Characterization of a multifunctional biocontrol agent, Streptomyces Misakimycin, against Newcastle Disease Virus

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Research Article

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Abstract

Background

The Newcastle Disease Virus (NDV) is present throughout the world, and outbreaks in Egypt caused serious economic losses in the poultry industry. Actinobacteria are a phylum of bacteria known for their potential in producing structurally diversified natural products which promising natural compounds used to combat viruses are presented and evaluated. *Streptomyces Misakimycin* isolated from Egyptian soil, and evaluated for their efficacy in controlling NDV. On the basis of the biochemical characteristics *S. Misakimycin* metabolites, identified by infrared (IR) and nuclear magnetic resonance (NMR) spectroscopy.

Results

In this investigation, NDV was found to have been isolated in February from a chicken farm in the Dakahlia Governorate of Egypt by the Animal Health Research Institute in Dokki, Giza. Diethylpthalate (DEP), a secondary metabolite of *S. misakimycin*, completely inhibited the hemagglutination (HI) activity of the NDV strain (MN635617) at log107 infectivity titers (EID50/mL). With 0.5 percent and 0.75 percent of chicken RBCs, the HA of the NDV strain was 2 log2 and 5 log2, respectively. In the treated group that received DEP and NDV inoculations, chorioallantoic-membranes (CAM) structures were preserved along with dilated capillary networks. Histological changes in SPF-ECE liver were examined after inoculation in ova to further characterize the DEP effect. Diethylpthalate and NDV mixture inoculated group showed preserved cytoarchitecture of hepatocytes with the presence of perivascular foci of lymphocytes. The group that was inoculated with telomycin alone showed normal histology of hepatic acini, central veins, and portal triads.

Conclusion

A potentially bioactive substance called diethylpthalate has the potential to be a biological weapon against a fatal chicken NDV that quickly increase cost losses for farmers.

Background

Natural products constitute a significant source of novel medications or act as models for the creation of new synthetic drugs. Many natural product medicines are actually created by microorganisms [1]. Actinobacteria are major bacterial groups that have capacity to produce useful secondary metabolites [2]. So Actinobacteria from a past and now are a key source for new medication development. Due to their extensive and potent biological activity, particularly their antiviral, antifungal, and anticancer properties from Streptomyces spp. have received considerable attention [3].

Newcastle disease (ND), also known as Ranikhet infection, is a highly contagious avian viral disease that affects both domestic and wild bird species [4]. The most typical symptoms of Newcastle disease are coughing, sneezing, nasal discharge, greenish/watery diarrhoea, and depression. Newcastle disease can cause sudden death without any evident signs and decrease in egg production or produce eggs with thin shells [4].

S. Misakimycin is a bacterium species from the genus of Kitasatospora which has been isolated from soil [6]. S. Misakimycin has ability for producing a bioactive secondary metabolites as tubermycin A, tubermycin B, misakimycin and the endothelin receptor antagonist BE-18257B [6]. The secondary metabolites from S. Misakimycin is Diethylpthalate (DEP) is members of the quinolone family, such as chloroquine and hydroxychloroquine which have antiviral efficacy against a variety of viruses, including coronaviruses, the human immunodeficiency virus and respiratory syncytial virus [5].

The purpose of the current study to identified natural compound with antiviral activities in vitro, from S. Misakimycin against NDV. Moreover, the study would extend to separate, analyze and characterize the crude extract using thin layer chromatography, and be identified by ultraviolet (UV-spectral) and infrared spectroscopic analysis.

Results

S. Misakimycin secondary metabolite extraction and characterization Chemo-physical tools like NMR, IR, and UV were used to define the structural molecules and functional groups of the DEP metabolite. The IR spectrum of DEP showed absorption spectra for (OH), (CH aliphatic), (C=O), (amide), and (C=C), respectively, at 2981, 2865, 1056, 1033, 1010 and 520. (Fig. 1). The conjugated system and N* transition were present in the UV spectrum at 232.00 nm (Fig. 2). Additionally, C13 NMR results showed that DEP produced signals at SP2 C=O, SP2 C=N, SP2 aromatic carbon, olefinic, SP2 of the aromatic system, and the final three aliphatic SP3 carbon, respectively, at 170.0, 140.0, 114.056, 132.418, 130.892, and 128.793. (Fig. 3). While the 1H1 NMR spectrum has the following ppm values: 0.8-0.9 (m, aliphatic, J=7.5Hz), 1.2-1.4 (m, O=H, J=97.5Hz), 2.0-2.1 (m, O=H, J=35.5Hz),

Virus titration 10-days-old-SPF-ECE

Domestically isolated NDV strains (Chicken / Egypt MN635617) (variant) were multiplied in 9–10 day old, SPF–ECE for 12 passages; allantoic fluids were collected and evaluated for sterility. Utilizing infectivity titration on SPF eggs, the virus was titrated, and the virus titer (EID50/mL) was estimated as EID50/mL = log105.

Assessment hemagglutination activity

The chicken erythrocytes were pre-treated with DEP in order to ascertain the effect of this drug on NDV's adhesion to these cells. The use of DEP pre-treated NDV strain and the HA test to evaluate agglutination (MN635617) as shown in Fig. 5, it is evident that chicken erythrocytes were not agglutinated, proving that

DEP can block chicken erythrocytes from agglutinating. By comparing the hemagglutination inhibition of chicken red blood cells at 0.5 and 0.75 percent, the activity of hemagglutination is evaluated with RBCs of 0.5 percent and 0.75 percent, respectively, the HA of the NDV strain (MN635617) was 8 log2 and 9 log2. The product produced from extremeophilic Streptomyces (Actinobacteria biomass extracts) and a mixture of NDV strains strongly inhibited the virus's ability to hemagglutinate with chicken RBCs at concentrations of 0.5 percent and 0.75 percent in vitro (shown in Table 1).

The effect of DEP on hemagglutination activity after SPF-ECE injection.

It is now typical to provide metabolites in vivo to assess the efficacy of a treatment. Table 1 and figure 4 provide our findings regarding the hemagglutination activity of collected allantoic fluid following mixture inoculation in SPF-ECE. Titers of HA varied from 2 to zero log2, which were 2 log lower than those of the parent strain before mixing and after egg inoculation. Our mixed inoculation results in eggs revealed that the viruses HA had reappeared but had decreased in their reading (five logs). HA of the original strain were 2 log2 and 5 log2, with respective RBC concentrations of 0.5 and 0.75 percent. Prior to inoculation in vitro, the P-value of NDV following DEP treatment was 0.5 for each of the two RBC concentrations (50 percent).

Table 1 Active hemagglutination NDV and DEP reading NDV and DEP mixing before and after SPF-ECE Inoculation

	RBCs concentration	V/V	1/2	1/4	1/8
Before	0.5%	zero	zero	zero	zero
	0.75%	zero	zero	zero	zero
After	0.5%	2	5	3	zero
	0.75%	2	7	5	zero

Liver chorioallantoic-membrane CAM and ECE histopathological changes

After inoculating in eggs, the effects of DEP and the histological changes to the liver were studied. Normal histology of hepatic parenchyma, sinusoids, central veins and portal triads in group received DEP only (Fig.5A). While, control received NDV group of liver showed extensive degenerative changes mainly steatosis which represented by clear cytoplasmic vacuoles with centrally or peripherally located nuclei. As well, congested sinusoids, central veins were seen (Fig.5B). Liver (treated group) showed mild hepatic degenerative changes with perivascular round cells infiltrations (Fig.5C).

Group received DEP showed normal chorio-allantoic epithelium and connective tissue layer (Fig.6A). While group received NDV Control +ve group revealed markedly increase thickness of both epithelial and

stromal layer. The stroma layer impacted by extensive hemorrhages ,congested vasculatures and some inflammatory cells .Moreover , the epithelial layer revealed vacuolated and hyperplastic epithelium with eosinophilic intracytoplasmic inclusions (Fig.6B,6C). Treated group revealed moderately thickened chorio-allantoic membrane due to edema and inflammatory cells infiltrate (Fig.6D).

Discussion

An urgent issue presently involves the search for antivirals to treat a number of viruses, including influenza, NDV, and paramyxoviruses that are developing multi-drug resistance [7]. Commercially available antiviral medications do not always work; therefore this poses an urgent concern. The development of novel antiviral medications using currently accessible natural product components offers a viable remedy for this growing problem. It is well known that the bulk of medications have been created utilising ingredients taken from naturally occurring gram-positive bacteria called Actinobacteria. Since a long time ago, many researchers throughout the world have turned their attention to the examination, isolation, and description of potential Actinobacteria strains that may produce secondary metabolites [8].

The isolation of a new Actinobacteria strain, *S. Misakimycin*, from a soil sample collected from Zagazig city, Sharkia, Egypt, agreed with by Bundle et al. [9]. The development of an effective fermentation process for producing secondary metabolites from Streptomyces species by adjusting several cultivation parameters like pH, incubation period, and temperature.

Misakimycin is a natural product found in *S. Misakimycin* has been reported from a variety of natural product source materials. DEP is the dimers of misakimycin which under quinolone family [6]. The findings are consistent with earlier research indicating that quinolone derivatives have the potential to serve as a promising framework for the creation of novel antivirals to combat this serious virus [10].

Assess this effect on the NDV strain (MN635617) (as a model) with 0.5 percent and 0.75 percent of chicken RBCs. The secondary metabolite of S. misakimycin (DEP) has a great amazing potential antiviral effect on the hemagglutination activity HA of viruses NDV. The findings shown that although HA was completely inhibited in vitro, in vivo reductions in these criteria reading three logs higher than the original were seen. Therefore, it may be concluded that, as suggested by multiple studies [11, 12]. HA should play a key role in viral entrance. The chemical (DEP) had an impact on the Newcastle virus NDV HA, which attacks fusion cells while ostensibly inhibiting the fusion of the virus with the host cell membrane. The function of is hampered by this interference.

Both the CAM and liver displayed significant histological alterations in the SPF-ECE negative control groups (DEP alone). In NDV injected embryos, the liver and CAM layers experienced varying degrees of congestion, bleeding, and hyperplasia. The information demonstrated that DEP our extract only possessed a broad and robust anti-NDV activity in vitro while in vivo displayed a distinct mode of action and may require further research to attain a novel antivirals activity in vivo result.

Material And Methods

S. Misakimycin cultivation and metabolite purification

The *S. Misakimycin* strain was cultured in a starch nitrate broth (pH 7.2) comprising starch (10 g), CaCO3 (3 g), MgSO4 (0.5 g), K2HPO4 (1 g), NaNO3 (2 g), and NaCl (5 g) per litre in order to explore the antiviral properties of S. *Misakimycin* secondary metabolites (Oxoid, UK). After being incubated for 5 days at 28 °C in a 100 mL total culture, the broth from 2 different conical flasks was combined (2 flasks were cultured for each strain), and the mycelium was removed to create cell-free culture broth by filtration through a coarse piece of clothing using Büchner porcelain funnels (Stonylab Egypt). Centrifugation was used to separate the *S. Misakimycin* cells' for 15 minutes at 13,000 rpm. After filtration and centrifugation, the metabolite solution was prepared [13].

DEP chemicals and structural characterization

At the Faculty of Science, Zagazig University, the chemical characteristics of the *S. Misakimycin* metabolite were investigated using the ultraviolet spectrum (UV), infrared spectrum (IR), nuclear magnetic radiation spectrum (NMR), and chemical shifts were referenced to tetramethylsilane (TMS Oxoid, UK) as an internal standard.

Thin-layer chromatography

Using pre-coated TLC sheets to identify antibiotics, thin layer chromatography (Sigma, St. Louis, MO) was used to separate the acquired metabolite. The bioactivity metabolite investigate activity by using the disc diffusion method, the resulting fraction was dissolved in diethyl ether [14].

UV Spectrum

A UV spectrophotometer (UV-Vis spectrophotometer, Thermo Scientific Multiskan Sky High Microplate Spectrophotometer, Germany) was used to scan the ultraviolet spectrum of the antibiotic. Using methanol solution, the spectrum was scanned at a wavelength of 200 nm. Each peak analysis' absorbance value was noted [15].

Fourier Transform Infrared Spectroscopy (FTIRS)

An IR spectrophotometer was used to scan the substance's infrared spectrum (ThermoFisher Nicolete IR IS10- USA) using methanol. The range of the spectrum was 4000-400 cm-1. Maximum and minimum resolution as well as the number of peaks were measured for this spectral range. [16].

Nuclear magnetic radiation spectrum (ECA-500II) (NMR)

Chloroform was used as a solvent in these spectra. The main analytical use of NMR spectroscopy is to gather precise structural and quantitative data on the synthesized metabolites inculde 1D-1H, 1D-1H

decoupled 13C, 2D 1H J-resolved, 1H-1H NOESY, 1H-1H COSY, 1H-1H TOCSY, 1H-13C HSQC, and 1H-13C HMBC. Data were compared to compounds made by *S. Misakimycin* that were similar [17].

Isolation and identification selected virus

NDV (GenBank accession number MN635617) was isolated from a chicken farm in the Dakahlia Governorate, Egypt, in February 2019. After speaking with farm owners who had gotten in touch with the Animal health research institution, Dokki, Giza, to discuss their current circumstance. This was done in February 2019.NDV primers (Forward 5'-TTG ATG GCA GGC CTC TTG C-3' and Revers, 5'-AGC GTY TCT GTC TCC T-3' were used to confirm the identification [18].

Virus propagation and titration

By injecting 0.1 ml of the virus into 10-day-old SPFECE (Kom Oshem, SPF Farm, Fayoum, Egypt) and daily candling, the NDV strain was titrated on allantoic fluid [19]. In order to determine infectivity titers using the Embryo Infective Dose 50 /mL, allantoic fluid was collected 72 hours after inoculation, according to Reed and Muench (EID50) [20].

DEP effect on NDV hemagglutination activity

An identical volume of 100 EID50/mL of the MN635617 NDV testing strain was combined with the researched DEP to produce the desired virus-inhibitory effect. With chicken red blood cells (RBCs) of 0.5 percent and 0.75 percent, the combination was tested for hemagglutination activity after 30 min of incubation at 37 °C. As a negative control, phosphate-buffered saline (pH 7.2) was employed [21].

Inoculation of SPF-ECE

Red blood cells were obtained from adult, healthy chickens via the Jugular vein of the neck using a sterile syringe and 4 percent sodium citrate as an anticoagulant. The cells were then washed three times with normal saline while rotating at 3000 rpm, and the packed cells were diluted as 10 percent and 0.5 percent before being used for plate-based and rapid tests for haemagglutination (HA) and haemagglutination inhibition (HI Using micro well plates for the HA activity assay, the allantoic fluid was examined for haemagglutination activity following SPF-ECE inoculation [22].

Inoculation of SPF eggs with two-fold dilutions of NDV

Strain was treated for 30 minutes at 37 °C with DEP (in phosphate-buffered saline, 1/2, 1/4, and 1/8) and DEP and NDV inoculation in SPF-ECE. The NDV strain was treated with three DEP dilutions (1/2, 1/4, and 1/8) for 30 min at room temperature before being inoculated into an allantoic sac of 10 day-old SPF-ECE/ 5 eggs/ 0.2 mL each, incubated at 37 °C in an egg incubator, and daily checked for mortality. The allantoic fluids from the live eggs were taken for analysis three days later after being kept at 4 °C overnight. [23].

SPF-ECE histopathological assessment

The collected CAM and liver of the inoculated SPF-ECE were fixed in 10% buffered neutral formalin solution, and paraffin Sects. (5 microns thickness) were prepared and stained with hematoxylin and eosin (H&E) [24].

Data analysis

A T-test was used to analyse the DEP and NDV strain results at the same RBCS concentration. $P \leq 0.05$ were considered statistically significant.

Abbreviations

NMR
Nuclear magnetic resonance
FTIRS
Fourier transform infrared spectroscopy
MS
Mass spectrometry
NDV
Newcastle Disease Virus
HI
Hemagglutination Inhibition
SPF
Specific Pathogen Free eggs, **HA**:Hemagglutination Activity
EID50
Egg Infective Dose.

Declarations

Ethics approval and consent to participate

The experimental protocol was approved by the Ethics of the Institutional Animal Care from el- dakhlia farming covenants and Use Committee of Zagazig University, Egypt (ZUIACUC–2019) and all animal experiments were performed in accordance with recommendations described in “The Guide for the Care and Use of Laboratory Animals in scientific investigations". All animal experiments were performed following the ARRIVE guidelines. Additionally, The permission of farm owners from farm has infection at dakhilia farming. Chicken RBC's obtained from Jugular vein of neck and use Committee of Zagazig University, Egypt (ZUIACUC–2019)."

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this article.

Competing interests

The authors declare that they have no conflict of interest.

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Contributions

Conceptualization: R.A., A.B.B., G.E., Y.H.T., M.M.G., and H.A.E. Methodology: R.A., A.B.B., G.E., Y.H.T., M.M.G and H.A.E. Resources and data curation: R.A. Writing —original draft: R.A. Writing—, review and editing: R.A. and Y. H.T. All authors have read and approved the manuscript.

Acknowledgments

To my dear mother Sanaa Affifi Emam Eelattar

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Figures

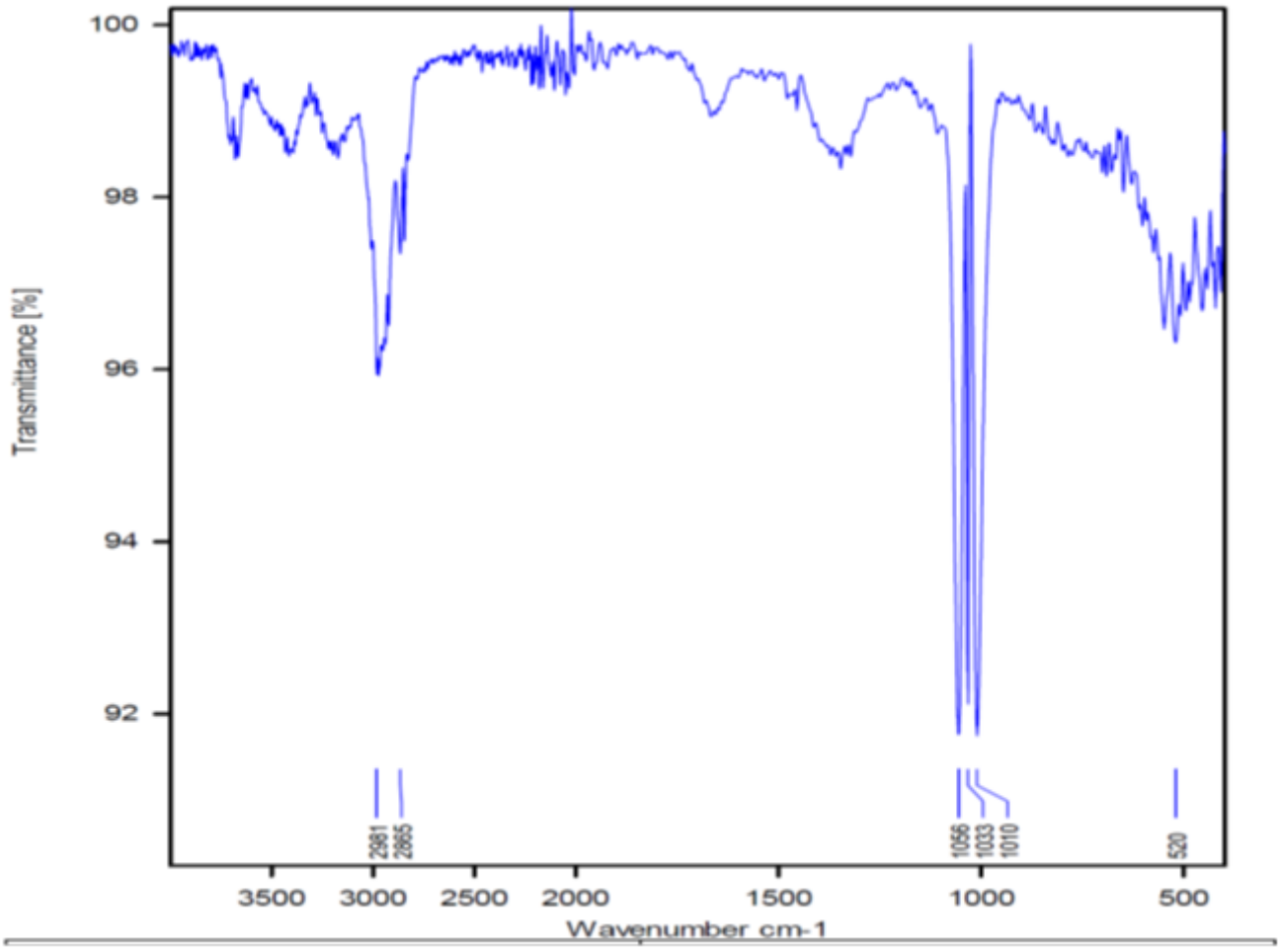

Figure 1

Using isoprbanol as a solvent, the antibiotic DEP was used in a peak report via IR spectrum.

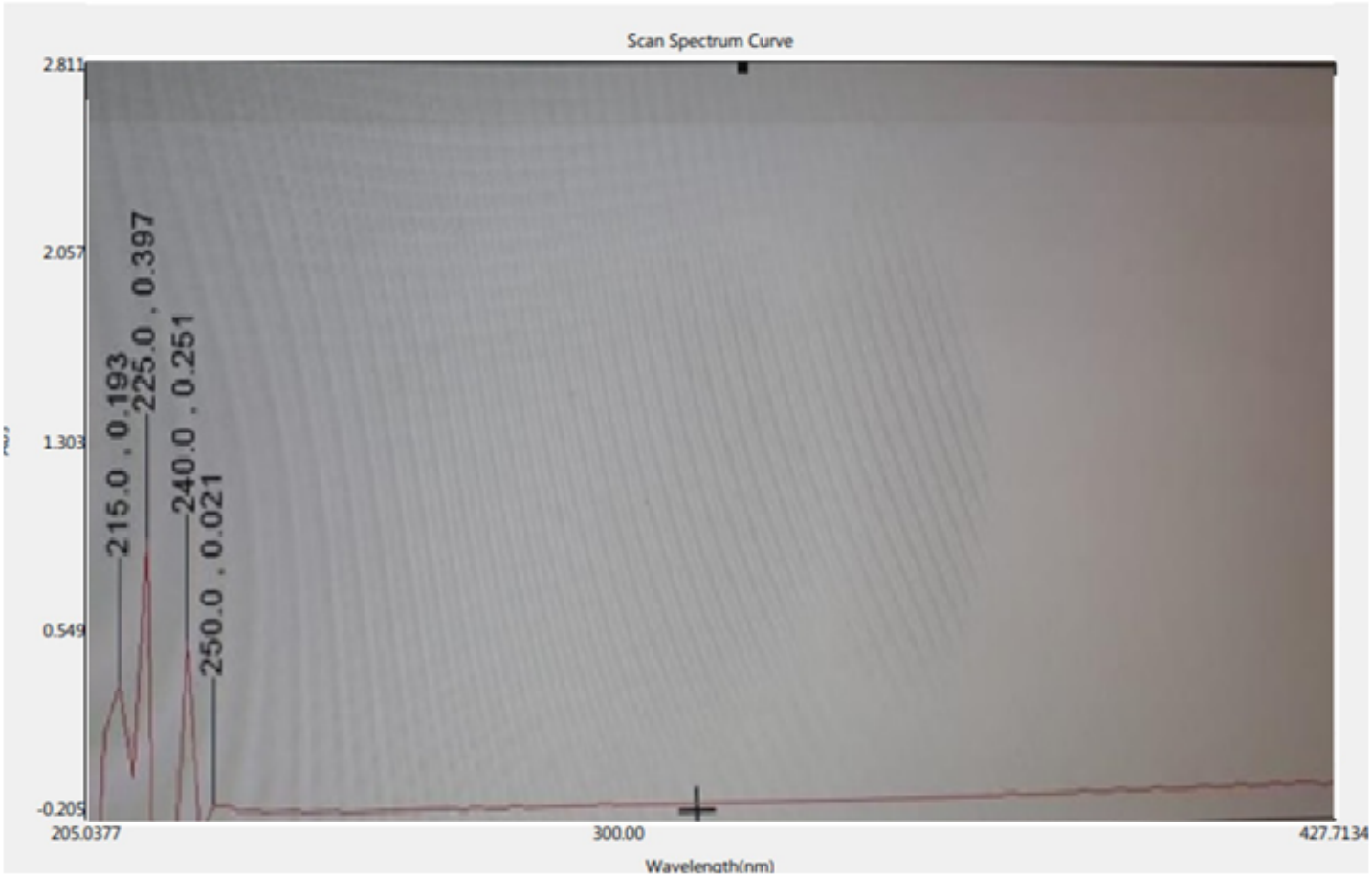

Figure 2

peak report for the UV spectrum of DEP antibiotic by ethylacetate as the solvent

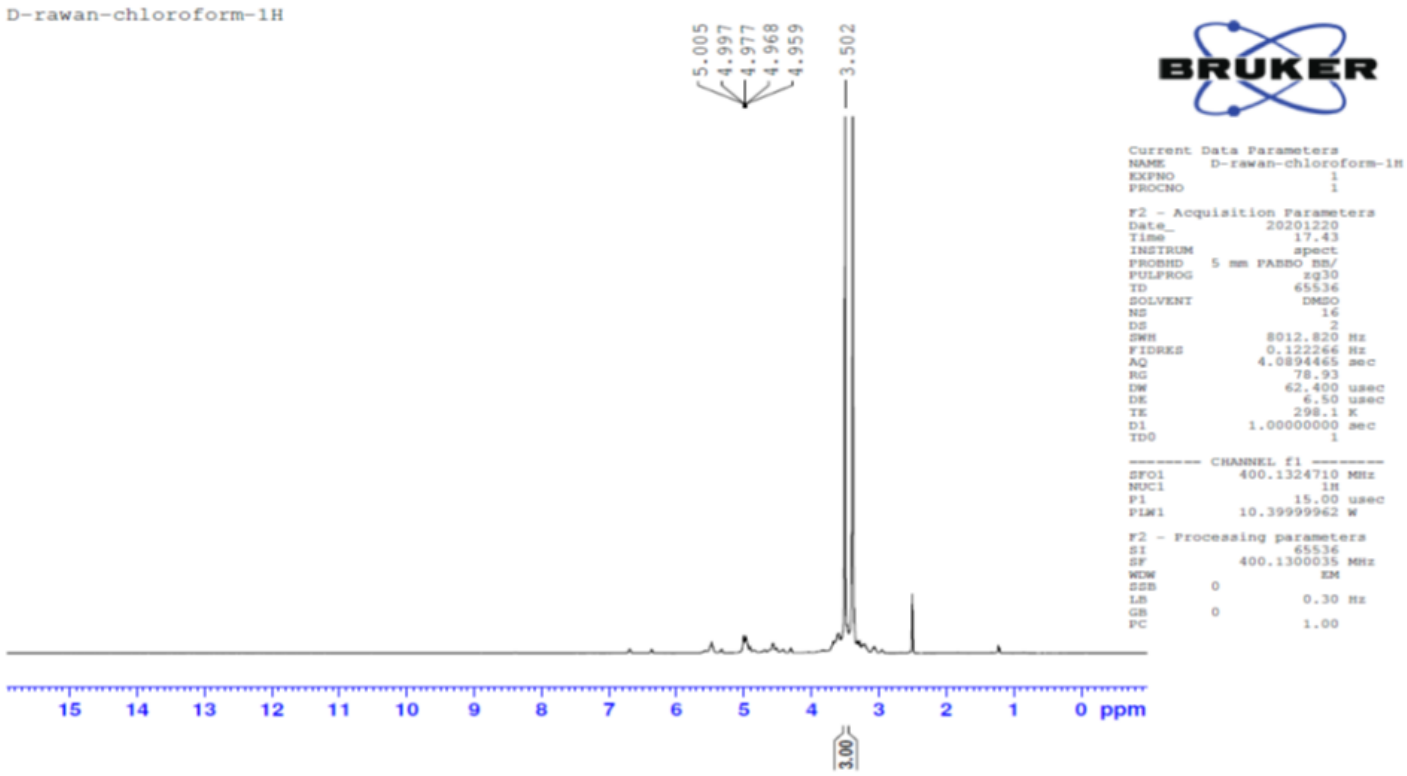

Figure 3

NMR-based H^1_1 Peak analysis range using chloroform as solvent

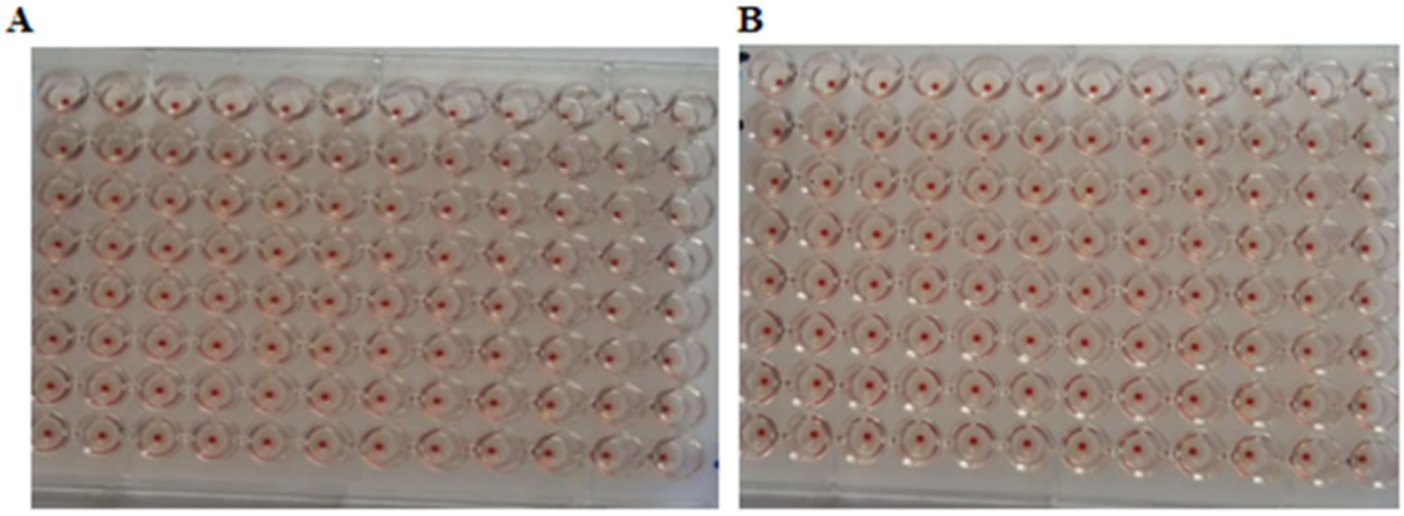

Figure 4

NDV and DEP mixture was inoculated into ova after 30 minutes at room temperature. After three days of collecting allotonic fluid and examining it, the results show that A: hemagglutination assay of harvested allantoic fluid after mixture inoculation NDV (MN635617) in SPF-ECE inhibits chicken. Red Blood Cells with 00.5 percent, and B: Hemagglutination assay of harvested allantoic fluid after mixture inoculation NDV (MN635617) in SPF-ECE inhibits chicken Red.

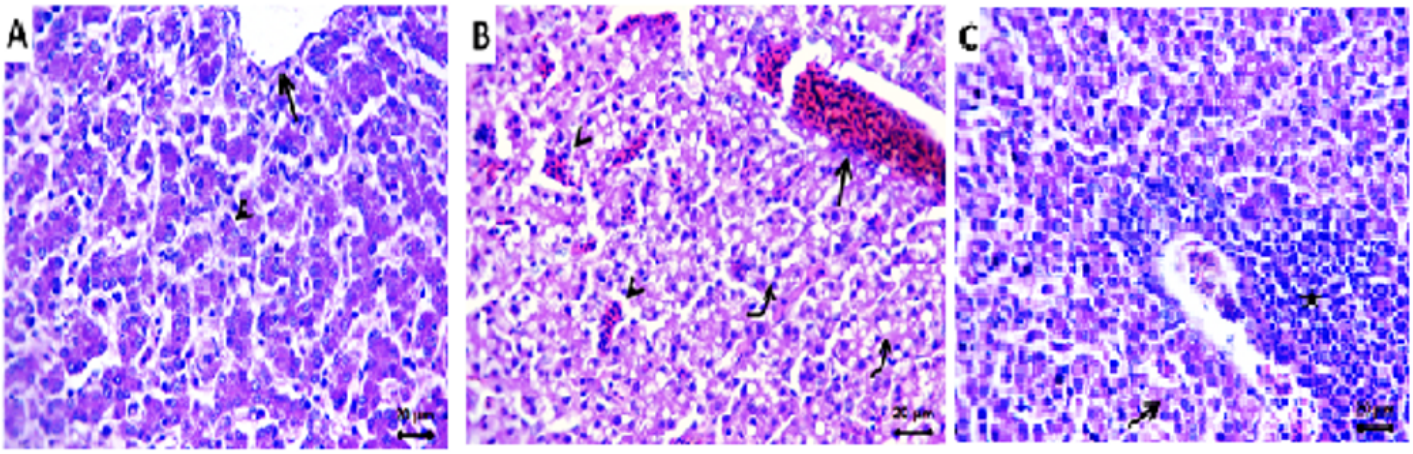

Figure 5

Representative photomicrograph of H&E stained sections of liver showing: Fig.A: Normal histology of hepatic parenchyma (arrowhead) ,sinusoids and central vein (arrow) in control-ve group.. Fig.B: Extensive steatosis (curved arrows), congested sinusoids (arrowhead) and central vein (arrow) in control +ve group. Fig.C: Few numbers of hydropic degenerated hepatocytes (arrow) and perivascular round cells infiltrations (star) in treated group. Scale bar 20µm.

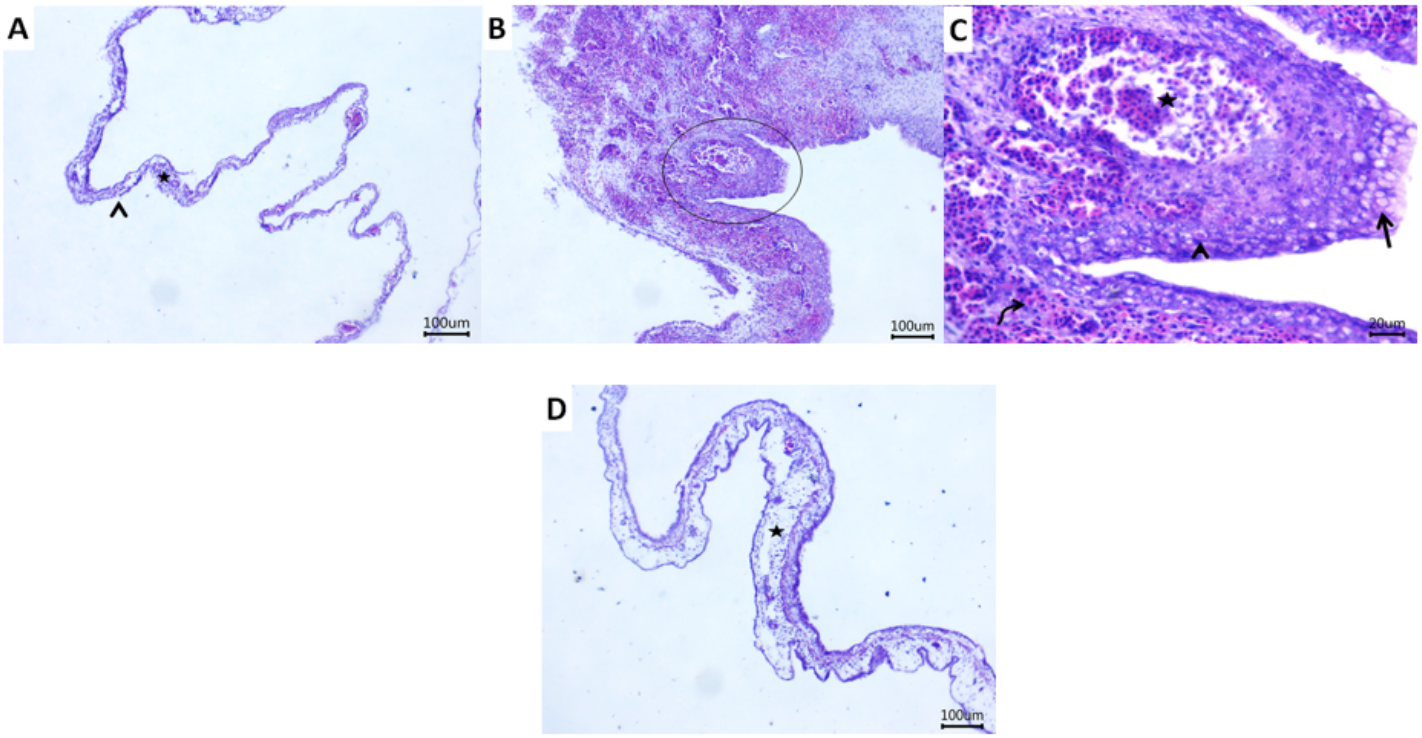

Figure 6

representative photomicrograph of H&E stained sections of CAM showing: **Fig.A**: Normal chorio-allantoic epithelium **(arrowhead)** and connective tissue layer **(star)** in control –ve group. **Scale bar 100 µm Fig. B, C**: Extensive hemorrhages **(curved arrow)** ,congested vasculatures **(star)** and some inflammatory cells within stroma layer. In addition to, hyperplastic vacuolated epithelium **(arrow)** with intracytoplasmic inclusions **(arrowhead)** within epithelial layer in control +ve group. **Scale bar 100,20 µm Fig.C**: Edema and inflammatory cells infiltrate **(star)** with moderately thickened chorio-allantoic membrane in treated group. **Scale bar 100 µm.**