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Research Article

Investigation of phytoconstituents of *Cardiospermum halicacabum* and its efficacy as a potential anti-cancer drug candidate

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ABSTRACT

On the basis of the traditional knowledge of treating solid tumor using *Cardiospermum halicacabum* gained attention to carry out the present work. The preliminary screening for determination of cytotoxicity of successive extract of *C halicacabum* (hexane, chloroform, ethyl acetate and ethanol) was performed through brine shrimp lethality assay and yeast growth rate cytotoxicity assay. Among the extracts, chloroform extract of *C halicacabum* exhibited highest cytotoxicity which was further investigated for anti-proliferative and cytotoxicity property in A-549 lung cancer cell line. Correspondingly, dose dependent effects on reduction of cell proliferation with prominent morphological abnormalities of A-549 cells were observed under treatment with chloroform extract. Hemocompatibility assessment of chloroform extract, by hemolysis assay, revealed its compatibility towards RBC which in turn may prevent heamolytic anemia (myelosuppression) the most adverse effects of cancer chemotherapy. Phytoconstituents of chloroform extract responsible for the cytotoxicity, anti-proliferative and hemocompatibiltiy was assessed by several chromatographic methods such as TLC, Column chromatography and HPLC which revealed the presence of flavonoids in chloroform extract of *C halicacabum*. Apparently the isolated flavonoids, in consistence with chloroform extract, exhibited similar effect on inhibition of proliferation of tumor cell line A-549. Therefore it could be evidenced from the current study that the *C halicacabum*, with its predisposed flavonoids, possesses anti-cancer property with least adverse effect on heamolysis. However detailed investigation on regulation of tumor cell proliferation and hemocompatability is required to bring *C halicacabum* as a potential candidate for cancer therapy.

Keywords: *C halicacabum*, Anti-cancer, Toxicity, Brine Shrimp Lethality Assay, Hemolysis.

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1. INTRODUCTION

In recent years, the usage of herbal based medicines and health supplements has been increased tremendously. Evidences for alternative and herbal based therapy are increasing exponentially worldwide[1]. In the last few decades resurgence of interest on plant derived natural products which forms a source of several drugs is elevated. Prevalence of cancer is being increased globally. High failure rate in obtaining successful cancer drugs with least adverse effect make the case further worsen. Drugs of natural origin especially plant derivatives gain advantage and being approved with the increased success rate. Almost fifty percentage of the anti-cancer drug available are from natural origin and natural derivatives.

Vinblastine and Vincristine both isolated from *Catharanthus roseus*[2] for treatment of Hodgkins disease, lymphosarcoma, leukemia in children. Teniposide and Etoposide developed from the anti-neoplastic lignans podophyllotoxins, a constituent of Podophyllum species are being used to treat testicular cancer, small cell lung cancer and lymphomas. Paclitaxel, a diterpenoid constituent of several Taxus species[3] is effective in the treatment of metastatic breast cancer, ovarian cancer and has potential uses in the treatment of lung cancer, malignant melanoma. Irenotecan (analogue of Quinoline alkaloid camptothecin) first isolated from the *Camptotheca acuminate*[4] but now obtained mostly from the Indian *Mappia foetida* Miers[4] is being used in Japan for lung, ovarian and cervical cancer treatment.

The pursuit of "bioactivity-guided fractionation" for plant extracts, using *in vitro* cell-based cytotoxicity assays, has served as the mainstay of anticancer drug discovery study, and has produced considerably valuable compounds thus far. In this regard, the aerial parts of *Cardiospermum*

halicacabum a tropical climber growing wild in Indian subcontinent are used for the treatment of hardened tumors in the Ayurvedic medicines. Hence in this present study the anti cancer activity of the selected fractions of the aerial parts of *Cardiospermum halicacabum* was carried to prove the traditional medical claim.

Various extracts of *Cardiospermum halicacabum* have been reported to possess anti-plasmodial, anti-bacterial, anti-inflammatory, anti-oxidant, anti-hyperglycemic and nephroprotective properties. It is also used as one of the major ingredient in traditional herbal formulations and products. Several scientific evidences are available for medicinal property of *C halicacabum* on various diseases, however no evidences are available for its anti-cancer property.

In the present study various extracts of *Cardiospermum halicacabum* prepared under maceration technique with solvents of increasing polarity (hexane, chloroform, ethyl acetate, alcohol), were screened for its cytotoxic activity using brine shrimp lethality assay and yeast growth rate cytotoxicity assay. The efficacy of selected extract and its active fraction on inhibition of tumor cell was evaluated in lung cancer cell line (A-549 cells) followed by safety assessment by human RBCs-hemolytic assay.

2. MATERIALS AND METHODS

2.1. Collection of Specimen

The plant specimens for the proposed study were collected from Sri Ramachandra University Campus. Care was taken to select healthy plants for the study. The Authentication of the plant specimen was carried out using standard methods[5]. It was authenticated by Prof. P. Jayaraman Director, Plant Anatomy Research Centre, Tambaram, Chennai.

2.2. Preparation of Extracts

The aerial parts of *Cardiospermum halicacabum* was washed with water, cut into small pieces, shade dried and powdered coarsely. The powdered aerial parts of *Cardiospermum halicacabum* (2 kg) were extracted exhaustively using different solvents with increasing polarity: Hexane>Chloroform>Ethyl acetate>Alcohol in an aspirator bottle at room temperature for 72 hours followed by 48 and 24 hours. The filtrates were collected and distilled over boiling water bath under reduced pressure.

2.3. Brine Shrimp Lethality Assay

The brine shrimp lethality assay, proposed by Michael et al.,[6], is a useful tool for preliminary assessment of toxicity[7]. The procedure of the brine shrimp lethality assay is as follows. Brine shrimp (cysts) tin can stored at 4°c was brought to room temperature the previous day before use. 100mg of cysts were weighed, soaked in distilled water for half an hour and was transferred into a separating funnel containing sea water of p^{H} 8.2 and the specific gravity 1.03, prepared from 33.33g of red sea rock salt dissolved in one liter of autoclaved distilled water. Aeration was provided through an aerator (motor). Illumination was provided through a 100 watt bulb. After 12hr, cysts were hatched and the active nauplii were collected and used for the assay.

Ten nauplii were taken vial containing 4 ml of brine (salt) solution and 1ml of each 6 different concentrations of test extracts. The standard was added to 4ml of brine solution and maintained at room temperature for 48 hr under light and surviving larvae were counted. The live ones and the dead ones were differentiated by their relative motilities, the one with low or no movements were counted as dead and those of normal movement were considered alive. Experiments were conducted along with control and different concentrations of the test substance in a set of three tubes per dose. The LC_{50} values were obtained by a plot of percentage of dead shrimp nauplii against the concentrations of the extracts.

2.4. Hemolytic Assay

The hemolytic assay of the active components was performed using the standard method. The hemolytic activity of extracts was determined using erythrocytes isolated from human red blood cells. Blood (4ml) collected from healthy individual was centrifuged at 450g for 10minutes and washed with phosphate buffered saline (X3) of pH-7.4 followed by treatment with different concentrations of test samples (50 µg, 100 µg, 150 µg, 200 µg). Final volume 50 µl of various concentrations of test samples with 40 µl PBS/ 90 µl of water (positive control) / 90 µl of normal saline (negative control) were incubate with 10 µl of RBC suspension for 1hr at 37°C and centrifuged at 200g for 5 minutes. The absorbency was measured in an UV spectrometer at 545nm wavelength. The percentage hemolysis was calculated using the below mentioned equation.

$$\text{Percentage hemolysis} = \frac{\text{OD (test)} - \text{OD (negative)} \quad \times 100}{\text{OD (positive)} - \text{OD (negative)}}$$

2.4. Yeast Growth Rate Cytotoxicity Assay

Yeast was reactivated in YEPD medium (Yeast Extract Peptone Dextrose) by a pure-culture (lyophilizated) and maintained in a molasses medium supplemented with KH_2PO_4 (8.36 mM), $(NH_4)2SO_4$ (5 mM), urea (38.75 mM), $MgSO_4.H_2O$ (3.57 mM), $ZnSO_4.7H_2O$ (0.10 mM), $MnSO_4.H_2O$ (0.12 mM) and linolenic acid (0.11 mM). Cells from the late-exponential growth phase were harvested by centrifugation at 800 gravity for 20 minutes and resuspended in double distilled water at a final concentration of 1g per 100 ml.

Fermentation was carried out with 250 ml of YED sterilized (yeast extract 1% and dextrose 2%) medium in 500 ml Erlenmeyer flasks sealed with aluminum foil. Media was transferred into various boiling tubes with or without 50µM of cadmium concentration/ different concentrations (50 µg, 100 µg, 300 µg, 500 µg) extracts with 1mL of final volume. All the tubes were inoculated with 1 mL of 1% yeast suspension and incubated in orbital shaker (70 RPM) at 30°C for 6 hours. Trypan blue staining was performed with 500 µl of cell suspension from each tubes at various time intervals (0, 60, 120, 180, 270 and 330 minutes) during fermentation.

2.5. High Performance Thin Layer Chromatography (HPTLC)

The fingerprinting of chloroform, extract of C halicacabum has been done using a 10×10cm preactivated HPTLC silica gel 60F 254 plate. The chloroform extracts of *C halicacabum* have been prepared at a concentration of 1mg/ml in ethyl acetate and were spotted using CAMAG Linomat 5 applicator. The method was optimized using solvent ratio and developed in a twin trough chamber, 20 ×10 cm at 25^{0}c. The plates were dried by hair dryer. The developed plates were scanned using CAMAG TLC scanner 3 and photo-documented using CAMAG REPROSTAR 3.

2.6. High Performance Liquid Chromatography (HPLC)

Alcohol, water and hydrochloric acid at ratio of 50:20:8 and methanol, water and phosphoric acid at ratio of 100:100:1

was used as extraction solvent and mobile phase respectively.

Standard and test solution of 20 µl was separately injected into the chromatography. Chromatogram was recorded and the areas for the major peaks were measured. The percentage of each flavonoid in the sample was calculated and expressed as flavones glycosides.

$$\text{Percentage content} = \frac{2\times F_1\times m_1\times 2.514\times p}{F_2\times m_2}$$

2.7. Column Chromatography of Chloroform Extract of *C. halicacabum*

The *C. halicacabum* chloroform extract 10gm was applied to a silica gel column (60 – 120 mesh) packed in hexane and eluted with solvents of varying polarity of hexane, hexane: chloroform, chloroform, chloroform: ethyl acetate, ethyl acetate, ethyl acetate: methanol and then methanol. Similar fractions were pooled based on TLC profiles. Fractions eluted with ethyl acetate: methanol (50:50) yielded crystals after concentration. It was soluble in methanol, ethyl acetate, and chloroform and insoluble in ether and hexane. It gave a pink color with concentrated hydrochloric acid and magnesium ribbon indicating it as a flavanoid. TLC of the fraction in BAW (4:1:5) gave a neat single streak of 0.77R_f .Since the isolated fraction showed a band instead of a spot it was suspected to be a mixture of flavanoids and hence the fraction was subjected for λmax determination.

Three prominent peaks were obtained at 254nm, 268nm and 366nm respectively which were similar to that of the Luteolin. Theoretically it is given that Luteolin have peaks at 255nm, 268nm and 350nm respectively (Harborne – Phytochemical Methods). The percentage content of flavanoids, expressed as flavones glycosides, was calculated using the below equation

$$\text{Percentage Content}= \frac{2\times F_1\times m_1\times 2.514\times p}{F_2\times m_2}$$

2.8. Microscopic Observation of A-549 Cell Morphology

The morphological changes in the A-549 cells after treatment with *Cardiospermum halicacabum* crude extract and flavonoid fraction were observed under 10X magnification in inverted light microscope (Nikon).

2.9. Anti-Proliferative Activity on Lung Cancer Cell Line

The A-549 cells were procured from National Centre for Cell Science (NCCS), Pune, India. Cells were seeded in 96 well plates at a concentration of 5×10^4 cells per plate and incubated for a period of 24 hrs at 37°C in 5% CO_2 regulated incubator. The cell were serum starved for 1 hr and treated with different concentrations of chloroform extract of *Cardiospermum halicacabum* isolated fraction in the range of 7.5µg, 15 µg, 30 µg, 60 µg, 120 µg respectively for 24 hours. After incubation treatment medium was replaced with 200µl of DMEM containing 0.5 mg/ml of MTT and cells were allowed for 4 hours at 37°c in 5% CO_2. The MTT containing medium was removed and the formazan crystals formed in the cells were dissolved in 200µl of DMSO. Plate was left on a shaker for 5 minutes and read at 570nm in multiwell-plate-reader.

3. RESULTS AND DISCUSSION

The brine shrimp lethality assay was performed to assess the cytotoxicity and anti-tumor properties of *C. halicacabum*. Figure 1 represents degree of lethality of brine shrimp in proportionate to the concentration of the respective *C. halicacabum* extracts. Determination of LC_{50} value revealed that the chloroform extract of *C halicacabum* exhibited prominent activity with LC_{50} value of 27.5 µg/ml, whereas hexane, ethanol and ethylacetate extract showed LC50 value of 92.1 µg/ml, 183.2 µg/ml and 915.2 µg/ml respectively. The significance of lethality of *C. halicacabum* extract on brine shrimp indicated the presence of cytotoxic components, which may probably possess anti-cancer activity. The above statement was supported by the cytotoxicity excreted by naturally derived potent anti-cancer drug curcumin (LC50 8.9 µg/ml), which was used as a positive control in the experiment.

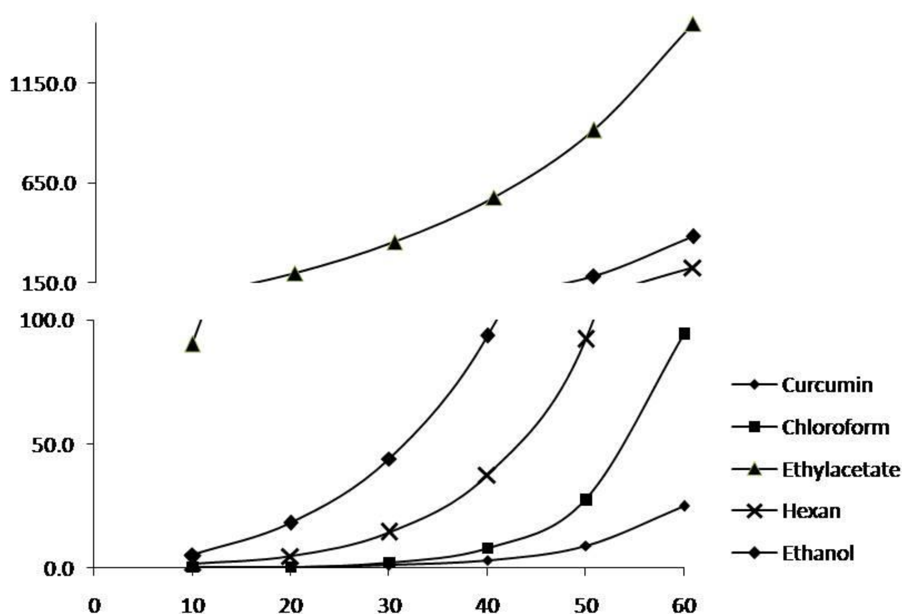

Figure 1: Brine Shrimp Lethality Assay. * p value < 0.05 compared with other extract of C halicacabum.

Further, the cytotoxicity of *C. halicacabum* extract was investigated in yeast system, *Saccharomyces cerevisiae* with cadmium as a positive control. Cadmium is an environmental toxicant, however its' complexes with supramolecules or other metals regulates proliferation and metastasis of several *in vitro* tumor cell lines and suppresses tumor growth in mouse xenograft models[8-10]. Yeast growth rate cytotoxicity assay represents lethality and decreased viability of *S. cerevisiae* mediated by cadmium or chloroform extract of *C. halicacabum*. Chloroform extract of *C. halicacabum*, as in close with cadmium, exhibited dose and time depended reduction in viability of *S. cerevisiae* under 50, 100, 300 or 500 µg/ml treatment concentrations for 1, 2, 3, or 4 hr.

The above mentioned preliminary cytotoxic evaluation of *C. halicacabum* extracts by brine shrimp lethality and yeast growth rate cytotoxicity assays represented the chloroform extracts to be effective among the other extracts (ethanol, hexane and ethyl acetate). The observation and significance of brine shrimp lethality and yeast growth rate cytotoxicity assays in screening anti-cancer drug was supported by the corresponding positive controls, curcumin and cadmium, which are established anti-cancer candidates.

The cytotoxic effect of chloroform extract of *C. halicacabum*, in concert with anti-cancer property, was validated in lung cancer cell line (A549 cells) though morphological analysis and MTT assay. Dose dependent morphological changes were observed under the treatment of 20, 50 and 100 µg/ml of chloroform extract. Morphological abnormalities mediated by chloroform extract were observed as more number of round cells with misshapen and shrunken cell membrane.

Correspondingly, proliferation of lung cancer line was also negatively affected by *C. halicacabum* chloroform extract with the increased rate of cell growth inhibition in dose dependent manner. Percentage of growth inhibition, calculated from MTT assay, indicated 16.3 to 63.0% reduced cell proliferation under the treatment concentration ranging from 7.5 to 120 µg/ml.

According to national cancer institute, anemia is one of the most common side effects experienced by almost many cancer patients undergoing chemotherapy (NIH-National Cancer Institute (https://www.cancer.gov/about-cancer/treatment/side-effects). It is necessary for any new anti cancer drug to possess lesser side effect. Therefore it was interested to assess the side effects of *C. halicacabum* chloroform extract especially on its' haemocompatible property. Because myelosuppression, a progressive hemolytic anemia (hemolysis of RBC), is the most common complication manifested in cancer patients undergoing chemotherapy[11, 12]. Even mild to moderate anemia associated with therapeutic interventions immensely affect the functionality and quality of life in cancer patients. Hence it was astounding to find the haemocompatible property of chloroform extract of *C. halicacabum*. As represented in the figure 2, chloroform extract of *C. halicacabum* did not exhibit notable hemolytic property even in the range of 50 to 200 µg/ml treatment concentrations. Thus, crude extract of *C. halicacabum* showed no hemolytic activity against human red blood cells which could be an added property for any anti-cancer drug candidate.

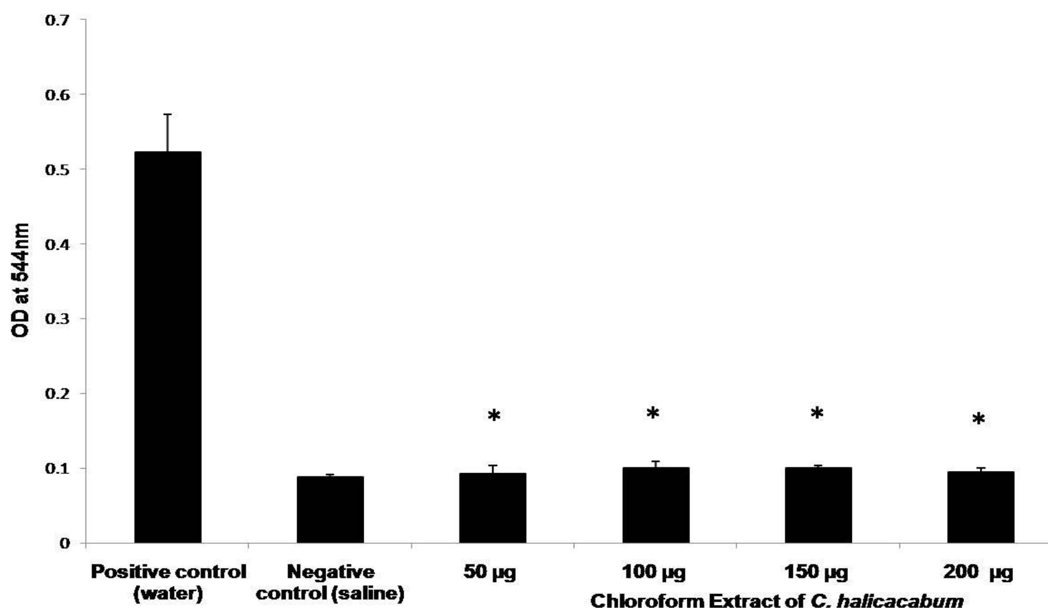

Figure 2: Hemolytic Assay: * p value ≤ 0.05 compared with negative control.

Phytochemical analyses were carried out to examine the chemical constituents of chloroform extract of C halicacabum using several chromatographic techniques. HPLC analysis revealed that the chloroform extract of *C. halicacabum* contains 13 compounds of which apigenin (4.12 mg), luteolin (0.871 mg) and chryseriol (0.112 mg) were estimated as 4.12 mg, 0.871 mg and 0.112 mg in 1g of chloroform extract. The flavonoid rich fraction of chloroform extract subjected to column chromatography for isolation of the favonoids.

The isolated fraction was then validated by its anti-proliferative property on A549 lung cancer cell line. However the isolated flavanoids exhibited lower anti-proliferative activity on A-549 lung cancer cell line when compared with the crude chloroform extract (Figure 4), which shows the influence of other non-flavanoids along

with flavanoids such as luteolin and apigenin. Luteolin and apigenin are potential anti-cancer agents[13,14] which possess hemocompatabile property of attenuating oxidative stress in erythrocytes[14,15]. Thus the current study provided the evidence for the presence of luteolin and apigenin along with non-flavonoids in chloroform extract of *C. halicacabum* which may be responsible for anti-cancer and haemoprotective activity of *C. halicacabum*.

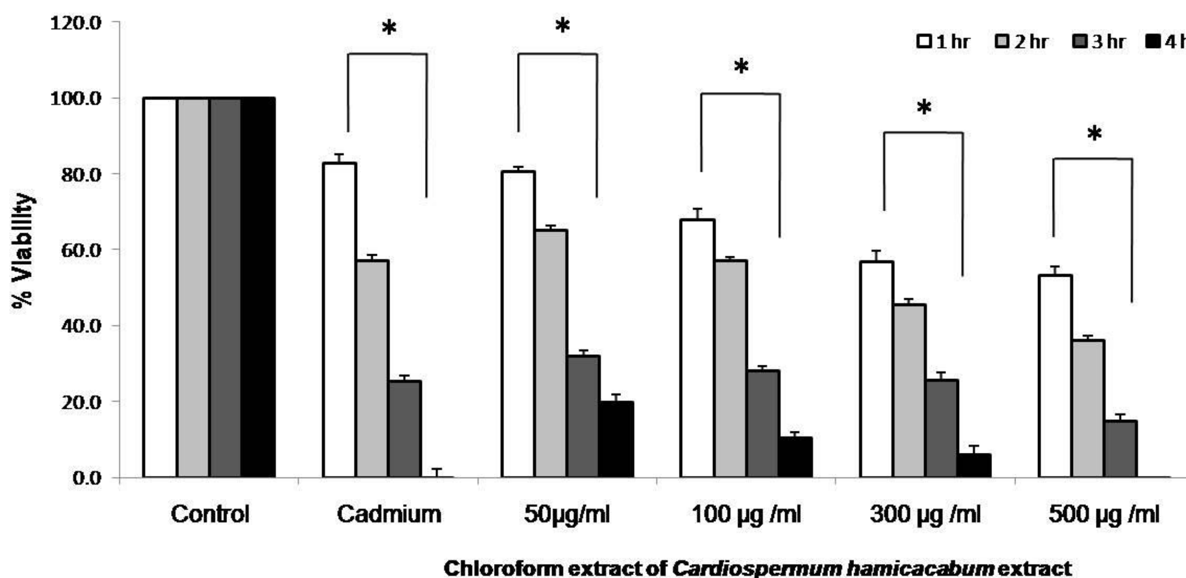

Figure 3: Yeast Growth Rate Cytotoxicity Assay: * p value ≤ 0.05 compared with control.

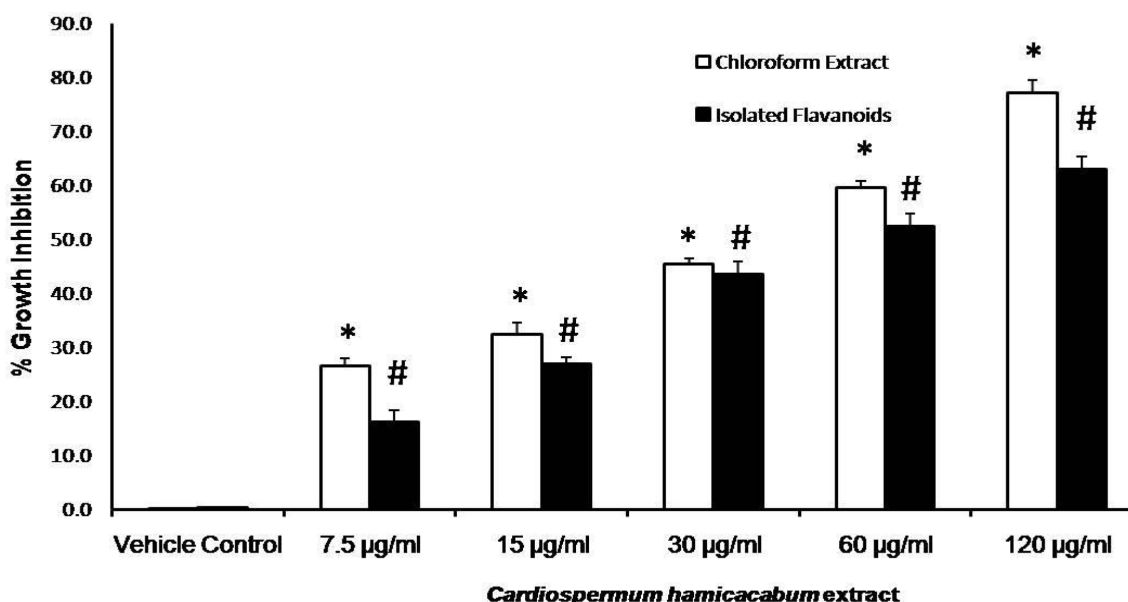

Figure 4: Anti-Proliferation Assay. * p value ≤ 0.05 compared with vehicle control. # p value ≤ 0.05 compared with chloroform extract.

CONCLUSIONS

The current study revisited the anti-cancer property of *C halicacabum* which was used traditionally for cancer therapy. Though detailed investigation is required to elucidate the exact mode of action of C halicacabum, as a preliminary observation, the results of the present study suggested that the chloroform extract of *C halicacabum* possessed cytotoxicity property on lung cancer cell line A549 due to the presence of non-flavonoids comppunds other than apigenin, luteolin and chryseriol. Further it can also be speculated from the results that the chloroform extract of *C halicacabum* may also be haemoprotective which is a substantial property for any anti-cancer drug to be considered for cancer chemotherapy.

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