

Original Article

The Effect of *Echinops lasiolepis* Extracts, Native Plant of Yazd Province, on Peripheral Blood Mononuclear Cell Proliferation and IFN- γ Secretion

Maryam Asadi¹ M.S., Hossein Hadinedoushan^{1,2*} Ph.D., Seyed Ali Mirghanizadeh¹ M.S., Alireza Karimollah³ Ph.D., Fatemehsadat Dashti¹ M.S., Saeid Malek-hosseini⁴ M.S.

¹ Department of Immunology, Faculty of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

² Department of Immunology, Reproductive Immunology Research Center, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

³ Department of Pharmacology, Faculty of Pharmacy, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

⁴ Department of Immunology, Faculty of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran.

ABSTRACT

Article history

Received 2 Nov 2014
 Accepted 30 Nov 2014
 Available online 17 Dec 2014

Keywords

Cell proliferation
Echinops lasiolepis
 IFN- γ
 Phytohaemagglutinin

Background and Aims: The present study was performed to investigate the effect of *Echinops lasiolepis* extract on peripheral blood mononuclear cells (PBMCs) proliferation and interferon (IFN)- γ secretion.

Materials and Methods: PBMCs were obtained from three healthy volunteers and cultured in presence of *Echinops lasiolepis* methanol extract in different concentrations of 0.1, 1, 10, 100 and 200 μ g/ml. Lipopolysaccharide (LPS) and Phytohaemagglutinin (PHA) were considered as mitogen. BrdU cell proliferation assay was used to determine cell proliferation. The concentration of IFN- γ in the PBMCs supernatant was determined using enzyme-linked immunosorbent assay.

Results: Most concentrations of *Echinops lasiolepis* methanol extract showed inhibitory effect on PBMCs proliferation. There were significant differences between the concentrations of 200, 1 and 0.1 μ g/ml ($p < 0.05$). The optimum proliferation (30.66 \pm 24.67%) was obtained at concentration 1 μ g/ml. The PBMCs supernatant IFN- γ concentrations in extract treated group without PHA and LPS and in PHA activated group were not significantly different.

Conclusion: Our findings indicated that *Echinops lasiolepis* extract has immunomodulating activity which may be potentially to used as an immunotherapy agent.

* **Corresponding Author:** Department of Immunology, Reproductive Immunology Research Center, Shahid Sadoughi University of Medical Sciences, Yazd, Iran, **Tel:** +983536285406, **E-mail address:** hhadin@ssu.ac.ir

Introduction

Plants have mostly had a vital role in human life as medicine. The application of herbal medicine to treat diseases has been significantly increased [1]. Immunomodulatory role of the plant extracts is one of the biological activities of the plants. This is due to the various functions of the plant mitogens and their diverse biological functions such as cell adhesion, cell migration, cellular growth and differentiation, and induction of apoptosis [2]. Acetylcholinesterase inhibitors and immune suppressants, cyclosporine and rapamycin are drugs that have been obtained from natural products [3]. *Echinops lasiolepis* is commonly known as “Shekartighal Ardestani” in Yazd, Iran. The decoction of the *Echinops* family also as a traditional herb to treat cough and lung irritation and has been used as sedative, though there is scant evidence to rely on [4]. In the Yazdian pharmacopoeia, there are still serious lack of information on the use of large numbers of plants and spices traditionally employed for treatment of several ailments. In many studies medicinal plants are used to stimulate the immune system [5-7].

In India immunomodulatory effect of *Echinops echinotus* was reviewed in 1989. The results of that study indicated that *Echinops echinotus* is effective on arthritis and inflammation in mice [8].

In a study performed on the modulatory effect of *Echinacea purpurea* extract on human dendritic cell, it reduced expression of chemokine CCL3 and CCL8 and their receptors at mRNA level [6].

The Present study was designed to evaluate the effect of *Echinops lasiolepis* on peripheral blood mononuclear cells (PBMCs) proliferation and interferon (INF)- γ secretion. INF- γ is a cytokine secreted from helper T cells type 1, natural killer cells, dendritic and cytotoxic T cells. This cytokine is a 35 KD protein consisting of 146 amino acids which have a regulatory effect on proliferation and differentiation of B and T lymphocyte as well as their response and performance [9]. Aberrant production of the INF- γ is associated with autoimmune and inflammatory diseases [10]. Some studies have shown that plant extracts can affect the secretion of INF- γ [5, 11].

Materials and Methods

Preparation of the extracts

Aerial parts of the *Echinops lasiolepis* were collected from different parts of Yazd province, center of Iran, from April to May. The plant was identified by Research Center of Natural Resources, Yazd, Iran. A sample of collected plant was deposited in the center's herbarium. Plant materials were shade dried, and then powdered and defatted with ether. A Methanol extract was obtained by maceration of the plant at room temperature, and then extracts were filtered. The yield of *Echinops lasiolepis* extracts (ELE) was 6.451%. ELE was solved in Dimethyl sulfoxide (DMSO), and we used Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma-Aldrich, USA) to get 2000 $\mu\text{g/ml}$ stock for preparing different

concentrations of plant extract (0.1, 1, 10, 100 and 200 $\mu\text{g/ml}$).

PBMC preparation and treatment

Peripheral blood from three healthy volunteers (25–40 years old) was obtained. PBMCs were prepared by Ficoll gradient centrifugation. Ten milliliters of Ficoll–Hypaque (Baharafshan, Iran) was stratified under 20 ml of peripheral blood and phosphate-buffered saline (PBS) mixture, and then the sample was centrifuged at 400g for 30 min at room temperature. PBMCs were collected from buffy coat layer and were washed two times with RPMI-1640 medium. The number of cells was counted by trypan blue 0.4% (Sigma-Aldrich, USA). Freshly isolated PBMC with viability more than 95% were used for all experiments. Cells were cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS) (Gibco Company). After counting, 100000/well cells were transferred as duplicate into the wells of a 96-well microculture plate with 10 $\mu\text{l/ml}$ of different ELE concentrations to a final volume of 100 $\mu\text{l/ml}$. There were three treatment groups as follows: ELE treated cells with 10 $\mu\text{g/ml}$ phytohaemagglutinin (PHA) (Sigma-Aldrich, USA), ELE treated cells with 1 $\mu\text{l/ml}$ lipopolysaccharide (LPS) (Sigma-Aldrich, USA) and ELE treated cells without mitogen. The Control group included of Con A, medium with DMSO (as solvent control) The medium consisted of cells or without cells (positive or negative control). The microculture plate was incubated at 37°C in an atmosphere of 5% CO_2 with 90% humidity.

BrdU cell proliferation assay

The proliferation of PBMCs was determined using BrdU cell proliferation kit according to the manufacture's instruction (Roch diagnostic, Indiana, USA). In briefly, after 48 hrs incubation period 10 $\mu\text{l/well}$ of labeling solution was added to wells and then microculture plate was incubated for 24 hrs in the previous mentioned condition. The microculture plate was centrifuged for 10 min. The supernatant was removed and the microculture plate was dried for 30 min at 60°C. two hundred μl of fix Denant was added and incubated for 30 min at 15-25°C. After adding 100 $\mu\text{l/well}$ anti-BrdU-PODS and 90 minute incubation, wells were washed three times and then 25 $\mu\text{l/well}$ H_2SO_4 was added as a stop solution. Finally the optical densities were determined at a test wavelength of 450 nm and 630 nm using microplate reader (Stat Fax, 3200 microplate reader, Awareness Technology, INC, USA). Cell growth or inhibition of cell proliferation was calculated from the following formula;

$$\text{Cell growth\%} = \frac{(\text{Sample OD} - \text{Control OD}) \times 100}{(\text{Control OD})}$$

IFN- γ assay

The cultivation and treatment of PBMCs was done as previously mentioned. We used 1 $\mu\text{g/ml}$ concentration of ELE. After 72 hrs incubation, particulates were removed from the supernatants by centrifugation. Sandwich enzyme-linked immunosorbent assay (ELISA) procedure was performed according to the standard protocol IFN- γ ELISA kit (E.Bioscience, San Diego, USA) for determining of IFN- γ concentration in

supernatant. The sensitivity of kit was 0.99 pg/ml.

The Ethics Committee of Shahid Sadoughi University of Medical Sciences approved this Study.

Statistical analysis

Statistical analysis was performed with SPSS version 16 (SPSS Inc, Chicago, IL, USA). Differences between means were evaluated using ANOVA test (one way) followed by Duncan test. Results are expressed as Mean \pm Standard Error (SE). $p < 0.05$ was considered as statistically significant.

Results

Although in all concentrations of (without PHA and LPS group) the extract had an inhibitory effect on PBMCs proliferation, significant differences were in 200, 1 and 0.1 $\mu\text{g/ml}$ ($p < 0.05$). The best effect ($30.66 \pm 24.67\%$) was at 1 $\mu\text{g/ml}$. The percentages of growth in all concentrations are mentioned in Table 1.

PHA-stimulated PBMCs with different concentrations of ELE showed insignificant inhibitory effect ($p > 0.05$). Concentration of 100 $\mu\text{g/ml}$ showed a mild additive effect on PBMCs (Fig 1). ELE reduced proliferation on the LPS activated cells. Only 100 $\mu\text{g/ml}$ concentration showed a significant difference ($p = 0.032$) with $265.09\% \pm 271.9\%$ of growth compare to the control (Fig. 2). The mean of IFN- γ concentration in extract treated group was 1.732 pg/ml, while the control group mean was 5.266 pg/ml (Fig. 3). There was no significant difference between the two groups ($p = 0.12$). The mean of IFN- γ in PBMCs supernatant of ELE in 1 $\mu\text{g/ml}$ concentration with PHA and the control group (only PHA) was not significant (554 pg/ml in PHA activated group vs. 573.5 pg/ml in the control group) ($p = 0.24$). The result is shown in Fig. 4.

Table 1. The percentage of PBMCs growth rate under the effect of different ELE concentration as compared to the control group.

Concentration ($\mu\text{g/ml}$)	Without mitogen	With PHA	With LPS
200	$30.19 \pm 23.20^*$	4.68 ± 5.37	101.13 ± 43.22
100	12.76 ± 32.4	11.32 ± 44.43	265.09 ± 271.9
10	22.91 ± 35.76	14.33 ± 29.8	37.92 ± 17.87
1	30.66 ± 24.67	20.32 ± 17.59	76.22 ± 58.70
0.1	29.8 ± 40.52	12.34 ± 35.63	2.6 ± 42.69

Data presented as Mean \pm SE

Significant differences in 200, 1 and 0.1 $\mu\text{g/ml}$ ($p < 0.05$).

PHA: Phytohemagglutinin; LPS; Lipopolysaccharide

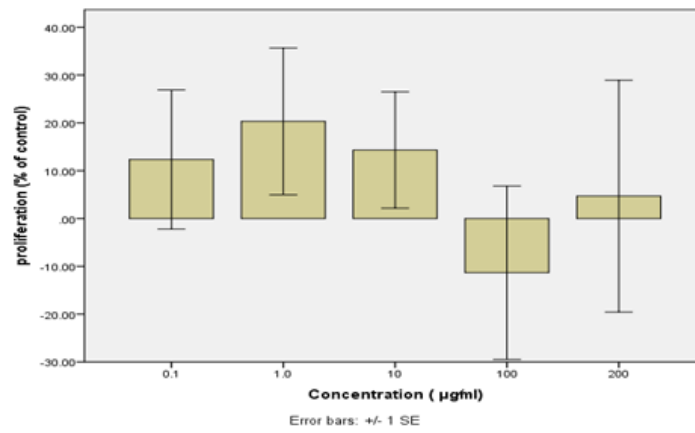


Fig. 1. The inhibitory effect of *Echinops lasiolepis* different concentrations on PHA activated PBMCs proliferation. Growth percentage was calculated compared to the control.

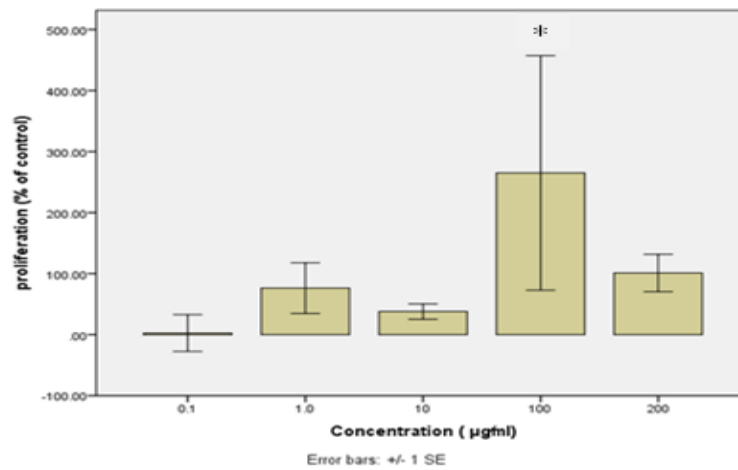


Fig. 2. The inhibitory effect of *Echinops lasiolepis* different concentrations on LPS activated PBMCs proliferation. Growth percentage was calculated compared to the control.

* The concentration of 100 µg/ml shows significant difference (p=0.032).

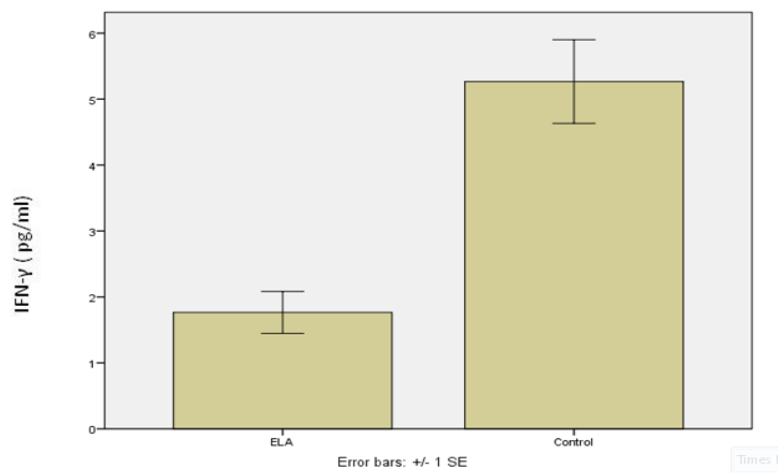


Fig. 3. The mean (\pm SE) value of IFN- γ in PBMCs supernatant of ELE at 1µg/ml concentration and the control group (p=0.12).

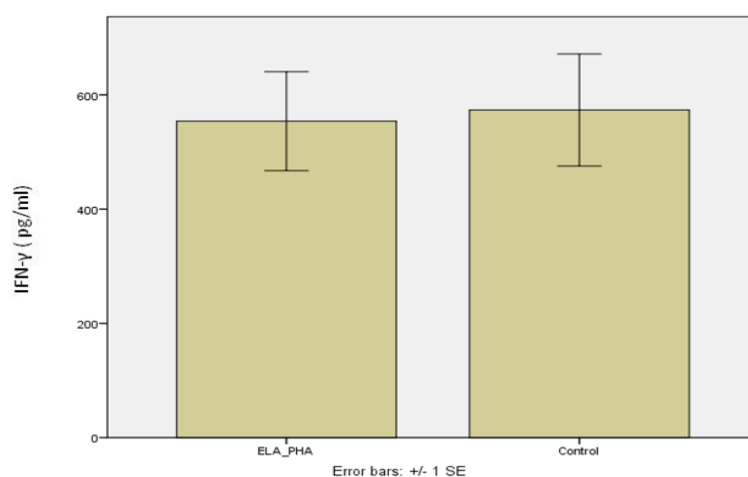


Fig. 4. The mean (\pm SE) value of IFN- γ in PBMCs supernatant of ELE at 1 μ g/ml concentration with PHA and the control group ($p=0.24$).

Discussion

Secondary plant products such as alkaloids, phenols and terpenoids protect plants against insects, bacterial and fungal agents, and are species specific [12, 13]. Several studies have shown anti-viral, anti-fungal, anti-bacterial, anti-inflammatory and anti-tumor activities that are related to secondary products [14]. As mentioned in the introduction, many plants have been shown to have immunomodulatory effects [13]. Although the constituents of this plant are not known, 22 substances of the other studied species plants, which can be involved in the obtained effects, were detected.

The cis-beta-farnesene and 5-3-buten-1-ynyl bithiophene are the most common of them [15]. Also, it had been shown that *Echinops ritro* has inulin-type fructans and alkaloid and lipid composition [16, 17]. The results of the present investigation suggest that plant fractions of methanolic extract at different dose levels inhibit PBMC proliferation while PHA activated cells at all concentrations showed no significant change and in the LPS

activated cells the extract induced proliferation at 100 μ g/ml concentration. This showed that the effect of this extract on various immune cells varies as LPS and PHA interact with different cells, indicating that extract is more effective on B cell than T cell. Since PHA and LPS have different effects and mechanisms of action on T cells and B cells [18, 19].

These differences on proliferation can occur due to mitogen. Almost highest percentage of growth was seen in LPS activated group (265.09 ± 271.9 , $p=0.032$), leading to greater B cell response. LPS binds to CD14/TLR4/MD2 receptor complex in many cells, especially monocytes, dendritic cells, macrophages and B cells, and causes release of pre-inflammatory cytokines and nitric oxide. Said et al have showed that LPS inhibits development of T CD4 cells through IL-10-dependent pathways [20]. This happens by increased expression of PD-1 in monocytes and its connection to the PDL, that leads to the production of IL-10. In this plant a composition or structure probably similar to LPS interferes with it and decreases

the proliferative effect of LPS by TLR4 pathway or others. As it has been shown, the A lipids which have less taper activate TLR2 pathway instead of TLR4 pathway [21, 22]. In this study, we found that this may be contributed by the modulation of cytokine secretion (IFN- γ). The experiment revealed that *lasiolepis* extracts with no significant effect on IFN- γ secretion induced apoptosis. To determine if it is related to internal or external apoptosis pathway, mRNA and Fas, Bcl-2 and Bax mRNA level examination is proposed. It is suggested that specific compounds of this plant which have not been yet investigated, be explored.

In few studies, the immunomodulatory and anti inflammatory effect of other spesces of *Echinops* has also been reported [23, 24]. Also other *Echinops* species showed anti tumor activity in human cancer cell lines [8, 25]. No other report was found in terms of the

biological activities of the *Echinops lasiolepis*.

Conclusion

our results clearly reflect the utility of *Echinops lasiolepis* at concentrations 0.1, 1 and 200 $\mu\text{g/ml}$ extract in inhibition of PBMCs proliferation, thereby causing insignificant change in IFN- γ secretion.

Conflict of Interest

The authors declare that they have no conflicts of interest.

Acknowledgement

Shahid Sadoughi University of Medical Sciences provided the grant for this research project. This manuscript is a part of in the M.Sc thesis of Maryam Asadi. Authors would like to thank the staff of Reproductive Immunology Research Center, Mrs. Fatemah Zare and Farzaneh Mirzaei for their technical assistance.

References

- [1]. Wachtel-Galor S, Benzie IFF. Herbal Medicine: An Introduction to Its History, Usage, Regulation, Current Trends, and Research Needs, in Herbal Medicine: Biomolecular and Clinical Aspects, I.F.F. Benzie and S. Wachtel-Galor, Editors 2011; Chapter 1.
- [2]. Yeap, S.K, Alitheen M, Noorjahan B, Ho W, Abdul Rahman O, Abdul Manaf A, et al. Immunomodulatory role of *Rhaphidophora korthalsii* methanol extract on human peripheral blood mononuclear cell proliferation, cytokine secretion and cytolytic activity. *Journal of Medicinal Plants Research* 2011; 5(6):958-965.
- [3]. Harvey A.L and Waterman P.G. The continuing contribution of biodiversity to drug discovery. *Curr Opin Drug Discov Devel* 1998; 1(1):71-76.
- [4]. Mozaffarian V. *Flora of Yazd*. 1998; Yazd: entesharate yazd: 437.
- [5]. Cherng J.M, Chiang W, Chiang L.C. Immunomodulatory activities of common vegetables and spices of Umbelliferae and its related coumarins and flavonoids. *Food Chemistry* 2008; 106(3):944-950.
- [6]. Wang CY, Chiao MT, Yen PJ, Huang WC, Hou CC, Chien SC, et al. Modulatory effects of *Echinacea purpurea* extracts on human dendritic cells: a cell- and gene-based study. *Genomics* 2006; 88(6): 801-8.
- [7]. Rimbau V, Cerdan C, Vila R, Iglesias J. Antiinflammatory activity of some extracts from plants used in the traditional medicine of North-African countries (II). *Phytotherapy Research* 1999; 13(2):128-132.
- [8]. Alaoui-Jamali M.A, Batist G, Zamir L. *Echinops* extract with anti-cancer activity 2002.
- [9]. Gray PW and Goeddel DV. Structure of the human immune interferon gene. *Nature* 1982; 298(5877):859-863.

- [10]. Schoenborn J.R and Wilson C.B. Regulation of Interferon- γ During Innate and Adaptive Immune Responses. In *Advances in Immunology*, W.A. Frederick, Editor 2007; 96:41-101.
- [11]. Hurinanthan V. Immune modulatory effect of *Dichrostachys cinerea*, *Carpobrotus dimidiatus*, *Capparis tomentosa* and *Leonotis leonurus* 2009.
- [12]. Gurib-Fakim, A. Medicinal plants: traditions of yesterday and drugs of tomorrow. *Molecular aspects of Medicine* 2006; 27(1):1-93.
- [13]. Briskin DP. Medicinal plants and phytomedicines. Linking plant biochemistry and physiology to human health. *Plant Physiology* 2000; 124(2):507-514.
- [14]. Manivasagan P, Venkatesana J, Sivakumar K, Kim S. Pharmaceutically active secondary metabolites of marine actinobacteria. *Microbiological research* 2014; 169(4):262-278.
- [15]. Guo D, Lou Z, Liu Z. Chemical components of volatile oil from *Echinops grijisii* Hance. *Zhongguo Zhong yao za, zhi Zhongguo zhongyao zazhi, China journal of Chinese materia medica* 1994; 19(2); 100.
- [16]. Van den Ende W, Clerens S, Vergauwen R, Boogaerts D, Le Roy K, Arckens L, et al. Cloning and functional analysis of a high DP fructan:fructan 1-fructosyl transferase from *Echinops ritro* (Asteraceae): comparison of the native and recombinant enzymes. *J Exp Bot* 2006; 57(4):775-789.
- [17]. Bhakuni, R.S, Shukla Y.N, and Thakur R.S. Alkaloids and lipid constituents of *Echinops niveus*. *Phytochemistry* 1990; 29(8):2697-2698.
- [18]. Benson K.F, Ruff K.J and Jensen G.S. Effects of natural eggshell membrane (NEM) on cytokine production in cultures of peripheral blood mononuclear cells: increased suppression of tumor necrosis factor-alpha levels after in vitro digestion. *J Med Food* 2012; 15(4):360-368.
- [19]. Williams R.M. and Benacerraf B. Comparison of T lymphocyte-dependent and B lymphocyte-dependent mitogen-stimulated DNA synthesis in serum-free medium with spleen cells from animals chosen for broad variation in genetically determined differences in T lymphocyte mitogen responsiveness. *The Journal of Immunology* 1974; 113(6):1844-1849.
- [20]. Said EA, Dupuy FP, Trautmann L, Zhang Y, Shi Y, El-Far M, et al. Programmed death-1-induced interleukin-10 production by monocytes impairs CD4+ T cell activation during HIV infection. *Nature medicine* 2010; 16(4):452-459.
- [21]. Netea MG, van Deuren M, Kullberg BJ, Cavaiillon JM, Van der Meer JW. Does the shape of lipid A determines the interaction of LPS with Toll-like receptors. *Trends Immunol* 2002; 23(3):135-139.
- [22]. Seydel U, Oikawa M, Fukase K, Kusumoto S, Brandenburg K. Intrinsic conformation of lipid A is responsible for agonistic and antagonistic activity. *European Journal of Biochemistry* 2000; 267(10):3032-3039.
- [23]. Singh B, Gambhir SS, Pandey VB, Joshi VK. Anti-inflammatory activity of *Echinops echinatus*. *Journal of Ethnopharmacology*. 1989; 25(2):189-199.
- [24]. Rimbau V, Cerdan C, Vila R, Iglesias J. Antiinflammatory activity of some extracts from plants used in the traditional medicine of north-African countries (II). *Phytother Res* 1999; 13(2):128-132.
- [25]. Kuete V, Kruscheb B, Younsb M, Voukenga H, Fankama AG, Tankeoa S, et al. Cytotoxicity of some Cameroonian spices and selected medicinal plant extracts. *J Ethnopharmacol* 2011;134(3):803-812.