ISSN: 0975 -8542

Journal of Global Pharma Technology

Available Online at: www.jgpt.co.in

RESEARCH ARTICLE

Investigation of Immunomodulation Effects of Ethanolic Neem Seeds (*Azadirachta indica*) Extract on Induced Immune Response in BALB/C Mice with *Vibrio alginolyticus* Whole Antigen

Ihsan Dhari Awad[1], Saif M. Abed[2]

1. *Microbiology Science, Department of Dentistry, Dijlah University College, Iraq.*

2. *Microbiology Science, Samawah, Iraq.*

Abstract

The Current study was studied the immunomoduatory potentials of Ethanolic Neem seed extract in treated mice with *Vibrio alginolyticus* whole antigen through estimation the phagocytic activity of and lymphocyte proliferations as well as estimation of the serum fractions to evaluate the humoral immune response . The study was divided into 4 groups: Group (I) was included treated mice with normal saline. Group II was included mice treated with *Vibrio alginolyticus* antigen only , Group,(III): included mice were treated with (200 ug /Kg) of the neem extract combination with *Vibrio alginolyticus* antigen ,finally , Group IV included mice were treated with (300 ug /Kg) of the neem extract combination with *Vibrio alginolyticus* antigen. The results of NBT index and MTT test were revealed group III and group IV showed highly significant increase ($P \leq 0.05$) comparison with other Groups .More further results of gel electrophoresis in current study were showed the level of Gamma globulin serum, Alpha-1 level, Alpha-2 level, Alpha-beta level were highly significantly in Group III and Group IV comparison with other groups. In other hands in this study the results were showed there is no significant difference between group III and group IV in NBT index and MTT test as well as Gel electrophoreses. Depending on the results of current study the Ethanolic Neem extract had a positive immunomodutory effect on the induced immune response.

Keywords: *Neem Seeds extract, Immunomodulatory Effects, Vibrio alginolyticus whole antigen, Immune response.*

Introduction

Immunomodulators are biological materials and plant products, which mediate many mechanisms of human body on of them is the immune system through stimulation to produce the cytokines or through enhance the cell proliferation which have direct effects on the immue response [1].Using many of these agents to promote the immunological defences as well as is exciting development in immunopharmacology.

The biological agents act on the immune systems by many levels such as, promotion and enhancement the humoral immune respose leads to increase antibody production and respond, increasing the phagocytic activity of macrophages, or enhance the cellular immunity. Some of The medical plants are very benefit as well as have no side effects to when treat the immune diseases to reduce the side effects of some synthetic drugs [1.2].

One of these plants is the Neem tree (*A zadirachta indica* A. Juss.) is very popular tree which is a medical plant with wide applications in medicine scopes [2]. An application of Neem (Azadirachta indica) is a herbal plant which is known to exhibit beneficial pharmacological properties including immunomodulatory effect in broilers [3].

V. alginolyticus Causes wound infections and otitis It is also present in animals and human as well as in fishs , the virulence factors are many toxins such as potent neurotoxin, tetrodotoxin *V. alginolyticus* was first identified as a pathogen of humans in 1973 [5].

Material and Methods

All experiments were done in the laboratories of the in Dijlah university collage and

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animals lab house in College of science, Al-Muthanna University, the research on male albino mice (Blab-c), which their average weight was 22-25 grams.

Isolation and Identification V. *alginolyticus*

The isolated was obtained and identified by laboratories of Dijlah university collage.

Preparation V. *alginolyticus* Antigen

V. alginolyticus whole cell antigen was prepared according to a method presented by [6] by using Ultra soncater system.

Preparion of Ethanolic Extract of Seed Neem

The ethanolic extract preparation was according to a method presented by [7] and prepare two concentrations which are (200 µg / kg), (300 µg / kg) for administration.

Determination *V. alginolyticus* Antigen Dose

In this Experiment 20 mice have been divided to four groups to evaluate the level of immunoglobulin IgG in serum of mice that have been injected subcutaneously with different *V alginolyticus* antigen doses by using Radial Immunodiffusion (RID).

- Group I: was injected with normal saline (0.2ml).
- Group II: injected with a single dose 30*ug / ml* of *V. alginolyticus* antigen.
- Group III: injected with a single dose 50 *ug / ml of V. alginolyticus* antigen.
- Group IV: injected with a single dose of 77 *ug / ml* of *V. alginolyticus* antigen.

The blood were collect from the mice after a month of the second boaster dose and the serum is obtained and froze in -20ºC and the dose of antigen measured by Radial Immunodiffusion (RID)

Groups of Study

This current study was included 4 groups each group was included 5 mice and divided as following

- Group I injected with normal saline.
- Group II injected with *V. alginolyticus* antigen.
- Group III: injected with *V. alginolyticus* antigen+ (200 µg / kg) of Ethanolic neem extract
- Group IV: injected with *V. alginolyticus* antigen + (300 µg / kg) of Ethanolic neem extract

Laboratory Assessments

Nitro blue Tetrazolium (NBT) Index

The NBT procedure was done depending to a method presented by [8].

Lymphocyte Transformation Test (MTT)

The procedure of MTT was done depending on [8].

Serum Agarose Gel Electrophoresis

Serum electrophoresis was carried out using a commercially available kit for estimation of proteins level in serum.

Statistical Analysis

The Statistical Analysis values by using the computer programmer SPSS by probability value equal or less than 0.05.

Results

The bacterial isolate and identified Dijlah university collage by using the biochemical test and Ap20 index. After the identification the isolated was inoculated in brain heart infusion broth for activation and cultured on brain heart infusion agar respectively to increase the growth for antigen preparation [9].

In other hand in this study the results of determination the antigen dose were showed the immunoglobulin concentration IgG was a significantly increasing ($P \leq 0.05$) in group III 440.4 ± 6.18^{a} comparison with other groups as in Table (1).

Table 1: IgG concentration V. *alginolyticus* antigen.

Groups	Dosage	IgG Mg/dl
Group I	0.2 ml	97 ± 1.8^{e}
Group II	30 *ug / ml*	200.2 ± 3.8^{c}
Group III	50 *ug / ml*	440.4 ± 6.18^{a}
Group IV	77 *ug / ml*	331.3 ± 5.3^{b}

*The different letters denoted that significant differences among the groups $<p \leq 0.05$

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For the immunological parameters, three the immunological assays were used to evaluate the immune response in treated mice. Were included NBT test to evaluate non specific immunity and MTT Test to evaluate the adaptive immunity and serum electrophoresis to measure the serum fractions level to evaluate the Humoral immune response. The NBT results were showed significant differences between Group III and Group (I,II) in addition there was significant differences between, Group IV with other Groups (Group I, Group II) but results were revealed no significant differences between Group III and Group IV but the values of Group IV higher than Group III .As in Table (2). In other side of this study .The results of MTT test showed same results as in The NBT where there was significant differences in Group III and Group IV comparison with other groups (Group I, Group II) as well as no significant differences between Group III and Group IV but the values of Group IV higher than Group III .As in Table (2).

Table 2: The Result of NBT index and MTT test

Assays	Group I	Group II	Group III	Group IV
	Control (Normal saline only)	*V. alginolyticus* antigen	200 µg / kg Ethanolic neem +*V. alginolyticus* antigen	300 µg / kg Ethanolic neem + *V. alginolyticus* antigen
NBT	1.72±0.14[a]	3.55±0.24[b]	2.10±0.30[c]	2.15±0.38[c]
MTT	0.259±0.02[a]	0.300±0.08[b]	0.274±0.010[c]	0.280±0.019[d]

*The different letters denoted a significant difference between the groups <p≤0.05

In addition, the results of Gel electrophoresis showed the Group III, Group IV have significant differences with other groups and the highest value recorded in Group IV as in Table (3).

Table 3: the Result of Gamma globulin serum, Alpha-1 level, Alpha-2 level, Alpha-beta level

Group	Gamma globulin level	Alpha-1 level	Alpha-2 level	Alpha-beta level
Group I	6.73 ± 0.13[a]	2.27 ± .0.15 [a]	9.60 ± 0.23 [a]	10.83 ± 0.10 [a]
Group II	17.85±1.06[b]	6.14 ±0.58[b]	17.84±1.07[b]	18.65±2.22[b]
Group III	20.94±0.38[c]	12.46±9.2[c]	28.62±0.47[c]	22.8±1.18[c]
Group IV	21.94±0.40[c]	12.90 ±10.2[c]	29.62±0.50[c]	20.8±2.18[bc]

*The different letters denoted that a significant difference between the groups <p≤0.05

Discussion

This study was focused to investigate the effects of Neem seed extract on the immune response and the results were revealed by estimation (non specific immunity and adaptive e.g cell mediated immunity and humoral immunity by the phagocyte activity index and Transformation lymphocyte index and Gel electrophoreses respectively.

The results of Determination of V. *alginolyticus* antigen dose program were revealed the immunoglobulin concentration IgG was a significant increasing (P≤0.01) in a group III (50 ug/ml) comparison with other groups, due to the antigen dose was able to induce the immune response while doses were showed no significant difference due to the high dose and low dose in group II and IV group respectively lead to tolerance [10]. Furthermore The NBT results were showed significant differences in Group III, Group IV compassion with Group I, Group II but there is no significant differences between Group III and Group IV but the values of Group IV higher than Group III those results obtained in the present study were agreed with those obtained by [11 ,12].The effect of Neen extract on the immune response is still under investigations but depending on the previous studies that revealed the Neem extraction was helped to increase releasing the immunity mediators such as the cytokines which helped to enhance activation of macrophage and neutrophile, the mechanism of effect extract neem is enhanced release IFN-γ.

IFN-γ helped to activated the macchrophage and neutrophiles and since the V. alginolyticus antigen is internalized, it will be killed by any of killing mechanisms which are; oxygen-dependent killing mechanisms (this pathway is also called reactive oxygen intermediates; ROIs) and reactive nitrogen intermediates (RNI) [13].

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NO has been shown to be the principal effector molecule produced by macrophages for cytotoxic activity and can be used as a quantitative index of macrophage activation [14]. The increase in carbon clearance index reflects the enhancement of phagocytic function of mononuclear macrophage in vivo and thus non-specific immunity. Phagocytosis by macrophages is important against the smaller parasites and its effectiveness is markedly enhanced by opsonisation of parasite with antibodies and complement C3b leading to more rapid clearance of parasite from blood [15].

The above results led to the assumption that the extract has the macrophage stimulatory abilities through release of chemical mediators in the lysis of foreign materials in terms of the engulfment by phagocytosis. In addition the results of MTT test showed the group IV and III which were treated with the extract and the vaccine showed a significant increasing comparison with group I, II. Our results showed a agreement with [16, 12, 11, 18] who obtained the neem oil acted no specific and active the cellular immune response mechanisms .And agreed with [16].

Who reported The Spleen cells of treated mice with neem oil and Con A or tetanus were showed the lymphocyte proliferative was increased significantly , and also agreed with [11] whose results revealed the splenic T lymphocytes CD4+ and CD8+ and Natural Killer cells were increased in mice injected (neem leaf product). According to our results the fact that ethanolic neem seed extract affects stimulates TH1 cells and as well as that leads to enhance the TNF-alpha and gamma interferon in serum [19].

Also enhance the "Killer T" cells which is the cell-mediated immune system is the body's first defense against infection. Killer T-cells are able to destroy microbes, viruses and cancer cells by injecting toxic chemicals into the invaders. Neem the mechanism is attachment with complexes of TCR and this will active T lymphocyte in same way the MHC and peptide active T lymphocyte [20]. The increasing T lymphocyte will helps to enhance activation of CD4, which play important roles in immunity, particularly in the adaptive immunity (Cellular immunity and humoral immunity) by helping other immune cells to release the cytokines, that are essential in B cells antibody class switching and this was observed in results of the serum gel electrophoresis where the gamma globulin fraction level Alpha-1 Fraction and Alpha-2.

Fraction and Alpha-beta Fraction was increased in group that treated with the Neem extract combination with the antigen the potentianal use of ethanolic neem seed extract for humoral immune response resulting in increased the antibodies level and gamma globulin these results were agreed with [21] who used dried Garlic powder and neem leaves in the broilers feeds, also [22] who gave neem leaves infusion at 30, 40 and 50 ml/ltr in fresh drinking water to the broiler chicken, [23] and [4] whose results were showed humoral immune response of broiler chicks was effected by different levels of neem extract in combination with a biological material such As (Flavofosfolipol).

Conclusion

The results of present study were revealed the ethanolic Neem seeds extract had a positive immunomodulatory as showed the ethanolic extract of Neem were acted as a non-specific immunostimulant and enhanced the cell mediated immune (CMI) and humoral immune response, and the researchers continue their study to investigate the other immunological effects of the plants in next studies.

Acknowledgments

The authors are grateful to Dijlah university collage, Iraq and Al-Muthanna University-College of science, Departments Biology, Iraq to support us during the study and for providing the facilities.

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