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CYTOTOXIC EFFECT OF ETHANOLIC EXTRACT OF CRATAEVA RELIGIOSA G. FORST LEAVES (CAPPARACEAE)

Mohanapriya R.[1]*, T. Venkata Ratina Kumar[2], Maruthamuthu Murugesan[3] and Sesha Kumar S. S.[4]

[1]JRF Siddha Central Research Institute, Arumbakkam, Chennai-600106.
[2] Department of Pharmacognosy, College of Pharmacy, Madurai Medial College, Madurai – 20.
[3]SRF, Captain Srinivasa murthy central Ayurveda research institute, Arumbakkam, Chennai-106.
[4]Assistant Professor, Department of Pharmacognosy, GRT Institute of Pharmaceutical Education and Research, Thiruttani, Tamilnadu.

***Corresponding Author: Mohanapriya R.**

JRF Siddha Central Research Institute, Arumbakkam, Chennai-600106.

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ABSTRACT

The Capparaceae, often known as Capparidacea or the Caper family, is the group of plants that includes *Crataeva religiosa*. The plant is known by the scientific name Crataeva, which honours the Greek botanist Crataevus who lived during the time of Hippocrates. The botanical name religiosa denotes the plant's profusion close to places of devotion. Examining *Crataeva religiosa 's* cytotoxic potential against HepG2 cell lines was the goal of the current investigation. The *Crataeva religiosa* leaves were gathered, and an ethanolic extract of the leaves was made, which was used to assess the cytotoxicity. Doxorubicin and 10% dimethyl sulfoxide were used as positive and negative controls, respectively, in the MTT experiment to assess the cytotoxic activity. HepG2 cell viability was considerably decreased by ethanol extracts of *Crataeva religiosa* leaves at concentrations of 10, 20, 40, 80, 160, and 320 g/ml. The IC50 value for doxorubicin and the ethanolic extracts of the leaves was computed using Graph Pad Prism, and it was discovered to be 18.52 g/ml and 51.61 g/ml, respectively. Due to the presence of quercetin in the extract, this study found that EECRL demonstrated considerable in vitro anticancer activity when compared to doxorubicin against HepG2 cell lines. Due to the fact that quercetin can cause HepG2 cells to apoptosis by reducing the expression and inhibiting intracellular FASN activity.

KEYWORDS: MTT assay, Cytotoxicity, HepG2, *Crataeva religiosa*, Quercetin.

INTRODUCTION

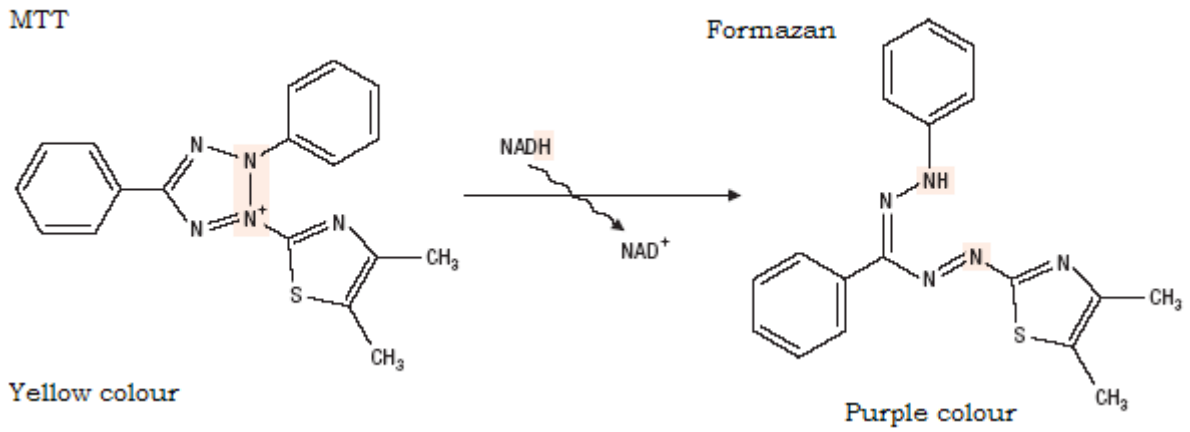
The term "cancer" describes the rapid development of aberrant cells that proliferate outside of their normal bounds, infiltrate nearby body components, and spread to other bodily organs.[1] In India, cancer is the second most prevalent disease and the leading cause of death, accounting for over 0.3 million fatalities annually.[2] According to a poll, 102 people worldwide die from cancer per year, or 182 people out of every 100,000. Cancer is the primary cause of death in both developing and industrialised nations.[3] Nearly one in six deaths worldwide are caused by cancer, with low- and middle-income nations accounting for about 70% of these deaths1. Despite significant progress in detection and the availability of numerous treatments, cancer continues to pose a serious threat to our society.[2]

There are many different strategies employed today to cure cancer, however many healthy cells will also be lost as a result of those approaches' non-selectivity of medication. The main challenge in treating cancer is eliminating tumour cells while leaving healthy cells alone without causing harm to healthy cells. Testing cytotoxic substances and screening raw extracts of plants are required in order to produce anticancer medications from natural resources like plants. As a result, natural products with more efficacy and fewer adverse effects are preferred. Herbal medicines are essential for treating cancer because they include a variety of chemical ingredients that can be used to create new cancer-fighting substances.[3]

The moderately big, attractive, deciduous, heavily branched *Crataeva religiosa* tree is 50 feet (15 metres) tall and 30 feets (9 metres) wide. It is a little spreading perennial tree with trifoliate leaves that, when buried, give off a strong odour. Since the time of the Vedas, this herb has been used. In general, it prefers moist, rich, neutral to acidic soil and does well in the sun or light shade. It can be found in frequently inundated forests, often below 100 metres in elevation but occasionally reaching 700 metres. typically seen around temples in India and Polynesia.[4]

There are many medical applications for *Crataeva religiosa*. Lithotriptic is *Crataeva religiosa* 's most popular application. The bark is utilised for calculous affection as well as urinary problems like kidney and bladder stones. Additionally, *Crataeva religiosa* is utilised as an insecticide, analgesic, anti-arthritic, anti-microbial, anti-inflammatory, and antifungal.[5]

The MTT technique uses mitochondrial dehydrogenases to gauge the activity of live cells. This assay technique is straightforward, precise, and produces repeatable findings. The main ingredient is 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide, a water-soluble tetrazolium salt that produces a yellowish solution when made in salt solutions or media without phenol red. The mitochondrial dehydrogenase enzymes of live cells cleave the tetrazolium ring of dissolved MTT to produce an insoluble purple formazan. If necessary, DMSO, acidified isopropanol, or other solvents can be used to dissolve this insoluble in water formazan (Pure propanol or ethanol). The resulting purple solution is measured spectrophotometrically. The amount of formazan generated changes depending on whether there are more or fewer cells present, reflecting the extent of the test material's effects.



Advantages

The MTT assay method is far more effective than dye ex clusion techniques. It is extensively used to detect both c ell viability and cytotoxicity tests because it is simple to use, safe, affordable, has a good repeatability, and offers these benefits.[6]

MATERIALS AND METHODS

Collection and authentication of leaves:

The leaves of *Crataeva religiosa*, a member of the Capparaceae family, were gathered in and near Madurai. Dr. Stephen, Professor, Department of Botany, American College, Madurai - 20, verified the gathered leaf. This specimen of a plant that was obtained was prepared for the herbarium and stored for future use.

Preparation of plant extract:

The collected leaves were properly rinsed in cold water and the water was entirely drained before the leaves were left to dry in the shade until they were completely free of moisture. They were then finely powdered and put through Sieve No. 40. Ethanol was used to make the extract (EECRL- Ethanolic extract of *Crataeva religiosa* leaves) using the cold maceration method. The content had previously undergone petroleum ether defatting. The defatted material was then given a chance to macerate in ethanol for around 72 hours. To remove all non-extractable material, including cellular components and other constitutions that are insoluble in the extraction solvent, the extract was filtered using Whatman filter paper No. 42. A buchi rotary evaporator was used to dry out the entire extract under reduced pressure.

***In vitro* cytotoxicity study of EECRL using HepG2 cell line by MTT assay[7-14]**

Requirements

1. MTT Powder (The solution is filtered through a 0.2µm filter and stored at 2–8 °C for frequent use or frozen for extended periods)
2. DMSO
3. CO_2 incubator
4. Tecan Plate reader

Preparation of test solutions

DMEM was used to create 32mg/ml stocks for cytotoxicity tests. From 3.2 mg/ml to 10 mg/ml, serial two-fold dilutions were made using DMEM plain medium.

Cell lines and Culture medium

Stock cells of the HepG2 cell line were purchased from ATCC and grown in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), and streptomycin (100 g/ml) at 37°C in a humidified environment of 5% CO2. Cell dissociating solution (0.2% trypsin, 0.02% EDTA, and 0.05% glucose in PBS) was used to separate the cell. Cell viability was examined, and centrifugation was performed. Additionally, a 96-well plate with 50,000 cells per well

was seeded and cultured for 24 hours in a 37°C, 5% CO2 incubator.

Source of reagents
DMEM, FBS, Penicillin, Streptomycin, Trypsin-procured from Invitrogen, DMSO.

Procedure
To gauge the rate of cell proliferation, the MTT test was performed. This assay is based on the transformation of MTT, which is water soluble, into formazan, a crystal that is insoluble in water. The number of viable cells is directly correlated with the amount of formazan that eventually forms. The following procedure was used to carry out the MTT assay. Using the appropriate media containing 10% FBS, the monolayer cell culture was trypsinized and the cell count was increased to 5x105 cells/ml. A 100-l portion of the diluted cell suspension (50,000 cells/well) was put to each well of the 96-well microtiter plate. When a partial monolayer had developed after 24 hours, the supernatant content was removed. The monolayer was then washed with media once, and 100µl of various test drug concentrations were added on to the partial monolayer plate. The plates were then incubated for 24 hours at 37°C in a 5% CO2 environment. Following incubation, 100 l of MTT (5mg/10ml of MTT in 1X PBS) was added to each well, replacing the test solutions that had been in the wells. At 37°C and with 5% CO2 in the air, the plates were incubated for 4 hours. To dissolve the formazan that had formed, 100 l of DMSO was added after the supernatant was drained from the plates. Utilizing a microplate reader, the absorbance was determined at a 590 nm wavelength. The formula below was used to calculate the percentage of growth inhibition, and the dose-response curves for each cell line were used to determine the concentration of test material needed to inhibit cell growth by 50% (IC50) values.

% Inhibition = ((OD of Control – OD of sample) / OD of Control) x 100

Statistical evaluation
IC_{50} Value
The ability of a drug to inhibit a biological or biochemical activity is measured by the half maximum inhibitory concentration (IC50). This quantitative indicator shows the amount of a specific medicine or other substance (inhibitor) required to completely stop a specific biological process (or component of a process, such as an enzyme, cell receptor, or microbe).

By building a dose-response curve and analysing the impact of different antagonist concentrations in reversing agonist activity, the IC50 value of a medication can be ascertained. The concentration of the substance required to inhibit 50% of the maximum biological response of the agonist can be used to compute the IC50 value for a specific antagonist. Using Graph Pad Prism 6, a nonlinear regression analysis (curve fit) based on a sigmoid dose response curve was used to determine IC50 values for cytotoxicity studies (Graph pad, SanDiego, CA, USA). The tables 1 and 2 displayed the values.

Nonlinear regression
A nonlinear combination of the model parameters that depends on one or more independent variables is used to model observational data in nonlinear regression, a type of regression analysis. A strategy of successive approximations was used to fit the data.[15,16]

RESULTS
HepG2 cell line (Human liver cancer cell line) MTT experiment was performed using doxorubicin as a reference and different concentrations (10, 20, 40, 80, 160, and 320 g/ml) for EECRL. A microplate reader was used to measure the absorbance at a 590 nm wavelength. The IC50 values and percentage of cells with growth inhibition were then computed and are shown in Tables 1 and 2, respectively.

Table 1: Percentage inhibition potential of doxorubicin against HepG2 cells.

Compound name	Conc µg/ml	OD at 590nm	% Inhibition	IC50µg/ml
Control	0	0.784	0.00	18.92
Doxorubicin	10	0.658	16.32	
	20	0.576	26.27	
	40	0.357	54.60	
	80	0.288	63.26	
	160	0.238	69.77	
	320	0.174	77.80	

Table 2: Percentage inhibition potential of EECRL against HepG2 cells.

Compound name	Conc µg/ml	OD at 590nm	% Inhibition	IC50µg/ml
Control	0	0.784	0.00	51.61
EECRL	10	0.678	13.52	
	20	0.545	30.48	
	40	0.478	39.03	
	80	0.358	54.33	

	160	0.275	64.79	
	320	0.168	78.57	

The half maximum inhibitory concentration, or IC50, is a measurement of a substance's ability to block a certain biological or metabolic function. The dose-response curves for each cell line were derived from a nonlinear regression analysis (curve fit) based on a sigmoid dose response curve (variable), and the results were computed using Graph Pad Prism 6. The results are shown in Tables 1 and 2. The concentration of the test drug required to inhibit cell growth by 50% (IC50) values were generated from the dose-response curves for each cell line.

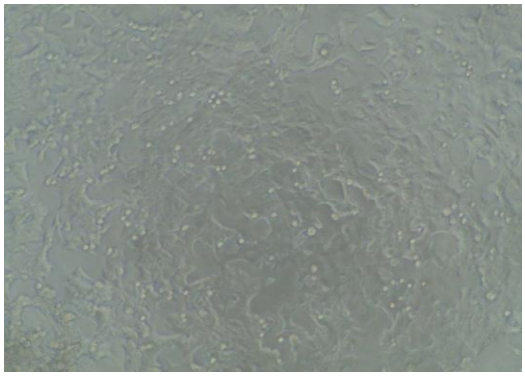
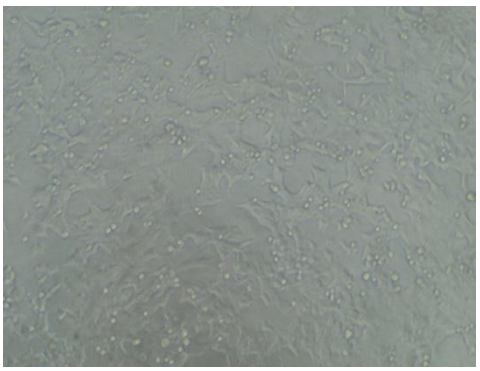


Fig. 1: Control.



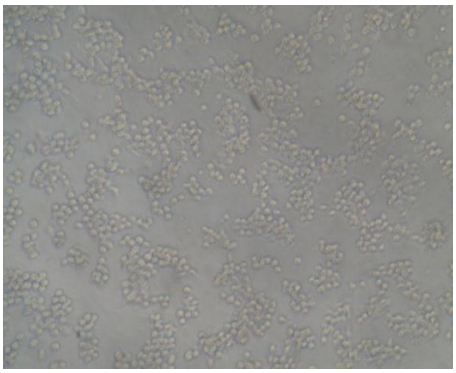
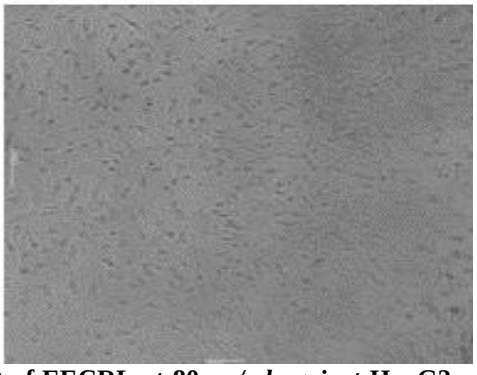


Fig. 2: Effect of doxorubicin at 80 *µg/ml* against HepG2 cells. Fig. 3: Effect of doxorubicin at 32*0 µg/ml* against HepG2 cells.



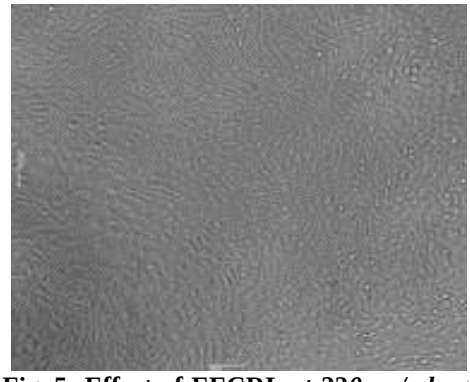


Fig. 4: Effect of EECRL at 80 *µg/ml* against HepG2 cells. Fig. 5: Effect of EECRL at 32*0 µg/ml* against HepG2 cells.

Cell viability assessed by MTT assay

HepG2 cell morphological changes as seen under an inverted light microscope. Figures 2 to 5 depict the effects of various doxorubicin and EECRL concentrations on HepG2 cells. Figure 1 depicts the control, which has a greater number of cells, and Figures 2 to 4 depict the cells treated with doxorubicin and EECRL, which demonstrate a reduction in the number of cells. Finally, EECRL demonstrated an IC50 value of 51.61 g/ml for HepG2 cells while Standard Doxorubicin shown an inhibition of 18.92 g/ml for HepG2 cells.

DISCUSSION

Quercetin, a flavonoid component, has been reported to have anticancer properties. The presence of quercetin in EECRL may be the cause of its anticancer properties. According to a study, quercetin can cause apoptosis in HepG2 cells by reducing the expression of FASN and inhibiting intracellular FASN activity. In addition to being highly expressed in adipose tissues and a number of human malignancies, such as liver, prostate, breast, endometrial, ovarian, colon, lung, and pancreatic cancer, fatty acid synthase (FASN) is a metabolic enzyme that catalyses the production of long chain fatty acids .[17] According to the study, EECRL significantly outperformed doxorubicin against HepG2 cell lines in terms of in vitro anticancer activities.

CONCLUSION

In this work, doxorubicin was used as a standard to assess the extract's (EECRL) anti-cancer (cytotoxicity) activity against the HepG2 tumour cell line. The IC50 value for HepG2 cells treated with EECRL was determined and found to be 51.61 g/ml, whereas Standard Doxorubicin for HepG2 Cells demonstrated inhibition at 18.92 g/ml. Due to the presence of quercetin in the extract, this study found that EECRL demonstrated considerable in vitro anticancer activity when compared to doxorubicin against HepG2 cell lines.

Conflict of interest

The authors declare no conflict of interest.

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