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Phytochemical Analysis of Bitter Melon Juice; Antiproliferative and Apoptosis Inducing Activity on Human Osteosarcoma Cells

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ABSTRACT: Osteosarcoma is one of the most common cancers among adolescents and young adults. Prognosis of osteosarcoma is particularly poor, suggesting critical needs for additional drugs to improve disease outcome. In this study, osteosarcoma cell SaOS-2 was used as an *in vitro* model to assess the effect of bitter melon (*Momordica charantia*) juice (BMJ) as an anticancer agent. Fruit juice of *Momordica charantia* was subjected to preliminary phytochemical analysis to identify various phytoconstituents present in them such as carbohydrates, starch, flavonoids, phenols, steroids, terpenoids, alkaloids, tanins and saponins. Cell viability (MTT) results revealed that 1 to 10% (v/v) of BMJ treatment significantly ($p<0.05$) reduced the proliferation of SaOS-2 cells in a dose dependent manner. Antiproliferative activity of BMJ was also coupled with morphological changes in the cells with the significant induction of apoptosis as quantified by DAPI stain. Reactive oxygen species (ROS) generation was also significantly induced by BMJ treatment. Furthermore, flow cytometric analysis revealed that BMJ induced cell cycle arrest in G2/M phase with the enhancement of apoptosis. The findings of this study suggest that BMJ modulates ROS generation and cell cycle arrest in G2/M phase of SaOS- 2 cells which lead to inhibition of cell proliferation and induction of apoptosis of osteosarcoma. These findings suggest that BMJ could be a potential agent for osteosarcoma treatment and anticancer drug discovery.

Keywords: *Momordica charantia,* osteosarcoma, apoptosis, reactive oxygen species, SaOS-2 cell, Bitter Melon juice (BMJ)

I. INTRODUCTION

Osteosarcoma is one of the most common forms of bone cancer in young adolescents from all over the world. It is the third most common cancer in adolescence, occurring less frequently than only lymphomas and brain tumors. It is thought to arise from a primitive mesenchymal bone-forming cell and is characterized by production of osteoid. Any area of bone or osteoid synthesized by malignant cells in the lesion establishes the diagnosis of osteosarcoma [1].

Osteosarcoma is malignant bone tumor that usually develops during the period of rapid growth. The main cause of occurrence of osteosarcoma is not known. The annual incidence of osteosarcoma reported in African Americans is 5.2 cases per million populations and in Caucasians are 4.6 cases per million populations younger than 20 years [2, 3]. Treatment options remain limited due to its various adverse side effects [4-7]. Therefore, non-toxic dietary agents are gaining interest in the treatment of various cancers and the use of natural agents is currently under investigation for its chemopreventive and chemotherapeutic role. In recent years, the products obtained from medicinal plants have gained much popularity in reducing the risk of several cancer types in humans. Identification of preliminary phytochemicals has become the most important tool for the identification of active components of the plant extracts [8]. Extensive laboratory studies in cell culture systems and in animal models have demonstrated that various medicinal plants and their products such as, major polyphenolic constituents, terpenoids, flavones, flavonoids, antioxidants etc. affords considerable protection against many cancer types [9, 10]. Studies conducted in several animal tumor model systems with Indian medicinal plants are encouraging, and suggest that more studies are warranted to decipher the molecular mechanism, by phytochemicals imparts its anti-carcinogenic effects. Unraveling the molecular mechanism(s) of anti-cancer effects of Indian medicinal plants may, therefore, provide new inside and better strategies to interfere with bone cancer development through its consumption or local administration thereby enhancing the quality of life and prognosis for survival of bone cancers.

Fortunately, India is a big resource of medicinal plants and natural products from various sources [10, 11]. However, the emphasis was not even given to develop and test anti-cancer properties of promising Indian medicinal plants. Epidemiological observations though inconclusive are, suggesting that Indian herbs, spices have potent anti-cancer properties, consumption is associated with reduced risk of some cancers.

Momordica charantia is commonly known as Bitter melon and belongs to Family Cucurbitaceae. It is a frequently consumed vegetable in the Asian and African continents [12]. Several studies have shown its beneficial effects on diabetes, obesity, hyperlipidemia etc. [13, 14]. Bitter melon has been evaluated in human population in several clinical trials for its anti-diabetic effects [15]. Apart from its anti-diabetic effects, bitter melon extract and several bioactive compounds obtained from it have shown anti-cancerous effect against leukemia, breast, prostate, and colon cancers [16-20]. Fruit juice of *Momordica charantia* was subjected to preliminary phytochemical analysis to identify various phytoconstituents present in them such as carbohydrates, starch, flavonoids, phenols, steroids, terpenoids, alkaloids, tanins and saponins. These phytoconstituents exhibits active role in the proliferation and apoptosis of cells [21]. The antiproliferative and apoptotic activity of BMJ were evaluated using MTT assay, ROS generation and cell cycle analysis in SaOS2 osteosarcoma cell lines. This study revealed the anti-proliferative and apoptotic effect of BMJ against SaOS2 cells.

II. MATERIALS AND METHODS

A. Reagents and Chemicals

McCoy's 5A Medium, L-Glutamine, Sodium bicarbonate, Non-essential amino acid, Antibiotic Solution 100X liquid, Trypsin-EDTA Solution 1X, Foetal Bovine Serum, Molisch's Reagent, Conc. H_2SO_4, Barfoed's Reagent, Fehling's Solution A and B, Ferric Chloride Solution, Acetic Acid, Ethanol, Magnesium ribbon, Conc. HCl, Sodium Hydroxide, Dil. HCl, Potassium mercuric iodide solution, Picric Acid, Potassium Hydroxide, Potassium Permanganate, Chloroform, RNase A, Phosphate Buffered Saline (PBS) and MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] dye were all purchased from Himedia Laboratories Pvt. Ltd. Mumbai, India. DMSO (Dimethyl sulfoxide) was purchased from Merck Specialities Pvt. Ltd. Mumbai, India and 2′,7′-dichlorodihydro fluorescein diacetate (H2DCF-DA), 4',6-diamidino-2-phenylindole (DAPI) was from Sigma – Aldrich Inc. St. Louis, USA. All the other reagents were of analytical grade.

B. Collection of Plant Material and Extract Preparation

Fruits of bitter melons were collected from nearby Lucknow. Bitter melon juice (BMJ) was prepared from the young bitter melons (raw and green) as discussed previously by Kaur *et al* 2013 [22]. Briefly, Fruits of bitter melons were washed and wiped to complete dryness and then fruits were sliced vertically and remove pulp and seeds. After deseeding, weight of fruits was taken and juice was extracted using a household juicer. Juice was then centrifuged at 3000g for 30 minutes to pellet down the particulate matter. The pellet was discarded and the remaining juice was stored in aliquots at -80°C. As needed, 1-10% (volume/volume in medium) of pure BMJ was used for cell culture studies.

(i) Preliminary Phytochemical Analysis of Bitter Melon Juice: BMJ were used for preliminary screening of phytochemicals. The various standard procedures were used for the phytochemical analysis such as carbohydrates (Molisch's test), reducing sugars (Fehling's test), combined reducing sugars (Fehling's test), tanins, alkaloid (Mayer's test and Hager's test), flavonoids (Alkaline reagent test and Shinoda test), phenolic compounds (Shinoda test and Ferric chloride test), steroids (Liebermann-Burchard test), terpenoids (Salkowsi's test), saponins, and soluble starch [23- 28].

Test for carbohydrates:

1. Molisch's test for carbohydrates: 2 ml of BMJ was taken and few drops of Molisch's reagent was added, then it was shaken well. 2 ml of con. H_2SO_4 was added on the sides of the test tube. The mixture was then allowed to stand for two minutes. Formation of a red or dull violet ring appeared at the interphase of two layers indicated the presence of carbohydrates.

2. Barfoed's test for monosaccharides: 1 ml of BMJ was taken and 1 ml of Barfoed's reagent was added to it, then the mixture was heated on a water bath for 2 minutes. The reddish precipitate was considered as a positive test for monosaccharides.

3. Fehling's test for free reducing sugars: 5 drops of test extract were heated with 5 ml of equal volumes of Fehling's solutions A and B. Formation of red precipitate indicated the presence of free reducing sugars.

Test for alkaloids:

1. Mayer's test: To the 2-3 ml of BMJ, added 1% aqueous HCl and few drops of Mayer's reagent (Potassium mercuric iodide solution) was added, occurrence of creamy white precipitate was an indication for the presence of alkaloids.

2. Hager's test: Few drops of Hager's reagent (saturated solution of Picric acid) were added in 2-3 ml of BMJ, appearance of yellow precipitate was an indication for the presence of alkaloids.

Test for flavonoids:

1. Shinoda's test: Few fragments of Magnesium ribbon was added to the 1 ml of BMJ and then HCl was added drop wise, pink, orange or red to purple colour was appear that indicate presence of flavonoids.

2. Ferric chloride test: 2 ml of BMJ was taken and few drops of 10% ferric chloride 10% ferric chloride were added to it, a green blue or violet colouration indicated the presence of flavonoids.

3. Sodium hydroxide test: 2 ml of BMJ was taken and 2ml of 10% aqueous sodium hydroxide was added in it, yellow colour was developed, yellow colour disappeared after addition of dilute HCl, indicated the presence of Flavonoids.

Test for phenolic compounds:

1. Ferric chloride test: Few drops of $FeCl_3$ was added to the 2 ml of BMJ, a blue- green colour was developed which indicated the presence of phenols.

2. Shinoda's Test: 1 ml of BMJ was taken and few fragments of Magnesium ribbon and conc. HCl was added drop wise. Yellowish, yellow orange or orange colour was appeared after few minutes.

Test for terpenoids:

2 ml of chloroform and 3 ml of con. H_2SO_4 was added to the 5 ml of BMJ, a monolayer of reddish brown colour formed at the interface indicated the presence of terpenoids.

1. Liebermann-Burchard test for steroids: 0.5 ml of BMJ was taken and 2 ml of acetic anhydride was added to it, the solution was cooled well in ice after that 2 ml of conc. H_2SO_4 was added carefully. Violet colour was developed which was turned into blue or bluish- green indicated the presence of steroids.

Test for saponins:

2 ml of BMJ was taken it was shaken vigorously with the 5 ml of distilled water for about 5 minutes. A 1cm layer of foam was formed which persisted on warming, indicated the presence of saponins.

Test for soluble starch:

1 ml of 5% KOH was added to the 1 ml of BMJ then the mixture was boiled, cooled and acidified with H2SO4. Yellow colour appeared which indicated the presence of soluble starch.

Test for tanins:

Few drops of 1% $FeCl_3$ was added to the 2 ml of BMJ, blue- black, green or blue green precipitate was appeared which indicated the presence of tanins.

(ii) In vitro Analysis:

1. Cell culture: Human osteosarcoma cells (SaOS2) were obtained from National Center for Cell Sciences NCCS, Pune, India and maintained in McCoy's 5a medium (Himedia) and supplemented with $NaHCO_3$, sodium pyruvate, 10% foetal calf serum (Himedia), and 1% penicillin and streptomycin. Cells were grown at 37° C, 5% CO_2 incubator till confluence.

2. Cell viability assay: The cytotoxic effect of Momordica charantia was assessed in human osteosarcoma cell line SaOS-2 by the MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide [29]. MTT is a quantitative colorimetric assay which is based on the phenomenon of enzymatic reduction of MTT dye. The assay provides a direct relationship between the cell viability and color formation (absorbance). The cells were seeded at an initial assay density of 1×10^4 cells/100µl complete culture media in 96-well plate and incubated for 24 h at 37ºC in humidified, 5% CO_2 atmosphere. Cells were treated with different concentrations of BME (0% - 10% v/v). After 24 h of the treatment, the medium was removed and cells were incubated with 20 µl of MTT (5mg/ml in PBS) in fresh medium for 4 h at 37ºC. After 4 h, formazan crystals, formed by mitochondrial reduction of MTT, were solubilized in DMSO (150 µl/well) and the absorbance was read at 540 nm with the help of microplate reader (BIO RAD Model 680). The percentage cell viability was calculated by using formula:

% Cell viability = [(Treated absorbance) / (controlled absorbance)] × 100

The cellular morphology was also observed after 24 h of BMJ treatment under inverted phase contrast microscopy (Nikon ECLIPSE Ti-S, Japan).

3. Nuclear apoptosis by DAPI (4', 6-diamidino-2-phenylindole) staining: The apoptotic effect of BMJ at the concentration 0, 2, and 4% was analyzed by using nuclear florescent dye DAPI [30]. The cells were seeded and treated for the period of 24 h in 48 well plates. After treatment period, cells were washed with PBS and fixed in 4% paraformaldehyde for 10 min. Subsequently the cells were permeabilized with permeabilizing buffer (3% paraformaldehyde and 0.5% Triton X-100) and stained with DAPI dye. After staining, the images were taken by using inverted fluorescent microscope (Nikon ECLIPSE Ti-S, Japan). The cells having fragmented and condensed nuclei were considered as apoptotic cells.

4. Measurement of ROS by DCF-DA: The fluorescent dye 2', 7'-dichlorofluorescein diacetate (DCFH-DA) is a cell membrane permeable and is changed to non-fluorescent and impermeable compound DCFH by intracellular esterases. Oxidation of DCFH by ROS produces a highly fluorescent DCF. ROS generation in osteosarcoma cells were measured by DCFH-DA dye with some modification [31]. In brief, SaOS-2 cells (1.5×10^4 per well) were seeded in 48-well culture plates for 24 h. Cells were then exposed to 0, 2 and 4% v/v BMJ concentrations for 24 h in triplicate. After exposure, cells were incubated with DCFH-DA dye (10 mM) for 30 min at 37°C. The reaction mixture was carefully replaced by 200 µl of phosphate-buffered saline (PBS) in each well. The plates were kept on a shaker for 10 minutes at room temperature in the dark. Photomicrographs were taken of plate by an inverted fluorescence microscope at an excitation wavelength of 330 nm and an emission wavelength of 420 nm to analyze intracellular fluorescence of cells.

5. Cell cycle analysis: Saos2 cells were seeded in two different 6 wells plate at a density of 1×10^6 cells/ml and treated with various concentrations of BMJ (0, 2 and 4% v/v) for 24h.

Cells were washed, trypsinized and fixed in ice-cold 70 % ethanol overnight at -20°C, cells were washed with PBS and then suspended in a staining buffer (10µg/ml propidium iodide, 0.5 Triton X-100 and 0.1% RNase A in PBS). The cells will be analyzed by flow cytometer equipped with Cell Quest acquisition and analysis software program (Becton Dickinson and Co., San Jose, CA)[19].

III. RESULTS AND DISCUSSION

The results demonstrated the presence of several phytochemicals in the fruit juice (Table 1). The flavonoids, steroids, phenols and alkaloids are important phytoconstituents, which affect the proliferation of cells and inhibit apoptosis by intrinsic and extrinsic pathway in cancer cells. Several secondary metabolites present in *M. charantia* fruit juice are known to possess different therapeutic activities for example, tannins have antimicrobial, antiviral, antiviral, molluscicidal and antitumoral; flavonoids have anticarcinogenic, antiviral, antihemorrhagic and antioxidant [22].

A. Cell Proliferation

The MTT assay is a method for quantifying metabolically viable cells through their ability to reduce a soluble yellow tetrazolium MTT by the action of dehydrogenase enzymes of mitochondria [32]. The proliferation of the osteosarcoma cells was inhibited by treatment with BMJ. A dose dependent decrease in percent cell viability was observed at1-10% (v/v) of BMJ treatment as compared to the control samples (Fig. 1). The IC50 (Inhibitory Concentration) was attained near the dosage of 4%v/v.

Table 1: Phytochemical Investigation of Ethanolic Extract of BMJ.

No.	Constituents	Test	BMJ
1	Carbohydrates	Molisch's test	+
		Barfoed's test	+
		Fehling's test	+
2	Soluble starch		+
3	Flavonoids	Shinoda's test	-
		Ferric chloride test	-
		Sodium hydroxide test	+
4	Phenols	Shinoda's test	+
		Ferric chloride test	+
5	Steroids	Liebermann-Burchardtest	+
		Salkowski's test	+
6	Terpenoids		-
7	Alkaloids	Mayer's test	-
		Hager's test	-
8	Tannins		-
9	Saponins		+

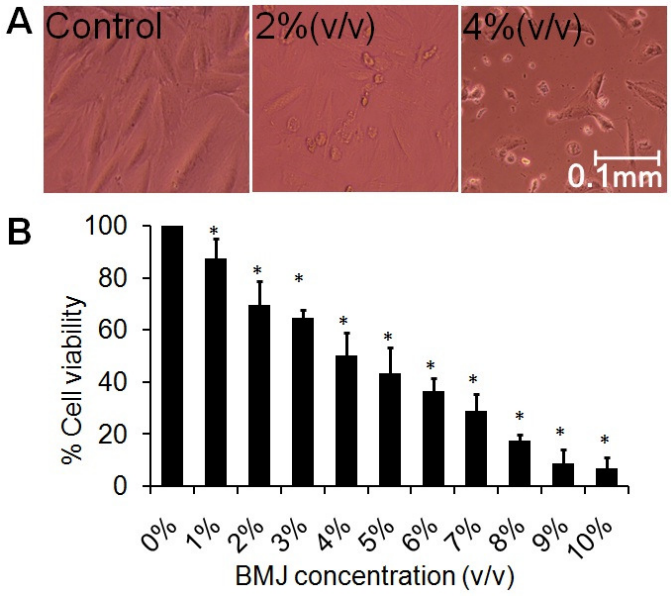

Fig. 1. % Cell viability assay of osteosarcoma SaOS-2 cells.

The cellular morphology was observed at IC50 (4% v/v) and below IC50 value (2% v/v). Cells treated with 2% (v/v) of BMJ exhibited changed morphology from normal to slightly spherical shape. However, 4% (v/v) of BMJ showed highest number of spherical shape with loss of membrane integrity and increased the cell death. Some other studies have also reported the anticancer activity of bitter melon extract against different cancerous cell lines viz. breast, prostate and colon cancers [18-20].

B. Nuclear Condensation and Apoptosis

DAPI is a blue florescence stain preferentially binds double-stranded DNA at AT clusters of the minor groove. Binding of DAPI to dsDNA produces a ~20 fold florescence increase, apparently due to the displacement of a water molecule from both DAPI and the minor groove. Nuclear condensation and fragmentation of nuclear envelope are characteristics of apoptosis. After treatment of BMJ, nuclear condensation increased with increasing concentration of BMJ. The fragmented and condensed nuclei were observed in the SaOS-2 cells treated with 2% and 4% (v/v) BMJ. The nuclear condensation of the cells suggests that the cell death was observed due to an apoptotic process [31]. Chromatin condensation was not observed in control cells. BMJ was found to be very efficient in case of osteosarcoma cells by visualizing the treated cells having condensed DNA with DAPI stain.

C. Determination of ROS activity

The ability of BMJ to induce oxidative stress was evaluated by measuring the levels of ROS in SaOS-2 cells. Results showed that intracellular ROS intensity was increased at doses 2% and 4% in a dose-dependent manner when compared to the untreated cells (Fig. 3). Excess of ROS can damage proteins, lipids and DNA which lead to induce the cellular apoptotic pathway and finally cell death. The H2DCF-DA fluorescent probe is commonly employed and may react with several ROS including hydrogen peroxide, hydroxyl radicals and peroxynitrite. The intracellular fluorescence intensity of DCF is proportional to the amount of ROS produced. [33].

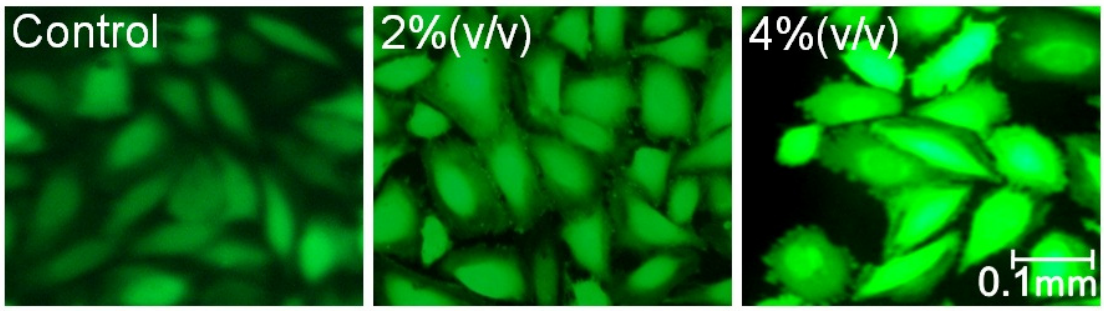

Fig. 2. Intracellular ROS Measurement.

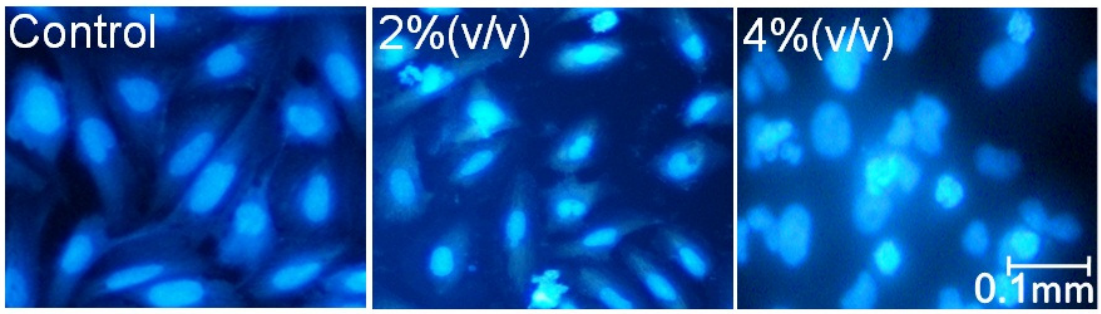

Fig. 3. Nuclear condensation in osteosarcoma cells.

D. Flow-Cytometry analysis

To understand the mechanism of BMJ mediated cell death and cell cycle progression, we examined the effect of BMJ on the cell cycle. This is very important to quantify cell cycle analysis in the study of molecular mechanism of cell cycle progression and cell death [34]. Cells divide into progenitor cells by four cell cycle phases- G1, S, G2 and M. G1 and G2 are the major check points and these are the critical points for cell division playing important role in cell cycle division [35].

Cell populations in the G0/G1 and S phases were 67.25 % and 25.15% respectively in control cells (Fig. 4). However, the S population was sharply increased to 31.28% at 2% BMJ treatment but noticeably decreased to 24.63% at 4% BMJ treatment which indicated that DNA content was significantly decreased at 4% of BMJ treatment. The G0/G1 population of cells was decreased by 60.19% and 57.0% at 2% and 4% in BMJ treated cells respectively, suggesting that BMJ treatment on SaOS-2 cells did not arrest the cells in G0/G1 phase. G2/M peak was found to be 7.6% in control cells whereas a significant increment of 8.53% and 17.47% at 2 and 4 % (v/v) of BMJ treatment respectively, were observed.

The novel finding in this study was that BMJ treatment arrests the osteosarcoma cells at G2/M phase of the cell cycle and eventually cell death. Apoptotic cells take up PI dye due to a change in membrane permeability as compared to control cells [36]. As evident from photomicrograph, negligible amount of apoptotic cells approximately 0.27% was observed in control samples while cells treated with 2 and 4% of BMJ have shown 8.75 and 13.95% of apoptotic cells respectively.

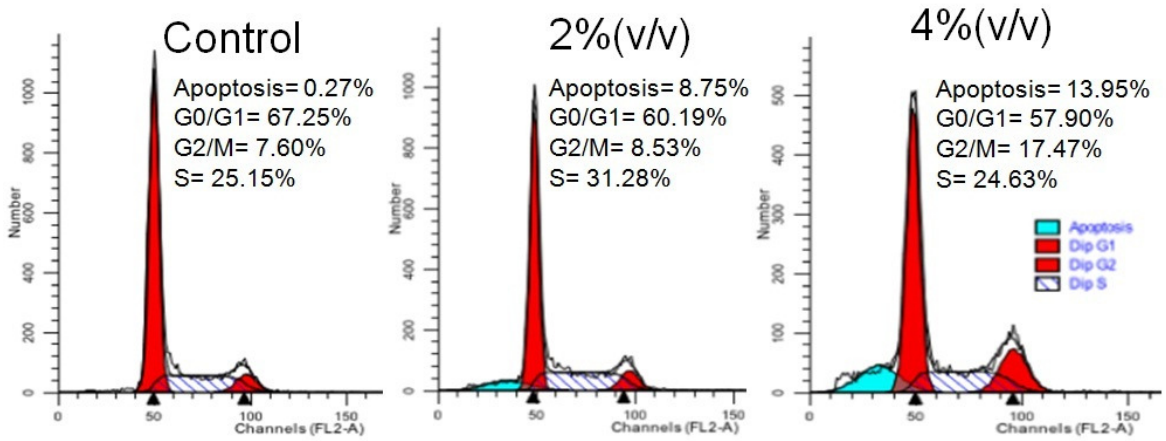

Fig. 4. Cell cycle analysis of osteosarcoma cell treated with BMJ.

BMJ was found to be effective on the osteosarcoma cells SaOS-2, which is inferred by the results of % cell viability, intracellular ROS generation, nuclear condensation, cell cycle arrest and apoptosis. These results suggest that BMJ could be prominent agent for osteosarcoma treatment and anticancer drug discovery. Further study will be carried out to analyze the total fate of BMJ against osteosarcoma.

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