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ANTIMITOTIC AND ANTIPROLIFERATIVE ACTIVITYOF LEAVES OF *CAESALPINIA PULCHERRIMA*

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ABSTRACT

Caesalpinia pulcherrima is a widely documented ethnomedicinal plant that locals have used in varied applications due to its antibacterial, antioxidant, antimutagenic, renoprotective, and other biological activities. The present study investigates the antimitotic and antiproliferative actions of leaf extract of *Caesalpinia pulcherrima*using onion (*Allium cepa*) root tip assay, *Vigna radiata* shoot inhibition and seed germination assay. In Allium assay and Seed germination assay ethanolic extract of leaves of *Caesalpinia pulcherrima* showed significant concentration dependant inhibitory influence against the dividing cells of Allium roots and *Vigna radiata shoots* which was observed by a decrease in root growth, shoot growth and mitotic index as compared to control. *A. cepa* assay and seed germination assay showed that the *Caesalpinia pulcherrima* leaf extract can block mitosis by causing spindle and chromosome damage. The extract is genotoxic based on the increasing frequency of chromosomal aberrations at higher concentrations. This study revealed that *Caesalpinia pulcherrima* crude ethanolic extract has antimitotic and antiproliferative properties. Further study could be performed focusing on identifying and isolating antimitotic, and antiproliferative components of leaves of *Caesalpinia pulcherrima* and conducting biological screening using other models.

Keywords: *Caesalpinia pulcherrima*; *Allium cepa* assay; seed germination assay; antimitotic

1. INTRODUCTION:

Plants have been used as an excellent source of medicine since beginning which established a foundation of traditional medicine. Such traditional medicinal plants play vital role to fulfil worldwide healthcare needs nowadays and their use will increase in the future **[1]**.

Cancer is among the most fatal diseases affecting human population and the major cause of death worldwide **[2]**. Cancer is thought to be caused by the toxic substances in the environment which may cause genetic changes in man. Biochemically cancer is any change or abnormality in the process of cell division that results in uncontrolled cell division leading to malignancy, *i.e.*, cancerous cell growth **[3]**. The suppression of abnormal cell division is therefore an important therapeutic strategy for treating uncontrolled cell growth in cancer patients **[4]**. Most of the chemotherapeutic drugs used for treating cancer actually work by inhibiting cell division (antimitotic) and by targeting fast-dividing cells (antiproliferative) hence called cytotoxic drugs. In the present scenario the research is being focused on use of cytotoxic drugs in cancer chemotherapy **[5]**. The researchers are, therefore, searching for substances which can prevent cells from division.

Exploration of traditionally medicinal plants is important on two levels: firstly, as a source of possible chemotherapeutic drugs and secondly, as an evaluate of safety for the continued use of medicinal plants **[6]**. The greater part of standard anticancer drugs has been derived from natural sources, based on their use in traditional medicine **[7]**. Knowledge of phytochemical constituents is requisite to understand the mechanism of bioactivity of plant extracts.

Antioxidant compounds such as phenolic acids, polyphenols, and flavonoids scavenge free radicals such as peroxide and hydroperoxide and thus inhibit oxidative stress. The free radical assumption supported the fact that the antioxidants effectively inhibit the DNA damage and uncontrolled division of cells **[8]**.

Plants belonging to the family *Leguminoceae* have wide folklore medicinal uses. *Caesalpinia pulcherrima* Swartz vernacularly known as Guletura is widely distributed in India and its leaves, flower, bark, and seeds are used in Indian medicine. The plant is considered as a tonic, stimulant, and emmenagogue. The bark is used as an abortifacient and an infusion of leaves is used as abortifacient and cathartic. *Caesalpinia pulcherrima* also possesses various pharmacological properties including antibacterial,

antifungal, antiviral, anticancer and anti-inflammatory effects. The plant is rich in active ingredients like caesalpin-type diterpenoids, sitosterol, pulcherrimin, lupeol, lupeol acetate, myricetin, quercetin and rutin, flavonoids, carotenoids, glycosides, peltogynoids, phenols, and steroids **[9, 10]**. An attempt has been made in the present study to evaluate the antimitotic and antiproliferative activity of the ethanolic extract of leaves of *C. pulcherrima* (Linn.).

2. MATERIAL AND METHODS:

2.1. Drugs and chemicals: Ethanol, glacial acetic acid and acetocarmine were obtained from obtained from MERCK, Mumbai, India. Other chemicals used in the present study were of analytical grade and purchased from the reputed manufacturers.

2.2. Collection and preparation of plant extract: The leaves of the plant *Caesalpinia pulcherrima* were collected from Joginpally group of institution campus and identified with the help of taxonomist from the Department of Botany Osmania University. The voucher specimen was deposited for future reference. The fresh leaves of *Caesalpinia pulcherrima* were washed, shade dried and powdered. The air dried powder materialwas extracted successively with ethanol by hot continuous method (soxhlation). The solvent was evaporated to dryness under pressure using rotary flash evaporator to obtain solid extracts. Working dilutions of all drugs were made in distilled water.

2.3. *In-vitro* assessment of anti-mitotic Activity (Allium Assay): The assay was conducted as per previously described methods with requisite modifications **[11, 12]**. Briefly, approximately equal size bulbs (40±10 g) of the onions (*Allium cepa* L.) were obtained from the local vegetable market Moinabad, India. The onion bulbs were grown in dark for 48 h over 50 ml of tap water at ambient temperature until the roots have grown to approximately 3 cm. The bulbs with root tips grown up to 2-3 cm were selected for the study. The bulbs were divided into five groups each containing 3 bulbs. The first group served as control. Immediately, the bulbs were placed in containers filled with test samples (one bulb in each) and control (tap water) and incubated at 22±2°C for 72 h away from direct sunlight. The test samples were changed daily with fresh ones. The length of roots grown during incubation (newly appearing roots not included), root number and the mitotic index were recorded after 72 h. The root tips (2-3 mm) were collected and were fixed in the fixing solution of acetic acid and ethanol (3:1). Squash preparation was made for microscopic studies by standard aceto-orcin stain squash

preparation technique **[13]**. For each root tip, the numbers of mitotic cells and total meristematic cells were counted manually in 5-8 fields of view using high resolution (100x) bright field light microscopy.

2.4. *In-vitro* anti mitotic and anti proliferative Activity seed germination assay: Experimental plant model *Vigna radiata* L. (Green-gram; Family: Fabaceae) was used as plant model. Good quality and free from any external injury *Vigna radiata* L. (Green-gram) were purchased from the local market. Green-gram seedling root apical meristem cells were used to determining the cell cycle delay and metaphase arresting activity for the validation of antiproliferative activities. Treatment of *Vigna radiata* L. (Green-gram) seedlings Germination test of *Vigna radiata* L. was performed according to ISTA (International Rules for Seed Testing) rules 2018. For the germination of seeds, four different concentrations of aqueous stem bark extract of *Caesalpinia pulcherrima* L. (250, 500, 1000 and 2500 µg/mL) were placed in a 24 well microplate and *Vigna radiata* L. seed (n=30) was dropped into each well **[14]**. The microplate was then covered with a lid and allowed to germinate at room temperature. Tap water alone in a microplate was considered as negative control. The dosage of colchicine was based on previous study. Seed germination and root length were measured at 24, 48 and 72 hour intervals both in control and test groups. The shoot growth microphotography was taken by using Nikon D3200 camera. The seeds that did not germinate were simply discarded and no other parameters could be measured on these seeds.

2.5. Mitotic index (MI) analysis: The analysis of total cell counted total cell in mitotic stage and mitotic index (MI) were determined by our previous report. Two doses of ethanolic extract of leaves of *Caesalpinia pulcherrima* L. (250 and 2500 µg/mL) were selected for the mitotic index analysis of *Vigna radiata* L. root apical meristem cells at 0, 24, 48 and 72 hour intervals. The roots were cut, and lengths were measured with standard scale and then fixed in Carnoy's fixative (aceto: methanol, 3 part methanol: 1 part acetic acid) for 24 hours. The root tips were then passed through 100%, 90% and 70% alcohol and preserved in 70% absolute alcohol for mitotic index analysis. The apical root tips were separated and stained in 2% acetocarmine and squashed under a cover slip. Slides were studied under the bright field light microscope at 400x, using Leica DM5000B ((Leica Microsystems, Germany) microscope for cell cycle phase (interphase, prophase, metaphase, anaphase, and telophase) observation. Each

experimental group of slides, 100 cells were analysed, calculating the total number of cells in whole cell cycle and total number of cells in mitotic stage of *Vigna radiata* L., and thereby determining the mitotic index (MI).

The Mitotic index (MI) was calculated by the following equation:

$$\text{Mitotic index} = \frac{P + M + A + T}{\text{Total number of cells}} \times 100$$

Where, P = Prophase, M = Metaphase, A = Anaphase, T = Telophase

2.6. Statistical Analysis: The data were analyzed with one-way ANOVA followed by Tukey's multiple comparison post hoc tests. A statistical difference of $P < .05$ was considered significant in all cases.

3. RESULT:

3.1. Phytochemical Screening: Phytochemical Screening of leaves of *Caesalpinia pulcherrima* revealed the presence of flavonoids, alkaloids, tannins, and phenolic compounds while carbohydrates, proteins, amino acids, and glycosides were absent.

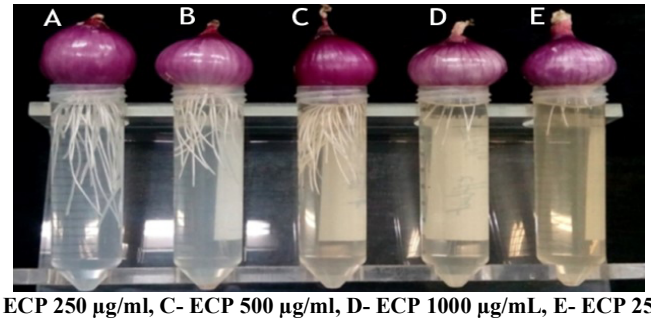
3.2. Effect of ethanolic extract of leaves of *Caesalpinia pulcherrima*treatment on the Mitotic Cell Division in Allium cepa Assay: The *A. cepa* root tip assay is the most common method for testing compounds with antimitotic activity. The mitotic division in root tip cells resembles cell division in normal human and cancer cells. Hence, onion root tip cells can be used for testing of drugs with anticancer activity **[15]**. An agent that prevents or disrupts mitosis is called as antimitotic agent. Antimitotic compounds can interrupt the process of mitosis in anywhere of the cell cycle. So, extracts of leaves of *Caesalpinia pulcherrima*were inhibitory against actively dividing cells and produced cytotoxic effect either by inhibiting spindle formation in M-phase or by damaging DNA in S-phase of the cell cycle. Low mitotic index reflected a direct genotoxic effect of plant extracts on living cells. This decrease in mitotic index indicates significant antimitotic activity of the extracts and this may be due to the interactions of the specific plant phytochemicals with the mitotic apparatus of the cells that causes delay in interphase resulting in increased number of cells at interphase stage, blocking transition from interphase to mitotic phase and decreasing mitotic index **[16]**. The results from the study showed that *Caesalpinia pulcherrima* had excellent antimitotic activity with lowest mitotic index at highest concentration.

The effect of test extract ELCP was studied on root growth and mitotic index. Two-way ANOVA indicated ELCP has significant influence on mitotic cell division of Allium root. ELCP at the concentration of (250, 500, 1000 and 2500 µg/mL) exhibited significant inhibitory influence on root

growth, (root length) in a concentration dependent manner compared to control group as shown in **Table 1**. The ELCP at higher concentration of 2500 µg/mL caused shrinking of roots and the color of roots turned blackish brown as shown in **Figure 1(a) and 1(b)**. Antimitotic compounds can interrupt the process of mitosis in anywhere of the cell cycle. So, extracts of leaves of *Caesalpinia pulcherrima* were inhibitory against actively dividing cells and produced cytotoxic effect either by inhibiting spindle formation in M-phase or by damaging DNA in S-phase of the cell cycle. Low mitotic index reflected a direct genotoxic effect of plant extracts on living cells. This decrease in mitotic index indicates significant antimitotic activity of the extracts and this may be due to the interactions of the specific plant phytochemicals with the mitotic apparatus of the cells that causes delay in interphase resulting in increased number of cells at interphase stage, blocking transition from interphase to mitotic phase and decreasing mitotic index **[16]** which is observed in **Table 3**. The results from the study showed that *Caesalpinia pulcherrima* had excellent antimitotic activity with lowest mitotic index at highest concentration. The mitotic index at 2500 µg/mL concentration of ELCP was lessas compared to in control group. Chromosomal abnormities were also observed in treated rootlets. Meristematic cells of treated group showed various alterations like, irregular prophase with decompressed chromosomes, irregular anaphase showing chromosomal breakage; irregular metaphase, with unorganized chromosome, showing chromosome with no orientation on the equatorial plate as shown in **Figure 2**.

Table 1: Effect of ethanol extract of leaves of *Caesalpinia pulcherrima* on root length of *Allium cepa*

Control/sample	Root length			
	0h (n=30)	24h (n=27)	48h (n=24)	72h (n=21)
Water	3.12±0.20	4.82±0.26	5.02±0.12	6.4±0.18
ECP 250 µg/mL	2.48±0.17	2.62±0.21	3.78±0.16	3.94±0.17
ECP 500 µg/mL	2.13±0.12	2.83±0.12	3.97±0.21	2.96±0.12
ECP 1000 µg/mL	2.94±0.15	3.59±0.18	3.17±0.24	2.42±0.16
ECP 2500 µg/mL	2.98±0.20	2.76±0.17	2.52±0.16	2.22±0.19



A-Normal control, B- ECP 250 µg/ml, C- ECP 500 µg/ml, D- ECP 1000 µg/mL, E- ECP 2500 µg/mL Figure 1(a): Effect of ethanol extract of leaves of *Caesalpinia pulcherrima* on root length of *Allium cepa*

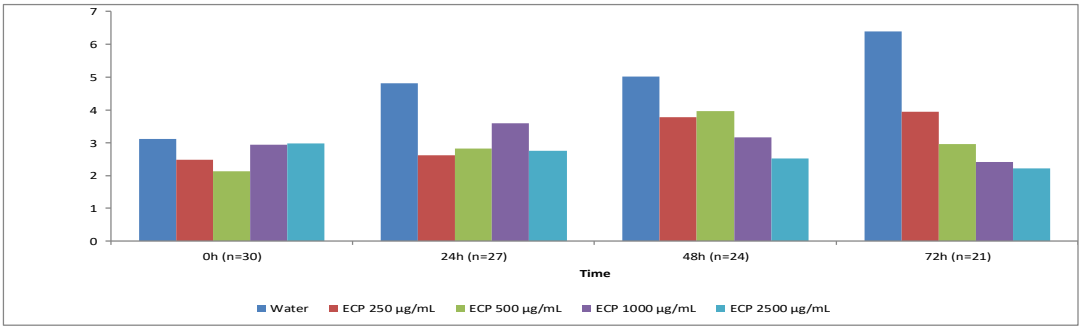


Figure 1(b): Graphical representation of effect of ethanol extract of leaves of *Caesalpinia pulcherrima* on root length of *Allium cepa*

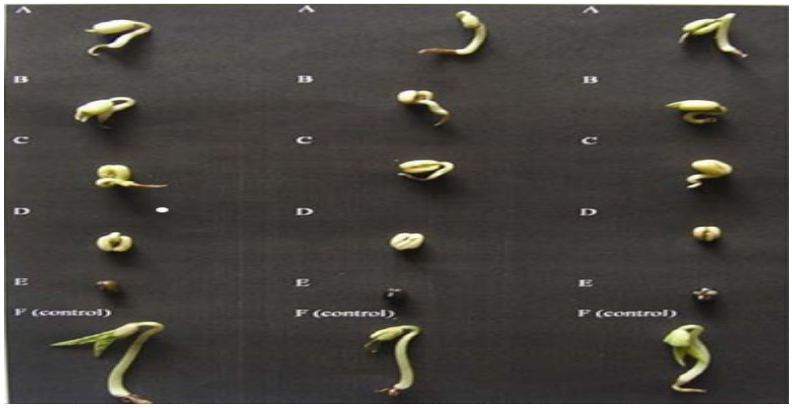
Figure 2: Normal mitotic phases A) interphase B) prophase C) metaphase D) anaphase E) telophase

Table 3: Effect of ethanol extract of leaves of *Caesalpinia pulcherrima* L. on the mitotic cell division of *Allium cepa* L. root apical meristem cells

Extract concentration mg/mL	Total no. of cells	No.of non-dividing cells	P	M	A	T	Total dividing cell	Mitotic index (%)
Water	100	4	86	6	2	2	96	96 ± 1.527
ECP 250 µg/mL	100	21	78	-	-	1	79	79 ± 0.577
ECP 500 µg/mL	100	29	68	2	1	-	71	71 ± 0.577
ECP1000 µg/mL	100	41	57	-	-	3	60	60 ± 0.577
ECP2500 µg/mL	100	47	43	-	-	-	43	43 ± 0.577

P = prophase; M = metaphase; A = anaphase; T = telophase. Values with different alphabets are significantly different from each other at P = 0.05

3.3. Effect of ethanol extract of leaves of *Caesalpinia pulcherrima* on shoot length of *Vigna radiata*: The ethanol extract of leaves of *Caesalpinia pulcherrima* L. investigated in this study revealed a dose and time dependent inhibitory effects on seed germination, shoot growth and root length in *Vigna radiata* L. At higher concentration of *Caesalpinia pulcherrima* L. leaf extract (2500 µg/mL) significantly inhibited seed germination at time dependant manner and the results were compared to the normal control **(Table 4 and Figure 4(a) and 4(b))**. Treatment with 1000 µg/mL and 2500 µg/mL of *Caesalpinia pulcherrima* L. leaf extract showed a significant effect on the root apical meristem growth of green gram seedlings at 48 and 72 hours' time interval. High concentration of *Caesalpinia pulcherrima* L. (2500 µg/mL) treatment significantly reduced the shoot length of green gram seedlings time dependently when compared to the negative control of the respective groups.



A- ECP 250 µg/mL, B- ECP 500 µg/mL, C- ECP 1000 µg/mL, D- ECP 2500 µg/mL, E-Colchicine treated (50 µg/mL), F-Normal Control

Figure 4(a): Effect of ethanol extract of leaves of *Caesalpinia pulcherrima* on shoot length of *Vigna radiata*

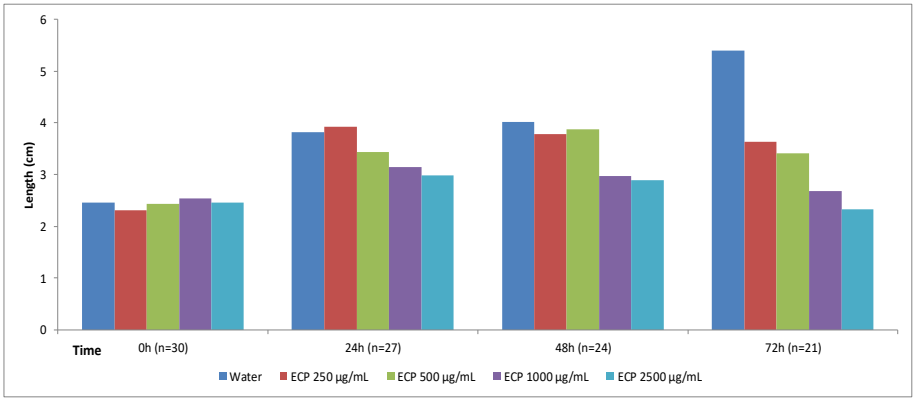


Figure 4(b): Effect of ethanol extract of *Caesalpinia pulcherrima* on the shoot length of *Vigna radiata*

Table 4: Effect of ethanol extract of leaves of *Caesalpinia pulcherrima* on shoot length of *Vigna radiata*

Control/sample	Shoot length			
	0h (n=30)	24h (n=27)	48h (n=24)	72h (n=21)
Water	2.46±0.20	3.82±0.26	4.02±0.12	5.4±0.18
ECP 250 µg/mL	2.31±0.17	3.92±0.21	3.78±0.16	3.64±0.17
ECP 500 µg/mL	2.43±0.12	3.43±0.12	3.87±0.21	3.41±0.12
ECP 1000 µg/mL	2.54±0.15	3.14±0.18	2.97±0.24	2.68±0.16
ECP 2500 µg/mL	2.46±0.20	2.98±0.17	2.90±0.16	2.32±0.19

DISCUSSION:

A simple plant model that has been widely used for such studies is the root tip meristem of *Allium cepa*, where cells are actively dividing. However, due to chemical treatment of the bulbs for long term storage, this model possesses problems in growing root tip meristem cells. The *A. cepa* root tip assay is the most common method for testing compounds with antimitotic activity. The mitotic division in root tip cells resembles cell division in normal human and cancer cells. Hence root tip cells can be used for testing of drugs with anticancer activity. An agent that prevents or disrupts mitosis is called as

antimitotic agent. Antimitotic compounds can interrupt the process of mitosis in anywhere of the cell cycle. So, extracts of *Caesalpinia pulcherrima* were inhibitory against actively dividing cells and produced cytotoxic effect either by inhibiting spindle formation in M-phase or by damaging DNA in S-phase of the cell cycle. Low mitotic index reflected a direct genotoxic effect of plant extracts on living cells. The mitotic index is used as an indicator of adequate cell proliferation, which can be measured using the *Allium cepa* L. In this study the treatment of ethanol extract of leaves of *Caesalpinia pulcherrima* L. (1000 and 2500 µg/mL) was found to be associated with a significant reduction in mitotic index in the root tip meristematic cells of *Allium cepa* L. and were compared to normal control. Reduction in the mitotic index could be due to inhibition of DNA synthesis or blocking in the G2 phase of the cell cycle, preventing the cell from entering mitosis. This result indicates that the plant extract possesses antiproliferative property. In the present investigation, the qualitative phytochemical screening of ethanol extract of leaves of *Caesalpinia pulcherrima*L. witnessed the presence various secondary metabolites of alkaloids, carbohydrates, glycosides, saponins, phytosterols, phenolic compounds and flavonoids. It is indicating that the inhibitory action against germination and seedling growth of *Vigna radiata* L and significant reduction in mitotic index and root inhibition in *Allium cepa* model may be due to the presence of secondary metabolites in the plant extract which have adverse effects on the cell cycle progression machinery.

The seed germination assay reported here is similar to the one recently reported by Kumar and Singhal **[17]**, Primary observation of inhibition in the sprouting of green-gram seeds by cytotoxic/antitumour drugs has been the basis in both cases. The method developed by Kumar and Singhal has demonstrated inhibition of the following parameters: (a) per cent sprouting of seeds, (b) imbibition of water, (c) length of radical in 24 h and (d) growth (cytotoxicity). In this study *Vigna radiata* L. was chosen as a plant model to evaluate the antiproliferative effect of the ethanol extract of leaves of *Caesalpinia pulcherrima* L. Many earlier studies havereported the antiproliferative activity of medicinal plant in *Vigna radiata* L. model system such as *Nigella sativa, Azadirachta indica, Parthenium hysterophorus,* and *Moringa oleifera*. In this study, the evaluation of antiproliferative effects of ethanol extract of leaves of *Caesalpinia pulcherrima* L. in *Vigna radiata* L. test system demonstrated that the species significantly inhibited the

rate of seed germination, root and shoot growth at dose and time dependent manner. Increasing concentration of plant extracts significantly exerted the growth inhibitory effects on all the parameters when compared to negative control groups. This indicates the antiproliferative capacity of the ethanol extract of leaves of *Caesalpinia pulcherrima* L. Moreover, these results agree with the previous study reports representing the seed germination, root and shoot growth retardation is a result of the suppression of cell division. A number of earlier studies have suggested that the level of root growth inhibition increases with the increasing plant extract concentrations.

CONCLUSION:

The present study we concluded that the secondary metabolites present in the ethanol extract of leaves of *Caesalpinia pulcherrima* L. significantly inhibited the rate of seed germination and seedling growth in *Vigna radiata* L. In root tip inhibition assay an interrupt the process of mitosis, decreased length of root tips and Low mitotic index reflected a direct genotoxic effect of plant extracts on living cells. It can signify that the use of leaf extract of *Caesalpinia pulcherrima*in high concentrations may potentially be therapeutic for inhibiting the cell cycle in eukaryotic organisms. Thus, the plant *Caesalpinia pulcherrima* L. can be a strong antiproliferative agent for the management of proliferative diseases and cancer progression. Further research will be needed to isolate the active compounds and to determine their influence on the cell cycle regulatory gene expression.

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