

Evaluation of *Clerodendrum inerme* (L.) Gaertn. on Burkitt's Lymphoma Cancer

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ABSTRACT

According to current census every year six million deaths occur in the world due to cancer. Hence, there is need of discovery and development of novel, safe and efficacious drugs for the treatment of various cancer. In the present study, an attempt has been made to explore the medicinal aspects of *Clerodendrum inerme* (L.) Gaertn.. Verbenaceae plant involving cytotoxic and antiproliferative potential as hydroalcoholic extract using methanol and water (70:30 V/V). Preliminary phytochemical investigation confirms the presence of the moiety in hydro-alcoholic extract and cytotoxic analysis using brine shrimp lethality assay, *Daudi* cell line culture, dye exclusion assay, MTT assay and animal model. The extract showed cytotoxicity below 100 ppm and LD₅₀ at 30 ppm level on brine shrimp lethality assay and on being subjected to MTT assay. A decrease in cell viability was observed at 213 µg/ml concentration and showing antitumor efficacy against Burkitt's lymphoma cells as well tumor model in female mice which showing the increased life span, as compared to control group, when treated with *C. inerme* (200 and 400 mg/kg body weight) and doxorubicin (2.5 mg/kg body weight) exhibited significant effect on tumor parameters. Hence it can be stated that *C. inerme* has potent antitumor properties.

Keywords: *Clerodendrum inerme*, Cytotoxicity, Antiproliferative, Lethality, MTT, *Daudi*

INTRODUCTION

Cancer is the most common cause of death of millions across the world. WHO statistics demonstrates the same as well since control and recurrence of cancer goes parallel, other contributions factors such as age, family history and also genetic abnormalities predominates.¹ Around 80% of the world's population depends upon traditional medicine system and primary health centers in a typical Indian scenario. Medicinal plants have the potent activity towards to maintain the healthcare.² In Ayurveda, the Indian medicine system is based on plant-based medicines. It played a pivot like role because most drugs are plant based. Camptothecin, Etoposide, Taxanes, Vincristine are plant derived compounds which have potent anti-

cancer activity.³ Conventional and traditional medicines also widely used as complementary or alternative therapy. Conventional therapies were like surgical removal, chemotherapy, and radiotherapy but sometimes these therapies are inadequate to cure the disease, so new drug discovery and development are in need to provide another alternative to provide better help to the community.⁴ Various plants are used in the various system of medicine like Ayurveda, Siddha, Unani and Homeopathy to treat cancer. The traditional system of medicine specially herbal medicine in India is directly linked to it's rich floral diversity, the Western Ghats of India is one such high biocultural diversity region, which is one of

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the global biodiversity hotspots. Medicine in contemporary India is a fascinating blend of a traditional system with conventional one and often been used for various historical, cultural and ecological and socioeconomically reasons. Even through the rate of medicinal plant utility is even increasing very little, is known about its, use patterns. It is very important to document, analyze and evaluate their knowledge not only for their cultural reasons but also for their commercial value, as ethnomedicinal uses of plants are one of the most essential critical used by the pharmaceutical industry in finding the new therapeutic agents.⁵ *Clerodendrum inerme* plant belongs to family Verbenaceae, and plants belong to this family plants have various useful ethnomedicinal properties.⁶ This plant is used as an ethnomedicine to treat cough, scrofulous infection, buboes problem, venereal infection, skin diseases, vermifuge and in beriberi.⁷ *Clerodendrum inerme* plant containing some important phytoconstituents viz. Pentadecanoic acid- β -D-glycoside, Stigmasterol, 4 α -methyl-24 β -ethyl-5 α -cholesta-14, 25-dien-3 β -ol, 24 β -ethylcholesta-5, 9(11), 22-trien-3 β -ol, Betulinic acid.^{8,9} These constituents have potential effects towards cancer cure.¹⁰ Dalton's ascitic lymphoma (DAL) is a very progressive cancer, which increases the lymphatic cells in the peritoneal cavity. However no proper systematic study was been evaluated for the lymphatic cancer activity using Burkitt's lymphoma (DAL) cells and in animal experimentation, comparison was made between effect of plant extract and standard drug doxorubicin on tumor parameters using Swiss mice. The present study is based on the ethnomedicinal claim of various species of *Clerodendrum* used in traditional system of medicine by various tribes. Hence the scientific validation of these claims with various evaluation parameters for anticancer potential has to be explored.

MATERIALS AND METHODS

Collection of Plant Material and Extract Preparation

Plant material *Clerodendrum inerme* aerial parts were identified and collected from Dandeli-Anshi forest, North Karnataka region in the month of January, and authenticated by comparing with earlier collection by taxonomist Dr. Harsha V. Hegde, Scientist -D Regional Medical Research Centre, (ICMR-RMRC), Belagavi, Karnataka with herbarium specimen no RMRC-1272. Shed dried plant material was crushed and subjected to extraction using maceration process, in methanol and water (70:30 V/V). The solvent was recovered using rotary evaporator (IKA) and the concentrated extract

was dried using hot air oven at 40 °C. The extract was kept at -20° C until its uses.

Phytochemical Investigation

Phytochemical screening was carried out on crude extract using appropriate procedure to evaluate phytochemical constituents such as carbohydrates, proteins, amino acids, steroids terpenoids, glycosides, flavonoids, alkaloids, tannins, phenolic compounds, fat, oil, and saponins.^{11, 12, 13}

Brine Shrimp Lethality Bioassay

The cytotoxic study was carried out by brine shrimp lethality bioassay, using the *Artemia salina*. Cysts were incubated in 1L of sea water for 24 hours, with aeration and light source to provide temperature and to attract nauplii. After hatching, matured nauplii were swimming and gathered near to light source. The plant extract was prepared concentration in triplicates ranging from 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 μ g/ml or ppm using sea water in 5ml test tubes. 10 nauplii were added into each of the test tubes. Without test, substance served as the control. A drop of dry yeast suspension (3 mg in 5 ml sea water) was added to each test tube as a food for shrimps. This setup was left for 24 hrs incubation under continuous illumination of light source near test tube to provide temperature. After 24 hrs the live and dead nauplii were counted. Extract have shown significant cytotoxic activity ($LC_{50} < 100$ ppm). The extract was almost 100% lethal to the brine shrimp at the concentration of 1000 μ g/ml. and $LC_{50} < 100$ ppm was considered significant. Results are expressed as the mean \pm standard error of the mean (S.E.M.) of three independent experiments. Cytotoxicity (mean % death after 24 h with LC_{50} values) of the extract was compared with those of the control and is shown.¹⁴

In-Vitro Cytotoxicity Activity- MTT Assay

Cell line and cell culture

The Burkitt's lymphoma (*Daudi*) cells were purchased from NCCS Pune, Maharashtra, INDIA. Cells were cultured in RPMI-1640 (SIGMA) medium, supplemented with 10% fetal bovine serum (FBS) (Gibco, Invitrogen, USA) 100 units penicillin-streptomycin (GIBCO, INVITROGEN, USA); gentamycin and amphoterecin (Himedia, India) at pH 7.4, 37°C and humidified atmosphere of 95% air and 5% CO₂ in incubator (NEW BRUNSWICK SCIENTIFIC, GERMANY), Doxorubicin hydrochloride obtained as sample from RPG Life Sciences Limited, Mumbai, India, and cells were grown in 75 semi-square tissue culture flask and used for experiments when in exponential growth phase.

Trypan Blue Dye Exclusion Assay

Burkitt's lymphoma cells *Daudi* were made in the density of 6×10^3 cells/ml cells in PBS (SIGMA) medium and $10 \mu\text{l}$ of cell suspension was mixed with $10 \mu\text{l}$ of 0.4% trypan blue solution in PBS (SIGMA) for 1min. The cells were counted using Neubauer chamber (Roheam, Germany) examined under an inverted light microscope (LABOMED INC., USA), the dead cells were visible blue in color whereas the live cells did not absorb the dye because of trypan blue dye differentiated the live cells and dead cells. Live cell does not absorb dye inside and in dead cells dye penetrate inside, so this difference easily differentiates the live and dead cells. Distinct cell type and cell viability were asses using Neubauer counting chamber.

MTT Assay

Hydro-alcoholic extract of *Clerodendrum inerme* on antiproliferative activity was determined by MTT assay, *Daudi* cells were seeded into flat bottom 96 well plate (Corning, Cell bind, U.S.A.) with lid were seeded with 6×10^3 cells per well with $150 \mu\text{l}$ of DMEM growth medium with 10% FBS (Gibco, Invitrogen, USA). The plate was incubated for 24 hrs at 37°C under 5% CO_2 in a humidified atmosphere. After 24 hrs test and standards in 1000, 500, 250, 125, 62.5, 31.25, 15.625 $\mu\text{g/ml}$ concentration were added for 24 hrs in triplicates, after incubation at 37°C under 5% CO_2 , $20 \mu\text{l}$ of 5 mg/ml MTT at pH 7.4 was added in per well, and kept in dark for another 4 hrs towards color reaction development. An equal volume $100 \mu\text{l}$ of DMSO (SIGMA-ALDRICH) was added to stop the reaction and to solubilize the purple-blue crystals. The absorbance was measured at 630 nm using Elisa plate reader (Lisa 300, Germany).¹⁵

$$\% \text{ cell viability} = \frac{\text{Absorbance of test}}{\text{Absorbance of negative control}} \times 100$$

Change in Cellular Morphology

Daudi cells 6×10^3 were seeded in 24 well plate and kept for incubation for 24 hrs at 37°C , 5% CO_2 . Cells were treated with IC_{50} of extract for 24 hrs. Cells were observed for cellular morphology changes using an inverted microscope (Labomad, USA) at $40\times$.

In-Vivo Antitumor Activity

For the animal experiment animals were procured from the central animal facility from the institute, female Swiss albino mice 30 ± 2 g were selected for the study to enhance sensitivity than male used, and housed under standard laboratory condition $25^\circ\text{C} \pm 2^\circ\text{C}$ temperature

RH 50%-60% and 12 hr natural light and dark condition, animals were free to access to standard pellet diet and water *ad libitum*. All animals were acclimatized for two weeks before the experiment. For acute oral toxicity and dose determination OECD guideline 423 was followed. Animals were divided into five groups and each group contains six animals.^{16, 17} Group I was a normal group, group II was a positive control group, group III was received doxorubicin (2.5 mg/kg body weight) and IV & V were received low and a high dose of *Clerodendrum inerme* (200 and 400 mg/kg body weight) respectively. Animal experiment protocol was performed by Institutional Animal Ethical Committee (IAEC) K.L.E. University's College of Pharmacy Belagavi, India. The Burkitt's lymphoma cell suspension (0.2 ml of 2×10^5 cells/ml) was injected intraperitoneally into respected groups of animals. Treatment was given after 24 hrs of tumor inoculation to animal groups. Group III received (Doxorubicin 2.5 mg/kg body weight) IV & V were received *Clerodendrum inerme* plant extract in low dose and high dose (200 and 400 mg/kg body weight). With prior calculations of dose as per body weight extract treatment were daily orally administered and doxorubicin once in 4 days for 4 dose treatment by intraperitoneal route. After experiment animals were sacrificed by CO_2 asphyxia and tumor parameters body weight, tumor volume, tumor weight, and hematological parameters were observed after 15 days. Body weights of all groups of mice were measured before the experiment and after the 15 days of treatment. Sacrificed the animal by CO_2 asphyxia ascitic fluid was collected into falcon tubes (Corning, USA) to measure the tumor volume and weight. Blood parameters like total blood count RBC, WBC, Haemoglobin count by using bright-field microscopy method to calculate, the number of RBC and WBC count in blood per liter Neubauer chamber (Haemocytometer) was used.

STATISTICAL ANALYSIS

Data were expressed as mean \pm Standard Deviation, transform data and non-linear regression analysis, one-way ANOVA followed by Tukey-Kramer multiple comparison tests using GraphPad Prism[®] version 5.01, GraphPad Software, San Diego, California, USA. $P < 0.05$ was considered as statistically significant

RESULTS AND DISCUSSION

Phytochemical Screening

Phytochemical screening of hydroalcoholic extract of *C. inerme* aerial part showed the presence of carbo-

Table 1 : The presence of phytoconstituent was representing using the symbol “+” and the absence was shown using the symbol “-”.

S.No.	Test	Observation
1.	Carbohydrate	+
2.	Proteins	+
3.	Amino acids	+
4.	Steroids	+
5.	Triterpenoids	+
6.	Glycosides	+
7.	Flavonoids	+
8.	Alkaloids	+
9.	Tannins and Phenolic compounds	+
10.	Fat and Oil	-
11.	Saponins	+

hydrate, protein, amino acid, steroids, triterpenoids, glycosides, flavonoids, alkaloids, tannins, phenolic compounds, and saponins. (Table 1)

Brine Shrimp Lethality Bioassay

Extract have shown significant cytotoxic activity ($LC_{50} < 100$ ppm). The extract was almost 100% lethal to the brine shrimp at the concentration of 1000 $\mu\text{g}/\text{ml}$. and $LC_{50} < 100$ ppm was considered significant. Results are expressed as the mean \pm standard deviation (S.D.) of three independent experiments. Cytotoxicity (mean % death after 24 h with LC_{50} values) of the extract was compared with those of the control and is shown $LC_{50} 30 \pm 0$ ppm was found.

MTT Assay

The percent cell viability of the hydroalcoholic extract of *Clerodendrum inerme* and doxorubicin were observed 213.2 $\mu\text{g}/\text{ml}$ and 62.36 $\mu\text{g}/\text{ml}$ which was the concentration that causes the significant decrease in proliferation by 50% ie. IC_{50} of each one showed in Figure 1. The optimal cell number were seeded for the study and was found to be promising results, in order to evaluate the dose-dependent effect against Burkitt's lymphoma (*Daudi*) cells showed.

Change in Cellular Morphology

Cellular morphology changes of treated and untreated Burkitt's lymphoma cells, Figure 2 were observed under inverted microscope at 40 \times after treatment with IC_{50} if plant extract for 24 hrs Figure 2 (a) represents normal morphology of control untreated cells and treated cells whether lost their normal morphology Figure 2 (b) & (c), due to cell death most of them were floating and rounded in the medium.

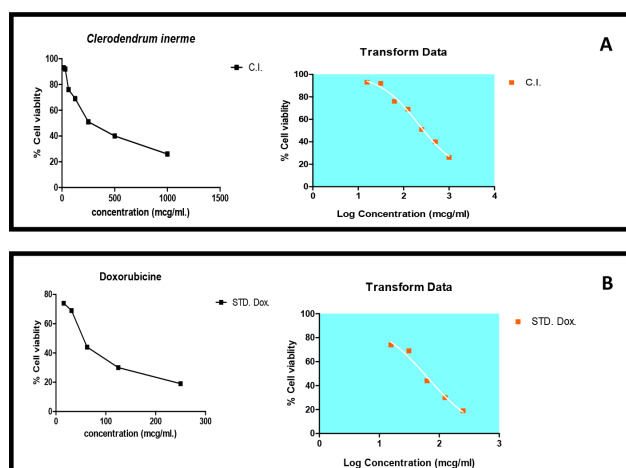


Figure 1: Representative graphs of the percentage of cell viability at different concentration (A) treatment with *C. inerme*, (B) treatment with doxorubicin, data were expressed as mean \pm Standard Deviation, transform of data and non-linear regression analysis

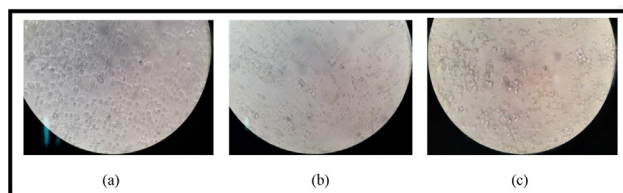


Figure 2: Representative images of cellular morphology of Burkitt's lymphoma cells (*Daudi*) (a) normal control cells, (b) treated with doxorubicin for 24 hrs (c) treated with *C. inerme* IC_{50} concentration for 24 hrs,.

In-Vivo Antitumor Activity

Both of the concentrations of *Clerodendrum inerme* extract revealed significant ($P < 0.05$) inhibition of ascitic tumor when the effect on body weight, tumor volume, tumor weight parameters compared with diseased control group. *Clerodendrum inerme* at low dose 200 mg/kg body weight showed lesser antitumor activity when compared with higher dose 400 mg/kg body weight. Hematological parameters also showing the effect on hemoglobin count of group III, IV and V compared with control group, showing decrease in hemoglobin count. MTT assay is the most important parameter to be evaluated for *in-vitro* anticancer effects. The present investigations provide the importance for biomedical research for safe and efficacious towards cancer. Antitumor activity of *C. inerme* extract at 200, 400 mg/kg body weight treated to burkitt's lymphoma induced female mice and effects revealed significant ($P < 0.05$) tumor inhibition (tumor volume, tumor weight) when compared with disease control group. Figure 3, the percent survival of all groups repre-

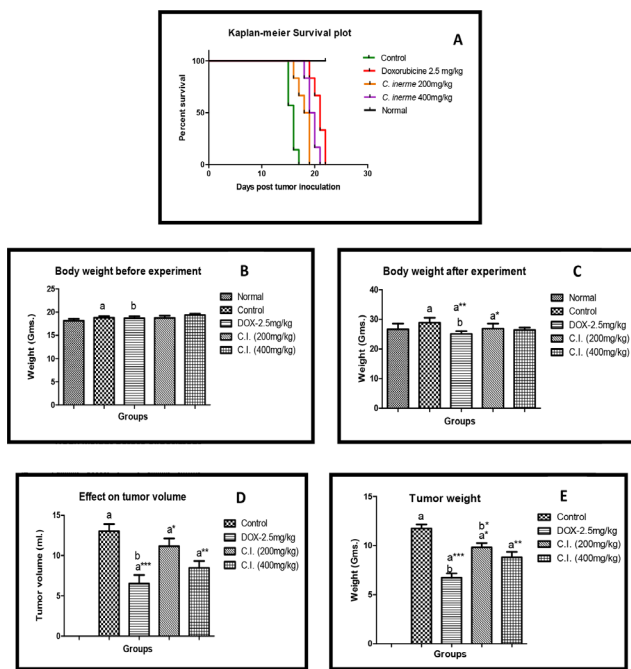


Figure 3: Therapeutic anticancer effect of *C. inerme* and doxorubicin; (A) Kaplan-meier survival plot of treatment with *C. inerme* extract and doxorubicin on different groups represents percent survival of animals after post treatment, (B) and (C) Difference in body weight before and after the treatment, (D) and (E) Tumor volume and tumor weight [I] Normal group [II] Control group [III] mice treated with Doxorubicin 2.5 mg/kg body weight [IV] and [V] mice treated with *C. inerme* 200 mg/kg and 400mg/kg body weight. *P< 0.001, **P<0.01, *P<0.05; a: disease control group, b: tumor induced group treated with doxorubicin, each data points represents mean \pm standard deviation, (n=6)**

sented by Kaplan-Meier survival plot of treatment with plant extracts and standard drug doxorubicin group. This antitumor effect could be due to presence of antitumor compounds present in the plant extract. Figure 3(A) shows the Kaplan-Meier survival plot of experimental group for the period of 30 days, which revealed that, there was 100% survival of normal group animals and treatment group III, doxorubicin 2.5 mg/kg body weight treated and group IV and group V at *C. inerme* 200, 400 mg/kg body weight treated also revealed increased the percentage survival life span, when compared with disease control group. However poor survival of disease control due to higher mortality of burkitt's lymphoma cancer. The suppression of blood component characterized by decrease in the production of WBC, RBC and Haemoglobin content which responsible for providing immunity and carrying oxygen. This type of condition showed, when infection in the body occurs or anticancer treatment done. In case of anticancer drug treatment like doxorubicin the reduction in the blood components due to toxicity of drug.¹⁸ Doxorubicin is the choice of drug in many cancer conditions, but it has severe side effects also. In the present investigation

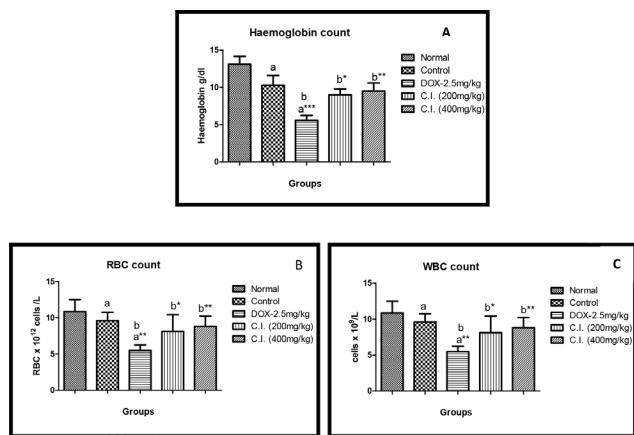


Figure 4: Representation of haemoglobin content and blood cell count, A: Haemoglobin content, B: red blood cells, C: white blood cells, mice treated with *C. inerme* extract at 200, 400 mg/Kg body weight and doxorubicin (2.5 mg/Kg body weight) *P<0.001, **P<0.01, *P<0.05 ; a: disease control group, b: tumor induced group treated with doxorubicin, each data points represents mean \pm standard deviation, (n=6).**

the extract was showing antitumor potential with less toxicity or side effects. Figure 4 represents haemoglobin content, WBC and RBC count, it was found that the significant ($P < 0.05$) decrease in percent haemoglobin count, WBC and RBC count, when doxorubicin group compared with disease control group, and the *C. inerme* extract 200 and 400 mg/kg body weight treated groups showing significant ($P < 0.05$) difference when compared with doxorubicin treated group. However it was observed that, the plant extract treated group showing not significant ($P < 0.05$) decrease blood components when compared with disease control group. In disease control group the higher level of WBC were observed, which could be due to different pathway and different mechanism in animals due to cancer.¹⁹ This study was found that the hydroalcoholic extract of *Clerodendrum inerme* extract has cytotoxic effect which demonstrated by Brine shrimp lethality bioassay and anti-proliferative effect has shown by MTT assay and morphological changes on Burkitt's lymphoma cell line. In the study, doxorubicin was used as standard drug, analyzed results are statistically significant. In conclusion, this study has been supporting the ethnomedicinal value of *Clerodendrum inerme* for the anti-cancer potential, and it can be used for the benefit for the sustainable use of traditional medicinal plants. The mechanism of action is still not cleared that how the active principle of the plant is showing effect. Further studies can be done to evaluate and demonstrate the mechanism pathway of biological anticancer activity of *Clerodendrum inerme* plant. The potent anticancer properties of *Clerodendrum inerme* hydroalcoholic extract showed the presence of antioxidant and anticancer principles. The

results of the study were consistent and support the earlier studies, which further support the anticancer potential of *Clerodendrum inerme* plant. Nowadays phytochemicals derived from plant source are being intensively investigated for cancer. Cell death and apoptosis is the important physiological and pathological condition, which involved in various importance protein-expression, condensation fragmentation of DNA and shrinkage of cells. As reported in literature the single compound could have more potential anticancer activity, the evaluation of anticancer compound is necessary for the proof of concept. The further scope of this study could be helpful to assess the exact mechanism of action.

CONCLUSION

The result of anti-proliferative or cytotoxic effect of *Clerodendrum inerme* inhibit the growth of cells and indicated the anticancer potential of plant extract, also showing a toxic effect on brine shrimp lethality assay, MTT assay morphological changes, and Daltons ascitic lymphoma model in Swiss mice. These all parameter showed the anticancer effect of plant extract. This is an indigenous plant and used as folk medicines for years together, this study provides the scientific validation to its traditional claims. Other anticancer activity are also done on this plant using other extracts but without using any standard anticancer drug treatment and this is the loop in research, so there was a need to fill up this gap and this study provides the anticancer effect of extract with the standard anticancer drug.

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