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Research Article

Assessment of Immunomodulatory Effects of Black Cumin Seed (*Nigella Sativa*) Extract on Macrophage Activity in Vitro

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ABSTRACT

This experimental study was proceeded to explore the immune-impact of black cumin seed "*Nigella sativa*" which is considered the most common and important medical herb known in Middle East and Islamic countries. The seed was dissolved in a mixture of (ethanol + water) mixture, then filtered, evaporated and dried under reduced pressure to obtain seed extract. The macrophages of three different origins; blood derived, splenic and peritoneal were provided from rats and mixed with two different seed extract doses and incubated then one part was stained with fluorescent acridine orange and examined under fluorescent microscope while the other part was inspected through transmission electron microscopy to assess the phagocytic and killing potency. The results showed a remarkable increase in both parameters and interestingly higher in peritoneal and splenic than blood derived macrophages. The obtained data indicated the immune-stimulant impact of the black cumin seeds.

Key words: Black cumin seed, Immunomodulation, Killing assay, Macrophages, *Nigella sativa*, Phagocytic activity.

INTRODUCTION

Immuno-tuning is critical for the conservation of immune system homeostasis and combating many diseases and disorders. The previous two decades showed raising studies concerned immunomodulatory impacts of medical plants and corresponding formulae. This includes their stimulatory effects on immune cells, immune organs, and cytokine production, as well as inhibitory effects on inflammation, allergy, and autoimmune diseases (Cheng *et al*. 2018). The widespread usage of spices, herbal extracts and active ingredients of vegetable origin support their ability for potentially replace the chemotherapeutic agents to avoid their known adverse reactions, stress on the beneficial microbiota and the developing antimicrobial resistance in pathogens (Mastan, 2015).

Nigella sativa, "commonly known as black cumin seed" is a well-known medicinal herb which has many curative properties. *N. sativa* is commonly cultivated in Middle Eastern Mediterranean countries. The seeds and oils have been popularly used for treatment of different illnesses related to the respiratory, digestive, renal and hepatic functions, as well as their immune system support (Ahmad *et al*., 2013).

Macrophages are major immune system regulators and implicated in many immunological activities. They engulf and assimilate microbes, cellular debris, cancer cells, and any non- self molecules, in a process called phagocytosis. These big eaters destroy the pathogens via production of bactericidal molecules (Murray and Wynn, 2011). In addition to phagocytosis, macrophages play not only an essential role in nonspecific defense (innate immunity) but also assist the initiation of specific acquired immunity (Bonnardel *et al*., 2015). Macrophages are found in all tissues but with different shapes and names, all forms are derived from monocyte; the primary kind of mononuclear phagocyte present in the blood. Monocytes are developed in the bone marrow, and some differentiate then migrate into various tissue and lymphoid organs (Davies *et al*., 2015).

Based on the above, the recent insight of medical plants is directed towards their extracts and bioactive components link to the immunological acts. The current study investigated the effect of the *N. sativa* seeds extract on different activities of macrophages; phagocytic and killing ability.

MATERIALS AND METHODS

Ethical approval: Anesthetic steps and handling with animals followed the ethical guidelines of the Ethical Committee of the National Research Centre in Egypt under number of 17119.

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Preparation of *N. Sativa* seeds extract

The hydroethanolic extract was prepared through the mixing of 200 g of chopped *N. sativa* seeds with 800 ml of 50% ethanol for 72 h at 40°C. The solutions after filtration were dried and the solvent (ethanol + water) was totally removed by rotary evaporation at 40°C under reduced pressure. The dried extract was solved in distilled water to achieve the final concentrations and stored at 4°C in dark closed container (Gholamnezhad *et al*., 2016).

Animals

Fifteen female Wistar albino rats of average weight (100- 130g) were divided randomly into 3 groups; each with 5 rats. They were obtained from the Animal House, National Research Centre, Egypt. All animals were kept in controlled environment of air and temp with access of water and fed with standard diet along the experimental period.

Assessment of macrophage phagocytic and killing activities in *vitro*

Isolation of monocytes from blood

The first group of rats was anesthetized with diethyl ether and blood samples were collected using heparinized falcon tubes. The heparinized (10 IU/ml) rat blood grouped samples were centrifuged at 3000 rpm for 10 minutes, then the buffy coat was aspirated and carefully layered on histopaque phagocytes isolation medium, pH 7.3 by a ratio 1:3 in siliconized centrifuge tubes and the gradient was centrifuged at 280 xg for 25 min at 4°C. The interphase layer that contained mononuclear cells was aseptically aspirated and centrifuged at 3000 rpm for 5 minutes (Winnicka *et al*., 2000).

Isolation of murine splenocytes

The second group of rats was anesthetized with diethyl ether. Splenocytes were obtained as described by Alonso Castro *et al*., (2012) and seeded in 96 well plates at a density of 2 $x10^5$ cells /well.

Isolation of murine peritoneal macrophages

The third group of rats was used for collection of peritoneal macrophages as described by (Sarkar et al., 2005). Each rat was injected i/p. in 0 day with 2 ml of 2% starch solution in phosphate buffered saline (PBS), 0.02 M, pH 7.2. After three days, rats were anesthetized with diethyl ether and the peritoneal cells "comprising macrophages principally" were lavaged with sterile 10 ml PBS. The exudate was centrifuged at 4000 rpm for 10 min resulted in cell pellet.

Preparation of bacterial suspension

Staphylococcus aureus ATCC 25923 was obtained from the reference laboratory of the Cairo Microbiological Resources Center (Cairo MIRCEN), Faculty of Agriculture, Ain-Shams University. The staphylococci were maintained on nutrient agar slopes (Difco) and grown overnight in BHI broth (Difco Laboratories, Detroit, MI). The overnight cultures served as inoculum; these bacteria were then harvested and washed twice in Hanke 's balanced salt solution (HBSS), pH 7.2 and the organism were suspended and adjusted to 52.5% transmission as each 1ml contained $50x10^6$ CFU. The bacteria were opsonized with 1ml of 10% inactivated homologous serum (obtained from 5 serum samples collected from 5 different rats) for 30 minutes at 37ºC with gentle shaking. After opsonization, the bacteria were centrifuged, washed once and suspended in 1ml HBSS (Silva et al., 1988).

Phagocytic and killing activities

All macrophage types were maintained in RPMI-1640 medium containing 10% fetal bovine serum, and traces of mercaptoethanol at 37℃ in a humidified 5% CO_2 atmosphere (ESPEC CO_2 Incubator) for 48 hours. The assays were done in 6- wells tissue culture plates, each plate contained one macrophage type and each well represented rat macrophage, staphylococcal suspension with 200mg /1ml of the seed extract and the sixth was control without seed extract. The plates were incubated and the wells contents were aspirated after 12 and 24 hrs. and centrifuged at 160 xg 4℃ for 7 min and divided into two sections; one was stained with acridine orange and the other was prepared for electron microscope examination.

Acridine orange staining

The first pellet part was stained with 15 mg/l acridine orange in phosphate buffered saline, pH 7.2 for 1 min, washed twice in ice-cold HBSS, and wet-mounted on microscope slides (Nagl *et al*., 2002). Intracellular phagocytosed staph cells then killed ones through determining viable (green) and non –viable (red /yellow) fluorescence through examination in a fluorescent microscope using transmitted light was equipped with x 100 oil immersion objective. Photographs were taken using Kodak color-print film with the shutter set for a two minutes.

Sample preparation for Transmission Electron Microscopy (TEM)

The second part of pellet was fixed in 3% (v/v) glutaraldehyde for 120 min, post-fixed in 2% osmium tetroxide, dehydrated in graded ethanol series and embedded in Eponate-12. Ultra-thin sections were stained with 0.5% uranyl acetate and examined using transmission electron microscopy (JEOL-JEM-1011, Japan) (Annette *et al*., 2000).

RESULTS

The Figures 1 b, c and d showed significant increasing in the phagocytosis of *S. aureus* cells after 12 hours in comparison with control 1 a. Furthermore, the Figures 2 b, c and d exhibited higher phagocytic and killing activities of peritoneal, splenic macrophages and blood derived monocytes after 24 hours incubation. On the other side, the Figure 3 displayed the phagocytosed *S. aureus* by macrophage through transmission electron microscope examination.

DISCUSSION

The implementation of medicinal plants and their extracts as immune-promoters is an efficient and entire method to boost the immune responses against pathogens. The major constituents of the innate immune system (nonspecific) are macrophages, monocytes, granulocytes and humoral elements (Artyomov *et al*., 2016).

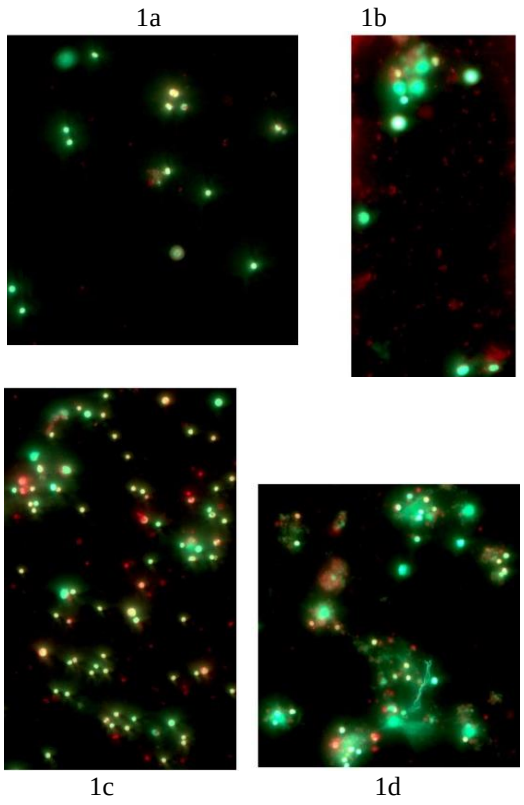

Fig. 1: Everywhere showed the increasing in phagocytic activity of macrophages after 12 hrs. of incubation with *N. sativa* compared with control. a) control b) blood derived macrophages c) splenic macrophages d) peritoneal macrophages. In the same manner the killing ability of these macrophages was determined by appearance of non –viable (red) fluorescence coccal cells, The Results shown in figure 2 b, c and d indicated a noticeable elevation of *S. aureus* killed cells in matching with control.

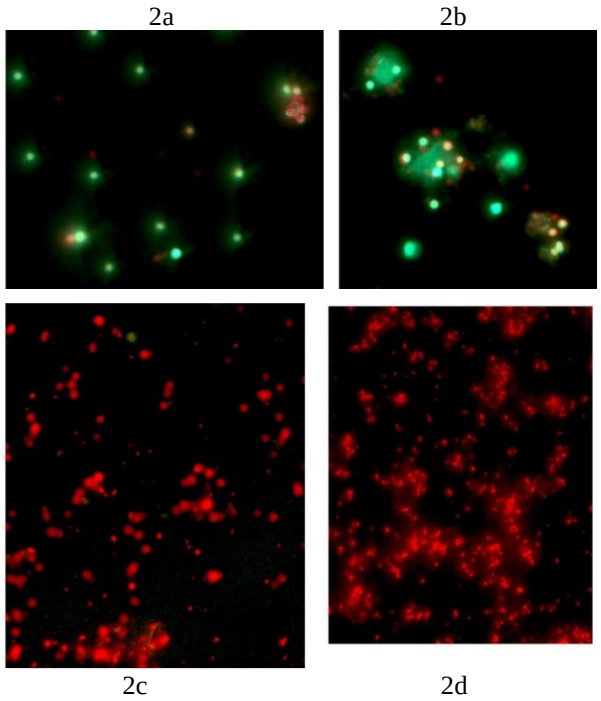

Fig. 2: Showed the increasing in phagocytic and killing activity of macrophages after 24 hours of incubation with *N. sativa* compared with control. a) control b) blood derived macrophages c) splenic macrophages d) peritoneal macrophages.

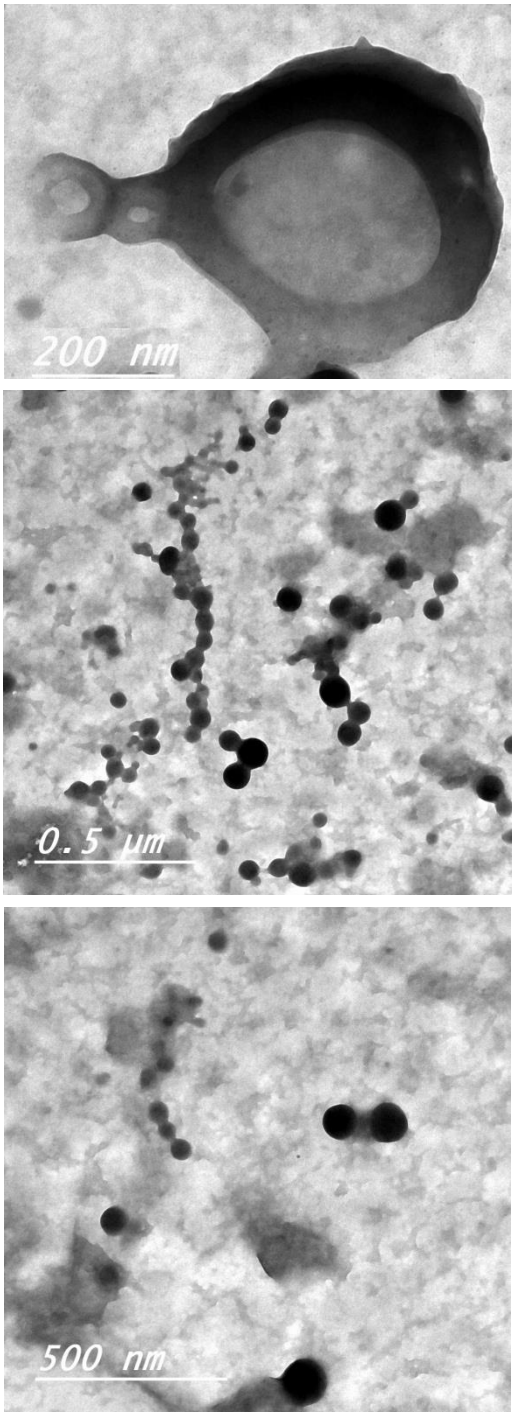

Fig. 3: showed the stages of phagocytic activity of macrophages with showing pseudopodia.

Nigella sativa (*N. Sativa*) represents a special dogma in Middle East and Islamic countries and constitutes a great way of recovery medicine. A great range of biological and pharmacological influences represents neuro-protective, hepato-protective, respiratory-protective, gastro-protective, anticancer activities and other useful unprecedented features (Ijaz *et al*., 2017).

It is known that macrophages play an essential role in host defense as they able to phagocytize pathogens. Thus, phagocytosis is a remarkable indicator of macrophage effector activity and it substitutes the definitive and most indispensable point of the immunological defense system (Guan *et al*., 2011).

In this study the phagocytosis of *S. aureus* by the three different macrophage types was monitored through checking the internalized viable (green) coccal cells. The Results shown in Figure 1 b, c and d indicated a marked increasing in the phagocytosis of *S. aureus* cells in comparison with control 1 a.

These results coincided with data obtained from previous studies; Ghonime *et al*. (2011) emphasized the immunomodulatory activity of black seed extract as it remarkably restored the resistance against lethal infection of granulocyte-conditioned *Candida albicans*.

Others elucidated the enhancement effect of *N. sativa* on immunological and bactericidal performances (Khatun *et al*. 2015; Khondoker *et al*. 2016; Celik Altunoglu 2017 and Fadeifared *et al*., 2018).

It is illustrated that most of the remedial characteristics of *N. sativa* are due to the entity of thymoquinone which the prime bioactive component of the plant essential oil is (Aftab *et al*., 2013 and Gholamnezhad *et al*., 2016).

Moreover, the figures 1 and 2 showed an interesting observation that the phagocytic and killing activities of peritoneal and splenic macrophages more than blood derived monocytes. Majdalawieh *et al*. (2010) demonstrated that the aqueous extract of *N. sativa* significantly enhances splenocyte proliferation in a dose-responsive manner.

However, there was more stunning catching; the peritoneal macrophage unlike bone marrow (blood) derived macrophage as its mitochondrial metabolic activities are not suppressed in presence of certain antigenic bacterial cell wall. Further, the peritoneal macrophages have a number of specific metabolic gene expression pathways and it is found that their nitric oxide production was respectably higher than that produced by blood derived macrophage (Artyomov *et al*., 2016).

On the other hand, the figure 3 demonstrated the phagocytosed *S. aureus* by macrophage through transmission electron microscope examination.

Conclusions

The present study gave light on the immune-stimulant potency of black cumin seed (*N. sativa*). Our data affirmed that the addition of seed extract to different types of macrophages increased their phagocytic and killing abilities in vitro under fluorescent and electron microscopical examination. Also, the obtained data exhibited that there was variation in between the three macrophages types in their response as both peritoneal and splenic macrophages showed a greater efficiency than blood derived macrophages.

Authors contributions

Ashraf S. Hakim has made the experimental design, prepared the initial draft of this manuscript, Hussien A Abouelhag has performed the incubation of macrophage and seed extract besides acridine orange assay, Amr M. Abdou has prepared the black cumin extract and has performed the electron microscopy, Ehab A Fouad has isolated the macrophages from rats and Doaa D Khalaf has prepared the bacterial suspension. All authors have shared in the manuscript revision.

REFERENCES

Aftab A, H Asif, M Mohd, AK Shah, N Abul Kalam, AS Nasir, AD Zoheir and A Firoz, 2013. A review on therapeutic potential of *Nigella sativa*: A miracle herb. Asian Pac J Trop Biomed, 3: 337–352.

Ahmad A, A Husain, M Mujeeb, SA Khan, AK Najmi, NA Siddique, Z Damanhouri and F Anwar, 2013. A review on therapeutic potential of *Nigella sativa*: A miracle herb. Asian Pacific J Trop Biomed, 3: 337-352.

Alonso-Castro AJ, E Ortiz-Sanchez E, F Domınguez F, V Arana-Argaez V, C Juarez- Vazquez C, M Chavez, C Carranza-Alvarez, O Gaspar-Ramırez, G Espinosa-Reyes, G Lopez-Toledo, R Ortiz-Andrade and A Garcıa-Carranca, 2012. Antitumor and immunomodulatory effects of Justicia spicigera Schltdl (*Acanthaceae*). J Ethnopharmacol, 141: 888–894.

Annette K, G Ivaylo, F Hans-Werner, F Franz, B Eva-Bettina, K Eckhart and G Werner, 2000. *Listeria monocytogenes*-Infected Human Dendritic Cells: Uptake and Host Cell Response. Infec Immun, 68: 3680–3688.

Artyomov M, A Sergushichev and JD Schilling, 2016. Integrating Immuno-metabolism and Macrophage Diversity. Semin Immunol, 28: 417–424.

Bonnardel J, C Da Silva, S Henri, S Tamoutounour, L Chasson, F Montañana-Sanchis, JP Gorvel and H Lelouard, 2015. Innate and adaptive immune functions of peyer's patch monocyte-derived cells. Cell Reports, 11: 770–84.

Celik Altunoglu, S Bilen, FU and G Biswas, 2017. Immune responses to methanolic extract of black cumin (*Nigella sativa*) in rainbow trout (*Oncorhynchus mykiss*). Fish Shellfish Immunol, 67: 103-109.

Cheng X, G Qingdong, T Yong, H Lifei and X Wuxiang, 2018. Medical Plants and Immunological Regulation. J Immunol Res, pp: 9172096.

Davies LC, SJ Jenkins, JE Allen and PR Taylor, 2013. Review: Tissue-resident macrophages. Nat Immunol, 14: 986-95.

Fadeifard F, M Raissy, M Jafarian, HR Boroujeni, M Rahimi and M Faghani, 2018. Effects of black seed (*Nigella sativa*), ginger (*Zingiber officinale*) and cone flower (*Echinacea angustifolia*) on the immune system of rainbow trout, *Oncorhynchus mykiss*. Arquivo Brasileiro de Medicina Veterinária e Zootecnia, 70: 199-204.

Gholamnezhad Z, S Havakhah and MH Boskabady, 2016. Preclinical and clinical effects of *Nigella sativa* and its constituent, thymoquinone: A review. J Ethnopharmacol, 190: 372-86.

Ghonime M, R Eldomany, A Abdelaziz and H Soliman, 2011. Evaluation of immunomodulatory effect of three herbal plants growing in Egypt. Immunopharmacol Immunotoxicol, 33: 141-5.

Guan D, Z Zhenya, Y Yingnan, X Guoqing and L Jiqiang, 2011. Immunomodulatory Activity of Polysaccharide from the Roots of *Actinidia kolomikta* on Macrophages. Int J Biol Arch, 3: 3-10.

Ijaz H, UR Tulain, J Qureshi, Z Danish, S Musayab, MF Akhtar, A Saleem, KK Khan, M Zaman, I Waheed, I Khan and M Abdel-Daim, 2017. Review: *Nigella sativa* (Prophetic Medicine): A Review. Pak J Pharm Sci, 30: 229-234.

Khatun A, MMM Hossain, MZ Rahman, ME Alam, F Yasmin, MS Islam and MM Islam, 2015. Effect of Black Cumin Seed Oil (*Nigella sativa*) on Enhancement of Immunity in the Climbing Perch, *Anabas testudineus*. Brit Microbiol Res J, 6: 331-339.

Khondoker S, MD Hossain, M Mer, M Hasan-Uj-Jaman, ME Alam, FU Zaman and N Tabassum, 2016. Effect of *N. sativa* to Enhance the Immunity of Common Carp (*Cyprinus carpio*) against *Pseudomonas fluorescens*. Am J Life Sci, 4: 87-92.

Majdalawieh AF, R Hmaidan and RI Carr, 2010. *Nigella sativa* modulates splenocyte proliferation, Th1/Th2 cytokine profile, macrophage function and NK anti-tumor activity. J Ethnopharmacol, 131: 268-75.

Mastan SA, 2015. Use of Immunostimulants in aquaculture disease management. In J Fish Aquatic Stud, 2: 277-280.

Murray PJ and TA Wynn, 2011. Protective and pathogenic functions of macrophage subsets. Nat Rev Immunol, 11: 723–37.

Nagl M, L Kacani, B Müllauer, EM Lemberger, H Stoiber, GM Sprinzl, H Schennach and MP Dierich, 2002. Phagocytosis and killing of bacteria by professional phagocytes and dendritic cells. Clin Diagn Lab Immunol, 9: 1165-8.

Sarkar D, A Dutta, M Das, K Sarkar, C Mandal and M Chatterjee, 2005. Effect of *Aloe vera* on nitric oxide production by macrophages during inflammation. 37: 371-375.

Silva ID, NC Jain, TB Farver and JG Zink, 1988. Phagocytic and post-phagocytic activities of bovine neutrophils for pure and mixed bacterial cultures. J Dairy Sci, 71: 2513-9.

Winnicka A, W Klucinski, J Kawiak, G Hoser and J Sikora, 2000. Effect of *Baypamun* on blood leucocytes in normal and dexamathasone treated goats. J Vet Med A Physiol Pathol Clin Med, 47: 385-94.