DETERMINATION SOME IMMUNOLOGICAL AND HISTOPATHOLOGICAL PARAMETERS RELATED WITH ROSEOMONAS MUCOSA AND ITS PIGMENT IN PHARMACEUTICAL FORMULAS.

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ABSTRACT

This study was aimed to conducted as a challenge test for *R. mucosa* ability to reduces atopic dermatitis induced in the skin of experimental animals and improve the fact that this kind of microorganism play important role as skin microflora to reduce some type of skin diseases, additionally to find the suitable pharmaceutical formula can be maintaining and stimulating bacterial isolate to produce a pigment. A probiotic application as a suitable cream formulas were prepared containing local *R. mucosa* biomass, Standard *R. mucosa* biomass and partial purified pigment to treat three groups of albino mice induced with atopic dermatitis. The main physical characteristics of the prepared cream formula are homogeneous with odorless, smooth texture, and clarity of its light yellowish color. Some immunological test including (CBC, lymphocyte, neutrophil, platelets), total serum IgE and histopathological analysis were also done. The *in vivo* result improve the effectivity of all prepared formulas particularly local *R. mucosa* biomass formula in reducing induced AD in mice either externally or internally. The findings suggested the safety of topical *R. mucosa* therapy of lab. animals induced with AD.

Keywords: *Roseomonas mucosa*, atopic dermatitis, lymphocyte and neutrophil.

تحديد بعض العوامل المناعية والنسيجية ذات العلاقة بالتركيبة الدوائية لبكتيريا *Roseomonas mucosa* وصبغتها.

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المستخلص

الهدف من هذه الدراسة هو أجراء اختبار التحدي لقابلية بكتيريا *Roseomonas mucosa* على التقليل من تأثير التهاب الجلد المستحث على جلد الحيوانات المختبرية واثبات حقيقة دور بعض الأحياء المجهرية التي تعيش بصورة طبيعية على الجلد في تقليل بعض انواع الامراض الجلدية، فضلاً عن ايجاد التركيبة الصيدلانية المناسبة للحفاظ على العزلة البكتيرية وتحفيزها لإنتاج صبغة. تم تحضير التراكيب الملائمة الحاوية على العزلة المحلية , العزلة القياسية والصبغة البكتيرية لعلاج 3 مجاميع من الحيوانات المحتبرية المستحثة بالتهاب الجلد التحسسي. الخصائص الفيزياوية للتركية المحضرة هي متجانسة, عديمة الرائحة, ذات قوام ناعمة ولون أصفر فاتح. بعض الطرق المناعية المتضمنة (فحص الدم الشامل, الخلايا اللمفاوية, الخلايا العدلة, الصفائح الدموية), اجمالي مستوى IgE في المصل والتحاليل النسيجية. برهنت نتائج اختبار التحدي على قابلية جميع التراكيب المحضرة وخصوصاً تركيبة البكتيريا المعزولة على تقليل استحثاث التهاب الجلد التحسسي في الفئران داخلياً وخارجياً. اظهرت النتائج سلامة العلاج الموضعي على الحيوانات المختبرية المستحثة بالتهابات الجلد التحسسي.

الكلمات المفتاحية: بكتيريا *Roseomonas*, التهاب الجلد، الخلايا اللمفاوية، الخلايا العدلة.

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INTRODUCTION

Atopic dermatitis is a common, complex, chronic, relapsing, inflammatory skin disease that primarily affects young children (3). Dermatitis and *eczema* are often used synonymously, although the term eczema is sometimes reserved for the acute manifestation of the disease. Its etiology involves interactions between the internal immune system and external factors (18). AD results in the epidermal barrier dysfunction and over activation of the immune response to increase inflammation when exposed to allergens. Eosinophils and mast cells contribute to IL-4 and IL-13 production and release that induces serum immunoglobulin E (IgE) concentrations (10). As a result, AD induces severe itching, dry skin, lichenification, eczema and edema by damaging skin barriers and inducing persistent inflammation (14). However, there are no effective interventions that address the fundamental causes of AD. The pathology of the skin lesions and immunological alterations of Nc/Nga mice are similar to human atopic dermatitis and they are commonly used animal models for AD research (21). Nc/Nga mice will spontaneously develop AD-like lesions and exhibit the characteristic elevation of plasma IgE concentrations but do not induce the skin lesion coverage over a normal circumference (14). Local application of 2, 4-dinitro-1-chlorobenzene (DNCB) can initiate the development of AD-like skin lesions in Nc/Nga mice in a defined region (15).

MATERIALS AND METHODS

***In vivo* test:** Depending on Teeba and Mourouj (19, 20) the following steps were done

Pharmaceutical formula including viable *Roseomonas mucosa* biomass

Preparation of *Roseomonas* biomass: Preparation of *Roseomonas* isolate and standard strain inoculums as follows: from an overnight culture on LB agar (India/Himedia), a few loopfuls of *Roseomonas* growth were inoculated into 10 ml of LB broth (India/Himedia), and then incubated under optimum conditions at 37°C for 24 hrs. After incubation, the numbers of cells were adjusted with 0.5 McFarland standard tube to be approximately $1X10^8$ cell/ml. Thereafter, biomass was collected using centrifuge at 12000 rpm for 15 min, and then a 12.5 ml of LB broth of the optimized medium was added to the precipitant. This cells suspension was kept as usable biomass to be mixed later with the formula.

Preparation of viable *Roseomonas mucosa* formula (100 gm)

An amount of 0.1 gm of methyl paraben was dissolved in 2 ml of absolute ethanol. Then 2.9 ml of glycerol was added with mixing until homogenization, the cells suspension (12.5 ml) in the previous section of $1X10^8$ cell/ml was added. And then an amount of 80 gm of white petroleum Vaseline was added gradually with continuous mixing to homogenize the mixture formula. The prepared ointment was stored in close container at 4°C.

Pharmaceutical formula including partially purified pigment

An amount of 0.1 gm of methyl paraben was dissolved in 1ml of ethanol (70%). A 2 gm of partially purified pigment was added with mixing until homogenization. Then 49 ml of olive oil was gradually added with continuous mixing. And then an amount of 50 gm of white petroleum Vaseline was added gradually with continuous mixing until homogenize all the formula components. The prepared cream was stored in close container at 4°C.

Laboratory animals

A number of (25) albino mice aged (12 weeks) and weighted between (23-25) grams were divided into five groups each group included (5) mice. Control group comprised mice without any stimulus but treated with (Acetone/olive oil) in a percentage 3:1 (v:v). DNCB group comprised mice were sensitized with 200 µL of DNCB (1%) (Sigma-Aldrich, St Louis, MO, USA) dissolved in acetone olive oil solution (acetone: olive oil =1:3) was swabbed on a shaved dorsal skin in the first day and followed by the treatment of exposed region to 100 µL of (0.2%) DNCB every day and continued for one month. Each dose was applied once daily and at the end of sensitizing period, the animals were treated with indicator formula. The 3rd, 4th and 5th groups were induced AD with DNCB as before and treated with a formula containing *R. mucosa* pigment, standard *R. mucosa* and local *R. mucosa* respectively. The experiment time was depended on the time of complete

disappearing of grossly AD signs and this new formula was applied once daily.

Immunological tests

Blood sample (2 ml), were collected from albino mice using disposable syringe. Each blood sample was divided into two parts; 1 ml was kept in EDTA tube and analyzed by automated methods within 4 h and not longer than 24 h after collection using hematology analyzer (Sysmex XP 300) to measure (Red blood cells, different white blood cells, Platelets). Another (1 ml) was kept in a gel tube then centrifuged for 10 min. at 400 rpm, then the serum was collected and estimation the total serum IgE using immunoassay vidas instrument.

Histopathological analyses

The fixed dorsal tissues were embedded in paraffin blocks; tissue sections (4-6 µm) were mounted on slides, Dewaxing with xylene, rehydrated through graded alcohols, and stained with hematoxylin and eosin. Finally, the slides were observed using microscope and examined under 100X.

Statistical analysis

The data were analyzed using the, Microsoft excel, and IBM SPSS V26. The results reported in this study were expressed as mean $\pm$ SD. one-way analysis of variance was used to examine the degree of significance between groups. The degree of significance and Probability values less than 0.05 were considered significantly different (5).

RESULTS AND DISSCUSSION

The *in vivo* experiment was conducted as a challenge test for *R. mucosa* ability to reduces atopic dermatitis induced in the skin of experimental animals and improve the fact that this kind of microorganism play important role as skin microflora to reduce some type of skin diseases, additionally to find the suitable pharmaceutical formula can be maintaining and stimulating bacterial isolate to produce a pigment. As well as this experiment was focused to compare between local and standard *R. mucosa* bacteria and its pigment to treat three groups of albino mice induced atopic dermatitis. The main physical characteristics of the prepared cream formula are homogeneous with odorless, smooth texture, and clarity as light yellowish cream. In this study, *R. mucosa* biomass and its pigment were successfully incorporated into cream (Figure 1). There is several advantage of using topical treatment delivery, including a wide range of serious systemic side effects would be avoided by using this method as (21) mentioned.

Figure 1. Pharmaceutical cream appearance for formulas (components without pigment or bacteria, containing Standard *R. mucosa*, containing *R. mucosa* isolate, and containing *R. mucosa* pigment).

The results of topical induced atopic dermatitis after 4 weeks showed that, all induced animals appeared grossly dark redness and scaly skin, this degree of atopic dermatitis is approximately between second and third degree as (13) documented. These grossly signs of atopic dermatitis are reflected the stimulation of topical inflammation that characterized by increases blood flow in the experimental region of skin that followed by swelling, and local itching. The results of using different formulas of *R. mucosa* biomass and its pigment through monitoring for 10 days approved the affectivity of *R. mucosa* biomass and its pigment to treat or decrease the local effects of induced atopic dermatitis. Complete recovery for all types of induced atopic dermatitis skin was occurred after 10 days of treatment compared with the control groups which appeared scaly skin with disappearance of skin coloration. Additionally, the results appeared no differences between treatment with bacterial biomass and bacterial pigment as shown in (Figure 2). As well as no difference between treatment with local and

standard *R. mucosa*. Therefore, it can be suggesting that the bacterial biomass and its production may be playing an important role in reducing induced atopic dermatitis. Also, this experiment reflected the suitability of formula composed material in maintaining and effectively of bacterial biomass and its pigment.

Types of groups	Induce atopic dermatitis	After treatment
1 st Control negative (without AD induction)		
2 ed Control positive (Induce AD and treated with all additive material of formula except *R. mucosa* or its pigment)		
3 rd group (Induce AD and treated with pigment formula)		
4 th group (Induce AD and treated with standard *R. mucosa* formula)		
5 th group (Induce AD and treated with local *R. mucosa* formula)		

Figure 2. Atopic dermatitis induction and treatment using different formulas according the experimental groups

Immunological tests

Complete blood count (CBC): The results in the recent study showed that there are variation among CBC parameters, first neutrophilia is considered as one of atopic dermatitis marker, The results showed an increase in this cell percentages in three groups of experiment included the following; positive control (78.67±1.15), treatment with standard bacteria (73.0±3.63), treatment with isolate bacteria (35.5±3.53) and treatment with pigment (66.20±2.00), all these groups are recorded significant increasing difference ($p \leq 0.01$) compared with the negative control group (24.17±1.53). Secondly lymphocytes are considered another internal AD parameter

that is elevated during AD induction as (22) documentation; they noted an increase of lymphocyte count in patients with AD. The recent results showed a significant decline ($p \leq 0.01$) in groups of positive control (18.20±0.95), treatment with standard bacteria (28.67±4.21) and treatment with pigment (29.63±3.75) compared with the negative control (67.90±11.75). Whilst no significant difference is recorded in lymphocytes percentages between local isolate treatment group (62.4±4.67) and negative control. The relation between the severity of AD and the lymphocytes count was determined by which stated that the lymphocytes counts are inversely correlated to the disease severity (8). Currently neutrophil and lymphocyte are reproducible laboratory marker, is used to quantify systemic inflammation.

Table 1. Complete Blood Count (CBC) for control and treatment groups.

Parameters	WBC Mean±SD	NEU% Mean±SD	LYM% Mean±SD	MON% Mean±SD	EOS% Mean±SD	BAS% Mean±SD
Control -	4.27±1.3 b	24.17±1.53 d	67.90±11.75 a	1.43±1.17a	1.10±0.10a	0.57±0.25a
Control +	5.81±1.63 a	78.67±1.15 a	18.20±0.95 b	2.07±0.15a	0.20±0.10a	0.90±0.10a
Treatment with pigment formula	10.50±4.7a	66.20±2.00 b	29.63±3.75 b	2.27±3.10a	0.97±0.95a	0.93±0.60a
Treatment with standard bacterial formula	6.91±3.76a	73.0±3.63ab	28.67±4.21 b	1.50±0.44a	0.10±0.10a	0.40±0.16a
Treatment with isolated bacterial formula	5.53±2.04a	35.5±3.53c	62.4±4.67a	0.82±0.91a	0.07±0.12a	1.13±0.74a
P-value	0.190	0.001**	0.001**	0.799	0.127	0.404

¥: One-way Anova was used to test between groups, **: highly significant (P<0.01), Means that do not share a letter horizontally are significantly different

Neutrophils were also essential for some key hallmarks of chronic itch, such as skin hyper innervation, heightened manifestation of itch signaling molecules, and upregulation of inflammatory cytokines, activity-induced genes, and markers of neuropathic itch. Platelets and its relation with other blood parameters are considered also as a marker for AD stimulation. The results in Table 2 revealed a significant increasing in the percentages of platelets in all experimental groups (725.0+195), (519.7+3.05), (625.0+31.0) and (618.0+170.9) for Control positive, Treatment with pigment formula, Treatment with standard bacteria formula and Treatment with isolated bacterial formula respectively compared with the negative control (366.7±1.53). These results are in agreement with (22) showed that platelet was significantly increased in patients with AD compatible to the control group. Therefore, it can be suggesting that the recent results indirectly reflected that both bacterial formulas are failed in reducing some important internal parameters related directly with AD and then modifying by inhibition of AD reactions as (16) mentioned. Activated platelets play an important role in the patho-mechanism of inflammatory diseases such as AD. Their results showed that blood platelets are activated in patients with AD. Other important factors that should be taken into consideration and at the same time considered as a Platelet sub factors, the first one is; the Mean platelet volume (MPV). The second factor is the Platelet distribution width (PDW), both factors are considered as inflammatory markers. The recent results in Table 2 revealed that, no significant differences are recorded among all experimental groups, additionally no significant differences between experimental groups and negative control group. In spite of a study by (12) clarified that, in the AD group, PDW was noticed to be lower than controls as well as in the patients group age having severe AD, they also concluded that, the mean MPV and PDW levels are correlated with Atopic Dermatitis severity in children.

Table 2. CBC parameters and total serum IgE of control groups and treatment groups.

Parameters	PLT Mean±SD	MPV Mean±SD	PDW Mean±SD	IgE Mean±SD
Control -	366.7±1.53 c	6.63±0.90 a	14.73±0.49 a	485.4±10.01 b
Control +	725.0±195 ab	6.10±0.46 a	14.90±0.44 a	861.3±55.3 a
Treatment with pigment formula	519.7±3.05 bc	5.83±0.35 a	13.60±1.13 a	491.0±7.94 b
Treatment with standard bacterial formula	625.0±31.0 ab	6.53±0.95 a	14.90±0.26 a	494.3±6.03 b
Treatment with isolated bacterial formula	618.0±170.9 ab	6.73±0.87 a	15.10±0.50 a	496.0±4.00 b
P-value	0.006**	0.560	0.095	0.001**

¥: One-way Anova was used to test between groups, **: highly significant (P<0.01), Means that do not share a letter horizontally are significantly different

Another test in a concern with the AD patients is considered to be very important in many aspects, especially, it can be considered as a predictable indication of AD. The test is carried out on serum and the total IgE result is to be recorded to show the difference in mean ± SD concentration as carried out in the present study. The recent results showed ordinary values, positive control group recorded a significant elevation (P<0.01) in IgE (861.3±55.3) compared with the negative control group (485.4±10.01) on the one hand, on another hand different treatment formula groups (491.0+7.94),(494.3+6.03) and (496.0+4.00) for treatment with pigment formula , treatment with standard bacteria formula and treatment with isolated bacterial formula respectively are succeeded significantly (P<0.01) in returning the IgE to a normal levels as in negative control value compared with the positive control. These results are compatible with (4) and (13) who they demonstrated that IgE levels were increased significantly in mice that induced AD using DNCB compared with normal control. These results indicate that DNCB acts as a good antigen to induce AD, however, in allergic skin reaction, IgE activates mast cells to induce inflammatory cytokines and the inflammatory response and this cause increase the serum IgE levels. Thus, the recent results are confirmed (7) conclusion that DNCB activates immunity to release IgE from the plasma cells, similar to the immune defense against parasites but it is associated with over-activating immunity. Also, IgE production results in the degranulation of activating mast cells to release histamine which causes itching and edema of the skin (2).

Histopathological analysis

The results of histopathological analysis for the dorsal region of the skin were performed throughout selected one animal for each experimental group at the end of *in vivo* experiment. A dry and scaly rough of positive AD mice (positive control) is hardened than control group. These characteristic signs are occurred due to epidermal and dermal thickness was increased in the DNCB- induced group compared with normal group. The histological section of skin (control positive group) shows folliculitis as hyperplastic squamous epithelium of hair follicles at dermis surrounded by mild-focal infiltration of mononuclear inflammatory cells (Figure 3). Additionally, the positive control improved dorsal skin AD symptoms. The lab. animal responses to different active ingredients formulas are varied according to the type of active ingredients. Positive control group animal was treated with formula containing additive components only, this formula was successes to prevent sever dryness of skin and preserve the slight moistening, simultaneously did not reduces internal factors including cellular infiltration, fibroblast accumulation, edema and decreasing adipose tissue contained. These results are translated in to

lichenification, hardening and thickening of the skin. As well Seunghee and his coworkers (17) explained this study finding that keratinocytes as a major epidermal cell have been stated to produce many pro-and anti-inflammatory cytokines. The pro-inflammatory cytokines such as IL-6 production by keratinocytes are upregulated by inducements such as sensitizer and activate the sensitization flow. The keratinocytes act as a key signal transducers liberating cytokines by chemicals such as DNCB as (11) mentioned. Whereas treatment with pigment is characterized by an increase the number of newly-formed hair follicles at dermis and hypodermis layer, also pigment formula has an inhibitory effect on the thickening and hardening of the dermis and epidermis of AD mice, throughout highly decreasing of edema and inflammatory cell infiltration in the dorsal area of skin. Furthermore, the grossly skin appearances are returned to normal condition. It can be suggesting that, epidermal administration of pigment formula patch significantly reduced epidermal thickness, which may due to reduction of ROS, and NO levels in skin lysate, also suppressed the levels of AD-involved (Th_1 and Th_2) cytokines such as IL-2, IFN-γ, and IL-4 in blood. In addition, the levels of other Th_1, and Th_2 and inflammatory cytokines such as IL-1β, TNF-α, IL-6, IL-12 and IL-10 may be reduced in this group of treatment than in the positive control group. Moreover, the decreasing in total serum IgE level in experimental groups and vice versa in positive control group in the recent study is improved the (9) conclusion that, using antihistaminic drug may reduce IgE level in serum of AD patients. While treatment with standard bacterial strain, the histological section of skin shows many sweet glands at dermis with no hair shift with irregular of reticular layer and increase of sebaceous glands at hypodermis layer. Finally, treatment with isolated bacterial formulas, the histological section of skin shows some area of epidermis was loosed the hair follicles at dermis with no hair shift with irregular of reticular layer, with congested blood vessels. In spite of all these histological characteristic changes for each formula, the newly formula compositions were significantly reduced the dermal and epidermal thickness compared with the positive control as shown in Figure (1-3). These results were agreed with (4) and (13) documentations who they explained the reasons that lead to increase the hardness of skin, which is that the epidermis and dermis in DNCB group showed strong edema and hyperplasia as well as massive infiltration of inflammatory cells while the treatment groups had significantly reduced numbers of infiltrated immune cells and thickness of the epidermis compared with the DNCB group.Also, this study showed that atopic dermatitis induction lead to severe itching, dry skin, lichenification, edema and primary eczema these signs are occurred due to damaging skin barriers and inducing persistent inflammation as (1) mentioned. As well as fillagrin gene plays an important role in maintaining moisture and the skin barrier function, while ceramide, a lipid degraded by ceramidase, performs similar functions. When deficiencies of filaggrin and ceramide impair skin barrier functions, the skin becomes rough and loses transparency, resulting in dry skin as (6) documented.

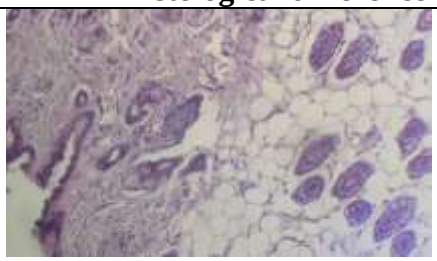
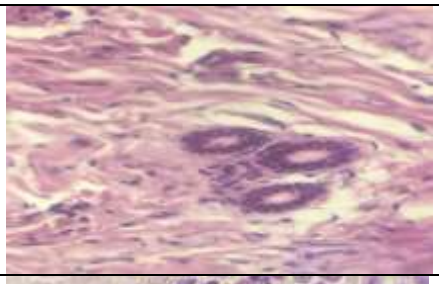
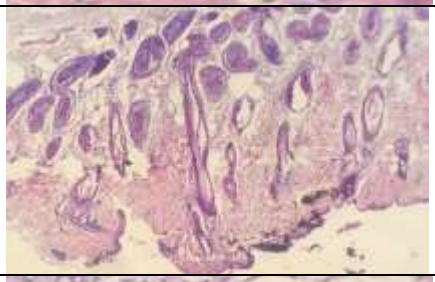
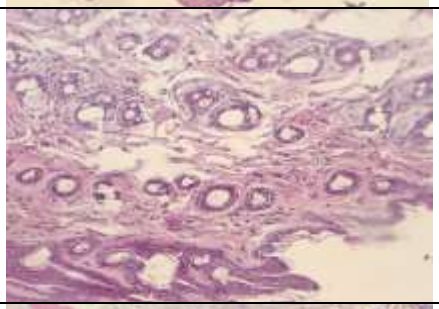
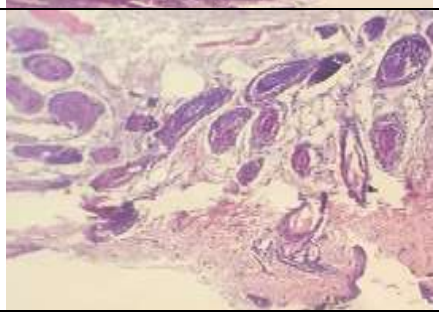
Type groups	Histological difference
Control negative	
Control positive (Induce atopic dermatitis and treated with indicator formula	
3 rd group (Induce atopic dermatitis and treated with pigment formula)	
4 th group (Induce atopic dermatitis and treated with standard *R.mucosa* formula)	
5 th group (Induce atopic dermatitis and treated with local *R.mucosa* formula)	

Figure 3. The histological differences in the dorsal skin

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