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Antiproliferative effects of total alkaloid extract of roots of *Chassalia curviflora* (Wall.) Thwaites on cancer cell lines

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Chassalia curviflora is used in folklore medicines for treating several ailments and infections owing to its anti-inflammatory properties. Though the plant has been reported to possess anti-inflammatory antihepatotoxic and analgesic activities, its anticancer potential has not been studied so far. In the present study, we investigated the antiproliferative effects of the total alkaloids isolated from the roots of *C. curviflora*. The total alkaloid was validated by MTT assay in three cancer cell lines, such as liver cancer cell line-A549, breast cancer cell line-MCF-7 and ovarian cancer cell line -HeLa. Significant antiproliferative effect (IC_{50} value 3.59±0.14*** µg/mL) was observed in A549 cells, and was taken for further studies. Cell cycle analysis showed that the cells got arrested in sub G0 phase and annexin V-FITC assay revealed that 27.4% cells were in early apoptosis and 7% cells in late apoptosis. The study revealed that the total alkaloids of *Chassalia curviflora* roots possess significant antiproliferative and apoptotic activity.

Keywords: Alkaloids, Anticancer, Anti-inflammatory, Apoptosis, Chemotherapeutic, Curved Flower Chasalis, Folklore, Immunomodulatory

Cancer is a group of diseases with multistage process involving abrupt cell division. According to World Health Organization (WHO), cancer accounts for the second leading cause of deaths globally. Although there is an overall decrease of 27%, since 1991 translating to approximately 2.6 million fewer cancer deaths, the cancer mortality rates of poor countries has increased to several folds during 2012-2016[1]. It has been estimated that one third of the chemicals used in cancer chemotherapy are derived from natural products[2,3]. About 20.2% of anticancer agents approved during the period 1981-2010, were synthetic and the rest were natural products or inspired by natural products[4]. For developing new drug, plants are selected based on their ethnopharmacologic or ethnobotanic knowledge or by random selection of different plants. Various plant derived anticancer drugs like topotecan (hycamtin), irrinotecan (camptosar), vinblastin (velban), teniposide (VM-26) were approved for clinical use[5]. Induction of apoptosis is one of the most important effects of cytotoxic antitumor agents and is a major anti-cancer mechanisms of plant derived drugs. Herbal drugs can be classified as an immune-modulatory drug or a chemopreventive medicine[6].

Both inflammation and infection are linked to cancer. There exists a strong correlation between the presence of inflammation and establishment, progression and aggressiveness of various malignancies[7,8] Inflammation affects the immune-surveillance as well as response of the body to therapy. Anti-inflammatory agents such as non-steroidal anti-inflammatory drug (NSAID), steroids and natural products are widely used in the treatment of cancer. Immunomodulatory natural products can suppress inflammation and associated tumor progression and metastasis as well as alleviate pain[9]. The contribution of natural products towards drug development has been scientifically documented. The structural diversity and biological activity of natural products make them the most valuable sources of drugs and drug leads.

The shrub *Chassalia curviflora* (Wall.) Thwaites [syn: *Psychotria curviflora* (Wall.)] belongs to the family Rubiaceae. The genus, commonly known as curved flower chasalis or curved flower woody chassalia, consists of more than 110 species with paleotropic distribution[10]. Chakma tribes of

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Bangladesh apply crushed leaves on wounds[11] for treating snake and insect bites, while Kani tribes of Agastyamalai, Kerala, India use roots as an effective medicine for treating jaundice[12]. In Peninsular Malaysia, root decoction is used to treat malaria, cough and remedy in phlegm, rheumatism and pneumonia[13,14] and both roots and leaves in combination are used to cure wounds, ulcers and to relieve headaches.

Ethanolic extract of leaves and roots of *C. curviflora* were reported to possess anti-inflammatory, analgesic[15], hepatoprotective activity[16] and phytochemical analysis revealed that roots have high content of alkaloid. The alkaloids present in the Rubiaceae family principally contribute to their pharmacological activities. Anti-inflammatory and immunomodulatory properties of a plant may contribute to its anticancer properties. Therefore, in this study, we tried to explore the antiproliferative activity of total alkaloid extracts of roots of *Chassalia curviflora* (Chr-alk) *in vitro* in cancer cells lines.

Materials and Methods

Plant material and extraction for alkaloids

Roots of *Chassalia curviflora* (Walls) Thwaites were collected from the medicinal plant garden of University of Kerala. The plant was identified and a voucher specimen was deposited in the Herbarium of Jawaharlal Nehru Tropical Botanic Garden and Research Institute, (JNTBGRI), Palode, Kerala, India (TBGT 57053 23/9/11. Alkaloid extraction was carried out according to Maldoni[17]. The powdered root (400 g) was extracted with ethanol for 24 h in a continuous extraction soxhlet apparatus. This ethanolic extract (30.5 g) was concentrated in a rotary vacuum evaporator, acidified with 2 % hydrochloric acid, HCl (pH 1-2) for salt formation and partitioned with chloroform-water (3:1). The alkaloid salts in aqueous phase was treated with dilute ammonium chloride, NH_4Cl (pH 9-10), to liberate tertiary alkaloids and partitioned in chloroform-water mixture. The organic layer was extracted until the last chloroform extract gave negative reaction for Dragendroff's reagent. The extract was tested for the presence of alkaloids. The w/w yield of the extract was 3.5% and was labeled as Chr-alk. The presence of indole group in the alkaloid was tested by p-dimethylaminobenzaldehyde reagent, which gave purple colour[18].

Cell lines

The human cancer cell lines A549, HeLa and MCF-7 and normal cell line 3T3L1 were grown in DMEM supplemented with 10% fetal bovine serum with 2 mM L-glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin. All media and supplements were purchased from Gibco™, Thermo Fischer Scientific, Waltham MA.

In vitro cytotoxic study –MTT assay

The cytotoxic activity of the Chr-alk was carried out using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay according to Mossman[19]and evaluated against three cancer cell lines panels consisting of breast cancer (MCF-7), lung cancer (A549), cervix cancer (HeLa) and normal fibroblast cell (3T3 L1). The cells were routinely maintained as monolayer cell cultures in Dulbeco's modified Eagle's medium containing fetal bovine serum (10%), glutamine, penicillin/streptomycin. The cells were trypsinized and seeded in a 96-well plate at a density of 5×10^3 cells/well and incubated in a humidified atmosphere of 5% CO_2 at 37°C for 24 h. For treatment various concentrations of Chr-alk (0.05, 0.1, 1, 5, 10, 25 µg/mL) were added and cells cultured in media alone served as positive control and media with DMSO as negative control. After 24 and 48 h time points, the medium was removed and cells were washed with PBS and fresh medium with MTT dye (5 mg/mL dissolved in PBS) was added. After 4 h of incubation in dark, the medium was removed, and 100 µL of isopropanol was added to each well to solubilize the formazan crystal and the optical density was measured at 570 nm in multiskan™ GO multiplate spectro-photometer[20]. Results were taken from three independent experiments, each performed in triplicates. The percentage inhibition of proliferation was calculated using the formula given below and the IC_{50} values were determined from log-dose response curves using Graph pad prism software version 5 for windows.

% Inhibition of proliferation =T/C×100, where

T = Absorbance of test at 570 nm; and

C = Absorbance of untreated control

Detection of apoptosis

Staining method: Acridine-ethidium bromide staining and Hoechst staining

Apoptosis was detected *in vitro* by acridine orange-ethidium bromide[21] and Hoechst staining methods[22]. A549 cells were grown in a 6-well plate at a cell density of 5×10^5 cell/mL and then treated with Chr-alk (2.0, 3.5 µg/mL) for 48 h. For acridine orange-ethidium bromide staining, mixture (1:1) of AO and EtBr (4 µg/mL) solution was added to each well. For

Hoechst staining, 100 µL of 10 mg/mL of Hoechst 33342 was added to each well and incubated for 5 min. Stained cells were visualized by fluorescent microscopy at 350-460 nm. Nuclei of cells manifesting apoptosis were visualized with morphological features such as nuclear fragmentation and chromatin condensation and photographed.

Cell cycle analysis

A549 cells (1×10^6) were seeded in a 60 mm tissue culture dish and treated with Chr-alk (2.0, 3.5 µg/mL) for 48 h. After incubation at room temperature (25°C), cells were trypsinized, pelleted and washed twice with ice cold PBS. It was suspended in ice-cold ethanol (70%) with gentle vortexing and kept in ice for 30 min. After incubation, the cells were washed with PBS and 50 µL of RNase was added directly to the pellet followed by addition of 400 µL of propidium iodide (1.0 mg/mL). Mixed well and incubated in the dark for 5-10 min. Cells were strained through a filter of 0.75 µM. Cell cycle distribution was analyzed using FACS Calibur (Becton-Dickinson)[23]. Data was analyzed using DIVA software program.

Detection of apoptosis by annexin V-FITC assay

Flow cytometric analysis were used to study the apoptotic cells[24]. Externalization of phosphatidyl serine is one of the indications of early stage of apoptosis. Annexin V-FITC quantitatively determines the percentage of cells within a population that are undergoing apoptosis[24]. Both apoptotic and necrotic cells from the same sample can be quantified apoptotic cells were quantified in A549 cells using Annexin V-FITC apoptosis detection kit II (BD Biosciences, Pharmingen) according to manufacturer's protocol. Briefly, A549 cells were treated with Chr-alk (2.0, 3.5 µg/mL) for 48 h. Cells were trypsinized, pelleted, washed twice with ice cold PBS and suspended in 100 µL 1X binding buffer at a concentration of (1×10^6 cells/mL). The cells were stained simultaneously with 5 µL FITC-conjugated annexin V and 5 µL PI. Gently vortexed the cells and incubated for 15 min at room temperature in the dark. To each tube 400 µL of binding buffer was added. The cells were analyzed immediately by flow cytometry (FACS Calibur, Becton Dickinson).

Statistical analysis

The results were expressed as the mean±S.D. of triplicate experiments. Statistical analysis was done by oneway ANOVA using Graph Pad Prism software and P <0.001 was considered as statistically significant.

Results

Alkaloid-rich extract (Chr-alk) is cytotoxic to cancer cells

The cytotoxic effect of the alkaloid rich extract (Chr-alk) on cancer cell lines was assessed by MTT assay. There was a concentration and time dependent increase in the percentage of cytotoxicity in MCF7 (Fig. 1A), A549 cells (Fig. 1B) HeLa (Fig. 1C) and 3T3L1 (Fig.1D) (P <0.001). There was more cell death treatment of extract on in A549 lung cancer cells with an IC_{50} value of 3.59±0.14 µg/mL (Table 1). The Chr-alk induced less toxicity in normal cells compared to cancer cells showing its specificity to cancer cells. The IC_{50} values were determined after 24 and 48 h of treatment.

Chr-alk induced apoptosis in cancer cells

Dual staining by acridine orange/ethidium bromide revealed the characteristic features of apoptosis, such

Table 1 — IC_{50} µg/mL values of *Chassalia curviflora* root alkaloid extract (Chr-alk) on different cell lines

Cell Lines	IC_{50} (µg/mL)	
	24 h	48 h
MCF7	15.6±0.12**	6.488± 1.23***
A549	8.26±0.152***	3.59± 0.14***
HeLa	12.56±0.05**	5.64±0.98***

[Values are expressed as mean ±SD by one way ANOVA using Graph Pad Prism software and P <0.001 was considered as statistically significant compared to negative control]

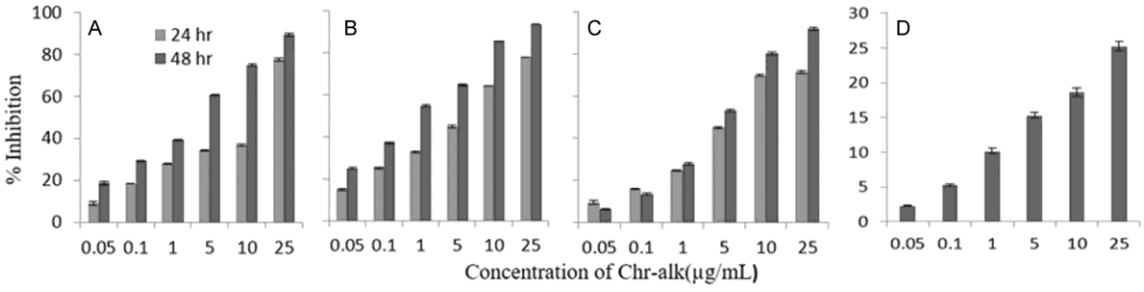

Fig. 1 — Cytotoxic assay (MTT) of Chr-alk on (A) MCF7 cells; (B) A549cells; (C) HeLa cells; and (D) 3T3L1 cells. [Three trials were done with thee replicate for each concentration. Data were expressed as ± SD by one way ANOVA using Graph Pad Prism software and P <0.001 was considered as statistically significant]

as nuclear fragmentation, chromatin condensation and membrane blebbing after 48 h of treatment with Chr-alk in A549 cells (Fig. 2 A-D). Viable cells appeared as green and apoptotic cells appeared as orange red. Hoechst staining revealed a greater intensity of blue colour in the treated cell compared to the untreated control cell, revealing condensed chromatin which is the major characteristic feature of apoptosis as shown in Fig. 3 (A-D). There was a concentration dependent increase in the percentage of apoptotic cells after 48 h of incubation. Microscopic studies to determine the growth inhibitory activities of Chr-alk by acridine orange-ethidium bromide dual staining and Hoechst staining revealed that Chr-alk induced morphological changes characteristic of apoptosis. Cells with chromatin condensation and nuclear fragmentation observed during the study clearly suggest apoptosis.

Cell cycle analysis

In the cell cycle analysis, it was found that cells were arrested in the sub G0 phase. A549 treated with Chr-alk (3.5 µg/mL), got arrested in sub G0 (Fig. 4 A-D) while 9.7% of cells were found to be in sub G0 phase, in control cultures only 2.5% of cells were in sub G0. Lower concentration of the extract also induced

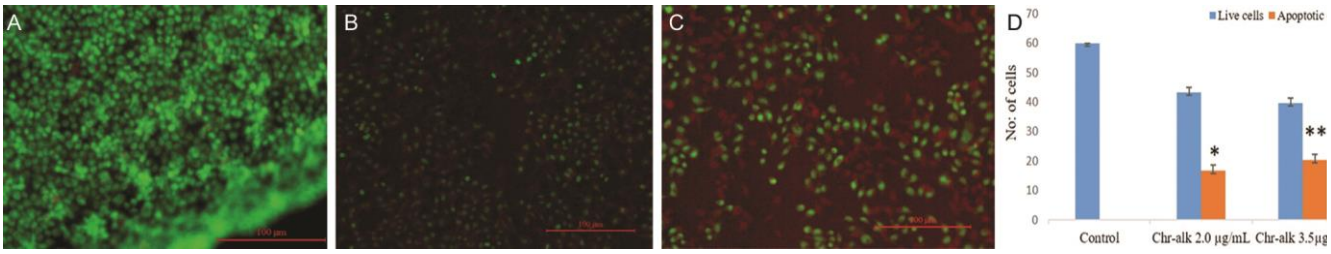

Fig. 2 — Fluorescent microscopic images of acridine orange-ethidium bromide dual stained A549 cells for visualization of apoptotic cells (A) Untreated cells; (B) Cells treated with Chr-alk (2.0 µg/mL); (C) Cells treated with Chr-alk (3.5 µg/mL); and (D) Data on number of apoptotic cells of A549 treated with Chr-alk 2.0 µg/mL) and Chr-alk 3.5 µg/mL). [A single field of 60 cells were counted for apoptotic/dead and live cells three times separately Significant increase (P <0.001) marked by asterisks in Acridine-orange staining were observed]

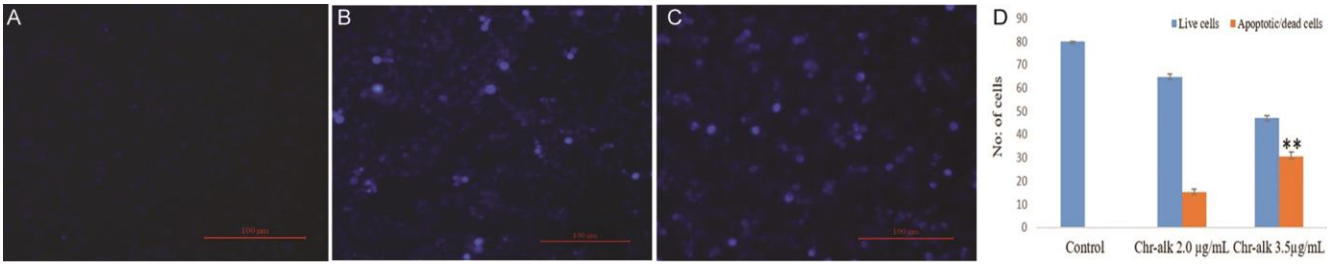

Fig. 3 — Fluorescent microscopic images of Hoechst stained A549 cells for visualization of apoptotic cells (A) Untreated cells; (B) Cells treated with Chr-alk (2.0 µg/mL); (C) Cells treated with Chr-alk (3.5 µg/mL); and (D) Data on number of apoptotic cells of A549 treated with Chr-alk 2.0 µg/mL) and Chr-alk 3.5 µg/mL). [A single field of 80 cells were counted for apoptotic/dead and live cells three times separately. Chr-alk caused apoptosis. Significant increase (P <0.001) marked by asterisks in Hoechst staining were observed]

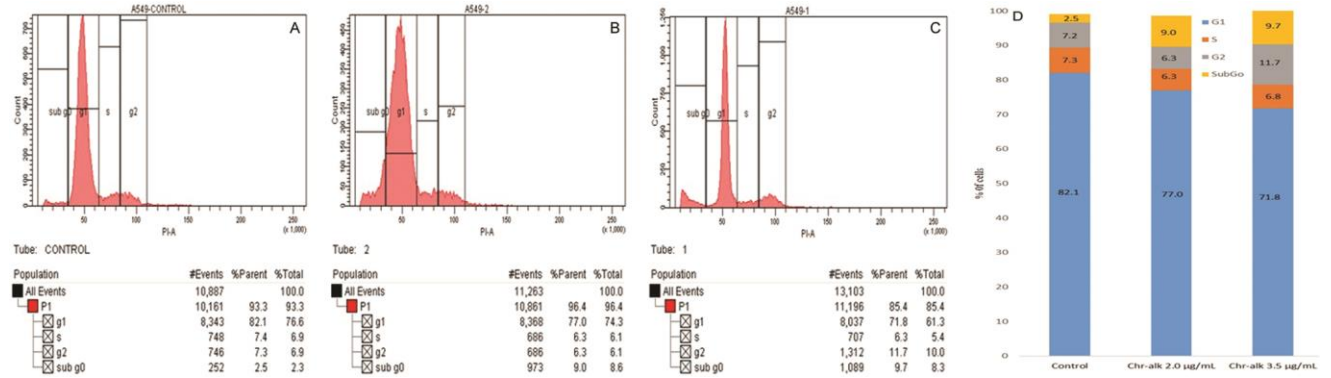

Fig 4 — Effect of Chr-alk treatment on the cell cycle of A549 cells. (A) Untreated A549; (B) A549 treated with Chr-alk (2.0 µg/mL); (C) A549 treated with Chr-alk (3.5 µg/mL); and (D) Cell cycle data of A549 cells after 48 h of treatment with Chr-alk at 2.0 and 3.5 µg/m. [Both control and treated cells after 48 h, were collected and stained with PI staining followed by FACS analysis The histogram shows there is an increase in subGo cells in both treatments compared to that of control. Chr-alk significantly (P <0.001) arrested cell cycle at sub G0 phase]

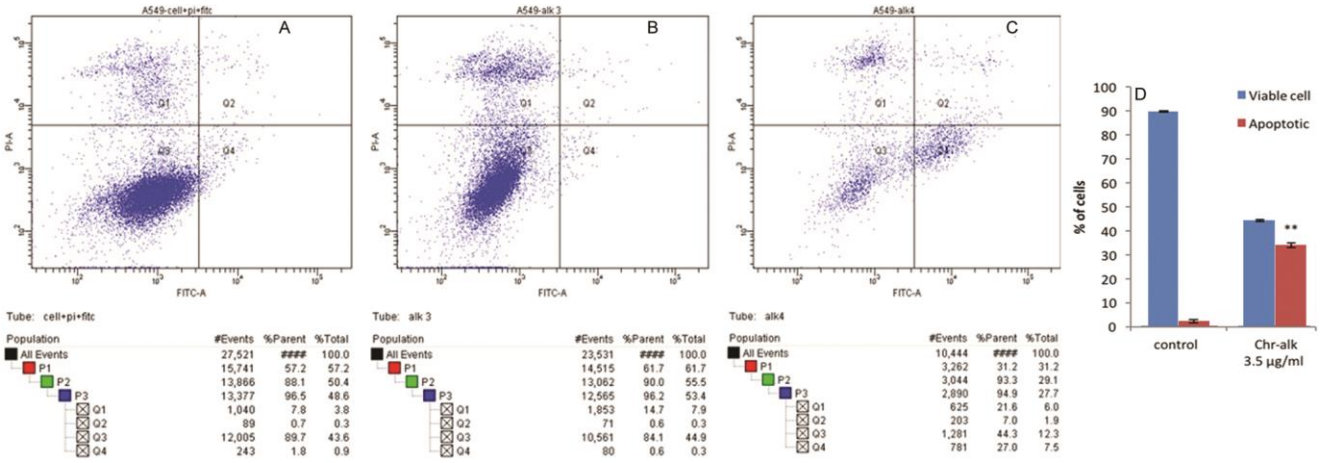

Fig. 5 — Flow Cytometric Analysis of FITC annexin V staining of A549 (A) control cells; (B) A549 cells treated with Chr-alk (2.0 µg/mL); (C) A549 cells treated with Chr-alk (3.5 µg/mL); and (D) Data on Percentage of Apoptotic cells of A549 cells treated with Chr-alk (3.5 µg/ml) after 48 h. [Significant increase (P <0.001) marked by asterisks in percentage distribution of cells were observed]

apoptosis with 9.0% of cells in sub G0. In cells treated with Chr-alk (3.5 µg/mL), 11.7% of cells also got arrested in G2 phase (Fig. 4D). The percentage of cells in sub G0 phase arrest undergoing apoptosis was determined by annexin V-FITC staining.

After exposure to Chr-alk (3.5 µg/mL for 48 h), proportion of early and late apoptotic cells increased significantly (P <0.001) as compared with control cells (Fig. 5A) with values of about 27.4% (Early apoptosis) and 7% (late apoptosis) (Fig. 5C). Chr-alk exerted significant early phase apoptotic effects by translocating phosphatidyl serine from inner to outer membrane. Lower concentration of Chr -alk did not give much apoptosis (Fig. 5B). The apoptosis index was quantitated by Annexin V-FITC assay and showed significant induction of early apoptosis against A549 cells. It was found that 34.4% of cells had undergone apoptosis.

Discussion

The present study demonstrated the potential effects of alkaloid extract of *Chassalia curviflora* (Chr-alk) on the viability and apoptosis of cancer cell lines. The extract reduced cell viability in a concentration dependent pattern as shown in MTT studies. All the three cell lines (MCF-7, HeLa and A549) used in the study responded to Chr-alk, independent of their lineage origin, with significant inhibition of growth. A549 cell line was significantly more sensitive to Chr-alk with an IC_{50} value of 3.59±0.14 µg/mL. Chr-alk induced less toxicity in normal adipocyte cell line, 3T3L, compared to cancer cells. Cancer cells being fast proliferating are more vulnerable to drugs compared to normal cells[25].

One of the factors regulating the population size of a clone is the induction of programmed cell death known as apoptosis, a highly regulated process that occurs as part of differentiation, proliferation and growth of normal and malignant cells and is characterized by morphological feature and DNA fragmentation. Microscopic studies to determine the grown inhibitory activities of Chr-alk by acridine orange-ethidium bromide dual staining and Hoechst staining revealed that Chr-alk induced morphological changes characteristic of apoptosis. There was a concentration dependent increase in the percentage of apoptotic cells after 48 h of incubation. Cells with chromatin condensation and nuclear fragmentation observed during the study clearly suggest apoptosis[26,27].

Cancers and many other human diseases are known as cell cycle disease due to the fact that many of regulatory factors of checkpoints are arrested or lost in tumorigenesis[28]. In this study, cell cycle analysis showed that increase in percentage of cell in sub G0 compared to the control cells, while 9.7% of cells were found to be in sub G0 phase, in control cultures only 2.5% of cells were in sub G0. This indicates that Chr-alk induced cell arrest at the sub G0 phase in a dose dependent manner, an Chr-alk arrested cells at sub G0 by inhibiting protein kinase complex consisting of cyclin and cyclin dependent kinase. It prevented DNA synthesis in cells. Thus, Chr-alk could act even at the initial stage of cell cycle, terminating further

proliferation. The apoptosis ratio induced by Chr-alk was further quantitatively assessed by annexin V-FITC assay where the extract showed significant apoptosis induction against A549 cells. It was found that 34.4% of cells had undergone apoptosis. The annexin V assay also confirmed the ability of Chr-alk to induce early apoptosis. Correlation between cell cycle arrest and apoptosis has been well established[29]. Apoptosis acts as a protective mechanism by removing ailing cells and thereby maintaining homeostasis.

Cancer cells shows resistance to apoptosis and thereby sustains uncontrolled proliferation of cells, therefore, any apoptosis modulating compound can act as a plausible chemotherapeutic agent against cancer cells[30]. Several natural products are in the standard repertoire of cancer therapy[31] and alkaloids are among the most promising chemotherapeutic agent[32]. Our above findings reveal that alkaloid rich extract of roots of *C. curviflora* is a strong inducer of early apoptosis and cell cycle arrest in A549 lung cancer cells.

Conclusion

The alkaloid rich extracts of roots of *Chassalia curviflora* exhibited significant antiproliferative as well as apoptotic effects on cancer cell lines and significant cytotoxicity was observed on A549 cell line with an IC_{50} value of 3.59±0.14 µg/mL. The cell cycle analysis and Annexin V-FITC assay revealed that Chr-alk induced early apoptosis in 34.4% cells. Bioactive phytochemicals with antioxidant, anti-inflammatory properties are potential leads to chemopreventive and chemoprotective activity. The anti-inflammatory and immunomodulatory roles of *C. curviflora* might have contributed to its anti-proliferative effects. Further studies are required to identify the major alkaloids contributing to the antiproliferative and apoptotic activity.

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Conflict of interest

Authors declare no conflict of interests.

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