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Assessment of Antiviral and Photodynamic Inactivation Activity of Different Compounds Against Hepatitis A Virus

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Abstract

Food contamination from hepatitis A virus (HAV) is a great concern to food producers worldwide. Finding an innovative approach to inactivate HAV on food contact surfaces and on different produce remains a challenge. Using chemical disinfectants (e.g. chlorine) is an effective way to inactivate HAV on fomites, but it maybe unfavorable for food products. While heat inactivation of HAV remains the most efficient way to inactivate HAV when present in foods, most foodborne outbreaks of HAV are related to ready-to-eat (RTE) foods including produce which do not undergo further heating. Therefore, finding compounds with effective anti-HAV activities will be of great benefit to the food sector. In our study, oleanolic acid (OA) and ursolic acid (UA) have been investigated for their anti-HAV properties. OA at 600 µg/ml and UA at 360 µg/ml showed 2.27±0.67 and 1.33±0.35 log PFU/ml reduction after a 1 h treatment, respectively. Furthermore, to increase virus inactivation, photodynamic inactivation (PDI) was applied, which uses oxygen, light and a photosensitizer to produce reactive oxygen species (ROS). Grape seed extract (GSE) and oleanolic acid with known antiviral properties were tested as photosensitizers. Conditions using UV light at 254 nm with a distance of 72 cm and doses (energy density) of 0.012±0.000, 0.020±0.001, 0.040±0.001, 0.061±0.002, 0.081±0.002 and 0.121±0.003 J/cm^2 for 3, 5, 10, 15, 20 and 30 min exposure times, respectively were applied for the PDI experiments. However, the acquired viral reductions by GSE and OA mediated PDI were attributed to UV light more than ROS production. Future work may include the use of different light sources for illumination, and the use of UA as a potential photosensitizer compound.

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Chapter I: Literature Review

The Picornaviridae Family: The family *Picornaviridae* comprises of nine genera, *Enterovirus* (Poliovirus and Coxsackievirus), *Rhinovirus* represented by *Rhinovirus*, *Cardiovirus*, *Aphthovirus*, *Erbovirus*, *Kobuvirus*, *Teschovirus*, *Parechovirus* and *Hepatovirus* (Whitton et al., 2005). Poliovirus is an *Enterovirus* spread by the oral-fecal route and replicates in the patient intestines then attacks the spinal cord motor neuron causing inflammation and necrosis that can cause permanent paralysis (poliomyelitis) (Whitton et al., 2005). Poliovirus has a long history in the human population, its records going back thousands of years. The paralytic poliomyelitis was depicted on an ancient Egyptian stele. Before the advent of the vaccine, the disease affected thousands all over the world every year. The *Picornavirus* life cycle begins with viral binding to a specific receptor, entry into the host cell, un-coating and the release of the virus genome. The RNA of all *Picornaviruses* is positive sense with one open reading frame (ORF) flanked by extended untranslated regions that contain an internal ribosome entry site (IRES); crucial for viral RNA ribosomal translation (Jang et al., 1988), with a fast mutation rate (Whitton et al., 2005; Ward et al., 1988). Most of the *Picornaviruses* can shut down the host translation machinery by the breakdown of a Eukaryotic translation initiation factor 4 G (eIF-4G) a cellular protein crucial for the cap-dependent translation process (Etchison et al., 1982). They do this by using protease enzymes which can create specific cleavages to the eIF 4G. These pieces then bind to the viral RNA and enhance its translation rate (Ohlmann et al., 1996). These proteases are not conserved in the *Picornaviruses* family; *Enterovirus* and *Rhinovirus* use the 2A protease and aphthovirus uses the L protein (Whitton et al., 2005). However, hepatitis A virus (HAV) does not arrest host cell translation, and it is non-cytopathic (Gauss-Muller et al., 1984).

Furthermore, the *Picornaviruses* are able to interfere with host cell transcription by using a 3C protease to cleave several cellular transcription factors of the host cell and reduce the level of transcription (Whitton et al., 2005). Some *Picornaviruses* are able to increase cell membrane permeability using the 2B protein, which is classified as a viroporin in the case of poliovirus (Doedens et al., 1995) and coxsackievirus (Van Kuppeveld et al., 1997). Interestingly these proteins are also able to inhibit the secretory pathway; poliovirus 3A protein can reduce the expression of the MHC class 1, thus hindering the immune system T cells' ability to identify the infected cell (Deitz et al., 2000). Vaccines against some *Picornavirus* are available; for example, with poliovirus, foot and mouth virus and hepatitis A virus; however, treatment of the infection is based on the transfer of immunoglobulin to the patients.

Hepatitis A Virus

In 1973 Feinstone and his team were the first to identify hepatitis A virus (HAV) using electron microscopy of the feces of an infected individual (Feinstone et al., 1973). Later HAV was classified in the order of *Picornavirales*, in the *Picornaviridae* family and genus *Hepatovirus* (Lemon et al., 1994). The 27-32 nm non-enveloped icosahedral particles enclose linear and positive sense single-stranded RNA, 7500 nucleotides in length (Lemon et al., 1992; Melnick et al., 1992; Hollinger et al., 2007). The genome can be divided into three main regions; the first region is the 59-nucleotide untranslated region (UTR) containing an extended secondary structure that is essential for the cap-independent translation, and that is covalently bonded to Vpg the viral protein (Costa-Mattioli et al., 2003). The second region consisting of the segments P1, P2, and P3 contains a single open reading frame and is responsible for encoding all the viral

proteins (Costa-Mattioli et al., 2003). In this location, all the structural proteins (namely VP1, VP2 and VP3 and the putative protein VP4) are encoded by the P1 segment and the nonstructural proteins responsible for replication are coded by the P2 and P3 segments, while the third region is the 39 UTR ending with a poly (A) tract (Costa-Mattioli et al., 2003). The 5' UTR has an internal ribosome entry segment that regulates the translation process (Brown et al., 1991). The coded RNA produces a poly-protein of 2200 amino acids (Cristina and Costa-Mattioli., 2007), which will be cleaved by the virus and the host cells proteases to give rise to 11 proteins, including the viral RNA dependent polymerase. This is responsible for producing the antigenomic negative-strand RNA that acts as the template for the production of the genomic positive-strand RNA, which gets packed into the viral capsids (Cuthbert et al., 2001). Moreover, the virion capsids are composed of three polypeptides encoded by VP1, VP2, and VP3. Variation in HAV isolates is detected by sequencing three specific regions of the viral genome that include the VP1/2A junction as well as the VP3 C and, the VP1 amino terminus, (Jansen et al., 1990). These regions are responsible for the genetic variation between different HAV strains (Bruisten et al., 2001; Byun et al., 2001; Apaire-Marchais et al., 1995; Arauz-Ruiz et al., 2001). HAV has only one serotype and six genetic groups (Cristina et al., 2007). The genotypes are labeled as I to VI; the genotypes I, II, III, are divided into A and B and are responsible for human disease (Cristina et al., 2007). Genotype I is broadly distributed worldwide, and sub genotype IA is more frequent in the United States and Western Europe, and IA has more occurrences than IB, and IIIA is ubiquitous in central Asia. HAV genotypes IV, V and VI infect simians (Desbois et al., 2010; Costa-Mattioli et al., 2003).

HAV Symptoms

The average incubation time from HAV exposure to symptoms of HAV illness is reported to be approximately 28 days (though can range from 15-50 days) (Krugman et al., 1970). HAV symptoms can be severe and include fever, anorexia, low appetite, vomiting, stomach pain, nausea, dark-colored urine or light-colored stools (Koff et al., 1992) and yellowish pigmentation of the skin and eyes resulting from an elevated level of bilirubin in the blood (jaundice) (Buttaro et al., 2012). In most cases, symptoms of HAV persist for several weeks; only 10% of cases have symptom relapses showing an increase in the serum aminotransferase levels that can continue for up to 6 months (Schiff, 1992). The average lethality rate of HAV infection is 0.3%, however, it can reach up to 1.8% for individuals age 50 or over, chronic liver disease patients, and otherwise immunocompromised individuals (CDC, 2018). It is common for HAV infected children younger than 6 years old to be asymptomatic. In these cases, only 10% develop jaundice (Gingrich et al., 1983). Approximately 76%–97% of young adults infected with HAV develop symptoms of which 40%–70% show jaundice symptoms (Lednar et al., 1985).

Hepatitis A Virus Diagnosis

The most often applied diagnostic test for HAV in clinical patient samples is based on the anti-HAV-IgM antibody detection using a highly sensitive and accurate enzyme-linked immunosorbent assay (ELISA) test and other biochemical enzyme tests (Cuthbert et al., 2001). However, in some cases of clinical discrepancy, there are other confirmation methods that can be used to detect the viral presence. One of these is to detect HAV RNA with the use of the reverse transcription (RT)-PCR (De Paula et al., 2004; Musana et al., 2004). Additionally, the liver

enzyme alanine transferase (ALT) is used to detect the virus that is present in the blood during the acute stage of infection, being produced from the damaged liver cells (Roque et al., 2004). However, for food samples, real-time PCR is an effective molecular technique used to detect HAV in shellfish, using primers and probes designed from the 5-prime untranslated region (600 nucleotides approximately), which is reported to be the most conserved region of the viral genome (Di Pasquale et al., 2010).

Life Cycle of HAV

When HAV is transmitted to the body through the fecal-oral or blood routes, the virus enters the bloodstream. Through the circulation of the blood, the virus will be carried to the liver, where it attaches to the host cell receptors of the hepatocytes (Gupta, 2018). Viral transcription occurs solely in hepatocytes, then the viral RNA is released from the capsid and translation of the viral proteins occurs by the host cell ribosomes followed by RNA replication and virion assembly, though only, when the virions are matured, they are secreted into the bile and then into the stool (Gupta, 2018).

HAV Distribution

There are approximately 1.4 million clinical cases of HAV worldwide each year, making it the most infectious hepatitis disease (Keeffe et al., 2006). However, it is anticipated that the actual number of infected persons is much higher than this due to cases that are not reported (Keeffe et al., 2006). Additionally, some of the exposed individuals may have immunity due to vaccination or previous infection and asymptomatic individuals can also spread the disease (Lednar et al.,

1985). HAV is a foodborne disease that has several routes of infection. It is spread mostly through contaminated food and water, close contact with infected persons, homosexual oral-anal practices, and to a lesser extent blood transfusion, and its geographical distribution of HAV is related to the economic and social conditions of different countries (Roque et al., 2004). The developed countries which have high hygiene and food safety standards and a wide application of HAV vaccinations and so have a low level of clinical cases. Conversely, the poorer, developing countries that lack basic hygiene and food safety have a high infection rate especially with children under 6 years old (Roque et al., 2004; Jacobsen et al., 2004). However, in high income countries, increasing international trade and travel contribute to the frequent occurrences of foodborne HAV outbreaks. Due to globalization, middle income countries are the major contributors to such incidents (Jacobsen, 2018).

HAV Transmission

The most prevalent form of HAV transmission is through infected food handlers (Dalton et al., 1996). It has been reported that a single HAV infected food handler can be the source of infection for hundreds of individuals, causing both sporadic cases and outbreaks (Dalton et al., 1996; Lucioni et al., 1998). Other than infection caused by food handlers through contamination of cooked food, the main source of infection is by the contamination of uncooked food or contaminated water. HAV transmission by contaminated food products can happen during any stage of food production including cultivation, harvest, distribution, and preparation. The detection of the source of the contamination is difficult due to the relatively long onset of the virus (2-6 weeks), making food history difficult for many patients to recall (Dalton et al., 1996;

Lucioni et al., 1998). In addition, low sensitivity of the current diagnostic tests food samples and high cost makes detection and tracking of transmission routes challenging.

HAV Prevention

In 2002, Purcell and his team used a gastric tube to inoculated tamarins and chimpanzees with a standard filtrate of human feces contaminated with HAV strain SD-11, using 0.5 ml of 10-fold dilutions of the filtrates (Purcell et al., 2002). After 14 days, it was found that the infectious dose for the primates was a viral titer of 10^4 PFU/ml (Purcell et al., 2002), while in humans the minimum infectious dose is still unknown (Acheson & Fiore, 2004). Freezing does not affect the HAV, as contaminated strawberries, raspberries, blueberries, parsley and basil with HAV HM-175 strain, titer of (1.6×10^6 $TCID_{50}$) were not affected by freezing at -20ºC and storage time of 90 days as reported (Butot, et al. 2008). A research study indicated that heating at 100ºC for 2 min in manila clams can cause total deactivation of HAV to ensure safer products (Pascoli et al., 2016). Also, cooking HAV contaminated turkey deli meat at 80ºC for 107 s was enough to cause a 6 log reduction in HAV (Bozkurt et al., 2015). Blanching HAV contaminated spinach at 100°C for at least 2 min is enough/sufficient to reduce HAV titer by 6 log PFU/ml (Bozkurt, Ye, et al., 2015). The undiluted surface disinfectant 2.0% glutaraldehyde with pH 7.5, and the diluted (1:11) 6% sodium hypochlorite of (5,000 ppm free chlorine) with pH 11.2, reduces HAV titer by > 99.9%, that can be used as contact surface disinfectants (Mbithi et al., 1990). While pre-exposure prophylaxis is available, vaccination for HAV provides protection against viral infection. The two main types of vaccines for HAV including; the inactivated HAV vaccine and the live attenuated HAV vaccine (Irving et al., 2012). The inactivated HAV vaccine provides at

least two years of protection and is considered to be safer than the live attenuated HAV vaccine, whose safety is not guaranteed (Irving et al., 2012). The inactivated vaccine is a preparation of an inactivated cell culture-adapted HAV virus which was licensed in 1995. The vaccine injection is taken in two doses; the first dose of the vaccine is enough to provide seroconversion in which the production of the viral antibodies can be detected after 2 weeks. This is measurable in 54–62% of the vaccinated individuals and this percentage increases to 90% after 4 months. In this time, the vaccine will show protective concentration levels of HAV antibodies, while the second dose (booster) taken after 6 months of the first dose can generate a long-term protection up to 20 years (CDC, 2018). Conversely, the post-exposure prophylaxis uses the immunoglobulin to counter HAV infection. The chances of the immunoglobulin to stop HAV is highest during the early incubation stage of the virus and are recommended only for those within 2 weeks of exposure, while after two weeks of HAV infection the immunoglobulin therapy is reported have only 85% efficacy to prevent HAV symptoms and illness (Winokur et al., 1992).

HAV Outbreaks

There have been several outbreaks reported worldwide due to HAV. An outbreak of HAV from raw clams was one of the worst recorded in history that occurred in Shanghai, China in 1988. There were 292,301 cases recorded (Halliday et al., 1991), meaning the rate of cases was 4,083 cases for every 100,000 people. The consumption of HAV contaminated raw clams was the reason for the outbreak, as the preparation method of steaming the clams until they open was not sufficient to inactivate the virus (Dismukes et al., 1969; Koff et al., 1967). The countermeasures taken by the Chinese government included prevention of the sale of clams in Shanghai, banning

of clamming in specific areas, and initiating investigations into sterilization methods of the shellfish during the time of the outbreak (Halliday et al., 1991).

Another notable HAV outbreak is the multistate outbreak of HAV infections linked to imported pomegranate seeds from Turkey. This outbreak occurred in May 2013 and was reported by the New Mexico Department of Health (Collier et al., 2014). The department reported to the Centers for Disease Control and Prevention (CDC) that the first two patients from the same city shopped at the same store. Both had bought fruits from the store and consumed them in smoothies. The outbreak included 165 cases in ten US states including "Arizona, California, Colorado, Hawaii, New Hampshire, New Jersey, New Mexico, Nevada, Utah, and Wisconsin" (Collier et al., 2014). Where 55% of the total number of cases included women aged between 40-64 years; 11 of the patients were children under 18 years old. 44% of patients were hospitalized, but no deaths were reported. All the patients exposed to HAV purchased the product from Costco markets, though this was also. sold at Harris Teeter stores, but no patients reported purchasing the product from this location (Collier et al., 2014). In 2016, another multistate outbreak occurred in the US which was investigated by the CDC and US Food and Drug Administration (FDA), where traceback and epidemiological analyses indicated that the International Company for Agricultural Production & Processing (ICAPP) imported frozen strawberries from Egypt which were the likely cause the outbreak. Patients mostly reported drinking smoothies that had strawberries at Tropical Smoothie Café in the regions of Maryland, North Carolina, Virginia, and West Virginia (CDC, 2016). The 143 cases were reported from nine involved states including Arkansas, California, Maryland, New York, North Carolina, Oregon, Virginia, West Virginia and

Wisconsin, and. since January 1st, 2016 ICAPP had recalled imported frozen strawberries sent to the USA (CDC, 2016). A recent HAV outbreak (2016) was caused by raw scallops in Hawaii. In January 11th, 2017, the confirmed number of cases of HAV from this outbreak in Hawaii was 292. The outbreak started with three lots of raw scallops imported by Koha Oriental Foods and True World Foods from the Philippines on November 2015; it was then distributed to three states including California, Hawaii, and Nevada. Genki Sushi restaurants on the islands of Oahu and Kauai in Hawaii, were reported to have served the contaminated scallops that were declared as the source of the outbreak by the Hawaii Department of Health (HDOH) (Health.hawaii.gov, 2018). The company recalled the three lots of the frozen scallops on August 18th, 2016. After August 19th, 2016, there were no HAV infection cases linked to the Hawaii outbreak (Health.hawaii.gov, 2018). Beginning in March 2017, a multistate HAV outbreak occurred affecting homeless people and those who use injection and non-injection drugs in California, New Mexico, Missouri, Kansas, Arkansas, Louisiana, Florida, Kentucky, Tennessee, Indiana, Ohio, North Carolina, West Virginia, Massachusetts, Michigan and Utah. The CDC and local health departments delivered HAV vaccinations to many homeless individuals who were not previously immunized (CDC, 2017).

Heat Inactivation of Hepatitis A Virus

There are many examples of studies using heat to inactivate HAV in food in order to make it safe for consumption. Cooking manila clams infected with 5 to 7 log $TCID_{50}$/ml HAV on a conventional stove for 2 min at 100ºC degree was sufficient to inactivate HAV. This was determined by extracting the virus from the digestive glands and testing it with real-time PCR

(Pascoli et al., 2016). Another study looked at spinach inoculated with HAV strain HM-175 titer of 7.34±1.28 log PFU/ml in plastic vacuum bags. The D-value at 50°C was 34.40±4.08 min; at 56°C was, 8.43±1.72 min; at 60°C was, 4.55±0.82 min; at 65°C was, 2.30±0.82 min and at 72°C was, 0.91±0.14 min (Bozkurt et al., 2015). The calculated D-value (50–72°C) was reported to range from 34.40±4.08 to 0.91±0.12 min, and the Z-value was reported as 13.92±0.87°C (Bozkurt et al., 2015). They reported the minimum energy required to begin inactivation of the microbial population (the activation energy) to be 162±11 kJ/mol calculated from the first order model. Blanching of spinach (placing in water at 100°C for 120–180s) resulted in a 6 log PFU/ml reduction of HAV; this cooking method is recommended to increase the safety of food (Bozkurt et al., 2015). When 25 g of mussels (*Mytilus edulis*) were inoculated with 5 ml HAV strain HM-175 at a titer of 7.04±1.34 log PFU/ml D-values of 54.17±4.94 min at 50°C, 9.32±3.26 min at 56°C, 3.25±0.72 min at 60°C, 2.16±0.17 min at 65°C and 1.07±0.24 min at 72°C, were reported using a linear model (Bozkurt et al., 2014). By the Weibull model, similar trends were obtained (Bozkurt et al., 2014).

In another study, 50 New Zealand Greenshell Mussels (*Perna canaliculus*) were inoculated at multiple sites in the guts by injection of 5 µL to 10 µL of cytopathic HM-175 strain of HAV at 5.7 log $TCID_{50}$.and boiling the mussels at 92ºC for 180 s was found to be sufficient to inactivate the virus to non-detectable levels (Hewitt et al., 2006). On the other hand, only a 1.5 log reduction of HAV resulted from a mean internal temperature of 63ºC after steaming for 180 s (Hewitt et al., 2006). Heating of dissected digestive glands of soft-shell clams (*Mya arenaria*) inoculated with 3×10^5 PFU/ml HAV strain HM-175 at 90ºC for 90 s resulted in reduction of 2.66

log cycles by RT-PCR and was reduced by 5.47 log cycles when similarly heated at 90ºC for 180 s, indicating the suitability of heat for HAV inactivation (Sow et al., 2011).

Inoculation with 100 µL of HAV strain HM-175 at a titer of 7.27±1.46 log PFU/ml on the surface of 6 g of turkey deli meat placed in vacuum plastic bags, using the first order model, showed a D-value of 42.0±5.6 min at 50ºC, 20.6±2.5 min at 56ºC, 5.9±1.3 min at 60ºC, 2.3±0.4 min at 65ºC and 1.0±0.1 min at 72ºC (Bozkurt et al., 2015). The z-value of HAV using first order model was reported to be 12.8±0.7ºC, while the calculated activation energy for the first order model for HAV was reported as 167±9 kJ/ mole. A 6 log reduction in HAV could be achieved by cooking at 80ºC for 107 s, at 85ºC for 46 s, at 90ºC for 20 s, at 95ºC for 9 s and 100ºC for 4 s (Bozkurt et al., 2015).

Chemical Inactivation of HAV

Chemical disinfectants are the most common method used to inactivate microorganisms during cleaning and sanitation. Different disinfectants such as chlorine, chlorine dioxide, 2% glutaraldehyde, 6% sodium hypochlorite, alcohols, peracetic acid, and citric acids have different modes of action against microorganisms. For example, chlorination is a widely used method for inactivation of microorganisms, particularly in aqueous solutions including municipal water, wastewater and swimming pools. Chlorine gas has fast and almost complete hydrolysis when dissolved in water and gives rise to two more species; hypochlorous acid (HOCl) and hypochlorite (OCl^-). In an aqueous chlorine solution, the three species Cl_2, HOCl, and OCl^- exist together with relative concentrations depending on the pH of the solution. The three species can oxidize organic compounds; however, the HOCl is the most efficient. It has 80 times more

germicidal potency than the OCl^-. The HOCl is formed at pH 6 and at 25°C where it represents about 90% of the chlorine species in the solution (Fukayama et al., 1986). Chlorine dioxide gas (ClO_2) can dissolve in water without reacting with it. ClO_2 has higher oxidation capacity than the HOCl but lower oxidizing potential. ClO_2 is most reactive in the aqueous solution containing phenolic compounds, aromatic and aliphatic tertiary amines aromatic and aliphatic tertiary amines (Fukayama et al., 1986). Chlorine affects bacteria by damaging and disrupting several cellular functions and organelles, which interrupts its metabolic processes (Shang & Blatchley, 1999). It also causes damage to the cytoplasmic membrane, altering its permeability (Venkobachar et al., 1977). Free chlorine has a strong oxidation stress effect and is used to inactivate HAV at 10 mg/L with 30 min of exposure that damages viral nucleic acid (Dennis et al., 1979). The 5' non-translating region (NTR) of HAV nucleic acid is associated with viral replication and translation, as it contains an internal ribosome entry site (IRES), and. the inactivated virus has a shorter 5` NTR, indicating that the chlorine inactivation effect on HAV is due to the disruption of nucleic acids at the 5' NTR of the virus (Li et al., 2002).

Another study in 2008 found that when individual strawberries (with the average weight of 21.93±4.09 g) were spotted with 2 to 4 µl of HAV strain HM-175 at 6–7 log PFU and exposed to 10 ppm free chlorine, inactivation of 0.7, 1.4, 1.7, 1.5 and 2.2 log PFU after 0.5, 1, 3, 5 and 10 min, respectively were reported (Casteel et al., 2008). However, increasing the free chlorine concentration dose to 20 ppm and treating the inoculated strawberries (average weight of 18.95±2.25 g), inactivation of 0.6, 0.7, 1, 1.2, 2.3 log PFU after 0.5, 1, 3, 5 and 10 min was obtained, respectively. Moreover, at a higher free chlorine dose of 200 ppm the strawberries

(average weight of 22.84±4.37 g) had HAV inactivation of 0.5, 0.6, 1.2 and 2.6 log PFU after 0.5, 1, 3 and 5 min exposure time, respectively. Similar to the strawberries, cherry tomatoes with an average weight of 17.21+2.51 g were exposed to 10 ppm free chlorine resulting in HAV inactivation of 0.6, 1.3, 1.1, ≥ 2.3, ≥ 2.3 log PFU after 0.5, 1, 3, 5 and 10 min, respectively. When exposed to 20 ppm free chlorine, cherry tomatoes (average weight of 16.57±3.61 g) had inactivation of 0.8, 1.4, ≥ 2.4, ≥ 2.4 and ≥ 2.4 log PFU after 0.5, 1, 3, 5 and 10 min respectively. Similarly, HAV spots inoculated on a head of lettuce (average weight of 1.33±0.40 g) were exposed to 10 ppm free chlorine achieved inactivation level of 0.8, 1.3, 1.4, ≥ 2.2, ≥ 2.2 log PFU after 0.5, 1, 3, 5 and 10 min, respectively. At a higher free chlorine concentration of 20 ppm, heads of lettuce (average weight of 1.23±0.32 g) achieved an HAV inactivation of 0.8, 1, 0.8, ≥ 1.7 and ≥ 1.7 log PFU after 0.5, 1, 3, 5 and 10 min, respectively (Casteel et al., 2008).

When 1 ml HAV HM-175 (titer of ~ 4-4.5 log PFU/ml) was, mixed with chlorine dioxide (ClO_2) solution at neutral pH at a concentration of 0.40 mg/l, reduction of 2, 3 and 4 log PFU/ml after a mean exposure time of 2.35, 6.79 and 19.58 min was obtained, respectively (Zoni et al., 2007). At a higher concentration of 0.60 mg/l ClO_2 solution, a viral reduction of 3 and 4 log PFU/ml after a mean exposure time of 0.53, 0.85 and 1.45 min was demonstrated, respectively. Furthermore, when the concentration was increased to 0.80 mg/l, a 2, 3 and 4 log PFU/ml viral reduction was reported after a mean exposure time of 0.26, 0.35 and 0.43 min respectively (Zoni et al., 2007). The results indicate for rapid inactivation of HAV in ClO_2 solution, a ≥ 0.6 mg/l aqueous ClO_2 concentration is required.

When individual strawberries with an average weight of 15 g were spotted with 10 spots of 50 µl containing 1.6×10^6 $TCID_{50}$ of HAV strain HM-175 and exposed to 200 ppm of free chlorine in water for 30 s, the HAV titer of the strawberries was reduced by -1.8 ± 0.26 $TCID_{50}$ (Butot et al., 2008). The same treatment was applied to other types of produce. In blueberries, the HAV titer was reduced by -2.4 ± 0.34 $TCID_{50}$, in raspberries, the HAV titer was reduced by -0.6 ± 0.21 $TCID_{50}$, in basil, the HAV titer was reduced by -2.4 ± 0.21 $TCID_{50}$, and in parsley the HAV titer was reduced by -1.4 ± 0.04 $TCID_{50}$. Individual raspberries with the same weight and conditions were exposed to 5 ppm ClO_2 for 10 minutes, in which the HAV titer was reduced by -0.97 ± 0.21 $TCID_{50}$. When exposed to 10 ppm ClO_2 for 10 minutes, the HAV titer was reduced by -0.79 ± 0.07 $TCID_{50}$. Similar to the raspberries, parsley exposed to 5 ppm ClO_2 for 10 minutes resulted in an HAV titer reduction of -1.05 ± 0.14 $TCID_{50}$ and exposure to 10 ppm ClO_2 in water for 10 minutes resulted in a reduction of -1.75 ± 0.19 $TCID_{50}$ (Butot et al., 2008). This research indicates that a high concentration of the disinfectant does not necessarily result in a higher virus reduction. It is dependent on the pH, produce and virus type; this research provides valuable information about the disinfection conditions of HAV in produce.

When 30 ml of 10^7 PFU/ml of HAV HM-175 was added to 270 ml of a solution of quaternary ammonium 10% with glutaraldehyde 5% (disinfectant #1, pH 7), sodium hypochlorite 12% (disinfectant #2, pH 13) or stabilized dioxide chlorine 2% (disinfectant #6, pH 8.3) and incubated for 5 min at 4 or 22ºC. disinfectant #1 at 1000 ppm and 4 and 22ºC, resulted in a 1.35 and 4.75 log PFU/ml reduction of HAV count, respectively (Jean et al., 2003). At 3000 ppm and 4 and 22ºC, a 1.95 and 7.25 log PFU/ml reduction were obtained, respectively. disinfectant #2 at 500,

1000 and 3000 ppm at 4 and 22ºC, for 5 min contact time resulted in a 7 log PFU/ml reduction for both 4 and 22ºC, independently of the concentration of the active ingredient. disinfectant #6 at 500 ppm, at 4 and 22ºC resulted in a 0.69 and 2.2 log PFU/ml reduction, respectively (Jean et al., 2003). When 20 µL of 10^6 PFU/ml HAV suspension was inoculated onto the surface of a disk (1 cm in diameter) made of polyvinyl chloride, high-density polyethylene, aluminum, stainless steel or copper (6.1 kg/m^2), 20 µL of disinfectants #1 and #2 were added to the disc for 5 min at 22ºC. A 3 log PFU/ml reduction resulted from treatment with 3000 ppm of disinfectant #1. A 5 log PFU/ml reduction resulted from treatment with 3000 ppm of disinfectant #2. There was a significantly higher veridical effect on the copper surface compared to other surfaces (Jean et al., 2003). This research proves the high efficacy of both quaternary ammonium 10% with glutaraldehyde 5% and sodium hypochlorite 12% to inactivate HAV in solution and on contact surfaces. When 50 ml of 10^5 $TCID_{50}$/ml HAV strain CF 53 suspension was mixed with glutaraldehyde to obtain final 0.50, 0.10, or 0.02% (w/v) concentrations and incubated at 23°C for 3 to 30 min using radioimmunoassay to measure antigenicity, 0.02% glutaraldehyde showed, a 0% to 57% antigen titer reduction after 3 and 30 min, respectively (Passagot et al., 1987). At 0.10% glutaraldehyde, a 40% to 68% antigen titer reduction resulted after 3 and 30 min, respectively. At 0.5% glutaraldehyde a 79% antigen titer reduction occurred after 3 min. The study also measured virus infectivity after treatments with different glutaraldehyde concentrations. At 0.02% glutaraldehyde, a 0.2 and 1.0 log $TCID_{50}$ viral titer reduction resulted after 3 and 30 min, respectively. At 0.10% glutaraldehyde a 1.4 and 2.9 log $TCID_{50}$ viral titer reduction after 3 and 30 min was obtained, respectively. At 0.5% glutaraldehyde a 3.5 and 4 log $TCID_{50}$ viral titer reduction occurred after 10 and 30 min respectively (Passagot et al., 1987).

The results indicate a significant HAV viral reduction using 0.5% glutaraldehyde for 10 and 30 min which can be utilized to decontaminate surfaces and tools.

When 20 µL of 2.0% glutaraldehyde with pH 7.5 was placed on stainless-steel disks inoculated with 10 µL of fecally suspended HAV for 1 min, the result was > 4 logs PFU reduction of the HAV titer (Springthorpe et al., 1990). Similarly, solutions of 6% sodium hypochlorite, 5,000 ppm free chlorine at a pH of 11.2, 0.4% quaternary ammonium compound (QAC) no. 1 and undiluted 23% HCl (toilet bowl cleaner) resulted in > 4 logs reduction of HAV PFU (Springthorpe et al., 1990). However, the tested phenolics, iodine-based products, solutions of acetic, citric, peracetic, and phosphoric acids, and alcohol, all showed <1 log PFU reduction of HAV titer (Springthorpe et al., 1990). This research provides important information about the efficacy of different disinfectant compounds against HAV.

When 1 ml of dialyzed HAV HM-175 at 10^6 - 10^7 RFU/ml (Relative Fluorescence Unit) was applied to 100 ml aliquots of ozone-treated buffer (ODF phosphate-carbonate buffer) at pH 6 with 0.1 mg/L ozone, a 1 log RFU/ml reduction of the virus was obtained after 120 s (Vaughn et al., 1990). At 0.5 mg/L ozone, a 1.5 and 3 log RFU/ml reduction was achieved after 30 s and 180 s, respectively, at pH 6, 7 and 8. At 1 mg/L ozone at pH 6 and 7, a 5 log RFU/ml reduction of the virus was obtained after 30 s and at pH 8 a 5 log RFU/ml reduction of the virus resulted after 60 s (Vaughn et al., 1990). The data indicates the ability of ozone at low concentrations and short times to inactivate HAV, which can be used in the decontamination of tools and surfaces in the food and medical sectors.

Photodynamic Inactivation

Photodynamic inactivation (PDI) utilizes a combination of visible light, oxygen and a photosensitizing chemical compound in order to produce reactive oxygen species (ROS). The mechanism of action depends on the photosensitizers' ability to absorb the light and transfer energy or electrons to the molecular oxygen (Hamblin et al., 2004). Examples of ROS include singlet oxygen superoxide anions and hydroxyl radicals, which are capable of destroying a broad spectrum of cellular targets like proteins, nucleic acids and lipids. As these targets are critical for cell survival, it is very unlikely for the microbes to develop resistance to treatments with ROS (Vatansever et al., 2013). A primary advantage of this approach is that there is no need for internalization of the drug to ensure cell death or inactivation, which again minimizes the microorganisms' chance to develop resistance (Lauro et al., 2002). Photo-antimicrobial action can reduce the expression of virulence factors such as protein toxins, proteases of *Pseudomonas aeruginosa*, α-hemolysin, sphingomyelinase, and lipopolysaccharides of *Escherichia coli* and *Staphylococcal aureus* by chemical oxidation (Tubby et al., 2009). This ability to degrade virulence factors is extremely beneficial, as these agents (i.e. endotoxins) may cause damage to the host cell even if there is no active infection (Komerik et al., 2000). Photo-antimicrobial action can also damage other phenotypical processes such as biofilm formation which is related to multidrug resistance (De Melo et al., 2013; Cieplik *et al*., 2014). Both chemical photo-sensitizer compounds, such as phenothiazinium salts, porphyrins, or phthalocyanines and natural photo-sensitizer products, such as curcumin (Dahll et al., 1994) (approved as a food additive E100), as well as riboflavin (vitamin B2) (Maisch et al., 2014), and hypericin (Yin et al., 2015) have a high biocompatibility in host tissues. They can be applied at low concentrations

(micromolar range), and without light activation they are harmless to host tissues. It is relatively easy to activate the photo-antimicrobial compounds using low powered lasers light emitting diodes or halogen lamps which should be enough to initiate the reaction. Moreover, with the advent of the fiber optic technology many infections inside the body can be reached endoscopically, which permits the application of the photo-antimicrobial agent with light. Even if the infection was deep-seated, a transcutaneous needle can potentially deliver both the photo-antimicrobial agent and light with a fiber (Cochrane et al., 2013).

Glueck et al (2017). Treated the surface of cucumber slices with 250 µL of 8.2×10^7 CFU (7 log CFU) *E coli* ATCC 25922 suspension and treated with 250 µL of the cationic curcumin derivative SACUR-3 at 10, 50 and 100 µM concentration, then illuminated from above with an LED-array consisting of 432 LEDs at a distance of 15 cm and radiant exposure 33.8 J cm^2, a reduction of 3, 4 and 4.5 log CFU were obtained, respectively (Glueck et al., 2017). Similarly, photodynamic inactivation (PDI) against the same bacteria on lettuce leaves with 10 µM SACUR-3 resulted in 3 log CFU reduction, while 50 µM and 100 µM SACUR-3 resulted in more than 6 log CFU reduction. In fact, the 50 µM SACUR-3 resulted in a relatively higher reduction than the 100 µM SACUR-3 concentration. PDI on tomatoes treated with 10 and 100 µM SACUR-3 resulted in a 3 log CFU reduction, while treatment with the 50 µM resulted in a 6 log CFU reduction (Glueck et al., 2017). Furthermore, fenugreek seeds inoculated with 7.3×10^5 CFU *E. coli* and treated with PDI and 10 µM SACUR-3 resulted in a 3 log CFU reduction, while the same conditions with 50 µM SACUR-3 resulted in a 5 log CFU reduction and with 100 µM, approximately 5 log CFU reduction (Glueck et al., 2017). Mung beans inoculated with 9.6×10^6

CFU *E. coli* and treated with PDI and 10 μM, 50 μM, and 100 μM SACUR-3 resulted in less than 1 log CFU reduction. This work shows that PDI treatments using the cationic curcumin derivative SACUR-3 is an effective antimicrobial tool against *E. coli* and other foodborne microorganisms (Glueck et al., 2017).

A bacterial suspension of the Gram-positive bacterium *Enterococcus faecalis* ATCC 29212 at 10^7 CFU/ml was treated with cationic Tri-Py+-Me-PF porphyrins of 0.5 μM, 1.0 μM and 5.0 μM and illuminated with white light PAR radiation, 13 OSRAM 21 lamps of 18 W each, wavelength of 380–700 nm at 20–25°C (Alves et al., 2009). The light fluence of 14.4 J/cm^2 resulted in a reduction of 6.80 log CFU/ml for the three tested concentrations. Similarly, 0.5 μM, 1.0 μM and 5.0 μM concentrations of the Tri-Py+-Me-CO_2 Me porphyrins resulted in a 7 log CFU/ml reduction under different light fluences, the lowest light fluence being 14.4 J/cm^2 with 5.0 μM. Moreover, the Tetra-Py+-Me porphyrins at 1.0 μM and 5.0 μM with 65 J cm^2 and 14.4 J/cm^2 light fluence respectively, resulted in a 7 log reduction of CFU/ml, while with 0.5 μM and 65 J/cm^2 light fluence 5 log CFU/ml reduction. A concentration of 0.5 μM Di-Py+-Me-Di-CO_2 H porphyrins and 64.8 J/cm^2 of light exposure resulted in a reduction of 7.03 log CFU/ml *E. faecalis* (Alves et al., 2009). Similarly, a bacterial suspension of *E. coli* ATCC 13706 at 7 log CFU/ml treated with 5.0 μM Tri-Py+-Me-PF and Tri-Py+-Me-CO_2 Me porphyrins with a light fluence of 21.6 J/cm^2 resulted in a 7 log reduction (Alves et al., 2009). Tetra-Py+-Me at 5.0 μM with 64.8 J/cm^2 light fluence resulted in a reduction of 7.50 log of *E. faecalis* count. Tricationic porphyrin Tri-Py+-Me-CO_2 H at of 5.0 μM under a light fluence of 64.8 J/cm^2 resulted in a reduction of 5.18 log (Alves et al., 2009).

When 10 ml of a 7 log CFU/ml bacterial suspension of *Listeria monocytogenes* ATCL3C 7644 was incubated for 2 h with 7.5 mM of 5-aminolevulinic acid (ALA) and illuminated for 20 minutes with visible light LED-based light source ($\lambda = 400$ nm) and energy density of 20 mWs/cm^2 reduction at 4 logs CFU/ml was obtained (Buchovec et al., 2010). When 5.9 log CFU/cm^2 of biofilm-associated cells of *L. monocytogenes* (adhered to a plastic coupon) were incubated in 10 mM ALA solution illuminated with light ($\lambda = 400$ nm) for 15 min with a light dose of 18 J/cm^2, a 3.1 log reduction was obtained (Buchovec et al., 2010). The results show that both vegetative cells and biofilms of *L. monocytogenes* can be inactivated with PDI, which will have a direct application in the food industry and other industrial and medical sectors. When 20 ml of a 1×10^7 CFU/ml *Salmonella enterica serovar* Typhimurium strain DS88 [SL5676 SmR (pLM32)] was incubated for 60 min with chlorophyllin–chitosan complex (1.5×10^{-5} M Chl–0.1% CHS) in vitro and illuminated by visible light ($\lambda = 405$ nm) at a light dose of 38 J/cm^2, a reduction of 7 log CFU/ml was obtained (Buchovec et al., 2016). When strawberries were soaked in a bacterial suspension of *S. enterica* ($\sim 1 \times 10^7$ CFU/ml) suspension for 30 min and soaked in Chl–CHS 1.5×10^{-5} M Chl–0.1% CHS for another 30 min, then illuminated with a light dose of 38 J cm^2 for 60 min, a 2.2 log CFU/ml reduction was obtained (Buchovec et al., 2016). When strawberries contaminated with mold and yeast at 4 log were soaked in chlorophyllin–chitosan complex Chl–CHS (1.5 × 10−5 M Chl–0.1% CHS) for 30 min, then illuminated with visible light (38 J/cm^2) for 60 min, a 1.4 log CFU/ml reduction of the mold and yeast populations occurred (Buchovec et al., 2016).

When 10 ml of four foodborne bacteria, *E. coli* O157: H7 EDL 933, *S.* Typhimurium ATCC 14028, *L. monocytogenes* BAA-679 and *S. aureus* ATCC 6538 at 10^6 CFU/ml in tryptic soy broth were illuminated for 7.5 h at 20, 15 and 10°C at a distance of 4.5 cm by blue LED of 461 nm wavelength, with dosages of 596.7, 431.2, and 688.0 J/cm^2 respectively, the blue light illumination wavelength 461 nm at 15°C resulted in 4.9, 5.0, 4.3 and 5.2 log CFU/ml reduction of *E. coli* O157:H7, *S.* Typhimurium, *L. monocytogenes* and *S. aureus*, respectively, while the green 521 nm was less effective showing 1.7 log CFU/ml reduction for *S.* Typhimurium and *S. aureus* and 1.0 and 0.9 log CFU/ml reduction of *E. coli* O157:H7 and *L. monocytogenes*, respectively (Ghate et al., 2013). Similarly, changing the temperature to 10°C and illumination to a light at 461 nm wavelength resulted in 5.1, 4.6, 5.2 and 4.7 log CFU/ml reductions of *E. coli* O157:H7, *S.* Typhimurium, *L. monocytogenes* and *S. aureus*, respectively, and 521 nm at the same temperature resulted in 1.8, 1.7, 1.5 and 1.5 log CFU/ml reduction of *E. coli* O157:H7, S. typhimurium, *L. monocytogenes* and *S. aureus*, respectively. Illumination at 20°C for all wavelengths did not show any reduction and 642 nm did not have efficacy against any of the tested bacteria (Ghate et al., 2013).

Light treatment against three strains of *E. coli* O157:H7 (ATCC 35150, C7927, and F12), three serotypes of *L. monocytogenes* (ATCC BAA-679 [ST 1/2a], ATCC BAA-839 [ST 1/2b], and ATCC 13932 [ST 4b]), and five serotypes of *Salmonella spp*. (*S.* Agona ATCC BAA-707, *S.* Newport ATCC 6962, *S.* Saintpaul ATCC 9712, *S.* Tennessee ATCC 10722, and *S.* Typhimurium ATCC 14028) at 10^9 CFU/ml using equal culture cocktails of each strain or serotype onto ten sites on the surface of a mango, by illumination using high intensity LED light (wavelength 405±5 nm) at a distance of 4.5 cm at 4, 10, or 20°C, for 24–48 h, with a total dose

of 2.6–3.5 kJ/cm^2 caused *E. coli* O157:H7 and *S.* spp. populations to be reduced to below detection levels of 1.0 log CFU/ml, while the *L. monocytogenes* strains were reduced to < 1.6 log CFU/cm^2 (Kim, Tang, Bang & Yuk, 2017). At 20°C, after 24 h, at a dose of 1.7 kJ/cm^2, *E. coli* O157: H7 was reduced to < 1.0 log CFU/ml and *S.* spp. population was reduced to 1.2 log CFU/cm^2, while on the other hand, *L. monocytogenes* population were not affected by illumination at this dose (Kim, Tang, Bang & Yuk, 2017). These data indicate that the illumination using 405±5 nm light at chilling temperatures has antibacterial effects against some foodborne bacteria for fruit preservation. When 10 µL of *S. enterica* serovar *S.* Agona (BAA-707), *S.* Newport (ATCC 6962), *S.* Saintpaul (ATCC 9712), or *S.* Typhimurium (ATCC 14028) suspensions at 10^5 CFU/ml were inoculated onto ten spots on cut papaya, illuminated for 24-48 h at 4, 10, or 20°C at a distance of 2.3 cm with LED light (wavelength 405±5 nm) at a dose of 0.9-1.7 kJ/cm^2, at 4°C a dose of 1.7 kJ/cm^2, after 24 h, caused 1.0-1.2 log CFU/cm^2 reduction of all *Salmonella* serovars (Kim, Bang & Yuk, 2017). At 10°C, after 36 h, a dose of 1.3 kJ/cm^2, caused 0.3, 0.6, and 1.3 log CFU/cm^2 reduction of *S.* Newport, *S.* Saintpaul, and *S.* Typhimurium, respectively. However, the treatment at 20°C was not effective against the bacteria (Kim, Bang & Yuk, 2017). These results indicate that the LED light at a wavelength of 405±5 nm together with chilling temperatures is a useful antibacterial treatment against several *S. enterica* serovars.

In another study, a pellet of 1.2×10^{10} CFU/ml *E. coli* was dissolved and mixed with 1000 µL of 30 µM photosensitizer Flavin Mononucleotide (FMN), and illuminated for 120 min with blue light-diode (wavelength 462 nm) at a dose 7.2 J/cm^2 and an illumination intensity of 1 mW/cm^2 (7.2 J/cm^2) causing a 96% inactivation rate of *E. coli* (Liang et al., 2015). Furthermore, 77%

inactivation of *E*. *coli* using 600 µM FMN for 60 min with the same illumination and light intensity was obtained. An 86% inactivation rate of *E. coli* was obtained with 120 µM FMN illumination for 120 min and 1 mW/ cm^2 light intensity was used (Liang et al., 2015). These results suggest that the combined treatment of the FMN at low concentration with blue light at 462 nm can efficiently inactivate *E. coli*. When 10 ml of orange juice inoculated with a cocktail of *S. enterica* serovars [*Salmonella* Gaminara (BAA 711), *Salmonella* Montevideo (BAA 710), *Salmonella* Newport (ATCC 6962), *Salmonella* Saintpaul (ATCC 9712), and *Salmonella* Typhimurium (ATCC 14028)], at an initial concentration 10^6 CFU/ml was illuminated from above by LED with a wavelength of 455 to 465 nm and irradiance of 92.0 mW/cm^2 for 13.58 h at 4 °C, a 3.3 log CFU/ml reduction resulted (Ghate et al., 2016). At 147.7 mW/ cm^2 for 8.46 h and 254.7 mW/ cm^2 irradiance for 4.91 h, a 2.2 and 2.1 log CFU/ml reduction resulted, respectively, and at 28°C with 92.0 mW/cm^2 irradiance for 13.58 h, a 3.6 log CFU/ml reduction was obtained; at 147.7 and 254.7 mW/cm^2 irradiance for 8.46 and 4.91 h, respectively, a 2.6 and 2.0 log CFU/ml reduction was obtained, respectively. Moreover, at 20°C, 92.0 mW/cm^2 irradiance for 13.58 h, a 4.8 log CFU/ml reduction resulted, at 147.7 and 254.7 mW/cm^2 irradiance for 8.46 h and 4.91 h respectively, a 3 and 2.5 log reduction were obtained, respectively (Ghate et al., 2016). The data point to the potential use of LED at 455 to 465 nm to preserve fruit juice.

When ten ml of five different foodborne bacterial suspension at 10^6 CFU/ml were illuminated for 9h at 10, 4 and 25°, at a distance of 33 mm from above by light-emitting diode (wavelength 405 nm) at a dose 306 J/cm^2, where 1.9, 2.1 and 4 log CFU/ml reduction of *S. aureus* at 4, 10 and 25ºC was obtained, respectively (Kumar et al., 2015). At similar treatment conditions, a 0.6 log

CFU/ml reduction of *S.* Typhimurium at 25ºC was obtained, and 2.3 and 1.9 log CFU/ml reduction of *B. cereus* and *L. monocytogenes* was obtained, respectively. A 1.2, 1.3 and 0.5 log CFU/ml reduction of *E. coli* O157:H7 at 4, 10 and 25ºC was achieved, respectively. While using 520 nm illumination at 25ºC and dose of 3,060 J/cm^2, a 1.2 log CFU/ml reduction of *S. aureus* obtained, while the 10 and 4ºC conditions did not cause reduction. A 0.7, 1.2 and 1.0 log CFU/ml reduction of *L. monocytogenes* at 25, 10 and 4 ºC was achieved, respectively. The 405 nm and 520 nm LEDs were not effective against *P. aeruginosa*. Moreover, the 520 nm LED was not effective against *E.coli* O157:H7 or *S.* Typhimurium (Kumar et al., 2015). The data indicates that 405 nm LED is the more effective in reducing bacterial population than the 520nm. Also, *S. aureus* had the highest reduction when illuminated with 405 nm and at 25ºC, followed by *B. cereus*.

When 0.1 ml of two foodborne bacteria (*S. aureus* strain 27853 and *P. aeruginosa* strain 27853) at 10^8 CFU/ml were mixed with 0.1 ml of 0 to 10 mM 5-Aminolevulinic acid, incubated for 60 min, and illuminated for 120 min with red light (wavelength 635±5 nm) at a dose of 216 J/cm^2, the ALA concentration of 1.0 mM with a light dose of 162 J/cm^2 achieved a complete reduction of *S. aureus* (Hsieh et al., 2014). At 1.0 mM ALA, a 2.3, 3.3, 4.0 and 4.7 log CFU/ml reduction of *P. aeruginosa* was obtained using 30, 60, 90 and 120 min illumination times, respectively. At 2.5 mM ALA, a 3.0, 4.1, 5.3 and 6.3 log CFU/ml reduction of *P. aeruginosa* using 30, 60, 90, and 120 minutes was obtained, respectively. A 6.5 log CFU/ml reduction at 5 mM ALA with a light dose of 162 J/cm^2 for 90 min resulted. At 10 mM ALA no surviving cells were detected (Hsieh et al., 2014). The data indicates that the Gram-positive *S. aureus* was more sensitive to

PDI than the Gram-negative *P. aeruginosa.* The major factor could be the complex outer membrane of the Gram-negative bacteria which hampers the photosensitizers' penetration of the bacterial cell membrane. When a 10^8 CFU/ml suspension of *Vibrio parahaemolyticus* ATCC 17802 was incubated for 5 min at 37ºC with 5, 10 and 20 µM curcumin dissolved in alcohol, illuminated for 60 s with blue light (wavelength of 470 nm), and a light dose of 3.6 J/cm^2, a 2 log CFU/ml reduction at 5 µM curcumin was obtained, while 10 and 20 µM curcumin resulted in a 6.5 log CFU/ml reduction of *V. parahaemolyticus* from oyster flesh (Wu et al., 2016). Similarly, the flesh of six oysters was contaminated with 5 log CFU/g *V. parahaemolyticus,* incubated for 3 h at 10ºC in an artificial seawater system with 10 µM curcumin, then illuminated at a wavelength of 470 nm and a light dose of 3.6 J/cm^2 from the bottom shell for 60 s. The oyster flesh was then removed, homogenized, filtered and plated. A 5 log CFU/g oyster flesh reduction of *V. parahaemolyticus* was obtained (Wu et al., 2016). This research indicated that PDI using curcumin as a photosensitizer shows improved inactivation effects against *V. parahaemolyticus* in oysters. It can have a direct application in the food sector.

When two ml of 7.6×10^7 CFU/ml *S. aureus* strain DSMZ 18587 or *E. coli* strain ATCC 25922 bacterial suspensions were incubated with 5 µM, 10 µM or 50 µM polyvinylpyrrolidone curcumin (PVP-C) at 5, 15 and 25 min followed by illumination from below with blue light (wavelength 432 nm) for 60 min at a fluence of 33.8 J cm^{-2}, at 5 µM PVP-C, 6, 5 and 1 log CFU/ml reduction of *S. aureus* were obtained, respectively (Winter et al., 2013). At 10 µM PVP-C, incubation for 5, 15 and 25 min resulted in 5.6, 5.8 and 4.9 log CFU/ml reduction of *S. aureus* was obtained, respectively. At 50 µM PVP-C, after 5, 15, and 25 min incubation with

illumination, a 6.9 log CFU/ml reduction was achieved after 5 min, with total eradication of the bacteria after 15 and 25 min. Similarly, preincubation of *E. coli* with 500 mM $CaCl_2$ for 10 min followed by incubation with 50 μM PVP-C for 15 and 25 min with similar illumination showed a 4 log CFU/ml reduction of *E. coli* colonies (Winter et al., 2013). This research demonstrates the ability of PVP-C to effectively reduce *S. aureus* and *E. coli* colonies. Moreover, PVP-C is water-soluble and does not require the addition of organic solvents such as Dimethyl sulfoxide (DMSO) for solubility as with underivatized curcumin. Overcoming curcumin's solubility problem opens the door for future employment of PVP-curcumin mediated PDI in clinical applications and food production.

When 250 μL of *S. aureus* (ATCC 25923) at 10^7 CFU/ml was mixed with 250 μL of 50 μM or 100 μM PVP-C, coated on cucumber pieces and illuminated from above with blue light (435±10 nm wavelength and a fluence of 33.8 J/ cm^2), a 2.6 log CFU/ml reduction was obtained (Tortik et al., 2014). Similarly, when green, red and yellow pepper pieces coated with *S. aureus* suspensions were incubated with 50 μM PVP-C followed by illumination at 435±10 nm 33.8 J/ cm^2, a mean reduction of 2.5 log CFU/ml was obtained (Tortik et al., 2014). Chicken pieces coated with *S. aureus* and incubated with 50 μM or 100 μM followed by illumination at 435±10 nm achieved a 1.7 log CFU/ml reduction of *S. aureus* (Tortik et al., 2014). The results of this research show the ability of curcumin-mediated PDI to decontaminate vegetables and poultry from *S. aureus*. Incubation of 50 μL of 5×10^5 CFU/50 ml methicillin-resistant *S. aureus* (MRSA/JD004, MRSA/L1, L2, and L4) for 10 min with 10 μg/ml indocyanine green (ICG; an inexpensive dye with a longer light absorption peak of light in the near infrared [NIR] region),

and NIR wavelength irradiation ranging from 700 to 2200 nm (light power of 65.54 mWs/cm^2), achieved a one log CFU/ml reduction with a light dose of 50 J/cm^2 after 25.4 min (Wong et al., 2018). Increased reduction of 2 and 3 log CFU/ml resulted when using 100 J/cm^2 after 25.4 min with 12.5 μg/ml and 25 μg/ml ICG, respectively. A reduction of 2 and 5 log CFU/ml after 50.8 min with a light dose of 200 J/cm^2 and 10 mg/ml ICG were also reported (Wong et al., 2018). These data indicate that increasing ICG concentration and increasing light doses using NIR lamps will give increased reductions suitable for use in medical environments to treat MRSA infections.

When two hundred-fifty μL of a 1.7×10^7 CFU/ml *S. aureus* (ATCC 25923) or a 4.3×10^7 CFU/ml *E. coli* (ATCC 25922) coated porcine skin sample was treated with an equal volume of 50 μM or 100 μM of Polyvinylpyrrolidone- curcumin (PVP-CUR) and illuminated by blue light LED lamp (wavelength 435±10 nm, light fluence 33.8 J cm^{-2}) a 1.7 and 1.3 log CFU/ml reduction of *S. aureus* at 50 μM and 100 μM PVP-CUR was obtained, respectively, and a 0.3 log CFU/ml reduction of *E. coli* with 100 μM PVP-CUR was reported (Tortik et al., 2016). In a similar approach, 50 μM and 100 μM of SACUR-3 (a four-fold cationic and water-soluble curcumin derivative) was applied to *S. aureus,* achieving a 2.2 and 1.8 log CFU/ml reduction, respectively. The same application to *E. coli* resulted in a 3.2 and 3.3 log CFU/ml reduction, respectively (Tortik et al., 2016). These data prove that the cationic SACUR-3 is a more effective photosensitizer against Gram-positive and Gram-negative bacteria than PVP-CUR, which shows relative effectiveness only against Gram-positive bacteria.

When 10 ml of 8 log CFU/ml *S. aureus* ATCC 6538 was incubated for 10 min with 5.0 μM Tetracationic Porphyrin (Tetra-Py+-Me) in phosphate buffered saline (PBS), and irradiated for 180 min with white light at wavelength 400–800 nm with a light dose of 43.2 J /cm^2, a complete 8 log CFU/ml reduction was obtained (Branco et al., 2018). Furthermore, these researchers showed that when 500 μL of 5 log CFU/ml *S. aureus* ATCC 6538 was distributed over 10 cm^2 (5×2 cm) of porcine skin coated with 500 μL of 50 μM Tetra-Py+-Me, incubated for 30 min in the dark, and illuminated with white light at irradiances of 150 mW/cm^2, a reduction of 4 log CFU/ml reduction was obtained (Branco et al., 2018). Tetra-Py+-Me is an efficient photo-inactivator against a wide range of microorganisms, which is readily available in pure form. Similarly, other researchers showed that when 50 μL of 7 log CFU/ml of *Aeromonas hydrophila* ATCC 7966 in suspension was mixed with 950 μL of the photosensitizer Erythrosine B (ERY) at 1×10^{-5} M in the dark for 10 min, and then illuminated with a green LED at a light dose of 78 J/cm^2 for 10 min, a 4.3 log CFU/ml reduction was observed (Yassunaka et al., 2015). A total reduction of *A. hydrophila* after 20 min at light dose of 156 J/cm^2 and 30 min at light dose 234 J/cm^2 was obtained. Using 1×10^{-5} M Erythrosine Methyl Ester (ERYMET)-mediated PDI with a light dose of 84 J/cm^2 after 30 min, *A. hydrophila* was reduced by a 4.4 log CFU/ml. Similarly, when 10^7 CFU/ml *S. aureus* was incubated for 10 min with ERY at 1×10^{-6} M, and illuminated with green light wavelength 510 nm, a 2.2 and 4 log CFU/ml reduction was obtained after 20 and 30 min at light dose of 26 J/cm^2 and 40 J/cm^2, respectively (Yassunaka et al., 2015). ERYMET and Erythrosine Butyl Ester (ERYBUT) at 1×10^{-6} M and a light dose of 4 and 3 J/cm^2 after 10 min resulted in total *S. aureus* reduction to non-detectable levels (Yassunaka et al., 2015). From

these experiments, it appears that the photosensitizer concentration, bacterial type and light dose are factors that contribute to and play a role in the overall inactivation effects.

Biofilms of *S. aureus* strain RN6390 incubated for 30 min with 0.5 mM toluidine blue (without light) resulted in a 2 log CFU reduction. Illumination by near-infrared laser technology (NIRT) at a wavelength of 980 nm on continuous mode for 15 to 100 s alone without toluidine resulted in a 3 log CFU reduction, using a light dose of 454 J/cm^2 (Gandara et al., 2017). When the biofilms were incubated with 0.5 mM toluidine blue for 30 min and illuminated from below with NIRT laser treatment at a 227 J/cm^2 light dose, a 3 log CFU reduction was obtained, and with a 454 J/cm^2 light dose a 5 log CFU reduction was obtained. Similarly, these researchers showed that when the biofilm was incubated for 30 min with 0.5 mM toluidine blue and illuminated from above with a laser light (wavelength of 635 nm) and a fluence of 157 J/cm^2 for 10 min, a 3 log reduction was obtained. Furthermore, biofilm incubation of toluidine blue combined with successive irradiations using the 980-nm laser and 635-nm laser resulted in a total reduction of 4.5 log (Gandara et al., 2017). This research demonstrated that treatments using lasers and toluidine blue efficiently inactivates *S. aureus* biofilms, which could be applied on a large-scale in different scientific and industrial fields. When 0.2 ml of 10^8 CFU/ml *S. aureus* ATCC 25923 suspension was mixed with either 0.1% of the photosensitizer radachlorin gel (a hydrosoluble chlorin composed of a set of sodium salts including chlorin e6, chlorin p6, and purpurine-5) illuminated by diode laser (wavelength of 662 nm) and energy density of 6 J/cm^2 on continuous mode or 0.1% of the photosensitizer toluidine blue O (TBO) (composed of phenothiazinium salts) illuminated by diode laser (wavelength of 633 nm) and energy density of 6 J/cm^2 on

continuous mode, reductions of a 6.1 log and log 5.83 CFU/ml were obtained, respectively (Fekrazad et al., 2016). This research indicates a similar bactericidal effect of the photosensitizers radachlorin and TBO on *S. aureus* which can be used for the inactivation of the bacteria in the industrial and health sectors.

With regards to viral inactivation using PDI human plasma (7 ml) inoculated with 3-4 log PFU/ml hepatitis A virus HM-175 clone (p24A) and 0.01 mM porphyrins tetratosylate (TMPyP4) followed by illumination with long-wavelength (365 nm) UV at a fluence rate of 2.2 mW/cm^2 from above, at a distance of 3.5 cm showed a reduction of 2.5 log PFU/ml after 1 min (Casteel et al., 2004). Increased exposure times of 10, 30, 90 min resulted in a > 4.2 log PFU/ml reduction, while exposure with 0.01 mM porphyrins (TPPS4) for 1, 10, 30 and 90 min resulted in a 0.2, 0.5, 0.7 and 1 log PFU/ml reduction, respectively. The 0.01 mM porphyrins (TBuPyP4) at 1, 10, 30 and 90 min of exposure resulted in 0.2, 1.6, 2.8 and > 3.4 log PFU/ml reductions. Furthermore, 0.01 mM porphyrins (TOcPyW) illuminated for 1, 10, and 30 and 90 min resulted in 0.4, 1.3, 2.2 and 2.5 log PFU/ml reductions, respectively (Casteel et al., 2004).

When murine norovirus 1 (MNV-1) at 8.5 log PFU/ml suspension was mixed with 5 µM and 20 µM curcumin (95% purity) and illuminated with LED blue light source (wavelength of 470 nm) of 3.6 J/cm^2 delivered in 60 s, a 1.32 log PFU/ml and a > 3 log PFU/ml reduction in viral titer was achieved, respectively (Wu et al., 2015). Similarly, when six oysters were exposed to seawater containing 10 µM and 20 µM curcumin (95% purity) for 6 h, allowed to bio-accumulate MNV-1, and illuminated with the same LED blue light source for 60 s, a 0.76 log

PFU/ml and a 1.15 log PFU/ml reduction were obtained, respectively. The data indicates that PDI using curcumin can help to inactivate MNV-1 in oysters (Wu et al., 2015).

When a 7 log $TCID_{50}$/ml FCV-F9 suspension was incubated at room temperature and exposed to LED blue light wavelength (464–476 nm) and 50–60 Hz frequency range, at a light dose of 3 J/cm^2 along with 5 µg/ml curcumin from *Curcuma longa L.* for 30 min, a reduction of 1.75 log $TCID_{50}$/ml was obtained, while similar treatment at 37ºC reduced FCV titer by 4.43 log $TCID_{50}$/ml (Randazzo et al., 2016). These researchers showed that increasing the concentration of curcumin to 50 µg/ml together with PDI resulted in a 3.29 log $TCID_{50}$/ml reduction at room temperature, and a 4.62 log $TCID_{50}$/ml reduction was obtained at 37ºC. When 7 log $TCID_{50}$/ml MNV-1 was exposed to LED blue light for 30 min with 50 µg/ml curcumin at 37 ºC, a 0.40 log $TCID_{50}$/ml reduction was obtained, when the illumination time increased to 120 min, a 0.03 log $TCID_{50}$/ml reduction resulted. A higher concentration of 100 µg/ml curcumin after 30 min illumination resulted in a 0.48 log $TCID_{50}$/ml reduction of MNV-1 and longer exposure of 120 min resulted in 0.73 log $TCID_{50}$/ml reduction (Randazzo et al., 2016). When a 6 log PFU/ml MNV-1 suspension was incubated for 72 h at 4ºC with 2.5 µL of 1 mg/ml concentration of phytochemicals, including curcumin, resveratrol, cinnamic acid, proanthocyanidin, ginsenoside Rh1, ginsenoside F2 or ginsenoside Rg3 (the ginsenosides are the active constituents of most ginseng species, the selected ginsenosides have different glycosidic moieties which gave different antimicrobial effects), a 90.88%, 80.05%, 79.58%, 60.19%, 59.26%, 52.14% and 48.71% viral neutralization (percentage of inactivated virus) was obtained, respectively (Yang et al., 2016). Incubating MNV-1 at 4ºC for 72 h with 0.25, 0.5, 0.75, 1 and 2 mg/ml curcumin

resulted in a 52.85%±11.39%, 68.35%±5.42, 68.96%±3.13%, 89.99%±5.88%, and 90.43%±9.135 viral neutralization, respectively, and 1 mg/ml curcumin for 10, 30, 60 and 120 min resulted in a 33.33%±3.85%, 36.79%±11.13%, 58.89%±2.94% and 82.50%±1.21% viral neutralization, respectively (Yang et al., 2016).

When 20 ml of 10% albumin containing 8.5 log EID_{50}/0.2 ml (50 Percent Embryo Infective Dose) of influenza virus A /Puerto Rico/8/34 H1N1, was mixed with 2 mg/ml solid phase fullerene-based photosensitizer (SPFPS) and irradiated with 324 J/cm^2 for 30 min, by double-sided panel (maximum wavelength 460 nm), complete inactivation (to non-detectable levels) was obtained (Belousova et al., 2014). The authors concluded that this method could potentially be used as an inactivation treatment for H1N1 virus in the plasma of blood donors.
When 6.5×10^6 focus forming units (FFU/ml) dengue virus type I was incubated with 0.01 μM 2,6-diiodo-1,3,5,7-tetramethyl-8-(N-methyl-4-pyridyl)-4,4′-difluoroboradiazaindacene photosensitizer (DIMPy-BODIPY) dye and illuminated for 30 min by a light source with a wavelength of 400–700 nm and fluence of 118 J/cm^2, a 2 log FFU/ml reduction was obtained (Carpenter et al., 2015). Higher concentrations (0.1 and 1 μM) of the DIMPy-BODIPY resulted in a 6 log FFU/ml reduction. Similarly, 7 log PFU/ml vesicular stomatitis virus (VSV) suspension incubated with 0.25 μM, 0.5 μM and 1 μM photosensitizer DIMPy-BODIPY and treated with light fluence of 118 J/cm^2, resulted in 4, 5 and 6 log PFU/ml reductions, respectively. Moreover, when 6.5×10^5 FFU/ml of human adenovirus-5 was incubated and treated with 1 μM and 5 μM DIMPy-BODIPY, 1 and 2 log FFU/ml reductions was obtained, respectively (Carpenter et al., 2015). These results prove that DIMPy-BODIPY is an effective

photosensitizer compound that can be used in PDI of human viruses at a low concentration. Based on this literature on HAV outbreaks, disease symptoms and current inactivation methods, the objectives of this research were to determine the effectiveness of PDI against HAV using grape seed extract solution as a photosensitizer and to find new compounds with anti HAV properties that can be used to inactivate HAV on produce and contact surfaces. The hypothesis was that these plant derived compounds will have antiviral activity against HAV, and that this activity will be enhanced in the presence of light of appropriate wavelengths.

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Chapter II: Photodynamic Inactivation of Hepatitis A Virus on a Formica Coupons, a Model Contact Surface by Grape Seed Extract and UV Light

Abstract

Hepatitis A virus (HAV) outbreaks are a major concern for food producers worldwide as it can withstand harsh environments and storage conditions. In this research, photodynamic inactivation (PDI) was applied against HAV using grape seed extract (GSE). PDI uses a photosensitizer compound, oxygen, and light to generate reactive oxygen species (ROS) to inactivate target microorganisms. The effectiveness of GSE alone and photoactivated GSE at two different concentrations and contact times to inactivate HAV dried on Formica coupons and the surface of tomatoes was investigated. Treatments of HAV on coupons with 0.91 mg/ml GSE showed no effects even after 30 min at room temperature, while treating HAV on tomatoes for at least 5 min with 10 mg/ml GSE resulted in ~1 log PFU/ml reduction. PDI of HAV treated with 0.91 mg/ml GSE and UV light at 254 nm for 3, 10, 20 and 30 min with dose (energy density) of 0.012±0.00, 0.040±0.001, 0.081±0.002 and 0.121±0.003 J/cm^2, respectively, caused 1.45±0.39, 1.74±0.37, 1.48±0.51 and 2.78±0.68 log PFU/ml reduction, respectively. PDI of HAV with GSE showed statistically significant reductions ($P<0.05$) from the control, that was not significant ($p>0.05$) from HAV treated with UV alone for the same time. This indicated that UV light was probably responsible for HAV reduction rather than the production of ROS. PDI of HAV with GSE on tomatoes for 5, 15 and 20 min, dose (energy density) were 0.020±0.001, 0.06±0.02, 0.081±0.002 J/cm^2, respectively causing1.88±0.38, 1.74±0.37 and 1.98±0.32 log PFU/ml reduction, respectively. Reductions of HAV with GSE, HAV with PBS and HAV with GSE without UV were statistically significant from the control but not significant from each other ($P>0.05$). This could be due to difficulties in virus recovery from tomatoes. These results showed

the antiviral effect of GSE against HAV. The data obtained can be used to optimize photodynamic conditions for use against HAV.

Introduction

Hepatitis A virus causes a serious concern for the global food manufacturing sector. An essential characteristic of the virus is its ability to survive harsh and severe environmental conditions. HAV virions are stable at a pH of 5; at this pH, release of the genomic RNA occurs at approximately 76°C and the viral protein melts at approximately 77°C (Wang et al., 2015). The empty proteins have similar characteristics of the full virions but can tolerate higher temperatures up to 81°C and a much lower pH level (2) (Walter et al., 2012; Wang et al., 2015). Furthermore, HAV can remain infectious for 5 h after exposure to a pH of 1 for 2 h at room temperature, while the highly purified virus can be infectious at room temperature for up to 8 h at a pH of 1 (Scholz et al., 1989). Other enteroviruses such as Poliovirus type 1 (PV l) and coxsackie B viruses are not stable under these same conditions (Scholz et al., 1989). Also, when feces contaminated with HAV are dried and stored at 25 °C at a relative humidity of 42% for 30 days, the virus remains viable and infectious (Mccaustland et al., 1982). This high level of survivability is a significant factor in the contamination of a wide variety of food products and the subsequent occurrence of several HAV outbreaks.

In 1988 the most massive HAV outbreak known to date occurred in Shanghai, China, due to the consumption of contaminated raw clams and approximately 292,301 cases were reported (Halliday et al., 1991). In 2003 in Pennsylvania, a large outbreak of HAV occurred among diners in a local restaurant, where clinical testing proved all workers to be free from HAV infection (Wheeler et al., 2005). The patients recalled eating salsa in the restaurant, which contained green onions from Mexico, that onions appeared to be the source of the infection and were

contaminated before arrival at the restaurant. There were 601 total cases in this outbreak; of these, 3 died and 124 were hospitalized (Wheeler et al., 2005). Another outbreak in 2016 related to frozen strawberries led to 143 cases in 9 states, with no fatalities recorded (CDC. 2016). In 2017, another multistate outbreak of HAV occurred in the U.S.; mainly intravenous drug users and the homeless were affected by the disease (CDC, 2018). The existence of HAV is ancient; its recorded history goes back to the ancient Greek and Roman times. Hippocrates referred to catarrhal jaundice and described it as the fourth kind of jaundice mentioning its etiology and frequency in the book De internis affectionibus (Cockayne et al., 1912). However, the most accepted reference to the disease in epidemic form was recorded by Cleghorn in Minorca in 1745. Others have referred to catarrhal jaundice cases during the American civil war, as the disease was common among the troops. Furthermore, similar cases were mentioned in the Boer war in South Africa, although its mortality rate was limited (Cockayne et al., 1912).

HAV belongs to the *Picornaviridae* family with, subtle but significant structural differences compared to the other picornaviruses (Wang et al., 2015). The virus capsid has a smooth surface with no canyons surrounding the 5-fold axis of symmetry. Canyons are essential to fit the host cell receptor as in the case of other enteroviruses (Rossmann et al., 1985). The capsid proteins are remarkably negatively charged with some positively charged fringes. A unique feature of HAV is that although it is a non-enveloped virus, it conceals itself with a host-derived membrane after the virions are released from infected liver cells, hijacking the identity of the host cell to escape the immune system, and this enveloped HAV (eHAV) particle size is approximately 50-100 nm in diameter (Feng et al., 2013). The eHAV circulates the bloodstream of the host using

the identity of the exosomes, small vesicles secreted by the cells that have a critical role in intracellular communications (Bobrie et al., 2011). Once the eHAV are released across the canalicular membrane, it loses its membrane by the effect of the bile acids inside the proximal biliary canaliculus (Hirai-Yuki et al., 2016). When HAV is shed in feces it is in the form of a non-enveloped virus (Feng et al., 2013). Due to HAV's ability to move from cell to cell by transcytosis (Dotzauer et al., 2000; Dotzauer et al., 2005), it is concluded that the virus enters the cell in an intact form. However, the exact mechanism of disassembly of the capsid and release of the virus RNA genome inside the host cell is still unclear (Wang et al., 2015).

HAV receptor was found in primates and, the expression cloning and sequence analysis of the c-DNA of the receptor indicated a mucin-like class I integral membrane glycoprotein comprising of 451 amino acids, that was named the HAV cellular receptor 1 (HAVcr-1) (Kaplan et al., 1996). In humans a homolog receptor was found, named (huHAVcr-1), that contains 13 hexameric repeats, while in monkeys (HAVcr-1) there are 27 hexameric repeats (Feigelstock et al., 1998). Moreover, the human (huHAVcr-1) lacks a 12 C-terminal amino acid in the cytoplasmic domain and was found to be expressed in the organs including the liver, small intestine, colon, and spleen, but most strongly in the kidney and testis (Feigelstock et al., 1998). In 2017, Das and his team concluded that HAVcr-1 cell receptor is not essential for HAV entry into the host cell, or for replication in permissive strains of mice, and suggested that HAV cr-1 may facilitate the early infection stages by the enveloped virus through binding between the TIM1 (HAVcr-1) receptor and the phosphatidylserine (PtdSer) residues on the eHAV membrane (Das et al., 2017). At present, the mechanism of HAV entry into the host cell for the both the

naked and enveloped virus is unknown; more research is required to discover the actual mechanism and the cell receptors used by the virus.

The immune response to HAV infection is through the production of serum antibodies IgM (Lemon et al., 1980). Usually, the immune response against the virus infection is delayed (Feng et al., 2013). However, in chimpanzees it was found that once the symptoms of the infection are present, the IgM antibodies are released from the plasmablasts (Hong et al., 2013) and several immunoglobulin genes in the B cells will be up-regulated in response to HAV infection that includes a CXCL13 gene which transcribes a chemokine, directing the B cells towards the liver (Lanford et al., 2011). Finally, the neutralizing IgG antibodies will be prevalent, and will protect against the virus infection (Lemon et al., 1983). Furthermore, the neutralizing IgG antibodies attach to specific epitopes on the surface of the viral protein capsid located at the highly conserved VP1, VP2, and VP3 regions (Wang et al., 2015; Lemon et al., 1983). Jaundice is an important symptom of HAV infection caused by the destruction of infected hepatocytes by the immune system, possibly when it recognizes the virus-specific CD8+ T cells on the infected cells (Kurane et al., 1985).

Spices and natural compounds were used since ancient times as preservatives and anti-microbial compounds. The Romans recorded the preservative properties of fermented grape juice (mustard) and used it to preserve fruit juice as it can prevent microbial growth in food (Shelef, 1984). In the modern era, the scientific community has begun to examine the different activities of natural products against a wide range of microbes. Wine and grape seed extracts are among many

natural compounds that possess antioxidant and antimicrobial properties, that are rich in proanthocyanidins and other phenolic compounds (Lau et al., 2003). These compounds possess antibacterial, antioxidant and anti-inflammatory properties (Perumalla et al., 2011). The antioxidant properties of GSE are due to the rich flavonoid contents which act as scavengers of free radicals and also as chelating agents (Perumalla et al., 2011). Different concentrations of GSE (10, 20, 30 and 40 mg/ml which represent 1, 2, 3, or 4%, wt./vol, respectively) showed antimicrobial properties against *Listeria monocytogenes* in tryptic soy broth after incubation for 24 h at 37°C (Sivarooban et al., 2007). Another team tested a commercially available GSE product (ActiVin™) at 1% concentration on cooked beef inoculated with 5 log CFU/g *Escherichia coli* O157:H7, *L. monocytogenes,* or *Salmonella* Typhimurium, then refrigerated and stored for nine days (Ahn et al., 2007). For *E. coli* O157:H7, the bacterial population was reduced to 3.18±0.37 log CFU/g in the GSE treated sample as compared to the control, which was reduced to 4.43±0.33 CFU/g, representing approximately a 1 log difference. Similarly, *S.* Typhimurium was reduced to 3.56±0.22 log CFU/g, while the control was reduced to 4.25±0.18 log CFU/g. In *L. monocytogenes,* the bacterial population increased to 6.80±0.30 log CFU/g in the GSE treated sample as compared to the control, which increased to 7.77±0 .19 log CFU/g (Ahn et al., 2007). The increase in bacterial counts of *L. monocytogenes* is consistent with the pathogen's ability to grow at refrigeration temperatures (Saldivar et al., 2018). These experiments indicate the significant antibacterial properties of GSE (Ahn et al., 2007).

At 37°C, high titer HAV (~7 log PFU/ml) in suspension treated with 0.25, 0.5, or 1 mg/ml GSE resulted in 1.81, 2.66 and 3.20 log PFU/ml reductions, respectively, while similar treatments for low titer HAV resulted in 1.86, 2.26, and 2.89 log PFU/ml reductions, respectively (Su & D'

Souza, 2011). Similar treatments of HAV at room temperature resulted in 0.86, 1.22 and 1.90 log PFU/ml reductions of high titer HAV, respectively, and 2.4, 2.62 and 3.01 log PFU/ml reductions of low titer HAV, respectively, showing dose-dependent effect. Also, increasing GSE wash temperature may increase the antiviral properties against gross contamination (Su & D' Souza, 2011). Further studies were conducted with 0.25, 0.5, or 1 mg/ml GSE for 30 s at room temperature that resulted in 0.84±0.22, 0.86±0.17 and 1.06±0.24 log PFU/ml reductions respectively on lettuce (Su & D'Souza, 2013). Slightly higher HAV reductions of 0.97±0.10, 1.02±0.16 and 1.12±0.09 log PFU/ml, respectively were obtained after 1 min treatments with same GSE concentrations, on lettuce. The lettuce contaminated with the lower HAV titer (~5 log10 PFU/ml) treated for 30 s with 0.25, 0.5, or 1 mg/ml GSE resulted in 0.98±0.06, 1.17±0.10 and 1.20±0.12 log PFU/ml reductions, respectively, with 1.14±0.10, 1.19±0.13 and 1.23±0.11 log PFU/ml reductions after 1 min, respectively (Su & D'Souza , 2013). Jalapeno peppers contaminated with high titer HAV showed 0.74±0.28, 0.80±0.17 and 1.03±0.23 log PFU/ml reductions after treatment with 0.25, 0.5, and 1 mg/ml GSE for 30 s, respectively, and 0.66±0.12, 0.93±0.15 and 1.13±0.18 log PFU/ml reductions resulted after 1 min treatment, respectively (Su & D'Souza, 2013). For the low titer virus, after 30 s treatment, 1.01±0.20, 1.11±0.12 and 1.21±0.21 log PFU/ml reduction were obtained, respectively and after 1 min treatment, 1.07±0.13, 1.15±0.07 and 1.29±0.19 log PFU/ml reductions were obtained, respectively. These researchers concluded that the HAV reduction by GSE for 30 s was similar to the reduction obtained at 1 min and that the produce type (lettuce or pepper) had no influence in HAV reduction by GSE (Su & D'Souza, 2013). Furthermore, GSE at 0.5, 1, or 2 mg/ml in apple juice at 37°C reduced 5 log PFU/ml HAV to undetectable level, and in milk reduced HAV by

1.12±0.01, 1.15±0.03 and 1.09±0.02 log PFU/ml respectively, after 24 h (Joshi, Su, & D'Souza, 2015).

A 4 log (99.99%) inactivation of HAV HM-175 in a petri dish (60 ×15 mm) with 10 ml (PBS) after exposure to ultraviolet (UV) irradiation at a wavelength of 254nm using four germicidal lamp at distance of 38 cm above the sample at 15 mWs/cm^2 was reported (Battigeli et al., 1993). This research concluded that the HAV was more sensitive to UV inactivation than other animal viruses (Coxsackievirus B5 and rotavirus SA-II) due to its relatively high uracil content of 33% which is susceptible to the UV light hydration (Battigeli et al., 1993). The ability of UV-C light to inactivate HAV (ATCC VR-1402) at titer of 10^7 to 10^9 $TCID_{50}$/ml, from the surfaces produce treated using a low-pressure lamp and G36T6 model 4136 germicidal light unit emitting UV-C light at 253.7 nm (Fuller Ultraviolet, Frankfort, IL) (Fino & Kniel, 2008), showed that HAV on lettuce was reduced by 4.29±0.59, 4.45±0.20 and 4.62±0.00 log TCID50/ml for the 40, 120 and 240 mWs/cm^2 UV doses, respectively, while green onions showed 4.16±0.42, 5.31±0.42 and 5.58±0.21 log $TCID_{50}$/ml reduction and strawberries showed a 1.28± 0.32, 1.79±0.05 and 2.60±0.73 log $TCID_{50}$/ml, respectively (Fino & Kniel, 2008). The team concluded that UV-C can be effective method to inactivate the HAV from the surface of produce. Park and his team investigated the effect of UV-C against HAV and murine norovirus (MNV) at titers of 5.85 and 6.2 log PFU/ml, respectively, that were dried on 810 mm diameter and 5 mm thick stainless-steel coupons (Park et al., 2015), using 10, 15- and 30-Watt low pressure UV lamps stationed in a bench scale collimated-beam UV reactor, emitting monochromatic radiation at 260 nm wavelength with a distance of 15 cm. The team recorded that doses of 180 and 240 mWs/cm^2

resulted in 3.9±0.0 and 4.4±0.2 log PFU/ml reductions of the MNV, respectively and HAV reductions of 2.1±0.0 and 2.6±0.1 log PFU/ml at the same UV-C dosage, respectively, indicating that HAV was more resistant to UV-C than MNV (Park et al., 2015). Thus, improved methods for the control and inactivation of HAV on produce and on food contact surfaces to prevent HAV transmission and foodborne outbreaks are needed.

The hypothesis of this research is that GSE that contains about (5-8%) polyphenols (varies dependent on variety) with ring structures have a maximum absorption of light at λ_{max} = 264 –280 nm (Shi et al., 2003), upon illumination with UV-C germicidal light wavelength (range is between 100-280 nm, Valero et al., 2007) of 254 nm will allow absorption of photon energy, generating enough reactive oxygen species to cause a significant reduction of HAV. Therefore, the objective of this study was to investigate the effectiveness of UV light and, GSE alone, and in combination to inactivate/reduce HAV on Formica coupons and on the surface of tomatoes.

Material and Methods

Viruses and Cell Lines

Hepatitis A virus (HAV; strain HM-175) and the host cells used for viral propagation, fetal rhesus monkey kidney (FRhK4) that were graciously provided by our collaborator, Dr. Kalmia Kniel at the University of Delaware were used in this study. Procedures that were described for the maintenance of these host FRhK4 cells at 37°C under 5% Co_2 were followed using 175 cm^2 flasks and Dulbecco's Modified Eagle's Medium/Ham's F-12 (DMEM-F12; HyClone

Laboratories, Logan, UT) that had 2% heat-inactivated Bovine Calf Serum (FBS, HyClone Laboratories) and 1×Antibiotic-Antimycotic (Invitrogen-Thermo Fisher Scientific) (Joshi et al., 2015; Su and D'Souza, 2013). Briefly, confluent FRhK-4 cells from these flasks were treated with trypsin, and then 0.5×10^6 to 1×10^6 cells/well were added to 6-well plates and maintained in DMEM F12 supplemented media as described above at 37°C in an atmosphere with 5% CO_2 (Water-jacketed CO_2 Incubator; Fisher Scientific, Waltham, Massachusetts) (Joshi, Su, & D'Souza, 2015).

Virus Propagation

When the FRhK4 cells reached > 90% confluency in 175 cm^2 cell culture flasks, washing with Dulbecco's phosphate-buffered saline (DPBS; pH 7.4) twice was carried out infected host cell monolayers with HAV stocks as described earlier (Su & D'Souza, 2013; Joshi, Su, & D'Souza, 2015), and incubated at 37°C in an atmosphere containing 5% CO_2 (Water-jacketed CO_2 Incubator) for 3 h, at 37°C and 10 ml of 10% FBS DMEM was added. After seven days of incubation, the infected cells were freeze thawed three times to break down the cells and release the virus, centrifuged at 5,000 × g for 10 min, then filtration through a 0.2-µm filter was carried out, and virus was aliquoted in 2 ml tubes, and stored at -80°C until use (Su & D'Souza, 2011).

Grape Seed Extract (GSE)

As described in earlier studies from our lab, Gravinol-S GSE in powder form that was kindly gifted from OptiPure®, Chemco Industries (Los Angeles, CA) was used in this study and previously described protocols were used. Briefly, four hundred mg of the powder was dissolved

in 2 ml absolute ethanol with 38 ml warm water and filtered using 0.2-mm filter to prepare a 10 mg/ml GSE solution containing a final concentration of 5% ethanol (Su & D'Souza, 2011).

Infectious Plaque Assays

Confluent FRhK4 cells were used HAV infectivity assays using standard plaque assays (Su & D'Souza, 2011; Joshi, Su, & D'Souza, 2015), in 6-well plates. After aspiration of the cell culture media, serially diluted treated and control (untreated) HAV (0.5 ml) was added to FRhK4 host cells and incubated for 3 h. After aspiration of the virus, each well overlaid with 2 ml of the mix of complete DMEM media and 1% Noble Agar at a 1:1 ratio. Five days later, another layer was added by using 1 ml of overlay media (DMEM 2X powder, bovine calf serum, sodium bicarbonate, non-essential amino acids, $MgCl_2$ (4M), hepes [4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid] buffer and gentamycin- kanamycin dissolved in sterile double deionized water and filtered through a 0.2-µm filter) to which neutral red (Sigma-Aldrich, St. Louis, MO) was added and after 24 h incubation the virus plaques were counted (Joshi, Su, & D'Souza, 2015; Su & D'Souza, 2013). The recovered virus was calculated by the multiplication of the plaque counts with correlated dilution factors. The reduction of the virus titer was calculated by the deduction of the recovered treated virus from the control, the plaque assay was executed in duplicates.

Photodynamic Inactivation of HAV Using GSE on Formica Coupons

A tube containing 700 µl of HAV suspension at 6-log plaque forming units (PFU/ml) mixed with 70 µl GSE at a concentration of 10 mg/ml for a final GSE concentration of 0.91 mg/ml was

prepared. Another tube with 700 µl HAV at the same virus titer mixed with 70 µl phosphate buffer saline (PBS) was prepared. The HAV with GSE suspension (100 µl) was applied to the surface of Formica coupons (6.5 cm × 4.5 cm), (3.1×10^4 PFU/ cm^2 HAV applied) and (3.1µg/cm^2 GSE applied) on coupons surface in duplicate and another 100 µl of the HAV with PBS suspension was applied to the surface of a Formica coupon in duplicate in sterile petri dishes. The coupons were left to dry within the LABCONCO class II biosafety cabinet for 10 minutes. They were then illuminated with UV light at 254 nm wavelength, with exposure times of 3, 10, 20 and 30 min at distance of 72 cm, the energy density for the 3, 10, 20 and 30 min treatments were 0.012±0.000, 0.040±0.001, 0.081±0.002 and 0.121±0.003 J/$cm^{2,}$ respectively. Another two coupons containing the HAV with GSE and HAV with PBS (control) were kept in closed petri dishes without illumination for the same time points. For each time point, three coupons were prepared (HAV with GSE-UV, HAV with PBS-UV and HAV with GSE only) except for the 30 min time point in which four coupons were prepared (HAV with GSE-UV, HAV with PBS-UV, HAV with GSE only and HAV with PBS only [control]). After illumination at the designated time points, the virus was recovered for each time point from the surface of the Formica coupons by repeated pipetting with 1200 µl DMEM with 8% Bovine Calf Serum (BCS) into a Petri dish inside the biosafety cabinet, then transferring this solution into a 15 ml tube containing 8.7 ml DMEM 8% BCS. The virus was serially diluted with 2% BCS DMEM media at a ratio of 1:10 of virus (167 µL) to media (1.5 ml), then the confluent FRhK-4 host cells were infected with recovered HAV suspensions in 6-well plates for 3 h, followed by virus aspiration and overlay using HAV overlay media and 1% Noble Agar at a 1:1 ratio. After 5 days of incubation at 37°C in an atmosphere containing 5% CO_2 (Water-jacketed CO_2 Incubator), the

cells were stained using neutral red and the virus plaques were counted, the recovered virus was calculated as mentioned previously. The treatment and control for all time points were repeated thrice.

Photodynamic Inactivation of HAV Using GSE on Tomatoes

Cherry tomatoes were bought from a local retail market (average of 9.2±0.78 gram), (average diameter of 2.54 cm). Four tomatoes were washed with distilled water and allowed to dry in the LABCONCO class II biosafety cabinet. They were then covered with 750 µL elution buffer (0.1 M Tris-HCl and 3% beef extract powder, and 0.05 M glycine) (Li et al., 2012) by repeated pipetting (30 times), allowed to dry for 10 min, inoculated the surface with 500 µL HAV titer of 6-7 log PFU/ml by repeated pipetting (30 times) and dried for another 10 min. Two of the tomatoes were each inoculated with 500 µL of GSE solution at a concentration of 10 mg/ml, by repeated pipetting of the surface (30 times) (maximum GSE concentration of 0.25 mg/cm^2), while the other two tomatoes were each inoculated with an equal volume of PBS. All tomatoes were then allowed to dry for 10 min and illuminated using UV light at 254 nm wavelength, at a distance of 72 cm in the biosafety cabinet, UV dose (energy density) of 0.020±0.001, 0.061±0.002 and 0.081±0.002 J/cm^2 for the 5, 15 and 20 min, respectively. The positions of the tomatoes receiving UV treatment in the biosafety cabinet were rotated 180° horizontally halfway through each time point (5, 15, and 20 min). The tomatoes without UV illumination were treated with GSE or buffer, respectively, for the same time points. Next, virus extraction was performed as described earlier (Bozkurt et al., 2015) with some modifications. The virus was recovered from all tomatoes by aseptically by repeated pipetting each tomato with 15 ml of the elution

buffer into glass beakers. The recovered buffer solution was adjusted to a pH of 9.5 (confirmed with pH indicator paper) followed by shaking for 20 min at 120 rpm at 4ºC. The samples were then centrifuged at 10000 × g for 15 min at 4 ºC, the recovered supernatant was adjusted to pH 7.2. Then 10% polyethylene glycol (PEG) 8000 and 0.3 M NaCl was added, and the samples were shaken overnight at 120 rpm at 4ºC. Following this, the samples were centrifuged at 10000 × g for 30 min at 4ºC and the pellet was dissolved in 1 ml PBS followed by subsequent dilution with 2% BCS DMEM media at a 1:10 ratio of recovered virus (167µL) to media (1.5 ml). Finally, the confluent FRhK-4 host cells were infected with the recovered HAV suspensions in 6-well plates, as reported above. The treatment and control for the 5, 15 and 20 min were replicated 4, 6 and three times, respectively.

UV-C Lamp

The UV illumination applied to the surface of the Formica coupons and tomatoes was conducted using a 30-Watt G30T8 fluorescent germicidal UV-C lamp (Philips, Somerset, NJ), emitting light at 254 nm wavelength.

Characterization of UV Light Irradiance

The irradiance of the UV light was measured using ILT5000 Research Lab Radiometer (International Light Technology, Peabody, MA) (Figure: 2.5). The irradiance (W/cm^2) was determined by taking the average of three irradiance measurements of four positions and then averaging those values. The received dosage (energy density) in J/cm^2 for each exposure time

calculated using the equation (E=Pt) where E is the dose and P is the calculated (irradiance power density) and t is the exposure time in seconds (Ghate et al., 2013).

Statistical Analysis

Results from the treatments and controls were statistically analyzed by one-way ANOVA; Tukey's Post Hoc test was used for mean separation; two-way ANOVA were used to analyze differences in means between treatments, time, and the treatment by time interaction and the means separate by Tukey's Post Hoc test, similar to those used in previous studies (Turner & Thayer, 2001; Iversen et al., 1987). All statistical assumptions regarding normality and equality of variances were met (SAS, version 9.4, release TS1M3) and used for all analyses; where P - value <0.05 was considered significant as reported in literature (Turner & Thayer, 2001; Iversen et al., 1987).

Results

Inactivation of HAV by Photoactivated Grape Seed Extract Dried on the Surface of Formica Coupons

Treatment of HAV (recovered titer of ~5-6 log PFU/ml) dried on Formica coupons with GSE at 0.91 mg/ml, did not result in any observable HAV reduction for all tested time points. The UV illumination of HAV with PBS at a wavelength 254 nm and a distance of 72 cm, resulted in 1.08±0.53, 1.23±0.61, 2.2±0.27 and 1.97±0.55 log PFU/ml reduction after treatment for 3, 10, 20 and 30 min, respectively. PDI of HAV with GSE at 0.91 mg/ml and similar illumination

conditions resulted in 1.45±0.39, 1.74±0.37, 1.48±0.51 and 2.78±0.68 log PFU/ml reduction after 3, 10, 20 and 30 min, respectively (Table 2.1).

Inactivation of HAV by Photoactivated Grape Seed Extract Dried on Tomato Surfaces

The recovered titer of HAV from the control tomatoes surfaces was ~4.69 -5.71 log PFU/ml using 15 ml of elution buffer. Since the average diameter of tomatoes was 2.54 cm and 500 µl of GSE solution at 10 mg/ml was applied, the maximum concentration of GSE was 0.25 mg/cm^2, assuming all the GSE solution dried on the surface of the tomatoes. The HAV with GSE without illumination showed 1.53±1.04, 1.23±0.99 and 1.24±0.56 log PFU/ml reductions after 5, 15 and 20 min, respectively. UV illumination of HAV with PBS at a wavelength of 254 nm and a distance of 72 cm resulted in 1.71±0.35, 1.24±0.40 and 1.00±0.16 log PFU/ml reductions after 5, 15 and 20 min, respectively. PDI of HAV with GSE at 10 mg/ml with similar illumination conditions resulted in 1.88±0.38, 1.74±0.37 and 1.98±0.32 log PFU/ml reductions after 5, 15 and 20 min, respectively (Table 2.3).

Characterization of UV Light Irradiance

The calculated UV light doses (energy density) for the 3, 5, 10, 15, 20 and 30 min exposure times were 0.012±0.000, 0.020±0.001, 0.040±0.001, 0.061±0.002, 0.081±0.002 and 0.121±0.003 J/cm^2, respectively.

Discussion

In this study, PDI against HAV inoculated Formica coupons as an example of a food contact surface as well as on tomato surfaces as an example of a produce surface was investigated. Treatment of HAV dried on Formica coupons with GSE at 0.91 mg/ml was not effective in reducing HAV titers, even after 30 min at room temperature. In contrast Su & D'Souza (2011) treated HAV in suspension with 0.25, 0.5 and 1 mg/ml GSE in suspension at room temperature for 2 h that resulted in reductions of 3.01 log PFU/ml from initial, 5 log PFU/ml, and 1.90 log PFU/ml from initial 7 log PFU/ml. The current study differs by treating dried HAV on Formica coupons for shorter time periods. Both factors could account for the lower reductions observed compared to those observed in suspension over 2 h as earlier reported by Su & D'Souza (2011).

Therefore, treatment of HAV with GSE for more extended times may have improved antiviral effects. Moreover, in the current study, PDI was applied to enhance the activity of GSE against HAV for treatment times of 3, 10, 20 and 30 min to determine if treatment time was statistically significant. However, illumination times of 3, 20 and 30 min were not statistically significant from each other ($P > 0.05$), yet the 10 min UV treatment time was significant from the 3 and 20 min but not from the 30 min. This significance for 10 min treatment time was possibly due to low mean recovery of the virus on control samples across the 10 min treatment. PDI of HAV with GSE for 3, 10, 20 and 30 min showed 1.45 ±0.39, 1.74±0.37, 1.48±0.51 and 2.78±0.68 log PFU/ml reduction, respectively and HAV with PBS treated with UV at the same time points showed reductions of 1.08±0.53, 1.23±0.61, 2.21±0.27 and 1.97±0.55 log PFU/ml, respectively (Table 2.1). PDI of HAV with GSE on Formica coupons for the 3, 10, 20 and 30 min treatments

is significantly different from the controls ($p<0.05$). However, this is not significantly different from the reduction of HAV with PBS treated with UV on Formica coupons for the same time points. These results suggest that HAV reduction from both treated coupons (with and without) is due to the effect of UV, and not the result of GSE absorption of photon energy to produce reactive oxygen species.

Park et al. (2015) conducted an experiment using stainless steel coupons to mimic food facility surface with smaller dimensions (diameter of 10 mm and thickness of 5 mm) compared to Formica coupons (6.5 × 4.5 cm) used in the current study. Both experiments used HAV titer around ~ 6 log PFU/ml. Moreover, the Park's work used UV-C lamps of 10, 15 and 30 W emitting light at 260 nm stationed in a bench scale collimated-beam UV reactor. However, Park did not report the illumination times, in our work we used UV-C lamp of 30 W emitting light at 254 nm inside a biosafety cabinet, the sample distance from the light source were 15 cm in Park et al, and 72 cm in our work. The virus recovery method used here was different from that used in the work of Park et al.(2015); where, 50 µl DMEM with 2% fetal bovine serum (FBS) was deposited in the middle of the coupon after the UV-C treatments. The coupons were then soaked in 450 µl DMEM with 2% FBS in a 15 ml conical tube, followed by vortexing and serial dilution. The virus recovery method used in our work involved rinsing the coupons by repeated pipetting (30 times) with 1200 µl DMEM with 8% BCS into a Petri dish inside the biosafety cabinet, then transferring this solution into a 15 ml tube containing 8.7 ml DMEM 8% BCS and followed by serial dilution. The very low standard deviations reported by Park et al. (2015) may be due to his virus recovery method (i.e. using stainless steel coupons and soaking the entire coupon in media) as compared to that used in our work. Comparison of the results of both

experiments reveals a similar viral reduction for the 30 min treatment time. In our work, a UV dose of 0.12 J/cm^2, achieved a 1.97±0.55 log PFU/ml (HAV with PBS) reduction in 30 min, while Park et al. (2015) using a similar UV dose of 120 mWs/cm^2 (0.12 J/ cm^2) reported, a 1.9±0.0 log PFU/ml reduction of infectious HAV (Table 2.1). Similarly, a 10 min treatment of HAV with a UV dose of 0.04 J/ cm^2 resulted in 1.23±0.61 log PFU/ml virus reduction, while a 0.7±0.0 log PFU/ml reduction of HAV was recorded by Park et al. (2015) using a similar UV dosage of 40 mWs/cm^2 (0.04 J/ cm^2). However, the current study with 3 min treatment showed higher viral reduction, where the UV dose of 0.01 J/cm^2 resulted in a 1.08±0.53 log PFU/ml virus reduction (Table 2.1) compared to the results of Park et al. (2015) that reported no reduction with a similar UV dose of 10 mWs/cm^2 (0.01 J/cm^2). However, in the current study this mean virus recovery for the 3 min treatment was not statistically different from the controls. Moreover, the experiments done by Park et al. (2015) also tested a much higher UV dose of 300 mWs/cm^2 (0.3 J/cm^2) that resulted in 2.6±0.0 log PFU/ml virus reduction.

The surface of cherry tomatoes (average of 9.6±0.21 grams) (average diameter of 2.54 cm) inoculated with HAV at a titer of 6 log PFU/ml and illuminated with UV light at 254 nm wavelength, UV dose (energy density) of 0.020±0.001, 0.061±0.002 and 0.081±0.002 J/cm^2 for the 5, 15 and 20 min treatment time, respectively, at a distance of 72 cm resulted in a 1.71±0.35, 1.24±0.40, and 1.00±0.16 log PFU/ml reductions of HAV respectively, for the photoactivated HAV with PBS (Table 2.3). PDI of HAV with GSE at 10 mg/ml under the same conditions for 5, 15 and 20 min resulted in a 1.88±0.38, 1.74±0.37 and 1.98±0.32 log PFU/ml reduction, respectively. HAV treated with GSE at 10 mg/ml without illumination has resulted in reduction

of 1.53±1.04, 1.23±0.98 and 1.24±0.56 log PFU/ml, respectively (Table 2.3). Statistical analysis indicated that the 5 min treatment was significantly different from the 15 and 20 min treatment, but there was no significant differences between the 15 and 20 min time points. This suggested that treatment of at least 15 min is needed for higher HAV reduction. Viral reduction values between HAV with GSE no UV illumination HAV with UV, and HAV with GSE and UV across the three time points are statistically significant from the control, however they are not significant from each other ($P > 0.05$). This insignificance between the three treatments may be explained by difficulties with virus recovery, as three-dimensional objects such as tomato surfaces make virus recovery more difficult than from a two-dimensional object such as the surface of the Formica coupons. Moreover, in the current work, significant viral reduction was only achieved using a high concentration of GSE (10 mg/ml) on the surface of tomatoes.

Earlier studies with lower GSE concentrations of 0.25, 0.5, and 1 mg/ml against HAV on lettuce and pepper contaminated with ~7 log PFU/ml HAV for shorter incubation times of 30 s and 1 min showed about 1 log PFU/ml reduction of HAV. The GSE at higher concentrations of 10 mg/ml for treatment times of 5, 15 and 20 min on tomatoes in this current study yielded similar 1 log reductions. However, Su & D'Souza, (2013) used approximately 2 ml of DMEM containing 10% heat inactivated fetal bovine serum (FBS) for HAV recovery, while the elution buffer (0.1 M Tris-HCl with 3% beef extract powder and 0.05 M glycine) was used in this study to recover the virus from tomatoes. In the current research with HAV inactivation on tomatoes, GSE concentration was increased to 10 mg/ml when applied on the tomatoes surface because the GSE at 0.91mg/ml on Formica coupons was not effective. Other researchers including Fino & Kniel,

(2008) used UV-C from a germicidal UV lamp of 253.7nm to inactivate HAV inoculated on the surfaces cut lettuce (square shape of 2.5 ×2.5 cm), green onions (2 cm segments), and strawberries (cut longitudinally). They spread 10 µl of HAV (titers of 10^7 to 10^9 $TCID_{50}$), evenly on demarcated 1 cm^2areas for lettuce and onions, whereas the strawberries were inoculated in three areas: the shoulder, midsection and the bottom and dried for 30 min, and treated in an enclosed chamber of one meter in length with highly reflective material placed on the interior to help minimize the amount of shadowing on irregularly shaped fruit pieces. They reported treatments with UV-C doses of 40mWs/cm^2 (0.04 J/cm^2), 120 mWs/cm^2(0.12 J/cm^2) and 240 mWs/cm^2 (0.24 J/cm^2).showed 4.29±0.59, 4.45±0.20 and 4.62±0.00 log $TCID_{50}$/ml HAV reduction on lettuce, respectively, while green onions showed 4.16±0.42, 5.31±0.42 and 5.58±0.21 log $TCID_{50}$/ml reduction, respectively, and strawberries showed 1.28±0.32, 1.79±0.05 and 2.60±0.73 log $TCID_{50}$/ml reduction, respectively (Fino & Kniel, 2008).

In the current work, tomatoes were used whole. The entire tomato was inoculated by repeated pipetting and spreading of 500 µl of HAV (titer 10^6 PFU/ml) over the surface of the tomato and dried for 10 min in the class II LABCONCO biosafety cabinet. Using positions shown in Fig, 2.5, the tomatoes treated with UV light in the biosafety cabinet were rotated 180° in horizontal plain half way through each time point to treat both sides of the top half of the surface. The tomatoes were not inverted to expose the bottom half to the UV. However, this rotation decreased the total amount of UV dose received by the HAV on each side of the top portion of the tomato. In our work with tomatoes, 1.71±0.35, 1.24±0.40 and 1.00±0.16 log PFU/ml reduction of HAV was obtained with UV doses of 0.02 J/cm^2, 0.06 J/cm^2 and 0.08 J/cm^2,

respectively (Table 2.3).These lower reductions and differences in reductions of HAV on tomatoes compared to lettuce and green onions (Fino & Kniel, 2008) could be due to various reasons. Besides, the difference in the surface of lettuce and green onions, different viral recovery methods were used that could account for the observed differences in reductions. Fino & Kniel, (2008) used a repeated pipetting (>25 times) with a total 1 ml minimal essential medium (MEM) with 2% (FBS) to recover HAV over the surface of the strawberries, while green onions and lettuce, samples were vortexed in 1 ml MEM with 2% FBS. In contrast, HAV was recovered from tomatoes in this study using a 15 ml elution buffer (0.1 M Tris-HCl with 3% beef extract powder, and 0.05 M glycine) by repeated pipetting over the surface of tomatoes (30 times). Besides, (Fino & Kniel, 2008) used 50% tissue culture infective doses ($TCID_{50}$) as a quantification method, while the plaque forming unit (PFU/ml) was used as a quantification method in our studies with tomatoes. Fino & Kniel, (2008) indicated that the shape of the produce affected the reduction, considering that the lettuce and green onions had higher viral reduction than the strawberries for the same UV treatment doses. The strawberries shape of the strawberries may have increased the shadowing effect, in that the presence of several grooves on the surface may have allowed the virus to be sheltered from the UV light, decreasing reduction. Moreover, lettuce has a smoother surface than strawberries which improved the UV light effect and increased virus reduction. In this study, the tomatoes share a similar shape with the strawberries thus a similar shadowing effect can occur. However, tomatoes are much smoother than strawberries. Finally, in the current protocol there was no UV treatment on the bottom half of the tomatoes and no reflective material. The rotation of the tomatoes by 180° halfway through each time point should also have decreased UV exposure and effectiveness on the top half.

Furthermore, perhaps, the UV doses used in this work were not enough to generate strong ROS production as the maximum UV dose tested in our work was 0.12 J/cm^2 (120 mWs/cm^2) against HAV dried on Formica coupons, compared to 180, 240 and 300 mWs/cm^2 against HAV on stainless steel surface tested by Park et al. (2015) and 240 mWs/cm^2 UV dose against HAV dried on produce by Fino & Kniel. (2008).

Conclusions

GSE at 0.91 mg/ml did not appear to affect HAV titers when dried on Formica coupons even after 30 min treatment. However, the application of a much higher concentration of GSE at 10 mg/ml, (GSE maximum concentration of 0.25 mg/cm^2) to the surface of tomatoes had an anti-HAV effect after 5 min. PDI of HAV with GSE and UV-C at 254 nm on Formica coupons and tomatoes did not show a significant difference compared to treatment with UV alone for all tested time points, suggesting that the reduction could be mainly due to the UV-C light effect rather than the effect of ROS production.

Therefore, illumination of HAV with GSE using UV at a wavelength of 254 nm and a distance of 72 cm was not suitable to initiate significant production of ROS. Furthermore, increased GSE concentrations could be used to possibly enhance the antiviral effect of GSE against HAV on Formica coupons and on the surface of Formica coupons and tomatoes for a longer time than 30 min can be tested in the future. It should be noted that a concentration of 0.8 mg/ml GSE or more on the cells will cause cytotoxic effects on the FRhK4 cell line (Su *et al*., 2011). Caution must be

taken when treating the virus with higher levels of GSE, as only the 10^{-2} virus dilution can be applied on the FRhK4 cell line before cell toxicity occurs. This study also suggests that the use of a different UV-C light source with a broader wavelength than the germicidal UV light used in our work, (most of its energy concentrated around 254 nm) (Qian, 2002) may be needed in order to initiate ROS to cause a significant reduction of infectious of HAV titer.

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Appendix

Table 2.1: Effect of photoactivated grape seed extract at 0.91 mg/ml at four time points using UV light with 254 nm wavelength, at a distance of 72 cm and dose (energy density) for the 3, 10, 20 and 30 min exposure times that were 0.012±0.000, 0.040±0.001, 0.081±0.002 and 0.121±0.003 J/cm^2, respectively, at room temperature against hepatitis A virus (titer of ~5-6 log PFU/ml) dried on Formica coupons. Values are displayed as the average of three replicates ±SD. Different lower case letters across columns denote significant differences between treatments of each time point. Different upper case letters across rows denote significant differences between treatments regardless of time points as determined by Two-way ANOVA ($P < 0.05$).

Time	**3 min 0.01 J/cm^2**		**10 min 0.04 J/cm^2**		**20 min 0.08 J/cm^2**		**30 min 0.12 J/cm^2**		TWO WAY ANOVA
Treatment	Average Recovered	Reduction	Average Recovered	Reduction	Average Recovered	Reduction	Average Recovered	Reduction	
Control	a 5.93±0.23	-	a 5.21±0.56	-	a 6.15±0.35	-	a 5.95±0.41	-	**A**
HAV with GSE at 0.91 mg/ml	a 5.98±0.11	0	a 5.48±0.31	0	a 5.89±0.20	0.31±0.25	a 5.83±0.25	0.27±0.50	**A**
HAV with PBS with UV	ab 4.73±0.49	1.08±0.53	b 4.20±0.40	1.23±0.61	b 4.10±0.49	2.21±0.27	b 4.12±0.24	1.97±0.55	**B**
HAV with GSE at 0.91 mg/ml and UV	b 4.44±0.46	1.45±0.39	b 3.75±0.38	1.74±0.37	b 4.75±0.21	1.48±0.51	b 3.66±0.79	2.78±0.68	**B**

Table 2.2:Effect of photoactivated grape seed extract at 0.91 mg/ml at four time points using UV light with 254 nm wavelength, at a distance of 72 cm and dose (energy density) for the 3, 10, 20 and 30 min exposure times that were 0.012±0.000, 0.040±0.001, 0.081±0.002 and 0.121±0.003 J/cm^2, respectively, at room temperature against hepatitis A virus (titer of ~5-6 log PFU/ml) dried on Formica coupons. Values are displayed as the average of five replicates ± SD for the 3 min time point, the average of 6 replicates ± SD for the 20 and 10 min time points, and the average of three replicates ± SD for the 30 min time point. Different lower case letters across columns denote significant differences between treatments of each time point. Different upper case letters across rows denote significant differences between treatments regardless of time points as determined by Two-way ANOVA ($P < 0.05$).

Time	**3 min 0.01 J/cm^2**		**10 min 0.04 J/cm^2**		**20 min 0.08 J/cm^2**		**30 min 0.12 J/cm^2**		TWO WAY ANOVA
Treatment	Average Recovered	Reduction	Average Recovered	Reduction	Average Recovered	Reduction	Average Recovered	Reduction	
Control	a 5.77± .33	-	a 5.21±0.66	-	a 5.86±0.43	-	a 5.95±0.41	-	**A**
HAV with GSE at 0.91 mg/ml	a 6.01±0.12	0	a 5.19±1.02	0.25±1.06	a 5.62±0.59	0.31±0.37	a 5.82±0.26	0.27±0.50	**A**
HAV with PBS with UV	b 4.78±0.43	0.93±0.46	ab 4.21±0.54	1.28±0.45	b 4.11±0.49	1.83±0.61	b 4.12±0.24	1.97±0.55	**B**
HAV with GSE at 0.91 mg/ml and UV	b 4.27±0.50	1.51±0.54	b 3.56±0.48	1.96±0.71	b 4.57±0.78	1.46±1.22	b 3.66±0.79	2.78±0.68	**B**

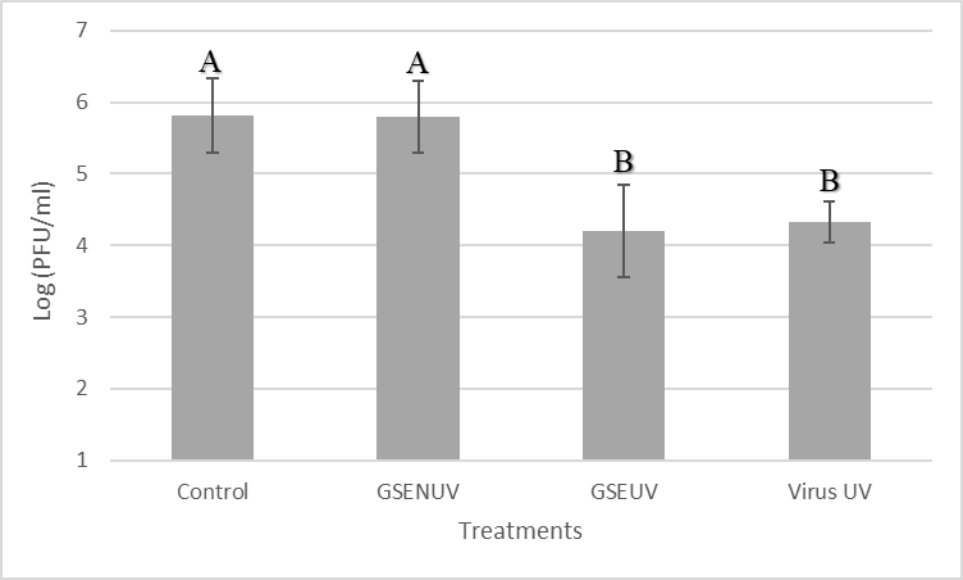

Figure 2.1: Illustration of the average recovered HAV titers in log PFU/ml (Data from table 2.1) from the surface of Formica coupons across all time points (3, 10, 20 and 30 min) of the different treatments, after UV illumination with 254 nm wavelength, at a distance of 72 cm and dose (energy density) for the 3, 10, 20 and 30 min exposure times were 0.012±0.000, 0.040±0.001, 0.081±0.002 and 0.121±0.003 J/cm^2, respectively, at room temperature, GSE at 0.91 mg/ml, against hepatitis A virus (titer of ~5-6 log PFU/ml), including HAV with PBS, No UV (Control); HAV with GSE 0.91 mg/ml, No UV(GSENUV); HAV with PBS and UV (HAVUV); and HAV with GSE at 0.91 mg/ml and UV (GSEUV). Different letters represent significant differences between different treatments for all time points.

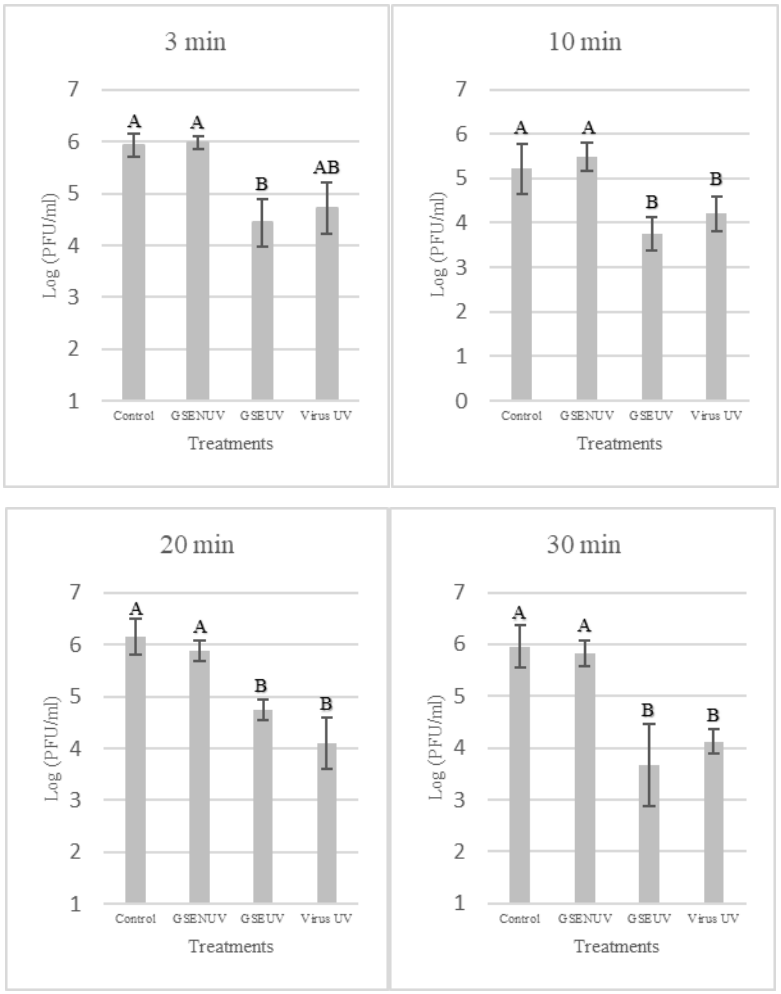

Figure 2.2: Illustration of the average recovered HAV titers in log PFU/ml (Data from table 2.1) from the surface of Formica coupons for each time point (3, 10, 20 and 30 min) of the different treatments, after UV illumination with 254 nm wavelength, at a distance of 72 cm and dose (energy density) for the 3, 10, 20 and 30 min exposure times were 0.012±0.000, 0.040±0.001, 0.081±0.002 and 0.121±0.003 J/cm^2, respectively, at room temperature, GSE at 0.91mg/ml, against hepatitis A virus (titer of ~5-6 log PFU/ml), including HAV with PBS, No UV (Control); HAV with GSE 0.91 µg/ml, No UV(GSENUV); HAV with PBS and UV (HAVUV); and HAV with GSE at 0.91 µg/ml and UV (GSEUV). Different letters represent significant differences between different treatments for each time point.

Table 2.3: Effect of photoactivated grape seed extract at 10 mg/ml at three time points using UV light with 254 nm wavelength, at a distance of 72 cm and dose (energy density) of 0.020±0.001, 0.061±0.002 and 0.081±0.002 J/cm^2 for the 5, 15 and 20 min, respectively, at room temperature against hepatitis A virus (titer of ~5-6 log PFU/ml) dried on tomato surfaces. Values are displayed as the average of 4, 6 and three replicates for the 5, 15 and 20 min ±S.D respectively. Different lower-case letters across columns denote significant differences between treatments of each time point. Different upper-case letters across rows denote significant differences between treatments regardless of time points as determined by Two-way ANOVA ($P < 0.05$).

Time	**5 min 0.02 J/cm^2**		**15 min 0.06 J/cm^2**		**20 min 0.08 J/cm^2**		TWO WAY ANOVA
Treatment	Average. Recovered	Reduction	Average. Recovered	Reduction	Average. Recovered	Reduction	
Control	**a** 5.71±0.63	-	**a** 4.69±0.40	-	**a** 4.86±0.42	-	A
HAV with GSE at 10 mg/mL	**b** 4.41±0.92	1.53±1.04	**b** 3.64±1.07	1.23±0.99	**ab** 3.61±0.45	1.24±0.56	B
HAV with PBS with UV	**b** 4.03±0.36	1.71±0.35	**b** 3.57±0.39	1.24±0.40	**b** 3.84±0.51	1.00±0.16	B
HAV with GSE at 10 mg/mL and UV	**b** 3.84±0.21	1.88±0.38	**b** 3.16±0.74	1.74±0.37	**ab** 2.90±0.60	1.98±0.32	B

Table 2.4: Effect of photoactivated grape seed extract at 10 mg/ml at three time points using UV light with 254 nm wavelength, at a distance of 72 cm and dose (energy density) of 0.020±0.001, 0.061±0.002 and 0.081±0.002 J/cm^2 for the 5, 15 and 20 min, respectively, at room temperature against hepatitis A virus (titer of ~5-6 log PFU/ml) dried on tomato surfaces. Values are displayed as the average of three replicates ±S.D except for the 15 min treatment was 4 replicates. Different lower-case letters across columns denote significant differences between treatments of each time point. Different upper-case letters across rows denote significant differences between treatments regardless of time points as determined by Two-way ANOVA ($P < 0.05$).

Time	**5 min 0.02 J/cm^2**		**15 min 0.06 J/cm^2**		**20 min 0.08 J/cm^2**		TWO WAY ANOVA
Treatment	Average. Recovered	Reduction	Average. Recovered	Reduction	Average. Recovered	Reduction	
Control	**a** 5.73±0.39	-	**a** 4.69±0.40	-	**a** 4.86±0.42	-	A
HAV with GSE at 10 mg/mL	**b** 3.97±0.61	2.02±0.42	**b** 3.00±1.07	1.59±0.65	**ab** 3.61±0.45	1.24±0.56	B
HAV with PBS with UV	**b** 4.04±0.34	1.72±0.43	**b** 3.45±0.39	1.35±0.37	**b** 3.84±0.51	1.00±0.16	B
HAV with GSE at 10 mg/mL and UV	**b** 3.81±0.22	1.93±0.45	**b** 2.94±0.72	1.75±0.43	**ab** 2.90±0.60	1.98±0.32	B

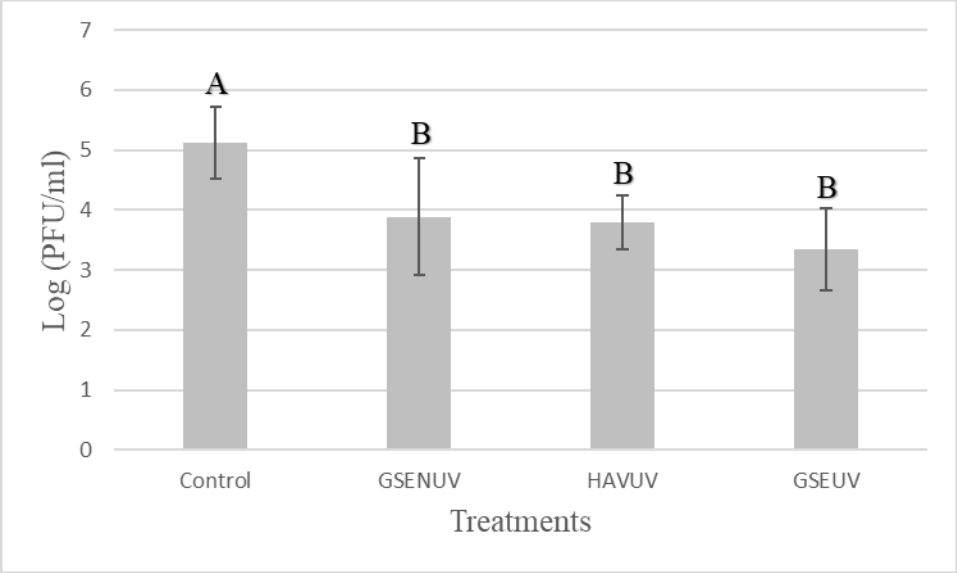

Figure 2.3: Illustration of the average recovered HAV titers in (log PFU/ml) from the surface of tomatoes across all time points (5, 15 and 20 min) of the different treatments (Data from table 2.3), the values are the average of 4, 6 and three replicates for the 5, 15 and 20 min ±S.D respectively, after UV illumination with 254 nm wavelength, at a distance of 72 cm dose (energy density) of 0.020±0.0011, 0.061±0.002 and 0.081±0.002 J/cm^2 for the 5, 15 and 20 min, respectively, at room temperature against hepatitis A virus (titer of ~5-6 log PFU/ml), including HAV with PBS, No UV (Control); HAV with GSE 10 mg/ml, No UV(GSENUV); HAV with PBS and UV (HAVUV); and HAV with GSE at 10 mg/ml and UV (GSEUV). Different letters represent significant differences between different treatments for all time points.

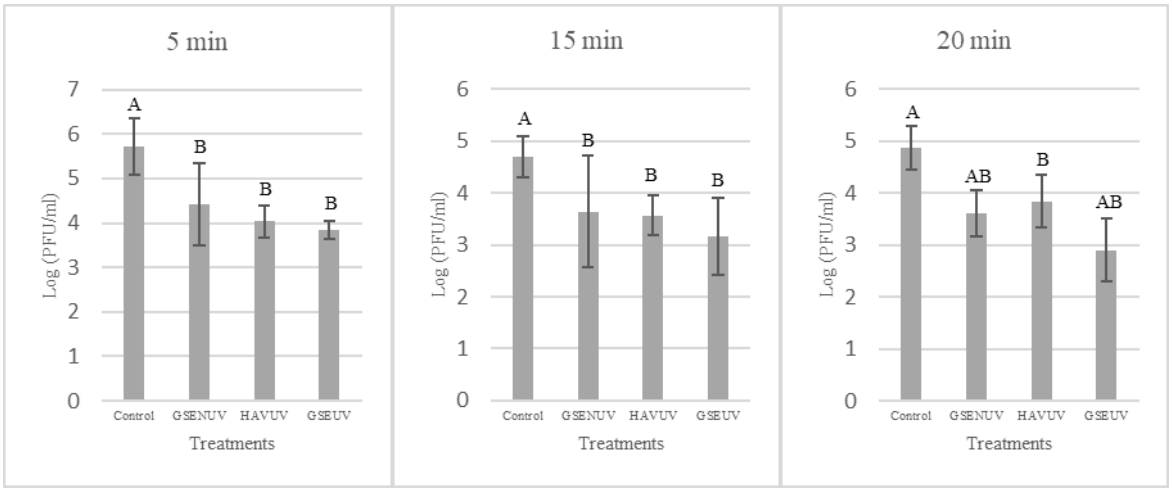

Figure 2.4: Illustration of the average recovered HAV titers in (log PFU/ml) from the surface of tomatoes for each time point of the different treatments (Data from table 2.3), the average of 4, 6 and three replicates for the 5, 15 and 20 min ±S.D respectively, after UV illumination with 254 nm wavelength, at a distance of 72 cm and dose (energy density) of 0.020±0.001, 0.061±0.002 and 0.081±0.00 J/cm^2 for the 5, 15 and 20 min, respectively, at room temperature against hepatitis A virus (titer of ~5-6 log PFU/ml), including HAV with PBS, No UV (Control); HAV with GSE 10 mg/ml, No UV(GSENUV); HAV with PBS and UV (HAVUV); and HAV with GSE at 10 mg/ml and UV (GSEUV). Different letters represent significant differences between different treatments for each time point.

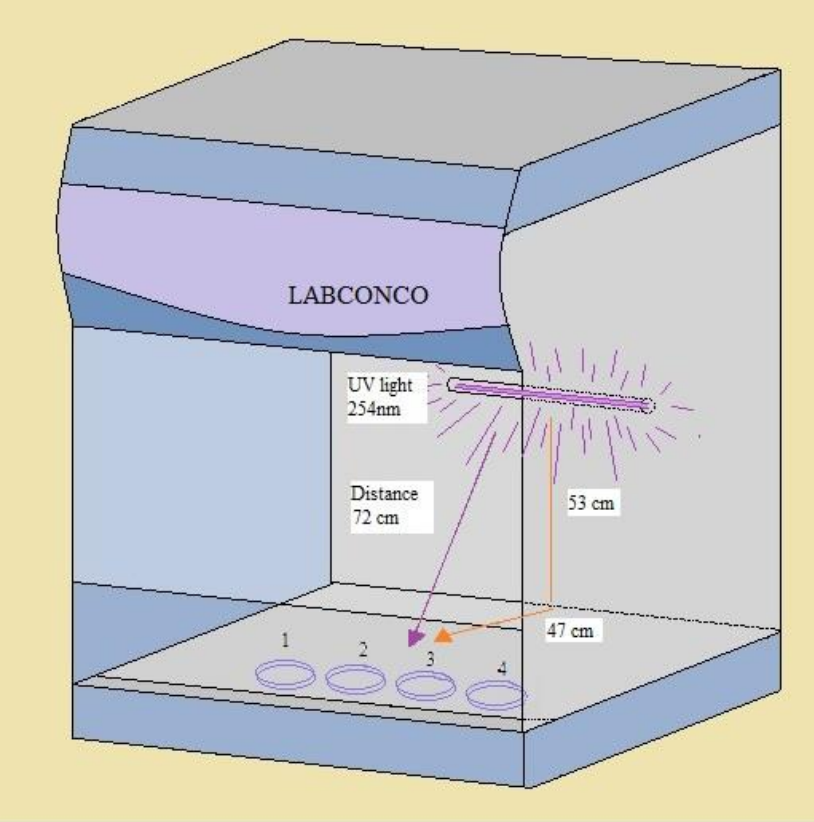

Figure 2.5: Illustration of the four positions of the petri dishes containing either coupons or tomatoes inside the LABCONCO class II biosafety cabinet. Specifications included UV light 30-Watt fluorescent light bulb (Philips), emitting at 254 nm, plates distance of 72 cm. the dose (energy density) for the 3, 5, 10, 15, 20 and 30 min exposure times were 0.012±0.000, 0.020±0.001, 0.040±0.001, 0.061±0.002, 0.081±0.002 and 0.121±0.003 J/cm^2, respectively. Irradiance was determined by taking the average of three irradiance measurements at each position and then averaging those values.

Chapter III: The Antiviral Activity of Oleanolic Acid, Ursolic Acid, and Photoactivated Oleanolic Acid Against HAV on Formica Coupons

Abstract

Literature on the anti-viral activity of different plant extracts against a variety of pathogenic microorganisms report that oleanolic acid (OA) and ursolic acid (UA) have activity against hepatitis C virus (HCV). Hepatitis A virus outbreaks continue to occur annually worldwide, demonstrating the need for improved control measures. In this study, we investigated the antiviral activities of OA at 60, 600 and 2000 µg/ml and UA at 180 and 360 µg/ml against HAV in a direct contact mode on Formica coupons (a model food contact surface). One-hundred µl of OA at 60, 600 and 2000 µg/ml and UA at 180 and 360 µg/ml were treated for various contact times directly on 100 µl of HAV dried on Formica coupons. After each treatment (replicated thrice), the viruses were eluted, ten-fold serially diluted and plaque assayed using confluent FRhK-4 host cells in 6-well plates. Recovered plaques were enumerated and data were statistically analyzed. OA at 60 and 600 µg/ml was shown to cause 1.23±0.27 and 2.27±0.67 PFU/ml reduction of HAV after 1 h, respectively with 1.79±0.61 log PFU/ml reduction after 10 min with 2000 µg/ml OA. UA at 180 and 360 µg/ml showed 0.66±0.16 and 1.33±0.35 log PFU/ml reduction of HAV after 1 h, respectively. Furthermore, photodynamic inactivation (PDI) of HAV using OA at 600 µg/ml with UV at 254 nm wavelength at a distance 72 cm for 10 min, and UV dose (energy density) of 0.040±0.0011 J/cm^2, resulted in 1.75±0.7 log PFU/ml reduction of HAV in distilled deionized water and a 2.39±0.31 PFU/ml reduction with OA. The two reduction values were statistically significant from the control ($P<0.05$), but not statistically significantly from each other, suggesting that UV rather than reactive oxygen species played a major role in HAV reduction. Both, OA and UA at the tested concentrations show possibility for use in hurdle approaches to control the spread of HAV from food contact surfaces.

Introduction

Oleanolic acid (3β-hydroxy-olea-12-en-28-oic acid) (OA) and its isomer ursolic acid (UA) (3β-hydroxy-urs-12-en-28-oic acid) belong to the class of pentacyclic triterpenoid compounds, the difference between them being the position of the methyl residue at carbon 19 or 20 of the cyclic ring system (Liu et al., 1995). They are secondary metabolites produced by numerous plant species that have several biological functions and beneficial properties, such as antifungal, antiviral, antibacterial, insecticidal, anti-tumor, anti-human immunodeficiency virus (HIV), hypoglycemic, hepatoprotective and anti-inflammatory activity (Fukushima et al., 2011; Guinda et al., 2004). OA and UA naturally exist together in plants in the form of free acids or aglycones; and are used in health and cosmetic products as they are relatively non-toxic (Liu et al., 1995). OA was isolated from approximately 1,620 plant species including several plants used for food or medicinal purposes (Pollier et al., 2012). The primary source of OA is from olive leaves (*Olea europaea*) which represents about 3% of the dry leaf weight (Guinda et al., 2004). Moreover, OA was used in clinical applications for more than 20 years in China as hepatic drug (Zhao et al., 2013).

Several studies to chemically modify OA and UA were performed to enhance its beneficial properties. In 1997, chemical modifications of both OA and UA were carried out in an attempt to obtain anti-ulcer compounds without mineralocorticoid activity (Farina et al., 1998), by the removal of ketone groups from 11 locations on the molecules' structures. They chemically modified the carboxylic functional groups at position 30 to include reduction to alcohol or changing to a ketone group. They produced carbenoxolone analogues in the β-amyrin series of

OA. These researchers found that the unsaturated compounds 14b and 23b and the one 1-methylene derivative 18 were less toxic and mineralocorticoids than the reference compound carbenoxolone. They found that several UA derivatives, such as dihemisuccinate sodium salt 35b, showed good separation between anti-ulcer and mineralocorticoid actions, however this compound was toxic to the liver and kidneys of monkeys. though, other derivatives such as uvaol dihemiphthalate sodium salt and diene analogues 39b and 38b were found to have a high level of anti-ulcer activity without being toxic to the kidneys or liver (Farina et al., 1998). In 1998 Suh et al. tried to enhance the anti-inflammatory and chemoprotective properties of OA and UA by chemically synthesizing more than 80 triterpenoids derived from them with the goal to decrease the formation of the inducible enzymes, nitric oxide synthase (iNOS) and the cyclooxygenase (COX-2) which are implicated in the inflammatory response and carcinogenesis (Suh et al., 1998). The research found that two derivatives have the ability to hinder the production of these enzymes; the oleananes 3, 12-dioxoolean-l-en-28-oic acid (TP-69) and 3, 11-dioxooleanl,12-dien-28-oic acid (TP-72) (Suh et al., 1998). OA has poor solubility in water, and when orally administered causes erratic pharmacological activity, a problem that hinders its effectiveness and its bioavailability. Attempts to overcome this problem included use of solid dispersion, cyclodextrin inclusions or nanosuspensions as methods of OA drug delivery (Chen et al., 2005). In 2018, Wang and his team explored the molecular mechanisms behind OA's hepatoprotective and anti-inflammatory properties. They used a recognized model to explore liver injury, Concanavalin A, (ConA) which is a plant lectin extract that induces liver injury in vivo (Wang et al., 2018). When they subcutaneously administered OA at 20, 40, or 80 mg/kg to mice once daily for 3 successive days, then injected them intravenously with ConA at 20 mg/kg to induce liver

injury, they found that OA inhibited liver damage mediated by ConA. They discovered the mechanism behind this is due to an increase in the expression of peroxisome proliferator-activated receptor alpha (PPARα), a group of nuclear receptors that act as a transcription factor regulating k gene expression, involved in lipid metabolism, regulation of inflammation and immunity and the decrease of the phosphorylation of c-Jun NH_2-terminal kinase (JNK) proteins, which mediate cell apoptosis and autophagy (Wang et al., 2018).

In 2005, Jiménez and his colleague reported antibacterial activity of UA against multi-drug resistant Mycobacterium tuberculosis H37Rv strain that had a 99% inhibition of growth with 100 µg/ml UA (Jiménez et al., 2005). Woldemichael and his research team reported antibacterial activities against *Mycobacterium tuberculosis* with an MIC of 8 µg/ml and 16 µg/ml for the triterpene 3-epi-UA and 3-epi-OA, respectively (Woldemichael et al., 2003). In 2011, Kim and his team reported antibacterial activity of UA against *Streptococcus mutans* and *S. sobrinus* with minimum inhibitory concentrations (MIC_{90}) of 2 µg/ml and 4 µg/ml, respectively, with no cytotoxic effects on the epithelial oral cells (KB) and subsequently suggested the use of UA in oral hygiene products (Kim et al., 2011). In 1998, Kashiwada and his team tested different plant extracts against Human Immunodeficiency Virus (HIV), with OA having an EC_{50} < 20 µg/ml. The team also produced different derivatives of OA and tested their activity against the HIV-1 infected human cell line, H9 that showed an EC_{50} of 1.7 µg/ml, an H9 cell growth inhibition IC_{50} of 21.8 µg/ml, and a therapeutic index (T.I.) of 12.8. An interesting finding is that OA derivative number 18 had the highest anti-HIV effect with a reported EC_{50} of 0.0005 µg/ml and a reported T.I. of 22,400 (Kashiwada et al., 1998). Though UA has an anti-HIV effect at EC_{50} of 2.0 µg/ml

and IC_{50} of 6.5 µg /ml it is reported to have some toxicity at these levels (Kashiwada et al., 1998). The plant extract of *Fructus Ligustri Lucidi* (FLL) had to activity against Hepatitis C virus (HCV) genotype NS5B through the inhibition of the action of viral RNA-dependent RNA polymerase (RdRp) (Kong et al., 2007). In 2013, Kong and his colleagues conducted another study that confirmed the ability of *Fructus Ligustri Lucidi* (FLL) and other reagents to decrease the intracellular and replication activities of HCV by 50% (IC_{50}) at concentrations of 33.8, 5.5, 0.8, and 3.1 µg/ml of fraction 1, fraction 2, OA and UA, respectively, reduced the activity and replication of NS5B HCV by 50% (IC_{50}) (Kong et al., 2013). Thus, it has been reported that oleanolic acid and ursolic acid possess anti-viral activity against HIV and hepatitis C virus (Kong et al., 2013; Kashiwada et al., 1998).

Therefore, the hypothesis of this research was OA and UA will demonstrate antiviral activity against HAV dried on Formica coupons. The highest light absorbance of OA is $\lambda_{max} = 259.09$ nm which is within the UV-C wavelength range of (100–280 nm) (Valero et al., 2007). Therefore, using germicidal UV light at 254 nm to illuminate the OA will allow absorption of photon energy, generating enough reactive oxygen species to cause a significant reduction to the HAV count. The objective of this study were to investigate the anti-viral activity of oleanolic acid and ursolic acid in the direct contact mode against HAV (HM-175) dried on the surface of Formica coupons and to investigate the effectiveness of UV light alone, OA alone, and a combination of UV light and OA to inactivate/reduce hepatitis A virus on Formica coupons.

Material and Methods

Viruses and Cell Lines

Hepatitis A virus (HAV; strain HM-175) and the host fetal rhesus monkey kidney (FRhK4) cells that were obtained from Dr. Kalmia Kniel (University of Delaware) were used as described earlier, (Joshi, Su, & D'Souza, 2015). As reported earlier, 0.5×10^6 to 1×10^6 FRhK-4 cells/well were added to 6 well plates and maintained at 37°C in Dulbecco's Modified Eagle's Medium/Ham's F-12 (DMEM-F12; HyClone Laboratories, Logan, UT) that was supplemented with 2% heat-inactivated Bovine Calf Serum (BCS, HyClone) and 1×Anti-Anti (containing Antibiotic and Antimycotic) under 5% CO_2 in a CO_2 incubator (Fisher Scientific, USA) (Joshi, Su & D'Souza, 2015).

Virus Propagation

Standardized procedures as described earlier were followed for HAV propagation (Bozkurt et al., 2014; Joshi, Su, & D'Souza, 2015). Briefly, when the FRhK4 cells reached > 90% confluency in cell culture flasks, they were washed twice with sterile Dulbecco's phosphate-buffered saline (DPBS; pH 7.4) and then HAV stocks were added to FRhK-4 monolayer cells These HAV infected host cells were incubated at 37°C for 3 h, and then 10 ml of DMEM-F12 containing 10% BCS was added. Flasks were incubated under 5% CO_2 (Water-jacketed CO_2 Incubator) until >90% cell lysis at 37°C. After seven days of incubation, the infected cells were freeze thawed three times to break down the host FRhK-4 cells and release HAV. HAV was recovered by initial centrifugation at 5,000 × g for 10 min, then filtered through

a 0.2-µm filter, and finally aliquoted into 2 ml tubes, and stored at -80°C for experiments as needed (Su & D'Souza, 2011).

Antiviral Reagents

Oleanolic acid was purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan), with a ≥ 98.0% purity. OA stock solution was prepared by dissolving 6 mg of the powder in 1 ml dimethyl sulfoxide (DMSO) to reach a final concentration of 6 mg/ml (13 mM) and (100% DMSO). Ursolic acid was purchased from Indofine Chemical Company (Hillsborough, NJ), at ≥ 99% purity. UA stock solution was prepared by dissolving 18 mg powder into 1 ml DMSO to reach a final concentration of 18 mg/ml (39.4 mM) and (100% DMSO).

UV-C Lamp

The UV illumination applied to the surface of the Formica coupons and tomatoes was conducted using a 30-Watt G30T8 fluorescent germicidal UV-C lamp (Philips, Holland), emitting light at 254 nm wavelength within a biosafety cabinet.

Absorbance Spectra

Solutions of 1 mg OA or UA per 1 ml DMSO were prepared. Light absorbance was measured within the range of 200 to 800 nm, using the Evolution 201 UV visible spectrophotometer (Thermo scientific).

Characterization of UV Light Irradiance

The irradiance of the UV light was measured using ILT5000 Research Lab Radiometer (International Light Technology, Peabody, USA) The irradiance (W/cm^2) was determined by taking the average of three irradiance measurements of four positions and then averaging those values (Figure: 3.1). The calculated dosage (energy density) in J/cm^2 received for the 10 min exposure time was calculated using the equation (E=Pt) where E is the dose and P is the calculated (irradiance power density) and t is the exposure time in s (Ghate et al., 2013).

Cytotoxicity Test

Cytotoxicity of OA and UA against the FRhK-4 cell line was measured using the MTS (3- [4,5, dimethylthiazol-2-yl]-5-[3-carboxymethoxy-phenyl]-2-[4-sulfophenyl]-2H -tetrazolium, inner salt), cell proliferation colorimetric assay kit as described by (Dia & Pangloli, 2017). Controls used were the untreated cell line with assigned 100% viability. Testing of all treatments and controls were replicated thrice.

Determination of Antiviral Activity of Oleanolic Acid and Ursolic Acid Against HAV

The tested compounds were serially diluted in Dulbecco's Modified Eagle Medium (DMEM) to reach the desired concentrations of 30, 60, 120, 600, 1000 and 2000 µg OA ml and concentrations of 60, 180, 360 and 600 µg UA per ml. Cleaned and autoclaved Formica coupons (6.5 × 4.5 cm) were inoculated with 100 µL HAV (~6 log PFU/ml) (3.41×10^3 PFU/cm^2 HAV on coupons), then placed in sterile petri dishes with open lid and left to air dry within the biosafety cabinet for 10 min. Next, 100 µL of the tested reagents or sterile deionized distilled

(dd) water (control) were applied to the surface of the coupons for 1 h or 10 min. The virus was recovered for each time point from the surface of the Formica coupons by repeated pipetting with 1200 µl DMEM-F12 containing 8% Bovine Calf Serum (BCS), then transferring this solution into a 15 ml tube containing 8.7 ml DMEM 8% BCS. The recovered virus was ten-fold serially diluted with 2% BCS DMEM media, and then infected on to confluent FRhK-4 host cells in 6-well plates for 3 h, followed by virus aspiration and overlay using overlay media (DMEM 2X powder, bovine calf serum, sodium bicarbonate, non-essential amino acids, $MgCl_2$ (4M), hepes buffer and gentamycin- kanamycin dissolved in sterile double deionized water and filtered through a 0.2-µm filter) and 1% Noble Agar at a 1:1 ratio. After 5 days of incubation at 37°C in an atmosphere containing 5% CO_2 (Water-jacketed CO_2 Incubator), the plaques were stained with a 1 ml of overlay media containing neutral red (Sigma-Aldrich, St. Louis, MO) incubated for 24 h and the virus plaques were counted. Each treatment was replicated thrice, and the data were statistically analyzed as described before.

Photodynamic Inactivation of HAV Using Oleanolic Acid on Contact Surfaces

Formica coupons (6.5 × 4.5 cm) were inoculated with 100 µL HAV (~6 log PFU/ml) (3.41×10^3 PFU/cm^2 HAV on coupons), then placed in sterile petri dishes and left to dry within the biosafety cabinet for 10 min as described above. Next, 100 µL of OA at 600 µg/ml was applied to the surface of the coupons (2µg/cm^2 OA on coupons) in duplicate. Similarly, 100 µl of sterile (dd) water was applied to the surface of Formica coupons in duplicate. The coupons were then placed in sterile petri dishes within the biosafety cabinet. They were illuminated with UV light at 254 nm wavelength for 10 min, at distance of 72 cm from the UV light source with UV dose of 0.04

J/cm^2. Another two coupons containing HAV with OA and HAV with sterile (dd) water (control) were kept in closed petri dishes without illumination for 10 min. After illumination, the virus was recovered from the surface of the Formica coupons by repeated pipetting with 1200 µl DMEM with 8% Bovine Calf Serum (BCS) into a Petri dish then transferring this solution into a 15 ml tube containing 8.7 ml DMEM 8% (BCS). The virus was serially diluted ten fold with DMEM-F12 containing 2% BCS and plaque assayed as described above.

Statistical Analysis

Results from the treatment (Oleanolic acid or Ursolic acid) and control were statistically analyzed by independent t-test, and one-way ANOVA was used to analyze PDI experiment; Tukey's Post Hoc test was used for mean separation, similar to those used in previous studies (Turner & Thayer, 2001; Iversen et al., 1987). All statistical assumptions regarding normality and equality of variances were met (SAS, version 9.4, release TS1M3) and used for all analyses; $P < 0.05$ was considered significant. All treatments and controls for the independent t-test were replicated thrice, except for OA at 2 mg/ml and PDI experiments, which were replicated five times, as reported in literature (Turner & Thayer, 2001; Iversen et al., 1987).

Results

Absorbance Spectra

OA showed highest light absorbance (lambda max) at 259.09 nm with 1.04 Absorbance unit (Abs) and lowest light absorbance at 256.32 nm with 0.29 Abs. UA showed lambda max at 216.50 nm with 2.06 Abs and the lowest light absorbance at 280.01 nm with 0.28 Abs.

Characterization of UV Light Irradiance

The irradiance value was determined by taking the average of three irradiance measurements at each position of the four used positions and then averaging those values. The calculated UV dose (energy density) value for 10 min exposure time was 0.040±0.0011 J/cm^2.

Cytotoxicity Test

The (MTS) assay did not detected cell toxicity of the FRhk-4 cell line using OA at 60 µg/ml and UA at 180 µg/ml.

Inactivation of HAV by Oleanolic Acid and Ursolic Acid on Formica Coupons surfaces

The stocks of OA and UA were dissolved in DMSO. Stocks of OA at 6 mg/ml DMSO were further diluted in DMEM to reach the desired concentrations of 30, 60, 120, 600, 1000 and 2000 µg/ml. UA stock of 18 mg/ml (dissolved in DMSO) was diluted in DMEM to prepare a range of tested concentrations (60, 180, 360 and 600 µg UA per ml), used for the treatment of HAV with a titer of ~6 log PFU/ml that was dried on Formica coupons for 1 h. The experiment was performed using one replicate for each concentration against HAV; those with significant viral reduction results were selected and repeated thrice. Kong et al., (2013) used a maximum DMSO level of 2%, as it is considered safe at this concentration when used on the HepG2 cell line (Kong et al., 2013). In this study, the direct contact treatment mode include an inactivation step, the virus and OA were diluted at 100x (10^{-2}) dilution factor in DMEM with 8% BCS, followed by serial dilution in DMEM with 2% BCS, at a 10x dilution factor for the next viral dilutions

Therefore, after diluting the 6000 µg/ml OA stock (100% DMSO) to 2000 µg/ml OA (33.33% DMSO) and when using the second viral dilution of (10^{-2}), the DMSO concentration applied on the FRhK-4 cells was 0.33% which is considered a safe level for the cells. Moreover, the OA at 2000 µg/ml in DMEM has a pH level of 7.3 when measured using pH test strips. The treatment of HAV (~6 log PFU/ml) dried on Formica coupons (6.5 × 4.5 cm) with UA at 180 and 360 µg/ml (applied concentrations of 0.62 and 1.23 µg/ cm^2, respectively), for 1 h resulted in 0.66±0.16 and 1.33±0.35 log PFU/ml reduction, respectively. Treatment with OA at 60 and 600 µg/ml on the surface of HAV dried on Formica coupons (applied concentrations were 0.21 and 2.1 µg/ cm^2, respectively) for 1 h resulted in reduction of 1.23±0.27 and 2.27±0.67 log PFU/ml, respectively. While OA at 2000 µg/ml for 10 min, (applied concentration of 6.84 µg/ cm^2) showed 1.79±0.61 log PFU/ml reduction of HAV (Table 3.1).

Inactivation of HAV with Photoactivated Oleanolic Acid

HAV (~6 log PFU/ml) dried on Formica coupons treated with 600 µg/ml oleanolic acid, (applied concentration of 2.1 µg/ cm^2), for 10 min did not result in any noticeable reduction of HAV. UV illumination of the control (HAV with dd water) for 10 min at a wavelength of 254 nm, distance of 72 cm, and UV-C dose (energy density) of 0.040±0.0011 J/cm^2 resulted in a 1.75±0.7 log PFU/ml HAV reduction. Photoactivated oleanolic acid at 600 µg/ml for 10 min and the same illumination conditions resulted in a 2.39±0.31 log PFU/ml reduction of HAV (Table 3.2).

Discussion

The treatment of HAV with OA at 30 µg/ml for one hour on Formica coupons did not show any viral reduction (using one replicate) compared to untreated control, while increasing the concentration to 60 µg/ml (0.13 mM) resulted in a statistically significant virus reduction of 1.23±0.27 log PFU/ml compared to control (using three replicates; $p<0.05$). The treatment of HAV with an increased OA concentration of 120 µg/ml for one hour did not show any enhanced reduction (using one replicate), while treatment of HAV with increased concentration of 600 µg/ml (1.31 mM) for one hour resulted in a statistically significant HAV reduction of 2.27±0.67 log PFU/ml ($p<0.05$) compared to control. However, the OA at 60 µg/ml HAV recovered reduction is not statistically significant from the OA at 600 µg/ml recovered HAV reduction for the one-hour treatment time ($p>0.05$). Treatment with a further increased OA concentration of 1 mg/ml did not show an increase in HAV reduction after one hour (using one replicate). However, increasing the OA concentration to 2 mg/ml (4.38 mM) resulted in a statistically significant HAV reduction of 1.79±0.61 log PFU/ml compared to control after a shorter treatment time of 10 min ($p<0.05$). It should also be noted that longer treatment times of one hour showed signs of cell toxicity at 2 mg/ml OA concentration. This cytotoxicity effect was not observed when using shorter treatment times of 10 min with 2 mg/ml OA (Table 3.1).

Contrary to treatment of HAV with OA at the same concentration, UA at 60 µg/ml (0.13 mM) did not reduce HAV titer (using one replicate), while an increased concentration to 180 µg/ml (0.39 mM) caused a reduction of 0.66±0.15 log PFU/ml after one hour. However, this reduction was not statistically significant from the control (using the average of three replicates; $p>0.05$).

Treatment of HAV with a higher UA concentration of 360 µg/ml (0.79 mM) for one hour resulted in an increased reduction of 1.32±0.35 log PFU/ml (using the average of three replicates). However, the UA at 180 µg/ml HAV recovered reduction is not statistically significant from the UA 360 µg/ml recovered HAV reduction for the one-hour treatment time ($p>0.05$). A further increase in the UA concentration to 600 µg/ml (1.3 mM) resulted in decreased HAV reduction of 0.6 log PFU/ml (using one replicate). Hence, we cannot conclude if anti-HAV of UA is dose dependent within this dosage range among the tested concentrations. UA at 360 µg/ml seems to be the most effective treatment concentration against HAV for 1 h (Table 3.1).

Therefore, OA, possesses the same level of HAV reduction at the lower concentration of 60 µg /ml compared to UA at 360 µg/ml. This work found that an increase in OA concentration leads to an enhanced HAV viral reduction indicating dose dependent effects; however, for UA, anti-HAV activity occurs within the range of 180-360 µg/ml with no improvement in antiviral activity at higher or lower concentrations. These results are supported by the previous study of Kong et al. in 2013, that reported a stronger antiviral activity of OA at lower concentrations than UA when applied to host cells after infection with HCV. In this current work, OA displayed a higher HAV reduction on Formica coupons compared to UA after a one-hour treatment. Furthermore, HAV recovered titer of both the OA at 60 µg/ml compared to 600 µg/ml was not statistical different ($p>0.05$). Likewise, recovered HAV titers of UA at 180 µg/ml compared to UA at 360 µg/ml was not statistical different. This insignificancy is perhaps due to high standard deviation among measurements. This could be improved in future experiments by a using

smaller stainless-steel coupons as a contact surface in order to measure and control surface roughness similar to those used by Park et al., 2015 or increasing the number of replicates (more than three replicates) in order to decrease the standard deviation of the HAV recovered titer.

Thus, OA at 600 µg/ml was used in the photodynamic inactivation (PDI) experiment to enhance its reduction level using a shorter treatment time of 10 min. OA has its highest absorbance at 259 nm with one absorbance unit. UV light with a wavelength of 254 nm was used as the light source, at a distance of 72 cm and UV-C dose (energy density) value of 0.040±0.001 J/cm^2. Although OA at 600 µg/ml had a 2.27±0.67 log PFU/ml reduction after one-hour, it did not affect HAV at a shorter treatment time of 10 min without UV illumination, which indicates the importance of longer treatment time for effective viral reduction at this concentration. Moreover, photoactivated HAV with coupons treated with OA at 600 µg/ml showed higher viral reduction levels of 2.39±0.31 PFU/ml than the photoactivated HAV with (dd) water, which recorded a 1.75±0.7 log PFU/ml reduction only. Although the recorded reductions of HAV with water and HAV with OA were statistically significant from the control, they were not statistically significantly($p>0.05$) from each other, which implies that the recorded reduction was probably due to the UV effect rather than the generation of reactive oxygen species.

Conclusions

This research shows that triterpenoid oleanolic acid and ursolic acid possesses antiviral activity against HAV, with OA exhibiting stronger anti-viral activity. It seems that the difference in the position of the methyl group proves to be crucial factor in the ability of these compounds to

inactivate HAV. It was observed that higher concentration of UA at 600 µg/ml (2.1 µg/ cm^2 applied on coupons) showed a decrease in the anti HAV activity. However, just one replicate was done which was not enough to confirm this finding. While this research does not give an answer to the question of the mechanisms of antiviral activity of OA and UA against HAV infectivity, or whether physical changes occur in the virus capsids, these questions could be answered in future research. By using electron microscopy images of OA treated HAV as compared to untreated HAV, as well as by treating the host cell with OA and UA pre-and post-HAV infection, the specific effects of these compounds in the virus' attachment to and replication within the host cells could be elucidated. In this study, the photodynamic inactivation of HAV using OA and UV-C light at 254 nm did not show a significant difference compared to the reduction caused by HAV with water and UV light Therefore-PDI treatment could potentially be successful if a different UV light source with broader wavelength is utilized than the germicidal UV light used in the experiment. The UV light used in this study has a narrow wavelength range, with most of the energy concentrated around 254 nm (Qian, 2002). However, UA has its highest light absorbance at 216 nm with 2 absorbance units, as measured by spectrophotometry. Although UA has more absorbance units than OA, suggesting better ability as a photosensitizing compound (by generating stronger ROS), there were no available, efficient light sources in our lab that could emit in this wavelength during the course of this experiment. Therefore, research with UA as a potentially promising photosensitizer compound could be used for future PDI research.

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Appendix

Table 3.1: Effect of oleanolic acid and ursolic acid at room temperature for one hour or 10 min treatment times against hepatitis A virus dried on Formica coupons compared to a control (water). Different letters within the recovered titer column denote significant differences between treatments ($p < 0.05$). Data are presented as the average of three replicates ±S.D.

Reagents	**HAV (log PFU/ml)**		
	Treatment for 1h	**Recovered titer**	**Reduction**
Ursolic Acid	Water	6.07±0.46 A	-
	180 µg/ml	5.49±0.31 A	0.66±0.16
	Water	6.36±0.25 A	-
	360 µg/ml	5.07±0.51 B	1.33±0.35
Oleanolic Acid	Water	6.07±0.46 A	-
	60 µg/ml	4.93±0.48 B	1.23±0.27
	Water	6.39±0.23 A	-
	600 µg/ml	4.35±0.78 B	2.27±0.67
	Treatment for 10 min	**Recovered titer**	**Reduction**
	Water	6.00±0.44 A	-
	2000 µg/ml	4.21±0.50 B	1.79±0.61

Table 3.2: Effects of photoactivated oleanolic acid at 600 µg/ml, with UV wavelength of 254 nm, distance of 72 cm, UV dose (energy density) of 0.040±0.0011 J/cm^2 for 10 min exposure time against hepatitis A virus (~6 log PFU/ml) dried on Formica coupons at room temperature. Different letters denote significant differences within the average recovered column ($p < 0.05$). Data are presented as the average of five replicates ±S.D.

Treatment	**Time 10 min 0.04 J/cm^2**	
	Average Recovered log (PFU/ml)	**Reduction log (PFU/ml)**
Control	A 6.24±0.18	-
HAV with Oleanolic Acid at 600 µg/ml	A 6.25±0.17	0
HAV with Water and UV	B 4.34±0.68	1.75±0.70
HAV with Oleanolic Acid at 600 µg/ml and U.V	B 3.82±0.49	2.39±0.31

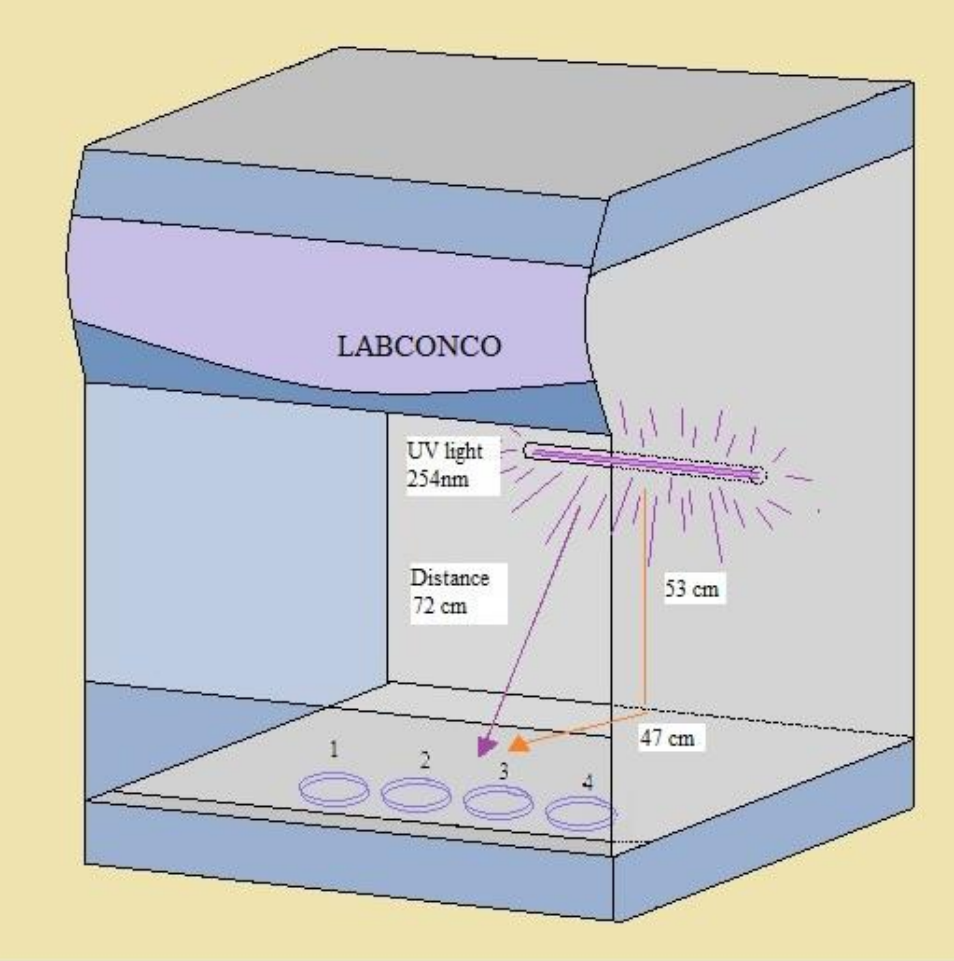

Figure 3. 1: Illustration of the four positions of the petri dishes containing either coupons or tomatoes inside the LABCONCO class II biosafety cabinet. Specifications included UV light 30-Watt fluorescent light bulb (Philips), emitting at 254 nm, with a plate distance of 72 cm from the light source. The UV dose (energy density) for the 10 min exposure time was 0.040±0.001 J/cm^2. Irradiance was determined by taking the average of three irradiance measurements at each position and then averaging those values.

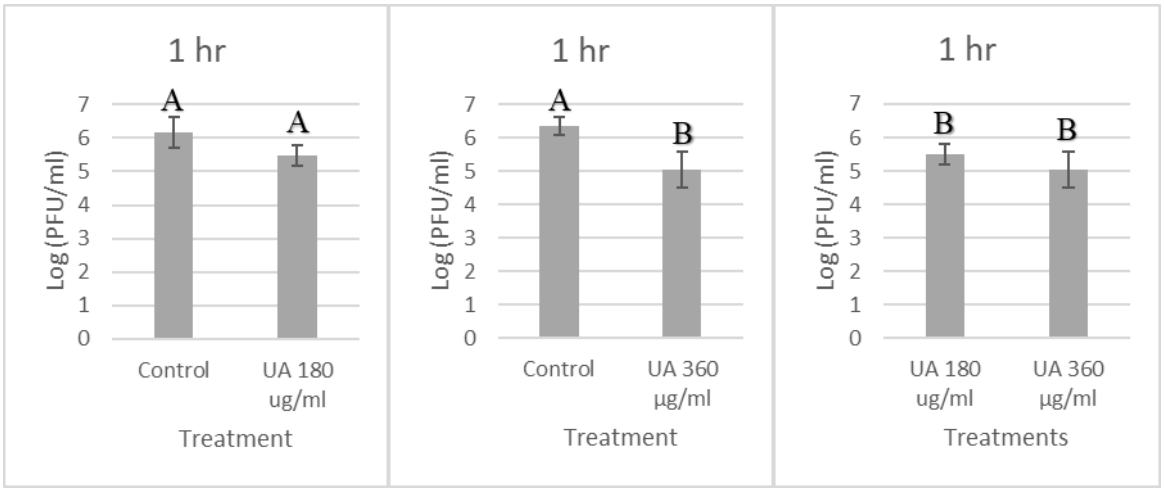

Figure 3.2: Treatment of HAV, dried on Formica coupons with ursolic acid at 180 µg/ml, 360 µg/ml for 1 h, resulting in 0.66±0.16 and 1.33±0.35 log PFU/ml reduction of HAV, respectively. Different letters denote significant differences between each treatment concentration and its control ($p < 0.05$). Data are presented as the average of three replicates ±S.D.

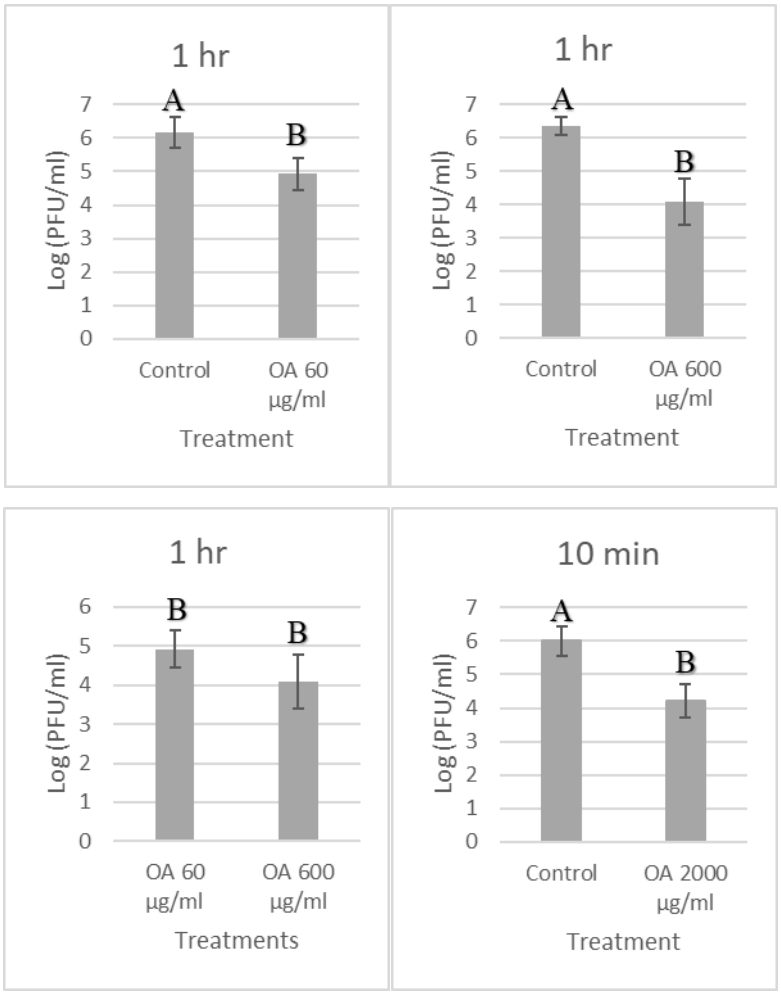

Figure 3.3:Treatment of HAV dried on Formica coupons with oleanolic acid at 60 and 600 µg/ml for 1 h and 2 mg/ml for 10 min, resulting in 1.23±0.27, 2.27±0.67, and 1.79±0.61 log PFU/ml virus reduction, respectively. Different letters denote significant differences between each treatment concentration and its control ($p < 0.05$). Data are presented as the average of three replicates ±S.D.

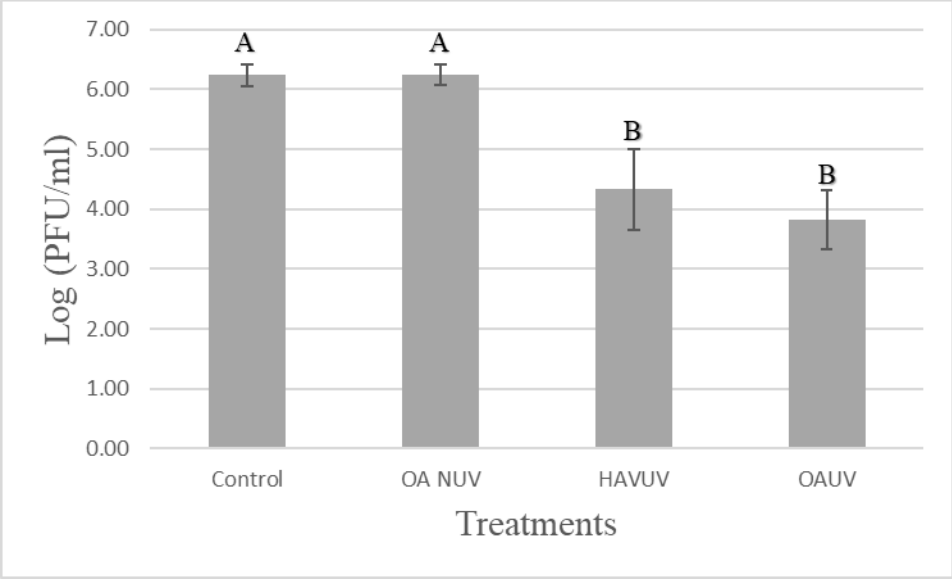

Figure 3.4: Effect of photoactivated oleanolic acid at 600 µg/ml against HAV (titers ~6 log PFU/ml) dried on the surface of Formica coupons after 10 min treatments and illumination with 254 nm wavelength, at a distance of 72 cm and dose (energy density) of 0.040±0.001 J/cm^2, at room temperature. Different treatments included HAV with (dd) water, No UV (Control); HAV with OA, No UV (OA-NUV); HAV with (dd) water and UV (HAV-UV); and HAV with OA and UV (OA-UV). Different letters represent significant differences of recovered titers between different treatments ($p<0.05$).

Chapter IV: Conclusion

This research investigated the anti-HAV activities of three compounds; grape seed extract (GSE), oleanolic acid (OA) and ursolic acid (UA) and in combination with ultraviolet light to explore potential enhanced inactivation. It was determined that GSE at a concentration of 0.91 mg/ml with a treatment time of 30 min had no effect on HAV dried on Formica coupons, while at a higher concentration of 10 mg/ml and a treatment time of 5 min, GSE showed 1.53±1.04 log PFU/ml reduction of HAV on the surface of tomatoes. Both OA at 60 and 600 µg/ml and UA at 180 and 360 µg/ml were found to possess anti-viral activities against HAV dried on Formica coupons. OA at 60 and 600 µg/ml after one-hour treatment time showed 1.23±0.27 and 2.2±0.67 log PFU/ml viral reduction, respectively. A higher OA concentration of 2 mg/ml and a shorter treatment time of 10 min, resulted in a 1.79±0.61 log PFU/ml reduction of HAV. UA at 180 and 360 µg/ml after one-hour treatment showed 0.66±0.16 and 1.33±0.35 log PFU/ml reduction of HAV, respectively. At the tested concentrations, OA was found to have a higher anti-viral activity against HAV than UA. Furthermore, photodynamic inactivation of HAV was investigated using GSE at 10 mg/ml and OA at 600 µg/ml to enhance viral reduction by the initiation of reactive oxygen species (ROS). The 30-Watt G30T8 fluorescent germicidal UV-C lamp (Philips, Holland) emitting light at 254 nm wavelength was used for the PDI experiments. The resulting reductions of HAV with 10 mg/ml GSE and HAV with 600 µg/ml OA for the different UV-C exposure times (3, 5, 10, 15, 20 and 30 min) were not statistically significant ($p>0.05$) from the reduction of HAV with PBS or water and UV-C which indicates that the resulting viral reduction was due to the effects of UV rather than the generation of reactive oxygen species. Moreover, the germicidal UV-C light used in the PDI experiments has most of

its energy concentrated around 254 nm; therefore, a different UV-C light source with a broader wavelength emission could be used in the future. Both OA and UA at the tested concentrations showed anti-HAV activities on Formica surfaces. However, OA at 2 mg/ml and a short treatment time of 10 min appears to be more applicable to the food sector for use on food contact surfaces. This approach can potentially reduce foodborne outbreaks associated with cross-contamination of ready-to-eat foods (RTE) such as produce.

Vita

Mostafa Mahmoud Ali Abotaleb was born in Cairo, Egypt in 1984. He attended and graduated high school from Holy Family School in Cairo in 2001, and then continued his college education at Faculty of Agriculture, Cairo University, earning a B.S. in Agricultural Science, Biotechnology in 2006. He went on to earn a M.S. of Horticultural Genetics and Biotechnology from the Mediterranean Agronomic Institute of Chania, Greece in 2012. He began the M.S. program in the Food Science department at the University of Tennessee, Knoxville in August of 2017 and will graduate in May 2019.