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Studies on the toxicity of mixtures of haloacetates and ethanol in AML-12 cells

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A Thesis

entitled

Studies on the Toxicity of Mixtures of Haloacetates and Ethanol in AML-12 Cells

by

Sukanto Salang Mamada

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Master of Science Degree in

Pharmaceutical Science

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May 2014

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An Abstract of

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The process of water chlorination results in production of different haloacetate by-products, such as dichloroacetate (DCA) and trichloroacetate (TCA). The compounds have been found to be hepatotoxic and hepatocarcinogenic in rodents through induction of oxidative stress (OS). Chronic ingestion of alcohol (ethanol) is also known to result in liver toxicity and cancer, with OS found to play an important role in that. This may suggest simultaneous exposure to mixtures of DCA, TCA and alcohol may result in interactivities that will increase their hepatotoxicity, and to test this hypothesis, mixtures of DCA, TCA and alcohol are to be studied. However, we found *in vivo* tests in animals can be challenged by ethical issues, as for the large number of animals required to test various mixtures, and the length of time and high cost required to complete that. Alternatively, we used mouse liver cells (AML12 cell line) as a screening model to test the effects of those compounds and their mixtures. Accordingly, various concentrations of each of the three compounds, as well as mixtures of the compounds were incubated with AML12 cells for 48 h, and cells were then assayed for viability and various biomarkers of OS, including production of nitric oxide (NO), superoxide anion (SA) and

advanced oxidation protein products (AOPPs). DCA, TCA and ethanol were found to result in concentration-dependent decreases in cellular viability, with respective concentrations of 770 ppm, 500 ppm and 1.5% resulted in 25% decreases. Concentrations corresponding to 25% decreases in cellular viability by the compounds were used to design binary mixtures of the compounds, as well as a mixture of the three compounds to assess possible interactivities between them. In general, binary mixtures containing DCA and TCA resulted in effects on cellular viability and induction of the tested biomarkers of OS that were additive, and binary mixtures of DCA or TCA with ethanol, as well as a mixture containing the three compounds resulted in greater than additive effects on those biomarkers. These results indicate significant contribution of alcohol to the hepatotoxic effects of DCA and TCA, and may suggest possible increase in the risk of hepatotoxicity from simultaneous consumption of chlorinated water and alcohol. The results may be used as basis for further investigation of that suggestion *in vivo*, in animals.

For my parents and family who always pray for me.

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Chapter 1

Introduction

1.1 Water chlorination

Chlorine has been used for years to disinfect drinking water (Chlorine Chemistry Council & Canadian Chlorine Coordinating Committee, 2003). However, it was found to react with certain organic compounds present in surface waters resulting in generation of haloacetates as by-products that include dichloroacetate (DCA) and trichloroacetate (TCA) (Zhang, et al., 2009). DCA and TCA can also be formed in vivo from metabolism of trichloroethylene, a solvent, which is used widely in industry and is found to contaminate the surface water in some areas (Ketcha, et al., 1996).

1.2 Dichloroacetate (DCA) and trichloroacetate (TCA)

Dichloroacetate (DCA), as a sodium salt (figure 1), has a molecular weight of 151 g/mol, and is stable, water soluble, and has a pKa of 1.29 (WHO, 2005). The compound is readily absorbed in the gastrointestinal tract (GIT) and is mostly metabolized in the liver generating glyoxylate, glycolate, oxalate, and CO₂ as main metabolites (Larson and Bull, 1992; Gonzalez-Leon et al., 1997; Bull, 2000; Schultz, et al., 2002). Approximately 40% of absorbed molecules is present in blood in unbound form (Toxopeus and Frazier, 1998). DCA is mainly excreted through first-order urinary excretion and is also removed

via biliary excretion (Larson and Bull, 1992; Abbas and Fisher, 1997; Toxopeus and Frazier, 1998).

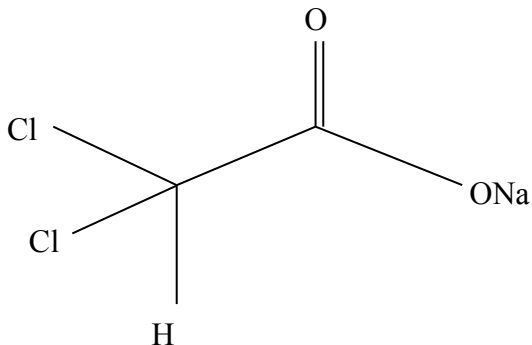


Figure 1. Chemical structure of sodium dichloroacetate (DCA)

Trichloroacetate (TCA), as a sodium salt (figure 2), has a molecular weight of 185.37 g/mol and is highly water-soluble (WHO, 2004). As DCA, TCA is immediately absorbed in the GIT and is mainly metabolized in the liver resulting in DCA and other metabolites (Larson and Bull, 1992; Abbas and Fisher, 1997; Merdink et al., 1998). Toxopeus and Frazier (1998) have demonstrated that 90% of TCA is bound to albumin. More than 50% of TCA dose is excreted in the urine as unchanged form, and a small fraction is removed through biliary excretion (Larson and Bull, 1992; Toxopeus and Frazier, 1998).

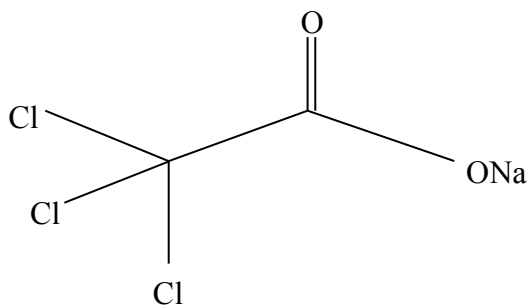


Figure 2. Chemical structure of sodium trichloroacetate (TCA)

DCA is known to have some beneficial effects in certain medical therapies. Stacpoole et al. (1988) found that DCA can be used as a therapy for patients suffering

from lactic acidosis, and Fox et al. (1996) have suggested the compound for use as an alternative therapy for stroke and head injury. Recently, it has been proven that DCA has a potential to induce apoptosis on endometrial cancer cells (Wong, et al. 2008), but this remains to be controversial as of the vast literature indicate the carcinogenic potential of the compound, in addition to several other toxic effects, as described below. Unlike DCA, TCA has no use in therapies. It is mostly used as a laboratory reagent, an antiseptic, and a peeling agent (Chlorine Chemistry Council & Canadian Chlorine Coordinating Committee, 2003)

DCA and TCA have been found to induce hepatocarcinogenic effects in B6C3F1 mice (DeAngelo et al., 1991; Herrend-Freund et al., 1987; Pereira, 1996; Daniel et al., 1992) and F344 rats (DeAngelo et al., 1996; Richmond et al., 1995). Long term exposure of mice to DCA resulted in development of liver neoplasia , and hepatocellular carcinoma in 80-90% of the exposed animals (Angelo et al. 1991; Herrend-Freund et al.,1987; Daniel et al., 1992; Bull et al., 2002); while long term administration of TCA resulted in hepatocellular carcinoma in 32% of treated mice (Herrend-Freund et al., 1987).

The hepatocarcinogenic effects of DCA and TCA were suggested to be due to the action of the compounds on peroxisome proliferator-activated receptor α (PPAR α) (Bull, 2000; Nakajima et al., 2000; Laughter et al., 2004; Walgren et al., 2004), but despite the same mode of action they may have, they produce different characteristics of liver changes or lesions. Bull et al. (1990) showed that DCA-treated mice undergo more massive accumulation of glycogen throughout the liver than mice treated with TCA after 52 weeks of treatment with the compounds. Also, focal hepatic necrosis was observed in mice treated with DCA, but TCA-treated mice experienced lower level of necrosis

(Sanchez and Bull, 1990). Pereira (1996) has indicated the predominance of eosinophil and GST- π in DCA-induced liver tumor, whereas predominance of basophil and lack of GST- π were observed in TCA-induced liver tumor.

The potentials of DCA and TCA as mutagens are still debatable. While Fox et al. (1996) concluded that both compounds have no mutagenic effects in in vitro and in vivo studies, other studies have demonstrated their mutagenic potentials in both systems (Bunya and Behera, 1987; Harrington-Brock et al., 1998)

Although liver is the major target organ that is found to be affected in animals exposed to DCA- and TCA, other toxic effects were also reported for the compounds, such as decrease in plasma glucose level and reduction of urine production in female rats (Davis, 1990), CNS, testicular, ocular, and gall bladder toxicities (Katz et al., 1981); erythrocyte, hemoglobin, leukocyte, lung, pancreas, and kidney toxicities (Cicmanec et al., 1991; Celik, 2007; Mather et al., 1990).

1.3 Ethanol

Ethanol (alcohol) is one of the most common beverages consumed by millions of people around the world. WHO (2011) reported approximately 2.5 million deaths, or 4% of all deaths worldwide per year are attributed to alcohol consumption. Although several serious toxic effects are found to be induced after drinking single high doses, more serious long term effects are commonly seen among alcoholics (Penny, 2013). Ethanol was also found to have a potential to be a carcinogen, teratogen, and mutagen (Obe and Ristow, 1979; Morgan, 2004; Baan et al., 2007). Accordingly, it has been classified by IARC as a group I human carcinogen (WHO, 2006).

Liver is the most affected organ after excessive alcohol drinking, since it is the main site for its metabolism (Lieber and DeCarli, 1991; Lieber, 1997). The absorbed ethanol is metabolized through the actions of alcohol dehydrogenase, cytochrome P450, and catalase (Mullen, 1977; Lieber, 1997; Rusyn and Bataller, 2013). A non-oxidative metabolism pathway is also known to have a limited role in alcohol metabolism (Lieber, 1997; Rusyn and Bataller, 2013). Acetaldehyde is the main metabolite produced and is recognized as the most contributing metabolite to the toxic effects of ethanol, such as glutathione depletion, increased collagen synthesis leading to fibrosis and cirrhosis, and decreased DNA repair (Lieber and DeCarli, 1991; Lieber, 1997; McKillop and Schrum, 2005)

Hepatocellular carcinoma (HCC) represents approximately 85% of liver cancers (Marra et al., 2011), and is known to be highly induced by chronic alcohol consumption (Donato et al., 2002; Morgan et al., 2004). Throughout the world, HCC accounts for more than 500,000 deaths per year (Parkin, 2001). Morgan et al. (2004) have indicated that the risk of HCC is enhanced, approximately 5-fold when daily alcohol consumption exceeds 80 g/L, for more than 10 years. This condition is found to be worsened by synergistic actions of hepatitis virus infection and diabetes mellitus (Donato et al., 2002; Hassan et al., 2002). Cirrhosis is recognized as the most common precursor in HCC development (McKillop and Schrum, 2005).

Although liver is known to be the main target organ for alcohol toxicity and carcinogenicity, other organs/ effects are also known to be produced in response to chronic alcohol consumption, such as lung cancer (Bandera et al., 1992) colorectal cancer (Glynn et al. 1996), oral cavity and pharynx cancer (Franceschi et al., 2000), and

breast cancer (Hiatt et al., 1988). While Novelli et al. (1997) have shown that alcohol can cause prostate injury in rat, alcohol was not found to be associated with prostate cancer in humans (Crispo et al., 2004). Alcohol was also found to be a teratogen, and has been associated with fetal alcohol syndrome and alcohol embryopathy (Obe and Ristow, 1979).

1.4 Oxidative Stress (OS)

Oxidative stress (OS) results from imbalance between oxidants and antioxidants in cells leading to overproduction of free radicals, including reactive oxygen species (ROS) (Davies, 2000; Valko et al., 2007; Palmieri et al., 2007). ROS are very toxic and can attack several important biomolecules, such as lipids, carbohydrates, protein, DNA and RNA, resulting in damage that lead to cellular toxicity, including mutations and cancer and also can lead to cellular death (Shacter, 2000; Valko et al., 2006; Kunwar et al., 2011; Sosa et al., 2013). Accordingly, ROS are associated with many diseases such as cardiovascular (Partasarathy et al., 2001), renal (Massy et al., 2009), pulmonary (Park et al., 2009), neurodegenerative (Parkinson and Alzheimer diseases) (Emerit et al., 2004), cerebrovascular (Gariballa and Sinclair, 1999), and hepatic (Wu and Cederbaum, 2009). It is also associated with erectile dysfunction (Agarwal et al., 2006) and aging process (Sastre et al., 1996).

In addition to their deleterious effects, ROS are also known to have beneficial effects, especially when they are produced at low or moderate concentrations, such as in cellular signaling, mitogenic response, and defense mechanism against attacking microbes (Valko et al., 2007; Hernandez-Garcia et al., 2010). Since ROS are generated during metabolic processes, especially during oxygen utilization in the respiratory chain

reaction, they are continually produced in the body (Valko et al., 2006). In order to protect against ROS over production, living systems are equipped with antioxidants for scavenging excessive ROS produced (deZwart et al., 1999).

1.5 Superoxide Anion (SA)

Superoxide anion (SA) is one form of ROS that is produced when an electron is added to a molecular oxygen. This reaction is mediated by NADPH enzyme, xanthine oxidase or by the action of complex I and III of mitochondria (Kunwar and Priyadarsini, 2011). SA can be dismutated by the antioxidant enzyme, superoxide dismutase (SOD), generating hydrogen peroxide (H₂O₂) which is another ROS that can be further converted to the most dangerous ROS, the hydroxyl radical, by Fenton reaction (Valko et al., 2007). However, H₂O₂ can be detoxified by the antioxidant enzymes, catalase (CAT) and glutathione peroxidase (GSH-PX), producing water (Valko, et al., 2006). Like any free radicals, SA is known to attack biomolecules in the body such as lipids, proteins and DNA, producing cellular damage.

1.6 Nitric Oxide (NO)

NO is a free radical produced from L-arginine through the action of nitric oxide synthase (NOS), and has been recognized to have pivotal roles in living systems involving large number of targeted organs (Ali et al., 2012). From the chemical biology aspect, NO has dual effects: direct and indirect effects (Wink and Mitchell, 1998). Direct effects usually occur at low concentration (< 1 μ M), and refer to any effect caused by direct interaction of NO with its molecular targets, resulting in either protective or regulatory effects. However, indirect effects would occur at slow rates, and higher concentrations (> 1 μ M), producing deleterious effects due to interaction between NO

and SA, generating reactive nitrogen oxide species (RNOS), such as peroxynitrite (ONOO-) (Wink and Mitchell, 1998).

When NO is produced at higher than normal levels, it results in nitrosative reactions with biomolecules, leading to nitrosative stress (Ridnour, et al., 2004; Wink and Mitchell, 1998). Nitrosative stress can result in deleterious effects associated with peroxynitrite and N₂O₃ production (Wink and Mitchell, 1998).

1.7 Advanced Oxidation Protein Products (AOPPs)

Production of advanced oxidation protein products (AOPP) is a marker used to evaluate OS-induced protein modification that results from interaction between ROS and protein (Witko-Sarsat et al., 1996; Shacter, 2000). Elevated levels of AOPPs were found to be associated with some pathological conditions, such as uremia (Witko-Sarsat et al., 1996), liver cirrhosis (Zuwala-Jagiello et al., 2009), acute coronary syndrome (Skvarilova et al., 2005), obesity (Krzystek-Korpacka et al., 2008), and diabetes mellitus (Cakatay, 2005).

AOPPs were suggested to be products of reaction of proteins with hypochlorous acid; a product of myeloperoxidase (MPO)-catalyzed reaction between hydrogen peroxide and chloride ion (Witko-Sarsat, et al. 1996; Capeillere-Blandin et al., 2004). Marsche et al., (2009) have indicated that this pathway contributes to up to 50% of the total AOPP produced. A more recent pathway for the production of AOPP was suggested by Servettaz et al, (2007) , which involves interaction of peroxynitrite with proteins.

While AOPPs are commonly used as biomarker of protein damage that is related to OS production, AOPPs were also found to induce production of free radicals, such as NO and SA, and act as mediators of OS (Witko-Sarsat, 1999). Added to that controversy

is the finding of Li et al. (2006) that AOPPs can act as inhibitors of NO production in mouse peritoneal macrophages.

1.8 Oxidative stress in DCA- and TCA-induced hepatotoxicity and hepatocarcinogenicity

DCA and TCA administration at single high doses to mice was found to induce the formation of thiobarbituric acid-reactive substances (TBARS) and 8-hydroxydeoxyguanosine (8-OHdG) in hepatic tissues, suggesting production of free radicals and /or ROS that lead to OS (Bull et al., 1990; Larson and Bull, 1992; Channel et al., 1998; Toraason et al., 1999, Hassoun et al. 2010, Austin et al., 1996). Also, acute administration of DCA and TCA to mice was found to induce production of SA, lipid peroxidation (LP) and DNA damage in hepatic tissues, after 6-12 h (Hassoun and Dey, 2008). Subacute and subchronic exposure of mice to DCA and TCA at doses ranging from those identified to be non-carcinogenic to those producing maximum hepatocarcinogenicity (DeAngelo et al., 1991) was also found to induce dose-dependent increases in production of SA, LP, and DNA damage in hepatic tissues (Hassoun et al., 2010), and also in modification of different hepatic antioxidant enzyme activities (Hassoun and Cearfoss, 2011). The studies have suggested OS as an early biomarker induced by DCA and TCA that can lead to the long term hepatotoxic and hepatocarcinogenic effects (Hassoun et al, 2010; Hassoun and Cearfoss, 2011).

Khan et al. (2009) have also demonstrated suppression of several hepatic antioxidant enzymes, such as GSH-Px, SOD, and CAT after subacute exposure of mice to trichloroethylene (TCE). TCE is an organic solvent that contaminates the surface water of several geographic areas and is known to be metabolized in vivo in animals and humans

to DCA and TCA (Merdink et al. 1998). Hence, it is believed that TCE-induced OS is contributed to DCA and TCA.

1.9 Oxidative stress in ethanol-induced hepatotoxicity and hepatocarcinogenicity

Ethanol is known to induce OS that is related to the production of free radicals and/ or ROS, especially in the liver, the main site for alcohol metabolism. Excessive alcohol consumption is found to be associated with production of hepatocellular carcinoma (Donato et al., 2002; Morgan et al. 2004). Hepatocellular carcinoma was found to be strongly associated with production of OS in the liver (Sasaki, 2006; Marra et al., 2011; Jo et al., 2011). Alcohol-induced OS was also found to be associated with other liver diseases such as alcoholic steatohepatitis and cirrhosis (Penny, 2013).

Several pathways have been proposed for induction of free radicals and ROS by alcohol. Alcohol is metabolized via several pathways generating acetaldehyde as the main metabolite, which is believed to be responsible for ROS induction (Bondy, 1992; Wu and Cederbaum, 2003), and suppression of antioxidants, such as glutathione, SOD, and GSH-Px (Schisler and Singh, 1989). This suggestion had been confirmed by studies of Videla et al. (1982) demonstrating hepatic glutathione depletion and LP in response to acetaldehyde administration to rats.

Jaeschke et al., 2002 have indicated that ethanol can result in expression of a cytochrome p-450 isoform that generates ROS, activates many toxicologically important substrates and may be the central pathway by which ethanol causes OS. This isoform was associated with CYP2E1 that is known to contribute to ROS production during alcohol metabolism (Wu and Cederbaum, 2003; Cederbaum, 2010). Alcohol-induced iron mobilization is another important oxidative pathway, since iron is involved in Fenton and

Haber-Weiss reactions that are responsible for ROS production (Stal et al., 1996; Valko et al., 2007). Alcohol also stimulates the movement of endotoxin to reach the liver leading to the production of SA via activation of TNF α in Kupffer cells (Wheeler et al., 2001; Wu and Cederbaum, 2003).

Chapter 2

Objectives

1. Dichloroacetate (DCA) and trichloroacetate (TCA) are known to induce hepatotoxic and hepatocarcinogenic effects *in vivo*, in rodents. Ethanol has also been proven to induce hepatotoxicity and hepatocarcinogenicity *in vivo*, in rodents, as well as in humans. DCA and TCA are present as by products in the drinking water, and chronic ethanol consumption has become a major concern among large segments of people, world-wide. Therefore, there is a great possibility of increasing the hepatotoxic/hepatocarcinogenic risks of the compounds upon simultaneous exposure to all of them through long term drinking of chlorinated water and alcohol consumption. This study was designed to test the outcomes of interaction between the three compounds *in vitro*, in Alpha Mouse Liver-12 cells.
2. While the *in vivo* toxicity tests are costly and time-consuming, the *in vitro* tests are much less costly and less time-consuming. In this study we used mouse hepatocytes (AML12 cells) as an *in vitro* model to achieve the goal of replacing, refining, and reducing the use of animals. The results generated will be used as basis for future *in vivo* studies that will need lower number of animals.

3. DCA, TCA, and ethanol are known to induce OS that result in hepatotoxic and hepatocarcinogenic effects through generation of ROS, such as SA. This study proposes a role for NO in the induction of these effects.
4. Induction of OS by DCA, TCA, and ethanol is known to be associated with the production of DNA damage and LP in the hepatic tissues. This study proposes a role for AOPP production, as well.

Chapter 3

Materials and Methods

The chemicals used for these studies The chemicals used for these studies were purchased from Sigma Aldrich (St. Louis, MO), at the highest grade available.

3.1 Alpha Mouse Liver-12 (AML-12) Cell Line

AML-12 is a hepatocyte line isolated from livers of Transforming Growth Factor (TGF α) – transgenic mice. The cell line has regular, flat, and polygonal cell shapes equipped with round nuclei, granular cytoplasm, peroxisomes and canalicular-like structures (Wu, 1994). The cells were purchased originally from the American Type Culture Collection (ATCC) (Manassas, VA), grown in our lab, and kept in liquid nitrogen. The medium used to culture the cells was 900 ml Dulbecco's Modified Eagle – Ham Medium (DMEM) enriched with 0.005 mg/mL insulin, 0.005 mg/mL transferrin, 40 ng/ml dexamethasone, 5 ng/ml selenium and 100 ml fetal bovine serum (FBS). Cells were cultured in 7 mL medium in flasks (Corning, NY), and incubated for 48 hours at 37°C in a humidified incubator containing 5% CO₂. The medium was then removed and 2.5 ml of 0.25% trypsin was added to the flask and incubated for 10 minutes to detach the cultured cells. Ten ml of medium were then added to each flask to neutralize trypsin, and cellular suspensions were then transferred to centrifuge tubes. Tubes were centrifuged for

10 minutes at 3000 rpm, and supernatants were then removed and 10 ml medium was added to each tube to re-suspend the cellular pellets. Cells were counted and 1.8 ml containing approximately 250,000 cells were added to 2 ml culture dishes. Dishes were incubated for 4 hours before they were treated.

3.2 Chemical Preparation and cellular treatment

Three chemicals were used for this study: dichloroacetate (DCA), trichloroacetate (TCA) and ethanol. Solutions of the chemicals were prepared in medium having a similar composition to that used for cell culture, and they were added to different culture dishes at a volume of 200 μ l to produce the following final concentrations:

- DCA: 4100, 1540, and 770 ppm
- TCA: 4100 , 1540 , 770, and 500 ppm
- Ethanol: 4%, 3%, 2%, 1.5% and 1%

Mixtures of the compounds were also prepared and added to different cultures at a volume of 200 μ l to produce final concentrations for DCA, TCA and ethanol of 770 ppm, 500 ppm and 1.5%, respectively.

Preliminary studies were conducted to test the effects of different concentrations of DCA, TCA and ethanol on cellular viability, where two cultures per treatment were used. Concentrations of DCA, TCA and ethanol producing approximately 25% of cellular death after 48 hours of incubation were used for the mixture studies. Four cell culture dishes/treatment were used to assess the effects of the followings:

- DCA 770 ppm
- TCA 500 ppm
- Ethanol 1.5%

- DCA 770 ppm + TCA 500 ppm
- DCA 770 ppm + ethanol 1.5%
- TCA 500 ppm + ethanol 1.5%
- DCA 770 ppm + TCA 500 ppm + ethanol 1.5%
- Control cultures were also used for the study, where 200 µl of medium were added to each dish.

Dishes were incubated for 48 hours at 37°C in a humidified incubator containing 5% CO₂. At the end of the incubation period, media were collected from each dish and were transferred to eppendorf tubes and frozen at -80°C. Cells were detached by scraping and were then suspended in 1 ml medium and counted. Cellular suspensions were transferred into eppendorf tubes and frozen at -80°C and used for the assays indicated in this study.

3.3 Determination of cellular viability

The number of viable cells was determined using the trypan blue staining method. Three hundred µl of 0.4% trypan blue in phosphate buffered saline was added to 300 µl of cellular suspensions and viable cells were counted 3 minutes later using hemocytometer under a light microscope.

3.4 Determination of Nitric Oxide (NO) Concentration

Nitric oxide was determined by the Griess reagent method (Griess, 1879). Griess reagent was prepared by mixing 0.1% n-(naphthyl) ethylene diamine and 1% sulfanilamide diluted in 5% phosphoric acid. A 0.5 ml of Griess reagent was then added to 0.5 ml of cellular suspension and the mixture was then incubated in a water bath at 37°C for 2 minutes. Reactions were then stopped by placing the reaction tubes on crushed

ice for 2 minutes. Absorbances of the mixtures were measured by Genesis 20® spectrophotometer (Thermo Fisher Scientific) at a wavelength of 543 nm. A blank (Griess reagent only) was also used and its absorbance was subtracted from the reading of each sample. The concentrations of NO were determined from a standard curve (Fig. 3) prepared follows:

Twelve concentrations of sodium nitrite were prepared from 30 mM stock solution. These concentrations were 5, 4, 3, 2, 1, 0.5, 0.25, and 0.125 μ M. Standard solution contained 1 ml of Griess reagent and 1 ml of sodium nitrite solutions. Mixtures were incubated at 37°C for 2 minutes and reactions were then stopped by placing the tubes in crushed ice for 2 minutes. The absorbances of the mixtures were then measured by Genesis 20® spectrophotometer at a wavelength of 543 nm against a blank containing Griess reagent. The equation of the standard curve was determined by regression and was used for determining the concentration of NO in each sample.

3.5 Determination of Advanced Oxidation Protein Products (AOPPs) Concentration

AOPPs were determined in cellular suspensions according to the method of Witko-Sarsat et. al. (1996). Aliquotes of cellular suspensions (200 μ l) were mixed with 800 μ l of Phosphate-Buffered Saline (PBS) containing 136.88 mmol/l NaCl, 2.68 mmol/l KCl, 8.10 mmol/l Na₂HPO₄, and 1.47 mmol/l KH₂PO₄, pH 7.2, followed by addition of 10 μ l of 1.16 M KI and 20 μ l acetic acid. Absorbances of mixtures were then determined by using spectrophotometer Genesis 20® (Thermo Fisher Scientific) at a wavelength of 340 nm. The absorbance of a blank containing PBS, CH₃COOH and 1.16 M KI was also

determined and subtracted from each reading. The concentrations of AOPPs were determined from a standard curve (Fig. 4) prepared as follows:

Eight concentrations of chloramine T were prepared from 1 mM stock solution. These concentrations were 100, 80, 60, 40, 20, 10, and 7.5 μM . One ml of each of the chloramine T solution was mixed with 10 μl of 1.16 M potassium iodide and 20 μl of acetic acid. Absorbances of the mixtures were then measured by Genesis 20® spectrophotometer at a wavelength of 340 nm against a blank containing 800 μl of PBS, 10 μl of 1.16 M potassium iodide and 20 μl of acetic acid. The equation of the standard curve was determined by regression and used for determining the concentrations of AOPP in the samples.

3.6 Determination of Superoxide Anion (SA) Concentration

Superoxide anion determination was based on the cytochrome c reduction method (Babior et al., 1973). A 50 μl of cellular suspensions were mixed with 1.5 mL of 0.05 mM cytochrome c oxidase in phosphate buffered saline (PBS), pH 7.2. The mixture tubes were incubated in a water bath at 37°C for 15 minutes and were then placed on crushed ice for 2 minutes to stop the reactions. Absorbances were measured at a wavelength of 550 nm, using Genesis 20® spectrophotometer (Thermo Fisher Scientific). Absorbances were converted into nmoles cytochrome c reduced/ min, using extinction coefficient of $4.76 \times 10^{-6} \text{ M}^{-1} \text{ cm}^{-1}$.

3.7 Statistical analysis

All data were presented as mean \pm SD of 4 cultures (samples)/ treatment. Data were compared using analysis of variance (ANOVA) and multiple t-test as post hoc test, and a significance level of $p < 0.05$ was used. Pearson's correlation coefficient and

multiple correlation coefficient were determined to assess correlations between the different studied biomarkers.

Chapter 4

Results

In an effort to select appropriate concentrations to test effects of mixtures of DCA, TCA and ethanol on cellular viability, control cells were found to be 99-100% viable, and cellular viability for the treated cells was calculated as % of control number, assuming control cells were 100% viable. Preliminary experiments were conducted to test different concentrations of the individual compounds using only two cultures/concentration. Results of these experiments with DCA, TCA and ethanol are demonstrated in figures 5,6 and 7, respectively. Concentration-dependent reductions in cellular viability were observed with all of the tested concentrations of the compounds. However, an approximate 25% reduction was observed with DCA, TCA and ethanol concentrations of 770 ppm, 500 ppm, and 1.5%, respectively (figures 5-7).

Figure 8 shows the effects of the individual compounds, as well as various mixtures of the compounds on cellular viability when added at concentrations producing 25% reduction of cellular viability by each. Significant reduction in cellular viability was produced in response to all of the treatments (individual compounds and mixtures), as compared with control, but no significant differences were observed between the effects

of the individual compounds when compared with each other. However, when the effects of binary mixtures were compared with each other,

DCA/ethanol and TCA/ethanol effects were found to be not significantly different, but they were greater than that of DCA/TCA mixture. Also, all of the effects of binary mixtures were found to be significantly greater than those of the individual compounds present in them. DCA/TCA/ethanol mixture effect was found to be significantly greater than any of the binary mixture effects.

Figure 9 shows the effects of individual compounds and mixtures of the compounds on NO production. Significant NO production was observed with all of the treatment groups when compared with the control. When comparing the effects of the individual compounds, ethanol effect was found to be significantly greater than those of DCA and TCA, and TCA was greater than that of DCA. Comparisons of the binary mixture effects with each other demonstrated no significant differences between DCA/ethanol and TCA/ethanol effects but the two mixtures effects were greater than that of the DCA/TCA. When comparing the binary mixture effects with those of the individual compounds present in them, TCA/ethanol effect was significantly greater than that of TCA but was not different from ethanol effect. However, DCA/ethanol, and TCA/ethanol effects were significantly greater than those of the individual compounds present in them. The DCA/TCA/ethanol mixture effect was found to be significantly greater than the effects of any of the binary mixtures.

The effects of the three compounds, binary mixtures of the compounds and a mixture of the three compounds on SA production are demonstrated in figure 10. Significant SA production was observed with all of the treatments when compared with

the control. When effects of the individual compounds were compared with each other, ethanol effect was found to be significantly greater than those of DCA and TCA, and DCA effect was significantly greater than that of TCA. When the binary mixture effects were compared with each other, TCA/ ethanol mixture effect was found to be significantly greater than those of ethanol/ DCA and DCA/TCA effects, and that of DCA/ethanol was greater than that of DCA/TCA. When the binary mixture effects were compared with those of the individual compounds present in them, TCA/ ethanol effect was found to be not significantly different from that of ethanol but greater than that of TCA. DCA/ethanol effect while was significantly lower than that of ethanol, it was not different from that of DCA. DCA/TCA effect was lower than that of DCA, but was not different from that of TCA. While the effect of DCA/TCA/Ethanol mixture was significantly greater than those of DCA/TCA and DCA/ethanol mixtures, it was not significantly different from that of TCA/ethanol mixture.

Figure 11 demonstrates the results of AOPPs production in response to treatment with individual compounds, binary mixtures of the compounds and a mixture of the three compounds. All the treatment groups resulted in significant AOPPs production when compared with the control. When effects of the individual compounds were compared with each other, ethanol effect was found to be significantly greater than those of DCA or TCA, and that of TCA was greater than DCA effect. Comparison of the binary mixtures effects with each other revealed significantly greater effect produced by TCA/ethanol mixture than those produced by DCA/ethanol or DCA/TCA mixtures, and that of DCA/ethanol was greater than DCA/TCA effect. Significantly greater effects produced by all of the binary mixtures when their effects were compared with those of the

individual compounds present in them. The effect of DCA/TCA/ethanol mixture was found to be significantly greater than any of the binary mixtures and of the individual compounds present in it.

Table 1 indicates correlations (R) between cellular viability and the various biomarkers, as well as between the biomarkers. The closer the R value to 1.0, the stronger correlation between the pair of variables will be, and negative R values indicate inverse relationships between the two variables. As can be seen, except for the correlation between SA and cellular viability that was revealed not to be strong, all other correlations were found to be strong, with those between NO and AOPP and NO, SA and AOPP are the strongest.

Chapter 5

Discussions

AML-12 cells were used in this study as an in vitro model to assess the effects of DCA, TCA, ethanol and mixtures of the compounds, because they possess organelles, such as round nuclei, granular cytoplasm, peroxisomes and canalicular-like structures similar to those of the humans (Wu, 1994).

One aim of this study was to determine possible interactivities between the compounds, where the types of these interactivities could be assessed. In environmental toxicology, there are three possible types of interactivities that may occur between different environmental pollutants: additive (net effect equal to the sum of the individual effects of the compounds), greater than additive effect (net effect is greater than the sum of the individual effects of the compounds), or less than additive (net effect is less than the sum of the individual effects of the compounds). Therefore, concentrations of the individual compounds were chosen so that each produces approximately 25% of maximal toxic response (cell death), and when the three are added together, they are expected to produce 75% effect of maximal response (if the effect is additive), > 75% of maximal response (if the effect is greater than additive), or < 75% of maximal response (if the

effect is less than additive). Had we chosen greater effects than 25% of maximal responses of individual compounds, greater than additive effects could not be assessed.

When added to AML-12 cells either individually, or in combination, DCA, TCA and ethanol produced significant reductions in cellular viability. These results are in line with those demonstrating induction of hepatocytes death by DCA, and TCA (Walgren et al. 2005, and by ethanol (Day, 2006; Jaeschke et al. 2002.). The significant decreases in cellular viability in response to the mixtures, as compared with the individual compounds may indicate additive or greater than additive effects. For example, DCA/TCA mixture induced approximately 50% cell death, which can be assessed as additive effect, while the three other mixtures produced > 75 % cellular death that can be assessed as greater than additive effect. Since the mixtures that produced greater than additive effects contained ethanol, as opposed to the DCA/TCA mixture that contained no ethanol, the greater than additive effect may be mainly attributed to ethanol.

The results of the study indicate induction of high levels of NO in hepatocytes, in response to the individual compounds, as well as to the mixtures. The results also revealed a strong correlation coefficient between reduction in cellular viability and NO production in response to the different treatments, suggesting a significant role of this NO in induction of cellular toxicity by the different treatments. The role of NO in hepatocyte toxicity was indicated by Richter et al (1994) who reported NO-induced mitochondrial de-energizing that was reversible at low, but was long-lasting at higher concentrations of this ROS. Also, the role of NO in liver diseases associated with chronic alcohol consumption has been assessed. For example, high NO levels have been demonstrated in livers of rats after chronic alcohol consumption (Wang et al, 1995), and high NO levels

are produced in Kupffer cells and hepatocytes of patients with alcoholic hepatitis (Hunt and Goldin, 1992). The mechanism by which chronic alcohol consumption increases NO levels has been suggested to be through lipopolysaccharide and several cytokines stimulation that are known to be NO inducers (Bode et al., 1987, Bigatello et al., 1987, Honchel et al., 1992). High NO production was also found to interfere with the activity of alcohol dehydrogenase, affecting alcohol metabolism (Gergel and Cederbaum, 1996). Further, combined effects of NO and ethanol was found to contribute to an increased susceptibility of mouse liver mitochondria to hypoxia through interference with the cytochrome c oxidase activity, leading to the deleterious effects of alcohol on liver (Zelickson et al., 2011). While the role of ethanol-induced NO production and hepatotoxicity has been assessed by several studies, the role of NO production by DCA and TCA is not known yet. However, our results show that ethanol and ethanol-containing mixtures induced significantly greater levels of NO in hepatocytes, as compared with those induced by DCA, TCA and TCA/DCA mixtures. These results may indicate a more significant contribution of ethanol than DCA and TCA to the production of NO, possibly via one of the aforementioned mechanisms (Bode et al., 1987, Bigatello et al., 1987, Honchel et al., 1992, Gergel and Cederbaum, 1996, Zelickson et al., 2011)

SA production was significantly increased in response to the individual compounds, as well as in response to mixtures of the compounds. These results are in line with studies demonstrating high SA production in response to DCA and TCA in the hepatic tissues of mice (Hassoun et al., 2011, Hassoun and Dey, 2008), and also with those demonstrating the role of ethanol and acetaldehyde in SA production in hepatic stellate cells (Novitskiy et al., 2006) and in isolated hepatocytes (Rajasinghe et al., 1990),

as well as in steatotic hepatocytes (Baraona et al, 2002). However, ethanol induced significantly greater levels of SA than those induced by DCA and TCA. Studies in mice have shown that DCA resulted in significant SOD induction hepatic tissues when administered at hepatotoxic doses, while TCA resulted in SOD induction at all tested doses (Hassoun et al. 2010). Also, recent in vitro studies in our lab in AML 12 cells have demonstrated significant SOD induction in response to DCA and TCA concentration in the range of those used for this study (Mettling, 2011). SOD results in SA dismutation, converting it to H₂O₂ (Davies, 1995). Therefore, the less significant induction of SA by DCA and TCA, as compared with ethanol can be due to SOD stimulation by the two compounds and the resultant conversion of SA to H₂O₂. Conversely, ethanol might have resulted in less stimulation, no stimulation, or inhibition of that enzyme activity. Modulation of SOD activity may have also contributed to the observed levels of SA in response to the binary mixtures, as well as to the mixture containing the three compounds. The non strong correlation between SA and cellular viability may also be attributed to lowering SA concentrations by SOD and can suggest a contribution of other species formed from SA, such as H₂O₂ to the compounds-induced cellular toxicity. Further studies to assess the role of this enzyme in the effects of the individual compounds, as well as the mixtures are required to confirm that.

The results of this study show significant increases in AOPP in response to the individual compounds, as well as to mixtures. AOPPs are a specific biomarker expressing certain level of protein damage leading to cellular toxicity (Witko-Sarsat et al., 1996). This can be confirmed by the strong correlation that was observed between AOPP and reduction in cellular viability in response to the different treatments. Also, peroxynitrite is

generated from reaction between NO and SA, and it has been proposed as one of the pathways responsible for AOPP production (Servettaz et al. 2007). This has been assessed in this study by determining the correlation between SA and NO, as well as between SA, NO and AOPP, which indicated strong correlations between all of those biomarkers. In addition, the contribution of AOPP to the observed reduction in cellular viability is confirmed by the strong correlation found between AOPP production and reduction in cellular viability, in response to the different treatment. Baraona et al. (2002) have indicated that ethanol consumption can increase the cytosolic activity of inducible NO synthase (iNOS), and also induce microsomal cytochrome p-450 that is capable of producing both NO and SA, and that when ethanol was consumed by rats for 24 days, it was associated with increases in nitrotyrosine protein residues, products of peroxynitrite toxicity that occurred predominantly in steatotic hepatocytes. Laskin et al, (2001) have indicated that NO donors or peroxynitrite can mimic the cytotoxic actions of liver toxins, such as ethanol, agents that prevent the generation of NO, antioxidants that bind reactive nitrogen intermediates, or knockout mice with reduced capacity to produce NO are protected from xenobiotic-induced tissue injury.

Therefore, the increases in AOPP may be due to DCA-, TCA- or ethanol-induced SA, NO or the products of reaction of SA with NO (peroxynitrite), which are all confirmed by the strong correlations between these biomarkers.

In conclusion, induction of production of NO, SA and AOPP by DCA, TCA and ethanol can all contribute to the observed cellular toxicity of the compounds and mixtures of the compounds. While NO and AOPP are suggested to have major contributions to the observed reduction of cellular viability, SA may have less direct contribution to that, but

may contribute through acting as a source for generation of other ROS, such as H₂O₂ and the peroxynitrite. While the effects observed in cells treated with binary mixtures are viewed as additive, the effect induced by the mixture containing the three compounds can be viewed as greater than additive. These interactivities could be attributed to changes in the concentrations of SA, NO and possibly the peroxynitrite in response to possible modulations of by the compounds of iNOS, SOD, or cytochrome p-450 enzymes. However, future studies to assess the roles of these enzymes in the mixtures outcomes are required to confirm that.

Chapter 6

Recommendations for Future Studies

1. Determination of myeloperoxidase Determination of myeloperoxidase, in order to assess its role or contribution to AOPP production, in response to DCA, TCA , ethanol and mixtures of the compounds
2. Determination of SOD activity in response to DCA, TCA, ethanol and mixtures of the compounds.
3. Determination of inducible-nitric oxide synthase (iNOS), in response to DCA, TCA , ethanol and mixtures of the compounds
4. This work can be expanded to test other haloacetates present as by-products in the chlorinated drinking water, as well as more complex mixtures of these compounds.
5. The study can be used as basis to test mixtures with the most significant effect in vivo, probably in mice. This is important since in vitro studies do not count for the contribution of the compounds toxicokinetics to the outcome of their toxic effects. However, those experiments would be minimal since the in vitro tests would screen the compounds and the mixtures to identify the most significant ones that need to be further tested.

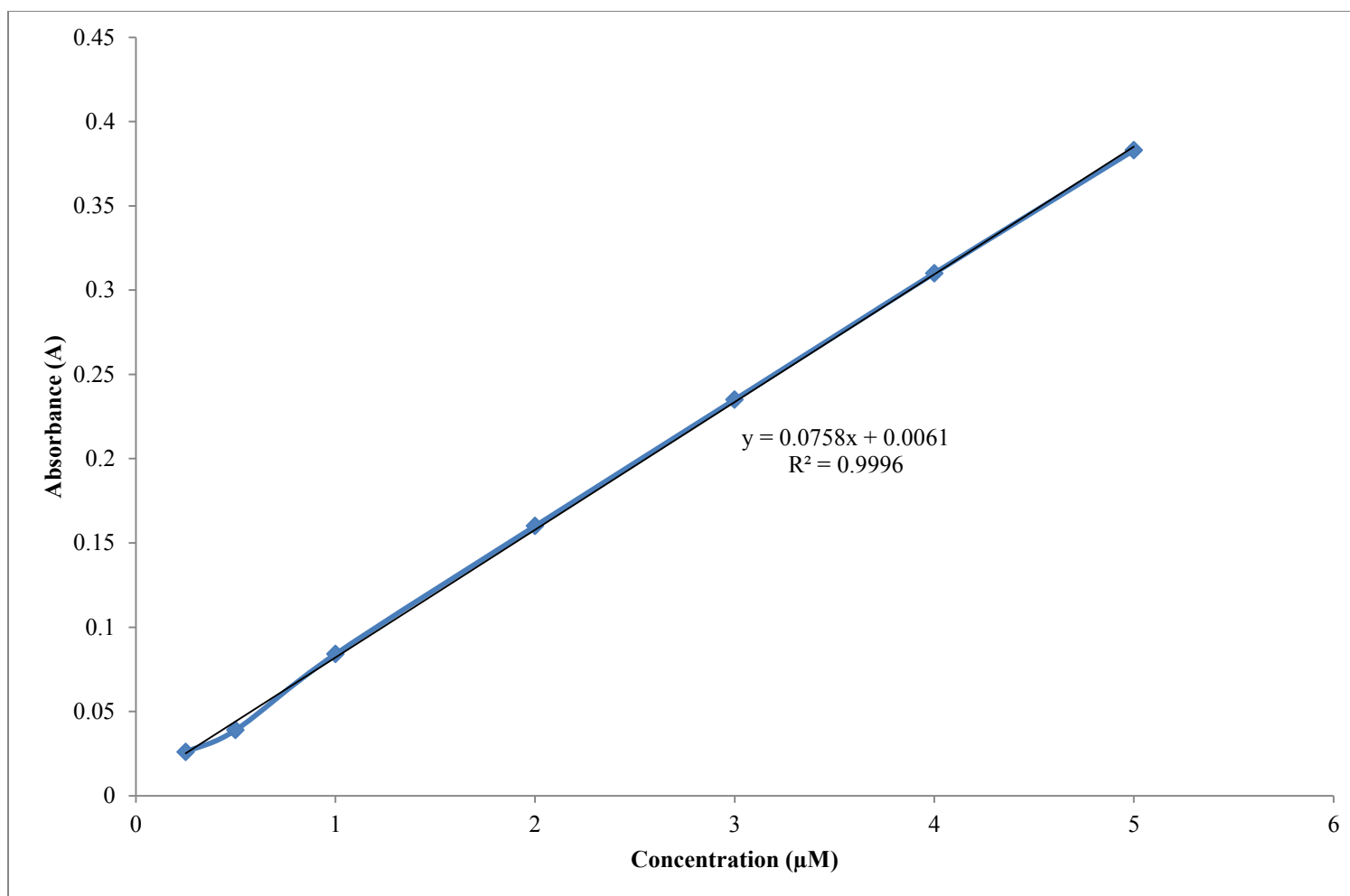


Figure 3. Standard curve of nitric oxide using sodium nitrite

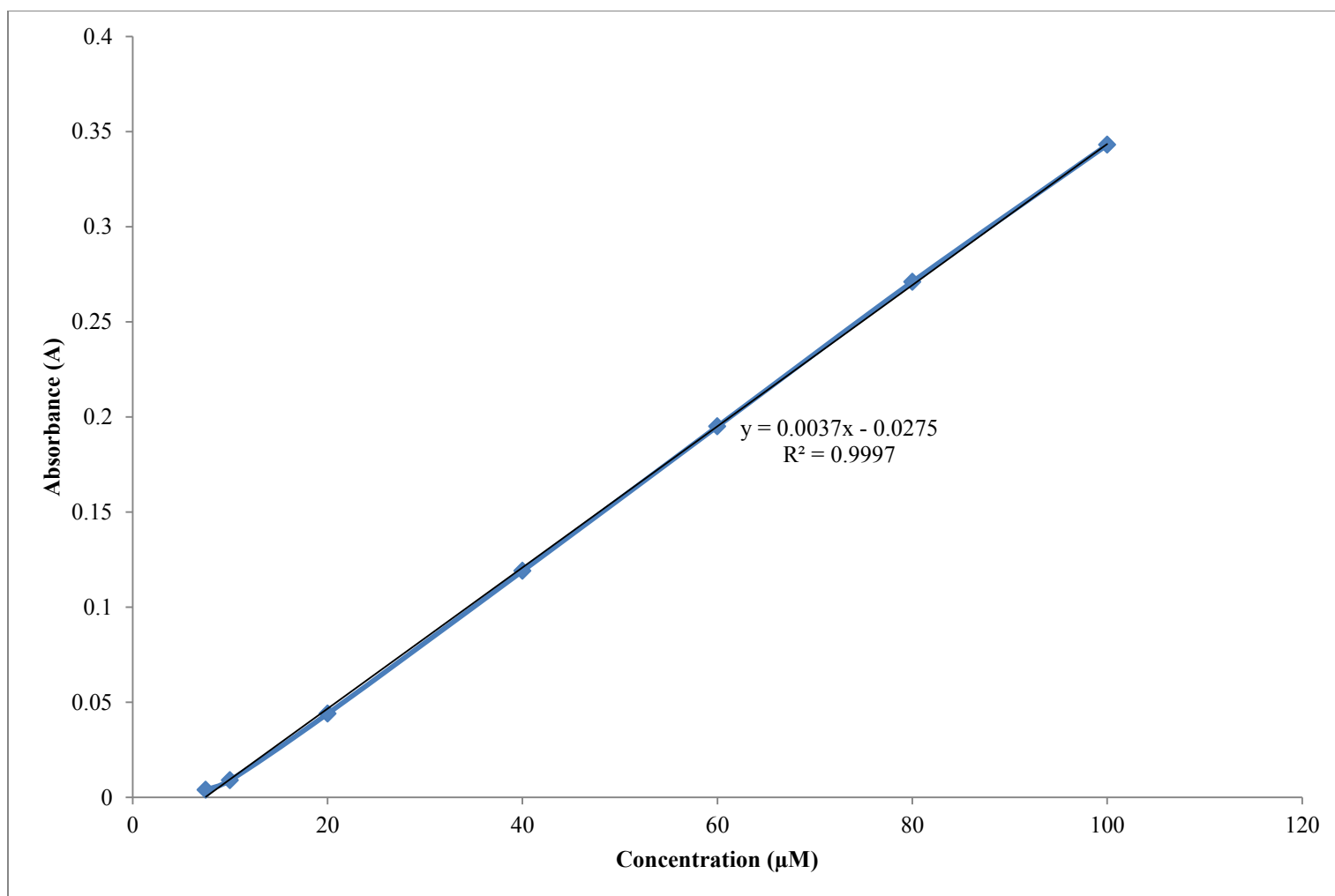


Figure 4. Standard curve of advanced oxidation protein products (AOPPs)

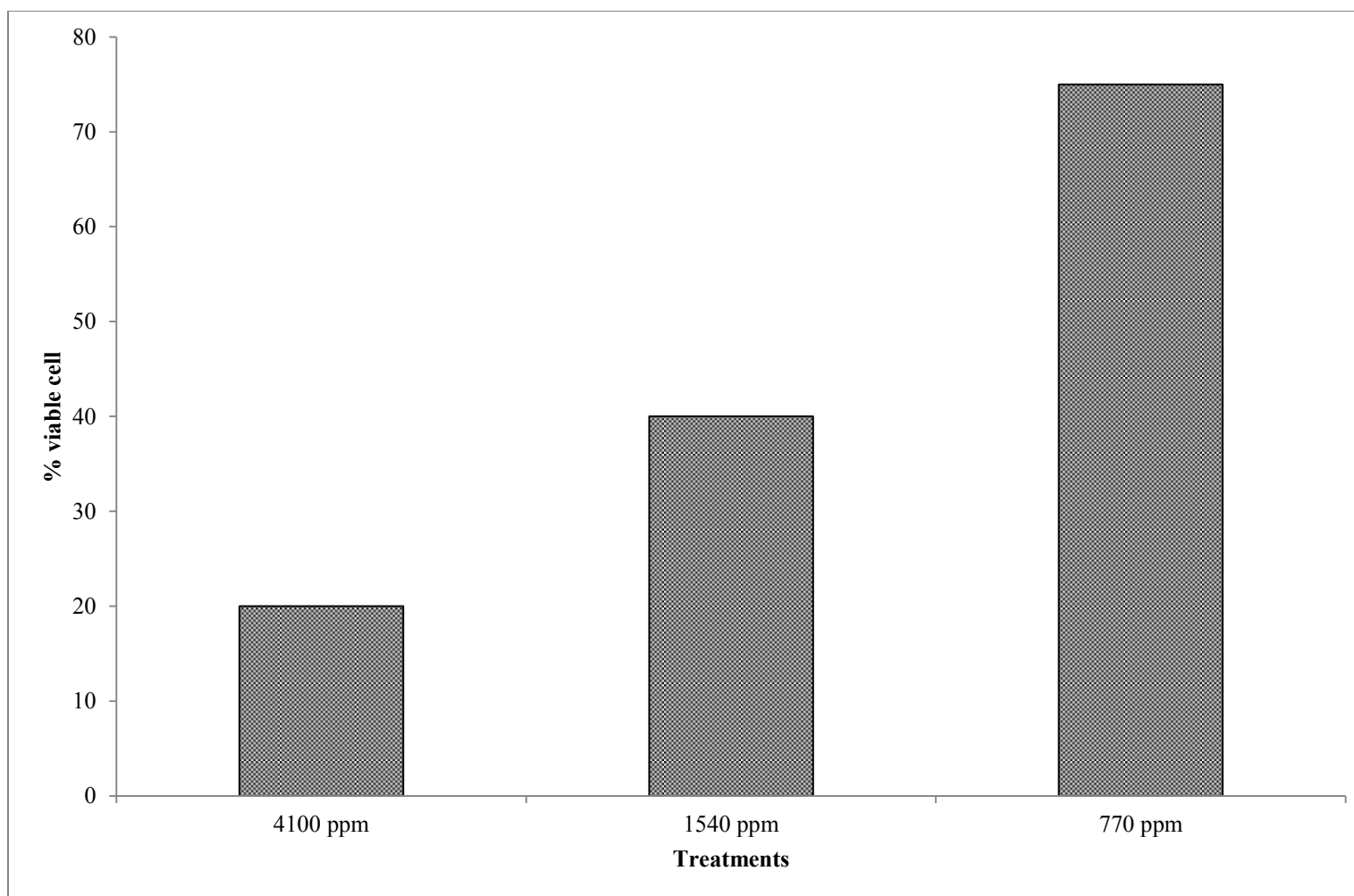


Figure 5. Cellular viability (percent of control, assuming control cells are 100% viable) in response to treatment with various concentrations of DCA for 48 h (2 cultures/concentrations).

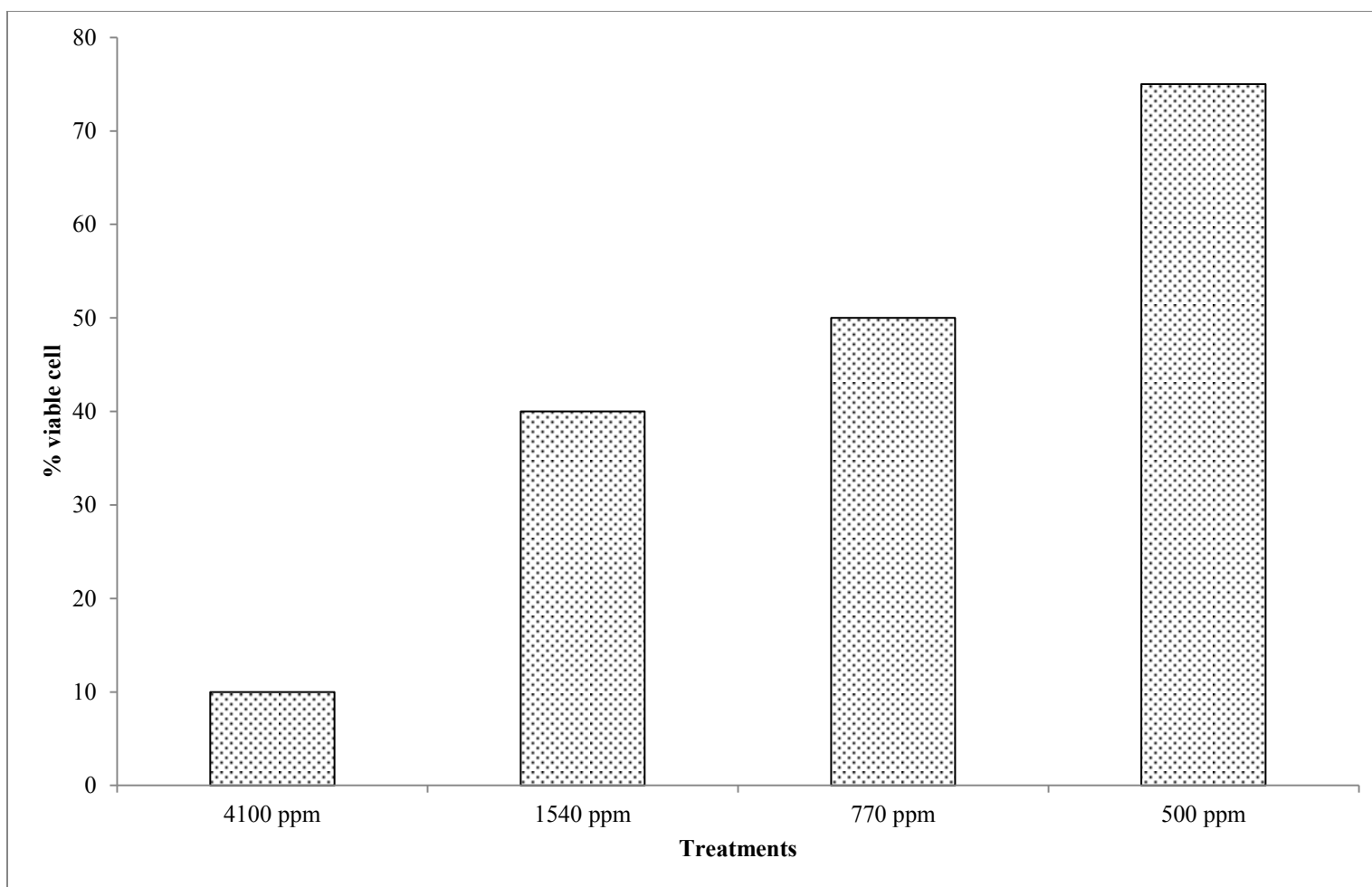


Figure 6. Cellular viability (percent of control, assuming control cells are 100% viable) in response to treatment with various concentrations of TCA for 48 h (average of 2 cultures/ concentrations)

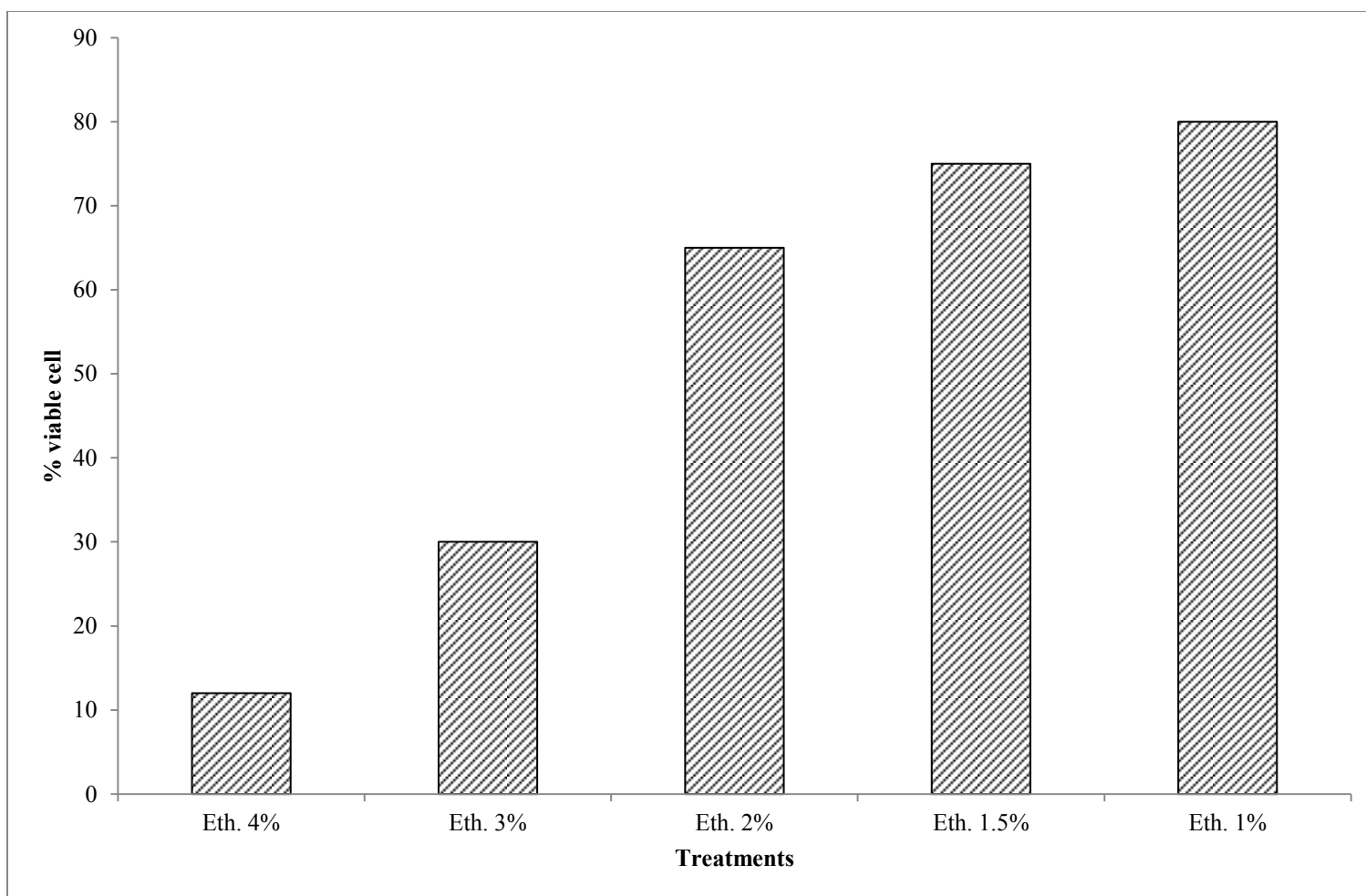


Figure 7. Cellular viability (percent of control, assuming control cells are 100% viable) in response to treatment with various concentrations of ethanol for 48 h (average of 2 cultures/ concentrations)

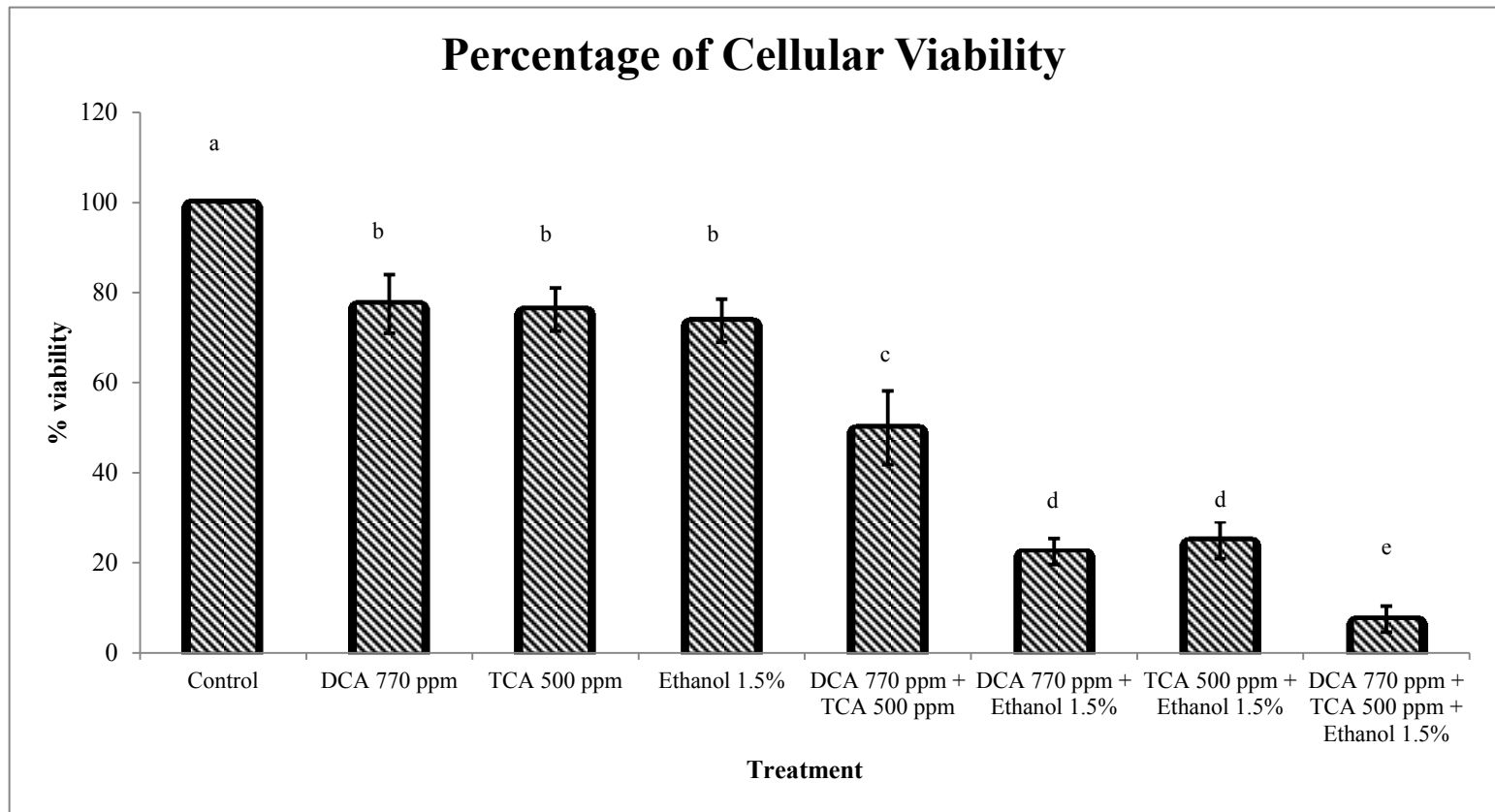


Figure 8. Cellular viability, calculated as % of control (assuming control cells are 100% viable), in response to treatment with DCA, TCA, ethanol, and mixtures of the compounds for 48 hours. Columns with different superscripts are significantly different, using single factor ANOVA with $p < 0.05$

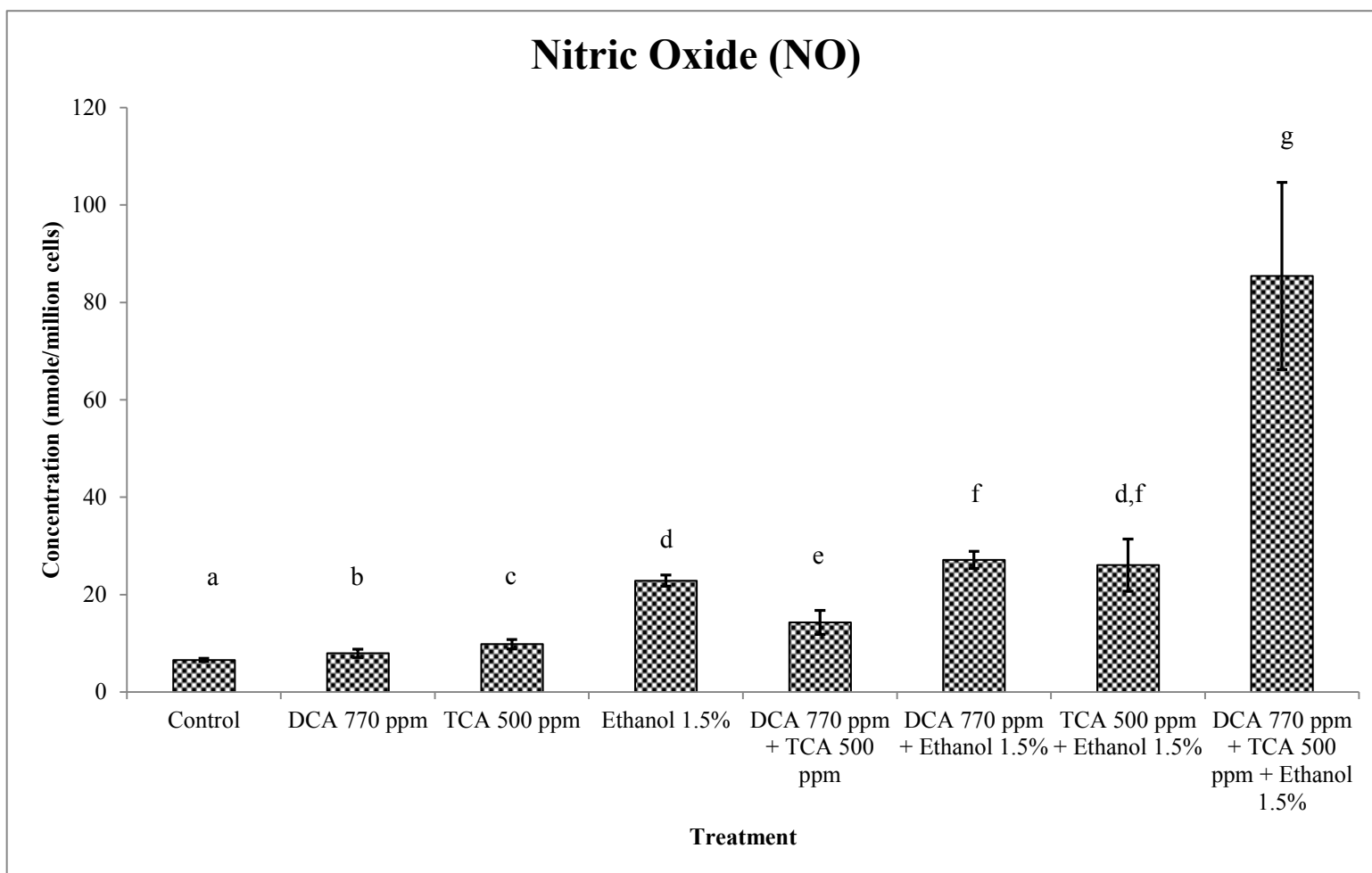


Figure 9. Nitric oxide production in response to treatment with DCA, TCA, ethanol, and mixtures of the compounds for 48 hours. Columns with different superscripts are significantly different, using single factor ANOVA with $p < 0.05$

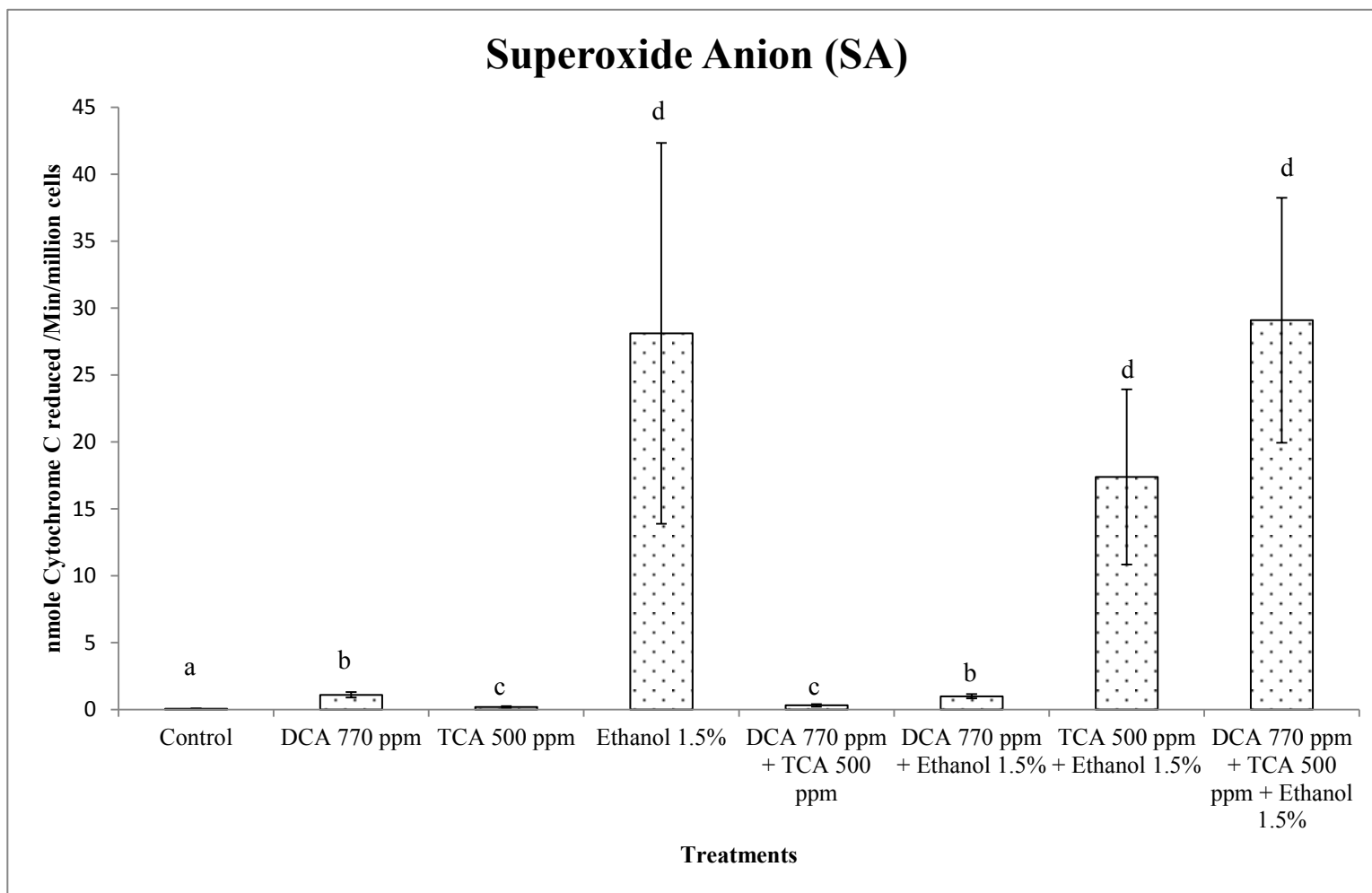


Figure 10. SA production (determined as cytochrome c reduced/min) in response to treatment with DCA, TCA, ethanol and mixtures of the compounds for 48 hours. Columns with different superscripts are significantly different, using single factor ANOVA with $p < 0.05$

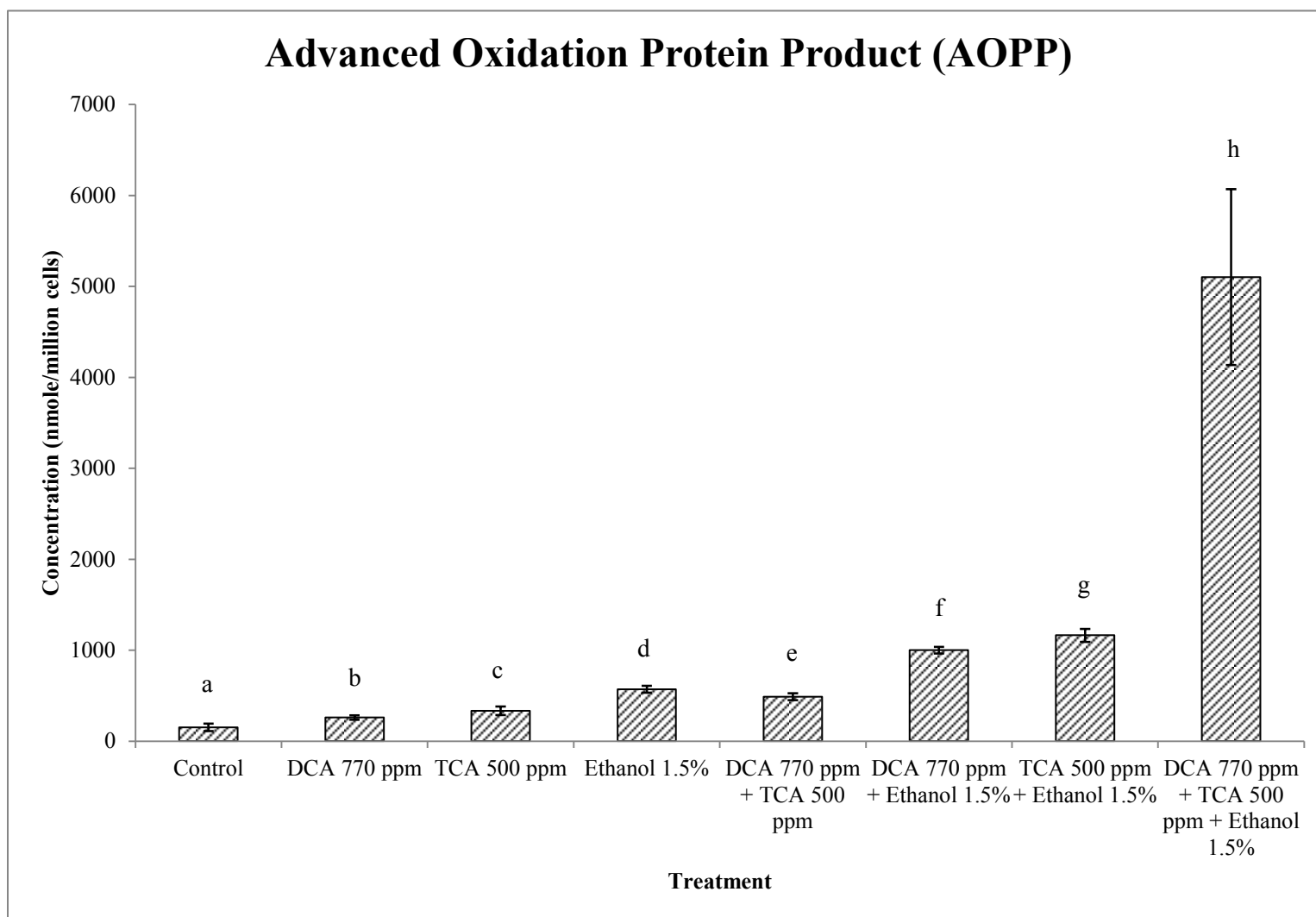


Figure 11. AOPP produced in response to treatment with DCA, TCA, ethanol and mixtures of the compounds for 48 hours. Columns with different superscripts are significantly different, using single factor ANOVA with $p < 0.05$

Table 1. Correlation between the biomarkers and cellular viability and also between the different biomarkers. R values for (1-6) were determined as Pearson's correlation coefficient, while R value between NO, SA and AOPP (7) was determined as multiple correlation coefficient

No	Biomarkers	Correlation coefficient (R)
1	NO and % viability	-0.7585
2	SA and % viability	-0.4342
3	AOPP and % viability	-0.7232
4	NO and AOPP	0.9899
5	SA and AOPP	0.6566
6	NO and SA	0.7183
7	NO, SA and AOPP	0.993

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