

Development and Evaluation of Anti-Cancer Activity of Phytosome Formulated from the Root Extract of Clerodendron Paniculatum Linn

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ABSTRACT

Formulation and Evaluation of anti-cancer activity of Clerodendron paniculatum Linn formulated phytosome from the extract by DAL cells. The root portions of the Clerodendron paniculatum Linn root extracted with ethanol by cold maceration process. The extracts were vacuum dried and subjected to phytochemical screening for the detection of various phyto constituents. The formulated phytosome from the extract exhibited potent activity against cancer cells. The study reveals that Clerodendron paniculatum phytosome possess better anti-cancer activity than the extract.

Keywords: Clerodendron paniculatum Linn root, Anticancer activity, DAL cells

INTRODUCTION

Preparation of plants or their parts have been widely used in medicine since ancient times and till today use of phytomedicines is widespread. Most of the biologically active constituents of plants are polar or water-soluble. However, water-soluble phytoconstituents like flavonoids, tannins, glycosides aglycones etc. are poorly absorbed either due to their large molecular size, which cannot be absorbed by passive diffusion or due to their poor lipid solubility, thus severely limiting their ability to transport across lipid-rich biological membranes, resulting in their poor bioavailability¹. Nano particle is a newly introduced patented technology developed to incorporate the standardized plant extracts or water-soluble phytoconstituents into phospholipids to produce lipid compatible molecular complexes, which improves their absorption and bioavailability². *Clerodendron paniculatum* Linn, (Family: Verbenaceae)³ was a species found in India, in this reported as folk remedy for tumours, leprosy, fever, infection, inflammation. The roots have been reported to possess laxative, diuretic, analgesic, anti-inflammatory, anti-cancer antibacterial activities⁴. To our knowledge there were no scientific reports on anti-tumour activities of phytosome formulated from Clerodendron paniculatum Linn, extract. The key objective of the present study is to develop the nanoparticle of Clerodendron paniculatum Linn to increase the solubility and bioavailability of drug. To prepared the phytosome of Clerodendron paniculatum Linn by specific method and evaluate its anti-cancer activities by assessing tumor volume, viable and nonviable tumor cell count, tumor weight, hematological parameters and biochemical estimations against DLA tumour cells.

MATERIALS AND METHODS

Experimental Methods

Plant material and Extraction

The plant *C.paniculatum* was collected from Pathanamthitta district of Kerala and identified by Thomas Mathew, HOD of Botany, Marthoma College Tiruvalla, Kerala. Voucher no. VSCp-14, were deposited in the Pharmacognosy department, Pushpagiri College of pharmacy, Tiruvalla. The root portion of the plant were washed with running water to remove soil and other matter and dried in shade for 20 days, powdered, extracted 500gm with ethanol (EECP) by cold extraction to yield the respective extract. The extracts were reduced to molten mass by rotary vacuum evaporator and the yield was 21% w/w Preliminary phytochemical screening was performed as per standard procedure and various phytochemical constituents were identified^{6,7}.

Preparation of Phytosome

Accurately weighed quantity of phosphatidylcholine and cholesterol were dissolved in 10 ml of chloroform in round bottom flask and sonicated for 10 min using bath sonicator. Organic solvent removal is done by Rotary evaporator (45-50°C). After complete removal of solvent thin layer of phospholipids mixture was formed. This film was hydrated with ethanolic extract of Clerodendron paniculatum root in rotary evaporator (37- 40°C for 1 hour). After hydration, mixture of lipid and plant extract was sonicated for 20 minutes in presence of ice bath for heat dissipation. Then prepared phytosomes were filled in amber colored bottle and stored in freezer (2-80C) until used⁸.

Evaluation of phytosome

Determination of % yield

(%) Yield = (Practical yield) × 100 (Theoretical yield)

Visualisation

The morphology of phytosomes was observed by digital microscopy, transmission electron microscope.

Digital microscopy

Phytosome formulation shaken in distilled water and viewed under digital microscope at 400X objective lens.

TEM analysis

The complex was shaken in distilled water and viewed using Transmission Electron Microscope

Determination of entrapment efficiency

Phytosome complex of extract was diluted 1-fold with 10 ml of methanol and then centrifuged at 18,000 rpm for 1/2 hr at -4°C using cooling centrifuge machine. The supernatant was isolated and the amount of free extract was determined by UV/Vis spectroscopy at 269 nm. To determine the total amount of extract, 0.1 ml of the extract phospholipid suspension was diluted in methanol, adjusting the volume to 10 ml. The Entrapment efficiency was calculated according to the following formula: 15-16
Entrapment efficiency (%) = $\frac{\text{Total amount of drug} - \text{Amount of free drug}}{\text{Total amount of drug}} \times 100$

Evaluation of in Vivo anti-cancer activity of formulated phytosome against DLA tumour cells.

Induction of cancer using DAL cells

Dalton's Lymphoma ascites (DAL) cell was supplied by Amala cancer research center, Trissur, Kerala, India. The cells maintained *in vivo* in Swiss albino mice by intra peritoneal transplantation. While transforming the tumor cells to the grouped animal the DAL cells were aspirated from peritoneal cavity of the mice using saline. The cell counts were done and further dilutions were made so that total cell should be 1×10^6 ; this dilution was given intra peritoneally. Let the tumor grow in the mice for minimum seven days before starting treatments.

Animals

Male Swiss albino mice (20-25 gm) Animal Ethical Committee no PCP/2013/IAEC/602/02 were produced from animal experimental laboratory, and used throughout the study. They were housed in micro nylon boxes in a control environment (temp $25 \pm 2^\circ\text{C}$) and 12 hours' dark/light cycle with standard laboratory diet and water *ad libitum*. The study was conducted after obtaining institutional animal ethical committee clearance. As per the standard practice, the mice were segregated based on their gender and quarantined for 15 days before the commencement of the experiment. They were fed on healthy diet and maintained in hygienic environment in our animal house.

Treatment Protocol

Swiss Albino mice were divided into five group of six each. All the animals in four groups were injected with DAL cells (1×10^6 cells per mouse) intra peritoneal, and the remaining one group is normal control group.

G₁ Served as the normal control.

G₂ Served as the tumor control. Group 1 and 2 receives normal diet and Water.

G₃ Served as the positive control; was treated with injection 5-FU(20mg/kg) Intra peritoneal.

G₄ Served as a low dose treatment control and was administered EECp (200mg/kg, n=6)

G₅ Served as a high dose treatment control and was administered EECp (400mg/kg, n=6)

G₅ Served as a high dose treatment control and was administered with formulated phytosome using Clerodendron paniculatum extract (20mg/kg)

Treatment

In this study, drug treatment was given after the 24 hrs of inoculation, once daily for 14 days. On day 14, after 24 hrs. the last dose, all mice from each group were sacrificed; blood was withdrawn from each mouse by retro orbital plexus method and the following parameters were checked.

Hematological parameters

White blood cells (WBC)

Red blood cells (RBC)

Hemoglobin content (Hb)

Platelet count

Packed cell volume (PCV)

Derived parameter

Body weight

Life span (%)

Cancer Cell Count

Cancer cell count

The fluid (0.1ml) from the peritoneal cavity of each mouse was withdrawn by sterile syringe and diluted with 0.8 ml of ice cold Normal saline or sterile Phosphate Buffer Solution and 0.1 ml of trypan blue (0.1 mg/ml) and total numbers of the living cells were counted using hemacytometer.

$$\text{Cell count} = \frac{\text{Number of cells} \times \text{Dilution}}{\text{Area} \times \text{Thickness of liquid film}}$$

Hematological parameters

WBC count

Total WBC count was found to be increased in cancer control, when compared with normal and treated tumor-bearing mice. The total WBC count was found to decrease significantly in animals treated with extract when compared with cancer control.

RBC and Hb

RBC and Hb content decreases with tumor bearing mice when compared with Normal control mice.

Platelets

In Hodgkin lymphoma, increase in platelet count was often reported in laboratory findings. Hence, we investigated this parameter in the study (Jacqueline, 1998).

Packed cell volume

In any case of anemia, the packed cell volume decreases.

Derived parameters

Body weight

All the mice were weighed, from the beginning to the 15th day of the study. Average increase in body weight on the 15th day was determined.

Percentage increase in life span (ILS)

% ILS was calculated by the following formula Life span of treated group

$$\% \text{ILS} = \frac{\text{Life span of treated group} - 1 \times 100}{\text{Life span of control group}}$$

All biochemical investigations were done by using COBAS MIRA PLUS-S Auto analyzer from Roche Switzerland.

Hematological test is carried out in COBAS MICROS OT 18 from Roche.

Table 1: Effect of EECp extract, Formulated phytosome using extract on tumor volume (ml), tumor weight (g), viable (cells × 106 cell/ ml) and nonviable cell count (cells × 106 cell/ ml), median survival time (MST), percentage increase life-span (% ILS) and haematological parameters like RBC (cells/106µl-1), WBC (cells × 103 µl-1) and HB content (g/dl) in DLA bearing mice.

Parameters	G ₁	G ₂	G ₃	G ₄	G ₅	G ₆
Tumor Volume	-	3.01±0.18	1.81±0.17b*	1.17±0.15 b*	0.30±0.05 b*	0.35±0.05b*
Tumor weight	-	2.92±0.21	1.33±0.14 b*	0.98±0.07 b*	0.19±0.05 b*	0.41±0.05b*
Viable cell	-	8.55±0.28	3.67±0.18 b*	1.40±0.16 b*	0.43±0.08 b*	0.48±0.05b*
Non-Viable cell	-	0.35±0.07	1.29±0.15 b*	2.57±0.18 b*	0.31±0.13 b*	
MST (days)	-	21.6±0.47	30.7±0.90	37±0.39	42.21±0.05 b*	40.01±0.05*
%ILS	-	00	42.87	74.41	90.10	83.13
RBC	6.08±0.06	3.14±0.38a*	4.13±0.24 b*	5.19±0.18 b*	1.31±0.06 b*	1.11±0.04 b*
WBC	5.18±0.06	12.14±0.76 a*	8.39±0.49 b*	6.39±1.12 b*	15.31±0.02 b*	4.81±1.02 b*
Hemoglobin	13.18±0.06	7.21±0.41 a*	9.18±0.96 b*	10.93±0.60 b*	13.31±0.05 b	12.89±0.02*

Group 1- Normal control (5ml/kg), Group 2- DLA Control (2x106cell/ ml), Group 3- DLA + EECp (200mg/kg), Group 4- DLA+EECp, (400mg/kg), Group 5- DLA+Formulated phytosome (20mg) Group 6- DLA+5-FU(20mg/kg) Values are represented as mean ±SEM, where n=6, a P<0.01 as compare to normal control, b P<0.01 as compare to DLA control.

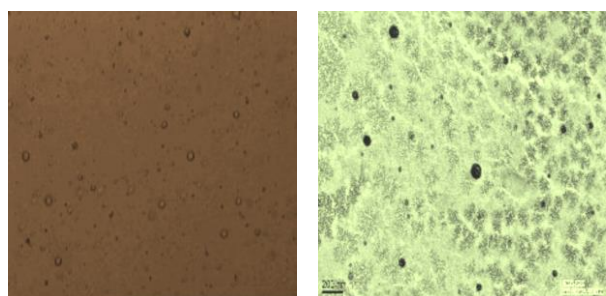


Figure 1: Digital microscopic, TEM view of phytosome

Newly added Hi-Tech instruments MAX MAT used for an auto analyzer for all biochemistry investigations in blood sample.

Effect of extract and the formulated phytosome on Survival Time

Animals were divided into five groups of six animals each. Except the normal control group, the remaining groups were inoculated with DAL cells (1x10⁶cells/mouse) intraperitoneally on day '0' and treatment with extract started 24 hrs after inoculation, at a dose of 200mg and 400mg/kg/day. *p.o.* The normal and tumor control group was treated with same volume of 0.9% sodium chloride solution. All the treatments were given for fourteen days. The increase in life span (ILS) of each group, consisting of 6 mice was noted. The antitumor efficacy of extract and the formulated phytosome was compared with that of 5-fluorouracil (Dabur pharmaceutical ltd. India; 5-FU, 20 mg/kg/day, *i.p.* for 14 days). The ILS of the treated groups was compared with that of the control group using the following calculation:

$$\text{Increase in lifespan} = [(T - C) / C] \times 100$$

Where T = number of days the treated animal survived.

C = number of days' control animals survived.

Statistical analysis

All the experimental data are expressed as the mean SEM. The data was statistically analyzed by using one-way Analysis of Variance (ANOVA) followed by Dunnett's post-hoc test.

RESULTS AND DISCUSSION

Preparation of phytosomes

Solvent evaporation method was used, percentage yield was found to be 89.81%, entrapment efficiency 96.4 ± 0.7

Evaluation of phytosome

Visualisation

Effect on Tumor Growth

The effect of EECp, and formulated phytosome on tumor growth responses were observed and shown in Table -1. In the DAL tumor control group, the average life span of animals was found to be 48% whereas, 200, and 400 mg/kg of EECp and formulated phytosome showed increase in life span to 42.87, and 90.10%

CONCLUSION

From above studies we are concluded that phytosomes has better physical characteristics than that of extract. In-vivo studies revealed that phytosomes showed more anticancer activity than that of 5-fluorouracil.

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