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ANTIVIRAL ACTIVITY AND MECHANISM OF ACTION OF EDIBLE BIRD'S NEST AGAINST INFLUENZA A VIRUS STRAIN A/PUERTO RICO/8/1934 (H1N1)

AMIN HAGHANI

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By

AMIN HAGHANI

Thesis Submitted to School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for Degree of Master of Science

April 2015

It is my genuine gratefulness and warmest regard that I dedicate my thesis to my parents and my beloved wife, Nikoo, without whom I could not pursue my dream and continue my education.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

ANTIVIRAL ACTIVITY AND MECHANISM OF ACTION OF EDIBLE BIRD'S NEST AGAINST INFLUENZA A VIRUS STRAIN A/PUERTO RICO/8/1934 (H1N1)

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April 2015

Chairman: Aini Bt Ideris, Professor
Faculty: Institute of Bioscience

Influenza infection is still a high-risk disease affecting human and different animal species by causative agent influenza A virus (IAV). Currently there is neither effective vaccine nor efficient drug to control this infection. Edible Bird's Nest (EBN) as a popular traditional Chinese medicine (TCM) is believed to have health enhancing effects like anti-tumor and immunomodulatory activities. These natural extracts also have shown antiviral properties against influenza viruses; however, the molecular mechanism of action of these compounds still is not well characterized. Hence, the first aim of this study was to highlight the inhibitory effects of EBNs against influenza A virus (IAV) infection. Accordingly, house EBNs were collected from Teluk Intan and cave nests from Gua Madai in Malaysia and the extractions were prepared based on the established methods with two different enzymatic treatments. The median cytotoxic concentration (CC_{50}) of the EBN extracts were determined on Madin-Darby canine kidney (MDCK) cell line using microculture tetrazolium (MTT) assay and later on the best exposure way and median inhibitory concentration (IC_{50}) of the EBNs were shown against IAV strain A/Puerto Rico/8/1934 (H1N1). The results showed that post inoculation of the EBNs had the highest antiviral effect against IAV. The CC_{50} of these compounds ranged from 27.5-32 mg/ml with IC_{50} of 2.5-4.9 mg/ml against IAV and EBNs from Gua Madai had higher selectivity index compared to Teluk Intan. The second aim of this study was to understand the mechanism of action of these natural compounds against different molecular processes of IAV life cycle. These processes included effect of EBN on four viral proteins, virus host immune interactions through cytokines, early endosomes formation and their trafficking, and lastly autophagy process during IAV infection. Consequently, four viral genes and six cytokines were selected to be analyzed by RT-qPCR and ELISA to elucidate the effect of EBNs on the virus and immune system. Later, Western blotting on three GTPases proteins, and immunofluorescent labeling of actin cytoskeleton and lysosomes were done to investigate the effects of EBNs on endocytosis, actin cytoskeleton and macroautophagy processes during influenza virus life cycle. Regarding the effect of EBNs on viral genes and cytokines, the results showed that depends on the EBN composition, EBNs could significantly decrease the extracellular NA and NS1 copy number ($p<0.05$) of the virus along with high immunomodulatory effects against IAV. EBNs showed anti-inflammatory effects through decrease of CCL2 and IL-6, and increase of IL-27. In addition, these compounds might affect the virus by increase of TNF-α and activation of NF-κB. Immunofluorescent staining and Western blot results revealed the effects of EBNs on endocytosis, actin filament polymerization and macroautophagy pathways against IAV. EBNs could affect the trafficking of early endosomes by significant ($p<0.05$) decrease in GTPase proteins like RAB5 and RhoA, also ameliorating the actin filaments distress. These natural mixtures could efficiently inhibit the autophagy process involved in IAV life cycle by decrease ($p<0.05$) in LC3-II protein and augmentation of lysosome activity. In conclusion, EBNs can inhibit influenza infection by affecting critical steps of the virus life cycle. EBNs from different locations would show different mechanisms against IAV. Hence, after screening for the composition, these natural remedies have the potential to be used as an alternative antiviral agent against future influenza disasters. Further in vitro and in vivo studies are required to detect the bioactive agents and investigate the clinical applications of this natural medicine against influenza.

Keywords: edible bird's nest (EBN) extract, influenza A virus, qPCR, ELISA, autophagy, Western blot, immunofluorescent

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia Sebagai memenuhi keperluan untuk ijazah Master Sains

AKTIVITI ANTIVIRUS DAN MEKANISME TINDAKAN SARANG BURUNG YANG BOLEH DIMAKAN TERHADAP VIRUS INFLUENZA A STRAIN A/PUERTO RICO/8/1934 (H1N1)

Oleh

AMIN HAGHANI

April 2015

Pengerusi: **Professor Aini Bt Ideris**
Fakulti: **Institut Biosains**

Jangkitan virus influenza A merupakan penyakit berisiko tinggi yang masih memberi impak kepada manusia dan pelbagai spesis haiwan. Pada masa kini, pengawalan terhadap jangkitan ini tidak berkesan samada melalui vaksin mahupun ubatan. Sarang burung yang boleh dimakan (EBN) adalah terkenal dalam Perubatan Tradisional Cina (TCM) dan dipercayai dapat meningkatkan taraf kesihatan, sebagai contoh, keberkesanannya dalam aktiviti anti-tumor dan imunomodulator. Ekstrak semulajadi ini juga menunjukkan aktiviti antivirus terhadap virus influenza, walau bagaimanapun, mekanisme molekul tindakan sebatian ini masih belum terungkai sepenuhnya. Oleh itu, tujuan utama kajian ini dijalankan adalah untuk memberi fokus kepada kesan rencatan EBN terhadap jangkitan influenza virus A dan juga kesan terhadap laluan molekul bertindih yang terlibat di dalam kitaran hidup virus ini. Selanjutnya, hasil pengumpulan ekstrak EBN yang diperolehi dari Teluk Intan dan gua sarang burung dari Gua Madai, Malaysia, dimana penyediaan ekstrak tersebut disediakan melalui dua rawatan enzim yang berbeza berdasarkan kaedah sedia ada. Nilai CC_{50} ekstrak ditentukan pada baris sel MDCK dengan menggunakan esei MTT, seterusnya kaedah pendedahan terbaik serta IC_{50} ekstrak yang diperolehi menunjukkan kesan terhadap IAV pada strain A/Puerto Rico/8/1934(H1N1). Pasca inokulasi didapati mempunyai kesan anti virus tertinggi terhadap IAV pada ekstrak EBN. Nilai purata bagi sebatian ini terhadap influenza adalah sekitar diantara 27.5-32 mg/ml bagi CC_{50} dan 2.5-4.9 mg/ml bagi IC_{50} dan EBNs, dari Gua Madai mempunyai indeks pemilihan yang lebih tinggi berbanding Teluk Intan. Tujuan kedua kajian ini adalah untuk memahami mekanisme tindakan oleh kompaun semula jadi atas proses molekula yang berbeza yang terlibat dalam kitaran hidup IAV. Proses-proses tersebut terkandung kesan EBN ke atas empat protein virus, interaksi antara sitokin dengan imun sistem, formasi endosom awal dan pengedarannya, dan akhirnya proses autofagi semasa jangkitan IAV. Selepas itu, mekanisme molekul tindakan EBN ke atas virus dan imun sistem dianalisi dengan RT-qPCR dan ELISA berdasarkan kepada empat (4) gen virus dan enam (6) sitokin terpilih. Sap Western dan pewarnaan imunofluoresen atas aktin sitoskeleton dan lisosom telah digunakan untuk menyiasat kesan EBN keatas endositosis, aktin sitoskeleton dan proses mikroautofagi terhadap virus influenza. Berdasarkan kesan komposisi EBN ke atas gen virus dan sitokin, penurunan ketara didapati dalam bilangan salinan ekstrasel NA dan NS1 virus ($p<0.05$) bersama-sama dengan kesan imunomodulatori tinggi terhadap IAV. EBN menunjukkan kesan anti-inflamasi menerusi penurunan CCL2 dan IL-6 serta peningkatan IL-27. Selain itu, sebatian ini juga memberi kesan ke atas virus dengan meningkatkan TNF-α dan pengaktifan NF-κB. Pewarnaan imunofluoresen dan sap Western menunjukkan kesan EBN pada endositosis, pempolimeran filamen aktin dan laluan makroautofagi terhadap IAV. EBNs boleh menjejaskan pengedaran endosomes awal dengan signifikan ($p <0.05$) penurunan dalam protein GTPase seperti RAB5 dan RhoA, serta mengurangkan tekanan ke atas filamen aktin. Campuran semula jadi ini juga berkesan dalam menghalang proses autofagi yang terlibat dalam kitaran hidup IAV menerusi penurunan ($p <0.05$) dalam protein LC3-II dan peningkatan aktiviti lisosome. Kesimpulannya, ekstrak EBN berkesan terhadap jangkitan influenza dengan merencat langkah-langkah kritikal terhadap kitaran hidup virus tersebut. Maka, penyaringan komposisi rawatan semula jadi ini berpotensi menjadi agen antivirus alternatif terhadap jangkitan influenza pada masa hadapan. Kajian in vitro dan in vivo yang mendalam perlu dijalankan bagi mengesan agen bioaktif dan menyiasat kegunaan klinikal ubat semula jadi ini terhadap influenza.

Kata Kunci: ekstrak sarang burung yang boleh dimakan (EBN), virus influenza A, qPCR, ELISA, autofagi, sap Western, imunofluoresen

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I certify that a Thesis Examination Committee has met on 24 April 2015 to conduct the final examination of Amin Haghani on his thesis entitled “Antiviral Activity and Mechanism of Action of Edible Bird’s Nest Against Influenza A Virus Strain A/Puerto Rico/8/1934(H1N1)” in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

Members of the Thesis Examination Committee were as follows:

Saleha Abdul Aziz, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Jalila Abu, PhD
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Internal Examiner)

Lim Yang Mooi, PhD
Associate Professor
Universiti Tunku Abdul Rahman
Malaysia
(External Examiner)

APPROVAL

ZULKARNAIN ZAINAL, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 17 July 2015

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

Aini Ideris, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Abdul Rahman Omar, PhD
Professor
Institute of Bioscience
Universiti Putra Malaysia
(Member)

BUJANG KIM HUAT, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

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Signature: ___________________________

Name of members of supervisory committee: Abdul Rahman Omar, PhD

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LIST OF ABBREVIATIONS

Ama	Amantadine hydrochloride
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
Atgs	Autophagy-related genes
CC_{50}	50% Cytotoxic Concentration
CCL2	Chemokine (C-C motif) Ligand 2
CDC	Center of Disease Control and Prevention
Cq	Threshold Cycle
Ct	Threshold Cycle
DMEM	Dulbecco's Modified Eagle's Medium
EBN	Edible Bird's Nest
EBN1 or E1	EBN collected from Teluk Intan with no enzymatic treatment
EBN2 or E2	EBN collected from Teluk Intan with Pancreatin F treatment
EBN3 or E3	EBN collected from Gua Madai with no enzymatic treatment
EBN4 or E3	EBN collected from Gua Madai with neuraminidase treatment
EDTA	Ethylene-Diamine-Tetra-Acetic Acid
EE	Early Endosome
EEA1	Endosomal Auto Antigen 1
EGF	Epidermal Growth Factor
ELISA	Enzyme-linked Immunosorbent Assay
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FTIR	Fourier Transform Infrared Spectroscopy
GAG	Glycosaminoglycans
GTPase	Guanosine Triphosphate
HA	Hemagglutinin
HA assay	Hemagglutination assay
HAU	Hemagglutination unit
hADSCs	Human Adipose-derived Stem Cells
Hr	Hour
IAV	Influenza A Virus
IC_{50}	50% Inhibitory Concentration
IFN	Interferon
IL	Interleukin
LC3	Light Chain 3 Protein
LE	Late Endosome
MDCK	Madin-Darby Canine Kidney
MEM	Modified Essential Medium
MIP	Macrophage Inflammatory Proteins
MOI	Multiplicity of Infection
MTT	Microculture Tetrazolium
NA	Neuraminidase
NF-κB	Nuclear Factor Kappa Beta
NS1	Non-structural protein 1
OPLS-DA	Orthogonal Projection to Latent Square Discriminant Analysis
Ose	Oseltamivir phosphate

PBS	Phosphate Buffer Saline
PCA	Principle Component Analysis
PR	Influenza A virus (A/Puerto Rico/8/1934 (H1N1))
RhoA	Ras Homolog Gene Family, member A
RIG-I	Retinoid acid-inducible Gene-I
RT-qPCR	Quantitative Reverse Transcription Polymerase Chain Reaction
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SPSS	Statistical Package for the Social Sciences
$TCID_{50}$	50% Tissue Culture Infectious Dose
TCM	Traditional Chinese Medicine
TLR	Toll-like Receptor
TNF-α	Tumor Necrosis Factor Alpha
TPCK	Tosylamide Phenylethyl Chloromethyl Keton-treated
TRAIL	Tumor Necrosis Factor Related Apoptosis Inducing Ligands
vRNPs	Viral Ribonucleoprotein Complexes

CHAPTER 1

INTRODUCTION

For centuries, human race and different animals have suffered from influenza disease. This includes the devastating experiences of last five pandemics in 20th century: Spanish influenza (H1N1) in 1918 and 1919, Asian influenza (H2N2) in 1957, Hong Kong influenza (H3N2) in 1968, Russian flu from H1N1 in 1977 and recently a new reassortment H1N1 influenza in 2009 (Fukuyama and Kawaoka, 2011). Influenza A virus, the cause of this disease, is an enveloped virus belongs to family *Orthomyxoviridae* (from the Greek myxa meaning "mucus") (Cox and Subbarao, 2000). It contains an eight-segmented negative sensed RNA (Julkunen et al., 2000) that code 14 different proteins by use of host cellular comportment (Liu et al., 2013). The antigenic instability and the ability of viral gene reassortment have given the virus the ability to dominate the immune system and become pandemic (Girard et al., 2010). Two most important strategies to encounter this virus are vaccines and antiviral drugs; Since pandemic-specific vaccines cannot be available several months after commencement of the pandemics, antiviral treatment is the first line of defense to encounter influenza (Van-Tam and Sellwood, 2009). However, widespread resistance of influenza A virus strains to current commercial antiviral agents has increased the concern regarding feasibility in usage of large scale treatment against influenza (Bright et al., 2006; Dharan et al., 2009; Fiore et al., 2009).

Hence, finding new therapeutic approaches and antiviral agents can be the only way for facing future pandemics. It is required to understand the influenza pathogenesis, which is a combination of several viral and host factors. In the infected host, Influenza virus induces a cytokine storm by activation of toll-like receptor (TLR) 7 and retinoid acid-inducible gene-I (RIG-I) (Fukuyama and Kawaoka, 2011). This process would be started by recruiting the GTPase proteins to move Nuclear Factor Kappa Beta (NF-κB) to nucleus and activate TLR7 (Kawai and Akira, 2006). Afterwards, type I interferons (IFNs) and other cytokines like TNF α/β, IL-6, MIP-1 (CCL2) will be produced (Osterholm et al., 2012; Piqueras et al., 2006) that lead to the inflammatory symptoms of influenza disease.

The life cycle of influenza virus would start with attachment of virus to the host cell and entering by endocytosis. During this process, the viral M2 channels produce a low pH environment in the endosomes to help release of the viral RNA in the cytoplasm. This viral protein will also inhibit the autophagosome degradation by blocking the fusion of lysosome (Gannage et al., 2009). In this process, the GTPase proteins like RAB5 and RhoA are required to accompany the endosomes to regulate vesicle formation and vesicle movement along with actin cytoskeleton (Lakadamyali et al., 2004). Consequently, all of these processes should be considered as a potential target for new antiviral agent development.

Recently, some scientists have shown the hemagglutination inhibition activity of edible bird's nest (EBN), a popular Chinese traditional medicine, against influenza virus (Guo et al., 2006). This natural medicine composed of a mixture of bioactive compounds that have been used for several purposes in Chinese medicine and cuisine (Lim and Cranbrook, 2002). The Chinese people believed that EBN is a remedy that can dissolve phlegm, improve the voice, raise libido, ameliorate gastric problems, help renal dysfunction, asthma, cough, and tuberculosis (Hobbs, 2004). In past decades, some scientists have tried to investigate the properties of EBNs. It has been shown that EBNs have the properties like inducing cell proliferation, immunomodulatory effects, helpful in wound healing and neurodegenerative diseases, improving bone strength and skin sickness, and may be helpful against influenza viruses (Abidin et al., 2011; Guo et al., 2006; Kong et al., 1987; Vimala et al., 2012). On the other hand, there are concerns about the possible anaphylaxis induction and tumor progression in cancer patients of these natural medicines (Goh et al., 2000; Herbst and Langer, 2002). Hence, several scientists are trying to elucidate the potencies of EBN and the content bioactive compounds to be used in modern medicine. Regarding the anti-influenza activity, there are still many ambiguous aspects in mechanism of action of EBN against this virus.

Hence, the main objective of this study is to investigate the efficacy and mechanism of action of EBN against influenza A virus (strain A/Puerto Rico/8/1934(H1N1)). This strain is one the most common laboratory strain of H1N1 IAV for antiviral and vaccine development with high growth ability in cells and eggs (Van-Tam and Sellwood, 2009). We have hypothesized that the EBN can actively inhibit influenza A virus through specific pathways in influenza life cycle.

The specific objectives of this study are:

1) To evaluate the in vitro antiviral activity of the EBNs with different source and enzymatic treatments against IAV (strain A/Puerto Rico/8/1934(H1N1));
2) To determine the effects of EBNs on cytokines against influenza;
3) To investigate the effects of EBN on amount of small GTPase proteins (Rab5 and RhoA) on modulating endocytosis and actin cytoskeleton polymerization in influenza life cycle; and
4) To study the effects of EBN on amount of LC3 protein and lysosomal activity involved in macroauthophagy process of influenza virus life cycle.

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LIST OF PUBLICATIONS

Submitted journal papers:

Haghani, A., Mehrbod, P., Safi, N., Omar, A. R., Aini, I., 2015. Edible bird's nest modulate intracellular molecular pathways of influenza A virus infected cells, submitted to Evidenced Based Complementary and Alternative Medicine.

Under submission journal papers:

Haghani, A., Mehrbod, P., Safi, N., Omar, A. R., Aini, I., 2015. Immunomodulatory and Antiviral Activity Mechanism of Edible Bird's Nest (EBN) against Influenza A Virus (IAV) Infection, under submission.

Proceedings/Conferences:

Haghani, A., Mehrbod, P., Safi, N., Omar, A. R., Aini, I., Evaluation of antiviral properties of edible bird nest (EBN) extracts on influenza A virus (IAV) attenuation. Abstract presented at 32nd Malaysian Society of Microbiology symposium, 2014.

Haghani, A., Mehrbod, P., Safi, N., Omar, A. R., Aini, I., The effects of edible bird nest (EBN) extracts on autophagy pathway against influenza A virus. Abstract presented in Edible Bird Nest Industry Conference - EBNIC 2014, Malaysia.