

ASSESSMENT OF PLANT PROTEINS / PEPTIDES AS POTENTIAL ANTIVIRAL AGENTS

THESIS SUBMITTED
UNIVERSITY OF MUHS

FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN MEDICAL MICROBIOLOGY

SUBMITTED BY
MUKHERJEE, SANDEEPAN

UNDER THE GUIDANCE OF
DR. ABHAY CHOWDHARY

HAFFKINE INSTITUTE FOR TRAINING, RESEARCH & TESTING
BOMBAY

500

2017

i.	Permanent Registration Number	:	2710100032
ii.	Name of the Candidate	:	Sandeepan Ranjit Kumar Mukherjee
iii.	Name of Faculty	:	Medicine
iv.	Name of the College/Institute	:	Haffkine Institute for Training, Research and Testing
v.	Name of the Guide	:	Dr. Abhay Chowdhary
vi.	Name of Examination	:	Doctor of Philosophy (Ph.D.)
vii.	Name of Subject/Speciality	:	Medical Microbiology
viii.	Title of the Thesis	:	Assessment of Plant Proteins/Peptides as Potential Antiviral Agents
ix.	Admission Year (Academic Year)	:	2010-2011
x.	Completion Year (Academic Year)	:	2016-2017

1	Maharashtra University of Health Sciences (MUHS), Nasik
2	Name of the Examination: Doctor of Philosophy (Ph.D)
3	Name of the Faculty: Medicine
4	Name of Subject/Speciality: Medical Microbiology
5	Admission Year (Academic Year): 2010-2011
6	Completion Year (Academic Year): 2016-2017
7	Title of the Thesis: Assessment of Plant Proteins/Peptides as Potential Antiviral Agents

TABLE OF CONTENTS

SR. NO.	PARTICULARS	PAGE NO.
1.	INTRODUCTION	01-04
2.	RATIONALE	05
3.	HYPOTHESIS	06
4.	AIMS & OBJECTIVES	07
5.	REVIEW OF LITERATURE	08-90
6.	MATERIALS & METHODS	91-116
7.	RESULTS	117-136
8.	DISCUSSION	137-149
9.	SUMMARY & CONCLUSION	150-152
10.	FUTURE APPLICATIONS	153
11.	REFERENCES	154-179

LIST OF FIGURES

Figure 1: Natural course of Viral Infections	10
Figure 2: Examples of Immunopathology of Viral Infections.	12
Figure 3: Anti-Influenza Treatment	29
Figure 4: Structure of Rabies Virus.....	31
Figure 5: Structure of Rabies Virus Genome.....	34
Figure 6: Transmission of the Rabies Virus	37
Figure 7: Pathogenesis of Rabies Virus.....	41
Figure 8: Demonstration of Negri Body in neuronal cell.....	45
Figure 9: Demonstration of Fluorescent Antibody Test showing apple green fluorescence of rabies antigen in brain tissue of rabid animals.	46
Figure 10: Demonstration of direct rapid immunohistochemical test (dRIT)	48
Figure 11 WHO classification of wounds	52
Figure 12 Demonstration of IDRV administration	54
Figure 13 Types of Phytochemicals and their activities.....	69
Figure 14 Isolation of antiviral phytochemicals from plants.	71
Figure 15: HPTLC profiles of the Extracts.....	117
Figure 16 Graph of in-vitro cytotoxicity assays on RAW cells.....	118
Figure 17 Graph of in-vitro cytotoxicity assays on BHK-21 cells	119
Figure 18 Graph of in-vitro cytotoxicity assays on MDCK cells	120
Figure 19: Fluorescent Foci Observed on BHK cell	121
Figure 20: Graph of Anti-Rabies Virus activity using modified RFFIT.....	122
Figure 21: Graph of Anti-Rabies Virus activity using qRT-PCR	123
Figure 22: Graph of HA inhibition activity assay.	124
Figure 23: Graph of HA inhibition activity assay.	125
Figure 24: Graph of Gene Expression analysis of IFN- γ	126
Figure 25: Graph of Gene Expression analysis of TNF- α	127
Figure 26: Graph of Gene Expression analysis of IL-4.....	127
Figure 27: Graph of Gene Expression analysis of IL-6.....	128
Figure 28: Cytokine gene expression in extract of <i>A. marmelos</i>	128
Figure 29: Cytokine gene expression in extract of <i>M. indica</i>	129
Figure 30: Cytokine gene expression in extract of <i>R. communis</i>	129
Figure 31: Graph of Nitric Oxide (NO) generated.	130
Figure 32: Graph of Membrane Stabilisation Assay	131
Figure 33: Graph of the concentration of tryptophan metabolites.....	132
Figure 34: Ligand interaction diagrams between compounds and AHR	134
Figure 35: Ligand interaction diagrams between compounds and AHR	135
Figure 36: Ligand interaction diagrams between compounds and AHR	136

LIST OF TABLES

Table 1: List of ICTV releases from 1971 until 2016.	9
Table 2: The first column lists viruses known to cause chronic infection in humans and their primary nucleic acid type.....	14
Table 3 Mode of action of specific phytochemicals with antiviral activity	70
Table 4 Mobile phase and derivatizing agents of specific phytochemicals	95
Table 5: Rabies Virus N gene primer sequences	105
Table 6: N gene PCR master Mix	106
Table 7: Cycling conditions for RABV N gene qRT-PCR.....	106
Table 8: Cytokine Gene Primer Sequences	110
Table 9: Cytokine Gene PCR master mix.....	110
Table 10: Cycling conditions for Cytokine Gene Expression PCR	111
Table 11: Phytochemical profiles of the extracts used in the study	117
Table 12: Median Cytotoxic Concentrations of the samples	121
Table 13: Anti-Rabies Virus activity using modified RFFIT'	121
Table 14: Anti-Rabies Virus activity using qRT-PCR.....	122
Table 15: Hemagglutination (HA) Inhibition activity (pre-exposure)	123
Table 16: Hemagglutination (HA) Inhibition activity (post-exposure)	125
Table 17: Gene Expression Analysis	126
Table 18: Concentration of Nitric Oxide	130
Table 19: Anti-Inflammatory activity	131
Table 20: Mean concentrations of Tryptophan metabolites	132
Table 21: Results of docking various phytochemicals	133

LIST OF ABBREVIATIONS

°C	degree celsius
5'HT	Tryptophan
ADE	Antibody Dependent Enhancement
AHR	Aryl Hydrocarbon Receptor
AM	<i>Agale marmelos</i> (Bael) Methanol Extract
ARDS	Acute Respiratory Distress Syndrome
ARV	Anti Rabies Vaccine
BHK-21	Baby Hamster Kidney Cells
CC50	Median Cytotoxic Concentration
CDC	Center for Disease Control & Prevention
CPE	Cytopathic Effect
CVS-11	Challenge Virus Standard
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
FITC	Fluorescein Isothiocyanate
FMDV	Foot and Mouth Disease Virus
GAP-31	Gelonium Anti-HIV Protein 31
GAPDH	Glyceraldehyde-3 phosphate dehydrogenase
HA	Hemagglutination Assay
HBV	Hepatitis B Virus
HCMV	Human Cytomegalovirus
HIV	Human Immunodeficiency Virus
HPTLC	High Performance Thin Layer Chromatography
HSV	Herpes Simplex Virus
IC50	Median Inhibitory Concentration
ICTV	International Committee on Taxonomy of Viruses
IDO	Indoleamine 2,3-Dioxygenase
IFN	Interferon Gamma
IL-4	Interleukin 4
IL-6	Interleukin 6
i-NOS	Inducible Nitric Oxide Synthase
JEV	Japanese Encephalitis Virus
KYN	Kynurenin
LCMV	Lymphocytic Chorio-Meningitis Virus
LPS	Lipopolysaccharide
LRIP	Luffa Ribosomal Inhibitory Protein
MDCK	Madin-Darby Canine Kidney Cells
MEM	Minimal Essential Medium
MIP	<i>Mangifera indica</i> (Mango) Protein fraction
MIP	<i>Mangifera indica</i> (Mango) Protein fraction
MP	<i>Agale marmelos</i> (Bael) Protein fraction
mRNA	messenger RNA
MTB	Mycobacterium tuberculosis
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide

NO	Nitric Oxide
NSAIDs	Non Steroidal Anti-Inflammatory Drugs
PAP	Pokeweed Antiviral Protein
PCR	Polymerase Chain Reaction
qRT-PCR	Real Time Reverse Transcriptase PCR
RABV	Rabies Virus
RC	<i>Ricinus communis</i> (Eranda) Methanol Extract
RFFIT	Rapid Fluorescence Foci Inhibition Test
RIG	Rabies Immunoglobulin
RIP	Ribosome Inhibitory Proteins
RNA	Ribonucleic Acid
RP	<i>Ricinus communis</i> (Eranda) Protein fraction
SI	Selectivity Index
Tc CELLS	Cytotoxic T Cells
TCID ₅₀	Median Tissue Culture Infectivity Dose
TLC	Thin Layer Chromatography
TMV	Tobacco Mosaic Virus
TNF	Tumor Necrosis Factor-Alpha
TPVG	Trypsine Phosphate Versene Glucose
TRP	Tryptophan
VHF	Viral Hemorrhagic Fever
WNV	West Nile Virus

INTRODUCTION

It wasn't something that we had not seen coming. But, what we had not anticipated was that it could get this big. So, although we were prepared to handle the situation face on, when it actually got to us we were overwhelmed, well at least initially! The 2009 pandemic H1N1 influenza episode was my first tryst with a viral epidemic and its impact will guide me forever...

The first set of samples from the designated Infectious Disease Hospital arrived on 6th August 2009. Little did we realize that this was only the tip of the iceberg! In the next 7 days, we had over 1500 samples!

At 100 samples per day that were to be reported, the target was tough, but tougher were screening the samples to determine the medically urgent ones and the 24×7 counselling of doctors, hospital staff, relatives of patients, patients and people who thought they were patients, about the dos and don'ts in that hour of emergency. The silver lining amidst the chaos however, was the live education that was being imparted. An experience that will influence my deliberations with medical research forever.

Two areas were highly intriguing; these, were the lack of a robust clinical differential diagnosis and the confusion over the use of specific anti-viral therapy. The lack of specific differential diagnosis meant initiation of empirical treatment regimes, consequently, most cases, which came, had already been treated with antibiotics. Initially, the option of using Oseltamivir (the specific anti-viral drug against influenza virus) was rather arduous, since it was a 'Schedule X' medicine and only patients testing positive for the virus would get access to the drug. Later however, the guidelines were modified and the physician could prescribe Oseltamivir on the basis of clinical presentations alone – if deemed appropriate – it was still under the 'Schedule X' category though^[1].

One of the most severe presentations in these patients was Acute Respiratory Distress Syndrome (ARDS). Over the next one year many studies indicating the roles of inflammatory pathways in the pathogenesis of H1N1 influenza were published^[2–4]. As more and more reports from across the world started emerging the role of inflammatory pathways in the pathophysiology of the disease was getting clearer. Antiviral therapy with Oseltaminivir was the mainstay in the management of the condition, however the

usage of Anti-inflammatory agents especially corticosteroids were also advocated for^[5,6]. Following several cases, over the years, across the globe, the use of corticosteroids has also been shown to not have any specific benefits. So as per the current guidelines the systemic use of corticosteroids in moderate to high dose are NOT recommended as adjunctive H1N1 treatment, by the WHO. In the meanwhile, the emergence of Oseltamivir resistant variants was being reported. This meant that the use of oseltamivir must become more judicious^[7].

Despite great progress in the management of ARDS with novel agents and sophisticated techniques, including antimicrobial drugs, extracorporeal membrane oxygenation, prostaglandins, nitric oxide, prostacyclin, exogenous surfactant administration and activated protein C, supportive treatment based mostly on advanced mechanical ventilation in the intensive care units seems to be the most important for the prognosis. Which is generally not available in resource limited settings. Novel anti-virals and better immune-management strategies are therefore the need of the hour.

Working at our institute also brought to our notice another disease, Rabies, highly noxious yet generally neglected. Although the disease finds a mention in the Ayurveda and also has a vaccine against it, the understanding of the pathophysiology of the disease is far from being achieved. Anté-mortem diagnostic assays are difficult to perform and therefore most cases go undiagnosed, especially in the rural set-ups, where the incidences are most abundant^[8]. Once clinical symptoms appear the disease is 100% fatal. The lack of an effective anti-viral agent is a huge gap in our preparedness against Rabies. Experimental therapy in the form of Milwaukee protocol looked encouraging to begin with, however over the years the success of this strategy has been abysmal. The positive results of Poly I:C and isoprinosine as immunomodulators in the management of Rabies in vivo, have also raised the possibilities of immunotherapy in

Rabies^[9]. Reports of enhanced Blood Brain Barrier (BBB) permeability in enhancing the effect of i.v Rabies Immuno-Globulin (RIG), has opened the possibilities of investigating the role of immunomodulatory agents as adjuvant therapy in management of clinical rabies^[10]. In their paper published in the Journal of Virology, Yin Huang and co-workers demonstrated that Parainfluenza virus 5 expressing the G protein of rabies virus protected 2 mice after rabies virus infection. They also demonstrated that recombinant RABVs expressing immune-modulating proteins (chemokines/cytokines), e.g. GM-CSF, can activate and recruit DCs in murine models and augment protective immune responses against RABV challenge when given before and after the challenge^[11]. Two major factors have been attributed behind this activity. First, the breaching of the BBB and second, the production of RABV neutralising antibodies in the brain. Such work further consolidated the hypothesis that a well-designed immune modulating system can become an effective tool to counter the effect of Rabies.

A variety of natural and synthetic molecules are known to modulate immune systems and/or have anti-viral activities. The system that I have been brought up, both personal and professional, has exposed me to a wide range of traditional and folklore medicines. The use of Neem (*Azadirachta indica*) or Haldi (*Curcuma longa*) over wounds, or the preparation of a concoction made of Tulsi (*Ocimum sanctum*) and honey to manage a sore throat has had an indelible impression on me. Using a paste of Doorva (*Cynodon dactylon*) or Ghritkumari (*Aloe barbadensis*) on skin rashes or an extract of Eranda (*Ricinus communis*) to treat fever or the quick use of Bael (*Aegle marmelos*) as an anti-spasmodic and carminative in weird hours of the day has always fascinated me. Hence it was an obvious choice to investigate from the wide and deep treasures of the Ayurveda and other Indian traditional medicinal systems, the potential of bringing about immune modulation or exhibiting anti-viral activities.

RATIONALE

Out of the known 200 viruses that infect humans only 9 have approved anti-virals. Given the fact that viruses are evolving rapidly, even these viruses might get resistant to the existing anti-viral drugs. Hence, discovery of novel anti-virals are a need of the hour. Also, many viral pathologies are as a result of a dis-oriented immune system of the host. Immunotherapy with antibodies and adjuvants have show promise with many conditions. Therefore, developing specific immunomodulating agents will help manage many viral diseases with or without anti-virals in the clinic.

Since the 3 plants – Mango, Bael and Eranda have been used in the management of many infections and other febrile illnesses. Also, most of the components exhibit promising anti-inflammatory activities. However, the signalling components modulated by these systems have not been studied. The present study assessed the potential anti-viral and immunomodulatory activities of the methanol extracts and protein extracts from the leaves of these plants. For the immunomodulatory activity, the effects were seen on the key signalling molecules which control many an important immune process.

HYPOTHESIS

Plant alkaloids, saponins, terpenoids have shown potential antiviral activities. The extracts of Mango, Bael and Eranda have been widely used in folklore and traditional medicine in the treatment of infections and inflammatory conditions. Many a viral-infections lead to hyper-inflammatory conditions along with breakdown of immune homeostasis.

We therefore hypothesize the following:

The methanolic and protein extracts of these plants if tested for their in-vitro antiviral activities, might inhibit Influenza and Rabies viruses in cell culture.

The extracts might also exhibit immunomodulatory activities, which might be assessed using various cellular, biochemical and molecular markers.

AIMS & OBJECTIVES

The aim of the project was to assess the anti-viral and immunomodulatory activities of the methanol extracts and protein extracts from the leaves of Mango, Bael and Eranda.

Objectives:

1. To collect and authenticate the plants
2. To prepare Methanol extracts and Protein extracts
3. To assess in-vitro anti-viral activity of the extracts against the Rabies Virus and the Influenza Virus
4. To assess the in-vitro immunomodulatory activity of the extracts
5. To perform in-silico docking analysis to study the interactions of known secondary metabolites from these plants against the Aryl Hydrocarbon Receptor (AHR) – an immunomodulatory soluble cytoplasmic receptor.

REVIEW OF LITERATURE

VIRUS DISEASE IN 21ST CENTURY

In less than 2 decades of its existence, the 21st Century has seen innumerable emerging and re-emerging viral infections. Not only are new species of viruses seen to infect humans, but many a previously known, existing viruses are presenting with unusual and complex pathologies. The fact that viruses – including human, animal, plant, fungal, bacterial etc. – have been emerging is endorsed by the listing in the International Committee on Taxonomy of Viruses (ICTV) reports. Wherein, in 1999 there were 1550 classified viral species in 64 families, the 2016 ICTV document lists 4404 viral species in 122 families. The table below enlists the data presented on the ICTV website regarding various ICTV releases across the years.

Year	Orders	Families	Subfam*	Genera	Species
2016	8	122	35	735	4404
2015	7	111	27	609	3704
2014	7	104	23	505	3185
2013	7	103	22	455	2827
2012	7	96	22	420	2617
2011	6	94	22	395	2480
2009	6	87	19	349	2285
2008	5	82	11	307	2079
2005	3	73	11	289	1899
2004	3	73	11	290	1832
2002	3	70	11	251	1619
1999	3	64	9	239	1550

Year	Orders	Families	Subfam*	Genera	Species
1998	3	63	9	233	2370
1997	2	56	9	197	2267
1996	2	53	9	182	2253
1995	1	50	9	166	2220
1993	1	49	9	160	1700
1991	1	40	9	142	1674
1990	1	40	9	137	1290
1987	0	37	8	116	1275
1984	0	35	8	103	1222
1982	0	29	8	97	1209
1981	0	29	8	93	1091
1979	0	24	8	84	1008
1978	0	24	7	76	760
1976	0	17	3	67	754
1975	0	17	1	63	309
1974	0	5	0	49	298
1971	0	2	0	43	290

Table 1: List of ICTV releases from 1971 until 2016. *Subfamily. Source: <https://talk.ictvonline.org/>^[12]

Although there has been great progress made in biomedical sciences in this period, most viral infections have 2 major clinical roadblocks. The first being the fact that specific clinical diagnosis of most viral infections is difficult since most clinical manifestations are non-specific and are shared among many a viral, bacterial and parasitic diseases. Also, the laboratory diagnosis of many viral infections is highly infrastructure intensive and are scarcely available in resource limited settings.

Nature and Course of Viral Infections

Viruses can exhibit varied nature of infection and pathological patterns. These include acute infections and chronic infections. Acute viral infections lead to a fast and dynamic interaction with the host, which ends in either resolution of the infection, or the virus kills the host. Chronic infections establish a dynamic equilibrium with the host. In chronic infections, the virus constantly adapts and evolves in respect to the immune pressures of the host. Eventually it establishes itself firmly in the host as a latent or persistent infection. However how some viruses manage to escape the host immune weaponry and cause an overt disease or remain associated inside the host over years together is still highly enigmatic.

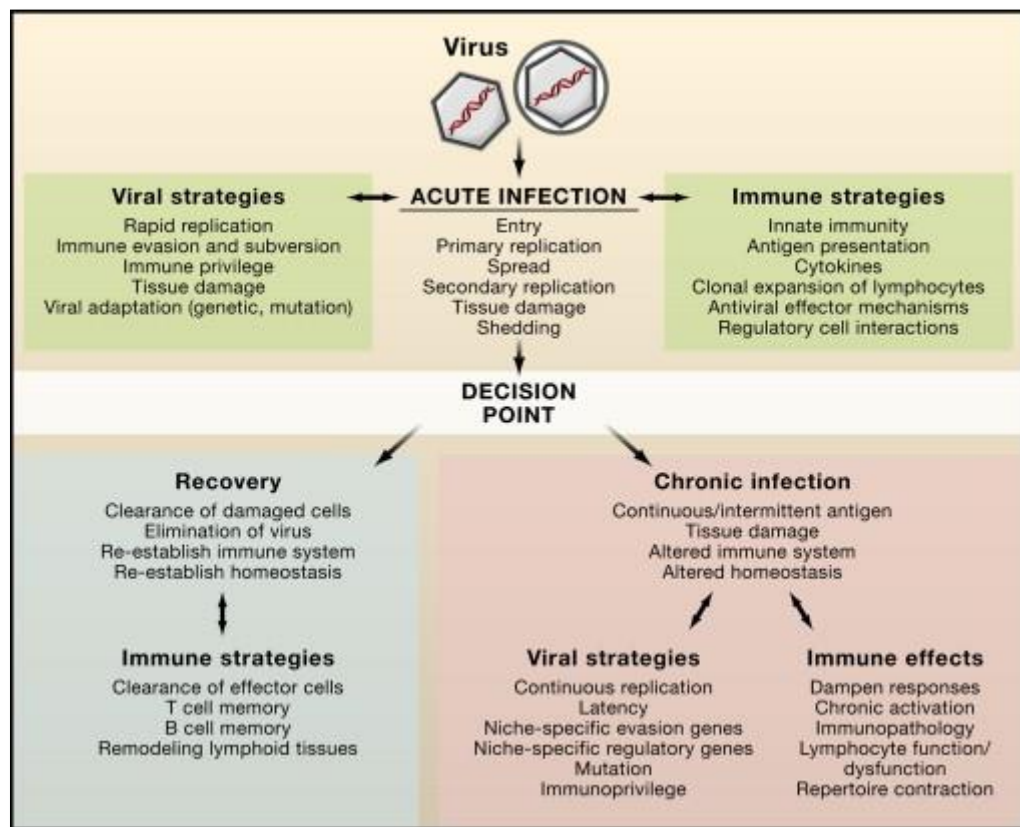


Figure 1: Natural course of Viral Infections. Viral infestations can lead to either acute or chronic infections. The outcomes of such infection are varied and based on the dynamic interaction between the viral and host genes. Either the infection is resolved or the virus kills the host or the virus establishes itself in the host as a latent entity^[13].

Most of our knowledge and understanding regarding virulence strategies of viruses and immune responses mounted by their hosts has come from studies on viral infections which are resolved by the host. Clearance of viral antigens using innate and adaptive defence mechanisms leads to the re-establishment of an uninfected state. Nevertheless, the formation virus specific memory T and B cells for long term immune response and antigen specific plasma cells to provide a continuous supply of antibodies provides for a much needed defence mechanism in event of a subsequent infection by the same virus. The host and viral factors, that direct acute versus chronic viral infections, however, differ distinctly^[13].

In order to establish chronic viral infection, two essential events must take place. First, the virus must be able to escape the neutralizing/sterilizing immune response mounted by the host. Second, an adjustment by the host immune system is needed in order to maintain status quo with the continuous inflammatory response elicited by the constant presence of the viral antigen. This inflammatory response is required to restrict the viral replication to tolerable levels without any major damage to the permanently infected tissue. In the event that the immune system fails to eliminate the virus, uncontrolled immune attack on virus antigen-bearing cells causes immunopathology. Hence, downregulating inflammatory cascades during chronic viral infections can result in reduced tissue damage, at least for noncytopathic viruses. Immunopathology can be serious in animal models and has been implicated in human chronic infections caused by hepatitis B virus (HBV) and hepatitis C virus (HCV) (Figure 2). It is critical that viruses that rely on a living but chronically infected host for their own survival must carefully circumvent mechanisms that overpower immunity and kill their hosts. In fact, given the swift pace of viral evolution, it is logical to say that viruses that are relatively

clinically benign, despite establishing chronic infection, would have evolved to greater virulence if this was advantageous.

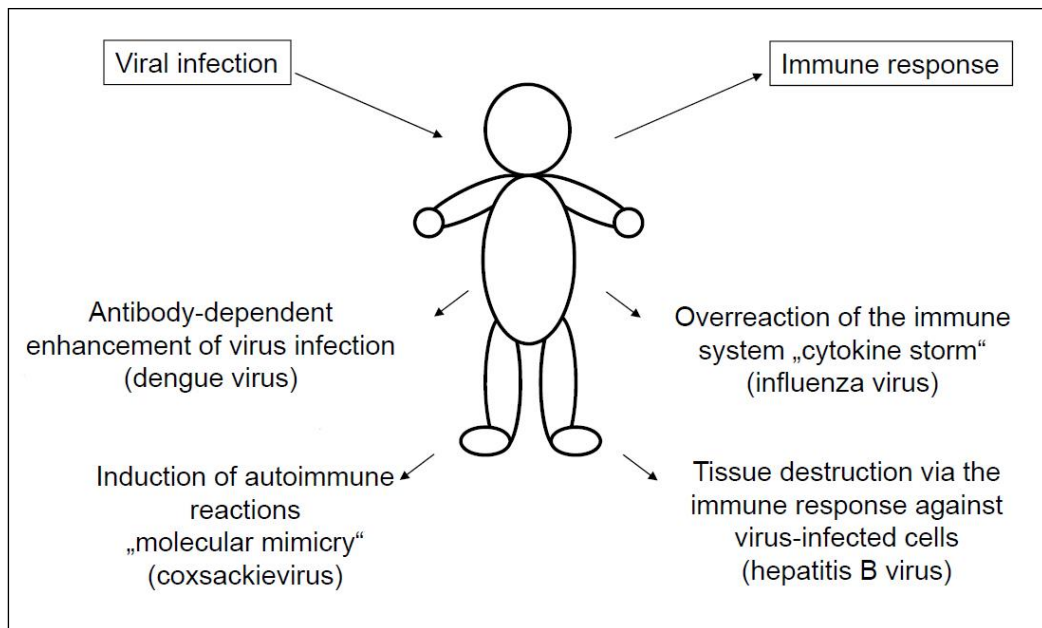


Figure 2: Examples of Immunopathology of Viral Infections. Source: <http://www.uniklinikum-jena.de>^[14]

There are multiple ways in which a virus can elicit immune reactions which may turn pathological. These include, Antibody Dependent Enhancement (ADE) as seen in Dengue Infection^[15], induction of Cytokine Storm reaction, as seen in influenza^[4], Induction of Auto immune reactions via molecular mimicry^[16], as seen with coxsackieviruses and tissue destruction via the immune response against virus-infected cells as seen in hepatitis B virus infection^[17].

Virus, Nucleic Acid type	1° Site of Persistence (Organ or Cell)	Acute Infection Examples	In Normal Hosts*	In IC Hosts*
Endogenous retroviruses (ERV), DNA	All	Not applicable	Unknown	Unknown
Anellovirus/Circovirus, DNA	Many tissues	Unknown	Unknown	Unknown
Human herpesvirus 6 (HHV-6)	Lymphocytes?	Roseola	Unknown	Meningoencephalitis, secondary infections, immunomodulatory?

Virus, Nucleic Acid type	1° Site of Persistence (Organ or Cell)	Acute Infection Examples	In Normal Hosts*	In IC Hosts*
Human herpesvirus 7 (HHV-7), DNA	Lymphocytes?	Roseola	Unknown	Unknown
Varicella zoster virus (VZV), DNA,	Sensory ganglia neurons and/or satellite cells, lymphocytes	Chicken pox	Herpes zoster	Disseminated disease, hepatitis, pneumonitis
Cytomegalovirus (CMV), DNA	Myelomonocytic cells	Mononucleosis	Rare	Disseminated disease, vasculitis, retinitis, pneumonitis, hepatitis, gastroenteritis, meningoencephalitis
Epstein-Barr virus (EBV), DNA	Pharyngeal epithelial cells, B cells	Mononucleosis	Burkitt's lymphoma, nasopharyngeal carcinoma, non-Hodgkin's lymphoma	CNS lymphomas, oral hairy leukoplakia, lymphoproliferative disease
Polyomavirus BK, DNA	Kidney	Unknown	Unknown	Hemorrhagic cystitis (post bone marrow transplantation), nephropathy (post kidney transplantation)
Polyomavirus JC, DNA	Kidney, CNS	Unknown	Unknown	Progressive multifocal leukoencephalopathy
Adeno-associated virus (AAV), DNA	Many tissues	Unknown	Unknown	Unknown
Herpes simplex type 1 (HSV-1), DNA	Sensory ganglia neurons	Pharyngitis, encephalitis, keratitis,	Cold sores, encephalitis, keratitis	Increased severity of same diseases, hepatitis pneumonitis
Adenovirus, DNA	Adenoids, tonsils, lymphocytes	Upper respiratory infection, gastroenteritis	Unknown	Enteritis, haemorrhagic cystitis, pneumonitis, hepatitis, others
Herpes simplex type 2 (HSV-2), DNA	Sensory ganglia neurons	Genital herpes	Genital herpes, encephalitis	Increased severity of same diseases
Kaposi's sarcoma herpesvirus (KSHV) or human herpesvirus 8, DNA	Endothelial cells, B cells	Unknown	Castleman's disease, Kaposi's sarcoma	Kaposi's sarcoma, primary effusion lymphoma
Hepatitis B virus (HBV), DNA	Hepatocytes	Hepatitis	Cirrhosis, hepatocellular carcinoma	Same diseases
GB virus C, RNA	Lymphocytes	Unknown	Unknown	Unknown

Virus, Nucleic Acid type	1° Site of Persistence (Organ or Cell)	Acute Infection Examples	In Normal Hosts*	In IC Hosts*
Papilloma virus, DNA	Epithelial skin cells	Unknown	Papilloma, cervical and other mucosal carcinomas	Increased severity and incidence of same diseases
Hepatitis C virus (HCV), RNA	Hepatocytes	Hepatitis	Cirrhosis, hepatocellular carcinoma	Same diseases
Human immunodeficiency virus (HIV-1 and HIV-2), RNA	CD4 ⁺ T cells, monocyte/macrophages	Acute febrile illness	AIDS	AIDS
Hepatitis D virus (HDV), RNA	Hepatocytes	Unknown	Exacerbation of chronic HBV infection	Unknown
Human T cell leukemia virus type 1 (HTLV1), RNA	T cells	Unknown	Adult T cell leukemia (2%–6% of carriers), tropical spastic paraparesis, myelopathy, uveitis, dermatitis	Unknown
HTLV II, III, IV, RNA	T cells	Unknown	Unknown	Unknown
Polyomavirus MC, DNA	Merkel cell carcinoma	Unknown	Merkel cell carcinoma?	Unknown
Rubella virus, German measles, RNA	CNS	Rubella, arthritis	Progressive rubella panencephalitis	Unknown
Parvovirus B19, DNA	Bone marrow erythroid progenitors	Fifth disease, arthritis	Aplastic crisis in hemolytic anemia, hydrops fetalis, chronic bone marrow deficiency	Red cell aplasia
Measles virus, RNA	Neurons and supporting cells in CNS	Measles	Subacute sclerosing panencephalitis, measles inclusion body encephalitis	Unknown
Coxsackie, RNA	Myocardial cells	Hand foot and mouth disease, herpangina	Myocarditis	Unknown

Table 2: The first column lists viruses known to cause chronic infection in humans and their primary nucleic acid type. Additional columns enlist identified sites of persistent infection and the diseases associated with infection. The exact sites of persistence are incompletely defined for many viruses. The associated clinical syndromes presented are not an exhaustive list. CNS, central nervous system. IC, Immunocompromised^[13]

THE INFLUENZA AND THE RABIES VIRUSES

Two viruses whose management strategies are very different, but which pose immense public health problems are the Influenza Virus and the Rabies Virus,

The Influenza Virus

The Influenza virus is a part of the *Orthomyxoviridae* family. Viruses of this family are enveloped viruses with segmented genomes. The genome consists of single stranded RNA with a negative orientation. Currently, Alphainfluenzavirus, Betainfluenzavirus, Deltainfluenzavirus, Gammainfluenza, Thogotovirus, Isavirus and Quaranjavirus are the seven genera that make up the *Orthomyxoviridae* family. Deltainfluenzavirus, Isavirus and Quaranjaviruses are relatively new addition to the family. From the public health perspective, Influenza A viruses, belonging to the Alphainfluenzavirus genus is the most important for humans. The Influenza A virus are present as various subtypes. In the present form of nomenclature subtypes are described as the host from which it was primarily isolated, geographic location of the primary isolation, strain number, year of isolation and, the serological characteristics of two viral proteins: hemagglutinin (HA) and neuraminidase (NA) are included in parentheses. When the strains isolated from humans, the host name is omitted. As an example, A/Sw/Taiwan/2/72 (H3N2) should be read: influenza A strain number 2, isolated from swine in Taiwan in 1972, which contains hemagglutinin HA3 and neuraminidase NA2. Till date, 18 serotypes of hemagglutinin and 11 serotypes of neuraminidase are described.

Structure of Influenza A Viruses

Orthomyxovirus particles have a shape which almost resembles a sphere, with size ranging from 100 – 200 nm in diameter. However, long threadlike structures, as long as

upto 1000 nm have also been described in certain laboratory strains. The virus particles are not very stable in the environmental conditions and the virions are easily inactivated by detergents, extreme pH, heat and desiccation. The viruses derive their envelope from the host cell plasma membrane in which they replicate. Embedded into the envelope, are about 500 projections per virions. These are primarily the rod-shaped hemagglutinins, which make up 80% of the total projection. The remaining molecules belong to neuraminidase, which roughly present with mushroom like structures. The envelope of the virus also contains some M2 proteins, which act as ion channels in the virus articles. The M1 protein acts as the matrix under the lipid envelope and is critical to the attachment of the ribonucleoprotein (RNP). It is the most abundant protein. The core of the virus is composed of the RNP, which is a complex structure made up of the Nucleoprotein (NP) binding to the 8 different segments of the viral genome. In addition, the RNP consists of the RNA-dependent RNA polymerase (RdRP). The RdRP of the Influenza A virus is constituted by 3 proteins, namely PA, PB1 and PB2.

Growth and Cultivation of Influenza virus

The first Influenza viruses were cultivated and isolated from pigs in 1930, thereafter the first human isolates were obtained in 1933. In the beginning, embryonated chicken eggs were the sole systems used for the virus cultivation. This method is still preferred for cultivation of the viruses for vaccine production, where large quantities of the virus stock is needed. Currently, most viruses are grown on the MDCK (Madin-Darby Canine Kidney cells). However, most subtypes of the influenza viruses require pre-treatment with trypsin to produce infectious particles. This protease cleaves hemagglutinin into active subunits, which is a prerequisite for the efficient propagation of the virus.

Replication of the Influenza A viruses

The major milestones of the influenza viruses are like most ssRNA virus with negative RNA polarity. These may be distinguished into the following steps:

1. Adsorption of the virus onto host cells, entry and uncoating
2. Synthesis of mRNA and replication of viral RNA
3. Post-transcriptional modification of viral mRNA
4. Synthesis and post-translational modification of viral proteins
5. Assembly of virus particles and release of virus from cells

One or more viral proteins play a crucial role in accomplishing the above steps. Also, it is important to note that certain host proteins are critical in aiding the viral proteins to carry out their functions.

The Virus Life Cycle

The influenza virus binds onto the host cell when the Hemagglutinin (HA) binds with the sialic acid associated with the glycoproteins or glycolipids on the host cell membrane.^[18] Most influenza virus strains infecting humans (viz. H1N1 and H3N2) selectively recognize the sialic acid linked to galactose by a α 2,6 Glycosidic linkage (SA α 2,6 Gal), abundant in the epithelial cells of the respiratory tract.^[19] On binding to the membrane receptor, the virus enters the host cell by a process of endocytosis. On being released into the cytoplasm, its membrane is fused to that of the endosome. Viral HA plays a vital part in this course, because when it is cleaved by the host proteases^[20] region known as “fusion peptide” is exposed. The fusion peptide cross talks with the endosome membrane, ensuing the fusion of the membranes and the release of the virion components into the cytoplasm.^[21–23] A critical requirement for this event is the acidification of the endosome. This is catalysed by the M2 ion channel protein. H^+

ions enter through this channel and make the M1 matrix protein to dissociate from the RNP complex of the virus, which is then released to the cytoplasm for consequent nuclear transport.^[24–26] On entering the nucleus, messenger RNA (mRNA) is transcribed from the viral RNA. The mRNA is then polyadenylated. This is essential for the expression of viral proteins.^[27] Translation of the Viral proteins are carried out using host cell machinery, and when the PA, PB and NP proteins have been made, they are transported into the nucleus to enable the transcription and replication of the viral RNA. RNP complexes are then exported from the nucleus, this process is facilitated by non-structural protein 2 (NS2), nuclear export protein (NEP) and M1. Glycosylation of HA, NA and M2 are performed in the endoplasmic reticulum and transported via the Golgi complex to the plasma membrane.^[28] The RNPs and the 8 viral RNA segments are transported to the plasma membrane, where they are packaged to generate viral particle buds. Several structural viral proteins, such as HA, NA and M2, interact with the cell lipid membrane, thus contributing to the process of budding.^[29] This process is achieved when the membranes fuse in the base of the virus bud and the virus is shed by the action of NA, which catalyses the release of sialic acid from the surface glycoproteins.

Treatment

Drugs Currently in Use

Of the 5 anti-influenza drugs currently available, only 3 are recommended by the U.S. Food and Drug Administration for this season: oral oseltamivir (Tamiflu®), inhaled zanamivir (Relenza®), and intravenous peramivir (Rapivab®), all of which are NA inhibitors. M2 ion channel inhibitors, amantadine and rimantadine, are not

recommended as circulating viruses have a high rate of resistance of these compounds and are ineffective against type B and C influenza viruses.

M2 Ion Channel Inhibitors

Adamantane derivatives, such as amantadine and rimantadine, have known anti-influenza A activity, and were the first choice in the treatment of influenza for many years. They act by binding to a specific pocket on the viral M2 protein, stabilizing its closed conformation and preventing the virus from releasing the ribonucleoprotein complex to the cytoplasm after fusion, thus halting the viral cycle.^[30] Amantadine has also been shown to affect the pH of the vesicles that transport the viral glycoproteins, thus interfering with the assembly process.^[31]

Viral resistance to these drugs has led to the development of new structurally related compounds, such as azoadamantanes.^[32] However, none of the drugs in this group has demonstrated activity against all circulating amantadine-resistant viruses.

Neuraminidase Inhibitors

These compounds are active against influenza A and B; oseltamivir and zanamivir are the oldest, and peramivir and laninamivir belong to the new generation. These drugs prevent the cleavage of sialic acid, inhibiting virion release and preventing the dissemination of new viral particles to other cells.^[33] Less than 2% of worldwide circulating viruses in 2013–2014 were resistant to NA inhibitors; however, viruses that are highly resistant to these drugs have been identified in localized communities (Japan, China, Australia).^[34] New, promising NA inhibitor molecules are being developed with

activity against strains which have shown resistance to currently available drugs.^[28,35–37]

In clinical practice, NA inhibitors are the only anti-influenza drugs currently recommended; the most widely used of these is oseltamivir, given its good oral bioavailability. However, the interpretation of clinical studies that support its efficacy is highly controversial. A metaanalysis by the Cochrane group in 2014 revealed a modest benefit from oseltamivir treatment in cases of mild influenza, with reduced symptom duration from 7 to 6.3 days in adults and reduced symptoms in healthy children; however, no benefit was demonstrated in children with asthma. Hospitalization rates in patients receiving oseltamivir was similar to that in untreated patients.^[38] A metaanalysis published in the Lancet in 2015 found that for mild cases of confirmed influenza infection, oseltamivir treatment reduced duration of symptoms, lower respiratory tract infections, and hospital admissions. Both studies reported nausea and vomiting as the main side effects.^[39] While no randomized clinical trials have been performed in severe forms of influenza, observational studies suggest that oseltamivir significantly reduces mortality in serious cases.^[40–42] Clinical practice guidelines recommend the early initiation of antivirals for the treatment of severe cases of confirmed influenza that require hospitalization or in patients with a risk of serious complications, such as those aged less than 2 or more than 65 years, with chronic lung disease, immunosuppression or morbid obesity, and pregnant women or women who have given birth within less than 2 weeks. Post-exposure chemoprophylaxis can be considered in individuals with a high risk of complications or who have not been immunized, and should always be started within 48 h of exposure. The first line of treatment is oseltamivir, while inhaled, nebulized or intravenous zanamivir is indicated for

oseltamivir-resistant strains or in subjects with poor intestinal absorption. The indicated dose of oseltamivir is 75 mg twice a day for treatment and once a day for chemoprophylaxis, while inhaled zanamivir should be administered at 10 mg twice a day for treatment and once a day for chemoprophylaxis; zanamivir is not recommended in patients with asthma or COPD. Treatment must be administered for 5 days, but duration can be extended in severe cases; recommended prophylaxis duration is 10 days, for both oseltamivir and zanamivir.^[43]

Other Pharmacological Groups

A considerable number of potential antiviral agents are currently being tested, but these are not yet available for use in the clinic. The most important groups are described below.

Drugs that Act Against Viral Components

Inhibitors of Viral Binding and Fusion. Synthetic viral HA inhibitors: This group consists of a variety of molecules recognizable by the viral HA, thus interfering in virus–cell interaction. Examples of these are MBX2329 (an aminoalkyl phenyl ester) and MBX2546 (a sulfonamide), which inhibit influenza A virus entry by binding to the HA stem region, interfering with viral fusion.^[44] Other studies have used synthetic sialic acid analog peptides that are recognized by the viral HA and inhibit its action,^[45] and fusion peptide antagonists that interrupt the conformational change in HA needed for the correct fusion of the virus to the cell.^[46]

Arbidol: A synthetic drug currently approved for the treatment of influenza in Russia and China, but not in the US, given the lack of clinical evidence.^[43] This is a

hydrophobic molecule that penetrates the virus lipid membrane and interacts with the membrane phospholipid and transmembrane proteins enriched in aromatic residues from the viral envelope, interfering with entry and host cell fusion processes.^[47],⁵⁴ A multicenter randomized clinical study (ARBITR) in influenza patients treated with arbidol showed a shorter disease duration with reduced severity and virus shedding.^[48]

Resistance to arbidol has been identified in some influenza strains with HA2 subunit mutations that allow the virus to continue the process of fusing to the endosome membrane.^[49] The synthesis of structurally related compounds paves the way for the development of new antivirals, such as synthetic indoles that have shown greater antiviral potency than arbidol against certain influenza A subtypes in in vitro studies.^[50]

Anti-HA antibodies: Monoclonal antibodies targeting highly conserved HA sites have been developed, permitting neutralization of influenza virus strains. For example, CH65 antibody obtained from a patient who received anti-influenza vaccination in 2007, has been shown to be effective in vitro against a wide spectrum of H1N1 influenza strains, due to its binding to the HA1 subunit receptor pocket.^[51] D1-8 antibody has shown efficacy in neutralizing different strains of H3N2 influenza virus in vitro and in vivo, achieving greater survival in mice compared to treatment with oseltamivir.^[52] Antibodies targeting the HA protein stem may also be effective against the influenza virus. HB36.6 showed efficacy against different strains of H1N1 and N5N1 in vitro, while in mice, it was able to reduce viral replication and improve survival.^[53]

Viral Polymerase Inhibitors. Viral polymerase is a protein that remains highly conserved among the various influenza strains, making it a therapeutic target of interest.

Favipiravir (T-705) is a molecule that can convert to a nucleoside analog, ribonucleotide T-705-4-ribofuranosyl-5'-monophosphate, within the cell, inhibiting viral RNA polymerase activity without affecting cell synthesis of RNA or DNA.⁶¹ It has demonstrated efficacy against various influenza strains.^[54–56]

PB2 inhibitors: A 5' cap initiator is needed for the translation of mRNA, which the virus “steals” from host pre-mRNA. This process requires the PB2 subunit to bind to the 5' cap of the host pre-mRNA, with subsequent cleavage of the 5' end by the PA protein with endonuclease activity. Drugs that interfere with this process are currently under study and show great antiviral potential.⁶⁷ One example is VX-787, which has shown in vitro activity against several classes of influenza A, and is effective both as prophylaxis and treatment.^[57]

Nucleoprotein Inhibitors. Nucleoprotein binds to viral RNA and forms part of the ribonucleoprotein complex. It is essential for the synthesis of viral RNA and also participates in the nuclear export of viral ribonucleoproteins and in cytoplasmic trafficking.^[58]

Nucleozin: This is the most widely studied drug of the group. It has demonstrated antiviral activity at various stages of the viral cycle: at early stages it inhibits viral RNA transcription and protein synthesis, and at the later stages it blocks cytoplasmic

trafficking in the new ribonucleoproteins.^[59] Nucleozin analogs showing antiviral potential against different influenza A species have recently been developed.⁷²

Naproxen: This is a cyclooxygenase 2 inhibitor which has recently revealed anti-influenza activity. It has been shown to form complexes with NP and to competitively inhibit NP binding to RNA. It has in vitro and in vivo activity against influenza A virus.^[60] Naproxen derivatives with antiviral potential have also been synthesized, and are currently under study.^[61]

NS1 Protein Inhibitors. NS1 is a multifunctional protein that binds to mRNA and regulates post-transcription processes: it prevents the activation of the PKR kinase,^[62]^[63] and inhibits nuclear export of polyadenylated host mRNA.^[64]–79 NS1 also protects the virus from antiviral cell defense by inhibiting the interferon-mediated response.^[65–67] Several other drugs that inhibit NS1 activity have also been developed.^[68] For example, NSC125044 and its derivatives^[69] including the compound JJ3297, affect viral replication in vitro, re-establishing the antiviral effect of interferon.^[70] Baicalin has shown in vitro and in vivo anti-influenza activity by altering the binding domain of the NS1 protein.^[71]

Drugs that Act Against Host Components

Insight into the cell functions on which the viral cycle depends has led to the identification of new therapeutic targets in the design of antiviral drugs. Host response modulation is a strategy that minimizes the development of resistant strains, which are not affected by genetic variability in the virus. However, there is a risk of treatment-related side effects. Research into host–influenza interaction has uncovered an

extensive network of more than 130 interactions between 10 viral proteins and 87 human proteins.^[72]

More recent analyses have identified 91 host factors that, if inhibited, prevent viral replication in vitro.^[73] Computerized analysis of drug databases returned hundreds of molecules that interact with host factors necessary for viral replication; most of these are drugs currently in use for other therapeutic purposes.⁸⁹

Viral Binding and Fusion. DAS181 (Fludase®): DAS181 is a sialidase catalytic domain/amphiregulin glycosaminoglycan binding sequence fusion protein which cleaves residual sialic acid from the cell surface, preventing the virus from entering the cell. It has shown activity against influenza A and B in animal experiments; however, clinical studies have shown no efficacy in improving influenza symptoms.^[74,75]

Aprotinin: A polypeptide that inhibits host proteases that cleave viral HA. This interferes with the process of viral cell binding and fusion, and anti-influenza activity has been observed both in vitro and in vivo.^[76] Other synthetic compounds that have been developed recently are respiratory epithelium serine protease inhibitors, showing anti-influenza activity in vitro.^[77]

Endocytosis and Fusion. Glycyrrhizin: This compound has shown anti-influenza A activity by altering the stability of the host cell lipid membrane, preventing endocytosis in enveloped viruses.^[78–80]

LJ001: This molecule has a similar mechanism of action to glycyrrhizin and acts on virus-cell fusion. It has wide-spectrum activity against enveloped viruses, including influenza A^[81]

Bafilomycin A1 and concanamycin A: Macrolide antibiotics that inhibit vacuolar H⁺ ATPase action that pumps protons from the cell cytoplasm to the interior of the endosome.^{99,100} More recently, saliphenylhalamide, another vacuolar H⁺ ATPase inhibitor has been studied, showing antiviral efficacy.^[82]

Viral Budding and Release. The enzyme farnesyl diphosphate synthase, which participates in the synthesis of lipid compounds of the plasma membrane, is inhibited by the protein viperin, the expression of which is induced by interferon. This inhibition has been shown to change the composition of the cell plasma membrane, interfering with assembly and budding of viral particles. The enzyme farnesyl diphosphate synthase has been proposed as a potential target for the development of drugs with anti-influenza activity.^[83]

Viral RNA Transcription and Transport. The chaperone protein Hsp90 participates in the nuclear import of viral RNA and in viral polymerase assembly, and has been shown to bind to PB2, with which it is transported into the nucleus, modulating interaction between polymerase basic protein 1 and PA.^[84] Hsp90 inhibitors, such as geldanamycin and its synthetic derivative 17-AAG, have shown activity against influenza virus in cell cultures.^[85]

Ribavirin and its precursor viramidine are wide-spectrum antivirals. Ribavirin (Virazole®), used in the treatment of hepatitis C, is a guanosine nucleotide analog. Its principal mechanism of action is competitive inhibition of the enzyme inosine 52 monophosphatase dehydrogenase, affecting GTP biosynthesis, and consequently, viral

RNA synthesis and viral protein production.^[86] Despite the positive results obtained in animal studies,^[87] no efficacy supporting its use has been demonstrated in clinical trials.^[88]

Viral Ribonucleoprotein Complex Export and Post-Transcriptional Processes. Verdinexor (KPT-335): A molecule that inhibits the protein exportin 1, needed for the release of the viral ribonucleoprotein complex from the nucleus to the cytoplasm. It has been shown to possess antiviral activity against several influenza strains.^[89]

Nitazoxanide: An antiparasitic drug that inhibits the maturation of viral HA, and is active against various strains of influenza A virus.^[90] A randomized clinical study showed a reduction of symptoms in patients with non-complicated influenza infection.^[91]

Intracellular Defence Mechanisms. The Raf/MEK/ERK pathway belongs to the family of mitogen-activated protein kinases – MAPK – and is activated by influenza viruses. Blocking this pathway inhibits the nuclear export of ribonucleoprotein due to the interaction with the viral nuclear export protein.^[92,93] In vivo experiments show that administration of U0126, an MEK inhibitor, reduces viral load in lung tissue and improves survival in mice infected with influenza.^[94] The combination of MEK inhibitors and oseltamivir increases the antiviral efficacy in vitro of the latter.^[95]

The NF- κ B transcription factor pathway regulates the expression of antiviral cytokines, and the activation of this pathway has been associated with an increase in influenza

virus replication.^[96,97] Acetylsalicylic acid, which inhibits IKK2, the activator kinase of I B, has an antiviral effect, modifying the nuclear export of viral ribonucleoprotein.^[98] Modulation of this pathway is a promising approach for the development of new treatments.

Other Agents with Potential Anti-Influenza Activity. A wide range of drugs used for other diseases have been identified as inhibitors of influenza virus replication in in vitro trials. For example, Na,K-ATPase inhibitors have been shown to possess antiviral activity against different types of DNA and RNA viruses. Cardiac glycosides, specific inhibitors of Na,K-ATPase, have potent in vitro activity against cytomegalovirus and other herpes viruses; ouabain in particular has been seen to inhibit viral protein synthesis.^[99,100] Ebola virus can also be inhibited in vitro by ouabain¹²¹ ; the anti-coronaviral effect of ouabain is mainly a result of its activating the Src kinase intracellular signalling pathway and inhibiting the fusion process.^[101] Reoviruses are also sensitive to the viral fusion-altering action of ouabain.^[102] Digoxin, another Na,K-ATPase inhibitor, is active in vitro against the vaccinia virus.^[103] Different members of the family of cardiac glycosides have recently been found to inhibit influenza A virus replication in vitro.^[104,105]

In summary, the treatment of influenza continues to be a challenge. Current drugs target viral components, so their efficacy may be affected by genetic changes in the influenza virus itself. In recent years, a great number of drugs aimed at modulating host cell response have been investigated and developed. Since these are effective against

different viral strains and minimize the emergence of resistant species, these compounds constitute promising new agents in antiviral therapy

Drugs currently in use	<i>Neuraminidase inhibitors</i>
<i>M2 ion channel inhibitors</i>	Oseltamivir
Amantadine	Zanamivir
Rimantadine	Peramivir
Other pharmacological groups	Laninamivir
<i>Drugs that act against viral components</i>	<i>Drugs that act against host components</i>
<i>Viral binding and fusion inhibitors</i>	<i>Binding inhibitors</i>
MBX2329	DAS181 (Fludase®)
Arbidol	Aprotinin
Antibodies: CH65, D1-8, HB36.6	<i>Endocytosis and fusion inhibitors</i>
<i>Viral polymerase inhibitors</i>	Glycyrrhizin
Favipiravir (T-705)	LJ001
VX-787	Bafilomycin A1
<i>Nucleoprotein inhibitors</i>	Concanamycin A
Nucleozin	Saliphenylhalamide
Naproxen	<i>Viral RNA transcription and transport inhibitors</i>
<i>NS1 protein inhibitors</i>	Geldanamycin
NSC125044	17-AAG
JJ3297	Ribavirin
Baicalin	Viramidine
	<i>Viral ribonucleoprotein complex export and post-transcriptional processes inhibitors</i>
	Verdinexor
	Nitazoxanide
	<i>Intracellular defense pathway inhibitors</i>
	U0126
	PD-0325901
	AZD-6244
	AZD-8330
	RDEA-119
	Acetylsalicylic acid
	<i>Other agents with anti-influenza activity</i>
	Ouabain

Figure 3: Anti-Influenza Treatment: Drugs Currently Used and Under Development^[106]

RABIES VIRUS

Rabies virus is unique in its mode of transmission via an animal bite and in its spread through the nervous system. After inoculation, the virus infects individual muscle cells and gains access to both sensory and motor nerve fibre endings. The neuromuscular junction is the major site of entry into neurons. The virus spreads to the CNS by retrograde axonal transport, replicates within neuronal cell bodies and rapidly disseminates through the CNS by axonal pathways, including trans-synaptic spread^[107]. Vaccines are not available for majority of the viruses causing encephalitis. There is a stable vaccine for rabies virus but once the virus gains entry into the CNS, vaccine is not effective. It is fatal. Antiviral drugs are the remedy for treatment of viral encephalitis but there are not many known potent antiviral drugs. Acyclovir and its derivative is an effective drug against Herpesviruses. Ribavirin has a broad antiviral effect against flavivirus, hepatitis and respiratory viruses. There are no effective antiviral drugs available for rabies virus. Rabies infection is fatal once the virus enters the CNS. There was only one reported case of a female survival from rabies virus by inducing coma and treating her with combination of known antiviral drugs^[108]. Therefore, the search for more effective antiviral agents against viral infections of the CNS is a necessary and highly desirable task.

Within the past decade therapeutic options for viral infections have improved significantly, however, there has also been emergence of resistant viruses as well. Furthermore, many of the licensed drugs are toxic as well as being expensive, thus the search for potential sources for the development of new drugs is very important. Based on ayurveda, siddha, unani and other traditional herbal medicine, several antiviral studies have been performed to detect active natural products in higher plants. The ayurvedic management of encephalitis consists of treating the basic cause for the

condition and preventing complications like neurological deficits and death. Viral, bacterial and fungal infection can be treated by using ayurvedic medicines in combination with modern (Allopathic) medicines. It is long known that many traditional medicinal plants have strong anti-microbial and antiviral activity.

Genome of the Rabies Virus:

The rabies virus (RABV) has an enveloped, bullet-shaped structure. RABV virions are typically 180 nm long (100-300 nm depending on strain) and 75 nm wide. Its genome is single-stranded, non-segmented and negative-sense. The length of the genome is approximately 12,000 nucleotides^[109].

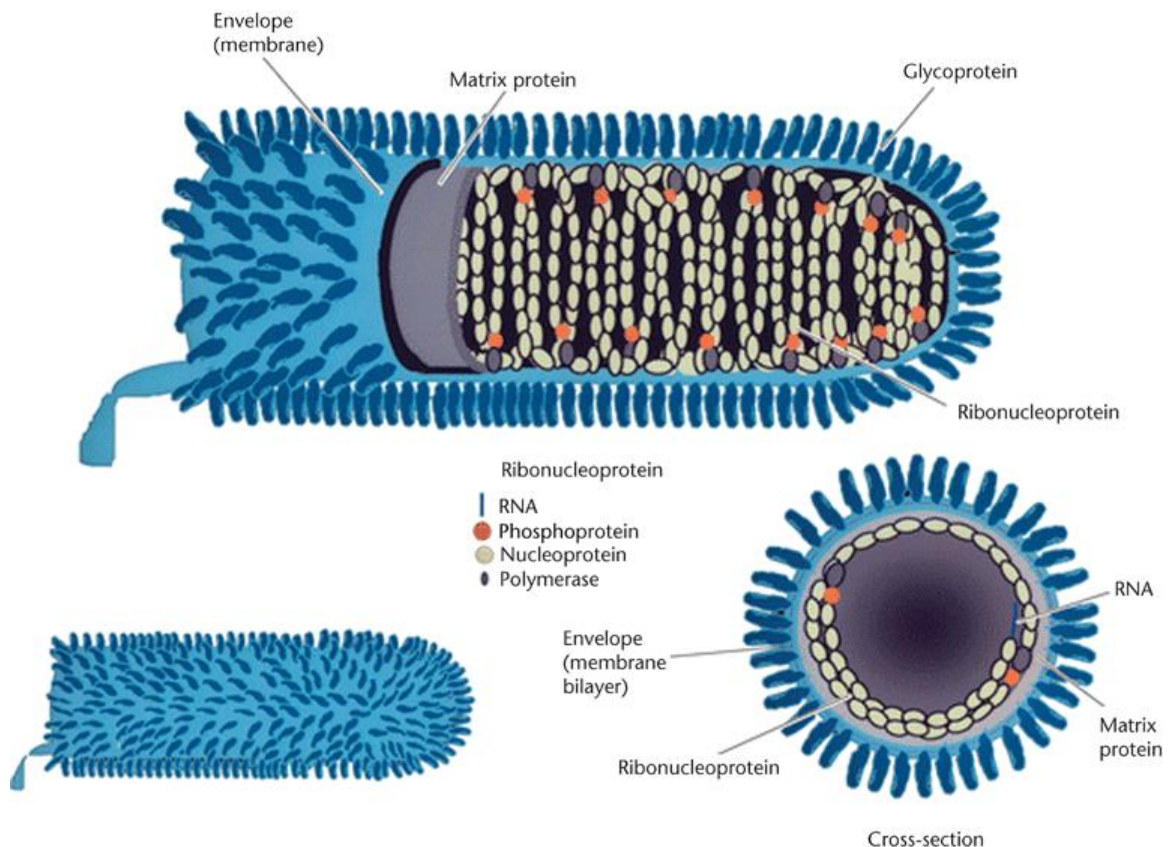


Figure 4: Structure of Rabies Virus. Adopted from: <http://www.healthdiseaseblog.com/>^[110]

The genome encodes for five different proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and large polymerase or large protein (L). Viral RNA is encapsidated by the nucleoprotein N to form a helical nucleocapsid. The nucleocapsid is further associated with phosphoprotein P and

some copies of large protein or polymerase L. The RNA together with N, P and L makes the ribonucleoprotein (RNP). The glycoprotein G and matrix protein M are membrane-associated proteins. They surround RNP by wrapping it into lipid bilayer. M protein is located beneath the viral membrane and connects the nucleocapsid and lipid bilayer. G protein plays a crucial role in stimulating virus neutralizing antibodies (VNA) and in process of RABV pathogenesis.

1. The RNA dependent RNA polymerase L protein (180KD):

This is the giant protein of 2142 and 2127 amino acids in the PV and SAD-B19 strains of rabies virus respectively, occupies more than half (54%) of the rabies genome. L protein shows the sequence homology with other members of the order *Mononegavirales*.

2. Glycoprotein G exists in two forms GI (65KD) and GII (70KD):

The G protein is the best studied of the rabies proteins, at both the structural and immunological levels. It is 524 and 522 amino acids long in rabies and Mokola virus, respectively. The G protein is involved in the pathogenesis of rabies and is believed to assume at least part of the neurotropism of the virus. At least eight antigenic sites have been located on the external domain of the G protein of the different rabies strains (I-VI, “a” and G1. The epitope site III of the rabies virus glycoprotein appears to be the major antigen responsible for protection. The proteolytic treatment of the virion removes the spike like projections and selectively affects the G protein. Only a small G protein remains linked with the spikeless particle indicating that the G protein is membrane anchored and constitutes the main component of the viral spikes. The spikes that contain the envelope glycoprotein are

attached to the core particles probably with the help of viral lipids. Each spike consists of three associated G proteins (trimers). There are about 1200 G protein (400 trimers) per virus particle. There are three sugar chains that are N-glycosidically attached. Penetration of the virus into the cytoplasm takes place in the endocytic pathway and not at the plasma membrane. This is because the G protein trimer undergoes a change in conformation at pH 6.1, thus stabilizing the trimer and probably allowing the hydrophobic region of the molecule to become exposed and to get embedded in the membrane of the cell to be infected.

The virus G protein is responsible for induction of virus neutralizing antibodies and protection of animals against challenge, stimulation of specific T cells, which express helper, suppressor or cytotoxic activities and determination of virulence. Because of its multiple immunogenic function, this G protein is potentially suitable for vaccination purposes. Antigenic determinants carried on the G protein vary substantially among the rabies virus strains.

3. Nucleoprotein:

This is a 450-amino acid long polypeptide which is phosphorylated on a serine residue in position 389. One function of this protein is to encapsidate and protect the genome. Monoclonal antibodies directed against the N protein have been used to differentiate and classify rabies isolates into types of lyssaviruses.

4. Phosphoprotein M1, exists in two forms: M1 I (38KD) and M1 II (41KD)

This is highly hydrophilic protein having 303 amino acids. In the year 1981, it was suggested that M1 protein is not a membrane protein but is found to be occupying an internal position within the virion in association with N protein.

5. Matrix or membrane protein M2, exists in two forms: M2 I (24KD) and M2 II (25KD)

This is a 202-amino acid long polypeptide. It plays an intermediate role between the ribonucleocapsid and the viral membrane, presupposing the presence of regions interacting with both elements. It is believed to play an important role in morphogenesis of the virus. The association of two membrane proteins with the nucleocapsid results in folding of the M2 protein and its enclosure by membrane.

Genome organization^[109]:

Rabies nucleic acid is a negative-sense, non-segmented, single-stranded RNA. The length of the genome is approximately 12,000 nucleotides (12 kb). At the 3' end of the genome, a non-coding leader (Le) is present. Similarly, at the 5' end, a non-coding trailer (Tr) sequence is present. In between them, in sequential order of 3'-N-P-M-G-L-5' five protein coding genes are found. Each gene is comprised of an internal coding region flanked by non-coding regions (NCRs). These NCRs are bordered by transcription initiation signals (TISs) and transcription termination & polyadenylation signals (TTS). Each of the genes is separated by intergenic region (IGR). The IGRs are short (2-6 nucleotides) except for G/L IGR. [43] The IGR between G and L (G/L IGR) is a stretch of 423 nucleotides, but does not encode for protein. It is believed that it may be a remnant of an ancient gene. Hence, it is often called as pseudogene (Ψ).

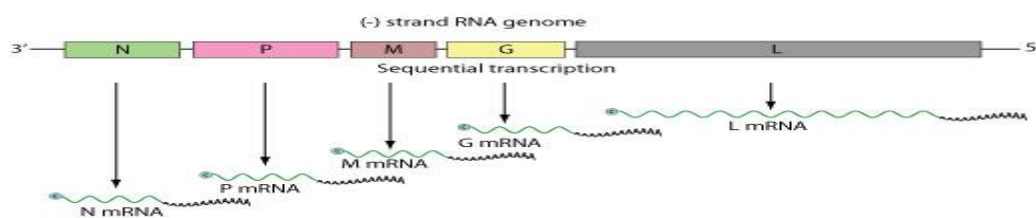


Figure 5: Structure of Rabies Virus Genome^[111]

HOST RANGE

Rabies is a disease of mammals caused by viral representatives in the genus *Lyssavirus*, family *Rhabdoviridae*. Rhabdoviruses as a group can replicate in vertebrates, plants, or

invertebrates. Mokola is the only lyssavirus considered as a remote possible bridge among such diverse host types because it is reported to be able to replicate in insect cells, at least under laboratory conditions. Similarly, rabies virus can replicate *in vitro* in certain cold-blooded vertebrate tissues, but no successful *in vivo* attempts have been reported. Birds are susceptible to experimental rabies infection, but the virus appears to be largely restricted to the central nervous system (CNS) and is apparently neutralized effectively by host defenses. Viral adaptation to certain common animals having a relatively narrow range of metabolically regulated internal temperatures might be a useful way, akin to a mobile incubator, for the virus to establish itself in a new environment. Abundant homeotherms with teeth and complex social repertoires seem ideal, particularly if relatively deep intramuscular inoculation of virus is needed to reach the vicinity of neuromuscular junctions or, alternatively, copious salivary contamination of mucous membranes and neural receptors with virus prior to direct infection of the CNS.

These proximate speculations do not begin to explain the divergence of lyssaviruses from other rhabdoviruses, the origins of neurotropism, or the ultimate selection of certain vertebrate groups over others. However, the paucity of recognized isolates in birds appears odd, considering rhabdovirus plasticity, adaptability, and persistence otherwise, as evidenced by their widespread occurrence in plants, invertebrates, fishes, amphibians, reptiles, and mammals. Similarly, given more than 4000 mammalian species, all of which are susceptible to rabies in theory, relatively few qualify as major reservoir hosts. All rabies reservoirs are also known vectors, capable of disease transmission, but not all mammalian vectors are also reservoirs.

TRANSMISSION:

Routes of Rabies Virus Transmission to Humans

Transmission of the disease usually requires bite-inflicted deposition of virus-laden saliva into tissue of the recipient animal. The virus cannot cross intact skin. The risk of rabies infection by a bite (5%-80%) is at least 50 times greater than that by a scratch (0.1%-1%). Inhalation of aerosolized RV occurred accidentally in laboratories of vaccine production or in caves inhabited by numerous infected bats. Infection through the digestive tract has also been reported. The contact with infected people could be a potential risk for their relatives and health workers when unprotected direct contact with secretions from a patient containing viable virus occurs. There are many reports of organ transplantation involved in the transmission of rabies. The most frequent cases have been observed in corneal transplantation. Although rare, human-to-human transmission of rabies has been well documented for eight recipients of transplanted corneas occurring in France, the United States, Thailand, India, and Iran. Rabies virus can be recovered from human fluids, including CSF, eye swabs, and sedimented urine, so appropriate precautions are required when treating humans with rabies. Two cases of human rabies possibly attributable to either human bite or infectious saliva exposure to mucous membranes have been described from Ethiopia. Although human transplacental transmission of rabies virus has been reported in a single case, infants have survived delivery from mothers infected with rabies when the child received (post exposure test) PET. Under exceptional circumstances, such as use of blenders to homogenize rabies virus-infected brain material, infections of humans with rabies virus by airborne droplets can occur. Rabies infection possibly acquired by droplets or aerosolized virus has been described in two persons visiting Frio cave in Texas. Millions of Mexican free-tailed bats congregate in this cave, and rabies virus is present

within the bat population. Experimental studies with animals and with an electrostatic precipitation device suggest that airborne transmission of rabies virus can occur under exceptional circumstances. There is little documentation for natural rabies transmission by simple contact with virus-infected tissue, although isolated reports suggest infection following butchering of infected carcasses. In the United States, ingestion of unpasteurized milk from rabid cows has been treated as a possible exposure to virus. Scratches from a rabid animal potentially could be contaminated with virus shed in saliva and in the United States are often treated as an exposure, but documented cases of this injury leading to rabies are rare^[112–114].

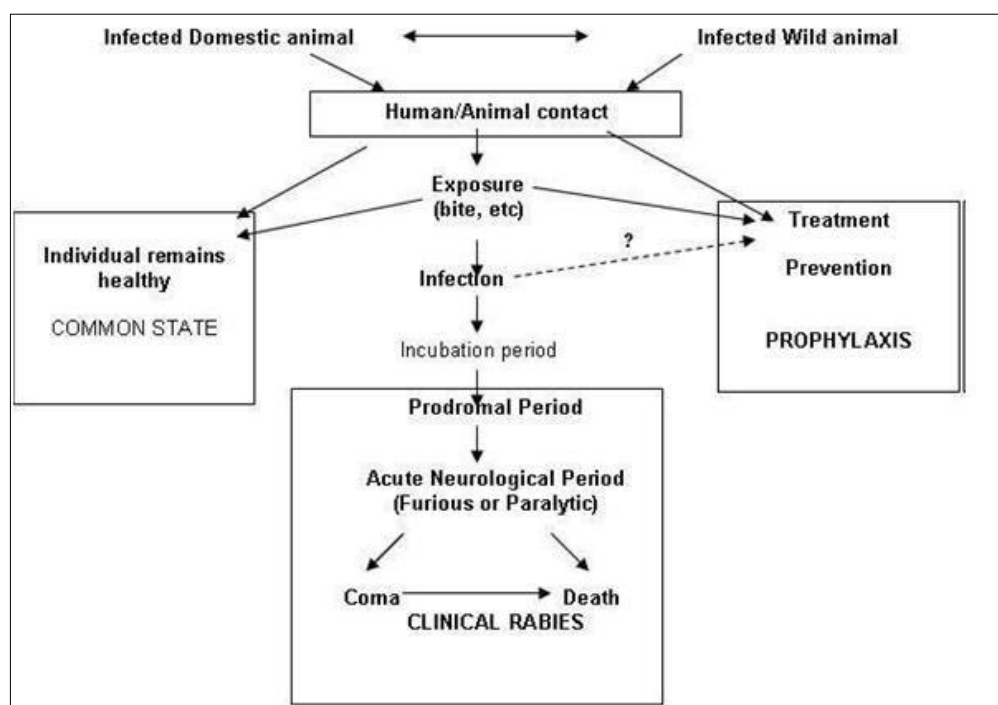


Figure 6: Transmission of the Rabies Virus^[115]

It has been noted previously that for many of the recent human rabies cases in the United States, no history of animal bite could be solicited from the patient, relatives, or close companions. Although unusual, this situation is not unique. In Yugoslavia, from 1935–1939 and from 1946–1952, 35 cases of rabies were reported among individuals who denied any injury or contact with rabid or suspected rabid animals.

REPLICATION OF RABIES VIRUS^[116–119]:

Absorption:

It is widely accepted that many viruses are taken into the cells by process of phagocytosis. In the case of enveloped viruses, fusion may occur between the envelope and the cell membrane. Both processes have been observed in cultures of baby hamster kidney (BHK-21) cells infected with rabies virus. The binding of the rabies virus to the cell membrane is thought to be mediated by the G protein. Based on sequence homology between the external of the G protein and the receptor binding site of the snake venom neurotoxins, it was postulated that the rabies virus could bind to the nicotinic acetylcholine receptor. In neuronal and fibroblastic cells, it has been shown that oligosaccharide and lipoprotein components such as sialic acids of gangliosides may also be involved.

Penetration and Uncoating:

Once fixed onto the cell, the rabies virion penetrates it by pinocytosis. The viral membrane then fuses with that of the lysosomal vacuole, releasing the ribonucleocapsid into the cytoplasm. The ribonucleocapsid is sufficient to assure the transcription of the virus.

Transcription, Replication and Budding:

The rabies virus possesses an unsegmented negative stranded RNA genome. The negative polarity means that the genome is unable to be directly translated into viral protein by the cell machinery. Therefore, a preliminary autonomous transcription step is necessary to produce the complementary positive stranded messenger RNA (mRNA), as soon as the ribonucleocapsid is liberated in the cytoplasm. This step is assured by a genome encoded enzyme, the RNA dependent RNA polymerase, which is included in the ribonucleocapsid. The viral polymerase does not recognize a naked RNA template.

The viral encoded polymerase (L gene) transcribes the genomic strand of rabies RNA into leader RNA and five capped and polyadenylated mRNAs, which are translated into proteins. Translation, which involves the synthesis of the N,P,M,G and L proteins, occurs on free ribosomes in the cytoplasm. Although G protein synthesis is initiated on free ribosomes, completion of synthesis and glycosylation (processing of the G protein) occurs in the endoplasmic reticulum (ER) and golgi apparatus. The intracellular ratio of leader RNA to N protein regulates the switch from transcription to replication. When this switch is activated, the replication of viral genome begins. The first step in viral replication is synthesis of full copies (positive stranded) viral genome. When the switch to replication occurs, RNA transcription becomes non-stop and stop codons are ignored. The viral polymerase enters a single site on the 3' end of the genome, and proceeds to synthesize the full-length copies of the genome. These positive strands of rabies RNA serve as a template for the synthesis of the full length of negative strand of viral genome. During the assembly process, the N-P-L complex encapsulates negative stranded genomic RNA to form RNP core, and the M protein forms a capsule around RNP. The RNP-M complex migrates to an area of the plasma membrane containing glycoprotein inserts, and the M protein initiates coiling. The M-RNP complex binds with the glycoprotein, and the completed virus buds from the plasma membrane. Within the central nervous system (CNS), there is preferential viral budding from plasma membranes. On the other hand, the virus in the salivary glands buds primarily from the cell membrane into the acinar lumen. Viral budding into salivary gland and virus induced aggressive biting behavior in the host animal maximize chances of viral infection of a new host.

PATHOGENESIS^[113,120–122]:

Rabies virus (RABV) is generally transmitted from the bite of a rabid animal. However, there are evidences that indicate transmission by aerosols, bite of bats (recognised or unrecognised) and by transplantation of tissues and organs. After exposure of bite, saliva containing the virus is deposited in muscle and tissues. The virus stays in the site for majority of the long incubation period. RABV binds to receptors, mainly nicotinic acetylcholine receptor in muscle, expressed on postsynaptic membrane of neuromuscular junction. Once neuromuscular junction is crossed, virus spreads within axons in peripheral nerves by retrograde axonal transport. After infecting the spinal cord, it reaches the neurons of CNS; dissemination occurs by fast axonal transport along neuroanatomical pathways. Centrifugal spread (away from CNS) involves parasympathetic system, to multiple organs including heart, gastrointestinal tract, adrenal medulla, skin and salivary gland. There is involvement of nerves in close proximity to hair follicles, which makes it suitable for diagnosis of rabies through skin biopsy and cardiac involvement may sometimes be associated with a myocarditis. The brain infection results in change of behaviour, possibly due to infection of neurons of limbic system and this facilitates transmission by biting other animals.

In transmission by bat, exposures are much more superficial and many times beyond recognition. It is likely that the virus gains access to small sensory or autonomic nerves in richly innervated skin. But there is no information available about sequential steps that proceed during infection. As silver-haired bat virus is said to be well adapted for efficient local replication in the dermis, it has been successful in majority of cases to cause rabies in developed countries. No strong evidences have been provided that haematogenous spread plays a significant role in spread of virus to the CNS

either under natural conditions or with organ transplantation.

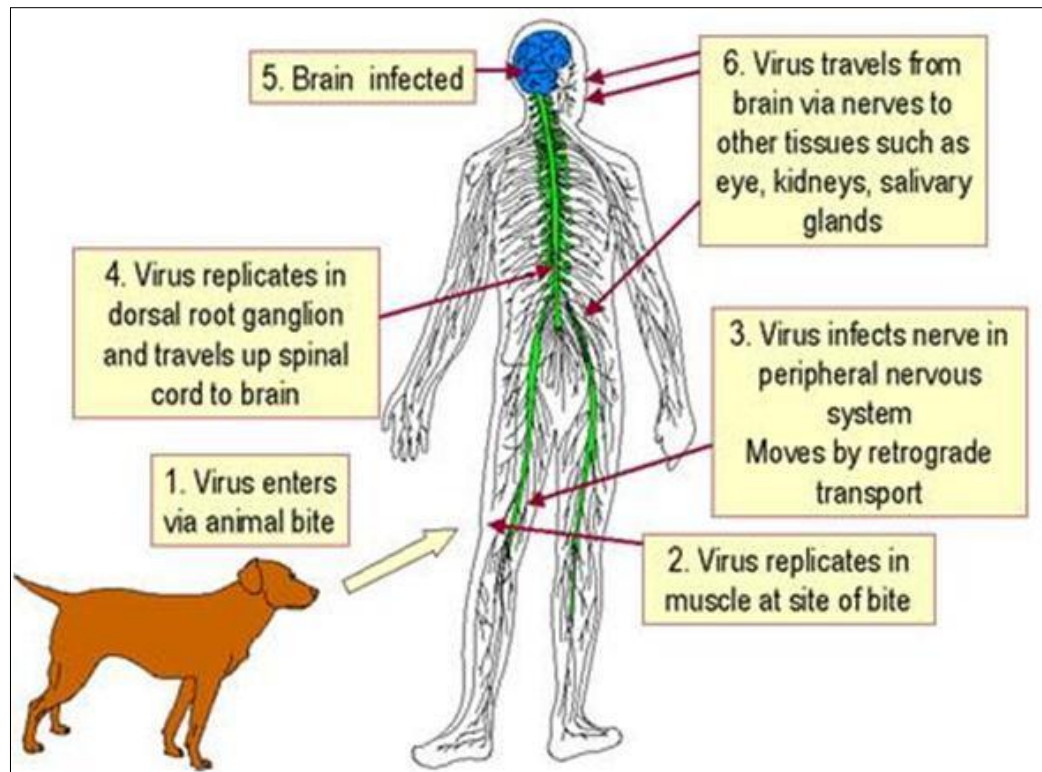


Figure 7: Pathogenesis of Rabies Virus. Adopted from: <http://www.healthdiseaseblog.com/>^[110]

Rabies is characterized by severe neurologic signs. Though relatively mild neuropathic changes in CNS occur, the outcome of rabies is almost always fatal. Also, mild inflammatory changes accompany with few degenerative neural changes. These suggest that neuronal dysfunction play an important role in disease. A variety of studies of infection of rabies both *in vivo* and *in vitro* have indicated that there are defects in neurotransmission especially of acetylcholine, serotonin and/or γ -amino-n-butyric acid (GABA). RABV may also impair ion channels and electrophysiology of neurons. Dysfunction of ion channels has been reported in cell cultures. Induction of inducible nitric oxide synthase mRNA and increased brain levels of nitric oxide have been demonstrated in rabies virus infected rodents. However, it is not clear whether these increased levels played any significant role in

producing neuronal dysfunction or not. Attenuated viruses are strong inducers of neuronal apoptosis both *in vivo* and *in vitro* than virulent virus strains. Recently, strong evidence has been documented suggesting neuronal apoptosis does not play important role in human rabies.

CLINICAL PRESENTATION^[123–126]:

Clinical rabies in man can be discussed by following five stages: incubation period, prodrome, acute neurologic phase, coma and death or rare recovery.

1. **Incubation period:** The reported incubation period for rabies ranges from as short as 5 to 6 days, but in most of cases, the incubation period is between 20 and 60 days. Although incubation period of longer than 6 months are reported in less than 1% cases. Incubation period are usually shorter when the site of the bite is on the head rather than an extremity and is shorter in children than adults. Without vaccination, the risk of death varies with the quantity of virus in the saliva of the biting animal and the severity and the location of the bite. During the incubation period, there are no symptoms and no means of diagnosis; clinical illness begins with non-specific prodrome like symptoms, slight fever, headache, malaise, anxiety lasting for few hours to few days.[57]
2. **Prodrome:** the first symptoms of clinical rabies are usually malaise, anorexia, fatigue, headache and fever. In about half of the individuals pain or paresthesias are reported at the site of the exposure. Apprehension, anxiety, agitation, irritability, nervousness, insomnia or depression may be prominent during this period.
3. **Acute neurologic phase:** Two to 10 days after onset of prodromal symptoms the patient develops objective signs of nervous system involvement usually including hyperactivity, disorientation, hallucination, seizures, bizarre behavior,

paralysis. As in animals, a period of hyperactivity lasting hours to days develops in most of cases. In humans, this hyperactivity is characteristically intermittent and consists of periods of agitation lasting 1 to 5 minutes. Hydrophobia (the fear of water) appears to develop in most cases of furious human rabies. Attempts to drink or eat may produce severe painful spasms of the pharynx and larynx producing choking, gagging and fear. Other abnormalities during this phase include muscle fasciculation, hyperventilation, hypersalivation, convulsions. Paralysis may be symmetric, asymmetric with maximal involvement in the bitten extremity or ascend as in the Guillain-Barre syndrome. During this phase the mental status gradually deteriorates from confusion to disorientation, stupor and finally come.

4. **Coma:** This period of coma may last for hour or months, depending on the intensity of care. Without supportive care, respiratory arrest usually develops shortly after the onset of coma and leads to death, within 5 days for furious rabies and 13 days for paralytic rabies.
5. **Death or rare recovery:** without PEP (post exposure prophylaxis) the disease is fatal, recovery is very rare after the onset of acute neurologic phase. There is no established treatment available for rabies and management is only by palliative support. Some experimental protocols to avoid mortalities have been reported. There was only one reported case of a female survival from rabies virus by inducing coma and treating her with combination of known antiviral drugs. But the same experimental protocol was not successful onto other patients.

WHO defines a clinical case of rabies as:

a subject presenting with an acute neurological syndrome (i.e. encephalitis) dominated by forms of hyperactivity (i.e. furious rabies) or paralytic syndromes (i.e. dumb rabies), progressing towards coma and death, usually by cardiac or respiratory failure, typically within 7-10 days after the first sign, if no intensive care is instituted^[127]

The definitive diagnosis of rabies can only be obtained by laboratory investigations as described by the OIE Terrestrial Manual- 2011^[128]. Laboratory diagnosis may be required in human beings when the clinical features are not unambiguous. It is of utmost importance in the era of organ transplantation^[129]. Though ante-mortem as well as post-mortem diagnostic techniques are available, the former do not have a sensitivity of more than 25% in best of hands. Battery of tests should be performed. A positive test by one of the standard procedures overrides negative reaction in the others. Negative tests do not rule out the possibility of rabies^[130].

Rabies is transmissible to humans by inoculation or inhalation of infectious virus, all suspected infected material must be handled under the appropriate safety conditions specified by the World Health Organization (WHO). As there is no gross pathognomonic lesion for rabies, diagnosis can only be made in the laboratory.

Laboratory Tests for Rabies Diagnosis:

Laboratory techniques are preferably conducted on central nervous system (CNS) tissue removed from the cranium. Specimen such as CSF, Nuchal biopsy, Saliva, skin biopsy, Corneal impression are collected for Ante-mortem detection of rabies while a Brain biopsy is done in case of post-mortem detection.

Several tests are necessary to diagnose rabies ante-mortem (before death) in humans; no single test is sufficient as detection of viral entity may be dependent on the

progression of disease. Tests are performed on samples of saliva, serum, spinal fluid, and skin biopsies of hair follicles at the nape of the neck. Following are the detection techniques employed for both ante-mortem and post-mortem rabies suspected sample and are performed in a combination of two or more techniques.

Direct microscopy (Demonstration of Negri bodies)

Histological test by Seller's Method was discovered by Adelchi Negri in 1903 in rabies infected brain tissue and marked the first milestone for laboratory diagnosis of rabies^[131]. Staining of infected neuronal cells reveals aggregates of viral particles referred to as "Negri Bodies" are intracytoplasmic inclusions that are specific to rabies and was considered as a gold standard for rabies diagnosis. Under light microscopy these Negri bodies are round or oval, acidophilic and strongly refringent, staining pink with haematoxylin-eosin and red with Mann's stain. However, this test had limitations attributed by its low sensitivity and time consuming especially when paraffin embedded sections was processed. This technique has eventually been replaced by more advanced and highly sensitive immunological methods.

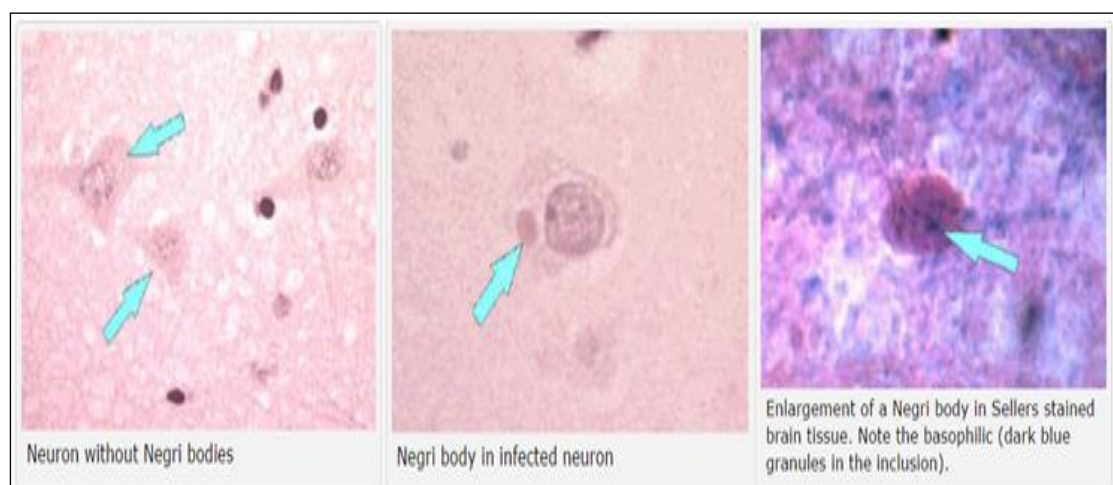


Figure 8: Demonstration of Negri Body in neuronal cell^[132].

Source: <http://www.cdc.gov/rabies/diagnosis/histologic.html>

Viral Antigen Detection

Detection of rabies virus antigen in suspected clinical samples is the first approach for laboratory based diagnosis of rabies. The viral antigenic target for rabies diagnosis is primarily the Nucleoprotein as it is the most abundantly present and is highly conserved. The nucleoprotein is intricately involved in the RNP core of the virus and thus becomes the highly coveted target for rabies detection in comparison to other viral proteins. Tests employed for antigen detection are as follows:

Fluorescent Antibody Test (FAT):

Developed by Goldwasser and Kissling in 1957, FAT is based on the microscopic examination of impressions of smear of brain tissue or cornea^[133,134]. The test detects the presence of rabies virus by targeting the rabies virus Nucleoprotein antigen with fluorescently labeled Nucleoprotein antibodies^[135]. Thin touch impression smears are fixed in cold acetone for one to four hour; air dried then stained using fluorescein isothiocyanate (FITC)-labeled rabies antibody. Examination at 400x to 1,000x magnification reveals punctiform of rabies antigen with apple green fluorescence. This test has largely replaced the Seller's technique and is recommended by both WHO and OIE, however, accuracy of this test depends on variables like expertise of the examiner, quality of the conjugate and basic equipment like Fluorescent Microscope^[136,137]

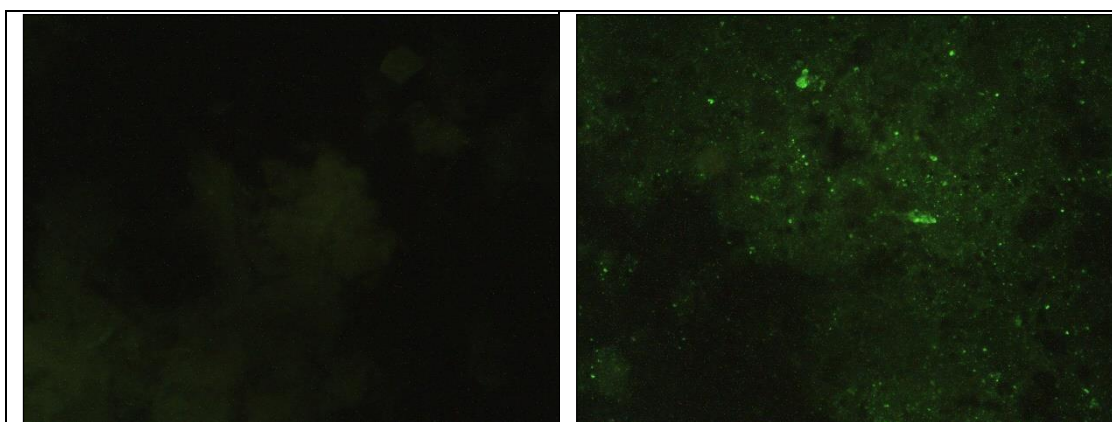


Figure 9: Demonstration of Fluorescent Antibody Test showing apple green fluorescence of rabies antigen in brain tissue of rabid animals.

Rapid Rabies Enzyme Immuno Diagnosis (RREID)

An ELISA-based technique was developed by Perrin et al. in 1986 which is known as rapid rabies enzyme immunodiagnosis (RREID)^[138]. The test is performed on the homogenates of brain samples. This test is based on the immunocapture of the rabies nucleocapsid antigen by an antinucleocapsid polyclonal globulin coated to the ELISA plates, followed by the addition of the same globulin conjugated to horse radish peroxidase enzyme. The results are obtained within three hours. The correlation between FAT and RREID is 96-99%.^[139] However, since these require brain tissue its use in antemortem diagnosis is eliminated.

direct Rapid Immunohistochemical test (dRIT):

The direct rapid immunohistochemical test (dRIT) was developed recently and validated as a field test for rabies surveillance. This test can be used on brain touch impressions and the reaction product is visible under an ordinary light microscope (magenta inclusions, while the neuronal background is blue). The test allows diagnosis within one hr. It can be used on frozen samples or samples preserved in glycerol, and have sensitivity similar to that of the direct FAT. Compared to the dFAT, the traditional standard in rabies diagnosis, the dRIT was 100% sensitive and specific^[140–143].

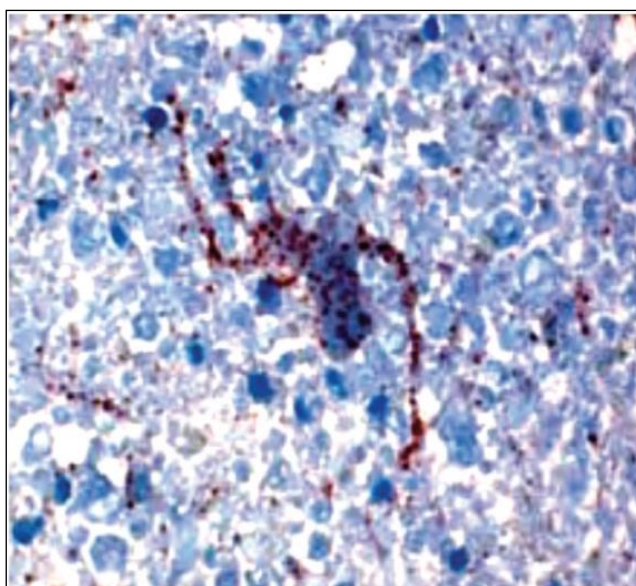


Figure 10: Demonstration of direct rapid immunohistochemical test (dRIT) ^[136]

Virus Isolation:

Virus isolation is required for confirmation of inconclusive FAT results or for molecular characterization studies to identify the circulating strain of the virus. It includes the following two techniques.

Mouse Inoculation Test (MIT) which involves intracerebral inoculation of mice by the clarified supernatant of the rabies suspected sample and observing the animals for a period of 28 days. Symptoms like ruffled fur, hunched back, toe walking, hind & forelimb paralysis followed by mortality are noted. Brain tissues are harvested after mortality has occurred and smears of these brain samples are tested by FAT^[136].

Rapid Tissue Culture Infection Test (RTCT) which in comparison to MIT is fast with results yielding in 24-48 hours. In this technique, the suspected clinical specimen supernatant is inoculated onto cell line susceptible to rabies virus infection like N2a, BHK-21, chick-embryo related and HEK 293 and incubated for 48 hours ^[144]. Post infection the cells are acetone fixed and subject to FAT.

Viral Antibody Detection

Presence of antibodies in the sera or CSF sample of a suspected patient in absence of previous immunization is considered as an indirect evidence of rabies virus infection. The likelihood of generation these virus neutralizing antibodies by the immune system can occur in an event of exposure to the virus or by immunization against the virus i.e. by vaccination. This approach is useful when there is no medical history of animal bite. This technique is also used to determine the vaccination response and the antibody titre in individuals. The tests used for antibody detection include:

Mouse Neutralization Test (MNT)

Webster and Dawson developed MNT which was a widely recommended test. The mouse protection test was the first serological test developed for rabies antibody detection but is now outdated. It involves mixing dilutions of serum and virus before inoculating the mixture into mice and noting whether or not the mice die from rabies. With the advent of cell culture techniques this test is no longer recommended by WHO or OIE^[136].

Rapid Fluorescent Foci Inhibition Test (RFFIT):

RFFIT is one of the most widely used substitutes of MNT. The principle of RFFIT is same with MNT except that the indicator system for virus infectivity in this case being a susceptible cell culture system usually BHK-21 or neuroblastoma cells. It is precisely a combination of cell culture and immunofluorescence. It is a rapid test and requires only 20 hr for completion. It involves the observation of foci of virus infected cells by fluorescent antibody staining^[135].

Viral RNA Detection

Nucleic acid detection and amplification have modernized the diagnosis of rabies and plays an important role in ante-mortem diagnosis. The Polymerase Chain Reaction

(PCR) techniques needs very little start volume of samples, are run in parallel to the conventional diagnosis method and mostly target the rabies nucleoprotein gene. This technique does not only find its application in detection of rabies but also conducting phylogenetic analysis, identifying lineage of circulating virus and for classification lyssaviruses based on genetic differences by processing the PCR products for sequencing and bioinformatic analysis^[145].

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR):

Nested RT-PCR is performed for viral nucleic acid detection targeting the N-gene and involves amplification of the N-gene using two different sets of primers. First, the outer primer is used to amplify a region of N-gene using extracted RNA from the rabies suspected sample as template and yields a PCR product^[146]. This is followed by another round of PCR using the PCR product of the outer PCR as the template for amplification by the inner primers that amplify a region within the outer PCR product. This PCR products thus obtained from both outer and inner pcr are subject to electrophoresis using 1.5% Agarose gel. The bands desired for the products are then viewed using imaging systems in parallel to a molecular weight ladder for marking the desired molecular weight.

Real Time RT-PCR:

A modification of the RT-PCR, this technique provides for real time diagnosis. Real time assays using SYBR green chemistry and TAQMAN PCR^[147] provide for highly specific detection of rabies virus ranging from 10-1000 times specificity as compared to nested PCR^[136].

Prevention

Rabies is a vaccine preventable disease and on exposure post exposure prophylaxis (PEP) can avert the chances of establishment of the disease. As per WHO classification,

there are three categories of exposure and its recommended PEP grading from a lick on intact skin to a deep bite wound^[148]. The PEP consists of:

- (1) Wound Cleansing in running water, cleaning the wound with a disinfectant or soap,^[128] Active Immunization by anti-rabies vaccine (ARV) and
- (3) passive immunization by rabies immunoglobulins (RIG).

Approach to Post Exposure Prophylaxis:

(1) Wound Cleansing & Management:

Animal bite wounds must be cleansed thoroughly using disinfectants like soap or detergent using generous amounts of running tap water. As the virus gains entry into the body through saliva, washing the wound in flowing or running water allows for removal of virus particles from the site of wound and disinfectants aids in chemical inactivation of the virus. In case of unavailability of soap the wound must be washed for a minimum of 10 minutes and then allowed to dry. Antiseptics like Povidine iodine, Dettol, Alcohol and Savlon can be used to further cleanse the wound after thorough washing and drying. Suturing of the wound should be avoided and in circumstances where it is inevitable loose sutures should be applied with adequate local treatment and rabies immunoglobulin administration.

Category	Type of contact	Type of exposure	Recommended post-exposure prophylaxis
I	Touching or feeding of animals Licks on intact skin	None	• None, if reliable case history is available
II	Nibbling of uncovered skin Minor scratches or abrasions without bleeding 	Minor	• Wound management • Anti-rabies vaccine
III	Single or multiple transdermal bites or scratches, licks on broken skin  Contamination of mucous membrane with saliva (i.e. licks) 	Severe	• Wound management • Rabies immunoglobulin • Anti-rabies vaccine

Figure 11 WHO classification of wounds^[149]

Source: http://www.ncdc.gov.in/Rabies_Guidelines.pdf

Active Immunization:

I. Vaccines:

AntiRabies vaccine (ARV) for humans should meet WHO recommendations for characterization, production and control as set out by WHO Expert Committee on Biological Standardization^[148]. The vaccines for rabies are of two types based on their mode of propagation: Cell Culture based vaccines and Nerve tissue based vaccines and out of the two WHO recommendations apply only to the former. The nerve tissue based vaccine induces severe adverse reactions and have low immunogenicity in comparison to its counterpart and has been strongly recommended to be replaced by the newer safer cell culture based vaccines since 1984 by WHO.

Cell Culture and Embryonated Egg based rabies Vaccine or CCEEVs as it is collectively called contain inactivated rabies virus propagated in cell culture using chick embryo and vero cells. Vaccines currently available in India are:

- (1) Human Diploid Cell Vaccine (HDCV);
- (2) Purified Chick Embryo Cell Vaccine (PCEC);
- (3) Purified Vero Cell Rabies Vaccine (PVRV);
- (4) Purified Duck Embryo Vaccine.

They are conferred safe and effective as human diploid cell vaccines and are also cost effective. The Nerve tissue based vaccines are prepared from animal central nervous system including suckling mouse brain which on administration is known to have severe adverse effects. Many countries, including India^[150], have adopted the recommendations and worked on acquiring the vaccines by importing them or by developing or acquiring the technology for manufacturing the cell culture based vaccine. However, certain developing countries like Peru and Ecuador in Latin America and Myanmar and Pakistan in Asia continue to use the Nerve tissue based vaccine while

still investigating alternative affordable and sustainable substitute for the nerve tissue vaccine.

II. Route & Dosage of Administration:

Current rabies vaccines are produced as individual doses for intramuscular (IM) injections. CCEEVs reconstituted with 0.5 or 1 mL of solvent in one IM dose vial with potency of ≥ 2.5 IU/dose can be used for both pre- and post- exposure prophylaxis. Vaccination via the intradermal route is an equally safe and immunogenic alternative with an advantage of only 1 or 2 vials required to complete a full dose of only 0.1mL per injection. It is characterized by the formation of an evident bleb at the site of inoculation. This reduces the cost of rabies vaccination by 60-80% in comparison to the standard IM administration. While antibody titres are higher and more sustained after an IM injection both routes induce rapid recall response upon booster immunization. However, ID is not recommended in immunocompromised individuals.

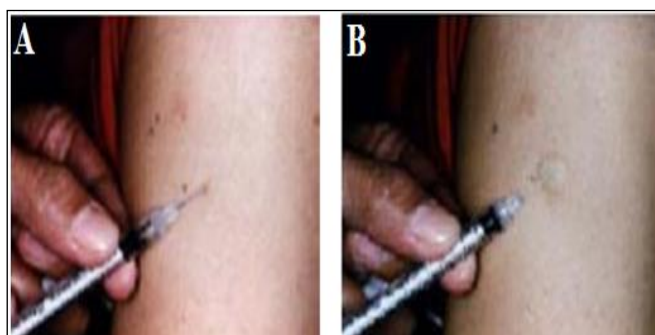


Figure 12 Demonstration of IDRV administration: (A) Insertion of needle for ID inoculation; (B) Formation of Bleb after successful ID administration^[130]

Source: http://www.ncdc.gov.in/Rabies_Guidelines.pdf

III. Vaccination Regime:

The vaccination schedule followed in India is the Essen Schedule which includes five ARV doses on days 0 (day of exposure), 3, 7, 14 and 28^[151]. Incomplete vaccination or no vaccination in the event of exposure may lead to infection and once established rabies is almost always fatal.

(2) Passive Immunization:

Rabies Immunoglobulins (RIGs) are administered as passive immunization along with anti-rabies vaccines. RIGs currently in use are- Human rabies immunoglobulin (HRIG), Equine rabies immunoglobulin (ERIG) and highly purified F(ab')₂ fragments produced from equine immunoglobulin. RIGs should be administered with the first dose of ARV and can be administered up to 7th day since the first dose of vaccination^[127]. It is presumed that an immune response may have been triggered in response to the ARV and hence is not indicated thereafter. Administration of RIGs is focused in and around the wound site and as much is anatomically feasible allowing for viral neutralization and the left over volume being administered at a site other than that of ARV inoculation. As per WHO recommendations, HRIG is given at 20 IU/Kg of body weight and is the preferred choice over its non-human origin counterpart as it is much safer with fewer side effects. ERIG is given at 40 IU/Kg of body weight and most of the new equine preparations are potent, highly purified and safe with few adverse reaction effects in comparison to its earlier versions. Although the risk of anaphylactic shock is low the patient must be observed post immunization with RIGs^[150].

PEP to previously vaccinated persons:

In case an individual who had received complete PEP with potent cell culture based vaccines is re-exposed should be given proper wound management care and be administered two booster dose of the ARV on days 0 & 3. RIG may not be necessary; however, it may be administered in case of severe deep bite wounds. As the cell culture based vaccines were introduced only in 2004, consideration of individuals who received the PEP using nerve tissue based vaccine as fresh cases should be done and be provided complete PEP depending on the category of exposure^[130].

Approach to Pre Exposure Prophylaxis:

Pre Exposure Prophylaxis (PrEP) is provided to individuals whose occupation brings them in close encounters with animals or those working in laboratories with fields or rabies research or rabies vaccine production. These individuals who are considered as at high risk are Veterinarians, Clinicians dealing with rabies patients, Laboratory staff handling virus or infectious material during testing, wildlife wardens, animal handlers and also travelers from rabies free to rabies endemic countries. Complete PrEP which includes vaccination with ARV on days 0, 7 & 21 or 28. These individuals should also have their neutralizing antibody titres checked every six months. In the event of exposure, a person who has received a complete successful PrEP should be given proper wound care and two dose of ARV on days 0 and 3. Administration of RIG is not necessary but may be considered depending on the case^[130].

Treatment:

Till date, no treatment is available for clinical rabies. It remains restricted to supportive care along with use of heavy sedatives and anti-convulsants that prolong life by a few weeks but is unable to prevent death^{[127][107]}. It has been observed that Due to under reporting and negligence by patients, medical practitioners do not encounter rabies patients on a routine basis which results in misdiagnosis and poor handling of rabies suspected patient. As no concrete line of treatment is available, strategies for treating rabies are carved on basis of research experimental data. Approach to patient care and therapy are often influenced by previous case reports of patients who have been treated by aggressive and palliative approach in critical care units and should be implemented with caution. Therapies involving use of sedatives, narcotic analgesics, antiepileptic medications and neuromuscular blockers may alleviate complications with neurological symptoms^[124].

Vaccination:

Reports to date suggest that survival rate for previously unvaccinated rabies patients is near about negligible with agonizing symptoms. Vaccination increases the chance of survival if received before or soon after exposure and before the onset of illness. Complete PEP along with passive immunization using HRIG or ERIG should be the first step as an approach to treating rabies followed by supportive medical care ^[148].

Monoclonal Antibodies (MAbs):

Of the various mechanisms involved in the virus neutralization, Monoclonal antibodies act by blocking the virus-receptor interaction. Monoclonal antibodies (mAbs) capable of neutralizing a diverse range of rabies isolates with high specificity could also offer a solution to address the cost, supply and safety issues associated with blood derived Rabies Immunoglobulin (RIG). Studies on animal model showed that monoclonal antibodies for RV were used for virus clearance study and showed prominent results^[152–155]. Clinical trials for studying the safety and efficacy of these MAbs are currently underway with collaborations between WHO and corporate organizations^[156].

Anti-virals:

Ribavirin and interferon-alpha are broad-spectrum antivirals that have significant in vitro activity against rabies but in vivo experiments and treatment of several human patients with rabies infection did not show any beneficial activity. Two other FDA approved drugs with some in vitro anti-rabies activity are the N-methyl-D-aspartate (NMDA) receptor antagonist ketamine and amantadine (this last one is also approved for treatment of influenza). Ketamine showed some effect on rabies infection in animal studies while for amantadine efficacy in animals could not be demonstrated. A combination of ketamine and amantadine was explored in patients, together with other treatment interventions, in the so-called 'Milwaukee Protocol'. While this

protocol had high coverage in the media as the first patient treated recovered, it has not been repeatable and is now considered to be ineffective.

Favipiravir

Another more recently approved drug that has been explored in rabies infection is favipiravir (T-705), a broad spectrum antiviral. Intracellular host enzymes convert favipiravir to its active form favipiravir-4-ribofuranosyl-5-triphosphate (T-705-RTP). The mechanism of action of T-705-RTP remains a topic of discussion. It mimics a purine nucleotide analog that selectively inhibits the RNA-dependent RNA polymerase (RdRp) or causes lethal mutagenesis upon incorporation into the viral RNA^[132]. Favipiravir is currently approved for treatment against influenza in Japan, and is in clinical development in the United States. Recently Yamada et al. showed that favipiravir also has antiviral activity against Rabies virus. In vivo experiments showed that it, under conditions of early start of treatment, improves the clinical signs and survival rate of mice infected intramuscularly. If the start of favipiravir treatment is delayed for 1 or 2 days no significant protective effect can be observed. This indicates that favipiravir might be ineffective against viruses located within the nervous systems and may be only effective before neuroinvasion.

Novel antivirals

In addition to repurposing existing drugs, novel antiviral strategies have been explored for rabies. These have been reviewed extensively in the recent paper by Appolinario and Jackson as well. In the following, we highlight some additional strategies.

Nucleosides in development for other negative strand RNA virus infections

While nucleosides, that selectively inhibit the polymerase of viruses, are approved for the treatment of Herpes virus, Human immunodeficiency virus (HIV), Hepatitis C virus (HCV) and Hepatitis B virus (HBV), the discovery of nucleosides that

specifically inhibit negative strand RNA virus polymerases has been more challenging. However, in recent years several nucleosides with activity against Respiratory syncytial virus (RSV) and Ebola virus have been discovered. As discussed below these are excellent candidates to be explored further on Rabies virus.

ALS-008176

ALS-008176 is a nucleoside against RSV that has entered clinical trials. ALS-008176, is the ester-prodrug of ALS-008112 which is a cytidine nucleoside analog. Biochemical studies revealed that ALS-008112-5'-triphosphate is a potent and selective inhibitor of RSV RNA-dependent RNA polymerase activity (K_i of $0.09\ \mu\text{M}$), via a classic chain termination mechanism. In vitro experiments also show that ALS-008112 is active on Parainfluenza virus and Vesicular stomatitis virus (VSV). The EC_{50} on VSV was reported as $3.4\ \mu\text{M}$, which is ~ 20 -fold less active as on RSV but still with a good selectivity ($\text{CC}_{50} > 100\ \mu\text{M}$). No activity was detected on negative strand RNA viruses beyond the Paramyxo- and Rhabdoviridae families, nor was activity noted against any of the tested positive strand RNA viruses. As indicated above ALS-008176 is a prodrug that needs metabolism to become active. In particular, the phosphorylation to the nucleoside-triphosphate is a step that can be cell type dependent and that should be explored in neurons in the case of rabies.

2'd2'F-nucleosides

Another recent series of anti-RSV nucleosides are the 2'd2'F-nucleosides. These have not yet been characterized in animal studies nor has their activity on other negative strand RNA viruses been reported, but given their activity on RSV it may be worthwhile to explore these molecules for anti-Rabies virus activity.

BCX4430

BCX4430 is a nucleoside with broad-spectrum activity against picorna-, flavi-, orthomyxo-, paramyxo- and filoviruses in vitro. Unfortunately, its activity against rhabdoviruses such as VSV or Rabies virus has not yet been reported. BCX4430 has shown efficacy against several virus infection in animal models and is currently in development for the treatment of Ebola virus disease. Given the broad-spectrum and the good efficacy in several animal models of virus disease, including infections with negative strand viruses, this molecule is a candidate to explore in rabies infections.

GS-5734

GS-5734 is a monophosphate prodrug that was discovered in a recent anti-Ebola virus screening campaign. It has broad-spectrum antiviral activity against several pathogenic RNA viruses, including filoviruses, arenaviruses, paramyxoviruses and coronaviruses. In a rhesus monkey model of Ebola virus disease GS-5734 showed very good efficacy even when treatments were initiated three days after infection. Given the broad-spectrum activity on filoviruses and paramyxoviruses, this nucleotide-prodrug may also inhibit rhabdoviruses including rabies.

Disrupting the viral ribonucleoprotein complex

A unique approach to investigate a potential Rabies virus antiviral strategy was the study of peptides that disrupt the interactions between the viral ribonucleoprotein complex (RNP) and the L- and P-protein. The rabies RNP is built around the N-protein that oligomerizes to form a helix around which the viral RNA is wrapped. This RNP is the template for transcription and replication by the RdRp (L protein). Viral RNA synthesis is mediated by the viral Phospho- (P) protein, which is a multifunctional protein that interacts with L and N. The P-protein allows efficient and specific recognition of the RNP template by the RdRp and subsequent

RNA polymerization. In this way, P permits the continued contact between the RdRp and the RNP as the RdRp progresses.

As the N-terminal domain of the P-protein interacts with both the N- and L-protein it was chosen as a tool to explore a novel antiviral strategy. Peptides derived from this domain were shown to interact directly with the N- and L-protein and to be able to block rabies virus replication in cell culture.

It is interesting to note that corresponding P-peptides have previously also been shown to inhibit replication of RSV and Nipah virus. Detailed characterization of the binding mode of these peptides on their N proteins could therefore lead to a general principle of inhibiting rhabdoviruses and paramyxoviruses.

Milwaukee Protocol:

An interesting case of complete recovery of a symptomatic patient after experimental therapy (Milwaukee Protocol) of antivirals during drug-induced coma has been reported^[125]. The Milwaukee protocol was conceived in 2004 by a team of medical professionals, led by Dr. Rodney Willoughby, after a 15-year-old girl was admitted to a Milwaukee hospital after a rabies diagnosis. After consulting with researchers at the Centers for Disease Control and Prevention in Atlanta, the team formulated and implemented a novel procedure.

Considering the theory that rabies pathology stems from central nervous system neurotransmitter dysfunction, doctors hypothesized suppressed brain activity would minimize damage while the patient's immune system developed an adequate response. The patient was placed in a drug-induced coma and given an antiviral cocktail composed of ketamine, ribavirin, and amantadine. Ribavirin increases the mutation rate of RV leading to a lower viral fitness, but a suppression on the production of antibodies

has been associated to this compound; amantadine increases the pH of the endosome after virus entry into the cytoplasm, avoiding the low pH-dependent virus uncoating, but no effectiveness has been definitely shown in mice; ketamine, a dissociative drug, has also been shown to inhibit RV transcription, but no definitive evidence comes from in vitro and in vivo trials^[157]. The patient was discharged from the hospital 76 days after admission with neurological impairment, however, it was observed to improve immensely overtime with physiotherapy and cognitive rehabilitation training.

A lot of debate has been triggered on total discontinuation of the Milwaukee protocol as a treatment for rabies^[157–159]. Reproducibility remains largely unsuccessful as out of 36 rabies patients treated with the original and revised version of Milwaukee protocol only 5 have survived^{[160][124]}. Crushing failure has prompted the health community to place a taboo on the protocol, encouraging experimentation that may lead to different treatment options.

The treatment, although with only few cases of success in human patients and inconsistent results obtained from in vitro and in vivo trials, has opened new avenues and possibilities for treating rabies^[157]. With the advent of RNA interference (RNAi) and its success in other viral diseases RNAi for Post-transcriptional Gene Silencing (PTGS) has witnessed an increasing interest from those concerned with a cure for rabies^[157,161]

ANTI-VIRAL CHEMOTHERAPY

In 1963, the first anti-viral drug, idoxuridine, was approved, since then 90 antiviral drugs categorized into 13 functional groups have been licensed for the treatment of the 9 human infections^[162]. These include:

1. HIV infections (protease inhibitors, integrase inhibitors, entry inhibitors, nucleoside reverse transcriptase inhibitors, nonnucleoside reverse transcriptase inhibitors, and acyclic nucleoside phosphonate analogues),
2. Hepatitis B virus (HBV) infections (lamivudine, interferons, nucleoside analogues, and acyclic nucleoside phosphonate analogues)
3. Hepatitis C virus (HCV) infections (ribavirin, interferons, NS3/4A protease inhibitors, NS5A inhibitors, and NS5B polymerase inhibitors)
4. Herpesvirus infections (5-substituted 2-deoxyuridine analogues, entry inhibitors, nucleoside analogues, pyrophosphate analogues, and acyclic guanosine analogues)
5. Influenza virus infections (ribavirin, matrix 2 protein inhibitors, RNA polymerase inhibitors, and neuraminidase inhibitors)
6. Human cytomegalovirus infections (acyclic guanosine analogues, acyclic nucleoside phosphonate analogues, pyrophosphate analogues, and oligonucleotides)
7. Varicella-zoster virus infections (acyclic guanosine analogues, nucleoside analogues, 5-substituted 2-deoxyuridine analogues, and antibodies)
8. Respiratory syncytial virus infections (ribavirin and antibodies)
9. External anogenital warts caused by human papillomavirus infections (imiquimod, sinecatechins, and podofilox).

These licensed antiviral drugs are arbitrarily categorised in 13 functional groups^[162]:

- i. 5-substituted 2-deoxyuridine analogues ($n=3$ drugs and drug combinations)
- ii. Nucleoside analogues ($n=3$)
- iii. Non-nucleoside pyrophosphate analogues ($n=1$)
- iv. Nucleoside reverse transcriptase (RT) inhibitors (NRTIs) ($n=9$)
- v. Non-nucleoside reverse transcriptase inhibitors (NNRTIs) ($n=5$)

- vi. Protease inhibitors (PIs) ($n=9$)
- vii. Integrase inhibitors ($n=5$)
- viii. Entry inhibitors ($n=7$)
- ix. Acyclic guanosine analogues ($n=6$)
- x. Acyclic nucleoside phosphonate (ANP) analogues ($n=10$)
- xi. Hepatitis C virus (HCV) NS5A and NS5B inhibitors ($n=8$)
- xii. Influenza virus inhibitors ($n=8$)
- xiii. Immunostimulators, interferons, oligonucleotides, and antimitotic inhibitors ($n=8$)

The activity spectrum of these licensed drugs against 9 human infectious diseases is summarized as follows:

- 1. Human Immunodeficiency Virus (HIV) (groups iv, v, vi, vii, viii, and x)
- 2. Human Cytomegalovirus (HCMV) (groups iii, ix, x, and xiii)
- 3. Hepatitis B virus (HBV) (groups ii, iv, x, and xiii)
- 4. HCV (groups vi, xi, xii, and xiii)
- 5. Herpes Simplex Virus (HSV) (groups i, ii, iii, viii, and ix)
- 6. Influenza virus (group xii)
- 7. Respiratory Syncytial virus (RSV) (groups viii and xii)
- 8. Varicellazoster virus (VZV) (groups i, ii, viii, and ix)
- 9. Human papillomavirus (HPV) (group xiii).

Enormous milestones have been reached in the field of antiviral drug discovery in the past 50 years. Many antiviral drugs and/or vaccines have been approved for the treatment of 22 infectious diseases: Mumps, Measles, Rubella, HIV, HBV, HCV, HCMV, HPV, HSV, RSV, VZV, Human Herpesvirus 6, Influenza virus, Smallpox, Rotavirus, Human Adenovirus, Poliovirus, Hepatitis A virus, Hepatitis E virus, Japanese Encephalitis virus, Yellow fever, and Rabies. However, there is still no

antiviral drug or vaccine for over 200 infectious diseases that are affecting humans worldwide.

Since the efficiency of most antiviral drugs is limited to only certain viral strains, novel antiviral compounds with broad-spectrum activity against different virus genotypes or subtypes are the need of the hour. Example, some molecules (e.g., amprenavir) inhibit only HIV-1 but not HIV-2. Most HCV inhibitors have been approved only for HCV genotype 1 but not for other genotypes. Nevertheless, many antiviral inhibitors (brivudine, acyclovir, foscarnet, famciclovir, lamivudine, ribavirin, valacyclovir, PegIFN α -2a, and PegIFN α - 2b) have been licensed for the treatment of more than one virus, supporting the idea of developing antiviral drugs against multiple infectious diseases in the future. Instead of the fast advancement of pharmaceutical and biotechnological approaches (e.g., RNA interference [RNAi]), the development of efficacious antiviral treatments have remained a task. There are many reasons for it. Firstly, strong antiviral drug that can neutralize the highly mutable nature of virus genomes are still required, because emerging drug resistance mutations remain a major reason for failure of therapies. Second, it is difficult to eliminate viral reservoirs using antiviral agents, since certain DNA viruses and retroviruses can remain in a proviral state by integrating their genomes into host genomes. Third, it is a challenge to speed up development of antiviral drugs and vaccines against emerging infectious diseases, calling for a joint effort between scientific and industrial partners. Fourth, it is a challenge to discover efficient, safe, and well-tolerated drugs that improve patient adherence and drug administration. Fifth, efficient antiviral treatments against viral coinfections (e.g., HIV/HBV coinfections) require further investigations. Sixth, access to and delivery of costly new therapies are becoming increasingly problematic in resource-limited settings. Political and financial commitment is also vital to eliminate

substandard and counterfeit antiviral drugs that threaten global public health. The possibility of finding potential novel anti-virals in natural environments like plants and the marine eco-system is enormous and highly encouraging^[163,164].

PLANTS AS ANTIVIRALS

Plants have naturally evolved over the years in diverse climate conditions on earth and have been endowed with rich complex of secondary metabolites/phytochemicals with wide pharmacokinetic spectrum. Very few of the phytochemicals have been purified and studies for their structure and therapeutic properties. Most of the crude plant products have been marketed as pharmaceutical products without certified quality and efficacy. Many traditional medicinal plants and herbs have been reported to have strong antiviral activity against HCV, HBV and H1N1, etc. These phytochemicals must be subjected to animal and human studies to determine their effectiveness in whole-organism systems including reactogenecity and toxicity studies

Many phytochemicals have been reported to have anti-viral activities. Mangiferin, a glycoside and the major constituent of alcoholic extracts from mango leaves has been shown to inhibit HSV-1 and HSV-2 in cell cultures^[165]. It is also known to antagonize cytopathic effects of HIV in cell culture^[166]. Similarly, many active microbial and bioactive compounds have been reported from Bael. Amongst these, Marmelide has exhibited anti-viral activities against the Ranikhet Virus (New Castle Disease Virus)^[167] and the Human Coxsackie Virus B1-B6^[168]. In this study the methanol extract from Eranda, RC exhibited potential Anti-Rabies Virus activity, while the protein fraction RP did not exhibit a promising anti-viral activity against the Rabies Virus. Much work on the anti-viral activities from plant sources are limited to the secondary metabolites of plants. However, there is a small body of work which draws our attention to the

potential anti-viral activities of proteins derived from plant sources. The *Momordica* Anti-HIV Protein 30 (MAP-30) has shown potent anti-HIV activity^[169]. Similarly, *Gelonium* Anti-HIV Protein 31 (GAP-31) isolated from *Gelonium multiflorum* has been shown to exhibit anti-HIV activity^[169]. GAP-31 has been found to inhibit HIV entry and viral genome integration. It is also known to have inhibitory activities against Herpes Simplex Virus (HSV) and Human Herpes Virus 8 (HHV-8)^[170]. The mechanisms of action of three important plant proteins – Ricin, isolated from *R. communis*, Pokeweed Antiviral Protein (PAP), isolated from *Phytolacca americana* and Luffa Ribosomal Inhibitory Protein (LRIP-I), isolated from *Luffa cylindrica* roem – have been elucidated and host Ribosomal Inhibition has been seen as the active principle behind their anti-viral properties^[171–173]. Apart from these, gp22 and gp35 – two proteins isolated from the Tobacco plant infected with Tobacco Mosaic Virus (TMV) have exhibited anti-viral activities against the TMV^[174]. Anti-viral activity, albeit to Plant viruses (TMV) has been reported in two proteins CIP-29 and CIP-34 isolated from the leaves of *Clerodendrum inerme* Gaertn^[175]. What is interesting is that these proteins, which have exhibited antagonistic properties against the plant viruses have been found to function as Ribosome Inhibitory Proteins (RIPs). There is therefore a great scope – and given the fact that there is a greater need for anti-virals – that plant proteins along with plant secondary metabolites be screened for their potential anti-viral properties. Gathering traditional information from local or indigenous people or using ethnomedicinally important plant(s) to extract bioactive molecules/phytochemicals for curing various diseases are quite challenging approaches. Many factors such as different solvents (polar, nonpolar) employed for the extraction of bioactive constituent(s), choice of plant part/tissue for extraction bioactive constituent(s) often play important roles in extracting the biologically active phytochemicals/natural

constituents from plants efficiently. To appraise the antiviral activity of plants systematic approach to isolate and characterize the bioactive molecules/phytochemicals and virus replication inhibition assays in animals or mammalian cell system are indeed needed before such phytomolecules could actually be employed to treat viral infection^[176]. Different methods for isolation, purification of bioactive molecules/phytochemicals from the extracts of plant to carry out biological activity such as their antibacterial, antifungal and antiviral properties are required to be established. A variety of biological assay such as antiviral properties such as cytopathic effect screen, neutralization assays, yield reduction assays and haemagglutination inhibition test have been successfully used to study the. Extraction of bioactive principle is an important first step in the analysis of plants to extract the desired bioactive molecules/phytochemicals. The traditional practices in isolation of these bioactive molecules/phytochemicals using different separation techniques such as TLC, column chromatography, flash chromatography, HPSCCC, HPLC, FTIR, NMR and MS have been extensively exploited to obtain and facilitate the identification of the bioactive molecules/phytochemicals. The ability to identify bioactive molecules/phytochemicals from large chemical libraries accurately and rapidly has been the goal in developing high throughput screening assays (Figure 2). To firmly establish the antiviral activity and adverse reactions like reactogenicity or toxicity of the purified phytomolecules, appropriate *in vivo* studies (animal models) and subsequent clinical trials are necessary. A bioactive flavonoid 'Baicalein' isolated from Chinese medicinal plant *Scutellaria baicalensis* Georgi showed antiviral properties using high-speed counter-current chromatography (HSCCC) technique^[177].



Figure 13 Types of Phytochemicals and their activities^[178]

Plant (part)	Phytochemicals	Target	Mode of action	Ref.
Artocarpus lakoocha (Heartwood)	Oxyresveratrol	HSV-1 HSV-2	Inhibitory activity at the early and late phase of viral replication of HSV-1 and HSV-2. Inhibition of late protein synthesis	[179]
Bupleurum kaoi (Root)	Saikosaponin b2 (Terpenoid)	HCV	Inhibiting early HCV entry, including neutralization of virus particles, preventing viral attachment	[180]
Citrus reticulata (Pericarps)	Tangeretin and nobiletin (Polymethoxylated flavones)	RSV	Affected the intracellular replication of RSV. Tangeretin down regulated the expression of RSV phosphoprotein (P protein)	[176]
Euphorbia amygdaloides spp. and semiperfoliata (Whole plant)	*Compound 3 (Jatrophone esters)	CHIKV HIV-1 HIV -2	Selective inhibitor of the replication and inducing down regulation of HIV receptors (CD4, CXCR4 and CCR5)	[181]
Glycyrrhiza radix (Roots)	Glycyrrhizic acid (GL)	EBV	GL interferes with an early step of EBV replication cycle.	[182]
Houttuynia cordata (Aerial parts)	Quercetin 3rhamnoside (Q3R)	Influenza A virus	Inhibit replication in the initial stage of virus infection by indirect interaction with virus particles	[183]
Limonium sinense (Root)	Samarangenin B	HSV-1	Inhibit HSV-1 α gene expression, including expression of the ICP0 and ICP4 genes, by blocking β transcripts such as DNA	[184]

			polymerase mRNA, and by arresting HSV-1 DNA synthesis and structural protein expression in Vero cells.	
Liriope platyphylla (Root)	LPRP-Et-97543	HBV	Inhibit viral gene expression and replication. Inhibit viral promoter activity.	[185]
Melia azedarach L. (Leaves)	Tetranortriterpenoid 1-cinnamoyl-3, 11-dihydroxymeliacarpin (CDM)	VSV HSV-1	CDM blocks VSV entry and the intracellular transport of VSV-G protein and confined it only to the Golgi apparatus and also modulates the NF- κ B signaling pathway by slowing down its activation in HSV-1-infected conjunctival cells which leads to the accumulation of p65 NF- κ B subunit in the cytoplasm of uninfected treated Vero cells	[186]
Prunella vulgaris (Fruit spikes)	Lignin-carbohydrate complex (PPS-2b)	HSV-1 HSV-2	Block HSV-1 binding and inhibiting penetration into Vero cells	[187]
Pterocarya stenoptera (Bark)	Pterocarnin A	HSV-2	Inhibit HSV-2 from attaching and penetrating into cells. It also actively suppressed HSV-2 multiplication in Vero cells even when added 12 h after infection	[188]
Ruta angustifolia (Leaves)	Chalepin and pseudane IX	HCV	Inhibited HCV at the post-entry step and decreased the levels of HCV RNA replication and viral protein synthesis	[189]
Saururus chinensis (Root)	Manassantin B (Dineolignans)	EBV	Inhibitory effects toward EBV lytic replication	[190]

Table 3 Mode of action of specific phytochemicals with antiviral activity.^[178]

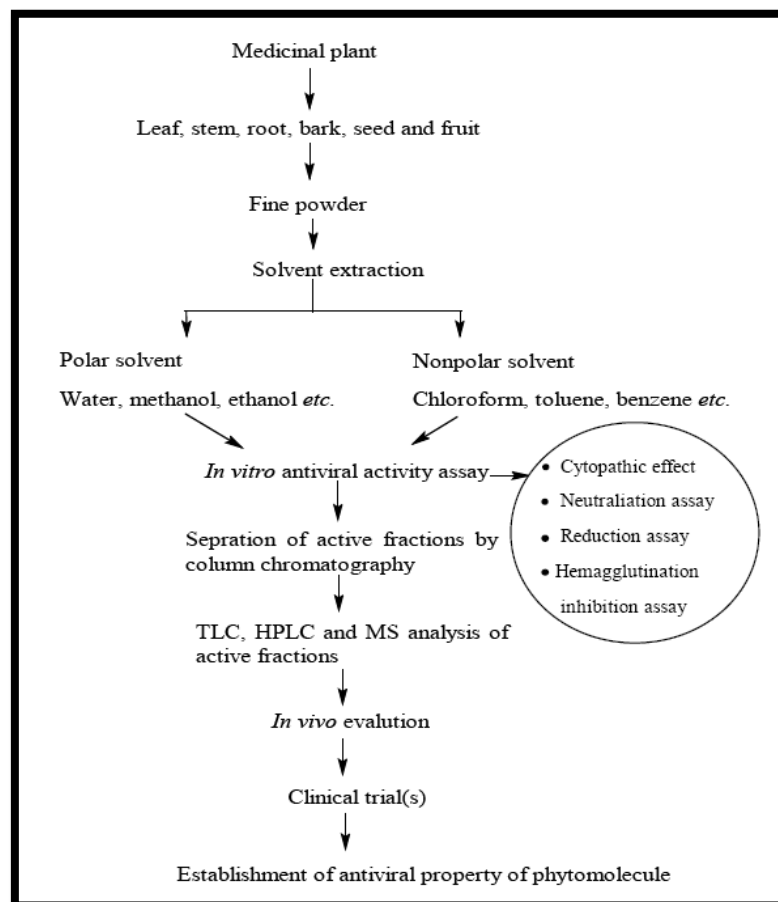


Figure 14 Isolation of antiviral phytochemicals from plants.^[178]

IMMUNOMODULATORS

Infections which lead to devastations in the past centuries are again re-emerging and newly emerging pathogens proficient of causing human diseases, continue to develop. Multidrug resistance has become a significant medical predicament. Smarter strategies for managing infections in the 21st century clinics have acquired wider elements. Immunomodulators of natural, synthetic, and recombinant origin can stimulate immune mechanisms of the host for the prophylaxis and treatment of diverse viral, bacterial, parasitic and fungal diseases. Some immunomodulator preparations have already been licensed for human use and many others are being broadly examined in preclinical and clinical studies. Immunomodulators offer a novel assistance to established antimicrobial therapies^[191,192].

Whereas vaccinations are used to manipulate the immune system specifically, non-specific but wider variation can be brought about by immunomodulators. Immunomodulators include both immunostimulatory and immunosuppressive agents. The mode of action of immunomodulators includes augmentation of the anti-infectious immunity by the cells of the immune system including lymphocyte subsets, macrophages, and natural killer cells. Other mechanisms involve induction or restoration of immune effector functions. Immunomodulators are alternatively also known as biological response modifiers, immunoaugmentors, or immunorestoratives. Few of the currently in-use immunomodulators include Microbial products, drugs of natural and synthetic origin, and proteins derived from the immune system represent.

Microbial immunomodulators

Whole microbes

Antibiotic associated diarrhea has been successfully prevented using whole microbes such as *Lactobacillus casei*, *Bifidobacterium* species, and *Saccharomyces boulardii*. They have also been used to treat other diarrheal illnesses caused by bacteria. *Bifidobacterium brede* has been shown to augment antibody production and induce significant reduction in the frequency of rotavirus shedding in stool samples of infants. The major *Lactobacillus* species present in the human gastrointestinal mucosa, *L. plantarum*, *L. rhamnosus* and *L. paracasei*, have been found to be potent stimulators of IL-12. More recently, efforts have been directed to construct recombinant *Bacillus Calmette & Guerin* (BCG) strains that secrete functional cytokines such as IL-2, IFN- γ or GM-CSF which have been shown to enhance immune responses to antigens^[191].

Bacterial lysates^[191]

Bacterial extracts have been widely used as immunomodulators to prevent recurrent infections of the respiratory tract. There exists a large body of primary literature on placebo-controlled and double-blind clinical trials which have been conducted with these agents. The current concept of the mucosal immune system postulates that stimulation of the gut-associated lymphoid tissue can lead to the induction of a generalized response by the whole mucosal-associated lymphoid tissue. Patients, most often children, having recurrent episodes of infections of the respiratory tract and of the ear, nose and throat have been treated with oral bacterial lysates. In most cases the frequency and the severity of infections was reported to be reduced and both the physician and the patient considered the treatment to be beneficial. It is of interest that many studies have reported a decrease in antibiotic consumption in bacterial lysate-treated patients.

Natural and synthetic immunomodulators

Muramyl peptides

N - acetyl - muramyl - L - alanyl - D - isoglutamine (MDP), is a small glycopeptide which signifies the minimal structure vital for mycobacterial adjuvanticity. Synthetic MDP and its analogs have pleiotropic properties with the potential to augment nonspecific resistance against diverse microbial infections. MDP-Lys(L18), a muramyl peptide analog, is a potent inducer of a variety of cytokines such as IL-1, IL-6, CSFs, TNF, and IFN- γ in mice and humans. MDP-Lys(L18) has been licensed in Japan under the trademark Romurtide[®], and has been shown to be effective for renewal of neutropenia and platelets in cancer patients. The incidence of infectious diseases in the MDP-Lys(L18)-treated group has been observed to be lower than in the control groups during the clinical trials. A placebo-controlled double blind clinical trial of glucosaminyl analog, GMDP, for immuno-therapy of septic complications arising after abdominal surgery has been reported. Reduced frequency of septic complications and lesser deaths were observed during prophylactic administration of GMDP and post-operative treatment of patients who acquired infections.

Resistance against experimental infections by HIV, influenza, herpes simplex, Sendai, Semliki-Forest, vaccinia and murine hepatitis viruses and against many bacterial infections has been observed with the administration of MDP and certain analogs, alone or in combination with other agents. Adamantylamide dipeptide consists of antiviral 1-amino-amantadane moiety linked to the D-isoglutamine portion of MDP. Adamantylamide dipeptide has shown significant activity against influenza virus in vitro cell cultures and preclinical studies demonstrated that the homotypic immunity induced by influenza subunit vaccines could be broadened to a heterologous immune response. Adamantylamide dipeptide also exhibited activity against in vitro HIV

infection^[193].

Thymic hormones and peptide analogs^[192,194]

Preparations containing thymus extracts are currently licensed in Europe for the adjunctive therapy of immune defects in infectious diseases, and are indicated for generally weakened host defense occurring in viral diseases and recidivating bacterial infections. Therapy with thymus extract preparations such as thymostimulin, has been shown to reduce the frequency and intensity of recurrences of acute respiratory infections. Combination therapy with thymosin- α 1, interferon- α and AZT in patients was associated with a substantial increase in the number and function of CD4 T cells. Thymosin- α 1, interferon- α and amantadine combination was shown to be effective for influenza virus infection. Clinical trials have shown that combination of thymosin and antiviral drug could significantly improve treatment of viral hepatic disease.

Methyl inosine monophosphate (MIMP) is an interesting thymomimetic immunomodulator. MIMP has been shown to enhance mitogen-induced proliferation of lymphocytes, augment IgM plaque-forming cells, induce delayed type hypersensitivity and normalize an impaired response to IL-2. The mean day death in mice infected with Friend leukemia virus, employed as a murine model of AIDS, could be significantly delayed by MIMP.

Isoprinosine^[195]

Isoprinosine has been found to be useful in treatment of several viral diseases such as herpes simplex, herpes zoster, influenza and rhinovirus infections and multicenter double-blind clinical trials of isoprinosine in asymptomatic HIV-positive patients showed a delay in the progression of HIV infection to AIDS in some trials. Isoprinosine increases survival in subacute sclerosing panencephalitis although it does not inhibit the replication of measles virus. Isoprinosine is currently licensed in Europe for

treatment of herpes simplex infections, subacute sclerosing panencephalitis, acute viral encephalitis caused by herpes simplex, Epstein ± Barr and measles viruses and for treatment of these viral infections in immunosuppressed patients.

Glucans^[196,197]

Poly-(1 ± 6)-b-glucotriosyl-(1 ± 3)-b-glucopyrano-seglucan (PGG-glucan) is derived from yeast cell walls and is marketed as Betafectin. Surgical patients at high risk for postoperative infection who underwent major thoracic or abdominal surgery and who received PGG-glucan had significantly fewer infectious complications, decreased intravenous antibiotic requirement and shorter intensive care unit length of stay. A recent multicenter, prospective, randomized, double-blind, placebo-controlled trial of 1249 patients showed that perioperative administration of PGG-glucan reduced serious postoperative infections or death by 39% after high-risk non-colorectal operations. Lentinan, a 1 ± 3-b-D-glucan, is licensed as an adjunctive for antitumor therapy in Japan. Experimental studies have demonstrated that lentinan can confer protection against influenza virus, *Listeria* and prevent re-lapse of *M. tuberculosis*. Yeast glucan could enhance resistance against herpes simplex virus types I and II, and murine hepatitis virus; induce nonspecific resistance against *Klebsiella pneumoniae* infection and could protect patients from sepsis, bacteremia and peritonitis resulting from *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* infections; and prolong survival against parasitic infections by *Plasmodium berghei* and *Leishmania donovani* and exert anti-fungal activity against *Candida*, *Cryptococcus* and *Sporotrichum*. Sulfated polysaccharides, such as curdlan sulfate, have been shown to exert inhibitory activity on HIV replication and are currently in clinical trials. Curdlan sulfate may act on HIV infection by interfering with the viral membrane fusion process and downregulation of tumor necrosis factor.

Cytokine immunomodulators

Cytokines are intimately involved in host antimicrobial immune responses. Certain cytokines stimulate the production of other cytokines and interact in complex synergistic or antagonistic networks. Local and systemic effects of cytokines produced by infiltrating cells appear to play an important role in control of infections. Recent advances in identification and cloning using monoclonal antibody and recombinant DNA technologies have led to the availability of ample quantities of cytokines with immunomodulatory activities.

Interferons^[198,199]

Interferons play an important role in host defense against infectious pathogens and in regulation of immune responses. There is a vast body of literature on activity of interferon- α as an antiviral agent. Natural interferon- α obtained from human serum and leukocytes is currently licensed for the treatment of a rare form of cancer, hairy cell leukemia. Recombinant interferon- α -2a is approved for treatment of chronic active hepatitis B and for hepatitis C virus infections. However, the number of patients with hepatitis B who are responsive to this therapy is very marginal and around 40% of cases with hepatitis C respond. Condyloma acuminata caused by human papilloma virus can also be treated with Interferon- α . It is also approved for treating and for Kaposi sarcoma in patients with HIV infection. Interferon- β obtained from human FS-4 fibroblast cell lines is indicated in severe uncontrolled virus-mediated diseases in cases of viral encephalitis, herpes zoster and varicella in immunosuppressed patients. A further indication is viral infection of the inner ear with loss of hearing. Interferon- β is also licensed for treatment of multiple sclerosis patients. Interferon- γ (IFN- γ) is a potent macrophage-activating factor produced by both CD4 and CD8 T cells and can induce class I and class II MHC products. Many research studies have exhibited a

significant role for IFN- γ in protection against *Listeria monocytogenes*, *Francisella tularemia*, *Toxoplasma gondii*, *Pneumocystis carinii* infection, asexual blood forms of *Plasmodium falciparum*, *Chlamydia trachomatis*, *Rickettsia conorii*, *Paracoccidioides brasiliensis* and primary *Shigella flexneri* infections. IFN- γ is approved as a therapeutic adjunct for use in patients with chronic (septic) granulomatosis for bringing down of the incidences of severe infections. *Tumor necrosis factor*^[200–202]

Tumor necrosis factor (TNF) has been shown exert antiviral activity against encephalomyocarditis virus or vesicular stomatitis virus, and respiratory syncytial virus. Administration of TNF in bacterial infections has been shown to confer protection against *K. pneumoniae*, *S. pneumoniae*, *Legionella pneumophila*, salmonellosis, *L. monocytogenes* and *My-cobacterium avium*. TNF has been also shown to inhibit experimental parasitic infections with *Plasmodium* species, cutaneous leishmaniasis, *Trypanosoma cruzi* and *T. gondii* in mice.

Colony stimulating factors^[203–205]

The colony stimulating factors (CSF) are closely involved in generation and differentiation of stem cells in the bone marrow to phagocytic cells. M-CSF has been shown to induce resistance against vesicular stomatitis virus, herpes simplex virus, *L. monocytogenes* and *Candida albicans*. G-CSF stimulated resistance in neutropenic and or non- neutropenic animals to infections by *P. aeruginosa*, *S. aureus*, *Serratia marcescens*, *L. monocytogenes* and disseminated *C. albicans*. G-CSF has been suggested as adjunctive therapy for treatment of serious bacterial and opportunistic fungal infections in nonneutropenic patients. G-CSF has been shown to induce increases of CD34 and CD4 in HIV patients. Human macrophages treated with GM-CSF exhibited significant inhibition of growth of *M. avium* complex and administration to AIDS patients enhanced their bactericidal activity against mycobacteria. GM-CSF

has been shown to be capable of inducing activity against *C. albicans*, *Leishmania*, *T. cruzi*, *Salmonella typhimurium*, and to improve monocyte functions in septic shock patients. GM-CSF produced by recombinant technology from *E. coli* has been licensed for achieving reduction of risk of infection by diminishing the severity of neutropenia in patients treated with usual doses of myelotoxic chemotherapy.

Interleukins^{[194,206][207]}

Treatment with interleukin-1 significantly increases resistance of mice to *L. monocytogenes*, *K. pneumoniae*, *Brucella abortus*, *S. typhimurium*, *P. aeruginosa*, and *C. albicans* lethal infections in normal and in neutropenic hosts.

Interleukin-12 (IL-12) has multiple effects on T and NK cells and is a potent inducer of IFN- γ . IL-12 has been shown to have protective activity against *L. donovani*, *Leishmania major*, and *Leishmania amazonensis* in a primate model of cutaneous leishmaniasis. IL-12 prolonged survival of immunodeficient SCID mice infected with *Histoplasma capsulatum* or *T. gondii* and was critical for resolution of *L. pneumophila* lung infection. IL-12 enhanced bone marrow and splenic erythropoiesis and alleviated malaria-induced anemia. IL-12 has been shown to contribute to the inhibition of early replication of influenza virus and when administered intranasally with influenza vaccine to enhance survival after lethal challenge with infectious influenza virus. Administration of IL-12 significantly increased survival after lethal systemic herpes simplex virus infection and has been shown to play an important role in the antiviral immune response to hepatitis B virus infection by preventing the assembly or triggering the degradation of the nucleocapsid particles. Infusion of IL-12 has been shown to enhance recovery from lethal vesicular stomatitis virus infection of the central nervous system. IL-12 is currently in clinical trials in cancer patients and HIV-infected patients. Interleukin-13 (IL-13) is an anti-inflammatory cytokine, which, as opposed to IL-4 or

IL-10, has limited consequences on T cell functions. IL-13, in addition to IL-4, has been recently shown to play an important role in resistance against intestinal nematode infection by *Trichuris muris*. IL-13 has been shown to act on macrophages before and after HIV-1 infection by blocking the completion of reverse transcription, decreasing virus production, and reducing the infectivity of the progeny virions. Interleukin-15 (IL-15) is produced primarily by macrophages and shares many biological activities with IL-2. IL-15 has been shown to upregulate the microbicidal activity of human monocytes against *C. albicans*. Treatment of monocytes from HIV-infected patients with IL-15 resulted in an increase in NK cell cytotoxicity and restoration of the deficient production of IL-12.

MANGO, BAELE AND ERANDA: THREE PLANTS MULTIPLE ACTIVITIES

Mango (*Mangifera indica*)

The mango is a rich source of various polyphenolic compounds. The major polyphenols in the mango in terms of antioxidative capacity and/or quantity are: mangiferin, catechins, quercetin, kaempferol, rhamnetin, anthocyanins, gallic and ellagic acids, propyl and methyl gallate, benzoic acid, and protocatechuic acid^[208,209]. The nutraceutical and pharmaceutical significance of mangiferin, which is a special polyphenol in the mango has been extensively demonstrated and continues to attract much attention especially in its potential to combat degenerative diseases like heart diseases and cancer. The amounts of the different polyphenolic compounds in the mango vary from part to part (pulp, peel, seed, bark, leaf, and flower) with most polyphenols being found in all the parts. Mango polyphenols, like other polyphenolic compounds, work mainly as antioxidants, a property that enables them to protect human cells against damage due to oxidative stress leading to lipid peroxidation, DNA damage, and many degenerative diseases. Use of pure isolated compounds has been found to be less effective than the use of crude mixtures from the particular mango part suggesting that synergism of the various mango polyphenols is important for maximum antioxidative activity. Antiviral activity against the HSV-2 has been exhibited with mangiferin, the major phytochemical in mango^[165]. Also mango extracts exhibit various anti-microbial activities^[210]. Different parts of Mango have been used widely for medicinal purposes in many countries. It has been widely used in cancer, diabetes, asthma, infertility, lupus, prostatitis, prostatic hyperplasia, gastric disorders, arthralgias, mouth sores and tooth pain. The phytochemical profile of mango stem barks has been reported. Mangiferin is the major component in mango stem bark. Other flavonoids and flavonol constituents include quercetin,

catachin and epicatechin.^[211] The phytochemical screening of the raw material showed that polyphenols, terpenoids, sugars and saponins were present in mango stem bark. Many phenolic constituents, benzoic acids and its propylester, three free sugars (galactose, glucose and arabinose) and three polyalcohols (sorbitol, myoinositol and xylitol) were identified and tested from the mango fruits and stem bark related constituents of mango stem barks. Volatile components of mango stem bark were extracted, and the major constituents identified were β -relemens, aromandrene, α -guaiene, β -endesmol, β -sitrosterol and β -campesterp. The amount of sesquiterpenoid hydrocarbons was higher than the oxygenated compounds^[211]. The composition of the fatty acid fraction was also determined as their trimethylsilyl esters derivatives and the major constituents were palmitic, oleic and linoleic acids with a minor proportion of myristic and estearic acids. Many polyunsaturated and dicarboxylic acids of biological importance such as eicosatrienicoic, succinic and malonic were found to be present in mango stem bark in trace levels. *Mangifera indica* stem bark has antioxidant, anti-inflammatory and immunodulatory properties and have been developed in Cuba as nutritional supplement or functional food in several formulations (antioxidant) or anti-inflammatory, analgesic and immunomodulator (tablet, capsule, syrup and cream) and have been extensively used to prevent disease progress or improve the patient's quality of life in diseases like HIV/AIDS, cancer, asthma, gastric and dermatological disorders^[211].

Bael (*Aegle marmelos*)

The therapeutic value of *Aegle marmelos* Correa (Rutaceae), commonly known as

“Bael,” has been recognized as a component of traditional medication for the treatment of various human ailments. The plant, though, being highly explored, still lacks sufficient evidences for the best variety possessing the highest degree of medicinal values.

Bael has been known to be one of the most important medicinal plants of India since Charak (1500 B.C). More than 100 phytochemical compounds have been isolated from various parts of the plant, namely phenols, flavonoids, alkaloids, cardiac glycosides, saponins, terpenoids, steroids, and tannins^[212,213]. These compounds are well known to possess biological and pharmacological activity against various chronic diseases such as cancer and cardiovascular and gastrointestinal disorders. Antioxidant, antiulcer, antidiabetic, anticancer, antihyperlipidaemic, anti-inflammatory, antimicrobial, antispermato-genic effects have also been reported on various animal models by the crude extracts of this plant^[214,215]. Every part of *Aegle marmelos* plant such as its fruits, stem, bark, and leaves possesses medicinal property and is used for treating various eye and skin infections. Leaf is considered to be one of the highest accumulatory parts of the plant containing bioactive compounds which are synthesized as secondary metabolites. Leaf extracts of bael have exhibited anti-viral activities against coxsackie virus B1-B6^[168]. Bael is one of the most important medicinal plants in Ayurveda. According to Charaka (1500 bc), the high priest of Ayurveda, Bael is one of the most important medicinal plants in Ayurveda, which has been in existence for a long time and is extensively used by inhabitants of India.^[216] Bael is referred to as an emblem of fertility and is a healing tree that strengthens the body. It is also used in various folk medicines in India, Nepal, Sri Lanka, Bangladesh, and Burma (now Myanmar).^[217] A sweet drink prepared from the ripe fruit pulp is supposed to be effective against bacillary dysentery. The unripe fruits of Bael

are reported to be useful in treating diarrhea, dysentery with spells of constipation, and stomach ache.^[217] The roots are also one of the important ingredients of the Ayurvedic traditional drug Dashamula, a panacea for colitis, dysentery, diarrhea, flatulence, and fever. The leaves are supposed to reduce bowel complaints, bleeding piles, dropsy (edema), diarrhea, and dysentery.^[217]

Chemical analysis suggests that Bael contains tannins, skimmianin, essential oils like caryophyllene, cineole, citral, cuminaldehyde, citronella, p-cymene, d-limonene, and eugenol, sterols and/or triterpenoids, including lupeol, β - and γ -sitosterol, α - and β -amyrin, flavonoids like rutin and coumarins, including aegeline, marmesin, umbelliferone marmelosine, marmelin, o-methyl halfordinol, alloimperatorin methyl ether, o-isopentenyl halfordinol, phlobatannins, flavon-3-ols, leucoanthocyanins, and anthocyanins.^[218,219] Most of these chemicals possess beneficial health effects and exhibit diverse pharmacological effects. Pharmacological studies have validated most of the ethnomedicinal uses of Bael such as antimicrobial effects, antimicrofilarial, antifungal, hypoglycemic, astringent, antidiarrheal, antidysenteric, demulcent, analgesic, anti-inflammatory, antipyretic, hypoglycemic, antidyslipidemic, immunomodulatory, antiproliferative, wound-healing, insecticidal, anticancer, antidiabetic, and cardioprotective properties.^[215]

Eranda (*Ricinus communis*)

Ricinus communis or castor plant has high traditional and medicinal value for its variety of biological activities. The Leaves have high levels of alkaloids, Flavanios, glycosides

and terpenoids^[220]. Traditionally the plant is used as laxative, purgative, fertilizer and fungicide etc. whereas the plant possess beneficial effects such as anti-oxidant, antihistamic, Antinociceptive, antiasthmatic, antiulcer, immunomodulatory, Antidiabetic, hepatoprotective, Antifertility, anti inflammatory, antimicrobial, central nervous system stimulant, lipolytic, wound healing, insecticidal and Larvicidal and many other medicinal properties^[221–224]. This activity of the plant possesses due to the important phytochemical constituents like flavonoids, saponins, glycosides, alkaloids and steroids etc. According to the Charak Samhita, from all the herbs which have the capability for pacifying vata dosha eranda is the best.

The plant has the following ethanobotanical uses:

Antioxidant activity: The DPPH (1,1-diphenyl-2- picrylhydrazyl)-mediated in vitro study reveals that gallic acid, quercetin, gentisic acid, rutin, epicatechin and ellagic acid are the major phenolic compounds responsible for the antioxidant activity of the dry leaves of *Ricinus communis* Linn^[225].

Antitumour activity: Ricin A, a lectin isolated from *R. communis* possess antitumor activity, it was more toxic to tumor cells than to nontransformed cells, judged from the ED50 of the lectin towards tumor cells and non-transformed cells^[226].

Bone Regeneration Activity: *Ricinus communis* polyurethane (RCP) has been studied for its biocompatibility and its ability to stimulate bone regeneration. Results showed that RCP blended with calcium carbonate or calcium phosphate could promote matrix mineralization and are biocompatible materials^[227].

Antiasthmatic activity: The ethanol extract of *R.communis* roots possess antiasthmatic activity, it significantly decreases milk induced leucocytosis and eosinophilia and protect degranulations of mast cells in mice^[228].

Antinociceptive activity: The methanol extract of *R. communis* leaves possesses

antinociceptive activity in writhing test, formalin induced paw licking and tail immersion method in mice^[228]. Previous phytochemical studies on *R. communis* revealed the presence of steroids, saponins, alkaloids, flavonoids and glycosides. The dried leaves of the plants have shown the presence of two alkaloids- ricinine and N-demethylricinine and flavones glycosides- kaempferol-3-O- β -D-xylopyranoside, kaempferol-3-O- β -D-glucopyranoside, quercetin-3-O- β -D-xylopyranoside, quercetine-3-O- β - glucopyranoside, kaempferol-3-O- β -rutinoside and quercetin-3-O- β -rutinoside. The monoterpenoids (1,8- cineole, camphor and α -pinene) and a sesquiterpenoid (β -caryophyllene), gallic acid, quercetin, gentisic acid, rutin, epicatechin and elingic acid are major phenolic compound isolated from leaves. Indole-3- acetic acid has been extracted from the roots. The seeds contain 45% of fixed oil, which consists glycosides of ricinoleic acid, isoricinoleic, stearic and dihydroxystearic acids and lipases and a crystalline alkaloid, ricinine. GLC (Gas Liquid Chromatography) studies of castor oil showed the presence of ester form of palmitic, stearic, arachidic hexadecenoic, oleic, linoleic, linolenic, ricinoleic and dihydroxy stearic acids. The stem also contains ricinine. The GC-MS analyses of *R. communis* essential oil (using capillary columns) have identified compounds like α -thujone and 1, 8- cineole, α -pinene, camphor and camphene. Lupeol and 30- Norlupan-3 β -ol-20-one are obtained from coat of castor bean^[229].

THE ARYL HYDROCARBON RECEPTOR (AHR)

The AHR is a cytosolic receptor interacting with low molecular weight molecules, it binds and becomes stimulated by sterically planar ligands approximately three benzene rings in size^[230]. It exhibits highest expressions in the liver, lungs, skin^[231], and the gastrointestinal tract^[232].

In the cytoplasm, AHR is present in a dormant state as part of a multiprotein complex. It remains associated with Chaperoning proteins including the heat shock protein 90 (hsp90), hsp23, and hepatitis B virus X-associated protein 2 (XAP2)^[233]. Pp60^{src} is an associated signaling partner that is released into the cytosol on ligand binding^[233]. This activates the mitogen-activated protein kinase (MAPK) signaling after binding to the epidermal growth factor receptor (EGFR)^[234,235].

On ligand binding, the receptor complex translocate to the nucleus^[236]. Here it binds to the aryl hydrocarbon receptor nuclear transporter (ARNT). Where the AHR-ARNT heterodimer networks with numerous histone acetyltransferases and chromatin remodeling factors^[237]. The transcription of genes containing xenobiotic response elements (XRE) in their promoters are initiated with this event.

Two feedback loops regulate AHR activity. First, AHR is released to the cytosol and degraded by the 26 S proteasome pathway^[238]. Second, binding to the XREs results in the transcription of the aryl hydrocarbon receptor repressor (AHRR), which regulates the activity of the AHR via a negative feedback loop^[239].

AHR also interacts with other pathways. These include the estrogen receptor, the retinoblastoma protein (Rb), thus inhibiting cell cycle progression and the retinoic signaling pathways. AHR can also bind the p65 subunit of nuclear factor kappa light chain enhancer of activated β cells (NF- κ B), thereby either suppressing or activating (depending on cellular context) the expression of NF- κ B-dependent genes^[240–244].

Tryptophan and the Aryl Hydrocarbon receptor

Many AHR ligands are tryptophan-based molecules. These include 6-formylindolo [3, 2-b] carbazole (FICZ) and 2-(1'-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE), a tryptophan-cysteine dimer^[245,246].

The kynurenine pathway is the metabolic pathway via which L-tryptophan is metabolized to nicotinamide adenine dinucleotide (NAD). In addition to generating endogenous supplies of nicotinamide, it has important immune- regulatory roles. Several metabolites known to act as endogenous ligands of the AHR include kynurenine, kynurenic acid, and cinnabarinic acid^[247–250].

Relationship with tryptophan metabolism

Tryptophan is metabolized by four primary pathways.

The kynurenine pathway

The kynurenine pathway is the metabolic pathway which L-tryptophan metabolises to NAD and the Kynurenine metabolites. Indoleamine 2,3-dioxygenase (IDO) is the rate-limiting enzyme of the kynurenine pathway (KP). It is induced by several proinflammatory molecules, especially interferon gamma (IFN- γ)^[251]. It is important that the inflammatory reaction be controlled and kynurenine which is KP downstream metabolite^[249], provides a negative feedback loop through AHR to promote the generation of T_{reg} cells^[249]. Other downstream AHR ligands include kynurenic^[248], cinnabarinic^[247], and xanthurenic acid^[252].

Reflecting the complexity and multilayered nature of the control mechanisms, it has been reported that kynurenic acid promotes the generation of inflammatory cytokines^[248]. In addition, IDO is upregulated by AHR activation, suggesting that positive feedback loops operate within the microenvironment^[253].

It has been demonstrated recently that the AHR chaperoning protein pp60^{src} can phosphorylate IDO-1, upregulating transforming growth factor beta 1 (TGFβ1) production by dendritic cells, leading to disease tolerance^[254]. TGFβ1 production in dendritic cells in response to the downstream KP metabolite quinolinic acid has also been reported^[255].

Pleiotropic responses are seen with AHR ligands, with some promoting the generation of T_{reg} cells (regulatory ligands), and others, TH₁₇ expansion (effector ligands). The former include TCDD and kynurenine, and the latter, FICZ. Effector ligands such as FICZ are rapidly metabolized and produce only transitory stimulation of AHR, whereas regulatory ligands such as TCDD are long-lived^[256–258].

Much research on the kynurenine pathway has focused on its neuro-inflammatory roles. The nervous system, however, expresses only low levels of the AHR compared to the integument^[231], and it is in the skin that the relationship is likely to be of greatest significance.

AHR is important in the development of the KP. Langerhans cells (LCs) from AHR-null mice display significantly reduced IDO activity^[259] compared to the wild phenotype. Also, the representative AHR ligand, TCDD has exhibited to induction of IDO in dendritic cells, which promotes T_{reg} generation^[248,260].

Several ligands of AHR are pharmacological agents known to modulate the KP also. These include tranilast and leflunomide^[261–263].

The tryptamine pathway

The tryptamine pathway, catalyzed by tryptophan hydroxylase and dopamine decarboxylase, has been implicated in AHR activation. Tryptamine activates AHR either as a direct ligand^[258] or as a precursor for downstream ligands such as indole

acetic acid. In addition, moving through this pathway via carcinoid tumors can result in nicotinamide deficiency (pellagra).

The serotonin pathway

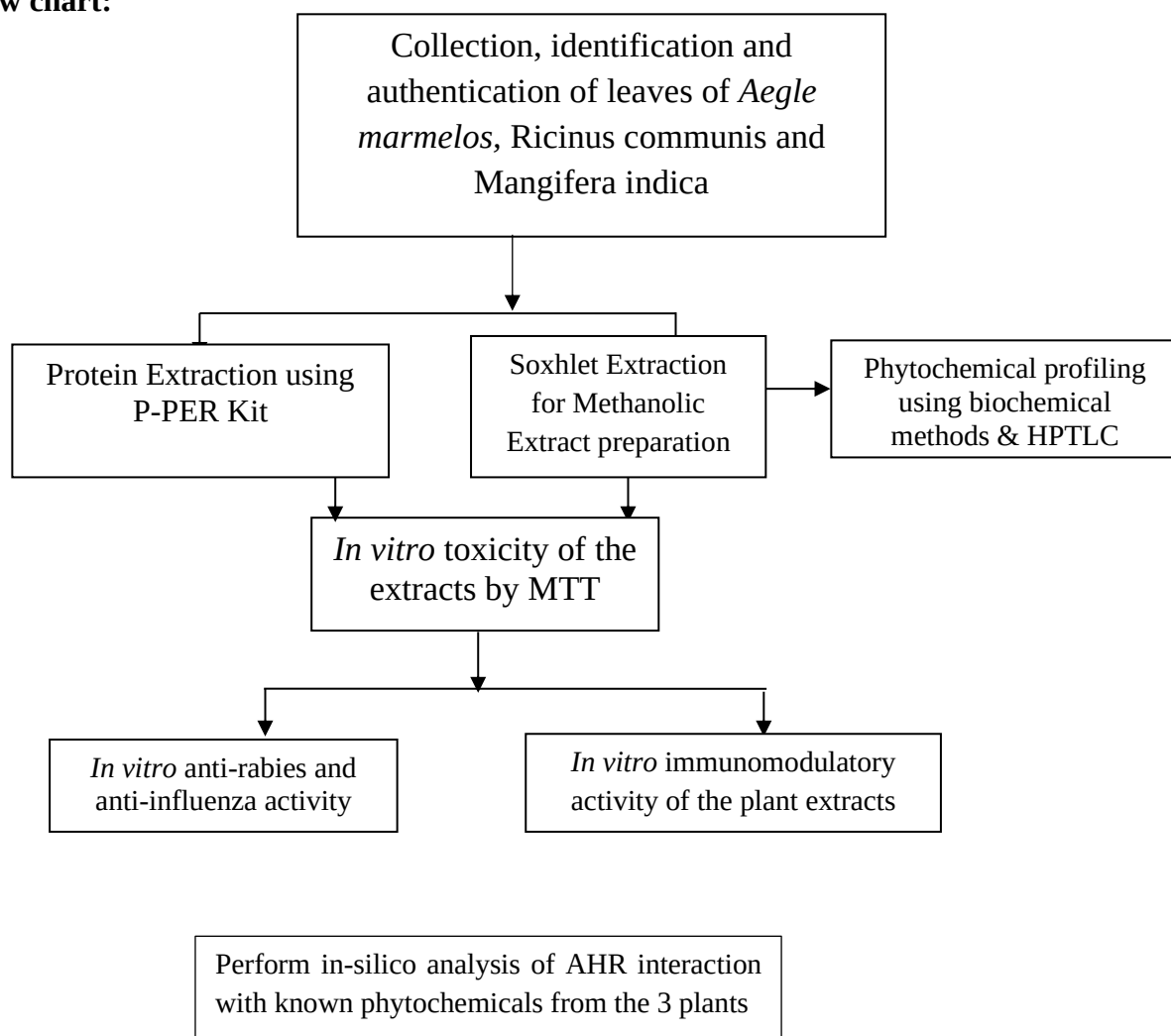
5-Hydroxy-tryptophan (Serotonin), is also an AHR agonist^[264].

Tryptophan photoproducts

Tryptophan is readily photooxidized by UV light. The products include kynurenine, tryptamine^[265], FICZ, and 6,12-di-formylindolo[3,2-*b*]carbazole (dFICZ), the latter two representing high affinity compounds^[266].

MATERIALS AND METHODS

Flow chart:



All experiments were done after obtaining due ethics committee clearance of the Institutional Ethics Committee of the Haffkine Institute, vide letter no. HITRT/IEC/13/2011, dated 24th January 2011.

COLLECTION AND PLANT IDENTIFICATION

The plant was collected from Haffkine Institute campus in the monsoon season and a specimen in the form of a twig sent to the 'Botanical Survey of India', Pune, for authentication.

PROCESSING OF THE PLANTS

The leaves of *Aegle marmelos*, *Ricinus communis* and *Mangifera indica* were collected and washed thoroughly with water. They were then kept for shade drying. The dried leaves were ground to fine powder and were used in the extraction.

Soxhlet Method:

- The dried plant powder (30g) was taken in a thimble made using Whatmann filter paper no. 3. The Soxhlet apparatus was assembled and 600 mL solvent (methanol and distilled water) was added.
- After subsequent number of cycles per solvent, as the solvent turned colorless, the extraction was stopped, the solvent was removed and the non-soluble part in the thimble was discarded. The solvent was recovered by double distillation method.
- Some amount of solvent was retained in the extract, which was then evaporated to concentrate the extract. The concentrated extract was then stored until further processing in a refrigerator at 4°C.

Protein Extraction^[267]:

Plant protein was extracted using the P-PER Plant Protein Extraction Kit (M/s. ThermoScientific). The method of extraction was as follows:

- Just before use the P-PER Working Solution (WS) was prepared by mixing 990 μ L of Reagent A with 10 μ L of Reagent B. Then 750 μ L of Reagent C was added and vortexed until a colloidal suspension forms.
- Plant tissue was placed into the polypropylene mesh bag. The bag contained an inner layer screen mesh that was sealed on three sides. With the open end of the bag facing up, the bag was pulled open and the tissue was placed between the inner screens.
- WS was poured into the mesh bag with the tissue.
- The open end of the bag was held upright and the lower portion was kept firmly on a flat firm surface. Using a glass rod
- the outside of the bag was rubbed and until the WS and the tissue are thoroughly mixed and no visibly intact tissue remained.
- Once a homogeneous mixture was achieved, the liquid was allowed to settle to the bottom of the bag and then retrieve liquid was retrieved with a pipette inserted into the clear strip edge of the bag.
- Mixture was kept into a centrifuge tube and centrifuged for 5 minutes at $5,000 \times g$ to partition organic and aqueous phases.
- Extracted, soluble protein was in the lower aqueous layer.
- Excess solvent was evaporated in a dry bath inside Bio-safety cabinet for 5-7 days at 37°C . The final concentrated protein sample was aliquoted and kept at -80°C until further use.

The samples are referred to as follows:

1. AM: *Agale marmelos* (Bael) Methanol Extract
2. RC: *Ricinus communis* (Eranda) Methanol Extract
3. MI: *Mangifera indica* (Mango) Methanol Extract
4. MIP: *Mangifera indica* (Mango) Protein fraction
5. MP: *Agale marmelos* (Bael) Protein fraction
6. RP: *Ricinus comunnis* (Eranda) Protein fraction

PHYTOCHEMICAL PROFILE^[268,269]

Phytochemical assessment and Fingerprinting were performed by HPTLC method. High Performance Thin Layer Chromatography (HPTLC) is a sophisticated and automated form of Thin layer chromatography (TLC). Fingerprinting of methanol extracts of the leaves were carried out to identify the components present in it.

The above samples were dissolved in their methanol solvents to give a final concentration of 1 mg/ mL. The samples were sonicated for 10 minutes for obtaining a homogenous mixture which was then loaded into the syringe. The samples were screened for Flavanoid, Phenolics, Tannins, Glycosides, Alkaloids and Sterols. CAMAG Linomat 5 system which is a semi-automated system was used for the loading of the sample and standards. 10µL of samples and standards was loaded onto a TLC silica gel aluminium sheet (20 mm x 10 mm) through a syringe.

Pre – conditioning and chromatographic development

In order to avoid high R_f values usually caused due to the use of an unsaturated chamber, a twin trough chamber (for 20 × 10 TLC plates) was filled at the base with 9 mL of the mobile phase solvent and lined with a filter paper in one trough side for approximately 30 minutes to allow saturation of the chamber prior to development. The mobile phase used was different of different phytochemiclas (Flavanoid, Phenolics, Alkaloids, Sterols). The chamber was covered with a lid to prevent evaporation of the mobile phase. On saturation, the TLC plate was placed in the chamber on the side of

the trough opposite to that of the filter paper and allowed to develop. The plate was removed after development and dried in vacuum desiccators.

Phytochemical	Stationary Phase	Mobile Phase	Derivatizing agent
Alkaloid	Silica F ₂₅₄	Ethyl acetate: methanol: glacial acetic acid (6.8:3.0:0.2)	Dragndroff reagent
Falvanoid	Silica F ₂₅₄	Ethyl acetate: formic acid: glacial acetic acid: water (10:0.5:0.5:1.3)	Anisaldehyde sulphuric acid
Phenolics	Silica F ₂₅₄	chloroform: methanol (9.5:0.5)	Anisaldehyde sulphuric acid
Sterols	Silica F ₂₅₄	Chloroform: Ethyl acetate (4:6)	Acetic anhydride: Sulphuric acid in ethanol
Tannins	Silica F ₂₅₄	Toluene-ethyl acetate-formic acid-methanol (3:3:0.8:0.2)	Ferric Chloride
Glycosides	Silica F ₂₅₄	Ethylacetate-Methanol (1.3:0.5)	Kedde Reagent

Table 4 Mobile phase and derivatizing agents of specific phytochemicals

Detection and visualization

- The bands were first detected under white light. Detection under UV light was the first choice as it is non destructive. Spots of fluorescent compounds were visualized at 254nm (short wave length) by D2 (Deuterium) lamp.
- The detection was carried out using CAMAG HPTLC TLC Visualizer and Camera and the plate was scanned using CAMAG TLC Scanner.

Derivatization

The derivatizing reagent was used as per the phytochemicals. The plate was immersed in a chamber containing the derivatizing reagent for a few seconds, air dried and placed on a heater at 70°C. The coloured spots developed after derivatization were then scanned using a CAMAG TLC Scanner at 546 nm.

CELL LINE AND VIRUS^[270]:

BHK-21 (Baby Hamster Kidney Cells), RAW 246.7 (Murine Macrophage Cell) and MDCK (Madin Darby Canine Kidney epithelial cells) were procured from NCCS, Pune. Challenge Virus Standard (CVS-11) of the Rabies virus used for in the study was provided by Department of Virology, Haffkine Institute. The CVS strain of rabies virus was cultivated in BHK-21 cell line.

All the three cell lines were maintained in 3 steps namely passaging, freezing and reviving.

Passaging

Same protocol was used for culturing all the three cells (BHK, RAW and MDCK). The cells in T25 tissue culture (TC) flask was observed under an inverted microscope (Nikon) for full monolayer, cell degeneration, pH and microbial contamination. Any contaminated flasks were discarded. Minimal Essential Medium (MEM), TPVG, (Trypsin phosphate versene glucose) and FBS (Fetal bovine serum) was removed from the fridge and kept at 37°C in water bath for 30 minutes. Cell lines were handled in a bio-safety cabinet. The MEM was decanted from tissue culture flask containing the cells cell line and 2 mL of TPVG was added on the side opposite to the cell sheet to wash cell layer and was discarded. Further, 1mL of TPVG solution was added on the side opposite to the cell sheet and was dispersed evenly on to the monolayer. Cells were observed every 15–20 seconds for signs of cells detachment. When first signs of dislodging is observed, TPVG was immediately removed and add 0.5 mL TPVG were added and the flask were kept in a 37°C, 5% CO₂ incubator until all the cells detached from the surface.

The cells were re-suspended in 10 mL of MEM containing 10% FBS and distributed in 2 flasks for 1:2 split. Each flask was labeled with cell name, passage number, and date of passage and initials of person preparing the culture. The flasks were incubated at 37°C, in 5% CO₂ incubator and sub-cultured every 3–4 days based on confluence of the monolayer.

Freezing

The flasks were observed under an inverted microscope for full monolayer, cell degeneration, for acidity of the medium and microbial contamination. Freezing mixture of 7:2:1 ratio was prepared (7 parts of MEM, 2 parts of FBS and 1 part of Dimethyl sulphoxide (DMSO)) in a sterile tube and kept at 4°C. Cells were trypsinised with TPVG treatment to detach cells and centrifuged at 1500 rpm, to get the cell pellet. The supernatant was discarded and freezing mixture was added slowly into the cell pellet and re-suspended. Cold chain was maintained during this step. The cells along with the freezing mixture were transferred into a labeled cryo vial and cryopreserved in a –196°C liquid nitrogen canister.[108]

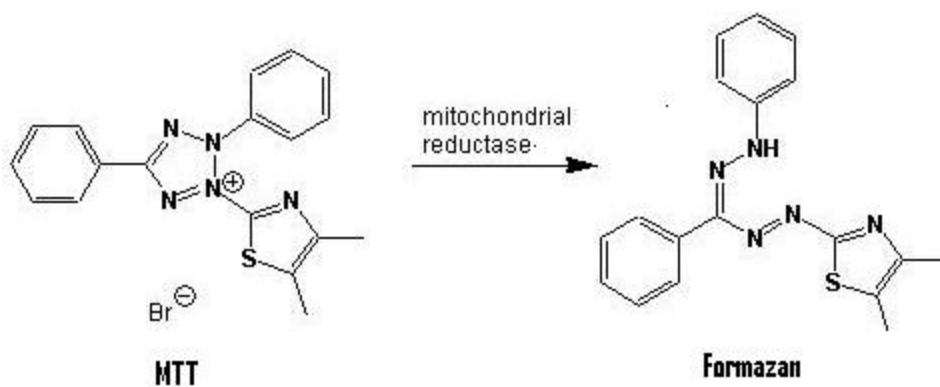
Reviving

The cryo vial containing the cells were removed from liquid nitrogen canister and was thawed in a 37°C water bath. The contents of the cryo vial were transferred in a centrifuge tube along with MEM with 20% FBS and was centrifuged at 1500 rpm. Supernatant was discarded and MEM along with 10% FBS was added and the cell line was transferred into the T25 tissue culture flask. After overnight incubation, the T25 flasks were observed for cell adherence under an inverted microscope.

IN-VITRO CYTOTOXICITY ASSAY^[271]

The *in-vitro* cytotoxicity (CC50) assay of the plant extracts was performed by MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay on all the three cell lines.

This is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilized with an organic solvent (eg. Isopropanol/Dimethyl sulfoxide (DMSO)) and the released, solubilized formazan reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells. These reductions take place only when reductase enzymes are active, and therefore conversion is often used as a measure of viable (living) cells. Changes in metabolic activity can give large changes in MTT results while the number of viable cells is constant. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing death, or changing metabolism of cells, can be deduced through the production of a curve.



Protocol:

The *In vitro* cytotoxicity assay of the plant extracts was performed by MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay in Vero cell line.

- A T-25 flask attaining monolayer of the cells were trypsinised and 100 μ L of 0.25×10^6 cells/ml was seeded per well into a 96 well microtitre plate. The plate was incubated at 37°C for 24 hours for the cells to attain a confluent monolayer.
- Methanolic and Protein extracts of the samples were dissolved in DMEM (without phenol red). Effective concentrations from 100 mg/mL-0.01 mg/mL were used in the study.
- The complete medium was then removed from the microtitre plate without disturbing the monolayer and 100 μ L of the serially diluted plant extract was added.
- A cell control and vehicle control was kept and the plates were incubated at 37°C in 5% CO₂ incubator for 16-18 hours.
- Extracts was removed and 100 μ L of 10% of 5 mg/ml MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) dye was added in each well and the plates were then incubated at 37°C for 4 hours.

- After incubation MTT was removed and 100 μ L of DMSO was added in each well and readings were taken at 540nm using a Multimode reader.
- All assays were carried out in triplicates

The cytotoxicity was calculated as:

$$\% \text{ Cytotoxicity} = \frac{\text{Control O.D} - \text{sample O.D}}{\text{Control O.D}} \times 100$$

ANTI VIRAL ASSAYS

Preparation of Rabies Virus Stock^[272]

CVS-11 stock was infected on BHK cells (confluent monolayer) and incubated for 3 days at 37°C in a CO₂ incubator. The cells were freeze thawed and the supernatant was collected by centrifuging at 2000 rpm for 10 min. The supernatant was aliquoted and stored at -80°C.

In-vitro titration of Rabies Challenge Virus Standard strain

Most of the viruses produce characteristic cytopathic effect (CPE) on cell lines but Rabies virus does not give any distinct CPE. The infectivity titres can thus be determined by infecting BHK cells with increasing dilutions of the virus and staining it with fluorescently labeled antibody. The 50% end point dilution is expressed as TCID₅₀/mL was calculated by Reed- Muench method.

- The CVS virus stock made by passaging the virus on BHK cell line and was titrated to determine its infectivity dose. The virus was serially diluted from 10⁻¹ to 10⁻⁵ in MEM with 2% FBS in a 96-well microtitre plate.
- Serially diluted virus (100 µL) was added on the 96 well microtitre plate. BHK cells were trypsinized and resuspended in medium with 2% serum and added (100 µL) over the diluted virus suspension. The assay was run with a cell control (100 µL of only trypsinised cell suspension) and a virus control (100 µL of neat virus). The plate was incubated at 37°C for 48 hours.
- All assays were carried out in triplicates.

Rapid Fluorescent Foci Inhibition Test (RFFIT)^[272]

The medium was decanted and the cells were fixed by adding cold acetone (50 µL per well) and kept at 4°C for 30 minutes. After discarding the acetone, cells were stained

by direct polyclonal FITC labeled antibody (1:20) for 30 minutes and then washed (3 times) with 0.01M phosphate buffer saline (PBS), air dried and visualized under an inverted fluorescence microscope. The titre was calculated using Reed & Muench method and expressed as TCID₅₀.

***In-vitro* Antiviral assay against Rabies Virus**

Rapid Fluorescent Focus Inhibition Test (RFFIT) is one of the most commonly used tests for estimation of rabies-virus neutralizing antibody. It has high sensitivity and specificity. The test involves reaction of a mixture of dilutions of the test serum and a fixed amount of Challenge Virus Standard (CVS), allowing for neutralization of the virus to occur and then detecting the extent of neutralization by inoculating cell culture and then detecting the presence or absence of viral antigen by doing Fluorescent Antibody Test (FAT). The samples were tested for its *in vitro* anti-viral activity based on the principle of RFFIT.

Modified RFFIT to test anti- rabies activity of plant extracts^[272]

- The minimal concentrations (1000 µg/mL – 1 µg/mL) of Methanol and Protein extracts of the samples based on the CC50 value were used in the antiviral assay.
- Varying concentrations of 50 µL of samples were mixed with 50 µL of 10 TCID₅₀ RV CVS and allowed to incubate for 1 hr in 96 well tissue culture plate. The reference serum (5 IU/ mL) was serially diluted two fold from 1:00 to 1:2800 using MEM with 2% FBS in sterile tubes which serve as a positive control.
- Vero cell line was trypsinized and 0.2×10⁶ cells/ml added onto 96 well tissue culture plate. This was incubated at 37°C in a CO₂ incubator for 72 hours.

- The medium was decanted and the cells were fixed by adding cold acetone (50 μ L per well) and kept at 4°C for 30 minutes. After discarding the acetone, cells were stained by direct polyclonal FITC labeled antibody (1:20) for 30 minute and then washed (3 times) with 0.01M phosphate buffer saline (PBS), air dried and visualized under an inverted fluorescence microscope.

RNA Extraction

The RNA was extracted manually using the QAIamp® Viral RNA mini kit (QIAGEN GmbH, Hilden, Germany) as per the manufacturer's instructions^[273].

Principle

The kit uses the properties of silica gel-based membrane with centrifuge technology. The sample is first lysed under highly denaturing condition to inactivate RNases and to ensure isolation of intact viral RNA. Buffering conditions are then adjusted to provide optimum binding of the RNA to the silica gel-based membrane.

The RNA was extracted from the clinical specimens. The RNA extraction protocol as per the manufacturer's instruction was followed.

Preparation of reagents:

- Carrier RNA: 310 μ L of buffer AVL was added to 310 μ g lyophilized RNA to obtain a solution of 1 μ g/ μ L. Ready to use AVL: 5.6 μ l of carrier RNA was added to per 560 μ L of AVL just before use.
- Buffer AW1: Supplied as concentrate diluted with 125 mL absolute alcohol to 95 mL of concentrate.
- Buffer AW2: supplied as concentrate diluted with 160 mL absolute alcohol to 250 mL of concentrate.

Procedure

Buffer AVL containing carrier RNA (1 µg/µL) was taken in 1.5 mL microcentrifuge tubes. Viral isolates were thawed and 140.0 µL was added and incubated at room temperature. A volume of 560.0 µL ethanol (96–100%) was added to the sample, mixed well by pulse vortexing for 15 seconds and centrifuged. Next, 630.0 µL of the sample was loaded on to the membrane in the spin column, which was in turn placed in a 2 mL collection tube and was centrifuged at 8000 rpm for 1 minute. The tube containing the filtrate was then discarded and replaced with new tube and 500.0 µL of buffer AW1 was added onto the membrane and centrifuged at 8000 rpm for 1 minute. The collection tube was discarded after every step. Further, 500.0 µL of Buffer AW2 was added to the above membrane and centrifuged at 14000 rpm for 3 minutes. The spin column was then placed in a clean micro-centrifuge tube and to the membrane 60.0 µL of elution buffer AVE was added; incubated at room temp for 1 minute and centrifuged at 8000 rpm for 1 minute. The eluted 60.0 µL contained the viral RNA of which 5 µL RNA was used for the rRT-PCR and remaining were stored in a –80°C deep freezer.

Real time Reverse Transcriptase PCR :

Reverse transcriptase polymerase chain reaction (RT-PCR) is a laboratory technique based on the PCR, which is used to amplify and simultaneously quantify a targeted DNA molecule. It enables both detection and quantification of one or more specific sequences in a DNA sample. In real time reverse transcriptase polymerase chain reaction (qRT-PCR) an RNA strand is first reverse transcribed into its DNA complement (*complementary DNA*, or *cDNA*) using the enzyme reverse transcriptase, and the resulting cDNA is amplified using traditional or real time PCR. The first step is reverse transcription (RT), in which RNA is reverse transcribed to cDNA using reverse transcriptase and primers. The next step involves the denaturation of the dsDNA

followed by annealing. The final step of PCR amplification is DNA extension from the primers. qRT-PCR was performed using SYBR Green[®] based chemistry by using a One step SYBR RT-PCR kit (perfect real time) from TAKARA BIO INC for the reactions.

RT PCR for *in vitro* anti rabies activity:

Procedure

qRT PCR: The viral RNA was extracted from vero cell line from the 96 well plate after in-vitro antiviral assay by using the spin column based QAIamp-Viral RNA mini kit (QIAGEN GmbH, Hilden, Germany) as per the manufacturer's instructions. Reverse transcriptase real time PCR (qRT-PCR) was carried out at a StepOne Real Time-PCR instrument (M/s ABI) by using the One step SYBR RT-PCR kit (perfect real time) from TAKARA BIO INC for the reactions. The rabies N (Nucleoprotein) gene primer sequence were procured from M/s. Eurofins.

Rabies N gene primer sequence

Gene	Sequence (5'-3')	Product length (bp)
N (F)	TTGT(AG)GA(TC)CAATATGAGTACAA	762
N (R)	CTGGCTCAAACATTCTTCTTA	

Table 5: Rabies Virus N gene primer sequences

The cycling condition for the qRT-PCR were 42°C for 5 min of reverse transcription followed by denaturation at 95° C for 10 sec and PCR amplification (38 cycles) of 95°C for 1min, 55°C for 1min and extension at 72°C for 1 min 30 sec. The anti-rabies activity was quantified by delta delta Ct method^[274].

The master mix (20.0 µL) was added into each well going down the row. The One step SYBR RT-PCR (perfect real time) from TAKARA BIO INC was used for the reactions. The master mix for rabies virus (N gene) was prepared as follows for the reaction:

Reagent	Volume (μL)/ reactions (1X)
2x One step RT- PCR mix	12.5
Nuclease free water	3.90
One step enzyme	0.80
Forward primer	0.40
Reverse primer	0.40
Total volume	18.0
RNA input	2.00

Table 6: N gene PCR master Mix

- 18 μL of master mix per reaction was added onto each the PCR tubes.
- 2.0 μL of nuclease free water was added as negative control (NTC) and 2.0 μL of Positive control (PC) and the plates were capped.
- 2.0 μL of extracted RNA sample was added to the respective wells. The plate was centrifuged at 3000 rpm for 10 seconds. Afterward the plates were kept in the ABI StepOne Real Time PCR instrument.

The rRT-PCR (ABI step one) was programmed as follows:

Reverse transcription	42°C for 5 min
Denaturation	95°C for 10 sec
PCR amplification (38 cycles)	95°C for 1min
Extension	72°C for 1 min 30 sec

Table 7: Cycling conditions for RABV N gene qRT-PCR

- All assays were carried out in triplicates

Preparation and Titration of Influenza Virus^[275]

Experiments were performed using Influenza Virus A/H1N1/PUNE/2009; standard virus obtained from National Institute of Virology, Pune, India.

Influenza virus stocks were grown in cell culture using Madin Darby canine kidney (MDCK) cells and titre of infectious virus were measured by median tissue culture infective dose (TCID₅₀) titration.

MDCK cells for viral titration were grown in complete MEM. Confluent MDCK cell monolayer 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 2 µg/mL L-(toslyamido 2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin and Nystatin (50 U/mL) were incubated in replicate wells (200 µL/well) for 4 days at 37 °C with 5 % CO₂.

Wells positive for virus growth were identified by staining the cells with 0.1 % trypan blue and TCID₅₀ titre were calculated by the method of Reed and Muench

Study on inhibition of Influenza hemagglutinin protein^[275]

Dried plant extracts were dissolved in DMEM without phenol red and required dilutions were prepared. Confluent MDCK cell cultures were treated with different dilutions of methanol and aqueous extract in three sets of experiments as follows:

Pre-exposure Assay

- Virus stock of 10^{2.5} TCID₅₀ (64 HA titre) was exposed to effective minimal cytotoxic concentrations of plant extracts for 1 h at 37 °C. Then 100 µL of the mixture was added to the cells cultured fluently in 96-well flat-bottom microtitre plate.

Following 1 h incubation at 37°C, the supernatants were removed and the cells were washed with phosphate buffered saline (PBS). Then 100 µL of the TPCK-containing medium was added to each well. All assays were carried out in triplicates.

Post- exposure Assay

Virus stock of $10^{2.5}$ TCID₅₀ (64 HA titre) (100 µL/well) was added to each well. After 1 h incubation at 37 °C the unabsorbed viruses were removed and cells were washed and 100 µL of plant extracts diluted with TPCK-containing medium was added to each well.

All the plates including MDCK cells (Cell control) and influenza virus inoculated MDCK cells were incubated at 37°C in CO₂ incubator for 48 h and the virus titration was carried out by Hemagglutination assay. All assays were carried out in triplicates.

Hemagglutination assay

In order to assess the presence of the virus in cell culture, serial dilutions of the cell culture media (50 µL/well) were added in 96-well U-shaped microtitre plate. Human O red blood cells (0.75 %) were added to each well (50 µL/well). Following incubation at least for 1 h at room temperature, the virus titre of each well was recorded. A diffused sheet of agglutinated erythrocytes covering the bottom was regarded as 100 % hemagglutination, while a well circumscribed button of non-agglutinated erythrocytes was regarded as non-hemagglutination. The HA titre of the supernatant was defined as the reciprocal of the highest dilution showing complete hemagglutination. Complete settling of the RBCs demonstrated absence of the virus while hemagglutination indicated the presence of the virus. All assays were carried out in triplicates.

IMMUNO MODULATORY ACTIVITY OF SAMPLES

RAW cells were cultured in 6 well plates. When cells reached confluency, the samples were added and incubated for 48 hours. After 48 hours the supernatants were collected and kept at -80°C. RNA was extracted from the cell monolayer using the QIAamp® Viral RNA mini kit (QIAGEN GmbH, Hilden, Germany) as per the manufacturer's instructions. The One step SYBR RT-PCR kit (perfect real time) from TAKARA BIO INC was procured for the reactions. The cytokine primers were procured from Eurofins and the cytokine (TNF- α , IFN- γ , IL-6, IL-4) along with GAPDH (house keeping gene) expression [123,124] was determined by qRT PCR and the expression of cytokines were estimated by delta delta Ct method. [122]

Real time RT-PCR for quantifying expression of cytokine genes at RNA level:

The extracted RNA samples are added as template in reaction mixture which are converted to cDNA in the first step with reverse transcriptase enzyme and later amplified by thermo stable enzyme Taq polymerase in presence of specific primers. SYBR Green I dye gives rise to emission of fluorescence at end of each cycle in extension step. Melt curve analysis is necessary to discriminate PCR products from primer-dimers as they tend to melt at lower temperature than products. Cycle threshold values of each sample RNA are further processed, normalized to a reference gene (i.e. GAPDH) and compared with control sample or calibrator (untreated sample). Relative expression can be measured by the comparative CT method (also known as the method). The final results are plotted in graphs. All assays were carried out in triplicates.

Cytokine primer sequences:

Gene	Sequence (5'-3')	Annealing Temperature
GAPDH	F: GGAGAAGCTGCCAATGGATA	54°C
	R: GTGGTCTTCACGTTTCGCATT	
TNF- α	F: CAG GAT GAG GAC ATG AGC ACC	50° C
	R: CTC TGC AGA CTC AAA CTC CAC	
IFN - γ	F: CTCAAGTGGCATAGATGTGGAAGA	56.4° C
	R: GAGATAATCTGGCTCTGCAGGATT	
IL-4	F:TCGGCATT TTTGAACGAGGTC	52 ° C
	R:GAAAAGCCCGAAAGAGTCTC	
IL-6	F:GTACTCCAGAAGACCAGAGG	55° C
	R:TGCTGGTGACAACCACGGCC	

Table 8: Cytokine Gene Primer Sequences

The reaction mixture is prepared in master tube (1ml or 1.5 ml) and distributed according to the number of samples. For each sample reaction mixture (20 μ L) can be prepared as follow:

Reagent	Volume (μ L)/ reactions (1X)
2x One step RT- PCR mix	5.0
Nuclease free water	1.0
One step enzyme	0.4
Forward primer	0.8
Reverse primer	0.8
Total volume	8.0
RNA input	2.0

Table 9: Cytokine Gene PCR master mix

- In negative control, instead of template (2 μ L) nuclease free water is to be added.
- Addition of template is carried out at last step. After adding template, the tubes covered with lid placed in stand are centrifuged at 4000 rpm, 12-15 °C for 3-4 minutes.
- In Real time system, on software program, necessary inputs are made as per specification by manufacture and other optimized parameters

The qRT-PCR (ABI step one) was programmed as follows:

Steps	Conditions	Temperature (°C)	Time	Optics	Cycles
Holding Stage		42	5 min	Off	1
		95	10 sec	Off	
Cycling Stage	Denaturation	94	1 min	Off	40
	Annealing	Varies with genes (Ref Table 7)	30 sec	Off	
	Extension	72	45 sec	On	
Melt Curve		95	15 sec	Off	1
		60	1 min	On	
		95	15 sec	Off	

Table 10: Cycling conditions for Cytokine Gene Expression PCR

The cycling condition for the r-RT-PCR were 42°C for 5 min of reverse transcription followed by denaturation at 95° C for 10 sec and PCR amplification (42 cycles) of 94° C for 1min, 55° C for 1min, the annealing temperature will vary according to the cytokine and extension at 72°C for 45 sec. The cytokine gene expression was quantified by delta delta Ct method.

Tubes covered with lid placed in stand are kept in wells available in machine and with the help of computer software program, the run is started. As the reactions progresses amplification plots progresses on the computer. Currently running step is also displayed. At the end, amplification plots are analysed. In final stage, melt curve analysis is carried out and plots appear and run is analysed. Those cycle threshold (CT) values of samples satisfying the melt curve analysis are taken up and further processed.

The formulas used for expression of each gene are as follow: [122]

$$\text{Fold change} = [(C_T \text{ gene of interest} - C_T \text{ internal control (housekeeping) sample}) - (C_T \text{ gene of interest} - C_T \text{ internal control (housekeeping) calibrator or untreated})]$$

The data achieved with the above formulas may be interpreted as ‘the expression of the gene of interest relative to the internal control (housekeeping) in the treated sample compared with the untreated control or calibrator’. Further they may be plotted in graphs.

Nitric Oxide Assay^[276]

Nitric Oxide was assayed as dissolved nitrites using the Griess Reagent. 1% Sulfanilamide Solution in 5% OPA and 0.1 % NED Solution was allowed to equilibrate to room temperature (15–30 minutes). First, 50 µL of sample was added to wells. Sodium Nitrite was used as the standard. Then, 50 µL of the 1% Sulfanilamide Solution was added to all wells. Plate was incubated 5–10 minutes at room temperature, protected from light. 50 µL of 0.1 % NED Solution was added to all wells. And, again plate was incubated at room temperature for 5–10 minutes, protected from light. A purple/magenta color of Azo - compound will begin to form immediately. Readings were taken on Multi-mode reader at 520 nm within 30 minutes. All assays were carried out in triplicates.

Standard Curve

0.1 M of Sodium Nitrite Standard was prepared and diluted with distilled water (i.e. 1:1000) to give working standard of 100µM. Using the working stock standards of 100 µM , 50 µM, 25 µM, 12.5 µM, 6.25 µM, 3.13 µM and 1.56 µM concentration were prepared.

RBC Membrane Stabilization Assay^[277,278]

The HRBC membrane stabilization has been used as method to study the anti-inflammatory activity. Blood was collected from healthy volunteer. The collected blood was mixed with equal volume of sterilized Alsever solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid and 0.42% sodium chloride in water). The blood was centrifuged at 3000 rpm and packed cell were washed with isosaline (0.90%, pH 7.2) and a 10% (v/v) suspension was made with isosaline. The assay mixture contained the drug, 1ml of phosphate buffer (0.15M, pH 7.4), and 2ml of hyposaline (0.36%) and 0.5 ml of HRBC suspension (4). Diclofenac sodium (50mg) was used as reference drug. Two controls were prepared, one with 1.0 ml of isotonic saline without red blood cells, (control 1) and the other one with 1 ml of extract solution without red blood cells (control 2). Instead of hyposaline 2 ml of distilled water was used in the control.

All the assay mixture were incubated at 37°C for 30 min and centrifuged. The hemoglobin content in the supernatant solution was estimated using spectrophotometer at 560 nm. The percentage hemolysis was calculated by assuming the hemolysis produced in presence of distilled water of as 100%. Percentage of haemolysis was calculated by using the following formula:

$$\text{Percentage of haemolysis} = \frac{\text{O. D of test sample}}{\text{O. D of control}} \times 100$$

The percentage of HRBC membrane stabilization or protection was calculated by using the following formula

$$\% \text{ Protection} = 100 - \frac{\text{Optical density of drugs treated sample}}{\text{Optical density of control}} \times 100$$

All assays were carried out in triplicates.

ESTIMATION OF TRYPTOPHAN METABOLITES

Estimation of Serotonin from supernatant

Biochemical estimation of serotonin content involved a pre-treatment procedure. 50 μ L cell culture supernatant was treated with 300 μ L 0.01N HCl followed by addition of 3ml n-butanol. This solution was then taken for estimation of serotonin.

200 μ L n-heptane and 400 μ L 0.1N HCl containing 0.1% cysteine was added to the above solution. It was shaken mechanically for 5mins followed by centrifugation at 2000 rpm for 10 mins. 100 μ L of aqueous phase was taken and was treated with 600 μ L of 10N HCL with 0.004% o-phthalaldehyde and 100ul of 0.1N HCl-cysteine. The solution was heated on water bath for 15mins. After cooling, fluorescence was measured at excitation of 360 nm and emission at 470 nm using a microtitre plate^[279]. All assays were carried out in triplicates.

Estimation of Kynurenine from supernatant

100 μ L pooled supernatants were treated with 100 μ L Ehrlich's Reagent (0.8% p-dimethylaminobenzaldehyde in glacial acetic acid) followed by incubation in dark for about 10 mins. OD was measured at 490 nm in a microtitre plate^[279]. All assays were carried out in triplicates.

Estimation of Tryptophan from supernatant

Fifty microlitres of sample supernatant was treated with 50 μ L of 25 mg/mL ninhydrin in Reagent A (Formic acid-Hydrochloric acid, 3:2, v/v). This solution was heated in boiling water bath for 15 mins. The tubes were cooled and 300 μ L absolute ethanol at room temperature was added. OD was measured at 385 nm using a microtitre plate^[280]. All assays were carried out in triplicates.

Statistical Analysis

Data are expressed as Mean $\pm$ Standard Deviation from three separate experiments.

These were calculated using Graph Pad Prism v5.0 software. The cytokine gene expressions are expressed as mean $\pm$ SEM for each group. Evaluation of the significances of the differences between the means of parameters was performed using one-way ANOVA. In all cases, $p < 0.05$ was considered significant. Graphs were plotted and statistics assessed using GraphPad Prism 5 Software (GraphPad Inc.)

IN-SILICO ANALYSIS OF DOCKING WITH AHR

Preparation of ligands:

The 2D structures of compound i.e. (R)-Aegeline, (S)-Aegeline, Rac Ricinoleic Acid-d13, Alpha-Thujone, Ricinine, N-Demethylricinine-13C3 and Mangiferin drawn and analyzed by Marvin view. The compounds were converted to 3D structure (.pdb) using Marvin suite 15^[281]. The 3D coordinates (.pdb) of each molecule were loaded on AutoDock tools 1.5.6 (ADT) for energy minimization. Gasteiger charges were added and the rotatable bonds were set by the ADT and all torsions were allowed to rotate.

Preparation of macromolecule:

The protein targets retrieved from RCSB Protein Data Bank are proteins associated with metabolic functioning and proliferation of Aryl hydrocarbon receptor. Aryl hydrocarbon receptor (PDB code 4M4X) served as docking receptor. All the bound ligands and water molecules were removed from the receptors. Polar hydrogen atoms were added and Kollman charges were assigned to the proteins using AutoDock tools (ADT).

Molecular docking:

The molecular docking was performed and analyzed using AutoDock Vina^[282]. A Lamarckian genetic algorithm method implemented in the program suite was employed to identify appropriate binding modes and conformation of the ligand molecules. The grid map was centered at the active site of the protein by Autogrid. The Lamarckian genetic algorithm, the pseudo-Solis and Wets methods were applied for minimization using default parameters. Binding energy, intermolecular amino acids and their distance were recorded in each ligand bound conformations.

RESULTS

PHYTOCHEMICAL PROFILING

Phytochemical profiling was performed using High Performance Thin Layer Chromatography (HPTLC). HPTLC plates were derivatized and developed using appropriate developing agents. The plates were scanned in a UV/Vis transilluminator. The presence of specific phytochemicals is indicated in Table 11.

SAMPLE	ALKALOID	FLAVANOID	TANNIN	GLYCOSIDE	PHENOLICS	STEROLS	TERPEOIDS
AM	++	+++	+	-	++++	+	-
RC	++	++	+	+	++	++++	+++
MI	+	+	++	++++	+++	++	-

Table 11: Phytochemical profiles of the extracts used in the study

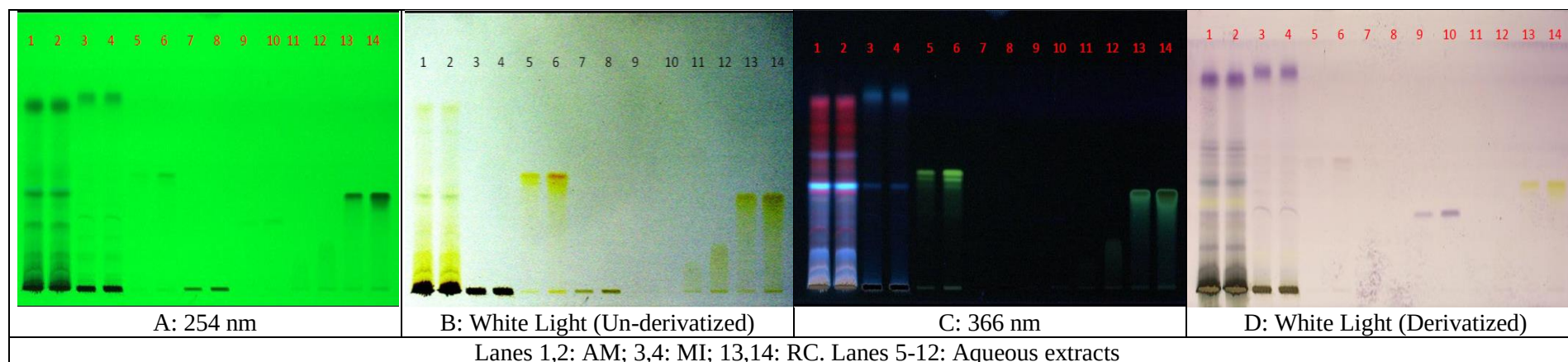


Figure 15: HPTLC profiles of the Extracts under various wavelengths of light. AM, MI and RC are methanol extracts of *Aegle marmelos*, *Mangifera indica* and *Ricinus communis*. The Aqueous extracts were not used in the study further

CYTOTOXICITY ASSAY

Cytotoxicity assay was performed on RAW, BHK and MDCK cells. The Median Cytotoxic values (CC50) was calculated using Graphpad Prism and shown in Figure 16-18 and Table 12

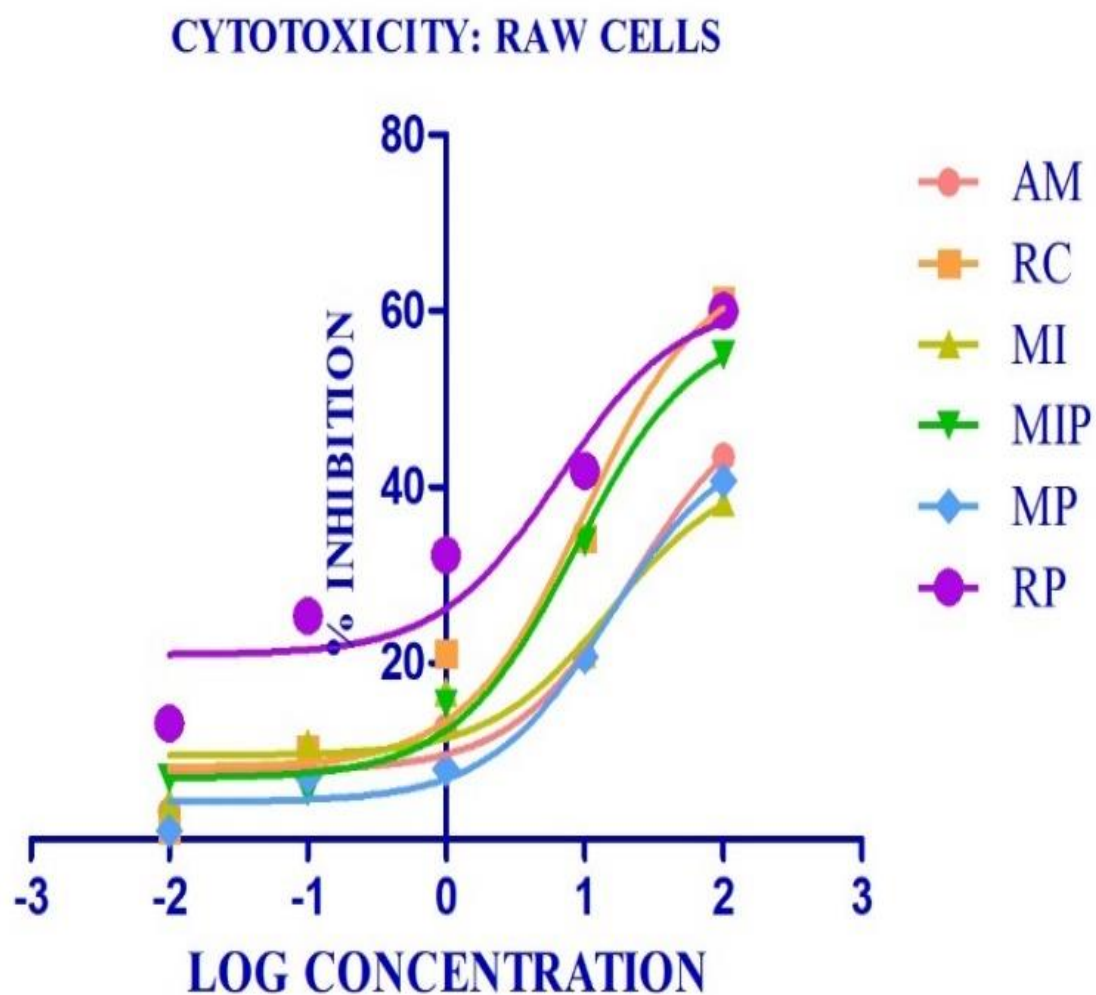


Figure 16 Graph of in-vitro cytotoxicity assays on RAW cells

CYTOTOXICITY: BHK

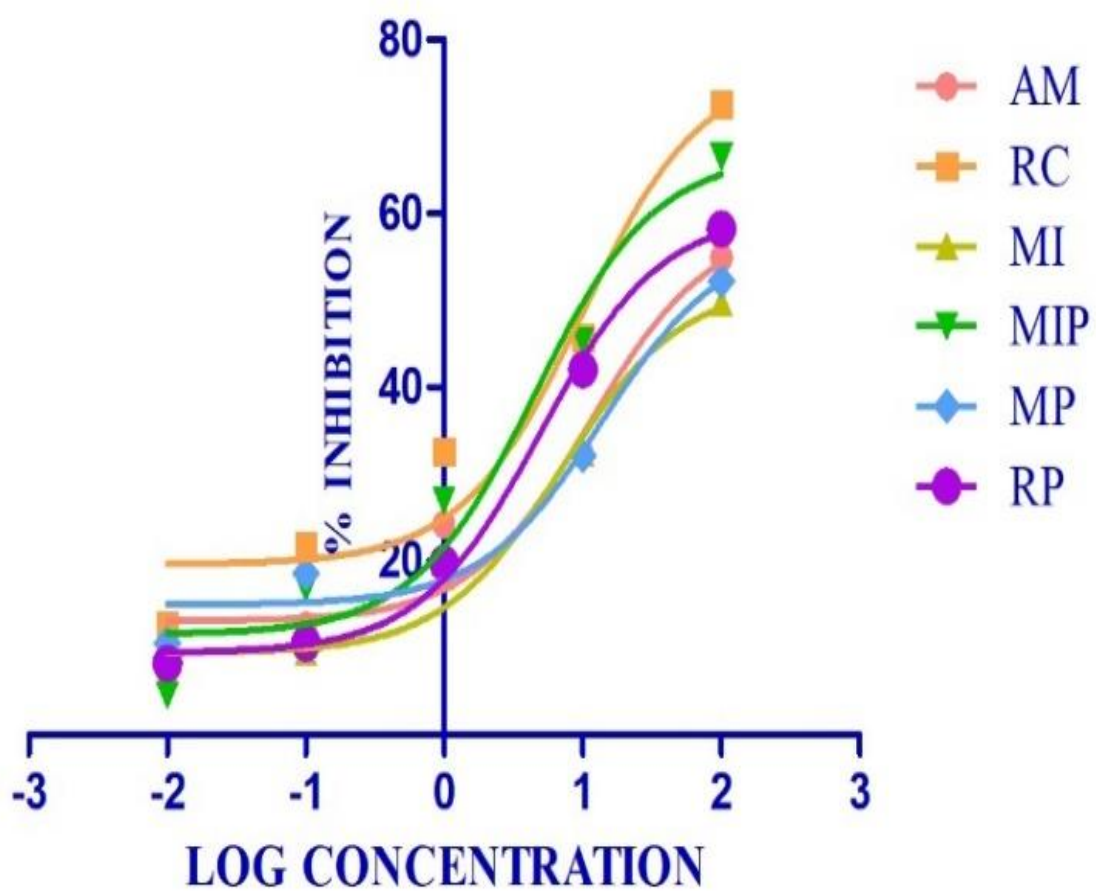


Figure 17 Graph of in-vitro cytotoxicity assays on BHK-21 cells

CYTOTOXICITY: MDCK

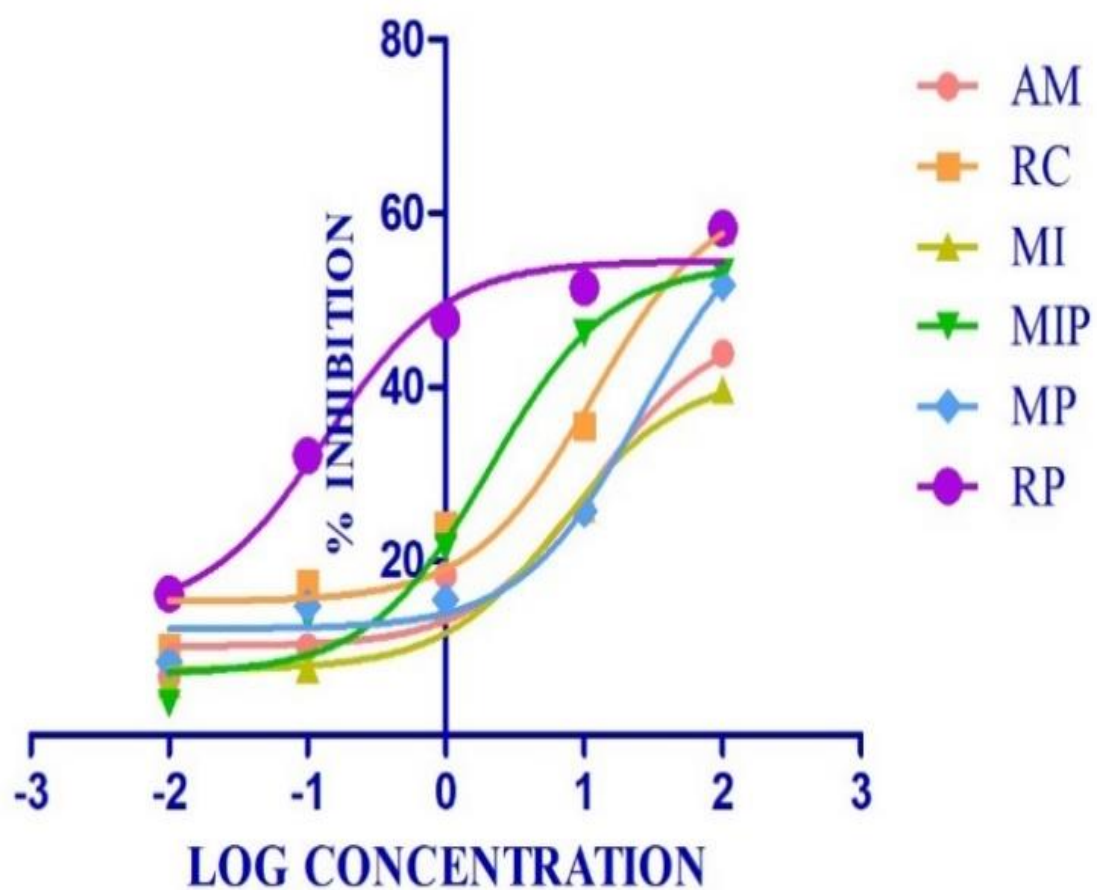


Figure 18 Graph of in-vitro cytotoxicity assays on MDCK cells

CELLS	MEDIAN CYTOTOXIC CONCENTRATION (mg/mL)					
	AM	RC	MI	MIP	MP	RP
RAW	21.69	9.795	15.21	8.533	14.89	6.577
BHK	11.33	9.79	7.06	4.5	13.79	4.94
MDCK	11.58	11.63	7.063	2.075	25.68	0.1362

Table 12: Median Cytotoxic Concentrations of the samples on different cell lines

ANTI-VIRAL ASSAYS

ANTI-RABIES VIRUS ACTIVITY

Antiviral activity against Rabies Virus was assessed using RFFIT and qRT-PCR. The results – IC50 and fold reduction in RNA levels are shown in Table 13 and Table 14 respectively.

	AM	RC	MI	MIP	MP	RP
IC50	55.71	19.70	24.66	134.20	39.37	310.30
SI	203.37	496.95	286.29	33.53	350.27	15.9

Table 13: Anti-Rabies Virus activity using modified RFFIT, results are expressed as Median Inhibitory Concentrations (IC50) of the Virus. The Selectivity Index (SI) is also mentioned. All concentrations are in µg/mL

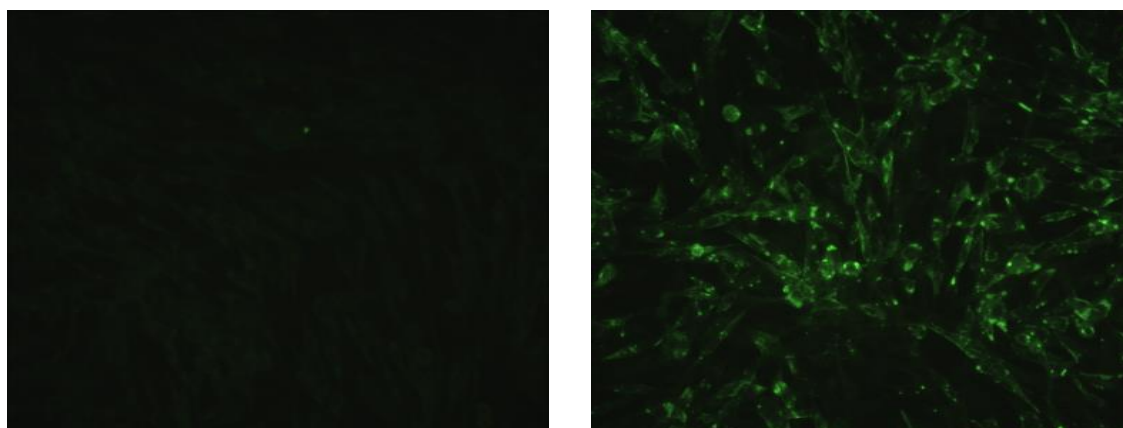


Figure 19: Fluorescent Foci Observed on BHK cells infected with the Rabies Virus (Right). Un-infected control cells (Left) did not exhibit any Fluorescent Foci

CONC. ($\mu\text{g/mL}$)	FOLD CHANGE					
	AM	RC	MI	MIP	MP	RP
1000	-166.57	-171.25	-286.03	-79.34	-188.71	-25.83
100	-74.54	-8.69	-52.71	-45.89	-121.1	-13.22
10	-39.95	-3.29	-32.45	-26.35	-37.27	-3.2
1	-8.11	-2.5	-3.29	-2.03	-8.11	-2.9

Table 14: Anti-Rabies Virus activity using qRT-PCR, results are expressed fold change w.r.t Virus Control.

ANTI-RABIES ACTIVITY: MODIFIED RFFIT

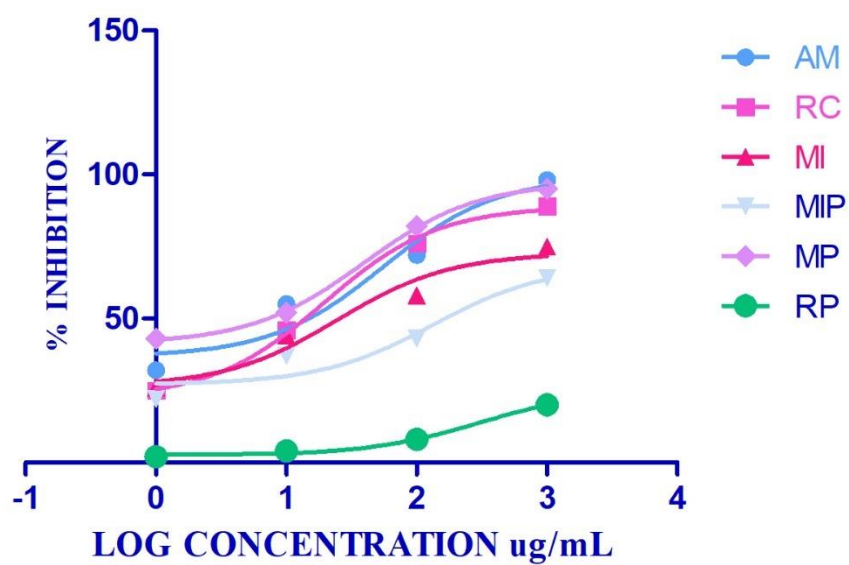


Figure 20: Graph of Anti-Rabies Virus activity using modified RFFIT

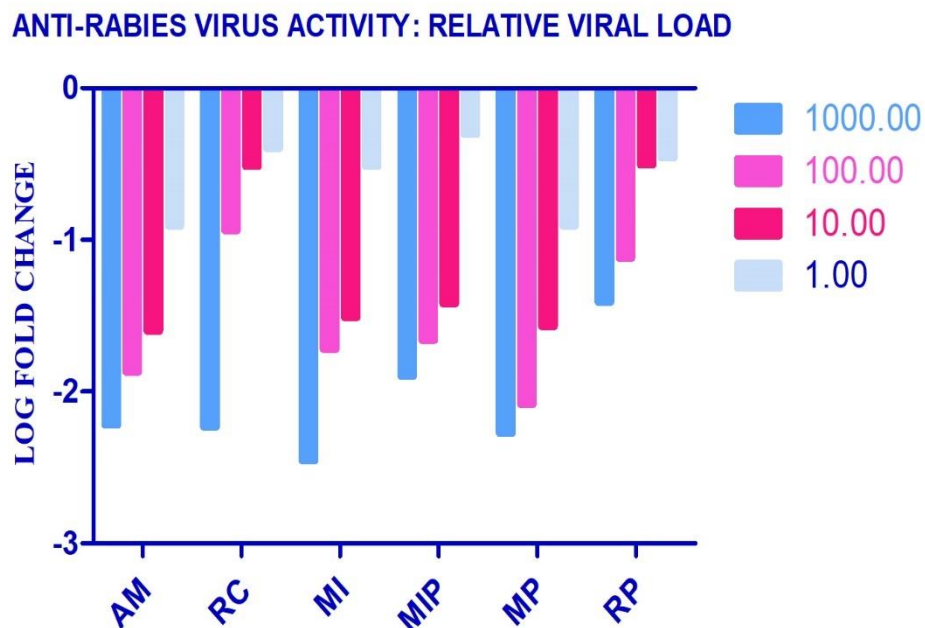


Figure 21: Graph of Anti-Rabies Virus activity using qRT-PCR

ANTI-INFLUENZA VIRUS ACTIVITY

HEMAGGLUTINATION INHIBITION

SAMPLES	HA UNITS AT VARIOUS CONCENTRATION ($\mu\text{g/mL}$)			
	1000	100	10	1
AM	16	32	64	64
RC	32	64	64	64
MI	32	32	32	64
MIP	32	32	64	64
MP	32	64	64	64
RP	64	64	64	64

Table 15: Hemagglutination (HA) Inhibition activity of the samples at various concentrations (1 $\mu\text{g/mL}$ -1000 $\mu\text{g/mL}$) in pre-exposure experiments. Values are HA units exhibited by the culture supernatants when virus was pre-incubated with extracts and then exposed to MDCK cells. Virus Control exhibited an HA titre of 64

HA INHIBITION ACTIVITY: PRE EXPOSURE

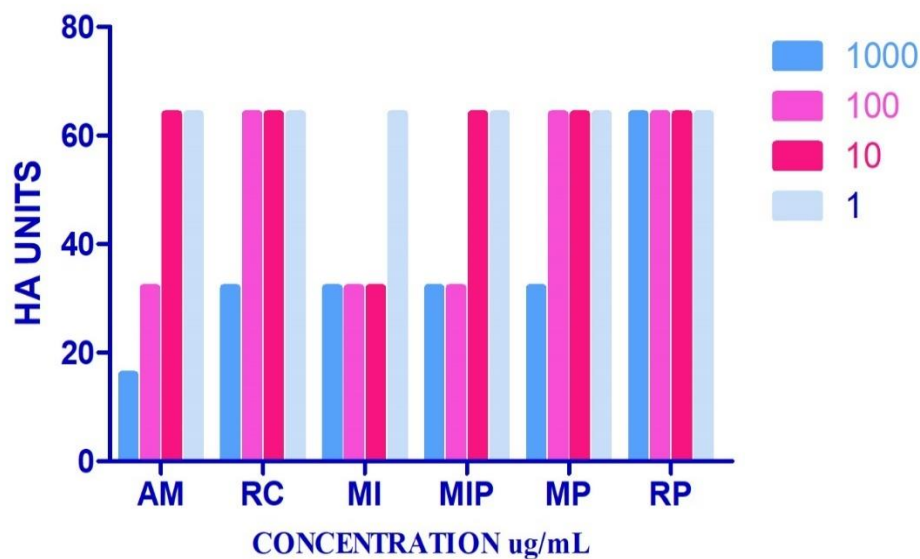


Figure 22: Graph of HA inhibition activity assay. Values on Y axis are HA units exhibited by the culture supernatants when virus was pre-incubated with extracts and then exposed to MDCK cells. Virus Control exhibited an HA titre of 64

In the pre-exposure experiments, anti-influenza virus activity was seen with the methanol extract of bael (AM) exhibiting a 4-fold reduction in virus titre (1:16) at 1000 µg/mL methanol extract of mango (MI) exhibiting a 2-fold reduction in virus titre (1:32) at 1 µg/mL concentration.

SAMPLES	HA UNITS AT VARIOUS CONCENTRATION ($\mu\text{g/mL}$)			
	1000	100	10	1
AM	16	32	64	64
RC	16	32	64	64
MI	8	8	32	64
MIP	32	32	64	64
MP	8	16	16	32
RP	64	64	64	64

Table 16: Hemagglutination (HA) Inhibition activity of the samples at various concentrations (1 $\mu\text{g/mL}$ -1000 $\mu\text{g/mL}$) in post-exposure experiments. Values are HA units exhibited by the culture supernatants when virus was incubated on cells for 1 hour and then the samples were added to MDCK cells. Virus Control exhibited an HA titre of 64

HA INHIBITION ACTIVITY: POST EXOSURE

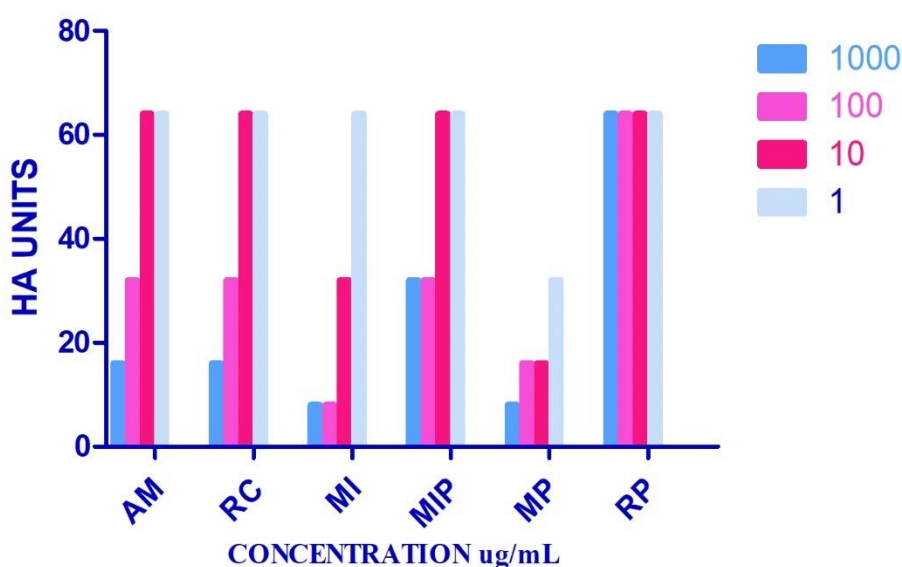


Figure 23: Graph of HA inhibition activity assay. Values on Y axis are HA units exhibited by the culture supernatants when virus was incubated on cells for 1 hour and then the samples were added to MDCK cells. Virus Control exhibited an HA titre of 64

In the post-exposure experiments anti-influenza virus activity was seen with the methanol extract of mango (MI) and protein extract of bael (MP) exhibiting a 8-fold reduction in virus titre (1:8) at 100 $\mu\text{g/mL}$ and 1000 $\mu\text{g/mL}$ respectively, methanol extract of bael (AM) and eranda (RC) exhibited a 2-fold reduction in virus titre (1:32) at 1000 $\mu\text{g/mL}$ concentration.

IMMUNOMODULATORY ACTIVITY

CYTOKINE GENE EXPRESSION

Cytokine Gene Expression was assessed using qRT-PCR. The results are shown in Table 17

GENES	MEAN FOLD CHANGE ($\pm$ SD)						
	LPS	AM	RC	MI	MIP	MP	RP
IFN-G	5.30 (0.10)	5.33* (0.45)	-3.16* (0.06)	3.26* (0.31)	5.23* (0.15)	2.40* (0.20)	-2.86 (0.25)
TNF-A	8.86 (0.31)	-4.46* (0.35)	-12.63** (0.45)	5.60* (0.79)	6.40* (1.05)	-5.66* (0.40)	-4.33* (0.38)
IL-6	4.20 (0.10)	7.30** (0.20)	5.20* (0.30)	4.50 (0.10)	4.3* (0.26)	5.86* (0.67)	2.00 (0.75)
IL-4	-2.30 (0.21)	5.80* (0.40)	6.36** (0.50)	1.56 (0.21)	2.10 (0.20)	3.63 (0.15)	4.06 (0.15)

Table 17: Gene Expression Analysis of the RAW cells exposed to the various samples and LPS.

Values are mean fold change $\pm$ SD w.r.t control (untreated) cells. * $p < 0.01$, ** $p < 0.001$

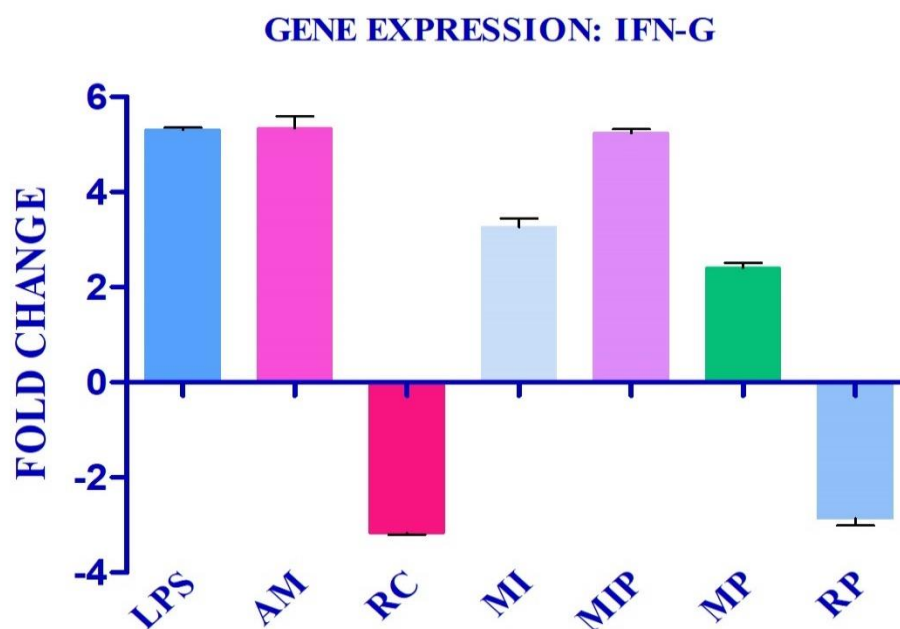


Figure 24: Graph of Gene Expression analysis of Interferon Gamma (IFN- γ) on RAW cells exposed to the various samples

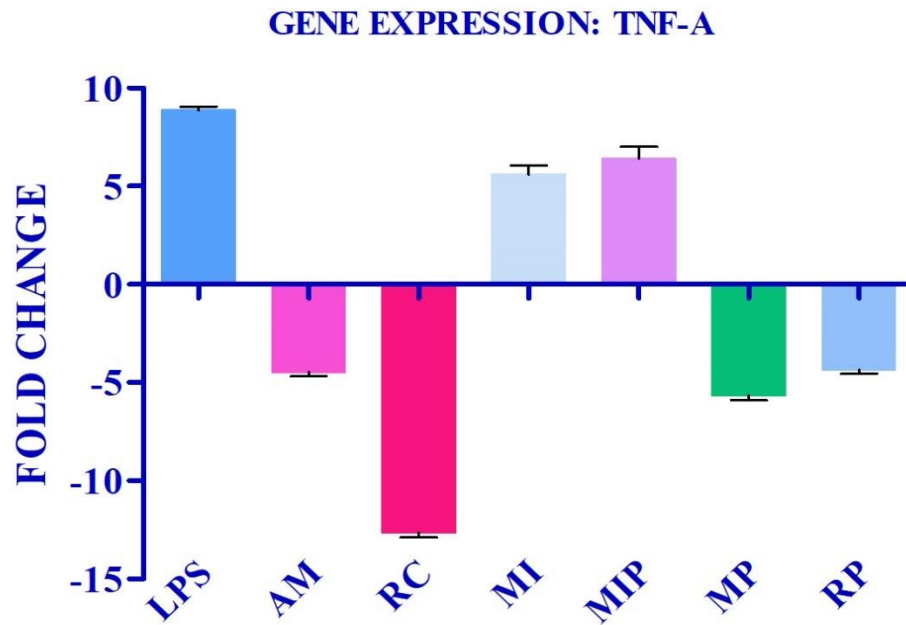


Figure 25: Graph of Gene Expression analysis of Tumor Necrosis Factor-Alpha (TNF- α) on RAW cells exposed to the various samples

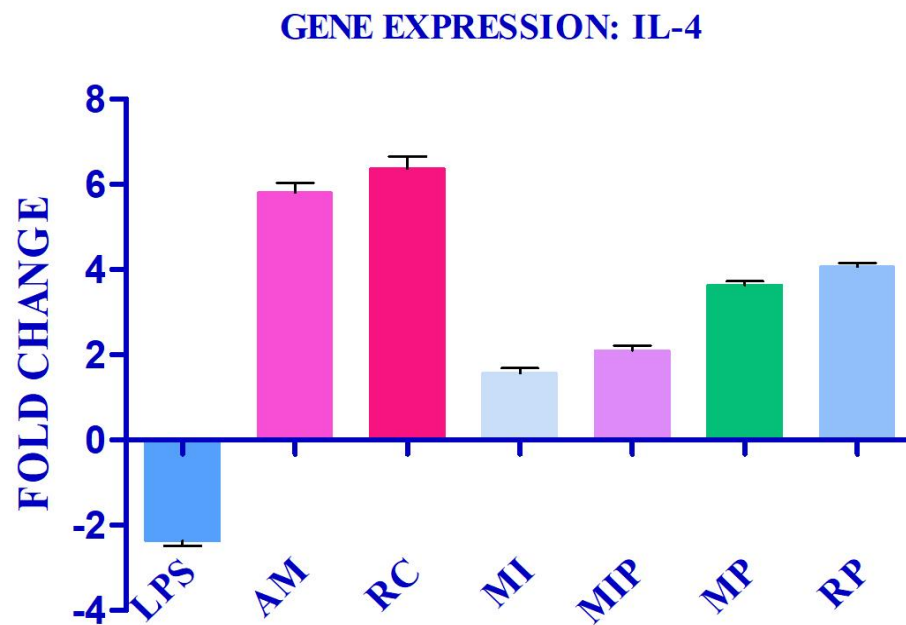


Figure 26: Graph of Gene Expression analysis of Interlukin-4 (IL-4) on RAW cells exposed to the various samples

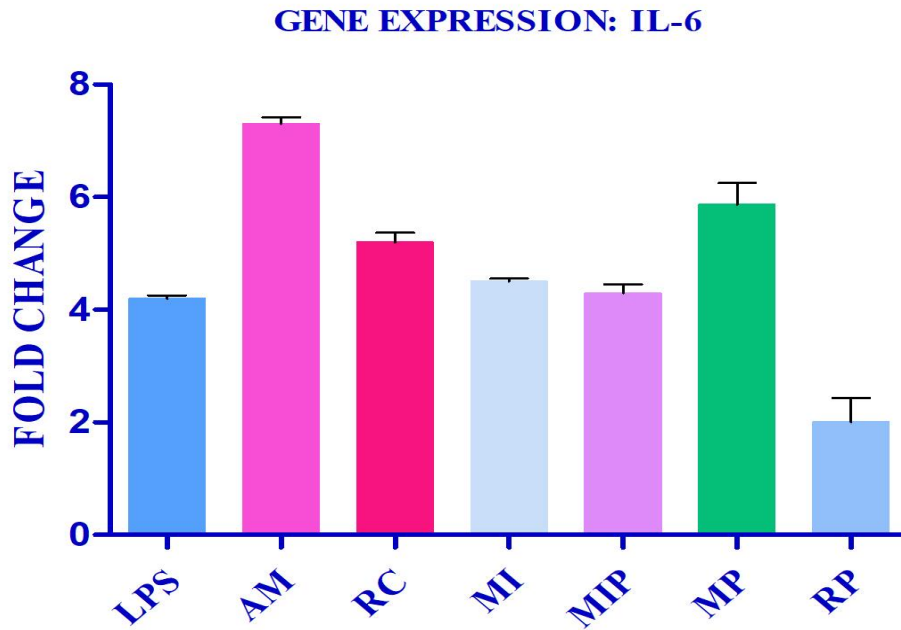


Figure 27: Graph of Gene Expression analysis of Interlukin-6 (IL-6) on RAW cells exposed to the various samples

PLANT WISE GENE EXPRESSION DATA

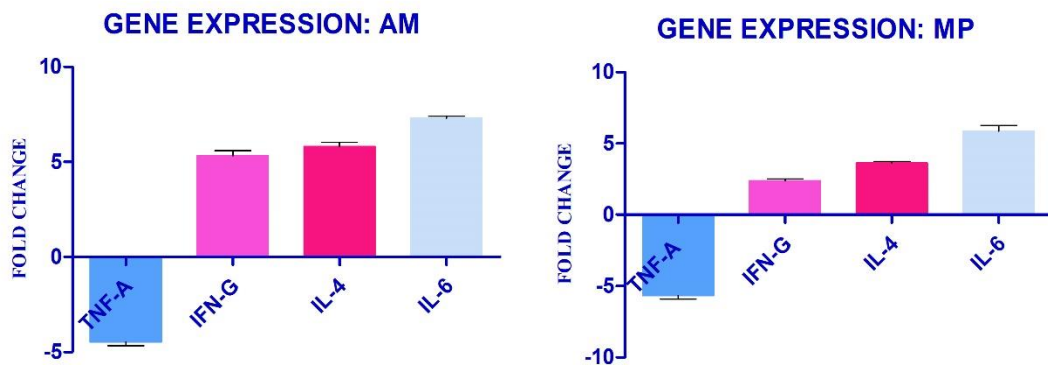


Figure 28: Cytokine gene expression in Methanol (AM) & Protein(MP) extract of *A. marmelos*

Figure 28 depicts the cytokine gene expression profile of Raw cells treated with the methanol extract and the protein extract of *A. marmelos*. Both the samples exhibit a downregulation of TNF- α genes. The M1/M2 polarisation seems well balanced.

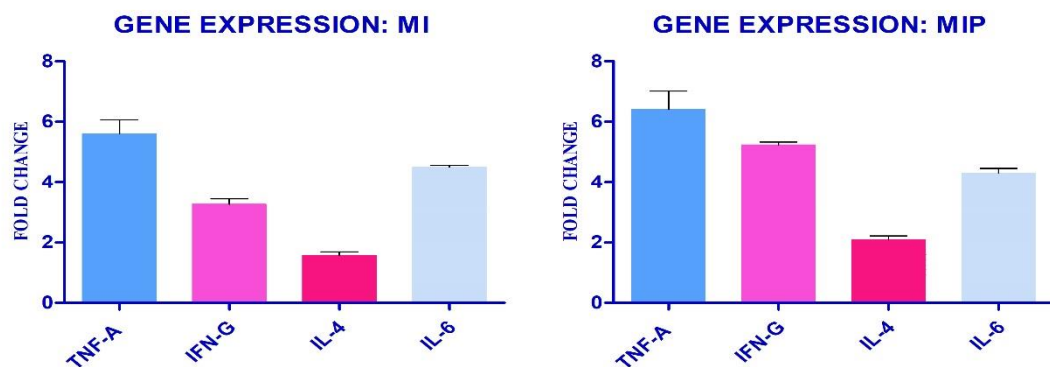


Figure 29: Cytokine gene expression in Methanol (MI) & Protein(MIP) extract of *M. indica*

Figure 29 depicts the cytokine gene expression profile of Raw cells treated with the methanol extract and the protein extract of *M. indica*. Both the samples exhibit a upregulation of TNF- α genes and IFN- γ genes markedly. The extracts orient the cells to an M1 phenotype.

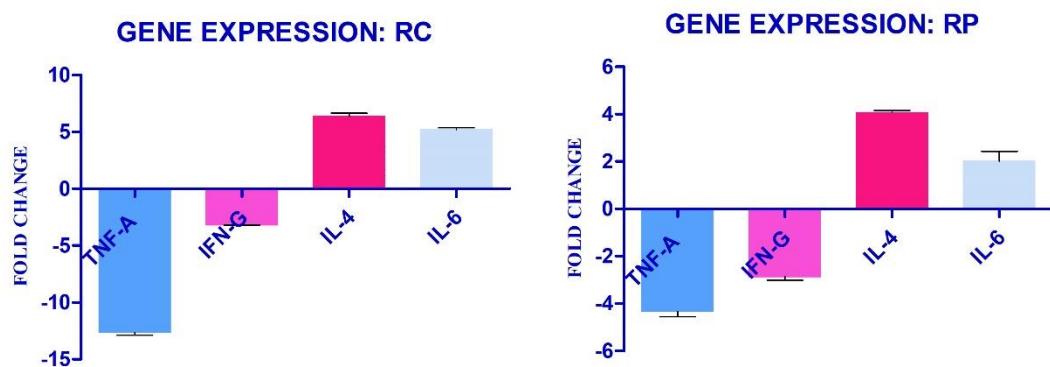


Figure 30: Cytokine gene expression in Methanol (RC) & Protein(RP) extract of *R. communis*

Figure 30 depicts the cytokine gene expression profile of RAW cells treated with the methanol extract and the protein extract of *R.comminis*. Both the samples exhibit a downregulation of TNF- α genes and IFN- γ genes markedly. The extracts orient the cells to an M2 phenotype.

NITRIC OXIDE ASSAY

Nitric Oxide was assessed as nitrates in the cell culture supernatants. The results are shown in Table 18.

MEAN CONCENTRATION IN μM ($\pm\text{SD}$)							
LPS	AM	RC	MI	MIP	MP	RP	CONTROL
73.22** (± 2.11)	31.09* (± 1.09)	25.44* (± 0.63)	66.76* (± 3.62)	54.59* (± 2.07)	40.95* (± 1.51)	25.87* (± 4.24)	6.94 (± 0.59)

Table 18: Concentration of Nitric Oxide in the Supernatants of RAW cells exposed to various samples. Concentrations in μM . * $p < 0.01$, ** $p < 0.001$

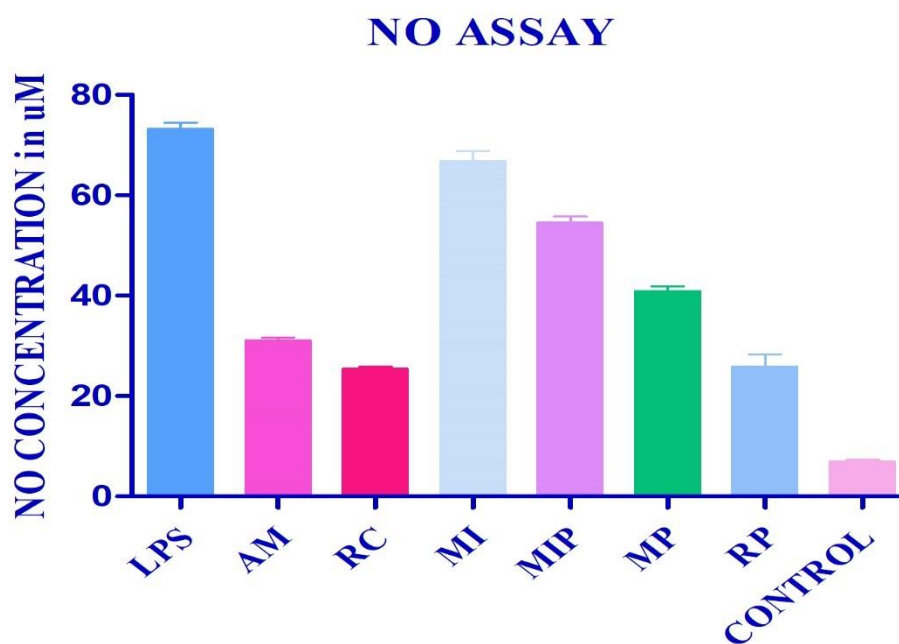


Figure 31: Graph of Nitric Oxide (NO) generated. Measured from the supernatants of RAW cells exposed to various samples.

MEMBRANE STABILISATION ASSAY

Anti-inflammatory activity was assessed as potential to stabilize RBC membranes. The results are shown in Table 19.

	AM	RC	MI	MIP	MP	RP
IC₅₀	96.2	147	152	131	101	602
SI	114.34	61.22	46.05	34.35	128.71	8.13

Table 19: Anti-Inflammatory activity was performed using the Membrane Stabilisation Assay, expressed as Median Inhibitory Concentrations (IC₅₀). The Selectivity Index (SI) is also mentioned. IC₅₀ concentrations are in µg/mL

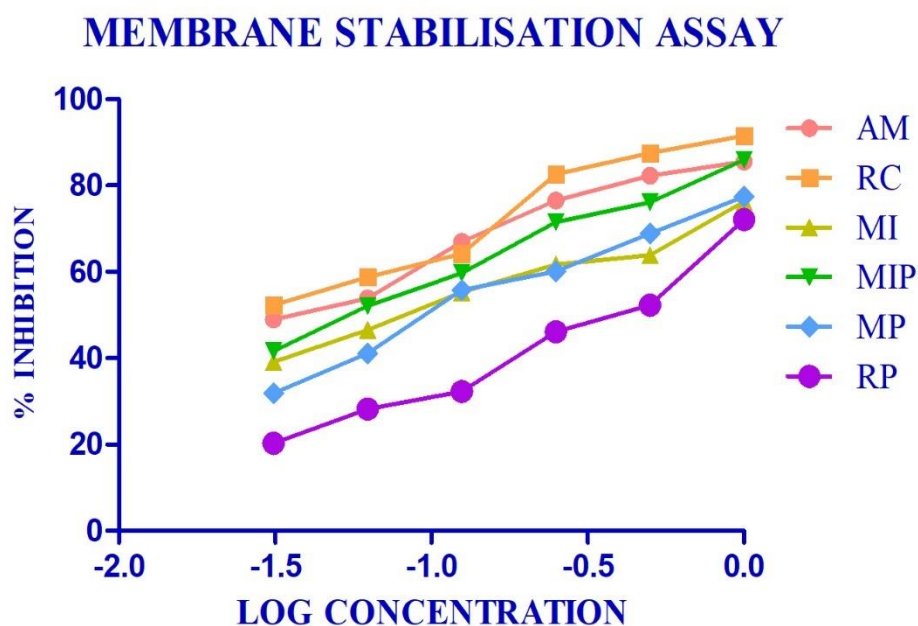


Figure 32: Graph of Membrane Stabilisation Assay, measured as percentage inhibition of haemolysis

ASSESSMENT OF TRYPTOPHAN METABOLITES

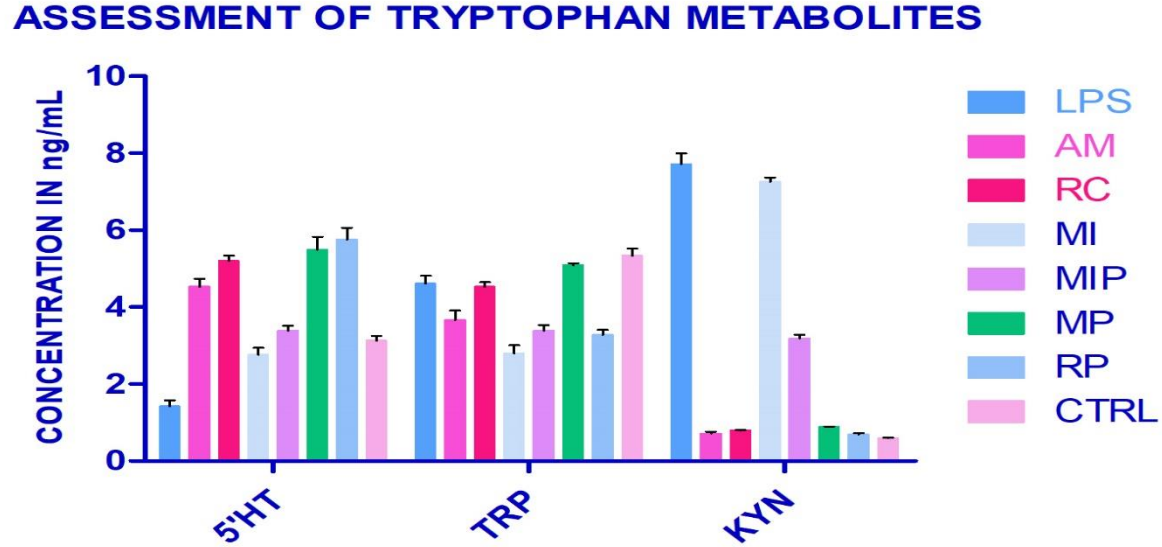


Figure 33: Graph of the concentration of tryptophan metabolites in RAW cell supernatants. 5'HT: Serotonin, TRP: Tryptophan, KYN: Kynurenine, CTRL: Control

ANAYLATE	MEAN CONCENTRATION IN ng/mL ($\pm$ SD)							
	CTRL	LPS	AM	RC	MI	MIP	MP	RP
5'HT	3.13 (0.21)	1.42* (0.27)	4.53* (0.36)	5.20** (0.25)	2.77 (0.31)	3.39* (0.22)	5.50* (0.57)	5.76* (0.52)
TRP	5.34 (0.32)	4.61* (0.35)	3.66* (0.44)	4.53* (0.20)	2.80** (0.36)	3.39* (0.25)	5.09* (0.10)	3.28* (0.23)
KYN	0.60 (0.03)	7.72** (0.47)	0.71 (0.09)	0.79 (0.03)	7.26** (0.18)	3.19* (0.18)	0.88 (0.02)	0.69 (0.06)

Table 20: Mean concentrations of Tryptophan metabolites in RAW cell supernatants. 5'HT: Serotonin, TRP: Tryptophan, KYN: Kynurenine. Concentrations expressed in ng/mL $\pm$ SD. * $p < 0.01$, ** $p < 0.001$

DOCKING STUDIES WITH AHR

The molecular docking was performed and analyzed using AutoDock Vina. The protein targets retrieved from RCSB Protein Data Bank are proteins associated with metabolic functioning and proliferation of Aryl hydrocarbon receptor. Aryl hydrocarbon receptor (PDB code 4M4X) served as docking receptor. The results are reported in Table 21.

Entry	Compound	Intermolecular distance (Å)	Amino Acids involved [†]	Binding Energy (kcal/mol)
1	(R)-Aegeline [@]	1.23	Leu110	-9.1
2	(S)-Aegeline [@]	2.92	Tyr135	-8.9
3	Catechin [#]	1.50	Tyr135	-8.8
4	Kaemferol [#]	1.67	Gly113	-8.6
5	Quercetin [#]	1.92	Gly113	-8.5
6	Imperatorin [@]	2.01	Tyr135	-8.2
7	Isoimperatorin [@]	1.97	Tyr135	-8.1
8	Marmesin [@]	2.14	Ala119	-7.4
9	Lupeol ^{\$}	2.80	Ser108	-7.2
10	Ricinoleic Acid-D13 ^{\$}	2.32 3.04	Leu110 Gly113	-7.2
11	α -Thujone ^{\$}	3.30	Tyr135	-6.7
12	Mangiferin [#]	1.40 1.01 2.48 2.62	Gln234 Thr264 Asn121 Ala119	-5.9
13	Ricinine ^{\$}	3.43	Met109	-5.5
14	N-Demethylricinine-13C3 ^{\$}	3.90 3.63	Leu110 Gly113	-5.2

Table 21: Results of docking various phytochemicals from the plants involved in this study as reported from literature. The compounds were docked against the Mouse Aryl Hydrocarbon Receptor (PDB ID: 4M4X).

[†]Amino acids of the protein, involved in intermolecular interactions with the compounds. @ reported in *A. marmelos*; # reported in *M. indica*; \$ reported in *R. communis*. Molecules with promising binding energies (>6 kcal/mol) are coloured in red.

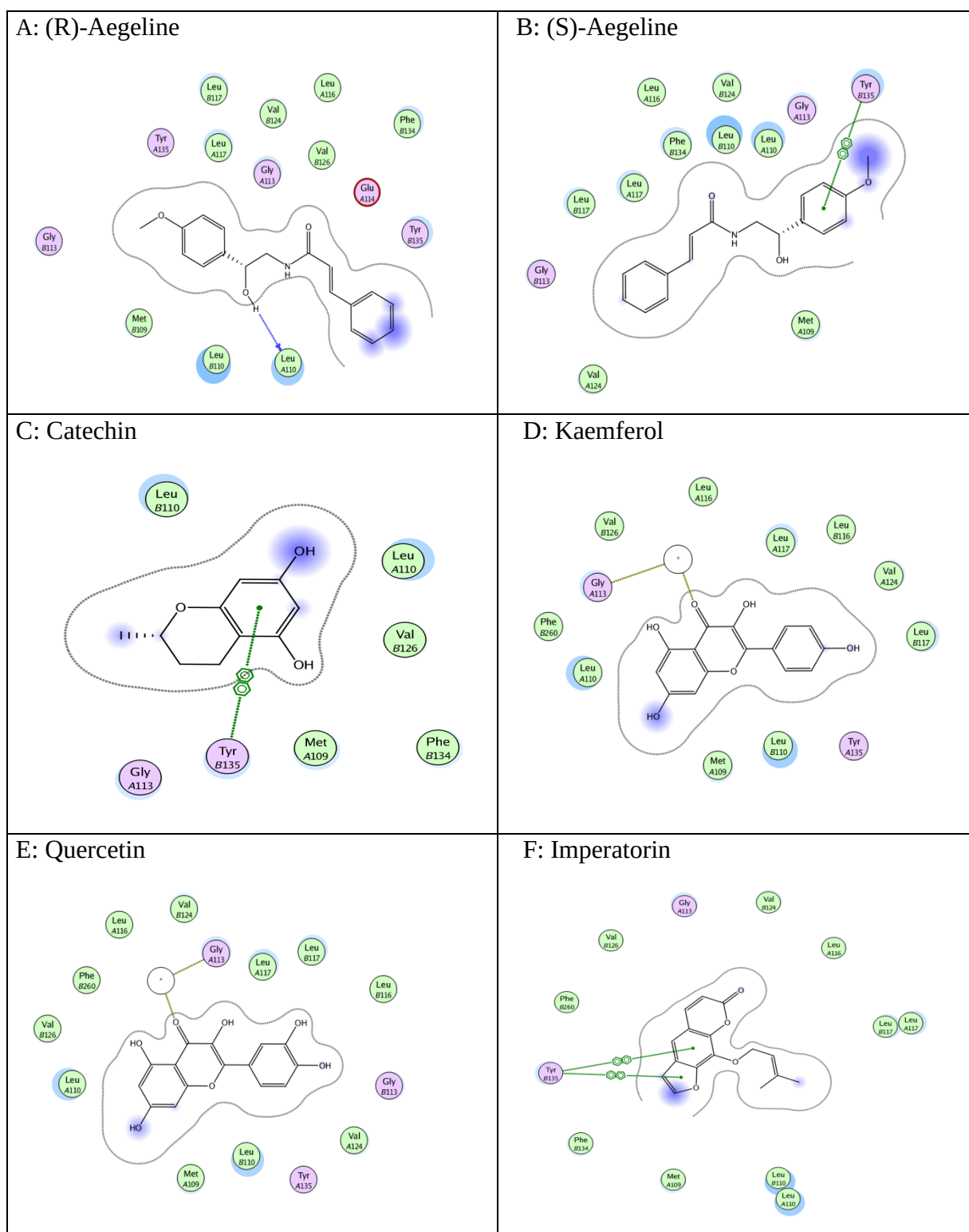


Figure 34: Ligand interaction diagrams between compounds and AHR

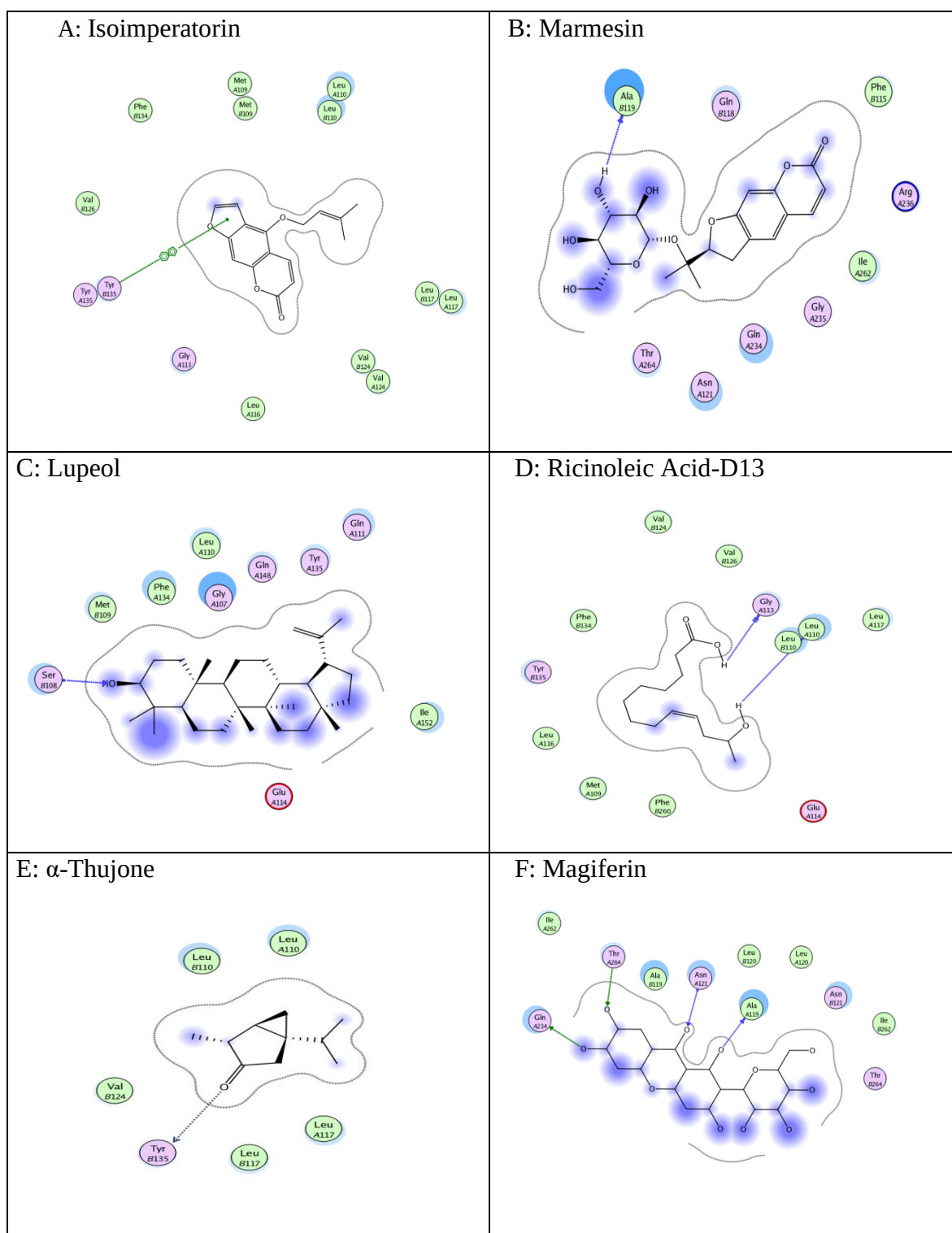


Figure 35: Ligand interaction diagrams between compounds and AHR

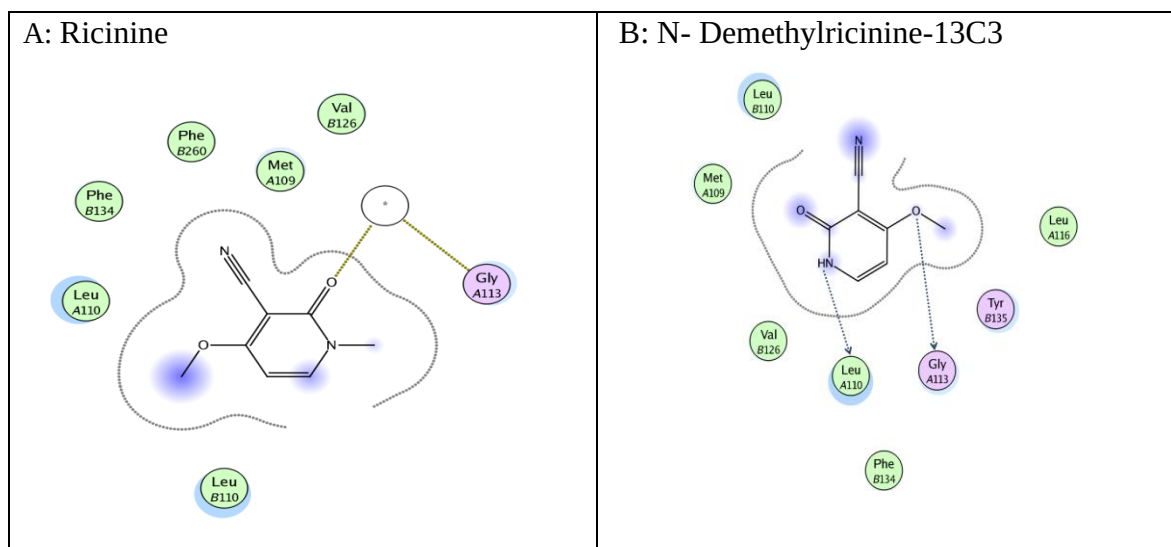


Figure 36: Ligand interaction diagrams between compounds and AHR

Figure 18-20 depict the molecular interactions between the small molecular ligands with the AHR. The critical amino acid residues of the AHR protein which form temporary bonds with the ligands are encircled.

DISCUSSION

Large number of drugs in use in the clinics today are of phytological origins or primarily derived from plant sources^[283]. The plethora of knowledge available in traditional and folklore medicines across the world may be used as starting points for screening and identification of potential anti-viral agents, especially against those that do not have an anti-viral. Hence taking a cue from Ayurveda, this study focussed on assessing the potential anti-viral activities of three important Indian plants against the rabies and the influenza virus.

Leaves of Mango (*Mangifera indica*), Bael (*Aegle marmelos*) and Eranda (*Ricinus communis*) were used in the study. Methanol extracts of the leaves were prepared using Soxhlet's extraction protocol and proteins were extracted using the P-PER plant protein extraction kit. Phytochemical profiling of the organic extracts was done using biochemical methods and confirmed by HPTLC.

Methanol extracts of *M. indica* (MI) exhibited the presence of Glycosides and Phenolics, whereas extracts of *A. marmelos* (AM) exhibited the presence of Flavonoids and Phenolics as the most abundance constituents. Terpenoids and Sterols were most abundant in the methanol extracts of *R. communis* (RC). The complete list of phytochemicals identified, was enlisted in Table 10 of the Results Section. Many other studies have reported similar phytochemical profiles from the leaves of these plants.

In-vitro cytotoxicity of the methanol extracts and protein extracts were assessed using the MTT assay on BHK-21, MDCK and RAW 264.7 cells. **The median cytotoxic concentrations were between 6.57 mg/mL for RP to 21.69 mg/mL for AM on RAW cells, 4.5 mg/mL for MIP to 13.79 mg/mL (MP) on BHK-21 and between 0.13 mg/mL for RP to 25.68 for MP on MDCK cells.** The Challenge Virus Standard (CVS-11) strain of the Rabies Virus was used in the study for assessing the anti-viral activity. Anti-viral activity against the rabies virus was evaluated using two methods; a modified RFFIT protocol and by assessing the relative change

in viral nucleic acid copy number using qRT-PCR. **Methanol extract from Eranda exhibited the most effective activity with an IC_{50} value of 19.70 $\mu\text{g/mL}$ (SI=496.95). This was followed by methanol extract of Mango followed by methanol extract of Bael – IC_{50} of 24.66 $\mu\text{g/mL}$ (SI=286.29) and protein extract of Bael at 39.37 $\mu\text{g/mL}$ (SI=350.27). Methanol extract of Bael also exhibited a promising IC_{50} at 55.71 $\mu\text{g/mL}$ (SI=203.37). Viral load estimation presented a similar picture with AM, RC, MI and MP exhibiting over 100-fold reduction in the viral RNA copies as compared to controls at 1000 $\mu\text{g/mL}$. AM, MI and MP exhibited over 30-fold reduction even at 10 $\mu\text{g/mL}$.**

Anti-viral effects were also carried out on the Influenza Virus. H3N2 strain of the influenza virus was cultured on MDCK cells. Methanol and Protein Extracts were added simultaneously and after infection of the cells. Potential anti-viral activity was assessed using the Hemagglutination assay. **The methanol extract of Mango and protein extract of Bael have potential anti-influenza virus activity, as exhibited by hemagglutination inhibition. Even at 100 $\mu\text{g/mL}$ the HA titre of 1:64 of the Virus control was reduced to 1:8 in case of MI. MP exhibited a 1:8 titre at 1000 $\mu\text{g/mL}$.**

Many phytochemicals have been reported to have anti-viral activities. Mangiferin, a glycoside and the major constituent of alcoholic extracts from mango leaves has been shown to inhibit HSV-1 and HSV-2 in cell cultures^[165]. It is also known to antagonize cytopathic effects of HIV in cell culture^[166]. Similarly, many active microbial and bioactive compounds have been reported from Bael. Amongst these, Marmelide has exhibited anti-viral activities against the Ranikhet Virus (New Castle Disease Virus)^[167] and the Human Coxsackie Virus B1-B6^[168]. In this study the methanol extract from Eranda, RC exhibited potential Anti-Rabies Virus activity, while the protein fraction RP did not exhibit a promising anti-viral activity against the Rabies Virus. Much work on the anti-viral activities from plant sources are limited to the secondary metabolites of plants. However, there is a small body of work which draws our attention to the

potential anti-viral activities of proteins derived from plant sources. The Momordica Anti-HIV Protein 30 (MAP-30) has shown potent anti-HIV activity^[169]. Similarly, Gelonium Anti-HIV Protein 31 (GAP-31) isolated from *Gelonium multiflorum* has been shown to exhibit anti-HIV activity^[169]. GAP-31 has been found to inhibit HIV entry and viral genome integration. It is also known to have inhibitory activities against Herpes Simplex Virus (HSV) and Human Herpes Virus 8 (HHV-8)^[170]. The mechanisms of action of three important plant proteins – Ricin, isolated from *R. communis*, Pokeweed Antiviral Protein (PAP), isolated from *Phytolacca americana* and Luffa Ribosomal Inhibitory Protein (LRIP-I), isolated from *Luffa cylindrica roem* – have been elucidated and host Ribosomal Inhibition has been seen as the active principle behind their anti-viral properties^[171–173]. Apart from these, gp22 and gp35 – two proteins isolated from the Tobacco plant infected with Tobacco Mosaic Virus (TMV) have exhibited anti-viral activities against the TMV^[174]. Anti-viral activity, albeit to Plant viruses (TMV) has been reported in two proteins CIP-29 and CIP-34 isolated from the leaves of *Clerodendrum inerme* Gaertn^[175]. What is interesting is that these proteins, which have exhibited antagonistic properties against the plant viruses have been found to function as Ribosome Inhibitory Proteins (RIPs). There is therefore a great scope – and given the fact that there is a greater need for anti-virals – that plant proteins along with plant secondary metabolites be screened for their potential anti-viral properties.

The emergence and re-emergence of viral diseases in the last couple of decades has been overwhelming. Also, the pathogenesis and hence the virulence of many an existing viral disease have become increasingly complex. With many viral pathologies being attributed to a disturbed immune homeostasis (hyper active host immune response/immune suppression), there has been a paradigm shift in the way viral diseases are managed clinically. Previously, a dysfunctional immune system was primarily considered to play a role in chronic/persistent viral infections like Herpes Zoster or HCMV mediated manifestations. However, in the present times, many

an acute viral manifestation are attributed to hyperactive immune interactions. These include West Nile Encephalitis, Chikungunya, ARDS associated with Influenza and the Dengue and other viral haemorrhagic fevers^[284–286]. A correction in the disturbed immune function is therefore a logical solution to amelioration of these conditions. Detailed descriptions of immunomodulatory agents have been described as early as the Ayurveda^[287–289].

Ayurveda has promulgated the idea of immunity as “*Vyadhikshamatwa*”. Acharya Chakrapanidatta has construed the term *Vyadhi-ksamatwa* as *Vyadhi bala Virodhitwa* i.e., antagonistic to the strength and virulence of the disease and *Vyadhyutpada Pratibandhakatwa* i.e., the capacity to inhibit and bind the causes and factors of the disease.

In effect, one of the therapeutic approaches in Ayurvedic medicines is to augment the body’s overall natural defence to the disease-causing agent rather than directly neutralizing the agent itself. The use of herbs for improving the overall resistance of body against common infections and pathogens has been a guiding principle of Ayurveda. Such herbs possessing immunomodulatory effects are referred to as *Rasayana* in Ayurvedic classics. They are supposed to have the ability of protecting the body against external factors that induce disease. This implied resistance against disease may represent the modern concept of immunity.

In this study, the *in vitro* immunomodulatory activities of the plant components were evaluated using a number of methods.

Since macrophages are central to innate as well as adaptive defences in the mammalian host, RAW 264.7 cell line (derived from murine macrophages) were cultured and exposed to the various secondary metabolite extracts and protein solutions. LPS treated cells were kept as controls. The cells were incubated for 48 hours, following which the supernatant was collected and stored at -80°C until further processing. The cells were lysed using the lysis buffer provided in the RNA extraction kit and processed for RNA extraction. Extracted RNA was

subjected to qRT PCR for assessing the gene expression of certain pivotal cytokines, which influence Macrophage polarization.

Interferon- γ (IFN- γ) is an immune interferon secreted by the T helper cells (specifically, T_H1 cells), cytotoxic T cells (T_C cells), macrophages, mucosal epithelial cells and NK cells. It is known to polarize macrophages towards the M1 lineage, which is important in inflammation and the Th cells towards the Th1 line, critical in the defence against intracellular parasites. Although, IFN- γ is a type II interferon (immune interferon), since IFN- γ operates via the activation of JAK/STAT pathway it is also crucial in the activation of anti-viral state in cells via activation of type I interferons^[290]. Replication of Duck Hepatitis B Virus has been inhibited in primary hepatocyte cultures treated with recombinant Chicken IFN- γ (chIFN- γ)^[291]. ch IFN- γ has also been shown to inhibit the replication of H1N1 human and H9N2 avian influenza viruses in chicken embryo fibroblasts^[292]. Studies on Vesicular Stomatitis Virus (VSV) has shown that IFN- γ induction as a result of synthetic cannabinoid, WIN55,212-2 exposure, decreases viral titres in infected neuronal cells in vitro by up to 100-fold compared with untreated cells^[293]. IFN- γ treatment has also been observed to inhibit replication of HSV-1^[294]. It also prevents virus re-activation in trigeminal neurons ex-vivo^[295] and inhibits HSV-1 induced apoptosis mice model^[296]. Poor interferon gamma (IFN γ) synthesis during respiratory syncytial infection (RSV) is related with extended viral clearance and enhanced disease severity in neonatal mice and humans^[297]. It has also been demonstrated that intra-nasal conveyance of IFN γ essentially improves RSV clearance from neonatal lungs preceding T-lymphocyte enrolment or enactment, suggesting an innate immune pathway of viral clearance. **In this study, the mRNA levels of IFN- γ in treated RAW cells were *up-regulated* in the methanol extract and protein extract of Mango leaves by 3.26-fold and 5.23-fold. Similar trend was observed in methanol extracts and protein extracts of Bael, 5.33-fold and 2.4-**

fold respectively. The methanol and protein extracts of Eranda downregulated IFN- γ mRNA in RAW cells by 3.16-fold and 2.86-fold respectively.

Tumor Necrosis Factor- α (TNF- α) is a pleiotropic cytokine that can affect various cellular functions. Although, it is produced primarily by activated macrophages many other cell types produce TNF- α . These include mast cells, eosinophils, NK cells, CD4+ lymphocytes, neutrophils and neurons. TNF- α has many important roles, the major ones being in induction of fever, apoptosis, inflammation and cachexia^[298]. It is a Th1 cytokine and has roles in killing of intracellular parasites. TNF- α has been reported to exhibit strong anti-influenza activity in lung epithelial cell model of the virus^[299]. The anti-viral activity of TNF- α in this case was found to be better than IFN- α and IFN- γ . TNF- α along with IFN- γ is needed to control HBV infection and HBV inactive carriers have exhibited re-activation of the virus replication when put on anti-TNF- α therapy for conditions like Rheumatoid Arthritis. TNF- α when used as an adjuvant, prompted development of dendritic cells. Su et al. have demonstrated that IL-6 and TNF- α , when used as molecular adjuvants can augment the antigen-specific cell-mediated responses produced by VP1 DNA vaccine against FMDV^[300]. Also, mTNF-K90R – a synthetic mutant of TNF- α , exhibited mucosal adjuvant activity for induction of both systemic and mucosal immune responses^[301]. However, TNF- α is also responsible for the pathological manifestations of many a condition. Apart from being the major therapeutic target in Rheumatoid arthritis and Ankylosing spondylitis, TNF- α related pathology has also been associated with HIV, JEV and experimental LCMV infections^[302]. **In this study mRNA levels of TNF- α in RAW cells were upregulated by the methanol extracts and protein extracts of Mango (5.6-fold and 6.4-fold respectively), whereas the levels were downregulated in the cells treated with samples of Bael (4.46-fold and 5.66-fold for ethanol and protein extracts) and Eranda (12.63-fold and 4.33-fold for methanol and protein extracts).**

This study also assessed the gene expression of IL-6 in the RAW cells treated with the samples. IL-6 is a multifunctional cytokine produced by large number of cells including macrophages and T cells. It is also secreted by non-immune cells including osteoblasts, smooth muscle cells and the neuron and glial cells. In the lymphoid system, IL-6 regulated cellular and humoral responses are vital in defining the outcome of viral infections. In HBV infection, for example, IL-6 promotes lymphocyte responses that are crucial for effective viral control^[303]. However, as an intermediary of inflammation, IL-6 is also engaged in the augmentation of HBV-induced liver cirrhosis and aggravating liver injury. Other than its involvement in the enrolment of T lymphocytes, IL-6 assumes a pivotal role in B and T cell differentiation. Initially IL-6 was categorized as a factor that augments antibody generation in a B cell line and supports growth of B lineage neoplasms. Presently there is enough evidence that, IL-6^{-/-} mice have a diminished IgG production upon immunization with a T cell-dependent antigen^[304]. It has also been demonstrated that IL-6 promotes B cell helper capabilities of CD4⁺ T cells through enhancing IL-21 production. Higher IL-6 levels have been associated with increased antibody production and better vaccine protection with vaccine against the Foot and mouth Disease Virus (FMDV)^[305]. Also, co-inoculation of *Mycobacterium tuberculosis* infected Macrophages along with IL-6, IL-1 β and TNF- α have exhibited enhanced immune outcome in protection against MTB compared to BCG vaccine^[306]. IL-6 also plays a significant role in the priming of a Th1 response to a tuberculosis vaccine^[307]. IL-6 brings about the expansion of Th17 cells from naïve T cells along with TGF- β ; contrary to that, IL-6 inhibits TGF- β induced Treg differentiation^[308]. Dysregulation or over-synthesis of IL-6 leads to autoimmune diseases such as multiple sclerosis (MS) and rheumatoid arthritis (RA)^[309]. **In this study, the mRNA levels of IL-6 have been upregulated in all the three plant systems (6 samples). The changes in cells exposed to Bael components – methanol and protein extract exhibited most**

upregulation at 7.3 and 5.86-fold respectively. Appropriate fractions from these plants may therefore serve as effective vaccine adjuvants in the future.

IL-4 is another polarising cytokine released by many cells, including macrophages and T cells. IL-4 orients macrophages or lymphocytes to a M2 or Th2 phenotype respectively^[310]. Among the many functions IL-4 has, the major ones are the stimulation of activated T and B cells, differentiation of B cells into antibody producing plasma cells and upregulation of MHC-II. It also has pivotal role in inflammation associated tissue repair^[311]. Th2 cell differentiation conducted by IL-4 is a pivotal step during immune responses against parasitic infections. This is demonstrated in mice lacking IL-4 receptor and STAT6 (Signal Transducer and Activator of Transcription-6), which exhibit severe forms of helminthic infections^[312]. Over-expression of IL-4 is associated with many an allergic reaction^[313]. Hence IL-4 is a critical cytokine in driving the Th1/Th2 or M1/M2 homeostasis. **In this study IL-4 gene expression was upregulated by all the samples. However, Methanol extract of Eranda exhibited the most upregulation at 6.36-fold, followed by the methanol extract of Bael at 5.8-fold.**

Supernatants from the cells exposed to the various samples were assayed for the production of Nitric Oxide (NO) via measuring the nitrates using Griess Reagent. **All the samples increased the levels of NO produced by the RAW cells. At 66.76 and 54.59 μ M the methanol and protein extracts of Mango exhibited the most prolific rise in the NO levels. These were almost comparable to LPS standard (73.22 μ M). the unexposed control cells produced 6.94 μ M of NO.**

Nitric oxide (NO) has been shown to have intricate and diverse functions in physiological and pathophysiological pathways. Over the past decade the mechanisms of many NO induced processes have been deciphered, hence a basic understanding of NO biology is almost established. Large number of reports indicate that NO and oxygen radicals especially superoxide, are key molecules involved in the pathogenesis of various infectious diseases.

Many microbial infections lead to the synthesis of NO via the induction of an inducible NO synthase (iNOS). While the antibacterial and anti-parasitic activities of NO are well documented, NO has contrasting consequences in virus infections such as influenza virus pneumonia and certain other neurotropic virus infections^[314]. One of the mechanism by which NO is known to exert its anti-viral activity is by inhibition of viral proteases^[315]. Studies on Coxsackieviruses and Human Rhinoviruses have been useful for gathering such evidence. NO can also inhibit the replication of other viruses that encode cysteine proteases, such as members of the Picornaviridae and Coronaviridae family^[315]. Many NO generating molecules have been found to have anti-viral effects. S-nitrosoglutathione (GSNO) and S-nitroso-N-acetylpenicillamine (SNAP) have exhibited anti-viral activities against porcine circovirus-2 and influenza A viruses respectively. However, NO also appears to have immunopathological consequences on the hosts immune system. For example, excess of NO generated in virus infections in mice is reported to suppress Th1 dependent immune responses, leading to Th2 host response^[314]. Also, production of an excessive amount of NO for long periods, allows synthesis of a highly reactive nitrogen species, peroxynitrite, via a radical coupling reaction of NO with superoxide. Oxidative tissue injury is caused by peroxynitrite via powerful oxidation and nitration reactions of various biomolecules. Thus, NO is considered as a potential immunomodulator rather than a simple antiviral agent.

Pathology of many viruses have been attributed to the hyper-inflammatory responses mounted by the host immune system in the presence of viral antigenic trigger. These include the hepatitis viruses, the encephalitis causing viruses – namely Japanese Encephalitis Virus, Herpes Simplex Viruses-1 & 2, West Nile Virus etc. – ARDS caused by Influenza Viruses and the viruses causing viral hemorrhagic fevers (VHF) – Dengue Virus, Lassa Fever Virus etc^[316–319]. In the case of these VHF's however the use of specific anti-inflammatory agents like certain NSAIDS is contraindicated. All this calls for the development of novel anti-inflammatory agents. In this

study, the anti-inflammatory activity of the samples was assessed using the RBC membrane stabilisation assay. The *in-vitro* RBC haemolysis assay is used for screening anti-inflammatory activity of molecules. Most of the anti-inflammatory molecules stabilize the plasma membrane of mammalian RBCs and thereby impede the heat-induced and the hypo tonicity-induced haemolysis. The RBC plasma membrane resembles the lysosomal membrane and hence the stabilizing effect of drugs on erythrocyte membrane may correlate with its lysosomal membrane stabilizing effect. **The methanol extract and protein extract of Bael exhibited the most promising anti-inflammatory activities at SI of 114.34 and 128.71 respectively. Methanol extract of Eranda also exhibited good anti-inflammatory activity with an SI of 61.22.** Phytochemicals like rosamarinic acid have been shown to have potent anti-inflammatory activity in experimental murine model of Japanese Encephalitis^[320]. Also, the use of naturally occurring phytochemicals as anti-inflammatory agents in the chemoprevention of many-a cancers have been advocated for.

Metabolism of certain small molecules and their modulation has been shown to affect the physiological status of cells. From the perspective of immune mechanisms metabolism of tryptophan in to serotonin or kynurenine plays a significant role in modulating the polarisation of macrophages, thereby having an impact on the overall immune homeostasis.

Besides its role as a neurotransmitter, serotonin (5-hydroxytryptamine, 5-HT) modulates inflammation and tissue repair via the Serotonin receptors (5-HT₁₋₇) whose pattern of expression varies among cell lineages^[321]. Considering the importance of macrophage polarization plasticity for inflammatory responses and tissue repair, 5-HT has been shown to hinder the LPS-induced proinflammatory cytokine release without influencing IL-10 production^[322], it also upregulated the expression of M2 polarization-associated genes (SERPINB2, THBS1, STAB1, COL23A1), and reduced the expression of M1-associated genes (INHBA, CCR2, MMP12, SERPINE1, CD1B, ALDH1A2). **In the present study, RAW cells**

treated with LPS were shown to have significantly reduced levels of 5-HT w.r.t control cells, whereas methanol and protein extracts of Bael and Eranda exhibited significantly enhanced levels of Serotonin. The metabolism of Tryptophan is regulated by the activity of two important enzymes namely, tryptophan hydroxylase-2 (TPH-2) and indoleamine 2,3-dioxygenase (IDO-2). TPH-2 is associated with activation of Serotonergic pathways. The kynurenine pathway (KP) is the other major metabolic pathway of tryptophan (TRP). Activation by inflammatory molecules, like IFN- γ , induces the induction of the KP, driving a intricate cascade of synthesis of both neuroprotective and neurotoxic metabolites, and in turn, controls the immune response and responses of brain cells to the KP metabolites. Consequently, substantial evidence has accumulated over the past couple of decades that dysregulation of the KP and the production of neurotoxic metabolites are associated with many neuroinflammatory and neurodegenerative diseases, including Parkinson's disease, AIDS-related dementia, motor neurone disease, schizophrenia, Huntington's disease, and brain cancers^[323,324]. Therefore, these molecules serve as pivotal markers in immune mechanisms. **In the present study levels of KYN were significantly increased w.r.t. control cells in cells exposed methanol and protein extracts of Mango (7.26 and 3.19 ng/mL respectively), these levels were comparable with cells treated with LPS (7.72 ng/mL). KYN levels were significantly reduced in cells exposed to the methanol and protein extracts of Bael (0.71 and 0.88 ng/mL) and Eranda (0.79 and 0.69 ng/mL). These levels were comparable with the KYN levels in control cells (0.60 ng/mL).**

Many studies have indicated the role of phytochemicals and other small molecules in the modulation of immune system. However, an understanding on the mechanism of their action is not well understood. Lately on cytosolic protein, the Aryl Hydrocarbon Receptor (AHR) has been indicated as a major receptor for interaction with these small molecules and regulation of downstream pathways, majorly immunomodulation. In this study, we performed in silico

analysis to evaluate the potential interactions of major phytochemicals ascribed to our plants of interest using docking studies. Automated docking was used to assess the binding modes and conformation of the compound with protein. The binding scores ranging from -9.1 to -5.2 kcal/mol against the resolved crystal structure of 4M4X. (R)-Aegeline chosen for computational studies showed better performance than the other drug molecules in terms of binding efficiencies with docking score of -9.1 kcal/mol (Table 20). The key intermolecular interactions between (R)-Aegeline and amino acids of Aryl hydrocarbon receptor are depicted in Figure1. (S)-Aegeline depicted a docking score of -8.9 kcal/mol. The least docking score was obtained for N-Demethylricinine-13C3 which is -5.2 kcal/mol. Both (R)-Aegeline and (S)-Aegeline are isomers of each other, but they both showed different docking scores. It was observed to that the compounds bound to the active site in different manner(Figure 24-26). The (S)-Aegeline was seen to flip in the active site of the protein, causing it to bind to different amino acids in receptor. Hydroxyl atom of (R)-Aegeline bound to the Leu110 amino acid, whereas phenol group of (S)-Aegeline bound to Tyr135 (Figure 24-26).

The interactions at the AHR PAS-A dimer interface involve two distinct regions, both of which involve the participation of the A' α -helix. The first region forms between the two reciprocating A' α -helices and consists of hydrophobic interactions between Leu110, Leu117, Ala119, and Leu120. The second one involves the A' α -helix forming contacts with the β -sheet of the other subunit and includes the hydrophobic residues Phe115, Leu116, Ala119, Gln118 (A' α), Val124 (A β), Phe260, Ile262, Thr264 (I β), and a set of polar residues, Ser108, Glu112, (A' α), Arg236, Lys238, Gln 234(H β) and His241 (HI loop). The (R)-Aegeline displayed good docking interactions with residues belonging to A' α -helix regions.. Hydroxyl atom of (R)-Aegeline bound to the Leu110 amino acid (Figure 2). Throughout the compounds good docking scores were observed for Leu110, Tyr135 and Gly113 amino acids. These are the hot spot residues in the active site which could be considered in docking for Aryl hydrocarbon receptor. Thus,

Molecular modelling suggested which possible residues are fundamental for small molecules to interact with target protein and which residues were irrelevant for their binding. This knowledge of the residues in the binding site will help us in developing better immunomodulatory regulators.

The 21st century has already seen the emergence and re-emergence of a large number of viral diseases. Most of these viral diseases do not have any known anti-viral. Also, the fact that most of these viral pathologies are attributed to a disturbed immune system, there is an urgent need to design and develop novel anti-virals and specialised immunomodulators. The plants used in this study have long been used in Ayurveda and other folklore medical systems, especially in the management of inflammatory conditions. Our study suggests that methanol extracts of all the three plants and protein extracts of Bael have promising in-vitro Anti-Rabies activity. Also, the methanol extract of Mango and protein extract of Bael have potential anti-influenza virus activity, as exhibited by hemagglutination inhibition. The samples also have promising immunomodulatory activities. Bael offers a good anti-inflammatory, balanced Th1/Th2 or M1/M2 response. Extracts of Eranda also exhibit potent anti-inflammatory activity polarising the macrophages to a M2 phenotype. The extracts of Mango potentiate a Th1 predominant response. Further detailed evaluation shall allow us to establish these systems in the regular management of viral diseases.

SUMMARY & CONCLUSION

The present study was undertaken to assess the anti-viral activity of methanol and protein extracts of Mango, Bael and Eranda against the rabies virus and the Influenza virus.

1. Plants were collected from the Institute campus. Specimens were sent to the Botanical Survey of India, Pune for authentication.
2. Methanol extracts were prepared using the Soxhlet's Apparatus.
3. Phytochemical analysis was performed using Biochemical Tests and HPTLC.
4. Protein extracts were made using commercially available kit.
5. In-vitro cytotoxicity was performed on RAW 264.7 cells, BHK-21 cells and MDCK cells using MTT assay
6. Anti Rabies Virus activity was performed with the CVS-11 strain of the virus on BHK-21 cells, using modified RFFIT method followed by Relative viral load estimation using qRT-PCR.
7. Anti-Influenza activity was performed using H3N2 strain of the influenza virus on MDCK cells. Results were interpreted as reduction in hemagglutination (HA) titres.
8. In-vitro immunomodulatory assay was performed on RAW cells. Samples were exposed to the RAW cells and incubated for 48 hours. Following which, supernatants were collected and stored at -80°C and cell monolayers were used for RNA extraction using commercially available kit
9. Extracted RNA was used to assess the relative gene expression of pivotal cytokine genes (IFN- γ , TNF- α , IL-6 and IL-4)
10. Part of the supernatants were used to assess the levels of Nitric Oxide using Griess Assay.
11. Part of the supernatants were used to assess the levels of tryptophan metabolites using specific biochemical assays for Tryptophan, Serotonin and Kynurenine.

12. RBC membrane stabilisation assay was used to assess the potential anti-inflammatory activity of the samples.
13. In-silico docking experiments were performed on the mouse AHR protein obtained from the PDB database using various metabolites of Mango, Bael and Eranda. These metabolite structures were obtained from PubChem. Docking was performed on the Auto Dock Vina software.
14. Phytochemical analysis revealed Methanol extracts of *M. indica* (MI) exhibited the presence of Glycosides and Phenolics, whereas extracts of *A. marmelos* (AM) exhibited the presence of Flavonoids and Phenolics as the most abundance constituents. Terpenoids and Sterols were most abundant in the methanol extracts of *R.communis* (RC).
15. The methanol extracts of all the three plants and protein extracts of Bael have promising in-vitro Anti-Rabies activity. The SI values of the same were 203.37, 496.95, 286.29 and 350.27 for AM, RC, MI and MP respectively. In the light of the fact that there exists no anti-viral chemotherapeutic agent against the rabies virus, the study could have significant implications. At least for initiating in vivo studies with these plants.
16. The methanol extract of Mango and protein extract of Bael have potential anti-influenza virus activity, as exhibited by hemagglutination inhibition. Even at 100 µg/mL the HA titre if 1:64 of the Virus control was reduced to 1:8 in case of MI. MP exhibited a 1:8 titre at 1000 µg/mL. In the event of constant mutations in the influenza virus novel antivirals will be useful in case of future epidemics.
17. The samples of bael and eranda exhibited a balance Th1/Th2 or M1/M2 response. With eranda exhibiting a Th2/M2 preference. This is critical for maintaining immune homeostasis, especially in immune compromised hosts. Also in the event of hyper immune activation like cytokine storm etc. these systems can be of immense benefit in correcting the immune function.

18. Extracts of mango elicited a Th1 dominant activity. This is critical for managing intracellular parasites and cancers. Also, these can be used as potential vaccine adjuvants.
19. The in-silico studies gave important clues to the interaction of principal phytochemical components from the 3 plants with AHR. The interaction of Aegeline, Catechin, Kaemferol Quercetin etc. exhibit a binding energy of >6 kcal/mol. This in-silico study will be an important starting point for systematic evaluation of small molecules especially those derived from plants and study their mechanism to bring about immunomodulation.

Limitations of the Study:

Since the Study was conducted on whole extracts instead of purified fractions, identification of potential active pharmaceutical leads was not possible.

FUTURE APPLICATIONS

1. Fractionation studies will help us identify potential pharmaceutical actives in these extracts. Assessing their individual activities will help us design specific anti-virals, whose mechanisms of action will be known.
2. Studies on Animal models of the disease will further help to establish the efficacy of the extracts or its actives thereof. Once identified, the studies can be further taken into human subjects of these diseases.
3. Viral infections in the animals (domesticated and wild) are also responsible for causing large number of morbidities and mortalities, leading to economic losses. It also increases the risk of sustenance of these viruses in nature. Therefore, the same systems of anti-virals and immunomodulators may then be applied to veterinary disease models leading to discovery of effective treatment and management strategies of viral disease in animals.

REFERENCES

1. Mahajan V, Saini SS, Fadnis VP, Shah N. Correspondence. Indian Pediatr 2010;47(1):101–4.
2. Hagau N, Slavcovici A, Gongnanu DN, Oltean S, Dirzu DS, Brezozski ES, et al. Clinical aspects and cytokine response in severe H1N1 influenza A virus infection. Crit Care [Internet] 2010;14(6):R203. Available from: <http://ccforum.com/content/14/6/R203>
3. To KKW, Hung IFN, Li IWS, Lee K, Koo C, Yan W, et al. Delayed Clearance of Viral Load and Marked Cytokine Activation in Severe Cases of Pandemic H1N1 2009 Influenza Virus Infection. Clin Infect Dis 2010;50:850–9.
4. Crowe CR, Chen K, Pociask DA, Alcorn F, Krivich C, Enelow RI, et al. Critical Role of IL-17RA in Immunopathology of Influenza Infection. J Immunol 2009;183(8):5301–10.
5. Kil H, Lee J, Lee K, Rhim J, Youn Y, Kang J. Early corticosteroid treatment for severe pneumonia caused by 2009 H1N1 influenza virus. Crit Care 2011;15:413.
6. Roberts C, Nirmalan M, O’Shea S. Steroid-sensitive Post-Viral Inflammatory Pneumonitis (PVIP). Am J Respir Crit Care Med 2010;182(8):1089–90.
7. Mariana B, Yacine A, Jesse P, Xavier B, Marie-Ève H, Guy B. Emergence of Oseltamivir-Resistant Pandemic H1N1 Virus during Prophylaxis. N Engl J Med 2009;361(23):2296–7.
8. Sudarshan MK, Madhusudana SN, Mahendra BJ, Rao NSN, Narayana DHA, Rahman SA, et al. Assessing the burden of human rabies in India : results of a national multi-center epidemiological survey. Int J Infect Dis 2007;11(1):29–35.
9. Bijlenga G. Virology: The efficacy of a new single post - exposure treatment of rabies in mice without vaccination. Vet Q 2001;23(4):187–90.
10. Huang C-T, Li Z, Huang Y, Zhang G, Zhou M, Chai Q, et al. Enhancement of blood–brain barrier permeability is required for intravenously administered virus neutralizing antibodies to clear an established rabies virus infection from the brain and prevent the development of rabies in mice. Antiviral Res [Internet] 2014 [cited 2017 Jun 1];110:132–41. Available from: <http://linkinghub.elsevier.com/retrieve/pii/S0166354214002125>
11. Huang Y, Chen Z, Huang J, Fu Z, He B. Parainfluenza Virus 5 Expressing the G Protein of Rabies Virus Protects Mice after Rabies Virus Infection. J Virol [Internet] 2015;89(6):3427–9. Available from: <http://jvi.asm.org/lookup/doi/10.1128/JVI.03656->

12. International Committee on Taxonomy of Viruses (ICTV) [Internet]. [cited 2017 Aug 3];Available from: https://talk.ictvonline.org/taxonomy/p/taxonomy_releases
13. Virgin HW, Wherry EJ, Ahmed R. Redefining chronic viral infection. *Cell* 2009;138(1):30–50.
14. Immunopathology of viral infections. [cited 2017 Aug 3];Available from: http://www.virologie.uniklinikum-jena.de/virologie_media/Script_Teil_3.pdf
15. Dejnirattisai W, Jumnainsong A, Onsirisakul N, Fitton P, Vasanawathana S, Limpitikul W, et al. Cross-reacting antibodies enhance dengue virus infection in humans. *Science* (80-) 2010;328(5979):745–8.
16. Oldstone MBA. Molecular mimicry and autoimmune diseases. *Cell* 1987;50(13):819–20.
17. Chisari F, Ferrari C. Hepatitis B virus immunopathology [Internet]. In: Springer Seminars in Immunopathology. Springer; 1995. page 261–81.Available from: <http://link.springer.com/10.1007/BF00196169>
18. Weis W, Brown JH, Cusack S, Paulson JC, Skehel JJ, Wiley DC. Structure of the influenza virus haemagglutinin complexed with its receptor, sialic acid. *Nature* 1988;333(6172):426.
19. Couceiro JNSS, Paulson JC, Baum LG. Influenza virus strains selectively recognize sialyloligosaccharides on human respiratory epithelium; the role of the host cell in selection of hemagglutinin receptor specificity. *Virus Res* 1993;29(2):155–65.
20. Böttcher E, Matrosovich T, Beyerle M, Klenk H-D, Garten W, Matrosovich M. Proteolytic activation of influenza viruses by serine proteases TMPRSS2 and HAT from human airway epithelium. *J Virol* 2006;80(19):9896–8.
21. White J, Kartenbeck J, Helenius A. Membrane fusion activity of influenza virus. *EMBO J* 1982;1(2):217–22.
22. Skehel JJ, Bayley PM, Brown EB, Martin SR, Waterfield MD, White JM, et al. Changes in the conformation of influenza virus hemagglutinin at the pH optimum of virus-mediated membrane fusion. *Proc Natl Acad Sci* 1982;79(4):968–72.
23. White J, Matlin K, Helenius A. Cell fusion by Semliki Forest, influenza, and vesicular stomatitis viruses. *J Cell Biol* 1981;89(3):674–9.
24. Stauffer S, Feng Y, Nebioglu F, Heilig R, Picotti P, Helenius A. Stepwise priming by acidic pH and high K⁺ is required for efficient uncoating of influenza A virus cores after penetration. *J Virol* 2014;JVI-01430.

25. Okada A, Miura T, Takeuchi H. Zinc-and pH-dependent conformational transition in a putative interdomain linker region of the influenza virus matrix protein M1. *Biochemistry* 2003;42(7):1978–84.
26. Bui M, Whittaker G, Helenius A. Effect of M1 protein and low pH on nuclear transport of influenza virus ribonucleoproteins. *J Virol* 1996;70(12):8391–401.
27. Emtage JS, Catlin GH, Carey NH. Polyadenylation and reverse transcription of influenza viral RNA. *Nucleic Acids Res* 1979;6(4):1221–39.
28. Kati WM, Montgomery D, Carrick R, Gubareva L, Maring C, McDaniel K, et al. In vitro characterization of A-315675, a highly potent inhibitor of A and B strain influenza virus neuraminidases and influenza virus replication. *Antimicrob Agents Chemother* 2002;46(4):1014–21.
29. Nayak DP, Hui EK-W, Barman S. Assembly and budding of influenza virus. *Virus Res* 2004;106(2):147–65.
30. Schnell JR, Chou JJ. Structure and mechanism of the M2 proton channel of influenza A virus. *Nature* 2008;451(7178):591.
31. Steinhauer DA, Wharton SA, Skehel JJ, Wiley DC, Hay AJ. Amantadine selection of a mutant influenza virus containing an acid-stable hemagglutinin glycoprotein: evidence for virus-specific regulation of the pH of glycoprotein transport vesicles. *Proc Natl Acad Sci* 1991;88(24):11525–9.
32. Zarubaev V V, Golod EL, Anfimov PM, Shtro AA, Saraev V V, Gavrilov AS, et al. Synthesis and anti-viral activity of azolo-adamantanes against influenza A virus. *Bioorg Med Chem* 2010;18(2):839–48.
33. Sidwell RW, Huffman JH, Barnard DL, Bailey KW, Wong M-H, Morrison A, et al. Inhibition of influenza virus infections in mice by GS4104, an orally effective influenza virus neuraminidase inhibitor. *Antiviral Res* 1998;37(2):107–20.
34. Takashita E, Meijer A, Lackenby A, Gubareva L, Rebelo-de-Andrade H, Besselaar T, et al. Global update on the susceptibility of human influenza viruses to neuraminidase inhibitors, 2013–2014. *Antiviral Res* 2015;117:27–38.
35. Abed Y, Nehmé B, Baz M, Boivin G. Activity of the neuraminidase inhibitor A-315675 against oseltamivir-resistant influenza neuraminidases of N1 and N2 subtypes. *Antiviral Res* 2008;77(2):163–6.
36. Chen B-L, Wang Y-J, Guo H, Zeng G-Y. Design, synthesis, and biological evaluation of crenatoside analogues as novel influenza neuraminidase inhibitors. *Eur J Med Chem* 2016;109:199–205.

37. El-Nezhawy AOH, Eweas AF, Maghrabi IA, Edalo AS, Abdelwahab SF. Design, Synthesis, and Molecular Docking of Novel Pyrrolooxazepinediol Derivatives with Anti-Influenza Neuraminidase Activity. *Arch Pharm (Weinheim)* 2015;348(11):786–95.
38. Jefferson T, Jones M, Doshi P, Spencer EA, Onakpoya I, Heneghan CJ. Oseltamivir for influenza in adults and children: systematic review of clinical study reports and summary of regulatory comments. *Bmj* 2014;348:g2545.
39. Dobson J, Whitley RJ, Pocock S, Monto AS. Oseltamivir treatment for influenza in adults: a meta-analysis of randomised controlled trials. *Lancet* 2015;385(9979):1729–37.
40. Adisasmito W, Chan PKS, Lee N, Oner AF, Gasimov V, Aghayev F, et al. Effectiveness of antiviral treatment in human influenza A (H5N1) infections: analysis of a Global Patient Registry. *J Infect Dis* 2010;202(8):1154–60.
41. McGeer A, Green KA, Plevneschi A, Shigayeva A, Siddiqi N, Raboud J, et al. Antiviral therapy and outcomes of influenza requiring hospitalization in Ontario, Canada. *Clin Infect Dis* 2007;45(12):1568–75.
42. Lee N, Choi KW, Chan PKS, Hui DSC, Lui GCY, Wong BCK, et al. Outcomes of adults hospitalised with severe influenza. *Thorax* 2010;65(6):510–5.
43. Organization WH. WHO guidelines for pharmacological management of pandemic (H1N1) 2009 influenza and other influenza viruses. 2010;
44. Basu A, Antanasijevic A, Wang M, Li B, Mills DM, Ames JA, et al. New small molecule entry inhibitors targeting hemagglutinin-mediated influenza a virus fusion. *J Virol* 2014;88(3):1447–60.
45. Matsubara T, Onishi A, Saito T, Shimada A, Inoue H, Taki T, et al. Sialic acid-mimic peptides as hemagglutinin inhibitors for anti-influenza therapy. *J Med Chem* 2010;53(11):4441–9.
46. Wu W, Lin D, Shen X, Li F, Fang Y, Li K, et al. New influenza A virus entry inhibitors derived from the viral fusion peptides. *PLoS One* 2015;10(9):e0138426.
47. Teissier E, Zandomeneghi G, Loquet A, Lavillette D, Lavergne J-P, Montserret R, et al. Mechanism of inhibition of enveloped virus membrane fusion by the antiviral drug arbidol. *PLoS One* 2011;6(1):e15874.
48. Киселев ОИ, Малеев ВВ, Деева ЭГ, Ленева ИА, Селькова ЕП, Осипова ЕА, et al. Клиническая эффективность препарата Арбидол (умифеновир) в терапии гриппа у взрослых: промежуточные результаты многоцентрового двойного слепого

- рандомизированного плацебо-контролируемого исследования АРБИТР. Терапевтический архив (архив до 2018 г) 2015;87(1):88–96.
49. Leneva IA, Russell RJ, Boriskin YS, Hay AJ. Characteristics of arbidol-resistant mutants of influenza virus: implications for the mechanism of anti-influenza action of arbidol. *Antiviral Res* 2009;81(2):132–40.
 50. Brancato V, Peduto A, Wharton S, Martin S, More V, Di Mola A, et al. Design of inhibitors of influenza virus membrane fusion: Synthesis, structure–activity relationship and in vitro antiviral activity of a novel indole series. *Antiviral Res* 2013;99(2):125–35.
 51. Whittle JRR, Zhang R, Khurana S, King LR, Manischewitz J, Golding H, et al. Broadly neutralizing human antibody that recognizes the receptor-binding pocket of influenza virus hemagglutinin. *Proc Natl Acad Sci* 2011;108(34):14216–21.
 52. Benjamin E, Wang W, McAuliffe J, Palmer-Hill F, Kallewaard N, Chen Z, et al. A broadly neutralizing human monoclonal antibody directed against a novel conserved epitope on the influenza virus H3 hemagglutinin globular head. *J Virol* 2014;JVI-03562.
 53. Koday MT, Nelson J, Chevalier A, Koday M, Kalinoski H, Stewart L, et al. A computationally designed hemagglutinin stem-binding protein provides in vivo protection from influenza independent of a host immune response. *PLoS Pathog* 2016;12(2):e1005409.
 54. Kiso M, Takahashi K, Sakai-Tagawa Y, Shinya K, Sakabe S, Le QM, et al. T-705 (favipiravir) activity against lethal H5N1 influenza A viruses. *Proc Natl Acad Sci* 2010;107(2):882–7.
 55. Smee DF, Hurst BL, Wong M-H, Bailey KW, Tarbet EB, Morrey JD, et al. Effects of the combination of favipiravir (T-705) and oseltamivir on influenza A virus infections in mice. *Antimicrob Agents Chemother* 2010;54(1):126–33.
 56. Tarbet EB, Maekawa M, Furuta Y, Babu YS, Morrey JD, Smee DF. Combinations of favipiravir and peramivir for the treatment of pandemic influenza A/California/04/2009 (H1N1) virus infections in mice. *Antiviral Res* 2012;94(1):103–10.
 57. Byrn RA, Jones SM, Bennett HB, Bral C, Clark MP, Jacobs MD, et al. Preclinical activity of VX-787, a first-in-class, orally bioavailable inhibitor of the influenza virus polymerase PB2 subunit. *Antimicrob Agents Chemother* 2015;59(3):1569–82.
 58. Portela A, Digard P. The influenza virus nucleoprotein: a multifunctional RNA-binding protein pivotal to virus replication. *J Gen Virol* 2002;83(4):723–34.
 59. Amorim MJ, Kao R, Digard P. Nucleozin targets cytoplasmic trafficking of vRNP-Rab11 complexes in influenza A virus infection. *J Virol* 2013;JVI-03123.

60. Lejal N, Tarus B, Bouguyon E, Chenavas S, Bertho N, Delmas B, et al. Structure-based discovery of the novel antiviral properties of naproxen against the nucleoprotein of influenza A virus. *Antimicrob Agents Chemother* 2013;AAC-02335.
61. Tarus B, Bertrand H, Zedda G, Di Primo C, Quideau S, Slama-Schwok A. Structure-based design of novel naproxen derivatives targeting monomeric nucleoprotein of Influenza A virus. *J Biomol Struct Dyn* 2015;33(9):1899–912.
62. Hatada E, Fukuda R. Binding of influenza A virus NS1 protein to dsRNA in vitro. *J Gen Virol* 1992;73(12):3325–9.
63. LU Y, WAMBACH M, KATZE MG, KRUG RM. Binding of the influenza virus NS1 protein to double-stranded RNA inhibits the activation of the protein kinase that phosphorylates the eIF-2 translation initiation factor. *Virology* 1995;214(1):222–8.
64. Alonso-Caplen F V, Nemeroff ME, Qiu Y, Krug RM. Nucleocytoplasmic transport: the influenza virus NS1 protein regulates the transport of spliced NS2 mRNA and its precursor NS1 mRNA. *Genes Dev* 1992;6(2):255–67.
65. García-Sastre A, Egorov A, Matassov D, Brandt S, Levy DE, Durbin JE, et al. Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. *Virology* 1998;252(2):324–30.
66. Gack MU, Albrecht RA, Urano T, Inn K-S, Huang I-C, Carnero E, et al. Influenza A virus NS1 targets the ubiquitin ligase TRIM25 to evade recognition by the host viral RNA sensor RIG-I. *Cell Host Microbe* 2009;5(5):439–49.
67. Kuo R-L, Li L-H, Lin S-J, Li Z-H, Chen G-W, Chang C-K, et al. The role of N-terminus-truncated NS1 proteins of influenza A virus in inhibiting IRF3 activation. *J Virol* 2016;JVI-02843.
68. Basu D, Walkiewicz MP, Frieman M, Baric RS, Auble DT, Engel DA. Novel influenza virus NS1 antagonists block replication and restore innate immune function. *J Virol* 2009;83(4):1881–91.
69. Jablonski JJ, Basu D, Engel DA, Geysen HM. Design, synthesis, and evaluation of novel small molecule inhibitors of the influenza virus protein NS1. *Bioorg Med Chem* 2012;20(1):487–97.
70. Walkiewicz MP, Basu D, Jablonski JJ, Geysen HM, Engel DA. Novel inhibitor of influenza non-structural protein 1 blocks multi-cycle replication in an RNase L-dependent manner. *J Gen Virol* 2011;92(1):60–70.
71. Nayak MK, Agrawal AS, Bose S, Naskar S, Bhowmick R, Chakrabarti S, et al. Antiviral activity of baicalin against influenza virus H1N1-pdm09 is due to modulation of NS1-

- mediated cellular innate immune responses. *J Antimicrob Chemother* 2014;69(5):1298–310.
72. Shapira SD, Gat-Viks I, Shum BO V, Dricot A, de Grace MM, Wu L, et al. A physical and regulatory map of host-influenza interactions reveals pathways in H1N1 infection. *Cell* 2009;139(7):1255–67.
 73. Watanabe T, Kawakami E, Shoemaker JE, Lopes TJS, Matsuoka Y, Tomita Y, et al. Influenza virus-host interactome screen as a platform for antiviral drug development. *Cell Host Microbe* 2014;16(6):795–805.
 74. Moss RB, Hansen C, Sanders RL, Hawley S, Li T, Steigbigel RT. A phase II study of DAS181, a novel host directed antiviral for the treatment of influenza infection. *J Infect Dis* 2012;206(12):1844–51.
 75. Malakhov MP, Aschenbrenner LM, Smee DF, Wandersee MK, Sidwell RW, Gubareva L V, et al. Sialidase fusion protein as a novel broad-spectrum inhibitor of influenza virus infection. *Antimicrob Agents Chemother* 2006;50(4):1470–9.
 76. Ovcharenko A V, Zhirnov OP. Aprotinin aerosol treatment of influenza and paramyxovirus bronchopneumonia of mice. *Antiviral Res* 1994;23(2):107–18.
 77. Meyer D, Sielaff F, Hammami M, Böttcher-Friebertshäuser E, Garten W, Steinmetzer T. Identification of the first synthetic inhibitors of the type II transmembrane serine protease TMPRSS2 suitable for inhibition of influenza virus activation. *Biochem J* 2013;452(2):331–43.
 78. Razinkov VI, Melikyan GB, Epand RM, Epand RF, Cohen FS. Effects of Spontaneous Bilayer Curvature on Influenza Virus-mediated Fusion Pores. *J Gen Physiol* 1998;112(4):409–22.
 79. Harada S. The broad anti-viral agent glycyrrhizin directly modulates the fluidity of plasma membrane and HIV-1 envelope. *Biochem J* 2005;392(1):191–9.
 80. Wolkerstorfer A, Kurz H, Bachhofner N, Szolar OHJ. Glycyrrhizin inhibits influenza A virus uptake into the cell. *Antiviral Res* 2009;83(2):171–8.
 81. Wolf MC, Freiberg AN, Zhang T, Akyol-Ataman Z, Grock A, Hong PW, et al. A broad-spectrum antiviral targeting entry of enveloped viruses. *Proc Natl Acad Sci* 2010;107(7):3157–62.
 82. Müller KH, Kainov DE, El Bakkouri K, Saelens X, De Brabander JK, Kittel C, et al. The proton translocation domain of cellular vacuolar ATPase provides a target for the treatment of influenza A virus infections. *Br J Pharmacol* 2011;164(2):344–57.
 83. Wang X, Hinson ER, Cresswell P. The interferon-inducible protein viperin inhibits

- influenza virus release by perturbing lipid rafts. *Cell Host Microbe* 2007;2(2):96–105.
84. Naito T, Momose F, Kawaguchi A, Nagata K. Involvement of Hsp90 in assembly and nuclear import of influenza virus RNA polymerase subunits. *J Virol* 2007;81(3):1339–49.
 85. Chase G, Deng T, Fodor E, Leung BW, Mayer D, Schwemmler M, et al. Hsp90 inhibitors reduce influenza virus replication in cell culture. *Virology* 2008;377(2):431–9.
 86. Streeter DG, Witkowski JT, Khare GP, Sidwell RW, Bauer RJ, Robins RK, et al. Mechanism of action of 1- β -D-ribofuranosyl-1, 2, 4-triazole-3-carboxamide (Virazole), a new broad-spectrum antiviral agent. *Proc Natl Acad Sci* 1973;70(4):1174–8.
 87. Stephen EL, Walker JS, Dominik JW, Young HW, Berendt RF. Aerosol therapy of influenza infections of mice and primates with rimantadine, ribavirin, and related compounds. *Ann N Y Acad Sci* 1977;284(1):264–71.
 88. Rodriguez WJ, Hall CB, Welliver R, Simoes EAF, Ryan ME, Stutman H, et al. Efficacy and safety of aerosolized ribavirin in young children hospitalized with influenza: a double-blind, multicenter, placebo-controlled trial. *J Pediatr* 1994;125(1):129–35.
 89. Perwitasari O, Johnson S, Yan X, Howerth E, Shacham S, Landesman Y, et al. Verdinexor, a novel Selective Inhibitor of Nuclear Export (SINE), reduces Influenza A virus replication in vitro and in vivo. *J Virol* 2014;JVI-01774.
 90. Rossignol J-F, La Frazia S, Chiappa L, Ciucci A, Santoro MG. Thiazolidines, a new class of anti-influenza molecules targeting viral hemagglutinin at post-translational level. *J Biol Chem* 2009;jbc-M109.
 91. Haffizulla J, Hartman A, Hoppers M, Resnick H, Samudrala S, Ginocchio C, et al. Effect of nitazoxanide in adults and adolescents with acute uncomplicated influenza: a double-blind, randomised, placebo-controlled, phase 2b/3 trial. *Lancet Infect Dis* 2014;14(7):609–18.
 92. Pleschka S, Wolff T, Ehrhardt C, Hobom G, Planz O, Rapp UR, et al. Influenza virus propagation is impaired by inhibition of the Raf/MEK/ERK signalling cascade. *Nat Cell Biol* 2001;3(3):301.
 93. Ludwig S, Wolff T, Ehrhardt C, Wurzer WJ, Reinhardt J, Planz O, et al. MEK inhibition impairs influenza B virus propagation without emergence of resistant variants. *FEBS Lett* 2004;561(1–3):37–43.
 94. Droebner K, Pleschka S, Ludwig S, Planz O. Antiviral activity of the MEK-inhibitor U0126 against pandemic H1N1v and highly pathogenic avian influenza virus in vitro and in vivo. *Antiviral Res* 2011;92(2):195–203.

95. Haasbach E, Hartmayer C, Planz O. Combination of MEK inhibitors and oseltamivir leads to synergistic antiviral effects after influenza A virus infection in vitro. *Antiviral Res* 2013;98(2):319–24.
96. Wurzer WJ, Ehrhardt C, Pleschka S, Berberich-Siebelt F, Wolff T, Walczak H, et al. NF- κ B-dependent induction of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas/FasL is crucial for efficient influenza virus propagation. *J Biol Chem* 2004;279(30):30931–7.
97. Nimmerjahn F, Dudziak D, Dirmeier U, Hobom G, Riedel A, Schlee M, et al. Active NF- κ B signalling is a prerequisite for influenza virus infection. *J Gen Virol* 2004;85(8):2347–56.
98. Mazur I, Wurzer WJ, Ehrhardt C, Pleschka S, Puthavathana P, Silberzahn T, et al. Acetylsalicylic acid (ASA) blocks influenza virus propagation via its NF- κ B-inhibiting activity. *Cell Microbiol* 2007;9(7):1683–94.
99. Dodson AW, Taylor TJ, Knipe DM, Coen DM. Inhibitors of the sodium potassium ATPase that impair herpes simplex virus replication identified via a chemical screening approach. *Virology* 2007;366(2):340–8.
100. Kapoor A, Cai H, Forman M, He R, Shamay M, Arav-Boger R. Human Cytomegalovirus Inhibition by Cardiac Glycosides—Evidence for Involvement of human Ether-a-go-go (hERG) Gene. *Antimicrob Agents Chemother* 2012;AAC-00898.
101. Burkard C, Verheije MH, Haagmans BL, van Kuppeveld FJ, Rottier PJM, Bosch B-J, et al. ATP1A1-mediated Src signaling inhibits coronavirus entry into host cells. *J Virol* 2015;JVI-03274.
102. Thete D, Danthi P. Conformational changes required for reovirus cell entry are sensitive to pH. *Virology* 2015;483:291–301.
103. Deng L, Dai P, Ciro A, Smee DF, Djaballah H, Shuman S. Identification of novel antipoxviral agents: mitoxantrone inhibits vaccinia virus replication by blocking virion assembly. *J Virol* 2007;81(24):13392–402.
104. Hoffmann H-H, Palese P, Shaw ML. Modulation of influenza virus replication by alteration of sodium ion transport and protein kinase C activity. *Antiviral Res* 2008;80(2):124–34.
105. Katzen J, Hurtado J, Lecuona E, Sznajder JI. B32 VIRAL INFECTION OF THE AIRWAY: The Cardioactive Glycoside Ouabain Inhibits Influenza A Viral Replication. *Am J Respir Crit Care Med* 2014;189:1.
106. Amarelle L, Lecuona E, Sznajder JI. Anti-influenza treatment: drugs currently used and

- under development. *Arch Bronconeumol* (English Ed 2017;53(1):19–26.
107. Hankins DG, Rosekrans JA. Overview, prevention, and treatment of rabies. In: *Mayo Clinic Proceedings*. Elsevier; 2004. page 671–6.
 108. Wilde H, Choomkasien P, Hemachudha T, Supich C, Chutivongse S. Failure of rabies postexposure treatment in Thailand. *Vaccine* 1989;7(1):49–52.
 109. Tordo N, Kouknetzoff A. The rabies virus genome: an overview. *Onderstepoort J Vet Res* [Internet] 1993;60(4):263–9. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=7777310
 110. Rabies virus: Properties, Mode of transmission, Pathogenesis, Types, Prevention, Vaccination, Lab diagnosis [Internet]. [cited 2017 Aug 3];Available from: <http://www.healthdiseaseblog.com/2013/02/rabies-virus-properties-disease-transmission-pathogenesis-neurotrophicvirus-types-of-rabies-virus-street-virus-fixed-virus-vaccine-lab-diagnosis.html>
 111. Weblet Importer [Internet]. [cited 2017 Aug 9];Available from: http://viralzone.expasy.org/2?outline=all_by_species
 112. Schneider MC, Romijn PC, Uieda W, Tamayo H, Silva DF da, Belotto A, et al. Rabies transmitted by vampire bats to humans: an emerging zoonotic disease in Latin America? *Rev Panam Salud Pública* 2009;25(3):260–9.
 113. Kruse H, Kirkemo A-M, Handeland K. Wildlife as source of zoonotic infections. *Emerg Infect Dis* 2004;10(12):2067.
 114. Srinivasan A, Burton EC, Kuehnert MJ, Rupprecht C, Sutker WL, Ksiazek TG, et al. Transmission of rabies virus from an organ donor to four transplant recipients. *N Engl J Med* 2005;352(11):1103–11.
 115. Consales CA, Bolzan VL. Rabies review: immunopathology, clinical aspects and treatment. *J Venom Anim Toxins Incl Trop Dis* 2007;13(1):5–38.
 116. Torcio N. Characteristics and molecular biology of the rabies virus. 1996;
 117. Finke S, Conzelmann K-K. Replication strategies of rabies virus. *Virus Res* 2005;111(2):120–31.
 118. Wunner WH, Larson JK, Dietzschold B, Smith CL. The molecular biology of rabies viruses. *Rev Infect Dis* 1988;10(Supplement_4):S771–84.
 119. Albertini AA V, Schoehn G, Weissenhorn W, Ruigrok RWH. Structural aspects of rabies virus replication. *Cell Mol Life Sci* 2008;65(2):282–94.
 120. Lentz TL, Burrage TG, Smith AL, Crick J, Tignor GH. Is the acetylcholine receptor a

- rabies virus receptor? *Science* (80-) 1982;215(4529):182–4.
121. Wang ZW, Sarmiento L, Wang Y, Li X, Dhingra V, Tsegai T, et al. Attenuated rabies virus activates, while pathogenic rabies virus evades, the host innate immune responses in the central nervous system. *J Virol* 2005;79(19):12554–65.
 122. Etessami R, Conzelmann K-K, Fadai-Ghotbi B, Natelson B, Tsiang H, Ceccaldi P-E. Spread and pathogenic characteristics of a G-deficient rabies virus recombinant: an in vitro and in vivo study. *J Gen Virol* 2000;81(9):2147–53.
 123. Hudson LC, Weinstock D, Jordan T, Bold-Fletcher NO. Clinical presentation of experimentally induced rabies in horses. *Zoonoses Public Health* 1996;43(1-10):277–85.
 124. Jackson AC, Warrell MJ, Rupprecht CE, Ertl HCJ, Dietzschold B, O'reilly M, et al. Management of rabies in humans. *Clin Infect Dis* 2003;36(1):60–3.
 125. Willoughby Jr RE, Tieves KS, Hoffman GM, Ghanayem NS, Amlie-Lefond CM, Schwabe MJ, et al. Survival after treatment of rabies with induction of coma. *N Engl J Med* 2005;352(24):2508–14.
 126. Hemachudha T, Laothamatas J, Rupprecht CE. Human rabies: a disease of complex neuropathogenetic mechanisms and diagnostic challenges. *Lancet Neurol* 2002;1(2):101–9.
 127. Organization WH. WHO expert consultation on rabies: second report. World Health Organization; 2013.
 128. Access online: OIE - World Organisation for Animal Health.
 129. Bronnert J, Wilde H, Tepsumethanon V, Lumlertdacha B, Hemachudha T. Organ transplantations and rabies transmission. *J Travel Med* 2007;14(3):177–80.
 130. Communicable Diseases Rabies - General Aspects & Laboratory Diagnostic Techniques | Rabies | Immune System.
 131. Young CC, Sellers TF. Laboratory: a new method for staining Negri bodies of rabies. *Am J Public Health* 1927;17(10):1080–1.
 132. CDC - Diagnosis: Histologic Examination - Rabies.
 133. Goldwasser RA, Kissling RE. Fluorescent antibody staining of street and fixed rabies virus antigens. *Proc Soc Exp Biol Med* 1958;98(2):219–23.
 134. Mathuranayagam D, Rao PV. Antemortem diagnosis of human rabies by corneal impression smears using immunofluorescent technique. *Indian J Med Res* 1984;79:463–7.
 135. Meslin F-X, Kaplan MM, Koprowski H, Organization WH. Laboratory techniques in

- rabies. Geneva: World Health Organization; 1996.
136. Mani RS, Madhusudana SN. Laboratory diagnosis of human rabies: recent advances. *Sci World J* 2013;2013.
 137. Protocol for Postmortem Diagnosis of Rabies in Animals by Direct Fluorescent Antibody Testing.
 138. Perring P, Rolling PE, Sureau P. A rapid rabies enzyme immuno-diagnosis (RREID): a useful and simple technique for the routine diagnosis of rabies. *J Biol Stand* 1986;14(3):217–22.
 139. Miranda NL, Robles CG. A comparative evaluation of a new immunoenzymatic test (RREID) with currently used diagnostic tests (DME and FAT) for dog rabies. *Southeast Asian J Trop Med Public Health* 1991;22(1):46–50.
 140. Madhusudana SN, Subha S, Thankappan U, Ashwin YB. Evaluation of a direct rapid immunohistochemical test (dRIT) for rapid diagnosis of rabies in animals and humans. *Virol Sin* 2012;27(5):299–302.
 141. Madhusudana SN, Mani R, Ashwin YB, Desai A. Rabid fox bites and human rabies in a village community in southern India: epidemiological and laboratory investigations, management and follow-up. *Vector-Borne Zoonotic Dis* 2013;13(5):324–9.
 142. Abraham SS, Antony MU, Viswanathan H. Evaluation of a direct rapid immunohistochemical test (dRIT) as a diagnostic tool for rabies in animals. *Indian J Vet Pathol* 2013;37(1):68–9.
 143. Lembo T, Niezgoda M, Velasco-Villa A, Cleaveland S, Ernest E, Rupprecht CE. Evaluation of a direct, rapid immunohistochemical test for rabies diagnosis. *Emerg Infect Dis* 2006;12(2):310.
 144. Madhusudana SN, Sundaramoorthy S, Ullas PT. Utility of human embryonic kidney cell line HEK-293 for rapid isolation of fixed and street rabies viruses: comparison with Neuro-2a and BHK-21 cell lines. *Int J Infect Dis* 2010;14(12):e1067–71.
 145. Mehta S, Charan P, Dahake R, Mukherjee S, Chowdhary A. Molecular characterization of nucleoprotein gene of rabies virus from Maharashtra, India. *J Postgrad Med* 2016;62(2).
 146. Nagaraj T, Vasanth JP, Desai A, Kamat A, Madhusudana SN, Ravi V. Ante mortem diagnosis of human rabies using saliva samples: comparison of real time and conventional RT-PCR techniques. *J Clin Virol* 2006;36(1):17–23.
 147. Hughes GJ, Smith JS, Hanlon CA, Rupprecht CE. Evaluation of a TaqMan PCR assay to detect rabies virus RNA: influence of sequence variation and application to

- quantification of viral loads. *J Clin Microbiol* 2004;42(1):299–306.
148. (WHO) WHO. WHO guide for rabies pre and post-exposure prophylaxis in human (updated 2013). Geneva WHO Available online http://www.who.int/rabies/WHO_Guide_Rabies_Pre_Post_Exposure_Prophylaxis_Humans_2013.pdf (accessed Febr 2014)
 149. (No Title). 2014;
 150. National Rabies Control Programme National Guidelines on Rabies Prophylaxis RABIES IS 100 PERCENT FATAL BUT PREVENTABLE.
 151. Organization WH. Rabies vaccines: WHO position paper–recommendations. *Vaccine* 28: 7140–7142. 2010;
 152. De Benedictis P, Minola A, Nodari ER, Aiello R, Zecchin B, Salomoni A, et al. Development of broad-spectrum human monoclonal antibodies for rabies post-exposure prophylaxis. *EMBO Mol Med* 2016;8(4):407–21.
 153. Enssle K, Kurrle R, Köhler R, Müller H, Kanzy EJ, Hilfenhaus J, et al. A rabies-specific human monoclonal antibody that protects mice against lethal rabies. *Hybridoma* 1991;10(5):547–56.
 154. Bakker ABH, Marissen WE, Kramer RA, Rice AB, Weldon WC, Niezgoda M, et al. Novel human monoclonal antibody combination effectively neutralizing natural rabies virus variants and individual in vitro escape mutants. *J Virol* 2005;79(14):9062–8.
 155. Dietzschold B, Gore M, Casali P, Ueki Y, Rupprecht CE, Notkins AL, et al. Biological characterization of human monoclonal antibodies to rabies virus. *J Virol* 1990;64(6):3087–90.
 156. Rabies monoclonal antibodies post exposure prophylaxis.
 157. Appolinario CM, Jackson AC. Antiviral therapy for human rabies. *Antivir Ther* 2015;20(1):1–10.
 158. Zeiler FA, Jackson AC. Critical appraisal of the Milwaukee protocol for rabies: this failed approach should be abandoned. *Can J Neurol Sci* 2016;43(1):44–51.
 159. Hemachudha T, Sunsaneewitayakul B, Desudchit T, Suankratay C, Sittipunt C, Wacharapluesadee S, et al. Failure of therapeutic coma and ketamine for therapy of human rabies. *J Neurovirol* 2006;12(5):407–9.
 160. Aramburo A, Willoughby RE, Bollen AW, Glaser CA, Hsieh CJ, Davis SL, et al. Failure of the Milwaukee protocol in a child with rabies. *Clin Infect Dis* 2011;53(6):572–4.
 161. Samir M, Pessler F. Small non-coding RNAs associated with viral infectious diseases of veterinary importance: potential clinical applications. *Front Vet Sci* 2016;3:22.

162. De Clercq E, Li G. Approved antiviral drugs over the past 50 years. *Clin Microbiol Rev* 2016;29(3):695–747.
163. Vanden Berghe DA, Vhetinck AJ, Van Hoof L. Plant products as potential antiviral agents. *Bull l’Institut Pasteur* 1986;
164. Gogineni V, Schinazi RF, Hamann MT. Role of marine natural products in the genesis of antiviral agents. *Chem Rev* 2015;115(18):9655–706.
165. Zhu XM, Song JX, Huang ZZ, Wu YM, Yu MJ. Antiviral activity of mangiferin against herpes simplex virus type 2 in vitro. *Zhongguo yao li xue bao= Acta Pharmacol Sin* 1993;14(5):452–4.
166. Guha S, Ghosal S, Chattopadhyay U. Antitumor, Immunomodulatory and Anti-HIV Effect of Mangiferin, a Naturally Occurring Glucosylxanthone. *Chemotherapy [Internet]* 1996;42(6):443–51. Available from: <http://www.karger.com/DOI/10.1159/000239478>
167. Gupta A. Flow cytometric evaluation of Res. *Pharm, L Sci* 2015;3(2):283–7.
168. Badam L, Bedekar SS, Sonawane KB, Joshi SP. In vitro antiviral activity of bael (*Aegle marmelos* Corr) upon human coxsackieviruses B1-B6. *J Commun Dis* 2002;34(2):88–99.
169. Lee-Huang S, Huang PL, Bourinbaiar AS, Chen HC, Kung HF. Inhibition of the integrase of human immunodeficiency virus (HIV) type 1 by anti-HIV plant proteins MAP30 and GAP31. *Proc Natl Acad Sci* 1995;92(19):8818–22.
170. Bourinbaiar AS, Lee-Huang S. The Activity of Plant-Derived Antiretroviral Proteins MAP30 and GAP31 against Herpes Simplex Virus Infection in Vitro. *Biochem Biophys Res Commun* 1996;219(3):923–9.
171. Di R, Tumer NE. Pokeweed antiviral protein: Its cytotoxicity mechanism and applications in plant disease resistance. *Toxins (Basel)* 2015;7(3):755–72.
172. Kishida K, Masuho Y, Hara T. Protein-synthesis inhibitory protein from seeds of *Luffa cylindrica* roem. *FEBS Lett* 1983;153(1):209–12.
173. Montanaro L, Sperti S, Stirpe F. Inhibition by ricin of protein synthesis in vitro. Ribosomes as the target of the toxin. *Biochem J* 1973;136(3):677–83.
174. Edelbaum O, Ilan N, Grafi G, Sher N, Stram Y, Novick D, et al. Two antiviral proteins from tobacco: purification and characterization by monoclonal antibodies to human beta-interferon. *Proc Natl Acad Sci* 1990;87(2):588–92.
175. Prasad V, Srivastava S, Verma HN. Two basic proteins isolated from *Clerodendrum inerme* Gaertn. are inducers of systemic antiviral resistance in susceptible plants. *Plant*

- Sci 1995;110(1):73–82.
176. Zhang N, Liu Z, Han Q, Chen J, Lv Y. Xanthohumol enhances antiviral effect of interferon α -2b against bovine viral diarrhea virus, a surrogate of hepatitis C virus. *Phytomedicine* 2010;17(5):310–6.
 177. Wu S, Sun A, Liu R. Separation and purification of baicalin and wogonoside from the Chinese medicinal plant *Scutellaria baicalensis* Georgi by high-speed counter-current chromatography. *J Chromatogr A* 2005;1066(1–2):243–7.
 178. Kapoor R, Sharma B, Kanwar SS. Antiviral phytochemicals: an overview. *Biochem Physiol* 2017;6(2):7.
 179. Álvarez ÁL, Habtemariam S, Moneim AEA, Melón S, Dalton KP, Parra F. A spiroketal-enol ether derivative from *Tanacetum vulgare* selectively inhibits HSV-1 and HSV-2 glycoprotein accumulation in Vero cells. *Antiviral Res* 2015;119:8–18.
 180. Geng C-A, Jiang Z-Y, Ma Y-B, Luo J, Zhang X-M, Wang H-L, et al. Swerilactones A and B, anti-HBV new lactones from a traditional Chinese herb: *Swertia mileensis* as a treatment for viral hepatitis. *Org Lett* 2009;11(18):4120–3.
 181. Nothias-Scaglia L-F, Retaillieu P, Paolini J, Pannecouque C, Neyts J, Dumontet V, et al. Jatrophone diterpenes as inhibitors of chikungunya virus replication: structure–activity relationship and discovery of a potent lead. *J Nat Prod* 2014;77(6):1505–12.
 182. Lin J-C. Mechanism of action of glycyrrhizic acid in inhibition of Epstein-Barr virus replication in vitro. *Antiviral Res* 2003;59(1):41–7.
 183. Choi HJ, Song JH, Park KS, Kwon DH. Inhibitory effects of quercetin 3-rhamnoside on influenza A virus replication. *Eur J Pharm Sci* 2009;37(3–4):329–33.
 184. Kuo Y-C, Lin L-C, Tsai W-J, Chou C-J, Kung S-H, Ho Y-H. Samarangenin B from *Limonium sinense* suppresses herpes simplex virus type 1 replication in Vero cells by regulation of viral macromolecular synthesis. *Antimicrob Agents Chemother* 2002;46(9):2854–64.
 185. Huang T-J, Tsai Y-C, Chiang S-Y, Wang G-J, Kuo Y-C, Chang Y-C, et al. Anti-viral effect of a compound isolated from *Liriope platyphylla* against hepatitis B virus in vitro. *Virus Res* 2014;192:16–24.
 186. Barquero AA, Alche LE, Coto CE. Block of vesicular stomatitis virus endocytic and exocytic pathways by 1-cinnamoyl-3, 11-dihydroxymeliacarpin, a tetranortriterpenoid of natural origin. *J Gen Virol* 2004;85(2):483–93.
 187. Zhang Y, But PP-H, Ooi VE-C, Xu H-X, Delaney GD, Lee SHS, et al. Chemical properties, mode of action, and in vivo anti-herpes activities of a lignin–carbohydrate

- complex from *Prunella vulgaris*. *Antiviral Res* 2007;75(3):242–9.
188. Cheng H-Y, Huang H-H, Yang C-M, Lin L-T, Lin C-C. The in vitro anti-herpes simplex virus type-1 and type-2 activity of Long Dan Xie Gan Tan, a prescription of traditional Chinese medicine. *Chemotherapy* 2008;54(2):77–83.
 189. Wahyuni TS, Widyawaruyanti A, Lusida MI, Fuad A, Fuchino H, Kawahara N, et al. Inhibition of hepatitis C virus replication by chalepin and pseudane IX isolated from *Ruta angustifolia* leaves. *Fitoterapia* 2014;99:276–83.
 190. Cui H, Xu B, Wu T, Xu J, Yuan Y, Gu Q. Potential antiviral lignans from the roots of *Saururus chinensis* with activity against Epstein–Barr virus lytic replication. *J Nat Prod* 2013;77(1):100–10.
 191. Masihi KN, Lange W. Immunomodulators and nonspecific host defence mechanisms against microbial infections. In: *International Symposium on Immunomodulators and Nonspecific Host Defence Mechanisms Against Microbial Infections* (1987: Robert Koch Institute). Pergamon Press; 1988.
 192. Mattia D De, Decandia P, Ferranle P, Pace D, Martire B, Ciccarelli M, et al. Effectiveness of thymostimulin and study of lymphocyte-dependent antibacterial activity in children with recurrent respiratory infections. *Immunopharmacol Immunotoxicol* 1993;15(4):447–59.
 193. Parnham MJ. Immunotherapy of infections. *Inflamm Res* 1995;44(6):227.
 194. Murray HW. Endogenous interleukin-12 regulates acquired resistance in experimental visceral leishmaniasis. *J Infect Dis* 1997;175(6):1477–9.
 195. De Simone C, Famularo G, Tzantzoglou S, Moretti S, Jirillo E. Inosine pranobex in the treatment of HIV infection: a review. *Int J Immunopharmacol* 1991;13:19–27.
 196. Babineau TJ, Marcello P, Swails W, Kenler A, Bistrian B, Forse RA. Randomized phase I/II trial of a macrophage-specific immunomodulator (PGG-glucan) in high-risk surgical patients. *Ann Surg* 1994;220(5):601.
 197. Maeda YY, Yonekawa H, Chihara G. Application of Lentinan as Cytokine Inducer and Host Defense Potentiator in Immunotherapy of. *Immunother Infect* 1994;15:261.
 198. Kumaratilake LM, Ferrante A, Rzepczyk C. The role of T lymphocytes in immunity to *Plasmodium falciparum*. Enhancement of neutrophil-mediated parasite killing by lymphotoxin and IFN-gamma: comparisons with tumor necrosis factor effects. *J Immunol* 1991;146(2):762–7.
 199. Li H, Jerrells TR, Spitalny GL, Walker DH. Gamma interferon as a crucial host defense against *Rickettsia conorii* in vivo. *Infect Immun* 1987;55(5):1252–5.

200. Liew FY, Parkinson C, Millott S, Severn A, Carrier M. Tumour necrosis factor (TNF alpha) in leishmaniasis. I. TNF alpha mediates host protection against cutaneous leishmaniasis. *Immunology* 1990;69(4):570.
201. Parant MA, Parant FJ, Le Contel C, Lefrancier P, Chedid L. MDP derivatives and resistance to bacterial infections in mice. *Adv Exp Med Biol* 1992;319:175.
202. Suzuki K, Lee WJ, Hashimoto T, Tanaka E, Murayama T, Amitani R, et al. Recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) or tumour necrosis factor-alpha (TNF- α) activate human alveolar macrophages to inhibit growth of *Mycobacterium avium* complex. *Clin Exp Immunol* 1994;98(1):169–73.
203. Mooney DP, Gamelli RL, O'Reilly M, Hebert JC. Recombinant human granulocyte colony-stimulating factor and *Pseudomonas* burn wound sepsis. *Arch Surg* 1988;123(11):1353–7.
204. Matsumoto M, Matsubara S, Matsuno T, Tamura M, Hattori K, Nomura H, et al. Protective effect of human granulocyte colony-stimulating factor on microbial infection in neutropenic mice. *Infect Immun* 1987;55(11):2715–20.
205. Lee MT, Warren MK. CSF-1-induced resistance to viral infection in murine macrophages. *J Immunol* 1987;138(9):3019–22.
206. Chehimi J, Marshall JD, Salvucci O, Frank I, Chehimi S, Kawecky S, et al. IL-15 enhances immune functions during HIV infection. *J Immunol* 1997;158(12):5978–87.
207. Cavanaugh VJ, Guidotti LG, Chisari F V. Interleukin-12 inhibits hepatitis B virus replication in transgenic mice. *J Virol* 1997;71(4):3236–43.
208. Rakholiya K, Chanda S. Pharmacognostic, physicochemical and phytochemical investigation of *Mangifera indica* L. var. Kesar leaf. *Asian Pac J Trop Biomed* 2012;2(2):S680–4.
209. Elzaawely AA, Tawata S. Preliminary phytochemical investigation on mango (*Mangifera indica* L.) leaves. *World J Agric Sci* 2010;6(6):735–9.
210. Negi P, John SK, Rao PU. Antimicrobial activity of mango sap. *Eur Food Res Technol* 2002;214(4):327–30.
211. Núñez-Sellés AJ. Antioxidant therapy: myth or reality? *J Braz Chem Soc* 2005;16(4):699–710.
212. Baliga MS, Bhat HP, Joseph N, Fazal F. Phytochemistry and medicinal uses of the bael fruit (*Aegle marmelos* Correa): A concise review. *Food Res Int* 2011;44(7):1768–75.
213. Mujeeb F, Bajpai P, Pathak N. Phytochemical evaluation, antimicrobial activity, and determination of bioactive components from leaves of *Aegle marmelos*. *Biomed Res Int*

- 2014;2014.
214. Ruhil S, Balhara M, Dhankhar S, Chhillar AK. *Aegle marmelos* (Linn.) Correa: A potential source of Phytomedicine. *J Med Plants Res* 2011;5(9):1497–507.
 215. Maity P, Hansda D, Bandyopadhyay U, Mishra DK. Biological activities of crude extracts and chemical constituents of Bael, *Aegle marmelos* (L.) Corr. 2009;
 216. Sharma P V. *Charaka-Samhita*, Vol I und II, Chaukambha orientalia, Delhi, Fourth. ISBN 81-7637-011-8;
 217. Kritikar KR, Basu BD. *Indian Medicinal Plants I-IV Vols.* Int B Distrib Books Publ Dehra Dun 1999;
 218. Karawya MS, Mirhom YW, Shehata IA. Sterols triterpenes, coumarins and alkaloids of *Aegle marmelos* correa, cultivated in Egypt. *Egypt J Pharm Sci* 1980;
 219. Rastogi RP, Mehrotra BN. *Compendium of Indian Medicinal Plants*, vol. I. CSIR. New Delhi 1990;
 220. Gopalkrishnan B, Rampally S. Pharmacognostical evaluation of castor leaves. *World J Pharm Pharm Sci* 2015;4:1260–6.
 221. Devanand P, Rani PU. Biological potency of certain plant extracts in management of two lepidopteran pests of *Ricinus communis* L. *J Biopestic* 2008;1(2):170–6.
 222. Visen PKS, Shukla B, Patnaik GK, Tripathi SC, Kulshreshtha DK, Srimal RC, et al. Hepatoprotective activity of *Ricinus communis* leaves. *Int J Pharmacogn* 1992;30(4):241–50.
 223. Bigi MF, Torkomian VL V, De Groote STCS, Hebling MJA, Bueno OC, Pagnocca FC, et al. Activity of *Ricinus communis* (Euphorbiaceae) and ricinine against the leaf-cutting ant *Atta sexdens rubropilosa* (Hymenoptera: Formicidae) and the symbiotic fungus *Leucoagaricus gongylophorus*. *Pest Manag Sci* 2004;60(9):933–8.
 224. Ilavarasan R, Mallika M, Venkataraman S. Anti-inflammatory and free radical scavenging activity of *Ricinus communis* root extract. *J Ethnopharmacol* 2006;103(3):478–80.
 225. Singh PP, Chauhan SMS. Activity guided isolation of antioxidants from the leaves of *Ricinus communis* L. *Food Chem* 2009;114(3):1069–72.
 226. Lin J-Y, Liu S-Y. Studies on the antitumor lectins isolated from the seeds of *Ricinus communis* (castor bean). *Toxicon* 1986;24(8):757–65.
 227. Beloti MM, de Oliveira PT, Tagliani MM, Rosa AL. Bone cell responses to the composite of *Ricinus communis* polyurethane and alkaline phosphatase. *J Biomed Mater Res Part A* 2008;84(2):435–41.

228. Taur DJ, Waghmare MG, Bandal RS, Patil RY. Antinociceptive activity of *Ricinus communis* L. leaves. *Asian Pac J Trop Biomed* 2011;1(2):139–41.
229. Bhakta S, Das SK. In praise of the medicinal plant *Ricinus communis* L.: A review. *Glob J Res Med Plants Indig Med* 2015;4(5):95.
230. Denison MS, Nagy SR. Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. *Annu Rev Pharmacol Toxicol* 2003;43(1):309–34.
231. Carlstedt-Duke JMB. Tissue distribution of the receptor for 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin in the rat. *Cancer Res* 1979;39(8):3172–6.
232. Johansson G, Gillner M, Högberg B, Gustafsson J. The TCDD receptor in rat intestinal mucosa and its possible dietary ligands. 1981;
233. Agostinis P, Garmyn M, Van Laethem A. The Aryl hydrocarbon receptor: an illuminating effector of the UVB response. *Sci Signal* 2007;2007(403):pe49–pe49.
234. Enan E, Matsumura F. Identification of c-Src as the integral component of the cytosolic Ah receptor complex, transducing the signal of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) through the protein phosphorylation pathway. *Biochem Pharmacol* 1996;52(10):1599–612.
235. Fritsche E, Schäfer C, Calles C, Bernsmann T, Bernshausen T, Wurm M, et al. Lightening up the UV response by identification of the arylhydrocarbon receptor as a cytoplasmatic target for ultraviolet B radiation. *Proc Natl Acad Sci* 2007;104(21):8851–6.
236. Furue M, Takahara M, Nakahara T, Uchi H. Role of AhR/ARNT system in skin homeostasis. *Arch Dermatol Res* 2014;306(9):769–79.
237. Schnekenburger M, Peng L, Puga A. HDAC1 bound to the Cyp1a1 promoter blocks histone acetylation associated with Ah receptor-mediated trans-activation. *Biochim Biophys Acta (BBA)-Gene Struct Expr* 2007;1769(9):569–78.
238. Pollenz RS. The mechanism of AH receptor protein down-regulation (degradation) and its impact on AH receptor-mediated gene regulation. *Chem Biol Interact* 2002;141(1):41–61.
239. Mimura J, Ema M, Sogawa K, Fujii-Kuriyama Y. Identification of a novel mechanism of regulation of Ah (dioxin) receptor function. *Genes Dev* 1999;13(1):20–5.
240. Vogel CFA, Khan EM, Leung PSC, Gershwin ME, Chang WLW, Wu D, et al. Cross-talk between Aryl hydrocarbon receptor and the inflammatory response a role for nuclear factor- κ B. *J Biol Chem* 2014;289(3):1866–75.

241. Tian Y. Ah receptor and NF- κ B interplay on the stage of epigenome. *Biochem Pharmacol* 2009;77(4):670–80.
242. Puga A, Ma C, Marlowe JL. The aryl hydrocarbon receptor cross-talks with multiple signal transduction pathways. *Biochem Pharmacol* 2009;77(4):713–22.
243. Murphy KA, Quadro L, White LA. The Intersection Between the Aryl Hydrocarbon Receptor (AhR)-and Retinoic Acid-Signaling Pathways. *Vitam Horm* 2007;75:33–67.
244. Matthews J, Gustafsson J-Å. Estrogen receptor and aryl hydrocarbon receptor signaling pathways. *Nucl Recept Signal* 2006;4.
245. Rannug U, Rannug A, Sjöberg U, Li H, Westerholm R, Bergman J. Structure elucidation of two tryptophan-derived, high affinity Ah receptor ligands. *Chem Biol* 1995;2(12):841–5.
246. Wang C, Ye Z, Kijlstra A, Zhou Y, Yang P. Activation of the aryl hydrocarbon receptor affects activation and function of human monocyte-derived dendritic cells. *Clin Exp Immunol* 2014;177(2):521–30.
247. Lowe MM, Mold JE, Kanwar B, Huang Y, Louie A, Pollastri MP, et al. Identification of cinnabarinic acid as a novel endogenous aryl hydrocarbon receptor ligand that drives IL-22 production. *PLoS One* 2014;9(2):e87877.
248. DiNatale BC, Murray IA, Schroeder JC, Flaveny CA, Lahoti TS, Laurenzana EM, et al. Kynurenic acid is a potent endogenous aryl hydrocarbon receptor ligand that synergistically induces interleukin-6 in the presence of inflammatory signaling. *Toxicol Sci* 2010;115(1):89–97.
249. Mezrich JD, Fechner JH, Zhang X, Johnson BP, Burlingham WJ, Bradfield CA. An interaction between kynurenine and the aryl hydrocarbon receptor can generate regulatory T cells. *J Immunol* 2010;185(6):3190–8.
250. Mándi Y, Vécsei L. The kynurenine system and immunoregulation. *J Neural Transm* 2012;119(2):197–209.
251. Widner B, Werner ER, Schennach H, Wachter H, Fuchs D. Simultaneous measurement of serum tryptophan and kynurenine by HPLC. *Clin Chem* 1997;43(12):2424–6.
252. Heath-Pagliuso S, Rogers WJ, Tullis K, Seidel SD, Cenijn PH, Brouwer A, et al. Activation of the Ah receptor by tryptophan and tryptophan metabolites. *Biochemistry* 1998;37(33):11508–15.
253. Bankoti J, Rase B, Simones T, Shepherd DM. Functional and phenotypic effects of AhR activation in inflammatory dendritic cells. *Toxicol Appl Pharmacol* 2010;246(1):18–28.
254. Quintana FJ. LeA (H) Rning self-control. *Cell Res* 2014;24(10):1155.

255. Noakes R, Spelman L, Williamson R. Is the L-tryptophan metabolite quinolinic acid responsible for eosinophilic fasciitis? *Clin Exp Med* 2006;6(2):60–4.
256. Van Voorhis M, Fechner JH, Zhang X, Mezrich JD. The aryl hydrocarbon receptor: a novel target for immunomodulation in organ transplantation. *Transplantation* 2013;95(8):983.
257. Quintana FJ, Basso AS, Iglesias AH, Korn T, Farez MF, Bettelli E, et al. Control of Treg and TH17 cell differentiation by the aryl hydrocarbon receptor. *Nature* 2008;453(7191):65–71.
258. Duarte JH, Di Meglio P, Hirota K, Ahlfors H, Stockinger B. Differential influences of the aryl hydrocarbon receptor on Th17 mediated responses in vitro and in vivo. *PLoS One* 2013;8(11):e79819.
259. Jux B, Kadow S, Esser C. Langerhans cell maturation and contact hypersensitivity are impaired in aryl hydrocarbon receptor-null mice. *J Immunol* 2009;182(11):6709–17.
260. Nguyen NT, Kimura A, Nakahama T, Chinen I, Masuda K, Nohara K, et al. Aryl hydrocarbon receptor negatively regulates dendritic cell immunogenicity via a kynurenine-dependent mechanism. *Proc Natl Acad Sci* 2010;107(46):19961–6.
261. Noakes RR. effects of Tranilast on the Urinary excretion of Kynurenic and Quinolinic Acid Under conditions of L Tryptophan Loading. *Int J tryptophan Res IJTR* 2013;6:67.
262. Hu W, Zhao J, Pei G. Activation of aryl hydrocarbon receptor (ahr) by tranilast, an anti-allergy drug, promotes miR-302 expression and cell reprogramming. *J Biol Chem* 2013;288(32):22972–84.
263. O'Donnell EF, Saili KS, Koch DC, Kopparapu PR, Farrer D, Bisson WH, et al. The anti-inflammatory drug leflunomide is an agonist of the aryl hydrocarbon receptor. *PLoS One* 2010;5(10):e13128.
264. Bittinger MA, Nguyen LP, Bradfield CA. Aspartate aminotransferase generates proagonists of the aryl hydrocarbon receptor. *Mol Pharmacol* 2003;64(3):550–6.
265. Creed D. The photophysics and photochemistry of the near-uv absorbing amino acids– i. Tryptophan and its simple derivatives. *Photochem Photobiol* 1984;39(4):537–62.
266. Diani-Moore S, Labitzke E, Brown R, Garvin A, Wong L, Rifkind AB. Sunlight generates multiple tryptophan photoproducts eliciting high efficacy CYP1A induction in chick hepatocytes and in vivo. *Toxicol Sci* 2005;90(1):96–110.
267. P-PER ® Plant Protein Extraction Kit. [cited 2017 Aug 3];Available from: https://tools.thermofisher.com/content/sfs/manuals/MAN0011557_PPER_Plant_Protein_Extract_UG.pdf

268. Karthika K, Jamuna S, Paulsamy S. TLC and HPTLC Fingerprint Profiles of Different Bioactive Components from the Tuber of *Solena amplexicaulis*. *J Pharmacogn Phytochem* 2014;3(1).
269. David T, George K V. HPTLC Analysis of the Leaf Extract of *Hydnocarpus macrocarpa* (Beddome) Warb. *J Pharmacogn Phytochem* 2014;3(1).
270. ATCC ® ANIMAL CELL CULTURE GUIDE tips and techniques for continuous cell lines. [cited 2017 Aug 3];Available from: https://www.atcc.org/~media/PDFs/Culture Guides/AnimCellCulture_Guide.ashx
271. Ferrari M, Fornasiero MC, Isetta AM. MTT colorimetric assay for testing macrophage cytotoxic activity in vitro. *J Immunol Methods* 1990;131(2):165–72.
272. Mehta S, Roy S, Chowdhary A. Use of rapid fluorescent focus inhibition test (RFFIT) for in vitro evaluation of anti-rabies activity. *VirusDisease* :1–6.
273. QIAamp Viral RNA Mini Handbook - QIAGEN [Internet]. [cited 2017 Aug 7];Available from: <https://www.qiagen.com/us/resources/resourcedetail?id=c80685c0-4103-49ea-aa72-8989420e3018&lang=en>
274. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2– $\Delta\Delta$ CT method. *methods* 2001;25(4):402–8.
275. Patil D, Roy S, Dahake R, Rajopadhye S, Kothari S, Deshmukh R, et al. Evaluation of *Jatropha curcas* Linn. leaf extracts for its cytotoxicity and potential to inhibit hemagglutinin protein of influenza virus. *Indian J Virol* 2013;24(2):220–6.
276. Sun J, Zhang X, Broderick M, Fein H. Measurement of nitric oxide production in biological systems by using Griess reaction assay. *Sensors* 2003;3(8):276–84.
277. Brown JH, Mackey HK, Riggilo DA. A novel in vitro assay for anti-inflammatory agents based on stabilization of erythrocytes. *Proc Soc Exp Biol Med* 1967;125(3):837–43.
278. Oyedapo OO, Akinpelu BA, Akinwunmi KF, Adeyinka MO, Sipeolu FO. Red blood cell membrane stabilizing potentials of extracts of *Lantana camara* and its fractions. *Int J Plant Physiol Biochem* 2010;2(4):46–51.
279. Kalshetti PB, Alluri R, Mohan V, Thakurdesai PA. Effects of 4-hydroxyisoleucine from Fenugreek Seeds on depression-like behavior in socially isolated olfactory bulbectomized rats. *Pharmacogn Mag* 2015;11(Suppl 3):S388.
280. Gaitonde MK, Dovey T. A rapid and direct method for the quantitative determination of tryptophan in the intact protein. *Biochem J* 1970;117(5):907–11.
281. Csizmadia P. MarvinSketch and MarvinView: molecule applets for the World Wide Web. In: *Proceedings of ECSOC-3, The Third International Electronic Conference on*

- Synthetic Organic Chemistry, September 1st 1999. page 367-369.
282. Vina A. Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading Trott, Oleg; Olson, Arthur J. *J Comput Chem* 2010;31(2):455–61.
 283. Atanasov AG, Waltenberger B, Pferschy-Wenzig E-M, Linder T, Wawrosch C, Uhrin P, et al. Discovery and resupply of pharmacologically active plant-derived natural products: A review. *Biotechnol Adv* 2015;33(8):1582–614.
 284. Wang Y, Lobigs M, Lee E, Müllbacher A. CD8⁺ T cells mediate recovery and immunopathology in West Nile virus encephalitis. *J Virol* 2003;77(24):13323–34.
 285. Green S, Rothman A. Immunopathological mechanisms in dengue and dengue hemorrhagic fever. *Curr Opin Infect Dis* 2006;19(5):429–36.
 286. Ng LFP. Immunopathology of Chikungunya Virus Infection: Lessons Learned from Patients and Animal Models. *Annu Rev Virol* 2017;(0).
 287. Rege NN, Thatte UM, Dahanukar SA. Adaptogenic properties of six rasayana herbs used in Ayurvedic medicine. *Phyther Res* 1999;13(4):275–91.
 288. Raj GRA, Shailaja U, Rao PN, Ajayan S. Review on the concept of Immunomodulation in Ayurveda with special emphasis on Prakara yoga. *Int J Pharm Sci Res* 2014;5(4):1116.
 289. Katiyar CK, Brindavanam NB, Tiwari P, Narayana DBA. Immunomodulator products from Ayurveda: current status and future perspectives. *Immunomodulation*, Narosa Publ House, New Delhi 1997;163–87.
 290. Nguyen KB, Watford WT, Salomon R, Hofmann SR, Pien GC, Morinobu A, et al. Critical role for STAT4 activation by type 1 interferons in the interferon- γ response to viral infection. *Science* (80-) 2002;297(5589):2063–6.
 291. Schultz U, Chisari F V. Recombinant duck interferon gamma inhibits duck hepatitis B virus replication in primary hepatocytes. *J Virol* 1999;73(4):3162–8.
 292. Yuk S-S, Lee D-H, Park J-K, Tseren-Ochir E-O, Kwon J-H, Noh J-Y, et al. Pre-immune state induced by chicken interferon gamma inhibits the replication of H1N1 human and H9N2 avian influenza viruses in chicken embryo fibroblasts. *Virol J* 2016;13(1):71.
 293. Herrera RA, Oved JH, Reiss CS. Disruption of IFN- γ -Mediated Antiviral Activity in Neurons: The Role of Cannabinoids. *Viral Immunol* 2008;21(2):141–52.
 294. Sainz B, Halford WP. Alpha/Beta interferon and gamma interferon synergize to inhibit the replication of herpes simplex virus type 1. *J Virol* 2002;76(22):11541–50.
 295. Liu T, Khanna KM, Carriere BN, Hendricks RL. Gamma interferon can prevent herpes

- simplex virus type 1 reactivation from latency in sensory neurons. *J Virol* 2001;75(22):11178–84.
296. Geiger KD, Nash TC, Sawyer S, Krah T, Patstone G, Reed JC, et al. Interferon- γ protects against herpes simplex virus type 1-mediated neuronal death. *Virology* 1997;238(2):189–97.
 297. Eichinger KM, Egaña L, Orend JG, Resetar E, Anderson KB, Patel R, et al. Alveolar macrophages support interferon gamma-mediated viral clearance in RSV-infected neonatal mice. *Respir Res* 2015;16(1):122.
 298. Beutler B, Cerami A. Tumor necrosis, cachexia, shock, and inflammation: a common mediator. *Annu Rev Biochem* 1988;57(1):505–18.
 299. Seo SH, Webster RG. Tumor necrosis factor alpha exerts powerful anti-influenza virus effects in lung epithelial cells. *J Virol* 2002;76(3):1071–6.
 300. Su B, Wang J, Wang X, Jin H, Zhao G, Ding Z, et al. The effects of IL-6 and TNF- α as molecular adjuvants on immune responses to FMDV and maturation of dendritic cells by DNA vaccination. *Vaccine* 2008;26(40):5111–22.
 301. Kayamuro H, Abe Y, Yoshioka Y, Katayama K, Nomura T, Yoshida T, et al. The use of a mutant TNF- α as a vaccine adjuvant for the induction of mucosal immune responses. *Biomaterials* 2009;30(29):5869–76.
 302. Beyer M, Abdullah Z, Chemnitz JM, Maisel D, Sander J, Lehmann C, et al. Tumor-necrosis factor impairs CD4⁺ T cell-mediated immunological control in chronic viral infection. *Nat Immunol* 2016;17(5):593–603.
 303. Lan T, Chang L, Wu L, Yuan Y-F. IL-6 plays a crucial role in HBV infection. *J Clin Transl Hepatol* 2015;3(4):271.
 304. Dienz O, Eaton SM, Bond JP, Neveu W, Moquin D, Noubade R, et al. The induction of antibody production by IL-6 is indirectly mediated by IL-21 produced by CD4⁺ T cells. *J Exp Med* 2009;206(1):69–78.
 305. Cox SJ, Gubbins S, Barnett P V. IL-6 production following vaccination in pigs—An additional immune response parameter for assessing FMD vaccine efficacy? *Vaccine* 2011;29(29):4704–8.
 306. Singh V, Jain S, Gowthaman U, Parihar P, Gupta P, Gupta UD, et al. Co-administration of IL-1+ IL-6+ TNF- α with Mycobacterium tuberculosis infected macrophages vaccine induces better protective T cell memory than BCG. *PLoS One* 2011;6(1):e16097.
 307. Leal IS, Flórido M, Andersen P, Appelberg R. Interleukin-6 regulates the phenotype of the immune response to a tuberculosis subunit vaccine. *Immunology* 2001;103(3):375–

- 81.
308. Kimura A, Kishimoto T. IL-6: regulator of Treg/Th17 balance. *Eur J Immunol* 2010;40(7):1830–5.
309. Tanaka T, Kishimoto T. Targeting interleukin-6: all the way to treat autoimmune and inflammatory diseases. *Int J Biol Sci* 2012;8(9):1227.
310. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol* 2002;23(11):549–55.
311. Mantovani A, Biswas SK, Galdiero MR, Sica A, Locati M. Macrophage plasticity and polarization in tissue repair and remodelling. *J Pathol* 2013;229(2):176–85.
312. Spencer L, Shultz L, Rajan T V. Interleukin-4 receptor–Stat6 signaling in murine infections with a tissue-dwelling nematode parasite. *Infect Immun* 2001;69(12):7743–52.
313. Tepper RI, Levinson DA, Stanger BZ, Campos-Torres J, Abbas AK, Leder P. IL-4 induces allergic-like inflammatory disease and alters T cell development in transgenic mice. *Cell* 1990;62(3):457–67.
314. Akaike T, Maeda H. Nitric oxide and virus infection. *Immunology* 2000;101(3):300–8.
315. Saura M, Zaragoza C, McMillan A, Quick RA, Hohenadl C, Lowenstein JM, et al. An antiviral mechanism of nitric oxide: inhibition of a viral protease. *Immunity* 1999;10(1):21–8.
316. Chaturvedi UC, Agarwal R, Elbishbishi EA, Mustafa AS. Cytokine cascade in dengue hemorrhagic fever: implications for pathogenesis. *Pathog Dis* 2000;28(3):183–8.
317. Saxena V, Mathur A, Krishnani N, Dhole TN. An insufficient anti-inflammatory cytokine response in mouse brain is associated with increased tissue pathology and viral load during Japanese encephalitis virus infection. *Arch Virol* 2008;153(2):283–92.
318. Hayes EB, Sejvar JJ, Zaki SR, Lanciotti RS, Bode A V, Campbell GL. Virology, pathology, and clinical manifestations of West Nile virus disease. *Emerg Infect Dis* 2005;11(8):1174.
319. Kuiken T, Taubenberger JK. Pathology of human influenza revisited. *Vaccine* 2008;26:D59–66.
320. Swarup V, Ghosh J, Ghosh S, Saxena A, Basu A. Antiviral and anti-inflammatory effects of rosmarinic acid in an experimental murine model of Japanese encephalitis. *Antimicrob Agents Chemother* 2007;51(9):3367–70.
321. de las Casas-Engel M, Corbí AL. Serotonin modulation of macrophage polarization:

- inflammation and beyond. In: *Oxidative Stress and Inflammation in Non-communicable Diseases-Molecular Mechanisms and Perspectives in Therapeutics*. Springer; 2014. page 89–115.
322. de las Casas-Engel M, Domínguez-Soto A, Sierra-Filardi E, Bragado R, Nieto C, Puig-Kroger A, et al. Serotonin skews human macrophage polarization through HTR2B and HTR7. *J Immunol* 2013;190(5):2301–10.
323. Bohár Z, Toldi J, Fülöp F, Vécsei L. Changing the face of kynurenines and neurotoxicity: therapeutic considerations. *Int J Mol Sci* 2015;16(5):9772–93.
324. Lovelace MD, Varney B, Sundaram G, Franco NF, Ng ML, Pai S, et al. Current evidence for a role of the kynurenine pathway of tryptophan metabolism in multiple sclerosis. *Front Immunol* 2016;7.

