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Research article

Evaluation of Immunity enhancing activity of *Cladophora glomerata* crude extracts and phytol in male albino mice

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ABSTRACT

Cladophora glomerata is the most widely distributed macroalga throughout the world's freshwater ecosystems. The immunological effects of *C. glomerata* in the present study were evaluated. The algal samples were collected from the fresh water in the canal around University of Baghdad. The GC- Mass analysis of crude algal extracts (aqueous and alcoholic) was carried out and the active chemical compounds in algal extracts were described. Phytol represented the highest one between the other compounds in alcoholic extract. Our study provides for the first time immunological effects of *C. glomerata* and phytol on interleukins (IL-2 and IL-4) and interferon-gamma levels. The production of IL-2 was increased as dose - dependent manner in all treated groups of mice. The production of IL-4 was also increased as dose-dependent manner in both alcoholic extract and phytol. The current study revealed that the aqueous extract increased IFN-γ as dose dependent manner, while the higher dose of alcoholic extract and phytol decreased IFN-γ. In addition, the lower dose of alcoholic extract increased IFN-γ. The data of this study suggested that the both crude extracts of *C. glomerata* and phytol can be used as immune-stimulator.

Keywords: Algae, *Cladophora*, Cytokines, Phytol.

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INTRODUCTION

Cladophora glomerata (L.) Kutz is potentially the most widely distributed macroalga throughout the world's freshwater ecosystems [1]. It covers over 70% of the water surface or of the bottom and it is the major contributor to biomass and plays an important role in primary production and nutrient cycling [2]. The active compounds in *C. glomerata* could be used as a novel source of natural antimicrobial and antioxidant agents for pharmaceutical industries [3]. Fabrowska *et al*. (2015) reported that *C. glomerata* could be used in cosmetic preparations because it contains fatty acids, polyphenols, macro- and microelements and terpenoids like phytol which is regenerating and rejuvenating the skin [4]. Phytol is one of the most important and simplest of the diterpenes is phytol or (3,7,11,15-tetramethyl- 2-hexadecen-1-ol), which forms the lipophilic side-chain of the chlorophyll [5]. It is highly significant among algal terpenes, as it has a definite role to play in the metabolism of algae [6]. Few studies focused on immunological applications of algae. The biological properties of immunological interest have been demonst-

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rated in 140 species of algae, and they have been found to have useful applications in human health, particularly in the fields of oncology and immunology [7].

Two major immune system components are innate immunity and acquired immunity which protect the body from the invasion of foreign substances. Innate immunity is the first line of defense for invading pathogens (non specific defense), while the acquired immunity (adaptive immunity) is specific immune response [8]. Cytokines are molecules secreted by cells that have been stimulated by potentially infectious materials. These are an important group of protein which include intrleukins (IL-1 – IL-18), Interferons (IFN) (alpha, beta and gamma), tumor necrosis factor, colony stimulating factors, basic and acid fibroblast factors and others [9].

The term 'interleukin' (IL) has been used to describe a group of cytokines with complex immunomodulatory functions including cell proliferation, maturation, migration and adhesion. Interleukins also play an important role in immune cell differentiation, activation and elicit a wide variety of responses in cells and tissues [10]. IL-2 is considered a key growth and death factor for antigen-activated T lymphocytes and it is the major inducer for the developmental production of suppressive T-regulatory cells. *In vivo* studies demonstrate that IL-2 controls autoimmunity through the production of CD4+ and CD25+ [11]. The signals of Interleukin-2 (IL-2) influence various lymphocyte subsets during differentiation and immune responses and it is crucial for the maintenance of (T-Reg) cells [12]. IL-4 was called the "prototypic immune-oregulatory cytokine." because it has an important role in regulating antibody production, inflammation, and the development of effector T-cell responses [13]. Chu and Paul (1997) referred that IL-4 is a cytokine that regulates growth and differentiation of lymphoid and non lymphoid cells. It was first recognized as a determinant of immune-globulin (Ig) class switching specificity of B lymphocytes stimulated with lipopolysa-ccharide (LPS) [14]. Interleukin-4 is a key cytokine in the development of allergic inflame-mation, it mediates important pro-inflammatory functions in asthma including induction of the IgE isotype switch, promotion of eosinophil transmigration across endothelium, mucus secretion, and differentiation of T helper type 2 lymphocytes leading to cytokine release [15]. The cellular effects of IFN-γ are up-regulation of pathogen recognition, antigen processing and presentation, inhibition of cellular proliferation, effects on apoptosis, immunomo-dulation, and leukocyte trafficking [16]. It modulates T and B cells activities, certain macroph-age activation processes, and natural killer reactivity of cells from various animal species.

However, the studies on immunological effects of *C. glomerata* in Iraq are scanty. That is why; we tried to cover the effects of its extract on some cytokines. Moreover, the effect of one of the active compound (Phytol) was also studied here.

MATERIALS and METHODS

Sample collection

C. glomerata was collected from the fresh water in the canal around University of Baghdad and stored in plastic bags prior to transport to the laboratory.

Isolation of macro algae

The collected algal samples were identified with the help of classical algal classification reference [17]. These samples were cleaned from epiphytes, necrotic parts and then washed with freshwater to remove sand, and any adhering substances. Samples were rinsed with sterile water to remove any associated debris and dried in oven with 38-40 ˚C and then these samples were stored in the refrigerator.

Preparation of alcoholic extract of *C. glomerata*

The dried algal samples were powdered with the help of the blender. The powder (10 gm) was filled in the thimble and extracted with ethanol 70%, by using a soxhlet apparatus at the temperature of 60 ˚C for 9 h [18]. The extract was dried in oven at 38-40 C˚. The dry extract was collected and weighted then preserved at 4ºC in refrigerator until required. A known weight of this extract was dissolved in Phosphate buffer saline (PBS) to make a stock solution for further concentrations.

Preparation of aqueous extract of *C. glomerata*

Dried algal samples were weighed followed by boiling at 70˚C for one hour. The extract was then filtered through filter paper (Whatman no.1). The filtrate was evaporated and then algal extract was stored at 4˚C [19]. A known weight of this extract was dissolved in PBS to make a stock solution for further concentrations. Furthermore 0.3 gm of crude extracts was dissolved in 2 ml ethanol. The solution was analyzed using Shimadzu GCMSQP2010Ultra.

Preparation of phytol stock solution

Phytol with purity (97%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Phytol was emulsified in 0.05% Tween-80, dissolved in 0.9% saline [20]. To make a stock solution for further concentrations**.**

Laboratory animals

Albino male mice in age 8-10 weeks and weight 23-25 gm were divided into three groups; each group was kept in a separate plastic cage. Mice were maintained at a temperature of 23-25 C˚, and they had free excess to food (standard pellets) and water. Mice of the first group were injected intraperitoneal with crude extracts (aqueous and alcoholic) in two doses (50 and 150 mg/kg), while the second one injected with pure phytol (50 and 150 mg/kg) and in the control group, mice were injected with normal saline.

Determination of interleukins in mice blood samples

Blood samples from control groups and treated mice were taken by using insulin syringe (1 ml), they were put in eppendrof microfuge tube and left for 15 min. The samples were centrifuged in a micro centrifuge to get the serum. The samples of serum were kept in a deep freeze until to be analyzed for IL-2, IL-4 and interferon gamma (IFN γ).

The current study followed the instructions which illustrated in **ABCAM'S** , which is *in vitro* ELISA (Enzyme-Linked Immunosorbent Assay) kit for the quantitative measurement of IL-2 and IL-4 in mouse serum, as well as to the **ADI's** Mouse IFG ELISA for measuring Mouse IFG in serum.

Statistical analysis

The results of a different parameters for the treatments (control, aqueous extract, alcoholic extract and phytol) in a current study, were analyzed by using the analysis of variance (ANOVA), F-test and T-test, carried out in complete randomized design (CRD). Difference between means of treatments were analyzed using least significant differences (LSD) at ($P \leq 0.05$), and expressed as (mean ±Sd). Programmings excel application and SPSS program (2010) was used to find the results and draw the figures with some effects to explain the statistical difference.

RESULTS

GC-MS analysis of *C. glomerata* aqueous extract

Thirty four chemical compounds were presence and identified in aqueous extract of *C. glomerata*. The highest peak of the screened components belonged to formic acid, butyl ester**.** The other compounds represented alcohols, acids, monoterpene Eucalyptol), hydrocarbons (undecane) and aromatics like (P-xylene and benzene 1,2,3 trimethyl).

GC-MS analysis of *C. glomerata* alcoholic extract

In this study thirty-seven constituents were identified in alcoholic extract of *C. glomerata*. The highest peak of the screened compounds belonged to phytol. This compound is the common occurrence diterpene alcohol in the green algae. The other compounds represented phenol, sterol, acids, alcohols, hydrocarbons (like hexadecane) and fatty acids.

Effect of *C. glomerata* crude extracts and phytol on Interleukin-2 level

The results in **table** 1 showed the significant difference in IL-2 production in all treated mice as compared with control group. In addition, there were significant differences between the two doses of phytol with the others in both crude extracts. The current results also showed that the production of IL-2 increased as dose dependent manner in all treated groups (**Fig. 1**).

Table 1. Levels of IL-2 (pg/ml) in blood sample of mice post treated with different doses of *C. glomerata* crude extracts. Capital letters refer to comparison in rows and small letters refer to comparison in columns, similar letters refer to non-significant differences between means at ($p \leq 0.05$), using (LSD test). The data presented in mean ± SE.

`	Control	150 mg / kg	50 mg / kg
Aqueous Extract	14.00 **C** ± 0.15 a	**37**.23 **A** ± 0.46 a	21.60 **B** ± 0.72 b
Alcoholic Extract	14.**00 C** ± 0.15 a	35.40 **A** ± 1.81 a	20.97 **B** ± 1.98 b
Phytol	14.00 **C** ± 0.15 a	29.17 **A** ± 1.65 b	**25**.10 **B** ± 1.**04** a
LSD P ≤ 0.05	**3.93**		

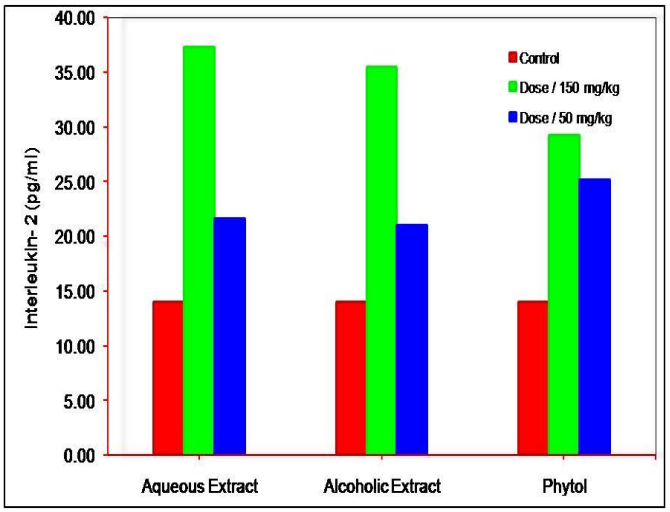

Fig 1. IL-2 level in mice post treated with two doses of *C. glomerata* crude extracts.

Effect of *C. glomerata* crude extracts and phytol on Interleukin-4 level

Table 2 shows a significant difference in IL-4 production in mice groups that administrated alcoholic extract and phytol as compared with control group. While the higher dose in aqueous extract showed the significant difference. In addition, the two doses of aqueous extract showed significant difference with the same doses in alcoholic extract and phytol. The present study also revealed that the production of IL-4 was increased as dose-dependent manner in both alcoholic extract and phytol. In contrast, at a higher dose of aqueous extract the production of IL-4 was reduced as shown in **Fig. 2**.

Table 2: Levels of IL-4 (pg/ml) in blood sample of mice post treated with different doses of *C. glomerata* crude extracts. Capital letters refer to comparison in rows and small letters refer to comparison in columns, similar letters refer to non-significant differences between means at ($p \leq 0.05$), using (LSD test). The data presented in mean ± SE.

`	Control	150 mg / kg	50 mg / kg
Aqueous Extract	24.67 **A** ± 0.35 a	12.80 **B** ± 0.71 b	22.33 **A** ± 1.24 b
Alcoholic Extract	24.67 **C** ± 0.35 a	35.67 **A** ± 1.17 a	32.20 **B** ± 0.70 a
Phytol	24.67 **C** ± 0.35 a	36.77 **A** ± 0.72 a	32.47 **B** ± 1.39 a
LSD P ≤ 0.05	**2.92**		

Effect of *C. glomerata* crude extracts and phytol on IFNγ

The results in **table** 3 showed a significant difference in level of IFN γ post administrating mice with both doses of crude extracts (aqueous and alcoholic), and the higher dose of phytol as compared with control group. The difference was significant in case of lower as well as higher

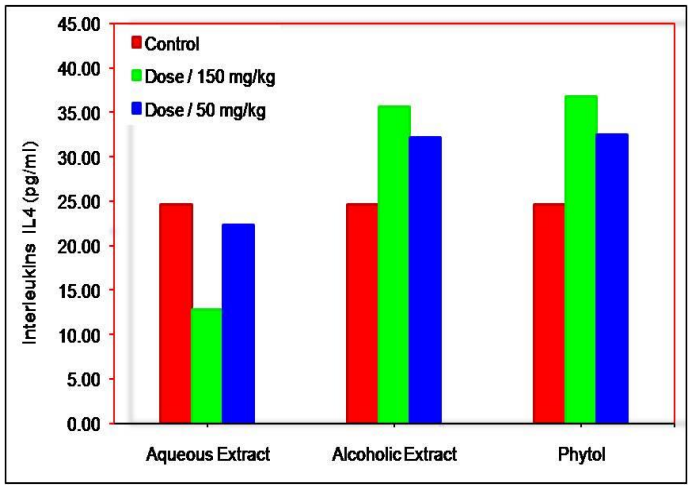

Fig 2. IL-4 level in mice post treated with two doses of *C. glomerata* crude extracts.

doses in all treated groups. The current results revealed that the aqueous extract increased IFN-γ as dose dependent manner, while the higher dose of alcoholic extract and phytol decreased IFN-γ. In addition, the lower dose of alcoholic extract increased IFN-γ (**Fig 3).**

DISCUSSION

The lower dose of phytol increased the production of IL-2 more than the lower doses in both crude extracts, this result is in accordance with previous study [21]. The investigators referred that only a small amounts of phytol is required to stimulate the immune responses. However, the activity of alcoholic extract may be resulted from phytol, which represented the main components in this extract, as well as the other compounds especially, fatty acids which modulate the immune response, by changing the cytokines biosynthesis [22].

Table 3. Levels of IFNγ (pg/ml) in blood sample of mice post treated with different doses of *C. glomerata* crude extracts. Capital letters refer to comparison in rows and small letters refer to comparison in columns, similar letters refer to non-significant differences between means at (p≤ 0.05), using (LSD test). The data presented in mean ± SE.

`	Control	150 mg / kg	50 mg / kg
Aqueous Extract	310.19 C ± 1.08 a	415.27 A ± 0.897 a	330.77 B ± 1.178 a
Alcoholic Extract	310.19 B ± 1.08 a	305.33 C ± 0.882 b	313.43 A ± 0.601 b
Phytol	310.19 A ± 1.08 a	295.33 B ± 0.33 c	308.33 A ± 0.882 c
LSD P ≤ 0.05	2.66		

According to the results, this study suggested that the two crude extracts of *C. glomerata* and phytol have an ability to stimulate the immune response by stimulating T-helper zero (Th_0). The aqueous extract of *C. glomerata* was able to activate Th_0 by increasing the production of IL-2 (**Table** 1), without transform these cells to T helper -2 by reducing IL-4 production, which indicated that the immune response stopped at Th_0. In contrast, the phytol and alcoholic extract have the ability to transform the Th_0 to Th-2 by increasing the level of IL-4.

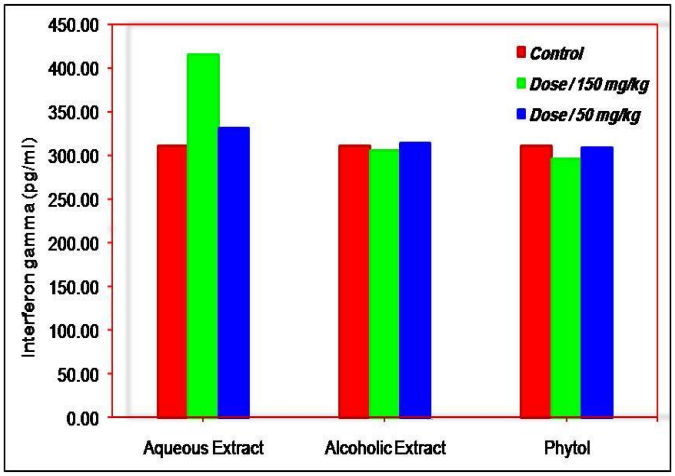

Fig 3. IFNγ level in mice post treated with two doses of *C. glomerata* crude extracts.

According to **tables** 2 and 3 the results suggested that the aqueous extract could stimulate the cellular immunity (cytotoxic T-cell) and humeral immune response by reducing the production of IL-4 and increasing IFN-γ. In contrast, alcoholic extract and phytol could stimulate the humeral immunity only by increasing IL-4 and decreasing IFN-γ.

From present study it can be concluded that the crude extracts (aqueous and alcoholic) of *C. glomerata* and phytol can be used as adjuvants with some vaccines to stimulate the immune response against antigens.

Conflict of interest

The authors declare that they have no conflict of interests.

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