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Isolation, Purification and Characterization of Novel Phytomitogen from Nigella sativa Seeds

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K E Y W O R D S

Mitogen, Nigella sativa, Phytomitogen, Protein extraction.

A B S T R A C T

The objective of the present study was to extract and purified proliferative factor for peripheral blood lymphocytes from Nigella sativa seeds. Dimeric 44, 32 and 26 -kDa glucosamine-specific lectins were extracted and purified from seeds of N. sativa L. by using different ionic strength solvents (deionized distilled water, phosphate buffer slain and 0.1 M HCl) purification procedure included, precipitation in saturated ammonium sulphate, then Sephadex G-100 gel filtration was used for further purification, finally electrophoresis by polyacrylamide gel to estimate the molecular weight in the presence of sodium dodecylsulphate. Single absorbance peak at 280 nm was recorded in each extracted method. The temperature and pH value on mitogenic activity was studied in temperature ranged from 4°C-60°C, the mitogenic activity reduced with gradual increase of temperatures, 50% of activity lost at temperature greater than 40°C, while the optimum pH for mitogenic activity ranged from 5-9, at pH value less than 3, and greater than 12 the activity disappeared. Interestingly, lectin extracted from N. sativa seeds have high activity especially that extracted with 0.1 M HCl and showed stability at temperature from 4°C to 10°C and pH from 5 to 9. The pure lectin is a homodimeric molecule of 26 kDa with subunit molecular mass of 12.9 kDa. The novel lectin exceed the PHA-Sigma in mitogenic activity.

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1. Introduction

From a previous study in PhD thesis for the researcher on Nigella sativa L. extracts, noticed that most extracts have mitogenic effects on peripheral blood lymphocytes of healthy and leukemic persons, so this study aims to investigate the mitogenic effects of proteins that extracted from N. sativa seeds, especially most of phytomitogens are extracted and produced from plants belong to Leguminosae [1]. Nigella sativa L. (Ranunculaceae,) a small herbaceous plant 30-50 cm in length, grows in the Mediterranean regions and cultivations in different parts of the world [2, 3]. Their fruits like small vesicles, and small-sized black seeds, about 3mm in length with acceptable aromatic odor and taste. N. sativa have potential therapeutic effects on various type of disease and have high power efficiency in antimicrobial infections, anti-tumor, antioxidant, detoxification of chemotoxic therapy In vivo, the

essential oil of N. sativa have good ability to heal injuries and deep wounds in less time[4, 5]. Chemical analysis of black seeds shows 23% protein, 38% carbohydrates, 35% essential and volatile oils and many types of vitamins like A, B, B2 and C, in addition to Ca^{+2}, K^{+1}, Fe^{+3}, Mg^{+2}, as well as 15 types of amino acids nine of them as essential, beside two types of alkaloids nigellamine-n-oxide and nigellicine and unique phenolic compound called thymoquinone which have significant therapeutic impacts[6-9]. From the numerous previous studies on N. sativa extracts, we notes high ability of these extracts to stimulate cell proliferation and increase cell mitotic in somatic and peripheral blood lymphocytes [9]. For thus, the present study aims to investigate the ability of proteins that extract from N. sativa seeds by various ionic strength solutions and test their ability on mitotic of peripheral blood lymphocytes In vitro as compared with standard mitogen like phytoheamagglutinin (PHA).

2. Materials and Methods

The present study was carrying out in Tissue Culture and Immunochemical Lab /Environmental Research Center / University of Technology/ Baghdad, Iraq, from January to June 2015, while blood samples were collected from healthy people after their written approval.

I. Chemicals

Tissue culture medium (RPMI-1640), Bovine Serum Albumin, Trypsin, Protease and phytoheaglutinine were purchased from Sigma Aldrich Co. USA. HCl, Ammonium sulfate, Abslute Methanol, Glacial Acetic Acid, polyacrylamide gel, Tri-HCl, and NaOH from BDH Co. UK. All materials that used in experiments utilized directly without further purification.

II. Seeds of Nigella sativa

Seeds of *N. sativa* were purchased from local markets and certified in the (General Authority for examination ratification seeds, Ministry of Agriculture, Baghdad, Iraq).

III. Extraction of mitogen

N. sativa seeds were cleaned manually by removing botanical impurities and dust, washed twice with tap water then by deionized distilled water, clean seeds were dried by air flow at room temperature and pulverized by laboratory ceramic mortar till obtain fine particulates. Powder was stored in dark cooled place until use. Five grams of powder were submerged in 250ml of different ionic strength solvents (0.1M HCl, PBS, DDW) for 24 hours at 4°C with continuous stirring. The extracted solution was filtered by (Wattman No. 1) filter paper, and centrifuged at 30000 g for 30 min at 4°C. The pellet was slighted while the supernatant used as crude protein source [1, 10].
For more purification of protein, ammonium sulfate (NH4)2SO4 was added to the crude extracts as consecutive batches with continued stirring at 4°C, practically, the equilibrate was achieved within two hours, the mixture was for 24hr at room temperature at sack 35 mm 1.4 inch MWCO 12,000 Da (BDH) [11]. Further protein purification obtained by Sephadex G-100 molecular column, was eluted with 150 mM NaCl (pH 7.2). 10ml of the semi purified protein was loaded into previously equilibrated column with flow rate reach to 0.5ml /min, 7 ml of 3 fractions were harvested and absorbance tested in UV-VIS spectrophotometer at 280 nm. Protein concentration was tested by using the Bradford method with BSA as standard [11]. Electrophoresis by polyacrylamide gel was used to estimate the molecular weight in the presence of sodium dodecyl sulphate (SDS) [12, 13], centrifuged at 30000 g for 30 min at 4°C, the deposited kept at 4°C, while the supernatant was saturated by adding more ammonium sulfate $(NH_4)_2SO_4$ to harvest more quantity of remaining protein, the output from ammonium sulfate, dissolved in PBS (pH 7.2), desalting the access ions by dialysis method against DDW with bovine serum albumin, trypsin, proteinase as standards. mitogenic activity of each protein was tested on peripheral blood lymphocytes (PBL), by measuring Mitotic index, lymphocytes have been cultivated for 70 hours at different concentrations (1, 2, 3, 4, 5, 10, 15, 25) µg/ml of purified proteins, the mitotic index calculated according to Verma and Babu [14].

IV. Effects of temperature and pH value on activity

The extracted mitogen samples were warmed at 4°C to 60°C for 30 minutes. Samples were quickly cooled on ice bath, and then the mitogenic activity measured on peripheral blood lymphocytes. Percentage of mitogenic activity was calculated by dividing the activity of the sample by the activity of that at room temperature .While pH effect was tested at different concentration of H+ ion at following reagents: pH 1-4: HCl; pH 5-7: PBS; pH 6–10: Tris-HCl and pH 11–14: NaOH, equal volumes of protein solution and pH solution were mixed and incubated at 25°C for 15 minutes [15, 16].

3. Results

Several steps of purification were applied to extract and purified of proteins from N. sativa seeds, the first step, was extracted by different ionic strength solvents (DDW, PBS and 0.1 M HCl), then precipitated by a gradual addition of ammonium sulphate, then Sephadex G-100 molecular column was used for more purification, finally electrophoresis by PAGE-SDS was used to determine the molecular weight of purified proteins. Table 1, shows extracted method and resulting fractions of each step, actually no further protein was precipitated up to 35% of ammonium sulphate saturation. From the results protein extracted by 0.1M HCl gave lowest molecular weight, highest absorbency at 280nm and showed highest mitotic activity (Figure 1 and Figure 4). The mitotic activity of (extract A) was higher than commercial PHA from Sigma at the same concentrations (Figure 5), while proteins were prepared from phosphate Buffer saline (PBS) and Deionized Distilled Water (DDW) gave less absorbency at 280 nm (Figure 2 and Figure 3) respectively and less mitotic index (Figure 4). Horizontal electrophoresis gel was used to examine the homogeneity of extracted proteins, one band of each extract appeared in alkaline medium (Figure 5), while in acid medium two bands appeared according to the time of passing between loading halls and the end point. Molecular weight of the extracted proteins by thin layer electrophoresis gel was not sufficient due to weak staining ability of the extracted proteins by bromophenol blue and the bands appeared as smear without clear sharp edge, experimentally the (A) protein has approximate molecular weight ranged from 20 to 60 kDa according to the electrophoretic conditions . The results of temperature and pH effects on mitogenic activity was summarized in (Figures 6 and 7) respectively, temperature ranged from 4°C-60°C was determined in the mitogenic activity the great activity was recorded at 4°C while 50% of activity was lost at temperature greater than 40°C, while the optimum pH for mitogenic activity ranged from 5-9, however, at pH value less than 3 and greater than 12 the activity was completely disappeared.

4. Discussion

Compared with purification of most Phaseolus coccineus and Phaseolus vulgaris mitogens that required more steps [10, 17] the preparation of purified N. sativa protein can be inhibited or stimulated by removing or adding the pointed carbohydrate, by this way can be controlling the activity of such protein, also protein can be nominated according to their carbohydrate binding specificity, such as mannose-binding, glucose-binding, galactose-binding and N-acetylgalactosamine-binding [18].

Frequent and multiple steps of purification of the raw extraction yielded acceptable purity of mitogen composed from one main substance, In SDS-PAGE at pH 8.5, protein moved as a single band of 25.8 kDa (Figure 5). Similarly, in acid medium pH 4.5 protein migrated as two bands of molecular weight 57 kDa, this may be due to that the lectin available as a homodimer of two identical subunits which are not linked together by disulphide linkages [19,20] or the lectin isomers result from variations side chains of oligosaccharide [21] or due to altered some amino acids in stem of lectins [22], the mitogenic activity of the isolated protein by 0.1 M HCl exceed the mitogenic activityt to that isolated from Phaseolus vulgaris L., such as PHA-Sigma at the same concentrations. Structurally, there is remarkable homology between P. vulgaris L lectin and N. Sativa lctins in N-terminal sequence, N. Sativa lectin illustration of some simple sugars like D(+) glucose, D(+) fructose, D(+) galactose, (+) ribose, D(+) lactose, D(+) sucrose D(+) arabinose and L(+) maltose. N. Sativa lectin is reasonably thermostable because its mitogenic activity is constant at 40°C, and but is dropped at 50°C. Actually, activity was kept even beyond heating at 50°C for 60 minutes [22].

Table1: Description of phytomitogen extraction steps from *N. sativa* seeds flour by different ionic strength solvents at 25°C with initial weight 20 gml^{-1}

Extraction by acid (0.1M HCl)			Extraction by PBS		Extraction by DDW	
Step	Protein	Yield/mg	Protein	Yield/	Protein	Yield/
Aqueous extraction	4275	40	2156	33	1876	30
(NH4)2S04	6754	34	3432	12	2156	11
Sephadex G-100	2135	6	1273	8	523	7
Polyacrylamide gel	1456	4	513	2	231	1.2

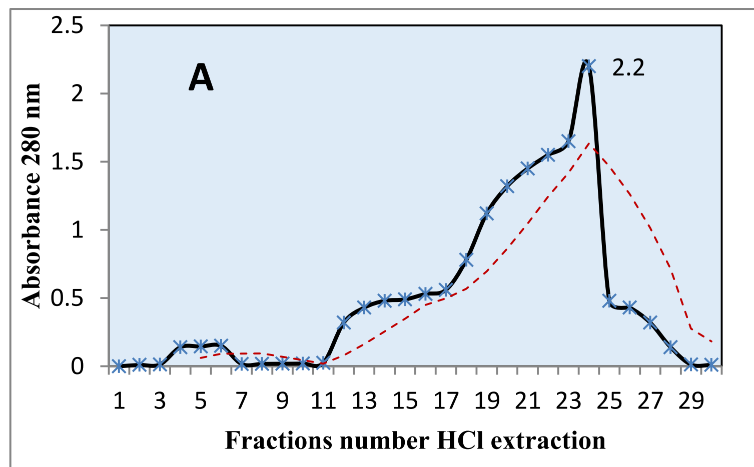

Figure 1: Absorbency curve at 280 nm of (0.1M HCl) extraction protein and elution profile of a freshly prepared solution of the 0-50% ammonium sulfate precipitate on Sephadex G-100 column

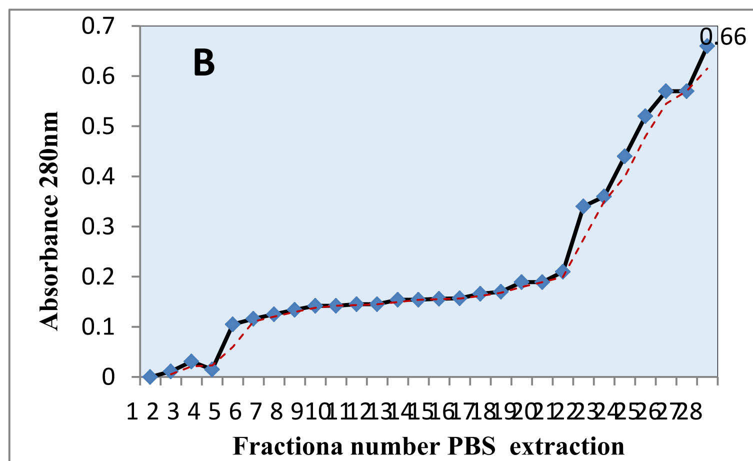

Figure 2: Absorbency curve at 280 nm of PBS extraction protein and elution profile of a freshly prepared solution of the 0-50% ammonium sulfate precipitate on Sephadex G-100 column

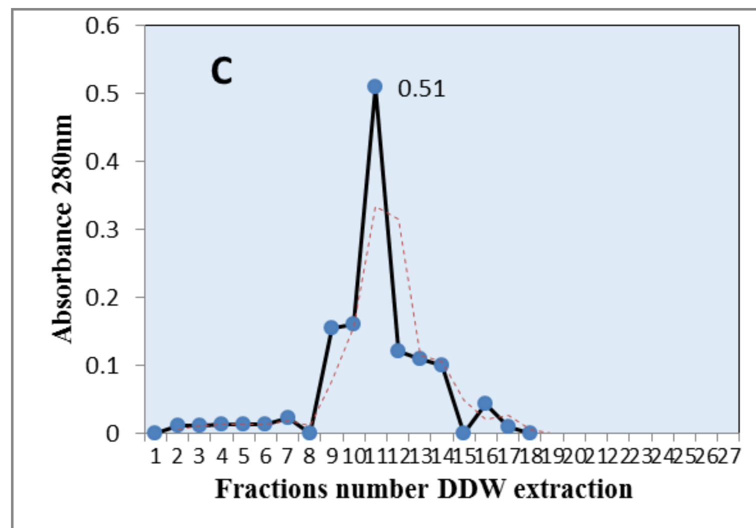

Figure 3: Absorbency curve at 280 nm of DDW extraction protein and elution profile of a freshly prepared solution of the 0-50% ammonium sulfate precipitate on

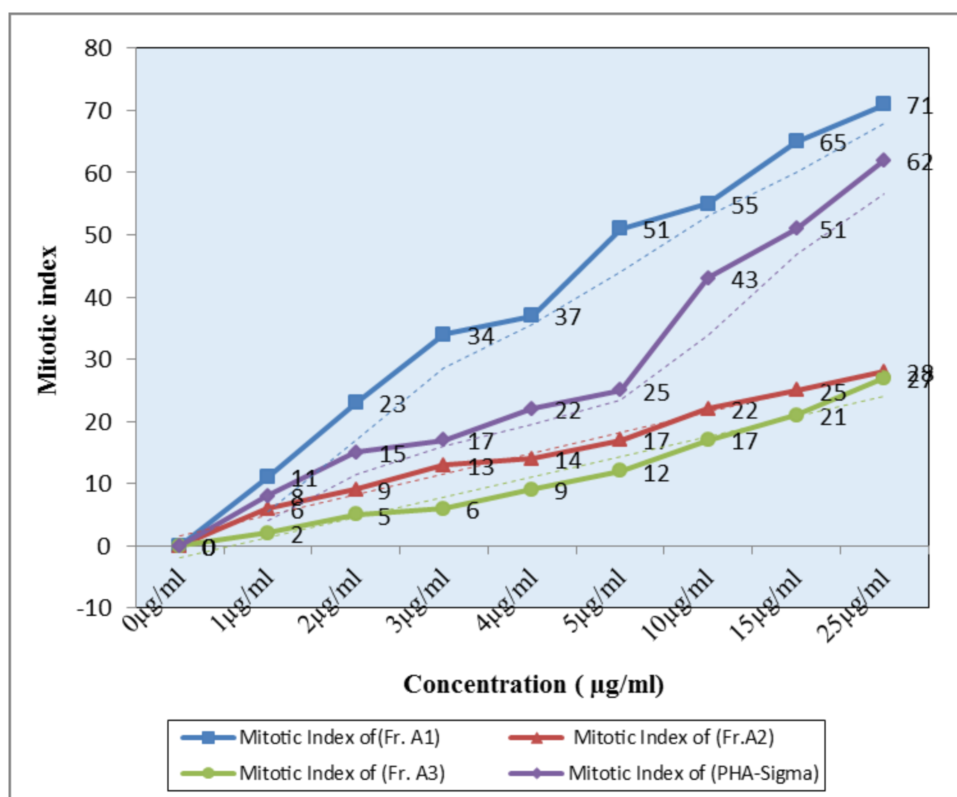

Figure 4: Dose depending mitogenic activity of the prepared lectins as a comparison with commercial mitogen

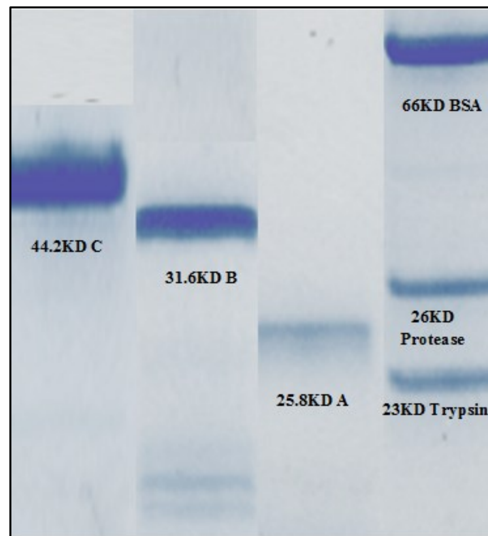

Figure 5: Purified N. sativa L. profile at SDS- PAGE lane 1 (A) protein extracted by 0.1 M HCl (B) protein extracted by PBS (C) protein extracted by DDW, lane2 standard proteins.

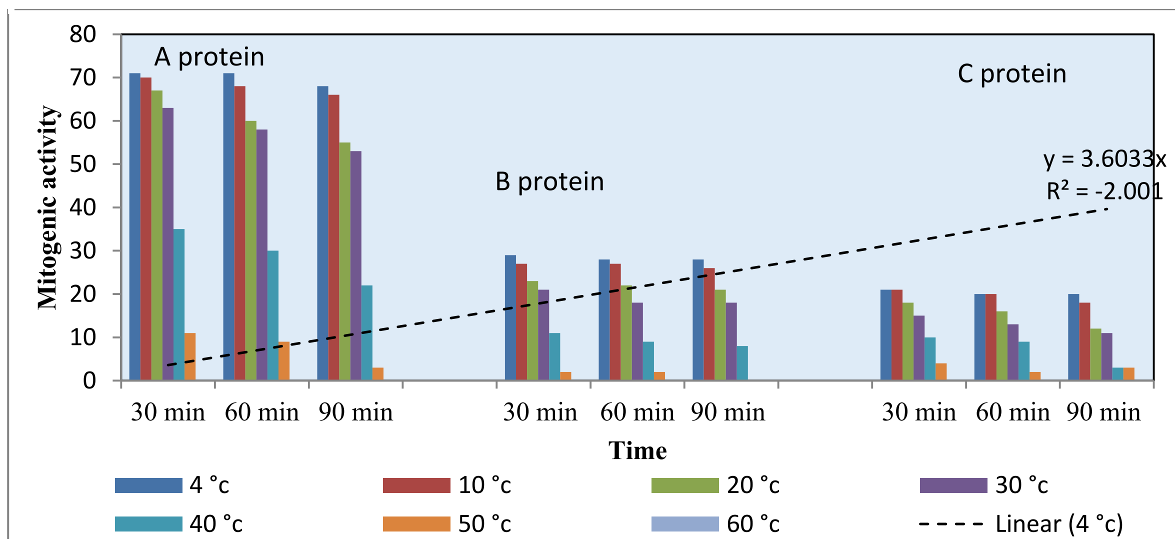

Figure 6: Effect of temperature value on the mitogenic activity of A, B, and C lectin with time, using human peripheral blood lymphocytes

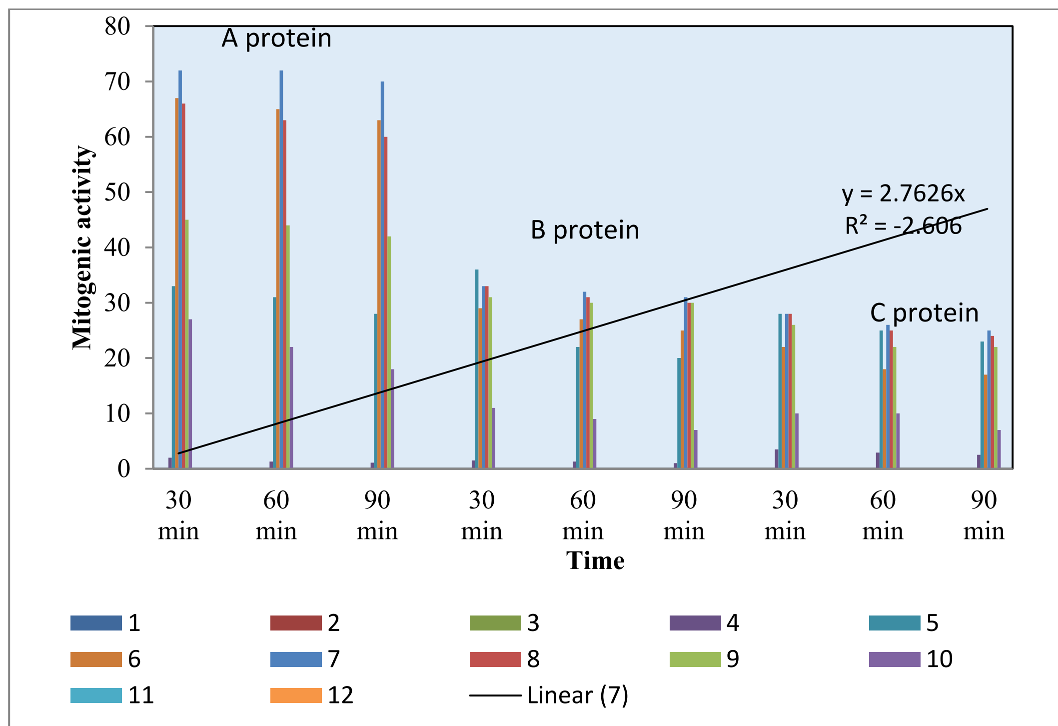

Figure 7: Effect of pH value on the mitogenic activity of A, B, and C lectin with time using human peripheral blood lymphocytes

5. Conclusions

The pure lectin is a homodimeric molecule of 26 kDa with subunit molecular mass of 12.9 kDa. The novel lectin exceed the PHA-Sigma in mitogenic activity this may be aided in explore the mechanism of lymphocyte activation as lectin bind to sugars of their specificity. So more research on pure lectin on molecular level or clinica trails may give us more information about their function and activity of such proteins on lymphocytes behavior.

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