

ANTIVIRAL EFFICACY OF GARLIC OIL AGAINST NEWCASTLE DISEASE VIRUS

Manar Mohammed Hizam* Firas Taha Mansour Al-Mubarak*

Wasfi Abood Al-Masoudi**

* Department of Microbiology, College of Veterinary Medicine , University of
Basrah, Basrah, Iraq.

** Department of Physiology , College of Veterinary Medicine , University of Basrah
Basrah ,Iraq.

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Corresponding Author: manarmh957@gmail.com

ABSTRACT

Newcastle disease is a highly contagious and devastating viral disease of poultry that distributed worldwide causing large economic losses in the poultry industry. Although vaccines are being used to control the disease, there is no effective antiviral drug used for the treatment of infections. The aim of this study is to test garlic oil for its antiviral activity against Newcastle disease virus. Garlic oil was incubated with the virus (LaSota strain) for 1 hr and 24 hrs and its antiviral effect was determined by performing hemagglutination and RT-PCR tests to detect viral surface proteins and viral genome, respectively. In addition, the toxicity of garlic oil was determined on the living organism by injecting it into chicken embryos with or without the virus. The results showed that this product played a role in the reduction of virus effectiveness through the destroying of viral surface receptors as well as the reduction of gene amplification as compared with the control group that included the treatment of the virus with a saline solution (phosphate buffer saline), which gave opposite results. In addition, there was no antiviral toxicity on the living organism since the injected embryos with the oil alone or the oil with virus were healthy and closely resemble those that have not been injected with anything. In comparison, the embryos that were injected with the virus only showed clear pathological signs that did not appear in the other groups containing the oil. These results suggest that garlic

oil would be a good potential antiviral and probably will have a role to eliminate the disease.

INTRODUCTION

Newcastle disease is a highly contagious and devastating viral disease of wide range of birds, notably the domestic poultry. It is caused by Newcastle disease virus which is belonged to *Paramyxoviride* family. The disease has the potential to cause large economic losses in the poultry industry ^(1,2). It is expected that all birds are susceptible to infection, with varying of clinical signs and outcome ⁽³⁾.

So far, there is no effective treatment for Newcastle disease, although secondary infections caused by bacteria may be treated by using antibiotics, so new alternative control measures are urgently required ^(4,5). Recent studies have supported the effect of garlic and its extracts in a wide range of medical applications. These studies raised the possibility of revival of garlic therapeutic values in different diseases. Different compounds in garlic have been thought to have anti-tumour and anti-microbial effects. In addition, it can reduce the risk for cardiovascular diseases and also show benefit on high blood glucose concentration ^(6,7). In comparison with the antibacterial effect of garlic compounds, very limited studies have been done to investigate their antiviral properties. The few studies have reported that garlic extract showed in vitro activity against influenza A and B, rhinovirus, human immunodeficiency virus, cytomegalovirus, viral pneumonia, herpes simplex virus 1, herpes simplex virus 2, and rotavirus ⁽⁶⁾.

The aim of the present work is concerned to test garlic oil for its antiviral activity against Newcastle disease virus. The aim was achieved by evaluating garlic oil activity on destroying the outer viral protein receptors and degrading of viral nucleic acid of the virus by performing haemagglutination and RT-PCR techniques, respectively. In addition, toxicity of the oil was measured via the inoculation of embryonated chicken eggs.

MATERIALS AND METHODS

Viruses

A stock of live Lentogenic Newcastle disease virus (LaSota vaccine) was used in this study. It was diluted 1:1000 with phosphate buffer saline (PBS) before use.

Extraction of oil from garlic

Dried garlic was obtained from the supermarket, then grinded with an electric mill and then stored in glass bottles until it was used. Seventy gram of dried garlic was placed in a paper container (thimble) in the extraction apparatus (succulet) using 500ml of ethanol solvent for about 6 hrs. The solution was then placed in a rotary evaporator at 50°C to evaporate the solvent and the remaining material was left at room temperature to dry and obtain garlic oil and then was put in a screw tube until use.

ANTIVIRAL EFFICACY OF GARLIC OIL

The role of garlic to inhibit virus effectiveness through the destroying of viral receptors or degrading viral nucleic acid was examined by performing haemagglutination and RT-PCR techniques, respectively. The concentration of garlic oil used to treat the virus was 50 µg/ml. Five groups were prepared for this purpose. Group 1 and 2 included the incubation of garlic oil with the virus for 1 hr or 24 hrs, respectively, while group 3 and 4 represented by using garlic oil without virus (negative control 1) or PBS (negative control 2). Group 5 was represented by using virus only without garlic (positive control).

Preparation of red blood cells

Five microliters of blood were collected from chickens in vacuum tubes (EDTA.K3) and RBCs were purified as follows: The whole blood was centrifuged at 3000rpm for 5mins and the supernatant was discarded. The RBCs were suspended in PBS by mixing the tube gently. The mixture was then centrifuged for 5 mins at 2000 rpm and the supernatant was discarded. This process was repeated for 3 to 4 times until the supernatant was cleared. The RBCs were than kept at 4°C or used directly for haemagglutination test.

Haemagglutination

The role of garlic oil to inhibit virus effectiveness through the destroying of virus receptors was examined by performing haemagglutination test. A ceramic agglutination plate which contains six wells was cleaned and prepared for this experiment. A drop of virus treated with garlic oil for 1 hr or 24 hrs was mixed with

another drop of RBC in the well. The negative controls 1 and 2 were represented by mixing a drop of RBC with another drop of garlic oil only or with PBS, respectively. The positive control sample was represented by mixing a drop of RBC with a drop of non-treated virus. the reaction was left for a few minutes to visualize the results.

Viral rna extraction and quantification

Viral RNA was extracted in the laboratory by using QIAamp viral RNA extraction kit (Qiagen, Germany) following the manufacturer's instructions. The concentration of the purified RNA was determined using NanoDrop spectrophotometer by UV absorption. Eluted viral RNA samples were either processed directly for RT-PCR or preserved at -20°C until use.

Reverse transcriptase polymerase chain reaction

Viruses were detected by using two-step RT-PCR kits (Bioneer, South Korea) following the manufacturer's protocol. cDNA synthesis and PCR amplification were performed in two separated tubes using this system. The components of cDNA reaction with their volumes and final concentrations were as follows: Four microliters of template RNA were used as a starting material accompanied with $0.4\ \mu\text{l}$ of forward and reverse primers (the final concentration of each primer was $0.2\ \text{pmole}/\ \mu\text{l}$). Fifteen point two microliters of nuclease-free water were then added to the reaction. The negative control reactions were prepared either without adding primers (negative control 1) or without RNA template (negative control 2). The conditions of the first step were as follows: primer annealing at 58°C for 10 min, followed by cDNA synthesis at 42°C for 30 min and then the a denaturation step at 95°C for 5 min.

Two microliters of the synthesized cDNA was then used to perform the second step (PCR amplification). Point four microliter of forward and reverse primers were added to the reaction with $17.2\ \mu\text{l}$ of nuclease-free water. The PCR conditions were as follows: initial denaturation at 95°C for 5 mins followed by 40 cycles of: denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min. The reaction was then held at 72°C for 5 mins, and then cooled down at 4°C for 5 mins. The amplified PCR product was then detected using 2% agarose gel prepared with agarose (Promega) in TBE buffer stained with ethidium bromide. The size of the band was estimated by comparison with a standard 100 bp DNA ladder (New England Biolabs).

Innoculation of embryonated chicken eggs

To determine the toxicity and effectiveness of garlic oil on live organisms, fertile hen's eggs provided by Fadak company, Basrah, Iraq were inoculated with the lowest concentration of the oil (50µg/ml), which inhibited virus infectivity prior egg inoculation. The fertilized eggs were first incubated for 9 days at 37.5°C then inoculated with the virus. Working virus concentration was prepared by diluting the stock virus 1:1000 in phosphate buffer saline containing 1% antibiotics (100 U/ mL penicillin and 100 ug/ mL streptomycin, Gibco). The volume of the inoculum was 100 µl per egg. Five groups of eggs were prepared for this purpose (10 eggs for each group). Group 1 and 2 were inoculated with a mixture of garlic oil and virus that had been incubated together for one 1 hr or 24 hrs, respectively. Group 3 and 4 were inoculated with garlic oil or virus, respectively. The eggs of group 5 were not inoculated at all. Following inoculation, the eggs were placed at 37.5°C for 48 hrs. The eggs were then chilled at 4°C for 30 mins before collecting the embryos and harvesting the virus. Allantoic fluid was carefully aspirated and then clarified at 1000 xg for 5 min, and the embryos were move to Petri dishes to visualize the pathological changes.

RESULTS

Determination of antiviral efficacy before injection of chicken embryos

Serological detection of virus by haemagglutination test

The role of garlic oil to inhibit virus effectiveness through the destroying of virus receptors was evaluated by performing haemagglutination test. The results were as follows: the incubation of garlic oil with the virus for 1 hr or 24 hrs showed complete inhibition of virus effectiveness. In addition, the use of garlic oil only (negative control 1) or PBS only (negative control 2) also showed no agglutination. In comparison, very clear agglutination was visualized following the use of virus only without garlic oil (positive control). The above results indicated that the garlic oil had an effective role in destroying viral surface receptors, which prevented the attachment of the virus with RBCs and thus inhibited the agglutination reaction (Figure 1).

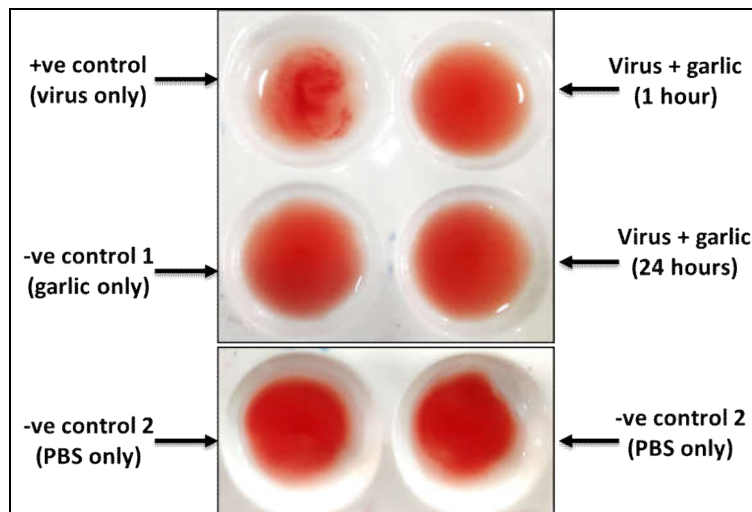


Figure 1: Antiviral efficacy of garlic oil on the surface receptors of Newcastle disease virus (pre-injection of egg embryos).

Molecular detection of virus by rt-pcr

The role of garlic oil to inhibit virus effectiveness through the destroying of viral nucleic acid was evaluated by performing RT-PCR test. The results were as follows: good amplification of viral nucleic acid with very clear bands of the positive control samples (virus without garlic oil). In addition, negative control group 1 (no PCR primers in the PCR reaction) and negative control group 2 (no viral RNA in the PCR reaction) showed no detectable bands. On the other hand, poor amplification (faint bands) and poor or no amplification (faint or no bands) of viral nucleic acid of viruses treated with the garlic oil for 1 hr and 24 hrs, respectively, was observed. The above results indicated that garlic oil had an effective role to destroying viral nucleic acid, which showed poor or no gene amplification (Figure 2).

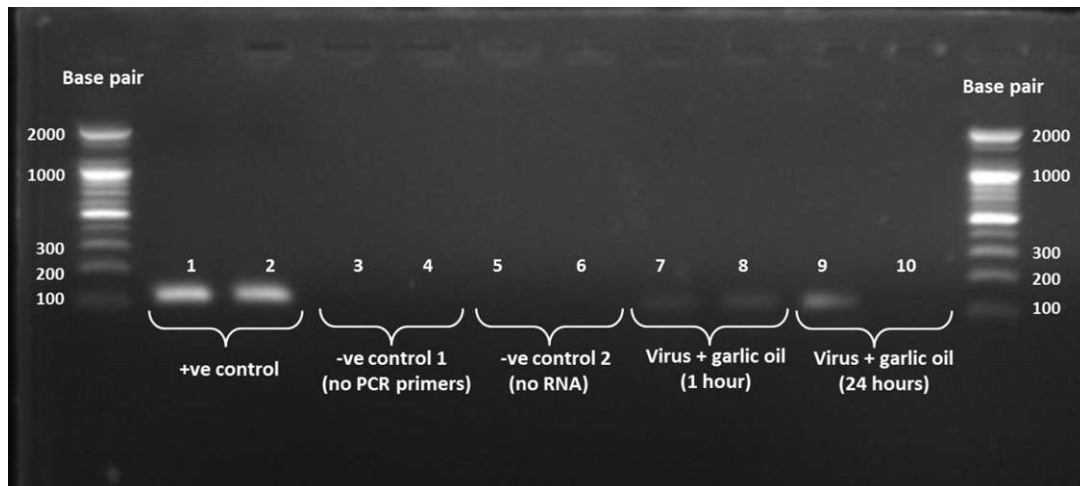


Figure 2 Antiviral efficacy of garlic oil on viral nucleic acid of Newcastle disease virus (pre-injection of egg embryos)

The figure shows the detection of Newcastle disease virus on agarose gel stained with ethidium bromide. The results showed good amplification of viral nucleic acid of positive control samples (lane 1 and 2), while the negative control samples (lane 3 and 4) and (lane 5 and 6), the nucleic acid was not amplified at all. The samples that were treated with garlic showed slight amplification of the nucleic acid (compared with positive control) after incubation the virus with drug for 1 hr (lane 7 and 8) and amplification was much less after incubating garlic with the virus for 24 hrs (lane 9 and 10).

Antiviral efficacy of garlic oil on newcastle disease virus after injection of chicken embryos:

Determination of toxicity and effectiveness of garlic oil on chicken embryos:

Toxicity and effectiveness of garlic oil was measured after inoculation of embryonated chicken eggs with the lowest oil concentration (50µg/ml), which inhibited virus infectivity prior egg inoculation. The results were as follows: following the inoculation of eggs with the mixture of garlic oil and virus that had been incubated together for 1 hr or 24 hrs, the embryos were normal and did not show any pathological signs such as haemorrhage, distortion or damage. Similar results were observed after inoculation of eggs with garlic oil only and also with the non-injected eggs. On the other hand, following the inoculation of eggs with the virus only, pathological signs such as haemorrhage or embryo distortion or damage were clearly observed on the embryos. The results toxicity and effectiveness are shown in (Figure 3).

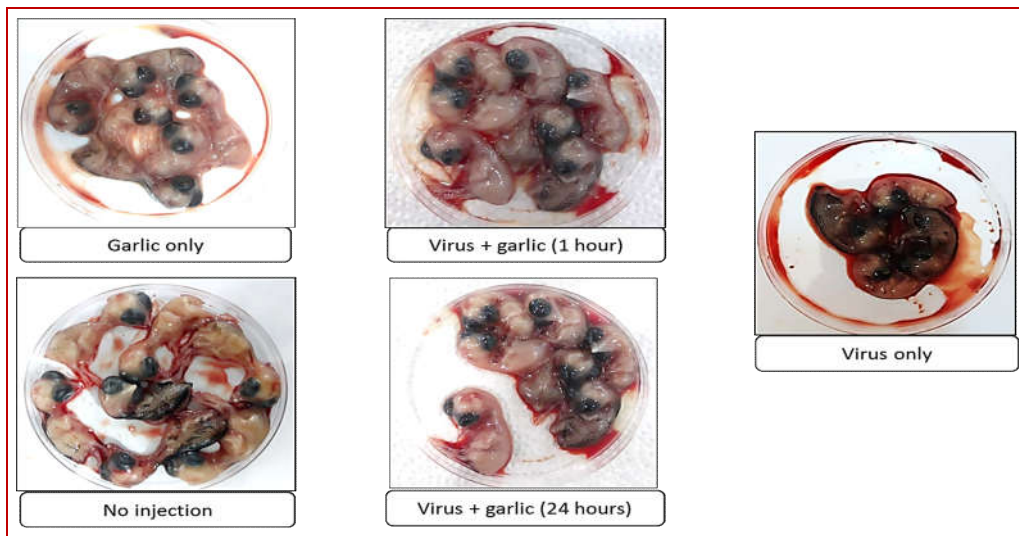


Figure 3 Detection of toxicity and effectiveness of garlic oil on chicken embryos.

The figure shows the effect of garlic oil on the virus as well as its effect on chicken embryos. Pathological signs such as petechial haemorrhage and distortion of the embryo were clearly observed following the injection of the eggs with the virus that was not treated with garlic oil. In contrast, the embryos look very normal after injection with the virus that was treated with garlic oil for one hr and 24 hrs, indicating that garlic played an important in decreasing virus infectivity. The embryos were also normal after injection with garlic oil only and there were no haemorrhage and distortions and they were similar to the non-injected group.

Detection of virus from allantoic fluid by haemagglutination test:

Haemagglutination test was performed to detect virus growth in the allantoic fluid that was collected from the inoculated eggs with virus only, virus treated with garlic oil, and non-inoculated eggs. The results were as follows: no evidence of haemagglutination in the allantoic fluid of eggs injected with virus treated with garlic oil for 1 hr or 24 hrs. Similar results were seen in eggs injected with garlic oil only (negative control 1) and the non-injected eggs (negative control 2). In contrast, very visible haemagglutination was observed in the allantoic fluid of eggs injected with virus only (positive control).

The above results indicated that garlic oil had an effective role in destroying viral surface receptors before the injection of embryonated chicken eggs, which eventually prevented virus replication inside the allantoic cells (**Figures 4**).

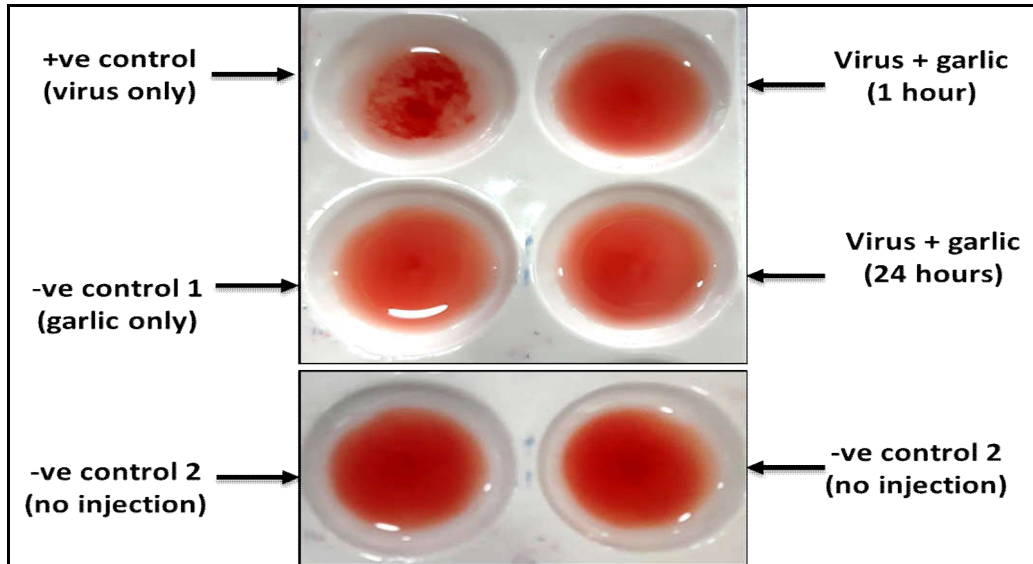


Figure 4 Antiviral efficacy of garlic oil after injection of embryonated chicken egg.

Detection of virus from allantoic fluid by rt-pcr:

The virus was detected from the allantoic fluid of the injected eggs by performing RT-PCR. The results were as follows: viral nucleic acid was detected successfully in the positive control samples (eggs injected with virus only) with very clear bands easily observed on the gel. In contrast, viral nucleic acid was not detected in eggs injected with garlic oil only, and also in eggs injected with virus treated with garlic oil for 1 hr and 24 hrs, and in the non-injected eggs.

The above results explained the role garlic oil to destroy viral nucleic acid more accurately. Although there was a slight amplification of viral nucleic acid of viruses treated with the oil before egg inoculation, this amplification was completely disappeared after inoculation (Figure 5).

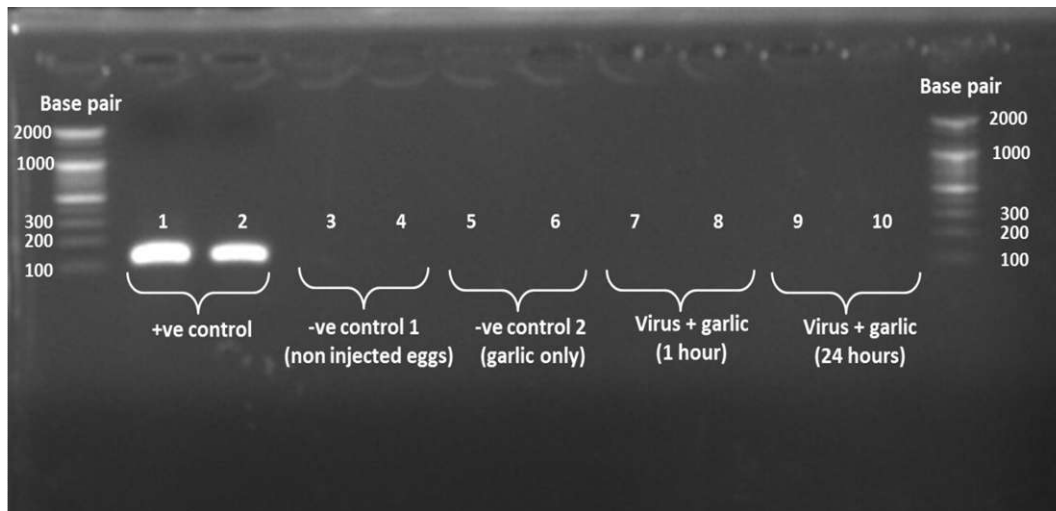


Figure 5: Molecular detection of Newcastle disease virus after injection of embryonated chicken eggs with garlic oil.

The figure shows the detection of Newcastle disease virus on agarose gel stained with ethidium bromide. The results showed good amplification of viral nucleic acid of positive control samples (lane 1 and 2), while the negative control samples (lane 3 and 4) and (lane 5 and 6), the nucleic acid was not amplified at all. No amplification was also noticed in samples treated with garlic for 1 hr (lane 7 and 8) and 24 hrs (lane 9 and 10).

DISCUSSION

Garlic oil was tested as an antiviral to Newcastle disease virus, and the results were evaluated using haemagglutination and RT-PCR techniques as well as the inoculation of embryonated chicken eggs with the virus. The results revealed that garlic oil has a great ability to eliminate the virus by destroying receptors on the surface of the virus and the genetic material inside. In addition, it was found that there was no toxicity or pathological effects of this oil on the organism as confirmed by the injection of chicken embryos, and it was effectively control the virus as no viruses were identified in the allantoic fluids of all treated groups.

A recent study has considered *Glycyrrhiza glabra* leave extract to be a potential antiviral in vivo against Newcastle disease virus. The study showed that this extract had a role in elimination of the virus with a high survival rate of the inoculated chicken embryos ⁽⁸⁾. This is agreed with the results of the current study as all the injected embryos were healthy and no pathological lesions were observed. On the

other hand, another study has evaluated the cytotoxicity and antiviral activity against Newcastle disease virus of ivermectin, a medication used to treat many types of parasite infestations such as scabies, head lice, strongyloidiasis; and ribavirin which is an antiviral medication used to treat RSV infection, hepatitis C, Lassa fever and severe cases of influenza. The study concluded that ivermectin has strong antiviral potential at 100µg/ml and higher but some concentrations were cytotoxic while ribavirin showed strong antiviral potential at all concentrations ⁽⁹⁾. In the current study, the concentration of garlic oil which was 50µg/ml gave good results in destroying the virus without toxicity seen in the chicken embryos.

A study suggested that fucoidan from *Cladosiphon okamuranus* exhibited antiviral activity against Newcastle disease virus LaSota strain, and represented a potential low-toxicity antiviral compound for the poultry industry ⁽¹⁰⁾. Another study revealed that ethanolic extracts of *Acacia cyanophylla* leaves, *Moringa peregrina* leaves, *Eucalyptus camaldulensis* fruits and *Pistacia atlantica* (leaves and stem) can inhibit Newcastle disease virus completely without causing death of the chicken embryo. However, the response of the viral-infected chicken embryos was different depending on the species of the medicinal plant, plant material concentration applied and the plant part. In addition, some plant extracts showed a dose dependent relationship with the degree of the virus inhibition, whereas other plant extracts showed some toxicity on the chicken embryo ⁽¹¹⁾. The results of our study revealed that the current doses of garlic oil has not shown any toxicity on chicken embryos and eliminate the virus completely, and therefore, garlic oil would be a potential antiviral drug against Newcastle disease virus.

In this study, it has been shown that garlic oil had a big role in destroying virus surface receptors and this was detected by haemagglutination test. On the other hand, some faints bands or no bands were appeared on the agarose gel after performing of RT-PCR on viruses treated with garlic oil, however, no detectible bands were seen after amplifying the virus genome taken from the allantoic fluids. The slight amplification of viral genome in the samples treated with the oil is likely to have traces of the RNA fragment of the virus where the primers have been associated with them and these fragments were amplified by RT-PCR, therefore this would not be biologically important.

In conclusion, based on the results gained in this study, it would be suggested that garlic oil is a good potential antiviral against Newcastle disease virus. However, further work is required to study the effect of this product in more depth such as determination of its effect on cell culture and employing other molecular and serological techniques for virus detection, and hence warrants further studies.

فعالية زيت الثوم ضد فيروس مرض نيوكاسل

منار محمد حزام* فراس طه منصور المبارك* وصفي عبود لعبيبي المسعودي**
* فرع الاحياء المجهرية، كلية الطب البيطري، جامعة البصرة، البصرة، العراق
** فرع الفسلجة، كلية الطب البيطري، جامعة البصرة، البصرة، العراق

الخلاصة

مرض نيوكاسل هو مرض فيروسي شديد العدوى في الدواجن في جميع أنحاء العالم وعادة ما يتسبب في خسائر اقتصادية كبيرة في صناعة الدواجن. على الرغم من استخدام اللقاحات للسيطرة على المرض، لا توجد أدوية فعالة مضادة للفيروسات تستخدم لعلاج الإصابة. الهدف من هذه الدراسة هو اختبار زيت الثوم كمضاد لفيروس مرض نيوكاسل. تم حضن زيت الثوم مع الفيروس (سلالة LaSota) لمدة ساعة و ٢٤ ساعة وتم تحديد تأثيره المضاد للفيروسات من خلال إجراء اختبارات التلازن الدموي واختبار تفاعل البلمرة المتسلسل العكسي للكشف عن البروتينات السطحية الفيروسية والمادة الوراثية للفيروس، على التوالي. بالإضافة إلى ذلك، تم تحديد سمية زيت الثوم على الكائن الحي عن طريق حقنه في أجنة بيض الدجاج مع أو بدون الفيروس. أظهرت النتائج أن زيت الثوم يلعب دوراً في تقليل فاعلية الفيروس من خلال تدمير مستقبلات الفيروس السطحية وكذلك تقليل تضخيم الجينات مقارنةً بمجموعة السيطرة التي شملت علاج الفيروس بمحلول ملحي فقط، والتي أعطت نتائج عكسية. بالإضافة إلى ذلك، لم يكن هناك أي سمية دوائية على الكائن الحي لأن الأجنة المحقونة مع الدواء وحده أو مع العقار الذي تم حضنه مع الفيروس كانت طبيعية وتشبه تلك التي لم يتم حقنها بأي شيء. من جانب آخر، فإن الأجنة التي تم حقنها بالفيروس فقط أظهرت علامات مرضية واضحة لم تظهر في المجموعات الأخرى التي تحتوي على زيت الثوم. هذه النتائج تشير إلى أن زيت الثوم مرشحاً كمضاد فيروسي جيد وربما سيكون له دور في القضاء على المرض.

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