MB-231 cell line were plotted to evaluate IC_{50} concentration. Present graph shows the cell viability at 24 hr,48 hr and 72 hr respectively for **methanolic extract** treatment.

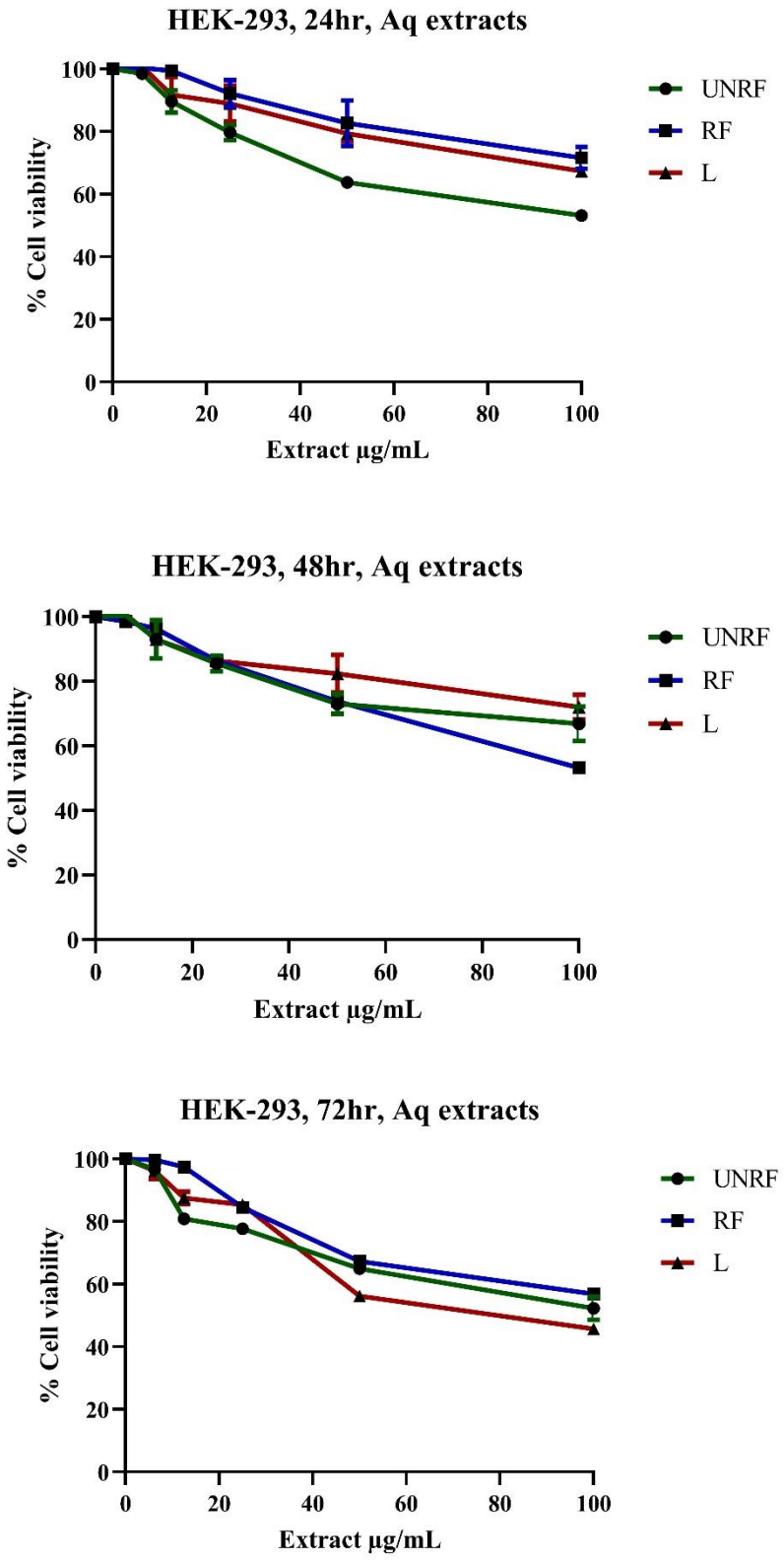

Figure 2.3.7(A): Cytotoxic evaluation of *Sv* plant extracts on HEK-293. The dose response curve of plant extracts (6.25-200 µg/ml) on HEK-293 cell line were plotted to evaluate IC_{50} concentration. Present graph shows the cell viability at 24 hr,48 hr and 72 hr respectively for **aqueous extract** treatment.

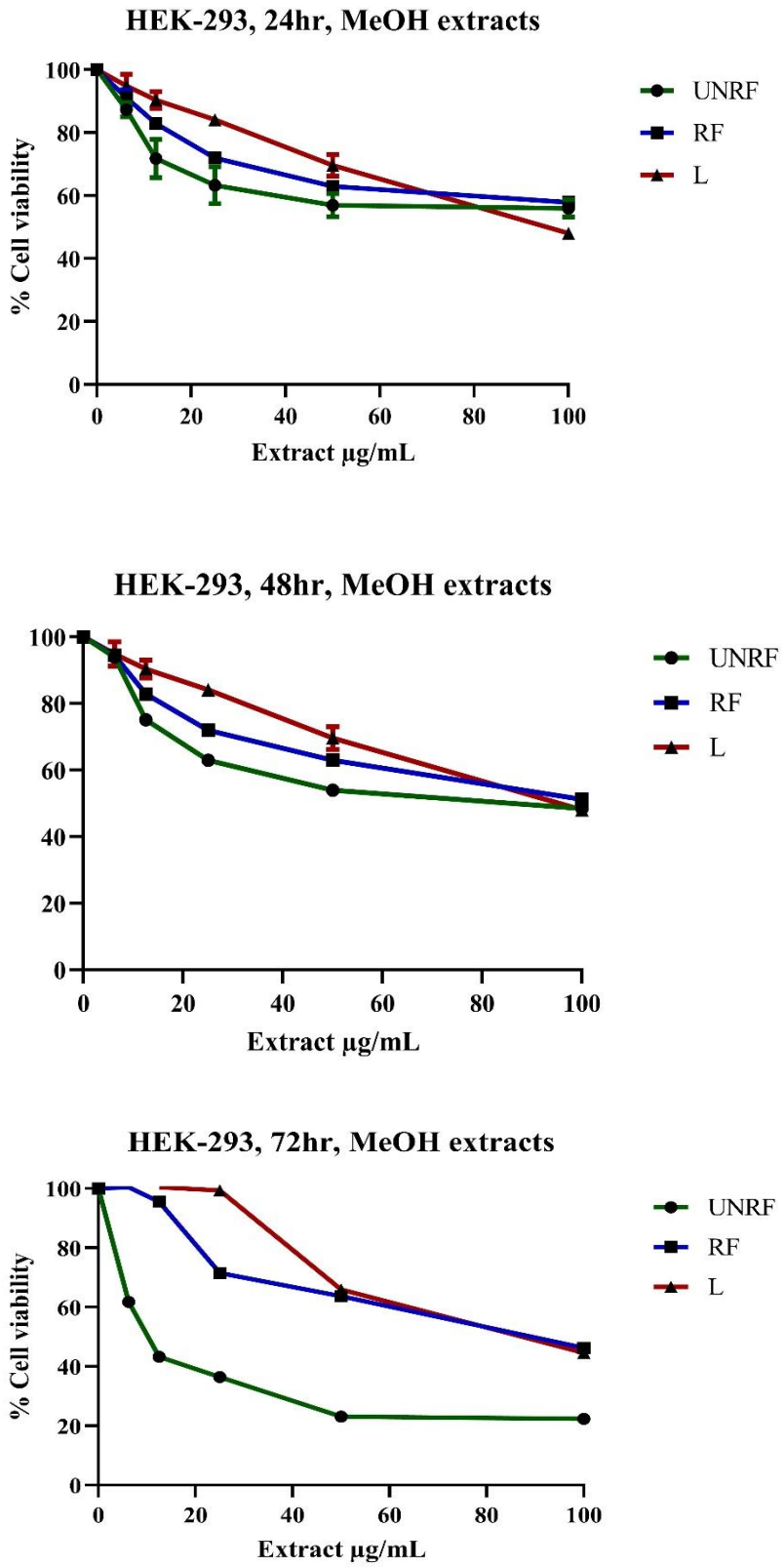

Figure 2.3.7(B): Cytotoxic evaluation of *Sv* plant extracts on HEK-293. The dose response curve of plant extracts (6.25-200 µg/ml) on HEK-293 cell line were plotted to evaluate IC_{50} concentration. Present graph shows the cell viability at 24 hr,48 hr and 72 hr respectively for **methanolic extract** treatment.

2.4 Discussion

The half minimal concentration (IC_{50}) evaluation of selected plants extract for its anti-proliferative activity against breast cancer was performed by anti-proliferative activity determination by colorimetric assay i.e. MTT assay (Corry et al.,1991 and Patel et al.,2024). The MTT assay is used to measures cell viability by assessing the metabolic activity of cells, providing valuable insights into the cytotoxic effects of the plant extract on cancer cells (Tolosa et al.,2015).

Previously, flower extracts were studied to evaluate the anti-cancer potential of *Bm* in which ethanolic extract showed anti-cancer activity against Ehrlich Ascites Carcinoma (EAC) in mice It increased the life span of the mice and restored hematological parameters. (J. and B.,2011). The methanol extract of *Bm* flowers exhibited significantly higher anti-proliferative activity against the MCF-7 human breast cancer cell line (Polina,2020). One of the previous study suggested that Isocoreopsin is an active constituent of the n-butanol extract of *Bm* flower possess various pharmacological activities, including antioxidant and anti-inflammatory properties (Subramaniyan et al.,2016). The cytotoxicity activity of New ring C-seco limonoids from Brazilian *Ma* fruit has been evaluated by Akihisa and group (Akihisa et al.,2013). In this study, researchers isolated and identified new ring C-seco limonoids from Brazilian *Ma.* One of the previous study has evaluated the cytotoxicity study of *Ma* leaf extracts o T47D cell line (Ervina et al.,2020). The ethanolic extract *of Sa* flowers exhibited dose-dependent inhibition of lung cancer cell viability, with an observed IC_{50} dose at a concentration of 60 µg/ml (Dharshini et al.,2021).

Findings from previous studies investigating the anti-cancer properties of *Sa* that inhibition of cell proliferation, induction of apoptosis, and suppression of tumour growth in

mouse mammary cells C127I. (Nair et al., 2023). In one of the previous study, researchers evaluated the in-vitro cancer activity of *Sa* extract on various cancer cell lines, including colon, and prostate cancer cells, has been shown to modulate various signalling pathways involved in cell survival and proliferation, such as the PI3K/Akt and MAPK pathways (Jeenathunisa,2021 & Choudhary et al.,2021).The phyto-compounds present in different plant parts of *Sv* are steroidal alkaoids, saponins, tannins, flavonoids, glycosides, cardiac glycosides, and anthraquinones (Chapter 2). The presence of these phyto-chemicals in *Sv* is responsible for its anti-proliferative activities against breast cancer cell lines. Previous studies have shown that the steroidal alkaloids included in *Sv* have the ability to cause cell death in several types of cancer cells (Kaunda et al.,2021). Some of the previous study included four extracts (methanol, aqueous, chloroform, benzene) obtained from the aerial vegetative parts of *Sv* has shown anti-bacterial and anti-oxidant activity (Rohilla et al., 2023). The plant parts leaf and bark of *Bm*, *Ma* and *Sa* was not much studied previously on breast cancer cell line. Previous phytochemical analysis has confirmed the presence of active metabolites e.g. buteaspermin A, buteaspermin B and buteasperminol, medicarpin, cajanin, formonentin, isoformonentin and cladrin, catachin, Epicatechin, β-sitosterol glucoside, Melianol, Melianolone, Melianone, Nimbolidin-A, Nimbolinin-A, Nimbolinin-B in leaf and bark of *Bm*, *Ma* and *Sa* plants (Kumari et ,2022; Salvi et al.,2022; Sharma et al.,2013; CT et al.,2020). Presence of these metabolites supports the anti-oxidant and cytotoxic activity observed in the results of this chapter.

Though *Sv* plant were used by folklore (Dalavi et al.,2023), unfortunately there is a lack of research in therapeutics direction. In present study, *Sv* leaf extracts were showing highest TPC and TFC (chapter 1) and at the same time also showing anti-proliferative activity against MCF-7 and MDA-MB-231 cell line. *Sv* leaf extracts were also showing higher anti-oxidant activity amongst all other extracts (chapter 1). This may be a possible reason for highest anti

proliferative activity of *Sv* leaf compare to other extracts (Milella et al.,2023). To evaluate the secondary metabolites, present in *Sv* leaf extracts, further advanced analytical methods (GC-MS and HR-LCMS/MS) were performed (details given in chapter 3).

Deeper mechanical insights of action of the active compounds from Sv needs to be deciphered to fully understand its potential in cancer therapy. The identification of this compounds and its cytotoxic activity in cancer cells paves the way for future studies to explore the development of novel anti-cancer drugs derived from natural sources.

The anti-proliferative and cytotoxicity activity explained in this chapter summarises, that amongst all extract, Sv leaf aqueous extract has good anti-proliferative activity against MCF-7 cell line while leaf methanolic extract were showing promising anti-proliferative activity against MDA-MB-231 cells without showing any toxicity study on HEK-293 at lower dosage. For upcoming in vitro studies, Sv leaf extract dosage and time durations were considered.