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Cytotoxicity and Microtubule Depolymerisation Effect of Curcumin on Oral Squamous Carcinoma Cells

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Abstract:- Epidemiological evidence indicates that plant derived flavonoids and other phenolic antioxidants can be used to treat cancers. Curcumin is one such flavonoid that is considerably more effective than other phenolic antioxidants; it is a potent drug against many diseases other than cancer. In the present study, the effects of curcumin on oral squamous carcinoma cell line AW 13516 was investigated. Curcumin induced cytotoxicity was measured by sulforhodamine B assay. Remarkably, the proliferation of oral squamous carcinoma cell lines were inhibited at IC50 of 8µM. Indeed, curcumin is generally considered to be a controller of microtubule polymerization; therefore, microtubule organization in growing and metaphase cells was analysed by immunofluorescence. Curcumin strongly depolymerized the interphase microtubules of AW 13516 cells at ≥ 20 µM. To track the phases of cell cycle, effect of curcumin on cell cycle distribution was analysed by Fluorescence activated cell sorting (FACS); cell cycle was arrested at G1/S phase checkpoint. This investigation has provided new findings about the effect of curcumin on AW 13516, oral squamous carcinoma.

Keywords:- *Curcumin, Oral cancer, Sulforhodamine B assay, Immunofluorescence, Flowcytometry.*

I. INTRODUCTION

Although several drugs are coming to the market every year, cancer remains incurable. According to Surveillance, Epidemiology, and End Results Program (SEER) of National Institute of Cancer, in all type of new cancer cases, the incidence of oral cancer is 2.5%. In the recent reports of SEER, it was estimated that 42,440 new cases of oral cavity and pharynx cancer were reported [1]. In fact, oral cancer is a subtype of head and neck cancer. It is most commonly reported in elder population of males than females because of poor oral hygiene, tobacco and alcohol use; the incidence rate is highest in Pakistan followed by France, India and Brazil [2]. In addition, human papillomavirus (HPV) also causes oral cancer in younger population of non-smokers and drinkers [3, 4].

Indeed, oral cancer is an epithelial neoplasia, 90% of the oral cancers occur in squamous cells in the oral mucosal linings [5]. It generally starts as a focal clonal overgrowth of altered stem cells near the basement membrane; replaces the normal epithelia by upward and lateral expansion [6]. Prior to oral squamous cell carcinoma, several premalignant lesions occur in the mouth: leukoplakia, erythroplakia and erythroleukoplakia [7], however, leukoplakia is more prone to cancer.

Phyto compounds are known to exhibit curing effect on several diseases. Previous studies have shown that phyto extracts isolated from Brazilian plants: Picrolemma spruce, Laetia suaveolens, and Abarema auriculata cavity [8]; Chinese herbal extracts: Drynaria baronii, Angelica sinensis, and Cornus officinalis Sieb [9]; and similarly, grape (Vitis vinifera) seed extracts [10] and freeze- dried black raspberry ethanol extracts [11] shown cytotoxic effect on oral squamous cell carcinomas. Alternatively, plant derived compounds, proanthocyanidin inhibited the progression of HPV-transfected oral squamous cell carcinoma proliferation [12]. Likewise, in the present study, a plant derived flavonoid curcumin was investigated on oral squamous cell carcinoma. It is known from the literature that curcumin is an effective inhibitor of tumor cell proliferation, migration and invasion by interfering the transcription factor STAT3 [13]. It also induces the apoptosis by suppressing the microtubule dynamics and activation of mitotic checkpoints by accumulation of Mad2 and BubR1 [14]. It was reported that curcumin could be used in several cancers that include lung cancer [15], breast cancer [16], liver cancer [17], cervical cancer [18], and prostate cancer [19]. Although curcumin treated head and neck cancers were reported on animal models like golden hamsters [20], however, the mechanism of action in oral squamous cells was not yet reported. Therefore, in the present study cytotoxicity effect curcumin and its molecular mechanism of cell cycle progression and apoptosis was investigated.

II. MATERIAL AND METHODOLOGY CELL LINES AND CULTURE

The effect of curcumin was investigated on human oral squamous carcinoma cell line AW 13516 and normal nonmalignant stratified squamous human keratinocyte line HaCaT was used for validation experiments; obtained from Advanced Centre for Treatment, Research and Education in Cancer (ACTREC, Navi Mumbai, India). AW13516 cells were grown in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% heat inactivated fetal bovine serum, 3.024 mg/ml sodium bicarbonate, antibiotic and antimycotic solution (streptomycin, amphotericin B and penicillin), and 10µg/ml

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human insulin. Whereas, HaCaT cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum, 0.6 mg/ml sodium bicarbonate, and antibiotic and antimycotic solution (streptomycin, amphotericin B and penicillin). Both the cell lines were cultured at 37°C in a humidified atmosphere containing 5% CO2.

- *Cytotoxicity Assay*

In this study, curcumin was solubilized in dimethyl sulfoxide (DMSO) and various concentrations were prepared for analysis. The proliferation of curcumin treated cell lines was investigated by Sulforhodamine B (SRB) cytotoxicity assay, described in detail previously [21-23]. Cells were fixed by 30µl of trichloro acetic acid (TCA) cultured in 24-well plate, after 45 min, gently washed and dried. 0.4% (Wt/Vol) of 100µl SRB which is prepared in 1% acetic acid was added and incubated for 30 min at room temperature; SRB binds to protein in presence of acidic groups. SRB was removed and wells were rinsed four times with 1% acetic acid to eliminate unbound dye. Finally, the bound dye was solubilized and incubated for 30 min with 100mM tris (pH 10.5).

- *Fluorescence-activated Cell Sorting*

Cells were treated with various concentrations of curcumin (0µM, 5µM, 10µM and 20µM) and nocodazole (10µM) control for 24 hrs. Then cells were washed with PBS, treated with trypsin- EDTA, and fixed in 70% ethanol. Cells were washed twice with PBS for analysis; resuspended in 10µg/ml Rnase and 400µg/ml propidium iodide at 37oC for 30 min in dark [24], to label the DNA. Fluorescent activated cell sorter (FACS) or flowcytormetry was used to analyze the DNA content of the cells in cell cycle progression by Modfit LT program.

- *Immunofluorescence*

For tubulin localization, cells were seeded on glass coverslips in 24-well plates for 24 hrs, then, different concentrations of curcumin were added and incubated for another 24 hrs. For cell fixation, 3.7% formaldehyde at 37oC was added. Prior to immunostaining, nonspecific antibody binding sites were blocked by 400-500 µL of 2% BSA, prepared in PBS, for 30 min. First, cells were stained by immunofluorescence using primary rabbit polyclonal anti-α-tubulin IgG (1:300 dilutions) and incubated for 2 hrs at 37ºC. Then, the cells were stained with secondary Fluorescein Isothiocyanate (FITC)-conjugated anti-rabbit IgG (1:300 dilutions) and incubated for another 2 h at 37ºC. The nucleus was stained using 1µg/ml of Hoechst 33258. The slides were observed under phase contrast and fluorescent microscope.

III. RESULTS AND DISCUSSION

Oral squamous carcinoma AW 13516 cells and nonmalignant HaCaT cells were cultured in 96- well plates to demonstrate the curcumin effect on cell proliferation in a dose dependent manner. In cytotoxicity assays, the average doses of IC50 values are shown in Figure 1. Interestingly, AW 13516 cells were inhibited at 8µM concentration, while, HaCaT cells were inhibited at 6.5µM concentration. Noticeably, AW 13516 cells were more sensitive than HeLa (13.8µM) and MCF-7 (12µM) cells [25]. It is evident from the results that curcumin effectively inhibiting proliferation of oral squamous carcinoma cells than nonmalignant squamous cells. Further, curcumin is more potent on oral squamous cells than other cancer cells (HeLa and MCF-7).

In cell cycle analysis, FACS of AW 13516 showed same distribution of cells in G1/S phases and low distribution in G2 phase. All the curcumin treated AW 13516 cells (5µM, 10µM and 20µM) exhibited increased distribution in G1 phase and decreased distribution in G2/S phases while compared with the control cells. Nocodazole treated AW 13516 cells showed increased distribution in G2 phase, indicating a cell cycle arrest at G2/M phase. The distribution of cells, in percentage, in each phase of the cell cycle is shown in Figure 2. In fact, there were no significant cell cycle changes were observed up to 20µM concentrations of curcumin. However, increased distribution of G1 phase observed in all concentrations, because, curcumin arresting the cell cycle of AW 13516 cells at G1/S phase [26].

Immunofluorescence analysis revealed that curcumin inhibits the cell cycle progression and induces apoptosis in AW 13516 cells. The effect of curcumin on the interphase microtubules of AW 13516 cells and HaCaT cells are shown in figure 3. Noticeably, curcumin at ≥10µM of concentration reduced the density of microtubules. At 20µM concentration of curcumin, the microtubule network at the centre was disorganized; peripheral density of microtubules was decreased. Same was observed in non-malignant HaCaT cells at lower concentrations when compared to AW13516 carcinogenic cells. In literature, it was reported that cervical cancer cells HeLa and breast cancer cells MCF-7 microtubules were depolymerized at 40µM concentration (Gupta eta al 2006). Interestingly, Control metaphase cells of AW13516 exhibited normal bipolar mitotic spindle organization with two prominent poles. The chromosomes aligned at the metaphase plate as shown in figure 4. However, there was no such spindle apparatus had observed in curcumin treated cells; suggesting that curcumin disrupted spindle microtubules even at lower concentrations (≥10µM) in oral cancer cells (AW13516).

IV. CONCLUSION

Curcumin commonly known as turmeric, has wide range of medicinal properties. It has been using as an antioxidant for years. In addition to that, it has anticancer property. Cytotoxicity of curcumin was investigated in several cancers, however, it was not reported on oral cancer. Therefore, in the present study, various concentrations of curcumin were tested on oral squamous carcinoma cells AW 13516 and non-malignant immortal squamous cells HaCaT. Remarkably, curcumin exhibited significant anti proliferation activity while compared to cervical cancer and breast cancer. Furthermore, cell cycle progress was arrested at G1/S phase and density of microtubules was reduced. Mitotic spindle fibers were disorganized. In conclusion, curcumin significantly inhibits the proliferation of oral squamous carcinoma cells, arrests the cell cycle progress, and depolymerizes the microtubules. However, continued investigation is required to characterize further insights into the molecular mechanisms of curcumin action on cancerous cells; molecular level targets need to be studied that would aid in drug discovery process.

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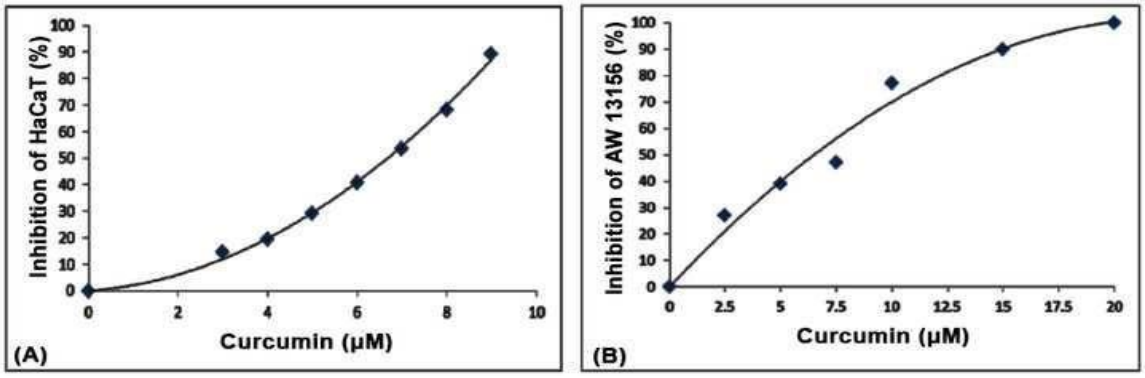

Fig 1

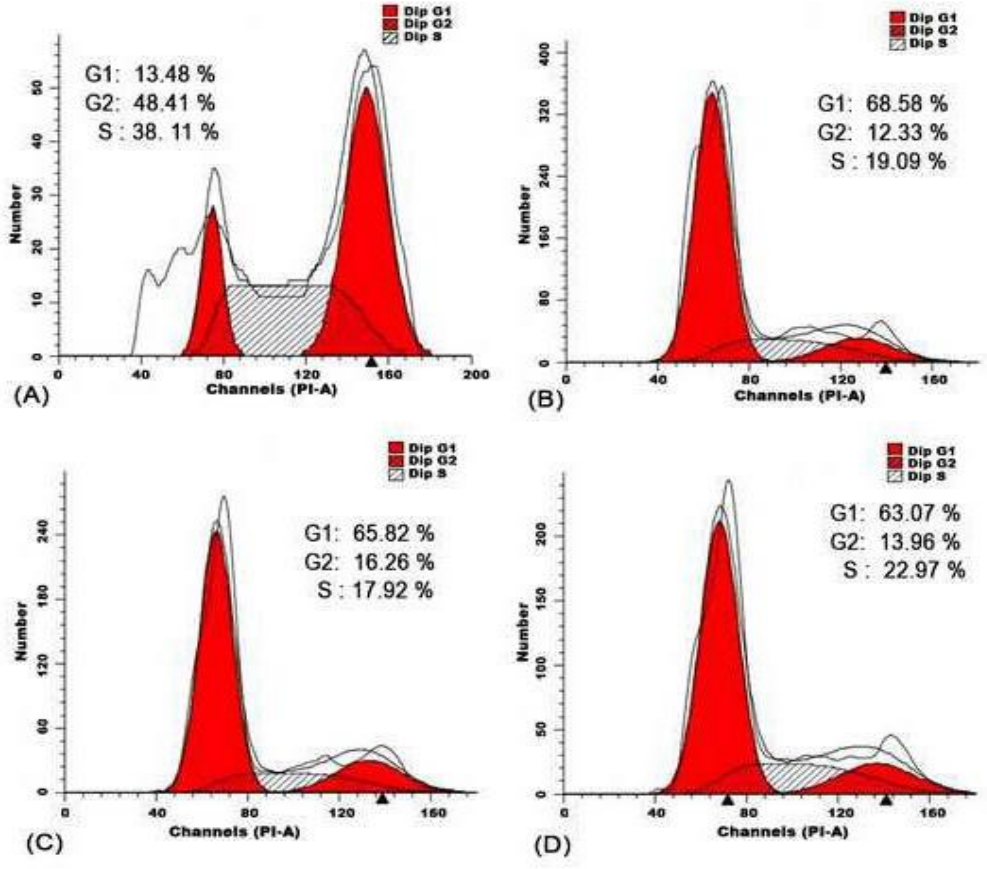

Fig 2

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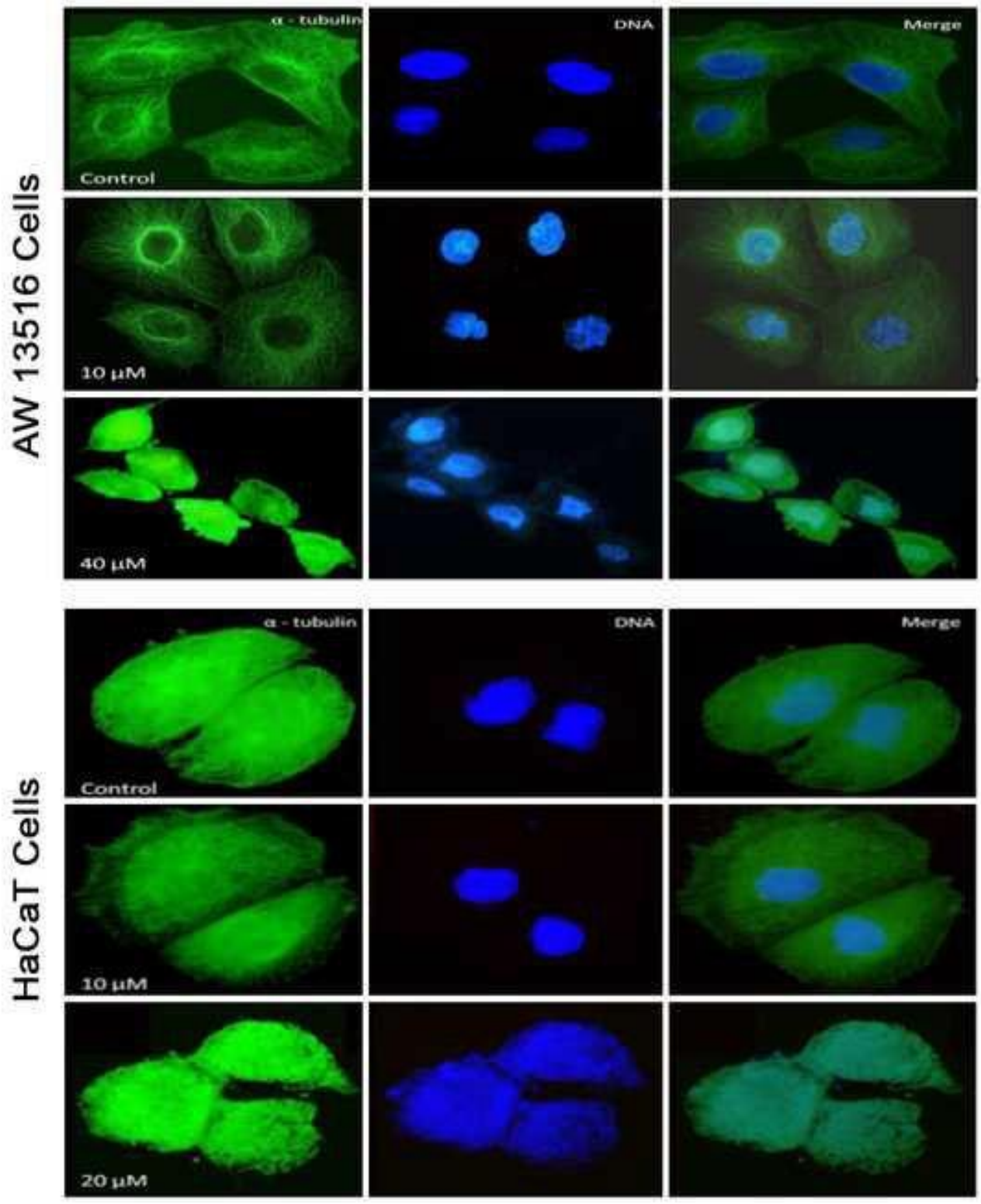

Fig 3

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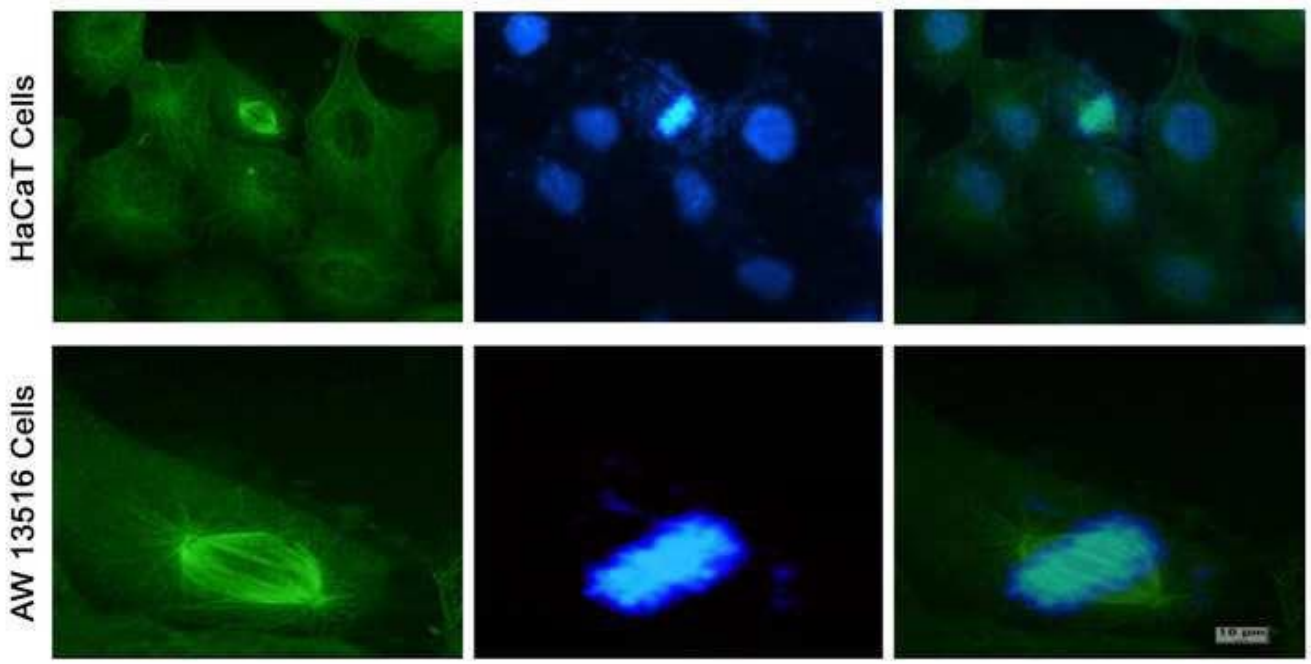

Fig 4

➢ *Figure Legends*

Figure 1: Cytotoxicity effect of curcumin, AW 13516 and HaCaT (control) cells were grown in 96-well plate; antiproliferation activity was measured by Sulforhodamine B cytotoxicity assay. Dose dependent iŶhiďitioŶ of ;AJ HaCaT Đells ;ICϱϬ is c.ϱμMJ, aŶd ;BJ AW ϭƚϱϭc Đells ;ICϱϬ is 8μMJ. CurĐuŵiŶ ;μMJ

Figure 2: Effect of curcumin on cell cycle distribution of AW 13516 cells that were stained with propedium iodide in flow cytometry analysis. AW 13516 cells were treated in the presence of curcumin for 24 hrs, however, no significant difference observed in the treatments. (A) Cell cycle of Nocodazole
;ĐoŶtrolJ treated Đells was arrested at GƬ/M phase. ;BJ Cell ĐyĐle of ϱμM ĐurĐuŵiŶ treated Đells. ;CJ Cell ĐyĐle of ϭϬμM ĐurĐuŵiŶ treated Đells. ;DJ Cell ĐyĐle of ƬϬμM ĐurĐuŵin treated cells.

Figure 3: In immunofluorescence, effect of curcumin on microtubules was investigated by primary anti- α-tubulin antibody and Fluorescein Isothiocyanate (FITC) conjugated secondary antibody that emits yellow green fluorescence; DNA was stained with blue fluorescent dye Hoechst 33258. Microtubules and DNA ĐoŶdeŶsatioŶ was altered iŶ ĐurĐuŵiŶ treatŵeŶt of ϭϬμM to ϰϬμM. At ϰϬμM ĐoŶĐeŶtratioŶ, microtubules and nuclear DNA were disorganized in oral squamous carcinoma (AW 13516) cells like non- malignant squamous (HaCaT) cells.

Figure 4: Mitotic spindle fibers and DNA organization was observed in curcumin treated HaCaT and AW ϭƚϱϭc Đells. MiĐrotuďules were poorly orgaŶized at ϰϬμM ĐurĐuŵiŶ treatŵeŶt.