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M.Sc. THESIS

EVALUATION OF ANTIVIRAL ACTIVITIES OF DIFFERENT TYPES OF PROPOLIS EXTRACTS

Sevda DEMİR

Department of Genetics and Bioengineering

Genetics and Bioengineering Programme

SUPERVISOR
Prof. Dr. Ali KARAGÖZ

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İSTANBUL

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Examining Committee Members

Prof. Dr. Ali KARAGÖZ(Supervisor)
İstanbul University
Faculty of Science

Prof. Dr. Ercan ARICAN
İstanbul University
Faculty of Science

Assoc. Prof. Dr. Cenk KIĞ
Yeni Yüzyıl University
Faculty of Medicine

Academic Title

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FOREWORD

After a long journey I am content with this study. I knew that transfer of knowledge into practice of such a research would not be so easy and it would call for patience. I have to admit that it would not have been possible without assistance of certain people from different fields. I would like to take this opportunity to express my gratitude and appreciation for the support they provided to my thesis.

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TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

LIST OF SYMBOLS AND ABBREVIATIONS

Symbol	Explanation
µg/mL	: Microgram/Mililiter
µL	: Microliter
µM	: Micromolar
mg/mL	: Miligram/Mililiter
mL	: Mililiter
ºC	: Centigrade Degree

Abbreviation	Explanation
CC_{50}	: The concentration of a compound that will kill half of the uninfected cells.
DMEM	: Dulbecco's Modified Eagle Medium
DMSO	: Dimethyl sulfoxide
DPBS	: Dulbecco's Phosphate-Buffered Saline
EDTA	: Ethylenediaminetetraacetic Acid
EC_{50}	: The concentration of a test compound that produces 50% inhibition of virus replication
FBS	: Fetal Bovine Serum
HaCaT	: Immortalized Human Keratinocytes Cell Line
HSV-1	: Herpes Simplex Type 1
HSV-2	: Herpes Simplex Type 2
MCC	: The concentration of compound that is necessary to cause minimal alterations in cell morphology as determined by microscopy
MTS	: 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NC	: Negative Control
NAD(P)H	: Nitrite Reductase
PSA	: Penicillin/Streptomycin/Ampicillin
qRT-PCR	: Quantitative Real Time Polymerase Chain Reaction

ÖZET

FARKLI TİP PROPOLİS EKSTRELERİNİN ANTİVİRAL AKTİVİTELERİNİN DEĞERLENDİRİLMESİ

YÜKSEK LİSANS TEZİ

Sevda DEMİR

İstanbul Üniversitesi

Fen Bilimleri Enstitüsü

Genetik ve Biyomühendislik Anabilim Dalı

Danışman : Prof. Dr. Ali KARAGÖZ

Bu tez çalışmasında, 4 farklı propolis ekstresinin Herpes Simpleks Tip 1 (HSV-1) ve Herpes Simpleks Tip 2 (HSV-2) virüslerine karşı *in vitro* antiviral potansiyeli değerlendirilmiştir.

Ekstrelerin *in vitro* sitotoksik etkilerinin belirlenmesi, İnsan İmmortalize Keratinosit (HaCaT) hücre hattı üzerinde Mitokondriyal Dehidrogenaz Enzim Aktivitesi (MTS) yöntemi ile gerçekleştirilmiştir. MTS yöntemi ile hesaplanan ve hücrelerin %50'sini öldüren sitotoksik konsantrasyon (CC_{50}) değerleri Propilen, Etanol, Gliserol ve Soya ekstreleri için sırasıyla 593 µg/mL, 375 µg/mL, 1723 µg/mL ve 1664 µg/mL'dir.

HSV-1 ve HSV-2 ile enfekte olan hücrelerin %50'sini öldüren etkili konsantrasyon (EC_{50}) değerleri kantitatif Gerçek Zamanlı PCR yöntemi ile analiz edilmiştir. HSV-1 için hesaplanan EC_{50} değerleri, Propilen, Etanol, Gliserol ve Soya ekstreleri için sırasıyla 86.64 µg/mL, 90.86 µg/mL, 768.6 µg/mL ve 501 µg/mL'dir. HSV-2 için hesaplanan EC_{50} değerleri, Propilen, Etanol, Gliserol ve Soya ekstreleri için sırasıyla 92.05 µg/mL, 48.99 µg/mL, 904.1 µg/mL ve 396.1 µg/mL'dir.

Herpes enfeksiyonlarının tedavisi için kullanılan antiviral ilaçlardan biri olan Asiklovir çalışmamızda kontrol ilacı olarak kullanılmıştır. Asiklovir için CC_{50} değeri 15.85 µM, EC_{50} değeri HSV-1 için 2.50 µM ve HSV-2 için 5.54 µM olarak belirlenmiştir.

Ekstrelerin, *in vitro* antiviral etkinliği yüksek Selektif İndeks (SI: CC_{50}/EC_{50}) değerleri ile ortaya konur. Çalışmamızda HSV-1 için SI değerleri Propilen, Etanol, Gliserol ve Soya ekstreleri için sırasıyla 6.84, 4.12, 2.24 ve 3.32 ve Asiklovir için 6.34 olarak hesaplanmıştır. HSV-2 için SI değerleri, Propilen, Etanol, Gliserol ve Soya ekstreleri için sırasıyla 6.44, 7.65, 1.90 ve 4.40 ve Asiklovir için 2.86 olarak hesaplanmıştır.

Propilen ekstresi, Asiklovir ile karşılaştırıldığında HSV-1 için anlamlı derecede daha yüksek SI değeri gösterirken Etanol, Soya ve Gliserol ekstrelerinin SI değerleri Asiklovir'den daha düşük ortaya çıkmıştır. HSV-2 için elde edilen SI değerleri karşılaştırıldığında Gliserol ekstresi Asiklovir'e göre anlamlı bir sonuç ortaya koymazken, Etanol, Propilen ve Soya ekstreleri Asiklovir'den anlamlı derecede yüksek olduğu belirlenmiştir.

Propolisin özellikle Etanol, Propilen ve Soya ekstrelerinin HSV-2'ye karşı antiviral aktivite potansiyeline sahip olduğu ortaya çıkmaktadır.

Haziran 2019, 85 sayfa.

Anahtar kelimeler: Propolis ekstraktı, Antiviral Aktivite, HSV-1, HSV-2, Kantitatif RT-PCR.

SUMMARY

EVALUATION OF ANTIVIRAL ACTIVITIES OF DIFFERENT TYPES OF PROPOLIS EXTRACTS

M.Sc. THESIS

Sevda DEMİR

İstanbul University

Institute of Graduate Studies in Science and Engineering

Department of Genetics and Bioengineering

Supervisor : Prof. Dr. Ali KARAGÖZ

In this thesis, the effect of 4 different propolis extracts were investigated against Herpes Simplex Type 1 (HSV-1) and Herpes Simplex Type 2 (HSV-2) viruses.

In-vitro cytotoxic effect of each extracts were determined by the Mitochondrial Dehydrogenase Enzyme Activity (MTS) method on Human Immortalized Keratinocyte (HaCaT) cell line and the cytotoxic concentration (CC_{50}) values that killed 50% of the uninfected cells were calculated. The CC_{50} values were determined as 593 µg/mL, 375 µg/mL, 1723 µg/mL and 1664 µg/mL for the extracts of Propylene, Ethanol, Glycerol and Soya, respectively.

Effective concentration (EC_{50}) values that kill 50% of cells infected with HSV-1 and HSV-2 were analyzed by quantitative Real Time PCR method. The EC_{50} values calculated for HSV-1 were 86.64 µg/mL, 90.86 µg/mL, 768.6 µg/m L and 501 µg/mL for the extracts of Propylene, Ethanol, Glycerol, and Soya, respectively. The EC_{50} values calculated for HSV-2 were 92.05

μg/mL, 48.99 μg/mL, 904.1 μg/mL and 396.1 μg/mL for the extracts of Propylene, Ethanol, Glycerol and Soya, respectively.

In vitro antiviral efficacy of an extract is determined by high of Selective Index values (SI:CC_{50}/EC_{50}). The SI values for HSV-1 were calculated as 6.84, 4.12, 2.24, 3.32 and 6.14 for the extracts of Propylene, Ethanol, Glycerol, Soya and Acyclovir, respectively. The SI values for HSV-2 were calculated as 6.44, 7.65, 1.90, 4.40 and 2.86 for the extracts of Propylene, Ethanol, Glycerol, Soya and Acyclovir, respectively.

In comparison with the Acyclovir, Propylene extract showed significantly higher SI values while SI values for Ethanol, Soya and Glycerol extracts were lower than the values of Acyclovir for HSV-1. The SI values for Ethanol, Propylene and Soya extracts were significantly higher than the value of Acyclovir while no significant results were obtained for glycerol extract, in comparison with Acyclovir for HSV-2.

As a conclusion it was revealed that in particular Propylene, Ethanol and Soya extracts of propolis having antiviral activity potantial against HSV-2.

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Keywords: Propolis extract, Antiviral Activity, HSV-1, HSV-2, Quantitative RT-PCR.

1. INTRODUCTION

Humans have suffered from infections caused by bacteria, viruses, fungi, parasites. Modern medicine, which depends on engineered drugs and anti-infection agents, have turned out to be accessible in recent years [1]. Previously, over centuries, people have used therapeutic agents as drugs from natural sources such as plants, fungi and animals.

Natural products inspire novel discoveries in biology, chemistry, and medicine and they are optimized as drug-like molecules and remain the best drugs sources. Mostly, plants are used as a source of starting materials for drug discovery, as of the substantially all of drugs in ancient medicine, were plant-derived extracts and these resulted in an information pool about plant species. In medical history there are remarkable discoveries such as the finding of Penicillin, emancipated from mould, in 1928, and Streptomycin, synthesized by the soil organism Streptomyces griseus, in 1943 they were awarded The Nobel Prize in 1945 and 1952. This period was the beginning of the start of a period from the 1950s to 1960s known as the Golden Age of natural product drug discovery [2, 3].

Over the last thirty years, modified plant products by animals have been attracting attention for drug discovery studies. Propolis is one of the modified plant products that honeybees (*Apis mellifera*) collect resinous substances from several plants like alder, poplar, birch, palm, pine, willow, *Baccharis dracunculifolia*, and *Dalbergia ecastaphyllum* and mix it with beeswax and salivary enzymes (*β-glucosidase*) [4, 5]. This resinous structure is also produced from other materials that are actively secreted by plants, or exuded from wounds in plants (lipophilic material on leaves, mucilage, gums, resins, lattices, etc.) [6].

Honeybees utilize propolis in their hives as a heat insulator, to repair impairment, as protection against predators and microorganisms and to build an aseptic environment for preventing microbial infection of larvae [7-9]. That is how the Propolis term was coined in Greek: pro (for ‘in front of’, ‘at the entrance to’) and polis (‘community’ or ‘city’) and means a substance in defense of the hive [10]. In the ancient times, humans were using propolis in folk medicine. Especially, in Egypt, because of the antiseptic properties of propolis, it has been used for mummification. The Greek and the Roman physicians have been using propolis as a mouth disinfectant and in wound healing treatments as an antiseptic and cauterizing

agent. The Persians have utilized propolis for rheumatism, eczemas, myalgia, and the Incas have used it as a fever reducing agent. In World War II, doctors have been using propolis to treat wounds. Propolis was used for tuberculosis treatment by the old Union of Soviet Socialist Republics (USSR) and it has been seen as a promising product for pharmacology and it is still being used in complementary medicine [10-13]. In the seventeenth century in London, propolis has been indexed as a functionary drug and it became famous because of its antibacterial features, between the seventeenth and twentieth century in Europe [10].

In the last 20 years, considerable studies have been performed to investigate biological functions of propolis and in many studies it has been proven that, propolis has antimicrobial, antifungal, antiviral, antiinflammatory, immunomodulatory, anticancer, wound healing, skin protection, antioxidant and hepato-protective properties. Because of its biologically active properties, modern herbalists also recommend the usage of propolis [10, 14].

1.1. CHEMICAL COMPOSITION OF PROPOLIS

Propolis is one of the most important bee product that is mainly composed of plant resin (50%), wax (30%), essential and aromatic oils (10%), pollen (5%), and other organic compounds (5%) [15]. Phenolics, aromatic aldehydes, beta-steroids, terpenes, esters, flavonoids and alcohols are important organic compounds found in the propolis [5]. Propolis also contains significant vitamins, minerals and several enzymes [12].

Development of technology, separation and purification techniques (high performance liquid chromatography, thin layer chromatography, gas chromatography), and identification techniques (mass spectroscopy, nuclear magnetic resonance, gas chromatography, mass spectroscopy) provides identifying more compounds including phenolics, terpenes, flavonoids and their hydrocarbons, esters, sugars and mineral elements from propolis. In the literature, over 300 identified chemical components have been reported for propolis [5].

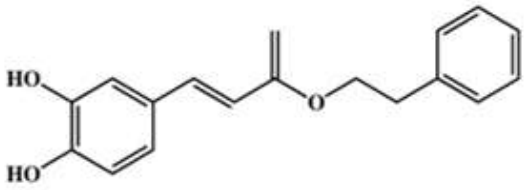
The chemical structure of propolis is subject to the bee species, botanical origin, and location [16]. For example, Chrysin, galangin, pinocembrin, pinobanksin are without B-ring substituents flavonoids that are characteristic components in temperate region propolis. Also, Caffeic acid phenethyl ester (CAPE) is the substantial component of the temperate region. Prenylated phenylpropanoids (e.g., artepillin C) and diterpenes are dominating component for tropical region [17].

1.1.1. Phenolic Compounds

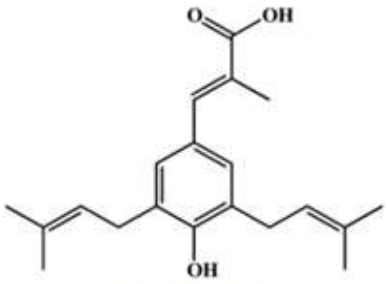
Phenolics are compounds that have one or more aromatic rings carrying one or more hydroxyl groups with over 8,000 structural variants. They are commonly classified as phenolic acids and analogs, curcuminoids, flavonoids, lignans, tannins, coumarins, stilbenes, quinones, and others based on the number of phenolic rings and of the structural elements that link these rings [18]. They mainly produce secondary metabolites in plants and shows diversity according to plant strain. In plants, phenolic compounds are produced with acetic acid pathway and shikimic acid pathway. For acetic acid pathway the simple phenols are produced as the main products. In shikimic acid pathway, phenylpropanoids are produced and mostly plant phenolic compounds are synthesized through this pathway [5, 18, 19]. These secondary metabolites have important properties in plants such as coloring for camouflage, accelerating pollination and defense against herbivores, as well as antibacterial and antifungal activities [20]. In literature, phenolic compounds in propolis also have several biological activities such as antiinflammatory, antibacterial, antioxidant, antitumoral and antiviral activities [21, 22].

The majority of the propolis content is composed of phenolic compounds. The main pharmacological properties of propolis are provided by flavonoids. In temperate regions the amount of flavonoids is used as a quality criteria for propolis [23]. Flavonoids in propolis are classified on the basis of their chemical structure such as chalcones, dihydrochalcones, flavones, flavanones, flavonols, flavanonols, isoflavones, isodihydroflavones, flavans, isoflavans and neoflavonoids.

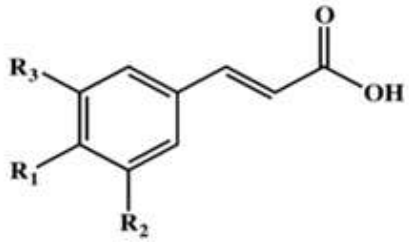
Phenolic acid, another class of phenolic compound, is found in the propolis. Naturally, phenolic acid is classified into two groups, being cinnamic acid and benzoic acids with their derivatives. For the benzoic acid group; gallic acid, gentisic acid, p-hydroxybenzoic acid, salicylic acid, protocatechuic acid, vanillic acid, and syringic acid, for cinnamic acid group ferulic acid, caffeic acid, p-coumaric acid, chlorogenic acid, and sinapic acid can be given as examples [19, 24]. In addition to these, especially in temperate regions, the phenolic acids found are benzyl-, methylbutenyl-, phenylethyl- and cinnamyl-esters, with caffeic acid phenyl ester (CAPE) [7, 25]. Some essential phenolic compounds in propolis are shown in Figure 1.1.

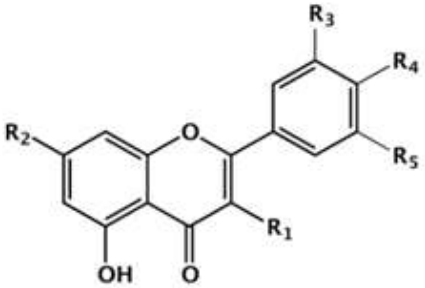


Caffeic acid phenethyl ester (CAPE)



Artepilin C





	R1	R2	R3
Cinnamic acid	H	H	H
p-Coumaric acid	OH	H	H
Caffeic acid	OH	OH	H
Ferulic acid	OH	OCH_3	H
Isoferulic acid	OCH_3	OH	H

	R1	R2	R3	R4	R5
Chrysin	H	OH	H	H	H
Galangin	OH	OH	H	H	H
Kampferol	OH	OH	H	OH	H
Quercetin	OH	OH	OH	OH	H

Figure 1.1: Some essential phenolic compounds in propolis.

1.1.2. Terpenoids

Terpenoids, also called isoprenoids, are legion and structurally varied natural products. Their names are formed from two parts, “terpene” is a given name to hydrocarbons found in turpentine, and the suffix “ene” point to the presence of olefinic bounds. Isoprene is the “unit” of terpenoids, 2-methylbuta-1,3-diene (C_5H_8), and one isoprene unit represents basic class of terpenoids, hemiterpenoids. Terpenoids are classified as monoterpenoids, diterpenoids, triterpenoids, sesquiterpenoids, iridoids, hemiterpenoids, sesterterpenoids, tetraterpenoids, polyterpenoids and irregular terpenoids [26]. In propolis, mainly mono- and sesquiterpenoids were identified till 2000 [6]. They are generated in two pathways. One is the mevalonate (MVA) pathway that occurs in the cytosol, the other is the plasticidal 2-C-methyl-D-erythriol 4-phosphate (MEP) pathway. While, there are exceptions and cross-talk between the two

pathways, generally, Hemi-, mono-, di-, and triterpenoids are produced in MEP pathway and sesqui and triterpenoids in the MVA pathway [27, 28]. Terpenoids are the main components of the essential oil and responsible for distinctive scent, smell and odor of many plants [29]. Although terpenoids constitute 10% of propolis, they are liable for the characteristic odor as well as to contribute to its pharmalogical effect. They also have a crucial role to differentiate original propolis from fake ones [18]. In the literature, anti-inflammatory, antitumoral, antinociceptive, antimicrobial and hepatoprotective activities of terpenoids are reported in *in-vitro* and *in-vivo* studies [26].

1.1.3. Sugars

In researches, it is reported that the exact source of sugar in propolis has not been specified yet. While some thesis suggest that honey and nectar can be a source of glucose, fructose and sucrose in propolis, others suggest that they are derived from hydrolyzed flavonoid glycosides [18]. In one study, plant adhesive liquids that comprise of several sugars, alcohols and acids of sugar have been listed as a potential sources of sugar in propolis. This idea has been supported by another study which identified many sugars, sugars alcohol and uronic acid in propolis [30, 31]. Also, gluconic acid, galacturonic acid, galactitol and 2-O-glycerylgalactose have been identified as a sugar alcohol, acid and sugar in propolis [32].

1.1.4. Hydrocarbons

Hydrocarbons are end products of the combination of only carbon and hydrogen. Aromatic hydrocarbons (arenes), alkanes, cycloalkanes and alkyne are basic compounds of different hydrocarbons and recently alkadienes, alkenes, alkanes, monoesters, aromatic esters, diesters, fatty acids and steroids have been categorized in several type of propolis that are belongs to Egypt, Brazil and Anatolia [31, 33, 34]. In one study, the wax of propolis has been analyzed and hydrocarbons and monoesters found the same with the comb wax. It has been suggested that wax in the propolis are secreted by bees, not only originating from plants [6].

1.1.5. Mineral Elements

In studies with propolis, it has been shown that Calcium, Magnesium, Sodium, Potassium, Aluminum, Barium, Boron, Chromium, Iron, Manganese, Nickel, Strontium and Zinc as trace elements and whereas Arsenic, Cadmium, Mercury and Lead were identified as a toxic

elements different propolis samples in Croatian region [35]. Another study has reported defining of Antimony, Bromine, Cobalt, Chromium, Iron, Rubidium, Samarium and Zinc from different Argentinean propolis. These data also claimed that profiles of the trace elements can be beneficial for identification of propolis according to geographic origin [36].

1.2. BIOLOGICAL ACTIVITIES OF PROPOLIS

Propolis has been used as an antiseptic, disinfectant, cauterizing and fever reducing agent in ancient times and it has gained popularity during this time because of its biological activities. However, in conventional medicine it has not been noted as a therapeutic agent because of the lacking of its chemical structure and biological activity standardization. In the health system, this kind of standardization is crucial for acceptance as a therapeutic agent. Thus, analyzing chemical profiles of propolis on the basis of its plant origin and corresponding biologic action have come into prominence. In other aspects, this kind of research will contribute to the development of new drug nominees [8, 37].

In terms of worldwide, chemical structure, biological action and pharmacological potential, this has been reported in many studies for different origins of propolis samples during the last decade. In this section, antioxidant, anti-inflammatory, immunomodulatory, antitumoral, antibacterial, antifungal and antiviral activities will be summarized from recent published article.

1.2.1. Antioxidant Activity

A known mechanism for oxidative stress, is that an endogenous stimulus, like cellular metabolism, and exogenous agents like toxins, drugs, and UV produce reactive oxygen species (ROS), such as hydroxyl ion (HO−), hydrogen peroxide (H_2O_2) and superoxide anion (O2−), and reactive nitrogen species (RNS), specially nitric oxide (NO). These reactive species cause oxidative modification in lipids, carbohydrates, proteins, and nucleic acids that leads to alteration of cell and its death [38-40]. As another defining oxidative stress is the alteration of balance between reactive species and antioxidant defense [41] or cardiovascular diseases [42, 43] cancer [38, 44] , diabetes [37], and atherosclerosis [45].

In the last decade, researchers have been focused on the natural product’s antioxidant capacity, and in various studies, propolis extracts which are comprised of different polyphenols have been clarified as having antioxidant potentials [40, 46, 47]. Also, regional

differentiation of propolis structure effects its antioxidant charateristic. In 2013, Fabris at al. reported that ethanolic extract of Russian and Italian propolis samples have similar phenolic content and antioxidant action, however low phenolic content Brazilian propolis extracts have low antioxidant charateristic [48]. The correlation of higher phenolic content with higher antioxidant charateristic also was reported from ethanolic extracts of different Transylvania propolis samples [49]. As a mechanism, antioxidant charateristic occurs by obstructing the charateristic of some enzymes such as cAMP phosphodiesterase, protein kinase C, xanthine oxidase, ascorbic acid oxidase, lipoxygenase, cyclooxygenase and Na^+/K^+ ATPase. These enzymes are responsible for obstruction of ROS species production by scavenging, disturbing lipid peroxidation reaction, by chelating metal ions that are activated in the process of free radical creation (mainly iron and copper) or by activating other antioxidants [50]. In literature, different antioxidant mechanisms of propolis have been reported in *in-vitro* studies. Compos et al. have analyzed antioxidant charateristic of Brazilian propolis ethanolic extracts in erythrocytes and reported that propolis effects free radical scavenging; obstruction of hemolysis and lipid peroxidation [51]. Silva et al. showed prevention of low-density lipoprotein peroxidation and NADPH oxidase and rise in nitric oxide synthase in Bovine aortic endothelial cells with ethanolic extracts of Uruguay propolis [52]. Mavri et al. revealed higher reduction power and potential to scavenge metal ions and free radicals for ethanolic extracts of Slovenia propolis [53]. Chen et al. examined cyclist's Peripheral blood mononuclear cells with commercial CAPE that has the same characteristic with European type propolis and found a reduction of intracellular superoxide, necrosis, superoxide production, glutathione depletion, and hyperthermia-induced survival suspension [54].

Yazıcıoğlu et al. showed that Mediterranean propolis from Turkey declined DNA damage inducing by H_2O_2 in fibroblast cells [55]. For different extraction of propolis, in 2018, it was reported that in glyceric extracts of propolis flavonols and flavones ranged from ~20% up to ~36%, while flavanones and diidroflavonols were between ~28% and ~41% and antioxidant charateristic, which was examined by DPPH method similar with ethanolic extracts [56]. Antioxidant charateristic also has been examined in in-vivo studies. Bolfa et al. observed in ultraviolet B exposure female swiss mice declining in malondialdehyde values and restoration of glutathione peroxidase activity with Romanian ethanolic propolis extract [57]. Yonar et al. analyzed ethanolic extract of Mediterranean propolis and found that declining in malondialdehyde values and superoxide dismutase (SOD) activity, rising of glutathione

peroxidase and catalase activity [58]. The same propolis type also have been examined in Male Wistar albino rats and announced that maintaining SOD activity, declining in xanthine oxidase (XO) activity, nitric oxidase and malondialdehyde values [59]. Propolis has variable structure for all one of its important component CAPE has significant role in antioxidant activity [54, 59, 60]. Antioxidant activity has been mostly studied characteristic of propolis, though there is no any specification with scientific data for safe dose in human beings [4].

1.2.2. Anti-Inflammatory Activity

Inflammation commonly occurs in reaction to the exposing any endogenous and environmental stimulus as well as accidental damage [61]. Two types of inflammation are known, acute inflammation and chronic inflammation. After any tissue injury, complex chemical signal cascade start for repairing that site. In acute inflammation is initiated with the cells of immune system that are emigrate to injured location and emancipate ROS/RNS species, growth factors and cytokines. Chronic inflammation is mediated with the unsuccessfully treated acute inflammation and has important role in several diseases such as cancer, asthma, atherosclerosis, Alzheimer, and Parkinson [62, 63].

In several studies, anti-inflammatory effect of different propolis and its components have been studied. In 1996, Mirzoeva and Calder analyzed several flavonoid of propolis with Peritoneal macrophages in inflammation which includes emancipating and oxygenation of arachidonic acid as a critical events. They found that lipoxygenase pathway of arachidonic acid metabolism have suspended by CAPE, caffeic acid, quercetin, and naringenin [64]. Funakoshi-Tago et al. reported that Nepalese propolis ethanolic extract suspends Interleukin 6, TNF-α and Interleukin 13 gene expression and declining the activation of IκB kinase resulting to NF-κB deactivation in mice Bone marrow-derived mast cells [65]. Juman et al. showed declining of the formation of IL-1β, monocyte chemoattractant protein 1, and the formation and assertion of TNF-α by CAPE in RAW264.7 macrophages [66]. In the same cell line, Bufalo et al. clarified downregulation of NF-κB, p38 mitogen-activated protein kinase, and c-Jun-N-terminal kinase with ethanolic ekstrakt of Brazilian propolis and organic Caffeic acid [67]. Boudreau reported that cape caffeic acid phenethyl ester is a potent leukotriene biosynthesis inhibitor that blocks 5-lipoxygenase activity and arachidonic acid emancipate in PMNs [68]. In *in-vivo* studies, anti-inflammatory effect of propolis has been analyzed. Koksel et al. found that polymorphonuclear neutrophilic leukocyte percolation declining in the lungs

tissues of Male Wistar albino rats by CAPE [69]. Hu et al. reported suspension of the mononuclear macrophages activation and differentiation, declining prostaglandin E2 and nitric oxide values with ethanolic and water extracts of European propolis in ICR mice and Wistar rats [61]. Teles et al. showed declining in renal macrophage percolation in chronic Male Wistar rats with kidney disease via ethanolic extract of red Brazilian propolis [70]. Rossi et al. stated that obstruction of cyclooxygenase 1 and 2 activity by CAPE in J774 macrophages and Male Wistar rats [71].

1.2.3. Immunomodulatory Activity

Natural products are used as a complementary adjuvant treatment because of their immunomodulatory effect in various diseases. Until 1990, there was little information about the propolis effect on immune system. However, in last decade numerous *in vitro* and *in vivo* studies have been published that were clarified this unknown subject.

Propolis immunomodulatory charateristic has been thought to be limited because of macrophages that have no effect on lymphocyte reproduction. In 1995, Ivanovska et al. clarified accelerating of lymphocyte expansion and emancipating of IL-1 and IL-2 cytokines by cinnamic acid in Female IRC mice [72]. Girgin et al. reported that ethanolic extract of Turkish propolis causes suppression of neopterin emancipate and tryptophan degradation, indoleamine 2,3-dioxygenase (IDO) enzyme downregulation and declining of IFN-ɣ and TNF-α values in healthy human beings peripheral blood mononuclear cells [73]. Conti et al. examined ethanolic extract of Brazilian propolis, especially cinnamic acid, in blood monocytes and reported that cinnamic acid downregulates assertion of Toll-like receptors 4 (TLR-4), CD80 and IL-10, TNF- α production by inhibiting TLR-4 in monocytes [74]. Same group also announced that excitation of monocytes charateristic against C. albicans; downregulation of HLA-DR, TLR-2 assertion and obstruction of cytokine production by Caffeic acid in Monocytes from blood [75]. Wang et al. reported that hinderance of Interleukin-12 p40, Interleukin-12 p70, Interleukin-10, Inteerferon gamma inducible protein values, obstruction of IκBα phosphorylation and NF-κB activation in monocyte-derived dendritic cells (MoDCs) by commercial CAPE that has European propolis characteristic [76]. For the same product, Marquez et al. identified suspension of transcription factors NF-κB and nuclear factor of activated T cells (NFAT), interleukin-2 gene transcription, interleukin-2 receptor assertion, and expansion of T cells and jurkat cells [77]. In *in-vivo* studies, on Male

BALB/c mice for Brazilian green propolis ethanol extracts, in several studies have been reported that accelerating of TLR-2, TLR-4 assertion and rising in IL-1, IL-6 production [78], accelerate of H2O2 generation and declining in the NO generation in peritoneal macrophages [79], accelerate in the interiorization and parasites Leishmania (Viannia) braziliensis killing by macrophages, accelerate in tumor necrosis factor alpha production and declining in IL-12 production [80]. For hydroalcoholic extraction of Brazilian propolis, it has been clarified that declining of splenocytes expansion and accelerate of IFN- ɣ production by spleen cells [81]. Also, it has been demonstrated that stimulation of interleukin-1β, interleukin-6 suspension and interleukin-10 productions on Male BALB/c mice by cinnamic and coumaric acids [82]. Park et al. found that upregulation of IgM antibody production, interleukin-2 and interleukin-4 production, T lymphocyte expansion in splenocytes, and IFN- ɣ production accelerate by CAPE in Female BALB/c mice [83].

In literature, there is information about immunstimulating effect of propolis in clinical studies which propolis has been applied as a prophylactic treatment. After propolis taking, cytokine value of patients accelerated during treatment time. These data have been reported that propolis showed an enhanced immune reactivity without side effect. For aqueous extract of propolis, it has been reported that accelerated protection against to Gram negative infections, presumably via macrophage activation [84].

1.2.4. Antitumoral Activity

Identifying and treatment of cancer have been popular topic for researchers during the time. Because of various active content and harmful effect of chemotherapy natural product become popular in anticancer research. Propolis is widely researched compound which has declared in *in vitro* and *in vivo* studies in this area and few of them debatable. In *in-vitro* studies, different originated propolis samples have been analyzed for cytotoxicity on various cancer cell. In *in-vivo* studies, it has been analyzed for improvement of new antitumoral agent without any side effect in mammals like rats [85]. Utmong et al. researched hexane extract of Thailand propolis on different cancer cell line such as breast (BT474), hepatic (Hep-G2), lung (Chago), colon (SW620), stomach (Kato-III) cells and as control normal cell lines; liver (CH-liver) and fibroblast (HS-27). They found that high antiproliferative charateristic against the cancer cell lines and lower toxicity on the normal cell lines [86]. Catchpole et al. examined propolis from Newzeland, which contains chrysin, CAPE, benzylferulate, benzyl isoferulate, pinostrobin,

galangin, 5-phenylpenta-2,4-dienoic acid, and tectochrysin, on colon cancer (DLD-1, HCT-116), esophageal squamous cancer (KYSE-30), and (NCI-N87) gastric carcinoma cells and showed antiproliferative charateristic [87]. In many studies, effects of CAPE, from different geographic origin and commercial, have been analyzed on various cancer cell line and found that its effect occurs through the obstruction of NF-κB [88, 89]. In MCF-7 cell line, Kamiya et al. showed that cell viability reducing through the process of mitochondrial dysfunction, DNA fragmentation and caspase-3 activity, also, acceleration in assertion CCAAT/enhancer-binding protein homologous protein (CHOP) [90]. In the same cell line, Watabe et al. demonstrated that process of apoptosis via Fas signal, process of Bax protein, caspases and MAPK family proteins activation [91]. Chu et al. analyzed effect of CAPE in prostate cell lines (LNCaP, DU-145, and PC-3) and reported that in dose dependent manner, CAPE repressed the cell expansion and LNCaP xenografs tumor growth in nude mice [92]. In some studies, relation of CAPE with tumor cell growth and survival genes via histone deacetylase inhibitor have been specified [93]. Yilmaz et al. studied on propolis from Aydın, Turkey and found that it has rich flavonoids and CAPE content and it shows dose dependent apoptotic effect on CCRFSB lymphoblastic leukemia cells [94]. Wu et al. demonstrated suspension of breast tumor growth (MCF-7 and MDA-MB- 231) via reduction of growth and transcription factors assertion, including NF-κB, in vitro and in vivo by CAPE [95]. Szliszka et al. reported triggering of caspase-3 and caspase-8 initiation and corruption of mitochondrial membrane potential by a co-treatment with Artepillin C and TRAIL on LNCaP cell line [96]. Ahn et al. examined commercial Artepilin C which has Brazilian propolis characteristics and found that repression of HUVECs expansion and rising of tube formation in Female ICR mice [97]. Alizadeh et al. studied with ethanolic extract of Iranian propolis in Male Wistar rats and reported that declining in number of lesions, tumor incidence, structural abnormalities, initiation of proapoptotic Bax assertion and reducing of antiapoptotic Bcl-2 assertion [98]. Dornelas et al. analyzed water extract of Brazilian propolis and specified that obstruction of angiogenesis in BBN (N-butyl-(-4-hydroxybutyl) nitrosamine) induced bladder cancer in Female Wistar rats [99].

In literature, as a mechanism, it has been reported that specific oncogene signaling pathways can be suspended by propolis, which cause to decline in cell growth and expansion. Also, it has been specified that propolis can lead to decline cancer stem cell population, increase apoptosis, exert antiangiogenic effects, and modulate the tumor microenvironment [88, 100,

101]. In several studies, mechanism of action have been studied for CAPE and reported that selective inihibition of cancerous cell viability in oral cancer [102], suspension of invasiveness and cell motility through voltage gated sodium channel in breast cancer [103], inhibiting of migration and invasiveness via Wnt suspension and ROR2 upregulation in prostate cancer [104], suppression of tyrosine kinase activity and leading to cell cycle arrest in G1 or G2/M phase [105]. Also, in breast cancer, Motawi et al. showed that CAPE has synergistic effect with tamoxifen on MCF-7 cells (T47D) [106] and Khoram et al. observed that triggered radio-sensitivity on estrogen receptor negative (MDA-MB-231) and estrogen receptor positive cancer cells [107].

Angiogenesis has pivotal role in cancer growth because of nutrients and oxygen necessity to prolong rapid unchecked expansion and metastasis. Cancer and stromal cells emancipate proangiogenic factors such as vascular endothelial growth factor, that inducing formation and sustention of new blood vessels [108]. Ahn et al. reported significant reduction of newly formed vessels and suppression of HUVEC's reproduction by ethanolic extract of Brazilian propolis [97]. This mechanism have been evaluated by Kunimasa et al. and reported that antiangiogenic effect occurs with triggering apoptosis in tube forming endothelial cells via inactivation of the survival signal ERK1/2 [109]. In chick embryo chorioallantoic membrane, *in vivo*, Yun et al. demonstrated angiogenesis suspension by CAPE [110].

1.2.5. Antimicrobial Activity

Antimicrobial effect is defined as killing or growth termination of microorganism by an agent. If an agent used for bacteria it is called antibiotic and for fungi, it is called antifungal. In literature, in various studies antibacterial and antifungal effect of propolis have been analyzed *in vitro*, *in vivo* and in some clinical researhes.

1.2.5.1. Antibacterial Activity

Effects of propolis have been studied for various bacterial strain and reported that propolis is more effective against Gram negative bacteria than Gram positive bacteria [9, 111]. The phenolic content is more important for antibacterial effect as in other biologic activities. In literature, it has been specified that flavonoids such as caffeic acid, quercetin, galangin, rutin, and naringenin cause accelerate permeability of bacterial membrane [112]. Also, bacterial RNA polymerase suspension has been reported for pinocembrin, galangin and CAPE [113].

Cui et al. studied effect of CAPE on *H. pylori* peptide deformylase, which is important enzyme for *H.Pylori* survival. It has been shown that CAPE is competitive hiderer for peptide deformylase, which is immobilizing the substrate entrance and prohibiting substrate from approaching the active site [114]. In literature, combination of propolis and antibiotics have been researched. Scazzocchio et al. analyzed combination effect of propolis and several antibiotics (ampicillin, gentamycin, streptomycin chloramphenicol, cefriaxone, vancomycin and erythromycin) with ethanolic extract of Italian propolis. They found that propolis meaningly increase the effect of streptomycin, ampicillin and gentamycin alleviate effect of chloramphenicol, cefriaxone, and vancomycin and no effect on erythromycin [115]. Orsi et al. studied with Brazilian and Bulgarian propolis for synergistic effect of ciprofloxacin and norfloxacin (effects bacterial DNA) and cotrimoxazole (effects bacterial mechanism) against *Salmonella typhi.* They determined antibacterial effect but not found any synergistic effect for Brazilian and Bulgarian propolis [116]. In, *in vivo* study, they showed accelerated bactericidal effect of Brazilian propolis ethanolic extract against S. typhimurium in Male BALB/c mice [117]. Resistance strains and propolis antibacterial effect also reported in literature. Wojtyczka et al. analyzed ethanolic extract of Polish propolis against clinically isolated methicillin-sensitive *S. aureus* and methicillin-resistant *S. aureus* and combination effectiveness with ten antistaphylococcal drugs. They found that different effect against twelve *S. aureus* strains and meaningly antibacterial impact to eight of all tested strains and no synergistic effect with ciprofloxacin and chloramphenicol [118]. Boisard et al. demonstrated antibacterial effect against methicillin susceptible and methicillin resistant *S. aureus* strains by dichloromethane extract of French propolis [119]. Antibacterial effect of propolis against Gram positive and negative bacterial strains have been confirmed clinically by Noronha et al. in 2014 [120].

1.2.5.2. Antifungal Activity

Propolis is used by bees to protect hive from any pathogen such as fungi. This situation is proven scientifically in many researches on different fungi, especially clinical interest. Dota et al. analyzed Argentina propolis as ethanolic extract and microparticles on clinical yeast isolates, which are important for vulvovaginal candidiasis. They showed that *Candida albicans* and non- *Candida albicans* are suspended by both type of propolis [121]. Falcao et al. studied with Portuguese propolis and reported that propolis shows highest activity against

Trichophyton rubrum and the lowest activity against *Aspergillus fumigatus* [122]. Szweda et al. demonstrated greatest activity against variety of *Candida* strains *(albicans, glabrata and krusei)* for ethanolic extract of Poland propolis [123]. Haghdoost et al. declared that Iranian propolis affects *Candida albicans* strongly and this effect attributed to formation of germ tube suspension [124]. In studied with Brazilian propolis extract, antifungal activity of propolis has been reported for different *Candida* strains, among them *Candida albicans* found to be the most sensitive and *Candida guilliermondii* the most resistant fungi against propolis [125]. Also, effect of green and red propolis of the Brazil have been analyzed on various type of Trichophyton which is cause dermatophytosis. It has been revealed that ethanolic extract of red propolis is more efficient than green one [126]. In another research, it has been specified that *Candida albicans* having mutation of metacaspase gene which supports cell death, are more sensitive to Brazilian propolis. They also showed controlling of vulvovaginal candidiasis with propolis based cream and gels in mouse model [127].

1.2.6. Antiviral Activity

Viral infection is another important topic that requires alternative treatment agent especially for immunosuppressive infected person. In literature, like other biological activities, antiviral charateristic of propolis have been analyzed and different activity results have been reported because of different geographic region. Especially in last decade, researchers have been focused on this area, so there is no large scientific data such as antitumoral or antioxidant activities. Among these few data, it has been reported remarkable antiviral charateristic by conducting at different values and interfering with some viruses' replication such as adenovirus type 2, influenza virus, herpes simplex types 1 and 2, or human immunodeficiency virus (HIV) [9]. Amoros et al. have been shown antiviral charateristic againts several DNA and RNA viruses such as herpes simplex types 1 and 2, vesicular stomatitis virus, adenovirus type 2 and poliovirus type 2 for propolis which has rich flavonols and flavones [128]. In another study, they reported that 30 μg/mL of France propolis extract (80% ethanolic) declines herpes virus (HSV-1 strain H29S, acyclovir resistant mutant HSV1-R strain H29R, HSV-2) and less effective for the vesicular stomatitis virus and adenovirus. Results have been interpreted that exertion of virucidal effect on enveloped viruses such as herpes and vesicular stomatitis virus [129]. Schnitzler et al. investigated ethanol and water extract of the Czech Republic propolis which is rich about CAPE, benzoic acid, p-coumaric acid, pinocembrin, chrysin, and galangin against HSV 1. They showed high antiviral effect for both extracts

when cells treated with propolis before viral infection [130]. Coelho et al. analyzed hydromethanolic extract of Brazilian propolis which is produced by stingless bee *Scaptotrigona postica* and found suspension of HSV replication and entrance to cells because of C-glycosyl flavones, 3,4-dicaffeoylquinic acid and catechin-3-O-gallate [131]. Tait et al. researched synthesized propolis ingredients having Brazilian green and red propolis characteristic. They demonstrated that suspension of viral particle decapsidation for picornavirus and significant antiviral effect against echovirus 30, coxsackie viruses B3, B4, and A9 [132]. Sartori et al. showed effectivity against HSV-2 infection and declining extravaginal lesions via oxidative and inflammatory processes on female BALB/c mice for brown Brazilian propolis hydroalcoholic extract [133]. In another research, antiviral charateristic of Brazilian green propolis ethanolic extract against influenza A/PR/8/34 (H1N1) virus have been analyzed on female DBA/2 Cr mice and found declining in virus yields in the bronchoalveolar lavage fluids of lungs and body weight loss of infected mice [134]. Ma et al. studied nanometer propolis flavone against Porcine parvovirus (PPV) on Britain white guinea pigs and demonstrated that suspension of infected kidney cells, declining in virus copy in lung, gonad, blood. Also, it was shown that reducing effectivity of virus on guinea pigs weight, PPV suspension in serum and accelerated quantitiy of interleukin 2, interleukin 2, and interferon gamma [135]. Yıldırım et al. identified effectivity of Hatay propolis against herpes simplex type 1 and type 2 [136]. Ito et al. investigated moronic acid, compound from Brazilian propolis, against HIV in H9 lymphocytes and showed substantial anti-HIV charateristic [137]. Gekker et al. researched ethanolic extraction of USA, China and Brazil propolis on CD4+ lymphocytes and microglial cell cultures against HIV-1_{AT}, HIV-1_{SF162} and reported suspension of both strains assertion in a dose dependent manner. The possible mechanism have been specified as inhibiting of viral entry [138].

1.3. HERPES SIMPLEX VIRUSES

Herpesviruses comprise large family of DNA viruses which have ability to infect variety of species such as Chordata (mammals, reptiles, birds, fishes, and amphibians) and the Mollusca (oysters). They are also ability to infect different type of cell in the same host [139]. Depending on their genome sequences and biological properties, Herpesviridae family composed of three subgroup such as Alphaherpesvirinae, Betaherpesvirinae and

Gammaherpesvirinae [140]. Among these, Alphaherpesvirinae include 5 identified genera featuring 37 different species. Herpes simplex virus type 1 and type 2 (HSV-1 and HSV-2) and varicella-zoster virus (VZV) are top three viruses in Alphaherpesvirinae that infect human routinely. Other 34 virus species have ability to infect different animals and cause mild to severe infections. All type of herpes, latent infection is characteristic feature that virus is reactivated and caused recurring diseases. For alphaherpesviruses, diseases can be ranged from mild skin lesions, reproductive disorders, respiratory and neurological disorders to tumors even death [141]. Structurally, all herpes viruses have linear **double stranded DNA** which is surrounded with icosahedral nucleocapsid, **envelope**, outer membrane of the viruses that comprise lipid bilayer embedded with glycoproteins, **tegument** proteinaceous structure which is found between capsid and envelope. The structure of viruses showed in Figure 1.2 [142].

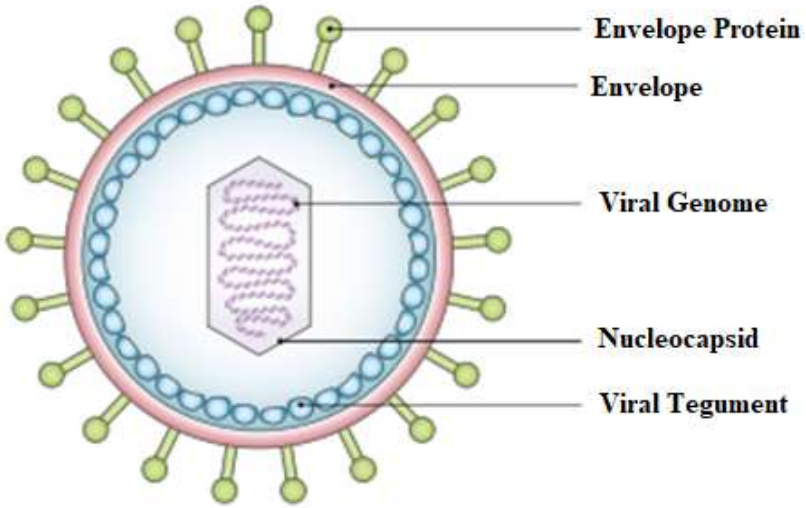


Figure 1.2: Structure of Herpes Viruses.

Herpes simplex type1 and type 2 are closely related viruses that belong to subfamily of Alphaherpesvirinae and both types cause widespread infections [143]. For HSV-1 symptoms are keratitis in the eyes and cold sores of around and in mouth while HSV-2 cause genital lesion. However, both viruses have potential of life-threatening diseases in newborns, HIV patients, immunocompromised and immunosuppressive individuals [143, 144]. As in the most

of the viruses HSV-1 and HSV-2 can be in lytic or lysogenic cycles. The viruses replicate actively and uncontrollably during lytic infection, that cell destruction occurs in the end and symptom of infection are seen like sore and irritations. In lysogenic cycle, viruses go in to latent phase in body nerve cells [145].

Entrance of the herpes viruses has complex mechanism and synergism is necessary between virus and cellular molecules. While small envelope viruses have one or two glycoproteins, herpes viruses have more than dozens of glycoprotein and several of them act to mediate viral entry. Viral entry can be occur in four process; (I) tethering or attachment or to the cell surface, (II) binding to specific cell receptors, (III) triggering of intracellular signaling, and (IV) viral envelope fusion with cellular membranes [146-148]. Attaching of viruses to cell surface is non-specific and charge based process that viral glycoproteins (mainly gB and gC for alphaherpesvirus) bind reversibly to cell surface heparin sulfate proteoglycans (HSPG) and chondroitin sulfate proteoglycans (CSPG) [148]. In the lack of glycoprotein C (gC), it was detected that declining in overall viral attachment to cell surface. This has been claimed that gC is not essential for virus entrance [144]. In dendritic cell (DC) gB and gC binds to C-type lectin (DC-SIGN) to facilitate HSV-1 attachment. In the case of HSV-2, this interaction was detected but there has been no direct conformation [149].

After attachment, penetration process begins which herpes simplex viruses achieve with two ways, which is depending on the host cell. First way is virus envelope fuse with plasma membrane move through the host cytoplasm. Second way is, virus go inside the host cell via endocytosis like pathway that plasma membrane surround virus and uptake it in [150, 151]. In both cases, viral glycoproteins (gB, gD, gH and gL, gD) and host receptors (herpesvirus entry mediator (HVEM), nectin-1 and -2, and 3-O sulfated heparan sulfate (3-O HS)) are essential for fusion [152, 153]. Glycoproteins and their cellular receptors of Alphaherpesviruses are shown in Table 1.1 [141].

Table 1.1: Glycoproteins of Alphaherpesviruses and their cellular receptors**.**

Virus	Glycoprotein	Function	Host Receptor
HSV-1	gC	* Attachment	* Heparan Sulfate * DC-SIGN
	gD	* Binds cells * Trigger fusion	* HVEM * Nectin-1 * Nectin-2 * 3-OS HS * ZF-3-OS HS
	gH/gL	* Regulates fusion * Activates gB	* αVβ3 integrin * αVβ6 integrin * αVβ8 integrin
	gB	* Attachment * Binds cells * Catalyzes membrane fusion	* Heparan Sulfate * DC-SIGN * PILR α * MAG * NMMHC-IIA * NMMHC-IIB
HSV-2	gC	* Attachment	* Heparan Sulfate
	gD	* Binds cells * Trigger fusion	* HVEM * Nectin-1 * Nectin-2
	gB	* Catalyzes membrane fusion	* Heparan Sulfate

In literature, accepted mechanism for membrane fusion indicating that glycoprotein D binding to its consanguine receptor cause conformational changes in glycoprotein D that activates glycoprotein complex, fusion complex, including gB, gD, gH and gL [154]. With fusion viral nucleocapsid and tegument proteins emancipates into the host cytoplasm. Then viral nucleocapsid dissociates from tegument proteins and binds to a microtubule (MT) directed motor, dynein [155]. Most of the tegument proteins are necessary for assertion of viral gene activation and modulation, inhibiting of host protein synthesis, some of them have role in nucleocapsid transporting along microtubules to nuclear membrane for uncoating and viral DNA emancipating into the nucleus. In host nucleus, viral DNA replication and gathering of progeny capsids occurs [156]. Molecular interaction that mediates Herpes simplex entry is illustrated in Figure 1.3 [157].

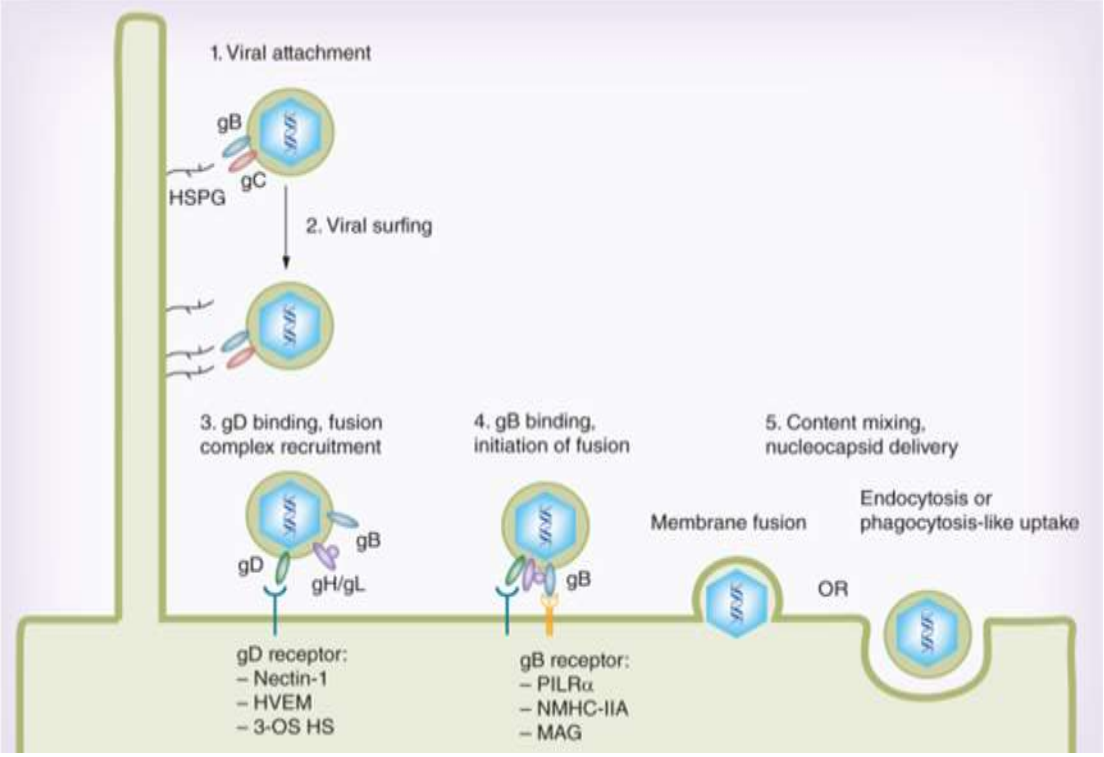


Figure 1.3: Molecular interaction arbitrates Herpes simplex virus entry.

Following virus entry and expansion cell-to-cell spreading initiates. For herpes simplex type 1 and type 2 cell to cell contact is important to propagate successfully. As in the initial infection by free virions, spreading process depends on glycoprotein D and its receptor interaction [158]. In this process, glycoprotein E and I heterodimer that are not seen in initial entry, proceeds from trans-Golgi network (TGN) to epithelial cell junctions with other virion particles and viral glycoproteins. In literature, for HSV-1 it has been shown that removing of early sorting genes through TGN, causing to prevent cell to cell spreading and moving of virus to apical surface in place of cell junctions. Also, it has been shown that glycoprotein K has important act in spreading in trigeminal ganglia and corneal cells [159]. In another study, declining of clinical signs and corneal spreading are observed when mice infected by glycoprotein K deleted virus [156].

After first infection from the epidermal layer viruses move to neurons and remain in latent phase because virus have ability to avoid immune detection. The ratio of the latency is reported for HSV-1 up to 80% adults and 40% for HSV-2 among the patient populations. Hosts have potential to spread viruses via asymptomatic shedding of the virions during the latent phase. Virus reactivation occurs when host suffers from environmental triggers that comprise physical and emotional stress. Triggers cause to movement of the virus to the

epithelial cells for its gene, replication and spread from cell to cell. Again, to move latency phase HSV-1 move to trigeminal nerve, HSV-2 move to sacral ganglia [156]. Life cycle of the herpes simplex is shown in Figure 1.4 [142].

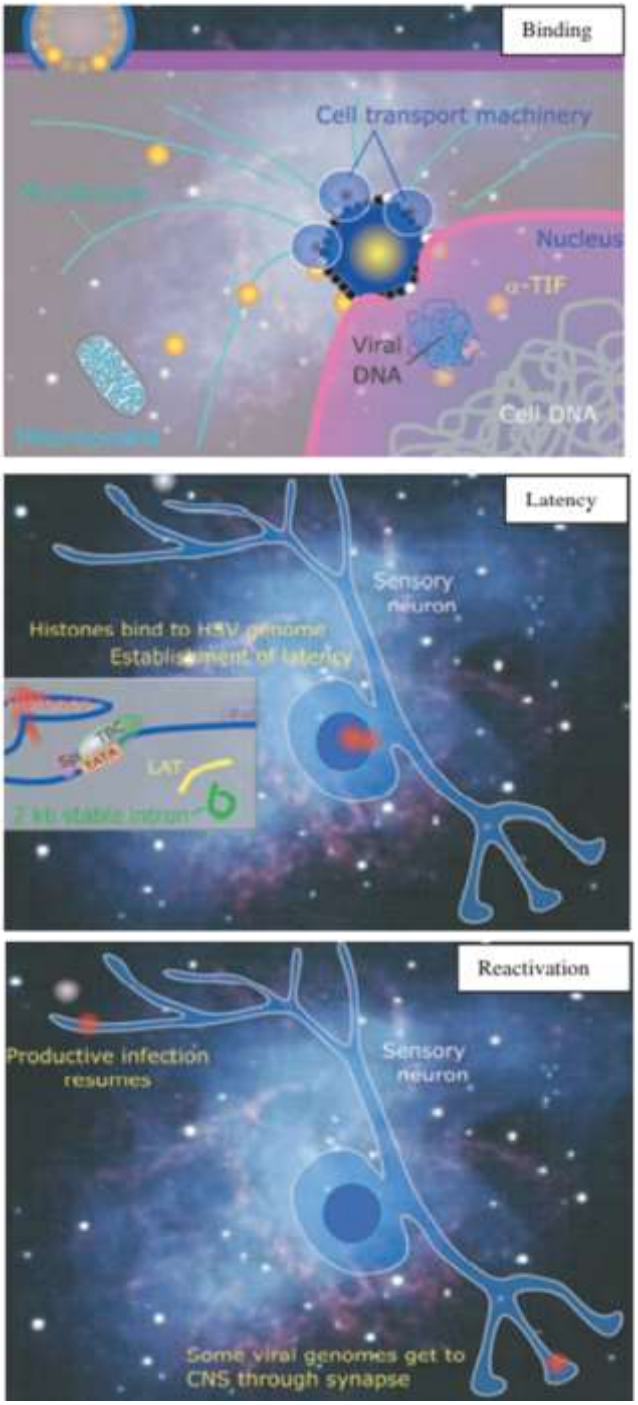


Figure 1.4: Herpes Simplex life cycle.

World Health Organization (WHO) reports that 90% of population is infected by different types of herpesviruses that develop latency or result in oral and genital herpes, eczema, conjunctivitis and other diseases [160]. Infection with the HSV commonly can be occurred by Herpes Simplex Type 1 which known as oral herpes or Herpes Simplex Type 2 which known as genital herpes [161]. HSV-1 commonly cause mucocutaneous infections which is resulting in recurrent orolabial lesions and cause ocular herpes and encephalitis in adults [162]. It is transmitted by contact with the virus in surfaces sores and saliva, in or around the mouth. In immunocompromised people, it shows more severe symptoms and more frequent recurrences. Sometimes, HSV-1 leads to genital symptoms via oral to genital contact. HSV-2 is widespread infection causing genital herpes. It is lifelong and incurable infection which is mainly transmitted during sex, contact with skin, sores, genital surfaces or fluids of infected person even without symptom. In immunocompromised people infection with HSV-1 and HSV-2 cause severe symptoms and complications such as keratitis, pneumonitis, retinal necrosis. Also, HSV infection can be serious, extensive and prolonged in immunocompromised individuals with occurrence of drug-resistant strains [161].

In past decades efficient antiviral agents have been improved for HSV infection such as acyclovir, valacyclovir, famciclovir, foscarnet, idoxuridine, trifluridine and vidarabine. Among them Acyclovir is widely used compound for HSV infection [163, 164]. HSV infection is lifelong diseases and patients used drugs over years. Prolonged antiviral treatment resulted occurrence of drug resistance strain [165]. Drug-resistance is most common in immunocompromised hosts, especially in transplant recipients and AIDS patients [166, 167]

Resistant herpes virus infection has become common in recent years therefore research on new active substances for the treatment of herpes viruses is growing rapidly. Researches show that;

 Propolis has various biological and physiological activities, such as antiviral, antibacterial, antioxidant, anticancer and antiinflammatory characteristics due to its chemical structure.

 Till now, anti-inflammatory, anti-tumor, anti-microbial, and anti-oxidative effect of propolis have been shown in vitro and animal model study. But there is no remarkable study for the anti-viral charateristic of propolis. Research for antiviral charateristic of propolis is virgin area.

 Frequent use of antiviral leads to the emergence of resistant virus associated with prolonged survival in patients. That is why new active molecule studies against to viruses are important and necessary.

For all of these reasons, antiviral charateristic of different propolis extracts will be analyzed for HSV-1 and HSV -2 in Human Immortalized Keratinocyte (HaCaT) cell line which is comprises 90% of cells in the skin epidermis layer and act as a barrier against environmental triggers by viruses, bacteria, fungi, parasites, heat, UV radiation and water loss [168].

2. MATERIALS AND METHODS

2.1. PROPOLIS EXTRACTS

In this study, propylene, ethanol, glycerol and soya extracts of propolis were examined against antiviral charateristic. Extracts prepared and characterized by B Natural srl (Milan, Italy). All extracts obtained in liquid form.

2.2. PREPARATION OF PROPOLIS EXTRACT

Main stocks of extracts sterilized with 0,22 μm milipore filter. They aliquoted in 1 mL volume and stored at +4 ^{o}C until using in cell culture. Each extract warmed 1 h at room temperature and vortexed just before diluting with media. Propylene and Ethanol extracts prepared 1 mg/mL, Glycerol and Soya extracts prepared 3.5 mg/mL in 5 mL as a stock solution with Dulbecco's Modified Eagle Medium (DMEM). Stock solutions of propolis extracts diluted with DMEM in different concentrations and added to cell culture.

2.3. ACYCLOVIR

Acyclovir (Acyclovir Sodium, Zovirax) is mainly used synthetic nucleoside analogue active against herpesviruses. It used as a control drug in our experiment. Each vial contain powder that equals to 250 mg acyclovir and solved with 10 mL saline solution (Sodium Chloride Intravenous Infusion BP (0.9% w/v)) to prepare concentration as 25 mg/mL. Dissolved Acyclovir diluted with sterile saline solution to prepare 20 μM working stock. It aliquoted as 500 mL and stored at -20 °C until use.

2.4. CELL LINE AND VIRUSES

In this study, immortalized Human keratinocytes (HaCaT) cell line, Herpes Simplex Type 1 (MacIntyre, # 0810005CF, Zeptometrix) and Herpes Simplex Type 2 (MS, # 0810006CF, Zeptometrix) viruses used to analyze in-vitro tests. Cell line and viruses obtained from Genetic and Bioengineering Department of Yeditepe University. Cell line was passaged in twice a week to provide cell continuity.

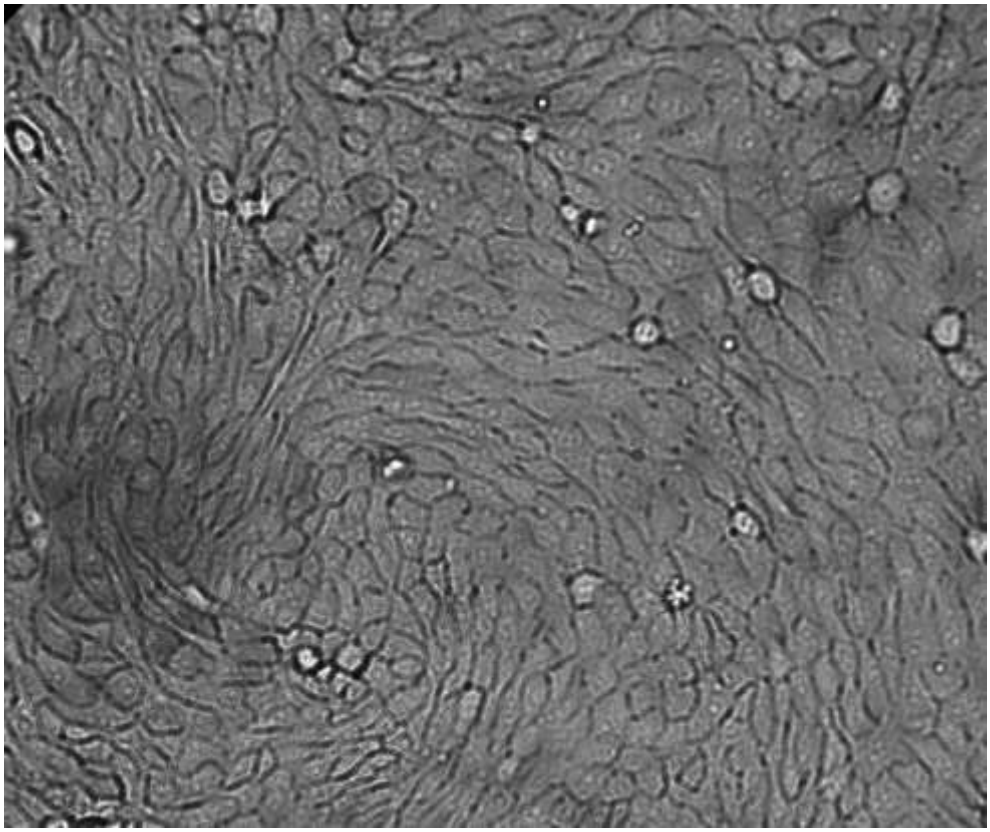


Figure 2.1: HaCaT cells monolayer incubated 37°C and 5% CO_2 for 72 h. Image taken with 20X objective.

2.4.1. Cell Culture

HaCaT cell line was grown in high glucose Dulbecco's Modified Eagle's Medium (DMEM, 4.5 g/L glucose) complete media containing 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin/ Ampicillin (PSA) and incubated at 37°C and 5% CO_2. Cell line passaged when it reached 80-90% confluency as a monolayer. Firstly, old medium discarded and cell line washed with Dulbecco's Phosphate Buffer Saline (DPBS) with 3 times to remove FBS residue. Then, 4 mL of 0.25% Trypsin-EDTA (Gibco™ 25200056) added on to cell line and incubated 10-15 minutes at 37 °C and 5% CO_2. Detached cells transferred to the falcon tube and centrifuged for 5 minutes at 1300 rpm. Cell pellet resuspended with fresh complete media. Cells counted with hemocytometer and seeded to new culture environment as a $1x10^5$ cell/mL. Then new culture incubated at 37 °C and 5% CO_2 with 80-90% relative humidity (NuAire, #NU-5810, USA). Also, cells froze at regular interval to overcome any risk at culture such as contamination, morphological changes etc. With this purpose, firstly cells

tyrpsinized and centrifuged for 5 minutes at 1300 rpm. Freezing media prepared that containing 10% Dimethyl Sulfoxide (DMSO, # D4540, Sigma-Aldrich, USA) and 90% complete media. Cell resuspended with freezing media and aliquoted with 1 mL volume to cryovials. Then, cryovials put into the freezing container that declines temperature 1 °C in every 1 minutes and it put immediately into -80 °C.

2.4.2. Virus Proliferation

At first step, cells seeded in 75 cm^2 tissue culture flask. When cell became monolayer, old media discarded and cells washed with DPBS for three times to remove FBS residue. Then cells infected with 2 mL of virus stock and incubated at 37 °C and 5% CO_2 for 1 h. Every 15 minutes flask shook smoothly. After 1 h, virus solution removed from cell culture flask and 12 mL of DMEM high glucose media containing 2% FBS and 1% PSA (virus media) added onto cells. Then flask incubated at 37 °C, 5% CO_2 for 72 h. At second step, after 72 h, cells collected into 15 mL falcon tubes and tubes put into -80°C for freezing then immediately put into 37 °C for thawing. This step repeated 2 times. Then, tubes centrifuged at 3000 rpm for 30 minutes and supernatant collected. At third step, 3 mL of collected supernatant put onto newly monolayer cells and incubated at 37 °C and 5% CO_2 for 1 h. Every 15 minutes flask shook smoothly. Then, 15 mL of virus media added onto cells and flask incubated at 37 °C, 5% CO_2 for 72 h. Flask put -80 °C for 24 h, then put into +4 till thawing. Cells collected in to falcon tubes and centrifuged at 3000 rpm for 30 minutes. Supernatant aliquoted as 1 mL into cryovials and put into -80 °C.

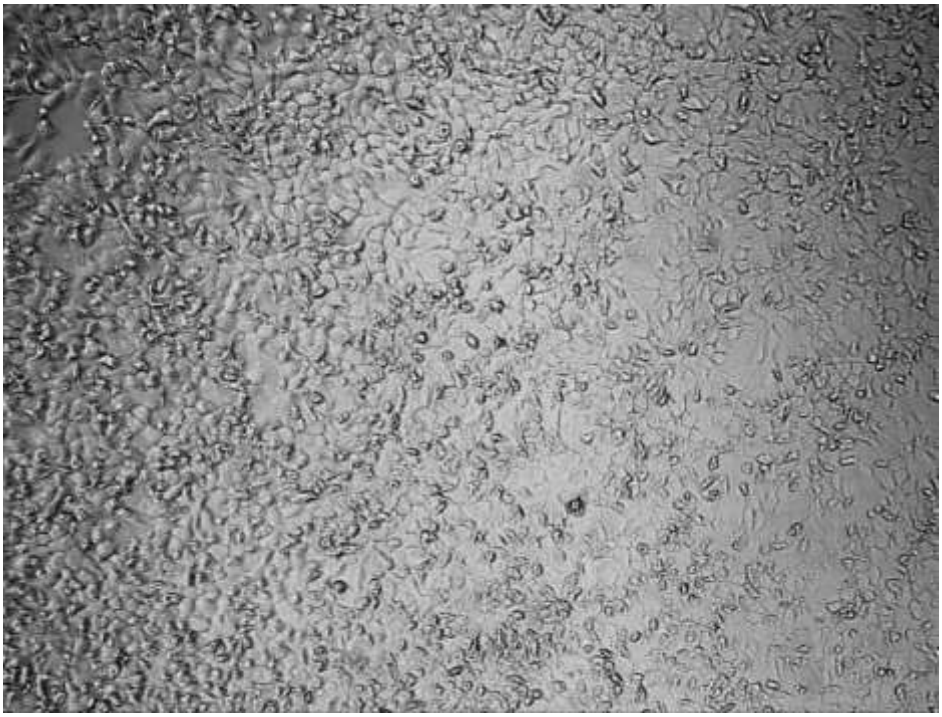


Figure 2.2: Uninfected HaCaT cells incubated 37 °C and 5% CO_2 for 72 h. Image taken with 10X objective.

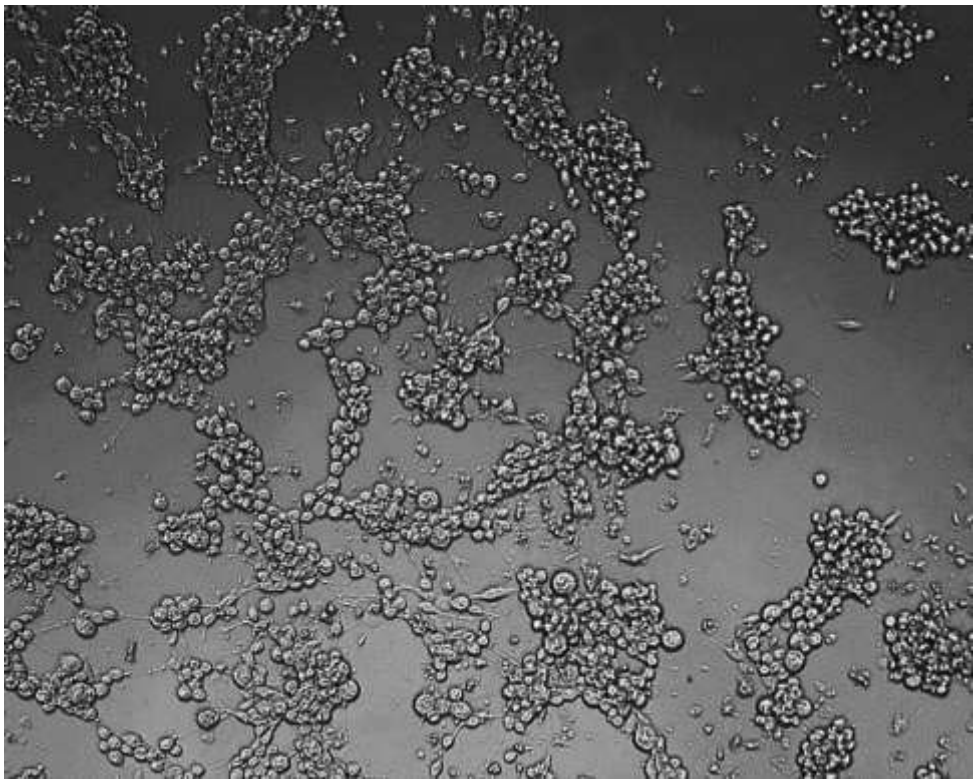


Figure 2.3: HSV Infected HaCaT cells incubated 37 °C and 5% CO_2 for 72 h. Image taken with 10X objective.

2.5. EFFECT OF DIFFERENT PROPOLIS EXTRACTS AND ACYCLOVIR ON CELL PROLIFERATION

2.5.1. Cytotoxicity Assay

Cytotoxicity assay is used to determine toxic and non-toxic concentration of the compounds. In this study, toxic effects of the different propolis extracts and acyclovir on immortalized HaCaT cell line analyzed with MTS (3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxy methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) method colorimetrically [169].

This colorimetric assay is based on the reduction of MTS tetrazolium compound to colored, aqueous soluble formazan product by NAD(P)H - dependent dehydrogenase enzyms in metabolically active viable cells. The colored formazan dye can be quantified by measuring absorbance at 490 nm.

In this direction, to perform this method HaCaT cells seeded as $5x10^3$ cell (100 µL/well) in 96 well plate and incubated at 37 °C, 5% CO_2 for 24 h. After incubation period, media on cell aspirated and diluted propolis extracts at different concentration added on cells. In one column of the plate only fresh media added as negative control and 4 well of the plate media with 20 % DMSO added as positive control. Plate incubated at 37 °C, 5% CO_2 for 72 h. After incubation period, old media discarded and 100 µL of MTS solution (10 % MTS with DPBS/Glucose Media) added onto cells and plate put incubator for 2h. Then, plate shook with shaker for 5 minutes to dissolve condensed dye at bottom of the well. The absorbance of colored dye measured using a Microplate Reader (Bio-Rad, Japan) at 490 nm. Quantification of the absorbance value performed by comparison of treated cells with control cells on the basis of equations at below.

Cell Viability % = (Treated cell absorbance / Negative control absorbance) x 100

Cell Inhibition % = (1 - Treated cell absorbance / Negative control absorbance) x 100

In antiviral activity study, methods are used to determine two important parameter which provide ascertainment of antiviral efficacy of compounds. One of them is cytotoxicity values. Under this term Minimum Cytotoxic Concentration (MCC - refers to compound concentration that is necessary to cause minimal alterations in cell morphology as shown by microscopy)

and Cytotoxic Concentration 50 (CC_{50} - refers to the concentration of a compound that will kill half of the cells in an uninfected cell culture) are specified [170].

In our study, MCC values shown on the basis of cell viability (%) and CC_{50} values calculated on the basis of cell inhibition values (%) with the obtained data from cytotoxicity assay. All experiment performed at least 3 times.

2.6. EFFECT OF DIFFERENT PROPOLIS EXTRACTS AND ACYCLOVIR ON VIRAL REPLICATION

The second important parameter for ascertainment of antiviral efficacy of compounds is Antiviral Activity values. Under this term Effective Concentration 50 (EC_{50} – refers to concentration of a test compound that produces 50% inhibition of virus replication) is defined. It is important for this term that virus amount should be 50% in treated cell culture when compared with the untreated virus infected culture [170]. In our study, EC_{50} values shown with Quantitative Real Time PCR (qRT-PCR) method that is accepted best and validated quantification method for ascertainment of antiviral activity [171]. To perform qRT-PCR following steps performed.

2.6.1. Viral Titration Assay

Virus titration is an important step of the any kind of virological research, especially when a specific virus amount need to be used in experimental procedures such as analyzing of potential antiviral drug effectiveness. Still, most common using methods for determining viral titers is 50 % tissue culture infectious dose (TCID50) by using microscopic observation of cytopathic effect (CPE) or counting viral plaque in culture plate. Because of conventional TCID50 methods are time consuming, produce quandantal data and open to subjective error, researchers improved colorimetric assay methods (MTT and MTS) which declined time of the assay, eliminated subjectivity of results and improving accuracy, reliability and reproducibility [172].

In our study, colorimetric MTS method used to determine viral titers with small modifications. HaCaT cells seeded as 3x104 cells (200 µL/well) in 96 well plate and incubated at 37 °C, 5% CO^2 for 24 h. Next day, cell seeded wells observed under microscope (Zeiss Axio Vert.A1, Germany) to check cell morphology and confluency. Cells should be

monolayer completely in each well. Then, different virus dilutions prepared on ice in Logarithmic scale (Log 2) from 10^{-1} diluted virus stock. For HSV-1 2^{-6} and HSV-2 2^{-5} tirations chose which gave 50% infectvity point for virus inoculation. Viral titration illustrated in Figure 2.4.

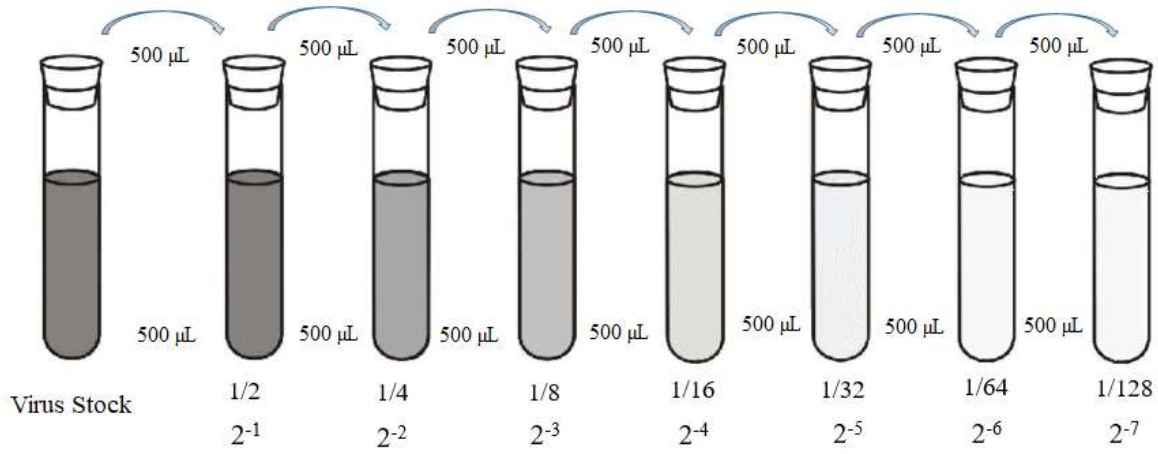


Figure 2.4: Viral Titration in logarithmic scale (Log2).

After preparation of virus dilution, old media aspirated from well and each well washed with DPBS for 3 times. Each viral suspension well pipetted 10 times before inoculated virus. Diluted virus inoculated to cells as 50 µL/well and at 37 °C, 5% CO_2 for 2 h. Plate shook smoothly with circular movement in every 20 minutes. After incubation time, unbound viruses aspirated and 200 µL of virus media (DMEM high with 2% FBS and 1% PSA) added to each well. Mock infected control which refers to uninfected cells with the virus, virus control refers to infected cells with virus stock and positive control refers to uninfected cell treated with 20% DMSO analyzed as a control for calculation. For uninfected control old media replaced with virus media and positive control old media replaced virus media with 20% DMSO. Then plate incubated at 37 °C, 5% CO_2 for 72 h. After incubation period, media aspirated from each well and 200 µL of virus media with 10% MTS added on to cells. Plate put in incubator for 3 h and then absorbance measured using a Microplate Reader (Bio-Rad, Japan) at 490 nm and absorbance quantified by using Cell Viability and Cell Inhibition equations which explained in Cytotoxicity Assay protocol. This protocol applied for ascertainment of HSV-1 and HSV-2 viral titration that specified 50% virus infection.

Working virus stock dilutions prepared according to determined virus titers for both viruses in 5 mL and put -80 ºC until use.

2.6.2. Viral DNA Isolation Method

For qRT-PCR analysis viral DNA isolated by using Roche High Pure Viral DNA Isolation Kit (#11858874001, Switzerland) from infected and treated cells. Firstly, HaCaT cells seeded as $1x10^5$ cell (400 µL/well) in 48 well plate and incubated at 37 °C, 5% CO_2 for 24 h. Next day, cell seeded wells observed under microscope (Zeiss Axio Vert.A1, Germany) to check cell morphology and confluency. Cells should be monolayer completely in each well. Then, old media aspirated from well and each well washed with DPBS for 3 times. Cells inoculated with 150 µL of working virus stock that prepared previously and incubated at 37 °C, 5% CO_2 for 2 h. Every 20 minutes plate shook smoothly. Meanwhile, propolis extracts warmed at room temperature to prepare different concentrations under minimum cytotoxic concentration. Concentrations prepared with virus media (DMEM high with 2% FBS and 1% PSA) and amount of concentrations showed in Table 2.1.

Table 2.1: Amount of different concentrations for Propolis extracts.

Extracts Name	Concentrations (µg/mL)				
	1	**2**	**3**	**4**	**5**
Propylene Extract	200	150	100	50	25
Ethanol Extract	200	150	100	50	25
Glycerol Extract	1300	1200	1100	1000	900
Soya Extract	800	700	600	500	400

Acyclovir working stock also warmed at room temperature to prepare different concentrations as 5 µM, 1 µM, 0.8 µM, 0.6 µM, 0.4 µM with virus media.

After incubation time of virus inoculation, unbound viruses aspirated and cells treated with 400 µL of propolis extracts and acyclovir. Virus infected cells, uninfected cell and 20% DMSO treated cells used as a control. Following treatment period cells incubated at 37 °C, 5% CO_2 for 72 h. End of the incubation period, supernatant was collected and centrifuged at

5000 rpm for 30 minutes to remove cell debris. Then, viral DNA isolated according to specified protocol in kit insert and isolated DNA stored at -20 until use. These procedure applied different concentration of each extracts and acyclovir for HSV-1 and HSV-2. All experiment performed 3 times.

2.6.3. Quantitative Real Time PCR (qRT-PCR) Method

After DNA isolation qRT-PCR analyze performed by using quantitative HSV-1 and HSV-2 Kit (R-Gene HSV-1 #71015, HSV-2 #71016, bioMérieux, France) according to procedure that specified in kit insert. Quantification for HSV-1 and HSV-2 is performed by real-time PCR using TaqMan 5' nuclease technology. For HSV-1 the targeted sequence is located in US7 gene and amplified fragment size is 142 base pairs. For HSV-2 the targeted sequence is located in US2 gene and amplified fragment size is 177 base pairs. Quantitative RT-PCR method steps summarized in Figure 2.5.

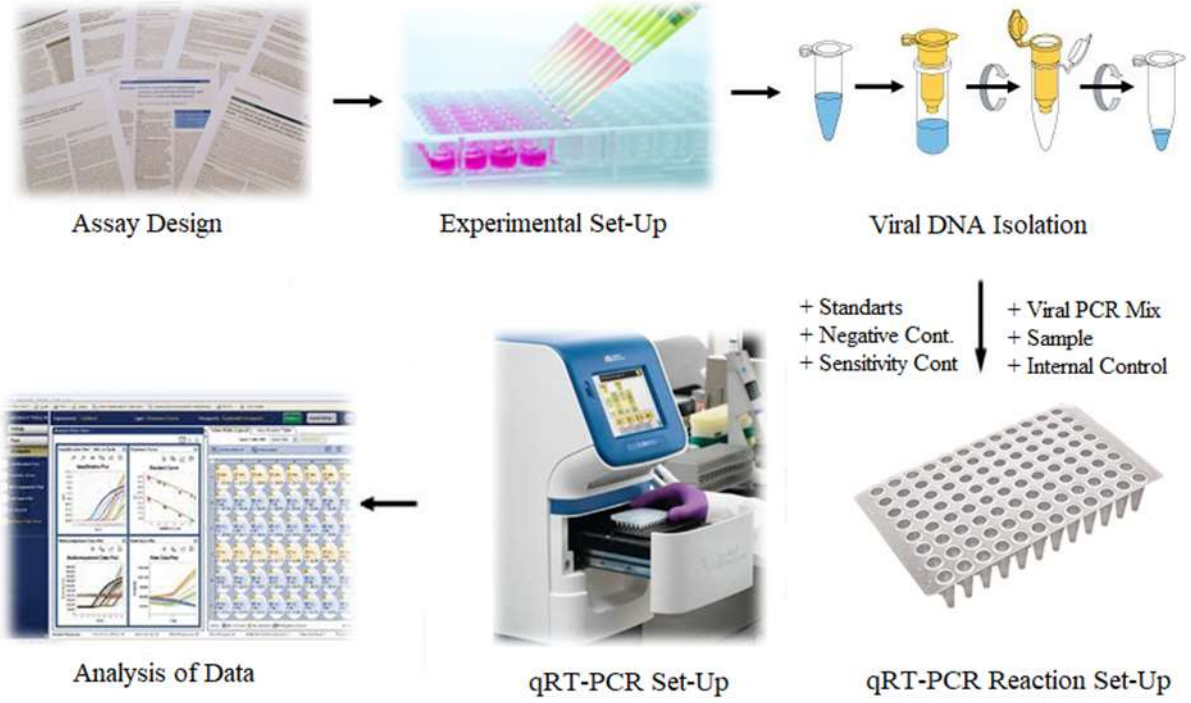


Figure 2.5: Quantitative Real Time PCR reaction steps.

EC_{50} values calculated with the obtained data from qRT-PCR (virus amount (Copy/mL)), concentrations of extracts and acyclovir.

The selectivity index (SI) of a compound is a widely accepted parameter used to express a compound's *in-vitro* efficacy in the suspension of virus replication [170].

Therefore, in our experiment, selectivity index was calculated on the basis of CC_{50} values of each extracts for HaCaT cell line and EC_{50} values of each extracts for HSV-1 and HSV-2.

Selectivity Index = Cytotoxic Concentration 50 (CC_{50}) / Effective Concentration 50 (EC_{50})

2.7. STATISTICAL ANALYSIS

All numerical data of the experiments were statistically evaluated with control group and each other by using GraphPad Prism (GraphPad Prism version 7.0, GraphPad Software, San Diego California, USA, Anonim-c). The significance of the groups according to control group were evaluated with one-way ANOVA and Dunnett's test. P <0.05 was taken as a significance value.

3. RESULTS

3.1. EVALUATION OF *IN VITRO* CYTOTOXICITY EFFECTS OF PROPOLIS EXTRACTS AND ACYCLOVIR WITH MTS METHOD

In our study, cytotoxic effects of 4 different propolis extracts and acyclovir on HaCaT cells were shown on the basis of determined concentrations preliminarily. Relative viability (%) of treated cells showed in Figure 3.1 – 3.5 and relative inhibition values (%) explained in Table 3.1 – 3.5. All data analyzed by comparing with negative control.

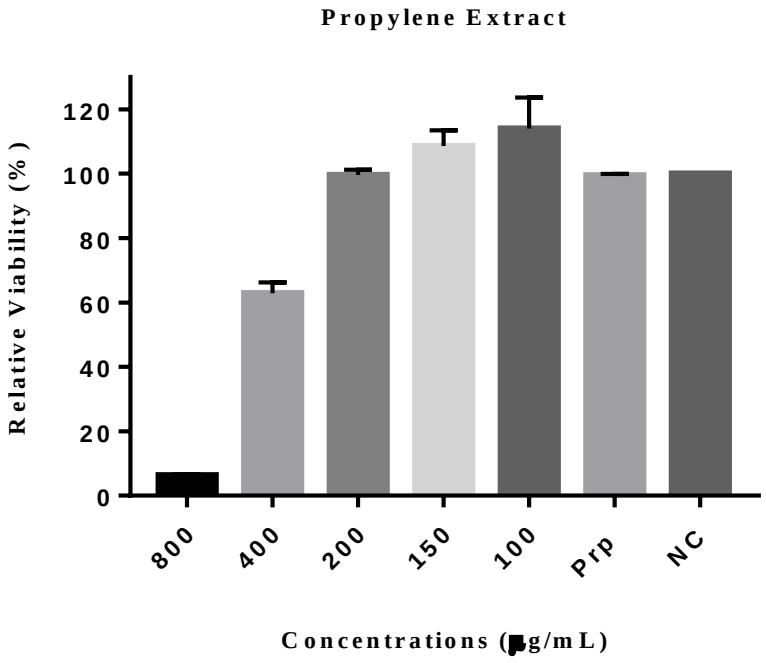


Figure 3.1: Cytotoxic effect of propylene extract on HaCaT cells viability (Prp: maximum propylene amount in highest concentration of extract, NC: Negative Control, P <0.0001, R^2: 0.9902).

Table 3.1: Different concentrations of Propylene extract and relative inhibition (%) values of HaCaT cells.

Concentrations (µg/mL)	Relative Inhibition (%)
800	93.66
400	37.09
200	0.36
100	-8.66
50	-14.08
Propylene	0,44

Propylene extract showed severe cytotoxic effect at 800 µg/mL concentration and moderate cytotoxic effect at 400 µg/mL concentration on HaCaT cells. At 200 µg/mL cytotoxic effect wasn't detected while proliferative effect was observed at 100 µg/mL and 50 µg/mL. Therefore 200 µg/mL was determined as minimum cytotoxic concentration (MCC or Maximum Non Toxic Dose). Propylene amount in highest concentration of propolis extract also was determined as non-toxic for HaCaT cells.

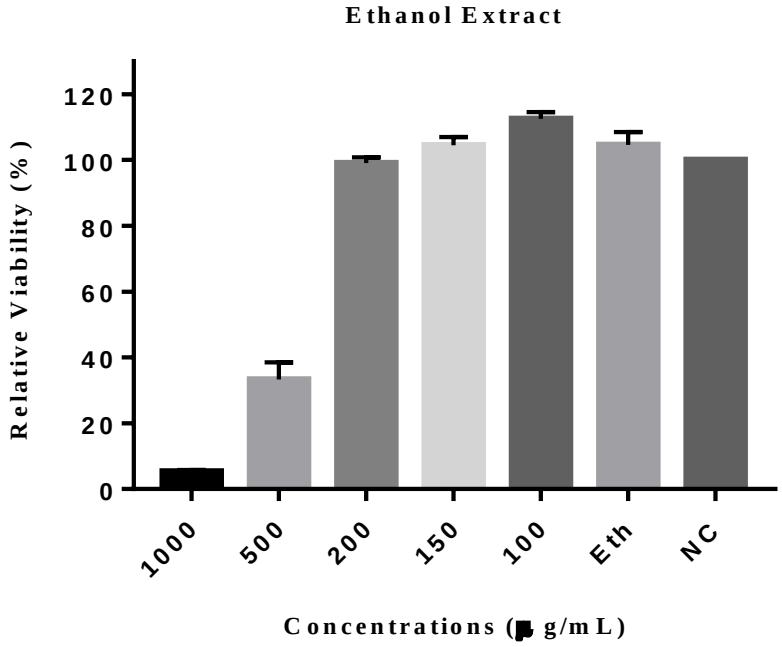


Figure 3.2: Cytotoxic effect of ethanol extract on HaCaT cells viability (Eth: maximum ethanol amount in highest concentration of extract, NC: Negative Control, P <0.0001, R2: 0.9966).

Table 3.2: Different concentrations of Ethanol extract and relative inhibition (%) values of HaCaT cells.

Concentrations (µg/mL)	Relative Inhibition (%)
1000	94.62
500	66.68
200	0.95
150	-4.47
100	-12.54
Ethanol	-4.64

Ethanol extract showed severe cytotoxic effect at 1000 µg/mL concentration and moderate cytotoxic effect at 500 µg/mL concentration on HaCaT cells. At 200 µg/mL cytotoxic effect wasn't detected while proliferative effect was observed at 150 µg/mL and 100 µg/mL. Therefore 200 µg/mL was determined as minimum cytotoxic concentration (MCC or Maximum Non Toxic Dose). Ethanol amount in highest concentration of propolis extract showed minimum proliferative effect on HaCaT cells.

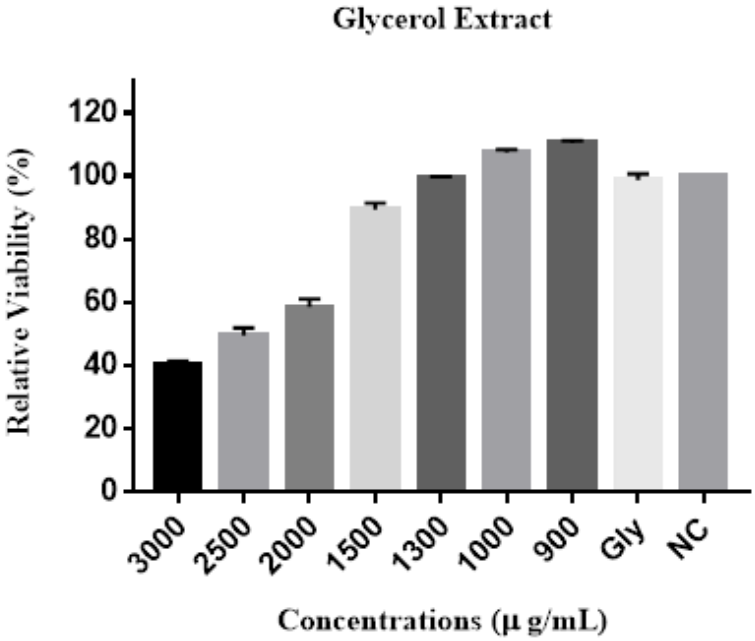


Figure 3.3: Cytotoxic effect of Glycerol extract on HaCaT cells viability (Gly: maximum glycerol amount in highest concentration of extract, NC: Negative Control, P <0.0001, R2: 0.9973).

Table 3.3: Different concentrations of Glycerol extract and relative inhibition (%) values of HaCaT cells.

Concentrations (µg/mL)	Relative Inhibition (%)
3000	59.78
2500	50.51
2000	41.40
1500	10.67
1300	0.64
1000	-7.43
900	-10.45
Glycerol	1.26

Glycerol extract showed moderate cytotoxic effect at 3000 µg/mL, 2500 µg/mL and 2000 µg/mL concentration on HaCaT cells. At 1300 µg/mL cytotoxic effect wasn't detected while proliferative effect was observed at 1000 µg/mL and 900 µg/mL. Therefore 1300 µg/mL was determined as minimum cytotoxic concentration (MCC or Maximum Non Toxic Dose). Glycerol amount in highest concentration of propolis extract also was determined as non-toxic for HaCaT cells.

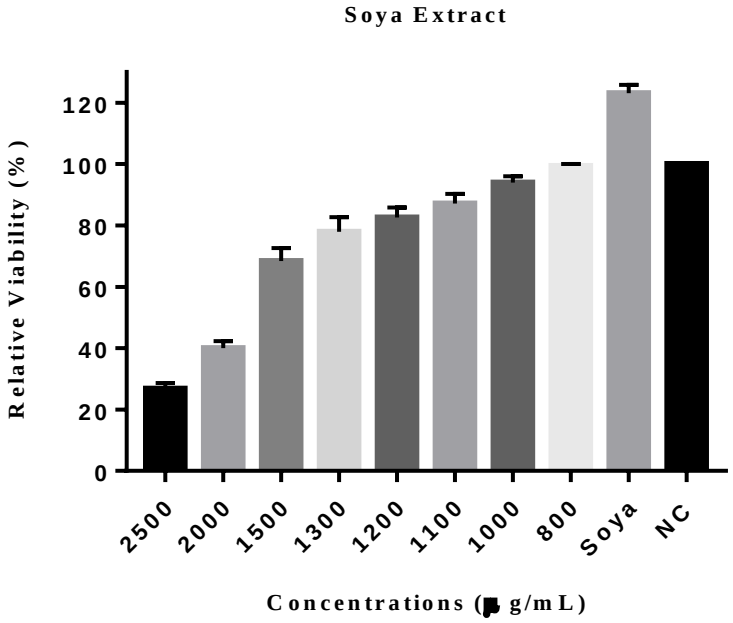


Figure 3.4: Cytotoxic effect of Soya extract on HaCaT cells viability (Soya: maximum soya amount in highest concentration of extract, NC: Negative Control, P <0.0001, R2: 0.9917).

Table 3.4: Different concentrations of Soya extract and relative inhibition (%) values of HaCaT cells.

Concentrations (µg/mL)	Relative Inhibition (%)
2500	73.21
2000	60.00
1500	31.63
1300	22.05
1200	17.39
1100	12.80
1000	6.05
800	0.63
Soya	-23.10

Soya extract showed cytotoxic effect at 2500 µg/mL and 2000 µg/mL concentration on HaCaT cells. Between 1300 µg/mL and 1000 µg/mL fairly well cytotoxic effect was observed. At 800 µg/mL cytotoxic effect wasn't detected while proliferative effect was observed at Soya control which is amount of soya in highest concentration of propolis extract. 800 µg/mL was determined as minimum cytotoxic concentration (MCC or Maximum Non Toxic Dose).

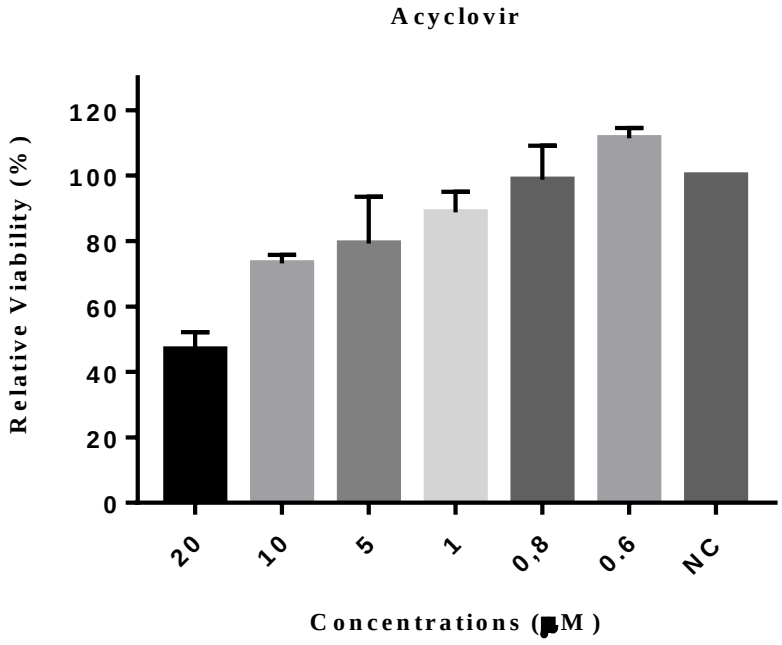


Figure 3.5: Cytotoxic effect of Acyclovir on HaCaT cells viability (NC: Negative Control, P<0.0001, R2: 0.8942).

Table 3.5: Different concentrations of Acyclovir and relative inhibition (%) values of HaCaT cells.

Concentrations (µM)	Relative Inhibition (%)
20	61.70
10	28.71
5	20.78
1	11.26
0.8	1.23
0.6	-11.47

Acyclovir showed cytotoxic effect which cause cell death over 50 % at 20 µM concentration and showed fairly well cytotoxic effect between 10 µM and 1 µM concentrations on HaCaT cells. At 0.8 µM significant cytotoxic effect wasn't detected. Therefore 0.8 µM was determined as minimum cytotoxic concentration (MCC or Maximum Non Toxic Dose). Also, proliferative effect was observed at 0.6 µM.

Also, Cytotoxic Concentration 50 (CC_{50}) which is the concentration of a compound that will kill half of the cells in an uninfected cell culture, calculated from suspension values graph. CC_{50} graphs showed in Figure 3.6 - 3.10. All data analyzed by comparing with negative control.

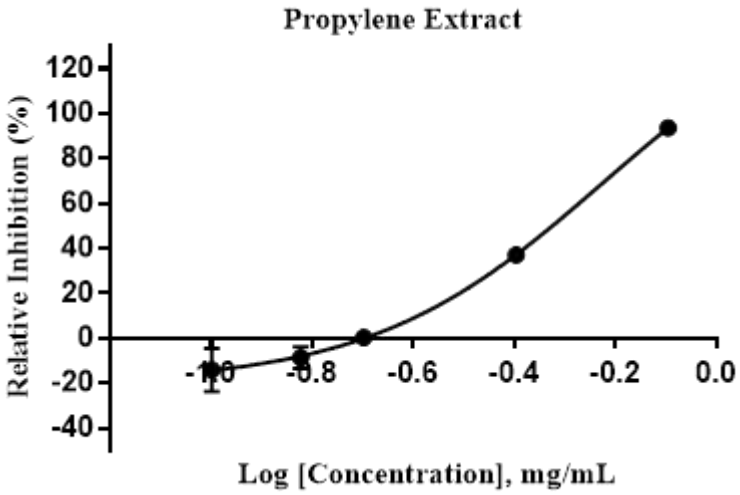


Figure 3.6: Cytotoxic Concentration 50 (CC_{50}) graph of Propylene extract for HaCaT cells.

Cytotoxic Concentration 50 (CC_{50}) of Propylene extract was calculated as 0.593 mg/mL (593 µg/ml) for HaCaT cells.

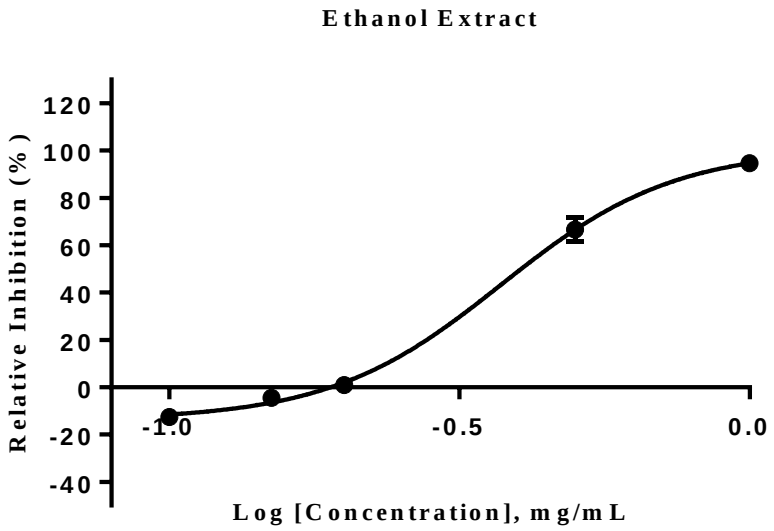


Figure 3.7: Cytotoxic Concentration 50 (CC_{50}) graph of Propylene extract for HaCaT cells.

Cytotoxic Concentration 50 (CC_{50}) of Ethanol extract was calculated as 0.375 mg/mL (375 µg/mL) for HaCaT cells.

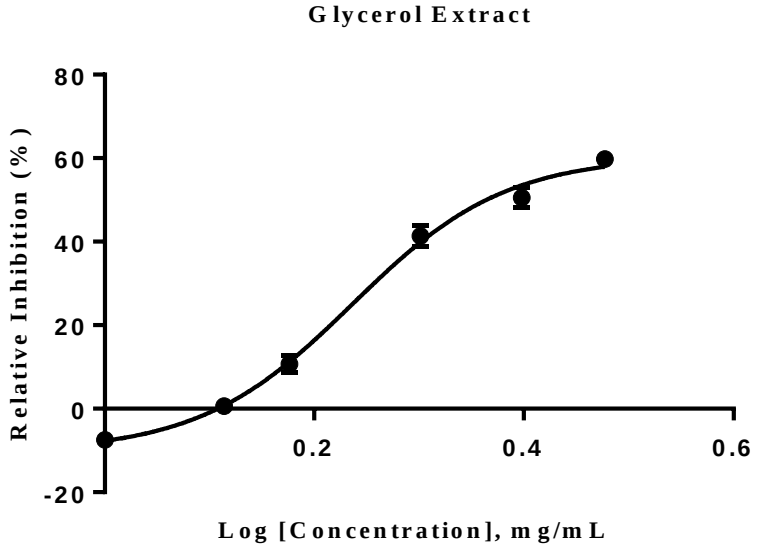


Figure 3.8: Cytotoxic Concentration 50 (CC_{50}) graph of Glycerol extract for HaCaT cells.

Cytotoxic Concentration 50 (CC_{50}) of Glycerol extract was calculated as 1.723 mg/mL (1723 µg/mL) for HaCaT cells.

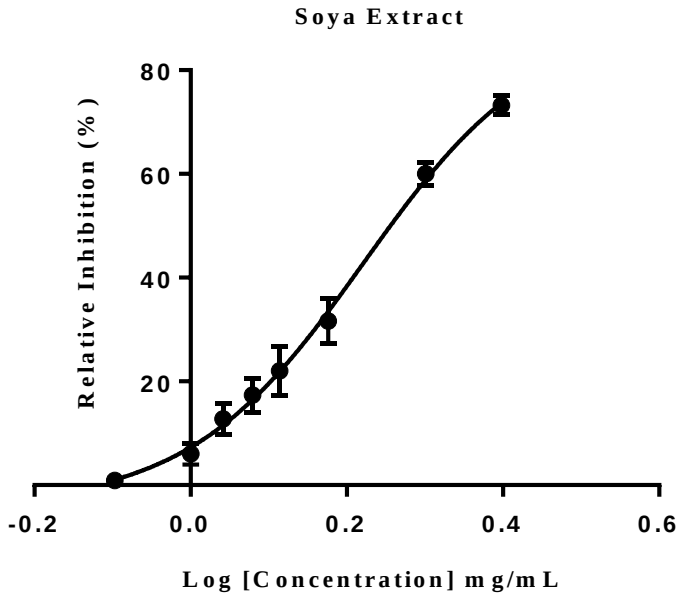


Figure 3.9: Cytotoxic Concentration 50 (CC_{50}) graph of Soya extract for HaCaT cells.

Cytotoxic Concentration 50 (CC_{50}) of Soya extract was calculated as 1.664 mg/mL (1664 µg/mL) for HaCaT cells.

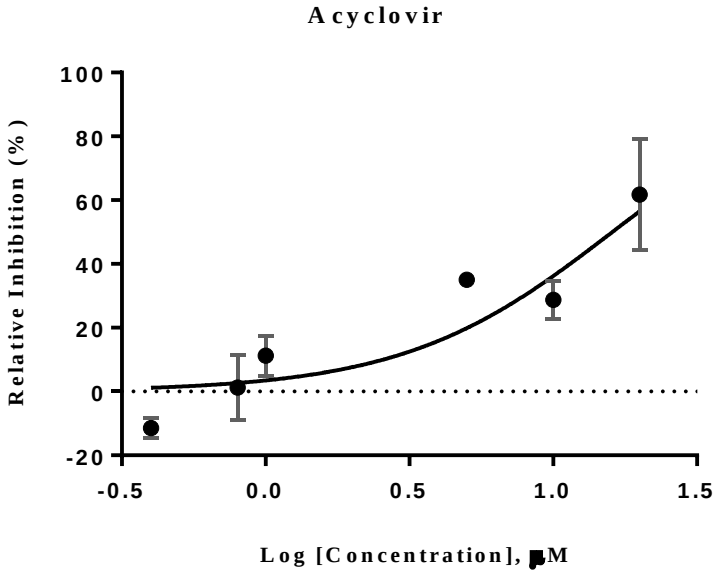


Figure 3.10: Cytotoxic Concentration 50 (CC_{50}) graph of Acyclovir for HaCaT cells.

Cytotoxic Concentration 50 (CC_{50}) of Acyclovir was calculated as 15.85 µM for HaCaT cells.

3.2. EVALUATION OF ANTIVIRAL ACTIVITY OF PROPOLIS EXTRACTS AND ACYCLOVIR WITH QUANTITATIVE REAL TIME PCR

In our study, antiviral activity of 4 different propolis extracts and acyclovir were analyzed for HSV-1 and HSV-2 on the basis of determined non-toxic concentration for HaCaT cell preliminarily.

Virus inhibition (Copy/mL) in treated cells for HSV-1 showed in figure 3.11 – 3.15. All data analyzed by comparing with negative control.

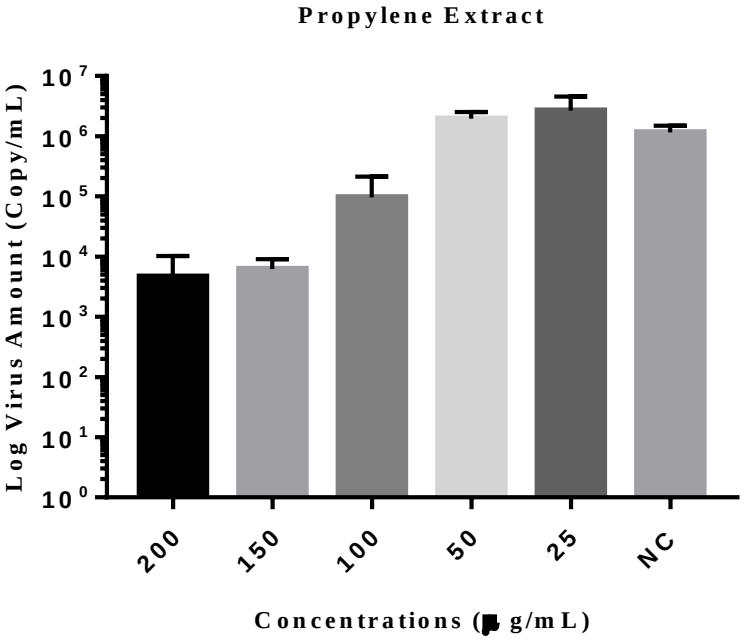


Figure 3.11: Viral inhibition rate of non-toxic concentrations of Propylene extract for HSV-1. (NC: Negative Control refers to virus infected cell only, P <0.05).

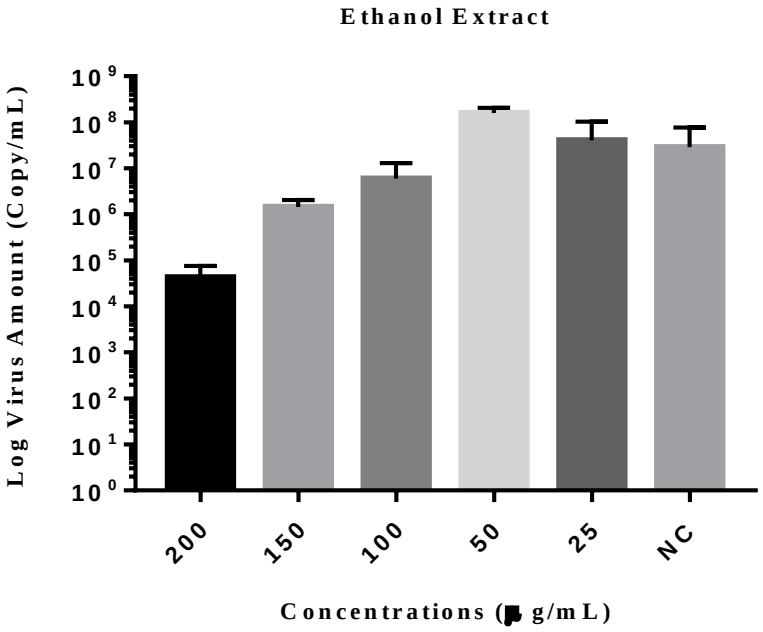


Figure 3.12: Viral inhibition rate of non-toxic concentrations of Ethanol extract for HSV-1. (NC: Negative Control refers to virus infected cell only, P <0.05).

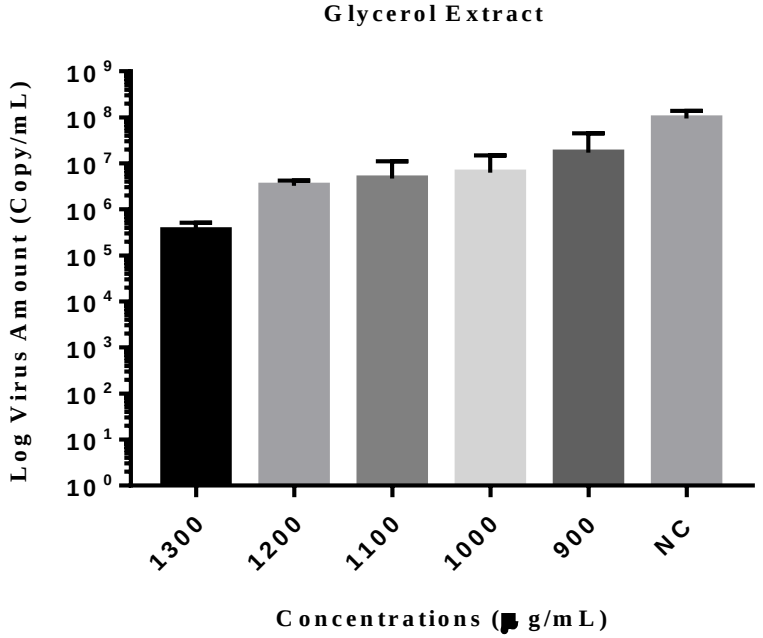


Figure 3.13: Viral inhibition rate of non-toxic concentrations of Glycerol extract for HSV-1. (NC: Negative Control refers to virus infected cell only, P <0.05).

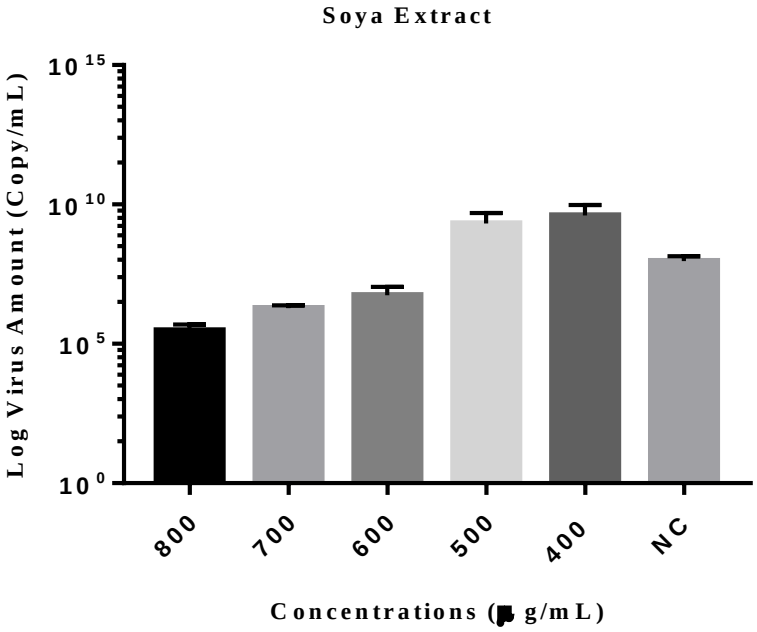


Figure 3.14: Viral inhibition rate of non-toxic concentrations of Glycerol extract for HSV-1. (NC: Negative Control refers to virus infected cell only, P <0.05).

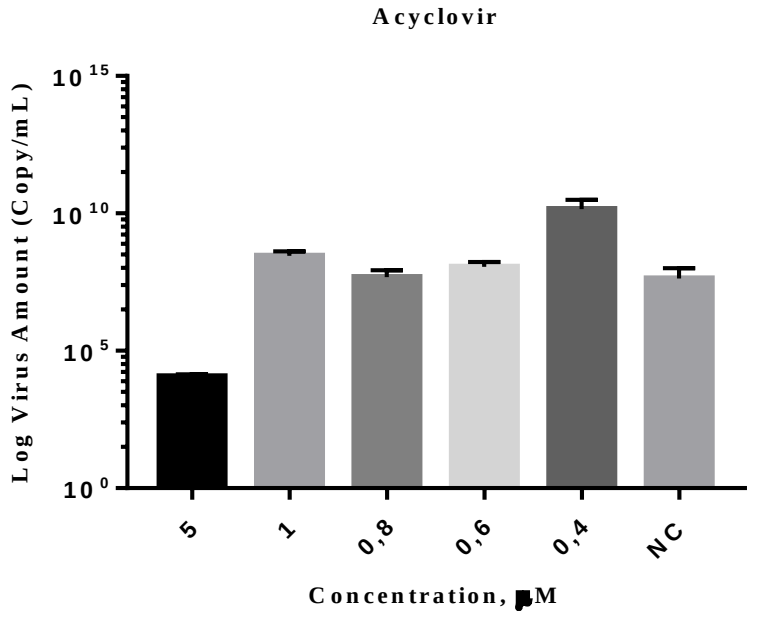


Figure 3.15: Viral inhibition rate of non-toxic concentrations of Acyclovir for HSV-1. (NC: Negative Control refers to virus infected cell only, P <0.05).

Virus inhibition (Copy/mL) in treated cells for HSV-2 Figure 3.16 – 3.20. All data analyzed by comparing with negative control.

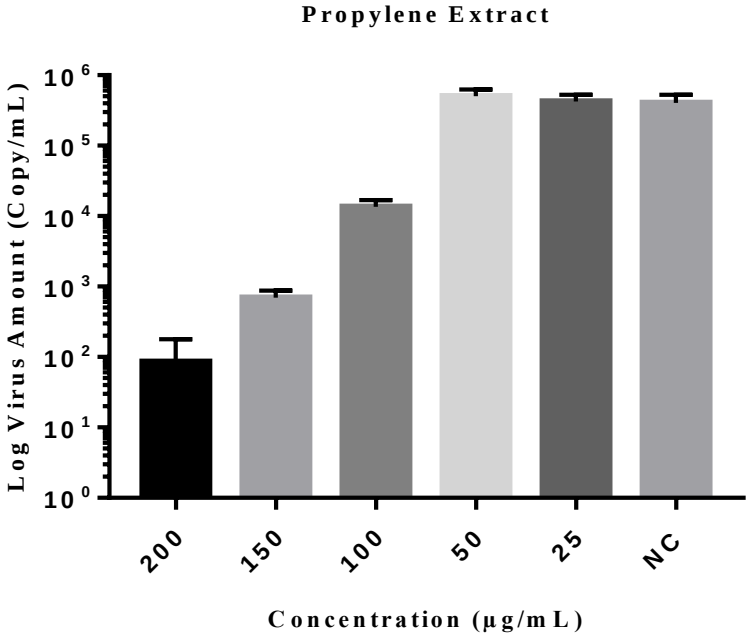


Figure 3.16: Viral inhibition rate of non-toxic concentrations of Propylene extract for HSV-2. (NC: Negative Control refers to virus infected cell only, P <0.05).

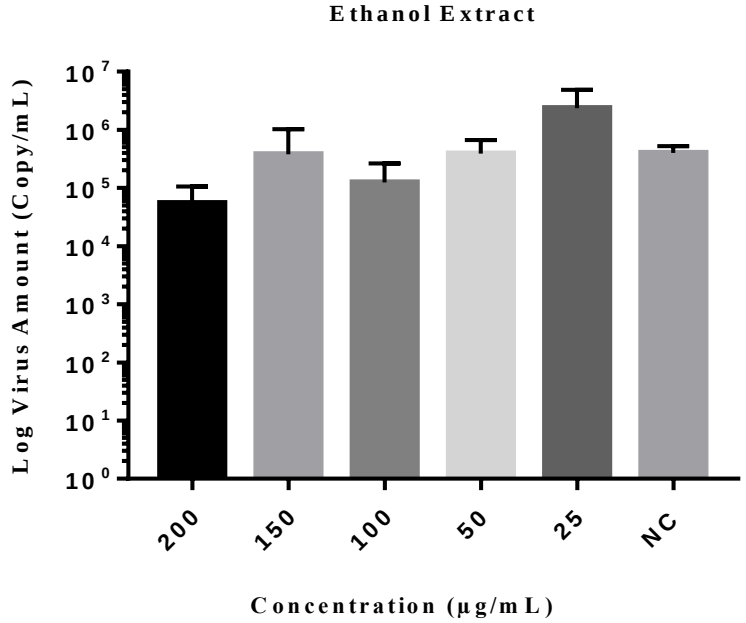


Figure 3.17: Viral inhibition rate of non-toxic concentrations of Ethanol extract for HSV-2. (NC: Negative Control refers to virus infected cell only, P <0.05).

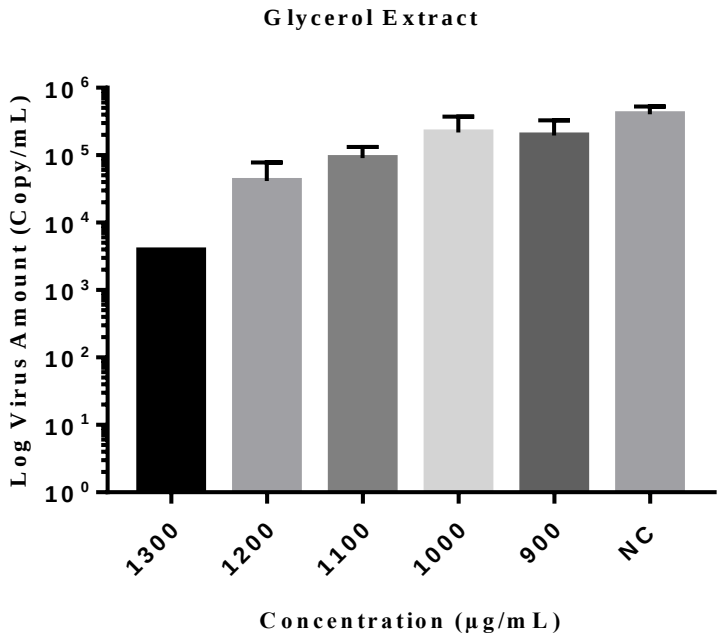


Figure 3.18: Viral inhibition rate of non-toxic concentrations of Glycerol extract for HSV-2. (NC: Negative Control refers to virus infected cell only, P <0.05).

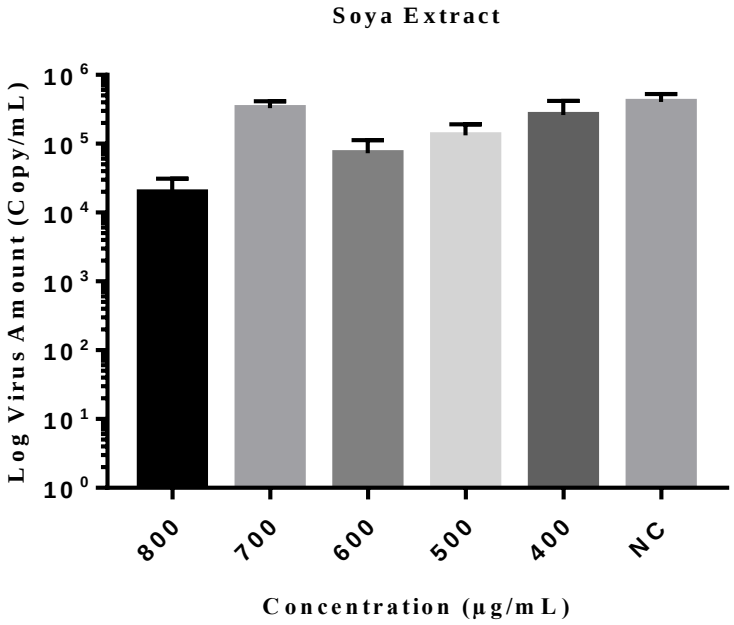


Figure 3.19: Viral inhibition rate of non-toxic concentrations of Soya extract for HSV-2. (NC: Negative Control refers to virus infected cell only, P <0.05).

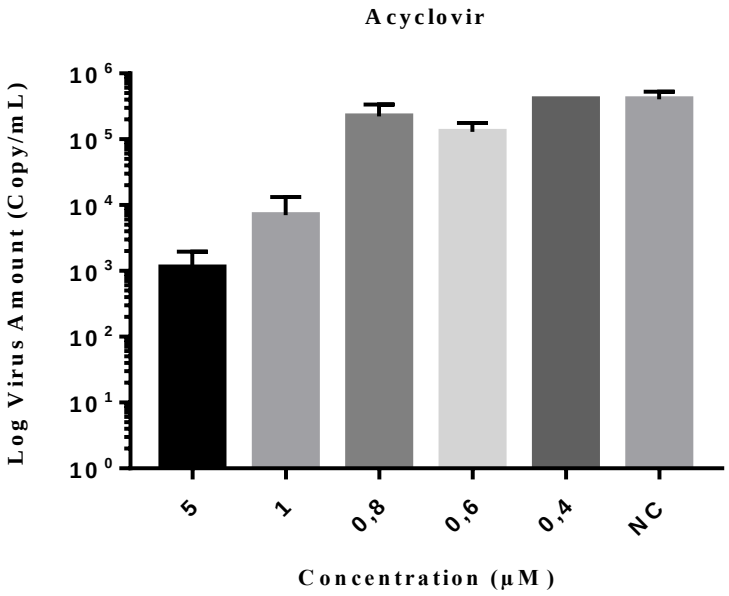


Figure 3.20: Viral inhibition rate of non-toxic concentrations of Acyclovir for HSV-2. (NC: Negative Control refers to virus infected cell only, P <0.05).

Effective Concentration 50 (EC_{50}) which is the concentration of a test compound that produces 50% suspension of virus replication. In the case of antiviral evaluation in cell culture, the EC_{50} is the compound concentration in which the amount of virus is 50% compared to what is detected in the untreated, virus-infected control [170]. Calculated EC_{50} values of each extracts and acyclovir for both viruses showed in Table 3.6.

Table 3.6: EC_{50} values of each propolis extracts and Acyclovir for HSV-1 and HSV-2.

Extracts	**EC_{50} (µg/mL)**	
	HSV-1	**HSV-2**
Propylene Extracts	86.64	92.05
Ethanol Extracts	90.86	48.99
Glycerol Extracts	768.6	904.1
Soya Extract	501	396.1
Control	**EC_{50} (µM)**	
Acyclovir	2.50	5.54

Viruses are obligate organisms which are living in cells of another organisms. Therefore, viral inhibitors should be effective without any toxic effects on cells, tissues and organs. This

concept is called “selective activity” against viruses. To determine selective activity, it is essential to compare replicating cells with replicating viruses [173]. Thus, the 50% concentration of cytotoxic concentration to 50 % viral inhibition concentration is defined as “selective index (SI)”. In another meaning, it measures the window between cytotoxicity and antiviral activity. The higher SI ratio, means theoretically compound would be more effective and safe during *in vivo* treatment for a given viral infection. The selectivity index of a compound is a widely accepted parameter used to express a compound’s *in vitro* efficacy in the suspension of virus replication [170]. According to the SI value, it can be concluded that the antiviral activity of a compound is real and not a result of its cytotoxic effect on cells. Therefore, the SI verifies the safety index of the material tested [174].

SI values of each propolis extracts calculated for HSV-1 and HSV-2 from obtained CC_{50} and EC_{50} data. Results showed table 3.7 for HSV-1 and 3.8 for HSV-2.

Table 3.7: EC_{50} and SI values of each propolis extracts and Acyclovir for HSV-1.

Propolis Extract	**HaCaT CC_{50} (µg/mL)**	**EC_{50} (µg/mL)**	**SI (CC_{50}/EC_{50})**
		HSV-1	
Propylene	593±3.94	86.64±4.97	6.84
Ethanol	375±2.39	90.86±6.71	4.12
Glycerol	1723±1.48	768.6±6.68	2.24
Soya	1664±3.14	501±7.44	3.32
Control	**HaCaT CC_{50} (µM)**	**EC_{50} (µM)**	**SI**
		HSV-1	
Acyclovir Sodium	15.85±8.61	2.50±7.33	6.34

SI values (CC_{50}/EC_{50}) of the Propolis Ethanol, Propylene, Glycerol, Soya extracts and Acyclovir were found as 6.84, 4.12, 2.24, 3.32 and 6.34 respectively.

Table 3.8: EC_{50} and SI values of each propolis extracts and Acyclovir for HSV-2.

Propolis Extract	**HaCaT CC_{50} (µg/mL)**	**EC_{50} (µg/mL)**	**SI**
		HSV-2	
Propylene	593±3.94	92.05±3.90	6.44
Ethanol	375±2.39	48.99±5.37	7.65
Glycerol	1723±1.48	904.1±4.90	1.90
Soya	1664±3.14	396.1±4.78	4.20
Control	**HaCaT CC_{50} (µM)**	**EC_{50} (µM)**	**SI**
		HSV-2	
Acyclovir Sodium	15.85±8.61	5.54±4.68	2.86

SI values (CC_{50}/EC_{50}) of the Propolis Ethanol, Propylene, Glycerol, Soya extracts and Acyclovir were found as 7.65, 6.44, 1.90, 4.20 and 2.86 respectively. Therefore, ethanol, propylene and soya extracts of propolis showed higher antiviral activity than Acyclovir against HSV-2.

4. DISCUSSION

Propolis is mainly composed of plant resin (50%), wax (30%), essential and aromatic oils (10%), pollen (5%), and other organic compounds (5%) [15]. Phenolics, aromatic aldehydes, esters, flavonoids, terpenes, beta-steroids, and alcohols are notible organic compounds found in the propolis [5]. It also contains significant vitamins, minerals and several enzymes [12]. In last 20 years, because of its rich content, numerous studies have been performed to investigate biological function of propolis and in many studies it has been proven that, propolis has antiviral, antimicrobial, antifungal, anti-inflammatory, immunomodulatory, anticancer, wound healing and skin protection, antioxidant and hepatoprotective properties [10, 14]. Among these biological function, antiviral activity is newly focused research area for propolis.

Viruses are small particles that they cannot reproduce itself and use host cell machinery to produce new viruses. They can cause mild to severe infections and even cause deaths of living organisms. Among them Herpes Simplex Viruses, cause life-long infection, which virus viruses go in to latent phase in body nerve cells after lysogenic cycle [175]. According to World Health Organization (WHO) 90% of population is infected by different types of herpesviruses that develop latency or result in oral and genital herpes, conjunctivitis eczema and other diseases [160]. Infection with the HSV commonly can be occurred by Herpes Simplex Type 1 which known as oral herpes or Herpes Simplex Type 2 which known as genital herpes. HSV-1 transmitted by contact with the virus in saliva, sores, and surfaces in or around the mouth. HSV-2 incurable infection which is mainly transmitted during sex, contact with skin, genital surfaces, sores or fluids of infected person even without symptom. In immunocompromised people HSV-1 and HSV-2, cause severe symptoms and complications such as keratitis, pneumonitis, and retinal necrosis. In addition, HSV infection can be serious, extensive and prolonged in immunocompromised individuals with occurrence of drug-resistant strains [161].

Recently, effective antiviral agents have been developed and among them widely used compound is Acyclovir for HSV infection that inhibits viral DNA polymerase. Viral infection is needed prolonged treatment especially for immunocompromised individuals, which leads to the emergence of resistant virus. Therefore, research on new active substances against herpes

viruses gained importance. With this aspect, in our study antiviral effect of Propylene, Ethanol, Glycerol and Soya extracts of propolis analyzed against HSV-1 and HSV-2 viruses in HaCaT cell line via ascertainment of CC_{50} and non-toxic dose with MTS and EC_{50} values with qRT-PCR. Acyclovir used as a control drug and antiviral effect compared according to selective index of each compound.

According to MTS result, Propylene extract showed severe cytotoxic effect that inhibited 93.66 % of the cells at 800 µg/mL while ethanol extract inhibited 94.62 % of the cell at 1000 µg/mL. Minimum cytotoxic concentration of both extracts obtained at 200 µg/mL. Propylene and ethanol are both polar solvent but dielectric constant of propylene (32.1) is higher than ethanol (25) which refers to propylene polar than ethanol [176]. It showed that flavonoid content of ethanol extract is lower than propylene extract and less polar substances can solved in ethanol extract [177].

The cytotoxic effect differences of both polar solvent can explained by differences between their contents. The same situation obtained for both non-polar oil extraction of propolis. The relative suspension rate obtained as 59.78 % for glycerol extract at 3000 µg/mL and 73.21 % for soya extract at 2500 µg/mL. Minimum cytotoxic concentration detected for glycerol extract 1300 µg/mL while it was 800 µg/mL for soya extract. In addition, the amount of propylene, ethanol and glycerol solvents in highest concentration of propolis extracts didn't show any cytotoxic effect while proliferative effect was obtained for soya extract on HaCaT cells. Cytotoxic Concentration (CC_{50}) calculated from obtained data and CC_{50} values of the Propolis Ethanol, Propylene, Glycerol, Soya extracts and Acyclovir were found as 0.593 mg/mL (593 µg/ml), 0.375 mg/mL (375 µg/ml), 1.723 mg/mL (1723 µg/ml), 1.664 mg/mL (1664 µg/ml) and 16.06 µM respectively Figure 3.6.1-3.6.10.

In our study, EC_{50} values for HSV-1 and HSV-2 viruses determined with Quantitative Real Time PCR (qRT-PCR) method that accepted best and validated quantification method for ascertainment of antiviral charateristic [171]. For HSV-1 EC_{50} values of the Propolis Propylene, Ethanol, Glycerol, Soya extracts and Acyclovir calculated as 86.64 µg/ml, 90.86 µg/ml, 501 µg/mL, 768.6 µg/mL and 2.50 µM respectively Table 3.2.2. For HSV-2 EC_{50} values of the Propolis and Acyclovir analyzed as 48.99 µg/mL, 92.05 µg/mL, 396.1 µg/ml, 904.1 µg/mL and 5.54 µM respectively Table 3.2.3. It was reported that HSV-1 and HSV-2 shows several type specific differences in interaction with their host cell. Particularly, HSV-2

infection of cells have been more adequately inhibited by polyanionic substances than HSV-1 infection while HSV-1 infection has been more efficiently inhibited by polycationic substances than HSV-2 [178]. This preoccupied that the differences between our results can be related with type specific differences of viruses. US Food and Drug Administration (FDA) approved EC_{50} range of acyclovir (Zovirax) for HSV-1 between 0.02 to 13.5 μg/mL and for HSV-2 between 0.01 to 9.9 μg/mL [179]. This data also supports our data for Acyclovir.

Selective Index (SI), measures the window between cytotoxicity and antiviral charateristic. The selectivity index of a compound is a widely accepted parameter used to express a compound's in vitro efficacy in the inhibition of virus replication [170]. Based on the SI value, it can be concluded that the antiviral charateristic of a compound is real and not a result of its cytotoxic effect on cells. In other words, the SI verifies the safety index of the material tested [174]. The higher SI ratio, means theoretically compound would be more effective and safe during in vivo treatment for a given viral infection [170].

In our study, SI (CC_{50}/EC_{50}) values of Propylene, Ethanol, Glycerol, Soya extracts and Acyclovir were found as 6.84, 4.12, 3.32, 2.24 and 6.34 respectively for HSV-1. When results compared with the Acyclovir, Ethanol extracts have approximately same SI value while Propylene, Glycerol and Soya extracts low SI values than Acyclovir. This results can be concluded that ethanol extract efficient than Acyclovir. Propylene, Glycerol and Soya extracts have lower antiviral charateristic than acyclovir against HSV-1. SI Values of Propylene, Ethanol, Glycerol, Soya extracts and Acyclovir calculated as 6.44, 7.65, 1.90, 4.20 and 2.86 for HSV-2. The result showed that Ethanol, Propylene and Soya extracts more efficient than Acyclovir against HSV-2 while Glycerol showed low antiviral charateristic. Extracts or compound with SI value >1 in the initial screen (tested at log dilution) are considered sufficiently active to warrant additional test and are subjected to further evaluation in the primary screen [180]. In this aspect, also, Glycerol extracts can evaluated as effective against HSV-2 for further analysis. Sensitivity testing result vary greatly depending upon a number of factors. This also explains differences between our results.

In 1992, Amaros et al. researched antiviral charateristic of resin balsam against HSV-1 via different treatment time. They detected significant virucidal charateristic when HSV-1 pretreated with the compound and also they found significant decline in plaque number when they add propolis during intra cellular replication [129]. This data also verifies our results

showing that viral copy is inhibited after treatment with propolis extracts for both viruses. In contrast, Huleihel and Isanu obtained significant suspension with pretreatment and at the time of infection but they don't obtained direct interaction between propolis and HSV-1. It was concluded that antiviral charateristic was probably due to prevention of virus adsorption to host cells [181]. Silke et al. reported high antiviral charateristic against HSV-2 in viral suspension test for aqueous and ethanolic extract of propolis. But, they didn't get antiviral effect during different time of the infection as pretreatment of cells or treatment of infected cells while obtained high anti herpetic effect virus pretreatment with both extract [182]. This situation also supports different propolis extract preparation revealed different modes of antiviral charateristic. It can be evaluated for our glycerol extract which shows lowest SI value against HSV-1. It can be more effective in different mode of action. Schnitzler et al. reports propolis with high contents have higher anti herpetic effect and selectivity index in comparison with that one isolated component [183]. This can also be an acceptable explanation for low SI values of the propolis in our study. In a clinical study, HSV-2 infected patients healed faster in propolis treated group and topical application of propolis promising for treatment of HSV-1, especially patients that are suffering from frequent recurrences [130].

Antiviral activity studies can be done therapeutically which refers to application of the compound after infection of the cell; in protective effect which refers to incubate cells with the compound before inoculation of virus; or virucidal affect which refers to virus pretreated with compound then inoculated to host cell. In therapeutic manner, antiviral agents can suspend transcription or replication of the viral genome; or can interrupt viral protein synthesis. In others, antiviral agents can impede virus entry into the host cell [146] or inhibit virus absorption into the host cell [181].

In our study, our extracts were tested in therapeutic method and they inoculated 2h after the infection of cell line. It was known that Acyclovir suspends specifically the viral DNA polymerase during the intracellular replication cycle when new viral DNA is synthesized [130]. When we compared SI values of propolis extracts which are higher than acyclovir, this effect can be concluded that functional components of the propolis extract could inactivate viral DNA polymerase. This supports the high antiviral potential of the propolis against HSV-1 and HSV-2.

In this study, we also obtained important data for Acyclovir (99% HPLC grade) which is used in cell culture. Acyclovir has poor oral viability and low aqueous solubility properties which delays its absorption [184]. That is why we did not get any cytotoxic effect of acyclovir even in 5 mM concentration for HaCaT cells. Moreover, cells continued their viability in a regular manner. To solve this problem Acyclovir was used in the form of sodium salt which can be dissolved and easily absorbed by cells. With this method we obtained cytotoxic effect in 5 μM.

As a result of this research which was conducted to reveal antiviral activity of different propolis extracts, it has been shown that propylene, ethanol, glycerol and soya extracts of Propolis are potential antiviral agents against HSV-1. Results also showed that propylene, ethanol and soya extracts of Propolis are potential antiviral agents against HSV-2.

In literature, we found supportive data for the anti-herpetic effect of ethanol extract of propolis; however, as far as we have researched, there is no data available regarding the effect of propylene, glycerol and soya extracts against HSV-1 and HSV-2. Therefore, high selective index values of propylene and soya extracts against HSV-1 and HSV-2 are considered as an important and novel finding.

Therefore, further researches are required to verify our data for other propolis extracts. It is suggested as a future plan that the effect mechanism of propolis extract should be clarified and it should be studied in in-vivo studies.

5. CONCLUSION AND RECOMMENDATIONS

Infection with HSV-1 and HSV-2 is a common in community and lifelong diseases which resulted in mild to severe symptoms and sometimes death. Viruses occasionally reactivate and can required prolonged antiviral treatment in high dose, especially in immunosuppressed people. Prolonged usage of antiviral drug cause occurrence of drug-resistant strains. Therefore, molecule that have antiviral charateristic should be found to overcome resistant strains. Natural products can be optimized as drug-like molecules and they remain the best sources of drugs and drug leads. Over last thirty years, modified plant products by animals have been attracted attention for drug discovery studies. Propolis is one of the modified plant products that have rich phenolic contents. In literature, anti-inflammatory, antioxidant, anti-cancer and anti-microbial reported in in-vitro and in-vivo studies. Because of its biological charateristic, propolis can be an effective antiviral agent for HSV-1 and HSV-2. In our study, propylene, ethanol and soya extracts showed good antiviral activity against HSV-1 and HSV-2 viruses when compared with the acyclovir. Therefore, we concluded that propolis is a promising natural product against HSV-1 and HSV-2 infections, especially for recurrent conditions.

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CURRICULUM VITAE

Personal Information	
Name Surname	Sevda DEMİR
Place of Birth	Sivas
Date of Birth	11.09 1985
Nationality	☑ T.C. ❒ Other:
Phone Number	0090536 271 71 70
Email	demirsvd@gmail.com
Web Page	

Educational Information	
B. Sc.	
University	Fatih University
Faculty	Faculty of Arts and Sciences
Department	Biology
Graduation Year	28.07.2009

M. Sc.	
University	Istanbul University
Institute	Institute of Science
Department	Genetic and Bioengineering
Programme	Genetic and Bioengineering Programme