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RESEARCH ARTICLE

Methyl Thiazolyl Tetrazolium Cell Viability Assay and Intracellular Reactive Oxygen Species Activity of an Ethanol Extract of *Eclipta prostrata* L.

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ABSTRACT

Anti neoplastic effect of Eclipta prostrata L. (HepG2) cell lines. Hepatocellular carcinoma (HCC) is a tumor of the liver. HCC is responsible for over 12,000 deaths per year in the United States. It is one of the serious health problems in most developing countries. The present probe proved that ethanol extract of *Eclipta prostrata* L. significantly suppressed the growth and induced the apoptosis in the liver cancer (HepG2) cell lines. IC50 dose was measured with methyl thiazolyl tetrazolium. 100 µg of extract showed 50% reduction of in HepG2 cell line growth at 48 h of incubation. The whole plant of *E. prostrata* L. extract-induced apoptotic features of cell death was stained with acridine orange. The intracellular enzymatic antioxidants such as superoxide dismutase, glutathione peroxidase, and catalase were slightly decreased in their activities when compared to control. Thus, the study resolves that *E. prostrata* L. extract is an effective to prevent or retard the spread of malignant cells and antineoplastic effect.

Keywords: Acridine orange, apoptosis, *Eclipta prostrata* L., HepG2 cell lines.

INTRODUCTION

Herbal medicine is also known as traditional medicine or indigenous medicine or folk medicine. In some Asian and African countries, up to 80% of the population relies on traditional medicine for their primary health-care needs. In countries such as Germany and Switzerland, roughly 600–700 plant-based medicines are available and are prescribed by approximately 70% of physicians. India is sitting on a gold mine of well-recorded and well-practiced knowledge of traditional herbal medicine. Ancient literature also mentions herbal medicines for age-related diseases, namely memory loss, osteoporosis, diabetic wounds, immune, and liver disorders.

Cancer is a leading cause of death worldwide and better tolerated anticancer drugs, especially from various parts of a plant. The oxidative stress including ultraviolet radiation (UV)-induced skin damage is involved in numerous diseases. About 80% of primary liver cancer is hepatocellular carcinoma (HCC). This is one of the less common cancers in Australia. About 1600 people are diagnosed with it every year. It is also common in Asia, the Pacific Islands, and Africa due to high rates of chronic hepatitis B infection. D-galactosamine is a hepatotoxic agent, which induces diffuse injury of liver tissue followed by the regeneration process. Our data showed a high increase of serum aminotransferase after D-galactosamine administration which indicates a high extent of liver injury.

The generation of free radicals is a feature of cellular function such as in the mitochondrial respiratory chain and phagocytosis. However, excessive production of free radicals impairs cell membrane integrity causes defects in the susceptible to redox deregulation and oxidative stress is associated with many diseases including liver damage, cancer, atherosclerosis, and chronic inflammatory disease. Excess reactive oxygen species (ROS) is also associated with aging processes. Biological systems involved endogenous defense mechanisms including employing antioxidants and antioxidative enzymes, to help protect against free radical-induced cell damage.

Eclipta prostrata L. has been used in traditional systems of medicine and also traditional healers, especially in South region of India for the treatment of cancer. Ethanolic extracts of *E. prostrata* L.

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showed that a significant increased amount of wedelolactone, cyclopentane carboxylic acid, N-decanoic acid, and heptanes has been determined as a marker of *E. prostrata* L. The aim of this study is to analyze ethanolic extracts of *E. prostrata* L. active ingredients and antioxidative activities. It is also play a key role the prevention of UV radiation-induced cytotoxicity.

MATERIALS AND METHODS

Preparation of media and reagents required for cell culture

Preparation of DMEM for cell culturing

The powdered DMEM media was dissolved in 900 ml of autoclaved distilled water in a conical flask under sterile conditions. Sodium bicarbonate of 3.7 g was added and stirred until it completely dissolved and the pH of the medium was adjusted to 7.2 by adding 3 ml of 1 N HCl. Following these antibiotics such as penicillin 120 U/ml, streptomycin 75 µg/ml, gentamycin 160 µg/ml, and amphotericin B 3 µg/ml were added. Finally, 10% FCS were added and the medium was sterilized using 0.2 micron filter under pressure and stored at 4°C (Joshua Shemer *et al*.).

Preparation of saline:trypsin:versene (STV)

The following solutions were prepared: ×10 saline: 8 g NaCl, 0.4 g KCl, 1.0 g D-glucose, and 0.35 g $NaHCO_3$ were dissolved in 100 ml water. ×10 saline was filter sterilized and stored at 4°C.

Versene

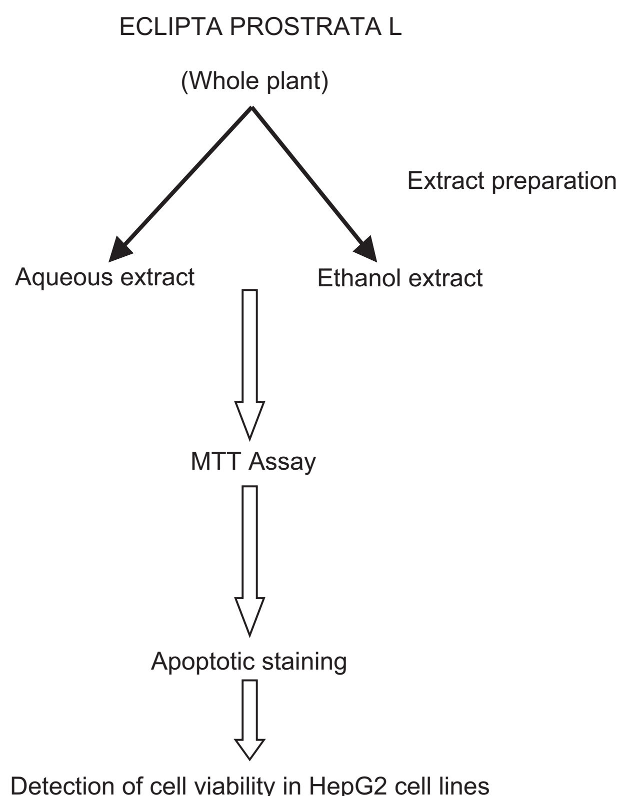
About 1 g of EDTA was weighed and added to 90 ml of distilled water. The solution was completely dissolved by adding 5 N NaOH in drops thereafter filter sterilized and stored at 4°C. For STV preparation, 100 mg of trypsin, 10 ml of ×10 saline, and 2.5 ml of versene were added and were made up to 100 ml using double distilled water. It was then filtered sterilized and stored at 4°C.

Cell culture

HepG2 cell lines were grown on polystyrene-coated flasks with DMEM as the growth medium. The cell line was passaged after attainment of confluence, that is, after every 2 days. Passaging was performed in a laminar flow hood as explained below. The culture medium was removed completely, and the traces of medium were removed by washing with STV after which 2.5 ml STV was added to the flask and incubated at 37°C for few minutes until the cells start detaching from the surface. After complete detachment, STV action was neutralized using DMEM containing serum. The cells were pelleted by centrifugation at 1500 rpm for 3 min and the supernatant was discarded. The cell pellet was resuspended in fresh medium and seeded into flask or plates according to the requirement and incubated at 37°C.

Extract preparation

Shade-dried powder was extracted with ethanol (1:3 w/v). Ethanol extract was prepared by cold percolation and it is concentrated under reduced pressure using rotatory evaporator at 4°C. Finally, crude extract was obtained. The crude extract was stored at 4°C until further use.



Drug preparation

E. prostrata L. extract was suspended in 1% dimethyl sulfoxide (DMSO) just before treatment and the final concentration of DMSO in the culture medium was 0.01% w/v. 0.01% DMSO was used as the control.

Cytotoxicity assay using methyl thiazolyl tetrazolium (MTT)

MTT assay is a calorimetric method used to measure cell viability. Yellow MTT (3-(4, 5-Dimethlthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore, conversion can be directly related to the number of viable (living) cells. Different concentration of extracts was prepared 100 ng, 10 ng, and 1 ng and 1 μg, 10 μg, and 100 μg. The cells were grown in 96-well plates and treated with different concentrations of the extracts. After 24 h of incubation, cells are treated with MTT reagent and incubated for 2–4 h. The reaction is terminated by aspiration of the media, the formed formazan crystals were dissolved in DMSO and the absorbance was read at 595 nm which is directly proportional to cell viability. Based on cell viability, percentage cytotoxicity of the extracts was calculated below (Slater *et al*.).

$$\frac{(\text{Mean absorbance of the control cells}) - \left(\text{Mean absorbance of the test cells}\right)}{\left(\text{Mean absorbance of control cells}\right)} \times 100$$

Apoptotic morphology analysis

Staining method

HepG2 cell lines were grown in 30 mm Petri plates of the concentration of 1.0×10^3 cells/plate. Then, the plates were incubated for 48 h supplemented with DMEM consisting 10% fetal bovine serum to obtain monolayer of cells, and then, it is treated with 30 μg of drug prepared. After 24 h, the cell suspension is washed with cold PBS and then added to DMEM medium. 2 μl of combined dye consisting acridine orange (100 μg/ml) was added to 20 μl of cell suspension. Then, 5 μl of stained suspension was transferred to a glass slide which is analyzed by fluorescence microscope.

Intracellular ROS assay

The effect of sample (NS1) on intracellular free radical production was assessed by 20, 70-dichlorofluorescein diacetate (DCFDA) fluorescent dye. HepG2 was seeded in 96-well plates and up to 70–80% confluence, following which the cells were treated with NS1 and NS1-positive control drug. After the stipulated time (24 h), cells were incubated with 20 mM of DCFDA for 30 min at 37°C. Finally, the supernatant was removed and relative fluorescence was measured using fluorimeter with excitation at 485 nm and emission at 530 nm.

Aspartate aminotransferase activity (AST)

The AST activity assay is the transfer of an amino group from aspartate to α-ketoglutarate results in the generation of glutamate, HepG2 cells (1 × 106) were homogenized in 200 μl of ice-cold AST assay buffer. Centrifuge the samples at ×13,000 g for 10 min to remove insoluble material. Add 50 μl of sample and 50 μl AST reaction mix and incubate the plate at 37°C. After 2–3 min, take the absorbance at 450 nm to product proportional to the AST enzymatic activity present. One unit of AST is the amount of enzyme that was generated 1.0 mmole of glutamate per minute at pH 8.0 at 37°C.

Alanine aminotransferase (ALT) activity = nmol/min/mL = IU/mL

Alanine transaminase (ALT)

ALT catalyzes the transfer of an amino group from alanine to α-ketoglutarate, the products of this reversible transamination reaction being pyruvate and glutamate, HepG2 cells (1 × 106) were homogenized in 200 μl of ice-cold AST assay buffer. Centrifuge the samples at ×13,000 g for 10 min to remove insoluble material. Add 50 μl of sample and 50 μl ALT reaction mix, measure output on a microplate reader in after 10 min at 37°C protected from light and its measure OD 570 nm.

ALT activity = nmol/min/mL = IU/mL

Alkaline phosphatase (ALP)

ALP catalyzes the hydrolysis of phosphate esters in alkaline buffer and produces an organic radical and inorganic phosphate, washed HepG2 cells (1 × 106) were homogenized in the assay buffer, centrifuge to remove insoluble material at ×13,000 g for 3 min, add the same amount of sample into separate wells, bring volume to 80 μl with ALT assay buffer, and incubate the reaction for 60 min at 25°C, protect from light. Add 20 μl stop solution and mix well to terminate ALP activity in the sample,

its uses p-nitrophenylphosphate as a phosphatase substrate which turns yellow (λ_{max} = 405 nm) when dephosphorylated by ALP.

ALP activity = nmol/min/mL = IU/mL

Catalase activity (CAT)

HepG2 cells (1 × 106) were homogenized in 200 µl of ice-cold CAT assay buffer. Centrifuge the samples at ×13,000 g for 10 min to remove insoluble material, add 10 µL stop solution into each sample, mix well and incubate at 25°C for 5 min to completely inhibit the CAT activity in the sample and add 12 µL of fresh 1 mM H_2O_2 solution to incubate reaction at 25°C for 30 min, add 50 µL of developer mix into each sample, and incubate at 25°C for 10 min protected from light, it was measured at OD 570 nm on a microplate reader.

CAT activity = nmol/min/mL = IU/m

Glutathione peroxidase (GPx)

GPx reduces cumene hydroperoxide while oxidizing GSH to GSSG. The generated GSSG is reduced to GSH with consumption of NADPH by GR. The decrease of NADPH (easily measured at 340 nm) is proportional to GPx activity, cell lysates with a detection sensitivity of ~0.5 IU/ml of GPx in samples. HepG2 cells (1 × 106) were homogenized and resuspend cells in 200 µL of cold assay buffer, resuspend were centrifuge 15 min at 4°C at 10,000 g using a cold microcentrifuge to remove any insoluble material, add 50 µL of reaction mix to 50 µL sample and mix well and incubate at room temperature for 15 min to deplete all GSSG in the samples. Then, add 10 µL cumene hydroperoxide solution to start the GPx reaction incubate at 25°C for 5 min (or longer if the GPx activity is low), and measure on a microplate reader at OD 340 nm.

Concentration of GPx in the test samples is calculated as nmol/min/mL = IU/mL.

Superoxide dismutase (SOD)

It catalyzes the dismutation of the superoxide anion into hydrogen peroxide and molecular oxygen. HepG2 cells (1 × 106) were homogenized and resuspend cells in 200 µL of lyses cells in ice-cold 0.1 M Tris/HCl, pH 7.4 containing 0.5% Triton X-100, 5 mM β-ME, 0.1 mg/ml PMSF and centrifuge at ×14,000 g for 5 min at +4°C, collect supernatant, and transfer to a clean tube, then add 200 µL of WST working solution to each well with 20 µL sample, mix and incubate at 37°C for 20 min and measure output (OD 450 nm) on a microplate reader.

SOD activity = (nmol/min/mL = IU/mL)

RESULTS AND DISCUSSION

The global acceptance of our traditional system is gaining prominence, thereby registering the steep rise in demand for various plants with medicinal

Table 1: Effect of *E. prostrata* L. extract on HepG2 cell line cytotoxicity

Concentration (µg/ml)	Cell viability inhibition/24 h	SD
SC	3.912148	0.854594
NS2-1 ng	21.82567	0.86472
NS2-10 ng	29.58133	0.434758
NS2-100 ng	42.69046	0.983703
NS2-S1 ug	57.58408	0.594112
NS2-10 ug	66.91833	0.937069
NS2-100 ug	72.20316	0.544547
P.Control-10 ng	41.59231	0.978373

E. prostrate: Eclipta prostrata

Table 2: Effect of *E. prostrata* L. extract on ROS activity

Concentration (µg/ml)	ROS activity/24 h	SD
SC	24.2915	4.448553
NS2-1 ng	19.19556	6.439352
NS2-10 ng	40.2482	7.379738
NS2-100 ng	48.09893	5.899336
NS2-1 ug	45.54656	7.780735
NS2-10 ug	32.92554	6.184057
NS2-100 ug	28.4809	6.184057
P.Control-10 ng	64.83014	1.823624

E. prostrate: Eclipta prostrate, ROS: Reactive oxygen species

Table 3: Effect of *Eclipta prostrata* L. extract on antioxidant enzymes

Antioxidant enzymes	Control	Positive control	Test (100 ng)	Test (1000 ng)
AST (SGOT)	95 IU/ml	145 IU/ml	131 IU/ml	125 IU/ml
ALT (SGPT)	32 IU/ml	90 IU/ml	84 IU/ml	70 IU/ml
ALP	21 IU/ml	35 IU/ml	32 IU/ml	30 IU/ml
GP	18 IU/ml	59 IU/ml	47 IU/ml	38 IU/ml
CAT	42 IU/ml	18 IU/ml	25 IU/ml	30 IU/ml
SOD	35 IU/ml	18 IU/ml	20 IU/ml	27 IU/ml

SGOT: Serum glutamic oxaloacetic transaminase, SGPT: Serum glutamate-pyruvate transaminase

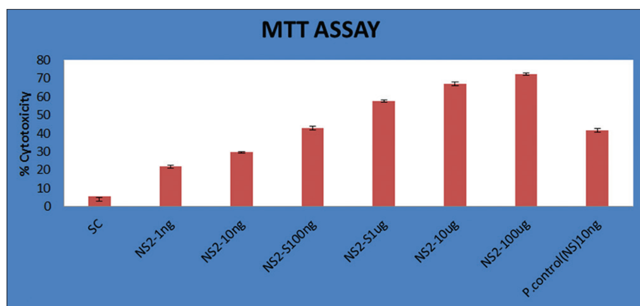


Figure 1: Effect of *Eclipta prostrata* L. extract on HepG2 cell line cytotoxicity

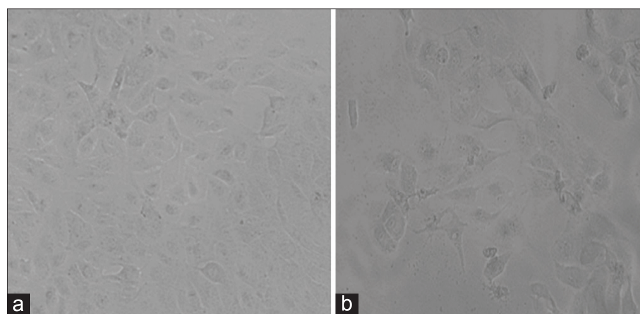


Figure 2: Control and test cells. (a) Control, (b) test

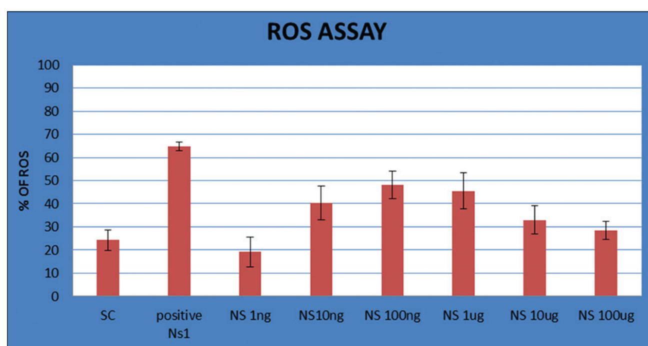


Figure 3: Effect of *Eclipta prostrata* L. extract on HepG2 cell line reactive oxygen species activity

properties. India is bestowed with enormous biodiversity of astonishing medicinal plants having more applications. Cytotoxicity generally possesses a broad spectrum of sensitivity and is able to detect many novel anticancer drugs, which potentially inhibit the biochemical activity of a variety of cancer cells of animal and human origin. The results clearly reported that when the cancer cells treated with whole plant of *E. prostrata* L. extract for long times. Addition of the extract with culture medium inhibits the growth of HepG2 cell lines in different concentrations of drug/24 h. In 100 ug of the extract induce 72.2% of cell death when compared to control [Table 1-3 and Figure 1-5].

Apoptotic morphological analysis

Apoptosis (means that “falling off”) is a process of programmed cell death that occurs in multicellular organisms. There are two ways that a cell can die: Apoptosis and necrosis. It is play a key role in causing and preventing some important medical processes. In humans, apoptosis plays a major role in preventing cancer by causing cells with damaged DNA to commit “suicide” before they can become cancerous. Biochemically and morphologically distinct from cellular necrosis, apoptosis involves chromatin condensation, cell shrinkage, DNA fragmentation, plasma membrane blebbing, and the formation of membrane-enclosed apoptotic cells.

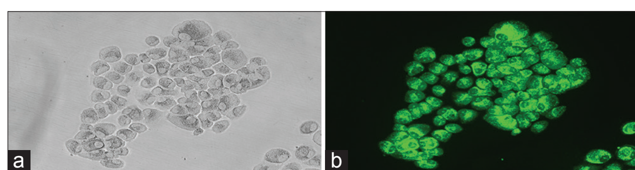


Figure 4: *Eclipta prostrata* L. extract on cell morphology with acridine orange. (a) Test with black and white image, (b) test with colored image

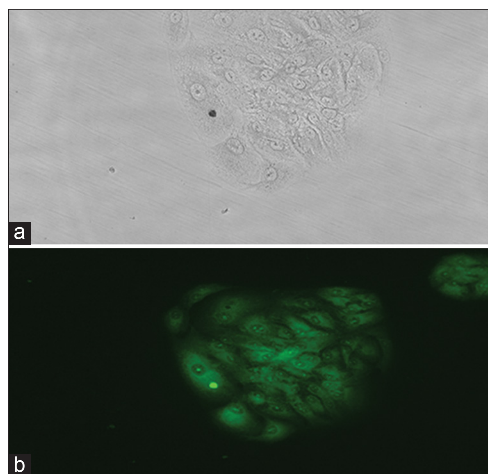


Figure 5: *Eclipta prostrata* L. extract on HepG2 cell line cytotoxicity with acridine orange. (a) Control with black and white image, (b) Control with colored image

In this study, the HepG2 cancer cells treated with *E. prostrata* L. stained using acridine orange. It reported that many morphological changes occurred, that is, cell shrinkage, loss of cell membrane integrity, impaction of nuclei, and more cells appeared in the granules in size and shape. However, no morphological changes were observed in control cells.

Intracellular ROS assay

Treatment with *E. prostrata* L. whole plant of ethanol extract to HepG2 cell lines indicates significant rise in the intracellular ROS production in a time dependent (24–48 h) as compared to control cells.

However, more amount of ROS production was observed at 48 h compared with 24 h. Besides, lipid peroxidation and decreased activities of antioxidant enzymes (SOD, GPx, and CAT) were observed and induced ROS production. Thus, overall, whole plant extract influences a beneficial action in the presence of low antioxidant defense. These effects revealed by *E. prostrata* L. whole plant of ethanol extract could be linked with inhibition of cell proliferation, stimulation of tumor cell death, and alterations in the oxidative stress markers. Induction of apoptosis is a perfect cancer therapy strategy. In our report stated that *E. prostrata* L. whole plant of ethanol extract selectively inducing cell death through ROS-dependent apoptotic pathway in HepG2 cells. In our reports suggest that *E. prostrata* L. extract selectively contains a key role in anticancer properties.

CONCLUSION

Our statistics clearly proves that *E. prostrata* L. whole plant of ethanol extract could exhibit the proliferation of HepG2 cells by stimulating programmed cell death in the powerhouse of the cell. In the ROS activity triggers that the cellular function's results increased the level of proapoptotic substances intracellularly. Furthermore, *E. prostrata* L. whole plant of ethanol extract could be a capability for the growth of an anticancer drug for the treatment of human HCC.

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