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Anticancer activity of *Eugenia jambolana* seeds against Hep2 cell lines

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ABSTRACT

Cancer is a life-threatening disease and leads to high rates of mortality worldwide, after cardiovascular disease, is the second leading cause of death. Investigations for finding new plant based anticancer compounds are imperative and interesting. There are many studies on anticancer herb/plant extracts in cell line models. *Eugenia jambolana* has been reported to contain phytochemicals like coumarin, flavanoids, glycosides, phenols, tannins and steroids. The various part of *Eugenia jambolana* have therapeutic applications. Plant active components were extracted using the decoction extraction method and the filtrate was obtained by means of filtering through a Whattman no.1 filter paper. The filtrate was evaporated in a weighed flask in a hot air oven set at 50°C. Extracts were reconstituted by re-dissolving in respective solvents. Different concentration i.e. 8, 15.6, 31.25, 62.5, 125, 250, 500 and 1000 µg. of the plant extracts were tested for the anticancer activity. The anticancer assay was performed on Human laryngeal epithiloma cells (Hep 2) obtained from King Institute of Preventive Medicine, Chennai, India. The cell viability was measured using MTT assay. Controls were maintained throughout the experiment (Untreated wells as cell control and diluent treated wells as diluent control). The assay was performed in triplicates for each of the extracts.

Keywords: *Eugenia jambolana*, Human laryngeal epithiloma cells (Hep2), MTT assay, Anticancer.

INTRODUCTION

Healing potential of plants has been known for thousands of years. Medicinal use of plants and their products was passed down from generation to generation in various parts of the world throughout its history and has significantly contributed to the development of different traditional systems of medicine. According to reports, it is estimated by World health organization that 80% of population of developing countries is dependent on the traditional medicinal plants as a source of drug. Herbal medicines have been used traditionally to treat infections since 3000 BC [1]. Plants produce a whole series of different compounds which are not of particular significance for primary metabolism, but represent an adaptive ability of a plant to adverse abiotic and biotic environmental conditions. They can have a remarkable effect to other plants, microorganisms and animals from their immediate or wider environment. All these organic compounds are defined as biologically active substances, and generally represent secondary metabolites, given the fact that they occur as an intermediate or end products of secondary plant metabolism.

These secondary metabolites, apart from determining unique plant traits, such as: color and scent of flowers and fruit, characteristic flavor of spices, vegetables, they also complete the functioning of plant organism, showing both biological and pharmacological activity of a plant. Therefore, medicinal properties of plants can be attributed to secondary metabolites [2]. Worldwide about 10 million people per year are diagnosed with cancer and more than 6 million die of the disease and over 22 million people in the world are cancer patients. It is a complex disease that is associated with a wide range of effects both at the molecular and cellular levels. Natural protection against cancer has recently been receiving a great deal of attention not only from cancer patients but also from physicians as well [3]. Varalakshmi states that cancer is a life-threatening disease and leads to high rates of mortality worldwide. Investigations for finding new plant based anticancer compounds are imperative and interesting. There are many studies on anticancer herb/plant extracts in cell line models [4].

Eugenia jambolana belongs to the member of family Myrtaceae, commonly known as jambul, black plum [5]. The Eugenia jambolana tree is large sized found in various countries like India, Bangladesh, Nepal, Pakistan, Srilanka, Indonesia, South-East Asia and Eastern Africa [6, 7]. *Eugenia jambolana* had been reported to contain phytochemicals like coumarin, flavanoids, glycosides, phenols, tannins and steroids. The various part of *Eugenia jambolana* had got therapeutic applications. Despite good understanding of the molecular basis of the disease and advances in treatment, globally cancer is still a major cause of death. Estimates are that it will surpass cardiovascular disease as the leading cause of

death, with higher incidences in the developing countries that have minimal resources. Chemotherapy and radiotherapy, the two most commonly used treatment modalities, are associated with untoward side effects. This has necessitated the search for alternatives that are effective, non toxic and easily affordable for patients and traditional medicinal plants are an ideal source [8].

Recent reports suggest that globally, in the year 2008, 12.7 million new cancer cases and 7.6 million cancer deaths occurred. More worryingly, predictions are that by the year 2020, the global incidence of the cancer will increase by threefold, with a disproportionate rise in cases from the developing world countries that have limited resources to tackle the problem [9]. Varalakshmi states that cancer is a life-threatening disease and leads to high rates of mortality worldwide. Investigations for finding new plant based antimicrobial and anticancer compounds are imperative and interesting [10]. There are many studies on anticancer herb/plant extracts in cell line models. Rentala reports that cancer after cardiovascular disease is the second leading cause of death. Worldwide about 10 million people per year are diagnosed with cancer and more than 6 million die of the disease and over 22 million people in the world are cancer patients. It is a complex disease that is associated with a wide range of effects both at the molecular and cellular levels. Natural protection against cancer has recently been receiving a great deal of attention not only from cancer patients but also from physicians as well [3].

Zhao reports that prostate cancer is the third most common cause of death from cancer in men of all ages and is the most common cause of death from cancer in men over age 75. Prostate cancer is rarely found in men younger than 40 [11]. MTT is considered to be a reliable assay to determine the extent of cell viability. The study by Venkatakrishnan reported that the ethanolic extract of *Pleurotus ostreatus* repressed the cell proliferation in a dose-dependent manner in leukemia cells (HL-60 cell line). The methanolic extract of *Piper sarmentosum* possessed anticarcinogenic properties in HepG2 cells [8]. Similarly, Zhang and Poporich estimated, using the MTT assay, the inhibition of cell proliferation in Hep G2 (liver carcinoma) cells by soya saponins, which was found to be dose-dependent [12]. Hajoori *et al*. reported on the anticarcinogenic activity of an ethanolic extract from *Piper sarmentosum* in HepG2 by MTT assay. The IC_{50} value for HepG2 cells was 12.5µg/ml. Treatment with 10, 12 and 14 µg/mL-1 of ethanolic extracts caused typical apoptotic morphological changes in HepG2 cells on staining with acridine orange and ethidium bromide [13]. Lingadurai *et al.* studied the cytotoxic effect of the leaves of *Bichofia javanica* extract on human leukemic cell lines (U937, K562, and HL60), 10 µg/ml methanolic extract of *Bichofia javanica* showed significant cytotoxicity [14].

Very recently Goyal *et al*. have also observed that administration of the Eugenia jambolana extract (25 mg/kg bodyweight/day) was effective in preventing benzo-a-pyrene-induced for stomach carcinogenesis. Eugenia jambolana reduced the tumor incidence, tumor burden and cumulative number of gastric carcinomas [15]. Additionally, recent observations also suggest that ellagitannin, a constituent of *Eugenia jambolana* and its colonic metabolite, urolithin A inhibit signalling crucial in the process of colon carcinogenesis. Urolithin A reduces proliferation of colon cancer cells, induces cell cycle arrest and modulates MAPK signalling [16]. The objectives of the current study are as follows: Screening of plant sample and studying the anticancer effect of the plant extract

MATERIALS AND METHODS

Plant material collection

The seeds of *Eugenia jambolana* were collected from in and around Namakkal district. The seeds of the plant were washed with de-ionized water and shade dried for 3 days. The shade dried seeds were powdered using a mechanical grinder and passed through 40 mesh sieve.

Experimental Design

Plant active components were extracted using the decoction extraction method (Sujatha *et al*., 2014). Acetone, methanol and ethanol were used for the extraction. 100ml of pure ethanol, acetone and methanol were added to 5g portions of the plant powder in sterile conical flasks individually and allowed to boil in a boiling water bath for 48 hours with a water bath set at 40°C. The filtrate was obtained by means of filtering through a Whattman no.1 filter paper. The filtrate was evaporated in a weighed flask in a hot air oven set at 50°C. Extracts were reconstituted by re-dissolving in respective solvents. Sterile extracts obtained were stored separately in labeled, sterile capped bottles, in a refrigerator at 4°C. Different concentration i.e. 8, 15.6, 31.25, 62.5, 125, 250, 500 and 1000 µg. of the plant extracts were tested for the anticancer activity.

Preparation of plates

The cells were maintained in Minimal Essential Media supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml CO_2 at 37 °C.). (1 × 105/well) Cells were plated in 0.2 ml of medium/well in 96-well plates. The cells were then incubated at 5 % CO_2 incubator for 72 hours. Then, various concentrations of the samples in 0.1% DMSO were added for 24hrs at 5 % CO_2 incubator. After removal of the sample solution and washing with phosphate-buffered saline (pH 7.4), 20µl/well (5mg/ml) of 0.5% 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl--tetrazolium bromide (MTT) in phosphate- buffered saline solution was added. After 4hrs incubation, 1ml of DMSO was added. Viable cells were determined by the absorbance at 540nm

Grouping of plates

The assay was performed in triplicates for each of the extracts. Cells (1 × 105/well) were plated in 0.2 ml of medium/well in 96-well plates.

Group I: 8, 15.6, 31.25, 62.5, 125, 250, 500 and 1000 µg. of methanolic plant extract in 0.1% DMSO was treated with the cells for 24hrs at 5 % CO2 incubator. After removal of the sample solution and washing with phosphate-buffered saline (pH 7.4), 20µl/well (5mg/ml) of 0.5% 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl--tetrazolium bromide (MTT) in phosphate- buffered saline solution was added. After 4hrs incubation, 1ml of DMSO was added.

Group II: 8, 15.6, 31.25, 62.5, 125, 250, 500 and 1000 µg. of ethanolic plant extract in 0.1% DMSO was treated with the cells for 24hrs at 5 % CO_2 incubator. After removal of the sample solution and washing with phosphate-buffered saline (pH 7.4), 20µl/well (5mg/ml) of 0.5% 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl--tetrazolium bromide (MTT) in phosphate- buffered saline solution was added. After 4hrs incubation, 1ml of DMSO was added.

Group III: 8, 15.6, 31.25, 62.5, 125, 250, 500 and 1000 µg. of acetone plant extract in 0.1% DMSO was treated with the cells for 24hrs at 5 % CO_2 incubator. After removal of the sample solution and washing with phosphate-buffered saline (pH 7.4), 20µl/well (5mg/ml) of 0.5% 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl--tetrazolium bromide (MTT) in phosphate- buffered saline solution was added. After 4hrs incubation, 1ml of DMSO was added.

Reagents and solvents

The following reagents and solvents were used in this study MEM, Fetal bovine serum (FBS), Trypsin, methylthiazolyl diphenyl-tetrazolium bromide (MTT), and HEP2 Dimethyl sulfoxide (DMSO), acetone, methanol and ethanol.

All chemical reagents were purchased from Hi media & Sigma Aldrich Mumbai, India.

Antitumor assay

The antitumor assay was performed on Human laryngeal epithiloma cells (Hep 2) obtained from King Institute of Preventive Medicine, Chennai, India with non-toxic dose of the plant extract and its dilutions. The cell viability was measured using MTT assay. Controls were maintained throughout the experiment (Untreated wells as cell control and diluent treated wells as diluent control). The assay was performed in triplicates for each of the extracts. Cells (1×10^5/well) were plated in 0.2 ml of medium/well in 96-well plates. Incubate at 5 % CO_2 incubator for 72 hours. Then, add various concentrations of the samples in 0.1% DMSO for 24hrs at 5 % CO_2 incubator. After removal of the sample solution and washing with phosphate-buffered saline (pH 7.4), 20µl/well (5mg/ml) of 0.5% 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl--tetrazolium bromide (MTT) in phosphate-buffered saline solution was added. After 4hrs incubation, 1ml of DMSO was added. Viable cells were determined by the absorbance at 540nm. Measurements were performed and the concentration required for a 50% inhibition of viability (IC_{50}) was determined. The effect of the samples on the proliferation of Hep2 cells was expressed as the % cell viability. The mean of the cell viability values was compared to the control to determine the effect of the extract on cells. The minimum concentration of plant extract that was non-toxic to Vero cells but toxic to Hep2 cells was recorded as the effective drug concentration.

RESULTS

Anticancer activities of plant extract

The methanol extract of *Eugenia jambolana* was reported to possess antiproliferative effects against human tumor cell lines. The results showed the inhibition of *in vitro* proliferation of human tumor cell lines against Hep 2 cell lines. This parameter was carryout with Methyl thiazolyl tetrazolium (MTT) assay. The cells were maintained in Minimal Essential Media supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml CO_2 at 37 °C.). (1×10^5/well) Cells were plated in 0.2 ml of medium/well in 96-well plates. The cells were then incubated at 5 % CO_2 incubator for 72 hours. Then, various concentrations of the samples in 0.1% DMSO were added for 24hrs at 5 % CO_2 incubator. After removal of the sample solution and washing with phosphate-buffered saline (pH 7.4), 20µl/well (5mg/ml) of 0.5% 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl--tetrazolium bromide (MTT) in phosphate- buffered saline solution was added. After 4hrs incubation, 1ml of DMSO was added. Viable cells were determined by the absorbance at 540nm

Different concentration i.e. 8, 15.6, 31.25, 62.5, 125, 250, 500 and 1000 µg (Fig. 2- Fig. 8) of the plant extracts were tested for the anticancer activity and all of these concentrations exhibited anticancer activity, the fifty percentage of cell death occurred when using 125 µg of plant extract and highest cell death occurred at 1000 µg (Fig. 8).

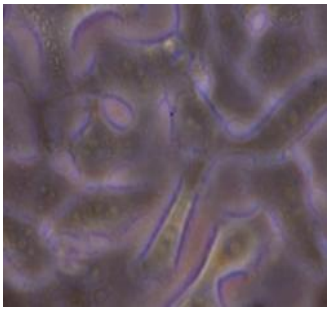

Figure 1: Hep2 cells Control

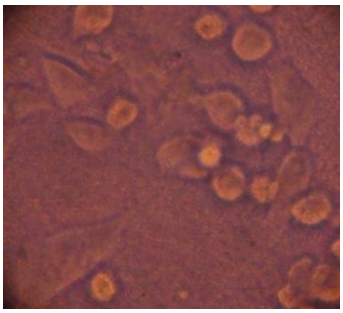

Figure 2: 8 µg/ml of Methanolic *E. jambolana* against Hep2 cells

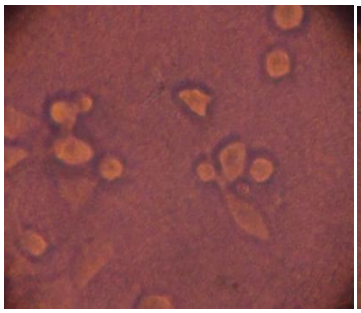

Figure 3: 15.6 µg/ml of Methanolic *E. jambolana* against Hep2 cells

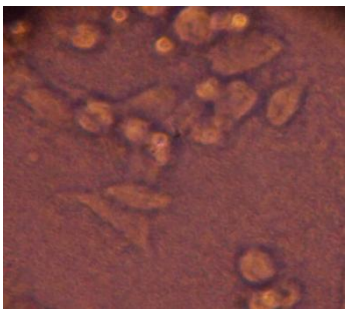

Figure 4: 31.25 µg/ml of Methanolic *E. jambolana* against Hep2 cells

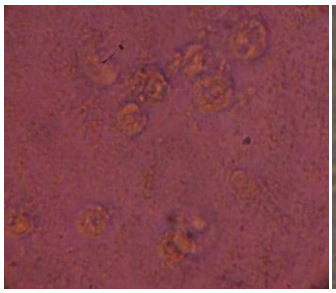

Figure 5: 125 µg/ml of Methanolic *E. jambolana* against Hep2 cells

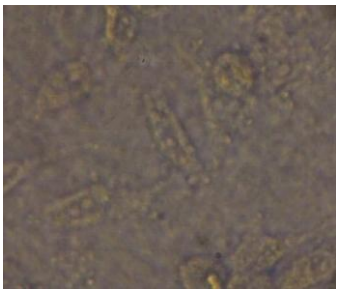

Figure 6: 250 µg/ml of Methanolic *E. jambolana* against Hep2 cells

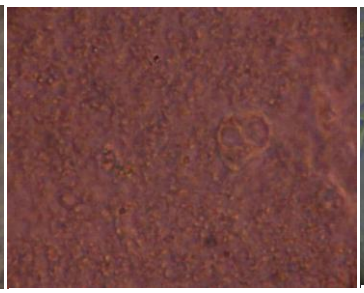

Figure 7: 500 µg/ml of Methanolic *E. jambolana* against Hep2 cells

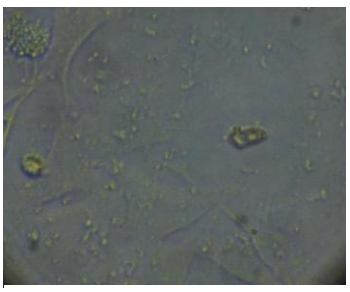

Figure 8: 1000 µg/ml of Methanolic *E. jambolana* against Hep2 cells

DISCUSSION

The results of anticancer activity of *Eugenia jambolana* extracts against Hep2 cell line was found to possess antiproliferative effects against human tumor cell lines and they showed the inhibition of in-vitro proliferation of human tumor cell lines against Hep 2 cell lines. The results of the study also showed that the induction of apoptosis by methanolic extract of *Eugenia jambolana* seeds in Human laryngeal epithiloma cells (Hep 2) indicates its anticancer activity. The study by Venkatakrishnan *et al*., (2010) reported that the ethanolic extract of *Pleurotus ostreatus* repressed the cell proliferation in a dose-dependent manner in leukemia cells (HL-60 cell line). The methanolic extract of *Piper sarmentosum* possessed anticarcinogenic properties in HepG2 cells.

The need to identify new compounds with anticancer properties that are more effective and less toxic than conventional drugs has motivated research on natural products isolated from plant species. These substances predominantly consist of alkaloids, terpenes, flavonoids, phenolics; have been identified in plant species with documented anticancer activity.

The study of the anticancer activity of *Eugenia jambolana* against human tumor cells was carried out with the methanol extract of *E. jambolana* and they were reported to possess ant proliferative effects against human tumor cell lines. The results showed the inhibition of in vitro proliferation of human tumor cell lines against Hep 2 cell lines. This parameter was carried out with Methyl thiazolyl tetrazolium (MTT) assay. Similarly, Goyal reported the anticancer effect of hydroalcoholic extract of bael leaves in the animal model of Ehrlich ascites carcinoma and proposed that induction of apoptosis may be due the presence of skimmianine in extract [3].

Studies in the recent past indicate the potential of *Eugenia jambolana* in cancer treatment and prevention. However, gaps in the studies conducted are apparent which need to be bridged in order to exploit the full medicinal potential of *Eugenia jambolana*.

Our results were congruent with the results of others.

CONCLUSION

Further studies are needed to find out the active compounds of this plant. It is possible to find the better therapies for many cancerous diseases from the plants. The *Eugenia jambolana* would be helpful in the treatment of cancerous diseases.

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