The Cytokines Activity in Mice Model Treated with *Withania somnifera*

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Abstract

Withania somnifera (WS) is one of the important herbs in the Ayurvedic and diagnosis medical systems. Mice were administered WS extracts in the dose of 1/20 of LD50 in drinking water. The influence of *Withania somnifera* on the cytokines, IL-2, IL-4, IL-10, IL-12, IFN-γ and GM-CSF in the spleen cells of mice model indicated a significant increase in their level. GM-CSF failed to show any detectable level in control and at all time periods. Mice cells pulsed with PHA and non-pulsed cells expressed a significant increase in their levels as measured by ELISA on day 5, 15, 20, and 30.The immunostimulatory effect of *Withania somnifera* was revealed further with RT-PCR which indicated a distinct difference between controls, day 5 and day 15. At day 15 genes of all cytokines were expressed in the cells pulsed with PHA and in the non-pulsed cells. The plant exerted clear influence on the cytokines activity measured in the spleen cells of treated mice.

Keywords: *Withania somnifera*, cytokines, mice, IL-10

Introduction:

Withania somnifera (WS) is one of the important herbs in the Ayurvedic and diagnosis medical systems. *Withania somnifera* is also known as ashwagandha, Indian ginseng and winter cherry. Ashwagandha is a member of Solanaceae family that is widely grown in Africa, the Mediterranean and India. The roots of this plant are considered the major portion that has the therapeutic use. The major active ingredients in WS are groups of alkaloids (isopelltierine, araferine) and steroidal lactones (withanolides, withaferins). There are 12 alkaloids, 35 withanolides and several sitoindosides (anonymous, 2004; Lakshmikumaran, 2000).

Ashwagandha or WS has been shown to have an overwhelming use as liver tonic, anti-inflammatory and in treatment of anxiety, cognitive neurological disorders and Parkinson's disease (no author, 2004). The plant is also found useful in enhancing the cancer radiotherapy (Devi *et al*., 1996) and has

bacteriological activity against Aspergillus fumigants and Salmonellosis (Dhuley, 1998; Owais *et al*., 2005). The therpeutical and immunostimulatory activity of WS was studied widely in mice model (Agarwal *et al*., 1999; Aphale *et al*., 1998; Davis and Kutton, 1999; Dhuley, 1997).

Treatment of Balb/c mice with 20 mg/dose/animal of WS root extract resulted with significant increase in white blood cells (WBC), bone marrow cellularity and enhanced the circulating anti-sheep red blood cells (SRBCs) antibodies. Treated mice expressed an enhancement in the phagocytosis of peritoneal macrophages and inhibited delayed type hypersensitivity (Davis and Kutton, 2000). Agarwal *et al.* (1999) reversed the immunosuppressive effect of cyclophosphamide in mice by treatment with WS. The treated mice also revealed significant increase in the hemagglutinating and hemolytic anti-sheep SRBCs antibodies. The lymphocytes cytotoxicity against thymoma cells was enhanced in vivo and in vitro after treatment with WS (Davis and Kutton, 2002). The study on the immunostimulatory effect of WS was taken further by measuring the cytokines in mice model (Davis and Kutton, 1999; Dhuley, 1997). Cytokines are crucial mediators in the regulation of immune responses and haemopoeisis (Nicola, 1994). Cytokines, interleukin-2 (IL-2) interferon-γ (IFN-γ) and granulocyte monocytes-colony stimulating factor (GM-CSF) were increased significantly in mice that were treated with powdered root of WS. The level of these cytokines reversed when these animals received cyclophosphamide (Davis and Kuttan, 1999). On the other hand, macrophages from mice that were suffered eminent suppression from the treatment with carcinogen Ochratoxin-A, indicated a significant increase in interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α) and chemotaxis when treated with WS (Dhuley, 1999).

This study is aimed to examine the immunostimulatory activity of WS in mice model by measuring wider range of cytokines, IL-2, IL-4, interleukin-10 (IL-10), interleukin-12 (IL-12), IFN-γ and GM-CSF by enzyme linked immunosorbent assay (ELISA) and their gene expression was examined using reverse transcriptase-polymerase chain reaction (RT-PCR).

Materials and Methods

Plant:

The aerial parts of Ws (leaves, stem & seeds) (Fig.1) were freshly collected from the local areas in the Al-Ahssa farms. Approximately one kg of dried plant material were grounded and extracted with 80% ethanol by shaking and

percolation for 24 hours at room temperature. The extract was centrifuged at 1000 rpm for 10 minutes. The solvent of supernatant evaporated at 40°C under vacuum condition. The residue was dissolved in drinking water at concentration of 1/20 of the LD50 (940 mg/100 g. body weight, Abdel-Magied *et al.,* 2001). Water adopted ad-libitum to the mice along the experimental period.

Mice:

A total of 60 Wister albino male mice used were housed in hygienic fiber glass cages. Animals were maintained in 6 groups of 10 mice each. Mice were apparently healthy and weighed 25 –30g. They were fed on commercial pellets, (Grain Silos and Flour mills Organization – Riyadh). The mice were allowed free access to treated drinking water and feed. Throughout the experimental study, all animals were observed daily for any signs. Three groups of total of 6 groups were treated by the drinking water containing 1/20 LD50 ethyl alcohol extract of WS. The other 3 groups were considered as control by drinking tap water only. The total period of the experiment was continued for 45 days. At each time point, 5 days, 15 days, 20 days, 30 days and 45 days, five mice of each of the two groups of the animal, one treated and one control, were killed by cervical dislocation and the spleens were removed aseptically. The extra 10 mice were kept as a reserve with the control groups.

Preparation of spleen cells:

Spleen cells of each group were harvested by squeezing the cells out by repeated injection of Dubelcco's modified Eagle's medium in the spleen. The cells were then washed once with the medium. The number of spleen cells were adjusted to a final concentration of 9 $X10^6$ cells/ml in growth medium RPMI-1640 (Sigma) containing 5% foetal calf serum, (Sigma) antibiotics and antimycotic (final concentration of 10 unit/ml penicillin G Sodium, 10 µg/ml streptomycin sulfate and 0.025 µg/ ml amphotericin B as fungizon) (Sigma Technology, USA). The cells then either incubated with final concentration of 10-12 µg/ml of phytohaemagglutinin (PHA) (Sigma) or without the presence of the mitogen at 37°C in the presence of 5% CO_2 for 48 hours. Control cells were also incubated in medium with and without PHA.

Monitoring the cytokines level with ELISA :

The cytokines, IL-2, IL-4, IL-10, IL-12, IFN-γ and GM-CSF levels were monitored using Quantkine mouse cytokines immunoassay kit (R&D Systems, USA). The harvested RPMI-1640 medium of spleen cells of each time points

was used to measure the level of the cytokines according to manufacturer direction.

Briefly, 50 µl of the assay diluent, were added to each well, then 50 µl of standard control and samples were added. After 2 hrs incubation, a 100 µl of conjugate were added to each well after thorough washing and incubated for overnight. After thorough washing, a 100 µl of the substrate solution was added to each well and incubated for 30 minutes. Finally a 100 µl of the stop solution was added to each well and the optical density was measured using ELISA reader with dual wavelength 450 and 570 nm (Thermo Labsystem, Finland).

The cytokines gene expression with RT-PCR.:

The Cytokine gene expression of IL-2, IL-4, IL-10, IL-12, IFN-γ and GM-CSF were examined with RT-PCR. The test was performed as follows:

RNA extraction :

Total RNA (toRNA) was extracted from the spleen cells that were incubated with or without the mitogen. The extraction was performed using total RNA extraction kit (Qiagen Ltd, Valencia, CA, USA). The procedures were carried out according to the manufacturer's directions.

RT-PCR:

Approximately 1µl of toRNA was reverse transcribed to Complementary DNA (cDNA) using 20 µl reverse transcription reaction mixture containing a final concentration of 5 mM $MgCl_2$, 1X of a 10 X PCR buffer II , 2.5µm Random Hexamers,1 mM of each of dGTP, dATP, dTTP and dCTP, 1U/µl RNase inhibitor , and finally 2.5 U/µl reverse transcriptase(GeneAmp® RNA PCR kits, Perkin Elmer Applied Biosystems, Branchburg, NJ, USA). The mixture was incubated at 42°C for 15 minutes heated to 99°C for 5 minutes and maintained at 5°C for 5 minutes Using GeneAmp® PCR system 2400 (Applied BioSystems, USA).

PCR amplification:

An 80 µl of PCR mixture was prepared for each cytokine containing a final concentration of 2mM $MgCl_2$, 1X of a 10X PCR buffer II, and 2.5U/100 µl of *Thermus aquticus* DNA polymerase (Ampli Taq® DNA polymerase) and approximately 40-45 pM of upstream and downstream primers (Proligo, USA) (Table-1) of each cytokine which were as those reported elsewhere [IL-2, IL-4, IL-10, and GM-CSF by (Bullock and Jahnson, 1996) IL-12p40 and IFN- γ (Nomura *et al,* 2002)]. PCR mixture of 100 µl was then amplified using

GeneAmp® PCR system 2400 Thermocycler (Applied BioSystems, USA) as follow, initial step for one minute and 45 seconds at 95 °C and then 35 cycles of amplification in which the melting was at 95 °C for 15 seconds and the anneal-extension at 60 °C for 30 seconds. Finally the reaction was kept at 72 °C for 7 minutes before it cooled down at 5 °C.

Agarose gel electrophoresis:

The products of RT-PCR were run on 1% agarose gels (Sigma chemical Co, Spain) dissolved in 1x tris-acetate EDTA (TAE) buffer (PH8) (50X TAE buffer is made of 40 mM Tris-acetate, 1 mM EDTA). The gel was fixed in the horizontal gel electrophoresis apparatus and run in 1L 1X TAE buffer containing 25 µl of 0.5µg/ml Ethidium bromide. The samples and the 100bp ladder marker (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) were Loaded with 30% glycerol, and run at 90V for 90min. The gel was then visualized with ultra-violet illuminator (Fisher Scientific, USA) and photographed by the C-4040 digital camera (Olympus, Japan).

The blood picture of mice treated with WS:

The analysis of blood picture, cells and proteins of control and treated mice, carried out at the hematology laboratory of King Fahad general hospital at Hofuf. The blood cells were counted in the coulter machine (Cell-Dyn-3700, USA).

The statistical analysis :

The data were analyzed with two-factor analysis of variance followed by Duncan multiple range test.

Results:

The cytokines levels in the WS treated-spleen cells of pulsed or not-pulsed with PHA.

The levels of cytokines concentration measured by ELISA test are summarized in Fig.2 and Fig.3.The GM-CSF failed to show any detectable levels. The two-factor analysis of variance of cytokines that were monitored in cells of treated mice (cells were either pulsed with PHA or grown in medium free of PHA) indicated a significant difference with time and response to the mitogen [IL-2 (P>0.006), IL-4 (P>5E-11), IL-10 (P>0.0002), IL-12 (P>2E-10)]. The results of the tow-factor analysis of variance were analyzed further by the Duncan multiple analyses (Table-2). The analyses revealed that the cytokine of spleen cells of treated mice but not pulsed with PHA, IL-2 and IFN-γ showed no significance difference with time. However, IL-4 and IL-12

indicated significant rise on day 5, 15 and 20, whereas; IL-10 indicated a significant rise on day 20. On the other hand, cytokines monitored in cells pulsed with PHA showed that IL-2 level was significantly increased on day 20 and 45, whereas, IL-4 was increased significantly on day 5 and 15. Interleukin-10 was significant on day 30. The level of IL-10 continued to increase until day 30. On day 5, 15 and 20, IL-12 was significantly increased (Table-2).

The cytokines gene expression.

The analysis of cytokines gene expression with RT-PCR indicated the gene expression of all cytokines on day 15 (Fig.4), which was clearly distinct from that was observed with control and day 5 (Fig.5). Cytokines of the control cells did not indicate any signals of gene expression. The analyses of cytokines gene expression on day 5 revealed that only cDNA of cytokines of cells that were pulsed with PHA were detectable. However, cytokines cDNA from both groups of cells, pulsed and non-pulsed, were detectable on day 15. The gene expression of GM-CSF was detectable despite its undetectable level by ELISA.

Although, examination of cytokines gene expression with RT-PCR is a qualitative method, a broad comparison with the cytokines level measured by ELISA (quantitative method) could exhibit a clearer picture of the cytokines activity. The absence of a distinct cytokines gene expression on day 5 of cells not pulsed with PHA correlates with the steady increase of most of the cytokines at the same period (Fig.2). On day 15, however, both PHA treated and non treated cells indicated obvious expression of all of the cytokines genes (Fig. 4), which correlates with the significant increase of most of the cytokines at this period (Table-2).

The blood pictures of mice treated with WS.

Complete hematological study on mice that were treated with WS revealed no significant difference from the control mice. The results are summarized in table-3.

Discussion:

Withania somnifera is an important herb in the Ayurvedic medical system. The immunostimulatory and anti-inflammatory properties of WS were studied extensively (Devi *et al.,* 1996; Dhuly, 1998; Owais *et al.,* 2005, Ziauddin *et al.,* 1996). The treated mice with WS expressed a significant cytokines activity in this study. The effect of WS on the IL-4, IL-10 and IL-12 is addressed for the first time. The statistical analysis indicated a significant increase in the cytokines activity with time. Non-pulsed cells from treated mice indicated

significant increase in IL-4, IL-10 and IL-12, whereas, in the Pulsed cells IL-2, IL-4, IL10 and IL-12 were significant. The increase in the cytokines activity in the treated mice was in accordance with other reported studies (Davis and Kutton, 1999; Dhuley, 1997). Davis and Kutton (1999) reported an enhancement of IL-2, IFN-γ and GM-CSF in the treated Balb/c mice. Therefore, the enhancement in the level of all studied cytokines can be envisaged in view of the reports on the potentiation of WS of innate immunity, cell mediated immunity and antibody production (Davis and Kutton, 2000, 2002, Agarwal, *et al.,* 1999). The continuous significant elevation in the level of IL-4, IL10 and IL-12 to day 20 and IL-10 to the day 30 indicates that the WS has a wide scale of immunoregulatory effect on the Th1 and Th2 cytokines. Furthermore, the gene expression of all cytokines on day 15 even that of the non pulsed cells endorse the mitogenic activity of this plant that was reported elsewhere (Devi *et al.,* 1996, Ziauddin *et al.,* 1996).

A distinct significant difference in cytokines activity between pulsed and non-pulsed cells indicates the imunopotentiating influence of this plant in enhancing the stimulatory response of the cells to the mitogen PHA. Nevertheless, failure in detection of any significant increase in the IFN-γ level within time is questionable. Interferon-γ is considered one of the important cytokines in enhancement of innate and cell mediated immunity (Gray, 1994). Although, the level of IFN-γ indicated a significant difference between control cells that were pulsed with PHA and the same cells that were non-pulsed, its level showed no significance at all time points in the treated cells. The failure in detection of any significant increase in IFN-γ level could be due to the immunoregulatory effect of IL-10 which has shown significant elevation.

The blood picture of mice treated with WS indicated no significant differences from that of control animals. It seems that the hematological analyses in this study suffered a certain set back. The non-significant changes in blood picture clearly contradict the reports that indicated the vast changes in the blood pictures brought about by WS treatment (Davis and Kutton, 2000; Agarwal, *et al.,* 1999). Elevation in the cytokines synthesis indicated by the ELISA and supported by their distinct gene expression should be associated with obvious physiological activity of the white blood cells as well as significant increase in their count. Hence, despite this unsatisfactory result of the blood picture analysis, the overall results of cytokines activity that was measured by ELISA and RT-PCR substantiate the immunostimulatory activity of this plant. However, further analysis on the effect of this plant on the

proinflammatory cytokines, interleukin-1, interleukin-6 and TNF-α, in parallel to the cytokines in this study seems important.

Acknowledgment:

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Table (1)
Oligonucleotides Sequences of upstream (sense) and downstream (antisense) primers of mouse Cytokines

Gene	Primers	Size of expected cDNA (bp)
IL-2	Sense 5′-GAAGATGAACTTGGACCTCTGC -3′ Antisense 5′-CAGAGTTGCTGACTCATCATCG -3′	167
IL-4	Sense 5′-TATTGATGGGTCTCAGCCCC-3′ Antisense 5′-GAGTCCCTTTTTTCTGTGACCTGG-3′	140
IL-10	Sense 5′-TAGAAGTGATGCCCCAGG -3′ Antisense 5′-TCATTCTTCACCTGCTCCACTGC -3′	159
IL-12p40	Sense 5′-TCCGGAGTAATTTGGTGCTTCACA -3′ Antisense 5′-ACTGTACAACCGCAGTAATACGG -3′	396
IFN-γ	Sense 5′- AGCGGCTGACTGAACTCAGATTGTAG -3′ Antisense 5′- GTCAGAGTTTTCAGCTGTATAGGG -3′	244
GM-CSF	Sense 5′-TGCTCTGGAGAACGAAAAGAACG -3′ Antisense 5′-ATTGAGTTTGGTGAGGTTGCCCC -3′	132

Figure.1 *Withania somnifera* leaves and fruit as they appear in their natural habitat.

Table (2) : Duncean multiple range test for means of cytokines levels at different time points.

IL-2 (Mean± SE)	IL-4 (Mean± SE)	IL-10 (Mean ±SE)	IL-12 (Mean± SE)	IFN-□(Mean± SE)
§W20 49.88±3.021$^{a\gamma}$	W15C 39.369±4.649^{a}	W30 664.6±2.54^{a}	W15 35.85±4.099^{a}	P 20.233±0.35^{a}
W45 42.293±1.845$^{b\gamma}$	W15 36.9±0.75^{a}	P 488.48±102.797^{b}	W5C 33.70±0.915^{a}	W15 16.50±0.00^{ab}
*p 33.067±0.377$^{c\gamma}$	W5 27.633±1.206^{b}	W15 409.1±1.378^{bc}	W20C 32.55±0.05^{a}	W5 12.223±0.315^{ac}
W5 27.787±1.738$^{cd\gamma}$	W5C 26.967±3.743^{b}	W45 379.05±10.775^{bc}	W20 32.45±4.05^{a}	W30 7.737±0.735^{c}
FW15C 26.81±5.343$^{cd\gamma}$	W20C 25.68±6.028^{b}	W20C 338.45±13.741^{bce}	W15C 25.00±1.837^{b}	W15C 1.933±0.00^{d}
W15 25.24±3.283$^{cde\gamma}$	W20 12.1±3.419^{c}	W30C 318.9±2.54^{ce}	W5 20.95±1.556^{b}	W5C 1.467±0.495^{d}
W30 21.723±2.292$^{de\gamma}$	P 11.92±1.195^{c}	W20 283.4±3.811^{cdef}	P 16.20±2.404^{c}	W30C 1.20±0.00^{d}
θC 20.9±0.735$^{de\gamma}$	W30C 8.355±2.045^{cd}	C 188.05±8.66^{efg}	W30C 6.55±2.925^{d}	C 0.76±0.705^{d}
W45C 20.343±2.271$^{de\gamma}$	W30 0.497±0.745^{d}	W5 149.9±13.4^{fg}	W30 5.45±2.155^{d}	W20 0.296±0.00^{d}
W20C 18.147±5.342$^{ef\gamma}$	W45 0.168±0.02^{d}	W5C 96.8±9.09^{g}	C 3.75±0.45^{d}	W20C -
W30C 17.27±3.413$^{ef\gamma}$	W45C 0.155±0.007^{d}	W15C 91.45±4.41^{g}	W45 -	W45 -
W5C 9.903±1.389$^{f\gamma}$	C 0.093±0.162^{d}	W45C 46.35±5.023^{g}	W45C -	W45C -

$^{\gamma}$. For each mean values with a superscript in common are not significantly different from each other with level significance 5% over all comparisons.
*. Control cells pulsed with PHA
$^{\theta}$. Control cells not pulsed with PHA
^{F}. Cells of treated mice but not treated with PHA
§. Cells of treated mice treated with PHA
5. 15, 20, 30, 45 referred to the time intervals.

Table (3) : The one-factor analysis of variance of the hematological pictures of mice treated with WS

	control		W5[§]		W15[§]		W20[§]		W30[§]		W45[§]		P value
	M[*]±St[§]		M ±St		M±St		M±St		M±St		M±St		
WBC	6.367	±1.503	6.275	±0.585	6.5	±1.63	5.22	±1.4	8.13	±1.49	6.91	±1.69	0.11
RBC	7.207	±0.554	6.854	±1.255	6.4	±0.35	6.95	±0.96	7.92	±0.88	6.92	±1.20	0.82
HGB	11.50	±0.886	10.20	±1.69	9.0	±0.66	10.46	±1.81	12.36	±1.74	9.02	±1.65	0.61
HCT	33.80	±2.246	30.74	±4.50	27.97	±2.35	32.36	±4.02	37.18	±4.83	32.07	±5.43	0.88

*.Mean
§. Standard deviation
§ 5. 15, 20, 30, 45 referred to the time intervals.
The cytokines activity in mice model treated with Withania somnifera
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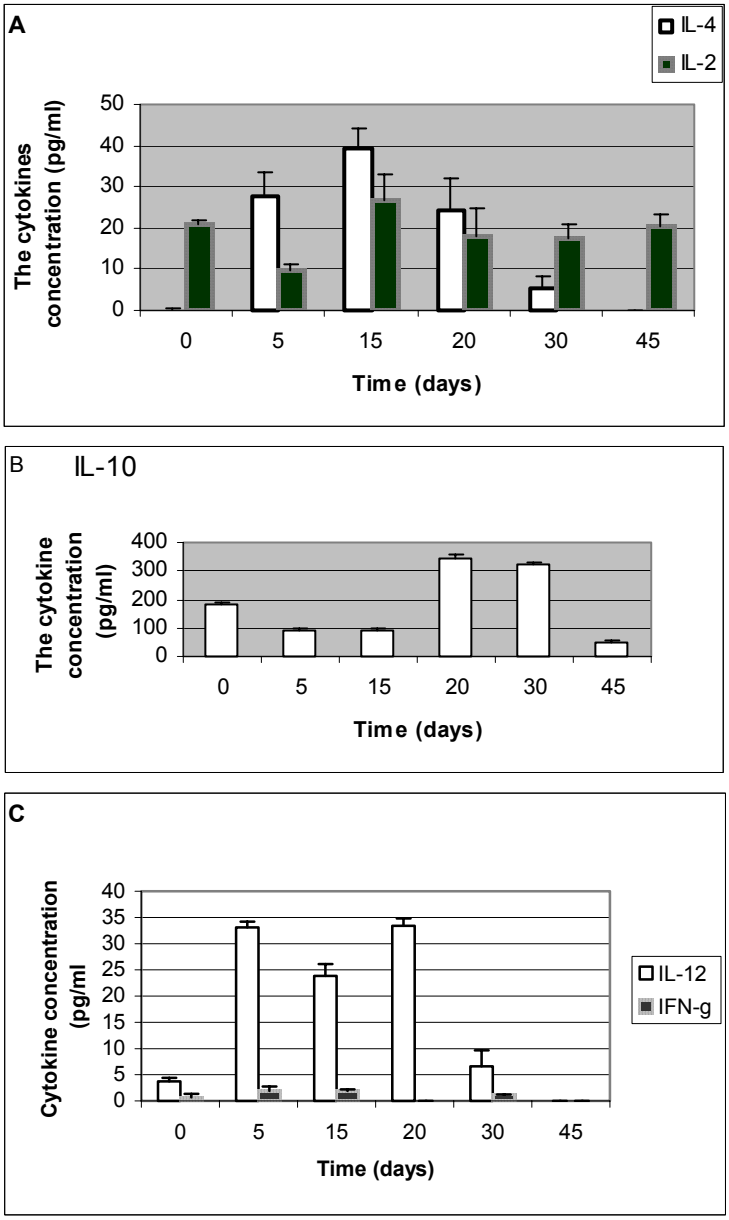

Fig.2: The level of the cytokines monitored by ELISA in spleen cells not pulsed with PHA. The level of cytokines IL-2, IL-4, IL-10, IL-12 and IFN-γ of control and mice model treated with *Withania somnifera*. The plate **A** depicts the level of IL-2 and IL-4, **B** indicates the level of IL-10 and **C** refers to the level of IL-12 and IFN-γ .

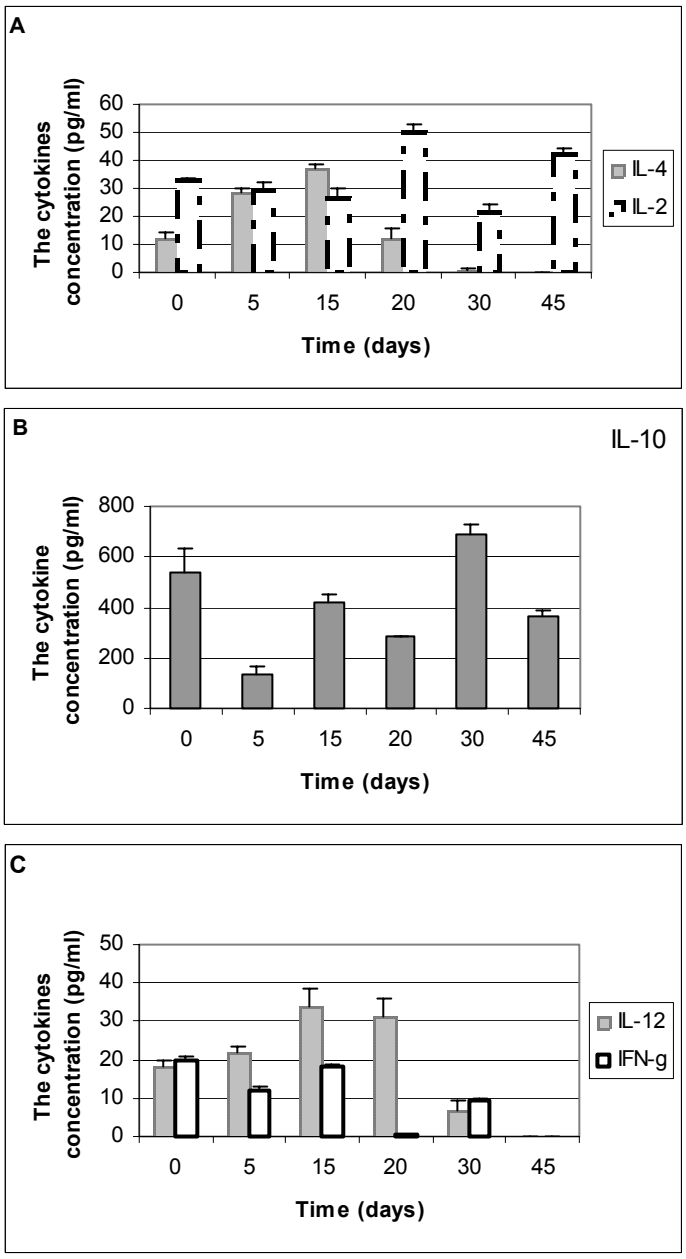

Fig.3: The level of the cytokines monitored by ELISA in spleen cells pulsed with PHA. The level of cytokines IL-2, IL-4, IL-10, IL-12 and IFN-γ of control and mice model treated with *Withania somnifera*. The plate **A** depicts the level of IL-2 and IL-4, **B** indicates the level of IL-10 and **C** refers to the level of IL-12 and IFN-γ .

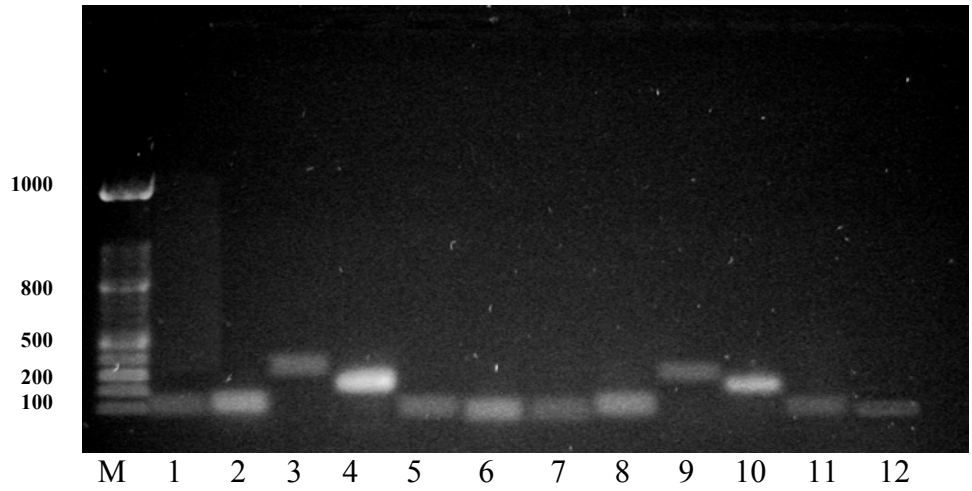

Figure.4 Effect of WS on mice cytokines gene expression using RT-PCR after 15 days of treatment:, M= 100 bp marker, (WS+PHA treatment) 1= IL-4, 2= IL-10, 3= IL-12, 4= INF-γ, 5= GM-CSF and 6= IL-2. (WS treatment only) 7= IL-4, 8= IL-10, 9= IL-12, 10= INF-γ, 11= GM-CSF and 12= IL-2.

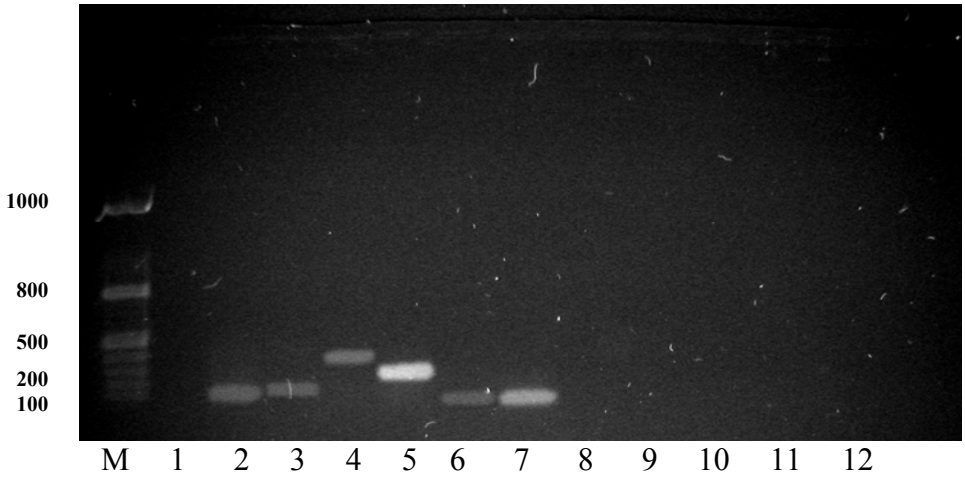

Figure.5: Effect of WS on mice cytokines gene expression using RT-PCR after 5 days of treatment: M= 100 base pair (bp) marker, (WS+PHA treatment) 1= IL-4, 2= IL-10, 3= IL-12, 4= INF-γ, 5= GM-CSF and 6= IL-2. No bands were detected for the cells treated with WS only.

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التعرف على نشاط الافرازات الخلوية في أنموذج الفأر المعالج بنبات سم الفأر

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**معهد الأبحاث ، دولة الكويت

الملخص :

يعتبر نبات سم الفأر من الأعشاب الطبية المهمة في تحفيز جهاز المناعة . و قد تم دراسة الإفرازات الخلوية التالية في أنموذج الفأر , IL-2, IL-4, IL-١٠, IL-12, IFN-γ , و GM-CSF. لقد تبين ارتفاع هذه الإفرازات في خلايا طحال الفأر المعالج و المستثارة بمادة الفايتوهيموجلاتنين (PHA) ما عدا الإفراز الخلوي GM-CSF . و قد تبين ارتفاع الإفرازات الخلوية في الأيام ٥ , ١٥ , ٢٠ , و ٣٠ . و قد تم فحص التأثير المناعي لنبات سم الفأر على إنتاج الإفرازات الخلوية بأسلوب آخر من خلال تقنية تكثير البصمة الوراثية (PCR). و قد اثبت الفحص فرق واضح بين البصمة الوراثية للإفرازات الخلوية في الأيام ٥ و١٥. حيث تم رصد نشاط البصمة الوراثية للإفرازات الخلوية في اليوم ١٥ للخلايا المستثارة بالفايتوهيموجلاتنين و غير المستثارة. أظهر العشب تأثيرا واضحا في زيادة إنتاج الإفرازات الخلوية لخلايا طحال الفأر.