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Anticancer activity of *Callicarpa arborea* Roxb. extracts against Type-II human lung adenocarcinoma cell line, A549

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Abstract

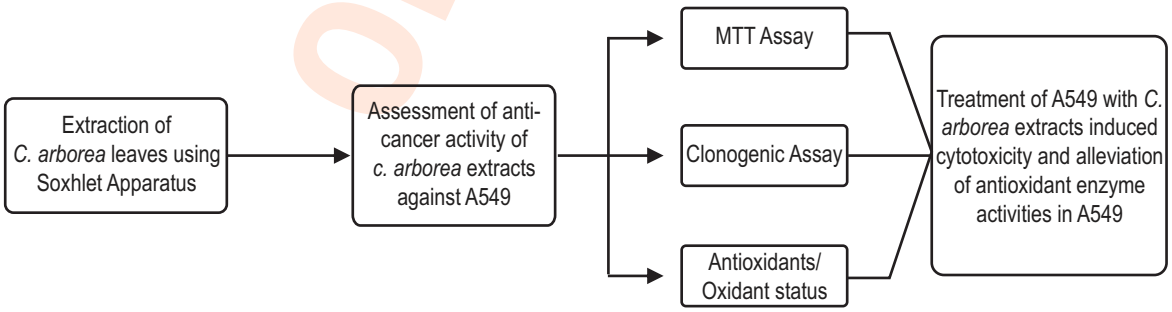
Aim: The study aims to evaluate the anticancer activity of *Callicarpa arborea* in A549 cancer cells.

Methodology: Fresh non-infected leaves of *Callicarpa arborea* were collected from Serkawn, Lunglei District, Mizoram and various solvents were used for Soxhlet extraction at their respective boiling points. The extracts were concentrated and the anticancer activity was tested in the human lung cancer cell line A549 using MTT and clonogenic assays. The effect of *C. arborea* on the antioxidant system was also assessed by measuring the levels of glutathione, glutathione s-transferases, superoxide dismutase as well as lipid peroxidation levels following standard protocols.

Results: Among the various solvent extracts of *C. arborea*, only chloroform extract showed significant cytotoxicity, and inhibited cell proliferation and survival against the A549 cancer cells. The chloroform extract of *C. arborea* induced cell death in A549 cells in a dose and time dependent manner with an IC_{50} of 52.8 μgml^{-1} and 20.4 μgml^{-1} at 24 hr and 48 hr, respectively. The clonogenic assay showed that the chloroform extract was able to inhibit cell proliferation in the A549 cells and the inhibition increased with increase in dose. The chloroform extract also alleviated the levels and activities of antioxidants glutathione, glutathione-s-transferase and superoxide dismutase, while elevating the lipid peroxidation level in the A549 cells.

Interpretation: The study shows that *Callicarpa arborea* possess both cytotoxic and anti-proliferative properties against the human lung cancer cell line A549. *Callicarpa arborea* is a potential candidate as a new anti-cancer agent and warrants further investigation.

Keywords: Anticancer agent, Antioxidants, *Callicarpa arborea*, Cell proliferation, Cytotoxicity



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Introduction

Cancer is a group of diseases characterized by uncontrolled proliferation of cells with the ability to invade or spread to other cells of the body (NCI, 2007) and it is the second leading cause of death globally next to cardiovascular diseases (Jemal *et al.,* 2007). According to the Global Cancer Statistics 2018, there were 2.1 million new cases and 1.8 million deaths due to lung cancer, representing 18.4% of all cancer deaths. In 2018, 67,795 new cases have been reported in India of which only 6.4% survived (Bray *et al*., 2018).

Despite the availability of certain cancer treatment modalities including chemotherapy, radiotherapy and surgical interventions, the mortality due to cancer does not seem to decline (Siegel *et al.,* 2015). Due to toxic side effects of chemotherapeutic drugs, exploration of plants with potential anticancer property is in high demand. Plants and natural products remain a prominent source of anticancer agents due to their safety, efficacy and lesser side effects (Thillaivanan and Samraj, 2014) and about 80% of individuals in the developing countries still depend on plants to treat different diseases (Kim and Kang, 2005). Plant-derived natural products such as flavonoids, terpenoids and alkaloids have received considerable attention in recent years due to their diverse pharmacological properties including cytotoxicity and anticancer effects (Osawa *et al.,* 1990). There is an increasing evidence for the potential of plant-derived compounds as inhibitors of various stages of tumorigenesis and associated inflammatory processes, indicating the importance of plant products in cancer prevention and therapy.

A549 cells are human alveolar basal squamous epithelial cells adenocarcinoma obtained from a 58-year-old Caucasian male (Giard, 1973) that grows adherently as a monolayer *in-vitro*. The cell line is hypotriploid with a modal chromosome number of 66, which occurs in 24% of cells. Naturally, these cells are responsible for the diffusion of substances such as water and electrolytes across the alveoli of lungs (Cooper *et al.,* 2016). A549 cells have been commonly used for the screening of anticancer properties of various plant extract *in-vitro* (An *et el.,* 2014; Venugopal *et al.*, 2017; Kumar *et al.,* 2017).

Callicarpa arborea Roxb. (Beautyberry) locally called as 'Hnahkiah' is a small evergreen tree belongs to family Verbanaceae and is widely distributed in the Asia-Pacific regions (Shihan *et al*., 2015). The leaves and bark of *C. arborea* have been extensively used for the treatment of inflammation (Mi *et al*., 1984), diabetes (Junejo *et al*., 2017) and intestinal disorder (Chen *et al*., 1986). The local people of Mizoram use the leaves and bark of *C. arborea* as haemostatic agent and for the treatment of abdominal colic (Sharma *et al*., 2016) as well as in cancer treatment (Lalfakzuala, 2007). Despite the traditional use of *C. arborea* as medicines, there has been no scientific validation on its potential activity against cancer. Therefore, the present study aims to evaluate the *in-vitro* anticancer activity of *C. arborea* in type II human lung adenocarcinoma (A549).

Materials and Methods

Chemicals used : Nitrobluetetrazolium (NBT), 5,5'dithio-2-nitrobenzoic acid (DTNB), reduced glutathione (GSH),1-chloro-2,4-dinitronbezene (CDNB), dimethyl sulphoxide (DMSO), ethylenediamine tetra-acetic acid (EDTA), bovine serum albumin (BSA), sodium pyruvate and thiobarbituric acid (TBA) were obtained from Sigma Chemical Co., Bangalore, India. Sodium bicarbonate (Na_2HCO_3), trichloroacetic acid (TCA), potassium chloride (KCl), potassium sodium-tartrate, hydrogen peroxide (H_2O_2), and Folin-Ciocalteu reagent were procured from SD Fine Chemicals, Mumbai, India. Trypsin EDTA, Eagle's Minimal Essential Medium (MEM), 3-(4,5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), Fetal Bovine Serum (FBS), and reduced nicotinamide adenine dinucleotide (NADH) were purchased from HiMedia, Mumbai, India. Doxorubicin (Getwell Oncology Pvt., Ltd., Haryana, India) was purchased from local pharmacy.

Collection of plant and preparation of extracts: The non-infected leaves of *Callicarpa arborea* were collected from Serkawn, Lunglei District, Mizoram. Identification and authentication of plant was done by the Department of Horticulture, Aromatic and Medicinal Plants, Mizoram University, Aizawl (voucher sample: MZU/HAMP/2019/023). The leaves of *C. arborea* were cleaned, shade dried at room temperature and grinded. The pulverized leaves were then sequentially extracted with petroleum ether, chloroform, methanol and distil water according to the increasing polarity using Soxhlet apparatus at their respective boiling points for a minimum of 40 cycles each. The liquid extracts were then filtered and evaporated in a hot air oven to obtain crude residue.

Cell culture: Type II human lung adenocarcinoma (A549) was obtained from the National Centre for Cell Science (NCCS), Pune, India. The cells were then cultured in MEM supplemented with 10% FBS, 1% L-glutamine and 50 μgml^{-1} gentamicin sulfate with loosened caps at 37 °C in an incubator containing 5% CO_2 (Eppendorf AG, Hamburg, Germany).

MTT assay: The cytotoxic effect of *C. arborea* was estimated by 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) reduction assay (Mossman, 1983). Briefly, 1 x 10^4 cancer cells were seeded in 96-well plate containing 100 µl MEM per well. The cells were allowed to attach for 24 hr and treated with different concentrations of various solvent extracts of *C. arborea* (20, 40 and 60 μgml^{-1}) or doxorubicin (50 μgml^{-1}; that serves as a positive control) for 24 hr and 48 hr. Untreated cells with either *C. arborea extract* or doxorubicin were used as negative control. After treatment with *C. arborea* extracts, 20 µl of MTT (5 $mgml^{-1}$)

was added to each wells and incubated for 3 hr. The drug-containing media were removed and cells were washed with FBS free media. Then the formazan crystals were dissolved in 200 µl of DMSO followed by incubation for 15 min after which the absorbance was measured at 560 nm using microplate reader (Spectramax m2e, Molecular Devices). Cytotoxicity was expressed as inhibition (%) which was calculated by the formula given below:

% inhibition = Control-Treatment/Control X 100.

Treatment of A549 cells with methanolic and aqueous extracts of *C. arborea* did not show statistically significant cytotoxicity, thus, only the chloroform extract was used for further estimation of anticancer activity.

Clonogenic assay: The effects of *C. arborea* extracts on the reproductive integrity of A549 cells was assessed using clonogenic assay (Franken *et al*., 2006). Briefly, 200 cells were seeded into several individual Petri dish containing 5 ml of media with different concentration of chloroform extract of *C. arborea* (20, 40 and 60 µgml^{-1}) or doxorubicin (50 µgml^{-1}). Cells were then allowed to grow for another 11 days. After removing the drug-containing media, the resultant colonies were then stained with 1% crystal violet in methanol and scored. Plating efficiency (PE) of the cells was determined and surviving fraction (SF) was calculated by the following formula:

PE = (Number of colonies countedx100) / (Number of cells seeded)

SF = (Number of colonies counted) / (Number of cells seeded) x (mean plating efficiency).

Antioxidants/Oxidant assays: For the estimation of antioxidant enzymes activities and lipid peroxidation level, 0.5 X 10^6 A549 cells were seeded in T-25 flasks containing 5 ml media. The cells were treated with different concentration of chloroform extract of *C. arborea* (20, 40 and 60 µgml^{-1}) or doxorubicin (50 µgml^{-1}) for 24 hr. After treatment, the drug-containing media were discarded and the cells were washed with sterile 1X PBS (phosphate buffered saline) and harvested with 1X trypsin EDTA. The cancer cells were pelleted, sonicated (PCI Analytics Pvt. Ltd., Mumbai, India) and 5% homogenate was prepared using cold sterile PBS (pH 7.4) and used for the biochemical estimations. Total protein contents were determined by the method of Lowry *et al*. (1951) using bovine serum albumin as standard.

Glutathione (GSH): The concentration of GSH was measured by its reaction with DTNB (Ellman's reaction) to give a compound that absorbs light at 412 nm (Moron *et al*., 1979). Briefly, 1.8 ml of 0.02 M Na_2HPO_4 (pH 8.0) and 40 µl of 10 mM DTNB were mixed with 160 µl of cell homogenate and incubated for 2 min at room temperature. The absorbance of the sample was read against blank, which consisted of distil water instead of cell homogenate, at 412 nm on a UV-Visible spectrophotometer (SW 3.5.1.0. Biospectrometer, Eppendorf India Ltd., Chennai), and the concentration of GSH was calculated from the standard curve and expressed in µmolmg^{-1} of total protein.

Glutathione-s-transferase (GST): The activity of GST was measured following the method of Beutler (1984). Briefly, 850 µl of phosphate buffer (pH 6.5) was mixed with 50 µl of 20 mM CDNB, incubated for 10 min at 37ÚC followed by the addition of 50 µl each of cell homogenate and 20 mM GSH. The blank consisted of all the reagents and distil water instead of cell homogenate. The absorbance of blank and sample was measured at 360 nm and the enzyme activity was expressed in units mg^{-1} of total protein.

Superoxide dismutase (SOD): The SOD activity was estimated by the method of Fried (1975). Briefly, 100 µl each of cell homogenate and 186 µM PMS were mixed with 300 µl of 3.0 mM NBT and 200 µl of 780 µM NADH. The mixture was incubated for 90 sec at 30°C and 1 ml of acetic acid and 4 ml of n-butanol were added to stop the reaction. The blank consisted of all the reagents, and distil water was added instead of cell homogenate. The absorbance of sample and blank was measured at 560 nm, and the enzyme activity was expressed in units (1U = 50% inhibition of NBT reduction) mg^{-1} protein.

% inhibition = (OD of blank – OD of test/ OD of blank) × 100

SOD unit = 1/50 × % inhibition.

Lipid peroxidation (LPO): Malondialdehyde (MDA) formed by the breakdown of polyunsaturated fatty acids, serve as a convenient index for determining the extent of peroxidation reaction of lipids. MDA has been identified as the product of lipid peroxidation (LPO) that reacts with TBA to give a red product. LPO was estimated by the method of Beuege and Aust (1978). Briefly, cell homogenate was added to a mixture containing 10% TCA, 0.8% TBA and 0.025N HCl in 1:2 ratio. The mixture was boiled for 10 min in a boiling water bath. After centrifugation, the supernatant was collected and its absorbance was read at 535 nm against the blank. The blank contained all the reagents and distil water was added instead of cell homogenate. The malondialdehyde (MDA) concentration of the sample was calculated using the extinction coefficient of 1.56 X 10^{-6} M^{-1} cm^{-1}.

Statistical analyses: Data are expressed as mean ± standard error. One-way analysis of variance (ANOVA) was performed to test significant variations on the cytotoxic effects and antioxidants/oxidant status of treatment groups followed by Tukey's multiple comparison of means using SPSS ver.20.0 software (SPSS Inc, Chicago, Illinois, USA). The IC_{50} was also calculated using Graph prism pad software ver. 6.0. Two-way ANOVA was also performed to assess the interacting effects of treatment duration and doses. A p-value <0.05 was considered statistically significant.

Results and Discussion

Effects of *C. arborea* extracts on cytotoxicity and clonogenicity of A549: To determine the effects of *C. arborea* on cell cytotoxicity, A549 cells were treated with different doses of *C. arborea* extracts for 24 hr and 48 hr. The amount of cell death was assessed by the MTT assay. Different solvent extracts of *C. arborea* – chloroform, methanol and aqueous were used to treat the cells. The results showed that only chloroform extract could significantly reduce the cell viability in a dose and time dependent manner. Therefore, in the subsequent experiments only chloroform extract of *C. arborea* was used and referred to as CACE. The cytotoxic effect of *C. arborea* was expressed as % inhibition of A549 cells and was plotted against log-doses for calculating IC_{50}. Treatment of cells with CACE showed a dose and time-dependent increase in cytotoxicity (Fig. 1A, B). The IC_{50} of CACE against A549 cells after 24 hr and 48 hr treatment were found to be 52.8 µgml^{-1} and 20.4 µgml^{-1}, respectively. Although prolonged treatment up to 72 hr was not performed, the IC_{50} at 24 hr and 48 hr showed the potency of CACE indicative of the potential use of plant extract as a suitable source of anticancer agent. According to the standard set by the U.S. National Cancer Institute (NCI), the cytotoxicity measure for potential anticancer agent must be an IC_{50} <20 ugml^{-1} (Vijayarathna and Sasidharan, 2012). Comparable effect of CACE with that of the standard chemotherapy drug doxorubicin at 50 µgml^{-1} was obtained when treating the cells with 60.0 µgml^{-1} of CACE for 24 hr and 48 hr (Fig. 1A, B). For each dose of CACE (20, 40 and 60 µgml^{-1}), longer duration of treatment resulted in increased toxicity (Fig. 2). The interacting effects of treatment duration and doses showed significant change in the inhibition (%) of A549 cells (Fig. 3). Treatment of A549 cells with methanolic and aqueous extracts of *C. arborea* did not induce significant ($p > 0.05$) toxicity, therefore IC_{50} against A549 could not be determined. Also, treatment duration, doses of treatment and their interacting effects showed insignificant changes in the inhibition (%) of A549 cells, indicating that only chloroform extract showed potential anticancer activity.

Inhibition of cancer growth has been a continuous effort for cancer treatment and reduction in cell growth and an induction in cell death are two major means to inhibit tumor growth (Huang *et al.,* 2003). According to the present cytotoxicity studies, CACE potentially worked as an anticancer agent. It is thus important to understand whether the cell death induced by CACE is via targeting the cell growth and cell division, as most anticancer drugs work by inhibiting cellular growth, thus affecting cell proliferation. Therefore, to determine the effect of CACE on cell growth and division, low concentration of A549 cells plated on

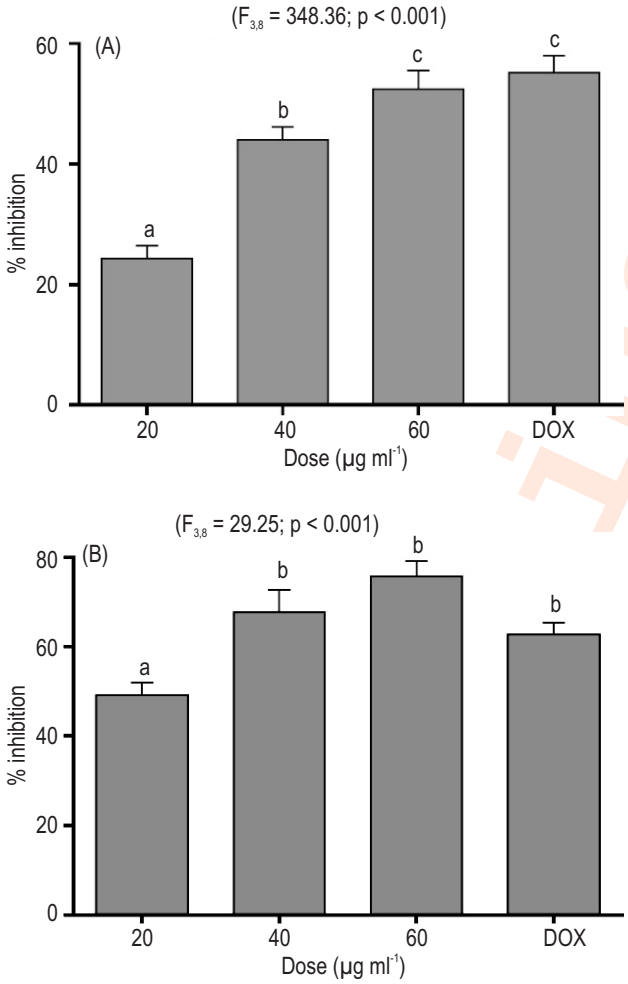

Fig. 1 : Cytotoxic effects of different concentration of chloroform extract of *C. arborea* (CACE; 20, 40 and 60 µg ml^{-1}) and doxorubicin (DOX; 50 µg ml^{-1}) on A549 cells for 24 hr (A) and 48 hr (B) treatment. Values are expressed as mean of three replicates ±SE. Different letters indicate significant variation.

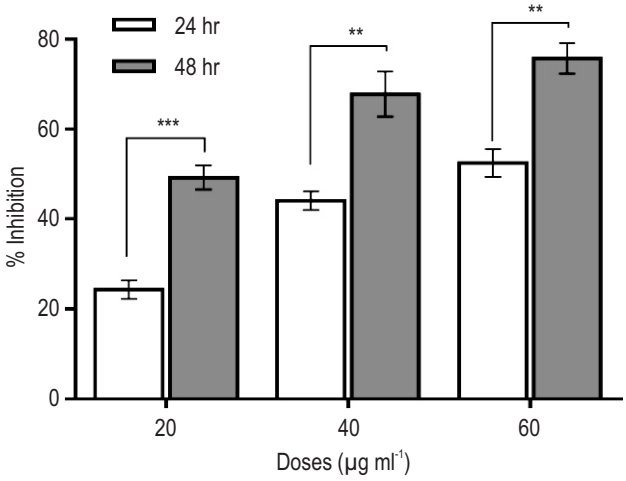

Fig. 2 : Effects of treatment duration at different concentration of chloroform extracts of *C. arborea* (CACE; 20, 40 and 60 µg ml^{-1}) on A549 cells. Values are expressed as mean of three replicates ±SE.** and *** indicate significant variation at $p < 0.01$ and $p < 0.001$ respectively.

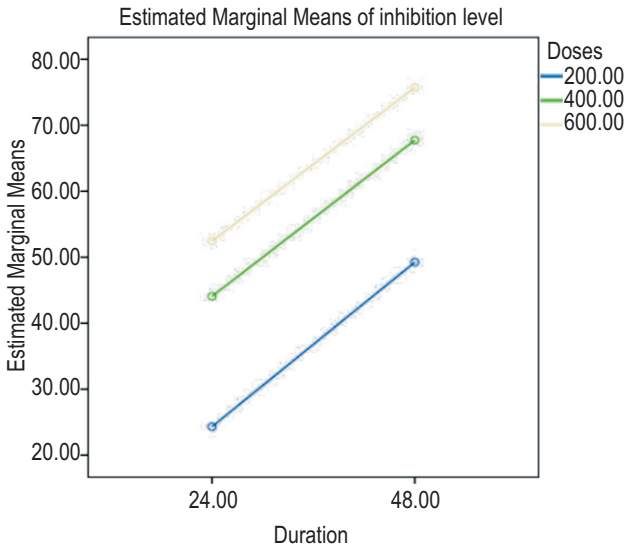

Fig. 3 : Interacting effects of treatment duration and doses of chloroform extract of *C. arborea* (CACE) in the inhibition (%) of A549 cells.

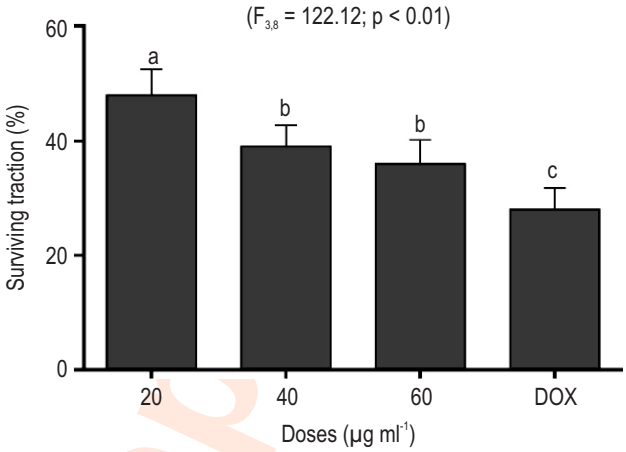

Fig. 4 : Effect of different concentration of chloroform extract of *C. arborea* (CACE, 20, 40 and 60 µg ml^{-1}) and doxorubicin (DOX; 50 µg ml^{-1}) on the reproductive integrity of A549 cells after 24 hr treatment. Values are expressed as mean of three replicates ±SE. Different letters indicate significant variation.

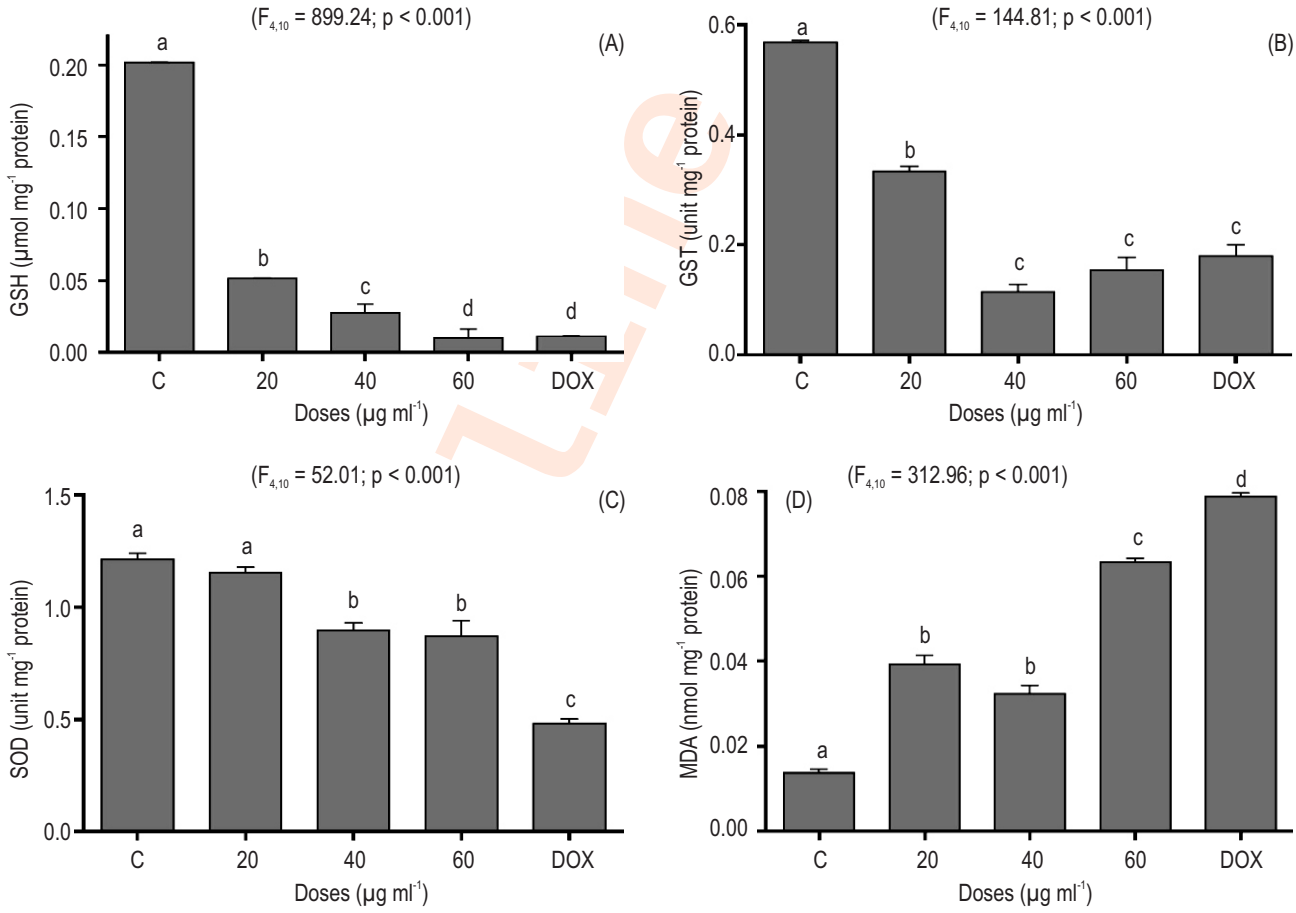

Fig. 5 : Effect of different concentration of chloroform extract of *C. arborea* (CACE, 20, 40 and 60 µg ml^{-1}) and doxorubicin (DOX; 50 µg ml^{-1}) on: (A) glutathione (GSH) level; (B) glutathione-s-transferase (GST) activity; (C) superoxide dismutase (SOD) activity; (D) malondialdehyde (MDA) level to assess lipid peroxidation. C-untreated control group. Means not sharing same letter are significantly different.

Petri dishes were treated with different doses of CACE and incubated for 11 days. Only single cells that were resilient enough to divide and form colonies survived the treatment were assessed. We found that CACE treatment effectively reduced the clonogenicity of A549 cells when compared with untreated control. The positive control doxorubicin (50 μgml^{-1}) treated cells showed higher efficacy than CACE. However, no significant difference was observed between 40 μgml^{-1} and 60 μgml^{-1} treatment groups (Fig.4). The results of this study suggest that reduction in cell viability caused by CACE exposure could be a result of its effect in reducing cell proliferation.

Effects of *C. arborea* extracts on antioxidants/oxidant status of A549: High levels of reactive oxygen species (ROS) were observed in most cancer cells. ROS in cancer cells are responsible for cancer cell dissemination and promoting disease progression by altering important cellular signaling pathways (Tochhawng *et al*., 2012). Interestingly, elevated levels of ROS in cancer cells also leads to cell death. Oxidative stress induced by many anticancer drugs including doxorubicin, paclitaxel, vinblastine etc., is thus an important strategy to induce cancer cell death (Yokoyama *et al.,* 2017). Induction of increased ROS levels in cancer cells by anticancer agents could be a result of interference in the antioxidant system. In order to assess whether *C. arborea* alter the antioxidant levels in A549 cells, cells were treated with different concentrations of CACE and the levels of GSH, GST and SOD were assessed. Glutathione (GSH) content of A549 cells in each treatment group was significantly ($p < 0.001$) lower than the untreated control. GSH level in A549 decreased with the increase in the concentrations of CACE (Fig. 5 A). Treatment of A549 with CACE at 60 μgml^{-1} reduced the GSH content by 3.2 folds when compared to control and showed similar effect with the standard doxorubicin (50 μgml^{-1}). A549 cells treated with different concentrations of CACE for 24 hr also resulted in significant reduction of GST and SOD activities when compared to untreated control ($p < 0.001$) (Fig. 5 B, C). CACE (40 $\mu gml^{-1}$and 60 μgml^{-1}) showed similar efficacy with the standard doxorubicin (50 μgml^{-1}) on GST activity (Fig. 5 B). However, CACE at 20 μgml^{-1} did not induce significant change ($p > 0.05$) in SOD activity when compared to untreated control (Fig. 5 C). In an effort to investigate whether CACE treatment induces rise in intracellular oxidant level, the level of lipid peroxidation (LPO) as a biomarker of oxidative stress was assessed. Treatment of A549 cells with different concentrations of CACE resulted in elevation of lipid peroxidation (Fig. 5 D). CACE at 60 μgml^{-1} enhanced MDA content 4.5 times in A549 cells when compared to control. Our study shows that *C. arborea* may lead to an increase in the intracellular ROS levels via disrupting the antioxidant system of A549 cells. It further implicates that cell death induced by CACE may be linked to downregulation of antioxidant activities. Plants such as *Momordica charantia* (Thiagarajan *et al*., 2019), *Rhynchosia rufescens* (Khader *et al*., 2019), *Malva pseudolavatera* (Khoury *et al*., 2020) have been reported to induced cytotoxic effects in cancer cells by increasing ROS production. Bioactive compounds such as phillygenin and plumbagin which are isolated from plants also induced cell death in cancer cells through the generation of intracellular ROS, resulting in apoptosis via a p53 dependent pathway (De *et al*., 2019; He *et al*., 2019). Understanding the nature of cell death induced by CACE and the mechanisms involved would be the next important step. The observations made from this study clearly suggests the potency of *C. arborea* as an anticancer agent and further analysis to determine the active ingredients preserved in the chloroform extract of *C. arborea* will provide more insight to the chemotherapeutic potential of this plant.

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