Int. J. Curr. Res. Biosci. Plant Biol. 2015, 2(9): 66-73

EXCELLENT PUBLISHERS

International Journal of Current Research in Biosciences and Plant Biology

ISSN: 2349-8080 Volume 2 Number 9 (September-2015) pp. 66-73

www.ijcrbp.com

Original Research Article

Inhibition of Wound Closure and Decreased Colony Formation by *Clerodendrum infortunatum* L. in Lung Cancer Cell Line

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Abstract

The use of synthetic drugs to cease the progression of cancer in human beings, is an enormously capable strategy for cancer prevention. *Clerodendrum infortunatum* L. is one of the most extensively used herbal medicines with various biological activities. In the present investigation, the *in vitro* clonogenic assay and wound closure assay was used to investigate the cytotoxic effect of hexane and chloroform root extracts of *C. infortunatum* against lung cancer cell line (A549). It was confirmed by colony forming assay that A549 cells are sensitive to these two samples in dose depending manner. Interestingly, at 200 µg/ml of hexane root (HR) and chloroform root (CR) extracts treatment, the number of soft agar colonies was reduced by ~4-fold ($p \leq 0.05$) compared to the untreated control cells. In wound closure assay it was observed that HR and CR effectively inhibited the migration of cells. The control cells filled-up the wound gap completely after 24 h whereas, the cells treated with HR and CR extract, wound gap was not completely filled. Interestingly, at 200 µg/ml, HR and CR extract treatment significantly decreased (~4-fold; $p \leq 0.01$) the migration rate of A549 cells thereby affecting their migration capability. In conclusion, we attributed that decrease in colony formation and wound closure of A549 cells by HR and CR extracts of *C. infortunatum* may contribute to the inhibition of migration in human lung cancer. From the results of this study, it is concluded, that decrease in colony formation and wound closure of A549 cells by HR and CR extracts of *C. infortunatum* may contribute to the inhibition of migration in human lung cancer.

Keywords

A549 cell line
Clerodendrum infortunatum
Clonogenic
Root extracts
Wound closure

Introduction

Dynamic approach for the development of new drugs from medicinal plants is growing considerably (Liu et al., 2005). Over the past decade, herbal medicines have developed into comprehensive attention, creating an influence correspondingly on both world health and international trade. Medicinal plants that have been in survival for thousands of years have designed the foundation of traditional medicine and continue to deliver civilization with novel remedies (Gurib-Fakim, 2006).

The genus *Clerodendrum* of the family Verbenaceae is a diverse genus that comprises 560 to 580 species of small trees, shrubs, lianas, or occasionally perennial herbs, mostly in the tropics and subtropics of the world (Munir, 1989). Many investigations have been performed to validate the traditional assertions of this genus by practising different *in vivo* and *in vitro* assays. These studies specified that the numerous species of this genus hold potent anti-inflammatory, antidiabetic, antimalarial, antiviral, and antioxidant activities and have prospects to be established as strong remedial agents from natural resources (Panthong et al., 2003; Khan et al., 1996; Hazekamp et al., 2001; Mehdi et al., 1997; Chae et al., 2005).

Although the developments anticipated in cancer treatment, there continues to be prerequisite for intervention strategies, comprising chemopreventive agents that perform as principal defensive agents by inhibiting, delaying or reversing preneoplastic lesions, as well as those that act on subordinate or periodic cancers as therapeutic agents (Kwon et al., 2007). Chemopreventive agents have lesser side effects and toxicity and are involved in abrogation of carcinogens as well as their effects on cells (Greenwald, 2002; Kakizoe, 2003; Johnson, 2007).

In the present study, *Clerodendrum infortunatum* Linn. (Family-Verbenaceae, Bhat in Hindi, Ghentu in Bengali, Bhania in Oriya) has been selected to evaluate anticancer activity on lung cancer by practising A549 cell line. Traditionally, its ethno botanical importance has been verified in the treatment of bronchitis, asthma, fever, diseases of the blood, inflammation (Das et al., 2010), burning sensation, tuberculosis, hepatoprotective (Sannigrahi et al., 2009) and antiepileptic in Indian folk medicine (Pal et al., 2009; Kapoor, 2001). Conventionally, the plant leaves extract is given orally in fever and bowel troubles among the Kuki and Rongmai Naga tribes of North-East India. Kachari, Hmar and Riang tribes of Barak Valley and North-Cachar hills use leaf extract for stomach pain and for diabetes (Baid, 2013). In swelling, the root paste is applied as bandage (Barbhuiya et al., 2009). For the cure of malaria fresh juice of the leaves has been employed (Chopra et al., 1992; Goswami et al., 1998).

Lung cancer is one of the most common basis of deaths associated with cancer all over the world (Jemal et al., 2008). The drug like cisplatin employed in the treatment of patients with lung cancer is highly toxic with a low survival profile against non-small cell lung cancer (NSCLC) (de Petris et al., 2006; Giaccone, 2000). Hence, a comprehensive understanding of the molecular mechanisms of more effective and less harmful therapies are needed to reduce lung cancer mortality. A549 cell line belongs to lung adenocarcinoma cell line, and has been extensively used in studies of cell cycle, apoptosis and adhesion mechanisms in cancer cells (Cottier et al., 2004). Here, using a combined approach of clonogenic assay, wound closure and nuclear morphology, we investigated anticancer activity of hexane and chloroform extracts from the roots of *C. infortunatum* against A549 cell line. The A549 cell line was first developed in 1972 by Giard et al. through the removal and culturing of cancerous lung tissue in the explanted tumour of 58-year-old caucausian male. The cells produced were adenocarcinomic alveolar basal epithelial cells with a model chromosome number of 66.

Materials and methods

Plant source and extraction

The plant *C. infortunatum* was collected from the road side of Bhadra River Channels, Shimoga, Karnataka, India. The roots (after cutting into small pieces) were shade dried for several days. The plant material was then oven dried for 24 h at considerably low temperature (40°C) for moisture free and better grinding. The air-dried and finely ground leaf root (500g) material of the plant was extracted in a Soxhlet apparatus successively with hexane and chloroform. A suitable solvent was added to the flask and the setup was heated under reflux. The steam of the solvent which, when contacts with the material will dissolve metabolites and brings back metabolites to the flask. The extracts were filtered, pooled and concentrated to dryness under reduced pressure in a rotary evaporator (Buchi, Flawil, Switzerland) to yield dried hexane, and chloroform extracts. The extracts thus obtained from root of plant using hexane as solvent was labeled as HR whereas, extract of chloroform was labelled as CR and yield was calculated in terms of grams/weight of the powdered material.

Solubility tests of plant extracts

Solubility tests were carried out for the analysis of solubility of crude extracts in different solvents like, hexane, chloroform, ethyl acetate, acetone, DMSO, ethanol, methanol, water, 1N NaOH, and 1N HCl.

Phytochemical analysis

Phytochemical screening of *C. infortunatum* root extracts was performed for the qualitative detection of reducing sugars, terpenoids, flavonoids, saponins, tannins, alkaloids, phlobatannins, steroids, amino acids and glycosides using standard procedures (Mehta et al., 2013; Ashok Kumar et al., 2012).

Chemicals

The chemicals utilized in this analysis comprises RPMI-1640, Minimum Essential Medium, fetal calf serum, trypsin, , DAPI stain, 4% paraformaldehyde, crystal violet, Triton X-100, DMSO, ethanol, penicillin, streptomycin, gentamycin, phosphate buffer saline (Merck, Darmstadt, Germany); trichloroacetic acid (TCA), distilled water, Tris-EDTA buffer, Tris buffer (Hi-Media, Mumbai, India), and Tris-acetate-EDTA buffer. All other chemicals used in this study were purchased locally and were of analytical grade.

Cell line and cell culture

The human cancer cell line used in this study, includes A549 (lung) was obtained from the National Centre for Cell Science, Pune, India. The cells were sustained in a CO_2 incubator with 5% CO_2 and 95% humidity, and supplemented with desired medium and 10% fetal bovine serum (FBS). Glutamine (2mM), penicillin (100 units/mL), and streptomycin (100 mg/mL) were also added to the medium to 1×final concentration from a 100 × stock. After attaining the confluent growth, the cells were trypsinized using Trypsin-EDTA (0.25%) before treating with the desired extracts.

Clonogenic Assay

The assay was performed according to the previously described method (Koppikar et al., 2010). Briefly, A549 cells were plated at a seeding density of (1×10^3 cells/well) in 6 well tissue culture grade plates. After 24 h the culture medium was changed and new medium was added and cells were exposed to various concentrations of Hexane root (HR) and chloroform root (CR) extracts (50, 100 and 200µg/mL) along with vehicle DMSO for 5 days at 37°C incubator in 5% CO_2. Later, the obtained colonies were fixed with 4% paraformaldehyde and were stained with 0.5% crystal violet solution. The colonies from the plates were counted and averaged from the observed fields randomly (n = 3) under phase contrast microscope and photographed with attached camera.

Scratch motility (wound healing) assay

The assay was performed as described previously (Chen et al., 2011). Briefly, A549 cells were plated in a 6 well plate at a concentration of (5.5×10^5 cells/well) and allowed to form a confluent monolayer for 24 h, it was then serum starved for 24 h. After that the monolayer was scratched with a sterile pipette tip (200 µL), washed with serum free medium to remove floated and detached cells and photographed (time 0 h). Cells were successively treated in medium containing low serum (1.0%) in presence of different concentrations of HR and CR extracts (50, 100, 200 µg/ml) along with vehicle DMSO for 24 h. Wounded areas were progressively photographed with microscope attached with camera (100x magnification).The percentage of wound closure was estimated by the following equation:

$$\text{Wound closure \%} = [1-(\text{wound area at } t_1/\text{wound area at } t_0) \times 100\%]$$

Where t_1 is the time after wounding and t_0 is the time immediately after wounding.

DAPI staining for nuclear morphology

DAPI staining for nuclear morphology was performed to assess the nuclear morphological changes on cells after treatment with HR and CR. A549 cells were incubated in 6-well plates and treated with varying concentrations of HR and CR for 24 h. After incubation, cells were washed with PBS, fixed and permeabilized with 4% paraformaldehyde and Triton X-100, respectively, followed by mounting with Ultracruz mounting medium, and analysed by using Nikon fluorescent microscope.

Results

Percentage yield and phytochemical analysis (Qualitative)

The hexane root (HR) and chloroform root (CR) extracts were found to be light yellow mass and brown sticky paste. It is estimated that yield of hexane and chloroform root extracts were found to be 1.0 and 1.5 % respectively. These extracts were subjected to preliminary qualitative phytochemical analysis. The major groups of phytochemical constituents present in

these extracts are alkaloids, flavonoids, terpenoids, carbohydrates, glycosides and amino acids.

Clonogenic assay

To test the effect of HR and CR extracts on the colony formation, A549 cells were treated with different concentrations of HR and CR (50, 100 and 200µg/mL) along with vehicle DMSO for 5 days at 37°C incubator in 5% CO_2. It was confirmed by colony forming assay that A549 cells are sensitive to these two samples depending on dose. At 50µg/mL of both HR and CR, the cells exhibited relatively lesser colonies compared to the control cells.

Consistent with the slow growth rate, it was observed that HR and CR extracts induced a dose-dependent decrease in the number of colonies at a concentration of 100µg/mL. Interestingly, at 200 µg/ml of HR and CR extracts treatment, the number of soft agar colonies was reduced by ~4-fold ($p \leq 0.05$) compared to the untreated control cells (Fig. 1A). The decrease in clonogenicity of A549 cells was found to be very significant by HR extract followed by CR extract. In this investigation, Staurosporine (20 nM) was used as a standard. The decrease in the colony formation ability of A549 cells after the treatment with HR, CR and standard in standard drug is found to be statistically significant ($p \leq 0.05$). The results have been graphically represented in Fig.1B.

Fig. 1A: Inhibition of colony forming ability of A549 cells by hexane root and chloroform root extracts of *C. infortunatum*.

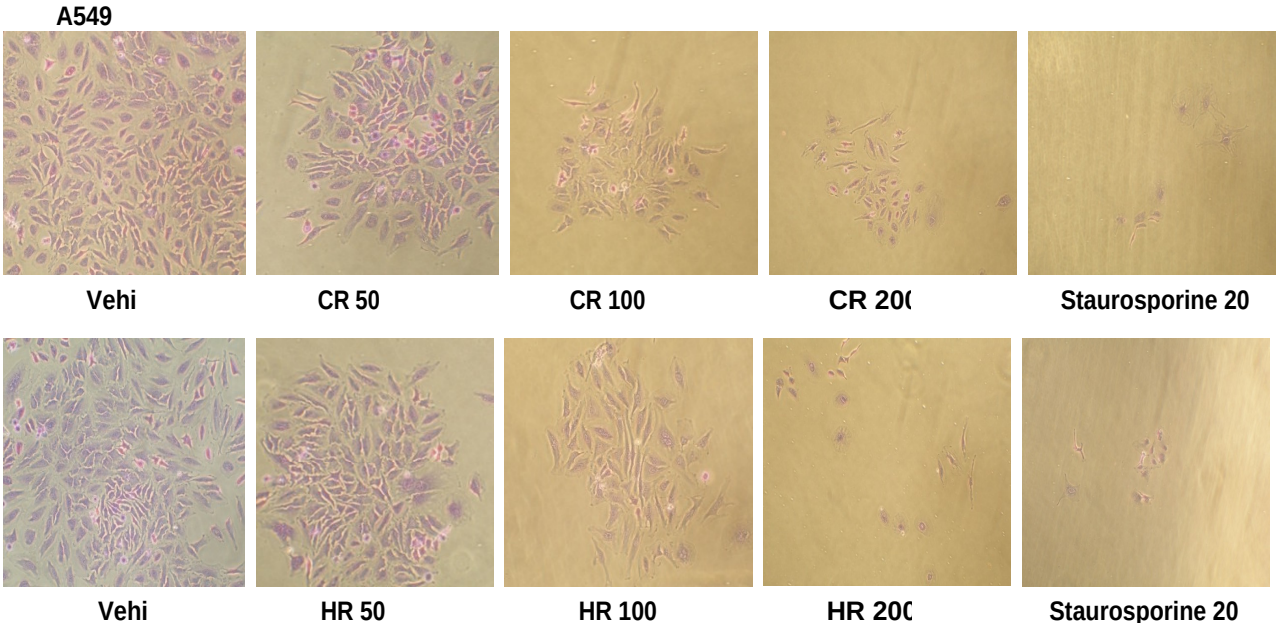
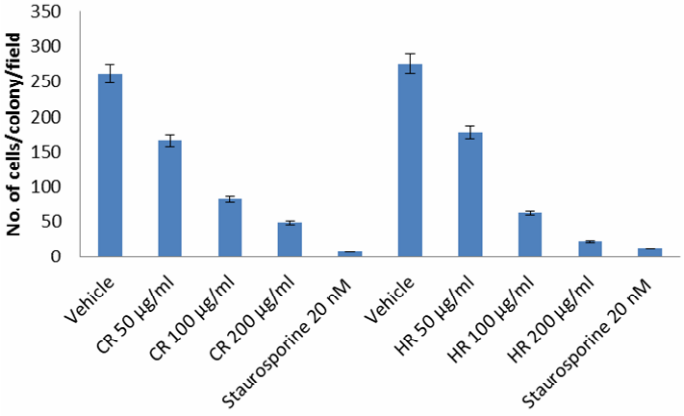


Fig. 1B. Decrease of cell colonies by hexane root and chloroform root extracts of *C. infortunatum* and standard drug in comparison with untreated control.



Scratch motility (wound healing) assay

To examine the effect of HR and CR on cell migration, it was intended to perform the wound healing assay on confluent monolayers of A549 cells to determine whether HR and CR extracts could inhibit motility of A549 cells. After incubation with different concentrations of HR and CR extracts for 24 h, it was observed that HR and CR effectively inhibited the migration of cells in a dose- and time-dependent manner compared to the untreated control cells (Fig. 2A).

The control cells filled-up the wound gap completely after 24 h whereas, the cells treated with HR and CR extract at 50 and 100 µg/ml concentrations, the wound gap was not completely filled. Interestingly, at 200

µg/ml, HR and CR extract treatment significantly decreased (~4-fold; $p \leq 0.01$) the migration rate of A549 cells thereby affecting their migration capability. Present investigation clearly suggests that HR and CR extracts significantly inhibited motility of A549 cells in statistically significant manner ($(p \leq 0.05)$ and are graphically represented in Fig. 1B.

Fig. 2A: HR and CR inhibits cell motility of A549 cells.

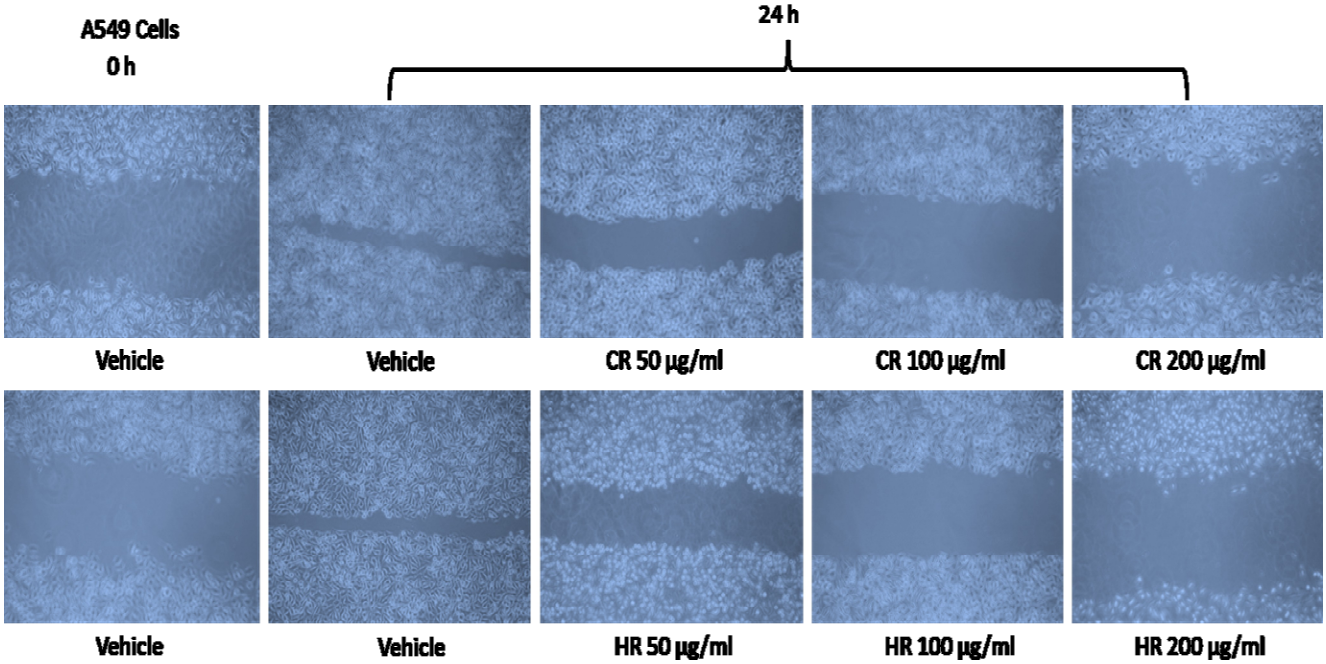
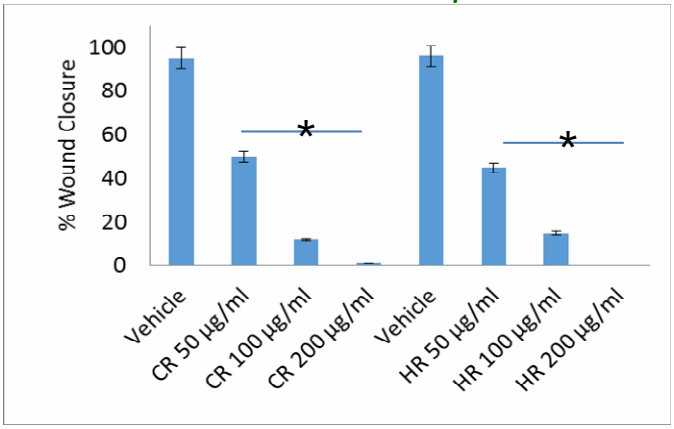


Fig. 2B: Percent wound closure of cells treated with hexane root and chloroform root extracts of *C. infortunatum.*



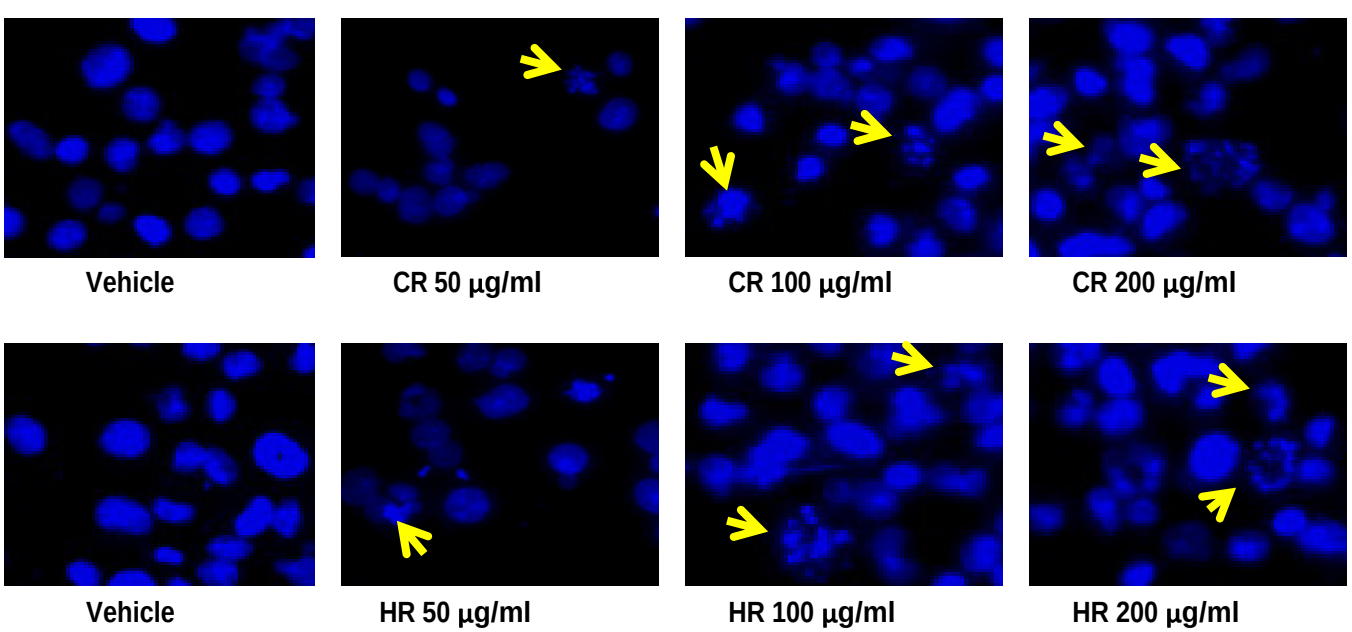
DAPI staining for nuclear morphology

DAPI staining for nuclear morphology was executed to evaluate the nuclear morphological variations on cells after treatment with HR and CR. It is evident from Fig. 3 that HR and CR extracts have direct influence on the nucleus of A549 cells. These extracts have malformed the nucleus of the cells in a dose-dependent manner compared to the untreated control cells. Interestingly, at a concentration of 200µg/mL, nuclei of the cells are considerably aborted with the treatment of HR and CR extracts. In Fig. 3 the yellow arrow clearly indicates apoptotic nuclei.

Discussion

The leading cause of death after the heart disease is because of cancer, and lung cancer is the predominant source of cancer related mortality. Development of therapeutic and preventive means of regulating this disease is obviously desired. Phytochemicals present in plant products play a vital role in cancer treatment and enormous number of these compounds has been perceived to act against tumours in experimental animals and demonstrated potential biological properties. A structural alteration of natural products characterizes a sophisticated method towards finding new biological activities. In the light of promising therapeutic prospective of *C. infortunatum* as biologically important medicinal plant (Bhaskar et al., 2014), different concentrations of hexane and chloroform root extracts have been employed to evaluate the colony formation of A549 cells, which demonstrated statistically significant reduction in the number of colonies compared to the untreated control cells in a dose dependent manner.

Fig. 3: Hexane root and chloroform root extracts of *C. infortunatum* are apoptosis inducing anti-proliferative agents.



The toxic effects of an anticancer agent in multiplying populations must be defined by its damage of the proliferative integrity of individual cells in cultured conditions. This impairment can be measured by the incompetence of cells to proliferate indefinitely and form colonies under the suitable experimental conditions (Puck et al., 1995). The surviving fraction of A549 cells declined in a dose-dependent manner with increasing concentrations of various HR and CR extracts. The most effective extract was the HR extract at the concentration of 200µg/mL followed by same concentration of CR extract. The effect of these extracts is almost similar to standard Staurosporine as far as cell killing is concerned. It can be stated monitoring of colony formation revealed that HR and CR were toxic at relatively low concentrations in A549 cell line.

It is well established that metastasis, being one of the major causes of mortality in cancer, involves a number of steps such as cancer cell adhesion, invasion, and migration (Liotta, 1986). Thus, to scrutinise the effect of HR and CR extract on migration of A549 cells, wound healing assays was performed on untreated control, HR and CR treated cells. Interestingly, HR and CR reduced the movement of cancer cells in a significant manner, indicating its potentiality for future use as an anti-cancer drug in lung cancer therapy. This suggests that HR and CR extracts might possess anti-metastatic potential. It is demonstrated that HR and CR suppressed migration of A549 cells expressively at the concentration of 200 µg/mL. Whereas, A549 cells when treated with HR and CR extracts (50 and 100 µg/mL), exhibited the inhibition of migration. These results implied that the inhibitory effects of these extracts on A549 cells cell migration may due to their cytotoxic effects. Further, these results are confirmed by nuclear morphology of A549 cells after staining the cells with DAPI. This observation suggests that the HR and CR extracts have direct impact on the nucleus of A549 cells. The plant extracts have deformed the nucleus of the cells in a dose-dependent manner compared to the untreated control cells. Interestingly,at a concentration of 200µg/mL, the extracts exerted their effects on the nuclei of the cells by considerably twisting them and thus altering the nuclear morphology which validates the effect of these extracts on the nucleus of the cancer cells.

Conclusion

In conclusion, the decrease in colony formation and wound closure of A549 cells by HR and CR extracts of *Clerodendrum infortunatum* which may contribute to the inhibition of migration in human lung cancer. These findings reveal the possession a new therapeutic potential in *C .infortunatum* as anti-metastatic therapy. Hence, *C. infortunatum* could be considered as favorable plant possessing anti-cancer properties.

Acknowledgement

The authors acknowledge the University Grants Commission for their financial support.

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