Anticancer Studies on the Leaves and Stem bark of *Cordia dichotoma G. Forst*

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Abstract **- The aim of the study was to evaluate anticancer activity of the methanol extract of leaves and stem bark of *Cordia dichotoma* G. Forst against a Human Breast Carcinoma Cell line(MDAMB231) by MTT assay. The coarse powder of leaf and stem were subjected to Soxhlet extraction using methanol and water as solvent. Phytochemical screening was performed to find out the secondary metabolite. Total Flavonoid and phenol content were determined by colorimetric method. The phytochemical screening of methanol and aqueous extract of leaf and stem bark showed the presence of alkaloids, flavonoids, tannins, carbohydrates, amino acids, saponins, steroids and triterpenoids. The total phenolic content and flavonoid content of methanol extract of leaf was 4.467µg/ml and 5.114µg/ml and stem bark was 4.739µg/ml and 4.762µg/ml respectively. The IC_{50} values of methanol extract of stem bark, aqueous extract of leaf and aqueous extract of stem bark were found to be 125µg/mL, 232.7µg/mL and 110.1µg/mL respectively in MDAMB231 cells in comparison to Standard Vincristine IC50 14.41µM whereas methanol extract of leaf did not possess any anticancer activity. This study suggests that *Cordia dichotoma* can be useful in the treatment of breast cancer.**

Index Terms **- Anticancer activity; Total Phenol content; Total Flavonoid content; *Cordia dichotoma;* MTT Assay; Breast cancer cell lines.**

INTRODUCTION

Now a days, breast cancer is the most frequently diagnosed life-threatening cancer in women and the leading cause of cancer death among women. Since last two decades, research related to the breast cancer has led to extraordinary progress in our understanding of the disease, resulting in more efficient and less toxic treatments, increased public awareness and improved screening have led to earlier diagnosis at stages amenable to complete surgical resection and curative therapies.[1]

Cancer cells are formed from normal cells due to a modification / mutation of DNA and / or RNA. These modifications/mutations can occur spontaneously, or they may be induced by other factors such as nuclear radiation, electromagnetic radiation, microorganisms, heat, chemicals in the air, water and food, mechanical cell-level injury, free radicals, evolution and ageing of DNA and RNA, etc. All these can produce mutations that may start cancer.

Cordia dichotoma is a small deciduous tree with a short bole and spreading crown.[2] Usually a small tree growing 3 - 4 meters tall, though some can reach a height of 20 metres or more.The tree is also often cultivated for its fruits. *Cordia dichotoma* has a long and proven history of medicinal use dating back to the time of the ancient Egyptians. In traditional system of Medicine, ripe fruit of *C. dichotoma* plant is used as antibacterial, antiviral, and antitussive. Leaves and stem bark are used in the treatment of fever, diarrhea, dyspepsia, leprosy, and gonorrhea. Leafs are used traditionally as astringent, anthelmintic, diuretic, purgative, expectorant, demulcent, tonic, and ulcer [3-7]. The anticancer activity of methanolic extract of leaf against HeLa, A 549, Cervical and Prostate cancer is already established [8]. There is no report on Breast cancer so the present aim of the study is to evaluate the anticancer activity of *C.dichotoma* against Human Breast Carcinoma Cell line(MDAMB231).

MATERIALS AND METHOD

Collection and authentication of plant material:

Leaves and stem bark of *C. dichotoma* G. Forst. for the proposed study were collected from nearby region of

Tirupati forest (Andhra Pradesh) and authenticated by Dr. K. Madhava Shetty, Sri Venkateshwara University, Tirupati (authentication reference number 0511 dated 02/08/2021). A voucher specimen (TOCOP/03/2020-21- *C. dichotoma* leaf and TOCOP/04/2020-21- *C. dichotoma* Stem Bark) were deposited in The Oxford College of Pharmacy, Bangalore. The leaves and stem bark were dried in shade and powdered coarsely, passed through sieve no. 40 and stored in airtight container for further use.

Preparation of extract:
Preparation of methanolic extract: The dried leaf powder (120gm) and dried stem bark powder (70gm) were extracted using methanol in a Soxhlet apparatus for two to three days till complete exhaustion. The percentage yield was calculated.

Preparation of water extract: The marc obtained by Soxhlet extraction was air dried and 50 g of drug each leaf and stem bark were weighed and extracted using enough distilled water. The prcentage yield was calculated. (Table 1).

Phytochemical Screening of different extracts of *C.dichotma*
Chemical tests for the screening and identification of bioactive chemical constituents (such as carbohydrates, alkaloids, glycosides, flavonoids, saponins, tannins and steroid) in different extracts were carried out using the standard procedures.[9]

DETERMINATION OF TOTAL PHENOLIC CONTENT

The phenolic contents of methanol extract of leaf and stem bark were determined as follows: 0.5 ml aliquot of extracts or gallic acid (standard) was added with 5 ml of Folin–Ciocalteu reagent and 4 ml of aqueous sodium carbonate (1 M). After incubation for 15 min at room temperature, the absorbance was read at 765 nm. The total phenolic contents were expressed in terms of µg/ml of gallic acid.[10]

DETERMINATION OF TOTAL FLAVONOID CONTENT

Flavonoid contents of methanol extract of leaf and stem bark were determined as follows: 0.5 mL of extracts diluted in 1.5 ml of 95% methanol solution, 0.1 ml of 10% $AlCl_3$ (w/v), 0.1 ml of 1 M potassium acetate, and 2.8 ml of distilled water were added. After 30 min at room temperature, the absorbance was measured at 415 nm. Quercetin was used as a positive standard for preparing the standard calibration curve. The total flavonoid contents were measured in terms of µg/ml of Quercetin. [10]

Cytotoxicity studies against MDAMB231 cell line
Preparation of test solutions:
For cytotoxicity studies, 32 mg/ml stocks were prepared using DMSO. Serial two-fold dilutions were prepared from 320 µg/ml to 10 µg/ml using DMEM media for treatment.

Cell lines and culture medium:
MDAMB231 cells were procured from ATCC, stock cells were cultured in DMEM supplemented with 10% inactivated Foetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100µg/ml) in a humidified atmosphere of 5% CO_2 at 37ºC until confluent. The cell was dissociated with cell dissociating solution (0.2 % trypsin, 0.02 % EDTA, 0.05 % glucose in PBS). The viability of the cells is checked and centrifuged. Further, 50,000 cells /well was seeded in a 96 well plate and incubated for 24 hrs at 37ºC, 5 % CO_2 incubator.

Determination of anticancer activity
The cells were trypsinized and the cell count was adjusted to $5x10^5$ cells/ml using respective media containing 10% FBS. To each well of the 96 well microtiter plate, 100µl of the diluted cell suspension (50,000cells/well) was added. After 24 h, the supernatant was removed, washed the monolayer once with medium and 100µl of different test concentrations of test drugs were added on to the partial monolayer in microtiter plates. The plates were then incubated at 37ºC for 24hrs in 5% CO_2 atmosphere. After incubation the test solutions in the wells were discarded and 0.05mg MTT was added to each well. The plates were incubated for 4 h at 37ºC in 5% CO_2 atmosphere. The supernatant was removed and 100 µl of DMSO was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 590 nm. The percentage growth inhibition was calculated 32 using the following formula and concentration of test drug needed to

inhibit cell growth by 50% (IC50) values is generated from the dose-response curves for each cell line. [11-18] % Inhibition = ((OD of Control – OD of sample)/OD of Control) x 100.

IC_{50} Value

The half maximal inhibitory concentration (IC_{50}) is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. This quantitative measure indicates how much of a particular drug or other substance (inhibitor) is needed to inhibit a given biological process (or component of a process, i.e., an enzyme, cell, cell receptor or microorganism) by half. The IC_{50} of a drug can be determined by constructing a dose-response curve and examining the effect of different concentrations of antagonist on reversing agonist activity. IC_{50} values can be calculated for a given antagonist by determining the concentration needed to inhibit half of the maximum biological response of the agonist.

Statistical evaluation:

IC_{50} values for cytotoxicity tests were derived from a nonlinear regression analysis (curve fit) based on sigmoid dose response curve (variable) and computed using Graph Pad Prism 6 (Graph pad, San Diego, CA, USA.

RESULT AND DISSUSION

Percentage yield of extract:

The percentage yield of methanol and aqueous extract of leaves and stem bark of *C.dichotoma* was calculated and given in the Table no 1. Aqueous extract of leaf gave the maximum yield when compared to all.

Preliminary Phytochemical screening:

Preliminary phytochemical screening revealed the presence of carbohydrates, alkaloids, amino acid, glycoside, flavonoids, tannins, saponins and triterpenoids which is given in table 2.

Total phenol content:

The total phenolic content of methanolic extract of leaf and stem bark was found to be 4.467µg/ml and 4.739µg/ml respectively as obtained from the standard gallic acid graph [Fig no 1]. Only methanol extract was used for this determination.

Total flavonoid content

The total flavonoid content of methanol extract of leaf and stem bark was found to be 5.114µg/ml and 4.762µg/ml respectively as obtained from the standard quercetin (Fig no 2). Only methanol extract was used for this determination.

Cytotoxicity studies against MDAMB231 cell line

Traditionally, the in vitro determinations of toxic effects of unknown compounds have been performed by counting viable cells after staining with a vital dye. Alternative methods used are measurement of radioisotope incorporation as a measure of DNA synthesis, counting by automated counters and others which rely on dyes and cellular activity. The MTT system is a means of measuring the activity of living cells via mitochondrial dehydrogenases. The MTT method is simple, accurate and yields reproducible results. The key component is (3- [4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) or MTT, is a water-soluble tetrazolium salt yielding a yellowish solution when prepared in media or salt solutions lacking phenol red. Dissolved MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by mitochondrial dehydrogenase enzymes of viable cells. This water insoluble formazan can be solubilized using DMSO, acidified isopropanol or other solvents (Pure propanol or ethanol). The resulting purple solution is spectrophotometrically measured. An increase or decrease in cell number results in a concomitant change in the amount of formazan formed, indicating the degree of effects caused by the test material. [14,18]

The anti-cancer activity was determined by MTT assay using methanolic and aqueous extract of leaf and stem bark of *C.dichotoma*. IC_{50} values was found to be 125µg/mL, 232.7µg/mL and 110.1µg/mL for methanol extract of stem bark, aqueous extract of leaf and aqueous extract of stem bark respectively in MDAMB231 cells in comparison to Standard Vincristine IC_{50} 14.41µM. Images also show that the cell morphology is disrupted which confirms the cytotoxicity effect. Methanol extract of leaf did not show any anticancer activity (Table no. 3; Fig no 3).

CONCLUSION

The result of the present study revealed that *C.dichotoma* showed anticancer activity against breast cancer. Methanolic extract of stem, aqueous

extract of leaf and aqueous extract of stem bark showed IC_{50} values was found to be 125µg/mL, 232.7µg/mL and 110.1µg/mL respectively in MDAMB231 cells in comparison to Standard Vincristine IC_{50} 14.41µM. thus *C. dichotoma* is a promising agent for nano chemoprevention of breast cancer cells used for the study. Further studies have to done to isolate the phytoconstiutents responsible for anticancer activity.

ACKNOWLEDGEMENT

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ETHICAL ISSUES

There is none to be applied.

CONFLICT OF INTEREST

None to be declared.

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Fig no. 1 Determination of total phenol content of standard gallic acid

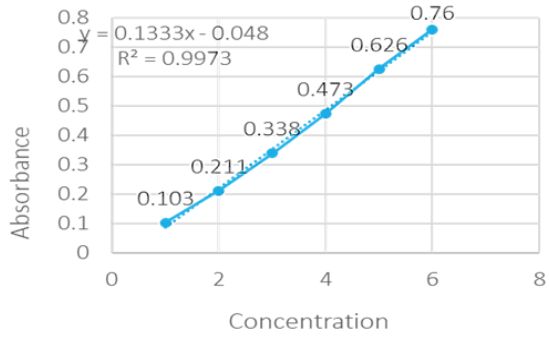


Fig no. 2 Determination of total flavonoid content of standard quercetin

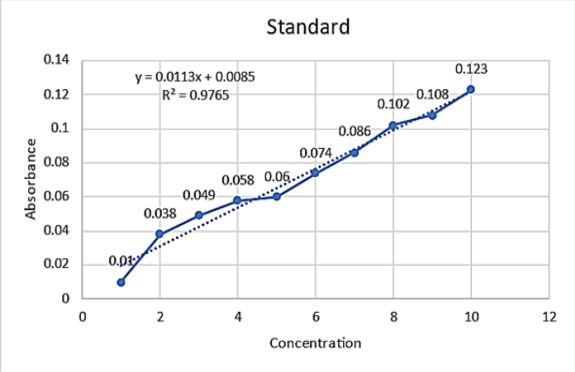
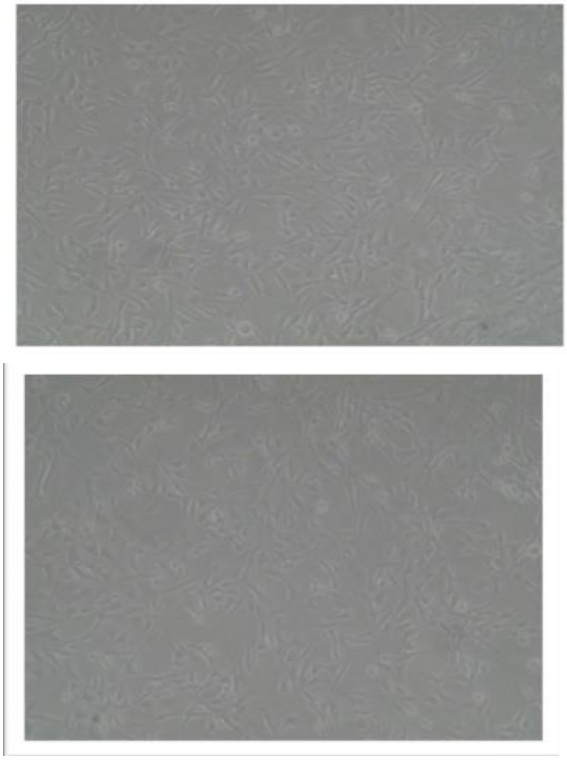
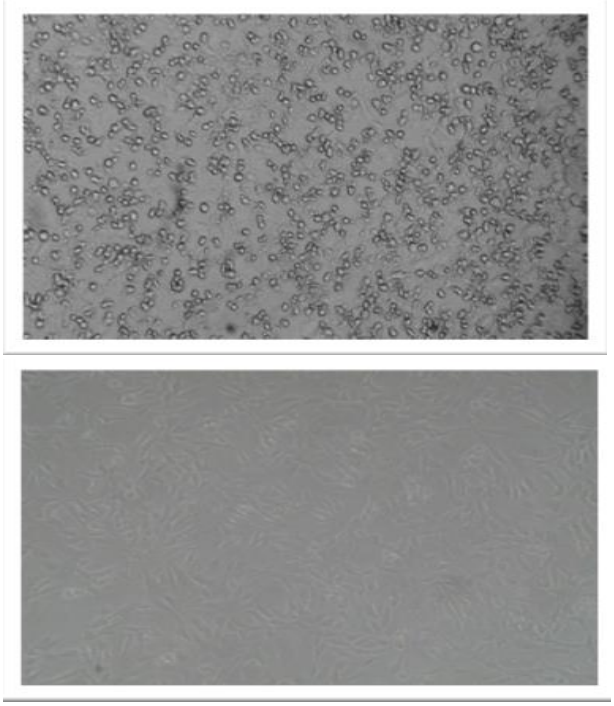


Fig no 3: Anticancer effect of different extracts of *C.dichotoma* leaf, stem bark and vincristine



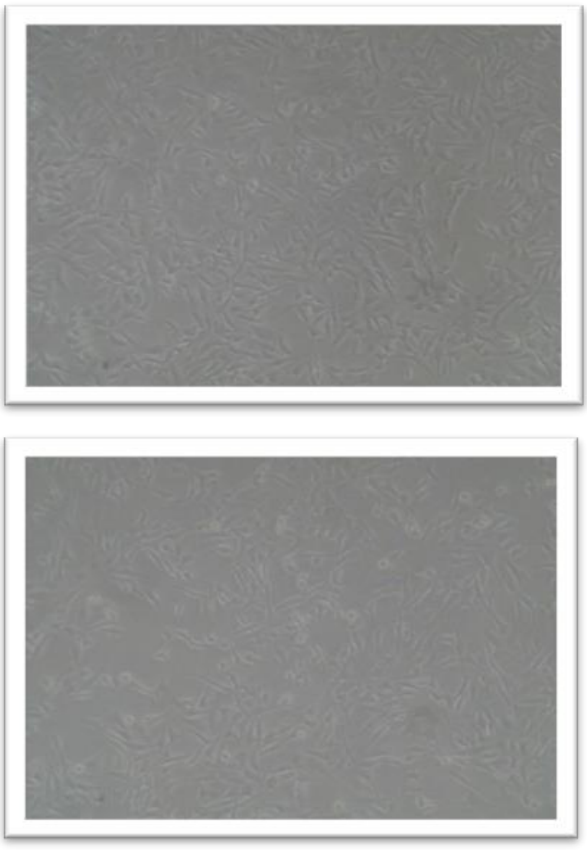
Control

Vincristine (3.125µM)

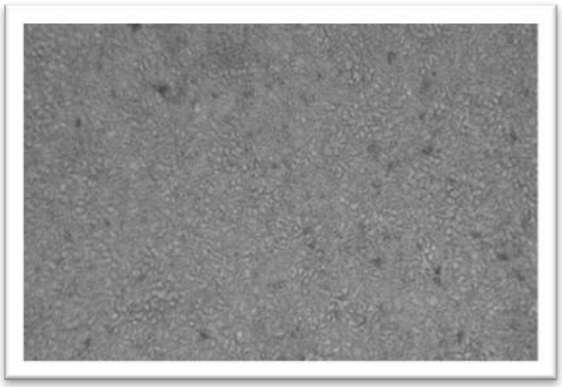


Vincristine (100 µM)

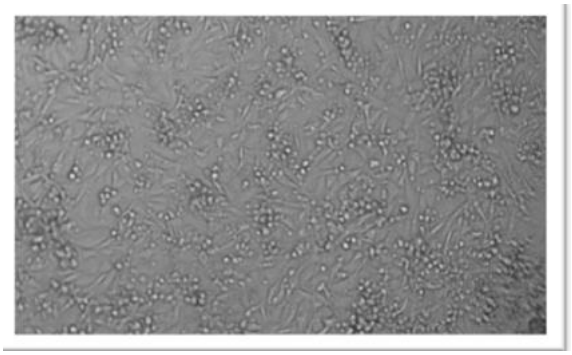
Methanolic extract of leaf (10µg/ml)



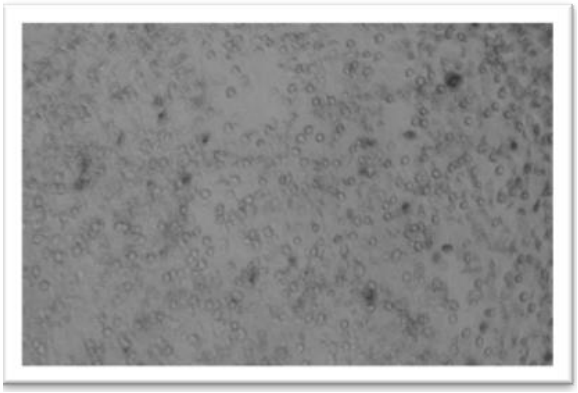
Methanolic extract of leaf (320µg/ml)
Methanolic extract of stem bark (10µg/ml)



Methanolic extract of stem bark (320µg/ml)
Water extract of leaf (10µg/ml)



Water extract of leaf (320µg/ml)
Water extract of Stem bark (10µg/ml)



Water extract of Stem bark (320µg/ml)

Table no.1 Percentage yield of different extract of *C. dichotoma*.

Sl No	Extract details	Percentage yield (% w/w)
1	Methanol extract of leaf	7.9
2	Water extract of leaf	29.3
3	Methanol extract of stem bark	4.66
4	Water extract of stem bark	8.6

Table no.2 Preliminary phytochemical screening of different extract of *C. dichotoma*

Sl No	Phytochemicals	Methanol extract of leaf	Water extract of leaf	Methanol extract of stem bark	Water extract of stem bark
1	Carbohydrate	+	+	+	+
2	Alkaloids	+	+	+	+
3	Amino acid	-	+	+	+
4	Glycosides	+	+	+	+
5	Flavonoids	-	+	+	+
6	Tannins	-	+	+	+
7	Saponins	+	+	+	+
8	Steroids and Triterpenoids	+	+	+	+

Table no.3: IC_{50} value of methanol and aqueous extracts of leaf and stem bark of *C.dichotoma* on MDAMB231 cell line

Sl No	Extract details	IC_{50} value(µg/ml)
1	Methanol extract of leaf	No inhibition at higher dose
2	Water extract of leaf	230.70
3	Methanol extract of stem bark	125.00
4	Water extract of stem bark	110.10
5	Standard Vincristine	14.41