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Evaluation of *Cichorium* Extract for the Growth Supporting Property in Rat Hepatocyte Primary Culture

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Abstract: *Cichorium intybus* a member of family Asteraceae, has been traditionally used as liver protectant and claimed as renal protective and antiinflammatory. It contains many essential lipids, vitamins and a variety of sugar. For routine primary culture and for optimum growth in several secondary cultures of animal cell serum is frequently used as a source of nutrients. Isolation of serum from fetal bovine being barbarous process as well as its use in culture media is also associated with several drawbacks. Available animal free media are having their own shortcomings. The objective of this study is to evaluate the plant's methanolic extract for *in vitro* cell growth supporting activity on rat hepatocyte primary culture. Plant extract was evaluated at five different concentrations in 96 well plates. At highest concentration of plant extract (50 μg mL^{-1}) cell viability was found to be 66.83%, whereas, at lowest concentration (10 μg mL^{-1}) cell viability was 24.12%. Plant extract was also found to overcome toxic effect of DMSO. Hence this work can be used for further characterization of the particular compound/s support the growth of rat primary hepatocyte culture as well as if cells of other origin and types. It will help in the formulation of novel animal cell culture medium based on plant origin/components, at least for the rat hepatocyte primary culture.

Key words: *Cichorium intybus*, serum, serum free media, methanolic extract, hepatocyte primary culture

INTRODUCTION

Although animal cell culture requires the medium in which they grow be supplemented with serum, the preferred source of serum in cell culture is fetal bovine serum (FBS) (Freshney, 2000) which is extracted via cardiac puncture of calves. This has several drawbacks such as physiological variability, shelf life and consistency, quality control, specificity, availability, downstream processing, contamination, growth inhibitors, standardization and cost (Freshney, 2000) and has raised many ethical issues. In recent years, the approach has been made to seek the alternatives of serum and varieties of formulations have been patented. They have been broadly classified as serum free media, protein free media and chemically defined media. The intriguing fact about certain animal origin free media is the presence of certain plant hydrolysates (FOA, 2003) and there has also been a report on *in vitro* growth supporting activity of aqueous extract of certain plants on *Lieshmania donovani*, a protozoan parasite (Suman, 2004).

Cichorium intybus, a member of family Asteraceae, seen as weeds at waysides, field borders, waste land (Ward, 1936) has been long claimed by ethonopharmacologist, as potent anti-inflammatory, renalprotective drug. It has also been used as stimulant. Traditionally, it has been used for hepatic conditions and liver rejuvenation (Nadkarni, 1994) and has shown protective effects in mice with high levels of liver damaging enzymes (Gilani *et al*., 1998). Besides, this has been a primary component of variety of herbal formulations, especially in cough relief. The roots of *C. intybus*, are roasted, ground and mixed with coffee for the benefit of drinkers of that beverage (Ward, 1936). The non-volatile oils of *C. intybus* are essential fatty acids for mammals, which could be the pathway intermediate and precursors for certain lipid hormones (Khan, 1999; Nelson and Cox, 2003). Its roots contain nucleotide sugar-Uridin-5-diphosphoglucose, series of glucofructosans between sucrose and inulin beside glucose and fructose. It also contains vitamins like, vitamin A, vitamin C, Riboflavin, Thiamine and Niacin (Prajapati *et al*., 2003).

Our present study focuses on the use of methanolic extract of *C. intybus* as a potent supplement to besal medium. The objective of the study is to evaluate methanolic extract of *C. intybus*, *in vitro*, in five different concentrations against the primary rat hepatocytes.

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MATERIALS AND METHODS

Plant material: Plants were selected on the basis of ethnopharmacology. Young plants were purchased from local market of Tiruchirappalli in February 2006. It was identified by Dr. Shahjahan A., Department of Botany Jamal Mohamed College, Tiruchirappalli, where a voucher specimen is deposited at department of Botany herbarium.

Preparation of crude plant extract: Whole plants were shade dried ground and soaked into methanol for extraction. The quantity of methanol was taken 10 times the quantity of plant material. Extraction was performed three times and each extraction was done for 24 h (Hoet *et al*., 2004; Maes *et al*., 2004). Methanolic filtrate was then evaporated to dryness at 30°C under reduced pressure. Extract was stored at 4°C before use (Bonjar and Nik, 2004).

Preparation of drug: A stock of plant extract was made with concentration of 1 mg mL^{-1} of DMSO and sterilized by autoclaving at 121°C and 15 lb for 15 min (Jarvis and Hungate, 1968). Then five concentrations of test drug, (50, 40, 30, 20 and 10 μg mL^{-1}) were prepared by diluting stock with RPMI 1640. To study toxic effect of DMSO, its five corresponding concentrations (50, 40, 30, 20 and 10 μL mL^{-1}) were also prepared by diluting it with RPMI 1640. RPMI 1640 supplemented with 10% FBS and RPMI 1640 alone used as Controls.

Animals: White albino rats were gifted by Laboratory of Department of Animal Science, Bharthidasan University, Tiruchirappalli. Animals were maintained in an environmentally controlled room with 12 h light/dark cycle and allowed free access to food and water (Bajt *et al*., 2004).

Hepatocytes preparation and primary culture: Primary hepatocytes were isolated from rat as described by Bajt *et al*. (2004) but with some modifications. Briefly, rat anesthetized with chloroform and liver was removed, washed three times with Phosphate-buffered saline (PBS, pH 7.4) and surface sterilized with 70% ethanol, chopped and gently minced and passed through syringe (Cho *et al*., 2001). Cell viability, determined by Trypan blue exclusion (James and Warburton, 1999; Laduca *et al*., 1989; Kuo *et al*., 2002), was generally>85% and cell purity was confirmed with light microscopy (Johnson *et al*., 1998). Purity was>95% for hepatocytes. Cells were plated on 96-well plates (Tarson) with the density of 2.5×10^5 cells/well in RPMI 1640 (supplemented with 20 mM L-glutamine, 16.5 mM $NaHCO_3$) containing 10% heat inactivated fetal bovine serum (Sigma) and cultured at 37°C with 5% CO_2 in humidified atmosphere. After incubation of 24 h, cultures were washed with PBS. RPMI 1640, RPMI 1640 (10% FBS) and media containing various concentrations of test drug and DMSO were added. Each combination and concentration was taken in triplet. Plate was incubated in CO_2 incubator at 37°C with 5% CO_2 for next 24 h (Bajt *et al*., 2004; Sest *et al*., 2005).

Determination of cell viability by neutral red assay (Babich and Borenfreund, 1999): Cell viability was determined by Neutral Red Assay. Neutral Red (Himedia) (4 mg mL^{-1} stock solution) was dissolved in RPMI-1640. Working solution was made by diluting stock (1:100), incubated overnight at 37°C and centrifuged before use. Treatment drugs and media were replaced by 200 μL of working solution and incubated for 3 h. At the end of the incubation period, the Neutral Red solution was removed and the cells were washed twice with PBS. And dye crystals were dissolved by adding 200 μL reaction stopping solution (1% acetic acid and 50% ethanol in distilled water). Absorbance was measured Spectrophotometerically (Bajt *et al*., 2004) at 540 nm and the results were expressed as a percentage of the absorbance of the samples in comparison to control.

Statistical analysis: Data were analyzed statistically by Student's t-test and p-values were determined for each.

RESULTS

The cell viability was 66.83% at highest concentration (50 μg mL^{-1}) and 24.12% at lowest concentration (10 μg mL^{-1}) of crude extract of *C. intybus* (Fig. 2). compared to cell viability in RPMI 1640 supplemented with 10% FBS. DMSO was completely growth inhibitory up to 40 μL mL^{-1} (4%) and onward concentration and it was allowing the viability of hepatocytes up to 18.34%

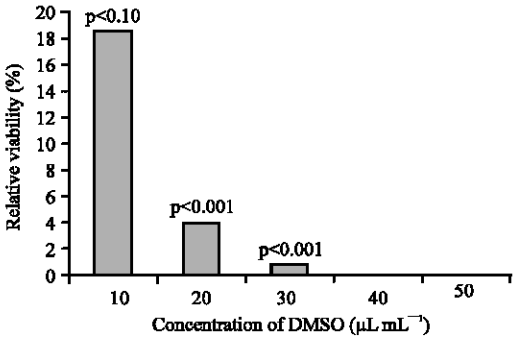

Fig. 1: Relative viability (%) of hepatocytes after 24 h incubation in presence of DMSO alone in RPMI 1640

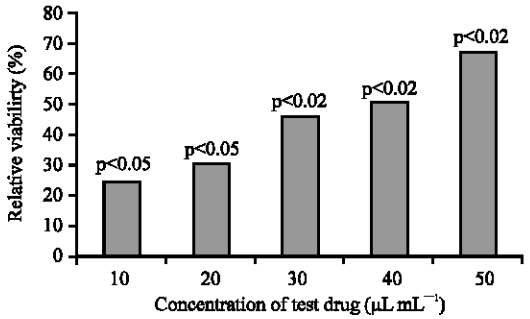

Fig. 2: Relative viability of hepatocytes after 24 h incubation in presence of Drug (DMSO + plant extract) in RPMI 1640

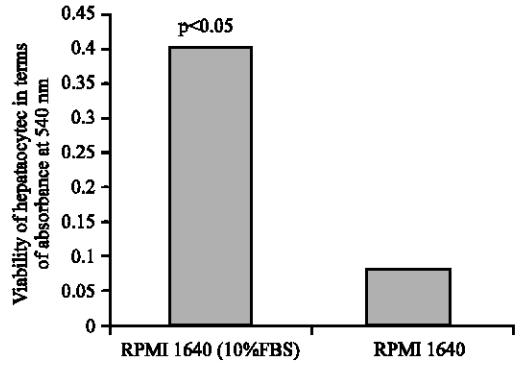

Fig. 3: Viability of hepatocytes in RPMI 1640 with 10% FBS and RPMI 1640 alone after 24 h incubation in terms of absorbance at 540 nm

at lowest concentration 10 µL mL^{-1} (1%) in absence of extract or FBS (Fig. 1). Viability of hepatocytes in RPMI 1640 alone was 20.6%. The cell viability in terms of absorbance at 540 nm in RPMI 1640 (10% FBS) was 0.398 and in RPMI 1640 was 0.082 (Fig. 3).

DISCUSSION

According to Tiuman *et al.* (2005) DMSO at lower concentration i.e., 0.1 to 0.05% is found to be non toxic. The results show that DMSO is toxic to animal cells at higher concentrations and its toxicity decreases only in low concentration in medium. Plant extract could not only overcome the toxic effect of DMSO even at higher concentrations (50 and 40 µL mL^{-1}) but also support the growth of hepatocyte primary culture. Reports suggest that the candidate plant has many types of lipids which are the precursors of many growth factors (Nelson and Cox, 2003; Prajapati *et al.*, 2003), as well as various essential vitamins and sugars. Use of serum in a medium has a number of disadvantages and much tedious process is involved in the production and storage of serum (Freshney, 2000). The present study may be used to explore and detect whether the same plant extract has the potential to support the growth of animal cells of different origins and type and the fraction that supports the growth of animal cells. May be it allows formulation of plant origin/component based animal origin free medium, at least for primary hepatocyte culture. This study may also form basis for promotion of the use of plants/components and reduce use of serum in animal cell culture works thereby solves the ethical issues and the problems associated with the use of serum.

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