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CYTOTOXIC EFFECT OF THE ETHANOLIC EXTRACT OF *LOPHOCEREUS SCHOTTII*: A MEXICAN MEDICINAL PLANT.

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Abstract

Lophocereus schottii is a Mexican cactus known as *garambullo* whose bark is used for the treatment of cancer, diabetes, ulcers, sores, stomach disorders and tuberculosis. The aim of this study was to evaluate the cytotoxic effect of the ethanolic extract of bark of *L. Schottii.* To assess these effects we established a flow of experiments in a model of BALB/c mice murine lymphoma. We value first survival of mice inoculated with 2×10^4 L5178Y murine lymphoma cells, orally treated with 10 mg/Kg of the extract for 10 consecutive days; the second assessment was to determine the influence of the immune system, we carry out studies of lymphoproliferation in mice with the same conditions of the previous study, only that the treatment was for 22 days before the completion cell cultures; the third study was to establish the cytotoxic effect of extract of *L. schottii* using different concentrations, by murine lymphoma cell cultures and splenocytes from healthy mice and finally we assessed the effect *in vivo* of extract of *L. Schottii* in a model of solid murine lymphoma inoculating 1×10^7 lymphoma cells in the gastrocnemius muscle observing the development of the tumor. We observed that oral treatment of 10 mg/kg of extract of *L. schottii* increased survival rate in treated mice; additionally, an intratumoral injection of 50 and 100 mg/kg in a solid murine lymphoma located in the gastrocnemius muscle, allowed a significantly slower tumor evolution. *In vitro* studies determined that extract inhibited 63% of lymphoma cell growth. With these evidences it is feasible to scientifically validate that ethanolic extract of *L. schottii* had an effect on L5178Y murine cells lymphoma and could have the same effect in human tumors.

Keywords: Cytotoxicity, murine lymphoma, *Lophocereus schottii*, medicinal plants, antitumor.

Introduction

The medicinal plants are a common alternative for cancer treatment in many countries, approximately 60% of drugs currently used for cancer treatment have been isolated from natural products (Gordaliza, 2007) ; in Mexico, 30 to 70% of diagnosed patients with cancer use herbal extracts as an alternative therapy for several types of cancer (Gerson-Cwilich et al., 2006; Gomez-Martinez et al., 2007). Plant extracts have been an important source of anticancerogenous agents used in medicine (Saklani, 2008; Jacobo-Salcedo et al., 2011), and Mexican medicinal plants play an important role in new treatment research (Alonso-Castro et al., 2011).

Lophocereus schottii (Engelm) Britton & Rose is a cactus which grows to 2 - 4 m tall, has 5 to 10 ribs, is an endemic species of Mexico, warm weather, associated with coastal dunes and xerophyl shrublands, found in Northwestern from Mexico and Arizona desert in United States of America; It's common names include cardona, muso, sina, sinita, cactus star and garambullo. *L. schottii* is traditionally used for treatment of cancer, diabetes, ulcers, sores, stomach disorders and tuberculosis (Argueta-Villamar et al., 1994; Encarnación-Dimayuga et al., 1987; Bravo-Hollis, 1999). However, there are no scientific studies demonstrating its anticancer activity. Alonso-Castro *et al*. (2011), in their study on 300 Mexican medicinal plants, recorded *L. schottii* in the group of plants that

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refer ethnopharmacological uses against cancer, without scientifically based research (Alonso-Castro et al., 2011).

Considering that the cytotoxic and immunomodulation effects of *L. schottii* are unexplored, the objective of this study is to evaluate *in vivo* and *in vitro* the ethanolic extract effect of the stem of *L. schottii* on a murine lymphoma model. We value first survival of mice inoculated with 2 × 10^4 L5178Y murine lymphoma cells, orally treated with 10 mg/Kg of the extract for 10 consecutive days; the second assessment was to determine the influence of the immune system, we carry out studies of lymphoproliferation in mice with the same conditions of the previous study, only that the treatment was for 22 days before of completion cell cultures; the third study was to establish the cytotoxic effect of ethanolic extract of *L. schottii* using different concentrations (125, 62.5, 31.25, 15.65, 7.81 y 0.97 µg/ml) by L5178Y murine lymphoma cell cultures and splenocytes from healthy mice and finally we assessed the effect *in vivo* of extract of *L. Schottii* in a model of solid murine lymphoma inoculating 1 x 10^7 lymphoma cells in the gastrocnemius muscle observing the development of the tumor.

Material and Methods

Plant materials and extraction and preparation of ethanol extract

Lophocereus schottii (Engelm) Britton & Rose was collected in July 2008 from Desert of Sonora, Mexico and identified by Dra. Hilda Julieta Arreola Nava (Institute of Botany University of Guadalajara). A voucher sample has been deposited in the Herbarium of Botany Department of University of Guadalajara under the number IBUG-189957. Stem from *L. schottii* (400g) was cut and dried into small pieces and extracted with ethanol absolute placing 10 g of powder in 100 ml absolute ethanol (1:10, w/v) under shaking for 48 h at room temperature in darkness. The extract was filtered and concentrated under reduced pressure in a rotary evaporator (MOD. RE47, Yamato Scientific CO LTD. Tokyo, Japan), and lyophilized (Freezone 4.5, Labconco, Kansas City, MO), finally the dry powder was resuspended in injectable water or culture medium AIM-V (GIBCO BRL, Grand Island, NY, USA) according to the *in vivo* or *in vitro* treatment respectively and sterilized by filtering through a 0.22 µm membrane (Acrodisc Siringe Filters Pall Corporation, Ann Arbor, MI).

Animals

Male BALB/c mice aged 6–8 weeks were maintained and bred under conventional laboratory conditions at the University of Guadalajara, Guadalajara, Mexico, according to the guidelines for the use and care of laboratory animals and World Medical Association Declaration of Helsinki (amended by the 52nd WMA General Assembly, Edinburgh, Scotland, October 2000). Animal protocols were approved by the Biomedicine Sciences Committee.

Lymphoma cell line

The murine lymphoma cell line L5178Y was derived from murine thymic lymphoma of DBA/2 mice (H-2d/d), and was maintained in ascetic form by weekly intraperitoneal (i.p.) passages of 1 x 10^6 cells in syngenic BALB/c mice (H-2d/d) (Perez-Puebla, 1998).

Lethal dose (LD_{50})

Oral single doses from ethanolic extract from *L. Schottii* (50, 500 and 5000 mg/Kg body weight) were administered in mice from three independent groups (n = 10 each) for the LD_{50} determination. The mice were kept in observation for 90 days to record clinical signs of acute toxicity such as, postration change behavior, respiratory and motor disturbances, piloerection and death (Loomis, 1982; Kent, 1998).

Studies on survival rate of mice with L5178Y lymphoma

The antitumor activity of ethanolic extract of *L. schottii* was evaluated *in vivo* using survival time (survival %) of mice inoculated with 2 x 10^4 cells of L5178Y murine lymphoma by intraperitoneal route and treated orally with 10 mg/kg daily, at the following day of tumor implantation for 10 days; mice were daily kept under observation to assess their general conditions and record the day of death. The survival time was valuated and represented with Kaplan- Meier curves of survival.

Lymphoproliferation with ex vivo MTT

In order to determine whether cell type immune response, in this murine lymphoma model, was modified by action of ethanolic extract of *L. schotti*, four experimental groups of 5 mice each were used. Two groups were inoculated intraperitoneally with 2 × 10^4 L5178Y murine lymphoma cells; one of them was treated with 10 mg/kg of ethanolic extract diluted in injectable water, and the other group only received injectable water (100 µl). The other 2 groups were also inoculated with only injectable water into the peritoneal cavity (100 µl), one of them also

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was treated with 10 mg/kg of ethanolic extract *L. schotti*. The four groups were treated for 22 consecutive days by oral route and after they were euthanized to obtain spleen cells, and lymphoproliferation assay was performed by using MTT method, according to the Hansen technique (Hansen, 1989), briefly: Concanavalin A (Sigma Chemical, St. Louis, MO) was used as mitogenic stimulus, the spleens were removed aseptically; cell proliferation was measured using 3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT). The method involves conversion of MTT to coloured formazan by the living cells. Then, 40 µl of MTT (5 mg/ml) was added to each well. After 2 h incubation at 37° C, 160 µl of extraction buffer pH 4.7 containing 20% w/v of SDS (Bio-Rad, Richmond, CA) in a solution of 50% N,N-dimethyl formamide (Sigma Chemical, St Louis, MO) in demineralized water were added. After overnight incubation at 37 ° C, optical densities at 570 nm were measured with an ELISA plate reader using the extraction buffer as blank. Cellular proliferation was determinated by Stimulation Index (SI) according to the following formula:
SI = (Optical Density with mitogen/ Optical Density without mitogen).

The proliferation index was statistically evaluated using the Kruscal-Wallis test.

Citotoxicity assay

In order to determine the direct *in vitro* effect of extract ethanolic on tumor cells growth and healthy spleen cells growth; L5178Y cells were collected from peritoneal liquid of mice bearing lymphoma and the cellular suspension obtained were washed three times with PBS solution and adjusted to 5 x 10^3 cell/well with AIM-V medium; in addition, spleen cells were obtained from healthy mice and seeded to flat bottom 96 well plates (4 x 10^5 cells/well) (Corning Incorporated, NY, USA), cultures were performed in triplicate, spleen cells were or not stimulated with 5 µg of Concanavalin A. All cultures (lymphoma cells, spleen cells with or without Concanavalin A) were incubated in the presence or absence of various concentrations of the extract ethanolic of *L. schottii* (125, 62.5, 31.25, 15.65, 7.81, 0.97 and 0.0 µg/ml). The culture assay was performed with colorimetric technique of MTT (Hansen, 1989, described above) and the optical density (OD) was measured at 570 nm in a microplate reader (OPSYS MR, Dynex Technologies, Inc. Chantilly, VA, USA). The viability of the culture cells was estimated from the relative growth as follows: % Citotoxicity = 100 – [(OD treated cells/OD not treated cells) x 100].

Inhibition of tumor development in solid phase

L5178Y cells were collected from peritoneal liquid of mice bearing lymphoma and the cellular suspension obtained were washed three times with PBS solution and adjusted to 1 x 10^7 cell/ml with AIM-V medium; four experimental groups of 10 mice each were used, all mice were inoculated with 100 µl of cellular suspension (1x10^6 lymphoma cells) in the left gastrocnemius muscle; two groups were treated with 50 (group 1) and 100 mg/Kg (group 2) of ethanolic extract of *L. schottii* diluted in injectable water by intratumoral route, other group (group 3) was treated with 100 mg/Kg of ethanolic extract by intramuscularly route and the last group (control group) was not treated. The four groups were treated for 30 consecutive days, 48 hours after tumor administration of lymphoma. Tumor volume was measured once every day using a caliper (Digimatic Caliper Mitutoyo Corporation, Japan), one day before the inoculated lymphoma cells and during 30 days. Tumor volumen was estimated according to the following formula: tumor volumen (mm^3) = (length x $width^2$)/2.

Statistical analysis

Experimental values are expressed for *in vivo* antitumor activity was evaluated by survival time with Kaplan-Meier curves; for lymphocyte proliferation *ex vivo* assay was evaluated by Kruscal-Wallis test; for cytotoxicity assay and inhibition of tumor development in solid phase were evaluated by Honestly Significant Difference Test of Tukey. The level of $p < 0.05$ was used to determine statistical significance. All calculations were performed using the Excel Statistical Microsoft Corporation Software.

Results

Lethal-dose 50 (LD_{50})

The ethanolic extract from *L. schottii* showed not lethal effects at doses (50-5000 mg/Kg body weight). Animals were observed until 90 days after administration. However, these animals did not die or behavioral and physiological alterations established by the toxicology standards, for more than 90 days of observation (Loomis, 1982; Kent, 1998).

Studies on survival rate of mice with L5178Y lymphoma

In order to determine the antitumor effect of ethanolic extract of *L. schottii*, 2 × 10^4 cells of L5178Y lymphoma were administered to two groups of mice (n = 10 for each one) in the peritoneal cavity, and one of the groups was treated with 10 mg/kg of ethanolic extract dissolved in injectable water by oral route, for the first 10 days after tumor administration; the other group only received the same sterile injectable water volume (100 µl), they were kept under observation until they died and the survival rate of each group was determined. Kaplan-

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Meier statistical test revealed that the survival rate increased significantly (33 days) in mice treated with *L. schottii* compared with the non-treated group (28 days) (Figure 1).

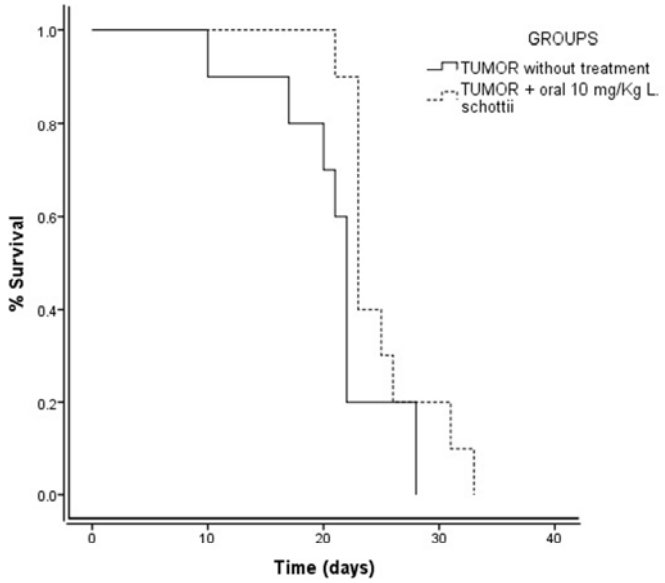

Figure 1: Antitumor effect of ethanolic extract of *L. schottii* evaluated by survival of mice with L5178Y murine lymphoma. Comparison of groups with or without the following treatment: 10 mg/kg/day/10 days (n = 10). The estimation of survival was significantly different $P < 0.05$ evaluated by the Kaplan-Meier test.

Lymphoproliferation with ex vivo MTT

It is known that L5178Y murine lymphoma model produces immunosuppression in treated mice with 1×10^7 cells (intraperitoneal route) at the sixth day of development (Oronzo-Barocio et al., 1999; Daneri-Navarro et al., 1995). In order to explain the survival increase due to *L. schottii* immunomodulator effect, *in vitro* tests of splenocyte lymphoproliferation were conducted in mice inoculated in the peritoneal cavity with 2×10^4 cells of L5178Y murine lymphoma for 22 days (2 groups n = 5). One of the groups was treated with 10 mg/kg of ethanolic extract of *L. schottii* diluted in sterile injectable water administered by oral route for 22 days; the other groups only received the vehicle (sterile injectable water) for the same period of time; lymphoproliferation tests were also conducted in two other groups of mice (n = 5 for each one) that did not received lymphoma cells and only ethanolic extract of *L. schottii* and vehicle were administered as previously described. MTT colorimetric assay was used to evaluate lymphoproliferation (Hansen, 1989). No significant difference (Kruskal-Wallis test) was observed between the 4 groups in regard to lymphoproliferation rate (Figure 2), which reveals that ethanolic extract of *L. schottii* has no effect on splenocyte proliferation of mice treated or not with the extract and survival increase can be explained by a direct cytotoxic action against murine lymphoma cells.

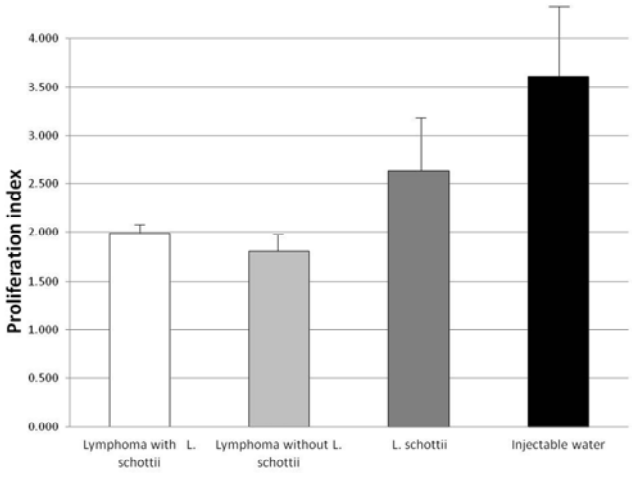

Figure 2: Effect of ethanolic extract of *L. schottii* on lymphoproliferation of mice with L5178Y murine lymphoma with or without the following treatment: 10 mg/kg/day/22 days (n = 5). There was no significant difference in proliferation indexes evaluated by the Kruskal-Wallis test.

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Cytotoxicity assay

Based on the previous results, the cytotoxic effect of ethanolic extract of *L. schottii* was determined using different concentrations (125, 62.5, 31.25, 15.65, 7.81 and 0.97 µg/ml), by L5178Y murine lymphoma cell cultures and splenocytes from healthy mice stimulated or not with Concanavalin-A (5 µg/ml). Plant extracts with cytotoxicity values of ED_{50} (Effective Dose 50) ≤ 30 µg/ml are considered active according to the parameters established by The National Cancer Institute (NCI) (Suffiness, 1990). According to these criteria, the ethanolic extract of *L. schottii* has toxic effects on L5178Y lymphoma cells (ED_{50} = 7.8 µg/ml with 63% of cytotoxicity); whereas, in splenocytes not stimulated with concanavalin-A from healthy mice, there was lower cytotoxic effect using the same concentration (38% of cell cytotoxicity, ED_{50} > 125 µg); however, the splenocytes stimulated with concanavalin-A experienced 80% of cytotoxicity (Table 1 and Figure 3).

Table 1.Effect of ethanolic extract of *L. schottii* on the cytotoxicity percentage of L5178Y murine lymphoma cells, healthy splenocytes and healthy splenocytes stimulated with Con-A.

Concentration of ethanolic extract µg/ml	L5178Y Lymphoma Cells	Healthy Cells without Con-A	Healthy Cells with Con-A
125	57	43	77
62.5	58	44	78
31.25	58	42	80
15.63	62	40	80
7.8	63	38	80
3.9	43	9	71
1.95	16	-8	61
0.97	2	-17	57

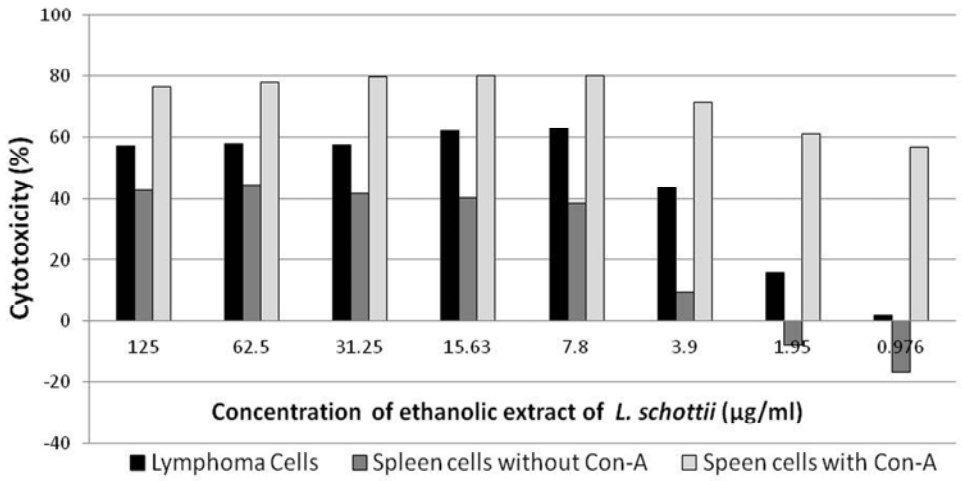

Figure 3: Effect of ethanolic extract of *L. schottii* on the cytotoxicity percentage of L5178Y murine lymphoma cells, healthy splenocytes and healthy splenocytes stimulated with Con-A. The cytotoxicity percentage, evaluated by Tukey's multiple comparison test, was significantly different $P < 0.05$.

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Inhibition of tumor development in solid phase

With these results, the *in vivo* cytotoxic effect of ethanolic extract of *L. schottii* was studied on a solid L5178Y murine lymphoma model, implanted in the left gastrocnemius of mice (1 x 10^6 cells), treated with ethanolic extract of *L. schotti* with daily doses of 50 and 100 mg/kg by intratumoral route and 100 mg/kg intramuscularly, starting 48 hours after first lymphoma inoculation. The development of the tumor volume, valued in mm^3, was slower in groups treated with ethanolic extract of *L. schottii* than in non-treated group, mostly in the group that received 50 mg/kg by intratumoral route. Likewise, there was no difference in the extract administration route (Tukey´s multiple comparison test) (Figures 4 and 5).

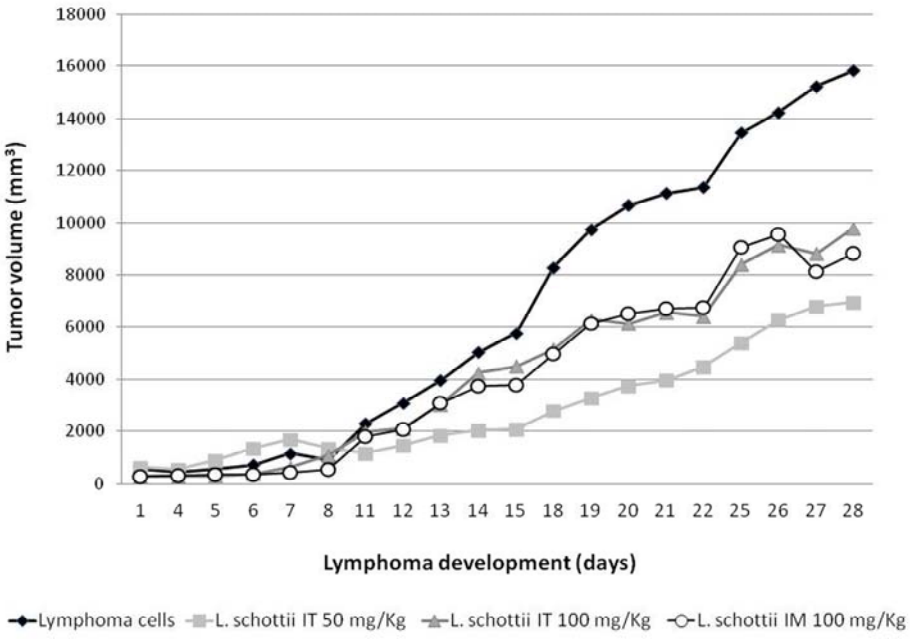

Figure 4: Effect of ethanolic extract of *L. schottii* on tumor growth (mm^3) in mice with L5178Y murine lymphoma implanted in gastrocnemius muscle, with or without treatment by intratumoral (IT) and intramuscular (IM) route, n=10. Tumor growth, evaluated by Tukey´s multiple comparison test, was significantly different $P < 0.05$.

Figure 5: Hind leg of a healthy mouse (left), another inoculated with L5178Y murine lymphoma in the gastrocnemius muscle at 30 days of evolution, without treatment (right), and with 50 mg/kg of ethanolic extract of *L. schottii* (center) by intratumoral route.

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Discussion

The results demonstrate unequivocally, the cytotoxic effect of ethanolic extract of *L. schottii* on cells undergoing mitosis (L5178Y murine lymphoma cells and splenocytes stimulated with Concanavalin-A), this cytotoxic effect being significantly different with regard to healthy splenocytes in resting stage of mitosis or not stimulated, as shown in Figure 3 and Table 1. Likewise, it can be observed that the tumor volume in solid state, is smaller when ethanolic extract treatment is administered (Figure 5), demonstrating that the dose of 100 mg/kg both intratumorally and intramuscularly injected, show the same effect with regard to tumor volume reduction; however, between 50 and 100 mg/kg treatments using the same route of administration, it, indeed, showed differences with respect to the tumor volume (Figure 4).

In summary, these results provide important biological data that ethanolic extract of *Lophocereus* schottii effect, has important implications in the ethnomedicinal use against tumor cells, since it has significantly greater cytotoxic effect on L5178Y murine lymphoma cells than in healthy splenocytes, allowing survival increase in treated mice, decrease in solid lymphoma growth and mitosis interference. References found stated that whole plant and stem contain alkaloids of Isoquinoline, Lophocerine and Pilocereine (Wani et al, 1980; Kircher, 1969), as well as triterpenes Lophenol and Lupeol, and sterol Schottenol (Argueta-Villamar et al., 1994). It is known that generally derived alkaloids such as the isoquinoline compounds have anti-cancer effects and reduce tumors development (Wang et al., 2009; Pettit et al., 2009; Li et al., 2009; Stærk et al., 2002); likewise, a number of triterpenoids have shown promise as antineoplastic agents and exhibit antiproliferative activity when tested against various cancer cell lines. Recent reports showed that triperpenes directly inhibit tumor growth, cell cycle progression and induce the apoptosis of tumor cells under *in vitro* and *in vivo* situations. (Palanimuthu et al., 2012; Misawa et al., 2012; Siddique et al., 2011; He et al., 2011; Saleem, 2009). On the other hand the schottenol presents anticarcinogenic properties (Khallouki et al., 2003; Arisawa et al., 1985). However, there is no information about the chemical derivatives of the generic compounds found in *L. schottii*. Therefore, it is necessary to conduct more phytochemical studies for isolation and identification of the active components of *L. schottii.* Likewise, further studies on the mechanisms of biological effects by which the extract exerts its anititumor effects are unavoidable.

Acknowledgement

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Declaration of Interest

The authors have declared that no conflicts of interest exist.

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