Journal of Applied Biological Sciences 9 (2): 19-22, 2015
ISSN: 1307-1130, E-ISSN: 2146-0108

The Effect of Standardized Extract of Echinacea Purpurea on Cytotoxicity and Proliferation of Rat Splenocytes

Razieh REZAEI[1*] Ali MOSTAFAIE[2,3] Ali GORGIN KARAJI[2] Kamran MANSOURI[3]
[1]Students Research Committee, Kermanshah University of Medical Sciences, Kermanshah, Iran
[2]Department of Immunology, School of Medicine, Kermanshah University of Medical Sciences, Kermanshah, Iran
[3]Medical Biology Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran

*Corresponding author:
Email: rezaei.razieh88@yahoo.com

Received: April 23, 2015
Accepted: June 02, 2015

Abstract

Objective: The main purpose of this research is to study the effect of standardized extract of Echinacea purpurea on viability and proliferation of rat splenocytes.

Materials and methods: The cytotoxic effect of various concentration of standardized extract of Echinacea purpurea (25, 50, 100, 200, 400, 800, 1600, 3200 µg/ml) on rat splenocytes were investigated by LDH method. Cells proliferation was performed at the aforementioned doses by MTT method at 24 to 72 hour. **Results:** Splenocytes treated with standardized extract showed no cytotoxicity in compare to control group; thus, the viability percentage between treated cells with various concentration of the extract and control group did not significant difference ($p>0.05$). Splenocytes proliferation under standardized extract was significantly ($p<0.05$) increased at concentrations of 800, 1600 and 3200 µg/ml in 24h and at 400, 800, 1600, and 3200 µg/ml concentrations in 72h compare to control group. This increase in proliferation depends on dosage and time. **Conclusion:** It is demonstrated that standardized extract of Echinacea purpurea has the proliferation effect without cytotoxicity effect on splenocytes, and it has immune enhancing feature.

Keyword: Echinacea purpurea, Cytotoxicity, Proliferation, Splenocytes, Rat.

INTRODUCTION

Each year millions of people, around the world, infected with common colds and flu. They lead to respiratory complications and significant fatality in children and immunocompromised individuals [1]. Considering the side effects of common and synthetic medications, using alternative combinations of herbal sources has a relatively long history in preventing and treatment of human diseases with less side effects and lower manufacturing costs. Applying phytomedications (medications derived from plants) as alternative remedies for traditional western medicine is sharply surged [2].

Echinacea purpurea, a member of the Asteraceae family (compositae), is the most popular traditional medicinal plant utilized for many years in the northern areas of America. This medicinal plant is applied for treatment of various symptoms of colds and flu, bronchitis, upper respiratory tract infections and some inflammations [3,4]. The Echinacea purpurea plant in different forms of dried root, capsules, tables, and liquid is used for producing commercial products immune boosting. Echinacea purpurea plant contains groups of essential ingredients including flavonoids, polysaccharides, derivatives of caffeic acid, essence, alkyl amides, poly acetylene and different chemicals [5]. In spite of studies conducted on Echinacea species, there exists still absence of adequate confidence about which main components of this plant can contribute in immunomodulatory performance. However, some studies concentrated on alkyl amides, it appears that a combination of essential ingredients influences Echinacea activities [6,7].

The isolated polysaccharides of Echinacea purpurea stimulate cytotoxic activity of macrophages and secretion of TNF-α, IL-6 and IL-1 from human and mice macrophages in vitro [8]. Moreover, these polysaccharides also enhance phagocytes proliferation in splenocytes and bone marrow tissue [9]. Immune-modulatory, antioxidant, antiviral, antibacterial effects of Echinacea purpurea have been seen in vitro and in vivo studies [10,11]. Echinacea purpurea extract stimulates proliferation of peripheral blood mononuclear cells and significantly increases numbers of circulating lymphocytes, monocytes, as well as natural-killer (NK) cells [12]. Although, the extract of Echinacea purpurea as immunomodulator is the most popular herbal medicine over the world; studies conducted on modulating effects of these products on immune system are not adequate.

The present study was investigated the effects of different concentration of standardized commercial extract of Echinacea purpurea on proliferation of rat splenocytes and to evaluate cytotoxicity of the standardized extract of Echinacea purpurea.

MATERIALS AND METHODS

Reagents

RPMI-1640 (GIBCO), concanavalin A (ConA; Sigma), fetal calf serum (FCS), LDH activity was measured using cytotoxicity detection kit LDH (Lactate dehydrogenase) cytotoxicity assay kit (Sigma Chemical Co, USA), DMSO (Sigma Chemical Co., USA), Teriton X100 (Merck), MTT (Sigma) and standardized extract of Echinacea purpurea obtained from North of Iran.

Animal

This study used male Wistar rats purchased from Institute Pasteur of Iran. All rats were 8–12 weeks of age at the beginning of experiments. Animals were housed in controlled conditions (20–25 °C, 12 h light cycle) with free access to diet and water. Animals were allowed to acclimate to laboratory conditions for at least 2 weeks before experimental manipulation.

Cell culture

Rat spleen removed in sterilized condition and spleen cells were isolated. Splenocytes suspended in RPMI (4ml) and centrifuged at 1100 g for 10 min. The supernatants to be discarded, 0.05 ml ACK buffer (EDTA, KHCO3, NH4CL, D.W) was added to the precipitate and incubated for 5 min in room temperature. Then, added culture medium contained RPMI+2% FCS for LDH assay and RPMI+5% FCS for MTT assay. Cells counted by neobar lam, 5×106 cells per ml along with 5µg/ml conA poured in 96 well plate and were treated with 25, 50, 100, 200, 400, 800, 1600, 3200 µg/ml concentrations of standardized extract of Echinacea purpurea. The plate was cultured in the incubator at certain times for testing at 37 °C with 5% CO2.

MTT proliferation assays

The MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay is widely employed for probing cytotoxicity, cell viability and proliferation of living cells. Tetrazolium bromide penetrates living cell, enters into mitochondria and reduces under reductase enzyme converting to formazan which is insoluble in water. The added DMSO causes formozan turn into solution form in purple color, and its absorption read at the wavelength 570 nm. The proliferation effect of the standardized extract of Echinacea purpurea was also determined using MTT assay as illustrated by Denizot with some modifications [13]. Splenocytes were treated with 25-3200 µg/ml concentrations standardized extract of Echinacea purpurea for 24 to 72h. The solution with a concentration of 5 mg/ml in PBS 1X buffer produced from MTT powder, and 20 µl of this mixture added to each well containing cells and 200 µl supernatants. The plate incubated for 4h in a 37 °C incubator with 5% CO2 and covered by foil. After this period, the supernatants removed out of wells and crystals were maintained. Then, 100 µl DMSO added to each well and placed on shaker for 10 min. The absorption read at 570 nm wavelength and 630 nm reference wavelength by Elisa plate reader.

LDH cytotoxicity assay

Lactate dehydrogenase enzyme is a stable cytoplasmic enzyme found in all living cells immediately released into cell culture supernatant due to plasma membrane damage. The activity of LDH can be determined through following enzymatic reaction:

$$NADH + Pyruvate \xleftrightarrow{LDH} NAD^{+} + Lactate$$

Following 24 hours of treating the cells with concentrations of 25 to 3200 µg/ml of standardized extract of Echinacea purpurea, the plate centrifuged and 100 µl of the cells supernatants transferred to a new 96-well plate. Then, 250 µl of catalyzed solution mixed with 11.25 ml of dye solution to prepare reaction mixture; 100 µl of it added to each well and incubated at room temperature for 30 min away from light. Added 20 µl HCL (1N) to each well so that the reaction stopped; samples absorption read at the wavelength of 490 nm using Elisa plate reader and the wavelength of 630 nm as reference. Two controls taken for this experiment including:

Low control: The wells receiving no treatment and medication, containing cell and RPMI+2% FCS.

High control: The wells receiving no treatment, containing cell and the whole culture medium with 40 µl of Triton X-100 solution 1% that added for cells complete lysis.

Cytotoxicity and viability percentages calculated according to the following formula:

Cytotoxicity (%) = [OD Test – OD Low / OD High – OD Low] ×100

Viability (%) = 100 – Cytotoxicity (%)

Statistical analysis

Statistical analysis was carried out using the students unpaired t test and one-way ANOVA. Values of $p<0.05$ were considered significant and all measurements were presented statically expressed as mean ± SD. All experiments were performed in triplicate unless otherwise noted.

RESULTS

Cytotoxic effect of Echinacea purpurea extract

Figure 1 shows the effect of different doses of the standardized extract on viability percent of rat splenocytes. According to the obtained results, in 24h after incubation of splenocytes in the extract vicinity did not differ significantly ($p>0.05$) between viability percentage of the cells treated with extracted and control cells (no treatment). Moreover, no cytotoxic effect seen in various concentrations of the extract on rat splenocytes following 24 hours in compare to control group.

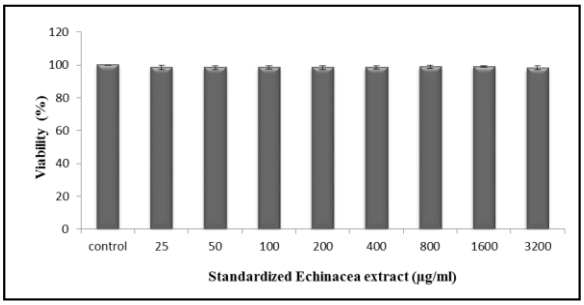

Figure 1. Effect of different doses of standardized extract of Echinacea purpurea on viability of rat spleen lymphocyte cells.

Proliferation effect of Echinacea purpurea extract

In order to study the proliferation effect of standardized extract of Echinacea purpurea on splenocytes through MTT method, wells absorptions determined after added MTT reagent at 24 and 72 h following incubation, at the wavelength 570 nm. Results showed that proliferation at 24 hours after treatment in concentration of 800, 1600, and 3200 µg/ml were significantly ($p<0.05$) higher as compared to the control group (Figure 2). Further, following 72 hours of treatment, proliferation in concentration of 400, 800, 1600, and 3200 µg/ml also showed significant increase ($p<0.05$) compared with the control group (Figure 3). Proliferation of the cells under treatment raises over time in 72h in compare to 24h, so that the proliferation effect seen at the lower dose (400 µg/ml) in 72h.

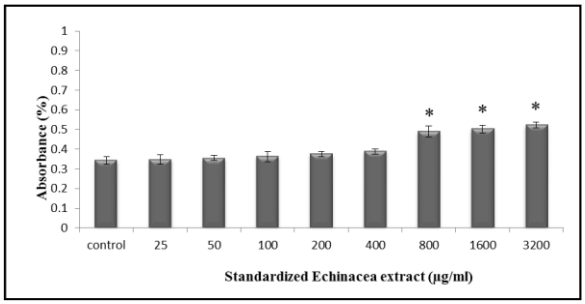

Figure 2. Effect of different doses of standardized extract of Echinacea purpurea on proliferation of rat spleen lymphocyte cells at 24h, (*p<0.05 V.S control).

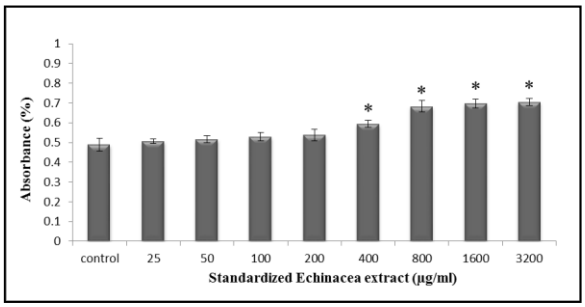

Figure 3. Effect of different doses of standardized extract of Echinacea purpurea on proliferation of rat spleen lymphocyte cells at 72h, (*p<0.05 V.S control).

DISCUSSION

This research applied on cytotoxic effects of a commercially available standardized extract of Echinacea purpurea on rat splenocytes, and results showed no cytotoxic effects (Figure 1). The obtained results are in agreement to previous research that according to MTT assays, trypan blue staining and microscopic examination was not observed cytotoxic effects on standardized Echinacea extract (Echinaforce) [14]. The different preparations for similar Echinacea species have demonstrated different effects in vitro studies [15,16]. Providing standard herbal medication is arduous and factors such as biological and therapeutic effects are important. Producing herbal products with high quality requires special attention to harvest season, harvest region, selecting proper plant, extraction process and purification [17]. There is no consensus on standard method applicable for extraction of Echinacea. Therefore, to use this plant as medicine requires more studies on selecting suitable plant species and purification method.

The results of various concentration of standardized extract of Echinacea purpurea on the proliferation of rat splenocytes showed that increased time and extract concentration improves the proliferation of the treated cells (Figures 2 and 3). In consistent with our results, Hashemi and coworkers studied the effect of Echinacea extract on spleen immune cells and reported that the viability and proliferation percentage of spleen cells increased in vitro and in vivo [18]. However, Parnham [19] demonstrated that high concentration of Echinacea purpurea extract has inhibitory effect on the proliferation of T lymphocyte cells in vitro. Echinacea spp. have shown contradictory effects in some cases. Therefore, it requires further studying to analyze standardized effect and the quality of commercial resources of Echinacea extracts. The commercial powder of Echinacea root in tumor bearing mice led to significant increase in stimulation NK cells production in spleen and bone marrow tissue [20]. Other studies revealed that E. angustifolia and E. purpurea may decrease the effectiveness of the chemotherapy or anticancer drugs and even Echinacea may have proliferative effect on cancer cells [21,22]. This difference in results may attribute to discrepancy in preparation steps and consequently the difference in extracts quality and effective compounds.

CONCLUSION

According to the findings, it concluded that the present standardized extract of Echinacea purpurea such as other Echinacea products possess immune stimulating properties. In addition, it had no toxic effect on splenocytes proliferation and increased in vitro cells proliferation. Regarding the obtained results, which are consistent with previous results, it appears that effective compounds of this plant are the main cause of difference in results of various studies. Therefore, it would be useful to study the role of these compounds in efficiency of this extract in future studies.

Acknowledgments

The authors greatly acknowledge the Research Council of Kermanshah University of Medical Sciences for financial support (Grant No. 94095). This article was performed in partial fulfillment of the requirements for the degree of immunology by Razieh Rezaei in School of Medicine, Kermanshah University of Medical Sciences, Kermanshah, Iran.

REFERENCES

[1] Augustin A, Shahum A, Kalvsky E, Liskova A, Kisac P, Krcmery V. Colonization of the respiratory tract by drug-resistant bacteria in HIV-infected children and prior exposure to antimicrobials. Med Sci Monit 2008; 14:19-22.

[2] Gilroy CM, Steiner JF, Byers T, Shapiro H, Georgian W. Echinacea and truth in labeling. Arch Intern Med 2003; 163:699-704.

[3] Barrett B. Medicinal properties of Echinacea: a critical review. Phytomedicine 2003; 10:66-86.

[4] Barnes J, Anderson LA, Gibbons S, Phillipson JD. Echinacea species (Echinacea angustifolia (DC.) Hell., Echinacea pallida (Nutt.) Nutt., Echinacea purpurea (L.) Moench): a review of their chemistry, pharmacology and clinical properties. J Pharm Pharmacol 2005; 57:929-954.

[5] Cozzolino R, Malvagna P, Spina E, Giori A, Fuzzati N, Anelli A, Garozzo D, Impallomeni G . Structural analysis of the polysaccharides from Echinacea angustifolia radix. Carbohyd Polym 2006; 65:263-272.

[6] Zheng Y, Dixon M, Saxena PK. Growing environment and nutrient availability affect the content of some phenolic compounds in Echinacea purpurea and Echinacea angustifolia. Planta Med. 2006; 72:1407-1414.

[7] Zhai Z, Solco A, Wu L, Wurtele ES, Kohut ML, Murphy PA, Cunnick JE. Echinacea increases arginase activity and has anti-inflammatory properties in RAW 264.7 macrophage cells, indicative of alternative macrophage activation. J Ethnopharmacol 2009; 122:76-85.

[8] Burger RA, Torres AR, Warren RP, Caldwell VD, Hughes BG. Echinacea induced cytokine production by human macrophages. Int J Immunopharmacol 1997; 19:371-379.

[9] Roesler J, Steinmuller C, Kiderlen A, Emmendorffer A, Wagner H, Lohmann-Matthes ML. Application of purified polysaccharides from cell cultures of the plant *Echinacea purpurea* to mice mediates protection against systemic infections with Listeria monocytogenes and Candida albicans. Int J Immunopharmacol 1991; 13:27-37.

[10] Lee TT, Huang CC, Shieh XH, Chen CL, Chen LJ, Yu B. Flavonoid, phenol and polysaccharide contents of Echinacea purpurea L. and its immunostimulant capacity in vitro. IJESD 2010; 1:5-9.

[11] Hudson JB. Applications of the phytomedicine Echinacea purpurea (Purple Coneflower) in infectious diseases. J. Biom. Biotechnol 2012:1-16.

[12] Cundell DR, Matrone MA, Ratajczak P, Pierce JD. The effect of aerial parts of Echinacea on the circulating white cell levels and selected immune functions of the aging male Sprague–Dawley rat. Int Immunopharmacol 2003; 3: 1041-1048.

[13] Denizot F, Lang R. Rapid colorimetric assay for cell growh and survival: modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol Methods 1986; 89:271-277.

[14] Sharma M, Anderson SA, Schoop R, Hudson JB. Induction of multiple pro-inflammatory cytokines by respiratory viruses and reversal by standardized Echinacea, a potent antiviral herbal extract. Antiviral Res 2009; 83:165-170.

[15] Rininger JA, Kickner S, Chigurupati P, McLean A, Franck Z. Immunopharmacological activity of Echinacea preparations following simulated digestion on murine macrophages and human peripheral blood mononuclear cells. J Leukoc Biol 2000; 68:503-510.

[16] Gertsch J, Schoop R, Kuenzle U, Suter A. Echinacea alkylamides modulate TNF-α gene expression via cannabinoid receptor CB2 and multiple signal transduction pathways. FEBS lett 2004; 577:563-569.

[17] Kokate CK, Purohit AP**,** Gokhale SB. "Analytical pharmacognosy", Pharmacognosy, 30th edition, Feb. 2005, pp.1-99.

[18] Hashemi SM, Soudi S, Ghaemi A, Soleimanjahi H, Hassan ZM. Study the Effect of Echinacea purpurea extract on cellular delayed type hypersensitivity and splenocyte proliferation in BALB/c mice. Yakhteh 2008; 9:254-261.

[19] Parnham MJ. Benefit-risk assessment of the squeezed sap of the purple coneflower (Echinacea purpurea) for long-term oral immunostimulation. Phytomedicine 1996; 3:95-102.

[20] Brousseau M, Miller SC. The effects of Echinacea root extract in plasmacytoma-bearing mice: Enhancement of non-specific immunity. J Herbs Spices Med Plants 2008; 13:11-23.

[21] Huntimer ED, Halaweish FT, Chase CC. Proliferative activity of Echinacea angustifolia root extracts on cancer cells: interference with doxorubicin cytotoxicity. Chem Biodivers 2006; 3:695-703.

[22] Cichello SA, Yao Q, He XQ. Proliferative activity of a blend of Echinacea angustifolia and Echinacea purpurea root extracts in human vein epithelial, HeLa, and QBC-939 cell lines, but not in Beas-2b cell lines. J Tradit Complement Med 2015. (In Press)