Studies on Antioxidant and Anticancer Activity from Leaf and Callus Extract of *Eupatorium triplinerve*

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ABSTRACT

The present study was performed to investigate the antioxidant activity, total phenol, terpenoids content and anticancer activity from the leaf of mother plant, *in vitro* leaf and callus extract of *Eupatorium triplinerve*. The leaf and callus extracts were evaluated for antioxidant activities by DPPH (1, 1– diphenyl -2- picryl - hydrazyl) radical scavenging assay. Among three different extracts with ethanol solvent used, maximum antioxidant activity was found in ethanol callus extract (19.76%) from Azhiyar accession followed by others. Total phenol and terpenoids content was quantitatively estimated which recorded maximum in Azhiyar accession 16.23 mg Gallic Acid Equivalents (GAE)/g and 8.36 mg Quercetin Equivalents (QE)/g). *In vitro* anticancer activity was evaluated using MTT assay at different concentrations. The maximum callus extract was found to be 75% at a dose of 250mg /ml. From the results, it is concluded that, the ethanol callus extract of *Eupatorium triplinerve* exhibited high level of *In vitro* anticancer activity, which may be due to the presence of phenol, Terpenoids and related polyphenols.

Keywords: *Eupatorium triplinerve,* antioxidant activity, DPPH, phenol and terpenoid.

1. Introduction

Antioxidant that scavenges these free radicals proves to be beneficial for these disorders as they prevent damage against cell proteins, Lipids and carbohydrates (Beris, 1991). Antioxidants are our first line of defence against free radical damage and critical for maintaining optimum health and well being. Antioxidant activity includes free radical scavenging capacity, inhibition of lipid per oxidation, metal ion chelating ability and reducing capacity. Compounds carrying antioxidant potential can be isolated and used as a remedy against oxidative stress and related diseases (Middleton, 2000). *Eupatorium triplinerve Vahl* is a valuable medicinal herb belonging to the family Asteraceae commonly known as Ayapana found in the Western and Eastern Ghats of India. The leaves are used to prepare infusions, decoctions, baths and plasters. It is a liver protectant, used to reduce the inflammation of the urinary tract, and cure tetanus. An infusion of the leaf and stem is used as a digestive stimulant and used to cure cancerous tumours.

Cancer is one of the major obstacles to human health around the world. It is a dreadful disease characterized by the irregular proliferation of the cells. Among all epidermis diseases, cancer holds the first place as a death causing diseases. As a cell progresses from normal to cancerous, the biological imperative to survive and perpetuate drives fundamental changes in cell behaviour (Ashworth, *et al.*, 2011).

The main reason behind the growing number of cancer cases is the changing life style of the population across the globe. Keeping in view the statistical data, the most prevalent cancer among females is breast cancer, accounting for about 23% of total cancer cases. In males the most prevalent is lung cancer, which accounts for 17% of total cancer case (Jemal, *et al*., 2011). Owing to potential benefits of plant based drugs for cancer treatments, their use is increasingly growing from 10% to 40% across the globe, specifically in the Asian continent it has reached 50% (Cassileth and Deng, 2004; Molassiotis *et al*., 2006).

The Chemical components of medicinal plants mainly possess antioxidant properties that contribute to their anticancer potential. Flavones, isoflavones, flavonoids, anthocyanins, coumarins, lignans, catechins and isocatechins are the major classes of bioactive constituents responsible for the antioxidant action (Nema *et al*., 2013). Medicinal plants constitute a common alternative to cancer treatments in many countries of the world (Gerson-cwilich *et al*., 2006 and Tascilar *et al*., 2006). Cytotoxic screening of a number of plants has been done to correlate their anticancer activity and further expand their scope for drug development (Akter *et al.*, 2014).

1. Materials and methods

Quantitative Determination of antioxidant activity

The antioxidant activities were determined using DPPH, (Sigma-Aldrich) as a free radical. Leaf extract of 100µl were mixed with 2.7mL of methanol and then 200µl of 0.1 % methanolic DPPH was added. The suspension was incubated for 30 minutes in dark condition. Initially, absorption of blank sample containing the same amount of methanol and DPPH solution was prepared and measured as control. Subsequently, at every 5 min interval, the absorption maxima of the solution were measured in UV double beam spectra scan at 517nm. The antioxidant activity of the sample was compared with known synthetic standard of (0.16%) Butylated Hydroxy Toluene (BHT) Lee *et al* (2005). The experiment was carried out in triplicate.

$$\% \text{ DPPH radical-scavenging} = \frac{(\text{Absorbance of control} - \text{Absorbance of Test Sample})}{(\text{Absorbance of control})} \times 100$$

Cell line culture:

In the present investigation, the cell viability and cytotoxicity assays were carried out with Liver cancer cell line ($HepG_2$) by MTT assay. Cancer cell line was obtained from National centre for cell sciences Pune (NCCS). The cells were maintained in Delbucco's Minimum essential medium (DMEM) supplemented with fetal bovine serum (FBS) and antibiotics Penicillin (100 U/ml), and streptomycin (100 µg/ml).

Reagents: MEM was purchased from Hi Media Laboratories Fetal bovine serum (FBS) was purchased from Cistron laboratories Trypsin, methylthiazolyl diphenyl- tetrazolium bromide (MTT), and Dimethyl sulfoxide (DMSO) were purchased from (Sisco research laboratory chemicals Mumbai). All of other chemicals and reagents were obtained from Sigma Aldrich Mumbai), in a humidified atmosphere of 50 µg/ml CO_2 at 37 °C.

2.2 In vitro assay for Cytotoxicity activity (MTT assay):

The Cytotoxicity of samples on HepG2 (liver) cell line was determined by the MTT assay (*Mosmann et al.*, 1983). Cells (1×10^5/well) were plated in 1ml of medium/well in 24-well plates (Costar Corning, Rochester, NY). After 48 hours incubation the cell reaches the confluence. Then, cells were incubated in the presence of various concentrations (250, 500, 1000 and 1500µg) of the samples in 0.1% DMSO for 48h at 37°C. After removal of the sample solution and washing with phosphate-buffered saline (pH 7.4), 200µl/well (5mg/ml) of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide cells (MTT) phosphate - buffered saline solution was added. After 4h incubation, 0.04M HCl/ isopropanol were added.

Viable cells were determined by the absorbance at 570nm. Measurements were performed and the concentration required for a 50% inhibition of viability (IC_{50}) was determined graphically. The absorbance at 570 nm was measured with a UV- Spectrophotometer using wells without sample containing cells as blanks. The effect of the samples on the proliferation of HepG 2 (liver cell line) was expressed as the % cell viability, using the following formula:

% cell viability = A570 of treated cells / A570 of control cells × 100%.

2. RESULTS and DISCUSSION

3.1Quantitative determination of Antioxidant activity

The quantitative estimation of the antioxidant activity was done (Table-7) and presented in graphical representative was given in Figure 1, 2 and 3. The ethanol extract of Azhiyar sample showed a higher antioxidant activity of 81% and aqueous extract showed 74.7%. The second higher activity was observed in chengalpet sample which was 74.1% in ethanol extract and 69.2% in aqueous extract, whereas the rest of the sample namely Pondicherry showed a lesser activity of 71%.

Eupatorium triplinerve (Azhiyar) antioxidant value being very close to synthetic antioxidant value which was 98% in BHT. Maximum amount of free radical scavenging activity was observed in ethanol leaf extract then in aqueous, petroleum ether, chloroform and acetone. Based on the results, the phytochemicals content and antioxidant activity of the three samples namely Azhiyar, Chengalpet and Pondicherry, the Azhiyar sample was found to be better.

3.2 ANTICANCER ANALYSIS OF THE MOTHER PLANT, CALLUS AND *IN VITRO* PLANT LEAF EXTRACT OF *EUPATORIUM TRIPLANERVE*

The mother leaf (fraction VI), callus (fraction IV) and *in vitro* leaf (fraction – III) extract of *Eupatorium triplinerve* was tested for anticancer activity against the human liver cancer HepG2 cells by the MTT (3-(4,5-dimethyl thiazolyl)-2,5-diphenyl-tetrazolium bromide) assay method. The ethanol mother plant leaf extract inhibited the growth of human liver cancer HepG2 cells and the reduction % was 82.57 ± 0.36, 76.39 ± 0.46, 49.02 ± 0.17, and 24.57±0.71 at the concentration of 250, 500, 1000 and 1500 mg/ ml (Table 26; Fig 21, 22, 23 and 24).

Whereas the ethanol callus extract inhibits the growth of human liver cancer HepG2 cells and the reduction % were 75.22 ± 1.59, 54.31 ± 0.15, 26.60 ± 0.56 and 17.65±0.29 at the concentration of 250, 500, 1000 and 1500 mg/ ml, (Table 26;Fig 2a, and *in vitro* leaf inhibits the growth of human liver cancer HepG2 cells and the reduction % was 86.05 ± 0.31, 77.55 ± 0.40, 61.69 ± 0.45, and 32.93±0.76 at the concentration of 250, 500 ,1000 and 1500 mg/ ml (Table 26;Fig 24,25and26)

The MTT assay of different fraction of mother leaf, callus and *in vitro* leaf extract of *Eupatorium triplinerve* shows anticancer activity. The ethanol callus extract show potent activities in which ethanol extracts shows highly potent anticancer activity.

The present study was designed to evaluate the potential capabilities of anticancer property of alcoholic extracts of *Eupatoriumtriplinerve* leaves in human liver cells. The cytotoxic responses of the extracts were determined by the 3-(4,5- dimethyl thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. The MTT assay of different fraction of *Eupatorium triplinerve* shows all fractions were having anticancer activity. In that ethanol are potent activities in which ethyl acetate shows highly potent anticancer activity. The maximum inhibition of mother plant leaf (fraction VI) callus (fraction – III) and *in vitro* plant (fraction IV) extract were in the concentration of 250mg/ml which showed anticancer activity against the human Liver cancer HePG2 cell line respectively. .

Phytochemical investigation of the ethanol extract of the aerial parts of *Andographis paniculata* has been reported with the isolation of 14 compounds, a majority of them are flavonoids and labdane diterponoids. The cytotoxic activites of these compounds have been evaluated against various cell lines and found that these isolates have a potent tumor inhibitor activity against all investigated cell lines (Geethangili *et al*., 2008). The MTT assay is based on the reduction of MTT 3-(4,5- dimethyl thiazolyl)-2,5-diphenyl-tetrazolium bromide) by mitochondrial dehydrogenase to purple formazan product. The different solvent fraction of whole plant *Merremiaemarginata* Burmf were subjected for MTT cell proliferation assay different parts the ethyl acetate fraction of *Merremia emarginata* was found to have cytotoxic activity although only fraction with an IC_{50} value lower than 200µg/ml were considered active (Kviecinskie *et al*., 2008). Experimental studies showed that the extracts of the various plants can also protect against breast cancer cells (Pratumvinit *et al*., 2009; Abu-Dahab

et al., 2012; Abdelhamed *et al.*, 2013). Anticancer property of alcoholic extract and oil of *Petroselinum sativum* (*P. sativum*) seeds in MCF-7 cells, a human breast cancer cell lines.

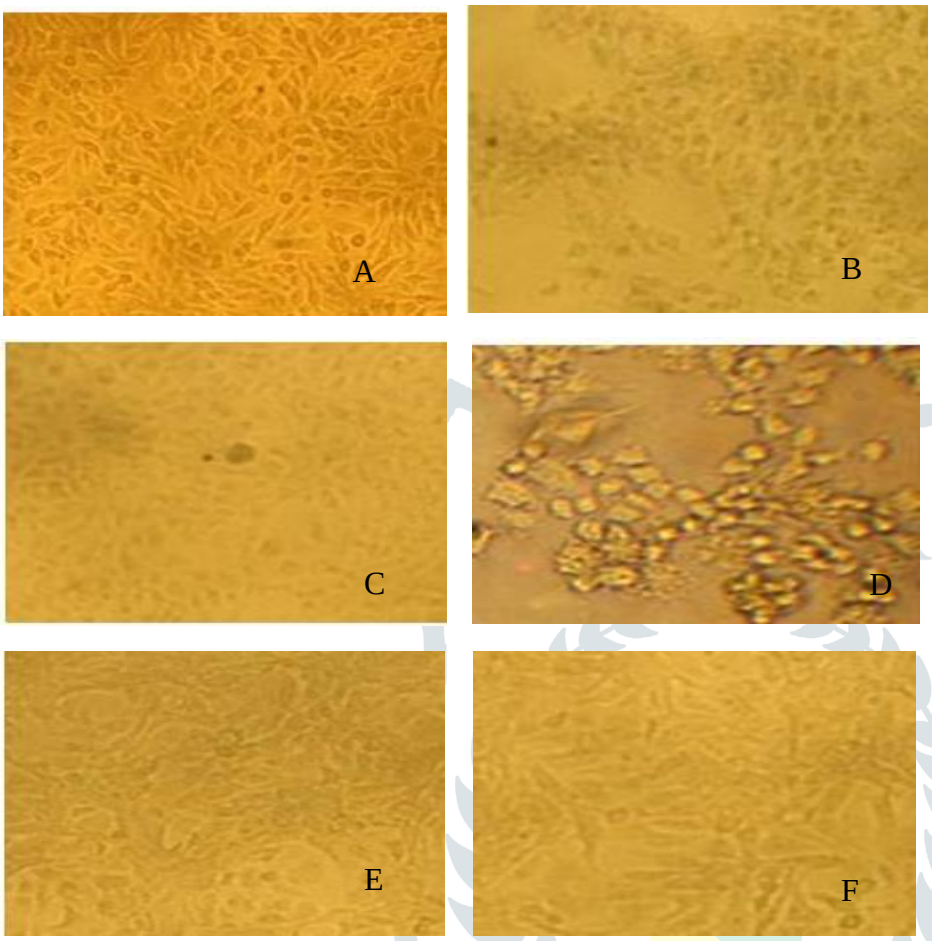
Table 1: MTT assay on HEPG 2 cells treated with Mother plant, *in vitro* plant and callus of *Eupatorium triplinerve*

S.No	Concentration (mg/ml)	MTT Reduction (%)		
		Mother PlantLeaf	**Callus**	***In vitro* Leaf**
1	Control	97.94±0.53	97.85±0.13	97.78±0.24
2	250	82.57±0.36	75.22±1.59	86.5±0.31
3	500	76.39±0.46	54.31±0.15	77.55±0.40
4	1000	49.02±0.71	26.60±0.56	61.69±0.45
5	1500	24.57±0.71	17.65±0.29	32.93±0.76
6	Pc	11.21±0.24	11.21±0.24	11.21±0.24
7	F value	16725.128	7160.956	17340.401
8	P value	<0.001**	<0.001**	<0.001**

Note: 1. **denotes significant at 1% level

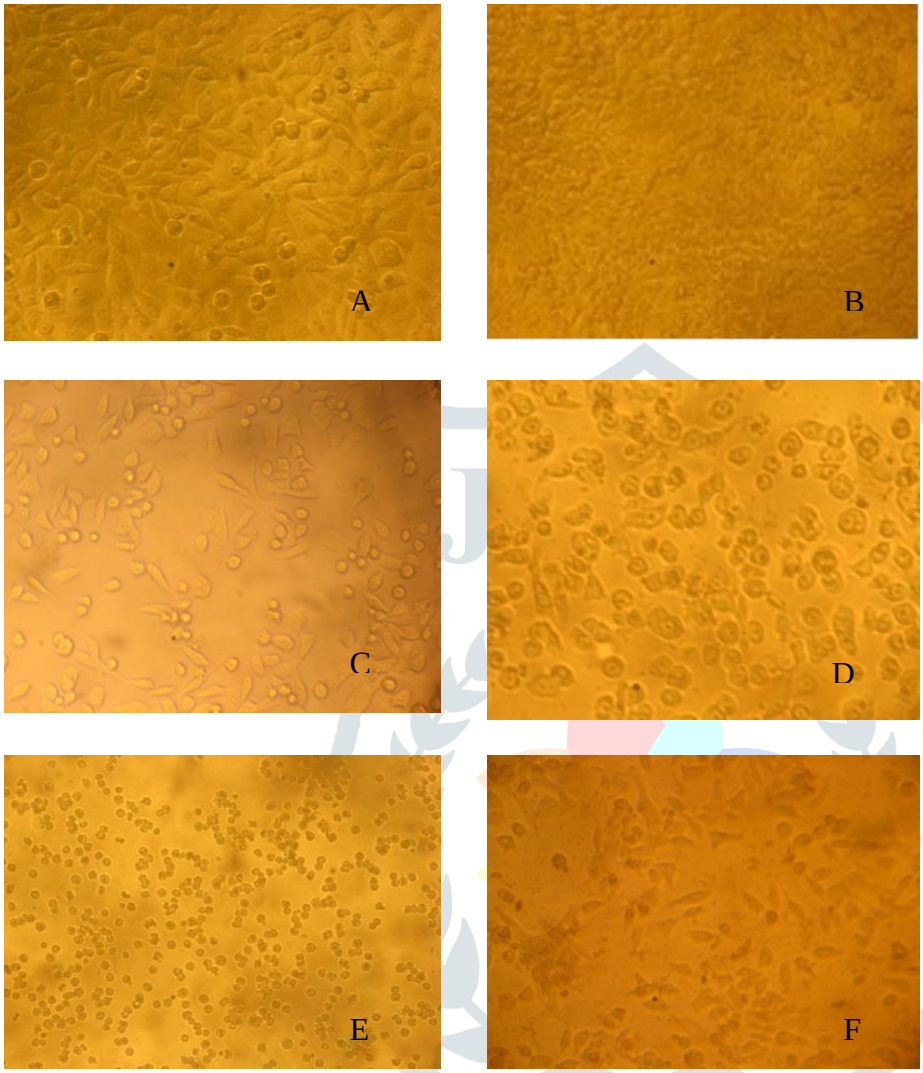
2. Different alphabets among concentration of extracts denotes significatents at 5% level using Duncan multiple range test (DMRT)

Figure 1: Reduction % of MTT on HepG 2 cells treated with ethanolic extract of *Eupatorium triplinerve* (mother plant)



A: Control cells (Untreated), B: Ethanol extract 250 µg/ml, C: Ethanol extract 500 µg/ml, D: Ethanol extract 1000 µg/ml, E: Ethanol extract 1500 µg/ml, F: Cyclophasphamide (Positive control) 180 µg/ml.

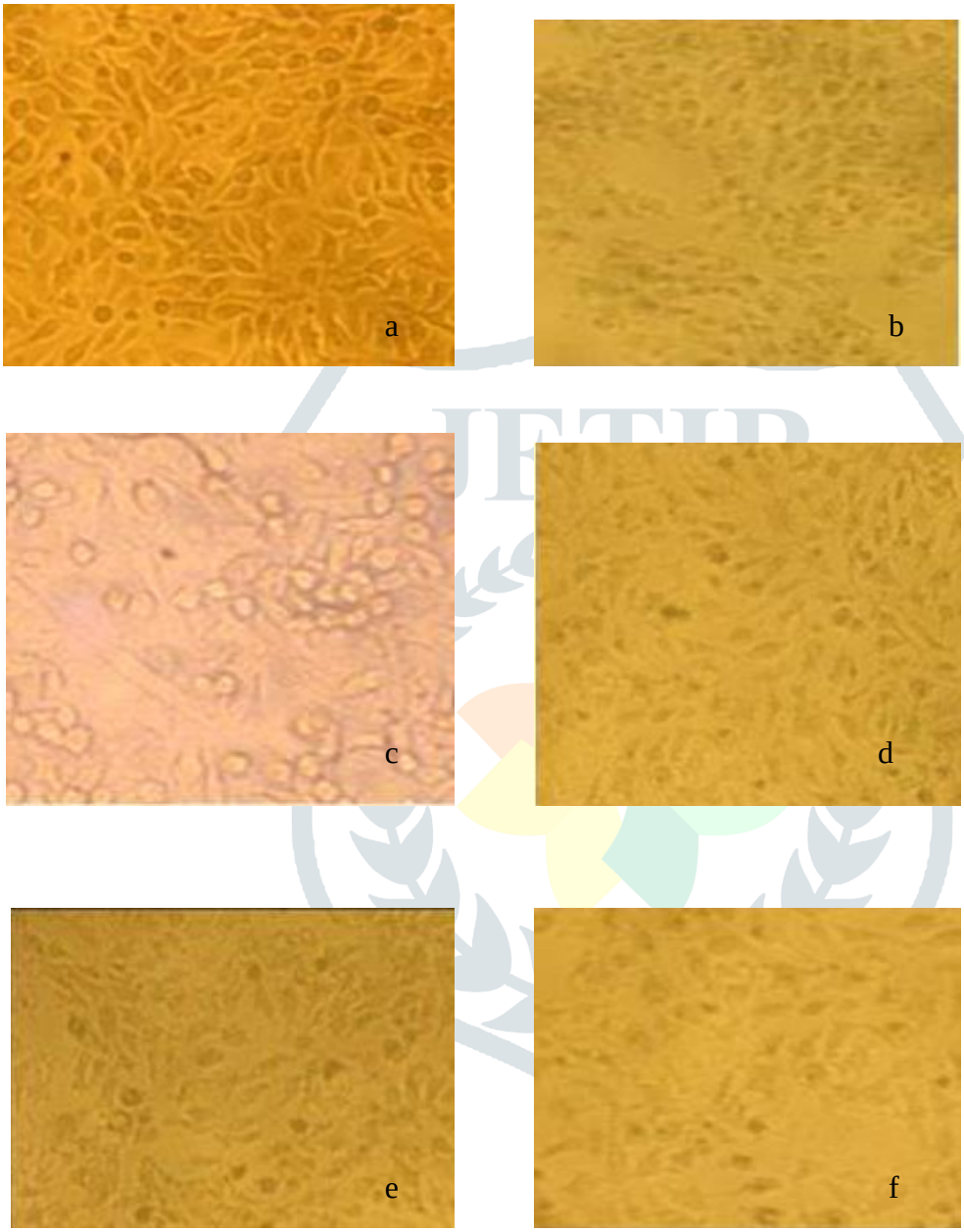
Figure 26: Reduction % of MTT on HepG 2 cells treated with ethanolic extract of *Eupatorium triplinerve* (*In vitro* plant)



A: Control cells (Untreated), B: Ethanol extract 250 µg/ml, C: Ethanol extract 500 µg/ml, D: Ethanol extract 1000 µg/ml, E: Ethanol extract 1500 µg/ml, F: Cyclophasphamide (Positive control) 180 µg/ml.

Figure 25: Reduction % of MTT on HepG 2 cells treated with ethanolic extract of *Eupatorium triplinerve* (callus culture)

Images of MTT Assay on HepG 2 Cell line (Ethanol Callus extract)



A: Control cells (Untreated), B: Ethanol extract 250 µg/ml, C: Ethanol extract 500 µg/ml, D: Ethanol extract 1000 µg/ml, E: Ethanol extract 1500 µg/ml, F: Cyclophasphamide (Positive control) 180 µg/ml.

Conclusion

The anticancer effect of mother plant leaf, *in vitro* plant leaf and callus extract of *Eupatorium triplinerve* was analysed using human liver cancer cell line (HepG2). The maximum inhibition was noted in the callus extract.

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