EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 11
Issue: 2
92-101
Year: 2024

PHYTOCHEMICAL DETERMINATION AND ANTI-CANCER ACTIVITY OF CALOTROPIS GIGANTEA

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Article Received on 25/11/2023 **Article Revised on 16/12/2023** **Article Accepted on 06/01/2024**

ABSTRACT
The present study was focused on the phytochemical screening, anti-microbial, anti-oxidant and cytotoxic activity of *Calotropis gigantea* plant extracts (stem, leaf & flower) on HCT-116 Human Colorectal adenocarcinoma cell lines. The crude extract of C. gigantea stems, leaves & flowers were prepared using distilled water as solvent. The plant extract was subjected to the phytochemical test; anti-microbial studies, anti-oxidant studies and the cytotoxic activity was tested using MTT assay. The phytochemical screening of *C. gigantea* plant extracts showed the presence of alkaloids, glycosides, tannins, steroids and tri terpenoids, flavonoids, proteins and phenols etc. Anti-microbial studies showed results for *Pseudonomos aeruginosa*, *Klebsiella pneumonia* and *Aspergillus niger*. The anti-oxidant study was performed by SOD assay. The IC 50 value with effective anticancer activity for stem extract was found to be 37.30%. The results suggest that the chemical compounds present in the extracts of *C. gigantea* could further be exploited as effective anticancer agents.

KEYWORDS: *Calotropis gigantea*, Cancer, Phytochemicals, MTT assay, SOD assay.

INTRODUCTION
Calotropis gigantea is a one of the six species of *Calotropis* weed that belongs to Apocynaceae family and has a lot of medicinal properties. Cancer is the growth of tissues characterized by the uncontrolled division of cells and they severely affect the human population. According to the World Health Organization, cancer is the second leading cause of death globally and was responsible for an estimated 9.6 million deaths in 2018.[1] Globally, about 1 in 6 deaths is due to cancer. In India, it is estimated that around 2.25 million people are suffering from different types of cancer according to the National Institute of Cancer Prevention and Research.[2] This plant is referred as "shallow wort", "giant milkweed" and "shallow wort". They have typical thick, wide leaves and odourless, purplish-coloured flowers that make their identification easier. The white milky latex produce by this plant has cardiac glycosides calotropin, uscharin, calotoxin, calactin, and uscharidin and gigantin, are found to have great wound healing activity.[3] While the leaves are that to possess antimicrobial and anti-inflammatory properties.[4] The entire tree is thought to have therapeutic benefits and is used to treat a variety of ailments, including syphilis, boils, inflammation, epilepsy, hysteria, fever, muscle spasms, warts, leprosy, gout, snakebites, and cancer. The extract of different parts of C. gigantea has been reported to have many promising primary and secondary metabolites and can be separated or combined for the development of effective drugs against various types of cancer.[5] Phytochemicals are biologically important molecules that play a critical role in the plant's defence system. Many selective phytochemicals including primary and secondary metabolites have been developed as drug molecules. Carbohydrates, chlorophyll, proteins, and lipids form the primary metabolites, while the secondary metabolites include alkaloids, tannins, saponins, phenols, flavonoids, and terpenoids. The secondary metabolites have been described as potent anticancerous agents and as important cancer chemo preventive agents.[6] Polyphenols, flavonoids, and steroids are some of the phytochemicals that have been extensively studied as anticancerous agents.[7,8] These secondary metabolites have also been described as pharmacological agents or noncytotoxic nutrients that boost the physiological mechanisms of an organism.[9] The aim of the study was to determine the phytochemical properties, anti-microbial properties, anti-oxidant properties of *C. gigantea* and to assess the cytotoxic activity of the stem, leaf and flower extracts using in vitro assays against HCT-116 Human Colorectal adenocarcinoma cell lines.

MATERIALS AND METHODS
Identification and Collection of plant
The medicinal plant *Calotropis gigantea* was collected in and around Coimbatore and Palakkad regions. The plant

was authenticated by Dr. M. U. Sharief, Scientist 'F'& Head of Office, Government of India, Ministry of Environment, Forest & Climate Change, Botanical Survey of India.

Preparation of aqueous extracts

Maceration is one of the oldest and simplest extraction techniques.[10] The solid to be extracted, once introduced into the container, is completely covered by the solvent, minimizing contact with the air, so that the liquid can enrich itself as much as possible with the substances contained in the solid matrix. Maceration is an extraction procedure in which coarsely powdered drug material, either leaves or stem is placed inside a container; the menstrum is poured on top until completely covered the drug material. The container is then closed and kept for at least three days. The content is stirred periodically, and if placed inside bottle it should be shaken time to time to ensure complete extraction. At the end of extraction, the micelle is separated from marc by filtration or decantation. Subsequently, the micelle is then separated from the menstrum by evaporation in an oven or on top of water bath. This method is convenient and very suitable for thermolabile plant material. The plant was washed several times in running water. Then the parts were dried in shade place at room temperature for about 2-3 weeks. After drying the stems, leaves & flowers were grinded into fine powder using grinding machine. The powder was then sieved through sieve no.66 &100. The powder was extracted by maceration. The powdered crude drug of *Calotropis gigantea's* leaf, stem and flower was extracted using distilled water. The powder (3 g) was measured and mixed with 30 mL of distilled water[11] in a conical flask. For extraction, the conical flask was plugged with a cotton plug and was placed in a shaker at 28°C for 24 h at 150 rpm. The mixture was filtered through muslin cloth and Whatman (No. 1) filter paper to obtain the extract. The green sticky mass of 150 g extract was labelled and preserved in the refrigerator at 4°C until use.[12] Suspensions of the extract were freshly prepared for experimental use.

Phytochemical screening

C. gigantea extracts (stem, leaf & flower) were subjected to successive standard phytochemical screening following the method of Harborne (1983) to identify alkaloids, glycosides, steroids, flavonoids, tannins, proteins and phenols.[13] All phytochemicals were carefully screened and identified by characteristic colour changes following standard procedures.

Anti-microbial activity

Agar well diffusion method was used to screen the antibacterial and antifungal activities of the extracts. 70µl of fresh bacterial (*P. aeruginosa* and K. *pneumoniae*) and fungi (*A. niger* and *A. flavus*) culture was pipetted in the centre of sterile (sterilization at 121^0C for 15 minutes) petri dishes (39g of Mueller Hinton agar in 1000ml of distilled water for bacteria and 38g of malt agar in 1000 ml of distilled water for fungi) containing solidified media. Followed by spreaded with cotton swab and, wells were made using a sterile cork borer (6 mm in diameter). Then, 50 µl of each extract was added to respective wells and the plates were incubated (24 hrs at 37°C for bacteria and 5 days at 30^0C for fungi). Antimicrobial activity was detected by measuring the zone of inhibition (excluding the wells diameter) appeared after the incubation time. Vancomycin disc was used as positive control for bacteria and fluconazole was used for fungi.[14]

Antioxidant activity

Superoxide dismutase (SOD) activity was determined by the inhibition in photo reduction of nitrobluetetrazolium by the SOD enzyme. To the 0.1ml of sample added 1ml of the reaction mixture I (1ml of 50mM PBS, 0.075ml of 20mM L Methionine, 0.04ml of 10mM hydroxyl amine hydrochloride, and 0.1ml of 50mMEDTA) and incubate the sample (extract of stem, flower & leaves) at 30^0C for 5minutes. After incubation added 50µM riboflavin and the sample was allowed to expose under 200W fluorescent light. 1ml of the reaction mixture II (1% Sulphanilamide in 5% phosphoric acid) was added and the measurement was read at 543nm under spectrophotometer.[15]

$$\% \text{ inhibition of nitrate formation} = 1 - \frac{AS}{AC} \text{ X } 10$$

Anticancer activity

The HCT-116 (Human Colorectal adenocarcinoma cell line) was purchased from NCCS, Pune, India. The cells were maintained in DMEM high glucose media supplemented with 10% FBS along with the 1% antibiotic-antimycotic solution in the atmosphere of 5% CO_2, 18-20% O_2 at 37^0C temperature in the CO_2 incubator and sub-cultured for every 2days. Passage No-48 was used for the present study. MTT assay is a colorimetric assay used for the determination of cell proliferation and cytotoxicity, based on reduction of the yellow-coloured water-soluble tetrazolium dye MTT to formazan crystals. Mitochondrial lactate dehydrogenase produced by live cells reduces MTT to insoluble formazan crystals, which upon dissolution into an appropriate solvent exhibits purple colour, the intensity of which is proportional to the number of viable cells and can be measured spectrophotometrically at 570nm.[16,17]

RESULTS AND DISCUSSION

The plant *Calotropis Gigantea* shows a lot of tremendous therapeutic effect and having a large economic value. In combination of past research and studies reports justify the use of *Calotropis gigantea* by traditional health care for professional for the treatment of various pathological alterations. The plant having huge number of phytochemical ingredients and various active constituents. plant having various organic and inorganic salts including macro and micronutrients.

Phytochemical screening

The value of the physiochemical parameters of the plant can be used as indicator in authentication and for assuring the quality of the powder form so that possibility of substitution and adulteration could be avoided. The results of preliminary phytochemical screening of stem, flower, leaf extracts of *Calotropis gigantea* showed presence of alkaloids, glycosides, flavonoids, proteins, phenol, steroids and triterpenoids and tannins.

Table 1: The phytochemical tests of *calotropis gigantea* extracts (Stem, Leave & Flower).

Phytochemical test	Stem	Leaf	Flower
Dragondroffs test	+	+	+
Wagner's test	+	-	-
Mayers test	_	+	-
Bontrager's test	_	+	+
Legals test	+	+	+
Killer-kellani test	+	-	-
Salkowski test	-	+	-
Gelatin test	-	+	+
Shinoda test	+	+	-
Lead acetate test	+	+	-
Alkaline test	+	-	-
Ferric chloride test	+	+	-
Lead acetate test	_	+	+
Biurette test	_	+	-

Anti-microbial activity

The presence of anti-bacterial and anti- fungal activity in the shade dry stem, leaf & flower extracts of *Calotropis gigantea* was obtained against human pathogenic organisms. The extracted was tested against bacterial pathogen such as *Pseudomonas aeruginosa* & *Klebsiella* pneumonia and fungal pathogen such as *Aspergillus flavus* & *Aspergillus niger.* The antimicrobial screening was done by agar well diffusion method. The efficiency of plant was determined by measuring zone inhibition.

Calotropis gigantea extracts of (stem, leaf & flower) showed antibacterial activity. Anti-fungal activity was presence only for the extracts of leaf & flower against Aspergillus niger.

***Klebsiella* pneumonia**

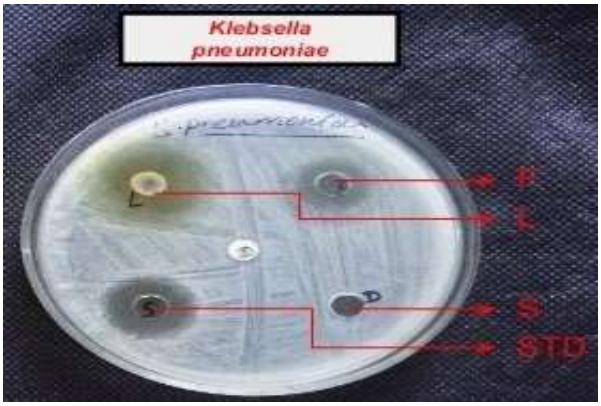
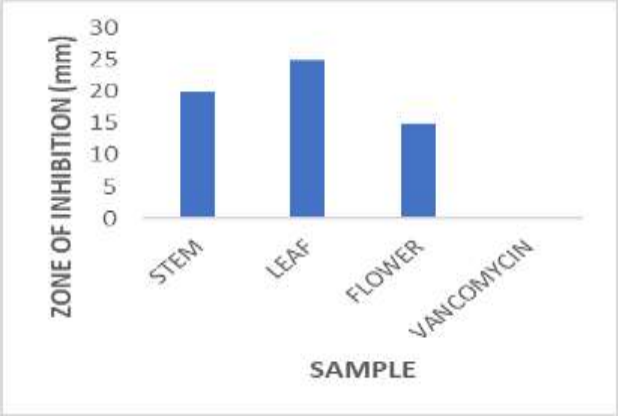


Table 2: Comparative studies of sample (Stem, Leaf and Flower extracts).

Sample	Zone of inhibition
Stem	20mm
Leaf	25mm
Flower	15mm
Vancomycin	0mm



Graph 1: Comparative studies of sample (Stem, Leaf and Flower extracts).

Pseudomonas aeruginosa

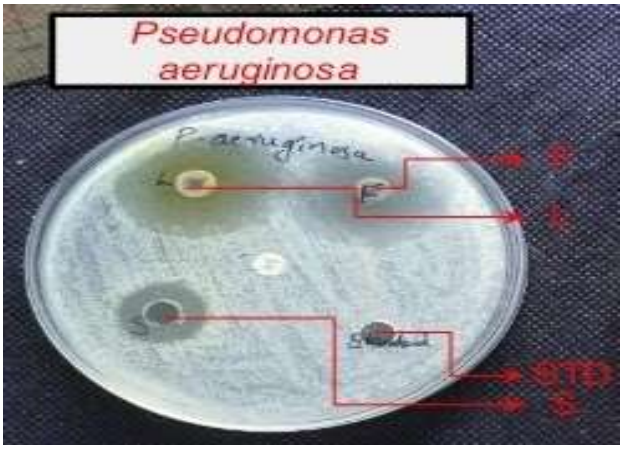
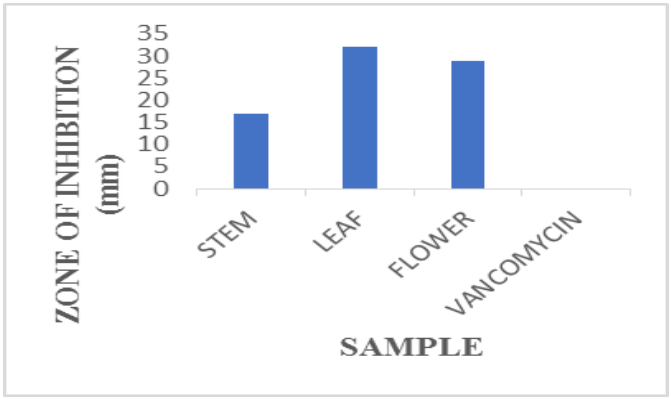


Table 3: Comparative studies of sample (Stem, Leaf and Flower extracts).

Sample	Zone of inhibition
Stem	17mm
Leaf	32mm
Flower	29mm
Vancomycin	0mm



Graph 2: Comparative studies of sample (Stem, Leaf and Flower extracts).

Aspergillus niger

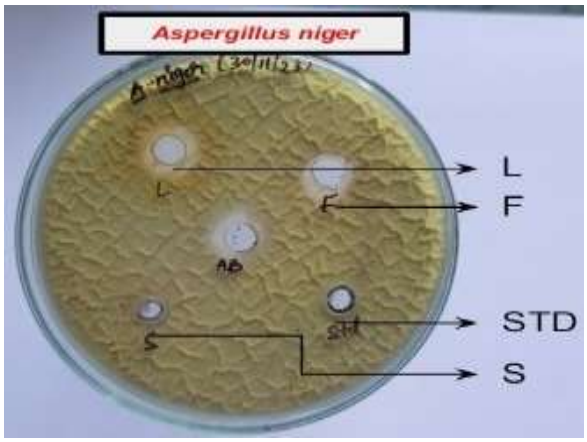
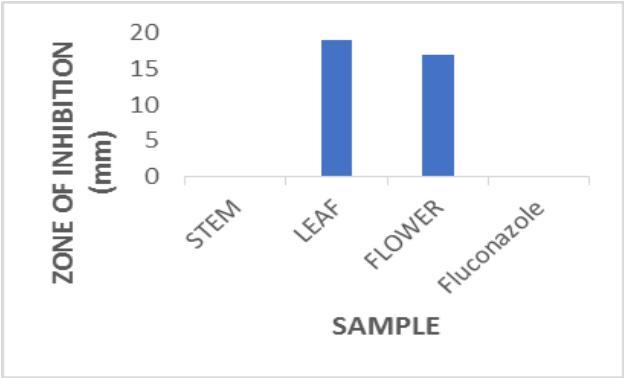


Table 4: Comparative studies of sample (Stem, Leaf and Flower extracts).

Sample	Zone of inhibition
Stem	0mm
Leaf	19mm
Flower	17mm
Fluconazole	0mm



Graph 3: Comparative studies of sample (Stem, Leaf and Flower extracts).

Aspergillus flavus

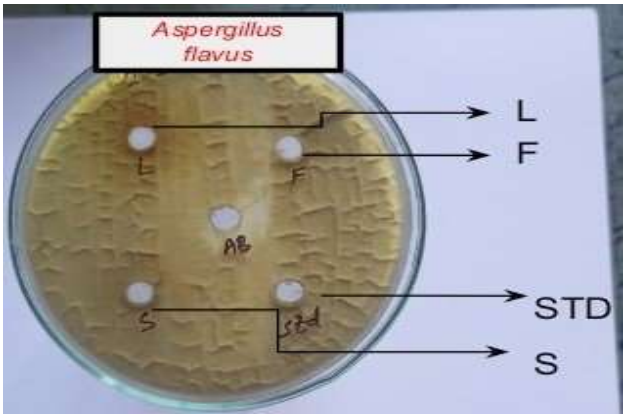


Table 5: Comparative studies of sample (Stem, Leaf and Flower extracts).

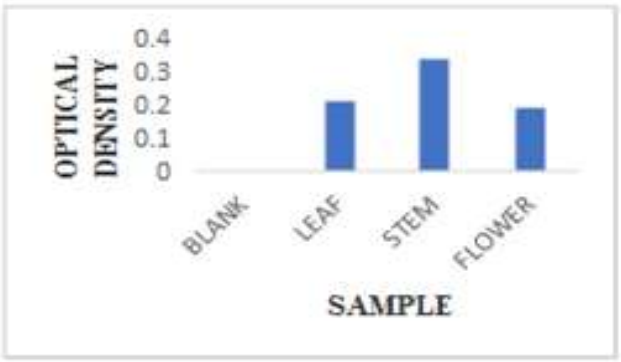
Sample	Zone of inhibition
Stem	0mm
Leaf	0mm
Flower	0mm
Fluconazole	0mm

Antioxidant activity: Sod assay

The plant extracts (Stem, leaf & flower) showed the presence of antioxidant activity. To screen the SOD activity the effect of the cauliflower extracts on enzyme activity was measured spectrophotometrically and the percentage of inhibition was calculated. The ability to reduce NBT by PMS-NADH coupling can measure the superoxide radicals generated from dissolved oxygen.

Table 6: Comparative studies of sample (Stem, leaf and flower extracts).

Sample	Optical density
Blank	0
Leaf	0.21
Stem	0.34
Flower	0.19



Graph 4: Comparative studies of sample (Stem, leaf and flower extracts).

Anticancer activity

The aqueous extracts of C. gigantea (stem, leaf & flower) exhibited cytotoxic effect against HCT-116 cancer cell lines. HCT-116 cell lines exhibited varying levels of viability upon treatment with different concentrations of the extract. IC50 value of the extract was also studied.

Cytotoxicity studies on stem extract

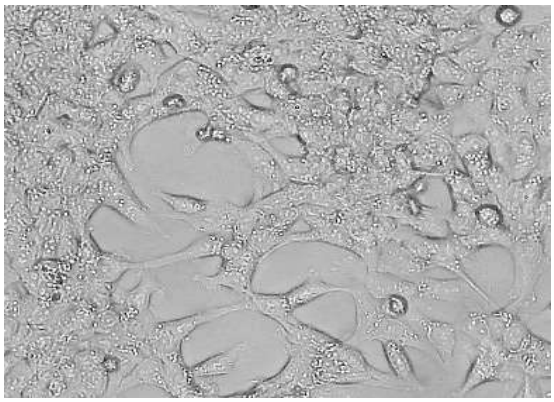


Fig. 1: Colon cancer cells.

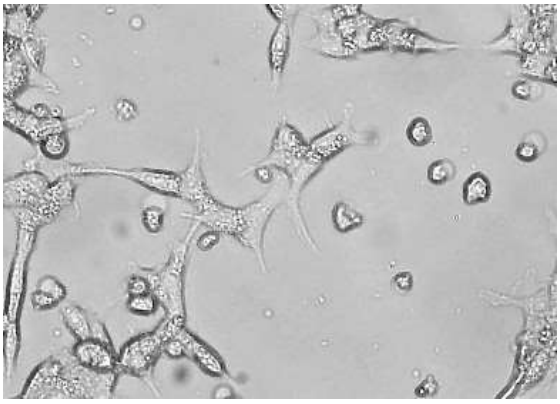
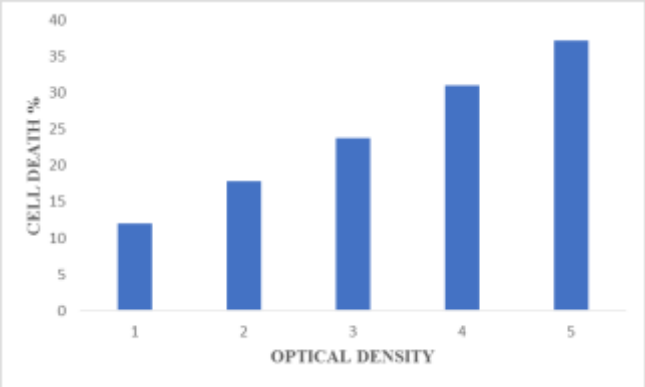


Fig. 2: Colon cancer cells after sample interaction.

Table 7 & 8: MTT Assay on Stem Extract.

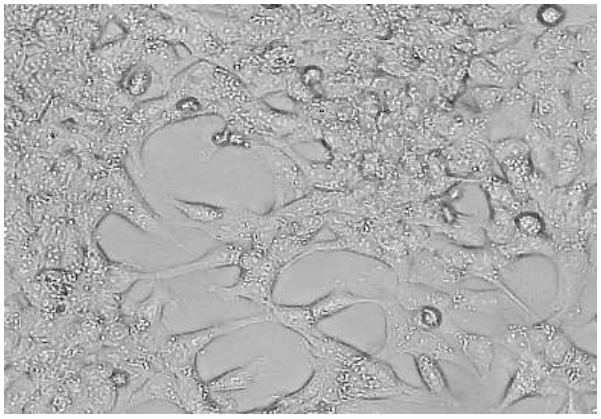
Sample	Optical density
Control	0.882
2µl	0.762
4 µl	0.725
6 µl	0.672
8 µl	0.608
10 µl	0.553

Sample optical density	Percentage of cell death (%)
0.762	12
0.725	17.80
0.672	23.80
0.608	31.06
0.553	37.30

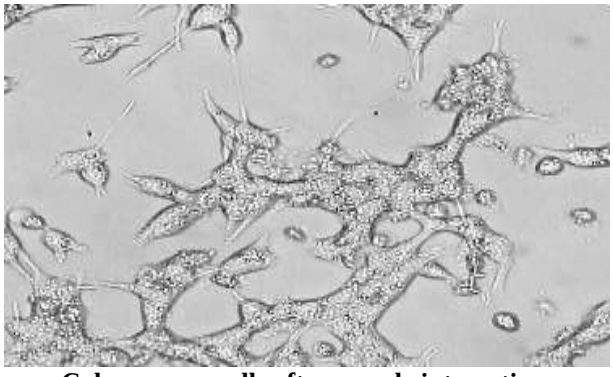


Graph 5: MTT Assay on Stem Extract.

Cytotoxicity studies on leaf extract



Colon cancer cells.

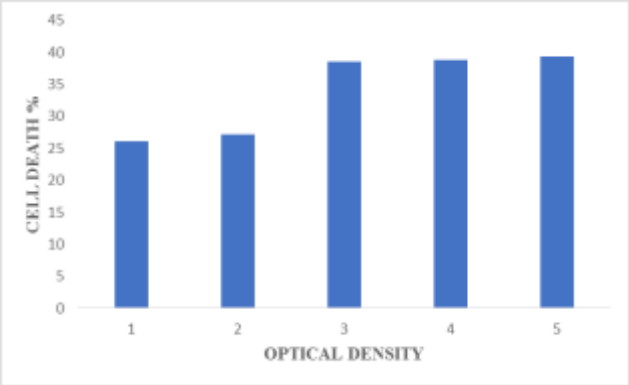


Colon cancer cells after sample interaction.

Table 9 & 10: MTT Assay on Leaf Extract.

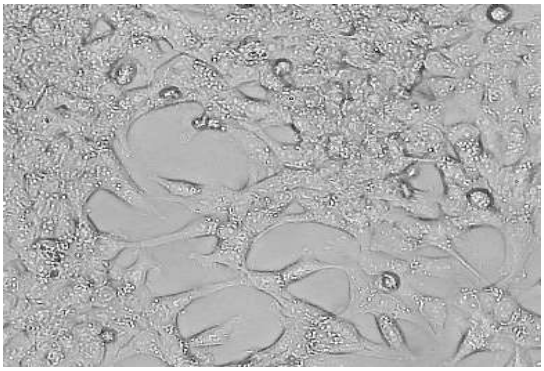
Sample	Optical density
Control	0.882
2µl	0.652
4 µl	0.642
6 µl	0.542
8 µl	0.540
10 µl	0.522

Sample optical density	Percentage of cell death (%)
0.652	26.07
0.642	27.2
0.542	38.54
0.540	38.77
0.522	39.4

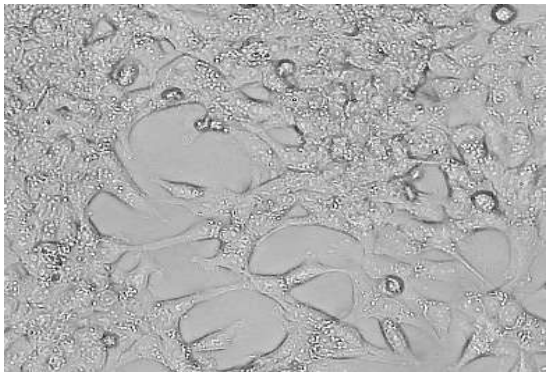


Graph 6: MTT Assay on Leaf Extract.

Cytotoxicity studies on Flower Extract



Colon cancer cells.

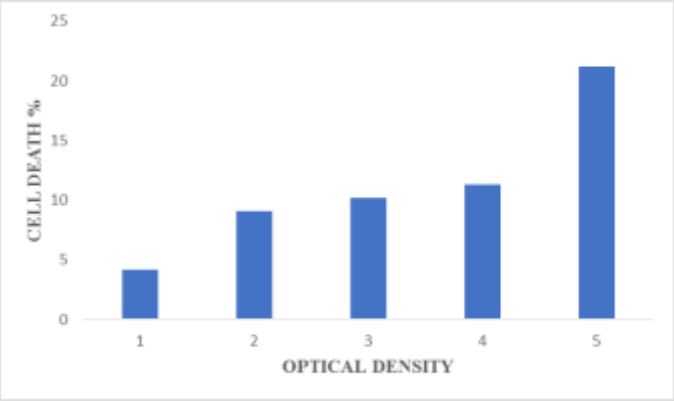


Colon cancer cells after sample interaction.

Table 11 & 12: MTT Assay on Flower Extract.

Sample	Optical density
Control	0.882
2µL	0.840
4 µL	0.802
6 µL	0.792
8 µL	0.782
10 µL	0.692

Sample optical density	Percentage of cell death (%)
0.840	4.2
0.802	9.1
0.792	10.2
0.782	11.3
0.692	21.2



Graph 7: MTT Assay on Flower Extract.

CONCLUSION

This study was screened for the phytochemical constituents of the aqueous extracts *C. gigantea* plant (stem, leaf & bark) extracts. The presence of phytochemicals as major constituents in C. gigantea may uphold the medicinal property of this plant. The plant extracts also expressed anti-microbial & antioxidant activities. *In vitro* cytotoxic assay assists the cytotoxic potential of the compounds present in the stem, leaf & flower extracts of *C. gigantea*. The extract induced apoptosis in Human Colorectal adenocarcinoma cell lines. Further screening of the active compounds of the plant extracts will help to understand the pharmacological activity of the compounds against various cancer cell lines.

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