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Research Article

COMMIPHORA MUKUL EXTRACT AND GUGGULSTERONE EXHIBIT ANTITUMOUR ACTIVITY THROUGH INHIBITION OF CYCLIN D1, NF-K*β* AND INDUCTION OF APOPTOSIS IN ORAL CANCER CELLS

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ABSTRACT

Comiphora mukul, a promising medicinal plant and its constituent Guggulsterone (GS) is used in Ayurveda since decades. This study was aimed to investigate the anticancer potential of *C. mukul* and GS on oral cancer cell lines (SCC-4, KB). MTT assay was used to determine tumour cell proliferation, propidium iodide labeling and annexin V- binding, followed by flow cytometry was used to determine cell cycle and apoptosis of tumor cells after treatment. Expression of regulatory proteins such as NF-κß, cyclin D1, p53 and vascular endothelial growth factor was determined by western blot. *C. mukul* and GS significantly inhibited tumor cell growth, caused cell cycle arrest and apoptosis in both tumor cells. Such activities appeared to be due to inhibition of NF-κß, cyclin D1and restoration of p53. Overall our data suggests that *C. mukul* and GS may be developed as chemopreventive and chemotherapeutic drug for oral cancer.

Keywords: *Commiphora mukul,* Oral cancer, Antitumor, Cell cycle, Apoptosis, NF-κß, Cyclin D1, P53.

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most commonly diagnosed malignancy and a leading cause of cancer-related deaths among men in developing countries. It includes carcinoma of the lip, tongue, mouth and oral cavity. A recent report showed that there were approximately 263,900 new cases, and 128,000 number of deaths occurred due to oral cancer in the year 2008 worldwide [1]. The current approach for controlling cancer which reverse, suppress or prevent malignancy have major side-effects such as toxicity and intolerance. Besides, the high cost of these medicines is also a limitation for the economically weak patient. Thus identification of novel, cost-effective and natural agents with lesser side-effects is highly desirable.

C. muku is one of the important medicinal plants that were first described in Atharva Veda (2000 B.C). Gum resin of *C. mukul* commonly known as Guggulu reportedly cure obesity, liver dysfunction, internal tumors**,** malignant sores and ulcers, intestinal worms, leucoderma, sinus, edema and sudden paralytic seizures as mentioned in Sushrut Samhita [2]. It has been safely used for wound healing, arthritis**,** and inflammation in Ayurveda and Unani medicine [3,4]. Components of Guggulu are steroids, diterpenoids, aliphatic esters, carbohydrates, amino acid**s,** and triglycerides used in the preparation of several medicine [5]. A very important sterol derived from the gum resin of Guggulu is Guggulusterone (GS) (4,17 [20]-pregnadie 3, 16-dione). GS is an antagonist of farnesoid X receptor, which is essential for normal glucose homeostasis and lipid metabolism [6]. In addition, trans-(z)-isomer of GS induced apoptosis and inhibited the growth of PC-3, DU145 and LNCaP human prostate cancer cells *in vitro* [7,8]. Anti-cancer properties of GS have also been reported in other cell types including human lung, acute myeloid leukemia, and breast cancer [9,10]. However, there are no reports on its effect on oral cancer.

In this study we investigated the anti-tumor activities of *C. mukul* and GS in OSCC cell lines their effects on some important molecular targets such as NF-κß, cyclin-D1, p53 and angiogenesis factor (vascular endothelial growth factor [VEGF]) in order to understand their mechanism of action.

METHODS

Maintenance of tumor cells

This study was performed on squamous cell carcinoma of tongue SCC-4 (ATCC, Manassas, VA) and epidermal carcinoma of mouth KB (National Center for Cell Sciences, Pune, India). These cell lines were maintained in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin-G and 100 µg/ml streptomycin (all from HiMedia Corporation Mumbai, India) at 37°C in CO_2 incubator.

Preparations of *C. mukul* extract and GS

Dried extract of *C. mukul* was provided and certified by Sanat product Ltd (Delhi, India). The powder extract was further suspended in 1% dimethyl sulfoxide (DMSO) in distilled water. Final concentration of DMSO in culture medium was <0.05% (non-toxic concentration). Z-GS was purchased from Sigma-Aldrich, USA.

Tumor cell growth inhibition by MTT assay

Tumor cell growth inhibition was determined by MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5 Diphenyltetrazolium bromide) dye reduction assay. Briefly, 5×10^3 SCC4 and 8×10^3 KB cells per 100 µl DMEM per well were seeded in triplicates in 96-well plates and kept overnight at 37°C in a humidified atmosphere of 5% CO_2. The cells were then treated with different concentration of *C. mukul* and GS in working solutions for 24, 48 and 72 hrs. Human peripheral blood mononuclear cells (PBMC) were used as normal control simultaneously. On the completion of the respective treatment period, 10 µl of MTT solution (Sigma-Aldrich, USA; 10 mg/ml) was added to each well and the plate was further incubated for 4 h. The formazan crystals were dissolved by adding 100 µl of 0.04 N HCl in 2-propanol. The color intensity was measured by $A_{570\ nm}$ and tumor cell growth inhibition was calculated using the formula: % growth inhibition = $(1\text{-}OD_{test}/OD_{control}) \times 100$. Subsequent experiment was performed using IC50 dose of extract at 48 hrs treatment.

Cell cycle analysis by propidium iodide (PI) labeling and flow cytometry

Cell cycle analysis was performed by PI (Sigma-Aldrich, USA) labeling and flow cytometry as described earlier [11]. Briefly cells were treated with

IC50 concentration of *C. mukul* and GS for 48 hrs in 6 well plates. After termination of the culture, cells were washed twice in phosphate buffered saline (PBS). Approximately, 0.5-1 × 10^6 cells were then fixed in 2-3 ml ice-cold 70% ethanol by incubation at 4°C for 4 hrs. After one wash in PBS, cells were further incubated in RNase (Sigma-Aldrich, USA; 1 mg/ml) for 30 minutes at 37°C to digest cellular RNA. The cells were finally incubated with 10 µl PI (Sigma Aldrich, USA; 50 µg/ml) for 10 minutes and 10,000 events were acquired in LSRII flow cytometer (BD Biosciences, CA, USA). The data was analyzed using the ModFit LT software.

Apoptosis by annexin-V binding assay and flow cytometry

The apoptotic activity of *C. mukul* and GS was assessed by annexin-V binding assay as described earlier [12].Briefly, tumor cells were treated with IC50 concentration of *C. mukul* and GS for 48 hrs as described above. After termination of the culture, cells were washed twice with PBS and 1 × 10^6 cells were resuspended in 100 µl annexin-V binding buffer. To the cell suspension, 5 µl of FITC-conjugated annexin-V (BD Biosciences, CA, USA) and 10 µl of PI (50 µg/ml) were added and further incubated for 15 minutes at room temperature. After staining, 400 µl of annexin binding buffer was added and samples were stored on ice until the acquisition. Approximately, 10,000 events were acquired on LSRII (BD Biosciences, CA, and USA) flow cytometer. The percentage of annexin-positive (early apoptotic), PI positive (necrotic) and double positive (apoptotic) cells were determined using BD FACSDiva software.

Immunobloting

Tumor cells were treated as described above, with IC50 value of *C. mukul* and GS for 48 hrs to study the effect on the expression of certain proteins by western blotting. Cells were harvested and washed two times with PBS by centrifugation at 1000 g for 5 minutes at 4°C. The cells were lysed in chilled RIPA buffer (Sigma-Aldrich, USA) along with protease inhibitor cocktail (Sigma-Aldrich, USA) on ice for 30 minutes with intermittent stirring, followed by freeze-thaw steps at −80°C for 15 minutes and then thawed at 37°C. Lysates were clarified by centrifugation (15000 g for 10 minutes at 4°C) and supernatants were stored at −80°C until use. Protein estimation was done by bradford assay using bovine serum albumin (Sigma-Aldrich, USA) as standard. Equal amounts of protein were loaded on a 10-12% SDS-polyacrylamide gel and transferred onto nitrocellulose membrane (Millipore Pvt. Ltd, India) with a mini protean® tetra system (Bio-Rad). Membranes were blocked for 2 hrs at room temperature in blocking buffer (5% BSA, 10 mM TrisCl pH = 7.5, 150 mM NaCl) with constant stirring. Proteins were detected by incubation with rabbit monoclonal antibodies for cyclin D1, P-p53 (ser 20), NF-κß, VEGF (Santa Cruz Biotechnology, Inc, USA) at appropriate dilutions in blocking buffer (1% BSA) overnight at 4°C. Beta-actin was used as loading control. The membranes were washed in buffer (20 mM TrisCl, 500 mM NaCl, 0.05% v/v tween 20) and incubated with goat anti-rabbit IgG-HRP antibody (Santa Cruz Biotechnology, Inc, USA) at room temperature for 2 hrs at appropriate dilutions. Proteins were visualized using a chemiluminescence kit (Thermo scientific, USA) and Cell Biosciences FluorChem® System (Santa Clara, CA). Their relative concentrations were determined by densitometry analysis by using the quantity one® image analysis software (Bio-Rad, USA).

Statistical analysis

Statistical analyses were performed using Graph Pad Prism Software, version 5 (La Jolla, CA, USA). Two-tailed t-test was used to compare observations between treated and untreated groups. $p<0.05$ with 95% confidence limit was considered significant.

RESULTS

C. mukul* and GS inhibits tumor cell growth *in vitro

We initially performed MTT assay to define the IC50 value of *C. mukul* and GS. In order to test the effect of *C. mukul* and GS on the growth kinetics, OSCC cells were treated with different concentrations and were grown for 24, 48 and 72 hrs. A dose-dependent decrease in the growth kinetics was observed as in treated cells as compared to the vehicle-treated cells. The 48 hrs IC50 values for SCC-4 and KB was found to be 50 µg/ml for *C. mukul* while that for GS it was 45 and 40 µM for SCC-4 and KB, respectively. The IC50 values for different time points have been shown in line graph (Fig. 1a-d). Morphologically treated cells were rounded, condensed, shrank and aggregated (Fig. 2). The cytotoxicity of *C. mukul* and GS did not differ significantly than the untreated controls (data not shown)

***C. mukul* and GS induced cell cycle arrest in G_0/G_1 phase**

The cell cycle distribution showed a profound increase in the proportion of cells in G_0/G_1 phase at the 48 hrs time point (Fig. 3). Treatment of *C. mukul* appeared to increase the percentage of cells in G_0/G_1 phase (Fig. 3) as compared to vehicle control (50-72% in SCC-4 and 50-83% in KB) with a concomitant decrease in S and G_2/M phase. Similarly, in

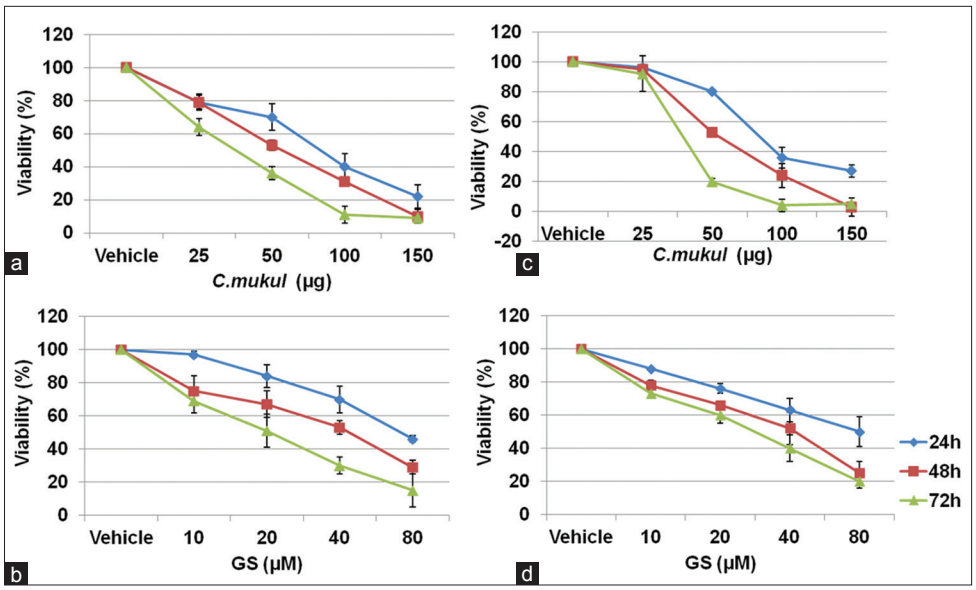

Fig. 1: The effects of *Comiphora mukul* and GS on cell growth inhibition in OSCC cell line (SCC-4. a, b, KB- c, and d). Cell growth inhibition activity of *C. mukul* and GS were assessed by MTT assay. MTT assay was performed at different concentrations of the above drugs and 24, 48, 72 hrs of treatment. % mean viability ± standard deviation has been shown in line graph (a-d). The data shown are the mean from three independent experiments

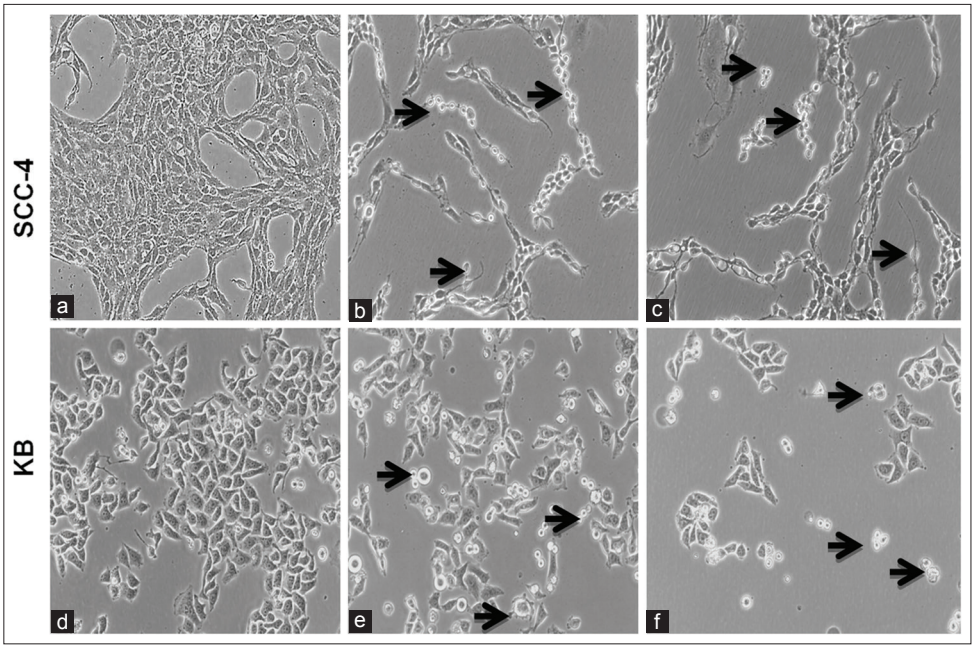

Fig. 2: Morphological changes in OSCC cell line in response to treatment with IC50 dose of *Comiphora mukul* (b and e) and GS (c and f) in comparison of vehicle control (a and d). Representative pictures (×40) showing morphological changes (shown with arrows) like detachment, rounding and shrinkage of cells after 48 h of treatment of *C. mukul* and GS. 0.05% dimethyl sulfoxide is considered as untreated

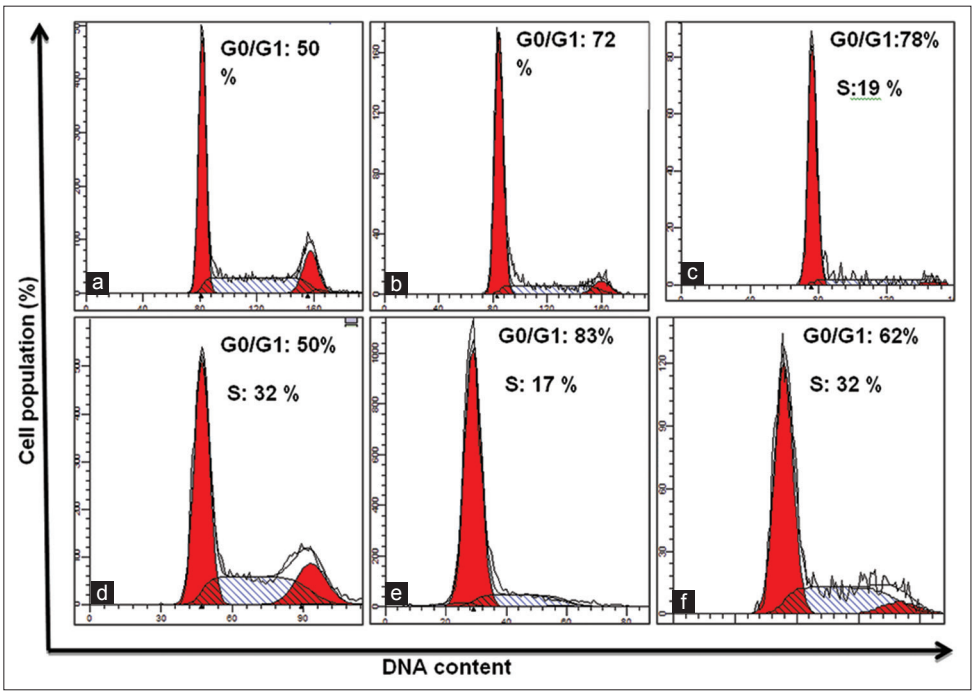

Fig. 3: The effect of *Comiphora mukul* (b and e) and GS (c and f) on the regulation of cell cycle arrest. *C. mukul* and GS induces cell cycle arrest in G1/G0 phase SCC-4 (a-c) and KB (d-f,) cell line, a and d were vehicle control

the case with GS a prominent G_0/G_1 phase arrest was observed at 48 hrs of treatment. Percentage of cells in G_0/G_1 phase increased to 50% from 78% in SCC-4 and 50% from 62% in KB cells.

Treatment of *C. mukul* and GS induces active cell death of OSCC cells *in vitro*

We further tested whether growth inhibition by treatment of *C. mukul* and GS is related with the induction of apoptosis (Fig. 4). *C. mukul* and GS induced the morphological changes like apoptosis after 48 hrs treatment (Fig. 2) and increased early, and the late apoptotic population was observed (Fig. 4). *C. mukul* increases the apoptotic cell by 27% in SCC-4 (Fig. 4a and b) and 68% in KB (Fig. 4d and e) cell line while GS increases the apoptotic cell by 42% in SCC-4 (Fig. 4a and c) and 27% in KB (Fig. 3d and f).

Effects of treatment with *C. mukul* and GS on the expression of NF-κß, cyclin-D1, P53 and VEGF

The treatment of both agents down regulate the expression of NF-κß and cyclin-D1 while no significant effects were observed on p53 and

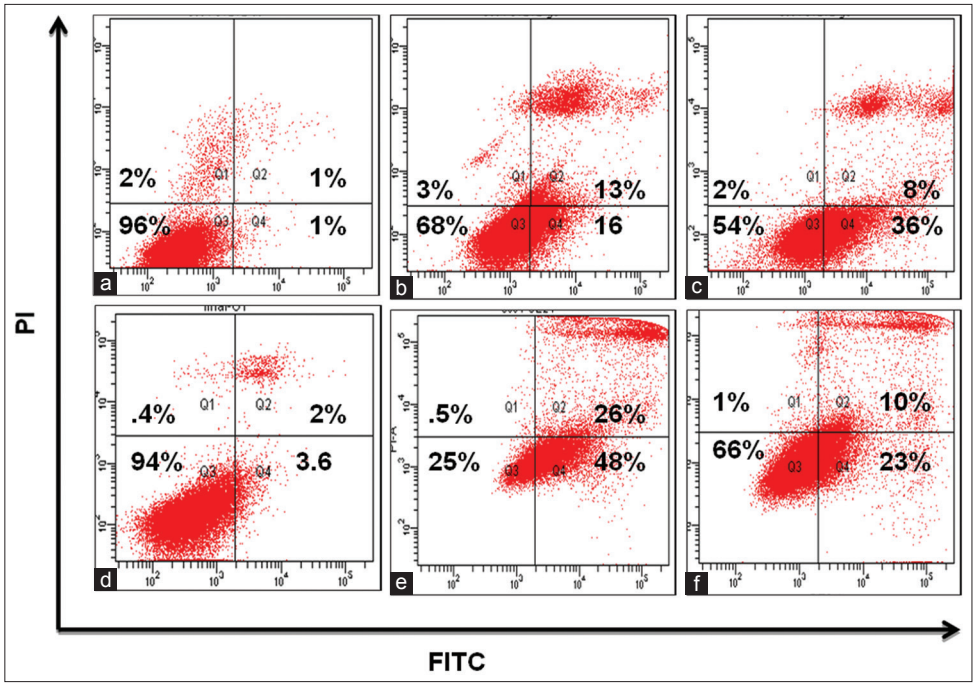

Fig. 4: *Comiphora mukul* and GS induces active cell death in oral cancer cell lines. Annexin V binding assay was performed to evaluate the apoptotic effect of *C. mukul* and GS) on SCC-4 (b and c) and KB (e and f) cells treated with their respective IC50 values for 48 hrs as compared to 0.05% dimethyl sulfoxide vehicle (a and d) control. Treatment of *C. mukul* and GS increases the number of early apoptotic cells in both cell line

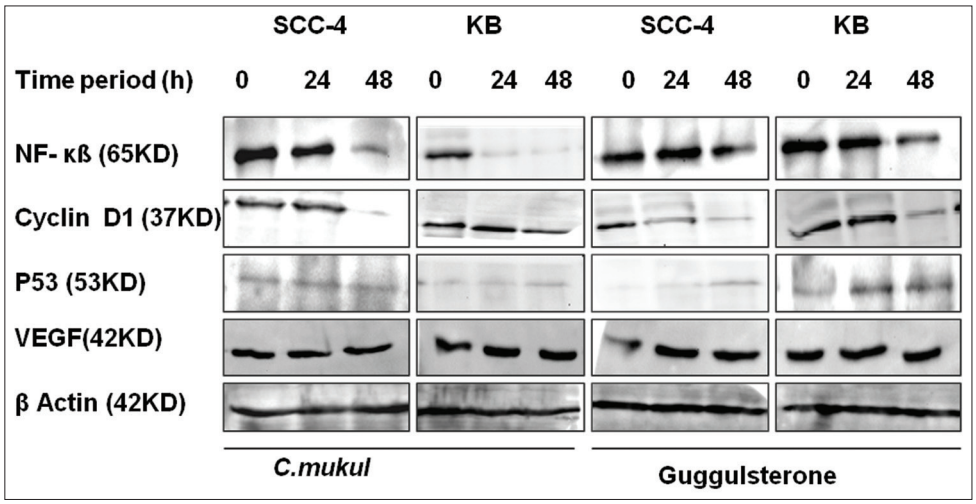

Fig. 5: Western blot analysis was performed to determine expression of different proteins involved in cell growth. Treatment of *Comiphora mukul* and GS down regulates the level of NF- κß and cyclin D1 in both cell lines. Treatment of GS increases the expression of P53 while no effect was seen in level of P53 after treatment of *C. mukul* at IC50 value after 48 hrs. No effect was seen in level of vascular endothelial growth factor after treatment of *C. mukul* and GS after 48 hrs

VEGF expression. The results have been shown in Fig. 5. Densitometric analysis showed that *C. mukul* could decrease the level of NF-κß protein by 80% in SCC-4 and 90% in KB cell line after 48 hrs, while GS could decrease its level by 50% in SCC-4 and 60% in KB. We also found that treatment of *C. mukul* decreases the expression of cyclin D1 in SCC-4 by 80% and in KB by 30%. Overall these results showed that *C. mukul* and GS targets NF-κß and cyclin D1 in OSCC cell lines.

DISCUSSION

Oral cancer is the sixth most common cancer reported globally. Conventional therapies play important roles, but therapeutic effects remain limited because of emerging cases of resistance. Therefore, finding a novel therapeutic agent against oral cancer is an urgent need. Despite different strategies, the use of individual targeted agents, have failed to provide significant improvements in the treatment and survival in oral cancer. It is documented that medicinal plant and natural products individually and/or in combination with known chemotherapeutic drugs may play a major role in therapy/prevention of oral cancer by targeting multiple signaling pathway [13]. In this study, we found that *C. mukul* and GS could suppress the growth of oral cancer cells by inducing apoptosis and G_0/G_1 arrest. Treatment with *C. mukul* triggered apoptosis in associated with down-regulation of NF-κß and cyclin-D1. In addition GS also induces apoptosis via restoration of p53 protein . However, *C. mukul* and GS did not exhibit any significant toxicity on normal cells (PBMC) at IC50 concentration, suggesting that both are specifically cytotoxic for cancer cell only.

NF-κß is overexpressed in all type of cancer and is related to poor prognosis [14]. It has been linked to the oral cancer development and metastasis through the activation of various genes responsible for tumor cell proliferation, cell survival and carcinogenesis [15]. *C. mukul* and GS suppressed the activation of NF-κß. According to the previous study, GS has therapeutic potential due to modulation of the growth factor receptors, cytokines, transcription factors, Farnesoid X receptor and enzymes [2,16]. In our study, treatment of *C. mukul* and GS down regulate NF-κß leading to suppression of tumor cell proliferation and induction of apoptosis in both the cell lines. It has been also reported that down-regulation of NF-κß, leads to decreased expression of anti-apoptotic gene (IAP1, xIAP, Bfl-1/A1, Bcl-2, cFLIP, survivin), proliferative gene (cyclin D1, c-Myc) and metastatic gene (MMP-9, COX-2) in pancreatic and prostate cell [7,17]. Besides, GS reportedly targeted smokeless tobacco-induced PI3K/Akt signaling in head and neck cancer cells [18].

Both *C. mukul* and GS treatment lead to cell cycle arrest in G_0/G_1 phase of the cell cycle that was further confirmed by impaired expression of cyclin D1. It is well-understood that Cyclin D1 is required for the progression of cells from G_1 phase to the S-phase of the cell cycle [19,20]. It's up-regulation was reported in 50% of cases of human head and neck cancer, and that up-regulation of this protein was shown to be a marker of poor prognosis in this disease [10,21].

CONCLUSION

Taken together, our results conclude that the antitumor activity of *C. mukul* and GS is mediated via cell cycle arrest and down-regulation of cyclin D1 and NF-κB expression. Treatment of GS also leads to the restoration of p53 expression in OSCC. The present study reveals that *C. mukul* and GS are a potent growth inhibitor of OSCC. However, the pharmacokinetic parameters for *C. mukul* and GS have not been determined in humans. These findings suggest that *C. mukul* and GS possess a strong anti-cancer activity, these can be developed as suitable chemotherapeutic and chemopreventive agent for OSCC.

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