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Evaluation Effect of Probiotic Cream in Carrageenan-Induced Inflammation Model in Male Rat Hind Paw

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ABSTRACT

Background: Inflammation is the first biological response produced by immune system against any infections or foreign allergen in order to protect the body. The main anti-inflammatory drugs that are used today are Non-steroidal anti-inflammatory drugs (NSAID), opioids, and corticosteroids. Despite the broad range of uses of these drugs, many side effects have been associated with their use such as digestive problems like ulcers and peptic bleeding. It has been suggested that products containing probiotics can be used as an effective anti-inflammatory drug, due to effects of Probiotics on immune responses such as reducing the inflammatory cytokines. In this study, we aim to investigate the anti-inflammatory effects of probiotics and to compare the effect with commonly used drugs such as Piroxicam and *Calandula* in an animal model.

Method: In this experimental study, after preparation of topical probiotic cream, used *Wistar* male rats weighing 180-200 g were used and randomly divided into 6 groups; negative control, positive control (piroxicam & calandula) and 3 treatment groups (2%, 4% and 8% w/w of LPE). Three groups, each received different doses (2%, 4% and 8% w/w) of LPE cream by topical use was 30 minutes before injecting carrageenan into plantar side of hind paw of the rats. Piroxicam gel and Calandula ointment were studied as positive control for anti-inflammatory activity, respectively. Finally, to observe the anti-inflammatory effect, the rat paw edema was measured every 15 min in the first hour and then hourly for 5 hours. In the end, the volume of intact rat's paw was measured in all groups by using plethysmometer. In the study, the IL-6 levels was measured by the ELIZA method.

Result: All groups under the administration of the probiotic extract of *Lactobacillus Casei* compared to the negative control group in the rat paw edema show significant difference ($P<0.05$). Dose 2% LPE (Lyophilized cell free Probiotic Extract), turned out to be less effective than 2% Piroxicam cream in reducing edema during the study. But there was no significant difference between the group receiving doses of 4% and 8% w/w. The 4% dose of LPE cream was selected as the most favorable. These effects may be due, at least in part, to the pro-inflammatory cytokine production of IL-6 and TNFα. Also, all the groups under the administration of probiotic extract of *Lactobacillus Casei* were compared to the *Calandula Officinalis* group in the male rat paw and it was seen that there was no significant difference between them.

Conclusion: The results of this study showed that LPE cream can improve inflammation effects in hand paw edema.

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INTRODUCTION

Inflammation is a complex biological response from body in response to harmful stimuli such as pathogens, damaged cells, or foreign organisms [1]. Inflammation is a protective response where the process of elimination of harmful stimuli happens in addition to treatment of damaged tissue [2]. All the skin's layer must prevent the infection and entry of harmful substances by controlling the exit of water and nutrients [3, 4]. At the beginning of this process normal microbiotics of skin will potentially initiate a competitive elimination of pathogens which, could be facilitated by use of probiotics [5]. The non-steroidal anti-inflammatory drugs (NSAIDs), opioids, and corticosteroids are the most commonly used drug in treatment of inflammation [6]. Various side effects are associated with the use of NSAIDs such as gastrointestinal problems like gastrointestinal bleeding and peptic ulcer, that could be due to direct or indirect stimulation [7]. Thus, there is a need for more anti-inflammatory agents with safer profile. Several researches have been focused on understanding the ways skin acts as a barrier and to increase the protective effects of skins. Similarly, the effects of probiotics on skin health and its mechanisms have been investigated.

The term "Probiotics" was first proposed as "microbial derived factor promoting the growth of other microorganism" in 1965 by Lilly and Stillwell [8]. Probiotics are defined as supplement containing live bacteria that have beneficial effects on host organism by restoring the balance of beneficial bacteria [9]. Probiotics have been successfully used in treatment of gastrointestinal disorders, such as inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), intestinal infection, and antibiotic-induced diarrhoea [10-12]. Moreover, probiotics by conditioning and increasing the mucosal immune response can improve the immune system and the intestinal microbial flora can be marketed as various immune boosting products [13].

Probiotic microorganism has shown to boost the skin health condition when used topically or orally. Oral uses of probiotics have been studied extensively in recent years. Probiotics may show preventive effect or use in treatment of skin conditions [14]. It has been shown that, probiotics can reduce the allergic skin reactions and increase the protective action of skin [15, 16].

IL-6 is a pre-inflammatory cytokine that is induced by LPS along with IL-1 and TNF. IL-6 is used as an indicator of activation of anti-inflammatory cytokines. It is necessary to mention that IL-6 show both pre-and/or anti-inflammatory actions [17].

The aim of this study is to confirm the anti-inflammatory effects of topically applied a probiotic extract on inflammation produced by carrageenan on paw of male *wistar* rat. Moreover, we aim to investigate if the *Lactobacillus casei* extract plays a role in reduction of IL-6 that has an important role in inflammatory responses.

MATERIALS AND METHODS

Microorganisms

The probiotic strain was *Lactobacillus Casei* (PTCC: 1608). The freeze dried sealed glass ampoules of microorganism used in this study were bought from Persian Type Culture Collection (PTCC).

Microbial Culture

The culture media use for propagation as well as identification of bacteria included, de Man Rogosa Sharpe (MRS) broth an agar, both obtained from Merck, Hemedia, Germany. Bacteria was cultured in MRS broth medium for 24 hours at 37°C and maintained on MRS agar, anaerobic glove box with 95%N_2, 5%H_2, 6%O_2, and 5%CO_2 (Anoximat incubator, Germany).

Growth Rate Determination of *Lactobacillus Casei*

For estimation of the growth rate of *Lactobacillus Casei*, 1 ml (equal to 100 Cfu/ml) of the fresh cultured of probiotic bacteria was inoculated to 100 ml of MRS broth. Then flask was shacked and incubated at 37°C for 42 hours, and the growth rate, every three hours were measured by Optical-Density [18].

Preparation of Supernatant Cell-free Probiotics Bacteria

After 24 hours, the *Lactobacillus* strain was grown anaerobically in 100 ml of MRS broth at 37°C. For this purpose, Supernatant was achieved by centrifuging the media at 4000 rpm (for 15 min at 20°C). Then supernatant was passed across a sterile filter (0.22 µ-pore-size), was collected as the mixture of metabolites of probiotic and was kept at 4°C [18].

Preparation of Stable Probiotic Powder

In order to increase the stability of probiotic supernatant obtained, as well as increase the strength of its impact, freeze–drying method was used. Finally, the samples were freeze-dried at -50°C (Alpha 2-4 LD plus CHRIST) and stable powder was obtained after 48 hours.

Cream Preparation

Water in oil emulsion base was chosen for its emollient and detergency properties. The base was mainly composed of liquid paraffin, tri ethanolamine, Glycerine, Tween 80, Stearic acid and water. Then, different amounts of the ingredients were incorporated together and formulations were compared regarding their extent of oil phase, the viscosity of the product, and the amount of the emulsifier added in the final preparation. Different formulations were evaluated regarding their appearance, particle size and phase homogeneity, emolliency and pH, and the best was chosen. Then, the required amounts 2%, 4% and 8% w/w of the LPE was added to make a proper formula having the best anti-inflammatory activity. Finally the best percentage of the LPE extract was determined and physicochemical stabilities were evaluated [19].

Long Term Stability Study

The acquired supernatant was stored for 9 months at 4°C. The value of pH was determined using a digital pH-meter (Met Rohm ®) during storage coarse and results were recorded.

Physical Examination

After the creams were set in the container, the homogeneity of each cream was tested by visual inspection. They were also examinated for their physical appearance and the presence of any aggregation [20].

Determination of pH

2g of each cream formulation was dissolved in 10 ml of deionized water and their pH was measured using a digital pH- meter (SL.901). Each test was performed in triplicate and the results were reported as Mean±SE…

Grittiness

Each formulation was microscopically evaluated if any no appreciable particle was seen under light microscope [21].

Thermal Cycle Test

The portion were stored at 5°C for 48 h and then at 25°C for 48 h. The procedure was repeated 6 times and their stability and appearance were evaluated [22].

Thermal Change Test

Three 20g portion of each formulation were stored at 4-6°C, 25°C and 45-50°C. After 24 h, one and three months, their stability and appearance were evaluated [22].

Freezing and Thawing

20g portion of each formula were stored periodically at 45-50°C and 4°C for 48 h. The procedure was repeated six times and their samples were checked regarding their appearance and stability [22].

Animals and Experimental Design

In this study, all of procedures were carried out in accordance with the ethical standards of Ahvaz *Jundishapur University of Medical Science*. About 36 Albino *Wistar* male rats (weighing between 180-200 gr) were purchased from the animal house of *Ahvaz Jundishapur University of Medical Science*. The animals were fed on conventional diets and tap water *ad libithum* and adapted during a ten-days period and maintained in under standard conditions, such as room temperature of 23±2 °C, relative air humidity 50%, and photoperiod light/dark of 12-hour.

In this study, the animals were randomly divided into 6 groups (n=6). At first, the volume of intact rats paw was measured in all groups using a plethysmometer. The first group served as a negative control while the second, third and fourth groups received different doses (2%, 4% and 8%) of LPE cream and fifth group as a positive control (piroxicam 2%). Each ointment at 0.3 g of cream containing 2-8% of LPE cream were applied to the plantar surface of the hind paw by gently rubbing 50 times with the index finger [23]. Carrageenan (1%) in 0.9% normal saline was prepared 30 minutes before each experiment session and a volume of 0.05 ml was injected into plantar side of hind paw of the rats. Carrageenan injection in all groups [24, 25]. The ability of anti-inflammatory agents to suppress paw inflammation was expressed as a percent inhibition of paw edema and calculated according to the following equation:

$$\%\ Relative\ Paw\ Edema = \frac{v1 - v.}{v.} \times 100$$

where v. = The animal paw volume before injection of irritant

V1 =The paw volume after drugs and injection at different time points.

Immediately after testing, in end fifth hour, the animals were anesthetized with ketamine (50 mg/kg) and xylazine (10mg/kg). For cytokine assay, a part of hand paw was excised, and the sample was subsequently homogenized in cold potassium phosphate buffer (PBS 0.05 M, pH=7.4). The homogenate was centrifuged at 5000 rpm for 10 minutes. Obtained clear supernatant was stored at -80°C prior to analysis for subsequent measurement of IL-6 [23].

Determination of IL-6

The anti-inflammatory cytokines are a series of immune regulatory molecules that control the pro-inflammatory cytokine response. In this study, the IL-6 levels were determined by the sandwich ELIZA method.

Histopathology

For histopathology assessment, tissue of rat paw from all groups were fixed in 10% formalin solution and embedded in paraffin. Sections were then deparaffinized, stained with hematoxylin and eosin [26].

Statistical Analysis

Results were presented as the Mean ± S.D. The statistical significance of differences between groups was determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. A probability value of $P< 0.05$ was assumed to indicate statistical significance.

RESULT

Growth Kinetic of *Lactobacillus Casei* in MRS Culture

Lactobacillus Casei took approximately 9 hours to reach the log phase with generation time (TG) in 1 hour (**Figure 1**). This figure also displays the time course of lactic acid production and pH gradient of culture medium during generation of probiotics.

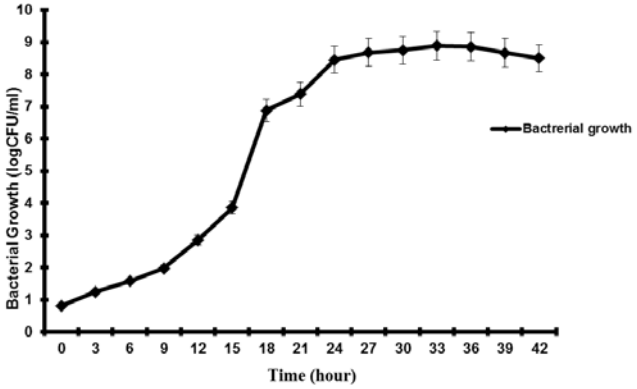

Figure 1. The Kinetics of Growth of in MRS Media Incubated at 37°C for 42 hours under defined microaerophillic condition

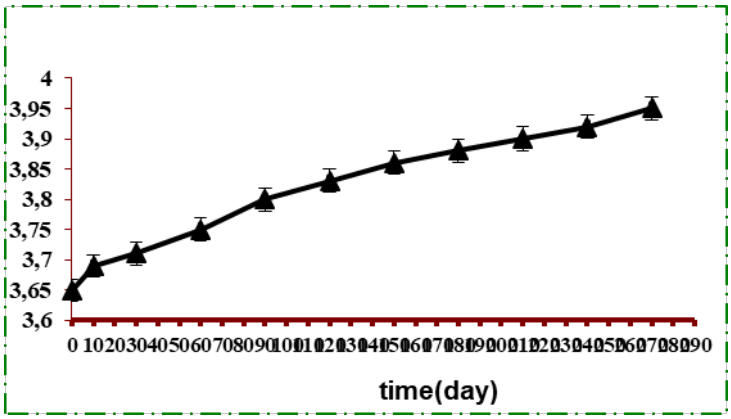

Figure 2. Long term stability of supernatant of *Lactobacillus casei* stored at 4°C for 9 months

Table 1. Evaluation data of pH of LPE creams (n=3)

Formulation code	pH				Storage time
	pH_1	pH_2	pH_3	Mean ±SD	
LPE 2%	5.48	5.48	5.48	5.48±0	After 48 hours
	5.38	5.38	5.39	5.38±0.005	After a week
	5.39	5.38	5.40	5.39±0.01	After a mount
	5.39	5.38	5.38	5.38±0.005	After3 mounts
LPE 4%	5.39	5.40	5.41	5.4±0.01	After 48 hours
	5.30	5.31	5.30	5.30±0.005	After a week
	5.30	5.32	5.31	5.31±0.01	After a mount
	5.31	5.30	5.31	5.30±0.005	After3 mounts
LPE 8%	5.32	5.33	5.33	5.32±0.005	After 48 hours
	5.27	5.28	5.29	5.28±0.01	After a week
	5.22	5.22	5.23	5.22±0.005	After a mount
	5.21	5.21	5.22	5.21±0.005	After3 mounts

Long Term Stability of Supernatant

Evaluation of pH values of acquired cell free supernatant showed a few variations during 9 months storage in 4°C, but even these low changes could bias the stability of supernatant properties (**Figure 2**).

Physicochemical Evaluation of Creams

The appearance of formulated creams remained clear and no significantly change in stability after 3 months' period. The result for pH, homogeneity and grittiness are shown in **Tables 1** & **2**.

Table 2. Evaluation data of homogeneity, grittiness and stability of formulations (n=3)

Property	Formulation code		
	LPE 2%	**LPE4%**	**LPE8%**
Homogeneity test	Homogenous	Homogenous	Homogenous
Creaming and coalescence	Stable	stable	stable
Thermal cycle test	Stable	stable	stable
Freezing and thawing	Stable	stable	stable
long term stability	Stable	stable	stable
appearance	clear, good flow	clear, good flow	clear, good flow

Carrageenan-induced Edema

The mean hind paw volumes at 5 h after the injection of carrageenan solution in the groups treated with control group, 2% ,4% and 8% w/w LPE, and 2% piroxicam were increased, and the swelling rations calculated for the groups was measured. In the 4% and 8% w/w LPE groups, the swelling ratio was significantly less than that in the control group ($P<0.05$). The swelling ratio in the positive control group was also significantly less than that in the control group ($P<0.05$).

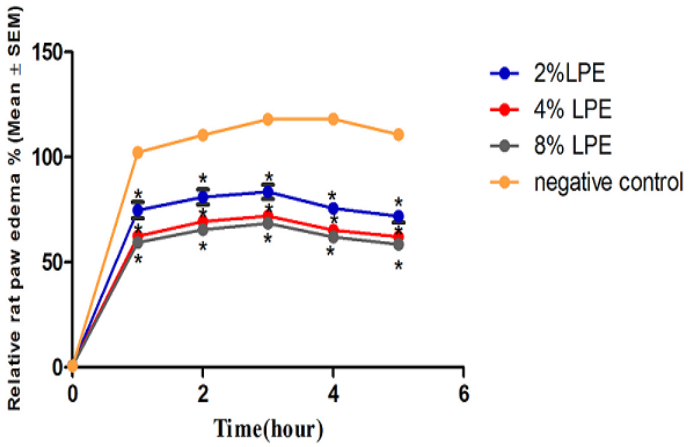

Figure 3. Comparison of reduction of carrageenan-induced paw edema in rat between different doses of LPE (2%, 4% & 8% w/w) and Negative control. Value are expressed as Mean±SEM; the number of rats in each group is 6 (n=6). *Significant when compared with respective negative control P≤0.05.

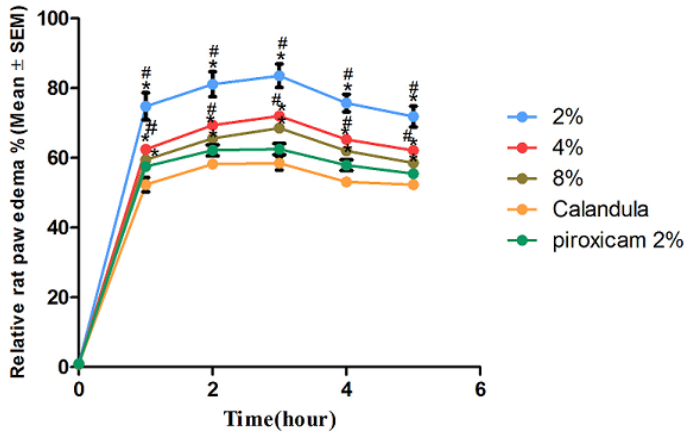

Figure 4. Comparison of reduction of carrageenan-induced paw edema in rat between different doses of LPE (2%, 4% & 8% w/w) and positive control (Piroxicam & Calandula). Value are expressed as Mean±SEM; the number of rats in each group is 6 (n=6). *Significant when compared with respective Calandula group P≤0.05. # Significant when compared with respective piroxicam P≤0.05.

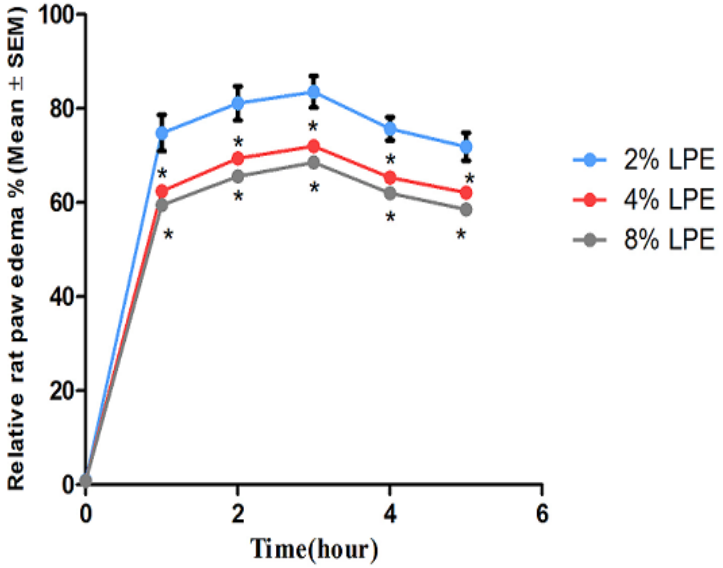

Figure 5. Comparison of reduction of carrageenan-induced paw edema in rat between different doses of LPE (2%, 4% & 8% w/w). Value are expressed as Mean±SEM; the number of rats in each group is 6 (n=6). *significant when compared with respective 2% w/w cream of LPE group P≤0.05.

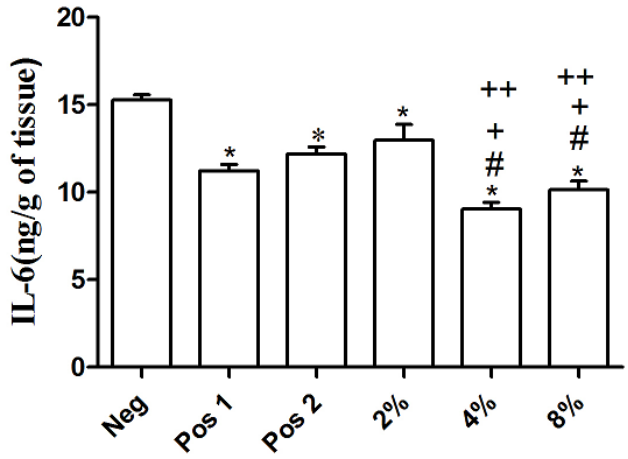

Figure 6. Effect of LPE formation in carrageenan-injected paws, different doses of LPE (2%, 4% & 8% w/w) was administered 30 min before carrageenan injection. At 5 hours after the carrageenan injection, the paw exudates were collected for IL-6 measurement, using EIA kits. Value are expressed as Mean±SEM; the number of rats in each group is 6 (n=6). *Significant when compared with respective negative control (normal saline) group P≤0.05. #Significant when compared with respective Pos1 (Calandula) group P≤0.05. +Significant when compared with respective Pos2 (piroxicam) group P≤0.05. ++Significant when compared with respective 2% LPE group P≤0.05.

Histopathological Results

Histopathologic results showed severe inflammation in the dermis of sham group. Diffuse and vast accumulation of inflammatory cells was seen. Also pink exudate filled spaces between connective tissue of the dermis (**Figure 7**). Section of paw in Cream 2% group showed severe inflammation in the dermis (**Figure 8**). Group Cream 4% showed mild inflammation with a few inflammatory cells accumulated in the dermis (**Figure 9**) and inflammation reaction were decreased in this group in comparison with sham and cream 2%. In cream 8% Group, pink exudate was accumulated in the dermis (**Figure 10**). Positive control showed relatively normal structure of paw skin (**Figure 11**).

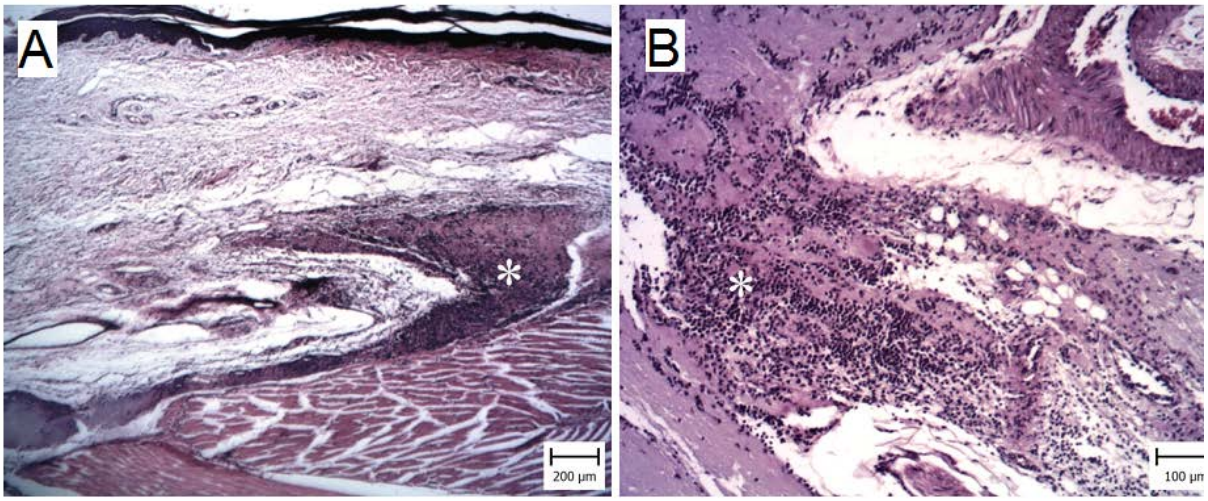

Figure 7. Paw skin of rat. Sham. (Hematoxylin and Eosin staining). A: Note to dark area in the dermis (asterisk) (Bar: 200 µm). B: Accumulation of inflammatory cells in the dermis (Asterisk) are seen (Bar: 100 µm).

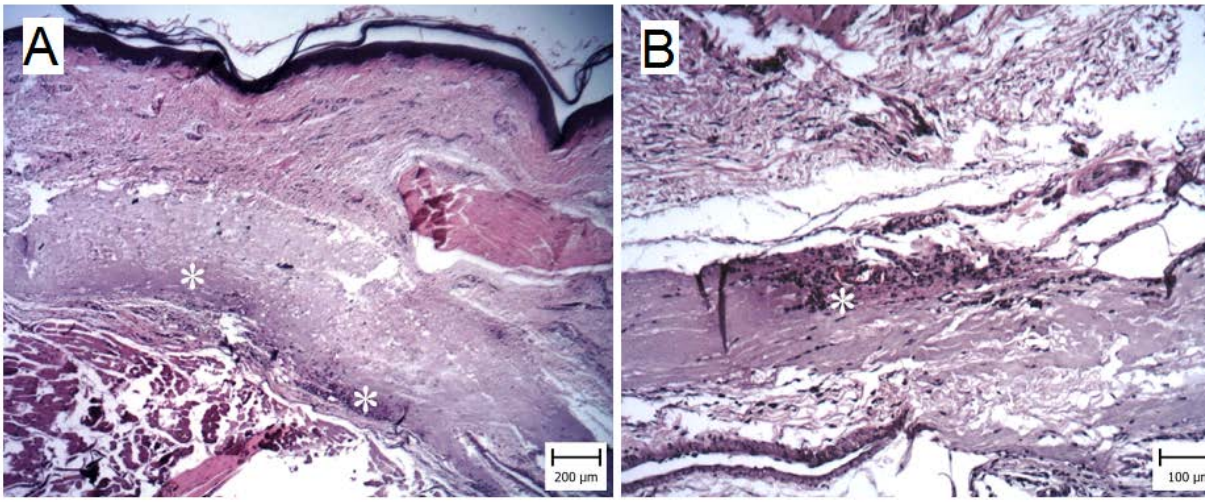

Figure 8. Paw skin of rat. Cream 2% group. (Hematoxylin and Eosin staining). A: Note to massive accumulation of pink materials in the dermis (asterisks) (Bar: 200 µm). B: Accumulation of inflammatory exudate in the dermis is seen (asterisk) (Bar: 100 µm).

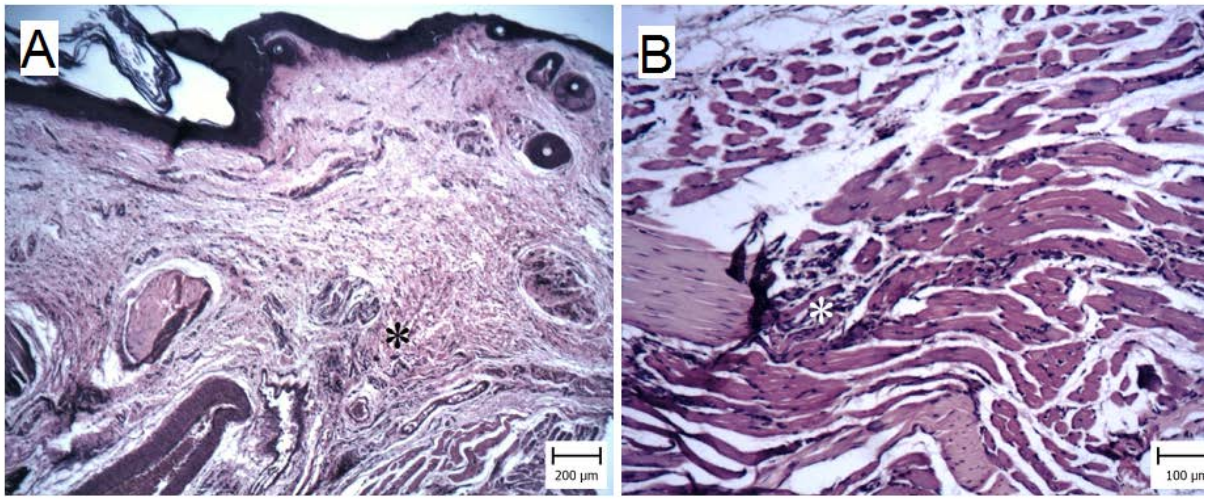

Figure 9. Paw skin of rat. Cream 4% group. (Hematoxylin and Eosin staining). A: Note to relatively normal structure of dermis (asterisk) (Bar: 200 µm). B: Low numbers of inflammatory cells (asterisk) are seen (Bar: 100 µm).

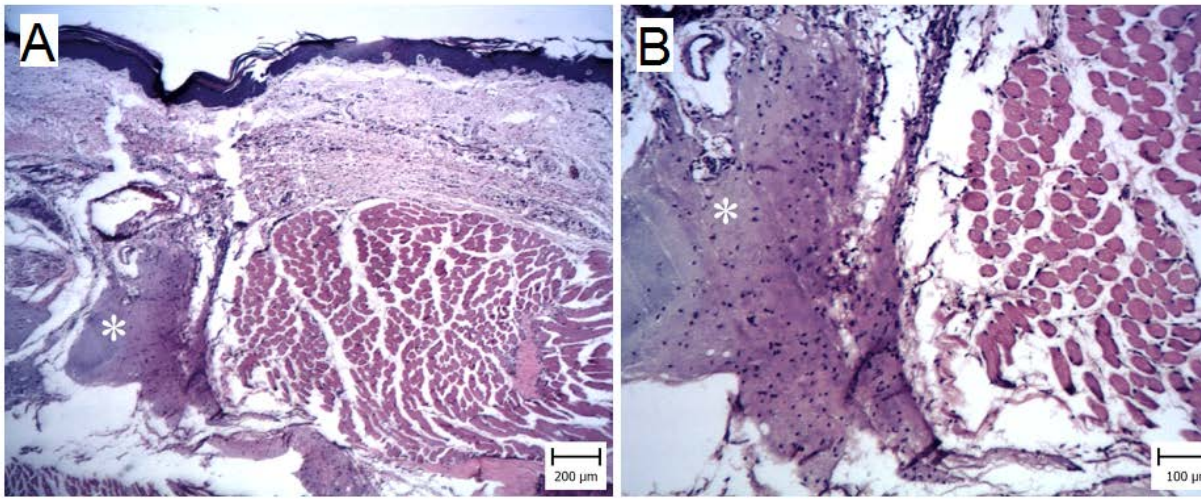

Figure 10. Paw skin of rat. Cream 8% group. (Hematoxylin and Eosin staining). A: Small area of pink materials accumulation is seen in the dermis (asterisk) (Bar: 200 µm). Note to inflammatory exudate accumulated around vessels (asterisk) (Bar: 100 µm).

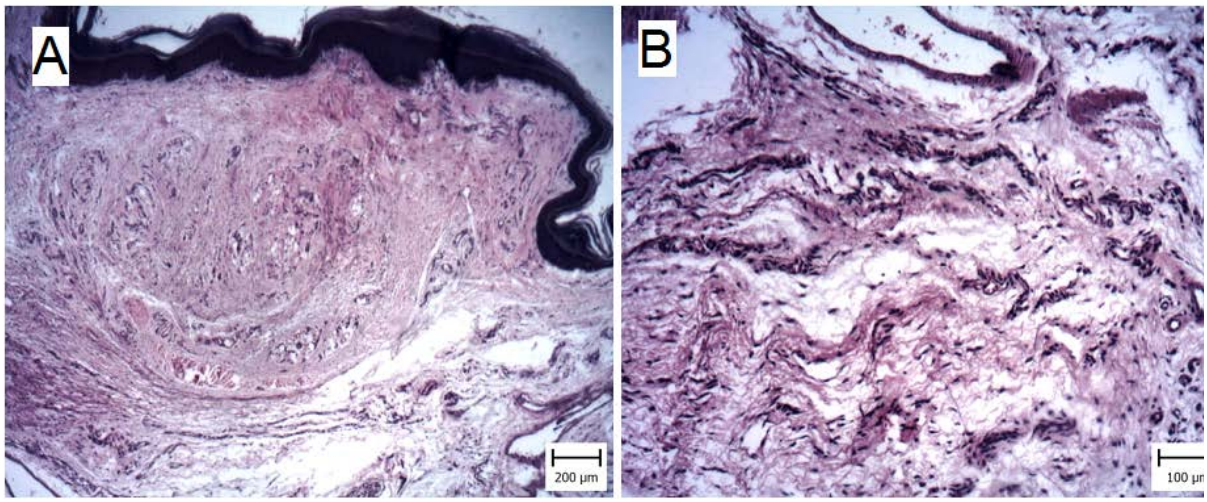

Figure 11. Paw skin of rat. Positive control. (Hematoxylin and Eosin staining) A: Note to normal structure of dermis (Bar: 200 µm). B: No accumulation of inflammatory cells and exudate are seen (Bar: 100 µm).

DISCUSSION

Inflammation is the first biological response produced by immune system against any infections, poisons, and foreign allergen in order to protect the body [27]. The main option for inflammation is Non-steroidal anti-inflammatory drugs (NSAID) such as piroxicam. Despite the broad range of uses of these drugs, the gastrointestinal side effects limit their use [28]. Thus, using natural anti-inflammatory compounds without these side effects can be an alternative option.

In conventional drug delivery methods such as oral or IV route, the drug is dispersed in the blood stream and to achieve the desired therapeutic effect larger dose of drug is required which results in an increase of the side effects. Many studies in recent years have focused on development of an ideal topical formulation for treatment of various skin conditions with a special focus on bio-friendly natural compounds with lower side effects [29].

In order to study the anti-inflammatory effects of *Lactobacillus casei* extract, a carrageenan induced (1% w/v) oedema model was used. Carrageenan induced oedema model is widely used to study the inflammatory process and to screen anti-inflammatory drugs. Carrageenan induces a two-phase inflammatory response. The initial phase starts within the first 2-3hrs following carrageenan injection and the second phase occurs 3hrs after injection [30].

Briefly, probiotics bacteria exert their beneficial effects via two mechanisms; I) direct effects of live cells, II) indirect effects via producing wide variety of metabolites or biogenics [31].

This study demonstrated that the main beneficial effects of *Lactobacillus casei* were related to their metabolites. To improve the stability of supernatant and also its concentrating, freeze-drying was applied as a suitable method. During freeze-drying, dehydration was occurred without exposing the obtained bioextract (or supernatant) to high temperatures which led to the preservation of its structure [32, 33].

The pre-inflammatory cytokines such as IL-6 are cytokines that have dual physiological actions. They facilitate the inflammatory process and result in immediate inflammatory response. On the other hand, they release anti-inflammatory mediators on other cells and alleviate the inflammation [34].

The control experiment and stability of formulation was conducted 72hrs after preparation. The formulations were microscopically uniform without any air bubble and palpable particles. In the stability studies that included temperature change, heating and cooling, and freezing and melting, the basic formula and other formulations were stable up to 3 months and no visible change such as change in colour or viscosity was observed.

In preparation of an emulsion types of additives, emulsifier, and pH are important factors to be considered. To prepare a stable and reproducible formulation the physiochemical properties of each ingredient must be analysed and comply with standards. The formulation component must not result in an undesirable chemical reaction during preparation or storage. To prepare the formulation in this study, we have used a basic formula containing 10% stearic acid, 30% organic oil, 1% glycerol, 2.5% tri-ethanolamine, and water for the remaining. The other formulations were then made by changing this basic formula.

In this study, all doses of 2%, 4% & 8% w/w topical *Lactobacillus casei* extract cream was rubbed thoroughly on the paw of *wistar* rats in treatment groups and after 30 min 100µl of carrageenan was injected S.C on the paw. The anti-inflammatory response was then compared with positive control groups (Piroxicam and *Calandula* treated) and negative control (placebo).

Our results suggested that the 4% *Lactobacillus casei* extract had the optimum anti-inflammatory response. The 2% w/w formulation did not reduce the initial oedema and thus was ineffective.

The inhibitory effect of pretreatment with LPE ointment on carrageenan-induced rat hind paw edema was examined at 5 h after injection of the polysaccharide, because the maximal swelling of the hind paw in response to carrageenan injection is found to be around 5 h after injection [31].

The present study showed that the administration of the probiotic extract, causes a significant difference ($p<0.05$) compared to the negative group in measuring the rat paw edema. Also, the 2% dose of LPE proved to be less effective than the same dose of Piroxicam cream in reducing inflammation. On the other hand, no significant difference was seen between the groups receiving 4% and 8% w/w of LPE. However, the 4% dose of LPE cream turned out to be the most favorable. In the end, comparing the groups under the administration of the probiotic extract of *Lactobacillus casei* with the *Calandula officinalis* group in measuring the male rat paw edema showed that there was no significant difference between them.

To confirm the research results and shed light on the importance of inflammatory cytokine in carrageenan-induced inflammation model in male rat hind paw, the amount of IL-6 content was also assessed. The results showed a significant increase in IL-6 content in plasma in the Neg. group in comparison with the LPE treated groups ($P<0.05$). On the other hand, the dose of 2% w/w of IL-6 in the treatment group shows no significant difference with the Neg. group, but 4% w/w and 8% w/w dose prove to be significantly effective in reducing inflammation in the treatment samples.

From this study, it can be concluded that the prepared probiotic topical cream, due to it's efficacy and safety can be used for the treatment of inflammation. However, it is needed for in vivo application to examine clinically the prepared cream containing LPE in more detail.

CONCLUSIONS

Generally, the anti-inflammatory response of *lactobacillus casei* extract is dose-dependent and the best response was observed in 4 and 8% w/w formulations. As there was no statistically significant difference observed between these two doses and the lower chances of side effect associated with smaller dose, the 4% w/w extract was chosen as the optimum dosage concentration.

From the observed results of from this study and other studies on probiotics, we proposed that probiotics could be a safe and effective alternative to NSAIDs. Although additional studies on different animal models and human are required to confirm this.

A statistically significant difference between the anti-inflammatory response of 2% and 4% LPE cream was observed. There was no statistically significant difference ($P>0.05$) observed between 4% LPE cream and Piroxicam. Although the anti-inflammatory response of Piroxicam was higher, the safer profile and lower side effects associated with probiotics makes them an appealing alternative in treatment of inflammation.

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AUTHORS' CONTRIBUTION

Saadatzadeh, Sistani karampour & Rezaie designed the experiments.

Imari & Pashmforosh carried out the analysis tests. Saadatzadeh & Sistani karampour prepared the manuscript.

FINANCIAL DISCLOSURE

None declared.

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