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In Vitro Analysis of the Anti-influenza Virus Activity of Pomegranate Products and Fulvic Acid

Radha Ganapathy
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a thesis written by Radha Ganapathy entitled "In Vitro Analysis of the Anti-influenza Virus Activity of Pomegranate Products and Fulvic Acid." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

Mark Sangster, Major Professor

We have read this thesis and recommend its acceptance:

Tim Sparer, Chunlei Su, Thandi Onami

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

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A Thesis for the

Master of Science Degree

The University of Tennessee, Knoxville

Radha Ganapathy

December 2009

DEDICATION

IN THE LORD I PUT MY TRUST (PSALM 11:1)

Dear Lord Father,

Thank you for coming into my heart and life in July 2009 and for being my Lord and Saviour.I dedicate this dissertation to you Lord. Thank you for the plans you have for me to prosper. Holy Spirit, lead me. Let me run at your commands. Your Word will be a lamp for me, a guide to light my ways, a solid place to set my feet. May I live my life to praise you, not for fortune, nor for fame, may everything I say and do bring glory to your name. I praise and thank you for giving me wisdom and for all the blessings I received from you. In Jesus Holy name I pray.

Amen.

Also I dedicate this dissertation to my beloved Husband and my heart throb "Prakash", my Family and my Friends who have lovingly and patiently stood by me through it all.

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I want to thank my family for their love and support and for encouraging me to pursue the higher education and to always set high goals.

And most importantly, I would like to thank my beloved Husband and my heart throb Prakash for his joy, love, patience and comfort throughout this project. Thanks for holding my hand and giving constant love and support all along the way.

My gratitude also goes to my Pastors and my Friends in the Church for the love and joy they are giving me.

Abstract

In traditional cuban medicine, pomegranate fruits have been used to treat acidosis, dysentery, microbial infections, diarrhoea, helminthiasis; haemorrhage and respiratory pathologies [Vuorela et al., 2003; Roig, 1974; Jimenez et al., 1979; Seoane, 1984].Pomegranates contain high levels of Polyphenolic compounds, which are largely responsible for the fruit's antioxidant properties. A number of studies have demonstrated that polyphenolic complexes derived from other plants have antiviral effects, suggesting that antiviral activity may also reside in the polyphenol (PP) fraction of pomegranates.

The decay of organic matter generates an extremely heterogeneous mixture of organic molecules referred to as humic substances. They are sub-classified on the basis of solubility characteristics. The Fulvic Acid (FA) fraction of humic substances includes a variety of low molecular acidic molecules that are soluble in water under all pH conditions. Antiviral activity of fulvic acid containing Secomet V against poxviruses and SARS has been demonstrated [Kotwal et al., 2006].

The current study was undertaken to evaluate the direct anti-influenza virus activity of pomegranate constituents present in Pomegranate Extracts: Pomegranate Juice (PJ) Pomegranate Liquid Extract (POMxl), Pomegranate Polyphenol enriched Powder Extract (POMxp) and Fulvic Acid (FA). Both Pomegranate Extracts and Fulvic Acid had anti-influenza activity. With regard to Pomegranate Extracts, all of the extracts had rapid antiviral activity when combined with influenza virus. The acidity of PJ and POMxl solutions contributed to anti-influenza activity, but this was not a factor with POMxp. Studies using POMxp showed that brief treatment at room

temperature with > 200 μg/ml PPs substantially reduced the infectivity of H1N1, H3N2, and H5N1 influenza viruses. Generally, the loss of infectivity was accompanied by loss of hemagglutinating activity. Electron microscopic examination of influenza particles neutralized by PP treatment identified a coating of amorphous material and some damage to virion integrity. Reassortant H5N1 viruses derived from avian isolates were less affected by PP treatment, indicating that PP susceptibility is modulated by small changes in surface glycoproteins. Our analysis supports the development of pomegranate-derived PPs as natural, rapidly active, broad-spectrum anti-influenza agents. Our finding demonstrates rapid anti-influenza virus activity in Pomegranate PPs and the Fulvic Acid.

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LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
BSA	Bovine Serum Albumin
CRBC	Chicken Red Blood Cells
DNA	Deoxyribonucleic Acid
FA	Fulvic Acid
FBS	Fetal Bovine Serum
FBS	Fetal Bovine Serum,
HA	Hemagglutinin
HBV	Hepatitis B Virus
HCMV	Human Cytomegalovirus
HCV	Hepatitis C Virus
HDL	High-Density Lipoprotein
HIV	Human Immunodeficiency Virus
HRV	Human Rotavirus
HSV	Herpes Simplex Virus
LDL	Low-Density Lipoprotein
MDCK Cells	Madin-Darby Canine Kidney Cells
MEM	Minimum Essential Medium
NA	Neuraminidase
PBS	Phosphate Buffered Saline

PJ	Pomegranate Juice
POMxl	Pomegranate Liquid Extract
POMxp	Pomegranate Powder Extract
PP	Polyphenol
PSA	Prostate-Specific Antigen
RNA	Ribonucleic Acid
RRV	Ross River Virus
RT	Room Temperature
RT	Reverse Transcriptase
SARS	Severe Acute Respiratory Syndrome
vRNA	viral RNA
VSV	Vesicular Stomatitis Virus

CHAPTER I: Introduction

Introduction:

Viruses:

Viruses are obligate intracellular parasites, which contain little more than bundles of gene strands of either RNA or DNA, and may be surrounded by a lipid-containing envelope [Wagner and Hewlett, 1999]. Unlike bacterial cells, which are free-living entities, viruses utilize the host cell environment to propagate new viruses. They use the reproductive machinery of cells they invade causing ailments as benign as a common wart, as irritating as a cold, or as deadly as what are known as the bloody African fever. The viruses that cause Lassa fever and Ebola fever and the retrovirus that causes acquired immunodeficiency syndrome (AIDS) are examples of viruses that spread easily, kill sometimes swiftly, and for which there is no cure or vaccine [Peter 1994]. Enveloped viruses like HIV, HBV, HCV, influenza and herpes viruses together contribute annually to over a billion infections and significant mortality worldwide**.**

Viruses have numerous invasion strategies. Each strain of virus has its own unique configuration of surface molecules [Wagner and Hewlett, 1999]. These surface molecules work like keys in a lock, enabling viruses to enter into hosts by precisely fitting the molecules on their surfaces to those on the membranes of target cells. The success of viruses in evolution has been assured by four general attributes: genetic variation, variety in means of transmission, efficient replication within host cells, and the ability to persist in the host [Wagner and Hewlett, 1999]. As a consequence viruses have adapted to all forms of life and have occupied numerous ecological niches resulting in widespread diseases in humans, livestock and plants.

Vaccine:

A vaccine is a biological preparation that improves immunity to a particular disease. A vaccine

typically contains a small amount of an agent that resembles a microorganism. The agent stimulates the body's immune system can more easily recognize and destroy any of these microorganisms that it later encounters. Vaccines can be prophylactic (e.g. to prevent or ameliorate the effects of a future infection by any natural pathogen), or therapeutic (e.g. vaccines against cancer are also being investigated). The term vaccine derives from Edward Jenner's 1796 use of the term cow pox which, when administered to humans, provided them protection against smallpox. Control of viral infections, like any other kind of infection control, can be affected either as a prophylactic measure or therapeutically, in order to control and alleviate a viral infection, which has already been established in the host. Unlike bacterial, fungal and parasitic infections, viruses are not autonomous organisms and therefore, require living cells in which to replicate. Consequently, most of the steps in their replication involve normal cellular metabolic pathways, and this makes it difficult to design a treatment to attack the virion directly, or its replication, without accompanying adverse effects on the infected cells [Wagner and Hewlett, 1999]. We do have vaccine and there have been some remarkable success stories. Smallpox has been eradicated. Polio has been controlled; and many once common childhood viral infections are rare. But Vaccines are not available for all viruses. There are viral infections that are poorly controlled by vaccination; HIV is the example of viral disease that does not have an effective vaccine. And there are the influenza viruses which mutate and change with time and this requires frequent modification of vaccine composition. Genetic diversity and hypermutation contribute to difficulties in developing a vaccine against viruses like HIV and influenza. There are currently no known immune correlates of protection against HIV. This has made the development of a vaccine against HIV that would provide sterilizing immunity in the near future an impossible

task. The abandonment of a recent AIDS vaccine human trial due to a failure to elicit a protective sterilizing immune response confirms that empirical attempts to develop a vaccine may result in failures. Also the difficulty in predicting the next pandemic strain of influenza may make it difficult to respond rapidly should there be an outbreak. And there are emerging viruses, which appear from nowhere and these viruses cause problems before we have a chance to develop and test vaccines. The example would be the emergence of the SARS Corona virus. Another example is the avian influenza viruses like H5N1 jumping directly from birds to humans. Common cold caused by a very large number of different viruses and it is impossible develop vaccines to all of them.

Antiviral Drugs:

Antiviral drugs are a class of medication used specifically for treating viral infections. Like antibiotics for bacteria, specific antivirals are used for specific viruses. Most of the antivirals now available are designed to help deal with HIV, herpes viruses, the hepatitis B and C viruses, which can cause liver cancer, and influenza A and B viruses. Designing safe and effective antiviral drugs is difficult, because viruses use the host's cells to replicate. This makes it difficult to find targets for the drug that would interfere with the virus without also harming the host organism's cells. The emergence of antivirals is the product of a greatly expanded knowledge of the genetic and molecular function of organisms, allowing biomedical researchers to understand the structure and function of viruses, major advances in the techniques for finding new drugs, and the intense pressure placed on the medical profession to deal with the human immunodeficiency virus (HIV), the cause of the deadly acquired immunodeficiency syndrome

(AIDS) pandemic. Almost all anti-microbials, including anti-virals, are subject to drug resistance as the pathogens mutate over time, becoming less susceptible to the treatment. For instance, influenza viruses rapidly develop resistance to antivirals when they are used extensively.

The ongoing HIV pandemic and the emergence of avian influenza viruses represent enormous challenges to worldwide control by vaccination and the currently available antivirals. These examples emphasize the importance of investigating all sources for potential antiviral drugs and particularly the largely unexplored resource of natural substances. An excellent outcome would be the identification of materials that are active against a range of different viruses, in contrast to vaccines and some antiviral drugs that target specific viral types.

Influenza Virus:

The first influenza virus to be isolated was from poultry, when in 1901 the agent causing a disease called “fowl plague" was shown to be able to pass through a Chamberland filter, which has pores that are too small for bacteria to pass through. [Heinen, 2003]. The etiological cause of influenza, the Orthomyxoviridae family of viruses, was first discovered in pigs by Richard Shope in 1931 [Shimizu, 1997] .This discovery was shortly followed by the isolation of the virus from humans by a group headed by Patrick Laidlaw at the Medical Research Council of the United Kingdom in 1933. [Laidlaw et al., 1933] However, it was not until Wendell Stanley first crystallized tobacco mosaic virus in 1935 that the non-cellular nature of viruses was appreciated.

Classification of influenza virus:

In virus classification the influenza virus is an RNA virus of the family Orthomyxoviridae

[Kawaoka, 2006], which comprises five genera - Influenza virus A, Influenza virus B, Influenza virus C, Isa virus and Thogotovirus

Influenza virus A

This genus has one species, influenza A virus. Wild aquatic birds are the natural hosts for a large variety of influenza A. Occasionally; viruses are transmitted to other species and may then cause devastating outbreaks in domestic poultry or give rise to human influenza pandemics [Klenk et al., 2008]. The type A viruses are the most virulent human pathogens among the three influenza types and cause the most severe disease. The influenza A virus can be subdivided into different serotypes based on the antibody response to these. Influenza A viruses are classified into subtypes based on antibody responses to HA and NA. These different types of HA and NA form the basis of the H and N distinctions in, for example, H5N1. [Hilleman, 2002] There are 16 H and 9 N subtypes known.

Influenza virus B

This genus has one species, influenza B virus. Influenza B almost exclusively infects humans [Hay et al., 2001] and is less common than influenza A. The only other animals known to be susceptible to influenza B infection are the seal [Osterhaus et al., 2000] and the ferret [Clive Sweet et al., 1994]. This type of influenza mutates at a rate 2–3 times lower than type A [Nobusawa and Sato,2006] and consequently is less genetically diverse, with only one influenza B serotype [Hay et al., 2001].

Influenza virus C

This genus has one species, influenza C virus, which infects humans, dogs and pigs, sometimes causing both severe illness and local epidemics [Matsuzaki et al, 2002] [Taubenberger & Morens, 2008]. However, influenza C is less common than the other types and usually seems to cause mild disease in children [Matsuzaki et al, 2006][Homma et al., 1983].

Structure and Properties of the virus:

Influenza viruses A, B and C are very similar in overall structure. [International Committee on Taxonomy of Viruses, 2006]. The virus particle is 80–120 nanometers in diameter and usually roughly spherical. The viral particles of all influenza viruses are similar in composition [Palese and Bouvier, 2008]. These are made of a viral envelope containing two main types of glycoproteins, wrapped around a central core. The central core contains the viral RNA genome and other viral proteins that package and protect this RNA.Its genome is not a single piece of nucleic acid; instead, it contains seven or eight pieces of segmented negative-sense RNA, each piece of RNA contains either one or two genes [Palese and Bouvier, 2008] . For example, the influenza A genome contains 11 genes on eight pieces of RNA, encoding for 11 proteins: hemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), M1, M2, NS1, NS2 (NEP), PA, PB1, PB1-F2 and PB2 [Salzberg et al, 2005] . Hemagglutinin (HA) and neuraminidase (NA) are the two large glycoproteins on the outside of the viral particles. HA is a lectin that mediates binding of the virus to target cells and entry of the viral genome into the target cell, while NA is involved in the release of progeny virus from infected cells, by cleaving sugars that bind the mature viral particles [Suzuki, 2005].Thus, these proteins are targets for antiviral drugs [Wilson and von Itzstein, 2003]. Furthermore, they are antigens to which antibodies can be raised.

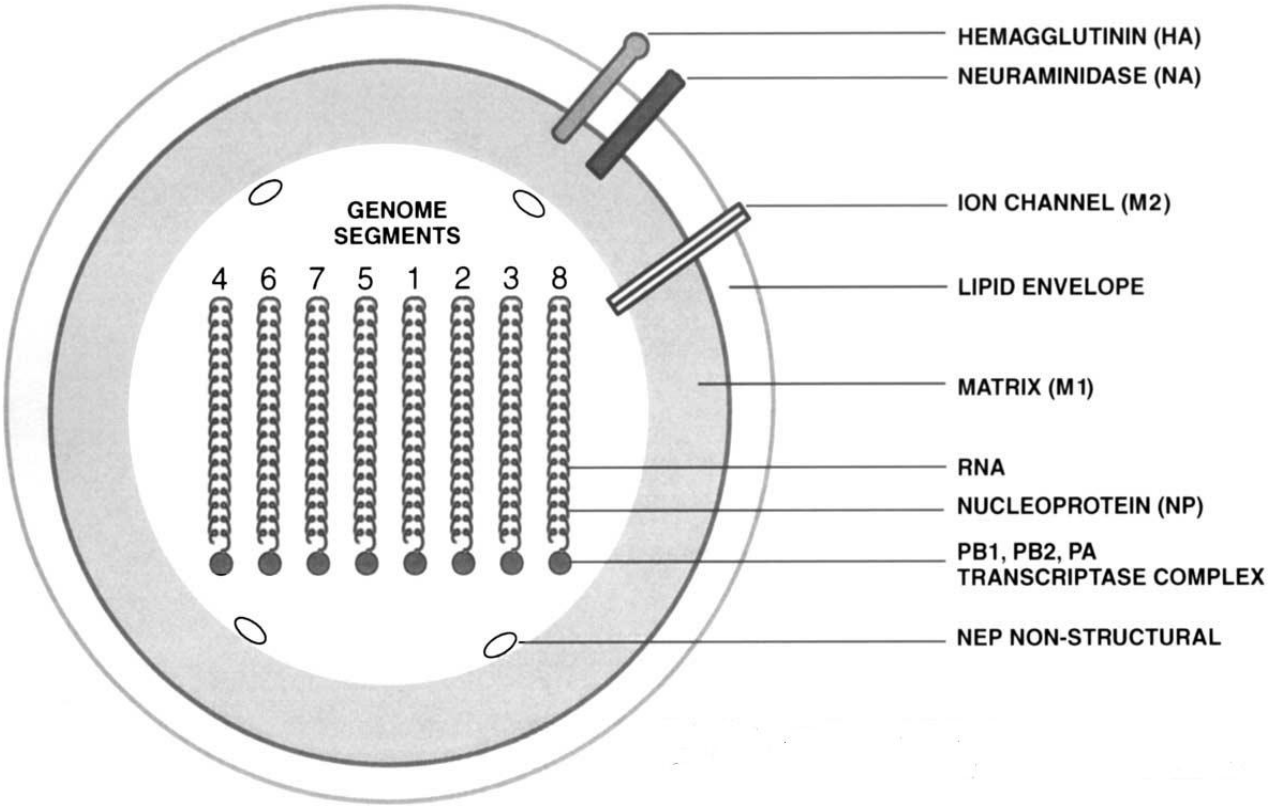

Figure 1: Structure of Influenza Virus [Hilleman, 2002].

Replication:

Viruses can only replicate in living cells [Helenius and Smith, 2004]. Influenza infection and replication is a multi-step process: firstly the virus has to bind to and enter the cell, then deliver its genome to a site where it can produce new copies of viral proteins and RNA, assemble these components into new viral particles and finally exit the host cell [Palese and Bouvier, 2008].

Viruses bind through hemagglutinin onto sialic acid sugars on the surfaces of epithelial cells; typically in the nose, throat and lungs of mammals and intestines of birds [Klenk et al., 2002].

After the hemagglutinin is cleaved by a protease, the cell imports the virus by endocytosis [Steinhauer, 1999].Once inside the cell, the acidic conditions in the endosome cause two events to happen: first part of the hemagglutinin protein fuses the viral envelope with the vacuole's membrane, then the M2 ion channel allows protons to move through the viral envelope and acidify the core of the virus, which causes the core to dissemble and release the viral RNA and core proteins [Palese and Bouvier,2008].

The viral RNA (vRNA) molecules, accessory proteins and RNA-dependent RNA polymerase are then released into the cytoplasm These core proteins and vRNA form a complex that is transported into the cell nucleus, where the RNA-dependent RNA polymerase begins transcribing complementary positive-sense vRNA [Palese and Cros, 2003]. The vRNA is both exported into the cytoplasm and translated, or remains in the nucleus.

Newly synthesised viral proteins are either secreted through the Golgi apparatus onto the cell surface or transported back into the nucleus to bind vRNA and form new viral genome particles.Hemagglutinin and neuraminidase molecules cluster into a bulge in the cell membrane. The vRNA and viral core proteins leave the nucleus and enter this membrane protrusion. The mature virus buds off from the cell in a sphere of host phospholipid membrane, acquiring hemagglutinin and neuraminidase with this membrane coat [Nayak et al., 2004].

As before, the viruses adhere to the cell through hemagglutinin; the mature viruses detach once their neuraminidase has cleaved sialic acid residues from the host cell [Klenk et al., 2002].

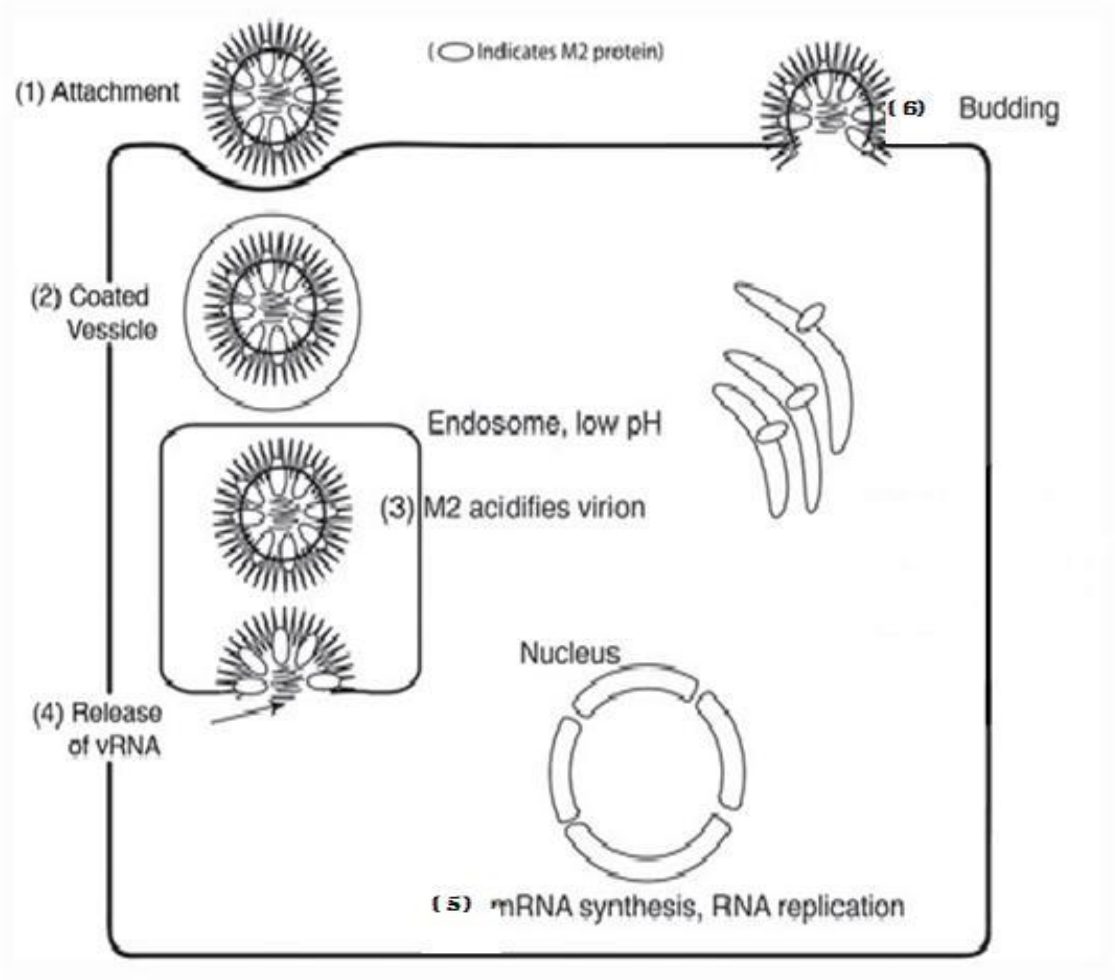

Figure 2: Influenza Virus Replication [Pinto, 2006].

Antigenic Changes:

Antigenic Drift:

Antigenic drift is the gradual evolution of viral strains, due to frequent mutations [Both et al., 1983]. It occurs on average every 2–8 years in response to selection pressure to evade human immunity [Smith et al., 2004; Plotkin et al., 2002; Koelle et al, 2006]. The process of antigenic drift is subtle, involving point mutations within antibody-binding sites in the HA protein, the NA protein, or both, which potentially occur each time the virus replicates [Finkenstadt et al., 2005; Koelle et al., 2006; Palese et al., 1991; Boni et al., 2004].

Most of these mutations are neutral as they do not affect the conformation of the proteins; however, some mutations cause changes to the viral proteins such that the binding of host antibodies is affected. Consequently, infecting viruses can no longer be inhibited effectively by host antibodies raised to previously circulating strains, allowing the virus to spread more rapidly among the population [Webster and Webby, 2001]. Antigenic drift occurs in all strains of A and B viruses, although the observed evolutionary patterns vary dependent on the strain [Carrat and Flahault, 2007].

Antigenic Shift:

Antigenic shift is only seen in influenza A viruses, and results from the replacement of HA (and less frequently NA) subtypes with novel ones [Cox and Subbarao, 2000]. This results in new viruses that have never been present in human circulation or last circulated decades before.

These can have a significant impact on disease burden, causing pandemics or worldwide

epidemics and resulting in hundreds of thousands, or possibly millions, of influenza-related deaths [Treanor, 2004]. Antigenic shift is estimated to occur approximately three times every 100 years [Potter, 2001], which is in line with the three antigenic shifts (and resulting pandemics) that occurred during the 20th century (1918, 1957 and 1968).

An important process that contributes to major shifts in influenza antigenicity is genetic reassortment (mixing of genetic material between different viral strains) which occurs due to co-circulation of different influenza A subtypes, and influenza B viruses. Although genetic reassortment can contribute to antigenic drift [Hay et al., 2004], it is primarily responsible for antigenic shift [Holmes, Ghedin, Miller, Taylor, Bao, et al., 2005; Holmes and Nelson, 2007]. Genetic reassortment is of particular importance in the evolution of A/H3N2 viruses [Schweiger et al., 2006], which emphasizes the need for comprehensive analysis of influenza viruses, particularly when considering the annual vaccine composition.

Genetic reassortment is also possible between co-infecting influenza A subtypes from different species, a process that has the potential to create new subtypes with substantial antigenic changes that can result in an influenza pandemic.

Thus, it is feasible that reassortment between human and avian virus strains will produce a virulent strain. Once a virus has undergone antigenic shift, it remains susceptible to antigenic drift, as occurs with any influenza virus. In fact, all current circulating influenza viruses are drift variants of previously pandemic influenza strains [Carrat and Flahault, 2007].

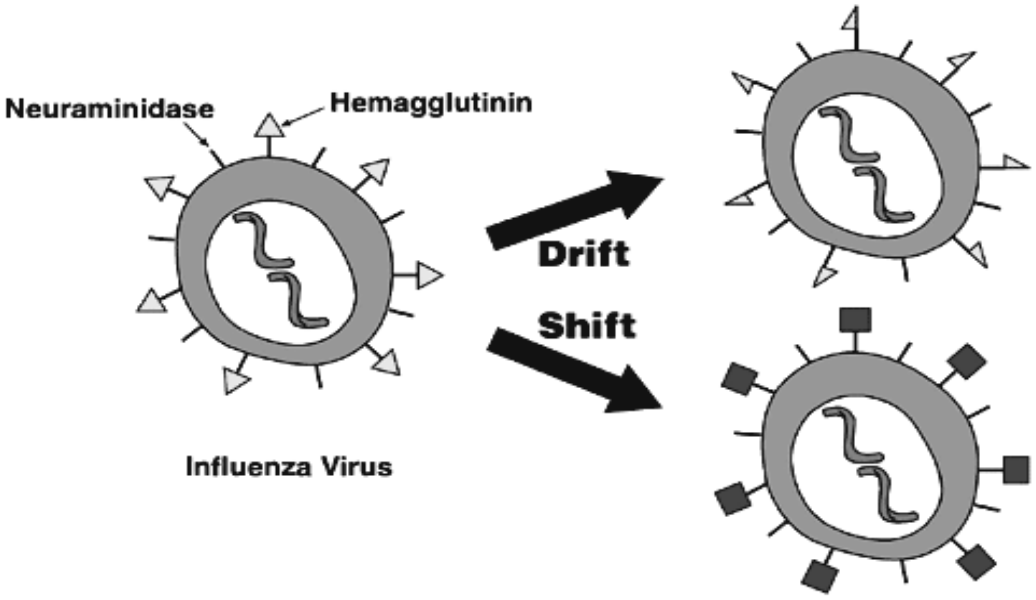

Figure 3: Influenza Virus Antigenic Changes

Influenza/Flu:

Influenza is a highly contagious, acute, febrile, respiratory disease attacking nose throat and lungs, which has been in circulation for centuries. The disease is caused by the influenza virus; Influenza has a high incidence in human populations and causes regular, large-scale morbidity and mortality. During seasonal epidemics, 5–15% of the worldwide population is typically infected, resulting in 3–5 million cases of severe illness and up to 500,000 deaths per year [WHO, 2003]. While all age groups are affected by the disease, most influenza-related hospitalization in industrialized countries occur in young children (<5 years of age) and in the elderly (≥65 years of age) [Advisory Committee on Immunization Practices Prevention and Control of Influenza, 2006] and most deaths occur among the elderly (≥65 years of age) [Kendal and Lui, 1987]. Seasonal flu occurs because the virus gradually changes as it spreads through a

population. As a result, our immunity to the virus becomes less and we become susceptible to infection again. Once every 10 – 50 years, a completely new influenza virus spreads to the human population. Humans have no immunity and the virus spreads worldwide causing a pandemic. The disease can be very severe and there can be more than one million deaths, the worst influenza pandemic occurred in 1918 and killed 40 – 50 million people. Taking into account work absenteeism as well as direct medical costs, the annual economic impact of influenza in the US has been estimated to be as high as US $12–14 billion [Monto, 2000]. The human population is currently contending with the most recent influenza pandemic, caused by an H1N1 virus that apparently spread to humans from a swine reservoir [Garten et al., 2009]. Although this does not represent the introduction of a novel subtype into the human population, the newly emergent H1N1 virus carries surface glycoproteins (perhaps particularly the H1) that are substantially different from those of recently circulating H1N1 viruses. Consequently, a high proportion of humans have little immunity to the new virus. Thus, influenza continues to have a major worldwide impact, resulting in significant human suffering and economic burden.

Transmission:

People who contract influenza are most infective between the second and third days after infection and infectivity lasts for around ten days [Carrat et al., 2006]. Influenza can be spread in three main ways: by direct transmission when an infected person sneezes mucus into the eyes, nose or mouth of another person; through people inhaling the aerosols produced by infected people coughing, sneezing and spitting; and through hand-to-mouth transmission from either contaminated surfaces or direct personal contact, such as a hand-shake[Weber and Stilianakis,

2008][Hall, 2007] and they may all contribute to the spread of the virus [Tellier , 2006][Gardam et al., 2007]. In the airborne route, the droplets that are small enough for people to inhale are 0.5 to 5 µm in diameter and inhaling just one droplet might be enough to cause an infection [Weber and Stilianakis, 2008]. Although a single sneeze releases up to 40,000 droplets, [Cole and Cook, 1998] most of these droplets are quite large and will quickly settle out of the air [Weber and Stilianakis, 2008].

Symptoms:

It can be difficult to distinguish between the common cold and influenza in the early stages of these infections [Eccles, 2005], but flu can be identified by a high fever with a sudden onset and extreme fatigue. Symptoms of influenza can start quite suddenly one to two days after infection. Usually the first symptoms are chills or a chilly sensation, but fever is also common early in the infection, with body temperatures ranging from 38-39 °C (approximately 100-103 °F) [Suzuki et al., 2007].Many people are so ill that they are confined to bed for several days, with aches and pains throughout their bodies, which are worse in their backs and legs. Symptoms of influenza may include: body aches, especially joints and throat, extreme coldness and fever, fatigue, headache, irritated watering eyes, reddened eyes, skin (especially face), mouth, throat and nose

Prophylaxis**:**

Vaccination:

Vaccination against influenza with an influenza vaccine is often recommended for high-risk groups, such as children and the elderly, or in people who have asthma, diabetes, or heart disease. The effectiveness of these influenza vaccines is variable. Due to the high mutation rate

of the virus, a particular influenza vaccine usually confers protection for no more than a few years. Every year, the World Health Organization predicts which strains of the virus are most likely to be circulating in the next year, allowing pharmaceutical companies to develop vaccines that will provide the best immunity against these strains [WHO, 2006]. It is possible to get vaccinated and still get influenza. The vaccine is reformulated each season for a few specific flu strains but cannot possibly include all the strains actively infecting people in the world for that season. It takes about six months for the manufacturers to formulate and produce the millions of doses required to deal with the seasonal epidemics; occasionally, a new or overlooked strain becomes prominent during that time and infects people although they have been vaccinated (as by the H3N2 Fujian flu in the 2003–2004 flu season) [Holmes, Ghedin, Miller, Taylor, Bao, et al., 2005]. It is also possible to get infected just before vaccination and get sick with the very strain that the vaccine is supposed to prevent, as the vaccine takes about two weeks to become effective[CDC publication, 2006] .

Treatment:

Anti Viral Drugs:

The two classes of antiviral drugs used against influenza are neuraminidase inhibitors and M2 protein inhibitors (adamantine derivatives).

Neuraminidase inhibitors

Antiviral drugs such as oseltamivir (trade name Tamiflu) and zanamivir (trade name Relenza) are neuraminidase inhibitors that are designed to halt the spread of the virus in the body

[Moscona, 2005].These drugs are often effective against both influenza A and B [Stephenson and Nicholson, 1999].

M2 inhibitors (adamantanes)

The antiviral drugs amantadine and rimantadine block a viral ion channel (M2 protein) and prevent the virus from infecting cells [Pinto and Lamb, 2006]. These drugs are sometimes effective against influenza A if given early in the infection but are always ineffective against influenza B because B viruses do not possess M2 molecules [Stephenson and Nicholson, 1999].

Influenza is a highly communicable acute respiratory disease, predisposes to a number of complications, resulting in a severe worldwide economic burden. Even though, prevention and control of both the annual influenza epidemics and its infrequent but severe pandemic outbreaks are achieved by the use of vaccines and newly emerging antiviral drugs, these vaccines provide sometimes lower than desirable protection, particularly in the immunocompromised and the elderly, the two most susceptible subpopulations [Keren et al., 1988; Admon et al., 1997]. Vaccines are generally unavailable in the early stages of a pandemic [WHO, 1999] and antiviral drugs are the only means of intervention. But these antiviral drugs are only 70 – 90 % effective and may shorten the duration of illness by 1.5 days when used within the first 48 hours [Treanor and Falsey, 1999; Hayden et al., 1999]. Also these antiviral drugs cause side effects in the hosts. So it is time to explore broad spectrum agents that can target either the lipid portion of the envelope or the sugar moieties of the glycoproteins or the rafts (regions within viral and cell envelopes where a higher concentration of the glycoproteins exist). Broad spectrum agents that can serve as disrafters or neutralize the viral infectivity by binding to the envelope lipid or sugar

moieties will not be affected by the vagaries of hypermutation of surface antigens. This is because the post-translation modification is a host function. The side effects caused by the antiviral drugs in the infected individuals (lactic acidosis, peripheral neuropathy and adipose deposition) are difficult to manage and there is an urgent need for alternate prophylactic and treatment approaches that can be easily delivered, are nontoxic, and are unaffected by the rapid mutation rates of the virus. Shikimic acid, one of the main starting materials of Tamiflu is obtained from star anise, a cooking spice from a tree grown in China but other sources of shikimic acid are being explored and fairly high concentrations of it are found in natural sources, e.g. pine, spruce and fir trees [Kotwal, 2008]. In a recent article, additional problems related to the use of shikimic acid, developments and the problems toward the synthesis of Tamiflu and the use of other starting materials have been discussed[Farina and Brown,2006].Complex molecules like lectins and mucins which can interfere with the attachment of enveloped viruses also seem to have a broad spectrum antiviral activity against HIV and poxviruses [Mall et al.,2006; Habte et al., 2007; Kaur et al.,2007]. These agents can be predicted to be effective against influenza but that remains to be proved and they may not be too practical because of their molecular size and due to interference of normal tissue functions, other than as possible additives to microbicides. Therefore a wonderful outcome would be the identification of materials, particularly the largely unexplored resource of natural substances that are active against a range of different viruses, in contrast to vaccines and some antiviral drugs that target specific viral types.

Antiviral Activity of Natural Substances:

Recently, both Pomegranate Juice and Fulvic Acid have been shown to inactivate genetically diverse strains of influenza including H5N1, further confirming the broad spectrum nature of

these agents [Kotwal, 2008].A number of studies have demonstrated that polyphenolic complexes derived from other plants have antiviral effects against DNA and RNA viruses [Serkedjieva and Manolova, 1992; Nakayama et al., 1993; Weiss et al., 2005; Ehrhardt et al., 2007; Schnitzler et al., 2008], suggesting that antiviral activity in pomegranates may also reside in the PP fraction. It is clear from some studies that plant PPs can exert an antiviral effect by interacting directly with viral particles [Nakayama et al., 1993; Song et al., 2005; Ehrhardt et al., 2007; Schnitzler et al., 2008], although the extent of PP binding to viral surface components may be influenced by the nature of the virus [Serkedjieva, 2003; Ehrhardt et al., 2007]. Plant PPs may also exert antiviral effects during intracellular replication [Palamara et al., 2005]. In part, this may be due to PPs opposing the pro-oxidant state induced in cells by the replication of some viruses [Fraternale et al., 2009].Also studies have shown that cranberry juice contains high molecular weight materials (NDM) that inhibit bacterial adhesion to host cells as well as the co-aggregation of many oral bacteria. Because of its broad-spectrum activity, researchers investigated NDM's potential for inhibiting influenza virus adhesion to cells, and subsequent infectivity. Their findings have indicated that the inhibitory effect of NDM on influenza virus adhesion and infectivity may have a therapeutic potential [Weiss et al., 2005].

Many traditional medicinal plants have been reported to have strong antiviral activity and some of them have already been used to treat animals and people who suffer from viral infection [Hudson, 1990; Venkateswaran et al., 1987; Thyagarajan et al., 1988, 1990]. Research interests for antiviral agent development was started after the Second World War in Europe and in 1952 the Boots drug company at Nottingham, England, examined the action of 288 plants against influenza A virus in embryonated eggs. They found that 12 of them suppressed virus

amplification [Chantrill et al., 1952]. Canadian researchers in the 1970s reported antiviral activities against herpes simplex virus (HSV), poliovirus type 1, coxsackievirus B5 and echovirus 7 from grape, apple, strawberry and other fruit juices [Konowalchuk and Speirs, 1976a,b, 1978a,b]. One hundred British Columbian medicinal plants were screened for antiviral activity against seven viruses [McCutcheon et al., 1995]; twelve extracts were found to have antiviral activity at the concentrations tested. The extracts of *Rosa nutkana* and *Amelanchier alnifolia* were very active against an enteric corona virus. A root extract of *Potentilla arguta* and a branch tip extract of *Sambucus racemosa* completely inhibited respiratory syncytial virus (RSV). An extract of *Ipomopsis aggregata* demonstrated good activity against parainfluenza virus type 3.

In addition to these, extracts prepared from *Cardamine angulata*, *Conocephalum conicum*, *Lysichiton americanum*, *Polypodium glycyrrhiza* and *Verbascum Thapsus* exhibited antiviral activity against herpes virus type 1. The extracts of 40 different plant species have been used in traditional medicine and were investigated for antiviral activity against a DNA virus, human cytomegalovirus (HCMV), and two RNA viruses, Ross River virus (RRV) and poliovirus type 1, at noncytotoxic concentrations [Semple et al., 1998]. The most active extracts were the aerial parts of *Pterocaulon sphacelatum* (Asteraceae) and roots of *Dianella longifolia* var. grandis (Liliaceae), which inhibited poliovirus type 1 at concentration of 52 and 250 $\mu g\ ml^{-1}$, respectively. The same authors concluded that the extracts of *Euphorbia australis* (Euphorbiaceae) and *Scaevola spinescens* (Goodeniaceae) were the most active against HCMV whilst, extracts of *Eremophila latrobei* subsp. glabra (Myoporaceae) and *Pittosporum phylliraeoides* var. microcarpa (Pittosporaceae) exhibited antiviral activity against RRV.The

human rotavirus (HRV), RSV and influenza A virus were susceptible to a liquid extract from *Eleutherococcus senticosus* roots. In contrast, the DNA viruses, adenovirus and HSV type 1 virus (HSV-1) were not inhibited by the same plant extract [Glatthaar-Saalmuller et al., 2001]. They concluded that the antiviral activity of *Eleutherococcus senticosus* extract is viral RNA dependant. Related studies also showed that influenza RNA was inhibited by a water-soluble extract of *Sanicula europaea* (L.) [Turan et al., 1996]. In a later study [Karagoz et al., 1999], it was shown that an acidic fraction obtained from the crude extract of *Sanicula europaea* was the most active fraction in inhibiting human parainfluenza virus type 2 replication at noncytotoxic concentrations.

Another example, *Myrcianthes cisplatensis* showed in vitro anti-RSV but not anti-HSV-1 or anti-adenovirus serotype 7 (DNA virus) [Kott et al., 1999]. In contrast, other medicinal plants, for example *Nepeta coerulea*, *Nepeta nepetella*, *Nepeta tuberosa*, *Sanguisorba minor magnolii* and *Dittrichia viscose* showed clear antiviral activity against DNA and RNA viruses, i.e. HSV-1 and VSV in addition to poliovirus type 1 in the case of *Dittrichia viscose* [Abad et al., 2000]. The *Azadirachta indica* leaf extract was found to be active against a number of viruses such as smallpox (DNA), chicken pox (DNA), poxvirus (DNA), poliomyelitis (RNA) and herpes viruses (DNA) [Rao et al., 1969; Kaii-a-Kamb et al., 1992]. An extract of the cactus plant *Opuntia streptacantha* inhibited intracellular DNA and RNA virus replication and inactivated extracellular virus, such as HSV, equine herpes virus, pseudorabies virus and influenza virus [Ahmad et al., 1996]. The *Bergenia ligulata*, *Nerium indicum* and *Holoptelia integrifolia* plants exhibited considerable antiviral activities against influenza virus (RNA) and HSV (DNA) [Rajbhandari et al., 2001].

The antiviral properties of marine algae have been addressed [Chamorro et al., 1996; Siddhanta et al., 1997; Berge et al., 1999; Nicoletti et al., 1999]. Preclinical testing suggests that *Spirulina*, a unicellular filamentous cyanobacteria (formerly called blue green algae), has several therapeutic attributes such as cholesterol regulation, immunological, antiviral and antimutagenic properties [Chamorro et al., 1996]. Strain-specific antiinfluenza virus inhibitory activity, based on the reproduction of influenza viruses in tissue cultures, was reported for marine algae of the Bulgarian Black Sea coast [Serkedjieva et al., 2000].

Polyphenols and the proanthocyanidins extracted from *Hamamelis virginiana* bark and two new hydrolysable tannins, shephagenins A and B, isolated along with hippophaenin A and strictinin from the leaf extract of *Shepherdia argentea*, showed a remarkable inhibitory activity against HSV-1 [Erdelmeier et al., 1996] and HIV-1 reverse transcriptase (RT) [Yoshida et al., 1996]. The inhibitory effect of the *Shepherdia argentea* leaf extract on HIV-1 RT was found to be caused by tannins, and their activities were stronger than that of (-))epigallocatechin gallate as a positive control [Yoshida et al., 1996].

In an early study of plant viral infection, Cadman [Cadman,1960] , suggested that polyphenolic extracts of the leaf of *Rubus idaeus* (raspberry) probably act against most viruses by clumping the virus particles together into complexes, which are largely noninfective. Hudson [Hudson, 1990], deduced that viral inactivation in vitro is directly attributable to preferential binding of the polyphenol to the protein coat of the virus, whereas, in a systematic study of the antiviral activity of a very wide range of natural products, Van den Berghe et al. [Van den Berghe et al., 1986] concluded that polyphenols act principally by binding to the virus and/or the protein of the host cell membrane and thus arrest absorption of the virus. Sakagami [Sakagami et al., 1995], have

put forward a number of possible mechanisms whereby polyphenols may exert their antiviral action. They suggested that the major part of the antiviral activity in polyphenols probably derives from their direct inactivation of the virus and/or from inhibition of the virus binding to the cells. They also noted that although polyphenols are known to inhibit viral replication enzymes (such as RT for HIV and RNA polymerase for influenza virus) and other enzymes (e.g. poly (ADP-ribose) glycohydrolase), these effects seem to be rather nonspecific. The most pronounced in vitro selectivity of anti-influenza and anti-herpes type 1 and type 2 actions were confirmed against polyphenolic complexes isolated from the Bulgarian medicinal plant *Geranium sanguineum* (L.) [Serkedjieva and Hay, 1998; Serkedjieva and Ivancheva, 1999]. Although polyphenols were shown to have a broad antiviral spectrum in vitro, their corresponding properties in vivo have not been well established [Sakagami et al., 1995].

The bioflavonoids comprise a large family of plantderived polyphenolic compounds of low molecular weight, which exhibit diverse biological activities. The naturally occurring benzo-d pyrone derivatives and phenylchromones are widely distributed throughout the plant kingdom, as components in fruits, vegetables, tea, grains, bark, roots, stems and flowers. Up to several hundred milligrams are consumed daily in the average Western diet. On balance, a considerable body of evidence suggests that plant flavonoids may be health promoting, disease-preventing dietary compounds [Middleton, 1998]. They are the basis of many traditional folk remedies which have many important biochemical effects, some of which have been applied in human therapy, as described in the review by Havsteen [Havsteen,1983], and are now being increasingly used as prototypes for the development of specific drug therapies [Berger et al., 1992].

The antiviral activities of bioflavonoids extracted from medicinal plants have been evaluated [Beladi et al., 1977; Tsuchiya et al., 1985]. The black tea flavonoid, theaflavin is a well-known antioxidant with free radical-scavenging activity and it was able to neutralize bovine rotavirus and bovine corona virus infections [Clark et al., 1998].

The flavonoid chrysosplenol C is one of a group of compounds known to be a potent and specific inhibitor of picornaviruses and rhinoviruses, the most frequent causative agents of the common cold [Semple et al., 1999]. The *Dianella longifolia* and *Pterocaulon sphacelatum* were found to contain flavonoid chrysosplenol C and anthraquinone chrysophanic acid, respectively, which inhibit the replication of poliovirus types 2 and 3 (Picornaviridae) in vitro [Semple et al., 1999, 2001]. Recently, new flavonol glycoside - the iridoid glycosides and three phenylpropanoid glycosides, named luteoside A, luteoside B and luteoside C were isolated from *Barleria prionitis* and from the roots of the medicinal plant *Markhamia lutea*, respectively, and shown to have potent in vitro activity against RSV [Chen et al., 1998; Kernan et al., 1998]. In another study, five groups of biflavonoids (amentoflavone, agathisflavone, robustaflavone, rhusflavanone and succedaneflavanone) were isolated from medicinal plants of *Rhus succedanea* and *Garcinia multiflora*, and exhibited various antiviral effects against a number of viruses including respiratory viruses (influenza A, influenza B, parainfluenza type 3, RSV, adenovirus type 5 and measles) and herpes viruses (HSV-1, HSV-2, HCMV and varicella zoster virus, VZV) [Lin et al., 1999]. Amentoflavone and robustaflavone demonstrated significant activity against anti-HSV-1 and anti-HSV-2 with only moderate anti-HSV- 2 from rhusflavanone. A significant anti influenza A and B activity was achieved by amentoflavone, robustaflavone and agathisflavone. By comparison, rhusflavanone and succedaneflavanone were found to produce a selective anti-

influenza type B only. The inhibitory activities against measles and VZV were demonstrated with rhusflavanone and succedaneflavanone, respectively. In general, none of groups of biflavonoids exhibited anti-HCMV [Lin et al., 1999].

Baicalein (BA), a flavonoid compound purified from the medicinal plant *Scutellaria baicalensis* Georgi, has been shown to possess anti-inflammatory and anti-HIV-1 activities. BA may interfere with the interaction of HIV-1 envelope proteins with chemokine co-receptors and block HIV-1 entry of target CD4 cells and BA could be used as a basis for developing novel anti-HIV-1 agent [Li et al., 2000].Morin is another type of flavonoid group extracted from *Maclura cochinchinensis* that exhibited a powerful anti-HSV-2 activity in contrast with a synthetized morin pentaacetate that was inactive [Bunyapraphatsara et al., 2000]. This would suggest that free hydroxyl groups are required for anti-HSV-activity, as demonstrated previously for the antiviral activity of other flavonoids [Hudson, 1990; Bunyapraphatsara et al., 2000]. Such studies clearly indicate that antiviral activity varies with the compound and the virus.

The mechanisms of binding the flavonoids extracted from medicinal plants received less attention. However, one stage of viral replication that may be inhibited by flavonoids is viral DNA synthesis. For example, SP-303 exhibited strong activity against herpes virus (HSV-1 and HSV-2) [Barnard et al., 1993]. Most of the potent anti-HIV flavonoids such as BA, quercentin and myricetin have shown inhibitory activity not only against the virus-associated RT but also against cellular DNA or RNA polymerase [Ono and Nakane, 1990].The inhibition of DNA and RNA polymerase by these flavonoids was extensively analysed to elucidate the inhibition mechanism(s) by Ono and Nakane [Ono and Nakane, 1990]. Once again the degree of inhibition also varied depending on the flavonoid [Jassim and Naji, 2003].

Pomegranate:

Origin and History:

Punica granatum L., the pomegranate belongs to the Punicacea family [Harde et al., 1970] and is one of the oldest edible fruits. It has been cultivated extensively in Mediterranean countries, Iran, India and to some extent in the U.S. (California), China, Japan and Russia. Iran is a native land of the pomegranate which is grown in every area, both coastal and mountainous areas. Introduced into Latin America and California by spanish settlers in 1769, pomegranate is now cultivated in parts of California and Arizona for juice production.

Climate:

Pomegranates can be grown in tropical to warm temperate climates. However, the best quality pomegranate fruits are produced in regions with cool winters and hot, dry summers. Pomegranates vary in frost tolerance, but in some cases temperatures down to 10°F may not severely injure the plants.

Pomegranate Tree:

Normally a dense, bushy, deciduous shrub, 2-4 m (6-12 ft.) tall, the plant has slender, somewhat thorny branches. It may be trained as a small tree reaching 7 m (20 ft.) in height. Pomegranate is an attractive ornamental.

Pomegranate Leaves:

Pomegranate leaves are glossy, dark green, oblong to oval, 2.5-3 cm (1-1.25 in.) long. Leaves are arranged opposite or nearly so and clustered on short branchlets.

Pomegranate Flowers:

Blooms are a flaming orange-red, 4-6 cm (1.5-2.5 in.) in diameter with crinkled petals and numerous stamens. Flowers are borne solitary or in small clusters angled towards the end of branchlets.

Pomegranate Fruit:

Pomegranates are brownish-yellow to purplish-red berries 5 - 12 cm (2-5 in.) in diameter with a smooth, leathery skin. Fruits are spherical, somewhat flattened, with a persistent calyx. The calyx may be 1-6 cm (1.5-2.5 in.) long. Numerous seeds are each surrounded by a pink to purplish-red, juicy, pulp (arils) which is the edible portion. The pulp is somewhat astringent.

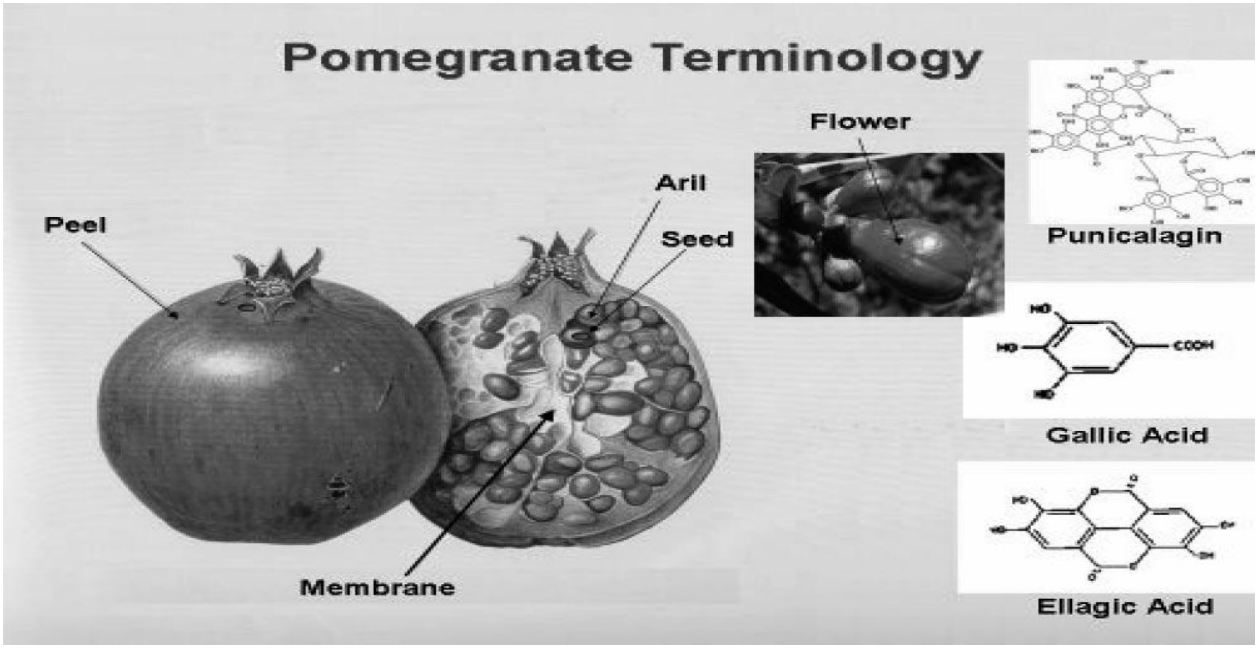

Figure 4: Schematic illustration of Pomegranate fruit parts [Aviram et al., 2008].

Benefits of Pomegranate:

The pomegranate fruit has a long history of traditional use as a folk remedy. Recent research has associated constituents of the fruit with a variety of medical benefits. Cardiovascular benefits, antiatherosclerotic effects, and inhibition of tumor initiation and growth have been attributed to the potent antioxidative activity of pomegranate components [Aviram et al., 2008; de Nigris et al., 2005; Ignarro et al., 2006; Adams et al., 2006; Malik et al., 2005].

Pomegranates contain high levels of polyphenolic compounds; including hydrolysable tannins (mainly punicalagin) and plant flavonoids such as the anthocyanins, which are largely responsible for the fruit's antioxidant properties [Gil et al., 2000; Tzulker et al., 2007].

Pomegranate Juice is a source of potent polyphenolic antioxidants (tannins, anthocyanins) which are considered to be anti-atherogenic. Athererogenesis involves lipoproteins modifications and arterial macrophage foam cell formation.

Invitro studies demonstrated a significant dose-dependent antioxidant capability of PJ against lipid peroxidation in plasma (by up to 33%), in low density lipoprotein (LDL, by up to 43%), and in high density lipoprotein (HDL, by up to 22%). Researchers have analyzed and compared polyphenols content and ability to reduce LDL oxidation of several juices obtained from fruits.

Pomegranate juice (wonderful variety) was found to have 6.1+/- 0.4 mM of total polyphenols in comparison to cranberry juice, red wine, blueberry juice, green tea and orange juice.

On a comparison based on similar total polyphenols content, pomegranate juice was again the

most potent antioxidant against LDL oxidation followed by cranberry juice, red wine, blue berry juice, green tea and orange juice, which were less potent than pomegranate juice by 10%, 13%, 30%, 40% and 70% respectively.

Pomegranate juice is the most potent antioxidant against LDL oxidation and this effect could be related to its high polyphenolic flavonoids content as well as to the specific potent flavonoids present in pomegranate juice such as tannins and anthocyanins. Researchers have demonstrated that a very potent anti-atherogenicity of pomegranate juice consumption which could be associated mainly with PJ hydrolysable tannins anti-oxidative properties [Aviram, 2002].

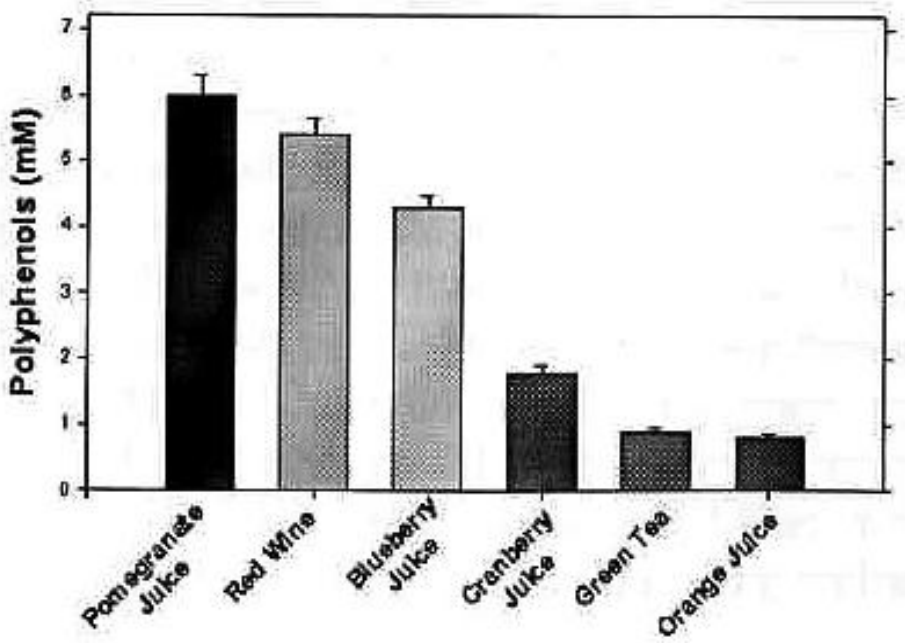

Figure 5: Total Polyphenols Content of Several Juices [Aviram, 2002]

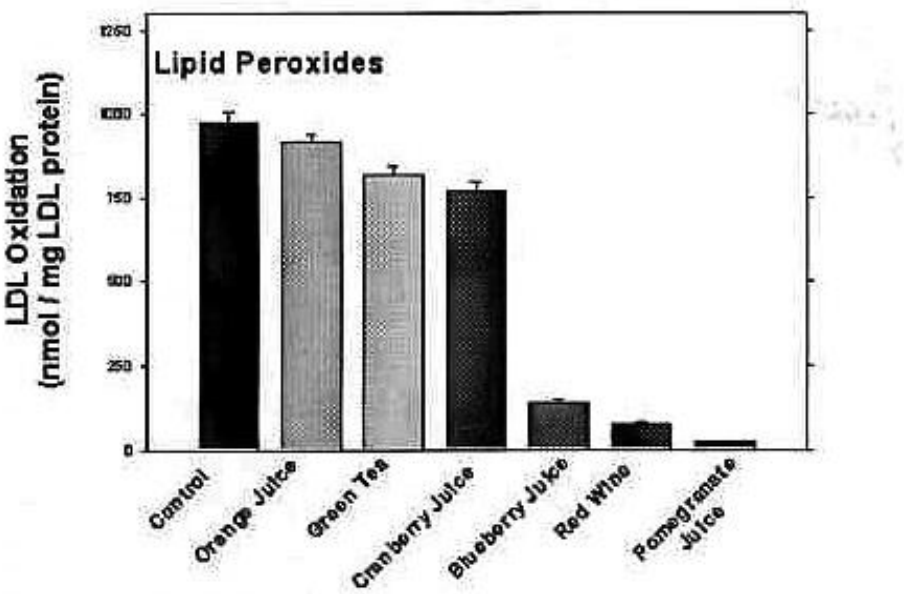

Figure 6: Anti-oxidant Activities of Several Juices against Cholesterol Oxidation [Aviram, 2002]

Pomegranate juice is neuroprotective in an animal model of neonatal hypoxic-ischemic brain injury. In this research paper, they determined whether protection of the neonatal brain against a hypoxic-ischemic insult could be attained through supplementation of the maternal diet with pomegranate juice, notable for its high polyphenol content. Dietary supplementation with pomegranate juice resulted in markedly decreased brain tissue loss (60%) in all three brain regions (hippocampus, cortex and striatum) assessed, with the highest pomegranate juice dose having greatest significance (p _ 0.0001). Pomegranate juice also diminished caspase-3 activation by 84% in the hippocampus and 64% in the cortex. These results demonstrate that maternal dietary supplementation with pomegranate juice is neuroprotective for the neonatal brain. [Holtzman et al., 2005]

Pomegranate juice is effective against prostate cancer. Research has determined the effects of Pomegranate juice consumption on prostate-specific antigen (PSA) progression in men with a rising PSA following primary therapy. A clinical trial for men with rising PSA after surgery or radiotherapy was conducted. Patients were treated with 8 ounces of pomegranate juice daily (wonderful variety, 570 mg total polyphenol gallic acid equivalents) until disease progression. Mean PSA doubling time significantly increased with treatment from a mean of 15 months at baseline to 54 months post treatment .In vitro assays comparing pretreatment and post treatment patient serum showed a12% decrease in cell proliferation and a17% increase in apoptosis, a 23% increase in serum nitric oxide, and significant reductions in oxidative state and sensitivity to oxidation of serum lipids after versus before pomegranate juice consumption [Pantuck et al., 2006].

Pomegranate components also have growth inhibiting activity against diverse microorganisms. Direct antibacterial, antifungal, and antiplasmodial activity was demonstrated for pomegranate polyphenol (PP) fractions [Reddy et al., 2007].

Pomegranate peel extracts contains substantial amounts of polyphenols such as ellagic tannins, ellagic acid and gallic acid. It has been used in the preparation of tinctures, cosmetics, therapeutic formulae and food recipes [Nasr, Ayed, &Metche, 1996].In a study the researchers dried pomegranate peels and they were powdered and extracted in a soxhlet extractor with ethyl acetate (EtOAc), acetone, methanol and water for 4 h each. The dried extracts were used to determine their antioxidant capacity by the formation of phosphomolybdenum complex and antimutagenicity against the mutagenicity of sodium azide by the ames test. All the peel extracts exhibited marked antioxidant capacity, but the water extract was the lowest. All the extracts decreased sodium azide mutagenicity in *Salmonella typhimurium* strains (TA100 and TA1535), either weakly or strongly. At 2500 mg/plate all the extracts showed strong antimutagenicity. The antimutagenicity of the water extract was followed by acetone, EtOAc and methanol extracts. The overall results showed that the pomegranate peel extracts have both antioxidant and antimutagenic properties and may be exploited as biopreservatives in food applications and neutraceuticals [Jena et al., 2002].

Binding by a pomegranate component to the viral envelope glycoprotein of HIV-1 blocked interaction with cell receptors and inhibited infection, but the chemical nature of the active component was not defined [Neurath et al., 2004].

Fulvic Acid:

The decay of organic matter generates an extremely heterogeneous mixture of organic molecules (referred to as humic substances) that are sub-classified on the basis of solubility characteristics. The Fulvic Acid (FA) fraction of humic substances includes a variety of low molecular acidic molecules that are soluble in water under all pH conditions

The hypothetical model structure of Fulvic acid (Buffle's model) contains both aromatic and aliphatic structures, both extensively substituted with oxygen - containing functional groups.

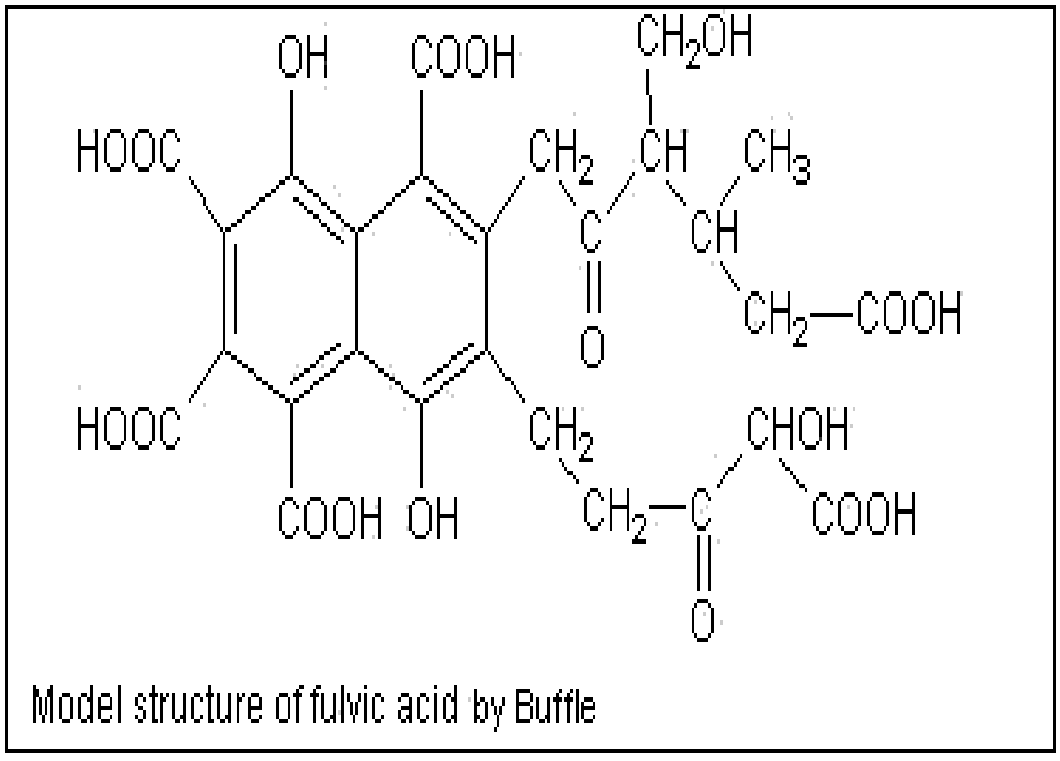

Figure 7: The model Structure of Fulvic Acid [Buffle's model].

Table 1: The elemental composition of humic substances and several plant materials [by Kononova].

Substances	% dry ash-free basis			
	C	H	O	N
Fulvic acids	44 - 49	3,5 - 5,0	44 - 49	2,0 - 4,0
Humic acids	52 - 62	3,0 - 5,5	30 - 33	3,5 - 5,0
Proteins	50 - 55	6,5 - 7,3	19 - 24	15,0 - 19,0
Lignin	62 - 69	5,0 - 6,5	26 - 33	-

Benefits of Fulvic acid:

A formulation called Secomet V has been reported to have a broad spectrum antiviral properties [Kotwal et al., 2006] and was subsequently revealed a year later by Stefan Coetzee from Fulvimed Pvt. Ltd. to have Fulvic Acid as the major active ingredient. A network of researchers have been investigating the antiviral properties of a formulation called Secomet V, whose active ingredient is fulvic acid, which is a complex mixture of compounds arising from decomposing organic matter. Antiviral activity of fulvic acid containing Secomet V against poxviruses and SARS has been demonstrated [Kotwal et al., 2006].

Fulvic acid has anti-inflammatory and antimicrobial effects and lends itself particularly to topical applications, such as for psoriasis and eczema. Research has described the anti-inflammatory properties in a mouse model of contact hypersensitivity as well as in atopic dermal reactions in humans [Snyman et al., 2002; Van Rensburg, Malfeld, and Dekker, 2001; Van Rensburg, Van Straten, and Dekker 2000]. Fulvic acid has been approved as a veterinary treatment for dogs and cats suffering from pyotraumatic dermatitis or eczema. Although it is not yet clear how exactly fulvic acid works, preliminary results indicate that it scavenges free radicals and inhibits specific phagocytic and lymphocytic cellular functions. The people involved in fulvic acid research always appear to have a direct or indirect connection to the now defunct Enerkom. For example, Pfeinsmith Ltd, the overseas company that bought the entire portfolio of Enerkom's intellectual property rights – some 128 patents in total – granted the exploitation of this intellectual property in Africa to the South African company that arranged the sale. This company is now called Secomet. (www.secomet.com). It operates out of the Techno Park in Stellenbosch near Cape Town. Secomet has identified a new source of fulvic acid, based on carbohydrates derived from

plants. The advantage of deriving fulvic acid from what is essentially a food source is that unlike coal it does not contain traces of toxic metals such as magnesium, chrome, or aluminum. These levels may be very low in fulvic acid derived from coal. Secomet markets a variety of extracts based on fulvic acid. It had the anti-microbial efficacy of the plant extracts it uses tested in vitro by the School of Child & Adolescent Health at the University of Cape Town. The lab reported growth inhibitions of 16 bacterial organisms to each of the four plant extracts tested, and recommended further research into the active component [www.secomet.com]. In addition, in 2005 Secomet contracted ViroLogic, a lab in California, to evaluate its product against ten strains of HIV in vitro. In vitro work undertaken at the Department of Medical Virology at the University of Cape Town also confirmed antiretroviral activity.

A possible antiviral effect of FA was raised by early studies demonstrating the inhibition of bacteriophage adsorption by FA [Bixby and O'Brien, 1979]. However, the potential of FA as an antiviral has received little attention, perhaps because of the difficulty in isolating standardized FA preparations from natural materials. More recently, high quality FA generated from defined starting materials by controlled oxidative processes [Bergh et al.,1997] has been shown to have broad in vitro antimicrobial activity[Van Rensburg, Van Straten, and Dekker, 2000], as well as anti-inflammatory activity following topical application[Van Rensburg, Malfeld, and Dekker, 2001]. Coal-derived humic acids and oxyfulvic acid, which are complex mixtures formed during the decomposition of organic matter including cellulose found naturally have been previously shown to have anti-HIV properties [Van Rensburg, Dekker, Weis, Smith T-L, Rensburg E.J and Schneider, 2002] and [Van Rensburg, Van Straten, and J. Dekker, 2000].

Our work with pomegranates developed from a small project in the laboratory when we were evaluating the in vitro antiviral activity of plant decomposition product - Fulvic Acid. We first included pomegranate juice in these experiments, and we were surprised to find that the juice had anti-influenza virus activity. This stimulated our interest in pomegranates and we evaluated the direct anti-influenza virus activity of three commercially available Pomegranate Extracts: Pomegranate Juice (PJ), Pomegranate Liquid Extract (POMxl), and a Pomegranate Polyphenol Powder Extract (POMxp). Extracts were prepared from California-grown and processed wonderful variety pomegranates and supplied by POM Wonderful (Los Angeles, CA).

Objectives of this Study:

1. The current study was undertaken to evaluate the direct anti-influenza virus activity of pomegranate constituents present in three commercially available Pomegranate Extracts: Pomegranate Juice (PJ), Pomegranate Liquid Extract (POMxl), and a Pomegranate Polyphenol Powder Extract (POMxp).

2. We also evaluated the anti-influenza virus activity of Fulvic Acid. We used the formulation called Secomet V which was supplied by Dr.Girish.J. Kotwal. Even though we included FA in our analysis, our main focus was on Pomegranate Extracts.

CHAPTER II Anti-Influenza Virus activity of Pomegranate Juice

CHAPTER II Anti-Influenza Virus activity of Pomegranate Juice

Abstract:

Pomegranates are one of the richest sources of plant polyphenols (PPs). Since antiviral activity has been attributed to plant PPs, we evaluated the anti-influenza activity of Pomegranate Juice (PJ).The current study was undertaken to evaluate the direct anti-influenza virus activity of pomegranate constituents present in commercially available pomegranate juice. A standard dose of infectious virus was incubated with the different concentration of pomegranate juice. Viral infectivity was measured by titration in susceptible MDCK cells, and hemagglutinating activity was determined using chicken red blood cells. Pomegranate juice had anti-influenza virus activity that was distinct from the effect of the acidic pH of the test materials. Our findings demonstrate rapid anti-influenza virus activity in pomegranate juice.

1. Introduction:

The high level of diverse polyphenolic compounds in pomegranates suggests that the fruit may be a rich source of molecules that exert antiviral activity via multiple mechanisms. Recently, evidence was presented that pomegranate PPs are directly virucidal for influenza viruses and also act at the intracellular level to inhibit influenza virus replication [Haidari et al., 2009]. In the current study, we investigated the direct anti-influenza virus activity of pomegranate juice. We demonstrate that components of the PP fraction of pomegranate juice rapidly inactivate influenza virus. Our findings suggest that inactivation is a consequence of PP interactions with the viral particles.

2. Materials and Methods:

2.1. Pomegranate Juice:

Pomegranate juice was prepared from California-grown and processed wonderful variety pomegranates and supplied by POM Wonderful (Los Angeles, CA).

Preparation of Wonderful Pomegranate Juice (PJ):

Pomegranate juice was prepared from the whole fruit that was cut to expose arils during the squeezing process. The juice was filtered, pasteurized, concentrated to 65 Brix and stored at -18 °C. Then, it was diluted 1:4 (v/v) to 16 Brix with water to obtain the single strength PJ which was used in the study [Aviram et al., 2008].

Chemical Composition of Pomegranate Juice:

Total phenolics (GAE), 3600 μg/mL (0.36%), including mainly hydrolyzable tannins (ellagitannins), such as oligomers and punicalagin / punicalin, with smaller amounts of ellagic acid and anthocyanins (delphinidin, cyaniding, and pelargonidin) and their glycosides [Aviram et al., 2008].

2.2 Chicken Red Blood Cells:

This reagent was stored in fridge. The 0.5% Chicken Red Blood Cells were prepared fresh in PBS each time we did the assay for hemagglutinating activity and stored it in ice until use.

2.3 Phosphate buffered saline (PBS):

We used PBS for diluting samples and Chicken Red Blood Cells

2.4 Citrate/Phosphate buffer:

These buffers were prepared matching the pH of test solutions.

2.5. Viruses and viral titration

Experiments were performed using the following influenza A viruses:

A/Puerto Rico/8/34 (H1N1) (PR8) and the reassortant virus A/HKx31 (H3N2) (x31), which expressed the H3 and N2 of A/Aichi/2/68 on the PR8 background [Kilbourne et al., 1971]. Influenza virus stocks were grown in the allantoic cavity of embryonated hen's eggs and titers of infectious virus were measured by 50% tissue culture infective dose ($TCID_{50}$) titration. Madin Darby canine kidney (MDCK) cells for viral titration were grown in MEM with L-glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), and gentamicin (10 μg/ml), and supplemented with 10% FBS.

Confluent MDCK cell monolayers in 96-well tissue culture plates were washed once with serum-free MEM before use. Serial 10-fold dilutions of virus in serum-free MEM containing 0.3% BSA and 1 μg/ml L-(toslyamido 2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin (Worthington, Lakewood, NJ) were incubated in replicate wells (200 μl/well) for 2-3 d at 37°C with 5% CO_2. Wells positive for virus growth were identified by the presence of hemagglutinating (HAg) activity in the supernatant, and 50% tissue culture infective dose ($TCID_{50}$) titers were calculated by the method of Reed and Muench.

2.6. Treatment of virus with Pomegranate Juice and evaluation of antiviral activity:

Confluent monolayers of MDCK cells for viral titration were grown in 96-well tissue culture

plates and were washed once with serum-free MEM before use. MEM containing 0.3% bovine serum albumin (Sigma) and 1 μg/ml L-(toslyamido 2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin [Worthington, Lakewood, NJ] was added to the wells and used for dilution of treated virus. Influenza virus at 5 x 10^7 $TCID_{50}$/ml was combined with different concentrations of PJ diluted in PBS, and incubated for 5 min at room temperature.

Immediately following incubation, treated virus was serially diluted 10-fold in triplicate wells containing MDCK cell monolayers, and plates were incubated for 2 days at 37°C with 5% CO2. Wells positive for virus growth were identified by the presence of hemagglutinating activity in the supernatant by using a fresh 96-well round bottom plate. We transferred 50μl of supernatant from the original plate to the fresh 96-well round bottom plate, carefully not disrupting the MDCK monolayer at the bottom of the wells, from the top row to the bottom row of the plate. Added the 50μl of 0.5%CRBC to all the wells.

The plates were briefly agitated with hand and set the plate on the bench for 30 minutes to one hour at room temperature. After the incubation, wells positive for virus growth were identified by the presence of hemagglutinating activity. In the absence of hemagglutinating activity, red blood cells roll to the bottom of the wells and form small round pellets, whereas no pellets are visible when the cells are agglutinated and 50% tissue culture infective dose (TCID50) titers were calculated by the method of Reed and Meunch. To control for antiviral effects due solely to pH, virus was treated with a citrate/phosphate buffer matching the pH of test solutions.(Table2)

3. Results:

3.1. Pomegranate Juice PPs eliminate influenza virus infectivity and hemagglutinating activity:

Initial experiments tested PJ for direct antiviral activity against influenza x31 and PR8. The virus was treated for 5 min at room temperature with different concentrations of PJ and titers of infectious virus were determined immediately thereafter.

A consistent trend was greater anti-influenza activity in PJ compared with the pH control, indicating antiviral activity that was distinct from the effect of pH alone. (Figure 8)

Table 2: This table shows the pH of different concentration of PJ, PomBlue Juice, Grape Juice and Coke.

Components	pH
Pomegranate Juice Neat	3.17
20% Pomegranate Juice	3.7
15% Pomegranate Juice	3.9
10% Pomegranate Juice	4.3
PomBlue Juice Neat	3.35
50% PomBlue Juice	3.49
20% PomBlue Juice	3.91
15% PomBlue Juice	4.14
10% PomBlue Juice	4.60
Grape Juice Neat	3.27
20% Grape Juice	3.88
10% Grape Juice	4.99
5% Grape Juice	6.33
Coke Neat	2.42
56% Coke	4.23

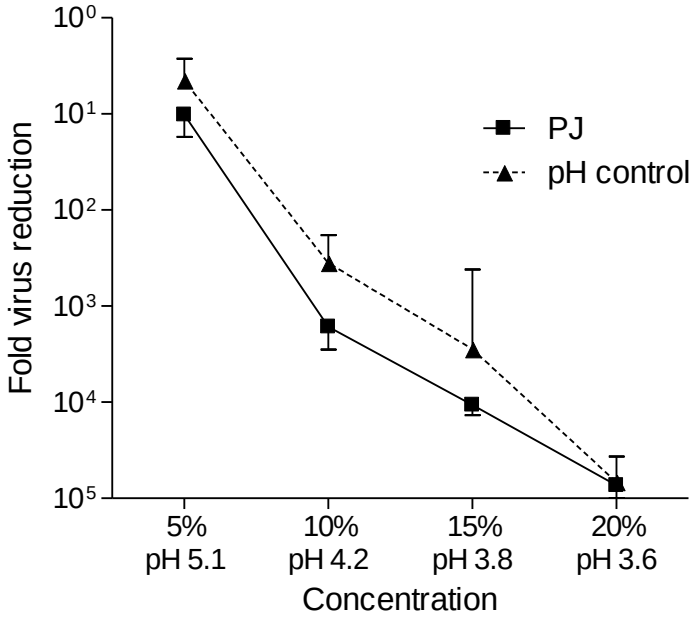

Figure 8: The antiviral activity of PJ against influenza virus.

Influenza x31 (H3N2) at 5 x 10^7 $TCID_{50}$/ml was treated for 5 min at room temperature with different concentrations of PJ, or with a pH-matched buffer. Infectious virus titers after treatment were determined by $TCID_{50}$ assay. Results are shown as the fold reduction in titer relative to untreated virus (incubated with PBS).

We compared anti-influenza virus activity of Pomegranate Blue Juice (PomBlue) against RGX31 virus. We treated the RGX31 virus with 20%, 10% and 5% of PomBlue along with the virus treated with PBS (untreated virus). We observed that RGX31 virus treated PomBlue at the concentration of 20% in PBS completely eliminated infectivity. (Figure 9)

When we were evaluating the anti-influenza virus activity of PJ against the influenza virus, we also included 10 % and 5% of Grape Juice and observed that 10% of Grape Juice completely eliminated infectivity.

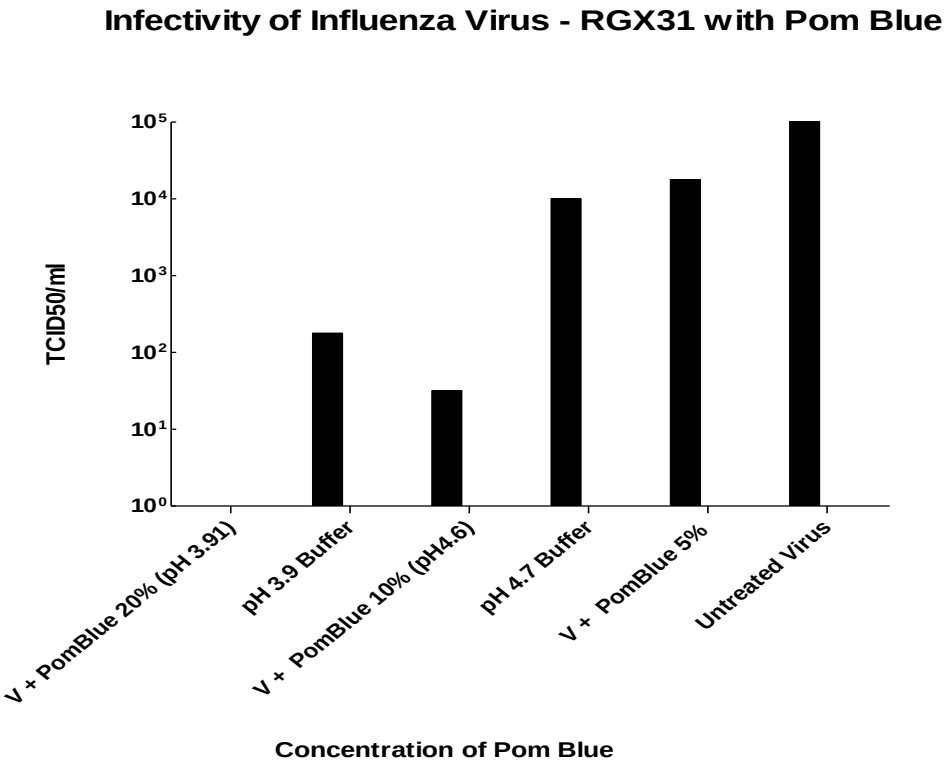

Figure 9: The antiviral activity of PomBlue Juice against influenza virus.

We also did an infectivity assay with a different approach. We evaluated the anti-influenza virus activity of 2% PJ to 0% PJ. In this procedure, everything remained the same as the usual procedure for the assay for infectivity but here we did not treat the samples. Also we first added different concentration of PJ (from 2%, to 0%) in viral growth media, added to the wells of the monolayer of MDCK cells, then added the RGX31 virus in the first row and then did tenfold dilution from top row to the bottom. The rest of the procedure is the same as we used to do. We expected a gradual increase in viral titer from 2% PJ to 0% PJ but we got an expected result.

4. Discussion:

These results indicate that the acidity of PJ contributed to anti-influenza virus activity and also a greater anti-influenza virus activity in PJ compared with the pH control, indicating anti-viral activity was distinct from the effect of pH alone. The anti-viral activity of PJ must be due to polyphenol component because comparable PP concentrations in POMxp solutions and in PJ produced similar fold reductions in viral titers (10% PJ ≈ 360 μg/ml PPs), indicating that the antiviral activity demonstrated in PJ was primarily due to the PP component also be dependent on a direct interaction between polyphenols (PP) in PJ and the viral particles.

Our analysis adds to the growing body of literature describing the antiviral activity of PP preparations derived from plants. We show that the PP component of pomegranate juice interacts directly with influenza virus particles and rapidly neutralizes infectivity. Previous studies demonstrating the anti-influenza activity of PPs from other plant sources have provided evidence

for a direct interaction of PPs with the viral HA and NA [Ehrhardt et al., 2007; Nagai et al., 1992; Nakayama et al., 1993; Serkedjieva and Manolova, 1992; Song et al., 2005].

PP preparations derived from plants are typically a complex mixture of molecular forms. There is evidence for differences in the antiviral activity of individual PP compounds [Haidari et al., 2009] and synergistic activity by PP mixtures [Song et al., 2005]. Presumably, the biochemical reactivity of PPs and their interactions with proteins and lipids play an important role [Diniz et al., 2008; Soares et al., 2007]. However, the molecular interactions that are the basis for the antiviral activity of PPs and how these relate to the characteristics of specific PPs are not well understood [Song et al., 2005]. Our analysis emphasizes the possibility that PPs derived from pomegranate juice and other plant sources may be utilized to combat influenza and other viral infections

CHAPTER III Anti-Influenza Virus activity of Pomegranate Liquid Extract

CHAPTER III Anti-Influenza Virus activity of Pomegranate Liquid Extract

Abstract:

Pomegranate are one of the richest sources of plant polyphenols (PPs), Since antiviral activity has been attributed to plant PPs, we evaluated the anti-influenza activity of Pomegranate Liquid Extract (POMxl).The current study was undertaken to evaluate the direct anti-influenza activity of pomegranate constituents present in Pomegranate Liquid Extract (POMxl). A standard dose of infectious virus was incubated with the different concentration of Pomegranate Liquid Extract (POMxl). Viral infectivity was measured by titration in susceptible MDCK cells. Pomegranate Liquid Extract (POMxl) had anti- influenza activity that was distinct from the effect of the acidic pH of the test materials. Our findings demonstrate rapid anti-influenza activity in Pomegranate Liquid Extract (POMxl).

1. Introduction:

Polyphenolic compounds present at a higher level in pomegranates suggests that the fruit may be a rich source of molecules that exert antiviral activity via multiple mechanisms. Research have shown that pomegranate PPs are directly virucidal for influenza viruses and also act at the intracellular level to inhibit influenza virus replication [Haidari et al., 2009]. In the current study, we investigated the direct anti-influenza virus activity of Pomegranate Liquid Extract. We demonstrate that components of the PP fraction of Pomegranate Liquid Extract rapidly inactivate influenza virus. Our findings suggest that inactivation is a consequence of PP interactions with the viral particles.

2. Materials and Methods:

2.1 Pomegranate Liquid Extract (POMxl):

Pomegranate Liquid Extract was prepared from California-grown and processed wonderful variety pomegranates and supplied by POM Wonderful (Los Angeles, CA).

Preparation of Pomegranate Liquid Extract (POMxl):

After expelling most of the juice from the pomegranate whole fruit, the remaining fruit (peels and membranes), which include aril residues, were collected and processed to remove the seeds before going through a screw press to produce a puree. The puree was enzymatically treated to break down the colloidal structure of the husks and to solubilize sugars, minerals, acids, and polyphenol compounds. The puree polyphenols were concentrated via membrane system and the resultant cloudy POMxl was filtered to produce a pomegranate polyphenol extract. The obtained extract was concentrated after passing through an evaporator and pasteurized. The final product has a 65–70 Brix concentration and was stored at 4°C [Aviram et al., 2008].

Chemical Composition of Pomegranate Liquid Extract (POMxl):

Total phenolics (GAE), 130 000 μg/mL (13%), including mainly hydrolysable tannins (ellagitannins), such as oligomers and punicalagin/punicalin, with a smaller amounts of ellagic acid and anthocyanins (delphinidin, cyaniding, pelargonidin) and their glycosides other components: sugars, 52%; organic acid, 2.0%; ash, 2.0%(70 Brix) [Aviram et al., 2008].

2.2 Chicken Red Blood Cells:

As described in Chapter 2

2.3 Phosphate buffered saline (PBS):

As described in Chapter 2

2.4 Citrate/Phosphate buffer:

As described in Chapter 2

2.5. Viruses and viral titration:

Experiments were performed using influenza A virus: Reassortant virus A/HKx31 (H3N2) (x31), which expressed the H3 and N2 of A/Aichi/2/68 on the PR8 background [Kilbourne et al., 1971]. Influenza virus stocks were grown in the allantoic cavity of embryonated hen's eggs and titers of infectious virus were measured by 50% tissue culture infective dose ($TCID_{50}$) titration. Madin Darby canine kidney (MDCK) cells for viral titration were grown in MEM with L-glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), and gentamicin (10 μg/ml), and supplemented with 10% FBS.

Confluent MDCK cell monolayers in 96-well tissue culture plates were washed once with serum-free MEM before use. Serial 10-fold dilutions of virus in serum-free MEM containing 0.3% BSA and 1 μg/ml L-(toslyamido 2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin (Worthington, Lakewood, NJ) were incubated in replicate wells (200 μl/well) for 2-3 d at 37°C with 5% CO_2. Wells positive for virus growth were identified by the presence of hemagglutinating (HAg) activity in the supernatant, and 50% tissue culture infective dose ($TCID_{50}$) titers were calculated by the method of Reed and Muench.

2.6. Treatment of virus with Pomegranate Liquid Extract and evaluation of antiviral activity:

As described in Chapter 2

3. Results:

3.1. Pomegranate Liquid Extract PPs eliminate influenza virus infectivity and hemagglutinating activity:

Initial experiments tested POMxl for direct antiviral activity against influenza x31. The virus was treated for 5 min at room temperature with different concentrations of POMxl and titers of infectious virus were determined immediately thereafter. A consistent trend was greater anti-influenza activity in POMxl compared with the pH control (statistically significant for 2.5% POMxl), indicating antiviral activity that was distinct from the effect of pH alone.(Figure 10)

Table 3: This table shows the pH of different concentration of POMxl.

Pomegranate Liquid Extract (POMxl)	**pH**
20% POMxl	3.21
15% POMxl	3.24
10% POMxl	3.26
5% POMxl	3.43
2.5% POMxl	3.65
1.25% POMxl	4.09
0.625% POMxl	4.96
0.3125% POMxl	6.16

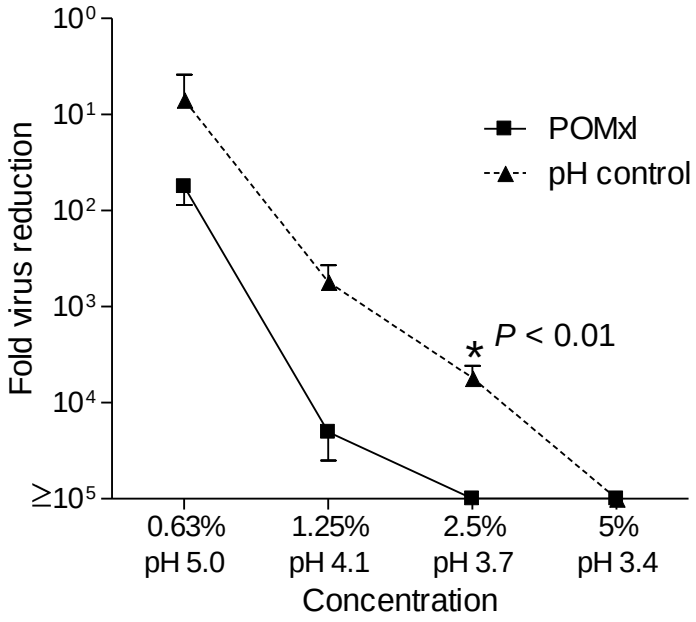

Figure 10: The antiviral activity of POMxl against influenza virus.

Influenza x31 (H3N2) at 5 x 10^7 $TCID_{50}$/ml was treated for 5 min at room temperature with different concentrations of POMxl, or with a pH-matched buffer. Infectious virus titers after treatment were determined by $TCID_{50}$ assay. Results are shown as the fold reduction in titer relative to untreated virus (incubated with PBS).

4. Discussion:

These results indicate that the acidity of POMxl contributed to anti-influenza virus activity and also a greater anti-influenza virus activity in POMxl compared with the pH control (statistically significant for 2.5% POMxl), indicating anti-viral activity was distinct from the effect of pH alone. The anti-viral activity may also be dependent on a direct interaction between Polyphenols (PP) in POMxl and the viral particles.

We show that the PP component of Pomegranates interacts directly with influenza virus particles and rapidly neutralizes infectivity. Previous studies demonstrating the anti-influenza activity of PPs from other plant sources have provided evidence for a direct interaction of PPs with the viral HA and NA [Ehrhardt et al., 2007; Nagai et al., 1992; Nakayama et al., 1993; Serkedjieva and Manolova, 1992; Song et al., 2005].

PP preparations derived from plants are typically a complex mixture of molecular forms. There is evidence for differences in the antiviral activity of individual PP compounds [Haidari et al., 2009] and synergistic activity by PP mixtures [Song et al., 2005]. Presumably, the biochemical reactivity of PPs and their interactions with proteins and lipids play an important role [Diniz et al., 2008; Soares et al., 2007]. However, the molecular interactions that are the basis for the antiviral activity of PPs and how these relate to the characteristics of specific PPs are not well understood [Song et al., 2005]. PP binding to cell surface molecules have been described [Williamson et al., 2006]. Any therapeutic benefits due to the antiviral mechanism we describe are likely to require direct application of PPs at the site of infection [Droebner et al., 2007]. Studies of mouse models have shown that PP administration to the lung reduced the effects of

influenza infection without toxicity to the host [Droebner et al., 2007; Nagai et al., 1992; Serkedjieva et al., 2008]. Interestingly, nasopharyngeal administration of pomegranate extracts is a Cuban folk medicine remedy for influenza [Vidal et al., 2003]. Our analysis emphasizes the possibility that PPs derived from Pomegranate Liquid Extract and other plant sources may be utilized to combat influenza and other viral infections

CHAPTER IV Anti-Influenza Virus activity of Pomegranate Powder Extracts

CHAPTER IV Anti-Influenza Virus activity of Pomegranate Powder Extracts

Abstract:

Pomegranates are one of the richest sources of plant polyphenols (PPs). Since antiviral activity has been attributed to plant PPs, we evaluated the anti-influenza activity of Pomegranate Polyphenol-enriched Powder Extract (POMxp).The current study was undertaken to evaluate the direct anti-influenza virus activity of pomegranate constituents present in Pomegranate Polyphenol-enriched Powder Extract (POMxp). A standard dose of infectious virus was incubated with the different concentration of filtered Pomegranate Polyphenol-enriched Powder Extract (POMxp). Viral infectivity was measured by titration in susceptible MDCK cells. Studies using POMxp showed that brief treatment at room temperature with > 200 μg/ml PPs substantially reduced the infectivity of H1N1, H3N2, and H5N1 influenza viruses. Generally, the loss of infectivity was accompanied by loss of hemagglutinating activity. Electron microscopic examination of influenza particles neutralized by PP treatment identified a coating of amorphous material and some damage to virion integrity. Reassortant H5N1 viruses derived from avian isolates were less affected by PP treatment, indicating that PP susceptibility is modulated by small changes in surface glycoproteins. Our analysis supports the development of pomegranate-derived PPs as natural, rapidly active, broad-spectrum anti-influenza agents.

1. Introduction:

The high level of diverse polyphenolic compounds in pomegranates suggests that the fruit may be a rich source of molecules that exert antiviral activity via multiple mechanisms. Recently, evidence was presented that pomegranate PPs are directly virucidal for influenza viruses and also

act at the intracellular level to inhibit influenza virus replication [Haidari et al., 2009]. In the current study, we investigated the direct anti-influenza virus activity of Pomegranate Polyphenol-enriched Powder Extract. We demonstrate that components of the PP fraction of Pomegranate Polyphenol-enriched Powder Extract rapidly inactivate different influenza virus subtypes, including emerging viruses with pandemic potential. Our findings suggest that inactivation is a consequence of PP interactions with the viral HA and NA, and may be substantially modulated by relatively small changes in these molecules.

2. Materials and Methods:

2.1 Pomegranate Polyphenol-enriched Powder Extract (POMxp):

Pomegranate Polyphenol-enriched Powder Extract (POMxp) was prepared from California-grown and processed wonderful variety pomegranates and supplied by POM Wonderful (Los Angeles, CA).

Preparation of Pomegranate Polyphenol-enriched Powder Extract (POMxp):

POMxl, diluted with water, was passed through a resin column, which preferentially absorbs polyphenols. Polyphenols adsorbed on the resin were recovered using ethanol in water. The recovered polyphenol solution was dried to produce POMxp [Aviram et al., 2008].

Chemical Composition of Pomegranate Polyphenol-enriched Powder Extract (POMxp):

Total phenolics (GAE), 930 μg/mg (93%), including mainly hydrolyzable tannins (ellagitannins), such as oligomers and punicalagin/punicalin, with smaller amounts of ellagic acid and trace anthocyanins other components: sugars, 3.2%; organic acids, 1.9%; ash, 2.5%; protein, 5.0%; and moisture, 1.2%[Aviram et al., 2008].

2.2 Chicken Red Blood Cells:

This reagent was stored in fridge. The 0.5% Chicken Red Blood Cells were prepared fresh in PBS / MEM containing 0.3% BSA / MEM containing 0.1% BSA each time we did the assay for hemagglutinating activity and stored it in ice until use.

2.3 Phosphate buffered saline (PBS):

We used PBS for both the assay for infectivity, the assay for hemagglutinating activity and toxicity assay.

2.4. Viruses and viral titration

Experiments were performed using the following influenza A viruses:

A/Puerto Rico/8/34 (H1N1) (PR8) and the reassortant virus A/HKx31 (H3N2) (x31), which expressed the H3 and N2 of A/Aichi/2/68 on the PR8 background [Kilbourne et al., 1971].[Haidari et al., 2009] Reassortant H5N1 viruses generated by reverse genetics (identified by the prefix rg) from A/Vietnam/1203/04 (VN/04), an isolate from a fatal human case, and from the avian isolates A/Duck/Hunan/795/02 (Dk/HN/02), A/Duck/Laos/3295/06 (Dk/LS/06), and A/Japanese White Eye/Hong Kong/1038/06 (JWE/HK/06). The reassortant H5N1 viruses were constructed with PR8 and expressed the H5 (from which polybasic amino acids that are associated with high virulence were removed) and N1 of the original isolates [Forrest et al., 2009].

Influenza virus stocks were grown in the allantoic cavity of embryonated hen's eggs and titers of infectious virus were measured by 50% tissue culture infective dose ($TCID_{50}$) titration. Madin Darby canine kidney (MDCK) cells for viral titration were grown in MEM with L-glutamine (2

mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), and gentamicin (10 μg/ml), and supplemented with 10% FBS. Confluent MDCK cell monolayers in 96-well tissue culture plates were washed once with serum-free MEM before use. Serial 10-fold dilutions of virus in serum-free MEM containing 0.3% BSA and 1 μg/ml L-(toslyamido 2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin (Worthington, Lakewood, NJ) were incubated in replicate wells (200 μl/well) for 2-3 d at 37°C with 5% CO_2. Wells positive for virus growth were identified by the presence of hemagglutinating (HAg) activity in the supernatant, and 50% tissue culture infective dose ($TCID_{50}$) titers were calculated by the method of Reed and Muench.

2.5. Treatment of virus with Pomegranate Polyphenol enriched Powder Extracts and evaluation of antiviral activity

POMxp was dissolved in PBS at a PP concentration of 4.0 mg/ml (= 4.3 mg/ml POMxp), passed through a 0.2 μm diameter filter, and diluted in PBS for testing. Influenza viruses at 5 x 10^7 $TCID_{50}$/ml were combined with different concentrations of POMxp (expressed as PP concentration) and incubated for 5 min at room temperature. Immediately following incubation, titers of infectious virus were determined by the assay for viral infectivity or $TCID_{50}$ assay as mentioned in Chapter 2 in MDCK cells.

The HAg activity of influenza viruses was also evaluated immediately after treatment with different concentrations of POMxp as described above. Serial 2-fold dilutions of treated virus were prepared with PBS in 96-well round-bottom plates (50 μl/well). An equal volume of 0.5% chicken RBC resuspended in PBS with 0.3% BSA was added to each well, plates were briefly

agitated, and hemagglutination was scored after 30 min incubation at room temperature. POMxp alone at a PP concentration of 4 mg/ml had no HAg activity when the assay was performed in the presence of 0.15% BSA, as was the case in all experiments. In the absence of BSA, PP concentrations greater than approximately 50 μg/ml had inherent HAg activity.

2.6. Electron Microscopy :(by Aarthi Sundararajan)

Influenza x31 was treated for 5 min at room temperature with different concentrations of POMxp and negatively stained for examination by transmission electron microscopy. Treated virus was adsorbed to freshly glow-discharged collodion and carbon films on 400 mesh copper grids, stained with 0.5% phosphotungstic acid, and examined with a Hitachi H-800 electron microscope at 75 kV.

2.7 Evaluation of Toxicity of POMxp on MDCK cells:

We evaluated the toxicity of different concentration of filtered POMxp on monolayers of MDCK cells. This was done to test if POMxp is toxic to MDCK cells. This assay is almost the same as the assay for infectivity described above. But we did several experiments by modifying the usual procedure each time we did the toxicity assay to see if POMxp is toxic to MDCK cells. We did not use the virus and we did not treat the samples at room temperature for 5 minutes. We used 6-well / 24- well plates. We made filtered POMxp as mentioned above. We also tried dissolving POMxp in PBS / Serum Free Growth Medium. Then we made different concentration of filtered POMxp in PBS / Serum Free Growth Media by diluting in PBS / MEM1X with 0.3% BSA / MEM1X with 5% FBS. We added different concentration of filtered POMxp samples to the wells containing the monolayer of MDCK cells. The different concentration of filtered POMxp

we used was from 40% to .078%. Even though we tested different concentration POMxp from 40% to .078%., most of the time we did toxicity assays with filtered POMxp concentration from 4% and below. After samples were added to the wells containing the monolayers of MDCK cells, the plates were incubated for 24 / 48 hours at 37°C with 5% CO_2. After incubation, initially we trypsinized cells and counted them and later on we just observed the MDCK cell monolayers with different concentration of filtered POMxp under the microscope.

3. Results:

3.1. Pomegranate Powder Extract PPs eliminate influenza virus infectivity and hemagglutinating activity

Initial experiments tested POMxp for direct antiviral activity against influenza x31. The virus was treated for 5 min at room temperature with different concentrations of PP-enriched POMxp. Test solutions of POMxp in PBS (Table 4) were at near-neutral pH, and matching pH control solutions had no effect on viral infectivity (not shown). Exposure of influenza x31 to > 100 μg/ml PPs rapidly reduced infectivity, with titers being lowered by more than 4 logs at 800 μg/ml (Figure 11). Control experiments established that the low PP levels in culture medium during titration of treated virus samples had no effect on MDCK cell viability or on $TCID_{50}$ titers (not shown). Thus, antiviral activity was dependent on a direct interaction between PPs and viral particles. To determine whether pomegranate PPs had antiviral activity against other influenza subtypes, we evaluated the activity of POMxp solutions against PR8 (H1N1) (by Aarthi Sundararajan)(Figure 12). As was the case for x31 (H3N2) (Figure 11), infectivity was rapidly eliminated by treatment with > 100 μg/ml PPs.

Table 4: This table shows the pH of different concentration of POMxp with near-neutral pH.

Pomegranate Powder Extract (POMxp)	pH
40% POMxp	6.48
20% POMxp	6.70
10% POMxp	6.82
5% POMxp	6.87
2.5% POMxp	6.89
1.25% POMxp	6.97

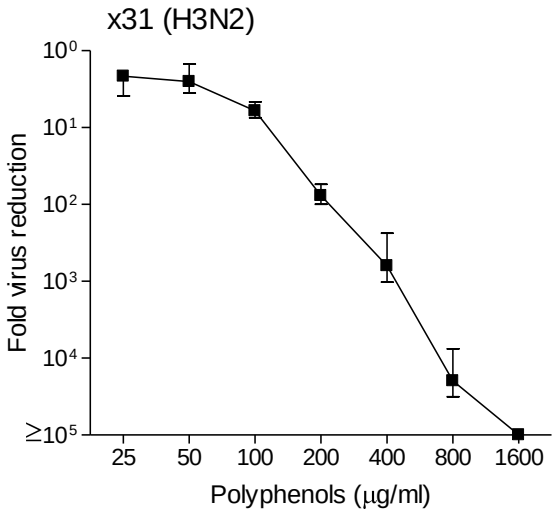

Figure 11: The antiviral activity of POMxp against influenza virus x31.

Influenza x31 (H3N2) was treated for 5 min at room temperature with different concentrations of POMxp (expressed as PP concentration). Infectious virus titers measured by $TCID_{50}$ assay were determined after treatment.

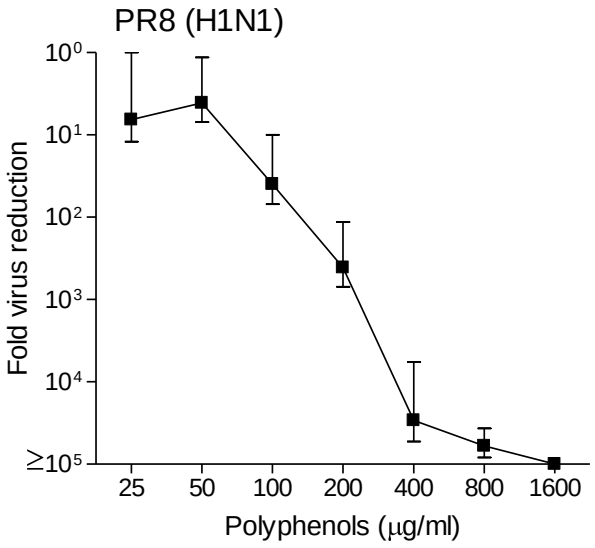

Figure 12: The antiviral activity of POMxp against influenza virus PR8.

Influenza PR8 (H1N1) was treated for 5 min at room temperature with different concentrations of POMxp (expressed as PP concentration). Infectious virus titers measured by $TCID_{50}$ assay were determined after treatment.

Influenza virus-induced hemagglutination results from binding of the viral HA to sialic acid residues on RBC surface molecules and reflects RBC cross-linking by intact virions. To determine the effect of POMxp treatment on the HAg activity of x31, viral preparations were treated as described above for the analysis of effects on viral infectivity. Hemagglutination titers were measured immediately following incubation with POMxp solutions. A loss of HAg activity accompanied the loss of infectivity of x31, raising the possibility of PP interactions with the viral HA or loss of virion integrity (Figure 13).

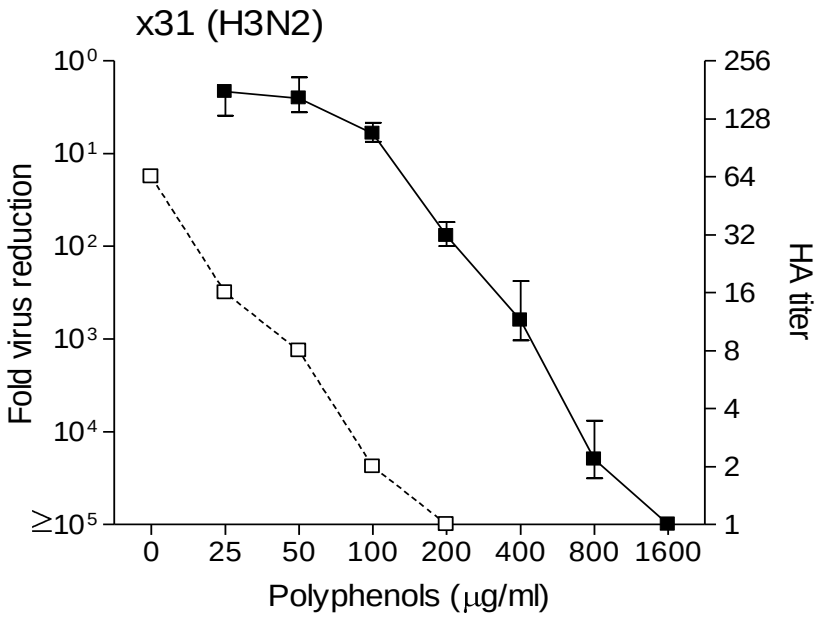

Figure 13: The antiviral activity of POMxp against influenza virus x31.

Influenza x31 (H3N2) was treated as above with different concentrations of POMxp (expressed as PP concentration). Infectious virus titers (filled squares, left axis) and hemagglutination titers (open squares, right axis) were determined after treatment.

Influenza virus-induced hemagglutination results from binding of the viral HA to sialic acid residues on RBC surface molecules and reflects RBC cross-linking by intact virions. To determine the effect of POMxp treatment on the HAg activity of PR8, viral preparations were treated as described above for the analysis of effects on viral infectivity. Hemagglutination titers were measured immediately following incubation with POMxp solutions. A loss of HAg activity accompanied the loss of infectivity of PR8 (Figure 14) as with x31(Figure13), raising the possibility of PP interactions with the viral HA or loss of virion integrity

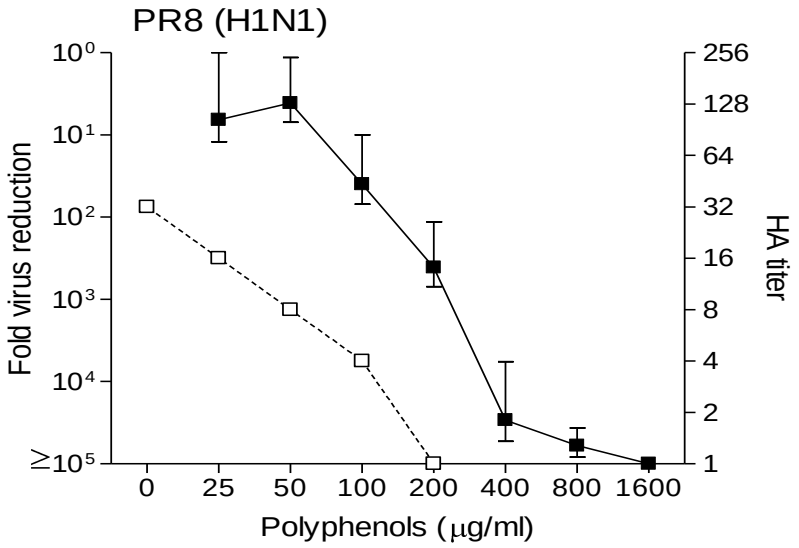

Figure 14: The antiviral activity of POMxp against influenza virus PR8.

Influenza PR8 (H1N1) was treated as above with different concentrations of POMxp (expressed as PP concentration). Infectious virus titers (filled squares, left axis) and hemagglutination titers (open squares, right axis) were determined after treatment.

3.2. Pomegranate PPs mask influenza virus envelope glycoproteins :(by Aarthi Sundararajan)

To gain insights into the mechanism of PP effects on influenza viruses, POMxp-treated and control influenza x31 particles were examined by transmission electron microscopy (Figure 15 A,B,C). Treated virions remained largely intact, but some damage to virion integrity was also evident. In addition, treated virions were surrounded by amorphous material that was increased in density after treatment with higher PP concentration.

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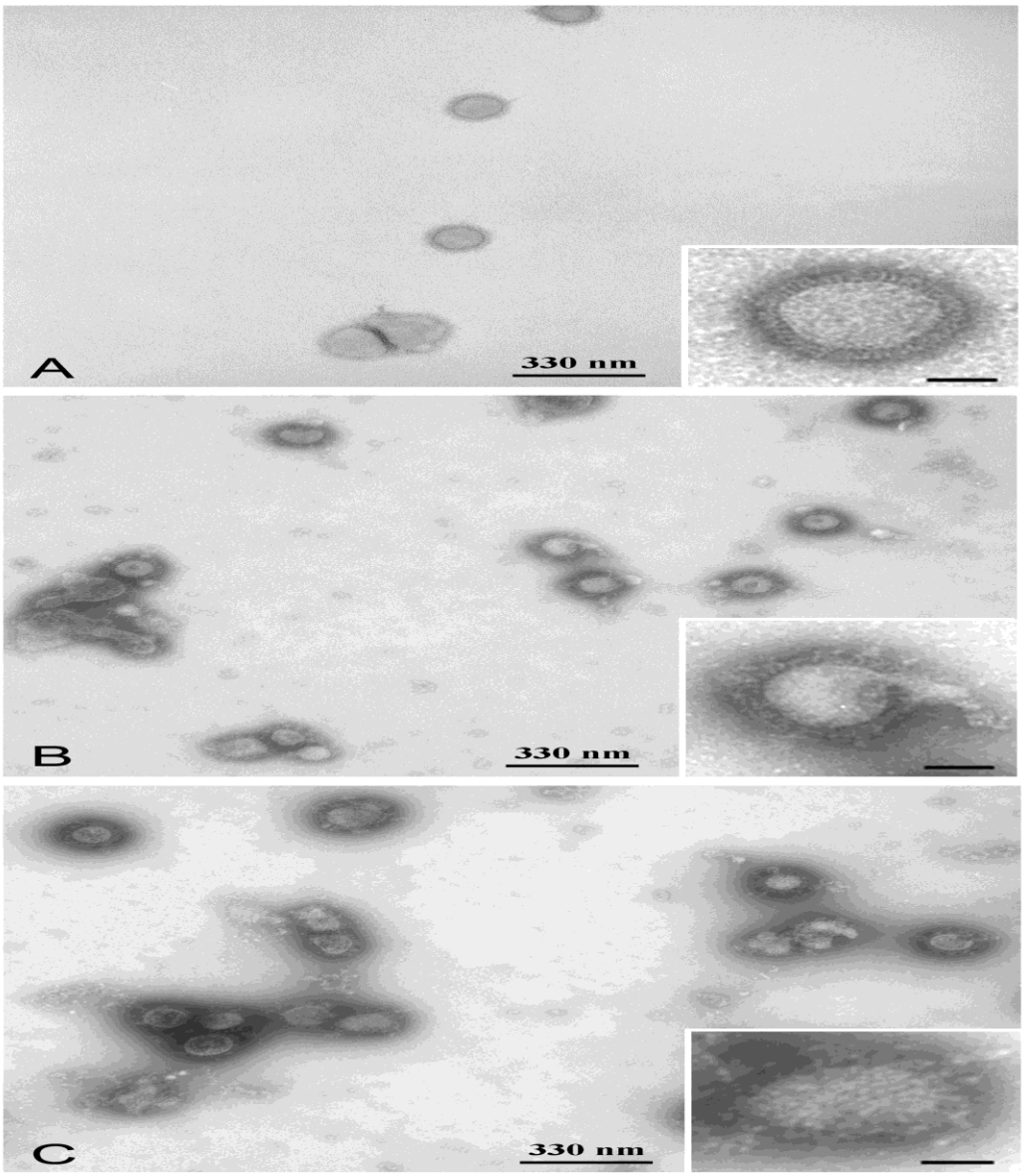


Figure 15 A, B, C: The electron micrographs of influenza x31 after treatment with POMxp.

Virus at 5 x 10^8 $TCID_{50}$/ml was incubated for 5 min at room temperature with PBS (A), 800 µg/ml PPs (B), or 1600 µg/ml PPs (C). Inset scale bar = 50 nm

3.3. Influenza virus variants differ in susceptibility to pomegranate PPs: (*by Dr.Richard* J. Webby - *Department of Infectious Diseases, St. Jude Children's Research Hospital*)

In our analysis, POMxp demonstrated potent antiviral activity against x31 and PR8, two long-established and now standard laboratory strains of influenza A virus. An important question was whether a similar level of antiviral activity would be evident against recent influenza isolates, including emerging viruses that present a threat to human health. To investigate this, POMxp activity was tested against reassortant influenza viruses expressing the HA and NA molecules of H5N1 isolates (the remaining viral components were derived from PR8). Compared with the effect against x31 and PR8, POMxp treatment resulted in only a minor reduction in the infectivity of rg-Dk/HN/02, rg-Dk/LS/06, and rg-JWE/HK/06. (Figure 16 B, C, D)

However, the infectivity of rg-VN/04 was markedly reduced (Figure 16 A), resembling the effect of POMxp on x31. Thus, the susceptibility of variant H5N1 subtype viruses to PP treatment is modulated by the nature of the expressed HA and NA. In contrast to the results for x31 and PR8, the loss of infectivity after POMxp treatment of rg-VN/04 did not appear to be associated with a loss of HAg activity. Overall, results could be taken to indicate that the effect of PP exposure on HAg activity is not a predictor of effects on infectivity. However, a cautious interpretation is warranted because POMxp itself has HAg activity when the assay is performed in the absence of BSA. It is not possible to predict the consequences of potential multidirectional interactions when PP-treated viruses are combined with RBC in a BSA solution. In experiments in this section using H5N1 viruses, x31 was tested in parallel to ensure the reproducibility of earlier findings. The results for x31 matched those shown in Figure 16A.

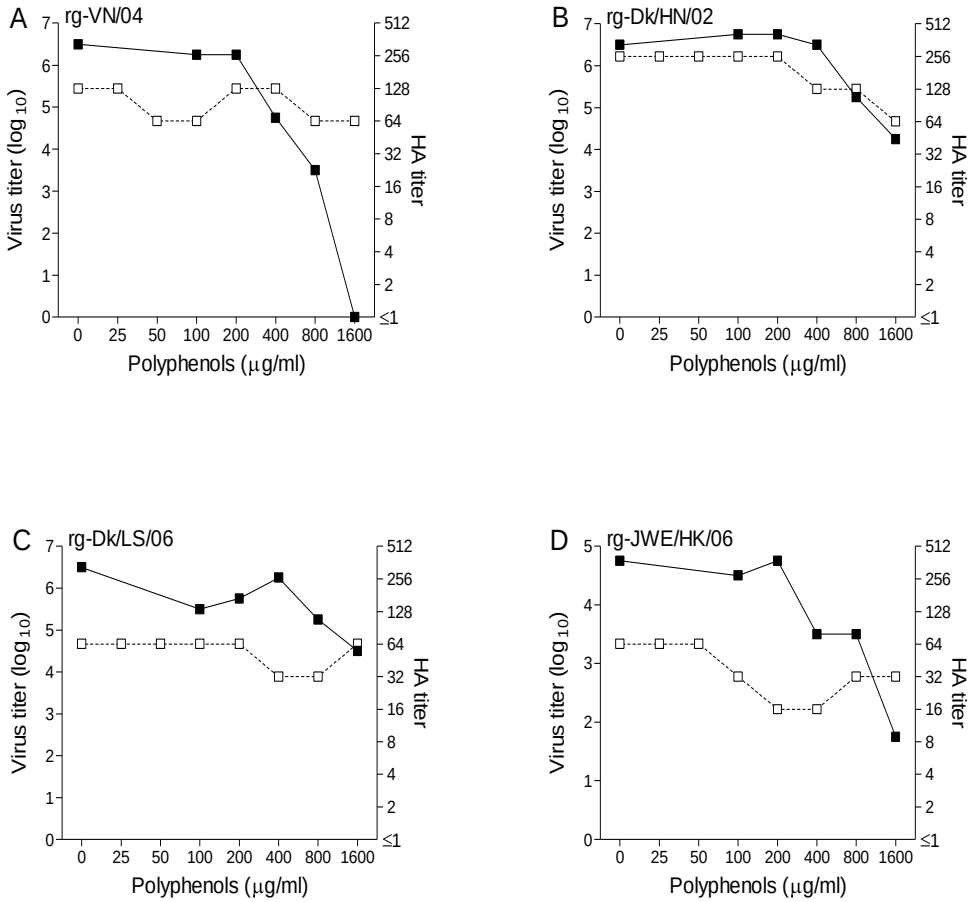


Figure 16 A, B, C, D: The antiviral activity of POMxp against variant H5N1 influenza viruses. The following influenza viruses at the indicated titers were treated for 5 min at room temperature with different concentrations of POMxp (expressed as PP concentration): rg-VN/04 (H5N1) at 5 x 10^7 $TCID_{50}$/ml (A), rg-Dk/HN/02 (H5N1) at 5 x 10^7 $TCID_{50}$/ml (B), rg-Dk/LS/06 (H5N1) at 2.8 x 10^7 $TCID_{50}$/ml (C), rg-JWE/HK/06 (H5N1) at 1 x 10^7 $TCID_{50}$/ml (D).Infectious virus titers measured by $TCID_{50}$ assay (filled squares, left axis) and hemagglutination titers (open squares, right axis) were determined after treatment.

We used a new approach of the infectivity assay when evaluating the anti influenza virus activity of POMxp. For this new method, we made different concentration of filtered POMxp in PBS. The filtered POMxp concentration we used in our infectivity assay were 40%, 20%, 10%, 5%, 2.5%, 1.25%, 0.625% in PBS. In this approach all the procedures were the same as the usual procedure of infectivity, except that we did not treat the samples at RT for 5 minutes. Also usually we add the sample and virus together into the wells containing monolayers but we added the sample first and then added the virus (equal amount of virus and sample) and after adding the sample and virus to the wells containing the monolayers of MDCK cells, we did the serial tenfold dilution. Rest of the procedure was the same. We observed that the viral titers were high for the different concentration of filtered POMxp. So we thought this is not the ideal approach.

We also evaluated the toxicity of different concentration of filtered POMxp on monolayers of MDCK cells. We found that monolayer was better when we used filtered POMxp diluted in MEM1X with 5% FBS than with MEM1X with 0.3% BSA.Overall, our findings indicate that POMxp is not toxic to MDCK cells.

4. Discussion:

Our analysis using POMxp showed that brief treatment at room temperature with > 200 μg/ml PPs substantially reduced the infectivity of H1N1, H3N2, and H5N1 influenza viruses. Generally, the loss of infectivity was accompanied by loss of hemagglutinating activity. Electron microscopic examination of influenza particles neutralized by PP treatment identified a coating of amorphous material and some damage to virion integrity. Reassortant H5N1 viruses derived from avian isolates were less affected by PP treatment, indicating that PP susceptibility is

modulated by small changes in surface glycoproteins. We show that the PP component of pomegranates interacts directly with influenza virus particles and rapidly neutralizes infectivity. The antiviral activity of pomegranate PPs was potent against diverse influenza virus subtypes, including emerging viruses that threaten human health.

Previous studies demonstrating the anti-influenza activity of PPs from other plant sources have provided evidence for a direct interaction of PPs with the viral HA and NA [Ehrhardt et al., 2007; Nagai et al., 1992; Nakayama et al., 1993; Serkedjieva and Manolova, 1992; Song et al., 2005]. Our analysis suggests that the accessibility of these molecules is impeded by PP treatment, but that at least some antigenic features remain intact.

Direct virus neutralization by PPs may simply result from physical interference with the interaction between the viral HA and cell receptors. This mechanism is consistent with the loss of virus-mediated hemagglutination after PP treatment, as was clearly seen for x31 and PR8. However, loss of infectivity after PP treatment was not associated with loss of HAg activity for all influenza viruses tested, raising the possibility of additional mechanisms of neutralization. This is supported by our electron microscopic analysis of PP-treated x31, which indicated at least some degree of envelope damage.

Pomegranate PPs were effective in reducing the infectivity of the H5N1 virus rg-VN/04. However, the effect was much smaller against three other H5N1 viruses, rg-Dk/HN/02 and rg-Dk/LS/06(in particular) and rg-JWE/HK/06. Differences in susceptibility can be attributed

entirely to the HA and NA molecules, since the H5N1 viruses were reassortants that expressed only the HA and NA of the original isolates and did not differ in the remaining viral components. Rg-Dk/HN/02, rg-Dk/LS/06, and rg-JWE/HK/06 were derived from avian H5N1 isolates, whereas rg-VN/04 was derived from an isolate from a fatal human case. This raises the possibility that features of the surface glycoproteins of influenza viruses adapted to maintenance in avian reservoirs may also confer resistance to the effects of PPs. It will be of interest to determine whether influenza isolates that are resistant to direct inactivation by PPs remain susceptible to the antiviral activity of PPs that operates during intracellular replication [Haidari et al., 2009].

PP preparations derived from plants are typically a complex mixture of molecular forms. There is evidence for differences in the antiviral activity of individual PP compounds [Haidari et al., 2009] and synergistic activity by PP mixtures [Song et al., 2005]. Presumably, the biochemical reactivity of PPs and their interactions with proteins and lipids play an important role [Diniz et al., 2008; Soares et al., 2007]. However, the molecular interactions that are the basis for the antiviral activity of PPs and how these relate to the characteristics of specific PPs are not well understood [Song et al., 2005].

Our analysis and other studies [Ehrhardt et al., 2007; Serkedjieva, 2003] indicate that reactivity with PPs is also influenced by relatively small changes in the molecular features of viral surface proteins. PP binding to cell surface molecules have been described [Williamson et al., 2006]. Nevertheless, there is evidence for some selectivity in PP reactivity and preferential binding to viral compared with host cell molecules [Nagai et al., 1992; Serkedjieva et al., 2008].

Studies of mouse models have shown that PP administration to the lung reduced the effects of influenza infection without toxicity to the host [Droebner et al., 2007; Nagai et al., 1992; Serkedjieva et al., 2008]. Interestingly, nasopharyngeal administration of pomegranate extracts is a Cuban folk medicine remedy for influenza [Vidal et al., 2003]. Our analysis emphasizes the possibility that PPs derived from Pomegranate Powder Extracts and other plant sources may be utilized to combat influenza and other viral infections.

CHAPTER V Anti-Influenza Virus activity of Fulvic Acid

CHAPTER V Anti-Influenza Virus activity of Fulvic Acid

Abstract:

The decay of organic matter generates an extremely heterogeneous mixture of organic molecules referred to as humic substances. They are sub-classified on the basis of solubility characteristics. The Fulvic Acid (FA) fraction of humic substances includes a variety of low molecular weight acidic molecules that are soluble in water under all pH conditions. The current study was undertaken to evaluate the direct anti-influenza activity of Fulvic Acid (FA).A standard dose of infectious virus was incubated with the different concentration of Fulvic Acid (FA). Viral infectivity was measured by titration in susceptible MDCK cells, and hemagglutinating activity was determined using chicken red blood cells. Our findings demonstrate rapid anti-influenza activity in Fulvic Acid (FA).

1. Introduction:

A network of researchers have been investigating the antiviral properties of a formulation called Secomet V, whose active ingredient is fulvic acid, which is a complex mixture of compounds arising from decomposing organic matter. Antiviral activity of fulvic acid containing Secomet V against poxviruses and SARS has been demonstrated [Kotwal et al., 2006].In the current study, we investigated the direct anti-influenza virus activity of fulvic acid. We demonstrate that components of fulvic acid rapidly inactivate influenza virus. Our findings suggest that inactivation is a consequence of fulvic acid with the viral particles.

2. Materials and Methods:

2.1. Fulvic Acid:

We used a formulation called Secomet V which was given / sent by Dr.Girish J. Kotwal. It has been reported to have a broad spectrum antiviral properties [Kotwal et al., 2006] and was subsequently revealed a year later by Stefan Coetzee from Fulvimed Pvt. Ltd. to have Fulvic Acid as the major active ingredient.

Chemical Properties of Fulvic Acid:

The bioactive Fulvic Acid found in the formulation was separated by ultra filtration using a 3000 Da filter and column chromatography and found to be in the range of 200–600 Da; with the peak activity observed around fractions corresponding to 113, 226 and 452 Da indicating that the active compounds may be repeats of a monomeric unit of 113 Da (Kotwal, unpublished). Fulvic acids are light yellow to yellow-brown in color. Physical and chemical analyses show that the formulation is acidic (around pH 2) and heat stable (survives autoclaving), and that the levels of iron and arsenic are well below the permissible levels [Kotwal, 2008].

2.2 Chicken Red Blood Cells:

As described in Chapter 2

2.3 Phosphate buffered saline (PBS):

As described in Chapter 2

2.4. Viruses and viral titration:

As described in Chapter 2

2.5. Treatment of virus with Fulvic Acid and evaluation of antiviral activity:

Confluent monolayers of MDCK cells for viral titration were grown in 96-well tissue culture plates and were washed once with serum-free MEM before use. MEM containing 0.3% bovine serum albumin (Sigma) and 1 µg/ml L-(toslyamido 2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin [Worthington, Lakewood, NJ] was added to the wells and used for dilution of treated virus. Influenza virus at 5 x 10^7 $TCID_{50}$/ml was combined with different concentrations of FA diluted in PBS, and incubated for 5 min at room temperature.

Immediately following incubation, treated virus was serially diluted 10-fold in triplicate wells containing MDCK cell monolayers, and plates were incubated for 2 days at 37°C with 5% CO2. Wells positive for virus growth were identified by the presence of hemagglutinating activity in the supernatant by using a fresh 96-well round bottom plate. We transferred 50µl of supernatant from the original plate to the fresh 96-well round bottom plate, carefully not disrupting the MDCK monolayer at the bottom of the wells, from the top row to the bottom row of the plate. Added the 50µl of 0.5% CRBC to all the wells. The plates were briefly agitated with hand and set the plate on the bench for 30 minutes to one hour at room temperature.

After the incubation, wells positive for virus growth were identified by the presence of hemagglutinating activity. In the absence of hemagglutinating activity, red blood cells roll to the bottom of the wells and form small round pellets, whereas no pellets are visible when the cells are agglutinated and 50% tissue culture infective dose (TCID50) titers were calculated by the method of Reed and Meunch.

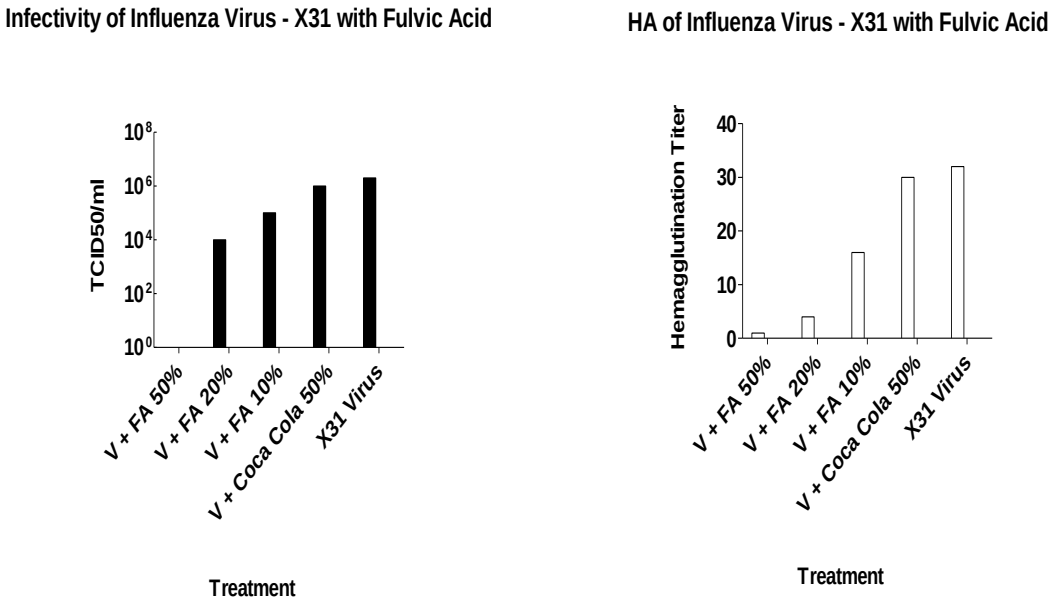
The HAg activity of influenza viruses was also evaluated immediately after treatment with different concentrations of FA as described above. Immediately following incubation with FA solutions, serial 2-fold dilutions of treated samples were prepared with PBS in 96-well round-bottom plates (50 µl/well).

An equal volume of 0.5% chicken RBC was added to each well, plates were briefly agitated, and hemagglutination was scored after 30 min to one hour incubation at room temperature. After the incubation, hemagglutiantion was scored. In the absence of hemagglutinating activity, red blood cells roll to the bottom of the wells and form small round pellets, whereas no pellets are visible when the cells are agglutinated.

3. Results:

3.1. Fulvic Acid eliminate influenza virus infectivity and hemagglutinating activity

Experiments tested FA for direct antiviral activity against influenza x31 and PR8. The virus was treated for 5 min at room temperature with different concentrations of FA and titers of infectious virus and hemagglutinating activity were determined .We observed that the viral infectivity and the hemagglutinating activity were inhibited in a dose (FA concentration) dependent manner indicating that FA possibly blocks entry of virus by interacting with the sugar or the lipid moiety or both of the surface glycoproteins of the influenza virus. (Figure 17)



Figures 17: The antiviral activity of FA.

4. Discussion:

The hemagglutinating activity of the virus was inhibited indicating that fulvic acid possibly blocks entry of the virus by interacting with the sugar or lipid moiety or both of surface glycoproteins of virus. Most of the time, formulation containing fulvic acid neutralized the infectivity of the virus in a dose-dependent manner when treated for 5 minutes at room temperature possibly by interacting with sugar chains on the viral surface protein and serving as an entry inhibitor.

A formulation consisting of fulvic acid, a complex mixture of compounds was previously reported to render vaccinia virus, HIV and SARS virus non-infectious. Recently, fulvic Acid has been shown to inactivate genetically diverse strains of influenza including H5N1, further confirming the broad spectrum nature of this agent. How fulvic acid will be used in developing a vaccine achieving sterilizing immunity or prophylaxis needs to be researched [Kotwal, 2008].

We suggest that compounds with antiviral activity from fulvic acid neutralize the infectivity of diverse enveloped viruses and a number of subtypes of a given enveloped virus, indicating potential for development as a treatment option that can be broadly effective against pandemic viruses like HIV potentially pandemic viruses like influenza and carcinogenic viruses like HBV and HCV.

CHAPTER VI CONCLUSION

CHAPTER VI CONCLUSION

All of the extracts of Pomegranate and Fulvic Acid had rapid antiviral activity. Both the Pomegranate Juice and the Pomegranate Liquid Extract has anti-influenza virus activity that was distinct from the effect of the acidic pH of the test materials. POMxp at a concentration of > 200 μg/ml PPs substantially reduced the infectivity of H1N1, H3N2, and H5N1 influenza viruses. Generally, the loss of infectivity was accompanied by loss of hemagglutinating activity. Electron microscopic examination of influenza particles neutralized by PP treatment identified a coating of amorphous material and some damage to virion integrity. Reassortant H5N1 viruses derived from avian isolates were less affected by PP treatment, indicating that PP susceptibility is modulated by small changes in surface glycoproteins.

We show that the PP component of pomegranates interacts directly with influenza virus particles and rapidly neutralizes infectivity. The antiviral activity of pomegranate PPs was potent against diverse influenza virus subtypes, including emerging viruses that threaten human health.Previous studies demonstrating the anti-influenza activity of PPs from other plant sources have provided evidence for a direct interaction of PPs with the viral HA and NA [Ehrhardt et al., 2007; Nagai et al., 1992; Nakayama et al., 1993; Serkedjieva and Manolova, 1992; Song et al., 2005]. Our analysis suggests that the accessibility of these molecules is impeded by PP treatment, but that at least some antigenic features remain intact.

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Pomegranate PPs were effective in reducing the infectivity of the H5N1 virus rg-VN/04. However, the effect was much smaller against three other H5N1 viruses, rg-Dk/HN/02 and rg-Dk/LS/06(in particular) and rg-JWE/HK/06. Differences in susceptibility can be attributed entirely to the HA and NA molecules, since the H5N1 viruses were reassortants that expressed only the HA and NA of the original isolates and did not differ in the remaining viral components. Rg-Dk/HN/02, rg-Dk/LS/06, and rg-JWE/HK/06 were derived from avian H5N1 isolates, whereas rg-VN/04 was derived from an isolate from a fatal human case. This raises the possibility that features of the surface glycoproteins of influenza viruses adapted to maintenance in avian reservoirs may also confer resistance to the effects of PPs.

It will be of interest to determine whether influenza isolates that are resistant to direct inactivation by PPs remain susceptible to the antiviral activity of PPs that operates during intracellular replication [Haidari et al., 2009].

PP preparations derived from plants are typically a complex mixture of molecular forms. There is evidence for differences in the antiviral activity of individual PP compounds [Haidari et al., 2009] and synergistic activity by PP mixtures [Song et al., 2005]. Presumably, the biochemical reactivity of PPs and their interactions with proteins and lipids play an important role [Diniz et al., 2008; Soares et al., 2007]. However, the molecular interactions that are the basis for the antiviral activity of PPs and how these relate to the characteristics of specific PPs are not well understood [Song et al., 2005].

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Studies of mouse models have shown that PP administration to the lung reduced the effects of influenza infection without toxicity to the host [Droebner et al., 2007; Nagai et al., 1992; Serkedjieva et al., 2008]. Interestingly, nasopharyngeal administration of pomegranate extracts is a Cuban folk medicine remedy for influenza [Vidal et al., 2003]. Our analysis emphasizes the possibility that PPs derived from pomegranates and other plant sources may be utilized to combat influenza and other viral infections

Studies have shown that virus neutralizing compounds in the body fluid could neutralize viruses in the blood stream and elicit an immune response to the neutralized authentically folded virus particle and thus facilitate development of a potentially protective immunity

[Kotwal and Kulkarni,1997].So the active antiviral components from Pomegranate Extracts and from Fulvic Acid could neutralize the infectivity of diverse viruses and a number of subtypes, indicating the potential for development of a treatment option that can be broadly effective against pandemic viruses like HIV, potentially pandemic viruses like influenza and carcinogenic viruses like HBV and HCV.

Thus the identification of optimally active components or combinations of components in Pomegranate Extracts and Fulvic Acid may lead to natural, rapidly active, broad-spectrum preparations for inhalation or topical application that aid in the control of seasonal and pandemic influenza.

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