In vitro Haploid Plant Production of *Calotropis gigantea* L. a Potential Medicinal Plant and Its Biological Activities

DEEPIKA.K

(Reg.No: 17PBO005)

Thesis submitted to the

Department Of Botany

Avinashilingam Institute For Home Science And Higher Education For Women, Coimbatore-641043

In partial Fulfilment of the Requirement for the

Degree of Master of Science in Botany

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Signature of the Head of the Department **Signature of the Supervisor**

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CHAPTER I

INTRODUCTION

1.1. GENERAL INTRODUCTION ABOUT MEDICINAL PLANTS

Medicinal plants are precious for human diseases. Herbs and plants have been in use as a source of therapeutic compounds in traditional medicinal system since ancient time. There is a continuous need of the development of new effective antimicrobial drugs because of the emergence of new infectious diseases and drug resistance. Most recently plants got a great attention of scientists for the development of alternative drugs to cure several lethal diseases (Kumar *et al.*, 2010). Some people value these plants due to the ancient belief which says plants are created to supply man with food, medical treatment and other effect. It is thought that about 80% of the 5.2 billion people of the world live in the less developed countries and the world health organization estimates that about 80% of these people rely almost exclusively traditional medicine for their primary health care needs (Maryam *et al*, 2012).

The ancient system of medicinal treatment is based on the rich experiences of innumerable vaidyas over thousands of year, having trials on hundreds and thousands of human being to its credit, to which no modern system of treatment in the world can ordinarily lay claim (Kapoor, 2017). In the ensuing 15 years, great strides were achieved by natural product chemistry all over the world. More and more new plants of varied properties and diverse uses for known plants were discovered mainly due to the leads taken from ethnobotany. Data on the chemical constituents on important plants increased by leaps and bounds. Experimental proof on the activities of individual compounds was also made available (Mammen Daniel, 2016).

1.2. MEDICINAL PROPERTIES

Traditional medicine is the sum total of knowledge, skills, and practices based on the theories, beliefs, and experiences indigenous to different culture that are used to maintain health, as well as to prevent, diagnose, improve, or treat physical and mental illness (Fawzi, 2013). Plants have played a central role in human health care since ancient times (Cragg and Newman, 2013). Medicinal plants are globally valuable sources of herbal product, and they are disappearing at a high speed (Shi-Lin Chen *et al*, 2016). Although ancient sages through trail and

error methods have developed herbal medicines, the reported uses of plant species do not certify the efficacy (Maryam *et al*, 2012). Herbal medicine which refers to the medicinal product of plant stem, roots, leaves, bark, seed, flower and fruit is one of the best source for extraction of chemopreventive agents as they are nontoxic in nature and causeless or no side effects to the patients (Arpita *et al*, 2018).

1.3. HISTORY OF PLANT TISSUE CULTURE

In 1902, Gottlieb Haberlandt proposed the idea to culture individual plant cells on artificial nutrient medium. Although he failed to culture them due to poor choice of experimental materials and inadequate nutrient supply, he made several valuable predictions about the nutrient requirement for *in vitro* conditions, which would possibly induce cell division, proliferation and embryo induction (Anwar *et al*, 2017). Plant tissue culture has become an invaluable aid in the field of experimental botany and has many practical applications in agricuture and horticulture (Bhojwani and Maharaj, 1986).

Plant tissue culture or the aseptic culture of cells, tissues, organs, and their components under defined laboratory conditions. Plant tissue culture is an important tool in both basic and applied studies as well as in commercial application. The early studies led to root cultures, embryo culture, and the first true callus / tissue cultures (Trevor, 2012). The field of plant tissue culture is based on the premise that plants can be separated into their parts (cells, tissues and organs), which can be manipulated *in vitro* and then grown back into complete plants (James *et al*, 2018).

1.4. CALLUS CULTURE

In the present study to determine the optimum level of plant growth regulator in different concentration of NAA and BA (mg/l) were used in MS medium for callus induction of stem and leaf explant. Callogenesis was observed in both types of explants (Narges *et al*, 2014). The growth of some calli showed high lignification, including of their hard texture, where as others were embryogenic and separated easily into small fragments (Ehsamdar *et al*, 2013). *In vitro* production of anthraquinone through callus cultures was standardized using *in vitro* leaves, derived from the nodal explant cultures maintain in Murashige and Skoog solid media containing

2mg/l 6-benzylaminopurine and 3% sucrose.

Leaves were harvested from the healthy shoot of axenic cultures and culture conditions were optimized for the establishment of callus (Anjusha and Gangaprasad, 2017). Callus culture can be used to study the stress physiology and genetic improvement at the cellular level and also to select mutants *in vitro.* This would enable the selection of new improved lines with better stress tolerance to be used in potato breeding programes (Laboney *et al,* 2013).

1.5. ANTHER CULTURE

Anther culture is one of the most widely used methods to induce gametic embryogenesis. Anthers were cultured at the vacuolated developmental stage, and seven cultivars, two culture media and two temperature treatments were assessed (Giuseppe *et al*, 2017). The culturability of both genotypes changed on colchicine - supplemented induction media. A high genotype dependency was recorded for callus induction, callus regenerating of green double haploid plantlets (Isidre *et al*, 2018). *In vitro,* the microspore inside the anther divides and follows the gametophytic program to form the mature pollen grain.

In vitro, upon the application of stress treatment the microspore can be deviated towards a proliferation process leading to embryogenesis, the so-called microspore embrogenesis that can be induced in anther and isolated microspore cultures (Pilar *et al*, 2018). Analysis of climatic parameters (temperature, sunshine hours and rainfall) during two growing seasons indicated that anther donor plants grown under better environmental conditions (Mayakaduwa *et al,* 2018).

1.6. FLORI CULTURE

Floriculture is the "culture of plants", in recent decades there has been increasing in demand of floriculture products with increasing income. Floriculture is an emerging area with great potential both in the domestic as well as export market in India commercial floriculture is ongoing development but have a long tradition of various type of flowers (Ninama *et al,* 2016). Today most flowers physically pass through the Dutch auction houses on their fixed routes from growers located all over the world to European customers. Physical presence is necessary to allow for inspection, quality control and break bulk activities (Jack *et al*, 2016).

Floriculture is a small, but important component of the agri-business. Critical and often limited resources such as water and land are required in the cultivation of flowers; therefore it is necessary to ensure that the cultivation and distribution impact of ornamental products on the environment is acceptable and sustainable. The floriculture industry itself is responsible to ensure that all flowers and potted plants meet acceptable levels set by social and environmental criteria (Coetzee and Hoffman, 2017). Floriculture crops affect substrate-pH and resulting solubility of micronutrients in the root zone during commercial production (Ryan, 2016).

1.7. EMBRYOGENESIS

A Somatic embryogenesis system was developed for *Sapindus mukorossi* Gaertn. From leaf explants obtained from fresh flushes of a mature tree. Callus was induced from the midrib region of explants on Murashige and Skoog (MS) medium containing different concentrations of 2,4-dichlorophenoxyacetic acid or 6- benzylaminopurine. Callus induction and somatic embryogenesis was significantly influenced by the size, physiological age, and orientation of leaf explants on the culture medium and plant growth regulators (Reetika *et al*, 2015).

Somatic embryos were obtained from both shoot apex and leaf explants of all three genotypes evaluated, although embroyogenic frequencies were significantly influenced by the species / genotype, auxin and explant type. Cotyledonary somatic embryos were differentiated after subculture of these nodular embryogenic structures on a medium lacking plant growth regulators (Corredoira *et al*, 2015). Somatic embryos were successfully regenerated from callus tissue of anthers and ovaries excised from inflorescences of grapevines. Production of pro-embryogenic masses (PEMS) was controlled by specific growth regulators and culture conditions, including heat incubation at 35˚C (Goussard *et al*, 2017).

1.8. MULTIPLE SHOOT FORMATION

An efficient protocol has been developed for inducing multiple shoots from nodal explants. The sterilized nodal explants were inoculated in MS medium containing various concentration (Balakrishnan *et al*, 2015). The cotyledons of both cultivars were used as explant source for *in vitro* shoot regeneration. The objective of this research was to examine the influence of the growth regulators thidiazuron (TDZ) and naphtheline acetic acid (NAA) on adventitious shoot

formation in these cultivars. This system of adventitious shoot regeneration from cotyledon explants could be useful as tool for transformation of the subspecies (Seyed *et al*, 2014)

Multiple shoot formation was induced from seed grown on agar-solidified Murashige and Skoog (MS)- based medium supplemented with 9.8 mm indole -3-butric acid (IBA). Shoot were used to regenerate plants directly, and some were then used as a source of root explants for callus induction and growth by transferring to MS medium (Wolfgang *et al*., 2015). The study suggest that multiple shoots of *Rugosa roses* can be used in commercial -scale bioreactors to produce useful bioactive compounds for the pharmaceutical and cosmetic industries (Hae-Rim Jang *et al*, 2016).

1.9. ENCAPSULATION

The process of spray drying consists of the transformation of a product in fluid form to the solid state in the form of powder. This is done through the dispersion of droplets from the product inside the chamber, in contact with hot air. In this process, substances known as carriers or encapsulating agents can be added, aiming at facilitating or even allowing the drying of certain products (Samantha *et al,* 2015).

Encapsulation is a critical step in LED packaging to ensure good light extraction and protection. We present a novel mold- free process using a specific functionalization of the substrate to master the shape of the encapsulant dome (Moulin *et al,* 2018). Under fill encapsulation play an important role to improve the reliability of flip chip package, yet the conventional under fill (CUF) encapsulation process is subjected to several drawbacks such as extended filling time, incomplete filling and voids formation (Aizat *et al*, 2016).

1.10. HARDENING

Hardening is simply means of gradual exposure of plantlets to an environmental conditions (Sujoy, 2017). The *in vitro* rooted plantlet were hardened and acclimatized by using different treatments. Plants transplanted at the age of four weeks after root initiation gave maximum survival (100%) during transplanting. These plants were hardened in glass beaker and polythene bags singly or in cluster. The maximum survival during hardening was observed by covering the plantlets with glass beaker individually and kept in culture room (Shahnawaz *et al*, 2014).

Hardening induced the increase in the frost tolerance of all cereals under study, and the resistance of rye and soft wheat was found to be significantly higher than that of durum wheat and barley. After freezing of both hardened and non-hardened seedlings, higher activities of all tested anti-oxidant enzymes were revealed in rye and soft wheat as compared to those in durum wheat and barley (Kolupaev *et al*, 2015).

1.11. AIMS & OBJECTIVES

Calotropis gigantea L. has a vast medicinal uses. Over exploitation of this species by mankind and Pharmaceutical purposes leads to extinction of this species, Due to these medicinal properties this species was selected for *in vitro* micropropagation through plant tissue culture.

- To standardize the *in vitro* propagation techniques for *Calotropis gigantea* L.
- To extract and analyze the secondary metabolities by preliminary quantitive phytochemical screening.
- To test the efficiently of crude extracts such as (HEX, CHLO, ETOAC, MEOH) of *Calotropis gigantea* L. for anticancer activity.

CHAPTER II

REVIEW OF LITERATURE

2.1. INTRODUCTION

Calotropis gigantea L. belongs to Asclepiadaceae family which includes more than 280 genera and approximately 2000 species (Ansari *et al*, 2016). *Calotropis gigantea* commonly known as giant milkweed or swallow wort is known for its medicinal properties. It grows wild in south and southeast of Iran and the fresh latex of the plant is used by local people for the treatment of cutaneous leishmaniasis (Rajesh *et al*, 2005). It is one of the plants classified under semi-poisonous category termed as Upavisha. Upavisha are the group of drugs which are less toxic in nature and not so lethal but they produce certain toxic symptoms on contact or on consumption (Namburi *et al*, 2013). Anti-dysentric and antidiarrheal properties of medicinal plants were found to be due to tannins, alkaloids, saponins, flavonoids, sterols and/or triterpenes and reducing sugars. The sesquiterpene lactones, a large group of compounds with anti-inflammatory properties have the ability to relax smooth muscles and thereby relieve gastrointestinal distress (Havagiray *et al*, 2004).

2.2. DISTRIBUTION

It is growing widely throughout the tropical and subtropical regions of Asia and Africa. Mostly found under cultivated conditions near temples in India (Ajai *et al*, 2017). The plant *C. gigantea* belonging to the family Asclepiadaceae, a common medicinal plant in Indian subcontinent. *C. gigantea* is a species of *Calotropis* native to Cambodia, Indonesia, Malaysia, Philippines, Thailand, Sri Lanka, India & China (Jitendra *et al*, 2015).

Calotropis gigantea **L.**

Sanskrit name : Arka

English name : Madar

Tamil name : Erukkam

Hindi name : Madara

Malayalam name : Errikke

Ayurveda : Sthavaravishavarga, Upavisha

Systematic position

Kingdom : Plantae

Class : Dicotyledonae

Division : Gamopetalae

Order : Gentianales

Family : Asclepiadaceae

Genus : *Calotropis*

Species : *gigantea*

2.3. BOTANICAL DESCRIPTION

Calotropis gigantea L. (Apocynaceae) is a glabrous or hoary, laticiferous shrubs or small trees, about 3-4 m tall commonly known as the swallow-wort or milkweed. Its stems are erect, up to 20 cm in diameter. The leaves are broadly elliptical to oblong-obovate in shape, with the size of 9-20 cm x 6-12.5 cm but sub-sessile. The cymes are 5-12.5 cm in diameter. The inflorescence stalk is between 5-12 cm long, the stalk of an individual flower is 2.5-4 cm long. Sepal lobes are broadly egg shaped with a size of 4-6 mm x 2-3 mm. Petal is 2.5-4 cm in diameter. It has clusters of waxy flowers that are either white or lavender in colour. Each flower consists of five pointed petals and a small, elegant "crown" rising from the center, which holds the stamens. The plant has oval, light green leaves and milky stem. The flower of the plant contains the cardiac glycosides, calotopin, uscharin, calotoxin, calactin, uscharidin and gigantin (Dhivya *et al*., 2013).

2.4. MEDICINAL PROPERTIES

According to Ajai *et al*., (2017) medicinal applications of both the taxa have been mentioned by many workers as abortifacient, analgesic, anthelmintic, antiangiogenic, anti-cancer, anti-coagulant, anti-convulsant, antidiabetic, anti-diarrheal, anti-dysentric, anti-dyspepsia, antifertility, anti-fungal, anti-inflammatory, anti-microbial, antiodontalgic, anti-oxidant, anti-purgative, anti-pyretic, antirheumatic, anti-sialagogue, anti-syphilitic, anti-tumor, antiviral, appetizer, carminative, diaphoretic, emetic, expectorant, fibrinolytic, gastroprotective, hepatic protective, hypolipidemic, larvicidal, mosquitocidal, molluscicidal, nematicidal, proteolytic, purgative, renal protective and spasmogenic, mostly by local people, like anaemia, anasarca, ascites, baldness, body pain, burn injuries, constipation, deafness, elephantiasis, flatulence, headache, indigestion, iridocyclites, jaundice, leprosy, leucoderma, migraine, mumps, nausea, paralysis, piles, otitis, sinus fistula, snake bite, tooth aches, vomiting, wound healing, and rheumatoid/joint pains.

2.5. Chemical constituent present in *Calotropis gigantea*

The hydroethanolic extract of root of *Calotropis gigantea* displayed a significant and dose dependent anti-diarrhoeal property. Previous studies showed that anti-dysenteric and anti-diarrhoeal properties of medicinal plants were mostly due to tannins, alkaloids, saponins, flavonoids, sterol and triterpenes. Sesquiterpenes, diterpenes, terpenes, flavonoids and terpenoid

derivatives are known for inhibiting release of autocoids and prostaglandins, thereby inhibit the motility and secretion induced by castor oil. When stem, branches & leaves are cut, crushed or incised, it yields thick, acrid milky white latex called Arkaksheera (madar juice).The juice forms into a white clot or coagulum leaving a clear straw coloured serum after it is heated or allowed to stand for few hours (Modi *et al*, 2013).

Ahmed *et al.*, (2003) analyzed the free radical scavenging activity of the ethanolic extracts of leaf and latex of *Calotropis procera* and *Calotropis gingantea* were tested of free radical scavenging activity using 1,1 Dipthenyl-Picryl-hydrazylradicas (DPPH). The latex extracts of *Calotropis procera* and *Calotropis gigeantea* (10 mg/ml) exhibited greater capacity to scavenge DPPH radicals whereas leaf extract showed moderate free radical scavenging activity.

Havagiray *et al.*, (2004) studied anti-diarrheal activity of *Calotropis gigantea.* The phytochemical analysis of the extract revealed the presence of sugars, flavonoids, flavonol glycosides, oxypregnane-oligoglycosides, terpenes, terpene derivatives, pentacyclic triterpenoids and triterpenoids and they have been isolated from *Calotropis gigantea*. These constituents may mediate the antidiarrhoeal property of the *Calotropis gigantea* extract. Although the antidiarrheal properties of the reported active terpenoids are well established, aspects of their mechanism of action remain poorly understood. Sesquiterpenes, diterpenes, terpenes, flavonoids and terpenoid derivatives are known for inhibiting release of autocoids and prostaglandins, thereby inhibit the motility and secretion induced by castor oil.

According to Adak *et al.*, (2006) evaluated the effect of Anti-inflammatory activity of *Calotropis gingantea* in various experimental animal models. The anti-inflammatory activity was evaluated using carrageenin-induced kaolin-induced rat paw oedema for acute and cotton-pellet granuloma, adjuvant-induced arthritis model for chronic inflammation. Antipyretic activity was carried out using yeast induced pyresis method. Ameeta *et al.,* (2006) was studied the alcoholic extract of peeled roots of *Calotropis gigantea* was tested orally in albino rats at the dose level of 250 and 500 mg/kg bodyweight for CNS activity. Subhas *et al.*, (2010) was evaluated on anti-convulsant activity of stem barks of *Calotropis gigantea* in experimental animals. Ramasubramania *et al.*, (2016) studied the interfere with heart function, Vomiting, Diarrhoea, Slow heartbeat, Convulsions and death.

Srivastava *et al.*, (2007) studied the ethanolic extract of the roots of *Calotropis gigantea* Linn. exhibited 100% pregnancy interceptive activity in rats when administered as a single oral dose of 100 mg/kg on Day 1 postcoitum. The extract also exhibited 100% efficacy at the dose of 12.5 mg/kg when administered in the Days 1-5 and 1-7 postcoitum schedules. When administered during the peri-cum-early post implantation period (i.e., Days 5-7 postcoitum at 250 mg/kg), most of the implantations showed signs of resorption.

According to Ashraful *et al.*, (2008) the methanol extract of root bark of *Calotropis gigantea* and its petroleum ether, chloroform and ethyl acetate fraction were tested for antibacterial activity by disc diffusion assay method (Vander and Vlietnck, 1991). Six pathogenic bacteria (*Bacillus megaterium* BTCC18, *Bacillus subtilis* BTCC19, *Sarcina lutea* ATCC27853, *Shigella sonnei* AJ8992, *Pseudomonas aeruginosa* ATCC27853 and *Escherichia coli* ATCC25922) were collected from the Institute of Biological Science (IBSC), University of Rajshahi, Bangladesh. Standard *Kanamycin* disc (30 µg/disc) and blank disc impregnated with the respective solvent, were used as positive and negative control respectively to study the antibacterial activity. The antibacterial activity of methanol extract and its petroleum ether, Chloroform and ethyl acetate fractions were tested against six bacteria at concentrations of 20 µg/disc, 30 µg/disc and 40 µ g/disc.

Saumya Das *et al.,* (2009) determine the anti-inflammatory action of the extract by the paw edema method. Paw edema was induced by a sub-plantar injection of 0.1 ml. of 1% carrageenan (in 5% gum acacia). The edema volume was determined using a Plethysmometer prior to and first, third and fifth hours after carrageenan injection. The animals were treated with the standard drug Ibuprofen at different dose levels (25, 50, 100 and 150 mg/kg. body weight). Other groups of animals were treated with the ethanolic extract of *Calotropis gigantea* (400mg/kg. body weight) and the fresh leaves extract of *T. procumbens* (300mg/kg. body weight) along with each doses of Ibuprofen respectively. The test drugs were given one hour prior to carrageenan injection. The control group received saline only.

Narendra nalwaya *et al.*, (2009) studied the wound healing activity of *Calotropis gigantea,* Excision and incision wound models were used to evaluate the wound-healing activity of latex of *Calotropis gigantea.*

Rowshanul *et al.*, (2009) evolved the ethyl acetate extract in *Calotropis gigantea,* compound 1 and compound 2 were tested separately for antibacterial and antifungal activity by

disc diffusion assay method. Kanamycin disc (30 µg/disc) and Nystatin disc (100 µg/disc) were used as positive antibacterial and antifungal control, respectively. Blank disc impregnated with the respective solvent was used as negative control. The antibacterial activity of each sample was tested against each bacterium at concentrations of 30 µg/disc, 60 µg/disc and 90 µg/disc. For antifungal screening, each sample was tested at concentrations of 100 µg/disc, 200 µg/disc and 400 µg/disc. Antibacterial assay plates were incubated at 37 ± 1°C for 24 h and antifungal assay plates were incubated at 37 ± 1°C for 48 h. Each experiment was carried out in triplicates, and diameter of the zone of inhibition surrounding each disc was recorded.

Kumar *et al.*, (2010) studied the antimicrobial activity of the crude latex extract was determined by agar well diffusion method. The concentration of the microbial suspensions was adjusted to 0.5 McFarland standards. The bacterial suspensions were seeded on MHA plates and fungal suspensions on SDA (intriplicates). In each of these plates two wells were cut out using a sterilize cork borer. Using a micropipette, 100 µl of crud extract and negative control was added in to different wells. A positive control antibiotic disc was placed in the plate. Bacterial plates were incubated for 24 hours at 37°C and fungal plates for 72 hours at room temperature. Antimicrobial activity was evaluated by measuring the zone of inhibition.

Kumar *et al*., (2010) evolved that the anti-Candida activity of the crude extracts were determined by the agar well diffusion method with some modifications. The concentrations of test suspensions were adjusted to 0.5 McFarland standards by using a spectrophotometer. The *Candida* cultures were inoculated on PDA plates by using sterilized cotton swabs. In each of these plates, five wells were cut out using a standard cork borer (7 mm). Using a micropipette, 100 µl of each extracts and negative control (DMSO) was added in to different wells. A positive control (Fluconazole) disc was placed in the plate. Plates were incubated for 24 hours at 37°C. Anti-Candida activity was evaluated by measuring the zone of inhibition. Experiment was performed in triplicates.

Gaurav *et al.*, (2010) studied that the antimicrobial activity *Calotropis gigantea* of the crude extracts was determined by the agar well diffusion method. All test organisms were inoculated in Mueller Hinton broth (pH 7.4) for 8 hours. The concentration of the suspensions was adjusted to 0.5 (optical density) by using a spectrophotometer. Isolates were seeded on Mueller Hinton agar plates by using sterilized cotton swabs. Agar surface was bored by using

sterilized gel borer to make wells (7 mm diameter). 100 µl of the test extract and 100 µl of sterilized distilled water (negative control) were poured in to separate wells. The standard antibiotic disc was placed on the agar surface as positive control. Plates were incubated at 37°C for 48 hours. Triplicate plates were maintained for each organism.

Baskaralingam *et al.*, (2012) was studied that the application of metallic nanoparticles to control microbial mediated diseases remains largely unexplored. Several methods have been employed to synthesize AgNps, including green chemistry. Although a number of physical and chemical methods for the synthesis of AgNps are available, there is a need to develop an eco-friendly method for the synthesis process. The green synthesis of AgNps has been reported in bacteria and angiosperms. In the present study, aqueous extracts of *Calotropis gigantea* were used to synthesize AgNps and turbing the cell permeability and respiratory functions. It has been suggested that the nanoparticles cause cell wall protein and other protein denaturation and prevent the replication process taking place. In conclusion, the green synthesis of metallic nanoparticles on overcoming multidrug resistant bacterial pathogens is still an unexplored area of research, and this study has made an attempt to show the capability of AgNps to control *Vibrio* as an experimental model. The study successfully demonstrated that AgNps synthesized from *C. gigantea* leaf extract can control *V. alginolyticus* in an *Artemia* culture system. Further studies are needed to assess the long-term toxic effects of AgNPs in the system, and to extend this knowledge to other experimental models.

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According to Jian Sun *et al.*, (2012) have studied the hairy root cultures were obtained by infecting leaf explants of *Calotropis gigantea* with *Agrobacterium rhizogenes* strain LBA 9402 (NCPPB 1855). Hairy roots emerged at wounding sites of the explants were isolated individually and grown on MS basal medium supplemented with 300 mg cefotaxime/l. After three subcultures on new antibiotic-containing medium, the bacterial-free hairy roots were transferred into liquid MMS-CG medium incubated on a gyratory shaker at 100 rpm and 25°C under 16 h photoperiod. The hairy roots were subcultured into fresh medium every 4 weeks. Transformed hairy roots were confirmed by PCR and selected for further experiments.

Dhivya *et* al., (2013) studied that the ethanol flower extracts of *Calotropis gigantea* revealed the presence of medicinally active constituents. The phytochemical constituents of the flower have alkaloids, tannins, phenol, flavonoids, sterols, anthroquinones, proteins and quinones were present in the flower extract. Terpenoids and saponins were found to be absent. The flower of *Calotropis gigantea* was preliminarily screened for the phytochemicals. The ethanolic extract was found to be rich in all the phytoconstituents. It was subjected to Gas Chromatography-Mass spectrometry (GCMS) analysis.

Amit gupta *et al.*, (2015) studied the antigen IBD specific immune response were evaluated in human peripheral blood mononuclear cells (PBMC) and was cultured for 48 h in presence of variable doses of *Calotropis gigantea* and *Terminalia arjuna* (0.5 – 30 mg/ml, 50 µl) along with standardized dose of IBD (1:100 dilution) in flat bottom tissue culture 96 well plates. After 48 h incubation, add MTT solution (2.5 mg/ml; 10 µl) and incubate 96 well plates for 2-3 h in carbon dioxide incubator. Again, the plates were centrifuged (1800 rpm for 5 minutes) and supernatant was eliminated. Add DMSO (100 µl solution) to the formazon crystals and the absorbance was evaluated in an ELISA reader at 570 nm.

Srimathi *et al.*, (2015) evolved the anti-proliferative activity of the taxol extracted from the experimental fungus was examined by the MTT assay against MCF-7 (human breast cancer cell line). The MCF-7 cell line was procured from the National Centre for Cell Sciences (NCCS), India and the cells were seeded in a 96 microwell plate. The cells were maintained in Dulbecco's

Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin and streptomycin and incubated for 24 h at 37°C. After incubation, the cells were washed with Phosphate buffered saline (PBS) and added with serum free medium (SFM).

Vishnu *et al.,* (2015) studied the anti-cancer activity of *Calotropis gigantea* . In this study, the antioxidant, antibacterial, antifungal and antitumour potential of its three root extracts viz. chloroform extract (CE), ethyl acetate extract (EE) and methanolic extract (ME) were investigate. Antiproliferative activity of the extract was examined on human hepatocellular carcinoma cells, HepG2 and human human breast cancer cells, MCF-7. Maximum cytotoxicity was obtained with ME on HepG2 cells with IC50 value of 85 µg/mL. ME was further investigated at 85µg/mL, for cell DNA damage using agarose gel electrophoresis and nuclear staining. RT-PCR analysis was done to study expression of bcl-2 family of genes viz, bax, bcl-2 and p53. Bax expression was significantly ($p<0.05$) lowered. Significant ($p<0.05$) increase of p53 expressed suggested damage to DNA of HepG2 cells . The study showed that methanolic root extract induces apoptosis in HepG2 cells by altering bax/bcl-2 expression.

Vishnu priya *et al.,* (2015) was studied the anti-cancer activity of methanolic Root extract of *Calotropis gigantea* induces apoptosis in Human Hepatocellular Carcinoma by Altering Bax /Bcl-2 Expression.

Roihatul *et al.,* (2016) evolved that the anticancer activity is determined by MTT method. Cancer cell used in this study was T47D breast cancer cells. T47D cells are *continuous cell line* isolated from the tumour tissue of breast ductal of a 54 woman. *Continuous cell line* is often used in the in vitro cancer research because it is easy to handle, unlimited replication capabilities, high homogeneity, and easily replaced by frozen stock if it is contaminated. Ethanol extract from the roots (IC50 89.76 µg/mL) of *Calotropis gigantea* has higher cytotoxic potential on the T47D breast cancer cell than ethanol extract from the leaves (IC50 459.51 µg/mL) and the flowers (IC50>1000). The roots can be developed to be chemotherapy agent, especially for breast cancer.

Neelima Sharma *et al.*, (2016) studied Thin Layer chromatography analysis was performed. This method is used for separation of the natural products such as steroids, terpenes, alkaloids, and flavonoids present in the mixture and various solvent systems are used for this purpose.

Eric *et al.*, (2017) studied that, besides cardenolides, other compounds of *Calotropis gigantea* and *Calotropis procera* such as oxypregnanes also exhibit cytotoxic activities.

Calotroposides from the root bark of *Calotropis gigantea* have also been reported to possess cytotoxic activity against cancer cell lines. Calotropone from the roots of *Calotropis gigantea* was cytotoxic towards K562 cells with IC_{50} value of 9.2 μg/mL. From the root bark of *Calotropis procera*, calotroposides K, M and S displayed the strong cytotoxic activity against A549, U373 and PC-3 cancer cells with IC_{50} values ranging from 0.5–4.4, 0.5–4.5 and 0.1–0.2 μM, respectively.

Varsha *et al.*, (2017) analyzed that the plant *Calotropis gigantea* is a traditional medicinal plant having many of phytochemical values with the antimicrobial, analgesic, antitumor, antioxidant, anti-diarrhoeal, anti-malarial activity, antiasthmatic, free radical scavenging, wound healing, vasodilation, pro-coagulant, antifertility, anti-inflammatory, anticancer, cytotoxic, analgesic, anti pyretic, anti convulsant and anti diarrheal activities. As a hydrocarbon rich plant this plant needs more investigation on the aspect of energy conversion. The quality and quantity of the active principle which are important for many ailments are subjected to many factors such as climate, soil, *etc*. In this way standardization of the phytochemicals by these factors are very important to establish the uses of the plant more effectively.

CHAPTER III

MATERIALS AND METHODS

General laboratory techniques recommend by Purvis *et al* (1966) Tuite (1969) and Booth (1971) were followed for the preparation of media inoculation and maintains of cultures.

3.1. CLEANING OF GLASSWARES

All the glassware (Borosil or corning) were immersed in leaning solution for 3 hrs., Then , the glassware's were washed thoroughly with tap water, followed by detergent solution and finally rinsed with distilled water .The cleaned glassware were dried in hot air oven and stored.

3.1.1. STERILIZATION

Dried glassware and media were sterilized in an autoclave for 15 min at 15lb /sq inch pressure.

3.1.2. COLLECTION OF PLANT SOURCE

Calotropis gigantea L. plants were collected from Coimbatore district, Tamilnadu, India.

3.2. STERILIZATION

The healthy seeds were washed under running tap water for 20 minutes, then treated with 70% ethanol for 2 minutes, then sterilized with 2% hypochlorite for 20 minutes and rinsed sterile distilled water.

3.3. SURFACE STERILIZATION OF EXPLANTS

Healthy plant leaves, node and internodes were collected and washed thoroughly under running tap water for 25 min without damage to the tissue. In order to avoid the interaction of microbes such as bacteria and fungi in plant tissue culture, the explants were washed with sodium hypochlorite (1.0%) and mercuric chloride (0.1-0.3 %) for different time duration. The explants were transferred to a beaker containing 1% sodium hypochlorite solution with rapid shaking for different time duration (15-30) min with changing the solution at min interval. Then, the explants was washed with distilled water and treated with sterile distilled water for 5 times (5

min each). The excess water on the explants was removed by using sterile tissue paper before culture. The explants were cut into small pieces (0.5-1.0 cm) barring the cut ends ad transferred to semi-solid culture medium under aseptic conditions in a laminar flow chamber.

3.4. PREPARATION OF TISSUE CULTURE MEDIA

For tissue culture, Murshige and Skoog (1962) medium was used. Six individual stock solution of macro, micro, iron, and vitamins were prepared and stored.

Iron stock was stored in a black bottle to prevent photolysis of chemicals. All the stock solutions were stored refrigerator and used within one year. Meso –inositol, cytokinin, and auxin stock solution were freshly prepared and used every month. For preparation of medium, all the 6 stock solution were mixed thoroughly with required amounts of sterile distilled water. Sucrose 3% (30g /L) 0.1% meso-inositol (100 mg /L) and required plant growth hormones were added to the medium and buffered by 1N Hcl or NaOH to adjust the medium pH to 5.6 before autoclaving. The medium was solidified by addition 0.8% agar (8 g/L). Sterile distilled water was used to make the final volume. The medium was poured into culture vials and autoclaved at 15 Ib pressure for 15 min at 121˚C. After inoculated with explants, all culture vials were kept under 16 / 8 h (light/ dark) photoperiod at 25± 2˚C.

3.4.1. CHEMICAL COMPOSITION OF MURSHIGE AND SKOOG (1962) MEDIUM

S.NO	Component	Stock conc (g)	Dissolved in water (mL)	For 1 litre
A	$(NH_3)No$	16.500		
	$KINo_3$	19.000		
	$CaCl_2.2H_2O$	4.400	500	50 ml
	$MgSo_47H_2O$	3.700		
	KH_2PO_4	1.700		
B	$MnSo_4.4H_2O$	2.230		
	$ZnSo_4.7H_2O$	0.860	250	2.5
	H_3Bo_3	0.620		
C	Na_2 $MoO_4.4H_2O$	0.125		
	$CuSo_4.5H_2O$	0.012	500	1.5
	$CoCl_2.6H_2O$	0.012		
D	KI	0.083	250	2.5
E	**Iron**			
	Na_2 EDTA	1.862	250	5.0
	$FeSo_4.7H_2O$	1.392		
F	**Vitamins**			
	Thiamine HCl	0.010		
	Pyrodixine HCl	0.050	100	1.0
	Nictonic acid	0.050		
	Glycine	0.200		

Meso-inositol - 100 mg

Sucrose - 30 mg

Agar - 8 g

pH - 5.6-5.8

3.4.2. PREPARATION OF HORMONE STOCK SOLUTION

Auxins 2,4-D (2,4-dichlorophenoxyacetic acid), IAA-(Indole -3- acetic acid),IBA (Indole -3- butyric acid) and NAA (α-Naphalene acetic acid) and cytokinins BAP (6 – benzylamino-purine), KIN –Kinetin (6-furferla –minoourine) and GA_3 (Gibberellic acid) were prepared and stored at 4°C.

1. **2, 4-D(2,4-dichlorophenoxyacetic acid)** (M.W.221.0),100 mg was disoved in 1.0 ml of 1N KOH and it was heated,water was added to make a final volume of 100 mL (1.0 mg / mL).

2. **IAA (Indole -3- acetic acid)** (M.W.175.2), 100 mg was dissolved in 1.0 mL 1N KOH , warm water was added to make final volume 100 mL (1.0 mg / mL).

3. **IBA (Indole-3- butyric acid)** (M.W.203.2), 100 mg was dissolved in 1.0 mL 1N NaOH warm water was added to make a final volume of 100 mL (1.0 mg / mL).

4. **NAA (α-Naphthalene acetic acid)** (M.W.186.2),100 m was dissolved in 1.0 mL 1N NaOH water was added to make final volume of 100 (1.0 mg / mL).

5. **BAP (Benzyl adenine)** (M.W.225.2), 100 mg was dissolved in 1.0 mL of 1N Hcl and heated slowly water as added to make final volume of 100 mL (1.0 mg / mL).

6. **KIN (Kinetin)** (M.W.215.2),100 mg was dissolved in 1.5 mL 1N KOH water was added to make a final volume of 100 mL (1.0 mg / mL).

7. **GA3 (Gibberellic acid)** (M.W.330.0), 100 mg dissolved in 1.0 mL 1N KOH water was added to make a final volume of 100 mL (1.0 mg / mL).

3.4.3. INOCULATION AND INCUBATION

Sterilized seeds were inolculated on MS basal media and the cultures were incubated in culture chamber at 25± 2°C for light condition. The culture vials were placed on the rack at a

distance of 25 cm from the light source. A 16 / 8 h (light / dark) photoperiod of cool white light was provided from 2000 lux fluorescent tubes dark incubation was kept in the dark chamber or by wrapping culture vials with aluminium foil.

3.5. STATISTICAL ANALYSIS

Data are presented as mean ± standard deviation and were analyzed using one – way analysis of variance (ANOVA). The different among means were tested by domet test at $P < 0.05$ level of significance. The analyses were carried out with the statistical software GRAPHPRISM (USA).

3.6. QUALITATIVE PHYTOCHEMICAL ANALYSIS

The different qualitative chemical tests were performed for establishing the profile of given extract of its chemical composition. The crude drug was redissolved in methanol and subjected to various phytochemical analyses. The following tests were performed on the extracts to detect various phytoconstituents present in them.

3.7. DETECTION OF ALKALOIDS (EVANS, 1997)

Solvent free extract (50 mg) was stirred with few mL of dilute hydrochloric acid and filterate. The filterate was tested carefully with various alkaloid reagents as follows:

A) MAYERS TESR (Evans, 1997)

To a few mL of filterate , a drop or two of mayers reagents was added by the sides of the test tube. A white creamy precipitate indicated the test as positive.

MAYERS REAGENT:

Mercuric chloride (1.358g) was dissolved in 60 mL of water and potassium chloride (5g) was dissolved in 10 mL of water. The two solution were mixed and made up to 100 mL distilled water.

B) WAGNERS TEST (Wagner, 1993)

To a few drops of filterate, few drops of wagners reagent was added by the side of the test tube. A reddish-brown precipitate confirmed the test as positive.

WAGNERS REAGENT

Iodine (1.27g) and potassium iodide (2g) were dissolved in 5 mL of water 44and made up to 100mL with distilled water.

C) DRAGENDORFF'S TEST (Waldi, 1965)

To a few mL of filterate, 1 or 2 mL of dragendorff''s reagent was added. A prominent yellow precipitate indicated the test as positive.

DRAGENDORFF'S REAGENT

STOCK SOLUTION

Bismuth carbonate (5.2g) and sodium acetate (4g) were boiled for a few min with 50 mL glacial acetic acid. After 12 hrs, the precipitated sodium acetate crystals were filtered off using sintered glass funnel. Clear and red-brown filterate (40 mL) was mixed with 160 mL of ethyl acetate and 1 mL of water stored in amber-coloured bottle.

WORKING SOLUTION

10 mL of stock solution was mixed with 20 mL of acetic acid and made up to 100 mL with water.

3.8. DETECTION OF CARBOHYDRATES AND GLYCOSIDES (Ramakrishnan *et al.*, 1994)

The extract (100 mg) was dissolved in 5 mL of water and filterate. The filterate was subjected to the following tests.

A. MOLISCH'S TEST

To 2 mL of filterate, two drops of alcoholic solution of α-naphthol were added, the mixture was shaken well and 1mL of concentrated sulphuric acid was added slowly along the sides of the test tube and allowed to stand. A violet ring indicated the presence of carbohydrates.

B. FEHLING'S TEST

One mL of filtrate was boiled on water bath with 1mL each of fehling solutions I and II. A red precipitated indicated the presence of sugar.

FEHLING'S SOLUTION

FEHLINGS SOLUTION I: copper sulphate (34.66g) was dissolved in distilled water and made up to 500 mL with distilled water.

FEHLINGS SOLUTION II: potassium sodium tartarate (173g) and sodium hydroxide (50 g) were dissolved in water and made up to 500 mL.

C. BARFOED'S TEST

To one mL filtrate, 1 mL of reagent was added and heated on a boiling water bath for 2 min. red precipitate indicated the presence of sugar.

D. BARFOED'S REAGENT

Copper acetate, 30.5 g was dissolved in 1.8 mL of glacial acetic acid.

E. BENDICT TEST

To 0.5 mL of filtrate, 0.5 mL of benedict's reagent was added. The mixture was heated on a boiling water bath for 2 min. A characteristic coloured precipitate indicated the presence of sugar.

BENDICT'S REAGENT

Sodium citrate (1739) and sodium carbonate (100g) were dissolved in 800 mL of distilled water and boiled to make it clear. Copper sulphate (17.3g) dissolved I 10mL distilled water was added to it.

For detection of glycosides, 50 mg of extract was hydrolysed with concentrated hydrochloric acid for 2 hrs on a water bath, filtrate and the hydrolysate was subjected to the following tests.

F. BORNTRAGER'S TEST

To 2 mL of filtrate hydrolysate, 3 mL of chloroform layer was separated and 10 % ammonia solution was added to it. Pink colour indicated the presence of glycosides.

3.9. DETECTION OF SAPONINS BY FOAM TEST (Kolkate, 1999)

The extract (50 mg) was diluted with distilled water and made up to 20 mL. The suspension was shaken in a graduated cylinder for 10 min. A two cm layer of foam indicated the presence of saponins.

DETECTION OF FIXED OILS AND FATS (Kolkate, 1999)

A. SPOT TEST

A small quantity of extract was pressed between two filter paper. Oil stain on the paper indicated the presence of fixed oil.

DETECTION OF PHENOLIC COMPOUNDS AND TANNINS

A. FERRIC CHLORIDE (Mace, 1963)

The extract (50 mg) was dissolved in 5mL of distilled water. To this a ferric chloride solution was added. A dark green colour indicated the presence of phenolic compounds.

B. GELATIN TEST (Evans, 1997)

The extract (50 mg) was dissolved in 5 mL of distilled water and 2 mL of 1 % way out of gelatin containing 10 % sodium chloride was added to it. White precipitate indicated the presence of phenolic compounds.

C. LEAD ACETATE TEST

The extract (50 mg) was dissolved in distilled water and to this, 3 mL of 10% lead acetate solution was added. A bulky white precipitate indicated the presence of phenolic compounds.

D. ALKALINE REAGENT TEST

An aqueous solution of the extract was treated with 10% ammonium hydroxide solution. Yellow flurosence indicated the presence of flavanoids.

E. MAGNESIUM AND HYDROCHLORIC ACID REDUCTION (Harbone, 1998)

The extract (50mg) was dissolved in 5mL of alcohol and few fragments of magnesium ribbon and concentrated hydrochloric acid (drop wise) were added. The presence of flavanol and glycosides were inferred by the development of pink to crimson colour.

3.10. THIN LAYER CHROMATOGRAPHY (TLC)

TLC can be used to identify compound present in a given substance and is less time consuming, low cost and can be performed with less complicated technique. It has a wide application and significantly used in pharmaceutical purposes. The mobile phase should be prepared freely at the time of experiment. The purity of the solvents and quality of the solvents mixture should be strightly followed. The mobile phase contain a solvent mixture of chloroform and ethanol in the form of different concentrations (4:1,3.5:1.5,3:2,2.5:2.5). TLC plate should be 8cm long in which spots were kept at above 1cm. Silica gel plate is used to separate the compounds.

3.11. ANTIOXIDANTS

The discovery of the implication of free radicals is used to identifying diseases has led to increased interest in functional food that contain many different dietary phytonutrients, including antioxidants. One of the most important issues in the natural antioxidants analysis is determination of their antioxidant activity. The total antioxidant activity of plant extract has been evaluated using a new TLC spot method that involves reaction between DPPH (2,2-dipheny1-1-picrylhydrazyl) and antioxidants from the extract.

3.12. ANTICANCER ACTIVITY

MATERIALS AND METHOD

1. 96-well Microplates
2. 0.45 Micron filter
3. Minimal essential medium (MEM)
4. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-phenyl tetrazolium bromide]
5. Tissue culture flasks 25cm^2
6. Trypsin-phosphate buffered saline-versene-glucose (TPVG)
7. Phosphate buffered saline 1x (PBS)
8. Hepes buffer (1M)
9. Ethidium bromide/ acridine orange (EBI/AO) staining

3.13. PREPARATION OF INGREDIENTS

PENICILLIN AND STREPTOMYCIN (P&S) MEDIUM

Crystalline penicillin G (1 × 106 units) and streptomycin sulphate (1g) were dissolved in 1oo mL of PBS. This solution was sterilized by filtration and stored at - 20°c. From this stock solution, 1 mL was added to 100 mL of medium to obtain a final concentration of 100 units of penicillin and 100 µg of streptomycin per mL.

A) KANAMYCIN SULPHATE SOLUTION

Kanamycin (1g) was dissolved in 50 mL of sterile Millipore double distilled water to obtain a final concentration of 20 mcg/mL and stored at -20°c.

B) FUNGIZONE SOLUTION

Amphotericin B (50 mg) was dissolved in 5 mL of sterile Millipore double distilled water. From this stock solution one mL was diluted to 100 mL of sterile Millipore double distilled water to obtain a final concentration of 20 mcg /mL and stored at 20°c.

C) L-GLUTAMINE SOLUTION (3%)

L-glutamine (6g) was dissolved in 200 mL of sterile Millipore double distilled water and filtered through 0.22 micron membrane filter and stored at 20°c.

D) FETAL BOVINE SERUM

Fetal bovine serum was thawed at room temperature and inactivated at 56°c in water bath for 30 min and cooled to room temperature. Floating particles were filtered through Seitz filter and stored at -20°c.

E) SODIUM BICARBONATE SOLUTION (7.5%)

Sodium bicarbonate (15g) was dissolved in 200 mL of sterile Millipore double distilled water, filtered through Whatmann filtered paper N0.4 and stored at -4 °c.

F) TRYPSIN, PBS, VERSENE, GLUCOSE SOLUTION (TPVG)

Phosphate buffered saline

Nac	- 8 g
Kcl A.R	- 0.2 g
Na_2HPo_4	- 2.88 g
$KH_2 Po_4$	- 0.2 g
pH	-7.4

All the above ingredients were dissolved in 1000 mL of sterile distilled water and filtered through Whatmann No.4 and autoclaved at 15 Ibs for 15 min

G) EDTA (VERSENE 0.2 %)

EDTA (200 mg) was dissolved in 100 mL sterile Millipore double distilled water and autoclaved at 15 Ibs/ 15 min.

H) GLUCOSE A.R. 10 mL (10 %)

One g of glucose was dissolved in 10 mL of sterile Millipore distilled water and filtered through Whatmann filter paper and autoclaved at 10 Ibs / 15 min.

I) TPVG-1000 ML

PBS	- 840 mL
2% trypsin	-50 mL
0.2 % EDTA	-100 mL
10% glucose	-5 mL
P&S	-5 mL

All the ingredients were mixed and pH was adjusted to 7.4 and stored at -20°c.

J) PHENOL RED (0.4 %)

Phenol red (0.04 g) was dissolved in 10mL of double distilled water sterilized by autoclaving at 15 Ibs, 121°c, for 15 min.

K) TRYPAN BLUE (0.1%)

Trypan blue (0.1 g) was dissolved in 100 mL of phosphate buffered saline, filtered through Whatmann filter paper No.4 and stored at -4°c.

PREPATION OF MEDIA FOR CELL CULTURE

Ingredients	**Growth medium (10%)**	**Growth medium(5%)**	**Maintenance medium (2%)**
MEM	870 mL	920 mL	950 mL
P&S (penicillin & streptomycin) solution	1 mL	1 mL	1 mL
Phenol red (0.4 %) solution	1 mL	1 mL	1 mL
Kanamycin solution	1 mL	1 mL	1 mL
Fungizone solution	1 mL	1 mL	1 mL
3% L-glutamine solution	1 mL	1 mL	1 mL
Fetal Bovine serum	100 mL	50 mL	20 mL
75% sodium bicarbonate solution	20 mL	20 mL	20 mL
Hepes buffer	5 mL	5 mL	5 mL
Total volume	1000 mL	1000 mL	1000 mL

All the ingredients were mixed well by shaking gently, pH was caliberated and adjusted to 7.2 to 7.4.

3.14. CELL LINES

MDAMB 231 – Breast Cancer cell line.

3.14.1. CELL MEMBRANE

MDAMB231 Breast cancer cells was cultured in open vented 75 cm^2 culture vessels (corning NY) in a standard horizontal laminar flow hood and incubated in a NUAIR cell incubator at 37°c in a atmosphere of 5% co_2 and 95% air. MDAMB231 Breast cancer cells were provided with growth media of 90% minimal essential medium (Eagle) (ATCC,VA) 10% fetal bovine serum (Gibco, CA) as well as 5% streptomycin – penicillin (sigma – Aldrich MO).

Media were refreshed atleast three times a week (15 mL) and aseptic techniques were Breast cancer cells were sub-cultured when 80% confluency was reached sub-culturing consisted of dispensing old media with a vaccum and addition of 5 mL of 0.25% Trypsin – 0.03% EDTA solution (Gibco, CA) to remaining cell layer followed by incubation for 6 min at 37°C TPVG was removed and the bottle was incubated at 37°C, until all the cells detach from the surface. The cells were resuspended in 5mL of growth media. The suspensor were aspirated few times to break cell clumps. The cell concentration was determined by counting the cells in haemocytometer.

3.14.2. SAMPLE USED

Various extracts of *Calotropis gigantea* such as Hexane, Chloroform, Ethyl acetate and Methanolic extracts was very meagre, it was not included for biological activities.

3.14.3. PREPARATION OF SAMPLES

To prepare stock solution, 5 mg of each sample was dissolved in 0.5mL of 2% MEM (minimal essential medium eagle). From this working solution with different concentrations (10-100 μg/mL) were prepared 96 well plate for cytotoxic study.

3.14.4. ASSAY OF CELL VIABILITY

(i) CELL VIABILITY

The of *in vitro* cytotoxicity assays were carried out by the need to rapidly evaluate the potential toxicity of large numbers of compounds, to limit animal experimentation whenever possible, and to carry out the tests with small quantaties of compound (Terry *et al*., 2005).

(ii) CELL COUNTING

A 0.2 mL of the cell suspension was diluted in 0.2 mL of trypan blue (0.1% trypan blue). It was mixed well with pipette and sufficient volume was aspirated to fill haemocytometer immediately. The viable cells (non-viable cells are stained blue and viable cells remain unstained) were counted in each of the four corners of both chambers, omitting cells lying on the top and to the left. If cell clumping was observed. It was discarded and original cell suspension

was re-suspended. The total number of cells in the suspension was calculated using the following formula.

Average cell count × dilution factor $\times 10^4$ = - million / mL or $c_1 = t\times tb \times 1/4 \times 10^4$

Where

C1= Initial cell concentration per mL

+ = Total viable cells count of 4 squares

Tb = Correction to the trypan blue dilution

1/4 = Correction to give mean cells per corner square

10^4= Conversation factor for counting chamber

Based on the cell count, sterile flasks/tubes were seeded and incubated at 36°C. For flasks (25 cm^2) and tubes, 1.0×10^5 cells were seeded. All the passages carried out were recorded. Cell lines were not passage beyond 15 times.

3.15. TRYPAN BLUE DYE EXCLUSION TECHNIQUE

3.15.1. PRINCIPLE

Trypan blue is a blue acid dye that has two azo chromophores group. Trypan blue will not enter into the cell wall of plant cells grown in culture. Trypan blue is an essential dye, use in estimating the number of viable cells present in a population (Phillips, 1975).

3.15.2. REAGENTS

10% Minimal Essential Medium

2% Minimal Essential Medium

TPVG Trypan blue

3.15.3. PROCEDURE

MDAMB231 Breast cancer cells and vero cells culture bottles were cultured before a week to the test to make the confluent growth of the cells. To the selected cell culture bottle 5mL of TPVG solution was added (for 25 cm^2 flasks) and dispersed evenly on to the monolayer. The bottle was kept flat with the cell surface side down on the table for 2-3 min. Then TPVG was removed using pipette and the flask was placed in incubator until the cells detached from the surface resuspended the cells in 5mL of growth medium. The suspension was aspirated few times with pipette to break cell clumps. Sterility was put up in nutrient agar tubes and the sterility tubes were labelled. To the 50μL of the cell suspension an equal volume of trypan blue was added and mixed well. From the mix, 50μL of solution was charged using a pipette to a haemocytometer. The cell suspension will passed under the cover slip by capillary action. The cell concentration per mL was determined by counting the cells are haemocytometer. The live cells are clear form were counted and dead cells as blue cells were left.

Percentage of cell viability was calculated using the formula

$$\% \text{ viability} = (\text{live cell count}/\text{total cell count}) \times 100$$

3.15.4. VISUAL OBSERVATION METHOD

PROCEDURE

After 80% confluency monolayers of MDAMB231 Breast cancer cells cultures, various concentrations (10 -100 μg/mL) of test samples were added in 96 well plates. The plates were incubated at 37˚C with 5% co_2 for 24 hrs. Control wells contained only the cell and the media. Each sample was assayed in triplicate. After 24 hrs, cell morphology was evaluated microscopically and the valued were recorded.

After 24 hrs, the plates were observed under inverted (florescence) microscope for any changes in the cell morphology. These changes were gives=n a designation of T (100% toxic), PVH (partially toxic –very heavy; 80%), pH (partially toxic; 40%), PS (partially toxic, slight; 20%), Normal or zero (no toxicity; 0%) conforming to the degree of cytotoxicity seen.

The maximum concentration of extract and compounds which showed toxic and non toxic to the cells were noted.

3.16. MTT METHOD

3.16.1. PRINCIPLE

MTT is a calorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tertazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazon product. The cells are then solubilised formazon reagent is measured spectrophotometerically. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells.

3.16.2. PROCEDURE

The MTT method as described by Mosmann (1983) was used to quantitatively detect living but not dead cells. In brief approximately 2×10^4 cells / well were seeded onto 96 well plate, 100µL of MEM medium was added and incubated at 37°C for 24 hrs. Then, the medium was discarded and fresh medium was added with different concentrations of extract and compounds, The set up was incubated for 1-3 l at 37°c in CO_2 incubator. After respective incubation period the medium was discarded and 100µL. Fresh medium was added with 10µL of MTT (5 kg/mL). After 2 hrs of incubation, the medium was discarded and 100µL of DMSO was added to dissolve the formazon crystals.

Then, the absorbance was noted using spectrophotometer at 570nm.

Viable cells (%) = Test OD/Control OD ×100

CHAPTER IV

RESULT AND DISCUSSION

4.1. SURFACE STERILIZATION OF EXPLANTS

Surface sterilization of leaf explant of *Calotropis gigantea* L. with sodium hypochlorite (1%) for 20 min and mercuric chloride (0.1%) 2 min yielded 98% contamination free explants (Table 4.1). In the nodal explant of *Calotropis gigantea* L. with sodium hypochlorite (1%) for 20 min and mercuric chloride (0.1%) 2 min yielded 98% contamination free explants (Table 4.2). Where as in the pollinium explant of *Calotropis gigantea* L. with sodium hypochlorite (1%) for 20 min and mercuric chloride (0.1%) 2 min yielded 98% contamination free explants (Table 4.3).

Similarly the same result were obtained by Raman *et al.*, (2013) surface sterilization of leaf explant of *Hibisicus sabdariffa* with sodium hypochlorite (1%) for 20 min and mercuric chloride (0.3%) 5 min yielded 96% of contamination free explants. Interestingly in our study showed 98% of contamination free explants were obtained sodium hypochlorite (1%) for 20 min and mercuric chloride (0.1%) 2min. In the internodal *Hibisicus sabdariffa* with sodium hypochlorite (1%) for 20 min and mercuric chloride (0.1%) 5 min yielded 94% of contamination free explants. Similarly in our study showed 98% of contamination free explants were obtained sodium hypochlorite (1%) for 20 min and mercuric chloride (0.1%) 2min. Where as in the anther explant of *Hibisicus sabdariffa* with sodium hypochlorite (1%) for 20 min and mercuric chloride (0.3%) 5 min yielded 92% of contamination free explants. Interestingly in our study showed 98% of contamination free explants with 2 min of surface sterilization with sodium hypochlorite (1%) for 20 min and mercuric chloride (0.1%) 2min.

4.2. HAPLOID CULTURE

The flower from the healthy plant was taken and after sterilization, the anther were dissected by the cross section technique. It leads to inoculate on MS basal medium supplemented with different growth hormone such as IAA, 2,4-D and combination of both the hormone. The haploid culture starts callus induction at the 10th day of the inoculation.

4.3. CALLUS INDUCTION

Callus induction of the leaf explant of *Calotropis gigantea* was observed in the 7th day of inoculation. Among the various growth regulators of IAA of (0.5 mg/mL) showed the maximum 94.2% of callus observed (Table. 4.4). When compared to the other hormones such as 2,4-D showed 94.2% (Table. 4.5) and callus induction of 2,4-D + IAA showed 94.3% (Table. 4.6, Fig.4.2).

Nodal explant showed maximum 81.7% on MS medium supplemented with 2,4-D + IAA (0.5mg/mL) when compared to the IAA and 2,4-D and it showed 77.2% and 77.0% of callus induction respectively (Table. 4.6; Fig. 4.3).

Pollinium explant showed the callus induction on 10th day of culture and showed 92.2% maximum growth on MS medium supplemented with 2,4-D (0.5mg/mL). IAA and 2,4-D + IAA (Table 4.3, Fig. 4.1) showed 84.6 % and 92% of callus induction respectively.

According to Raman (2013) the callus induction of leaf explant of *Hibiscus sabdariffa* yields a 25% in IAA and IBA and 2, 4-D showed the 35% and 80% at (0.5mg/mL). Interestingly in our studies IAA + 2,4-D showed 92.3% of callus induction and IAA showed 92.2% whereas 2,4-D showed 92.2% respectively. The callus induction of internodal region of *Hibiscus sabdariffa* showed the 28% and IBA and 2, 4-D showed the 26% and 75% of callus induction in (0.5mg/mL). Similarly our study showed the maximum 81.7% of callus growth on MS supplemented with IAA +2,4-D (0.5mg/ mL) when compare to the IAA and it showed 2,4-D 77.2% and 77% of callus induction respectively.

4.4. QUALITATIVE PHYTOCHEMICAL ANALYSIS

The different qualitative chemical test were performed for establishing the profile of hexane, ethyl acetate, chloroform and methanolic crude extract of *Calotropis gigantea*. The crude extract were dissolved in methanol and subjected to the various phytochemical analysis were performed. The phytochemical such as alkaloids, carbohydrates, glycosides, oils and fats, phenolic compounds and tannins were present (Table.4.7).

Interestingly in our studies the methanolic extracts of *Calotropis gigantea* showed the positive result for alkaloids and carbohydrates. All the result showed negative result for saponins and oils and fats test by qualitative methods. According to the Vennila *et al.,* (2012) the phytochemical analysis of the *H. ixora* extracts showed the presence of carbohydrates, proteins, phenols, tannins, terrines and flavonoids by simple qualitative phytochemical screening methods. The methanolic extracts shows positive for the flavonoids and terpenes.

4.5. THIN LAYER CHROMATOGRAPHY (TLC)

TLC was performed with various crude extract such as such as hexane, chloroform, ethylacetate and methanol of *Calotropis gigantea*. For each extracts, four different solvent system were used as developing systems. These are 4:1, 3.5:1.5, 3:2 and 2.5:2.5. The retention factor (R_f) for each of the extracts of *Calotropis gigantea* in different solvent system showed (Table no: 4.8, Fig. No.4.4).

Talukdar *et al.,* (2010) reported that TLC profiling of all the *Calotropis gigantea* extracts indicated that diverse type of phytochemical were present. The different R_f value of the compounds provides an ideas about this polarity. This information will help to select appropriate solvent system for further separation of biocompound from plant extract of *Calotropis gigantea.*

4.6. ANTIOXIDANTS

The discovery of the implication of free radicals is used to identifying diseases has led to increased interest in functional food that contain many different dietary phytonutrients, including antioxidants. One of the most important issues in the natural antioxidants analysis is determination of their antioxidant activity. The total antioxidant activity of plant extract has been evaluated using a new TLC spot method that involves reaction between DPPH (2, 2-dipheny1-1-picrylhydrazyl) and antioxidants from the extract. The purple colour of the DPPH has been changed into yellow coloured due the presence of antioxidants activity after adding the extract of hexane, chloroform, Ethylacetate and methanol of *Calotropis gigantea* leaf and fruit. The yellow colour indicate the presence of antioxidants activity in the given plant sample (Fig. no. 4.5).

4.7. ANTICANCER

The *in vitro* cytotoxicity was carried out with various extracts such as hexane extract (HEX), chloroform extract (CHLO), ethyl acetate (ETOAC), methanolic extract (MEOH) of *Calotropis gigantea* L. by using MTT as in breast cancer cell line (MDAMB231). The toxicity was observed microscopically for the changes of cells and the viability of cells were observed by MTT assay method. Among the four extracts (HEX, CHLO, ETOAC and MEOH) HEX (67.49 µg/mL) and MEOH (71.30 µg/mL) displayed higher cytotoxic potential against Breast cancer cell line MDAMB231with IC_{50} value ranging from 84.05 µg/mL and 72.27 µg/mL respectively. Further studies are in progress in order to identify *Calotropis gigantea* cytotoxic compounds and its mechanism of action responsible for this anticancer potential (Table 4.9 and Fig. no. 4.6, 4.7, 4.8, 4.12).

Table 4.1 Effect of Sodium hypochlorite 1% and Mercuric chloride 0.1% *Calotropis gigantea* L. Leaf

S.no	$NaHCl_3$/ Min	$HgCl_2$/ Min	Germination%
1.	10	1	95.8±0.5
2.	15	2	94.9±0.7
3.	20	3	94.5±0.1

Table 4.2 Effect of Sodium hypochlorite 1% and Mercuric chloride 0.1% *Calotropis gigantea* L. Node

S.no	$NaHCl_3$/ Min	$HgCl_2$/ Min	Germination%
1.	10	1	94.5±0.1
2.	15	2	94.7±0.6
3.	20	3	94.2±1.0

Table 4.3 Effect of Sodium hypochlorite 1% and Mercuric chloride 0.1% *Calotropis gigantea* L. Pollinium

S.no	$NaHCl_3$/ Min	$HgCl_2$/ Min	Germination%
1.	10	1	94.7±0.7
2.	15	2	93.5±0.2
3.	20	3	94.3±0.3

Table 4.4. Effect of different concentration of IAA on callus induction from leaf, nodal and pollinium explants of *Calotropis gigantea* L.

Hormone	Concentration	Leaf	Node	Pollinium
IAA	0.1	67.1±1.6^{e}	63.3±1.1^{e}	44.9±0.1^{e}
IAA	0.2	74.8±0.2^{d}	72.3±0.4^{d}	55.4±0.5^{d}
IAA	0.3	83.5±0.8^{c}	75.4±0.4^{c}	65.5±0.3^{c}
IAA	0.4	86.6±0.3^{b}	82.5±0.6^{b}	66.8±0.2^{b}
IAA	0.5	**93.4±0.5^{a}**	**85.8±0.3^{a}**	**75.8±0.7^{a}**

Values represent mean ± standard deviation of three replicates per treatment. Mean followed same letter in same column did not differ significantly at $p < 0.05$ according to Dunnet's HSD test.

Table 4.5. Effect of different concentration of 2-4 D on callus induction from leaf, nodal and pollinium explants of *Calotropis gigantea* L.

Hormone	Concentration	Leaf	Node	Pollinium
2,4-D	0.1	65.1±0.7^{e}	66.6±1.3^{e}	58.0±1.9^{e}
2,4-D	0.2	75±0.1^{d}	73.9±0.3^{d}	61.2±3.7^{d}
2,4-D	0.3	76.8±0.8^{c}	77.7±1.6^{c}	68.5±0.7^{c}
2,4-D	0.4	84.9±0.3^{b}	85±0.3^{b}	75.4±0.05^{b}
2,4-D	0.5	**90.2±2.6^{a}**	**89.4±3.9^{a}**	**80.0±1.1^{a}**

Values represent mean ± standard deviation of three replicates per treatment. Mean followed same letter in same column did not differ significantly at $p < 0.05$ according to Dunnet's HSD test.

Table 4.6. Effect of different concentration of IAA, 2-4 D on callus induction from leaf, nodal and pollinium explants of *Calotropis gigantea* L.

Hormone	Concentration	Leaf	Node	Pollinium
IAA, 2-4 D	0.1	73.3±2.6[e]	60.2±0.5[e]	45.7±0.4[e]
IAA, 2-4 D	0.2	77.4±0.4[d]	65.5±0.7[d]	54.5±1.1[d]
IAA, 2-4 D	0.3	83.9±1.2[c]	73.6±0.6[c]	58.8±1.9[c]
IAA, 2-4 D	0.4	83±3.8[b]	77.5±0.6[b]	65.1±0.2[b]
IAA, 2-4 D	0.5	**91.5±0.7[a]**	**82.5±0.6[a]**	**77.2±1.9[a]**

Values represent mean ± standard deviation of three replicates per treatment. Mean followed same letter in same column did not differ significantly at $p < 0.05$ according to Dunnet's HSD test.

Table 4.7 Qualitative preliminary phytochemical analysis of *Calotropis gigantea* L.

S.No	Tests	Hexane crude extract	Chloroform crude extract	Ethyl acetate crude extracts	Methanol crude extracts
1	**Alkaloids**				
A	Mayer's Test	+	-	+	+
B	Wagner's Test	+	-	+	+
C	Drangendorff's Test	+	-	+	+
2	**Carbohydrates And Glycosides**				
A	Molisch's Test	+	+	+	+
B	Fehling Test	+	+	+	+
C	Barfoed Test	+	+	+	+
D	Bendict's Test	+	+	+	+
E	Borntrager's Test	+	+	+	+
3	**Saponin Test**	-	-	-	-
4	**Oils And Fats Test**				
A	Spot Test	-	-	-	
5	**Phenolic And Tannin Test**				
A	Ferric chloride Test		-	-	+
B	Gelatin Test	-	-	-	+
C	Lead Acetate Test	+	-	-	++
D	Alkaline Reagent Test	-	-	-	+
E	Magnesium and Hydrochloric Acid Test	-	-	-	+

Table 4.8 Thin layer chromatography of various crude extracts of *Calotropis gigantea* L.

SL.No.	Solvent system/ Extracts	CHL: MEOH (3.5:1.5)	CHL: MEOH (3:2)	CHL: MEOH (2.5:2.5)
1	**Hexane extract**	1.0,0.71	1.0,0.68	0.98,0.73
2	**Chloroform extract**	0.98,0.56,0.3	0.98,0.68,0.55	0.98,0.78,0.68
3	**Ethyl acetate extract**	0.91,0.68,0.58,0.46	0.85,0.71,0.51	0.96,0.81,0.68
4	**Methanol extract**	0.98	0.93	0.96

ANTI CANCER

Table 4.9. Cytotoxicity percentage of MDAMB231 (Human Breast Cancer Cell Line) Against Hexane, Chloroform, Ethyl acetate and methanol crude extracts of *Calotropis gigantea* L.

S.no.	Concentration (µg/mL)	Hexane extract	Choroform extract	Ethyl acetate extract	Methanol extract
1.	20	17.27± 0.014	12.59± 0.007	11.71± 0.001	15.08± 0.004
2.	40	25.91± 0.0007	38.21± 0.012	35.57± 0.003	34.99± 0.006
3.	60	36.16± 0.006	44.80± 0.007	41.43± 0.006	47.14± 0.009
4.	80	47.14± 0.008	53.29± 0.009	52.85± 0.006	55.34± 0.005
5.	100	**67.49± 0.010**	67.34± 0.007	65.15± 0.006	**71.30± 0.010**
	IC_{50}	**84.07**	**75.05**	**75.67**	**72.27**

CHAPTER V

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

The present investigation was carried out to standardize *in vitro* micropropagation techniques of *Calotropis gigantea* L. Different explant such as leaf, node and pollinium were carried out. Murshige and Skoog medium was used for this investigation *Calotropis gigantea* L. is an important medicinal plant, various extracts such as hexane, chloroform, Ethylacetate and methanol were used for phytochemical analysis and antioxidants and anticancer studies.

In the study of leaf, node and pollinium were used for regeneration of *Calotropis gigantea* L.

- The callus induction was observed on leaf explants of *Calotropis gigantea* L. was observed on 7th day of inoculation. Among the various growth regulators of IAA of (0.5mg/mL) showed the maximum 94.2% of callus observed. When compared to the other hormones such as 2,4-D and 2,4-D+IAA showed only 94.2% and 93.9% of callus induction on MS media (0.5mg/mL) respectively.
- The nodal explant showed maximum of 77.9% on MS medium supplemented with 2,4-D +IAA (0.5mg/mL)when compared to the IAA and 2,4-D and it showed 77.2% and 77.0% of callus induction respectively.
- The pollinium explants showed the maximum growth of 89.8% was observed on MS medium supplemented with 2,4-D (0.5mg/mL)when compared to IAA and 2,4-D +IAA and it showed the 84.6% and 92% of callus induction respectively.
- The different qualitative chemical test were performed for establishing the profile of hexane, chloroform, Ethylacetate and methanolic crude extract of *Calotropis gigantea* L. The crude extract were dissolved in methanol and subjected to various phytochemical analysis were performed. The phytochemical such as alkaloids, carbohydrates, glycosides, oils and fats, phenolic compounds and tannins were present. The alkaloids such as serpentine, reserpine and serpentine were identified.

- TLC were performed with various crude extract such as hexane, chloroform, Ethylacetate and methanol of *Calotropis gigantea* L. For each extract four different solvent system were used as a developing system. These are 4:2, 3.5:1.5, 3:2 and 2.5:2.5. The retention factor (Rf) for each of the extract of *Calotropis gigantea* L. in different solvent system showed different values.

- The discovery of the implication of free radicals is used to identifying diseases has led to increased interest in functional food that contain many different dietary phytonutrients, including antioxidants. One of the most important issues in the natural antioxidants analysis is determination of their antioxidant activity. The total antioxidant activity of plant extract has been evaluated using a new TLC spot method that involves reaction between DPPH (2,2-dipheny1-1-picrylhydrazyl) and antioxidants from the extract. The purple colour of the DPPH has been changed into yellow coloured due the presence of antioxidants activity after adding the extract of hexane, chloroform, Ethylacetate and methanol of *Calotropis gigantea* L. leaf and fruit. The yellow colour indicate the presence of antioxidants activity in the given plant sample.

- The in vitro cytotoxicity was carried out with various crude extracts such as hexane extract (HEX), chloroform extract (CHLO), ethyl acetate (ETOAC), methanolic extract(MEOH) of *Calotropis gigantea* L. by using MTT as in MDAMB231 (Breast Cancer cell line). The toxicity was observed microscopically for the changes of cells and the viability of cells were obser using by MTT assay method. Cell viability was assessed by MTT assay, which is a calorimetric assay for measuring the activity of cellular enzymes. MTT to its insoluble formazon, giving purple colour. IC_{50} Value of hexane, chloroform, ethyl acetate and methanolic extracts of wild plant and µg/mL concentrations respectively. The compounds were treated and incubated with 24 hrs, after the incubation caused cell damage and induced cell death which characterized morphology of necrosis such as cell blebbing were observed.

CONCLUSION

The present investigation is a preliminary study, *Calotropis gigantea* L. *in vitro* haploid plants production, callus induction and as many active phytoconstituents with various bioactivities including antioxidant, antimicrobial and anticancer. Our results showed that various extracts of *Calotropis gigantea possess* promising *in vitro* antiproliferative activity on Breast cancer cell lines. Exploitation for commercial purpose of *Calotropis gigantea* may also going to extinct. Hence we suggest that *in vitro* conservation measure must be taken.

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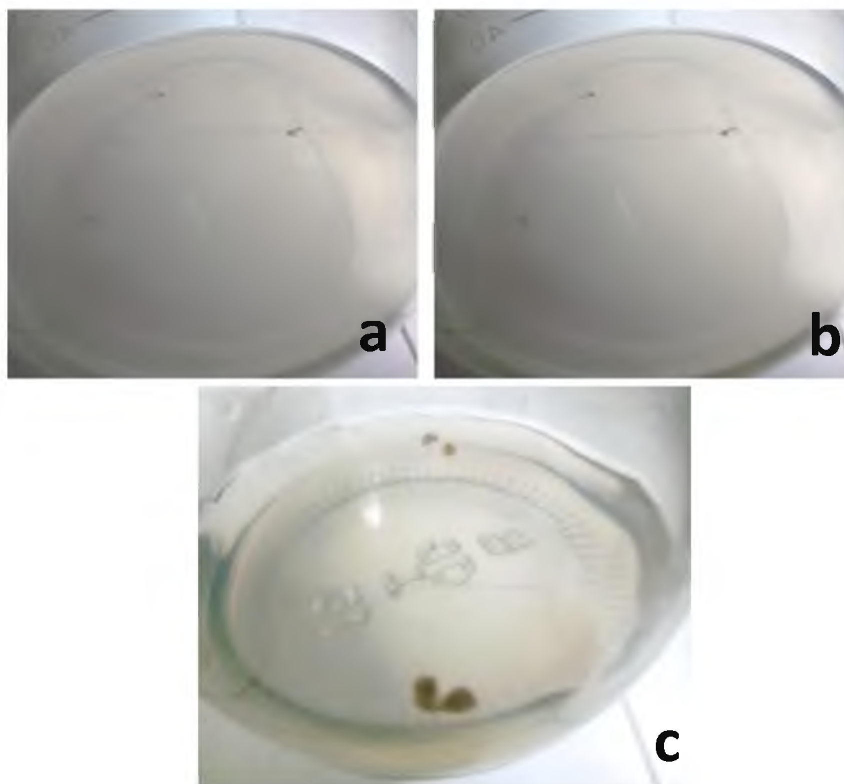
ABBREVIATIONS

λ – Lamda
$(NH_4)_2NO_3$ – Ammonium nitrate
< – Lessor than
> – Greater than
1M – Molarity
1N – Normality
2,4-D – 2,4-Dichloro phenoxy acetic acid
2-iP – 2-isopenteyladenine
5MT – Methyltryptophan (5MT)
ABA – Abscisic acid
ACE – Acetyl choline esterase
AhR – Arylhydrocarbon receptor
ANOVA – Analysis of Variancep
ATP – Adenosine triphosphate
BA – Benzyl adenine
BAP – Benzyl amino purine
BHA – Butylated hydroxyanisole
BHT – Butylated hydroxytoluene
BSI – Botanical Survey of India
$CaCl_2.2H_2O$ – Calcium chloride
CAT – Catalase
C-C – Carbon-carbon
CH – Casein Hydrolysate
CHD – Coronary heart disease
CHX – Cycloheximide
cm – Centi meter
cm^2 – Square centimeter
CNS – Central nerve system
CO_2 – Carbon-di-oxide
$CoCl_2.6H_2O$ – Cobalt chloride
Conc. – Concentration
Conc. $H_2 SO_4$ – Concentrated sulphuric acid
CR – Chrysin
$CuSO_4.5H_2O$ – Cupper sulphate
Da – Dalton
D-Glucose – Dextroglucose
DHEA – Dehydroepiandrosterone
Dis. – Distilled
dL – Deciliter
DMSO – Dimethyl sulfoxide
DNA – Deoxy ribonucleic acid
DPPH – 2,2-diphenyl-1-picrylhydrazyl
EDTA – Ethelene diamine tetra acetic acid

FCS	–	Fetal calf serum
$FeSO_4.7H_2O$	–	Ferrous sulphate
GA_3	–	Gibberellic acid
Gly	–	Glysine
H_3BO_3	–	Boric acid
HCl	–	Hydrochloric acid
HeLa	–	Human cervical carcinoma cell line
HT 29	–	Human colon carcinoma cell line
HIV	–	Human immunodeficiency virus
HSV	–	Herpes simplex virus
IAA	–	Indole-3-acetic acid
IBA	–	Indole-3-butyric acid
IC_{50}	–	Inhibitory concentration
ICP	–	Intracavernous pressure
ISM	–	Indian System of Medicine
KH_2PO_4	–	Potassium hydrogen phosphate
KI	–	Potassium iodide
Kn	–	Kinetin
KNO_3	–	Potassium nitrate
L	–	Litre
MEM	–	Minimal essential medium
mg	–	Milligram
$MgSO_4.7H_2O$	–	Magnesium sulphate
Min	–	Minutes
mL	–	Mililitre
mm	–	Millimeter
$MnSO_4.4H_2O$	–	Manganese sulphate
MS	–	Murashige and Skoog medium
MSO	–	Methionine sulfoximine
MSW	–	Medicine System of Europe
MTT	–	3-(4,5-Dimethyl Thiazol-2yl)-2,5-diphenyl tetrazolium bromide
Na_2CO_3	–	Sodium carbonate
Na_2EDTA	–	Di-sodium ethylene di-amine tetra acetic acid
$Na_2MoO_4.4H_2O$	–	Sodium molypdate
NaCl	–	Sodium chloride
NaOH	–	Sodium hydroxide
nM	–	Nano molar
NO	–	Nitric oxide
PBS	–	Phosphate buffered saline
PEG	–	Polyethylene glycol
PGR	–	Plant growth regulator
pH	–	Negative logerthem of hydrogen ion
PS	–	Penicilin and Streptomycine
RAAS	–	Renin-angiotensin-aldosterone system

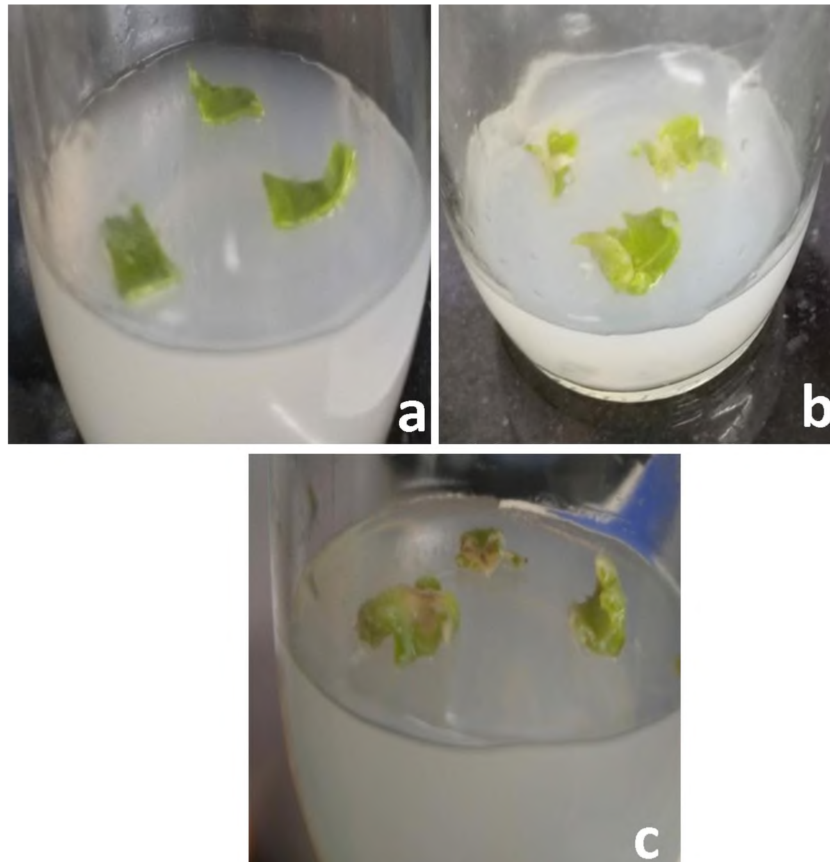
RH	–	Relative humidity
RNS	–	Reactive nitrogen species
ROS	–	Reactive oxygen species
Rpm	–	Revolutions per minute
S.D	–	Standard deviation
SAAs	–	Sugar amino acids
SOD	–	Superoxide dismutase
SPSS	–	Statistical Package for Social Sciences
TBHQ	–	Tert-butylated hydroxyquinone
TDZ	–	Thiazuron
U/L	–	Unit per litre
UGC	–	University Grants Commission
UTI	–	Urinary tract infections
WHO	–	World Health Organization
Zn	–	Zeatin
$ZnSO_4.7H_2O$	–	Zinc sulphate
α	–	Alfa
β	–	Beta
γ	–	Gamma
μL	–	microlitre
μM	–	micro molar

Fig. No. 4.1. *Calotropis gigantea* L. pollinium explants inoculated on MS medium supplemented with 0.5 µL of IAA, 2,4 D



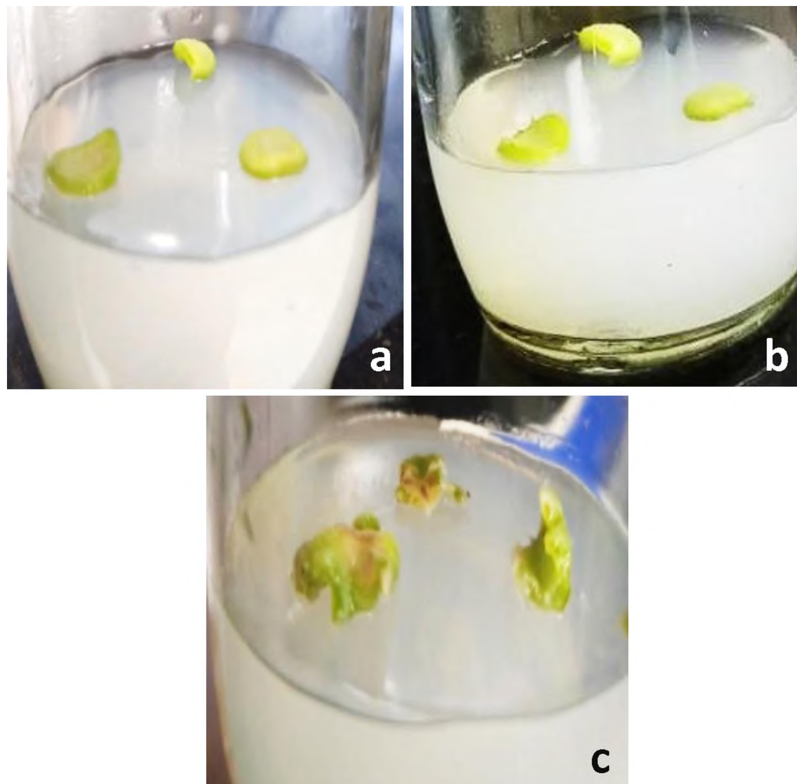
a. On 1st day of inoculation b. On 10th day of inoculation
c. On 30th day of inoculation

Fig No. 4.2. *Calotropis gigantea* L. leaf explants inoculated on MS medium supplemented with 0.5 µL of IAA and 2,4 D.



a. On the day of inoculation b. On 10th day of inoculation
c. On 30th day of inoculation

Fig. No. 4.3 *Calotropis gigantea* L. node explants inoculated on MS medium supplemented with IAA and 2,4 D



a. On the day of inoculation b. On 10th day of inoculation

b. c. On 30th day of inoculation

Fig. No. 4.4. a. TLC profile of *Calotropis gigantea* L. on Hexane crude extract solvent system Chloroform: methanol 2.5: 2.5

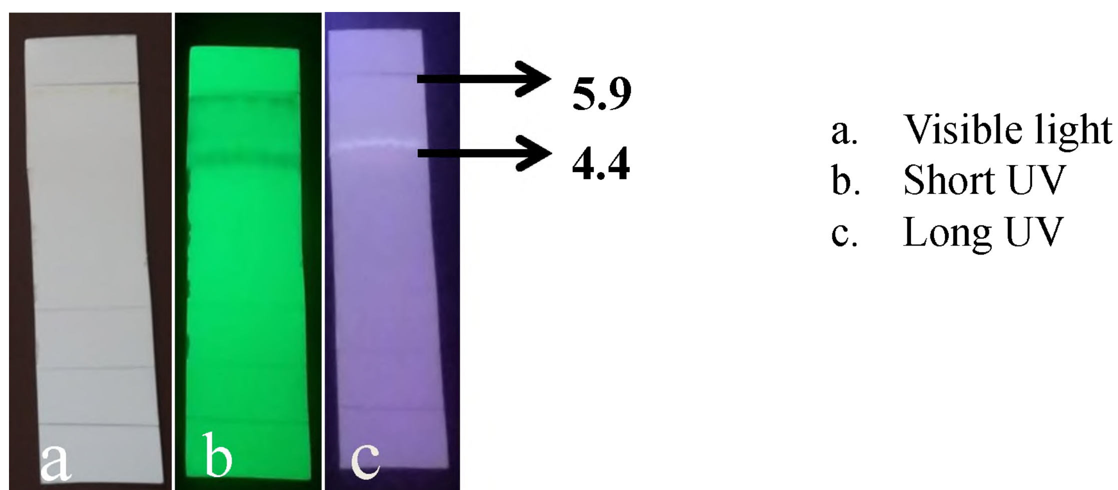


Fig. No. 4.4. b. TLC profile of *Calotropis gigantea* L. on Hexane crude Extract solvent system Chloroform: Methanol 3:2

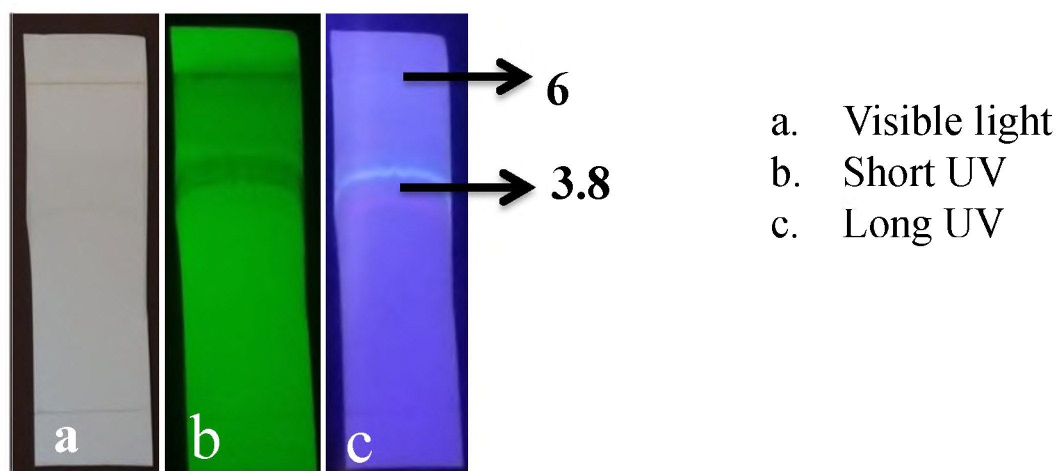


Fig. No. 4.4. c. TLC profile of *Calotropis gigantea* L. *on* Hexane crude extract solvent system Chloroform: methanol 3.5 : 1.5

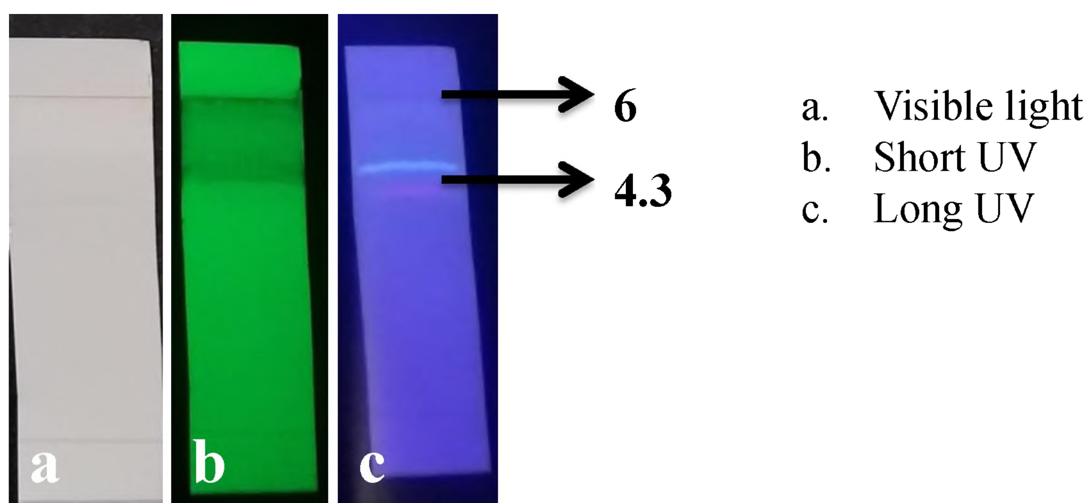


Fig. No. 4.4. d.TLC profile of *Calotropis gigantea* L. on chloroform crude extract solvent system Chloroform: methanol 2.5:2.5

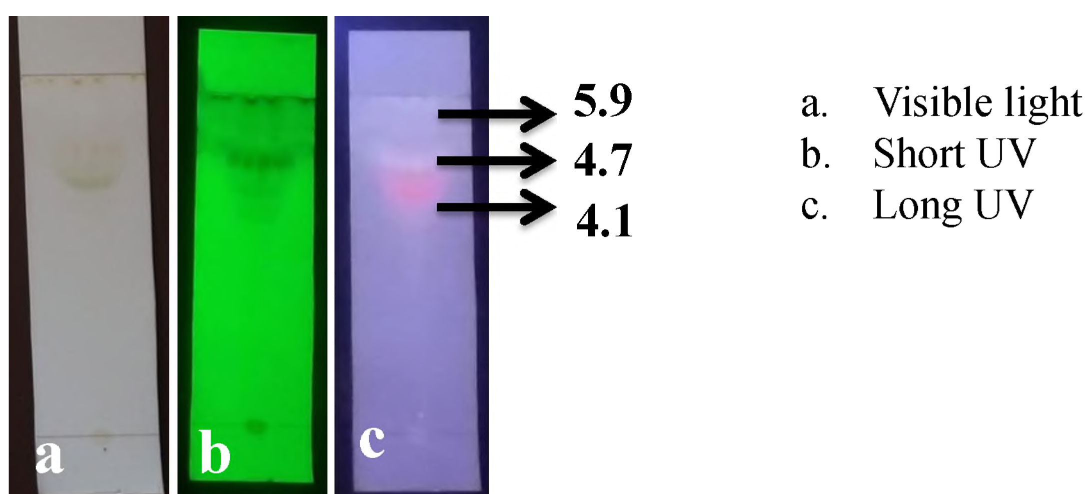


Fig. No. 4.4. e.TLC profile of *Calotropis gigantea* L. on chloroform crude extract solvent system Chloroform: methanol 3:2

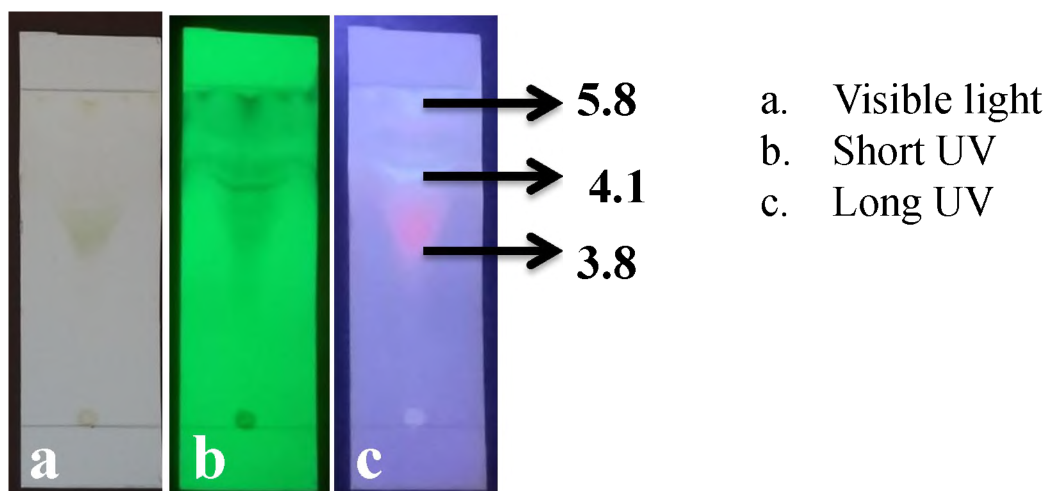


Fig. No. 4.4. f.TLC profile of *Calotropis gigantea* L. on chloroform crude extract solvent system Chloroform: methanol 3.5:1.5

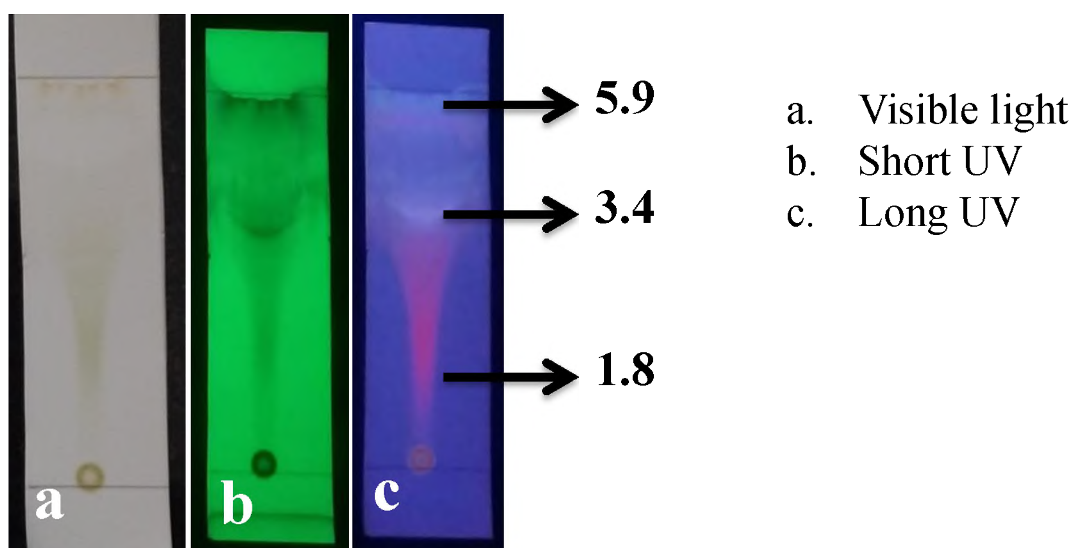


Fig. No. 4.4. g. TLC profile of *Calotropis gigantea* L. on Ethylacetate crude extract solvent system Chloroform: methanol 2.5:2.5

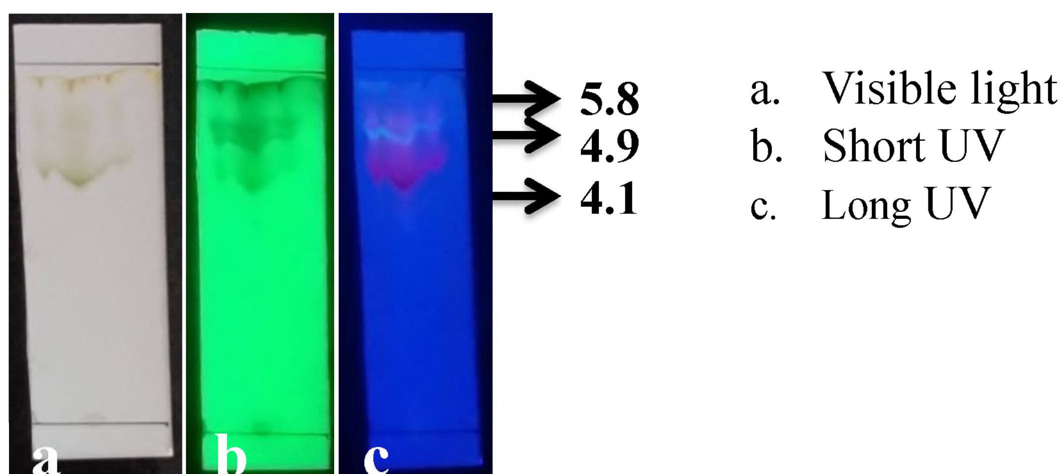


Fig. No. 4.4. h.TLC profile of *Calotropis gigantea* L. on Ethylacetate crude extract solvent system Chloroform: methanol 3:2

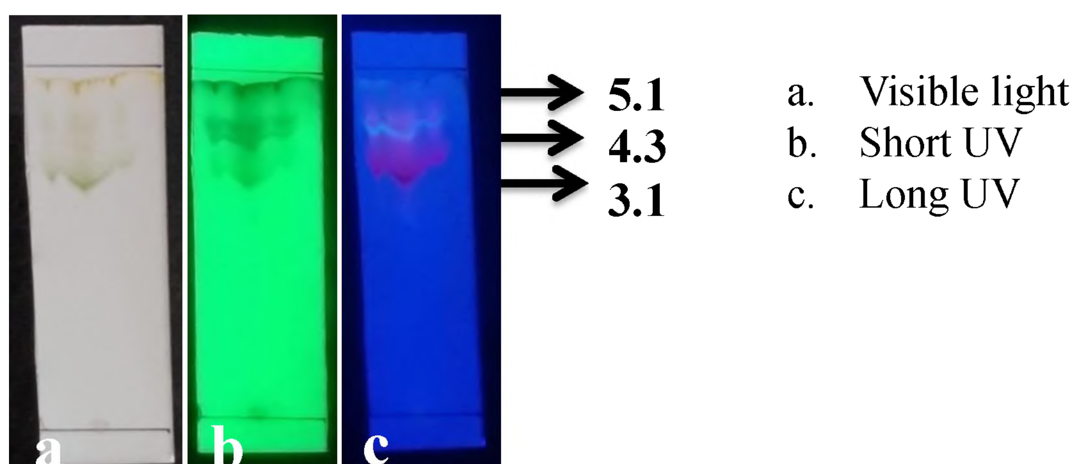


Fig. No. 4.4. i.TLC profile of *Calotropis gigantea* L. on Ethylacetate crude extract solvent system Chloroform: methanol 3.5:1.5

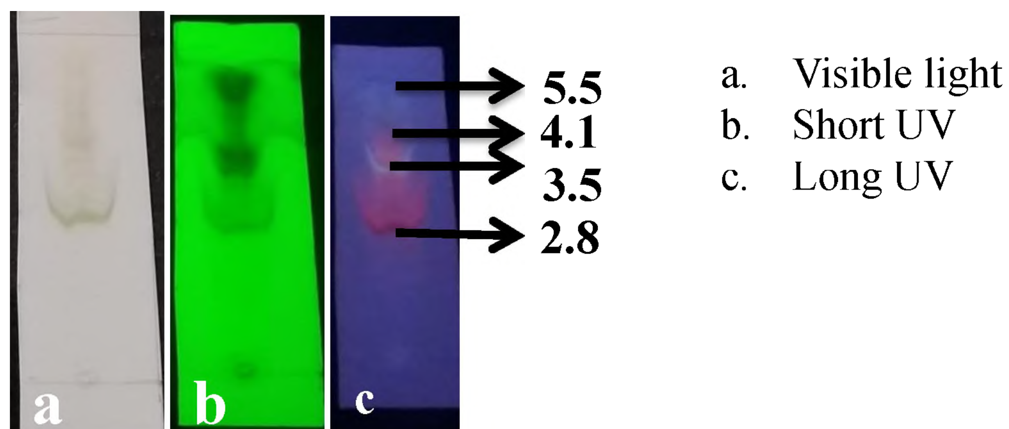


Fig. No. 4.4. j.TLC profile of *Calotropis gigantea* L. on Methanol crude extract solvent system Chloroform: methanol 2.5: 2.5

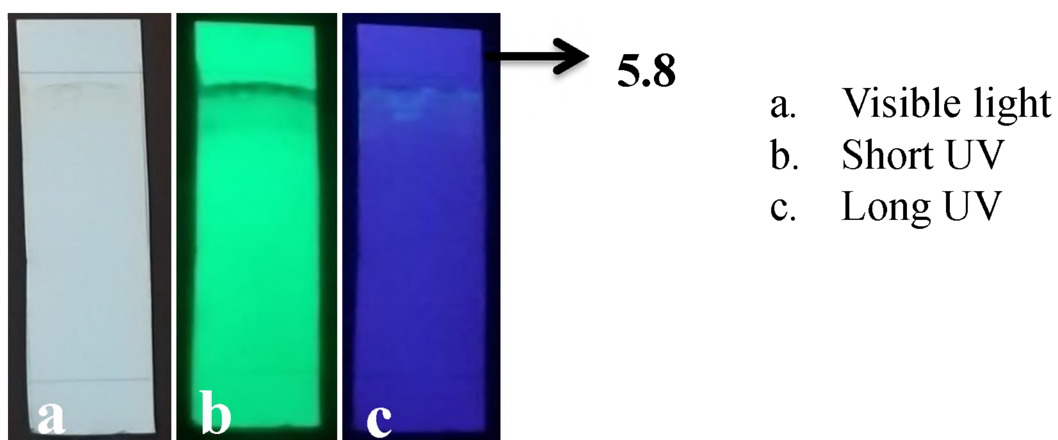


Fig. No. 4.4. k.TLC profile of *Calotropis gigantea* L. on Methanol crude extract solvent system Chloroform: methanol 3:2

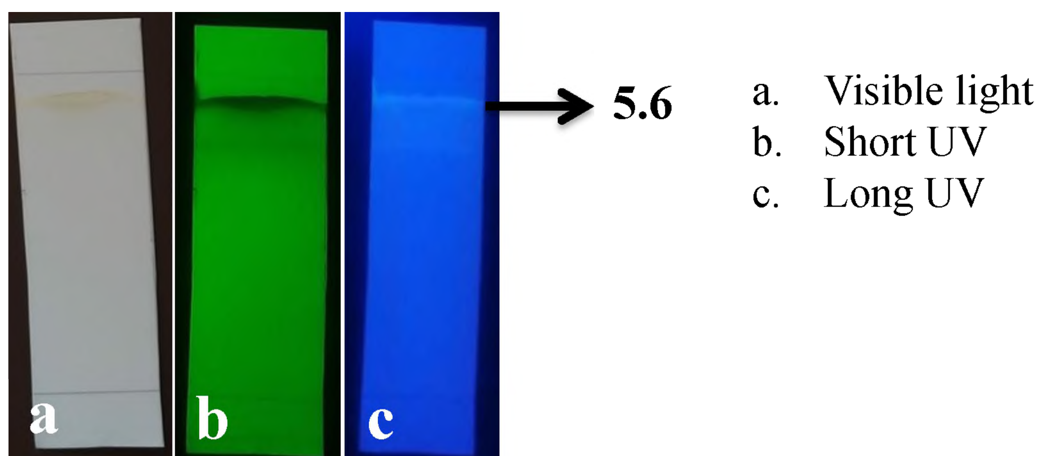


Fig. No. 4.4. l.TLC profile of *Calotropis gigantea* L. on Methanol crude extract solvent system Chloroform: methanol 1.5:1:5

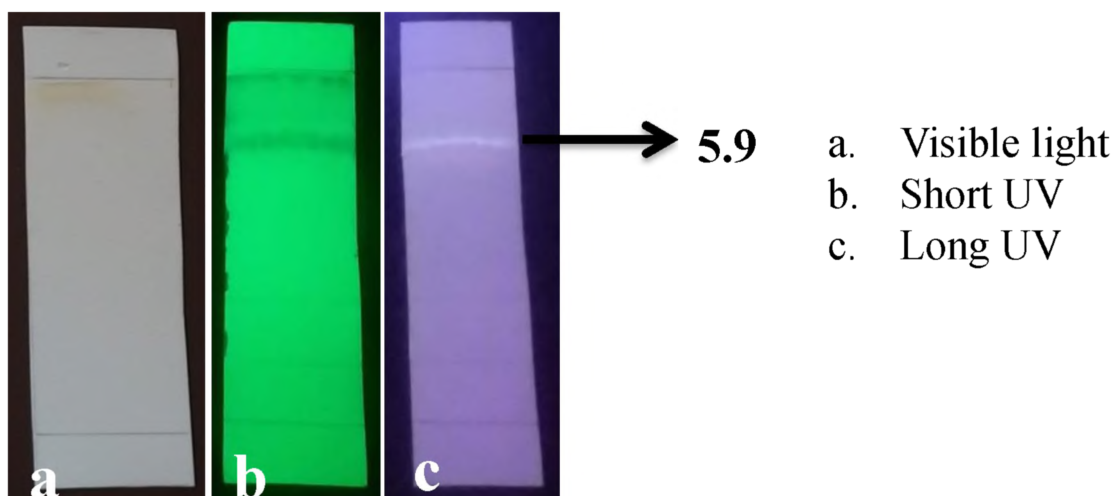


Fig. no. 4.5. Antioxidant activity of various crude extracts of *Calotropis gigantea* L. (DPPH Spot assay)

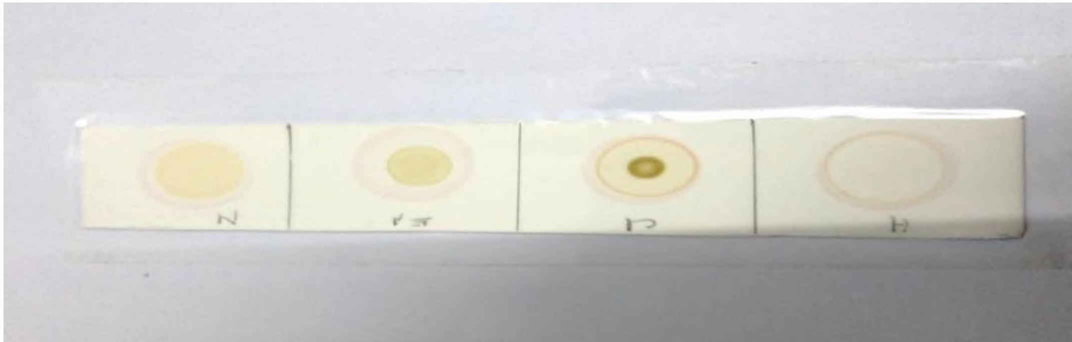
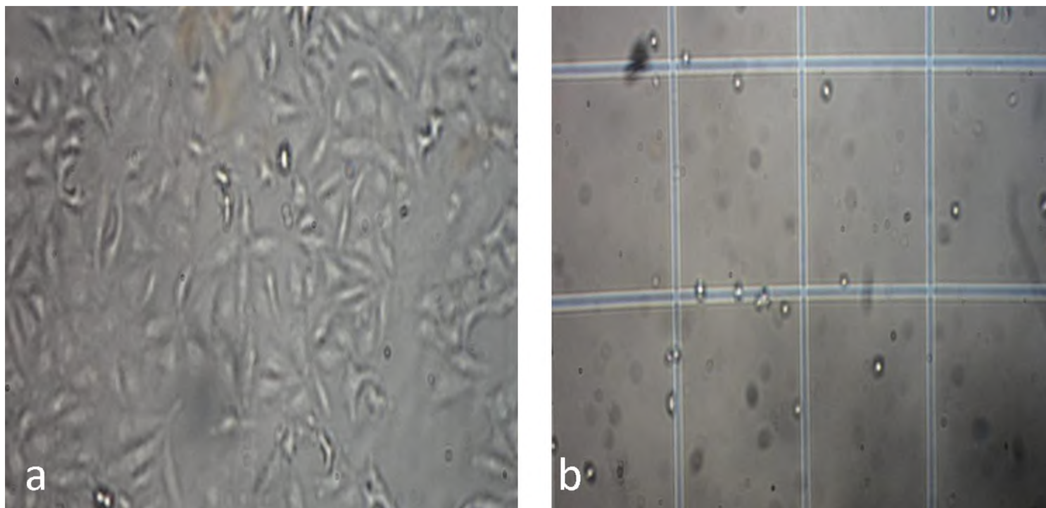
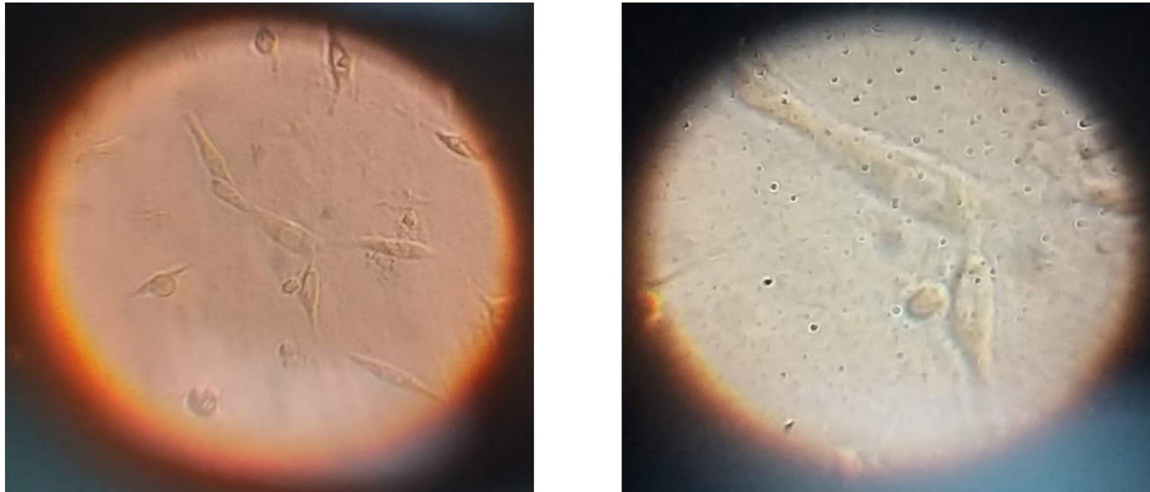


Fig. No. 4.6. Trypan blue extraction technique for cell counting



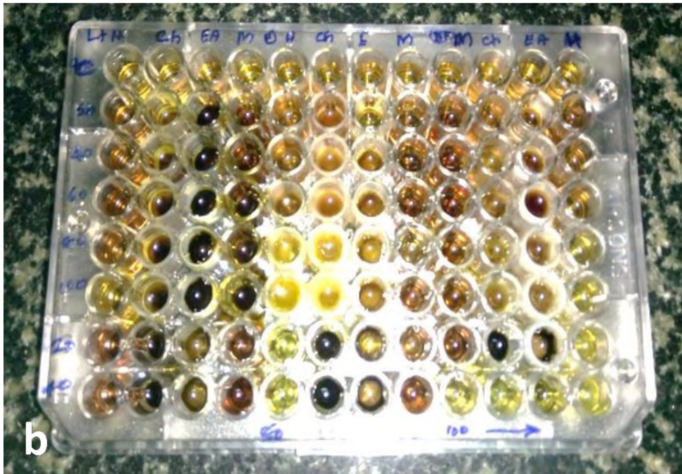
a. MDAMB231 (Breast cancer cell line)
b. Living cell count in hemocytometer

Fig. No. 4.7. MDAMB231 Cells (Breast Cancer Cell Line) with different extracts of *Calotropis gigantea* L.



MDAMB231 Cells (Breast Cancer Cell Line)

Fig. No. 4.8. MTT assay of various extracts of *Calotropis gigantea* L.



a. Before incubation
b. After incubation

Fig. No. 4.9. Effect of different concentration of IAA on callus induction from leaf, nodal and pollinium explants of *Calotropis gigantea* L.

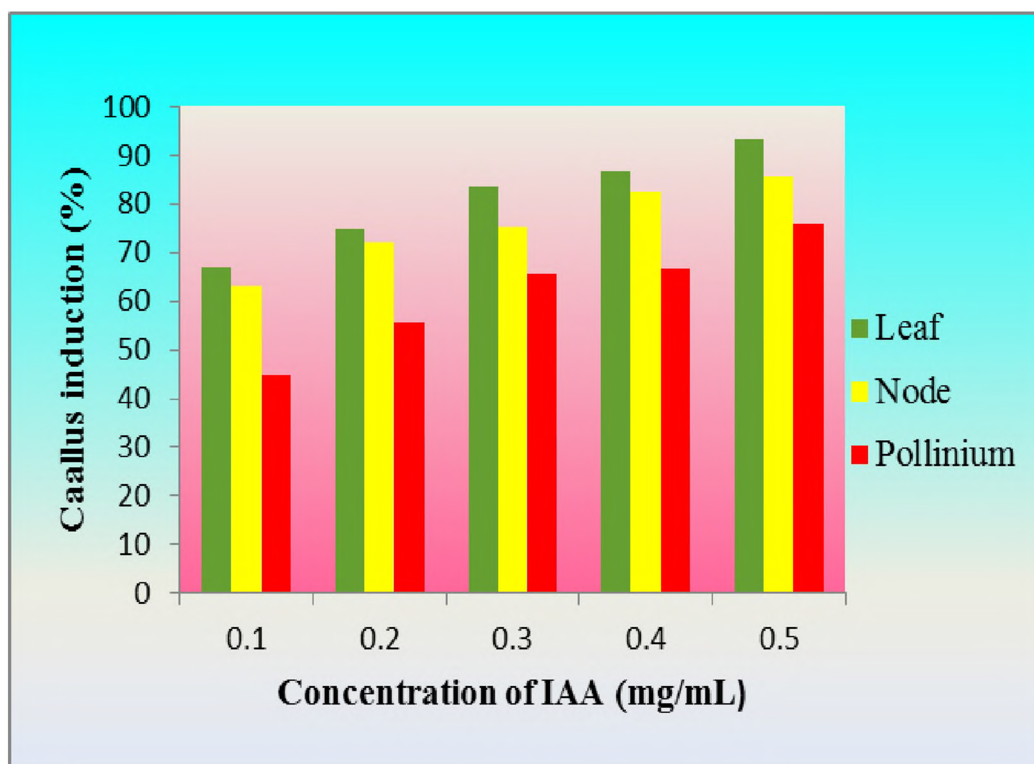


Fig. No. 4.10. Effect of different concentration of 2,4D on callus induction from leaf, node and pollinium explants of *Calotropis gigantea* L.

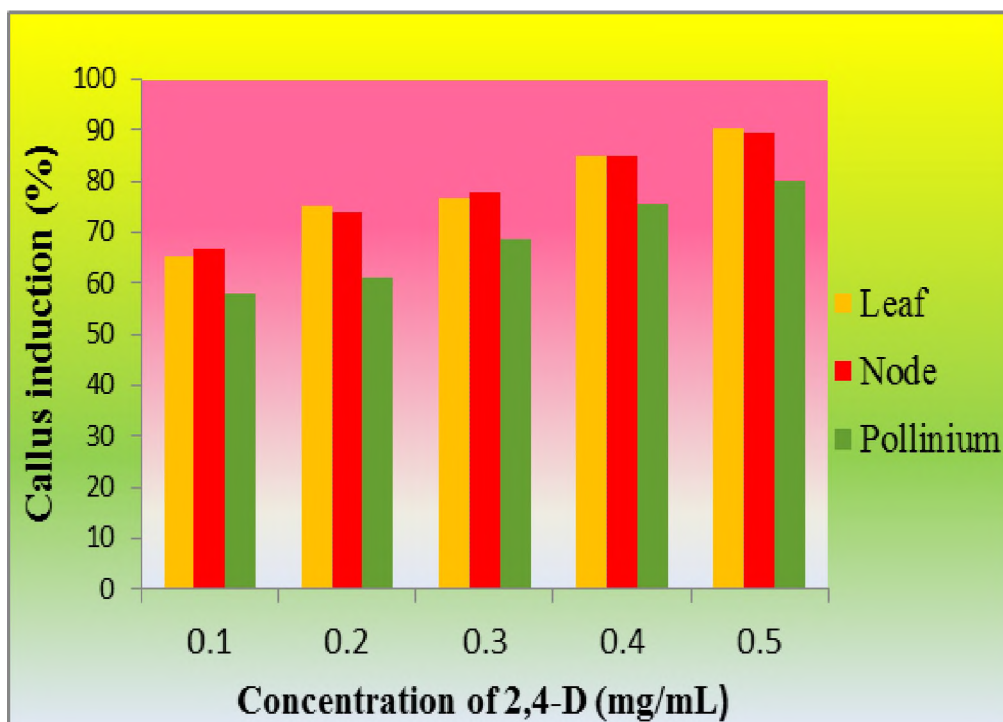


Fig. No. 4.12. Effect of different concentration of 2,4 D and IAA on callus induction from leaf, node and pollinium explants of *Calotropis gigantea* L.

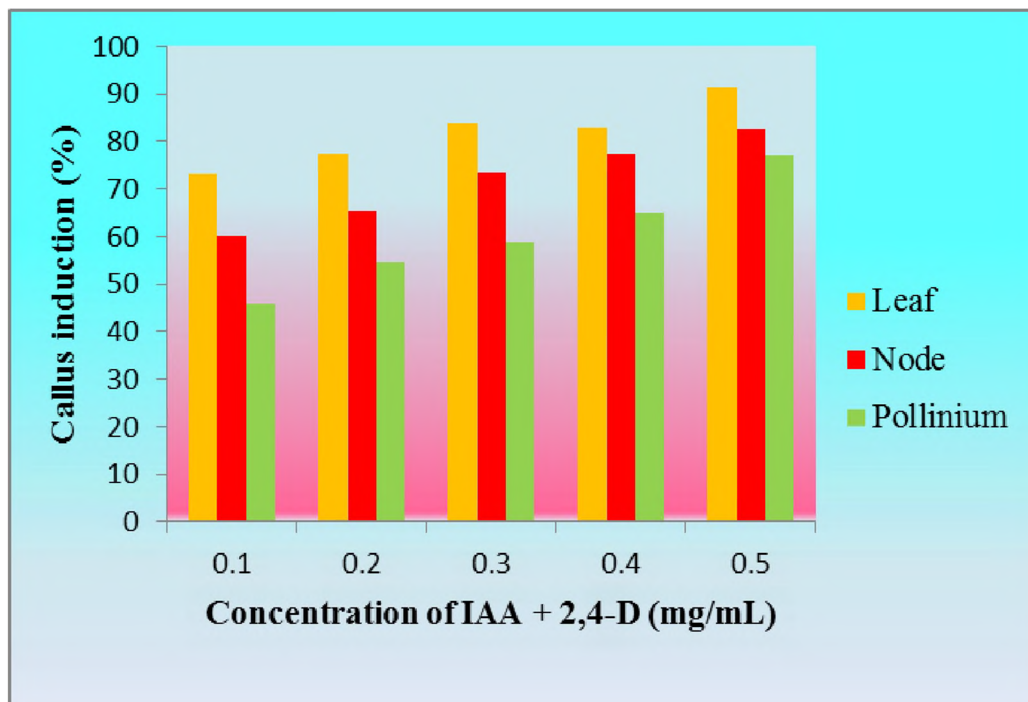


Fig.no. 4.12. Cytotoxicity percentage of MDAMB231 (Breast Cancer cell line) against HEX, CHLO, ETOAC, MEOH crude extracts of *Calotropis gigantea* L.