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CYTOTOXIC EFFECT OF *Brassica oleracea* L. var. *italica* FLORETS AND MICROGREENS ON ACUTE LEUKEMIC CANCER CELL LINE

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ABSTRACT

The bioactivity of broccoli microgreens might be valuable for understanding the association between diet and cancer along with searching for healthy and functional food options. The present study was centred on broccoli microgreens as a functional food. The anticancer activity was measured in broccoli florets and microgreens. The cytotoxicity of microgreens extract was confirmed by flow cytometry assays. Significantly higher anticancer activity was observed in microgreens as compared to the florets. The IC_{50} value for Thp-1 cell line was 772.47 and 446.34 µg mL^{-1} for florets and microgreens, respectively. In cell cycle analysis, 69% cells were arrested at G_0/G_1 phase. The 8.58% of dead cells were detected in early apoptosis. As the cells were in early apoptosis, DNA damage was observed only in 3.53% cells. Thus, the increased consumption of broccoli microgreens could have significantly positive impact on human health.

Keywords: Apoptosis, broccoli microgreens, cell cycle arrest, cytotoxic effect, Thp-1 cell line

INTRODUCTION

Brassica vegetables, often referred to as cruciferous vegetables, comprise a diverse and nutritionally rich group of crops that have widely been grown and cherished for centuries. The family *Brassicaceae* includes a wide array of popular and nutritious crops such as cabbage, cauliflower, kale, broccoli, Brussels sprouts, and mustard greens (Ağagündüz *et al.,* 2022). Brassica vegetables are abundantly rich in several key phytochemicals like glucosinolates (GLSs), isothiocyanates (ITCs), carotenoids, vitamin C and fibers that have gained attention for their various health-promoting properties (Fuente *et al.,* 2020; Ağagündüz *et al.,* 2022). These compounds have extensively been studied for their potential to reduce the risk of chronic diseases, like cancer, cardiovascular diseases, and diabetes (Fuente *et al.,* 2020). Microgreens (MG), essentially young and tender edible plants harvested at an early growth stage, represent a treasure trove of bioactive compounds that offer potential health benefits (Tomas *et al.,* 2021; Kowitcharoen *et al.,* 2021). Their concentrated nutritional profile is attributed to their rapid growth and higher photosynthesis rates during this phase, where they accumulate a higher contents of vitamins, minerals, and antioxidants as compared to the mature plant (Fuente *et al*., 2020; Kowitcharoen *et al*., 2021 Tomas *et al*., 2021). Microgreens have piqued the interest of health-conscious individuals and researchers due to their potential role in promoting wellness and preventing disease (Kowitcharoen *et al*., 2021).

Acute monocytic leukemia is a type of acute myeloid leukemia (AML). More than 80% of AML cells have monocytic lineage. AML, a systemic and diverse hematologic malignancy, stands as the predominant form of acute leukemia in elders, contributing significantly to morbidity and mortality rates (Mohammadi *et al.,* 2017). The prevailing therapeutic tactics predominantly hinge on aggressive

interventions like chemotherapy and radiotherapy. Nevertheless, these approaches demonstrate restricted effectiveness in securing long-term survival for AML and are often fraught with various side effects. Conventional drug associated toxicities such as gastrointestinal, musculoskeletal or constitutional symptoms, hair loss, heart or kidney problems, lung tissue damage or nerve damage, infertility and so on pose additional challenges. Consequently, there is a pressing need to explore alternative treatment modalities and identify potential therapeutic targets that offer minimal side effects and enhanced efficiency.

The role of brassica vegetables in cancer prevention and potential therapeutic applications has been a subject of scientific debate. Broccoli (*Brassica oleracea* L. var. *italica)*, specifically in the form of sprouts and microgreens, has garnered significant attention for its potential health benefits (Jang *et al*., 2015; Le *et al*., 2020a). The young seedlings of broccoli are an exceptional source of health-promoting phytochemicals (Marchioni *et al*., 2021). In recent years, research has provided insight into the bioactive compounds found abundantly in broccoli and their profound impact on cancer-related processes (Le *et al*., 2020a). These compounds, primarily glucosinolates, are present in higher concentration in broccoli as compared to the other brassica crops which are responsible for pungent taste and distinctive aroma of these vegetables (Bhandari *et al*., 2015). When consumed, they undergo enzymatic breakdown to form biologically active substances, including isothiocyanates and indoles, which demonstrate potent anticancer properties (Tomas *et al*., 2021; Ağagündüz *et al*., 2022). The ongoing clinical and epidemiological research has produced convincing evidence suggesting that a diet rich in broccoli may be linked with a reduced risk of certain cancers, like colon, breast, lung and prostate cancer (Le *et al*., 2020b). The link between food and cancer has been a topic of scientific inquiry and public fascination for a considerable period. In the quest for natural ways to prevent and even potentially cure cancer, brassica vegetables have emerged as a fascinating and promising area of study. In our literature study, we did not find sufficient articles related to effect of broccoli microgreens on leukemia. Therefore, the present study explores the multifaced relationship between broccoli microgreens and leukemia, delving into the mechanisms through which the microgreens may demonstrate protective effects against cancer.

MATERIALS AND METHODS

Standard sulforaphane (SFN) was purchased from Sigma Aldrich. SFN is one of the major ITCs present in broccoli. SFN (5 mg) was dissolved in 10 mL 70% methanol to prepare a stock solution of 500 µg mL^{-1} concentration, and stored at 4°C before use. Acute myeloid leukemia cell line (Thp-1) was procured from the National Centre for Cell Science (NCCS), Pune, India. The cell line was subcultured and maintained at 5% CO_2 and 37°C during the whole experiment.

Plant material

Broccoli florets (*Brassica oleracea* L. var. *italica*) at its edible stage were purchased from a local market of Surat, Gujarat (India). They were rinsed under flowing tap water until the dust and debris were removed, followed by a rinse with deionized water. Fresh florets were ground in a mixer grinder to prepare a pulp and immediately used for hydrolysis extraction (Chauhan *et al.*, 2016).

Microgreens cultivation

Broccoli "Omaxe" hybrid seeds (*Brassica oleracea* L. var. *italica*) were purchased from a local market in Surat, Gujarat. The seeds were surface sterilized by 70% ethanol for 1 min, followed by 1.5% sodium hypochlorite (NaOCl) treatment for 15 min. The ethanol and NaOCl were removed by two rinses of distilled water (Ninh Le *et al.*, 2019). The seeds were soaked in distilled water for 10 h. Then, 5 g seeds were spread evenly on pots having soil enriched with worm compost, and irrigated twice a day by tap water. The pots were kept under natural conditions of aeration and light. A photo-

period cycles of 14 h light and 10 h darkness was maintained. Microgreens were collected after 10 days seed sowing when two cotyledons stage was clearly visible. After harvest, the microgreens were rinsed under flowing tap water and then with distilled water (Ninh Le *et al.*, 2019). Fresh microgreens were ground in a mixer grinder to prepare juice and immediately used for hydrolysis extraction.

Hydrolysis extraction

Hydrolysis was conducted as per the method of Lee *et al.* (2017) with slight modifications (Saengha *et al.*, 2021). The 200 g broccoli florets and microgreens were ground and homogenized with a multi-food processor to prepare a pulp. To this pulp, 100 mL distilled water (pH 7.0) was added, kept in a water bath for 3 h at 45°C and then left outside the water bath for 30 min to reach room temperature. Then 100 mL dichloromethane (DCM) was added, the mixture was stirred for 15-20 min and filtered through a Buchner funnel with Whatman filter paper grade 1. The filtrate was collected in different beaker and the extraction from residual pulp was repeated with another 2 portions of 100 mL DCM. The organic layers were combined and excessive water was removed by adding 4 g anhydrous sodium sulphate. The solvent was then evaporated on a rotary evaporator until dry. The dry extract was dissolved in 70% methanol to get a final concentration of 1 mg mL^{-1}.

Proliferation assay

Each well of 96-well microtiter plate received 200 µL cell suspension or around 40,000 cells and was subsequently incubated at 37ºC for 24 h with 5% CO_2. The utilized medium was aspirated after a day and then 100 µL of various sample concentrations were introduced into their corresponding wells. Again, incubation was done at 37ºC for 48 h with 5% CO_2. The sample-containing media was aspirated when microtiter plate was taken out of the incubator. To get 0.5 mg mL^{-1} of final concentration in each well, 100 µL medium having 10% MTT reagent was added (Pozarowski and Darzynkiewicz, 2004) and incubated at 37ºC for 3 h in 5% CO_2. The medium was fully withdrawn without causing any disruption to the formazan crystals. After adding DMSO (100 µL) solubilization solution, the plate was carefully agitated to dissolve formazan that had formed (Le *et al*., 2020b). The absorbance was noted at 570 nm using an ELISA reader (BK-EL 10A, Biobase China) (Tajalli *et al.*, 2020). The quantity of test sample required to block 50% cell growth (IC_{50}) was ascertained from a dose-response curve for cell line.

Cell cycle arrest

To confirm whether the cancer cells were arrested at various stages after treatment with broccoli microgreens extract (IC_{50}), a cell cycle study was performed (Arora *et al.*, 2016)**.** The cells were cultured in a six well plate at a population of 3.0 x 10^5 cells 2 mL^{-1}. The cells were treated with broccoli microgreens (IC_{50}) and SFN (IC_{50}) for 48 h (Pozarowski and Darzynkiewicz, 2004)**.** The cells treated with SFN were considered positive control. Centrifugation was done to remove cells from the plate following the treatment. After the pellet has been created, it was cleaned twice with 1X Dulbecco's phosphate buffered saline (DPBS), preserved in 70% cooled ethanol at -20°C and then resuspended in 400 µL PI-RNase solution per million cells (Ormerod *et al.*, 1998)**.** The samples were thoroughly combined and examined using a USA-made Cytomics FC500 flow cytometer. The results were expressed as the percentage of cells arrested in each phase of cell cycle. (Fuente *et al.*, 2020)**.**

Annexin V apoptosis assay

The various apoptotic stages of cells were identified by using propidium iodide (PI) fluorochrome and Annexin V-AbFlourTM 488 (KTA0002) apoptosis detection kit (Abbkine Inc**.)** (Fuente *et al.*, 2020)**.** A 6-well plate was used for the assay, which had a count of 3 x 10^5 cells 2 mL^{-1}. The cells were treated with broccoli microgreens (IC_{50}) and SFN (IC_{50}) for 48 h, followed by centrifugation (300 × g for 5 min) to remove the cells from plate. The pellets were washed twice with PBS. The cell pellets were re-suspended with 100 µL binding buffer and then diluted with distilled water (1:10). After adding annexin V (5 µL), the mixture was left to incubate in dark for 15 min at ambient temperature (Vermes *et al.*, 1995)**.** Then 2 µL PI and 400 µL binding buffer were put in each tube and vortexed carefully.

Early apoptotic cells show green fluorescence of the cellular membrane, dead cells show red fluorescence of the nucleus and green fluorescence of the cellular membrane, and live cells show little or no fluorescence. Detection was done by flow cytometry. The results were recorded immediately after the addition of PI**.**

TUNEL assay

DNA fragmentation is among the final stages of apoptosis, which is facilitated by endonucleases. The nucleases breakdown the large chromatin structure into small fragments of 300 kb length, and then into DNA fragments with roughly 50 base pairs. This assay is used to identify fragmented DNA (Darzynkiewicz *et al*., 1997). It is triggered by exogenous terminal deoxynucleotidyl transferase (TdT). The assay was conducted using APO-DirectTM kit (Catalog No. 556381; Pharmingen, BD Biosciences) adopting *in situ* cell death detection kit's methodology. Briefly, the cells were grown to a population of 3 x 10^5 cells mL^{-1} in a six-well plate. After treatment with samples, the cells were collected as pellets and fixed for 60 min at ambient temperature using paraformaldehyde (1% w/v) in PBS (pH 7.4) at cell density of 2 x 10^6 mL^{-1}. After rinsing the cells with PBS and washing buffer, the DNA labelling solution (50 µl) was added and kept for 60 min. After incubation, cells were washed with rinse solution and stained for 30-35 min using PI/RNase staining buffer at room temperature in dark. The fluorescence (green) intensity was recorded to estimate the fraction of TUNEL+ cells.

Statistical analysis

All the experiments were conducted in triplicate. The data was analysed by one-way analysis of variance test ($p < 0.05$) and expressed as mean ± standard deviation (SD).

RESULTS AND DISCUSSION

Proliferation assay

Anticancer activities of hydrolysed extracts of broccoli florets and microgreens were assessed against Thp-1 cell line using MTT assay (Fig. 1; Table 1). Cell line was subjected to increasing concentrations of sample extracts for 48 h. All samples triggered cellular demise in a dose-dependent manner. For

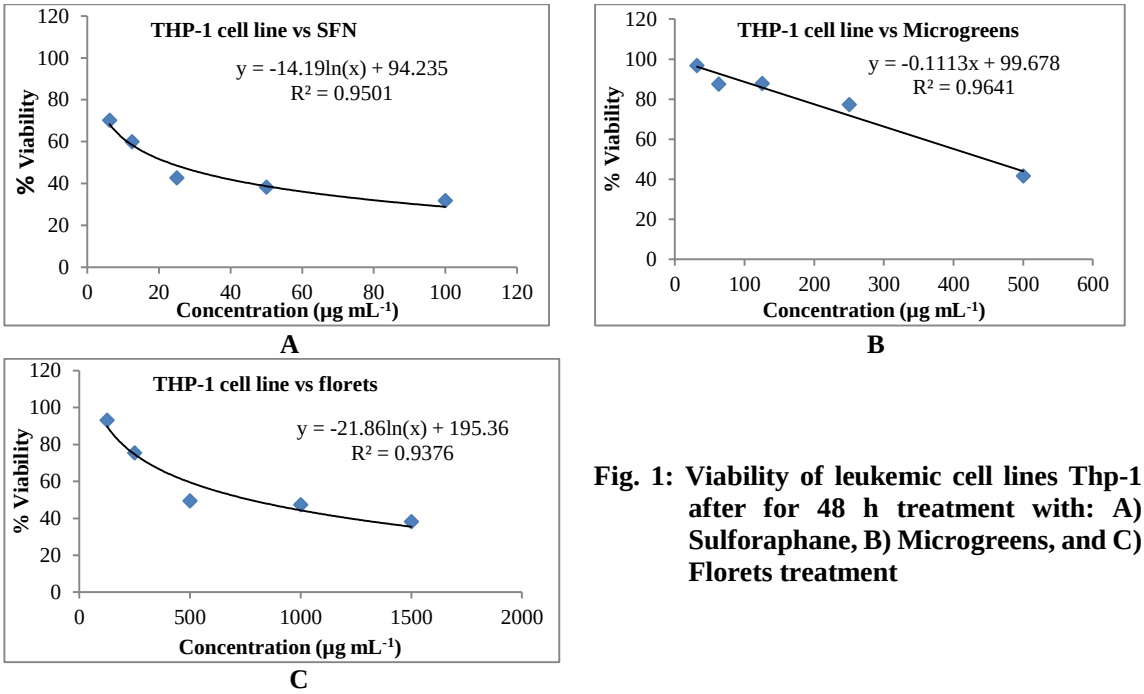

Fig. 1: Viability of leukemic cell lines Thp-1 after for 48 h treatment with: A) Sulforaphane, B) Microgreens, and C) Florets treatment

Table 1: The viability, mean absorbance and SD for sulforaphane, broccoli microgreens and for broccoli florets treatments (Thp-1 cell line)

Treatments	Test concentration µg mL^{-1}				
	6.25	12.5	25	50	100
Sulforaphane					
Mean absorbance	0.5553	0.4743	0.3370	0.3027	0.2520
SD	0.0150	0.0351	0.0010	0.0121	0.0115
Viability (%)	70.18	59.94	42.59	38.25	31.84
Broccoli microgreens					
Mean absorbance	1.3467	1.2167	1.2217	1.0740	0.5780
SD	0.0449	0.0096	0.0469	0.0441	0.0277
Viability (%)	96.74	87.40	87.76	77.16	41.52
Broccoli florets					
Mean absorbance	1.4303	1.1583	0.7597	0.7280	0.5877
SD	0.0541	0.0439	0.0352	0.0645	0.0619
Viability (%)	93.16	75.45	49.48	47.42	38.28

MG, 31.25 to 500 µg mL^{-1} concentrations were taken along with standard SFN (6.25 to 100 µg mL^{-1}) and florets (125 to 1500 µg mL^{-1}). The results revealed effective IC_{50} values of 446.34 and 772.47 µg mL^{-1} for broccoli microgreens and florets, respectively (Table 2). Broccoli in various forms (florets, sprouts, microgreens) exhibited cytotoxic activity against a range of cancer cell lines, including 786-O (renal adenocarcinoma), MCF7 (breast adenocarcinoma), NCI-H460 (lung carcinoma), HT29 (colorectal adenocarcinoma) HepG2 (hepatocellular carcinoma cells), SW480 (colorectal adenocarcinoma), Caco-2 (colorectal adenocarcinoma), PC-3 (prostate carcinoma) and AGS (gastric adenocarcinoma) (Paśko *et al.,* 2018; Ninh Le *et al.*, 2019; Nandini *et al.,* 2020). Multiple research findings consistently demonstrate that the intake of cruciferous vegetables offers robust protective benefits against various cancers.

Table 2: IC_{50} values (µg mL^{-1}) for cytotoxic activity of test samples for Thp-1 cell line

Samples	IC_{50} values
Sulforaphane	22.59
Broccoli microgreens	446.34
Broccoli florets	772.47

The study revealed the anticancer potential of broccoli microgreens**.** Natural product-based cancer prevention has drawn a lot of curiosity. Multiple carcinogenesis models have been used to assess the potentially preventive role of brassica vegetables along with their active phytochemicals, such as GLSs and phenols.(Baenas *et al*., 2015; Paśko *et al.,* 2018; Ninh Le *et al*., 2019). Broccoli microgreens are considered an excellent source of GLSs and hydrolysed compounds (isothiocyanates, particularly sulforaphane), which may induce apoptosis and stop the headway of cell cycle, hence lower the cancer risk. Several *in vitro* studies have demonstrated anti-cancerogenic impact of antiproliferative activities of broccoli microgreens (Wang *et al*., 2018; Wang *et al*., 2021).

Cell cycle arrest

Our work demonstrated cell cycle arrest (clearly in G_0/G_1 phase) to support whether the cytotoxicity observed was enough to arrest the cell cycle progression. PI stained Thp-1 cells were used to study the cell population at three distinctive phases of cell cycle comprising G_0/G_1 phase, S phase, and G_2/M phase. G_1/S and G_2/M transitions are two important checkpoints in cell cycle (Le *et al*., 2020b). The cells treated with standard SFN showed increased G_0/G_1 phase to a level of 55.1% and MG extracts showed an increase up to 69.0% (Fig. 2). Compared to the untreated control cells, MG treated cells had higher percentage of cells in G_0/G_1 phase and reduced percentage of cells in S and G_2 phase (Fig. 2), implicating MG extract has efficiently arrested the cell cycle at G_0/G_1 phase. A recent area of interest is the molecular connections among cell cycle, cell death, and cell survival. Many anticancer drugs work by obstructing various phases of cell cycle, which ultimately lead to apoptosis (Ninh Le *et al*., 2019; Wang *et al*., 2021).

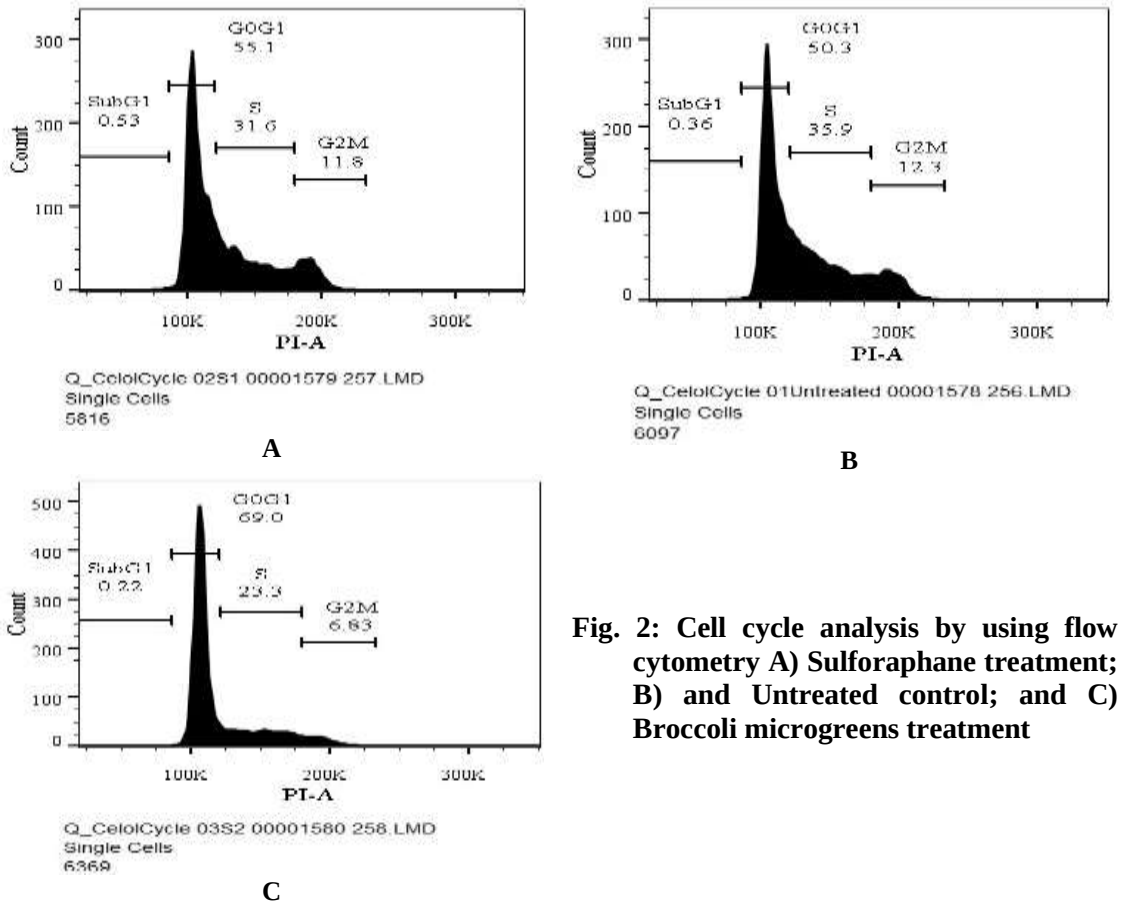

Fig. 2: Cell cycle analysis by using flow cytometry A) Sulforaphane treatment; B) and Untreated control; and C) Broccoli microgreens treatment

Annexin V apoptosis assay

We performed apoptosis assay to confirm the stage of apoptosis which clearly showed early apoptosis in cells (Koolivand *et al*., 2018; Wang *et al*., 2021). Thp-1 cells were treated with IC_{50} (22.59, 446.34 µg mL^{-1}) concentrations of SFN and MG for 48 h. Cell were stained with Annexin V and propidium iodide and then detected by flow cytometry (Fig. 3). The untreated control cells demonstrated 4.6% dead cells, 0.1% late apoptosis, and 4.28% early apoptosis. Similarly, the cells treated with SFN and MG extract exhibited different cell populations in different stages (Fig. 3). In present study sulforaphane and microgreens treated cell lines had higher population of cells under early apoptosis as compared to the control cells (14.6 and 8.58%, respectively). Cellular death is characterized by increased release of ROS and morphological changes that can be tracked using flow cytometer (Gurudhathan *et al.*, 2023). Annexin V is a Ca2+-dependent protein with prominent affinity for phospholipid phosphatidyl-serine, which forms cellular membranes. This lining is translocated outward in dead cells, which aid in the binding of FITC-labelled Annexin V protein. The PI stain differentiates between living and non-viable cells, and easily penetrates into the cell membrane of dead cells (Gurudhathan *et al*., 2023)...

TUNEL assay

We did not find any substantial percentage of cells with fragmented DNA in this instance because the cell population was in early apoptosis, while DNA damage happens in late apoptosis (Fig. 4). DNA fragmentation is among the last events in nucleus during apoptosis (Kiraz *et al*., 2016). At higher concentration, SFN reportedly induces ferroptosis, activated and accompanied by the depletion of intracellular glutathione (GSH) and decreased GSH peroxidase 4 protein expression levels (Greco *et al*., 2021). This study was conducted on U-937 and MV4-11 human AML cell lines.

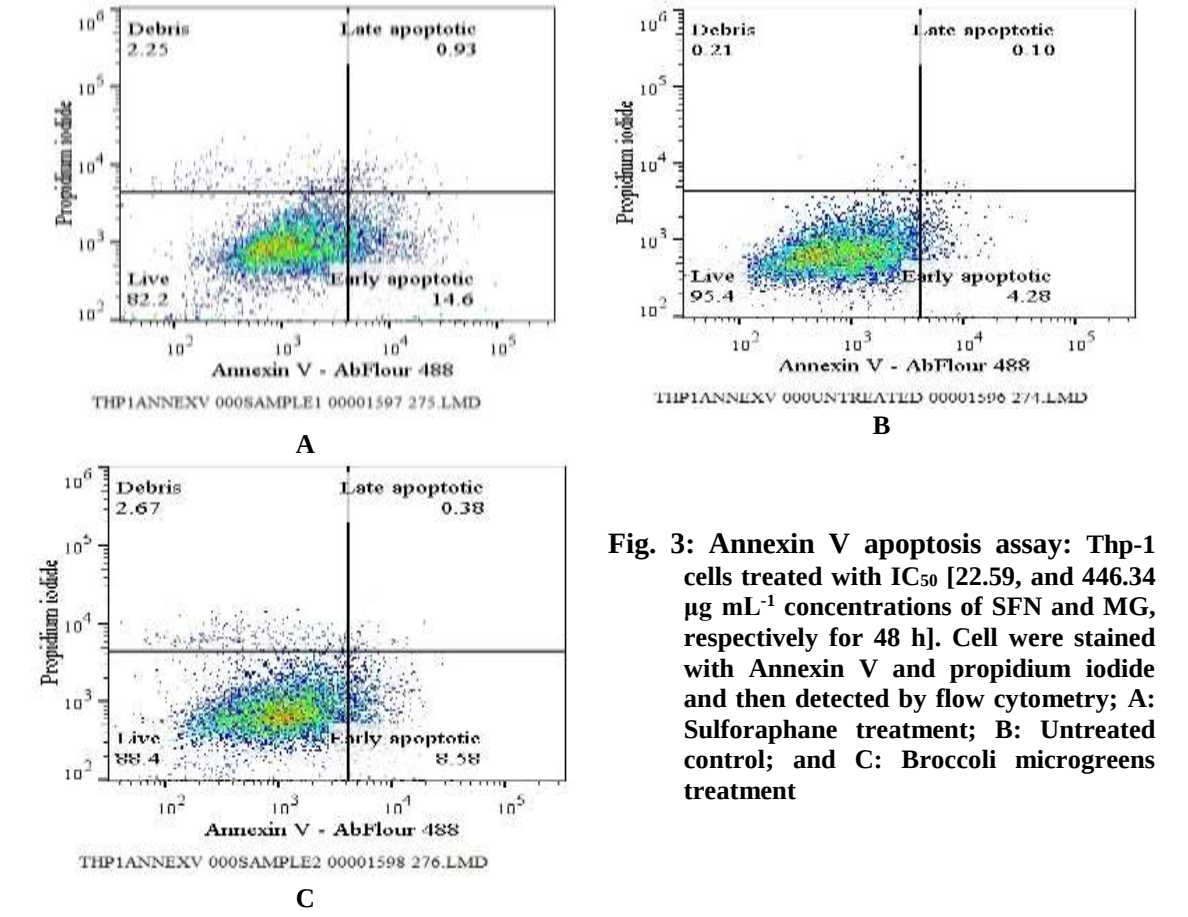

Fig. 3: Annexin V apoptosis assay: Thp-1 cells treated with IC_{50} [22.59, and 446.34 µg mL^{-1} concentrations of SFN and MG, respectively for 48 h]. Cell were stained with Annexin V and propidium iodide and then detected by flow cytometry; A: Sulforaphane treatment; B: Untreated control; and C: Broccoli microgreens treatment

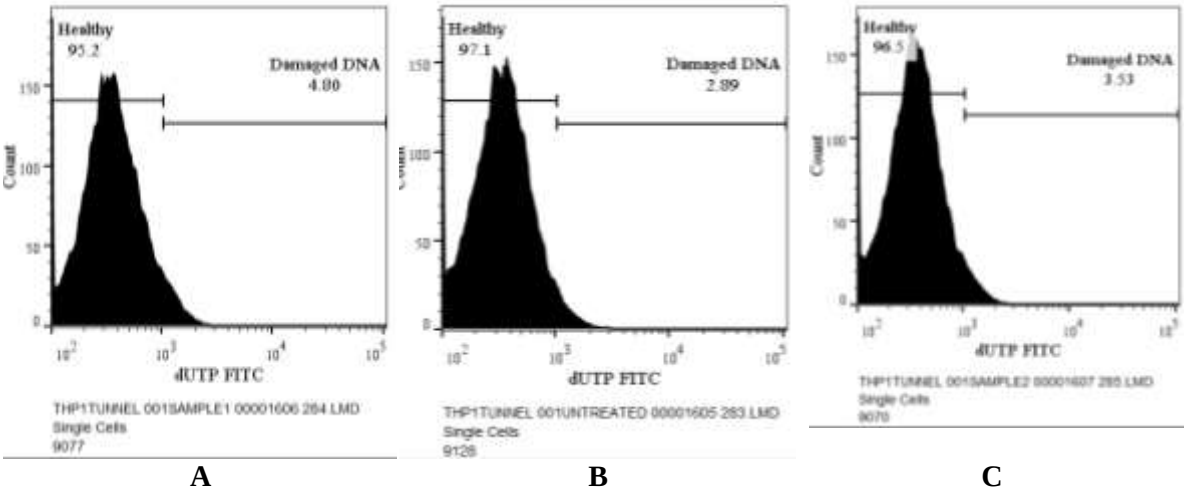

Fig. 4: TUNEL assay showing meagre percentage of cells with fragmented DNA; A) Sulforaphane treatment, B) Untreated control, and C) Broccoli microgreens treatment

Anti-leukemic activity of brassica derived bioactive compounds has previously been reported in HL-60 cell line in which SFN showed the most potent anti-leukemic activity (Núñez-Sánchez *et al*., 2022). In another study, fresh kale juice induced apoptosis through caspases dependent pathway in U-937 cell line (Pungpuag *et al*., 2023). The effect of broccoli sprouts and microgreens on the activation of apoptosis in leukemic cells was partially reported in past. Therefore, it is imperative to focus on to explore the underlying molecular mechanism in both *in vitro* and *in vivo* models.

Conclusion: The study provides better understanding about the benefits of consuming broccoli microgreens over florets. Fresh microgreens hydrolysed extract and showed good cytotoxic activity on acute leukemic cell line in various experiments of flow cytometry. Such properties could be a strong argument to recommend broccoli microgreens as a functional food.

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Authors contribution: The first author Ami Shah was involved in execution of experiments, visualization, data curation; and drafted the original paper, while Dr. Rekha Gadhvi supervised, conceptualized, and validated the study.

Conflict of interest: The authors have no conflict of interest regarding research work.

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