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IN VITRO CYTOTOXICITY AND ANTIOXIDANT STUDY OF DIFFERENT EXTRACTS OF *BOERHAVIA DIFFUSA LINN* IN CARDIAC H9c2 CELL LINE

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ABSTRACT

Boerhavia diffusa linn (BD) is known as a health food but its beneficial effects in protecting cardiomyocytes remain elusive. The aim of the present study was to evaluate the in vitro cytotoxic activity of methanolic, hexane and ethylacetate extract of a whole part of Boerhavia diffusa linn on H9c2 cardiomyocytes cell lines. Antioxidant activity of the plant extracts were characterized by using SOD, catalase and glutathione peroxidase method. The cytotoxic activity of the extracts of Boerhavia diffusa linn on H9c2 cells was investigated in vitro through MTT assay. The results showed Antioxidant activity using SOD, catalase and glutathione peroxidase were found to be increased in a concentration dependent manner and decreased cell viability and cell growth inhibition in a dose dependent manner. The findings from this study indicated that methanolic extract of Boerhavia diffusa linn possessed vast potential as a medicinal drug especially in cardioprotective treatment.

KEY WORDS

Boerhavia diffusa linn, SOD, catalase, glutathione peroxidase, MTT assay, Antioxidant, Cytotoxicity

INTRODUCTION:

Natural products of plants possess several biological activities including antioxidant and anti-inflammatory activity. Medicinal plants are rich in active phytochemical compounds with various biological activities. Researchers are highly interested in studying plants with the aim of isolating novel active drugs to replace synthetic drugs present in the market. The availability of these plants constituents provides a source of natural drugs for modern medicine. Hence, cytotoxic level of medicinal plants must also be evaluated against host cells. The safety of plants as a potential therapeutically agents must be ascertained, and the side effects should be acceptable to the host. Bioactive compounds with no or less toxic effect to the host are the good candidates for formulation of drugs.

Antioxidants play an important role in neutralizing free radical species which are produced as end or byproducts of normal biochemical reactions in normal system. High amounts of free radical molecules cause oxidative stress in cells which result in damaging essential macromolecules including DNA, lipids, and proteins. The damage of macromolecules leads to inflammation and many degenerative conditions such as Parkinson's diseases, atherosclerosis, aging, immunosuppression, ischemic heart disease, diabetes, hair loss, membrane lipid peroxidation, and decreased membrane fluidity. Reactive oxygen species are also reported as carcinogenic and mutagenic agents[1].

Boerhaavia diffusa L. (Nyctaginaceae), figure-1 commonly known as 'Punarnava' in the Indian system of medicine, is a perennial creeping herb found throughout the waste land of India. The plant has gained

lot of importance in the field of phytochemistry because of its various pharmacological and biological activities such as immunomodulatory effects, immunosuppressive activity, antimetastatic activity, antioxidant activity, antidiabetic activity antiproliferative and antiestrogenic activity, analgesic and anti-inflammatory activity, antibacterial activity, anti-stress and adoptogenic activity, antil-lympho proliferative activity, nitric oxide scavenging activity, hepatoprotective activity, anti-viral activity, bronchial asthma, anti-fibrinolytic activity, chemo-preventive action, genetic diversity analysis, anticonvulsant activity[2]. The aim of this study was to evaluate the cytotoxic effects of extracts *Boerhavia diffusa linn* against H9c2 cell line as well as its antioxidant activity using SOD, Catalase and glutathione peroxidase assay.

MATERIALS AND METHODS:

Plant collection and processing:

Healthy, ailment free whole *Boerhaavia diffusa L*. plant were collected from Samanthipuram village, Arcot, Tamil Nadu. The taxonomical identification was done by Dr. P. JAYARAMAN, Director, Plant anatomy research center, Chennai and voucher specimen was kept for further reference with register number PARC/2014/2078. The dried plant materials were milled to fine powder and stored at room temperature in closed bottles in the dark until use for the extraction.

Preparation of extracts:

Each sample was separately prepared by air drying, grinding and finally kept in paper bags. The resulting powder of each plant powder (10 g) was extracted in 150mL of methanol, hexane and ethyl acetate using a soxhlet for 18 h. The solution obtained from each powder was concentrated under vacuum to have a crude dried extract.

***In vitro* Cardioprotective determination by MTT assay:**

H9c2 (cardiomyoblast cell line) was initially procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained Dulbecos modified Eagles medium (Gibco, Invitrogen). The cell line was cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphotericin B (2.5µg/ml). Cultured cell lines were kept at 37°C in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany). The viability of cells was evaluated by direct observation of cells by Inverted phase contrast microscope and followed by MTT assay method.

Cytotoxicity Assay by MTT Method:

Viability test was performed by MTT dye-reduction assay. The log phase cells (H9c2) were suspended in the complete DMEM media to make a final cell concentration of 2 x 10^3/ml cells. In a tissue-culture coated 96-well plate, 150 µl of these cells were seeded and incubated at 37°C overnight supplemented with 5% CO_2. After that, when cells were adhered to plate surface, the given samples was supplemented at different concentration to make the final volume of 200 µl. The concentrations used were 0µL, 20 µL, 40 µL, 60 µL, 80 µL and 100 µL. All the concentrations were added as triplicates along with control i.e. media without cells and cells without test samples. After 24h, 20 µl of MTT solution (5 mg/ml in PBS) was added to each well and incubated for 4h at 37°C. Then, 100 µl of DMSO (to solubilize the purple formazon) was added, incubated for 30 min, after which the absorbance at 570 nm was recorded with a microplate reader (Thermoscientific Multiskan FC). Growth was expressed as the percentage of cell viability[3].

% cell viability = Absorbance of sample (Average of triplicates) * 100 / Absorbance of control

Invitro antioxidant assay: Superoxide dismutase assay

Measurement of superoxide radical scavenging activity was done by using Standard method. The superoxide anions generated by phenazinmethosulfate (PMN)/ nicotinamide-adenine-dinucleotidephosphate reduced form (NADPH) system, were detected by the reaction with 2,2'-di-p-nitrophenyl)-5,5'-diphenyl-(3,3'-dimethoxy-4,4'-diphenylene) ditetrazolium chloride (nitro blue tetrazolium – NBT). Stock solution of plant extracts and Quercitin (standard) was prepared to the concentration of 1mg/ml. The reaction mixture contained 1ml of Nitro blue tetrazolium (NBT) solution (312µM prepared in phosphate buffer, pH-7.4), 1ml of Nicotinamide adenine dinucleotide (NAD) solution (936 µM prepared in phosphate buffer, pH-7.4) and samples at different concentration (200, 600 and 1000 µg/mL) obtained from stock solution were added and finally the reaction was accelerated by adding 100µl phenazinemethosulfate (PMS) solution (120 µM prepared in phosphate buffer, pH-7.4). The reaction was incubated at 25°C for 5 minutes and absorbance was measured at 560nm against the corresponding blank solutions. Blank consist of all the reagents, except for the extract or standard solution is substituted with

water[4]. The activity of free radicals was calculated in % inhibition according to the following relation:

$$\textbf{Inhibition \%} = \frac{(\text{Absorbance of control} - \text{Absorbance of sample})}{\text{Absorbance of control}} \times 100$$

Estimation of Catalase

The estimation of catalase was done according to the method described by Luck, 1974. Each sample was homogenized in a blender with M ⁄ 150 phosphate buffer (assay buffer diluted 10 times) at 4˚C and centrifuged. The sediment was stirred with cold phosphate buffer, allowed to stand in the cold with occasional shaking and then the extraction was repeated. The combined supernatants were used for the assay. Read against a control cuvette containing the enzyme solution as in the experimental cuvette but containing H_2O_2 free phosphate buffer (M ⁄150). 3 ml of H_2O_2 phosphate buffer was pipetted out into the experimental cuvette and 0.01-0.04 of the sample was added and mixed with a glass or plastic rod flattened at one end. Note the time (Δt) required for a decrease in absorption from 0.45 to 0.40. The value was used for the calculations. If 't' is more than 60 sec. The measurements have to be repeated with a more concentrated solution of the samples[5].

Estimation of Glutathione Peroxidase:

To 2ml of Tris buffer, 0.1ml of sodium azide, 0.2ml of EDTA and 0.5ml of plant extracts were added. 0.2ml of glutathione followed by 0.1ml of hydrogen peroxide were added to the mixture, mixed well and incubated at 37°C for 10 minutes along with a tube containing all the reagents except sample. The reaction was arrested after 10 minutes by the addition of 0.5 ml of 10% TCA. The samples were centrifuged, and the supernatant was assayed for glutathione. The activities are expressed as µg GSH consumed /minute/mg protein[6].

Statistical Analysis

All results data are presented as mean and standard deviation of three consecutive technical repetitions on the statistical tool; GraphPad Prism (GraphPad Software, Inc., USA) was used to analyse the data via one-way analysis of variance (ANOVA).

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Result and discussion:

Free radicals are known as major contributors to several clinical disorders such as diabetes mellitus, cancer, liver diseases, renal failure, and degenerative diseases as a result of deficient natural antioxidant defense mechanism. A systematic search for useful bioactivities from medicinal plants is now considered to be a rational approach in pharmaceutical and drug research. The results of the effect of extracts of plants on the H9c2 cells at different concentrations showed the significant decrease of the viability of cells in a concentration-dependent manner after 24 h incubation. The increasing of the concentration of extracts almost completely blocked the growth H9c2 cells. The activity of each extract was expressed as CC50 (50 cytotoxic concentration values) with a lower CC50 value indicating a higher activity. The cytotoxic concentration (CC50) values varied from one extract to another at different concentration. According to the results of CC50 indicated in figure 1, methanol extract of *Boerhavia diffusa linn* was having more potent among the three extracts tested. The methanolic extract was used for the evaluation of the antioxidant activity.

Cytotoxicity of *Boerhavia diffusa linn* was assessed using MTT assay, after exposing the H9c2 cardio myoblast cells at 20 to 100µg/ml concentrations using methanolic, hexane and ethyl acetate extract, induced significant increase in cell viability in a concentration dependent manner. Methanolic extract Concentration of 80 µg/ml shows moderate activity whereas 100 µg/ml was found to have potent activity. Hexane extract concentration of 40 µg/ml shows moderate activity whereas 20 µg/ml was found to have potent activity. Ethyl acetate extract concentration of 60 µg/ml shows moderate activity whereas 20 µg/ml was found to have potent activity Cell viability at 20, 40, 60, 80 and 100 µg/ml was recorded as 95, 96, 109, 120 and 132% for methanolic extract, 104, 100, 94, 78 and 55 for ethyl acetate extract and 82, 73, 72, 73 and 71 for hexane extract respectively by MTT assay. Finally, it is concluded that methanolic extract is considered as a good cytotoxic activity.

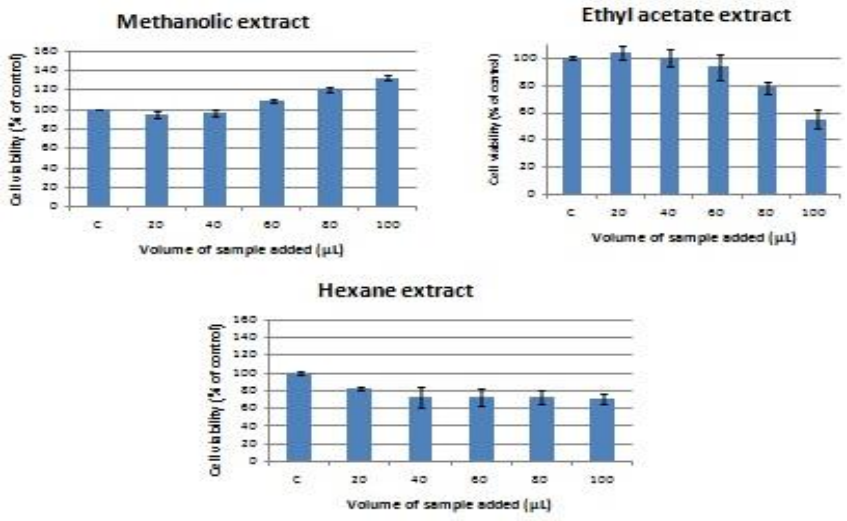

Figure 1: Shows cell viability report of three different extracts using MTT Assay.

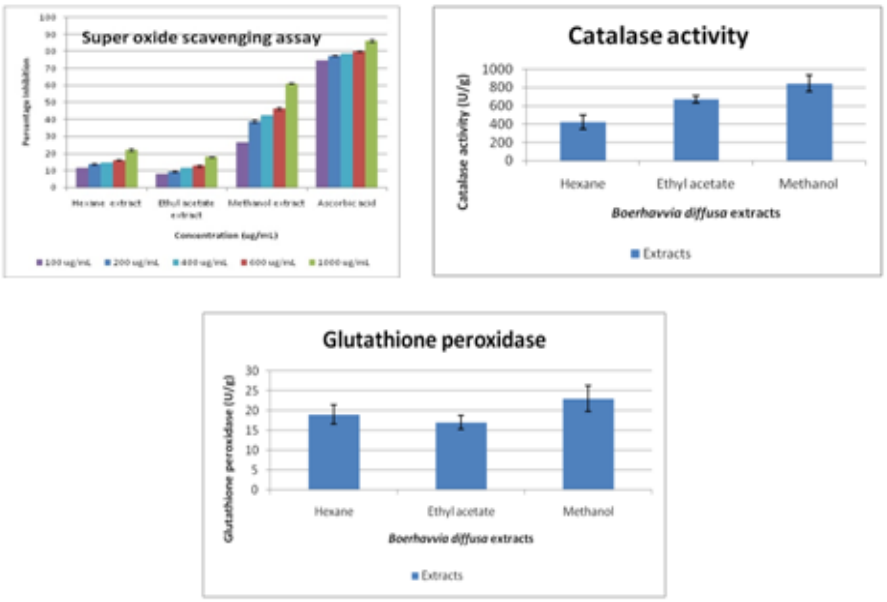

Figure 2: SOD, Catalise and Glutathione Peroxidase activity different extracts of *Boerhavia diffusa linn*

Antioxidant enzymes:

The results of the study of the antioxidant activity (SOD, Catalase and glutathione peroxidase) of each extract at different concentration in the presence of H9c2 cells after 24, 48, and 72 h treatment evoked a significant variation compare to the control. After 24 h of treatment with each extract, the enzymes (SOD, Catalase and glutathione peroxidase) exhibited a weak increase of their activities compare with the control. The methanolic extract induced significant ($p < 0.05$) increase of the activities of SOD after 48 h compared with their control and decrease after 72 h. However, methanol extract of *Boerhavia diffusa linn* significantly stimulated the augmentation of the SOD activity after 48 h and 72 h compared to their control. After 48 h the activity of SOD is increased. The scientific methods for the evaluation of natural products with biological property require the implementation of large-scale screening programs.

The intracellular concentration of ROS depends on the production and/or removal by the antioxidant system. Cells contain a large number of antioxidants to prevent or repair the damage caused by ROS, as well as to regulate redox-sensitive signaling pathways. Three of the primary antioxidant enzymes contained in mammalian cells that are thought to be necessary for

life in all oxygen metabolizing cells[6] are superoxide dismutase (SOD), catalase, and a substrate specific peroxidase, glutathione peroxidase (GPx). The SODs convert superoxide radical into hydrogen peroxide and molecular oxygen (O_2), while the catalase and peroxidases convert hydrogen peroxide into water and in the case of catalase to oxygen and water. The net result is that two potentially harmful species, superoxide and hydrogen peroxide, are converted to water. SOD and catalase do not need co-factors to function, while GPx not only requires several co-factors and proteins but also has five isoenzymes. Thus, the many forms of each of these enzymes reduces oxidative stress in the various parts of the cell. Thus, antioxidant proteins with similar enzymatic activity may have different effects after modulation due to different localizations within cells.It was reported that plant-derived extracts containing antioxidant principle showed cytotoxicity toward tumor cells[7].

From the result of the current study, it seems that the methanolic extracts of *Boerhavia diffusa linn* utilize their antioxidant properties by increasing SOD activity nearly to its normal level in order to protect cells against negative effects of stress produced by the proliferation of H9c2 cells[8]. Ali et al. (2005) have reported that the increased level of enzymic antioxidants glutathione peroxidase and catalase led to the breakdown of oxidants such as H_2O_2, organic hydroperoxides and lipid hydroperoxide resulting in greater protection against oxidative damage.[9]

CONCLUSION:

Finally, these results make it highly interesting to investigate the potential and protective effect of different extracts of *Boerhavia diffusa Linn* on H9c2 Cells. The various enzymic antioxidants have been evaluated in the methanolic, hexane and ethyl acetate extracts of *Boerhavia diffusa Linn*. Hence methanolic extact have proved to be potent source of antioxidants in eradicating the free radicals. Further purification can be done, and drug designing can be focussed from methanolic extract for the future benefit of the society

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