The Sensitivity of Adenovirus and Herpes simplex virus to Honey

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Abstract

Honey has been used for centuries as a medicine to treat various ailments and infections. A large amount of research has established that honey has potent antibacterial activity. However, the sensitivity to honey of viral species that cause infections has been studied in only a small number of cases. The aim of this study was to obtain data to clarify and extend knowledge obtained from these previous studies of honey's antiviral activity, and especially study those viruses that cause localised infections which have limited or no therapy available, which are suitable to treatment with topically applied honey.

The susceptible A549 cell line and viral isolates of Adenovirus serotypes 1, 3, and 8, and Herpes simplex virus serotypes 1 and 2, were provided by the Waikato Hospital Virology Laboratory. A number of types of honey were investigated from a range of sources: Manuka honey with high concentrations of methylglyoxal, "unique manuka factor" activity, and phenolics, Honeydew and Rewarewa honeys which have high antioxidant activity, and Ling Heather honey which is high in phenolic compounds. These honeys were selected due to their range of characteristic activities in order to make comparisons with antiviral activity.

A variety of tests using cell culture were developed to evaluate the sensitivity of the viruses to whole honey. Each test scored and monitored the development of morphological changes to the cells, to observe whether the honey treatment can prevent the development of these changes known as viral cytopathic effect. These included tests for: protection, in which the cells were pre-treated with, and

incubated either with or without honey; prevention, where honey was used to treat infected cells, and in plaque reduction assays, to examine whether it can reduce the resultant number of plaques; and neutralisation, in which the virus was directly exposed to the honey for a defined period.

It was found with each type of test using cell culture that many of the honeys studied can lower the severity of viral cytopathic effect or delay its onset compared with the development observed with virus that was not treated with honey. This can suggest that the antiviral activity may be a feature of more than one type of honey. In general the antiviral effect increased with the concentration of honey and time the virus was exposed to it. Manuka honey M116 at a concentration of 10% was effective in preventing the development of viral cytopathic effect of each of virus, after the viruses at concentrations in excess of the tissue culture infectious dose had been exposed to the honey for 8 hours.

Enzyme-linked immunosorbant assays were used to measure the effect the successful treatments found in the extended neutralisation experiments had on viral surface proteins necessary for viral entry into the cells. The results using this technique suggested that there was very little virus present in the samples that had been treated with honey and with the untreated virus. Therefore it could not be shown whether the honey was acting via this mechanism.

It is concluded from the findings in this study that honey is likely to be an effective antiviral treatment for the therapy of localised viral infections, this needs to be verified by clinical trials.

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Chapter 1

Introduction and Literature review

This chapter aims to introduce the research area of this thesis, outlining the traditional and more recent therapeutic properties of honey. It describes honey's established antimicrobial activity, as well as methylglyoxal the speculative causative antimicrobial agent present in especially high levels in Manuka honey. It finally reports on previous investigations of honey's antiviral activity and gives statements of the hypotheses and aims of this study.

Many viruses cause localised infections, few of which have therapeutic treatments available for the elimination of the infection or alleviation of the symptoms of the infection. Studies have indicated that honey may have antiviral activity, which has raised the possibility of honey being an effective treatment for such infections caused by viruses. Much research has been done on the antibacterial activity of honey but little on its antiviral activity. Therefore this study has been undertaken to investigate honeys' antiviral capacity.

1.1 HONEY

1.1.1 Traditional to more recent uses of honey

Honey has been used for centuries as a traditional medicine for a range of ailments, Aristotle (384-322 BC) referred to pale honey being a "good salve for sore eyes and wounds". The ancient Egyptians, Assyrians, Chinese, Greeks and Romans all used honey in combination with other ingredients and alone to treat wounds and infections of the gut (Zumla & Lulat, 1989). In 50 AD, Dioscorides described honey as being "good for all rotten and hollow ulcers" and "good for sunburn and spots on the face" (Gunther, 1934). He further wrote that "honey heals inflammation of the throat and tonsils, and cures coughs." The use of honey has been sustained into present day folk medicine. In India, lotus honey was said to be a panacea for diseases of the eye (Fotidar & Fotidar, 1945) and it has further been reported that honey can be used to treat eyes discharging pus (Meier & Freitag, 1955). Traditionally honey has been used to treat colds and coughs (Beck & Smedley, 1944). Honey also has a history of being used as therapy for infected leg ulcers (Ankra-Badu, 1992), for earache (Obi *et al.*, 1994) and in the eyes in measles to prevent corneal scarring (Imperato & Traore, 1969).

The dominance of "superbugs", those species of bacteria that contain multiple antibiotic-resistance genes, has led to a rediscovery of the therapeutic use of old remedies, including a re-evaluation of honey (Zumla & Lulat, 1989).

More recently, it has been reported that honey has an inhibitory action against about 60 species of bacteria including both anaerobic and aerobic as well as both gram positive and negative (Molan, 1992a). Antifungal activity in honey has been noted for some yeasts (Molan, 1992a) and dermatophytes (Brady *et al.*, 1997).

1.1.2 Constituents, antimicrobial, and antioxidant activity of honey

It has been established through the volume of published literature that honey has significant antibacterial activity which has been comprehensively reviewed by Molan (1992a, 1992b).

The antibacterial property of honey was firstly observed in 1892, by van Ketel (Dustmann, 1979). It was assumed that this antibacterial activity was entirely due to the osmotic effect of honey, due to its high sugar content (Bose, 1982; Chirife *et al.*, 1983; Condon, 1993; Keast-Butler, 1980; Mossel, 1980; Seymour & West, 1951; Somerfield, 1991; Tovey, 1991). Honey like other saturated sugar solutions has an osmolarity capable of inhibition of microbial growth (Chirife et al., 1983). If used to treat a wound, the wound exudate would dilute the osmolarity and cease the healing, however, it was found that upon dilution, the antibacterial action increased beyond the point where osmolarity would have been inhibitory (Cooper *et al.*, 1999). It was first observed and reported that the dilution of honey

increased antibacterial activity in 1919 (Sackett, 1919). The explanation of this, that an enzyme within honey produces hydrogen peroxide when diluted was founded by White (1963). Hydrogen peroxide is a well known antimicrobial agent. It has been used for its antibacterial and cleansing properties in clinical practice (Turner, 1983). Hydrogen peroxide is, however, harmful to tissues, causing inflammation and damage (Saissy *et al.*, 1995). The level of hydrogen peroxide in honey produced after dilution is typically 1000 times less than a 3% solution commonly used as an antiseptic. The effects of hydrogen peroxide are more greatly reduced due to the sequestration and inactivation of free iron, which catalyses the formation of oxygen free radicals by hydrogen peroxide (Bunting, 2001), as well as the ability to mop up oxygen free radicals (Frankel *et al.*, 1998). It is possible that the antioxidant activity of honey may have an antiviral effect by decreasing the activation of NFκB. Although the hydrogen peroxide level is low, it is still effective as an antimicrobial agent.

In some honeys an additional antibacterial agent can be revealed by the removal of hydrogen peroxide by catalase (Adcock, 1962; Allen *et al.*, 1991; Bogdanov, 1984; Molan & Russell, 1988; Roth *et al.*, 1986). Usually such activity is at a low level, but in Manuka honey it was found to be at a high level (Allen et al., 1991). This component has become known as the unique manuka factor (UMF). The level of this antibacterial activity can however show variation between honeys. Molan and Russell (1988) found a range of honey samples that showed varying additional activity from nil to almost whole of the activity of some Manuka honeys. They also observed a correlation between the level of additional

antibacterial activity and the overall antibacterial activity of individual honey samples. Very recently, it has been proposed that methylglyoxal described in Section 1.2 is the UMF as reported by Henle (2008) and Adams (2008).

In addition to hydrogen peroxide and additional non-peroxide, antibacterial activity, the glucose content, and the acidic pH, may be aiding the increased lymphocyte and phagocytic activity of honey (Ryan & Manjno, 1977).

1.1.3 Ailments and infections that can be treated with honey

Honey has shown to be an effective therapeutic agent against a range of ailments and similarly a range of infections. Many studies have shown the ability of honey to hasten the healing time of wounds and burns, where honey has been used as a dressing to treat a range of wounds as reviewed by Molan, 2006.

Honey has shown to quicken the healing time of superficial to more severe burns compared with the control treatment silver sulfadiazine (reviewed by Molan, 2006).

With regard to the healing of wound infections, honey has shown with severe postoperative abdominal surgical wounds to more than half the: time to attain a negative swab of culture, duration of antibiotic treatment, resulting scar size, and days of hospitalisation (reviewed by Molan, 2006). Likewise, honey has shown to be effective in treating large infected surgical wounds. When other treatments had failed to heal the wounds after 14 days of therapy, the use of honey as a dressing

caused a marked clinical improvement after 5 days, and all wounds were closed, clean and sterile after 21 days (reviewed by Molan, 2006). When honey was used to treat non-healing venous leg ulcers as a dressing under compression as the standard treatments for 12 weeks, honey caused significant reduction of ulcer size (32%), and complete healing in 7 out of 40 cases (reviewed by Molan, 2006).

Honey also has few adverse effects when used as a therapeutic treatment. Allergic reactions are rare (Kiistala *et al.*, 1995) and there have been numerous reports of honey being used clinically on open wounds where no adverse reactions have been recorded other than a transient stinging described by some patients (Al-Waili & Saloom, 1999; Betts & Molan 2001; Dunford *et al.*, 2000a; Dunford *et al.*, 2000b; Natarajan *et al.*, 2001; Robson *et al.*, 2000; Subrahmanyam, 1996). This could be due to the acidity of the honey, as the stinging has not been noted when the acidity has been neutralised (Betts & Molan 2001).

There have also been many reports of honey has been used for the treatment of eye infections (reviewed by Molan, 1999), and Al-Waili (2003) has shown that natural honey can be safely administered by inhalation using an ultrasonic nebulizer.

1.1.4 Previous investigations of the antiviral activity of honey

The antiviral activity of honey has been demonstrated *in vivo* by the research of Al-Waili (2004). He investigated the effect of raw honey, topically applied, on recurrent attacks of herpes lesions, labial and genital, and compared this with the

effect of Acyclovir cream. It was concluded that topical honey application is safe and effective in the management of signs and symptoms of recurrent lesions, of labial and genital herpes. It is, however, unclear if this research showed that honey is antiviral or simply capable of reducing the associated symptoms by means of reducing inflammation, or both. This study leaves open to investigation *in vitro* the effect of honey on Herpes simplex virus to confirm that it has an antiviral activity.

Another study investigating the effect of honey, compared with thyme, on Rubella virus survival *in vitro*, by Zeina et al. (1996), showed that honey had anti-rubella activity, while thyme did not. This investigation showed that it is possible to use cell culture (*in vitro*) to examine the antiviral activity of honey.

Further, in a study by French (2002) Manuka honey and pasture honey at concentrations up to 25% were tested for their antiviral activity against HSV-1 *in vitro*. The results of this investigation were suggestive of antiviral activity with Manuka honey and pasture honey at concentrations of 10% and 15% respectively.

The French and Zeina studies did not reveal nature of honey's antiviral activity thus it remains to be examined.

1.2 METHYLGLYOXAL

Methylglyoxyl has been found at a much higher level in Manuka honey than in any other honey and it has been speculated by Weigel (2004) and more recently

by Adams (2008) and Henle (2008) that the pronounced antibacterial activity of New Zealand Manuka honey directly originates from methylglyoxal.

Methylglyoxal, a 1,2-dicarbonyl compound, is a cytotoxic metabolite produced during glycolysis that can react with cysteine, lysine and arginine residues on proteins. This leads to the production of advanced glycation end products (AGEs). The cytotoxicity of this compound was assessed by Weigel (2004) using HepG2 and HT29 cell lines. Concentrations for 50% inhibition of cell growth, as measured by neutral red assay were 1.5 mmol/l for HepG2 and 0.9 mmol/l for HT29.

Methylglyoxal, as a synthetic compound, has been found to have antiviral activity against Newcastle disease and influenza (Tiffany *et al.*, 1957) as well as foot and mouth disease (Ghizatullina, 1976), which are all caused by RNA viral infections.

With influenza a 2 mmol/l methylglyoxal solution was found to lower the 50% tissue culture infectious dose (TCID 50) levels to negative values after 5 hours at 37°C *in vitro* (Tiffany *et al.*, 1957).

In the case of the foot and mouth disease virus (FMDV), the rate of inactivation of the virus was dependent upon drug concentration, incubation temperature, and the pH of the medium (Ghizatullina, 1976). It was shown that treatment of the viral suspension with 0.1% (16.25 mM) methylglyoxal, at pH 7.6 at 23°C for 5 hours, resulted in complete inactivation of the virus shown by the reduction in TCID 50

levels with the methylglyoxal treatment. It appeared that the effect of methylglyoxal on the virus increased with temperature: a concentration of 0.1% at pH 7.6 caused inactivation of the virus within 3 hours at 37°C and within 5 hours at 23°C. It has been reported that methylglyoxal interacts with nucleic acid, specifically guanosine in nucleic acid, producing modified RNA (Staehelin, 1959). Egyud and Szent-Gyogyi (1966) showed that the reaction of methylglyoxal with nucleic acids was reversible thus in the Ghizatullina study the treated viral preparations were observed for increases in viral titre or other signs of re-activation, no such re-activation was observed. The infectiousness of RNA isolated from methylglyoxal-treated and untreated virus was examined and it was found that RNA treated with methylglyoxal was not infectious on subcutaneous inoculation of white mice (Ghizatullina, 1976).

Methylglyoxal has been reported to inhibit TNF-induced Nuclear Factor Kappa-B (NFκB) p65 activation and NFκB-dependent reporter gene expression, by inhibiting the DNA binding capacity of NFκB p65 (Laga *et al.*, 2007). This suggests that methylglyoxal can provide an additional control mechanism for modulating the expression of NFκB- responsive genes and that it may be responsible for inducing TNF-induced cell death in cells with NFκB activation. This maybe important in relation to the effect honey may have with inhibition of viral activity. It is possible that methylglyoxal present in the honey suppresses the viruses' ability to induce the transcription of genes important for its own replication and survival and so prevents viral activity.

A number of viral proteins activate NFκB as part of their life cycle due to NFκB's anti-apoptotic activity, in order to prolong cell survival so as to maximise viral replication (Gilmore & Mosialos, 2003). In other cases, activation of NFκB may be part of the host's response to viral infection due to the role NFκB has in regulating genes of the innate immune response, which is achieved partially by NFκB-activated cytokine genes.

1.3 AIMS OF THIS THESIS

The hypothesis of this study is that honey has antiviral activity against Adenovirus and Herpes simplex virus.

This study aims to confirm honey's antiviral activity and to compare different types of honey for their antiviral activity. By comparing the effects of honey on two DNA viruses that differ in structural characteristics it is aimed to evaluate the similarities and differences in the action of honey on these. This should identify whether it is likely that honey's action could be applied to treat other DNA viruses. A further aim is to begin characterising honey's antiviral activity via protein detection after treatment of the virus with and without honey.

This thesis is intended to determine primarily, whether honey can provide protection from viral infection, prevent the spread of infection from infected to healthy cells, and neutralise each virus. Also to be examined is whether methylglyoxal, present more greatly in some honeys, is contributing to or is responsible for the observed antiviral effects.

The study aims to provide a better understanding of the scope of honey's antiviral activity and uncover the groundwork of this activity.

Cultures of the susceptible human cell line A549 (lung epithelial cells) will be used as an *in vitro* model to observe any viral infection as morphological changes that have occurred to the cells caused by the virus known as cytopathic effect (CPE). The experiments are to be carried using a range of honey concentrations to treat each virus and include controls to detect changes that occur to the cells after treatment with virus in the absence of honey and without virus at each of the honey concentrations. The developing CPE is observed using phase contrast microscopy. By detecting for changes to viral proteins the successful cell based experiments can be confirmed using immunochemical techniques, leading to a better understanding of the mechanism behind the antiviral activity.

Chapter 2

Viruses studied in this thesis

This chapter reviews characteristics of the two viruses studied in this thesis, and explains why these viruses were chosen for this study. It gives some detail of the structure, entry, and replication cycle, and outlines epidemiology as well as current treatment.

Both Adenovirus and Herpes simplex cause in addition to systemic infection, localised infections which are amenable to treatment with honey, and when used at a pharmacological level this could ensure effective treatment for such ailments.

Where sources are not cited, the information in this chapter came from Fields Virology (2007).

2.1 ADENOVIRUS

2.1.1 Introduction and history

Adenoviruses (Ad) were first isolated and characterised in 1953 by two groups searching for etiologic agents of acute respiratory infections (Hilleman & Werner, 1954; Rowe *et al.*, 1953). The two isolated viruses were found to be related, and were named adenovirus after the tissue in which the prototype viral strain was identified (adenoid tissue) (Enders *et al.*, 1956). It was soon found after adenovirus was first cultured that multiple serotypes of these agents existed, and that they were antigenically related, by a group-specific complementation fixation antigen. A nomenclature for adenoviruses was adopted in 1956, then adenoviruses were reclassified in 1999 (van Regenmortel *et al.*, 2000). *Adenoviridae* is the accepted family name, and there are four accepted genera: *Mastadenovirus,* from mammals; *Aviadenovirus,* from birds; and *Atadenovirus* and *Siadenovirus,* from a broad range of hosts (Davison *et al.*, 2003; van Regenmortel et al., 2000). Human adenoviruses are split into six species, named A through F. Fifty-one human serotypes have been identified on the basis of their resistance to neutralisation by antisera to other known human adenoviruses (De

Jong *et al.*, 1999; Hierholzer *et al.*, 1991), and are classified into these six species (Regenmortal, 2000) based on their ability to agglutinate red blood cells (Rosen, 1960).

The variety of adenoviruses can cause a wide range of common and sporadic infections, and there is no strict one-to-one relationship between the serotype and disease as seen in Table 2.1.

Table 2.1 Classification of human Adenoviruses

Subgroup/Species	Serotype	Tropism
A	12, 18, 31	-
B1	3, 7, 16, 21, 50	Respiratory and Ocular
B2	11, 14, 34, 35	Renal
C	1, 2, 5, 6	Respiratory and ocular
D	8, 9, 10, 13, 17, 19, 20, 22, 23, 24, 25, 26, 27, 28, 29, 30, 32, 33, 36, 37, 38, 39, 42, 43, 45, 46, 47, 48, 49, 51	Ocular and other (hepatitis, pneumonia, encephalitis)
E	4	Respiratory
F	40, 41	Intestinal

Traditionally, laboratory diagnosis has involved use of cell culture to identify adenoviral infection, through the development of a characteristic CPE. The cells swell up, detach from the culture surface into grapelike clusters, and the nuclei become enlarged (Kasel, 1979). Eventually the cells lyse, leaving cell debris. This CPE is a result of the infection passing into the late stage of infection, when adenoviral DNA, messenger RNA (mRNA), and protein are being made in large quantities and virions are assembling in the cell nucleus. The time from infection until CPE is present is dependent upon the amount of adenovirus present in the

inoculating specimen: the CPE can be seen in as little as one day and after periods as long as 14 days. More rapid approaches to diagnosis can be made using immunomicroscopy of adenovirus antigens in tissue samples. And more recently, assays based on the polymerase chain reaction (PCR) are of high specificity and sensitivity can be used clinically (Pehler-Harrington *et al.*, 2004).

2.1.2 Structure

Adenoviruses are non-enveloped, icosahedral particles, approximately 90 nm in diameter, with fiber proteins projecting from the vertices of the icosahedrons, the capsid (protein shell) surrounds the DNA containing core.

2.1.3 Adenovirus genomes

The human adenovirus genomes contain five early transcription units (E1A, E1B, E2, E3, and E4), three delayed early transcription units (IX, Iva2 and E2 late) and one late transcription unit that is processed to generate five families of late mRNA (L1 to L5), all of which are transcribed by RNA polymerase II (Pettersson & Roberts, 1986).

2.1.4 Entry into the host

Adenoviruses enter susceptible hosts either via the mouth, the nasopharynx, or the ocular conjunctiva. The cell protein CAR (coxsackie-adenovirus receptor) is a receptor shared by two groups of unrelated viruses, is the target of the fiber protein in many, if not all, adenovirus serotypes in species A, C, D, E, and F (Bergelson *et al.*, 1997; Roelvink *et al.*, 1998). The cell protein CD46 is a cellular

receptor for all species B adenoviruses except Ad3 and 7 (Gustafsson *et al.*, 2006; Marttila *et al.*, 2005), Ad3 has been shown to use cell protein CD80 and CD86 as cellular attachment receptors (Short *et al.*, 2004). Three species D adenoviruses including Ad8 appear to bind to sialic acid rather than a cell surface protein (Arnberg *et al.*, 2000).

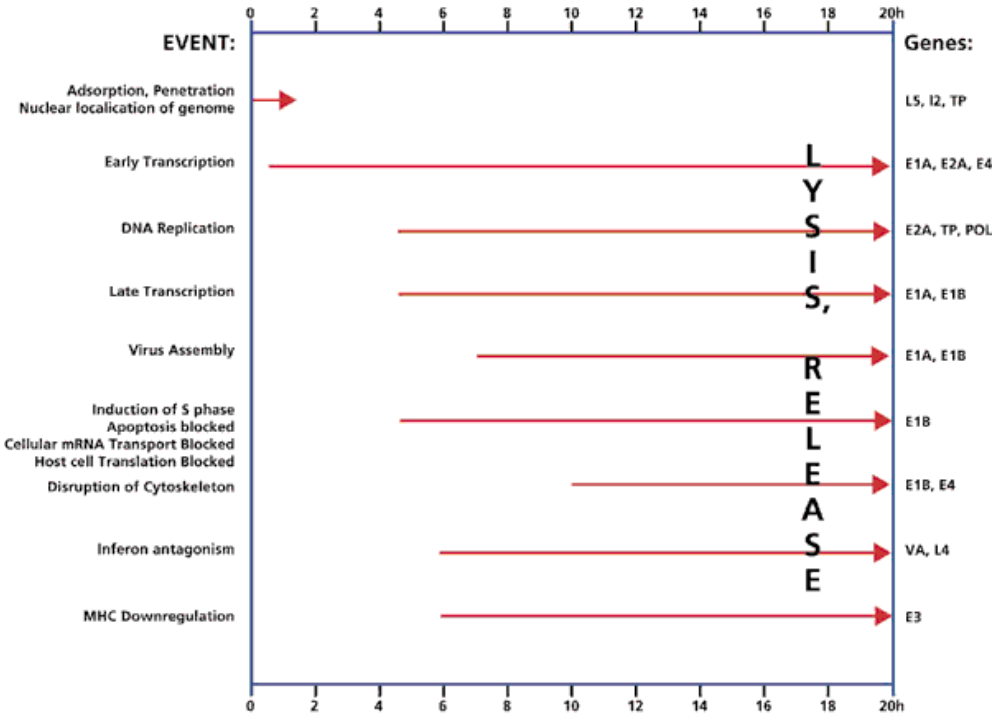
Several integrins participate in adenovirus uptake into the cells, by interacting with the RGD motif on the adenovirus penton base protein (Zhang & Bergelson, 2005). The penton base binds to integrin types $a_v\beta_3$- and $a_v\beta_5$- that are abundant on most epithelial cells (Wickman *et al.*, 1993). This interaction leads to detachment of fibers, allowing a fiber-free virion to be endocytosed (Nakano *et al.*, 2000). Li (1998b) showed the clustering of intergrins induced by the penton base-integrin interaction that are activated by these transmembrane receptors, lead to induction of phosphatidylinositol-3-OH kinase (PI3K). Li (1998a) also showed that once activated, PI3K induces Rac, Cdc42, and RhoA, stimulating rearrangements of the local actin cytoskeletal network to promote endocytosis of the virion into clathrin-coated vesicles (Guilfoyle & Weinmann, 1981; Varga *et al.*, 1991). Clatherin-coated vesicles mature into endosomes, and subviral particles free of fibers escape from endosomes into the cytosol (Greber *et al.*, 1996). During this time virion surface polypeptides dissociate, and the intracellular reducing environment causes reactivation of the virion-associated viral protease and cleavage of protein VI that links the viral core to the capsid. This is necessary for the final disassembly of subvirion particles at the nuclear pore complexes and DNA import into the nucleus (Greber et al., 1996).

2.1.5 Activation of early viral genes

E1A is the first transcriptional unit to be expressed after the viral chromosome reaches the nucleus (Moran *et al.*, 1986). E1A proteins influence transcription by binding to a variety of cellular transcription factors and regulatory proteins. The large E1A protein is primarily responsible for activating transcription from early viral promoters (Montell *et al.*, 1984; Montell *et al.*, 1982; Moran et al., 1986; Ricciardi *et al.*, 1981; Winberg & Shenk, 1984), and the small E1A protein activates transcription from the E2 early promoter specifically (Bagchi *et al.*, 1990; Kitajewski *et al.*, 1986). Figure 2.1 illustrates the order of transcription.

Figure 2.1 Transcriptional map of Adenovirus

From: Clonetech (2008)



2.1.6 Activation of the host cell

Adenovirus activates the host cell to gain an optimal environment for virus production by inducing quiescent cells to enter the S phase of growth by the E1A proteins. Open reading frames (orf) E4orf1 and E4orf4 function cooperatively to activate mTOR (protein kinase, mammalian target of rapamycin), leading to a high rate of protein synthesis in the absence of mitogens and nutrients, conditions which would otherwise greatly suppress translation in uninfected cells (O'Shea *et al.*, 2005). Functions also exist to inhibit apoptosis, for example, to suppress the level and activity of tumour suppressor p53, a transcription factor that activates genes leading to cell cycle arrest or apoptosis, is activated in response to abnormal stimulation of cell cycling, including the activation via E1A proteins (Debbas & White, 1993; Lowe & Ruley, 1993). p53, E1B-55K, and an additional protein encoded in E4 (E4orf6) associate with each other (Sarnow *et al.*, 1984), and a number of cellular proteins, to form an ubiquitin ligase complex that binds p53 and directs its polyubiquination (Harada *et al.*, 2002; Querido *et al.*, 2001), this results in proteosomal degradation of p53 (Boyer & Ketner, 2000; Cathomen & Weitzman, 2000; Querido *et al.*, 1997; Roth *et al.*, 1998; Steegenga *et al.*, 1998). By studying E4 mutants, it has been revealed that adenovirus inhibits the cellular DNA damage response (Carson *et al.*, 2003).

2.1.7 Viral DNA replication

As the E2 proteins accumulate in response to transcriptional activation of the E2 early promoter, by the large E1A (Berk *et al.*, 1979; Jones & Shenk, 1979), and stimulation of E2F transcription factor binding by E4orf6/7 (Bagchi et al., 1990),

(Cress & Nevins, 1994; Marton *et al.*, 1990; Obert *et al.*, 1994), viral DNA replication can now commence, and continues until the host cell dies.

In vivo studies suggest that DNA replication takes place in two stages (Lechner & Kelly, 1977). Adenoviral late genes are expressed efficiently at the onset of viral DNA replication, where poly (A) site utilisation and splicing generate at least twenty unique mRNAs (Evans *et al.*, 1977; Nevins & Darnell, 1978). These are grouped based on the use of common poly (A) addition sites into L1 to L5 (Shenk, 2001). This large family of late mRNA is expressed via the major late promoter (MLP), which becomes more active compared with early activity after initial infection, as the DNA level increases (Shaw & Ziff, 1980). Cellular mRNA is blocked as DNA replication begins and when all the late mRNAs are synthesised (Beltz & Flint, 1979), and in addition to their facilitated transport from the nucleus, viral mRNAs are preferentially translated when they reach the cytoplasm (Cuesta *et al.*, 2000).

2.1.8 Viral assembly and release from the cell

Mutations in a variety of viral genes can interfere with the assembly of virions. A number of systems exist that allow release of viral progeny, one mechanism leads the cell to more likely suffer from lysis, another mechanism involves an E3 11.6 kd protein, which kills cells as it accumulates during the late stage of infection (Tollefson *et al.*, 1996). Further, free fiber trimers that interfere with CAR oligomerisation at the tight junctions of epithelial cells could promote the release of progeny to the airway in the respiratory tract (Walters *et al.*, 2002).

2.1.9 Interactions with the host

Adenovirus can maintain a long-term association with its human host, persisting for years after the initial infection. Adenoviruses encode several proteins that can facilitate both initial primary infection and persistence by antagonising the antiviral responses of the host: E1A proteins and VA RNA repress the cellular response to interferon (IFN)-α and -β; VA RNA can in addition, repress the RNA interference process; and E3 coded proteins protect infected cells from destruction by cytotoxic lymphocytes, and apoptosis inducing cytokines.

2.1.10 Immune response

The virus must survive both immune and chemical defences of the host. Defensins are cationic peptides of 3 to 4.5 kd: some have been reported to inhibit replication of some adenovirus serotypes in cell culture (Harvey *et al.*, 2005; Nazir & Metcalf, 2005). Cellular defences include alveolar macrophages and Kupffer cells which can eliminate virus from the lung and liver respectively in murine models (Liu & Muruve, 2003; Nazir & Metcalf, 2005). These cells can take up vectors quickly and release inflammatory cytokines such as TNFα, IL-6 and Il-8. Increased levels of TNFα, IL-6 and 8 have also been found in adenovirus infection of children (Mistchenko *et al.*, 1994). Adenovirus infection of cultured respiratory epithelial cells activates NFκB and mitogen-activated protein kinase pathways (Palmer *et al.*, 2005). Stimulation of type 1 interferons α and β is part of the innate immune response to adenoviral infection. Adenovirus however can overcome these via at least two mechanisms as stated previously.

2.1.11 Other E3 protein functions

E3-14.7K can bind to FIP-1, -2, and -3 (Carter & Roizman, 1996). Li (2005) showed that FIP is identical to NEMO, a subunit of the IKB kinase complex (IKK) that regulates the NFκB transcription factor that is critical to immune cell function. Action of E3-14.7K with NEMO may contribute to the block in activation of NFκB induced by E3 functions (Friedman & Horwitz, 2002). It is stated that this may be the mechanism by which E3 functions to repress TNFα induced expression of chemokines that are chemotactic for leukocytes (Lesokhin *et al.*, 2002). It is interesting that the E3 promoter includes NFκB binding sites and that NFκB is a potent inducer of E3 expression in T lymphocytes (Mahr *et al.*, 2003; Windheim *et al.*, 2004). This may result in a negative feedback loop whereby activation of NFκB in acutely and latently infected lymphocytes, results in activation of E3 functions that in turn repress NFκB, keeping NFκB activity at a low level in the face of cellular responses that normally activate NFκB.

As noted in Chapter One (see Section 1.2), methylglyoxal can inhibit TNF-induced NFκB p65 activation and NFκB-dependent reporter gene expression, by inhibiting the DNA binding capacity of NFκB p65. The E3 proteins function in part to regulate the expression of NFκB as stated above, it is possible that methylglyoxal disrupts these mechanisms and subsequently reduces the activity of the virus.

2.1.12 Epidemiology

Adenoviral infections are present worldwide in humans as well as in some animals. The transmission of adenovirus infection can differ from sporadic to epidemic where the pattern often correlates with the viral serotype and the age of children or adults of the susceptible population.

A survey undertaken in Manchester, United Kingdom, indicated that 61.3% of patients with adenovirus infections were under the age of five, 24.2% were adults, and 5.6% were children aged between 5 and 18 years (Cooper *et al.*, 2000). The most prevalent serotypes were Ad2 (18.6%), Ad3 (14.9%), Ad1 (12.1%) and Ad41 (10.9%).

Serological investigations have given some estimates of prevalence of adenovirus infection in various populations. Antibodies to Ad1, 2 and 5 are most common and present in 40% to 60% of children and the presence of antibodies to Ad3, 4, and 7 was found to be much lower at the same ages (Badger *et al.*, 1956; Brandt *et al.*, 1969; Huebner *et al.*, 1954). This would explain the greater susceptibility to serotypes Ad3, 4, and 7. It was also found that only about 75% of adenovirus infections were accompanied by an antibody response, as measured in a complement fixation (CF) test (Fox *et al.*, 1969). This could have been related to the insensitivity of the CF test or the lack of antibody production by some children.

2.1.13 Treatment

Cidofovir is a potent, non-toxic inhibitor of adenovirus replication in cell culture (Kinchington *et al.*, 2005; Naesens *et al.*, 2005). It is one of a group of drugs known as acrylic nucleoside phosphonates which are effective against a range of viruses (De Clercq & Holy, 2005). Cidofovir is an analogue if 2',3'-dideoxycytosine. As it is already exists as monophosphate, it does not require a viral enzyme, such as thymidine kinase, required to phospohorylate acyclovir, and cidovovir can be converted to the di- and triphosphate forms by cellular enzymes. Such compounds have a much higher affinity for DNA polymerase and so act as DNA synthesis chain terminators (De Clercq & Holy, 2005).

The use of cidofovir against adenoviral ocular infections of humans was tested in a trial in the United States where it was shown that cidofovir was an efficient treatment. However, the trial was discontinued due to the narrow efficacy-to-toxicity ratio as well as associated rare cases of lachrymal canalicular blockade with off label use at higher doses for greater than one week (Kinchington, et al., 2005). Additionally, the finding of resistant adenovirus isolates to cidofovir raised concerns of the possible emergence of clinical resistance following widespread usage (Kinchington, et al., 2005).

2.2 HERPES SIMPLEX VIRUS

2.2.1 Introduction and history

Herpes simplex virus (HSV) was recognised as an infectious agent from the last quarter of the 19th century (from 1875), and was the first human virus to be

discovered. They are also one of the most commonly studied human viruses due to their ability to cause a range of infections, remain latent in their host, and reactivate to cause lesions at or near the initial site of infection. The clinical symptoms of primary HSV-1 infections varies greatly: infection can be asymptomatic, or, result in a combination of fever, sore throat, ulcerative and vesicular lesions, gingivostomatitis, odema, localised lymphadenopathy, anorexia, and malaise. The onset of recurrent orolabial lesions is distinguished by pain, burning, tingling, or itching, which lasts for usually 6 hours before a vesicle begins to form. The total area of involvement is usually localised where there may be three to five vesicles. Within 72 to 96 hours, lesions progress to pustular or ulcerative, crusting stages. Viral infections of the eye are usually caused by HSV-1 (Binder, 1977; Ostler, 1977; Scott, 1957). Recurrent infections are common, and parallel that of herpes labialis infection. Infections can also occur in the skin.

Prior to viral DNA and amino acid sequencing, members of the *Herpesviridae* family were classified into three subfamilies (*Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae*) based on biological properties (Roizman *et al.*, 1981). Now the viruses have been further classified into genera based on DNA sequence similarity, similarities in genome sequence arrangement, and relatedness of important viral proteins shown by immunological methods. The members of the *Alphaherpesvirinae* subfamily were classified on the basis of a variable host range, reasonably short reproductive cycle, rapid spread in cell culture, efficient destruction of the host cells, and the capacity to develop latent

infections primarily in sensory ganglia. This subfamily contains the genus *Simplexvirus*.

Herpesviruses are widely distributed in nature and most animal species are susceptible to at least one herpesvirus. The following herpesviruses, that have humans as their primary host, have been identified as: herpes simplex virus 1 (HSV-1); herpes simplex virus 2 (HSV-2); human cytomegalovirus (HCMV); varicella-zoster virus (VZV); Epstein-Barr virus (EBV); and *Human herpesviruses 6*, *7* and *8* (HHV-6, HHV-7, and HHV-8).

HSV like Ad is grown in cell culture where a characteristic CPE can be observed within 24 hours depending on the level of virus in the original inoculum. CPE is presented as ballooned cells that over the course of infection can destroy the monolayer of cells. Immunohistochemical techniques can also be used to identify viral infection of cells as well as PCR and more recently like Ad, real time PCR as diagnostic tools to identify the virus.

2.2.2 Structure

The herpes virion consists of four parts: an electronopaque core containing viral DNA; an icosahedral capsid surrounding the core; a largely unstructured proteinaceous layer known as tegument that surrounds the capsid; and an outer lipid bilayer envelope exhibiting spikes on the surface (Roizman & Furlong, 1974).

Mature herpes virions can vary in size from 120 nm to as large as 260 nm (Roizman & Furlong, 1974). The core contains a double stranded DNA (dsDNA) genome wrapped as a toroid (Furlong *et al.*, 1972) or as shown by Zhou (1999) as a spool, in a liquid crystalline state (Booy *et al.*, 1991). Zhou (1999) further showed that the tegument is largely unstructured, apart from some icosahedral structure around the pentons and is made up of at least 20 viral proteins. The most common of the proteins associated with the tegument are the viral protein (VP) VP16 virion transactivator protein (also known as α-trans-inducing factor (αTIF)), the virion host shut off (VHS) protein, VP22, and a very large protein (VP1-2) which may play a role in DNA release at the nuclear pore during viral entry (Batterson & Roizman, 1983). One purpose of the tegument is to carry into newly infected cells an assortment of already synthesised proteins that can immediately begin to manage the host environment, such as shutting down host protein synthesis, inhibiting infection-triggered cell defences, and stimulating viral gene expression.

Electron microscope studies show that the viral envelope has a typical trilaminar appearance (Epstein, 1962). It is made up of a lipid bilayer with approximately 11 different viral glycoproteins embedded in it, gB, gC, gD, gE, gG, gH, gI, gL, and gM. The capsid is made up of 162 capsomeres (150 hexons and 12 pentons) and the outer shell of the capsid is composed of four viral proteins, VP5, VP26, VP23, and VP19C. It is assumed that HSV gains the envelope lipids from its host: this hypothesis is supported by Spear and Roizman (1967).

2.2.3 Replicative cycle overview

To begin infection the virus must attach to the cell surface receptors, to allow fusion of the envelope with the plasma membrane. The de-enveloped tegument-capsid can then be transported to the nuclear pores where viral DNA is released into the nucleus. Transcription of the viral genome, replication of viral DNA, and assembly of the new capsids takes place in the nucleus. Viral DNA is transcribed throughout productive infection through the action of the host RNA polymerase II, with the involvement of viral factors at all stages of infection. Viral gene expression is co-ordinately regulated and sequentially ordered in a cascade fashion. The α genes (immediate early) expressed first, are produced in the absence of *de novo* viral replication, the products of which are involved in activating the expression of β or delayed early genes. Many of these gene products are involved in DNA replication and include enzymes and DNA binding proteins. The γ genes are then transcribed, and these gene products are largely involved in assembly of progeny virions. Assembly of the virus occurs over several stages, after packaging of DNA into preassembled capsids, the filled capsid or nucleocapsid matures into a virion and acquires full infectivity by budding through the inner lamella of the nuclear membrane. In fully permissive cell culture this takes approximately 18-20 hours.

2.2.4 Entry into the host cell

Transmission of herpesvirus is dependent upon intimate, personal contact between a susceptible individual (seronegative) and someone excreting HSV. The virus must come into contact with mucosal surfaces or abraded skin for infection to be

initiated. Clinical experience would suggest that after primary infection, the replication of the virus at the entry portal, mucosa of the oral or genital region, results in infection of the sensory nerve endings. The virus then travels to the dorsal root ganglia (Baringer & Swoveland, 1973; Bastian *et al.*, 1972; Stevens & Cook, 1974). The fundamental principle of disease pathogenesis is: the tendency of the virus to replicate at the mucosal surface; be transported to dorsal root ganglia; and to become latent. With reactivation, the virus is detected at mucocutaneous sites, appearing as skin vesicles or mucosal ulcers.

HSV can enter the host cell via attachment to the cell surface. This is a reversible process and involves the binding of virion gC and gB to glycosaminoglycans (GAG) (Herold *et al.*, 1991; Shieh *et al.*, 1992; WuDunn & Spear, 1989). The next step in one pathway involves the interaction of gD with a specific receptor and fusion of the envelope with the plasma membrane. gD can interact with nectins, herpes virus mediator (HVEM), and a selected form of 3-O-sulfated heparin sulphate (3-OS HS) (Cocchi *et al.*, 2000; Geraghty *et al.*, 1998; Montgomery *et al.*, 1996; Shukla *et al.*, 1999; Warner *et al.*, 1998) and together with gB, gH, and gL, allow the fusion of the envelope with the plasma membrane (Campadelli-Fiume *et al.*, 2000; Spear & Longnecker, 2003).

The translocation of the capsids to the nucleus was found by two studies (Miyamoto & Morgan, 1971; Morgan *et al.*, 1968) to not require the dissolution of the capsids from the membrane where release of viral DNA takes place through some port in the capsids. Evidence of release at nuclear pores came from studies

of a *ts*B7 mutant (Batterson *et al.*, 1983; Knipe *et al.*, 1981), and several laboratory studies show that the capsids are transported from the site of de-envelopment to the nuclear pore along the microtubular network (Kristenson *et al.*, 1986; Sodeik *et al.*, 1997; Topp *et al.*, 1996).

Once viral DNA enters the nucleus, viral transcription, DNA replication, encapsidation, and egress take place. By remodelling the host cell nucleus, the virus then has a structurally sound base for efficient viral DNA replication and late DNA transcription.

2.2.5 Viral gene expression

During productive infection, over 80 HSV proteins are expressed. The host RNA polymerase II mediates transcription of all viral genes on the viral DNA during infection (Alvine *et al.*, 1974; Costanzo *et al.*, 1977), even though viral gene products may modify its activity and structure.

Very soon after infection (2-4 hours), the viral α or immediate early (IE) genes are expressed. These genes code for six proteins known as ICP0, ICP4, ICP22, ICP27, ICP47 and $U_S1.5$. Although transcription of the α genes requires no previous viral protein synthesis, an HSV protein brought in with the virion tegument, VP16, stimulates the transcription of the α genes. Five of the six α genes products, including ICP4, ICP0, ICP27, ICP22 and $U_S1.5$ stimulate β gene expression. Overall, HSV encodes one site-specific transcriptional activator (VP16), three proteins whose function is to block cellular silencing machinery

(ICP0, U_S3, $U_S3.5$), one sequence-specific repressor (ICP4), and one sequence independent transcription factor (ICP4). HSV infection prevents host transcription, RNA splicing and transport, and protein synthesis, to enable the transition from cellular to viral gene expression. Expression of the next subset (β genes) requires at least the presence of functional ICP4 but not the onset of viral DNA synthesis, and between 4 to 8 hours after infection these β proteins are expressed. These proteins are involved in replication of viral DNA and nucleotide metabolism and promote viral DNA replication and the expression of late γ or late genes. Two general groups of β proteins exist. B_1 genes are expressed within a short time after or almost concurrently with the onset of synthesis of α proteins, and are exemplified by U_L23 encoding thymidine kinase (TK). Some β genes require ICP27 for their expression. The γ or late genes are expressed at peak times after viral DNA synthesis has begun, and their expression is enhanced by viral DNA synthesis. They, like the β genes, have been categorised into two groups, γ_1, early/late, or leaky late, and one called γ_2, late, or true late genes. The typical γ_1 gene (ICP5, glycoproteins B and D, and γ34.5) is expressed relatively early in infection and is stimulated a few fold by viral DNA synthesis. ICP5 is made both early and late in infection (major capsid protein). In contrast, typical γ_2 genes (UL44, the gene encoding glycoprotein C, U_L41, U_L36, U_L38, and U_L11) are expressed late in infection and are not expressed in the presence of effective concentrations of inhibitors of viral DNA synthesis.

2.2.6 Initiation of viral gene expression

There are at least two sequences of events that take place in the first few minutes after infection. The first, and least well understood, is the activation of calcium signalling (Cheshenko *et al.*, 2003), activation of NFκB (Mogensen & Paludan, 2001; Patel *et al.*, 1998), and induction of the IFN pathway (Mossman et al., 2001; Nicholl *et al.*, 2000; Preston *et al.*, 2001). The second sequence of events takes place after entry of viral DNA into the nucleus and shapes the outcome of infection. Cellular proteins either with affinity for viral DNA silence it, or transcriptional activators attempt to express its genes. The finding that residues 537 to 613 of ICP0 are homologous without gaps to those of amino acids 3 to 79 of CoREST led to evidence that ICP0 plays a direct role in blocking the silencing of HSV DNA (Gu *et al.*, 2005; Roizman *et al.*, 2005). U_S3 and $U_S3.5$ kinases, whether newly synthesised or brought into the cells as components of the tegument, further enhance the expression of post-α genes by phosphorylating histone deacetylase (HDAC) I and II. The synthesis of ICP8 enables viral DNA synthesis and enhances γ gene expression.

2.2.7 Viral DNA Replication

Once the β proteins are expressed, a number of these proteins localise into the nucleus and assemble in DNA replication complexes at pre-replicative sites.

The first steps in HSV DNA replication involve the binding of U_L9 protein, and/or ICP8, or both, to the origin sequences, where the looping and distortion of the origins by U_L9 helicase activity unwinds the DNA. The helicase-primase

complex is then recruited to the origin by interactions with U_L9, ICP8, or both. Leading strand synthesis involves the unwinding of DNA and synthesis of a primer by the HSV helicase-primase complex, from which leading strand synthesis can be accomplished by the HSV pol-U_L42 holoenzyme. Alternatively, primers may be synthesised by cellular polymerase α-primase. Lagging strand synthesis is then accomplished by primer synthesis and pol-U_L42 extension of the DNA strand. Seven viral proteins are required for viral DNA replication: viral DNA polymerase catalytic subunit (U_L30) and its processivity factor (U_L42); an origin binding protein (U_L9); the ICP8 ssDNA binding protein (SSB; U_L29); and the helicase-primase complex of three proteins, U_L5, U_L8 and U_L52 (Challberg, 1986; Conley *et al.*, 1981; Purifoy *et al.*, 1977; Wu *et al.*, 1988). Host cell factors are presumed to be involved also but have not been identified largely due to HSV DNA replication involving origin-dependent initiation, and synthesis having not been achieved *in vitro*.

2.2.8 Viral assembly and release from the cell

After the γ capsid proteins are synthesised they are localised into the infected cell nucleus where capsid assembly occurs. Empty shells containing scaffolding are assembled first, and the scaffolding is lost on insertion of viral DNA into the capsid. As the DNA is being transferred into the capsid, a head full sensing mechanism causes cleavage of the genome at conserved sequences that identify genomic termini. The conserved viral proteins are responsible for causing rearrangement of the nuclear lamina that allows for nuclear egress. There is fundamental agreement that herpesviruses can be enveloped at the inner nuclear

membrane, and evidence that the nuclear membrane and nuclear lamina must undergo extensive modification by at least three viral proteins (U_S3, PK, U_L31, and U_L34) (Reynolds *et al.*, 2000; Reynolds *et al.*, 2001; Reynolds *et al.*, 2002; Simpson-Holley *et al.*, 2004). The exact mechanism of viral egress from the nucleus to the extracellular space is a hotly debated issue. Three models exist to describe virion egress (Epstein, 1962; Stackpole, 1969; Wild *et al.*, 2005).

2.2.9 Interactions with the host

DNA microarray studies validated by northern blot analysis and real-time PCR indicated that a substantial fraction of total mRNA was up or down regulated (Esclatine *et al.*, 2004; Taddeo *et al.*, 2002; Taddeo *et al.*, 2003a), and a substantial amount of the upregulated mRNA was NFκB dependent. The activation of NFκB occurs at two stages, one early after exposure of virus to cells and at a later time. The major stimulus for NFκB activation at later times is activated PKR (Taddeo *et al.*, 2003b). Also, HSV-1 requires NFκB proteins for its replication (Taddeo *et al.*, 2006a). It has been shown that U_S11 protein expressed early in infection blocks the activation of PKR (Cassady *et al.*, 1998). In cells infected with viruses lacking U_L11 NFκB was not activated (Taddeo et al., 2003b). Likewise NFκB was not activated in PKR $^{-/-}$ cells infected with wildtype virus, but was in PKR $^{+/+}$ cells (Taddeo et al., 2003b). In murine cell lines lacking p50, p65, or both NFκB genes yielded 10 to 30 times less wildtype virus than $p50^{+/+}/p65^{+/+}$ (Taddeo *et al.*, 2004). The activation of PKR is a way for HSV-1 to ensure that NFκB is induced. These findings relate to earlier observations that cells expressing a dominant negative form of IκB protein yield less virus than

wild type cells (Amici *et al.*, 2001; Patel et al., 1998). This can lead to a general question of how NFκB functions in viral replication. It has been debated that NFκB induces antiapoptotic functions (Goodkin *et al.*, 2003; Gregory *et al.*, 2004).

2.2.10 Reactivation of virus from a latent state

In humans, latent viral infection is reactivated after local stimuli such as injury to tissues innervated by neurons harbouring latent virus, or by systemic stimuli, which may reactivate virus simultaneously in neurons of diverse ganglia. Evidence shows that the most plausible common denominator is that injury or stimulation of cells innervated by dorsal root neurons harbouring latent virus is a common trigger of recrudescence of lesions caused by reactivated virus. At the time of reactivation, LAT transcript levels decrease (Spivack & Fraser, 1988) and the histones associated with the LAT gene become deacetylated (Amelio *et al.*, 2006). At the same time, lytic genes including ICP0 gene become associated with acetylated histones (Amelio et al., 2006), and lytic gene transcripts accumulate (Devi-Rao *et al.*, 1994; Kosz-Vnenchak *et al.*, 1993).

2.2.11 Epidemiology

Orolabial herpes simplex virus infection ranges from the more usual mild illness, non-discernable in most individuals, to sporadic, severe, and life-threatening disease in a few infants, children, and adults. Much overlap in epidemiology is seen between HSV-1 and HSV-2, although they involve transmission via different routes and different areas of the body and humans are the sole reservoir for

transmission of HSV to other humans. Due to the latency effect of this virus, over half the world's population has been infected by HSV and so exists the capability of transmitting HSV during episodes of productive infection.

Using serological assays, the seroprevalence of HSV-1 and HSV-2 was redefined: HSV-1 is more common than HSV-2. The trigeminal ganglia houses HSV-1 latent infection, which reactivates, leading to recurrent herpes labialis. Studies accessing the frequency and severity of the immunocompentent host are limited. A positive history of recurrent herpes labialis was observed in 38% of 1800 graduate students (Ship *et al.*, 1960; 1961). The new lesions occurred at a frequency of one per month in 5% of the affected students, and in intervals of 2 – 11 months in 34%. Recurrences of one per year or less were found in 61%.

Both HSV-1 and HSV-2 can cause genital herpes. Antibodies to this virus generally do not occur before the age of sexual activity (Adam *et al.*, 1979; Schillinger *et al.*, 2004; Xu *et al.*, 2002). The overall seroprevalence rates of HSV-2 in the United States, is about 22%, a 30% increase over the past decade (Fleming *et al.*, 1997). The importance of serological observations is the potential for women to reactivate HSV-2 at the time of delivery and transmit infection to their children. Likewise with orolabial lesions, latent infection of the sacral ganglia is the largest reservoir for HSV-2, and reactivation leads to recurrent HSV-2 infection. Overall, nearly 90% of patients infected with HSV-2 will have one or more recurrences per year, 38% more than six recurrences, and 20% more

than ten (Benedetti *et al.*, 1994). HSV-1 infection recurs less frequently than HSV-2 infection (Benedetti et al., 1994; Lafferty *et al.*, 1987).

2.2.12 Prevention and treatment

Presently the only mechanism for prevention of mucocutaneous HSV infection is to avoid contact with infected secretions. No proven post-exposure antiviral therapy or vaccines are presently available.

In the 1970s, virarabine (an adenine analogue) became the first licensed antiviral therapeutic treatment for herpes simplex encephalitis and neonatal infections. However, virarabine was soon replaced by acyclovir for the treatment of all herpesvirus infections. Now, acyclovir, and its prodrug valacyclovir, as well as peneciclovir and famiciclovir, are the most useful and widely used therapeutics for the treatment of HSV infections. Ocular infections are typically treated with toptrifluorothymidine, with or without concomitant oral acyclovir, valacyclovir, or famiciclovir. Resistance to these drugs is possible through mutations in the thymidine kinase TK gene and HSV DNA polymerase which as a consequence causes these treatments to be inactive.

2.3 HOW HONEY COULD BE USED TO TREAT SUCH INFECTIONS

From the evidence given in Chapter One, indicating the ancient to more recent uses of honey, a range of infections can be potentially treated with honey. This can include the common cold, eye infections such as conjunctivitis, as well as Herpes coldsores and genital lesions. As described these viruses cause localised

infections, and these are amendable to treatment with an effective level of honey that can be maintained through re-application, to ensure efficient treatment.

Chapter 3

Materials and Methods

This chapter outlines the general materials and methods used throughout this study.

3.1 MATERIALS

3.1.1 Honey

A number of New Zealand honeys were used throughout this investigation:

Manuka honey which contains high concentrations of methylgyloxal, UMF activity, and phenolics, sourced from Summer Glow apiaries, Denis Watson, Spirits Bay, and Waikuku; Ling Heather honey which is generally high in phenolics sourced from Waikato Honey Products; Rewarewa honey and Honeydew honey which both show a high level of antibacterial activity due to hydrogen peroxide, and high iron-binding activity sourced from Mossops and Tom Penrose Rangiora respectively. Each was stored at 4°C.

These honeys show a range of antibacterial and antioxidant activity levels (see Chapter 4) and were selected to allow comparisons to be made between characteristic properties and antiviral activity.

3.1.2 Viral isolates

Isolates of Ad1, Ad3, Ad8, HSV-1 and HSV-2 were from patients of Waikato Hospital, they were samples that had been cultured in the Virology Laboratory that had been approved for use in accepted research. They were obtained as swab samples within a cell culture medium.

3.1.3 Cell culture materials

The A549 cell line (human lung carcinoma epithelial cells) was kindly provided by the Waikato Hospital Virology Laboratory. This cell line was cultured in

RPMI 1640 containing Hepes, obtained from Invitrogen. This culture medium was supplemented with foetal calf serum from ICP Biologicals or Invitrogen at 10% or 1% to make growth or maintenance medium respectively, with the following antimicrobials added: gentamycin from Bomac Laboratories Ltd (final concentration 50 μg/ml), ampicillin from Roche pharmaceuticals (final concentration 50 μg/ml), vancomycin from Eli Lilly (final concentration 50 μg/ml), and amphotericin B from Gibco (final concentration 5 μg/ml).

3.2 METHODS

3.2.1 Growth, maintenance, and freezing down of cell line

Once a confluent monolayer of A549 cells had been attained in a tissue culture flask after growth within 10% growth medium at 37°C for a minimum of 24 hours based on cell density, the cells were detached from the culture vessel using 1 mM trypsin EDTA from Invitrogen (1-3 ml based on the size of the flask) with incubation at 37°C for approximately 5-20 minutes. After detachment of the cells, for further growth the cell suspension was diluted 1:3 with growth medium (using 15 ml for small flasks and 45 ml for large flasks), then distributed appropriately into flasks or plates from Greiner Bio-One at an average density of 4.1×10^5 cells per ml. To freeze down the cell line, 2-6 ml of growth medium was added to the cell suspension and the cells were distributed into 1.5 ml Eppendorf centrifuge tubes then centrifuged for 2 minutes at 6000 rpm. The cells were re-suspended using a pasture pipette in 1 ml 10% foetal calf serum in Dimethyl sulphoxide (10% FCS/DMSO), and transferred to 1.5 ml freezing tubes which were frozen at

-70°C initially within a cell freezing chamber (surrounded by isopropanol), then without a freezing chamber.

3.2.2 Growth, maintenance, and freezing down of viral isolates

As described in Section 3.2.1 cells were detached from the tissue culture flask and supplemented with growth medium. These were distributed in appropriate volumes to a number of 10 ml securely capped tubes or 25 ml flasks and were incubated overnight at 37°C. Once the cells were confluent, the medium was replaced with maintenance medium and inoculated with 200 µl of the appropriate viral isolate from the samples obtained from the Waikato Hospital Virology Laboratory. The cells were further incubated at 37ºC and observed daily for characteristic morphological changes caused by the virus (CPE). Once the level of CPE was quite severe (see Chapter 4, Figure 4.6, CPE 3-5) the tubes or flasks were frozen at -70°C overnight. After freezing, the tubes or flasks were thawed at room temperature for further lysis of the cells to release viral particles, the medium was then removed from the flasks or tubes and re-frozen in a number of 1.5 ml Eppendorf centrifuge tubes at -70°C for further use.

3.2.3 Determining viral titre

Tubes of viral stock suspension prepared as described in Section 3.2.2 were thawed slowly in the fridge at 4°C. Once thawed, 100 µl of the stock viral solution was serially diluted from 10^{-1} to 10^{-9} in maintenance medium. Confluent A549 cells prepared in 24 well plates were inoculated with 1 ml of 10^{-5}-10^{-9}

dilutions in triplicate. The plates of inoculated A549 cells were then incubated at 37°C and observed daily for development of characteristic CPE.

The dilution of the stock viral suspension that gave CPE in 1/3 of the replicates followed by absence of CPE in the sequential dilution was taken as the maximum dilution that would lead to infection. This was termed the TCID as it was the minimum concentration of virus required to detect viral CPE, and gave a rough indication of the overall concentration of virus present within the stock suspension.

3.2.4 Measuring the density of honey

To determine each type of honey's density, 5 ml of distilled water was measured into a 25 ml measuring cylinder and tared on a balance beam. Five grams of honey at room temperature was added to the cylinder with special care taken not to touch the sides of the cylinder with the honey. The new volume within the measuring cylinder was read and recorded. The densities were expressed as grams of honey per ml.

3.2.5 Measuring the antibacterial activity of honey

The antibacterial activity with and without catalase added to remove activity due to hydrogen peroxide, was measured according to the method described by Allen et al. (1991). The activity measured by this method is expressed as the concentration of phenol with equivalent antibacterial activity.

3.2.6 Measuring the methylglyoxal level of honey

Methylglyoxal levels were determined by Dr. C. Adams by means of RP-HPLC using an indirect orthophenylenediamine (OPD) derivatisation method as described in Adams et al. (2008) which was based on the method outlined in Weigel et al. (2004) with minor changes to the chromatography. The methylglyoxal levels were given in mg per kg of honey.

3.2.7 Measuring the antioxidant activity of honey

The antioxidant activity of the honeys was measured by a modification of the method of Baltrusaityte (2007) in which the removal of the coloured ABTS radical was measured spectrophotmetrically. The trolox equivalent antioxidant capacity (TEAC) values were expressed as mmol trolox per kg of honey.

Chapter 4

Initial experiments and results

This chapter gives the results of the preliminary experiments investigating: the characteristic properties of each honey, the development of viral CPE and scoring system, viral titre, and the A549 cell line toleration of honey.

The first part of this study involved determining the characteristics of each honey type to be tested including the: density; total antibacterial activity; non-peroxide antibacterial activity; methylglyoxal levels; and antioxidant capacity. It also involved the culture of each viral isolate within the A549 cell line, followed by development of a CPE scoring system on which to base measurement of future observations of viral infection. This preliminary investigation further included finding the concentrations of each honey that could be tolerated by the cells in culture.

4.1 GROWTH OF VIRAL ISOLATES AND CYTOPATHIC EFFECT SCORING

4.1.1 Introduction

To detect the presence of viral infection, the susceptible A549 cell line was used as it clearly exhibits the development of characteristic CPE. By examining viral CPE observations are inexpensive as they can be made visually using phase contrast microscopy. The CPE can be qualitatively measured by comparing visually the level of infection against a specifically developed CPE scoring system.

Thus, after the cell line had been successfully grown, each viral isolate was cultured to establish viral stocks, determine an approximate time period from point of infection until viral infection was apparent at a moderate level in the form of CPE, and to establish a scoring system and viral titre.

4.1.2 Methods

As outlined in Section 3.2.2 A549 cells were cultured in tubes and flasks which once confluent were inoculated with virus. The inoculated cells were further incubated at 37°C and observed daily for CPE development.

4.1.3 Results

The results of CPE development of each viral isolate are shown in Figures 4.1 to 4.5. The results for the CPE scoring system are shown in Figure 4.6 where the score is outlined with each illustration.

CPE development was observed for a number of days until a moderate to high level was obtained with each viral isolate.

Figure 4.1 CPE resulting from infection of A549 cells with Ad1: (a) cultured in tubes, and (b) cultured in flasks

(a) Cultured in tubes (b) Cultured in flasks

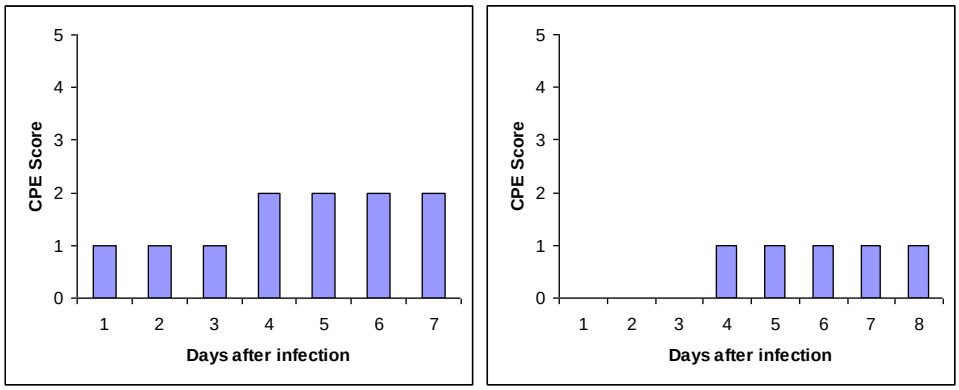


Figure 4.2 CPE resulting from infection of A549 cells with Ad3: (a) cultured in tubes, and (b) cultured in flasks

(a) Cultured in tubes (b) Cultured in flasks

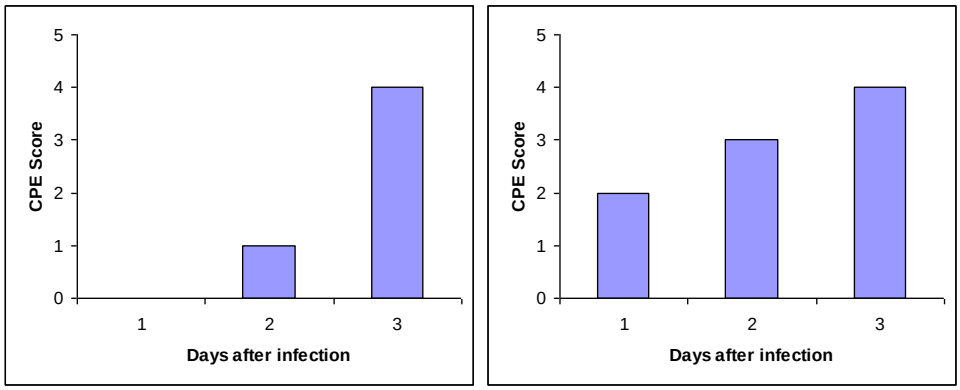
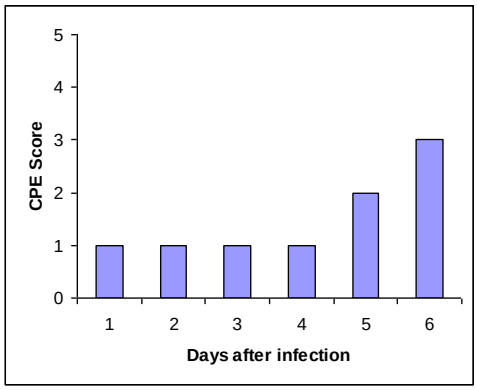


Figure 4.3 CPE resulting from infection of A549 cells with Ad8: (a) cultured in tubes, and (b) cultured in flasks

(a) Cultured in tubes



(b) Cultured in flasks

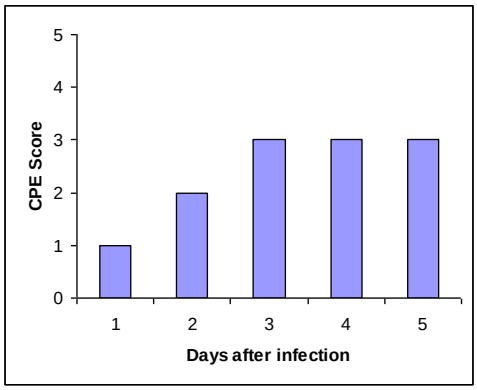


Figure 4.4 CPE resulting from infection of A549 cells with HSV-1 cultured in tubes

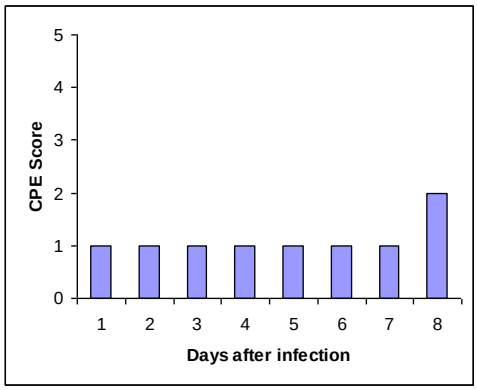
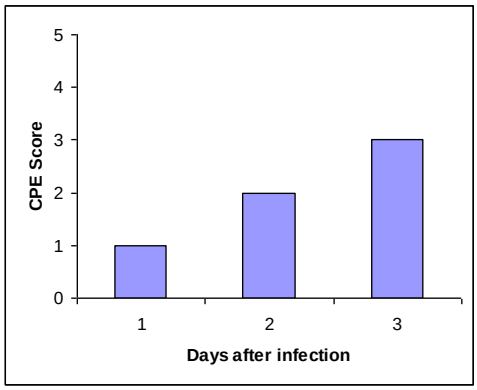


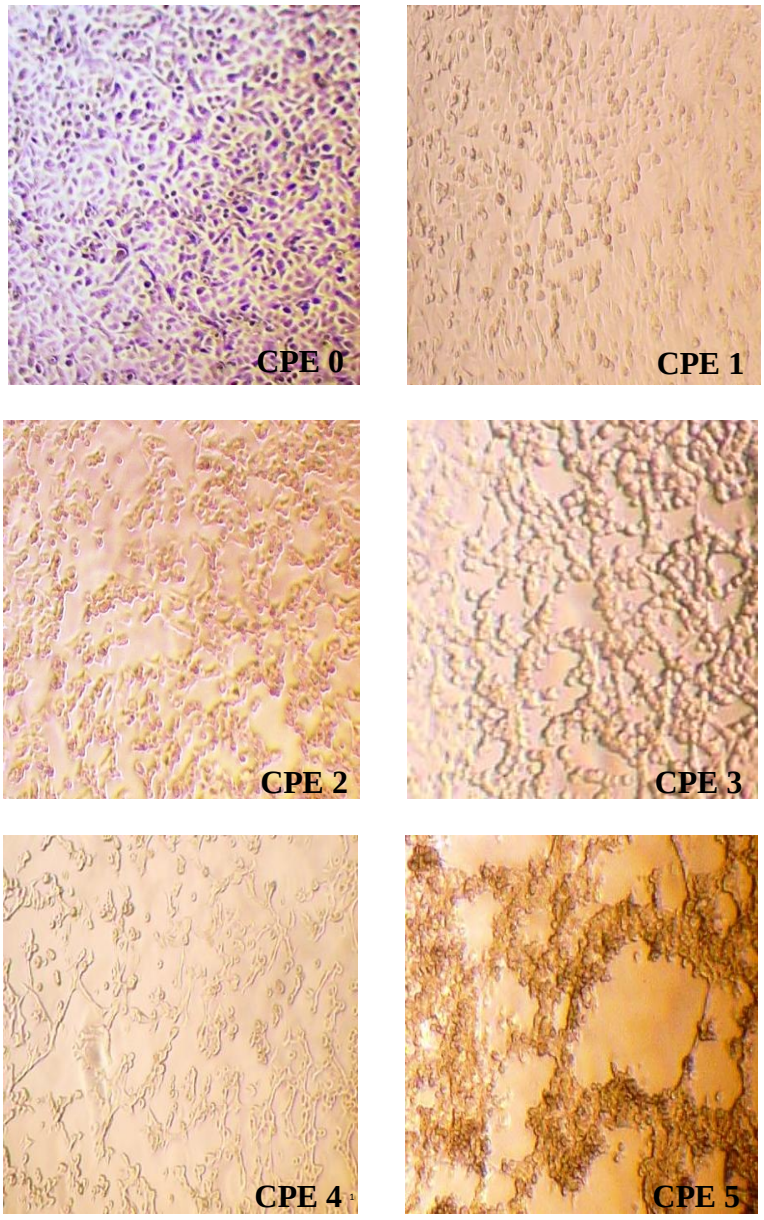
Figure 4.5 CPE resulting from infection of A549 cells with HSV-2 cultured in tubes



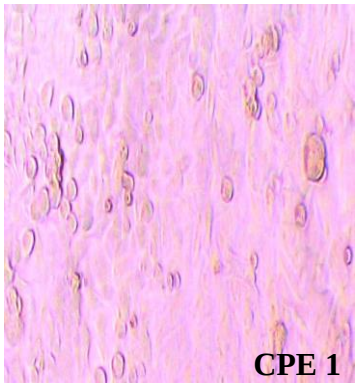
With HSV-1 and HSV-2 a CPE score of 3 was obtained by 24 hours after infection using A549 cells cultured in flasks. No further observations were made as a moderate level has been attained within a short period.

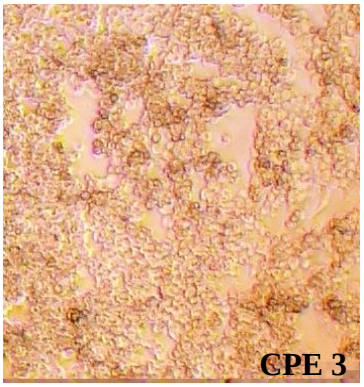
Figure 4.6 CPE scoring system of: (a) Adenovirus, and (b) Herpes simplex virus

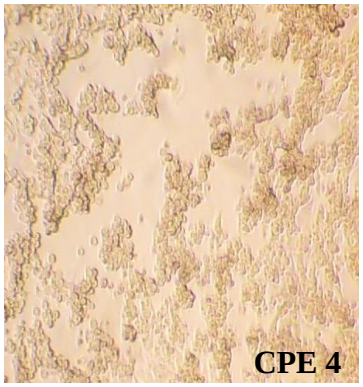
(a) Adenovirus

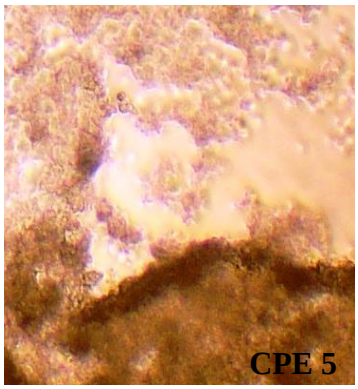


(b) Herpes simplex virus









4.1.4 Discussion

4.3.4.1 Evaluation of viral culture using A549 cells grown in tubes

Figures 4.1 to 4.3 show the CPE development of the adenoviral isolates. With Ad1 low levels of CPE were recorded over the observation period, more moderate levels were obtained with Ad8, and Ad3 in contrast within 3 days had reached a high level of CPE as shown by Figure 4.2.

Both Herpes virus strains caused evident infection from an early stage and with HSV-2 a characteristic CPE level was seen from the second day of observation. HSV-1 CPE was delayed and did not increase to as high a level as seen with HSV-2 until day seven.

Due to the delay in the onset of more lucid viral infection with HSV-1 compared with HSV-2, the medium of HSV-1 was tested for potential contaminants. A yeast contaminant was identified by the presence of "budding" at 400 times magnification and therefore the antifungal agent amphotericin B (final concentration 5 μg/ml) was added to the medium. It is likely that the contaminant was present in the viral inoculum, and its presence might explain the delay of viral growth.

Once a CPE level of 2-3 was obtained with each virus, the viral medium was frozen for further use at -70°C.

From these growth plots, preliminary experimental periods were able to be estimated for each viral isolate. It was clear that experiments with adenovirus needed to be run for two to seven days, in which evident CPE can be expected from day two. With HSV, experiments need to be run for one to seven days, where from days one to two CPE can be expected.

4.3.4.2 Evaluation of viral culture using A549 cells grown in flasks

Further culture of each viral isolate was required to establish stocks. The stock viral solutions were grown up in flasks containing prepared confluent cells. A 1:10 dilution of the original viral inoculum was cultured. The growth of larger volumes of virus enabled greater accuracy in the detection of viral infection as a CPE score, and a better approximation of the period after infection to reach such levels.

The data showed for Ad1 (Figure 4.1 (b)), that the degree of CPE did not increase over the course of infection, where only early levels were observe. Ad1 would require further growth if it was to be used as a test viral sample.

Figure 4.2 showed that Ad3 grew successfully with clearly visible infection present from 24 hours after inoculation of the cells which increased to CPE 4 by day three.

Ad8 took one day longer to obtain CPE 2 compared with Ad3, however, the level of infection increased to CPE 3 by day four (Figure 4.3 (b)).

The herpes isolates both reached CPE levels of 3 from 24 hours after infection. This observation supports the lack of growth seen previously of HSV-1 being likely caused by the presence of a fungal contaminant.

Each of the viral isolates upon satisfying a moderate to high level of CPE (CPE 2-3) were frozen at -70°C in their flask to allow for freeze-thawing the following day. The freezing then thawing aimed to release further viral particles from the cells due to cellular lysis. Each viral solution was then distributed into smaller volumes for storage at -70°C for experimental use.

4.3.4.3 Overall evaluation of viral culture

From these initial attempts to culture each viral isolate, it was found that each had a different growth rate and CPE within the A549 cells. Owing in addition to the limited time span of this study, it was for this reason that, Ad1 was omitted from further examination based on its delay in onset of viral infection compared with the other adenoviral isolates Ad3 and Ad8. Viral isolates to be assessed in this study that could be tested over the course of one week were more ideal.

4.2 VIRAL TITRE

4.2.1 Introduction

To find the titre of each viral isolate, tissue culture infectious doses (TCID) were tested for.

4.2.2 Methods

As described in Section 3.2.3 viral stock suspension was thawed and used to serially inoculate confluent A549 cells prepared in 24 well plates in triplicate. The cells were then incubated at 37°C and observed daily for the development of characteristic CPE.

4.2.3 Results

The results of the CPE scores observed with serial dilutions of each virus are shown in Tables 4.1 to 4.5.

Table 4.1 CPE resulting from serially diluted Ad3 at (a) 24 and (b) 48 hours after infection

(a) 24 hours after infection

Viral Dilution	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
Replicate 1	1	1	1	1	0
Replicate 2	1	1	1	0	0
Replicate 3	1	1	1	1	0

(b) 48 hours after infection

Viral Dilution	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
Replicate 1	2	2	2	2	1
Replicate 2	2	2	2	2	2
Replicate 3	2	2	2	2	1

Table 4.2 CPE resulting from serially diluted Ad8 at (a) 24, (b) 48, and (c) 72 hours after infection

(a) 24 hours after infection

Viral Dilution	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
Replicate 1	0	0	0	0	0
Replicate 2	0	0	0	0	0
Replicate 3	0	0	0	0	0

(b) 48 hours post infection

Viral Dilution	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
Replicate 1	1	2	1	1	0
Replicate 2	2	2	1	1	0
Replicate 3	2	2	1	1	1

(c) 72 hours after infection

Viral Dilution	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
Replicate 1	3	2	2	2	1
Replicate 2	2	3	2	2	1
Replicate 3	2	2	2	2	2

Table 4.3 CPE resulting from serially diluted HSV-1 at (a) 24, (b) 48, and (c) 72 hours after infection

(a) 24 hours after infection

Viral Dilution	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
Replicate 1	1	1	1	1	1
Replicate 2	1	1	1	1	0
Replicate 3	1	1	1	1	0

(b) 48 hours after infection

Viral Dilution	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
Replicate 1	2	1	1	0	1
Replicate 2	2	1	1	1	1
Replicate 3	2	1	1	1	0

(c) 72 hours after infection

Viral Dilution	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
Replicate 1	2	2	2	1	1
Replicate 2	3	2	2	2	1
Replicate 3	3	2	2	2	1

Table 4.4 CPE resulting from serially diluted HSV-2 at (a) 24 and (b) 48 hours after infection

(a) 24 hours after infection

Viral Dilution	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Replicate 1	0	0	0	0	0
Replicate 2	0	0	0	0	0
Replicate 3	0	0	0	0	0

(b) 48 hours after infection

Viral Dilution	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Replicate 1	3	3	2	2	2
Replicate 2	3	3	3	2	2
Replicate 3	3	3	3	2	2

Table 4.5 CPE resulting from further serially diluted HSV-2 at (a) 24, (b) 48, and (c) 72 hours after infection

(a) 24 hours after infection

Viral Dilution	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}
Replicate 1	0	0	0	0	0
Replicate 2	0	0	0	0	0
Replicate 3	0	0	0	0	0

(b) 48 hours after infection

Viral Dilution	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}
Replicate 1	1	1	0	0	0
Replicate 2	1	1	1	0	0
Replicate 3	1	1	1	0	0

(c) 72 hours after infection

Viral Dilution	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}
Replicate 1	2	1	0	0	0
Replicate 2	2	1	1	0	0
Replicate 3	2	1	1	0	0

4.2.4 Discussion

Determining the TCID or viral titre aimed to ascertain suitable dilutions to investigate each virus with, in one million or one thousand fold excesses of this value.

This was achieved as described in Section 4.2.2 and the TCID was determined as the dilution of virus that from which visible viral infection was absent, or the dilution that showed substantially less infection across its replicates compared with the preceding dilution at 48-72 hours after inoculation of the cells.

The first virus examined was HSV-2. Serial dilutions from 10^{-5} through 10^{-9} were investigated as a gauge of the TCID. It was shown by this initial experiment that with each dilution a CPE 2 score was obtained. In this case the viral stock needed to be further diluted to detect the TCID value, and thus the experiment was repeated using dilutions from 10^{-8} to 10^{-12}. It was shown that the 10^{-10} dilution was the point from which visible viral infection was undetectable. Thus the tissue infectious dose for HSV-2 was 10^{-10} and indicated a rough viral titre of 10^{10} viral particles per ml within the stock solution.

The other three viruses were also tested, using dilutions from 10^{-6} to 10^{-10}, their results likewise gave TCID values of 10^{-10}.

In further experiments each viral isolate was to be tested at one million fold, then if unsuccessful one thousand fold excess of 10^{-10}, thus at dilutions of 10^{-4} and 10^{-7}.

4.3 HONEY DENSITY

4.3.1 Introduction

To accurately measure out honey for the preparation of different concentrations of honey as percentages by volume for the use in further experiments investigating antiviral activity, it was necessary to determine the density of each honey so that the required volume could be obtained by measuring out an appropriate weight of honey.

4.3.2 Methods

As described in Section 3.2.4, a known volume of distilled water was measured in a measuring cylinder and tared on a balance beam. A specific weight of honey was added to the cylinder and the new volume measured and recorded. Calculations of the required grams of honey per ml of solution were determined as shown in Table 4.6.

4.3.3 Results

Honey density calculations are shown in Table 4.6.

Table 4.6 Honey density calculations

Honey type	NV (ml)	NV/OW (ml/g)	NV-OV (ml)	Density OW/NV-OV (g/ml)
Manuka M116, M112, M157, M151, M155	8.5	8.5/5 = 1.7	8.5 - 5 = 3.5	5/3.5 = 1.4
Honeydew HD19	8.5	8.5/5 = 1.7	8.5 – 5 = 3.5	5/3.5 = 1.4
Rewarewa R19/06	8.5	8.5/5 = 1.7	8.5 – 5 = 3.5	5/3.5 = 1.4
Ling Heather LH27	9	9/5 = 1.8	9 - 5 = 4	5/4 = 1.2

Key: NV new volume, OV original volume, OW original weight.

4.4 INVESTIGATING THE CHARACTERISTIC PROPERTIES OF HONEY

4.4.1 Introduction

The range of honeys used were chosen to have different characteristics so that comparisons could be made between antiviral activity and any particular characteristic. These characteristics were measured for repeatedly over the course of this study in case they changed.

4.4.2 Methods

Using the methods outlined in Chapter 3, Sections 3.2.5 to 3.2.7, the standard method for the assay of antibacterial activity of honey, the method described by Adams (2008) to measure methylglyoxal levels, and the trolox equivalent antioxidant capacity (TEAC) method to detect free radical scavenging activity,

were used to detect the total activity and non-peroxide activity, methlyglyoxal and antioxidant capacity levels of each honey at 0, 6, and 12 monthly intervals.

4.4.3 Results

Results are shown in Table 4.7.

Table 4.7 Average activity levels over course of study

Honey	TA (w/v) phenol %	NPA (w/v) phenol %	TEAC mmol/kg	MGO mg/kg
M116	28.10	27.25	0.62	1241.70
M112	23.73	22.02	0.97	772.40
M157	20.85	20.25	1.27	745.50
M151/M155	0	0	1.09	116.95
LH27	19.50	0	1.08	2.45
R19/06	17.10	0	1.42	25.70
HD19	15.10	0	1.73	6.80

Key: TA total antibacterial activity, NPA non-peroxide antibacterial activity, TEAC trolox equivalent antioxidant capacity, MGO methylglyoxal.

4.4.4 Discussion

The Manuka honeys have generally high total (the majority of which is non-peroxide) and non-peroxide antibacterial activity, and methylglyoxal levels. The other honeys generally have lower levels of total antibacterial activity and methylglyoxal levels, and do not have non-peroxide antibacterial activity, however, they characteristically have high levels of antioxidant capacity.

4.5 THE A549 CELL LINE TOLERANCE OF HONEY

4.5.1 Introduction

There was a possibility that morphological changes of the cells could be due to the highly osmotic nature of honey. The maximum concentration of each honey that was tolerable by the A549 cell line was therefore determined.

In order to detect morphological changes, confluent A549 cells were prepared then treated with 0% to 5% concentrations of honey. Observations were made over two days to record any morphological changes of the cells and to allocate a specific score so as to monitor the level of change.

4.5.2 Methods

First and second examinations

A549 cells were seeded in 24 well plates as described in Section 3.2.1. Once confluent, 0% to 5% honey solutions of the first honeys to be tested were prepared in maintenance medium using the densities outlined in Table 4.1, at 1% intervals, and then in the next experiment 0% to 3% concentrations at 0.5% intervals, these were mixed and filter sterilised. The cell medium was removed from the cells and replaced with 1 ml of these various honey solutions. Observations were made daily to record any changes that occurred to the cells due to the presence of honey within the cell culture medium and change was recorded using the scoring system outlined in Table 4.8. The first experiment was done twice in duplicate, and the second experiment done once in duplicate.

Table 4.8 Morphological scoring system

Morphology	Score
100% dividing, 0% swollen	0
75% dividing, 25% swollen	1
50% dividing, 50% swollen	2
25% dividing, 75% swollen	3
0% dividing, 100% swollen	4
All cells dead	5

4.5.3 Results

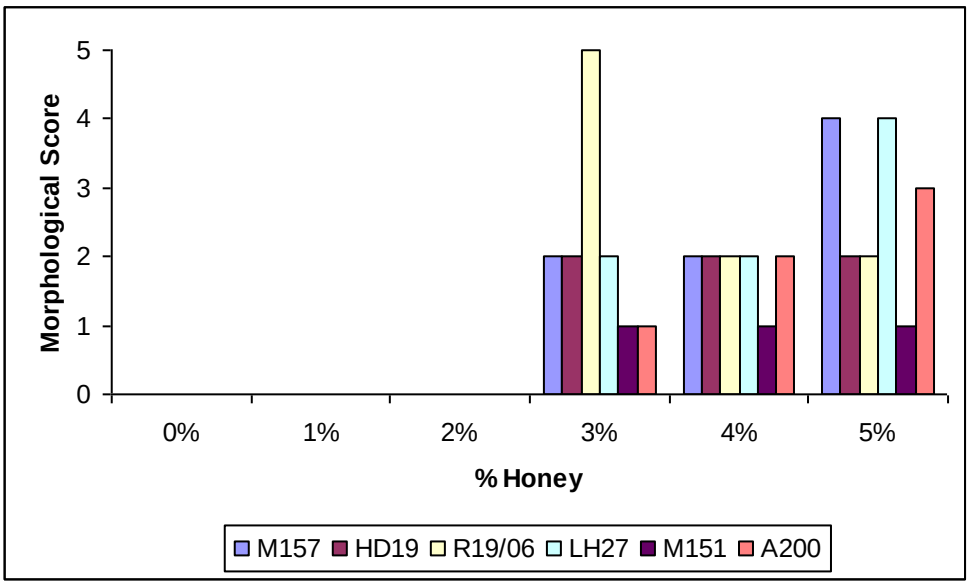
The observed morphological changes of the cells due to the honey solutions are shown in Figures 4.7 to 4.8. Where the duplicates gave identical results one bar indicates the level of morphological change. Table 4.9 summarises the maximum concentrations of honey that can be tolerated by the A549 cells.

First examination of the A549 cell line tolerance to honey

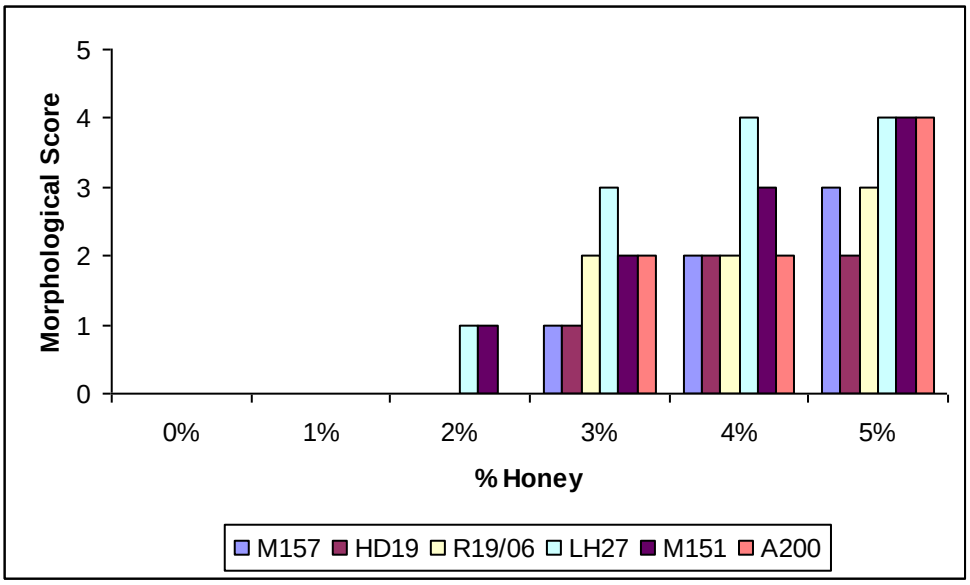
After 1 hour exposure of the cells to the various honey solutions no changes had yet occurred.

Figure 4.7 Morphological changes caused by each honey treatment at: (a) 24 hours, (b) 24 hours repeat, (c) 48 hours, and (d) 48 hours repeat

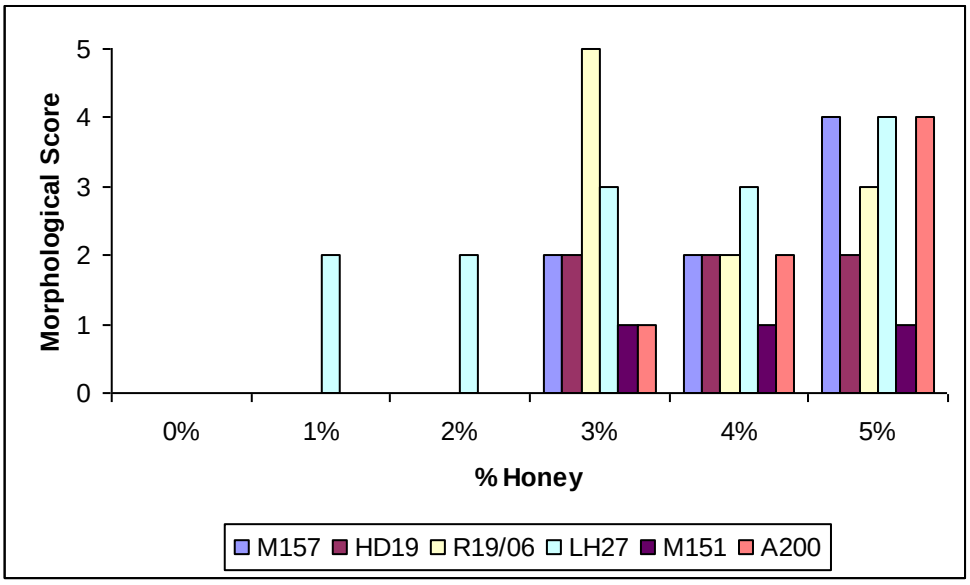
(a) 24 hours



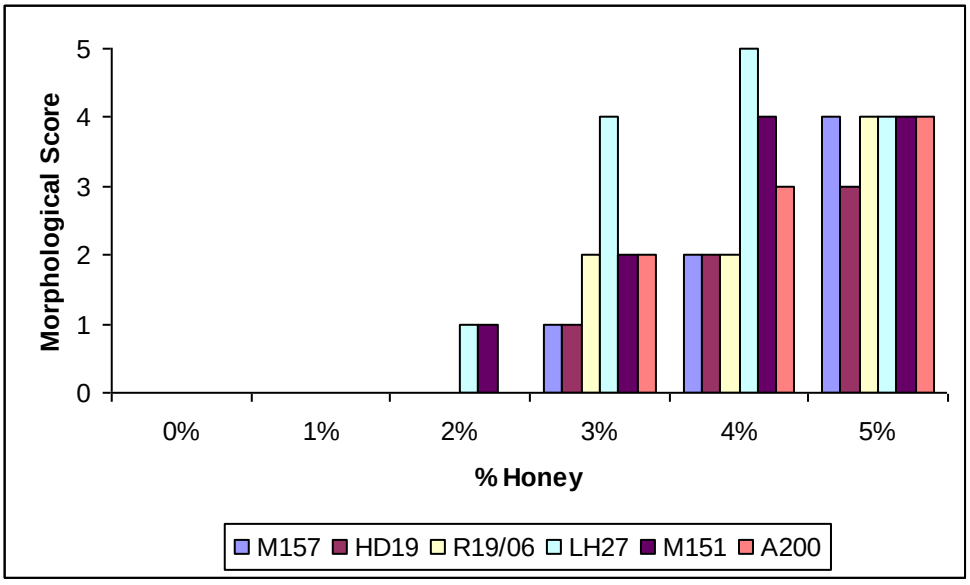
(b) 24 hours repeat



(c) 48 hours



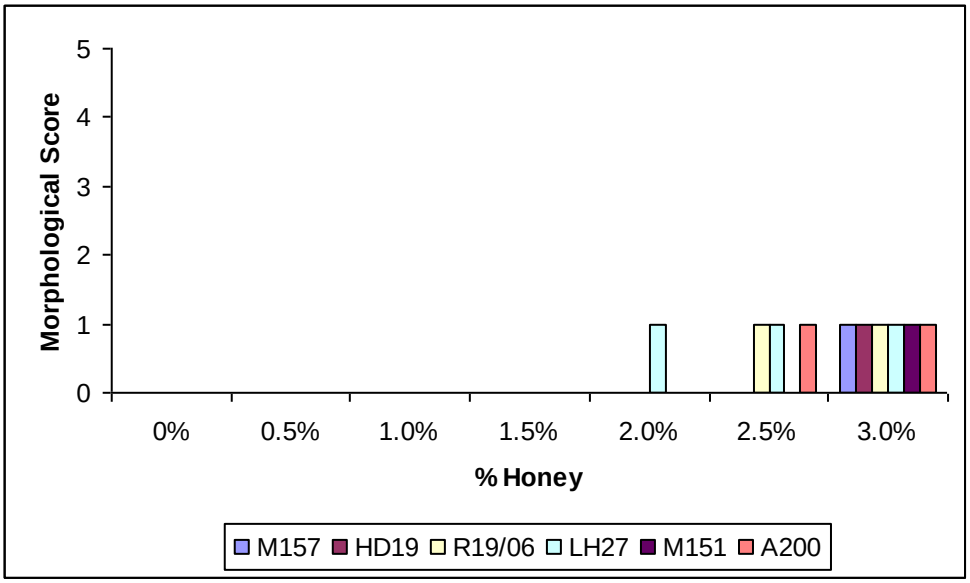
(d) 48 hours repeat



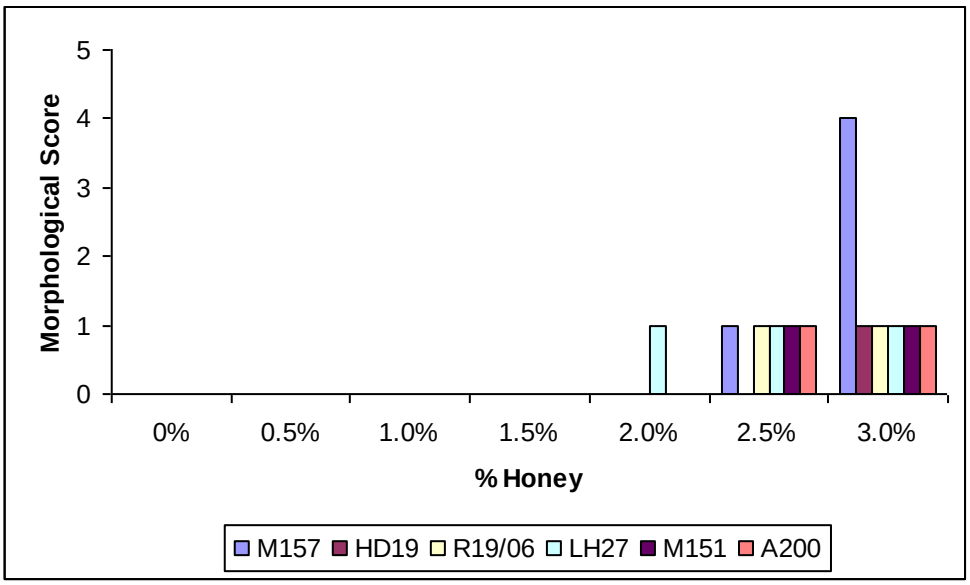
Second examination of the A549 cell line tolerance to honey

Figure 4.8 Morphological changes caused by each honey treatment at: (a) 24 hours, (b) 48 hours, and (c) 72 hours

(a) 24 hours



(b) 48 hours



(c) 72 hours

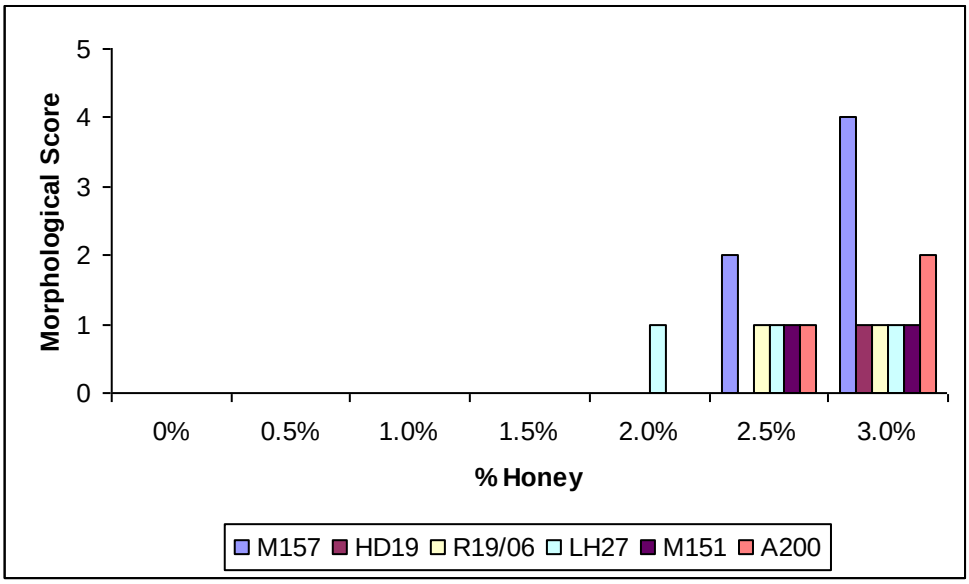


Table 4.9 Maximum concentrations of honey that can be tolerated without causing morphological changes shown by: (a) the first examination, and (b) the second examination

(a) The first examination

Honey	Experiment 1	Experiment 2
M157	2%	2%
HD19	2%	2%
R19/06	2%	2%
LH27	1%	1%
M151	2%	1%
A200	2%	2%

(b) The second examination

Honey	
M157	2%
HD19	2%
R19/06	2%
LH27	1.5%
M151	2%
A200	2%

4.5.4 Conclusion

The maximum concentration of each honey type that could be used without causing any morphological changes in the A549 cells is shown in Table 4.9.

4.5.5 Effect of hydrogen peroxide from honey on the A549 cell line

In order to eliminate the possibility that hydrogen peroxide (a product of diluted honey) was being responsible for the observed effects on cell morphology, an experiment was done with and without catalase using Ling Heather honey LH27 which has the highest antibacterial activity made up of hydrogen peroxide of the non-Manuka honeys.

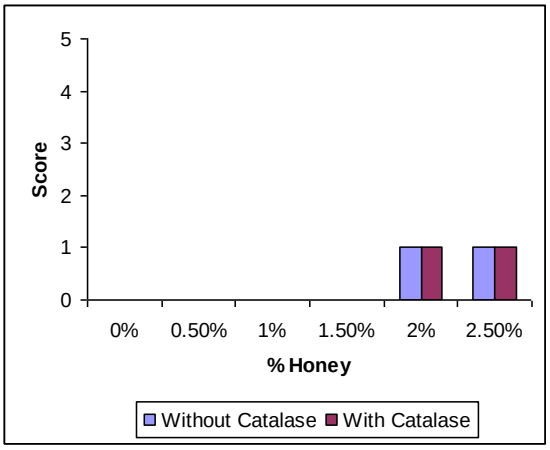
4.3.5.1 Methods

Confluent A549 cells were prepared as described in Section 3.2.1. Catalase solution was made to 2 mg/ml in distilled water and was filter sterilised. Honey solutions from 0% to 2.5% were prepared at 0.5% intervals in maintenance medium, and maintenance medium plus catalase (maintenance medium:catalase equal volumes designated by total volume required for the concentration of the honey solution), these were mixed and filter sterilised.

4.3.5.2 Results

The results are shown in Figure 4.9.

Figure 4.9 Effect of hydrogen peroxide on cell morphology 24 hours after treatment of Ling Heather honey LH27 with or without catalase



4.3.5.3 Discussion

There was no difference recorded in the presence or absence of catalase, therefore the previous observations that Ling Heather honey (LH27) caused changes to the cells from a concentration of 2% were reconfirmed, and it was shown that hydrogen peroxide was not the cause of the morphological changes to the cells.

The level of catalase used in this trial was at the same concentration as used in the experiments testing for non-peroxide antibacterial activity. It was thus a sufficient level to mop up any artefact activity due to hydrogen peroxide. The more damaging honeys may be more osmotic or may have cytotoxic components that may be responsible for the morphological changes to the cells.

Chapter 5

The antiviral activity of honey: Preliminary studies

This chapter describes the first investigations of the antiviral activity of honey.

5.1 INTRODUCTION

In order to evaluate the antiviral activity of honey, a range of methods were used to examine different aspects of the hypothesised antiviral effect. The methods aim to investigate the protective, preventative, and neutralisation capacities, with the goal of gaining a better understanding of the underlying nature of honey's antiviral activity.

These primary experiments tried using cell culture and one type of honey to investigate the feasibility of methodology, to ascertain methods that were most suitable to be used with a range of honey types.

The ability of honey to protect the cells from infection by viruses was evaluated through the culturing of confluent A549 cells within honey-containing growth medium, followed by removal of this medium for inoculation of the cells with a particular viral isolate. This approach aimed to test whether pre-treatment of the cells with honey can prevent the development of viral CPE following inoculation of the cells with virus. Honey was also included into culture medium after inoculation of the cells, to test whether continued exposure of the cells to honey gave better protection by means of more greatly inhibiting the development of viral CPE.

A positive result obtained in such an investigation can provide support towards the use of honey as a preventative treatment for viral infections.

The ability of honey to prevent infection of the cells developing after the initial infection with virus was examined through the exposure of the cells to honey after

inoculation. Honey was included in the medium continuously after inoculation, to test further whether frequent replacement of honey can allow for even lower levels of viral CPE.

This type of investigation can show whether honey can be used as a treatment for viral infections once infection has taken place.

The neutralisation effect of honey on viruses was evaluated through the exposure of each viral isolate to honey prior to inoculation of prepared A549 cells with the honey-treated virus. This experiment can show whether honey could be used as a therapy for treating viral infections, as if neutralisation is found to occur with a short period of exposure of virus to honey, it may be possible for the virus to be inactivated between being shed and infecting other cells.

5.2 PROTECTION OF THE CELLS FROM VIRAL INFECTION

5.2.1 Protection of the cells from viral infection: by pre-exposure of the cells to honey

The first experiments investigating the protective effects of honey against viral infection aimed to evaluate the differences in the consequential levels of viral CPE when the cells were infected with viral culture prepared in medium with or without honey. These experiments further examined the influence of washing off the residual honey-containing growth medium from the cells prior to inoculation with virus compared with only removing the honey-containing growth medium. The aim of this

was to determine whether the remaining traces of honey had an effect on the development of viral CPE.

5.2.1.1 Methods

Growth medium was prepared containing 0%, 0.5%, 1%, and 2% concentrations of Manuka honey M157, these were mixed and filter sterilised. Stocks of A549 cells were prepared for seeding into 24 well plates as described in Section 3.2.1. Appropriate volumes of the cell suspension in trypsin EDTA were mixed with the honey solutions as done in Section 3.2.1 based on the size of the flask of cells. One ml volumes of the cell-honey solutions were distributed into 24 well plates and incubated overnight at 37°C until confluent. The viral inoculum (diluted to 10^{-4}) was prepared in (1) maintenance medium or (2) honey solutions prepared in maintenance medium immediately prior to inoculation of the cells. The honey-containing growth medium was removed and (a) the cells were washed twice with PBS or (b) the cells were left un-washed. The viral inoculum was then used to infect the cells for 1 hour at 37°C. Controls of each honey concentration without virus were also tested. The inoculum was removed and the cells were supplemented with 1 ml of maintenance medium. The plates of cells were incubated at 37°C and observations of the development of viral CPE were made daily and a score allocated based on the severity using the system outlined in Section 4.1 Figure 4.6. Each experiment was completed in duplicate.

5.2.1.2 Results

The results of the experiments are presented as plots of developing CPE in Figures 5.1 to 5.10. The controls of each honey concentration without virus were shown to not cause morphological changes of the cells over the observation period. Where the different treatments gave identical results, one graph illustrates the levels of CPE observed.

Figure 5.1 CPE from Ad3 after pre-treatment of the cells with honey

A549 cells were pre-treated with various concentrations of honey, with the honey washed off the cells, and with the honey just aspirated, not washed off the cells, with the viral inoculum prepared in medium without honey.

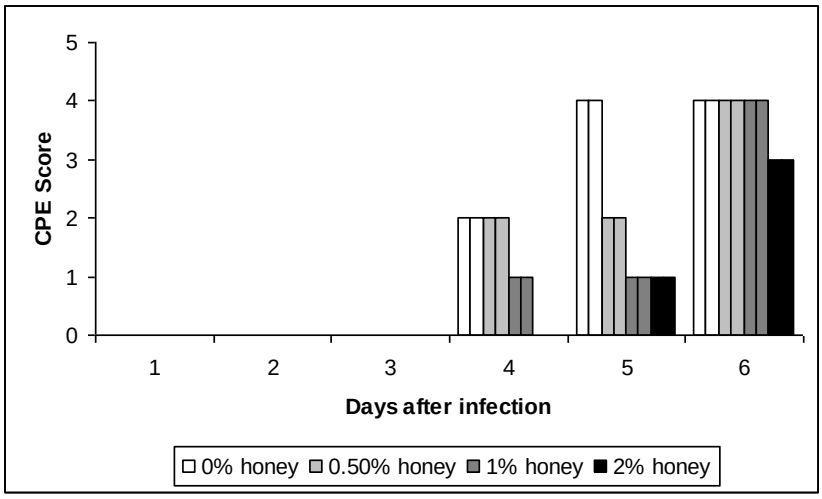


Figure 5.2 CPE from Ad3 after pre-treatment of the cells with honey

A549 cells were pre-treated with various concentrations of honey, with the honey washed off the cells, and with the honey just aspirated, not washed off the cells, with the viral inoculum prepared in honey solutions.

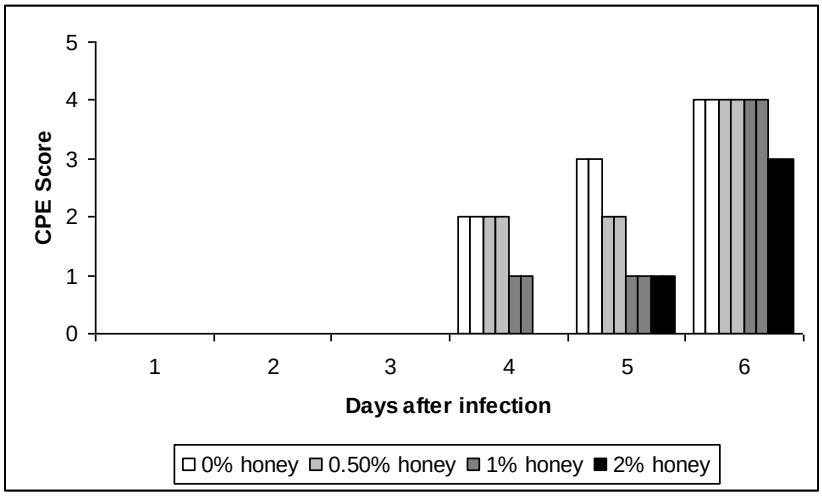


Figure 5.3 CPE from HSV-1 after pre-treatment of the cells with honey (washed off and inoculum without honey)

A549 cells were pre-treated with various concentrations of honey, with the honey washed off the cells and viral inoculum prepared in medium without honey.

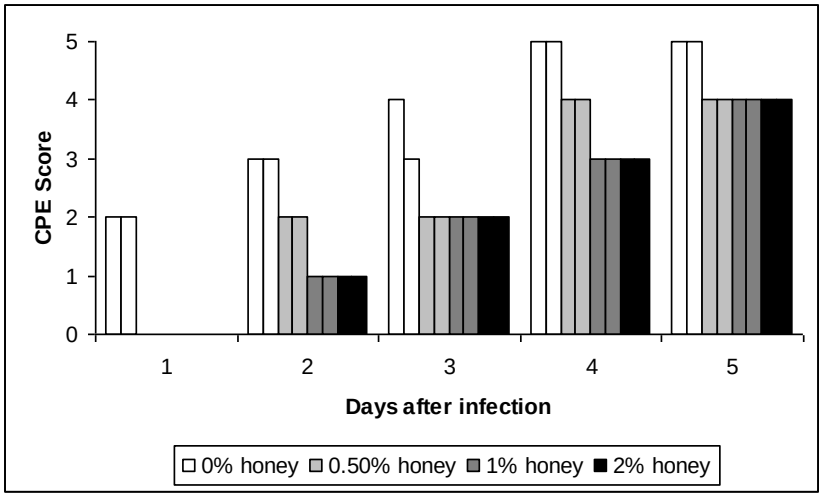


Figure 5.4 CPE from HSV-1 after pre-treatment of the cells with honey (not washed and inoculum without honey)

A549 cells were pre-treated with various concentrations of honey, with the honey just aspirated, not washed off the cells and viral inoculum prepared in medium without honey.

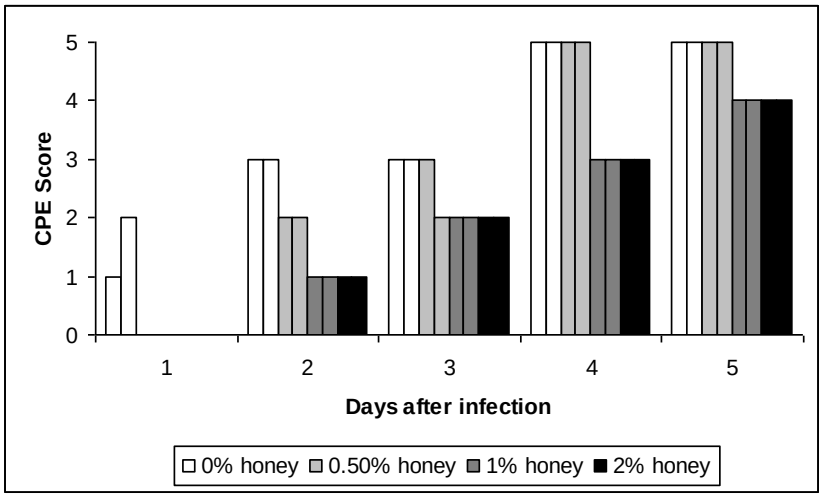


Figure 5.5 CPE from HSV-1 after pre-treatment of the cells with honey (washed off and inoculum with honey)

A549 cells were pre-treated with various concentrations of honey, with the honey washed off the cells and viral inoculum prepared in honey solutions.

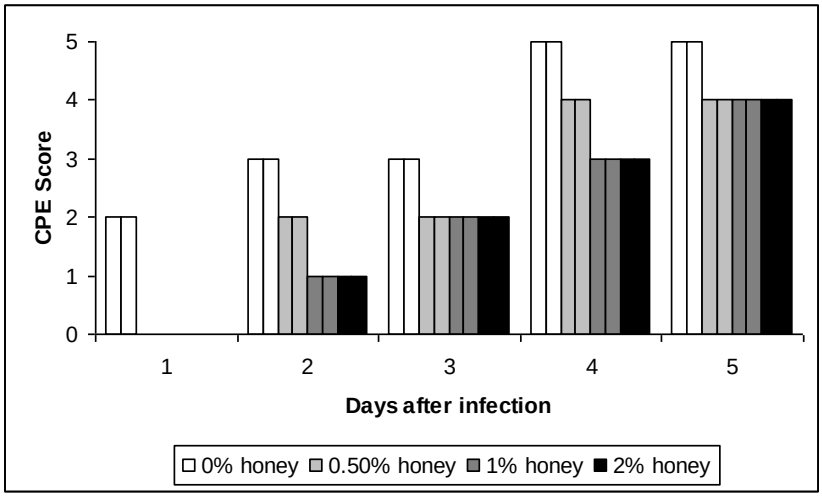


Figure 5.6 CPE from HSV-1 after pre-treatment of the cells with honey (not washed and inoculum with honey)

A549 cells were pre-treated with various concentrations of honey, with the honey solution just aspirated, not washed off the cells and viral inoculum prepared in honey solutions.

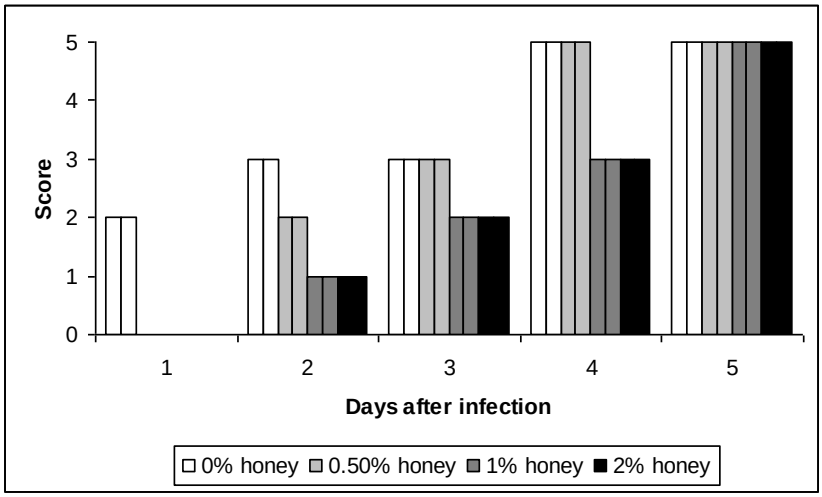


Figure 5.7 CPE from HSV-2 after pre-treatment of the cells with honey (washed off and inoculum without honey)

A549 cells were pre-treated with various concentrations of honey, with the honey washed off the cells and viral inoculum prepared in medium without honey.

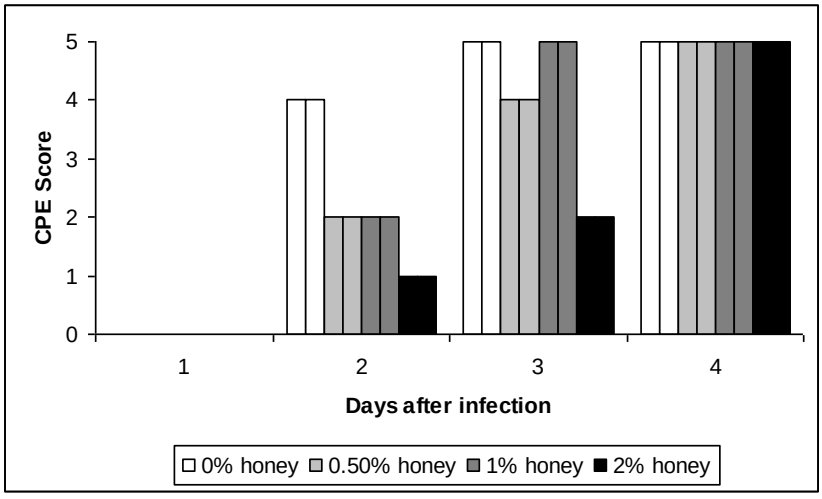


Figure 5.8 CPE from HSV-2 after pre-treatment of the cells with honey (not washed and inoculum without honey)

A549 cells with HSV-2 virus were pre-treated with various concentrations of honey, with the honeys just aspirated, not washed off the cells and viral inoculum prepared in medium without honey.

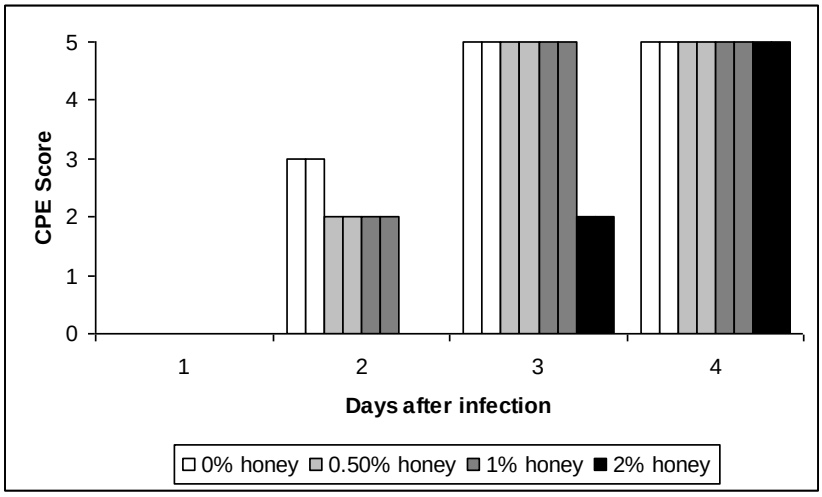


Figure 5.9 CPE from HSV-2 after pre-treatment of the cells with honey (washed off and inoculum with honey)

A549 cells were pre-treated with various concentrations of honey, with the honey washed off the cells and viral inoculum prepared in honey solutions.

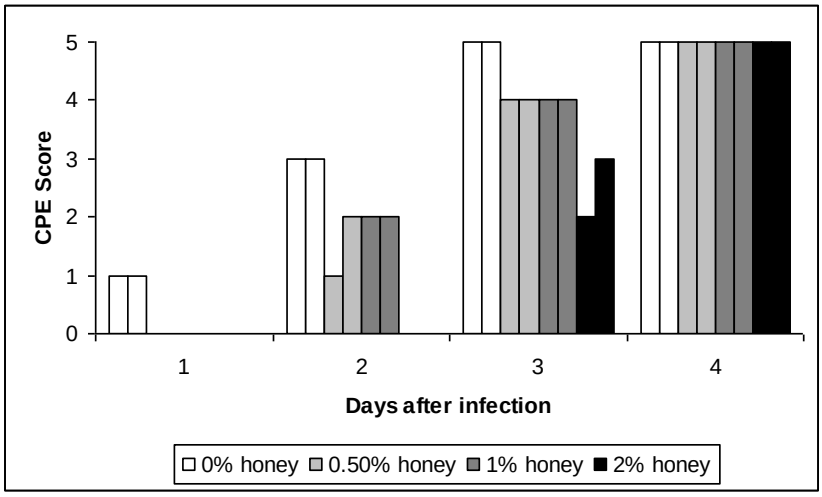
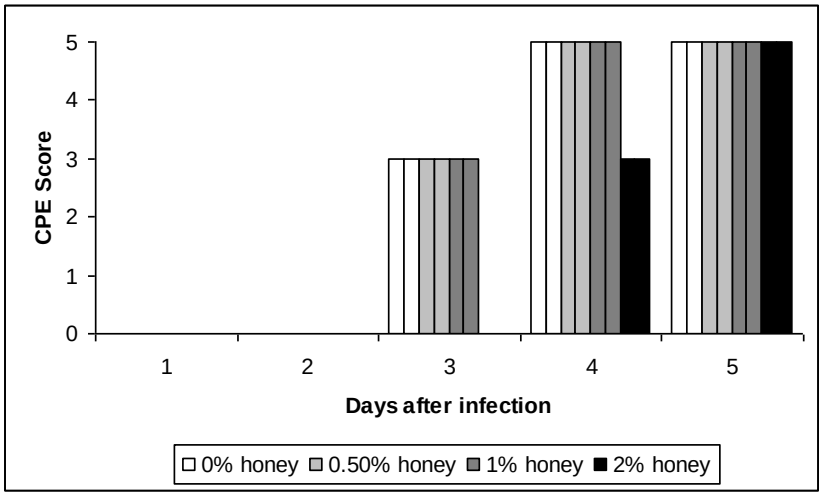


Figure 5.10 CPE from HSV-2 after pre-treatment of the cells with honey (not washed and inoculum with honey)

A549 cells were pre-treated with various concentrations of honey, with the honey solution just aspirated, not washed off the cells and viral inoculum prepared in honey solutions.



5.2.1.3 Summary of results

As shown with each figure, there was a temporal delay observed in the development of CPE when the cells had been pre-exposed to honey. In general the higher concentrations of honey reduced the severity of CPE for the longest period.

With Ad3 and HSV-1 there were no differences observed with the resulting CPE levels between washing the honey off the cells, or, just removing it without washing, prior to inoculation of the cells with virus. Nor, with Ad3, were there any differences in CPE resulting from the preparation of the viral inoculum with or without honey in the maintenance medium. With HSV-2 there were similar observations between the washing off of the honey from the cells and just removing it. However, with the virus

prepared in medium containing honey, greater differences were observed between the washing off and removing of the honey from the cells, shown by the levels of resulting CPE observed unseen with the other experiments. These observations can suggest little effect of the residual honey on the development of CPE. Likewise, the brief exposure of virus to honey in the solutions used to prepare the virus for inoculation of the cells made little to no difference to the resulting CPE.

5.2.2 Protection of the cells from viral infection: by continued exposure to honey

Experiments were carried out in which honey was included in the medium after inoculation of the cells with virus. These experiments were done to examine the effect of continued exposure of the cells to honey on the development of viral CPE. It was expected that if the honey was having a protective effect on the cells then if honey was present continuously after inoculation that lower levels of viral CPE would be observed compared with the first protection experiments.

5.2.2.1 Methods

The method used was similar to that described in Section 5.2.1.1 The honey was removed from the cells, not washed off, and the viral inoculum (diluted to 10^{-4}) was prepared in maintenance medium. The cells however, were incubated with honey-containing maintenance medium after inoculation with virus, with the honey at the same concentrations as used during pre-exposure. Each experiment was completed in duplicate.

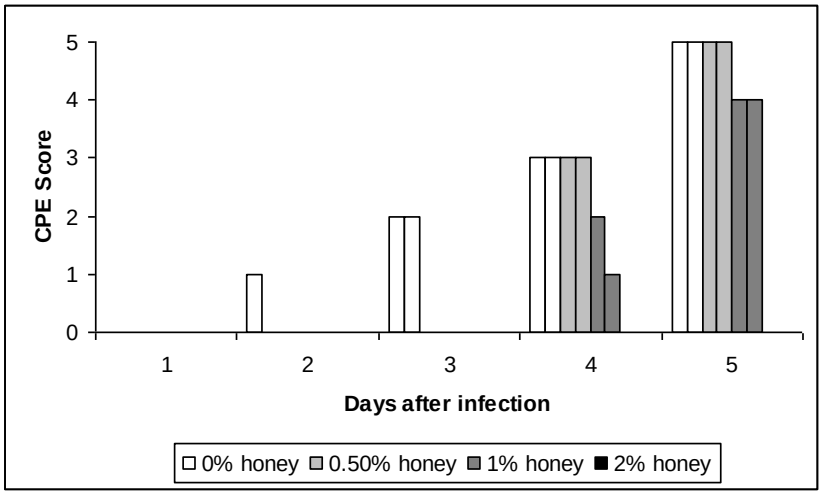
5.2.2.2 Results

The results of the experiments are presented as plots of developing CPE in Figure 5.11. The controls of each honey concentration without virus were shown to not cause morphological changes of the cells over the observation period.

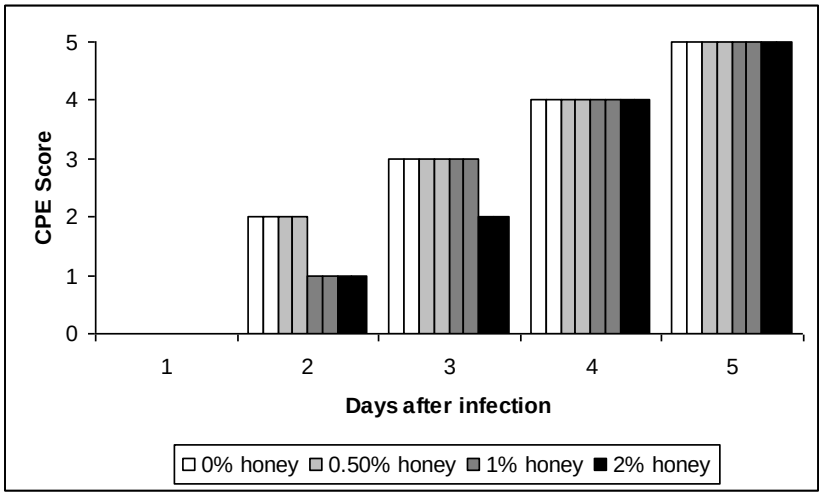
Figure 5.11 CPE from (a) Ad3, (b) HSV-1, and (c) HSV-2 after pre-treatment of the cells with honey and continued exposure to honey after inoculation with virus

A549 cells were pre-treated with various concentrations of honey, and after inoculation with virus were continuously exposed to honey at the same concentrations as in the pre-treatment.

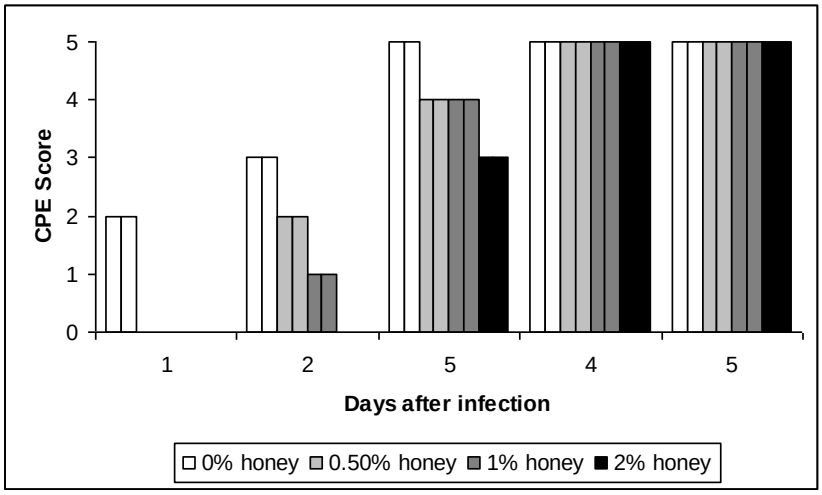
(a) Ad3



(b) HSV-1


CPE Score
0
1
2
3
4
5
1
2
3
4
5
Days after infection
0% honey
0.50% honey
1% honey
2% honey

(c) HSV-2


CPE Score
0
1
2
3
4
5
1
2
5
4
5
Days after infection
0% honey
0.50% honey
1% honey
2% honey

5.2.2.3 Summary of results

As with the first protection experiments a temporal delay was observed with the honey treatments, where generally the higher concentrations of honey used the more greatly reduced was the severity of the CPE.

It was hypothesised that the honey would have a greater effect against the development of a CPE when the cells were continuously exposed to it after the inoculation with virus. However, this was found not to be the case as lower levels of CPE were observed only in the Adenovirus experiment as shown in Figure 5.11 (a) in which the 2% honey concentration prevented the development of viral CPE.

5.2.3 Protection of the cells from viral infection: by frequent supplement of honey

These experiments which involved frequently replacing the cell medium with fresh honey-containing medium aimed to investigate the impact of repetitive re-supplementation of the cells with honey on the development of CPE. It was reasoned that even lower levels may be observed compared with the previous methodologies, as a daily dose of antiviral agent was being used on the cells. It was thought possible that during the observation period antiviral factors in the honey may be destroyed by chemical reaction or cellular metabolism.

5.2.3.1 Methods

The method used was the same as that described in Section 5.2.2.1. The cells however, were incubated with honey-containing maintenance medium which was

removed each day and replaced with fresh honey-containing maintenance medium, the honey was used at the same concentrations as during the pre-exposure.

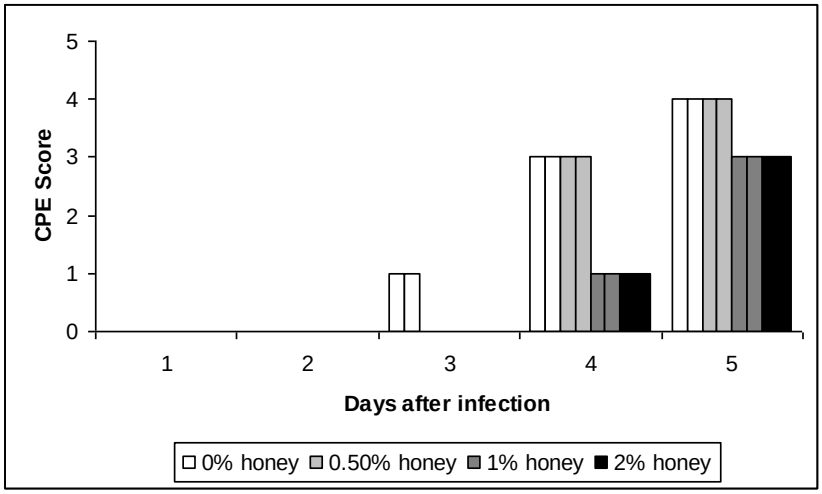
5.2.3.2 Results

The results are presented in Figure 5.12 as plots of developing CPE. The controls of each honey concentration without virus were shown to not cause morphological changes of the cells over the observation period.

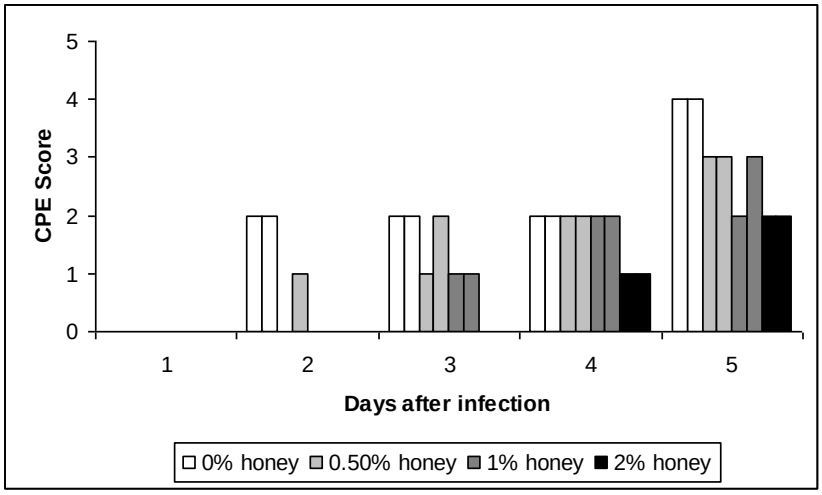
Figure 5.12 CPE from (a) Ad3, (b) HSV-1, and (c) HSV-2 after pre-treatment of the cells with honey and continued exposure to fresh honey each day

A549 cells were pre-treated with various concentrations of honey, and after inoculation with virus were continuously exposed to honey which was replaced each day with fresh honey solution.

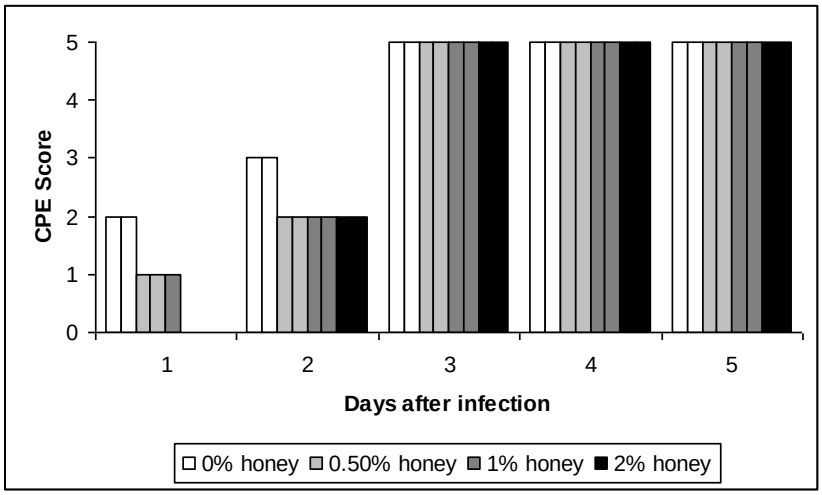
(a) Ad3



(b) HSV-1



(c) HSV-2



5.2.3.3 Summary of results

With these experiments, greater suppression of viral CPE was hypothesised to be seen. With each virus the observations that were made were similar to those of the

first protection experiments, suggesting very little effect from the frequent replacement of the medium with fresh honey-containing medium.

5.3 PREVENTION OF VIRAL INFECTION

These experiments aimed to test whether incubation of the cells with honey can prevent the spread of the virus from infected cells to uninfected cells and whether frequent replacement of the honey-containing medium would give lower levels of viral CPE. In these experiments the cells were firstly infected with virus before the honey was added as part of the medium.

5.3.1 Methods

A549 cells were seeded into 24 well plates and incubated at 37°C overnight until confluent as described in Section 3.2.1. The virus (diluted to 10^{-4}) was prepared in maintenance medium. The growth medium was removed from the cells, and 200 µl of viral solution was used to inoculate the cells for 1 hour at 37°C, including controls without virus using each of the honey concentrations tested. The residual inoculum was removed from the cells at the conclusion of the inoculation period, and maintenance medium containing honey at 0%, 0.5%, 1%, and 2% was added to the cells. Each experiment was completed in duplicate.

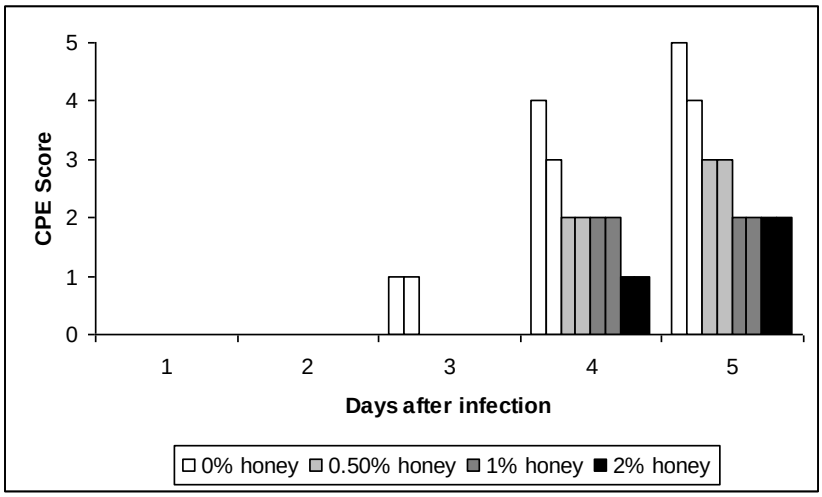
Further experiments were done in the same way but with the daily replacement of the honey solutions with freshly made ones.

5.3.2 Results

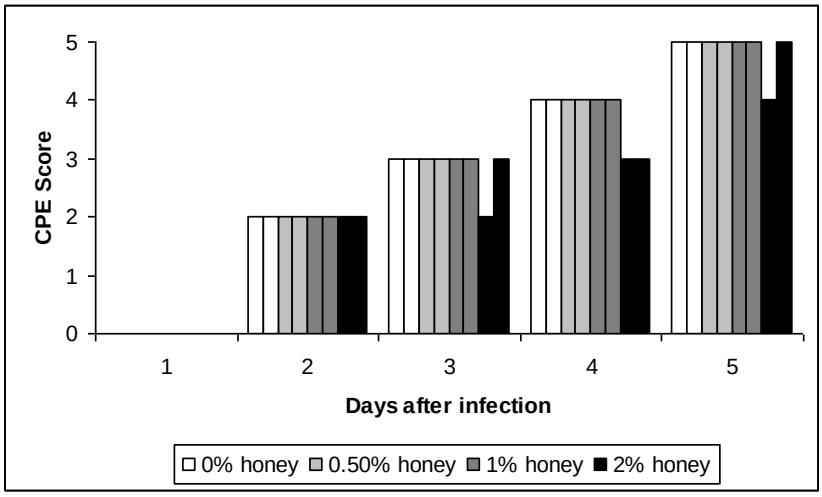
The results are presented in Figures 5.13 and 5.14 as plots of CPE development. No morphological changes of the cells were observed in the controls without virus.

Figure 5.13 CPE from infection of A549 cells with: (a) Ad3, (b) HSV-1, and (c) HSV-2, with the cells treated after inoculation with various concentrations of honey

(a) Ad3



(b) HSV-1



(c) HSV-2

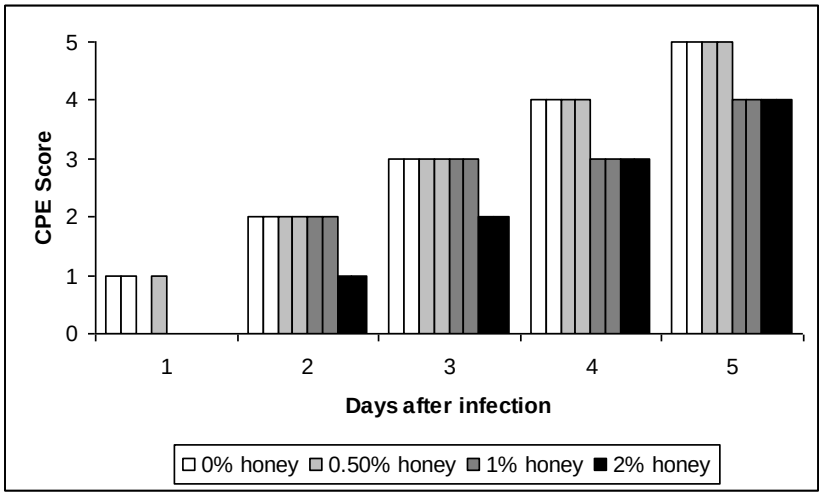
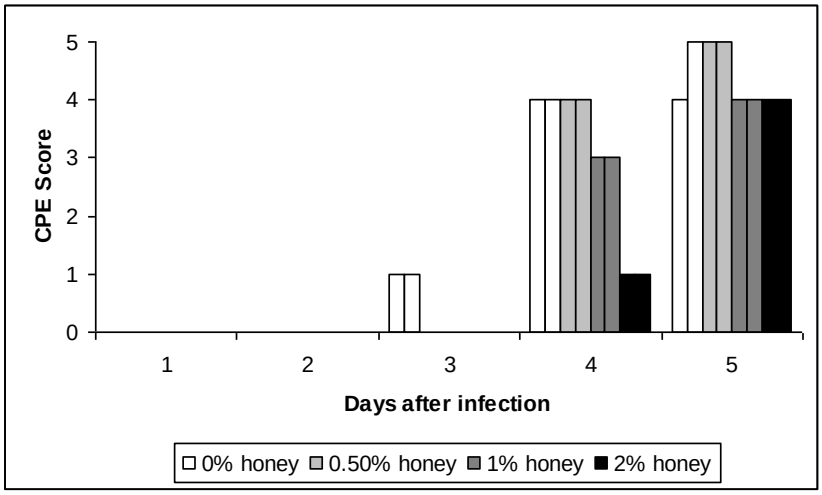
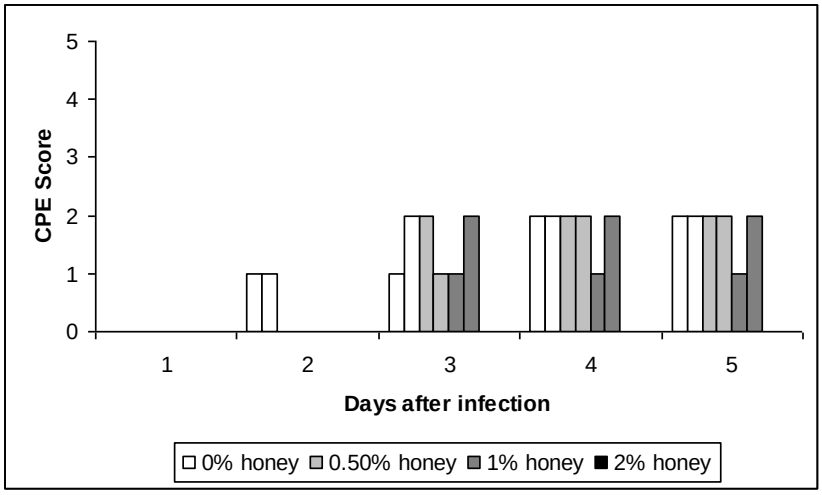


Figure 5.14 CPE from infection of A549 cells with: (a) Ad3, (b) HSV-1, and (c) HSV-2, with the cells treated after inoculation with various concentrations of honey which were replaced daily with fresh ones

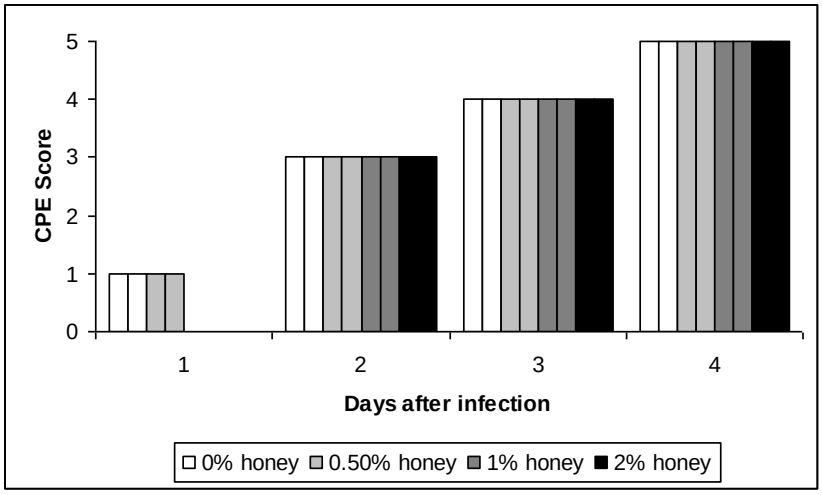
(a) Ad3



(b) HSV-1



(c) HSV-2



5.3.3 Discussion

In these experiments, the honey has been shown to have an effect against the spread of virus from infected cells to uninfected cells, as shown by the lower levels of viral CPE observed with the honey treatments compared with the untreated virus over the course of observation.

In the first experiments, treating the cells after inoculation with various concentrations of honey, each honey treatment suppressed the severity of viral CPE, where the higher concentrations had the greatest effect with each virus. In the second experiments involving frequent replacement of honey-containing medium, with Ad3 overall higher levels of viral CPE were observed with each treatment compared with the first experiments. Similarly, with HSV-2, the honey treatments had little effect. With HSV-1, however, the replacement of the medium with fresh honey solutions caused suppression of the virus with honey at a concentration of 2% over the observation period. The other honey treatments had little effect.

5.4 NEUTRALISATION

By exposing each viral isolate to solutions of honey for a defined period, this aimed to test whether honey had a direct effect on the virus. Neutralisation can be observed as the absence of viral CPE after inoculation of the cells with the honey-treated viruses.

5.4.1 Methods

A549 cells were seeded into 24 well plates and incubated overnight at 37°C until confluent as described in Section 3.2.1. Honey at concentrations of 0%, 0.5%, 1%, and 2%, and viruses diluted to 10^{-6} (in initial experiments) and to 10^{-3} (in subsequent experiments) were prepared in maintenance medium. One hundred µl of the diluted virus (10^{-6} or 10^{-3}) was added to 900 µl of honey solution to give an appropriate final dilution of the virus at 10^{-7} or 10^{-4}, these viral-honey solutions, the 0% honey-viral solution and honey solutions without virus serving as controls, were incubated with

shaking for 1 hour (in the initial experiments) or 1, 2, 4, 8, 12, 24, or 48 hours (in the subsequent experiments) using a shaking incubator at level 3-4 set at 37°C. At the conclusion of the neutralisation period, the growth medium was removed from the growing cells, and 200 µl of viral-honey solution was used to inoculate the cells for 1 hour at 37°C. The residual viral-honey inoculum was removed from the cells and the cells were washed with PBS, the cells were then supplemented with maintenance medium. Observations were made daily to record the development of CPE for up to 7 days or until a high level of CPE had developed with untreated virus (0% honey-viral solution). Each experiment was completed in duplicate.

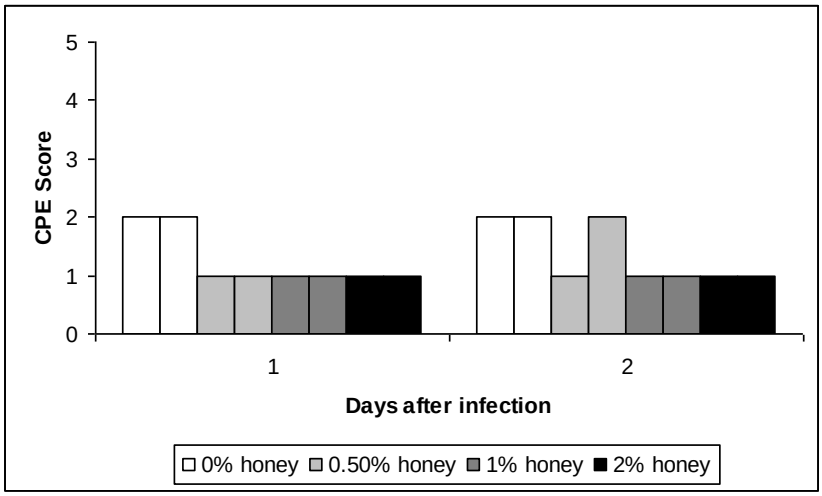
5.4.2 Results

The results are presented in Figures 5.15 to 5.23 as plots of the development of viral CPE after inoculation of the cells with virus. Figures 5.15 to 5.18 show the results after exposure of the viruses to honey for 1 hour. Figures 5.19 to 5.23 show the results after exposure for longer periods. With these figures, the results for each replicate have been given and each successive bar with each concentration of honey indicates a different exposure period of the viruses to honey. These exposure periods are shown above the appropriate bars with the first concentration of honey, to indicate which bar corresponds to which period.

In each experiment the honey solutions without virus caused no morphological changes to the cells over the course of observation.

Figure 5.15 CPE resulting from infection of A549 cells with HSV-2 at a dilution of: (a) 10^{-7} and (b) 10^{-4}, after 1 hour exposure of the virus to honey

(a) 10^{-7}



(b) 10^{-4}

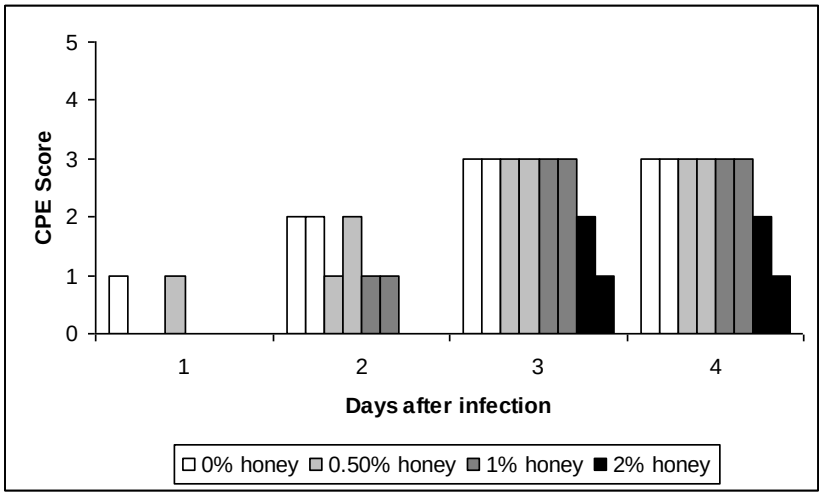
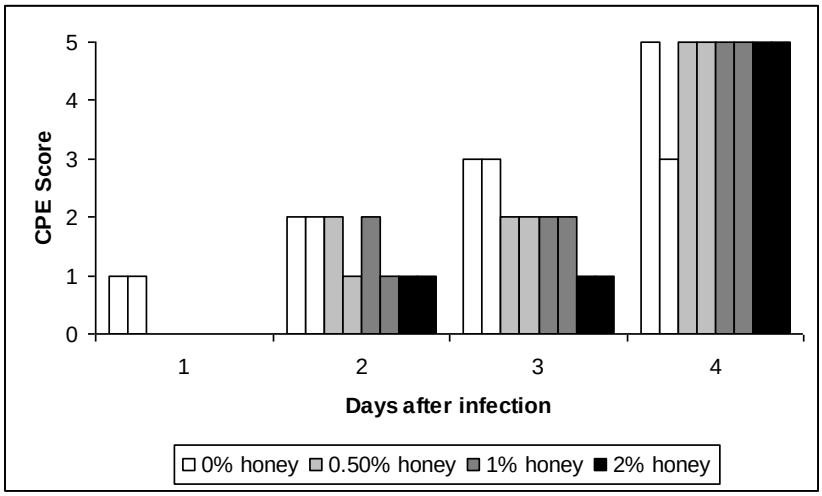


Figure 5.16 CPE resulting from infection of A549 cells with HSV-1 at a dilution of: (a) 10^{-7} and (b) 10^{-4} after 1 hour exposure of the virus to honey

(a) 10^{-7}



(b) 10^{-4}

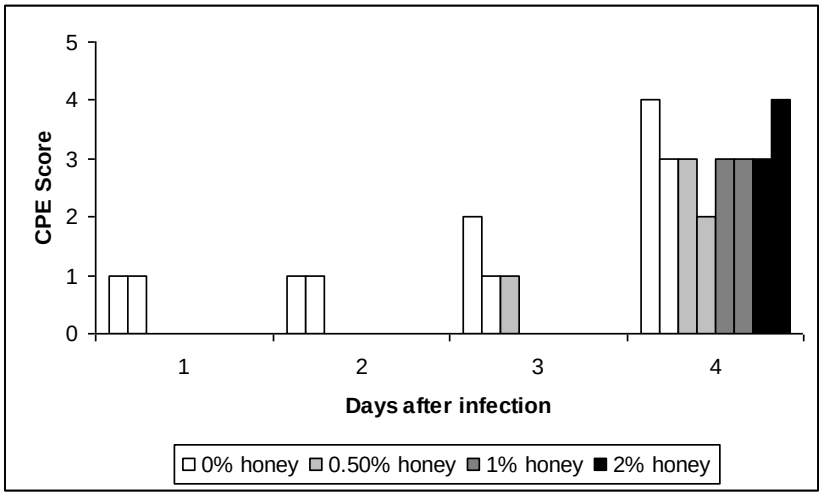
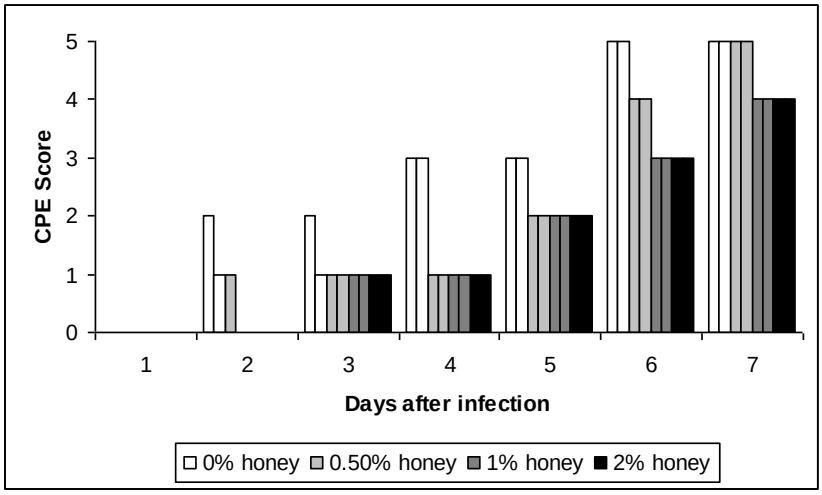


Figure 5.17 CPE resulting from infection of A549 cells with Ad3 at a dilution of: (a) 10^{-7} and (b) 10^{-4}, after 1 hour exposure of the virus to honey

(a) 10^{-7}



(b) 10^{-4}

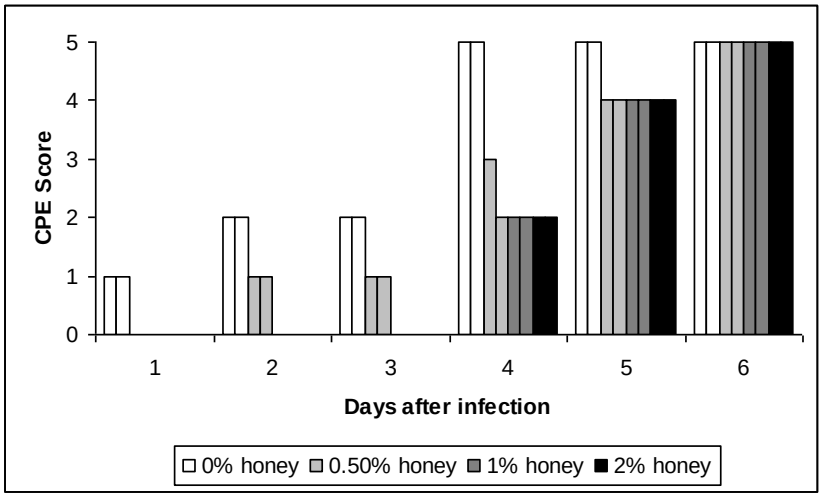
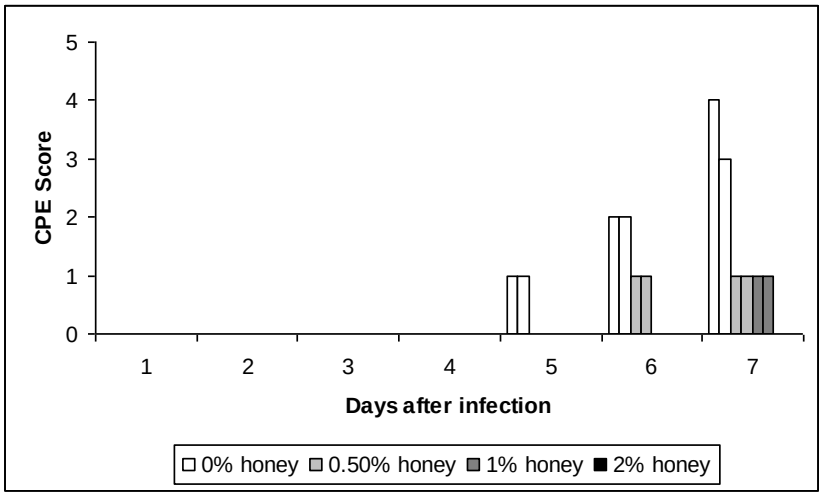


Figure 5.18 CPE resulting from infection of A549 cells with Ad8 at a dilution of: (a) 10^{-7} and (b) 10^{-4}, after 1 hour exposure of the virus to honey

(a) 10^{-7}



(b) 10^{-4}

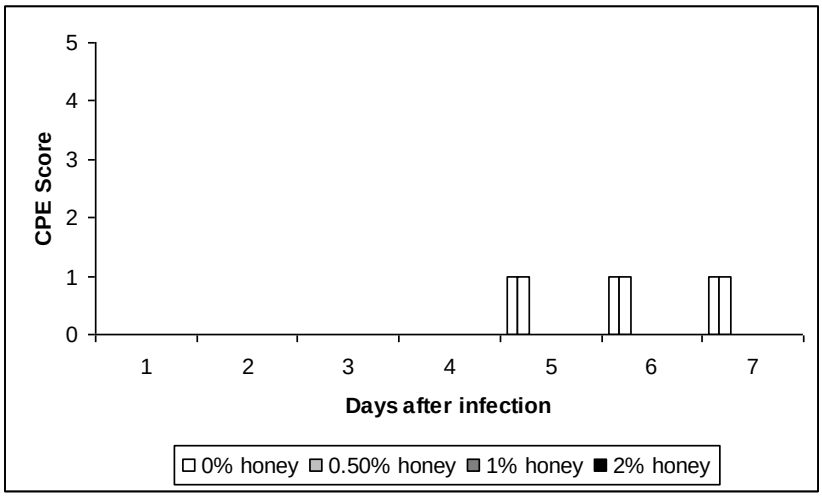
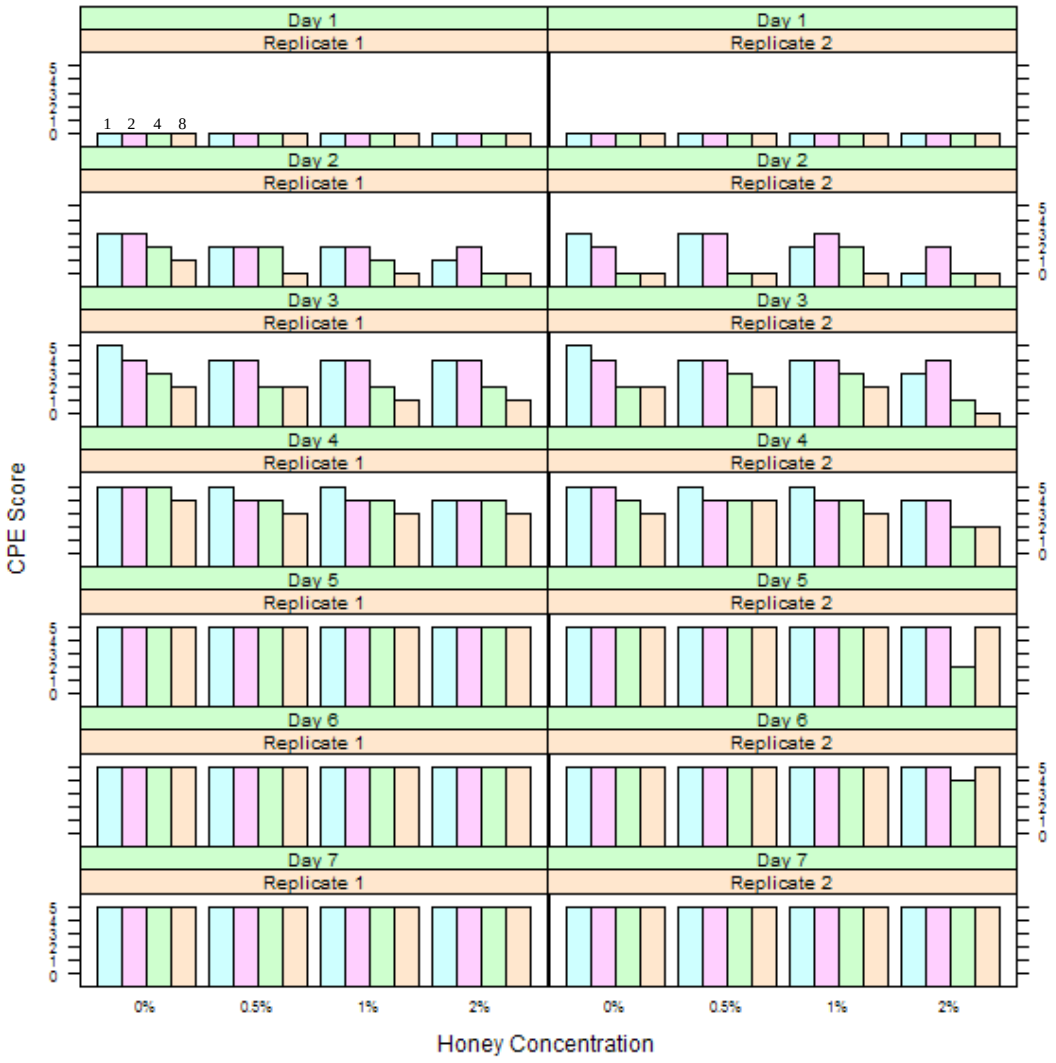


Figure 5.19 CPE resulting from infection of A549 cells with HSV-2 at a dilution of 10^{-4} after: (a) 1 to 8 hours and (b) 12 to 48 hours exposure of the virus to honey

(a) 1 to 8 hours



(b) 12 to 48 hours

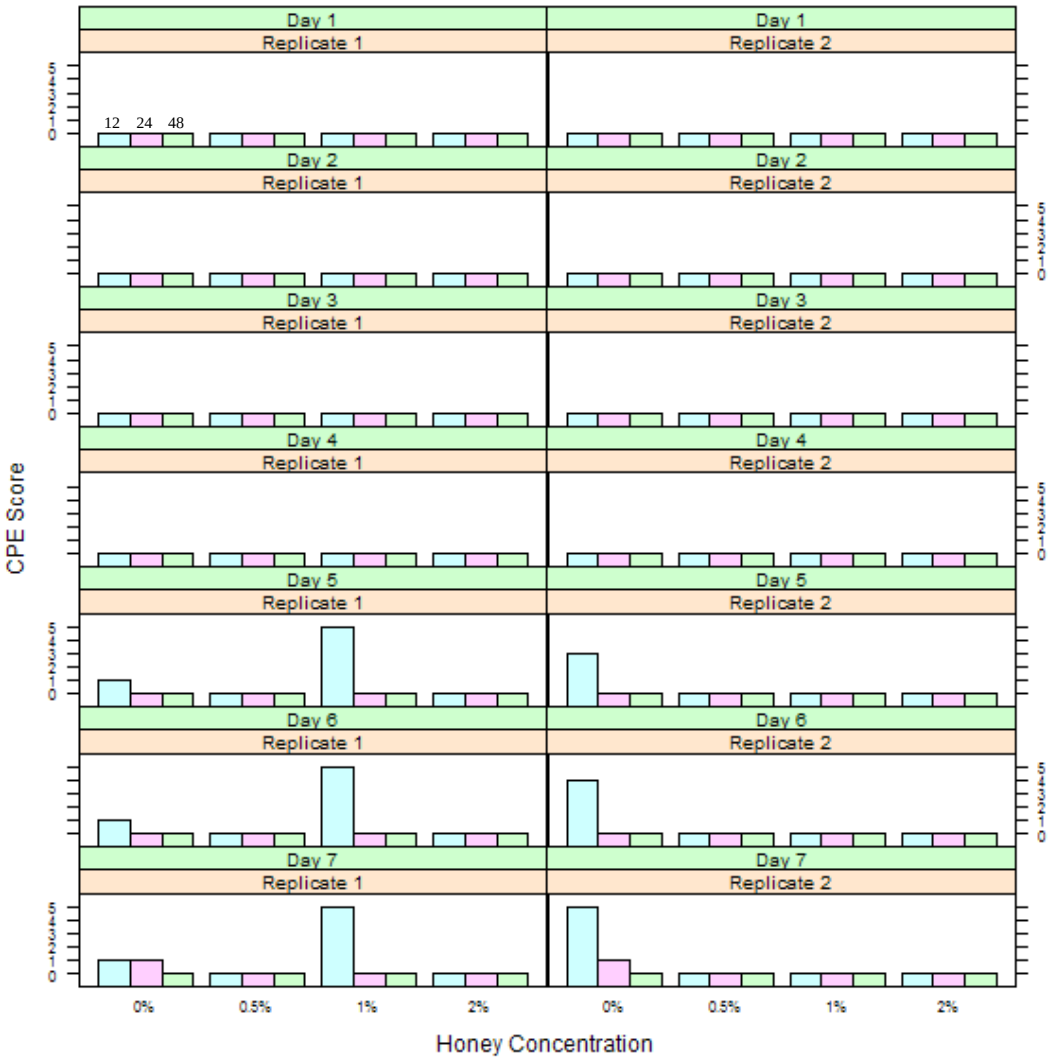
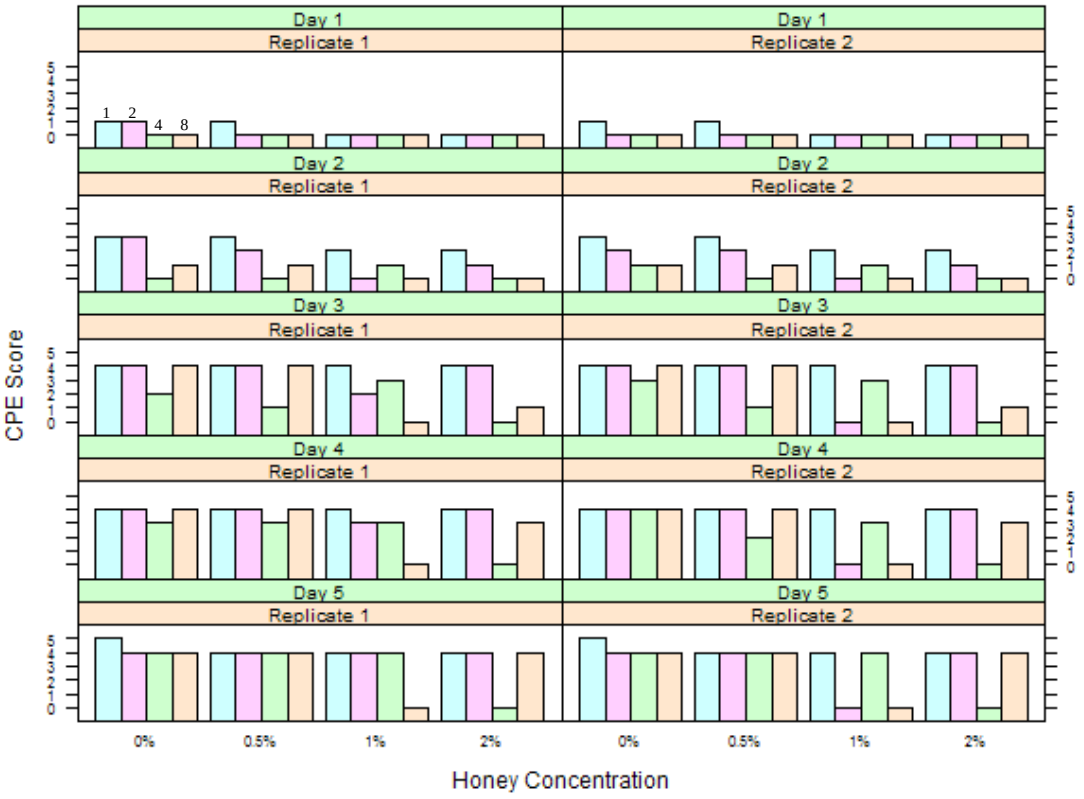


Figure 5.20 CPE resulting from infection of A549 cells with HSV-2 at a dilution of 10^{-4} after: (a) 1 to 8 hours and (b) 12 hours exposure of the virus to honey

(a) 1 to 8 hours



(b) 12 hours

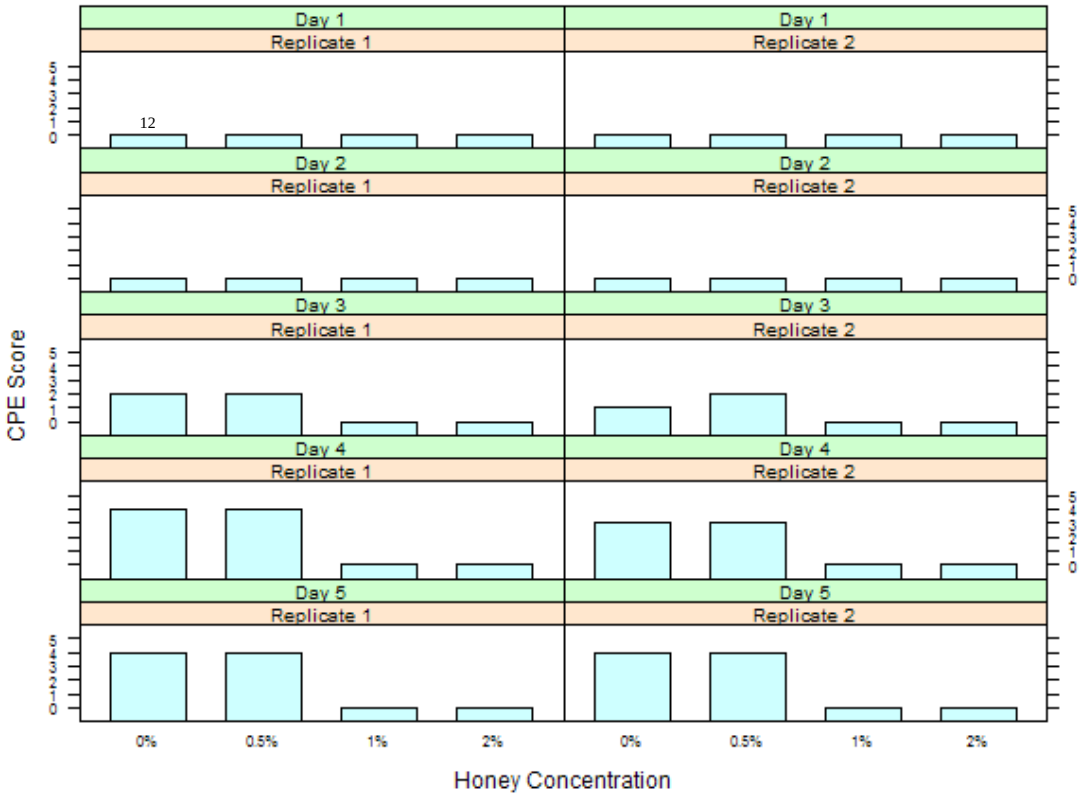
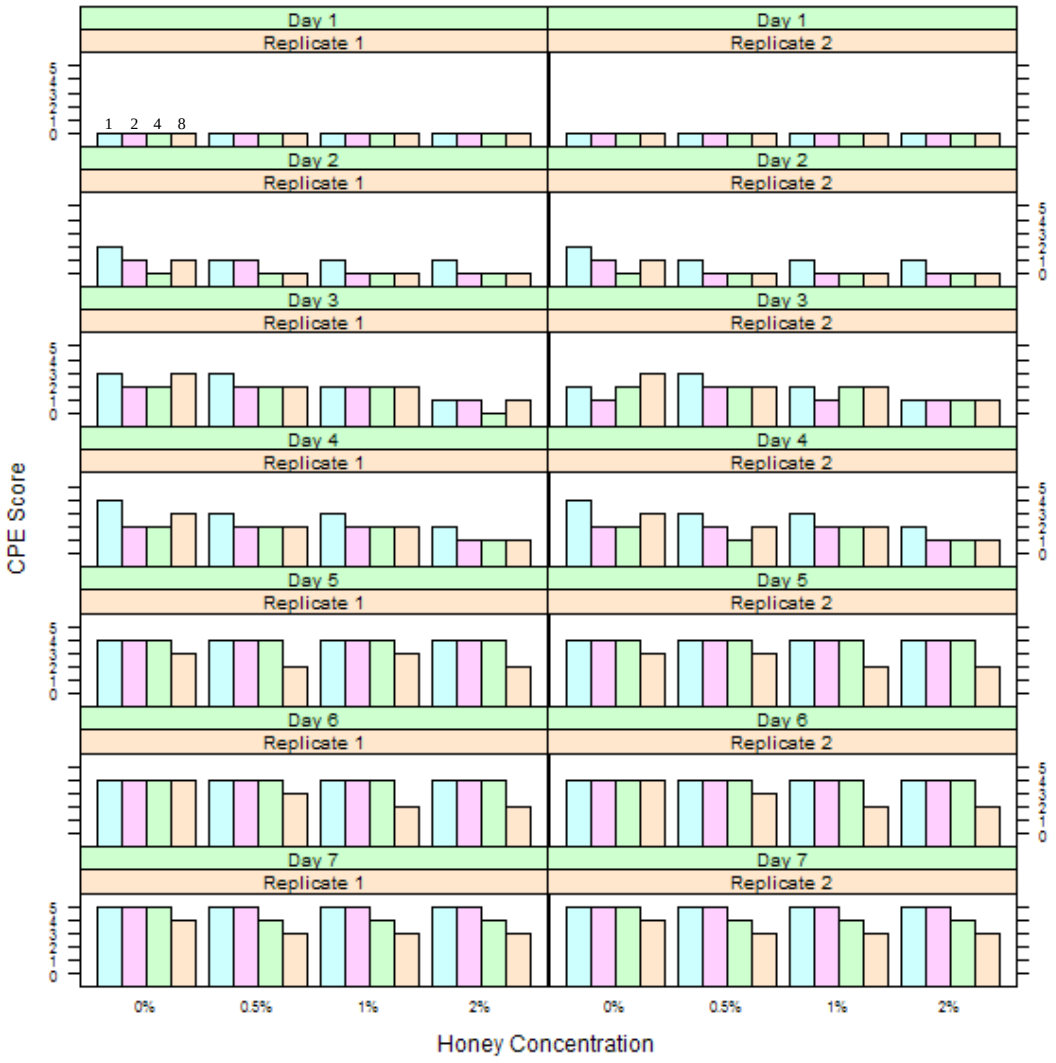

Day 1
Replicate 1
Day 1
Replicate 2
12
Day 2
Replicate 1
Day 2
Replicate 2
Day 3
Replicate 1
Day 3
Replicate 2
Day 4
Replicate 1
Day 4
Replicate 2
Day 5
Replicate 1
Day 5
Replicate 2
CPE Score
0%
0.5%
1%
2%
0%
0.5%
1%
2%
Honey Concentration

Figure 5.21 CPE resulting from infection of A549 cells with HSV-1 at a dilution of 10^{-4} after: (a) 1 to 8 hours and (b) 12 to 48 hours exposure of the virus to honey

(a) 1 to 8 hours



(b) 12 to 48 hours

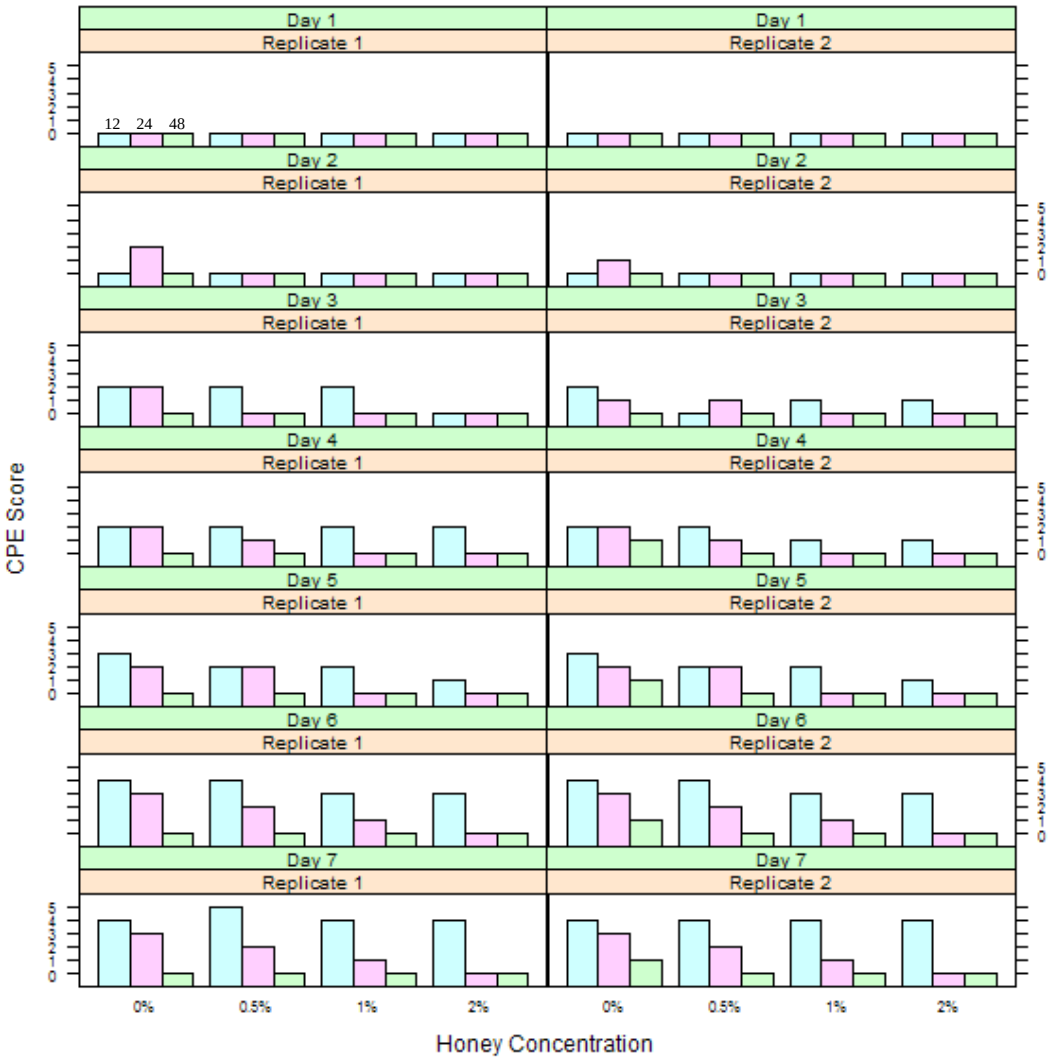
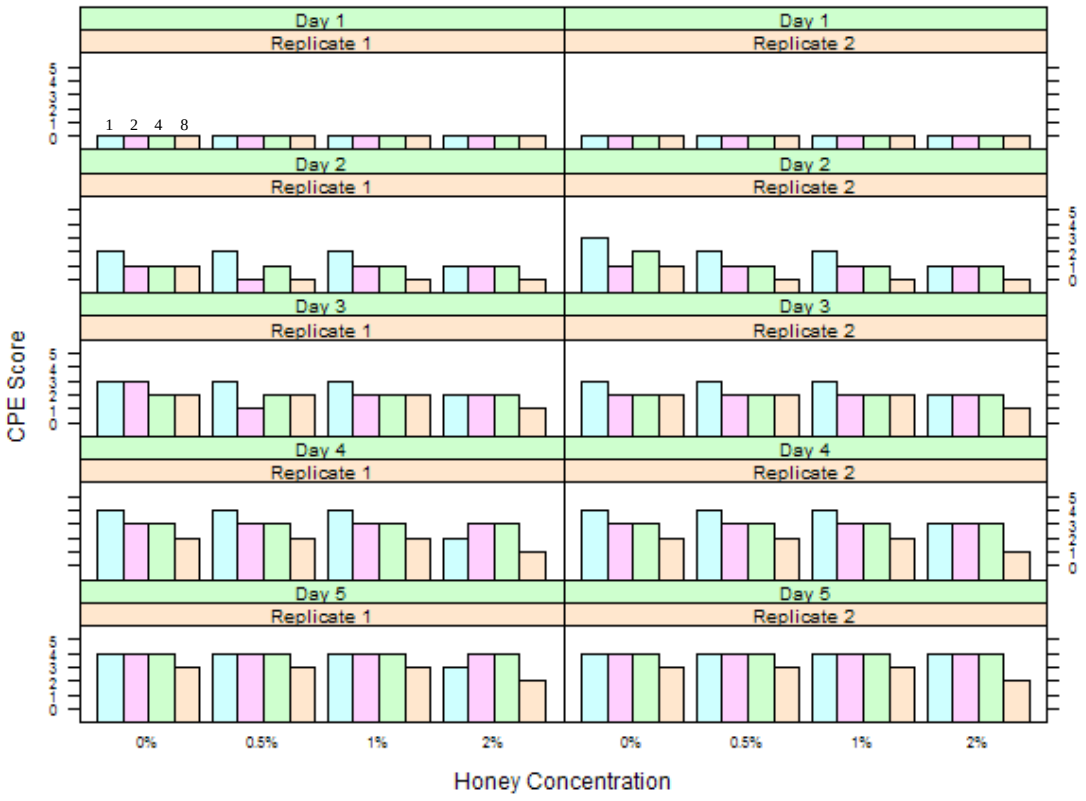


Figure 5.22 CPE resulting from infection of A549 cells with HSV-1 at a dilution of 10^{-4} after: (a) 1 to 8 hours and (b) 12 to 48 hours exposure of the virus to honey

(a) 1 to 8 hours



(b) 12 to 48 hours

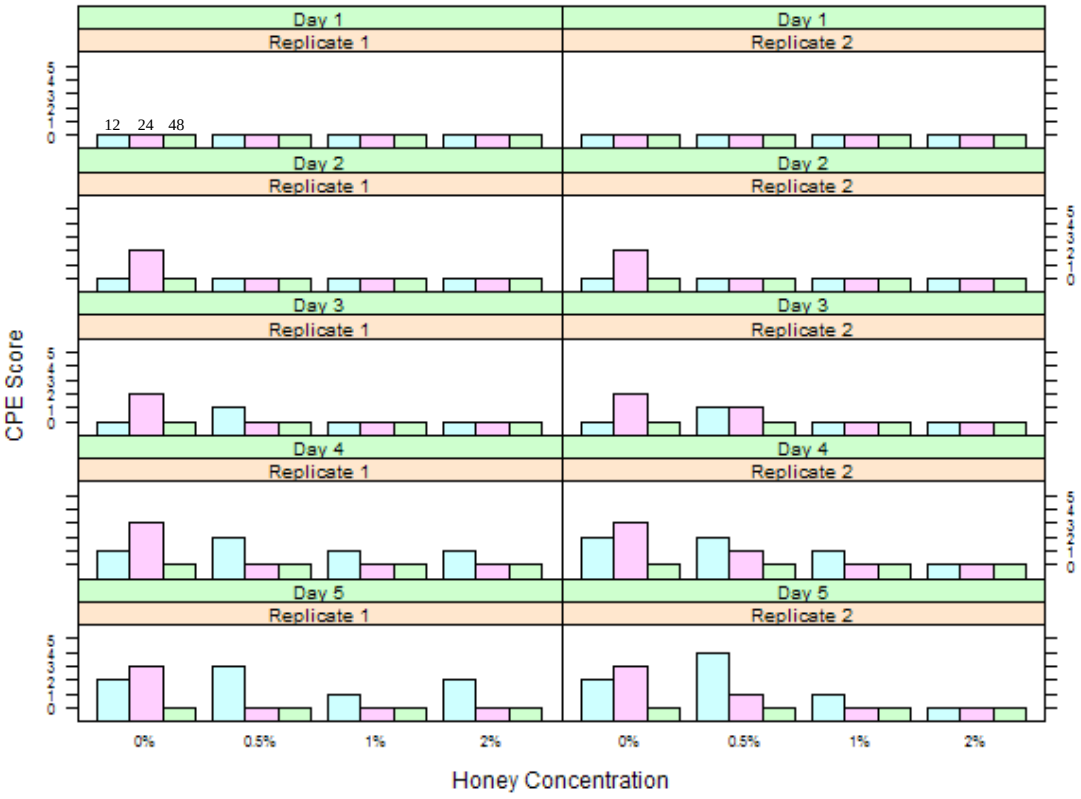
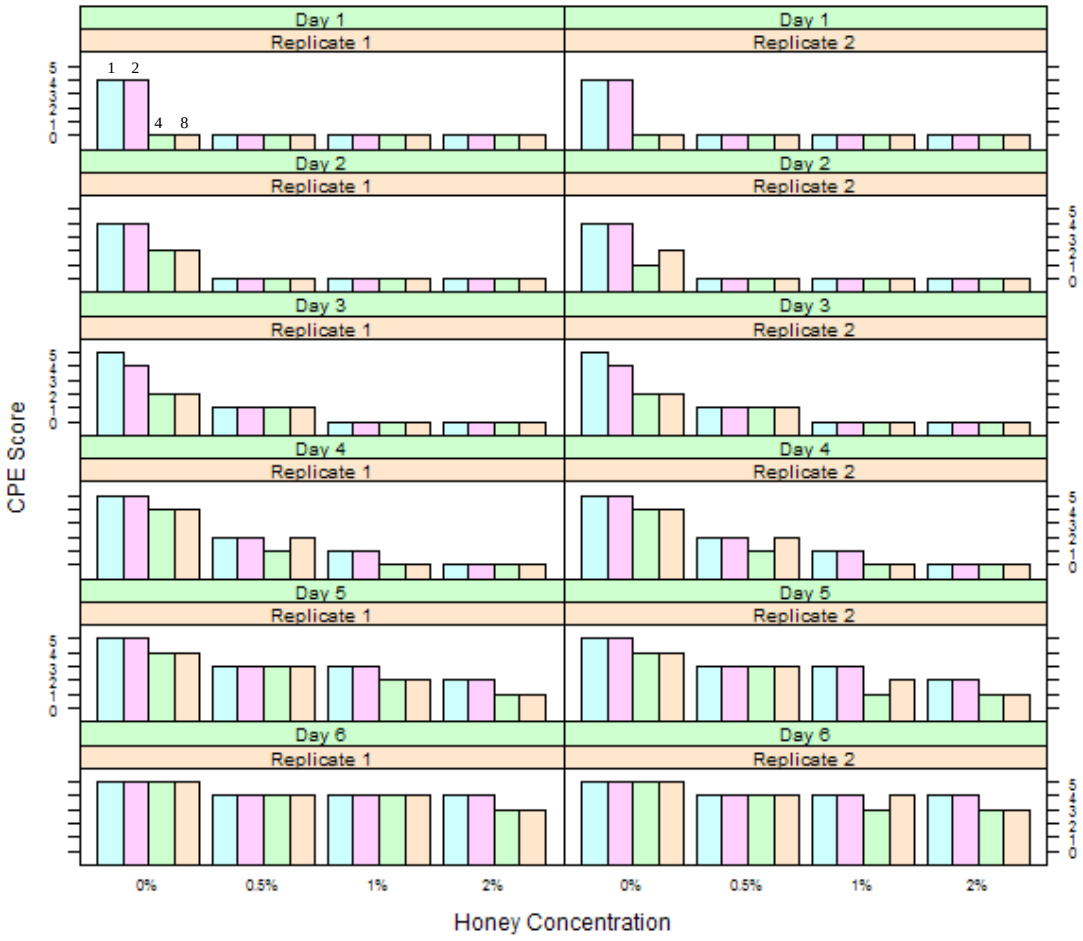
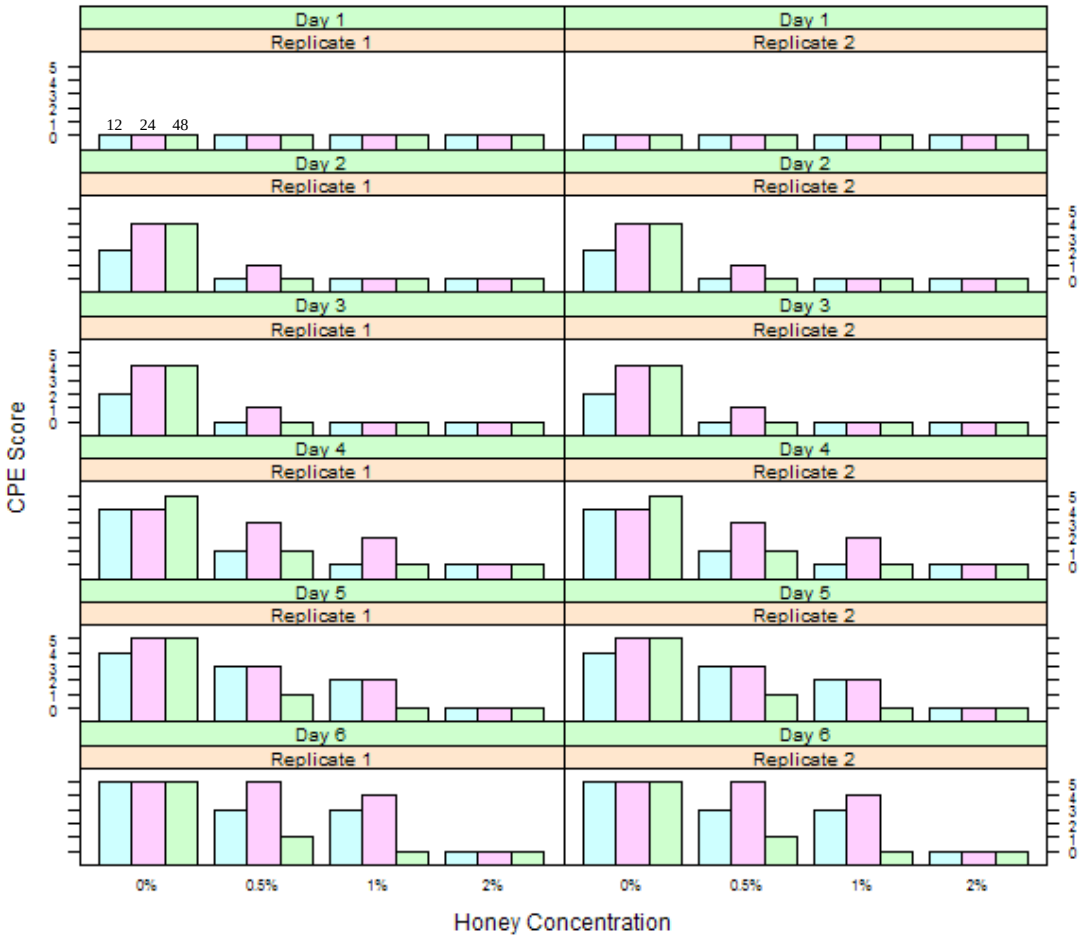


Figure 5.23 CPE resulting from infection of A549 cells with Ad3 at a dilution of 10^{-4} after: (a) 1 to 8 hours and (b) 12 to 48 hours exposure of the virus to honey

(a) 1 to 8 hours



(b) 12 to 48 hours



5.4.3 Summary of results

The neutralisation method was firstly tested with HSV-2 at a dilution of 10^{-7}. These experiments indicated that the method was able to show CPE and an effect of honey on the level of CPE when compared with the untreated virus. However, it was shown that a low level of CPE was obtained using this dilution of virus even when the virus had not been treated with honey. With the experiments using virus diluted to 10^{-4}, a higher level of viral CPE was observed, and the honey was shown to temporarily suppress the level of CPE compared with the untreated virus.

With HSV-1 and Ad3 it was further seen that the honey temporarily suppressed CPE levels compared with the untreated virus. Ad8 took a longer period post-inoculation of the cells to display signs of CPE. Due to the desire to have the CPE reach a high level within a short period and with the difficulty in the culture and recognition of Ad8 viral infection, it was decided that Ad8 be excluded from further investigation at this stage, if time permitted, Ad8 would be re-cultured and examined at a later time.

As described, the results shown by Figures 5.15 to 5.18 revealed temporal suppression of development of viral CPE after treatment of the virus with honey. To establish whether greater neutralisation periods were sufficient to inhibit the development of CPE at these honey concentrations, exposure periods of up to 48 hours were tested with each of the viral isolates using the virus diluted to 10^{-4}, this was to ensure accurate recognition and development of a high level of CPE with the untreated virus which served as a control.

In the first extended neutralisation experiment with HSV-2, shown in Figure 5.19, it was observed with the viruses untreated with honey that were exposed to longer incubation (4 hours or more) that lower levels of CPE were seen compared with the shorter incubation treatments when observed the same length of time after inoculation of the cells. With the untreated virus one would expect CPE to be present from similar times after inoculation of the cells whatever the period of incubation. The temperature of the shaking incubator was checked and was found to fluctuate to 39°C during the course of the neutralisation period, although the thermostat was set to 37°C. Despite setting the thermostat 2°C lower, the correct temperature was still not attained. The high temperature during the longer neutralisation periods was likely to be inactivating the virus, and this may be able to explain the absence of CPE with even the untreated virus, with the long incubation treatments.

The repeat work with HSV-2 shown in Figure 5.20 using a more insulated incubator during the neutralisation period gave temporal suppression of the development of CPE, and with the longer periods of exposure of the virus to honey there was evidence of neutralisation activity. The 24 and 48 hour neutralisation treatments did not show any resultant viral CPE. However, nor did any CPE develop with the virus not exposed to honey. Therefore even with the correct temperature within the incubator there was an effect of prolonged incubation on the virus.

Observations of viral CPE after the 1 hour exposure period were very similar to those seen in the initial experiments, thus the method was shown to be quite reproducible.

With HSV-1, CPE was observed much later after infection with virus exposed honey after the 24 hour neutralisation treatment compared with the shorter treatments. As with HSV-2, no CPE was seen with the 48 hour neutralisation treatment. With the honey treatments, suppression of CPE was observed compared with the untreated virus, and as with HSV-2, evidence of neutralisation was seen with the honey treatments after longer neutralisation periods. Again, similar observations were made after the 1 hour exposure to honey as within the first examination.

Ad3 with the 1 to 8 hour neutralisation treatments showed the most apparent trends of a reduction of CPE related to the concentration of honey and time of exposure to it. Again, with the longer neutralisation treatments at higher honey concentrations, evidence of neutralisation was observed. Further, similar suppression of viral CPE was observed after the 1 hour exposure period as within the first examination.

In each experiment, suppression of CPE was observed with the honey treatments, and further evidence of neutralisation was seen with the longer neutralisation treatments. The method produced similar results in both sets of experiments and indicated the feasibility to further evaluate honey's antiviral activity by following the development of CPE for 7 days. The observation from the 12 hour neutralisation treatment and longer revealed that less CPE developed after prolonged incubation of the virus with

no honey present, therefore further investigations can be done with incubation up to only 8 hours. To further evaluate honey's neutralisation capacity, the use of higher concentrations of honey is desirable. The osmotic effect on the A549 cells of using higher concentrations of honey, however, would need to be overcome.

5.5 DISCUSSION

The results from this Chapter suggest that honey acts by more than one mode of action. It was observed with the first protection experiments, that there was less CPE after pre-treatment of the cells with the higher concentrations of honey compared with the untreated virus. These observations can suggest that the honey has an effect on the cells by possibly penetrating the cell and working internally, acting against the virus during its replication. The honey may be causing an antiviral response of the cells which then works on the virus after infection. Alternatively, the honey may be coating the cellular receptors preventing the virus from attaching to the cell which is necessary for viral infection. The consequent CPE observed after infection could be explained by the shedding of the cell membrane which in effect removes the honey from the cell. Further, the toxic nature of honey on the cells may be thought to slow cellular growth eliminating the optimal environment for viral infection which is most efficient when the cells are actively dividing, this could explain the delay of CPE. In each case, however, it would be expected that the continued exposure to honey, and the further daily replacement of the medium with fresh honey solution would cause lower levels of CPE to be observed, this was not always the case.

The neutralisation experiments were undertaken to test whether the honey had a direct effect on the virus by treating the virus with honey in the absence of cells, followed by inoculation and observation of CPE development. The results show that the honey treatments temporally suppressed CPE development and in some cases prevented CPE from developing over the observation period. The direct effect of the honey on the virus may be being caused by the honey targeting internal or external proteins of the viral particle, which causes unsuccessful attachment to, penetration of, or replication within the cell.

These two sets of experiments (protection and neutralisation) have shown that the honey acts both on the cells and directly on the virus in the absence of the cells.

The results of the prevention of viral infection experiments further propose an effect of the honey on the cells and directly on the virus. These experiments were undertaken to evaluate the potential therapeutic benefit of honey as a treatment for viral infection once infection has taken place. As with the other experiments looking to test protective and neutralisation capacities of honey, temporal suppression of CPE development was observed with the honey treatments most greatly with the 1% and 2% concentrations. These effects can be explained by the same theories used to describe the protective and neutralisation antiviral activity: the honey may be acting on the virus being released from the cell, on cell surface receptors preventing further infection, or possibly by causing a cellular antiviral response.

With each of these experiments honey has shown an antiviral effect. Honey can potentially be used as an antiviral agent to aid the immune system in response to viral infection by adenovirus and herpes simplex virus, by slowing the rate of infection to allow the body's own defences to help eliminate the virus.

Chapter 6

The antiviral activity of honey: Neutralisation

This chapter describes the extended investigation of the neutralisation capacity of a range of New Zealand honeys on viruses.

Neutralisation has been used classically to test drug sensitivity of viruses. It involves directly exposing the virus to various concentrations of the drug of interest for specified periods. The evaluation of sensitivity can be done by observing the development of CPE within susceptible cells.

6.1 INTRODUCTION

It was shown by the preliminary results in Chapter 5 that honey can temporarily suppress the development of CPE. It was desirable to test a range of honeys at higher concentrations to determine whether greater suppression or neutralisation of the viruses can be achieved, and whether some honeys are more effective than others.

These previous experiments used honey up to a concentration of 2% (v/v), as at this concentration the cells remain microscopically unaffected by the honey. In these experiments however, higher concentrations of honey were to be investigated for their ability to neutralise each virus, as these potentially would have a greater effect on the development of viral CPE due to the reduced levels of viral CPE associated with the concentration of honey and time of exposure to it observed in the earlier experiments. By using higher concentrations of honey the shorter neutralisation periods may give successful results to what was achieved with the longer neutralisation periods which used lower concentrations of honey. This would help avoid the complication of reduced levels of viral infection with the untreated viruses which was observed with some of the >8 hour treatments in earlier investigations.

To ensure the health of the cells was not jeopardised by the increased honey concentration the inoculation period of the cells was reduced, and involved using centrifugation to hasten the viral attachment process, rather than allowing the attachment to take place over an hour of incubation. This was to overcome and so minimise the osmotic effect the increased concentrations of honey may have had on the cells during this inoculation period.

After inoculation the cells were incubated at 37°C for 7 days and daily observations were made to detect viral CPE. Likewise, any presence of osmotic effects in both the tests and controls (each honey concentration without virus) were looked for over the observation period. The cells were allocated a numerical score for CPE as described in Section 4.1, Figure 4.6. The honeys tested for neutralisation capacity are described in Chapter 3.

The experiments in this chapter aimed to compare the neutralisation capacity of a range of New Zealand honeys, to determine concentrations of honey and duration of treatment required to eliminate, delay, and/or reduce the severity of CPE resulting from infection of the cells with the treated virus. Further to: determine whether honey's antiviral activity was a found with more than one type of honey; and to ascertain a range of treatments suitable for further experiments.

It was hypothesised: that honey from a range of floral sources has neutralisation activity against Ad3, HSV-1, and HSV-2 and that there will be differences within and between different types of honey in the potency of their antiviral activity; that the reduced CPE will be related to the concentration and time of exposure of the

virus to the honey; that the honeys (other than Manuka) high in phenolic compounds may display similar antiviral activity to Manuka honey; and that the observed antiviral activity can be linked to a characteristic property within the honey.

6.2 METHODS

A549 cells were prepared and grown overnight in 24 well plates at 37°C until confluent as described in Section 3.2.1. Bijoux or 1.5 ml Eppendorf tubes were prepared by labelling: 0%, 1%, 2%, 2.5%, 5%, 10% (for honey concentration) and 1, 2, 4, and 8 hour (for neutralisation periods).

Once the cells were confluent, a total volume of 15 ml of stock honey solutions were prepared in sterile universals as shown in Table 6.1 (based on honey density calculations: see Section 4.3 Table 4.6).

Table 6.1 Dilutions to prepare 15 ml honey solutions

Honey Conc. (v/v)	Wt. honey (g) most honeys	Vol. medium (ml) most honeys	Wt. honey (g) for LH27	Vol. medium (ml) for LH27
0%	0	15	0	15
1%	0.214	14.785	0.187	14.812
2%	0.428	14.571	0.375	14.625
2.5%	0.535	14.464	0.468	14.531
5%	1.071	13.928	0.937	14.062
10%	2.142	12.857	1.875	13.125

The honey solutions were mixed and filter sterilised then added to the appropriately labelled tubes. Serial dilutions of each viral isolate (Ad3, HSV-1, and HSV-2) were prepared in maintenance medium to a dilution of 10^{-3} taking

100 µl each time to 900 µl of maintenance medium. The honey solutions were inoculated with 100 µl of viral dilution to make the final dilution of 10^{-4} and honey solutions at 9/10 of the stated concentration. The viral-honey solutions were then incubated at 37°C with shaking (using the shaking incubator at level 3 to 4 for 1, 2, 4, or 8 hours depending on treatment). Upon completion of the neutralisation period, the viral-honey solutions were either immediately used to inoculate the cells, or were frozen at -70°C for later examination.

To inoculate the cells, the growth medium was removed from the cells and 200 µl of the appropriate viral-honey solution was added to the wells in duplicate, this included testing each concentration of honey without virus. The plates were centrifuged for 15 minutes at 2000 rpm at 25°C. After centrifugation, the residual viral-honey solution was removed from the cells and replaced with 1 ml of maintenance medium. The plates were incubated at 37°C and observed for the development of CPE daily. Any spare viral/honey solutions were frozen at -70°C for use in later experiments testing for virucidal or virustatic activity.

Two experiments for each honey and virus type were done on separate days in duplicate to compare consistency of this experimental method.

6.3 RESULTS

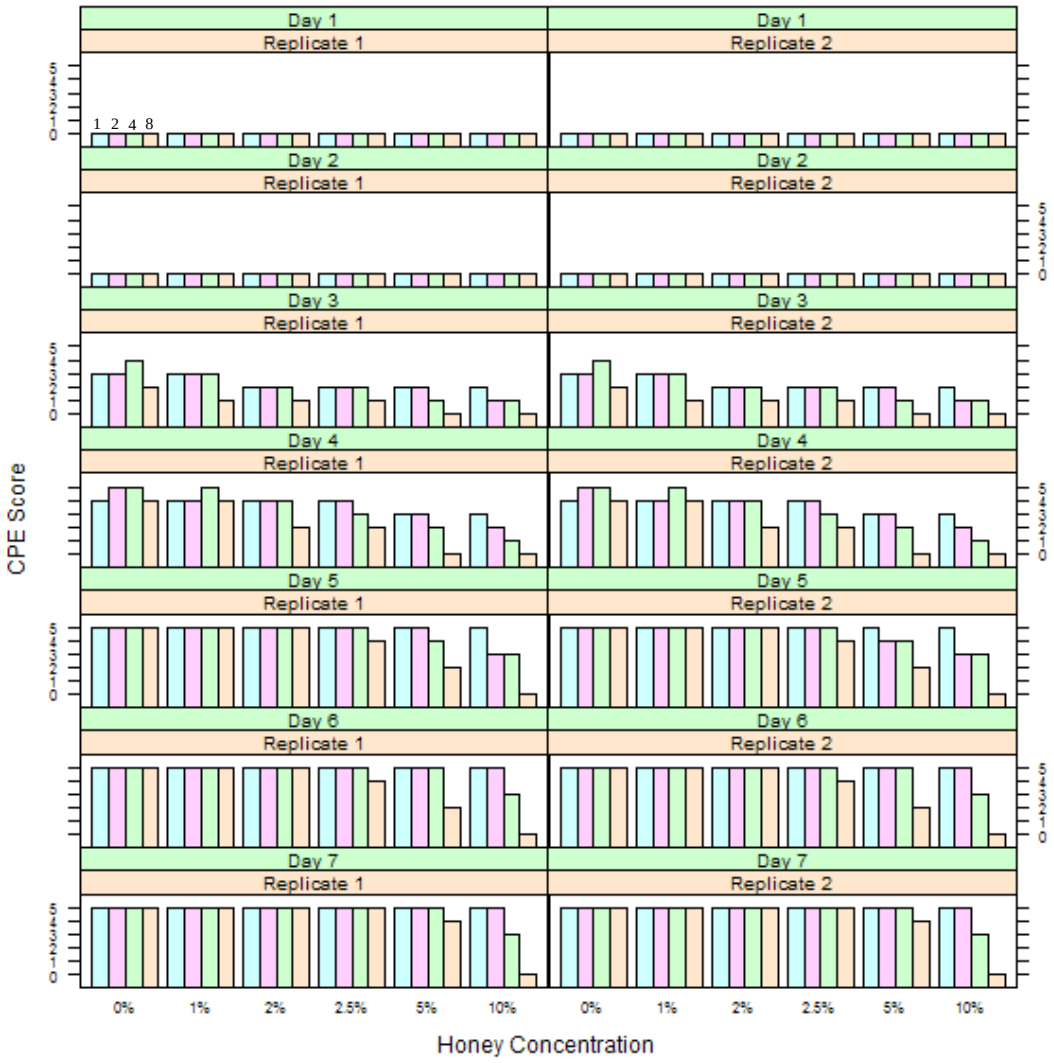
Figures 6.1 to 6.12 show the development of CPE with each virus and Manuka honey treatment and Figures 6.13 to 6.21 show CPE development with each virus and treatment with other honeys for each of the 1, 2, 4, and 8 hour neutralisation periods. With these figures, the results for each replicate have been given and

each successive bar with each concentration of honey indicates a different exposure period of the viruses to honey. These exposure periods are outlined above the appropriate bars with the first concentration of honey, to indicate which bar corresponds to which period. The plots are followed by a summary of the combinations of treatments that suggested neutralisation had been achieved, by the absence of any CPE after inoculation of the cells over the observation period. The first column of this table gives details of the type, concentration, and time of exposure to the honey. The average activity levels of each of the honeys tested has also been included for comparisons to be made.

The honey solutions without virus in each experiment did not cause any morphological changes to the cells over the course of observation, and those treatments that did not show any kind of viral CPE at any time during the observation period were omitted from graphing.

Figure 6.1 CPE resulting from infection of A549 cells with Ad3 after treatment of the virus with Manuka honey M116 at various concentrations for 1, 2, 4, or 8 hours: (a) experiment 1 and (b) experiment 2

(a) Experiment 1



(b) Experiment 2

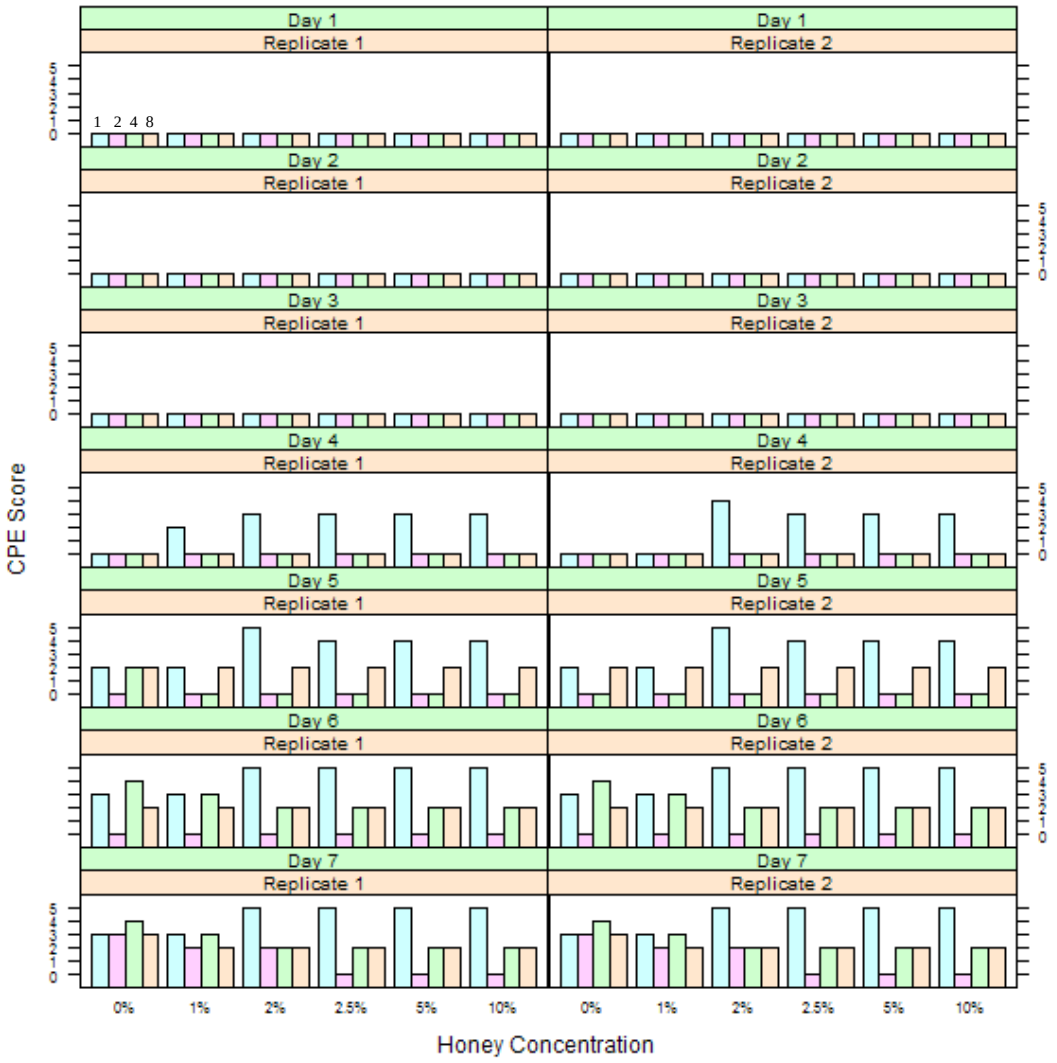
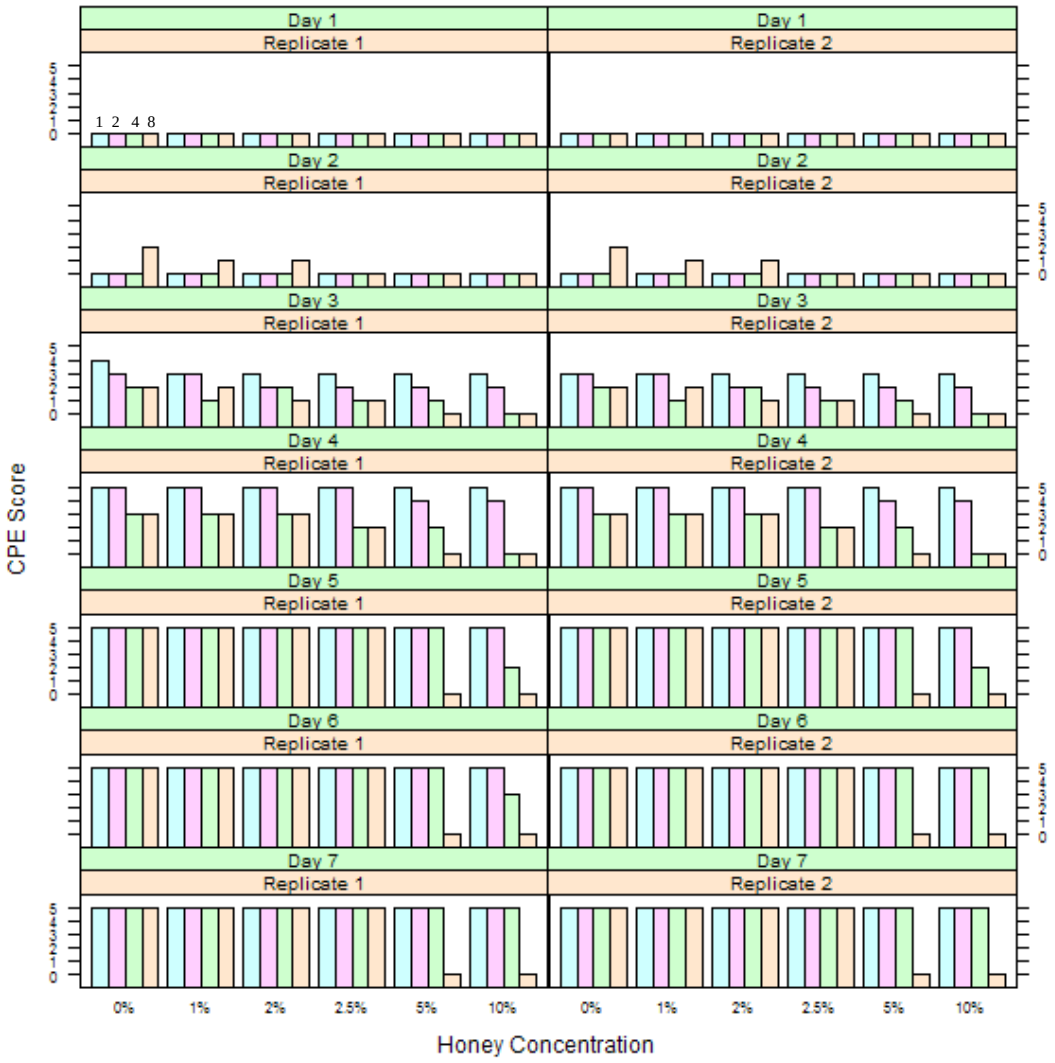


Figure 6.2 CPE resulting from infection of A549 cells with HSV-1 after treatment of the virus with Manuka honey M116 at various concentrations for 1, 2, 4, or 8 hours: (a) experiment 1 and (b) experiment 2

(a) Experiment 1



(b) Experiment 2

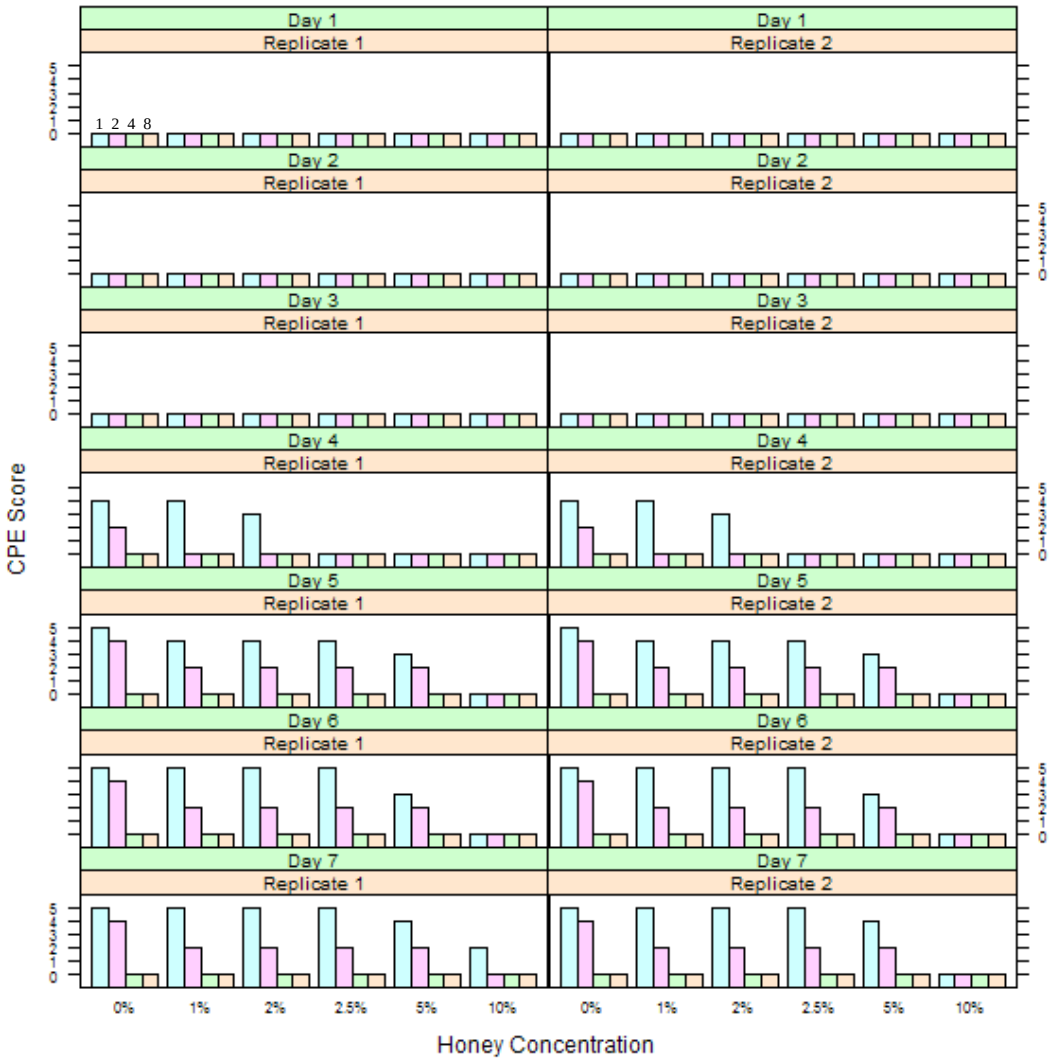
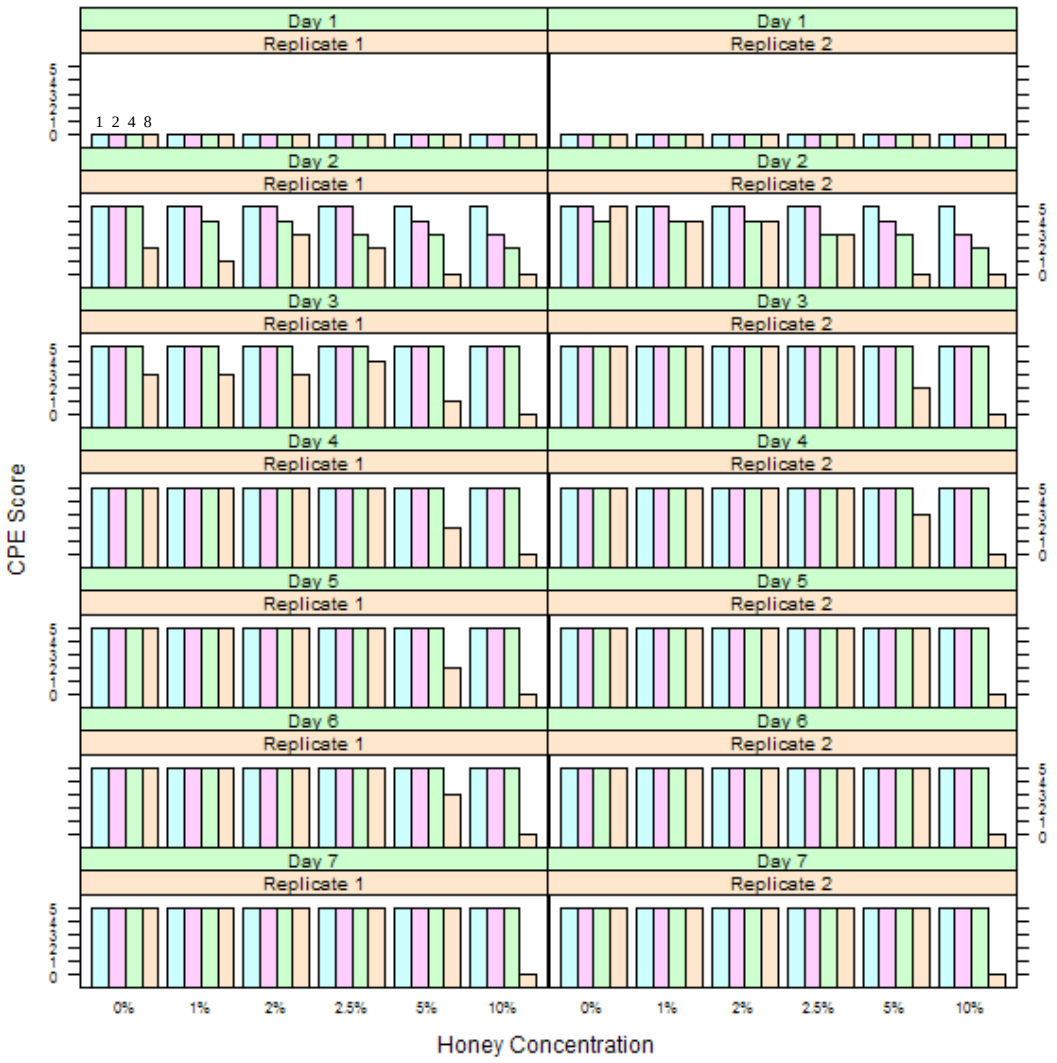


Figure 6.3 CPE resulting from infection of A549 cells with HSV-2 after treatment of the virus with Manuka honey M116 at various concentrations for 1, 2, 4, or 8 hours: (a) Experiment 1 and (b) experiment 2

(a) Experiment 1



(b) Experiment 2

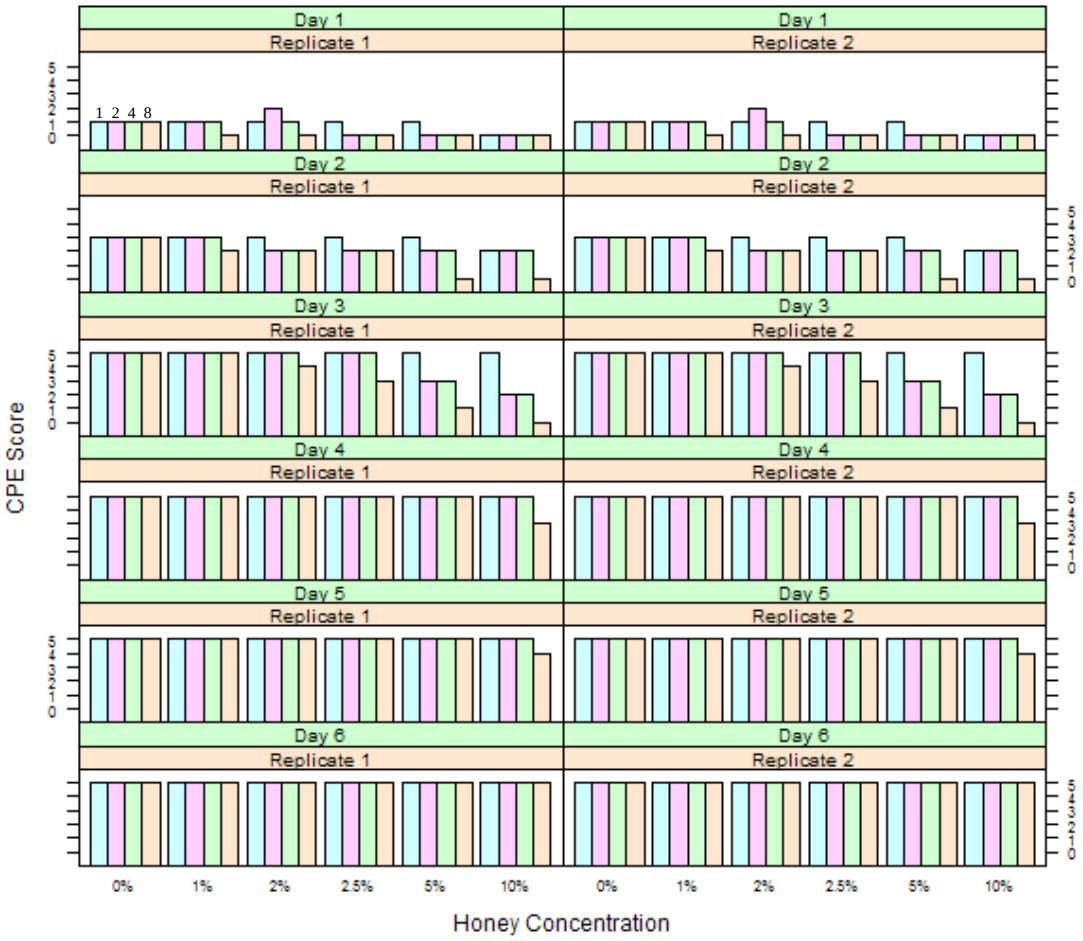
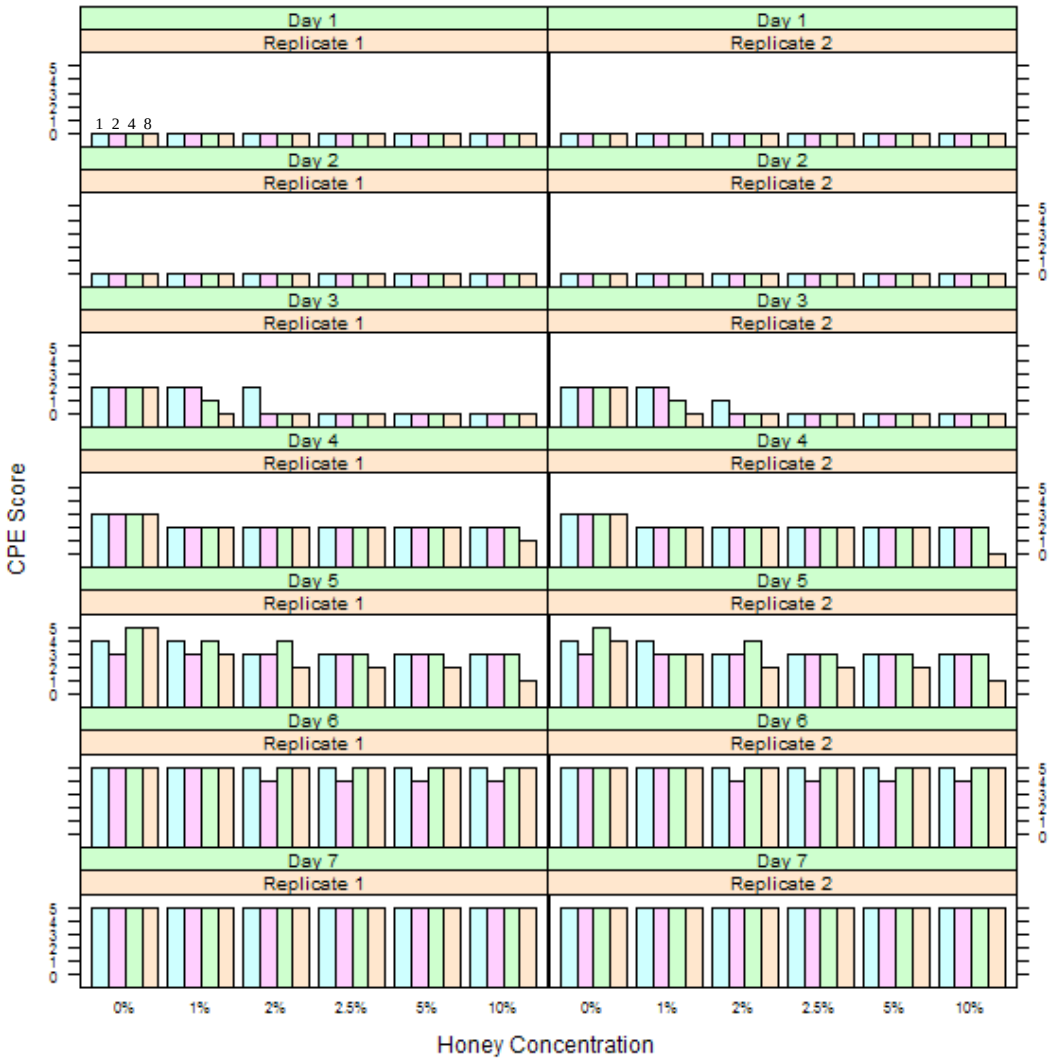


Figure 6.4 CPE resulting from infection of A549 cells with Ad3 after treatment of the virus with Manuka honey M112 at various concentrations for 1, 2, 4, or 8 hours: (a) experiment 1 and (b) experiment 2

(a) Experiment 1



(b) Experiment 2

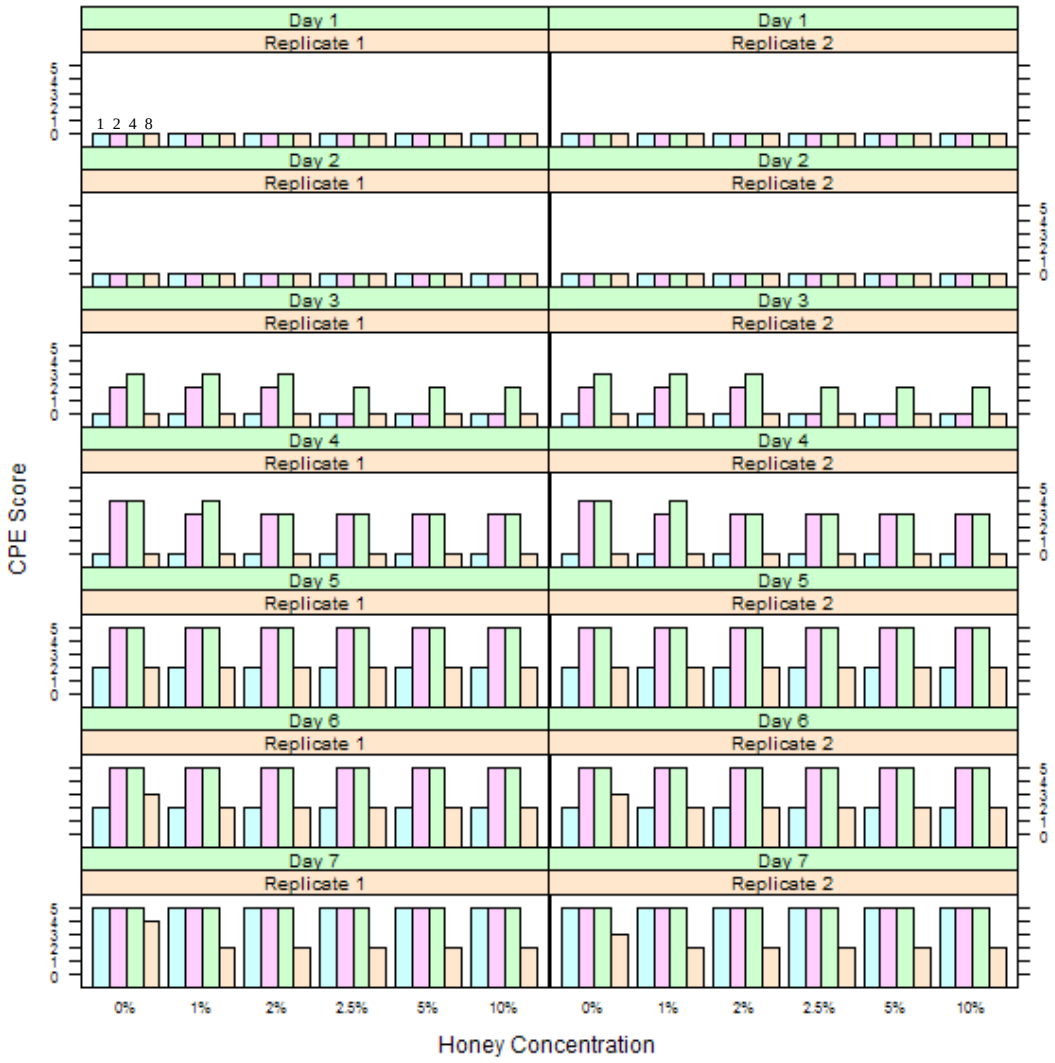
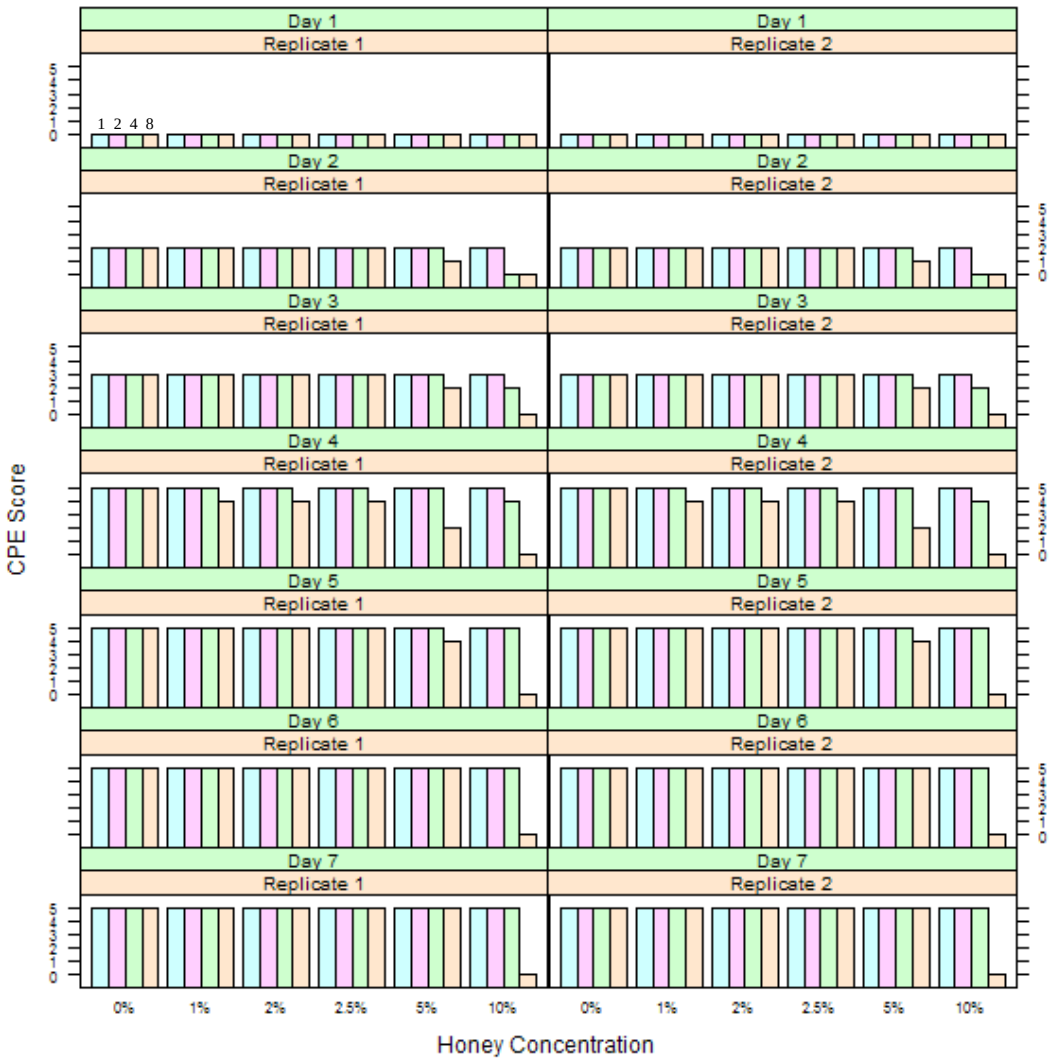


Figure 6.5 CPE resulting from infection of A549 cells with HSV-1 after treatment of the virus with Manuka honey M112 at various concentrations for 1, 2, 4, or 8 hours: (a) experiment 1 and (b) experiment 2

(a) Experiment 1



(b) Experiment 2

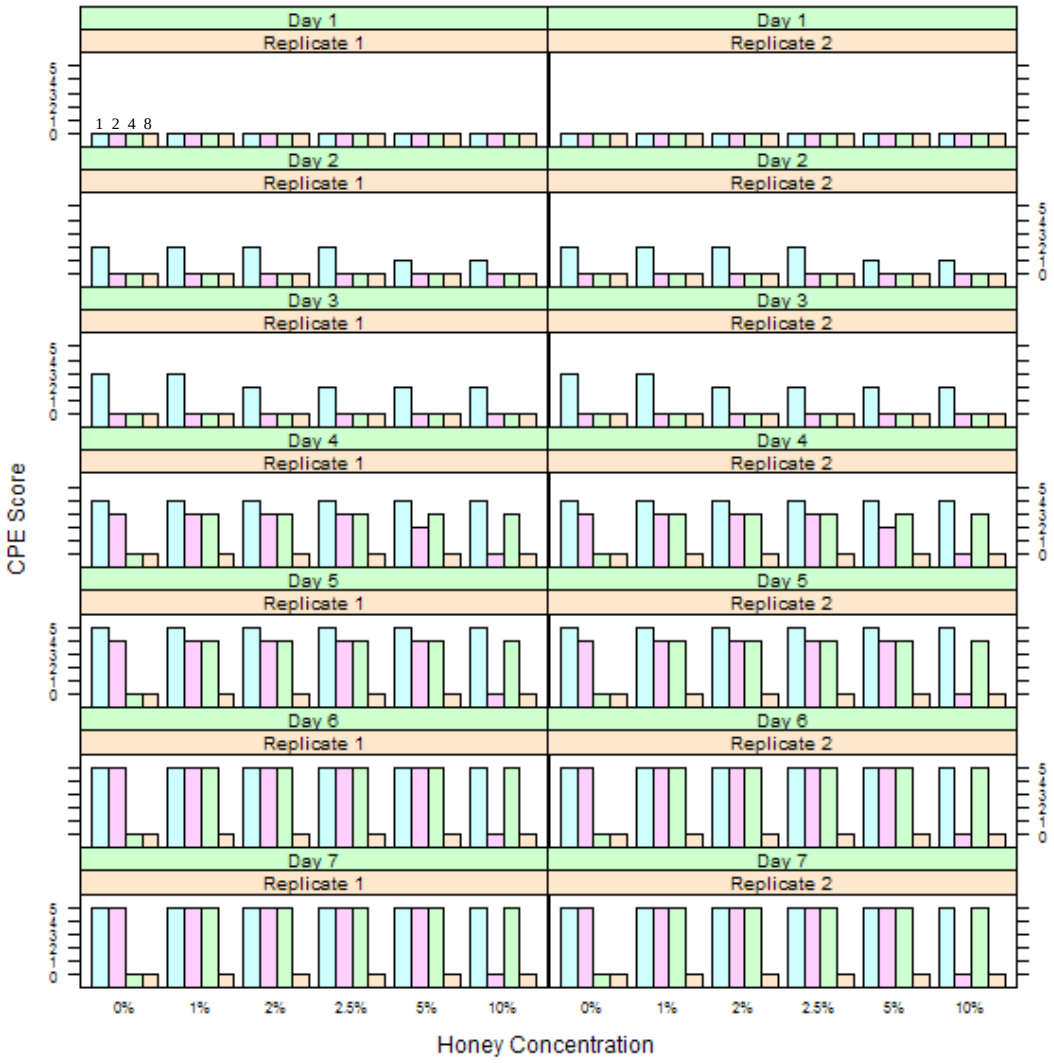
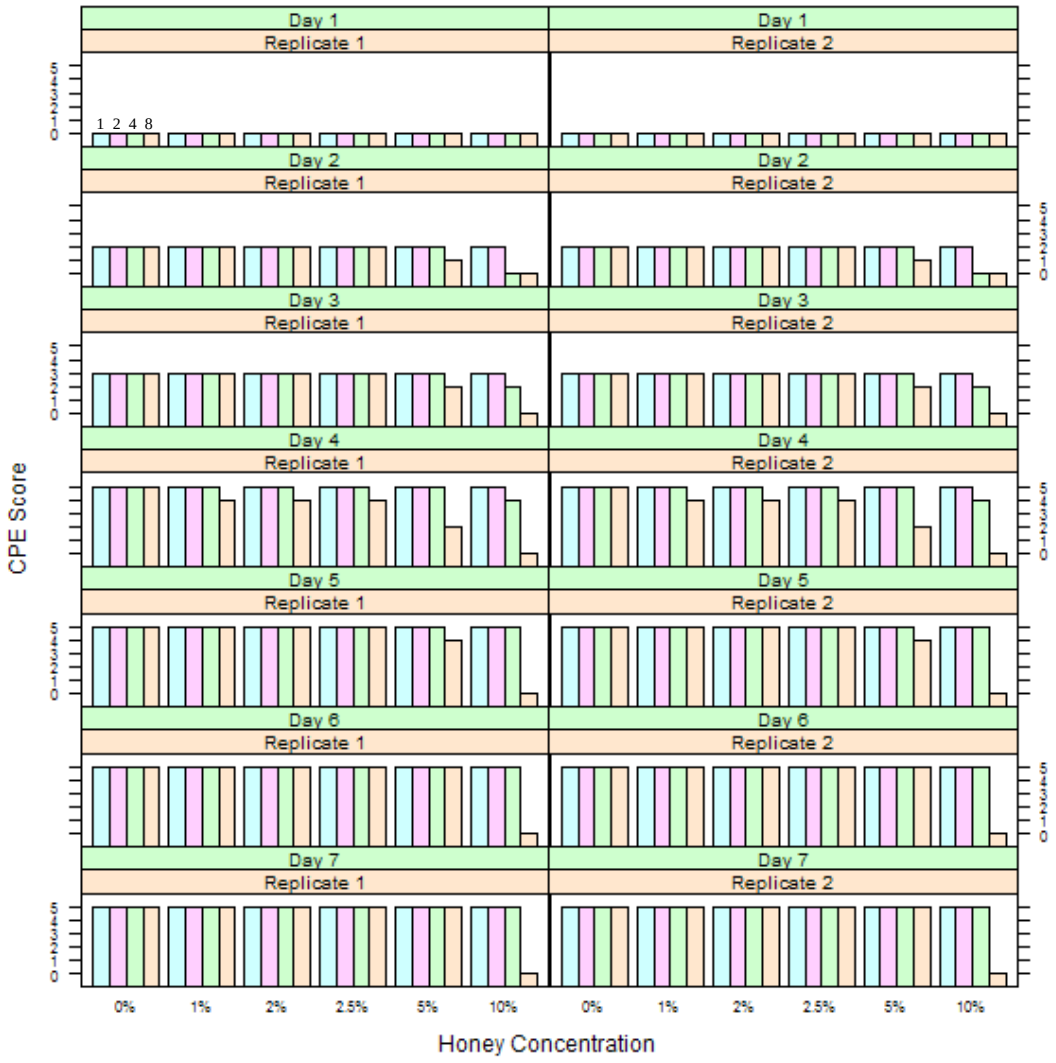


Figure 6.6 CPE resulting from infection of A549 cells with HSV-2 after treatment of the virus with Manuka honey M112 at various concentrations for 1, 2, 4, or 8 hours: (a) experiment 1 and (b) experiment 2

(a) Experiment 1



(b) Experiment 2

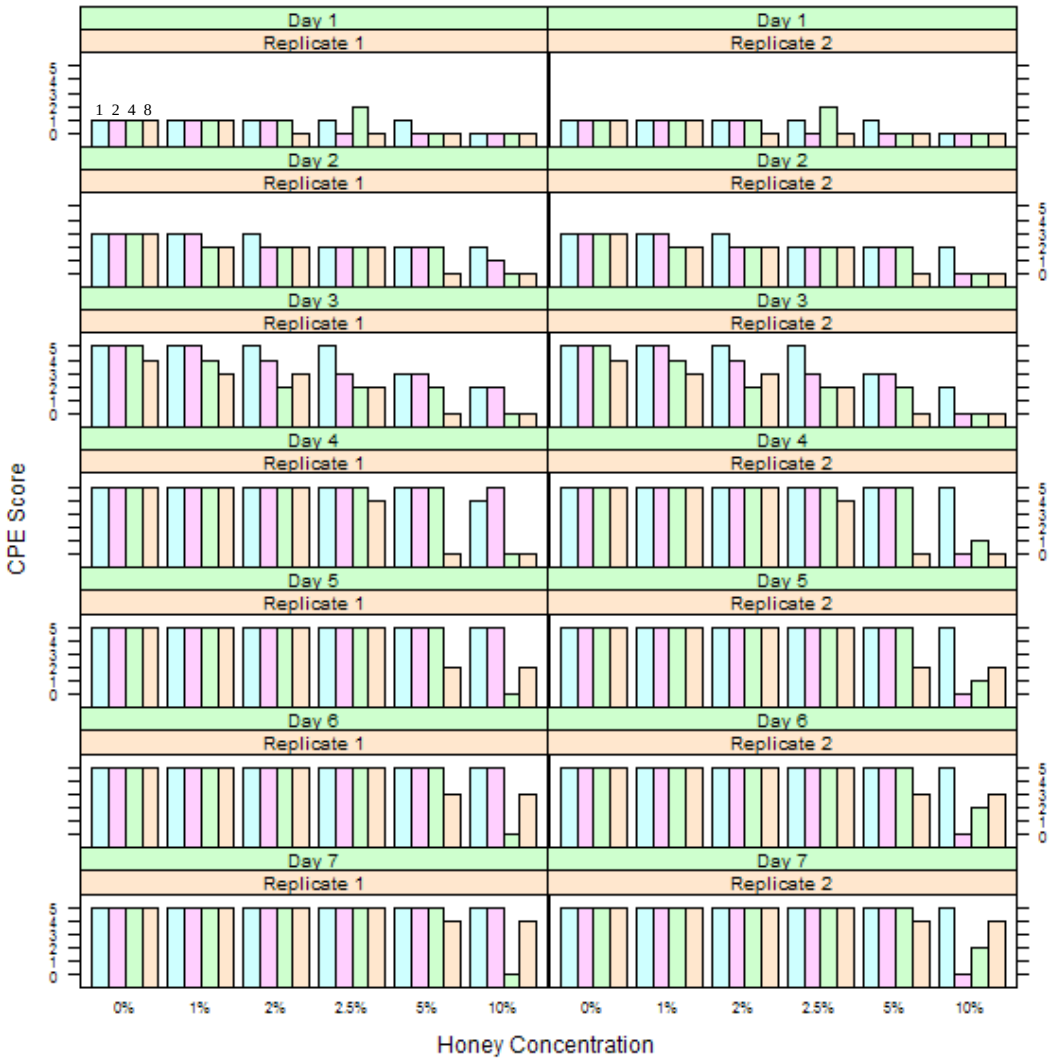
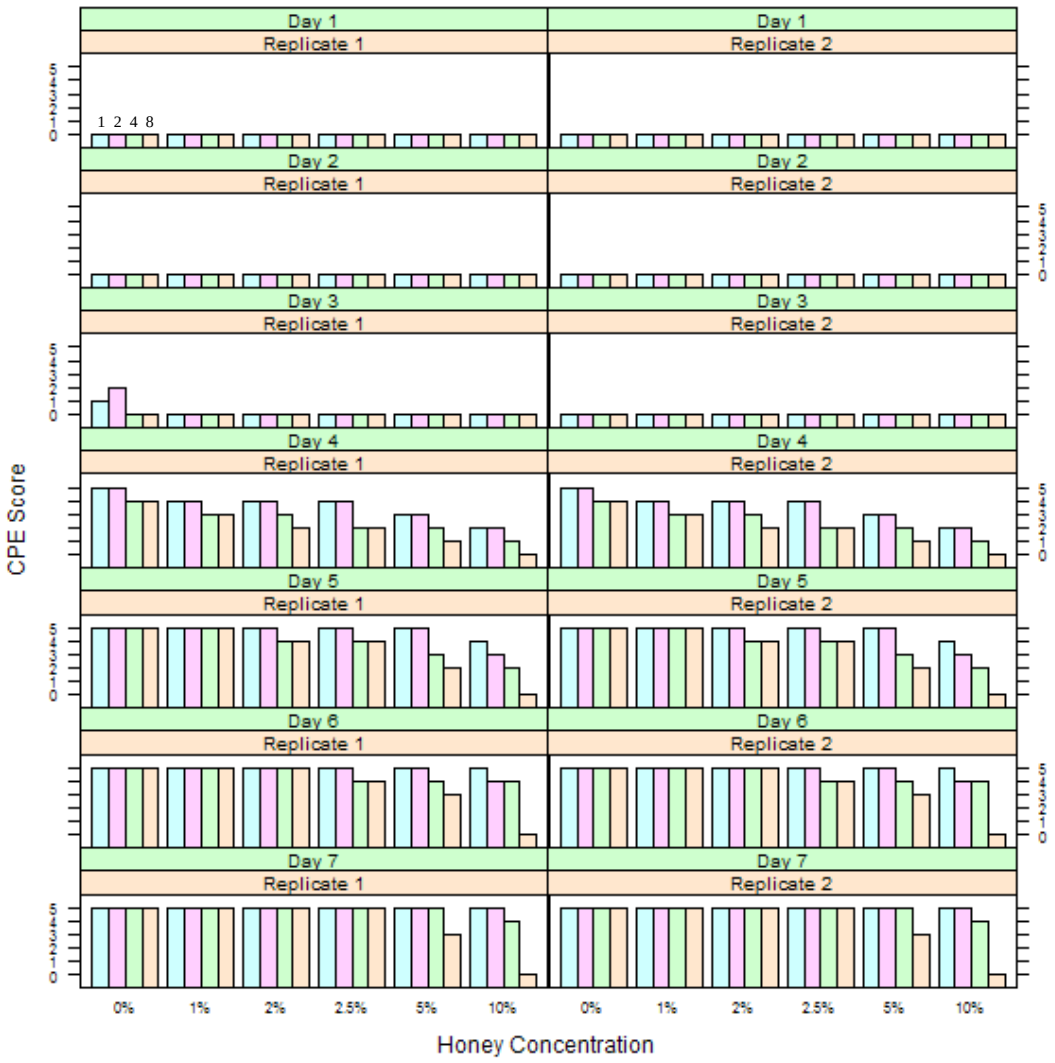


Figure 6.7 CPE resulting from infection of A549 cells with Ad3 after treatment of the virus with Manuka honey M157 at various concentrations for 1, 2, 4, or 8 hours: (a) experiment 1 and (b) experiment 2

(a) Experiment 1



(b) Experiment 2

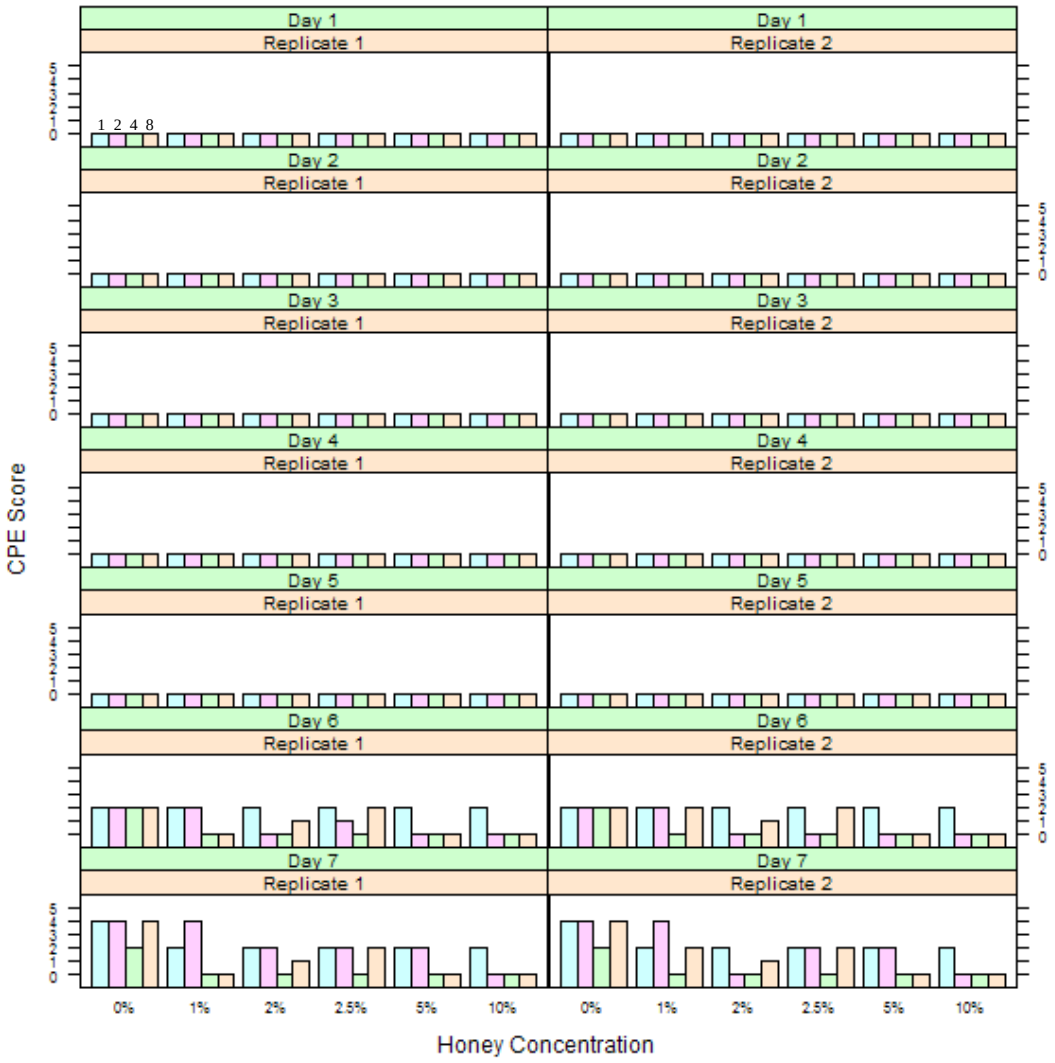


Figure 6.8 CPE resulting from infection of A549 cells with HSV-1 after treatment of the virus with Manuka honey M157 at various concentrations for 1, 2, 4, or 8 hours

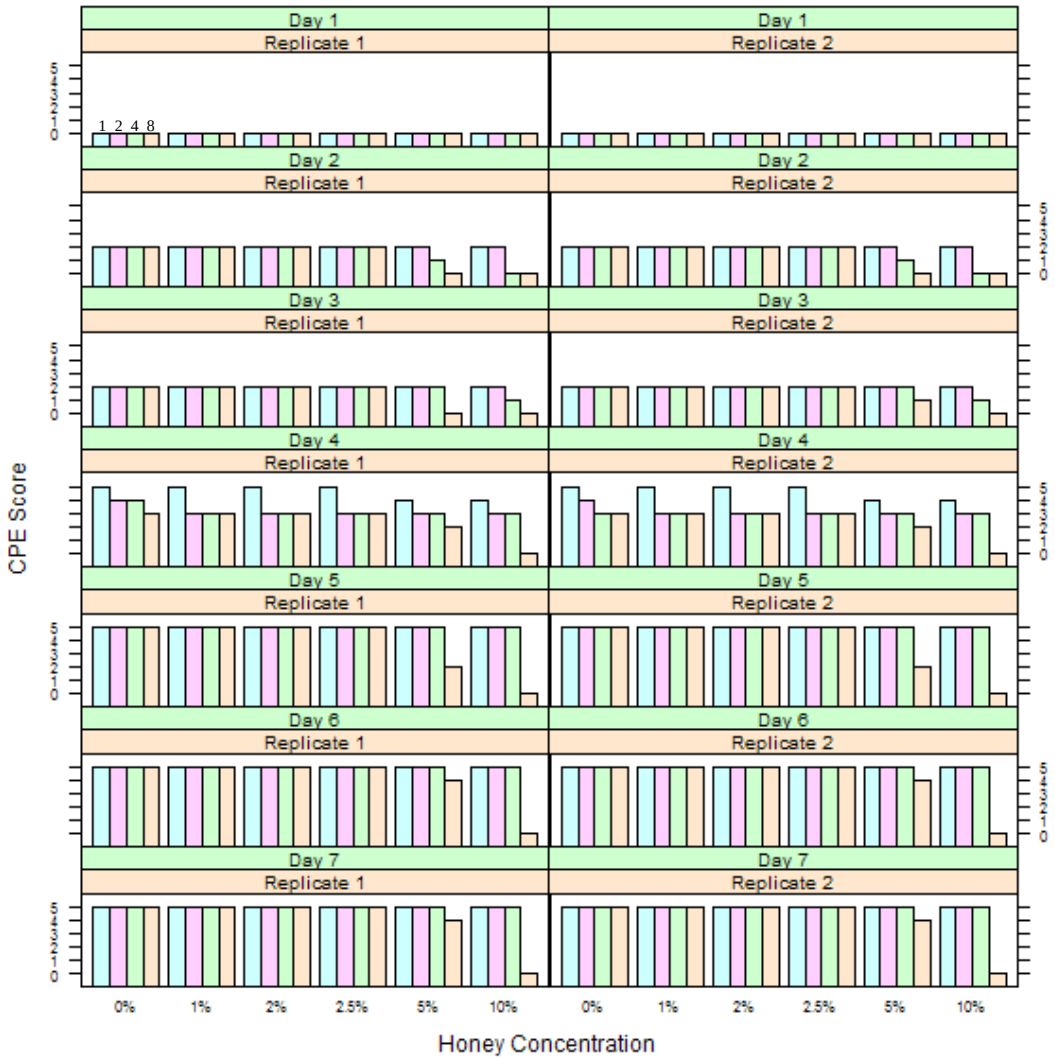
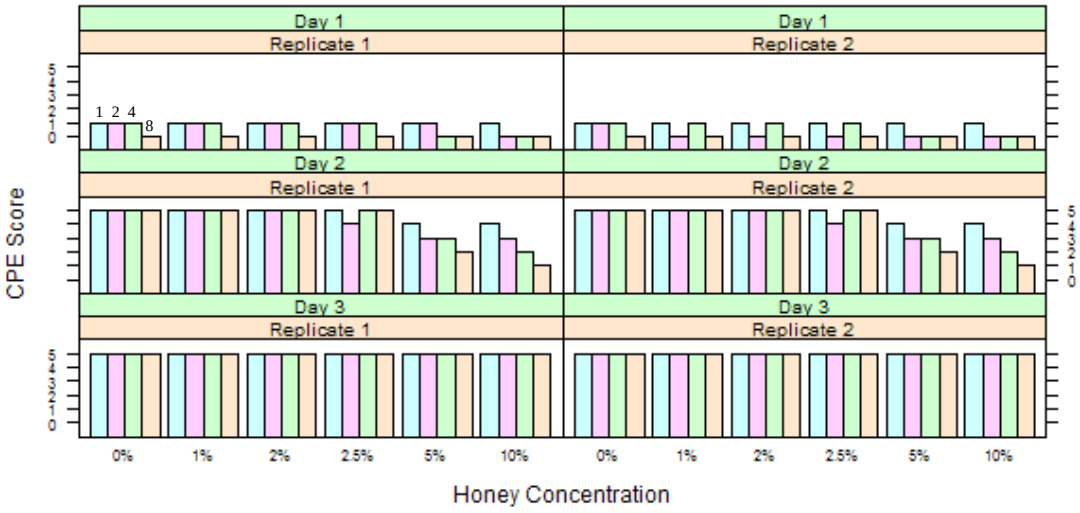


Figure 6.9 CPE resulting from infection of A549 cells with HSV-2 after treatment of the virus with Manuka honey M157 at various concentrations for 1, 2, 4, or 8 hours: (a) experiment 1 and (b) experiment 2

(a) Experiment 1



(b) Experiment 2

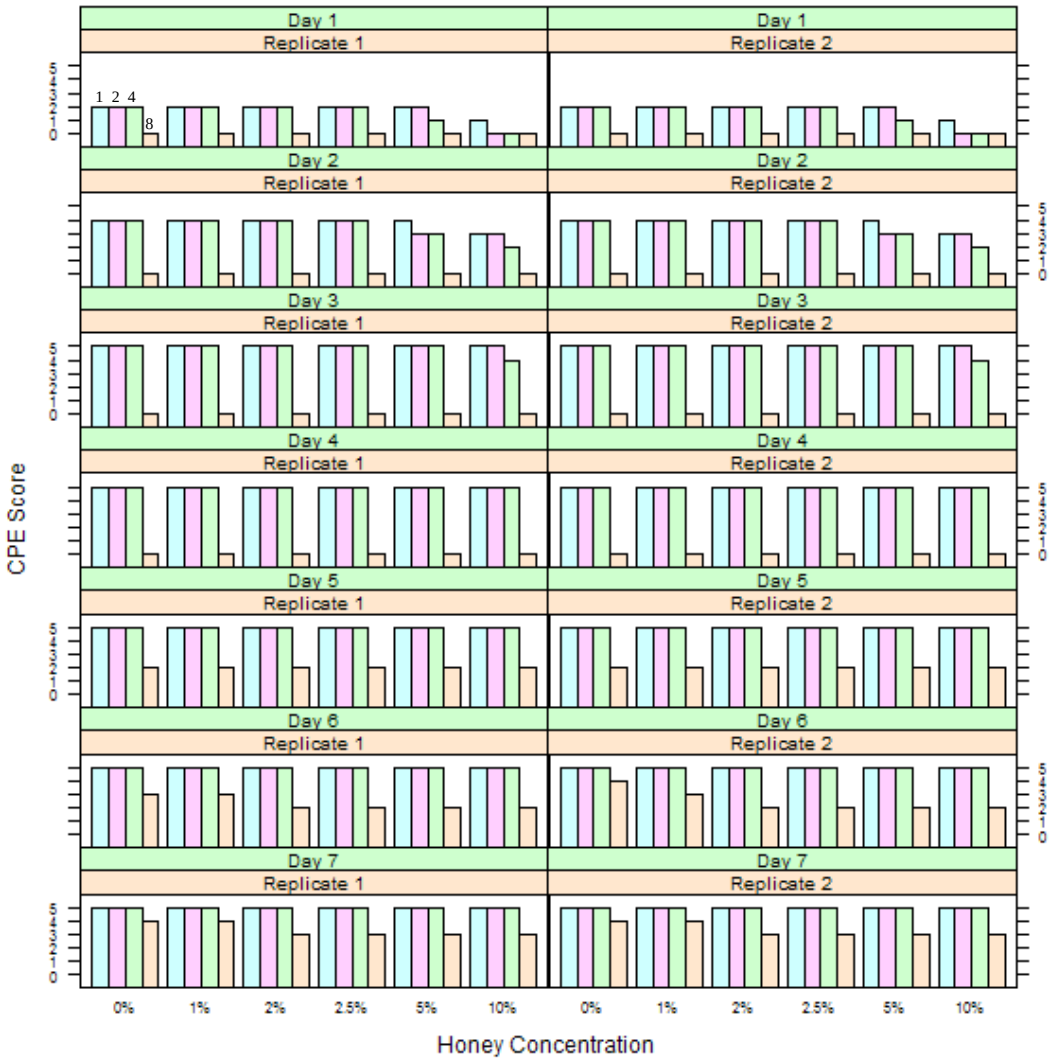
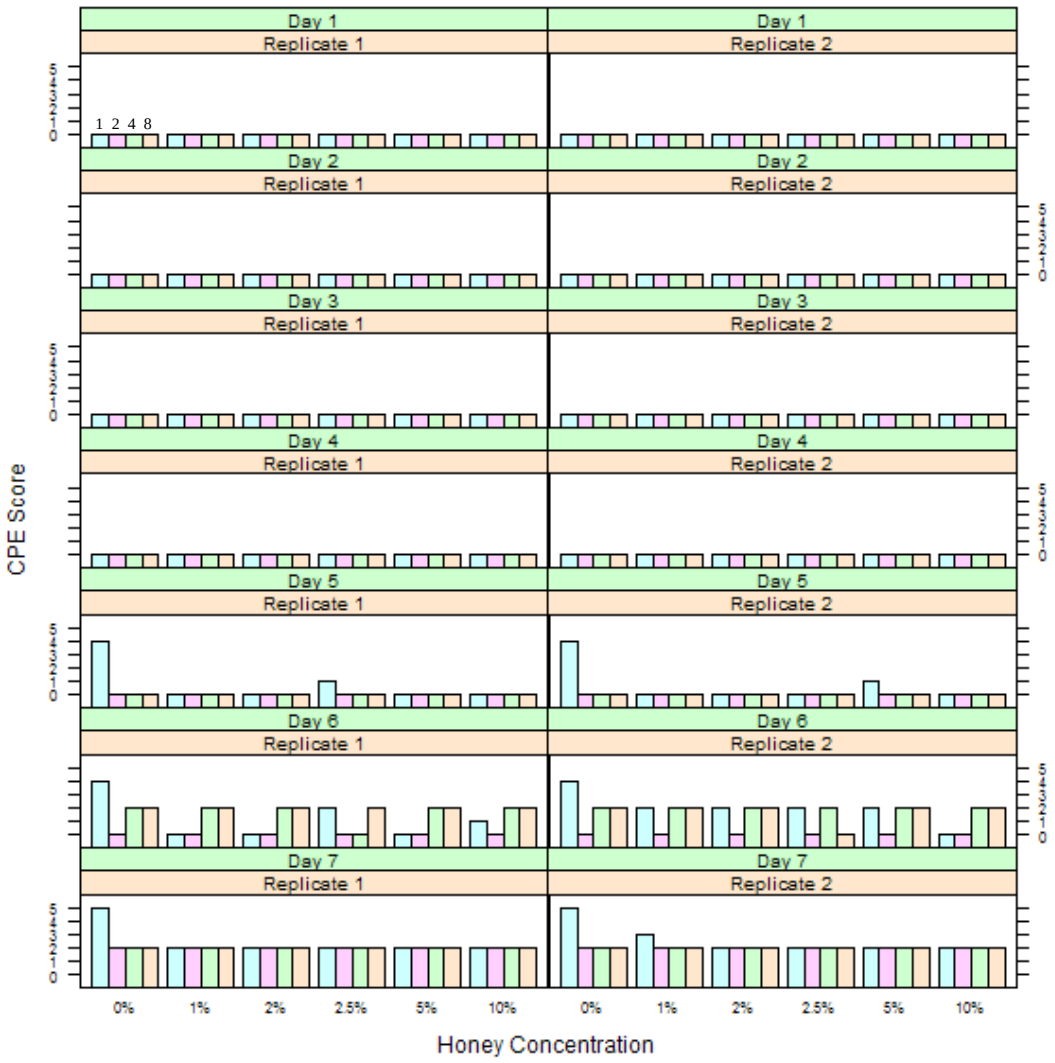


Figure 6.10 CPE resulting from infection of A549 cells with Ad3 after treatment of the virus with Manuka honey M151 at various concentrations for 1, 2, 4, or 8 hours: (a) experiment 1 and (b) experiment 2

(a) Experiment 1



(b) Experiment 2

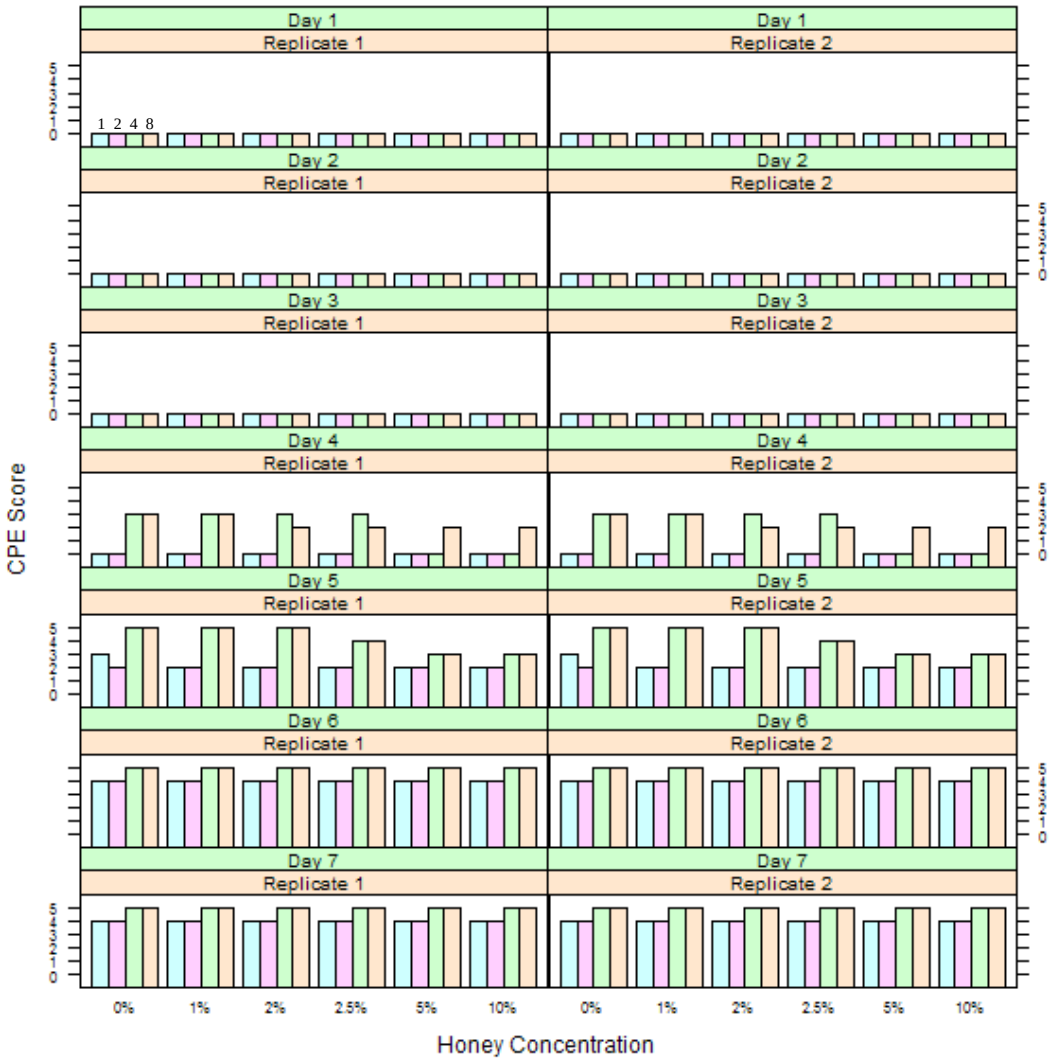


Figure 6.11 CPE resulting from infection of A549 cells with HSV-1 after treatment of the virus with Manuka honey M151 at various concentrations for 1, 2, 4, or 8 hours

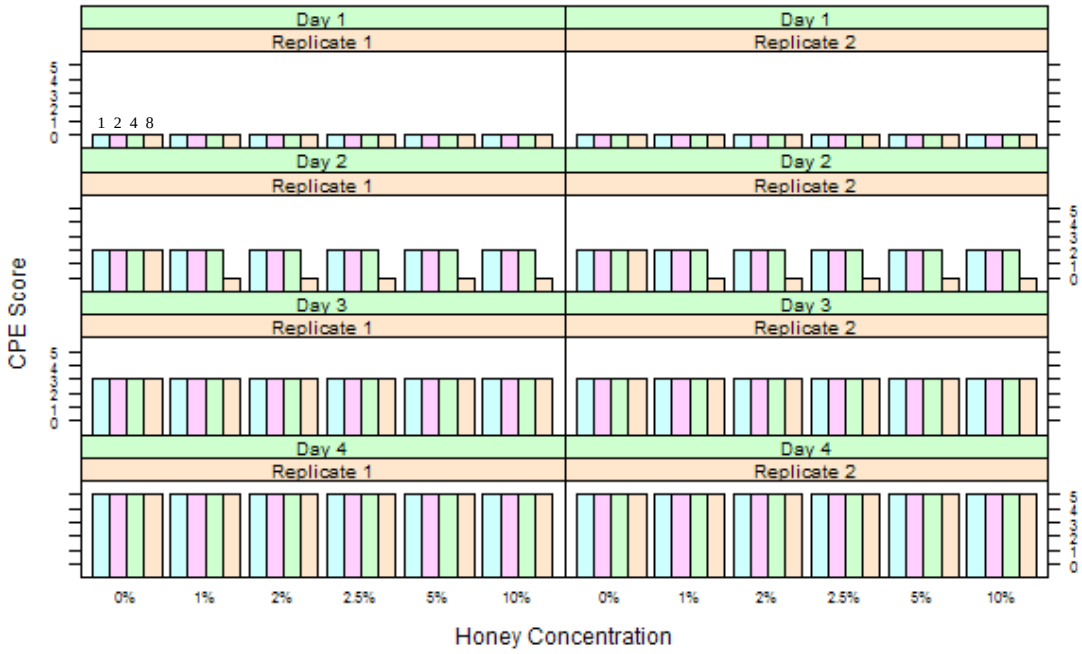
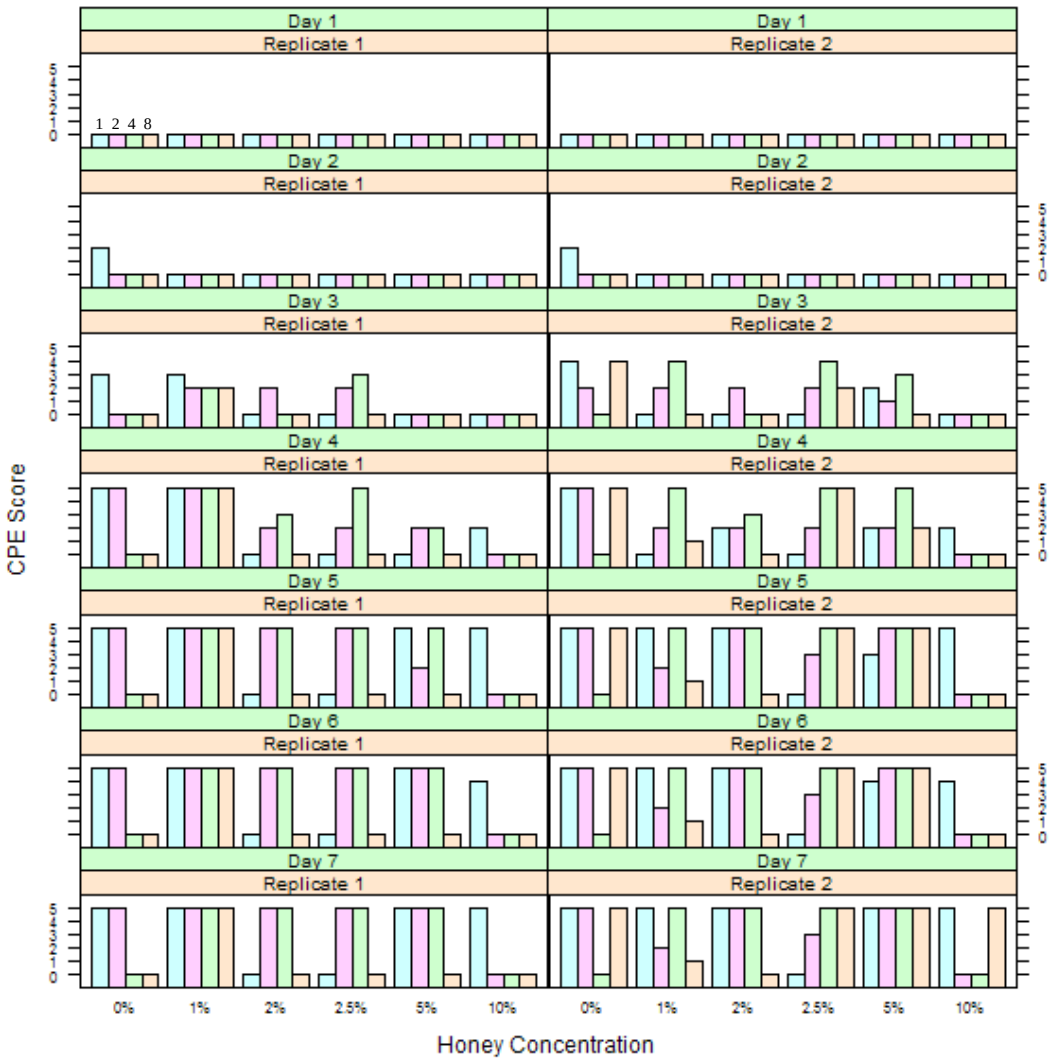


Figure 6.12 CPE resulting from infection of A549 cells with HSV-2 after treatment of the virus with Manuka honey M151 at various concentrations for 1, 2, 4, or 8 hours: (a) experiment 1 and (b) experiment 2

(a) Experiment 1



(b) Experiment 2

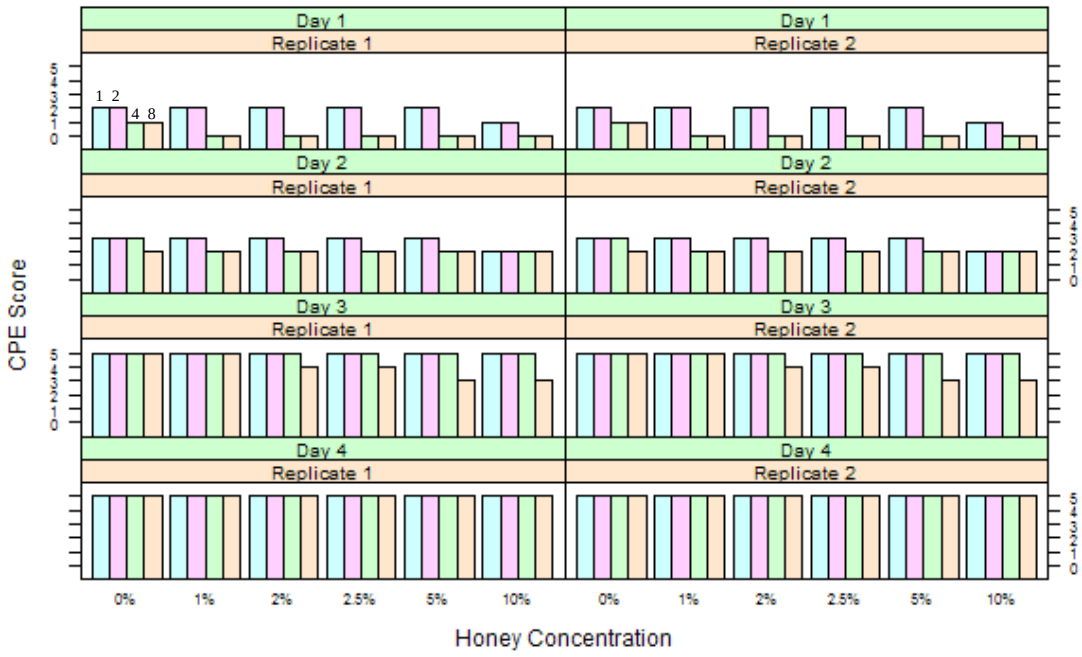


Figure 6.13 CPE resulting from infection of A549 cells with Ad3 after treatment of the virus with Ling Heather honey LH27 at various concentrations for 1, 2, 4, or 8 hours

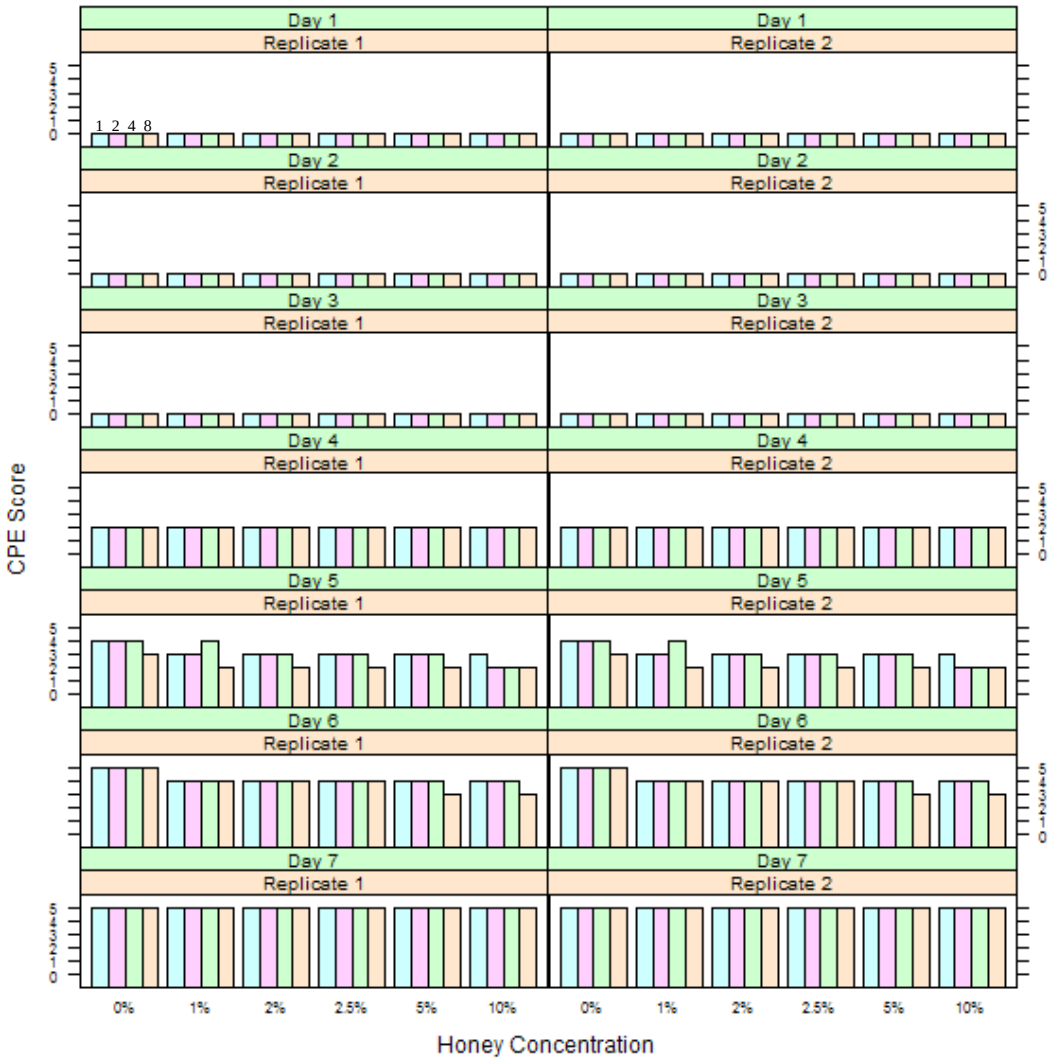


Figure 6.14 CPE resulting from infection of A549 cells with HSV-1 after treatment of the virus with Ling Heather honey LH27 at various concentrations for 1, 2, 4, or 8 hours

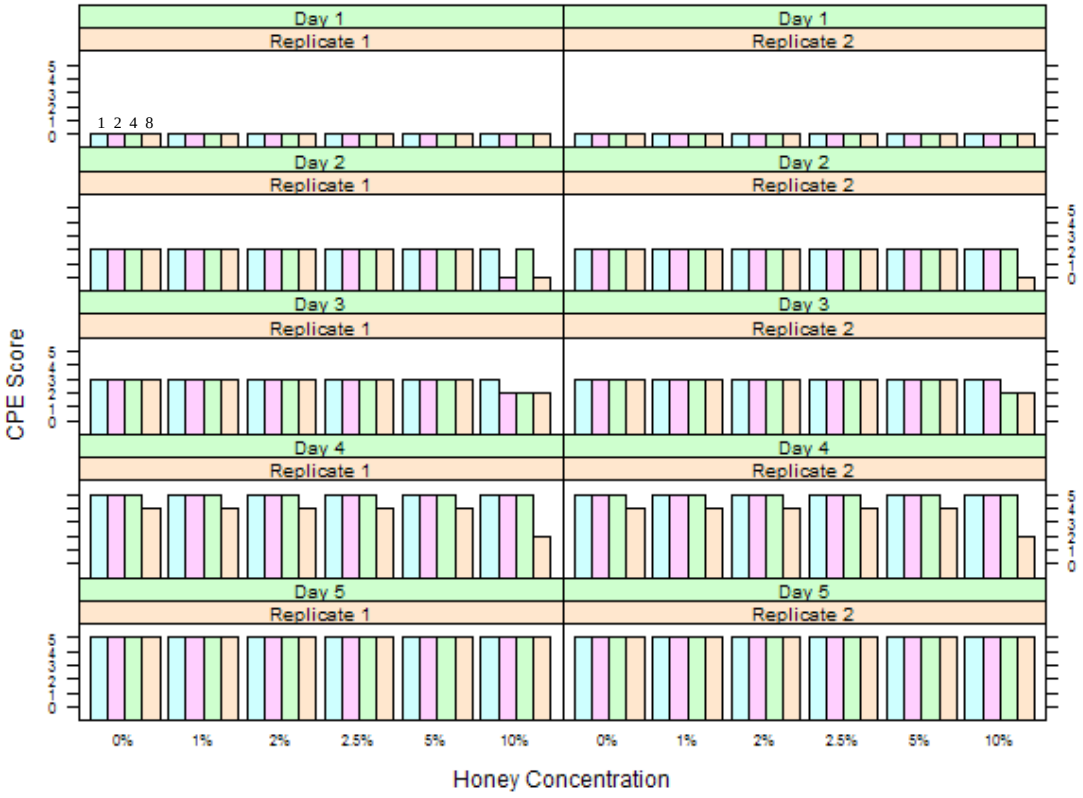
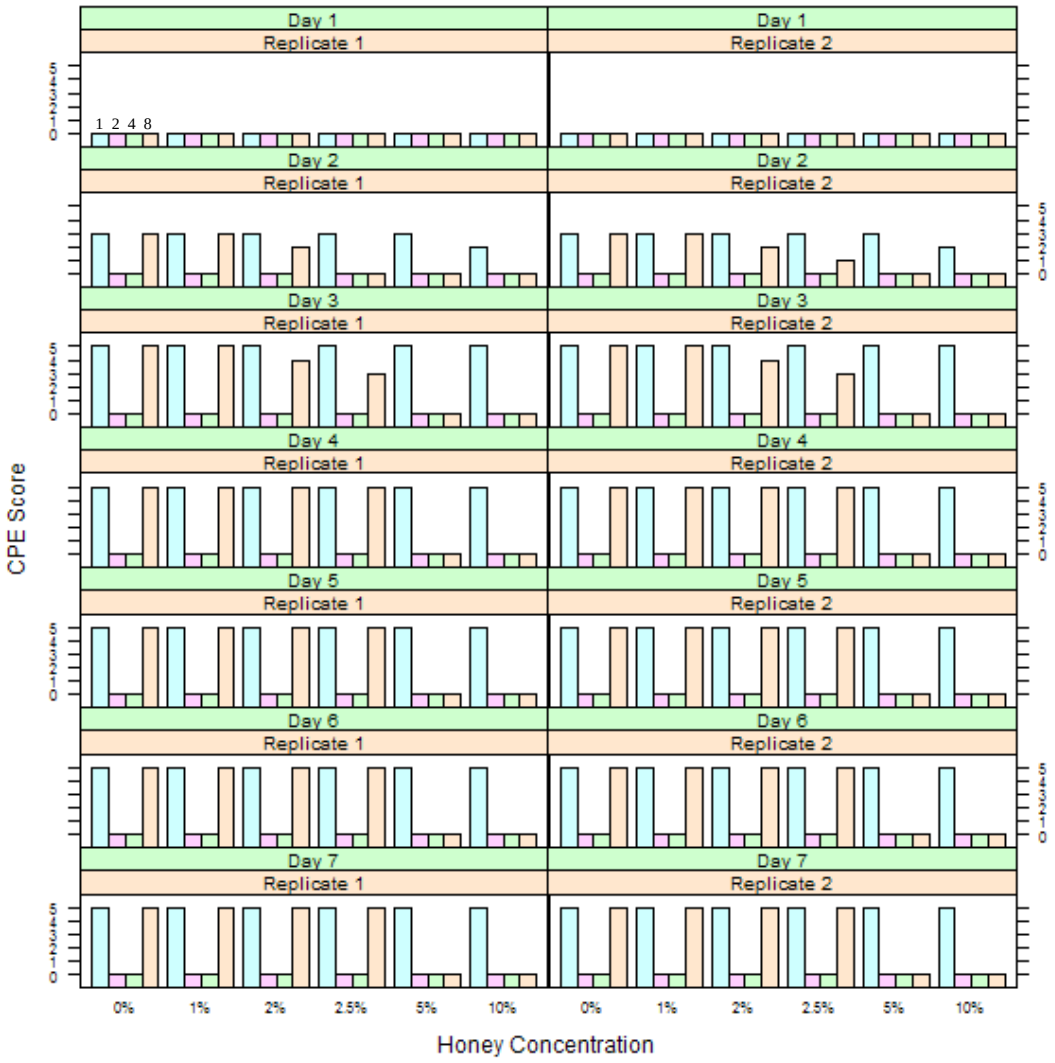


Figure 6.15 CPE resulting from infection of A549 cells with HSV-2 after treatment of the virus with Ling Heather honey LH27 at various concentrations for 1, 2, 4, or 8 hours: (a) experiment 1 and (b) experiment 2

(a) Experiment 1



(b) Experiment 2

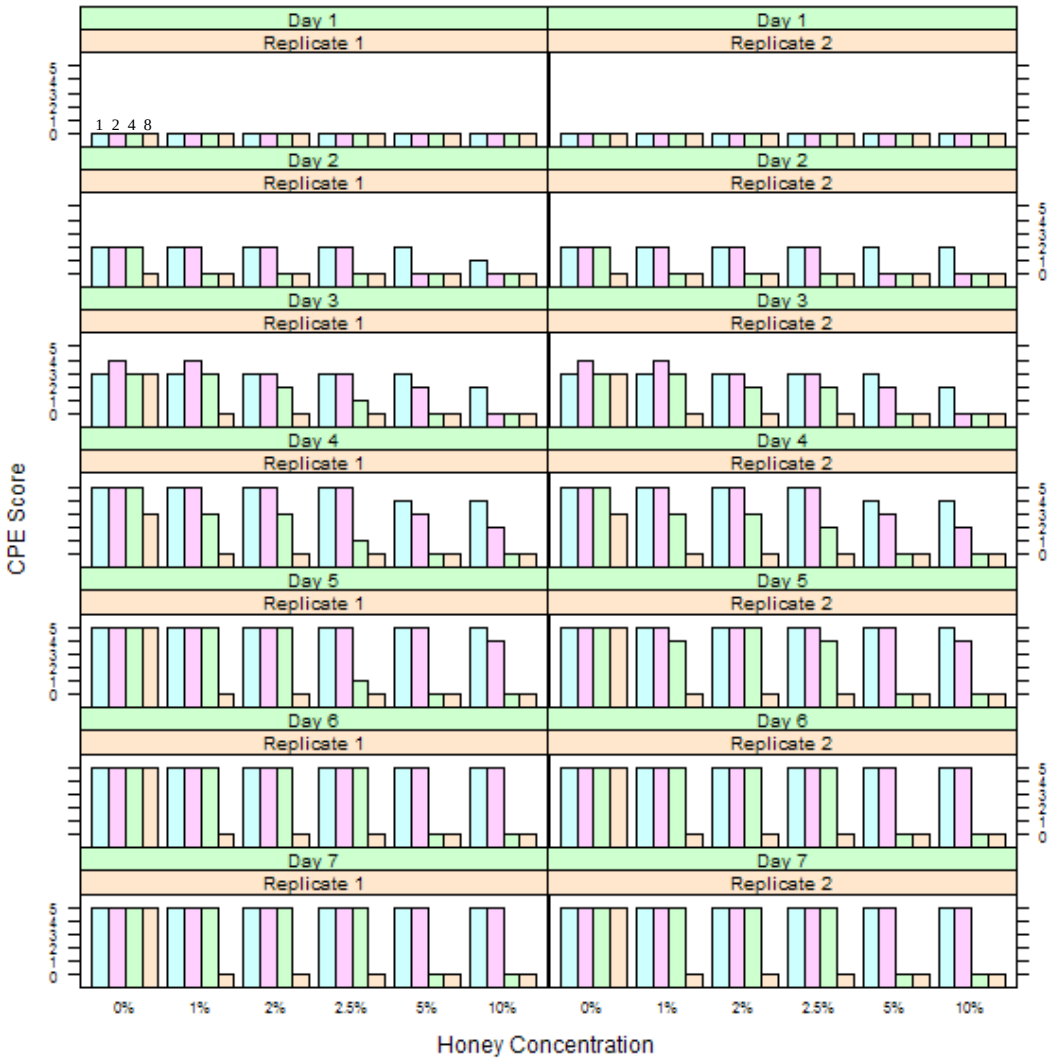
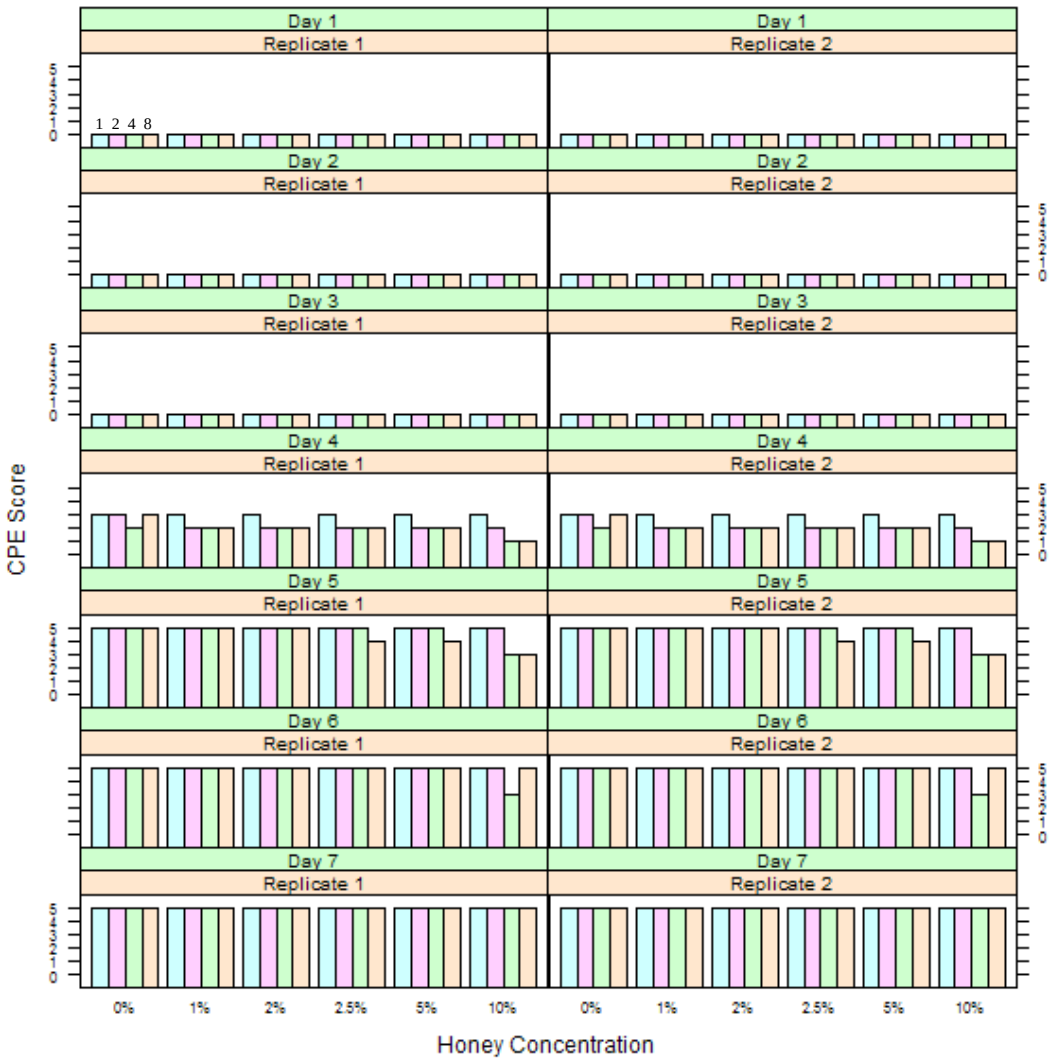


Figure 6.16 CPE resulting from infection of A549 cells with Ad3 after treatment of the virus with Rewarewa honey R19/06 at various concentrations for 1, 2, 4, or 8 hours: (a) experiment 1 and (b) experiment 2

(a) Experiment 1



(b) Experiment 2

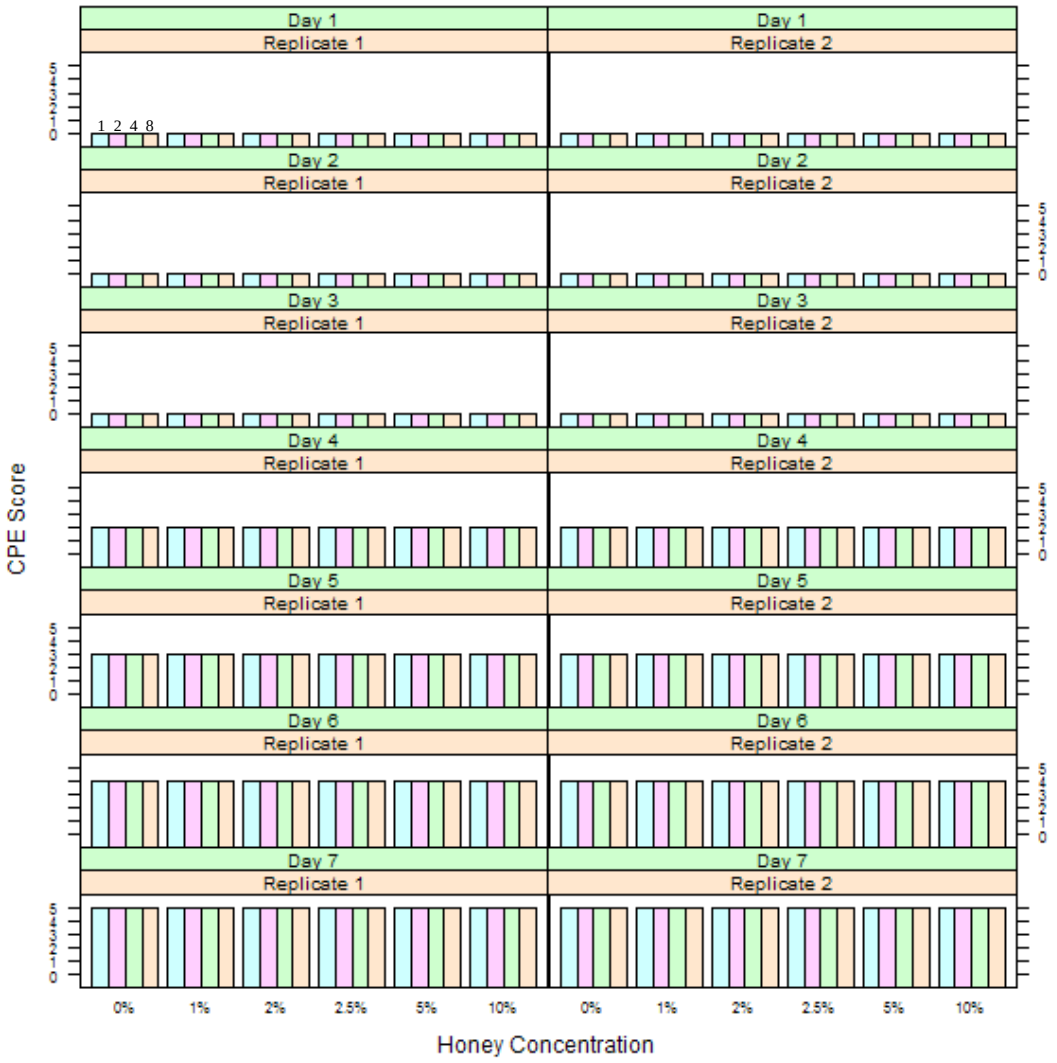
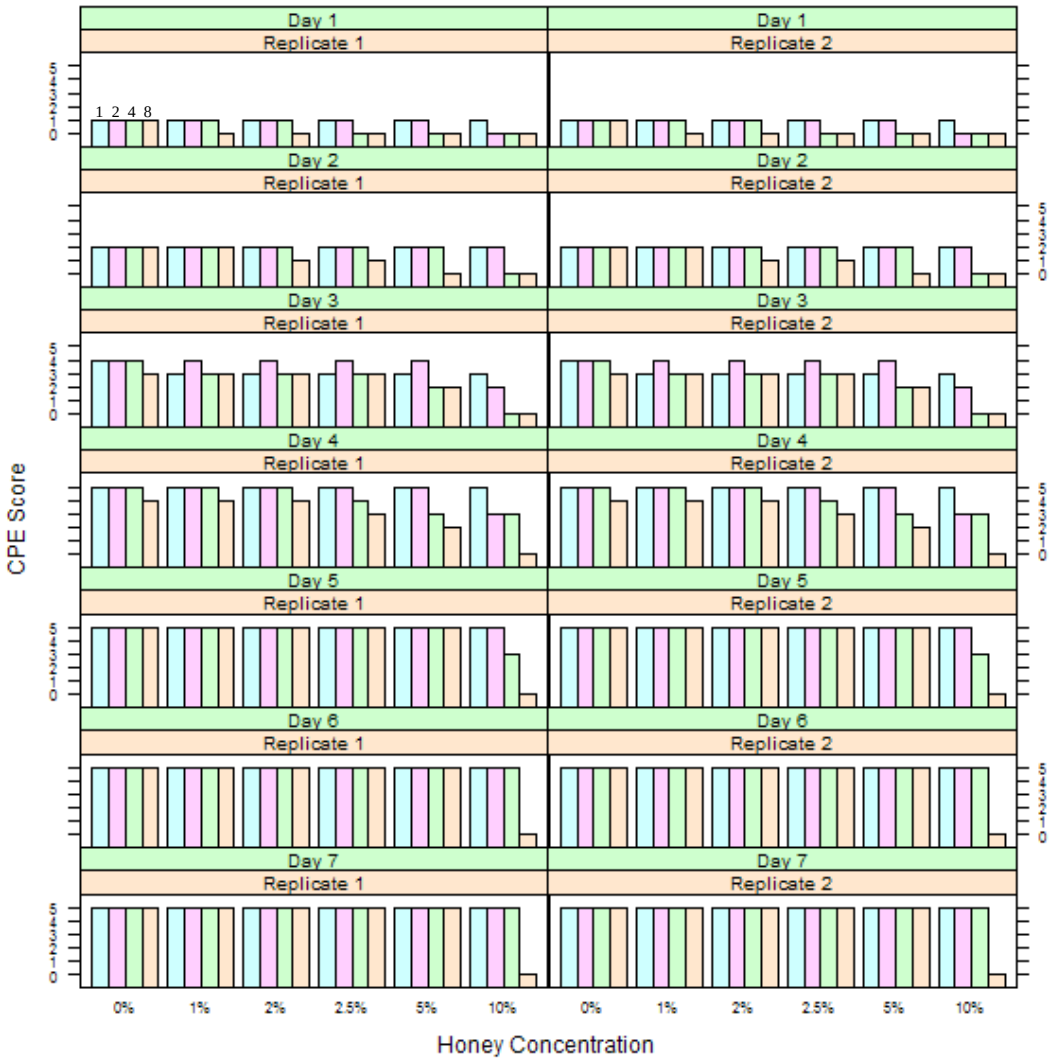


Figure 6.17 CPE resulting from infection of A549 cells with HSV-1 after treatment of the virus with Rewarewa honey R19/06 at various concentrations for 1, 2, 4, or 8 hours: (a) experiment 1 and (b) experiment 2

(a) Experiment 1



(b) Experiment 2

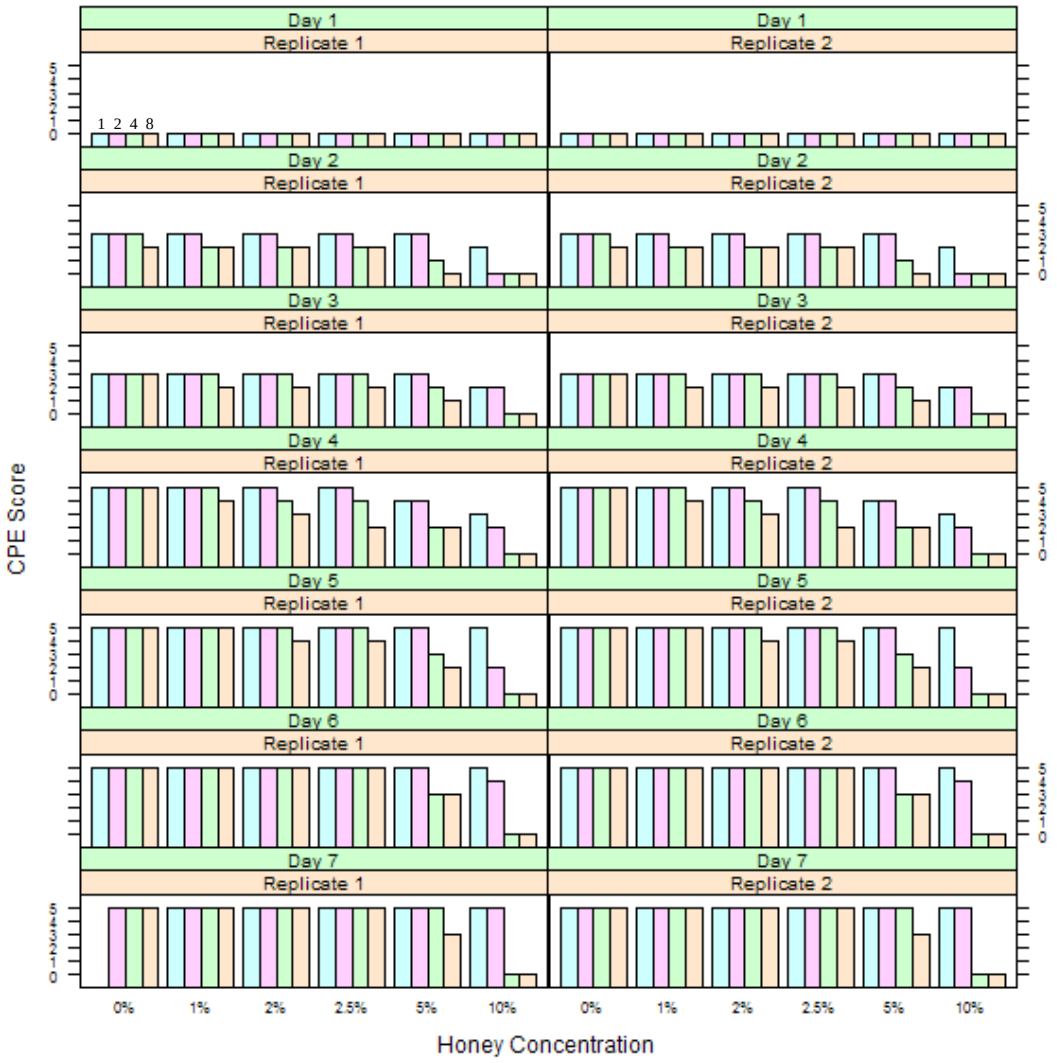
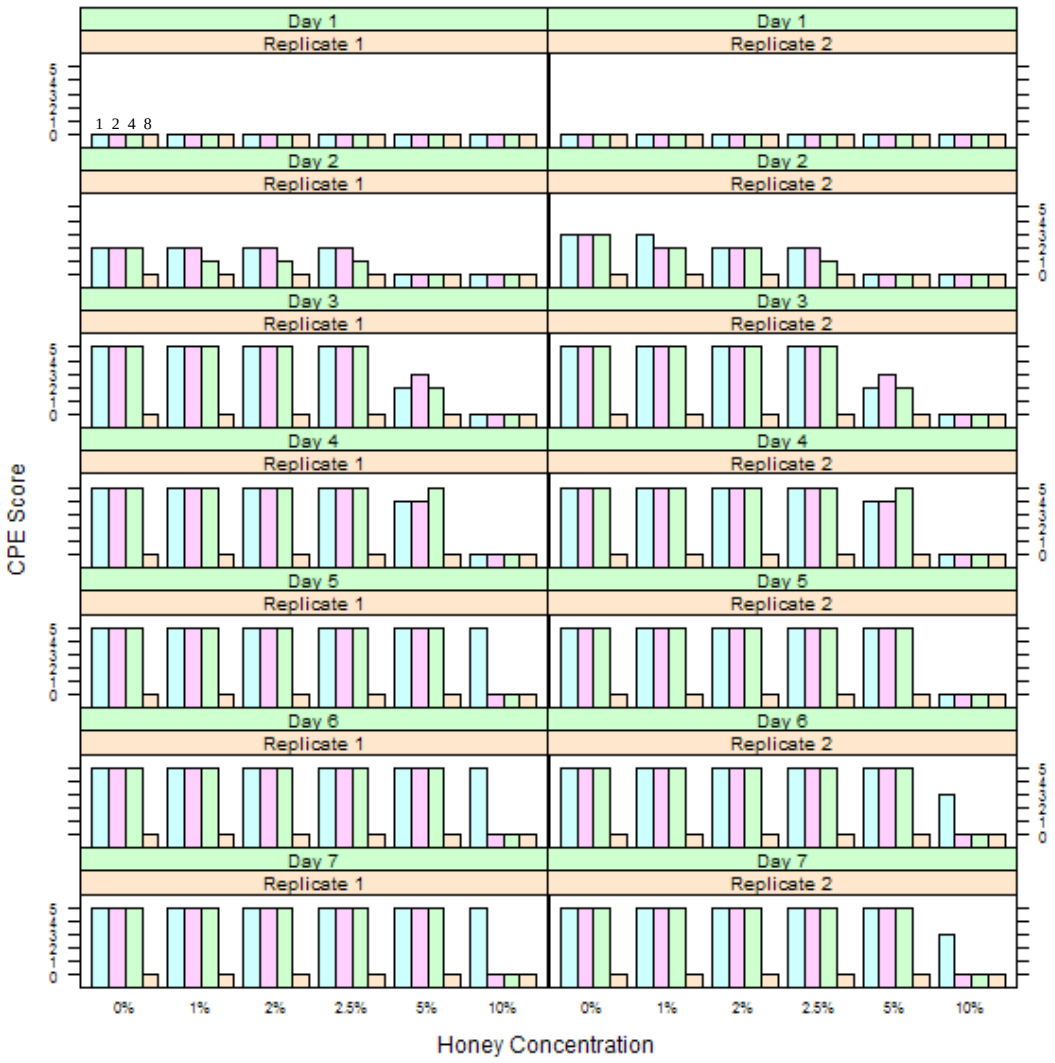


Figure 6.18 CPE resulting from infection of A549 cells with HSV-2 after treatment of the virus with Rewarewa honey R19/06 at various concentrations for 1, 2, 4, or 8 hours: (a) experiment 1 and (b) experiment 2

(a) Experiment 1



(b) Experiment 2

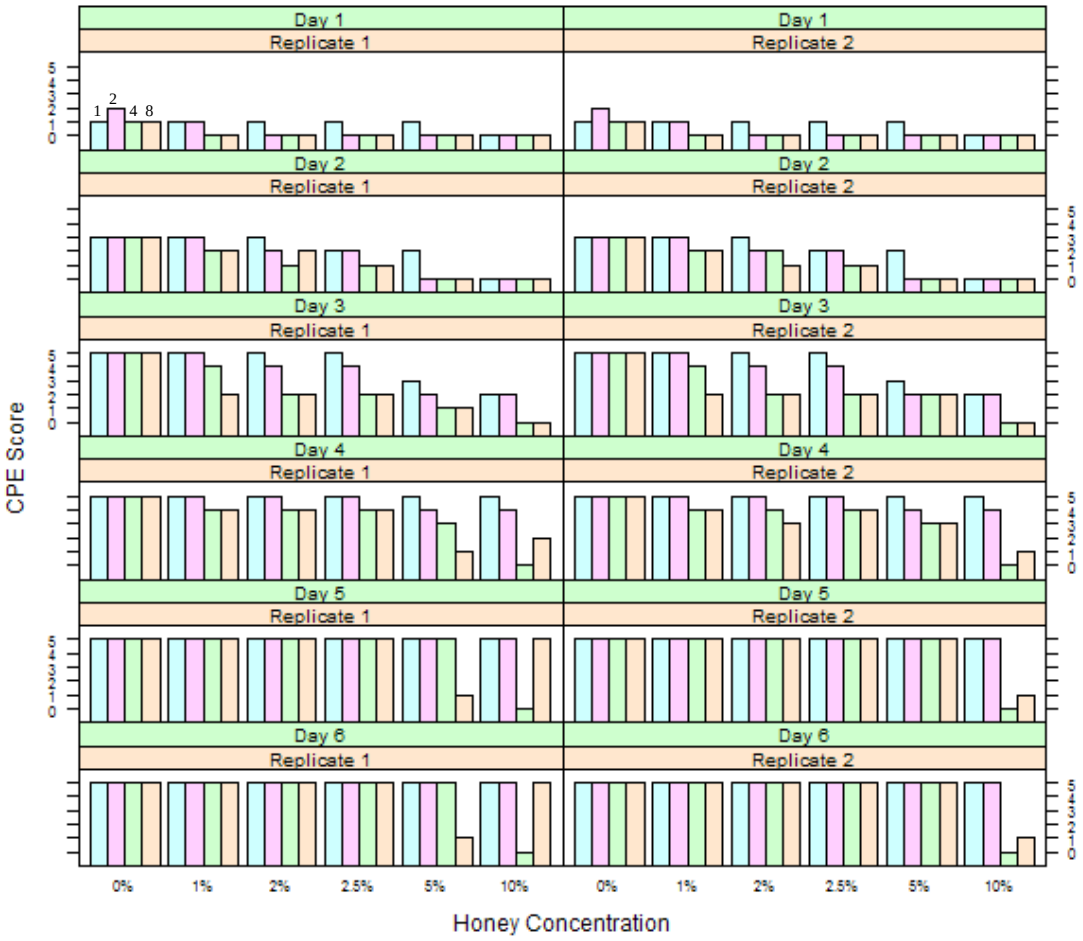
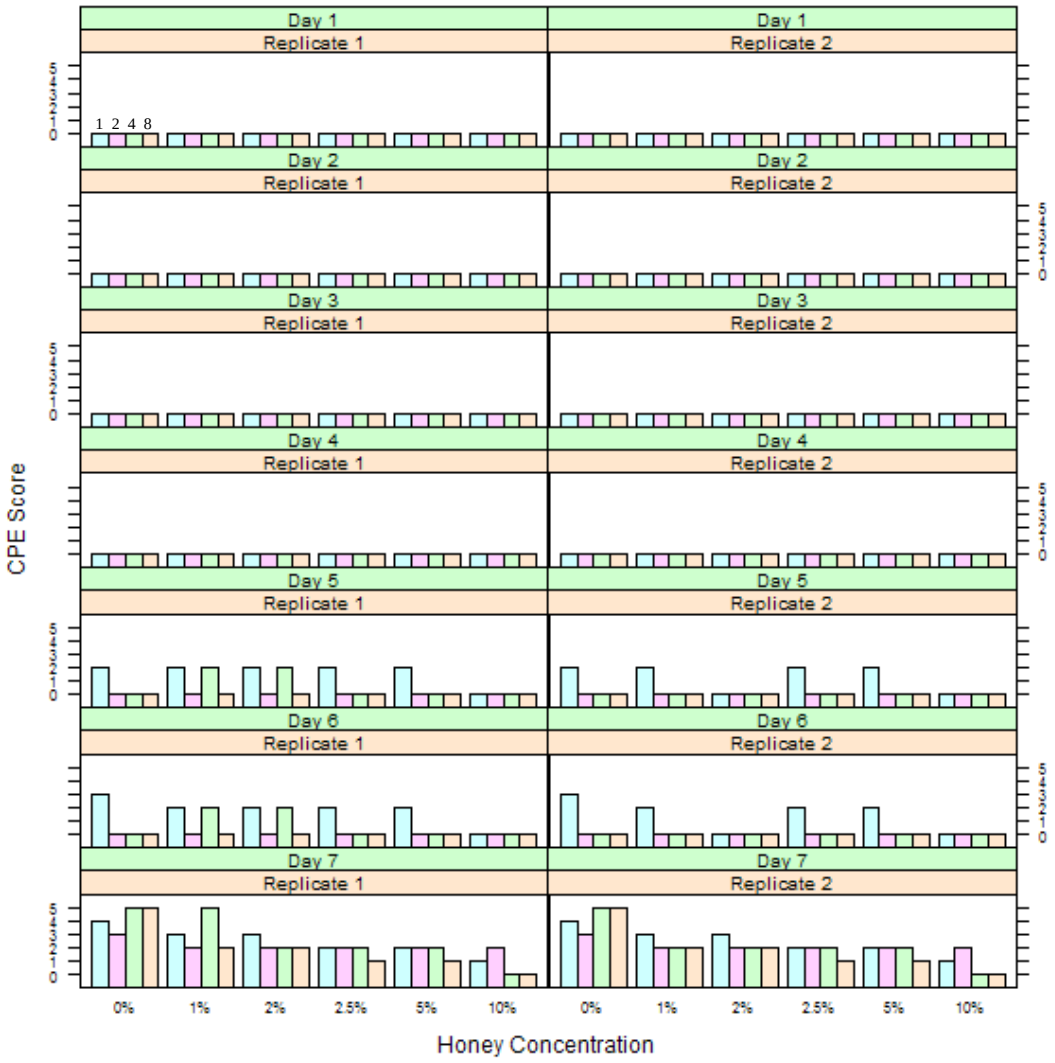


Figure 6.19 CPE resulting from infection of A549 cells with Ad3 after treatment of the virus with Honeydew honey HD19 at various concentrations for 1, 2, 4, or 8 hours: (a) experiment 1 and (b) experiment 2

(a) Experiment 1



(b) Experiment 2

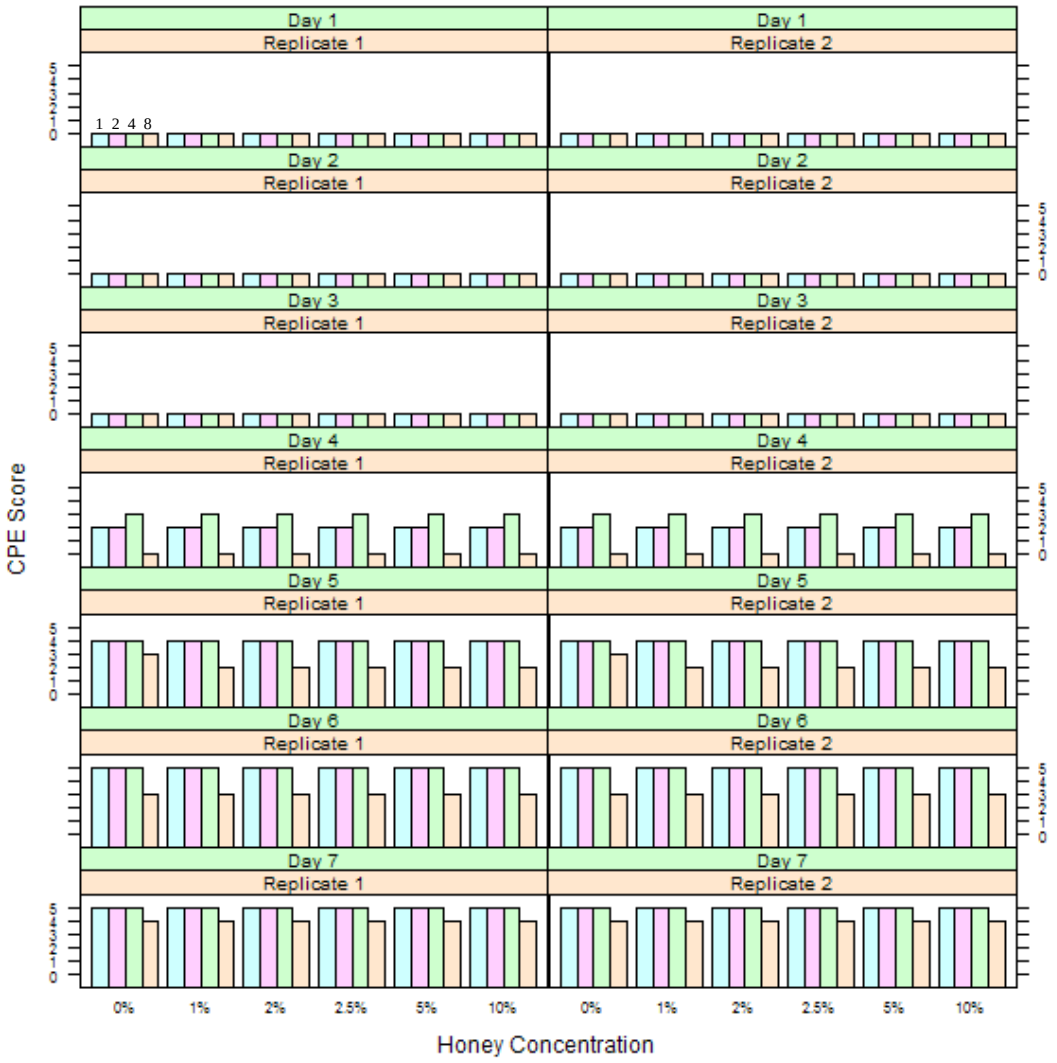
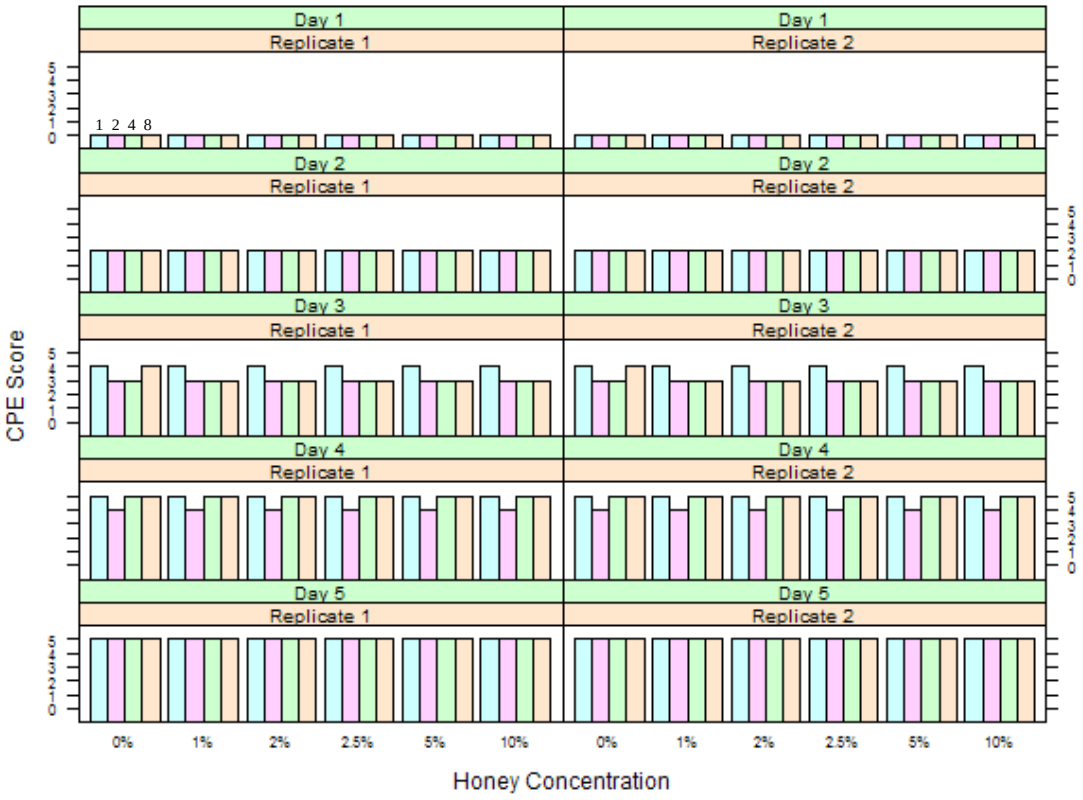


Figure 6.20 CPE resulting from infection of A549 cells with HSV-1 after treatment of the virus with Honeydew honey HD19 at various concentrations for 1, 2, 4, or 8 hours: (a) experiment 1 and (b) experiment 2

(a) Experiment 1



(b) Experiment 2

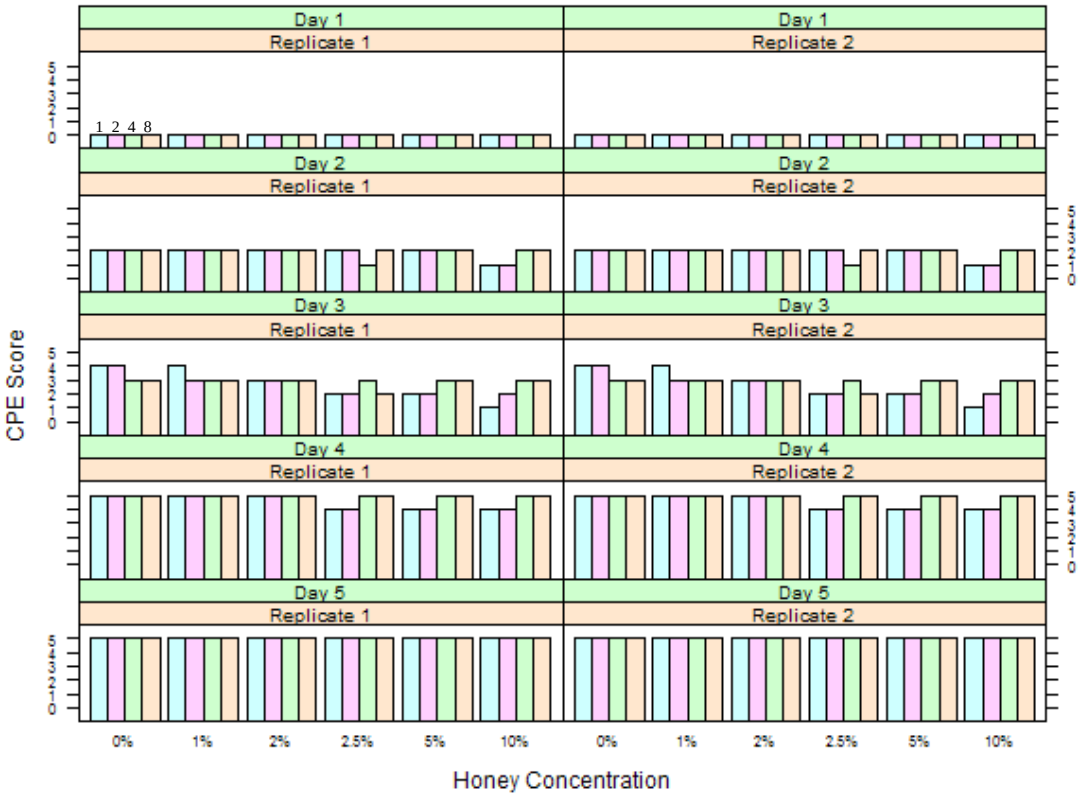
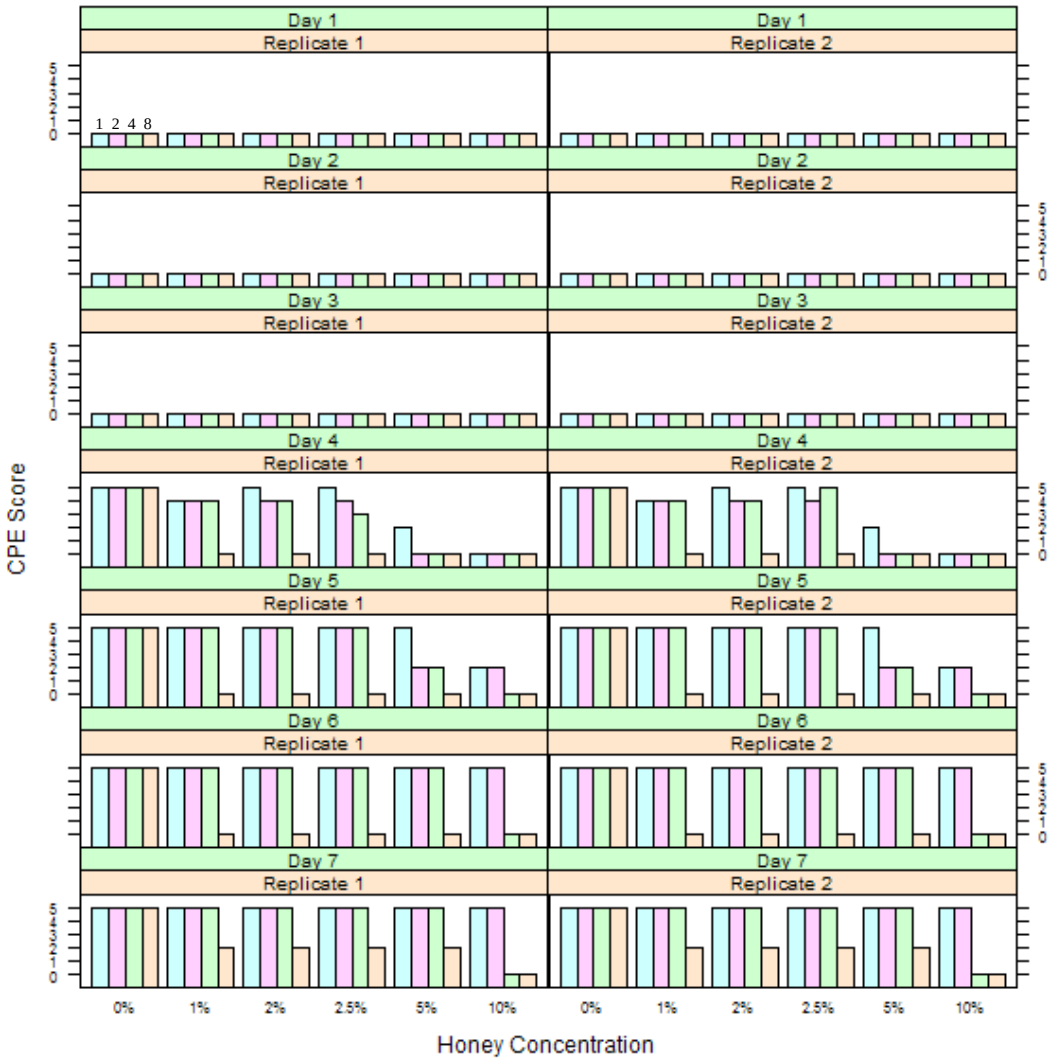


Figure 6.21 CPE resulting from infection of A549 cells with HSV-2 after treatment of the virus with Honeydew honey HD19 at various concentrations for 1, 2, 4, or 8 hours: (a) experiment 1 and (b) experiment 2

(a) Experiment 1



(b) Experiment 2

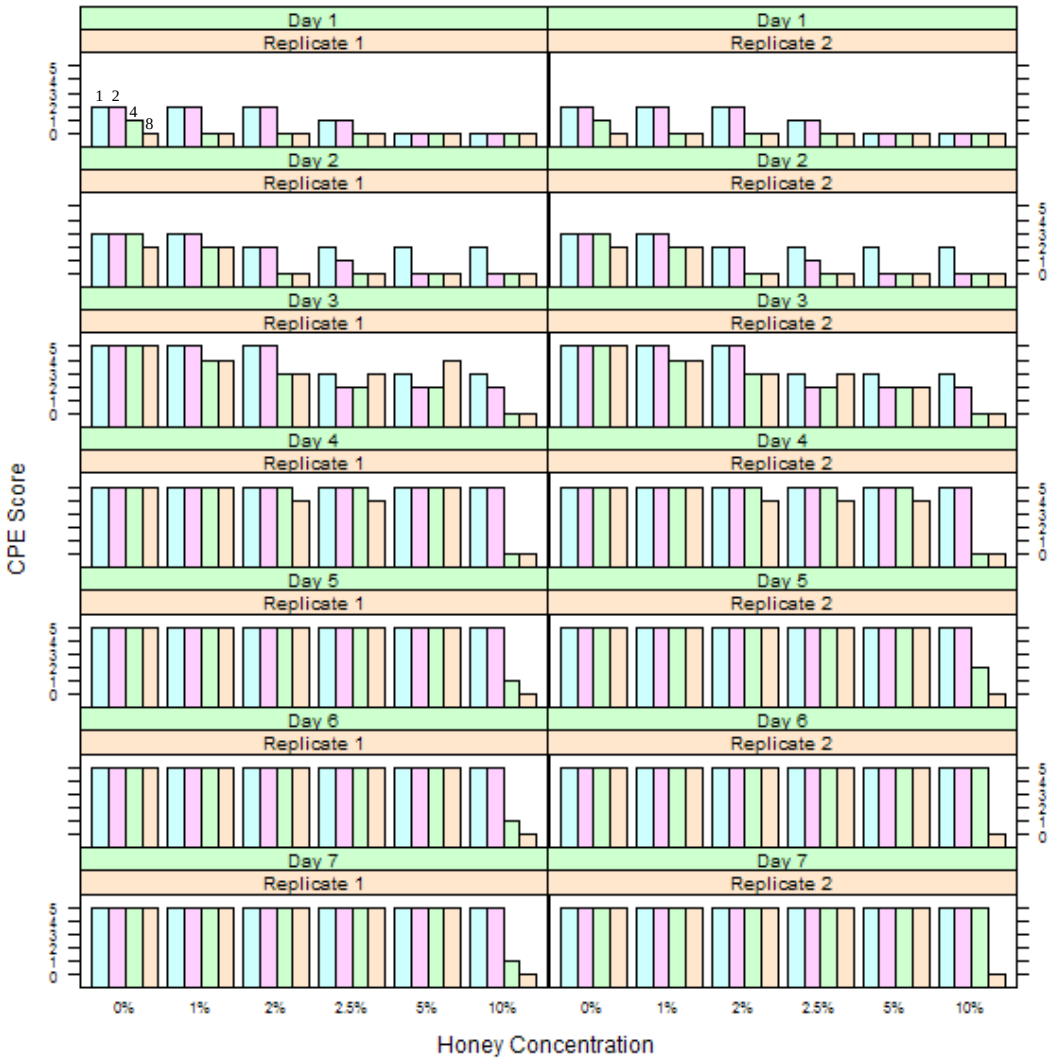


Table 6.2 Summary of the results shown in Figures 6.1 to 6.21

Honey Treatment	Ad3	HSV-1	HSV-2
Manuka M116 8 hr treat. 10%	✓	✓	✓
Manuka M116 8 hr treat. 5%		✓	
Manuka M112 8 hr treat. 10%		✓	✓
Manuka M112 4 hr treat. 10%			✓
Manuka M112 2 hr treat. 10%			✓
Manuka M157 8 hr treat. 10%	✓	✓	
Ling Heather LH27 8 hr treat. 10%			✓
Ling Heather LH27 8 hr treat. 5%			✓
Ling Heather LH27 8 hr treat. 1% - 10%			✓ *
Ling Heather LH27 4 hr treat. 10%			✓
Ling Heather LH27 4 hr treat. 5%			✓
Rewarewa R19/06 8 hr treat. 10%		✓	
Rewarewa R19/06 8 hr treat. 5%		✓	
Rewarewa R19/06 4 hr treat. 10%		✓	
Honeydew HD19 8 hr treat. 10%	✓ *[1]		✓
Honeydew HD19 8 hr treat. 5%	✓ *[1]		

* Neutralisation shown in Figure 6.15 (b), some not shown in Figure 6.15 (a).

*[1] Neutralisation shown in Figure 6.18 (a), not in Figure 6.18 (b).

Table 6.3 Summary of the average activity levels of each honey

Honey ID	Total Activity (w/v) phenol %	Non-peroxide Activity (w/v) phenol % (UMF)	TEAC mmol/kg	MGO mg/kg
Manuka M116	28.10	27.25	0.62	1241.70
Manuka M112	23.73	22.02	0.97	772.40
Manuka M157	20.85	20.25	1.27	745.50
Manuka M151	0	0	1.09	116.95
Ling Heather LH27	19.50	0	1.08	2.45
Rewarewa R19/06	17.10	0	1.42	25.70
Honeydew HD19	15.10	0	1.73	6.80

6.4 DISCUSSION

6.4.1 Comparisons between neutralisation treatments

The results show that in most experiments that the reduction of CPE was related to the concentration of honey and the time of exposure to it: lower levels of CPE compared with the untreated virus were observed with increasing honey concentration and duration of neutralisation period. The higher concentrations of honey, 5% and 10%, with the longest neutralisation periods, 4 and 8 hour, had the greatest effect on the resulting severity of viral infection by causing delays in the onset of viral infection and lower levels of infection over the course of observation compared with the shorter treatments and to their respective control of untreated virus.

In some cases the longer neutralisation treatments resulted in no or great delay in the onset of CPE with both the treated and untreated virus, suggesting an effect of prolonged heating at 37°C during the neutralisation period on the virus. If prolonged heating of the virus was not a factor, signs of viral infection with the untreated virus should have been expected to appear from the same time after inoculation of the cells as with the shorter treatments. In most experiments this was not the case and delays in the onset of CPE were observed. This impact of heating may have then accounted for some of the supposed greater antiviral activity of the longer neutralisation treatments, especially the 8 hour treatment with honey. To counteract this, the differences in CPE scores between the untreated virus and honey-treated virus were looked at, and in most experiments the 8 hour treatments seemed to give a greater difference between honey-treated virus and untreated virus than the shorter honey treatments.

6.4.2 Comparisons between neutralisation-causing treatments

Table 6.2 shows that the Manuka honeys were generally more effective against more than one viral isolate. It would seem that HSV was more greatly affected by the other honeys than Ad3 was. Ad3 was neutralised by just one of the other honeys studied, Honeydew honey HD19. It would further seem that the other honeys studied work effectively against specific viral isolates for example Ling Heather honey with HSV-2 suggested a range of successful treatments in one or both experiments more so than the Manuka honeys did.

6.4.3 Evaluation of experimental design

These experiments aimed to compare the neutralisation capacity of many honeys, with the goal of determining successful neutralisation treatments for each virus, measured by means of absence of any kind of viral CPE over the course of observation which was compared with the untreated virus. It has been shown with these experiments completed on different days that many of the honeys tested were able to achieve this with the 10% honey concentration.

6.4.3.1 Evaluation of modified neutralisation methodology

It was clear that by increasing the concentration of honey from 2% (v/v) up to 10% (v/v) that a greater range of honeys showed antiviral activity.

Based on the earlier experiments testing the tolerance of the cells to honey, these higher concentrations of honey were likely to have potentially caused changes to cells morphology over the observation period, if the inoculation period had remained at 1 hour. However, the shorter inoculation period of the cells incorporating centrifuging overcame the negative effects of using these concentrations of honey. It was shown with this shorter inoculation period that viral CPE appeared after the usual time after infection of the cells, indicating that it was a sufficient time period to allow for viral entry. This short inoculation period was also likely to have minimised any effect the honey present in the inoculum may have had on the cells, in previous work (Chapter 4, Section 4.5) it was not until 24 hours that changes to the cells were apparent, and with the replacement of the inoculum with maintenance medium any remaining honey would have been greatly diluted to well below inhibitory levels.

In almost all experiments the replicates matched. It was found though that many results between the experiments were dissimilar, even between the untreated viruses, including: reverse trends as seen in Figures 6.1 (b), 6.4 (a), 6.5 (b), 6.6 (b), 6.7 (b), 6.10 (a) and (b), 6.12 (a), 6.15 (a), 6.18 (a), 6.20 (a) and (b), with one or more of the treatments; random variance as shown in Figures 6.4 (b), 6.13, 6.14, 6.16 (a), 6.18 (a), 6.19 (a) and (b), with one or more of the treatments; unconfirmed neutralisation as shown in Figures 6.1 (b), 6.3 (b), 6.6 (b), 6.19 (b); or additional neutralisation treatments unseen in the first experiment as shown in Figures 6.7 (b), 6.15 (b), 6.17 (b); absence of any CPE with the untreated virus in Figures 6.5 (b), 6.15 (a), 6.18 (a), and complete absence of viral CPE over the course of observation with four experiments testing Manuka honeys M157 and M151, and Ling Heather honey with HSV-1, and Ling Heather honey with Ad3 (data not graphed). It would have been ideal to observe that with a specific honey-treatment on a particular day after inoculation of the cells that a certain level of CPE would be shown. Despite in most cases the overall result being the same between experiments whether or not neutralisation had occurred, the discrepancies show that this method can not be used to specifically designate scores; this method simply gives an idea of whether or not honey had any activity against the virus of interest.

6.4.4 Comparisons with the activity table

Table 6.3 outlines the activity levels of the honeys investigated for neutralisation capacity. Manuka honeys M116 and M112 with the highest antibacterial activity ratings, especially the highest levels of methylglyoxal, had the greatest effect and so neutralised HSV-2. It was further observed that neutralisation occurred with 8

hours treatment with Honeydew honey HD19 at 10%. Perhaps in addition to high methylglyoxal requirements, exceptional antioxidant activity has an effect on viral infection of HSV-2. Likewise, Ling Heather honey LH27 too caused neutralisation of HSV-2 suggesting perhaps an effect of phenolic compounds known to be found at high levels in Ling Heather honey (Castro-Vazquez, 2009). Similarly, high levels of phenolic compounds are found in Manuka honey, suggesting that it may be the phenolics rather than methylglyoxal that are responsible for the neutralising action of Manuka honey.

HSV-1 too appears to be affected by the honeys containing the highest levels of methylglyoxal and was also prevented from causing infection with Rewarewa honey, despite the far lower methylglyoxal levels (lower than levels of other honeys that did not eliminate this virus) and lower levels of antioxidant capacity, compared with Honeydew honey HD19 which did not eliminate HSV-1 viral infection.

Ad3 was neutralised by Manuka honeys M157, M116, and Honeydew honey HD19, therefore there was no correlation between increasing methylglyoxal and non-peroxide antibacterial activity and the prevention of viral infection. It cannot be assumed then that high methylglyoxal and non-peroxide antibacterial activity dictate a reduction of adenoviral infection, as Manuka honey M112 which did not cause neutralisation of Ad3, has higher activity levels than Manuka honey M157. This can suggest that either a combination of components or some other component within honey may be wholly or partly responsible for the observed antiviral effects.

Honeydew honey HD19 caused neutralisation of Ad3 after 8 hours exposure of the virus to 5% and 10% solutions of honey, further suggesting some effect of this high antioxidant honey on viral infection. This honey acted most broadly against the viral isolates, compared with the other non-Manuka honeys tested.

6.4.5 Further study

Due to the limited time span of this study it was appropriate at this stage to reduce the number of honey types and samples used in the subsequent part of this study, and so focus the remainder of the investigation on those honeys that showed most promise and that had characteristic properties that could be potentially responsible in part for the antiviral activity.

As there was no correlation between an increase in methylglyoxal level and antiviral activity Manuka honeys M112 and M157 can be excluded from further analysis, leaving the extremes of non-peroxide antibacterial activity for investigation. The honeys amenable to further study were the highest and lowest non-peroxide antibacterial activity Manuka honeys, and the non-Maunka honeys with the highest antioxidant content; similarly the highest total antibacterial activity honey, Ling Heather which was effective against HSV-2. It is for these reasons that Manuka honeys M116 and M151, and Honeydew honey HD19 and Ling Heather LH27 were chosen as being suitable for further study.

Chapter 7

The antiviral activity of honey: virustatic or virucidal

This chapter aims to describe results of the virucidal activity of honey, an investigation subsequent to the results of the neutralisation experiments in Chapter 6.

Virucidal activity of a drug implies that it has caused deactivation of the virus, which prevents it from causing successful infection within a host cell. This can suggest that the drug perhaps has a greater effect on a virus than observed with neutralisation experiments, as the absence of CPE caused by the neutralisation effect may be due to either virustatic (temporary prevention of infection) or virucidal (permanent prevention of infection) activity. These types of activity can be tested for through the diluting of the drug of interest to sub-inhibitory levels and observing for any consequential viral growth within susceptible cells.

7.1 INTRODUCTION

The investigation described in Chapter 6 extended the preliminary experiments and has shown that after the direct treatment of the virus with honey, viral CPE can be inhibited as shown in Table 6.2 or temporarily suppressed. It was unidentified whether the honey treatments had deactivated the viruses and so permanently prevented any kind of further viral growth and therefore CPE. In order to determine whether the viruses had been deactivated by the neutralisation treatments, after the 8 hour treatment with 5% and 10% solutions of each honey type, the honey solutions containing the viruses were diluted in maintenance medium. The aim was to decease to an ineffective level any residual honey which may be inhibiting the virus from attaching to, or replicating within the cells and so preventing CPE. This can assess whether the honey is a virucidal agent against the viruses being tested.

7.2 METHODS

Confluent A549 cells were prepared in 24 well plates as described in Section 3.2.1. The growth medium was removed from the cells and was replaced with 0.8

ml of maintenance medium and 0.2 ml of thawed 5% and 10% honey solutions containing virus that had been frozen at -70°C at the conclusion of the 8 hour neutralisation period (see Section 6.2 for details of the neutralisation methodology). This diluted each virus to 1/15 of the original 10^{-4} viral dilution, 6.6^{-6} (to approximately 6.6^{4} viral particles per ml) and a 9/50 dilution of the original honey concentration to 0.9% or 1.8% respectively. The cells were incubated at 37°C and observed daily for the development of viral CPE for 15 days. Each experiment was completed in quadruplet.

7.3 RESULTS

The results of CPE development after inoculation of the cells with diluted honey-treated virus after treatment with 5% and 10% solutions of honey are presented in Tables 7.1 to 7.6. The bold values in the tables outline where viral CPE became apparent, and the captions indicate the virus and honey type being tested.

Table 7.1 CPE from Ad3 after treatment of the virus with Manuka honeys: (a) M116 and M157, (b) M112, and (c) M151

(a) Manuka honeys M116 and M157

	0% honey (without virus)				5% honey				10% honey			
Day 1	0	0	0	0	0	0	0	0	0	0	0	0
Day 2	0	0	0	0	0	0	0	0	0	0	0	0
Day 3	0	0	0	0	0	0	0	0	0	0	0	0
Day 4	0	0	0	0	0	0	0	0	0	0	0	0
Day 5	0	0	0	0	0	0	0	0	0	0	0	0
Day 6	0	0	0	0	0	0	0	0	0	0	0	0
Day 7	0	0	0	0	0	0	0	0	0	0	0	0
Day 8	0	0	0	0	0	0	0	0	0	0	0	0
Day 9	0	0	0	0	0	0	0	0	0	0	0	0
Day 10	0	0	0	0	0	0	0	0	0	0	0	0
Day 11	0	0	0	0	0	0	0	0	0	0	0	0
Day 12	0	0	0	0	0	0	0	0	0	0	0	0
Day 13	0	0	0	0	0	0	0	0	0	0	0	0
Day 14	0	0	0	0	0	0	0	0	0	0	0	0
Day 15	0	0	0	0	0	0	0	0	0	0	0	0

(b) Manuka honey M112

	0% honey (without virus)				5% honey				10% honey			
Day 1	0	0	0	0	0	0	0	0	0	0	0	0
Day 2	0	0	0	0	0	0	0	0	0	0	0	0
Day 3	0	0	0	0	0	0	0	0	0	0	0	0
Day 4	0	0	0	0	0	0	0	0	0	0	0	0
Day 5	0	0	0	0	0	0	0	0	0	0	0	0
Day 6	0	0	0	0	0	0	0	0	0	0	0	0
Day 7	0	0	0	0	0	0	0	0	0	0	0	0
Day 8	0	0	0	0	0	0	0	0	0	0	0	0
Day 9	0	0	0	0	0	0	0	0	0	0	0	0
Day 10	0	0	0	0	0	0	0	0	0	0	0	0
Day 11	0	0	0	0	0	0	0	0	0	0	0	0
Day 12	0	0	0	0	0	0	**1**	0	0	0	0	0
Day 13	0	0	0	0	0	0	**2**	**1**	0	0	0	0
Day 14	0	0	0	0	0	0	**3**	**2**	0	0	0	0
Day 15	0	0	0	0	0	0	**4**	**3**	0	0	0	0

(c) Manuka honey M151

	0% honey (without virus)				5% honey				10% honey			
Day 1	0	0	0	0	0	0	0	0	0	0	0	0
Day 2	0	0	0	0	0	0	0	0	0	0	0	0
Day 3	0	0	0	0	0	0	0	0	0	0	0	0
Day 4	0	0	0	0	0	0	0	0	0	0	0	0
Day 5	0	0	0	0	0	0	0	0	0	0	0	0
Day 6	0	0	0	0	0	0	0	0	0	0	0	0
Day 7	0	0	0	0	0	0	**2**	**2**	0	0	0	0
Day 8	0	0	0	0	0	0	**3**	**3**	0	0	0	0
Day 9	0	0	0	0	0	0	**3**	**3**	0	0	**2**	**2**
Day 10	0	0	0	0	0	0	**4**	**4**	0	0	**2**	**2**
Day 11	0	0	0	0	0	0	**4**	**4**	0	0	**3**	**4**
Day 12	0	0	0	0	**3**	**3**	**5**	**4**	**2**	**2**	**3**	**4**
Day 13	0	0	0	0	**3**	**3**	**5**	**4**	**3**	**3**	**4**	**4**
Day 14	0	0	0	0	**4**	**4**	**5**	**4**	**2**	**2**	**4**	**5**
Day 15	0	0	0	0	**4**	**4**	**5**	**5**	**2**	**2**	**5**	**5**

Table 7.2 CPE from Ad3 after treatment of the virus with: (a) Honeydew honey HD19, (b) Rewarewa honey R19/06, and (c) Ling Heather honey LH27

(a) Honeydew honey HD19

	0% honey (without virus)				5% honey				10% honey			
Day 1	0	0	0	0	0	0	0	0	0	0	0	0
Day 2	0	0	0	0	0	0	0	0	0	0	0	0
Day 3	0	0	0	0	0	0	0	0	0	0	0	0
Day 4	0	0	0	0	0	0	0	0	0	0	0	0
Day 5	0	0	0	0	0	0	0	0	0	0	0	0
Day 6	0	0	0	0	**2**	**2**	0	0	**2**	**2**	0	0
Day 7	0	0	0	0	**3**	**3**	0	0	**3**	**3**	0	0
Day 8	0	0	0	0	**4**	**4**	0	0	**4**	**4**	0	0
Day 9	0	0	0	0	**4**	**4**	0	0	**4**	**4**	0	0
Day 10	0	0	0	0	**4**	**4**	0	0	**4**	**4**	0	0
Day 11	0	0	0	0	**5**	**5**	0	0	**5**	**5**	0	0
Day 12	0	0	0	0	**5**	**5**	0	0	**5**	**5**	0	0
Day 13	0	0	0	0	**5**	**5**	**2**	**2**	**5**	**5**	0	0
Day 14	0	0	0	0	**5**	**5**	**2**	**2**	**5**	**5**	0	0
Day 15	0	0	0	0	**5**	**5**	**3**	**3**	**5**	**5**	**2**	**2**

(b) Rewarewa honey R19/06

	0% honey (without virus)				5% honey				10% honey			
Day 1	0	0	0	0	0	0	0	0	0	0	0	0
Day 2	0	0	0	0	0	0	0	0	0	0	0	0
Day 3	0	0	0	0	0	0	0	0	0	0	0	0
Day 4	0	0	0	0	0	0	0	0	0	0	0	0
Day 5	0	0	0	0	0	0	0	0	0	0	0	0
Day 6	0	0	0	0	**2**	**2**	0	0	**2**	**2**	0	0
Day 7	0	0	0	0	**3**	**3**	0	0	**3**	**3**	0	0
Day 8	0	0	0	0	**4**	**4**	**2**	**2**	**4**	**4**	0	0
Day 9	0	0	0	0	**4**	**4**	**3**	**3**	**4**	**4**	**2**	**2**
Day 10	0	0	0	0	**4**	**4**	**4**	**4**	**4**	**4**	**3**	**3**
Day 11	0	0	0	0	**4**	**4**	**4**	**4**	**4**	**4**	**3**	**3**
Day 12	0	0	0	0	**4**	**4**	**4**	**4**	**4**	**4**	**4**	**4**
Day 13	0	0	0	0	**5**	**5**	**4**	**4**	**5**	**5**	**4**	**4**
Day 14	0	0	0	0	**5**	**5**	**5**	**4**	**5**	**5**	**4**	**4**
Day 15	0	0	0	0	**5**	**5**	**5**	**4**	**5**	**5**	**4**	**4**

(c) Ling Heather honey LH27

	0% honey (without virus)				5% honey				10% honey			
Day 1	0	0	0	0	0	0	0	0	0	0	0	0
Day 2	0	0	0	0	0	0	0	0	0	0	0	0
Day 3	0	0	0	0	0	0	0	0	0	0	0	0
Day 4	0	0	0	0	0	0	0	0	0	0	0	0
Day 5	0	0	0	0	0	0	0	0	0	0	0	0
Day 6	0	0	0	0	0	0	0	0	0	0	0	0
Day 7	0	0	0	0	0	0	**2**	**2**	0	0	0	0
Day 8	0	0	0	0	0	0	**2**	**2**	0	0	**2**	**2**
Day 9	0	0	0	0	0	0	**3**	**3**	0	0	**3**	**3**
Day 10	0	0	0	0	0	0	**4**	**4**	0	0	**4**	**4**
Day 11	0	0	0	0	0	0	**4**	**4**	0	0	**4**	**4**
Day 12	0	0	0	0	**2**	**2**	**5**	**4**	**2**	**2**	**4**	**4**
Day 13	0	0	0	0	**3**	**3**	**5**	**4**	**2**	**2**	**4**	**4**
Day 14	0	0	0	0	**3**	**3**	**5**	**5**	**2**	**2**	**4**	**4**
Day 15	0	0	0	0	**4**	**4**	**5**	**5**	**3**	**2**	**5**	**5**

Table 7.3 CPE from HSV-1 after treatment of the virus with Manuka honeys: (a) M116, M112, and M157, and (b) M151

(a) Manuka honeys M116, M112, and M157

	0% honey (without virus)				5% honey				10% honey			
Day 1	0	0	0	0	0	0	0	0	0	0	0	0
Day 2	0	0	0	0	0	0	0	0	0	0	0	0
Day 3	0	0	0	0	0	0	0	0	0	0	0	0
Day 4	0	0	0	0	0	0	0	0	0	0	0	0
Day 5	0	0	0	0	0	0	0	0	0	0	0	0
Day 6	0	0	0	0	0	0	0	0	0	0	0	0
Day 7	0	0	0	0	0	0	0	0	0	0	0	0
Day 8	0	0	0	0	0	0	0	0	0	0	0	0
Day 9	0	0	0	0	0	0	0	0	0	0	0	0
Day 10	0	0	0	0	0	0	0	0	0	0	0	0
Day 11	0	0	0	0	0	0	0	0	0	0	0	0
Day 12	0	0	0	0	0	0	0	0	0	0	0	0
Day 13	0	0	0	0	0	0	0	0	0	0	0	0
Day 14	0	0	0	0	0	0	0	0	0	0	0	0
Day 15	0	0	0	0	0	0	0	0	0	0	0	0

(b) Manuka honey M151

	0% honey (without virus)				5% honey				10% honey			
Day 1	0	0	0	0	0	0	0	0	0	0	0	0
Day 2	0	0	0	0	0	0	0	0	0	0	0	0
Day 3	0	0	0	0	0	0	0	0	0	0	0	0
Day 4	0	0	0	0	0	0	0	0	0	0	0	0
Day 5	0	0	0	0	0	0	0	0	0	0	0	0
Day 6	0	0	0	0	0	0	**2**	**2**	0	0	**2**	**2**
Day 7	0	0	0	0	0	0	**2**	**2**	0	0	**3**	**3**
Day 8	0	0	0	0	0	0	**3**	**3**	0	0	**3**	**3**
Day 9	0	0	0	0	0	0	**3**	**3**	0	0	**3**	**3**
Day 10	0	0	0	0	0	0	**3**	**3**	0	0	**3**	**3**
Day 11	0	0	0	0	0	0	**3**	**3**	0	0	**3**	**3**
Day 12	0	0	0	0	0	0	**3**	**3**	0	0	**4**	**4**
Day 13	0	0	0	0	0	0	**4**	**4**	0	0	**4**	**4**
Day 14	0	0	0	0	0	0	**4**	**4**	0	0	**4**	**4**
Day 15	0	0	0	0	0	0	**4**	**4**	0	0	**4**	**4**

Table 7.4 CPE from HSV-1 after treatment of the virus with: (a) Honeydew honey HD19, (b) Rewarewa honey R19/06, and (c) Ling Heather honey LH27

(a) Honeydew honey HD19

	0% honey (without virus)				5% honey				10% honey			
Day 1	0	0	0	0	0	0	0	0	0	0	0	0
Day 2	0	0	0	0	0	0	0	0	0	0	0	0
Day 3	0	0	0	0	0	0	0	0	0	0	0	0
Day 4	0	0	0	0	0	0	0	0	0	0	0	0
Day 5	0	0	0	0	**3**	**3**	0	0	**3**	**2**	0	0
Day 6	0	0	0	0	**3**	**3**	**2**	**2**	**3**	**3**	**2**	**2**
Day 7	0	0	0	0	**4**	**4**	**2**	**2**	**4**	**4**	**2**	**2**
Day 8	0	0	0	0	**5**	**5**	**2**	**2**	**5**	**5**	**2**	**2**
Day 9	0	0	0	0	**5**	**5**	**3**	**3**	**5**	**5**	**3**	**3**
Day 10	0	0	0	0	**5**	**5**	**3**	**3**	**5**	**5**	**3**	**3**
Day 11	0	0	0	0	**5**	**5**	**4**	**3**	**5**	**5**	**3**	**3**
Day 12	0	0	0	0	**5**	**5**	**4**	**3**	**5**	**5**	**3**	**3**
Day 13	0	0	0	0	**5**	**5**	**4**	**4**	**5**	**5**	**4**	**4**
Day 14	0	0	0	0	**5**	**5**	**4**	**4**	**5**	**5**	**4**	**4**
Day 15	0	0	0	0	**5**	**5**	**4**	**4**	**5**	**5**	**4**	**4**

(b) Rewarewa honey R19/06

	0% honey (without virus)				5% honey				10% honey			
Day 1	0	0	0	0	0	0	0	0	0	0	0	0
Day 2	0	0	0	0	0	0	0	0	0	0	0	0
Day 3	0	0	0	0	0	0	0	0	0	0	0	0
Day 4	0	0	0	0	0	0	0	0	0	0	0	0
Day 5	0	0	0	0	0	0	0	0	0	0	0	0
Day 6	0	0	0	0	0	0	0	0	0	0	0	0
Day 7	0	0	0	0	**1**	**1**	0	0	0	0	0	0
Day 8	0	0	0	0	**1**	**1**	**2**	**2**	0	0	0	0
Day 9	0	0	0	0	**1**	**1**	**2**	**2**	0	0	0	0
Day 10	0	0	0	0	**1**	**1**	**3**	**3**	0	0	0	0
Day 11	0	0	0	0	**1**	**1**	**3**	**3**	0	0	0	0
Day 12	0	0	0	0	**1**	**1**	**3**	**3**	0	0	0	0
Day 13	0	0	0	0	**2**	**2**	**3**	**3**	**2**	**2**	0	0
Day 14	0	0	0	0	**3**	**3**	**3**	**3**	**2**	**2**	0	0
Day 15	0	0	0	0	**3**	**3**	**5**	**4**	**3**	**3**	**2**	**2**

(c) Ling Heather honey LH27

	0% honey (without virus)				5% honey				10% honey			
Day 1	0	0	0	0	0	0	0	0	0	0	0	0
Day 2	0	0	0	0	0	0	0	0	0	0	0	0
Day 3	0	0	0	0	0	0	0	0	0	0	0	0
Day 4	0	0	0	0	0	0	0	0	0	0	0	0
Day 5	0	0	0	0	0	0	0	0	0	0	0	0
Day 6	0	0	0	0	0	0	**2**	**2**	0	0	0	0
Day 7	0	0	0	0	0	0	**2**	**2**	0	0	0	0
Day 8	0	0	0	0	0	0	**2**	**2**	0	0	0	0
Day 9	0	0	0	0	0	0	**2**	**2**	0	0	0	0
Day 10	0	0	0	0	0	0	**2**	**2**	0	0	0	0
Day 11	0	0	0	0	0	0	**3**	**3**	0	0	0	0
Day 12	0	0	0	0	0	0	**3**	**3**	0	0	0	0
Day 13	0	0	0	0	0	0	**3**	**3**	0	0	**2**	**2**
Day 14	0	0	0	0	0	0	**3**	**3**	0	0	**2**	**2**
Day 15	0	0	0	0	0	0	**3**	**3**	0	0	**2**	**2**

Table 7.5 CPE from HSV-2 after treatment of the virus with Manuka honeys: (a) M116, M112, and M157, and (b) M151

(a) Manuka honeys M116, M112, and M157

	0% honey (without virus)				5% honey				10% honey			
Day 1	0	0	0	0	0	0	0	0	0	0	0	0
Day 2	0	0	0	0	0	0	0	0	0	0	0	0
Day 3	0	0	0	0	0	0	0	0	0	0	0	0
Day 4	0	0	0	0	0	0	0	0	0	0	0	0
Day 5	0	0	0	0	0	0	0	0	0	0	0	0
Day 6	0	0	0	0	0	0	0	0	0	0	0	0
Day 7	0	0	0	0	0	0	0	0	0	0	0	0
Day 8	0	0	0	0	0	0	0	0	0	0	0	0
Day 9	0	0	0	0	0	0	0	0	0	0	0	0
Day 10	0	0	0	0	0	0	0	0	0	0	0	0
Day 11	0	0	0	0	0	0	0	0	0	0	0	0
Day 12	0	0	0	0	0	0	0	0	0	0	0	0
Day 13	0	0	0	0	0	0	0	0	0	0	0	0
Day 14	0	0	0	0	0	0	0	0	0	0	0	0
Day 15	0	0	0	0	0	0	0	0	0	0	0	0

(b) Manuka honey M151

	0% honey (without virus)				5% honey				10% honey			
Day 1	0	0	0	0	0	0	0	0	0	0	0	0
Day 2	0	0	0	0	0	0	0	0	0	0	0	0
Day 3	0	0	0	0	0	0	0	0	0	0	0	0
Day 4	0	0	0	0	0	0	0	0	0	0	0	0
Day 5	0	0	0	0	**1**	**2**	0	0	0	0	0	0
Day 6	0	0	0	0	**2**	**3**	0	0	0	0	0	0
Day 7	0	0	0	0	**2**	**4**	**2**	**2**	0	0	0	0
Day 8	0	0	0	0	**2**	**5**	**3**	**3**	**2**	**2**	**3**	**3**
Day 9	0	0	0	0	**2**	**5**	**3**	**3**	**2**	**2**	**3**	**3**
Day 10	0	0	0	0	**2**	**5**	**3**	**3**	**2**	**2**	**3**	**3**
Day 11	0	0	0	0	**2**	**5**	**3**	**3**	**2**	**2**	**3**	**3**
Day 12	0	0	0	0	**2**	**5**	**3**	**4**	**2**	**2**	**3**	**4**
Day 13	0	0	0	0	**2**	**5**	**3**	**4**	**2**	**2**	**3**	**4**
Day 14	0	0	0	0	**3**	**5**	**3**	**5**	**2**	**2**	**4**	**4**
Day 15	0	0	0	0	**3**	**5**	**3**	**5**	**2**	**2**	**4**	**4**

Table 7.6 CPE from HSV-2 after treatment of the virus with: (a) Honeydew honey HD19, (b) Rewarewa honey R19/06, and (c) Ling Heather honey LH27

(a) Honeydew honey HD19

	0% honey (without virus)				5% honey				10% honey			
Day 1	0	0	0	0	0	0	0	0	0	0	0	0
Day 2	0	0	0	0	0	0	0	0	0	0	0	0
Day 3	0	0	0	0	0	0	0	0	0	0	0	0
Day 4	0	0	0	0	**2**	**2**	0	0	0	0	0	0
Day 5	0	0	0	0	**3**	**3**	0	0	0	0	0	0
Day 6	0	0	0	0	**4**	**4**	0	0	0	0	0	0
Day 7	0	0	0	0	**4**	**4**	0	0	0	0	0	0
Day 8	0	0	0	0	**5**	**5**	0	0	**2**	**2**	0	0
Day 9	0	0	0	0	**5**	**5**	0	0	**2**	**2**	0	0
Day 10	0	0	0	0	**5**	**5**	0	0	**2**	**2**	0	0
Day 11	0	0	0	0	**5**	**5**	0	0	**2**	**3**	0	0
Day 12	0	0	0	0	**5**	**5**	0	0	**2**	**3**	0	0
Day 13	0	0	0	0	**5**	**5**	0	0	**2**	**3**	0	0
Day 14	0	0	0	0	**5**	**5**	0	0	**2**	**3**	0	0
Day 15	0	0	0	0	**5**	**5**	0	0	**3**	**4**	0	0

(b) Rewarewa honey R19/06

	0% honey (without virus)				5% honey				10% honey			
Day 1	0	0	0	0	0	0	0	0	0	0	0	0
Day 2	0	0	0	0	0	0	0	0	0	0	0	0
Day 3	0	0	0	0	0	0	0	0	0	0	0	0
Day 4	0	0	0	0	0	0	0	0	0	0	0	0
Day 5	0	0	0	0	**2**	**2**	0	0	0	0	0	0
Day 6	0	0	0	0	**2**	**2**	**2**	**2**	0	0	0	0
Day 7	0	0	0	0	**2**	**2**	**2**	**2**	0	0	0	0
Day 8	0	0	0	0	**2**	**2**	**2**	**3**	0	0	0	0
Day 9	0	0	0	0	**2**	**2**	**3**	**3**	0	0	**2**	**2**
Day 10	0	0	0	0	**2**	**2**	**3**	**3**	0	0	**2**	**2**
Day 11	0	0	0	0	**2**	**2**	**3**	**4**	0	0	**2**	**2**
Day 12	0	0	0	0	**2**	**2**	**3**	**4**	0	0	**2**	**2**
Day 13	0	0	0	0	**2**	**2**	**3**	**4**	0	0	**2**	**2**
Day 14	0	0	0	0	**2**	**2**	**3**	**5**	0	0	**2**	**2**
Day 15	0	0	0	0	**3**	**3**	**3**	**5**	0	0	**2**	**2**

(c) Ling Heather honey LH27

	0% honey (without virus)				5% honey				10% honey			
Day 1	0	0	0	0	0	0	0	0	0	0	0	0
Day 2	0	0	0	0	0	0	0	0	0	0	0	0
Day 3	0	0	0	0	0	0	0	0	0	0	0	0
Day 4	0	0	0	0	0	0	0	0	0	0	0	0
Day 5	0	0	0	0	0	0	0	0	0	0	0	0
Day 6	0	0	0	0	0	0	0	0	0	0	0	**3**
Day 7	0	0	0	0	0	0	0	0	0	0	0	**3**
Day 8	0	0	0	0	0	0	**2**	**2**	0	0	**2**	**3**
Day 9	0	0	0	0	0	0	**2**	**2**	0	0	**2**	**4**
Day 10	0	0	0	0	0	0	**3**	**2**	0	0	**3**	**4**
Day 11	0	0	0	0	0	0	**3**	**3**	0	0	**3**	**5**
Day 12	0	0	0	0	0	0	**3**	**3**	0	0	**3**	**5**
Day 13	0	0	0	0	0	0	**3**	**3**	0	0	**4**	**5**
Day 14	0	0	0	0	0	0	**3**	**3**	0	0	**4**	**5**
Day 15	0	0	0	0	0	0	**4**	**3**	0	0	**4**	**5**

7.4 DISCUSSION

As described in the Section 7.2 the honey-treated virus solutions were diluted with the aim of removing any residual honey from the viruses. The final dilution of the virus was within the TCID thus it was expected to display CPE. The final honey concentrations previously have shown to be unable to prevent the development of CPE when used under these experimental conditions (see Section 5.3), it was therefore likely that the remaining honey would be too dilute to inhibit the development of CPE.

7.4.1 Ad3

The Manuka honeys M116 and M157 that caused neutralisation of Ad3 in the previous work were shown to have virucidal activity in the current experiments shown by the absence of CPE over the observation period in Table 7.1 (a).

Manuka honey M112, however, although also having a high level of non-peroxide antibacterial activity and methylglyoxal, in the neutralisation experiment did not cause neutralisation of Ad3 after treatment with either the 5% honey or 10% honey solutions, so it was reasoned that some CPE may have been seen over the observation period in this experiment. As expected CPE was detected, it was seen from day 12 after treatment of the virus with 5% honey concentration as shown in Table 7.1 (b).

Virus treated with 5% Manuka M151 likewise displayed CPE from day 7 after infection as seen in Table 7.1 (c). This honey in the neutralisation experiment did not cause neutralisation at either the 5% honey or 10% honey concentrations, thus

it was expected to show some evidence of CPE at some stage over the observation period.

With of the other honeys tested CPE was evident from days 6 to 7 after infection following the treatment with 5% honey, and from day 8 with some of the treatments with 10% honey (see Table 7.2) this was despite the neutralisation observed previously with Honeydew HD19 at 10%: this result can suggest virustatic activity of this honey against Ad3.

7.4.2 HSV-1 and HSV-2

Tables 7.3 (a) and 7.5 (a) suggest that each of the Manuka honeys have virucidal activity that caused neutralisation in the previous work. As expected CPE developed with Manuka honey M151 (see Tables 7.3 (b) and 7.5 (b)) which previously did not prevent CPE development. The CPE, however, developed with just one set of replicates with HSV-1 exposed 5% and 10% honey.

With the other honeys tested CPE developed despite the previous neutralisation of HSV-1 observed with Rewarewa honey R19/06, and with each of the other honeys with HSV-2, this can suggest virustatic activity of these honeys against HSV-1 and HSV-2.

7.4.3 Comparisons with the activity table

When comparing the above trends with the activity levels of each honey type (see Table 6.3) it appears that those honeys which show virucidal activity typically have the highest levels of methylglyoxal. This can suggest that perhaps

methylglyoxal contributes to the virucidal activity of honey as the honeys with lower methylglyoxal levels were unable to prevent viral CPE developing over the 15 days of observation, and with many treatments CPE was seen within the usual 7 day observation period.

7.5 TESTING METHYLGLYOXAL BY ITSELF

It was necessary to confirm whether methylglyoxal found in the honey was acting alone to deliver the observed virucidal activity. This was achieved by using commercially available methylglyoxal at the equivalent levels found within whole honey as 5% and 10% solutions, and exposing each of the viruses to these equivalent concentrations for 8 hours as done in the neutralisation experiments, the same method then being followed as in this present investigation to determine the virucidal activity.

7.5.1 Methods

A stock solution was prepared as a 100 fold dilution in water of synthetic methylglyoxal obtained as 40% (6500 mM) solution from the Sigma-Aldrich chemical company. The stock solution was diluted further to prepare 4 ml methylglyoxal solutions of concentrations equivalent to that in 5% and 10% Manuka M116, M112, M157, and M151 as shown in Table 7.7. These are based on the concentrations of methylglyoxal found in whole honey. The concentration of methylglyoxal in 5% and 10% solutions of honey was determined then converted to mmol/l by dividing the new concentration by the molecular weight of methylglyoxal 72.06, then further dividing this figure by the density of honey 1.4 g/ml (1/1.4 l) to give the final concentration.

Table 7.7 Calculations to prepare methylglyoxal at equivalent concentrations to that in 5% and 10% Manuka honeys

Honey ID	MGO mg/kg	MGO in 5% solution of honey mg/kg	Final concentration mmol/l	MGO in 10% solution of honey mg/kg	Final concentration mmol/l
M116	1241.7	62.1	1.2	124.2	2.4
M112	772.4	38.6	0.8	77.2	1.5
M157	745.5	37.3	0.7	74.6	1.5
M151	116.4	5.9	0.1	11.7	0.2

Key: MGO methylglyoxal

Serially diluted stock viral solutions of Ad3, HSV-1, and HSV-2 (diluted to 10^{-3}) were made in maintenance medium, and the final serial dilution was added to an appropriate volume (based on numbers of wells inoculated) of equivalent methylglyoxal solution to give 10^{-4} viral dilution. The viral-methylglyoxal solutions and methylglyoxal solutions without virus were incubated with shaking using a shaking incubator at level 3-4 set at 37°C for 8 hours. Confluent A549 cells were prepared and supplemented with 800 µl of maintenance medium and 200 µl of methylglyoxal-viral solution or methylglyoxal solution without virus. This was done in quadruplet.

7.5.2 Results

Tables 7.8 to 7.10 present the development of CPE recorded over a number of days after the inoculation of the cells with virus that had been treated for 8 hours with equivalent concentrations of methylglyoxal to that found in 5% and 10% solutions of Manuka honey. Where the scores were found to be identical across the quadruplets, one value is given in the table. The methylglyoxal solutions

without virus did not cause morphological changes to the cells over the course of observation.

Table 7.8 CPE resulting from infection of the cells with Ad3 treated with methylglyoxal at levels equivalent to that found in Manuka honey: (a) M116, (b) M112, (c) M157, and (d) M151

(a) Manuka honey M116

	0% MGO (no virus)	0% MGO (virus)	5% Equivalent	10% Equivalent
Day 1	0	0	0	0
Day 2	0	2	2	2
Day 3	0	3	3	3
Day 4	0	3	3	3

(b) Manuka honey M112

	0% MGO (no virus)	0% MGO (virus)	5% Equivalent	10% Equivalent
Day 1	0	0	0	0
Day 2	0	2	2	2
Day 3	0	3	3	3
Day 4	0	3	3	3

(c) Manuka M157

	0% MGO (no virus)	0% MGO (virus)	5% Equivalent	10% Equivalent
Day 1	0	0	0	0
Day 2	0	2	2	2
Day 3	0	3	3	3
Day 4	0	3	3	3

(d) Manuka M151

	0% MGO (no virus)	0% MGO (virus)	5% Equivalent	10% Equivalent
Day 1	0	0	0	0
Day 2	0	2	2	2
Day 3	0	3	3	3
Day 4	0	3	3	3

Table 7.9 CPE resulting from infection of the cells with HSV-1 treated with methylglyoxal at levels equivalent to that found in Manuka honeys: (a) M116, (b) M112, (c) M157, and (d) M151

(a) Manuka M116

	0% MGO (no virus)	0% MGO (virus)	5% Equivalent	10% Equivalent
Day 1	0	0	0	0
Day 2	0	0	0	0
Day 3	0	2 1 1 1	2	2 2 1 1
Day 4	0	2 2 1 1	2	2 2 2 1
Day 5	0	2	2	2 2 2 1
Day 6	0	3 2 1 1	3 3 2 3	2 2 2 2
Day 7	0	3 2 1 1	3	3 2 2 2
Day 8	0	4 2 1 1	4	3 3 2 2

(b) Manuka honey M112

	0% MGO (no virus)	0% MGO (virus)	5% Equivalent	10% Equivalent
Day 1	0	0	0	0
Day 2	0	0	0	0
Day 3	0	2 1 1 1	2	2
Day 4	0	2 2 1 1	2	2
Day 5	0	2	2	2 2 2 3
Day 6	0	3 2 1 1	3 3 2 3	3 2 2 3
Day 7	0	3 2 1 1	3	3
Day 8	0	4 2 1 1	4	4 3 3 4

(c) Manuka honey M157

	0% MGO (no virus)	0% MGO (virus)	5% Equivalent	10% Equivalent
Day 1	0	0	0	0
Day 2	0	0	0	0
Day 3	0	2	2	1
Day 4	0	2	2	2 1 1 1
Day 5	0	2	2	2 1 1 1
Day 6	0	3	2	2 2 1 1
Day 7	0	3	3	2
Day 8	0	4	4 3 3 4	2

(d) Manuka M151

	0% MGO (no virus)	0% MGO (virus)	5% Equivalent	10% Equivalent
Day 1	0	0	0	0
Day 2	0	0	0	0
Day 3	0	2	2	2
Day 4	0	2	2	2
Day 5	0	2	2	2
Day 6	0	3	3 3 2 3	3
Day 7	0	3	3 3 2 3	3
Day 8	0	4	4	4

Table 7.10 CPE resulting from infection of the cells with HSV-2 treated with methylglyoxal at levels equivalent to that found in Manuka honeys: (a) M116, (b) M112, (c) M157, (d) M151

(a) Manuka honey M116

	0% MGO (no virus)	0% MGO (virus)	5% Equivalent	10% Equivalent
Day 1	0	0	0	0
Day 2	0	2 1 1 1	0	0
Day 3	0	2	0	0
Day 4	0	2 3 2 2	2 2 3 1	0
Day 5	0	3	2 2 3 2	0
Day 6	0	4	4	0
Day 7	0	4	4	2 0 2 2
Day 8	0	4	4	3

(b) Manuka honey M112

	0% MGO (no virus)	0% MGO (virus)	5% Equivalent	10% Equivalent
Day 1	0	0	0	0
Day 2	0	2 1 1 1	0	0
Day 3	0	2	0 1 2 2	0
Day 4	0	2 3 2 2	2 2 3 3	0
Day 5	0	3	3	0
Day 6	0	4	4	0
Day 7	0	4	4	2
Day 8	0	4	4	4

(c) Manuka honey M157

	0% MGO (no virus)	0% MGO (virus)	5% Equivalent	10% Equivalent
Day 1	0	0	0	0
Day 2	0	2 1 1 2	2	0
Day 3	0	3 3 2 2	3 3 2 2	0
Day 4	0	3	3	0
Day 5	0	4	4	0
Day 6	0	4	4	0
Day 7	0	4	4	0 2 2 2
Day 8	0	4	4	1 2 2 2

(d) Manuka honey M151

	0% MGO (no virus)	0% MGO (virus)	5% Equivalent	10% Equivalent
Day 1	0	0	0	0
Day 2	0	2 1 1 2	1 2 1 1	2 2 2 1
Day 3	0	3 3 2 2	3 3 2 2	2
Day 4	0	3	3	3
Day 5	0	4	4	4 3 4 3
Day 6	0	4	4	4
Day 7	0	4	4	4
Day 8	0	4	4	4

7.5.3 Discussion

The results in Tables 7.8 with Ad3 and 7.9 with HSV-1 show that methylglyoxal at equivalent concentrations to that in Manuka honeys M116, M112, M157, and M151 at 5% and 10% concentrations was unable to suppress the development of CPE. Therefore it cannot be concluded that methylglyoxal is solely responsible for the observed virucidal effects of Manuka honeys M116, M112 and M157.

With HSV-2, methylglyoxal treatments at levels equivalent to that in Manuka honeys M116, M112, and M157, results shown in Table 7.10 indicate a temporal delay of CPE. This suggests that perhaps there is a virustatic effect of methylglyoxal at these higher concentrations against HSV-2.

Methylglyoxal used at 2 mmol/l previously had been shown to reduce the TCID of influenza virus by 50% (Tiffany *et al.*, 1957). In the current study many of the concentrations used were lower than this concentration and as shown were unable to inhibit the development of CPE compared with the untreated virus. The concentrations of methylglyoxal used in the current experiments at equivalent levels to that found 10% solutions of Manuka honeys M116, M112, and M157 were higher than what had been shown by Weigel (2004) to cause inhibition of cell growth of HepG2 and HT29 cell lines. However, as indicated, the methylglyoxal solutions without virus did not cause any morphological changes to the cells over the observation period.

Chapter 8

The antiviral activity of honey: Prevention of viral infection

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This chapter describes the results of subsequent work continuing that in Chapter 5, testing the effect honey has on the spread of viral infection using two cell based methods: the mixing of infected and healthy cells treated and untreated with honey, and plaque reduction assays. This chapter also describes further testing of the duration of tolerance of the cells to the higher concentrations of honey and whether the cells revive from the exposure to honey, work that was done first to find if higher concentrations of honey could be used to treat the infected cells for long periods.

8.1 INTRODUCTION

Viral spread experiments test to see whether the drug of interest can inhibit the spread of virus within susceptible cells. This can be achieved as described in Chapter 5 by treating inoculated cells immediately with the drug, honey-containing medium then following the development of CPE. Or, as done in this chapter, by inoculating the cells with virus and then treating these infected cells with honey for a defined period, the mixing of these treated-infected cells with healthy uninfected cells allows CPE development to be followed, to test whether the honey treatment can prevent or delay infection spreading to further cells compared with those infected cells that were not treated. Further, plaque reduction assays can be used to test quantitatively whether the honey when included in an agarose overlay can cause fewer viral plaques from forming compared with the plaques numbers produced with the untreated viruses.

For therapeutic relevance it was of interest to observe whether honey can prevent the virus from spreading from infected cells to healthy uninfected cells. This was to find whether honey would be a suitable treatment for infections caused by viruses once infection has taken place.

In the previous work, treatment of the viruses with a 10% (v/v) honey solution caused inhibition of viral CPE with some of the honeys studied. The length of exposure of the cells to this concentration and lower concentrations of honey was 15 minutes during the inoculation of the cells with honey-treated virus (from which the honey could not be removed). Afterward, the viral-honey solution was removed from the cells and replaced with maintenance medium. It was important

to observe the tolerance of the cells to this concentration of honey before the osmotic nature of honey caused the cells to change morphologically so as to give the length of time before the changes would occur. It was also of interest to test whether the cells can revive from their honey treatment, by allowing the cells to change to a specific morphological level, then replacing the honey-containing medium with maintenance medium, and observing for any changes.

After the additional experiments testing the tolerance of the cells to honey, honeys at two concentrations were used to treat virally infected cells for up to 4 hours. The cells were then detached from the culture flask and mixed in specific ratios with healthy uninfected cells. The development of viral CPE was followed for a number of days and a score given based on the level observed. Further, plaque reduction assays were undertaken to test the effect of honey against each virus quantitatively. Once CPE had been identified with the untreated-agarose overlay, the cells were fixed then stained with crystal violet to observe for plaque formation. Plaques were likely to form with the treated viruses and represent those cells that were infected during the period of inoculation prior to overlay.

8.2 THE A549 CELL LINE TOLERANCE OF HONEY

The prolonged exposure of the cells to 5% and 10% concentrations of honey were likely to cause morphological changes to the cells. To find whether the cells can resume a more healthy morphology after exposure to honey, a specified level of morphological change was obtained before the honey-containing medium was removed and replaced with maintenance medium (that did not contain honey).

8.2.1 Methods

Confluent A549 cells were seeded in 24 well plates as described in Section 3.2.1. Honey solutions of Manuka honeys M116, and M151, Honeydew honey HD19, and Ling Heather honey LH27 were prepared in maintenance medium at concentrations of 0%, 5%, and 10%. These were mixed for 5 minutes and filter sterilised. The culture medium was removed from the cells, and was replaced with 1 ml of the appropriate honey solution in duplicate for each morphological score to be tested (scores 1 to 4). Morphological changes were observed and recorded hourly, and allocated an appropriate score based on the criteria shown in Table 8.1.

Table 8.1 Morphological scoring system

Morphology	Score
100% dividing, 0% swollen	0
75% dividing, 25% swollen	1
50% dividing, 50% swollen	2
25% dividing, 75% swollen	3
0% dividing, 100% swollen	4
All cells dead	5

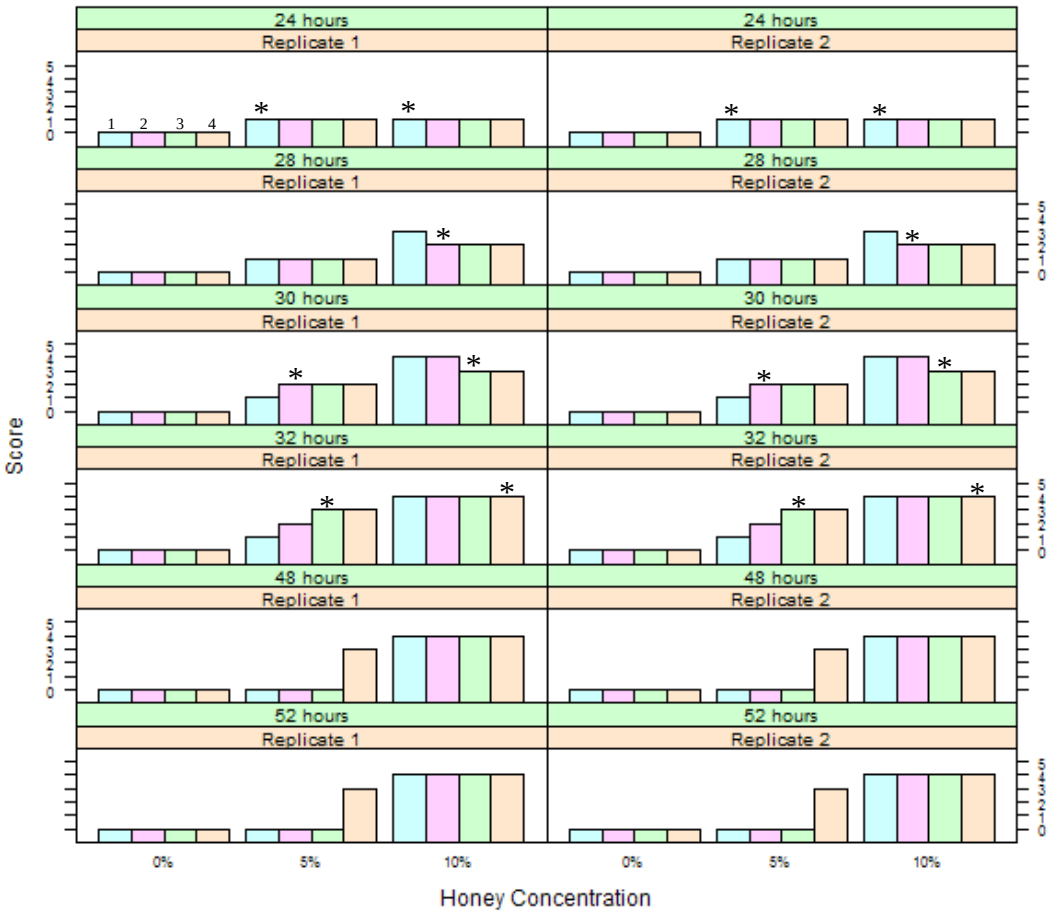
Once morphological changes had occurred to scores of 1, 2, 3, and 4, the honey solution was removed from the appropriate cells and replaced with 1 ml of maintenance medium. The cells were observed to test whether they could resume a more healthy morphology and scores were appropriately allocated. Each experiment was completed in duplicate.

8.2.2 Results

Figures 8.1 to 8.4 show the morphological scores recorded over 7 days with each honey treatment. The first graph with each type of honey represents the 24 to 52 hour observation period, the second graph with each type of honey represents the 54 to 168 hour observation period. The four successive bars at each honey concentration indicate which cells were to reach which score before medium replacement. The score values are outlined above the appropriate bars with the first concentration of honey, to indicate which bar corresponds to which score. The asterisks indicate when the medium was replaced, from which point on the observations were monitoring cell revival.

Figure 8.1 Morphological changes caused by Manuka honey M116 over: (a) 24 to 52 hours, and (b) 54 to 168 hours

(a) 24 to 52 hours



(b) 54 to 168 hours

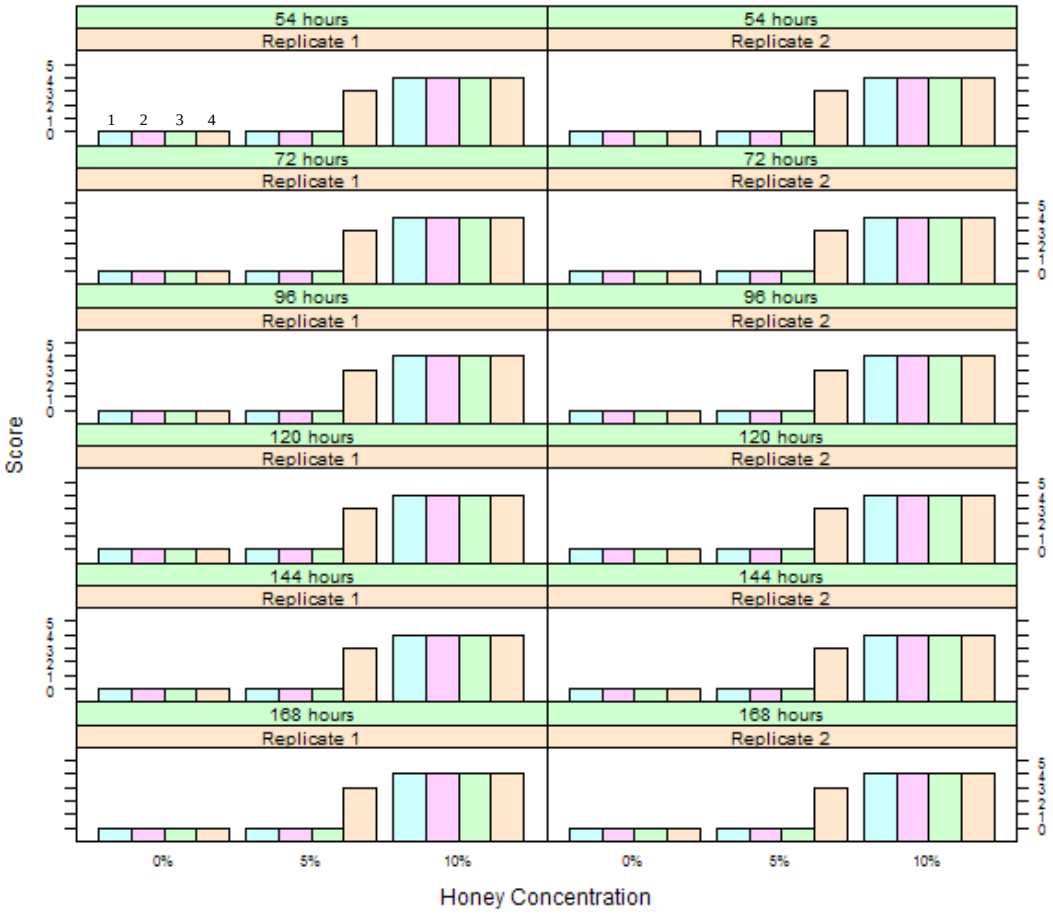
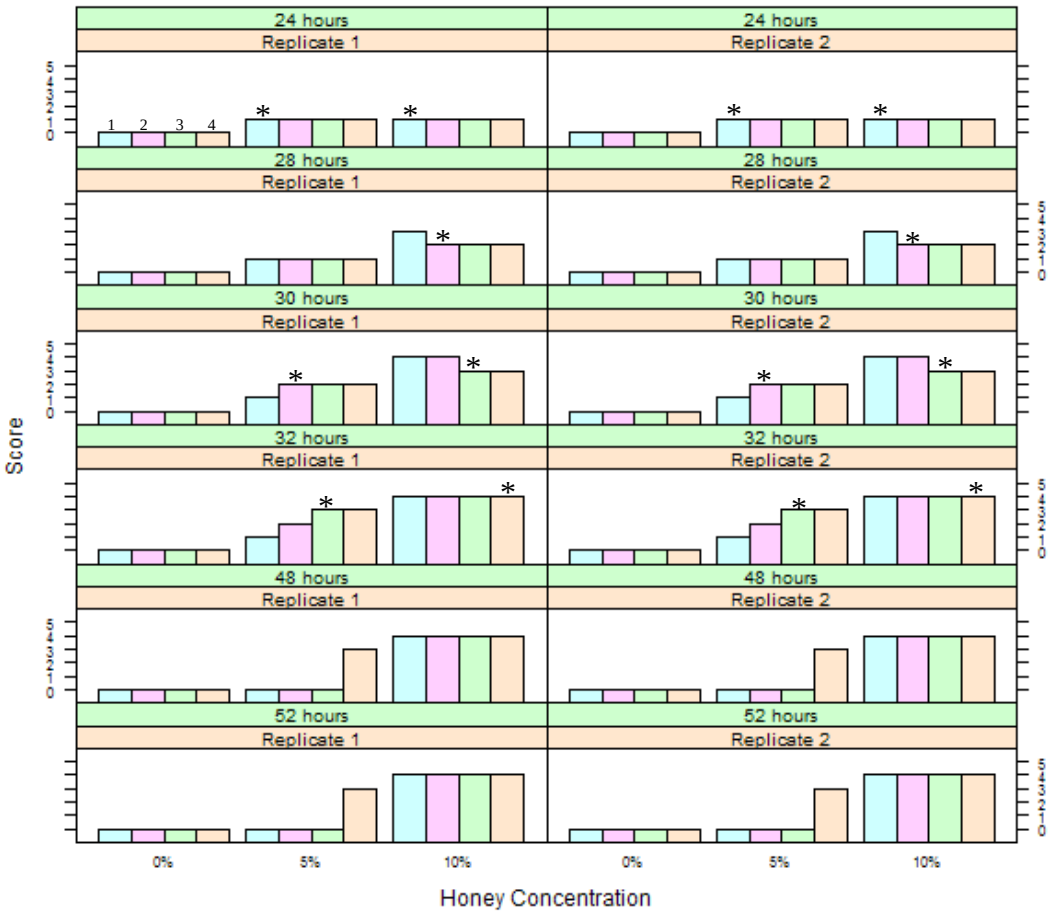


Figure 8.2 Morphological changes caused by Manuka honey M151 over: (a) 24 to 52 hours, and b) 54 to 168 hours

(a) 25 to 52 hours



(b) 54 to 168 hours

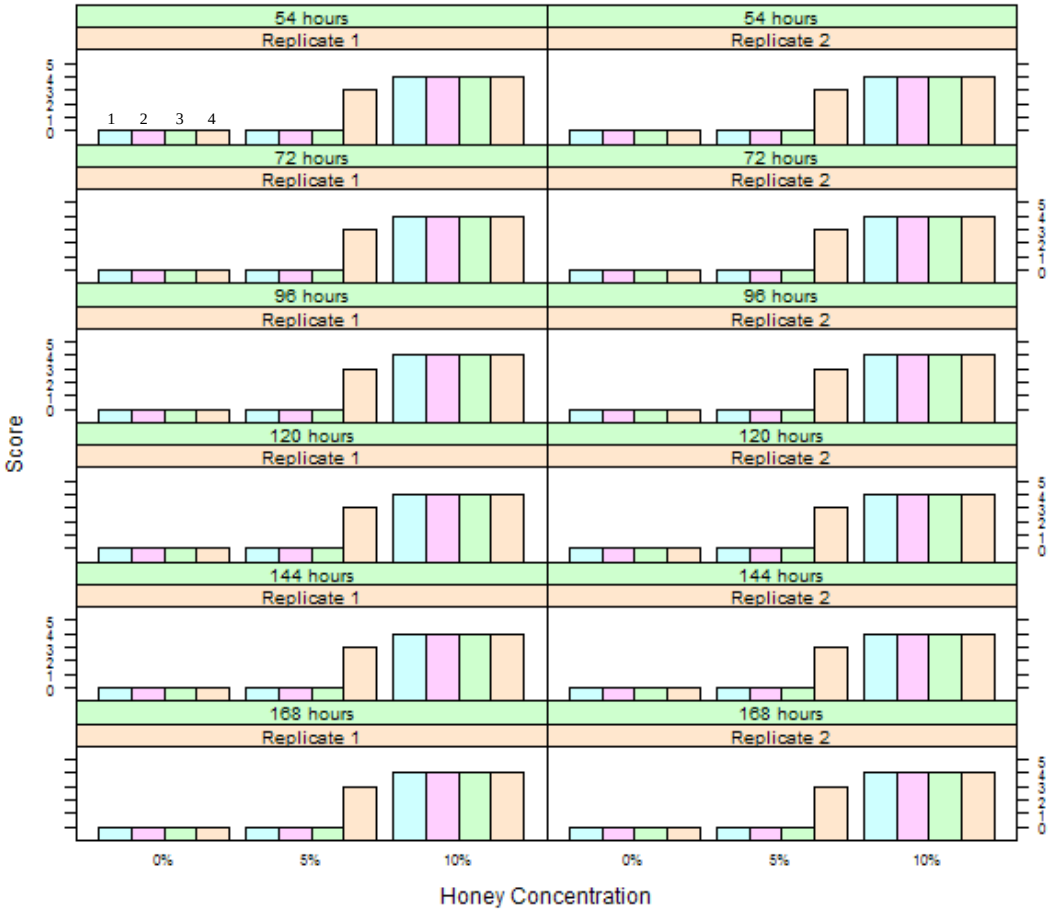
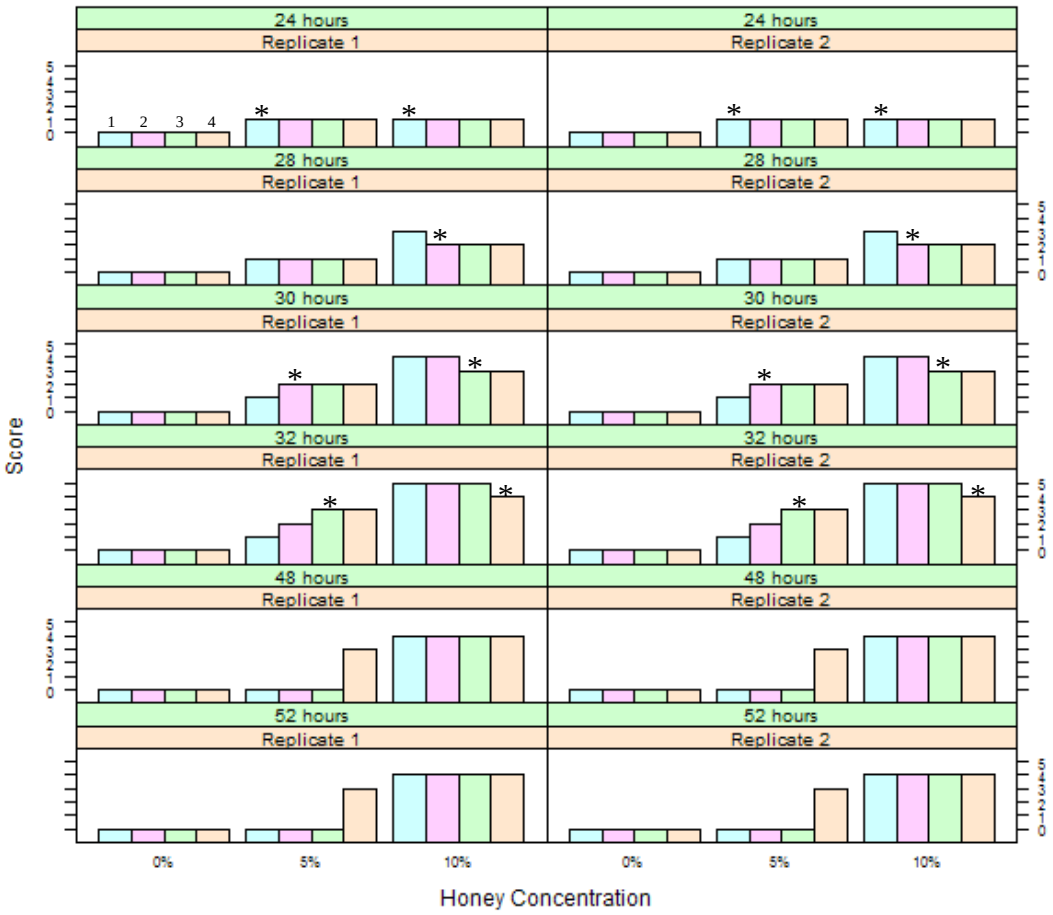


Figure 8.3 Morphological changes caused by Honeydew honey HD19 over: (a) 24 to 52 hours, and (b) 54 to 168 hours

(a) 24 to 52 hours



(b) 54 to 168 hours

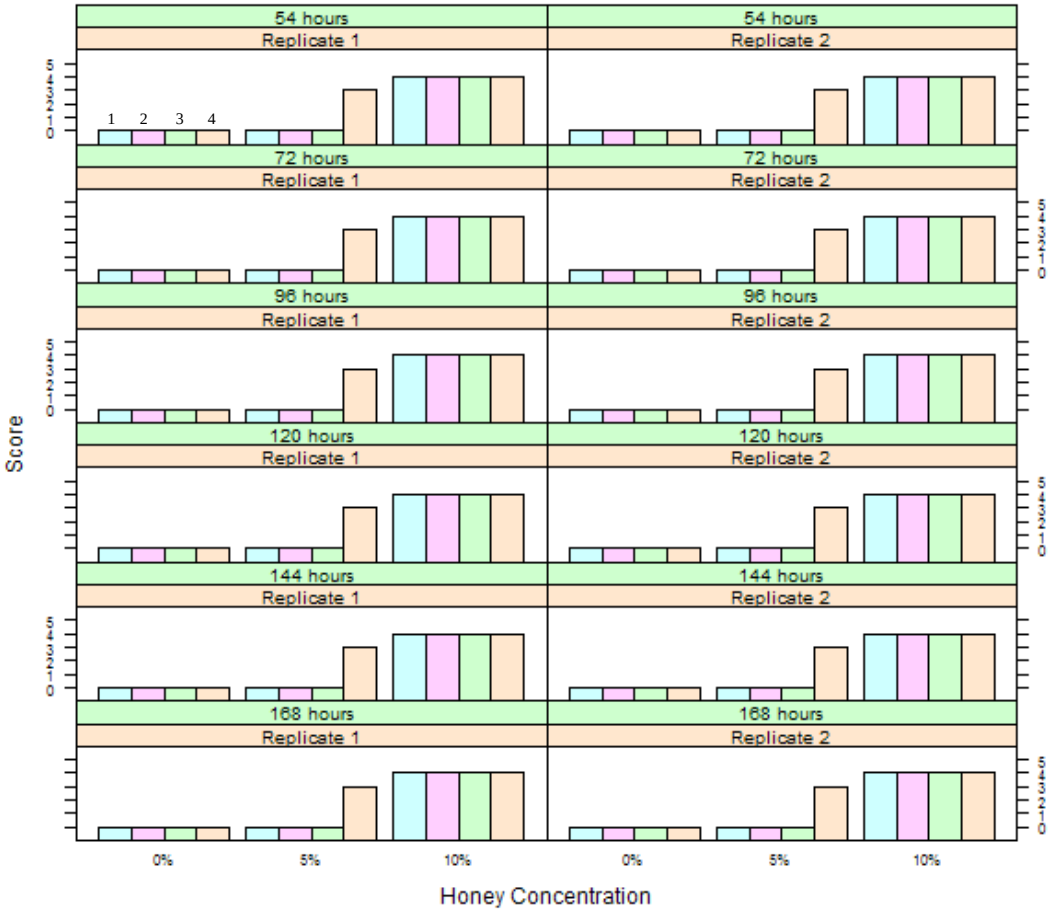
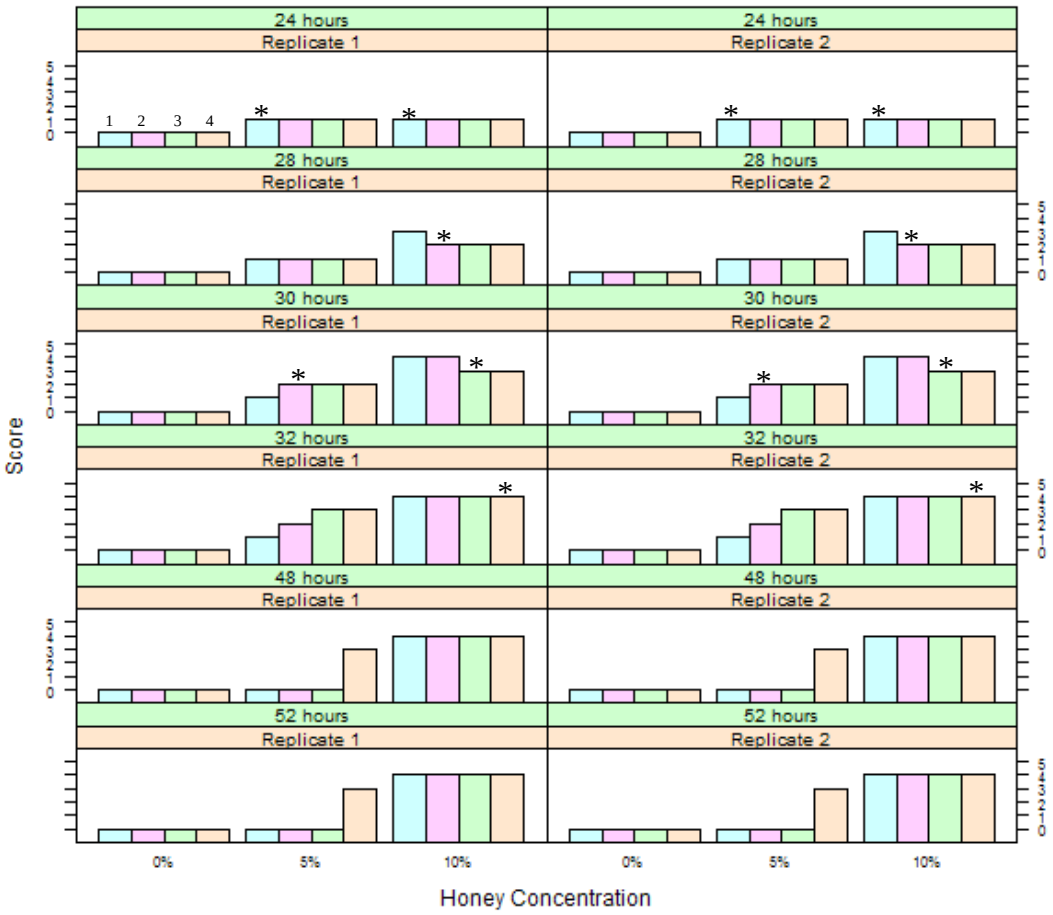
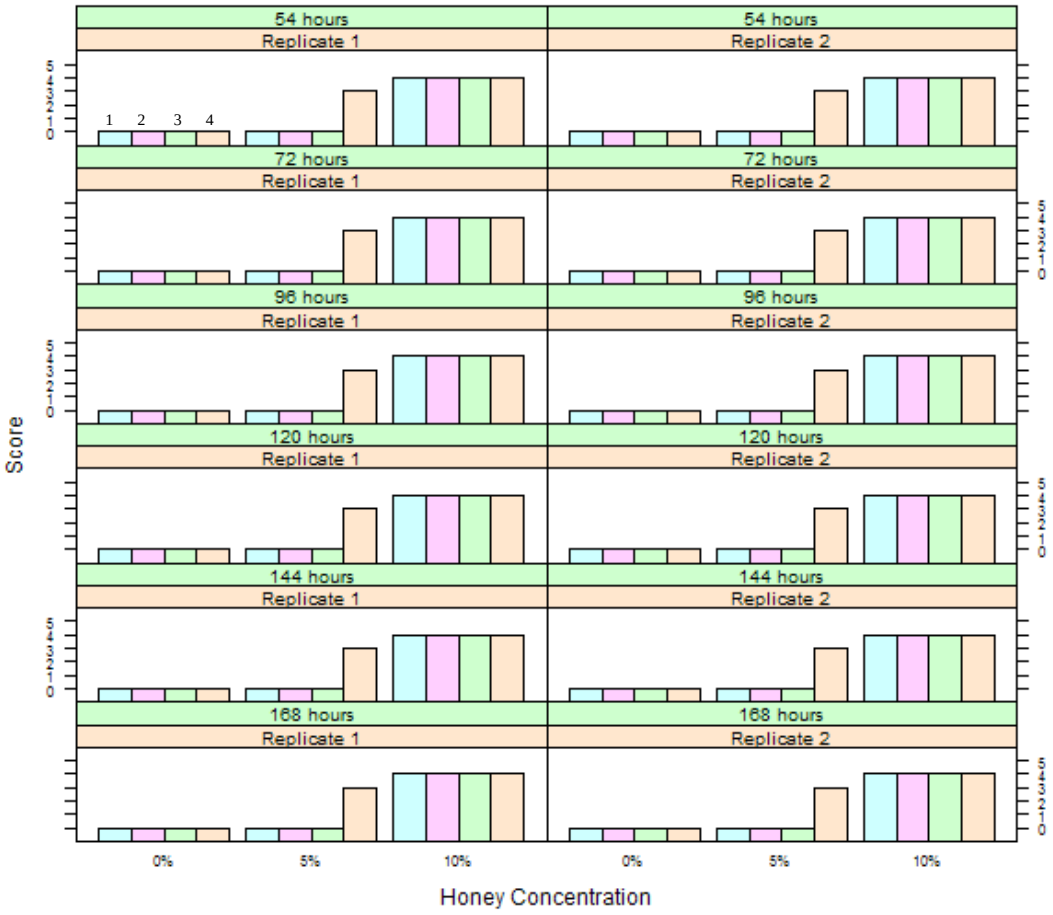


Figure 8.4 Morphological changes caused by Ling Heather honey LH27 over: (a) 24 to 52 hours, and (b) 54 to 168 hours

(a) 24 to 52 hours



(b) 54 to 168 hours



8.2.3 Discussion

With each of the 5% honey treatments after the honey solution had been replaced with maintenance medium, revival to a more normal morphology was seen within 24 hours. This was not observed with the treatments using honey at a concentration of 10%, the cells after the honey solution had been replaced either progressively worsened with respect to morphology or remained at the morphological level to which they had deteriorated. The treatments with 10% honey caused a score of 4 within 32 hours and this score was seen to be observed earlier or at the same time with the cells that had already had their honey treatment replaced with maintenance medium. The 5% honey treatment did not cause a morphological score of 4 within the observation period. It can be suggested then that the cells can tolerate treatment of 5% honey for a much longer period than a treatment of 10% honey.

It can be concluded that treatment of the cells with 10% honey causes great change to cell morphology that can not be reversed by the removal of the honey solution from the cells. Treatment of the cells with a 5% honey solution causes far less change to the cells and the morphology of the cells can be restored through removal of the honey.

8.3 PREVENTION OF THE SPREAD OF VIRAL INFECTION

Experiments on the preventative effects aim to determine whether the honey has an effect on the spread of the virus by limiting the development of viral CPE over the course of observation.

8.3.1 Methods

Confluent A549 cells were prepared in flasks or tubes for treatment with virus and honey, and for the mixing with treated-infected cells, as described in Section 3.2.1. Each viral isolate was prepared at a dilution of 10^{-4} in maintenance medium, and was used to inoculate the confluent cells (200 µl of viral dilution per 1 ml cell culture) for 1 hour at 37°C. During this time honey solutions of Manuka honeys M116 and M151, Honeydew honey HD19, and Ling Heather honey LH27 at concentrations of 0%, 2%, and 5% were prepared in maintenance medium. These were mixed and filter sterilised. At the conclusion of the inoculation period, the viral inoculum was removed and the cells were washed with PBS. The cells were supplemented with 1 ml of honey solution. These were incubated at 37°C for 1, 2, or 4 hours. At the conclusion of each exposure period, the honey solution was removed and the cells were washed with PBS. Trypsin EDTA was used to detach the treated and untreated cells, which were then mixed together to make 1 ml volumes of 1:0, 0:1, 1:1, 1:2, 1:5, 1:10, ratios of infected:healthy cells in duplicate in 24 well plates. These were incubated at 37°C and observed daily for developing CPE and a score was recorded as described in Section 4.1, Figure 4.6.

The first experiments were done without honey to test for CPE development after mixing the infected cells with healthy cells.

8.3.2 Results

Table 8.2 show the results of the first experiments without honey and give the CPE scores recorded over 7 days. Each of the replicates gave identical scores.

Table 8.2 CPE resulting from mixing healthy cells with cells infected with virus in various ratios: a) Ad3, b) HSV-1, and c) HSV-2

(a) Ad3

Ratio of infected:healthy cells	0:1	1:1	1:2	1:5	1:10
Day 1	0	0	0	0	0
Day 2	0	0	0	0	0
Day 3	0	0	0	0	0
Day 4	0	0	0	0	0
Day 5	0	0	0	0	0
Day 6	0	0	0	0	0
Day 7	0	2	2	1	0

(b) HSV-1

Ratio of infected:healthy cells	0:1	1:1	1:2	1:5	1:10
Day 1	0	0	0	0	0
Day 2	0	0	0	0	0
Day 3	0	0	0	0	0
Day 4	0	0	0	0	0
Day 5	0	0	0	0	0
Day 6	0	0	0	0	0
Day 7	0	0	0	0	0

(c) HSV-2

Ratio of infected:healthy cells	0:1	1:1	1:2	1:5	1:10
Day 1	0	0	0	0	0
Day 2	0	0	0	0	0
Day 3	0	0	0	0	0
Day 4	0	2	2	2	0
Day 5	0	4	3	2	2
Day 6	0	4	3	3	2
Day 7	0	5	5	5	2

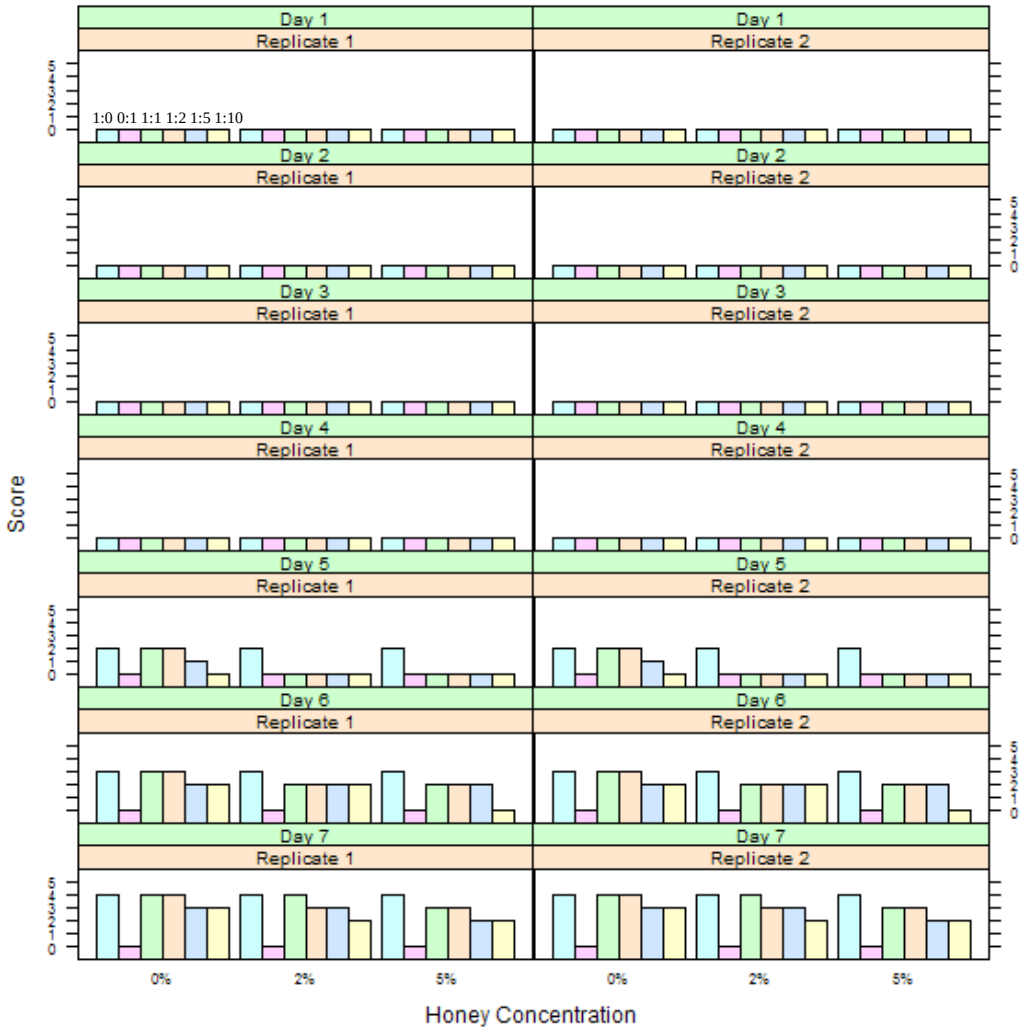
8.3.2.1 Experiments with honey

Figures 8.5 to 8.7 describe the results with each virus and Manuka honey M116 for each period of exposure, Figures 8.8 to 8.10 describe the results with each virus and Manuka honey M155, Figures 8.11 to 8.13 describe the results with each virus and Honeydew honey HD19, and Figures 8.14 to 8.16 describe the results with each virus and Ling Heather honey LH27. The observations of developing viral CPE from 1 to 7 days are plotted against honey concentration, and results are given for each replicate. With each honey concentration, each successive bar represents a different ratio of infected:healthy cells, these are outlined above the appropriate bars with the first concentration of honey, to indicate which bar corresponds to which ratio.

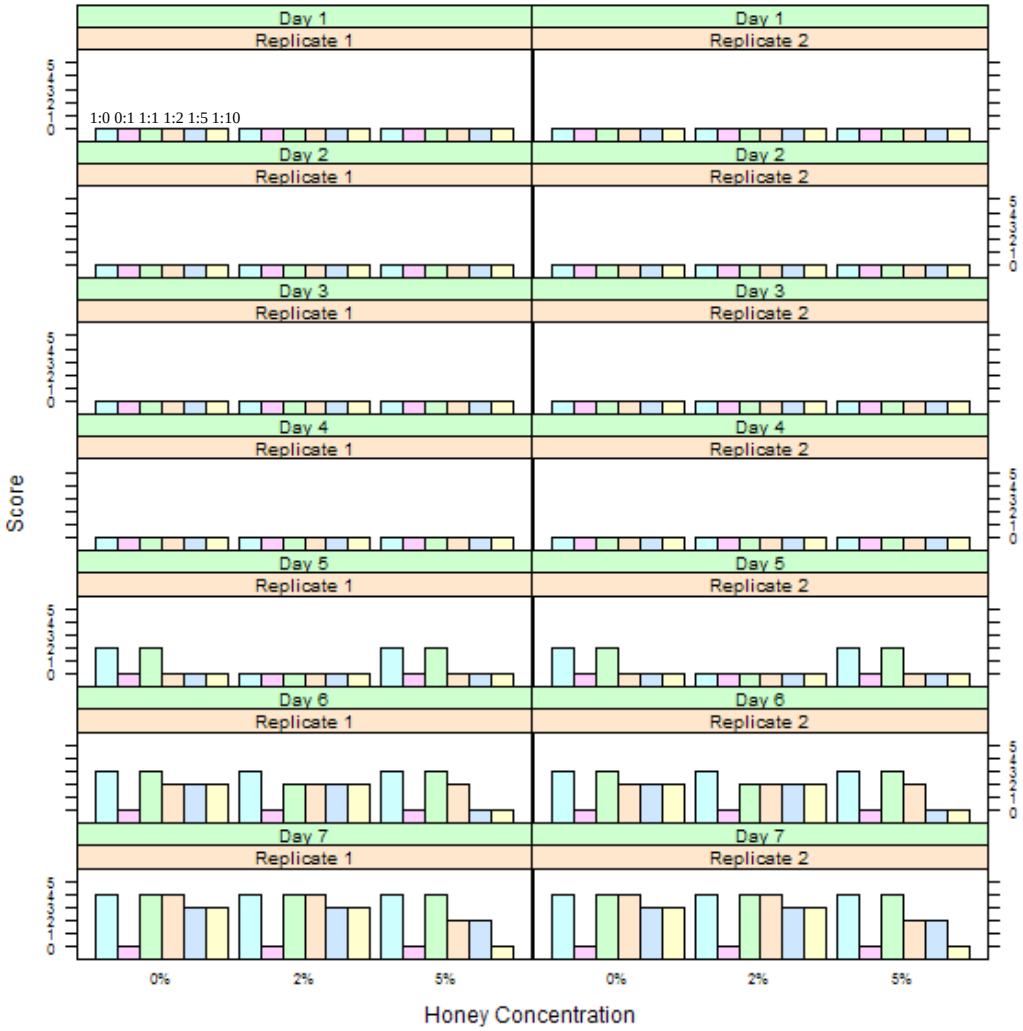
Figure 8.5 CPE from Ad3 after treatment with Manuka honey M116 for: a) 1 hour, b) 2 hours, and c) 4 hours

Healthy uninfected A549 cells were mixed at varying ratios with A549 cells infected with virus treated with 0%, 2%, or 5% Manuka honey M116 for: (a) 1 hour, (b) 2 hours, and (c) 4 hours.

(a) 1 hour



(b) 2 hours


Day 1
Replicate 1
Day 1
Replicate 2
1:0 0:1 1:1 1:2 1:5 1:10
Day 2
Replicate 1
Day 2
Replicate 2
Day 3
Replicate 1
Day 3
Replicate 2
Day 4
Replicate 1
Day 4
Replicate 2
Day 5
Replicate 1
Day 5
Replicate 2
Day 6
Replicate 1
Day 6
Replicate 2
Day 7
Replicate 1
Day 7
Replicate 2
Score
0%
2%
5%
0%
2%
5%
Honey Concentration

(c) 4 hours

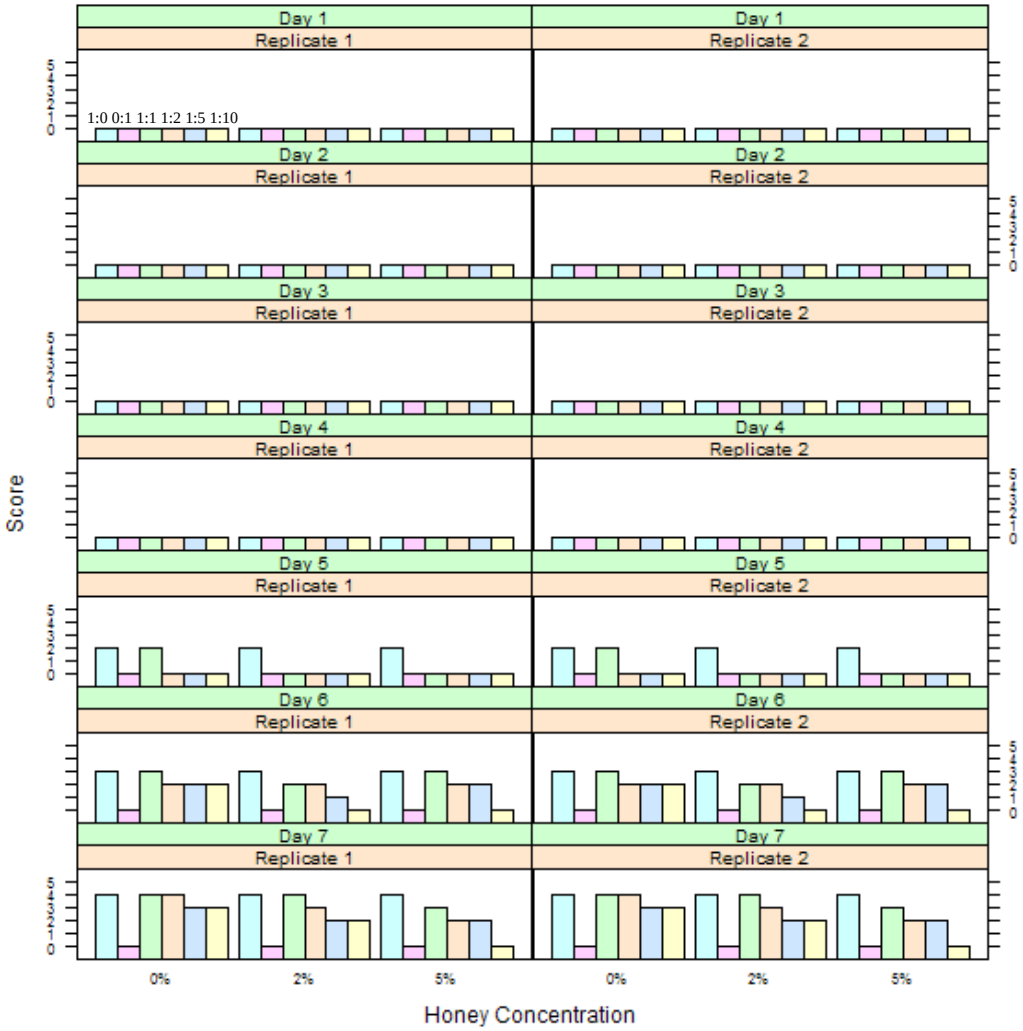
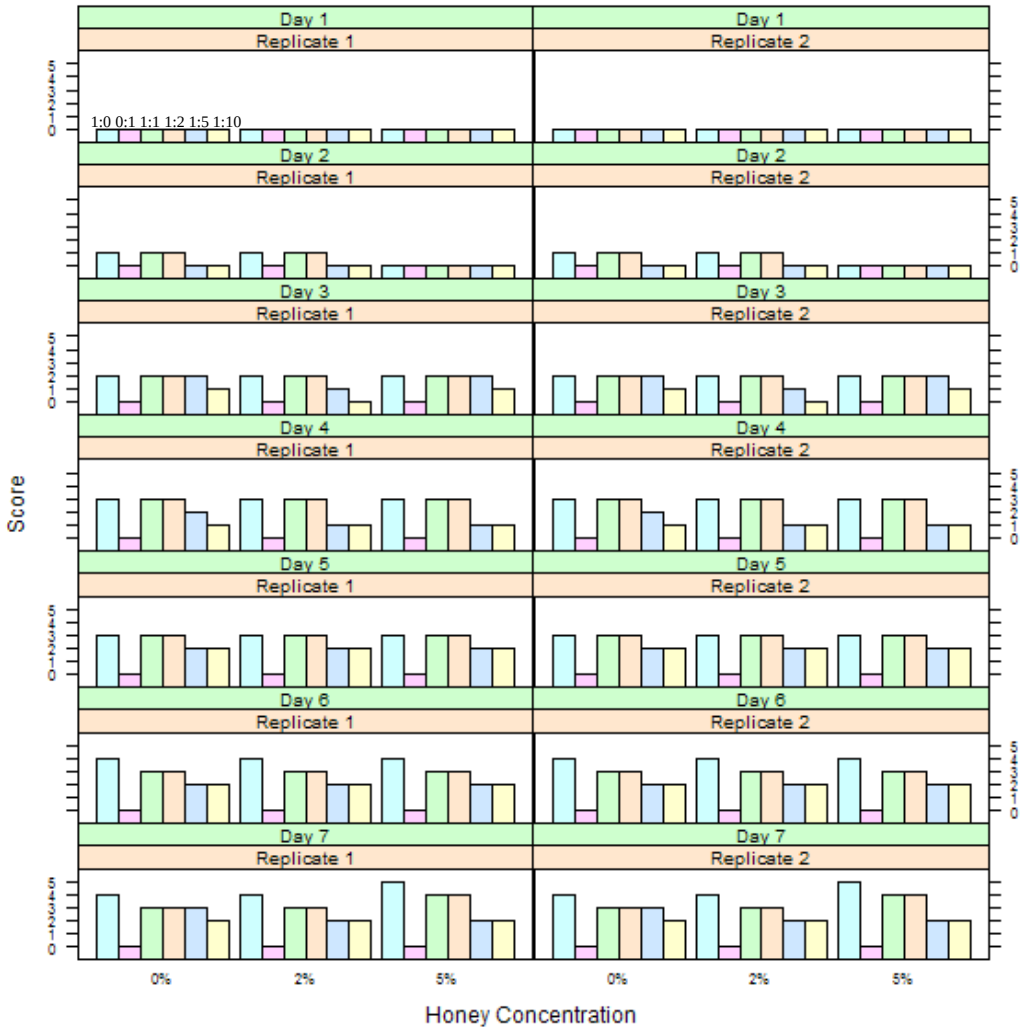

Day 1
Replicate 1
Day 1
Replicate 2
1:0 0:1 1:1 1:2 1:5 1:10
Day 2
Replicate 1
Day 2
Replicate 2
Day 3
Replicate 1
Day 3
Replicate 2
Day 4
Replicate 1
Day 4
Replicate 2
Day 5
Replicate 1
Day 5
Replicate 2
Day 6
Replicate 1
Day 6
Replicate 2
Day 7
Replicate 1
Day 7
Replicate 2
Score
0%
2%
5%
0%
2%
5%
Honey Concentration

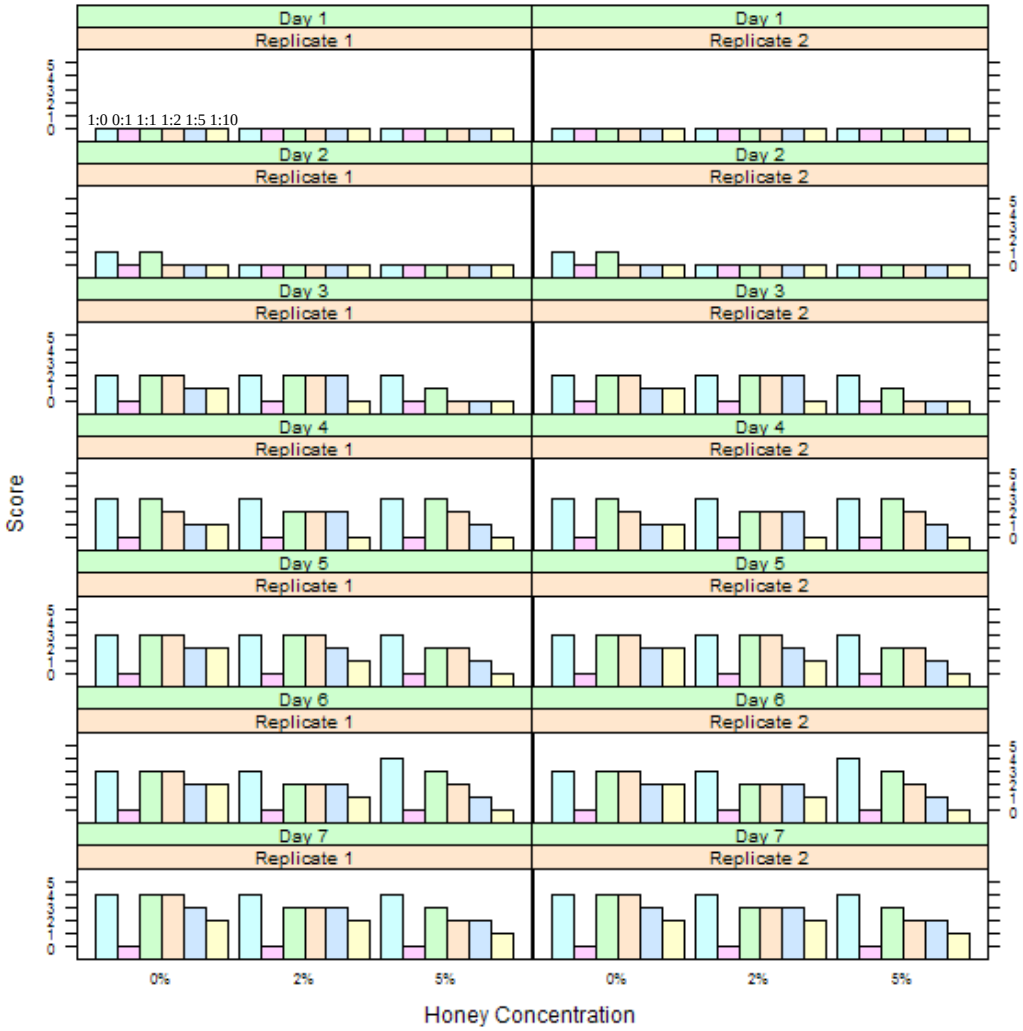
Figure 8.6 CPE from HSV-1 after treatment with Manuka honey M116 for: a) 1 hour, b) 2 hours, and c) 4 hours

Healthy uninfected A549 cells were mixed at varying ratios with A549 cells infected with virus treated with 0%, 2%, or 5% Manuka honey M116 for: (a) 1 hour, (b) 2 hours, and (c) 4 hours.

(a) 1 hour



(b) 2 hours


Day 1
Replicate 1
Day 1
Replicate 2
1:0 0:1 1:1 1:2 1:5 1:10
Day 2
Replicate 1
Day 2
Replicate 2
Day 3
Replicate 1
Day 3
Replicate 2
Day 4
Replicate 1
Day 4
Replicate 2
Day 5
Replicate 1
Day 5
Replicate 2
Day 6
Replicate 1
Day 6
Replicate 2
Day 7
Replicate 1
Day 7
Replicate 2
Score
0%
2%
5%
0%
2%
5%
Honey Concentration

(c) 4 hours

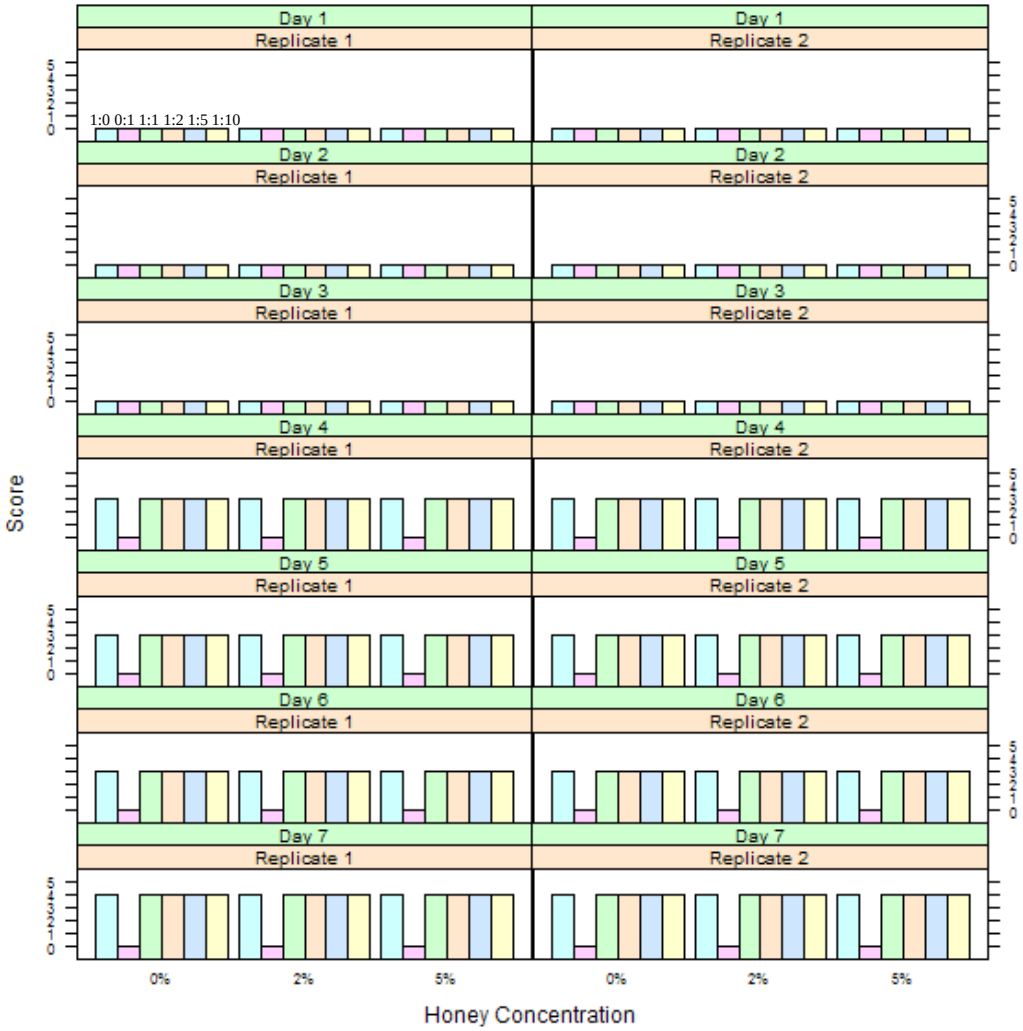
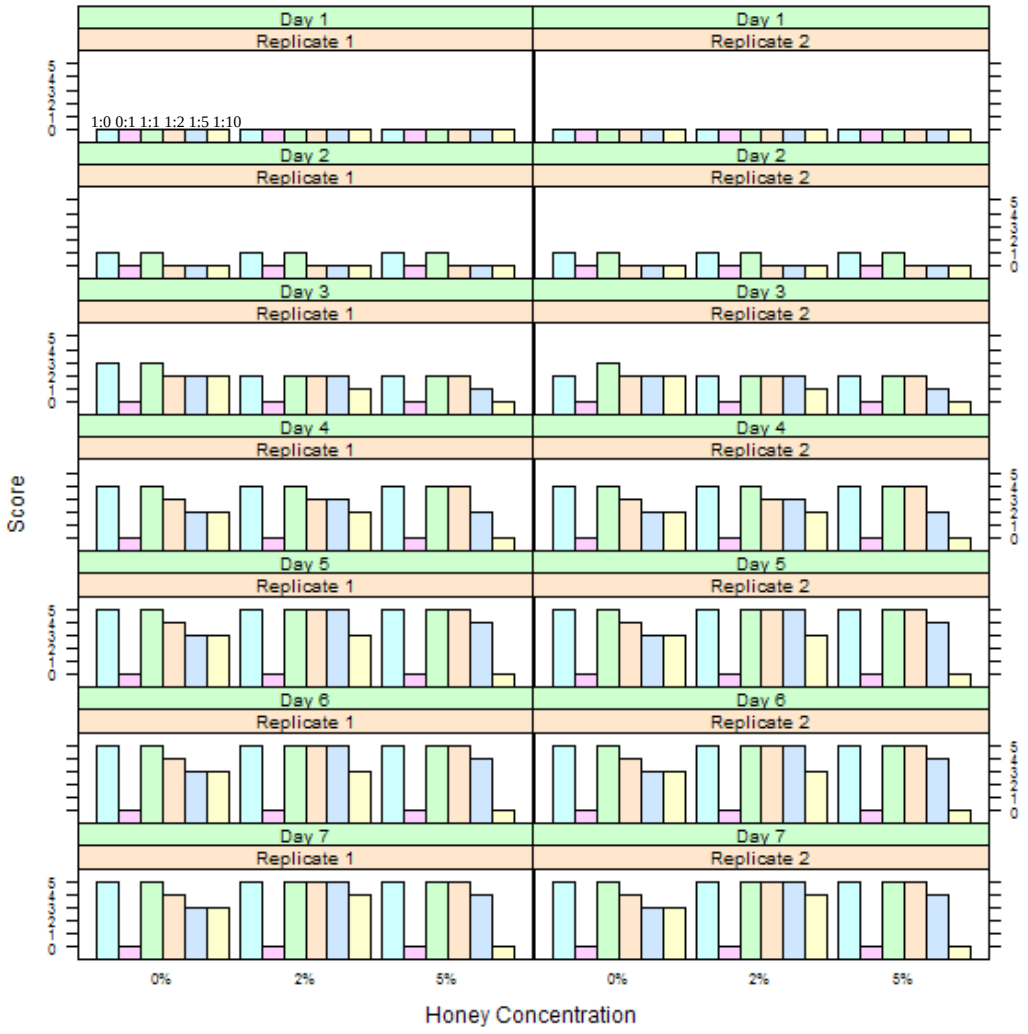

Day 1
Replicate 1
Day 1
Replicate 2
1:0 0:1 1:1 1:2 1:5 1:10
Day 2
Replicate 1
Day 2
Replicate 2
Day 3
Replicate 1
Day 3
Replicate 2
Day 4
Replicate 1
Day 4
Replicate 2
Day 5
Replicate 1
Day 5
Replicate 2
Day 6
Replicate 1
Day 6
Replicate 2
Day 7
Replicate 1
Day 7
Replicate 2
Score
0%
2%
5%
0%
2%
5%
Honey Concentration

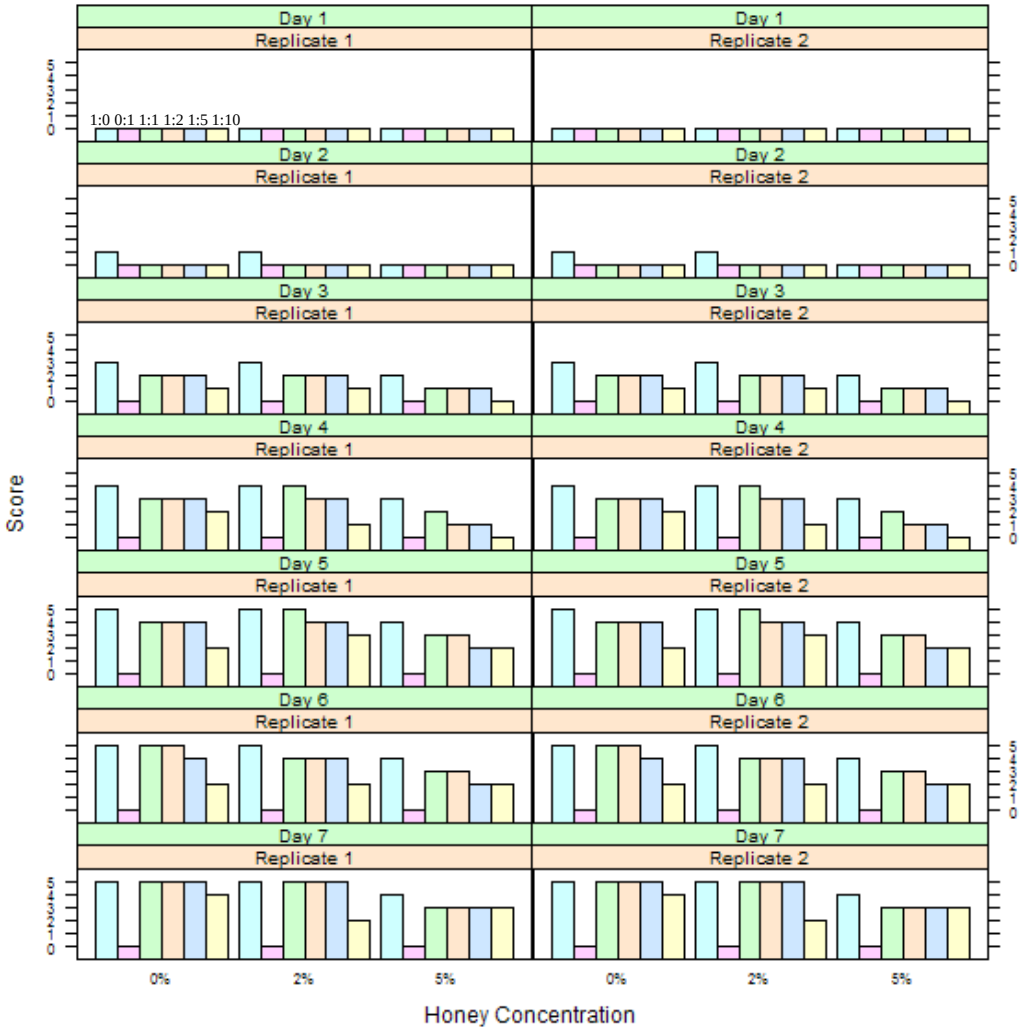
Figure 8.7 CPE from HSV-2 after treatment with Manuka honey M116 for: (a) 1 hour, (b) 2 hours, and (c) 4 hours

Healthy uninfected A549 cells were mixed at varying ratios with A549 cells infected with virus treated with 0%, 2%, or 5% Manuka honey M116 for: (a) 1 hour, (b) 2 hours, and (c) 4 hours.

(a) 1 hour



(b) 2 hours


Day 1
Replicate 1
Day 1
Replicate 2
1:0 0:1 1:1 1:2 1:5 1:10
Day 2
Replicate 1
Day 2
Replicate 2
Day 3
Replicate 1
Day 3
Replicate 2
Day 4
Replicate 1
Day 4
Replicate 2
Day 5
Replicate 1
Day 5
Replicate 2
Day 6
Replicate 1
Day 6
Replicate 2
Day 7
Replicate 1
Day 7
Replicate 2
Score
0%
2%
5%
0%
2%
5%
Honey Concentration

(c) 4 hours

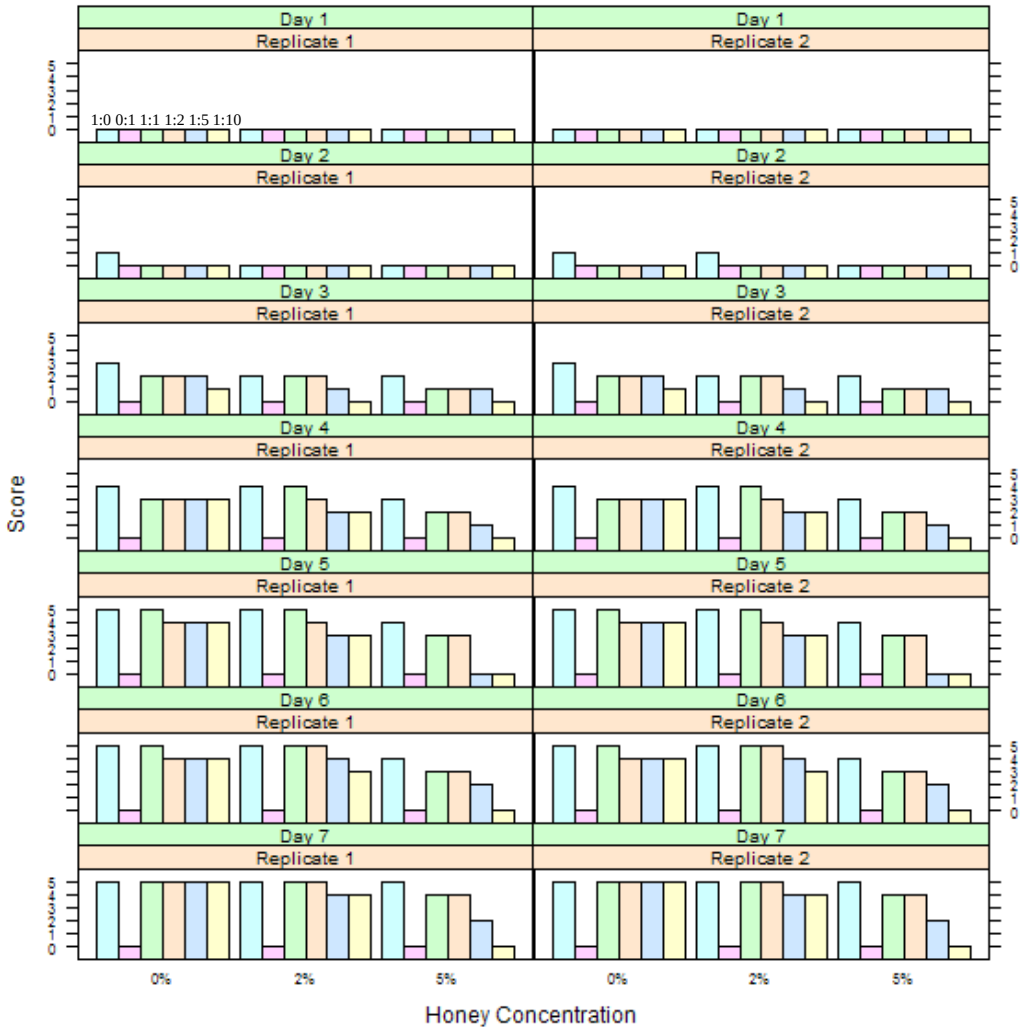

Day 1
Replicate 1
Day 1
Replicate 2
1:0 0:1 1:1 1:2 1:5 1:10
Day 2
Replicate 1
Day 2
Replicate 2
Day 3
Replicate 1
Day 3
Replicate 2
Day 4
Replicate 1
Day 4
Replicate 2
Day 5
Replicate 1
Day 5
Replicate 2
Day 6
Replicate 1
Day 6
Replicate 2
Day 7
Replicate 1
Day 7
Replicate 2
Score
0%
2%
5%
0%
2%
5%
Honey Concentration

Figure 8.8 CPE from Ad3 after treatment with Manuka honey M155 for: 1 hour, 2 hours, and 4 hours

Healthy uninfected A549 cells were mixed at varying ratios with A549 cells infected with virus treated with 0%, 2%, or 5% Manuka honey M155 for: 1 hour, 2 hours, and 4 hours.

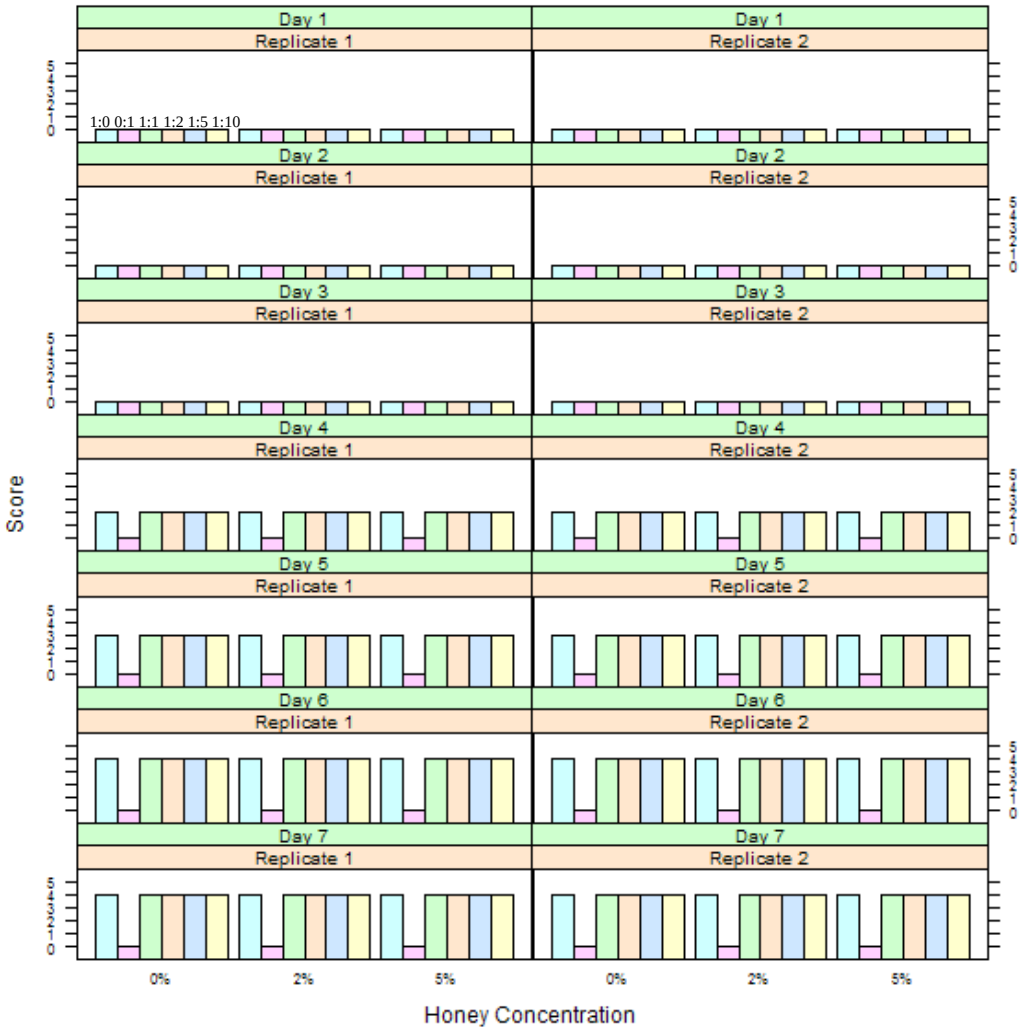


Figure 8.9 CPE from HSV-1 after treatment with Manuka honey M155 for: 1 hour, 2 hours, and 4 hours

Healthy uninfected A549 cells were mixed at varying ratios with A549 cells infected with virus treated with 0%, 2%, or 5% Manuka honey M155 for: 1 hour, 2 hours, and 4 hours.

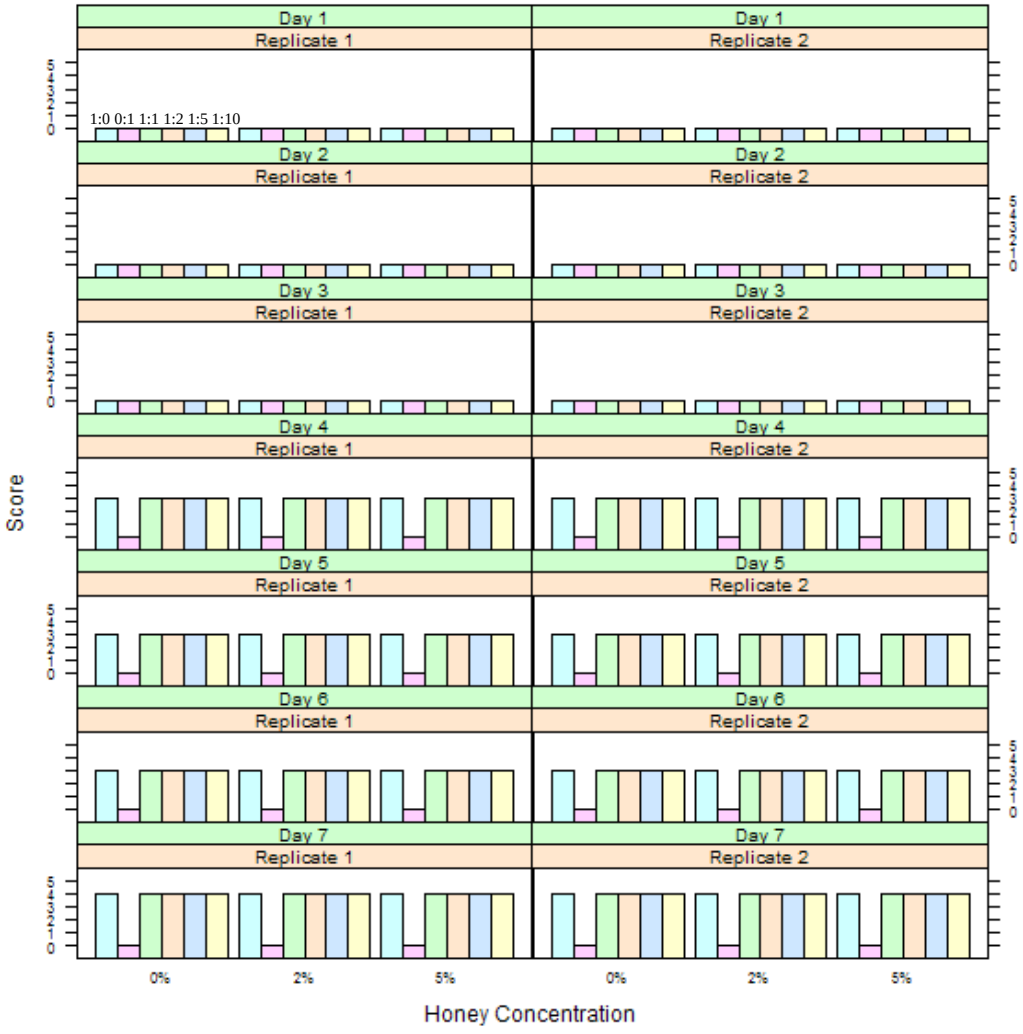


Figure 8.10 CPE from HSV-2 after treatment with Manuka honey M155 for: 1 hour, 2 hours, and 4 hours

Healthy uninfected A549 cells were mixed at varying ratios with A549 cells infected with virus treated with 0%, 2%, or 5% Manuka honey M155 for: 1 hour, 2 hours, and 4 hours.

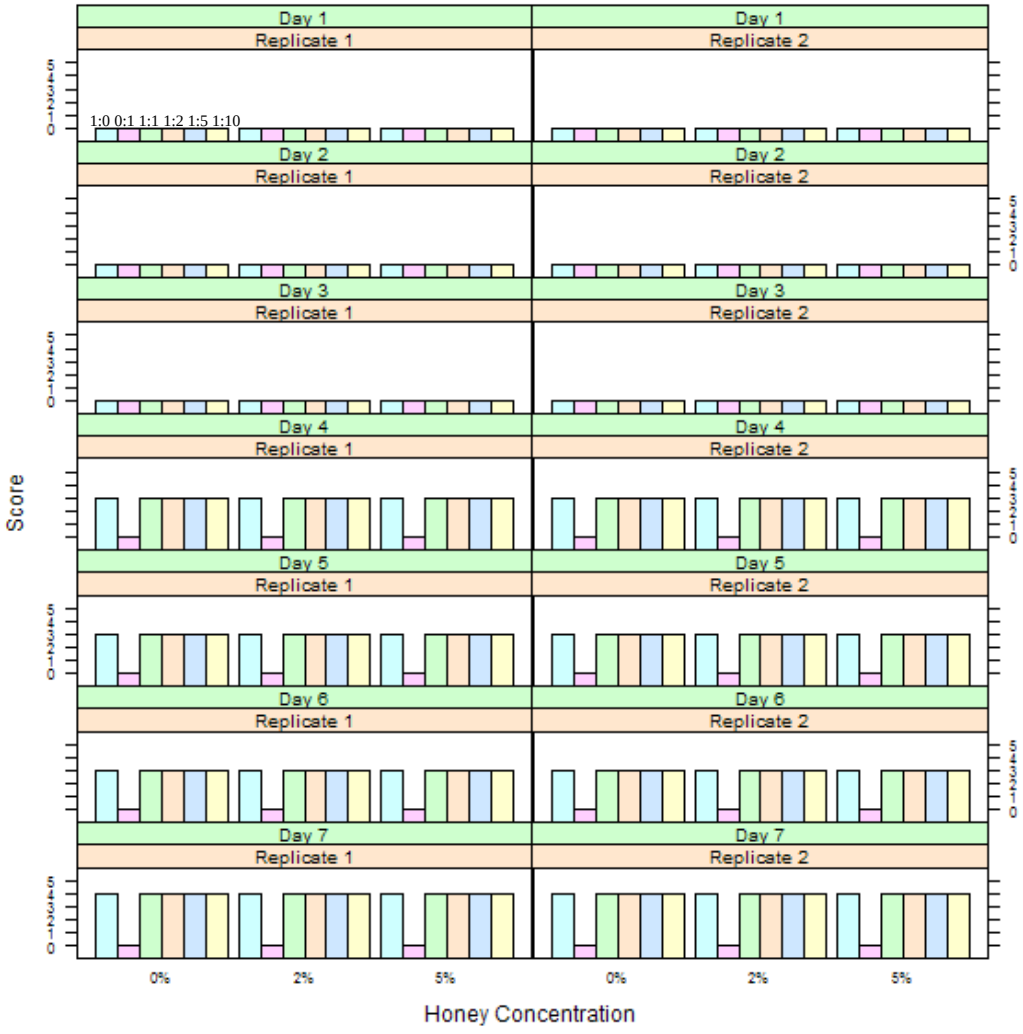
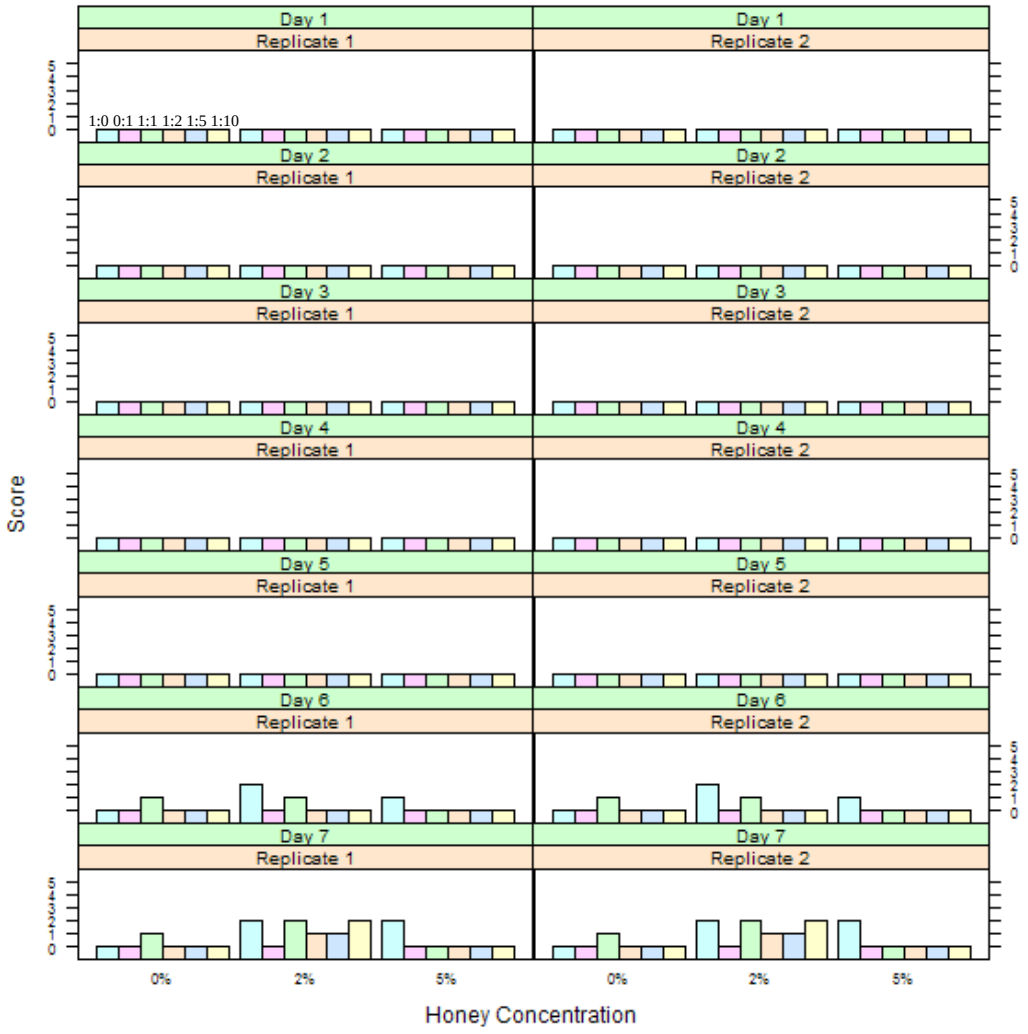


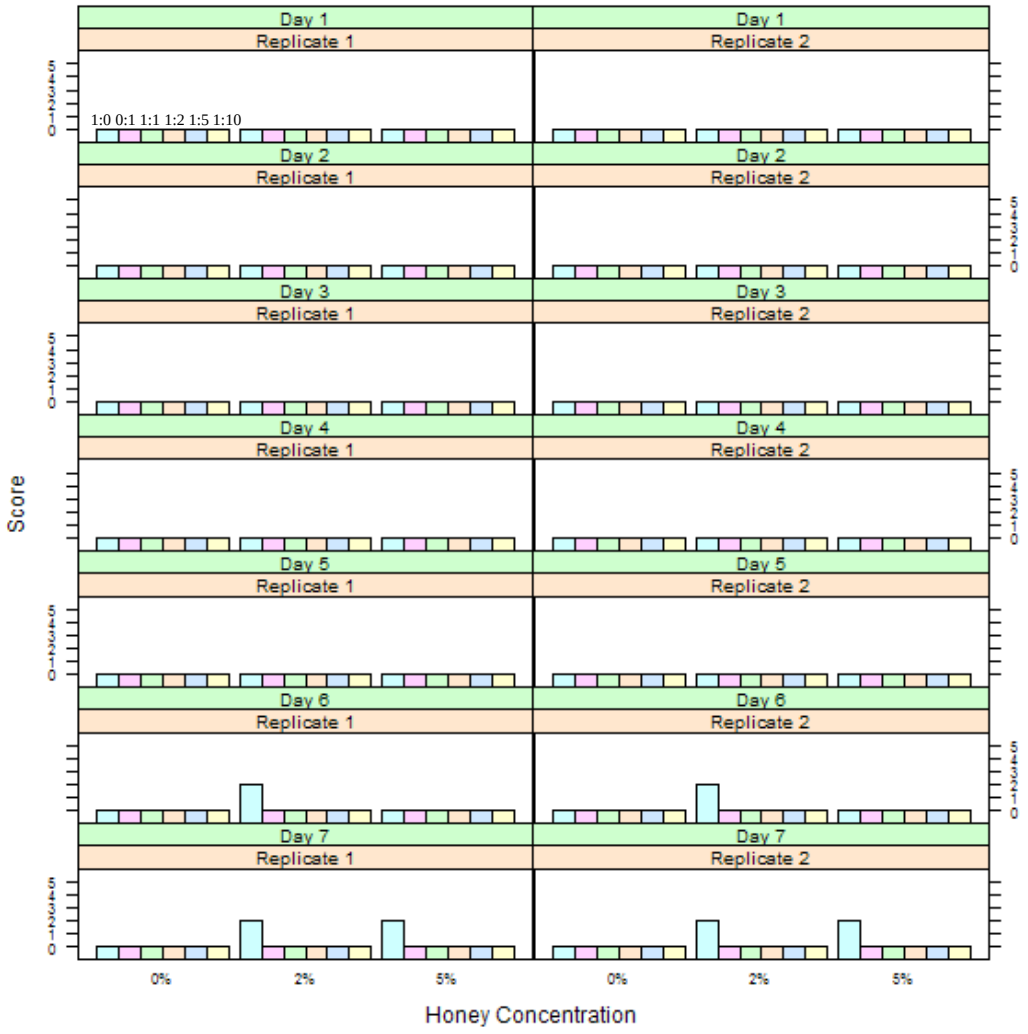
Figure 8.11 CPE from Ad3 after treatment with Honeydew honey HD19 for: (a) 1 hour, (b) 2 hours, and (c) 4 hours

Healthy uninfected A549 cells were mixed at varying ratios with A549 cells infected with virus treated with 0%, 2%, or 5% Honeydew honey HD19 for: (a) 1 hour, (b) 2 hours, and (c) 4 hours.

(a) 1 hour



(b) 2 hours


Day 1
Replicate 1
Day 1
Replicate 2
1:0 0:1 1:1 1:2 1:5 1:10
Day 2
Replicate 1
Day 2
Replicate 2
Day 3
Replicate 1
Day 3
Replicate 2
Day 4
Replicate 1
Day 4
Replicate 2
Day 5
Replicate 1
Day 5
Replicate 2
Day 6
Replicate 1
Day 6
Replicate 2
Day 7
Replicate 1
Day 7
Replicate 2
Score
0%
2%
5%
0%
2%
5%
Honey Concentration

(c) 4 hours

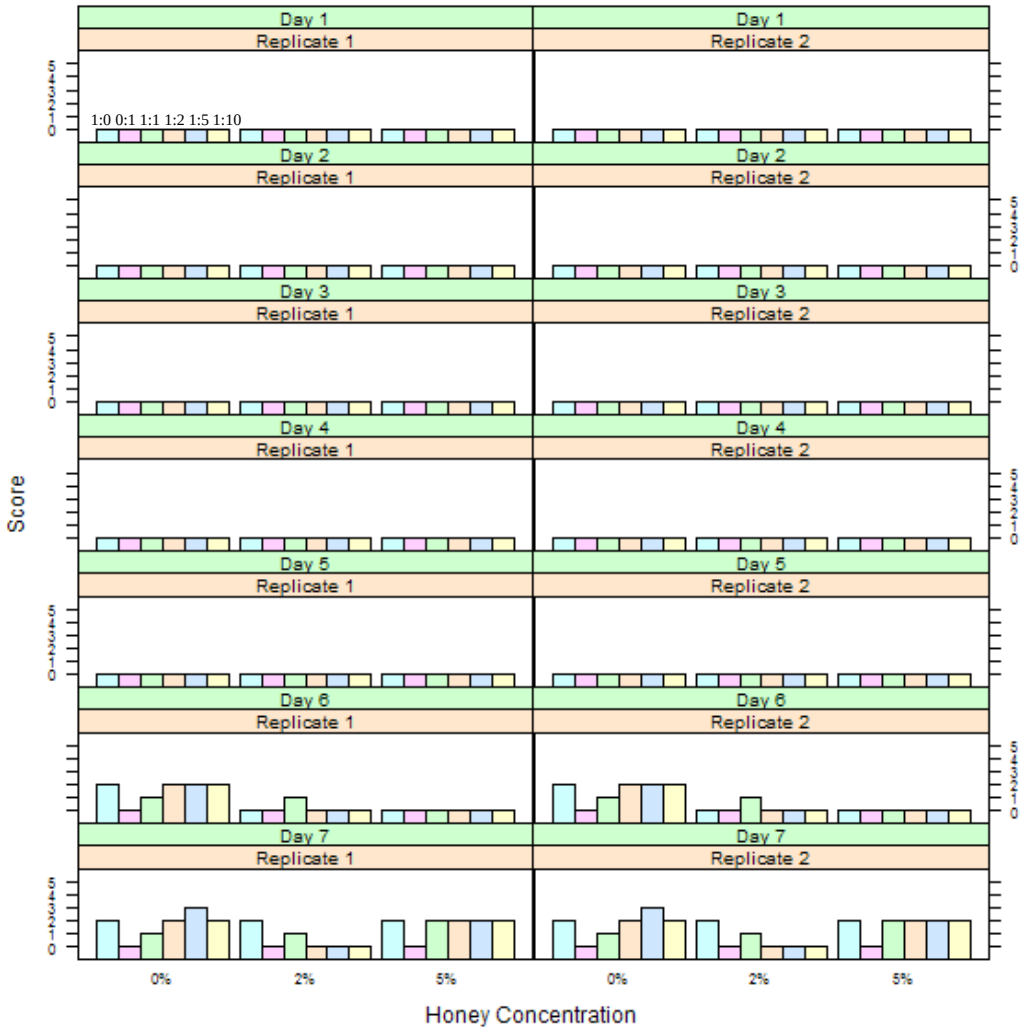
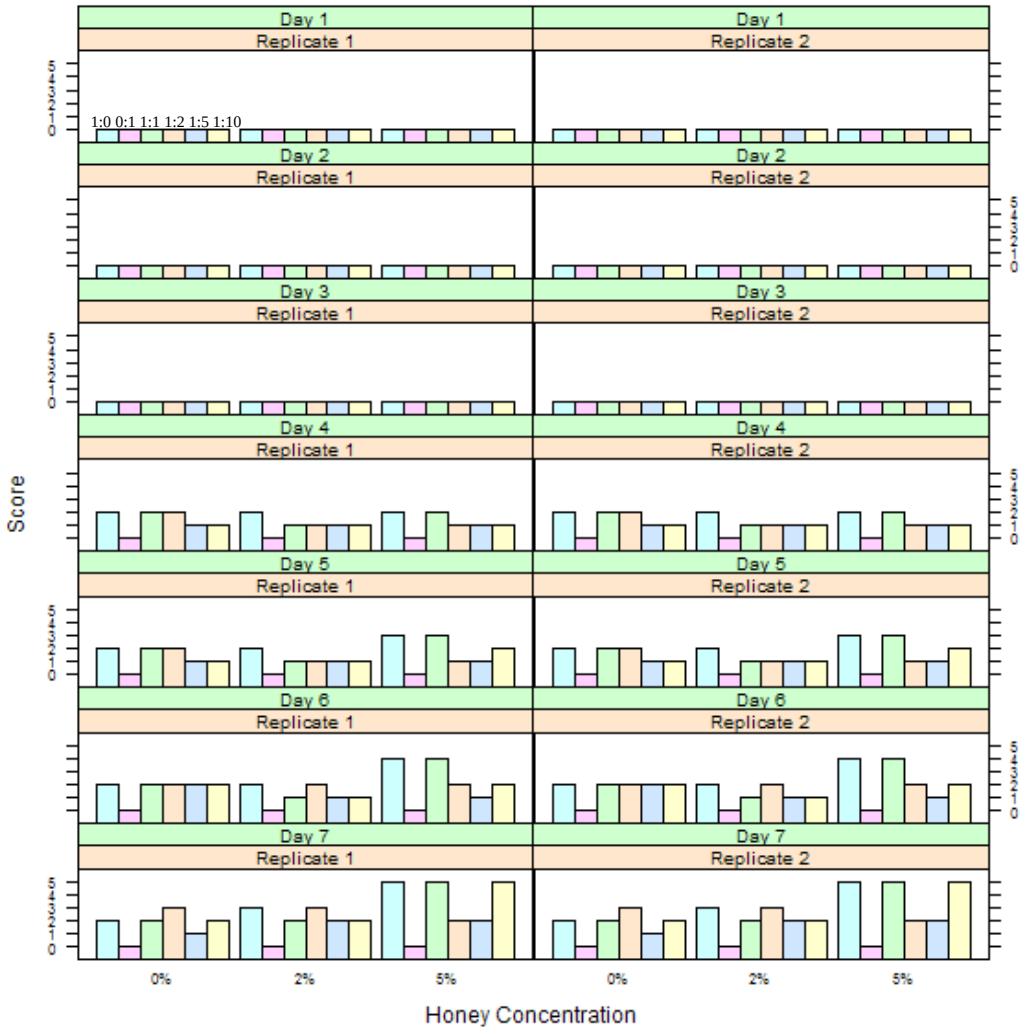

Day 1
Replicate 1
Day 1
Replicate 2
1:0 0:1 1:1 1:2 1:5 1:10
Day 2
Replicate 1
Day 2
Replicate 2
Day 3
Replicate 1
Day 3
Replicate 2
Day 4
Replicate 1
Day 4
Replicate 2
Day 5
Replicate 1
Day 5
Replicate 2
Day 6
Replicate 1
Day 6
Replicate 2
Day 7
Replicate 1
Day 7
Replicate 2
Score
0%
2%
5%
0%
2%
5%
Honey Concentration

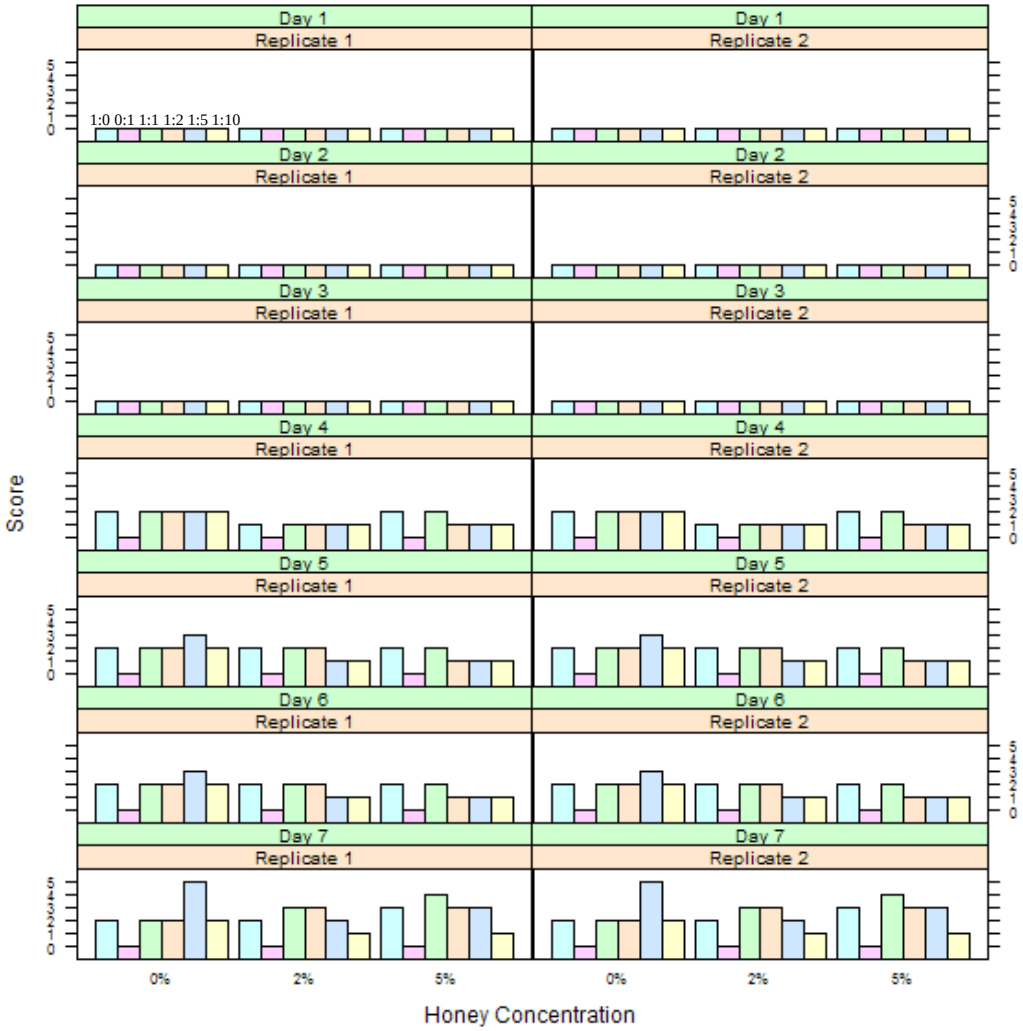
Figure 8.12 CPE from HSV-1 after treatment with Honeydew honey HD19 for: (a) 1 hour, (b) 2 hours, and (c) 4 hours

Healthy uninfected A549 cells were mixed at varying ratios with A549 cells infected with virus treated with 0%, 2%, or 5% Honeydew honey HD19 for: (a) 1 hour, (b) 2 hours, and (c) 4 hours.

(a) 1 hour



(b) 2 hours


Day 1
Replicate 1
Day 1
Replicate 2
1:0 0:1 1:1 1:2 1:5 1:10
Day 2
Replicate 1
Day 2
Replicate 2
Day 3
Replicate 1
Day 3
Replicate 2
Day 4
Replicate 1
Day 4
Replicate 2
Day 5
Replicate 1
Day 5
Replicate 2
Day 6
Replicate 1
Day 6
Replicate 2
Day 7
Replicate 1
Day 7
Replicate 2
Score
0%
2%
5%
0%
2%
5%
Honey Concentration

(c) 4 hours

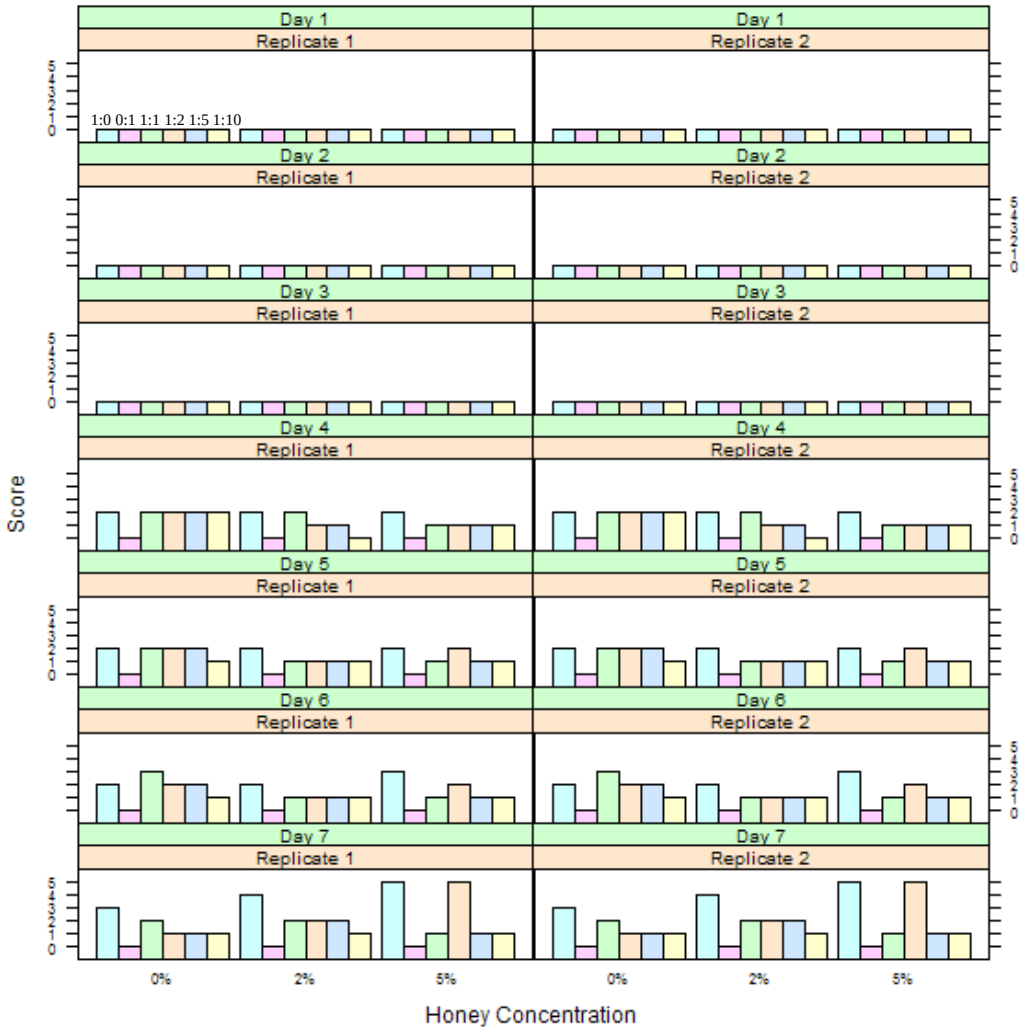
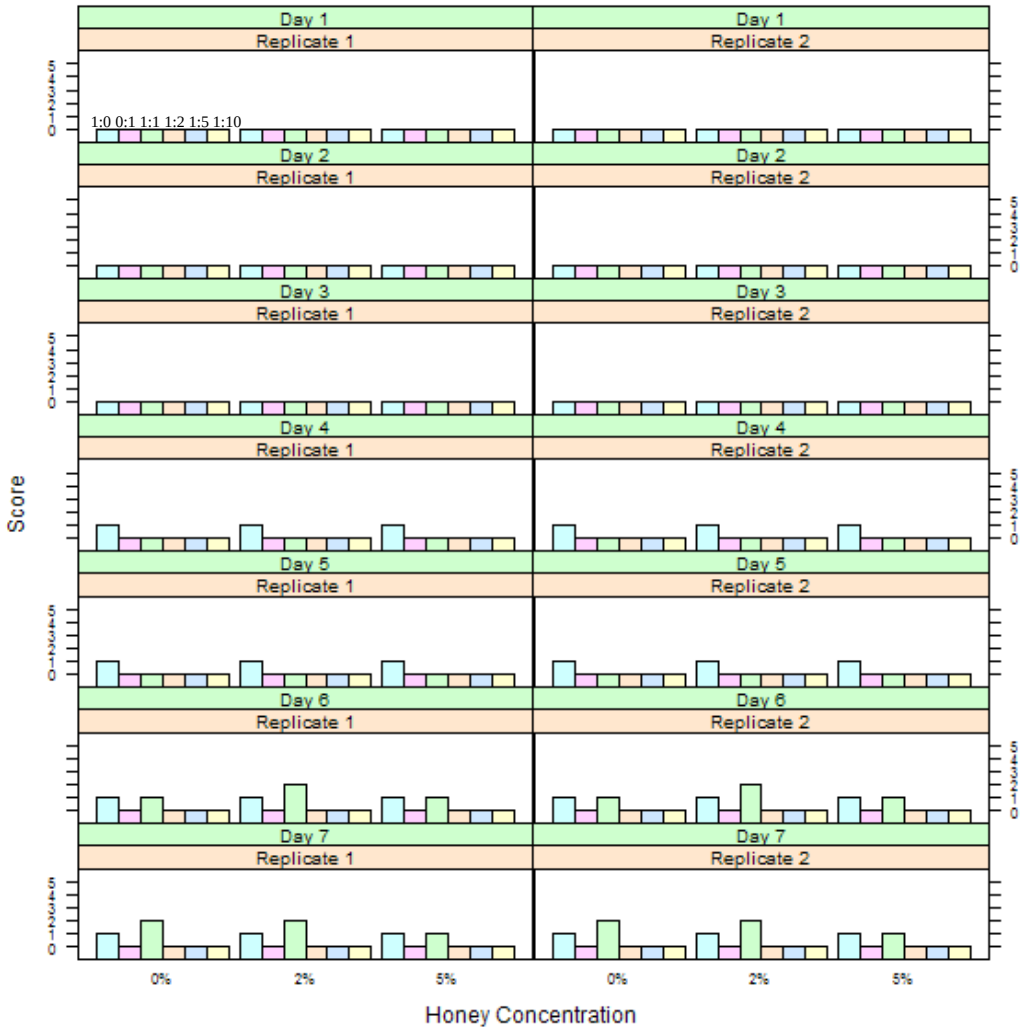

Day 1
Replicate 1
Day 1
Replicate 2
1:0 0:1 1:1 1:2 1:5 1:10
Day 2
Replicate 1
Day 2
Replicate 2
Day 3
Replicate 1
Day 3
Replicate 2
Day 4
Replicate 1
Day 4
Replicate 2
Day 5
Replicate 1
Day 5
Replicate 2
Day 6
Replicate 1
Day 6
Replicate 2
Day 7
Replicate 1
Day 7
Replicate 2
Score
0%
2%
5%
0%
2%
5%
Honey Concentration

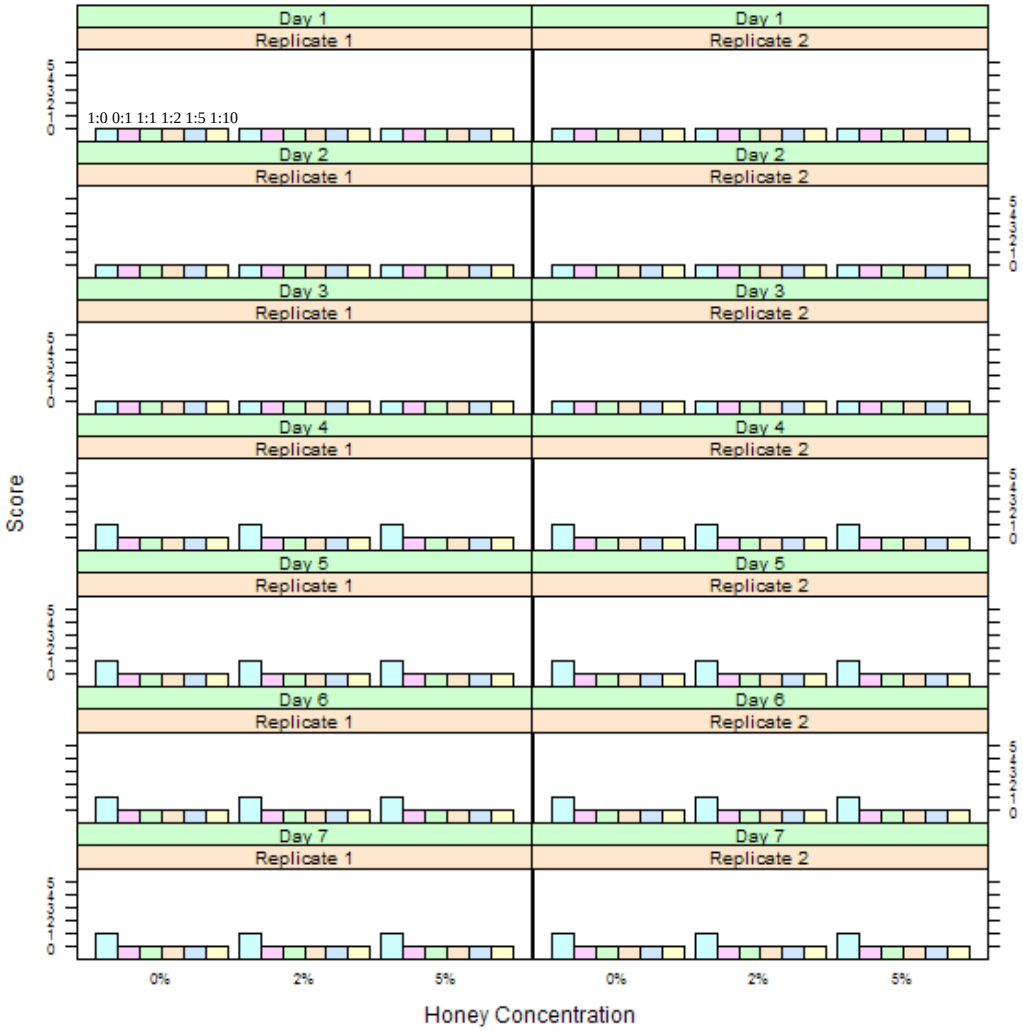
Figure 8.13 CPE from HSV-2 after treatment with Honeydew honey HD19 for: (a) 1 hour, (b) 2 hours, and (c) 4 hours

Healthy uninfected A549 cells were mixed at varying ratios with A549 cells infected with virus treated with 0%, 2%, or 5% Honeydew honey HD19 for: (a) 1 hour, (b) 2 hours, and (c) 4 hours.

(a) 1 hour



(b) 2 hours


Day 1
Replicate 1
Day 1
Replicate 2
1:0 0:1 1:1 1:2 1:5 1:10
Day 2
Replicate 1
Day 2
Replicate 2
Day 3
Replicate 1
Day 3
Replicate 2
Day 4
Replicate 1
Day 4
Replicate 2
Day 5
Replicate 1
Day 5
Replicate 2
Day 6
Replicate 1
Day 6
Replicate 2
Day 7
Replicate 1
Day 7
Replicate 2
Score
0%
2%
5%
0%
2%
5%
Honey Concentration

(c) 4 hours

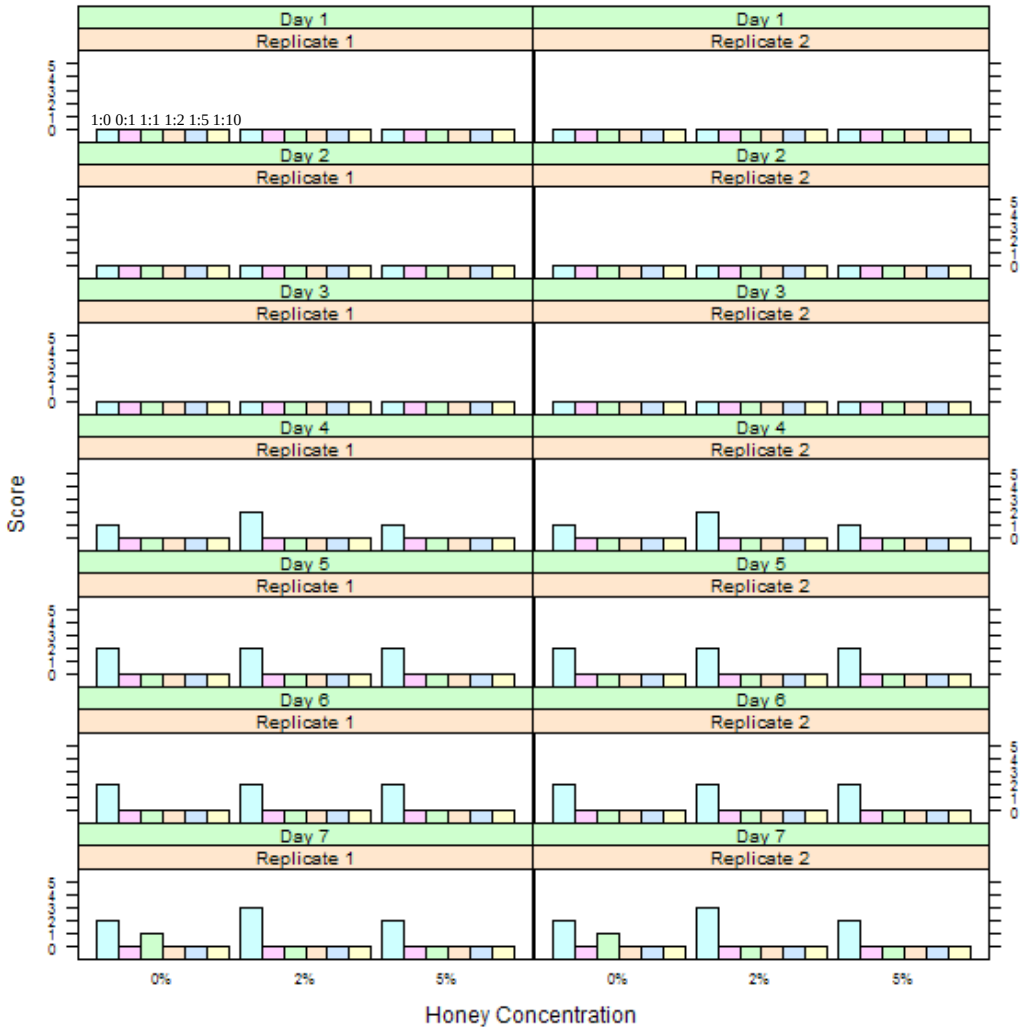
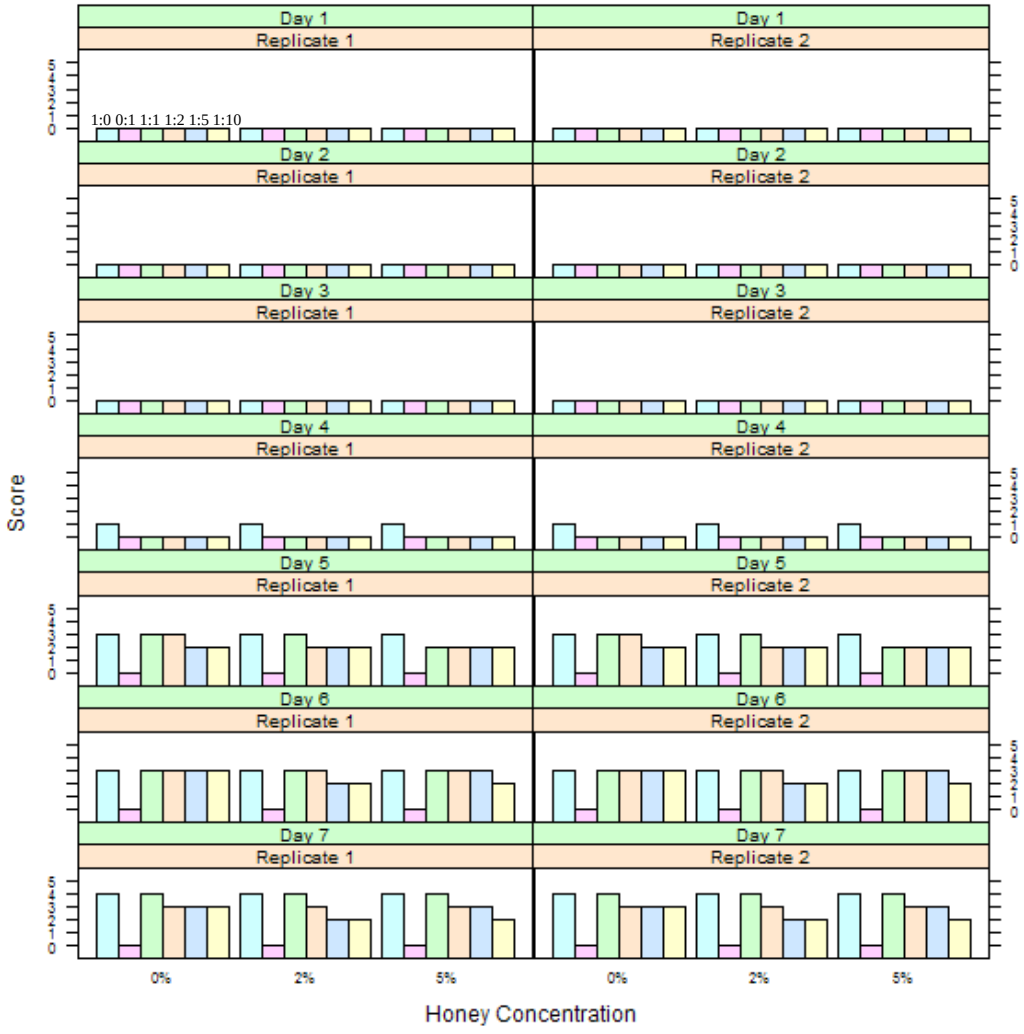

Day 1
Replicate 1
Day 1
Replicate 2
1:0 0:1 1:1 1:2 1:5 1:10
Day 2
Replicate 1
Day 2
Replicate 2
Day 3
Replicate 1
Day 3
Replicate 2
Day 4
Replicate 1
Day 4
Replicate 2
Day 5
Replicate 1
Day 5
Replicate 2
Day 6
Replicate 1
Day 6
Replicate 2
Day 7
Replicate 1
Day 7
Replicate 2
Score
0%
2%
5%
0%
2%
5%
Honey Concentration

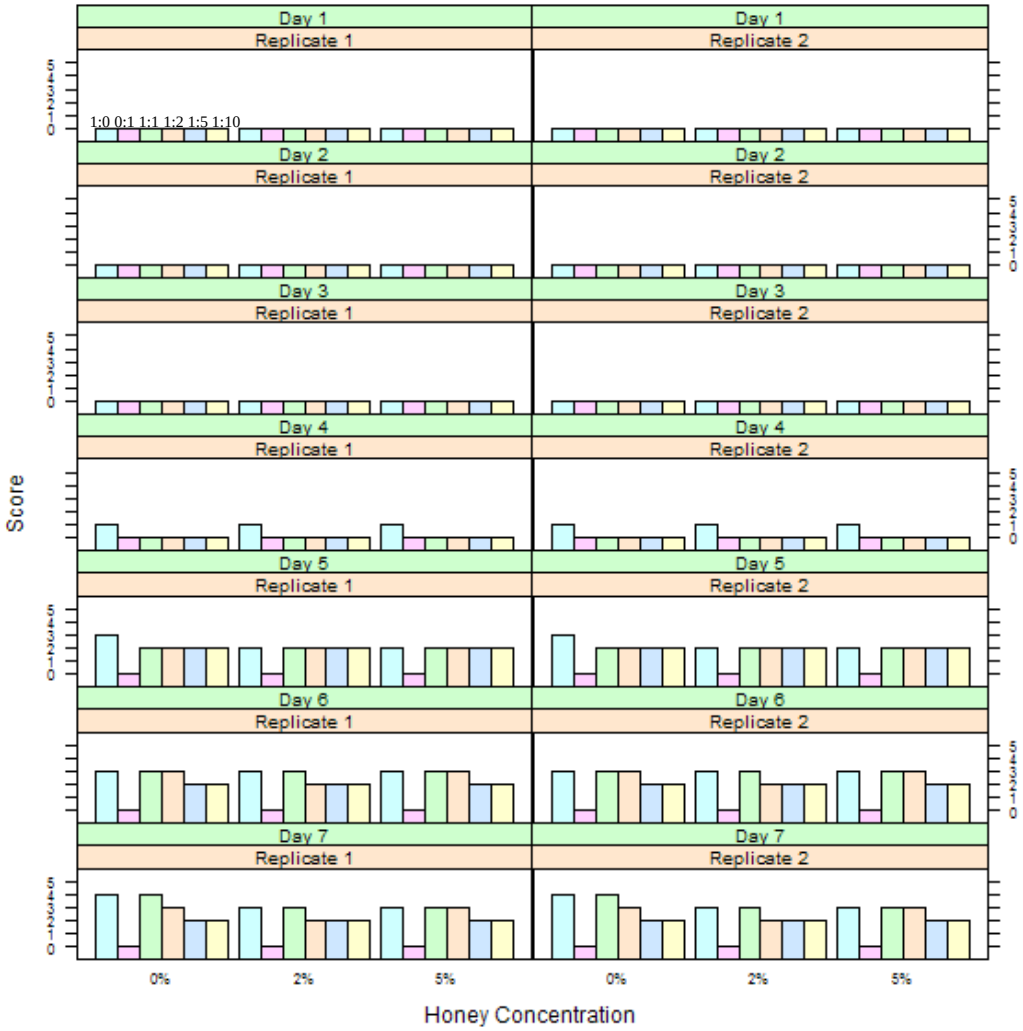
Figure 8.14 CPE from Ad3 after treatment with Ling Heather honey LH27 for: (a) 1 hour, (b) 2 hours, and (c) 4 hours

Healthy uninfected A549 cells were mixed at varying ratios with A549 cells infected with virus treated with 0%, 2%, or 5% Ling Heather honey for: (a) 1 hour, (b) 2 hours, and (c) 4 hours.

(a) 1 hour



(b) 2 hours


Day 1
Replicate 1
Day 1
Replicate 2
1:0 0:1 1:1 1:2 1:5 1:10
Day 2
Replicate 1
Day 2
Replicate 2
Day 3
Replicate 1
Day 3
Replicate 2
Day 4
Replicate 1
Day 4
Replicate 2
Day 5
Replicate 1
Day 5
Replicate 2
Day 6
Replicate 1
Day 6
Replicate 2
Day 7
Replicate 1
Day 7
Replicate 2
Score
0%
2%
5%
0%
2%
5%
Honey Concentration

(c) 4 hours

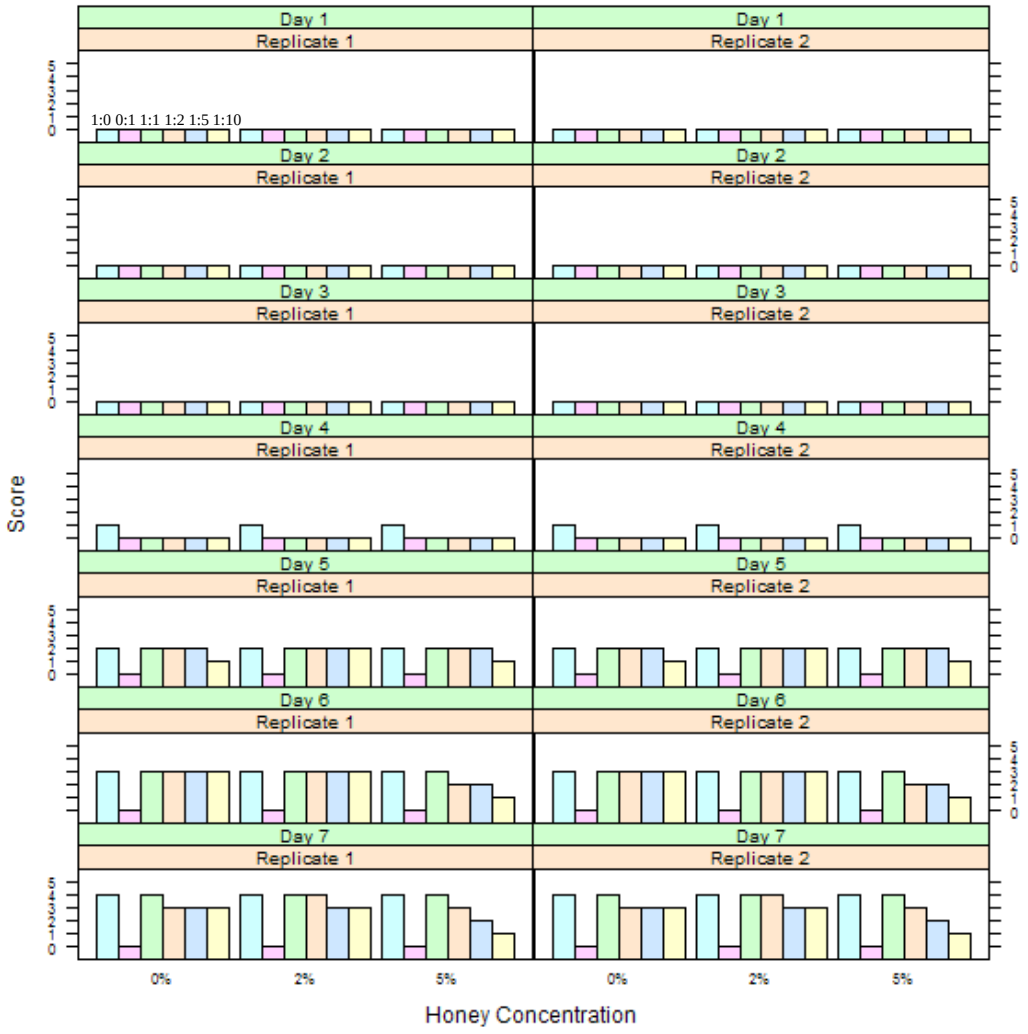
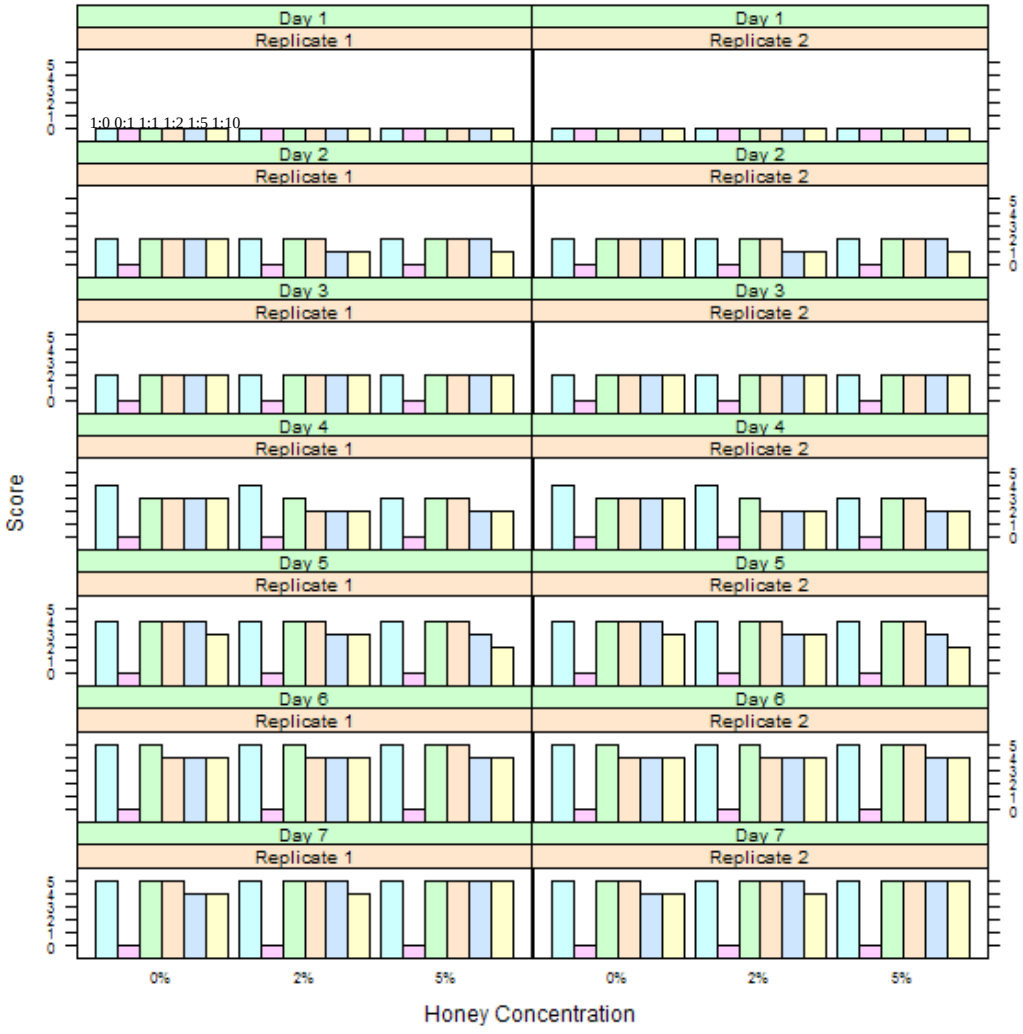

Day 1
Replicate 1
Day 1
Replicate 2
1:0 0:1 1:1 1:2 1:5 1:10
Day 2
Replicate 1
Day 2
Replicate 2
Day 3
Replicate 1
Day 3
Replicate 2
Day 4
Replicate 1
Day 4
Replicate 2
Day 5
Replicate 1
Day 5
Replicate 2
Day 6
Replicate 1
Day 6
Replicate 2
Day 7
Replicate 1
Day 7
Replicate 2
Score
0%
2%
5%
0%
2%
5%
Honey Concentration

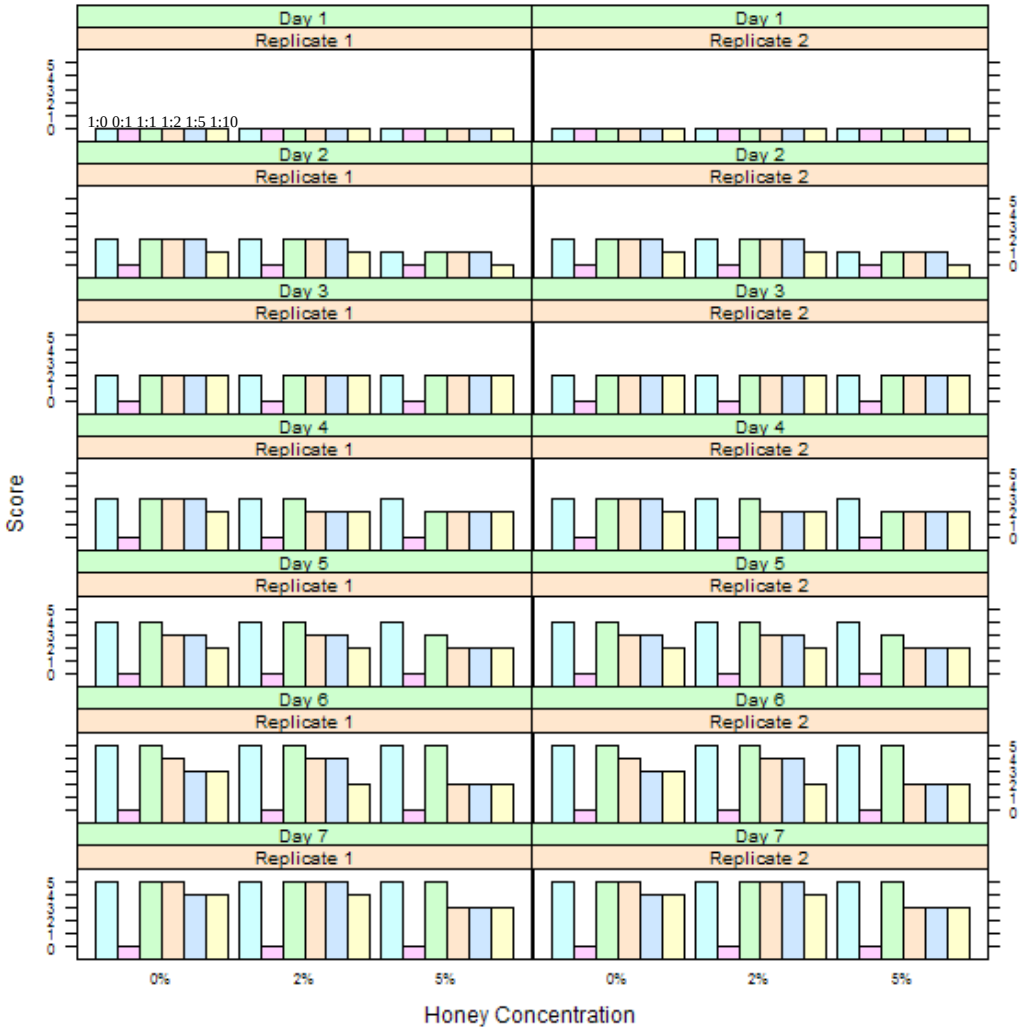
Figure 8.15 CPE from HSV-1 after treatment with Ling Heather honey LH27 for: (a) 1 hour, (b) 2 hours, and (c) 4 hours

Healthy uninfected A549 cells were mixed at varying ratios with A549 cells infected with virus treated with 0%, 2%, or 5% Ling Heather honey for: (a) 1 hour, (b) 2 hours, and (c) 4 hours.

(a) 1 hour



(b) 2 hours


Day 1
Replicate 1
Day 1
Replicate 2
1:0 0:1 1:1 1:2 1:5 1:10
Day 2
Replicate 1
Day 2
Replicate 2
Day 3
Replicate 1
Day 3
Replicate 2
Day 4
Replicate 1
Day 4
Replicate 2
Day 5
Replicate 1
Day 5
Replicate 2
Day 6
Replicate 1
Day 6
Replicate 2
Day 7
Replicate 1
Day 7
Replicate 2
Score
0%
2%
5%
0%
2%
5%
Honey Concentration

(c) 4 hours

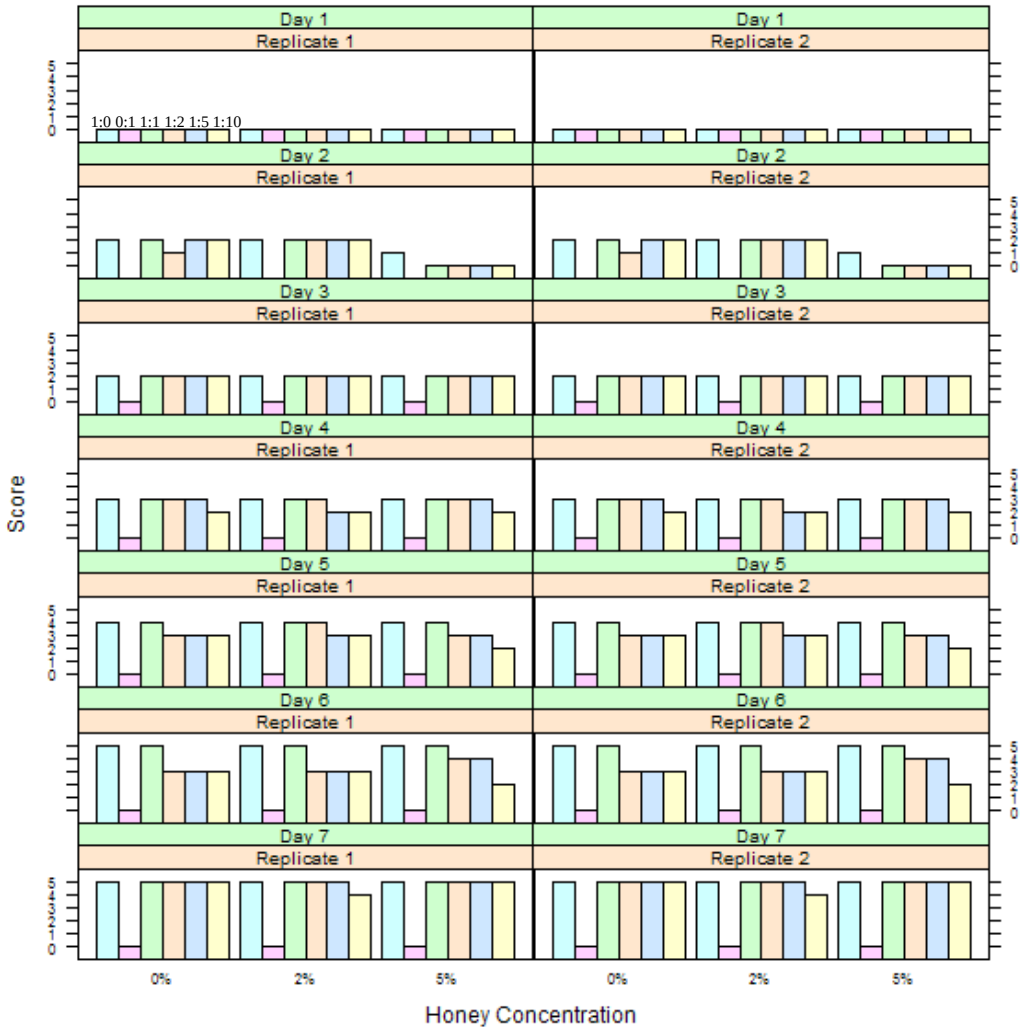
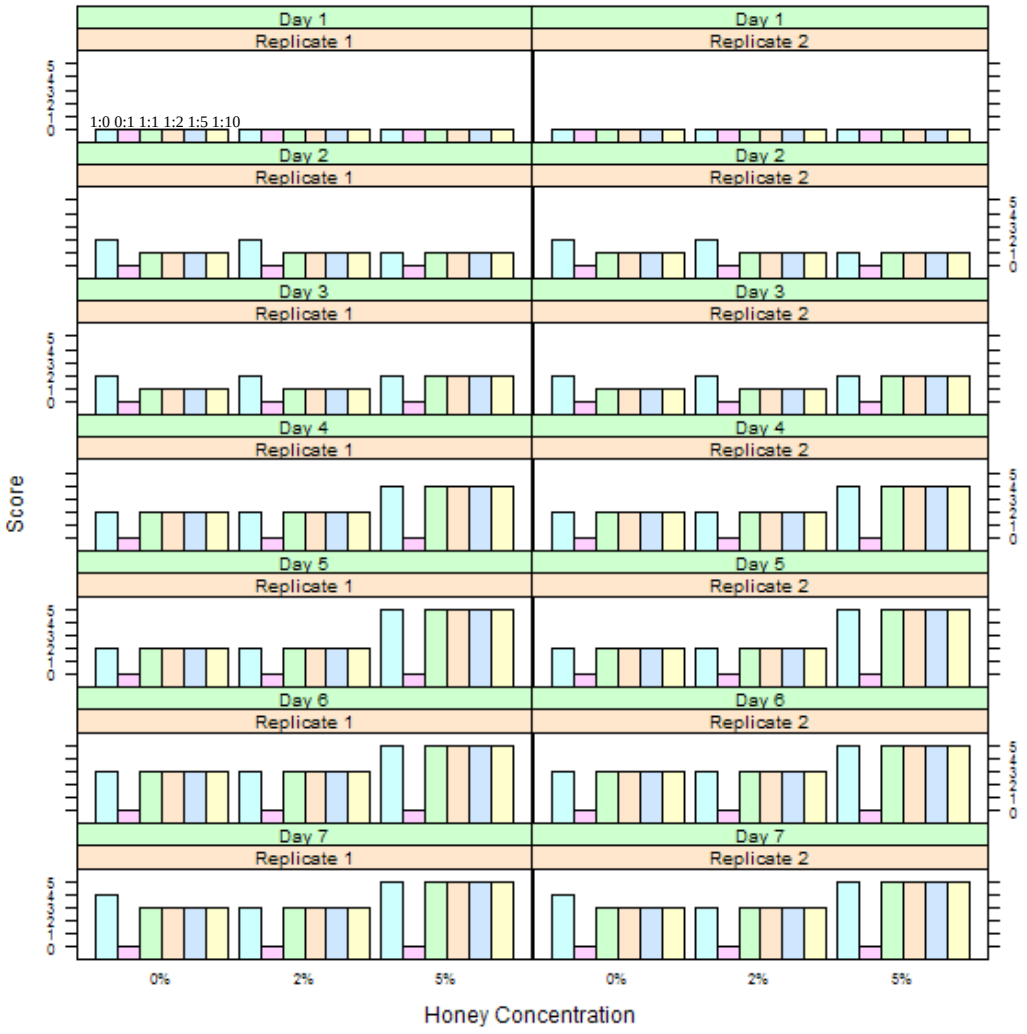

Day 1
Replicate 1
Day 1
Replicate 2
1:0 0:1 1:1 1:2 1:5 1:10
Day 2
Replicate 1
Day 2
Replicate 2
Day 3
Replicate 1
Day 3
Replicate 2
Day 4
Replicate 1
Day 4
Replicate 2
Day 5
Replicate 1
Day 5
Replicate 2
Day 6
Replicate 1
Day 6
Replicate 2
Day 7
Replicate 1
Day 7
Replicate 2
Score
0%
2%
5%
0%
2%
5%
Honey Concentration

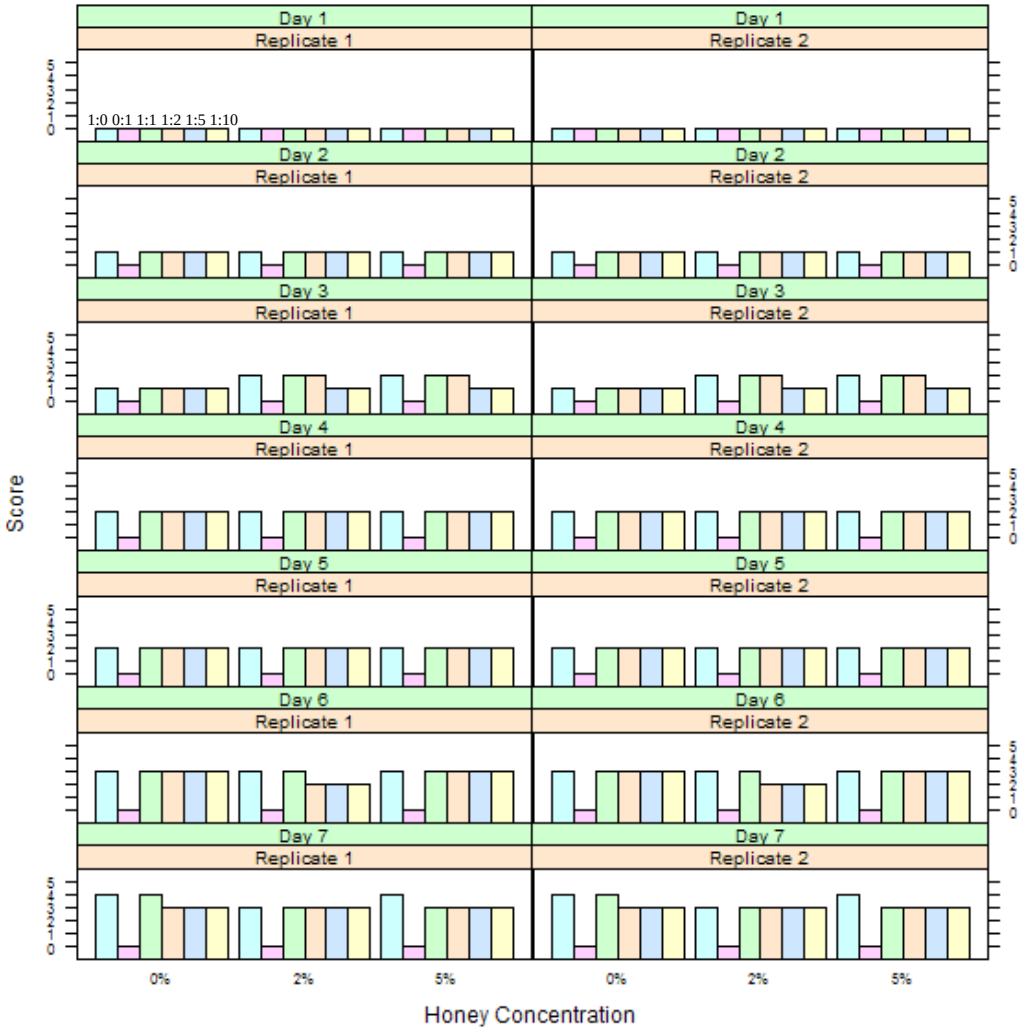
Figure 8.16 CPE from HSV-2 after treatment with Ling Heather honey LH27 for: (a) 1 hour, (b) 2 hours, (c) 4 hours

Healthy uninfected A549 cells were mixed at varying ratios with A549 cells infected with virus treated with 0%, 2%, or 5% Ling Heather honey for: (a) 1 hour, (b) 2 hours, and (c) 4 hours.

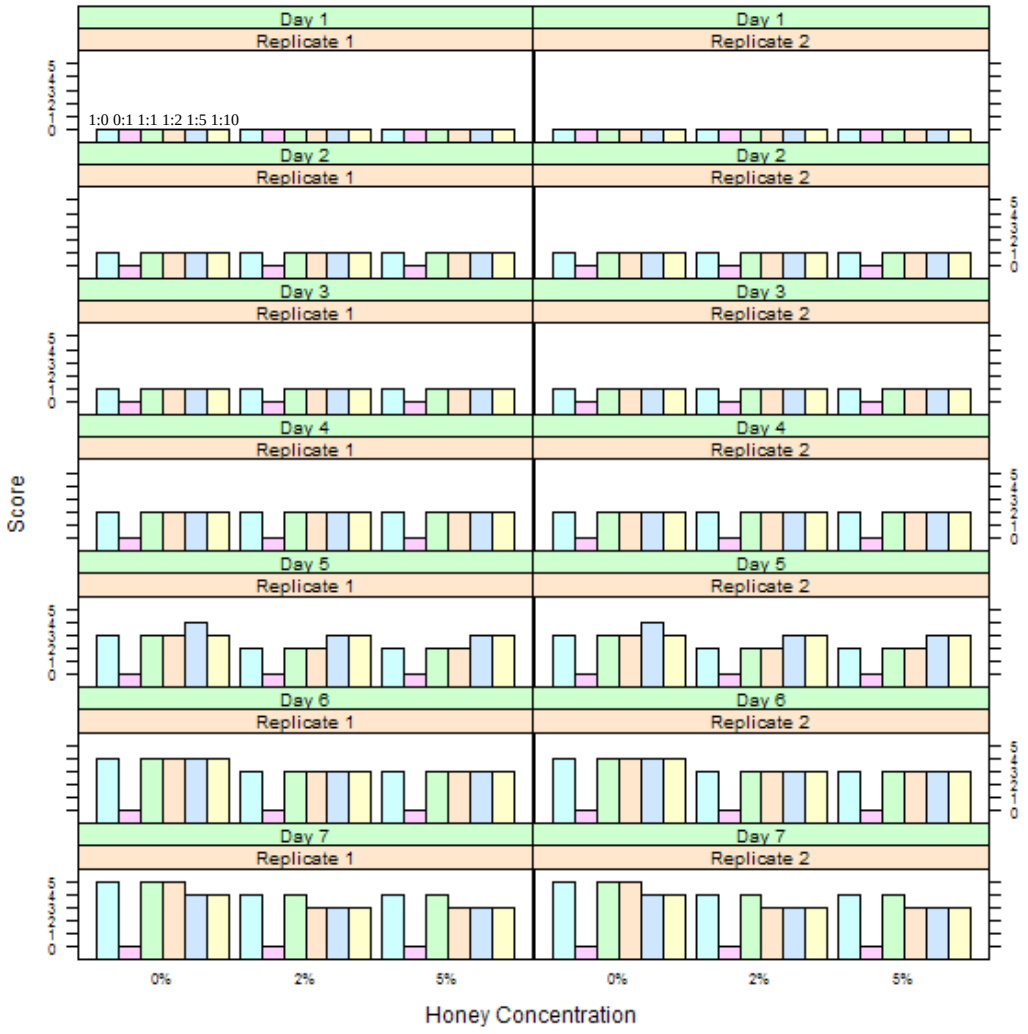
(a) 1 hour



(b) 2 hours


Day 1
Replicate 1
Day 1
Replicate 2
1:0 0:1 1:1 1:2 1:5 1:10
Day 2
Replicate 1
Day 2
Replicate 2
Day 3
Replicate 1
Day 3
Replicate 2
Day 4
Replicate 1
Day 4
Replicate 2
Day 5
Replicate 1
Day 5
Replicate 2
Day 6
Replicate 1
Day 6
Replicate 2
Day 7
Replicate 1
Day 7
Replicate 2
Score
0%
2%
5%
0%
2%
5%
Honey Concentration

(c) 4 hours


Day 1
Replicate 1
Day 1
Replicate 2
1:0 0:1 1:1 1:2 1:5 1:10
Day 2
Replicate 1
Day 2
Replicate 2
Day 3
Replicate 1
Day 3
Replicate 2
Day 4
Replicate 1
Day 4
Replicate 2
Day 5
Replicate 1
Day 5
Replicate 2
Day 6
Replicate 1
Day 6
Replicate 2
Day 7
Replicate 1
Day 7
Replicate 2
Score
0%
2%
5%
0%
2%
5%
Honey Concentration

8.3.3 Discussion

The results from the preliminary experiments shown in Table 8.2 (without treatment with honey) show that CPE appeared much later after infection of the cells with virus compared with previous experiments (in Chapters 4, 5, 6) even with the 1:0 ratio of infected to healthy cells. HSV-1 did not display any CPE over the usual 7 days of observation likewise Ad3 gave signs of viral infection only from the final day of observation. It was thus expected using this method for the detection of viral spread of infection that high levels of CPE may not be seen. However, in most of the subsequent experiments higher CPE scores were obtained.

Many of the honeys were able to delay or prevent the development of viral CPE over the course of observation. Figures 8.5 (b) and (c), 8.7 (a) and (c), 8.11 (a), (b), and (c), 8.13 (a), (b), and (c) show inhibition of spread of Ad3, and HSV-2 infection with treatments of Manuka honey M116 and Honeydew honey HD19. None of the honeys were able to inhibit the development of HSV-1 CPE. With the previous neutralisation experiments, HSV-1 was affected by higher concentrations of Manuka honeys M116 and M112, and Rewarewa honey R19/06 only, whereas Ad3 and HSV-2 were affected by Manuka honey M116, Honeydew honey HD19, and Ling Heather honey LH27 (with HSV-2).

Reverse trends were observed (higher CPE scores with high concentrations of honey and low CPE scores with low concentrations of honey) between ratios of infected to healthy cells (see Figures 8.12 (a), (b), and (c), and 8.16 (a) and (c)) and no effect of the honey on CPE development was seen in Figures 8.6 (c), 8.8,

8.9 or 8.10. This further suggests the minimal effect of Manuka honey M155 on each virus.

Manuka honey M116 and Honeydew honey HD19 were the most successful treatments and Ling Heather in these experiments had little impact on the development of CPE showing its greatest effect after the 4 hour treatment with each honey concentration.

The aim of varying the length of exposure of the infected cells to the honey solutions was to test if greater exposure to honey could have a greater effect on inhibition of CPE development. It appears that the increased period of exposure had little influence on the consequent development of CPE, and if the honey was to be effective against CPE development, it showed these effects after one hour of exposure of the infected cells to honey.

It was reasoned that with the greater dilutions of infected cells to healthy cells that less viral infection can be observed given the lower amount of virus present. The CPE scores resulting from the mixing of infected cells with healthy cells show clearly that infection was spreading to the healthy cells from the infected cells, with similar CPE levels reached by the conclusion of the observation period. With Manuka honey M116, however, the 5% treatment with the 1:10 ratio of infected to healthy cells gave low levels of CPE compared with the untreated virus (shown in Figure 8.5, and 8.7 (a) and (c)). In most other cases little difference was observed between the two concentrations of honey and ratios tested.

From these results, it is concluded that when honey is used to treat infected cells it can only delay CPE developing in healthy cells, it cannot prevent it.

8.4 PREVENTION OF VIRAL SPREAD: PLAQUE REDUCTION ASSAYS

Plaque reduction assays can be used to measure quantitatively the effect an antiviral agent has against a virus. A plaque can be defined as a cleared zone within the monolayer of cells that does not stain with crystal violet.

8.4.1 Methods

A 4% agarose in double distilled H_2O was prepared and sterilised by autoclaving. Confluent A549 cells were prepared as described in Section 3.2.1 in 24 well plates and these were inoculated for the preliminary experiment with HSV-2 serially diluted to 10^{-6} for 1 hour at 37°C. In subsequent experiments with honey, the virus was used at a dilution of 10^{-4} and 0%, 10% and 20% honey solutions of Manuka honeys M116 and M155, Honeydew honey HD19, and Ling Heather honey LH27 were prepared in doubly concentrated maintenance medium (RPMI 1640 with Hepes, 2% FCS, supplemented with antimicrobials ampicillin and gentamycin at final concentrations of 100 µg/ml and amphotericin B at a final concentration of 10 µg/ml), these were mixed and filter sterilised. The previously prepared agarose was melted in a 45°C water bath. The viral inoculum was removed from the cells and was replaced with either 0.25 ml of doubly concentrated maintenance medium alone, or, 0.25 ml of honey solution with 0.25 of agarose to obtain 5% and 10% concentrations of honey. The overlay was allowed to set on the cells and the plates were incubated at 37°C. CPE development was observed through the thin overlay and was allocated an

appropriate score over a number of days. Once a moderate level of CPE had been reached with the viruses that had not had honey included in their agarose overlay, the cells were fixed with 20% trichloroacetic acid (TCA) for 5 minutes. After fixing the TCA was aspirated off and the overlay was removed, the cells were then stained with crystal violet working solution (20 ml stock (1% crystal violet made in 20% ethanol), 40 ml 95% ethanol, and 150 ml distilled water) for 30 minutes. The stain was washed off with distilled water and the plaques were counted at low power. In further experiments honey was used at a final concentration of 2% with virus diluted from 10^{-1} to 10^{-4}. Each experiment was completed in duplicate, and with the testing honey two experiments were completed on separate days.

8.4.2 Results

Table 8.3 describes the plaque counts of the first experiment using HSV-2 diluted from 10^{-1} to 10^{-6}. Figure 8.17 gives the plaque counts of each virus and honey type, this is followed by Table 8.4 which describes the mean, standard deviation, coefficient of variation and average percent reduction in plaque numbers with the honey treatments for each of the plaque counts.

The preliminary experiments using honey at a final concentration of 5% and 10% were too concentrated for the cells to tolerate, and resulted in destruction of the monolayer. The Figure below outlines the counts using honey at a final concentration of 2%.

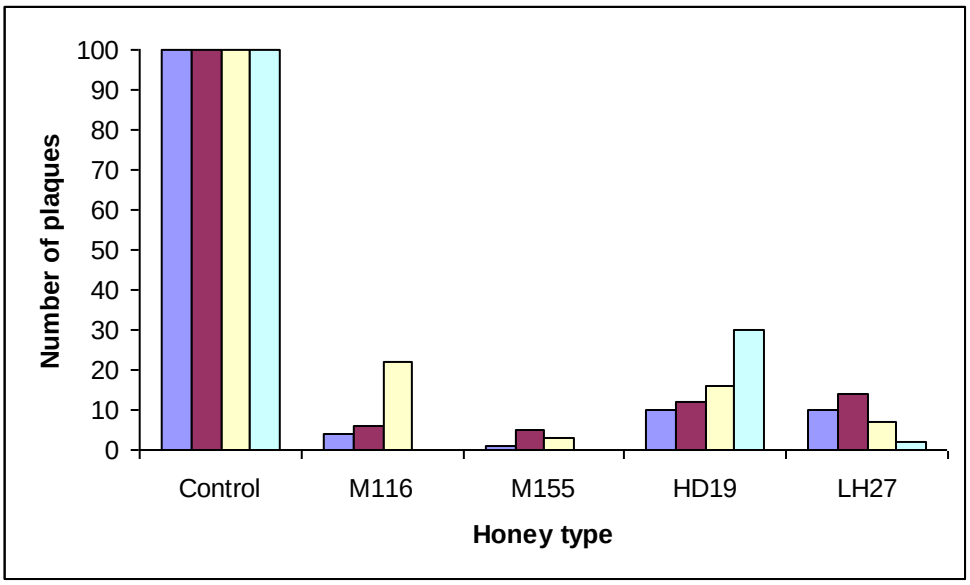
The further experiments using a range of viral dilutions from 10^{-1} to 10^{-4} were unsuccessful due to continual contamination of the honey-agarose overlay and consequent destruction of the monolayer.

Table 8.3 Plaque counts after infection of A549 cells with HSV-2, counted on day 7 after infection

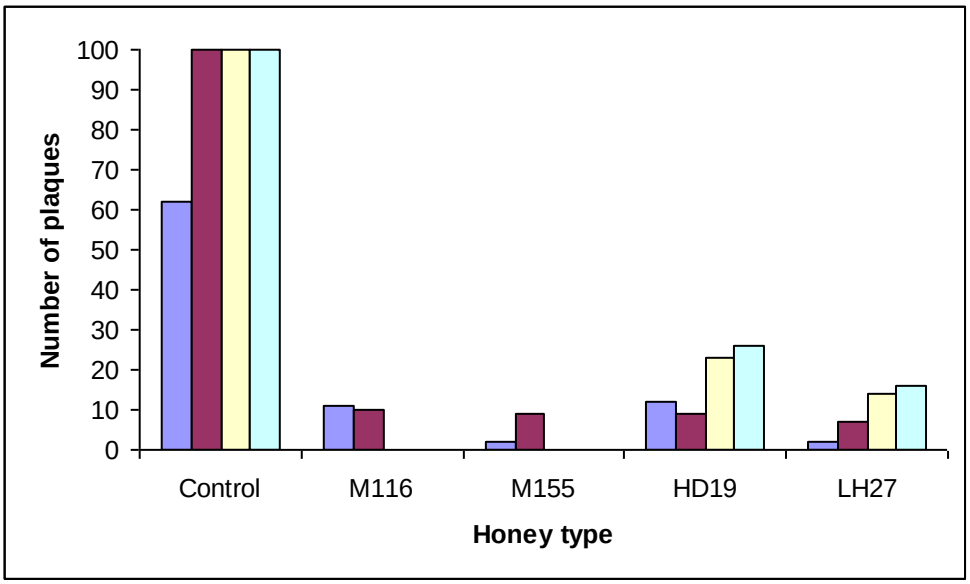
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Replicate 1	Monolayer destroyed	>100 plaques	33 plaques	30 plaques	20 plaques	15 plaques
Replicate 2	Monolayer destroyed	>100 plaques	30 plaques	30 plaques	18 plaques	12 plaques

Figure 8.17 Plaque counts of each replicate after infection of A549 cells with: (a) Ad3, (b) HSV-1, (c) HSV-2, diluted to 10^{-4}, treated with agarose containing various types of honey at final concentrations of 2%, counted on day 7 after infection

(a) Ad3



(b) HSV-1



(c) HSV-2

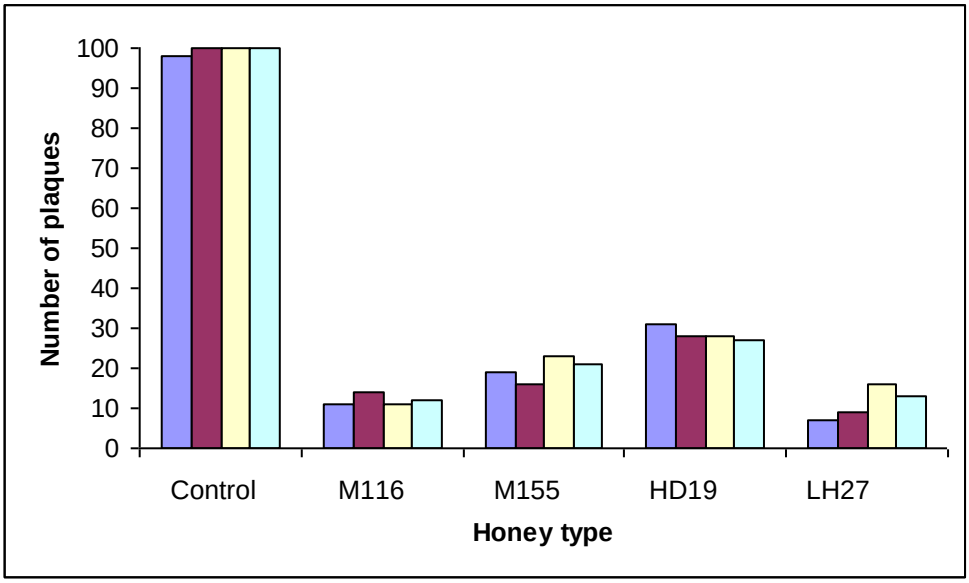

100
90
80
70
60
50
40
30
20
10
0
Number of plaques
Control
M116
M155
HD19
LH27
Honey type

Table 8.4 Plaque count means, standard deviations, coefficients of variation, and % inhibition with each honey (data from Figure 8.17) with (a) Ad3, (b) HSV-1, (c) HSV-2

(a) Ad3

	Control	M116	M155	HD19	LH27
Mean	100	8	2.25	17	8.25
SD	0	9.66	2.22	9.02	5.06
CV	0	120.8	98.7	53.1	61.3
% Inhibition	-	92.0	97.0	83.0	91.7

(b) HSV-1

	Control	M116	M155	HD19	LH27
Mean	90.5	5.25	2.75	17.75	9.75
SD	19	6.07	4.27	8.06	6.45
CV	20.99	115.62	155.27	45.51	66.15
% Inhibition	-	94.2	97.0	80.4	89.2

(c) HSV-2

	Control	M116	M155	HD19	LH27
Mean	99.5	12	19.75	28.5	11.25
SD	1	1.41	2.98	1.73	4.03
CV	1.01	11.75	15.09	6.07	35.82
% Inhibition	-	87.9	80.2	71.4	88.7

8.4.3 Discussion

The results shown in Table 8.3 provide the plaque numbers with the preliminary experiment using HSV-2 only. This experiment aimed to optimise the plaque assay technique, and it was shown that plaques were detectable with each of the viral dilutions.

The subsequent experiments, with the viruses at a dilution of 10^{-4} as used in the previous cell-based experiments, honey was used initially at final concentrations of 5% and 10%. These concentrations were found to destroy the monolayer of cells thus a final concentration of 2% honey was used in the subsequent experiments. The plaque counts with each of the 2% honey treatments are shown in Figure 8.17 (a), (b) and (c). It can be seen that each of the honeys gave a marked reduction in plaque formation and that each of the viruses were susceptible to the honey within the overlay. It was interesting to observe such an effect of Manuka honey M155 given the previous observations indicating little antiviral activity. There is however some variability between numbers counted, which makes the results somewhat unreliable. Nevertheless, the degree of inhibition seen 71.4% to 97.0 % makes it conclusive that the honeys decreased to a large degree the ability of the viral infection to spread from cell to cell.

Chapter 9

The antiviral activity of honey: Study using ELISA

This Chapter describes the work done to investigate the mode of action of the previously successful neutralisation treatments against each virus, using ELISA to detect specific proteins important for viral attachment to the host cell.

9.1 INTRODUCTION

Enzyme-linked immunosorbant assays (ELISA) can be used as a sensitive measure to detect the amount of a specific antigen within a sample. This technique can therefore be used to test for changes to the proteins on a virus caused by a preceding treatment.

By firstly optimising experimental conditions for the detection of antigens on each virus using ELISA, followed by testing the virus after treatment with honey (as in Chapter 6), it is possible to see whether the successful neutralisation treatments have caused specific alterations to the virus.

Observations in earlier chapters have suggested that when honey is used to treat a virus it causes delays of development of severe CPE compared with the untreated virus. This has been shown with each of the cell-based experiments which suggests either a direct effect of the honey on the virus or an effect of the honey on the cells or both. If honey is interacting with viral surface proteins, it would prevent viral attachment to the host cell and therefore prevent viral infection. The trend of reduced CPE related to the increasing concentration of honey and duration of exposure to it observed early after infection with the neutralisation experiments with many of the honey treatments, could be explained by viral surface protein modification. To test whether the honey causes a direct effect on the virus by potentially attaching to and/or causing protein modifications of the viral surface proteins involved in the attachment of the virus to the host cell (see Sections 2.1.4 and 2.2.4) ELISA was used to measure the amount of protein on the viruses before and after treatment with honey.

This method is not limited by the concentration of honey used or qualitative observations. This technique can provide justification of the previous reports of antiviral activity.

9.2 EXPERIMENTS

The antibodies to detect adenovirus and herpes simplex virus surface proteins were obtained from AbD Serotec, Mouse anti-Adenovirus hexon, and Mouse anti-Herpes simplex virus 1 & 2 specific to an epitope within glycoprotein D. The enzyme-linked secondary antibody was also from AdD Serotec, Goat anti-mouse IgG, A, M:HRP (human absorbed).

9.2.1 Determining optimal dilutions of the primary and secondary antibodies for the detection of high concentrations of antigen

9.2.1.1 Methods

One hundred µl of the appropriate antigen (stock virus diluted to 10^{-1} in 0% medium (RPMI 1640, without the addition of FCS)) was used to coat the wells of ELISA modules (NUNC-Immuno modules) these were left overnight at 4ºC. The antigen was removed from the modules and each well was washed once with wash buffer (0.05% Tween 20 in phosphate buffered saline pH 7.4 (PBS)) using a multichannel pipette. Two hundred µl of blocking buffer (1% bovine serum albumin (BSA) from Sigma chemical company prepared in PBS pH 7.4) was added to each well and this was incubated at 37ºC for 30 minutes. After incubation with the blocking buffer, the wells were washed 3 times with wash buffer for 5 minutes and air dried, 100 µl of the primary antibody was prepared in 0.02% sodium azide in PBS pH 7.4 was then added

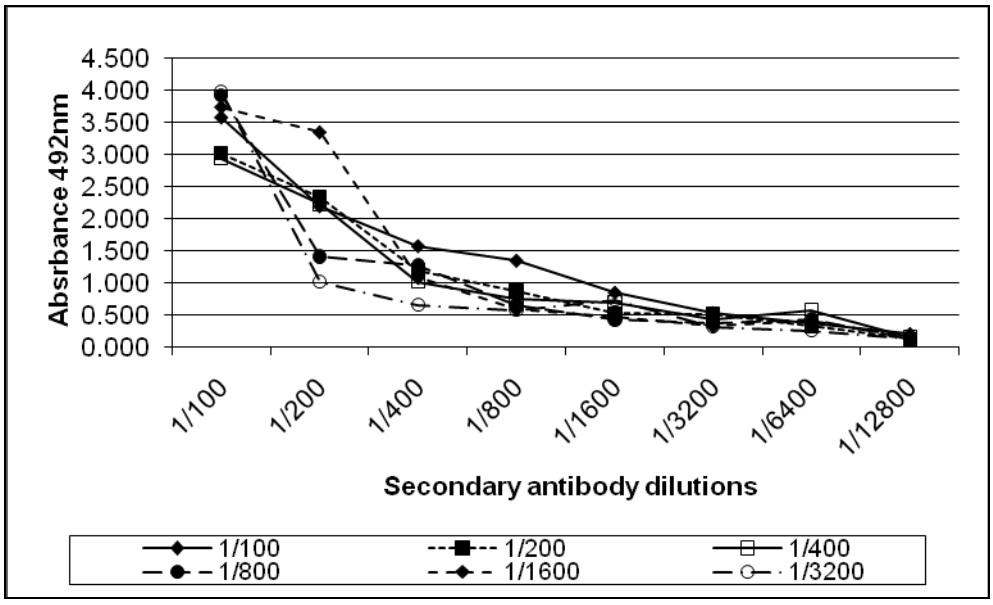
to each well (dilutions from 1/100 to 1/3200 were used: 10 µg/ml, 5 µg/ml, 2.5 µg/ml, 1.25 µg/ml, 0.625 µg/ml, 0.3125 µg/ml) and incubated at 37ºC for 1 hour with gentle shaking. After incubation with primary antibody, the wells were washed 3 times with wash buffer for 5 minutes, and 100 µl of the secondary antibody prepared in PBS was added to each well (dilutions 1/100 to 1/6400: 10 µg/ml, 5 µg/ml, 2.5 µg/ml, 1.25 µg/ml, 0.625 µg/ml, 0.3125 µg/ml, 0.156 µg/ml) and incubated at 37ºC for 1 hour with gentle shaking. After incubation with the secondary antibody, the wells were washed 3 times with wash buffer for 5 minutes and once with distilled water. During the last wash the substrate was prepared by mixing 30 ml of substrate buffer (citric acid prepared with di-sodium hydrogen phosphate pH 5) with a 30 mg Ortho-phenylenediamine dihydrochloride (OPD) tablet from Sigma chemical company, once the tablet had dissolved, 200 µl 6% H_2O_2 was added. One hundred µl of substrate was added to each well and incubated at 37º for 30 minutes in the dark. After incubation, 50 µl 2 mol/L H_2SO_4 was added to each well. The absorbance was then read at 492 nm using a plate reader.

9.2.1.2 Results

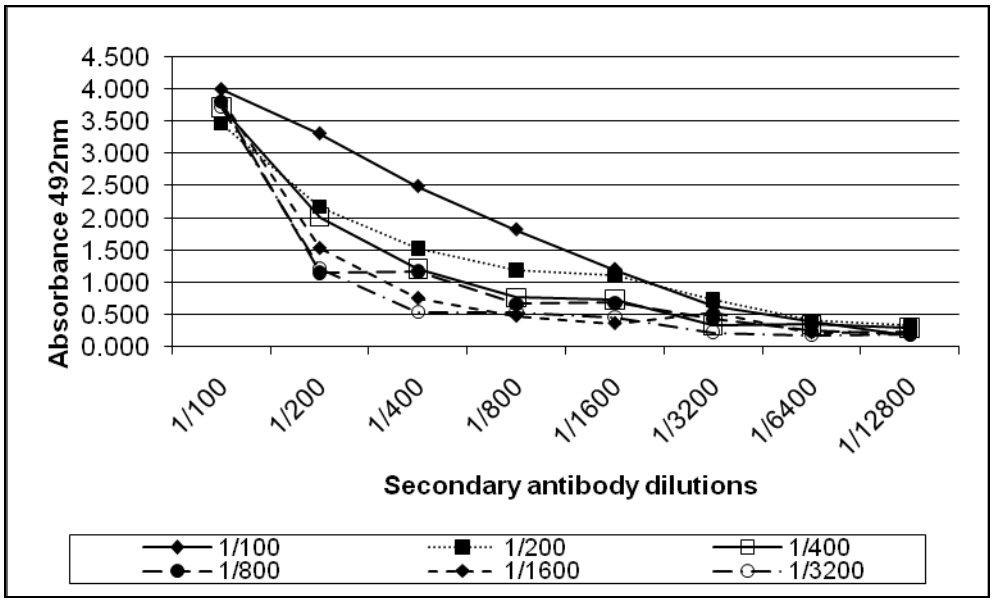
The results of the first experiments are shown in Figures 9.1 to 9.4 as plots of absorbance against secondary antibody dilution. Each plotted line represents a different primary antibody dilution as indicated by the key.

Figure 9.1 ELISA absorbance readings using various dilutions of both the primary and secondary antibodies with: (a) Ad3, (b) HSV-1, (c) HSV-2 at dilutions of 10^{-1}, and (d) no virus

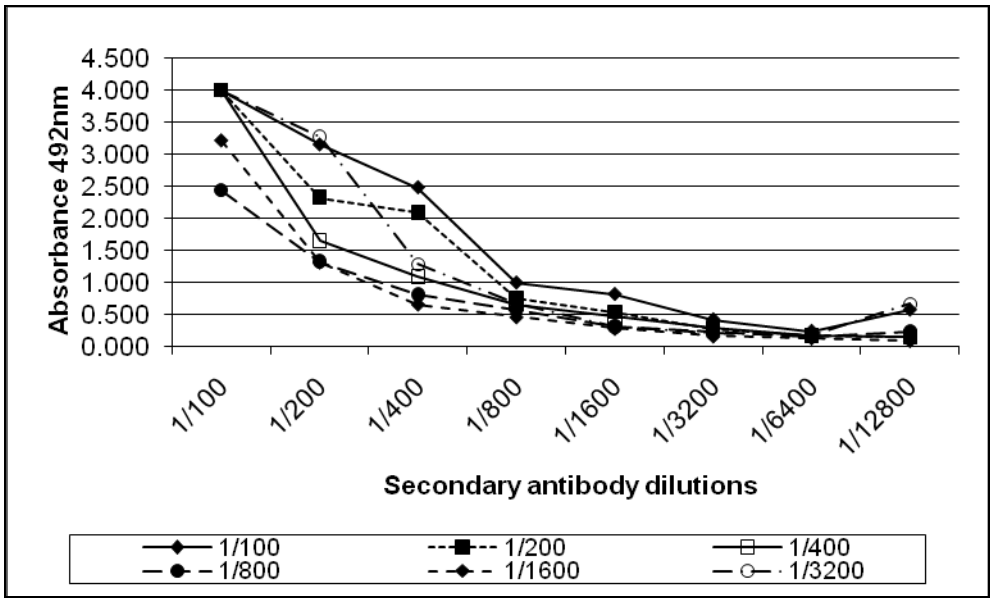
(a) Ad3



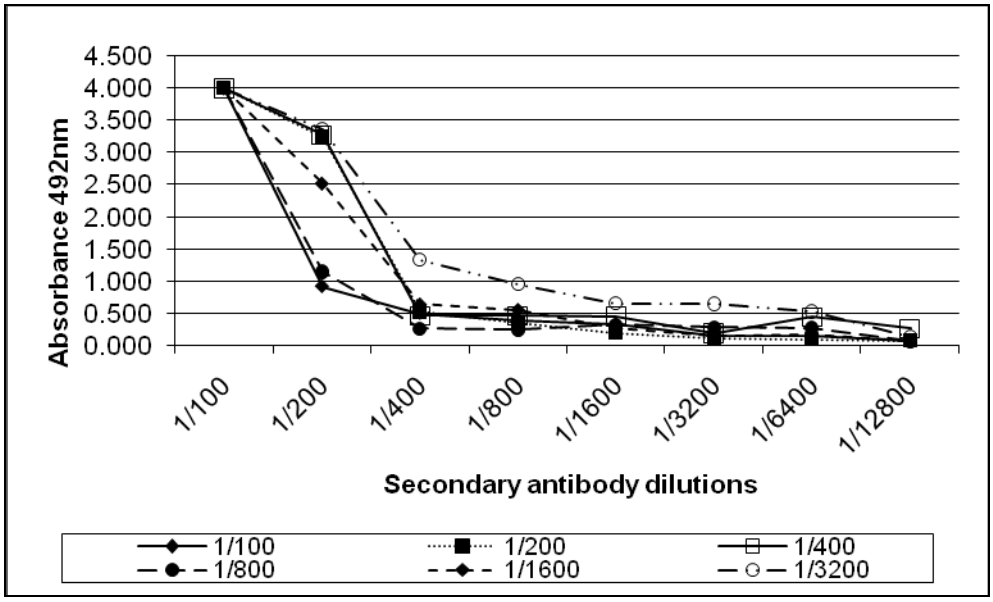
(b) HSV-1



(c) HSV-2



(d) No virus



9.2.1.3 Discussion

This experiment using combinations of primary and secondary antibody dilutions provided an overview of the specificity and absorbance range with a high amount of antigen. It was shown that the antibodies used to detect HSV were binding non-specifically as indicated by the colour development in the absence of antigen in Figure 9.4.

From the absorbance readings from these experiments with different combinations of the primary and secondary antibodies, a selection of dilutions were chosen to be tested with and without the antigen and in the presence and absence of one or both antibodies. From the raw data (used in Figure 9.1), dilutions of the antibodies that gave absorbance readings of about 1.5 were selected. For Ad3, the primary antibody dilutions, 1/100 to 1/1600 with secondary antibody dilution 1/400 gave readings in this range thus 1/100, 1/200, and 1/400 dilutions of the primary antibody were chosen to use with a 1/400 dilution of the secondary antibody. For HSV-1 and HSV-2, 1/400, 1/800, and 1/1600 primary dilutions with 1/200 dilution of the secondary antibody were chosen.

9.2.2 Further examination of antibody dilutions, with increased time of exposure to and concentration of blocking buffer

9.2.2.1 Methods

The method used was as described in Section 9.2.1.1 and was completed in triplicate with the following changes: The incubation with blocking buffer was increased to 1

hour and the concentration of BSA within the blocking buffer was increased to 2% BSA in PBS to reduce the background colour development. For Ad3, primary antibody dilutions 1/100, 1/200, and 1/400 (10 µg/ml, 5 µg/ml, and 2.5 µg/ml) were used with the secondary antibody at 1/400 dilution (1.25 µg/ml).

For Herpes simplex type 1, primary antibody dilutions 1/400, 1/800, and 1/1600 (2.5 µg/ml, 1.25 µg/ml, and 0.625 µg/ml) were used with the secondary antibody at 1/200 dilution (5 µg/ml).

This experiment included test wells with and without the primary and/or secondary antibody to detect non-specific background caused by the antibodies with and without the antigen.

The means, SD, and % CV were calculated for each combination of antigen and antibody dilution.

9.2.2.2 Results

The results using different combinations of antibodies are shown in Tables 9.1 to 9.3. Abbreviations Y (Yes) and N (No) indicate whether the virus or antibody was included in the test combination. If the antibody was included the dilution used has been given.

Table 9.1 Absorbance readings using various dilutions of the primary antibody with one dilution of the secondary antibody, with: (a) Ad3, (b) HSV-1, and (c) HSV-2 at dilutions of 10^{-1}

(a) Ad3

			Absorbance		
Ad3	**Primary antibody dilution**	**Secondary antibody dilution**	**Mean**	**SD**	**% CV**
Y	1/100	1/400	1.930	0.024	1.2
Y	1/200	1/400	1.691	0.076	4.5
Y	1/400	1/400	1.247	0.040	3.2
N	1/100	1/400	1.871	1.119	59.8
N	1/200	1/400	0.895	0.387	43.2
N	1/400	1/400	0.437	0.097	22.2
Y	1/100	N	0.059	0.019	32.6
Y	1/200	N	0.057	0.004	6.3
Y	1/400	N	0.049	0.007	14.4
N	1/100	N	0.051	0.002	4.2
N	1/200	N	0.051	0.008	16.6
N	1/400	N	0.053	0.004	6.7
Y	N	1/400	0.374	0.093	25.0
N	N	1/400	0.072	0.002	3.0
Y	N	N	0.040	0.003	7.1
N	N	N	0.043	0.004	9.9

(b) HSV-1

			Absorbance		
HSV-1	**Primary antibody dilution**	**Secondary antibody dilution**	**Mean**	**SD**	**% CV**
Y	1/400	1/200	1.177	0.410	34.8
Y	1/800	1/200	1.215	0.124	10.2
Y	1/1600	1/200	1.090	0.505	46.3
N	1/400	1/200	0.209	0.142	67.8
N	1/800	1/200	0.185	0.047	25.2
N	1/1600	1/200	0.429	0.401	93.6
Y	1/400	N	0.065	0.006	8.7
Y	1/800	N	0.065	0.014	21.8
Y	1/1600	N	0.073	0.016	22.4
N	1/400	N	0.063	0.006	10.2
N	1/800	N	0.064	0.009	14.5
N	1/1600	N	0.061	0.007	11.6
Y	N	1/200	0.186	0.032	17.2
N	N	1/200	0.161	0.004	2.6
Y	N	N	0.048	0.002	4.5
N	N	N	0.046	0.003	6.2

(c) HSV-2

			Absorbance		
HSV-2	**Primary antibody dilution**	**Secondary antibody dilution**	**Mean**	**SD**	**% CV**
Y	1/400	1/200	0.672	0.333	49.6
Y	1/800	1/200	0.799	0.307	38.4
Y	1/1600	1/200	0.898	0.268	29.8
N	1/400	1/200	0.227	0.159	70.3
N	1/800	1/200	0.243	0.163	67.1
N	1/1600	1/200	0.467	0.164	35.2
Y	1/400	N	0.067	0.038	56.7
Y	1/800	N	0.063	0.040	63.9
Y	1/1600	N	0.062	0.042	68.2
N	1/400	N	0.059	0.030	50.2
N	1/800	N	0.059	0.032	55.0
N	1/1600	N	0.056	0.036	63.6
Y	N	1/200	0.158	0.044	28.1
N	N	1/200	0.122	0.040	33.1
Y	N	N	0.059	0.010	16.2
N	N	N	0.055	0.015	27.2

9.2.2.3 Discussion

Table 9.1 shows the results using the dilutions of antibody found to be suitable in Section 9.2.1 with each virus at a dilution of 10^{-1}. It is apparent that the increased incubation period and increased concentration of BSA in the blocking buffer lowered the non-specific binding seen with the absence of the antigen. When the primary antibody was excluded from the test combination with the antigen present, moderate readings were obtained suggesting that the secondary antibody was non-specifically binding to the wells.

9.2.3 Repeats of specific combinations with or without antigen/antibodies

9.2.3.1 Methods

The method used was as described in Section 9.2.1.1 with the modifications outlined in Section 9.2.2.1. In addition, the substrate incubation period was reduced to 10 minutes due to rapid colour development. Combinations tested were for Ad3, with and without antigen, and without the antigen including the primary antibody, and for HSV-1, with and without antigen, and with and without antigen excluding the primary antibody, each completed in triplicate.

9.2.3.2 Results

The results repeating specific combinations of the antibodies are shown in Tables 9.4 to 9.5. Abbreviations Y (Yes) and N (No) indicate whether the virus or antibody was included in the test combination. If the antibody was included the dilution used has been given.

Table 9.2 Absorbance readings using various dilutions of the primary antibody and one dilution of the secondary antibody, with: (a) Ad3 and (b) at dilutions of 10^{-1}

(a) Ad3

			Absorbance		
Ad3	**Primary antibody dilution**	**Secondary antibody dilution**	**Mean**	**SD**	**% CV**
Y	1/100	1/400	1.856	0.028	1.5
Y	1/200	1/400	1.387	0.457	33.0
Y	1/400	1/400	0.838	0.348	41.6
N	1/100	1/400	1.853	0.424	22.9
N	1/200	1/400	0.701	0.072	10.2
N	1/400	1/400	0.391	0.066	16.9
Y	N	1/400	0.193	0.002	1.1

(b) HSV-1

			Absorbance		
HSV-1	**Primary antibody dilution**	**Secondary antibody dilution**	**Mean**	**SD**	**% CV**
Y	1/400	1/200	0.660	0.098	14.8
Y	1/800	1/200	0.545	0.134	24.6
Y	1/1600	1/200	0.397	0.163	41.2
N	1/400	1/200	0.152	0.012	8.0
N	1/800	1/200	0.104	0.010	9.6
N	1/1600	1/200	0.103	0.012	11.7
Y	N	1/200	0.121	0.004	4.1
N	N	1/200	0.081	0.006	8.0

9.2.4 Measurement of antigen on serially diluted virus using previously successful dilutions of each antibody

9.2.4.1 Methods

Using the method described in Section 9.2.1.1 with the modifications outlined in Sections 9.2.2.1, and 9.2.3.1, for Ad3, a 1/100 dilution (10 µg/ml) of the primary antibody was used with a 1/400 dilution (2.5 µg/ml) of the secondary antibody to detect the antigens on the virus diluted from 10^{-1} to 10^{-4}. For HSV-1, a 1/400 dilution (2.5 µg/ml) of the primary antibody was used with a 1/200 dilution (5 µg/ml) of the secondary antibody to detect the antigens on the virus diluted from 10^{-1} to 10^{-4}. Each combination was done in triplicate. In addition, skim milk powder was added to the blocking buffer at a concentration of 5% (to make the composition of the blocking buffer 5% skim milk powder, 2% BSA in PBS) to reduce the background colour development.

In addition to the means, SD, and % CV the equation of the trend line and R^2 value were calculated using Excel.

9.2.4.2 Results

The results are presented in Figures 9.2 to 9.3 and Tables 9.3 to 9.4.

Figure 9.2 Absorbance readings of Ad3 serially diluted from 10^{-1} to 10^{-4} with a 1/100 dilution of the primary antibody and a 1/400 dilution of the secondary antibody

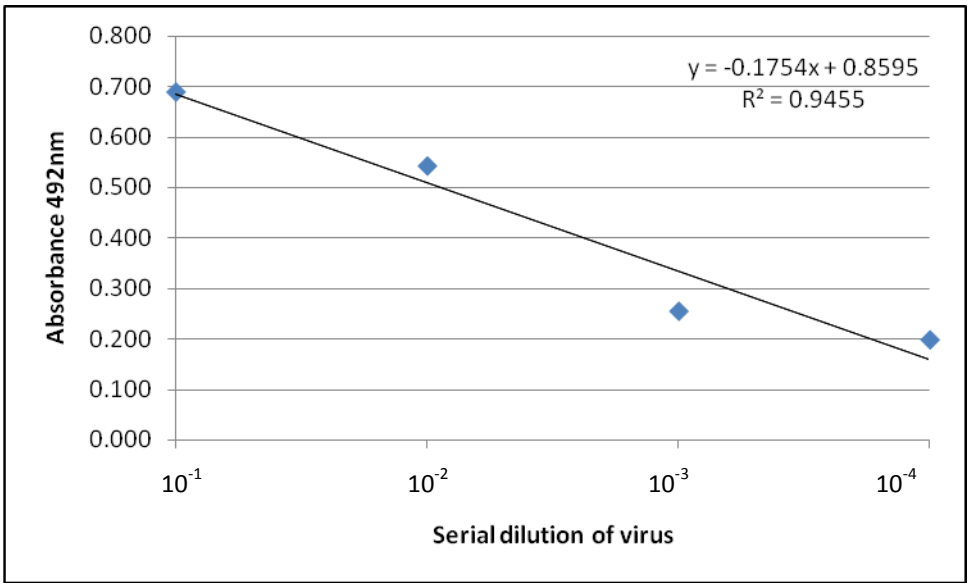


Table 9.3 Absorbance means, SD, and % CV of the data shown in Figure 9.2

Ad3 dilution	Mean	SD	% CV
10^{-1}	0.688	0.074	10.7
10^{-2}	0.542	0.074	13.7
10^{-3}	0.256	0.013	5.3
10^{-4}	0.199	0.029	14.3
N	0.187	0.040	21.4
N	0.193	0.028	14.7
N	0.183	0.011	5.8
N	0.128	0.009	6.8

Figure 9.3 Absorbance readings of serially diluted HSV-1 with a 1/400 dilution of the primary antibody and a 1/200 dilution of the secondary antibody

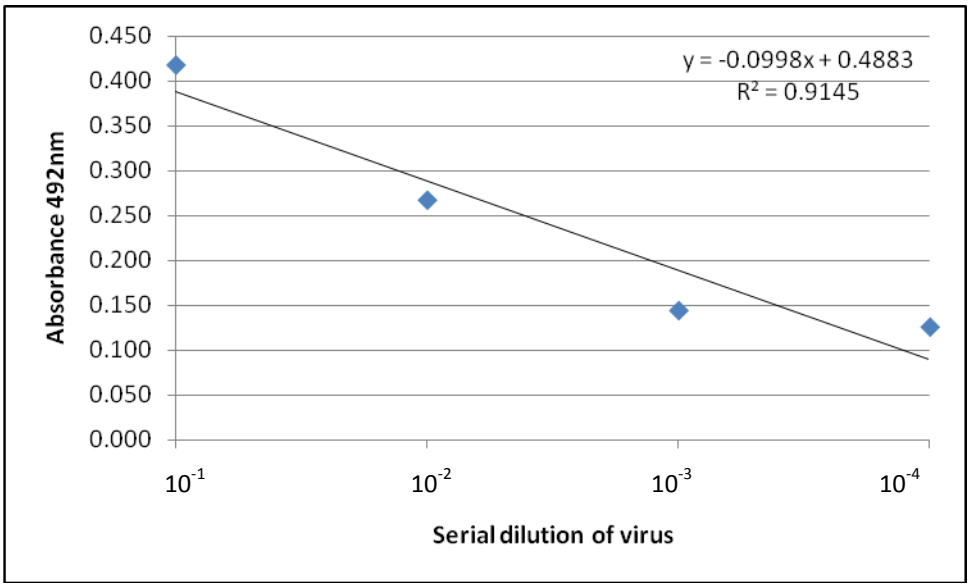


Table 9.4 Absorbance means, SD, and % CV of the data shown in Figure 9.3

HSV-1 dilution	Mean	SD	% CV
10^{-1}	0.418	0.019	4.6
10^{-2}	0.267	0.018	7.0
10^{-3}	0.144	0.014	9.8
10^{-4}	0.126	0.003	2.4
N	0.128	0.006	4.3
N	0.126	0.017	13.5
N	0.139	0.029	20.7
N	0.122	0.010	8.4

9.2.4.3 Discussion

In these experiments 5% skim milk powder was added to the blocking buffer to try to reduce the background colour development. It was shown that the dilutions of the

antibodies that were used previously to detect high amounts of antigen were not able to give very high absorbance readings when the virus was diluted to 10^{-4}. Even with the virus diluted to 10^{-1} as used previously, lower absorbance readings were observed in this experiment than seen before using the same dilution.

9.2.5 Measurement of antigens on serially diluted virus using higher concentrations of each antibody

9.2.5.1 Methods

The method used was as described in Sections 9.2.1.1, 9.2.2.1, and 9.2.3.1, however, the period of incubation with blocking buffer was increased to overnight at 4°C. With Ad3, 1/100 and 1/200 dilutions (10 µg/ml and 5 µg/ml) of the primary antibody were used, with 1/100 and 1/200 dilutions (10 µg/ml and 5 µg/ml) of the secondary antibody. With HSV-1 and HSV-2, 1/400 and 1/800 dilutions (2.5 µg/ml and 1.125 µg/ml) of the primary antibody were used, with 1/100 and 1/200 dilutions (10 µg/ml and 5 µg/ml) of the secondary antibody. Each combination was completed in triplicate.

9.2.5.2 Results

The results are presented in Table 9.5.

Table 9.5 Absorbance readings using serially diluted (a) Ad3, (b) HSV-1, and (c) HSV-2, with higher concentrations of primary and secondary antibodies

(a) Ad3

			Absorbance		
Ad3 dilution	**Primary antibody dilution**	**Secondary antibody dilution**	**Mean**	**SD**	**% CV**
10^{-1}	1/100	1/100	2.813	0.102	3.6
10^{-2}	1/100	1/100	1.484	0.105	7.1
10^{-3}	1/100	1/100	0.471	0.011	2.4
10^{-4}	1/100	1/100	0.312	0.008	2.5
10^{-1}	1/100	1/200	1.594	0.006	0.4
10^{-2}	1/100	1/200	1.255	0.076	6.2
10^{-3}	1/100	1/200	0.664	0.035	5.3
10^{-4}	1/100	1/200	0.642	0.028	4.3
10^{-1}	1/200	1/100	1.455	0.323	22.2
10^{-2}	1/200	1/100	0.785	0.042	5.4
10^{-3}	1/200	1/100	0.438	0.013	3.0
10^{-4}	1/200	1/100	0.316	0.008	2.7
10^{-1}	1/200	1/200	1.204	0.119	9.9
10^{-2}	1/200	1/200	1.008	0.125	12.4
10^{-3}	1/200	1/200	0.516	0.067	12.9
10^{-4}	1/200	1/200	0.427	0.083	19.5

(b) HSV-1

			Absorbance		
HSV-1 dilution	**Primary antibody dilution**	**Secondary antibody dilution**	**Mean**	**SD**	**% CV**
10^{-1}	1/400	1/100	0.512	0.033	6.4
10^{-2}	1/400	1/100	0.292	0.019	6.5
10^{-3}	1/400	1/100	0.178	0.045	25.5
10^{-4}	1/400	1/100	0.095	0.006	6.3
10^{-1}	1/400	1/200	0.381	0.023	6.0
10^{-2}	1/400	1/200	0.265	0.014	5.2
10^{-3}	1/400	1/200	0.129	0.004	3.1
10^{-4}	1/400	1/200	0.104	0.015	14.4
10^{-1}	1/800	1/100	0.427	0.025	6.0
10^{-2}	1/800	1/100	0.324	0.010	3.2
10^{-3}	1/800	1/100	0.207	0.022	10.4
10^{-4}	1/800	1/100	0.121	0.008	6.5
10^{-1}	1/800	1/200	0.354	0.070	19.9
10^{-2}	1/800	1/200	0.211	0.011	5.3
10^{-3}	1/800	1/200	0.118	0.013	11.1
10^{-4}	1/800	1/200	0.086	0.001	1.3

(c) HSV-2

			Absorbance		
HSV-2 dilution	**Primary antibody dilution**	**Secondary antibody dilution**	**Mean**	**SD**	**% CV**
10^{-1}	1/400	1/100	0.397	0.031	7.8
10^{-2}	1/400	1/100	0.268	0.023	8.6
10^{-3}	1/400	1/100	0.136	0.012	8.8
10^{-4}	1/400	1/100	0.097	0.007	7.3
10^{-1}	1/400	1/200	0.377	0.059	15.6
10^{-2}	1/400	1/200	0.243	0.033	13.6
10^{-3}	1/400	1/200	0.138	0.020	14.1
10^{-4}	1/400	1/200	0.115	0.007	6.1
10^{-1}	1/800	1/100	0.315	0.042	13.4
10^{-2}	1/800	1/100	0.223	0.040	18.1
10^{-3}	1/800	1/100	0.142	0.027	19.1
10^{-4}	1/800	1/100	0.117	0.021	18.1
10^{-1}	1/800	1/200	0.236	0.017	7.1
10^{-2}	1/800	1/200	0.160	0.027	17.1
10^{-3}	1/800	1/200	0.122	0.002	1.2
10^{-4}	1/800	1/200	0.122	0.027	22.0

9.2.5.3 Discussion

With the successful neutralisation treatments that were to be tested, the virus was used at a dilution of 10^{-4} thus higher concentrations of antibodies needed to be used to detect this lower level of antigen. The experiment described in Section 9.2.4 was repeated using higher antibody concentrations and increased incubation with blocking buffer (overnight at 4°C). Higher absorbance readings were observed with Ad3 however, with HSV-1 and HSV-2 the absorbance readings remained quite low.

9.2.6 First experiments with honey

9.2.6.1 Methods

Using the method described in Section 9.2.1.1, with the modifications outlined in Section 9.2.4.1 and 9.2.5.1 (changes to blocking buffer), the previously successful neutralisation treatments at each exposure period found in Chapter 6, Table 6.2, plus the associated controls of untreated virus, medium only (used to prepare the viral solution), and honey solutions without virus, were used to coat the wells overnight at 4°C. For Ad3, 1/100 of the primary antibody was used with 1/200 of the secondary antibody, and for HSV-1 and HSV-2, a 1/400 dilution of the primary antibody was used, with a 1/200 dilution of the secondary antibody. Each of the combinations was completed in triplicate. The means, SD, and % CV were calculated as well as the final absorbance as [mean absorbance of the treatment] – [mean absorbance of the appropriate control].

9.2.6.2 Results

The results are presented in Table 9.6. The type, concentration of honey, and time of exposure are shown in column 1 of each Table.

Table 9.6 Effect of honey on the detection of antigens of (a) Ad3, (b) HSV-1, and (c) HSV-2 absorbance readings with virus treated and not treated with honey

(a) Ad3

Treatment	Mean	SD	% CV	Final absorbance
Medium only	0.322	0.011	3.272	
Ad3 only	0.393	0.031	7.771	0.071
Honey solutions				
10% M116	1.860	0.146	7.831	
10% M157	1.092	0.215	19.736	
10% HD19	2.065	0.315	15.252	
5% HD19	0.572	0.046	7.986	
Honey plus virus				
Ad3, 10% M116, 8 hour	0.412	0.018	4.429	-1.448
Ad3, 10% M157, 8 hour	0.255	0.039	15.189	-0.837
Ad3, 10% HD19, 8 hour	0.591	0.072	12.186	-1.474
Ad3, 5% HD19, 8 hour	0.317	0.062	19.681	-0.255

(b) HSV-1

Treatment	Mean	SD	% CV	Final absorbance
Medium only	0.130	0.012	8.9	
HSV-1 only	0.176	0.001	0.4	0.046
HSV-1 (4H) only	0.131	0.001	0.5	0.001
Honey solutions				
10% M116	0.247	0.037	15.0	
5% M116	0.247	0.027	10.8	
10% M112	0.382	0.047	12.4	
10% M157	0.276	0.036	13.0	
10% R19/06	0.350	0.047	13.3	
5% R19/06	0.397	0.119	30.0	
10% R19/06 (4H)	0.480	0.129	26.8	
Honey plus virus				
HSV-1 10% M116 8H	0.262	0.015	5.7	0.015
HSV-1 5% M116 8H	0.276	0.024	8.7	0.029
HSV-1 10% M112 8H	0.446	0.024	5.3	0.064
HSV-1 10% M157 8H	0.244	0.009	3.5	-0.032
HSV-1 10% R19/06 8H	0.315	0.036	11.3	-0.035
HSV-1 5% R19/06 8H	0.299	0.015	4.9	-0.098
HSV-1 10% R19/06 4H	0.361	0.006	1.5	-0.119

(c) HSV-2

Treatment	Mean	SD	% CV
Medium only	0.174	0.042	24.1
HSV-2 only	0.178	0.036	20.3
HSV-2 (4H) only	0.195	0.027	13.6
HSV-2 (2H) only	0.160	0.018	11.3
Honey solutions			
10% M116	0.320	0.052	16.2
10% M112	0.570	0.060	10.5
10 % M112 (4H)	0.711	0.067	9.5
10 % M112 (2H)	0.346	0.051	14.7
10% HD19	0.617	0.038	6.1
10% LH27	0.513	0.084	16.5
5% LH27	0.357	0.101	28.3
1% LH27	0.252	0.075	29.6
2% LH27	0.106	0.012	11.2
2.5% LH27	0.154	0.021	13.5
10% LH27 (4H)	0.192	0.008	4.1
5% LH27 (4H)	0.158	0.011	7.2
Honey plus virus			
HSV-2 10% M116 8H	0.249	0.029	11.7
HSV-2 10% M112 8H	0.373	0.005	1.3
HSV-2 10 % M112 4H	0.642	0.024	3.7
HSV-2 10 % M112 2H	0.828	0.030	3.6
HSV-2 10% HD19 8H	0.560	0.072	12.8
HSV-2 10% LH27 8H	0.498	0.049	9.9
HSV-2 5% LH27 8H	0.324	0.052	16.1
HSV-2 1% LH27 8H	0.249	0.063	25.4
HSV-2 2% LH27 8H	0.159	0.019	11.729
HSV-2 2.5% LH27 8H	0.147	0.008	5.772
HSV-2 10% LH27 4H	0.262	0.087	33.088
HSV-2 5% LH27 4H	0.278	0.049	17.7

9.2.6.3 Discussion

These experiments testing each of the viruses that had been subjected to successful neutralisation treatments, with each virus the honey solutions that did not contain antigen caused higher absorbance readings than the untreated virus, and with the

medium only (used in preparation of the virus) high absorbance readings were recorded which caused very low final absorbance results. This further suggested non-specific binding.

9.2.7 Further optimisation to detect antigens on viruses treated with honey

9.2.7.1 Methods

The method used was the similar to that outlined in Sections 9.2.1.1, with the modifications described in Sections 9.2.4.1 and 9.2.5.1. However, the wash step between the coating and blocking was omitted and the antibodies were prepared in growth medium (that contained 10% FCS) to try to reduce the non-specific binding. Virus diluted to 10^{-4} was used to coat the wells, as well as the honey solutions that had previously caused the greatest absorbance readings with each virus. The primary antibody was diluted to 1/100 (10 µg/ml) and used with secondary antibody diluted to 1/500 (2 µg/ml). This experiment was completed with six replicates.

9.2.7.2 Results

The results are presented in Table 9.7. The first column gives the virus, and type and concentration of honey.

Table 9.7 Absorbance readings with the antibodies prepared in growth medium, with Ad3, HSV-1, HSV-2, and honey solutions

Treatment	Mean	SD	% CV
Ad3 only	0.212	0.018	8.6
10% M157	0.094	0.010	11.1
HSV-1 only	0.258	0.020	7.9
10% R19/06	0.099	0.018	17.8
HSV-2 only	0.246	0.021	8.7
10% M112	0.091	0.015	16.9

9.2.7.3 Discussion

To overcome the non-specific colour development, the solutions used in the preparation of the antibody dilutions were changed to growth medium which provided high amounts of protein that could competitively bind and so eliminate the non-specific binding. This experiment was undertaken to test each virus at a dilution of 10^{-4} and the honey solutions that had previously caused high absorbance readings (shown in Table 9.6). A 1/200 dilution of the primary antibody was used with a 1/500 dilution of the secondary antibody, due to the use of a higher protein content solution to prepare the antibodies higher dilutions of each antibody should give suitable absorbance readings. The results in Table 9.7 clearly show detectable absorbance levels with each of the viruses, and with the honey solutions low absorbance readings indicating a reduction of the non-specific binding.

9.2.8 Further experiments with honey

9.2.8.1 Methods

Using the method described in Section 9.2.7 with the associated modifications, the successful neutralisation treatments of Chapter 6 plus the honey solutions were re-tested in triplicate.

9.2.8.2 Results

The results are presented in Table 9.8 and 9.9. The first column indicates the virus, type and concentration of honey, and the time of exposure. The final absorbance is given as the [mean absorbance of the treatment] – [mean absorbance of the appropriate control].

Table 9.8 Effect of honey on the detection of antigens of (a) Ad3, (b) HSV-1, and (c) HSV-2, absorbance readings with virus treated and not treated with honey

(a) Ad3

Treatment	Mean	SD	% CV	Final absorbance
Medium only	0.105	0.001	1.3	
Ad3 only	0.151	0.006	4.2	0.046
Honey solutions				
10% M116	0.123	0.023	19.0	
10% M157	0.051	0.009	18.2	
10% HD19	0.157	0.033	21.2	
5% HD19	0.127	0.024	18.9	
Honey plus virus				
Ad3 10% M116 8H	0.080	0.002	2.2	-0.043
Ad3 10% M157 8H	0.136	0.005	3.7	0.085
Ad3 10% HD19 8H	0.110	0.047	42.7	-0.047
Ad3 5% HD19 8H	0.142	0.023	16.1	0.015

(b) HSV-1

Treatment	Mean	SD	% CV	Final absorbance
Medium only	0.106	0.013	12.2	
HSV-1 only	0.116	0.012	10.5	0.010
HSV-1 (4H) only	0.127	0.018	13.8	0.021
Honey solutions				
10% M116	0.063	0.005	7.3	
5% M116	0.110	0.025	22.7	
10% M112	0.139	0.009	6.2	
10% M157	0.101	0.014	13.6	
10% R19/06	0.121	0.017	13.7	
5% R19/06	0.131	0.021	16.2	
10% R19/06 (4H)	0.117	0.025	21.6	
Honey plus virus				
HSV-1 10% M116 8H	0.109	0.007	6.8	0.046
HSV-1 5% M116 8H	0.067	0.011	15.8	-0.043
HSV-1 10% M112 8H	0.092	0.034	37.2	-0.047
HSV-1 10% M157 8H	0.110	0.030	27.6	0.009
HSV-1 10% R19/06 8H	0.098	0.031	32.1	-0.023
HSV-1 5% R19/06 8H	0.108	0.023	21.2	-0.023
HSV-1 10% R19/06 4H	0.100	0.014	14.0	-0.017

(c) HSV-2

Treatment	Mean	SD	% CV	Final absorbance
Medium only	0.080	0.023	29.3	
HSV-2 only	0.083	0.025	29.9	0.003
HSV-2 (4H) only	0.070	0.002	3.0	-0.01
HSV-2 (2H) only	0.066	0.002	2.6	-0.014
Honey solutions				
10% M116	0.088	0.031	35.8	
10% M112	0.061	0.011	17.1	
10 % M112 (4H)	0.098	0.019	19.1	
10 % M112 (2H)	0.066	0.006	8.4	
10% HD19	0.068	0.011	16.2	
10% LH27	0.078	0.005	6.8	
5% LH27	0.092	0.024	26.4	
1% LH27	0.095	0.008	8.4	
2% LH27	0.088	0.011	12.7	
2.5% LH27	0.063	0.018	27.7	
10% LH27 (4H)	0.060	0.012	20.5	
5% LH27 (4H)	0.067	0.017	25.3	
Honey plus virus				
HSV-2 10% M116 8H	0.097	0.022	22.3	0.009
HSV-2 10% M112 8H	0.089	0.022	24.6	0.028
HSV-2 10 % M112 4H	0.096	0.014	14.0	-0.002
HSV-2 10 % M112 2H	0.075	0.017	22.6	0.009
HSV-2 10% HD19 8H	0.120	0.022	18.5	0.052
HSV-2 10% LH27 8H	0.089	0.017	19.2	0.011
HSV-2 5% LH27 8H	0.114	0.060	52.5	0.022
HSV-2 1% LH27 8H	0.088	0.027	30.4	-0.007
HSV-2 2% LH27 8H	0.083	0.003	3.4	-0.005
HSV-2 2.5% LH27 8H	0.094	0.017	17.9	0.031
HSV-2 10% LH27 4H	0.067	0.000	0.0	0.007
HSV-2 5% LH27 4H	0.092	0.007	7.7	0.025

Table 9.9 Effect of honey on the detection of antigens of (a) HSV-1 and (b) HSV-2 absorbance readings with virus treated and not treated with honey (repeats)

(a) HSV-1

Treatment	Mean	SD	% CV
HSV-1 only	0.061	0.012	18.9
Honey plus virus			
HSV-1 10% M116 8H	0.047	0.004	8.5
HSV-1 5% M116 8H	0.053	0.014	26.2
HSV-1 10% M112 8H	0.044	0.003	5.7
HSV-1 10% M157 8H	0.037	0.003	7.2
HSV-1 10% R19/06 8H	0.065	0.024	36.2
HSV-1 5% R19/06 8H	0.052	0.013	25.8
HSV-1 10% R19/06 4H	0.061	0.012	19.0

(b) HSV-2

Treatment	Mean	SD	% CV
Medium only	0.039	0.004	10.6
HSV-2 only	0.060	0.006	9.4
HSV-2 (4H) only	0.051	0.015	29.3
HSV-2 (2H) only	0.042	0.007	17.0
Honey plus virus			
HSV-2 10% M116 8H	0.047	0.004	8.5
HSV-2 10% M112 8H	0.033	0.009	28.4
HSV-2 10 % M112 4H	0.037	0.002	4.2
HSV-2 10 % M112 2H	0.042	0.011	26.4
HSV-2 10% HD19 8H	0.051	0.010	19.9
HSV-2 10% LH27 8H	0.047	0.003	5.4
HSV-2 5% LH27 8H	0.042	0.007	16.6
HSV-2 1% LH27 8H	0.036	0.002	6.4
HSV-2 2% LH27 8H	0.057	0.012	21.2
HSV-2 2.5% LH27 8H	0.034	0.005	14.9
HSV-2 10% LH27 4H	0.051	0.015	29.6
HSV-2 5% LH27 4H	0.046	0.008	17.3

9.2.8.3 Discussion

The experiments with honey used 1/200 and 1/500 dilutions of the antibodies prepared in growth medium shown in Tables 9.8 and 9.9 caused low absorbance readings with the controls that did not contain antigen. However, as with the first experiments with honey (Table 9.6) low absorbance readings were obtained with each of the viruses that were not treated with honey. These low readings could be explained by the heating at 37°C during the neutralisation period which as a consequence may have caused alterations to the epitope of the surface protein thus making it undetectable by these antibodies. Table 9.7 shows that the virus at a dilution of 10^{-4} can be detected when it had been freshly prepared prior to coating the plates, and in the neutralisation experiments, the untreated virus caused a CPE suggesting that there was virus present after the neutralisation period at 37°C. The successful honey treatments against HSV-1 and HSV-2 were re-tested without the control honey solutions to see whether similar absorbance readings would be recorded, again low absorbance readings were observed as shown in Table 9.9.

Due to the very low absorbance readings, comparisons between the final absorbance results of the untreated virus and the honey-treated virus are inconclusive as to whether the honey or the heating is responsible for the resulting absorbance readings, and it has not shown whether the honey has caused alterations to the surface proteins.

This investigation using ELISA has shown that this technique with additional optimisation may be feasible for the detection of proteins after treatment with honey.

Chapter 10

Discussion and further work

The aim of the present study was to extend knowledge of the sensitivity of viral species to whole honey. This chapter is a final discussion of the effect each type of honey had against the viruses tested in this thesis. The observations are compared with work done by other researchers, and suggestions of the possible modes of action are outlined as well as recommendations for further work. The benefits of honey over current therapy are also discussed.

10.1 SUMMARY OF THE IMPACT THE HONEYS HAD ON THE VIRUSES

The preliminary investigations using Manuka honey M157 in Chapter 5 provided some evidence that the honey was working both on or within the uninfected and infected cells, and directly on the virus. It was shown further by results of Chapter 6 a direct effect of the honeys at various concentrations on the prevention of CPE development after infection of the cells with each virus. On determining if the successful neutralisation treatments were virustatic or virucidal it was shown that the previously successful treatments using Manuka honey against HSV-1 and HSV-2 were virucidal, as were the Manuka honey treatments against Ad3 with the exception of M112. The other honeys that had been successful in the neutralisation experiments were shown to have virustatic activity, as there was development of viral infection at some stage over the observation period. as there was development of viral infection at some stage over the observation period. This suggested an effect of the high levels of methylglyoxal present in the Manuka honeys as Manuka honey M151 (very low activity levels) was unsuccessful in causing an antiviral effect. The experiments testing methylglyoxal at equivalent concentrations to what was present in each of the honeys showed that it was not solely responsible for the antiviral activity.

In the experiments studying the prevention of viral infection, Manuka honey M116 and Honeydew honey HD19 were the most successful in inhibiting the spread of virus. It was shown that when the honey was used to treat virally infected cells that it can delay the development of CPE when the infected cells are mixed with uninfected cells. The plaque reduction assays confirmed that honey

can decrease the spread of infection from infected cells to uninfected cells by 71.4% to 97.0%. The final experiments using ELISA in this study failed to indicate whether the viral surface proteins were being modified by the preceding honey treatment. It is therefore unknown whether honey acts in this way.

10.2 COMPARISONS WITH PREVIOUS RESREACH

The Manuka or pasture honey used in previous investigations by French (2002) to test the antiviral activity of honey against HSV-1 were indicated to be close to the median antibacterial activity for each type of antibacterial activity (peroxide/non-peroxide), equivalent to 17.5% (w/v) phenol for peroxide activity with no detectable non-peroxide activity for the pasture honey, and the Manuka honey used had a non-peroxide activity equivalent to 16.8% (w/v) phenol.

French's (2002) results suggested neutralisation of HSV-1, which was defined as the absence of subsequent viral infection (CPE) after treatment of the virus with 10% Manuka honey. Likewise neutralisation was suggested with one replicate of 10% pasture honey and with all replicates after treatment of the virus with 15% pasture honey. The period of exposure of the virus to honey in French's investigation was 1 hour followed by dialysis. The table of results given did not give details of how long after inoculation of the cells the observations of viral CPE were made or whether they were all taken at the same time. Despite this, results are comparable with these in the present study as HSV-1, although examined under slightly different methodology, was neutralised by 10% Manuka honey. Similarly, Rewarewa honey (which has antibacterial activity due to

hydrogen peroxide) was shown to cause neutralisation at 10% in the present study, like one replicate of the pasture honey did.

When comparing the current results with those of the study undertaken by Zeina et al. (1996) it was apparent that far lower concentrations of honey were able to eliminate Rubella infection than found in the present study against Adenovirus or Herpes virus. In the study of Zeina concentrations of honey from 0.001% to 50% were found to cause absence of viral infection and retained the viability of the vero cells over the course of infection (four days). This suggests that Rubella virus was particularly susceptible to the honey treatments, and that the vero cell line can withstand very high concentrations of honey for a prolonged period compared with the A549 cells in the present study which were seen to withstand no more than 2% honey. There is a possibility that the vero cells were rendered non-viable by the osmolarity (hence infection could not develop) but that the monolayer remained intact so that the non-viability was not detected. The method of how the viability was tested for was not provided.

The current study has verified the study undertaken by Al-Waili (2004) that tested the topical application of raw honey versus acyclovir for the treatment of recurrent herpes lesions. It has been now shown *in vitro* that the clinical observations could have been due to an effect of honey on the virus and not just on the symptoms of infection.

From the results of previous investigations and those found in the current study, the antiviral activity has been shown to be a property of more than one type of honey. Although the efficacy differs within and between honeys of various floral sources against the different viral isolates, in each case the magnitude of viral infection is usually reduced with a treatment of honey. Manuka honey was found to have the greatest effect against all three types of virus studied, where the other honeys seemed to be slightly more specific with the virus they acted most successfully upon. There is potential then for other dsDNA viruses to be susceptible to treatment with honey.

10.3 POSSIBLE MODES OF ACTION

The preliminary experiments and further testing of the antiviral activity of a range of honeys has shown in each of the different experiments that honey had an effect on the development of viral CPE. This suggests that the honey acts on the cells, on the surface or within, as well as directly on the virus in the absence of the cells.

As discussed in Chapter 5, the honey maybe entering the cell and working against the virus during its replication, it may be causing an antiviral response, or it maybe coating cellular receptors preventing the attachment of the virus. It may be thought that the toxic nature of honey may be slowing cellular growth eliminating the optimal environment for viral infection. However, when considering the evidence for honey increasing the rate of cell growth in wound healing (as reviewed by Molan, 2006) it is unlikely. The delay rather than prevention of CPE can suggest a possible loss of the inhibition factor in the honey through chemical

reaction or metabolism over the observation period, although it is also possible that it is due to the progress of viral infection being only partially inhibited and thus slowed.

The results of Chapter 6 indicate that honey at the appropriate concentrations (see Table 6.2) can neutralise the virus and so prevent it from displaying CPE within susceptible cells. The period of which the cells were exposed to the viral-honey inoculum was 15 minutes followed by replacement of the inoculum with maintenance medium. It is reasoned that during this short period that the honey would have had very little effect on the cells and with the removal of the inoculum any remaining honey would have been greatly diluted to well below inhibitory levels.

By the delays observed in the onset of viral infection the honey may be preventing the spread of infection from cell to cell. This could have been due to the honey blocking viral or cellular receptors, or by slowing the replication and release of viral particles. A further possibility is that the honey causes the induction of antiviral cytokines in the cells.

With the experimental conditions testing the protective and neutralisation effects of honey, the virus may have bound to and entered an original cell during the inoculation period, then taken a prolonged period to replicate or release viral particles to infect surrounding cells due to the preceding honey treatment (on the cells or on the virus).

The honey could be bound in some way to the cell surface where it may not only be blocking the appropriate cellular receptors, but may be modifying them in some way and so preventing viral attachment. Ad3 attaches to coxsachie-adenovirus receptors (CARs), CD80 and CD86 cellular proteins, and integrins. HSV-1 and HSV-2 attach to glycosaminoglycans (GAGs) and interact with nectins, herpes virus mediator, and specific forms of heparin sulphate (3-OS HS). Honey may enter the cell and alter viral entry and transportation mechanisms to the nucleus.

Alternatively, the honey may cause alterations to the viral particle preventing successful attachment and penetration into the susceptible cell, thus the infection observed after inoculation was due to those viral particles that were not altered by the honey. This fits with the reduction of CPE related to the concentration of honey and time of exposure to it, as in effect a proportion of the viral particles would have been removed. Experiments were undertaken to try to find, by ELISA immunochemistry, if there was binding to the virus but unfortunately the results were inconclusive because of practical problems.

It is possible that the morphology of the viral particle was not altered by the honey treatment but that the honey had some affect on the viral life cycle within the cell, such as damage to the DNA therefore preventing the expression of genes essential to viral replication, particle formation, and viral release (egress). Due to the sequential manner in which these viruses are expressed (see Chapter 2), damage to the DNA at any stage would prevent viral release and so would prevent further

infection of other cells. This possibility again fits with the reduction of CPE with increasing concentration of honey and time exposed to it, as greater DNA damage due to prolonged exposure may have a greater effect than damage over a short period, similarly the infection observed may be due to those viral particles that escaped the effects of the honey.

These possibilities may be being caused by methylglyoxal present in high levels in especially Manuka honeys that may be binding to DNA or amino groups on the viruses or on the cellular receptor proteins. The hydrogen peroxide responsible for the antibacterial activity of non-Manuka honeys may oxidatively modify viral or cellular proteins, and the antioxidants may prevent oxidative activation of NFκB.

10.4 RECOMMENDATIONS FOR FURTHER WORK

In order to identify the component(s) within honey that are responsible for the antiviral action, the honey could be fractionated and the fractions then tested separately and in combination against the viruses. It is also important to address the modifications that may be occurring to the host cell receptors suggested by experiments which showed protection and prevention of viral infection. This could be done by optimising the ELISA methodology. PCR and real time PCR may be further used to see if honey alters the gene expression of either the virus or the cells and so determine the impact of honey of the replication of the virus. Also suitable for further investigation is the effect of honey on cellular pathways such as NFκB crucial for viral replication known to be effected by methylglyoxal

which is found within honey. The NFκB pathway is also possibly affected by honey antioxidants.

It would be of therapeutic benefit to test the honeys *in vivo* against the infections caused by these viruses (cold sores, genital infection, and eye infections) to compare the healing with the conventional treatments. The results of this study do not show whether honey will work clinically, but do justify the running of clinical trials. Further, cells on the surface of living tissue have circulation that can replace osmotically withdrawn water they can therefore withstand 100% honey unlike the A549 cells used in this investigation, it is likely that when honey is used at this concentration *in vivo* that it would be effective against these infections.

10.5 POTENTIAL BENEFITS OF HONEY OVER CURRENT THERAPY

Cidofovir as discussed in Chapter 2 can have toxic effects and is ineffective against some viral isolates due to resistance (Kinchington et al., 2005). For this reason honey is more likely to be a better therapeutic option for the treatment of Adenoviral infections if clinical trials prove the effectiveness indicated by the *in vitro* results in the present study. It is feasible to use honey for eye infections as honey has been used in numerous cases to treat ailments of the eyes (Fotidar & Fotidar, 1945; Imperato & Traore, 1969; Molan, 1999). No allergic reactions (Kiistala, et al., 1995) or other reactions have been recorded other than a transient stinging described by some patients with honey (Betts & Molan 2001).

With the current therapy for herpes infections, acyclovir, resistance is possible through mutations of thymidine kinase and HSV DNA polymerase rendering these treatments inactive. Honey therefore is a promising treatment for herpes infections as with this study it was found to inhibit or delay viral infection *in vitro*.

Due to the observations that honey when used topically to treat wounds accelerates healing such as with the healing of chronic leg ulcers, post-operative wounds, and burns (Molan, 2001; Molan, 2006), it has been reasoned that honey may help combat viral infections of both eye infections and Herpes lesions. This stimulatory effect of honey may help with the repair of tissue damage caused by the viral infection.

The evidence of an immunostimulatory action of honey (Tonks, 2003; 2007) would augment the direct antiviral action and so can contribute to the body's own defences to clear the virus and heal the infection within a short period. The antibacterial activity of honey in addition will provide protection from secondary infections commonly associated with infections.

The anti-inflammatory activity will help reduce the pain associated with the viral infections. Honey has been shown to reduce odema associated with wounds through a direct action and not a secondary result of removal of infection (as reviewed by Molan, 2006).

In conclusion this thesis has confirmed the antiviral activity of honey against HSV and extended the scope of susceptible viral species. It has been confirmed that the action of honey observed previously in clinical trials which could have been an effect on the symptoms, actually could have been a direct antiviral action. It has been shown that honey has an antiviral action on other dsDNA viruses as well.

References

Adam, E., Kaufman, R. H., & Mirkovic, R. R. (1979). Persistence of virus shedding in asymptomatic women after recovery from herpes genitalis. *Obstetrics and Gynecology, 54*, 171-173.

Adams, C. J., Boult, C. H., Deadman, B. J., Farr, J. M., Grainger, M. N. C., Manley-Harris, M., et al. (2008). Isolation by HPLC and characterisation of the bioactive fraction of New Zealand manuka (Leptospermum scoparium) honey. *Carbohydrate Research 343*(4), 651-659.

Adcock, D. (1962). The effect of catalase on the inhibine and peroxide values of various honeys. *Apicultural Research, 1*, 38-40.

Akusjarvi, G., & Stevenin, J. (2003). Remodelling of the host cell RNA splicing machinery during an adenovirus infection. *Current Topics in Micorbiological Immunology, 272*, 243-286.

Allen, K. L., Molan , P. C., & Reid, G. M. (1991). A survey of the antibacterial activity of some New Zealand honeys. *Journal of Pharmacy and Pharmocology, 43*(12), 817-822.

Alvine, J. C., Steinhart, W. L., & Hill, C. W. (1974). Transcription of herpes simplex type 1 DNA in nuclei isolated from infected Hep-2 and KB cells. *Virology, 60*, 302-307.

Al-Waili, N. S. (2003). Intrapulmonary administration of natural honey solution, hyperosmolaar dextrose or hypoosmolar distill water to normal individuals and to patients with type-2 diabetes mellitus or hypertension: their effects on blood glucose level, plasma insulin and C-peptide, blood pressure and peaked expiratory flow rate. *European Journal of Medical Research, 8,* 295-303.

Al-Waili, N. S. (2004). Topical honey application vs. acyclovir for the treatment of recurrent herpes simplex lesions. *Medical Science Monitor, 10*(8), 94-98.

Al-Waili, N. S., & Saloom, K. Y. (1999). Effects of topical honey on post-operative wound infections due to gram positive and gram negative bacteria following caesarean sections and hysterectomies. *European Journal of Medical Research, 4*(3), 126-130.

Amelio, A. L., Giordani, N. V., & Kubat, N. J. (2006). Deacetylation of the herpes simplex virus type 1 latency-associated transcript (LAT) enhancer and a decrease in LAT abundance precede an increase in ICP0 transcriptional permissiveness at early times postexplant. *Journal of Virology, 80,* 2063-2068.

Amici, C., Belardo, G., & Rossi, A. (2001). Activation of IkB kinase by herpes simplex virus type 1: a novel target for anti-herpetic therapy. *Journal of Biological Chemistry, 276*, 28759-28766.

Ankra-Badu, G. A. (1992). Sickle cell leg ulcers in Ghana. *East African Medical Journal, 69*(7), 366-369.

Aristotle. (350 BC). *Historia Animalium*. Oxford, UK: Oxford University.

Arnberg, N., Edlund, K., & Kidd, A. H. (2000). Adenovirus type 37 uses sialic acid as a cellular receptor. *Journal of Virology, 74*, 42-48.

Badger, G. F., Curtiss, C., & Dingle, J. H. (1956). A study of illness in a group of Cleveland families. *American Journal of Hygiene, 64*, 336-348.

Bagchi, S., Raychaudhuri, P., & Nevins, J. R. (1990). Adenovirus E1A proteins can dissociate heteromeric complexes involving the E2F transcription factor; a novel mechanism for E1A trans-activation. *Cell, 62*, 659-669.

Baltrusaityte. V., Venskutonis, P.R., & Ceksteryte, V. (2007). Radical scavenging activity of different floral origin honey and beebread phenolic extracts. *Food Chemistry, 101(2),* 502-514.

Baringer, J. R., & Swoveland, P. (1973). Recovery of herpes simplex virus infection from human trigeminal ganglions. *New England Medical, 288,* 648-650.

Bastian, F. O., Rabson, A. S., & Yee, C. L. (1972). Herpesvirus hominins: Isolation from human trigeminal ganglion. *Science, 178,* 306.

Batterson, W., & Roizman, B. (1983). Characterisation of the herpes simplex virion-associated factor responsible for the induction of alpha genes. *Journal of Virology, 46*, 371-377.

Batterson, W., Furlong, D., & Roizman, B. (1983). Molecular genetics of herpes simplex virus. VIII. Further characterization of a temperature-sensitive mutant defective in release of viral DNA and in other stages of viral reproductive cycle. *Journal of Virology, 45*, 397-407.

Beck, B. F., & Smedley, D. (1944). *Honey and your health* (2nd ed.). New York: McBride.

Beltz, G. A., & Flint, S. J. (1979). Inhibition of HeLa cell protein synthesis during adenovirus infection. Restriction of cellular messenger RNA sequences to the nucleus. *Journal of Molecular Biology, 131*, 353-373.

Benedetti, J., Corey, L., & Ashley, R. (1994). Recurrence rates in genital herpes after symptomatic first-episode infection. *Annals of Internal Medicine, 121*, 847-854.

Bergelson, J., Cunningham, J. A., & Droguett, G. (1997). Isolation of a common receptor for coxsackie B viruses and adenoviruses 2 and 5. *Science, 275*, 1320-1323.

Berk, A. J., Lee, F., & Harrison, T. (1979). Pre-early adenovirus 5 gene product regulates synthesis of early viral messenger RNAs. *Cell, 17*, 935-944.

Betts, J. A., & Molan , P. C. (2001). *A pilot trial of honey as a wound dressing has shown the importance of the way honey is applied to wounds*. Paper presented at the 11th Conference of the European Wound Management Association.

Binder, P. S. (1977). Herpes Simplex Keratitis. *Survey of Ophthalmology, 21*, 313-331.

Bogdanov, S. (1984). Characterisation of antibacterial substances in honey. *Lebensm Wiss Technology, 17*(2), 74-76.

Booy, F. P., Newcomb, W. W., & Trus, B. L. (1991). Liquid-crystalline, phage-like packing of encapsidated DNA in herpes simplex virus. *Cell, 64*(5), 1007-1015.

Bose, B. (1982). Honey or sugar in the treatment of infected wounds? *Lancet, 1*, 963.

Boyer, J. L., & Ketner, G. (2000). Genetic analysis of the adenovirus E4 34k protein. *Journal of Biological Chemistry, 275*, 14969-14978.

Brady, N. F., Molan , P. C., & Hartfoot, C. G. (1997). The sensitivity of dermatophytes to the antimicrobial activity of manuka honey and other honey. *Pharmaceutical Science, 2*, 1-3.

Brandt, C. D., Kim, H. W., & Vargosko, A. J. (1969). Infections in 18,000 infants and children in a controlled study of respiratory tract disease. I. Adenovirus pathogenicity in relation to serologic type and illness syndrome. *American Journal of Epidemiology*, *90*, 484-500.

Bunting, C. M. (2001). *The production of hydrogen peroxide by honey and its relevance to wound healing.* University of Waikato, Hamilton.

Campadelli-Fiume, G., Cocchi, F., & Menotti, L. (2000). The novel receptors that mediate the entry of herpes simplex viruses and animal alphaherpesviruses into cells. *Reviews in Medical Virology, 10*, 305-319.

Carson, C. T., Schwartz, R. A., & Stracker, T. H. (2003). The Mre11 complex is required for ATM activation and the G2/M checkpoint. *Embo Journal, 22*, 6610-6620.

Carter, K. A., & Roizman, B. (1996). The promoter and transcriptional unit of a novel herpes simplex virus 1 alpha gene are contained in, and encode a protein in frame with, the open reading frame of the alpha 22 gene. *Journal of Virology, 70*, 172-178.

Cassady, K. A., Gross, M., & Roizman, B. (1998). The herpes simplex virus US11 protein effectively compensates for the gamma1 (34.5) gene if present before activation of protein kinase R by precluding its phosphorylation and that of the alpha subunit of eukaryotic translation initiation factor 2. *Journal of Virology, 72*, 8620-8626.

Castro-Vazquez, L., Diaz-Maroto, M.C, Gonzales-Vinas, M.A., & Perez-Coello, M.S. (2009). Differentiation of monofloral citris, rosemary, eucalyptus, lavender, thyme and heather honeys based on volatile composition and sensory descriptive analysis. *Food Chemistry, 112, 1022-1030.*

Cathomen, T., & Weitzman, M. D. (2000). A functional complex of adenovirus proteins E1B-55kDa and E4orf6 is necessary to modulate the expression level of p53 but not its transcriptional activity. *Journal of Virology, 74*, 11407-11412.

Challberg, M. D. (1986). A method for identifying the viral genes required for herpesvirus DNA replication. *PNAS, 83*, 9094-9098.

Cheshenko, N., Del Rosario, B., & Woda, C. V. (2003). Herpes simplex virus triggers activation of calcium-signalling pathways. *Journal of Cellular Biology, 163*, 283-293.

Chirife, J., Herszage, L., Joseph, A., & Kohn, E. S. (1983). in vitro study of bacterial growth inhibition in concentrated sugar solutions: microbiological basis for the use of sugar in treating infected wounds. *Antimicrobial Agents and Chemotherapy, 23*(5), 766-773.

Clontech. (2008). *Transcriptional map of Adenovirus.* Retrieved April 21, 2008, from www.clonetech.com/upload/images/tools/adeno/inages/adeno5.gif

Cocchi, F., Menotti, L., & Dubreuil, P. (2000). Cell-to-cell spread of wild-type herpes simplex virus type 1, but not syncytial strains, is mediated by the immunoglobulin-like receptors that mediate virion entry, nectin 1 (PRR/HveC/HIgR) and nectin 2 (PRR2/HveB). *Journal of Virology, 74*, 3909-3917.

Condon, R. E. (1993). Curious interaction of bugs and bees. *Surgery, 113*(2), 234-235.

Conley, A. J., Knipe, D. M., & Jones, P. C. (1981). Molecular genetics of herpes simplex virus. VII. Characterisation of a temperature-sensitive mutant produced by in vitro mutagenesis and defective in DNA synthesis and accumulation of gamma polypeptides. *Journal of Virology, 37*, 1282-1295.

Cooper, R. A., Molan , P. C., & Harding, K. G. (1999). Antibacterial activity of honey against strains of Staphlococcus aureus from infected wounds. *Journal of the Royal Society of Medicine, 92*(6), 283-285.

Cooper, R. J., Hallett, R., & Tullo, A. B. (2000). The epidemiology of adenovirus infections in Greater Manchester, UK 1982-96. *Epidemiology and Infection, 125*, 333-345.

Costanzo, F., Campadelli-Fiume, G., & Foa-Tomasi, L. (1977). Evidence that herpes simplex virus DNA is transcribed by cellular RNA polymerase B. *Journal of Virology, 21*, 996-1001.

Cress, W. D., & Nevins, J. R. (1994). Interacting domains of E2F1, DP1, and the adenovirus E4 protein. *Journal of Virology, 68*, 4213-4219.

Cuesta, R., Xi, Q., & Schneider, R. J. (2000). Adenovirus-specific translation by displacement of kinase Mnk1 from cap-inhibition complex eIF4F. *Embo Journal, 19*, 3465-3474.

Davison, A. J., Benko, M., & Harrach, B. (2003). Genetic content and evolution of adenoviruses. *Journal of General Virology, 84*, 2895-2908.

De Clercq, E., & Holy, A. (2005). Acrylic nucleoside phosphonates: a key class of antiviral drugs. *Nature Reviews Drug Discovery, 4*, 928-940.

De Jong, J. C., Wermenbol, A. G., & Verweij-Uijterwaal, M. W. (1999). adenoviruses f0rom human immunodeficiency virus-infected individuals, including two strains that represent new candidate serotypes Ad50 and Ad51 of species B1 and D, respectively. *Journal of Clinical Microbiology, 37*, 3940-3945.

Debbas, M., & White, E. (1993). Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. *Genes and Development, 7*, 546-554.

Devi-Rao, G. B., Bloom, D. C., & Stevens, J. G. (1994). Herpes simplex virus type 1 DNA replication and gene expression during explant-induced reactivation of latency infected murine sensory ganglia. *Journal of Virology, 68*, 1271-1282.

Dunford, C., Cooper, R. A., & Molan , P. C. (2000b). Using honey as a dressing for infected skin lesions. *Nursing Times, 96*(14 suppl), 7-9.

Dunford, C., Cooper, R., White, R. J., & Molan , P. C. (2000a). The use of honey in wound management. *Nursing Standard, 15*(11), 63-68.

Dustmann, J. H. (1979). Antibacterial effect of honey. *Apiacta, 14*(1), 7-11.

Egyud, L. G., & Szent-Gyorgyi, A. (1966). The effect of heat on the structure of foot-and-mouth disease virus and the viral ribonucleic acid. *Biochemica et Biophysica Acta, 55*, 388-393.

Enders, J. F., Bell, J. A., & Dingle, J. H. (1956). Adenoviruses: group name proposed for new respiratory-tract viruses. *Science, 124*, 119-120.

Epstein, M. A. (1962). Observations on the mode of release of herpes virus from infected HeLa cells. *Journal of Cellular Biology*, *12*, 589-597.

Esclatine, A., Taddeo, B., & Evans, L. (2004). The herpes simplex virus 1 UL41 gene-dependent destabilization of cellular RNAs is selective and may be sequence-specific. *PNAS, 78*, 8582-8592.

Evans, R. M., Fraser, N., & Ziff, E. (1977). The initiation sites for RNA transcription in Ad2 DNA. *Cell, 12*, 733-739.

Fleming, D. T., McQuillian, G. M., & Johnson, R. E. (1997). Herpes simplex virus type 2 in the United States, 1976 to 1994. *New England Medical, 337*, 1105-1111.

Fotidar, M. R., & Fotidar, S. N. (1945). 'Lotus' honey. *Indian Bee Journal 7*(102).

Fox, J. P., Brandt, C. D., & Wassermann, F. E. (1969). The virus watch program: a continuing surveillance of viral infections in metropolitan New York families. VI. Observations of adenovirus infections: virus excretion patterns, antibody response, efficiency of surveillance, patterns of infections, and relation to illness. *American Journal of Epidemiology, 89*, 25-50.

Frankel, S., Robinson, G. E., & Berenbaum, M. R. (1998). Antioxidant capacity and correlated characteristics of 14 unifloral honeys. *Journal of Apicultural Research, 37*(1), 27-20.

French, V. M. (2002). *Investigating the Sensitivty of Medically Important Micro-organisms to the Antimicrobial Activity of Honey.* University of Waikato, Hamilton.

Friedman, J. M., & Horwitz, M. S. (2002). Inhibition of tumour necrosis factor alpha-induced NF-kappa B activation by the adenovirus E3-10.4/14.5K complex. *Journal of Virology, 76*, 5515-5521.

Furlong, D., Swift, H., & Roizman, B. (1972). Arrangement of herpes-deoxyribonucleic acid in the core. *Journal of Virology, 10,* 1071-1074.

Geraghty, R. J., Krummenacher, C., & Cohen, G. H. (1998). Entry of alpha-herpesviruses mediated by poliovirus receptor-related protein 1 and poliovirus receptor. *Science, 280,* 1618-1620.

Ghizatullina, N. K. (1976). Effect of methylglyoxyl on infectivity and antigenicity of foot and mouth disease virus. *Acta Virologica, 20,* 380-386.

Gilmore, T. D., & Mosialos, G. (2003). Viruses as Intruders in the Rel/NF-kB Signaling Pathway. In R. Beyaert (Ed.), *Nuclear Factor kB Regulation and Role in Disease*: Springer.

Goodkin, M. L., Ting, A. T., & Blaho, J. A. (2003). NF-kappaB is required for apoptosis prevention during herpes simplex virus type 1 infection. *Journal of Virology, 77*, 7261-7280.

Greber, U. F., Webster, P., & Weber, J. (1996). The role of the adenovirus protease on virus entry into cells. *Embo Journal, 15*, 1766-1777.

Gregory, D., Hargett, D., & Holmes, D. (2004). Efficient replication by herpes simplex virus type 1 involves activation of the IkB kinase-IkB-p65 pathway. *Journal of Virology, 78*, 13582-13590.

Gu, H., Liang, Y., Mandel, G., & Roizman, B. (2005). Components of the REST/CoREST/histone deacetylase repressor complex are disrupted, modified, and translocated in HSV-1-infected cells. *PNAS, 102*, 7571-7576.

Guilfoyle, R., & Weinmann, R. (1981). Control region for adenovirus VA RNA transcription. *PNAS, 78*, 3378-3382.

Gunther, R. T. (1934). *The Greek Herbal of Dioscorides*. New York: Hafner.

Gustafsson, D. J., Segerman, A., & Lindman, K. (2006). The Arg279Glu substitution in the adenovirus type 11p (Ad11p) fiber knob abolishes EDTA-resistant binding to A549 and CHO-CD46 cells, converting the phenotype to that of Ad7p. *Journal of Virology, 80*, 1897-1905.

Harada, J. N., Shevchenko, A., & Pallas, D. C. (2002). Analysis of the adenovirus E1B-55K-anchored proteome reveals its link to ubiquination machinery. *Journal of Virology, 76*, 9194-9206.

Harvey, S. A., Romanowski, E. G., & Yates, K. A. (2005). Adenovirus-directed ocular innate immunity: the role of conjunctival defensin-like chemokines (IP-10, I-TAC) and phagocytic human defensin-alpha. *Investigative Ophthalmology and Visual Science, 46*, 3657-3665.

Henle, T., Mavric, E., Wittmann, S., & Barth, G. (2008). Identification and quantification of methylglyoxal as the dominant antibacterial constituent of Manuka (Leptospermum scoparium) honeys from New Zealand. *Molecular Nutrition and Food Research, 52*, 1-7.

Herold, B. C., WuDunn, D., & Soltys, N. (1991). Glycoprotein C of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells in infectivity. *Journal of Virology, 65*, 1090-1098.

Hierholzer, J. C., Stone, Y. O., & Broderson, J. R. (1991). Antigenic relationships among the 47 human adenoviruses determined in reference horse antisera. *Archives of Virology, 121*, 179-197.

Hilleman, M. R., & Werner, J. H. (1954). Recovery of new agent from pateints with acute respiratory illness. *Proceedings of the Society for Experimental Biology and Medicine, 85*, 183-188.

Huebner, R. J., Rowe, W. P., & Ward, T. G. (1954). Adenoidal-pharyngoconjunctival agents. *New England Medical 251*, 1077-1086.

Imperato, P. J., & Traore. (1969). Traditional beliefs about measles and its treatment among the Bambara of Mali. *Tropical and Geographical Medicine, 21*, 62-67.

Jones, N., & Shenk, T. E. (1979). An adenovirus type 5 early gene function regulates expression of other early viral genes. *PNAS, 76,* 3665-3669.

Kasel, J. A. (1979). Adenoviruses. In E. H. Lennette & N. J. Schmidt (Eds.), *Diagnostic Procedures for Viral, Rickettsial and Chlamydial Infections* (pp. 229-256). Washington, DC: American Public Health Association.

Keast-Butler, J. (1980). Honey for necrotic malignant breast ulcers. *Lancet, 2,* 809.

Kiistala, R., Hannuksela, M., Makinen-Kiljunen, S., Niinimaki, A., & Haahtela, T. (1995). Honey allergy is rare in patients sensitive to pollens. *Allergy, 50*(10), 844-847.

Kinchington, P. R., Romanowski, E. G., & Jerold, G. Y. (2005). Prospects for adenovirus antivirals. *Journal of Antimicrobial Chemotherapy, 55,* 424-429.

Kitajewski, J., Schneider, R. J., & Safer, B. (1986). Adenovirus VA1 RNA antagonises the antiviral action of interferon by preventing activation of the interferon-induced eIF-2 alpha kinase. *Cell, 45,* 195-200.

Knipe, D. M., Batterson, W., & Nosal, C. (1981). Molecular genetic of herpes simplex virus. VI. Characterisation of a temperature-sensitive mutant defective in the expression of all early viral gene products. *Journal of Virology, 38*, 539-547.

Knipe, D. M., Howley, P. M., Griffin, D. E., Lamb, R. A., Martin, M. A., Roizman, B., et al. (2007). *Field's Virology* (5 ed. Vol. 2): Wolters Kluwer, Lippincott Williams & Wilkins.

Kosz-Vnenchak, M., Jacobson, J., & Coen, D. M. (1993). Evidence of a novel regulatory pathway for herpes simplex virus gene expression in trigeminal ganglion neurons. *Journal of Virology, 67*, 5383 -5393.

Kristenson, K., Lycke, E., & Roytta, M. (1986). Neuritic transport of herpes simplex virus in rat sensory neurons in vitro. Effects of substances interacting with microtubular functional and axonal flow [nocodazole, taxol and erythro-9-3-(2-hydroxynonyl)adenine]. *Journal of General Virology, 67*, 2023-2028.

Lafferty, W. E., Coombs, R. W., & Benedetti, J. (1987). Recurrences after oral and genital herpes simplex virus infection. Influence of site of infection and viral type. *New England Medical, 316*, 1444-1449.

Laga, M., Cottyn, A., Van Herreweghe, F., Vanden Berghe, W., Haegeman, G., Van Oostveldt, P., et al. (2007). Methylglyoxal suppresses TNF-a-induced NF-kB activation by inhibiting NFkB DNA-binding *Biochemical Pharmacology, 74*, 579-589.

Lechner, R. L., & Kelly, J. T. J. (1977). The structure of replicating adenovirus 2 DNA molecules. *Cell, 12*, 1007-1020.

Lesokhin, A. M., Delgado-Lopez, F., & Horwitz, M. S. (2002). Inhibition of chemokine expression by adenovirus early region three (E3) genes. *Journal of Virology, 76*, 8236-8243.

Li, E., Stupack, D., & Bokock, G. M. (1998a). Adenovirus endocytosis requires actin cytoskeleton reorganisation mediated by Rho family GTPases. *Journal of Virology, 72*, 8806-8812.

Li, E., Stupack, D., & Klemke, R. (1998b). Adenovirus endocytosis via alpha(v) integrins requires phoshoinositide-3-OH kinase. *Journal of Virology, 72*, 2055-2061.

Li, Q., Withoff, S., & Verma, I. M. (2005). Inflammation-associated cancer NF-kappaB is the lynchpin. *Trends in Immunology, 26*, 318-325.

Liu, Q., & Muruve, D. A. (2003). Molecular basis of the inflammatory response to adenovirus vectors. *Gene therapy, 10*(11), 935-940.

Lowe, S. W., & Ruley, H. E. (1993). Stabilisation of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis. *Genes and Development, 7*, 535-545.

Mahr, J. A., Boss, J. M., & Gooding, L. R. (2003). The adenovirus e3 promoter is sensitive to activation signals in human T cells. *Journal of Virology, 77*, 1112-1119.

Marton, M. J., Baim, S. B., & Ornelles, D. A. (1990). The adenovirus E4 17-kilodalton protein complexes with the cellular transcription factor E2F, altering its DNA-binding properties and stimulating E1A-independent accumulation of E2 mRNA. *Journal of Virology, 64*, 2345-2359.

Marttila, M., Persson, D., & Gustafsson, D. J. (2005). CD46 is a cellular receptor for species B adenoviruses except types 3 and 7. *Journal of Virology, 79*, 14429-14436.

Meier, K. E., & Freitag, G. (1955). Uber die antibiotischen Eigenchaften von Sacchariden und Bienenhonig. *Zeitschrift fur Hygiene und Infektionkrankheiten, 141*, 326-332.

Mistchenko, A. S., Diez, R. A., & Mariani, A. L. (1994). Cytokines in adenoviral disease in children: association of interleukin-6, interleukin-8, and tumour necrosis factor alpha levels with clinical outcome. *Journal of Pediatrics, 124*, 714-720.

Miyamoto, K., & Morgan, C. (1971). Structure and development of viruses as observed in the electron microscope. XI. Entry and uncoating of herpes simplex virus. *Journal of Virology, 8*(910-918).

Mogensen, T. H., & Paludan, S. R. (2001). Molecular pathways in virus induced cytokine production. *Microbiology and Molecular Biology Reviews, 65*, 131-150.

Molan, P. C., & Russell, K. M. (1988). Non-peroxide antibacterial activity in some New Zealand honeys. *Journal of Apicultural Research, 27*, 62-67.

Molan, P. C. (1992a). The antibacterial activity of honey. 1. The nature of the antibacterial activity. *Bee World, 73*(1), 5-28.

Molan, P. C. (1992b). The antibacterial activity of honey. 2. Variation in the potency of the antibacterial activity. *Bee World, 73*(2), 59-76.

Molan, P. C. (1999). Why honey is effective as a medicine. 1. Its use in modern medicine. *Bee World, 80*(2), 80-92.

Molan, P. C. (2001). Why honey is effective as a medicine. 2. The scientific explanation of its effects. *Bee World, 82*(1), 22-40.

Molan , P. C. (2006). The Evidence Supporting the Use of Honey as a Wound Dressing. *Lower Extremity Wounds, 5*(1), 40-54.

Montell, C., Courtois, G., & Eng, C. (1984). Complete transformation by adenovirus 2 requires both E1A proteins. *Cell, 36*, 951-961.

Montell, C., Fisher, E. F., & Caruthers, M. H. (1982). Resolving the functions of overlapping viral genes by site-specific mutagenesis at mRNA splice site. *Nature, 295*, 380-384.

Montgomery, R. I., Warner, M. S., & Lum, B. J. (1996). Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. *Cell, 87*, 427-436.

Moran, E., Grodzicker, T., & Roberts, R. J. (1986). Lytic and transforming functions of individual products of the adenovirus E1A gene. *Journal of Virology, 57*, 765-775.

Morgan, C., Rose, H. M., & Mednis, B. (1968). Electron microscopy of Herpes simplex virus. I. Entry. *Journal of Virology, 2*(507-516).

Mossel, D. A. (1980). Honey for necrotic breast ulcers. *Lancet, 2*, 1091.

Mossman, K. L., Macgregor, P. F., & Rozmus, J. J. (2001). Herpes simplex virus triggers and then disarms a host antiviral response. *Journal of Virology, 75*(2), 750-758.

Naesens, L., Lenearts, L., & Andrei, G. (2005). Antiadenovirus activities of several classes of nucleoside and nucleotide analogues. *Antimicrobial Agents Chemotherapy, 49*, 1010-1016.

Nakano, M. Y., Boucke, K., & Suomalainen, M. (2000). The first step of adenovirus type 2 disassembly occurs at the cell surface, independently of endocytosis and escape to the cytosol. *Journal of Virology, 74*, 7085-7095.

Natarajan, S., Williamson, D., Grey, J. A., Harding, K. G., & Cooper, R. J. (2001). Healing of an MRAS-colonised, hydroxyurea-induced leg ulcer with honey. *Journal of Dermatological Treatment, 12*, 33-36.

Nazir, S. A., & Metcalf, J. P. (2005). Innate immune response to adenovirus. *Journal of Investigative Medicine, 53*, 292-304.

Nevins, J. R., & Darnell, J. E. (1978). Groups of adenovirus type 2 mRNA's derived from a large primary transcript: probable nuclear origin and possible common 3' ends. *Journal of Virology, 25*, 811-823.

Nicholl, M. J., Robinson, L. H., & Preston, C. M. (2000). Activation of cellular interferon-responsive genes after infection of human cells with herpes simplex virus type 1. *Journal of General Virology, 81*, 2215-2218.

Obert, S., O'Connor, R. J., & Schmid, S. (1994). The adenovirus E4-6/7 protein transactivates the E2 promoter by inducing dimerisation of a heteromeric E2F complex. *Molecular and Cellular Biology, 14*, 1333-1346.

Obi, C. L., Ugoji, E. O., Edun, S. A., Lawal, S. F., & Anyiwo, C. E. (1994). The antibacterial effect of honey on diarrhoea causing bacterial agents isolated in Lagos, Nigeria. *African journal of Medical Sciences, 23*, 257-260.

O'Shea, C. C., Choi, S., & McCormick, F. (2005). Adenovirus overides cellular checkpoints for protein translation *Cell Cycle, 4*, 883-888.

Ostler, H. B. (1977). Herpes simplex: the primary infection. *Survey of Ophthalmology, 21*, 91-99.

Palmer, D. H., Chen, M. J., & Searle, P. F. (2005). Inhibition of NF-kappaB enhances the cytotoxicity of virus-directed enzyme prodrug therapy and oncolytic andenovirus cancer gene therapy. *Gene Therapy, 12*, 1187-1197.

Patel, A., Hanson, J., & McLean, R. J. (1998). Herpes simplex virus type 1 induction of persistent Nf-kB nuclear translocation increases the efficiency of virus replication. *Virology, 247,* 212-222.

Pehler-Harrington, K., Khanna, M., & Waters, C. R. (2004). Rapid detection and identification of human adenovirus species by adenoplex, a multiplex PCR-enzyme hybridisation assay. *Journal of Clinical Microbiology, 42,* 40972-44076.

Pettersson, U., & Roberts, R. J. (1986). Adenovirus gene expression and replication: a historical review. *Cancer Cells, 4,* 37-57.

Preston, C. M., Hartman, A. N., & NJM. (2001). Activation of interferon response factor-3 in human cells infected with herpes simplex virus type 1 or human cytomegalovirus. *Journal of Virology, 28,* 499-517.

Purifoy, D. J., Lewis, R. B., & Powell, K. L. (1977). Identification of the herpes simplex virus DNA polymerase gene. *Nature, 269,* 621-723.

Querido, E., Blanchette, P., & Yan, Q. (2001). Degradation of p53 by adenovirus E4orf6 and E1B55K proteins occurs by a novel mechanism involving a Cullin-containing complex. *Genes and Development, 15,* 3104-3117.

Querido, E., Marcellus, R. C., & Lai, A. (1997). Regulation of p53 levels by the E1B 55-kilodalton protein and E4orf6 in adenovirus infected cells. *Journal of Virology, 71,* 3788-3798.

Regenmortal, M. H. V. (2000). Virus Taxonomy. In M. H. V. Regenmortal (Ed.), *The seventh report of the ICTV* (pp. 227-238): Academic Press.

Reynolds, A. E., Liang, L., & Baines, J. D. (2000). Conformational changes in the nuclear lamina induced by herpes simplex virus type 1 require genes U(L)31 and U(L)34. *Journal of Virology*, *78*, 5564-5575.

Reynolds, A. E., Ryckman, B. J., & Baines, J. D. (2001). UL31 and UL34 proteins of herpes simplex virus type 1 form a complex that accumulates at the nuclear rim and is required for envelopement of nucleocapsids. *Journal of Virology, 75*, 8803-8817.

Reynolds, A. E., Wills, E. G., & Roller, R. J. (2002). Ultrastructural localization of the herpes simplex virus type 1 u(l)31, u(l)34, and u(s)3 proteins suggests specific roles in primary envelopement and egress of nucleocapsids. *Journal of Virology, 76*, 8939-8952.

Ricciardi, R. P., Jones, R. L., & Cepko, C. L. (1981). Expression of early adenovirus genes requires a viral encoded acidic polypeptide. *PNAS, 78*, 6121-6125.

Robson, V., Ward, R. G., & Molan , P. C. (2000). The use of honey in split-skin grafting, *Conference of the European Wound Management Association*. Harrogate, UK.

Roelvink, P. W., Lizonova, A., & Lee, J. G. (1998). The coxsackievirus-adenovirus receptor for adenovirus serotypes from subgroups A, C, D, E, and F. *Journal of Virology, 72,* 7909-7915.

Roizman, B., & Furlong, D. (1974). The replication of herpesviruses. In Fraenkel-Conrat & R. R. Wagner (Eds.), *Comprehensive Virology* (pp. 229-403). NY: Plenum Press.

Roizman, B., Carmicheal, L. E., & Deinhardt, F. (1981). Herpesviridae: definition, provisional nomenclature, and taxonomy. *Intervirology, 16*, 201-217.

Roizman, B., Gu, H., & Mandel, G. (2005). The First 30 minutes in the Life of a Virus: unREST in the Nucleus. *Cell Cycle, 4*, 1019-1021.

Rosen, I. (1960). A hemagglutination-inhibitor technique for typing adenoviruses. *American Journal of Hygiene, 71*, 120-128.

Roth, L. A., Kwan, S., & Sporns, P. (1986). Use of a disc-assay system to detect oxytetracycline residues in honey. *Journal of Food Protection, 49*(6), 436-441.

Roth, W. P., Konig, C., & Wienzek, S. (1998). Inactivation of p53 but not p73 by adenovirus type 5 E1B 55-kilodalton and E4 34-kilodalton oncoproteins. *Journal of Virology, 72*, 8510-8516.

Rowe, W. P., Huebner, R. J., & Gilmore, L. K. (1953). Isolation of a cytopathogenic agent from human adenoidsundergoing spontaneous degeneration in tissue culture. *Proceedings of the Society for Experimental Biology and Medicine, 84*, 570-573.

Ryan, G. B., & Manjno, G. (1977). *Inflammation*. Michigan: Upjohn.

Sackett, W. G. (1919). Honey as a carrier of intestinal diseases. *Bull Colorado State University Agricultural Experiment Station, 252*, 1-18.

Saissy, J. M., Guignard, B., Guiavarch, M., & Rouvier, B. (1995). Pulmonary edema after hydrogen peroxide irrigation of a war wound. *Intensive Care Medical, 21*(3), 287-288.

Sarnow, P., Hearing, P., & Anderson, C. W. (1984). Adenovirus early region 1 B 58,000-dalton tumour antigen is physically associated with an early region 4 25,000-dalton protein in productively infected cells. *Journal of Virology, 49*, 692-700.

Schillinger, J. A., Xu, F., & Sternberg, M. (2004). National seroprevalence and trends in herpes simplex virus type 1 in the United States 1976-1994. *Sexually Transmitted Diseases, 31*, 753-760.

Scott, T. F. (1957). Epidemiology of herpetic infections. *American Journal of Ophthalmology, 43*, 134.

Seymour, F. I., & West, K. S. (1951). Honey - its role in medicine. *Medical Times, 79*, 104-107.

Shaw, A. R., & Ziff, E. B. (1980). Transcripts from the adenovirus-2 major late promoter yeild a single early family of 3' coterminal mRNAs and five late families. *Cell, 22*, 905-916.

Shenk, T. (2001). *Adenoviruses*: Lippincott Williams & Wilkins.

Shieh, M. T., WuDunn, D., & Montgomery, R. I. (1992). Cell surface receptors for herpes simplex virus are heparan sulfate proteoglycans. *Journal of Cellular Biology, 116*, 1273-1281.

Ship, I., Morris, A. L., & Durocher, R. T. (1960). Recurrent aphthous ulcerations and recurrent herpes labialis in a professional school student population. 1. Experience. *Oral Surgery Oral Medicine Oral Pathology, 13,* 1191-1202.

Ship, I., Morris, A. L., & Durocher, R. T. (1961). Recurrent aphthous ulcerations and recurrent labialis in a professional school student population. IV. Twelve month study of natural disease patterns. *Oral Surgery Oral Medicine Oral Pathology, 14*, 39.

Short, J. J., Pereboev, A. V., & Kawakami, Y. (2004). Adenovirus serotype 3 utilizes CD80 (B7.1) and CD86 (B7.2) as cellular attachment receptors. *Virology, 322*, 349-359.

Shukla, D., Liu, J., & Blaiklock, P. (1999). A novel role for 3-O-sulfated heparan sulfate in herpes simplex virus 1 entry. *Cell, 99*, 13-22.

Simpson-Holley, M., Baines, J. D., & Roller, R. J. (2004). Herpes simplex virus type 1 U(L)31 and U(L)34 gene products promote the late maturation of viral replication compartments to the nuclear periphery. *Journal of Virology, 78*, 5591-5600.

Sodeik, B., Ebersold, M. W., & Helenius, A. (1997). Microtubule-mediated transport of incoming herpes simplex virus 1 capsids to the nucleus. *Journal of Cellular Biology, 136*, 1007-1021.

Somerfield, S. D. (1991). Honey and healing. *Royal Society of Medicine, 84*(3), 179.

Spear, P. G., & Longnecker, R. (2003). Herpesvirus entry: an update. *Journal of Virology, 73*, 10179-10185.

Spear, P. G., & Roizman, B. (1967). Buoyant density of herpes simplex virus in solutions of caesium chloride. *Nature, 214*, 713-714.

Spivack, J. G., & Fraser, N. W. (1988). Expression of herpes simplex virus type 1 latency-associated transcripts in the trigeminal ganglia of mice during acute infection and reactivation of latent infection. *Journal of Virology, 62*, 1479-1485.

Stackpole, C. W. (1969). Herpes-type virus of the frof renal adenocarcinoma. I. Virus development in tumour transplants maintained at low temperature. *Journal of Virology, 4*, 75-93.

Staehelin, M. (1959). Inactivation of virus nucleic acid with glyoxal derivatives. *Biochemica et Biophysica Acta, 31*, 448-454.

Steegenga, W. T., Riteco, N., & Jochemsen, A. G. (1998). The large E1B protein together with the E4orf6 protein target p53 for active degradation in adenovirus infected cells. *Oncogene, 16*, 349-357.

Stevens, J. G., & Cook, M. L. (1974). Latent herpes simplex virus in sensory ganglia. *Perspectives in Virology, 8*, 171.

Subrahmanyam, M. (1996). Honey dressing versus boiled potato peel in the treatment of burns: a prospective randomized study. *Burns, 22*(6), 491-493.

Taddeo, B., Esclatine, A., & Roizman, B. (2002). The patterns of accumulation of cellular RNAs in cells infected with a wild-type and a mutant herpes simplex virus 1 lacking the virion host shutoff gene. *PNAS, 99*, 17031-17036.

Taddeo, B., Esclatine, A., & Zhang, W. (2003a). The stress-inducible immediate-early responsive gene IEX-1 is activated in cells infected with herpes simplex virus 1, but several viral mechanisms, including 3' degradation of its RNA, preclude expression of the gene. *Journal of Virology, 77*, 6178-6187.

Taddeo, B., Luo, T. R., & Zhang, W. (2003b). Activation of NF-kappaB in cells productively infected with HSV-1 depends on activated protein kinase R and plays no apparent role in blocking apoptosis. *PNAS, 100*, 12408-12413.

Taddeo, B., Zhang, W., & Lakeman, F. (2004). Cells lacking NFkB or in which NFkB is not activated vary with respect to ability to sustain herpes simplex virus 1 replication and are not susceptible to apoptosis induced by a replication-incompetent mutant virus. *Journal of Virology, 78*, 11615-11621.

Taddeo, B., Zhang, W., & Roizman, B. (2006a). The UL41 protein of herpes simplex virus 1 degrades RNA by endonucleolytic cleavage in absence of other cellular or viral proteins. *PNAS, 103*, 2827-2832.

Tiffany, B. D., Wright, J. B., Heinzelman, R. V., Strube, B. D., & Aspergren, B. D. (1957). Antiviral compounds. Aliphalic glyoxyls, alpha-hydroxyaldehydes and related compounds. *Journal of American Chemical Society, 79*, 1682-1687.

Tollefson, A. E., Scaria, A., & Hermiston, T. W. (1996). The adenovirus death protein (E3-11.6K) is required at very late stages of infection for efficient cell lysis and release of adenovirus from infected cells. *Journal of Virology, 70*, 2296-2306.

Tonks, A. J., Cooper, R. A., Jones, K. P., Blair, S., Parton, J., & Tonks, A. (2003). Honey stimulates inflammatory cytokine production from monocytes. *Cytokine, 21*, 242-247.

Tonks, A. J., Dudley, E., Porter, N. G., Brazier, J., Smith, E. L., & Tonks, A. (2007). A 5.8-kDa component of manuka honey stimulates immune cells via TLR4. *Journal of Leukocyte Biology, 82*.

Topp, K. S., Meade, L. B., & LaVail, J. H. (1996). Microtubule polarity in the peripheral processes of trigeminal ganglion cells: relevance for the retrograde transport of herpes simplex virus. *Journal of Neuroscience, 14*, 318-325.

Tovey, F. I. (1991). Honey and healing. *Royal Society of Medicine, 84*(7), 447.

Turner, F. J. (1983). *Hydrogen Peroxide and Other Oxidant Disinfectants*. Philadelphia: Lea and Febiger.

van Regenmortel, M. H. V., Fauquet, C. M., & Bishop, D. H. L. (2000). Virus taxonomy: classification and nomenclature of viruses. Seventh Report of the International Committee on Taxonomy of Viruses In *Family Adenoviridae* (pp. 227-237). San Diego, CA: Academic Press.

Varga, M. J., Weibull, C., & Everitt, E. (1991). Infectious entry pathway of adenovirus type 2. *Journal of Virology, 65*, 6061-6070.

Walters, R. W., Freimuth, P., & Moninger, T. O. (2002). Adenovirus fiber disrupts CAR-mediated intercellular adhesion allowing virus escape. *Cell, 110*, 789-799.

Warner, M. S., Geraghty, R. J., & Martinez, W. M. (1998). A cell surface protein with herpesvirus entry activity (HveB) confers susceptiblity to infection by mutants of herpes simplex virus type 1, herpes simplex virus type 2 and pseudorabies virus. *Virology, 246*, 179-189.

Weigel, K. U., Opitz, T., & Henle, T. (2004). Studies on the occurence and formation of 1,2-dicarbonyls in honey. *European Food Reseach and Technology, 218*(5), 147-151.

White, J. W., Subers, M. H., & Schepartz, A. I. (1963). The identification of inhibine, the antibacterial factor in honey, as hydrogen peroxide and its origin in a honey glucose-oxidase system. *Biochemica et Biophysica Acta, 73*, 57-70.

Wickman, T. J., Mathias, P., & Cheresh, D. A. (1993). Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalisation but not virus attachment. *Cell, 73*, 309-319.

Wild, P., Engels, M., & Senn, C. (2005). Impairment of nuclear pores in bovine herpesvirus 1-infected MDBK cells. *Journal of Virology, 78*, 4783-4796.

Winberg, G., & Shenk, T. (1984). Dissection of overlapping functions within the adenovirus type 5 gene. *Embo Journal, 3*, 1907-1912.

Windheim, M., Hilgendorf, A., & Burgert, H. G. (2004). Immune evasion by adenovirus E3 proteins: exploitation of intracellular-trafficking pathways. *Current Clinical Topics in Microbiology and Immunology, 273*, 29-85.

Wu, C. A., Nelson, N. J., & McGeoch, D. J. (1988). Identification of herpes simplex virus type 1 genes required for origin-dependent DNA synthesis. *Journal of Virology, 62*, 435-443.

WuDunn, D., & Spear, P. G. (1989). Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. *Journal of Virology, 63*, 52-58.

Xu, F., Schillinger, J. A., & Sternberg, M. R. (2002). Seroprevalence and coinfection with herpes simplex virus type 1 and type 2 in the United States, 1988-1994. *Infectious Diseases, 185*, 1019-1024.

Zeina, B., Othman, O., & Al-Assad, S. (1996). Effect of Honey versus Thyme on Rubella Virus Survival in vitro. *The journal of alternative and complementary medicine, 2*(3), 345-348.

Zhang, Y., & Bergelson, J. M. (2005). Adenovirus Receptors. *Journal of Virology, 79*(19), 12125-12131.

Zhou, Z. H., Chen, D. H., & Jakana, J. (1999). Visualisation of tegument-capsid interactions and DNA in intact herpes simplex type 1 virions. *Journal of Virology, 73*, 3210-3218.

Zumla, A., & Lulat, A. (1989). Honey - a remedy rediscovered. *Journal of the Royal Society of Medicine, 82*, 384-385.