Assessment of potency of PC-complexed *Ocimum sanctum* methanol extract in embryonated eggs against Influenza virus (H1N1)

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Submitted: 23-08-2012 Revised: 27-09-2012 Published: 21-02-2014

ABSTRACT

Background: Despite of new vaccines, the threat of influenza infection persists. In addition, availability, cost, duration of protection rendered and effectiveness of vaccines additional to the need of effective drug therapy makes influenza a challenge, which the globe faces. Traditionally used herbs and their decoctions are used for ages to cure symptoms similar to influenza. *Tulsi* or *Ocimum sanctum* is one of these major herbs used for influenza-like disease treatment. We attempted to explore a new methodology for assessing phosphatidyl choline (PC)-complexed *O. sanctum* methanol extract in embryonated vaccine quality eggs model. **Materials and Methods:** The PC-complexed *O. sanctum* methanol extract was prepared and standardized using High-Performance Liquid Chromatography (HPLC). (Data not provided here) Nine to 11 days embryonated eggs were inoculated with the virus and drug mixture and then harvested to perform a hemagglutination (HA) test on the allantoic fluid. The experiments were performed at three different concentrations of ursolic acid with various virus concentration and dose levels of drugs. The HA titer was calculated from all experiments and observed for any inhibition of virus. **Results:** In initial experiments, matrix method for drug and virus concentration was employed. It was observed that the drug exhibited some response for 3log EID_{50} (egg infective dose) in few samples at 1:2 HA titer, but no response was observed at 4log EID_{50}. In subsequent experiment, all the virus titers from 7log EID_{50} to 2log EID_{50} demonstrated positive HA titer of 1:64. However, the drug failed to exhibit any significant inhibition at any level of demonstrable virus titer. At all the concentrations, *O. sanctum* extracts were found to be safe. **Conclusion:** The embryonated egg model may be utilized further to screen other drugs, which possess direct inhibitory properties like neuraminidase inhibition, and *O. sanctum* does not inhibit the influenza virus in this model at the given concentration.

Key words: Embryonated eggs, hemagglutination test, influenza, *Ocimum sanctum,* phosphatidyl choline

Access this article online

Website: www.phcog.com

DOI: 10.4103/0973-1296.127352

Quick Response Code:

INTRODUCTION

Threat of Influenza prevails throughout the globe as one of the most devastating and life-threatening viral infection. The ability of this virus to undergo genetic re-assortments causes unpredictable changes in its antigens, and the consequent immune response leads to recurrent epidemics of febrile respiratory disease every 1-3 years. In the 20th century, three influenza pandemics occurred and killed tens of millions of people, with each of these pandemics being caused by the appearance of a new strain of the virus in humans.[1] According to data from World Health Organization (WHO), 10-20% of the world population fall victim to Influenza every year. Despite success in the development of new antiviral agents such as oseltamivir and ribavirin, in recent years, problems regarding these chemotherapeutic drugs have been reported: Adverse effects, emergent risk of viral resistance, and loss of efficacy owing to serotype variation of viruses.[2] Therefore, the development of effective low-toxicity anti-influenza virus agents is still urgently needed. The search for viral inhibitors from plant origins may be a promising approach, since traditional herbal medicines have been used to

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treat influenza in India for centuries. *Ocimum sanctum* also known as *Tulsi* (Hindi) and Holy Basil (English) is an aromatic plant of the family Lamiaceae. The plant, as a whole, is a treasure house of potent compounds with its leaves, seeds, and roots, as well as flower being medicinally important and is considered divine by the Hindus. The main chemical constituents of *O. sanctum* are oleanolic acid, ursolic acid, rosmarinic acid, eugenol, carvacrol, linalool, and β-caryophyllene.[3,4] The antiviral activity of eugenol has been reported. *Ocimum* extracts are used in ayurvedic remedies for common colds, headaches, stomach disorders, inflammation, heart disease, various forms of poisoning, and malaria.[4] The embryonated eggs are widely utilized for Influenza vaccine production, yet they have not been widely explored before to assay drugs from natural origin. It has been shown that embryonated egg model can be utilized for *in-vivo* evaluation of anti-influenza activity of neuraminidase inhibitors.[5] Based on this, we screened *O. sanctum in-ovo* to assess its antiviral activity against Influenza.

MATERIALS AND METHODS

Plant materials

The plant selected, *O. sanctum* (leaves, stem), was collected from Gujarat state of India. The plant species was taxonomically identified and confirmed using morphological and anatomical techniques. They were authenticated at the Botanical Survey of India, Pune, and voucher specimens were deposited. The extracts were standardized using the high-performance thin-layer chromatographic (HPTLC) method (data not provided here).

Preparation of samples

For screening in eggs, *O. sanctum* extracts were complexed with phosphatidyl choline (PC) (1:1) and dissolved in an appropriate amount of water to get solution containing 0.0051%, 0.025%, and 0.012% of ursolic acid, as analyzed by High-Performance Liquid Chromatography (HPLC). The solution was filtered through 0.45 μm membrane filter and stored at 4°C in a sterile container. To ensure the sterility, the samples were screened to pass sterility testing of 14 days. The clean eggs were fertilized and incubated at 37.2 ± 0.1°C and 60 ± 5% relative humidity and candled every day to eliminate infertile eggs and dead embryos.

Virus titration

Inoculation of eggs

The 9-11 day embryonated eggs were inoculated with A/H1N1 Solomon Island Influenza virus strain. The virus titration was calculated by using Reed and Muench method.[6] The incubated eggs were cleaned with 0.5% sodium hypochlorite. The 50% egg infective dose (EID_{50}) calculated by the Reed-Muench method was $10^{8.3}$. This virus concentration of seed stock was serially diluted to acquire the desired virus concentrations. The virus and the drug mixture were incubated for 60 minutes before inoculation in the eggs. Punching was carefully performed and a volume of 200 μl was administered and incubated for 48 hours. Before harvesting, they were stored in cold storage for 12 hours to facilitate the harvest procedure.

Harvesting of eggs

The eggs were decapped and allantoic membrane was carefully moved to sides to pipette out the upper allantoic fluid. After harvesting, the allantoic fluid was subjected to hemagglutination (HA) test.

Hemagglutination test

A U-bottom 96 well microtiter plate was labeled horizontally 1:2 to 1:64 dilutions (column 1 to 4). Using a multi-channel pipette, 50 μL of Phosphate Buffer Saline (PBS) (pH 7.2) was added to all the wells. 50 μL of allantoic fluid (test harvest) was added to the first well of each row (A to D) and only PBS is added to the last row. Two-fold dilutions were made by transferring 50 μL from the first well of each column A1-D1 to A2-D2 using multi-channel micropipette. This was done until last column and the remaining 50 μL was discarded. Fifty microliter of 0.5% Guinea pig red blood cells (RBC) were added to all the wells using a multi-channel micropipette and were mixed by manual agitation to the plates thoroughly. The plates were incubated for 60 minutes at room temperature. The plates were checked for complete settling of RBC's, and the results were recorded to calculate the EID_{50}. In the wells, where the Guinea-pig RBCs formed a button or a ring at the bottom of the wells, were recorded as negative. If HA occurred, i.e. RBCs remained in suspension, it was recorded using a "+" symbol. The highest dilution of virus that caused complete HA was considered as the end-point in HA titration. The HA titre was the reciprocal of the dilution of virus in the last well with complete HA. Eggs showing HA activity were scored as positive and virus titer was calculated.

Experiment design

PC-complexed *O. sanctum* methanol extract was assessed at various concentrations at three dose levels in different experiments. In initial experiments, the concentrations were 0.0051% and 0.012% of ursolic acid, which was assessed in 160 and 128 eggs, respectively, at three dose levels with virus concentration, 3log EID_{50} and 4log EID_{50}. The next experiment in 310 eggs was with higher concentration of drug (0.025% ursolic acid) assayed against all the virus concentrations from 7log EID_{50} to 2log EID_{50}. The experiment design was intended to optimize the embryonated egg model for screening of drugs against Influenza virus [Experiment 1].

Experiment : HA test results of anti-influenzascreening

Expt: Assessment of potency of PC complexed *O.sanctum*

Strain: Solomon island H1N1 influenza virus

E	Material	No.of eggs	1	2	3	4
		HA unit ®	2	4	8	16
1	2 log EID_{50}/2ml+1OSH	1	O			
		2	O			
		3	O			
		4	O			
		5	O			
		6	O			
		7	O			
		8	O			
		9	O			
		10	O			
2	2 log EID_{50}/1ml+1OSH	1	O			
		2	O			
		3	O			
		4	O			
		5	O			
		6	O			
		7	O			
		8	O			
		9	O			
		10	O			
3	1 log EID_{50}/1ml+1OSH	1	O			
		2	O			
		3	O			
		4	O			
		5	O			
		6	O			
		7	O			
		8	O			
		9	O			
		10	O			
4	1 log EID_{50}/2ml+1OSH	1	O			
		2	O			
		3	O			
		4	O			
		5	O			
		6	O			
		7	O			
		8	O			
		9	O			
		10	O			
5	2 log EID_{50}/1ml+1OSR	1	O			
		2	O			
		3	O			
		4	O			
		5	O			
		6	O			
		7	O			
		8	O			
		9	O			
		10	O			
6	1 log EID_{50}/1ml+1OSR	1	O			
		2	O			

Contd...

Experiment : Contd

Expt: Assessment of potency of PC complexed *O.sanctum*

Strain: Solomon island H1N1 influenza virus

E	Material	No.of eggs	1	2	3	4
		HA unit ®	2	4	8	16
		3	O			
		4	O			
		5	O			
		6	O			
		7	O			
		8	O			
		9	O			
		10	O			
7	2 log EID_{50}/2ml+1OSF	1	O			
		2	O			
		3	O			
		4	O			
		5	O			
		6	O			
		7	O			
		8	O			
		9	O			
		10	O			
8	1 log EID_{50}/2ml+1OSF	1	O			
		2	O			
		3	O			
		4	O			
		5	O			
		6	O			
		7	O			
		8	O			
		9	O			
		10	O			
9	2 log EID_{50}/1ml+1OSF	1	O			
		2	O			
		3	O			
		4	O			
		5	O			
		6	O			
		7	O			
		8	O			
		9	O			
		10	O			
10	2 log EID_{50}/1ml (virus control)	1	O			
		2				+
		3	O			
		4				+
		5	O			
		6	O			
		7	O			
		8	O			
		9	O			
		10	O			
11	1 log EID_{50}/1ml (virus control)	1	O			
		2	O			
		3	O			
		4				+
		5	O			

Contd...

Experiment : Contd

Expt: Assessment of potency of PC complexed *O.sanctum*

Strain: Solomon island H1N1 influenza virus

E	Material	No.of eggs	1	2	3	4
		HA unit ®	2	4	8	16
		6	O			
		7	O			
		9	O			
		10	O			
12	1OSF drug control	1	O			
		2	O			
		3	O			
		4	O			
		5	O			
		6	O			
		7	O			
		8	O			
		9	O			
		10	O			
13	1OSH drug control	1	O			
		2	O			
		3	O			
		4	O			
		5	O			
		6	O			
		7	O			
		8	O			
		9	O			
		10	O			
14	Vehicle control	1	O			
		2	O			
		3	O			
		4	O			
		5	O			
		6	O			
		7	O			
		8	O			
		9	O			
		10	O			
15	Uninoculated eggs	1	O			
		2	O			
		3	O			
		4	O			
		5	O			
		6	O			
		7	O			
		8	O			
		9	O			
		10	O			
		11	O			
		12	O			
		13	O			
		14	O			
		15	O			
		16	O			
		17	O			
		18	O			
		19	O			
		20	O			
	Total eggs	160				

EID: Egg infective dose, OSH:Ocimum sanctum half dose, OSF: Ocimum sanctum full dose, OSR: Ocimum sanctum reduced dose, PC: Phosphatidyl choline

RESULTS

Interpretation of results of HA test

The embryonated egg model was established for the screening of crude herb powders and extracts from medicinal plants by using matrix method for drug and virus concentrations. It was observed that the drug exhibited some response for 3log EID_{50} in few samples at 1:2 HA titer, but no response was observed at 4log EID_{50}. Since very fewer responses were observed in the initial experiment by the drug, it was further concentrated and a higher dose was used for the next experiment. In this experiment, virus titer from maximum to minimum was utilized to observe the HA response and to test the potency of *O. sanctum* extracts. All the virus titers from 7log EID_{50} to 2log EID_{50} demonstrated positive HA titer of 1:64. However, the drug failed to exhibit any significant inhibition at any level of demonstrable virus titer. At all the concentrations, *O. sanctum* extracts were found to be safe [Experiment 1].

DISCUSSION

To our knowledge, *O. sanctum* and its constituents have not been studied before in an embryonated egg model for screening against Influenza virus. *O. sanctum* is extensively used in ayurveda and has shown to possess adaptogenic, anti-inflammatory, antibacterial, antiplasmodial, immunomodulatory, and antioxidant properties.[7-15] A double-blind randomized control trial to assess the immunomodulatory activities of *O. sanctum* in healthy volunteers have shown increased levels of Interferon-γ and Interleukin-4 along with T-helper cells and Natural killer cells.[16] Ursolic acid is one of the major constituents of *O. sanctum*,[17,18] and has demonstrated activities like protection against lipid peroxidation,[19] antiviral activity,[20,21] including Influenza, when extracted from *Mosla scabra in-vitro* and *in-vivo*.[22] Also, this drug has been traditionally used for symptoms of fever resembling Influenza, and many ayurvedic practitioners used this drug prophylactically during the pandemic. This model assesses the direct inhibitory properties

of drug while is not useful for prodrug assessment. Currently, United States Food and Drug Administration (US FDA) has approved two classes of drugs for treatment of prevention of influenza virus infection: M2 ion channel blockers and neuraminidase inhibitors (NAIs).[23] Oseltamivir of the NAIs approved by US FDA is a prodrug. The *O. sanctum* extract was complexed with PC to facilitate the solubility of the drug. Other drug extracts like curcumin complexed with PC have proven to exhibit better absorption, enhanced bioavailability, and improved pharmacokinetics.[24] Thus, this study indicates that *O. sanctum* may exert its anti-influenza activity by some other mechanism than directly inhibiting the virus through neuraminidase inhibition. Immunomodulatory effects of *O. sanctum* are widely studied, and it may exert its clinical efficacy via modulating immune system to combat the virus.[25] This experiment in which we have optimized the virus titer for the embryonated egg model and excluded one of the main speculated mechanism of action, may form a basis for other such studies. It also indicates that the traditionally used medicine needs to be assessed in a manner, which can explore various modes of action, unless to render precious drugs ineffective.

CONCLUSION

This experiment has demonstrated that embryonated egg model can be used for screening of Influenza virus. *O. sanctum* extract did not show any significant activity against A/H1N1 Solomon Island virus strain, which can be correlated to the probable mechanism of action. Like oseltamivir, if the drug acts as a prodrug, it needs to undergo metabolism for activation, which does not happen in this model. In addition, *O. sanctum* has been proven to possess immunomodulatory properties, which can contribute to the efficacy of this drug. *Tulsi* is used traditionally and by many senior ayurvedic physicians to treat fever and other symptoms resembling Influenza. It can be concluded that the embryonated egg model can be utilized further to screen other drugs, which possess direct inhibitory properties, and *O. sanctum* did not inhibit the influenza virus in this model at the given concentration.

ACKNOWLEDGEMENT

We sincerely thank Dr. Rajeev Dhere, Dr. Milan Ganguly, and the team at Serum Institute of India, Pune, for the technical and intellectual support and guidance.

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Cite this article as: Jadhav P, Lal H, Kshirsagar N. Assessment of potency of PC-complexed *Ocimum sanctum* methanol extract in embryonated eggs against Influenza virus (H1N1). Phcog Mag 2014;10:S86-91.

Source of Support: Nil, **Conflict of Interest:** None declared.