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BIOCHEMICAL ACTIVITY AND *IN VITRO* ANTICANCER PROPERTY ANALYSIS OF *CALOTROPIS GIGANTEA* L.

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Abstract

Plants are nature's treasure, providing oxygen, food shelter and medicinal properties while also playing a vital role in maintaining the balance of our ecosystem and supporting life on Earth. They possess immense potential in medicine, with many species containing bioactive compounds that can be used to develop new treatments, drugs and therapies for various diseases. The phytochemicals present in a particular plant are the basis of its curative properties. *Calotropis gigantea* L. belongs to the family Asclepiadaceae is a medicinal plant which is used for treating different ailments. The present study aimed to evaluate phytochemical, nutritional, antioxidant and *in vitro* anticancer activity analysis and *in vitro* conservation of *Calotropis gigantea.* The plant is highly known for its therapeutic activities. Crude methanolic leaf extract is used for the preliminary phytochemical analysis. Different phytochemicals such as reducing sugar, alkaloids, flavonoids, terpenoids, steroids, glycosides, tannins and saponins were qualitatively analysed. Phytochemicals can have direct or indirect effect on the medicinal properties attributed to the plant. Nutritional factors like reducing sugar, total carbohydrates, total proteins, pigments and starch were analysed by standard estimation methods and found very low amount of nutritional factors in *Calotropis gigantea*. The non-enzymatic and enzymatic antioxidants were also estimated. Different non enzymatic antioxidants like proline, lycopene, total polyphenol and carotenoids and enzymatic antioxidants like superoxide dismutase, amylase, polyphenol oxidase and lipid peroxidase were estimated quantitatively by standard estimation procedures and found to be higher. Higher amount of antioxidants was found in *Calotropis gigantea*, satisfying its use as a potential source of antioxidants. Anticancer analysis of crude methanolic leaf extract in EAC (Ehrlich's Ascites carcinoma) and DLA (Dalton's Lymphoma Ascites) showed higher cytotoxicity in EAC compared to DLA against standard drug revealing promising anticancer effects of the leaf extract. *In vitro* conservation of different explants on Murashige and Skoog (MS) medium supplemented 2 mg/L BAP showed less chance of survival rate. Present study evaluated various nutritional, medicinal and regenerative aspects of the plant *Calotropis gigantea* exhibiting diverse potentialities of the plant and provide supporting information for its use as an ethnomedicinal plant.

Keywords: *Calotropis gigantea*, Asclepiadaceae, EAC, DLA , *In vitro* conservation

Introduction

Since ancient times, plants have been a valuable source of drugs; nature has always played a major role in catering for the health of man. A large proportion of the world population depends on herbal medicine for primary health care (Haque *et al*., 2021). Secondary metabolites are chemically and taxonomically extremely diverse compounds with obscure function. They are widely used in the human therapy, veterinary, agriculture, scientific research and countless other areas (Vasu *et al*., 2009). Many herbaceous and medicinal plants contain important vitamins and phytochemicals such as alkaloids, flavonoids, tannins, cyanogenic glycosides, phenolic compounds, saponins, lignin and lignans. Vitamin-C, Vitamin-E and carotenoid which are utilized both by humans and animals as important components of diets (Okwu *et al*., 2005). Those plants having superior genotypes, medicinal properties and those under the threat of extinction, can be regenerated using the scope of biotechnological tool of micro-propagation or tissue culture. Micro propagation, slow or reduced growth cultures and cryopreservation are some of the *in vitro* techniques under ex-situ conservation method. Therefore, the need of *in vitro* production as an alternative can ensure quality

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plant materials in large scale without the destruction of natural habitat and also satisfying the growing demand as it enables propagation of plant material with high multiplication rates (Wala *et al*., 2003). The present study intends to highlight medicinal value of plant *Calotropis gigantea* and its application, as well as significant resources for future research.

Materials and methods

Plant Material Collection

The plant *Calotropis gigantea* was collected as fresh from Kesavadasapuram, Thiruvananthapuram district of Kerala. The plant specimen was made into herbarium and deposited in the herbarium repository of Botanical Survey of India (BSI), Southern Regional Centre (SRC), Coimbatore-3 and authenticated (Plant authentication No.-336). The plant material was washed and dried in a shady environment at room temperature before being crushed to powder using a mixer grinder.

Preparation of Plant Extract

The dried plant materials were extracted with methanol for 8 hours by soxhelt apparatus and extract obtained as green, black solid respectively. After which, the residues were transferred to pre-weighted sample container for storage and later used for phytochemical screening.

Preliminary Phytochemical analysis

Qualitative phytochemical analysis of *Calotropis gigantea* extract was done by the standard protocol to determine whether certain bioactive chemicals are present or not (Harborne, 1977).

Quantitative Analysis.

The fresh samples of plants were used for the analysis of nutritional, non-enzymatic and enzymatic antioxidant properties of *Calotropis gigantea* and experiment was repeated thrice to confirm the result.

In vitro Conservation

In vitro conservation of the plant was carried out by using explants like leaf, stem, petiole, node with axillary bud and terminal bud. The explants were pre-sterilized in tap water for 15 minutes, trimmed and washed with tap water and three drops of labolene for 10 minutes. Surface sterilization was done using 1% sodium hypo-chloride solution and 0.1% mercuric chloride solution for 5 minutes followed by washing with distilled water. MS (Murashige and Skooge) medium supplemented with 2 mg/L BAP adjusted to *p*H 5.8 was used for inoculation. The culture was maintained under 16 hours' photoperiod at a temperature of 26℃.

Results and Discussion

Preliminary phytohemical analysis

The preliminary phytochemical analysis of *Calotropis gigantea* showed the presence of alkaloids, flavonoids, terpenoids, steroids and tannins. But the presence of reducing sugar, glycosides and saponins were not detected (Table 1).

Table 1. Preliminary phytochemical analysis in crude methanolic leaf extract of *Calotropis gigantea*.

Sl. No.	Phytochemicals	Methanolic extract of *Calotropis gigantea*
1	Reducing sugar	-
2	Alkaloids	+
3	Flavonoids	+
4	Terpenoids	+
5	Steroids	+
6	Glycosides	-
7	Tannins	+
8	Saponins	-

(+ denotes presence, - denotes absence)

Quantitative Analysis

Nutritional Evaluation

Nutritional factors present in *Calotropis gigantea* like reducing sugar, total carbohydrates, reducing sugar from the leaves of *Calotropis gigantea* were extracted and analysed by Dinitrosalycylic acid method and the results were also found to be low (0.04 mg g-1) as shown in

figure 1. Total protein, starch and pigments were also analysed quantitatively. Excessive consumption of reducing sugars can contribute to various health issues, including obesity, diabetes and heart disease. Thus, there has been a growing interest in reducing sugar intake, which has led to the development of sugar alternatives and the reformulation of food products to lower their sugar content (Gropper *etal*., 2018). Carbohydrates play several vital roles including providing energy, regulating blood glucose levels and sparing the use of proteins for energy. Additionally, they are involved in the synthesis of certain amino acids and fatty acids (Whitney and Rolfes, 2018). The amount of total carbohydrates present in leaf of *Calotropis gigantea* was estimated by using Anthrone Method and found to be lower (0.313 mg g-1) as shown in figure 2.

Leaves when consumed in adequate quantities can supplement protein with other sources are lacking, since they have vitamins minerals and of the essential amino acids (Ghali and Alkoaik, 2010). Estimation of proteins from the leaves of *Calotropis gigantea* was done by Lowry's method and the amount of proteins was found to be lower (0.558 mg g-1) as shown in figure 2. Foods rich in starch are a staple in many diets around the world and are important for providing sustained energy (Brown, 2017). The estimation of starch from the leaves of *Calotropis gigantea* was found to be lower (0.05352 mg g-1) as shown in figure 4. The first plants, which appeared in the Mesozoic era, were probably cream coloured and only with time developed sharper colours, increasing the concentration of various pigments (Raven, 2005). The different pigments in *Calotropis gigantea* was estimated using Arnon's formula and found alow quantity of chlorophyll-a (0.00742 mg g-1), chlorophyll-b (0.003403 mg g-1) and total chlorophyll (0.00941 mg g-1) as shown in figure 3. The nutritional analysis of leaves of *Calotropis gigantea* showed the presence of very low amount of nutritional factors such as, reducing sugars, carbohydrates, proteins, starch and pigments.

Evaluation of antioxidant properties

Plants are a rich source of antioxidants, which help protect cells from damage caused by free radicals. Antioxidants play a crucial role in maintaining overall health by neutralizing free radicals, reducing inflammation, protecting cells, supporting immune function etc. Evaluation of enzymatic and non-enzymatic antioxidants in *Calotropis gigantea* can help in understanding the therapeutic potential of the plant in terms of its antioxidant properties. Present study evaluated non-enzymatic antioxidants like proline, lycopene, carotenoids and polyphenols and enzymatic antioxidants such as superoxide dismutases (SOD), polyphenol oxidase (PPO), amylase and lipid peroxidase (LP_X) by standard estimation methods.

Non-Enzymatic Antioxidant

Proline catabolism in mitochondria is linked to oxidative respiration and it gives out energy for growth resumed after stress (Szabados and Arnould, 2010). The amount of proline in *Calotropis gigantea* (0.891mg g-1) was found to be higher (figure 5). Lycopene is a powerful antioxidant and carotenoid pigment found in certain fruits and vegetables. It has been shown to have anti-cancer properties, particularly in reducing the growth and proliferation of cancer cells (David and Lu, 2002). The estimated amount of lycopene in methanolic leaf extract of *Calotropis gigantea* (0.671mg g-1) is shown in figure 5 and found to be higher. Carotenoids can quench-highly reactive singlet oxygen and block free radical mediated reactions (Bendich and Olson,1989). The amount of carotenoids in *Calotropis gigantea* (0.518 mg g-1) is found to be higher (figure 5). Polyphenol compounds are diverse group of bioactive organic compounds that have been known for their remarkable health benefits, antioxidant properties and potential to prevent chronic diseases. They help in prevention of hypercholesterolemia, hyperglycemia, hyperlipidemia and cancer insurgence (Abbas *et al*., 2017). The phytochemical estimation revealed that the total polyphenol content in *Calotropis gigantea* was found to be higher

(0.913 mg g-1) as shown in figure 6.

Enzymatic Antioxidants:

Super Oxide Dismutase (SOD) is essential for protecting cells from oxidative stress, maintaining cellular homeostasis, preventing cell damage and death and reducing inflammation. Intracellular SOD may play key role protection of cancer cells against reactive oxygen species generated by anticancer drugs and radiation (Shingo *et al*., 1994). The amount of superoxide dismutases present in *Calotropis gigantea* (2.950 mg g -1) is found to be higher as shown in figure 6. Increased PPO activity in oxidative browning in wounded or infected indicates its importance in plant defense against infection or wounding (Yoruk and Marshall, 2003). Enzymatic antioxidant polyphenol oxidase in *Calotropis gigantea* is found to be higher (0.987 mg g-1) as shown in figure 6.

There are three types of amylases namely salivary amylase, pancreatic amylase and microbial amylase. Amylase play important role in inducing growth of embryo by the breakdown of starch to sugar in the seeds (Pradeesh and Swapna, 2018). The estimated amount of enzymatic antioxidant amylases in *Calotropis gigantea* (0.957 mg g-1) is shown in figure 6 and found to be higher. Lipid peroxidase (LPx) an enzyme that catalyses the oxidation of lipids, leading to the formation of lipid peroxides. This process can cause cellular damage, inflammation and contribute to various diseases. (Pradeesh and Swapna, 2018). The result revealed that the amount of enzymatic antioxidant lipid peroxidase in *Calotropis gigantea* is found to be higher (0.991 mg g-1) as shown in Fig. 6.

Evaluation of Pharmacological Property

***In vitro* Anticancer Activity in Crude Methanolic Extract of *C. gigantea*.**

Plants have been used for centuries in traditional medicine to treat various diseases and health conditions. Cancer is a complex and multifaceted disease and plants have been found to have potential in its treatment and prevention. Present study evaluated *in vitro* anticancer activity of *Calotropis gigantea* leaf extract in methanol. Anticancer effect was analysed using Dalton's Lymphoma Ascites (DLA) and Ehrlich Ascites Carcinoma (EAC) cell lines. Viability was determined by Trypan blue dye exclusion method. The viable cell suspension 1×10^6 cells in 0.1 ml was added in the tubes containing various concentrations (100, 500 and 1000 µg/ml) of test compounds and the volume was made up to 1 ml using phosphate buffer saline (PBS). The mixtures were incubated for 3 hours at 37°C and were added with 2 drops of Trypan blue dye. Dead cells take up the blue colour of the dye while the live cells do not. Reduction in the viable cell count and increased non-viable cancer cell count towards normal in tumour-host suggest antitumor effect against EAC and DLA cells in mice. Cyclophosphamide is used as standard anticancer compound. The result obtained from anticancer study revealed that the methanol extract of *Calotrpis gigantea* showed 50.034, 82.917, 90.103 %cytotoxicity in EAC compared to 46.981, 71.531, 83.694% (figure 7).Fijesh (2011), reported that the extract treated cells showed membrane blebbing, vacuole formation and nuclear condensation which was absent in untreated cells. Thus the cytotoxic and antitumor effects of the leaf extract can provide possibilities to novel therapeutic findings for treating cancer cells.Result obtained in the present study demonstrated that the methanol extract of leaf of *Calotropis gigantea* exhibits *in vitro* anticancer activity against DLA and EAC cell lines. The leaf extracts showed concentration dependent cytotoxicity which was found to be effective against solid tumour induced by DLA and ascites tumour induced by EAC.

Table 2. *In vitro* anticancer activity in leaves of *Calotropis gigantea*

Concentration	**Standard**	**DLA**	**EAC**
100µg/ml	60.908	46.981	50.034
500µg/ml	86.39	71.531	82.917
1000µg/ml	98.19	83.694	90.103

***In vitro* Conservation of *Calotropis gigantea*.**

In vitro conservation of *Calotropis gigantea* was carried out with explants such as leaf and petiole, node with auxiliary bud and terminal bud in MS medium supplemented with 2 mg/L BAP. The results revealed that the leaf explant have lesser survival chance and they were highly vulnerable to fungal infection from the first week itself. The results of *in vitro* conservation of leaf explants of *Calotropis gigantea*was disappointing (Plates 1a & b).

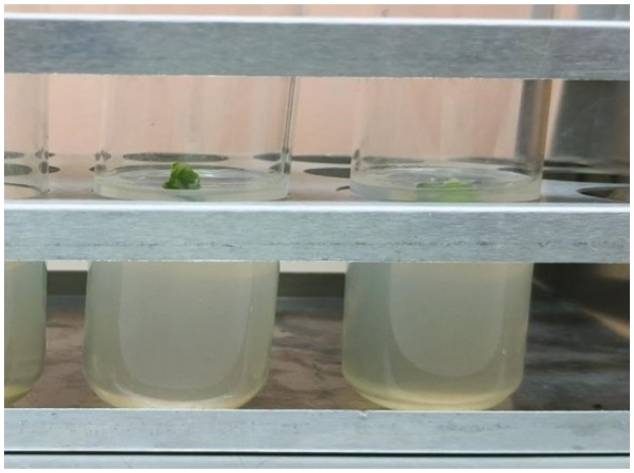


Plate 1a. Inoculated leaf explants of *Calotropis gigantean*

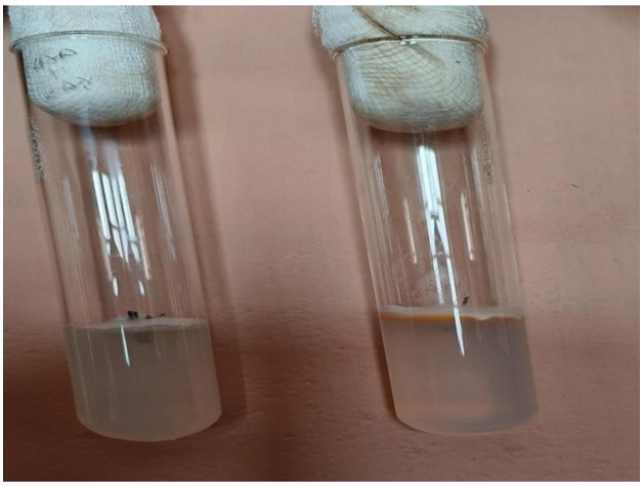


Plate 1b. Infected explant of *Calotropis gigantea*

Fig. 1. Reducing sugar in leaves of *Calotropis gigantea*

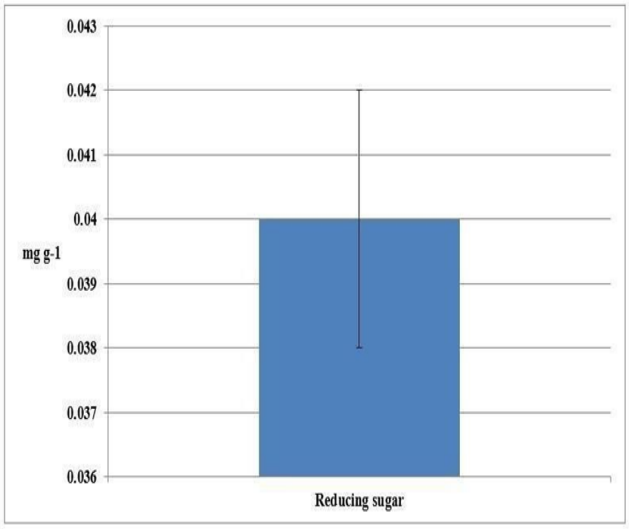


Fig. 2. Total carbohydrates and Total protein in leaves of *Calotropis gigantea*

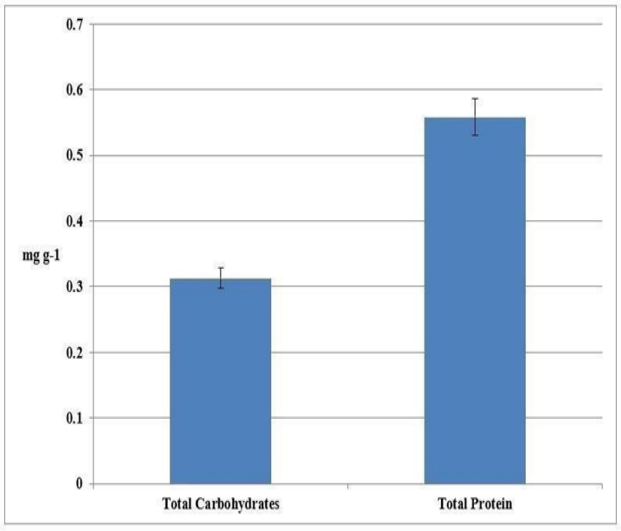


Fig. 3. Pigments in leaves of *Calotropis gigantea*

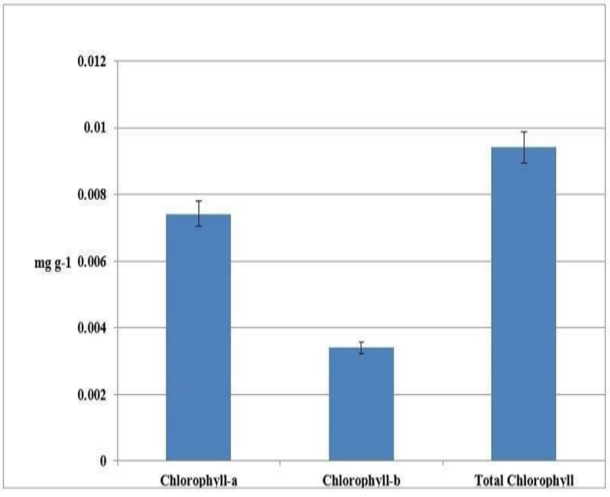


Fig. 4. Starch in leaves of *Calotropis gigantea*

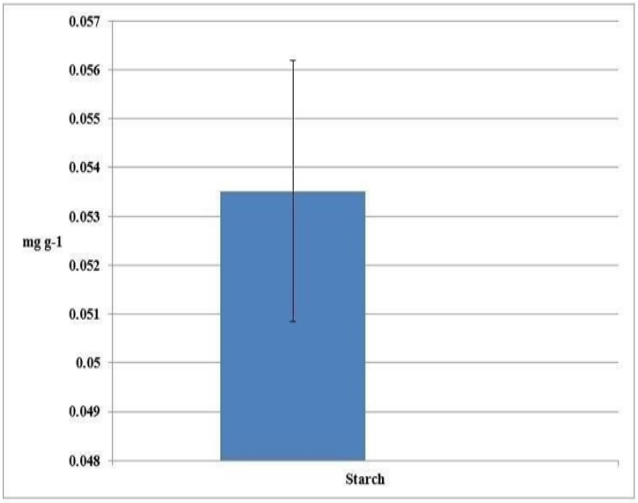
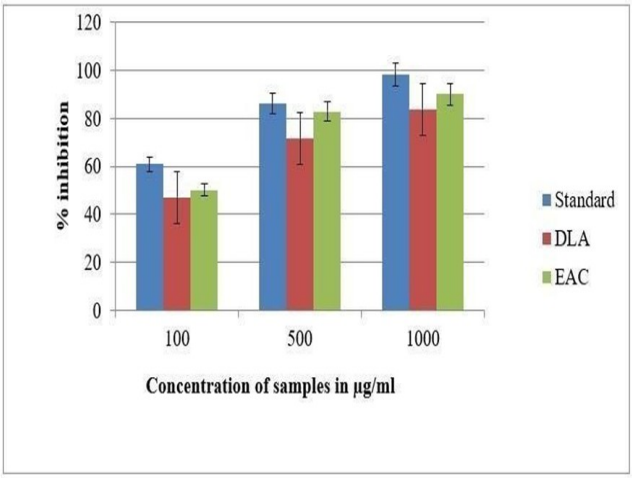


Fig. 7. *In vitro* anticancer activity in leaves of *Calotropis gigantea*



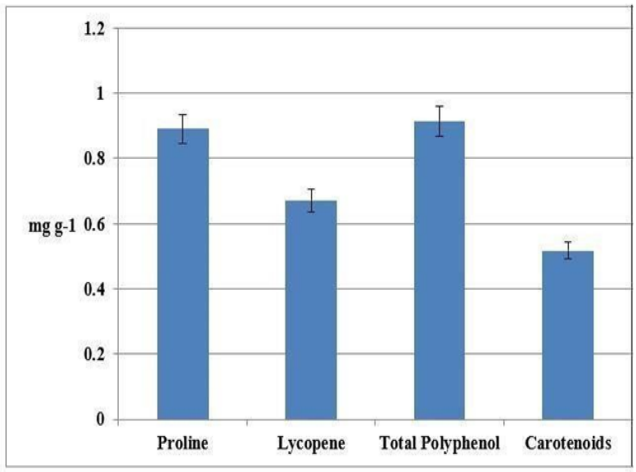
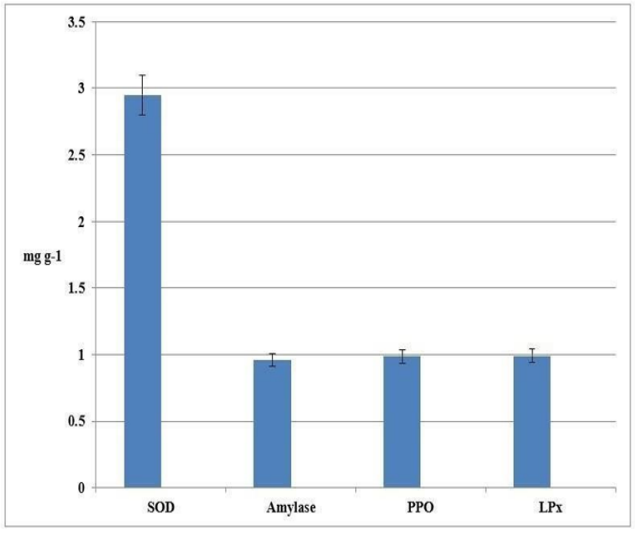


Figure 5. Non-Enzymatic Antioxidants in leaves of *Calotropis gigantea*

Fig. 6. Enzymatic Antioxidants in leaves of *Calotropis gigantea*



Summary and Conclusion

The leaves of *Calotropis gigantea* screened for phytochemical constituents seemed to have potential as source of useful drugs and also to improve the health status of its users as a result of the presence of various compounds that are vital for good health. Quantitative analysis of the phytochemicals of these plant leaves and also the anti-fungal and antimicrobial activities should be investigated. Results obtained from the *in vitro* anticancer analysis of crude methanolic leaf extract of *Calotropis gigantea* showed that the concentration dependent anticancer effect in DLA and EAC cell lines were found to be higher. High cytotoxicity was showed in EAC than in DLA on increased concentration. This reveals the anticancerous potential of this plant in the field of cancer therapy. *In vitro* conservation of *Calotropis gigantea* was done with different explants such as leaf and petiole, node with auxiliary bud and terminal bud. The culture was decontaminated several times but the chances of survival was very low. All the explants failed to persist in the culture medium and the results of *in vitro* conservation of *Calotropis gigantea* was disappointing. This generated information on phytochemical, nutritional and medicinal characteristics and therapeutic potential of *Calotropis gigantea* provide scientific evidence for identifying the plant as a potential bioresources and its effective utililisation in the future.

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