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Evaluation of *In-vitro* Anti-cancer activity of Ethanolic Extract of *Corallocarpus epigaeus* on Chronic Myeloid Leukaemia K562 Cell lines

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ABSTRACT:

Aim: The tremendous chemical diversity of nature is an attractive source of myriad chemotherapeutic agents. This fact aided by the virtual revolution in tissue culture technology facilitates employment of high throughput, *In-vitro* cytotoxic assays using human derived cancer cell lines to discover meticulous drug molecules. The present study was initiated to evaluate the anti proliferative potential of whole plant ethanolic extract of *Corallocarpus epigaeus* against human chronic myeloid leukaemia K562 cell lines by Trypan blue and MTT assay.

Method: A trend in the reduction of total viable cell count was evident following a 24 hr single exposure to different concentrations of test extract in Trypan blue as well as in MTT assay.

Result: Thus the ethanolic extract of *Corallocarpus epigaeus* has appreciable growth inhibitory activity on K562 cell lines with an IC50 value of 78.84µg/ml.

Conclusion: Further studies on various extracts of *Corallocarpus epigaeus* can be initiated to explore its potential as a promising phytochemical in anti-cancer therapy.

Key words: Chronic Myeloid Leukaemia, K562 Cell lines, Trypan blue assay, MTT assay.

INTRODUCTION:

Health is a universal human aspiration and a basic human need. International Health conference, held in New York in June 1946 defined health as “a state of complete physical, mental and social wellbeing, and not merely the absence of disease or infirmity” [1]. The development of society whether rich or poor can be judged by the quality of its population health, distribution of health across the social spectrum and the degree of protection provided from ill-health. Non-communicable diseases such as cancer, Heart disease, diabetes, stroke account for nearly half of the global burden of disease at all stages and the burden is rapidly escalating in low and middle income countries [2]. As the science of therapeutics has been evolving through

an imperative phase of research and development, it may possibly bring about unprecedented cures and palliative treatments. But devastating illness such as cancer, diabetes and a number of neurodegenerative disorders renders enduring challenges to the health and wellbeing of people world over. Cancer, medically a multifactorial malignant neoplasm characterized by uncontrolled proliferation of normal cells due to the stimulation by a myriad of carcinogens. On a worldwide basis cancer represents the single leading cause of death among men and women and so cancer is one of the major thrust area for which effective drugs at affordable prices are not available probably due to the lack of understanding of mysterious cancer cell biology. Approximately 5-10% of Cancers are entirely

hereditary [3]. Chronic Myelogenous leukaemia (CML) is an uncommon type of cancer of blood cells characterized by increased and unregulated growth of predominantly myeloid cells in the bone marrow and the accumulation of these cells in the blood. It is a kind of clonal myoproliferative disease associated with a chromosomal translocation called the Philadelphia chromosome. It occurs in all age groups, but most commonly in middle-aged and elderly with men more likely to be affected than women. The widely used drugs that are effective cancer chemotherapeutic agents suffer from the drawback of enormous organ toxicity. Therefore the challenging task at this juncture is to identify the quick and novel methods to develop molecules, which can be as potential drug candidates with less toxicity towards normal healthy cells.

Plant derived natural products such as flavonoids, terpenoids and steroids have received considerable attention due to their diverse pharmacological properties, among which the cytotoxic and chemo preventive effects are thoroughly researched by many scientists [4]. Medicinal plants, shows the presence of many antioxidants such as carotenoids, flavonoids, polyphenols, saponins, enzymes and minerals which either singly or synergistically exhibit wide array of pharmacological actions. As per ethnobotanical sources most of the medicinal plants have been found to be effective in cancer chemotherapy. *Corallocarpus epigaeus* (Cucurbitaceae) is the species globally distributed and within India it is recorded in Andhra Pradesh, Gujarat, Karnataka. Some of the folk medicinal uses of the plant are in the cleaning of wounds, treatment of obesity, rheumatism etc. Roots of the plant have been extensively studied as it contains sesquiterpene lactones, a bitter principle bryonin [5].

Currently, in vitro cytotoxicity assays are being largely employed for research purpose because of their wide range of advantages such as feasibility in using small amount of test substance, facilitate to do mechanistical studies, less time consuming and are proved to be excellent models for the study of the biological mechanisms involved in cancer cell proliferation, deregulation, apoptosis and progression [6]. In vitro methods like Trypan blue exclusion method, MTT assay, Lactate Dehydrogenase assay are based on various cell functions such as enzyme activity, cell membrane permeability, cell adherence, ATP production and nucleotide uptake activity. Cancer cell lines have been widely used for research purposes and proved to be a useful tool in the genetic approach and its characterization. In fact it is an excellent model for the study of cell proliferation mechanisms involved in the pathogenesis of cancer [7]. Different cancer cell lines display diverse morphologies and responses to cytotoxic anticancer drugs and K562 cell lines are one such having growth kinetics most suitable for research

purposes. They were the first human immortalised Myelogenous leukaemia lines derived from a 53 yr old female CML patient and they exhibit less clumping in culture than other suspension lines [8]. According to the International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use 3R's i.e. Reduction and Replacement of animals and Refinement of existing procedures in drug screening procedures, we have selected K562 cell lines as an alternative approach to in vivo animal screening methods to determine the cell viability and antiproliferative potential of ethanolic extract of *Corallocarpus epigaeus* at different concentrations by Trypan blue and MTT assay along with preliminary phytochemical evaluation of plant extract as there is no literature review on similar kind of activity. Therefore the present study is designed with an aim to evaluate the anticancer potential of *Corallocarpus epigaeus* against human chronic myeloid leukaemia K562 cell line by employing Trypan blue exclusion method and MTT assay.

MATERIALS & METHODS:

Collection and Identification of Plant material

The plant material was collected from the hills of Tirumala, A.P, India and was taxonomically authenticated by Prof. Dr. K. Madhava Chetty, Department of Botany, SV University, Tirupathi and a voucher specimen has been prepared.

Preparation of whole plant ethanolic extracts of *Corallocarpus epigaeus*

Whole plant of *Corallocarpus epigaeus* was dried under shade, powdered and extracted with 95% ethanol using soxhlet apparatus for about 72 hrs. After extraction the marc was pressed and the extract was dried below 50°C in a hot air oven. The dried extract was then subjected to preliminary phytochemical screening for the detection of phytoconstituents in accordance to the standard protocol [9].

Trypan blue exclusion assay

The study designed consists of three groups viz: Negative control, Control, Test. In the negative control cell lines are incubated with the medium for a period of 48 hrs to rule out the growth inhibitory effects of components of medium. Control group was designed to rule out the effect of growth inhibitory effects of solvent and the test group was used to test the efficacy of the test extract.

- Negative control- K562 cell lines with RPMI (Rose Bengal Parker Medium)
- Control- K562 cell lines with RPMI along with 95% ethanol at a concentration of 0.1% v/v.

- c. Test-K562 cell lines with culture medium and plant extract at different concentration.

Procedure of assay:

- ✓ An aliquot of cell suspension tested for viability was centrifuged and supernatant discarded and the aliquot is taken and suspended in 1ml PBS (Phosphate Buffered saline).
- ✓ To this 1 part of 0.4% Trypan blue was added, mixed and incubated at room temperature for 3 minutes.
- ✓ A drop of Trypan blue /cell mixture was applied to haemocytometer and the haemocytometer was placed on a binocular microscope and the number of unstained (viable) and stained (non-viable) cells were counted.

Drug Treatment:

Cells were maintained in 24 well plates in triplicate for every concentration and treated with 10, 25, 50, 75, 100µg of extract, control groups were treated with medium and ethanol. The treated cells were incubated for 48hrs in 5% CO₂ incubator at 37°C.

In vitro Cytotoxicity assay:

After 48 hrs of incubation the cells were collected and centrifuged at 3000rpm for 10 min to get a pellet. To the pellet 50µl of each medium and Trypan blue was added and mixed well to suspend the pellet. Percent of growth inhibition was calculated by the formula,

$$\% \text{ Growth inhibition} = \frac{\text{Cells in control/ml} - \text{Cells in test/ml}}{\text{Cells in control/ml}} \times 100$$

Microculture Tetrazolium Assay

Procedure for cell assay:

Cell lines in the exponential growth phase were trypsinized and suspended in RPMI culture media. Cells were plated at 10,000 cells/well in 96 well microtitre plate and incubated for 24 hrs. Test were then exposed to different concentrations of the extract where as the control receives only the culture medium. After 48 hrs the sample solution was flicked off and 50 µl of MTT dye was added to each well and the plates were incubated for 4hrs at 37°C in 5% CO₂ incubator. The supernatant was removed and 50 µl of DMSO was added. The plates were gently shaken and absorbance was measured at 540nm. The percentage growth inhibition was calculated using the following formula,

$$\% \text{ Growth inhibition} = \frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \times 100$$

$$\% \text{ Viability} = 100 - \% \text{ of growth inhibition}$$

Statistical Analysis:

Statistical evaluation was done by one-way analysis of variance (ANOVA) followed by Turkey's multiple comparison tests on viability using computer based fitting programme (prism graph pad version 6.03) Statistical significance was set at p<0.05.

Calculation of IC₅₀:

It was calculated by the linear interpolation method using the formula

$$IC_{50} = 50 - A/B - A \times (D - C) + C$$

Where

A- First point on the curve, expressed in % inhibition that is less than 50 %

B- First point on the curve, expressed in % inhibition that is greater than or equal to 50 %

C-Concentration of inhibitor that gives A% inhibition

D- Concentration of inhibitor that gives B% inhibition

RESULTS & DISCUSSION

Phytochemical investigation

Phytochemical analysis of the ethanol extract of whole plant of *Corallocarpus epigaeus* revealed the presence of saponins, flavonoids, tannins, alkaloids, glycosides, steroids, carbohydrates, triterpenoids, gums, mucilage and phenolic compounds. The phytochemical profile was tabulated in Table 1.

Table. 1: Phytochemical screening of *Corallocarpus epigaeus* whole plant ethanolic extract

S.No	Phytoconstituent	In 95% ethanol extract
1.	Carbohydrate	Present
2.	Alkaloids	Present
3.	Flavonoids	Present
4.	Triterpenoids	Present
5.	Phenols	Present
6.	Tannins	Present
7.	Saponins	Present
8.	Steroids	Present
9.	Glycosides	Present
10.	Fixed oils and fats	Absent
11.	Proteins and aminoacids	Absent
12.	Gums and Mucilage	Present

Trypan Blue Assay

There is a dose dependent reduction in number of viable cells in this assay which indicate that the plant extract may be responsible for disturbing the cell membrane permeability. A very little reduction of viability in control group might be attributed to the ethanol slightly damaging the cell membrane. Effect of different concentrations of extract on % viability of cell lines was given in Table 2 & Figure1.

MTT Assay

The results from this assay showed that at 10,25,50,75,100 µg/ml of test extract there was 4.06%, 16.1%, 17.2%, 22.5%, 44%, 51.6% inhibition of growth. This indicates a significant and dose dependent reduction in the growth of cell lines.

IC₅₀ is one of the commonly determined parameter in *In vitro* cytotoxic assay. The IC₅₀ value of ethanolic extract of *Corallocarpus epigaeus* on K562 cell lines was found to be 94.73 µg/ml by MTT assay.

The reduction in survivability of K562 cell lines by the ethanolic extract of *Corallocarpus epigaeus* for a single exposure may be due to various secondary metabolites acting either singly or in synergy.

However, Flavonoids have been proven to inhibit proliferation in many kinds of cultured human cancer cell lines causing no harm to the healthy cells. Effect of different concentrations of extract on % viability of cell lines using MTT assay was given in Table 3 & Figure 2.

CONCLUSION

Tissue culture technology has virtually revolutionized cancer biology in discovering cytotoxic molecules. Our current study is a preliminary effort to evaluate cytotoxic potential of whole plant ethanolic extract of *Corallocarpus epigaeus* against K562 cell lines employing Trypan blue and MTT In-vitro cytotoxic assay. The study revealed a dose dependent reduction in number of K562 cell lines upon single exposure for 48hrs which indicates that the extract may be capable of altering the membrane permeability and functioning of mitochondrial dehydrogenase synthase. Further studies were implicated to identify individual phytoconstituents responsible for cytotoxicity and elucidation of probable mechanism of action may lead to the development of promising natural agents in the treatment of Chronic Myeloid leukaemia.

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Table. 2: Effect of ethanolic extract of *Corallocarpus epigaeus* on survival of K562 cell lines by Trypan blue assay

S.No	Groups	Concentration of ethanolic extract of <i>Corallocarpus epigaeus</i> (µg/ml)	% of cell viability
1.	Control		95.4±0.9539
		10	85 ±0.5 ^a
		25	81.43 ± 0.2333 ^a
2.	Test	50	79 ±0.2887 ^a
		75	50.20 ± 1.528 ^a
		100	51 ±0.5774 ^a

Note: All values were expressed as Mean ± SEM of 3 observations, a=p< 0.0001 when compared to that of control.

Table. 3: Effect of ethanolic extract of *Corallocarpus epigaeus* on survival of K562 cell lines by MTT assay

S.No	Groups	Concentration of ethanolic extract of <i>Corallocarpus epigaeus</i> (µg/ml)	% of cell viability
1.	Control		95.93±0.2404 ^a
		10	83.90 ±0.1 ^a
		25	82.77 ± 0.56 ^a
2.	Test	50	77.5 ±0.2877 ^a
		75	56 ± 2.082 ^a
		100	48.33 ±0.8819 ^a

Note: All values were expressed as Mean ± SEM of 3 observations, a=p< 0.0001 when compared to that of control.

Figure. 1: Effect of ethanolic extract of *Corallocarpus epigaeus* on % viability of cancer cell lines by Trypan blue assay

Figure. 2: Effect of ethanolic extract of *Corallocarpus epigaeus* on % viability of cancer cell lines by MTT assay

CONFLICT OF INTEREST:

NIL

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