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Antiviral Strategies in the Treatment of Human and Animal Viral Infections

Edited by Arli Aditya Parikesit

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Infectious Diseases

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Aims and Scope of the Series

This series will provide a comprehensive overview of recent research trends in various Infectious Diseases (as per the most recent Baltimore classification). Topics will include general overviews of infections, immunopathology, diagnosis, treatment, epidemiology, etiology, and current clinical recommendations for managing infectious diseases. Ongoing issues, recent advances, and future diagnostic approaches and therapeutic strategies will also be discussed. This book series will focus on various aspects and properties of infectious diseases whose deep understanding is essential for safeguarding the human race from losing resources and economies due to pathogens.

Meet the Series Editor

Dr. Rodriguez-Morales is an expert in tropical and emerging diseases, particularly zoonotic and vector-borne diseases (notably arboviral diseases), and more recently COVID-19 and Monkeypox. He is the president of the Publications and Research Committee of the Pan-American Infectious Diseases Association (API), as well as the president of the Colombian Association of Infectious Diseases (ACIN). He is a member of the Committee on Tropical Medicine, Zoonoses, and Travel Medicine of ACIN. Dr. Rodriguez-Morales is a vice-president of the Latin American Society for Travel Medicine (SLAMVI) and a member of the Council of the International Society for Infectious Diseases (ISID). Since 2014, he has been recognized as a senior researcher at the Ministry of Science of Colombia. He is a professor at the Faculty of Medicine of the Fundacion Universitaria Autonoma de las Americas, in Pereira, Risaralda, Colombia, and a professor, Master in Clinical Epidemiology and Biostatistics, at Universidad Científica del Sur, Lima, Peru. He is also a non-resident adjunct faculty member at the Gilbert and Rose-Marie Chagoury School of Medicine, Lebanese American University, Beirut, Lebanon, and an external professor, Master in Research on Tropical Medicine and International Health, at Universitat de Barcelona, Spain. Additionally, an invited professor, Master in Biomedicine, at Universidad Internacional SEK, Quito, Ecuador, and a visiting professor, Master Program of Epidemiology, at Diponegoro University, Indonesia. In 2021 he was awarded the "Raul Isturiz Award" Medal of the API and, the same year, the "Jose Felix Patiño" Asclepius Staff Medal of the Colombian Medical College due to his scientific contributions to the topic of COVID-19 during the pandemic. He is currently the Editor in Chief of the journal Travel Medicine and Infectious Diseases. His Scopus H index is 55 (Google Scholar H index 77) with a total of 725 publications indexed in Scopus.

Meet the Volume Editor

Dr. rer. nat. Arli Aditya is Vice Rector of Research and Innovation at Indonesia International Institute for Life Sciences (I3L). He obtained bachelor's and master's degrees in chemistry at the Faculty of Mathematics and Natural Sciences, University of Indonesia. In order to pursue a degree in bioinformatics, Dr. Parikesit accepted an offer from DAAD (German Academic Exchange Service) to conduct doctorate research at the Bioinformatics Group, Faculty of Informatics and Mathematics, University of Leipzig, Germany. His doctoral research is focused on the utilization of modern protein domain annotation techniques in the three domains of life. In addition, Dr. Parikesit is also an expert on immunoinformatics, bioinformatics algorithms, structural bioinformatics, silico drug design, and in silico transcriptomics. Currently, he is devising a pipeline to apply his expertise to COVID-19 drug and vaccine designs.

Contents

Preface

Infections caused by viruses pose a serious threat to both human and animal health, with significant morbidity and mortality on a global scale. To stop and manage viral outbreaks as well as to treat persistent viral infections, efficient antiviral therapies must be developed. However, the development and propagation of viral strains that are resistant to medication presents a significant obstacle for the current antiviral treatments. In addition, a multidisciplinary and all-encompassing approach is necessary to comprehend the biology, pathology, and host interactions of viral infections due to their diversity and complexity. This book examines numerous elements of viral infections in humans and animals, such as epidemiology, diagnosis, prevention, therapy, and immunology, in addition to the most recent developments and difficulties in the field of antiviral research. Additionally covered in the text are the molecular mechanisms underlying antiviral resistance, the creation of innovative vaccinations and antiviral drugs, as well as the possible use of nanotechnology and gene therapy. In addition to serving as a useful resource for students, educators, and health professionals interested in this quickly developing topic, the book intends to encourage additional study and collaboration among scientists and clinicians working on various aspects of viral infections. The first section of the book introduces the theme in general, while other sections focus on more specific topics. I would like to thank the Department of Research and Community Services of Indonesia International Institute for Life Sciences (RCS i3L) for their heartfelt support of this initiative.

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Section 1

Introduction

Chapter 1

Introductory Chapter: Current Landscape of Animal and Human Virus Treatments

Arli Aditya Parikesit

1. Introduction

As the world is currently watching the COVID-19 pandemic unravel to a controlled course, there are still many instances of viral infection outbreaks that occurred. Although there is a downward trajectory, the influenza virus is still a potential threat due to its history of causing pandemics [1]. Moreover, some other viral infections such as HIV/AIDS and Hepatitis are still causing sporadic outbreaks worldwide [2, 3]. However, the discussion about the current situation of human and animal viral outbreaks will not be complete without mentioning the COVID-19 pandemic. As a zoonosis infection, the SARS-CoV-2 virus could infect both humans and animals. Besides humans, it is known to infect cats, dogs, tigers, minks, and others [4–6]. Henceforth, zoonosis infection will continue to be a challenging research topic. In this respect, several developments in the field of biomedicine and medical biotechnology for dealing with COVID-19 pandemics should be mentioned, as they could be possibly catered to another type of viral infections as well.

2. Current status of COVID-19 drug and vaccine development

As soon as WHO declared the ongoing COVID-19 pandemic in March 2020, several repurposed drugs are prepared. One of them is remdesivir, that previously developed for treating hepatitis C, Ebola, and Marburg virus infections [7–11]. Although there are mixed results in the clinical settings, remdesivir is currently one of the standard options in COVID-19 treatments [12]. Moreover, more drugs have been rolled out for COVID-19, namely molnupiravir and paxlovid [13, 14]. The aforementioned drugs are working by targeting specific proteins of the SARS-COV-2 virus, such as Mpro and RdRp [15]. Hence, biologics such as antibody treatment are also developed with regeneron ® as one of the examples [16]. However, one of the game changers in the COVID-19 pandemic is always the vaccine development. The successful deployment of new mRNA vaccines, as well as the existing ones such as live attenuated and vectors, have successfully slowed down the infection rate of COVID-19 [17–19]. While highly promising, some therapeutic approaches are still in their infancy. The development of lead compounds for COVID-19 from natural products is currently still ongoing, mainly in both *in vitro* and *in vivo* stages [20]. Moreover, the same condition also occurred with siRNA-based therapeutics [21]. However, the application of natural products and siRNA-based therapeutic for COVID-19 faces several challenges, such

as low bioavailability, poor stability, off-target effects, and delivery barriers [22, 23]. Therefore, more research is needed to overcome these limitations and optimize the efficacy and safety of these potential agents. Furthermore, the development of combinatorial strategies that target multiple viral genes or pathways may enhance antiviral activity and reduce the risk of viral resistance [22, 24]. Thus, natural products and siRNA-based therapeutics could offer promising alternatives or adjuncts to conventional treatments for COVID-19 and other coronaviruses. In this regard, the current research progress for COVID-19 will eventually serve as a benchmarking trend that could be applied in other cases.

3. Structural bioinformatics as virology studies instrument

Structural bioinformatics is currently an integral part of the research pipeline in virology studies, as it has provided detailed information about virus' protein and nucleic acid structures [25–27]. As such, the annotated information is useful to design drugs and vaccines and gives a push to the development of rational drug design and immunoinformatics as possible solutions for combating viral infections [28–30]. Moreover, structural bioinformatics is currently under trial for examining phages molecular mechanism [31]. Some of the structural bioinformatics methods and tools for virology research include visualization and analysis of protein structures [32], comparative protein structure analysis [33], protein–protein modeling using cryo-EM restraints [34], biological assembly comparison, and integrating molecular simulation and experimental data. These methods and tools can help to identify and characterize viral proteins and their interactions with host factors, drugs, antibodies, and other viruses. They can also facilitate the discovery of novel targets, inhibitors, and vaccines for viral diseases. However, there are still many challenges and limitations in applying structural bioinformatics to virology, such as the scarcity of experimental data, the complexity of viral dynamics and evolution, and the uncertainty of molecular interactions and mechanisms [35–37]. Therefore, more efforts are needed to improve the accuracy, efficiency, and applicability of structural bioinformatics methods and tools for virology research.

4. Alternative viral infection diagnostics methods

In the early phase of the COVID-19 pandemic, WHO has elevated NAAT (Nucleic acid amplification test) method such as RT-PCR as a standard instrument for diagnosing the disease. WHO's stance has been enacted in several of their official documents that dictate the diagnostics protocol in the clinical setting [38–40]. Due to the limitation of RT-PCR deployment in the early phase of pandemic, WHO also provides guidelines for NAAT alternatives, such as antigen-detecting rapid diagnostic tests [41]. All of WHO's guidelines on COVID-19 diagnostics are totally in line with their already published guidelines on the establishment of virology laboratory [42]. However, the WHO's establishment of standard operating procedure (SOP) for virology diagnostics did not deter the development of alternatives method. One of them is the breath analysis by electronic nose (enose) that has been trialed in Indonesia [43, 44]. Similar technology also has been applied in the Netherlands [45]. Unlike standard NAAT and non-NAAT-based diagnostics, enose was developed to detect Volatile Organic Compounds (VOC) that are produced by human cells. It is deemed

as a unique and creative application of diagnostics and has huge potential [46]. The other alternative is the bioelectrochemistry-based method. It has been applied in animal viruses with nanoparticle deployment, such as with African Swine Fever Virus (ASFV) [47, 48]. Moreover, it is also trialed in detecting SARS-CoV-2 as well [49]. Despite the development of alternatives, WHO has not changed its stance pertaining to its virology SOPs. In the end, the status of RT-PCR as a standard method for COVID-19 diagnostics is still unchanged up to the publication of this chapter. This condition could serve as a standing ground for more research in this area, as WHO's SOP will serve as a benchmark for any developed method in virology.

5. Outlook: Bioinformatics as instrument to resolve biomedical problem

Staring in the post-COVID-19 lockdown period, there will be massive integration of Bioinformatics into biological sciences, especially in virology studies. Bioinformatics will serve as an indispensable instrument to resolve biomedical and medical biotechnology problems. Moreover, methods of structural bioinformatics such as molecular simulation is a crucial method to understand the biochemical properties of viruses ([50–52], p. 201). Thus, the COVID-19 pandemic has taught us that SARS-CoV-2 virus is indeed evolving. In order to comprehend viral evolution, a more sophisticated instrument is needed. The standard tool to do that is definitely the New Generation Sequencing (NGS) instrument. NGS is crucial in the discovery of new SARS-CoV-2 variants such as delta and omicron [53]. Moreover, portable NGS instrumentation such as Oxford Nanopore ® has made the integration of bioinformatics into wet laboratory pipelines more assured than ever. Its versatility and portability have enabled such tools to be used by any life scientist with minimal training time [54]. Sequence analysis, as an integral part of bioinformatics, also plays an important part in Loop-mediated Isothermal Amplification (LAMP) and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) diagnostics development [55, 56]. Both of these methods are also trialed for COVID-19 diagnostics as well [57, 58]. Lastly, phages and biologics will possibly play an important role in dealing with viral infection in general [16, 59–62]. An improved and updated bioinformatics pipeline should be devised to comprehend the potential role of phages and biologics in biomedical applications.

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Chapter 2

Antiviral Strategies in the Treatment of Viral Infections in Humans and Animals: Surface and Space Disinfection Strategies

Shouguo Wang, Bin Yan and Li Song

Abstract

The 2019 coronavirus (COVID-19) epidemic is rampant, making people's awareness of virus elimination and prevention gradually increase, and giving more attention to the cleanliness of the indoor air environment and the use of goods. According to the World Health Organization (WHO) estimates, the annual global influenza cases can reach 1 billion, including 3 million to 5 million severe cases, and the number of deaths from influenza-related respiratory diseases recorded is as high as 290,000 to 650,000. The virus is generally transmitted through respiratory droplet transmission, airborne transmission, and contact transmission. For most infectious diseases, disinfection, isolation, and personal protection remain the most effective means, especially in the prevention and control of influenza, intestinal infectious diseases, contact-transmitted diseases, bloodborne diseases, and sexually transmitted diseases, which are most effective in spring, such as in 2019 coronavirus, severe acute respiratory syndrome (SARS), Middle East respiratory syndrome (MERS), influenza, and other. In respiratory infectious diseases, early disinfection is an important means of prevention and control; viruses can also be transmitted by contact and through a variety of ways, which occur in indoor spaces and on the surfaces of indoor objects. As a result, many different methods of disinfection have been conducted for indoor spaces and surfaces of objects.

Keywords: viruses, surface sterilization, space sterilization, physical disinfection, chemical disinfection, comprehensive disinfection

1. Introduction

Disinfection is an effective measure to cut off the transmission of infectious diseases. Most countries specify the concentration of bacterial and fungal colonies, only Korea specifies the total number of colonies, the U.S. Food and Drug Administration (FDA) does not provide specific limits, and China requires that "the logarithm of the extinction of natural bacteria in the air is ≥1.00." The choice of disinfection method depends not only on the disinfection product itself and external controllable factors

such as temperature, concentration, exposure time, etc., but also on the subjective will of the user. Physical, chemical, and other disinfection methods can be used to eliminate bacteria and viruses present in space and on surfaces with objects, such as temperature, electrostatic adsorption, air laminar flow purification, filtration, ultraviolet (UV), photocatalytic, plasma, and other physical methods, chlorine dioxide, sodium hypochlorite, hydrogen peroxide, and peroxyacetic acid, and other common disinfection means such as ozone and herbal disinfection (**Table 1**) [1]. Different disinfection methods differ in their application forms and inactivation effects depending on the environment in which they are used. In addition, in terms of the development path of the disinfection technology itself, it can be divided into static and dynamic disinfection technologies based on whether it supports human-machine coexistence as a basic feature. Static disinfection technology is widely used in the final disinfection and preventive disinfection process, which means that disinfection is carried out in an unoccupied environment and personnel must leave the site before entering the disinfection state, often using ultraviolet (UV) lamp disinfection, ozone disinfection, hydrogen peroxide spray disinfection, and peroxyacetic acid fumigation; dynamic disinfection technology is widely used in all types of environments at any time of disinfection, which means that disinfection can be carried out in a human environment, so disinfection staff do not need to leave the site. Dynamic disinfection technologies are widely used in all types of environments to disinfect at any time. Air disinfection can weaken the airborne capacity and transmission of viruses, while surface disinfection can eliminate the source of infection and cut off the transmission pathway.

This chapter will systematically discuss the principles and advantages of the above-mentioned antiviral methods and compare them with contemporary environmentally friendly and economical materials to propose viable strategies for making surface and space disinfection more efficient and economical, including novel products such as wearable protective devices.

2. Physical disinfection

Physical disinfection refers to the use of physical methods such as inactivation or destruction of microbial structures through heating, mechanical damage, irradiation, etc., to achieve disinfection effects. The common disinfection used for air and object surfaces can be categorized as ventilation, heating disinfection, plasma disinfection, ozone disinfection, ultraviolet (UV) disinfection, and other disinfection methods, which are described below according to the different subjects used for disinfection.

2.1 Ventilation and disinfection

The function of ventilation is to make the air circulate and take away some of the bacteria in the air or blow away the bacteria attached to the surface of objects. In schools, libraries, and other public places, as well as in the daily life of families, this disinfection method is preferred. In hospitals, treatment rooms, operating rooms, and other environments, ventilation plus air disinfection can ensure the quality of indoor disinfection.

Disinfection category	**Heating**	**Plasma**	**Ozone**	**Ultraviolet**	**Adsorption**	**Negative ions**	**Photocatalysis**	**Chemical reagents**
Principle	High Temperature Inactivation	High Energy Ion Breakdown	Oxidation	Radiation denaturation	Filter adsorption	Oxidation decomposition	Hydroxyl radical decomposition	Reaction product abatement
Mode of action	Machine	Machine	Ozone release	Irradiation	Passive adsorption	Machine	Machine	Wipe/Spray
Disinfection range	Object table/air	Object table/air	Air	Object surface/ air	Air	Air	Air	Object table/air
Disinfection efficiency	Low	High	High	High	Low	Low	High	High
Harmful reagents	/	/	/	/	/	/	/	√
Harmful rays	/	/	/	√	/	/	/	/
Harmful gases	/	/	√	/	/	√	/	/

Table 1.
Common disinfection techniques and comparison.

2.2 Heating and disinfection

According to the characteristics of COVID-19 and other viruses that are not heat-resistant, high temperatures can be used to inactivate them. The principle is: in the indoor environment, it is not possible to heat the environment as a whole to the virus inactivation temperature (56°C), but continuous heating of a small amount of air above 100°C is still easy to do, however in the environment above 100°C virus life will be greatly reduced, the temperature is high to a certain extent, and the new crown-like virus may be in an "instant" manner burned to death. High-temperature conditions will also reduce the humidity of the air, so that the droplets wrapped in the virus periphery undergo moisture evaporation, which also deprives the virus of survival conditions. Heating is mostly done with a specific electric heater, so that the ambient air enters through the entrance of the electric heater, passes through the heater at 200°C or even higher, and then exits, during which the virus is inactivated by the high temperature. This process of heating and inactivation is continuous. As shown in **Figure 1** below.

And the electric heater that can achieve the above ability can be mainly divided into (1) a resistance wire heater (heating wire surface temperature up to 1000°C or more); (2) positive temperature coefficient (PTC) ceramic heater (heater surface temperature is generally 275°C); and (3) any other high surface temperature heat conduction-type electric heater. Theoretically, the heater can kill all kinds of germs, especially for the temperature sensitive virus for example the 2019 coronavirus. However, the specific sterilization time, power, environment, and space adapted to further research are needed. Such as a home electric hair dryer whose internal temperature can also reach more than 56°, can produce dry hot air, and also has the potential and ability to sterilize the new coronavirus, it needs a certain role time. Later, a small home-type high-temperature sterilizer can be developed based on the portable characteristics of the hair dryer. In addition, most nonspore bacilli are 80 ~ 100°C under a few minutes almost all dead, 70°C need 10 ~ 15 min to kill, and 60°C must be more than 30 min to be killed. The spores of mold usually 86 ~ 88°C heated for 30 min can die. Therefore, with the help of an indoor furnace, the incineration method, the dry baking method, as well as high-energy infrared and other high-temperature methods it is possible to kill bacterial germ cells, to achieve a sterilization effect.

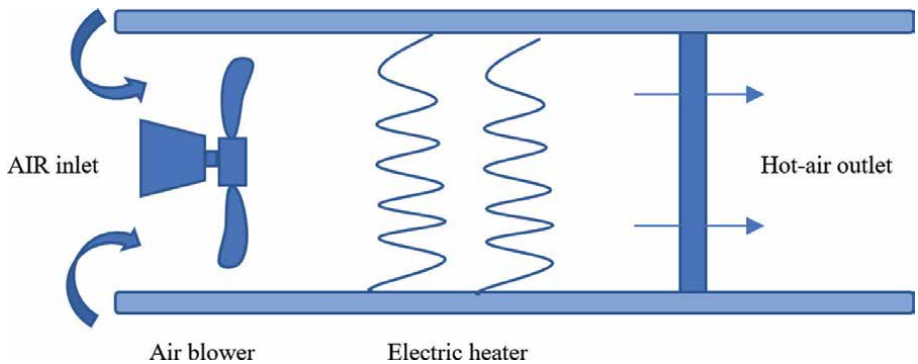

Figure 1.
Electric heating wire channel sterilization.

High-temperature disinfection is a commonly used means of disinfection for medical devices. Medical devices can be classified as high-, medium-, and low-risk medical devices based on their risk of causing infection in use. High-risk medical devices must be sterilized before use, medium-risk devices should be disinfected at a high or medium level before use, and low-risk medical devices should be disinfected at a medium or low level before use or kept clean daily. One study treated duodenoscopes with three different levels of disinfection and found no significant difference between the three groups mentioned above [2]. Therefore, it is sufficient to disinfect medical devices and equipment according to the level in which they are located, without the need to overdisinfect and cause a waste of resources. Among them, medical devices that are resistant to moisture, heat, and reusability should be preferred to autoclaving; glass devices, oils, and dry powders are generally selected for dry heat sterilization; and those that are not resistant to heat or moisture are selected for low-temperature sterilization.

Boiling disinfection is a frequently used and effective method for disinfecting the surface of hospital objects, which can cause most of the nonbudding pathogenic microorganisms in the objects to die rapidly in boiling water at 100°C. Most of the budding spores can be killed in 15–30 min of boiling, and all pathogens can be eliminated by boiling for 1–2 h. However, when the object is exposed to hot and humid air, it will lose its antifouling properties, resulting in secondary contamination [3]. Various metals, glass equipment, and clothing can be disinfected by boiling. Steam disinfection is a method of disinfection using water vapor at about 100°C under atmospheric pressure and is mainly used for the disinfection of hospital aids, such as mattresses, bedding, and bedsheets.

2.3 Plasma disinfection

In 1996, Laroussi, an American scientist, found that atmospheric pressure low-temperature plasma has a good killing effect on *Pseudomonas aeruginosa*, which is the earliest report on sterilization by atmospheric pressure low-temperature plasma. Plasma air sterilizers can significantly reduce the infection rate in surgical ports compared to UV and chemical disinfection. Plasma sterilization also decomposes polymeric toxic organic compounds such as formaldehyde and smoke and converts them into nontoxic, odorless inorganic substances such as carbon and water, which are not harmful to humans or medical equipment. This technology has been shown to inactivate a wide range of microorganisms such as bacteria, fungi, viruses, and spores, and has received increasing attention due to the above-mentioned efficient disinfection capabilities and environmental friendliness [4]. The principle of atmospheric pressure low-temperature plasma sterilization is to achieve rapid killing of bacteria and viruses using the compound action of electrons, ions, photons, and free radicals contained in the plasma. Low-temperature plasma sterilization can not only kill germs in space but also achieve rapid sterilization of the surface of the object, as well as the surface of human skin. Because its unique advantages have attracted the attention of more and more researchers, the killing effect on microorganisms is also heavily studied and applied. The plasma channel made using atmospheric pressure low-temperature cold plasma technology (such as medium blocking plasma discharge dielectric barrier discharge (DBD)), as shown in **Figure 2**, and by combining other machine materials, such as combining fans and purification filter materials, can make air purification and sterilization products such as circulating air plasma sterilizer (**Figure 3**). The machine is mostly used for preventive disinfection in crowded

Figure 2.
Atmospheric pressure low-temperature cold plasma discharge channel.

Figure 3.
Circulating air plasma sterilizer.

places such as operating rooms, fever clinics, intensive care units, etc. The common equipment is contact, and the positive and negative ions in the interface capture microorganisms to kill them and achieve air disinfection. Low-temperature plasma air disinfection machines can inactivate bacteria and viruses in artificially generated aerosols very well, with purification efficiencies (removal rates) of 99.98, 99.98, and 99.95% for *Staphylococcus aureus*, *Micrococcus garcinia*, and ΦX174 phage aerosols. However, due to the complexity of the machine system, problems, such as the whole machine not working, plasma not working, fan not working, small airflow at the outlet, machine not working according to the set time, or remote control not responding, often occur in daily use. In daily maintenance, it is necessary to regularly maintain and repair its filter motor, to improve the quality of equipment operation and ensure the safe and effective operation of the air disinfection machine and provide a strong guarantee for clinical diagnosis and treatment services.

Atmospheric pressure low-temperature cold plasma beam can eliminate pathogenic bacteria such as *Escherichia coli*, *Candida albicans*, *Staphylococcus aureus*, and *Bacillus subtilis* attached to different materials (**Figure 4**).

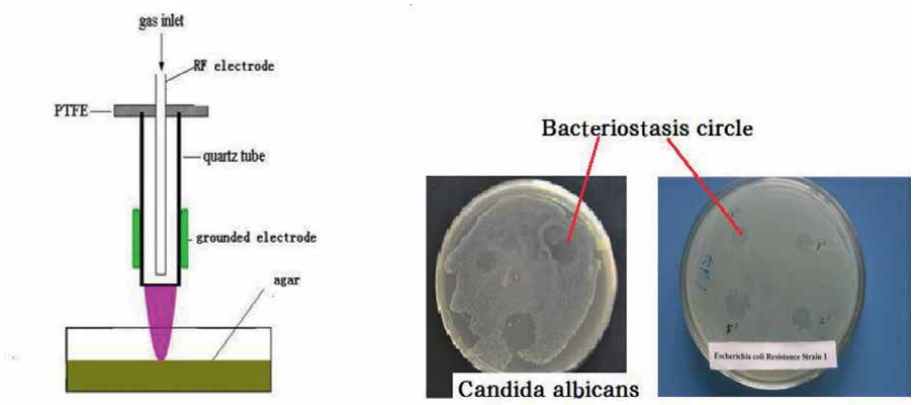

Figure 4.
Experiment of atmospheric pressure plasma killing bacteria.

Figure 5.
Atmospheric pressure plasma beam can interact with human skin.

In a study using helium and argon plasma beams as working gases, it was found that atmospheric pressure low-temperature plasma beams sterilize quickly, killing germs on the surface of objects quickly and without damaging human skin within 1–2 min (**Figure 5**) [5].

Meanwhile, more and more researchers have successfully discovered that atmospheric pressure low-temperature plasma can act on Gram-positive bacteria, Gram-negative bacteria, spores, microbial biofilms, fungi, viruses, etc., and have a good killing effect on all of them, unveiling a new picture as physical sterilization of human skin and object surfaces (**Figure 6**).

Atmospheric air plasma can also treat *Escherichia coli*, *Staphylococcus aureus*, yeast, and phage ΦX174 attached to different materials, the survival number of microorganisms attached to the surface of the treated materials was significantly reduced, the surface of the bacteria was broken after 10–25 s of discharge treatment and fragments could be seen under the microscope, and after 59–90 s of treatment, the survival number of bacteria was reduced by at least 5. The mechanism is the hypothesis of cell membrane fragmentation due to reactive oxygen species (ROS) [6]. The accumulation of experiments on the disinfection of microorganisms on the surface of objects by low-temperature plasma gradually unveiled the wide application of atmospheric pressure low-temperature plasma sterilization technology. *In vitro*, the action of

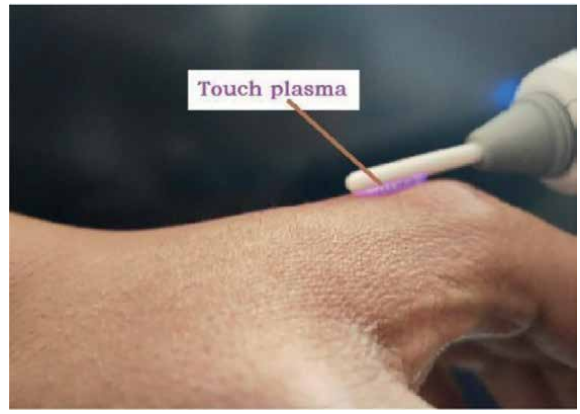

Figure 6.
A plasma in touch with human skin.

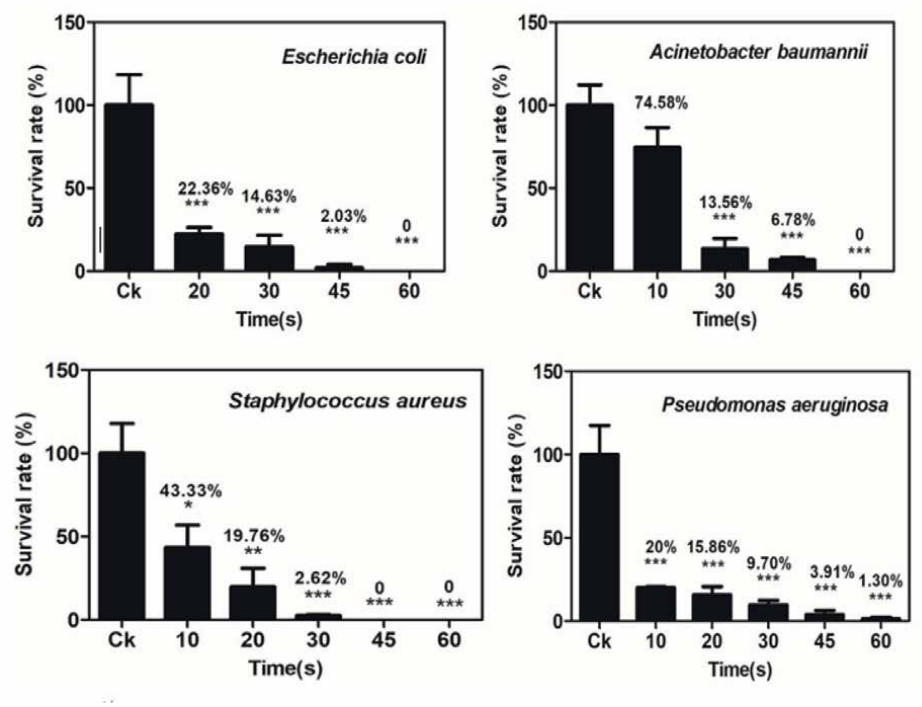

Figure 7.
The remarkable killing effects on common bacteria and fungi in the clinic.

pathogenic microorganisms on the surface of skin wounds using a low-temperature cold plasma beam obtained the following sterilization effects (**Figure 7**).

2.4 Ozone disinfection

Commonly used ozone generators are generated by atmospheric pressure low-temperature plasma discharge, generally using a medium to block the plasma discharge

can generate ozone, its generation principle is shown in **Figure 8** (left), and **Figure 8** (right) for indoor space with a small ozone generator photograph.

The mechanism of action of ozone disinfection (**Figure 9**) is that ozone can diffuse into the surface of microbial membranes or protein shells for rapid oxidation reactions with pathogens, degrading biofilm and shell structures, and leading to loss of cytoplasm and intershell substrates. The ozone that penetrates the membrane and shell and the reactive oxygen species it generates can further attack the genetic material DNA or RNA, disrupting the structure of bacteria and viruses leading to the destruction of genetic material and inactivation of coenzymes, thus achieving disinfection [7].

Compared to chlorinated disinfectants, ozone has a shorter reaction time and a lower regeneration rate of microorganisms, and it can decompose itself into oxygen without residual contamination. Therefore, it is commonly used for air disinfection in large workshops and laboratories. However, excessive ozone can not only remove organic micro-pollutants, but also corrode pipeline equipment, and incomplete oxidation reaction with dissolved organic micro-pollutants will produce toxic and harmful disinfection by-products, thus causing risks to the surrounding environment and human body. Therefore, the optimization of ozone disinfection technology and the development of the ozone co-disinfection process will also be an important development direction in the future.

The use of ozone treatment reported by B. Clavo et al. [8] was able to provide good inactivation of personal protective gear contaminated with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2 virus) after only 30 s treatment when the volume fraction of ozone reached 10,000 × 10^{-6}.

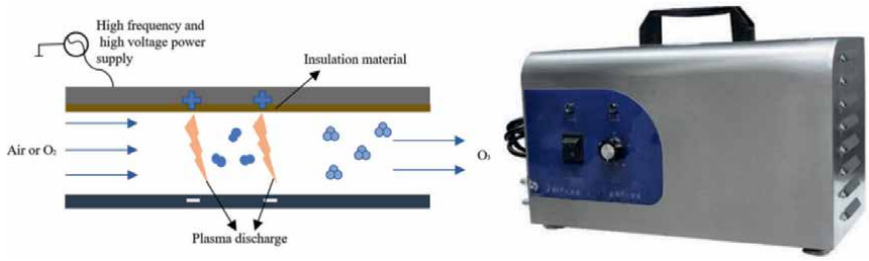

Figure 8.
Generation principle (lift) ozone generator (right).

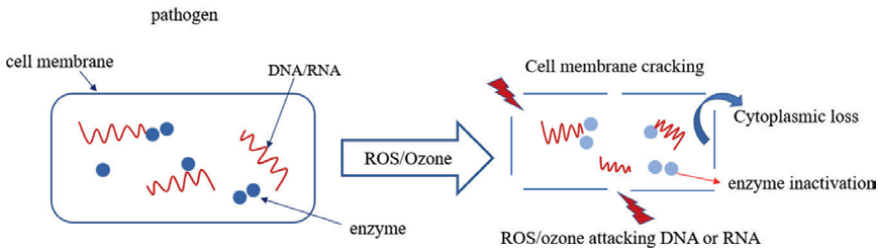

Figure 9.
Mechanism of ozone action.

2.5 UV disinfection

Germicidal disinfection by ultraviolet C (UVC) shows that the use of UVC can damage the molecular structure of DNA (deoxyribonucleic acid) or RNA (ribonucleic acid) in the cells of microbial organisms, resulting in growth cell death and (or) regenerative cell death to achieve the effect of germicidal disinfection. The action of UV on nucleic acids can lead to bond and strand breaks, interstrand cross-linking, and formation of photochemical products, which alter the biological activity of DNA and make the microorganism itself unable to replicate and proliferate, and this UV damage is also lethal [9].

In an unoccupied environment, UV disinfection is currently the most commonly used physical disinfection method in medical units and the most used disinfection instrument in households since the outbreak of the new crown-like virus. Since viruses absorb the largest amount of photons at a wavelength of 260 nm, 260 nm UV is generally chosen to destroy the nucleic acids and envelope proteins of viruses and microorganisms to kill microorganisms. However, its disinfection effect is also easily affected by the temperature and humidity of the surrounding environment. When the room temperature is higher than 40°C, lower than 20°C, or the humidity is greater than 60%, it is necessary to extend the irradiation time of the UV lamp. Usually, UV is used in combination with other substances. Common joint processes include UV + H_2O_2, UV + H_2O_2 + O_3, UV + TiO_2, UV+ negative ions, air filtration systems, etc., work together to kill pathogenic microorganisms in the air, and the joint use can achieve better disinfection effects. Of course, the final effect of UV sterilization is related to the intensity (power) of UV irradiation, irradiation time, and space size.

The germicidal effect is determined by the dose of irradiation received by the microorganism, and also by the output energy of the UVC, which is related to the type of lamp, light intensity, and time of use; as the lamp ages, it will lose 30–50% of its intensity. UV irradiation dose is the amount of specific wavelength UV needed to achieve a certain bacterial inactivation rate: the higher the germicidal efficiency required, the greater the required irradiation dose.

Disinfection of indoor air can be carried out by the indirect irradiation method and the direct irradiation method. Among them, the indirect irradiation method is preferred with a high-intensity UV air disinfector, as it not only has a reliable disinfection effect and can be used during indoor activities, generally turning on the disinfection 30 min can reach qualified disinfection level; the direct irradiation method in indoor unoccupied conditions can take the UV lamp of the hanging type or mobile direct irradiation. When using indoor suspended UV disinfection or indoor installation of UV disinfection lamp (30 W UV lamp), the intensity at 1.0 m > 70 uW/cm^2 number of not less than 1.5 W per cubic meter on average irradiation time of not less than 30 min is recorded.

While using ultraviolet disinfection, it should be noted that:

1. In the process of use, the surface of the UV lamp should be kept clean, generally wiped once every 2 weeks with an alcoholic cotton ball, and the surface of the lamp should be wiped at any time when dust and oil are found.

2. When using UV lamps to disinfect indoor air, the room should be kept clean and dry to reduce dust and water mist, and the irradiation time should be extended

appropriately when the temperature is lower than 20°C or higher than 40°C and the relative humidity is greater than 60%.

3. When disinfecting the surface of articles with UV light, the irradiated surface should be exposed to direct UV light and should reach a sufficient dose of irradiation.

4. When using UV light source, we shall not make the UV light source irradiate people to avoid causing injury;

5. The UV intensity meter is calibrated at least once a year.

2.6 Other physical disinfection

In addition to the physical disinfection methods mentioned above, high-pressure electrostatic adsorption, negative ion disinfection, ultrasound sterilization, microwave sterilization, ionizing radiation sterilization such as X-rays and γ-rays, electron beam sterilization and other sterilization methods are also common in the food industry, medical devices, and pharmaceutical industry. High-pressure electrostatic adsorption air disinfection is also mostly used in closed medical operation areas such as emergency surgery or outpatient drug change rooms, but the effect and application of high-pressure electrostatic adsorption on SARS-CoV-2 are to be confirmed by further clinical studies. These methods include thorough sterilization and high health safety, but the radiation sources are generally large and can only be installed and used in fixed settings.

In addition, nanometer antibacterial material is a new class of healthcare antibacterial materials developed in recent years and is the focus of nanotechnology and antibacterial technology research. Nanomaterials have a large specific surface, bond mismatch, and many active centers so nanomaterials have a strong electrical capture ability, which can strongly attract the sulfhydryl groups of proteases in bacteria, and quickly combine to make the bacterial genetic enzymes inactive, resulting in the death of bacteria.

Nanometer antibacterial products continue to enter people's daily life. For example, nano antibacterial thermal underwear and antibacterial shoes and socks are now available in the market. Adding nanoparticles to chemical fiber products and textiles also has the effect of deodorizing and sterilizing property, adding silver nanoparticles to socks can remove foot odor; adding silver nanoparticles to medical gauze has the sterilizing effect.

3. Chemical disinfection

Chemical disinfection is the use of chemical disinfectants to act on microorganisms and pathogens to denature their proteins and cause them to lose their normal function and die. The disinfectants used in air and surface disinfection can be divided into four categories: chlorinated disinfectants, nonchlorinated disinfectants and chemical surfactants, and plant disinfectants. In the use of chemical disinfectants, it is necessary to consider not only the impact of the type of chemical disinfectant, the dose and ratio used, the duration of action, pH, temperature, and other factors on the efficiency of virus inactivation but also the impact on human health.

3.1 Chlorinated disinfectants

When used for air disinfection, commonly used chlorine-containing chemical disinfectants include over 84 disinfectants, bleach, chlorine-containing effervescent tablets, hypochlorous acid, chlorine dioxide, and other reagents in the form of liquid or spray for disinfection operations of ambient air. Among them, spray disinfection refers to spraying the disinfectant through mechanical force or any other means of action, so that the disinfectant forms a water mist of fine droplets or atomized into aerosols, and the object surface or air disinfection way is the site of environmental disinfection which is a commonly used disinfection method. Chlorine dioxide performs the functions of disinfection and sterilization, formaldehyde removal, odor removal, nicotine purification, etc. It is an internationally recognized green and efficient disinfectant, commonly used in hospital sewage disinfection and air disinfection and widely used in Europe, America, Japan, and other developed countries. However, chlorine dioxide and hypochlorite disinfection solution is very volatile and will lose its effect during a long time of light in the process of storage, so it should not be stored for a long time and needs to be dispensed as it is used.

3.2 Nonchlorine disinfectants

Peroxyacetic acid and hydrogen peroxide are often used in air disinfection by spraying or fumigation because of their strong oxidizing properties. By making the active ingredients in the disinfectant interact with microorganisms' outer layer proteins, glycoproteins, membrane lipids, and other macromolecules, the synthesis of RNA, DNA, and proteins is inhibited, thus killing the microorganisms in the air. Currently, dry mist hydrogen peroxide disinfection systems are mostly used, and the Chinese Technical Specification for Disinfection in Medical Institutions also proposes that indoor air disinfection can be carried out by spraying hydrogen peroxide. Hydrogen peroxide has many advantages as a highly effective disinfectant, such as a concentration below 3% can be used for disinfection, 6% or more can be used for sterilization and the sterilization effect is fast, sterilization ability is strong, the sterilization spectrum is wide; at the same time, it has the advantages that other low-temperature disinfectants do not have, such as less irritation, low corrosion, easy to undergo gasification into oxygen and water, no pollution to the environment, no residual toxic substances, etc., and aerosol disinfection, both. It can ensure uniform disinfection area and wide coverage, and avoid the problems of limited irradiation distance and dead angle of physical UV method, and solve the problems of consumables' replacement, microbial enrichment, and secondary pollution of electrostatic adsorption and filtration method.

In recent years, chemical disinfectants for terminal disinfection in medical institutions and epidemic source sites are commonly used for disinfection by hydrogen peroxide fogging. However, it is necessary to consider that the chemical components in the spray may cause allergy, asthma, nausea, headache, memory loss, and other symptoms in some people in the air. And chemical disinfectants are strong disinfectants, which are only suitable for indoor air disinfection in unoccupied situations, such as laboratory or end-of-life air disinfection at the source of the epidemic, but for disinfection at any time inwards, homes, offices, etc., it is necessary to coordinate the time of human and machine disinfection, and to use human-machine simultaneous operation of disinfection in the same field. Moreover, chemical disinfectants are generally irritating and corrosive, with a certain lag and decay in prevention and

control effects, so they are mostly suitable for indoor air disinfection in unoccupied conditions.

3.3 Chemical surfactants

Surfactants are also one of the commonly used disinfectants, which can be used not only for disinfection and sterilization of the surface of articles but also for topical clinical skin application and prevention of postoperative infections, and are widely used in daily life, medical hygiene, wound treatment, and other fields. These agents are low in toxicity, noncorrosive, stable for long-term storage, have a wide range of disinfection, are effective and fast, and have a strong killing effect on pathogenic bacteria in general. In people's daily life, chemical disinfection is mainly reflected in bare skin such as hands, furniture, and electronic equipment. For disinfection of human hands, it was found that soap washing has the best total bacteria removal effect compared to alcohol. Repeated hand washing and the use of disinfectants can cause significant skin dryness and contact dermatitis.

4. Other disinfection methods

Some studies have compounded with low toxicity, residue-free permethrin, compound quaternary ammonium salts, guanidine, and other safe raw materials to develop a new compounded fogging disinfectant that can effectively disinfect the environmental air of containers used for transportation.

In addition, Chinese herbal air disinfection is also commonly used in medical, home, and other indoor places. Chinese medicine fumigation for air disinfection method has a long history, "the elbow after the preparation of the urgent formula" will be the first to propose the air disinfection method of Chinese medicine fumigation, such as "Taiyi Liujin formula ... in the court of burning, warm patients also burn fumigation." Chinese medicine disinfection has strong broad-spectrum antibacterial and antiviral effects and can achieve effective and safe disinfection at the same time, but is also nontoxic to humans and the environment, has abundant resources, is stable, low priced, and is simple to use. Mo Qian [5] et al. found that mugwort, atractylodes, patchouli, peppermint, honeysuckle, forsythia, and other heat-clearing and damp-relief drugs were mostly used for air disinfection in Chinese medicine. This method is inexpensive, simple to operate, overcomes the disadvantages of chemical disinfectants, safe and effective, nonirritating to humans, noncorrosive to objects, and is more suitable for use in environments with high personnel density and easy contamination. Application in the medical environment: In clinical studies on disinfection of hospital wards, it was found that mugwort leaves, when used for air disinfection, could significantly reduce the number of harmful microorganisms in the environment, with comparable or even better effects than ultraviolet light and chemical disinfectants, but the disinfection time of herbal medicine is longer and there may be problems of secondary contamination. The newly developed *Atractylodes macrocephala* oil microcapsules also have a certain killing effect on natural indoor air bacteria by heating and fumigation. During the COVID-19 airborne outbreak, indoor air disinfection can make essential indoor spaces, such as clinical and daily homes, safer. In daily household life, the use of herbal disinfection both ensures its ideal disinfection effect and enhances indoor comfort, which is becoming increasingly popular for household disinfection.

5. Summary

Traditional single physical and chemical disinfection is prone to secondary air pollution and certain toxic side effects, each with its advantages and disadvantages. Therefore, it is necessary to develop a reasonable disinfection process and strategy that combines physical, chemical, and biological methods to synergize disinfection. Overdisinfection can be avoided while ensuring the disinfection effect [10]. This is because overdisinfection can affect the environment and the balance of microorganisms in the plant and animal, water and soil environments, and even bring about the spread of antimicrobial resistance.

In future research, the study of biomedical mechanisms of the action of physical means and bacterial viruses should be strengthened to avoid environmental pollution by applying cost-effective sterilization and disinfection techniques. Reducing the cost of sterilization and sterilization equipment, achieving green, environmentally friendly, fast, and portable characteristics, ensuring no damage to the human body, and realizing the completion of sterilization and disinfection of indoor air and objects under human conditions still need further research and improvement.

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Section 2

Natural Products and Repurposed Drugs

Chapter 3

Antiviral Plant Extracts: A Treasure for Treating Viral Diseases

Gamil S.G. Zeedan and Abeer M. Abdalhamed

Abstract

Viral infections remain a challenge in human and veterinary medicine due to factors such as viral mutations, new viruses, toxic effects, disease severity, intracellular viability, high costs, and limited availability of antiviral drugs. Despite advancements in immunization and antiviral drugs, there is a need for new and more effective antiviral compounds. Plants produce secondary metabolites that have shown antiviral activity, such as alkaloids, flavonoids, and essential oils. Advanced analytical techniques like HPLC, GC-MS, and NMR spectroscopy are used to identify and characterize these bioactive compounds. Flavonoids, terpenoids, lignans, sulphides, polyphenolics, coumarins, and saponins are among the groups of bioactive compounds found in plants that have demonstrated antiviral activity against viruses like HIV, influenza, herpes simplex, and hepatitis. Screening plant extracts and isolating active compounds allow scientists to identify potential new antiviral drugs. *In vitro* and *in vivo* studies have shown significant antiviral activity of plant extracts and their bioactive compounds. However, further research is needed to ensure safety, investigate drug interactions, and explore combination therapies with other natural products. The use of advanced analytical techniques helps identify and characterize bioactive compounds that target different stages of the viral life cycle. Examples of plant extracts and compounds with antiviral activity against specific viruses are mentioned, including SARS-CoV-2 and various veterinary viruses. The abstract emphasizes the ongoing research on natural sources, particularly plants, for the discovery of new and effective antiviral compounds, while highlighting the need for extensive studies on safety, drug interactions, and combination therapies.

Keywords: antiviral plant extracts, bovine herpes virus type-1, bovine, foot-and-mouth disease, rotaviruses, buffalo pox virus

1. Introduction

Viral diseases continue to cause significant morbidity and mortality globally, posing a persistent threat to public health for both humans and animals [1, 2]. Antivirals are medication drugs used specifically for treating viral diseases or substances that can produce either a protective or therapeutic effect on the virus-infected host [1, 3–5]. The uses of antiviral drugs in human and veterinary medicine are limited in comparison with the use of antimicrobial agents due to viral mutants resistant to existing antiviral diseases. Furthermore, new viral pathogens have been discovered as

having side effects and high costs [6–9], the severity of viral diseases, and the ability of viruses to survive intracellularly [10].

Approximately 80% of the people as well as domestic animals in developing countries use traditional and medicinal plants for maintaining their health [11, 12]. Medicinal plants became a new source of drug discovery due to advent of today's advanced analytical chemistry, developed standardized and extraction procedure, as well as standard assays [13, 14]. Twenty-five percent of the drugs in common use are of plant origin, and more than 4,22,000 species of flowering plants have been reported, only 5000 species among them are used for medicinal purposes [14]. Medicinal plants with strong antiviral activity to treat viral infections in humans and animals and those containing novel plant-derived antiviral agents have been identified [15–17]. Herbal medicines and purified natural products provide a rich resource for novel antiviral drug development [18, 19]. Identification of antiviral mechanism of natural agents has shed light on interaction with the viral life cycle such as viral entry, replication, assembly, and release, as well as targeting of virus–host specific interactions [20].

The antiviral activity of several natural products of the following species *Ipomopsis* aggregate, *Aloe Vera*, A *L. dissectum*, *Achillea millefolium*, *Achillea tenuifolia*, *Achillea talagonica* with *Ania somnifera*, Ashwagandha, *Allium sativum*, and *Azadirachta indica* were examined in vitro and in vivo and found all of these plants' extracts had antiviral against DNA or RNA viruses [8, 9, 21, 22]. Many phytochemicals showed dose-dependent viral inhibitory effect against common veterinary viral pathogens [23–25]. This review study aimed to spotlight on antiviral activities of some medicinal plants against viral pathogens including families *Herpesviridae*, *Flaviviridae*, *Retroviridae*, *Picornaviridae*, *Hepadnaviridae*, and *Paramyxoviridae in vitro* and *in vivo*.

1.1 Viral infection control

Viruses differ from bacterial and fungal infections in that they require living cells to replicate, and this makes controlling them a difficult task. Viruses integrate into host cells both functionally and physically, making it extremely challenging to distinguish them. Some viruses can persist as latent infections, which is a concerning problem. Moreover, many viral infections lack effective treatments, and antiviral drugs can have potential toxic effects, along with the emergence of cross-resistant mutants. As a result, plant extracts and phytochemicals are receiving increasing attention as alternative approaches for controlling contagious diseases in livestock, with scientists continuing to study their potential as antivirals [26, 27].

1.2 Antiviral drugs limitations

The limited efficacy of antiviral drugs compared to antimicrobial agents is due to the difficulty of identifying specific viral targets with high selectivity and low side effects. However, in recent years, a more rational approach has been taken to the development of new antiviral drugs. Currently, only one antiviral compound, feline interferon-omega (IFN-), has been licensed for use in veterinary medicine due to its undefined mechanism. Although several antiviral drugs are licensed for use in cats with feline herpes virus-1 (FeHV-1), such as idoxuridine, trifluridine, and acyclovir, or against feline immunodeficiency virus (FIV), there are many reasons why antiviral agents are not widely used in veterinary medicine. These include their high cost, especially for use in food species, their lower cytotoxicity in animals, and the lack of

rapid diagnostic techniques. Despite these factors, animal viruses have been used as models for developing antiviral drugs for humans, with bovine viral diarrhea (BVD) considered a valuable surrogate for the hepatitis C virus.

1.3 Plants are a source of antiviral agents

Plants possess a natural ability to synthesize medicinal compounds, leading to the discovery of new drugs with potent therapeutic effects. Traditional medicine still serves as a primary source of healthcare for almost 80% of the world's population, with plants and plant products being utilized for centuries to treat diseases even before the active constituents were identified. It is noteworthy that approximately 50% of all prescribed medications are derived from plants or their derivatives [28]. Medicinal plants in combination therapy have been shown to be effective against various viruses, including herpes and influenza viruses. Additionally, many plant extracts, such as those obtained from *Agrimonia apilosa* and *Ocimumba silica*, have demonstrated antiviral activity against a broad range of DNA and RNA viruses [29]. In addition to their traditional use in medicine, plants have also been a source of new drugs with potent therapeutic effects. In fact, about half of the drugs that are prescribed today are either derived from plants or are plant-produced. Combining different medicinal plants has also proven effective against various viruses, including herpes and influenza viruses. Many plant extracts, such as *A. apilosa* and *O. silica*, have demonstrated antiviral effects against a broad spectrum of DNA and RNA viruses. This highlights the potential of plant-based therapies as an alternative approach to treating viral infections.

1.4 Coronavirus and medicinal plants

The coronavirus (CoV) is a type of single-stranded, positive-sense RNA (ssRNA) virus that belongs to the family *Coronaviridae*. This family of viruses is responsible for causing respiratory and gastrointestinal infections in both mammals and birds. Although in humans, it usually causes mild symptoms such as a cold or flu, it can lead to more severe complications such as pneumonia and severe acute respiratory syndrome (SARS). There are several documented types of human coronavirus (HCoV), including HCoV-229E, -OC43, -NL63, and -HKU1. The most widely known member of the family is the severe acute respiratory coronavirus syndrome (SARS-CoV), which caused a high mortality rate in 2003. In 2012, the World Health Organization (WHO) reported a sixth highly lethal form of HCoV infection known as the Middle East Respiratory Syndrome Coronavirus (MERS-CoV) [30, 31]. The severe acute coronavirus 2 respiratory syndromes (SARS-CoV-2) were first reported in December 2019 in Wuhan, Hubei, China, and declared a pandemic by the World Health Organization (WHO) on March 11, 2020.

The World Health Organization proposed the name 2019-nCoV; later, the International Committee on Taxonomy of Viruses renamed it SARS-CoV-2 (coronavirus disease 2019). The Wuhan strain was identified as a new Group 2B Betacorona virus strain with nearly 70% genetic similarity to SARS-CoV. The virus seems to have a 96% resemblance to the coronavirus bat, and therefore it is generally believed to emanate from bats. There seem to be no precise medications or treatment options for COVID-19 [32]. Coronaviruses are large, pleomorphic, spherical particles with a bulbous surface projection. The diameter of the virus particles is approximately 120 nm.

In electron micrographs, the virus membrane was observed as a distinct double-layered structure. This viral envelope is composed of a lipid bilayer, which houses the membrane, envelope, and spike structural proteins. A specific subtype of beta coronaviruses known as subgroup A features a truncated spike protein, known as hemagglutinin esterase (HE). Moreover, nucleocapsids develop copies of the nucleocapsid protein attached to the positive-sense single-stranded RNA genome. The genome size for coronaviruses ranges from 27 to 34 kilobases, the largest among documented RNA viruses [33].

The lipid bilayer envelope, membrane protein, and nucleocapsid safeguard the virus outside the host cell. A specific genome sequence analysis of viruses found in pangolins and humans has revealed only one amino acid variation. Currently, only about 92% of the genetic material between pangolin coronavirus and SARS-CoV-2 has been compared as a complete genome, which is not enough evidence to establish pangolins as intermediate hosts. The virus is assumed to have originated in bats as it is 96% identical to the bat coronavirus. The name coronavirus originates from the Latin word "corona, meaning" "crown" or "halo," as the virus particles exhibit a crown-like appearance due to the presence of club-shaped protein spikes on their surface, as observed under two-dimensional electron microscopy. Currently, there are no specific treatments or preventive vaccines for CoV infection, and there is a need for the development of new antivirals to prevent and treat CoV infection, as highlighted by Agarwal et al. [34]. The complete list of potent plant extracts and their bioactive compounds that inhibit coronaviruses Ginsenoside Rb1 (Gynosaponin C), one of the bioactive ginsenosides extrapolated from Panax ginseng, displayed antiviral activity. Tetra-O-galloyl-beta-d-glucose, luteolin, and tetra-O-galloyl-beta-d-glucose blocked the SARS-CoV host cell entry. Herbal extracts have been studied for their potential antiviral properties. Among the 200 extracts analyzed, *Lycoris radiata*, *Artemisia annua*, *Pyrrosia lingua*, and *Lindera* aggregate were found to have anti-SARS-CoV effects with an EC50 range of 2.4–88.2 g/ml. Black tea phenolics such as tannic acid, 3-isotheaflavin-3-gallate, and theaflavin-3,3′-digallate have also exhibited inhibitory effects. These compounds have IC50 values of 3, 7, and 9.5 M, respectively, against SARS-CoV 3CLpro. On the other hand, phenolic compounds from *Isatis indigotica* have been shown to inhibit SARS-CoV 3CLpro with IC50 values of 217, 752, 8.3, 365, and 1210 M for sinigrine, indigo, aloe emodin, hesperetine, and sitosterol, respectively [35, 36].

1.5 Antiviral activity targeting plant extracts

Antiviral compounds found in medicinal plants have the potential to inhibit various stages of virus replication, including viral attachment to cells, virus-specific enzymes, and egress of viruses from infected cells. Such compounds may also target the virus itself. However, specific information about the viruses causing respiratory infections is often limited. One potential drawback of targeting specific antivirals directed at viral genes or products is the emergence of virus-resistant mutations [37]. People have used combinations of two or more antiviral drugs to solve the problem. However, there is an alternative approach that has the capacity to inhibit many different respiratory viruses [38, 39]. These targets include virus attachment, entry inhibitors, modifiers of the viral genome, protein processing, virus assembly, release inhibitors, and immunomedulators, as shown in **Figure 1**.

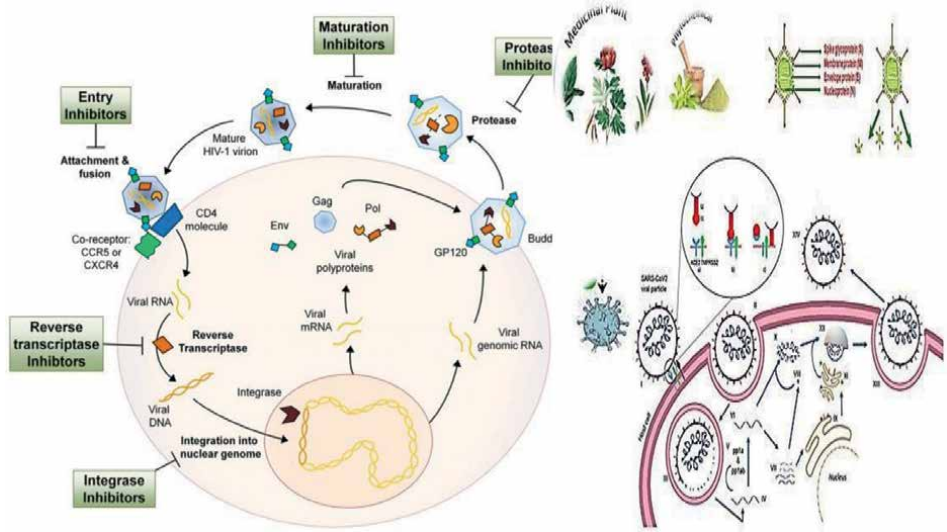

Figure 1.
Virus life cycle and possible antivirus targets.

The life cycle of a virus can be summarized as follows:

I. Attachment: The virus attaches to a host cell using its spike protein, which binds to specific receptors on the cell surface. II. Entry: The virus enters the host cell either by fusing with the host cell membrane or by endocytosis. III. Uncoating: Once inside the host cell, the viral genome (usually RNA) is released from the capsid. IV. Replication: The viral genome is copied by viral RNA-dependent RNA polymerase (RdRp), which produces new viral RNA strands. V. Translation: The viral RNA is translated into viral proteins, including the structural proteins and enzymes required for viral replication. VI. Assembly: The new viral RNA and proteins assemble into new virus particles. Release: The newly assembled virus particles are released from the host cell, usually by budding through the host cell membrane. To develop antiviral drugs, scientists often target specific steps in the viral life cycle. For example: Attachment: Drugs can be developed to block the binding of the viral spike protein to host cell receptors, preventing the virus from entering the cell. Replication: Drugs can be developed to inhibit RdRp activity, preventing the virus from replicating its genome. Assembly: Drugs can be developed to disrupt the formation of new virus particles, preventing their release from the host cell. Release: Drugs can be developed to inhibit the budding or release of new virus particles, preventing their spread to other cells. Overall, understanding the life cycle of a virus and its interactions with the host cell is essential for developing effective antiviral drugs.

2. Inhibition of virus attachment and entry

Antiviral therapy targets the attachment and entry of viruses into host cells. The virus enters the host cell by interacting with surface receptors or co-receptors, leading to fusion of the viral envelope to the host cell membrane and release of the viral genome. Various antiviral plant products have shown similar mechanisms in

inhibiting viral replication by targeting virus attachment and entry. For instance, plant-derived mannose-specific lectins from the *Galanthus* and *Hippeastrum* genera can inhibit viral envelope glycoproteins, thereby preventing viral entry into the cell. These agents may also interfere with viral attachment to the host cell, as shown in **Figure 2**. Also, extracts from seaweeds, carrageenans, and sea-weed-derived heparin sulphate molecules have an antiviral inhibitory effect against the dengue virus by preventing the uncoating of the virus. The viral envelope is a good target for antiviral drugs on enveloped and nonenveloped viruses, including herpes viruses, orthomyxoviruses, paramyxoviruses, rhabdoviruses, coronaviruses, retroviruses, arenaviruses, togaviruses, flaviviruses, and bunyaviruses [40, 41].

2.1 Modifiers of viral genome and protein processing

The next target for an antiviral strategy that targets viral transcription and translation processes. DNA viruses are getting directly integrated into the host genome or may be processed cellular machinery in RNA viruses. The antiviral agents can inhibit reverse transcription, integration, replication, transcription and translation that providing potential targets [42, 43]. The viral nucleic acid targets for chemotherapy and medicinal plant overlap and inhibit the viral protein synthesis. The binding of *Calophyllum lanigerum* to the active site of reverse transcriptase enzyme is irreversible, thereby inhibiting the activity of the enzyme [44, 45].

2.2 Virus assembly and release inhibitors

Antiviral drugs are known to prevent the assembly of newly synthesized viral proteins and inhibit their release from host cells. Protease inhibitors are a class of antiviral drugs that prevent the cleavage of polypeptides, thereby disrupting viral assembly.

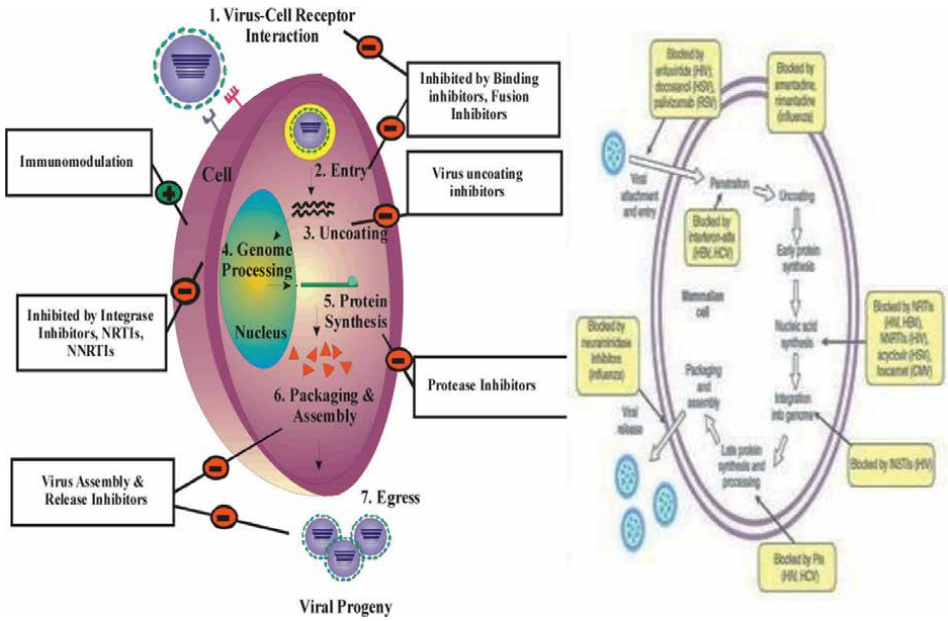

Figure 2.
Targets of antiviral agents' therapy.

Neuraminidase inhibitors, on the other hand, block the release of influenza virus from infected cells, and antiviral drugs that prevent virus transmission from cell-to-cell, such as oseltamivir and zanamivir. There are currently more than 30 different protease inhibitors derived from plant sources, such as *Eclipta prostrata*, *Alpinia galanga*, *Zingiber zerumbet*, *Coccinia grandis*, *Boesenbergia* [46, 47], and *Pandurata*, *Cassia garretiana*, and *Orostachys japonicus*, which have antiretroviral efficacy [46, 48, 49].

2.3 Other targets as immune-modulators

The increased attempt to synthesize antiviral agents that will stimulate the defense mechanism of the host is exemplified by the variety of biological response modifiers and interferon inducers. The induction of a protective immune response is one of the primary targets of antiviral therapy. Many of the currently registered products adapt this mechanism against viral infections. Interferons, interleukins, and colony-stimulating factors are the most prominent immunostimulants. Interferon-induced polypeptides and glycoproteins that serve as mediators to induce the production of certain enzymes that inhibit viral replication in the cell [8, 9].

Interleukin are involved in the stimulation, growth, differentiation, maturation, and regulation of immune cells that can help in the neutralization of the virus [50]. The β-sitosterol obtained principally from the plants of genus *Nigella*, enhance the cellular immune response by enhancing the activity of natural killer (NK) cells, CTLs, and by the increased secretion of cytokines. *Tinosp sitosterolia* plant extracts have the ability to cause lymphocytic activation [8, 9, 38, 39]. The flowering plant *Echinacea purpurea* has been used for its immune-stimulating properties, and it is believed that

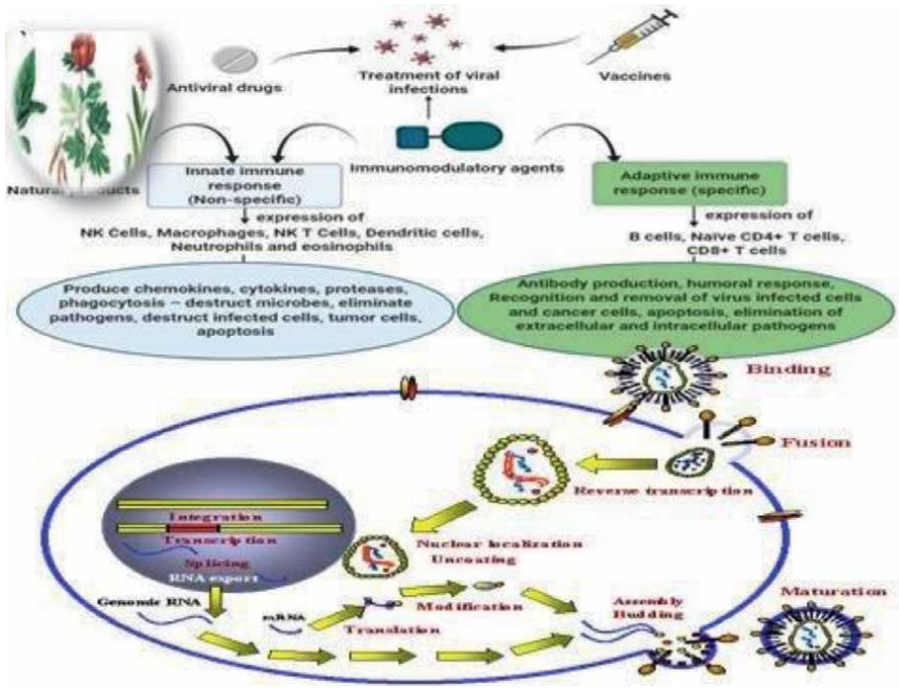

Figure 3.
Various receptors through which the subtypes of virus enter the target cells. The presence of medicinal plant.

the *Asteraceae* family of plants possess the largest number of plants with immunomodulatory activity. Proteins derived from *Allium sativum*, also known as garlic, have been found to exhibit mitogenic activity toward human lymphocytes, splenocytes, and thymocytes (**Figure 3**) [51].

3. Mechanism of active antiviral compound from medicinal plants extracts

3.1 Antiviral plant extracts and their impact on selected veterinary viruses

3.1.1 Antiviral effects of herbal plants against a range of DNA and RNA veterinary viruses

These include herpesviruses such as bovine herpesvirus type-1 (BHV-1), pseudorabies virus, and equine herpesvirus-1 (EHV-1), as well as poxviruses such as poxvirus, parapoxvirus (PPV), and lumpy skin disease virus (LSDV). Canine parvovirus type 2 (CPV-2) from the *Parvoviridae* family and RNA viruses such as FMD from the *Picornaviridae* family, bovine viral diarrhea virus (BVDV), and classical swine fever virus (CSFV) from the *Flaviviridae* family, rotaviruses from the *Reoviridae* family, and *Influenza* A from the *Orthomyxoviridae* family. Additionally, antiviral compounds derived from natural sources have demonstrated broad-spectrum activity against a variety of DNA and RNA viruses as shown in **Tables 1–3** [72].

Active of plant compound	Mechanism virus target	Example of plant source	References
Flavonoids: Amentoflavone, theaflavin, iridoids, phenylpropanoid glycosides, agathisflavone, robustaflavone, rhusflavanone, succedaneflavanone, chrysosplenol C, morin, coumarins, galangin (3,5,7-trihydroxyflavone), baicalin	Disrupt viral enveloped Blocking RNA synthesis. Exhibited HIV-inhibitory activity	These active component isolated from the ethanol extract of *Selaginella sinensis*. *Scutellaria baicalensi*, *Agastache rugosa*, *Euphorbia grantii*, *Barleria prionitis*, *Calophyllum cerasiferum, Calophyllum inophyllum*, *Cal.teysmannii*, *Garcinia multiflora*, *Helichrysumureo nitens*, *Maclura cochinchinensis*, *Markhamia lutea*, *Monotes africanus*, *Pterocaulon sphacelatum*, *Rhus succedanea*, *Scutellaria baicalensis*, *Selaginella sinensis*, *Sophora moorcroftiana*, *Sophora tomentosa*, *Tephrosi* sp.	[52–56]
Tannins	Inhibition of viral RNA and DNA replication by inhibition of prooxidative enzymes	*Prunella vulgaris* L. (Lamiaceae) and *Rhizomacibotte*	[54]
Apigenin, Linalool, and Urolic Acid	Blocking virus replication have a broad spectrum of anti-DNA and anti-RNA virus activities	*Elsholtziarugulosa* Hemsl. (Lamiaceae), a common Chinese herb	[57, 58]

Active of plant compound	Mechanism virus target	Example of plant source	References
Polysaccharides	Blocking virus binding	*Achyrocline flaccida*, *Bostrychia montagnei*, *Cedrela tubiflora*, *Prunella vulgaris*, *Sclerotiumglucanicum*, *Stevia rebaudiana*, *Rhizophora mucronata*	[55]
Alkaloids	DNA and other polynucleotides and virions proteins.	Rutaceae, *Camptotheca acuminate*, *Atropa belladona* (L.), *Swainsona canescens*, *Astragalus lentiginosus*, *Castanospermum australe*, *Aglaia roxburghiana*	[54]
Terpenoids Terpenoids: sesquiterpene, triterpenoids (moronic acid, ursolic acid, maslinic acid and saponin)	Membrane-mediated mechanisms. Inhibition of viral DNA synthesis	*Cokanthera sp.*, *Anagallisarvensis* (Primulaceae), *Cannabis sativa*,*Geum japonicum*, *Glycyrrhiza glabra*, *Glycyrrhiza radix*, *Glyptopetalum sclerocarpum*, *Gymnema sylvestre*, *Maesa lanceolata*, *Olea europa*, *Quillaja saponaria*, *Rhus javanica*, *Strophanthus gratus*	[59]
Miscellaneous phenolic compounds: anthraquinonechrysophanic acid, caffic acid, eugeniin, hypericin, tannins (condensed polymers), proanthocyanidins, salicylates and quinines (naphthoquinones, naphthoquinones and anthraquinones in particular aloe emodin)	Inhibition of viral RNA and DNA replication	*Aloe barbadensis*, *Aster scaber*, *Cassia angustifolia*, *Dianella longifolia*, *Euodia roxburghiana*, *Geum japonicum*, *Hamamelis virginiana*, *Hypericum* sp., *Melissa officinalis*, *Phyllanthus myrtifolius*, *Phyllanthus urinaria*, *Punica granatum*, *Rhamnus frangula*, *Rhamnus purshianus*, *Rheum officinale*, *Rhinacanthus nasutus*, *Shepherdia argentea*, *Syzgium aromatica*, St. John's wort	[53, 56, 60]
Thiophenes	Membrane interaction Phototoxic activity	*Aspilia*, *Chenactis douglasii*, *Dyssodia anthemidifolia*, *Eclipta alba*, *Eriophyllum lanatum*	[61]
Lectins	Viral membrane interactions	*Canavalia ensiformis*, *Lens culinaris*, *Phaseolus vulgaris*, *Triticum vulgaris*	[62]
Antiviral factor	Mechanism of action is not known	*Nicotianaglutinosa*	[63]

Table 1.
Exploring the antiviral potential of plant extract-derived compounds.

4. Conclusion

Viral infections remain a significant challenge in human and animal health, as many viruses still lack protective vaccines and efficient antiviral therapies. However, natural products have shown promising antiviral effects against various viruses, including *Herpesviridae, Flaviviridae, Retroviridae, and Picornaviridae*. Further research into the bioactive compounds and mechanisms of action of these natural products can help to develop effective antiviral drugs. Combination therapy with

DNA Viruses	Mode of action	Plant extracts	References
1. *Herpesviridae* 2. *Bovine herpes virus* 3. *Bovine herpesvirus type 1* (BHV-1) 4. *Equine herpesvirus 1* 5. *Feline herpesvirus-1* (FHV-1) 6. *Pesudorabies virus*	Inhibited viral replication by interfering with the early stages of viral adsorption and replication	1. *Erythroxylum deciduum* 2. *Lacistemahasslerianum (chodat)* 3. *Xylopia aromatica* 4. *Heteropteris aphrodisiaca* 5. *Acacia nilotica (gum arabic tree)* 6. *Lippia graveolens (Mexican oregano or red brush lippia),* 7. *Guettarda angelica (Velvetseed),* 8. *Prunus myrtifolia (West Indian cherry),* 9. *Symphyopappus compressus* 10. *Pimpinella anisum (Anise) T* 11. *Thymus capitata*	[64–67]
Poxviridae 1. *Cowpox virus* 2. *Buffalopox virus* 3. *Parapoxvirus* 4. *Lumpy skin disease virus* (LSDV)	Unknown	1. *Podocarpus henkelii* 2. *Achilleafragrantissima* 3. *Artemisia herba alba plant is known as* 4. *Jasiona Montana*	[11, 12]

Table 2.
Antiviral effects from several plant extracts against specific viruses.

RNA viruses	Mode of action	Plant extracts	References
Picornaviridae Foot-and-mouth disease (FMD)	Combined effects of this polyherbal drug phytochemicals against FMDV and individual plant extracts against FMDV	*Ashwagandha, Tulsi, Turmeric MorindaellipticaL. M. citrifolia L.*	[68]
Flaviviridae: Bovine viral diarrhea virus (BVDV) Classical swine fever virus.	Antiviral activity as on HCV. Antiviral effect partly due to enhancement of the IFN-associated JAK–STAT pathway. Inhibitors of viral replication	*Phylantus orbicularis, Meliaazedarach, Perseaamericana, Acanthospermumhispidum Guazumaulmifolia Stryphnodendronadstringes*	[69]
Reoviridae, rotaviruses, bluetongue virus	Saikosaponin B2 inhibits viral attachment and penetration stages	*Black tea, Citrus aurantium, Marine sponges, Stevia rebaudiana, Alpinia katsumadai (AK), Zingiberaceae*	[70]
Orthmyxoviridae Influenza A.	Inhibits viral entry and release; inhibits viral hem-agglutination and NA activity. Inhibits viral NP RNA levels and polymeraseactivity	*H. erectum, Terminalia chebula Momordica cochinchinensis*	[71]

Plant extracts such as black tea, Citrus aurantium*, marine sponges,* Stevia rebaudiana, Alpinia katsumadai *(AK), and* Zingiberaceae *contain saikosaponin B2, which inhibits viral attachment and penetration stages of Reoviridae, rotaviruses, and bluetongue virus.*
Orthomyxoviridae: Plant extracts from H. erectum, Terminalia chebula*, and* Momordica cochinchinensis *exhibit antiviral effects against influenza A virus. They inhibit viral entry and release, reduce viral hem-agglutination and neuraminidase (NA) activity, and decrease viral nucleoprotein (NP) RNA levels and polymerase activity.*

Table 3.
Antiviral activity of plant extracts against RNA viruses.

natural plants also holds potential for reducing the risk of viral drug resistance. However, extensive safety and drug interaction studies are needed to ensure the effectiveness of these natural remedies. Overall, the exploration of natural products and their bioactivity can aid in supporting global health systems and improving viral treatment.

Recommendation

1. Medicinal plants could be serving as essential sources of antiviral agents for humans and animals diseases but still need further extensive studies for exploration of plants bioactive ingredients consider top global priorities.

2. Indeed, different research studies have been done to increase the antiviral activity of plant extracts and increase its water solubility.

3. Studies of the efficacy of plant extracts *in vivo* is encouraged to help developing effective antiviral drugs as well as studies of natural agent's combination with chemical antiviral therapeutics as multitarget therapy for reducing viral escape mutant.

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Chapter 4

HBV and HCV Infection Prophylaxis in Liver Transplant Recipients

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Abstract

Liver transplantation is the treatment of choice for patients with liver cirrhosis caused by viruses (HCV, HBV, and HBV + HDV) in end-stage liver disease. However, liver transplantation is complicated by the risk of recurrent infection, which significantly affects the graft and patient survival, the main risk factor for the development of recurrent viral infection being the viral load at the time of transplant. The development of antiviral drugs and effective prophylactic regimens using hepatitis B immunoglobulins have significantly improved hepatitis B outcomes after liver transplantation. Hepatitis B virus (HBV) reinfection after liver transplantation (LT) may favor the recurrence of hepatocellular carcinoma (HCC), and combination therapy with hepatitis B immunoglobulin (HBIG) and nucleoside/nucleotide analog may reduce HBV recurrence after LT. In patients transplanted for HCV infection due to the availability of direct-acting antivirals, the survival of transplanted patients is comparable to that of transplants performed for alcoholic liver disease and even better than the survival evaluated in patients with hemochromatosis or hepatitis B infection. However, current approaches continue to be hampered by the extremely high cost of treatment and the emergence of drug-resistant viral mutations.

Keywords: hepatitis B virus, hepatitis C virus, liver transplant recipients, infection, hepatocellular carcinoma

1. Introduction

Chronic hepatitis B (CHB), which is the primary cause of hepatocellular carcinoma, currently represents a major threat to public health since it may result in potentially fatal complications [1]. According to the World Health Organization epidemiological reports, 296 million people were living with CHB infection in 2019, with an annual incidence of 1.5 million and an estimated mortality of 820,000 people [2].

Current CHB therapies based on nucleos(t)ide analogues (NUCs) are effective in minimizing viral load but are curable in less than 5% of cases [3]. The only effective treatment for decompensated CHB cirrhosis, whether or not it is exacerbated by

HCC, is orthotopic liver transplantation (OLT) [4]. A common indication for OLT at the moment is CHB, which accounts for 30% of all OLT procedures in Asia and 10% in Europe and the USA [5]. The key indicators for liver transplantation (LT) in patients with chronic hepatitis B (CHB) infection are severe episodes of infection and long-term consequences, such as decompensated cirrhosis and hepatocellular carcinoma (HCC) [1]. However, in areas where infection persists to be prevalent, CHB tends to be among the most prevalent primary liver conditions for LT [6].

Due to the high likelihood of HBV recurrence following OLT, which causes graft losses and a dismal survival rate of less than 40% after 5 years, CHB was historically regarded as a contraindication [4]. A long-term antiviral preventative medication is required since liver transplantation is not a curative treatment for CHB infection [5]. The primary objective of antiviral prophylaxis is to avoid reactivation, rather than recurrence or reinfection, because the virus is never entirely destroyed after transplant [7]. Oral nucleos(t)ide analogs (NUCs), which have been accessible since 1998, are considered to be the most efficient HBV suppressants with a small probability of developing resistance [8]. A nucleotide analogue (NA) was used as an antiviral medication both pre- and post-transplant when the spontaneous risk for HBV re-infection was previously over 80% [8]. The introduction of hepatitis B immunoglobulins (HBIG) and several generations of NUC over the past three decades has modified the prophylaxis schedule advised by the majority of worldwide hepatic societies [9].

In 90–100% of individuals, the combination of both prevents HBV recurrence. According to theory, ongoing therapy could reduce the risk of HCC while additionally delaying the onset of cirrhosis, decompensation, and acute flares [10].

In worldwide studies, 5% of HBV-infected people were additionally discovered to have exposure to the hepatitis delta virus (HDV) [10]. This article discusses the present level of research regarding the treatment and prevention of post-LT recurrent HBV and HDV infections, as well as the effect that these infections have on liver allograft outcomes [10].

HCV infection is one of the most common causes of liver transplantation, as it currently accounts for 40% of persistent liver damage in the United States. The newly transplanted graft will become infected during transplant reperfusion if the infection is neglected before surgery [11]. Eliminating the viral infection before transplant is now the greatest way to avoid the recurrence of the graft itself with HCV after the procedure [12].

In HBV/HCV-coinfected LT recipients, reinfection was decreased by hepatitis B immune globulin (HBIG) [13]. Due to their ability to prevent HCV infection within human hepatocytes and lower viral loads in experimental animals, two HCV-neutralizing human monoclonal antibodies showed an in vitro advantage [12]. There is an urgent need for more research on HCIGs, and several studies are already being conducted. Following a liver transplant, what is referred to as "prophylaxis" for recurrent HBV and HCV is disputable, suppressive rather than preventative, and is likely to continue for a lifetime [14].

2. Chronic hepatitis B infection

The Hepadnaviridae family includes the small DNA virus known as the hepatitis B virus (HBV) [15]. Eight genotypes of HBV, ranging from A to H, have been identified, each with a unique geographic distribution [16]. The infectious HBV virion (Dane particle) is a spherical, double-shelled structure with a diameter of 42 nm [15]. It

is made up of an inner nucleocapsid which is composed of hepatitis B core antigen (HBcAg) combined with virally encoded polymerase and the viral DNA genome, as well as a lipid envelope containing HBsAg [17]. The partly double-stranded DNA virus HBV reverse converts pre-genomic ribonucleic acid (RNA) into DNA throughout its life cycle [17]. The genome comprises an external phospholipid envelope and an internal nucleocapsid core encoded by the overlapping C, X, P, and S open reading frames [18].

The pathogenic virion known as Dane particles expresses the envelope protein HBsAg on their surface. The presence of HBsAg in the bloodstream is evidence of active HBV infection [15]. Anti-HBc antigen identification suggests acute, chronic, or cured infection but not immunity caused by vaccination and demonstrates previous contact with HBV [19]. The detection of HBeAg along with viral replication in progress is suggestive of the individual's infectiousness. Anti-HBs are a sign of healing and vaccination against HB infection, either through the HB vaccine or disease [19].

HBV is transmitted from mother to child after exposure being exposed to infected blood or bodily fluids or through sexual contact. Additionally, HBV can live and remain contagious on wet surfaces at ambient temperature for a couple of weeks [20]. The primary and most reliable method of preventing HBV exposure is vaccination (WHO 2019a). Since its introduction in 1982, the HBV vaccination has significantly reduced the number of HBV infections worldwide [2].

Hepatitis B (HB) is a serious health threat that can manifest as acute, chronic, severe liver failure, and malignancy, resulting in high morbidity and mortality despite the existence of efficient immunizations and therapeutic options [15]. Chronic infections are most prevalent in immunocompromised patients and are age-related in terms of risk and prognosis. It is generally accepted the theory according to which the infection contracted in youth will evolve into chronic infection [19]. Acute infections are typically cleared in individuals with impaired immunity, while 90% of babies, 30–50% of children under the age of five, and 5–10% of adults suffer from chronic infections [19]. The typical manifestations of an acute infection, which may persist for 2–3 months, include fever, jaundice, abdominal discomfort, nausea, and vomiting [5]. The age of the person at the moment of infection, the manner of acquiring, ethnic background, and the genotype all affect the typical course of chronic HBV in children, which is characterized by the persistence of HBsAg for a period of time exceeding 6 months. Although acute liver disease may develop in young adults, chronic hepatitis is infrequent in this demographic group [21]. A high probability of the occurrence of chronic infections with evolution towards liver cirrhosis is specific to hemodialysis patients who have coinfection with the HIV virus [21].

Chronic HBV develops in an unpredictable way via four stages with clinically distinct symptoms, while there is occasionally overlapping [4]. The immune-tolerant stage, which may persist for years but the majority of patients exit around the teenage years, is characterized by low hepatic inflammation in the majority of children regardless of an elevated rate of HBV replication [22]. The immune clearance phase, which comes after this one, is marked by liver damage as well as phases involving moderate and elevated HBV replication as the body's defense mechanism works to eliminate HBV [23]. A child may reach the chronic inactive carrier stage if they seroconvert (go from being HBeAg negative to HBeAb positive) after eliminating the virus. Despite the fact that this condition is typically regarded as benign, 20–30% of individuals may experience HBV reactivation in the presence of immunodeficiency or HBV mutation. To determine the stage of infection and to assess the host

immunological response, it is required to continually track levels of HBV genetic material and alanine transaminase (ALT) [23].

Considering the clinical manifestations and symptoms of chronic HBV may prove nonexistent or subtle, a complete evaluation of the patient's risk elements and medical records is necessary for the diagnosis.

The most effective way to evaluate inflammatory processes, fibrosis extent, the existence of additional sources of liver illnesses, and to track the progression of the disease following therapy is by liver biopsy [24]. The immune clearance phase is marked by portal inflammatory disorders, interface hepatitis, and varied fibrosis, although biopsy evidence of liver inflammation is limited in the immunological-tolerant phase [24]. Whilst neither of the noninvasive indicators is currently regularly advised as standard testing, they are being studied as a means of determining the degree of liver fibrosis in HBV patients [21]. Simple or non-invasive indicators have been demonstrated to be helpful in patients with chronic HBV, such as the aspartate aminotransferase (AST)-platelet ratio index and the fibrosis-4 index based on platelets, ALT, AST, and age [25].

Patients suffering from cirrhosis from an infection with HBV are at increased risk for developing HCC. On an annual basis, 600,000 people die globally from cirrhosis, hepatocellular cancer, acute liver failure from recent infections, or reactivation in chronic carriers [26]. 10% of liver transplants are for chronic hepatitis B infections, although the graft could become reinfected as a result [26].

Infections with HBV can occur in liver transplant recipients in two distinct manners [10]:

- De-Novo infection: in patients without previous serological proof of HBV infection. This generally happens as a result of HBV transmission from a donor who has tested positive for the hepatitis B core antibody (anti-HBc). Additionally, seronegative donors may spread an "occult" HBV infection [10].

- Re-infection: following a liver transplant, a compromised immune system leads to a spike in HBV multiplication. Re-infection probability has a direct connection with the replication of HBV prior to LT. Two scenarios may lead to a re-infection: (1) circulating HBV particles or (2) excessive production of immunological HBV particles (escaped mutants) in extrahepatic places like the peripheral blood mononuclear cells, which is referred to as the "Immune Pressure mechanism" [10].

Increased HBV DNA levels than those from de novo infections are linked to re-infection.

Serum HBsAg and HBV DNA are biomarkers of recurrence cancer of the liver, one of the side effects of recurrent HBV. Re-infection can, in rare instances, progress into fibrosing cholestatic hepatitis, a subtype of the disease marked by early fibrosis [27]. Nevertheless, candidates for LT for chronic HBV illness must have anti-viral prophylaxis.

3. Prevention of post-transplant HBV recurrence

Because of the substantial likelihood of HBV reappearance following OLT, which can lead to graft failures and a dismal survival rate after 5 years of below 40%, CHB was formerly regarded as a contraindication [28]. Hepatitis B immunoglobulins (HBIG) and

several generations of NUC have modified the prophylactic protocol adopted to avoid HBV recurrence during the past thirty years. The risk of HBV recurrence following OLT, determined by HBsAg positive in the serum, is presently below 10% when third-generation NUC and HBIG perfusions are used together. In consideration of this, the majority of worldwide hepatic societies advise using this combination preventive strategy [29]. Due to the substantial expense of the administration of HBIG by i.v. infusion or s.c. injection, minimizing the HBIG preventive measures period is taken into consideration in certain groups of patients according to the absence of risk factors like pre-OLT viral load, HDV coinfections, and when optimal patient compliance to treatment is ensured [30]. For the prevention of HBV recurrence, NUC monotherapy without HBIG has been documented in various trials. After using NUCs with an elevated barrier to resistance, these research investigations stated a rate of detectable HBsAg in serum of 8–10% after a period of time of 2–8 years post OLT, which was linked to sustained suppression of the virus and a lack of virologic recurrence [2].

There are a number of established risk factors for HBV recurrence before LT: HBeAg positive and a detectable blood HBV DNA level. Additional warning signs for HBV re-infection include simultaneous HDV infection or human immunodeficiency virus (HIV) infection, HCC before LT, limited adherence to antiviral therapy, and antiviral medication resistance [10].

Prior to the development of HBV prevention, confirmations of recurrence were frequent. The prophylactic period first began with HBIG alone, which was later utilized alongside antivirals. Antivirals are now also utilized on their own. The therapy combinations, however, vary per facility and are always changing [10].

In order to prevent HBV recurrence, HBIG, a polyclonal antibody targeting HBsAg, was developed in the last decades of the nineteenth century. It was groundbreaking research by Samuel et al. that indicated a significant decrease in HBV recurrence, from 75% in patients getting no medication or short-term HBIG therapy to 33% in those undergoing long-term IV HBIG medication, and was linked to better graft and patient outcomes. As a result, there was no agreement on the frequency, dosage, or length of HBIG [31].

Additionally, it presented drawbacks such as higher expenses, resistance, the need for intravenous infusion, a lack of accessibility, the requirement for follow-ups and lab monitoring, local and systemic adverse reactions, and the inability to achieve appropriate protective anti-HBs concentrations for individuals [18]. As a result of these limitations and the development of antivirals that work in concert with HBIG, their usage as monotherapy was discontinued [32].

Initial investigations on LAM as a monotherapy revealed up to 18% recurrence over 3 years of treatment [33]. However, monotherapy with strong antivirals showed encouraging outcomes. These outcomes offer a more cost-effective approach to reduce HBIG consumption. Benefits should not be extrapolated,However, monotherapy should only be administered to a small number of lowrisk individuals under close observation [2].

NAs altered the HBV LT management environment. They reduced the recurrence incidence by less than 10% when combined with HBIG [34]. The initial type of NA was LAM, and its inaugural successful investigation was made public in 1998 by Markowitz and associates from the UCLA Medical Center [35]. A more extensive patient follow-up in Italian research by Marzano et al. that revealed 4% recurrence with the same combo treatment provided more evidence for this [35]. This motivated doctors to combine different NAs, and a meta-analysis revealed that a combo of adefovir dipivoxil (ADV) and HBIG is better than HBIG/LAM [10]. The adoption of more recent NAs with strong

genetic resistance barriers, such as entecavir (ETV) or tenofovir disoproxil fumarate (TDF), followed. Nevertheless, among individuals who have been exposed to LAM, ETV is linked to high resistance, and TDF safety concerns regarding bone and renal function are quite important [36]. Tenofovir alafenamide (TAF), a tenofovir prodrug, is, therefore, an option for patients with these worries [36].

Different reduced doses and methods were investigated, which were provided intramuscularly (IM) every day for 1-week post-transplantation and monthly due to HBIG's restrictions (cost, IV mode of administration) [37]. Additionally, it was more than 90% less expensive than a high-dose prescription. Furthermore, trials using subcutaneous (SC) injections of HBIG revealed improved satisfaction among patients, less pain, and over 90% adherence to maintaining protective anti-HBs blood concentration [38]. Only individuals with minimal viral load before to transplant have been shown to benefit from low-dosage HBIG, according to studies. Therefore, the dose should be customized for each patient while anti-HBs levels are continuously monitored [10].

Until NAs were developed, HBIG was administered as monoprophylaxis at a high dose to stop the recurrence of HBV following LT. LMV, ADV, or any one of these medications plus HBIG were initially administered to avoid HBV recurrence after LT following the introduction of NAs [39]. Since LMV and ADV have poor effectiveness and a high incidence of resistance development, they are currently not suggested as the best primary preventive treatment [40]. Effective and well-tolerated in preventing HBV recurrence after LT is ETV or TDF combined with HBIG. Another approach that is currently used involves starting an HBIG/NA combination, stopping HBIG, and continuing NA as a monotherapy [41].

As a result, the findings demonstrate the effectiveness of high susceptibility barrier antivirals and provide evidence in favor of the possibility of stopping HBIG if the cost is minimal. In individuals who had high levels of detectable HBV DNA after transplant, had HCC as a sign of needing a transplant, or were coinfected with HDV, it should be maintained for a longer period [41]. After LT, there are center-specific rehabilitation alternatives, but there are also several suggestions from societies all across the world. The American Association for the Study of Liver Diseases (AASLD) advises against ever using HBIG monotherapy [42]. They emphasize that treatment should last a lifetime and that ETV or TDF should constitute the chosen antiviral medication due to reduced resistance with prolonged usage [42].

For end-stage liver disease/HCC caused by HBV/HDV, liver transplantation is the gold standard therapy, with results that are on parity with those of the other criteria. The proper care and primary prevention of viral recurrence are essential to its effectiveness [43]. Presently, either the transplant center or the transplant region determines the prevention and treatment of recurrent HBV/HDV [2]. However, considering the information together, it is reasonable to assume that the strategy for therapy should be customized to the particular features of every patient. Importantly, the dosage and duration of HBIG should be based on the patient's HBV viral level at the time of transplantation [37]. However, the stronger antivirals (ETV, TDF, and TAF) have to be used as first-line medications and kept up throughout life, whether in combination or as monotherapy [20].

4. Recipients of anti-hepatitis B core-positive livers

Due to the enormous number of patients on the waiting list for liver transplantation and the limited number of donors, the facilities were built to accommodate

the use of liver transplants from donors who had positive anti-HBc antibodies [44]. Most of these donors had previously been infected with HBV (anti-HBc positive and hepatitis B surface antigen [HBsAg] negative) [45]. Liver grafts from HBc antibody-positive patients should be transplanted into HBsAg-positive patients before transplantation as they will usually receive post-transplant prophylaxis to prevent re-infection with HBV due to the shortage of adequate liver grafts, there are cases in which patients without HBV infection received livers from donors with positive anti-HBc antibodies [41]. For recipients without a history of chronic HBV infection, the risk of graft-related HBV infection is decreased with antiviral prophylaxis. Graft-related infection is defined as the appearance of HBV surface antigen and detectable HBV DNA in the recipient [10]. HBV infection can be acquired from the liver of the anti-HBc-positive donor due to the persistence of HBV covalently closed circular DNA (cccDNA) in the donor's liver, which can be reactivated secondary to recipient immunosuppression [2].

The risk of graft-related hepatitis B infection from an anti-HBc-positive donor liver varies with the recipient's HBV status. Patients who have not been infected with HBV (HBs Agnegative, anti-HBc negative, and anti-HBs antibodies negative), people with immunity due to vaccination (HBs Ag negative, anti-HBc negative, and anti-HBs positive), and patients who have passed through HBV infection with spontaneous healing (anti-HBc positive, HBsAg negative) [46].

The combined administration of Human anti-hepatitis B Immunoglobulin (HBIG) and nucleotide(z)ide analogues (tenofovir, entecavir, and lamivudine) for an indefinite period, with the maintenance of an anti-HBs titer of more than 100–150 IU/ml in people with HBV DNA negative and 500 IU/ml in people with positive HBV DNA in pre-TH represents the universal prophylactic regimen recommended for preventing HBV reinfection of the graft [41].

The recommended initial regimen consists of the administration of HBIG 10,000 IU i/v during the anhepatic phase, followed by 2000–10,000 IU i.v. daily in the first post-transplant week, with subsequent administrations being adjusted to maintain the Anti-HBs titer above 100–150 IU/ml in pre-transplant HBV DNA negative patients and over 500 IU/ml in pre-transplant HBV DNA positive patients [6].

All recipients of anti-HBc-positive liver grafts should receive lifelong antiviral prophylaxis and the antiviral agent should be initiated at the time of transplantation. Initially, an antiviral agent with a low resistance barrier (Lamivudine) was used for antiviral prophylaxis, then entecavir or tenofovir alafenamide (TAF), due to its efficacy, tolerability, and high barrier to resistance [44].

Cholongitas, Papatheodoridis, and Burroughs showed in a study published in 2010, on a group of >900 patients who received a positive HBc graft, that approximately 20% of recipients with negative HBsAg left without antiviral prophylaxis presented de novo infection and that patient the probability was lower in those who went through HBV infection (anti-HBc/anti-HBs) [47].

5. HVB/HDV coinfection

Reinfection prophylaxis in patients transplanted for HBV and HDV co-infection is not very clear due to the fact that the presence of HBsAg is mandatory for viral replication of HDV [10]. Thus, the recurrence of post-transplant HBV infection, in patients with pre-transplant HBV/HDV co-infection, can lead to re-infection of the liver graft, including delta virus, which complicates the post-transplant

evolution [48]. Some guidelines recommend long-term administration of HBIG prophylaxis usually combined with a nucleotide/nucleoside inhibitor. Older studies evaluated the efficacy of long-term combination HBIG with LAM as prophylaxis for transplant patients for HBV/HDV co-infection [41]. No recurrence of HBV/HDV co-infection was observed in either group, however, the authors concluded that a preference for the HBIG-LAM combination was a cost-effective treatment. Option [49].

Approved treatment for HBV monoinfection such as nucleoside/nucleotide inhibitors does not affect HDV viral load and currently, there is no globally approved treatment for HDV [50]. Pegylated Interferon Alpha (PEG-IFN-alpha) has been used for decades to treat HDV infections and has shown a virologic response rate of up to 30%, with use limited by severe adverse effects that have compromised therapy and its use among patients with decompensated liver disease or in the post-transplant setting [51]. Recently, Bulevirtide, a lipopeptide that blocks the binding of HBsAg-coated particles to the sodium taurocholate cotransporter polypeptide (NTCP), which is the cellular entry receptor for both HBV and HDV, was approved by the EMA at a dose of 2 mg/day, subcutaneous injection, in patients with HDV coinfection [52]. Clinical studies have confirmed the efficacy and safety of Bulevirtide, even in patients with advanced-stage compensated cirrhosis [51].

However, the duration and dose of the antiviral treatment have not yet been precisely established. More studies are needed to understand the mechanisms of virologic response, build models to predict therapeutic response, determine discontinuation criteria, and test drug efficacy and safety in special populations such as transplant patients [41]. Two investigational drugs, PEG-IFN and Lonafarnib, are currently in phase III, but these drugs, as well as Bulevirtide, are not been tested in transplant patients; specific prophylaxis for transplant patients for HBV/HDV co-infection is not yet available, but because HBV needs HBV to infect hepatocytes and replicate, the primary goal of post-transplant prophylaxis in patients with HDV co-infection is to prevent HBV relapse [52].

Caccamo et al. compared HBIG monotherapy with the combination of lamivudine and HBIG and found no cases of HDV recurrence in either group and suggested combination therapy as a more cost-effective option because this strategy allows the use of lower doses of expensive HBIG [53]. The use of NUC alone rarely leads to the loss of HBsAg, and this increases the chances of HDV infection developing in new hepatocytes, although the manifestation of HDV infection is difficult without complete HBV reactivation [53]. However, there is no known effective treatment for HDV infection in the post-transplant setting, and lifelong combination therapy of high-barrier NUC and HBIG is the recommended prophylaxis [2].

6. Hepatocellular carcinoma

Patients with HBV infection and hepatocarcinoma have a higher risk of recurrence of HBV infection than those without neoplasia at the time of transplantation [54]. The way in which a neoplasia recurrence occurs could be explained by the persistence of extrahepatic metastatic cells, which are difficult to eradicate and act as a reservoir for the hepatitis B virus [55].

Hepatitis B virus (HBV) reinfection after liver transplantation (LT) may favor the recurrence of hepatocellular carcinoma (HCC), and combination therapy with hepatitis B immunoglobulin (HBIG) and nucleoside/nucleotide analogues may reduce

HBV recurrence after LT [5]. Before effective prophylaxis was available, HBV reinfection often led to early graft cirrhosis with a 2-year mortality rate of 50% [26].

The current antiviral prophylactic regimen of hepatitis B immunoglobulin (HBIG) and nucleoside/nucleotide analogues has decreased viral recurrence and dramatically improved clinical outcomes after LT for HBV [25]. The pathological progression of HBV-induced liver injury to hepatocellular carcinoma (HCC) is well documented. Unfortunately, historically, up to 40% of patients have experienced tumor recurrence after LT [56].

Lamivudine (LAM) monotherapy has recently been shown to delay the progression of advanced liver disease and the subsequent development of HCC in nontransplant patients. Moreover, survival has been improved with a combination of HBIG and nucleoside/nucleotide analogues in patients undergoing liver transplants for HBV-associated disease [5]. Previous research suggests that the successful treatment of HBV is associated with improved survival and a reduction in HCC recurrence after transplantation. Whether the combination of HBIG and antivirals has a direct antineoplastic effect that is distinct from the suppression of viral replication is not known [37].

7. Patients receiving HBsAg-positive grafts

Since the early nineties, researchers have been studying the potential of liver transplant from HBsAg-positive patients because of the lack of patients who could have become donors; so, they decided to use grafts from sources they have not used before for patients who have been diagnosed with cirrhosis and hepatocellular carcinoma [57].

From 1994 to 2006, 8.5% of about 35,000 adult transplant recipients in the United States received anti-HBc-positive grafts due to the presence of occult infection of some of the grafts [58]. The first transplant with an HBsAg-positive graft was reported in 1994 when a critically ill woman received a seropositive graft [44]. She has done the profilaxis with HBV immunoglobuline but shortly after that she was found with liver dysfunction and diagnosed with chronic hepatitis B [59]. This case report has been followed by an unicentric study on 10 patients from 2005 to 2009 [59]. Patients who underwent surgery for liver transplant for HBV–related disease have never cleared the HBs Antigen; some of them cleared the HBs Antigen and others tested positive for HBV whereas they never tested positive for it before [38]. All in all, none of the patients have had hepatitis, mostly because HBV replication was controlled by the antiviral therapy [38].

A retrospective analysis of a larger study back in the early 2000 has also confirmed that 78 of 92 patients have received HBs antigen-positive grafts, but the recipients already had a history of HBV infection [44].

These results were confirmed by Chinese researchers who have done a retrospective study on about 370 patients who underwent liver transplants from 2010 to 2013, and a few of them (N = 42) received an HBs-positive graft. Post-transplant follow–up has shown similar liver function in both of the two groups and the complications—such as acute rejection, liver dysfunction, and biliary complications—were similar. They concluded that doctors all over the world should abandon the administration of hepatitis B immunoglobuline in favor of antiviral therapy [60].

On the other hand, other case report suggested that even after immunoglobuline profilaxis was used in association with Lamivudine and Adenofovir, some patients

remained positive for HBs antigen [61]. The therapy has been switched to pegylated interferon and after that to Tenofovir. None of them seemed to work so the patient has revealed symptoms of cirrhosis and has been listed again for liver transplant. Nowadays, the British transplant society's guidelines suggests that HBs antigen-positive liver donation is possible because of antiviral drugs such as Entecavir or Tenofovir because they can successfully stop the replication of the virus and prevent graft damage [61]. All the patients should be tested for VHD co-infection because a positive test means the transplant will not be possible because of the high risk of liver cirrhosis after transplant.

Therefore, the British researchers recommend [62]:

- that only in case of emergency HBs antigen-positive liver will be used for a HBs antigen-negative patient
- patient who underwent surgery for a liver transplant and got a positive graft, should be treated with entecavir or tenofovir since the surgery
- HBs antigen-positive livers can be transplanted to HBs antigen-negative patients only if the co-infection with VHD virus is not present
- HB immunoglobuline is not recommended anymore

8. Hepatitis C infection and recurrence post-transplant

The initial evaluation of HVC-infected patients involves a full clinical and biological evaluation of the patient so that we can start antiviral therapy. Sometimes patients discover the infection when the fibrosis is already advanced [63]. Furthermore, patients should get an ultrasonography and an upper endoscopy twice every year so that their medical team can screen them for hepatocellular carcinoma or spot the esophageal varices [63].

Since 40% of the chronic liver disease in the United States is caused by HCV, this infection becomes one of the most common indications for liver transplant. If the infection is not treated before the surgery, the new liver will get infected when the graft is reperfused and it could cause graft failure [11]. Without antiviral therapy before the transplant, the evolution of the graft is variable. In most cases, patients who have not been cured before the surgery will develop histologic damage. On the other hand, some of them will develop cirrhosis shortly after the transplant [48].

Although disease disease-free patient is the targeted patient for transplant, there a critically ill patients on the transplant list who have decompensated cirrhosis, and the antiviral therapy is not suitable for them, so it would be a lot more beneficial to postpone treatment until after the transplant [64]. Furthermore, patients with child C cirrhosis have a lower response to antiviral therapy so this implies there is a higher risk of resistance to the antivirals. The virus could also mutate and the therapy regimen would get even more complicated. All in all, the decision to treat HCV should be individualized. The target is to increase life expectancy with treatment before or after liver transplant (**Table 1**) [65].

Antiviral therapy BEFORE transplant	Postponed therapy – after transplant
• Compensated cirrhosis and HCC • Decompensated cirrhosis but no HCC when the MELD score is relatively low (e.g., <20) and there are no other conditions such as severe portal hypertension that warrant prompt transplantation • Decompensated cirrhosis and HCC when expected wait time for transplantation is more than 3–6 months	• Advanced decompensated cirrhosis with an anticipated wait time less than 3 months • Decompensated cirrhosis and HCC with an anticipated wait time less than 3–6 months

Table 1.
International liver transplantation society recommends [66].

Due to the availability of Direct Acting Antivirals, transplanted patient's survival is comparable with the one regarding the transplants performed for alcohol liver disease and even better than the survival rate for patients with hemochromatosis or hepatitis B infection [66].

The only test necessary for patient medical management is the direct identification of the HCV virus to determine whether a patient has an active infection with HCV. Regardless of this, HCV is currently diagnosed in two stages: first, HCV antibodies (anti-HCV) are detected using both centralized laboratory tests or rapid diagnostic tests to identify exposure to the virus secondly, for individuals with anti-HCV positive, active HCV infection has been established by nucleic acid testing (NAT) or HCV core antigen (HCVcAg) detection [67].

After patients are stabilized post-transplant, it is recommended to test for HCV RNA [68].

The specific expenses of each test determine whether anti-HCV screening is necessary before HCV RNA and/or HCVcAg testing. This two-step diagnosis process will probably continue until the price of an HCV RNA or HCVcAg test is drastically decreased [67].

For the treatment of hepatitis C, telaprevir (TVR), a protease inhibitor, is used with pegylated interferon alfa-2b and ribavirin in a Japanese study. TVR significantly inhibits CYP3A4 and CYP3A5. Effective TVR therapy for liver transplant recipients who experienced a relapse of hepatitis C while undergoing immunosuppressive medication was reported [69].

All liver transplant recipients with detectable viremia should be treated because they can achieve sustained virologic response and the rate of liver fibrosis progression will be lower. If the conventional therapy does not work, the patients could be listed again for transplant, although the prognosis is generally poor [68].

Patients with detectable HCV viremia at the time of transplant are certain to have recurrent infection of the transplanted liver allograft, which can result in a variety of diseases ranging from asymptomatic chronic infection to an aggressive fibrosing cholestatic hepatitis. Recurrent HCV is a primary cause of allograft loss, morbidity, and death in the post-transplant population because it is more frequent in this group [70].

American and European Associations of Studying Liver Diseases (AASLD and EASL) recommendations for treating recurrent HCV infection after transplant are listed in the **Table 2** [71].

Genotype	AASLD/IDSA	EASL
1	Ledipasvir + sofosbuvir + ribavirin for 12 weeks	Sofosbuvir + daclatasvir +/− ribavirin for 12–24 weeks
	Ledipasvir + sofosbuvir for 24 weeks (alternate regimen if ribavirin intolerant or ineligible)	Sofosbuvir + simeprevir +/− ribavirin for 12 weeks (alternate regimen)
	Sofosbuvir + simeprevir +/− ribavirin for 12 weeks (alternate regimen)	—
	Paritaprevir + ritonavir + ombitasvir + dasabuvir for 24 weeks (alternate regimen)	—
2	Sofosbuvir + ribavirin for 24 weeks	Sofosbuvir + ribavirin for 12–24 weeks
3	Sofosbuvir + ribavirin for 24 weeks	Sofosbuvir + daclatasvir +/− ribavirin for 12–24 weeks
4	Ledipasvir + sofosbuvir + ribavirin for 12 weeks	Sofosbuvir + daclatasvir +/− ribavirin for 12–24 weeks
	Ledipasvir + sofosbuvir for 24 weeks (alternate regimen if ribavirin intolerant or ineligible)	Sofosbuvir + simeprevir +/− ribavirin for 12 weeks (alternate regimen)
5	—	Sofosbuvir + daclatasvir +/− ribavirin for 12–24 weeks
6	—	Sofosbuvir + daclatasvir +/− ribavirin for 12–24 weeks

Table 2.
Antiviral strategies in the treatment of human and animal viral infections.

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Chapter 5

Natural Phenolic Acids and Their Derivatives against Human Viral Infections

Yi-Hang Wu, Yan Chen, An-Qi Zhuang and Shan-Mei Chen

Abstract

Natural compounds with structural diversity and complexity offer a great chance to find new antiviral agents. Phenolic acids have attracted considerable attention due to their potent antiviral abilities and unique mechanisms. The aim of this review is to report new discoveries and update pertaining to antiviral phenolic acids. The antiviral phenolic acids were classified according to their structural properties and antiviral types. Meanwhile, the antiviral characteristics and structure-activity relationships of phenolic acids and their derivatives were summarized. Natural phenolic acids and their derivatives possess potent inhibitory effects on multiple viruses in humans such as human immunodeficiency virus, hepatitis C virus, hepatitis B virus, herpes simplex virus, influenza virus and respiratory syncytial virus etc. In particular, caffeic acid/gallic acid and their derivatives exhibit outstanding antiviral properties through a variety of modes of action. In conclusion, naturally derived phenolic acids especially caffeic acid/gallic acid and their derivatives may be regarded as novel promising antiviral leads or candidates. Additionally, scarcely any of these compounds have been used as antiviral treatments in clinical practice. Therefore, these phenolic acids with diverse skeletons and mechanisms provide us an excellent resource for finding novel antiviral drugs.

Keywords: natural phenolic acid, viral infection, structure property, antiviral mechanism, structure-activity relationship

1. Introduction

Viral diseases are caused by pathogenic viruses invading the body of human. The basic process of infection includes: the infectious virions firstly attaching to the membrane of susceptible cells and then entering host cells to begin the replication of viruses [1]. Some viruses cause serious and deadly diseases including human immunodeficiency virus (HIV), hepatitis C virus (HCV), hepatitis B virus (HBV), herpes simplex virus (HSV), influenza virus (IV) and respiratory syncytial virus (RSV) etc. However, the current antiviral agents can only inhibit or reduce viral replication, while cannot clear virus infection thoroughly. Therefore, in the research area of fighting viral disorders, especially those involving potential of fatal development,

there is an urgent need for improved treatment by new antiviral drugs in the whole world.

Phenolic acids, a subclass of polyphenols, are the secondary metabolites from plants or fungi for preventing aggression by pathogens or ultraviolet radiation [2]. Recently, phenolic acids have aroused wide interest owing to their beneficial biological properties such as antiviral and anti-inflammatory activities etc., especially in the treatment of human viral diseases.

2. Structural types of phenolic acids

Phenolic acids are the various types of naturally derived aromatic acid compounds containing a phenolic ring and an organic carboxylic acid function [3]. Naturally occurring phenolic acids include two important types of derivatives of cinnamic acid (C6-C3 skeleton) and derivatives of benzoic acid (C6-C1 skeleton), which originated from non-phenolic compounds of cinnamic and benzoic acids, respectively [4]. Chemically, these compounds have at least one aromatic ring in which at any rate one hydrogen is substituted by a hydroxyl group (**Figure 1**). Phenolic acids are found to be abundant in plants. Furthermore, hydroxycinnamic acid derivatives are more common than hydroxybenzoic acid derivatives [2].

3. Antiviral effects of phenolic acids

3.1 Phenolic acids with anti-HIV activity

HIV is a retrovirus that invades human immune cells and causes acquired immunodeficiency syndrome (AIDS) [5]. Currently, the anti-HIV therapies include the inhibitors targeted at reverse transcriptase (RT), protease (PR) and integrase (IN). The fundamental role of RT in retroviruses replication has made the enzyme a key target in the chemotherapy of HIV infection [6]. The treatment with combinations of RT and PR inhibitors has been proven effective in reducing the levels of circulating virus to below detectable levels. HIV replication depends on the IN that mediates integration of an HIV DNA copy into the host cell genome. This enzyme represents a novel target to which antiviral agents might be directed [7].

3.1.1 Anti-HIV activities of caffeic acid derivatives

The anti-HIV effects of caffeoylquinic acids (CQAs) and caffeoyltartaric acids (CTAs) have attracted extensive attention in recent years. Thereinto, 3,5-di-*O*-CQA,

Cinamic acid derivatives (C6-C3 skeleton) Benzoic acid derivatives (C6-C1 skeleton)

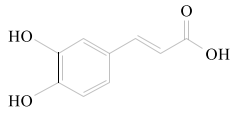
Figure 1.
Structure types of natural phenolic acids.

1-methoxyoxalyl-3,5-di-*O*-CQA (1-MO-3,5-di-*O*-CQA) and L-chicoric acid (**Figure 2**) inhibited HIV-1 IN at 0.06–0.66 μg.ml^{-1} and HIV-1 replication at 1–4 μg.ml^{-1} *in vitro*, respectively [8]. To determine whether the inhibition of IN by CQAs was limited to the 3,5 substitution, 3,4-, 4,5-, and 1,5-di-*O*-CQAs were measured for inhibition of HIV-1 replication and HIV-1 IN *in vitro*. All of the CQAs were found to inhibit HIV-1 replication at 1 to 6 μM in T cell lines. Meanwhile, these compounds suppressed HIV-1 IN at submicromolar concentrations [9]. In addition, molecular modeling of CQAs with IN showed that the most potent inhibitors filled a groove within the predicted catalytic site of IN. The change of internal free energy of the ligand/IN complex is correlated with the ability of CQAs to inhibit HIV-1 IN [9]. Thus, the CQAs are promising leads to new anti-HIV drug discovery.

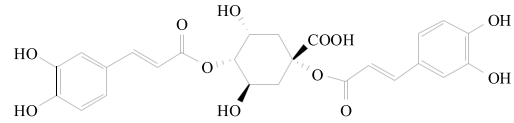
The CQAs and CTAs are highly selective HIV-1 IN inhibitors [8–10]. For instance, 4,5-di-*O*-CQA and 3,4,5-tri-*O*-CQA from *Securidaka longipedunculata* inhibited selectively the replication of HIV and inactivated viral infectivity by specific binding to gp120 which blocks HIV's interaction with CD4 on T cells [11]. However, the specificity of the CQAs and CTAs against HIV-IN remains unknown. Further studies indicated that the CQAs (3,5-di-*O*-CQA, 1-methoxyoxalyl-3,5-di-*O*-CQA, 1,3-di-*O*-CQA, 1,5-di-*O*-CQA, 3,4-di-*O*-CQA and 4,5-di-*O*-CQA) and CTA (L-chicoric acid) (**Figure 2**) inhibited HIV-1 IN at 150–840 nM and HIV replication at 2–12 μM. Their activity against RT ranged from 7 μM to greater than 100 μM. Concentrations that inhibited gp120 binding to CD4 exceeded 80 μM. No compound blocked HIV-1 RNase H by the 50% inhibition concentration (IC_{50}) value exceeding 80 μM. Furthermore, the CTAs were no effects on RT in acutely infected cells. The CQAs and CTAs exhibit >10- to >100-fold specificity for HIV IN [11, 12]. Hence, CQAs and CTAs are potential HIV inhibitors that act at a site distinct from current anti-HIV agents.

Titration experiments with HIV-1 IN or DNA substrate found that the effects of 3,4-di-*O*-CQA, 1-MO-3,5-di-*O*-CQA, and L-chicoric acid were exerted on the enzyme and not the DNA. The inhibition of retroviral INs was relatively specific, and CQAs had no effect on other DNA-modifying enzymes and phosphoryltransferases. Kinetic experiments indicated that the effect of CQAs on IN was irreversible. The inhibition was not affected by preassembling IN onto viral DNA. It suggested that the irreversible inhibition by CQAs on IN is directed toward conserved amino acid residues in the central core domain during catalysis [13]. L-Chicoric acid is a potent IN inhibitor and also inhibits entry at above 1 μM. Kinetic analyses using recombinant HIV IN showed that L-chicoric acid was consistent with a non-competitive or irreversible mechanism of inhibition. Further research demonstrated that L-chicoric acid was reversibly bound to the protein. Thus, L-chicoric acid is a noncompetitive but reversible inhibitor of HIV integration and likely interacts with amino acids other than those which bind substrate [14–16].

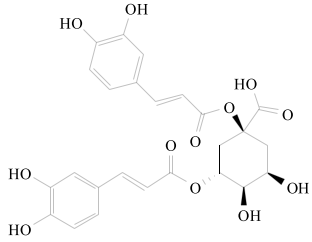
The structure-activity relationships (SAR) analyses suggested that biscatechol moieties were absolutely required for inhibition of IN, while at least one free carboxyl group was required for anti-HIV effect. These data demonstrated that the CTAs and CQAs analogs can be synthesized which have improved activity against HIV IN [17]. The CQAs and chicoric acid, both of which contain two catechol moieties, exhibit remarkable antiviral activity with high potency against IN. Among these inhibitors, hydroxylated aromatics which are contained in all sorts of natural components, have consistently shown marked potency for IN *in vitro*. Two aryl units separated by a central linker, as a common structural feature, are shared by the majority of these inhibitors [18].



Caffeic acid



1,4-di-*O*-Caffeoylquinic acid



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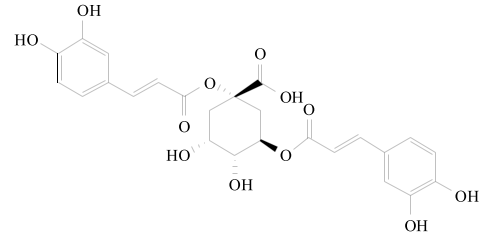

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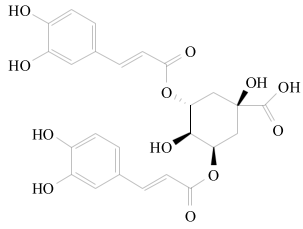
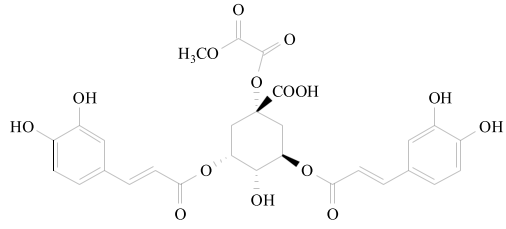
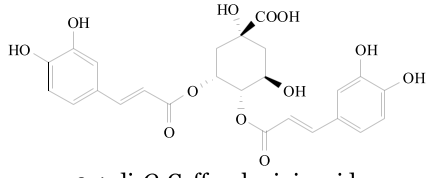


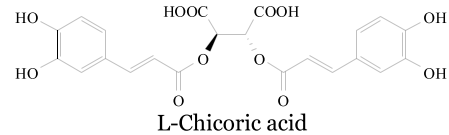
3,5-di-*O*-Caffeoylquinic acid



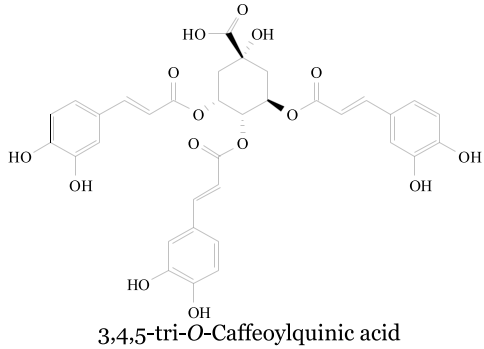
1-Methoxyoxalyl-3,5-di-*O*-caffeoylquinic acid



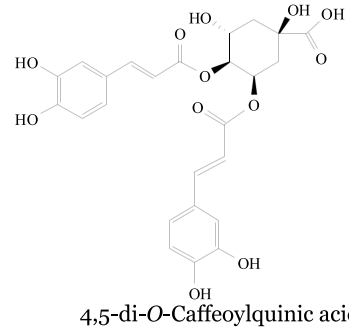
3,4-di-*O*-Caffeoylquinic acid



L-Chicoric acid



3,4,5-tri-*O*-Caffeoylquinic acid



4,5-di-*O*-Caffeoylquinic acid

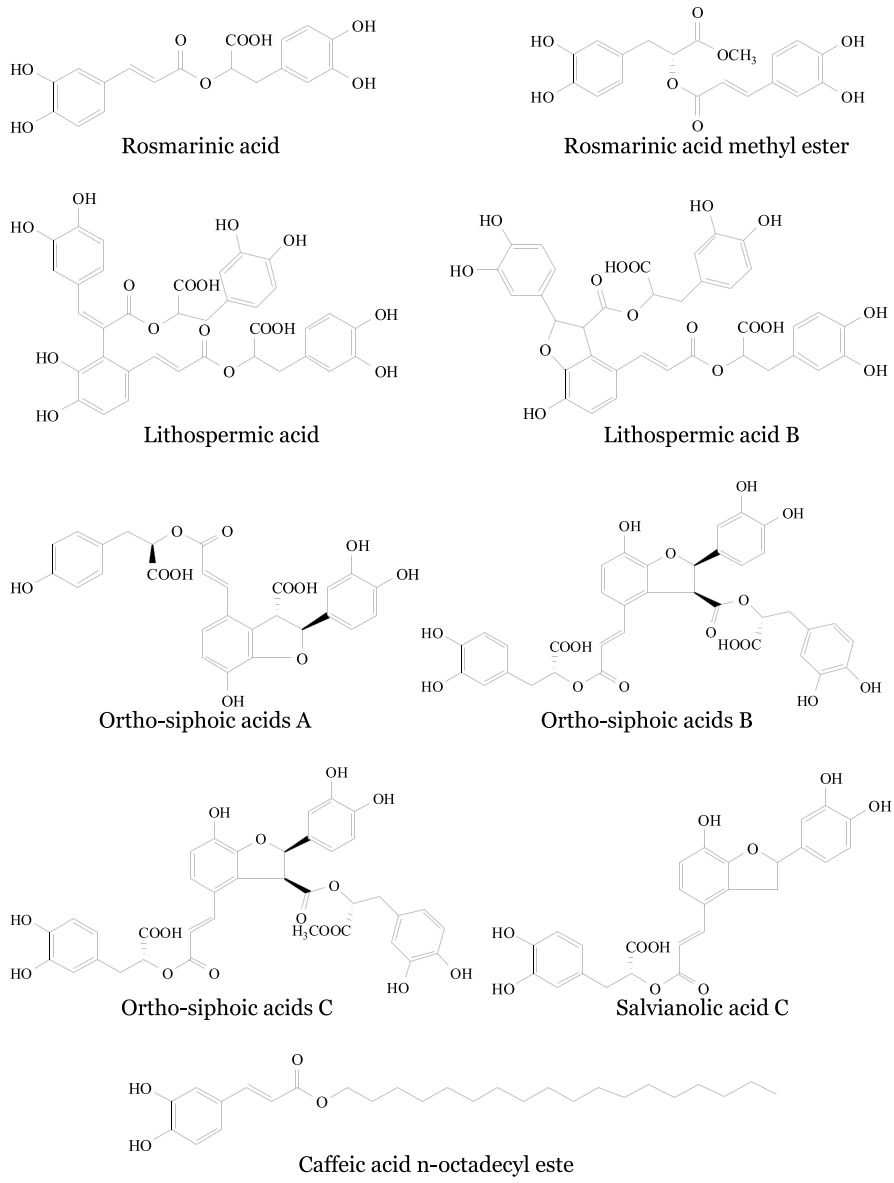


Figure 2.
Structures of natural caffeic acid and its derivatives with anti-HIV activity.

Other caffeic acid derivatives also displayed anti-HIV effects. Rosmarinic acid and rosmarinic acid methyl ester (**Figure 2**) from medicinal plants exhibited inhibitions against HIV-1 IN with IC_{50} values of 5.0 and 3.1 μM, respectively. The dimer, trimer, and tetramer of rosmarinic acid suppressed HIV-1 IN with IC_{50} values of 5.0, 1.4 and 1.0 μM, respectively [19]. Additionally, rosmarinic acid also inhibited RT directly [20]. Caffeic acid n-octadecyl ester (**Figure 2**) from *Daphne acutiloba* Rehd. showed anti-HIV activity with the 50% effective concentration (EC_{50}) value of 0.16 $\mu g.ml^{-1}$ [21]. The monopotassium and monosodium salts of isomeric caffeic acid tetramers from *Arnebia euchroma* displayed potent inhibitory activity against HIV. Furthermore, the potassium and sodium salts were proved to be important to enhance the anti-HIV

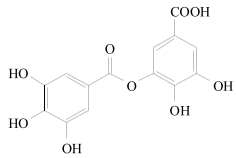
activity [22]. Acteoside and acteoside isomer from *Clerodendron trichotomum* exhibited potent inhibitory effects on HIV-1 IN with IC_{50} values of 7.8 and 13.7 μM, respectively [23].

Lithospermic acid and lithospermic acid B (**Figure 2**) from *Salvia miltiorrhiza* roots are potent anti-HIV inhibitors. The IC_{50} for inhibition of 3′-end-processing by HIV-1 IN was found to be 0.83 and 0.48 μM for lithospermic acid and lithospermic acid B, respectively. In addition, lithospermic acid and lithospermic acid B suppressed HIV-1 IN catalytic activities of 3′-joining to the target DNA with IC_{50} values of 0.48 and 0.37 μM, respectively [24]. The acute HIV-1 infection of H9 cells was strongly inhibited by lithospermic acid and lithospermic acid B with IC_{50} values of 2 and 6.9 μM, respectively. Thus, the two IN inhibitors hold promise as a novel class of anti-HIV agents [24]. Additionally, four phenolic acids (orthosiphoic acids A-C and salvianolic acid C) (**Figure 2**) from *Clerodendranthus spicatus* exhibited anti-HIV-1 protease activity with IC_{50} values of 86.9, 35.9, 38.4 and 74.2 μM, respectively [25].

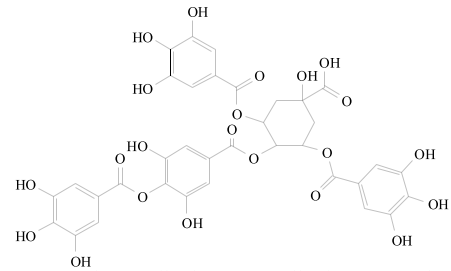
3.1.2 Anti-HIV activities of gallic acid derivatives

In searching for potential anti-HIV agents in natural products, galloylquinic acids (GQAs) were found to show potent anti-HIV activity. Four GQAs (**Figure 3**), 3,5-di-*O*-galloyl-4-*O*-diGQA, 3,4-di-*O*-galloyl-5-*O*-diGQA, 3-*O*-digalloyl-4,5-di-*O*-GQA, and 1,3,4,5-tetra-*O*-GQA, exhibited inhibitory effects on HIV RT and virus reproduction in cells at the concentrations of 10–30 μM [26]. 1,3,4-tri-*O*-GQA (**Figure 3**) was found to inhibit HIV replication and virus-cell interactions in infected H9 lymphocytes [27]. 3,4,5-tri-*O*-GQA (**Figure 3**) from *Guiera senegalensis* selectively suppressed HIV replication and RT by interaction with gp120 to prevent virus binding to CD4 receptor [11]. 3,4,5-tri-*O*-GQA from *Myrothamnus flabellifolia* was shown to inhibit HIV-1 RT. Kinetic monitoring of HIV-1 RT revealed the non-competitive inhibition of 3,4,5 tri-*O*-GQA with an IC_{50} value of 34 μM [28]. These findings suggested that GQAs and related derivatives have potential as indigenous agents for anti-HIV therapy.

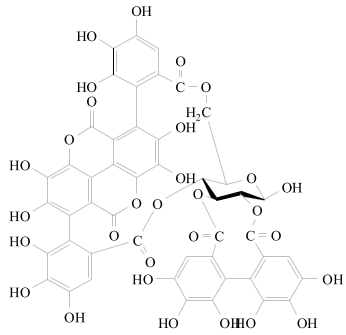
Gallic acid derivatives, 3,5-di-*O*-galloyl-shikimic acid, 3,4,5-tri-*O*-galloylshikimic acid, punicalin, punicalagin, punicacortein C, chebulagic acid and ellagitannin (**Figure 3**), were found to inhibit HIV-cell interactions. 3,5-di-*O*-Galloyl-shikimic acid, 3,4,5-tri-*O*-galloylshikimic acid, punicalin, and punicalagin suppressed the replication of HIV in infected H9 lymphocytes. The purified HIV RT was inhibited by two gallic acid derivatives punicalin and punicacortein C with IC_{50} values of 8 and 5 μM, respectively. Further research indicated that punicalin and chebulagic acid did not directly inactivate HIV in H9 lymphocytes. However, 3,5-di-*O*-galloylshikimic acid was shown to be more efficacious inhibitor among these compounds [27]. Digallic acid (**Figure 3**) from *Acacia farnesiana* inhibited significantly HIV RT by an IC_{90} value of 0.5 $\mu g.ml^{-1}$. The mode of action of digallic acid was partially competitive relative to the template, primer, and noncompetitive to the triphosphate substrate, dTTP. The Ki value of digallic acid was determined to be 0.58 μM for HIV RT [29, 30]. Further studies showed that three hydroxyl groups at the 3, 4, and 5 positions seem to be required for the inhibition of digallic acid derivatives. Besides RT, digallic acid moderately inhibited DNA polymerases α and β, whereas terminal deoxynucleotidyltransferase and DNA polymerase γ were virtually unaffected by this compound [30]. Epigallocatechin-3-gallate (EGCG) (**Figure 3**) from green tea destructed HIV-1 particles and markedly inhibited post-adsorption entry, RT, PT kinetics, and mRNA



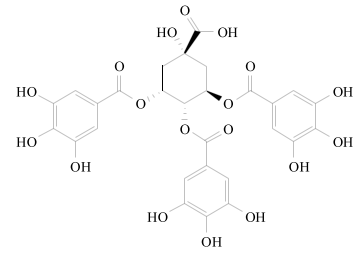
Digallic acid



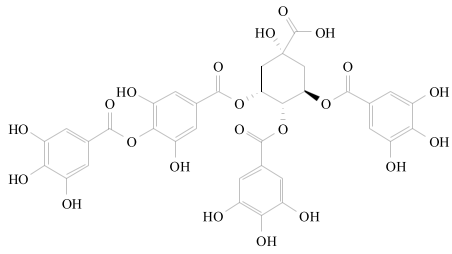
3,5-di-*O*-galloyl-4-*O*-Digalloylquinic acid



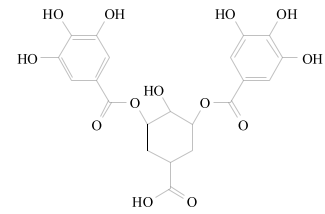
Punicalagin



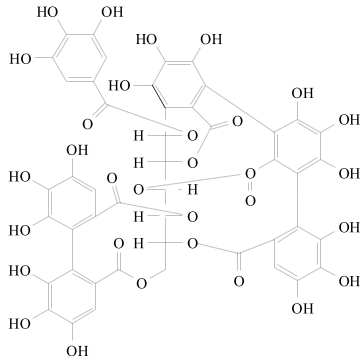
3,4,5-tri-*O*-Galloylquinic acid



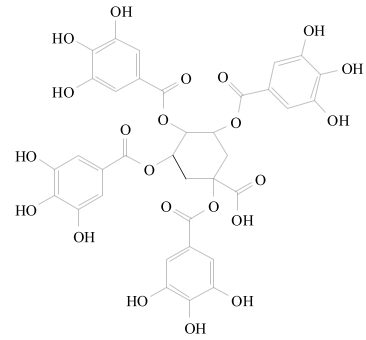
3,4-di-*O*-galloyl-5-*O*-Digalloylquinic acid



3,5-di-*O*-Galloyl-shikimic acid



Ellagitannin



1,3,4,5-tetra-*O*-Galloylquinic acid

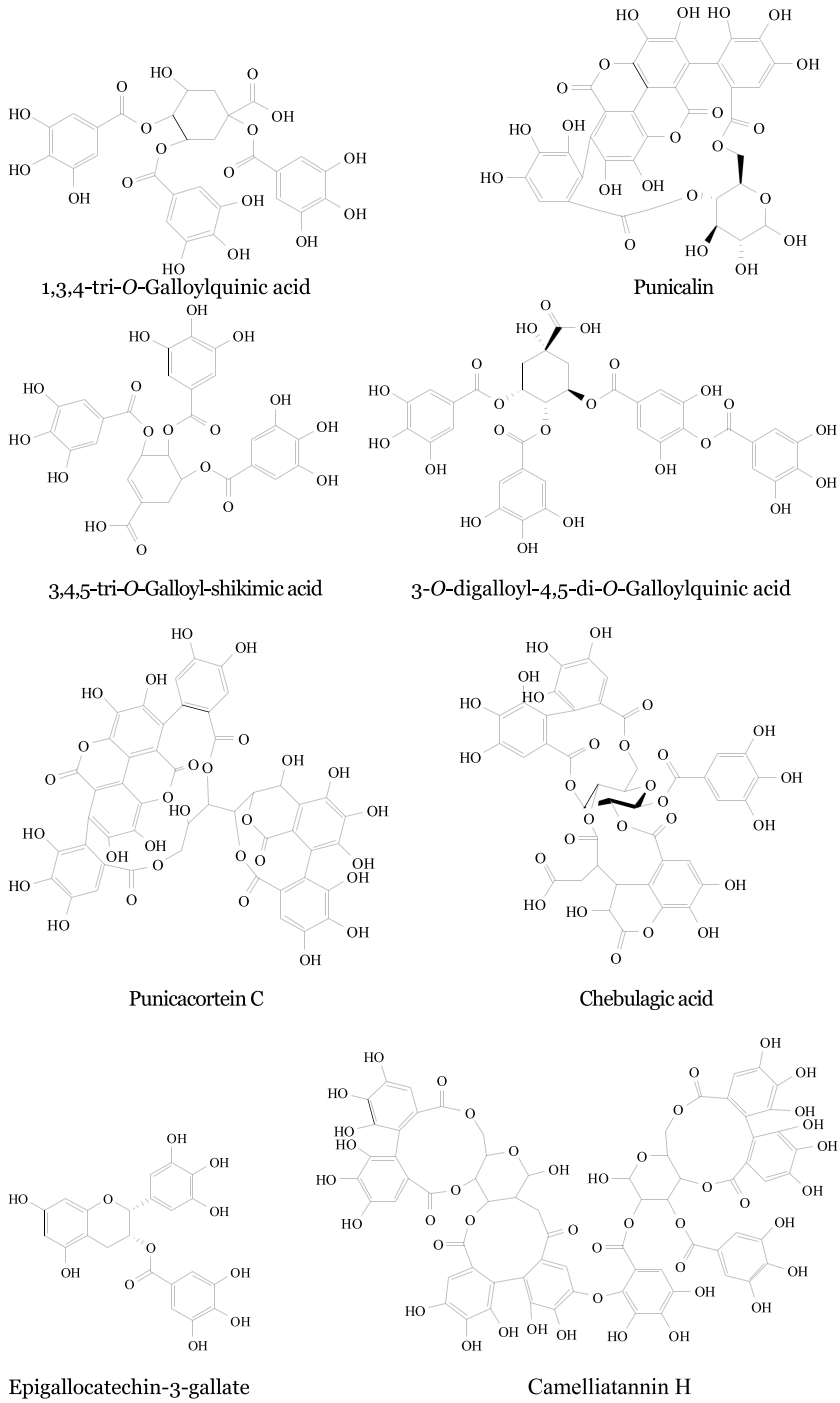


Figure 3.
Structures of natural gallic acid derivatives with anti-HIV activity.

production at micromolar concentrations. Its anti-HIV effect may result from interactions with multiple steps in the viral cycle [31]. Camelliatannin H (**Figure 3**) from *Camellia japonica* potently inhibited HIV-1 PR with an IC_{50} value of 0.9 μM [32].

3.2 Phenolic acids with anti-HCV activity

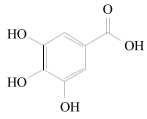
Hepatitis C is an infectious disease caused by HCV and is one of the primary causes of hepatocellular carcinoma. There are about 130–150 million people with chronic hepatitis C globally [33]. Approximately 20–30% of the patients with chronic hepatitis C develop cirrhosis, but only several antiviral agents have been approved against HCV to date [34]. General therapy is often difficult for some HCV genotypes. Hence, new anti-HCV drugs are needed. Natural products provide an abundant resource to screen for potential anti-HCV compounds for promising candidates in the clinic and to improve treatments. The nonstructural protein NS3, NS4A, and NS 5A proteases and RNA polymerase represent the key targets as they are essential for HCV replication [35].

3.2.1 Anti-HCV activities of gallic acid derivatives

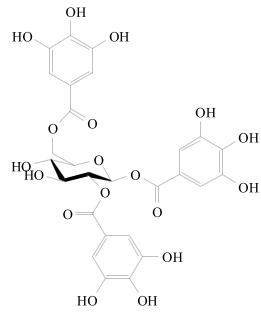
Gallic acid (**Figure 4**) is a natural phenolic acid from plants [36]. A subgenomic HCV replicon cell system was employed to study the effect of gallic acid on HCV expression. The results showed that gallic acid decreased the expression levels of HCV-RNA (∼50%) and NS5A-HCV protein (∼55%). Particularly, gallic acid reduced ROS production at the early time points of exposure in cells expressing HCV proteins. It indicated that the antioxidant ability of gallic acid might be associated with the downregulation of HCV replication [37].

Gallic acid glucosides (**Figure 4**) showed remarkable anti-HCV effect. Three gallic acid glucosides, 1,2,6-tri-*O*-galloyl-β-d-glucose, 1,2,3,6-tetra-*O*-galloyl-β-d-glucose, and 1,2,3,4,6-penta-*O*-galloyl-β-d-glucose from *Rhus chinensis* (Mill.) gallnut, inhibited HCV NS3 serine protease with IC_{50} values of 1.89, 0.75, and 1.60 μM, respectively [38]. Additionally, gallic acid and its derivatives 1,2,3,4,6-penta-*O*-galloyl-β-D-glucoside, tercatain (1,4-di-*O*-galloyl-3,6-(R)-hexahydroxydiphenoyl-β-D-glucose), 1,3,4,6-tetra-*O*-galloyl-β-D-glucose, punicafolin (1,2,4-tri-*O*-galloyl-3,6-(R)-hexahydroxydiphenoyl-β-D-glucose) and 1,3,6-tri-*O*-galloyl-β-D-glucose from *Saxifraga melanocentra* Franch possessed potent inhibition against HCV NS3 protease. The IC_{50} values of these compounds were 1.76, 0.68, 0.76, 0.81, 0.85, and 1.01 μM, respecitively. And their inhibition rates on HCV NS3 protease were 34.9, 98.7, 98.1, 95.8, 99.5%, and 94.7 at 100 $\mu g.ml^{-1}$, respectively [39].

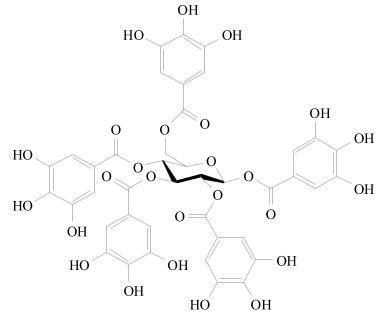
Excoecariphenol D, corilagin, geraniin, and chebulagic acid (**Figure 4**) were isolated from *Excoecaria agallocha* L. These gallic acid derivatives were measured against HCV NS3/4A proteases and HCV RNA in Huh 7.5 cells. The results showed that corilagin, excoecariphenol D, geraniin, and chebulagic acid possessed potential inhibition toward HCV NS3/4A proteases with IC_{50} values of 3.45, 6.93, 8.91, and 9.03 μM, respectively. Furthermore, corilagin and excoecariphenol D significantly inhibited HCV RNA replication with EC_{50} values of 13.59 and 12.61 μM, respectively, whereas chebulagic acid and geraniin showed moderate inhibition on HCV RNA by EC_{50} values of 22.25 and 33.19 μM, respectively [40]. Two novel gallic acid derivatives, SCH 644343 and SCH 644342 (**Figure 5**) from *Stylogne cauliflora*, suppressed HCV NS3 protease with IC_{50} values of 0.3 and 0.8 μM, respectively. Subsequent studies indicated that SCH 644343 was also active with an IC_{50} value of 2.8 μM in the HCV protease binding assay [41]. These findings suggested that HCV NS3, NS 4A, and NS5A serine proteases may be regarded as a possible pathway for anti-HCV effects of gallic acid derivatives.



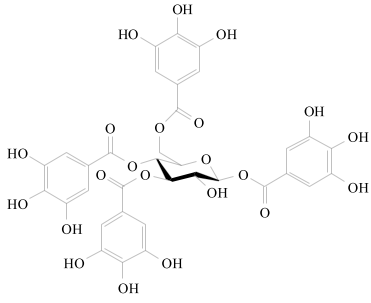
Gallic acid



1,2,6-tri-*O*-Galloyl-β-D-glucose



1,2,3,4,6-penta-*O*-Galloyl-β-D-glucose



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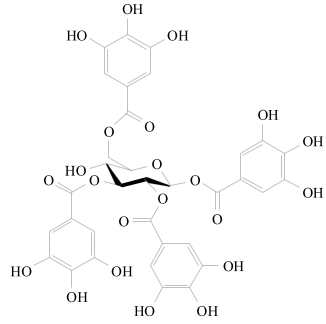
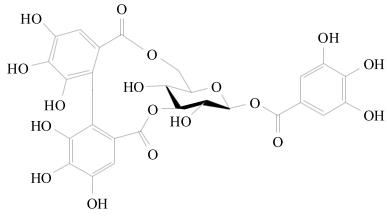
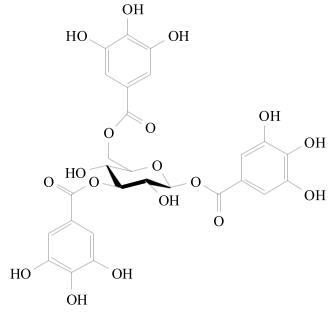


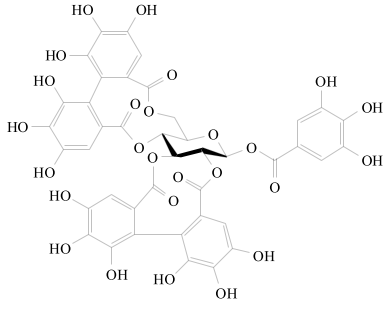
1,2,3,6-tetra-*O*-Galloyl-β-D-glucose



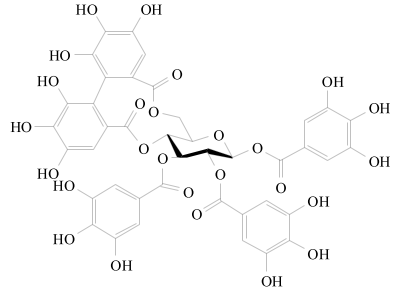
Corilagin



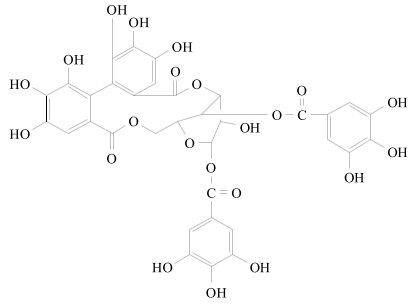
1,3,6-tri-*O*-Galloyl-β-D-glucose



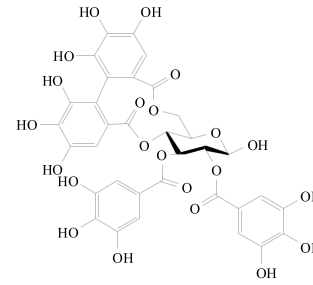
Casuarictin



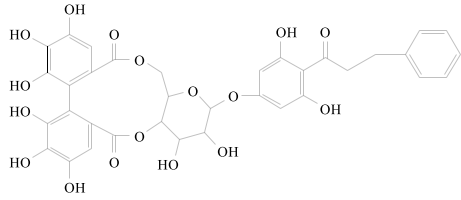
Eugeniin



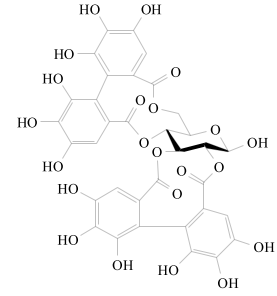
Tercatain



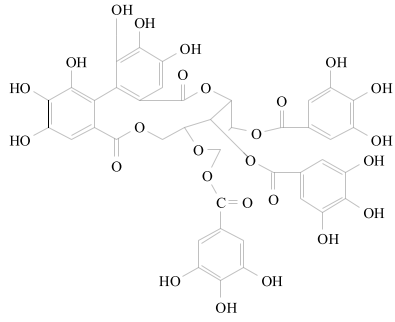
Tellimagrandin I



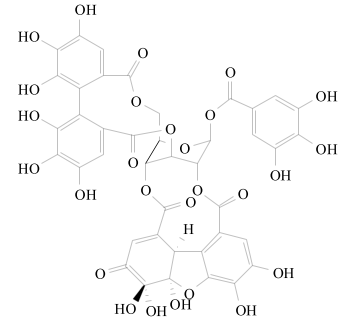
SCH 644342



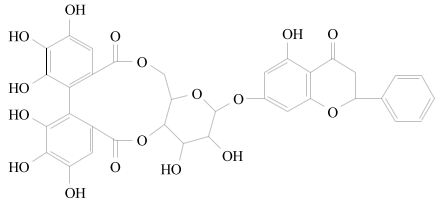
Pedunculagin



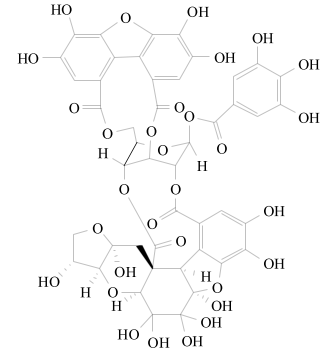
Punicafolin



Geraniin



SCH 644343



Excoecariphenol D

Figure 4.
Structures of natural gallic acid and its derivatives with anti-HCV activity.

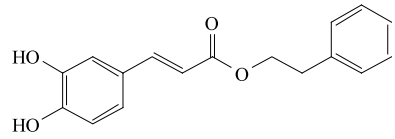


Figure 5.
Structure of natural caffeic acid phenethyl ester (CAPE) with anti-HCV activity.

Four gallic acid analogs (**Figure 4**), tellimagrandin I, eugeniin, and casuarictin from *Rosa rugosa* Thunb together with pedunculagin from *Juglans regia*, were proved to be potent HCV invasion inhibitors using the model virus that expressed HCV envelope proteins E1 and E2 [42]. The mechanisms of anti-HCV action of chebulagic acid and punicalagin (**Figure 4**) from *Terminalia chebula* Retz. were studied during the attachment and entry steps of infection. The results showed that two gallic acid derivatives were effective in abrogating HCV infection at micromolar concentrations [43]. EGCG at a concentration of 50 μM inhibited HCV infectivity at an early step by over 90%. EGCG disrupted the initial step of HCV entry to block viral attachment to the cell, probably by acting on viral particle directly. Besides, it also suppressed cell-to-cell virus spread. Therefore, EGCG as an HCV entry inhibitor might be a promising antiviral strategy aimed at HCV reinfection [44–46]. Taken together, it suggests the potential of gallic acid and its derivatives for developing anti-HCV drugs.

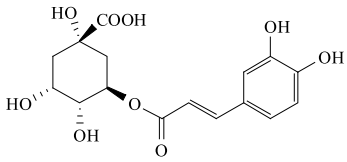
3.2.2 Anti-HCV activities of caffeic acid derivatives

The effect of caffeic acid (**Figure 2**) on HCV propagation was evaluated using a naïve HCV particle infection and production system in Huh 7.5.1–8 cells. The amount of HCV particles released into the medium was significantly reduced at 3 and 4 days post-infection when the cells were cultured with 0.1% caffeic acid for 1 h after HCV infection. HCV-infected cells were treated with 0.001% caffeic acid for 4 days, which was adequate to decrease the amount of HCV particles released into the medium. Caffeic acid treatment suppressed the initial stage of HCV infection including HCV genotypes 1b and 2a, thus suggesting the inhibition of caffeic acid on HCV propagation [47].

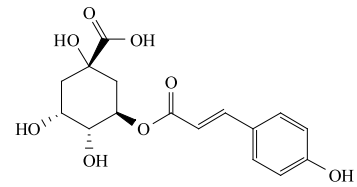
Caffeic acid phenethyl ester (CAPE) (**Figure 5**) and CAPE derivatives exhibited anti-HCV activity in HCV replicon cell line of genotype 1b with EC_{50} values from 1.0 to 109.6 μM. Caffeic acid n-octyl ester showed the strongest anti-HCV activity with an EC_{50} value of 1.0 μM and a selectivity index (SI) value of 63.1. SAR analyses indicated that the length of the n-alkyl side chain and catechol moiety are responsible for the anti-HCV activities of these derivatives [48].

3.3 Phenolic acids with anti-HBV activity

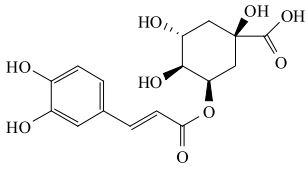
Hepatitis B is a very harmful and epidemic disease caused by HBV. It can cause chronic infection and puts patients at high risk of death from cirrhosis and hepatocellular carcinoma [49]. Although an effective vaccine can prevent HBV infection at present, chronic HBV infection poses still a huge health burden in the whole world [50]. The current anti-HBV drugs have their limitations without exception. There is no effective drug or therapeutic method that can really and truly cure hepatitis B so far [49].



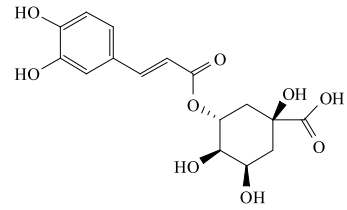
Chlorogenic acid



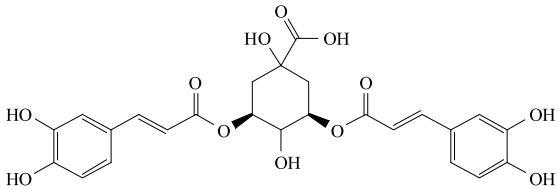
5-*O*-(E)-*p*-Coumaroylquinic acid



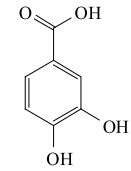
5-*O*- Caffeoylquinic acid



3-*O*-Caffeoylquinic acid



3,5-di-*O*-Caffeoyl-muco-quinic acid



Protocatechuic acid

Figure 6.
Structures of anti-HBV phenolic acids.

Some naturally originated phenolic acids have potent anti-HBV activity. Seven caffeoylquinic acid derivatives from *Lactuca indica L.*, including 3-*O*-CQA, 5-*O*-CQA, 5-*O*-(*E*)-*p*-coumaroylquinic acid, 3,4-di-*O*-CQA, 3,5-di-*O*-CQA, 3,5-di-*O*-caffeoyl-muco-quinic acid, and 4,5-di-*O*-CQA (**Figures 2** and **6**), significantly decreased the level of HBV DNA in HepG2.2.15 cells, and treatment with 5-*O*-(*E*)-*p*-coumaroylquinic acid, 3,4-di-*O*-CQA, and 3,5-di-*O*-caffeoyl-muco-quinic acid respectively caused a remarkable decline in the level of the extracellular HBV DNA [51]. Caffeic acid and chlorogenic acid (**Figures 2** and **6**) in the fruits and leaves of dicotyledonous plants have a variety of antiviral activities. Caffeic acid and chlorogenic acid suppressed HBV DNA replication as well as hepatitis B surface antigen (HBsAg) production in HepG2.2.15 cells. Furthermore, the two phenolic acids also reduced the serum level of duck hepatitis B virus (DHBV) in a DHBV-infected duck model [52].

Two caffeic acid derivatives, 3,4-di-*O*-CQA and 3,5-di-*O*-CQA (**Figure 2**) from *Laggera alata*, markedly inhibited hepatitis B envelope antigen (HBeAg) and HBsAg expressions with inhibitory rates of 81.01, 72.90 and 89.96, 86.90%, respectively. Moreover, 3,4-di-*O*-CQA significantly reduced the content of HBV covalently closed circular DNA (HBV cccDNA) and markedly upregulated the expression of heme oxygenase-1 (HO-1) in HepG2.2.15 cells and HBV transgenic mice [53]. 3,5-di-*O*-CQA exhibited a similar effect as 3,4-di-*O*-CQA [54]. Due to the destabilization of HO-1 on

the HBV core protein, suggests that the overexpression of HO-1 may be involved in the anti-HBV activities of two CQAs by reducing the stabilization of HBV core protein, which blocks the replenishing of HBV cccDNA in nuclear [53, 54]. Cichoric acid from *Cichorium intybus* leaves at 10–100 $\mu g.ml^{-1}$ decreased markedly the expression levels of HBsAg and HBeAg in HepG2.2.15 cells and produced the maximum inhibitory ratios of 76.41% and 79.94%, respectively [55].

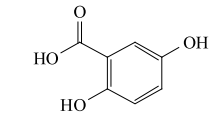
Gallic acid and its derivatives punicalagin and punicalin could be used for suppressing the expressions of HBsAg and HBeAg [56]. EGCG down-regulated the HBeAg and HBV pre-core mRNA expressions, and reduced the levels of both HBV cccDNA and DNA replicative intermediates in HepG2.2.15 cells, thus suggesting that the inhibition of EGCG on HBV replication results in decreasing production of HBV cccDNA by impairing the synthesis of HBV DNA replication intermediates [57]. Additionally, the inhibitory effect of protocatechuic acid on HBV replication was exhibited by activating the extracellular-signal-related kinase 1/2 pathway and then downregulating the HNF4α and HNF1α expressions in HepG2.2.15 cells [58].

3.4 Phenolic acids with anti-HSV activity

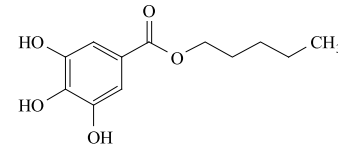
HSV-1 and HSV-2 are two members of herpesvirus family that infect humans [59]. There are about 3.7 billion people infected with HSV-1 worldwide, whereas approximately 417 million people with HSV-2 infection globally. Some antiviral agents such as valacyclovir, acyclovir, and famciclovir can reduce the frequency and severity of symptoms of people with HSV, but they cannot cure the infections [60]. Besides, human herpesvirus 4, also called Epstein-Barr virus (EBV), as a common human virus, is an important member of the herpes-virus family.

Gallic acid (**Figure 4**) and its derivative pentyl gallate (**Figure 7**) decreased the replication of HSV-2 when either incubated with HSV-2 prior to the addition of the mixture to cells or added to and cultured with cells after infection [61]. The virucidal effects of gallic acid and pentyl gallate on virus particles may contribute to their anti-HSV-2 activities by partial inhibition of HSV-2 attachment to cells and subsequent cell-to-cell spread [61, 62]. Eugeniin (**Figure 4**) from *Syzygium aromaticum* and *Geum japonicum* exhibited anti-HSV activity in mice and suppressed the reproduction of thymidine kinase-deficient HSV-1, acyclovir-phosphonoacetic acid-resistant HSV-1 and wild HSV-2, also inhibited purified HSV-1 DNA polymerase activity, viral DNA and late viral protein syntheses in HSV-infected Vero cells. Thus, viral DNA synthesis is one of its major targets of inhibitory action. Therefore, eugeniin may be developed as a novel anti-HSV agent which is different from anti-HSV nucleoside analogs [63].

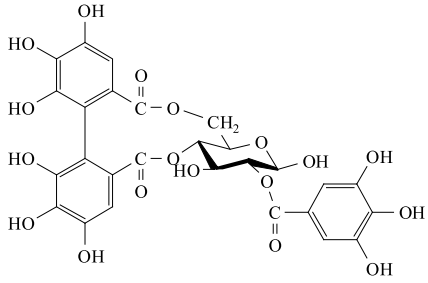
Hippomanin A (**Figure 7**) from *Phyllanthus urinaria* Linnea was shown to suppress HSV-2 infection by the plaque reduction assay and its inhibitory effect on HSV-2 multiplication was exhibited with an IC_{50} value of 28.2 μM [64]. Geraniin and 1,3,4,6-tetra-*O*-galloyl-β-D-glucose (**Figure 4**) from *P. urinaria* were tested for their inhibitory activities against HSV-1 and HSV-2 *in vitro*. The results showed that geraniin actively suppressed HSV-2 infection with an IC_{50} value of 18.4 μM, whereas 1,3,4,6-tetra-*O*-galloyl-β-D-glucose effectively inhibited HSV-1 infection with an IC_{50} value of 19.2 μM. Hence, the two gallic acid derivatives have different magnitudes of potency against HSV-1 and HSV-2 multiplications [65]. Excoecarianin (**Figure 7**) from *P. urinaria* Linnea protected Vero cells from HSV-2 infection with an IC_{50} value of 1.4 μM. Moreover, its inhibitory effect on HSV-2 infection was the strongest when excoecarianin was simultaneously added to the virus. Further studies showed that excoecarianin prevented viral infection by inactivation of HSV-2 virus particles.



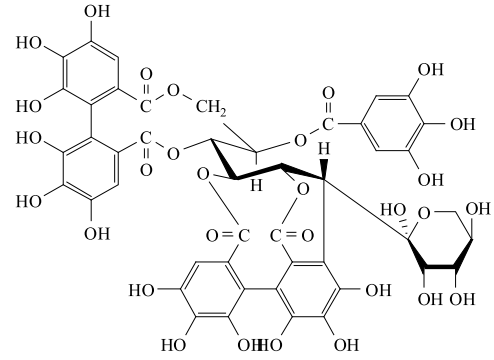
2,5-Dihydroxybenzoic acid



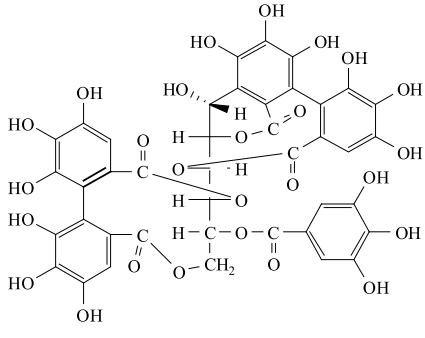
Pentyl gallate



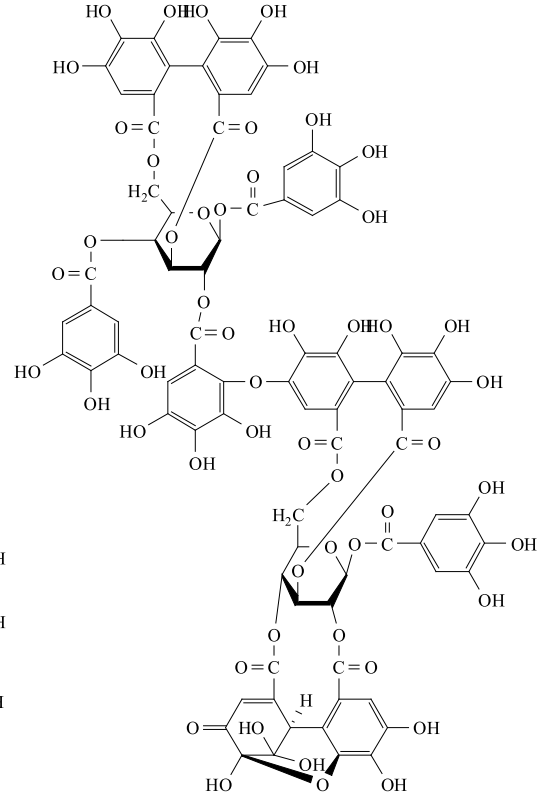
Hippomanin A



Pterocarnin A



Casuarinin



Excoecarianin

Figure 7.
Structures of natural phenolic acids and their derivatives with anti-HSV activity.

Additionally, a synergistic antiviral assay indicated that excoecarianin could be potential for combinatorial therapy with nucleoside analogs such as acyclovir in HSV-2 infection. These data revealed that excoecarianin as an entry inhibitor against HSV-2 merits to be further investigated [66].

Chebulagic acid and punicalagin (**Figure 3**) from *T. chebula* Retz. suppressed HSV-1 entry in A549 human lung cells. Two derivatives could inactivate HSV-1 viral particles and prevent binding, penetration, and cell-to-cell spread, as well as secondary infection. Both compounds targeted HSV-1 glycoproteins and blocked interactions between HSV-1 glycoproteins and cell surface glycosaminoglycans. Consequently, chebulagic acid and punicalagin may be used as competitors for glycosaminoglycans in HSV-1 infection and help decrease the risk for development of viral resistance to nucleoside analogs [67]. Casuarinin (**Figure 7**) from *Terminalia arjuna* Linn., an isomer of casuarictin, exhibited anti-HSV-2 activity with IC_{50} values of 3.6, 1.5 μM and SI values of 25, 59 for XTT and plaque reduction assays, respectively. Casuarinin also showed to prevent the attachment of HSV-2 to cells and inhibit viral penetration. Interestingly, casuarinin at 25 μM reduced viral titers up to 100,000-fold. Thus the anti-HSV activity of casuarinin was performed by disturbing the late events of infection and inhibiting viral attachment and penetration [68]. Pterocarnin A (**Figure 7**) from *Pterocarya stenoptera* was shown to actively inhibit HSV-2 multiplication from attaching and penetrating into cells. These observations suggested that pterocarnin A suppressed both early and late stages in the HSV-2 replication cycle [69]. The anti-HSV activity of EGCG exhibited a direct effect on the virion by binding to the envelope glycoproteins gB and gD or another envelope glycoprotein [70]. Ellagitannins from *P. urinaria* and *P. myrtifolius* showed an anti-EBV effect at micromolar concentration [71]. These studies reveal that gallic acid derivatives may be regarded as potential candidates for developing anti-HSV agents.

Caffeic acid (**Figure 2**) from *Plantago major* L. exhibited potent activity against HSV-1 (EC_{50} = 15.3 μg.ml^{-1}, SI = 67.1) and HSV-2 (EC_{50} = 87.3 μg.ml^{-1}, SI = 118). Its mode of action against HSV-2 was at multiplication stages at 0–12 h postinfection of HSV-1, suggesting the potential use of this compound for treatment of HSV infection [72, 73]. Rosmarinic acid (**Figure 2**) from *Melissa officinalis* inhibited HSV-1 attachment to host cells for acyclovir-sensitive and resistant strains [74]. In addition, 2,5-dihydroxybenzoic acid (**Figure 7**) from *Origanum vulgare* had a weak effect against HSV-1 with an IC_{50} value of 32.7 μM [75]. Protocatechuic acid (**Figure 6**) from *Hibiscus sabdariffa* displayed anti-HSV-2 activity *in vitro* (EC_{50} = 0.92 μg•ml^{-1}, SI > 217) [76].

3.5 Phenolic acids with anti-IV activity

Influenza is an infectious disease caused by IV and spreads around the world in a yearly outbreak, resulting in about 3–5 million severe cases and 250,000 to 500,000 deaths [77]. IVs that infect people include three types of A, B, and C. The vaccine made for 1 year may not be effective in the following year, since the virus evolves rapidly [78]. Despite anti-IV drugs such as oseltamivir and zanamivir have been used to treat influenza, the lack of excellent agents intensifies the importance of novel anti-IV drugs development.

Caffeic acid (**Figure 2**), which is abundant in nature, has a variety of potential pharmacological effects especially antiviral activity [79]. Some natural products containing the fragment of caffeic acids, such as chlorogenic acid and its analogs also show inhibitory effects on influenza neuroaminidases (NAs) [80]. Chlorogenic acid,

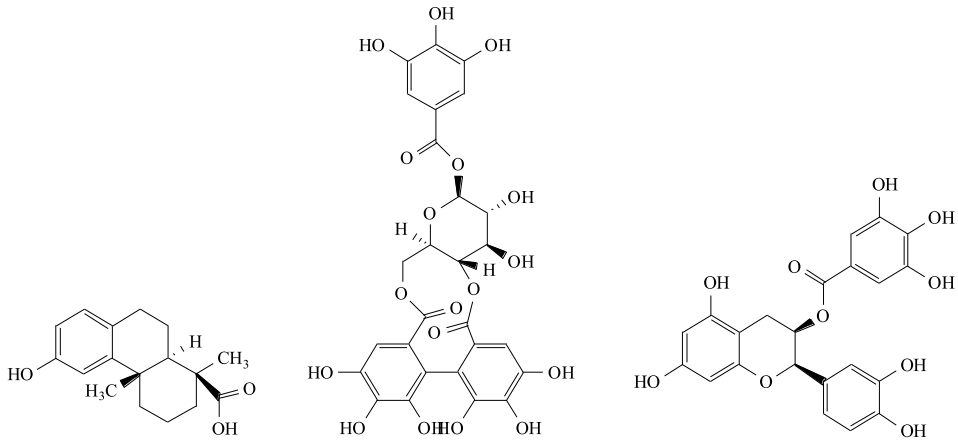


Figure 8.
Structures of natural phenolic acids and their derivatives with anti-IV activity.

caffeic acid, and their derivatives (**Figures 2** and **6**) have been found to exert antiviral effects against NAs from H5N1. Chlorogenic acid and related derivatives exhibited high activities against NAs. The catechol group from caffeic acid may be important for the activity [81]. Caffeic acid, chlorogenic acid, 3,4-di-*O*-CQA, 3,5-di-*O*-CQA, 4,5-di-*O*-CQA, and 3,4,5-tri-*O*-CQA (**Figures 2** and **6**) from the propolis showed effective anti-IV activity [82]. A caffeic acid derivative, caffeic acid phenethyl ester (**Figure 5**) was found to have anti-IV properties *in vitro*. Caffeic acid derivatives seem to be a promising source to find potent NA inhibitors [83, 84].

Rosmarinic acid methyl ester (**Figure 2**) from *Salvia plebeian* exhibited potent inhibition against H1N1 NA with an IC_{50} value of 16.65 μM and reduced cytopathic effect of H1N1 during replication, thus suggesting the potential of the compound to be new lead for developing novel NA inhibitor [85]. Gallic acid and its derivatives possess an inhibitory effect on human avian influenza [56]. Strictinin (**Figure 8**), which is a natural gallic acid derivative, prevented influenza A viruses (IAV) replication with IC_{50} values from 0.09 to 0.28 μM. Further studies showed that strictinin inhibited IAV-induced hemifusion and also exhibited antiviral activities against influenza B virus (IBV) and parainfluenza virus type-1 *in vitro*. Thus strictinin may be a useful antiviral agent [86, 87]. Podocarpic acid (**Figure 8**) from *Dacrydium cupressinum* and its derivatives exhibited inhibitory activity against H1N1 IAV at nanomolar concentrations and suppressed multicycle replication of influenza A/Kawasaki/86 (H1N1) virus *in vitro* [88, 89]. EGCG and (−)-epicatechin gallate (ECG) (**Figures 3** and **8**) from green tea showed potent inhibitory effects on IV replication and effectively inhibited neuraminidase activity [90].

3.6 Phenolic acids with anti-RSV activity

RSV is a syncytial virus that causes respiratory tract infections. It is also a significant pathogen in infants, young children, the elderly, and the immunocompromised [91]. Despite its global impact on human health, there are relatively few therapeutic options available to prevent or treat RSV infection. To date, no effective vaccine or therapeutic agent has been developed [92].

The inhibitory activities of 3,4-di-*O*-CQA and 3,5-di-*O*-CQA (**Figure 2**) from *Schefflera heptaphylla* against RSV were verified by a plaque reduction assay with IC_{50} values of 2.33 and 1.16 μM, respectively. Their anti-RSV modes were performed by the suppression of virus-cell and cell-cell fusion in the early stage and at the end of viral reproduction in turn [93]. Five caffeic acid derivatives from *Markhamia lutea*, luteoside A, luteoside B, luteoside C, verbascoside, and isoverbascoside, exhibited potent antiviral activity against RSV *in vitro* [94]. Two gallic acid derivatives, chebulagic acid and punicalagin (**Figure 3**), abrogated RSV infection at micromolar concentrations by inhibiting viral attachment, penetration, and spread. Two compounds may be of value as antivirals for limiting emerging/recurring viruses to engage host cell GAGs for entry [43].

Carnosic acid (**Figure 9**) from *Rosmarinus officinalis* displayed potent activities against both RSV A- and B-type viruses. The compound efficiently suppressed the replication of RSV and inhibited viral gene expression without inducing type-I interferon production. Furthermore, the addition of carnosic acid at 8 h after infection still blocked the expression of RSV genes, further suggesting that carnosic acid might directly inhibit the replication of RSV [95]. Sekikaic acid (**Figure 9**) from *Ramalina farinacea* exhibited potent inhibition toward a recombinant RSV strain with an IC_{50} value of 5.69 $\mu g.ml^{-1}$ and RSV A2 strain with an IC_{50} value of 7.73 $\mu g.ml^{-1}$, and clearly interfered with viral replication at a post-entry step [96].

3.7 Phenolic acids against other viruses

Enterovirus 71 (EV71) is a causative agent that causes hand, foot, and mouth disease, a highly contagious viral infection that affects young children. It can also cause severe neurological or cardiac complications [97]. To date, no approved antiviral agents have been developed for the treatment of EV71 infection. The anti-EV71 activity of gallic acid (**Figure 4**) from *Woodfordia fruticosa* Kurz flowers was evaluated in Vero cells. Gallic acid exhibited a high anti-EV71 activity with an IC_{50} value of 0.76 $\mu g.ml^{-1}$, thus suggesting that gallic acid may be used as a potential anti-EV71 agent [98].

Adenoviruses (ADVs) can cause mild infections involving the gastrointestinal tract, upper or lower respiratory tract, and conjunctiva. The infections of ADVs are more common in young children, owing to lack of humoral immunity. No reliable therapy or vaccine is available to civilians [99]. Caffeic acid (**Figure 2**) from *P. major* L. exhibited the strongest activity against ADV-3 (EC_{50} = 14.2 $\mu g.ml^{-1}$, SI = 727), whereas chlorogenic acid (**Figure 6**) from *P. major* L. possessed potent anti-ADV-11 activity (EC_{50} = 13.3 $\mu g.ml^{-1}$, SI = 301). The mode of action of caffeic acid against ADV-3 was at multiplication stage, suggesting the potential of the compound against ADV infection [72, 73]. EGCG reduced ADV yield with an IC_{50} value of 25 μM in Hep2 cells and its anti-ADV activity indicated itself through several mechanisms, including multiple steps both outside and inside the cell in virus infection [100].

Dengue virus (DENV) causes a spectrum of human diseases ranging from mild dengue fever to dengue hemorrhagic fever and dengue shock syndrome in severe cases [101]. Measles virus (MV) can cause a severe infection characterized by high fever, coryza, cough, exanthema, and conjunctivitis [102]. Cytomegalovirus (CMV) is a member of the herpes family of viruses. Most of the patients with CMV do not cause symptoms, but it can be fatal for the immunocompromised such as newborn infants or HIV-infected patients [103]. Currently, there is no effective antiviral therapy available for DENV, MV, and CMV. Hence, it is very important of finding an effective

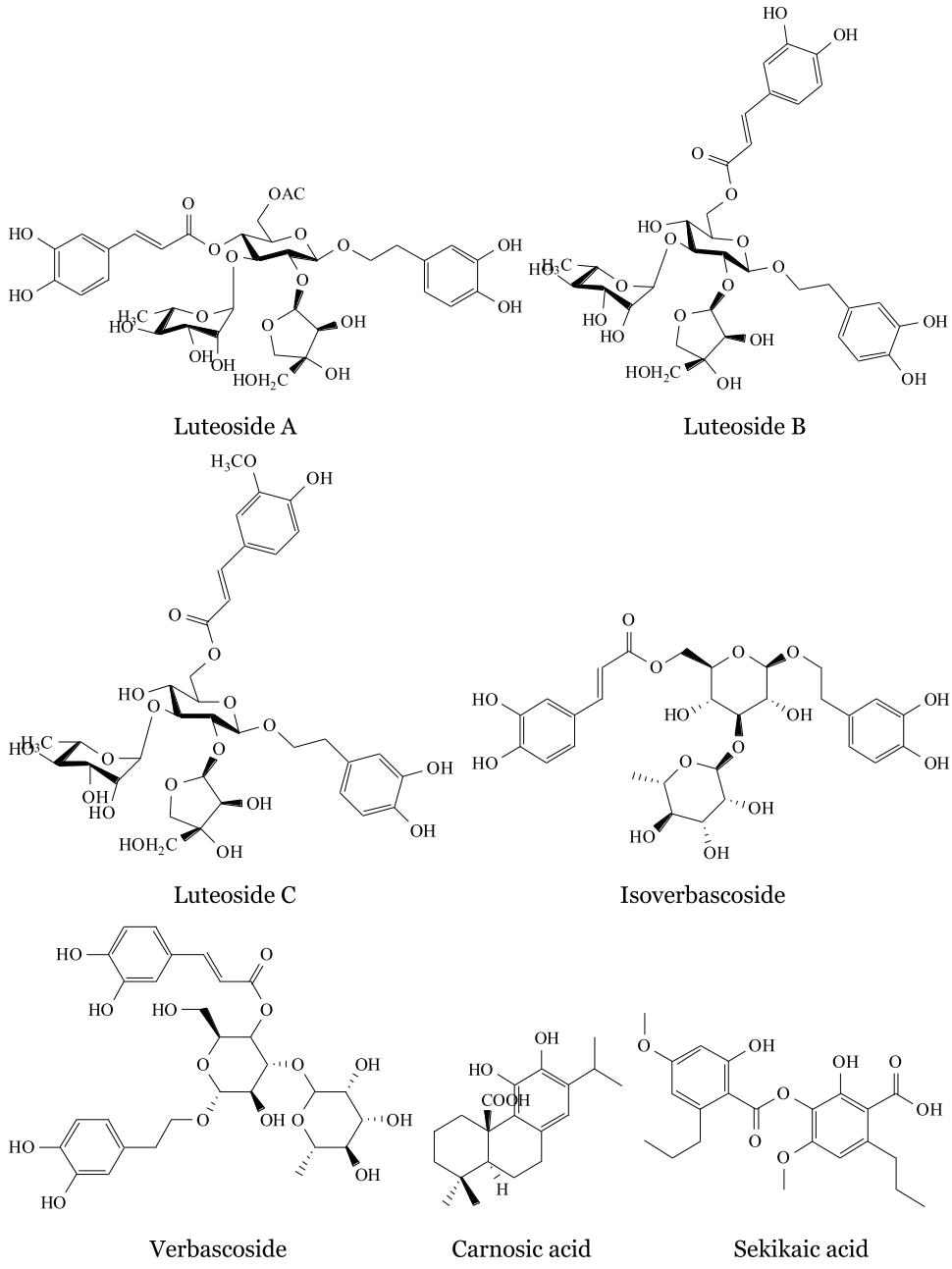


Figure 9.
Structures of anti-RSV phenolic acids.

compound against these viruses. Chebulagic acid and punicalagin (**Figure 3**) were effective in abrogating infections by DENV, MV, and HCMV at micromolar concentrations. Furthermore, these compounds blocked viral attachment, penetration, and spread for MV and HCMV infections. Hence, as the broad-spectrum antivirals, two gallic acid derivatives may be useful for limiting emerging/recurring viruses to engage host cell GAGs for entry [43].

Human papillomaviruses (HPVs), as a family of more than 180 related viruses, cause lots of diseases including condyloma acuminatum by HPV type 6 and 11 infection mainly [104, 105]. EGCG exhibited an anti-HPV effect by inhibiting the HPV11 E6 and E7 mRNA expressions in the recombinant HPV11.HaCaT cells [106].

4. Structural and antiviral properties

From the perspective of the structural properties of natural phenolic acids, caffeic acid, and its derivatives exhibited strong inhibitory activities against multiple viruses (**Table 1**), such as caffeic acid derivatives, including 1,3-di-*O*-CQA, 1,4-di-*O*-CQA, 1,5-di-*O*-CQA, 3,4-di-*O*-CQA, 3,5-di-*O*-CQA, 1-MO-3,5-di-*O*-CQA, 4,5-di-*O*-CQA, L-chicoric acid, rosmarinic acid, rosmarinic acid methyl ester, caffeic acid n-octadecyl ester, lithospermic acid, lithospermic acid B, orthosiphoic acids A-C and salvianolic acid C, possessed remarkable anti-HIV acitvity [8–16, 19–21, 23–25]. SAR suggested that two catechol moieties were required to inhibit HIV IN, while at least one free

Stuctural type	Compounds	Antiviral types	Structure-activity relationship	Reference
Caffeic acid and its derivatives	Caffeic acid	Anti-HCV; Anti-HBV; Anti-HSV; Anti-IV; Anti-ADV	① Biscatechol moieties were required for inhibition of IN, while at least one free carboxyl group was required for anti-HIV effect; Two aryl units separated by a central linker, as a common structural feature, is shared by the majority of these inhibitors. ② The potassium and sodium salts were found to be essential to increase the anti-HIV abilities of caffeic acid tetramers. ③ Chicoric acid derivatives lacking one carboxyl group and with 3,4,5-trihydroxycinnamoyl sidechains replacing caffeoyl group had the most strongest inhibition of HIV replication and end-processing activity ④ The length of the n-alkyl side chain and catechol moiety are responsible for the anti-HCV activities of caffeic acid derivatives. ⑤ The catechol group from caffeic acid seems to be important for the anti-IV activity. ⑥ The caffeoyl group may be indispensable for the anti-IV effects of CQAs.	[45, 51, 70, 71, 77, 79, 80]
	Chlorogenic acid	Anti-HBV; Anti-IV; Anti-ADV		[50, 70, 71, 78–80]
	3-*O*-CQA	Anti-HBV		[45]
	5-*O*-CQA	Anti-HBV; Anti-IV		[49, 82]
	5-*O*-(*E*)-*p*-Coumaroylquinic acid	Anti-HBV		[49]
	1,3-di-*O*-CQA	Anti-HIV		[12]
	1,4-di-*O*-CQA	Anti-HIV		[16]
	1,5-di-*O*-CQA	Anti-HIV		[12]
	3,4-di-*O*-CQA	Anti-HIV; Anti-HBV; Anti-IV; Anti-RSV		[12, 13, 49, 51, 80, 91, 107]
	3,5-di-*O*-CQA	Anti-HIV; Anti-HBV; Anti-IV; Anti-RSV		[12, 49, 52, 80, 91]
	1-MO-3,5-di-*O*-CQA	Anti-HIV		[12, 13]
	3,5-*O*-Dicaffeoyl-muco-quinic acid	Anti-HBV		[49]
	4,5-di-*O*-CQA	Anti-HIV; Anti-HBV; Anti-IV		[11, 12, 49, 80]
	3,4,5,-tri-*O*-CQA	Anti-HIV; Anti-IV		[11, 80]

Stuctural type	Compounds	Antiviral types	Structure-activity relationship	Reference
	Chicoric acid	Anti-HIV, Anti-HBV		[12, 14, 55, 108]
	Rosmarinic acid,	Anti-HIV; Anti-HSV		[17, 18, 72]
	Rosmarinic acid methyl ester	Anti-HIV; Anti-IV		[17, 83]
	Caffeic acid n-octadecyl ester	Anti-HIV; Anti-HCV		[19, 46]
	Caffeic acid phenethyl ester	Anti-HCV; Anti-IV		[46, 81, 82]
	Lithospermic acid	Anti-HIV		[21]
	Lithospermic acid B	Anti-HIV		[21]
	Orthosiphoic acids A-C	Anti-HIV		[22]
	Salvianolic acid C	Anti-HIV		
	Caffeic acid tetramers	Anti-HIV		[20]
	Luteoside A, B, C	Anti-RSV		[94]
	Verbascoside/ Acteoside, Isoverbascoside/ Isoacteoside	Anti-HIV, Anti-RSV		[23, 94]

Table 1.
Antiviral types and structure-activity relationship of natural caffeic acid and its derivatives.

carboxyl group was required for anti-HIV effect. Most of the inhibitors have two aryl units separated by a central linker. Meanwhile, hydroxylated aromatics seem to play an important role in inhibiting IN [17, 18]. The salts of isomeric caffeic acid tetramers showed potent anti-HIV activity. Moreover, the potassium and sodium salts were found to be essential to increase their anti-HIV abilities [22]. SAR of chicoric acid indicated that chicoric acid derivatives lacking one carboxyl group and with 3,4,5-trihydroxycinnamoyl sidechains replacing caffeoyl group had the strongest inhibition of HIV replication and end-processing activity [109]. Caffeic acid and its related compounds also displayed anti-HCV effects, while SAR demonstrated that the length of the n-alkyl side chain and catechol moiety is responsible for their anti-HCV activities [47, 48]. The derivatives of caffeic acid, including 3-*O*-CQA, 5-*O*-CQA, 5-*O*-(*E*)-*p*-coumaroylquinic acid, 3,4-di-*O*-CQA, 3,5-di-*O*-CQA, 3,5-di-*O*-caffeoyl-muco-quinic acid, 4,5-di-*O*-CQA and chicoric acid, had potent anti-HBV activity [51, 52, 55]. Caffeic acid exhibited potent inhibitory activity against HSV [72, 73]. Rosmarinic acid inhibited HSV-1 attachment to host cells [74]. Caffeic acid and its analogs such as chlorogenic acid, 5-*O*-CQA, 3,4-di-*O*-CQA, 3,5-di-*O*-CQA, 4,5-di-*O*-CQA, 3,4,5-tri-*O*-CQA, and CAPE showed inhibitory effect on IV [79–84]. The catechol group from caffeic acid seems to be important for the anti-IAV activity [81, 84]. The caffeoyl group may be indispensable for the anti-IV effects of CQAs [82]. 3,4-di-*O*-CQA and

3,5-di-*O*-CQA exhibited inhibitory activity against RSV infection [93]. Luteoside A-C, verbascoside and isoverbascoside displayed potent anti-RSV effect [94]. Additionally, caffeic acid and chlorogenic acid displayed anti-ADV activity [72, 73]. Other caffeoyl conjugates such as echinacoside also have antiviral effect [110].

In addition, gallic acid and its derivatives exhibited potent inhibitory effects on several viral infections (**Table 2**), such as gallic acid derivatives, including 3,5-di-*O*-galloyl-4-*O*-diGQA, 3,4-di-*O*-galloyl-5-*O*-diGQA, 3-*O*-digalloyl-4,5-di-*O*-GQA, 1,3,4,5-tetra-*O*-GQA, 1,3,4-tri-*O*-GQA, 3,4,5-tri-*O*-GQA, 3,5-di-*O*-galloyl-shikimic acid, 3,4,5-tri-*O*-galloylshikimic acid, punicalin, punicalagin, punicacortein C, chebulagic acid, ellagitannin, EGCG and camelliatannin H, were found to possess potent anti-HIV effect [11, 26–28, 31, 32, 111]. Digallic acid and its derivatives

Stuctural type	Compounds	Antiviral types	Structure-activity relationship	Reference
Gallic acid and its derivatives	Gallic acid	Anti-HCV; Anti-HBV; Anti-HSV-2; Anti-IV; Anti-EV71	① The docking analysis of gallic acid derivatives indicated that the gallic acid-based inhibitor could be effectively targeted for designing HIV-1 PR inhibitors. ② Three hydroxyl groups at the 3, 4, and 5 positions seem to be required for the inhibition of digallic acid derivatives. ③ SAR analysis of the hydrolysable tannins elucidated that the galloyl groups on C-2 and C-3 and the hexahydroxydiphenyl group bridged between C-4 and C-6 increased inhibitory ability for HCV invasion. ④ The 3-galloyl group of EGCG skeleton plays a significant role on its antiviral effect, whereas the 5'-OH at the trihydroxy benzyl moiety at 2-position plays a secondary role. ⑤ The essential pharmacophore of ellagitannins exits in the corilagin moiety and the outer carboxylic acid moieties seem to serve only as auxopharmacore.	[35, 37, 53, 59, 60, 93]
	3,4-di-*O*-galloyl-5-*O*-diGQA	Anti-HIV		[24]
	3,5-di-*O*-galloyl-4-*O*-diGQA	Anti-HIV		[24]
	3-*O*-digalloyl-4,5-di-*O*-GQA	Anti-HIV		[24]
	1,3,4-tri-*O*-GQA	Anti-HIV		[25]
	3,4,5-tri-*O*-GQA	Anti-HIV		[11, 26]
	1,3,4,5-tetra-*O*-GQA	Anti-HIV		[24]
	3,5-di-*O*-galloyl-shikimic acid	Anti-HIV		[25]
	3,4,5-tri-*O*-galloylshikimic acid	Anti-HIV		[25]
	Punicalin,	Anti-HIV; Anti-HBV		[25, 53]
	Punicalagin	Anti-HIV; Anti-HBV; Anti-HSV-1; Anti-RSV; Anti-DENV; Anti-MV; Anti-HCMV		[25, 41, 53, 65]
	Punicacortein C	Anti-HIV		[25]
	Chebulagic acid	Anti-HIV; Anti-HSV-1; Anti-RSV; Anti-DENV; Anti-MV; Anti-HCMV		[25, 38, 41, 65]
	Ellagitannin	Anti-HIV		[25]
	Digallic acid	Anti-HIV		[27, 28]
	Camelliatannin H	Anti-HIV-1		[32]
	1,2,6-tri-*O*-galloyl-β-d-glucose	Anti-HCV		[36]

Stuctural type	Compounds	Antiviral types	Structure-activity relationship	Reference
	1,3,6-tri-*O*-galloyl-β-D-glucose	Anti-HCV		[37]
	1,2,3,6-tetra-*O*-galloyl-β-d-glucose	Anti-HCV		[36]
	1,3,4,6-tetra-*O*-galloyl-β-D-glucose	Anti-HCV; Anti-HSV		[37, 63]
	1,2,3,4,6-penta-*O*-galloyl-β-D-glucoside	Anti-HCV		[36, 37]
	Tercatain	Anti-HCV		[37]
	Punicafolin	Anti-HCV		[37]
	Excoecariphenol D	Anti-HCV		[38]
	Corilagin,	Anti-HCV		[38]
	Geraniin	Anti-HCV; Anti-HSV		[38, 63]
	SCH 644343	Anti-HCV		[39]
	SCH 644342	Anti-HCV		[39]
	Tellimagrandin I	Anti-HCV		[40]
	Eugeniin	Anti-HCV; Anti-HSV		[40, 61]
	Casuarictin	Anti-HCV		[40]
	Pentyl gallate	Anti-HSV-2		[59, 60]
	Hippomanin A	Anti-HSV-2		[62]
	Excoecarianin	Anti-HSV-2		[64]
	Casuarinin	Anti-HSV-2		[65]
	Pterocarnin A	Anti-HSV-2		[66]
	Strictinin	Anti-IV		[84, 85]
	Epigallocatechin-3-gallate	Anti-HIV; Anti-HCV; Anti-HBV; Anti-HSV; Anti-IV; Anti-ADV; Anti-HPV		[29, 42–44, 54, 68, 88, 99, 105]

Table 2.
Antiviral types and structure-activity relationship of natural gallic acid and its derivatives.

displayed significant anti-HIV and anti-HSV activities. All three hydroxyl groups at the 3, 4, and 5 positions seem to be responsible for their inhibitory activities [29, 30, 61, 62]. The docking analysis of gallic acid derivatives indicated that the gallic acid-based inhibitor could be effectively targeted for designing HIV-1 PR inhibitors [108]. Gallic acid glucosides and other derivatives, including 1,2,6-tri-*O*-galloyl-β-D-glucose, 1,3,6-tri-*O*-galloyl-β-D-glucose, 1,2,3,6-tetra-*O*-galloyl-β-D-glucose, 1,3,4,6-tetra-*O*-galloyl-β-D-glucose, punicafolin, 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose, 1,2,3,4,6-

penta-*O*-galloyl-β-D-glucoside, excoecariphenol D, tercatain, corilagin, geraniin, chebulagic acid, tellimagrandin I, eugeniin, casuarictin, chebulagic acid, punicalagin, SCH 644343, SCH 644342 and EGCG, possessed marked anti-HCV activity [38–45]. Gallic acid and its derivatives punicalagin, punicalin, and EGCG were found to inhibit HBV replication [56, 57]. Ten gallic acid derivatives such as eugeniin, hippomanin A, casuarinin, geraniin, 1,3,4,6-tetra-*O*-galloyl-β-D-glucose, excoecarianin, chebulagic acid, punicalagin, pterocarnin A and EGCG showed remarkable inhibitory activity against HSV infection [63–70]. Ellagitannins exhibited anti-EBV activity. SAR analysis indicated that their essential pharmacophores exist in the corilagin moiety and the outer carboxylic acid moieties seem to serve only as auxopharmacore [71]. Gallic acid and its derivatives also showed antiviral activity against human avian influenza [56]. Strictinin, EGCG, and ECG have potent inhibitory activity against IAV infection [86, 87, 90]. Two gallic acid derivatives, chebulagic acid and punicalagin, exhibited strong inhibitory effects on RSV, DENV, MV, and HCMV infections, respectively [43]. Gallic acid had high potency against EV71 [98]. EGCG showed potent anti-ADV and anti-HPV activities [100, 106].

As regards antiviral characteristics or mechanism of action, several naturally originated phenolic acids exhibited new targets or modes of antiviral action. Firstly, caffeic acid and its derivatives have special antiviral mechanisms. CQAs and CTAs, as highly selective HIV IN inhibitors, act at a site distinct from that of current anti-HIV agents [8–12]. The irreversible inhibition of CQAs on HIV IN is directed toward conserved amino acid residues in the central core domain during catalysis [13]. The reversible and noncompetitive inhibition of L-chicoric acid on HIV IN may interact with amino acids other than those which bind substrate [14]. Rosmarinic acid suppressed HIV-1 IN and also inhibited RT directly [19, 20]. The primary target of L-chicoric acid and its analogs against HIV is the viral entry in cells [107]. Salvianolic acid C and orthosiphoic acids A-C displayed anti-HIV-1 PR effect [25]. The anti-HBV effects of 3,4-di-*O*-CQA and 3,5-di-*O*-CQA are associated with the upregulation of HO-1 by decreasing HBV core protein stability, which blocks HBV cccDNA refill [51, 52]. The antiviral effects of caffeic acid against HSV-2 and ADV-3 were at viral multiplication stages [72, 73]. Caffeic acid, chlorogenic acid, and their derivatives exerted antiviral effects against Nas from H5N1 [81–84]. The ability of 3,4-di-*O*-CQA to clear IAV infection was performed by increasing tumor necrosis factor-related apoptosis-inducing ligand [112]. The anti-RSV effects of 3,4-di-*O*-CQA and 3,5-di-*O*-CQA were exerted by the suppression of virus-cell and cell-cell fusion in the early stage and at the end of viral reproduction in turn [93].

Secondly, gallic acid and its derivatives possess new antiviral characteristics. GQAs and other gallic acid derivatives displayed inhibitory activities against HIV RT, virus reproduction, and virus-cell interactions [26, 27]. 3,4,5-tri-*O*-GQA selectively inhibited HIV replication and non-competitively suppressed HIV RT by interaction with gp120 to block virus binding to CD4 receptor [11, 28]. Digallic acid inhibited HIV RT and DNA polymerases α/β. Its mode of action against HIV was partially competitive relative to the template and primer, and also noncompetitive to the triphosphate substrate and dTTP [29, 30]. Gallic acid reduced ROS production at the early time points of exposure in cells expressing HCV proteins, thus suggesting that the antioxidant capacity of the compound may be involved in anti-HCV replication [37]. Meanwhile, HCV NS3/4A and NS5A proteases are possible pathways for gallic acid derivatives to inhibit HCV [37–41]. The anti-HCV mechanisms of gallic acid derivatives chebulagic acid and punicalagin may be related to suppressing the attachment and entry steps of infection [43]. EGCG inhibited HCV entry by acting directly on the

virions, leading to the structural alteration of viral particles, which could impair the attachment to the surface of hepatocyte [113]. Gallic acid and its derivatives against HSV infection were ascribed to their virucidal effects on virus particles by partial inhibition of the virus attachment to cells and its subsequent cell-to-cell spread [61, 62]. Eugeniin may be a promising novel anti-HSV agent by targeting viral DNA synthesis [63]. Excoecarianin, as an entry inhibitor against HSV-2, contributes to improving combinatorial drug treatment with nucleoside analogs [66]. Chebulagic acid and punicalagin blocked interactions between cell surface glycosaminoglycans and HSV-1 glycoproteins, and thus they may be used as competitors for glycosaminoglycans and improve drug resistance to nucleoside analogs [67]. Casuarinin exhibited anti-HSV activity by inhibiting viral attachment and penetration and also disturbing the late events of infection [68]. Pterocarnin A inhibited both early and late stages in HSV-2 replication cycle [69]. Strictinin suppressed IAV-induced hemifusion and prevented IAV replication, and also showed antiviral activities against IBV and human parainfluenza virus type-1 infections [86, 87]. Chebulagic acid and punicalagin blocked viral attachment, penetration, and spread for RSV, MV, and HCMV infections [43]. The anti-HPV effect of EGCG was performed by inhibiting HPV11 E6 and E7 mRNA expressions [106].

Additionally, other phenolic acids also possess antiviral properties. Protocatechuic acid showed anti-HBV and anti-HSV-2 activities [58, 76]. 2,5-Dihydroxybenzoic acid had a weak anti-HSV-1 effect [75]. Podocarpic acid and its derivatives suppressed the replication of H1N1 IAV and influenza A/Kawasaki/86 (H1N1) virus [88, 89]. Carnosic acid suppressed viral gene expression and RSV replication [95]. Sekikaic acid showed potent inhibition toward RSV and clearly interfered with viral replication at a viral post-entry step [96].

5. Conclusion

Viral infections are an important part of human disorders and their treatments are still difficult. The approved antiviral drugs have their limitations without exception. Many viral diseases lack efficient vaccines and antiviral therapies so far, which are often perplexed by the development of drug resistance and the generation of viral mutation. Hence, it is urgently needed to discover novel antiviral drugs. Naturally originated compounds especially phenolic acids are an excellent source for finding new antiviral agents because of their potent activities and unique antiviral mechanisms [114, 115]. In this review, the naturally occurring phenolic acids with antiviral activity are discussed according to their structure properties and antiviral types such as anti-HIV, anti-HCV, anti-HBV, anti-HSV, anti-IV, anti-RSV, *etc*. These natural phenolic acids and their derivatives may be cited as promising antiviral leads or candidates.

To summarize, naturally originated phenolic acids and their derivatives exerted potent antiviral effects on multiple viruses in humans. In particular, caffeic acid/gallic acid and their derivatives exhibited prominent antiviral properties and special targets or mechanisms of action, thus suggesting these compounds can be regarded as novel promising leads or candidates for the development of new antiviral agents. In addition, these natural phenolic acids with antiviral effects are mostly limited to the *in vitro* results to date. Furthermore, scarcely any of them have been used as antiviral drugs in clinical practice. Therefore, naturally derived phenolic acids with diverse skeletons and different targets or mechanisms, as a powerful resource for novel antiviral agent development, are worthy to be further studied and explored in the future.

Conflict of interest

The authors have no conflict of interest. The partial content of this manuscript was published in *Current Medicinal Chemistry* (2017, 24(38): 4279–4302).

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Chapter 6

Antiviral Targets and Known Antivirals (HAART)

Nma Helen Ifedilichukwu and Oladimeji-Salami Joy

Abstract

In 2021, the number of HIV-positive people worldwide was estimated to be 38.4 million. Since its discovery four decades ago, the scope of the HIV infection has outstripped all predictions, necessitating the urgent need to develop novel antivirals against the virus that target crucial stages in the virus' life cycle. New antiviral drug classes that were developed in response to the HIV epidemic were coupled to offer very highly active antiretroviral treatment. These novel highly active antiretroviral therapies (HAART) were developed as a result of the emergence of drug-resistant strains of the virus. By inhibiting these enzymes, reverse transcriptase, integrase, and protease that are essential for viral attachment, entry, integration, and maturation, antiretroviral therapy (ART) strategies can suppress the virus, lower the viral load, boost CD4 count, and ultimately halt the progression of the disease. Advances in research on the biology of both the immature and the mature forms of the HIV capsid in terms of its structure and function have made it possible to discover and/or design small molecules and peptides that interfere with the virus's assembly and maturation. This article presents and reviews HAART's current state and strategies as a very active antiviral.

Keywords: antiviral targets, HIV, AIDS, antiviral agents, HAART, epidemic

1. Introduction

A virus is composed of a protective capsid that surrounds a core of genetic material, either DNA or RNA. The envelope protein is what makes up the capsid. Each virion is made up of a viral envelope, a matrix, and a capsid, which contains two copies of the single-stranded RNA genome as well as a number of enzymes (**Figure 1**). A small number of viral proteins, encoded by the HIV genome, always form cooperative relationships with host proteins and other HIV proteins in order to enter host cells and take over their internal machinery. The structure of HIV is distinct from that of other retroviruses. The HIV virion has a diameter of about 100 nm. Its innermost portion is made up of a cone-shaped core that contains [1] the principal core protein, some minor proteins, reverse transcriptase, integrase, and protease enzymes, as well as two copies of the (positive sense) ssRNA genome. Eight viral proteins critical to the HIV life cycle are encoded in the HIV genome, according to research [2].

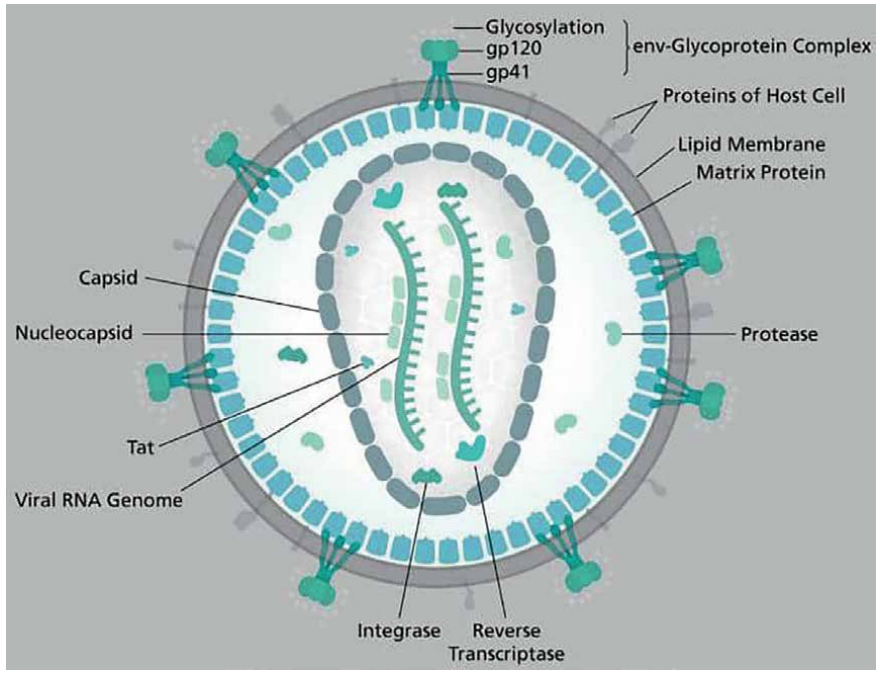

Figure 1.
Human immuno virus. Source: [3].

Viruses and their hosts have similar metabolic processes because they are obligatory cellular parasites, that is, they depend on a living host for existence, viruses can either encode proteins that are closely related to and similar to host proteins to carry out crucial metabolic and survival activities, or they may have evolved the capacity to directly integrate their genome into that of the host, co-opting the host's cellular genetic factors [4].

Although many of these viral infections can be successfully averted through vaccination [4], there is currently an inadequate understanding of vaccinations against several significant pathogens that have been linked to numerous human viral disorders like HIV and hepatitis C virus (HCV). Interest in the creation of novel antiviral chemicals is typically fueled by two crucial aspects and considerations: the prerequisites for a specific antiviral medicine against a particular viral infection are essential in assuming that the proposed antiviral would be able to reasonably control the particular viral infection. Second, what antiviral treatments or preventative measures are now available for the specific viral infection, as well as what antiviral tactics might be used to address this demand [5]?

In 2019, there were roughly 38 million HIV infections worldwide, according to U.S. statistics [6]. Although combination antiretroviral therapy (cART) has improved the success rate being recorded in the management of HIV infection, there is currently no known cure for the illness. Data show that cART has been able to suppress the viral load to the point where it is undetectable, and carriers frequently lead nearly normal lives with measurable increased average life expectancies compared to historical data [7]. Though highly active antiretroviral therapy (cART), also known as HAART (highly active anti-retroviral therapy), has been successful in treating HIV-1 infections, the rise of resistance forces the development of new antiviral medicines that would be more effective and efficient in viral suppression.

Despite this success in treating HIV-1 infections that have been attributed to cART, also known as HAART (highly active antiretroviral therapy), the emergence of resistance drives a pressing need to create new antiviral agents that are more effective and efficient in viral suppression. Furthermore, cross-resistance (a phenomenon in which the emergence of resistance to one therapeutic agent simultaneously results in the emergence of resistance to other agents in that class) has prompted the need to develop novel compounds as well as ones that are active against novel targets [8].

In some cases where HIV infection becomes chronic, the specifics of a pattern of its progression in a patient may completely differ from the average, thereby manifesting differently in different patients, suggesting complexity in the pattern of infection in different individuals. A lot of factors ranging from genetics to environment have been implicated in such a scenario. This variability is of great public health concern and has huge clinical importance, as well as raising the question of whether or not an average treatment regimen is optimal for a given patient [7].

Recent research has shown that the mechanism of action as well as the pathogenesis of HIV-1 is complex and dynamic being characterized by the interplay and modulation of both viral and host cellular factors. Since the identification of HIV as the causative agent of AIDS, a lot of efforts and resources have been committed to keeping the disease under control. A lot of comprehensive research into understanding the individual steps of the viral replication/life cycle and the dynamics during infection has led to the development of a wide range of antiviral compounds exploiting different molecular strategies. Every stage in the viral life cycle/replication stage and every gene product of the virus remains a potential target for therapeutics and prevention [9].

The pathophysiology and mode of action of HIV-1 are complicated and dynamic, as evidenced by the interaction and modification of both viral and host cellular components, according to a recent study. Many resources and efforts have been devoted to controlling the disease ever since HIV was discovered to be the primary cause of AIDS. A variety of antiviral drugs utilizing various molecular methods have been developed as a result of extensive research into the dynamics of infection as well as the individual steps of viral replication and life cycle. Every stage of the viral life cycle/replication as well as every virus gene product continue to be potential targets for treatment and averting disease [9].

The primary molecular-based strategies against viruses, with a focus on HIV-I, that have been described in the literature are outlined in this review, along with their potential to lead to the creation of stronger and more potent antiviral drugs for clinical use.

2. HIV-1 infection and therapeutic targets

The ability of HIV to propagate and cause pathogenesis in patients is essentially determined by the efficacy of HIV entry into a host cell. Prior to HIV-1 entering its target cells (CD4-expressing T-lymphocytes, macrophages, and monocytes), the viral envelope glycoprotein (Env) gp120 fuses with the host cell membrane via the primary cellular receptor CD4 [10]. This causes changes in Env conformation that make the interaction between the gp120 V3 loop and coreceptor CCR5 or CXCR4 easier. The trimeric spikes that make up the HIV-1 envelope are each composed of three gp120 surface subunits and three gp41 trans-membrane subunits. The CD4 receptor and either the CCR5 or the CXCR4 co-receptor are often needed for viral binding

and entrance into the cell [11]. The machinery that drives the fusing of the viral and host cell membranes is set off by coreceptor binding, which also causes the six-helix bundle of Env to form. Effectively hindering HIV entry and dissemination is possible by functionally blocking receptors, coreceptors, or HIV Env [10].

The viral reverse transcriptase is used to convert the viral RNA genome to double-stranded DNA following receptor-mediated binding and internalization. Following the integration of the viral genomic DNA into the host genome in the nucleus, the full 9.2-kb mRNA transcript is produced from the imported viral genomic DNA. This transcript can be doubly spliced to produce 1.8-kb mRNAs that code for the proteins Tat, Rev, and Nef. Tat and Rev move to the nucleus where Rev mediates the export of mRNAs during the late phase while Tat triggers transcription.

Env, Vpu, Vpr, and Vif proteins are encoded by single-spliced 4.0 kb transcripts, while 9.2 kb unspliced mRNA transcripts are responsible for Rev. is required for the nuclear export of Gag and Gag-Pol. These unspliced mRNAs serve as the genomic RNA for virus progeny as well. A number of Gag molecules bind to the viral DNA before being transported to lipid rafts on the host cell's plasma membrane. Viral proteins are used to assemble the viral genome as a dimer, which then suddenly leaves the cell [11]. Once the viral particles have reached maturity, they are expelled from the host cell. The above-described processes offer several options for various therapeutic treatments, interventions and tactics.

3. Virus-receptor interaction and entry

The ability of HIV to propagate and cause pathogenesis in patients is essentially determined by the efficacy of HIV entry into a host cell. Prior to HIV-1 entering its target cells (CD4-expressing T-lymphocytes, macrophages, and monocytes), the viral envelope glycoprotein (Env) gp120 fuses with the host cell membrane via the primary cellular receptor CD4 [10]. This causes changes in Env conformation that make the interaction between the gp120 V3 loop and coreceptor CCR5 or CXCR4 easier. The trimeric spikes that make up the HIV-1 envelope are each composed of three gp120 surface subunits and three gp41 trans-membrane subunits. The CD4 receptor and either the CCR5 or the CXCR4 co-receptor are often needed for viral binding and entrance into the cell [11].

The virion gp120 surface subunit (SU protein) binds to the CD4 receptor to start the HIV-1 infection. A non-covalent bond between the SU protein and the gp41 transmembrane subunit (TM protein) holds the SU protein to the virus. A cellular convertase called furin located in the endoplasmic reticulum (ER) proteolytically separates SU and TM from the Envelope (Env) precursor protein. Both continue to be noncovalently linked and are transported by vesicles to the host plasma membrane. The TM protein facilitates the fusion of the viral membrane with the host cell membrane, while the SU protein is in charge of receptor recognition on CD4+ T-lymphocytes [12].

A structural change in SU brought on by binding to the CD4 receptor makes the binding site for a co-chemokine family receptor visible. Chemokine receptors CCR-5 (R5 HIV-1 isolates) and CXCR-4 (X4 HIV-1 isolates), which are employed by HIV-1 viruses that are monocytes/macrophage- and T-cell-tropic, respectively, are the two main co-receptors needed for HIV-1 entry [13]. Another structural change that results from the SU protein's binding to the co-receptor exposes the N-terminal portion of TM. This component also referred to as the fusion peptide, facilitates the fusing of the

host and viral membranes. Through a process called syncytium formation, the Env protein is also able to mediate the union of infected and uninfected cells [14, 15]. The CD4-SU interaction, SU-chemokine co-receptor interaction, and the TM-mediated virus-cell membrane fusion process are the three main targets of current methods.

4. Antiretrovirals

Antiretroviral (ARV) drugs, as defined by the World Health Organization, are drugs used to treat HIV. Antiretroviral therapy, or ART, is the term used to describe the use of three or more ARV medications in combination to treat HIV infection. ART requires ongoing care. Combination ART and highly active ART are synonyms [16]. Currently, over twenty-four antiretroviral medications that have been licensed by the FDA are being used to treat HIV infection [17]. These are divided into many classes based on how they work, particularly when it comes to virus replication [18].

They include:

1. Specific CD4-directed post-attachment inhibitors bind to chemokines co-receptors, thereby preventing HIV from attaching to and entering host cells;

2. Chemokine receptor antagonists (CRAs) selectively block interactions between the human CCR5 receptor and the HIV-1 gp120 protein which, in turn, prevents HIV entry into cells;

3. Fusion inhibitors (FIs) disrupt HIV binding and, ultimately, fusion with host cells;

4. The transcription of viral RNA into double-stranded DNA can be prevented with nucleoside or nucleotide reverse transcriptase inhibitors (NRTIs);

5. Targeting the same process, the activity of the key enzyme HIV-1 reverse transcriptase may be reduced or blocked with non-nucleotide reverse transcriptase inhibitors (NNRTIs);

6. Integrase inhibitors (IIs) hinder the transport and attachment of pro-viral DNA to host-cell chromosomes;

7. HIV replication and the formation of mature, infectious viral particles can be prevented with protease inhibitors (PIs);

8. Pharmacokinetic enhancers do not directly interfere with viral replication but rather boost the concentration of antiretrovirals in the blood to make them more effective. Numerous variations of the cart have been approved.

The initial therapy usually starts with a combination of three antiretrovirals, including two NRTIs plus an NNRTI, or two NRTIs plus a protease inhibitor. The use of four antiretrovirals was shown not to improve outcomes over a combination of three compounds [19].

The ability of the virus to maintain latency or persist in dormant memory CD4+ T cells (reservoirs) and other cell types at various sites throughout the body

presents the biggest challenge for cART or HAART [17, 20]. When treatment is stopped or instructions are not followed as directed, the virus can re-emerge and become active. Its mechanism, viral integration location, pro-viral orientation, genomic architecture, and stochastic gene expression have all been suggested as significant factors [17, 21, 22] despite the fact that there is a dearth of knowledge and understanding of it.

Reactivation and subsequent depletion ("shock and kill") of the virus in latent reservoirs has been one therapeutic strategy [23–25], while manipulation of the signaling pathways necessary for latency establishment has been another strategy [26]. It is important to highlight that despite the use of all these techniques, cART has not yet completely eliminated or eradicated the virus [27]. As a result, cART must be continued for the entirety of a carrier's life.

The HIV-1 genome is composed of nine viral genes (gag, pol, vif, vpr, tat, rev, vpu, env, and nef) that are required for all processes and stages of the viral life cycle, including viral assembly, viral entry and receptor binding, membrane fusion, reverse transcription, integration, and proteolytic protein processing [11]. HIV-1 is diploid and has two plus-stranded RNA copies of its genome (**Figure 2**) [28]. It is now necessary to develop alternative therapeutic strategies that are safer, more effective, and more resistant to viral escape because the current cART regimens are unable to cure HIV-1 patients, as these drugs cannot eradicate latent viral reservoirs, may also fall

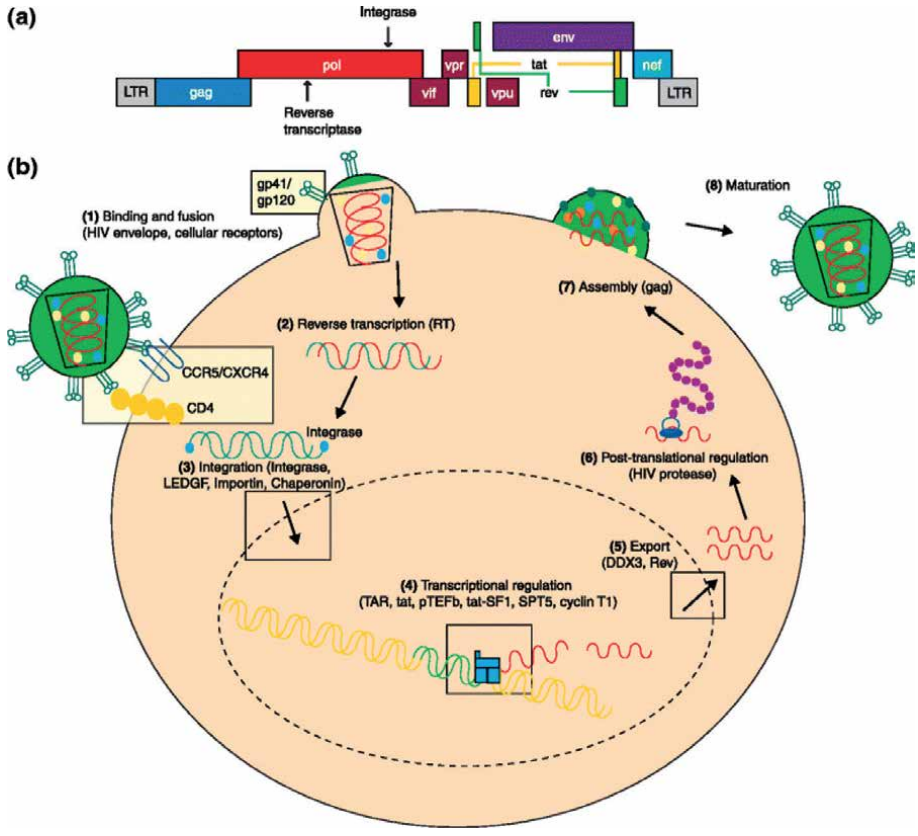

Figure 2.
The HIV-1 genome and strategies for antiviral targeting. Source: [11].

short in completely suppressing viral replication despite drug intensification, and also because there is a possibility that resistance will develop and that there will be toxic side effects. Gene-based and nucleic acid-based therapeutics based on gene editing, ribozymes, and RNA interference (RNAi) [11] are examples of such cutting-edge therapeutic approaches.

5. Interfering strategies against HIV-1

Over the past 30 years, antiviral agents that target viral proteins or host factors have been successfully developed. Based on their inhibitory mechanisms, antiviral reagents can be divided into two groups:

1. Inhibitors that target the viruses themselves.

2. Inhibitors that target host cell factors.

Virus-targeting antivirals (VTAs) can function directly (DVTAs) or indirectly (InDVTAs) to inhibit biological functions of viral proteins, mostly enzymatic activities, or they can also prevent the proper development of the viral replication machinery or protein(s), depending on the situation.

Host-targeting antivirals (HTAs) include reagents that target the host proteins that are involved in the viral life cycle (**Figure 2**), regulating the function of the immune system or other biological/cellular processes in host cells [28].

6. Molecular strategies to inhibit HIV replication in host cells

6.1 Direct virus-targeting antivirals

Attachment inhibitors: The first stage of viral invasion is the attachment to host cells by contact with the functional receptor(s). For enveloped viruses, receptor identification and host cell attachment are carried out by viral proteins found on the virion's outer envelope. The envelope proteins gp120 and gp41, which are organized on the viral membrane as a trimer of three trans-membrane gp41 and three noncovalently linked gp120 surface subunits, mediate the invasion and entry of HIV, a typical enveloped virus that belongs to the family of Retroviridae (**Figure 2**). In response to the CD4 receptor being recognized, gp120 initiates conformational changes that reveal the binding sites for the co-receptors (CCR5 and CXCR4). As a result, anti-HIV medicines have been created that function as antagonists to prevent interactions between HIV and its receptor and co-receptors [28].

6.2 Entry inhibitors

Following direct membrane fusion or endocytosis, a virus releases its genome into the cytoplasm of its host cells after being attached to host cells. Entry inhibitors have been successfully developed for antiviral medicines due to viral entry being one of the crucial early stages in the viral life cycle. When gp120 binds to the receptor, it activates gp41, which then aids HIV-1 in entering cells as an enveloped virus. Two heptad repeat domains (HR1 and HR2) plus a hydrophobic fusion peptide (FP) at the

N terminus make up the extracellular component of gp41. A meta-stable prefusion intermediate is created when the FP of gp41 is incorporated into the host cell membrane and HR1 forms a triple-stranded coiled-coil structure. After then, HR2 folds into the hydrophobic grooves of HR1's coiled-coil to create a stable six-helix bundle that positions the viral and cellular membranes next to each other in preparation for fusion. Inhibitors of membrane fusion are created to prevent the conformational alterations necessary for membrane fusion. The first and only clinically licensed fusion inhibitor is the T20 peptide (Enfuvirtide), which is a peptidic mimic of HR2 and functions by competitively binding to HR1 [29]. T20 can block a wide variety of HIV strains at the nanomolar level, but due to the medication's poor bioavailability (its plasma half-life is 4 hours), make the clinical application of this drug difficult [28].

6.3 Fusion inhibitors

Theywork to prevent the conformational alterations necessary for membrane fusion. The first and only clinically licensed fusion inhibitor is the T20 peptide (Enfuvirtide), which is a peptidic mimic of HR2 and functions by competitively binding to HR1 [29].

Structure of the HIV-1 genome. The genome contains nine genes and two long terminal repeats (LTRs) that can be targeted by RNA interference (RNAi). Certain genomic regions are more conserved than others, making them better targets. In addition, many of the genes are alternatively spliced, requiring careful target design.

HIV-1 targeting. Several steps of the HIV-1 viral replication cycle can be targeted by RNAi. Current drug targets are in parentheses [1]. The first step is receptor binding and membrane fusion by the HIV envelope glycoproteins gp120 and gp41 to host receptors CD4 and either CCR5 or CXCR4. This step can be inhibited by knocking down the HIV-1 co-receptors, CCR5 or CXCR4 [2]. Next, the viral genome must be reverse transcribed by the viral reverse transcriptase (RT) and [3] integrated into the cellular genome which is mediated by the viral integrase protein and host factors LEDGF, Importin, and Chaperonin. After integration [4]. The virus is transcribed, which is mediated by viral (TAR and tat) and host (pTEFb, tat-SF1, SPT5, cyclin T1) factors, [5] exported to the cytoplasm (dependent on DDX3 and Rev) and then translated and [6] subjected to post-translational processing by the viral protease [7]. Finally, the proteins are processed and [8] packaged into new viral particles.

Over the past few decades, research has focused heavily on the development of potential means of inhibiting the enzymes implicated in the replication of HIV-1, thereby inhibiting the corresponding pathway leading to a delay in progression or complete elimination of the infection in infected individuals. It's important to keep in mind that HIV-1 uses the host cell's replication apparatus, which reduces the number of potential viral targets. The evolutionary freedom for some virus components that interact with host cell molecules has been constrained as a result of the intimate host-virus relationship [30].

6.3.1 Targeting the viral enzymes

6.3.1.1 HIV-1 reverse transcriptase (RT)

Early 1980s revelation that HIV-1, a human retrovirus, causes AIDS sparked retroviral research and drew attention to viral enzymes, which are now the main targets of anti-AIDS medications [31]. Of the 26 medications now approved to treat

HIV-1 infections, 14 are RT inhibitors, including the first anti-HIV medication, AZT, which targets RT [32]. Reverse transcription is carried out by the two enzymes DNA Polymerase and RNase, which are both present in RT. Reverse transcription is influenced by other viral and cellular variables, it is important to note [31]. Of the 26 medications now approved to treat HIV-1 infections, 14 are RT inhibitors, including the first anti-HIV medication, AZT, which targets RT [32]. Reverse transcription is carried out by the two enzymes DNA polymerase and RNase, which are both present in RT. Reverse transcription is influenced by other viral and cellular variables, it is important to note [31]. Transcribed backwards [31]. By inserting mutations into the viral genome, HIV-1 reverse transcriptase (RT), which is created from a Gag-Pol polyprotein by cleavage with the viral protease (PR), aids in the emergence of drug resistance to all anti-AIDS medications. The Reverse Transcription complex, or RTC, has been shown to contain a number of viral proteins, including MA, CA, NC, IN, and Vpr. RNA- and DNA-dependent DNA synthesis, RNase H activity, strand transfer, and strand displacement synthesis are the primary functions of RT and are all critical to the retrotranscription process [33]. RT is primarily responsible for several distinct activities that are all indispensable for the retrotranscription process: RNA- and DNA-dependent DNA synthesis, RNase H activity, strand transfer, and strand displacement synthesis [33].

Mutations in RT cause resistance to RT inhibitors at the molecular level. HIV-1 medications include five non-nucleoside inhibitors (NNRTIs) and eight nucleoside/nucleotide analogs (NRTIs). Different conformational and functional states of the enzyme have been revealed by the structures of RT that have been identified in complexes with substrates and/or inhibitors [32]. Two kinds of RTIs that target the viral enzyme with two separate modes of action are part of the approved combination therapies for HIV-1. The second class consists of substances known as nonnucleoside RT inhibitors (NNRTIs), while the first class consists of substances known as nucleoside/nucleotide RT inhibitors (NRTIs/NtRTIs). When supplied as prodrugs, NRTIs and NtRTIs can be integrated into viral DNA by RT after being taken up by cells and phosphorylated by the host cell enzymes. Since NRTIs do not include a 30 hydroxyl group, their inclusion prevents the synthesis of viral DNA (**Figure 3**).

The common NRTI resistance mutations cause resistance by two general mechanisms:

1. Mutations that reduce the incorporation of the NRTITP relative to the normal dNTPs.

2. Mutations that lead to a selective excision of the incorporated NRTIs by RT, unblocking the viral DNA. NNRTIs bind to RT and block the chemical step of DNA synthesis [31]. RT enzyme inhibitors such as NRTIs and NNRTIs block its enzymatic function and prevent the completion of synthesis of the double-stranded viral DNA, thus preventing HIV from proliferating [35].

6.3.1.2 Nucleoside RT inhibitors

There are currently eight NRTIs clinically available, structurally resembling both pyrimidine and purine analogues [33]. These agents, in order to inhibit reverse transcription, have to be phosphorylated by cellular kinases to their triphosphate derivatives. All NRTIs follow the same mechanism of RT inhibition: once activated to their triphosphate form, they are incorporated by RT into the growing primer (**Figure 4**), competing with the natural dNTPs and terminating DNA synthesis due to their lack of

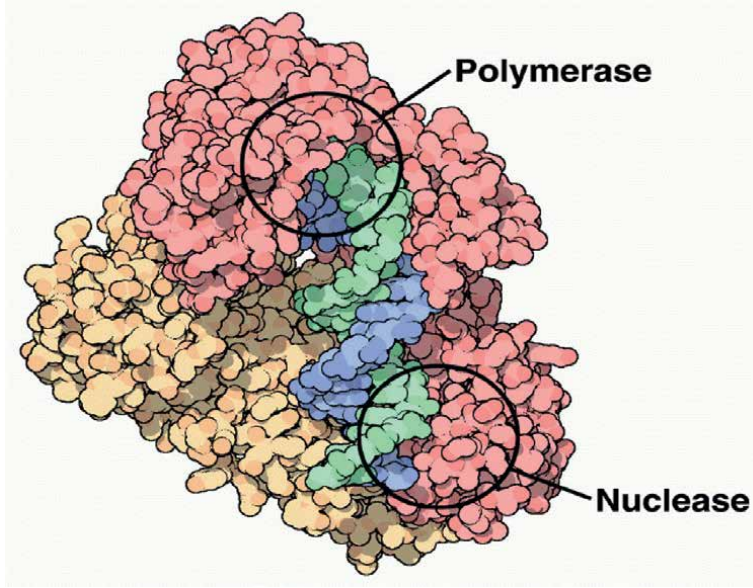

Figure 3.
HIV reverse transcriptase with a short piece of DNA bound in the active sites. Source: [34].

the 3-hydroxyl group (**Figure 5**). Therefore, once incorporated into dsDNA they prevent the incorporation of the incoming nucleotide. Importantly, while HIV-1 RT uses these NRTIs as substrates, the cellular DNA polymerases do not recognize them with the same affinity [33]. HIV-1 resistance to NRTIs usually involves two general mechanisms: NRTI discrimination, which reduces the NRTI incorporation rate, and NRTI excision which unblocks NRTI-terminated primer [33]. HIV-1 resistance to NRTIs usually involves two general pathways: NRTI discrimination, which reduces the NRTI incorporation rate, and NRTI excision which unblocks NRTI-terminated primers [33].

6.3.1.3 Nucleotide RT inhibitors (NtRTIs)

These are substances, such as [R]-9-[2phosphonylmethoxypropyl]-adenine (tenofovir, PMPA), that already have a phosphonate group that is resistant to hydrolysis. Therefore, the intracellular activation pathway is shortened, allowing for a more quick and thorough conversion to the active agent. They only require two phosphorylation steps to transform into their active diphosphate derivatives. Similarly to NRTIs, NtRTIs are phosphorylated to the corresponding diphosphates by cellular enzymes and serve as alternative substrates (competitive inhibitors); once incorporated into the growing viral DNA, they act as obligatory chain terminators. NtRTIs such as tenofovir are taken as prodrugs to facilitate penetration of target cell membranes. Subsequently, endogenous chemolytic enzymes release the original nucleoside monophosphate analogue that exerts its action [33].

6.3.1.4 Nonnucleoside RT inhibitors NNRTIs

Nonnucleoside RT inhibitors, or NNRTIs, are chemically and structurally unrelated substances that bind noncompetitively to a hydrophobic RT pocket near

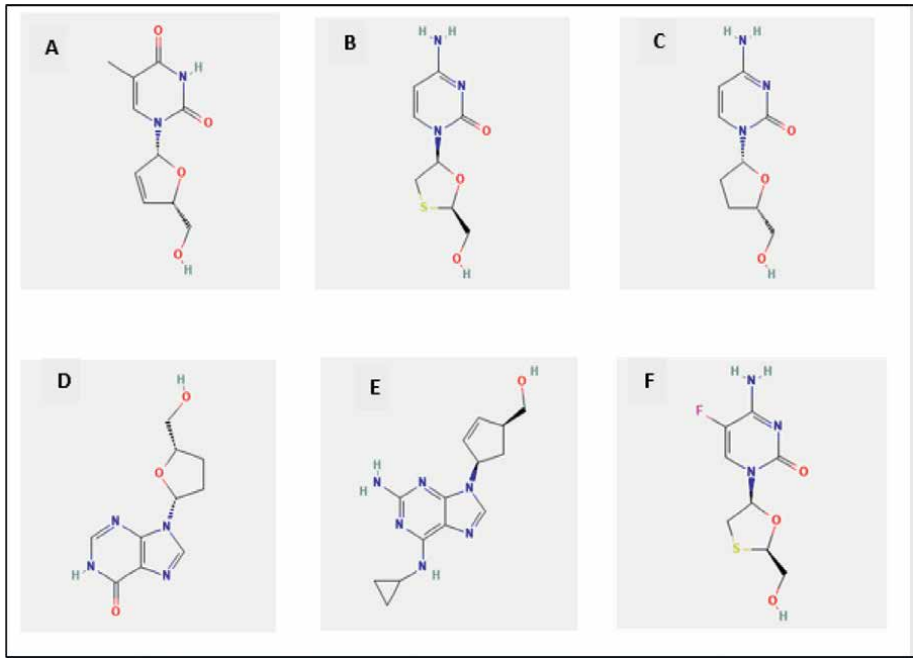

Figure 4.
Chemical structures and PubChem identification numbers of approved NRTIs: A: Stavudine (PubChem CID 18283), B: Lamivudine (PubChem CID 60825), C: Zalcitabine (PubChem CID 24066), D: Didanosine (PubChem CID 135398739), E: Abacavir (PubChem CID 441300) and F: Emtricitabine (PubChem CID 60877) respectively. Source: https://pubchem.ncbi.nlm.nih.gov.

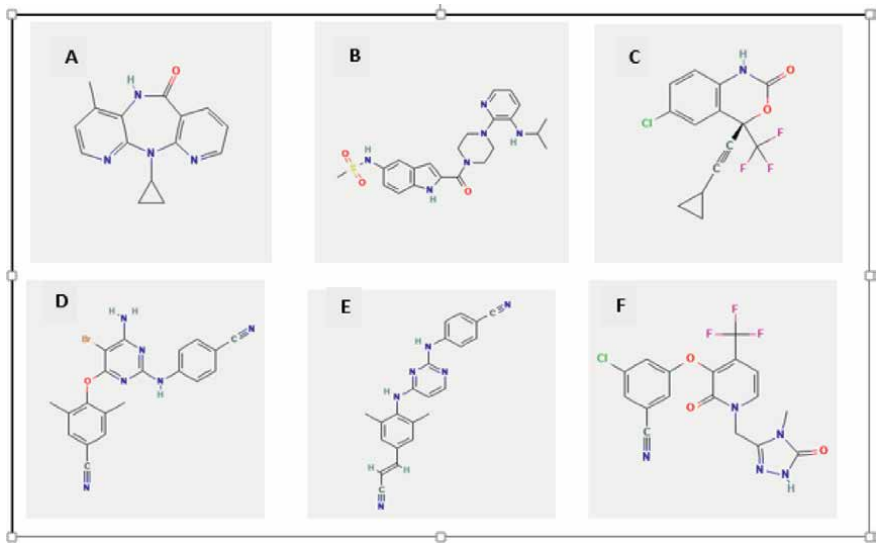

Figure 5.
Chemical structures and PubChem identification numbers of new NRTIs acting as chain terminators; A: Nevirapine (PubChem CID 4463), B: Delavirdine (PubChem CID 5625), C: Efavirenz (PubChem CID 64139), D: Etravirine (PubChem CID 193962), E: Rilpivirine (PubChem CID 6451164), and F: Doravirine (PubChem CID 58460047) respectively. Source: https://pubchem.ncbi.nlm.nih.gov.

the polymerase active site. This causes the protein to deform and prevents the chemical process of polymerization [36]. When NNRTIs bind to RT, they modify the conformation of certain residues (Y181 and Y188) in the rotamer and stiffen the thumb region, which prevents DNA synthesis. It's significant to note that NNRTIs, unlike NRTIs, do not depend on intracellular metabolism to function [33]. According to research in docking, molecular modeling, and crystallography, first-generation NNRTIs adopt a butterfly-like conformation [37]. The stabilization of the NNRTI binding in the allosteric site is accomplished through (i) stacking interactions between the NNRTIs aromatic rings and the side chains of Y181, Y188, W229, and Y318 residues in the RT lipophilic pocket; (ii) electrostatic forces (particularly significant for K101, K103, and E138 residues); (iii) van der Waals interactions with L100, V106, V179, Y181, G190, W229, L234, and Y318 residues; (iv) hydrogen bonds between NNRTI and the main chain (carbonyl/amino) peptide bonds of RT.

6.3.1.5 Protease inhibitors (PIs)

The majority of viruses contain one or more proteases that are essential to the progression of the virus/viral life cycle. In order for the viral proteins to function properly, release functional viral proteins and individually during replication, transcription, and maturation, the viral proteases perform the proteolysis of a polyprotein precursor [28]. Viral proteases can also modify host cell processes including ubiquitination and ISGylation to protect viral proteins in an efficient manner [38]. In contrast to the diversity of viral protease functions and structures, the catalytic active site of viral proteases generates stringent substrate specificity in protein cleavage. Synthetic substrate peptides, which can be designed according to the natural substrates of individual viral proteases, usually generate high-affinity binding, hence, provide potent candidates for further drug discovery. One of the great successes is the HIV-1 protease inhibitors (PIs). There are ten PIs currently approved to treat HIV-1 infection: amprenavir (APV), atazanavir (ATZ), darunavir (TMC114), fosamprenavir (Lexiva), indinavir (IDV), lopinavir (LPV), nelfinavir (NFV), ritonavir (RTV), saquinavir (SQV), and tipranavir (TPV). The cross-resistance to PIs occurs in the active site of HIV-1 protease because all HIV-1 PIs have small identity chemical structures derived from their natural peptidic substrate. Because of this, PIs are frequently used in conjunction with other anti-HIV medications to prevent drug resistance. PIs are now the most effective antiviral medication kinds because the majority of their pharmacological drawbacks have been resolved [39].

6.3.1.6 Integrase inhibitors (INIs)

Integrase inhibitors (INIs) are the latest class of antiretroviral drugs approved for the treatment of HIV infection and are becoming 'standard' drugs in the treatment of both naïve as well as heavily pretreated individuals with HIV [40]. Integrase enzyme inhibition is an appealing therapeutic target for HIV-1 treatment due to the lack of a homolog in human cells and its critical involvement in HIV-1 replication [41]. INIs have the potential to reduce the likelihood of the virus adapting or becoming resistant due to their distinct mechanism of action, when administered alone or in conjunction with other classes of anti-HIV medications. Their structural complexity and mechanism of action suggest several therapeutic interventions, including interfering with integrase binding to viral cDNA ends, interfering with integrase oligomerization,

interfering with strand transfer activity, interfering with 3-P activity, and interfering with IN-protein interactions with cellular cofactors [42].

INIs are classified in 10 scaffolds based on their structures including hydroxylated aromatics, diketo acids, naphthyridinecarboxamides, pyrrolloquinolones, dihydroxypyrimidinecarboxamides, azaindolehydrixamic acids, 2-hydroxyisoquinoline-1,3[2H,4H]-diones, 6,7-dihydroxy-1-oxoisoindolines, quinolone-3-carboxylic acids and carbamoyl pyridines [41]. INIs fall into one of two categories based on their mechanism of action: protein-protein interaction inhibitors (PPIIs) and integrase strand transfer inhibitors (INSTIs). INSTIs bind to Mg^{2+} ions and target the enzyme's active site. Competitive inhibitors prevent 3′-end processing by engaging in direct competition with viral DNA for binding to integrase [43]. Significant progress has been made since integrase was identified as a potential therapeutic target, leading to the approval of three INIs, notably dolutegravir, elvitegravir, and raltegravir (**Figure 6**).

INSTIs are all IN inhibitors with FDA approval [42]. PPIIs work by preventing integrase, which is necessary for viral replication, from interacting with the host protein lens epithelial-derived growth factor (LEDGF/p75). Data indicate that LEDGF/p75 instructs integrase to insert viral DNA into transcriptionally active areas in the host genome, despite the fact that the exact mechanism of action is unknown. The inhibitors of this protein are already being created and patented. They are less likely to acquire resistance and are more likely to be highly target selective [44]. Integrase binding inhibitors (INBIs) like V-165, however, might also be categorized as INIs because of their capacity to interfere with integration while having no noticeable impact on viral DNA synthesis. Investigation into the mechanism of action revealed that V-165 prevents the development of viral DNA-IN complexes. Because of its interference activity, it is categorized as an IN binding inhibitor. Similar actions are shared by other substances, such as styrylquinolines, which compete with the LTR substrate for IN binding [45].

6.3.1.7 Polymerase inhibitors

Most viruses encode polymerases in the central steps of replication and transcription. Viral-encoded polymerases perform essential enzymatic steps through amplification- or transformation of the viral genome during the viral life cycle [46]. Therefore, viral polymerases are becoming the most attractive and suitable targets

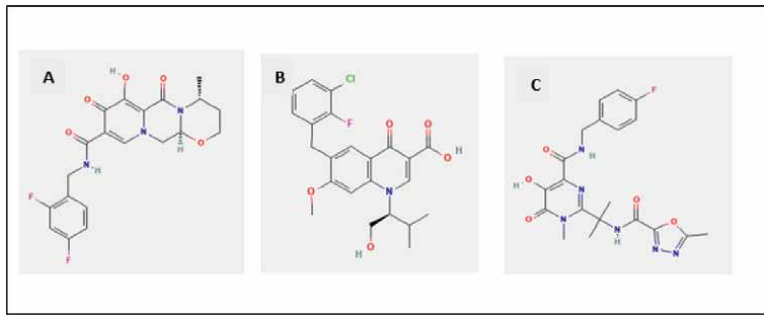

Figure 6.
Chemical structures and PubChem identification numbers of INIs approved for the treatment of HIV; A: Dolutegravir (PubChem CID 54726191), B: Elvitegravir (PubChem CID 5277135), and C: Raltegravir (PubChem CID 54671008) respectively. Source: https://pubchem.ncbi.nlm.nih.gov).

for antiviral design and development against many viral infections. Based on the function and structure of viral polymerases, there are two major types of polymerase inhibitors: (i) nucleoside and nucleotide substrate analogs and (ii) allosteric inhibitors. Nucleoside/nucleotide analogs (NAs) play a dominant role in antiviral drugs targeting viral polymerases [28], and were among the first polymerase inhibitors that showed clinical efficacy and are nowadays broadly used to treat hepatitis B-, herpes simplex- and HIV-1 infection [41] and constitute the typical backbone components of modern highly active antiretroviral treatment (HAART). The majority of the time, nucleoside analogs are created as pro-drugs that must undergo intracellular phosphorylation in order to create an analog of [deoxy-] nucleoside-triphosphate (NA-TP) that can be incorporated by the viral polymerase into nascent viral DNA. NA-TP can mimic adenosine, thymidine, guanine, cytosine, or uracil. Nucleoside analogs stop the polymerization process after inclusion because they lack the chemical group required to connect the subsequent incoming nucleotide [46]. The host cell first triphosphates nucleoside analogs to create the active inhibitor, which then competes with the natural nucleoside triphosphates to stop the viral nucleic acids from growing [22]. However, some viral polymerases have the ability to selectively remove or excise incorporated analog, saving the nascent viral DNA and causing a temporary rather than long-lasting method of inhibition. The balance between viral clearance by the immune system and viral replication can be tipped in favor of the immune system by inhibiting the critical step of viral DNA polymerization, which can decrease the likelihood by which circulating virus can successfully infect host cells and the number of viral progeny produced per unit of time [46].

One of the demerits of nucleoside analogs is that the initial phosphorylation step, (production of the monophosphorylated form), required for the activation of a triphosphate may not correctly take in the host cell [22]. Therefore, monophosphate nucleotide analogs were developed as polymerase inhibitors to avoid this problem. To date, most of the approved antiviral drugs for anti-HIV therapy utilize this mechanism, including Zidovudine (AZT, 30-azido-20,30-dideoxythymidine), Didanosine (ddi, 20,30-dideoxyinosine), Zalcitabine (ddC, 20,30-dideoxycytidine), Stavudine (d4T, 20,30-dideoxy-20,30-didehydrothymidine), Lamivudine (3TC, [−]-b-L-30-thia-20,30-dideoxycytidine), and others [22]. Secondly, the inhibition of DNA polymerization by the NAs is not restricted to viral polymerase, but can also affect cellular polymerases, leading to unwanted side effects [46]. The molecular kinetics of the relevant enzymes in relation to a specific inhibitor heavily influences the therapeutic window of NAs. Therefore, for NAs to have a therapeutic benefit, the targeted viral enzyme must be very specific. By altering the kinetics of the viral enzyme, viral resistance development can reverse this selectivity [46].

6.3.2 Prospect for new nucleoside RT inhibitors

There are various variables that restrict the therapeutic usage of NRTIs. First off, interactions between other NRTIs utilized in combination therapies, including the one between AZT and D4T, share the same phosphorylation route and exhibit a less-than-additive effect [33]. Second, interactions between drugs and other molecules, such as those that occur when a protease inhibitor is used in conjunction with the administration of ABC or tenofovir or when ABC is given in combination with ethanol. Thirdly, NRTIs have been connected to a number of negative side effects, including renal dysfunctions, drug hypersensitivity reactions, and mitochondrial toxicity (tied to myopathy, cardiomyopathy, anemia, and lipoatrophy) [47]. Fourthly,

given the requirement for ongoing antiviral therapy, the choice of NRTI-resistant strains continues to be the key restriction. Particularly, it has been reported that 6–16% of patients with viremia have viruses resistant to at least one drug, which results in a worse response to therapy and a lower barrier to the selection of additional drug-resistant strains [47]. Additionally, it has been reported that almost 50% of viremic patients actually harbor M184V RT mutant strains. In light of this situation, it is sought that the new NRTIs under research have a favorable resistance profile, diminished side effects, and/or a unique mechanism of action.

7. Conclusion

The increase in knowledge regarding the HIV life cycle and its biochemistry has led to many insights in the field of antiviral design and development. The virus being smart and highly infidel has further grown the quest for more information on the need for effective and efficient antiviral drugs. This has led to the emergence of new strategies and targets against the virus and or the host cell that would ultimately culminate in the development of many novel antiviral agents in the nearest future.

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Section 3

Biologics and Genome Editing System

Chapter 7

Biobetters: IFN-α2b Variants with Reduced Immunogenicity for the Treatment of Human Viral Diseases

Eduardo F. Mufarrege, Lucía C. Peña and Marina Etcheverrigaray

Abstract

For more than three decades, IFN-α2b has been widely used for the treatment of multiple human viral infections such as chronic hepatitis B and C, and certain types of cancers. However, IFN-α2b can be immunogenic, and these undesired immune responses can lead to a decrease in therapeutic efficacy. In addition, IFN-α therapy has also been associated with the progression of certain autoimmune diseases. For these reasons, the development of new IFN-α2b versions with reduced (or even null) immunogenicity has become the focus of several investigations. The "de-immunization" strategies usually involve several steps starting with T cell epitope identification and mutation of those immunogenic residues using immuno-informatics tools. Then, further experimental validation through *in vitro* and *in vivo* experimental platforms is needed to confirm *in silico* predictions. In this chapter, we will review the main strategies addressed so far to develop more effective and safer IFN-based therapies.

Keywords: interferon alpha, antiviral therapy, immunogenicity, de-immunization, biobetters, *in silico* epitope prediction, *in vitro* assay, *in vivo* assay

1. Introduction

Interferons (IFN) were initially discovered in 1957 by Lindenmann and Isaacs while performing infection experiments on chicken cells with the influenza virus [1]. In this study, the investigators observed a factor secreted by the infected cells that induced an antiviral state in the cell itself and in neighboring cells. This and other further studies [2] established the basis for elucidating the precise mechanism of action of IFNs. These secreted factors were later named "interferon" because of their ability to interfere with viral infections.

IFNs constitute a family of species-specific cytokines secreted in an autocrine and paracrine manner by host cells in response to pathogens, mainly viruses, and microbial products such as endotoxins, pyrans, and double-stranded polyribonucleotides, among others. They are composed of polypeptides ranging in molecular weights from 20 to 100 kDa. Like most proteins, they are sensitive to proteolytic enzymes and heat, although relatively stable at low pH values [3]. Essentially, the main function of IFNs

is to increase the transcription of hundreds of IFN-stimulated genes (ISGs) whose products play a key role in the innate and adaptive immune response to viral infections, producing profound metabolic changes [4].

Due to the antiviral and immunomodulatory function of IFN-α2b, this protein was developed as a recombinant version (rhIFN-α2b), which was first approved by the Food and Drug Administration (FDA) in June 1986 for the treatment of hairy cell leukemia. Then, in 1988, its use was also approved for the treatment of AIDS-related Kaposi's Sarcoma and in 1991 and 1992 to treat chronic hepatitis B and C virus infections, respectively.

Since its approval as an antiviral agent more than three decades ago, the use of rhIFN-α2b in the clinic has grown markedly. However, some adverse effects can be observed during rhIFN-α2b-based therapy. Among the most common unwanted effects are flu-like symptoms such as fever, fatigue, myalgia, chills, and headache. Also, subacute and chronic side effects such as myelosuppression, neuropsychiatric effects, and immunogenicity events can occur [5]. Regarding product immunogenicity, the development of antidrug antibodies is the most frequently detected response in patients. As an example, more than 40% of patients with malignant intestinal tumors showed anti-rhIFN-α2b antibody formation [6]. In addition, a similar proportion of patients suffering from renal cell carcinoma showed the presence of neutralizing antibodies after being treated with this biologic [7–9]. These rhIFN-α2b-based therapy unwanted effects represented a major challenge in the biopharmaceutical industry and prompted the development of new rhIFN-α2b variants with reduced immunogenicity. In the present chapter, we will discuss the main strategies addressed to develop reduced immunogenicity (de-immunized) versions of the cytokine.

Another drawback related to rhIFN-α2b therapy is the protein's short circulating half-life, which poses the need for repeated high doses to reach the therapeutic window. This can lead to the appearance of treatment adverse effects. In general, the higher the dose of rhIFN-α2b administered and the longer the treatment, the more frequent and severe adverse effects are observed. To circumvent this issue, numerous platforms based on glycoengineering and pegylation have been approached with encouraging results. Here, we will describe the strategies that led to more stable and safer rhIFN-α2b-based products.

2. Antiviral action of IFN-α

IFN-α triggers intracellular signaling cascades that activate multiple immune functions, affecting numerous cellular processes such as proliferation, differentiation, activation, migration, and apoptosis [10].

In the canonical signaling pathway, known as the JAK–STAT pathway (Janus kinase pathway, JAK, and the signal transducer and activator of transcription, STAT), the binding of IFN-α promotes the IFNAR heterodimeric receptor complex dimerization, which enables phosphorylation and activation of the Janus family kinases JAK1 and TYK2. Then, JAK1 and TYK2 recruit and activate STAT1 and STAT2 proteins, which allow their dimerization and consequently the recruitment of IFN regulatory factor nine (IRF9). These three proteins form the ternary ISGF3 complex, capable of translocating to the cell nucleus where they bind to IFN-stimulated response elements (ISREs) in the DNA and mediate the expression of different IFN-stimulated genes (ISGs) involved in the development of antiviral, proinflammatory, and immunomodulatory responses. Additionally, through the noncanonical signaling pathway, IFN-α

binding induces the expression of ISGs from the activation of other STAT proteins (such as STAT 3, 5, and 6) or through the mitogen-activated kinases (MAPKs) or phosphatidylinositol 3-kinase (PI3K) pathway [10].

Thus, IFN-α mediates antiviral actions by inhibiting the virion cycle and promoting enzyme synthesis, which interferes with viral particle transport, viral DNA transcription, replication, or RNA translation [11, 12].

Also, IFN-α elicits a powerful immunomodulatory action aiming to promote an effective antiviral immune response. For instance, IFN-α induces the activation of macrophages and NK cells, enhancing their cytotoxic activity, which helps promote the development of adaptive immune responses by increasing the expression of major histocompatibility complex (MHC) class l and ll molecules. As a result, this enhances the cytotoxic T cell-mediated killing efficiency of infected cells. Furthermore, IFN-α favors cell sequestration in lymph nodes, which promotes their activation and acts directly on T and B cells, guiding the progression of the antiviral response [13].

In addition, IFN-α inhibits cell proliferation through the regulation of different genes involved in the cell cycle, such as c-myc oncogenes, cyclin D3, phosphatase CDC25A [14], and cyclin-dependent kinase CDK-2 [15], or through the activation of numerous proapoptotic genes and proteins [16].

3. Immunogenicity of rhIFN-α2b in the clinic

rhIFN-α2b therapy can lead to the formation of antidrug antibodies. These antibodies can have varied consequences on the patient and the therapy efficacy. For example, these antibodies can bind to the biologic without having a significant impact on its biological effect or, on the contrary, neutralize its function or affect its pharmacokinetic properties [17, 18]. Regarding the nature of the antibody response, there are cases where rhIFN-α2b treatment resulted in the appearance of neutralizing IgG1 antibodies [19]. However, there is also evidence demonstrating the emergence of anti-rhIFN-α2b IgM antibodies [20]. Although the persistence and affinity of these antibodies are lower than the IgG antibody-mediated response, the consequences are potentially more severe due to their multivalent nature and the capacity to engage complement molecules and promote Fc-receptor cross-linking [20].

rhIFN-α2b immunogenicity was also evidenced by the appearance or exacerbation of certain autoimmune diseases. This is the case of autoimmune thyroiditis associated with rhIFN-α2b therapy. Initially, the occurrence of this disease was attributed to impurities in the biologic [21, 22]. However, the persistence of this disease after interruption of treatment and replacement with a product of higher purity showed that the observed autoimmunity was a direct consequence of the rhIFN-α2b proinflammatory capacity [23, 24]. The product's proinflammatory properties could explain the immunogenicity events observed in the clinic. There is growing evidence showing that the presence of T cell epitopes in the molecule can facilitate a proinflammatory context and also contribute to the development of antidrug antibodies [25–28]. Specifically, three regions enriched in T cell epitopes were identified in the rhIFN-α2b sequence, of which the region located at the c-terminal end of the molecule was found to be the most immunogenic [29].

The relevance of rhIFN-α2b immunogenicity in the clinic highlights the many efforts made to mitigate the effect of this unwanted drug attribute during treatment. The most promising strategies developed so far will be discussed in detail below.

4. Strategies aimed at reducing rhIFN-α2b immunogenicity

Because of the risk to patient health and therapy efficacy, several strategies are now available to reduce or mitigate the effects of rhIFN-α2b immunogenicity. Some of these involve modification of the protein, whereas others include coadministration of immunosuppressors.

Among the developments involving product modification, changes in the glycosylation pattern and the addition of polyethylene glycol (PEG) residues in the molecule deserve special mention. While the initial goal of these strategies was to increase the plasma half-life through an increase in apparent size and changes in the molecule's overall charge, these modifications could also exert a masking effect on potentially immunogenic epitopes. More recently, the advent of immuno-informatics and *in vitro* and *in vivo* experimental platforms have further enabled the development of de-immunized versions of biologics. These new biotherapeutic variants had modified the most immunogenic residues, thus reducing the binding of the biologic-derived peptides to human MHC, and consequently, the activation of specific T and B cells.

We will now review these strategies, highlighting their main advantages and limitations.

4.1 Development of rhIFN-α2b variants by glycoengineering

Glycoengineering is a modification strategy that is based on the linkage of glycan residues to the protein structure through covalent bonds [30]. This technology allows optimizing several macromolecular properties, such as increased solubility in aqueous solvents, thermal stability, as well as proteolytic and chemical degradation. In addition, the attachment of glycans can also lead to changes in specificity, affinity, and protein biological activity. Furthermore, there are studies showing that the presence of glycans could reduce the formation of aggregates and improve polypeptide folding and final protein conformation [31–34].

Protein glycosylation can take place in a variety of ways, on the side chains of the amino acids that constitute the polypeptide chain. However, the main forms of sugar linkage are through binding to the N atoms of the asparagine (Asn) side chain or the oxygen atom of the amino acids serine and threonine. In the first case, glycosylation is called N-glycosylation, while in the second it is known as O-glycosylation.

4.1.1 Development of N and O-glycosylated versions of rhIFN-α2b

rhIFN-α2b is a low molecular mass molecule and, therefore, susceptible to plasma clearance once it reaches the blood circulation. Indeed, the protein is nearly undetectable after 1-day post subcutaneous administration [35]. As a result, multiple doses of the product are required to reach the therapeutic window. For this reason, several approaches have been addressed to increase the apparent molecular size and, consequently, the stability, half-life, and *in vivo* biological activity of the molecule. Among the most widely used strategies, the incorporation of amino acid residues that allows the addition of specific glycans during posttranslational protein processing stands out [31, 36]. This not only increases cytokine size but also the overall protein charge, properties that are closely linked to renal clearance [37]. An additional effect is exerted by the sialic acid that decorates the glycans attached to the protein. This sugar residue restricts the binding of the cytokine to the liver receptors and consequently prolongs the half-life and *in vivo* biological activity [38, 39].

An interesting study using glycoengineering as a strategy to increase plasma half-life was addressed by Ceaglio et al. [40]. The authors modified the rhIFN-α2b sequence to add N-glycosylation sites to the molecule. These mutations were selected so as not to negatively impact the structure/function of the cytokine. Thus, the new hyperglycosylated version, known as 4 N-IN, evidenced a marked increase in plasma half-life and a significant improvement in systemic clearance in animal experiments. However, the *in vitro* antiviral activity showed a significant decrease as compared to the original molecule [40].

Similarly, another hyperglycosylated rhIFN-α2b was recently developed and referred to as GMOP-IFN. This new variant consisted of the addition at the N-terminal end of the cytokine of a peptide tag derived from the human granulocyte and macrophage colony-stimulating factor (GM-CSF), which contained additional O-glycosylation sites. As a result, the therapeutic protein was endowed with a higher sialic acid content and larger apparent molecular size. These properties allowed improved pharmacokinetic parameters in rats and increased thermal and plasma stability. In addition, unlike what was observed for 4 N-IFN, GMOP-IFN evidenced an antiviral activity similar to that of the wild-type molecule [41].

However, we further demonstrated using T cell proliferation and cytokine profiling assays that 4 N-IFN and GMOP-IFN were immunogenic. Moreover, through an immuno-informatics toolkit, we not only identified the potentially immunogenic amino acids and regions for both molecules but also the specific mutations that allow for disruption in the binding of IFN-derived peptides to the most prevalent human MHC (HLA, human leukocyte antigen) molecules in the world population [42, 43]. The development of these new variants will be discussed in detail below.

4.2 Use of pegylation for the development of rhIFN-α2b versions with enhanced stability

In addition to glycoengineering, another successful strategy to increase the circulating half-life of proteins for human therapeutic use is through the addition of polyethylene glycol (PEG) molecules. One case that exemplifies this is PEGIntron® produced by Schering Corporation. PEGIntron is a pegylated version of rhIFN-α2b which, due to the addition of a small PEG structure (approximately 12 kDa), exhibits increased residual biological activity. However, PEGIntron achieves only a modest improvement in circulating half-life over the original protein [44–47]. In this product, almost half of the PEG residues added to the molecule are exclusively bound to histidine and show high instability in water. This could explain, at least partially, the limited improvement in the stability of the product with respect to the nonpegylated molecule [44, 46].

To circumvent this drawback, another product based on rhIFN-α2b was developed by Roche and includes the addition of a 40 kDa molecule, called PEGASYS®. This product is more heterogeneous and consists of different isoforms with varying biological potency [47, 48]. As a consequence of the addition of a larger PEG molecule, the plasma half-life is increased. However, this also results in a lower biological activity, which ranges between 1 and 7% with respect to the unmodified cytokine [47].

A remarkable improvement in rhIFN-α2b pegylation technology was addressed by Rosendahl et al. [49]. In this study, the authors demonstrated that the addition of only one cysteine in the rhIFN-α2b sequence followed by pegylation at that residue allowed the development of a homogeneous mono-pegylated product. Among the cysteine residues whose mutation does not lead to a significantly increased immunogenicity,

mutein Q5C showed the most encouraging results. In fact, pharmacokinetic studies in rats inoculated subcutaneously with the product showed a plasma half-life 20 to 40 times higher than that of the unmodified protein [49].

In addition to facilitating an increased rhIFN-α2b *in vivo* stability, pegylation is frequently associated with reducing product immunogenicity [50–52]. Despite this, the presence of neutralizing anti-rhIFN-α2b antibodies has been detected in patients with chronic hepatitis C treated with pegylated rhIFN-α2b. Moreover, these antibodies caused a lack of response to IFN-based therapy, and consequently, these patients failed viral clearance [53].

4.3 Development of de-immunized therapeutic protein variants

As previously mentioned, although the development of improved versions of rhIFN-α2b with increased plasma half-life proved challenging, the results obtained were encouraging. Moreover, some of the rhIFN-α2b pegylated versions are commercially available on the market. However, the immunogenicity risk in the clinic still prevails [53]. As a result, this raises the need for reduced immunogenicity versions of the product. An interesting strategy is the socalled de-immunization for functional therapeutics (DeFT) [54]. The biotherapeutic de-immunization process involves several stages that will be discussed below. Finally, the de-immunization process of human rhIFN-α2b will be addressed, highlighting the challenges and achievements so far.

4.3.1 In silico analysis

The de-immunization process usually begins with the identification of potentially immunogenic protein regions. To accomplish this task, several *in silico* platforms are now available [55]. Usually, this identification involves testing the binding of potentially immunogenic regions of the molecule to the most relevant HLA class II alleles in the world population [56]. The results obtained from this analysis are summarized as an immunogenicity score that allows inferring the probability of binding of that peptide sequence to one or more HLA class II alleles. This information can be used to establish which HLA alleles/patients are most susceptible to developing an unwanted immune response against the therapeutic product.

Also, the computational study carries out an iterative process for the identification of changes or mutations that leads to greater disruption in peptide-HLA binding. The selection of which mutations are more appropriate should also be made taking into account the available protein structural information, to avoid significant disruptions in the molecule structure/function. This information can be obtained from a literature search, if available, or through protein molecular modeling.

4.3.2 In vitro *studies*

Although the results achieved through *in silico* analysis allow reducing the number and complexity of experimental assays, they are still predictions and consequently must be validated through *in vitro* studies.

For this purpose, peptides defined as potentially immunogenic are synthesized, including the "original" sequences, that is, those derived from the wild-type protein and the "modified" ones, which include the mutations defined from the *in silico* and structural study.

Then, the synthesized peptides must be tested using binding assays to specific HLA molecules. These assays allow estimating the binding affinity of the original and modified peptides to multiple HLA molecules and inferring which mutations actually produce a significant disruption in the antigenicity of the tested peptide. One of the most widely used assays consists of a competitive binding assay [57] that allows assaying various selected alleles in order to cover a wide repertoire of HLA pockets [56]. This experiment establishes competition between original and modified untagged peptides at different concentrations and a biotinylated standard peptide for binding to HLA class II molecules. The complexes formed are then captured on multi-well plates using immobilized anti-HLA class II antibodies. Assay development is performed by incubating the immuno-complexes with a streptavidin-enzyme conjugate and subsequent treatment with the enzyme substrate.

This *in vitro* binding assay provides valuable information that allows the identification of specific changes in the molecule that lead to a disruption of the interaction between the antigenic peptide and the HLA molecule. However, it is still an artificial assay that does not consider critical issues of antigenic protein processing. For example, peptides analyzed in binding assays are designed based on the clustering of highly immunogenic epitopes and do not take into account that such peptides are formed by the action of proteases (lysosomal cathepsins). As a result, the peptides actually formed inside the endosome and then loaded onto HLA molecules may differ from those designed on the basis of epitope content. Moreover, while binding assays provide information on the antigenicity of therapeutic protein-derived peptides, they do not establish whether those peptides will ultimately be recognized by the T cell receptor (TCR). Consequently, a peptide could be highly antigenic but not necessarily immunogenic. To overcome these limitations, *ex vivo* assays are now available and will be described below.

4.3.3 Ex vivo *assays*

Considering the relevance of achieving clinical trials with effective and safe biotherapeutics, it is necessary to rely on preclinical assays to anticipate the risks in the clinic. As a result, in the last decades, much effort has been devoted to the development of experimental platforms based on cell cultures (bioassays). These bioassays engage immune system mediators that could detect the product and consequently trigger an unwanted response in the patient.

One of the most commonly used assays involves the utilization of human peripheral blood mononuclear cells (PBMCs). PBMC samples typically engage monocytes, B cells, T cells, NK cells, and to a lesser extent dendritic cells. Therefore, these cell cultures include antigen-presenting cells (APCs) and T cells, which are the cellular mediators engaged in developing an immune response against immunogenic proteins. Wullner and collaborators [58] developed an experimental strategy based on the utilization of PBMC samples in which the therapeutic product is used to successively "challenge" the cell cultures. Initially, the APCs present in the PBMC sample internalize the product, process it at the lysosomal level, and the resulting peptides are presented in the context of HLA molecules. Thus, if the biotherapeutic contains T cell epitopes, repeated stimulation with the product will lead to activation, proliferation, and differentiation of specific naïve T cells into a specific T helper (Th) profile. In particular, the most frequently observed effector Th profiles are Th1, Th2, and Th17, by ELISA and/or ELISPot assays.

Due to the HLA allelic diversity in the world population and the potential differences in the elicited immune response, this assay should be performed using PBMC

samples from donors expressing different HLA alleles. In particular, it has been reported that HLA-DRB1 alleles are the most frequently involved in antigenic presentation and, therefore, required for T cell-mediated responses [59, 60].

Although this experimental strategy allows predicting with good accuracy the risk of immunogenicity of certain biologics in preclinical stages [27], it has a limitation associated with the direct effect that some biotherapeutics may have on T cells and, consequently, on the results derived from this assay. For example, IFN-α exerts a potent antiproliferative action on naïve T cells. Therefore, this experimental approach cannot be used to analyze the immunogenicity of rhIFN-α2b-based products.

To circumvent this issue, an interesting approach derived from this bioassay includes an initial step based on the development of immature dendritic cells as antigen-presenting cells [60]. Immature dendritic cells can be obtained *in vitro* from the differentiation of monocytes isolated from PBMC samples. Differentiation can be achieved by culturing monocytes with GM-CSF and IL-4 for 6 days [61]. The generation of dendritic cells can then be confirmed by microscopic inspection and flow cytometry by analyzing CD11 and CD14 cell marker expression. Immature dendritic cells are cocultured with the biotherapeutic and then maturation of the Ag pulsed-DCs is induced with human tumor necrosis factor (TNF) or LPS [42]. During the maturation process, there is an increase in the expression of costimulatory molecules CD80 and CD86 required for T cell activation. Finally, mature dendritic cells presenting biologic-derived peptides are cocultured with autologous T cells and the specific cell activation is analyzed as mentioned above.

In vitro and *ex vivo* assays provide valuable information on biologic immunogenicity risks and allow validation of results derived from *in silico* predictions [27]. However, on occasion, it is also necessary to confirm these data through *in vivo* assays.

4.3.4 In vivo *studies*

Initially, toxicity and immunogenicity tests of biological products were carried out using "wild-type" animals (mainly mice). However, these animal models exhibit species-specific characteristics. In particular, the results achieved when studying the immune response developed by these animals may differ considerably from those in humans.

The advent of molecular biology and genetic engineering techniques has allowed the development of animals with particular characteristics of interest for this type of study. Currently, the most commonly used models for immunogenicity analysis are therapeutic protein-tolerant animals and transgenic animals expressing HLA molecules.

4.3.4.1 Mice immune tolerant to human proteins

Human protein-tolerant mice are engineered to express the therapeutic protein of interest. Hence, during cell ontogeny, tolerance processes allow the maintenance of immune homeostasis toward that specific protein.

These mouse strains are useful for evaluating the presence of neo-epitopes as well as subtle changes in product attributes such as formulation.

Several tolerant mouse strains are available. Among them are human tissue plasminogen activator (tPA)-tolerant murine models [62], mice producing human preproinsulin [63], and human IFN-α [64] and IFN-β [65] tolerant animals. Currently, other tolerant murine models that are of great utility for *in vivo* analysis of different

biologic formulations include human growth hormone (GH)-producing mice [66] and human IgG-tolerant mice [67].

Although human protein-tolerant mouse strains have proven useful, they have some limitations. These mice express the murine antigenic processing and presentation system components and, consequently, the biologic-derived peptides that are exhibited by APCs and the immune response developed are still mouse-specific. Therefore, this may sometimes lead to results that do not necessarily correlate with those addressed during human clinical trials. In addition, this strategy raises the need to develop and validate a tolerant mouse strain for each therapeutic protein under study.

These issues could be addressed through the use of transgenic animals expressing HLA molecules, or combinations of these mice with tolerant animals (if available).

4.3.4.2 Mice expressing HLA molecules

In addition to mice tolerant to human therapeutic proteins, genetic engineering strategies have allowed the development of animals that have had the murine antigenic presentation system knocked out and express specific HLA molecules instead. In humans, numerous HLA genes have been identified and classified into three classes. HLA class I comprises the A, B, and C genes; HLA class II contains the DR, DQ, and DP genes; while HLA class III includes complement molecules. Class I and II molecules are involved in the selection processes of T cells in the thymus. Thus, HLA class I is responsible for the negative and positive selection of CD8 T cells, whereas HLA class II is involved in the selection processes of CD4 T cells [68]. Therefore, HLA transgenic mice exhibit an antigenic presentation system analogous to the human one (at least in those specific HLA molecules). Thus, peptides derived from antigenic proteins, including therapeutic proteins, will be identical to those presented by humans expressing those HLA molecules. In addition, due to the selection processes in the thymus mentioned above, these animals possess T cells endowed with TCRs capable of recognizing peptides presented in the context of these HLA molecules. As a result, these animals develop an immune response that mimics the human immune response. The close correlation of T cell-mediated responses observed in patients and in animals expressing HLA molecules represents clear evidence of the utility of this *in vivo* strategy [69].

These transgenic mice strains can be used for multiple purposes, including, in addition to the analysis of the immunogenicity of biological products, autoimmune diseases, vaccine development, certain infectious diseases, and oncology studies. In the immunogenicity analysis of therapeutic proteins, HLA-DR3 and HLA-DR4 mice have proven to be particularly useful [54, 70].

Despite the high correlation that *in vivo* studies involving transgenic animals may show, their execution and the number of animals required must be strongly justified. For this reason, before reaching *in vivo* assays, it is recommended to carry out all available *in silico*, *in vitro*, and *ex vivo* stages of the biologic immunogenicity analysis.

4.3.5 De-immunization of human rhIFN-α2b

To de-immunize rhIFN-α2b a multi-step strategy was approached [42]. First, the most immunogenic regions of the protein were identified by using the immunocomputational algorithms of EpiVax Inc. Then, mutations leading to a substantial disruption of IFN-derived peptide binding were selected from a group of eight HLA class II archetypes that combined exhibited a coverage of more than 90% of the allelic

diversity of the world population. In particular, the DRB1 alleles analyzed in this study were *0101, *0301, *0401, *0701, *0801, *1101, *1301, and *1501.

The results were validated by *in vitro* binding assays following the experimental method described above and the same eight HLA alleles used for the *in silico* analysis. Most of the computational predictions were experimentally confirmed, which highlights the accuracy of the computational method used in this study. Then, with the knowledge of the most relevant changes to be introduced in the rhIFN-α2b sequence and an exhaustive search in the literature of the most relevant residues for protein structure/function, we developed two variants containing these mutations in different combinations. Both muteins evidenced a dramatic decrease in *ex vivo* immunogenicity assays using human PBMC samples [56]. Moreover, a recent study demonstrated that whereas rhIFN-α2b induced a robust neutralizing antibody response in HLA-DRB1 transgenic animals, both rhIFN-α2b variants induced a markedly lower response. In addition, this study also revealed that these biobetters showed improved stability against several physical factors such as heat stress, repeated freeze/thaw cycles, and low pH [70].

However, both muteins exhibited only a residual *in vitro* antiviral biological activity of 28.4 and 16.9%. These results indicate that despite selecting mutations while avoiding introducing changes in critical residues of the molecule, a reduction of *in vitro* antiviral activity was evident [56]. To address this issue, five of the mutations identified as key in reducing rhIFN-α2b immunogenicity were introduced into a hyperglycosylated variant of the cytokine that exhibits improved plasma stability with respect to the unmodified protein. Thus, the new rhIFN-α2b variant retained not only the improved pharmacokinetic properties of the original molecule but also reduced immunogenicity, and a high residual antiviral activity (72%) [43].

5. Conclusion

For more than 30 years, rhIFN-α2b has been used for the treatment of chronic and emerging viral infections. However, rhIFN-α2b is potentially immunogenic, which may compromise the efficacy and safety of rhIFN-α2b-based therapy. In addition, rhIFN-α2b is a low molecular size cytokine, which correlates with its reduced plasma half-life in patients.

For these reasons, several strategies have been addressed to reduce the impact of protein immunogenicity as well as increase the product's plasma stability.

Glycoengineering and protein pegylation have been proposed as strategies aimed at increasing the apparent macromolecule size and, consequently, reducing the rhIFN-α2b clearance rate. The results obtained so far have been very encouraging, with remarkable pharmacokinetic parameters for these biobetters. In addition, both approaches would allow the addition of glycans or large PEG residues that would generate a shielding effect of protein epitopes. However, neither of these strategies has allowed the development of products that significantly ameliorate the negative impact associated with product immunogenicity.

In contrast, de-immunization for functional therapeutics (DeFT) of rhIFN-α2b has emerged as a promising alternative for markedly reducing product immunogenicity. The success of this strategy is based on the careful selection of the target residues, which will rely on the impact of such a change on the antigenicity/immunogenicity as well as on protein structure/function.

The individual or combined use of these strategies will enable the development of more effective and safer rhIFN-α2b-based antiviral therapies.

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