

Original Research Article

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Growth Inhibition and Induction of Apoptosis in Different Carcinoma Cell Lines by *Euphorbia tirucalli* Stem Extracts

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ABSTRACT

The present study was designed to evaluate *in vitro* antimicrobial activity and antioxidant activity and anticancer activity of different five extracts of *Euphorbia tirucalli* Stem. Plant roots were extracted in different five solvents viz. Hexane, Chloroform, Methanol, Water and Methanol: Water (90:10) through Soxtherm according to polarity gradients. Aiming to investigate antimicrobial activities, agar well diffusion method was followed using five pathogenic bacteria and five fungi as test organisms. The plant stem extracts showed good antibacterial activities against *Bacillus subtilis* and *Pseudomonas aeruginosa* which was compared with standard Chloramphenicol and Streptomycin, while 80% MeOH extract showed good antifungal activities (ZOI-13) against *Fusarium oxysporum* and (ZOI-18) against *Sclerotium rolfsii*. We assessed the antioxidant potential of all five extracts of *Euphorbia tirucalli* Stem using test involving inhibition of DPPH activities. The highest antioxidant activity of hexane and chloroform extracts were noticed at IC₅₀ (Inhibition concentration at 50%) of 240 µg/ml and 350.480 µg/ml respectively, compared to those of ascorbic acid (7.5 µg/ml). The effects of plant stem extracts on four cancer cells (HeLa 229, A549, ACHN and KB 3-1) were studied. The highest IC₅₀ (502.55 µg/ml to 118µg/ml.) values were found for the hexane and chloroform extracts respectively against A549 and ACHN Cell lines. This study validates the traditional use of the plant in management of Cancer. Current studies indicated that plant stem extracts possessed moderate antimicrobial activities and good antioxidant activity. So our findings revealed that the hexane and chloroform extracts of *Euphorbia tirucalli* Stem possess antioxidant and anticancer properties and could serve as free radical inhibitors or scavenger or, acting possibly as natural antioxidants, which may reduce the growth of Cancer cells and induce apoptosis in A549 and ACHN cell lines.

Keywords

Euphorbia tirucalli Stem,
Antimicrobial activity,
Antioxidant activity,
DPPH, Anticancer
activity, Trypan blue cell
exclusion assay, MTT
Assay

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Introduction

Ever since the prehistoric era, nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been derived from natural sources, many of these isolations were based on the

uses of the agents in traditional medicine. Plants, especially used in Ayurveda can provide biologically active molecules and lead structures for the development of modified derivatives with enhanced activity and /or reduced toxicity. Secondary plant metabolites have biological properties such as antioxidant

activity, antimicrobial effect, modulation of detoxification enzymes, stimulation of the immune system, decrease of platelet aggregation and modulation of hormone metabolism and anticancer property. There are more than thousand known and many unknown phytochemicals in plants. It is well-known that plants produce these chemicals to protect themselves, but recent researches demonstrate that many phytochemicals can also protect human against diseases.

Secondary constituents are the remaining plant chemicals such as alkaloids, terpenes, flavonoids, lignans, plant steroids, curcumines, saponins, phenolics, flavonoids and glucosides. Phytochemicals such as vitamins (A, C, E and K), carotenoids, terpenoids, flavonoids, polyphenols, alkaloids, tannins, saponins, pigments, enzymes and minerals that have antimicrobial and antioxidant activity (Madhuri and Pandey, 2009).

Free radicals induce Oxidative damage to lipids, proteins and nucleic acids, which eventually cause atherosclerosis, ageing, cancer, diabetes, inflammation, AIDS and several degenerative diseases in humans are well documented (Halliwell *et al.*, 1994; Maxwell *et al.*, 1997).

Several classes of anticancer drugs have been developed and many of them are of from natural origin. Natural products have been the mainstay of cancer chemotherapy for the past 30 years (Mann, 2002). It is well established that plants have been a useful source of clinically relevant antitumor compounds (Cragg *et al.*, 1994). Plants have long history of use in the treatment of cancer.

The use of *Euphorbia tirucalli* in traditional medicine as a treatment for cancer has attracted the recent interest of the West. However, this must be treated with caution, as

whole plant extracts have been shown to interact with antioxidant enzyme systems in human leukocytes via up regulation of key antioxidant enzyme genes. This leads to increased cytotoxicity, confirming the need for precise investigations into growth inhibition and apoptosis induced by *Euphorbia tirucalli* extracts for medicinal purposes (Jahan *et al.*, 2011).

Materials and Methods

The *Euphorbia tirucalli* Stem were collected from Junagadh region (Fig. 1). Using standard taxonomical methods, Dept. of Botany, JAU, Junagadh provided information regarding identification of the plant's parts used in this work. The samples were then separated and cleaned from impurities.

Extraction of plant material

The Stem of plants were separated and washed with tap water to remove the impurities. The Stem were cut into small pieces and were subjected to air dry for 10 days. The air-dried samples were then transferred into oven for drying and then were crushed. Dried powder of experimental material was extracted in Soxhlet apparatus successively with hexane, ethyl acetate, acetone, methanol and water, respectively due to their nature of polarity. 130ml solvent required per 10gm dried powder of experimental material. Plant materials were extracted in the mixture of methanol and water in 9:1 ratio. Desired sample was weighted and dissolved in a reasonable amount of the corresponding solvent (typically about 1.5 ml for every 10 mg of sample). The solution was filtered through a 0.2 micron filter to ensure that no particles were present in the solution.

After extraction, the hexane, chloroform, methanol, water and methanol: water extracts were concentrated using rotary evaporator and

dried in hot air oven at 500°C to get the solid mass and remaining sample weighted yield was collected after lyophilisation for further use.

Biological activity

Antimicrobial activity

The antimicrobial activity of the crude extracts were determined by the agar well diffusion method (Bauer *et al.*, 1966) against the microbial strains given in Table 5 whereas Chloramphenicol (1 mg/ml) and Streptomycin (1 mg/ml) were used as the standard for antibacterial, Ketoconazole (1 mg/ml) and fluconazole (1 mg/ml) were used as the standard for antifungal.

The extracts were dissolved separately in DMSO concentration of 1000µg/ml and carefully load into the well. The plates were then incubated at 37°C for 24 h to allow maximum growth of the organisms. The test material having antimicrobial activity inhibited the growth of the microorganisms and a clear, distinct zone of inhibition was visualized surrounding the well. The antimicrobial activity of the test agents was determined by measuring the diameter of zone of inhibition expressed in mm. Activity index was calculated compare to standard result.

Collection of microorganism

All the Bacteria *i.e.* *Bacillus subtilis*, *Escherichia coli*, *Streptococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhi*, and all the Fungi *i.e.* *Macrophomina phasolina*, *Aspergillus niger*, *Aspergillus flavus*, *Fusarium oxysporum* and *Sclerotium rolfsii* were provided by Department of Biotechnology, Junagadh Agricultural University, Junagadh. Microorganisms were stored at 4°C on Nutrient agar slant and potato dextrose agar slant before use.

In vitro antioxidant assays

The DPPH (2, 2-Diphenyl-1-Picrylhydrazyl) free radical scavenging activity was measured by the modified method of McCune and Johns (2002). The reaction mixture (3.0 ml) consisted of 1.0 ml DPPH in methanol (0.3 mM), 1.0 ml methanol and 1.0 ml different concentrations of the extracts were incubated in dark for 10 min, after which the absorbance was measured at 517 nm against blank. For control, 1.0 ml of methanol was used in place of extract. Ascorbic acid was used as positive control (Yamaguchi *et al.*, 1998). Percentage of inhibition was calculated using the formula:

$$\text{Inhibition (\%)} = (A_0 - A_1 / A_0) \times 100$$

Where, A₀ is the absorbance of control and A₁ is the absorbance of sample.

In order to calculate IC₅₀ value, plant extract solution in methanol was further diluted and tested for DPPH assay to find out 50% inhibition. IC₅₀ value was calculated by graph method.

In vitro anticancer activity

Cell culture and treatment

Four carcinoma cell lines (HeLa 229, KB 3-1, ACHN and A549) were cultured in 100 µl of DMEM media containing 10% fetal bovine serum (FBS). Carcinoma cell Lines were incubated overnight at 37°C in 5% CO₂ for cells attachment (Table 1).

Trypan blue cell exclusion method

Trypan blue dye assay method was carried out to evaluate the in vitro cytotoxicity potentials of all the extracts of *E. tirucalli*. In a test tube, 100µl of plant extract was mixed with 800µl of phosphate buffer saline and 100µl (1X10⁶ in 1ml) of carcinoma cell lines were added.

Each concentration of the extracts was tested in triplicate. All the samples were incubated at 37°C in an incubator for 30min. About 100µl of trypan blue dye was added to all the test tubes and the number of dead cells was counted in a haemocytometer under a compound microscope. Percentage of cytotoxicity was calculated by the following formula. % dead cells = Number of dead cells/Sum of dead cells and living cell × 100 (Raj Kapoor *et al.*, 2003).

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

The experiment was conducted as follows: Briefly, cancer cells were seeded in 96-well plates at a density of 1×10^4 cells/well in 100 µl DMEM. 24 h after seeding, the medium was removed and then the cells were incubated for 3 days with DMEM in the absence and the presence of various concentration of extracts. Extract was added at various concentrations ranging from 100, 200, 300, 400 and 500 µg/ml. After incubation, 20 µl of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reagent was added into each well. This plate was incubated again for 4 h in CO₂ incubator at 37°C. The resulting MTT-products were determined by measuring the absorbance at 570 nm using ELISA reader (Lau *et al.*, 2004). Each point represents the mean of triplicate experiments. The cell viability was determined using the formula: Viability (%) = (optical density of sample/optical density of control) × 100 IC₅₀ values were calculated as the concentrations that show 50% inhibition of proliferation on any tested cell line.

Results and Discussion

Antimicrobial activity

The antimicrobial potential of all the experimental plants was evaluated according

to their zone of inhibition against various pathogens and the results (zone of inhibition) were compared with the activity of the antibacterial standards (viz., Chloramphenicol 1.0 mg/ml, Streptomycin 1.0 mg/ml) and antifungal standards (viz., Ketoconazole 1.0 mg/ml, Fluconazole 1.0 mg/ml). The bioassays results for antimicrobial activity of the various extracts against five bacteria and five fungi are presented in Table 2 and 3 with Activity index compared to 2 Antibiotics standards of bacteria and fungi. The growth of *Sclerotium rolfsii* was inhibited by many extracts such as 80% methanol extract of *Euphorbia tirucalli*. *Fusarium oxysporum* was found resistant to most the extracts as well as Standard antibiotics, but *Euphorbia tirucalli* stem 80% methanol extract. The antimicrobial potency of Medicinal plant extracts may be due to the presence of important secondary metabolites.

Antioxidant activity

To neutralize the free radicals the amount of antioxidant principles present under normal physiological condition may be insufficient. So natural antioxidants from plant materials are included in the traditionally designed indigenous drug and it is evaluated for its antioxidant activity. In the presence of an electron donating antioxidant, the purple color of the DPPH free radical diminishes in intensity a change that can be followed spectrophotometrically at 517 nm. The radical scavenging activity of the extract measured as decolorizing activity following the trapping of the unpaired electron of the DPPH (Deepa and Kannappan, 2012).

It was observed that the extracts of *Euphorbia tirucalli* (Dandaliyo Thor) stem had higher activity in chloroform extract than that of the other extracts (Table 4). At a concentration of 1000 µg/mL, the scavenging activity of the Hexane, Chloroform, Methanol, Water and 80% Methanol extracts reached 75.14, 83.16,

10.56, 27.566 and 8.8% respectively. The result showed that hexane and chloroform extracts are good scavengers, and the IC₅₀ of Hexane and Chloroform extracts were 240 and 350.480µg/ml, respectively (Table 5).

Anticancer activity

For Screening Plants for Their Anticancer Activity, all extracts were tested a variety of cancer cell lines originating from various human tumors including HeLa Cell line, KB 3-1 Cell line, A549 Cell line and ACHN Cell line. This was done by closely monitoring the viability of cultured human cells exposed to the plant extracts (Fig. 2). The plant extracts inhibited the growth of the various human tumor cell lines eventually leading to cell death. Efficacy of cell death varied, depending on the specific plant extract. Based on repeated assays, the most effective anticancer plant extracts per plant were selected for further studies.

Trypan blue cell exclusion assay

In a continuing search for naturally occurring antineoplastic agents from higher plants, cytotoxic activities against human cancer cell lines could be considered as a reliable source of information. It is reported that many plant derivatives have antiproliferative and cytotoxic effects against cultured human cancer cell lines (Ye *et al.*, 1999).

Table 5 shows the results of the preliminary screening of selected medicinal plant extracts for their cytotoxicity by the trypan blue method. From the table, it is evident that maximum cytotoxicity was observed by the hexane (10.1030%) and Chloroform (23.5865%) extracts of *Euphorbia tirucalli*-stem against A549 Cell Line and ACHN cell lines respectively. At the 1000 µg/ml concentration, cytotoxic effect of all other extracts in *Euphorbia tirucalli*- Stem was found very low against all four cell lines.

Table.1 Cell line, origin and appropriate medium

Cell line	Origin	Medium
Hela 229	human cervical carcinoma cell line	D- MEM + 10 % FBS + 10 µg/mL gentamicin
KB 3-1	human oral carcinoma cell line	E-MEM + 10 % FBS + 1 % NEAA
ACHN	human Kidney carcinoma cell line	E- MEM + 10 % FBS + 1 % NEAA
A549	human lung adenocarcinoma epithelial cell line	DMEM + + 10% Foetal Bovine Serum FBS / FCS.+ 1% Antibiotics

Table.2 Antibacterial activity and activity index of *E. tirucalli* extracts

Extract	Bacillus subtilis			Escherichia coli			Staphylococcus aureus			Pseudomonas aeruginosa			Salmonella typhi		
	ZI	AI (CHL)	AI (STR)	ZI	AI (CHL)	AI (STR)	ZI	AI (CHL)	AI (STR)	ZI	AI (CHL)	AI (STR)	ZI	AI (CHL)	AI (STR)
Hex	12	0.75	0.60	-	-	-	-	-	-	15	0.834	0.714	-	-	-
Chl	15	0.93	0.75	-	-	-	10	-	0.5	14	0.78	0.64	-	-	-
MeoH	11	0.687	0.55	-	-	-	-	-	-	16	0.87	0.762	09	0.529	0.473
Water	11	0.687	0.55	-	-	-	-	-	-	18	1.00	0.857	-	-	-
80% MeoH	16	1.00	0.80	-	-	-	-	-	-	-	-	-	-	-	-

ZI = Zone of inhibition (in mm) includes the diameter of well 6mm; Standards- chloramphenicol 1mg/ml, streptomycin 1mg/ml; AI-Activity index

Table.3 Qualitative antifungal activity and activity index of *E. tirucalli* extracts

Extract	<i>Macrophomina Phaseolina</i>			<i>Aspergillus Niger</i>			<i>Aspergillus Flavus</i>			<i>Fusarium Oxysporum</i>			<i>Sclerotium Rolfsii</i>		
	ZI	AI (KT)	AI (FLC)	ZI	AI (KT)	AI (FLC)	ZI	AI (KT)	AI (FLC)	ZI	AI (KT)	AI (FLC)	ZI	AI (KT)	AI (FLC)
Hex	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Chl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MeoH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Water	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
80% MeoH	-	-	-	-	-	-	-	-	-	13	-	-	18	0.9	-

ZI = Zone of inhibition (in mm) includes the diameter of well 6mm; Standards- Ketoconazole 1mg/ml, Flucanazole 1mg/ml; AI-Activity index

Table.4 DPPH scavenging activities of *E. tirucalli* extracts

Extract	Concentration (µg/ml)	DPPH scavenging activities
Hex	1000 µg/ml	75.13±1.90(a)
Chl	1000 µg/ml	83.16±0.81(a)
MeoH	1000 µg/ml	10.56±0.46(b)
Water	1000 µg/ml	27.56±0.31(b)
80% MeoH	1000 µg/ml	8.8±0.17(b)

The values are mean ± standard error of Mean, n=3 followed by Lowercase letters show Duncan's grouping are significantly different at $P < 0.05$.

Table.5 IC50 Values (µg/ml) for DPPH scavenging activities of selected *E. tirucalli* extracts

Extracts	Hexane	Chloroform
<i>Euphorbia tirucalli</i> - Stem	240	350.480

Table.6 Trypan blue cell exclusion assay of *E. tirucalli* extracts

Extract	HeLa 229	KB 3-1	ACHN	A459
Hex	1.02±0.20	3.78±0.30	14.42±0.23	10.10±0.85
Chl	4.07±0.12	6.68±0.44	23.59±0.54	2.10±0.52
MeoH	0.40±0.15	3.28±0.78	-0.54±0.52	3.94±0.26
Water	0.90±0.23	3.82±0.69	-2.03±0.51	3.29±0.23
80% MeoH	1.73±0.12	1.89±0.48	0.34±0.42	4.45±0.49

Result showed by % inhibition ± SEM

Table.7 MTT reduction cytotoxic assay for evaluation of *E. tirucalli* extracts

Extract	HeLa 229	KB 3-1	ACHN	A459
Hex	-	-	13.039± 0.94	17.49± 2.55
Chl	-	-	27.97±0.87	8.07± 4.48
MeoH	-	-	-	-
Water	-	-	-	-
80% MeoH	-	-	-	-

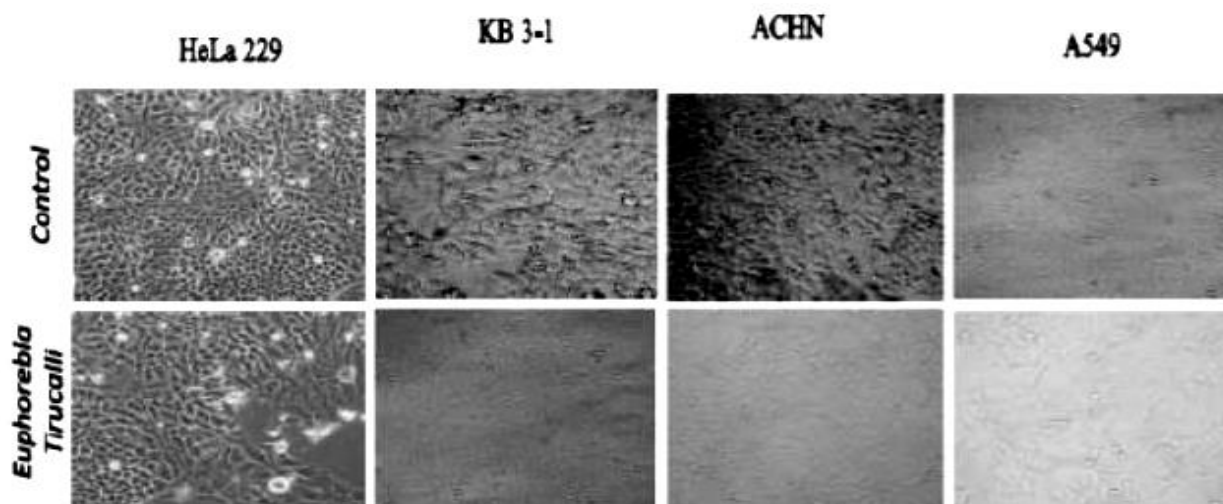
Table.8 IC₅₀ (µg/ml) evolution of highest MTT reduction cytotoxic extracts

Extract	HeLa 229	KB 3-1	ACHN	A459
Hex	-	-	-	502.55
Chl	-	-	118.99	-

Fig.1 *Euphorbia tirucalli* stem



Fig.2 Microscopic view of various control and different *E. tirucalli* extract treated cell lines



From Table 6, it is seen that the Chloroform extract of *Euphorbia tirucalli* (Dandaliyo Thor) stem was more effective against ACHN cell line (23.5865%) than the other extracts, namely hexane, methanol, water and 80% methanol extracts. Hexane extract also inhibited both the ACHN (14.4199%) and A459 (10.1030%) cancer cell lines. This effect was less pronounced with the other extracts. The cytotoxic effect of the all extracts was negligible against the HeLa and KB 3-1 Cell line.

MTT reduction cytotoxic assay for evaluation of medicinal plant extracts against four different cell lines

The MTT assay is based on the reduction of MTT into formazan crystals by living cells. The intracellular reducing power is mainly provided by NAD(P)H which is derived from dehydrogenase activity in mitochondria, endoplasmic reticulum and plasma membrane (Stockert *et al.*, 2012).

An antiproliferative assay on four human cancer cell lines (HeLa, KB 3-1, ACHN and A549 cell line) was performed. Table 6 presents the MTT reduction cytotoxic assay of the all plant extracts. Cytotoxicity activity (IC₅₀) of the 5 plant extracts was shown in Table 7. Extracts with IC₅₀ > 550 µg.mL⁻¹ in MTT assay were considered inactive.

The anticancer activity of *Euphorbia tirucalli* stem extracts against all four cell line was evaluated by MTT assay. Treatment with Hexane and chloroform extract at concentration of 100 µg/ml for 24 h resulted in reduction in cell viability of ACHN and A459 cells. Inhibition of ACHN cell line was 13.04% and 27.97% by hexane and chloroform extracts respectively. Treatment with hexane and chloroform extracts resulted in reduction in cell viability of A459 cell line up to 17.49% and 8.07% respectively.

Estimated IC₅₀ value for suppression of cell proliferation at 24 h was 118.99 µg/ml by chloroform extract against ACHN cell line and 502.55 µg/ml by Hexane extract against A459 cell line (Table 8).

On the basis of Trypan blue cell viability assay and MTT reduction cytotoxic Assay results, it clearly indicates that Growth of ACHN Cell line was effectively inhibited by *Euphorbia tirucalli* chloroform extract, A549 Cell line growth was highly reduced by *Euphorbia tirucalli* hexane extract.

All of the five extracts of selected plants examined in this study possess varying levels of anticancer activity in vitro. This is evident by the concentration dependent manner reduction in the final number of cancer cells as a consequence to treatment. Two kinds of anticancer effects were examined and found to take part in this study. The first is anti-cell proliferation effect (decreased number of metabolically active cells) and the second is cytotoxicity (decreased number of live cells). All The extracts examined possess both of the effects with various degrees.

Euphorbia tirucalli Hexane and chloroform extracts reported as good inhibitor of *Bacillus subtilis* and *Pseudomonas aeruginosa* and also found as good antioxidant extracts with IC₅₀ 240 and 350.480 µg/ml, while anticancer activity revealed the truth that hexane and chloroform must contain certain important phytochemicals which induce apoptosis effect in A549 and ACHN carcinoma cell lines with IC₅₀ 502.55 and 118.99 µg/ml respectively. Result revealed that plant contain anti cell proliferative as well as cytotoxic effect on A549 and ACHN Cell lines.

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