CHAPTER II

LITERATURE REVIEW

1. Herpes Simplex Virus

The infections of herpes simplex viruses in humans have been documented since ancient Greek times. Records of human HSV infections began with descriptions of cutaneous spreading lesions thought to be of herpetic etiology, particularly in the writings of Hippocrates (Nahmias and Dowdle, 1968). Scholars of Greek civilization define the word "herpes" to mean creep or crawl, in reference to the spreading nature of the visualized skin lesions (Beswick, 1962). Several observations made at the beginning of the 20th century brought an end to the early imprecise descriptive era of HSV infections. Several points substantiate this conclusion. First, histopathologic studies described multinucleated giant cells associated with herpesvirus infections (Unna, 1886). Second, the unequivocally infectious nature of HSV was recognized (Lowenstein ,1919).

The advances in major laboratory in the past 25 years have provided a foundation for the recent application of molecular biology to the study of human disease. One significant advance was the detection of antigenic differences between HSV types. Although suggested by Lipschitz (1921) on clinical grounds more than 60 years ago and by others from laboratory observations (Plummer, 1964), in 1968 Nahmias and Dowdle demonstrated antigenic and biologic differences between HSV-1 and HSV-2. These investigators demonstrated that HSV-1 was more frequently associated with nongenital infection, whereas HSV-2 was associated with genital disease. This observation was pivotal for many of the clinical, serologic, immunologic, and epidemiologic studies. Obviously, other critical advances made over the past decade have contributed to our understanding of the natural history of HSV infections. These include the following, among others. First, successful antiviral therapy was established unequivocally for HSV encephalitis (Whitley, 1977) and, subsequently, for genital HSV infections (Bryson et al.,

1983) and HSV infections in the immunocompromised host (Meyers et al., 1980). Second, differences between strains of HSV were demonstrated by restriction endonuclease technology, which has become an important molecular epidemiologic tool (Buchman et al., 1978). Third, the use of type-specific antigens for seroepidemiologic surveys has advanced our understanding of the clinical epidemiology of infection (Roizman et al., 1984). Fourth, much work has focused on the replication of HSV and the resultant gene products. A principal goal of these efforts is to define the biologic properties of these gene products, a goal that is in the very early stages of accomplishment. Fifth, the engineering of HSV and the expression of specific genes will provide technology for new vaccines (Roizman et al., 1985 Stevens et al., 1987;), as well as for the use of such viruses for gene therapy. Finally, extensive effort was devoted to the study of latency with incremental advances (Roizman and Sears, 1993).

2. Characteristics of Herpes Simplex Virus

Herpes simplex viruses (HSV) are classified in the subfamily *alphaherpesvirinae*. (Roizman, 1996). The members of this subfamily are classified on the basis of variable host range, relatively short reproductive cycle, rapid spread in culture, efficient destruction of infected cells, and capacity to establish latent infections primarily but not exclusively in sensory ganglia.

It has been reported that the size of herpesvirions varied from 120 to nearly 300 nm. The precise number of polypeptide species contained in the virions is not known and may vary from one virus to another. The estimates generally range from 30 to 35 polypeptides. The virions consist of four elements: (i) an electron-opaque core, (ii) an icosahedral capsid surrounding the core, (iii) an amorphous tegument surrounding the capsid, and (iv) an outer envelope exhibiting spikes on its surface (Figure 1). The core of the mature virion contains the viral DNA in the form of a torus. The precise arrangement of the DNA in the toroid is not known. The capsid is approximately 100 nm in diameter and the number of capsomers is 162. The pentameric capsomeres at the vertices have not been well characterized. The hexameric capsomeres are 9.5 x 12.5

nm in longitudinal section; a channel 4 nm in diameter runs from the surface along the long axis. The tegument, a term introduced by Roizman and Furlong to describe the structures between the capsid and envelope, has no distinctive features in thin sections but may appear to be fibrous on negative staining. The thickness of tegument may vary, depending on the location of the virion within the infected cell. When the amount is variable, there is more of it in virions accumulating in cytoplasmic vacuoles than in those accumulating in the perinuclear space. The available evidence suggests the amount of tegument is more likely to be determined by the virus than by the host. The tegument is frequently distributed asymmetrically. The envelope of the virus has a typical trilaminar appearance; it appears to be derived from patches of altered cellular membranes. The presence of lipids was demonstrated by analyses of virions and by the sensitivity of the virions to lipid solvents and detergents. The herpesvirus envelope contains numerous protrusions of spikes, which are more numerous and shorter than those appearing on the surface of many other enveloped viruses. Wildy and Watson estimates that the spikes on HSV virions are approximately 8 nm long. The spikes consist of glycoproteins. The number and relative amounts of viral glycoproteins vary; HSV specifies at least 11 glycoproteins (Roizman, 1996)

The packaged HSV DNA is linear and double stranded. In the virion, HSV DNA is packaged in the form of a toroid. The ends of the genome are probably held together or are in close proximity in as much as a small fraction of the packaged DNA appears to be circular and a large fraction of the linear DNA circularizes rapidly in the absence of protein synthesis after it enters the nuclei of infected cells. DNA extracted from virions contains ribonucleotides, nicks, and gaps.

The size of HSV genome is approximately 150 kbp, with a G+C content of 68% for HSV-1 and 69% for HSV-2. (Morse, et al., 1978). The homology between the two type of HSV genome is about 50%. The HSV genome codes for approximately 100 proteins (Roizman, et al., 1975). Most of the polypeptides specified by one virus type are antigenically related to the polypeptides of the other type (Corey and Spear, 1986). They are designated as either infected cell specific polypeptides ($ICSP_S$) or infected cell

polypeptides (ICP_S). The three groups of HSV proteins, α, β and γ, are synthesized in a sequential order. There are five α-proteins namely ICP 0, 4, 22, 27 and 47. The synthesis of α-polypeptides reaches peak rate at approximately 2-4 hours post infection but some α-proteins continue to be produced throughout the period of infection (Honess and Roizman 1974). The β-proteins comprise two groups; β_1 and β_2. They reach peak rates of synthesis at about 5-7 hours post infection. The β_1 protein is synthesized earlier, its peak synthesis is overlapping slightly with α-class. β_1 proteins include the major DNA-binding protein and the large component of the viral ribonucleotide reductase (Huszar and Bacchetti, 1981). β_2 proteins are synthesized later, they include the viral thymidine kinase and DNA polymerase. All of these β-proteins involve in viral nucleic acid metabolism. The γ-proteins are primarily structural polypeptides and include the viral glycoprotein, capsid, and some tegument components. They are divided into two classes, γ_1 and γ_2. γ_1 proteins can be synthesized early in the absence of viral DNA replication. In contrast, the γ_2 protein are expressed late in the infection and absolutely require viral DNA replication.These γ-proteins also act as a major target for host immune response(Silver and Roizman, 1985).

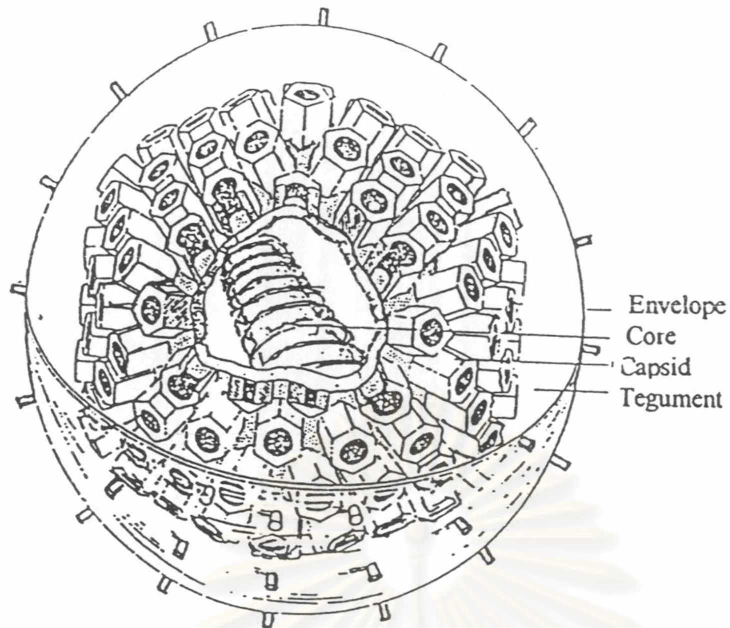

Figure 1. Diagram of a virion of herpes simplex virus (From Longson, 1990.)

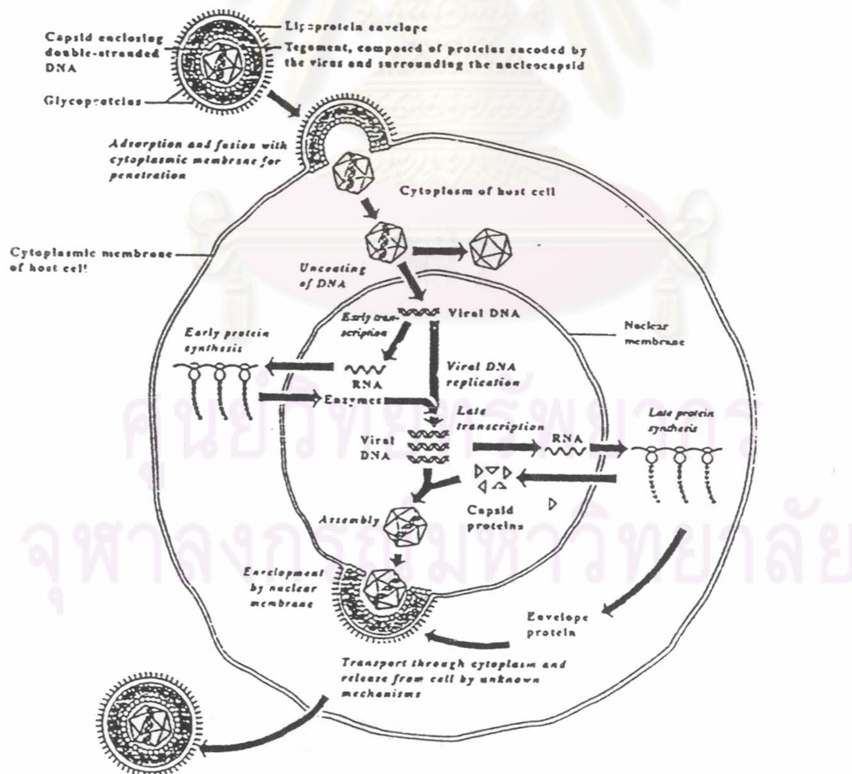

Figure 2. Diagram of replicative cycle of herpes simplex virus

(From Ackerman and Tremblay, 1988.)

3. Multiplication of herpes simplex virus

The envelope lycoproteins of the virus provide the normal attachment of the virions to susceptible cells. Following attachment, the viral envelope glycoprotein B (gp B) induces its fusion with the cellular plasma membrane, permitting the nucleocapsid to enter directiy into the cytoplasm. Intact virions may also enter via endocytosis, from which they are released into the cytoplasm by similar viral envelope-membrane fusion. In the cytoplasm the capsid migrates to a nuclear pore, where the viral DNA is released into the nucleus and initiates viral multiplication. The eclipse period is 5 to 6 hours in monolayer cell cultures, and virus increases exponentially until approximately 17 hours after infection; each cell has then made 10^4 to 10^5 physical particles, of which about 100 are infectious. Virions are released by slow leakage from the cells.

The biochemical events , as with other DNA-containing viruses are sequentially regulated, presenting a cascade like effect. Thus, after the viral DNA enters the nucleoplasm, even in the absence of protein synthesis, the host cell RNA polymerase II transcribes noncontiguous, restricted regions of the viral genome to produce five immediate early (α) mRNAs (Figure 2). If translation is blocked, only these α mRNAs accumulate in the cytoplasm, and the larger, unprocessed transcripts remain in the nucleus. If protein synthesis is permitted, α proteins are made, leading to transcription of other regions of the genome and production of delayed early (β) mRNAs. The β proteins block further synthesis of α proteins and lead to transcription of a third set of RNAs and their processing into late γ mRNAs.

Thus, the synthesis and translation of the mRNAs are coordinately regulated: formation of the α proteins is necessary for synthesis of the β proteins, and both of these nonstructural and minor structural proteins are necessary for synthesis of the late major structural γ proteins. It is noteworthy that synthesis of all the γ proteins is not dependent on viral DNA replication: γ1 proteins such as gp B and the major capsid protein VP5 are made in the absence of viral DNA synthesis, although they are synthesized in relatively low abundance; but γ2 proteins (e.g., glycoprotein C) strictly require amplification of

viral DNA. The three sets of mRNAs produce an aggregate of about 50 encoded proteins of virus.

DNA replication of virus is carried out by both viral α and β proteins, which include a DNA polymerase and DNA binding protein, and host cellular enzymes. The reactions are not yet precisely understood, but they appear to involve complex replicative intermediates, including "head-to-tail" concatemeric circular and linear-circular forms generated by the reiterated nucleotide sequences. Three origins of DNA replication have been described: two in the terminal *c* reiterated sequences of the S segment and one in the middle of the L segment near the genes encoding the DNA polymerase and DNA-binding protein. The concatemeric viral DNA is cleaved at a terminal reiterated *a* sequence, and it is packaged in preformed capsids.

These particles are noninfectious and unstable until they acquire an envelope. Envelopment is initiated at sites on the inner nuclear membrane into which viral glycoproteins have been inserted. The nuclear membrane reduplicates to permit egress of viral particles into the cytoplasm, where it associates with the endoplasmic reticulum, and the viral particle appears either to complete envelopment or to become enveloped anew. Mature, infectious virus is slowly liberated from infected cells through the endoplasmic reticulum, but occasionally it also escapes by a process to reverse phagocytosis.

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Unlike the process with other enveloped viruses, envelopment and release of viral particles does not occur by budding from the plasma membrane. Rather, the plasma membrane is changed morphologically and contains virus-specific glycoproteins; this consequently makes the membrane a target for the attack of immune response. The movement of viral particles from the nucleus into the cytoplasm is accompanied by the transport of soluble antigens into the cytoplasm; concomitantly the originally basophilic intranuclear inclusion body is converted into an eosinophilic, Feuigen-negative mass. Thus, the eosinophilic inclusion body that is usually observed in

infected cells does not contain viral particles or specific viral antigens (detectable by immunofluorescence) but actually is the burnt-out remnant of a viral factory (Ginsberg, 1980).

4. Pathology of herpes simplex virus infection

Pathologic changes are due to necrosis of infected cells together with the inflammatory response. because HSV causes cytolytic infections, Lesions induced in the skin and mucous membranes by HSV-1 and HSV-2 are the same and resemble those of varicella-zoster virus. Changes induced by HSV are similar for primary and recurrent infections but vary in degree, reflecting the extent of viral cytopathology.

The characteristic in histopathologic changes include ballooning of infected cells, production of Cowdry type A intranuclear inclusion bodies, margination of chromatin, and formation of multinucleated giant cells. The early inclusions virtually fill the nucleus but later condense and are separated by a halo from the chromatin at the nuclear margin. Cell fusion provides an efficient method for cell-to-cell spread for HSV, even in the presence of neutralizing antibody (Brooks, Butel, and Morse, 2001).

HSV is transmitted by contact of a susceptible person with an individual excreting virus.in primary Infection. The virus must encounter mucosal surfaces or broken skin in order for an infection to be initiated (unbroken skin is resistant). HSV-1 infections are usually limited to the oropharynx, and virus is spread by respiratory droplets or by direct contact with infected saliva. HSV-2 is usually transmitted by genital routes.

Due to infection with HSV-1 generally to oropharynx and is transmitted by direct contact of a susceptible individual with infected secretion . Thus initial replication of virus will occur in the oropharyngeal mucosa. The trigerminal ganglion becomes colonized and harbors latent virus. Acquisition of HSV-2 infection is usually the consequence of transmission by genital contact. Virus replicates in genital, perigenital and anal skin sites with seeding of the sacral ganglia. After the establishment of latency,

a recurrence of HSV is known as reactivated infection or recurrent infection. This form of infection leads to recurrent vesicular lesions of the skin such as HSV labialis or recurrent HSV genitalis. Reinfection with a different strain of HSV can occur.but uncommon in the normal host and is called exogenous reinfection(Brooks et al., 2001).

Primary HSV infections are usually mild; in fact, most are asymptomatic. Only rarely does systemic disease develop. Widespread organ involvement can result when an immunocompromised host is not able to limit viral replication and viremia ensues (Brooks et al., 2001).

Virus resides in latently infected ganglia in a nonreplicating state; only a very few viral genes are expressed in latent Infection. Viral persistence in latently infected ganglia lasts for the lifetime of the host. No virus can be recovered between recurrences at or near the usual site of recurrent lesions. Provocative stimuli can reactivate virus from the latent state, including axonal injury, fever, physical or emotional stress, and exposure to ultraviolet light. The virus follows axons back to the peripheral site; and replication proceeds at the skin or mucous membranes.

Spontaneous reactivations occur in spite of HSV-specific humoral and cellular immunity in the host. However, this immunity limits local viral replication, so that recurrent infections are less extensive and less severe. Many recurrences are asymptomatic, reflected only by viral shedding in secretions. When symptomatic, episodes of recurrent HSV-1 infection are usually manifested as cold sores (fever blisters) near the lip. More than 80% of the human population harbors HSV-1 in a latent form, but only a small portion experience recurrences.

Passively transferred maternal antibodies are acquired in many newborns These antibodies are lost during the first 6 months of life, and the period of greatest susceptibility to primary herpes infection occurs between ages 6 months and 2 years. Transplacentally acquired antibodies from the mother are not totally protective against infection of newborns, but they seem to ameliorate infection if not prevent it. HSV-1 antibodies begin to appear in the population in early childhood; by adolescence, they

are present in most persons. Antibodies to HSV-2 rise during the age of sexual activity and adolescence.

During primary infections, IgM antibodies appear transiently and are followed by IgG and IgA antibodies that persist for long periods. The more severe the primary infection or the more frequent the recurrences, the greater the level of antibody response. However, the pattern of antibody response has not correlated with the frequency of disease recurrence. Cell-mediated immunity and nonspecific host factors (natural killer cells, interferon) are important in controlling both primary infection and recurrent infections of HSV .After recovery from a primary infection (inapparent, mild, or severe), the virus is carried in a latent state in the presence of antibodies. These antibodies do not prevent reinfection or reactivation of latent virus but may modify subsequent disease (Brooks et al., 2001).

5. Epidemiology of herpes simplex virus infection

The distribution of herpes simplex viruses are worldwide . No animal reservoirs or vectors are involved with the human viruses. Transmission is by contact with infected secretions. The epidemiology of type 1 and type 2 herpes simplex virus differs. HSV-1 is probably more constantly present in humans than any other virus. Primary infection occurs early in life and is usually asymptomatic; occasionally, it produces oropharyngeal disease (gingivostomatitis in young children, pharyngitis in young adults). Antibodies develop, but the virus is not eliminated from the body; a carrier state is established that lasts throughout life and is punctuated by transient recurrent attacks of herpes infection.

The incidence of HSV-1 infection is highest among children 6 months to 3 years of age. By adulthood, 70-90% of persons have type 1 antibodies. Middle-class individuals in developed countries acquire antibodies later in life than those in lower socioeconomic populations. Presumably this reflects more crowded living conditions and poorer hygiene among the latter. The virus is spread by direct contact with infected saliva or through utensils contaminated with the saliva of a virus shedder. The source of infection for children is usually an adult with a symptomatic viral shedding in saliva. The

frequency of recurrent HSV-1 infections varies widely among individuals. At any given time, 1-5% of normal adults will be excreting virus.

Usually, HSV-2 is acquired as a sexually transmitted disease, so antibodies to this virus are seldom found before puberty. It is estimated that there are about 45 million infected individuals in the USA. Antibody prevalence studies have been complicated by the cross-reactivity between HSV types 1 and 2. Surveys using type-specific glycoprotein antigens recently determined that 20% of adults in the USA possess HSV-2 antibodies, with seroprevalence higher among women than men and higher among blacks than whites. Recurrent genital infections may be symptomatic or asymptomatic. Either situation provides a reservoir of virus for transmission to susceptible persons. HSV-2 tends to recur more often than HSV-1.

Maternal infections of genital HSV pose risks to both mother and fetus. Rarely, pregnant women may develop disseminated disease after primary infection, with a high mortality rate. Primary infection before 20 weeks of gestation has been associated with spontaneous abortion. The fetus may acquire infection as a result of viral shedding from recurrent lesions in the mother's birth canal at the time of delivery. Estimates of the frequency of cervical shedding of virus among pregnant women vary widely. However, the majority of infants (≈ 70%) who develop neonatal disease are born to women who do not have a history of genital herpes and are asymptomatic at the time of delivery (Brooks et al., 2001).

Genital herpes can also be due to HSV-1 infection . Both HSV-1 and HSV-2 are able to infect and reactivate in the same anatomic area, although the natural history of these infections is markedly different, with HSV-2 recurring more frequently than HSV-1, so most clinical reactivations are likely to be due to HSV-2 (Brooks et al., 2001).

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6. *In vitro* method for antiviral test

The methodology used in the determination of the antiviral activity as well as the interpretation of the results have been virtually specific to each laboratory and are consequently not comparable to one another, simple procedures and guidelines for evaluating antiviral and/or virucidal activities of single compounds are urgently needed. This is even more true for the antiviral testing of crude extracts, containing a complicated mixture of different compounds present in various proportions.

Many cell culture-based assays are currently available and can be successfully applied for the antiviral or virucidal determination of single substances or mixtures of compounds e.g. plant extracts. Antiviral agents interfere with one or more dynamic processes during virus biosynthesis and are consequently candidates as clinically useful antiviral drugs, whereas virucidal substances inactivate virus infectivity extracellularly and are rather candidates as antiseptics, exhibiting a broad spectrum of germicidal activities.

The method commonly used for evaluation of *in vitro* antiviral activities are based on the different abilities of viruses to replicate in cultured cells. Some viruses can cause cytopathic effects (CPE) or form plaques. Others are capable of producing specialized functions or cell transformation. Virus replications in cell cultures may also be monitored by the detection of viral products, i.e. viral DNA, RNA or polypeptides. Thus, the antiviral test selected may be based on inhibition of CPE, reduction or inhibition of plaque formation and reducing virus yield or other viral functions (Vlietinck and Berghe, 1991).

The various *in vitro* antiviral tests and their possible suitability for the antiviral and/or virucidal screening of plant extracts and plant products is presented in Table 1.

It should be emphasized that the toxic effects of an antiviral agent on the host cells must be considered since a substance may exhibit an apparent antiviral activity by virtue of its toxic effects on the cells. The cytotoxicity assay on cell cultures is usually done by the cell viability assay and the cell growth rate test, although other parameters

such as destruction of cell morphology under microscopic examination or measurement of cellular DNA synthesis have been used as indicators of toxicity.

The therapeutic index of the antiviral compound in a given virus-cell system can be calculated after the antiviral potency of a test substance together with its cytotoxicity is determined. Taking into account that the cell growth rate test has been claimed to be the most stringent method for measuring cytotoxicity, the therapeutic index (TI_x) can be defined as the ratio of the maximum drug concentration at which 50% of the growth of normal cells is inhibited (CD_{50}) to the minimum drug concentration at which *x*% (50%, 90% or 99%) of the virus is inhibited (ED_{50}).

It should be noted that the calculation of the therapeutic index of a mixture of compounds e.g. crude extracts is irrelevant since cytotoxicity and antiviral activity of the mixture are not necessarily due to the same components of the mixture. On the contrary, without the cytotoxicity data reports of antiviral activity of a single compound even at very low concentrations are of limited value. In addition, the relative potency of a new antiviral product should also be compared with the approved drugs.

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Table 1 *In vitro* antiviral screening assays (Vlietinck and Berghe, 1991)

Determination of the viral infectivity in cultured cells during virus multiplication in the presence of a single compound (A-S) or a mixture of compounds e.g. plant extracts (A-M) or after extracellular incubation with a single compound (V-S) or a mixture of compounds (V-M).

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(1) *Plaque inhibition assay*

Only for viruses which form plaques in suitable cell systems.

Titration of a limited number of viruses in the presence of a non-toxic dose of the test substance.

Applicability: A-S.

(2) *Plaque reduction assay*

Only for viruses which form plaques in suitable cell systems.

Titration of residual virus infectivity after extracellular action of test substance(s). Cytotoxicity should be eliminated e.g. by dilution, filtration etc. before the titration.

Applicability: V-S; V-M.

(3) *Inhibition of virus-induced cytopathic effect (CPE)*

For viruses that induce CPE but do not readily from plaques in cell cultures.

Determination of virus-induced CPE in monolayers, cultures in liquid medium, infected with a limited dose if virus and treated with a non-toxic dose of the test substance(s).

Applicability: A-S; A-M.

(4) *Virus yield reduction assay*

Determination of the virus yield in tissue cultures, infected with a given amount of virus and treated with a non-toxic dose of the test substance(s).

Virus titration is carried out after virus multiplication by the plaque test (PT) or the 50% tissue culture dose end point test (TCD50).

Applicability: A-S; A-M.

(5) *End point titration technique (EPTT)*

Determination of virus titer reduction in the presence of two-fold dilutions of test compound(s).

Applicability: A-S; A-M. This method has been especially designed for the antiviral screening of crude extracts.

(6) *Assays based on measurement of specialized functions and viral products*

For viruses that do not induce CPE or form plaques in cell cultures.

Determination of virus specific parameters e.g. hemagglutination and hemadsorption tests (myxoviruses), inhibition of cell transformation (EBV), immunological tests detecting antiviral antigens in cell cultures (EBV, HIV, HSV and CMV).

Reduction or inhibition of the synthesis of virus specific polypeptides in infected cell cultures e.g. viral nucleic acids, determination of the uptake of radioactive isotope labeled precursors or viral genome copy numbers.

Applicability: A-S; A-M; V-S; V-M.

A: antiviral; V: virucidal; S: single substances; M: mixtures of compounds

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Generally, plaque reduction/inhibition assay is considered as a reference or standard for antiviral assay (Ellis, et al. 1987). This assay uses a constant number of viral particles and varying the non-toxic concentrations of test substance (Abou-Karam and Shier, 1990). Typically, a monolayer of cultured cells is allowed to bind virus and then overlaid with a layer of semi-solid medium which prevents spreading of virus from the area of originally infected cells. The test substance can be added into cell monolayers before or after virus adsorption is accomplished. The infected cultures are incubated further for an appropriate period of time, then they are fixed, stained with dye, and plaques (areas of infected cells) are counted. By reference to the number of plaques observed in virus control (untreated culture), the effective concentration or dose of test compound which inhibited plaque number by 50% (EC_{50}, ED_{50}, IC_{50} or ID_{50}) is calculated and expressed

Histochemical staining for plaque assay using a cell line, Vero ICP6LacZ#7, that expresses β-galactosidase activity was developed.(Tebas, et al.,1995) Antiviral compound was added into HSV-infected cell monolayers, followed by pooled human immunoglobulin which limits the spread of virus to surrounding cells Tebas, et al.,(1995) developed. Two days later, the cell monolayers were stained for β-galactosidase activity. The plaques appeared blue against a clear background of unstained, uninfected cells. The EC_{50} determined by this method correlated with that determined by the plaque inhibition assay. The procedure of the CPE inhibition assay is similar to the plaque inhibition assay except the semi-solid substance is not included in the culture medium. The infectivity of virus could be revealed through microscopic observations of characteristics of viral CPE (Yip et al., 1991). or determined by the dye uptake assay (Marchetti, et al 1996). Treated and untreated infected cells are stained with neutral red 2 days post-infection. The uptake dye is then extracted and the optical density at 550 nm measured. Then, the concentration required to inhibit CPE by 50% is calculated.

In virus yield inhibition assay, cell monolayers are infected with virus and serial dilution of test compound are added after virus adsorption. Following a cycle of virus replication, the harvested cultures are disrupted by freezing and thawing, supernatants

are kept, and virus yields are determined by plaque assay. The drug concentrations required to reduce 90% (1 $\log_{10}$ reduction) are determined for antiviral activity in this assay. The plaque inhibition and CPE inhibition methods have limitations from the low or limited amount of virus input so virus yield reduction assay have been used instead. The multiplicity of infection (MOI) of virus in yield reduction assay is usually carried out with one or more PFU per cell. Thus, progeny virus could be recovered at a much greater range, i.e., 1-10^6 PFU/ml in yield reduction compared to 1-10^2 PFU in plaque reduction/inhibition assay (Prichard, et al 1990). However, yield reduction assay is not routinely utilized due to its labor-intensive nature and time consuming. The antiviral assays based on measurement of specialized functions and viral products have been studied using methods such as flow cytometric analysis , nucleic acid hybridization, and enzyme-linked immunosorbent assay (ELISA).

7. Antiherperpes virus agents

Three categories of antiherpesvirus agents can be discerned (Andrei et al., 1995). The first category consists of some pyrophosphate analogs such as phosphonoacetic acid (PAA) and phosphonoformic acid (PFA; foscarnet). These drugs inhibit a viral DNA polymerase directly by binding to the site involved in releasing the pyrophosphate product of DNA synthesis. The second category comprises a variety of nucleoside analogs including acyclovir (ACV), ganciclovir (GCV), brivudin (BVDU), and penciclovir (PCV). These agents depend on virus-induced thymidine kinase (TK) to affect their antiviral action. Formation of monophosphate (for BVDU, also further phosphorylation to the diphosphate) forms of these nucleoside analogs is catalyzed by viral TK. Following further phosphorylation to their triphosphate forms by cellular enzymes, they inhibit the DNA polymerase reaction. The third class consists of those drugs that are independent of viral TK for their activation. This class includes vidarabine (araA) and the acyclic nucleoside phosphonates, i.e., phosphonylmethoxyethyl (PME) and 3-hydroxy-2-phosphonylmethoxypropyl (HPMP) derivatives of purines (i.e., adenine [PMEA and HPMPA] and 2,6-diaminopurine [PMEDAP]) and pyrimidines (i.e., cytosine [HPMPC]). AraA is converted to araA triphosphate by cellular enzymes and then inhibits

HSV DNA polymerase. Phosphorylation of acyclic nucleoside phosphonates to their mono- and diphosphoryl derivatives is also carried out by cellular enzymes, and then the resulting diphosphoryl derivatives interact at the DNA polymerase level Also, the novel N-7 isomeric acyclic nucleoside 2-amino-7-(1,3-dihydroxy-2-propoxymethyl)purine (S2242) has proved to be a potent and selective inhibitor of HSV replication, acting independently of virus-induced TK ((Balzarini and De Clercq, 1991,. Neyts et al., 1994).

Acyclovir (ACV), an acyclic guanosine analogue, is a selective inhibitor of the replication of HSV types 1 and 2 and varicella-zoster virus. In virally- infected cells, it is initially monophosphorylated by HSV-specific viral thymidine kinase (TK), then converted to its di- and triphosphate forms by cellular enzymes. The active form is acyclovir triphosphate , which lacks the 3'-hydroxyl group required to elongate the DNA chain. Acyclovir triphosphate competes with deoxyguanosine triphosphate as a substrate for viral DNA polymerase, thereby terminating viral DNA replication. This results in inactivation of viral DNA polymerase (Figure 3, Scholar and Pratt, 2000). Acyclovir is a highly selective inhibitor of HSV replication. The concentration required for 50% inhibition of Vero cell growth is 300 µM and for WI-38 human fibroblast it is greater than 3000 µM. This highly selective toxicity is based on a large degree of selective activation of the drug by the viral TK.

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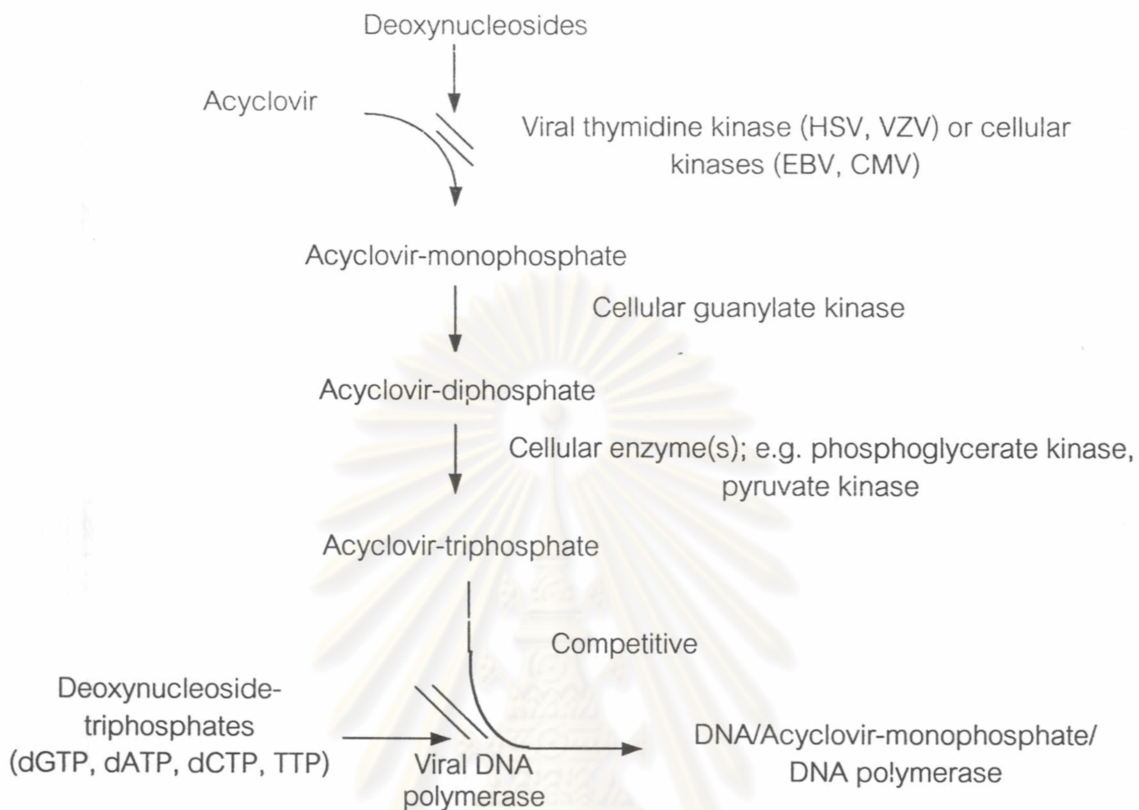

Figure 3 Acyclovir inhibition of viral DNA synthesis

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HSV-2 strains generally are *in vitro* less susceptible to acyclovir than HSV-1 strains, although there nas been considerable overlap in the range of concentrations reported to inhibit the CPE of these 2 types of herpes simplex viruses by 50% (ID_{50} as measured by the plaque reduction assay or reduction in viral-induced cytopathogenicity) (O'Brien et al., 1989). As reviewed by Richards et al. (1983), in most studies ID_{50}s have been in the range 0.01 to 0.7 mg/l for herpes simplex type 1, and 0.01 to 3.2 mg/l for herpes simplex type 2. The 10- to 100-fold reduction in susceptibility of herpes simplex viruses growing in African green monkey kidney (Vero) cells compared with that in human fibroblast cells appears to be due, at least in part, to the substantially higher concentrations of thymidine in Vero cells ((Harmenberg et al., 1980, Harmenberg et al., 1985)

8. **Antiviral activity of medicinal plants**

Medicinal plants have a long history of use and their use is widespread in both developing and developed countries. Herbal medicines provide rational means for the treatment of many diseases that are obstinate and incurable in other systems of medicine. These are gaining popularity because of several advantages such as often fewer side effects, better patient tolerance, relatively expensive and acceptance due to long history of use. Medicinal effects of plants tend to normalize physiological function and correct the underlying cause of the disorder. (Murray and Pizzomo, 1999). Medicinal plants are renewable in nature unlike the synthetic drugs that are obtained from non-renewable sources of basic raw materials such as fossil soueces and petrochemicals (Samanta et al., 2000). Cultivation and processing of plants often is environment friendly unlike the pollution by chemical industry. Cultivation of medicinal plants can also be a source of income for poor families. Many of the medicinal plants are locally available, especially in developing and underdeveloped countries. Also, plants are often less prone to the emergence of drug resistance/ Due to all these advantages. Plants continue to be a major source of new lead compounds.

Many traditional medicinal plants have been reported to have strong antiviral activity and some of them have already been used to treat animals and people who suffer from viral and influenza virus (Ahmad et al., 1996). The *Bergienia ligulata*, *Nerium indicum* and *Holoptelia integnifolia* exhibited considerable antiviral activities against influenza virus and HSV .(Jassim and Naji, 2003). infection. The examples of these medicinal plants are presented. Mycianthes showed in vitro anti-RSV and not anti-HSV-1 or anti-adenovirus serotype 7. The *Azadirachta indica* leaf extract was found to be active against a number of viruses such as smallpox , chickenpox,poxvirus, poliovirus and herpes viruses. An extract of the cactus plant *Opuntia streptacantha* inhibited intracellular DNA and RNA virus replication and inactivated extracellular virus, such as HSV, equine herpes virus, pseudorabies virua

A wide variety of active phytochemicals including the flavonoids, terpenoids, lignans, sulphides, polyphenolics, coumarins, saponins, furyl compounds, alkaloids, polyines, thiopenes, proteins and peptides have been identified,. Some volatile essential oils of commonly used culinary herbs, spices and herbal teas have also exhibited a high level of antiviral activity. However, given the few classes of compounds investigated, most of the pharmacopoeia of compounds in medicinal plants with antiviral activity is still not known. Several of these phytochemicals have complementary and overlapping mechanisms of action, including the activity of viral reproduction. Assays methods to determine antiviral activity include multiple-arm trials, randomized crossover studies, and more compromised designs such as nonrandomized crossovers and pre- and post-treatment analyses. Methods are needed to link antiviral efficacy/potency- and laboratory-based research. Nevertheless, the relative success achieved recently using medicinal plant/herb extracts of various species that are capable of acting therapeutically in various viral infections has raised optimism about the future of phyto-viral agents. Ther are innumerable potentially useful medicinal plants and herbs waiting to be evaluated and exploited for therapeutic applications against genetically and functionally diverse viruses families such as Retroviridae.(Jassim and Naji, 2003).

Medicinal plants are used in vaginal formulations. Viracea, a proprietary formula of Destiny BioMediX Cooperation, is a topical microbicide consisting of benzalkonium chloride and phytochemicals derived from *Echinacea purpurea.* Viracea has been reported to possess antiviral activity against acyclovir resistance as well as susceptible strains of HSV-1 and HSV-2(Thompson, 1998). Praneem polyhedral cream, tablets and suppositories are under clinical development and possess wide spectrum antibacterial, antifungal and antiviral effects against sexually transmitted pathogens. Praneem contains purified extract of A. *indica* (neem) and saponins extracted from *Sapindus mukerrossi* . Intravaginal inoculation of these formulations prevented lesions and vaginal transmission of HSV-2 and *C. trachomatis* in progestin-sensitized mice. In addition, they have also been found to possess virucidal activity against HIV at doses non-toxic to cells in culture. Praneem polyherbal had completed phase I safety and acceptability trials in India. (Vermani and Garg, 2002).

There is considerable interest in developing topical microbicides, products to be used intravaginally by women for protection against sexually transmitted diseases. Many compounds derived from plants have been shown to have antimicrobial properties. Nineteen compounds were examined in vitro by plaque reduction assay to determine their activity against a common sexually transmitted pathogen, herpes simplex virus type2. Four compounds, carrageenan , cineole , and eugenol provided significant protection in a mouse model of intravaginal HSV-2 challenge.(Bourne et al., 1999). The antiviral activity of eugenol may be due to the phenolic nature of the compound. Polyphenolic complexes have been shown to damage protein envelopes in newly synthesized HSV virions (Serkedjieva and Manolova, 1992). Eugenol also has capsaicin-like properties that might contribute to its in vivo anti-HSV activity. Like capsaicin, eugenol activates C-type sensory nerve fibers, has antinociceptive properties, and activates a Ca+2-permeable ion channel in dorsal root ganglion neurons . Capsaicin, while lacking in vitro anti-HSV activity, has been shown to protect animals from genital HSV disease (Stanberry, 1990). and

from HSV infection of the central nervous system. It has been hypothesized that capsaicin alters the pathogenesis of HSV infection by interfering with essential virus-neuron interaction (Stanberry, 1990).

Various herbs used topically for genital herpes have been reported. Those are aloe vera, lemon balm (Melissa officinalis), cloves, eucalyptus, licorice root (*Glycyrrhiza globra*), seaweed, turmeric, olive leaf extract, *Prunella vulgaris* (Wolberg and Leonhardt, 1994, Bourne et al., 1999, Lee et al., 1999)

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Table 2 List of medicinal plants with antiviral activities against herpes simplex viruses

Medicinal plants	Family	Type of extracts	Reference
1. *Achyrocline flaccida*	Asteraceae	EtOH, aqueous	Garcia et al., 1995; 1999
2. *Adansonia digitata*	Bombacaceae	MeOH	Anani et al., 2000.
3. *Annona cherimola*	Annonaceae	MeOH	Betancur-Galvis et al., 1999.
4. *Annona muricata*	Annonaceae	EtOH MeOH	Padma et al., 1998; Betancur-Galvis et al., 1999
5. *Artemisia princeps*	Compositae	aqueous	Kurokawa et al., 1996.
6. *Atalantia monophylla*	Rutaceae	$CHCl_3$	Chansakaow, et al., 1996.
7. *Baccharis genistelloides*	Asteraceae	aqueous	Abad et al., 1999a.
8. *Baccharis trinervis*	Asteraceae	aqueous	Abad et al., 1999b.
9. *Bauhinia vahlii*	Fabaceae	MeOH	Taylor et al., 1996.
10. *Beta vulgaris*	Chenopodiaceae	aqueous	Betancur-Galvis et al., 1999.
11.*Callisia gracilis*	Commelinaceae	EtOH	Betancur-Galvis et al., 1999.
12. *Cardamine angulata*	Cruciferae	MeOH	McCutcheon et al., 1995.
13. *Carissa carandas*	Apocynaceae	MeOH	Taylor et al., 1996.
14. *Centella asiatica*	Umbelliferae	aqueous, EtOH	Yoosook et al., 2000
15. *Cerbera odollam*	Apocynaceae	aqueous-hexane	Lipipun,et al., 1999
16. *Clausena excavata*	Rutaceae	CHCl3	Lipipun et al., 1999
17. *Clerodendron myricoides*	Verbenaceae	EtOH	Vlietinck et al., 1995.
18. *Coleus amboinicus*	Labiatae	$CHCl_3$	Lipipun et al., 1999
19. *Conocephalum conicum*	Conocephalaceae	MeOH	McCutcheon et al., 1995.
20. *Conyza aegyptiaca*	Asteraceae	MeOH	Anani et al., 2000.
21. *Crassocephalum rymbosamulticorymbosum*	Asteraceae	EtOH	Vlietinck et al., 1995.
22.*Cryptolepis sanguinolenta*	Periplocaceae	EtOH	Cimanga et al., 1996.
23. *Cynometra cloiselii*	Fabaceae	EtOH	Hudson et al., 2000.
24. *Cynometra madagascariensis*	Fabaceae	EtOH	Hudson, 2000.
.25 *Dittrichia viscosa [Inula viscosa]*	Asteraceae	aqueous	Abad et al., 2000.
26 .*Dryopteris inaequalis*	Asteraceae	EtOH	Vlietinck et al., 1995.
27. *Erythrina abyssinica*	Fabaceae	EtOH	Vlietinck et al., 1995.

EtOH=ethanol, MeOH=methanol, $CHCl_3$=chloroform

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Table 2 (continued)

Medicinal plants	Family	Type of extracts	Reference
28. *Evonymopsis longipes*	Celastraceae	MeOH	Nawawi, et al., 1999.
29. *Filicium decipiens*	Sapindaceae	EtOH	Hudson , 2000.
30. *Gamochaeta simplicicaulis*	Asteraceae	MeOH	Nawawi, et al., 1999.
31. *Garcinia mangostana*	Guttiferae	aqueous	Cavallaro et al., 1995.
32. *Geranium sanguineum*	Geraniaceae	MeOH	Nawawi, et al., 1999.
33. *Glycine javanica*	Fabaceae	aqueous	Serkedjieva and Ivancheva, 1999.
34. *Hamamelis virginiana*	Hamamelidaceae	EtOH	Vlietinck et al., 1995.
35. *Heisteria acuminata*	Olacaceae	EtOH	Erdelmeier et al., 1996.
36. *Helichrysum aureonitens*	Asteraceae	EtOH	Abad et al., 1999b.
37. *Holoptelia integrifolia* (*Holoptelea integrifolia*)	Ulmaceae	aqueous,	Meyer et al., 1996; Afolayan and Meyer, 1995
38. *Houttuynia cordata*	Saururaceae	steam distillate	Hayashi et al., 1995.
39. *Hypericum cordifolium*	Clusiaceae	MeOH	Taylor et al., 1996.
40. *Lithraea molleoides*	Anacardiaceae	aqueous	Kott et al., 1999.
41. *Lychnis coronata*	Caryophyllaceae	fresh leaf juice	Yue-Yu et al., 1998.
42. *Lysichiton americanum*	Araceae	MeOH	McCutcheon et al., 1995.
43. *Machilus thunbergii*	Lauraceae	aqueous	Kurokawa et al., 1996.
44. *Maclura cochinchinensis*	Moraceae	ethyl acetate, MeOH,aqueous	Bunyapraphatsara et al., 2000
45 *Maesa lanceolata*	Maesaceae (Myrsinaceae)	MeOH	Sindambiwe et al., 1998; 1999
46. *Mallotus philippensis*	Euphorbiaceae	MeOH	Taylor et al., 1996.
47. *Mangifera indica*	Anacardiaceae	aqueous	Yoosook et al., 2000
48. *Markhamia lutea*	Bignoniaceae	EtOH	Vlietinck et al., 1995.
49. *Melia azedarach*	Meliaceae	aqueous	Claus et al. , 1998.
50. *Millettia extensa*	Fabaceae	MeOH	Taylor et al., 1996.
51 *Nepeta coerulea*	Lamiaceae	aqueous	Abad et al., 2000.
52. *Nepeta nepetella*	Lamiaceae	aqueous	Abad et al., 2000.

EtOH=ethanol, MeOH=methanol, $CHCl_3$=chloroform

Table 2(continued)

Medicinal plants	Family	Type of extrracts	Reference
53. *Palisota hirsuta*	Commelinaceae	MeOH	Anani et al., 2000.
54. *Persea americana*	Lauraceae	aqueous	Almeida et al., 1998; Miranda et al., 1997
55. *Petunia axillaris* (*P. nyctaginiflora*)	Solanaceae	aqueous	Padma et al., 1998.
56. *Phyla nodiflora*	Verbenaceae	hexane	Lipipun et al., 1999
57. *Phyllanthus orbicularis*	Euphorbiaceae	aqueous	Barrio, 2000.
58. *Piliostigma thonningii*	Fabaceae	MeOH	Cristoni et al., 1996.
59. *Plectranthus hereroensis*	Lamiaceae	acetone	Batista et al., 1995.
60. *Polygonum glycyrrhiza*	Polygonaceae	MeOH	McCutcheon et al., 1995.
61. *Polygonum punctatum*	Polygonaceae	aqueous	Kott et al., 1999.
62. *Prunella vulgaris*	Lamiaceae	aqueous	Hong-Xi et al., 1999.
63. *Psychotria serpens*	Rubiaceae	EtOH	Kuo et al., 2001.
64. *Punica granatum*	Punicaceae	MeOH, aqueous	Nawawi, et al., 1999.
65. *Ravensara retusa*	Lauraceae	EtOH	Hudson,, 2000.
66. *Rhus vulgaris*	Anacardiaceae	EtOH	Vlietinck et al., 1995.
67. *Rumex hastatulus*	Polygonaceae	MeOH	Taylor et al., 1996.
68. *Sanguisorba minor*	Rosaceae	aqueous	Abad et al., 2000.
69. *Satureja boliviana*	Lamiaceae	aqueous	Abad et al., 1999a
70. *Sebastiania brasiliensis*	Euphorbiaceae	aqueous	Kott et al., 1999.
71. *Sebastiania klotzschiana*	Euphorbiaceae	aqueous	Kott et al., 1999.
72. *Spatholobus suberectus*	Papillionaceae	aqueous	Kurokawa et al., 1996.
73. *Stephania cepharantha*	Menispermaceae	MeOH	Nawawi, et al., 1999.
74. *Streblus asper*	Moraceae	MeOH	Taylor et al., 1996.
75. *Terminalia alata*	Combretaceae	MeOH	Taylor et al., 1996.
76. *Terminalia monoceros*	Combretaceae	MeOH	Hudson,, 2000.
77. *Thevetia peruviana*	Apocynaceae	$CHCl_3$	Lipipun, et al., 1999
78. *Toona sureni*	Meliaceae	MeOH	Nawawi, et al., 1999.
79. *Tridax procumbens*	Asteraceae	MeOH	Taylor et al., 1996.
80. *Zanthoxylum bungeanum*	Rutaceae	aqueous	Kurokawa et al., 1996

EtOH=ethanol, MeOH=methanol, $CHCl_3$=chloroform