

**Immunostimulant Effect of Different Fractions of *Nigella sativa* L. Seeds against Rabies Vaccine**Abeer A.H. Boseila<sup>1</sup> and Afaf A.H. Messalam<sup>2</sup><sup>1</sup>Department of Virology, <sup>2</sup>Department of Phytochemistry, National Organization for Drug Control and Research (NODCAR), Giza 12553, Egypt.[abeerboseila1971@yahoo.com](mailto:abeerboseila1971@yahoo.com).

**ABSTRACT:** Interest in new methods of potentiating the immune response against vaccine antigens has increased considerably over the past decade for improving existing vaccines. The present study was designed to evaluate the immunostimulant effect of oils, n-hexane and methanol fractions of *Nigella sativa* L. seeds in combination with vitamin E and selenium as new adjuvant compared with aluminum hydroxide (alum) as established adjuvant against rabies vaccine in male Swiss albino mice. Inoculation was done intraperitoneally in the form of two doses, two weeks apart. Five samples of sera were collected for every two weeks beginning from two weeks after the last vaccination till the 12<sup>th</sup> week and the antibody were detected using indirect ELISA technique. Our results revealed that both methanol and volatile oil fractions of *Nigella sativa* L. seeds can improve the immune response against rabies vaccine save and suggested that they could be used as an alternative adjuvant to alum in rabies vaccine.

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**Key words:** rabies vaccine; adjuvant; immunostimulant effect; *Nigella sativa* L. seeds; vitamin E and selenium.

**INTRODUCTION**

Vaccines were designed to protect against diseases by inducing specific immunity. Immunization is a proven tool for controlling and even eradicating diseases. The densely populated countries especially in Africa and Asia, the rabies is endemic and remains a major health problem. Rabies is a viral disease of mammals and is most commonly transmitted through the bite of a rabid animal. Rabies disease is caused by a neurotropic virus belonging to the family *Rhabdoviridae*. Rabies is one of the oldest and most devastating viral diseases affecting humans and animals. It was recognized in Egypt before 2300 B.C. and was described by Aristotle in ancient Greece. It is the most lethal of all infectious diseases and has the widest host range of any virus (**Fenner and White, 1994**). Globally, according to WHO Fact Sheet, an estimation of 10 million people receive post exposure antiserum treatment each year, after being exposed to rabies-suspect animal (**Gómez et al. 2010**).

Adjuvants are highly valuable additions to vaccines. Adjuvants may modulate the quality and quantity of the immune response following vaccination. Most of the cell culture rabies vaccines commercially produced for animals and sometimes for human was adjuvanted principally with aluminum salts (alum), which held the antigen at its site of deposition, delaying its adsorption and subsequently released antigen in a deduced secondary response (**Glenny et al. 1931 and Nakashima et al. 1981**).

*Nigella sativa* Linn. (a dicotyledon of *Ranunculaceae* family), commonly known as black seed or black cumin, is a grassy plant grows in temperate and cold climate areas and has green to blue flowers and small black seeds. It is an annual herbaceous plant native to Asia, and cultivated and naturalized in Europe and North Africa. In Egypt, *Nigella sativa* has been steadily increasing for the strong demand to volatile oils for pharmaceutical purpose (**Tohamy et al., 2010**). It has been traditionally used for culinary and medicinal purposes as a natural remedy for a number of illnesses and conditions that include diuretic, appetitive, hemorrhagic and anti-dandruff therapy, asthma, hypertension, diabetes, inflammation, cough, bronchitis, headache, eczema, fever, dizziness, and influenza a carminative, lactagogue, and vermifuge, as well as in food as a spice or condiment (**Baytop, 1999; Ali and Blunden, 2003; Isik et al. 2010**).

The general chemical composition of the *Nigella sativa* seeds is fats (31-35.5% w/w), proteins (16-19.9% w/w), carbohydrates (33.9%), fibers (4.5-6.5%) and moisture (5-7%) (**Ansari and Sadiy, 1989**). The fatty component of the seed consists mainly of fixed oil and volatile oil. The fixed oil consists of high percentage of unsaturated fatty acids (74.4-82.5%), including arachidonic and eicosadienoic acids, while saturated fatty acids are only 14.9-17.3%. Steam distillation of the whole oil yields 1.4-1.9% volatile oils based on the weight of total oil extracted (**Rathee et al. 1982**). This is equivalent to 0.40-0.45% w/w of the weight of the

seeds used to extract the oil (Juknevičienė et al. 1977). This oil is also rich in fatty acids (oleic, linoleic and linolenic acid) and carotene (Al-Jassir, 1992). The active constituent of the volatile oil, nigellone was first isolated by Mahfouz and El Dakhakhny (1960) and thymoquinone which was then isolated. When thymoquinone exposed to air, dimerization occurs with the formation of dithymoquinone (El Dakhakhny, 1965). El Alfy et al. (1975) isolated a white crystalline compound identified as thymohydroquinone, which is probably a reduction product of thymoquinone. Also, *Nigella sativa* seeds contain monotropens such as *p*-cymene and -pinene (El-Dakhakhny, 1965), nigellidine (Atta and Malik, 1993), nigellimine (Atta and Malik, 1985) and a saponine (Ansari and Sadiq, 1989). The chemical composition of the plant was summarized in a recent review (Labib, 2005). Consequently, the present study was aimed to evaluate the effect of different fractions of *Nigella sativa* seeds on the immune response against rabies vaccine in Swiss albino mice.

## MATERIALS AND METHODS

### *Plant material:*

Dried seeds of *Nigella sativa* L. (black seed) were purchased from a local market, and authenticated by botanists from faculty of Science, Cairo University.

### *Nigella sativa fixed oil:*

*Nigella sativa* fixed oil, the natural oil of the black seed, was purchased from the local market. It was intra-peritoneally injected in a dose of 2.06 mg/Kg body weight (Zaoui, et al. 2002).

### *E-SELEN:*

E-SELEN, a mixture of vitamin E acetate 150 mg/ml and sodium selenite 1.67 mg/ml, produced by MAM Egypt, M.O.H.Reg. No.: 2597/2005 was diluted depending upon their LD<sub>50</sub> of sodium selenite in mice (0.9 mg/Kg body weight) according to (Toxic Report Series, 1994) and vitamin E acetate (100 mg/kg body weight) according to (Toutain et al. 1992) was mixed with the inactivated rabies vaccine in a ratio of 1:1 to make a homogenous mixture.

### *Extraction of volatile oils from Nigella sativa L. seeds:*

According to Samsam-Shariat and Moatar, (1996), 500 grams of crushed seeds of *Nigella sativa* seeds were placed in a rounded flask of a quick fit steam distillation apparatus in which steam was passed from steam generator and sufficient water

was added. A 30 ml of glycerin was added to the content of the flask to raise the boiling point. The content of the flask were boiled gently until all the volatile oil has been distilled, saving guard against any charring of the material in the flask. The oil fraction (F1), being lighter than water, was separated by a separating funnel, dried over anhydrous sodium sulphate and kept in a dark away from light and moisture.

### *Extraction of n-hexane and methanol fractions from Nigella sativa L. seeds:*

According to Boskabady et al. (2008), 300 ml of n-hexane was added to 500 grams of the chopped, dried *Nigella sativa* L. seeds and the solution was kept at room temperature for 48 hours. The solution was decanted and the solvent was evaporated and this represented the n-hexane fraction (F2). Methanol was added to the remaining powder and the mixture was allowed to remain at room temperature for 48 hours. The solution was then decanted and the solvent was evaporated and this represented the methanol fraction (F3) of the *Nigella sativa* seeds.

### *Rabies vaccine:*

Aluminum salts have become the reference preparations for evaluation of new adjuvants for human vaccines. Therefore, it is important that aluminum adjuvants be used optimally to allow correct evaluation of the experimental adjuvant (Gupta and Siber, 1995). Therefore, two types of Rabies vaccines for human vaccination against rabies virus infection were used:

- 1) Inactivated, purified and adsorbed rabies vaccine prepared on VERO cells and adsorbed onto aluminum hydroxide salts was used as control (VC).
- 2) Inactivated and purified rabies vaccine prepared on VERO cells and produced by Sanofi Pasteur, France. According to the method described by Madbouly et al (2006), this inactivated rabies vaccine was mixed with the new adjuvants as follows: Four parts of the water phase (vaccine and vitamin E and selenium combination is mixed thoroughly with 1% tween 20) was mixed with one part of the oil phase (one part of span 80 was thoroughly mixed with 9 parts of the *Nigella sativa* fraction).

### *Animals:*

Male Swiss albino mice weighing 20-25 gram were supplied by National Organization for Drug Control and Research (NODCAR). Animals were kept under standard laboratory conditions of light/dark cycle (12/12h.), temperature (25 ± 2°C)

and fed on normal laboratory diet and water ad libidum. They were acclimatized for a week in the new environment before initiation of experiment.

#### Experimental Design:

A total of 150 mice were assigned into 6 groups (each containing 25 animals). Each group was intraperitoneally vaccinated by 0.5 ml per mice contained different Rabies vaccine adjuvants in the form of two doses, two weeks apart as follows:

**Group 1:** Rabies vaccine mixed with aluminum hydroxide salts (VC- group).

**Group 2:** Rabies vaccine adjuvanted with E-SELEN (VE- group)

**Group 3:** Rabies vaccine adjuvanted with a mixture of E-SELEN and *Nigella sativa* fixed oil (VEFO- group).

**Group 4:** Rabies vaccine adjuvanted with a mixture of E-SELEN and volatile oils fraction of *Nigella sativa* seeds (VEF1- group).

**Group 5:** Rabies vaccine adjuvanted with a mixture of E-SELEN and n-hexane fraction of *Nigella sativa* seeds (VEF2- group).

**Group 6:** Rabies vaccine adjuvanted with a mixture of E-SELEN and methanol fraction of *Nigella sativa* seeds (VEF3- group).

Blood samples were collected every two weeks beginning from two weeks after the first vaccination dose and continue for 3 months. Then the antibody titres in the serum were evaluated using ELISA technique.

#### ELISA Technique:

The indirect enzyme-linked immunosorbent assay (ELISA) technique, a sensitive and simple method was used for quantitative determination of antibodies. The 96-well micortitre plates coated with

inactivated rabies antigen were incubated with diluted antisera (1:100) followed by incubation with an enzyme labeled preparation of anti-immunoglobulin. The color change in each well was estimated spectrophotometrically at 490/630 nm according to the method described by **Hubschle et al. (1981)**.

#### Statistical Analysis:

The data are expressed as means  $\pm$  S.E. The enhancement effects of the different adjuvants were statistically analyzed by One-way Analysis of Variance (ANOVA) followed by Dunnet's tests for multiple comparisons with the level of significance accepted at  $P < 0.05$ .

#### RESULTS

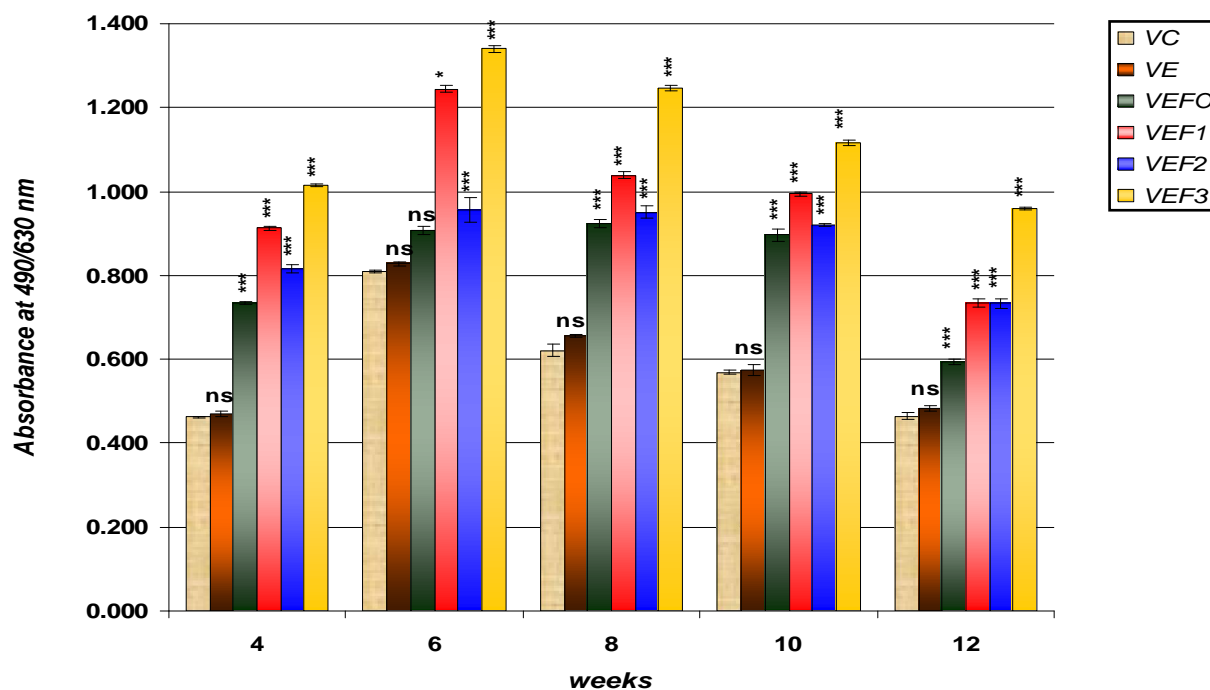
The use of an adjuvant to enhance antibody titer is an attractive option to improve the performance of an existing vaccine. Figure 1 illustrates the results of the indirect ELISA for the measurement of antibodies using inactivated rabies antigen coated plates with antisera collected from different vaccinated groups. After the second doses, the result showed that there is no significant difference between the immune response of rabies vaccine adjuvanted with alum (VC) and mixture of E-SELEN (VE). While, significant ( $P < 0.001$ ) increase specific immune response and was observed in the groups (3-6) which received the formulation with the novel adjuvant systems; all *Nigella sativa* fractions were generally effective in increasing the antibody against the rabies vaccine. Among the *Nigella sativa* seed fractions, the highest antibody was detected in the group treated with rabies vaccine adjuvanted with a mixture of E-SELEN and methanol fraction (VEF3) followed by VEF1, VEF2 and VEFO groups, respectively.

**Table (1) :** The results of the optical density (O.D.) for (1:100) dilution of the collected sera of the inoculated groups measured at wave length of 490/630 nm .

Group name	4 <sup>th</sup> week	6 <sup>th</sup> week	8 <sup>th</sup> week	10 <sup>th</sup> week	12 <sup>th</sup> week
VC	0.462	0.809	0.621	0.569	0.465
VE	0.471	0.828	0.655	0.575	0.483
VEFO	0.733	0.908	0.923	0.896	0.596
VEF1	0.913	1.244	1.040	0.995	0.735
VEF2	0.815	0.956	0.951	0.920	0.733
VEF3	1.014	1.340	1.247	1.115	0.960

**Table (2):** The standard errors and the significance and non-significance (ns) between groups.

Group name	4 <sup>th</sup> week	6 <sup>th</sup> week	8 <sup>th</sup> week	10 <sup>th</sup> week	12 <sup>th</sup> week
VC	0.462 ± 0.002	0.809 ± 0.005	0.621 ± 0.005	0.569 ± 0.004	0.465 ± 0.010
VE	0.471 ± 0.004ns	0.828 ± 0.003ns	0.655 ± 0.005ns	0.575 ± 0.005ns	0.483 ± 0.008ns
VEFO	0.733 ± 0.002***	0.908 ± 0.002***	0.923 ± 0.006***	0.896 ± 0.003***	0.596 ± 0.006***
VEF1	0.913 ± 0.004***	1.244 ± 0.003***	1.040 ± 0.004***	0.995 ± 0.004***	0.735 ± 0.005***
VEF2	0.815 ± 0.002***	0.956 ± 0.006***	0.951 ± 0.006***	0.920 ± 0.004***	0.733 ± 0.006***
VEF3	1.014 ± 0.003***	1.340 ± 0.009***	1.247 ± 0.007***	1.115 ± 0.002***	0.960 ± 0.005***

**Figure 1** the mean immune response of different groups against rabies vaccine detected by indirect ELISA technique

## DISCUSSION

Vaccines require optimal adjuvants including immuno-stimulants and delivery systems to offer long term protection from infectious diseases in animals and man. The current study was aimed to investigate the immunostimulant effect of different fractions of *Nigella sativa L.* seeds against rabies vaccine.

The ideal method for controlling rabies virus in both man and animals is by using active immunization through injecting either live attenuated or inactivated rabies vaccines. The WHO's experts

(WHO, 1973) recommended that "No vaccines containing living virus could be used in man". The U.S. authorities recommended the use of inactivated vaccines in animals (CDC, 1978) because of the residual neuro-virulence of the virus which is very dangerous due to its auto-interference phenomena which could occur at non detectable level and the undesirable effect in case of using the living attenuated viral vaccines in immune-suppressed animals where it may act as avirulent strain (Wachendorfer, 1976).

Although the inactivated cell culture rabies vaccines cause no or fewer reactions than the nervous tissue or the chicken embryo derived vaccines, they are relatively free from aggregates that could contain ineffective virus particles, but the virus titre obtained requires further concentration (Sokol, 1973). Thus choose of natural immune-stimulants as adjuvant in this study based upon:

- 1) Its effective stimulation of the immune cells like macrophage (Basil and Erwa, 1993) and T-lymphocyte (El-Kadi et al. 1990) as shown in *Nigella sativa* adjuvant vaccines (Madbouly et al. 2006). Moreover, the *Nigella sativa* oil has shown potent antioxidant and anti-inflammatory effects in several inflammation-based models, including experimental encephalomyelitis, colitis, peritonitis, edema, and arthritis, through suppression of the inflammatory mediators' prostaglandins and leukotrienes. Also, the oil and certain active ingredients showed beneficial immunomodulatory properties, augmenting T-cell and natural killer cell-mediated immune responses (Salem, 2005; Terzi et al. 2010).
- 2) The powerful role of the combination of vitamin E and selenium as antioxidant protecting the sensitive rapidly proliferating cells of the immune system from oxidation damage, an immunopotentiating agent (Yasunaga et al. 1982), increasing cell-cell interaction by membrane alteration (Tengerdy and Lacetera 1991) and significant enhancement for the formation of IgM & IgG in contrast to alum (Inagaki et al. 1984). *Nigella sativa* oil is so beneficial due to its content of over 100 components (such as aromatic oils, trace elements, vitamins, and enzymes) and contains about 58% of essential fatty acids, including omega-6 and omega-3. These are necessary for the formation of prostaglandin E1 that balances and strengthens the immune system, enabling it to prevent infections and allergies and control chronic illnesses (Terzi et al. 2010).
- 3) To overcome the disadvantage that associated with the aluminum hydroxide salts (alum) in rabies vaccines. Redhead et al. (1992) reported a transient rise in the level of brain tissue aluminum that peaks around the second and third day after intraperitoneally injection of alum adsorbed vaccines into mice that not observed in saline control group and with vaccine not containing aluminum salt. Also Jefferson et al. (2004) noticed that alum adsorbed vaccine associated with local pain lasting up to 14 days in older children administered such vaccines. Moreover, Verdier et al. (2005) observed

histopathological lesions similar to the Macrophagic Myofascitis (MMF) described in humans, and was still present 3 months after aluminum phosphate and 12 months after aluminum hydroxide adjuvanted vaccine administration.

Throughout history, black seed has been one of the most revered medicinal seeds. The Islamic Prophet, *Muhammad* (Sal Allahu Alayhi Wasallam) recommended its use over 1400 years ago. Regarding to the previous studies that revealed that *Nigella sativa* oil and seeds are scientific techniques, a number of pharmacological actions of *Nigella sativa* have been investigated including immunostimulant, anti-inflammatory, antioxidant (Labib, 2005). Black seed oil and its derivatives inhibit eicosanoid generation in leukocytes and membrane lipid peroxidation (El-Dakhakhny et al., 2002). Besides, the seeds contain eight essential amino acids that improve natural immune system activity (Omar et al., 1999).

In the current study, fixed oil, volatile oils, n-hexane and methanolic fractions of *Nigella sativa* L. seeds were studied as new adjuvants to the rabies vaccine in the presence of vitamin E and selenium. As compared with control group (VC), there was a significant ( $P < 0.001$ ) increase in the antibodies against rabies antigen in all vaccines adjuvanted with *Nigella sativa* fractions. The most superior effect of the used adjuvants on the immune response of mice against rabies vaccine was belonged to the groups vaccinated with the methanol and volatile oils fractions, respectively. Our results are in agreement with that of Kanter et al., (2005) who showed that the thymoquinone which is the major active principle of *Nigella sativa* has immuno-potentiating activities via increasing neutrophil percentage and hence increasing the defense mechanism of the body against infection. Concerning the first and second groups the first group was vaccinated with the alum (VC) and the second one (VE) was vaccinated with vitamin E and selenium (E-SELEN) adsorbed rabies vaccine, we noticed that the immune response in both groups were quite similar. But regarding to the side effect of the alum reported by Verdier et al. (2005), the use of both vitamin E and selenium is safer than alum. This result is in agreement with that obtained by Madbouly et al. (2006). Also the immune stimulant effect of the methanol fraction to rabies antigen was showed to be more potent than that of the n-hexane fraction and whole oils which means that lipid soluble ingredients of the *Nigella sativa*. seeds are mainly responsible for their immunostimulant effect. Our results indicate that the new formulations were safe, well-tolerated, and immunogenic and promote

more rapid and prolonged protection against rabies infection.

### CONCLUSION

The use of an adjuvant to enhance antibody production is an attractive option to increase the vaccine efficacy. A potent immunostimulant effect of mainly methanol and volatile oil fractions of *Nigella sativa* L. seeds in combination with vitamin E and selenium allow them to be used as new adjuvants for rabies vaccine. More studies are still needed in order to isolate and identify the effective compounds in the *Nigella sativa* L. seeds that responsible for the immunostimulant effect.

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