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In vitro Cytotoxic potential of *Clerodendrum colebrookianum* Walp. against Dalton's Lymphoma cells

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Abstract

Traditionally used medicinal plants are of interest among researchers due to their rich diversity of phytochemicals with varied pharmacological importance. In the present study, we aimed to investigate the phytochemical constituents, antioxidant potential, and *in vitro* cytotoxicity of *Clerodendrum colebrookeanum* against Dalton's Lymphoma (DL) cells. The standard phytochemical tests revealed the presence of important phytoconstituents in the methanol leaf extract such as carbohydrates, glycosides, phenols, tannins, polyphenols, flavonoids, terpenoids, and saponins. The antioxidant activity determined using DPPH as a free radical showed potent antioxidant ability compared to standard ascorbic acid. Moreover, the *in vitro* antitumor potential was determined using cell viability assays trypan blue and MTT whose results exhibited the cytotoxic potential of the extract against DL cells. A significantly ($p<0.0001$) low IC_{50} value was found compared to the half-maximal reduction ability of the reference drug, cisplatin. However, the viability of normal cells (splenocytes) was higher, indicating the specificity of extract towards cancerous cells. Thus, the outcome of the present study will lead to further exploration of the anticancer potential of *C. colebrookianum* through in vivo studies and isolation of active principle(s)

Keywords: anticancer; antioxidant; cytotoxicity; Dalton's lymphoma; phytochemicals

Introduction

The use of plants in traditional medicine can be evidenced from the earlier centuries which mentioned the preparation of various herbal recipes using medicinal plants [1]. The traditional medicine system of India includes Ayurveda, Unani, Siddha, Yoga, naturopathy, and homeopathy. In Ayurveda, the majority of the drugs are prepared using traditional medicinal plants to treat various ailments since ancient times[2]. Statistically around 60% of the

population globally and 70% of the rural population in India relies on traditional medicines [3]. With the rise in cancer cases, researchers currently focus on the development of drugs with fewer side effects compared to the conventional treatment methods which have severe side effects[4].

Clerodendrum colebrookeanum Walp. belonging to the family Lamiaceae is an evergreen perennial shrub that grows up to a height of about 1.5 to 3 meters. It has quadrangular stems, leaves are broad, and ovate with terminal inflorescence which is compact with corymbose cymes [5]. It is known by different local names among the ethnic communities of the Northeastern states such as Nefafu (Assam), Orematong and Umrem (Nagaland), Phuinum (Mizoram), Jarem and Sia-long (Meghalaya), Kuthab (Manipur), Tapen, Papua-toh, Poto, Huikam (Arunachal Pradesh) [6]. The plant covers a geographical area of the tropical and sub-tropical regions and mostly endemic to the Northeastern parts of India [7]. It is traditionally used in the Northeastern region of India to cure various ailments such as headache, dysentery, anti-helminthic, to reduce blood pressure, diabetes, cough, and to treat colic pains, stomach disorders, and skin diseases [8]. The Khasi and the Jaintia tribes consume boiled leaves to treat hypertension, liver problems, malaria and to reduce rheumatic pains [9]. Further, it has also been used to treat conditions of syphilis, thyroid disorders, cancer, jaundice, obesity, and asthma [10].

The various parts of the plant are also found to be rich in diverse phytochemicals such as flavonoids, alkaloids, phenols, polyphenols, terpenoids, glycosides, diterpenes, and steroids [7], [11]. Further, five steroids Colebrin A-E and β- sitosterol were isolated from the plant [6, 8]. Moreover, the plant was also found to exhibit various pharmacological properties such as anti-inflammatory and analgesic activity [12], antioxidant properties [13], antihypertensive activity [14], anti-microbial [15], anti-diabetic [7]. Furthermore, anti-helminthic, antipyretic, hepatoprotective, and CNS depressant activity was also reported [9, 16]. The methanolic extract was also found to exhibit a cytotoxic response against the HepG2 cell line [17]. The current study, aimed to explore the anticancer potential of *C. colebrookeanum* against Dalton's lymphoma (DL) cells. The outcome of the study will provide insight into the anticancer potential of the plant and lead to further exploration of the molecular mechanisms involved and isolation of active principle(s).

METHODS

***Collection and identification of plant sample*:**

The plant was collected from the wild during the flowering season from the Tipam gaon area of Jeypore under Dibrugarh district, Assam, India. The collection was performed during October 2022 and herbarium sheets were prepared for its authentication and identification. The identification was done at the Botanical Survey of India, Shillong, Meghalaya, and provided with an accession No. 101357.

***Extract preparation*:**

The leaves were washed properly and shade-dried for about 10-15 days. The dried leaves were then grinded to a coarse powder and then dissolved in 70% methanol and macerated for about 72h. The mixture was filtered at an interval of 24 hours to collect the filtrate. The collected filtrate is then deried through evaporation of the solvent in a rotary evaporator at 50^0C and stored in glass vials at 4^0C for further experimental purposes[18].

***Animal model and cell culture*:**

To carry out the *in vitro* experiments Swiss albino mice were maintained in the laboratory to propagate the DL cells. The mice were kept in cages with paddy husk as bedding and the temperature was maintained at 23 ± 2^0C and was provided with proper mice feed and water *ad libitum*. The care and maintenance of the animals were as per the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) and the approval for the experimentation on animals was obtained from the Institutional Ethics Committee of Cotton University and ethics approval number is 7/IAEC/CU/05/01/2021 dated 16/01/21.

The Dalton's Lymphoma (DL) cells were cultured in RPMI-1640 growth medium accompanied with antibiotic solution, Fetal bovine serum (FBS), vitamin solution, and Minimum essential medium (MEM). After the cells reached a confluency of ~80% they were sub-cultured in 24 well plates and treated with different concentrations of the plant extract for 24h to observe its cytotoxic effects [19].

***Preparation of single-cell suspension of splenocytes*:**

To check the cytotoxic effect of the extract on normal lymphocytes, we extracted spleen from normal mice, separated the cells to prepare single cell suspension, and treated them with varying concentrations of the extract (100, 150, 200, 500 and 1000) µg/ml. The cell viability was assessed to determine the cytotoxic effect.[20].

***Preliminary phytochemical screening*:**

The methanolic extract of *C. colebrookianum* was subjected to qualitative phytochemical screening for the presence of important primary and secondary metabolites using standard phytochemical tests [21].

***Free radical scavenging assay using DPPH*:**

DPPH ((2,2-diphenyl 1-picrylhydrazyl) is a free radical that can donate hydrogen atoms. The assay determines the scavenging of DPPH by the extract which on reduction produces decolorization to the methanolic solution of DPPH [22]. The assay was performed following the procedures of Blois [23] with slight variations. A 0.1mM DPPH solution of different concentrations in methanol and plant extract of concentrations from 10-250µg/ml was prepared. In a 96well plate, 50µl of the extract was mixed with 195µl of DPPH solution and incubated in the dark for 1h. Ascorbic acid used as standard was prepared in the same concentration range and a control group containing a mixture of methanol and DPPH solution was also run. All the doses were run in triplicates. The absorbance was then recorded at 492nm and the percentage of the DPPH scavenged was calculated as follows:

$$\% \text{ scavenging effect} = \frac{Absorbance\ of\ control - Absorbance\ of\ treated\ group}{Absorbance\ of\ control} \times 100$$

Moreover, the IC_{50} dose was also calculated for the extract and the standard, ascorbic acid.

***Determination of cell viability*:**

***Trypan blue method*:**

To estimate the viability of DL cells in comparison to normal cells (splenocytes) on treatment with *C. colebrookianum* extract trypan blue dye method was used. The assay is based on the principle that the trypan blue dye penetrates the dead and damaged cells and appears blue whereas the live cells appear with clear cytoplasm due to intact plasma membrane. The experiment was carried out as per the study of Kuttan et al. [24]. The subcultured DL and normal cells in a 24-well plate were treated with extract of various concentrations for 24h along with a positive control group of cells treated with cisplatin (a reference drug). The cells were then stained with 0.4% trypan blue dye and incubated for 3 minutes. The viability was then observed in a microscope and images were captured. About 4000 cells of each treatment dose were analyzed using Image J software. The number of viable and non-viable cells was counted and the percentage viability of cells was determined using the formulae as follows:

$$\% \text{ of viable cells} = 1 - \frac{Number\ of\ non-vaible\ cells}{Total\ number\ of\ cells} \times 100$$

Furthermore, the concentration representing 50% cell viability (IC_{50}) was also determined for both the extract and cisplatin-treated cells.

***MTT assay*:**

The viability of the DL cells was further assessed through a sensitive colorimetric assay that uses MTT (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium), a yellow dye of tetrazolium salts. The cell viability is measured based on the formation of purple formazan crystals with the help of mitochondrial reductase enzyme by the live cells. The experiment was conducted following the procedures of [25]. Briefly, the cells treated with extract for 24h were mixed with 20µl of MTT of concentration 5mg/ml dissolved in PBS and incubated for 2h at 5% CO_2 and 37^{O}C. Post incubation 100µl of DMSO (Dimethyl sulphoxide) was added in each tube for the solubilization of formazan crystals. The absorbance was measured at 550nm by plating the mixture in a 96-well microtiter plate. A control and a blank group were also run. The blank group contained cells with only PBS (Phosphate buffer saline) and the control group contained PBS along with MTT dye. A positive control group treated with cisplatin at the same concentration range as extract was also conducted in the experiment. The IC_{50} dose was calculated to assess the effectiveness of the extract in comparison to the reference drug, cisplatin. All readings were calculated in triplicates and the viability of cells was calculated as follows:

$$\% \text{ Cell viability} = \frac{Absorbance\ of\ sample - Absorbance\ of\ control}{Absorbance\ of\ blank - Absorbance\ of\ control} \times 100$$

LDH assay:

To assess the cytotoxicity potential of the extract against the DL cells the activity of the Lactate Dehydrogenase enzyme (LDH) present inside the cells is measured. The dead and damaged cells release LDH enzyme in the cell supernatant which can be measured to determine the cell cytotoxicity [26]. The experiment was performed using the Lactate Dehydrogenase Kit of HiMedia as per the manufacturer's instructions. The DL cells treated with extract and reference drug cisplatin at different concentrations were incubated for 24h at 5% CO_2 and 37^{O}C conditions. Furthermore, a group of cells treated with lysis solution which released maximum LDH served as a standard, the control group receiving no treatment, ascitic fluid of DL cells was used as background control and another group of cells treated with PBS and lysis solution was used as the volume correction group. The incubated cells were centrifuged to collect the cell supernatant. 50µl of supernatant was mixed with 50µl of LDH reagent and incubated for half an hour at room temperature. The reaction was stopped by adding 50µl of stop solution

and absorbance was taken at a wavelength of 450nm. The percentage of cytotoxicity was measured by calculating the following formula:

$$\% \text{ Cell cytotoxicity} = 100\times A_{t}- A_{C} / A_{m}- A_{C}$$

Here, Final absorbance of treated sample (A_t) = absorbance of treated sample -absorbance of untreated control.

Final absorbance of untreated control (A_C)= absorbance of untreated control- absorbance of background control.

Final maximum LDH release (A_m)= absorbance of maximum LDH – absorbance of volume correction control.

***Statistical analysis*:**

The statistical tool Graph Pad Prism version 8.0 was used to carry out the statistical analysis. The means of the control and the treated group were compared using a two-way analysis of variance and the multiple comparisons were performed using Bonferroni post hoc test. Student's t-test was employed to compare the IC_{50} dose and a linear regression analysis was performed to show a positive correlation between the dose with the increase in activity. A significance level of $p<0.05$ was considered to be significant. The data were represented as Mean±SEM and run in triplicates.

RESULTS

***Phytochemical analysis*:**

The outcome of the phytochemical screening showed the presence of phytoconstituents in various degrees as depicted in the heat map (Figure 1). The color key represents the concentration of the phytochemicals at various concentrations. The extract is found to be rich in carbohydrates, glycosides, phenols, tannins, polyphenols, flavonoids, and terpenoids. A moderate concentration of saponins is also detected.

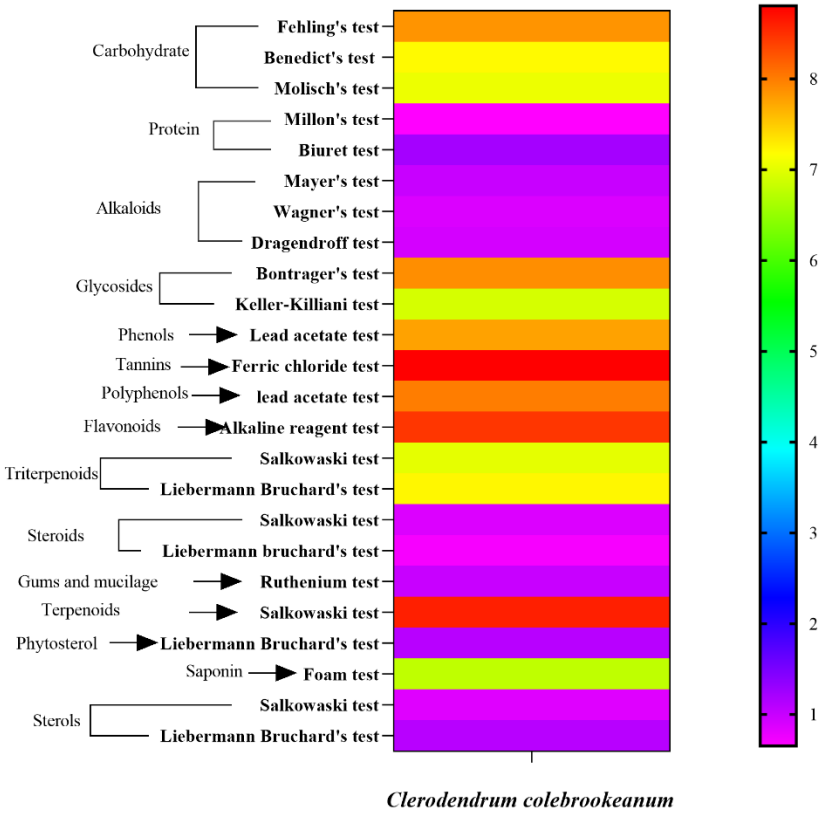

Figure 1: Heap map representing the phytochemicals in *C. colebrookeanum* methanol extract.

***Antioxidant activity using DPPH*:**

The antioxidant activity was determined using DPPH as a free radical and the scavenging effect of the extract was determined. The results of the assay indicated a significant ($p<0.0001$) increase in the scavenging effect of the extract compared to ascorbic acid. The scavenging effect of the standard, ascorbic acid increased up to 300µg/ml of dose and further higher concentrations exhibited a steady scavenging effect. On the other hand, the extract showed an increase in free radical scavenging effect with an increase in dose. At the highest concentration of 500µg/ml, the scavenging effect of the extract was found to be 44% (Figure 2A). Moreover, the IC_{50} value of the extract was found to be 101.3± 1.92 µg/ml and that of the ascorbic was 27.24± 1.55 µg/ml which showed significant ($p<0.001$; t-test) differences (Figure 2B).

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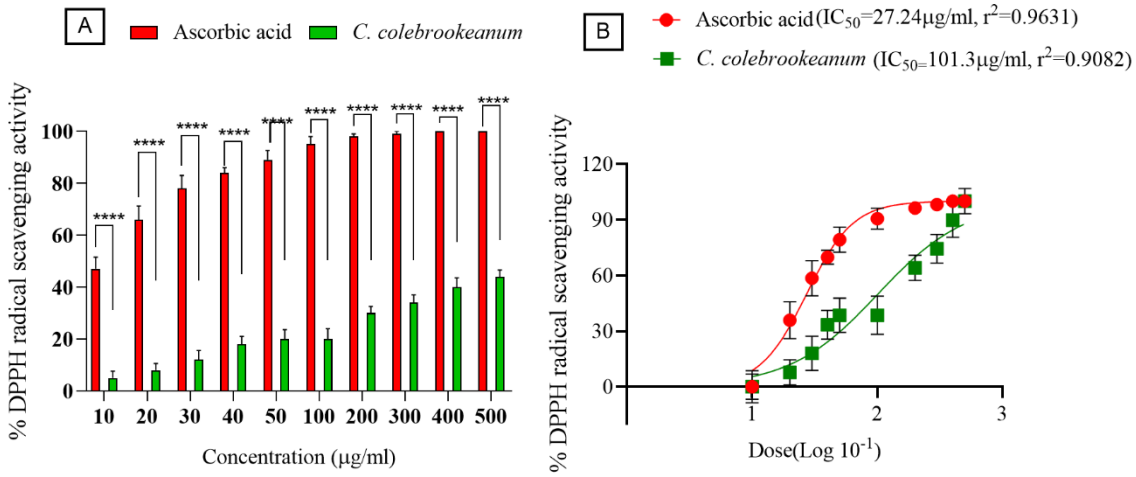


Figure 2: Graph representing the DPPH radical scavenging activity of the extract and standard, ascorbic acid. (A) Two-way ANOVA analysis representing the increase in scavenging activity of the extract compared to ascorbic acid (B) Log transformed and normalized IC_{50} value of extract and standard. Results are represented as mean± SEM and the asterisk represents significance level ****$p<0.0001$.

***Determination of in vitro antitumor potential*:**

***Trypan blue assay*:**

The trypan blue assay showed the viability of DL and normal cells on treatment with extract. The results indicated that the viability of DL cells was found to decrease on treatment with extract at different concentrations which showed significance ($p<0.0001$) in comparison to the viability of cells treated with cisplatin. However, the normal cells(splenocytes) showed significantly greater cell viability compared to DL cells even at the higher dose of treatment. However, the change in cell viability of the extract and normal cells was found to be insignificant ($p>0.05$) at the lowest dose of 25µg/ml (Figure 3A). Moreover, the half maximal reduction in cell viability (IC_{50}) of the extract was found to be at a dose of 100.8± 2.21 µg/ml which is statistically different (t-test, $p<0.001$) from the IC_{50} dose of cisplatin at 91.18± 0.72µg/ml (Figure 3B). Moreover, the decrease in cell viability was found to be proportional to dose (Figure 3C). Furthermore, the observation of membrane blebbing, cell shrinkage, and

cell membrane disintegration indicates the occurrence of apoptosis which substantiates its observed cytotoxic effect (Figure 4).

Figure 3: Cell viability of DL and normal cells on treatment with extract for 24h was determined using the trypan blue method. (A) Viability of cells (DL and splenocytes) compared to cisplatin-treated cells analysed through two-way ANOVA (B) Log transformed and normalized IC_{50} values of extract and cisplatin. (C) Linear regression analysis exhibiting proportionality of dose with the viability of cells. Here, all the data are represented as mean± SEM and the asterisk represents significance level ****p<0.0001, ***p<0.001, **p<0.01.

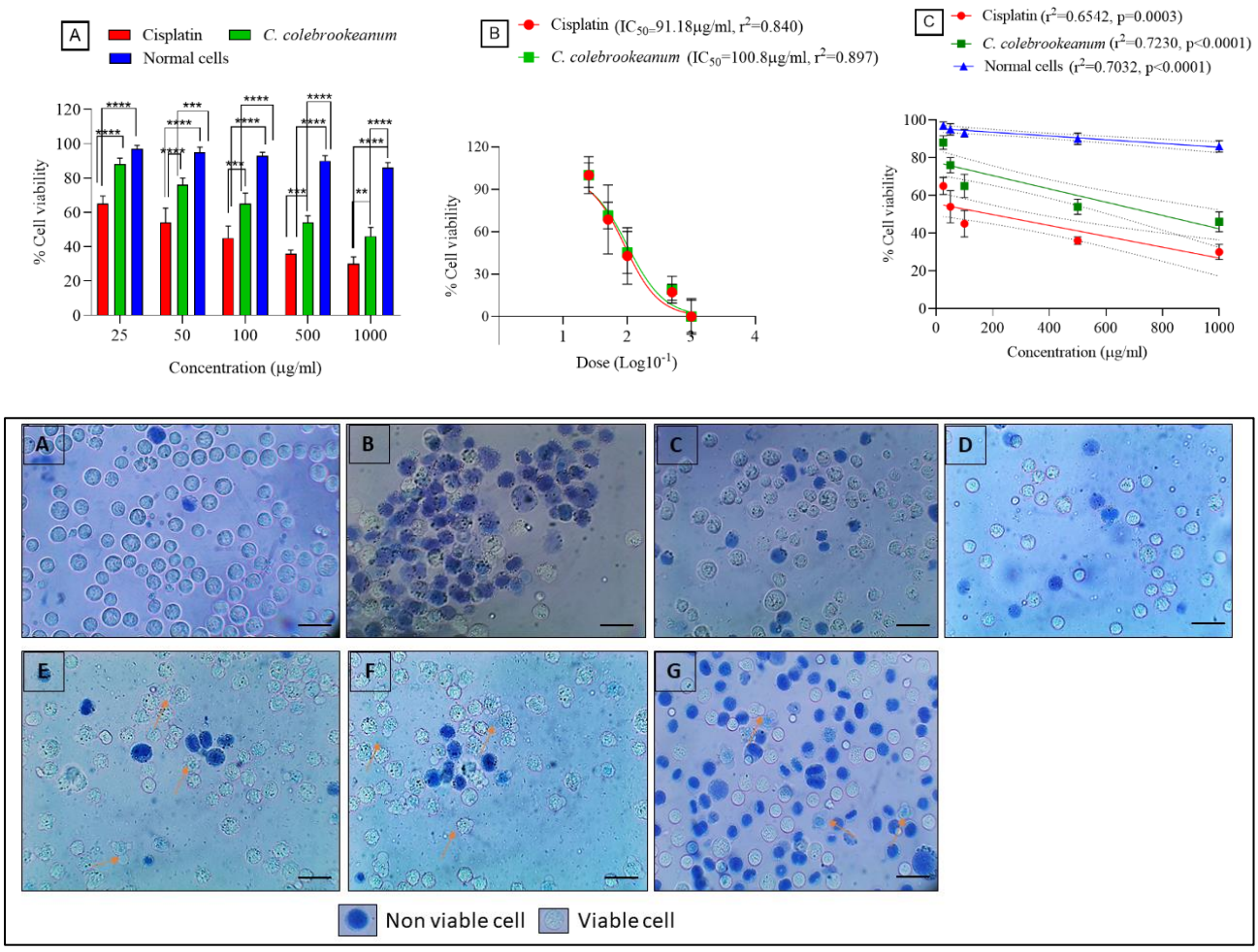
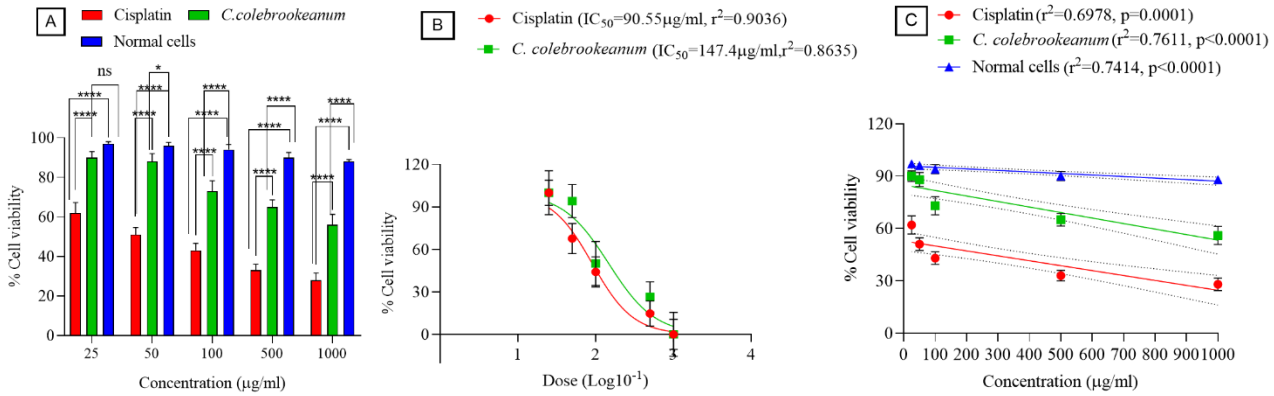


Figure 4: Photographs representing the cell viability and morphological changes of DL cells observed on treatment with *Clerodendrum colebrookeanum* at different concentrations. (A) Negative control (B) Cisplatin treated (C) 25µg/ml (D) 50µg/ml (E) 100µg/ml (F) 500µg/ml

(G) 1000µg/ml. The orange arrows represent the apoptotic features such as membrane blebbing, membrane disintegration, and cell shrinkage. The images are represented in 40x magnification. Each bar in the image represents 10µm.

MTT assay:

An MTT assay was performed to further affirm the extract's cytotoxic effect. The results revealed that the extract exhibited a positive cytotoxic response against DL cells indicated with a reduction in the percentage of viable cells at various treatment concentrations. Further, the



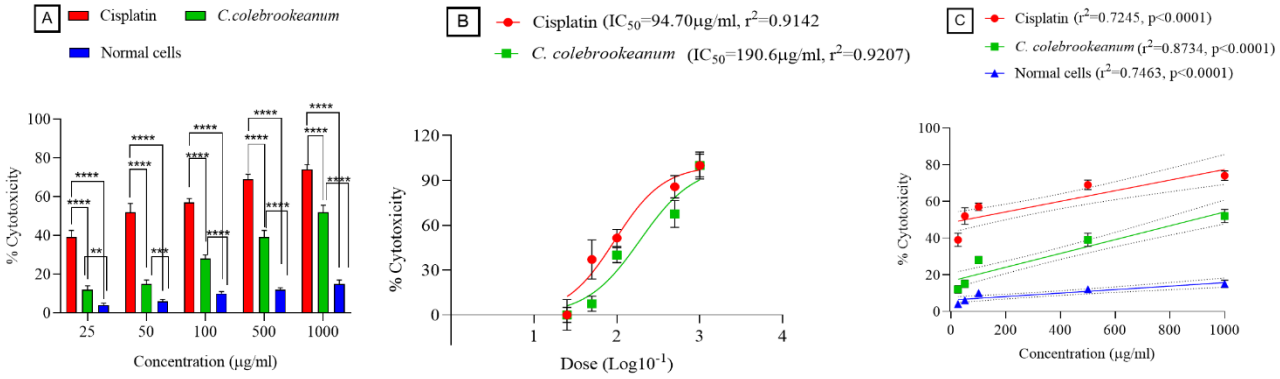
decrease in cell viability was found to be significant ($p<0.0001$) compared to that of the viability of cisplatin-treated cells. However, normal cells were unaffected by the extract treatment and exhibited higher cell viability of about 88% even at the maximum treatment concentration. At the lowest dose of 25µg/ml the differences in cell viability of DL and normal cells(splenocytes) treated with extract were found to be non-significant ($p>0.05$) (Figure 5A). The IC_{50} dose of the extract, 147.4 ± 1.10 µg/ml showed significant differences ($p<0.05$, t-test) as compared to that of the cisplatin treatment with an IC_{50} dose of 90.55± 0.76 µg/ml (Figure 5B). Additionally, the decrease in cell viability was found to be dose-dependent (Figure 5C).

Figure 5: Estimation of cell viability through MTT assay. (A) Analysis of cell viability of DL and normal cells on treatment with extract and cisplatin using two-way ANOVA((B) Log transformed and normalized IC_{50} dose (C) Linear regression analysis exhibiting correspondence in the decrease of cell viability with an increase in dose. All data are represented as mean± SEM and the asterisk represents significance level ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05.

LDH (Lactate Dehydrogenase) assay:

The percentage of cell cytotoxicity was further assessed by measuring the amount of lactate dehydrogenase enzyme in the cell supernatant using LDH assay. The outcome of the

assay presented the effective cytotoxic response of the extract indicated by a significant ($p<0.0001$) increase in % cytotoxicity compared to that of the cytotoxicity of the cisplatin-treated cells at each dose of treatment. However, the normal cells represented the lowest % of



cytotoxicity compared to the DL cells treated with extract. The % cytotoxicity of the normal cells at the highest dose of treatment was about 15% whereas the extract-treated DL cells showed 52% cytotoxicity at the same dose (Figure 6A). Further, the IC_{50} dose of extract at 190.6±1.24µg/ml represented statistical significance (t-test, $p<0.001$) from that of the 50% cytotoxicity dose of cisplatin shown at 94.70± 1.07µg/ml (Figure 6B). Furthermore, a positive proportionality was found between dose and increase in cytotoxicity (Figure 6C).

Figure 6: Cell cytotoxicity determined through LDH assay. (A) Analysis of cell cytotoxicity exhibited by the DL and normal cells on treatment with extract and reference drug cisplatin through two-way ANOVA (B) Log transformed and normalized IC_{50} dose (C) Analysis of proportionality of cytotoxicity with dose using linear regression. Results are expressed as mean ±SEM and the asterisk represents significance level ****$p<0.0001$, ***$p<0.001$, **$p<0.01$.

DISCUSSION

Traditionally used medicinal plants are the subject of interest among researchers for drug discovery. Plants are found to be rich sources of naturally occurring phytochemical compounds which are found to have various biological activities. Hence, identifying compounds in the plant sample is growing inquisitiveness among researchers to estimate its pharmaceutical potential[27]. The present study evaluated the occurrence of phytochemicals, antioxidant activity and assessed the *in vitro* cytotoxic potential of a traditionally used medicinal plant, *C. colebrookianum* methanol extract against the murine tumor model, Dalton's Lymphoma (DL) cells. The qualitative phytochemical analysis revealed that the plant is rich in phytoconstituents such as carbohydrates, glycosides, phenols, tannins, polyphenols,

flavonoids, and terpenoids (Figure 1). The study's findings corroborate with the studies conducted by [11,14]. Further, studies have reported that these phytoconstituents have various pharmacological properties. Flavonoids are an important class of secondary metabolites exhibiting an array of biological activities which include anti-cancer, anti-inflammatory, anti-ulcer, anti-bacterial, anti-hypertensive, anti-depressant, anti-diabetic, and also effective in Parkinson's disease [28]. Moreover, polyphenols are also reported to have an array of biological activities such as antioxidant, anti-inflammatory, anticancer, anti-microbial, and protection against heart diseases [29]. Similarly, another group of metabolite terpenoids is the most abundant and found with diverse structures. These compounds are also pharmacologically active and exhibit potent anticancer, antiviral, antibacterial, antimalarial, anti-inflammatory, cardioprotective, and hypoglycaemic activities [30]. Thus, the existence of such phytochemicals in the methanol extract of *C. colebrookeanum* suggests that it is rich in active phytochemicals and supports its traditional use to treat various ailments.

Further, the antioxidant activity of the extract determined through the scavenging effect of the free radical, DPPH showed potent results in comparison to standard ascorbic acid. The scavenging effect was found to increase significantly compared to ascorbic acid (Figure 2A). Further, a moderately low IC_{50} dose closer to the standard was obtained indicating its potent antioxidant potential (Figure 2B). A similar observation was concluded by the previous experiments where the antioxidant activity was found to increase with dose concentrations [11, 15]. However, a variation in IC_{50} dose was observed which could be due to the difference in the place of collection. Moreover, the presence of phytoconstituents such as phenols, polyphenols, and flavonoids which have natural antioxidant properties might contribute to the observation of such antioxidant potency of *C. colebrookeanum* methanol extract.

The *in vitro* cytotoxicity potential of the extract determined through the various cell viability assays such as trypan blue dye method, MTT, and LDH showed that the methanol extract of *C. colebrookianum* exhibited a positive cytotoxic response against DL cells. The results of the trypan blue method showed an increment in the occurrence of non-viable DL cells on treatment with extract (Figure 3). Moreover, the observation of morphological features such as extensive membrane blebbing, cell shrinkage, and membrane disintegration substantiating the cell death observed (Figure 4). The development of the tumor is accompanied by suppression of apoptosis accomplished through anti-apoptotic proteins or the occurrence of mutations in the proteins causing apoptosis [31]. Thus, the observation of apoptotic features in DL cells indicates the apoptosis-inducing potential of the extract. Similarly, the outcome of the

MTT assay showed that the cell viability of DL cells decreased significantly compared to cisplatin-treated cells in a dose-dependent manner (Figure 5), and an increase in the percentage of cell cytotoxicity with dose was also observed in the LDH assay (Figure 6). Furthermore, in all the assays a low IC_{50} value was observed for the extract which significantly differed from that of the IC_{50} dose of cisplatin (reference drug) indicating the *in vitro* antitumor potential of the extract. Interestingly, the normal cells (splenocytes) remain unaffected by extract treatment as higher cell viability and low cytotoxicity were observed at all the treatment concentrations. This observation indicated the specificity of extract towards carcinoma cells. A similar observation of the *in vitro* antitumor potential of *C. colebrookianum* was observed against HepG2 carcinoma cells [17]. Thus, the results of the *in vitro* antitumor studies indicate the potency of the extract as an anticancer agent which could lead to further exploration through *in vivo* studies.

CONCLUSION

The outcome of the current study showed the rich diversity of phytochemicals in the *C. colebrookianum* leaves methanol extract which are found with different pharmacological activities. Moreover, the potent antioxidant activity was observed indicating it to be a natural source of antioxidants. Further, a positive cytotoxic effect was observed against the DL cells revealing its antitumor potential. This study will lead to further *in vivo* exploration of the plant to affirm its anticancer potential and isolation of the active components which might help in the drug discovery process.

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AUTHOR CONTRIBUTIONS: NSS and AKV conceive the idea and correct the manuscript, MD perform the experiment and MD draft the manuscript, RKS collect the sample and help in experiments.

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