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Review

Anti-influenza virus effect and mechanism of edible bird's nest: A review

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Abstract

Edible bird's nest (EBN) is made from the saliva of swiftlets, and has been consumed in China for more than 600 years. In recent years, as influenza virus has continued to cause serious damage to human health, the anti-influenza virus effect of EBN has become a research hotspot. In this paper, the antiviral effect of EBN is comprehensively reviewed. The types and components of influenza viruses are first introduced, and the main process of influenza virus infection is briefly summarised. The active components and related mechanisms of EBN are then described, and its anti-influenza virus activity is discussed. The components of EBN mainly responsible for its antiviral activity are sialic acid and protein; these exert antiviral effects by inhibiting virus adsorption and binding to host cells, blocking the release of virus on the surfaces of cell membranes, and reducing virus replication and transport in host cells. This paper provides theoretical and scientific evidence for the development and application of EBN products.

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Introduction

Influenza virus is one of the most common respiratory pathogens. Human beings are extremely susceptible to infection because influenza virus is highly contagious (Moradi *et al*., 2018). According to a study by the World Health Organization (WHO), seasonal influenza epidemics cause 5 to 10% of the global population to be infected each year, resulting in considerable mortality and morbidity, as well as economic losses. Influenza virus is therefore a serious medical and social problem facing human beings (Liu *et al*., 2021). Influenza virus is mainly transmitted by droplets and by human contact; these cause the clinical manifestations of sneezing, sore throat, fever, headache, muscle fatigue, and mild respiratory symptoms in infected people (Kapoor and Dhama, 2014). At present, various anti-influenza drugs and vaccines are used clinically for the prevention and treatment of influenza virus (Caceres *et al*., 2022). However, with increased numbers of adverse reactions to related drugs and vaccines, the emergence of drug-resistant virus strains, and the mismatching of vaccine antigens, there is now an urgent need for new methods to prevent and treat influenza (Amarelle *et al*., 2017). The therapeutic value of many natural foods is now well established, and researchers have identified a number of natural food products as potential options for the prevention and treatment of influenza virus infection (Haidari *et al*., 2009).

Edible bird's nest (EBN) is a nest made by swiftlets using its saliva secretion. It has been consumed in China for more than 600 years, and continues to be highly sought-after by Chinese consumers. EBN is mainly produced in Indonesia, Malaysia, Thailand, and other regions of Southeast Asia. On the basis of its source location, it can be classified as either house EBN or cave EBN. On the basis of its colour, it can be classified as either white EBN or blood EBN (Jamalluddin *et al*., 2019). EBN contains protein, sialic acid, minerals, and other

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nutrients (Fan *et al*., 2020). The level of protein content in EBN has been reported to be 42 - 63% (Lee *et al*., 2021), and the level of sialic acid content to be 7 - 12%. Sialic acid has been reported to play important roles in various biological activities of EBN (Wu, 2019). Other recent studies have shown that EBN can be used as a potential functional food, not only for the prevention and treatment of influenza virus infection, but also for regulating the immune system, improving the skin, antioxidation, promoting the development of brain nerves, and maintaining the health of bones and joints (Chok *et al*., 2021; Li *et al*., 2021).

Although researchers have made some progress in assessing the antiviral efficacy of EBN, there has been no systematic discussion on the active ingredients and mechanisms, by which EBN achieves any anti-influenza virus effect. In this paper, therefore, we review these active ingredients and mechanisms in detail, based on the type and composition of the influenza virus. We also consider the means by which the virus infects the human body, as well as the functional characteristics of EBN. The findings presented herein provide scientific reference for subsequent research on the efficacy of EBN and the development of related products.

Influenza virus types and structure

Influenza viruses belong to the Orthomyxidae family, whose genome consists of segmented negative-sense single-stranded RNA fragments. It is an encapsulated pathogen that can cause acute respiratory diseases. Based on the antigenic and genetic properties of viral matrix proteins and nucleoproteins, influenza viruses are classified as influenza A virus (IVA), influenza B virus (IVB), influenza C virus (IVC), or influenza D virus (IVD). Of these four types, the most clinically important pathogen is IVA, which has repeatedly caused severe epidemics in humans and livestock, and continues to pose a substantial risk to public health. Consequently, most influenza virus research has focused on analysis of IVA (Pleschka, 2012).

Influenza virus particles are spherical, with diameters of about 80 - 120 nm. Influenza viruses are mainly composed of lipid envelopes and viral structural proteins. The lipid envelope is derived from the plasma membrane of infected host cells, and serves as a protective barrier in the outermost layer of the influenza virion. Two different varieties of glycoprotein spike are embedded in the envelope. These are hemagglutinin (HA) and neuraminidase (NA). HA plays an important role in the attachment of viruses to host-cell receptors. NA is primarily involved in facilitating the release of newly produced viral particles from host cells (Yi Tsang *et al*., 2017; Pan, 2022). Based on the different HA and NA antigens, IVA can be divided into different subtypes. To date, 18 HA subtypes (H1 - H18) and 11 NA subtypes (N1 - N11) have been identified. Common human influenza virus subtypes include H1N1, H2N2, and H3N2, which may cause zoonotic infection. Avian influenza virus subtypes include H5N1, H7N2, H7N3, H7N9, and H9N2 (Gramza-Michałowska *et al*., 2017).

The structure of the influenza virus is shown in Figure 1. It can be seen that a small number of matrix (M2) ion channels traverse the lipid envelope. Under the lipid envelope is the matrix of the matrix protein (M1), which surrounds the core genome of the virion. The viral genome consists of eight single-stranded RNA fragments encapsulated by negative nucleoproteins. These RNA fragments are packaged together with nucleoproteins into viral ribonucleoprotein (vRNP) complexes. Fragments of the viral genome encode more than ten viral proteins, including seven virion structural proteins (PB1, PB2, PA, HA, NA, NP, and M1) and three non-structural proteins (M2, NS1, and NS2) (Luo, 2012). The functions of these proteins are shown in Table 1. Transcription and replication of the influenza virus RNA genome is catalysed by the viral heterotrimeric RNA-dependent RNA polymerase. RNA polymerase is a heterotrimeric complex consisting of the three subunits PA, PB1, and PB2 (Liu *et al*., 2009).

Influenza virus infection route

Influenza viruses are mainly transmitted by aerosol infection when relatively large droplets produced by a person when coughing or sneezing enter the mucous membranes of another person through short-range contact. Transmission can also occur through direct contact with virus-contaminated surfaces. The main parts of the body infected with influenza virus are the epithelium of the nose, the larynx, the trachea, and the alveoli. After infection, the virus begins to replicate at these sites. As the infection progresses, this replication also affects the lower respiratory tract (Hutchinson, 2018). Influenza virus is usually incubated for two to three days, with

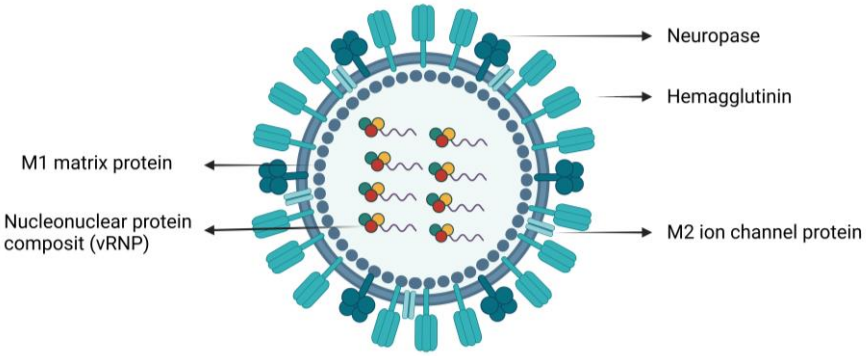

Figure 1. Structure of influenza virus.

Table 1. Functions of influenza virus genome segment encoded proteins.

Encoded protein	Protein function
HA	Surface glycoproteins which bind to host-cell sialic acid receptors
PA	Polymerase subunits which exhibit protease activity, and cut host-cell mRNA cap
PB1	Polymerase subunits which exhibit endonuclease activity and catalytic RNA elongation
PB2	Polymerase subunits which recognise and bind mRNA cap
NP	RNA-binding protein which is a component of vRNP complexes
NS1	Main antagonist of host innate immunity
NS2	Nuclear export of vRNP
M1	Matrix proteins which are responsible for vRNP interaction, RNA nuclear export regulation, and viral budding
M2	Ion channel proteins which are responsible for viral uncoating and assembly
NA	Surface glycoproteins which exhibit sialidase activity, and cleave the sialic acid binding site on the host-cell surface to release the virus

acute respiratory symptoms appearing one to five days after the incubation period, accompanied by unpleasant clinical symptoms such as headache, muscle pain, high fever, and nausea. The process of influenza virus infection in the human body mainly includes the adsorption and binding, internalisation, entry into the nucleus, replication, assembly, and release of the virus (Mercer *et al*., 2020).

Influenza virus adsorption and invasion

The cell membrane is the first barrier to be overcome before the influenza virus can invade cells. Influenza virus first recognises and adsorbs on sialic acid residue receptors on the corresponding glycoproteins or glycolipids of host cells through HA on its surface. The binding affinity of IAVs to host-cell surface receptors determines virus tropism and host specificity. For example, HA of avian influenza virus binds to α-2,3-sialic acid receptors on host cells, and HA of human influenza virus binds to α-2,6-sialic acid receptors on host cells (Yi Tsang *et al*., 2017; Pan, 2022). The binding of the virus to the receptor triggers cellular pinocytosis, where viral particles are engulfed by intracellular clathrin-mediated endocytosis at the cytoplasmic membrane, and rapidly enter intracellularly coated vesicles (endosomes) (Luo, 2012; Chen and Ye, 2022).

Influenza virus replication and transport

The low pH of the late endosome triggers a conformational change in HA, exposing a fusion peptide; this results in fusion of the viral envelope and the endosomal membrane, releasing vRNPs into the cytoplasm of the host cell. At the same time, the low

pH environment of the endosome activates the ion channel protein M2, which generates proton influx relative to the inner membrane of the virus, and guides the transport of vRNP into the nucleus for the viral replication program (Basler, 2007; Xu *et al*., 2014; Yin *et al*., 2021). After the vRNP enters the nucleus, the viral mRNA is first transcribed and synthesised in a primer-dependent manner. After entering the nucleus, vRNP first passes through the primer-dependent formula to transrecord, and then synthesises into virus mRNA. Some of the newly translated proteins (PA, PB1, PB2, NP, M1, *etc*.) are transported back to the nucleus, while other proteins such as HA, NA, and M2 are transported to the host-cell plasma membrane.

When PA, PB1, PB2, and NP are transported back to the nucleus, they combine with cRNA and vRNA to form cRNPs and vRNPs. Viral M1 and NS2 then bind to vRNPs, and mediate their export from the nucleus to the cytoplasm. Finally, by interacting with circulating endosomes, they migrate to the plasma membrane (Zhang *et al*., 2019).

Influenza virus assembly and release

Assembly and release of viruses are the final steps of viral infection. Viral vRNPs are transported to the cell membrane surface to assemble progeny viruses. A portion of the host-cell membrane folds to encapsulate the virion, and form the viral lipid envelope. During the assembly step, progeny viruses vRNP, HA, NA, M2, and M1 bud from the cell membrane. When budding is nearly complete, M2 moves to the neck of the budding virion at the lipid phase boundary, separating the virion from the host cell. NA begins to cut off the bond between the viral HA and the host-cell sialic acid residues, preventing the aggregation of virus particles, and the progeny virus is released from the host-cell surface (Yi Tsang *et al*., 2017; Zhang *et al*., 2019; Yin *et al*., 2021).

Potential active ingredients of edible bird's nest responsible for antiviral effects

Sialic acid

EBN is a natural food with a high level of sialic acid content. Sialic acid, as one of the important bioactive components of EBN, is closely related to antiviral activity (Ling *et al*., 2022). Sialic acid is a 9-carbon carboxylated monosaccharide derivative, the main form of which is N-acetylneuraminic acid (NANA) (Wu, 2019). There are two forms of NANA in EBN: a free form and a bound form. The free form is loosely attached to the surface of EBN; the bound form is covalently bound to glycan molecules, and linked to proteins. Recent research has proved that 7% of the sialic acid in EBN exists in the form of conjugated glycoproteins. In one study, the sialoglycoprotein structure of eight different types of EBN (including Baiyanzhan, Xueyan, and Dongyan) was analysed by lectin-specific affinity blotting, and it was found that the sialylation of glycoprotein sugar-chain ends varied among different EBN samples, with N-acetylneuraminic acid-α-2-3-Gal being mainly connected in EBN, and N-acetylneuraminic acid-α-2-6-Gal also included (Cao, 2012).

The sialic acid in EBN is similar to the sugar chain structure of the receptor sialic acid on the surface of the cell membrane. Because the influenza virus needs to recognise and bind the sialic acid residues on the cell membrane to enter the cell, the sialic acid in EBN can inhibit infection by competitively binding to the influenza virus. Guo *et al*. (2006) showed that the sialic acid hydrolysed by EBN exhibits antiviral activity, and that this activity is positively correlated with the content of sialic acid.

Protein

The protein in EBN mainly exists in the form of glycoprotein which is mostly water-soluble. In recent studies, researchers have used proteomic technology to identify the protein in EBN. Published results show that EBN content includes acidic mammalian chitinase, mucin-5AC, ovoinhibitor, nucleobindin, calcium-binding protein, lysyl oxidase homolog, lactoferrin, and ovotransferrin (Mohamad Nasir *et al*., 2021). Research findings in the protein research literature confirm that both mucin-5AC and lactoferrin exhibit antiviral activity.

Mucin-5AC

The results of a number of EBN proteomic analyses show that EBN contains mucin-5AC protein (Wong *et al*., 2018). Mucin-5AC is a secreted, gel-forming mucin which is expressed primarily in the airways, lungs, and stomach. Muc5ac protects the body from infection by trapping and eliminating inhaled viruses (Ehre *et al*., 2012). Influenza virus must bind sialic acid residues on the surface of epithelial cells to facilitate internalisation and subsequent infection processes when it invades the human body; however, in order to reach the surfaces of epithelial cells, the virus must pass through the

airway mucus, which is mainly composed of Muc-5ac and Muc-5b (Chang *et al*., 2020). Mucin has always been regarded as a barrier which prevents mucosal infections in the body, and studies on the efficacy of this protein have shown that it may be a potential active ingredient in the antiviral efficacy of EBN (Hou *et al*., 2015).

Chang *et al*. (2020) studied the roles of the mucins Muc5ac and Muc5b in the process of parainfluenza virus infection. By knocking out the Muc5ac and Muc5b genes in mice, four days after inoculation with the virus, they found that the virus titre in mice with knocked-out related genes was significantly higher than that in the blank control mice, indicating that the airway mucins Muc5ac and Muc5b contribute to the antiviral immunity of the airway to parainfluenza virus, and that the antiviral activity of Muc5ac mucin may be associated with mucociliary clearance (Chang *et al*., 2020). In addition, Ehre *et al*. (2012) found that the purified Muc5ac mucin can effectively inhibit the infection of MDCK cells by influenza virus, *i.e*., the Muc5ac protein has a direct antiviral effect. Muc5ac can protect mice from influenza virus infection by acting as "bait" to bind to the virus during the mobile phase. The increased secretion of Muc5ac produces a stronger anti-influenza virus infection effect; however, this high secretion of Muc5ac mucin is not sufficient by itself to trigger airway mucus obstruction or airway inflammation. These results suggest that Muc5ac mucin may serve as a protective barrier against influenza virus (Ehre *et al*., 2012).

Lactoferrin

In addition to the Muc-5ac protein, LF can also serve as a bioactive component of EBN's antiviral properties. The LF content in EBN is about 4.68 μg/mg (Hou *et al*., 2015). LF is an iron-binding protein from the transferrin family which consists of about 690 amino acid residues. It is mainly found in mucous secretions such as milk, tears, and saliva, but it can also be found in multiple organs and blood. LF has antibacterial, antifungal, antiviral, immunomodulatory, and anti-inflammatory properties. Since 1994, LF has been shown to demonstrate antiviral activity against both enveloped and naked viruses (Berlutti *et al*., 2011).

Studies have shown that LF can act in the early stages of viral infection, preventing the virus from binding to host cells by directly binding to viral particles or blocking surface receptors on host cells. Superti *et al*. (2019) found that LF can interact with hemagglutinin on the surface of IVA at low pH. By stabilising the conformation of hemagglutinin, LF prevents the exposure of the viral fusion peptide, so that the viral envelope and the host endosomal membrane cannot be fused, and the viral vRNA cannot be released in the host cell, preventing the replication process of the virus in the host cell (Superti *et al*., 2019). Pietrantoni *et al*. (2010) analysed the effect of LF on IVA infection *in vitro*, and showed that, in a MDCK cell model, IVA-infected cells died due to apoptosis. LF can inhibit programmed cell death by interfering with the function of the virus-induced apoptosis effector caspase 3, and prevent the virus assembly program by effectively blocking the export of vRNP from the host-cell nucleus to the cytoplasm (Pietrantoni *et al*., 2010).

Other potentially active ingredients

Chua *et al*. (2014) identified a metabolite called thymol-β-glucopyranoside in EBN using GC/MS analysis. Thymol derivatives are effective against foodborne microbial infections such as *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella* Typhimurium.

We may say, then, that sialic acid, mucin Muc-5ac, lactoferrin, and thymol-β-glucopyranoside are all important potential active ingredients of EBN which exhibit anti-influenza virus effects.

Inhibitory activity of edible bird's nest against influenza virus

By reviewing EBN-related research, it may be discerned that researchers have investigated the anti-influenza virus activity of EBN using cell experiments and animal experimental models, and found that EBN can safely and effectively prevent H1N1, H3N2, and H5N1 subtypes of influenza virus. Recent research into the anti-influenza virus effect of EBN is summarised in Table 2. From the table, it can be seen that, in *in vitro* studies of EBN anti-influenza virus activity, MDCK cells are the most widely used cell line. The hemagglutinin on the surface of influenza virus has the ability to agglutinate blood cells. The hemagglutination inhibition test can be used to evaluate the current antiviral activity. In certain animals, viruses or virus hemagglutinin can selectively agglutinate red blood cells. This

Table 2. Research on anti-influenza virus activity of edible bird's nest.

EBN type	Origin	Sample handling method	Influenza A virus type	Cell or animal model	Reference
House EBN, Cave EBN	Indonesia	Aqueous extracts treated with trypsin	A/Aichi/2/68(H3N2)	MDCK cell, A549 cell	Guo *et al*. (2006)
House EBN	Perak, Malaysia	Aqueous extract and its trypsin treatment	A/PR/8/34 (H1N1)	MDCK cell, BALB/c mice	Haghani *et al*. (2016)
Cave EBN	Sabah, Malaysia	Aqueous extract and its neuraminidase treatment			
House EBN	Perak, Malaysia	Aqueous extract and its trypsin treatment	A/Puerto Rico/8/1934 (H1N1)	MDCK cell	Haghani *et al*. (2017)
Cave EBN	Sabah, Malaysia	Aqueous extract and its neuraminidase treatment			
EBN	/	Aqueous extract	A/DK/BNY/F.2014P1 (H5N1)	Vero cell	Nuradji *et al*. (2018)
EBN	Indonesia	Aqueous extract and its digestion by gastric juice or intestinal juice	H5N1 Avian Influenza Pseudovirus	293T cell	Lin *et al*. (2016)
House EBN	Malaysia	Bird's nest trypsin extract	A/Aichi/2/68(H3N2)	MDCK cell, guinea pig red blood cells, ICR mice	Guo *et al*. (2017)
Cave EBN	Malaysia				
EBN	/	Bird's nest neutral protease extract	A/Memphis/1/71(H3N2)	MDCK cell, RAW264.7 cell	Lu *et al*. (2022)

aggregation of red blood cells is called hemagglutination (HA), also known as direct hemagglutination. In all hemagglutination assay experiments carried out to date, the results have shown that EBN can inhibit red blood cell hemagglutination. In addition, EBN aqueous extract and enzymatic hydrolysate (trypsin, neutral protease) have shown the potential to inhibit influenza virus infection.

In *in vivo* studies of the anti-influenza virus activity of EBN, two animal models of influenza virus infection have been used to date: normal BALB/c mice and aplastic anaemia ICR mice (Haghani *et al*., 2016; Guo *et al*., 2017). In the normal BALB/c mouse model, the antiviral activity of EBN was compared with that of the neuraminidase commercial antiviral drug oseltamivir phosphate. It was found that the anti-influenza virus effect of the two was similar, and that the IVA-pretreated viral load in the lungs of mice in the EBN administration group was zero, indicating that EBN can be used as a preventive anti-influenza virus food. In addition, in terms of cytokine changes, compared with oseltamivir, the EBN pair showed higher IFN-γ levels. This is noteworthy because IFN-γ plays an important role in the body's antiviral-related immune regulation (Haghani *et al*., 2016). In the aplastic ICR mouse model, the protective effect of EBN on influenza virus infection in aplastic anaemia mice was studied. It was found that EBN effectively improve peripheral blood circulation and body weight in aplastic mice, as well as improve the survival rates of aplastic mice infected with influenza virus (Guo *et al*., 2017).

Inhibitory mechanism of edible bird's nest against influenza virus

Direct action mechanism of edible bird's nest

In the initial stage of influenza virus infection, the hemagglutinin on the surface of the virus needs to bind to the sialic acid residues of glycoproteins or glycolipids on the cell membrane surface, so as to enter the cell through endocytosis. Western blot experiments have shown that, due to the abundance of sialic acid outside the cell, the Neu5Ac residue of the sialic acid sugar chain in the EBN extract can competitively inhibit the binding of influenza virus to the host-cell surface receptor by binding to the hemagglutinin of influenza virus. The study by Guo *et al*. (2006) found that, after enzymatic hydrolysis of EBN water extract, inhibitory activity against influenza virus was significantly enhanced. This may be because enzymatic hydrolysis fully exposes the sialic acid residues of EBN, and the binding ability to influenza virus becomes stronger.

After the influenza virus enters the cell through endocytosis, it is transported through the endosome. Rab5 is a key protein involved in the directional transport of the endosome. Studies have shown that reducing Rab5 to control the transport pathway can effectively reduce influenza virus infection (Zhu and Wang, 2019). RhoA belongs to the family of small GTPase proteins. In host cells, small G proteins are mainly involved in the regulation of important physiological processes such as cell polarity, vesicle transport, and protein transport. In the transfer process, actin filaments play a key role in the trafficking of virus particles in polarised epithelial cells. EBN can affect early endosomal trafficking of viruses by reducing Rab5 and RhoA proteins, and redirecting the actin cytoskeleton of IAV-infected cells (Haghani *et al*., 2017).

After the influenza virus enters the cell, the viral RNP is released into the cytoplasm, and the genome is transcribed and translated to synthesise important proteins such as HA, NA, M1, and M2 (Chua *et al*., 2021). EBN can directly reduce copy numbers and transcription levels of the viral genes M, NP, and NS1, thereby directly inhibiting the virus replication process. In addition, the host-cell autophagy response is a key mechanism to defend against IVA infection; this can induce virus clearance by degrading virus particles, and activating innate or acquired immunity, while IVA can induce virus clearance by blocking autophagic maturation, and further interfering with host antiviral signalling. EBN can also increase the degradation of lysosomes, and prevent the autophagy process of influenza virus from interfering with host cells, thereby effectively reducing virus replication (Haghani *et al*., 2017).

Due to the interaction between HA and sialic acid, the synthesised virus particles attach to the host-cell membrane, and are released by the catalytic hydrolysis of terminal sialic acid residues by NA (Chua *et al*., 2021). NA is a glycoside hydrolase that can cleave the neuraminic-acid glycosidic bond between the virus and the cell surface, and plays an important role in the release of new virus particles from the host-cell membrane during the cycle of influenza virus infection. The aqueous extract of EBN without enzyme (pancreatin) treatment can significantly reduce NA gene copy numbers of extracellular influenza virus. Therefore, by inhibiting

the activity of NA, the virus particles are aggregated on the surface of infected cells, and cannot be released, thereby reducing the infectivity of the virus. A general illustration of the mechanism by which EBN acts against influenza virus infection is shown in Figure 2.

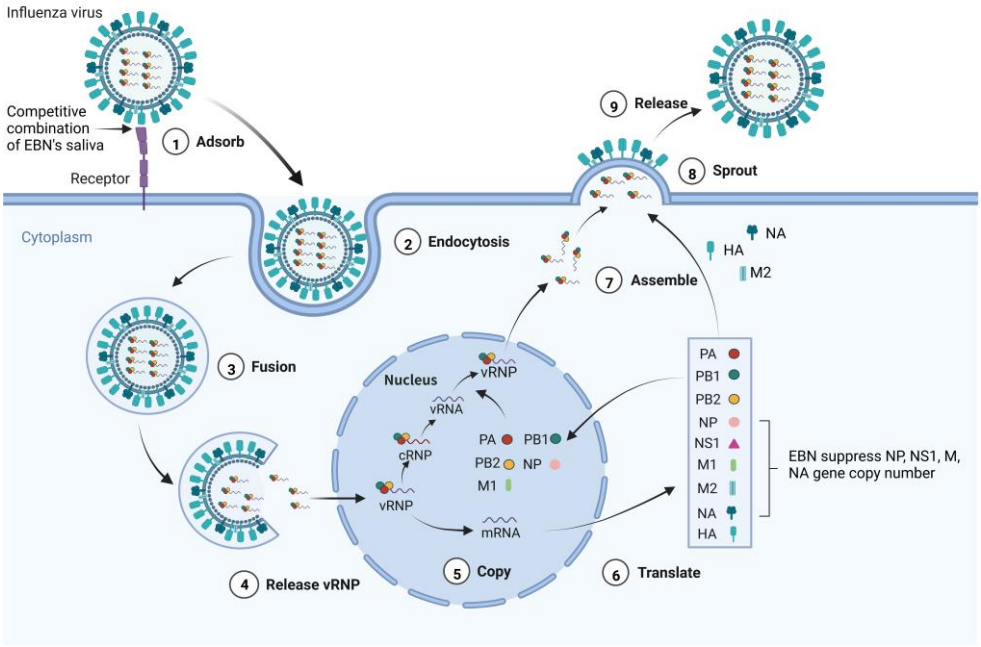

Figure 2. Mechanism of edible bird's nest against influenza virus infection.

Indirect action mechanism of edible bird's nest

EBN also produces an anti-influenza effect by regulating the level of immune factors in the body. EBN has been shown to play an antiviral role by regulating IFN-γ factors. In one study, this cytokine showed a high correlation with NA copy number in a group treated with EBN, but this was not observed in oseltamivir-treated or virus control groups. IFN-γ factor is mainly secreted by NK cells and T cells, which can directly inhibit virus replication by destroying the replication and accumulation of viral RNA (Haghani *et al*., 2016).

Conclusion

Influenza viruses are highly contagious, and their infection can cause serious harm to the body. Although there are existing drugs and vaccines against influenza virus infection, they are prone to cause adverse reactions. Therefore, the increased use of natural foods against influenza virus infection represents a new option for influenza virus prevention and treatment. As a traditional Chinese tonic, EBN has been consumed in China since the time of the Ming Dynasty. Recent scientific research has shown that EBN contains active ingredients such as sialic acid, mucin Muc-5ac, lactoferrin, and thymol-β-glucopyranoside; and that EBN can be used as a potential anti-influenza virus food. However, research into EBN is still in the pioneering stage, and its key active ingredients need to be further investigated by researchers. At present, research on the anti-influenza virus activity of EBN has mainly focused on cell models, with few studies involving *in vivo* models. Systematic explorations of mechanism pathways are also lacking. Further studies involving *in vivo* research models and systematic reviews of mechanism pathways can thus be regarded as very important areas for future research.

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