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PHYTOCHEMICAL ANALYSIS, ANTI OXIDENT, *IN VITRO* ANTI-CANCER ACTIVITY OF *ERYTHRINA VARIEGATA* L. LEAVES

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ABSTRACT

Objective: Erythrina variegata L. species have been reported to be used in the treatment of cancer in traditional/folk medicine, which may explore their anticancer potential. **Methods:** A preliminary qualitative phytochemical screening presence of flavonoids, phenolic compounds, tannins, terpenoids, carotenoids, chlorophyll and alkaloids. Chemical principles of 1,1-diphenyl-2-picrylhydrazyl (DPPH•) radical scavenging, Fe3+–Fe2+ transformation assay, ferric reducing antioxidant power (FRAP) assay, superoxide radical ion (O2•−), scavenging, superoxidizing assay, hydroxyl Radicals, nitric oxide (NO) scavenging, metal chelating and reducing power function. These various antioxidant activities were compared with standard antioxidants such as L-ascorbic acid. **Results:** Erythrina variegata leaf extract exhibited the greatestantioxidant activity via DPPH, superoxide anion scavenging, metal chelator (iron chelator and iron reducing power) and nitric oxide scavenging activity. The antioxidant activity of Erythrina variegata leaves the extract due to the presence of flavonoids and polyphenols. Anticancer activity of Erythrina variegata leaves was confirmed against human colon cancer cell line (HT-29). Anticancer activity was directly stimulated by concentrations and maximum growth inhibition was 81.38% at 400 µg/ml. **Conclusion:** Overall, it can be concluded from the present study that Erythrina variegata leaves are rich in phytochemicals and natural antioxidants, which have anticancer activity.

KEYWORDS: Erythrina variegata, Antioxidant, Anti cancer.

INTRODUCTION

Cancer is one of the most common non communicable diseases in developing countries and is the world's second leading cause of death after heart disease, with the burden increasing day by day.[1] Cancer is defined as the abnormal growth of cells caused by multiple changes in gene expression, leading to an uncontrolled balance of cell proliferation and cell death. Cancer is those tumors[2] They have developed the ability to invade surrounding normal tissue. Cancers are caused by exogenous chemical, physical or biological carcinogens in humans and the mechanisms of carcinogenesis are often multifaceted and complex. Different factors may act by different mechanisms and at different stages of tumor development[3] The histology of breast cancer shows the heterogeneity of cells and, like any cancer, growth is regulated by a large number of genes.[4,5] Therefore compounds that arrest the cell cycle, suppress cell proliferation, induce cell death and/or activate apoptosis are ideal for cancer therapy.[6-11]

MATERIALS AND METHODS

Collection and preparation of plant sample

Erythrina variegata was collected from waste lands in and around Poondi village, Thanjavur district, Tamil Nadu, India, where it was naturally available. Fresh leaves of *Erythrina variegata* were collected and stored. Soak the fresh leaves in ethanol for 24 hours and then strain. The filtered supernatant was collected in a reagent bottle, tightly capped and stored at 4°C until use.

Phytochemical Screening

A preliminary qualitative phytochemical screening of the aqueous extract of Erythrina variegata was performed to detect alkaloids, phenolic compounds, flavonoids, saponins, tannins, glycosides, steroids, carbohydrates and various phytochemical constituents using standards.[13]

Test for alkaloids

About 50 mg of solvent-free aqueous extract was stirred with 5 ml of dilute hydrochloric acid and filtered. To the filtrate, 2 mL of Hager's reagent (aqueous solution of

picric acid) was added. A yellow precipitate appears, indicating the presence of alkaloids.[14]

Test for phenolic compound
About 50 mg of solvent-free aqueous extract was stirred with 5 ml of dilute hydrochloric acid and filtered. To the filtrate, 2 mL of Hager's reagent (aqueous solution of picric acid) was added. A yellow precipitate appears, indicating the presence of alkaloids.[12]

Test for tannins
The aqueous extract (500 mg) was added to 10 ml of freshly prepared 10% potassium hydroxide (KOH) in a beaker and shaken well to dissolve. The formation of a dirty precipitate indicates the presence of tannins in the sample.[13]

Test for flavonoids
Aqueous solution of plant extract was treated with 10% ammonium hydroxide solution. The appearance of a thick white precipitate indicates the presence of flavonoids.[14]

Test for terpenoids
About 50 mg in 1mL chloroform. This was mixed well and added to acetic anhydride with concentrated sulfuric acid from the sides of the tubes. The appearance of red and blue-green color indicates the presence of steroids and triterpenoids.

Test for saponins
The aqueous extract (50 mg) was diluted with distilled water to 10 ml. The suspension was shaken in a graduated cylinder for 15 min; An increase in the foam layer indicates the presence of saponins.[15]

Test for glycosides
About 50mg extract was hydrolyzed with concentrated hydrochloric acid on a water bath for 2 hours and filtered. To 2 ml of the filtrate was added 3 ml of chloroform and shaken well. The chloroform layer was separated and 10% ammoniasolution was added to it. The formation of pink color indicates the presence of glycosides.

Test for steroids determination
Two ml of chloroform and 1 ml of concentrated sulfuric acid were mixed with 10 drops of aqueous extract of isopropyl alcohol, slowly until a double phase was formed. A dish-brown color in the middle layer indicates the presence of a steroidalring.

Estimation of total phenolics
Quantitative estimation of phenolics in *Erythrina variegata* extract was determined based on the method of Chithuraju and Becker, (2003).[16] About 0.5 ml of 1N Folin-Ciocalteu reagent and 2.5 ml of 20% sodium carbonate solutions were added and then the volume was made up to 10 ml with water. Followed by 40 min dark incubation and the absorbance was recorded at 725nm against blank for the estimation of phenolics. The results were based on the calibration curve: $y = 0.029x – 0.065$, $R2 = 0.955$ where x was the absorbance and y was the Gallic acid equivalents (mg/g) and were expressed in terms of milligrams Gallic acid equivalents (GAE) per gram of extract.

Antioxidant activity of *Erythrina variegata*
1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals scavenging activity was assayed by the method of Shimada *et al*. (1992).[16]

Determination of total antioxidant capacity
The antioxidant activity of the *Erythrina variegata* leaves extract was evaluated as per the method of Prieto *et al.* (1999).[17]

Superoxide anion scavenging activity assay
The superoxide radicals scavenging activity was determined as per the method of Liu *et al.* (1997).[18]

Fe^{2+}chelating activity assay
The metal chelating activity of the *Erythrina variegata* leaves extractwas assayed by the method of Dinis *et al*., (1994).[19]

Nitricoxide scavenging activity assay
Nitric oxideradical scavenging activity was determined according to themethod reported by Garrat(1964).[20]

In vitro* anti-Cancer Activity of *Erythrina Variegata
Cellline and culture
The human colorectal adenocarcinoma cell line (HT-29) was obtained from the NCCS, Pune. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 1% antibiotic (50,000 units/L of penicillin and 50 mg/L of streptomycin) and 2 mM glutamine. Once the cells attained confluent growth, the cells were trypsinized using Trypsin-EDTA and the required number of cells like 10^5 and 10^3 cells/ml was seeded into 6-well and 96-well plates respectively for carrying out various assays. Cultures were grown in 25cm flasks at 37°C, 5% CO_2 and 95% relative humidity, changing media at least twice a week. Cytotoxic assay was evaluated by the MTT reduction assay[3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium] (**Mosmann, 1983**; **Monks *et al*., 1991) in cell line as HT-29.**[21-22]

Reagents
Minimum essential medium (MEM) was purchased from Hi Media Laboratories. FBS was purchased from Systron Laboratories. Trypsin, 3-(4,5- dimethyl-2- thiazolyl)-2,5-diphenyl-tetrazolium bromide (MDT) and dimethyl sulfoxide (DMSO) were purchased from Cisco Research Laboratory Chemicals, Mumbai. All other chemicals and reagents were obtained from Sigma Aldrich, Mumbai.

Cytotoxic assay
Cytotoxic assay was evaluated by the MTT reduction

assay [3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] (Mosmann, 1983; Monks et al., 1991). The monolayer cells were detached and single cell suspensions were made using trypsin-ethylene diamine tetraacetic acid (EDTA). A hemo cytometer was used to count the viable cells and the cell suspension was diluted with a medium containing 5% FBS in order to obtain final density of 1x105 cells/ml. 96-well plates at plating density of 10,000 cells/well were seeded with one hundred microlitres per well of cell suspension and incubated for cell attachment at 37° C, 5% CO2, 95% air and 100% relative humidity. Aliquots of 100 µl of different concentrations of leaf extracts (12.5, 25, 50, 100, 200 and 400µg/ml) dissolved in DMSO (1%) were added to the appropriate wells already containing 100 µl of medium, resulted the required final sample concentrations for 48h at 37°C, 5% CO2, 95% air and 100% relative humidity. After 48h of incubation, to each well 20µl/well (5mg/ml) of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl--tetrazolium bromide (MTT) phosphate- buffered saline solution was added and incubated at 37°C for 4 h. Then, 100µl of 0.1% DMSO is added to each well to dissolve the MTT metabolicproduct. Then the plate is shaken at 150 rpm for 5 min. Viable cells were determined by the absorbance at 570nm. Measurements were performed and the concentration required for inhibition Concentration (IC_{50}) was determined graphically. The absorbance at 570nm was measured with a UV- Spectrophotometer. The medium without samples served as control and triplicate was maintained for all concentrations. The effect of the samples on the anticancer activity of HT-29 was expressed as the % Cytotoxicity using the following formulas.

% Cytotoxicity = 100-[Abs(sample)/Abs (control)] x100.

% Cell Viability = [Abs (sample) / Abs (control)] x 100.

Statistical Analysis

Experimental experiments were performed in triplicate for 3–5 separate experiments. The *amount* of extract required to inhibit the concentration of free radicals by 50%, IC_{50}, was estimated graphically by a non-linear regression algorithm.

Phytochemical analysis of leaves extract of *Erythrina variegata*

Qualitative analysis

Phytochemical characters of *Erythrina variegata* leaves were studied and summarized in Table 1. *Erythrina variegata* leaves showed the presence of tannin, saponins, flavonoids, steroids, terpenoids, tri terpenodis, alkaloids, anthroquinone, polyphenol and glycosides while ethanol and aqueous extracts did not. Of the two extracts, the ethanol extract showed the richest content of phytochemicals and was used for further experimental studies.

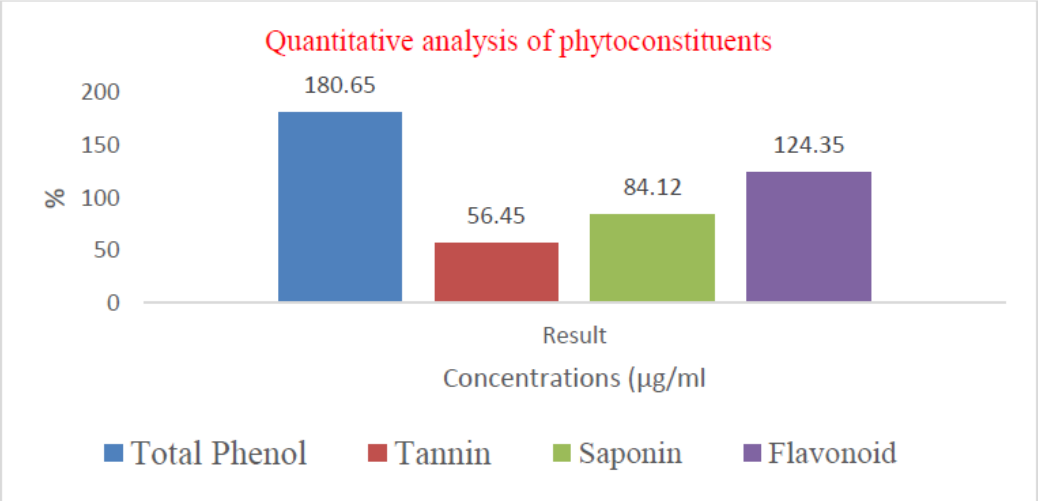
Table 1: Preliminary Qualitative phytochemical screening.

S.No	Phytochemical Analysis	Ethanol extract	Aqueous extract
1	Tannin	+	+
2	Saponin	++	+
3	Flavonoids	++	+
4	Steroids	+	+
5	Terpenoids	++	+
6	Tri terpenodis	+	+
7	Alkaloids	+	+
8	Anthroquinone	+	+
9	Polyphenol	++	+
10	Glycoside	+	+

(+)Presence,(++)High concentration,(-)Absence.

Quantitative analysis

Quantitative analysis of *Erythrina variegata* revealed that total phenol (180.65 ± 21.25mg/gm), tannins (56.45 ± 8.45mg/gm), saponin (84.12 ± 13.26mg/gm) and flavonoids (124.35 ± 16.25mg/gm) were present (Table 4.1 and Fig. 4.1). The above phytoconstituents were tested as per the standard methods.



Values are expressed as Mean ± SD for triplicates.

Fig 1: Quantitative analysis of phytoconstituents.

DPPH radical scavenging activity

The DPPH radical scavenging activity of *Erythrina variegata* leaf extract and standard ascorbic acid is presented in Figure 2. The half inhibitory concentration (IC_{50}) of *Erythrina variegata* leaves extract and ascorbic acid was 48.20µg/ml-1 and 40.20µg/ml-1 respectively. *Erythrina variegata* leaf extract exhibited significant dose-dependent inhibition of DPPH activity. The ability of L-ascorbic acid to scavenge the DPPH radical is directly proportional to the concentration. DPPH assay activity is close to that of ascorbic acid.

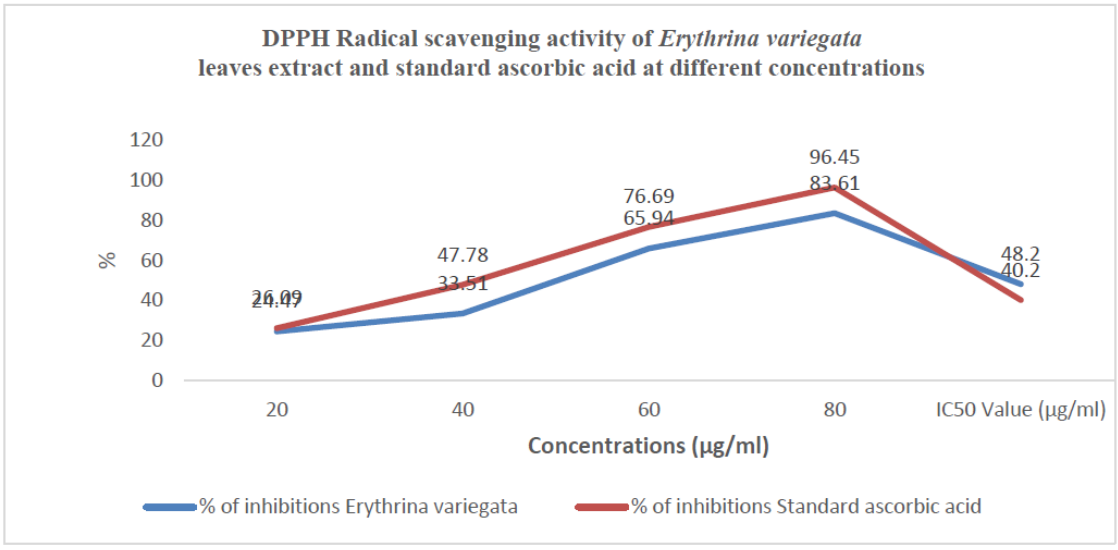


Fig. 2: DPPH Radical scavenging activity of *Erythrina variegata* leaves extract and standard ascorbic acid at different concentrations Total antioxidant activity.

The yield of the ethanol extract of *Erythrina variegata* leaves extract and its total antioxidant capacity are given in Fig. 2. The study reveals that the antioxidant activityof the *Erythrina variegata* leaves extract is in the increasing trend with the increasing concentration of the leaves extract. The half inhibition concentration (IC_{50}) of *Erythrina variegata* leaves extract and ascorbic acid were 49.06µg/ml-1 and 40.55µg/ml-1 respectively.

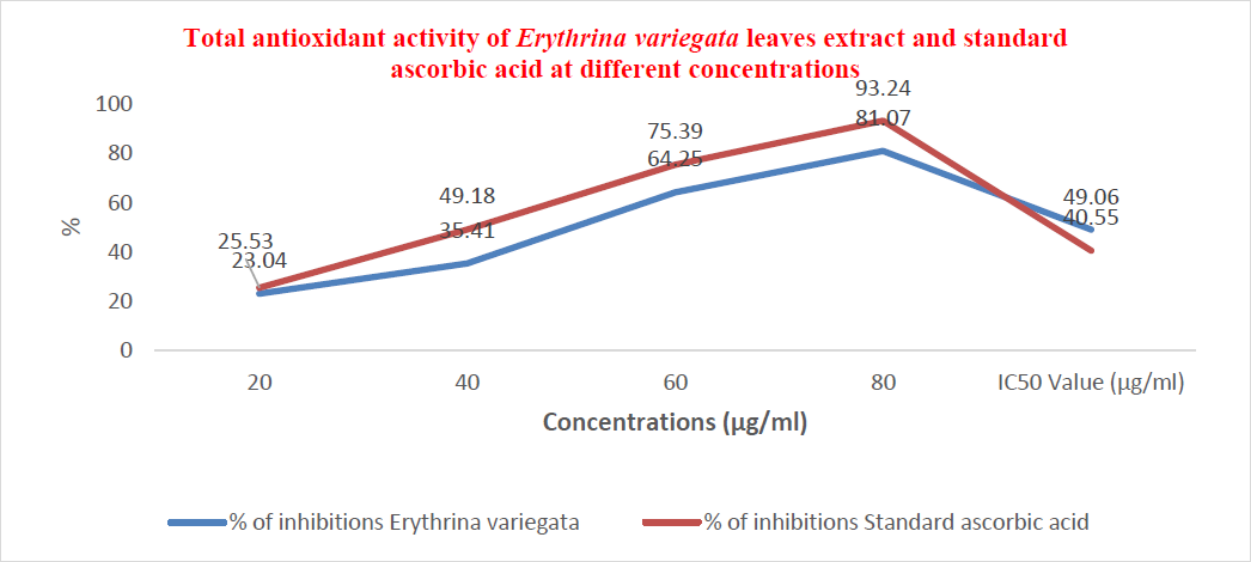


Fig 3: Total antioxidant activity of *Erythrina variegate* leaves extract and standard ascorbic acid at different concentrations.

Superoxide scavenging activity

The superoxide anion radical scavenging activities of the leaves extract from *Erythrina variegata* assayed by the PMS-NADH system were shown in Fig 4.8. The superoxide scavenging activity of *Erythrina variegata* was increased markedly with the increase of concentrations. The half inhibition concentration (IC_{50}) of *Erythrina variegata* was 48.58µg/ml-1 and ascorbic acid were 39.69µg/ml-1 respectively.

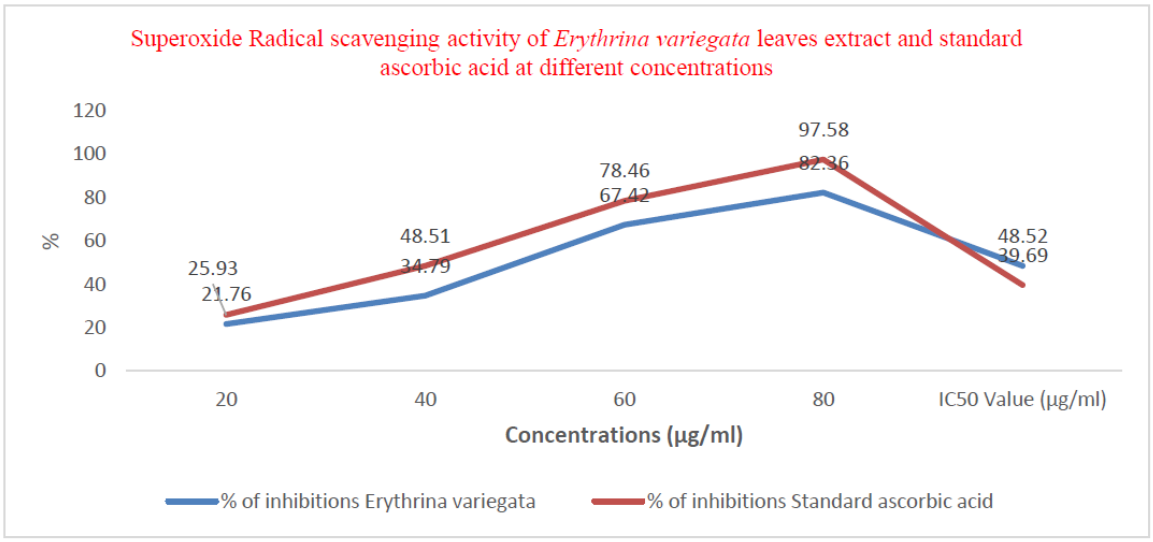
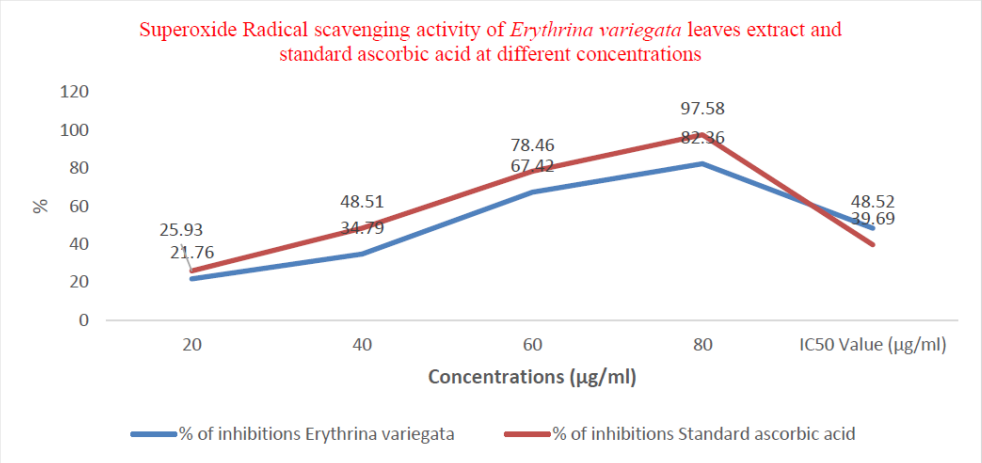


Fig. 4: Superoxide Radical scavenging activity of *Erythrina variegate.*

Leaves extract and standard ascorbic acid at different concentrations.

The ferrous ion chelating activity of leaves extract of *Erythrina variegata*

The formation of the ferrous Fe2+ complex is interrupted in the presenceof aqueous extract of *Erythrina variegata*, indicating that have chelating activity with an IC_{50} of 49.64µg/ml and ascorbic acid was 41.82µg/ml respectively (Fig. 4).



Values were expressed as Mean ± Standard deviation for triplicates

Fig. 5: Iron chelating activity of *Erythrina variegata* leaves extract and standard ascorbic acid at different concentrations.

Nitric oxide scavenging activity

Figure 6 depicts the Nitric oxide scavenging activity of *Erythrina variegata*. The nitric oxide scavenging activity of *Erythrina variegata* increased with increasing concentrations. All the doses showed significant activities near to the control. The half inhibition concentration (IC_{50}) of *Erythrina variegata* leaves extract and ascorbic acid were 49.93µg/ml and 40.47µg/ml respectively.

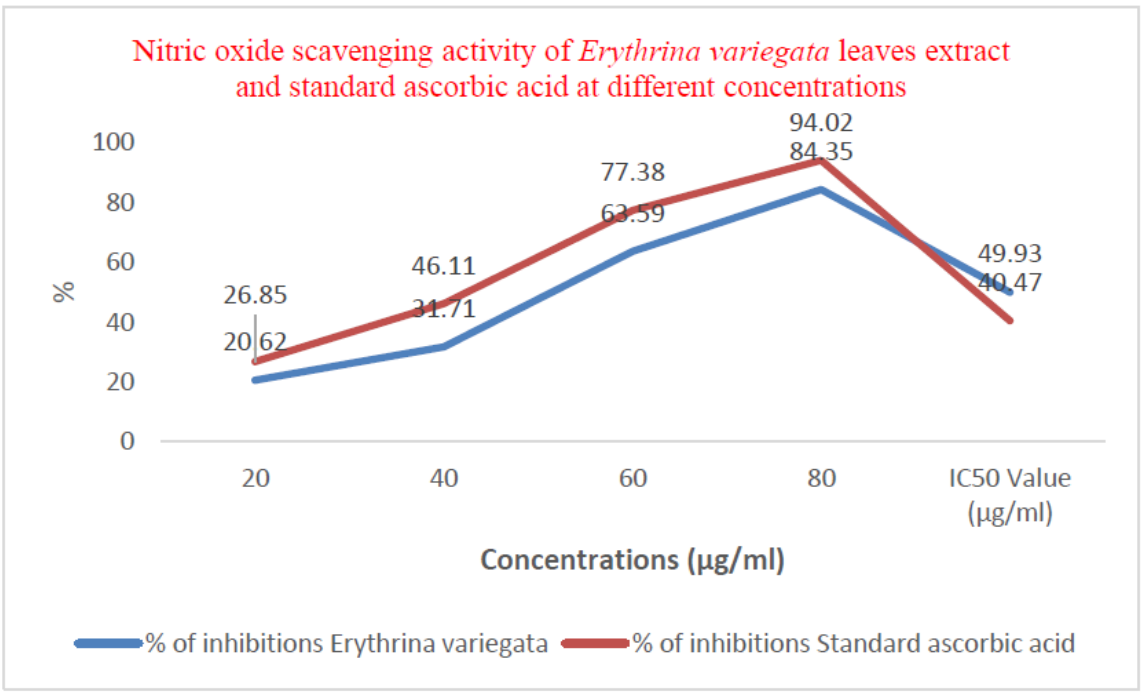


Fig. 6: Nitric oxide scavenging activity of *Erythrina variegata* leaves extract and standard ascorbic acid at different concentrations.

In vitro anti-cancer activity of *Erythrina variegata* in cell lines

Cancer cell lines have been widely used for research purposes and proved to be a useful tool in the genetic approach and its characterization shows that they are, in fact, an excellent model for the study of the biological mechanisms involved in cancer. The use of the cell model was in the origin of the development and testingof anticancer drugs presently used and in the development of new therapies but also as an alternative to transplantable animal tumours in chemotherapeutics testing. Keeping this in view, the present study has been undertaken to investigate the anticancer activity of *Erythrina variegata* leaves extract in HT-29 cell line.

In vitro anti-cancer activity of *Erythrina variegata* in human colon cancer cell line (HT-29)

The cell growth inhibition of the *Erythrina variegata* leaf

extract tested against HT-29 cell line at different concentrations (12.5, 25, 50, 100, 200 and 400 µg/ml). The results of the study observed that the concentrations increases with increase in the cell growth inhibition. The lowest growth inhibition was found at 6.39% at 12.5 µg/ml and highest growth inhibition was 81.38% at 400 µg/ml. The IC_{50} value was more than 195.35µg/ml (Fig 4.11 and 4.12). Normal cells showing surface architecture. Cytotoxic cells shows the cells became rounder, shrunken and showed signs of detachment from the surface of the wells denoting cell death (Apoptosis).

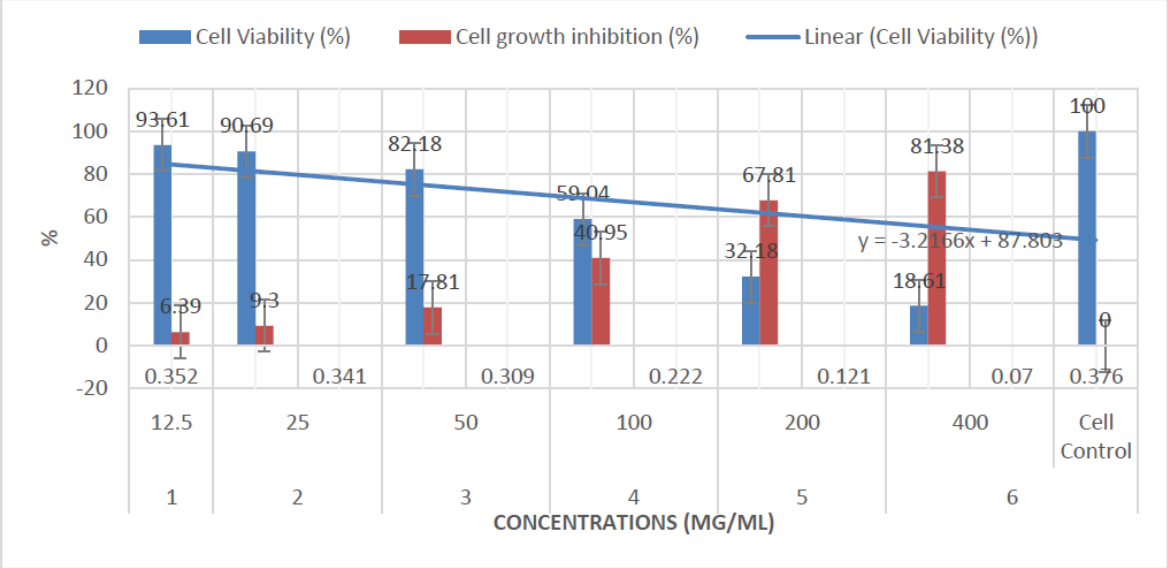
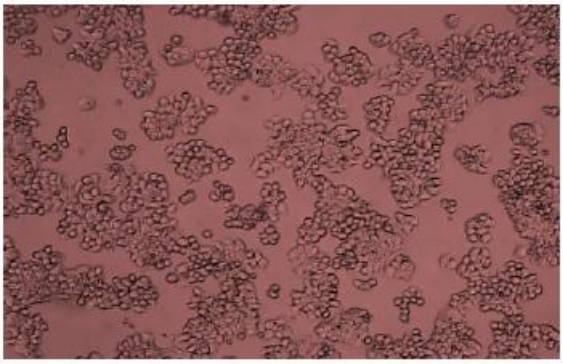
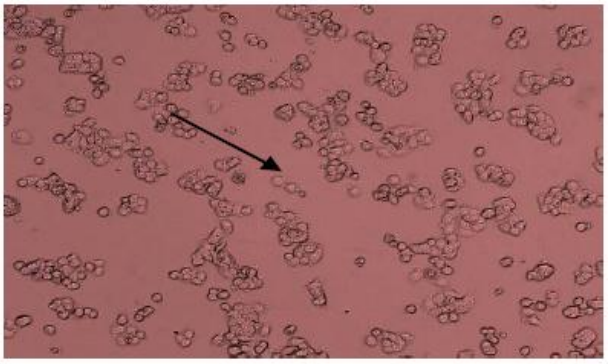


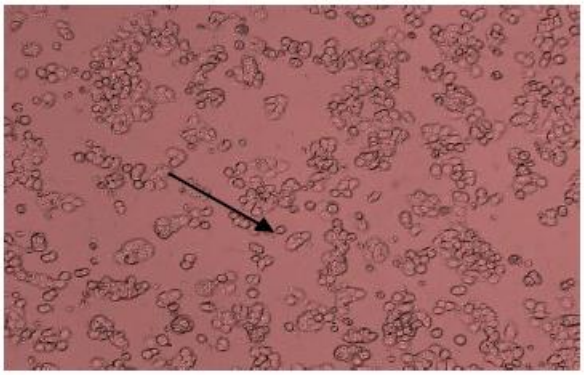
Fig. 7: Percentage of cell growth inhibition (Cytotoxic) of leaf extract on HT-29 cellline by MTTassay.



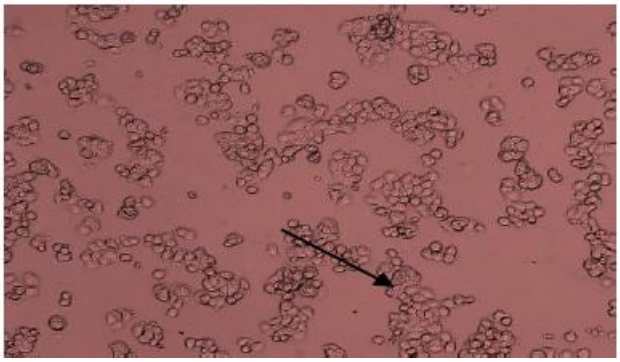
Control



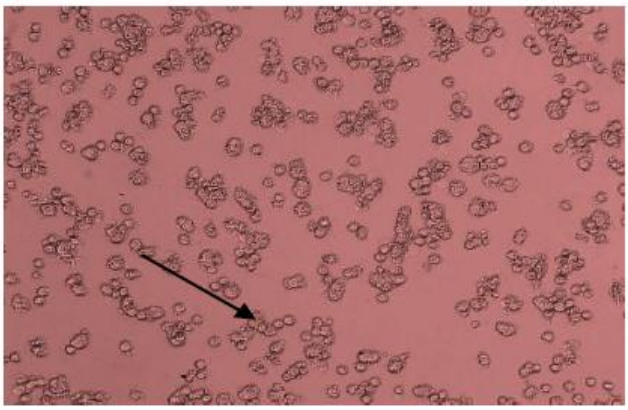
12.5µg/ml



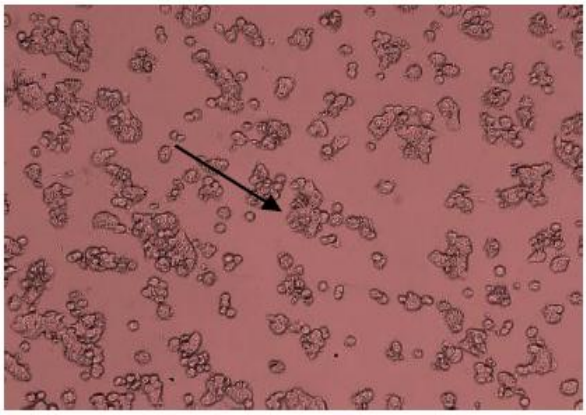
25µg/ml



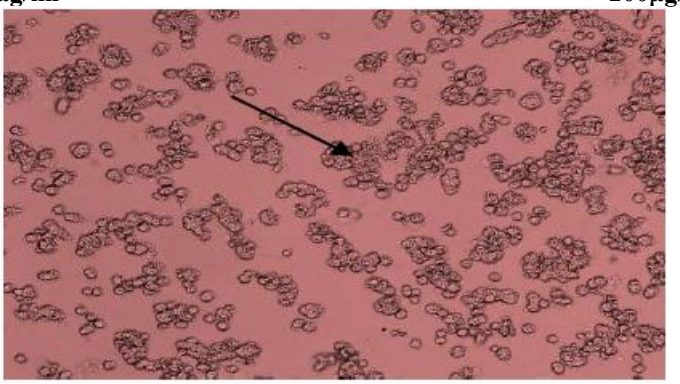
50 µg/ml



100µg/ml



200µg/ml



400µg/ml

Plate 4.1: Morphology of HT-29 cell line on different concentrations of leaf extract treatment.
Fig 8: Percentage of cell viability of leaf extract on HT-29 cell line by MTTassay.

CONCLUSION

The qualitative phytochemical characters of *Erythrina variegata* leaf extract include tannin, saponins, flavonoids, steroids, terpenoids, triterpenoids, alkaloids, anthro quinone, polyphenol and glycosides. Total phenol (180.65 ± 21.25mg/gm), tannins (56.45 ± 8.45mg/gm), saponin (84.12 ± 13.26mg/gm) and flavonoids (124.35 ± 1m/gm) were provided. *Erythrina variegata* leaf extract exhibited the greatest antioxidant activity through DPPH, superoxide anion scavenging, metal chelator (iron chelator and iron reducing power) and nitric oxide scavenging activity. The antioxidant activity of *Erythrina variegata* due to the presence of flavonoids and polyphenols in the extract was attributed to the confirmed anticancer activity of *Erythrina variegata* leaves against human colon cancer cell line (HT- 29). Anticancer activity was directly stimulated by concentrations and maximum growth inhibition was 81.38% at 400 µg/ml. The IC_{50} value was greater than 195.35µg/ml. Overall, it can be concluded from the present study that *Erythrina variegata* leaves are rich sources of phytochemicals and natural antioxidants with anticancer activity.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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