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The Cytogenetic Study of Soluble Beta Glucan of *Saccharomyces cerevisiae* on Human Circulating Blood Lymphocytes

Ali J. Jabber[1], Nahi Y. Yaseen[2], Nidhal R. Mahdi[3]

1 *Department Of Microbiology / Medicine Collage / University of Missan .*
2 *Iraqi Center for Cancer & Medical Genetic Research / University of AL-Mustansiriy .*
3 *Department of Microbiology /Collage of Veterinary medicine /Baghdad university*

Abstract:

This study was designed to evaluate the cytogenetic effects of the soluble beta glucan (ß-glucan) local and commercial extracts of the Saccharomyces cerevisiae on human circulating blood lymphocytes at the concentration (5, 50, 500, 1000) µg/ml .The results showed that the effect of beta glucan local extract, commercial extract on mitotic index were significant increased , and the best effect on mitotic and blast indexes at the highest concentration of the Local extract and Commercial .

The mitotic index of control group was represented (20.2) was lower than Local extract (22, 23.8 ,26.2) and (20, 21.6 , 28.8) for commercial extract at (50, 500 , 1000µg/ml) in concentration ,The value of both (Local and commercial extracts) at high concentration is lower than control group that treated with (PHA) which was represented (32.4). While the blast index of control group was (23.3) and the value of treated group exhibited low significant decrease in a concentration dependant manner (19.5, 16.6 and 15) for Local extract, (24, 19 and 17.4) for commercial extract at the concentrations (5,50,500,1000)µg/ml respectively. In commercial extract the blast index value (24, 24.1) was high at concentration (5 , 1000)µg/ml than (50 , 500) µg/ml which were (19,17.4) respectively.

Key word: *ß- glucan (β-G) , Cytogenetic study , Leukemia , Lymphocytes subset , TH1 and TH2 type response , beta glucan immune cells receptors .*

Introduction:

Beta glucan is a scientifically proven biological defence modifier (BDM) that nutritionally potentiates and modulates the immune response (1). through immune response potentiation and modulation, in many instances various therapeutic healing effects generated by the immune cells. For many years glucan have been investigated (History) for these immune enhancing properties (2,3) . β-glucans (beta-glucans) are polysaccharides of D-glucose monomers linked by β-glycosidic bonds. β-glucans are a diverse group of molecules that can vary with respect to molecular mass, solubility, viscosity, and three-dimensional configuration. They occur most commonly as cellulose in plants, the bran of cereal grains, the cell wall of baker's yeast, certain fungi, mushrooms and bacteria (4). β-glucan refers to yeast-derived, isolated from Saccharomyces cerevisiae has been shown to act as a potent non-specific immune-activator , and further efforts resulted in the development of a water-soluble, pharmaceutical grade yeast β-glucan whose biological effects have been extensively in different studies in vivo and in vitro (5,6).

Polysaccharides from fungi have attracted attention in the fields of biochemistry and pharmacology for their immunopotentiation and anti-tumor effects (7) . It can stimulate the polymorph nuclear cells (PMNs) to trigger the secretion of cytokines that will modulate the immune system (8,9) , due to recognition of these compounds by certain receptors located on the leukocytes and other immune cells that lead to enhance the innate and cell mediate immune responses (10,11).

Dendritic cells (DCs) have been recognized as important mediators of immune response in vivo. They are specialized antigen-presenting cells that are highly potent in their presentation of antigen to naïve or quiescent CD4+ and CD8+ T

Corresponding Address:
Ali J. Jabber
University of Missan/ Collage of medicine
Email: dr.alijwad80@yahoo.com
Tel: 07705544996

cells. They capture, process, and present antigens in combination with Major Histocompatibility Complex (MHC) class I and II molecules, activating specific Cytotoxic T lymphocytes (CTLs). This ability to stimulate CTLs directly and effectively makes DCs ideal targets to exploit for manipulation of the immune system for immunotherapy purposes (12).

The best known effects of glucan consist of the direct stimulation of phagocytosis of professional phagocytes (13), and direct activation of natural killer cells , it stimulate macrophages to secrete cytokines such as TNFα and IL-6 . These pro-inflammatory cytokines can potentially enhance the acti¬vation of adaptive immunity, such as antigen presentation and T cell activation , the administration of β-glucan could link the activation of both innate and adaptive immunity , considered to be the basic effector cells in host defense against bacteria, viruses, parasites and tumor cells (14,15,16) . The immunological effects are manifested through its binding to several specific receptors, most of all complement receptor-3 (CR3) and Dectin-1(17,18). The general aims of the study are designed to:

1 - Preparation of solube β-glucan and the determination of their polysaccharides .

2 - Evaluate the activities of the soluble β-glucan extract and commercial from Saccharomyceses cerevisiae on normal peripheral human lymphocytes .

Materials & Methods:

Preparation of soluble beta glucan extract (Local extract)

The soluble β-glucan was prepared as follow :

A - Preparation of particulate β-glucan (from baker yeast)

Laboratory extract of beta-glucan was prepared by extraction procedure from baker yeast (Saccharomyces cerevisiea) as follow :

According to the (19 and 20) the baker's yeast (S. cerevisiae) β-glucan material was obtained from the Market, This material was processed from common, active dry yeast (500gm) then added to one liter 0.1 mol of NaOH and stirred for 30 min at 60 °C. The material was then heated to 115 °C at 8.5 pressure /inch for 45 minute and then allowed to settle for 72 h. The sediment was resuspended and washed in D.W. by centrifugation (350 g for 20 min). The alkali insoluble solids were combined with 0.1 mol of 1L of acetic acid and heated to 85 °C for 1 h, then allowed to settle at 38 °C. The acid insoluble solids were drawn off and centrifuged as above. The compacted solid material was mixed with 3% H2O2 and refrigerated for 3 h with periodic mixing. Then the material centrifuged and the pellet washed twice with D.W. followed by two washes in 100% acetone. The harvested solid material was dispersed on drying trays and dried under vacuum at 38 °C for 2 h in the presence of Ca2SO4, and then further dried overnight under vacuum at room temperature. This procedure yielded a bright yellow powder .

B- Preparation of soluble beta glucan extract (LE) of Saccharomyces cerevisiae

The β-glucan was phosphorylated individually by the improved method for (20) .The fraction (4gm) of β-glucan powder was dissolved in (200) ml of Dimethyl sulfoxide Me2SO containing (72)gm of urea. With stirrer, about (40) ml of phosphoric acid 85% H3PO4 was added drop wise slowly to the above solution at ambient temperature. Then the solution was heated to 100 ◦C , and the reaction was carried out for 6h with stirring. A crystalline precipitate (presumed ammonium phosphate) formed at 1–2 h of reaction. Following heating, the reaction mixture was cooled to ambient temperature and diluted in distilled-water to form a yellow bright solution. Finally, the resulting phosphate derivative was dialyzed (3000 – 5000) Millipore in size against double D.W. for seven days to remove endotoxin (includingMe2SO,H3PO4 and salt).

Carbohydrates Measurement :

Samples containing 1 ml of solvent were mixed with 1 ml of 5% phenol in a test tube cuvette with a 19-mm path length; 5 mL of concentrated sulphuric acid was added rapidly to generate heat to drive the reaction. The reaction mixture was allowed to cool to room temperature, and absorbance was measured at 490 nm in a spectrophotometer (Sequoia-Turner, model 690) against a water blank as described by Dubois (21), with some modification.

Determination of LE β-glucan by high performance liquid chromatography (HPLC) Technique :

Procedure was carried according to (22); (23) .

1 - HPLC colum : Lichrospher C 18 (4.6 mm x 50 mm , 3 µm), particle size , mobile phase : deionized water

2- Detection ; refractive index detector RF shimadzu .

3- Flow rate ; 1.2 ml/minute. at 30 C°.

Preparation of sample :

Ten mg (commercial extract capsule) were dissolved in 250 ml to get 40 µg/ml standard , then 10 drops of lichenase (endo-beta (1-3)D-glucan – 4 – glucanhydrolase) were added to hydrolase the beta – glucan to oligosaccharide .

1 – Beta – cellobiosyl – d- glucose DP3.

2 – Beta – cello triose – D – glucose DP4.

And then 20 µl were injected into HPLC analyzer ,The sequences of the eluted material of the standard were as follow, each standard was 40 µl/ml.

Sequences	Subject	Retention time	area	Concentration µg/ml
1	Beta–cellobiosyl –d glucose DP3	2.51	13265	40 µl/ml each
2	Beta-cello triose-D-glucose DP4	3.35	21171	

The separation was occurred on liquid chromatography shimadzu 10 Av-LC equipped with binary delivery pump model LC – 10 A shimadzu , the spectrophotometer .

This procedures was done according to (24) ; HPLC colum , Lichrospher C 18 (4.6 mm x 50 mm , 3 µm) was performed using with binary delivery pump model LC – 10 A shimadzu ,These pump was used of high pressure that speed the movement of the molecules down the column ,as well as higer quality chromatographic materials that abled withstand the crushing forces of the pressurized flow , by reducing the transit time on the column (25,24). And they were connected with a Spectra-physics AS3500 auto injector and a Shimadzu RID6A refractive index detector controlled with Chromeleon 6.80 software. Serially HPLC columns were connected to a Shodex OHPack SB-LG precolumn and eluted at 30°C with 50 mM Na2SO4 (1.2ml/min), and (20 µl) of CE β-glucan (40µg/ml) prepared were injected using a 100 µL loop. Beta glucan molecular were estimated offline by the software WINGPC −6.2 using CE for calibration (the peak of LE ß-glucan as compared with the peak of CE as seen in fig.(1) .

Calculation :

Concentration of samples (µg/ml) = (area of sample / area of standard) × conc. of standard × dilution factor

Commercial extract (CE) (Pure Pharmaceutical Grade β-glucan)

Imunic (10 mg/capsule) was purchased, Istanbul TURKEY, and 1 mg/ml from imunic was prepared by (20) methods .

Cytogenetic study of human circulating lymphocyte

1 - Blood collection

Blood was taken from normal adult of human by puncturing; using disposable syringe. blood was transferred into heparinized tubes.

2 - Determination of Mitotic Index (In vitro mitotic index assay)

Procedure :

Blood culture with Local and Commercial extract of soluble beta glucan :

The whole blood culturing method described by (26,27) was used as follows:-

1) The both LE and CE of S. cereviese at concentrations (5 , 50 , 500 , 1000 µg/ml) was added to each test tube containing the whole media which prepared , (three replicates for each concentration. Total=24 test tubes). Also PBS was added for other three tubes and these consider as control negative (-) (28,26) .

2) Three hundred µl of PHA was added into one sets tubes (It was supplied as liquid by Iraqi Centre for Cancer and Medical Genetics Research ,ICCMGR), Baghdad, Iraq , mixed the components very well, these consider as control positive (+).

3) Five hundred µl (8 – 10 drops) of peripheral blood was added into all test tubes containing (5 ml) of culture medium and then transferred to incubator at 37C° put like slant for 72 hours , and gently the tubes were shacked each 24 hours (twicly)

Harvesting :

1. Just 1 hour prior to the end of incubation period (After 71 h of incubation) , 0.1ml colcemid Solution (10µg/ml/ Kreatch (Netherland) was added to each culture tube and mixed by shaking gently to all tubes that returned to incubator to complete the 72hr period.

2. The tubes were centrifuged at 2000 rpm for 5min, the supernatant was discarded and leaving about 0.5ml of it over the precipitated pellet, which are mixed well.

3. The cells were resuspended in 10ml of pre-warmed (37c°) hypotonic solution Potassium chloride (0.075 M KCL) and left for 20 minute at 37˚c.

4. Centrifugation at 2000 rpm for 5min has been done, the supernatant was discarded and leaving about 0.5ml of it over the pellet, which are mixed well.

5. Gradually, drop by drop, 4ml of freshly prepared ice-cold fixative solution was added (freshly prepared fixative methanol: glacial acetic acid (3:1) with continuous agitation on a vortex mixer, the tubes were centrifuged and the supernatant was discarded.

6 . Step 5 was repeated (3-4) times until the suspension became colorless (clear), then the supernatant was discarded while the remaining pellet is referred as the cell suspension 0.5-1 mL of fresh, cold fixative solution was added .

7 . By using a Pasteur pipette, 4 - 5 drops of cell suspension was dropped on a cold slide chilled, oil-free slides , let the drop run down the slide as it spreads (this can be performed by dropping from 60 cm height and tilting the slide to 45˚), the slides were left to dry at room temperature.

Staining:

The slides stained using freshly made Giemsa stain (stock solution was prepared by dissolving 2 g of Giemsa stain powder in 100 ml of methanol then stirred constantly using a magnetic stirrer at room temperature for two hours .The solution was filtered by filter paper (Whatmann no.1) and stored in dark light bottle. On staining 1 ml of the stock solution was added to 4ml of Sorenson's buffer(1:4 V/V).which was applied for 2 min., then rapidly washed with warmed Sorenson's buffer, after that left to dry at room temperature. Microscopic examination was performed to determine mitotic index (MI %) and blast index (BI %) .

MI % analysis: The MI %was determined as a ratio of the mitotic cells to the cells in interphase in 1000 calculated cells.

M.I. %=No. of dividing cells in metaphase / {Total No. of dividing cells +No. of non- dividing cells (1000) cells} X 100. (27) .

BI % analysis: The BI %was determined as a ratio of the cells in blast form to the other cells in 1000 calculated cells.(27).

M.I. = No. of cells in blast form / Total No. of dividing cells +No. of non- dividing cells (1000) cells} X 100.

Results:

Local extract (LE) :

The dried particulate of LE of Saccharomyces cerevisiae , in 500g of backer's yeast was given 67g (13.4%) of β-glucan, a bright yellow product , which became powder upon drying.

Chemical detection of active compounds:

samples containing carbohydrate developed a red-orange colour rather than the amber colour typical of the phenol-sulphuric acid assay. Intensity of the red colour increased with increasing the concentration and compared with immuneks, absorbance at 490 nm (the wavelength of maximum absorbance for glucose and starch). High performance liquid chromatography (HPLC) method was used for determination of LE ß-glucan as compared to CE (fig.-1) .

Cytogenesis study of human circulating lymphocytes:

The results in table (1) show the effect of beta glucan Local extract (LE) , Commercial extract (CE) on mitotic were significant increase at the level (P<0.05), and the best effect on mitotic and blast indexes was at the highest concentration of the LE and CE .

The mitotic index of control group is (20.2),While (26.2) for LE and (28.8) for CE at (1000µg/ml) in concentration ,The value of both at high concentration is lower than control group that treated with (PHA) was represented (32.4) as seen as in table (1). Mitotic index value of treated group revealed high significant decrease in a concentration depend manner (22, 23.8) for LE at the concentrations (50, 500)µg/ml respectively, and high significant decrease (20, 21.6) for CE at the concentration (5,50µg/ml). The mitotic index value (22 , 23.8) of the concentration 50 , 500 µg/ml of LE that significant exhibited at level (P<0.05) as compared with concentration of CE (50 , 500) µg/ml which were (21.6 , 25.4) respectively. The results shown that the concentrations 1000 µg/ml of LE, CE have best on mitotic index when compared with the control group (figures 2,3).

Table (1) referred that blast index of control group was (23.3). The blast index value of treated group exhibited low significant decrease in a concentration dependant manner (19.5, 16.6 and 15) for LE, (24, 19 and 17.4) for CE at the concentrations (5,50,500,1000)µg/ml respectively. In CE the blast index value (24, 24.1) was represented high value at concentration (5 , 1000)µg/ml than (50 , 500)µg/ml which were (19,17.4) respectively.

Table (1): Mean values of mitotic index percentage (MI) % and blast index percentage (BI) % of normal human blood lymphocytes after treated with different concentrations of soluble β-glucan (Local and Commercial extracts) of S. cerevisiae with control groups.

Concentration of β- glucan µg/ml	Mitotic Index(MI) % Mean±SE	
	Local extract	Commercial
5	19.2 ± 0.42 d	20 ± 1.22 d
50	22 ± 1.22 c	21.6 ± 0.25 d
500	23.8 ± 1.22 c	*25.4 ± 0.65 c
1000	26.2 ± 0.75 b	*28.8 ± 0.81 b
Control group –ve	20.6 ± 0.61 d	
Control +ve (PHA)	32.4 ± 0.65 a	
Concentration of µg/ml β-glucan	Blast Index(BI)% Mean±SE	
	Local extracted	Commercial
5	19.5 ± 0.41 d	*24 ± 0.82 b
50	16.6 ± 0.36 e	*19 ± 0.42 c
500	15 ± 0.42 e	*17.4 ± 0.25 d
1000	21.3 ± 0.64 c	*24.1 ± 0.82 b
Control group	23.3 ± 0.81 b	
Control (PHA)	27.2 ± 0.73 a	

Small different letter denoted that significant differences between concentration at level (P≤ 0.05 , 0.01)
***Significant different at level P ≤ 0.05**

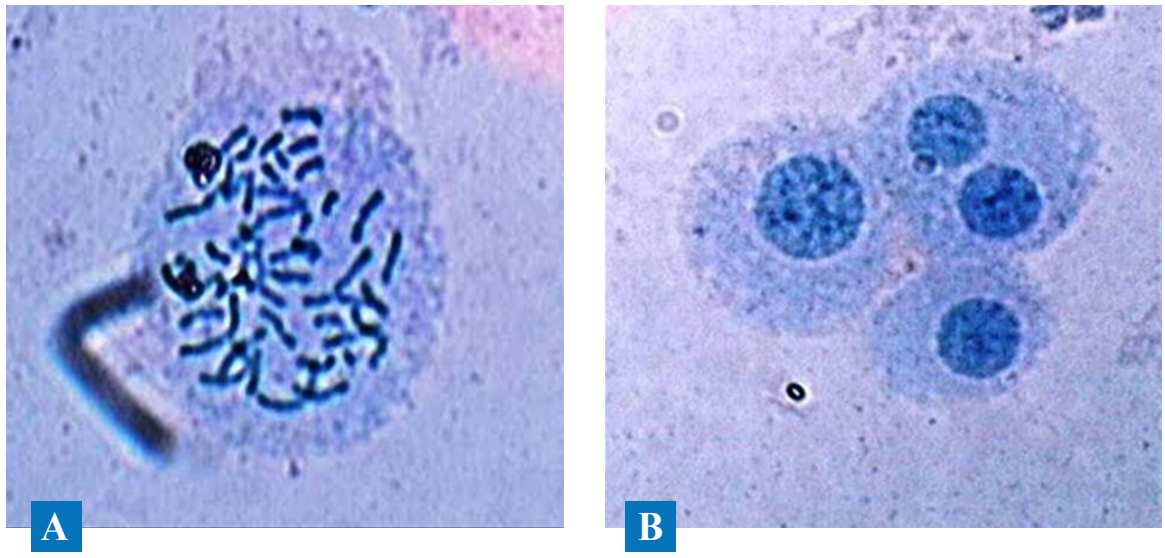

Figure (2) : The effect of high concentration of β-glucan extracts, Showing metaphase of chromosomes (A) , and lymphoblast (B) of blood peripheral lymphocytes Exposure to high concentration of Local extract (1000 µg / ml) (Giemsa stain , X1000)

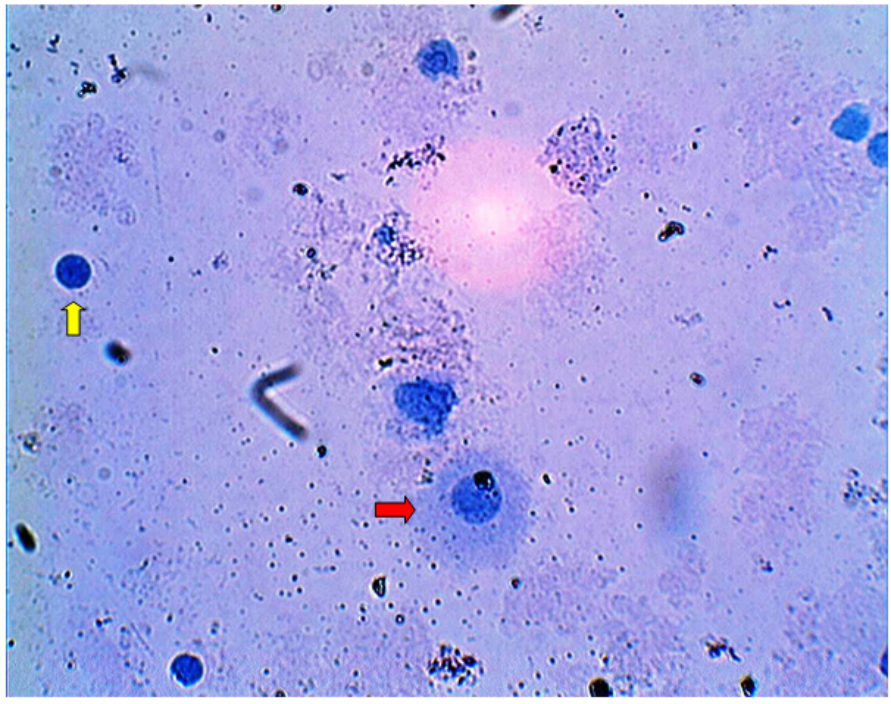

Figure (3): the effect of high concentration of β-glucan extracts on normal human blood lymphocytes after exposure to the concentration (500 µg/ml), showing the lymphoplast (➡) and the number of lymphocyte (⇧) (Giemsa stain , X400)

Discussion:

The extract of Saccharomyces cerevisiea yielded crude extract (13.4%) was in agreement with (29) ; The yield was (14.4%) of extract β-glucan from Saccharomyces cerevisiae , difference may be due to some has been lost in this yield during processing of extraction (Fractionated and hydrolysis), depending on the type of preparation to each one. The local

extract (LE) β-glucan in the study showed fine bright yellow powder and sticky extract.

According to results in concern with the mitotic index (MI), blast index (BI), both the Local extract & Commercial of Saccharomyces cerevisiae exhibited dual effects of immunomodulatory activity ,enhancing lymphocyte proliferation at low concentrations(5µg/ml), and increasing in there proliferation with duto the effect at high concentrations(1000µg/ml). This immunomodulatory effect was stronger on stimulated lymphocytes (15) .Based on in vitro studies, β-glucans act on several immune receptors including Dectin-1, complement receptor (CR3) and TLR-2/6 and trigger a group of immune cells including macrophages, neutrophils, monocytes, natural killer cells and dendritic cells. As a consequence, both innate and adaptive response can be modulated by β-glucans and they can also enhance opsonic and no opsonic phagocytosis (4) .Chiang et al., (30) revealed the aqueous of Plantago major and P. asiatica both exhibited dual effected immunomodulatory activity enhancing lymphocyte proliferation and secretion of interferon –γ-.at low concentration, but inhibiting this effect at high concentrations. The effect of both β-glucan (LE & CE) of S. cerevisiae depends on the concentration in which the high concentrations enhancing the transformation of lymphocyte to enter mitosis and also increased the nuclear division index .In addition, the high concentrations enhancing cell to continue replicate reaching third mitotic stage (M3).The parent cells replicated to liberate the first mitotic stage (M1) and several number of lymphocyte entered second mitotic stage (M2) but very few number of lymphocyte entered third mitotic stage (M3)and other failed completely.

The MI depends on two factors: first the proportion of the cell population that participates in the whole cycle of interphase leading to division, and second the relative lengths of interphase and recognizable mitotic stages (31,32,33).This assay requires the addition of colchicine or colcemid to arrest the progression of cells from metaphase to anaphase ensuring suffi cient number of metaphases for cytogenetic analysis. The MI was used to characterize proliferating cells and identify compounds that inhibit or induce mitotic progression (34,35) . The suggestions may explain the proliferative effect of β-glucan on lymphocyte; the first one is the ability of glucan or its constituents to act as mitogenic agent, or to act as immuno-regulatory factors that modulate the secretion of certain cytokines particularly those needed in proliferation process (36).

The inhibitory effect of the mitogenic stimulation of cells by colcemid could not be accounted, by diminution in cell viability or by metaphase arrest of mitosis in the stimulated cells (27,38) . So that, due to this reason the soluble ß-glucan have the stimulatory effect on mitotic and blast indexes , these findings support the hypothesis that cytoplasmic microtubular function plays a role in the commitment of resting cells to undergo mitotic division (38). In previous study by (39) who demonstrated that orally administered whole glucan particulate functions to accelerate hematopoiesis following irradiation in an analogous manner as intravenous administered β-glucan.

the mitogenic factor (PHA) which stimulates lymphocyte division and this result denoted in increasing in proliferation of lymphocytes dueto the mitogen (PHA) than the β-glucan results, and the competition effect between mitogenic factor (PHA) and β-glucan in present study not doing it .In previous study As a result of interaction with reproduction of lymphocyte in vitro (40) found that addition of a commercial preparation of nettle leaf extracts to whole human blood resulted in an inhibition of phytohaemoagglutinin-stimulated production of T helper cell 1 (Th1)-specific interleukin-2 (IL-2) and interferon-gamma (IFN-g) in culture in a dose-dependent manner up to 50%and 4%, respectively. They may interact with metabolism source on cell membrane .

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الدراسة الخلوية الوراثية للبيتا كلوكان الذائب لخميرة لخبز *Saccharom-ces cerevisiae* في الخلايا اللمفاوية للإنسان

علي جواد جابر[1]، ناهي يوسف ياسين[2]، نضال رؤوف مهدي[3]

1 فرع الإحياء المجهرية / كلية الطب / جامعة ميسان
2 المركز العراقي لبحوث السرطان والوراثة الطبية
3 فرع الإحياء المجهرية /كلية الطب البيطري /جامعة بغداد

الخلاصة:

صممت الدراسة للتحري عن التأثير الوراثي الخلوي للبيتا كلوكان الذائب للمستخلص المحلي والتجاري لخميرة الخبز S. cerevisiae في الخلايا اللمفاوية للإنسان للتراكيز المستخدمة (5 , 50 , 500 , 1000) مايكرو غرام / مل .

بينت نتائج الدراسة الخلوية الوراثية على خلايا اللمفاوية بعد معاملتها بالبيتا كلوكان الذائب (المستخلص المحلي والتجاري) لجميع المجاميع فروقات معنوية في معامل الانقسام الخيطي ومعامل التحول الأرومي ,أذ اظهر كلا العلاجين زيادة في نسبة الانقسام الخيطي للخلايا اللمفاوية على حد سواء وفي جميع الفترات الزمنية مقارنة لمجموعة السيطرة , إذ بلغت نسبة الانقسام الخيطي لمجاميع السيطرة السالبة (20.2) وهي اقل من نسبة المستخلص المحلي والتجاري المحضر على حد سواء والتي بلغت (22, 23.8 ,26.2) , (20, 21.6 , 28.8) مقارنتا مع مجاميع السيطرة الموجبة المعاملة (PHA) والتي كانت قيمتها (32.4)اعلى من قيمة المستخلص المحلي والتجاري عند التركيز العالي ((1000µg/ml المستخدم في الدراسة , اما قيمة معامل التحول الارومي لمجاميع السيطرة (23.3) اعلى من قيمة المستخلص المحلي والتجاري والتي اظهرت فرقا معنويا للتراكيز المستخدمة في التجربة والتي تمثلت (19.5 , 16.6 , 15) للمستخلص المحلي و (24 , 19 , 17.4) للمستخلص التجاري .