

Measurement of Serum Levels of Interleukin 17 and 23 in the Immune System with Different Amounts of Spleens White Pulp Size after Inoculation of New *Leishmania* Vaccine in Susceptible Mice

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Abstract

Objective: Different species of *Leishmania* parasite can cause leishmaniasis and its prevalence is growing in Iran and same as in other parts of the world is twice every ten years. According to studies conducted an effective vaccine is better way to prevent this disease than antihistamines.

Method: In our study, six groups were vaccinated and the seventh group was control group. The vaccine groups received two injection doses (100 and 200 micrograms/0.1 ml) of cocktail *leishmania* vaccine respectively both with two adjuvants (Teucrium Polium or BCG).

Results: Results showed that mice survived along study period in all vaccinated groups. The serum cytokine's results showed that IL-23 was highest in group LT100 µg/0.1 ml and group LBT 200 µg/0.1 ml was lowest, and, highest IL-17 related to control and LBT 100 µg/0.1 ml and lowest to LBT 200 µg/0.1 ml. Spleen results show that highest mean of spleen weight related to LT100 µg/0.1 ml and lowest to LBT 100 and 200 µg/0.1 ml. The highest percentage of median spleen divided by the mean body weight related to same three groups: LB 100, LB200, LT200 µg/0.1 ml and lowest to LBT200 µg/0.1 ml. The largest number of spleen's lymphoid follicles: related to LB 200 µg/0.1 ml and lowest to LT100.

Conclusion: Our findings about health, decline of mortality and undesirable immunological changes of this new *leishmania* vaccine show that no significant changes were observed in its IL-17, IL-23. Besides that, It was safe, harmless, without any complications and clinical pathological macroscopic and microscopic dangerous in animal models, and one of the most important points was survival of mice along study. The experience with the vaccine was repeated for the third time and the results were satisfactory and confirmed our previous experience on this new formulation of *leishmania* vaccine.

Keywords: *Leishmania* new vaccine; IL-17; IL-23; Spleen changes; Animal model

Introduction

Leishmaniasis infection is transmitted by the vector of *leishmania* species. The promastigote form of the protozoan, which has an extracellular flagellum, in the carrier insect called phlebotomus sand-flies. The protozoan amastigote form is infected inside human macrophages, or susceptible animals and dogs [1,2]. Leishmaniasis can be seen in various forms including cutaneous, visceral and mucosal leishmaniasis. Leishmaniasis is endemic in Asia, Africa, North-South, and Central American and Europe [3,4]. Dogs can be infected and parasite carrier between susceptible species [5,6]. More than 30 species of Leishmaniasis have been diagnosed so far [2,6]. Dogs can be infected and parasite carrier between susceptible species [5,6]. More than 30 species of Leishmaniasis have been diagnosed so far [2,6] which 10 items of them are medically important. Three species (*L. major*, *L. tropica*, *L. infantum*) in Iran and Asia produce cutaneous and visceral leishmaniasis. Regarding the feature of this infection, it was understood that only way to control the disease is to have a protective vaccine, which many efforts being made so far. Two major immune cells MQ and DC help to remove infection agents. Sensitivity or persistence to *leishmania* is begin with macrophage and dendritic cells which recognize pathogen and producing immune cytokines and lead to cleaning up of intracellular pathogens. Macrophage and neutrophils phagocytized promastigotes and digest it for terminate infection. If it

deficient in digestion promastigotes change to amastigote, proliferate and induce development of leishmaniasis [6-8]. Reported of Panaro et al. showed that Lipophosphoglycan on the *Leishmania* promastigote's surface can causes killing of parasites through phagocytosis killing mechanism. In leishmaniasis various factors, including host gene and internal environment of body, may help the survival of the protozoan within the macrophage and the living body. Roma et al. shown that *in vitro* induction of ROS production by infected peritoneal macrophages cannot help to kill micro-organisms [9]. In report of Assureuy et al. Phagocytosis cells can induce reactive oxidase after swallowing *L. major* and killing it [10]. In the presence of interleukin 17 nitrite oxide synthase (iNOS) can produce nitrite oxide [11]. It also

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stimulates production of pro inflammatory cytokines (TNF-alpha, IL-1-beta), inflammatory chemokines to elevation of phagocytosis and effect on neutrophils and macrophages [12,13]. Neutrophils in leishmaniasis are very important in remove of parasites [13], but its short life cause serve only as "Trojan Horse" to avoid destruction by immune system [14]. TLRs on surface of MQ and DC are critical for induce immune response against *leishmania* infection and or vaccine. First it was said that the relationship between TLRs [2,4,9] and TLRsL play an important role in the onset of leishmaniasis disease [15], but later it was observed that mice withTLR9 defects is more sensitive to leishmaniasis than mice deficient in TLRs 2 and 4 [8,16]. After recognition of *leishmania* by TLRs-TLRsL, signals initiated leading to production of pro-inflammatory, chemical and inflammatory peptides [17]. It seems that the preparation of an appropriate protective vaccine is the best solution for controlling the cutaneous leishmaniasis disease. The various environment and environmental factors involved in the response to leishmaniasis are not interfered with it. Therefore, it is not possible to predict the optimal immune response against this parasite, and this is an important point to be considered in the development of the vaccine. Because of factors such as antigenic characteristics of a vaccine, general condition of vaccinated individual and host genetic factors can influence the vaccine's protectiv capacity. In this study, we investigated new formulation of leishmaniasis with two different adjuvants (BCG and Teucrium pulium). Teucrium Pulium was used in Iran for abdominal pain, mal digestion, colds, and type II diabetes. It seems that this adjuvant has anti-inflammatory and antioxidant effects and has protective effects against infections, cancers and fungi [18]. For this reason, we used of alcoholic extract of Teucrium Pulium beside to BCG as an adjuvant with the new *Leishmania* vaccine in Balb/C mice. In fact, we also measured for the third time the survival power, spleen changes, and for the first time inflammatory cytokines (IL-23 and 17) in the susceptible animal model (Balb/C mice).

Material and Method

This study was conducted in accordance with the Helsinki Declaration. The protocol is supported by both the research fellows of the School of Medicine and Deputy of Research of Tehran University of Medical Sciences. Tehran, Iran.

Culture and isolation of leishmania parasites

Leishmania parasites and promastigote antigens from the *L. major*, WHO strain prepared from the Pasteur Institute. It was cultured and grown in mediuNNN (Novy-MacNealNicoll) medium supplemented with Hemin, Homa, RPMI 1640, normal saline, and/or 5–10% heat inactivated fetal calf serum.

The harvested parasites were washed three times with normal saline (0.9%) or phosphate/salt serum. The parasites were counted using a Neubauer chamber and then kept at 80°C until use. The collected parasites then reached the concentration of 5.92×10^{10} , and were divided into five equal volume tubes. The first group was killed by pasteurization in water at 56°C for 30 min. The second bottle was autoclaved at 121°C for 30 min. The third batch was merthiolated for 30 min at a dilution of 1:10,000. The fourth batch was freeze-thawed three times and the fifth batch was left innate.

Vaccine preparation

The five batches were mixed and centrifuged, and the sediments were dispensed into sterile vials. *Leishmania* components Vaccines were tested for complete parasitic infections .The culture was carried out on a plate of blood agar and injected into the footpads Balb/c mice.

Detailed procedures have already been described [9]. In short, just before the injection, the BCG vaccine "SSI" was suspended in a suspension of SST and 0.1 mg BCG (first dose) or 0.01 mg BCG (consecutive dose) was added to each bottle containing promastigote. Based on previous studies, 100 mg/0.1 ml or 200 mg/0.1 ml of *leishmania* protein was selected at each dose of the temporary vaccine to formulate and prepare the vaccine. The protein content of each dose was estimated by the Lowry method [19]. The vaccine was kept at 4°C until injected. BCG adjuvant for each injection dose is included 2×10^5 CFU/0.1 ml. To prepare Teucrium adjuvant Palm, 400 mg of Teucrium polium alcoholic extract in 1 ml distilled water without endotoxin deionized, 205 mg/0.1 ml was used for each of the doses of the *leishmania* antigens given above, and two injection doses containing 100 µg/0.1 ml antigens or 200 µg/0.1 ml containing adjuvants [20-22].

Animal model

They included 40 Balb/c mice kept at the Faculty of Medicine's animal House. For detailed procedures please refer to Latifynia and collaborations [20-22] [23,24,25]. In brief, Balb/C and traditional white laboratory mice (n=40) were obtained at three months old from the Pasture institute.

Vaccination

All doses of vaccine antigens of *Leishmania* and the bold reminder to the tail (or legs) of mice were intra dermally injected.

Dosages (100, 200 µg protein) were used as follow: Group LB (100, 200) received 100, 200 µg/0.1 ml antigen combined with BCG Group LT (100, 200) received 100, 200 µg/ 0.1 ml antigen combined with BCG alcoholic extract of Teucrium polium.

Group LBT (100, 200) received 100, 200 µg/0.1 ml antigen combined with BCG and alcoholic extract of Teucrium polium.

The control group did not receive any injections.

Hematoxylin and Eosin staining

All groups were injected subcutaneously with vaccine preparation at the foot of the tail and increased one week later using similar doses.

Twenty five days after the booster injection, all animals, including the control group, were weighed and Blood sampling was performed and then the spleens were harvested. All spleens were cut in equal length and width and fixed in 10% formaldehyde buffer solution. Stable protein tissue was processed in tissue processing. Paraffin blocks were made and tissue sections of 4 to 5 microns were prepared and stained with Harias Hematocillin and Eosin. The fixed spleen was stained in paraffin blocks and stained in a tissue processor (Tissue sections (5–6 µm thick) and stained with hematoxylin and eosin.

The number and diameter of the white pulp of the spleen (lymphoid spleen follicles) were examined using a microscope of light with the fragment of the eye. The diameter of the SWP sections was measured and compared with each other and with the controls.

Cytokine assay

ELISA and sandwich methods were used to evaluate the IL-17 and IL-23 in animal serum and their serum levels were measured using an auto-reader at 405 nm.

Statistical analysis

Data were obtained using statistical software (SPSS Inc., Chicago, IL, USA). The means were analyzed using standard variance analysis/

simple factorial test with two-way Student–Newman–Keuls methods. The correlation coefficient was determined using a Pearson bivariate, two tests.

Result

More than one month after vaccination and twenty-five days later to all the injected groups (LB, LT and LBT) and doses (100 and 200 µg/0.1 ml) that had been injected with the vaccine, booster dose was administered again.

At the end of the experiment, the survival rates were 100%. In the autopsy, no microscopic and macroscopic pathological changes were seen in the internal, visceral and external organs, and were normal in all respects. The results of cytokines and also the microscopic and macroscopic spleen are as follows:

IL-23 of serum: The highest was for group LT 100 µg/0.1 ml and (29.55 pg/ml) and was the lowest for LBT-200 µg/0.1 ml group (16.36 pg/ml) (Figures 2 and 15) (table 1).

The levels of serum IL-23 in all groups were as follow: LT-100

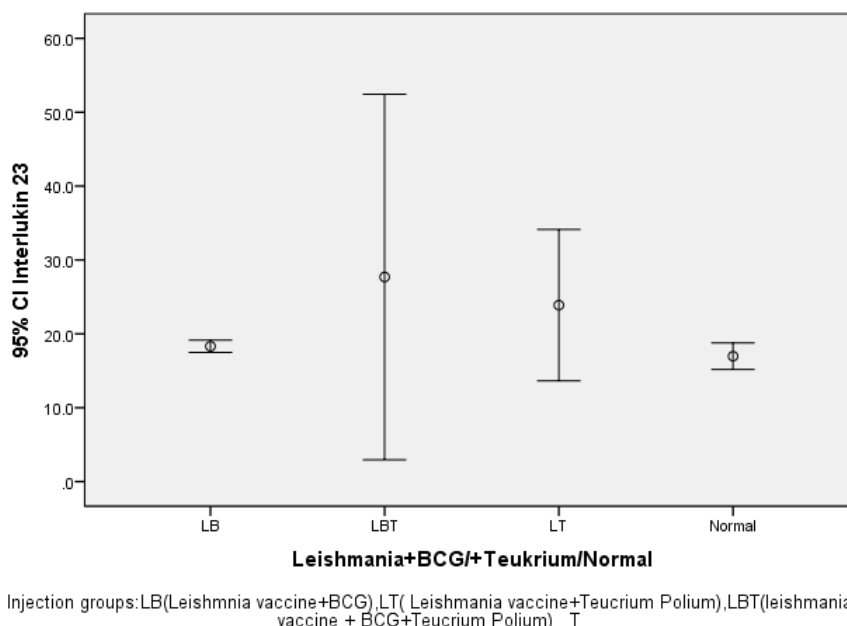


Figure 1: Mean of IL-23 for three ijection groups: LB, LT, LBT with normal group in serum of Balb/c mice after vaccination with new *leishmania* vaccine.

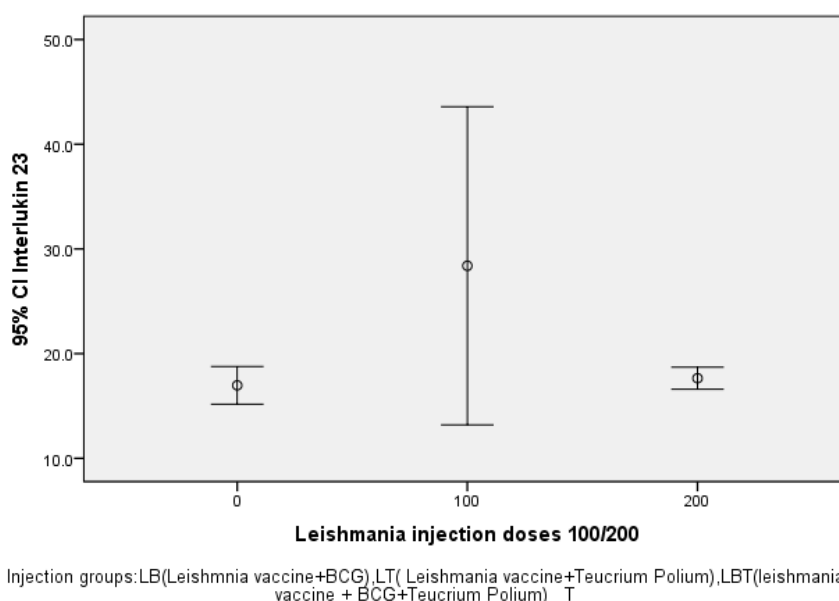


Figure 2: Mean of IL-23 (IL-23) for two ijection doses: 100 and 200 microgram/0.01 ml with normal group 0.00 micro/ml serum of Balb/c mice after vaccination with new *leishmania* vaccine.

Group	Dosages Of Antigen µg/0.1 ml	Number of Balb/C mice	M.B.W _i (gr) x̄ Min.-max.	M.S.W _i (gr) x̄ Min.-max.	MPSW(gr./BW) x̄ Min.-max.	Meant of Serum IL17 (pg/ml) x̄ Min.-max.	Meant of Serum IL23 (pg/ml) x̄ Min.-max.	MNSWP x̄ Min.-max.	MSWPS (micron) x̄ Min.-max.	Survival rates (percent)
LB	100	6	21.312 19.064-22.707	0.118 0.095-0.341	0.55 0.45-0.64	11.77 5.70-30	18.48 17.6-19.2	35.50 30-38	1785.50 1455-1982	100%
LT	100	6	20.881 18.590-23.0825	0.112 0.111-0.960	0.53 0.111-0.960	19.67 5-55	29.55 15.2-73.2	29.5 27-32	1697.52 13980-2234	100%
LBT	100	6	18.223 11-109-21.1675	0.102 0.038-0.134	0.56 0.34-0.64	22.90 6.20-60	20.5 14.4-38.6	33.5 25-40	1777.53 1180-2375	100%
LB	200	6	20.538 15.772-22.697	0.108 0.057-0.910	0.52 0.36-0.74	14.32 4.60-29.60	18.15 15.7-20.8	41.67 27-54	1895.82 1718.-2110	100%
LT	200	6	21.103 18.519-24.200	0.116 0.099-0.141	0.545 0.49-0.64	13.80 4.80-29.60	18.22 14.7-21.4	39.50 35-44	2022 1855.-2222	100%
LBT	200	5	20.440 19.677-21.315	0.106 0.90-0.122	0.500 0.46-0.57	9.86 0.00-30	16.36 13.5-17.6	41.2 35-52	1800.98 1466-2107	100%
Control	0	5	20.993 19.165-24.779	0.108 0.96-0.121	0.514 0.49-0.56	24.56 0.00-69.80	16.96 15.0-18.7	35.00 30-56	1897.22 1535- 2223	100%

MBW: Mean Body weight; MSW: Mean Spleen Weight; MPSW (g)/BW: Mean Percentage of Spleen Weight to Body weight.

MNSWP: Mean Number of Spleen white Pulp; MSWPS: Mean Spleen White Pulp Size.

Table 1: Effects of provisional *Leishmania* Vaccine on spleen parameters, IL-17, IL-23 and Survival Rates of the Female Balb/C Mice in mean of injection groups: LB, LT, LBT and control group and injection doses 100 and 200 µg/0.1 ml. Mean within column with no common superscripts are significantly different (P<0.005)

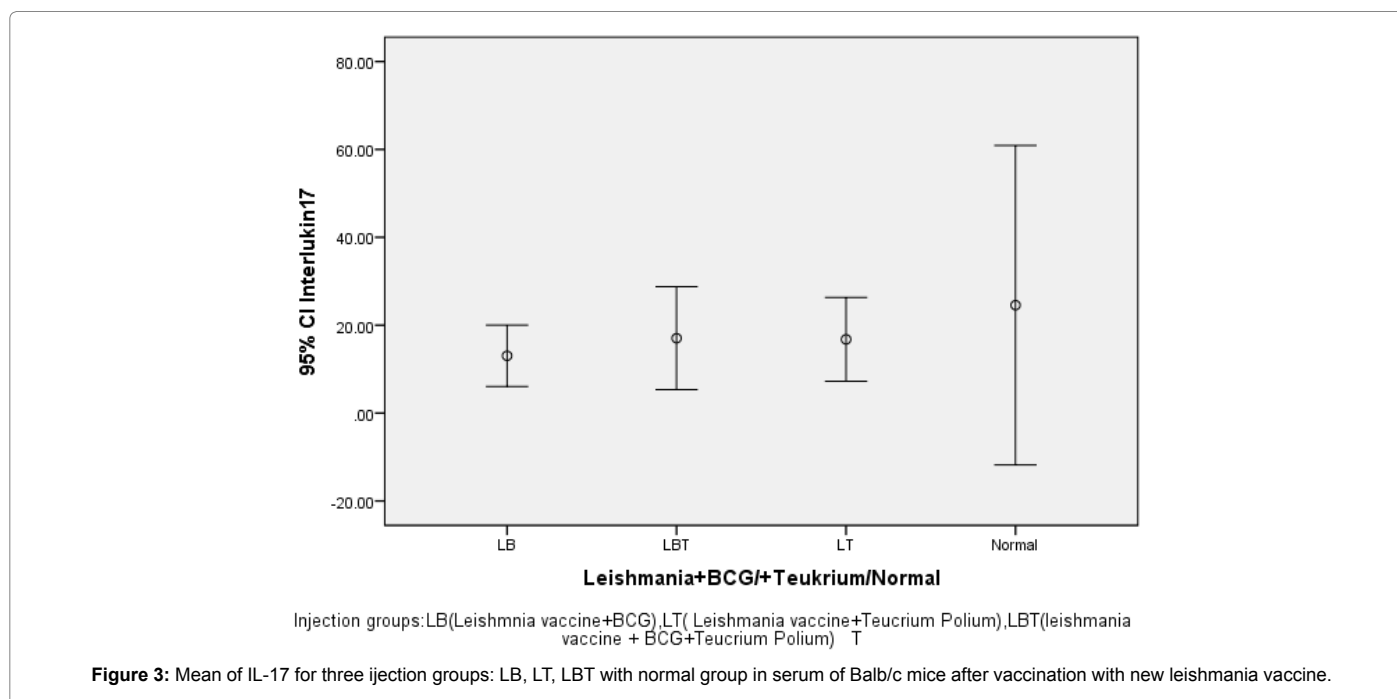


Figure 3: Mean of IL-17 for three ijection groups: LB, LT, LBT with normal group in serum of Balb/c mice after vaccination with new leishmania vaccine.

(29.55 pg/ml), LBT-100 (20.50), LB-100 (18.48), LT-200 (18.22), LB 200 (18.15), control (16.96) and LBT-200 (16.36 pg/ml) (Table 1). **Differences between groups were not statistically significant (P=0.268) IL-17 of Serum:** The highest was for control group (24.56 pg/ml), and was the lowest for LBT-200 (9.86 pg/ml).

The levels of IL-17 in all groups were as follow: Control (24 pg/ml), LBT 100 µg/0.1 ml (22.90), LT 100 (19.67), LB 200 (14.32), LT 200 (13.8), LB 100 (11.77) and LBT 200 µg/0.1 ml (9.86 pg/ml) (Figures 3, 4 and 15) (Table 1). Statistic differences between all groups were not significant (P=0.344). **Mean body weight (MBW):** The highest for group LT-200 µg/0.1 ml (21.103 g) and was the lowest for LBT 100 µg/0.1 ml (18.223 g).

The MBW in all groups were as follow: LB-200[0] µg/0.1 ml (21.312 g) LT 200 (21.103 g) control (20.993 g) LT 100 (20.881 g) LB 200 (20.538 g) LBT 200 (20.44 g) and LT-100 µg/0.1 ml (18.233 g) (Figures 5, 6 and 15) (Table 1). Differences between groups were not significant (P=0.880).

The mean of spleen weight (MSW): The highest level seen in LT 100 µg/0.1 ml (0.391 g) and lowest related to LBT 100 (0.102 g) (MSW) were as follow: LT-100 µg/0.1 ml (0.391 g), LB 100 (0.264) LB 100 (0.264) LB 200 (0.247) LT 200 (0.116) control (0.108) LBT 200 (106) and LBT 100 µg/0.1 ml (0.102 g) (Figures 7, 8 and 15) (Table 1). There was not significant differences between animal groups (p=0.398) (Table 2).

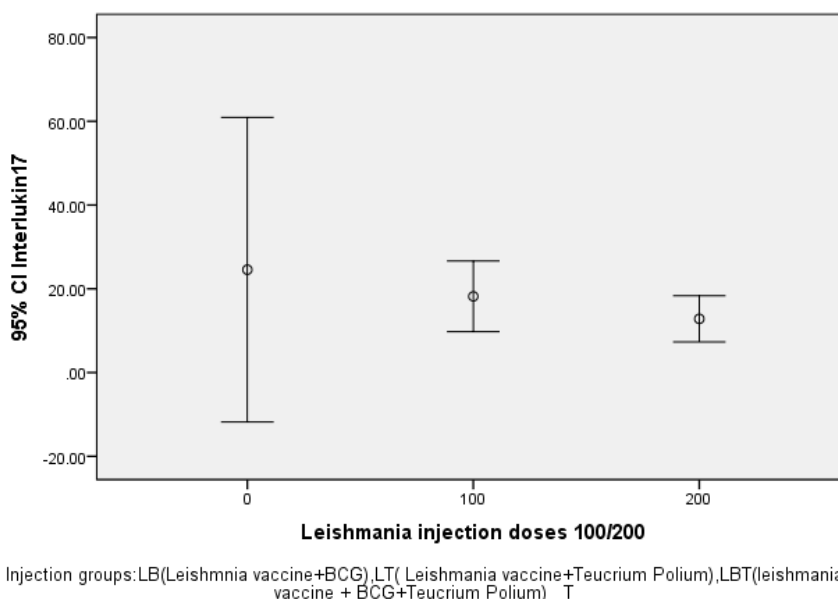


Figure 4: Mean of IL-17 (IL-17) for two ijection doses: 100 and 200 microgram/0.01 ml with normal group 0.00 micro/ml serum of Balb/c mice after vaccination with new leishmania vaccine.

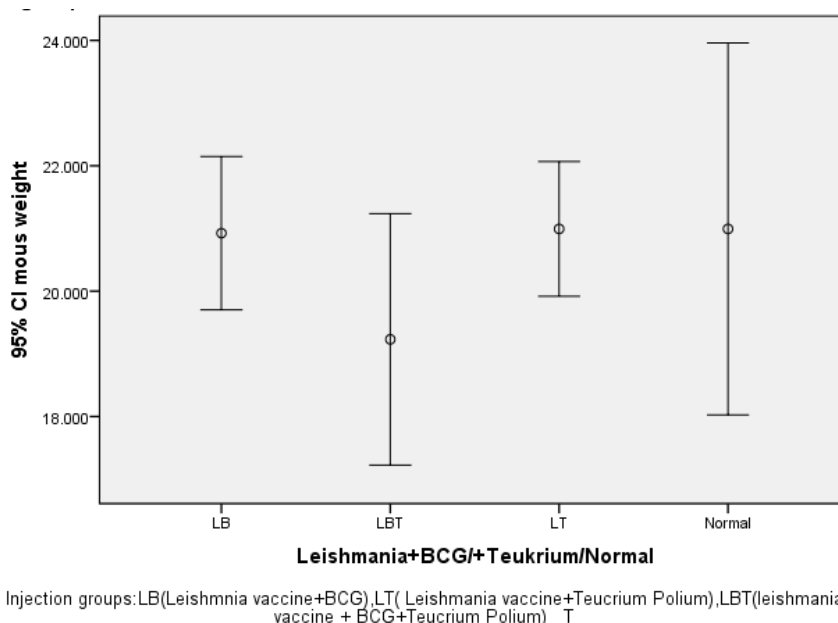


Figure 5: Mean of mouse weight for three ijection groups: LB, LT, LBT with normal group in serum of Balb/c mice after vaccination with new leishmania vaccine.

Percentage of mean spleen weight/mean body weight (%MSW/MBW): The highest level seen in three groups of LB 100 µg/0.1 ml, LBT 100 and LT 200 (0.55%) and LBT 200 µg/0.1 ml (0.52%) had lowest levels (Figures 10 and 15) (Table 1).The percentages were as follow: LB 100 µg/0.1 ml=LB 200=LT 200 (0.55%) LT 100 = LT 200 (0.53) LBT 200 (0.52) (Figures 9, 10 and 15) (Table 1).

No significant differences of %MSW/BW was observed for all groups (Table 2). Number of Spleen White Pulp Unit (NPU): The highest mean seen in LB 200 µg/0.1 ml (41.67) and lowest was for LT 100 µg/0.1 ml (29.5). (NPU) were as follow: LB 200 µg/0.1 ml (41.67)

LBT 200 (41.2) LT 200 (39.50) LB 100 (35.50) control (35.00) LBT 100 (33.50) <100 µg/0.1ml (29.5) (Figures 11, 12 and 15) (Table 1). ANOVA test for NPU indicated a significant difference (P=0.001) (Table 2).

Mean of spleen white pulp size or diameter (MSWPS): The highest was related to LT 200 µg/0.1 ml (2022 micron) and lowest was to LT 100 (1697.52 micron). (MSWPS) was as follow: LT 200 µg/0.1 ml (2022 micron) control (1897.22) LB 200 (1895.82) LBT 200 (1800.98) LB 100 (1785.50) LBT 100 (1777.8) and LT 100 (1697.52 micron) (Figures 13-15) (Table 1). ANOVA test for (MSWPS) did not showed significant (P=0.152) (Table 2).

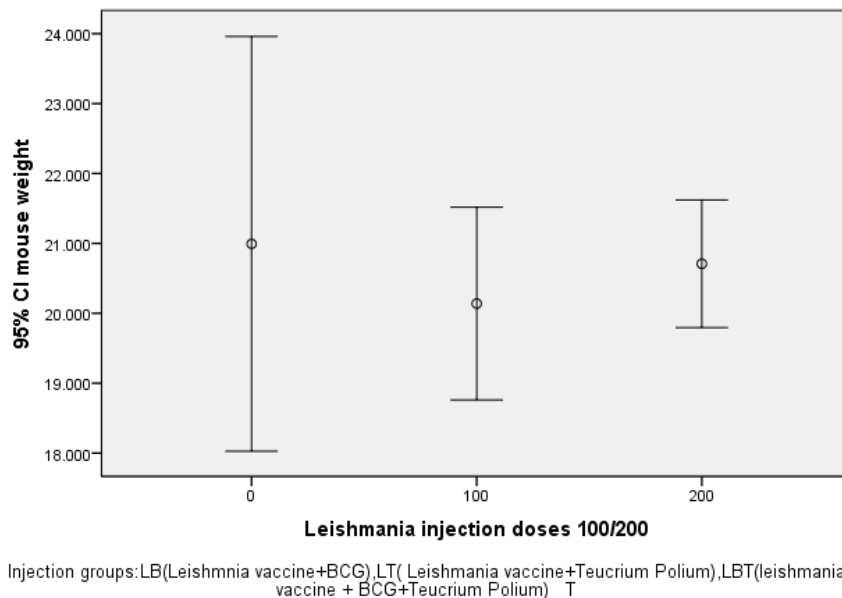


Figure 6: Mean of mouse Weight (MV) for two ijection doses: 100 and 200 microgram/0.01 ml with normal group 0.00 micro/ml serum of Balb/c mice after vaccination with new leishmania vaccine.

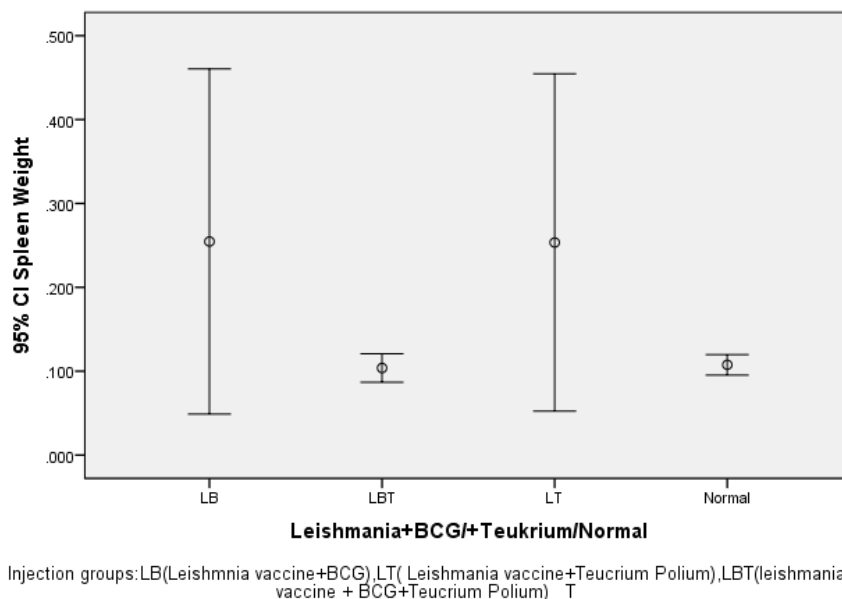
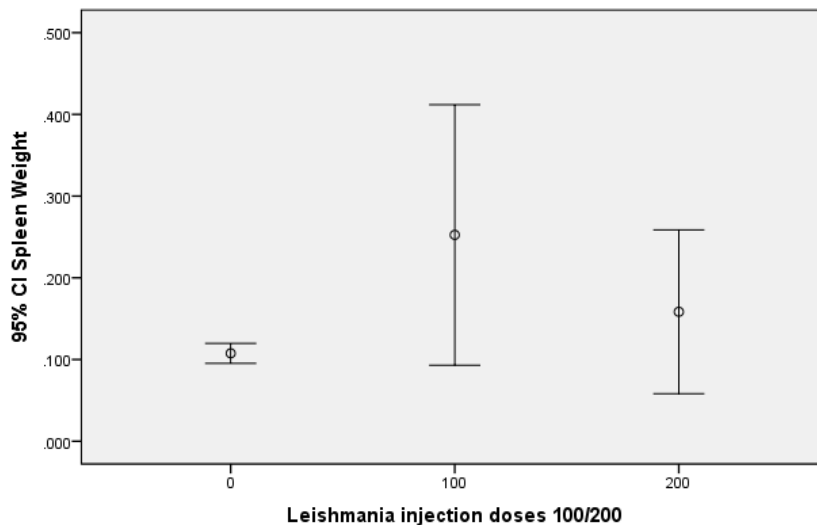


Figure 7: Mean of spleen (SPW) weight for three ijection groups: LB, LT, LBT with normal group in serum of Balb/c mice after vaccination with new leishmania vaccine.

Discussion

The components of the effective immune system at the site of entry of the parasite are potentially important in the immune response and the elimination of microorganisms. Macrophage activity by killed agents includes PRR (unnecessary receptors), local tissue matrix and intrinsic immunity, including T helper cells, DCs and tissue macrophages that provide effective and useful cytokine networks that are relevant before and after leishmanial parasitic infiltration to the host cells (macrophages). In this regard, optimal production of local cytokines such as IFN- γ , TNF α , IL12, IL17 and IL23, IL-8 in

the immune response against parasites of the entrance to the site is important. In the present study, vaccines and mice were evaluated for more than one month after vaccination and 25 days after booster dose. IL-17, IL-23 and white spleen measured in 6 groups. According to the data in Tables 1 and 2 and Figures 1-15, new vaccines for vaccinated animals create immunity and provide satisfactory reactions (safety and toxicity). As shown in Tables 1 and 2 and Figures 1-15, the vaccine can induce systemic immune responses. The microscopic changes of the spleen in the vaccinated groups of Balb/c mice showed that the vaccine activates the secondary lymphoid tissues (white pulp). It is

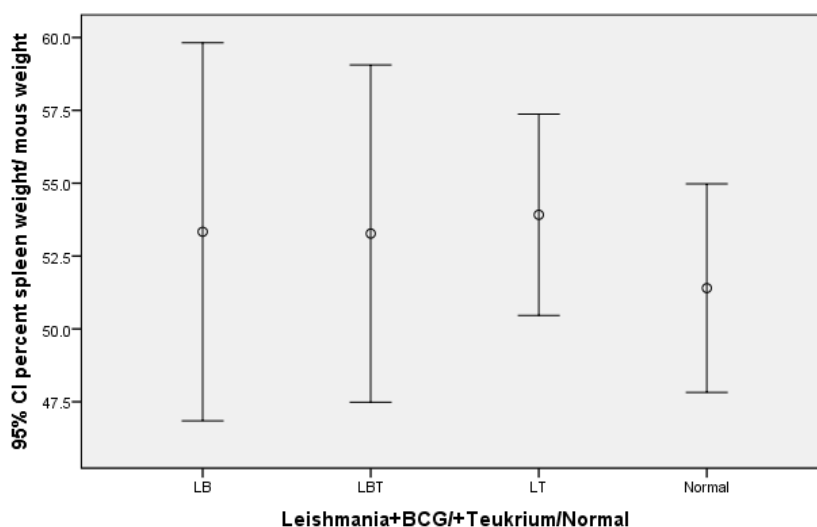


Injection groups: LB(Leishmania vaccine+BCG), LT(Leishmania vaccine+Teucrium Polium), LBT(leishmania vaccine + BCG+Teucrium Polium) T

Figure 8: Mean of spleen (SPW) weight for two ijection doses: 100 and 200 microgram/0.01 ml with normal group 0.00 micro/ml serum of Balb/c mice after vaccination with new leishmania vaccine.

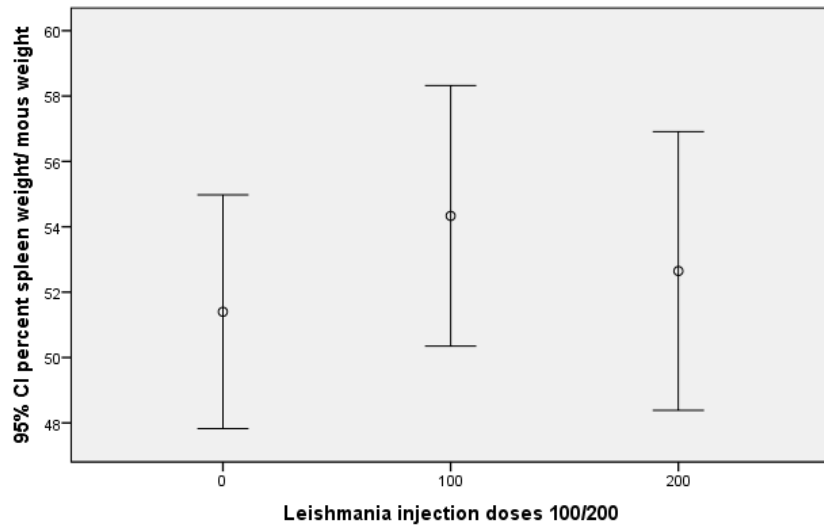
Group	Number of Balb/C mice	Meant of Serum IL17 (pg/ml) \bar{x} Min.-max.	Meant of Serum IL23 (pg/ml) \bar{x} Min.-max.	M.B.W ₁ (gr.) \bar{x} Min.-max.	M.S.W ₁ (gr.) \bar{x} Min.-max.	MPSW(gr.)/ BW \bar{x} % Min.-max.	MNSWP \bar{x} Min.-max.	Multiplication of MNSWP& MSWPS	Survival rates (percent)
LB	12	13.05	18.33	20.93	0.113	0.54	38.6	71191.45	100%
LT	12	16.74	23.89	20.99	0.114	0.54	34.5	64973.3	100%
LBT	12	16.38	18.43	19.33	0.104	0.53	37.5	67144.0	100%
Control	5	24.56	16.96	20.99	0.108	0.51	35.0	66402.7	100%
Dose 100µg/0.1ml	18	18.11	22.48	20.139	0.111	0.55	32.8	57669.7	100%
Dose 200µg/0.1ml	18	12.66	17.58	20.69	0.110	0.53	40.8	77869.7	100%

Table 2: Effects of provisional *Leishmania* Vaccine on spleen parameters, IL-17, IL-23 and Survival Rates of the Female Balb/C Mice in mean of injection groups: LB, LT, LBT and control group and injection doses 100 and 200 µg/0.1 ml.



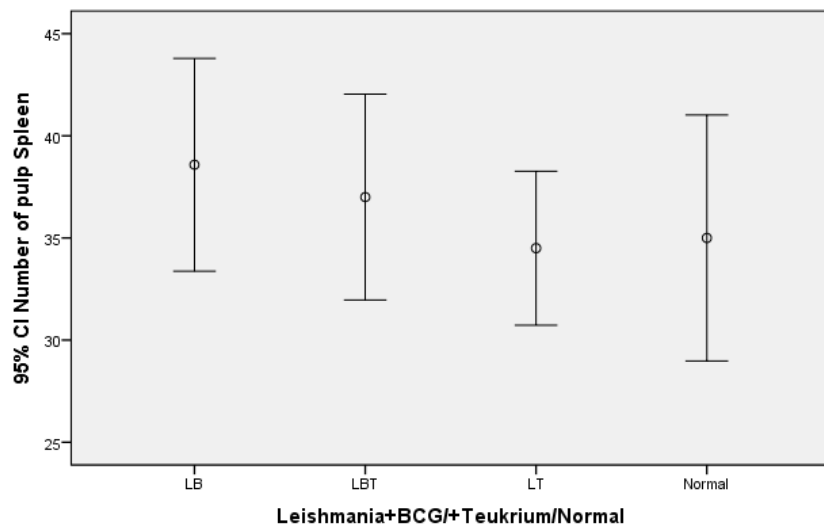
Injection groups: LB(Leishmania vaccine+BCG), LT(Leishmania vaccine+Teucrium Polium), LBT(leishmania vaccine + BCG+Teucrium Polium) T

Figure 9: Mean of percent spleen weight/mouse weight (PSM) for three ijection groups: LB, LT, LBT with normal group in serum of Balb/c mice after vaccination with new leishmania vaccine.



Injection groups: LB(Leishmania vaccine+BCG), LT(Leishmania vaccine+Teucrium Polium), LBT(leishmania vaccine + BCG+Teucrium Polium) T

Figure 10: Mean of percent spleen weight/mouse weight (PSM) for two ijection doses: 100 and 200 microgram/0.01 ml with normal group 0.00 micro/ml serum of Balb/c mice after vaccination with new leishmania vaccine.

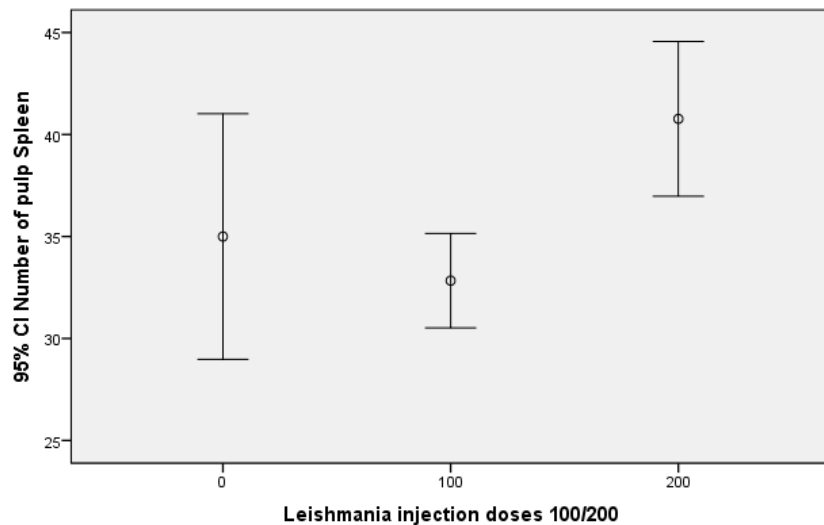


Injection groups: LB(Leishmania vaccine+BCG), LT(Leishmania vaccine+Teucrium Polium), LBT(leishmania vaccine + BCG+Teucrium Polium) T

Figure 11: MEAN OF NUMBER OF PULP SPLEN (NPU) FOR THREE IJECTION GROUPS: LB, LT,LBT WITH NORMAL GROUP IN SERUM OF BALB/C MICE AFTER VACCINATION WITH NEW LEISHMANIA VACCINE.

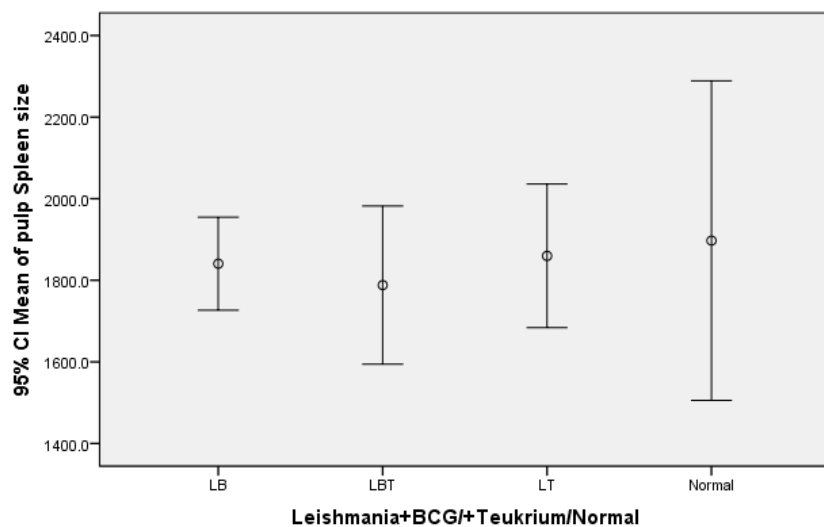
suggested that the spleen lymphoid tissue can respond effectively to the anti-leishmaniasis response, which includes the following: when the parasite is systematically (visceral) or local (skin or cutaneous, mucocutaneous). In this connection, According to the references appeared in the literature, spleen lymphoid cells including B cell can produce IL-10 which suppress the cytokines production required to activate CMI which damaging to leishmania parasites [26,27]. B10 cells have also been identified in mesenteric lymph nodes, peritoneal cavity and the peripheral blood and lymph nodes of mice; however the spleen is the predominant location for B10 cells [28]. These B10 cells are capable of producing IL-10 which acts as a potent regulatory cytokine [27]. IFN-gamma and TNF-alpha and absence of IL-10 is associated with tissue

destruction and development of the lesions observed in cutaneous and mucosal leishmaniasis despite low parasites number in the lesion [27]. Incubation of splenic B cells with LPS, CPG or apoptotic cells induced significantly elevated IL-10 secretion indicating that B cells are primed to respond to mitogenic stimulate [28]. Also, in [29] proposed the role of B cell regulation of immune response in tuberculosis *via* suppression of delayed type hypersensitivity. They concluded that the suppression is immunologically specific and is dependent on B cells. At a glance a double functions could be proposed when induction of immune responses against *leishmania* parasite is the principle interest: firstly, the immune responses must provide the body with a well harmonized cytokines-chemokines networks, reasonable cellular activation and



Injection groups: LB(Leishmania vaccine+BCG), LT(Leishmania vaccine+Teucrium Polium), LBT(leishmania vaccine + BCG+Teucrium Polium)

Figure 12: Mean of Num Pulp spleen (NPU) for two ijection doses: 100 and 200 microgram/0.01 ml with normal group 0.00 micro/ml serum of Balb/c mice after vaccination with new leishmania vaccine.



Injection groups: LB(Leishmania vaccine+BCG), LT(Leishmania vaccine+Teucrium Polium), LBT(leishmania vaccine + BCG+Teucrium Polium)

Figure 13: Mean of Pulp spleen size (MPS) for three ijection groups: LB, LT, LBT with normal group in serum of Balb/c mice after vaccination with new *leishmania* vaccine.

well-conditioned tissue matrix capable to confront with pathogenic *leishmania* parasite, secondly the induced immune responses must either be harmless, or if produce harm must be mild and well tolerated to the naïve subjects or in the patient during the course of infection. In essence, induction or fortification of a satisfactory immune response against *leishmania* parasites requires a potent and harmless vaccine which could be used to immunized susceptible species. According to our present and previous experiments on Balb/C mice [20-24], the vaccine seem to be safe and effective; 100% viability was recorded in all groups vaccinated with different doses of antigen (100 µg/0.1 ml or 200 µg/0.1 ml) with BCG or alcoholic extract of *T. polium* and or both as adjuvant. The provisional vaccine induced satisfactory cytokine

responses in such a way that are not only safe to the vaccinated animals (Balb/C mice), could protect vaccinated subjects against challenging with live *leishmania* parasite as shown previously [30,31]. There are some suggestions related to the vaccine: The immune responses against organism leading to intracellular destruction or blocking some unknown ligands necessary to the course of the life cycle continuity or survival of organisms within the vertebrate host.

Nevertheless, the parasite maybe protected from hostile extracellular environment, helping survival of the organisms. These host- parasite interactions are basically complicated and may be affected by various parameter related to host (genetic, nutrition, body physiological



Injection groups: LB(Leishmania vaccine+BCG), LT(Leishmania vaccine+Teucrium Polium), LBT(leishmania vaccine + BCG+Teucrium Polium) T

Figure 14: Mean of Pulp spleen size (MPS) for two injection doses: 100 and 200 microgram/0.01 ml with normal group 0.00 micro/ml serum of Balb/c mice after vaccination with new leishmania vaccine.

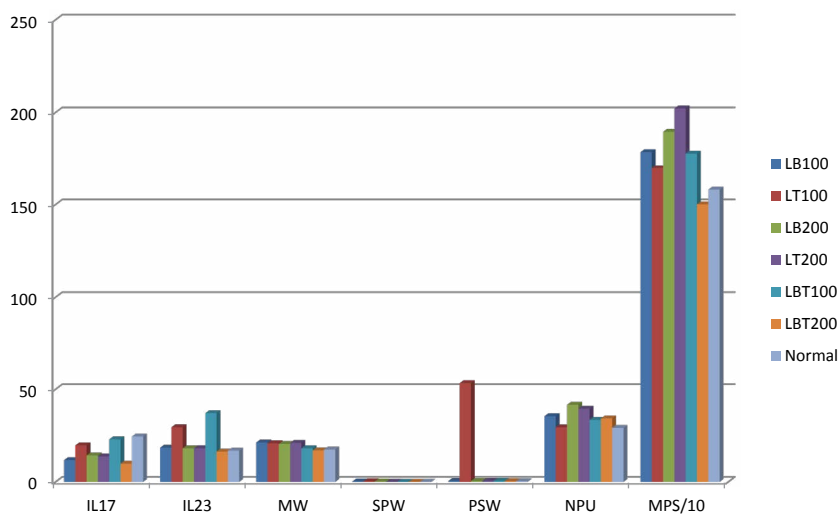


Figure 15: The effects of the treatment of crude cocktail *L. major* antigen preparation. On, mean mouse weight, Spleen Pulp Weight, Number of Pulp Unit and mean Pulp Size/10, IL-23 and IL-17 in two injection dose (100, 200 µg/0.1 ml) and three injection groups (LT, LB and LBT) compared to control group.

conditions, the body immunological potency at the same time when the organisms penetrate the vertebrate host, and the standard of living, etc.), and parasite (virulence, or ability of organism to survive within vertebrate host). Following vaccination, the quality and quantity of immune components and the biochemical conditions of local microenvironment in which the parasites penetrate, are important to confer immunity against leishmaniasis. An ideal vaccine which enable the vaccinated subjects to deal with the pathogen, must provide local innate immune responses which detrimental to *leishmania* organisms at the time of entry. Also the vaccine could mount an effective acquired immune response against the penetrated organisms in such a condition that if the organisms evade innate immune responses, would face effective acquired immune responses which could destroy intracellular organism, *via* activation of effective cell mediated immunity orchestrated

by IL12, IL17, IL23 and IFN-γ cytokines. The present findings indicate that the provisional vaccine could be administrated safety at least in mice. Beside the safety of the vaccine, it may induce production of a series of protective immune components which could be transferred *via* the blood stream to the cutaneous *leishmania* lesions resulted in activation of anti-*leishmania* macrophages response or could destroy metacyclic promastigotes released from the ruptured parasite-laden phagocytes (macrophages or neutrophils) present within cutaneous perhaps mucocutaneous ulcers. It is postulated that, the Teucrium adjuvant could solely or together with *leishmania* antigens could provide body with effective anti-*leishmania* immune components. The percentage of survival in the present experiment was 100% at 25-30 days post-vaccination which is in the favor of our previous findings [20,32,33] Regards to our results related to the body weight and spleen

parameters showed that with the exception of mean number of spleen white pulp (lymphoid follicles), the differences between the groups were not significant post vaccination. The findings can be summarized as follow: [mean mouse weight ($P=0.68$), mean of spleen weight ($P=0.398$), mean of percentage of spleen weight/mouse weight ($P=0.694$), and mean of spleen white pulp size ($P=0.152$)], however when mean number of spleen white pulp (follicles) in seven groups of mice were compared, the differences between groups were highly significant ($P=0.001$). These findings were showed that our new *leishmania* vaccine could affect the spleen tissue; however the effect was moderate as shown previously. The interesting point is that the group LB which received 100 or 200 $\mu\text{g}/0.1$ ml had lowest mean of spleen white pulp size, but they had the highest mean number of spleen white pulp and thus had highest levels of white pulp expansion (71191.45 micron). The results confirmed our previous theory about regulation of homeostasis *in vivo* in different situations [34] but injection group LT for both injection doses 100 and 200 $\mu\text{g}/0.1$ ml had lowest levels of spleen white pulp expansion (64923. 0).

Lowest space occupied by the white pulp lymphoid follicle was related to injection dose 100 $\mu\text{g}/0.1$ ml (57669.7 micron) and the highest was related to injection dose 200 $\mu\text{g}/0.1$ ml (77869.7micron). Lowest levels for IL-17 related to injection group LB (13.05 pg) and injection dose 200 (12.66 pg.), and highest related to injection group LT (16.74 pg) and injection dose 100 (18.11 pg.) (Figures 3 and 4) (table 1). IL-23 had lowest level in group LB (18.33 pg.) was highest in LT group (23.89 pg.), and similarity had lowest level in dose 200 (17.58 pg.) and highest level in dose 100 $\mu\text{g}/0.1$ ml (22.84 pg) (Table 1) (Figures 1 and 2).

In a summary it can be concluded that: 1-LB group had, highest mean number spleen white pulp (38.5), highest mean spleen white pulp expansion (71191.45 micron), lowest mean IL-17 (13.05 pg.), lowest mean IL-23 (18.33 pg), 2-LT group had lowest mean number spleen white pulp (34.5), lowest mean spleen white pulp expansion (64923 micron), highest mean IL-17 (16.74 pg.), highest mean IL-23 (23.89 pg.), highest mean of body weight (20.99 g), 3-LBT group had highest mean IL-17 (16.38 pg.), lowest mean IL-23 (18.45 pg), lowest mean of body weight (19.33 g), 4-Injection dose 100 $\mu\text{g}/0.1$ ml had lowest mean expansion of spleen white pulp (57669.7 micron), highest mean IL-17 (18.11 pg.), highest mean IL-23 (22.84 pg.), lowest mean of body weight (20.14 g), 5-Injection dose 200 $\mu\text{g}/0.1$ ml had highest mean expansion of spleen white pulp (77869.7 micron), lowest mean IL-17 (12.66 pg), lowest mean IL-23 (17.58 pg.) and highest mean of body weight (20.69 g).

Our results from post vaccination and pre challenging are desirable and as it was demonstrated in the present experiment, the best vaccinated group was LT group and the best injection dose was 100 $\mu\text{g}/0.1$ ml nominated LT100 which had lowest spleen white pulp expansion and highest levels of IL-17 and IL-23. These finding confirmed our previous results concerning expansion of spleen white pulp [35] and the levels of serum IL17 and IL-23 in the vaccinated animals [36-40,41]. This is a satisfactory results for this new *leishmania* Vaccine, since IL-23 and IL-17 axis and activation of JAK-STAT is essential for immunity against Chronic Mucocutaneous Candidiasis (CMCC) and CMCC also is Mendelian disease that leishmaniasis is one of its Complications. Fortunately our vaccine was safe and its survival rate following vaccination was 100%. Also that no complications or skin nodule formation at the site of the vaccine injection were noticed following vaccination. Besides that, It was safe, harmless, without any complications and clinical pathological macroscopic and microscopic dangerous in animal models. The experience with the vaccine was repeated for the third time and the results were satisfactory and confirmed the previous experience.

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