تم تدقيق البحث من قبل
الاستاذ:
بتاريخ:
و قد تم تصحيح كافة الاخطاء و كان البحث وفق متطلبات النشر.
توقيع الاستاذ:

THE USAGE OF ß-GLUCAN WHICH EXTRACTED FROM *Saccharomyces cerevisiae* YEAST CELLS WALL AS IMMUNOMODULATOR

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Abstract

Carbohydrates (polysaccharides) were extracted from *Saccharomyces cerevisiae* cell wall by hot alkaline solution, the polysaccharides concentration was 96 mg /ml , while protein concentration was 500 µg /ml ,then primary purification method was done to separated carbohydrates from other protein materials, at this time , carbohydrates concentration was 250 mg /ml, while protein concentration was 148 µg /ml .Then partial purification using Ion exchange technique was done, and the results revealed ,four peaks were separated ,every peak was collected ,dialysed , and measured the carbohydrates concentration which were 300, 50, 33.4 and 32 mg/ml respectively, while Lawery method did not detect any protein materials in samples. The four peaks were examined as immunomodulator through the animal (mice) that were divided into 4 groups which depend upon the peaks series, were injected intraperitoneal and orally administration with constant concentration 500 µg /ml twice, then read the blood picture for immune cells, the second group was reported a significant increasing in all cells which are (2.968 ± 0.316), (1.56± 0.3), (0.546 ± 0.291) Lymphocyte, Granulocytes and monocyte respectively while the cell percentage for first, third, and fourth peaks were for lymphocyte (2.292 ± 0.3)، (1.83 ±0.32) and (2.67 ± 0.5) respectively, for Granulocytes (0.83± 0.23)، (0.53 ± 0.22) and (0.6 ± 0.35) respectively, while for Monocytes (0.3 ± 0.19)، (0.03 ± 0.01) and (0.3± 0.02) respectively when compared with control group. Organs crossly examination was showed hypertrophy of liver and spleen, while microscopically, there were infiltration of lymphocytes around the blood vessels in liver, lung, and peritonea, and in white pulp of spleen.

استعمال ß-glucan المستخلص من جدار خلايا خميرة *Saccharomyces cerevisiae* كمحفز مناعي

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الخلاصة

استخلصت المواد الكاربوهيدراتية (متعدد السكريد) من جدار خميرة الخبز *Saccharomyces cerevisiae* باستعمال المحلول القاعدي الساخن، وكانت نسبة المواد الكاربوهيدراتي ٩٦ مليغرام /مل، في حين بلغت نسبة المواد البروتينية ٥٠٠ مايكرو غرام /مل، تم إجراء عملية تنقية أولية لفصل المواد الكاربوهيدراتية عن ما موجود من مواد بروتينية مرافقه وكانت قيمة تركيز المواد الكاربوهيدراتية ٢٥٠ مليغرام / مل، في حين بلغت نسبة المواد البروتينية ١٤٨ مايكروغرام /مل. ومن ثم أجريت عملية التنقية

الجزيئية للمواد الكاروهيدراتية باستعمال تقنية التبادل الأيوني (DEAE) وأظهرت النتائج انفصال ناتج الاستخلاص الى اربع قمم شكلت أول قمة أعلى تركيز، ثم جمعت القمم وأجريت لها عملية ديلزة وقيس تركيز المواد الكاربوهيدراتية فكان ٣٠٠، ٥٠، ٣٣,٤، ٣٢ مليغرام /مل على التوالي، في حين لم تعطي طريقة لوري مؤشر عن وجود مواد بروتيني، اختبرت القمم الأربعة كمواد مطورة مناعيا من خلال حقن حيوانات التجربة (الفئران) والتي قسمت إلى أربع مجاميع وفق ترتيب القمم بتركيز ثابت من المستخلص والبالغ ٥٠٠ مايكروغرام /مل ولمرتين، ومن ثم قراءة الصورة الدموية للخلايا المناعية، إذ سجلت المجموعة الأولى زيادة معنوية في نسبة الخلايا اللمفاوية والحبيبية وأحادية النواة إذ بلغت، (2.968 ±٠,٣١٦)، (1.56 ±٠,٣) و(0.546 ± 0.29) خلية/ سم^3 على التوالي، في حين بلغت نسبة الخلايا اللمفاوية للمجاميع الأولى والثالثة والرابعة (2.292 ± 0.3)، (1.83 ± 0.32) و(2.67 ± 0.5) خلية/ سم^3، وللخلايا الحبيبية (0.83 ±0.23)، (0.53 ± 0.22) و(0.6 ± 0.35) خلية/ سم^3 وللخلايا أحادية النواة (0.3 ± 0.19)، (0.03± 0.01) و(0.3 ±0.02) خلية/ سم^3 على التوالي بالمقارنة بمجموعة السيطرة. الفحص العضوي اظهر حصول تضخم في الكبد والطحال ومجهريا حصول ارتشاح للخلايا اللمفاوية حول الأوعية الدموية في كل من الكبد والرئة والبريتون، واللب الأبيض في الطحال.

Introduction

The emergence of multiple antibiotics - resistant microorganisms has led to a search for alternative to traditional therapeutic regimens, beta- glucan, an immunomodulater, can selectively enhance the microbicidal activities of neutrophils and macrophages without stimulating pro-inflammatory cytokine production.

yeast glucan is constituted predominantly from 1,3-ß-glucan which is synthesized by 1,3-ß-glucan synthase which is turn consists of a catalytic and a regulatory subunit .ß-1, 3- D-glucan is a complex carbohydrate (i.e., comprised of sugar molecules) which has been demonstrated to have powerful immune boosting and anticancer effects. ß-glucan has been isolated from a Variety of fungi such as mushrooms (1) Yeast cell walls including brewers and baker yeasts (*Saccharomyces cerevisiae*) and from oat and barley bran (2). Research efforts have focused on developing cytokine-mediated immunoprophylactic strategies. A complementary strategy to up-regulate the innate immune response in mice (3). Cells of the innate immunity such as macrophages, phagocytes and natural killer cells have surface beta-glucan receptors, which specifically recognize and bind the beta- 1,3-glucan linkage of the beta-glucan molecule. Lymphocytes belong to the acquired immunity and play a key role in defending the body against disease(4).

ß-glucan enhances immunity through a number of mechanisms, it binds to leukocytes (phagocytes and macrophages) at specific receptor sites and activates their function and tumor fighting activity by stimulating the production of free radicals(5). The WBC signals to engulf disease in tissues that were caused by microorganisms including bacteria, viruses and tumor cells (6) ß-glucan also has efficacy as an effective immune stimulant in a variety of infection diseases (including anthrax) as well as radioprotectant and anti-toxic substance. Glucan is a potent reticuloendothelial – modulating agent whose immunobiological activity is mediated in part by an increase in the number and function of macrophages.

The purpose of this study was to determine the effects of extractable b- glucans obtained from baker's yeast, barley,as immunostimulating agent for leukocytes when used as stimulants in mice.

Materials and methods:

Materials:

Baker ´s yeast (pakmia) from Turkey , H_2SO_4 (analar) European product, Standard glucose sugar (BDH), Standard Acetone European product, Phenol (BDH), Bovine serum albumin (Merk), Coomassai blue stain G-250, NaOH (Merk), Acetic acid (analar) BDH , Hydrogen peroxide (BDH) ,NaCl (BDH), KCl (BDH), K_2HPO_4 (BDH) , Na_2HPO_4.

Methods:

Extraction and purification

This procedure was done according to the method described by Naohito,*et.al*.(7) and modified by researchers

- Homogenization:

Two hundred ml of sodium hydroxide 0.1 M was added to 50 g of baker's yeast with shaking to mix the content with each other for 30 min in water bath at 60 ˚C then left the mixture in refrigerator at 4 ˚C for 18 hrs. after that the mixture was autoclaved for 5 min under 8 psi then cooled.

- Precipitation:

The previous mixture was centrifuged with 4000 rpm for 15 min. The pellet was taken and Acetic acid solution 0.1 M two fold was added, then the mixture was heated by water
bath at 85 ˚C for 30 min to give the time for the reaction to occurred between NaOH and Acetic acid (equilibration) .Then this mixture was placed in incubator at 37˚ C for 15 min, the pellet was taken, and was suspended in distilled water and washed twice then centrifuged at 4000 rpm for 15 min.

- Oxidation:

Hydrogen peroxide solution 50% was added to the pellet in ratio of (2: 1), then mixed well by vortex and placed in refrigerator 4˚ C for 90 min .The mixture was centrifuged with cooling at 10000 rpm for 20 min.

- Drying and Grinding

Acetone was added to the pellet in ratio of (2:1), plus a few drops of distilled water and the mixture was dispersed in petri dishes at room temperature and were left for several days until drying .Then the dried extract was removed from dishes and grined, the yield was soft, off white powder.

- purification

The yield from previous step was taken and resuspended in equal volume of triacetic acid 20%.Then the suspension was filtered by filter paper (Whitman no 1), the precipitant on filter paper was washed with ethanol 98% in three fold as filtered solution .The solution was centrifuged at 3000 rpm/min for 10 min, at 4C in a cooling centrifuge, then the pellet was removed and dissolved in distilled water and dialyzed against distilled water for 3 days (water changed daily).

More purification process was done using Ion exchange (DEAE cellulose) to obtain as possible pure yield.

- Carbohydrate analysis:

Carbohydrate content was determined by phenol - sulfuric acid method (8).
Standard curve for carbohydrate was prepared by using different concentration of glucose (**20, 40, 60, 80, 100**) µg/ml.

- Protein analysis:

The Protein content was determine by the Bradford method (9 and 10).
The protein standard curve was prepared by using different concentration of bovine serum albumin(20, 40, 60, 80, 100) µg/ml.

- Invivo experiment:

Thirty mice were divided into 5 groups, each one contain 6 mice. One of these groups used as control, while other groups were divided according to the peaks of separation (Ion exchange curve).

- Immunization procedure:

This procedure was done according to the method described by (7).
Each animals group except control was injected with 500 µg/ml of each separated peaks intraperitonally. After one week, each animal group was administrated orally with the same dose. The animals were scarified after one week of the last dose. Some organs (Lung, Small and Large intestine, Spleen) were separated and preserved with 10% formalin, in the same time blood smear was prepared from each animal to study the blood picture (Lymphocyte, Monocyte, Granulocyte), after stained by Gimza stain.

- The percent of leukocytes calculation:

The percent of leukocytes was determined microscopically examination for groups were compared to control group using the following formula(11) (L= leukocytes):

$$L\ \% = \frac{\text{L ratio in experiment}}{\text{L ratio in control}} \times 100$$

Results and Discussion:

The yield of Polysaccharide fraction of *Saccharomyces cerevisiae* that were extracted is 7 g/50 g (yeast) and the concentration of protein was 500 µg/ml, while the concentration of Polysaccharide was 96 mg/ml. After primary purification, the protein and carbohydrate of the fraction was measured by a phenol –sulfuric acid method with glucose as a reference (figure 3) using Bradford and Lowry method with Bovine serum albumin as a reference (figure 2), the extract was mainly composed of carbohydrate and contained significant amounts

of protein substances. The results of primary purification showed increasing in the level of carbohydrate concentration which reached to 250 mg /ml, while the protein concentration was decreased to 148 µg /ml, this variation in the concentration of protein was due to the denaturizing effect of the material that were used in primary purification and this lead to increasing in concentration of carbohydrate in solution. The purification procedure was continued using ion exchange DEAE chromatography to increase the purity of polysaccharide in solution. The result of eluted fractions were showed the separation of four peaks (Figure 4), every peak was collected, dialyzed against distilled water and measured the carbohydrates concentration which were (300, 50, 33.4, 32 mg/ml) respectively, while Lowery method did not detect any protein materials in samples this indicates that the procedure was efficient in purification for carbohydrate.

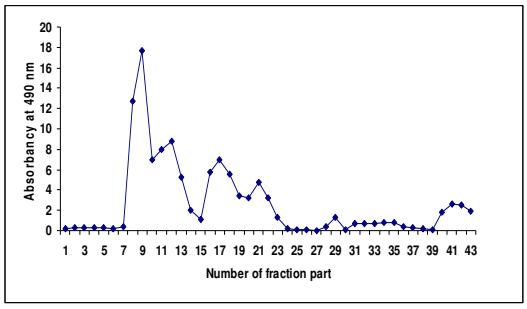

Figure 3: Ion exchange diagram for carbohydrates fractions by usage column (DEAE) in dimension 50 × 2.5 c m .washed with PBS plus KCl 0.1 buffer in flow rate 3 ml / fraction with flow rate speed 36 ml/hr.

Controversial data have been published regarding the immunomodulating effect of yeast derived beta-glucans. Acontribution to this research field has been tried to approach focusing on the viability of lymphocytes, monocytes, granulocytes**.**

The results of invivo procedure were showed a significant differences among the four groups and with the control group in the other hand. The four groups showed increasing in all leukocytes, but the second one was the best (Table 1).

Table 1: represent the mean of leukocytes in mice.

Group	Lymphocyte	Monocyte	Graulocyte
Group 1	**2.292 ± 0.297**	**0.3 ± o.19**	**0.83 ± 0.231**
Group 2	**2.968 ± 0.316**	**0.546 ± 0.291**	**1.56 ± 0.3**
Group 3	**1.83 ± 0.316**	**0.03 ± 0.01**	**0.53 ± 0.22**
Group 4	**2.67 ± 0.5**	**0.3 ± 0.02**	**0.6 ± 0.35**
Group 5 (control)	**1.2**	**0.04**	**0.3**

That indicated, the second peak in the curve of ion exchange analysis was contain the active compound which as act as immunomodulator (picture 1-B), while the other groups (other peaks) were also contain immunomodulator compound but with different activity (Picture 1-A, C, D).

Table 2: represent the ratio of leukocytes in mice groups

	Lymphocyte	Monocyte	Graulocyte
Group 1	**191%**	**750%**	**276.7%**
Group 2	**247%**	**1363%**	**520%**
Group 3	**152.5%**	**750%**	**176.7%**
Group 4	**222.5%**	**750%**	**200%**

Table (2) showed a significant increase in leukocytes and leukocyte ratios for the duration of treatment (21 days), and remained significantly higher than the control levels after exposure to beta-glucan.

This indicated that the yeast cell wall contain active carbohydrate (ß-glucan), but with varied shapes, the active one may be 1, 3-ß-glucan, or 1, 6 -ß-glucan, these two types may appeared as dispersed groups or as a compound groups. This result was in agreement with(11, 12, 13, 14), which they certified the effects of beta- glucan as immunomodulater. On the other hand there was cross enlargement in some organs of animals spleen, liver and several payers batches in the small intestine which mean that there immuno reaction were effected by organs due to the effect of the extract and this result agreed with those of (15, 16).

Otherwise, the results of macroscopical examination showed, that in liver there was perivesicular leukocyte couphing mainly lymphocyte (figure 2A), in lung there was hyperplasia of the peribronchiol associated lymphoid tissue with perivascular lymphoid couphing (figure 2B), in spleen there was hyperplasia of white pulp characterized by infiltration of lymphocyte(Figure 2C), and in peritonea there was infiltration of inflammatory cells mainly lymphocytes and macrophages around the blood vessels in the peritoneal tissues

and there was congestion of blood vessels as show in (Figure 2D).

In this study the group two after one week from last dose, showed increased viability number of lymphocyte, monocytes, and granulocytes in blood and infiltration of lymphocytes in different tissues is higher than other groups, in comparison with control.

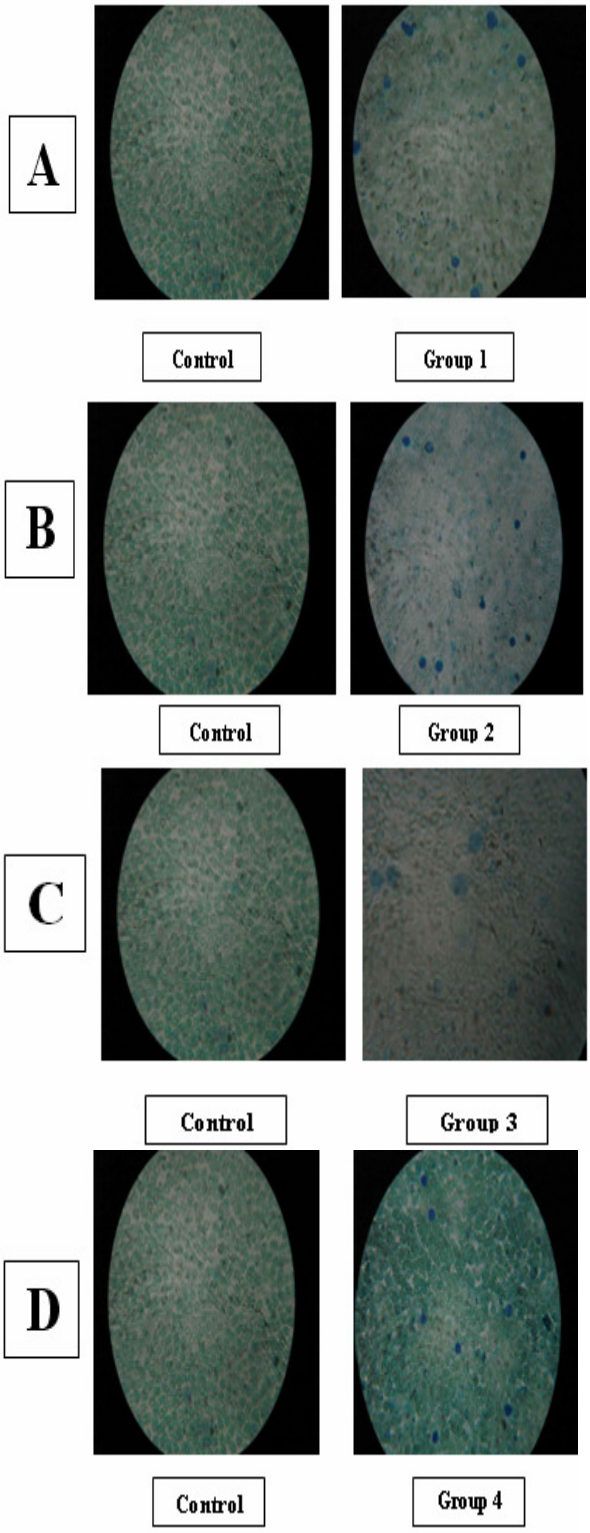

Figure 1: Represent the differentiation in blood picture between different groups

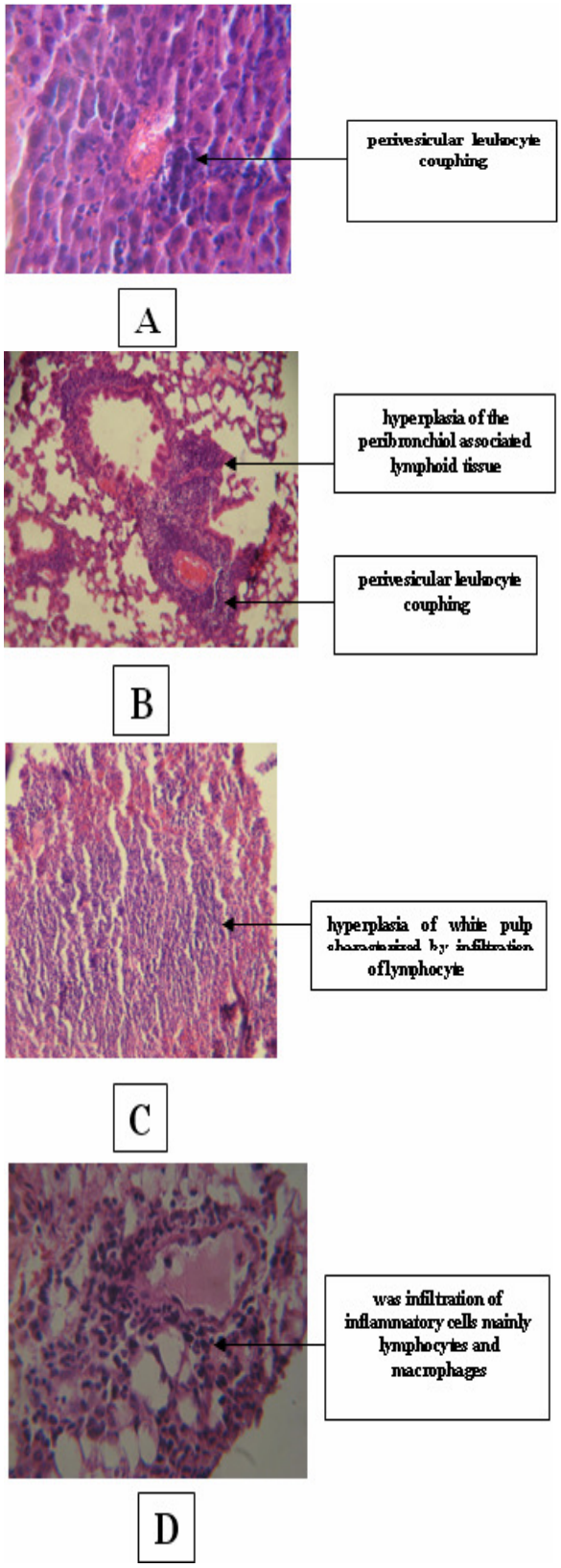

Figure 2: Show the microscopically examination for Liver, Lung, Spleen, and Peritonea tissues

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