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Impact of UV-C irradiation on the quality, safety, and cytotoxicity of cranberry-flavored water using a novel continuous flow UV system

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Abstract

The influence of short wave-length UV-C irradiation at 254 nm on microbial inactivation, anthocyanins stability, ascorbic acid, and cytotoxicity of formulated cranberry flavored water was studied. *Escherichia coli* ATCC 25922 and *Salmonella enterica* serovar Typhimurium ATCC 13311 were inactivated by more than 5 $\log_{10}$ at UV-C fluence of 21 $mJ \cdot cm^{-2}$. At UV-C fluence of 40 $mJ \cdot cm^{-2}$ the content of ascorbic acid was 82% of that in the untreated beverage. The concentrations of the anthocyanins (Cy_3Ar, Cy_3Ga, Pe_3Ar, and Pe_3Ga) were not significantly affected at the same treatment level. Cytotoxicity evaluation of the irradiated beverage on normal colon (CCD-18Co), colon cancer (HCT-116), and healthy mice liver (AML-12) cells showed that UV-C irradiation had no cytotoxic effects on all three cell lines. This research study suggests that UV-C treatment of formulated cranberry flavored water can achieve high levels of microbial inactivation without significantly decreasing the concentration of anthocyanins, ascorbic acid content or generating cytotoxic effects. These results suggest that UV-C irradiation can be an alternative to thermal pasteurization in producing high quality beverages.

1. Introduction

Literature suggests that the consumption of fruits and their products may lessen the impact from several types of cancer and other chronic diseases, and that the phytochemical constituents present in these foods are mainly responsible for these health benefits (Arts & Hollman, 2005). Cranberries are an abundant source of flavonoids, which exhibit strong antimicrobial and antioxidant properties. These flavonoids have the ability to inactivate pathogenic microbes (Vollmannova et al., 2014). Phenolic acids, which are abundantly available in cranberries, are responsible for neutralizing the oxidation process and decreasing inflammation in humans (Neto, 2007; Vinson, Zubik, Bose, Samman, & Proch, 2005). Unlike phenolic acids, anthocyanins are accountable for the color of the fruit and are the primary antioxidant compounds in cranberries, which are used in a variety of products such as juices, juice cocktails, and flavored water. The popularity of bottled flavored waters has been increasing worldwide (Lawlor et al., 2009). These products have preservatives, bioactive compounds, and/or sweeteners that convey good sense of taste and flavor. The increasing consumption of flavored water is due to the health benefits such as low in calories, reduced sugar intake, and the concept of "all natural" and minimally processed. However, quality and safety remain crucial aspects for consumers (Grunert, 2005; Rohr et al., 2005).

FDA requires that juice or beverage processors attain a 5-$\log_{10}$ reduction in the most resistant and pathogenic microorganism in their products (FDA, 2001). In fruit juices, *E. coli* and *Salmonella* have been the bacterial pathogens responsible for most outbreaks (Zhao et al., 1993; Cook et al., 1998; CDC, 1996). While thermal processing such as pasteurization can effectively inactivate pathogens in fruit juices, thermal treatment can impact organoleptic properties and reduce the content of bioactive compounds (Patras et al., 2009). In order to avoid the adverse

effects of thermal processing on liquid foods, non-thermal disinfection methods including ultraviolet (UV) treatment have increasingly been considered over the last few years (Rawson et al., 2011).

UV-C irradiation is a promising food preservation technology that may be a substitute for thermal processing (Worobo, 1999). UV-C irradiation is increasingly being considered for the treatment of liquid foods (Bhullar et al., 2018; Islam et al., 2016a; Islam et al., 2016b, Chandra et al., 2017; Ward., et al 2017). The short wavelength UV-C spectrum is effective for inactivating a range of microorganisms. UV-C light can penetrate into the cell, and is strongly absorbed by nucleic acids, forming pyrimidine dimers, and thereby preventing replication of microorganisms (Azimi, Allen, & Farnood, 2012). Additionally, FDA has approved the use of UV-C irradiation for treatment of juices in order to inactivate pathogenic microbes (U.S FDA 2001).

Acceptance of UV as a treatment method for consumer beverages has been hampered by absent or inconclusive literature on the effectiveness of the treatment. Most of the published literature did not follow standard methods to validate UV dose delivery in absorbing liquid foods (Unluturk et al., 2010; Caminiti et al., 2012). Some authors reported dose levels up to 1,269,000 $mJ \cdot cm^{-2}$ for bacterial inactivation, even though it is well established that *E. coli* requires less than 20 $mJ \cdot cm^{-2}$, to achieve 5-$\log_{10}$ inactivation (Sommer et al, 1995). The variances in the recorded UV sensitivity values of these organisms leads to uncertainty in setting a UV-C treatment target for liquid foods. Many published studies had questionable methods of ensuring accurate UV-C dose delivery, and did not include verification of the applied dose. The use of modern methods to determine UV-C dose in inactivation studies could greatly reduce the uncertainty in the sensitivity and establish the design dose values for inactivating pathogenic and spoilage organisms.

Practical UV treatment for liquid foods must be achieved with flow-through reactors. However, any practical flow-through reactor will have non-uniform dose delivery, (intensity x time) because there will be gradients in both intensity and velocity that will not be perfectly offsetting. These effects become more severe when the optical absorbance is high. For liquids with high optical absorption, such as flavored water, most UV reactors will be unable to deliver equal UV treatment to the entire fluid volume, leading to poor performance dominated by flow paths where intensity is low. When absorbing fluid such as flavored water is to be treated by UV, unique reactor designs must be used to overcome the very short penetration of UV-C irradiation into bulk liquid.

UV irradiation studies in beverages usually do not consider the optical attenuation of the fluid, even though this factor will strongly affect performance (Unluturk et al., 2010; Caminiti et al., 2012). UV dose (fluence) can be described as the number of photons absorbed by an irradiated object per surface area. It is the product of UV intensity and exposure time, for each fluid element. However, in most experiments the optical absorbance of the fluid results in a non-uniform intensity field. As a result, there will be a distribution of doses delivered to a sample. Since disinfection is a logarithmic function of dose, the low-dose regions will dominate the measured disinfection performance.

It has been reported that UV irradiation of fruit juices can affect enzyme activity and may even form toxic byproducts. Koutchma et al. (2016) reported that UV irradiation affects enzymes associated with fresh juices, such as polyphenol oxidase (PPO), peroxidase (POD), lipoxygenase (LOX) and pectin methylesterase (PME). While there has been some investigation of the effects of UV on polyphenols, anthocyanins, and ascorbic acid, none of the studies verified the UV dose

delivered. Extreme UV doses could be expected to result in fluid damage, but this may be irrelevant at the doses suitable for disinfection.

None of the prior studies on UV treatment have evaluated the cytotoxicity of irradiated cranberry flavored water. UV-C photons can break chemical bonds, and could result in modifying compounds in the beverage. UV disinfection itself is the result of forming dimers (bonds) between adjacent pyrimidines in the nucleic acids of bacteria and viruses. Cytotoxicity of irradiated beverages must be evaluated to ensure that a novel food processing technique such as UV irradiation does not produce toxic chemical compounds.

Any practical UV-treatment system must utilize a flow-through reactor, and when highly-opaque fluids are to be treated by UV light, unique reactor designs are necessary. Most UV irradiation research studies have utilized batch reactors (i.e., collimated beam devices). By contrast, in this study a novel continuous-flow reactor was utilized; the fluid was pumped around a central monochromatic UV lamp in curved paths, forming Dean Vortices to achieve high mixing for uniform UV dose delivery. The reactor uses conventional, low-cost UV lamps along with a PTFE flow-path. The materials are all food-grade, and can be easily cleaned using conventional Clean-In-Place procedures. In addition, the wetted parts can be replaced as needed and autoclaved as required to ensure cleanliness and sterility. The flow will primarily be in laminar regime but the formation of Dean vortices will assist in high mixing. Higher the flowrate, higher will be the magnitude of these dean vortices. In this research study, the absorption coefficient of the fluid was accounted for, and dose delivery was verified through bio-dosimetery, ensuring that target levels of disinfection are achieved, and allowing direct comparisons with other UV treatment studies. The present study investigates the effect of UV

irradiation on the individual anthocyanins, ascorbic acid (quality markers) including microbial inactivation in cranberry flavored water.

2. Materials and Methods

2.1 Preparation of cranberry flavored water and its properties:

A cranberry-flavored beverage was formulated in the laboratory, in order to fully control the composition. Organic cranberry juice (R.W. Knudsen, market available, Nashville, TN, US) was diluted with sterile water in the ratio of 1:10 v/v. The brix of the test fluid was adjusted to 8% by the addition of sucrose (Alfa Aesar, Haverhill, MA, US). Ascorbic acid (Alfa Aesar, Haverhill, MA, US) was added to the test fluid in the concentration of 25 µg/mL. The absorption coefficient of the cranberry flavored water was matched to that of a store bought cranberry flavored water.

Due to the high absorbance of the beverage, optical measurements were conducted with diluted samples. For absorption measurements, the test fluid was diluted 1:10 v/v and the absorption coefficient at 254 nm was determined with a UV-Visible Spectrophotometer with 1 cm path length quartz cuvette (Thermo scientific, Genesys 10S, Milwakee, WI, US). The dilution factor was incorporated in the calculations to determine the absorption coefficient of the beverage. A pH meter (Jenway, Cole Palmer, OSA, UK) was used to measure the pH of the cranberry flavored water beverage. The total soluble solids (TSS) of the cranberry flavored water were measured using a refractometer (Schmidt + Haensch GmbH & Co. Berlin, Germany). All measurements were done in triplicate to reduce the random error. The optical and physical properties of formulated beverage are shown in Table 1.

2.2. Bacterial strains and culture conditions

Escherichia coli ATCC 25922 and *Salmonella enterica* ser. Typhimurium ATCC 13311 were investigated in this study. These cultures were stored in 25% glycerol in cryovials at a temperature of -80^{o} C. These strains were developed by two consecutive loop transfers of individual strains followed by incubation at temperature of 37° C for 18 hours using Tryptic Soy Broth (TSB) as a culture media (15 mL) (Oxoid Ltd., Basingstoke, UK). After incubation, the cultures were shifted to 30 mL of TSB for incubation at 37° C for 18 hours to stationary phase. Later, the bacterial cells were harvested using a centrifuge (Thermo Scientific Sorvall ST 16 R., New Jersey, US) at 3000 rpm for 15 minutes. After centrifugation, the cell pellets were collected and suspended two times with 0.1% (w/v) phosphate buffer saline (PBS, Becton Dickinson, New Jersey, US) and re-suspended in 50 mL of PBS. To enumerate the original population densities in each cell suspension, appropriate dilutions in peptone water (in 0.1% PW) were plated in duplicate onto Eosine Methylene Blue (EMB) (Oxoid Ltd., Basingstoke, UK) agar and *Salmonella* Chromogenic Agar (SCA) (Oxoid Ltd., Basingstoke, UK) plates for *E. coli* and *Salmonella* suspensions respectively, and kept for incubation at a temperature of 37° C for 24 hours.

2.3. Cranberry flavored water inoculation

Aliquots of 400 mL of bacterial cultures (*E. coli*, *Salmonella*) were inoculated into 3600 mL of cranberry flavored water targeting a concentration of 10^{8} CFU/mL. To determine the initial titres, inoculated cranberry flavored water was plated on Eosine Methylene Blue (EMB) agar (Oxoid Ltd., Basingstoke, UK); *Salmonella* Chromogenic Agar (SCA) (Oxoid Ltd.,

Basingstoke, UK) plates respectively and kept for incubation at a temperature of 37° C for 24 hours.

2.4. UV-C irradiation treatment & fluence verification

Cranberry flavored water was exposed to UV-C irradiation by means of a continuous-flow-through system. Test fluid was pumped through a Teflon tubing bundle arranged around a central low-pressure mercury UV lamp emitting at 254 nm wave-length (Trojan Technologies, London, ON, Canada). The system was designed and developed to achieve strong mixing in order to deliver uniform fluence to strongly-absorbing fluids (Bhullar et al., 2017; Ward et al., 2017). Flow regime plays an integral part in inactivating pathogens using continuous flow UV reactors. The flow in curved tubes results in secondary flow called Dean Vortices (Dean, 1927). It can generate strong mixing, resulting in uniform UV fluence (Koutchma et al., 2007). The flow can be characterized by the Dean number (D_e), which can be expressed as;

$D_e = R_e \sqrt{D/Dc}$ equation 1

$R_e = (\rho/\mu) \times V \times D$ equation 2

where *D* denotes the diameter of the tube, D_c is the coil diameter, R_e is Reynolds number of tube, μ is the dynamic viscosity of fluid, *V* is the velocity of the fluid, and ρ denotes fluid density (Mansor et al., 2014). Simple Dean Flow arises if (D/ D_c) in equation (1) is in the range of 0.03 and 0.1 (Dean, 1927). In the current study, the reactor design induced dean vortices in the test fluid that were effective in inducing high mixing thus allowing efficient inactivation of *E. coli* and *Salmonella.* A schematic view of flow through system is shown in Fig. 1.

Cleanliness and sterility of the UV system were ensured by performing a Clean-In-Place (CIP) procedure before and after use. This CIP consisted of flushing the system with sterile water followed by 0.1 N HCl, sterile water again, then 0.1 N NaOH, and final rinse by sterile water. Flushing was performed at the highest flow-rate.

Flow rates and average exposure times are inversely related. The exposure times were calculated based on the known flow rates and the total system volume. To deliver fluence between 6 and 21 $mJ \cdot cm^{-2}$, the exposure times for *E. coli* ATCC 25922 were between 115s, and 403s. For *Salmonella enterica* ser. Typhimurium ATCC 13311 the exposure times ranged from 94s to 376s for the UV-C fluence range of 5 to 20 $mJ \cdot cm^{-2}$. Higher UV fluence levels were chosen for the analysis of the impact of UV on anthocyanins, ascorbic acid and cytotoxicity. In this case the formulated cranberry flavored water was irradiated with UV-C fluence of 15 to 240 $mJ \cdot cm^{-2}$. All experiments were conducted in triplicate to reduce error.

It is important to validate UV dose delivery in order to ensure that fluid properties or systematic errors have not affected the UV dose. Reduction equivalent fluence (REF) delivered was validated using MS2 bacteriophage (GAP Enviro Microbial Services, London, ON, Canada) as a challenge organism. MS2 is a well-characterized virus that is commonly used in validating UV systems, since it can be grown at high titres and has consistent and known UV sensitivity. However, cranberry flavored water is acidic and would inactivate MS2. Accordingly, aqueous solutions of organic humic acid were used as a surrogate fluid to verify UV dosage as in earlier studies (Bhullar et al., 2017; Ward et al., 2017; Islam et al., 2016). Organic humic acid was diluted with distilled water to match the optical absorbance of the formulated cranberry flavored water, and this surrogate was then inoculated with MS2. This challenge fluid was then treated at

identical flow rates (UV doses) and the inactivation used to calculate the UV dose received by the fluid.

The log reduction obtained from MS2 bacteriophage was evaluated by GAP Enviro Microbial Services (London, ON, Canada). A linear trend was established between reduction equivalent fluence (REF) and target fluence up to 240 $mJ \cdot cm^{-2}$.

2.5. Organism sensitivity test

The UV sensitivity of the *Salmonella* and *E. coli* strains was verified through UV irradiations in buffered water. This approach, with high optical transparency, minimizes the intensity gradient in the fluid sample, thereby reducing the mixing necessary to achieve constant delivery of average fluence, reducing the uncertainty in the delivered REF. The following UV-C fluence values were delivered: 0, 4, 8, 12 $mJ \cdot cm^{-2}$ for *E. coli* ATCC 25922 and 0, 10, 20 $mJ \cdot cm^{-2}$ for *Salmonella* ATCC 13311. The UV-C fluence per log inactivation, or D_{10} values, is shown in Table 2. These values match the D_{10} values reported in previous studies (Sommer et al., 2000; Ward et al., 2017).

2.6. Enumeration of pathogens in cranberry flavored water after UV-C treatment

Enumeration of the viable bacteria was performed through dilution, culture, and counting colonies. After UV-C irradiation of cranberry flavored water, decimal dilutions of the treated samples and control were prepared in 0.1% buffered peptone water (Oxoid Ltd., Basingstoke, UK). Samples of *E. coli* and *Salmonella* Typhimurium inoculated cranberry flavored water were diluted to between 10^{0} and 10^{-6}. Viable counts of *E. coli* and *Salmonella* cells were obtained by culturing on Eosine Methylene Blue (EMB) agar and *Salmonella* Chromogenic Agar (SCA) plates respectively. Plate counts within the range of 25-250 or 30-300 were considered for

analysis. The bacterial colonies were counted and expressed as log CFU mL^{-1} of cranberry flavored water (undiluted).

2.7. Inactivation kinetics - Log-Linear model

The Log-Linear model is known to describe microbial inactivation after UV treatment and some other disinfection processes. The model is known as the Chick-Watson model and has been widely used for a century. For UV disinfection, the model is given in the following equation in which k_1 denotes the inactivation constant of first order (cm^{-2}·mJ). It is a property of the microbe that is being modelled. *D* is the UV-C fluence received by the organism or fluid element (Chick-Watson 1908).

$$\log_{10}\left(\frac{N}{No}\right) = -k1D \qquad (3)$$

where *N* is number (concentration) of organisms and N_o is initial number of organisms

Log reduction is calculated as $\log_{10}\left(\frac{N}{No}\right)$. The UV dose required for one log of inactivation, denoted D_{10}, is the reciprocal of the inactivation constant, *k1*.

2.8. Anthocyanins analysis

The quantification of cyanidin 3-arabinoside (C_3Ar), cyanidin 3-galactoside (C_3Ga), peonidin 3-arabinoside (P_3Ar), and peonidin 3-galactoside (P_3Ga) was carried out with calibration curves prepared with C_3Ga (Polyphenols Laboratories Sandes, Norway) as an authentic anthocyanin standard. A UHPLC system (Shimadzu Scientific Instruments, Columbia, MD) equipped with two Shimadzu LC-20ADXR pumps, a SIL-20AXR autosampler and an SPD-M20A photodiode array UV detector was utilized for the study (Patras et al., 2017). The injection volume was 10 μL and flow rate was 0.5 mL·min^{-1}. Initially, the concentration of

solvent B was 10%, and later increased linearly to 20% until 4.01 minutes. At 4.01 minutes, solvent B concentration was increased to 100%, and continued at 100% until 4.21 minutes. At 4.22 minutes, solvent B concentration was reverted to 10%, and continued at 10% until the end of the program at 6.0 minutes. The cyanidin-3-galactoside standard concentrations were serially diluted to create the calibration curve. Selected ion monitoring (SIM) MS methods were established for observing the following ions: *m/z* 419, *m/z* 449, *m/z* 433, *m/z* 463, corresponding to cyanidin-3-arabinoside, cyanidin-3-galactoside, peonidin-3-arabinoside, and peonidin-3-galactoside respectively. The areas of the peaks in the UV chromatogram (520 nm) were used to determine the concentrations of anthocyanins based on the Cy_3Ga calibration curve.

2.9. Ascorbic acid analysis

External calibration standards for ascorbic acid were prepared at concentrations from 25 μg/mL to 500 μg/mL. Analysis of ascorbic acid was carried out using a high-pressure liquid chromatography (HPLC) system (Shimadzu Corporation, USA). The system consisted of a pump, a vacuum degasser, and a Diode-Array Detector, and it was controlled through EZ Start 7.3 software (Shimadzu) at 40 °C. A hypersil ODS column (15 cm×4.6 cm, 5 μm, Supelco., US) fitted with hypersil ODS guard column (Gemini C_{18} [4 mm L×3.0 mm ID], Phenomenex., UK) was used with a mobile phase (isocratic) of 25 mM monobasic potassium phosphate adjusted to pH 3 at a flow rate of 1 mL/min (Patras et al., 2009).

2.10. Cell Cytotoxicity

UV-C irradiated cranberry flavored water samples of 5 mL each were reconstituted in PBS solution and stored at -20°C until use. Sample was added to cell culture media to a final

concentration of 5% v/v ratio for cytotoxicity analysis. HCT-116 cells were seeded in a 24 well plate with 2×10^5 cells per well in Eagle's minimum essential medium (EMEM) containing 10% fetal bovine serum (FBS) and kept for incubation in a humidified chamber at a temperature of 37°C and 5% CO_2 condition. After 24 hours, the cells were serum-starved overnight in a 1 mL EMEM containing 1% FBS (Patras et al., 2017).The cells were treated with 10 µL (less than 5% v/v) of reconstituted UV-C irradiated cranberry flavored water for 24 hours. At the end of the exposure time, CCK-8 reagent was used as per manufacturer's instruction (Dojindo Molecular Technologies, Inc., Rockville, MD) to measure the cell viability.

For CCD-18Co testing, 75,000 CCD-18Co cells in 15% FBS and EMEM medium were seeded in a 24 well plate for 48 hours. Growth medium was exchanged after 48 hours to 1.5% FBS and EMEM and left overnight. Each sample (10 µL) treatment was added to the corresponding well and incubated for 24 hours. Following that, 40 µL of CCK-8 reagent was added and incubated for an additional 2 hours. A Synergy 2 multi-mode microplate reader (BioTek, Winooski, VT) was utilized to measure the absorbance at a wavelength of 450 nm with reference wavelength of 650 nm for both the cell lines.

Healthy mice liver cells (AML-12, from ATCC, Manassas, VA) were cultured in DMEM enhanced with 10% FBS, at 37 °C with 5% carbon dioxide. The cells were cultivated regularly in 10 mL tissue culture dish from Corning (Corning, USA). The cell culture medium was changed every other day, i.e., three times a week. UV-C irradiated cranberry flavored water was extracted with ethyl acetate before cytotoxicity analysis and was diluted with cell culture media at various concentrations compared to that of the control (without UV treatment). After seeding in a 96-well plate for 24 hours, cells were subjected to various concentrations ranging from 50-fold dilution to 6.25-fold dilution for 3 days. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-

tetrazolium bromide (MTT) was used to determine cell viability. Cells in each well were incubated with 0.1 mL of culture medium comprising 0.5 mg/mL MTT at a temperature of 37 °C for an hour. MTT-containing media was removed prior to the solvation of reduced formazan dye using 0.1 mL of DMSO (Dimethyl sulfoxide) per well. Cell concentration was measured via absorbance at 570 nm.

2.11. Statistical analysis

A balanced experimental design was used, with three replicates for each treatment level (UV dose). Each sample was independent and assigned randomly to a treatment. All microbial data was fitted to log-linear models in Microsoft Excel 2016. Model fit statistics including R^2 (coefficient of determination) and Root Mean Square Error (RMSE) were calculated.

One-way ANOVA with Tukey's HSD multiple comparison tests were performed to assess the effects of UV in SAS statistical computing environment (SAS, 2016). Data are presented as means ± one standard deviation from the mean. Statistical significance were tested at 5 percent significance level ($p<0.05$).

3. Results and Discussion

3.1. UV Dose validation

Survival curves (“Dose-response”) were plotted and the log inactivation vs. UV dose was determined (Fig. 2). Log inactivation of MS2 at each flow rate, along with the UV-sensitivity of MS2 determined through the batch testing in PBS, was used to determine the Reduction Equivalent Fluence (REF) delivered to the fluid at each flow rate in the flow-through reactor. It was found that in the flow-through reactor the UV dose was directly proportional to average

residence time, (inversely proportional to flow rate) indicating good dose uniformity over the applied range. (Poor uniformity would result in “tailing”, or decreasing slope at higher doses.)

3.2. Bactericidal effect of UV

The optical and physical properties of cranberry flavored water are summarized in Table 1. From the optical data, it can be seen that cranberry flavored water was a strong absorber of UV-C light; UV light has very little transmission through the test fluid. The D_{10} values of *E. coli* and *Salmonella* Typhimurium derived from the collimated beam tests in buffer are shown in table 2.

The log reduction of *E. coli* and *Salmonella* as a function of UV dose (fluence) delivered in the Dean-flow UV reactor (Fig. 1) are illustrated in Fig. 3. The inactivation curves for *E. coli* and *Salmonella* Typhimurium were built by marking Log reduction (N/N_o) versus UV-C fluence ($mJ \cdot cm^{-2}$) as determined from the validation testing with MS2 phage. Based on the literature (UV sensitivity of microbes), it was predicted that low UV fluence ranging from 0-21 $mJ \cdot cm^{-2}$ would achieve up to about 5 log inactivation of *E. coli* and *Salmonella enterica sero.* Typhimurium. The number of cells in *E. coli* and *Salmonella* present in inoculated cranberry flavored water was about 8 $\log_{10}$ CFU/mL, ensuring that there were adequate surviving organisms to allow determination of log inactivation at the highest doses.

In this study, *E. coli* was inactivated by more than 5 logs at a UV fluence of 21 $mJ \cdot cm^{-2}$. The initial concentration for the untreated flavored cranberry water was 7.94 $\log_{10}$ CFU/ml and after maximum dose of 21 $mJ \cdot cm^{-2}$, the viable *E. coli* cells were reduced to 2.64 $\log_{10}$ CFU/ml. In this study, five different fluence levels of 6, 12, 15, 18, 21 $mJ \cdot cm^{-2}$ achieved inactivation of *E. coli* by 1.33, 2.99, 4.00, 4.85, 5.30 $\log_{10}$ CFU/ml, respectively. The inactivation curve followed a

log linear model with R^2 =0.99 and D_{10} value of 3.86 mJ·cm^{-2} (Fig. 3) with a low RMSE value of 0.15. The data adequately fit the model as depicted in Fig. 2. Goodness of fit for both microbes is listed in Table 3. The D_{10} values determined in this study are consistent with values reported in the literature. The D_{10} values of *E. coli* O157:H7 cells in the literature were between 0.4 and 3.5 mJ·cm^{-2} (Sommer et al., 2000, Tosa & Hirata, 1999; Yuan et al., 2003). Our data is in agreement with the literature, which used carefully controlled UV dosing that accounted for optical absorbance and other factors. Our results suggest that an excellent reduction of viable bacteria could be achieved when using a continuous flow UV reactor. This was despite the fact that the cranberry flavored water had very high absorption.

Some authors have found very high resistance of *E. coli* in opaque media, but these values were likely the effect of improper dose calculation and poor dose distribution in opaque media. A study by Unluturk et al., (2008) on liquid egg products (LEP) contaminated with non-pathogenic *E. coli* K-12 ATCC 25253 and pathogenic *E. coli* O157:H7 irradiated with UV-C via a collimated beam apparatus described that at the maximum claimed dose of 98 mJ·cm^{-2}, only an inactivation of 0.675-log$_{10}$ CFU/ml and 0.316-log$_{10}$ CFU/ml reduction in bacteria for liquid egg yolk and liquid whole egg respectively was observed. This would correspond to a D_{10} of more than 145 mJ·cm^{-2}, an extraordinarily high resistance to UV. The high optical absorbance of the liquid egg would result in a very strong intensity gradient in the sample. Further, high viscosity would prevent adequate mixing, resulting in non-uniform UV dose delivery, so the results could be dominated by the surviving organisms in the dark regions of the sample.

Keyser et al (2008) reported that *E. coli* K12 in apple juice was inactivated by 7.42 log at a claimed dose of 1377 mJ·cm^{-2} (D_{10}~186 mJ/cm^2) using a continuous-flow commercial UV system. In a different study, Guerrero-Beltran and Barbosa-Canovas (2005) observed 5.1 log

reduction for *E. coli* in pasteurized juice at a claimed dose of 450 KJ·m^{-2}(45,000 mJ·cm^{-2}), equivalent to a D_{10} of nearly 5,000 mJ·cm^{-2}. It is critical to note that the authors in this study calculated UV dose as surface fluence times the treatment time (hydraulic retention time). The opacity of the fluid and the hydraulic flow path of the fluid likely resulted in poor dose distributions and consequently poor inactivation. It is of vital importance to consider the optical attenuation coefficients of the test fluids. (Caminiti et al. 2012, Unluturk et al. 2010).

The flow-through reactor in the present work also achieved high levels of inactivation for *Salmonella* inoculated into cranberry flavored water. Just over 5 $\log_{10}$ reduction was observed for *Salmonella* at 20 mJ·cm^{-2}. The initial concentration for the untreated flavored cranberry water was 7.96 $\log_{10}$ CFU/ml and after maximum dose of 20 mJ·cm^{-2}, the concentration of viable *Salmonella* cells was reduced to 2.61 $\log_{10}$ CFU/ml. According to our study, the population of *Salmonella* were decreased by 1.21, 3.36, 4.84, 5.35 logs at a UV-C irradiation fluence levels of 5, 10, 15, 20 mJ·cm^{-2} respectively. The inactivation curve showed a log linear model with R^2 =0.97 and D_{10} value of 3.41 mJ·cm^{-2} and low RMSE value of 0.40. The data adequately fit the model as depicted in Fig. 3. These results agree with the published results of Tosa and Hirata (1998) who found that different strains of *Salmonella* have D_{10} values ranging from <2 to 7.5 mJ·cm^{-2}.

It is apparent that system design of the continuous flow UV-C reactor provided adequate mixing that resulted in log linear inactivation of microbes even up to 5 logs or more for *E. coli* and *Salmonella*. The reactor also achieved good result with the more resistant MS2 challenge organism. The UV-C irradiation treatment resulted in greater than 5 $\log_{10}$ reduction of pathogen microbial strains such as *E. coli* and *Salmonella* in formulated cranberry flavored water at the

fluence threshold of 40 $mJ \cdot cm^{-2}$ set by the FDA. This study has evidently attained 5-log reduction mandated by US Food and Drug Administration for refrigerated fruit juices.

3.3. Effect of UV on ascorbic acid

Exposure to light can degrade vitamin C, which is naturally present in some juices and is added to beverages both to promote health and as a chelating agent to prevent color change (Maillard's reaction). Tikekar et al. (2011) found that UV-C irradiation resulted in vitamin C degradation in liquid food due to induced molecular excitation and subsequent photochemical reactions. Accordingly, the effect of the UV treatment on ascorbic acid concentration was investigated. Cranberry flavored water was subjected to UV-C fluence of 0, 15, 30, 60, 120 and 240 $mJ \cdot cm^{-2}$ and ascorbic acid was quantified. A plot of ascorbic acid concentrations with different UV fluence levels is shown in Fig. 4. A monotonic decrease in the concentration of ascorbic acid was observed. The FDA has recommended that UV radiant exposure must be at least 40 $mJ \cdot cm^{-2}$ for microbial disinfection for acidic fluids. UV treatment resulted in a statistically significant change ($p<0.05$) in ascorbic acid concentration at a fluence of 60 $mJ.cm^{-2}$. At the dose of 60 $mJ \cdot cm^{-2}$ there was about 20% reduction in ascorbic acid concentration. While there were additional small decreases at higher UV dose, these changes were not statistically significant.

Limited studies on the vitamin stability in UV-C treated juices are available in the scientific literature. Adzahan (2006) reported that UV treatment of apple cider using a continuous flow UV system at dosage of 14.3 $mJ \cdot cm^{-2}$ resulted in 30% loss in vitamin C content, but the authors did not verify and validate the UV doses: biodosimetry studies were not conducted. By comparison with thermal pasteurization studies, Santhirasegaram et al. (2015) reported a degradation of 65 % of ascorbic acid in mango juice using thermal treatment. The

presence of oxygen contributes to the heat-degradation of ascorbic acid (Oms-Oliu et al., 2012). It is quite evident that when compared with heat treatment, ascorbic was well retained in UV irradiated cranberry flavored water even at high fluence levels used in this study.

3.4. Anthocyanins stability

Plots of anthocyanin concentrations with different UV fluence levels are shown in Fig. 5 (a, b, c, d). Cranberry flavored water was exposed to UV-C fluence up to 240 $mJ \cdot cm^{-2}$ and four anthocyanins were quantified (Cy_3Ar, Cy_3Ga, Pe_3Ar, Pe_3Ga). No statistically significant changes in concentration of any of the anthocyanins were observed at a UV-C fluence of 60 $mJ \cdot cm^{-2}$, which exceeds the FDA-mandated dose of 40 $mJ \cdot cm^{-2}$ to achieve 5 log reduction of *E. coli* and *Salmonella*.

Even at higher UV doses, the impact on anthocyanins was small. The concentration of Cy_3Ar was not affected by UV-C irradiation treatment ($p<0.05$) at any of tested levels. The concentration of Cy_3Ga, Pe_3Ar and Pe_3Ga in cranberry flavored water had no significant difference up to the UV-C fluence of 120 $mJ \cdot cm^{-2}$ (Fig. 5b, 5c and 5d). However, the concentration of cyanidin-3-galactoside (Cy_3Ga), peonidin-3-arabinoside (Pe_3Ar) and peonidin-3-galactoside (Pe_3Ga) was reduced at a UV-C dose of 240 $mJ \cdot cm^{-2}$ ($p<0.05$). While it has been reported that long term absorption of UV-C light (at 253.7 nm) by anthocyanins may affect conjugated bonds such as aromatic, double rings, and disulfide compounds, and may decrease the anthocyanin content (Koutchma et al., 2009), these results were not observed in the present study.

There are conflicting reports of UV damage to anthocyanins, but these differences may relate to uncertainties in UV dose application. It has been reported that the loss of individual

anthocyanin pigments in the range of 8.1-16.3% occurs after UV-C treatment in pomegranate juice with a dose of 62.35 J/mL (Pala et al., 2011). However, in that study, UV-C dose/fluence is reported as Joules/Liter, which cannot be correlated with disinfection. For example, the same Joules/Liter in an absorbing beverage like cranberry water will result in a completely different disinfection than the same Joules/Liter in clear water, because of much different penetration of UV (due to varying optics). The authors also did not take the fluid optics into account. Other studies have reported that anthocyanins are very heat sensitive. Dorko et al., (2015) reported that heat treatment of cherry juice caused significant reduction in the content of cyanidin-3-glucosyl-rutinoside (Cy_3Gr) by 18, 29 and 38% at 70, 80 and 90° C respectively. In a different study, the stability of polyphenols (Chlorogenic, phloridzin and catechins) as function of UV dose was investigated. Minor changes were induced at 40 $mJ.cm^{-2}$. Biodosimetry studies were conducted to verify the UV dose or fluence (Islam et al 2016a). Similar results were reported for catechins in green tea. Continuous-flow UV-C irradiation of the green tea beverage at a relevant commercial disinfection dose of 68 $mJ \cdot cm^{-2}$ induced a minor reduction in the concentration of the most abundant catechin in green tea, (-)-epigallocatechin gallate (EGCG), from 145 to 131.1 $\mu g \cdot mL^{-1}$. The total phenolic content of the green tea beverage remained constant at all UV fluence levels (Vergne et al., 2018).

To summarize, the concentration of all the four anthocyanins in cranberry flavored water were not affected at the FDA recommended fluence of 40 $mJ \cdot cm^{-2}$ respectively.

3.5. Cell Cytotoxicity

Cytotoxicity studies were done on UV-C treated cranberry flavored water at fluence levels of 0, 15, 30, 60, 120, 240 $mJ \cdot cm^{-2}$ on normal colon (CCD-18Co) and colon cancer (HCT-116) cells (Fig. 6 a and b). Cells were exposed to cranberry flavored water for 24 hours of

treatment time. These results reveal that there is no significant difference in the cell viability (%) of cranberry flavored water at any UV treatment level on both normal colon cells and colon cancer cells ($p<0.05$). Furthermore, it was observed that cranberry flavored water alone, without UV treatment, slightly reduced the cell viability of cancer cells. However, the effect of UV-C irradiation was not significant within the two cell lines used for the study. In short, UV-C irradiation at any UV fluence up to a maximum fluence of 240 $mJ \cdot cm^{-2}$ did not affect the cytotoxicity of cranberry-flavored water on these two cell lines.

Cytotoxicity studies were also done on UV-C treated cranberry flavored water at a fluence of 0, 15, 30, 60, 120 and 240 $mJ \cdot cm^{-2}$ in healthy mice liver cells (AML-12, Fig. 6 c). The results demonstrated that there was no increased inhibition that could be found from the entire dilution range of UV-C treated cranberry flavored water. Moreover, it showed that different UV-C fluence (0, 15, 30, 60, 120 and 240 $mJ \cdot cm^{-2}$) did not cause an increased inhibition of viability of the AML-12 cells in comparison to that of untreated cranberry flavored water. Our results also demonstrated that cell viability of UV-C treated cranberry flavored water had no significant difference in all dilutions when compared to control ($p<0.05$). Therefore, it is quite evident that cranberry flavored water showed no cytotoxic effects on healthy mice liver cells.

Literature showcasing the effects of fruit juice on cancer *in vivo* or *in vitro* is lacking. Studies on citrus fruit juices, for example grapefruit, pomelo, orange, and lime were conducted on Caco-2 adenocarcinoma cells (Lim and Lim, 2006). These studies reported that lime and lemon juices exhibited cytotoxicity towards Caco-2 cells compared to other fruit juices. Similarly, the anticancer and antiproliferative effects of cranberry phytochemicals have been investigated *in vitro* and *in vivo* (McMurdo et al., 2008; Neto et al., 2008; He and Liu, 2006; Ferguson et al., 2006). The results achieved in our study showed that cranberry flavored water

alone has imparted slightly higher antiproliferative effects on HCT-116 colon cancer cells than normal colon cells. However, UV-C irradiation did not show any significant trend caused due to UV-C fluence. Therefore, this cytotoxicity study signifies that UV-C irradiation of cranberry flavored water does not affect the toxicological properties on the three cell lines that were examined.

4. Conclusion

This study indicates that UV fluence of less than 40mJ.cm^{-2} can successfully reduce the microbial load in cranberry flavored water, suggesting that UV-C irradiation is a plausible treatment method to inactivate microbes in cranberry flavored water. Log linear kinetics described the inactivation of the microorganisms chosen for study. Inactivation UV-C doses for a 5-log reduction (99.999%) were around 21 mJ·cm^{-2} for *E. coli* and *Salmonella enterica* ser. Typhimurium. UV-C irradiation induced minor reductions in the concentration of ascorbic acid and anthocyanins in cranberry flavored water at the FDA-recommended fluence of 40 mJ·cm^{-2}. UV-C treatment did not affect the cytotoxicity of cranberry flavored water on normal colon cells, cancer cells and healthy mice cell lines. The findings were consistent across various fluence levels, even at high exposure levels about 6 times the FDA recommended level. This study demonstrated that the UV treatment of cranberry flavored water did not generate cytotoxic compounds in cranberry flavored water nor did it cause degradation of bioactive compounds in cranberry flavored water. Results suggested that UV-C irradiation can be an alternative to thermal pasteurization in producing high quality flavored water.

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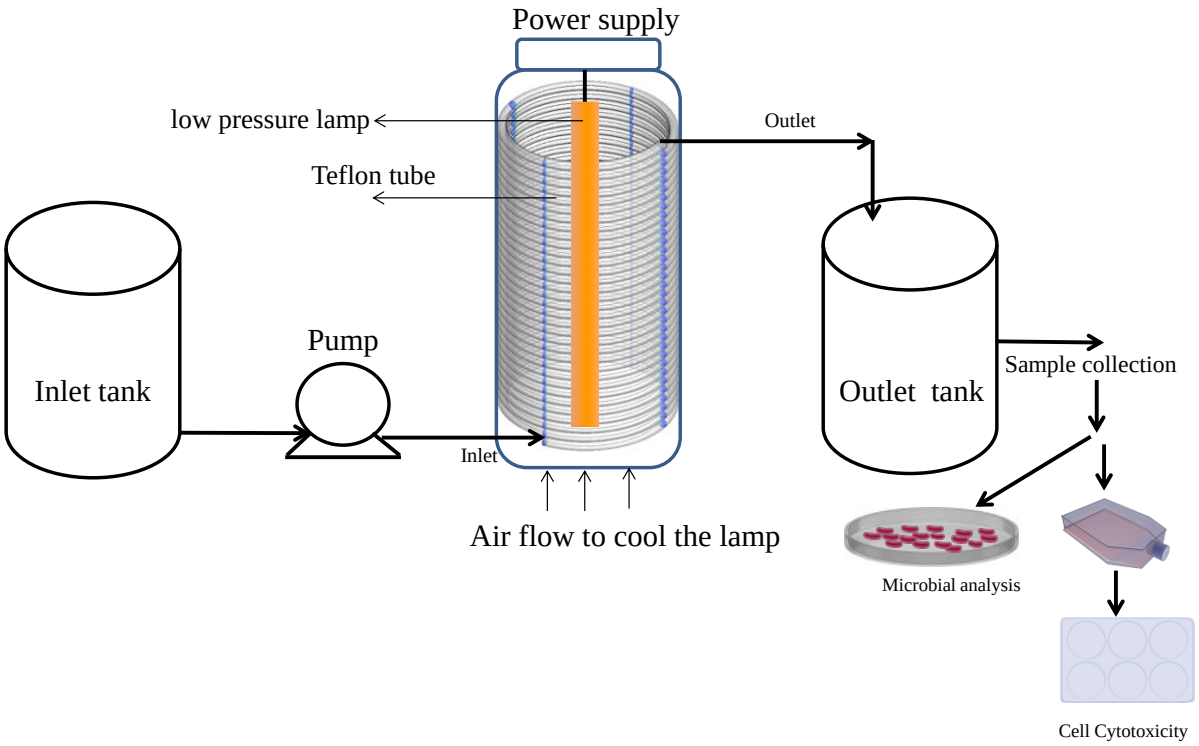


Figure 1: Schematic view of UV flow-through system

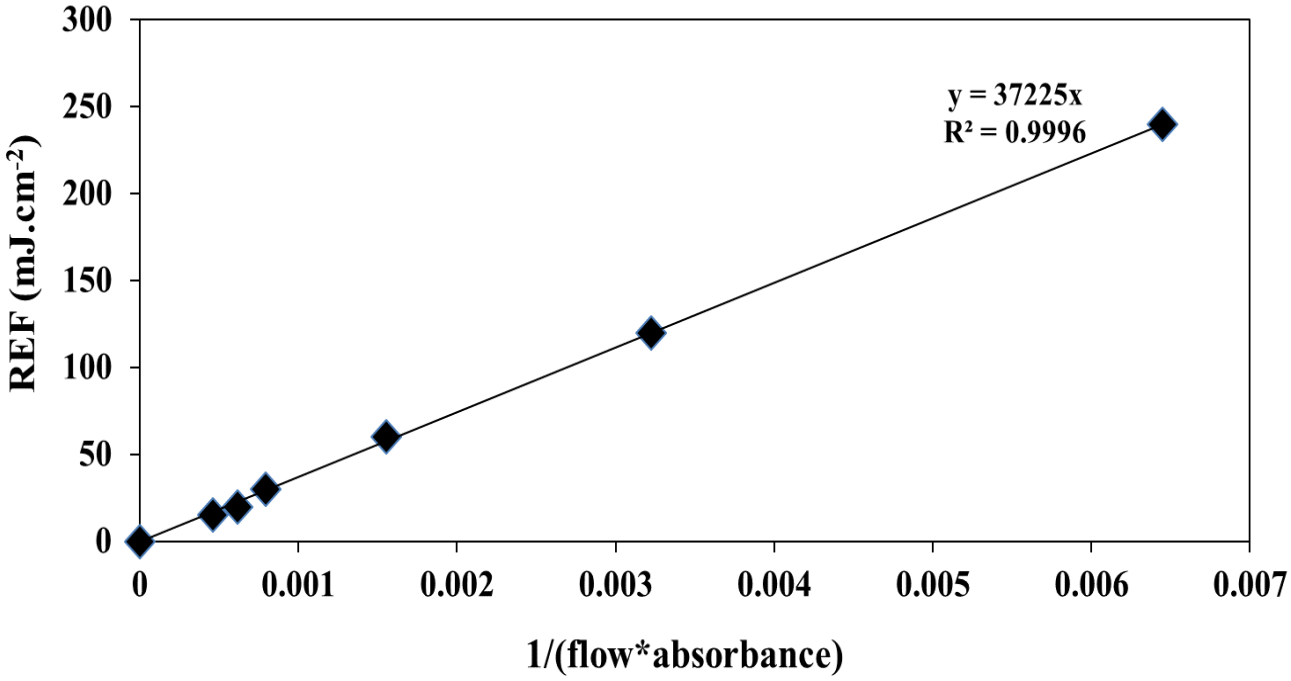


Figure 2: MS2 Reduction Equivalent Fluence vs. flow rate for surrogate fluid pumped through the UV flow-through system

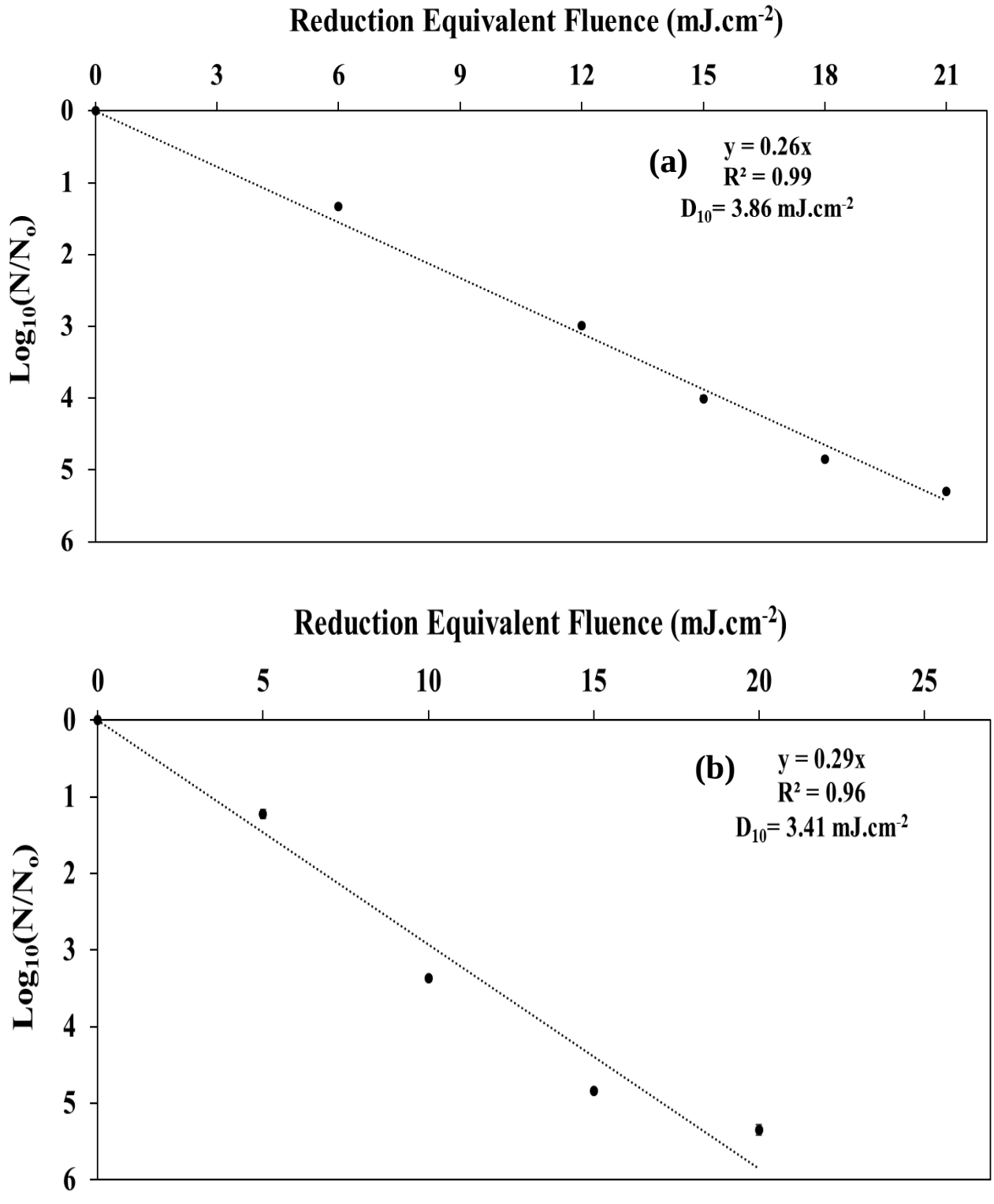


Figure 3: UV inactivation rate of (a) *Escherichia coli* ATCC 25922 and (b) *Salmonella* Typhimurium ATCC 13311 in cranberry flavored water.

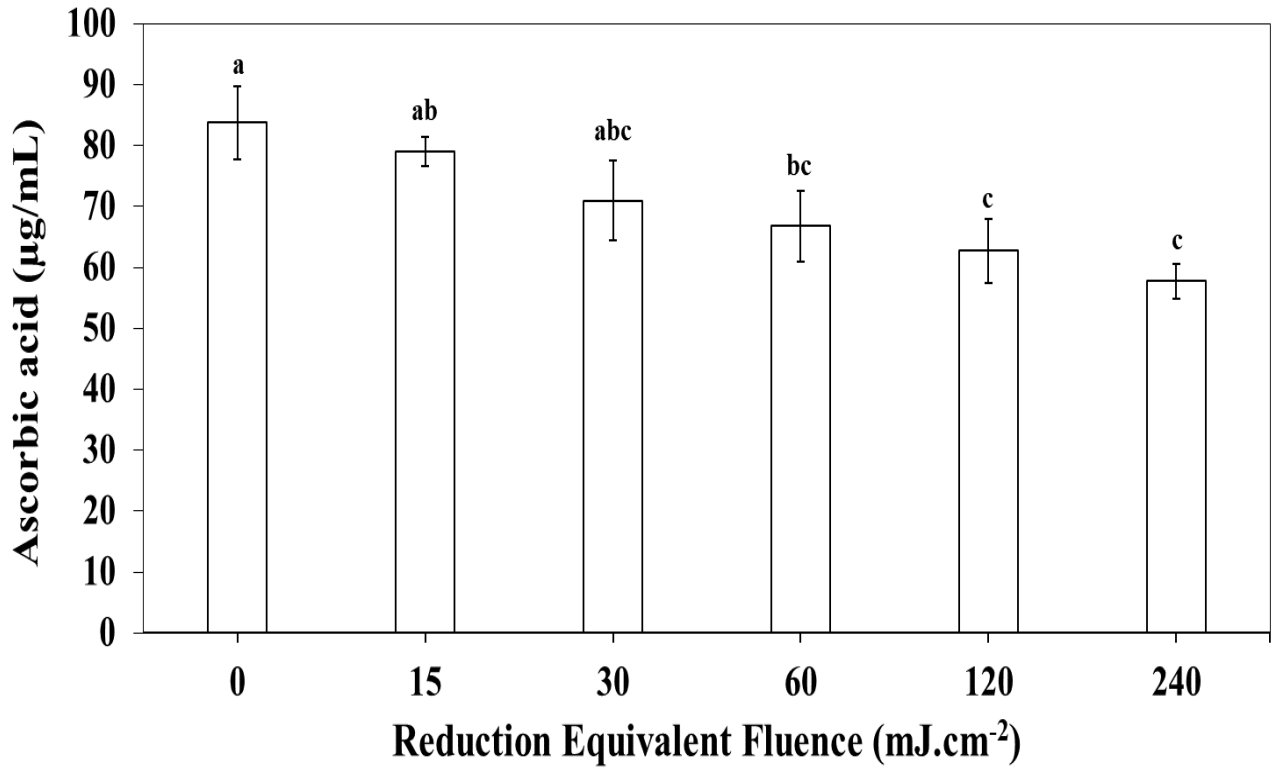
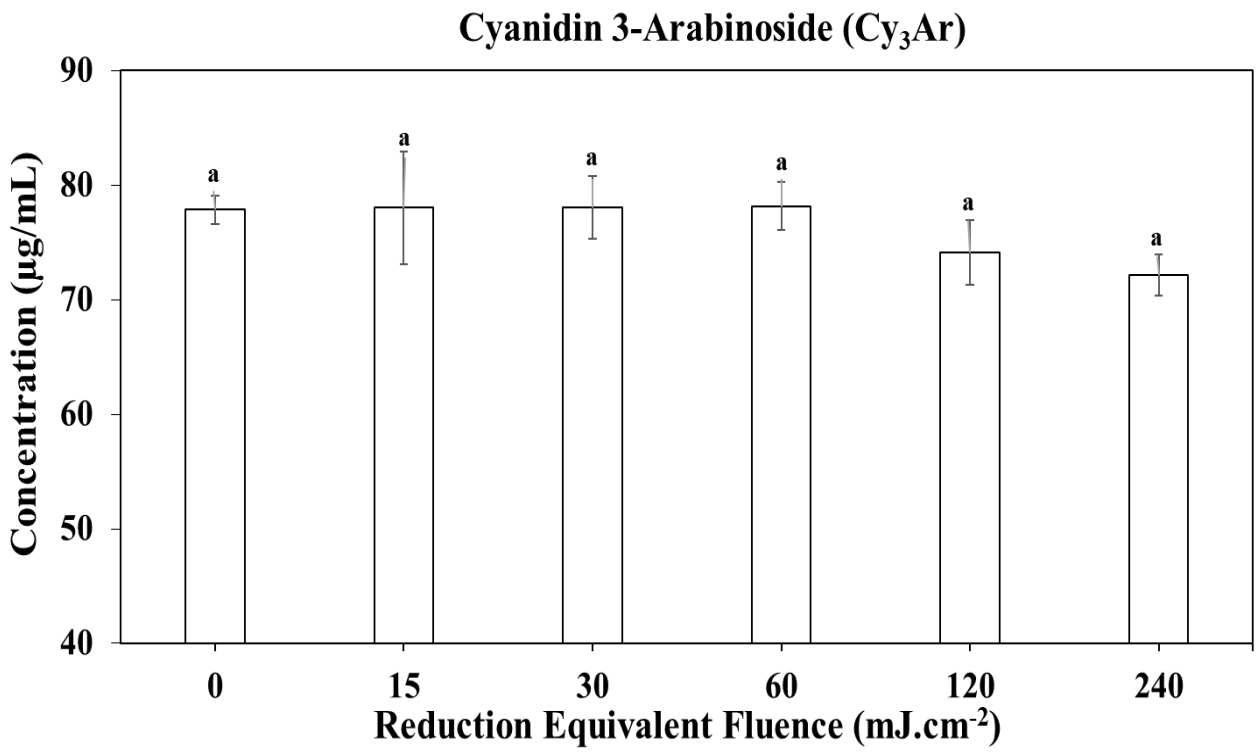
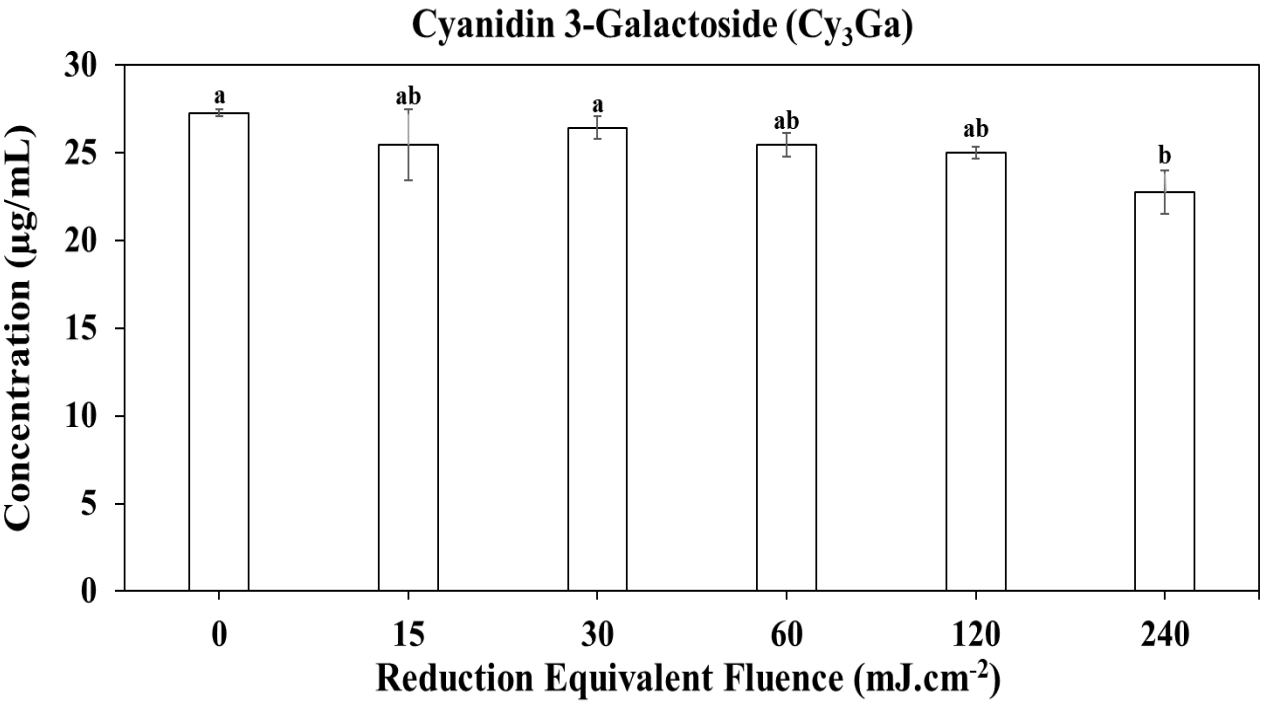


Figure 4: Effect of UV-C irradiation on ascorbic acid (mean value ± SD, n = 3) in cranberry flavored water at UV-C fluence of 0 (control) 15, 30, 60, 120, and 240 $mJ \cdot cm^{-2}$. Within each figure, different letters denote significant differences at $p<0.05$.

(a)


Cyanidin 3-Arabinoside (Cy3Ar)
Concentration (µg/mL)
90
80
70
60
50
40
a
a
a
a
a
a
0
15
30
60
120
240
Reduction Equivalent Fluence (mJ.cm-2)

(b)


Cyanidin 3-Galactoside (Cy3Ga)
Concentration (µg/mL)
30
25
20
15
10
5
0
a
ab
a
ab
ab
b
0
15
30
60
120
240
Reduction Equivalent Fluence (mJ.cm-2)

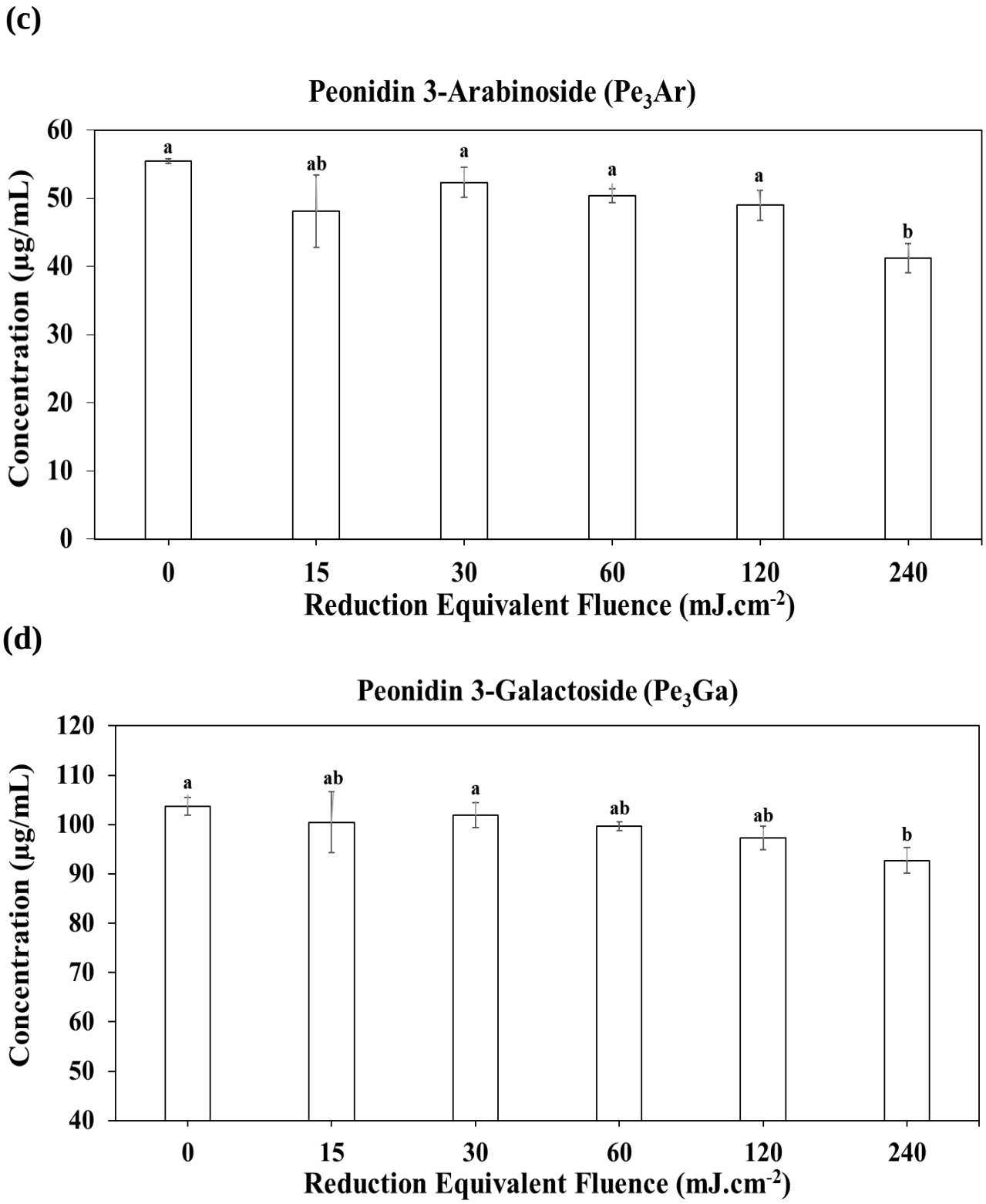
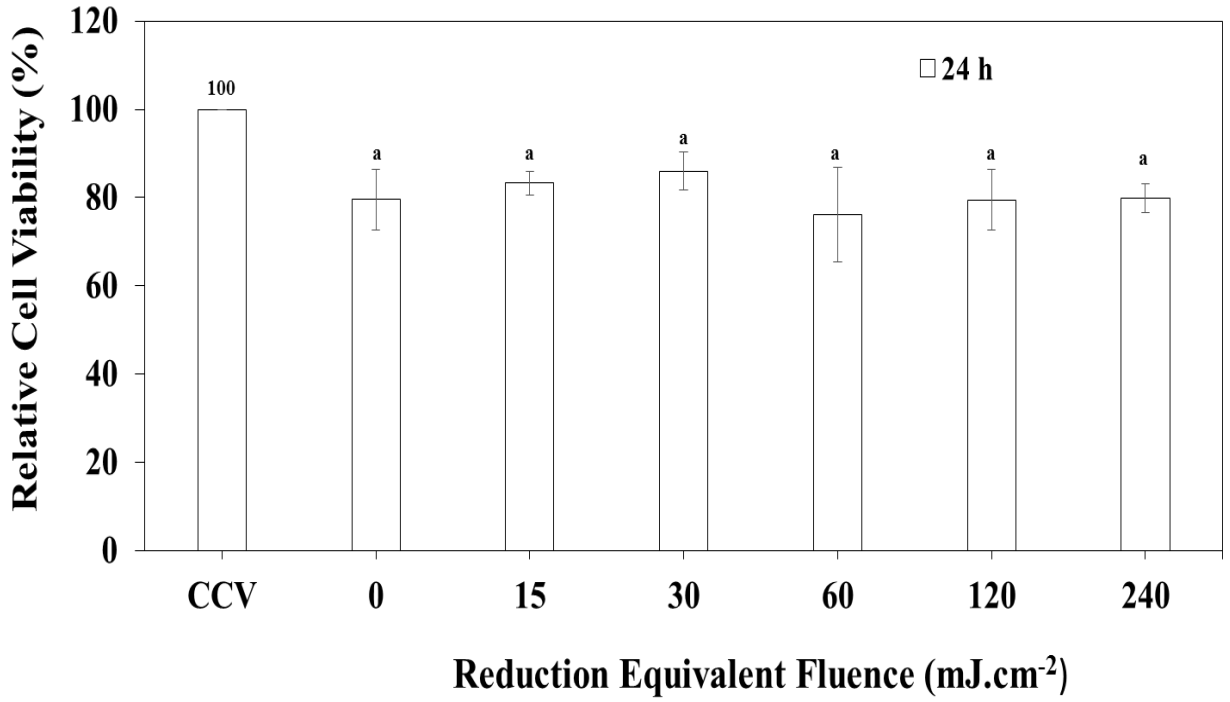
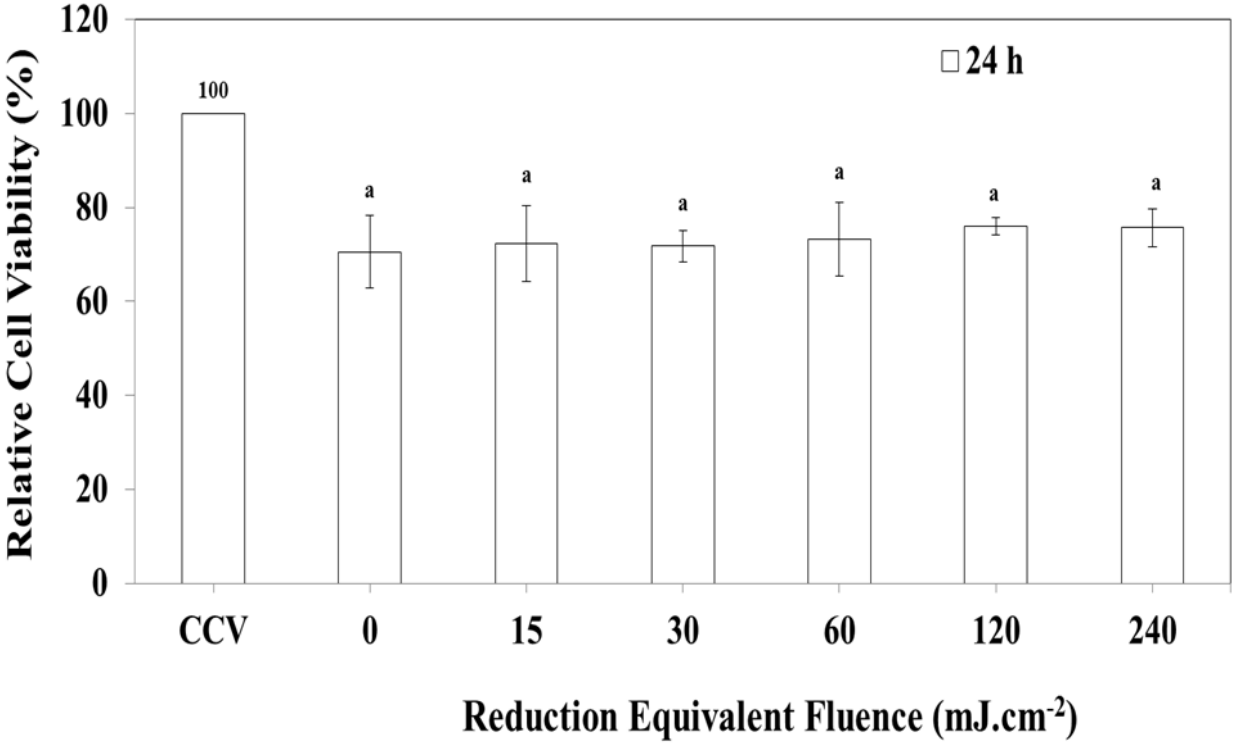


Figure 5: Effect of UV-C irradiation on anthocyanins (mean value ± SD, n = 3) in cranberry flavoured water at UV- C fluence of 0 (control) 15, 30, 60, 120, and 240 mJ·cm^{-2} : (a), (b), (c), (d). Within each figure, different letters denote significant differences at $p<0.05$.

(a)


Relative Cell Viability (%)
120
100
80
60
40
20
0
24 h
100
a
a
a
a
a
a
CCV
0
15
30
60
120
240
Reduction Equivalent Fluence (mJ.cm-2)

(b)


Relative Cell Viability (%)
120
100
80
60
40
20
0
24 h
100
a
a
a
a
a
a
CCV
0
15
30
60
120
240
Reduction Equivalent Fluence (mJ.cm-2)

(c)

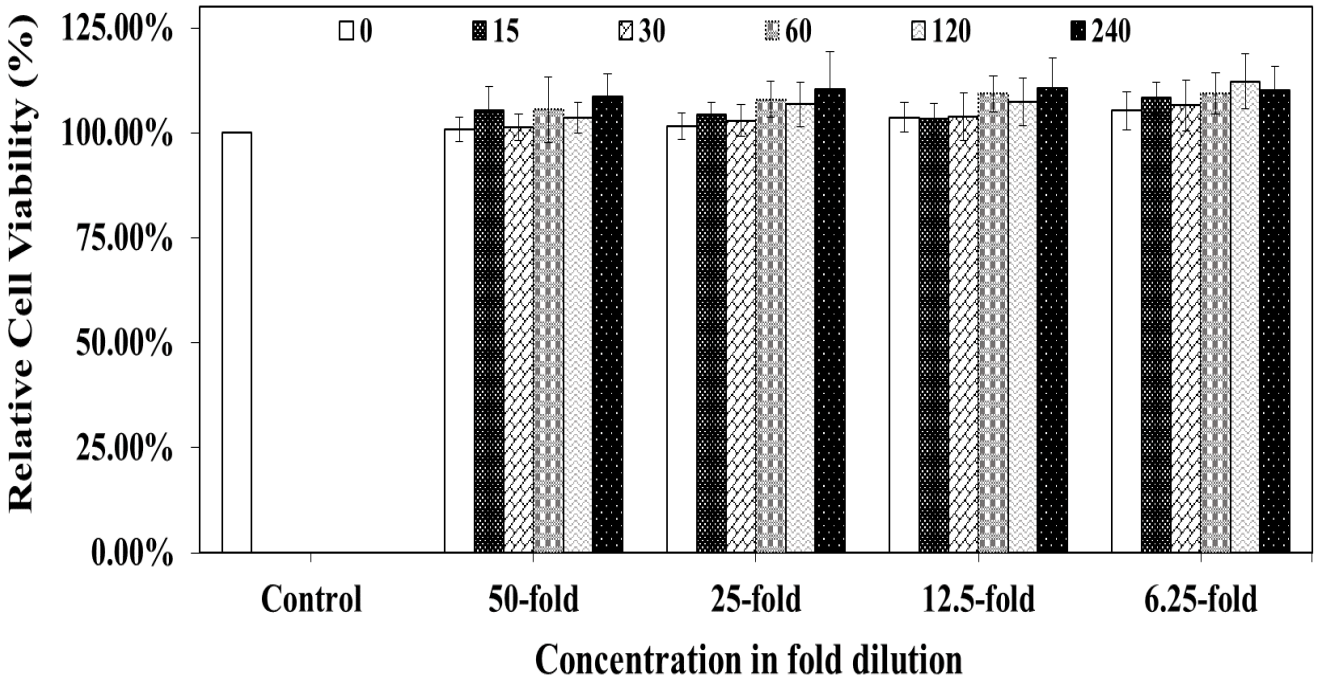


Figure 6: Effect of UV-C irradiation on cell viability of (a) normal colon cells (CCD-18Co), (b) cancer colon cells (HCT-116), and (c) healthy mice liver cells (AML-12). CCV denotes Cell Control-Viability

List of Tables

Table 1: Optical and physical properties of formulated Cranberry flavored water

Parameters	**Values**
pH	2.9 ± 0.05
Absorbance (1/cm)	4.43 ± 0.05
Transmittance (%)	3.72E-03± 0.0004
Total soluble solids (^{0} brix)	8.0 ± 0.15

Data reported as means±standard deviation

Table 2: D_{10} values for micro-organisms obtained after UV-C treatment

Micro-organisms	D_{10} ($mJ \cdot cm^{-2}$)[a]
Escherichia coli (ATCC 25922)	2.74 ± 0.06
Salmonella Typhimurium (ATCC 13311)	5.47 ± 0.02

[a]Data reported as means standard deviation; D_{10}- 10% survival in microbial population

Table 3: Goodness of fit and model parameters

Micro-organisms under study	R_{Square}	[a]RMSE	[b]K_{max}	F Ratio
Escherichia coli ATCC 25922	0.99	0.15	0.61	<0.0001
Salmonella typhimurium ATCC 13311	0.96	0.40	0.66	<0.0001

[a] Root Mean Squared Error; [b]Maximum death rate specific inactivation