STRONG REDUCTION POTENTIAL OF ALKALINE ELECTROLYZED (EO) WATER FOR PREVENTING OXIDATIVE DAMAGE AND ITS ANTIBROWNING, ANTIOXIDATION, AND ANTICANCER EFFECTS

by

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(Under the Direction of Yen- Con Hung)

ABSTRACT

The objectives of this study were to investigate strong reduction potential of alkaline electrolyzed (EO) water and its antibrowning, antioxidant, and anticancer activities. The antibrowning effects of alkaline EO water, ascorbic acid, citric acid, sodium metabisulfite, and their combination on polyphenol oxidase (PPO) activity, total color difference, hue angle, chroma, and browning index (BI) of apple slices were determined. Alkaline EO water inhibited apple PPO by about 66%. Combination of other antibrowning agents and alkaline EO water inhibited browning on apple slices than antibrowning agents alone.

To investigate the antioxidant and anticancer activity of alkaline EO water, alkaline EO water was used for reconstitution of apple juice. Total antioxidant capacities were evaluated by the ORAC and TEAC assays. Apple juice reconstituted with alkaline EO water had higher antioxidant values (ORAC: 11 ± 2.9 μM TE and TEAC: 3.2 ± 0.1 μM TE) than apple juice reconstituted with deionized water (ORAC: 9.8 ± 1.6 μM TE and TEAC: 2.8 ± 0.4 μM TE). ORAC values of apple juice reconstituted with alkaline EO water also maintained for 7 days at 4^{o}C. Redox potential (ORP) has been used as an important indicator for antioxidant activity.

The apple juices reconstituted with alkaline EO water had higher negative ORP and higher ORAC values than apple juice reconstituted with deionized water.

The MTT assay was used to determine cell proliferation and apoptosis was assessed using a DNA fragmentation. Apple juice reconstituted with alkaline EO water had greater inhibition on cancer cell proliferation and induced apoptosis in HT-29 cells than apple juice reconstituted with deionized water. In addition, the comet assay was used to determine DNA damage. HT-29 cells treated with apple juice reconstituted with alkaline EO water had less DNA damage than cell treated with apple juice reconstituted with deionized water.

INDEX WORDS: Alkaline electrolyzed water, Antibrowning effect, ORAC, TEAC, Cell proliferation, DNA fragmentation, DNA damage, Fresh-cut fruits, Reconstituted apple juice

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DEDICATION

To my beloved Dad, Jaecheon Kim and Mom, Duckyim Seo who offered me unconditional love and support, and to my beloved brother, Hyunho Kim.

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CHAPTER 1

INTRODUCTION

Consumer interest in the health contributions of food and drink is increasing rates of chronic disease increase in the general population over time. During life, a human is exposed to various harmful external factors, such as poor environment, unhealthy nutrition, poor quality drinking water, stress, smoking, alcohol abuse, medications, diseases and many others (Goncharuk and others, 2010). All these factors impact the oxidation-reduction system of the organism at the cellular level (Goncharuk and others, 2010). One direct cause of cell damage and death may be extremely reactive substances containing oxygen or free radicals: $O_2^{-\cdot}$, O_2, $OH^{\cdot}$, $OOH^{\cdot}$, $NO^{\cdot}$, and H_2O_2 (WCRF/AICR, 2008). Alkaline EO water has been shown to possess the potential to scavenge reactive oxygen species (ROS) so that consumption of alkaline EO water, instead of regular tap or bottled water, is increasing.

Alkaline electrolyzed (EO) water, which is produced near a cathode during electrolysis of water in the presence of a dilute salt solution, has significant negative redox potential (approximately -400 to -900 mV) (Sun and others, 2007). Ability to scavenge ROS may be caused by negative redox potential and molecular hydrogen dissolved in alkaline EO water. Molecular hydrogen in alkaline EO water is a main factor for the scavenging ability of ROS (Shirahata and others, 1977; Miyashita and others, 1999; Hiraoka and others, 2004). Also, molecular hydrogen is closely associated with the redox potential value because negative redox potential activated during electrolysis is determined by the hydrogen release (Goncharuk and

others, 2010). Thus, alkaline EO water could be applied to reduce oxidation or to scavenge ROS as antibrowning agents or antioxidants.

The occurrence of browning in fresh-cut produce is one of the main problems in the food industry and its prevention is important for maintaining the quality and safety of minimally processed products (Tortoe and others, 2007; Lu and others, 2007). The most used antibrowning agent is ascorbic acid, a type of reducing agent, and its key role is inactivation of polyphenol oxidase (PPO) (McEvily and Iyengar, 1992). Due to its strong reduction potential, alkaline EO water could have strong antibrowning activity and also be used as a solution instead of deionized water to dissolve antibrowning agents, suggesting synergy effects.

Another potential application of alkaline EO water that has been reported is as a drinking water because hydrogen and electrons in alkaline EO water can bind radicals and convert them to non-radical species (Su and others, 2007). Use of alkaline EO water as a scavenger of ROS could be convenient because humans drink water every day since it is essential for life. Researchers have begun to study the ability of alkaline EO water to prevent oxidative stress. However, their studies are limited to in vitro studies and have any shown a weak effectiveness of alkaline EO water on antioxidant or anticancer activities. In the current study, we, thus, used alkaline EO water to reconstitute apple juice instead of regular tap or bottled water.

Consumption of apples has been linked to reduce risk of some cancer (put references). Apple peels have been shown to have a high content of phenolic compounds, antioxidant activity, and antiproliferative activity against cancer cells (Wolfe and others, 2003). Whole apple extracts also have inhibited the growth of colon cancer cells (Eberhardt and others, 2000). It was proposed that the additive and synergistic effects of phytochemicals in apples and apple juice are responsible for the potent antioxidant activity and anticancer activities and that the complex

mixture of phytochemicals contributes to the health benefit of apple products (Eberhardt and others, 2000; Liu, 2004; Liu and others, 2005).

Alkaline EO water, therefore, has scavenging activity against free radicals or ROS, and may also have synergistic effects if combined with other substances, thus functioning as an antioxidant. One example of an effective application would be to use alkaline EO water to reconstitute apple juice, which is already known as a good antioxidant.

Six chapters compose this dissertation, including the introduction, summary, and conclusions. The second chapter presents a literature review of topics related to alkaline electrolyzed (EO) water, its properties and potential health benefits, including antibrowning, antioxidant, and anticancer effects. The third chapter investigates the antibrowning effects of alkaline EO water and the synergistic effect with other antibrowning agents. The fourth chapter evaluates the potential antioxidant capacity of alkaline EO water and synergistic effect when alkaline EO water is used to reconstitute apple juice. The preventive effect of alkaline EO water and reconstituted apple juice on oxidative damage, cell proliferation, and apoptosis is studied in Chapter 5. The sixth chapter summarizes Chapters 3, 4, and 5, and represents overall conclusions.

LITERATURE CITED

Eberhardt, M.V.; Lee, C.Y.; Liu, R.H. Antioxidant activity of fresh apples. Nature, **2000**, 405, 903-904.

Goncharuk, V.V.; Bagrii, V.A.; Mel'nik, L. A.; Chebotareva, R.D.; Bashtan, S.Y. The use of redox potential in water treatment processes. Journal of Water Chemistry and Technology **2010**, 32, 1-9.

Huang, K.C.; Yang, C.C.; Lee, K.T.; Chien, C.T. Reduced hemodialysis-induced oxidative stress in end-stage renal disease patients by electrolyzed reduced water. Kidney International **2003**, 64, 704-714.

Hiraoka, A.; Takemoto, M.; Suzuki, T.; Shinohara, A.; Chiba, M.; Shirao, M.; Yoshimura, Y. Studies on the properties and real existence on aqueous solution systems that are assumed to have antioxidant activities by the action of "Active Hydrogen". Journal of Health Science, **2004**, 50(5), 456-465.

Kajiyama, S.; Hasegawa, G.; Asano, M.; Hosoda, H.; Fukui, M.; Nakamura, N.; Kitawaki, J.; Imai, S.; Nakano, K.; Ohta, M.; Adachi, T.; Obayashi, H.; Yoshikawa, T. Supplementation of hydrogen-rich water improves lipid and glucose metabolism in patients with type 2 diabetes or impaired glucose tolerance. Nutrition Research, **2008**, 28, 137-143.

Liu, R.H. Potential synergy of phytochemicals and their health benefits. Nutrition Journal. **2004**, 3, 5.

Lu, S.M.; Luo, Y.G.; Turner, E.; Feng, H. Efficacy of sodium chlorite as an inhibitor of enzymatic browning in apple slices. Food Chemistry **2007**, 104, 824-829.

McEvily, A.J.; Iyengar, R. Inhibition of enzymatic browning in foods and beverages. Critical Reviews in Food Science and Nutrition, **1992**, 32, 253-273.

Miyashita, K.; Yasuda, M.; Ota, T.; Suzuki, T. Antioxidative activity of a cathodic solution produced by the electrolysis of a dilute NaCl solution. Bioscience Biotechnology and Biochemistry, **1999**, 63, 421-423.

Nishikawa, R.; Teruya, K; Katakura, Y.; Osada, K.; Hamasaki, T.; Kashiwagi, T.; Komatsu, T.; Li, T.; Ye, J.; Ichikawa, A.; Otsubo, K.; Morisawa, S.; Xu, Q.; Shirahata, S. Electrolyzed reduced water supplemented with platinum nanoparticles suppresses promotion of two-stage transformation. Cytotechnology, **2005**, 47, 97-105.

Park, S.K; Qi, X.F.; Song, S.B.; Kim, D.H.; Teng, Y.C.; Yoon, Y.S.; Kim, K.Y.; Li, J.H.; Jin, D.; Lee, K.J. Electrolyzed reduced water inhibits acute ethanol induced hangovers in Sprague-Dawley rats. Biomedical Research, **2009**, 30, 263-269.

Shirahata, S.; Kabayama, S.; Nakano, M.; Miura, T.; Kusumoto, K.; Gotoh, M.; Hayashi, H.; Otsubo, K.; Morisawa, S.; Katakura, Y. Electrolyzed-reduced water scavenges active oxygen species and protects DNA from oxidative damage. Biochemical and Biophysical Research Communications, **1997**, 234, 269-274.

Su, Y.C.; Liu, C.; Hung, Y.C. Electrolyzed water: principles and applications. ACS book on "New biocides development: The combined approach of chemistry and microbiology." Ed: P.Zhu. American Chemical Society. Washington, DC., **2007**, 309-322.

Tortoe, C.; Orchard, J.; Beezer, A. Prevention of enzymatic browning of apple cylinders using different solution. International Journal of Food Science and Technology, **2007**, 42, 1475-1481.

WCRF/AICR. Food, nutrition, physical activity, and the prevention of cancer: a global perspective. Washington DC: American Institute for Cancer Research, **2008**.

Wolfe, K.; Wu, X.; Liu, R.H. Antioxidant activity of apple peels. Journal of Agriculture and Food Chemistry, **2003**, 51, 609-614.

CHAPTER 2

LITERATURE REVIEW

2.1 Electrolyzed water

Water electrolysis technology has first been introduced around 1900 in the soda industry and since the 1980s, also has been used for sanitation control in the food industry to eliminate bacterial on food products, food processing surfaces, and non food contact surfaces (Al-haq and others, 2005; Hricova and others, 2008) and as an antioxidant rich water to scavenge reactive oxygen species (ROS) (Hiraoka and others, 2004; Huang and others, 2006). Electrolyzed water generators also has approved for use in the food industry by the U.S. Environmental Production Agency (EPA) (Park and others, 2002; Hricova and others, 2008). During water electrolysis, two types (alkaline and acidic) of water are produced from each electrode side. Acidic electrolyzed water has been classified as functional water; some scientists use term electrolyzed oxidizing (EO) water, strongly acidic electrolyzed water (SAEW), or electrolyzed strong acid aqueous solution (ESAAS) (Al-haq and others, 2005; Huang and others, 2008). On the other hand, alkaline electrolyzed water has been referred to as electrolyzed reduced water or basic electrolyzed water (Al-haq and others, 2005).

2.2 Generation of electrolyzed water

Electrolyzed water is produced by an electrolyzed water generator, which is composed inside of two differently charged electrodes and a membrane, and outside two supply lines of tap

water and diluted salt solution. Ampere, voltage and pH are controlled to collect two kinds of water, acidic electrolyzed water and alkaline electrolyzed water. Fig. 1 show simple schematics of electrolyzed water generator and produced compounds. The water and the diluted salt solution are put into the machine and then electrolyzed by applying voltage and current. During electrolysis inside the machine, water and sodium chloride are first decomposed into hydrogen (H^+), chloride (Cl^-), hydroxide (OH^-) and sodium ions (Na^+). Negatively charged ions such as chloride (Cl^-) and hydroxide (OH^-) migrate toward the anode to give up electrons and become oxygen gas (O_2), chlorine gas (Cl_2), hypochlorite ion (OCl^-), hypochlorous acid (HOCl), and hydrochloric acid(HCl). Likewise, positively charged ions such as hydrogen (H^+) and sodium (Na^+) migrate toward the cathode to take up electrons and become hydrogen gas (H_2) and sodium hydroxide (NaOH) (Huang and others, 2008). During electrolysis inside the machine, the primary chemical reactions are the following.

At the anode:

$2Cl^- \rightarrow 2Cl + 2e^- \rightarrow Cl_2 \uparrow + 2e^-$

$Cl_2 + H_2O \leftrightarrow HOCl + HCl$

$HOCl \leftrightarrow H^+ + ClO^-$

$2H_2O \rightarrow 4H^+ + O_2 \uparrow$

At the cathode:

$H_2O \rightarrow H^+ + OH^-$

$4OH^- \rightarrow H_2O + O_2 + 4e\text{-}$

$H_2O + Na \rightarrow NaOH + H^+$

$2H_2O + 2e^- \rightarrow 2OH^- + H_2\uparrow$

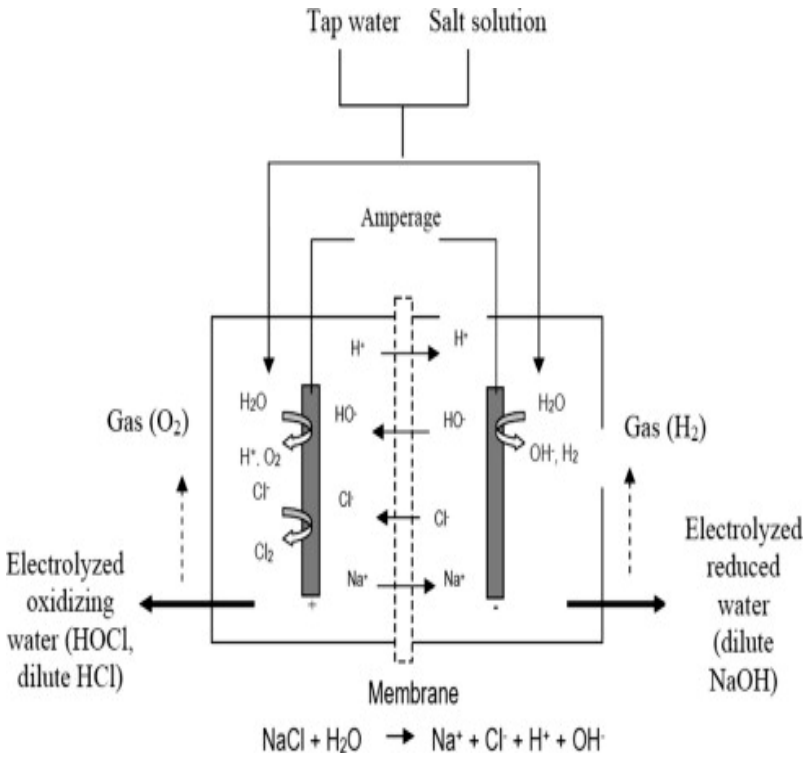

Fig. 2.1: Schematic of electrolyzed water generator and produced compounds (Source: Huang and others, 2008)

2.3 Properties of electrolyzed (EO) water

Water electrolysis generates two different types of water: acidic electrolyzed water and alkaline electrolyzed water. The physical properties of electrolyzed water that are generally observed are pH, Oxidation-reduction potential (ORP), chlorine concentration, dissolved oxygen (DO), and dissolved hydrogen (DH). In addition, alkaline electrolyzed water and acidic electrolyzed water have different functional properties.

2.3.1 Acidic electrolyzed water

Acidic electrolyzed water produced from the anode side has a low pH value (about pH 2.6-2.8), positive redox potential (about +1,100 mV), high dissolved oxygen (DO) and it also contains hypochlorous acid (HOCl). Acidic electrolyzed water has been widely used in medical applications, for various agricultural purposes such as sterilization of vegetables, and by the food processing industry and household kitchens for the disinfection of food materials and food processing equipment (Hsu, S-Y., 2005). These applications of acidic electrolyzed water demonstrate that it has a strong bactericidal activity against a variety of microorganisms (Hricova and others, 2008; Su and others, 2007; Huang and others, 2008). Factors influencing the bactericidal activity of acidic electrolyzed water are pH, available chlorine compounds (Cl_2, HOCl, and OCl^-), ORP, or combinations of these factors. Some studies report that hypochlorous acid (HOCl) is related to antimicrobial properties of acidic electrolyzed water. According to Hricova and others (2008), hypochlorous acid (HOCl) can penetrate the bacterial cell wall and produces hydroxyl radicals that exert their antimicrobial activity through the oxidation of key metabolism (Hricova and others, 2008). Also, high ORP is an important factor contributing to

the antibacterial activity of electrolyzed water because high ORP makes the environment unsuitable for growth of some bacteria (Kim and others, 2000). In addition, the pH value of acidic electrolyzed water is responsible for the bactericidal effect (Len and others, 2002). The growth of most bacteria is limited by pH 4 to 9 and the pH of acidic electrolyzed water is usually around 2.5. Thus, acidic electrolyzed water has an inhibitory effect on bacteria growth (Su and others, 2007) and pH, ORP, and chlorine concentration are important factors to eliminate bacteria from foods or food processing equipments. Hsu (2005) reported that ORP, total chlorine concentration, and electrical conductivity of the electrolyzed water were affected by water flow rate and salt concentration. Increasing water flow rate decreased total residual chlorine concentration and ORP, and increasing salt concentration increased total chlorine concentration and electrical conductivity in electrolyzed water (Hsu, 2005).

2.3.2 Alkaline electrolyzed water

Alkaline electrolyzed (EO) water, which is produced near a cathode during electrolysis of water, is characterized by higher pH, lower oxidation reduction potential (ORP), lower dissolved oxygen, and higher dissolved hydrogen than tap water or deionized water (Kim and others, 2007; Su and others, 2007; Tasi and others, 2009).

Alkaline electrolyzed water has been originally used as drinking or cooking water in Japan (Koseki and others, 2005; Koseki and others, 2007) and alkaline electrolyzed water generators are made by many electric companies as home electronic devices (Hiraoka and others, 2004). Koseki and others studied the effect of water hardness and pH on the taste of alkaline electrolyzed (EO) water through a sensory test (Koseki and others, 2003; Koseki and others,

2005; Koseki and others, 2007). Their studies reported three important results of sensory tests. First, the taste of drinking water depended on its concentrations of Cl and Na ions (Koseki and others, 2003). Second, alkaline water electrolyzed from tap water showed good taste when hardness ranged from approximately 50mg/L to 80mg/L (Koseki and others, 2005). In addition, the taste of alkaline electrolyzed water with pH 9.5 was considered better than that of the unelectrolyzed water (Koseki and others, 2007).

Goncharuk and others (2010) demonstrated that electrolysis is the main technique to obtain water having negative ORP values. Recently this technology was introduced to the USA and other countries, and bottled alkaline electrolyzed water has been sold in the market (Hiraoka and others, 2004). According to Hiraoka and others (2004), such bottled water or home electronic devices are advertised to have antioxidant activities, scavenging harmful reactive oxygen species (ROS). Some studies have been carried out to examine antioxidant activities of alkaline electrolyzed water in vitro or vivo (Shirahata and others, 1999; Miyashita and others, 1999; Hanaoka 2001; Hanaoka and others, 2003; Yanagihara and others, 2005; Lee and others, 2006) to support potential as healthy drinking water.

The most important physical properties for antioxidant activity in alkaline electrolyzed water are molecular hydrogen released at the cathode and negative redox potential (ORP) achieved during electrolysis of water. Redox potential is already known as an indicator of the antioxidant efficiency of food products (Nicoli and others, 2004) and alkaline electrolyzed water has significantly negative ORP at around -400 to -800 mV. Goncharuk and others (2010) reported that the molecular hydrogen in alkaline electrolyzed water is a strong reducer and negative ORP is determined by the hydrogen release process of the following reactions (1) and (2):

$$2H^+ + 2e \rightarrow H_2 \quad (1)$$

$$2H_2O + 2e \rightarrow H_2 + 2OH^- \quad (2)$$

Due to such health benefits, the relationship between the amount of hydrogen gas and other parameters, which can affect the amount of hydrogen, also is important when alkaline EO water is produced by electrolysis of water. Kikuchi and others (2001) studied the relationship between the concentration of hydrogen produced and the electrolysis conditions such as flow rate or concentration of NaCl solution. Kikuchi and others (2001) demonstrated that the concentration of hydrogen decreased with an increase in flow rate and decrease of the concentration of NaCl solution. Their findings will be useful, in further studies, to control the concentration of hydrogen in the generation of alkaline electrolyzed water.

2.4 Health benefits of alkaline electrolyzed water

Alkaline EO water has been studied for clinical improvement of various diseases (Hanaoka, 2001). Reactive oxygen species generated through normal oxidative metabolism have the potential to cause extensive DNA damage (WCRF/AICR, 2008). The body has several mechanisms, which can scavenge reactive oxygen species to prevent such damage from occurring or to block its effects (WCRF/AICR, 2008). The direct cause of the cell damage and death may be extremely reactive substances containing oxygen or free radicals: $O_2^{-\cdot}$, O^2, $OH^{\cdot}$, $OOH^{\cdot}$, $NO^{\cdot}$, and H_2O_2. As reported by Goncharuk and others (2010), oxidative stress represents the state of unbalance caused by the formation of excess free radicals and the reduced activity of the antioxidant protective body system. Such oxidative stress is closely related to many chronic and inflammatory diseases such as renal disease, diabetes, cancer, hangovers, and immune

system problems**.** Alkaline electrolyzed water has been known to be a scavenger of free radical or reactive oxygen and a few studies have been conducted to show the effects of alkaline electrolyzed water in diseases such as renal disease, diabetes, cancer, and hangovers.

2.4.1 Renal disease

Hemodialysis is usually used for removal of excessive toxins, metabolic products, and blood components from patients with end-stage renal disease (ESRD). The hemodialysis process, however, increases production of reactive oxygen species (ROS) and consequently increases oxidative stress, which may lead to cardiovascular events (Huang and others, 2003). Several researchers have used alkaline electrolyzed water to manufacture reverse osmosis (RO) water and hemodialysis (HD) solutions. Huang and others (2003) demonstrated that hemodialysis using alkaline electrolyzed water may efficiently strengthen the antioxidant defense system and reduce H_2O_2 (hydrogen peroxide) and HOCl (hypochlorite) induced oxidative stress. Huang and others (2006) also studied the long-term effect of alkaline electrolyzed water during hemodialysis in end-stage renal disease patients. Their results reported that the application of alkaline electrolyzed water can decrease blood ROS production and RBC lipid peroxidation for six months during hemodialysis. In addition, Nakayama and others (2009) examined the biological actions of alkaline electrolyzed water on human polymorphonuclear leukocytes (PMNs), which may play a central role in excess inflammation and oxidative stress in HD patients, when alkaline electrolyzed water is used to manufacture reverse osmosis (RO) water instead of control RO water. In their study, e-RO (manufactured reverse osmosis water with alkaline electrolyzed water) was better at preserving cellular viability than control RO water. In

conclusion, these studies demonstrated that alkaline electrolyzed water could be useful to use for hemodialysis in end-stage renal disease patients, but future study is needed.

2.4.2 Diabetes mellitus

Diabetes mellitus is a disease caused by high blood sugar (glucose) levels and is classified into two types, insulin dependent (type 1) and non-insulin dependent diabetes mellitus (type 2). The chronic presence of high glucose levels produces reactive oxygen species (ROS) from protein glycation and glucose autoxidation (Kim and Kim, 2006). Oxidative stress is produced under diabetic conditions, and alkaline electrolyzed water may have a potential effect on diabetic mellitus due to ROS scavenging ability (Kim and Kim, 2006).

Li and others (2002) reported that alkaline electrolyzed water strongly protects pancreatic β cells in vitro from damage induced by the diabetogenic agent alloxan, suggesting that alkaline electrolyzed water may be effective in preventing alloxan-induced type 1 diabetes mellitus. Their study demonstrated that alkaline electrolyzed water could scavenge intracellular ROS in pancreatic β cells (HIT-T15) and inhibit cytotoxicitc effects of alloxan, leading to the glucose-stimulated increase of ATP levels and to insulin release (Li and others, 2002).

Recently, the effects of alkaline electrolyzed water were studied in diabetic animal models (Kim and Kim, 2006; Kim and others, 2007). Their results demonstrated that alkaline electrolyzed water, provided as a drinking water, significantly reduced the blood glucose concentration and improved glucose tolerance. Kajiyama and others (2008) reported that alkaline electrolyzed water had beneficial effects on the progression of diabetes and insulin resistance in humans. The results of their study showed that a sufficient supply of alkaline

electrolyzed water prevented or delayed development and progression of diabetes by providing protection against oxidative stress.

2.4.3 Antioxidant

Recently a few studies have studied antioxidant properties of alkaline electrolyzed water (Shirahata and others, 1999; Miyashita and others, 1999; Hanaoka 2001; Hanaoka and others, 2004; Hiraoka and others, 2004; Yanagihara and others, 2005; Lee and others, 2006; Goncharuk and others, 2010).

Although there are various reasons for the effect, one of the reasons cited was due to an increase in the dissociation activity of the reduced water (Hanaoka 2001, Hanaoka and others, 2004). Antioxidants dissolved in alkaline electrolyzed water showed higher dismutation activity for superoxide anion radicals than those dissolved in pure water, although alkaline electrolyzed water itself did not show superoxide dismutation activity (Hanaoka 2001). In addition, Hanaoka and others (2004) demonstrated that the enhancement of antioxidant effects may be due to the increase of the ionic product of water as solvent. They determined that if a sufficient amount of alkaline electrolyzed water is taken into the body, it will increase the dissociation activity for the water-soluble antioxidant substances of relatively lower dissociation activity (Hanaoka, 2001; Hanaoka and others, 2004). Both studies may support the results of Lee and others (2006). Lee and others (2006) discovered that enhancement of the antioxidant activity of ascorbic acid dissolved in alkaline electrolyzed water was about three fold higher than ascorbic acid dissolved in non-electrolyzed deionized water, as measured by a xanthine-xanthine oxidase superoxide

scavenging assay. This suggested that alkaline electrolyzed water has an inhibitory effect on the oxidation of ascorbic acid.

Other researchers reported that alkaline electrolyzed water can scavenge not only $O_2^{\cdot -}$ and H_2O_2, but also $^1O_2$ and $^{\cdot}OH$ due to high atomic hydrogen in the solution and it also exhibited superoxide dismutation activity (Shirahata and others, 1997). Miyashita and others (1999) also tested antioxidant activity of alkaline electrolyzed water and the effectiveness showed the decrease in lipid oxidation. Miyashita and others (1999), however, concluded the effectiveness was due to low dissolved oxygen level of alkaline electrolyzed water. Their conclusion opposes the study by Shirahata and others (1997). On the other hand, two studies showed the antioxidant activity of alkaline electrolyzed water was partly reasoned from molecular hydrogen (Hiraoka and others, 2004; Goncharuk and others, 2010). Hiraoka and others (2004) suggested that such antioxidant effects of alkaline electrolyzed water were derived from ordinary molecular hydrogen (hydrogen gas) and/or reductive vanadium ions. Goncharuk and others (2010) explained that antioxidant effect in alkaline electrolyzed water is due to the negative value of oxidation reduction potential (ORP), and ORP is determined by dissolving hydrogen in water. Therefore, both studies determine such effectiveness is caused by molecular hydrogen.

2.4.4 Others

Alkaline electrolyzed water created through electrolysis has been studied for clinical improvement of various diseases in which reactive oxygen species (ROS) are known to play an essential role. During the development of cancer, cells undergo initiation through DNA damage, and then enter a promotion stage, which continues the transformation of cells. Nishikawa and

others (2005) determined that alkaline electrolyzed water with platinum nanoparticles (Pt nps) can suppress the promotion stage during cancer progress. They suggested that Pt nps stably disperse in aqueous solution for a long time and convert hydrogen molecules to active hydrogen that can scavenge ROS (Nishikawa and others, 2005). Reactive oxygen species (ROS) are known to play an important role in angiogenesis because tumor cells are exposed to higher oxidative stress compared to normal cells (Ye and others, 2008). In their experiment, intracellular H_2O_2 levels were reduced by alkaline electrolyzed water treatment, suggesting that alkaline electrolyzed water can scavenge intracellular H_2O_2 in A549 cells (Ye and others, 2008).

Moreover, oxidative stress is known to be related to ethanol consumption. Park and others (2009) investigated the effect of alkaline electrolyzed water on acute ethanol-induced hangovers in Sprague-Dawley rats. Their results suggested that drinking alkaline EO water has an effect of alcohol detoxification by antioxidant mechanism and has potential to relieve ethanol-induced hangover symptoms (Park and others, 2009).

2.5 Polyphenol oxidase (PPO)

Browning of raw fruits and vegetables is a major problem in the food industry and believed to be one of the main causes of quality loss during postharvest handling and processing (Ohlsson, 1994). The most important browning reactions in fruits and vegetables, especially in fresh-cut produce, are caused by enzymatically catalyzed reactions (Arias and others, 2007). The formation of pigments via enzymatic browning is initiated by the enzyme polyphenol oxidase (McEvily and Iyengar, 1992). Polyphenol oxidase (1,2-benzenediol:oxygen oxidoreductase; EC1.10.3.1), which can mediate the browning reaction, is known as tyrosinase, polyphenolase,

phenolase, catechol oxidase, cresolase, or catecholase (Yoruk and Marshall, 2003; Lei and others, 2004). PPO is present in some bacteria and fungi, in most plants, in some arthropods, and in all mammals (Martinez and Whitaker, 1995). PPO also catalyzes the oxidation of phenolic compounds to quinones, which produce brown pigments in wounded tissues (Queiroz and others, 2008). Enzymatic oxidation occurs severely in destroyed cell walls and cellular membranes from cutting, peeling, pureeing, pitting, pulping, or freezing (McEvily and Iyengar, 1992; Martinez and Whitaker, 1995).

The pH optimum value of PPO is generally in the range of 4.0 to 8.0 even if it varies widely by plant source (Yoruk and Marshall, 2003). In the case of apples, PPO has broad acidic pH optima values from pH 3.5 to 4.5, and it is below the normal optima compared to other fruits (Marques and others, 1995). The changes in ionization of prototropic groups in the active site of an enzyme at lower acid and higher alkali pH values may prevent proper conformation of the active site, binding of substrates, and/or catalysis of the reaction (Whitaker, 1994; Yoruk and Marshall, 2003). Another important factor of PPO activity is temperature, and the optimum temperature of PPO varies for different plant sources (25 to 45 ^{o}C) (Yoruk and Marshall, 2003). Zhou and others (1993) reported that the optimum temperature of apple PPO is 30 ^{o}C.

2.6 Mechanisms of enzymatic browning

Enzymatic browning is initiated with the action of the monooxygenase (polyphenol oxidase, PPO). In the presence of oxygen, PPO hydroxylates the colorless monophenols to *o*-diphenols, which are subsequently oxidized to colored *o*-quinones (Pilizota and Drago, 1998). The reason for browning is mainly the enzymatic oxidation of phenolic compounds by

polyphenol oxidase (PPO). The active site of PPO consists of two copper atoms, and the enzyme catalyzes two different reactions in the presence of molecular oxygen: the hydroxylation of monophenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity) (Figure 2.2).

2.7 Control of browning

Browning of fruits and vegetables has traditionally been a key indicator of diminishing produce quality in fresh-cut produce. Because of its involvement in adverse browning of plant products, PPO has received much attention from researchers in the field of food science (Yoruk and Marshall, 2003). Methods to control enzymatic browning can be divided usually into two categories: physical methods and the use of chemical antibrowning inhibitors.

Physical methods may include reduction of temperature or oxygen during handling, processing, and storage, as well as high pressure, irradiation, and electric pulses. Removing oxygen can delay enzymatic browning in the presence of active enzyme and phenolic substrates because a key mechanism of browning involved in existence of oxygen. However, fresh-cut products cannot be exposed to environments with complete removal of oxygen because oxygen is a requirement for living tissues. In addition, temperature control plays a key role for inhibition of browning because of the ability of low temperatures to reduce metabolic reactions. A common practice in the preparation of fresh-cut produce is rinsing the peeled and/or cut produce in cold water, which helps low the temperature, as well as remove cellular exudates released during the peeling and/or cutting of produce (Garcia and Barrett, 2002).

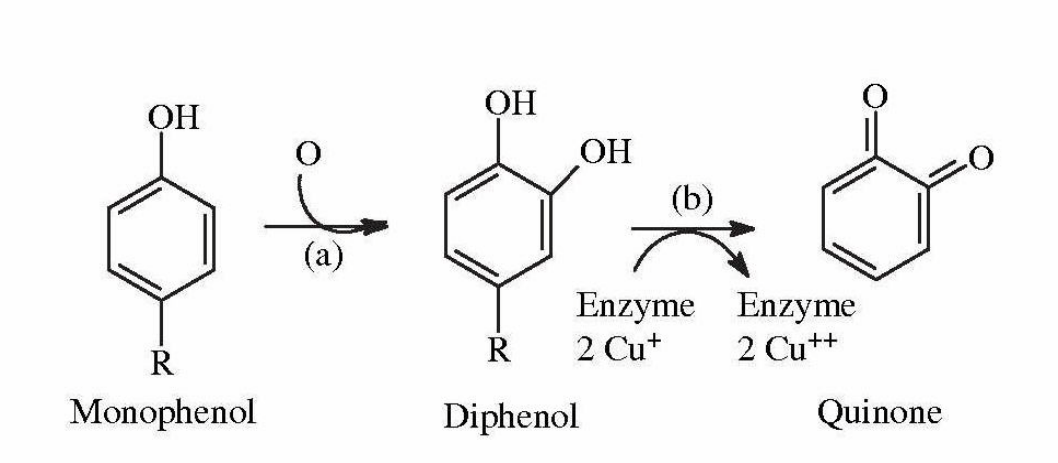

Figure 2.2: Reactions of (a) hydroxylation and (b) oxidation catalyzed by PPO (Source: Queiroz, 2008)

Physical methods have limitations due to the possibility of changes in quality. Therefore, chemical antibrowning inhibitors have been widely used to control the PPO activity in fruits and vegetables. Six major chemical groups (reductants, chelating agents, enzyme inhibitors, acidulants, enzyme treatment and complexing agents) are used to prevent enzymatic browning on fresh-cut fruits and vegetables (Table 1) (Garcia and Barrett, 2002; McEvily and Iyengar, 1992).

Reductants: The primary role of reducing agents is to reduce *o*-quinone to less reactive diphenols, or react permanently with the o-quinones to produce stably colorless products, and these actions prevent the development of browning compounds (Fig.2.3) (Nicolas and others, 1994). Reductants, however, react with pigment intermediates (*o*-quinones) or metals such as copper so that they have only a temporary effect in the control of browning (McEvily and Iyengar, 1992). Sulfur dioxide (SO_2) or sulfites such as sodium bisulfite and sodium metabisulfite are the best inhibitors for enzymatic browning (Ozoglu and Bayindirli, 2002); however, due to serious allergic reactions, their use in fruits and vegetables was forbidden by the FDA (Fan and others, 2009). As shown in Table 1, ascorbic acid or thiol-containing compounds (such as cystein) also are reducing agents and are widely used antibrowning agents.

***Chelating agents*:** The primary action of chelating agents is to bind with the active site of PPO, which contains copper atoms, or to reduce the level of copper available for incorporation into the browning occurrence (McEvily and Iyengar, 1992; Pilizota and Subaric, 1998). As shown Table 1, chelating agents are polyphosphate, EDTA, organic acid, and citric acid. Chelating agents are commonly used in combination with other antibrowning chemicals.

Table 2.1 Chemical agents of browning inhibitors

Chemical groups	Inhibitors
Reductants (Reducing agents; antioxidants)	Sulfiting agents Ascorbic acid and analogs Glutathione Cysteine
Chelating agents	Polyphosphate EDTA Organic acid
Enzyme inhibitors	Aromatic carboxylic acid Aliphatic alcohols Substituted resorcinols Anions: Chloride (NaCl, $CaCl_2$, $ZnCl_2$) Peptides
Acidulants	Citric acid Other organic acids: Tartaric acid, Malic acid, Lactic acid Inorganic acids: Phosphoric acid, Hydrochloric acid
Enzyme treatments	Oxygenases o-Methyl transferases Proteases
Complexing agents	Cyclodextrins (cyclic oligosaccharides) β-cyclodextrin maltosyl-β-cyclodextrin hydroxyethyl-β-cyclodextrin

Source: McEvily and Iyengar, 1992

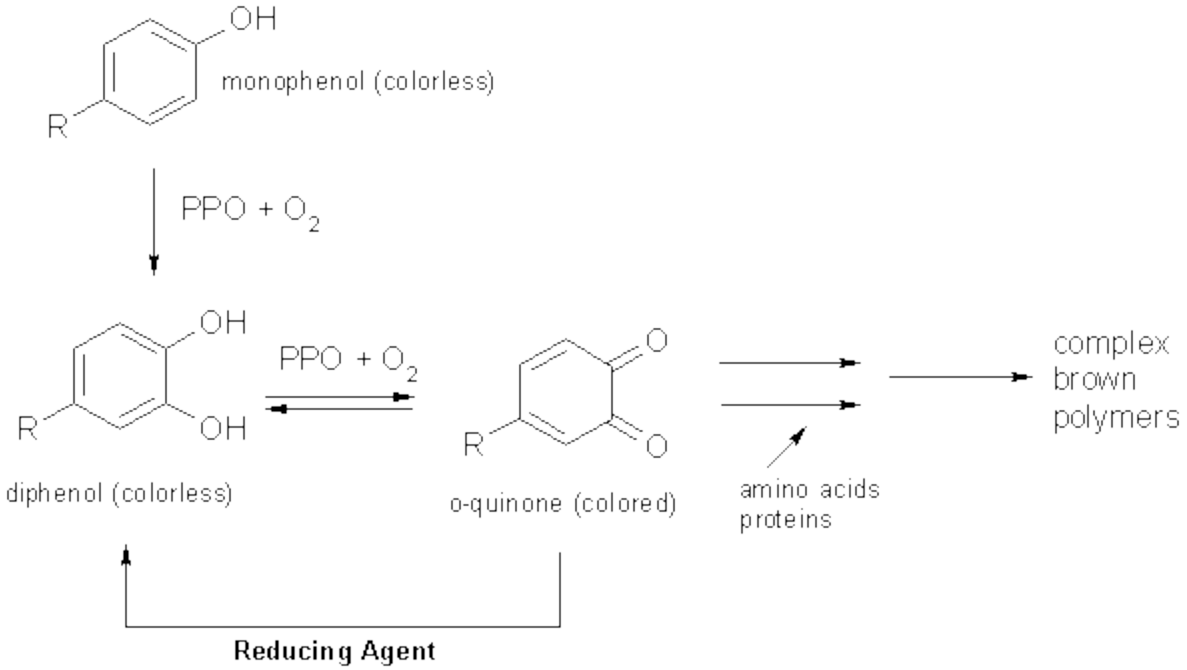

Figure 2.3: The primary role of reducing agents in the Browning Reaction

(Source: McEvily and Iyengar, 1992)

***Enzyme inhibitors*:** Aromatic carboxylic acid, aliphatic alcohols, substituted resorcinols, anions, and peptides play a role as PPO inhibitors (McEvily and Iyengar, 1992). 4-hexylresorcinol, one of the substituted resorcinols, is widely used as a PPO inhibitor, and inactivation of PPO by 4-HR can be explained by the high affinity of 4-HR for PPO (Arias and others, 2007). 4-HR binds preferentially intermediate forms of the enzyme in the catalytic cycle (Arias and others, 2007; Whitaker, 1995). 4-HR effectively improved browning control in fruits and vegetables when combined with ascorbic acid (Arias and others, 2007; Luo and Barbosa-Canovas, 1995).

***Acidulants*:** Acidulants are also used as browning inhibitors and the role of acidulants is to maintain the pH well below that which is necessary for optimal catalytic activity of PPO. The pH of optimum PPO activity ranges from pH 6.0 - 6.5 in most fruits and vegetables, and the pH to effectively inhibit enzyme is below pH 3. The most commonly used acidulants is citric acid.

***Enzyme treatments*:** Enzymatic treatments, such as proteases, *o*-methyl transferases and oxygenases, have been suggested as alternative prevention treatments for enzymatic browning (McEvily and Iyengar, 1992). In case of proteases, PPO inhibition was due to proteolysis or binding at specific sites required for activation (Garcia and Barrett, 2002).

2.8 Free radical and diseases

A radical is any molecule that contains one or more unpaired electrons, and radicals are normally generated in many metabolic pathways. This unpaired electron(s) usually gives a considerable degree of reactivity to the free radicals (Valko and others, 2007). There are six major ROS and RNS that regularly interact and damage the major macromolecules in

physiological and food-related systems: (1) the superoxide anion ($O_2^{\cdot -}$), (2) hydrogen peroxide (H_2O_2), (3) the peroxyl radical ($ROO^{\cdot}$), (4) the hydroxyl radical ($OH^{\cdot}$), (5) singlet oxygen ($^1O_2$), and (6) peroxynitrite ($ONOO^-$) (MacDonald-Wicks, 2006).

Once a free radical interacts with a tissue, many changes can occur, and these changes are a major cause of tissue injury and human disease (Kehrer, 1993). For example, the production of superoxide occurs mostly within the mitochondria of a cell (Cadenas and Sies, 1998). The mitochondrial electron transport chain is the main source of ATP in the mammalian cell and thus is essential for life. Superoxide anion is considered the "primary" reactive oxygen species (ROS) and generates "secondary" ROS, such as hydroxyl radical or peroxyl radical, by interaction with other molecules. Overproduction of ROS results in oxidative stress, which can be an important mediator of damage to cell structures including lipids and membranes, proteins, and DNA (Valko and others, 2007).

Oxidative stress is induced by an imbalance between the production of reactive oxygen species (prooxidant) and a biological antioxidant defense system (antioxidant). Oxidative stress is linked to either the primary or secondary pathophysiologic mechanisms of multiple acute and chronic human diseases (Dalle-Donne and others, 2006). Valko and others (2007) categorized these diseases into two groups: (i) "mitochondrial oxidative stress" conditions (cancer and diabetes mellitus); (ii) "inflammatory oxidative conditions" (leading to atherosclerosis and chronic inflammation). In addition, the process of aging may be due, in part, to the damaging consequences of free radical action (lipid peroxidation, DNA damage, protein oxidation) (Harman, 1956).

2.9 Antioxidant

An antioxidant is defined as a substance that in small quantities is able to delay or prevent the oxidation of easily oxidisable materials (MacDonald-Wicks and others, 2006). Any substance able to donate a hydrogen atom or to promote an electron transfer to a radical also can be considered as an antioxidant (Nicoli and others. 2004). Diets rich in antioxidants may have especially protective effects against several diseases including cancer and cardiovascular disease, both of which have been associated with oxidative stress (Wu and others, 2004). Antioxidants also perform physiological defenses against oxidative stress and hence prevent damage to cell membranes and structures such as cellular proteins, lipids, and DNA. For these reasons, the consumption of dietary antioxidant supplements has been constantly increasing, and numerous new supplements have come onto the market (Monagas and others, 2005).

Antioxidant protection systems include endogenous enzymatic antioxidant protection and exogenous non-enzymatic dietary compounds. For example, the superoxide anion is converted to oxygen and hydrogen peroxide by the enzyme superoxide dismutase, and hydrogen peroxide is converted to water and oxygen by the enzyme catalase (MacDonald-Wicks and others, 2006). And as an example of non-enzymatic antioxidants, antioxidants in foods include vitamins A, C, E and phytochemicals in plants and zoochemicals in animals. There are also synthetic antioxidants such as BHA (butylated hydroxylanisol), BHT (butylated hydroxyl toluene), and tert-butylhydroquinone. Many studies have focused on seeking natural antioxidants for use in foods or medicinal materials (Lee and others, 2007).

2.10 Measurement of Total Antioxidant Capacity

Total antioxidant capacity assays can be divided into two categories, those on the transfer of hydrogen atoms (HTA) and those based on the transfer of electrons (ET) (Huang and others, 2005).

The oxygen radical absorbance capacity (ORAC) method, which is based on the transfer of hydrogen atoms, has been applied extensively to evaluate the antioxidant capacity of a large variety of foods, and many supplemental and functional food companies compare their products, including juices, favorably to fruits and vegetables using the ORAC results from those studies (Seeram and others, 2008). The ORAC assay is based on the measurement scavenging activity against the peroxyl radical formed by heating AAPH (2, 2'-azobis (2-amidino-propane) dihydrochloride). The peroxyl radical has been the most commonly used compound in assays to measure antioxidant capacity as it is a key component of autoxidation and can be easily produced by the decomposition of azo compounds (MacDonald-Wicks, 2006). In general, sample, control, and standards (trolox of four different concentrations is used to construct a standard curve) are mixed with the FL solution and incubated at 37^{o}C before AAPH initiates the reaction. The reaction is measured at 485 nm (ex) and 525 nm (em) for changes to fluorescence. In the basic assay, the peroxyl radical reacts with a fluorescent probe to form a nonfluorescent product, which can be quantitated easily by fluorescence (Fig. 2.4). In the reaction processes, FL is consumed and the fluorescence diminishes (MacDonald and others, 2006). In the presence of an antioxidant, the decay of FL is prevented (Fig. 2.4). Antioxidant capacity is determined by a decreased rate and amount of product formed over time (Prior and others, 2005).

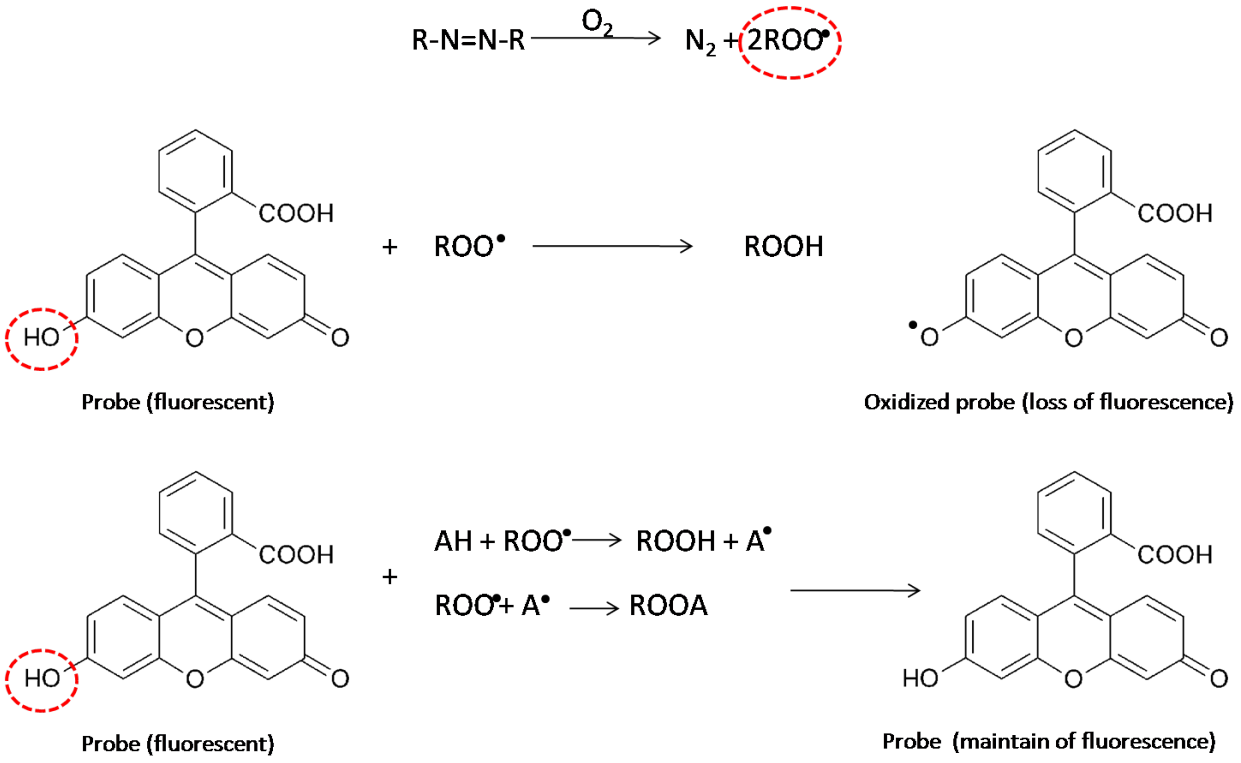

Fig. 2.4: Chemical reaction of fluorescent in ORAC assay.

(Source: Prior and others, 2005; Ou and others, 2001)

The trolox equivalent antioxidant capacity (TEAC) assay, which is based on the transfer of electron (ET), has gained popularity due to its simplicity and speed (Ou and others, 2001). It is based on the following reaction and electron transfer

Probe (oxidant) + e (from antioxidant) → reduced probe + oxidized antioxidant

In TEAC assay, $ABTS^{\cdot+}$, the oxidant, was generated by reaction with potassium persulfate and ABTS (2, 2'-azinobis (3-ethyl-benzothiazolline-6-sulfonic acid)) and is intensely colored to blue/green (Fig. 2.5). Colored $ABTS^{\cdot+}$ radical is reduced to colorless ABTS by antioxidant (Fig. 2.5).

2.11 Cancer

Cancer is defined as a group of diseases characterized by uncontrolled cellular growth and spread of abnormal cells as a result of alterations or damage accumulated over time to the genetic material within cells. All cancers start as a single cell that has lost control of its normal growth and replication processes (Fig. 2.6) (WCRF/AICR, 2008). The development of cancer in humans or animals is a multistep process. The complex series of cellular and molecular changes involved in cancer development are mediated by a variety of endogenous and exogenous stimuli (Valko and others, 2004). Exogenous factors include tobacco smoking and its use, infectious agents, medication, radiation, industrial chemicals, and carcinogenic agents in food. In addition, endogenous causes are inherited germ line mutations, oxidative stress, inflammation, and hormones.

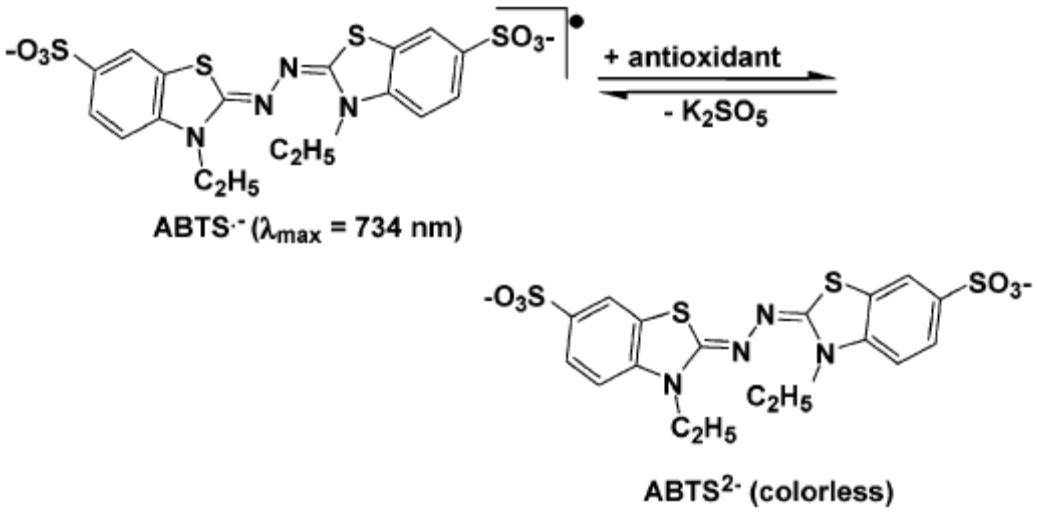

Fig. 2.5: Chemical reactions of ABTS in TEAC assay.

(Source: Huang and others, 2005)

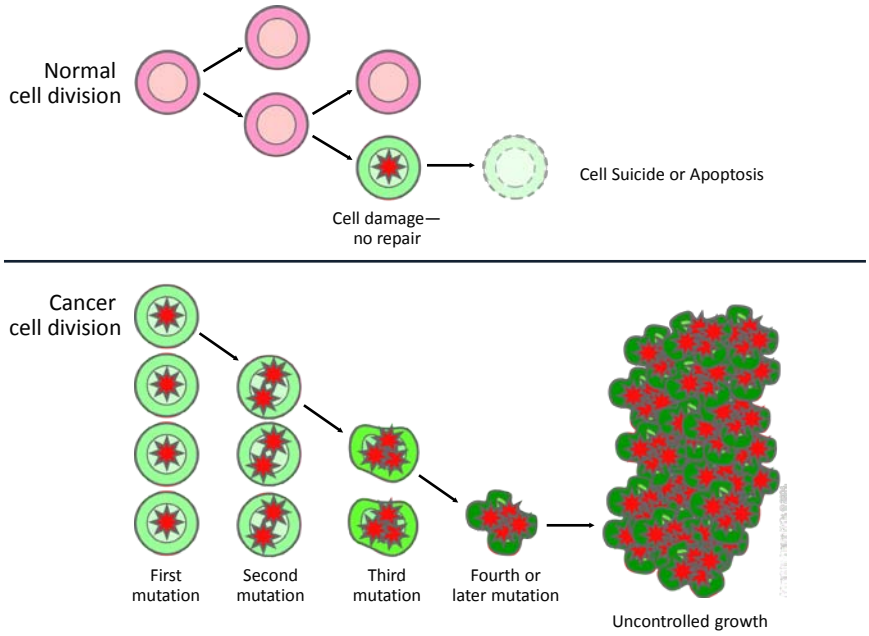

Fig. 2.6: Loss of normal growth control

(Source: National Cancer Institute)

Hanahan and Weinberg (2002) suggested that all types of cancer showed a manifestation of six essential alterations in cell physiology. These hallmarks are self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Fig. 2.7).

Three hallmarks of cancer, which are self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, and limitless replicative potential, enhance cell proliferation. Once normal cells have replicated 60 - 70 times, they reach the end of their telomeres and apoptosis begins. In contrast, cancer cells maintain the length of their telomeres and thus replicate endlessly. Normally, apoptosis controls cell numbers, removes damaged cells, and prevents damaged cells from being replicated; however, cancer cells evade apoptosis.

The supply of nutrients and oxygen to any growing tissue including tumors is important for cell function and survival. Angiogenesis forms new blood vessels, which supply the oxygen and nutrients. Normal cells maintain their position in the body and generally do not migrate. As a cancer increases, it eventually reaches the membrane encapsulating the organ. Cancer cells secrete enzymes such as matrix metalloproteases (MMPs), which digest the membrane and allow cancer cells to invade adjacent tissue.

2.12 Apoptosis and cell proliferation

Apoptosis (programmed cell death) is a kind of physiologically programmed ‘cell-suicide’ (Fulda and Debatin, 2006). Apoptosis plays a fundamental role in maintenance of tissues and organ systems by providing a controlled cell deletion to balance cell proliferation; thus, it

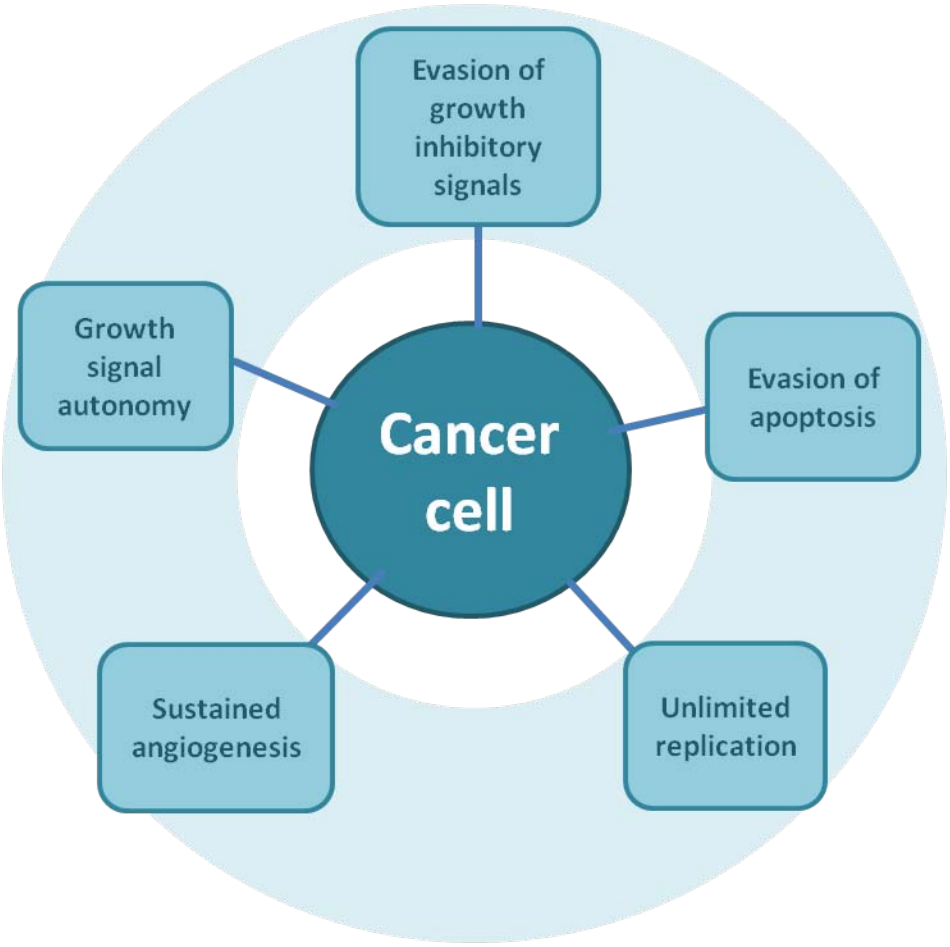

Fig. 2.7: Critical elements of cancer development
(Source: Adapted from Hanahan and Weinberg, 2002)

composes a common mechanism of cell replacement, tissue remodeling, and removal of damaged cells (Ozben, 2007; Walter and others, 2000). Altered rates of apoptosis are central to many disease processes including cancer, and many dietary factors are associated with activation or inhibition of apoptosis. Generally, proapoptotic compounds could protect against cancer by enhancing elimination of initiated, precancerous cells; in contrast, antiapoptotic compounds could promote tumor formation by inhibiting apoptosis in genetically damaged cells. It has been proposed that oxidative stress activates apoptosis, and antioxidants protect against apoptosis in vitro; thus, a central role of dietary antioxidants may be to protect against oxidative stress induced apoptosis (Walter and others, 2000).

Apoptosis is stimulated by two pathways -- the intrinsic and extrinsic pathways (Fig. 2.8) (Ashkenazi, 2002). In the extrinsic pathway, apoptosis is activated by death ligands of the cell surface death receptors (Fulda and Debatin, 2006). Binding of ligands induces the formation of the death induced signaling complex (DISC), which recruits caspase-8 and promotes the cascade of procaspase activation (Okada and Mak, 2004). The extrinsic pathway triggers apoptosis independently of the p53 protein (Okada and Mak, 2004). On the other hand, the intrinsic pathway is activated by various extracellular and intracellular stresses (Okada and Mak, 2004). Destruction of the cell is ultimately carried out by intracellular protease enzymes (caspases). Caspases are activated depending on the intrinsic and/or extrinsic pathways and play a central role in apoptosis (Lavrik and others, 2005). Also, p53, tumour suppressor gene, is involved in apoptosis and functions as a transcriptional activator of genes, encoding apoptosis effectors (WCRF/AICR, 2008). Cancer cells have acquired mutations in genes regulating apoptosis and therefore can evade apoptotic signals.

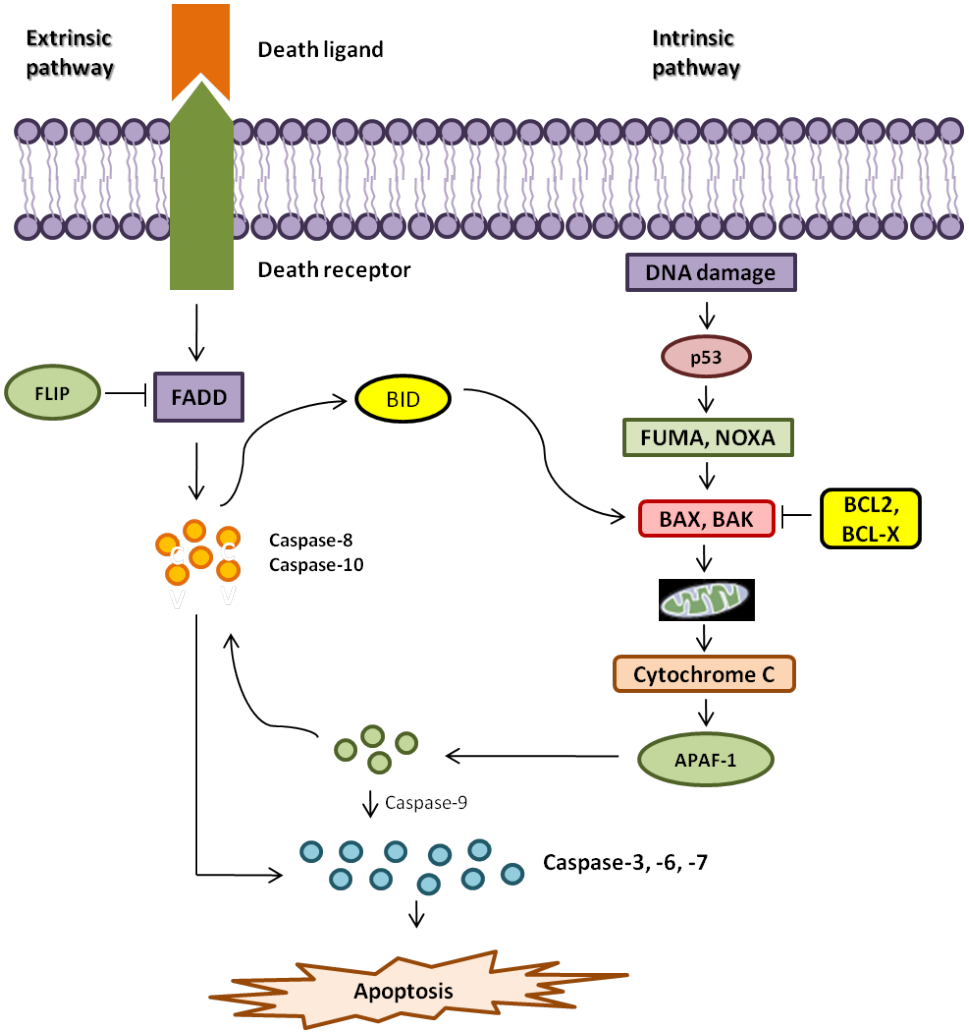

Fig. 2.8: Apoptotic pathways
(Source: Adapted from Ashkenazi, 2002)

LITERATURE CITED

Al-haq, M.I.; Sugiyama, J.; Isobe, S. Applications of electrolyzed water in agriculture and food industries. Food Science Technology Research, **2005**, 11(2), 135-150.

Arias, E.; Gonzlez, J.; Peiro, J.M.; Oria, R.; Lopez-Buesa, P. Browning prevention by ascorbic acid and 4-Hexylresorcinol: Different mechanisms of action on polyphenol oxidase in the presence and in the absence of substrates. Journal of Food Science, **2007**, 72, C464-C470.

Ashkenazi, A. Targeting death and decoy receptors of the tumour-necrosis factor superfamily. Nature Reviews. Cancer, **2008**, 7, 1001-1012.

Cadenas, E.; Sies, H. The lag phase. Free Radical Research, **1998**, 28, 601-609.

Dalle-Donne, I.; Rossi, R.; Colombo, R.; Giustarini, D.; Milzani, A. Biomarkers of oxidative damage in human disease. Clinical Chemistry, **2006**, 52, 601-623.

Fan, X.; Sokorai, K. J.B. ; Liao, C.H.; Cooke, P.; Zhang, H.Q. Antibrowning and antimicrobial properties of sodium acid sulfate in apple slices. Journal of Food Science, **2009**, 74, M485-M492.

Fulda, S.; Debatin, KM. Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. Oncogene, **2006**, 25(34), 4798-4811.

Garcia, E.; Barrett, D.M. Preservative treatments for fresh-cut fruits and vegetables. CRC book on “Fresh-cut Fruits and Vegetables: Science, Technology, and Market.” Ed: Olusola Lamikanra. Washington, DC. **2002**, 267-303.

Goncharuk, V.V.; Bagrii, V.A.; Mel'nik, L. A.; Chebotareva, R.D.; Bashtan, S.Y. The use of redox potential in water treatment processes. Journal of Water Chemistry and Technology, **2010**, 32, 1-9.

Halliwell, B.; Gutteridge, J.M.C. Free radicals, antioxidants, and human disease: where are we now? Journal of Laboratory and Clinical Medicine, **1992**, 119(6), 598-620.

Harman, D. Aging – A theory based on free radical and radiation – chemistry. Journal of Gerontology, **1956**, 11, 298-300.

Hanaoka, K. Antioxidant effects of reduced water produced by electrolysis of sodium chloride solutions. Journal of Applied Electrochemistry, **2001**, 31, 1307-1313.

Hanaoka, K.; Sun, D.; Lawrence, R.; Kamitani, K.; Fernandes, G. The mechanism of the enhanced antioxidant effects against superoxide anion radicals of reduced water produced by electrolysis. Biophysical Chemistry, **2004**, 107, 71-82.

Hanahan, D.; Weinberg, R.A. The Hallmark of cancer. Cell, **2000**, 100(1), 57-70.

Hiraoka, A.; Takemoto, M.; Suzuki, T.; Shinohara, A.; Chiba, M.; Shirao, M.; Yoshimura, Y. Studies on the properties and real existence of aqueous solution systems that are assumed to have antioxidant activities b**y** the action of "Active Hydrogen". Journal of Health Science, **2004**, 50, 456-465.

Hricova, D.; Stephan, R.; Zweifel, C. Electrolyzed water and its application in the food industry. Journal of Food Protection, **2008**, 71(9), 1934-1947.

Hsu, S-Y. Effects of flow rate, temperature and salt concentration on chemical and physical properties of electrolyzed oxidizing water. Journal of Food Engineering, **2005**, (66), 171-176.

Huang, D.J.; Ou, B.X.; Prior, R.L. The chemistry behind antioxidant capacity assays. Journal of Agricultural and Food Chemistry, **2005**, 53(6), 1841-1856.

Huang, Y-R., Y-C. Hung, S-Y. Hsu, Y-W. Huang and D-F. Hwang. Application of electrolyzed water in the food industry. Food Control, **2008**, 19, 329-345.

Huang, K-C., C-C. Yang, S-P. Hsu, K-T Lee, H-W Lie, S. Morisawa, K. Otasubo and C-T. Chien. Electrolyzed-reduced water reduced hemodialysis-induced erythrocyte impairment in end-stage renal disease patients. Kidney International, **2006**, 70, 391-398.

Huang, K.C.; Yang, C.C.; Lee, K.T.; Chien, C.T. Reduced hemodialysis-induced oxidative stress in end-stage renal disease patients by electrolyzed reduced water. Kidney International, **2003**, 64, 704-714.

Kajiyama, S.; Hasegawa, G.; Asano, M.; Hosoda, H.; Fukui, M.; Nakamura, N.; Kitawaki, J.; Imai, S.; Nakano, K.; Ohta, M.; Adachi, T.; Obayashi, H.; Yoshikawa, T. Supplementation of hydrogen-rich water improves lipid and glucose metabolism in patients with type 2 diabetes or impaired glucose tolerance. Nutrition Research, **2008**, 28, 137-143.

Kehrer, J.P. Free radicals as mediators of tissue injury and disease. Critical Reviews in Toxicology, **1993**, 23(1), 21-48.

Kikuchi, K., H. Takeda, B. Racolt, T. Okaya, Z. Ogumi, Y. Saihara, and H. Noguchi. Hydrogen concentration in water from an alkali-ion-water electrolyzer having a platinum-electroplated titanium electrode. Journal of Applied Electrochemistry, **2001**, 31, 1301-1306.

Kim, C., Y-C. Hung and R.E. Brackett. Roles of oxidation-reduction potential in electrolyzed oxidizing and chemically modified water for the inactivation of food-related pathogens. Journal of Food Protection, **2000**, 63, 19-24.

Kim, M.J.; Kim, H.K. Anti-diabetic effects of electrolyzed reduced water in streptozotocin-induced and genetic diabetic mice. Life Sciences, **2006**, 79, 2288-2292.

Kim, M-J., K.H. Jung, Y.K. Uhm, K-H. Leem and H.K. Kim. Preservative effect of electrolyzed reduced water on pancreatic β-cell mass in diabetic *db/db* mice. Biological and Pharmaceutical Bulletin, **2007**, 30(2), 234-236.

Koseki, M., A. Nakagawa, Y. Tanaka, H. Noguchi and T. Omochi. Sensory evaluation of taste of alkali-ion water and bottled mineral water. Journal of Food Science, **2003**, 68(1), 354-358.

Koseki, M., S. Fujiki, Y. Tanaka, H. Noguchi and T. Nishikawa. Effect of water hardness on the taste of alkaline electrolyzed water. Journal of Food Science, **2005**, 70(4), S249-S253.

Koseki, M., Y. Tanaka, H. Noguchi and T. Nishikawa. Effect of pH on the taste of alkaline electrolyzed water. Journal of Food Science, **2007**, 72(5), S298-S302.

Lavrik, I.N.; Golks, A.; Krammer, P.H. Caspases: pharmacological manipulation of cell death. The Journal of Clinical Investigation, **2005**, 115(10), 2665-2672.

Lee, B.B.; Cha, M.R.; Kim, S.Y.; Park E.; Park, H.R.; Lee, S.C. Antioxidative and anticancer activity of extracts of cherry (prunnus serrulata var. spontanea) blossoms. Plant Foods for Human Nutrition, **2007**, 62, 79-84.

Lee, M. Y., Y. K. Kim, K. K. Ryoo, Y. B. Lee and E. J. Park. Electrolyzed-reduced water protects against oxidative damage to DNA, RNA, and protein. Applied Biochemistry and Biotechnology, **2006**, 135, 133-144.

Lei, D.F.; Feng, Y.; Jiang, D.Z. Characterization of polyphenol oxidase from plants. Progress in Natural Science, **2004**, 14, 553-561.

Len, S-V., Y-C. Hung, D. Chung, J.L. Anderson, M. Erickson and K. Morita. Effects of storage conditions and pH on chlorine loss in electrolyzed oxidizing (EO) water. Journal of Agriculture and Food Chemistry, **2002**, 50(1), 209-212.

Li, Y.; Nishimura, T.; Teruya, K.; Maki, T.; Komatsu, T.; Hamasaki, T.; Kashiwagi, T.; Kabayama, S.; Shim, S.Y.; Katakura, Y.; Osada, K.; Kawahara, T.; Otsubo, K.; Morisawa, S.; Ishii, Y.; Gadek, Z.; Shirahata, S. Protective mechanism of reduced water against alloxan-induced pancreatic β cell damage: Scavenging effect against reactive oxygen species. Cytotechnology, **2002**, 40, 139-149.

Luo, Y.; Barbosa-Canovas, G.V. Inhibition of apple slice browning by 4-hexylresorcinol. In C.Y. Le and J.R. Whitaker, eds., Enzymatic browning and its prevention. ACS Symp. Ser. 600, Washington, D.C., **2002**, 240-250.

MacDonald-Wicks, L.K.; Wood, L.G.; Garg, M.L. Methodology for the determination of biological antioxidant capacity in vitro: a review. Journal of the Science of Food and Agriculture, **2006**, 86 (13), 2046-2056.

Marques, L.; Fleuriet, A.; Macheix, J. Characterization of multiple forms of polyphenoloxidase from apple fruit. Plant Physiology and Biochemistry, **1995**, 33, 193-200.

Martinez, M.V.; Whitaker, J.R. The biochemistry and control of enzymatic browning. Trends in Food Science and Technology, **1995**, 6, 195-200.

McEvily, A.J.; Iyengar, R. Inhibition of enzymatic browning in foods and beverages. Critical Reviews in Food Science and Nutrition, **1992**, 32, 253-273.

Miyashita, K., M. Yasuda, T. Ota and T. Suzuki. Antioxidative activity of a cathodic solution produced by the electrolysis of a dilute NaCl solution. Bioscience, Biotechnology, and Biochemistry, **1999**, 63(2), 421-423.

Monagas, M.; Hernandez-Ledesma, B.; Garrido, I.; Martin-Alvarez, P.J.; Gomez-Cordoves, C.; Bartolome, B. Quality assessment of commercial dietary antioxidant products from Vitis vinifera L. grape seeds. Nutrition Cancer, **2005**, 53(2), 244-254.

Nakayama, M.; Kabayama, S.; Nakano, H.; Zhu, W.J.; Terawaki, H.; Nakayama, K.; Katoh, K.; Satoh, T.; Ito, S. Biological effects of electrolyzed water in hemodialysis. Nephron Clincal Practice, **2009,** 112, C9-C15.

Nicolas, J.J.; Richard-Forget, F.C.; Goupy, P.M.; Amiot, M.; Aubert, S.Y. Enzymatic browning reactions in apple and apple products. Critical Review Food Science and Nutrition, **1994**, 34, 109-157.

Nicoli, M.C.; Toniolo, R.; Anese, M. Relationship between redox potential and chain breaking activity of model systems and foods. Food Chemistry, **2004**, 79-83.

Nishikawa, R.; Teruya, K; Katakura, Y.; Osada, K.; Hamasaki, T.; Kashiwagi, T.; Komatsu, T.; Li, T.; Ye, J.; Ichikawa, A.; Otsubo, K.; Morisawa, S.; Xu, Q.; Shirahata, S. Electrolyzed reduced water supplemented with platinum nanoparticles suppresses promotion of two-stage transformation. Cytotechnology, **2005**, 47, 97-105.

Ohlsson, T. Minimal processing preservation methods of the future: an overview. Trends in Food Science and Technology, **1994**, 5, 341-344.

Ou, B.; Hampsch-Woodill, M.; Prior, R.L. Development and validation of improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. Journal of Agriculture and Food Chemistry, **2001**, 49, 4619-4626.

Ozben, T. Oxidative stress and apoptosis: impact on cancer therapy. Journal of Pharmaceutical Science, **2007**, 96, 2181-2196.

Ozoglu, H.; Bayindirli, A. Inhibition of enzymic browning in cloudy apple juice with selected antibrowning agents. Food Control, **2002**, 13, 213-221.

Park, H.; Hung, Y.C.; Kim, C. Effectiveness of electrolyzed water as a sanitizer for treating different surfaces. Journal of Food Protection, **2002**, 65, 1276-1280.

Park, S.K; Qi, X.F.; Song, S.B.; Kim, D.H.; Teng, Y.C.; Yoon, Y.S.; Kim, K.Y.; Li, J.H.; Jin, D.; Lee, K.J. Electrolyzed reduced water inhibits acute ethanol induced hangovers in Sprague-Dawley rats. Biomedical Research, **2009**, 30, 263-269.

Pilizota, V.; Subaric, D. Control of enzymatic browning of foods. Food technology and Biotechnology, **1998**, 36, 219-227.

Prior, R.L.; Wu, X.; Schaich, K. Standardized methods for determination of antioxidant capacity and phenolics in food and dietary supplements. Journal of Agricultural and Food Chemistry, **2005**, 53(10), 4290-4302.

Queiroz, C.; Lopes, M.L.M.; Fialho, E.; Valente-Mesquita, V.L. Polyphenol oxidase: characteristics and mechanisms of browning control. Food Reviews International, **2008**, 24, 361-375.

Sanchez-Moreno, C. Review: methods used to evaluate the free radical scavenging activity in foods and biological samples. Food Science and Technology International, **2002**, 8(3), 121-137.

Seeram, N.P.; Aviram, M.; Zhang, Y.; Henning, S.M.; Feng, L.; Dreher, M.; Heber, D. Comparison of antioxidant potency of commonly consumed polyphenol-rich beverages in the United States. Journal of Agriculture and Food Chemistry, **2008**, 56, 1415-1422.

Shirahata, S., S. Kabayama, M. Nakano, T. Miura, K. Kusumoto, M. Gotoh, H. Hayashi, K. Otsubo, S. Morisawa and Y. Katakura. Electrolyzed-reduced water scavenges active oxygen species and protects DNA from oxidative damage. Biochemical and Biophysical research communications, **1997**, 234, 269-274.

Simic, M.G.; Taylor, K.A. Introduction to peroxidation and antioxidantion mechanism. Basic Life Science, **1998**, 49, 1-10.

Singh, S.; Singh, R.P. In vitro methods of assay of antioxidants: an overview. Food Reviews International, **2008**, 24, 392-415.

Su, Y-C., C. Liu and Y-C. Hung. Electrolyzed water: principles and applications. ACS book on "New Biocides Development: The Combined Approach of Chemistry and Microbiology." Ed: P. Zhu. American Chemical Society. Washington, DC., **2007**, 309-322.

Tsai, C.-F., Y.-W. Hsu, W.-K Chen, Y.-C Ho, and F.-J Lu. Enhanced induction of mitochondrial damage and apoptosis in human leukemia HL-60 Cells due to electrolyzed reduced water. Bioscience Biotechnology and Biochemistry, **2009**, 73(2), 280-287.

Valko, M.; Leibfritz, D.; Moncol, J.; Cronin, M.T.D.; Mazur M.; Telser J. Free radicals and antioxidants in normal physiological functions and human disease. The International Journal of Biochemistry and Cell Biology, **2007**, 39, 44-84.

Valko, M.; Izakovic, M.; Mazur, M.; Rhodes, C.J.; Telser, J. Role of oxygen radicals in DNA damage and cancer incidence. Molecular and Cellular Biology, **2004**, 37-56.

WCRF/AICR. Food, nutrition, physical activity, and the prevention of cancer: a global perspective. Washington DC: American Institute for Cancer Research. **2008**.

Whitaker, J.R. Principles of enzymology for the food science, Second ED., Marcel Dekker, New York, **1994**, 271-556.

Wu, X.; Beecher, G.R.; Holden, J.M.; Haytowitz, D.H.; Gebhardt, S.E.; Prior, R.L. Lipophilic and hydrophilic antioxidant capacities of common foods in the United States. Journal of Agricultural and Food Chemistry, **2004**, 52, 4026-4037.

Yanagihara T., K. Arai, K. Miyamae, B. Sato, T. Shudo, M. Yamada, and M. Aoyama. Electrolyzed hydrogen-saturated water for drinking use elicits an antioxidative effect: A feeding test with rats. Bioscience, Biotechnology and Biochemistry, **2005**, 69(10), 1985-1987.

Ye, J.; Li, Y.; Hamasaki, T.; Nakamichi, N.; Komatsu, T.; Kashiwagi, T.; Teruya, K.; Nishikawa, R.; Kawahara, T.; Osaka, K.; Toh, K.; Abe, M.; Tian, H.; Kabayama, S.; Otsubo, K.; Morisawa, S.; Katakura, Y.; Shirahata, S. Inhibitory effect of electrolyzed reduced water on tumor angiogenesis. Biological and Pharmaceutical Bulletin, **2008**, 31, 19-26.

Yoruk, R.; Marshall, M.R. Physicochemical properties and function of plant polyphenol oxidase: a review. Journal of Food Biochemistry, **2003**, 27, 361-422.

Zhou, P.; Smith, N.L.; Lee, C.Y. Potential purification and some properties of Monroe apple peel polyphenol oxidase. Journal of Agriculture and Food Chemistry, **1993**, 41, 532-536.

CHAPTER 3

EFFECT OF ALKALINE ELECTROLYZED WATER ON POLYPHENOL OXIDASE AND COLOR CHANGE IN RED DELICIOUS APPLES

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ABSTRACT

This study was conducted to investigate the effectiveness of alkaline EO water in preventing browning of fresh-cut apples. Enzymatic extracts and apple slices were prepared from Red Delicious apples. Anti-browning properties of alkaline EO water treated apples (polyphenol oxidase (PPO) activity, total color change, hue angle, chroma, and browning index) were compared with apples treated with other anti-browning agents (ascorbic acid, citric acid, and sodium metabisulfite) treated apples.

Alkaline EO water was shown to reduce the enzymatic activity of apple PPO by about 66%. The alkaline EO water, when used in combination with other anti-browning agents, inhibited browning on apple slices better than individual anti-browning agents alone. Apple slices treated with alkaline EO water showed less total color difference than those treated with ascorbic acid or citric acid. Hue angle values of apple slices treated with alkaline EO water reduced less after 24h of dipped treatment than treated with deionized water (97 to 91 vs. 93 to 84) and hence less brown. In addition, the effectiveness of alkaline EO water to reduce browning index was better than that of ascorbic acid (40.84 vs. 47.62 after 24h). When combined with other anti-browning agents, alkaline EO water was more effective on reducing the browning index (indicates less brown) than those individual agents alone. This study demonstrated that alkaline EO water can be a promising treatment solution to prevent browning and thereby enhance the quality of fresh cut apples.

INTRODUCTION

In recent years, consumption of fresh-cut produce as ready-to-eat food is increasing because of its convenience; thus, preservation of the fresh quality and natural ingredients of fruits and vegetables is an important issue in the food industry (Tortoe and others, 2007). The prevention of browning and growth of microorganisms in fresh-cut produce is important for maintaining the quality and safety of minimally processed products (Lu and others, 2007). Enzymatic browning of fruits and vegetables is usually caused by activity of the enzyme, polyphenol oxidase (PPO; EC 1.10.3.1) (McEvily and Iyengar, 1992). PPO is a copper containing metalloprotein and catalyzes the oxidation of phenolic compounds to *o*-quinines (Queiroz and others, 2008). Quinones produce brown color in broken tissues of fruits or vegetables. Reduction of *o*-quinone might consider a key role to inhibit browning on fruits and vegetables.

Common anti-browning agents are either reducing quinones production or chelating copper atoms which are subsequently oxidized to colored *o*-quinone. Traditionally, anti-browning agents and browning prevention methods for fruits and vegetables have been developed to specifically control the PPO activity (McEvily and Iyengar, 1992). The most common agents used historically as inhibitors of enzymatic browning are sulfiting agents (Ozoglu and Bayindirli, 2002); however, sodium bisulfite, one of sulfiting agents, was forbidden by FDA on many fruits and vegetables because it may cause serious allergic reactions (Fan and others, 2009). In addition, one of the largest costs in producing fresh-cut apples is the antibrowning solution. So, alternative inhibitors are being explored (Ozoglu and Bayindirli, 2002; Fan and others, 2009).

Anti-browning compounds are classified into six major chemical groups based on the inhibition mechanism as reducing agents (sulfiting agents and ascorbic acid), chelating agents (EDTA), acidulants (citric acid), enzyme inhibitors (aromatic carboxylic acid), enzyme treatments (oxygenases and proteases) and complexing agents (cyclodextrins) (McEvily and Iyengar, 1992).

Ascorbic acid (AA) is a reducing agent, which reduces *o*-quinones formed by the oxidation reaction of PPO, has been widely used to control browning reaction on many food products (Pilizota and Subaric, 1998; Queiroz and others, 2008). Tortoe and others (2007) reported apple cylinders treated with AA had higher browning inhibition effect than other solutions (cystein, sodium chloride, calcium chloride, citric acid and sodium ascorbate). Arias and others (2007) demonstrated AA prevented browning by 2 different mechanisms: it can reverse the oxidation of PPO reaction or inactivate the enzyme through binding its active site. However, Eissa and others (2006) reported low inhibitory activity of ascorbic acid when compared with thiol or sulfite containing compounds on Red delicious apple slices.

Citric acid (CA) has also been used commercially as an antibrowning agent by chelating copper from the PPO active site and reducing the pH (McEvily and Iyengar, 1992). Son and others (2001) showed that citric acid had the moderate inhibitory activity on apple slices by chelating copper at the PPO active site. Other studies have reported similar findings on citric acid antibrowning activity when compared with other antibrowning agents for apple slices (Lu and others, 2007; Fan and others, 2009; Tortoe and others, 2007).

Electrolyzed (EO) water is conveniently produced by electrolysis of tap water and diluted salt (NaCl) solution. Acidic EO water and alkaline EO water are obtained from the anode and cathode side respectively (Kim and others, 2000). The main advantages of EO water are

environmentally friendly, safe for the human contact and cheaper than other chemicals (AL-Haq and others, 2005; Huang and others 2008). Acidic EO water has a strong bactericidal effect due to the presence of high levels of hypochlorous acid (HOCl), a low pH value (approximately pH 2.6-2.8) and a strong oxidation potential (approximately +1100mV) (Huang and others, 2008). On the other hand, alkaline EO water has a high pH value (approximately pH 11.2-11.4), negative redox potential (approximately -800mV), low dissolved oxygen (DO), and high dissolved molecular hydrogen (DH) (Su and others, 2007). Because the hydrogen molecules in alkaline EO water can bind radicals and converting them to non-radical species, alkaline EO water has been widely reported as an anti-oxidant drinking water. Shirahata and others (1997) reported that alkaline EO water has superoxide dismutase (SOD) activity and antioxidant dissolved in alkaline EO water has shown higher dismutase activity for superoxide anion radicals than those in pure water (Hanaoka, 2001). Additionally, Hanaoka and others (2004) demonstrated the enhancement of superoxide anion radical dismutation activity was due to change in the ionic product of water in the alkaline EO water. Miyashita and others (1999) reported that high dissolved hydrogen concentration in alkaline EO water was the main factor responsible for its antioxidative activity.

Because of its strong reduction potential and antioxidant effect, alkaline EO water should have strong antibrowning effect. However, no research on antibrowning effect of alkaline EO water on fresh-cut produces is available. Therefore, the main objective of the present study was to investigate the anti-browning effect of alkaline EO water on red delicious apples. In addition, alkaline EO water was compared with other commonly used anti-browning compounds, including ascorbic acid (reducing agent), citric acid (acidulants agent), and sodium metabisulfite

(sulfite compound). Benefit of combining alkaline EO water with other anti-browning for inhibiting apple browning was also evaluated.

MATERIALS AND METHODS

Materials

Red Delicious apples were purchased from a local super market in Griffin, Georgia, USA and stored at 4℃ until use. EO water was produced from an EO water generator (ROX-20TA, Hoshizaki) by electrolysis of dilute NaCl solution at currents of 10A, 14A and 19A. Alkaline EO water, obtained from the cathode side, was used for the experiment. Ascorbic acid (Fisher), citric acid (Sigma-Aldrich), and sodium metabisulfite (Sigma-Aldrich) were used as anti-browning agents.

Polyphenoloxidase (PPO) activity measurement

The PPO activity of apple extract was measured according to Ozoglu and Bayindirli (2002) with slight modification. Fifty grams of apple pulp was homogenized with 50 ml of McIlvaine buffer (pH 6.5) for 2 minutes using a food processor (Hamilton Beach). The homogenate was then filtered through cheesecloth. The filtrate was centrifuged at 4000 rpm and 4℃ for 10 minutes in a centrifuge (Centra CL3R, IEC, Thermo scientific, MA, USA). The supernatant was filtered through Whatman paper No.1 and analyzed for PPO activity.

A mixture of 0.5mL of apple PPO extract, 1mL of McIlvaine buffer pH 6.5 and 1mL of anti-browning solution served as inhibitor was reacted for 5 min at 25 ^{o}C. One milliliter of 0.2M

catechol was then added to the reaction mixtures. The absorbance values were recorded at 420 nm using a spectrophotometer (DU® 520 Ceneral Purpose UV/Vis Spectrophotometer, Beckman coulter, CA, USA) for 1min. One unit of PPO activity was defined as 0.001 ΔA_{420} / min.

Apple slice preparation and treatments

The apples selected were of uniform size and color. After being washed in tap water, apples were sliced into ten equal slices using a stainless steel knife. Apple slices were either dipped in solutions for 5 min or soaked in ten different treatment solutions for 2 h, respectively. They were then drained and placed into Petri dishes. Controls were apple dipped or soaked in deionized water. Alkaline EO water generated at Amperage setting (A) of 10, 14 and 19 were need within 1 h of production. Anti-browning agent solutions were prepared by dissolving 2mM ascorbic acid, 2mM citric acid, and 2mM sodium metabisulfite in deionized water. Anti-browning agent solutions (2mM ascorbic acid, 2mM citric acid, and 2mM sodium metabisulfite) prepared using alkaline EO water (19 A) were also used as inhibitor solutions in order to evaluate whether synergic effect exist by combining anti-browning agents with different mode of activity.

Color measurement

The color of apple slices was measured using a Minolta colorimeter (CR-200b, Japan) according to Rocha and Morais (2003). The Hunter L^*, a^*, and b^* color readings representing lightness, red to green color dimension, and yellow to blue color dimension were recorded. The instrument was standardized using a white ceramic plate: Y= 92.6, x= 0.3129 and y= 0.3190.

Color readings were obtained from the middle section of cut surface of apple slices. Three apple slices were used for each treatment solution and the whole experiment were replicated five times. Total color difference (ΔE; Eq. (1)), Hue (Eq. (2)), chroma (saturation index; Eq (3)), and browning index (Eq (4)) values (Askari and others, 2008) were calculated using Hunter L^*, a^*, and b^* values as follows:

$$\Delta E = [(L^*_0-L^*)^2 + (a^*_0-a^*)^2 + (b^*_0-b^*)^2]^{1/2} \quad (1)$$

$$\text{Hue angle} = \arctan (b^*/a^*) \quad (2)$$

$$\text{Chroma} = [a^{*2} + b^{*2}]^{1/2} \quad (3)$$

$$BI = [100(X-0.31)]/0.172 \quad (4)$$

where:

$$X= (a^*+1.75L^*)/ (5.645L^*+a^*-3.012b^*)$$

Statistical analysis

The data was analyzed by using the statistical analysis system (SAS). Comparison of storage time and treatment variations was determined using analysis of variance (ANOVA) with the least significant difference (LSD) ($\alpha = 0.05$). Results were expressed as the mean plus/minus standard deviation.

RESULTS AND DISCUSSIONS

Polyphenol oxidase (PPO) activity

In order to investigate the anti-browning effect of alkaline EO water on apple PPO, anti-browning effect of ascorbic acid, citric acid, and sodium metabisulfite were compared with alkaline EO water. For all tested samples, relative PPO activities were significantly decreased ($p<0.001$). As shown in Fig. 3.1, alkaline EO water had a similar inhibitory effect on the apple PPO activity as citric acid. Apple extract treated with ascorbic acid and sodium metabisulfite had significant lower PPO activity than treated with alkaline EO water at 19A. This trend is in agreement with previous studies (Eissa and others, 2006; Son and others, 2001; Ozoglu and Bayindirli, 2002; Rojas-Grau and others 2008). Son and others (2001) suggested citric acid prevents enzymatic browning by its metal-chelating characteristics. Eissa and others (2006), Ozoglu and Bayindirli (2002), Son others (2001), and Rojas-Grau and others (2008) reported ascorbic acid inhibited polyphenol oxidase activity of apple products by reduction of *o*-quinone compounds. The active site of PPO is composed of two copper atoms and the enzyme catalyzes the hydroxylation of monophenols and the oxidation of *o*-diphenols to *o*-quinones (Queiroz and others, 2008). Chemicals that can either react with copper atoms or binding the active site of the enzyme can prevent enzymatic browning.

Color measurement

Table 3.1 and Fig. 3.2 showed values of total color difference (ΔE) on apple slices when dipped in respective treatment solutions for 5 min. ΔE of apple slices treated with control (DW)

increased with storage time, indicating more color change with increasing storage time. Alkaline EO water at 10A showed similar ΔE patterns as DW for 4 h storage time; however, remained unchanged for up to 24h. Apples treated with 14A alkaline EO water had similar ΔE as control after 2h storage then remain unchanged for up to 24h. Apples treated with 19A alkaline EO water had lower ΔE than DW (9.07 v.s 20.4 after 24h storage) or other alkaline EO water treatments. In addition, ΔE for apple treated with 19A alkaline EO water increased from 1.66 to 8.93 after 2h then remain unchanged for up to 24h (Table 3.1). Apples treated with 19A alkaline EO water (9.07 after 24h) also had lower ΔE than apples treated with citric acid (10.2 after 24h) or ascorbic acid (12.8 after 24h), however, showed higher ΔE than apples treated with sodium metabisulfite (Table 3.1 and Fig. 3.2).

The ΔE of apple slices treated with the combination of 19A alkaline EO water and ascorbic acid or citric acid had lower ΔE (indicating better antibrowning effect) than the color change of apple slices treated with ascorbic acid or citric acid alone (Fig. 3.2), indicating less color change with combination treatment. The combination of citric acid and 19A alkaline EO water treatment was only slightly lower than 19A alkaline EO water treatment alone (6.65 v.s 9.07 after 24h). However, ΔE of apple treated with combination of ascorbic acid and 19A alkaline EO water was significantly lower than citric acid and 19A alkaline EO water combination and had value similar to the sodium metabisulfite treatment (Fig. 3.2).

The browning indexes on apple slices dipped with respective treatment solutions for 5 min (Table 3.2 and Fig. 3.3) follow the similar patterns as ΔE reported in Table 3.1 and Fig. 3.2. Apple slices treated with 19A alkaline EO water had the lowest browning index than other antibrowning solutions except sodium metabisulfite. Apples treated with the combination of ascorbic acid and 19A alkaline EO water had lower browning index (28.92) than either of the

treatment alone (40.84 for 19A alkaline EO water and 47.62 for ascorbic acid) (Table 3. and Fig. 3.3). Apple slices treated with the combination of 19A alkaline EO water and ascorbic acid had lower browning index value than the 19A alkaline EO water and citric acid combination, however, it still had slightly higher browning index (indicating more brown color development) values than apple slices treated with sodium metabisulfite alone (Fig. 3.3).

Ascorbic acid and citric acid are widely used to prevent browning development on fresh-cut fruits or vegetables and studied by many researchers. Garcia and Barrett (2002) reported that citric acid, which is classified as acidulants, had dual effects: lowering pH and chelating Cu from PPO active site. Citric acid is often used in combination with other antibrowning agents, because it is difficult to prevent effectively browning development through pH (Garcia and Barrett, 2002). Zuo and Lee (2004) reported that combination of 0.5% ascorbic acid and 0.5% citric acid had the most effective inhibition on browning of apple cubes when compared with other antibrowning agents. Jiang and others (2008) reported the use of L-cystein and citric acid combination significantly reduced the browning index in harvested litchi fruit. Such studies demonstrated that the combination with citric acid and other antibrowning agents inhibited effectively browning development on apple products. In addition, ascorbic acid is a powerful reducing agent and can convert the enzymatically formed *o*-quinones back to their precursor diphenols and hence preventing the formation of pigments (Nicolas and others, 1994). Several researchers, however, reported that ascorbic acid treatment is not strong inhibitor to control enzymatic browning of fresh cut apples (Rojas-Grau and others, 2008; Son and others, 2001; Rojas-Grau and others, 2006; Ozoglu and Bayindirli, 2002). McEvily and Iyengar (1992) and Garcia and Barrett (2002) suggested the effect of ascorbic acid is only temporary because ascorbic acid itself oxidized irreversibly when react with polyphenoloxidase and metals such as

copper. Their reports can support our findings that apple slices treated with ascorbic acid developed more brown color than treated with 19A alkaline EO water after 2h of storage (Table 3.1 and Fig. 3.2).

The current study found the combination of ascorbic acid with 19A alkaline EO water significantly reduced browning development than either treatment alone as indicated on ΔE and browning index (Table 3.1-3.2 and Fig. 3.2-3.3). Ozoglu and Bayindirli (2002) reported that a combination of ascorbic acid, L-cystein and cinnamic acid provided better browning inhibition of cloudy apple juice (Golden delicious) than the individual compounds alone. Wang and others (2007) also found that the treatment of fresh-cut apples with acidic EO water followed by treatment with calcium ascorbate was very effective at browning prevention. In addition, a combination of N-acetyl-cysteine and glutathione (Rojas-grau and others, 2006) and the combination of L-cysteine, kojic acid and 4-hexylresorcinol (Iyidogan and Bayindirli, 2006) proved to be the most effective treatments for preventing browning on fresh-cut 'Fuji' apple slices and amasya apple juice, respectively. Their findings showed the combination treatments of chemicals, which have antibrowning activity, had more synergistic antibrowning effect than applied individually. In current study, we applied alkaline EO water in combination with other antibrowning agents to prevent browning reactions on apple slices. Combination of ascorbic acid and 19A EO water had stronger antibrowning effect than citric acid and 19A EO water combination. The reason might be from citric acid, which is acidulants inhibitor. In addition, pH value of the combination of citric acid and 19A alkaline EO water might be increased when it combined with 19A alkaline EO water (pH=approximately 11).

The antibrowning effect of alkaline EO water might be due to its significantly negative oxidation reduction potential (ORP) value (about -800 mV) as well as hydrogen molecules (Su

and others, 2007). Goncharuk and others (2010) reported the molecular hydrogen released at the cathode during electrolysis of water is a strong reducer in alkaline EO water and the amount of hydrogen is related with negative ORP value. In addition, Hanaoka and others (2004), Shirahata and others (1997), and Miyashita and others (1999) reported that alkaline EO water plays a role like antioxidants. Hanaoka and others (2004) reported that the ionic product of water in the alkaline EO water enhanced reducing properties of ascorbic acid and is supportive of our result that combination treatment (19A EOW + AA) had a strong antibrowning effects than individual treatment alone. According to these studies and findings reported in current studies, alkaline EO water has the potential to be used as an anti-browning agent alone or enhance the anti-browning effect when combined with other anti-browning compounds like ascorbic acid.

In order to evaluate whether longer treatment time with antibrowning solutions can further prevent browning on apple slices, apple slices were soaked in treatment solution for 2 h. Table 3.3 shows ΔE of apple slices when soaked in respective treatment solutions for 2 h. ΔE of apple slices soaked in DW for 2 h (control) significantly increased during soaking. Alkaline EO water (10A and 14A) soaking treatment showed similar ΔE patterns as control during storage. Apples treated with 19A alkaline EO water had lower ΔE than control (26.79 v.s 30.64 after 24 h storage) and other alkaline EO water treatments (Table 3.3).

The combination of citric acid and 19A alkaline EO water treatment had a lower ΔE than citric acid alone (4.08 v.s 23.67) immediately after soaking and ΔE only increased slightly to 7.13 after 3 h and then increased significantly to 20 after 24 h of storage (Fig. 3.4). The combination of ascorbic acid and 19A alkaline EO water treatment had better effect to maintain the ΔE than apple slices treated with ascorbic acid alone (13.21 v.s 23.21 after 24h storage) (Fig.

3.4). Sodium metabisulfite, however, showed the strongest inhibition in ΔE change than other treatment solutions.

The browning index (Table 3.4 and Fig. 3.5) of soaked apple slides followed a similar patterns as dipped apples Δ E (Table 3.3 and Fig. 3.4). Browning index of apple slices treated with control (DW), 10A and 14A EO water significantly increased with storage time (Table 3.4). Apples treated with 19A alkaline EO water had the lowest browning index compared to DW or other EO water treatments. The combination of ascorbic acid and 19A EO water or citric acid and 19A EO water had lower browning index values than ascorbic acid or citric acid alone (Fig. 3.5). Although sodium metabisulfite is still the best antibrowning agent in regent to browning index value, combination treatment of ascorbic acid and 19A EO water had lower browning index after 24 h storage than the citric acid and 19A EO water combination treatment (50 vs. 65). As shown in Tables 3.1-3.2 and Figs. 3.2-3.3, apple slices soaked with inhibitors developed more brown color than dipped. Although the overall pattern of Δ E and browning index for dipped (5min) and soaked (2 h) are almost the same (Tables 3.1 to 3.4 and Figs. 3.2 to 3.5), the absolute values of ΔE and browning index for soaked apples were significantly higher than dipped apple slices. This might possibly be due to tissue damage caused by the long exposure time in treatment solutions. Queiroz and others (2008) suggested that PPO is released by rupture of the plastids and vacuole when the tissue is damaged. The longer exposure of apple slices in treatment solutions, thus, might accelerate PPO activity of wounded or cut surfaces of apple slides and hence more browning development in soaking treatments.

Hue angle and chroma also have been used as indicator of browning (Bozkurt and Bayram (2006). Bozkurt and Bayram (2006) reported that a decrease in hue angle values indicates the production of an orange-red color and increase chroma values indicates the increasing intensity

or purity of the hue (Rocha and Morais, 2003). The hue is an angle in a color wheel of 360^{o}; with 0^{o} (red), 90^{o} (yellow), 180^{o} (green), and 270^{o} (blue) respectively (Rojas-Grau and others, 2006). The hue angle value could represent true color, which is effective for visualizing the color appearance of food products (Rojas-Grau and others, 2006). Thus, hue angle is one of the main properties to distinguish light brown (80^{o}) and dark brown (60^{o}).

Table 3.5 shows values of hue angle on apple slices when dipped in respective treatment solutions for 5 min. The hue angle values of apple slices immediately after treatment (0 h) had hue angle between 103 and 93 (yellow). Apples treated with DW (control) and 10A or 14A EO water had hue angle values in the lower 90^{o} range (dark yellow) while apples treated with other treatment solutions had hue angle values in the high 90^{o} or over 100^{o} (lighter yellow). Control apple slides had the lowest hue angle among other treatments and decreased with increased storage time, indicating more browning development on apple surface, whereas the values of apples treated with 10A alkaline EO water had similar tendency as DW although the hue angle values of 10A alkaline EO water were slightly higher than that of DW (Table 3.5). Hue angle values of apple slices treated with 14A alkaline EO water were similar as control (DW) immediately after treatment with 14A EO water had higher hue angle value than the control or 10A EO water treated apples. Hue values for apples treated with 19A alkaline EO water decreased from 97 to 91 after 24 h storage and had similar change as the citric acid treatment (96 to 91) or the combination of 19A alkaline EO water and citric acid. Apple slides had the highest hue angle value immediately after treatment (103); however, if decreased to 92 after 24 h storage indicating ascorbic acid can inhibit browning at beginning but cannot maintain its efficacy during storage. Apple slices dipped in ascorbic acid dissolved in 19A alkaline EO water had less change in hue angle value during 24 h storage (100 to 97) except sodium metabisulfite alone or

combination treatments, indicating that the combination of 19A alkaline EO water and ascorbic acid is an effective antibrowning agent (Table 3.5).

Table 3.6 shows hue angle values of apple slices during 24 h storage after soaked in treatment solutions for 2 h. Sodium metabisulfite, still the best treatment, had hue angle value of 104 immediately after treatment and did not change during storage. Ascorbic acid is also a good browning inhibition treatment with hue angle of 104 immediately after soaking. However, hue angle quickly decreased (become brown in color) with increasing storage time to 84 after 24 h. The combination of ascorbic acid and 19A EO water can slow down the browning development (from 99 to 91 during 24 h storage). However, the amount of decrease was higher than the 5min dipping in the same treatment solution (100 to 97, Table 5). This support the suggestion from Queiroz and others (2008) and our finding on ΔE and browning index that longer treatment time (soaking) may accelerate the release of PPO from damaged apple cells and hence more browning development. Apple slides treated with DW, EO water alone, citric acid, and combination of citric acid and 19A EO water all had significant higher decrease on hue angle value after 24 h storage (77 to 84, Table 6) than corresponded dipping treatment (84 to 92, Table 5).

Table 3.7 and Table 3.8 show chroma value of apple slices dipped (5min) or soaked (2h) in treatment solutions, respectively. Chroma value of apple slices dipped with DW (21 to 35) were the highest after 24h storage, indicating increasing color intensity. Similar trends were observed as hue angle (Tables 3.5 and 3.6) that sodium metabisulfite or the combination treatments were the best as indicated no increase of chroma value. Combination of ascorbic acid and 19A EO water was the next best treatment with chroma increased from 20 to 23 (Table 7) for dipped apple slides and apple slices soaked in treatment solutions also had higher increase in chroma than the respective dipped apple slides.

As demonstrated in the color parameters (ΔE, hue angle, and chroma) and browning index values, combination treatments of 19A alkaline EO water and ascorbic acid showed synergistic effect to prevent browning in apples, suggesting that alkaline EO water is an effective antibrowning agent. The mechanism of inhibition is quite different for each inhibitor so that these mechanistic differences may allow the use of combinations of antibrowning agents that resulted in enhancements of antibrowning activity than individual antibrowning (McEvily and Iyengar, 1992). In current study, antibrowning property of alkaline EO water was reported due to its strong reducing effects (negative oxidation reduction potential) and change in ionic products of alkaline EO water for antibrowning activity. Ascorbic acid also reduces *o*-quinones formed by the oxidation reaction of PPO. Then because of the different antibrowning mechanism, the combination treatment has the synergistic effect. Therefore, our results suggest that the application of alkaline EO water as antibrowning agent might be able to contribute in fresh-cut produce processing by the benefits such as low cost, non-thermal method, safety for the human contact compared to other chemicals or in combination with other antibrowning agents at a lower concentration than use individually.

CONCLUSIONS

Strong alkaline EO water produced at 19A can effectively delay browning development in apples by application alone or by mixing with other anti-browning agents. The combination treatment with ascorbic acid achieved a strong anti-browning effect equivalent to that of sodium metabisulfite. This better anti-browning effect may be a result of synergy between the activity of anti-browning agents and the reducing activity of alkaline EO water. Results indicated that the use of alkaline EO water for the preservation of minimally processed fruit is promising.

LITERATURE CITED

Al-Haq, M. I.; Sugiyama, J.; Isobe, S. Applications of electrolyzed water in agriculture and food industries. Food Science and Technology Research, **2005**, 11, 135-150.

Arias, E.; Gonzlez, J.; Peiro, J.M.; Oria, R.; Lopez-Buesa, P. Browning prevention by ascorbic acid and 4-Hexylresorcinol: Different mechanisms of action on polyphenol oxidase in the presence and in the absence of substrates. Journal of Food Science, **2007**, 72, C464 - C470.

Askari, G.R.; Emam-Djomeh, Z.; Mousavi, S.M. Investigation of the effects of microwave treatment on the optical properties of apple slices during drying. Drying Technology, **2008**, 26, 1362-1368.

Bozkurt, H.; Bayram, M. Colour and textural attributes of sucuk during ripening. Meat Science, **2006**, 73, 344-350.

Eissa, H.A.; Fadel, H.H.M.; Ibrahim, G.E.; Hassan, I.M.; Elrashid, A.A. Thiol containing compounds as controlling agents of enzymatic browning in some apple products. Food Research International, **2006**, 39, 855-863.

Fan, X.; Sokorai, K. J.B. ; Liao, C.H.; Cooke, P.; Zhang, H.Q. Antibrowning and antimicrobial properties of sodium acid sulfate in apple slices. Journal of Food Science, **2009**, 74, M485-M492.

Garcia, E.; Barrett, D.M. Preservative treatments for fresh-cut fruits and vegetables. CRC book on “Fresh-cut Fruits and Vegetables: Science, Technology, and Market.” Ed: Olusola Lamikanra. Washington, DC, **2002**, 267-303.

Goncharuk, V.V.; Bagrii, V.A. ; Mel'nik, L.A. ; Chebotareva, R.D.; Bashtan, S.Y. The use of redox potential in water treatment processes. Journal of Water Chemistry and Technology, **2010**, 32, 1-9.

Hanaoka, K. Antioxidant effects of reduced water produced by electrolysis of sodium chloride solutions. Journal of Applied Electrochemistry, **2001**, 31, 1307-1313.

Hanaoka, K.; Sun, D.; Lawrence, R.; Kamitani, Y.; Fernandes, G. The mechanism of the enhanced antioxidant effects against superoxide anion radicals of reduced water produced by electrolysis. Biophysical Chemistry, **2004**, 107, 71-82.

Huang, Y.R.; Hung, Y.C.; Hsu, S.Y.; Huang, Y.W.; Hwang, D.F. Application of electrolyzed oxidizing water in the food industry. Food Control, **2008**, 19, 329-334.

Iyidogen, N.F.; Bayindirh, A. Effect of L-cystein, kojic acid ad 4-hexylresorcinol combination on inhibition of enzymatic browning in amasya apple juice. Journal of Food Engineering, **2006**, 62, 299-304.

Jiang, Y.M.; Li, J.R.; Shi, J. Enhanced effect of L-cysteine and citric acid combination on browning inhibition and quality maintenance in harvested litchi fruit. Journal of Food Science and Technology-Mysore, **2008**, 45, 75-77.

Kim, C.; Hung, Y.C.; Brackett, R.E. Roles of oxidation-reduction potential in electrolyzed oxidizing and chemically modified water for the inactivation of food-related pathogens. Journal of Food Protection, **2000**, 63, 19-24.

Lu, S.M.; Luo, Y.G.; Turner, E.; Feng, H. Efficacy of sodium chlorite as an inhibitor of enzymatic browning in apple slices. Food Chemistry, **2007**, 104, 824-829.

McEvily, A.J.; Iyengar, R. Inhibition of enzymatic browning in foods and beverages. Critical Reviews in Food Science and Nutrition, **1992**, 32, 253-273.

Miyashita, K.; Yasuda, M.; Ota, T.; Suzuki, T. Antioxidative activity of a cathodic solution produced by the electrolysis of a dilute NaCl solution. Bioscience Biotechnology and Biochemistry, **1999**, 63, 421-423.

Nicolas, J.J.; Richardforget, F.C.; Goupy, P.M.; Amiot, M.J.; Aubert, S.Y. Enzymatic browning reactions in apple and apple products. Critical Reviews in Food Science and Nutrition, **1994**, 34, 109-157.

Ozoglu, H.; Bayindirli, A. Inhibition of enzymic browning in cloudy apple juice with selected antibrowning agents. Food Control, **2002**, 13, 213-221.

Pilizota, V.; Subaric, D. Control of enzymatic browning of foods. Food technology and Biotechnology, **1998**, 36, 219-227.

Queiroz, C.; Lopes, M.L.M.; Fialho, E.; Valente-Mesquita, V.L. Polyphenol oxidase: characteristics and mechanisms of browning control. Food Reviews International, **2008**, 24, 361-375.

Rocha, A.M.C.N; Morais, A.M.M.B. Shelf life of minimally processed apple (cv. Jonagored) determined by colour changes. Food Control, **2003**, 14, 13-20.

Rojas-Grau, M.A.; Souliva-Fortuny, R.; Martin-Belloso, O. Effect of natural antibrowning agents on color and related enzymes in fresh-cut fuji apples as an alternative to the use of ascorbic acid. Journal of Food Science, **2008**, 73, S267-S272.

Rojas-Grau, M.A.; Sobrino-Lopez, A.; Tapia, M.S.; Martin-Belloso, O. Browning inhibition in fresh-cut 'Fuji' apple slices by natural antibrowning agents. Journal of Food Science, **2006**, 71, S59-S65.

Shirahata, S.; Kabayama, S.; Nakano, M.; Miura, T.; Kusumoto, K.; Gotoh, M.; Hayashi, H.; Otsubo, K.; Morisawa, S.; Katakura, Y. Electrolyzed-reduced water scavenges active oxygen species and protects DNA from oxidative damage. Biochemical and Biophysical Research Communications, **1997**, 234, 269-274.

Son, S.M.; Moon, K.D.; Lee, C.Y. Inhibitory effects of various antibrowning agents on apple slices. Food Chemistry, **2001**, 73, 23-30.

Su, Y.-C.; Liu, C.; Hung, Y.C. Electrolyzed water: principles and applications. ACS book on "New biocides Development: The Combined Approach of Chemistry and Microbiology." Ed: P.Zhu. American Chemical Society. Washington, DC., **2007**, 309-322.

Tortoe, C.; Orchard, J.; Beezer, A. Prevention of enzymatic browning of apple cylinders using different solution. International Journal of Food Science and Technology, **2007**, 42, 1475-1481.

Wang, H.; Feng, H.; Luo, Y.G. Control of browning and microbial growth on fresh-cut apples by sequential treatment of sanitizers and calcium ascorbate. Journal of Food Science, **2007**, 72, M1-M7.

Zuo L.; Lee J.H. Effect of anti-browning agents on the quality of minimally processed apple cubes. Food Science and Biotechnology, **2004**, 13, 40-45.

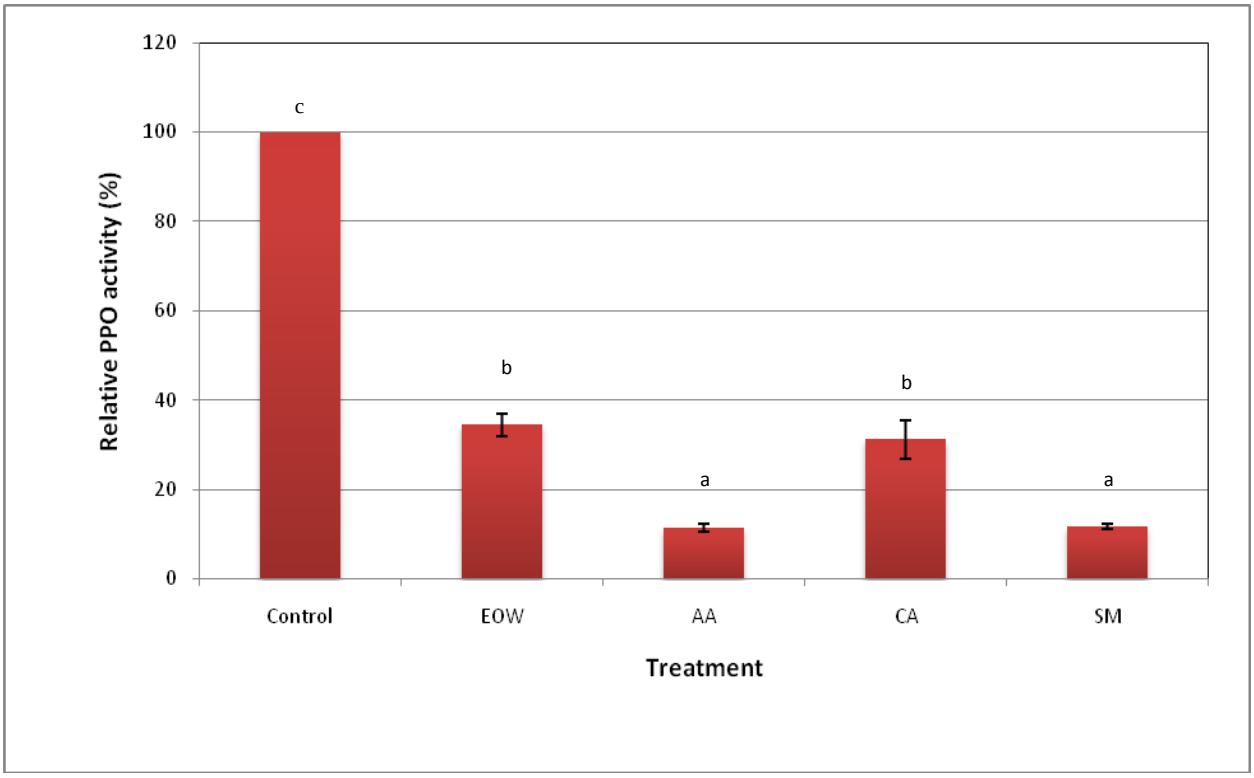

Fig. 3.1. The inhibitory effects of alkaline electrolyzed water on apple polyphenol oxidase.

Data are means ± SD of three replicates. Control is deionized water. EOW is alkaline electrolyzed water at 19A. AA is ascorbic acid. CA is citric acid. SM is sodium metabisulfite. Means with the same letter are not significantly different (p value < 0.05).

Table 3.1. Total color difference of apple slices dipped in EOW. [1]

	Total Color Difference			
	After 0h	After 2h	After 4h	After 24h
DW(Control)	3.21±1.2 A d	15.17±0.84 A c	16.72±0.95B b	20.4±1.9A a
EOW(10A)	3.84±1.21 A c	13.1±1.38 B b	17.92±0.87 A a	17.27±1.3B a
EOW(14A)	4.18±1.38 A b	14.4±1.7 A B a	14.66±0.72C a	15.18±1.57C a
EOW(19A)	1.66±0.5 B b	8.93±1.47 C a	8.55±1.35D a	9.07±0.4D a

[1]Same capital letter after mean value in the same column and the same lower-case letter after each mean value in the same row indicate not significantly different at p values <0.05. DW is deionzed water. EOW is alkaline EO water and produced at current of 10A, 14A, and 19A. Data are means ± SD of five replicates.

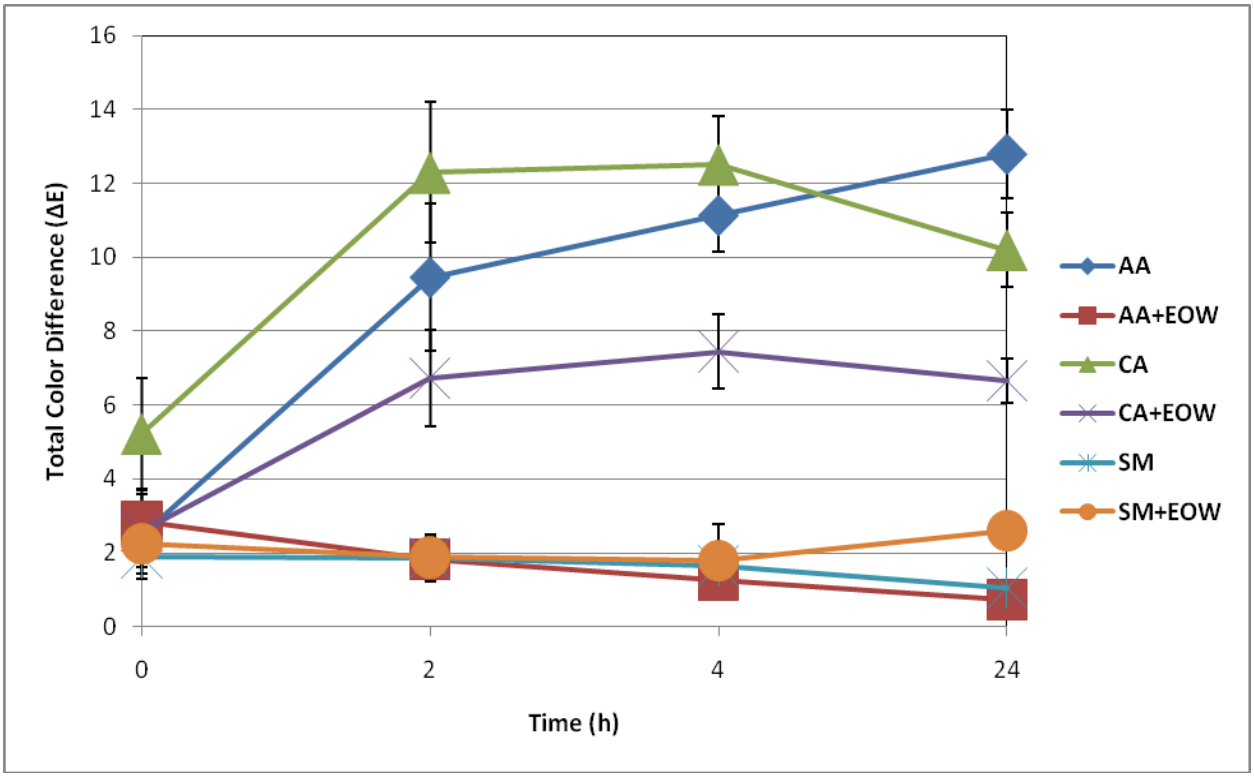

Fig. 3.2. Total color difference of apple slices dipped in combination treatment with EOW and various antibrowning solutions.

AA is ascorbic acid. CA is citric acid. SM is sodium metabisulfite. AA+EOW is a combination of ascorbic acid and alkaline EO water (19A). CA+EOW is a combination of citric acid and alkaline EO water (19A). SM+EOW is a combination of sodium metabisulfite and alkaline EO water (19A). Data are means ± SD of five replicates.

Table 3.2. Browning index values of apple slices dipped in EO water. [1]

	Browning Index			
	After 0h	After 2h	After 4h	After 24h
DW	28.86±1.68A c	55.07±2.03A b	58.27±1.8A b	69.36±5.67A a
EOW(10A)	27.98±1.94AB c	47.46±3.35B b	58.41±3.1A a	56.28±2.26B a
EOW(14A)	29.18±2.33A b	50.16±4.32A B a	50.11±2.58B a	51.36±4.14C a
EOW(19A)	25.62±1.03 C c	32.89±2.67C b	39.91±2.9C a	40.84±0.95D a

[1]Same capital letter after mean value in the same column and the same lower-case letter after each mean value in the same row indicate not significantly different at p values <0.05. DW is deionzed water. EOW is alkaline EO water and produced at current of 10A, 14A, and 19A. Data are means ± SD of five replicates.

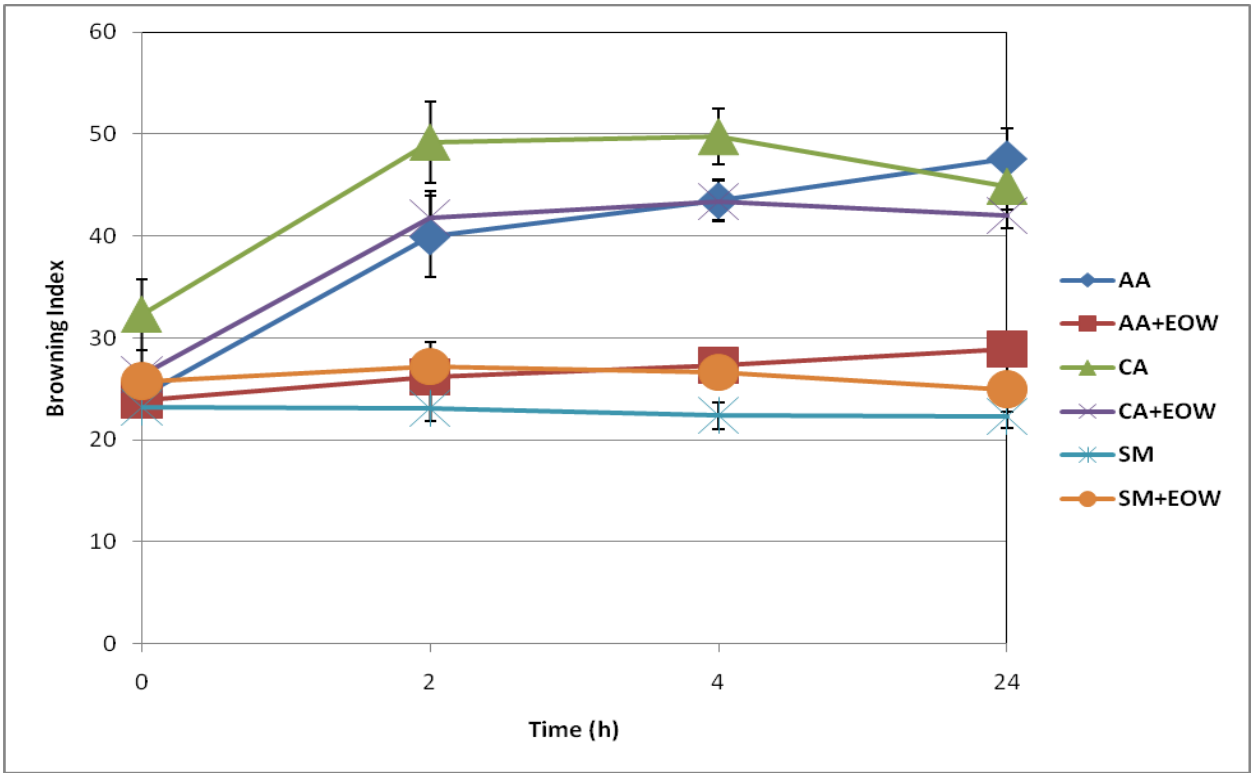

Fig. 3.3. Browning index of apple slices dipped in combination treatments with EO water and various antibrowning solutions.

AA is ascorbic acid. CA is citric acid. SM is sodium metabisulfite. AA+EOW is a combination of ascorbic acid and alkaline EO water (19A). CA+EOW is a combination of citric acid and alkaline EO water (19A). SM+EOW is a combination of sodium metabisulfite and alkaline EO water (19A). Data are means ± SD of five replicates.

Table 3.3. Effect of storage on total color difference of apple slices after soaked in EO water for 2 h. [1]

	Total Color Difference			
	After 0h	After 1h	After 3h	After 24h
DW(Control)	11.17±0.81A d	23.27±1.35A c	26.93±1.44A b	30.64±1.32 BC a
EOW(10A)	10.22±0.44A d	17.73±1.54B c	23.04±1.31B b	34.24±2.37A a
EOW(14A)	10.7±2.14A c	24.07±1.51A b	27.74±2.67A a	32.66±0.7 AB a
EOW(19A)	9.58±1.23A d	21.51±0.6A c	22.68±0.7B b	26.79±2.14C a

[1]Same capital letter after mean value in the same column and the same lower-case letter after each mean value in the same row indicate not significantly different at p values <0.05. DW is deionzed water. EOW is alkaline EO water and produced at current of 10A, 14A, and 19A. Data are means ± SD of five replicates.

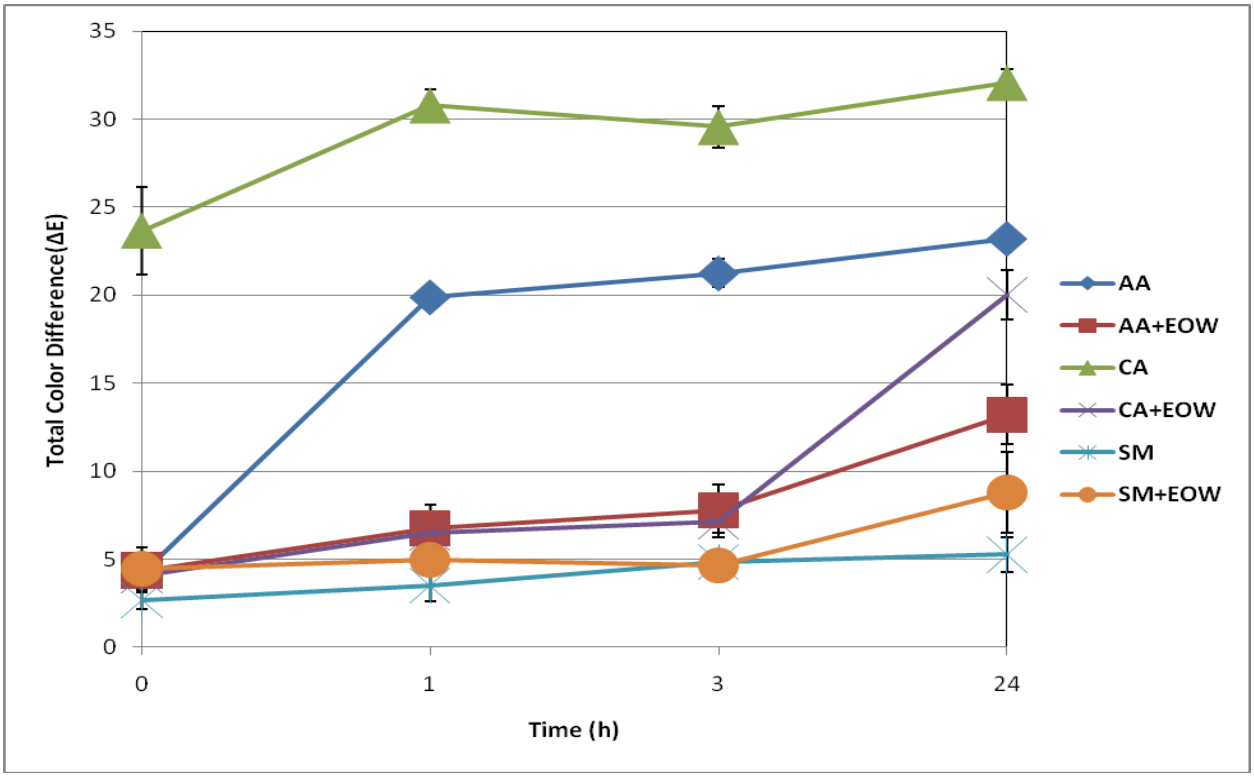

Fig. 3.4. Effect of storage on total color difference of apple slices after soaked in combination treatment with EO water and various antibrowning solutions for 2 h.

AA is ascorbic acid. CA is citric acid. SM is sodium metabisulfite. AA+EOW is a combination of ascorbic acid and alkaline EO water (19A). CA+EOW is a combination of citric acid and alkaline EO water (19A). SM+EOW is a combination of sodium metabisulfite and alkaline EO water (19A). Data are means ± SD of five replicates.

Table 3.4. Effect of storage on browning index of apple slices after soaked in EO water for 2 h.[1]

	Browning Index			
	After 0h	After 1h	After 3h	After 24h
DW	43.05±2.19A d	69.6±2.47A c	87.46±4.12A b	101.55±5.26B a
EOW(10A)	40.36±2.73A d	56.04±4.14B c	72.52±4.38B b	114.98±10.6A a
EOW(14A)	39.3±3.73A d	68.05±3.81A c	87.24±11.23A b	106.42±5.77B a
EOW(19A)	35.02±3.05B c	68.5±2.09A b	73.35±2.9 B b	86.11±7.91C a

[1]Same capital letter after mean value in the same column and the same lower-case letter after each mean value in the same row indicate not significantly different at p values <0.05. DW is deionzed water. EOW is alkaline EO water and produced at current of 10A, 14A, and 19A. Data are means ± SD of five replicates.

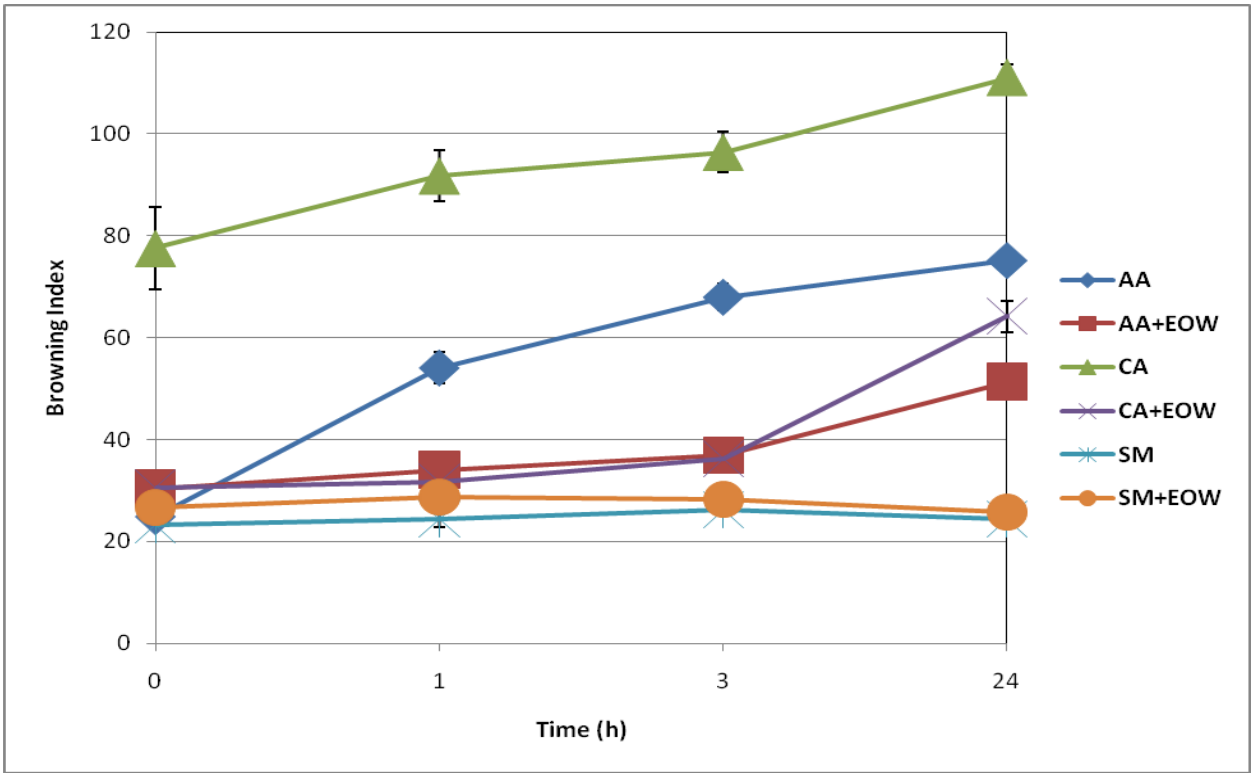

Fig. 3.5. Effect of storage on browning index of apple slices after soaked in combination treatments with EOW and various antibrowning solutions for 2 h.

AA is ascorbic acid. CA is citric acid. SM is sodium metabisulfite. AA+EOW is a combination of ascorbic acid and alkaline EO water (19A). CA+EOW is a combination of citric acid and alkaline EO water (19A). SM+EOW is a combination of sodium metabisulfite and alkaline EO water (19A). Data are means ± SD of five replicates.

Table 3.5. Hue angle values of apple slices dipped in various anti-browning solutions.[1]

	Hue Angle			
	After 0h	After 2h	After 4h	After 24h
DW(Control)	93.36±2.25 E a	87±0.28 F b	84.75±3.04 F bc	83.71±1.16G, c
EOW(10A)	93.95±1.8 E a	87.72±1.62 F b	85.95±2.26 F bc	84.59±1.73 G c
EOW(14A)	93.7±0.93 E a	86.86±2.16 F b	88.05±1.33 E b	88.28±1.24 F b
EOW(19A)	96.99±0.22 CD a	91.63±0.53 D b	91.12±0.85 D b	91.13±1.21 DE b
AA	103.11±0.55 A a	94.15±0.41 C b	93.73±0.35 C b	92.32±0.57 D c
AA+EOW	99.83±0.28 B a	99.48±0.54 B ab	98.91±0.27 B b	97.44±1.11 C c
CA	96.04±0.23 D a	89.16±1.2 E c	89.9±1.25 DE bc	90.91±0.25 E b
CA+EOW	98.07±1 C a	91.29±0.8 D b	90.5±0.6 D b	91.2±0.58 DE b
SM	102.52±0.38 A a	102.9±0.47 A a	102.86±0.43 A a	102.82±0.56 A a
SM+EOW	99.91±0.4 B ab	100.13±0.53 B ab	101.2±1.97 A a	99.7±0.41 B b

1Same capital letter after mean value in the same column and the same lower-case letter after each mean value in the same row indicate not significantly different at p values <0.05. DW is deionzed water. EOW is alkaline EO water and produced at current of 10A, 14A, and 19A. AA is ascorbic acid. CA is citric acid. SM is sodium metabisulfite. AA+EOW is a combination of ascorbic acid and alkaline EO water (19A). CA+EOW is a combination of citric acid and alkaline EO water (19A). SM+EOW is a combination of sodium metabisulfite and alkaline EO water (19A). Data are means ± SD of five replicates.

Table 3.6. Hue angle values of apple slices soaked in various anti-browning solutions.[1]

	Hue Angle			
	After 0h	After 1h	After 3h	After 24h
DW(Control)	90.42±0.76 E a	83.92±0.73 D b	80.1±0.88 H c	78.22±0.76 EF d
EOW(10A)	91.25±1.56 E a	81.96±12.08 D b	84.27±0.64 E ab	77.39±1.99 F b
EOW(14A)	93.72±1.55 D a	83.92±0.68 D b	81.88±1.39 G c	79.35±1.35 E d
EOW(19A)	93.39±0.56 D a	83.33±0.52 D b	82.93±0.38 F b	81.79±1.25 D c
AA	103.64±0.3 A a	88.99±0.38 C b	85.13±0.31 E c	84.43±0.46 C d
AA+EOW	99.29±0.15 C a	99.12±0.33 B a	97.93±1.18 C b	90.54±0.29 B c
CA	80.55±0.62 F a	76.85±0.65 D a	75.42±0.65 I b	75.73±0.98 G b
CA+EOW	98.8±0.45 C a	98.56±0.51 B a	96.51±0.89 D b	82.6±0.81 D c
SM	103.83±0.83 A ab	104.36±0.53 A a	104.25±0.45 A ab	103.54±0.38 A b
SM+EOW	100.46±0.58 B b	100.42±0.22 AB b	100.59±0.85 B b	102.7±0.65 A a

[1]Same capital letter after mean value in the same column and the same lower-case letter after each mean value in the same row indicate not significantly different at p values <0.05. DW is deionzed water. EOW is alkaline EO water and produced at current of 10A, 14A, and 19A. AA is ascorbic acid. CA is citric acid. SM is sodium metabisulfite. AA+EOW is a combination of ascorbic acid and alkaline EO water (19A). CA+EOW is a combination of citric acid and alkaline EO water (19A). SM+EOW is a combination of sodium metabisulfite and alkaline EO water (19A). Data are means ± SD of five replicates.

Table 3.7. Chroma values of apple slices dipped in various anti-browning solutions.[1]

	Chroma			
	After 0h	After 2h	After 4h	After 24h
DW	21.17±0.48 B c	31.32±0.67 A b	31.42±0.96 A b	34.52±1.32 A a
EOW(10A)	20.58±1.05 BC c	28.63±1 B b	31.66±1.96 A a	30.25±0.74 B ab
EOW(14A)	21.13±1.39 B b	28.88±1.5 B a	28.94±1.07 B a	29.38±1.24 B a
EOW(19A)	20.21±0.48 BC b	26.13±1.1 D a	25.57±1.25 C a	26.03±0.72 D a
AA	21.05±0.91 BC c	26.87±1.98 CD b	28.32±0.63 B ab	29.69±1.08 B a
AA+EOW	20.4±0.34 BC c	21.98±0.66 E b	22.47±0.32 D b	23.18±0.2 Ea
CA	23.41±1.91 A b	28.89±1.13 B a	29.3±0.51 B a	28±0.8 C a
CA+EOW	21.34±0.98 B b	27.73±1.02 BC a	28.22±0.72 B a	27.97±0.45 C a
SM	19.94±0.11 C a	20.05±1.19 F a	19.57±0.98 E a	19.71±1.1 F a
SM+EOW	21.3±1.11 B ab	22.23±1.38 E a	22.36±1.43 D a	20.57±1.31 F b

[1]Same capital letter after mean value in the same column and the same lower-case letter after each mean value in the same row indicate not significantly different at p values <0.05. DW is deionzed water. EOW is alkaline EO water and produced at current of 10A, 14A, and 19A. AA is ascorbic acid. CA is citric acid. SM is sodium metabisulfite. AA+EOW is a combination of ascorbic acid and alkaline EO water (19A). CA+EOW is a combination of citric acid and alkaline EO water (19A). SM+EOW is a combination of sodium metabisulfite and alkaline EO water (19A). Data are means ± SD of five replicates.

Table 3.8. Chroma values of apple slices soaked in various anti-browning solutions. [1]

	Chroma			
	After 0h	After 1h	After 3h	After 24h
DW	26.5±0.94 B d	33.77±0.37 B c	35.94±0.37 A b	37.62±0.61 B a
EOW(10A)	25.59±1.08 B d	30.04±1.12 D c	34.35±1.01 BC b	39.58±0.97 A a
EOW(14A)	19.23±1.18 E d	32.22±1.34 C c	35.59±2.14 AB b	37.76±0.5 B a
EOW(19A)	20.1±1.4 DE c	32±0.59 C b	32.63±0.95 D b	34.14±1.5 D a
AA	21.2±0.66 D d	30.31±1.35 D c	33.18±0.73 CD b	35.25±0.54 C a
AA+EOW	23.53±0.37 C c	25.1±0.81 E b	25.97±1.46 E b	29.64±0.84 E a
CA	34.04±1.41 A c	35.48±0.91 A b	35.88±0.94 A b	38.41±0.41 B a
CA+EOW	23.6±1.43 C c	23.93±1.13 E bc	25.3±0.57 E b	30.3±0.64 E a
SM	20.17±0.29 DE b	21.04±0.94 F a	21.87±0.5 F a	20.16±0.23 F b
SM+EOW	21.17±1.39 D ab	22.13±1.2 F a	22.18±1.65 F a	20.13±0.67 F b

[1]Same capital letter after mean value in the same column and the same lower-case letter after each mean value in the same row indicate not significantly different at p values <0.05. DW is deionzed water. EOW is alkaline EO water and produced at current of 10A, 14A, and 19A. AA is ascorbic acid. CA is citric acid. SM is sodium metabisulfite. AA+EOW is a combination of ascorbic acid and alkaline EO water (19A). CA+EOW is a combination of citric acid and alkaline EO water (19A). SM+EOW is a combination of sodium metabisulfite and alkaline EO water (19A). Data are means ± SD of five replicates.

CHAPTER 4

ANTIOXIDANT PROPERTIES OF APPLE JUICE RECONSTITUTED WITH ALKALINE ELECTROLYZED WATER

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To be submitted to *Journal of Food Science*.

ABSTRACT

This study was conducted to investigate enhanced antioxidant activity of reconstituted apple juice with alkaline electrolyzed (EO) water. Reconstituted apple juice was prepared from commercial frozen concentrated apple juice and alkaline EO water, which was produced by electrolysis of dilute NaCl and KCl solutions. The total antioxidant capacity (ORAC and TEAC) and other characteristics (pH, ORP, and mineral contents) were measured.

The antioxidant activity of ascorbic acid dissolved in alkaline EO water were enhanced more than that of ascorbic acid dissolved in deionized water by five times, as measured by an ORAC assay. The ORAC values of alkaline EO water (NaCl) and alkaline EO water (KCl) were 1.4 ± 0.3 μM TE/L and not detected, respectively. The TEAC values were 55.2 ± 11 μM TE (EOW-Na) and 23 ± 6.11 μM TE (EOW-K). In addition, the reconstituted apple juice, when mixed with EOW-Na, had higher total antioxidant capacity values (ORAC: 11000 ± 2900 μM TE and TEAC: 3200 ± 100 μM TE) than others. In subsequent studies, the antioxidant stability of reconstituted apple juices during storage at 4^{o}C was evaluated for 2 weeks. TEAC values showed a decrement according to storage time. In contrast, the antioxidant capacities in reconstituted apple juices measured with ORAC assay slightly increased until 7 days, after which point the antioxidant capacities in all reconstituted apple juices are decreased.

The apple juices reconstituted with alkaline EO water had the more negative redox potential values, and higher antioxidant capacity values, than those reconstituted with deionized water. Also, the apple juice reconstituted with alkaline EO water (pH 9) had the highest antioxidant activity. Therefore, this study demonstrated that alkaline EO water could have potential as antioxidant water to enhance total antioxidant capacity.

INTRODUCTION

Recent research on antioxidants in foods and beverages is increasing and the use of antioxidants and their positive effects have been demonstrated in a large variety of foods and beverages (Becker and others, 2004). Antioxidants have been broadly defined as 'substances that in small quantities are able to prevent or greatly retard the oxidation of easily oxidisable materials such as fats' (Chipault, 1962). Mechanistic definitions of antioxidants are usually focused on the ability to donate hydrogen or promote an electron transfer toward a radical (MacDonald and others, 2006). Diets rich in antioxidants may have especially protective effects against several diseases including cancer and chronic disease (Kanakis and others, 2007), both of which have been associated with oxidative stress (Wu and others, 2004). Oxidative stress is induced by an imbalance between the production of reactive oxygen species and a biological system's ability to repair the oxidative damage. Reactive oxygen species, including superoxide radicals ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot}$), and singlet oxygen ($´O_2$), are generated as byproducts of normal metabolism. Excess of these reactive oxygen species or free radicals causes oxidative damage, which leads to the disease development process (Wang and Jiao 2000). Antioxidants also perform physiological defenses against oxidative stress and hence prevent damage of cell membranes and structures such as cellular proteins, lipids, and DNA. For these reasons, the consumption of dietary antioxidant supplements has been constantly increasing, and numerous new supplements have come onto the market (Monagas and others, 2005).

Antioxidant-rich water created through electrolysis has been studied for clinical improvement of various diseases, and reactive oxygen species (ROS) are known to play an essential role in such diseases. Shirahata and others (1997) have demonstrated that alkaline EO water produced by electrolysis scavenged reactive oxygen species (ROS) such as superoxide

anions ($O_2^{-\cdot}$) and hydrogen peroxide molecules (H_2O_2), and as a result had a preventive effect against cell injury and DNA damage. To further develop research of Shirahata and other (1997), Hanaoka (2001) has discovered that the increase in superoxide dismutation activity is due to an increase in the dissociation activity of water when a proton donor such as L-ascorbic acid is dissolved in alkaline EO water. The common discovery of both researchers was synergistic antioxidant activity, which showed greater effect on antioxidant activity when using alkaline EO water and other antioxidant compounds such as ascorbic acid or ethylenediaminetetraacetic acid (EDTA) (Shirahata and others, 1997; Hanaoka, 2001). Based on these results, Yanagihara and others (2005) conducted a feeding test of alkaline EO water with rats to investigate its antioxidative effect using the oxidative stress biomarkers 8-hydroxyldeoxyguanosine (8-OHdG) and thiobarbituric acid reactive substances (TBARS). They reported that the use of alkaline EO water lessened the DNA damage on 8-OHdG markers and inhibited lipid peroxidation in liver of rats on TBARS test (Yanagihara and others, 2005).

Consumption of alkaline EO water is less common than commercial drinking water. Koseki and others (2003; 2005; 2007) studied hardness and pH and conducted a sensory test on taste of alkaline EO water to consider whether the taste of alkaline EO water is acceptable to consumers. Their sensory evaluation of alkaline EO water and commercial bottled mineral water showed that alkaline EO water was more highly rated than commercial bottled mineral water (Koseki and others, 2003). In addition, Koseki and others (2007) have reported that the taste of alkaline EO water with a pH of 9.5 was considered better than that of the unelectrolyzed water. Furthermore, the taste of alkaline EO water should be acceptable because the hardness range (50 ppm to 80 ppm) of alkaline EO water electrolyzed from tap water was similar as that (40 ppm to 90 ppm) of tap water (Koseki and others, 2005). From reports of Koseki and others (2003; 2005;

2007), alkaline EO water was acceptable for drinking, and it has potential application as drinking water by contributing to health benefits

On the other hand, alkaline EO water did not show antioxidant effects by itself (Hanaoka and others, 2004). Other studies, thus, have demonstrated that combining alkaline EO water and other antioxidants such as ascorbic acid or glutathione showed a synergistic scavenging effect against reactive oxygen species (ROS) (Hanaoka, 2001; Hanaoka and others, 2004; Tsai and others, 2009). Still, the benefit of alkaline EO water is obvious, at least potentially: if combined with other substances known to scavenge reactive oxygen species (ROS), it could be an alternative to tap or bottled drinking water. An effective application could be to use alkaline EO water to reconstitute apple juice, which is already known as a good antioxidant, so that apple juice reconstituted with alkaline EO water could be a stronger antioxidant-rich beverage.

The objectives of the current study were (1) to evaluate the antioxidant properties of alkaline EO water when EO water is used to reconstitute commercial frozen concentrated apple juice, (2) to study the stability of reconstituted apple juice's antioxidant capacity during storage, and (3) to investigate the relationship of antioxidant capacity with oxidation reduction potential (ORP) of alkaline EO water to consider whether the antioxidant effect in apple juice reconstituted with alkaline EO water is related to oxidation reduction potential (ORP) of alkaline EO water.

MATERIALS and METHODS

Materials and Chemicals

Commercial frozen concentrated apple juices were purchased from a grocery store in Griffin, Georgia, and stored at -20^{o}C until used. Alkaline EO water was prepared using a ROX-20TA EO water generator (Hoshizaki Electric Inc., Japan) with the electrolysis of 2mM NaCl or KCl solutions at 14A and 10V setting. Monopotassium phosphate, dipotassium phosphate, potassium chloride and ascorbic acid were purchased from Fisher Scientific (Pittsburgh, PA). To conduct total antioxidant capacity assays, fluorescein (FL) sodium salt, 2, 2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH) and 6-hydroxy-2, 5, 7, 8-tetrame-thylchroman-2-carboxylic acid (Trolox) were obtained from Sigma-Aldrich (Milwaukee, WI). ABTS substrate (Southern Biotech, Birmingham, AL) was used to generate the ABTS radical.

Preparation of reconstituted apple juice

Apple juice from concentrate was reconstituted according to the recipe on the carton, which required 3 parts water to 1 part frozen juice concentrate. Alkaline EO water obtained from the electrolysis of NaCl (EOW-Na) and KCl (EOW-K) solutions, negative control, and positive control were used to reconstitute commercial frozen concentrated apple juice. Deionized water was used as a negative control and deionized water brought to the same pH as the alkaline EO water by addition of NaOH was used as positive control. Each apple juice sample was prepared within 30 minutes of use.

Characteristics of alkaline electrolyzed water

pH and ORP values were determined using a pH/ion/conductivity meter (Accumet model 50, Fisher Scientific Co., Fair Lawn, NJ) with a pH and ORP electrode for alkaline EO water. Mineral contents were analyzed using an inductively coupled plasma-optical emission spectrometer (ICP-OES) and conducted by the Chemical Analysis Laboratory at the University of Georgia, Athens.

Oxygen Radical Observance Capacity- fluorescein assay

The ORAC-FL assay was carried out on the FLUOstar OPTIMA plate reader (BMG Lab Technologies, Durham, NC) equipped with an incubator and two injection pumps. The temperature of the incubator was set at 37^{o}C. Fluorescence filters were set to pass the light with an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The procedure was based on the modified procedures of Prior and others (2003). Reconstituted apple juice was diluted 700 fold in phosphate buffer (pH 7.4) prior to analysis. Briefly, 20 µL of diluted reconstituted apple juices (as antioxidants), 20 µL of 75mM phosphate buffer (as blank; pH 7.4), or 20 µL of Trolox (as standard calibration solution at 6.25, 12.5, 25, and 50µM concentration) were placed in the 96-well microplates, respectively, and the plate was preincubated for 30 min at 37 ^{o}C. Fluorescein solution of 200 µL (as oxidizable substrate; 3.75 µ mole per well concentration) was then added by injectors in the microplate reader, followed by 20 µL of AAPH solution (as peroxyl radical generator; 1.6 µmole per well concentration). The microplate was automatically shaken for 8s following each injection and/or readings and fluorescence readings

were taken continuously at each well every 245s for 154 min. Three independent experiments were performed for each sample.

The raw data were exported from the FLUOstar OPTIMA plate reader to Microsoft Excel (Microsoft, Roselle, IL) and area under the fluorescence decay curve (AUC) was calculated using equation (1).

$$AUC = [0.5 + \sum_{i=4}^{i=35} f_i/f_4] \times CT \quad (1)$$

where f_4 = the initial fluorescence reading at cycle 4, f_i = fluorescence reading at cycle i, and CT = cycle time in seconds (245s).

The net AUC for each sample was calculated by subtracting the AUC of the blank. Standard curve of Trolox (μM) and AUC was used to calculate the ORAC reading of test samples. Final ORAC value of reconstituted apple juices was converted to 700 times the calculated ORAC reading and expressed as micromoles of Trolox equivalents (TE) per liter of reconstituted apple juice (μM TE).

Trolox Equivalent Antioxidant Capacity assay

TEAC assay was conducted using a modified method described by Srivastava and others, (2007). The ABTS radical cation ($ABTS^{\bullet+}$) was generated by reacting 7.4mM ABTS and 2.45 mM potassium persulfate after incubation at room temperature in the dark for 16h. The $ABTS^{\bullet+}$

solution was then diluted with ethanol to obtain an absorbance of 0.70 ± 0.1 at 734 nm on a spectrophotometer (DU®520 Ceneral Purpose UV/Vis Spectrophotometer, Beckman). A 40 µL aliquot of test sample (ethanol as blank, reconstituted apple juice, alkaline EO water, and Trolox as the standard) was mixed with 1960 µL $ABTS^{\bullet+}$ solution. After exactly 6 min, the absorbance was read at 734 nm. Trolox standards were prepared at concentrations ranging from 0 to 2mM for the development of a standard curve. The Trolox equivalent antioxidant capacity of apple juice was calculated based on the Trolox standard curve as Trolox equivalents (TE) per liter of apple juice (µM TE).

Enhanced effect of EOW-Na on antioxidant capacity of ascorbic acid

The enhanced antioxidant capacity of EOW-Na was indirectly determined by comparing the antioxidant capacity of ascorbic acid dissolved in EOW-Na with that in deionized water. This procedure was partly determined according to Lee and others (2006). Ascorbic acid was prepared with phosphate buffer (pH 7.4) based on EOW-Na or deionized water. Antioxidant capacity of different concentrations of ascorbic acid (25µM and 50µM) was measured using ORAC assay and expressed as micromoles of Trolox equivalents (TE) per liter (µM TE).

Stability effect of reconstituted apple juice

To evaluate the stability effect of reconstituted apple juice, concentrated frozen apple juice was reconstituted with EOW-Na, EOW-K, negative control, and positive control. Each reconstituted apple juice was packed in a glass bottle and stored at 4^{o}C for 2 weeks. Stability

effect of reconstituted apple juice was determined using ORAC assay and expressed as micromoles of Trolox equivalents (TE) per liter of reconstituted apple juice (μM TE).

The relationship of Redox Potential, pH, and antioxidant capacity

To evaluate the relationship of ORP and antioxidant capacity, concentrated frozen apple juice was reconstituted with deionized water. Reconstituted apple juice was diluted 600, 800, 1000 fold in EOW-Na or deionized water. From different dilution factors (600, 800, 1000 fold), diluted apple juice with EOW-Na showed different ORP values, but diluted apple juice with deionized water had similar ORP values. The antioxidant capacity of diluted apple juices was measured using ORAC assay. Final ORAC value of reconstituted apple juice was converted to dilution factor times the calculated ORAC reading and expressed as micromoles of Trolox equivalents (TE) per liter of reconstituted apple juice (μM TE).

pH value of alkaline EO water used in the current study was 11.97 ± 0.23 when EOW-Na was produced with electrolysis of 2 mM NaCl at 14 A and 10V setting using a ROX-20TA EO water generator (Table 4.2). To evaluate the relationship of pH (EOW-Na) and antioxidant capacity, concentrated frozen apple juice was reconstituted with EOW-Na, which was adjusted to pH 7, 8, 9, 10, and 11 by adding diluted HCl solutions. Each reconstituted apple juice was diluted 700 fold in phosphate buffer (pH 7.4), and antioxidant capacity was measured using ORAC. Final ORAC value of reconstituted apple juice was converted to 700 times the calculated ORAC reading and expressed as micromoles of Trolox equivalents (TE) per liter of reconstituted apple juice (μM TE).

STATISTICAL ANALYSIS

The data was analyzed by using the statistical analysis system (SAS). Comparison of treatment variations was determined using general linear models (GLM) with Fisher's least significant difference (LSD). A value of $p < .05$ was considered to be statistically significant. Results were expressed as the mean plus/minus standard deviation.

RESULTS AND DISCUSSIONS

Characteristics of alkaline EO water

The results of mineral contents in alkaline EO water are shown in Table 4.1. As shown in Table 4.1, the concentrations of potassium (K) and sodium (Na) were significantly higher than other elements due to the addition of NaCl and KCl solutions during electrolysis. Also, the concentrations of aluminum (Al), iron (Fe), magnesium (Mg), selenium (Se), and silicon (Si) were higher than other elements. Hiraoka and others (2004) reported that alkaline EO water has antioxidant activities in vitro and that such effects were derived from ordinary molecular hydrogen and reductive vanadium (V) ions. In their study, V^{2+} and V^{3+} generally are reductive cations and the antioxidant activities of some alkaline EO water might be derived at least partly from such reductive V ions (Hiraoka and others (2004). In their study, the water with the strongest antioxidant effect had the highest values (18.5μg/L) of vanadium (V), and alkaline EO water used in the current study also had high V values 30μg/L and 20μg/L for EOW-Na and EOW-K, respectively (Table 4.1).

When the diluted salt solutions are electrolyzed, decomposition of a diluted salt solution and water produces chloride, hydroxide, hydrogen, sodium, potassium ions (Huang and others,

2008). Anions migrate to the anode and release electrons, which are moved to the cathode. Hydrogen ions receive electrons from the cathode and change into active atomic hydrogen (H), which is then changed to hydrogen molecules (H_2). The molecular hydrogen released at the cathode is a strong reducer, and ORP value of alkaline EO water generated in the cathode side is determined by dissolving hydrogen in water (Goncharuk and others, 2010).

In the current study, oxidation reduction potential of alkaline EO water shows significantly negative values (EOW-Na = -849 mV and EOW-K = -850 mV) (Table 4.2). The ORP value achieved during electrolysis is determined with the hydrogen molecules release by two reactions ($2H^+ + 2e \rightarrow H_2$; $2H_2O + 2e \rightarrow H_2 + 2OH^-$) and successive dissolution and diffusion of hydrogen molecules over the entire volume of liquid (Goncharuk and others, 2010). Therefore, we could assume alkaline EO water used in the current study contains abundant hydrogen molecules, and it plays an important role as antioxidant rich water. In addition, apple juice reconstituted with EOW-Na showed the least ORP value (ORP value: -67 mV) among reconstituted apple juices indicating the highest antioxidant capacity.

Total antioxidant capacity and its stability during storage

The total antioxidant capacities of reconstituted apple juices and alkaline EO water were determined with the ORAC and TEAC assay. The total antioxidant capacities of EOW-Na were 1.4 μM TE on ORAC and 55.2 μM TE on TEAC, respectively, and were significantly higher than EOW-K (ORAC not detectable and TEAC = 23 μM TE) (Table 4.3). There were no statistically differences between EOW-Na and EOW-K for both ORAC and TEAC readings (Table 4.3). Apple juice reconstituted with EOW-Na had the highest values on ORAC (11000

μM TE) and TEAC (3200 μM TE) of all samples although this is not significantly different than other apple juices. The reconstituted apple juice with EOW-K showed lower ORAC values than that with deionized water (9300 μM TE vs. 9800 μM TE) but had similar TEAC values (2900 μM TE vs. 2800 μM TE). The apple juice reconstituted with positive control, which was adjusted at the same pH as alkaline EO water, showed the least total antioxidant capacity for ORAC (9000 μM TE) and TEAC (2600 μM TE) of others.

As shown in Table 4.3, the total antioxidant capacities of EOW-Na were higher than that of EOW-K. It is assumed that alkaline EO water obtained from electrolysis of NaCl solutions had more hydrogen molecules than that electrolyzed with KCl due to lower electric potential. Hanaoka and others (2004) found when electrolysis was carried out in electrolyte solution as 2 mM NaCl and 2 mM KCl solutions, the different ionic mobility between cations and anions will be proportional to the electric potential. The ratio of Cl^- ions to Na^+ ions showed lower electric potential than that of Cl^- ions to K^+ ions. As a result, it is assumed that Na^+ ions are higher than K^+ and produce more hydrogen in NaCl solutions when electrolysis is carried out in NaCl and KCl solutions, respectively (Hanaoka and others, 2004). This may be why apple juice reconstituted with EOW-Na had higher total antioxidant capacities than apple juice reconstituted with EOW-K although they are not significantly different. In addition, vanadium (V) concentration in EOW-Na was higher than EOW-K (30 μg /L and 20 μg/L, respectively) (Table 4.1). As discussed, in mineral content, vanadium ions may also influence antioxidant capacity of alkaline EO water (Hiraoka and others, 2004).

Apple juice was known as a good antioxidant source and many studies have been conducted to identify why apple juice as an antioxidant-rich beverage also has health benefits. Some researchers have thought the reason comes from polyphenol compounds in apple juice and

studied the correlation between polyphenol compounds and antioxidant activity. They found juices having different polyphenol compounds showed different antioxidant capacities when quantified by the TEAC value. Gliszczynska-Swiglo and Tyrakowska (2003) demonstrated that phenolic compounds of apple juice were major contributors to its TEAC antioxidant activity. Based on the these studies, polyphenol content may play a key role for antioxidant activity of apple juices, and many studies showed the individual polyphenolic constituents in the apple juices (Gliszczynska-Swiglo and Tyrakowska, 2003; Oszmianski and others, 2007; Kahle and others, 2005; Seeram and others, 2008). According to their reports, primary antioxidant compounds in apple juices were polyphenolic acid, flavonoids, and proanthocyanidins (Gliszczynska-Swiglo and Tyrakowska 2003; Oszmianski and others, 2007; Kahle and others, 2005). Because apple juice is produced through filtration and pasteurization, the amount of polyphenol contents in apple juices may vary due to different processing step.

In our study, we used commercially concentrated frozen apple juice, which can be ready to reconstitute and drink at home using either bottled drinking water or tap water. In the current study, we didn't identify the polyphenol contents and individual polyphenolic compounds of the apple juices used. Instead, our results suggest that apple juice reconstituted with EOW-Na, which itself was used as antioxidant drinking water, may alter polyphenol contents of apple juice because ORAC and TEAC values of reconstituted apple juice with EOW-Na was higher than other apple juice samples (Table 4.3), even if this is not significantly different.

Hanaoka (2002) demonstrated that ascorbic acid dissolved in alkaline EO water (NaCl) had higher antioxidant activity than ascorbic acid alone. Lee and others (2006) also reported that enhancement of the antioxidant activity of ascorbic acid dissolved in alkaline EO water (EOW-Na) was about threefold that of ascorbic acid dissolved in deionized water, as measured by

xanthine-xanthine oxidase superoxide scavenging assay system. The results of Hanaoka (2002) and Lee and others (2006) were consistent with our result (Fig. 4.1), which shows ORAC values in ascorbic acid alone and ascorbic acid dissolved in alkaline EO water. In Fig. 4.1, the antioxidant activity of ascorbic acid dissolved in alkaline EO water was compared to that in deionized water by using ORAC assay. The ORAC value of 25μM ascorbic acid dissolved in EOW-Na was five times higher than that dissolved in deionized water (11.45 μM TE vs. 2.15 μM TE). The ORAC value of 50 μM ascorbic acid dissolved in EOW-Na was higher than in deionized water (23.25 μM vs. 13.5 μM). The result indicated that alkaline EO water indirectly enhanced antioxidant activity of ascorbic acid. Vitamin C was already added to the concentrated apple juice used in the current study, and vitamin C and drinking water might be interacted when concentrated apple juice is mixed by drinking water. Based on our result (Fig.4.1) and report of Hanaoka, (2002), we can suggest that apple juice reconstituted with EOW-Na has the highest ORAC value because of the enhancing effect of alkaline EO water on antioxidant capacities.

In subsequent studies, the antioxidant stability of reconstituted apple juices during storage at 4^{o}C was evaluated for 2 weeks and the results are given in Fig. 4.2 and Table 4.4. Fig. 4.2 presents the effect of storage on ORAC reading during storage. The ORAC values of reconstituted apple juices slightly increased until 7 days. Apple juice reconstituted with EOW-Na increased ORAC values with storage time up to 7 days (16 mM TE) and then decreased with further storage (Fig. 4.1). In contrast, apple juice reconstituted with deionized water (negative control) showed the second higher values on ORAC (15 mM TE) at day 7; however, they dramatically decreased after 14 days of storage. Apple juice reconstituted with EOW-K showed less antioxidant capacity on ORAC than apple juice reconstituted with deionized water after 7 days; and then, it maintained up to 14 days of storage. Apple juice reconstituted with positive

control, which was adjusted to the same pH as the EOW by addition of NaOH, had the lowest ORAC values (13 mM TE) after 7 days and remained about the same for up to 14 days (ORAC reading of 13 mM TE) and higher than apple juice reconstituted with deionized water (negative control).

Table 4.4 shows the TEAC values of reconstituted apple juice for 2-week storages. The results had significantly different values according to treatment (p values = 0.0234) and storage time (p values = < 0.0001). TEAC values of apple juice reconstituted with EOW-Na decreased from 3.2 μM TE to 2.2 μM TE after 2 weeks of storage at 4^{o}C. In the case of reconstituted apple juice with EOW-K, the TEAC values showed similar as reconstituted apple juice (EOW-Na) (3.2 μM TE vs. 2.9 μM TE); however, they decreased for 3 days of storage and then maintained until 14 days of storage. TEAC values in apple juice reconstituted with deionized water (negative control) were statistically similar as apple juice reconstituted with EOW-Na. In addition, apple juice reconstituted with positive control showed lower values on TEAC than others immediately after the juice was made; then similar values as apple juice reconstituted with EOW-Na between 7 days and 14 days of storage time.

The result on TEAC in reconstituted apple juices is in good agreement with other studies (Gliszczynska-Swiglo and Tyrakowska, 2003). They demonstrated that the decrease of antioxidant capacity was related to the reduction of phenolic acid and flavonoids as well as total polyphenols after storage (Gliszczynska-Swiglo and Tyrakowska, 2003). Srivastava and others (2007) also suggested that the reduction of antioxidant capacity of blueberry was due to the hydroxylation and glycosylation in flavonoids, but the rate of reduction in antioxidant activity was different from that of TPP (Total polyphenols) and TACY (Total antocyanins). The difference may be due to other compounds such as ascorbic acid which is partly responsible for

the antioxidant activity (Srivastava and others, 2007). Their suggestion may explain why ORAC readings for the current study slightly increased up to 7 days during storage. Fig. 4.1 further demonstrates that alkaline EO water can enhance antioxidant activity of ascorbic acid as indicated by the ORAC readings.

Relationship of ORP, pH, and ORAC value of reconstituted apple juice

Fig 4.3 shows that the ORAC values of diluted apple juice were affected by dilution factors. When reconstituted apple juice was further diluted using EOW-Na, ORAC values (11700 to 18800 μM TE) of reconstituted apple juices increased with increasing dilution factor; however, apple juice diluted with deionized water had the almost same ORAC values (5400 to 6200 μM TE), further suggesting alkaline EO water may increase antioxidant capacity.

Generally, an antioxidant is able to donate a hydrogen atom or promote an electron transfer toward a radical (MacDonald and others, 2006). Nicoli and others (2004) suggested that redox potential may represent an interesting indicator of the antioxidant efficiency of food products because oxidation reduction potential (ORP) measurements are suitable to evaluate the ability of reducing compounds to promote electron transfer. Although they do not give information about the ability of molecules to donate hydrogen atoms (Nicoli and others, 2004), oxidation reduction potential (ORP) values are determined by dissolving hydrogen in alkaline electrolyzed (EO) water, which are involved in the two reactions ($2H^+ + 2e \rightarrow H_2$; $2H_2O + 2e \rightarrow H_2 + 2OH^-$) (Goncharuk and others, 2010). In addition, Okouchi and others (2002) studied water evaluation method based on the relationship between ORP and pH values. They demonstrated that water having high ORP values is in the oxidative state and water having low ORP values is

in the reductive state. Lee and others (2004) developed a new method for antioxidant capacity with ORP – pH system. They demonstrated that the relationships between the ORAC values and the ORP system values have high correlation coefficients, suggesting the possibility of measurement of antioxidant capacity with simplicity and correctness. Thus, ORP values might be related to antioxidant capacity and our results (Figs. 4.3-4.5) are a good example to support their theory based on studies of Nicoli and others (2004), Lee and others (2004), Okouchi and others (2002), and Goncharuk and others (2010).

Fig. 4.4 (alkaline EO water) and Fig. 4.5 (deionized water) represent the ORAC values of reconstituted apple juice plotted against their redox potential values. Apple juice with higher negative redox potential values also had higher antioxidant capacity (ORAC) as indicated in Fig. 4.4. Apple juice with -290 mV ORP value had the highest ORAC values of 18800 μM TE. Apple juice with -220 mV ORP value showed higher values on ORAC than that with -180 mV ORP (ORAC: 14600 μM TE vs. 11700 μM TE), suggesting more negative ORP values might enhance antioxidant capacity. In contrast, Fig. 4.5 shows that reconstituted apple juices diluted with different amounts of deionized water have similar redox potential values (ORP: 178 to 200 mV) and similar ORAC values (ORAC: 5400 μM TE vs. 6200 μM TE).

Figure 4.6 presents the ORAC values of apple juices reconstituted with alkaline EO water having different pH values (pH 7 to 11). To investigate the contribution to antioxidant capacity of alkaline EO water having different pH values (pH 7 to 11), pH of alkaline EO water was adjusted to pH 7, 8, 9, 10, and 11 by adding diluted HCl solutions and was then used to reconstitute mixed concentrated frozen apple juice according to the recipe on the carton. The reconstituted apple juice made with EOW-Na at pH 9 had the highest antioxidant activity (ORAC values: 15770 μM TE). In contrast, apple juices reconstituted with pH 10 or pH 11

alkaline EO water showed lower values on ORAC than others. Koseki and others (2007) reported the taste of alkaline EO water was dependent on pH values, and the taste of alkaline EO water may be dependent on pH values, and the taste of alkaline EO water with a pH 9.5 was preferred to the regular water. In the current study, apple juice reconstituted with pH 9 alkaline EO water indicates possibility of the best antioxidant rich beverage, showing the highest antioxidant capacity values.

CONCLUSION

Alkaline EO water can enhance the total antioxidant capacity of reconstituted apple juice. Also, the antioxidant capacity of apple juice, when reconstituted with alkaline EO water, maintained for 2 weeks at 4^{o}C. Such enhancement on antioxidant activity of reconstituted apple juice may be a result of negative redox potential of alkaline EO water. Results indicate that the use of alkaline EO water as antioxidant water might contribute to scavenge reactive oxygen species.

LITERATURE CITED

Becker E.M.; Nissen, L.R.; Skibsted, L.H. Antioxidant evaluation protocols: Food quality of health effects. European Food Research and Technology, **2004**, 219, 561-571.

Chipault, JR. Antioxidants for food use. In Lunberg WO(ed) Autoxidation and Antioxidants, Wiley, New York, **1962**, 477-542.

Gliszczynska-Swiglo, A.; Tyrakowska, B. Quality of commercial apple juices evaluated on the basis of the polyphenol content and the TEAC antioxidant activity. Journal of Food Science, **2003**, 68, 1844-1849.

Goncharuk, V.V.; Bagrii, V.A.; Mel'nik, L.A.; Chebotareva, R.D.; Bashtan, S.Y. The Use of redox potenial in water treatment processes. Journal of Water Chemistry and Technology, **2010**, 32, 1-9.

Hanaoka, K. Antioxidant effects of reduced water produced by electrolysis of sdium chloride solutions. Journal of Applied Electrochemistry, **2001**, 31,1307-1313.

Hanaoka, K.; Sun, D.; Lawrence, R.; Kamitani, Y.; Fernandes, G. The mechanism of the enhanced antioxidant effects against superoxide anion radicals of reduced water produced by electrolysis. Biophysical Chemistry, **2004**, 107, 71-82.

Hiraoka, A.; Takemoto, M.; Suzuki, T.; Shinohara, A.; Chiba, M.; Shirao, M.; Yoshimura, Y. Studies on the properties and real existence of aqueous solution systems that are assumed to have antioxidant activities by the action of "active hydrogen". Journal of Health Science, **2004**, 50, 454-465.

Huang, Y.R; Hung, Y.C.; Hsu, S.Y.; Huang, Y.W.; Hwang, D.F. Appleication of electrolyzed water in the food industry. Food Control, **2008**, 19, 329-345.

Kahle, K.; Kraus, M.; Rcihling, E. Polyphenol profiles of apple juices. Molecular Nutrition and Food Research, **2005**, 49, 797-806.

Kanakis, C.D.; Tarantilis, P.A.; Polissiou, M.G.; Diamantoglou, S.; Tajmir-Riahi, H.A. An overview of DNA and RNA bindings to antioxidant flavonoids. Cell Biochemistry and Biophysics, **2007**, 49, 29-36.

Koseki, M.; Nakagawa, A.; Tanaka, Y.; Noguchi, H.; Omochi, T. Sensory evaluation of taste of alkali-ion water and bottled mineral water. Journal of Food Science, **2003**, 68, 354-358.

Koseki, M.; Fujiki, S.; Tanaka, Y.; Noguchi, H.; Nishikawa, T. Effect of water hardness on the taste of alakline electrolyzed water. Journal of Food Science, **2005**, 70, S249-S253.

Koseki, M.; Tanaka, Y.; Nogughi, H.; Nishikawa, T. Effect of pH on the taste of alkaline electrolyzed water. Journal of Food Science, **2007**, 72, S298-S302.

Mac Donald-Wicks, L-K; Wood, L.G.; Garg, M.L. Methodology for the determination of biological antioxidant capacity in vitro: a review. Journal of the Science of Food and Agriculture, **2006**, 86, 2046-2056.

Lee, M.Y.; Kim, Y.K.; Ryoo, K.K.; Lee, Y.B.; Park, E.J. Electrolyzed-reduced water protects against oxidative damage to DNA, RNA, and Protein. Applied Biochemistry and Biotechnology, **2006**, 135, 133-144

Monagas, M.; Blanca, H.L.; Ignacio, G.; Pefro J, M.A.; Carmen, G.C; Begona, B. Quality assessment of commercial dietary antioxidant products from vitis vinifera L.Grape seeds. Nutrition and Cancer, **2005**, 53, 244-254.

Nicoli, M.; Toniolo, C.R.; Anese, M. Relationship between redox potential and chain-breaking activity of model systems and foods. Food Chemistry, **2004**, 88, 79-83.

Oszmianski, J.; Woliniak, M.; Wojdylo, A.; Wawer, I. Comparative study of polyphenolic content and antiradical activity of cloudy and clear apple juices. Journal of Science and Food Agricultural, **2007**, 87, 573-579.

Prior, R.L.; Hoang, H.; Gu, L.; Wu, X.; Bacchiocca, M.; Howard, L.; Hampsch-Woodill, M.; Huang, D.; Ou, B.; Jacob, R. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity ($ORAC_{FL}$)) of plasma and other biological and food samples. Journal of Agriculture and Food Chemistry, **2003**, 51, 3273-3279.

Seeram, N.P.; Aviram, M.; Zhang, Y.; Henning, S.M.; Feng, L.; Dreher, M.; Heber, D. Comparison of antioxidant potency of commonly consumed polyphenol-rich beverages in the United States. Journal of Agriculture and Food Chemistry, **2008**, 56, 1415-1422.

Shirahata, S.; Kabayama, S.; Nakano, M.; Miura, T.; Kusumoto, K.; Gotoh, M.; Hayashi, H.; Otsubo, K.; Morisawa, S.; Katakura, Y. Electrolyzed-reduced water scavenges active oxygen species and protects DNA from oxidative damage. Biochemical and Biophsical Research Communications, **1997**, 234, 269-274.

Srivastava, A.; Akoh, C.C.; Yi, W.; Fischer, J.; Krewer, G. Effect of storage conditions on the biological activity of phenolic compounds of blueberry extract packed in glass bottles. Journal of Agriculture and Food Chemistry, **2007**, 55, 2705-2713.

Tsai, C. F.; Hsu, Y.W.; Chen, Y.K.; Ho, Y.C.; Lu, F.J. Enhanced induction of mitochondrial damage and apoptosis in human leukemia HL-60 cells due to electrolyzed-reduced water and glutathione. Bioscience Biotechnology and Biochemistry, **2009**, 73, 280-287.

Wang, S.Y; Jiao, H. Scavenging capacity of berry crops on superoxide radicals, hydrogen peroxide, hydroxyl radicals, and singlet oxygen. Journal of Agriculture and Food Chemistry, **2000**, 48, 5677-5688.

Wu, X.; Beecher, G.R.; Holden, J.M.; Haytowitx, D.B.; Gebhardt, S.E.; Prior, R.L. Lipophilic and hydrophilic antioxidant capacities of common foods in the United States. Journal of Agriculture and Food Chemistry, **2004**, 52, 4026-4037.

Yanagihara, T.; Arai, K.; Miyamae, K.; Sato, B.; Shudo, T.; Yamada, M.; Aoyama, M. Electrolyzed hydrogen-saturated water for drinking use elicits an antioxidative effect: A feeding test with rats. Bioscience Biotechnology and Biocheistry, **2005**, 69, 1985-1987.

Table 4.1. Mineral contents of alkaline electrolyzed water.

	mg/L		
	EOW-Na	EOW-K	Tap Water
Ag	0.012±0.003	0.014±0.001	
Al	0.212±0.001	0.223±0.007	
As	0.061±0.015	0.094±0.007	
B	0.035±0.002	0.044±0.002	
Ba	0.007±0.0003	0.008±0.0002	
Be	0.001±0	0.001±0	
Ca	0.088±0	0.092±0.007	
Cd	0.012±0.002	0.01±0.005	
Co	0.018±0.002	0.022±0	
Cr	0.06±0.005	0.063±0.001	
Cu	0.041±0	0.049±0	310
Fe	0.146±0.04	0.085±0.013	
K	5.72±1	1019±5	
Mg	0.167±0	0.21±0.02	
Mn	0.007±0.0004	0.007±0	
Mo	0.074±0.06	0.033±0.002	
Na	730±9	8.49±3.2	
Ni	0.07±0.02	0.054±0.003	
P	0.24±0.08	0.27±0.03	
Pb	0.12±0.01	0.17±0.18	7.8
Sb	0.16±0.03	0.16±0.01	
Se	0.2±0.03	0.18±0.02	
Si	1.73±0.33	1.19±0.04	
Sr	0.003±0	0.003±0.0001	
V	0.03±0.01	0.02±0.001	
Zn	0.02±0.003	0.02±0	

Data are means ± standard deviation duplicated. EOW-Na is alkaline electrolyzed (EO) water procedure by using 2mM NaCl solution. EOW-K is alkaline electrolyzed (EO) water procedure by using 2mM KCl solution. Tab Water is from Public Works and Utilities Department.

Table 4.2. pH and ORP values of alkaline electrolyzed (EO) water and reconstituted apple juices.

	ORP value (mV)	pH
Negative Control	468 ± 13.9	7.8 ± 0.2
Positive Control	119 ± 0.95	12.04 ± 0.11
EOW-Na	-849 ± 4.5	11.97 ± 0.23
EOW-K	-851 ± 12.1	12.1 ± 0.03
AJ1	-67.73 ± 1.9	3.95 ± 0.04
AJ2	20.63 ± 2.1	4.06 ± 0.08
AJ3	165 ± 5.9	4.15 ± 0.12
AJ4	118 ± 0.9	3.9 ± 0.1

Data are means ± standard deviations of three replicates. Negative control is deionized water. Positive control is deionized water brought to the same pH as the alkaline EO water. EOW-Na is alkaline EO water obtained from the electrolysis of NaCl solution. EOW-K is alkaline EO water obtained from the electrolysis of KCl solution. AJ1 is apple juice reconstituted with EOW-Na. AJ2 is apple juice reconstituted with EOW-K. AJ3 is apple juice reconstituted with positive control. AJ4 is apple juice reconstituted with negative control.

Table 4.3. Total antioxidant capacity of alkaline electrolyzed (EO) water. [1] [2]

	Samples	ORAC value (μM TE)	TEAC value (μM TE)
Water	Negative Control	N.D [a] B	N.D [a] C
	Positive Control	N.D [a] B	N.D [a] C
	EOW-Na	1.4 ± 0.3 A	55.2 ± 11 A
	EOW-K	N.D B a	23 ± 6.11 B a
Apple Juice	AJ-EOW-Na	11000 ± 2900 A	3200 ± 100 A
	AJ-EOW-K	9300 ± 6700 A	2900 ± 200 A B
	AJ-PC	9000 ± 1100 A	2600 ± 300 B
	AJ-NC	9800 ± 1600 A	2800 ± 400 A B

[1]Mean with same capital letter in the same column of each group (water and apple juice) indicates not significantly different at p value < 0.05. [2]N.D[a] is not detected. Data are means ± standard deviations of three replicates. Negative control is deionized water. Positive control is deionized water brought to the same pH as the alkaline EO water. EOW-Na is alkaline EO water obtained from the electrolysis of NaCl solution. EOW-K is alkaline EO water obtained from the electrolysis of KCl solution. AJ-EOW-Na is apple juice reconstituted with EOW-Na. AJ-EOW-K is apple juice reconstituted with EOW-K. AJ-PC is apple juice reconstituted with positive control. AJ-NC is apple juice reconstituted with negative control.

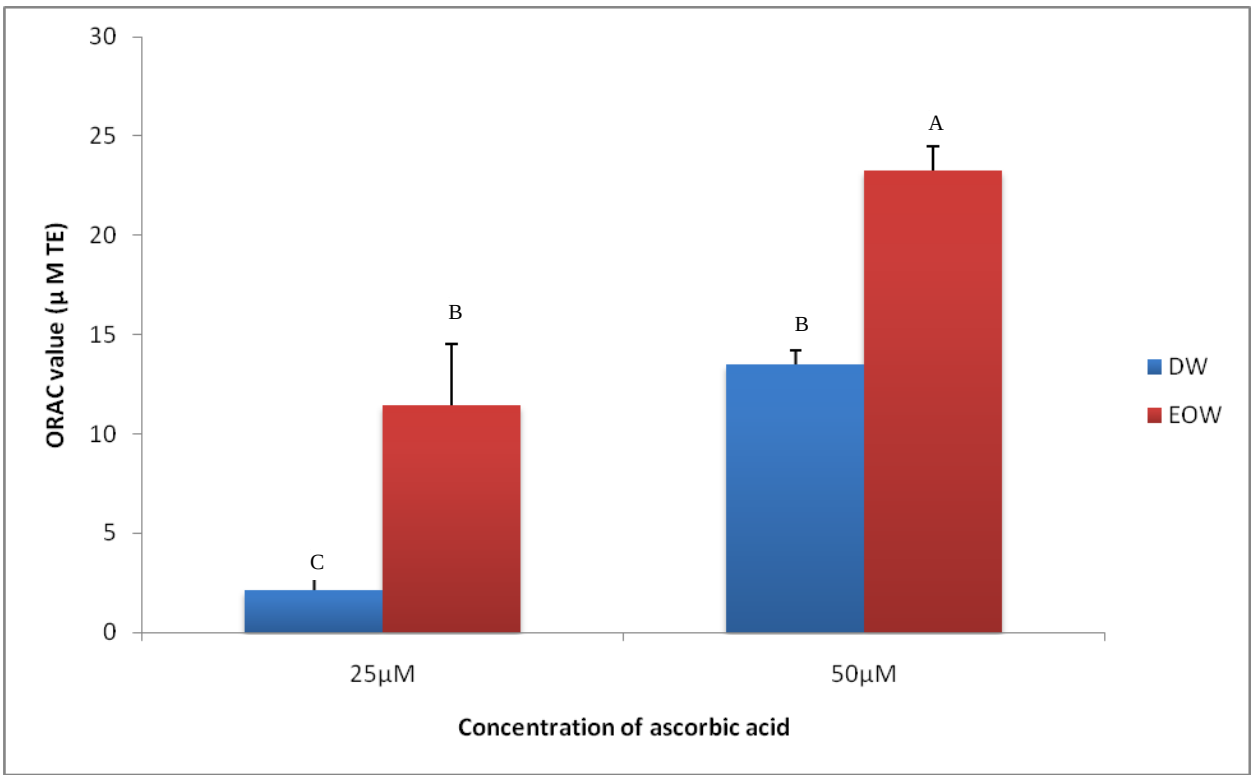

Fig. 4.1. Enhanced effect of alklaine EO water on antioxidant activity of ascorbic acid.

Data are means ± standard deviations of duplicated. DW means that ascorbic acid was dissolved in deionized water. EOW means that ascorbic acid was dissolved in EOW. Means with the same letter are not significantly different at p vale < 0.05. [1]25 μM and 50 μM ascorbic acid were prepared with phosphate buffer (pH 7.4) based on EOW or deionized water. Antioxidant capacity of different concentrations of ascorbic acid (25 μM and 50 μM) was measured using ORAC assay.

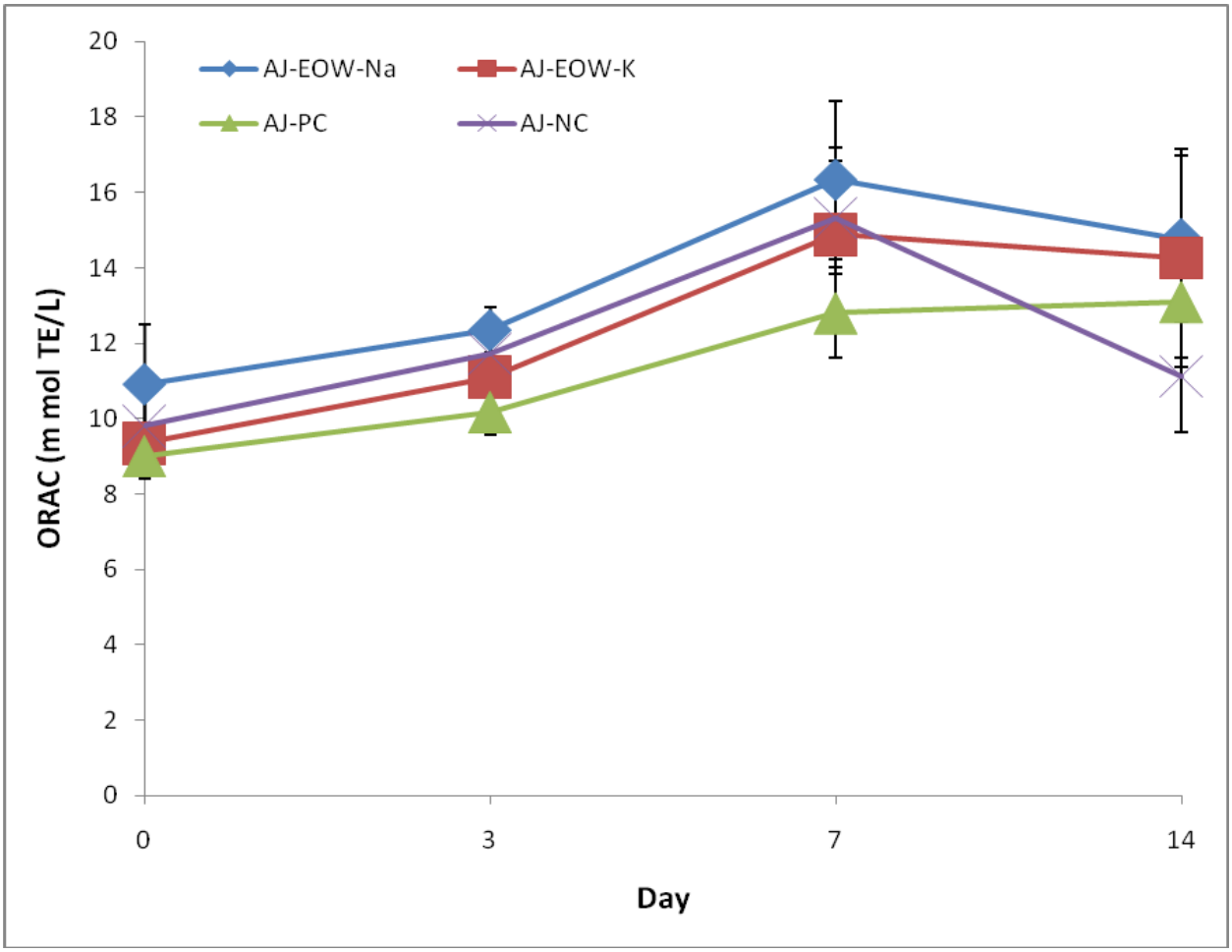

Fig. 4.2. Oxygen Radical Absorbance Capacity of reconstituted apple juice during storage.

Data are means ± standard deviations of three replicates. AJ-EOW-Na is apple juice reconstituted with EOW-Na. AJ-EOW-K is apple juice reconstituted with EOW-K. AJ-PC is apple juice reconstituted with positive control. AJ-NC is apple juice reconstituted with negative control.

Table 4.4. Trolox Equivalent Antioxidant Capacity of reconstituted apple juice during storage.[1]

	TEAC (mM TE)			
	0 day	3 days	7 days	14 days
AJ-EOW-Na	3.2 ± 0.06 A a	2.5 ± 0.05 A b	2.1 ± 0.1 A c	2.2 ± 0.06 A b c
AJ-EOW-K	2.9 ± 0.1 AB a	1.9 ± 0.03 C b	1.7 ± 0.07 B c	1.9 ± 0.09 A b c
AJ-PC	2.6 ± 0.1 B a	2.2 ± 0.11 B a	2.1 ± 0.1 A a	2.1 ± 0.28 A a
AJ-NC	2.8 ± 0.2 AB a	2.6 ± 0.09 A a	2.1 ± 0.15 A a b	1.7 ± 0.4 A a

[1]Mean with same capital letter in the same column and with the same lower-case letter in the same row indicate not significantly different at $p < 0.05$. AJ-EOW-Na is apple juice reconstituted with EOW-Na. AJ-EOW-K is apple juice reconstituted with EOW-K. AJ-PC is apple juice reconstituted with positive control. AJ-NC is apple juice reconstituted with negative control. Data are means ± standard deviations of three replicates.

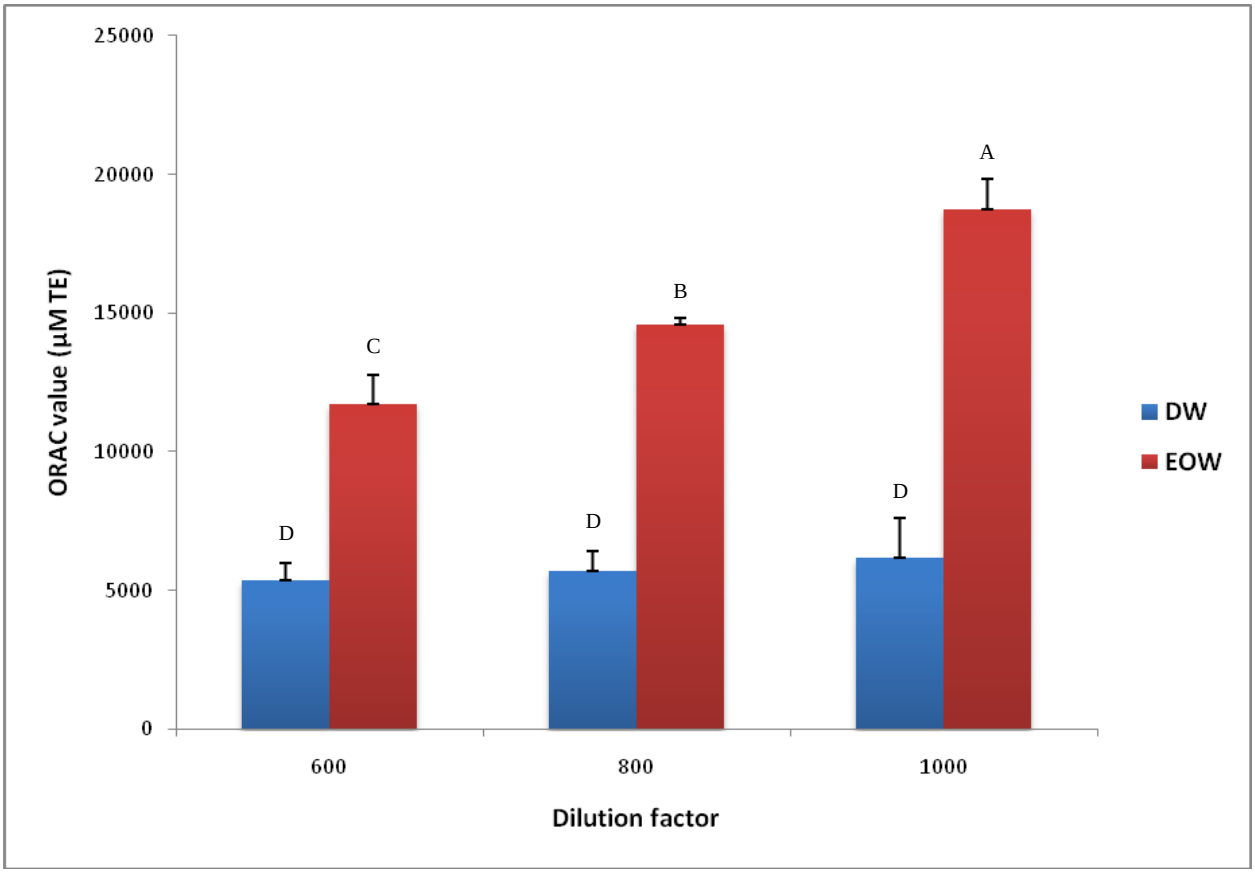

Fig. 4.3. ORAC values of overall total reconstituted apple juice in different dilution.

Data are means ± standard deviations of three replicates. DW means that reconstituted apple juice was diluted with deionized water. EOW means that reconstituted apple juice was diluted with EOW-Na. Means with the same letter are not significantly different at p value < 0.05.

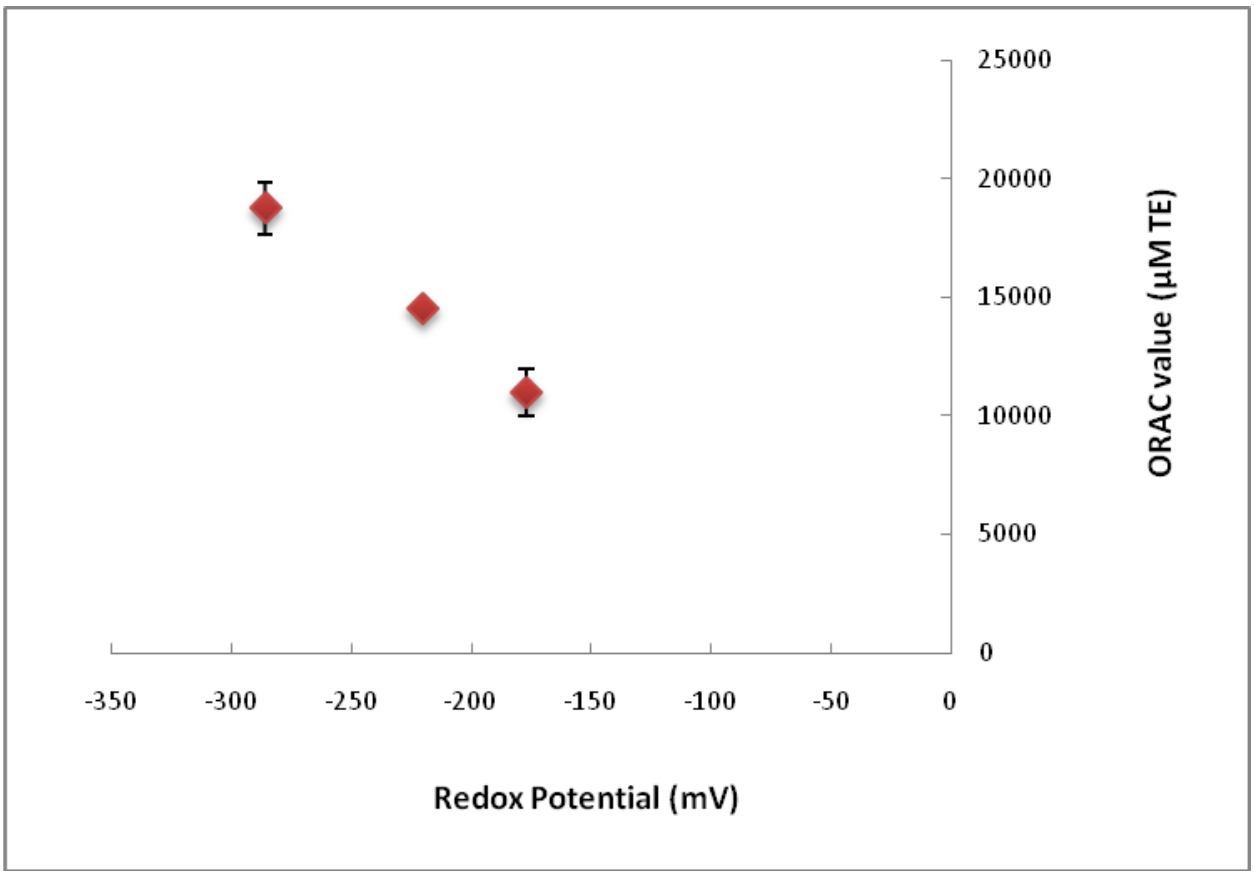

Fig. 4.4. The relationship between Redox Potential and antioxidant capacity of apple juice reconstituted with EOW-Na.

Data are means ± standard deviations of three replicates.

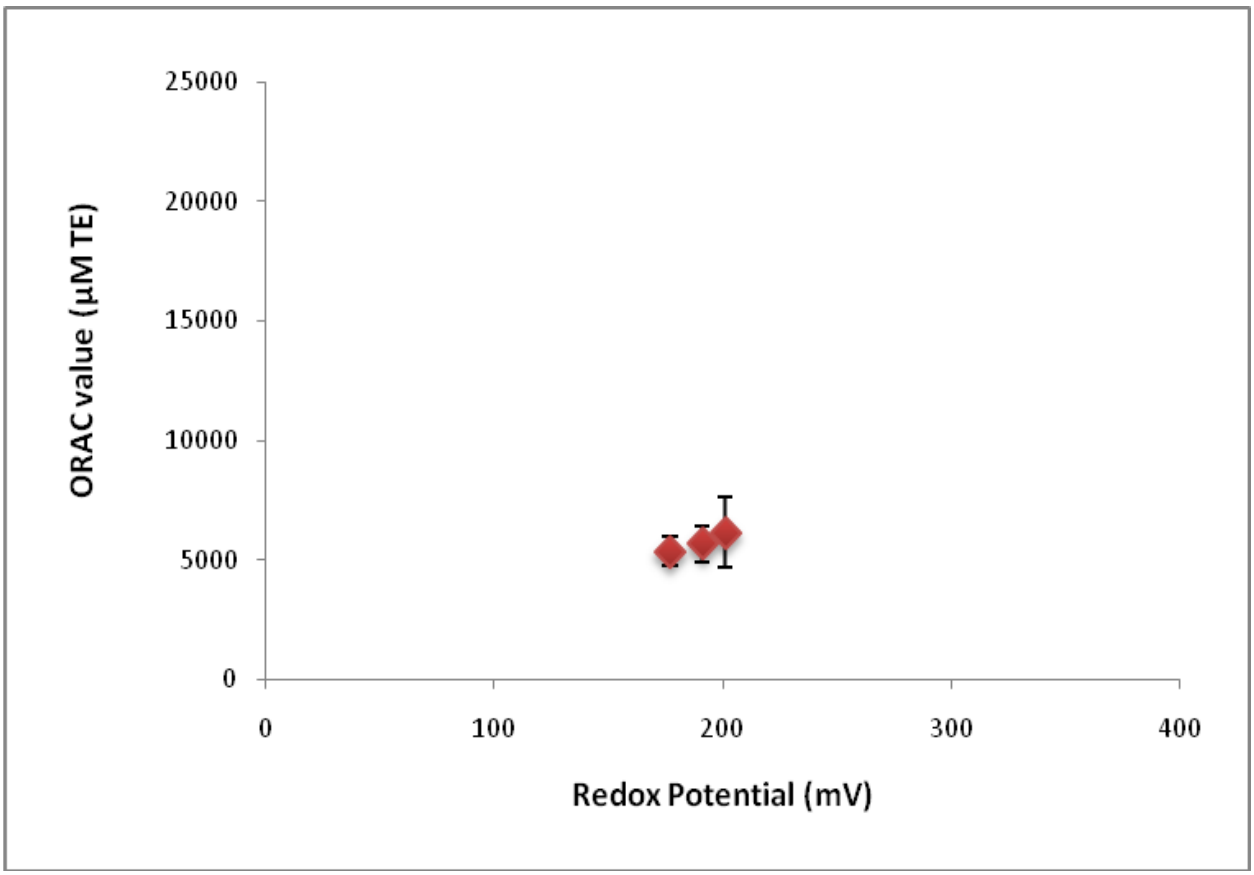

Fig. 4.5. The relationship between Redox Potential and antioxidant capacity of apple juice reconstituted with deionized water.

Data are means ± standard deviations of three replicates.

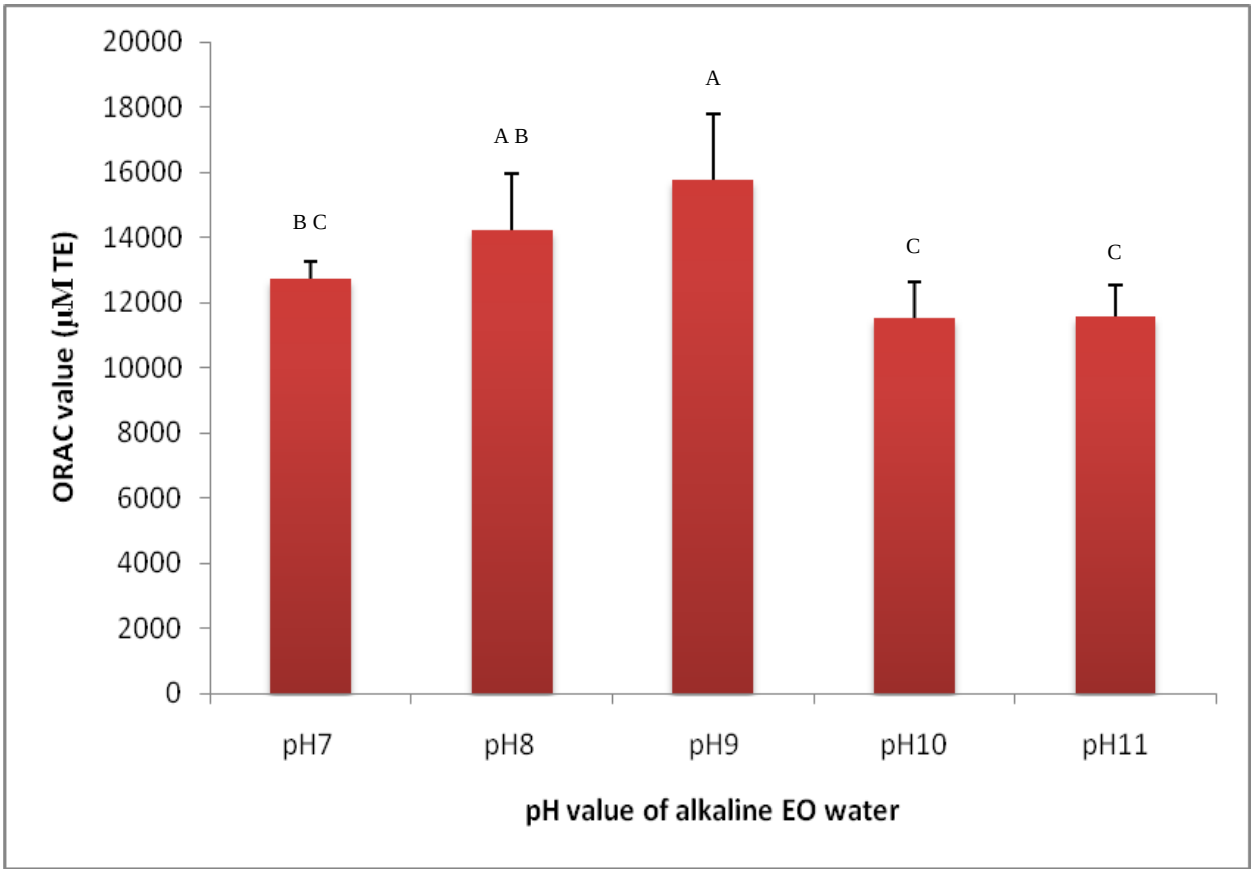

Fig. 4.6. The relationship between antioxidant capacity and apple juice reconstituted with EOW-Na having different pH.

Data are means ± standard deviations of three replicates. pH 7 indicates apple juice reconstituted with EOW-Na having pH 7. pH 8 indicates apple juice reconstituted with EOW-Na having pH 8. pH 9 indicates apple juice reconstituted with EOW-Na having pH 9. pH 10 indicates apple juice reconstituted with EOW-Na having pH 10. pH 11 indicates apple juice reconstituted with EOW-Na having pH 11. Means with the same letter are not significantly different at p value < 0.05.

CHAPTER 5

PREVENTIVE EFFECT ON OXIDATIVE DAMAGE, CELL PROLIFERATION, AND APOPTOSIS BY APPLE JUICE RECONSTITUTED WITH ALKALINE ELECTROLYZED WATER IN HT-29 CELLS

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ABSTRACT

Apple juice, known as a good antioxidant source, may partially protect the body from oxidative stress. When consumed orally, alkaline electrolyzed (EO) water may have antioxidant properties. In this study, we investigated the effect of apple juice reconstituted with alkaline EO water on human colon adenocarcinoma (HT-29) cells. Specifically, we examined the total antioxidant capacity (ORAC and TEAC) of apple juice combined with EO water, and the effects of apple juice mixed with EO water on cell proliferation (MTT assay), DNA fragmentation, and oxidative DNA damage (comet assay) of HT-29 cells.

The TEAC value of apple juice reconstituted with alkaline EO water was higher compared to apple juice reconstituted with ultra pure water (2773μM TE vs. 1793 μM TE). Apple juice reconstituted with alkaline EO water had a higher ORAC value (15446 μM TE) than that with ultra pure water (13908 μM TE) but this was not significant. After 72 h incubation, HT-29 cell proliferation was more effectively reduced by the apple juice reconstituted with alkaline EO water than the apple juice reconstituted with ultra pure water. The HT-29 cells treated with 0.5μL/mL apple juice, which was reconstituted with alkaline EO water, showed the greatest effect (Rel. cell viability (%): 58.57 ± 5.07) on cell proliferation. Induction of apoptosis was determined using a DNA fragmentation assay, and apoptotics increased in a dose-dependent manner after treatment with both reconstituted apple juices. The greatest induction of apoptosis was exhibited in the apple juice reconstituted with alkaline EO water (concentration 0.4 μL/mL). In addition, prevention of oxidative DNA damage by reconstituted apple juice was conducted using the COMET assay. Cells treated with apple juice reconstituted with alkaline EO water had less DNA damage than those treated with apple juice, which was reconstituted with ultra pure

water, suggesting an antioxidant effect. In conclusion, apple juice reconstituted with alkaline EO water had a stronger antioxidant effect than apple juice reconstituted with ultra pure water.

INTRODUCTION

Cancer is defined as a group of diseases characterized by uncontrolled cellular growth and spread of abnormal cells as a result of alterations or damage to the genetic material within cells that accumulate over time (WCRF/AICR, 2008). A balance between cell proliferation (division of cells) and apoptosis (death of cells) is found in healthy tissues. Unlimited cell proliferation and evasion of apoptosis within a cell cycle can lead to the development of cancer; the initial stage is characterized by DNA damage and the need for cellular repair. A prolonged imbalance of cell proliferation and apoptosis may result in tumor development with continued DNA damage (WCRF/AICR. 2008; Hanahan and Weinberg. 2000).

Cancers of the gastrointestinal tract are a very significant health problem in the United States (Macdonald 1999; WCRF/AICR, 2008) and colon cancer is the second leading cause of cancer death in all Western countries (Roncucci, 2000; Koch and others, 2009; Kim and others, 2009). Some epidemiological studies have suggested that risk of gastrointestinal cancers may be reduced with high intakes of fruits and vegetables, which are rich sources of polyphenolic flavonoids (Koch and others, 2009; Barth and others, 2005). Compounds such as flavonoids can induce differentiation, cause apoptosis, and enhance anti-inflammatory responses in colorectal cancer cell lines (Veeriah and others, 2007).

Apples and apple juice are a rich dietary sources of polyphenolic flavonoids (Bellion and others, 2008), and recent studies have focused on the capacity of flavonoids to act as cancer-

preventing compounds. Flavonoids may act as antioxidants, which scavenge free radicals and reduce oxidative stress (Schaefer and others, 2006a; Schaefer and others, 2006b; Bellion and others, 2008; Wolfe and others, 2008); inhibit cell proliferation (Veeriah and others, 2006; Veeriah and others, 2007; Olsson and others, 2004); induce apoptosis (Gosse and others, 2005; Goss and others, 2006; Kern and others, 2007; Fini and others, 2007; Maldonado-Celisa and others, 2008); and prevent DNA damage (Schaefer and others, 2006a; Veeriah and others, 2008; McCann and others, 2007). Some studies have also reported that complex mixtures of phytochemicals in fresh fruits have a greater preventive effect on colon cancer cells than the sum of individual ingredients when tested alone (Veeriah and others, 2007).

Electrolysis of water produces two types of water: alkaline water at the cathode and acid water at the anode (Ye and others, 2008). Recently, alkaline electrolyzed (EO) water has received attention due to its ability to scavenge reactive oxygen species (ROS). Alkaline EO water, which has a high pH, low oxidation-reduction potential, low dissolved oxygen, and high dissolved hydrogen, has been used for drinking water in several Asian countries, including Japan. Manufacturers of household electrical appliances have developed devices which can produce alkaline EO water in the home (Koseki and others, 2007). Moreover, some companies have developed and sold alkaline EO water commercially, advertising its health properties (Hiraoka and others, 2004).

Researchers studying alkaline EO water have sought to determine its health benefits. Hanaoka and others (2001 and 2004) found that alkaline EO water enhanced the antioxidant effects of ascorbic acid using measurement of superoxide dismutation activity due to changes in the ionic content of water during EO water production. Further, Hiraoka and others (2004) suggested that hydrogen gas (molecular hydrogen), which is generated by the electrolysis of

water, is the effective component responsible for the antioxidant activities of alkaline EO water. In addition, Shirahata and others (1999) reported that alkaline EO water can scavenge reactive oxygen species (ROS), such as superoxide anions, and also provide a protective effect against oxidative damage to DNA. Based on these results, Yanagihara and others (2005) tested the effect of alkaline EO water using the oxidative stress biomarkers 8-hydroxyldeoxyguanosine (8-OHdG) and thiobarbituric acid reactive substances (TBARS) to investigate its antioxidative effect when orally ingested by rats. They reported that the use of alkaline EO water lessened the DNA damage on 8-OHdG markers and inhibited lipid peroxidation in liver of rats on TBARS test. Other studies have investigated the effects of alkaline EO water on alloxan-induced pancreatic β-cell damage (Li and others, 2002); end-stage renal disease (Nishikawa and others, 2005); mitochondrial damage and apoptosis in human leukemia HL-60 cells (Tsai and others, 2009); and tumor angiogenesis (Ye and others, 2008).

The aim of the present research was to study the effects of alkaline EO water on cell proliferation, DNA fragmentation and oxidative damage in intestinal cells when EO water is used to reconstitute apple juice from concentrate. HT-29 cells, a human colorectal cancer cell was chosen for the studies as a representative intestinal cancer cell line and because previous studies with apple juice have been conducted in colorectal cancer cell lines (Veeriah and others, 2006; Schaefer and others, 2006a; Schaefer and others, 2006b).

MATERIALS AND MEHTODS

Materials and Chemicals

Commercial frozen concentrated apple juice (Kroger, Cincinnati, Ohio) was purchased from a grocery store in Athens, Georgia, and stored at -20°C until used. Alkaline EO water was prepared by electrolyzing tap water using a HOX-40A EO water generator (Hoshizaki Electric Inc.) at a high pH setting. The MTT cell proliferation assay kits, HT-29 colon adenocarcinoma cells, and fetal bovine serum (FBS) were obtained from America Type Culture Collection (ATCC: Manassas, VA). The cell death detection ELISA ^{plus} kit (Boehringer Mannheim, Roche) was purchased from Roche (Indianapolis, IN). The comet assay kit, silver staining kit, and phosphate-buffered saline (PBS) were purchased from Trevigen ® (Gaithersburg, MD). McCoy's 5a medium, trypsin-EDTA, and sodium bicarbonate were purchased from Sigma-Aldrich (Milwaukee, WI). Monopotassium phosphate and dipotassium phosphate were purchased from Fisher Scientific (Pittsburgh, PA). To conduct total antioxidant capacity assays, fluorescein (FL) disodium salt, 2, 2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH) and 6-hydroxy-2, 5, 7, 8-tetrame-thylchroman-2-carboxylic acid (Trolox) were obtained from Sigma-Aldrich (Milwaukee, WI). ABTS substrate (Southern Biotech, Birmingham, AL) was used to generate the ABTS radical.

Characteristics of alkaline electrolyzed (EO) water

pH and ORP values were determined using a pH/ion/conductivity meter (Accumet model 50, Fischer Scientific Co., Fair Lawn, NJ) with a pH and ORP electrode for alkaline EO water.

Mineral content of the EO water was analyzed using an inductively coupled plasma-optical emission spectrometer (ICP-OES) and conducted by the Chemical Analysis Laboratory at the University of Georgia, Athens.

Cell culture and preparation of medium

McCoy's 5a medium was prepared with ultra pure water. In order to study the effects of alkaline EO water on HT-29 cells, media was also prepared using alkaline EO water (pH 10.59) instead of ultra pure water. The pH of media prepared with alkaline EO water was adjusted to the same pH as regular media by adding diluted HCl. These media were filtered through a 0.22 μm filter (Nalgene, Rochester, NY) before use. The HT-29 cells were cultivated in 75 cm^2 flasks with McCoy's 5a medium containing 10% fetal bovine serum in an incubator with 5% CO_2 at 37 ^oC. Fresh media was replaced every 2-3 days.

Reconstitution of apple juice and HT-29 cell treatment

Apple juice from concentrate was reconstituted using either alkaline EO water or ultra pure water with 3 parts water to 1 part frozen juice concentrate. Each apple juice sample was prepared within 30 minutes of use. Juice samples were diluted to 10μL/mL using phosphate buffered saline (PBS) and then further diluted to final concentrations (0.1, 0.2, 0.3, 0.4, 0.5 μL/mL) with McCoy's 5a medium. Apple juices of each final concentration had same pH as PBS. Cells were seeded at 1 x 10^4 per well for the MTT assay and 1 x 10^5 per well for the DNA fragmentation or comet assays with regular McCoy's 5a medium. Regular media was then

replaced with media containing different concentrations of apple juice reconstituted with alkaline EO water or ultra pure water (0.1 to 0.5µL/mL), or media prepared using an equivalent amount of alkaline EO water instead of ultra pure water for each assay.

ORAC (Oxygen Radical Observance Capacity)-fluorescein assay

The ORAC-FL assay was carried out on the FLUOstar OPTIMA plate reader (BMG Lab Technologies, Duraham, NC) equipped with an incubator and two injection pumps. The temperature of the incubator was set at 37^{o}C. Fluorescence filters were set to pass the light with an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The procedure was based on the modified procedures of Prior and others (2003). Reconstituted apple juice was diluted 700 fold in phosphate buffer (pH 7.4) prior to analysis. Briefly, 20 µL of diluted reconstituted apple juices, 20 µL of 75mM phosphate buffer (as blank; pH 7.4), or 20 µL of Trolox (as standard calibration solution; 6.25, 12.5, 25, and 50µM concentration) were placed in the 96-well microplates, respectively, and the plate was preincubated for 30 min at 37 ^{o}C. Fluorescein solution of 200µL (as oxidizable substrate; 3.75 µ mole per well concentration) was then added by injectors in the microplate reader, followed by 20 µL of AAPH solution (as peroxyl radical generator; 1.6 µmole per well concentration). The microplate was automatically shaken for 8s following each injection and/ or readings and fluorescence readings were taken continuously at each well every 245 s for 154 min. Three independent experiments were performed for each sample.

The raw data were exported from the FLUOstar OPTIMA plate reader to Microsoft Excel (Microsoft, Roselle, IL) and area under the fluorescence decay curve (AUC) was calculated using equation (1).

$$AUC = [0.5 + \sum_{i=4}^{i=35} f_i/f_4] \times CT \quad (1)$$

where f_4 is the initial fluorescence reading at cycle 4, f_i = fluorescence reading at cycle i, and CT = cycle time in seconds (245 s).

The net AUC for each sample was calculated by subtracting the AUC of the blank. Standard curve of Trolox (μM) and AUC was used to calculate the ORAC reading of test samples as micromoles of Trolox equivalents (TE) per liter of reconstituted apple juice (uM TE).

TEAC (Trolox Equivalent Antioxidant Capacity) assay

TEAC assay was conducted using a modified method described by Srivastava and others (2007). The ABTS radical cation ($ABTS^{\bullet+}$) was generated by reacting 7.4 mM ABTS and 2.45 mM potassium persulfate after incubation at room temperature in the dark for 16h. The $ABTS^{\bullet+}$ solution was then diluted with ethanol to obtain an absorbance of 0.70 ± 0.1 at 734 nm on a spectrophotometer (DU®520 Ceneral Purpose UV/Vis Spectrophotometer, Beckman). A 40 μL aliquot of test sample (ethanol as blank, reconstituted apple juice, alkaline EO water, and Trolox as the standard) was mixed with 1960 μL $ABTS^{\bullet+}$ solution. After exactly 6 minutes, the absorbance was read at 734 nm. Trolox standards were prepared at concentration ranging from 0 to 2mM for the development of a standard curve. The Trolox equivalent antioxidant capacity of

apple juice was calculated based on the Trolox standard curve as Trolox equivalents (TE) per liter of apple juice (μM TE).

Measurement of cell viability

The MTT reduction assay (n=3 per treatment group) was used to measure cell proliferation using the MTT assay kit (ATCC: Manassas, VA) according to the modified procedures of Yi and others (2005). Uniform amounts (100 μL) of HT-29 cells in McCoy 5a medium were plated at a density of 1.0×10^4 cells per well onto 96 well plates and incubated for 24 h at 37 °C in 5% CO_2. The medium was then replaced with 100 μL of McCoy's 5a medium containing apple juice reconstituted with alkaline EO water or ultra pure water (concentration of 0.1, 0.2, 0.3, 0.4, or 0.5 μL/mL), or media prepared with alkaline EO water and then incubated for 72h at 37 °C in 5% CO_2. The cells were then incubated with 10 μL MTT regents for additional 4 h at 37 °C and then the reaction was stopped by adding 100 μL detergent regents. The amount of MTT formazan product was determined by measuring absorbance using a microplate reader (Bio-Rad Model 680 Microplate Reader, Hercules, CA) at a test wavelength of 595 nm and a reference wavelength of 655 nm. The results of the MTT assay were expressed as relative cell viability using equation (2).

$$\text{Rel. Cell viability \%} = [(\text{treatment} - \text{blank}) / (\text{control} - \text{blank})] \times 100 \quad (2)$$

where the blank was McCoy's 5a medium only (no cells and no apple juice), control cells were HT-29 cells in McCoy's 5a medium (no apple juice), and treatment was HT-29 cells in McCoy's

5a medium with various amount of apple juice reconstituted with alkaline EO water or ultra pure water, or media prepared with alkaline EO water.

Measurement of DNA fragmentation

DNA fragmentation (n=3 per treatment group) was measured using a Cell Death detection ELISA ^{plus} kit (Boehringer Mannheim, Roche) according to the modified procedure of Yi and others (2005) and Kern and others (2007). A uniform amount of cells (1mL) were plated at a density of $1.5x10^5$/mL in twelve-well plates and incubated for 24 h at 37°C in 5% CO_2. The medium was then replaced with 1mL McCoy's 5a medium containing apple juice samples (0.1, 0.2, 0.3, 0.4, and 0.5 μL/mL) and incubated for 72 h at 37°C in 5% CO_2. HT-29 cells were then treated with 100 μM H_2O_2 for 1 h to induce oxidative stress and incubated with fresh medium for 24 h. After incubation, HT-29 cells were harvested and 1.5 x 10^4 HT 29 cells were centrifuged at 200 x *g* for 10 min. Twenty μL of supernatant (cytosolic fraction) was used as an antigen source in the sandwich enzyme-linked immunosorbant assay (ELISA) with biotin-labeled primary anti-histone antibody linked to a streptabidin-coated plate and a secondary anti-DNA antibody conjugated with a peroxidase. The DNA fragmentation was measured using a microplate reader (ELx 800^{TM}, Bio-Tek) at a test wavelength of 405 nm and a reference wavelength of 490 nm. Each absorbance reading was calculated using the equation:

Induction of apoptosis = (treatment – blank)/ (control-blank) (3)

where the treatment was HT 29 cells in McCoy's 5a medium with reconstituted apple juice, the blank was McCoy's 5a medium only, and the control was HT 29 cells in regular McCoy's 5a medium (no reconstituted apple juice). Results were recalculated as the relative induction apoptosis of cells treated with H_2O_2.

Alkaline single cell gel electrophoresis (Comet assay)

Alkaline single-cell gel electrophoresis (n=3 per treatment group) was performed according to the modified procedure of Pisha and others (2001), Schaefer and others (2006), and Waston and others (2009). 1.5 x 10^5 cells were placed in each well of twelve well plates and incubated for 24 h at 37°C in 5% CO_2. After incubation, the medium was replaced with 1mL McCoy's 5a medium containing apple juice samples reconstituted with alkaline EO water or ultra pure water (0.1, 0.2, 0.3, 0.4, and 0.5 µL/mL), or media prepared with alkaline EO water, and then incubated for 24 h at 37°C in 5% CO_2. After treatment, the comet assay was conducted using the comet assay kit (Trevigen®, Gaithersburg, MD). Briefly, 1x10^6 HT 29 cells were centrifuged (5 min at 1250 x *g*) and 20 µL of the pellet was mixed with 200 µL of low melting agarouse. This mixture (80 µL) was placed onto the comet slide, solidified at 4°C, and treated with 100 µL of 75 µM H_2O_2 for 5 min on ice to induce oxidative stress. The slices were placed in ice-cold lysis buffer for 4 h and then incubated in electrophoresis buffer (0.3 M NaOH, 1mM EDTA) for 30 min at 4°C. Electrophoresis was carried out at 25 V and 295 mA for 30 minutes. After electrophoresis, the slides were placed in 70% ethanol for 5 min, then dried, and stained using a silver staining kit (Trevigen®, Gaithersburg, MD). The DNA of each cell migrated according to the amount of DNA damage resulting in a head (original location of DNA) and tail

(migration of damaged DNA). The slides were then photographed and analyzed using the CometSore software (TriTek, Sumerduck, VA), at least 50 -100 cells per slide. DNA damage was calculated as mean % DNA in the tail, which corresponds to percentage of DNA damage:

% DNA in the tail = I_T/I_C x 100

,where I_T means the sum of pixel intensity values in the Tail and I_C means the sum of pixel intensity values in the Comet when images were analyzed.

STATISTICAL ANALYSIS

Results are expressed as the mean plus/minus standard error. The difference between the control and each experimental test condition was determined using general linear models (GLM), with Fisher's least significant difference (LSD). A value of $p < 0.05$ was considered to be statistically significant. Statistical analysis was conducted using the statistical analysis system (SAS).

RESULTS AND DISCUSSION

Characteristics of alkaline EO water and total antioxidant capacity

Alkaline EO water used in this study had the following physical properties: pH 10.59 and oxidation reduction potential (ORP) of -139.2 mV. As shown in Table 5.1, the concentrations of silicon (Si), sodium (Na), potassium (K), calcium (Ca), and magnesium (Mg) were significantly higher than other elements. Alkaline EO water contains various minerals compare to ultra pure water, which contains no minerals. When media is made with alkaline EO water instead of ultra

pure water, the media will contain additional minerals. In addition, iron (Fe) and copper (Cu) concentration were 0.04 ± 0.01mg/L and 0.07 ± 0 mg/L in the alkaline EO water, respectively. Hiraoka and others (2004) reported that the presence of Fe or Cu may act as a weak prooxidants at concentrations of 0.04 mg/L (Fe) and 0.09 mg/L (Cu) in Tap water based on the Fenton reaction. Alkaline EO water used in current study is electrolyzed Tap water and the amount of iron and copper was similar as results of Hiraoka and others (2004). These mineral contents of alkaline EO water, thus, should be considered in results on related to oxidative damage because Fe and Cu can act as prooxidants based on the Fenton reaction in cell growth.

The total antioxidant capacities of reconstituted apple juice and alkaline EO water are shown in Table 5.2. Results show that the total antioxidant capacity of alkaline EO water using the ORAC assay was 37.38 ± 9.32 μM TE, but was not detectable using the TEAC assay. The ORAC assay, which is based on the transfer of hydrogen atoms, measures scavenging activity of the peroxyl radicals formed by heating AAPH (Huang and others, 2005). In contrast, the TEAC assay, which is based on the transfer of electron, measures changed color from colored ABTS radical to colorless ABTS by antioxidant (Huang and others, 2005). The total antioxidant capacity of alkaline EO water just showed the effects on the ORAC assay. Alkaline EO water can transfer a hydrogen atom to peroxyl radical because alkaline EO water contains molecular hydrogen.

Alkaline EO water can scavenge ROS (reactive oxygen species) and protect DNA from oxidative damage (Shirahata and others, 1997). It has been theorized that active atomic hydrogen in alkaline EO water can contribute to ROS scavenging activity and may also be engaged in the redox regulation of cellular function (Shirahata and others, 1997). Alkaline EO water contains abundant molecular hydrogen, which can be converted to atomic hydrogen by

hydrogenases (Happe and others, 1997). In contrast, Huang and others (2003) examined antioxidant activity of alkaline EO water and found no antioxidant effect of alkaline EO water. They suggested this was due to the direct adsorption of ROS by active atomic hydrogen. Hiraoka and others (2004) demonstrated that the most important components for antioxidant activity in alkaline EO water are molecular hydrogen and the presence of reductive vanadium (V). Water having the strongest antioxidant effect among the water tested in their study had the highest values (18.5μg/L) of vanadium (V); alkaline EO water used in our study had similar values (20μg/L) (Table 5.1).

The TEAC reading of apple juice reconstituted with alkaline EO water was significantly higher than that of apple juice reconstituted with ultra pure water (2773 μM TE vs. 1793 μM TE) ($p < .0001$). In contrast, apple juice reconstituted with alkaline EO water was only slightly higher than apple juice reconstituted with ultra pure water for ORAC readings (15446 vs. 13980 μM TE) but they are not significantly different ($p = 0.67$). The results demonstrate that alkaline EO water has low antioxidant capacity compared to apple juice but may enhance the antioxidant properties of apple juice on TEAC assay. Alkaline EO water or ultra pure water will alter polyphenol contents of concentrated apple juice when was reconstituted with them. From our results, we may assume that alkaline EO water alters less polyphenol contents than ultra pure water. In addition, the results of this study showing an enhancement of the TEAC value of apple juice could be supported by the findings of Hanaoka and others (2004) and Hanaoka (2001). They demonstrated that the higher dissociation activity of alkaline EO water might increase the dissociation activity of antioxidant substances such as vitamin C with relatively lower dissociation activity and hence enhance their antioxidant capacity (Hanaoka and others, 2004; Hanaoka, 2001). Their reporting showed that ascorbic acid dissolved in alkaline EO water had

higher dismutation activity for superoxide anion radicals than it dissolved in pure water (Hanaoka, 2001; Hanaoka and others, 2004).

Cytotoxicity of reconstituted apple juice and alkaline EO water on HT-29 cells

The effects of reconstituted apple juice and alkaline EO water on the cell proliferation of HT-29 cells are shown in Table 5.3, Figs. 5.1. When cells were treated with a medium made from a combination water mixture of ultra pure water and alkaline EO water, a significant dose-dependent relationship was shown with cell viability decreasing with increasing concentrations of alkaline EO water. Relative cell viability when treated with a medium made with alkaline EO waters and ultra pure water at ratio of 5:5, 4:6, 3:7, 2:8, and 1:9 for 72 h was 80 %, 83 %, 85 %, 88 %, and 92 % of control (100%), respectively (Table 5.3). Fig. 5.1 shows the cell viability when cells were treated with different concentrations of reconstituted apple juice. Cell proliferation decreased with increasing concentrations of apple juice in medium, however the concentration effect was not significant (p value = 0.1282). Medium containing apple juice reconstituted with alkaline EO water resulted in lower cell viability than medium made with apple juice reconstituted with ultra pure water (p value = 0.0005). The HT-29 cells treated with 0.5 µL/mL apple juice reconstituted with alkaline EO water had the greatest reduction (Cell viability %: 58.57 ± 5.07) on cell population.

Results from the current studies (Table 5.3 and Fig. 5.1) are in line with other studies showing polyphenol-rich apple juice extract led to the inhibition of cell growth of HT-29 cells (Kern and others, 2005).

Cell cycle control in cancer cells plays a key role in cell proliferation (Sun and Liu, 2008). In the previous studies, apple has been shown to have effective antioxidant activity and antiproliferation effects upon cancer cells in vitro and in vivo (Wolfe and others, 2003; Liu and others, 2009; Barth and others, 2007; Schaefer and others, 2006; Veeriah and others, 2006). Olsson and others (2004) also investigated the association between antioxidant compounds from fruits and berries and cell proliferation, demonstrating that cancer cell proliferation was inhibited by increased concentration of antioxidant compounds including vitamin C. Sun and Liu (2008) reported that the antiproliferative activity of apple extracts in human breast cancer cells could result from induced G1 arrest in the cell cycle. These studies suggest that although fruit extracts can act as antioxidants, they also may alter other cell functions to inhibit cell proliferation, leading to cell cycle arrest (Wolfe and others, 2003; Liu and others, 2009; Olsson and others, 2004).

Tsai and others (2009) found that alkaline EO water or GSH inhibited cell proliferation of HL-60 cells in a dose- and time-dependent manner. In addition, cell viability was significantly lower when GSH and alkaline EO water were combined than treated with GSH alone (Tsai and others, 2009). Our study similarly showed alkaline EO water enhanced the inhibition of cell proliferation of HT-29 cells when alkaline EO water was used to reconstitute concentrated frozen apple juice.

Apoptosis

DNA fragmentation in HT-29 cells treated with reconstituted apple juice shows apoptosis was dependent on whether apple juice was reconstituted with alkaline EO water ($p<0.0001$) and

apple juice concentration ($p<0.0001$) (Fig. 5.2). Medium containing apple juice at all concentration showed higher induction of apoptosis than control (treated with H_2O_2 alone and no apple juices). In addition, DNA fragmentation increased with increasing concentration of apple juices reconstituted with both alkaline EO water and ultra pure water up to a concentration of 0.4 μL/mL.

Apple juice mixed with ultra pure or EO water showed 50% greater induction of apoptosis than control at an apple juice concentration 0.1μL/mL. Apple juice reconstituted with alkaline EO water had higher DNA fragmentation than apple juice reconstituted with ultra pure water only at 0.4 μL/mL (p value = 0.0006). Apple juice with alkaline EO water showed the greatest induction of apoptosis at 0.4 μL/mL (Fig. 5.2). Also, at a concentration of 0.5μl/mL, apoptosis, which occurred in apple juice reconstituted with ultra pure water, was higher than that with alkaline EO water. Kern and others (2007) also reported that fragmented DNA of HT-29 cells was increased at sudden point (high concentration of apple extract) and then decreased with higher concentration of apple extract for prolonged treatment time. Prolonged incubation time may induce more apoptotic cells but also induced necrotic cells. Thus, after concentration 0.4 μL/mL, apoptosis may be decreased due to increased necrotic cells because cell death was increased even if apoptotic cells are decreased (Fig. 5.1 and 5.2).

The ability of tumor cell populations to expand in number is determined not only by the rate of cell proliferation but also by the rate of apoptosis (Hanahan and Weinberg, 2000), which is a kind of physiologically programmed 'cell-suicide' (Okada and Tak, 2004). Deregulated cell proliferation and suppressed cell death together, therefore, provide the underlying platform for neoplastic progression (Evan and Karen, 2001). Our finding on the effect of apple juice on apoptosis induction is in agreement with several previous studies. In an evaluation of the

apoptotic effect of alkaline EO water, Tsai and others (2009) indicated that a treatment of GSH combined with alkaline EO water significantly induced DNA fragmentation of HL-60 cells. They also found that the manifestation of pro-caspase-3 was significantly decreased after a treatment of the GSH and alkaline EO water combination (Tsai and others 2009). Activation of caspase-3, a cysteine-dependent aspartase, is a characteristic early event of the apoptotic process, whereas DNA fragmentation is linked to a late apoptotic stage (Kern and others, 2007). As shown in study by Kern and others (2007), DNA fragmentation was induced at a high concentration of apple extract; the concentrations resulting in significant induction of DNA fragmentation were higher than those found with the caspase-3 assay, which might be due to the fact that DNA fragmentation represents a late apoptotic event (Kern and others, 2007).

As suggested by Tsak and others (2009) and Kern and others (2007), both alkaline EO water and apple juice have shown the induction effect of apoptosis. Current studies found apple juice reconstituted with alkaline EO water shown an enhancement of apoptosis induction on HT29 cells only at one concentration. The induction of apoptosis in HT-29 cells treated with apple juice, which was reconstituted with alkaline EO water, could result from several potential mechanisms. Apoptosis is stimulated by the intrinsic and extrinsic pathways (Ashkenazi, 2002). The extrinsic pathway triggers apoptosis independently of the p53 protein (Okada and Mak, 2004) and by death ligands of the cell surface death receptors (Fulda and Debatin, 2006). On the other hand, the intrinsic pathway is activated by various extracellular and intracellular stresses including oxidative stress (Okada and Mak, 2004). Liu and others (2009) reported that induction of apoptosis by apple extract in mammary cancer may regulate through the down-regulation of Bcl-2 expression as well as the up-regulation of Bax expression. Death receptors can activate the cell intrinsic pathway by caspases-8-mediated cleavage and BID, which interacts with the Bcl2

and then increases Bax protein, triggering apoptosis induction through the cell-extrinsic pathway (Ashkenazi, 2002). As shown the oxidative DNA damage (Fig. 5.4), reconstituted apple juice was functioned as an antioxidant, thus, reconstituted apple juice treated in current study may induce apoptotic cells through extrinsic pathway.

Oxidative DNA damage of reconstituted apple juice and alkaline EO water

The effects of reconstituted apple juice and alkaline EO water on H_2O_2-induced oxidative DNA damage in HT-29 cells are shown in Fig. 5.3 and Fig. 5.4. Cells treated with alkaline EO water showed a significant dose-dependent decrease in DNA damage as alkaline EO water increased in the medium (Fig. 5.3). Treatment with alkaline EO water decreased DNA damage below that of the positive control, suggesting an anti-oxidant effect to prevent DNA damage. As shown Fig. 5.3B, comet shape with increase of alkaline EO water was unbroken compared to positive control.

Fig. 5.4 shows the effect of reconstituted apple juice on DNA damage in HT-29 cells. A significant decrease in H_2O_2- induced DNA damage was observed in apple juice reconstituted with alkaline EO water (Fig. 5.4). DNA damage was reduced by 47% when HT-29 cells were pre-incubated with apple juice at concentration of 0.3 to 0.5 μL/mL (Fig. 5.4). Throughout the concentration range (0.1 to 0.5 μL/mL), treatment with apple juice reconstituted with alkaline EO water reduced DNA damage below that of the positive control suggesting an anti-oxidant effect on DNA damage. Cells treated with reconstituted apple juice with alkaline EO water were had less DNA damage than that those that were treated with apple juice reconstituted with ultra pure water at 0.1 μL/mL.

Oxidative damage is linked to the formation of tumors through several mechanisms. Free radicals cause DNA damage, which results in a wide range of chromosomal abnormalities, causing abnormal DNA replication and wide cytotoxicity (Liu, 2004; Valko, 2004). The cancer induced by oxidative damage might be prevented or limited by dietary antioxidants found in fruits and vegetables (Liu, 2004). Other studies report that apple extract alone or mixed with other compounds significantly inhibits DNA damage. McCann and others (2007) reported that apple extract significantly inhibited H_2O_2-induced damage in HT29 cells ($p=0.02$). In addition, Schaefer and others (2006) demonstrated that menadione-induced (oxidative) DNA damage was more effectively reduced by reconstituted mixtures of cider and table apples compared to the original extracts. In the current study, apple juice reconstituted with alkaline EO water reduced DNA damage more than apple juice reconstituted with ultra pure water at 0.1 and 0.2 μL/mL. Lee and others (2006) reported that alkaline EO water had a protective effect on H_2O_2 induced DNA damage, but their findings showed that increasing the amount of alkaline electrolyzed water did not affect DNA damage. Our results, however, show treatment with increased levels of alkaline EO water reduced relative H_2O_2-induced DNA damage (Fig. 5.4). Considering the results of Fig. 5.3 and Fig. 5.4, alkaline EO water, however, may enhance the antioxidant activity of apple juice on DNA damage in HT-29 cells.

CONCLUSION

In conclusion, apple juice reconstituted with alkaline EO water showed higher antioxidant capacity (TEAC), and enhanced the effect of apple juice on HT-29 cell proliferation, cell apoptosis, and DNA damage at some, but not all concentrations of apple juice. In part, some

effects are related to its antioxidant capacity to prevent oxidative DNA damage. In addition, alkaline EO water and apple juice reconstituted with alkaline EO water may affect other mechanisms leading to the inhibition of cell proliferation and induction of apoptosis.

LITERATURE CITED

Ashkenazi, A. Targeting death and decoy receptors of the tumour-necrosis factor superfamily. Nature Reviews. Cancer, **2008**, 7, 1001-1012.

Barth, S.W.; Faehndrich, C.; Bub, A.; Watzl, B.; Will, F.; Briviba, K.; Rechkemmer, G. Cloudy apple juice decreases DNA damage, hyperproliferation and aberrant crypt foci development in the distal colon of DMH-initiated rats. Carcinogenesis, **2005**, 26(8), 1414-1421.

Barth, S. W.; Faehndrich, C.; Bub, A.; Watzl, B.; Will, F.; Dietrich, H.; Rechkemmer, G.; Briviba, K. Cloudy apple juice is more effective than apple polyphenols and an apple juice derived cloud fraction in a rat model of colon carcinogenesis. Journal of Agricultural and Food Chemistry, **2007**, 55, 1181-1187.

Bellion, P.; Hofman, T.; Pool-Zobel, B.L.; Will, F.; Dietrich, H.; Knaup, B.; Richling, E.; Baum, M.; Eisenbrand, G.; Janzowski, C. Antioxidant effectiveness of phenolic apple juice extracts and their cut fermentation products in the human colon carcinoma cell line Caco-2. Journal of Agricultural and Food Chemistry, **2008**, 56, 6310-6317.

Evan, G.I.; Vousden, K.H. Proliferation, cell cycle and apoptosis in cancer. Nature, **2001**, 411(17), 342-348.

Fini, L.; Selgrad, M.; Fogliano, V.; Graziani, G.; Romano, M.; Hotechkiss, E.; Daoud, Y.A.; De Vol, E.B.; Richard Boland, C.; Ricciardiello, L. Annurca apple polyphenols have potent demethylationg activity and can reactivate silenced tumor suppressor genes in colorectal cancer cells. The Journal of Nutrition, **2007**, 137 (12), 2622-2628.

Fulda, S.; Debatin, KM. Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. Oncogene, **2006**, 25(34), 4798-4811.

Gosse, F.; Guyot, S.; Roussi, S. Lobstein, B.; Fischer, N. S.; Raul, F. Chemopreventive properties of apple procyanidins on human colon cancer-derived metastatic SW620 cells and in a rat model of colon carcinogenesis. Carcinogenesis, **2005**, 26 (7), 1291-1295.

Gosse, F.; Roussi, S.; Guyot, S.; Schoenfelder, A.; Mann, A.; Bergerat, J.P.; Seiler, N.; Raul, F. Potentiation of apple procyanidin-triggered apoptosis by the polyamine oxidase inactivator MDL 72527 in human colon cancer-derived metastatic cells. International Journal of Oncology, **2006**, 29, 423-428.

Hanahan, D.; Weinberg, R.A. The Hallmark of cancer. Cell, **2000**, 100(1), 57-70.

Hanaoka, K. Antioxidant effects of reduced water produced by electrolysis of sodium chloride solutions. Journal of Applied Electrochemistry, **2001**, 31, 1307-1313.

Hanaoka, K.; Sun, D.; Lawrence, R.; Kamitani, Y.; Fernandes, G. The mechanism of the enhanced antioxidant effects against superoxide anion radicals of reduced water produced by electrolysis. Biophysical Chemistry, **2004**, 107, 71-82.

Happe, R. P.; Roseboom, W.; Pierik, A. J.; Albracht. S. P. J. Biological activation of hydrogen. Nature, **1997**, 385(9), 126.

Hiraoka, A.; Takemoto, M.; Suzuki, T.; Shinohara, A.; Chiba, M.; Shirao, M.; Yoshimura, Y. Studies on the properties and real existence on aqueous solution systems that are assumed to have antioxidant activities by the action of “Active Hydrogen”. Journal of Health Science, **2004**, 50(5), 456-465.

Huang, K. C.; Yang, C. C.; Lee, K. T.; Chien, C. T. Reduced hemodialysis induced oxidative stress in end-stage renal disease patients by electrolyzed reduced water. Kidney International, **2003**, 64, 704-714.

Huang, D.J.; Ou, B.X.; Prior, R.L. The chemistry behind antioxidant capacity assays. Journal of Agricultural and Food Chemistry, **2005**, 53(6), 1841-1856.

Kern, M.; Tjaden, Z.; Ngiewih, Y.; Puppel, N.; Will, F.; Dietrich, H.; Pahlke, G.; Marko D. Inhibitors of the epidermal growth factor receptor in apple juice extract. Molecular Nutrition and Food Research, **2005**, 49, 317-328.

Kern, M.; Pahlke, G.; Balavenkatraman, K.K.; Bohmer, F.D.; Marko, D. Apple polyphenols affect protein kinase C activity and the onset of apoptosis in human colon carcinoma cells. Journal of Agricultural and Food Chemistry, **2007**, 55, 4999-5006.

Kim, M.J.; Jeong, M.K.; Chang, P.S.; Lee, J.H. Radical scavenging activity and apoptotic effects in HT-29 human colon cancer cells of black sesame seed extract. International Journal of Food Science and Technology, **2009**, 44, 2106-2112.

Koch, T.C.L.; Briviba, K.; Watzl, B.; Fahndrich, C.; Bub, A.; Rechkemmer, G.; Barth, S.W. Prevention of colon carcinogenesis by apple juice in vivo: Impact of juice constituents and obesity. Molecular Nutrition and Food Research, **2009**, 53, 1289-1302.

Koseki, M.; Tanaka, Y.; Noguchi, H.; Nishikawa, T. Effect of pH on the taste of alkaline electrolyzed water. Journal of Food Science, **2007**, 72, S298-S302.

Lee, M.Y.; Kim, Y.K.; Ryoo, K.K.; Lee, Y.B.; Park, E.J. Electrolyzed-reduced water protects against oxidative damage to DNA, RNA, and Protein. Applied Biochemistry and Biotechnology, **2006**, 135, 133-144

Li, Y.; Nishimura, T.; Teruya, K.; Maki, T.; Komatsu, T.; Hamasaki, T.; Kashiwagi, T.; Kabayama, S.; Shim, S.Y.; Katakura, Y.; Osada, K.; Kawahara, T.; Otsubo, K.; Morisawa, S.; Ishii, Y.; Gadek, Z.; Shirahata, S. Protective mechanism of reduced water against alloxan-induced pancreatic β-cell damage: Scavenging effect against reactive oxygen species. Cytotechnology, **2002**, 40, 139-149.

Liu, J.R.; Dong, H.W.; Chen, B.Q.; Zhao, P.; Liu, R.H. Fresh apples suppress mammary carcinogenesis and proliferative activity and induce apoptosis in mammary tumors of the Sprague-dawley rat. Journal of Agriculture and Food Chemistry, **2009**, 57, 297-304.

Macdonald, J.S. Adjuvant therapy of colon cancer. CA Cancer Journal for Clinicians, **1999**, 49 (4), 202-219.

Maldonado-Celis, M. E.; Roussi, S.; Foltzer-Jourdainne, C.; Gosse, F.; Lobstein, A.; Habold, C.; Roessner, A.; Schneider-Stock, R.; Raul, F. Modulation by polyamines of apoptotic pathways triggered by procyanidins in human metastatic SW620 cells. Cellular and Molecular Life Science, **2008**, 65(9), 1425-1434.

McCann, M. J.; Gill, C.R.I.; Brien, G.O; Rao, J.R.; McRobers, W.C.; Hughes, P.; McEntee, R.; Rowland, I.R. Anti-cancer properties of phenolics from apple waste on colon carcinogenesis in vitro. Food and Chemical Toxicology, **2007**, 45(7), 1224-1230.

Nishikawa, R.; Teruya, K.; Katakura, Y.; Osada, K.; Hamasaki, T.; Kashiwagi, T.; Komatsu, T.; Li, Y.; Ye, J.; Ichikawa, A.; Otsubo, K.; Morisawa, S.; Xu, Q; Shirahata, S. Electrolyzed reduced

water supplemented with platinum nanoparticles suppresses promotion of two-stage cell transformation. Cytotechnology, **2005**, 47, 97-105.

Okada, H.; Mak, T.W. Pathways of apoptotic and non-apoptotic death in tumour cells. Nature, **2004**, 4, 592-602.

Olsson, M.E.; Gustavsson, K.E.; Andersson, S.; Nilsson, A.; Duan, R.D. Inhibition of cancer cell proliferation in vitro by fruit and berry extracts and correlations with antioxidant levels. Journal of Agricultural and Food Chemistry, **2004**, 52, 7264-7271.

Pisha, E.; Lui, X.; Constantinou, A.I.; Bolton, J.L. Evidence that a metabolite of equine estrogens, 4-hydroxyequilenin, induces cellular transformation in vitro. Chemistry Research in Toxicology, **2001**, 14, 82-90.

Prior, R.L.; Hoang, H.; Gu, L.; Wu, X.; Bacchiocca, M.; Howard, L.; Hampsch-Woodill, M.; Huang, D.; Ou, B.; Jacob, R. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity ($ORAC_{FL}$)) of plasma and other biological and food samples. Journal of Agriculture and Food Chemistry, **2003**, 51, 3273-3279.

Roncucci, M.P.L.L., The cause of colorectal cancer. Digest Liver Disease, **2000**, 32, 426-439.

Schaefer, S.; Baum, M.; Eisenbrand, G.; Dietrich, H.; Will, F.; Janzowski, C. Polyphenolic apple juice extracts and their major constituents reduce oxidative damage in human colon cell lines. Molecular Nutrition and Food Research, **2006a**, 50, 24-34.

Schaefer, S.; Baum, M.; Eisenbrand, G.; Janzowski, C. Modulation of oxidative cell damage by reconstituted mixtures of phenolic apple juice extracts in human colon cell lines. Molecular Nutrition and Food Research, **2006b**, 50, 413-417.

Shirahata, S.; Kabayama, S.; Nakano, M.; Miura, T.; Kusumoto, K.; Gotoh, M.; Hayashi, H.; Otsubo, K.; Morisawa, S.; Katakura, Y. Electrolyzed-reduced water scavenges active oxygen species and protects DNA from oxidative damage. Biochemical and Biophysical Research Communications, **1997**, 234, 269-274.

Srivastava, A.; Akoh, C.C.; Yi, W.; Fishcer, J.; Krewer, G. Effect of storage conditions on the biological activity of phenolic compounds of blueberry extract packed in glass bottles. Journal of Agriculture and Food Chemistry, **2007**, 55, 2705-2713.

Sun, J.; Liu, R.H. Apple phytochemical extracts inhibit proliferation of estrogen-dependent and estrogen-independent human breast cancer cells through cell cycle modulation. Journal of Agriculture and Food Chemistry, **2008**, 56, 11661-11667.

Tsai, C.F.; Hsu, Y.W.; Chen, W.K.; Ho, W.C.; Lu, F.J. Enhanced induction of mitochondrial damage and apoptosis in human leukemia HL-60 cells due to electrolyzed-reduced water and glutathione. Bioscience Biotechnology and Biochemistry, **2009**, 73(2), 280-287.

Valko, M.; Izakovic, M.; Mazur, M.; Rhodes, C.J.; Telser, J. Role of oxygen radicals in DNA damage and cancer incidence. Molecular and Cellular Biochemistry, **2004**, 266, 37-56.

Veeriah, S.; Kautenburger, T.; Habermann, N.; Sauer, J.; Dietrich, H.; Will, F.; Pool-Zobel, B.L. Apple flavonoids inhibit growth of HT29 human colon cancer cells and modulate expression of genes involved in the biotransformation of xenobiotics. Molecular Carcinogenesis, **2006**, 45, 164-174.

Veeriah, S.; Hofmann, T.; Glei, M.; Detrich, H.; Will, F.; Schreier, P.; Knaup, B.; Pool-Zobel, B.L. Apple polyphenols and products formed in the gut differently inhibit survival of human cell lines derived from colon adenoma (LT97) and carcinoma (HT29). Journal of Agricultural and Food Chemistry, **2007**, 55, 2892-2900.

Veeriah S.; Balavenkatraman, K.K.; Bohmer, F.D.; Kahle, K.; Glei, M.; Richling, E.; Scheppach, W.; Pool-Zobel, B.L. Intervention with cloudy apple juice results in altered biological activities of ileostomy samples collected from individual volunteers. European Journal of Nutrition, **2008**, 47(5), 226-234.

Watson, W.H.; Cai, J.; Jones, D.P. Diet and apoptosis. Annual Review of Nutrition, **2000**, 20, 485-505.

WCRF/AICR. Food, Nutrition, Physical Activity, and the Prevention of Cancer. Global Prespective. Washington DC: American Institute for Cancer Research. **2008**

Wolfe K.L.; Kang, X.; He, X.; Dong, M.; Zhang, Q; Liu, R.H. Cellular Antioxidant Activity of Common Fruits. Journal of Agricultural and Food Chemistry, **2008**, 56, 8418-8426.

Yanagihara T.; Arai, K.; Miyamae, K.; Sato, B.; Shudo, T.; Yamada, M.; Aoyama, M. Electrolyzed hydrogen-saturated water for drinking use elicits an antioxidative effect: a feeding test with rats. Bioscience Biotechnology and Biochemistry, **2005**, 69(10), 1985-1987.

Yi, W.; Fishcer, J.; Krewer, G.; Akoh, C.C. Phenolic compounds from blueberries can inhibit colon cancer cell proliferation and induce apoptosis. Journal of Agricultural and Food Chemistry, **2005**, 53(18), 7320-7329.

Ye, J.; Li, Y.; Hamasaki, T.; Nakamichi, N.; Komatsu, T.; Kashiwagi, T.; Teruya, K.; Nishikawa, R.; Kawahara, T.; Osada, K.; Toh, K.; Abe, M.; Tian, H.; Kabayama, S.; Otsubo, K.; Morisawa, S.; Katakura, Y.; Shirahata, S. Inhibitory effect of electrolyzed reduced water on tumor angiogenesis. Biolological and Pharmaceutical Bulletin, **2008**, 31(1), 19-26.

Table 5.1. Mineral contents of alkaline electrolyzed water.

Element	mg/L	Element	mg/L
Ag	0.0031±0.002	Mg	1.85±0.67
Al	0.16±0.01	Mn	0.006±0.0006
As	0.073±0.002	Mo	0.016±0.001
B	0.035±0.001	Na	7.9±0.13
Ba	0.012±0.001	Ni	0.03±0.001
Be	0.0007	P	0.33±0.06
Ca	5.84±2.04	Pb	0.12±0.02
Cd	0.0047±0.003	Sb	0.09
Co	0.02±0.001	Se	0.19±0.06
Cr	0.04±0.0003	Si	5.5±0.06
Cu	0.07	Sr	0.02±0.004
Fe	0.04±0.01	V	0.02±0.002
K	7.94±6.12	Zn	0.01±0.001

Data are means ± standard deviation duplicated.

Table 5.2. Total antioxidant capacity of reconstituted apple juice.

	TEAC value (μM TE/L)	ORAC (μM TE/L)
AJ-DW	1793±105 b	13908±2102 a
AJ-EOW	2773±88 a	15446±2723 a
EOW	N.D c	37.38±9.32 b
DW	N.D c	N.D b

Data are means ± SEM of three replicates. AJ-DW is apple juice reconstituted with ultra pure water. AJ-EOW is apple juice reconstituted with alkaline electrolyzed water. EOW is alkaline electrolyzed water. DW is ultra pure water. Means with the same letter are not significantly different at p value < 0.05.

Table 5.3. Inhibition of HT-29 cell proliferation by alkaline electrolyzed water.

	Cell Viability % of Control
Control	100±0 a
EOW-10	91.63±0.5 b
EOW-20	87.94±1.07 c
EOW-30	85.08±1.7 c, d
EOW-40	82.62±3.08 d, e
EOW-50	79.85±2.17 e

Data are means ± SEM of three replicates. EOW-10 is medium made with a water mixture ratio of ultra pure water to alkaline EO water = 9:1. EOW-20 is medium made with a water mixture ratio of ultra pure water to alkaline EO water = 8:2. EOW-30 is medium made with a water mixture ratio of ultra pure water to alkaline EO water = 7:3. EOW-40 is medium made with a water mixture ratio of ultra pure water to alkaline EO water = 6:4. EOW-50 is medium made with a water mixture ratio of ultra pure water to alkaline EO water = 5:5. Means with the same letter are not significantly different at p value < 0.05.

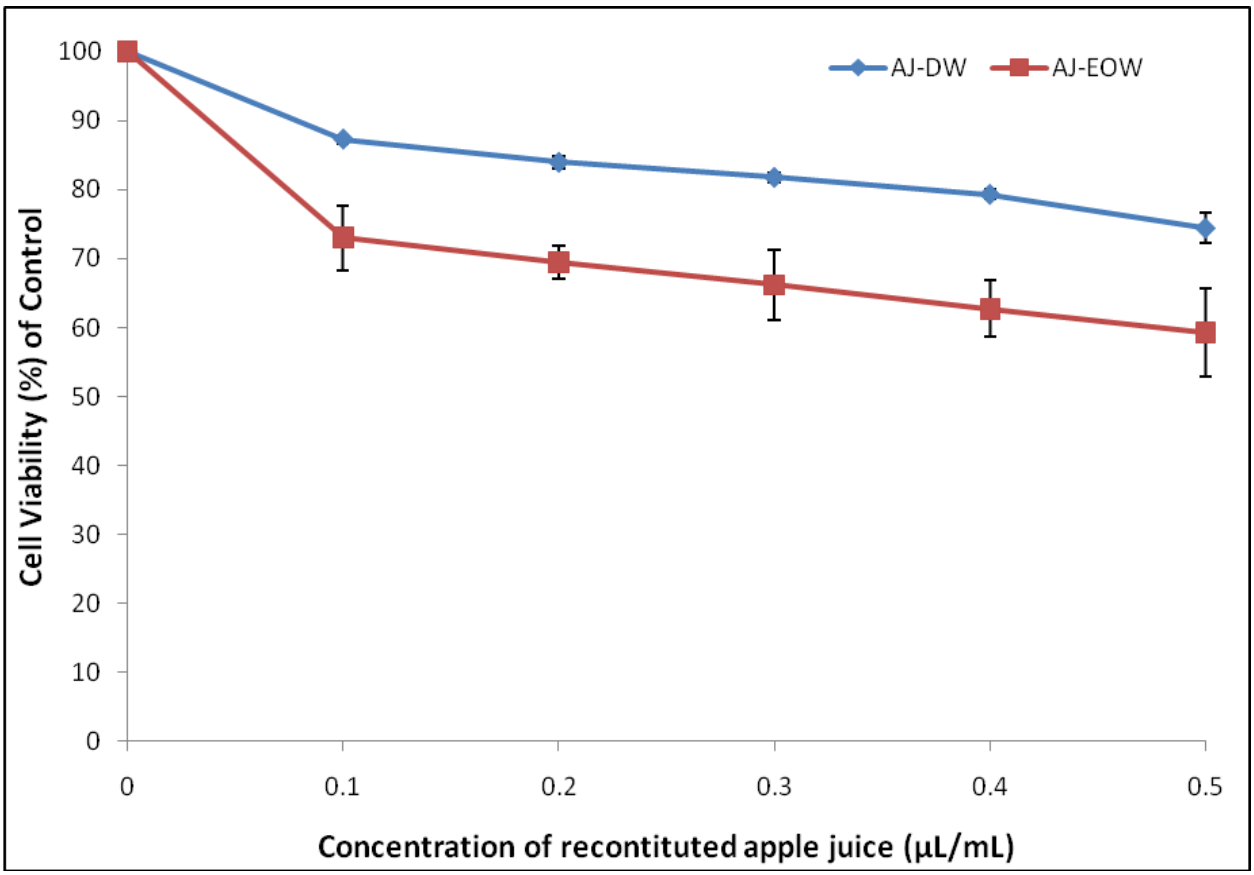

Fig. 5.1. Effect of reconstituted apple juice in HT-29 cells on cell viability.

Data are means ± SEM of three replicates. AJ-DW is apple juice reconstituted with ultra pure water. AJ-EOW is apple juice reconstituted with alkaline electrolyzed water.

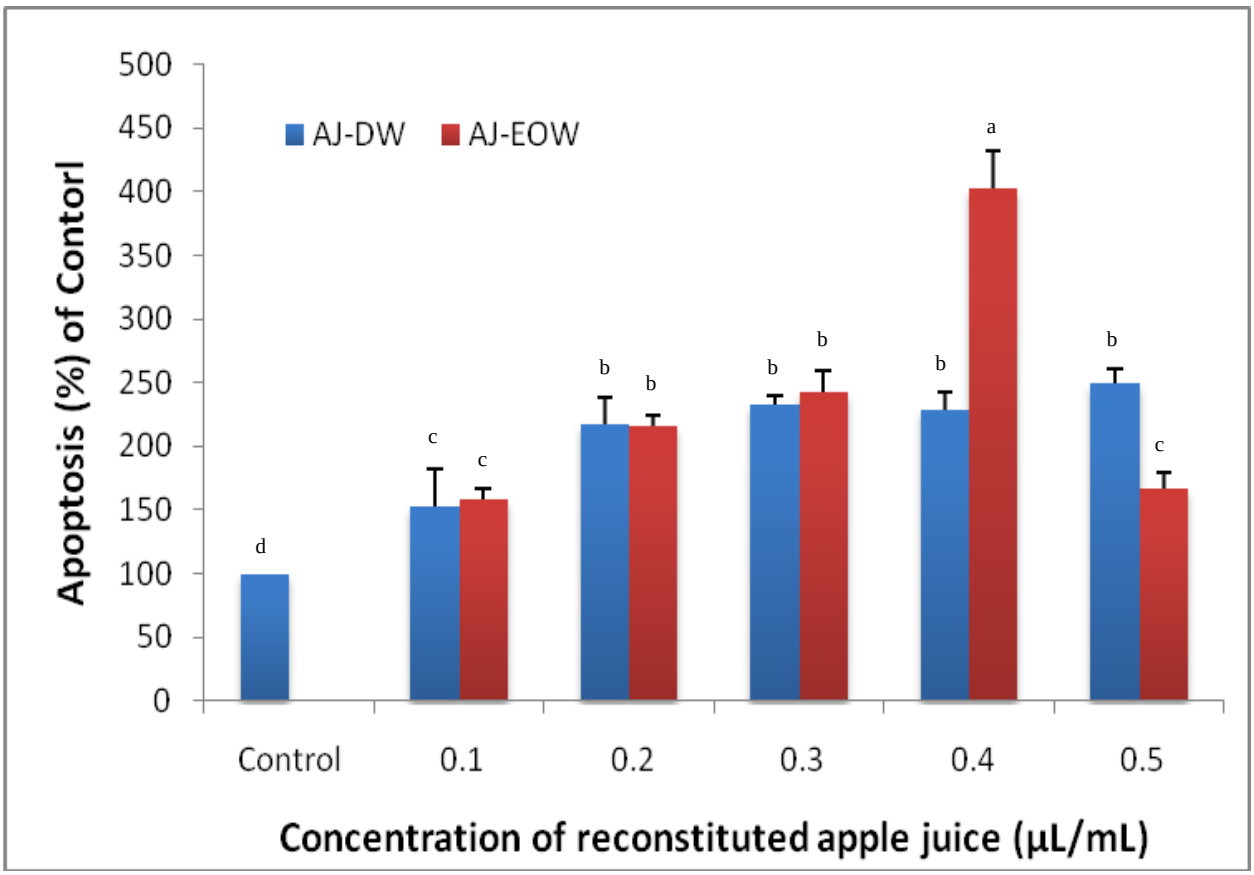

Fig. 5.2. Effect of reconstituted apple juice with ultra pure water in HT-29 cells on apoptosis.

Data are means ± SEM of three replicates. AJ-DW is apple juice reconstituted with ultra pure water. AJ-EOW is apple juice reconstituted with alkaline electrolyzed water.

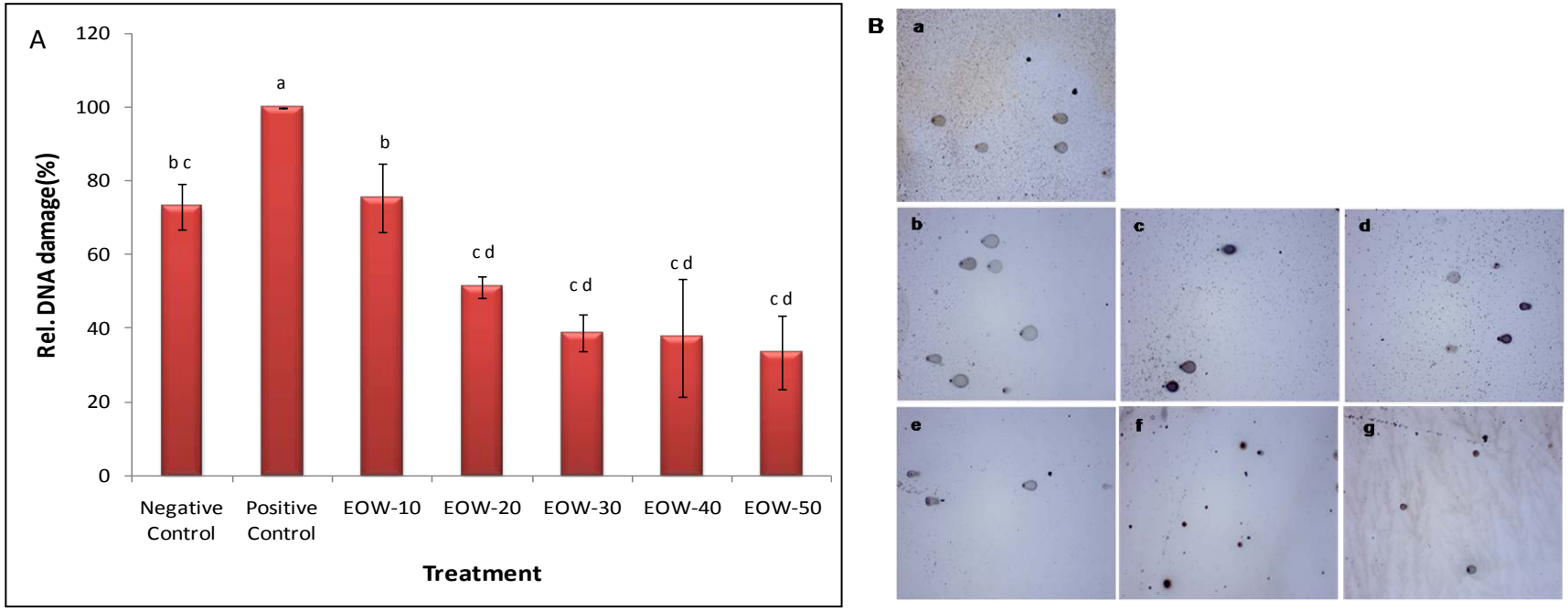

Fig. 5. 3. Effect of alkaline EO water in HT-29 cells on DNA damage.

Datas are means ± SEM of three replicates. A indicates relative DNA damage and B is comet slides under microscopy. EOW-10 is medium made with a combination water mixture of ratio in ultra pure water and alkaline EO water = 9:1. EOW-20 is medium made with a combination water mixture of ratio in ultra pure water and alkaline EO water = 8:2. EOW-30 is medium made with a combination water mixture of ratio in ultra pure water and alkaline EO water = 7:3. EOW-40 is medium made with a combination water mixture of ratio in ultra pure water and alkaline EO water = 6:4. EOW-50 is medium made with a combination water mixture of ratio in ultra pure water and alkaline EO water = 5:5. Negative control treatment received no juice and no H_2O_2. Positive control received no juice, but did receive H_2O_2. In the B, each slides indicate follow as a: negative control, b: positive control, c: EOW-10, d: EOW-20, e: EOW-30, f: EOW-40, and g: EOW-50. Means with the same letter are not significantly different at p value < 0.05.

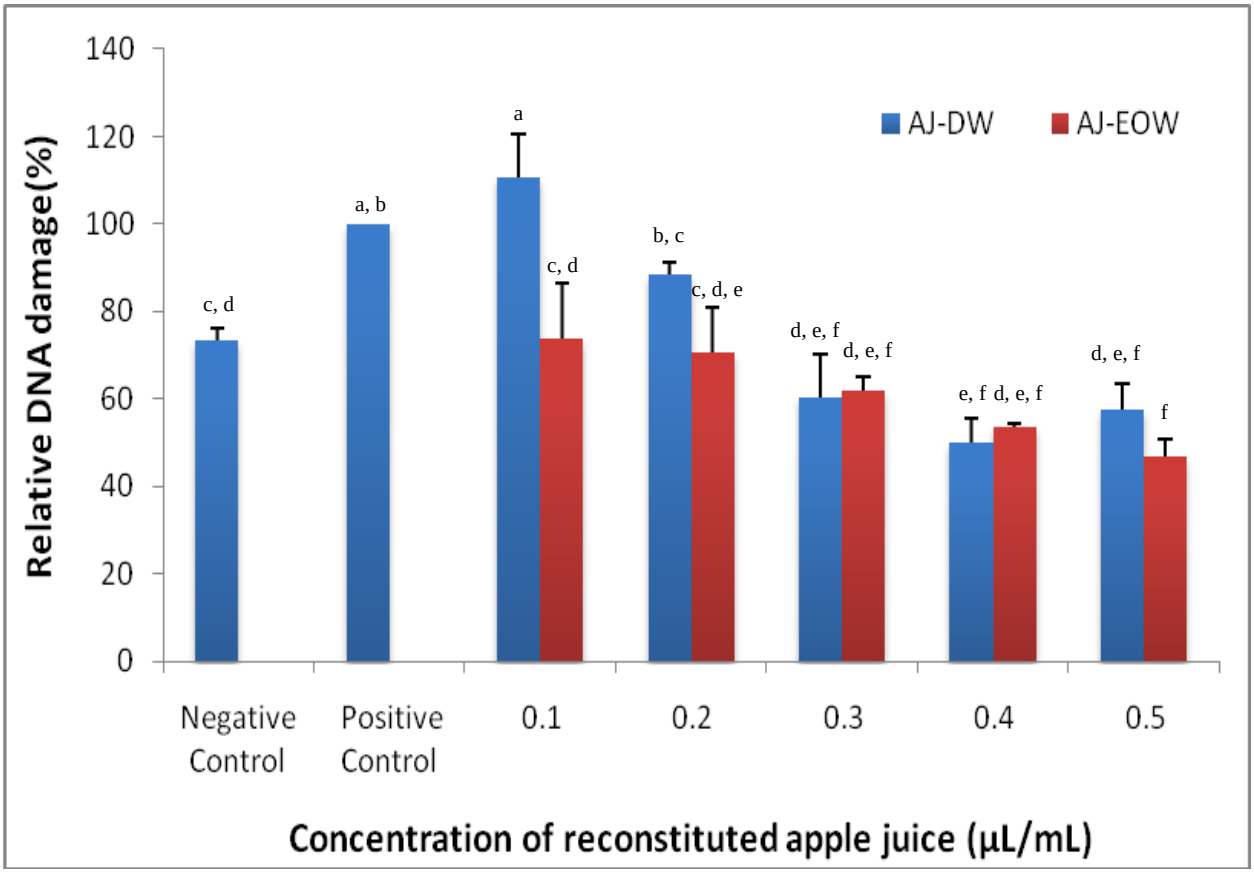

Fig. 5. 4. Effect of reconstituted apple juice in HT-29 cells on DNA damage.

Data are means ± SEM of three replicates. AJ-DW is reconstituted apple juice with ultra pure water. AJ-EOW is reconstituted apple juice with alkaline EO water. Negative control treatment received no juice and no H_2O_2. Positive control received no juice, but did receive H_2O_2. Means with the same letter are not significantly different at p value < 0.05.

CHAPTER 6

SUMMARY AND CONCLUSION

The goal of current study was to determine whether alkaline EO water has health contributions as antibrowning agent or drinking water. Alkaline electrolyzed (EO) water was used to investigate antibrowning and antioxidant, and cell proliferation, apoptosis, and oxidative damage in HT-29 cells. Results from these studies indicate that alkaline electrolyzed (EO) water had strong reduction potential, which is a main factor for health contributions of alkaline EO water.

In third chapter, the antibrowning effect of alkaline EO water was investigated on apple slices. Alkaline EO water played a role as effective browning agent, suggesting the use of alkaline EO water for the prevention of minimally processed fruit. Especially, it showed better antibrowning effect when alkaline EO water instead of deionized water used as solutions to dissolve antibrowning agents such as ascorbic acid, citric acid, and sodium metabisulfite.

In addition, fourth and fifth chapters evaluated the possibility of alkaline EO water as drinking water for health. To investigate enhanced antioxidant capacity of alkaline EO water, it used to reconstitute concentrated frozen apple juice, which can be purchased from grocery as ready to beverage. Apple juice reconstituted with alkaline EO water had higher ORAC and TEAC values than apple juice reconstituted with deionized water. Such enhancement on antioxidant activity of reconstituted apple juice may be a result of negative redox potential of

alkaline EO water. Apple juice with higher negative redox potential values also had higher antioxidant capacity value compared to lower negative redox potential values.

Such antioxidant effect can be supported by results of oxidative DNA damage (Comet assay). Apple juice reconstituted with alkaline EO water prevented effectively oxidative DNA damage of HT 29 cells. Also, apple juice reconstituted with alkaline EO water had effective reduction of cell proliferation and induction of DNA fragmentation in HT 29 cells, suggesting preventive effect on colon cancer.

APPENDICES

APPENDIX A
STANDARD OPERATING PROCEDURE OF OXYGEN RADICAL ABSORBANCE CAPACITY ($ORAC_{FL}$)

REAGENT PREPARATION

1.1. Phosphate Buffer

- *Buffer Stock Solution:*

  Solution A: 75 mMolar Monopotassium phosphate

  - Accurately weigh 10.21 grams of Monopotassium Phoshate into a weigh dish
  - Transfer the monopotassium phosphate into a 1000 mL volumetric flask
  - Add approximately 700 mL DI water and a magnetic stir bar. Stir on a magnetic stirplate until the phosphate is dissolved
  - Remove the stir bar and make up to volume with DI water

  Solution B: 75 m Molar Dipotassium phosphate

  - Accurately weigh 13.06 grams of Dipotassium Phosphate into a weigh dish
  - Transfer the Dipotassium phosphate into a 1000 mL volumetric flask
  - Add approximately 700 mL DI water and a magnetic stir bar. Stir on a magnetic stirplate until the phosphate is dissolved
  - Remove the stir bar and make up to volume with DI water

- *Buffer Working Solution:*

  - Place a magnetic stir bar into a 1000 mL beaker. Pour 800 mL of solution B into the 1000 mL beaker. Insert the pH electrode and begin reading the pH of the solution.

- Transfer 200 mL of solution A into a graduated cylinder. Transfer the A solution into the 1000 mL beaker containing B and monitor the change in pH.
- Slowly add additional solution A as needed to reach a final solution pH of 7.4
- Store the 7.4 Buffer in a one liter amber glass bottle.

1.2. Trolox Standards

- *Trolox Stock Solution:*

  Dissolve 25 mg (0.025 g) of Trolox in 100 mL phosphate buffer working solution to make 1 mM trolox, then dilute to 500 µM. Aliquot 1.5 mL in 1.8 mL Eppendorf tubes and store at –70°C until use.

- *Trolox Working Solution:*
- Thaw an aliquot of Trolox stock solution. Take 1 mL of 500 µM Trolox in a 15 mL tube; add 9 mL of working buffer and vortex to make 50 µM Trolox solutions. Make serial dilutions of the 50 µM Trolox solution with working buffer solution to produce 25, 12.5, 6.25 µM Trolox standards. Keep remaining Trolox solution at 4°C.

1.3. Fluorescein Solution

- *Fluorescein Stock solution:*
  - Stock solution #1: dissolve 0.0225 g in 50 mL of working phosphate buffer and mix well.
  - Stock solution #2: dissolve 50 µL of stock solution #1 in 10 mL of working buffer and vortex. Aliquot stock solution #2 into 1.8 mL Eppendorf tubes and store at –20°C until use.

- *Fluorescein Working Solution:*

  Pipette 800 µL of stock solution #2 into 50 mL phosphate buffer (for two runs) in a 50 mL conical tube. Before use, incubate in the water bath at 37°C until thoroughly heated. This solution can be kept in the water bath for many hours.

1.4. AAPH Solution

The phosphate buffer is preincubated at 25 °C. Dissolve 0.108 g of AAPH into 5 mL of incubated phosphate buffer immediately before the start of the assay. This produces an AAPH solution containing 79.6 μmol/mL. A 20 μL aliquot provides 1.6 μmol AAPH per well. It is important to keep the AAPH in the refrigerator at 4^{o}C before adding the warm buffer. The AAPH reaction is temperature-dependent. This procedure was proven with the BMG plate reader to significantly reduce apparent temperature effects.

NOTE: If –20°C and –70°C storage facilities are not available, fresh reagent solutions have to be made freshly on a daily basis.

2. PREPARE AND LOAD 96-WELL MICROPLATE

- Set up a paper layout to determine the order of your samples. In order to avoid possible positional errors, a "forward-then-reverse" order is recommended. It is highly recommended to leave the edge wells empty or blank (phosphate buffer working solution) to reduce the impact of "edge effect" on samples and standards particularly if your instrument exhibits some temperature effects on the outside wells. The following is an example of sample layout in a 96-well microplate:

X	B	B	B	B	B	B	B	B	B	B	B
B	T1	T2	T3	T4	B	S1	S2	S3	S4	S5	B
B	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	B
B	B	B	B	T1	T2	T3	T4	B	B	B	B
B	S16	S17	S18	S19	S20	S20	S19	S18	S17	S16	B
B	S15	S14	S13	S12	S11	S10	S9	S8	S7	S6	B
B	S5	S4	S3	S2	S1	B	T4	T3	T2	T1	B
B	B	B	B	B	B	B	B	B	B	B	B

X: 200 µL Fluorescein working solution for gain adjustment of plate reader.

B: Blank, phosphate buffer working solution.

S: Sample

T: Trolox standards, from T1 to T4, from 6.25 µM to 50 µM.

- Pipette 20 µL of sample, blank and Trolox standard solutions into appropriate wells. Also pipette 200 µL of fluorescein working solution into the selected well for the gain adjustment.
- Place 25 mL Fluorescein working solution in the plate reader so that it corresponds to Pump #1.
- Place the correct amount of AAPH solution in position so that it corresponds to Pump #2.

3. SETTING THE PLATE READER

Plate Reader Startup

- Click “incubator” icon  and set the temperature at 37 °C.
-  Click on the “open door” icon after the color of the “incubator” icon turns from red to green. Insert the microplate, and click the “close door” icon. Leave the plate in the chamber for ten minutes for pre-heating.  Click on the “Test Protocol” icon to create ORAC Test Protocol.

Basic Parameters

Positioning delay: 0.3s

No. of kinetic windows: 1

No. of cycles: 35

Measurement Starting Time: 0.0 s

No. of flashes per cycle: 15

Cycle time: Depending on the layout.

Filters and integration: Fluorescence Intensity

No. of multichromatics: 1

Gain: will change depending on each Gain Adjustment

Pause before cycle: 0

Excitation filter: 485 nm

Emission filter: 520 nm

Calculation range: start from 1, stop at 35

Click on “check timing” icon (lower left corner). Note the cycle time.

Concentration/Volume/Shaking

Volume 1: 200 µL, Pump speed: 420 µL/s

Volume 2: 20 µL, Pump speed: 420 µL/s

Shaking mode: orbital

Shaking width: 4 mm

Additional Shaking: before each cycle

Shaking time: 8 s

Injection and timing for instruments with automatic pipettors as part of the plate reader:

Volume group 1 injection cycle: 2

Volume group 2 injection cycle: 4

Volume group 1 injection start time: 0.0 s

Volume group 2 injection start time: 0.0 s.

- Place the injection needles and the tubes into their appropriate solution. The solution should pump back into its tube. Pump 1 is for Fluorescein and pump 2 is for AAPH. Next, click on the ‘Prime’ icon  to prime the two pumps and remove any air bubbles. Prime each needle at l times.
- Click the ‘measure’ icon or select ‘measure’ under the Measure menu. Select the correct test protocol. Enter plate and sample identifications and check the gain adjustment for the fluorescein well. The normal range of gain value is 1700 ± 100, at 90% required value.

- Open the microplate chamber, cover the plate and leave it in the instrument for ten minutes. Then, open the chamber once more, remove the cover, close the chamber, and start the test run.

- NOTE
  - ⇗ Make sure the incubator is on and the temperature is set to 37°C.
  - ⇗ Make sure the cables are turned so as to read from the bottom of the plate.
  - ⇗ Back flush old reagents from pumps after each run.
  - ⇗ Two injection needles and measurement head need to be washed thoroughly at least every two days if the machine is fully used.

APPENDIX B

DNA FRAGMENTATION USING ELISA CELL DEATH DETECTION KIT

Procedure:

1. Harvesting cells

1) Remove media containing treatment and trypsinize cells.
(rinse with 0.2 mL trypsin, treat with 0.3 mL trypsin, and stop reaction with 0.7 Ml media)

2) Add 10µL cells to respective eppendorf tube and count cell to be 1 x 10^5

3) Repeat until all wells have been transferred.

4) Centrifuge the tubes 10 min at 2700 rpm (200 x g).

5) Carefully remove supernatant.

2. Lysis

1) Resuspend pellet in 200 µL lysis buffer (bottle 5 in ELISA Cell Death Detection Kit) and incubate 30 min at 15-25^{o}C.

2) During incubation, prepare ELISA solutions.

Bottle #	Content	Preparation	For use in	Storage and stability
1	Anti-histone biotin	add 450ul double distilled water, mix thoroughly	Immunoreagent	at 2-8 C (fridge) for 2 mo.
2	Anti-DNA POD	add 450ul double distilled water, mix thoroughly	Immunoreagent	Fridge for 2 mo.
3	Positive control	add 450 ul double distilled water, mix thoroughly	ELISA	Fridge for 2 mo.
7	ABTS	dissolve ***1***,2 or 3 tablets in ***5***,10, or 15 ml Subtrate Buffer (bottle 6) depending on the number of samples tested	ELISA	1 month, Store protected from light! (wrap bottle in foil) Allow to come to RT before use.

3) Centrifuge lysate at 2700 rpm for 10 min (200 x g) and mix up Immunoreagent during centrifugation.

Bottle #	# of tests	10	20	***40***	50
4	incubation buffer	720 ul	1440 ul	***2880 ul***	3600 ul
1	Anti-Histone buffer	40 ul	80 ul	***160 ul***	200 ul
2	Anti-DNA-POD	40 ul	80 ul	***160 ul***	200 ul
	final volume	800 ul	1600 ul	***3200ul***	4000 ul

3. ELISA

1) Transfer 20 µL from supernatant (which is the cytoplasmic fraction) carefully into the streptavidin coated microplate for analysis. Also transfer 20 µL from positive control (bottle 3), negative control (untreated cells), and background control (incubation buffer, bottle 4) into microplate.

2) Add 80 µL of Immunoreagent and cover MP with adhesive cover foil.
Incubate plate on shaker under gently shaking (300 rpm) for 2 h at 15-25^{o}C.

3) After 1.5 h, remove ABTS from fridge so it can warm to RT. Turn on 37 ^{o}C water bath to warm up.

4) After the 2h incubation period is complete, remove solution thoroughly by tapping and rinse each well three times with 250-300µL incubation buffer (bottle 4).

5) Pipette each well with 100 µL ABTS solution and incubate on the plate shaker at 250 rpm until color development is sufficient.

6) Place ABTS stop solution into the water bath to warm immediately after placing the MP on the shaker, until solute goes into solution.

7) Pipette 100 µL ABTS stop solution into each well.

8) Measure at 405 nm against ABTS solution and 100 µL stop solution as a blank (reference wavelength 490 nm).

APPENDIX C

COMET ASSAY USING TREVIGEN KIT

PROCEDURE:

P*erform steps 1-3 30 minutes prior to the end of treatment.*

1) Prepare lysis solution:

120ml	Lysis solution
12ml	DMSO

Chill in fridge until use.

2) Agarose:

Turn on 42^{o}C water bath and boiling water bath.

Boil LM agarose for 5 minutes with the cap loosened and then place in 42 ^{o}C water bath until needed. (*Place ~3.3 ml in a bottle and put the rest back in the fridge in case of spillage)*

3) Label 1 set of eppendorf tubes for the samples.

Label comet slides and then put back in slide box until needed.

Warm trypsin in the 37^{o}C water bath for 10 minutes.

4) Harvest Cells:

Remove media containing extracts and trypsinize cells.

(rinse with 0.2 ml trypsin, treat with 0.3 ml, stop reaction with 0.7 ml media)

Use the 1ml pipettes and remember that they fill VERY quickly!

Transfer 100 ul cells + 400 ul PBS to labeled eppendorf tubes.

This dilutes the cells which are roughly at 1X10^{6} to the desired 1-2X10^{5}cells/ml.

Spin 5 min at 5000rpm (1250xg)

Wash with and then resuspend cells in PBS.

5) Slide Prep:

In an Eppendorf tube, half submerged in water bath, mix 200 ul LMagarose
20 ul cells

Pipette 80ul of mixture onto each of the 2 slide wells and spread with pipette tip

(Note: be careful not to drag tip on slide as it will remove the coating!)

Dry slides flat in fridge for 10 min (or until clear ring around edge is visible)

6) Hydrogen peroxide treatment:

While slides are drying, set up for serial dilution, but don't add H_2O_2 until immediately before treating the slides.

Also fill tray with ice.

Pour lysis solution into a plastic container.

Serial dilution to make 75 uM H_2O_2:

	H_2O_2	PBS	
1	85 ul	915 ul	
2	10 ul	990 ul	
3	20 ul	1980 ul	*Pipette 990 ul twice*

Lay slides on ice.

Pipette 100ul of 75uM H_2O_2 onto each slide well. *You can time the treatment of the slides, for example the second slide it treated exactly 10 seconds after the first, similar to what we do with the Lowry assay, to ensure that all the slides are treated for exactly 5 min.*

Tap slides on a paper towel to remove excess H_2O_2 and place in lysis solution. *Remember to continue spacing the slides by 10 seconds.*

7) Incubation:

Close lid and incubate the slides in lysis solution for 4 hours in fridge.

8) Electrophoresis prep: (*30 minutes before lysis incubation is complete)*

Prepare denaturing and electrophoresis solution

For 2 L 24 g NaOH

0.744 g EDTA

About 1.8 L distilled water

Add DI water to 2 L , after NaOH has completely dissolved.

Set up electrophoresis tank in fridge so solution will chill.

When incubation is complete:

9) Line up slides at center of tank. Denature *(incubate)* for 30 minutes.

10) Electrophoresis**:**

Set voltage at 25 V and adjust buffer level to get as close to 300mA as possible (usually around 295 mA)

(Note: Start with buffer just barely covering slides, and add more to increase ampage, take away to increase voltage.)

Run for 30 minutes.

Tap excess buffer off slides and dip in 70% alcohol for 5 minutes.

Air dry slides over night, then store at RT with desiccant in the dark.

SILVER STAINING PROCEDURE

1) Fixation

Solution preparation (mix in this order and only immediately before use)

Per sample,

30 μL DI water

50 μL methanol

10 μL glacial acetic acid

10 μL fixation additive, 10x

Pipette 100 μL on each sample and incubate for 20 min at RT.

Get Regent 4 out of fridge to warm, and mix up 100 μL of 5% acetic acid per sample.

Rinse slides with DI water for 30 min.

2) Staining

Solution preparation

Per sample,

35 μL DI water

5 μL Reagent 1

5 μL Reagent 2

5 μL Reagent 3

Mix by tapping tube

50 μL Reagent 4

Quickly pipette to mix and flood each sample with 100 μL of staining solution.

Incubate approximately 11min at RT. Monitor staining progress under 10x lens of microscope and allow developing until the DNA tails are easily visible.

Stop the reaction by pipetting 100 μL of 5% acetic acid on each sample and incubate for 15 min, and rinse slides with DI water, air dry and store in dark with desiccant.