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IN VITRO HEPATOPROTECTIVE ACTIVITY OF ROOTS OF *DRACAENA TERNIFLORA* ROXB. AGAINST ETHANOL INDUCED HEPATIC DAMAGE IN HEPG2 CELL LINES

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ABSTRACT

This study was undertaken to validate the efficacy by evaluating the hepatoprotective activity of ethanolic root extract of *Dracaena terniflora* Roxb.(DTR) on Hep G2 cell line. Four doses of DTR root extract (6.25, 12.5, 25, 50 and 100 µg/ml) were tested on ethanol induced HepG2 cell toxicity model. The cytotoxicity study was conducted on the DTR root extract, using MTT assay to determine the CTC_{50} value which was 188.81%. The ethanolic root extract was also evaluated for their hepatoprotective activity on human liver hepatocellular carcinoma (Hep G2) cell line against ethanol as a liver damage inducing agent by quantifying the release of lactate dehydrogenase (LDH) in the medium. Of all the DTR treated groups, the Hep G2 cell lines treated with 6.25 µg/ml concentration showed maximum cell viability (91.79%) and minimum LDH leakage (0.0401U/ml). The DTR root extract exhibited significant ($P < .001$) cytoprotective activity with a maximum protection of 91.79 % at dose 6.25µg/ml, and the activity was comparable with that of the standard, Silymarin. The results of the present study indicate that the *Dracaena terniflora* Roxb. demonstrated a significant hepatoprotective activity and could be used as an active herbal alternative for the treatment of liver ailments.

Keywords: *Dracaena terniflora* Roxb. ethanol, HepG2, MTT, hepatocytes, Hepatoprotective activity, Silymarin

INTRODUCTION

Alcohol is a hepatotoxin that is commonly consumed worldwide and excessive alcohol consumption is a global healthcare problem with enormous social, economic, and clinical consequences. The liver sustains the greatest degree of tissue injury by heavy drinking because it is the primary site of ethanol metabolism. Chronic and excessive alcohol consumption produces a wide spectrum of hepatic lesions, the most characteristic of which are steatosis, hepatitis, and fibrosis/cirrhosis **[1]**.

Chronic viral hepatitis B and C, alcoholic liver disease, non-alcoholic fatty liver disease, and hepatocellular carcinoma are some of the major chronic liver diseases which represent a major health burden worldwide. Treating liver diseases with plant-derived compounds are accessible and do not require laborious pharmaceutical synthesis which seems highly attractive. In spite of various limitations, a number of herbals show promising effects of hepato protective efficacy, either experimentally in cell culture, in animal studies, or even in clinical trials **[2]**. The pharmacological treatment of alcohol liver disease is associated with free radicals. Reactive oxygen and nitrogen species (ROS and RNS) are produced by metabolism of normal cells. However, in liver diseases, redox is increased thereby damaging the hepatic tissue; the capability of ethanol to increase both ROS/RNS and peroxidation of lipids, DNA, and proteins was demonstrated in a variety of systems, cells, and species, including humans. ROS/RNS can activate hepatic stellate cells, which are characterized by the enhanced production of extracellular matrix and accelerated proliferation. ROS play an important role in fibrogenesis throughout increasing platelet-derived growth factor **[3]**.

Human hepatoma cell lines (HepG2) have been proposed as an alternative to human hepatocytes for in vitro models of normal liver cells. The potential advantages of hepatoma cells are that, as an immortalized cell line, they are readily available in large quantities, they are easy to maintain because they can be cryopreserved, and their drug-metabolizing enzyme activities do not decrease in cultivation, as happens in primary cultures of human hepatocytes **[4]**. The HepG2 hepatoma cell line is used widely in various studies of liver function, metabolism, and drug toxicity. They also possess many of the biochemical and morphological characteristics of normal hepatocytes and are used in studies to determine whether medicinal plants have hepatoprotective activities **[5]**.

Dracaena terniflora Roxb. (*Pleomele terniflora* Roxb.) is a traditional medicinal herb; also known as Elathaani,

Dwarf dracaena or Wild dracaena belongs to the family *Liliaceae*. This plant is widely distributed in the evergreen and semi-evergreen forests of South East Asia, India, Myanmar, Thailand, and Vietnam etc **[6]**. It has wide spread uses in ethno medicine. The various parts of the plant are used for diverse health ailments in traditional and folklore remedies. The various parts of the plant are used for diverse health ailments in traditional and folklore remedies. The decoction of the roots of this plant is used for treating spermaturia **[7]**. The fresh juice of this plant is used for the treatment of diabetes by the Kurunarippullu tribes of Wayanad **[8]**. Roots boiled with rice are taken internally for jaundice. Fruits boiled in coconut oil are used against head ache **[9]**. Roots of this plant is used for the treatment of various liver disorders especially jaundice by the various tribal communities of Kerala **[10]**. Traditionally the Root and fruit are used in bed sores **[11]**. Despite of the widespread folklore uses of *Dracaena terniflora* Roxb. there are no results on the scientific validation of its traditional medicinal claim. Thus the aim of the present study was to evaluate proposed claim of hepatoprotective activity of the roots of *Dracaena terniflora* Roxb. using Hep G2 cell lines *in vitro*.

MATERIALS AND METHODS

Collection and preparation of extracts

The whole plant of *Dracaena terniflora* Roxb. were collected in March 2017 from the semi forest regions of Kerala, India and identified by Dr. Sr. Tessy Joseph, H.O.D department of botany, Nirmala College, Muvattupuzha, kerala, India where a herbarium specimen was deposited (Voucher number NCH/2017/538). Fresh plant materials were washed thoroughly in running tap water to remove adhering impurities, shade dried to constant weight. The roots and aerial parts were separated.

Roots were coarsely powdered separately and passed through a 40-mesh sieve. It was stored in a tightly closed container. Fifty grams of the dried root powder was extracted with ethanol by using Soxhlet apparatus. To ensure the complete extraction process, exhaustive extraction was applied for 10 hours. After that the extract was recovered from the solvent by evaporation in a rotary evaporator at 60ºC and final drying was done by keeping the extract in desiccators for 1 hour to yield a reddish brown coloured semisolid sticky mass. The percentage yield of the ethanolic extract was obtained to 6.31%.

Preliminary Qualitative phytochemical analysis [12] [13]

The preliminary phytochemical analysis of extract was carried out using standard procedures to identify the various phytoconstituents present in the roots.

Determination of Total phenolic content (TPC) [14] [15]

Folin-Ciocalteau method was used for the determination of the total phenolic content of the root extract using expressed as milligram of gallic acid equivalent (GAE) per gram of the extracts with minor modifications as previously reported and was carried out in triplicates.

Determination of total flavonoid content (TFC) [15] [16]

The total flavonoid content was determined according to the aluminium chloride colorimetric method and expressed as milligram of quercetin equivalent (QE) per gram of extract. The determination of total flavonoid in the extract was also carried out in triplicates.

***Invitro* cytotoxic activity by MTT assay [17] [18]**

The *in vitro* cytotoxic and hepatoprotective activity of the ethanolic root extract of *Dracaena terniflora* Roxb. was assessed by MTT Assay (3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide) using Hep G2cell line. The 50% cytotoxic concentration (CTC_{50}) was determined by estimating mitochondrial synthesis using tetrazolium assay.

Culturing and maintenance of Hep G2 cells [18]

Hep G2 (Human Hepatic Cells) cells were initially procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained Dulbecco's modified Eagles medium, DMEM (Sigmaaldrich, USA).

The cell line was cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate (Merck, Germany) and antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphotericin B (2.5µg/ml). Cultured cell lines were kept at 37ºC in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany). The viability of cells were evaluated by direct observation of cells by Inverted phase contrast microscope and followed by MTT assay method.

Cells seeding in 96 well plate:

Two days old confluent monolayer of cells were trypsinized and the cells were suspended in 10% growth medium, 100µl cell suspension ($5x10^3$ cells/well) was seeded in 96 well tissue culture plate and incubated at 37ºC in a humidified 5% CO_2 incubator.

Preparation of compound stock:

1mg of sample was weighed and dissolved in 1mL DMEM using a cyclomixer. The sample solution was filtered through 0.22 µm Millipore syringe filter to ensure the sterility. Ethanol was added to induce toxicity.

Hepatotoxicity Evaluation:[18]

After attaining sufficient growth, Alcohol (40%) was added to induce toxicity and incubated for one hour, freshly prepared

each compounds in 5% DMEM were five times serially diluted by two fold dilution (100µg, 50µg, 25µg, 12.5µg, 6.25µg in 500µl of DMEM) and each concentration of 100µl were added in triplicates to the respective wells and incubated at 37ºC in a humidified 5% CO_2 incubator. Non treated control cells were also maintained.

- **Hepatotoxicity Assay by Direct Microscopic observation:**

Entire plate was observed after 24 hours of treatment upto 72 hours in an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) and microscopic observation were recorded as images. Any detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity.

- **Hepatotoxicity Assay by MTT Method:**

15 mg of MTT (Sigma, M-5655) was reconstituted in 3 ml PBS until completely dissolved and sterilized by filter sterilization.

After 24 hours of incubation period, the sample content in wells were removed and 30µl of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well, then again incubated at 37ºC in a humidified 5% CO_2 incubator for 4 hours. After the incubation period, the supernatant was removed and 100µl of MTT Solubilization Solution (Dimethyl sulphoxide **(**DMSO**)**, Sigma Aldrich, USA) was added and the wells were mixed gently by pipetting up and down in order to solubilize the formazan crystals. The absorbance values [optical density (OD)] were measured by using microplate reader at a wavelength of 540 nm (Laura B. Talarico *et al*., 2004).

The percentage of growth inhibition was calculated using the formula:

% Growth inhibition = (Mean OD of normal control − Mean OD of test group/Mean OD of Normal control) × 100

CTC_{50} (50% cytotoxic concentration), the concentration of the test drug needed to inhibit cell growth by 50% is generated from the dose- response curves for test samples. The dose-response curve was generated using % growth inhibition on Y axis and the extract concentration (µg/ml) on X-axis.

Invitro hepatoprotective effect determination by MTT assay [19]

MTT assay is a colorimetric assay that measures the reduction of yellow 3-(4, 5dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an

insoluble, coloured (dark purple) formazan product. The cells are then solubilized with an organic solvent (isopropanol) and the released, solubilized formazan reagent. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells.

***In vitro* Assessment of hepatoprotective activity of *Dracaena terniflora* Roxb. [20] [21] [22]**

The hepatoprotective activity of ethanolic extract of *Dracaena terniflora* Roxb (DTR) was evaluated using well-maintained HepG2 cells. Ethanol was used to induce hepatotoxicity and Silymarin was used as a standard positive control. The toxic concentration of ethanol taken was 100 mM. The doses of DTR and standard were chosen based on the results of the MTT assay. The experimental groups were carried out in triplicate as follows:

- **Group I (control)**

Normal control: The cells were treated with 100 µl of serum-free culture medium for 24 hours.

Dimethyl sulfoxide (DMSO) control: The cells were treated with 100 µl of serum-free culture medium containing DMSO (0.3% v/v) for 24 hours

- **Group II (toxin treatment):** The cells were treated with 100 µl of serum-free culture medium containing 100 mM ethanol for 24 hours.
- **Group III (Silymarin treatment):** The cells were treated with 100 µl of serum-free culture medium containing 100 mM ethanol with Silymarin at a concentration of 6.25, 12.5, 25, and 50µg/ml for 24 hours.
- **Group IV (DTR treatment):** The cells were treated with 100 µl of serum-free culture medium containing 100 mM ethanol with DTR at a concentration of 6.25, 12.5, 25, 50 and 100 µg/ml for 24 hours.

Later, cell viability and lactate dehydrogenase (LDH) leakage assays were performed for all groups according to standard methods. The absorbance values were measured at a wavelength of 540 nm using microplate reader.

Measurement of LDH activities [23] [24]

After attaining sufficient growth of HepG2 cells, ethanol (40%) was added to induce toxicity and incubated for one hour. LDH release assay was performed with cell free supernatant collected from tissue culture plates induced with ethanol was exposed to different concentrations of ethanolic extract of *Dracaena terniflora* Roxb.(DTR) such as 6.25 µg/ml, 12.5µg/ml, 25µg/ml, 50µg/ml and 100µg/ml. To this, added 2.7ml potassium phosphate buffer, 0.1ml 6mM NADH solution, and 0.1ml sodium

pyruvate solution. The decrease of OD was measured at 340nm in a spectrophotometer; thermo stated at 250̊ C. A blank solution is also prepared by adding enzyme dilution buffer instead of sample. Untreated control and Ethanol induced cells were also maintained.

Lactate dehydrogenase activity can be calculated by using the formula,

Activity of LDH (U/ml) = [(Abs – Ab0) x 3 (ml) x df] ÷ [6.2 x 0.1 (ml)]

Statistical analysis:

The results were expressed as mean ± standard deviation. Statistical analysis was carried out by one-way analysis of variance followed by Dunnett's t-test, using Graph pad Prism 6.0, San Diego, California, USA.

Result and discussion:

The present study revealed that the root extract of *Dracaena terniflora* Roxb. showed the presence of alkaloids, flavonoids, phenols, saponins, steroids, tannins, terpenoids, carbohydrates, proteins, coumarins and triterpenoids and absence of anthocyanins and glycosides.

Total phenolic (TPC) and flavonoid (TFC) content:

The flavonoid and phenolic compounds play an important role in culminating the oxidative stress produced by reactive oxygen species and hydroxyl radicals generated in alcohol metabolism. Total phenolic and flavonoid contents of root extracts of *D.terniflora* Roxb were calculated. Ethanolic root extract (DTR) showed the flavonoid content of 72 mg quercetin equivalent/g and phenolic content of 31 mg GAE/g.

***In vitro* cytotoxicity assay of ethanolic root extract of *Dracaena terniflora* Roxb.**

The percentage cell viability with respect to the normal control (NC) cell lines (HEPG2) at different concentrations of DTR root extract was determined and the results are shown in **Figure 1**. The normal control cells showed 100% cell viability. The ethanolic root extract of *Dracaena terniflora* Roxb. at concentrations 100 µg/ml, 50 µg/ml, 25 µg/ml and 12.5 µg/ml and 6.25 µg/ml showed 72.98%, 78.05%, 83.89%, 88.01% and 98.95 %cell viability, respectively. The inhibitory concentration (CTC_{50}%) value of DTR was found to be 188.81µg/ml (Calculated using ED50 PLUS V1.0 Software).

In MTT assay, the cell viability was >70 % up to a concentration of 100µg/ml. Thus, DTR root extract showed less cytotoxic effect to hepatic cells due to the presence of phytoconstituents. Therefore, these extract concentrations were selected to further evaluate the cytoprotective activity against ethanol-induced cell damage.

***In vitro* hepatoprotective activity of ethanolic extract of roots of *Dracaena terniflora* Roxb.**

The ethanol-exposed $HepG_2$ cells showed a viability of 43.93%. These exposed cells, when treated with different concentrations of DTR ethanolic root extracts, showed a reasonable change in the viability and the results were highly significant (*P <.001*, when compared to ethanol intoxicated group). The increase in percentage viability of the HepG2 cells treated with DTR extract at 6.25µg/ml was significant (*P <.001*, when compared to standard Silymarin) and are almost comparable with the percentage viability that produced by the standard Silymarin at 25 µg/ml. The results of the study are shown in **Table 1**. There was a significant decrease in cell viability and a significant increase in the levels of LDH in the Group II, i.e., treated with 100 mM ethanol as compared with normal control (Group I). HepG2 cells when treated with different concentrations of DTR (6.25, 12.5, 25, 50 and 100 µg/ml) showed a significant restoration of the altered levels of hepatic enzyme and improved cell viability which was comparable to that of standard drug Silymarin. Of all the doses tested, DTR at6.25µg/ml showed better cytoprotective activity **(Figure 2 and 3)**.

Lactate Dehydrogenase Assay

Lactate dehydrogenase (LDH) leakage assays were performed for all groups according to the standard method using Ecoline diagnostic kits. The results of the study are shown in **Table 2**.

Morphological changes:

Hep G2 Cells exposed to 100 mM of ethanol and increasing concentrations of various extracts of *Dracaena terniflora* Roxb were shown in **Figure 4. Figure 4(A)** is the image of normal control cell lines. Ethanol induced toxicity to hepatic cells (Hep G2) showed detachment of cells from the surface of plate, rounding up of cells and alteration in cellular meshwork indicating cytotoxicity and necrosis. Most of the cells exposed to ethanol lost their typical morphology and appeared smaller in size **[Figure 4(G)]**. But Hep G2cells previously treated with ethanol and exposed to increasing concentrations of root extracts of *Dracaena terniflora* Roxb. convincingly restore their original morphology in a dose dependent manner **[Figure 4(B to F)]**.

Hepatoprotective Effects in the HepG2 Cell Line:

The ethanol exposed HepG2 cells showed a percentage viability of 43.93 %. These exposed cells, when treated with different concentrations of DTR root extracts showed a rational increase in percentage viability and the results were highly significant (*P < 0.001*, when compared to ethanol intoxicated group). The percentage viability has ranged between 55.30 and 91.79 % at 100 to 6.25

µg/ml concentration of the ethanolic root extract of *Dracaena terniflora* Roxb. **(Table: 1)**. The increase in percentage viability of the HepG2 cells treated with DTR root extract at 6.25 µg/ml was significant ($P < 0.001$, when compared to standard Silymarin) and comparable to that produced by the standard Silymarin at 12.5 µg/ml. The DTR root extract is supposed to prevent the apoptosis of HepG2 cells through suppression of reactive oxygen species production. The results are shown in **Figure 5**.

Effect of DTR root extract on LDH leakage in Ethanol -induced HEPG2 cells:

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme that is found in all cells. LDH is rapidly released into the cell culture supernatant when the plasma membrane is damaged, a key feature of cells undergoing apoptosis, necrosis, and other forms of cellular damage. LDH activity can be easily quantified by using the NADH produced during the conversion of lactate to pyruvate to reduce a second compound in a coupled reaction into a product with properties that are easily quantified. This protocol measures the reduction of a yellow tetrazolium salt, INT, by NADH into a red, water-soluble formazan-class dye by absorbance at 492 nm. The amount of formazan is directly proportional to the amount of LDH in the culture, which is, in turn, directly proportional to the number of dead or damaged cells **[25]**.

We have evaluated the % cell viability by checking LDH release of control, ethanol treated as well as DTR treated cell lines. LDH release was significantly increased in ethanol intoxicated group when compared with the normal control group from 0.0034 to 0.3381 U/ml. Group IV (DTR treated group) showed variation in the LDH release while comparing with control and ethanol intoxicated group. DTR treated cell lines at concentrations of 6.25, 12.5, 25, 50 and100 µg/ml significantly reduced the elevated level of this enzyme in comparison with ethanol treated groups indicating the hepatoprotective activity of the extract. Of all the DTR treated groups, the Hep G2 cell lines treated with 6.25 µg/ml concentration showed maximum cell viability (91.79%) and minimum LDH leakage (0.0401U/ml).

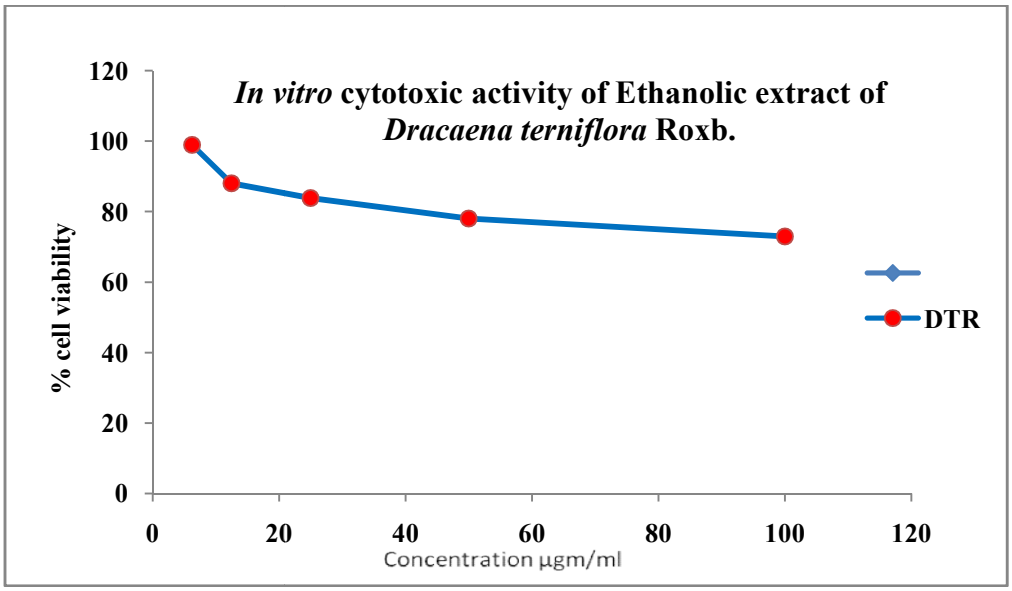


Figure 1: ***In vitro*** **cytotoxic activity of ethanolic extract of** ***Dracaena terniflora*** **Roxb. roots**

Table 1: ***In vitro*** **hepatoprotective activities of DTR root extract using HepG2 cell lines**

Groups	%Cell viability
Group I (control)	
Normal control	100.00
Group II (toxin treatment)	
100 mM ethanol	43.93 ±0.015
Group III Silymarin treatment	
100 mM ethanol + Silymarin (6.25 µg/ml)	65.69±0.040
100 mM ethanol + Silymarin (12.5 µg/ml)	83.96±0.081
100 mM ethanol + Silymarin (25 µg/ml)	95.53±0.602
100 mM ethanol + Silymarin (50 µg/ml)	96.06±0.020
Group IV (DTR treatment)	
100 mM ethanol + DTR(6.25 µg/ml)	91.79±0.920
100 mM ethanol + DTR (12.5 µg/ml)	75.37±0.321
100 mM ethanol + DTR (25 µg/ml)	70.90±0.730
100 mM ethanol + DTR (50 µg/ml)	68.98±0.569
100 mM ethanol + DTR (100 µg/ml)	55.30±0.453

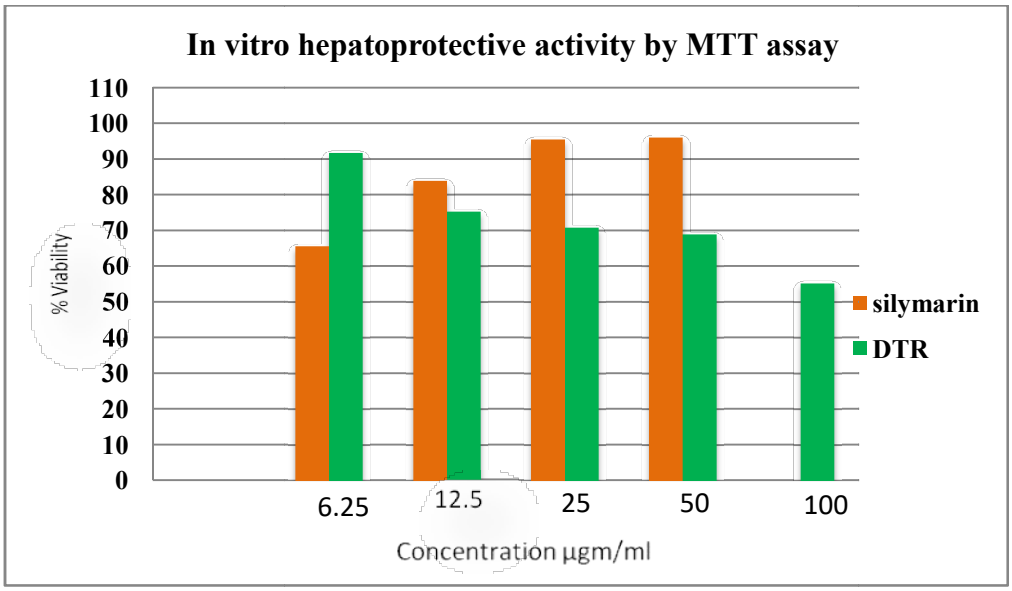


Figure 2: ***In vitro*** **hepatoprotective activity of DTR root extract using HepG2 cell lines**

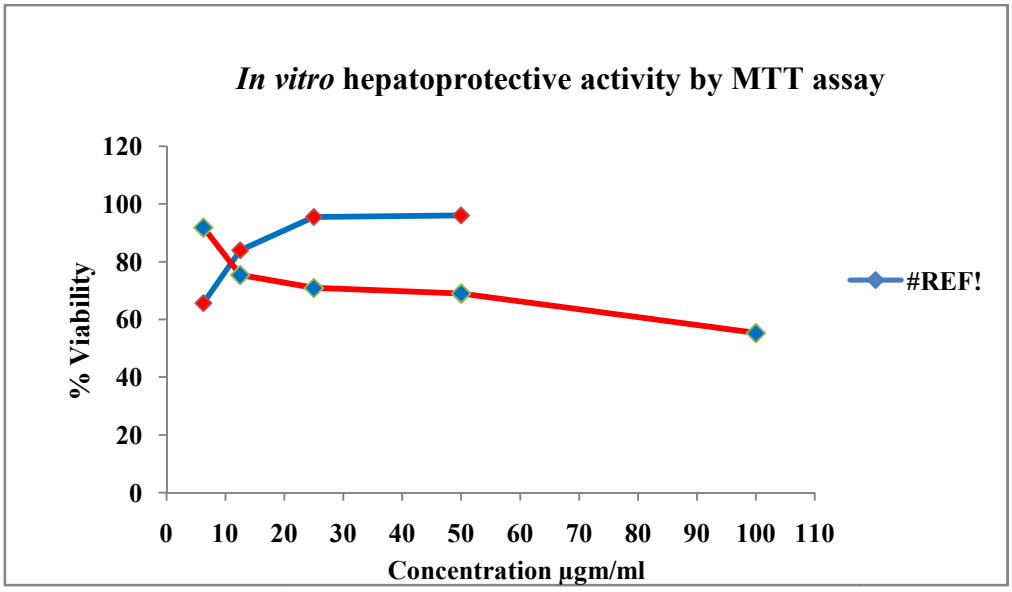
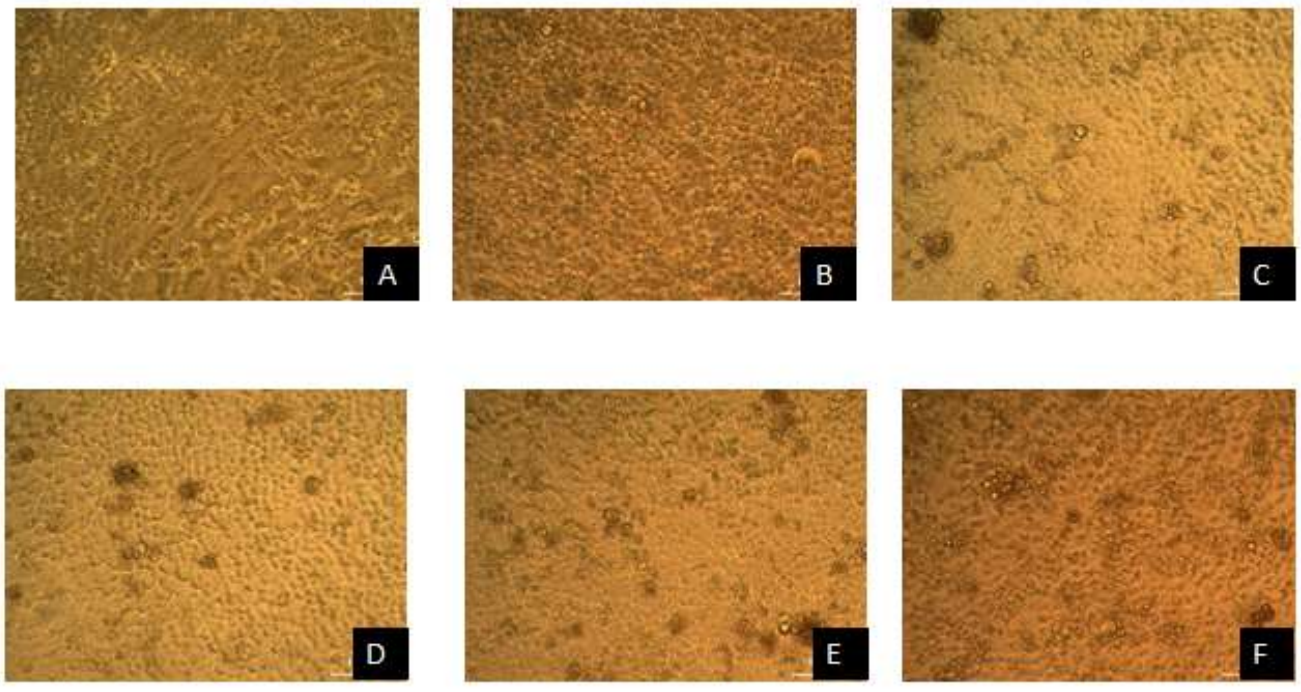


Figure: 3 *In vitro* hepatoprotective activity of DTR root extract using HepG2 cell lines

Table 2: Lactate Dehydrogenase Assay

Concentration (µg/mL)	►OD	Activity of LDH (U/ml)
Control	0.0007	0.0034
Ethanol	0.0700	0.3381
Sample code: DTR		
6.25	0.0083	0.0401
12.5	0.0174	0.0840
25	0.0271	0.1309
50	0.0314	0.1517
100	0.0491	0.2372



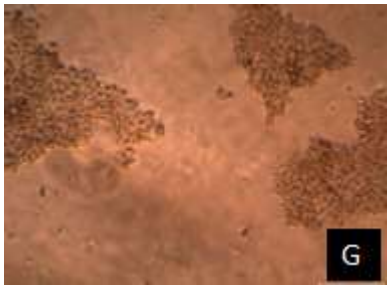


Figure: 4(A): Normal control cells. The $HepG_2$ cells pre-treated with Dulbecco's Modified Eagles Medium showing normal cells surface architecture (×40); Figure 4(B): Protection of HEPG2 pre-treated DTR at concentration 6.25 µg/ml, Figure 4(C): Protection of HEPG2 pre-treated with DTR at concentration 12.5 µg/ml, Figure 4(D): Protection of HEPG2 pre-treated with DTR at concentration 25 µg/ml, Figure 4(E): Protection of HEPG2 pre-treated with DTR at concentration 50 µg/ml, Figure 4(F): Protection of HepG2 pre-treated with DTR at concentration 100 µg/ml, Figure: 4(G) Ethanol intoxicated group

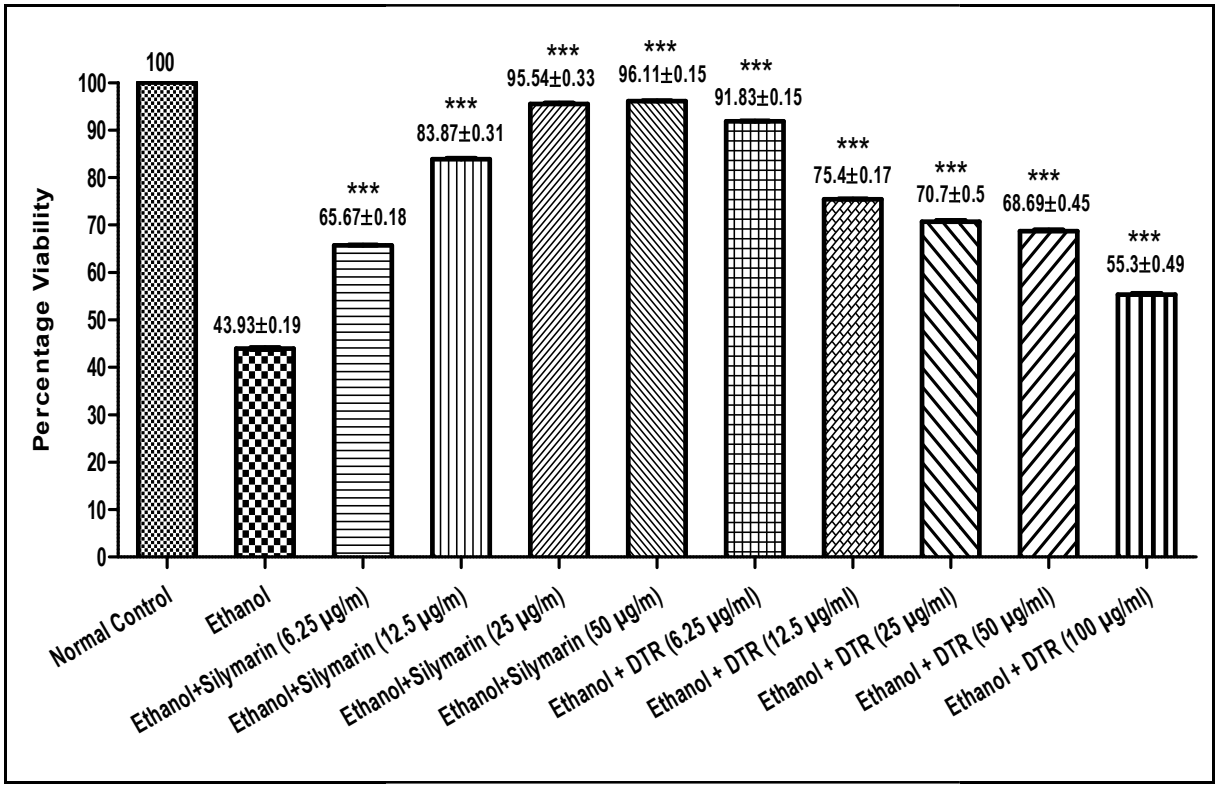


Figure 5: Data represented in graph mean ± SD(n=3). Ethanol versus other groups; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

CONCLUSION

Apoptotic hepatotoxicity has been implicated in the pathogenesis of several liver diseases including that involving ethanol abuse. Insights into the cellular mechanisms involved in the initiation and propagation of apoptosis will significantly impact our understanding of alcohol-induced liver disease and may lead to the potential development of therapeutic interventions **[26]**. The reason behind the hepatoprotective effect of this plant extract may be due to its ability to scavenge-free radicals that are produced during the metabolism of ethanol in Hep G2 cells. The antioxidant active ingredients like flavonoids and polyphenolic compounds available in the DTR root extract,

contribute the hepatoprotective activity of the extract. The findings of the present study could validate the practice of the plant in traditional medicine for the treatment of jaundice.

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