2024

COMBINED CYTOTOXICITY OF THE HERBICIDE GLYPHOSATE AND THE FUNGICIDE MANCOZEB IN MOUSE NEUROBLASTOMA CELLS

Heidi Ebid

COMBINED CYTOTOXICITY OF THE HERBICIDE GLYPHOSATE AND THE FUNGICIDE MANCOZEB IN MOUSE NEUROBLASTOMA CELLS

A dissertation submitted in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

to the faculty of the

DEPARTMENT OF PHARMACEUTICAL SCIENCES

of

COLLEGE OF PHARMACY AND HEALTH SCIENCES

at

ST. JOHN'S UNIVERSITY
New York
by
Heidi Ebid

Date Submitted 01/16/2024 Date Approved 01/30/2024

_____	_____
Heidi Ebid	Dr. Louis D. Trombetta

ABSTRACT

COMBINED CYTOTOXICITY OF THE HERBICIDE GLYPHOSATE AND THE FUNGICIDE MANCOZEB IN MOUSE NEUROBLASTOMA CELLS

Heidi Ebid

The herbicide glyphosate and the fungicide mancozeb are globally applied on a variety of agricultural crops. Residues of both pesticides are frequently detected in food and water. Combined exposure to both compounds is therefore possible. Glyphosate and mancozeb have been separately investigated in previous studies and were shown to induce neurotoxicity. The current study investigates the cytotoxicity of different glyphosate and mancozeb combinations including mixture of mancozeb + glyphosate, combination of mancozeb followed by glyphosate and combination of glyphosate followed by mancozeb and compares neurotoxicity seen with the combinations to that of glyphosate or mancozeb using mouse neuroblastoma Neuro-2a cells. Significant decreases in cell viability were observed with glyphosate (500-10,000 μM), mancozeb (4-10 μM) and with glyphosate (100 μM) and mancozeb (6 μM) combinations. Glyphosate and mancozeb combinations resulted in greater decrease in cells viability than that induced by the same concentration of glyphosate when tested alone. Light microscopy observations were consistent with cell viability data. Scanning electron microscopy (SEM) observations showed more prominent morphological alterations in glyphosate and mancozeb combination groups compared to

either glyphosate or mancozeb including, neuretic processes disruptions, loss of retraction fibers, blebbing and distorted plasmalemma. Metal analysis was done using Inductively Coupled Plasma-Optical emission spectroscopy (ICP-OES). Mancozeb and combinations of glyphosate and mancozeb resulted in intracellular elevations in copper, manganese and zinc levels. Intracellular metal levels elevations caused by combination treatments were induced at a glyphosate concentration that didn't affect metal levels when glyphosate was tested alone. Mancozeb and combinations of glyphosate and mancozeb induced a decrease in GSH/GSSG ratio. Combinations of glyphosate and mancozeb induced a greater decrease in GSH/GSSG ratio than that exerted by glyphosate alone. Antioxidant post-treatment with butylated hydroxytoluene did not alleviate cytotoxicity of either mancozeb or glyphosate + mancozeb mixture. These findings concluded that mancozeb and combinations of glyphosate and mancozeb cause cytotoxicity in Neuro-2a cells through inducing intracellular metal levels elevations leading to redox balance disruptions and oxidative stress. It is further concluded that glyphosate and mancozeb combinations induce greater cytotoxicity, more intracellular metal elevations and redox balance disruptions in Neuro-2a cells than glyphosate alone.

DEDICATION

This thesis is dedicated to my husband Ammar and my children Taha, Yunus and Sidra. Your Companionship added meaning to every moment and step of this journey, and gave birth to an added level of determination even prior to his own.

ACKNOWLEDGMENTS

My deepest and most sincere gratitude goes to Dr. Louis D. Trombetta, my mentor and guide throughout my journey at St. John's University. His constant guidance, encouragement and support has made this dissertation possible. Most importantly was what I learned from him over the years for which I will be eternally thankful.

I would like to thank my committee members Dr. Joseph Cerreta, Dr. Sue Ford, Dr. Diane Hardej, and Dr. Blase Billack for their invaluable advice and their guidance in shaping this project. I would also like to thank Dr. Nitesh Kunda for serving as the Chair of my defense committee.

My gratitude goes to my lab mates, Mumtaz Akhtar, Ali Shohatee and Olivia Stephenson for their friendship, encouragement and continuous support throughout the ventures of this research.

Thank you to my friends from the Graduate Program, Amanda, Rachel, Ben, Belkys, Randy and Jeff who have helped me maintain my drive and inspiration to keep going through toughest of times.

I would like to thank the PHS department for awarding me the opportunity to be a part of its program and for all their support.

Thank you to my beloved parents, Hanaa and Abdelhameed Ebid, for their unconditional love and support that has helped me define who I am and constantly bring out my best to cross yet another milestone.

My gratitude goes to my aunt, Hasnaa Abdelrahman, for her boundless love and support throughout the years. Will forever be grateful and there will never be enough words to describe my indebtedness to her.

Lastly, I would like to thank my husband, Ammar. His support and encouragement were always to be found, especially when it mattered most. From him I was able to draw the inspiration and confidence to take on each challenge and overcome every obstacle.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1. INTRODUCTION

1.1 Pesticide Combinations

Pesticides are compounds used to enhance agricultural crop production by preventing and mitigating pests, weeds and plant diseases (Sharma et al., 2019; Tudi et al., 2022). There are several types of pesticides targeting a wide range of unwanted organisms including insecticides, fungicides, herbicides, and rodenticides (Tudi et al., 2021). As reported by U.S. Environmental Protection Agency (US EPA), pesticide consumption was about 6 billion pounds globally and around 1.1 billion pounds in the United States in the years 2011 and 2012 (Atwood & Paisley-Jones, 2017). Due to their extensive use, residues of pesticides are frequently found in water, food and soil (Crépet et al., 2013). In 2020, about 27% of fruits and vegetables analyzed in Europe and around 48% of food products tested in the United States contained residues of two pesticides or more (EFSA, 2022; USDA, 2022). Surface water sampling from different regions across the United States, showed detection of an average of 17 pesticide per site between the years 2013 and 2017 (Stackpoole et al., 2021). Humans are therefore continuously exposed to various combinations of pesticides (Jellali et al., 2018).

Exposure to pesticides has been associated with carcinogenicity, endocrine disruption, immunotoxicity and neurodegeneration (Blair & Zahm, 1995; G. H. Lee & Choi, 2020; Mnif et al., 2011; Vellingiri et al., 2022). Most toxicological research evaluates the effects of one pesticide at a time, recently however more research has been directed towards studying the impacts of exposure to environmentally relevant combinations of pesticides on human health. Evidence shows pesticides in combinations

result in effects that are different from those reported when pesticides are tested individually (Gómez-Giménez et al., 2018; Gordon et al., 2006). Astiz et al. (2009) observed more pronounced antioxidant disruptions in brain, kidneys, liver and plasma of Wistar rats treated with combinations of glyphosate, dimethoate and zineb compared to rats treated with each pesticide separately (Astiz, de Alaniz, et al., 2009). Combinations of aldicarb, atrazine and nitrate resulted in endocrine, immune, and behavioral changes in mice that were not reported when pesticides were tested separately (Jaeger et al., 1999). Atrazine, chlorpyrifos and endosulfan mixture induced higher cytotoxicity on human primary hepatocytes compared to each of these compounds alone (Nawaz et al., 2014). Fan et al. (2021) reported higher mortality rate and increased deformities in zebra fish embryos as a result of dimethomorph and difenoconazole combinations compared to either dimethomorph or difenoconazole alone (Fan et al., 2021). Spatial memory impairments in male rats, associative learning deteriorations in female rats, and hyper motor activity in male and female rats were noted upon exposure to cypermethrin and endosulfan combinations and not each pesticide alone (Gómez-Giménez et al., 2018).

1.2 Glyphosate

Glyphosate or N - (phosphonomethyl) glycine is a non-selective systemic herbicide (Duke & Powles, 2008; USEPA, 1993, 2015). Glyphosate was synthesized in 1950 by Henri Martin, a Swiss chemist at Cilag pharmaceutical company (Kőmíves & Schröder, 2016; Duke & Powles, 2008). In 1960, glyphosate was patented by Stauffer Chemical company as a metal chelator (Kőmíves & Schröder, 2016). It, however, wasn't until 1970 when the herbicidal activity of glyphosate was tested and discovered by a

Monsanto Co. chemist named John E Franz (Duke & Powles, 2008). Glyphosate was then patented for herbicidal use and in 1974 the first glyphosate-based formulation "Roundup®" was commercially available (Benbrook, 2016; Kőmíves & Schröder, 2016; Duke & Powles, 2008).

The herbicidal activity of glyphosate is mainly due to the inhibition of 5-enoylpyruvyl-shikimate-3-phosphate synthase (EPSPS) enzyme and therefore it interferes with the shikimate pathway and the biosynthesis of several aromatic amino acids including tryptophan, tyrosine, and phenylalanine (Kőmíves & Schröder, 2016). In addition to EPSPS inhibition, glyphosate is believed to chelate important co-factors that are required for antioxidant enzymes (Gomes et al., 2014). Glyphosate – mediated reduction in aromatic amino acids as well as metal chelation results in oxidative stress, photosynthesis inhibition and plant growth retardation (Gomes et al., 2014; Kőmíves & Schröder, 2016; USEPA, 2015).

Following its initial introduction to the market, the use of glyphosate as a herbicide was limited due to its non-selective nature where it wasn't only killing weed but it was also destroying agricultural crops (Dill, 2005; Kőmíves & Schröder, 2016). In 1996, however, with the introduction of glyphosate resistant crops (GRCs) developed through genetic modification of EPSPS enzyme, a massive increase in glyphosate use as a herbicide was achieved (Dill, CaJacob, Padgette, 2008; Dill, 2005; Duke, 2005). Currently, glyphosate is the most widely used herbicide in the world (Duke & Powles, 2008; Woodburn, 2000). In U.S.A., the annual use of glyphosate on agricultural crops is estimated to be around 280 million pounds mainly on soybeans, corn and cotton (USEPA,

2019). Glyphosate is also used for non-agricultural purposes including weed control in residential areas, railways, roadways, forestry and aquatic systems (USEPA, 2015, 2019). As a result of being excessively applied, glyphosate residues have been detected in food products such as flour, oats, cereals, and bread, in drinking water as well as daily used items such as tampons and medical gauze (Torretta et al., 2018). Glyphosate have been detected in organic and non-organic honey in concentrations exceeding the Limit of Quantification (LOQ) (Rubio et al., 2014). Traces of glyphosate have also been reported in human urine samples (Grau et al., 2022).

For years, the use of glyphosate has been backed up with assertions claiming its safety to mammals and that it only targets the shikimate pathway which is absent in mammalian cells. These claims however have been disputed by recent studies showing wide range of effects in response to exposure to glyphosate and glyphosate - based formulations (Benedetti et al., 2004; Cattani et al., 2014; Dallegrave et al., 2003; De Liz Oliveira Cavalli et al., 2013a; Dedeke et al., 2018; Gasnier et al., 2009; Gui et al., 2012a; Marrie, 2004; Myers et al., 2016; Peixoto, 2005; Romano et al., 2012a; Tizhe et al., 2014; Wunnapuk et al., 2014; L. Zhang et al., 2019). In rats, glyphosate - based formulations induced histopathological changes in hepatocytes, degeneration of hepatic cells in portal areas as well as leakage of hepatic aspartate amino transferase (AST) and alanine amino transferase (ALT) (Benedetti et al., 2004; Tizhe et al., 2014). Histopathological changes in renal tissues, glomerular degeneration, tubular necrosis and renal biomarkers alterations were all manifested in rats exposed to glyphosate- based formulations (Dedeke et al., 2018; Tizhe et al., 2014; Wunnapuk et al., 2014). Gestational exposure of Wistar rats to glyphosate – based formulation, Roundup® was associated with increased

mortality and abnormal skeletal development in dams (Dallegrave et al., 2003). Gestational exposure to Roundup® also resulted in behavioral changes, increased testosterone and estradiol levels, and histological changes in seminiferous epithelium in male rats offsprings (Romano et al., 2012). In 2015, the International Agency for Research on Cancer (IARC) classified glyphosate as "probably carcinogenic to humans (group 2A)" based on epidemiological evidence showing a link between occupational exposure to glyphosate – based formulations and non-Hodgkin lymphoma (Fan et al., 2021; Myers et al., 2016). The EPA and EFSA however opposed the IARC classification and regarded glyphosate as not likely to "be carcinogenic to humans" (Benbrook, 2019; Weisenburger, 2021).

1.3 Mancozeb

Dithiocarbamates (DTCs) are a group of organic sulfur compounds that are mainly used as pesticides. Most DTC compounds with pesticidal properties have been introduced during and after World War II, a few DTCs however including thiram and ziram were developed earlier in the 1930s (World Health Organization, 1988). DTCs are highly effective against plant diseases caused by fungi and are therefore widely applied as fungicides (World Health Organization, 1988). In addition to their agricultural significance, DTCs have a number of industrial purposes including their use as accelerators for rubber vulcanization, as slimicides for water cooling systems, as metal scavengers in waste water treatment as well as in pulp and paper manufacturing (World Health Organization, 1988). DTCs are also used in clinical settings for the treatment of

chronic alcoholism, in cancer therapy and as antimicrobials (Kaul et al., 2021; Szolar, 2007).

Based on chemical structure, DTCs can be classified into thiurams, dimethyl dithiocarbamates (DMDCs), ethylene bis-dithiocarbamates (EBDCs) and propylene bis-dihiocarbamate (PBDCs) (Liesivuori and Savolainen, 1994).

Mancozeb is an EBDC compound that is linked to transition metals, manganese and zinc, with manganese to zinc ratio of 9:1 (USEPA, 2005). Mancozeb was first registered in the United States in 1948 to be used as a non-selective contact fungicide (Hoffman & Hardej, 2012; USEPA, 2005). The Fungicidal activity of mancozeb is mediated by its ability to attack sulfhydryl group – containing enzymes and disruption of cell metabolism in the target organism (Gullino et al., 2010; USEPA, 2005). Currently, mancozeb is amongst the 20 most globally used pesticides (Maggi et al., 2019). In the United States, an average of 7 million pounds of mancozeb are applied annually on agricultural crops (USGS, 2022). Mancozeb is applied over a wide variety of agricultural crops including pears, tomatoes, cucumbers, onions, rice, grapes, wheat, and cotton, with the highest rate of application on potatoes and apples. Mancozeb is also used on ornamentals, in residential areas and in golf courses (USEPA, 2005).

Being extensively applied, environmental exposure to mancozeb and other EBDCs is not impossible. Residues of EBDCs have been detected in food and tobacco products (Rossi et al., 2006). Mancozeb and other DTCs have been found in levels exceeding Maximum Residue Limit (MRL) in fruits and vegetables especially lettuce and tomatoes (Kaye et al., 2015; López-Fernández et al., 2013). Traces of mancozeb exceeding the

acceptable daily intake (ADI) were reported by the European Food Safety Authority (EFSA) in samples of pears and oranges (EFSA, 2022). Increased urinary excretion of mancozeb metabolite, ethylene thiourea (ETU), and elevated levels of manganese in urine among workers exposed to mancozeb were also documented (Colosio et al., 2002; Dall'Agnol et al., 2021; Maroni et al., 2000).

For the past few years, reregistration of mancozeb has been under scrutiny by regulatory agencies around the world. In 2020, the European Union revoked license renewal for mancozeb due to health risks associated with exposure to mancozeb mainly endocrine disruption and reproductive toxicity (European Union, 2020). Increased thyroid weight, hypertrophy and hyperplasia of follicular cells, reduced thyroid peroxidase activity and decreased levels of thyroxine (T4) were all reported in rats orally exposed to mancozeb (Axelstad et al., 2011; Kackar et al., 1997). Increased prevalence of thyroid diseases among wives of pesticide applicators has also been documented (Goldner et al., 2010). Female rats treated orally with mancozeb showed disruption in estrous cycle and lower number of healthy follicles in the ovaries with reduction in the ovary size (Baligar and Kaliwal, 2001). Decreased testicular weight, spermatogenesis inhibition with decreased number of spermatogenic cells and reduced sperm count have also been observed in mancozeb treated male rats (Ksheerasagar and Kaliwal, 2003). In addition to endocrine and reproductive toxicity, mancozeb has been reported to adversely affect other organ systems. Mancozeb induced increases in liver weight, histopathological changes as well as elevations in plasma levels of liver enzymes including aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), gamma glutamyl transpeptidase (GGT) and lactate dehydrogenase (LDH) in rats (Aprioku et al.,

2023). Pirozzi et al. (2016) reported exacerbated fatty acid – induced steatosis in HepG2 cells treated with mancozeb (Pirozzi et al., 2016). Mice exposed to mancozeb demonstrated histopathological renal abnormalities including hyaline degeneration in the tubular epithelial envelope, renal function markers, creatinine and uric acid were elevated, levels of reactive oxygen species were increased with a down regulation in antioxidant enzymes, superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX) (Y. Zhang et al., 2023). Bioaccumulation of copper in the renal cortex, increased 4-hydroxynonenal (4-HNE) adduct formation in proximal and convoluted tubules as well as increased tubular injury immunoreactivity markers including kidney injury molecule-1 (KIM-1) and neutrophil gelatinase – associated lipocalin (NGAL) were all reported in rats orally exposed to mancozeb (Akhtar and Trombetta, 2023). Mancozeb induced apoptotic cell death and disrupted mitochondrial activity via mitochondrial complexes inhibition in transformed human colon cells (HT-29) (Dhaneshwar and Hardej, 2021).

1.4 Neurotoxicity

Pesticide exposure has long been implicated in neurotoxicity and development of neurodegenerative diseases (Franco et al., 2010; Kamel and Hoppin, 2004). Both glyphosate and mancozeb have been established in numerous studies as inducers of neurotoxicity (Cattani et al., 2017; Domico et al., 2006; Martínez et al., 2018; Miranda-Contreras et al., 2005; Tsang and Trombetta, 2007).

Glyphosate induced cell death of differentiated PC12 cells through autophagic and apoptotic pathways (Gui et al., 2012). Glutamate excitotoxicity, oxidative stress and lipid peroxidation were observed in the hippocampus of immature rats upon acute exposure to

the glyphosate – based formulation, Round-up® for 30 minutes as well as chronic exposure to Round-up® during pregnancy and lactation (Cattani et al., 2014). Monoaminergic neurotransmitters changes were induced in rat brains upon exposure to glyphosate (Martínez et al., 2018). Glyphosate was also shown to alter neuronal differentiation and axonal growth of hippocampal cultured neurons (Coullery et al., 2016).

Mancozeb has also been associated with neurotoxicity. Tsang and Trombetta (2007) observed a concentration - dependent decrease in the viability of rat hippocampal astrocytes exposed to mancozeb for 1 hour (Tsang and Trombetta, 2007). Mancozeb treatment induced cytotoxicity and uncoupling of mitochondrial respiration in dopaminergic and GABAergic mesencephalic neurons (Domico et al., 2006). Prenatal exposure to mancozeb caused alterations in the synaptic transmission in the cerebellar cortex of developing mouse (Miranda-Contreras et al., 2005).

1.5 Metals, Oxidative Stress and Neurotoxicity

Essential elements are those necessary for human life. Human's health, growth, maintenance and reproduction are dependent upon these elements (Momčilović et al., 2010) In humans and other mammals, about 23 elements have been identified and their physiological roles have been recognized. Among the essential elements are the metals, chromium, cobalt, copper, iron, lithium, manganese, magnesium, nickel, selenium and zinc (Chen et al., 2016; Fraga, 2005). Essential metals serve as co-factors in several enzymes for example copper acts as a cofactor in Cu/Zn-superoxide dismutase and cytochrome c oxidase, manganese is a cofactor in arginase, glutamate synthase as well as

Mn-superoxide dismutase and zinc that is involved in the function of several enzymes including RNA polymerase, angiotensin I converting enzyme and Cu/Zn-superoxide dismutase. In addition to serving as an enzymatic cofactor, zinc ions also participate in the structure of zinc finger proteins that interact with DNA and act as transcription factors (Chen et al., 2016; Fraga, 2005; Laity et al., 2001). Essential metals are therefore incorporated in a wide range of cellular processes such as redox reactions, oxygen transport, electron transport, cell proliferation and neurotransmitter synthesis (Chen et al., 2016; Garza-Lombó et al., 2018). In humans, only trace amounts of essential metals are required. When however present in amounts exceeding those required to perform their biological functions, these metals can induce oxidative damage and toxicity (Farina et al., 2013; Fraga, 2005). In the brain, elevated levels of metals are linked to neurotoxicity and development of neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, manganism, amyotrophic lateral sclerosis and Huntington's disease (Chen et al., 2016; Farina et al., 2013).

Metals are recognized of their ability to generate reactive oxygen species (ROS) (Ercal et al., 2001; Garza-Lombó et al., 2018). ROS are highly reactive molecules that are derived from molecular oxygen and include superoxide anions ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$) (Beckhauser et al., 2016; Garza-Lombó et al., 2018). ROS act as signaling molecules and are associated with biological functions such as smooth muscle relaxation, immune regulation, inflammation, neuronal differentiation and synaptic plasticity (Beckhauser et al., 2016; Boldt, 1999; Suzuki et al., 1996). Though being imperative for several cellular processes, levels of ROS must be finely tuned and

this is accomplished with the help of a number of antioxidants (Adwas et al., 2019; Birben et al., 2012; Gandhi and Abramov, 2012; Sreedhar and Csermely, 2004).

Metal-induced production of ROS is mediated by Fenton- like reactions and Haber-Weiss reaction (Ercal et al., 2001; Karihtala and Soini, 2007). In Fenton –like reactions, a metal (Fe^{+2} or Cu^{+}) reacts with hydrogen peroxide (H_2O_2) with the formation of hydroxyl radical ($OH^{\cdot}$), hydroxide ion (OH^{-}) and oxidized metal (Fe^{3+} or Cu^{2+}). Haber-Weiss reaction is a two-step reaction involving metal catalyzed interaction between superoxide and hydrogen peroxide with the generation of hydroxyl radical (Karihtala and Soini, 2007).

Failure to regulate levels of metals can stimulate an over production of ROS and can result in oxidative stress where the elevated levels of ROS can no longer be eliminated by the available antioxidant defense systems (Adwas et al., 2019; Ercal et al., 2001). ROS attack different cellular substrates including proteins, DNA, RNA and lipids promoting their oxidation and hence disrupting function of proteins, damaging cellular organelles and eventually leading to cell death (Gandhi and Abramov, 2012; Garza-Lombó et al., 2018). Metal catalyzed generation of ROS is associated with oxidation of backbone and the side chains of proteins that then attacks nearby amino acid side chains with the formation of carbonyl groups leading to altered polypeptide chain conformation and subsequent inactivation of proteins (Gandhi and Abramov, 2012; Gonos et al., 2018). Protein carbonyls have been implicated in aging as well as a number of disease states including amyotrophic lateral sclerosis (ALS), Parkinson's disease and Alzheimer's disease (Gonos et al., 2018; Lajtha, 2009). ROS can also target nucleic acid provoking

DNA-protein crosslink formation, strand breaks and base-pair modification that can in turn lead to mutations (Gandhi and Abramov, 2012). Membrane lipids such as polyunsaturated fatty acids (PUFA) are major targets for ROS attack. Hydroxyl radicals generated through Fenton-like reactions can initiate the process of lipid peroxidation in a membrane lipid by extracting a hydrogen atom from a PUFA side chain leaving behind a carbon centered lipid radical. The lipid radical then reacts with oxygen forming a peroxyl radical. Peroxyl radical can then extract hydrogen atom from adjacent PUFA side chain with the formation of more lipid and peroxyl radicals. Peroxyl radicals can then be converted into lipid hydroperoxides. Peroxidation of lipid membranes severely disrupts their integrity and, therefore, leads to cellular damage (Halliwell, 1992).

Brain particularly is considered prone to oxidative damage and this is related to its increased oxygen consumption and its high lipid content (Jelinek et al., 2021). Oxidative neuronal damage has been implicated in Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, psychiatric disorders and cerebrovascular disorders (Gonzalez-Pinto et al., 2012; Lee et al., 2020).

1.6 Antioxidant Defense

In order to halt oxidants-induced damage, antioxidant defense systems exist in cells to counterbalance the effects of ROS (Birben et al., 2012). Antioxidants can either act by blocking ROS generation or capturing the generated ROS and these include, the enzymes catalase, superoxide dismutase (SOD), glutathione peroxidase, glutathione-S-transferase, glutathione reductase, thioredoxin as well as non-enzymatic antioxidants glutathione (GSH), and stress proteins (Adwas et al., 2019; Birben et al., 2012; Gandhi

and Abramov, 2012; Sreedhar and Csermely, 2004). A part of antioxidant defenses are oxidized lipids-repairing enzymes including phospholipases, peroxidases and acyl transferases as well as proteolytic enzymes that function to remove oxidized proteins (Pisoschi and Pop, 2015).

One of the most significant antioxidants is glutathione (GSH). GSH or gamma glutamyl cysteinyl glycine is a thiol containing tri-peptide and it is the most abundant small molecule in cells (Sies, 1999). The synthesis of GSH occurs in the cytosol in two ATP-dependent steps. The first, the rate limiting step, involves bonding of L-glutamate and L-cysteine in a reaction catalyzed by gamma-glutamyl-cysteine synthase. The second step involves adding glycine to gamma glutamyl cysteine in a reaction catalyzed by glutathione synthase (Pizzorno, 2014; Sies, 1999).

GSH as an antioxidant can act directly by capturing ROS such as hydroxyl radical and superoxide anion (Pizzorno, 2014). GSH also acts as a cofactor in antioxidant enzymes involved in eliminating hydrogen and lipid peroxides including glutathione peroxidase where GSH gives away an electron and is converted to its oxidized form, glutathione disulfide (GSSG) (Birben et al., 2012; Pizzorno, 2014). GSH is then regenerated from GSSG by glutathione reductase enzyme using NADPH as a cofactor (Pizzorno, 2014). In addition to being a cofactor for antioxidant enzymes and to directly eliminating ROS, GSH plays a role in the recycling of vitamin C and vitamin E into their active forms (Birben et al., 2012; Pizzorno, 2014).

Under healthy cellular conditions, high levels of GSH and low levels of GSSG are maintained. During oxidative stress, however, levels of GSH are depleted with an increase in GSSG. In neurodegenerative disorders such as Parkinson's disease and

Alzheimer's disease, depleted stores of GSH has been a common finding (Aoyama, 2021). Ratio of reduced glutathione to oxidized glutathione, GSH/GSSG, is a sensitive index of cellular redox status and therefore can be applied as a biomarker for cells undergoing oxidative stress (Birben et al., 2012; Merad-Boudia et al., 1994; Pizzorno, 2014).

Antioxidant defenses that are endogenously found in the cells are not always able to counterbalance ROS-mediated injury. And hence, exogenous consumption of antioxidants might be helpful in resisting oxidative damage (Pisoschi and Pop, 2015). Dietary consumption of fruits and vegetables is a major source of exogenous antioxidants such as retinol, beta-carotene, vitamin C, vitamin E, vitamin B6 and phenolics including phenolic acids, cinnamic and hydroxycinnamic acid derivatives as well as flavonoids (Pisoschi and Pop, 2015; Zhu et al., 2023).

Butylated hydroxytoluene or BHT is a synthetic phenolic compound, patented in 1947 to be utilized as an antioxidant. In 1954, BHT use as a preservative in food products was approved by the United States Food and Drug administration (FDA). By 1998, the FDA approved its use as an antioxidant in cosmetic formulations (Yehye et al., 2015). Currently, BHT is amongst the most commonly used antioxidants and its applications range from a preservative in food, food packaging, cosmetics and pharmaceuticals to an additive in plastic and rubber manufacturing and in the production of petroleum (Yehye et al., 2015). Antioxidant capacity of BHT is owed to its ability to capture ROS and terminate lipid peroxidation chain reactions (Hossain et al., 2020). BHT has demonstrated efficiency in protecting against metal and DTC mediated oxidative insult (Hossain et al., 2020; Tsang and Trombetta, 2007). BHT reduced atherosclerotic lesions in the aorta of

cholesterol treated rabbits by providing protection against cholesterol generated auto-oxidation products (Bjorkhem et al., 1991). BHT was also shown to prevent ethanol-mediated brain damage in rats by mitigating oxidative damage and inflammation (Crews et al., 2006).

1.7 Glyphosate and Mancozeb: Mechanisms of Toxicity

Glyphosate and DTC compounds such as mancozeb are recognized for their metal chelating abilities (Cheng and Trombetta, 2004; Delmaestro and Trombetta, 1995; Mertens et al., 2018; Sunderman et al., 1984; Yahfoufi et al., 2020). Glyphosate was identified as a metal chelator long before discovering its herbicidal potential. Glyphosate can bind divalent metals such as copper, iron, manganese and zinc to form stable complexes and this is due to the presence of amino (NH), carboxylate (COOH) and phosphonate (H_2PO_3) functional groups (Caetano et al., 2012; Glass, 1984; Mertens et al., 2018; Yahfoufi et al., 2020). DTC compounds such as sodium diethyldithiocarbamate have been used in treatment of metal poisoning and Wilson's disease (Cheng and Trombetta, 2004; Lakomaa et al., 1982; Sunderman et al., 1984). Due to the presence of sulfer atoms within their molecules, DTC compounds can bind most metals in the periodic table forming stable complexes (Adeyemi and Onwudiwe, 2020).

Toxicity of glyphosate and mancozeb might be attributed to their metal-chelating potential. Altered metal levels have been reported upon exposure to either glyphosate or mancozeb (Stephenson and Trombetta, 2020; Tsang and Trombetta, 2007; Yahfoufi et al., 2020). Tsang and Trombetta (2007) demonstrated elevated levels of manganese and zinc in rat hippocampal astrocytes upon 1hour exposure to mancozeb (Tsang and Trombetta,

2007). Stephenson and Trombetta (2020) showed that mancozeb resulted in an increase in zinc levels and a decrease in manganese levels in the myocardium of Long Evans rats (Stephenson and Trombetta, 2020). Hoffman et al. (2016) reported that 24 hour treatment with EBDCs, maneb, mancozeb, zineb and nabam all resulted in elevations in copper levels in human colon cells in addition to elevations in manganese and zinc levels upon 24 hour treatment with mancozeb or maneb (Hoffman et al., 2016). Yahfoufi et al. (2020) showed an reduced levels of zinc in mouse oocyte after glyphosate treatment (Yahfoufi et al., 2020).

The contribution of oxidative stress in the toxicity of glyphosate and mancozeb have been demonstrated in a few studies (Cavalli et al., 2013; Domico et al., 2006; Iorio et al., 2015; Martínez et al., 2020). Glyphosate induced cytotoxicity, lipid peroxidation and elevated levels of ROS in human neuroblastoma (SH-SY5Y) cells (Martínez et al., 2020). Glyphosate and Roundup® resulted in lipid peroxidation and reduced GSH levels in Sertoli cells and also in the livers of albino male rats (Cavalli et al., 2013; El-Shenawy, 2009).

Treatment of mesencephalic cells with mancozeb induced elevations in levels of ROS (Domico et al., 2006). Mancozeb treatments resulted in lipid peroxidation as well as a decrease in GSH/GSSG ratios in colon cells (Hoffman et al., 2016; Hoffman and Hardej, 2012). Iorio et. Al. (2015) observed a reduction in GSH levels and an elevation in ROS levels in mouse granulosa cells upon treatment with mancozeb (Iorio et al., 2015). Exposure of RAT-1 fibroblasts and peripheral blood mononucleated (PBMC) cells to mancozeb for 1 hour caused an increase in levels of ROS, DNA oxidation as

demonstrated by increase in DNA adduct 8-hydroxy-2'-deoxyguanosine (8-OHdG) as well as DNA damage observed by the increase in DNA single strand break formation (Calviello et al., 2006).

1.8 Neuroblastoma (Neuro-2a) Cells: *In Vitro* Model for Neurotoxicity

Neuroblastoma cell lines are neural crest derived transformed cells. Under *in vitro* conditions, these cells can undergo unlimited proliferation (Shastry et al., 2001). Neuroblastoma cell lines have been successfully utilized in neurotoxicity studies, as well as cancer and virus research (Shastry et al., 2001).

In the current study, a murine neuroblastoma cell line, Neuro-2a was used. Morphological and ultrastructural observations demonstrated similarity of Neuro-2a cells to normal neurons. When grown in monolayer, Neuro-2a cells extend long processes also known as neurites, that are similar in appearance to axons and dendrites that are found in normal neurons (Ross et al., 1975). Organelles and organelles groupings in Neuro-2a cells are comparable to those seen in neuronal cells. Microtubules, microfilaments and neurofilaments were found to be similarly distributed in Neuro-2a cells and in normal neurons (Ross et al., 1975).

In addition to morphological similarities, neuroblastoma cells also share electrophysiological and biochemical features with sympathetic neurons including generation of action potential in response to electrical stimulation and to undergo spontaneous and repetitive depolarization (Haffke and Seeds, 1975). Neuro-2A cells have been successfully employed in studying neurotoxicants including compounds such as

methylmercury as well as metals such as aluminum and cadmium (Ge et al., 2019; Johnson et al., 2005; Trombetta et al., 1988; Trombetta and Kromidas, 1992).

1.9 Purpose of Study

The purpose of the current study is to assess the neurotoxic effects of the pesticides, glyphosate and mancozeb using a murine neuroblastoma cell line, Neuro-2a as a model system. To test whether the combined exposure to environmentally relevant pesticides is more damaging than the exposure to each pesticide separately. Neurotoxic effects of glyphosate and mancozeb combinations are evaluated as well and compared to that of glyphosate and mancozeb. In this study, Neuro-2a cells are exposed to three forms of combinations including exposure to mixture of both mancozeb plus glyphosate, exposure to mancozeb followed by glyphosate or exposure to glyphosate followed by mancozeb. Alterations in metal levels and disruptions in redox balance are also investigated as potential underlying mechanisms of toxicity of glyphosate, mancozeb and their combinations. Finally, recovery of Neuro-2a cells after being treated with glyphosate, mancozeb or their combination was evaluated in the presence of the antioxidant, butylated hydroxytoluene (BHT).

1.10 Hypotheses

1. Exposure to glyphosate or mancozeb induces reduction in cell viability and results in morphological changes in Neuro-2a cells.

2. Combinations of glyphosate and mancozeb result in decreased Neuro-2a cells viability and more pronounced morphological changes compared to mancozeb or glyphosate alone.

3. Exposure to glyphosate or mancozeb alters metal levels in Neuro-2a cells.

4. Combinations of glyphosate and mancozeb induce greater metal level alterations in Neuro-2a cells compared to mancozeb or glyphosate alone.

5. Exposure to glyphosate or mancozeb disrupts redox balance in Neuro-2a cells.

6. Combinations of glyphosate and mancozeb induce greater disruption in redox balance compared to mancozeb or glyphosate alone.

7. Post-treatment with the antioxidant butylated hydroxytoluene (BHT) alleviates cytotoxicity caused by mancozeb or glyphosate and mancozeb combination.

1.11 Specific Aims

1. Assessment of cell viability in Neuro-2a cells exposed to glyphosate, mancozeb or their combinations using trypan blue exclusion assay.

2. Observing morphological changes in Neuro-2a cells exposed to glyphosate, mancozeb or their combinations using phase-contrast microscopy and scanning electron microscopy (SEM).

3. Measuring levels of copper, magnesium, manganese, iron and zinc in Neuro-2a cells exposed to glyphosate, mancozeb and their combinations using inductively coupled plasma optical emission spectroscopy (ICP-OES).

4. Evaluate redox balance disruptions in Neuro-2a cells exposed to glyphosate, mancozeb and their combinations by measuring reduced glutathione to oxidized glutathione ratio.

5. Examine role of the antioxidant butylated hydroxytoluene as a post-treatment to alleviate cytotoxicity associated with mancozeb or combination of glyphosate and mancozeb in Neuro-2a cells using trypan blue exclusion assay

CHAPTER 2. MATERIALS AND METHODS

2.1 Materials

Glyphosate (GL; MW: 169.07 g/mole), mancozeb (MZ; MW: 266.51 g/mole) and Dulbecco's phosphate buffer powder were obtained from Sigma-Aldrich (St. Louis, MO). Butylated hydroxytoluene (2,6-Di-tert-butyl-p-cresol; BHT; MW: 220.36 g/mol) was purchased from Spectrum Chemical MFG Corporation (New Brunswick, NJ). Dulbecco's Modified Eagle's Medium (DMEM) powder was acquired from Gibco Life Technologies (Grand Island, NY). HEPES (4-(2- hydroxyethyl-)1-piperizineethane sulfonic acid (MW: 238.31 g/mole), sodium bicarbonate (MW: 84.01 g/mole) and sodium phosphate monobasic monohydrate (MW: 137.99 g/mole) and sodium phosphate dibasic anhydrous (MW: 141.96) were obtained from J.T. Baker (Phillipsburg, NJ). Premium, heat inactivated fetal bovine serum (FBS) was purchased from R&D Systems (Flowery Branch, GA). Gentamycin solution (50 mg/mL) was obtained from Lonza Biowhittaker® (Walkersville, MD). Trypsin EDTA 1X solution (0.25% Trypsin/2.21mM EDTA in HBSS without sodium bicarbonate, calcium and magnesium) was purchased from Corning (Corning, NY). Dimethyl Sulfoxide (DMSO) was acquired from ATCC (Manassas, VA). Trypan blue for Trypan blue assay was purchased from The Coleman & Bell CO. (Norwood, Ohio). Bright Line scientific hemocytometer was purchased from Hausser Scientific (Horsham, PA). GSH/GSSG-Glo luminescence kit was purchased from Promega (Madison, WI). For metal analysis, atomic spectroscopy-grade ICP standards were obtained from Perkin Elmer (Waltham, MA) and ultrapure nitric acid was acquired from Millipore sigma (Burlington, MA). Cell culture flasks were purchased from Greiner-Bio-One (Monroe, NC). Culture slides were obtained from BD Biosciences

(Bedford, MA). Sterile 96-well plates and 6-well plates with clear bottom as well as centrifuge tubes were obtained from Costar (Corning, NY). Disposable culture tubes, disposable pipette tips, disposable sterile pipettes, 12 mm glass coverslips and 0.2 µm PTFE syringe filter were purchased from VWR International (West Chester, PA). Rapid-flow top filters (0.2 µm SFCA membrane) were obtained from ThermoFisher Scientific (Waltham, MA).

2.2 Dulbecco's Modified Eagle Medium (DMEM)

DMEM was prepared in a 1 L volume by adding 13.374 g of DMEM powder, 5.957 g of HEPES, and 3.7 g of sodium bicarbonate into 1 L of ultrapure water. The solution was left to vortex for 10 minutes, 1 mL of gentamycin 50 mg/mL was then added to the medium and the pH was adjusted to 7.4. Finally, the medium was filtered using 500 mL rapid-flow bottle top filters into 500 mL sterile bottles.

2.3 Phosphate Buffer Saline (PBS)

PBS saline was prepared in a 1 L volume by adding 9.6 g of Dulbecco's phosphate buffer powder into 1 L of ultrapure water. The solution is left to vortex for 10 minutes and the pH was then adjusted to 7.4. Finally, the solution was filtered using 500 mL rapid-flow bottle top filters into 500 mL sterile bottles.

2.4 Cell Culture and Treatments

Murine neuroblastoma cell line, Neuro-2a (CCL-131™) was obtained at a passage number 182 from ATCC (Manassas, VA). Neuro-2a cells were grown in T75 culture flasks at a density of 1,000,000 cell/ flask and were maintained in 15 mL of complete medium, DMEM supplemented with 10% fetal bovine serum (FBS). Cells were incubated at 37°C with 5% CO_2 and 95% air in a ThermoFisher Scientific series II water

jacketed CO_2 incubator (ThermoFisher Scientific; Waltham, MA). Cells were fed with complete medium once every 2 days and were collected at 80% sub-confluency by trypsinization for 2 minutes using 0.25% Trypsin solution. For experiments, cell passage numbers 189 through 196 were utilized.

Stock pesticide solutions were freshly prepared before each treatment. Stock solution of 10 mM of mancozeb (MZ) was prepared in a 10 mL volume and this required 26.6 mg of MZ powder brought into solution with the help of 0.5 mL DMSO and sonication for 10 minutes using Branson 1510 sonicator (Branson; Danbury, CT), 9.5 mL of media was then added and the stock solution was adequately mixed. The final DMSO concentration never exceeded 0.5%. Stock solution of 40 mM of glyphosate (GL) was prepared in a 5 ml volume by dissolving 33.8 mg of GL powder directly into 5 mL of media. Stock solutions of both pesticides were then diluted with media to appropriate concentrations.

Cells were treated at 70% sub-confluency with GL (100-10,000 μM) for 24 hours, MZ (2-10 μM) for 24 hours or various combinations of GL and MZ including, GL (100 μM) plus MZ (2μM) for 24 hours, GL (100 μM) plus MZ (4 μM) for 24 hours, GL (100 μM) plus MZ (6 μM) for 24 hours, MZ (6 μM) for 30 minutes followed by GL (100 μM) for 24 hours as well as GL (100 μM) for 30 minutes followed by MZ (6 μM) for 24 hours. For the combinations of MZ (6 μM) for 30 minutes followed by GL (100 μM) for 24 hours and GL (100 μM) for 30 minutes followed by MZ (6 μM) for 24 hours, cells were washed twice with PBS after the 30 minutes incubation with the first pesticide and before adding the second pesticide. Concentrations of MZ and GL assessed in

combination groups are within reference dose limits (RfD) of either pesticide as set by the U.S. EPA (USEPA, 1993, 2005).

2.5 Trypan Blue Exclusion Assay

Cell viability was assessed in Neuro-2a cells treated with GL, MZ or combinations of GL and MZ using trypan blue exclusion assay. Trypan blue exclusion assay distinguishes viable cells from non-viable ones by the ability of the later to get stained by trypan blue dye. Trypan blue dye (0.4%) was prepared in a 100 mL volume by dissolving 0.4 g of trypan blue powder in 100 mL of PBS, the solution was then filtered using rapid-flow bottle top filter into 100 ml bottle.

Neuro-2a cells were seeded in T25 flasks at initial density of 300,000 in 5 mL of complete media and were grown until reaching 70% sub-confluency. Cells were then treated with GL (100, 500, 1000, 5000 and 10,000 μM) or MZ (2, 4, 6, 8 and 10 μM) for 24 hours. In order to determine concentrations of GL and MZ to be used in the combination treatments, cells were treated with mixture of GL (100 μM) + MZ (2 μM) for 24 hours and mixture of GL (100 μM) + MZ (4 μM) for 24 hours. Finally, GL concentration of 100 μM and MZ concentration of 6 μM were picked to be used in combination treatments in this assay and the rest of the study. Cells were exposed to three forms of 100 μM GL and 6 μM MZ combinations which include mixture of MZ plus GL for 24 hours, MZ + GL (24 h), combination of MZ for 30 minutes followed by GL for 24 hours, MZ (30 min) + GL (24 h) and combination of GL for 30 minutes followed by MZ for 24 hours, GL (30 min) + MZ (24 h).

Following the treatment period, cells were washed by PBS and cell pellets were collected into 15 mL centrifuge tube via trypsinization. Cells were then resuspended in

complete media and approximately 200 µL of the cell suspension was mixed with 500 µL of 0.4 % trypan blue dye and 300 µL of PBS in a disposable culture tube. Following incubation in room temperature for 30 seconds, 10 µL of trypan blue cell suspension were loaded into each chamber of a Bright Line hemocytometer (Hausser Scientific; Horsham, PA) and immediately examined at 10X magnification under an OLYMPUS CK2 inverted microscope (Olympus Optical CO; Tokyo, Japan). Finally, the number of viable cells in each chamber was counted and the total number of viable cells in the cell suspension was estimated.

2.6 Phase Contrast Microscopy

To observe morphological changes in Neuro-2a cells after exposure to GL, MZ or their combinations, phase contrast micrographs were taken. Cells were seeded in one-chambered culture slides at a density of 20,000 cells/chamber and were left to grow until reaching 70% sub-confluency. Cells were then treated with 100 µM GL for 30 minutes, GL (30 min), 100 µM GL for 24 hours, GL (24 h), 6 µM of MZ for 30 minutes, MZ (30 min), or 6 µM of MZ for 24 hours, MZ (24 h). Cells were also treated with combinations of GL (100 µM) and MZ (6 µM) including, mixture of MZ plus GL, MZ + GL (24 h), combination of GL for 30 minutes followed by MZ for 24 hours, GL (30 min) + MZ (24 h) or combination of MZ for 30 minutes followed by GL for 24 hours, MZ (30 min) + GL (24 h). After treatments, micrographs were taken at objective power of 20X using Nikon Eclipse Ts2R microscope (Nikon Instruments Inc., Melville, NY).

2.7 Scanning Electron Microscopy

Scanning electron microscopy (SEM) was utilized to observe structural changes in Neuro-2a cells after exposure to GL, MZ or their combinations. Cells were seeded at a

density of 25,000 cells/well in 6-well plates, with two round 12 mm glass coverslips placed in each well and were left to grow until reaching 70% sub-confluency. Cells were then treated with 100 μM GL for 30 minutes, GL (30 min), 100 μM GL for 24 hours, GL (24 h), 6 μM of MZ for 30 minutes, MZ (30 min), or 6 μM of MZ for 24 hours, MZ (24 h) as well as combinations of GL (100 μM) and MZ (6 μM) including, mixture of MZ plus GL, MZ + GL (24 h), combination of GL for 30 minutes followed by MZ for 24 hours, GL (30 min) + MZ (24 h) or combination of MZ for 30 minutes followed by GL for 24 hours, MZ (30 min) + GL (24 h).

Following treatment period, cells were washed three times with Sorensen's phosphate buffer (pH 7.4) and then fixed in 1.5% Sorensen's phosphate buffered (pH 7.4) glutaraldehyde for 1 hour on ice. After fixation, cells were washed three times on ice using Sorensen's phosphate buffer (pH 7.4) where each wash lasted for 5 minutes and were serially dehydrated in 30%, 60% and 90% ethanol each of which lasted for 10 minutes. Cells were then dehydrated for 5 minutes in 100% ethanol, followed by chemical drying using Hexamethyldisilazane Reagent (HMDS; Electron Microscopy Sciences; Hatfield, PA). For chemical drying, cells were placed at room temperature in HMDS:100% ethanol at a ratio of 1:1 for 10 minutes and then cells were placed in 100% HMDS for another 10 minutes. Following chemical drying, excess liquid is wicked away using filter paper and cells were allowed to dry overnight at room temperature. After chemical drying, cells-containing coverslips were removed from the 6-well plates and placed on sticky cleaned aluminum stubs, then silver paint dots were added on the top and bottom of each coverslip. Afterwards, coverslips were sputter coated with platinum-palladium using Cressington 108 Auto/SE Sputter coater (Ted Pella Inc.; Redding,

California). Micrographs were then viewed and captured using JEOL JSM-6010LA scanning electron microscope (JEOL Ltd; Tokyo', Japan).

2.8 Inductively Coupled Plasma-Optical Emission Spectroscopy

Inductively coupled plasma-optical emission spectroscopy (ICP-OES) was utilized to determine concentrations of copper, magnesium, manganese, iron and zinc in neuro-2a cells. neuro-2a cells were seeded in T75 flasks at initial seeding of 1,000,000 cells per each flask and were left to grow in complete media until reaching 70% sub-confluency. Cells were then treated with 100 μM of GL, 6 μM MZ or their combinations. Treatments included were GL (30 min), GL (24 h), MZ (30 min), MZ (24 h), mixture MZ + GL (24 h), combination GL (30 min) + MZ (24 h) or combination MZ (30 min) + GL (24 h). Depending on the treatment, the number of flasks ranged from 6 to 12 per treatment group for every time the experiment was repeated.

Following the treatment period, media were taken out of the flasks and transferred into snap cap vials. Cells were then collected via trypsinization, cells from each treatment group were pooled together, pelleted and then washed three times in PBS. After the final rinse, cells were transferred in 5 mL of PBS into snap cap vials and stored in -80°C along with the media collected from the treatments. Prior to analysis, samples were lyophilized for 48 hours using Labconco Free Zone 4.5 lyophilizer (Kansas City, MO), lyophilized samples were then weighed in acid-washed beakers and dry weights were recorded. Samples were then left overnight in 3 mL of concentrated ultrapure nitric acid per beaker and covered with acid-washed watch glass. Afterwards, samples were digested on hot plates until dryness. Following digestion, samples were reconstituted in 6.5 mL of 2% ultrapure nitric acid solution per sample and particulate matter was removed by filtering

the samples using 0.2 µm PTFE syringe filter into 15 mL conical tubes. For the analysis, standard curve dilutions 10, 1, 0.1 and 0.01 ppm were created for copper, magnesium, manganese, iron and zinc using atomic spectroscopy grade ICP standards (1000 ppb) and 2% nitric acid solution as a blank. Standard curves were then constructed and metal levels were analyzed using Perkin Elmer Optima 2100 DV Optical Emission Spectrometer (Waltham, MA). Metal levels were normalized to dry weight and results were expressed as the concentration of metal in each sample (ppb) divided by the dry weight of the sample (mg), ppb/mg.

2.9 GSH/GSSG-Glo Glutathione Assay

Reduced glutathione (GSH) and oxidized glutathione (GSSG) levels were determined and GSH/GSSG ratio was calculated in Neuro-2a cells using the luminescence-based assay, GSH/GSSG-Glo ™ assay. Neuro-2a were seeded at a density of 4,500 cell/well in white walled 96-well plates and were incubated for 48 hours until reaching 70% sub-confluency. Cells were then treated 100 µM GL, 6 µM MZ or their combinations. Treatments included were GL (30 min), GL (24 h), MZ (30 min), MZ (24 h), mixture MZ + GL (24 h), combination GL (30 min) + MZ (24 h) or combination MZ (30 min) + GL (24 h).

Following treatments, media and all treatments were removed from the wells, 50 µL of total glutathione lysis reagent or oxidized glutathione lysis reagent were added to their designated wells for measuring of total glutathione (GSH+GSSG) and oxidized glutathione (GSSG) and the plates were shaken at room temperature for 5 minutes using orbital shaker. Luciferin generation reagent was then added to all wells at volume of 50 µL per well, the plates were shaken briefly and were incubated at room temperature for

30 minutes. Finally, luciferin detection reagent is added to all wells at a volume of 100 µL per well, the plates were shaken briefly and were left to incubate for additional 15 minutes after which luminescence signal was read using Promega Glomax® - Multi Detection System (Promega; Madison; WI). Amount of reduced glutathione (GSH) was determined by subtracting the net relative luminescence units (RLU) of oxidized glutathione (GSSG) from the net RLU of total glutathione (GSH+GSSG) and the ratio of GSH/GSSG was calculated by dividing the net RLU of reduced glutathione (GSH) by the net RLU of oxidized glutathione (GSSG).

2.10 Post-Treatment with Antioxidant

Post-treatment of Neuro-2a cells with the antioxidant butylated hydroxytoluene (BHT) was examined for its ability to alleviate cytotoxicity associated with MZ and mixture of MZ+GL using Trypan blue exclusion assay. Stock solution of BHT (10 mM) was freshly prepared before each treatment in a volume of 10 mL by dissolving 22.03 mg of BHT using 0.5 mL of DMSO and then adjusting the volume to 10 mL using media. Stock BHT solution is then diluted with media to achieve the desired concentrations.

Cells were seeded in T25 flasks at a density of 300,000 cells per flask and were left to incubate in 5 mL of complete media until reaching 70% sub-confluency. Cells were then treated for 24 hours with 100 µM of GL, 6 µM of MZ or mixture of 100 µM GL plus 6 µM MZ. Following 24 hours incubation, treatments were removed from the flasks, cells were washed twice with 5 mL of PBS and then cells were incubated for another 24 hours in just media or in media containing 50 µM of BHT. Accordingly, treatment groups included in this assay were GL (24 h) + media (24 h), GL (24 h) + BHT

(24 h), MZ (24 h) + media (24 h), MZ (24 h) + BHT (24 h), mixture of GL + MZ (24 h) + media (24 h) or mixture of GL + MZ (24 h) + BHT (24 h).

Following treatments and incubation periods with or without BHT, cells were washed twice with PBS and collected via trypsinization. Finally, cell viability was assessed using Trypan blue exclusion assay.

2.11 Statistical Analysis

Statistical analysis was performed using GraphPad Prism® version 9.0. Results are expressed as the mean ± standard error of the mean (SEM). Data sets from Trypan blue exclusion assay and GSH/GSSG – Glo ™ assay were analyzed using one-way analysis of variance (ANOVA) followed by Tukey post-hoc test. Data sets from inductively coupled plasma – optical emission spectroscopy were compared using one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test or Student's t-test. Results were considered statistically significant when $p<0.05$.

CHAPTER 3. RESULTS

3.1 Cytotoxicity of GL, MZ or their combinations on Neuro-2a cells using Trypan blue exclusion assay

3.1.1 Cytotoxicity of glyphosate (GL) on Neuro-2a cells

To evaluate cytotoxicity of GL, Neuro-2a cells were incubated for 24 hours with GL at concentrations ranging from 100 to 10,000 µM. Significant reductions in cells viability were observed with GL concentrations 500, 1000, 5000 and 10,000 µM when compared to control group. GL concentration of 100 µM, on the other hand did not result in any significant changes in cells viability when compared to control group (Figure 1).

3.1.2 Cytotoxicity of mancozeb (MZ) on Neuro-2a cells

To evaluate cytotoxicity of MZ, Neuro-2a cells were incubated for 24 hours with MZ at concentrations ranging from 2 to 10 µM. Significant reductions in cells viability were observed with MZ concentrations 4, 6, 8 and 10 µM when compared to control group. MZ concentration of 2 µM did not result in significant changes in cells viability compared to control group (Figure 2).

3.1.3 Cytotoxicity of GL and MZ combinations on Neuro-2a cells

To evaluate cytotoxicity of GL and MZ combinations, mixture of 100 µM GL plus 2 µM MZ was initially assessed. Neuro-2a cells were incubated for 24 hours with GL (100 µM), MZ (2 µM) or GL (100 µM) + MZ (2 µM) mixture. None of the treatment groups resulted in significant changes in cells viability compared to the control group. Mixture of GL (100 µM) + MZ (2 µM) did not result in significant changes in

cells viability when compared to GL (100 μM) or MZ (2 μM) treatment groups (Figure 3).

Cytotoxicity of 100 μM GL plus 4 μM MZ was then assessed. Neuro-2a cells were incubated for 24 hours with GL (100 μM), MZ (4 μM) or GL (100 μM) + MZ (4 μM) mixture. Significant reductions in cells viability were observed with MZ (4 μM) treatment group compared to control. GL (100 μM) and GL (100 μM) + MZ (4 μM) mixture however didn't result in significant changes in neuro-2a cells viability when compared to control. Mixture of GL (100 μM) + MZ (4 μM) also didn't result in significant changes in cells viability when compared to GL (100 μM) or MZ (4 μM) (Figure 4).

Finally, cytotoxicity of 100 μM GL and 6 μM MZ combinations was assessed. Neuro-2a cells were incubated with:

a. 100 μM GL for 30 minutes, GL (30 min)
b. 100 μM GL for 24 hours, GL (24 h)
c. 6 μM MZ for 30 minutes, MZ (30 min)
d. 6 μM MZ for 24 hours, MZ (24 h)
e. Mixture of 100 μM GL plus 6 μM MZ for 24 hours, GL + MZ (24 h)
f. Combination of 6 μM MZ for 30 minutes followed by 100 μM GL for 24 hours, MZ (30 min) + GL (24 h)
g. Combination of 100 μM GL for 30 minutes followed by 6 μM MZ for 24 hours, GL (30 min) + MZ (24 h)

Significant reductions in cells viability were observed with treatment groups, MZ (30 min), MZ (24 h), GL + MZ (24 h) mixture, MZ (30 min) + GL (24 h) combination

and GL (30 min) + MZ (24 h) combination when compared to control. While there were no significant changes in cells viability observed with GL (30 min) or GL (24 h) treatment groups when compared to control. Mixture of GL + MZ (24 h) resulted in significant reduction in cells viability compared to GL (24 h) treatment group. There however were no significant changes in cells viability when the mixture of GL + MZ (24 h) was compared to MZ (24 h) treatment group. Combination of MZ (30 min) + GL (24 h) resulted in significant reductions in cells viability compared to MZ (30 min) and GL (24 h) treatment groups. Combination of GL (30 min) + MZ (24 h) also resulted in significant reductions in cells viability compared to GL (30 min) and MZ (24 h) treatment groups (Figure 5).

3.2 Phase contrast light microscopy of Neuro-2a cells treated with GL, MZ or their combinations

Phase contrast micrographs of Neuro-2a cells (Figures 6-13) were taken to observe morphological changes following treatment with:

a. 100 µM GL for 30 minutes, GL (30 min)
b. 100 µM GL for 24 hours, GL (24 h)
c. 6 µM MZ for 30 minutes, MZ (30 min)
d. 6 µM MZ for 24 hours, MZ (24 h)
e. Mixture of 100 µM GL plus 6 µM MZ for 24 hours, GL + MZ (24 h)
f. Combination of 6 µM MZ for 30 minutes followed by 100 µM GL for 24 hours, MZ (30 min) + GL (24 h)
g. Combination of 100 µM GL for 30 minutes followed by 6 µM MZ for 24 hours, GL (30 min) + MZ (24 h)

Figure 6 shows control Neuro-2a cells demonstrating polymorphic appearance with fusiform and stellate shaped cells. Control Neuro-2a cells also showed long neuretic processes characteristic of this cell line (Figure 6).

Neuro-2a cells treated with 100 μM GL for 30 minutes, GL (30 min) had translucent appearance, but they still showed an extensive network of neuretic processes similar to that seen in control Neuro-2a cells (Figure 7). Neuro-2a cells treated with 100 μM GL for 24 hours, GL (24 h) had similar appearance to GL (30 min) treatment except that cells were more rounded with a loss of neuretic processes following the GL (24 h) treatment (Figure 8).

Neuro-2a cells treated with 6 μM of MZ for 30 minutes, MZ (30 min) or for 24 hours, MZ (24 h) showed altered neuretic processes and round floating cells. Cellular debris were also noted with MZ (24 h) treatment (Figure 9 and Figure 10).

Neuro-2a cells treated with combinations of 100 μM GL and 6 μM MZ including GL + MZ (24 h), MZ (30 min) + GL (24 h) and GL (30 min) + MZ (24 h) demonstrated altered neuretic processes, round floating cells, and numerous cellular debris (Figures 11-13).

3.3 Scanning electron microscopy of Neuro-2a cells treated with GL, MZ or their combinations

Scanning electron micrographs were taken for control and treated Neuro-2a cells to observe morphological changes. Neuro-2a cells were treated with GL, MZ or their combinations. GL and MZ concentrations used were 100 μM and 6 μM, respectively. Figures 14-16 are representative micrographs showing control Neuro-2a cells extending

long neuretic processes which appear as thin tubular structures or as flattened projections. Control cells also demonstrate abundance of retraction fibers (Figures 14-16).

Figures 17 and 18 are representative micrographs showing Neuro-2a cells treated with GL for 30 minutes, GL (30 min). GL (30 min) treated cells had a rounded appearance (Figure 17 and Figure 18). Figures 19 and 20 are representative micrographs showing Neuro-2a cells treated with GL for 24 hours, GL (24 h). GL (24 h) treated cells demonstrated rounded appearance with few retraction fibers and blebbing (Figure 19 and Figure 20).

Figures 21-23 are representative micrographs showing Neuro-2a cells treated with MZ for 30 minutes, MZ (30 min). MZ (30 min) treated cells appeared round with surface blebbing and few retraction fibers. Cytoplasmic fragments were also noted (Figures 21-23). Figures 24 and 25 are representative micrographs showing Neuro-2a cells treated with MZ for 24 hours, MZ (24 h). MZ (24 h) treated cells showed disrupted processes and loss of retraction fibers (Figure 24 and Figure 25).

Figures 26 and 27 are representative micrographs for Neuro-2a cells treated for 24 hours with GL + MZ mixture, GL + MZ (24 h). Mixture treated cells demonstrated rounded appearance with plasmalemmal toroids, disrupted neuretic processes and cytoplasmic fragments (Figure 26 and Figure 27). Figures 28 and 29 are representative micrographs of cells treated with MZ for 30 minutes followed by GL for 24 hours, MZ (30 min) + GL (24 h). MZ (30 min) + GL (24 h) combination treated cells demonstrated plasmalemmal toroids and loss of retraction fibers (Figure 28). Blebbing along the neuretic processes were also noted (Figure 29). Figures 30 and 31 are representative micrographs of cells treated with GL for 30 minutes followed by MZ for 24 hours, GL

(30 min) + MZ (24 h). GL (30 min) + MZ (24 h) combination treated cells demonstrated plasmalemmal toroids, disrupted neuretic processes and loss of retraction fibers (Figure 30 and Figure 31).

3.4 Metal analysis using Inductively Coupled-Optical Emission Spectroscopy

Copper, zinc, manganese, magnesium and iron levels were measured using Inductively coupled plasma-optical emission spectroscopy (ICP-OES) (Tables 1-6) in 100 μM GL and 6 μM MZ treatment solutions, in culture media before and after treatments and in Neuro-2a cells after treatment with:

a. 100 μM GL for 30 minutes, GL (30 min)
b. 100 μM GL for 24 hours, GL (24 h)
c. 6 μM MZ for 30 minutes, MZ (30 min)
d. 6 μM MZ for 24 hours, MZ (24 h)
e. Mixture of 100 μM GL plus 6 μM MZ for 24 hours, GL + MZ (24 h)
f. Combination of 6 μM MZ for 30 minutes followed by 100 μM GL for 24 hours, MZ (30 min) + GL (24 h)
g. Combination of 100 μM GL for 30 minutes followed by 6 μM MZ for 24 hours, GL (30 min) + MZ (24 h)

3.4.1 Metal levels in media, GL and MZ solutions

Levels of copper, magnesium, manganese, iron and zinc were measured using ICP-OES in the media and in solutions of 100 μM GL and 6 μM MZ which were prepared by diluting stock solutions of these compounds in media. No significant changes were observed in levels of copper, magnesium, iron and zinc with the treatment solutions

when compared to media. Levels of manganese however were significantly higher in 6 μM MZ treatment solution when compared to media (Table 1).

3.4.2 Copper levels in Neuro-2a cells and in media following treatment with GL, MZ or their combinations

Copper levels were measured in Neuro-2a cells and in the media collected from the flasks after treatments. Intracellular copper levels were significantly increased in MZ (24 h) treatment and in combinations MZ (30 min) + GL (24 h) and GL (30 min) + MZ (24 h) compared to control cells. Intracellular copper levels were significantly higher in MZ (30 min) + GL (24 h) combination treatment compared to GL (24 h) and MZ (30 min) treatment groups. Intracellular copper levels were significantly higher in GL (30 min) + MZ (24 h) combination treatment compared to GL (30 min) treatment group, while the increase in intracellular copper levels was not significant compared to MZ (24 h) treatment group. GL treatments, GL (30 min) and GL (24 h), MZ (30 min) treatment group as well as GL + MZ (24 h) mixture treatment group did not result in significant changes in intracellular copper levels compared to control Neuro-2a cells (Table 2).

In media collected after treatments, copper levels were significantly decreased in media collected from MZ (24 h) treatment and GL (30 min) + MZ (24 h) combination treatment compared to media collected from control cells. No significant changes in copper levels were observed in media collected from GL (30 min), GL (24 h), MZ (30 min), GL +MZ (24 h) mixture and MZ (30 min) + GL (24 h) combination treatments compared to media collected from control cells (Table 2).

3.4.3 Zinc levels in Neuro-2a cells and in media following treatment with GL, MZ or their combinations

Zinc levels were measured in Neuro-2a cells and in the media collected from the flasks after treatments. Intracellular zinc levels were significantly increased in MZ (24 h) treatment and in all GL and MZ combination treatments, MZ + GL (24 h), MZ (30 min) + GL (24 h) and GL (30 min) + MZ (24 h) compared to control cells. Intracellular zinc levels were significantly higher in MZ (30 min) + GL (24 h) combination treatment compared to GL (24 h) and MZ (30 min) treatment groups. Intracellular zinc levels were significantly higher in GL (30 min) + MZ (24 h) combination treatment and in GL + MZ (24 h) mixture compared to GL (30 min) treatment and GL (24 h) treatment, respectively, while the increases in intracellular zinc levels with both combination treatments were not significant compared to MZ (24 h) treatment group. GL treatments, GL (30 min) and GL (24 h) and MZ (30 min) treatment group did not result in significant changes in intracellular zinc levels compared to control Neuro-2a cells (Table 3).

In media collected after treatments, zinc levels were significantly decreased in media collected from combination treatments MZ (30 min) + GL (24 h) and GL (30 min) + MZ (24 h) compared to media collected from control cells. No significant changes in zinc levels were observed in media collected from GL (30 min), GL (24 h), MZ (30 min), MZ (24 h) and GL +MZ (24 h) mixture compared to media collected from control cells (Table 3).

3.4.4 Manganese levels in Neuro-2a cells and in media following treatment with GL, MZ or their combinations

Manganese levels were measured in Neuro-2a cells and in the media collected from the flasks after treatments. Intracellular manganese levels were significantly increased in MZ (24 h) treatment and in the combination treatments, MZ + GL (24 h) and GL (30 min) + MZ (24 h) compared to control cells. Intracellular manganese levels were significantly higher in GL (30 min) + MZ (24 h) combination treatment and in GL + MZ (24 h) mixture compared to GL (30 min) treatment and GL (24 h) treatment, respectively, while the increases in intracellular manganese levels with both combination treatments were not significant compared to MZ (24 h) treatment group. GL treatments, GL (30 min) and GL (24 h), MZ (30 min) treatment group and MZ (30 min) + GL (24 h) combination treatment did not result in significant changes in intracellular manganese levels compared to control Neuro-2a cells (Table 4).

In media collected after treatments, manganese levels were significantly higher in media collected from MZ (24 h) treatment group and from combination treatments GL + MZ (24 h) and GL (30 min) + MZ (24 h) compared to media collected from control cells. No significant changes in manganese levels were observed in media collected from GL (30 min), GL (24 h), MZ (30 min) and MZ (30 min) + GL (24 h) treatment groups compared to media collected from control cells (Table 4).

3.4.5 Magnesium levels in Neuro-2a cells and in media following treatment with GL, MZ or their combinations

Magnesium levels were measured in Neuro-2a cells and in media collected from the flasks after treatments. No significant changes in intracellular magnesium levels were

observed with any of the treatment groups compared to control cells. There were also no significant changes in magnesium levels in media collected from treatment groups compared to media collected from control cells (Table 5).

3.4.6 Iron levels in Neuro-2a cells and in media following treatment with GL, MZ or their combinations

Iron levels were measured in Neuro-2a cells and in media collected from the flasks after treatments. No significant changes in intracellular iron levels were observed with any of the treatment groups compared to control cells. There were also no significant changes in iron levels in media collected from treatment groups compared to media collected from control cells (Table 6).

3.5 GSH/GSSG ratio in Neuro-2a cells treated with GL, MZ or their combinations

To determine if exposure to GL, MZ or their combinations will disrupt redox balance, reduced glutathione to oxidized glutathione ratios (GSH/GSSG) were evaluated using GSH/GSSG-glo glutathione assay in Neuro-2a cells treated with:

a. 100 µM GL for 30 minutes, GL (30 min)
b. 100 µM GL for 24 hours, GL (24 h)
c. 6 µM MZ for 30 minutes, MZ (30 min)
d. 6 µM MZ for 24 hours, MZ (24 h)
e. Mixture of 100 µM GL plus 6 µM MZ for 24 hours, GL + MZ (24 h)
f. Combination of 6 µM MZ for 30 minutes followed by 100 µM GL for 24 hours, MZ (30 min) + GL (24 h)
g. Combination of 100 µM GL for 30 minutes followed by 6 µM MZ for 24 hours, GL (30 min) + MZ (24 h)

No significant changes in GSH/GSSG ratios were observed with GL treatment groups, GL (30 min) and GL (24 h) compared to control group. Significant decreases in GSH/GSSG ratios were observed with MZ treatment groups, MZ (30 min) and MZ (24 h) as well as all three forms of GL and MZ combinations including GL + MZ (24 h), MZ (30 min) + GL (24 h) and GL (30 min) + MZ (24 h) when compared to control group. The decreases in GSH/GSSG ratios observed with GL + MZ (24 h) mixture and with MZ (30 min) + GL (24 h) were significant when compared to GL (24 h) treatment group and were however not significant when compared to MZ (24 h) and MZ (30 min) treatment groups, respectively. On the other hand, decreases in GSH/GSSG ratios observed with GL (30 min) + MZ (24 h) combination treatment were significant when compared to GL (30 min) and MZ (24 h) treatment groups (Figure 32).

3.6 Post-treatment with butylated hydroxytoluene (BHT)

Antioxidant butylated hydroxytoluene (BHT) was tested as a post-treatment for its ability to alleviate cytotoxicity associated with MZ and GL + MZ mixture. Neuro-2a cells were treated for 24 hours with 100 μM GL, 6 μM MZ or mixture of GL + MZ. Cells were then post-treated for 24 hours with 50 μM BHT or were left to recover in media. Cell viability was assessed afterwards using Trypan blue exclusion assay.

3.6.1 GL exposed Neuro-2a cells post-treated with BHT

No significant changes in Neuro-2a cells viability were observed with control BHT group, 50 μM BHT when compared to control group, untreated cells (Figure 33). No significant changes were observed in Neuro-2a cells viability with GL groups, whether cells were post-treated with BHT for 24 hours, GL (24 h) – BHT (24 h) or were left in media for 24 hours, GL (24 h) – media (24 h) compared to control group (Figure

33). There were also no significant changes in cells viability with GL group post- treated with BHT, GL (24 h) – BHT (24 h), when compared to control BHT group (Figure 33).

3.6.2 MZ exposed Neuro-2a cells post-treated with BHT

Significant decreases in Neuro-2a cells viability were observed with MZ groups whether post-treated with BHT, MZ (24 h) – BHT (24 h) or were left to recover in media, MZ (24 h) – media (24 h) when compared to control group. (Figure 34). There were also significant decreases in cells viability with MZ group post-treated with BHT, MZ (24 h) – BHT (24 h) when compared to control BHT (Figure 34).

3.6.3 GL + MZ mixture exposed Neuro-2a cells post-treated with BHT

Significant decreases in Neuro-2a cells viability were observed with GL + MZ mixture groups whether post-treated with BHT, GL + MZ mix (24 h) – BHT (24 h) or were left to recover in media, GL + MZ mix (24 h) – media (24 h) when compared to control group. (Figure 35). There were also significant decreases in cells viability with GL + MZ mixture group post-treated with BHT, GL + MZ mix (24 h) – BHT (24 h) when compared to control BHT (Figure 35).

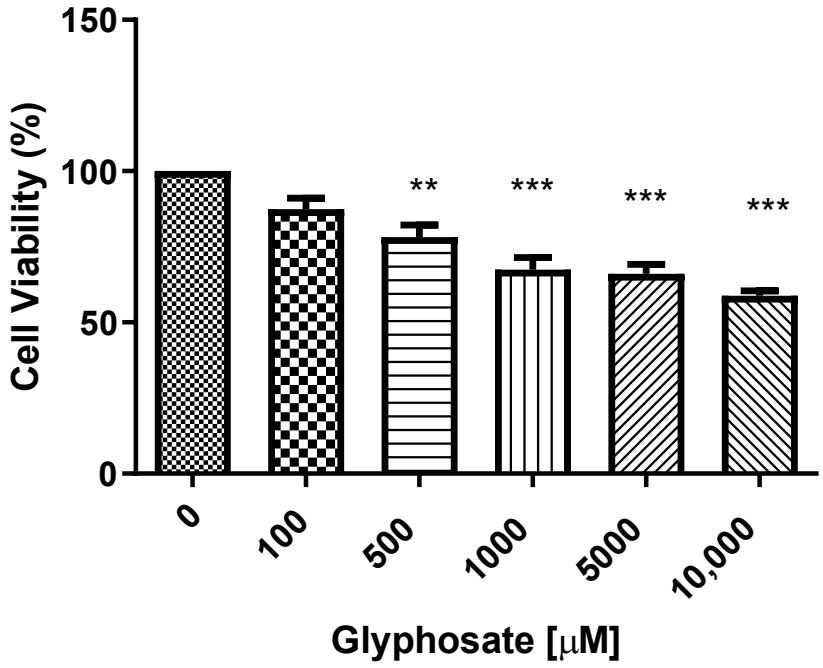

Figure 1. Cytotoxicity of glyphosate on Neuro-2a cells as determined by Tryban blue exclusion assay. Neuro-2a cells were treated for 24 hours with various concentrations of glyphosate (GL) ranging from 100 to 10,000 μM. Significant decreases in cell viability were observed at GL concentrations 500 to 10,000 μM as compared to the control. All values are expressed as percentage of control group. The experiment was repeated in triplicate, n=3. Data were analyzed using one-way ANOVA followed by Tukey's post-hoc test. **$p<0.01$, ***$p<0.001$.

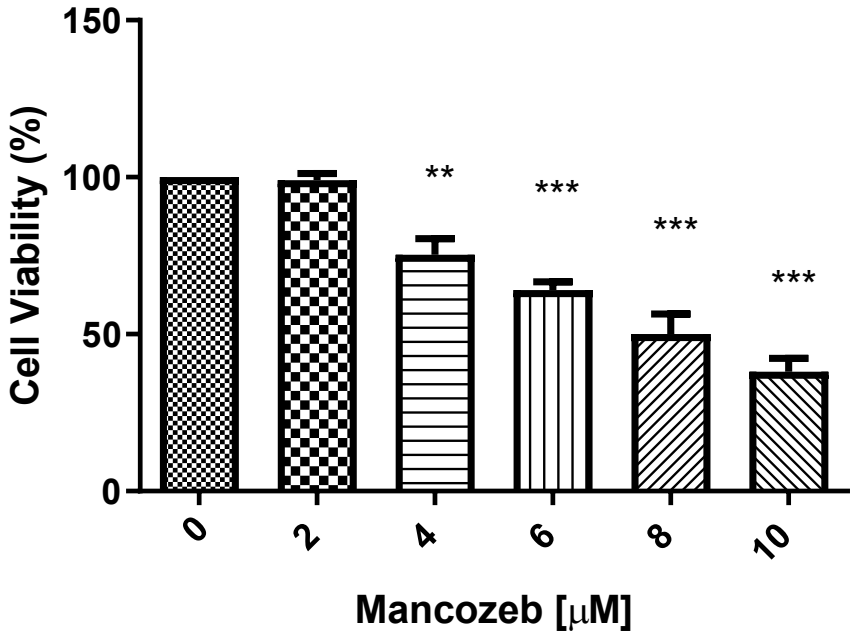

Figure 2. Cytotoxicity of mancozeb on Neuro-2a cells as determined by Tryban blue exclusion assay. Neuro-2a cells were treated for 24 hours with various concentrations of mancozeb (MZ) ranging from 2 to 10 μM. Significant decreases in cell viability were observed at MZ concentrations 4 to 10 μM as compared to the control. All values are expressed as percentage of control group. The experiment was repeated in triplicate, n=3. Data were analyzed using one-way ANOVA followed by Tukey's post-hoc test. **$p<0.01$, ***$p<0.001$.

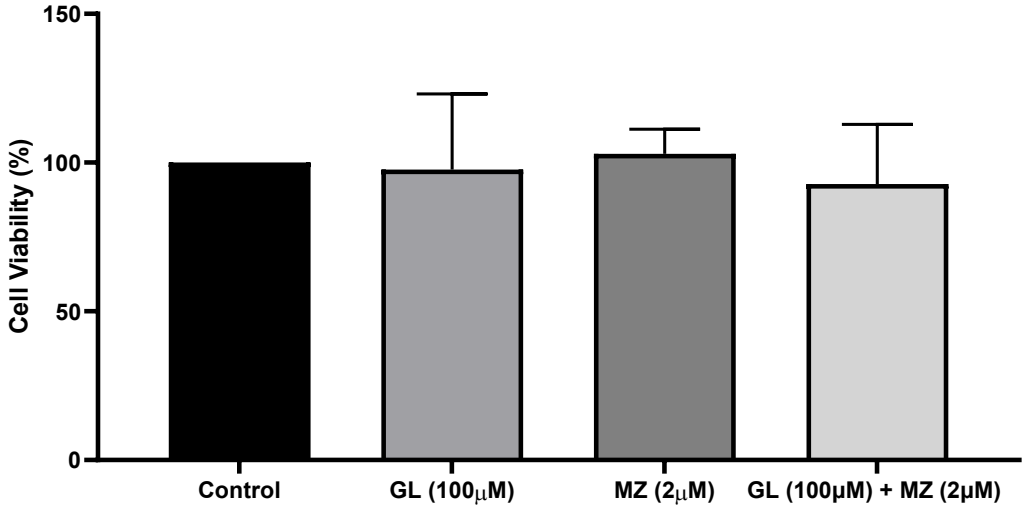

Figure 3. Cytotoxicity of glyphosate (100 μM) plus mancozeb (2 μM) mixture on Neuro-2a cells as determined by Tryban blue exclusion assay. To determine GL and MZ concentrations to be used in combination treatments, a mixture of 100 μM GL + 2 μM MZ was assessed. Neuro-2a cells were treated for 24 hours with GL at a concentration of 100 μM, MZ at a concentration of 2 μM or mixture of 100 μM GL + 2 μM MZ. No significant changes in cell viability were observed when treatment groups were compared to control. No significant changes in cell viability were observed when comparing GL (100 μM) + MZ (2 μM) mixture to GL (100 μM) or MZ (2 μM). All values are expressed as percentage of control group. The experiment was repeated in triplicate, n=3. Data were analyzed using one-way ANOVA followed by Tukey's post-hoc test.

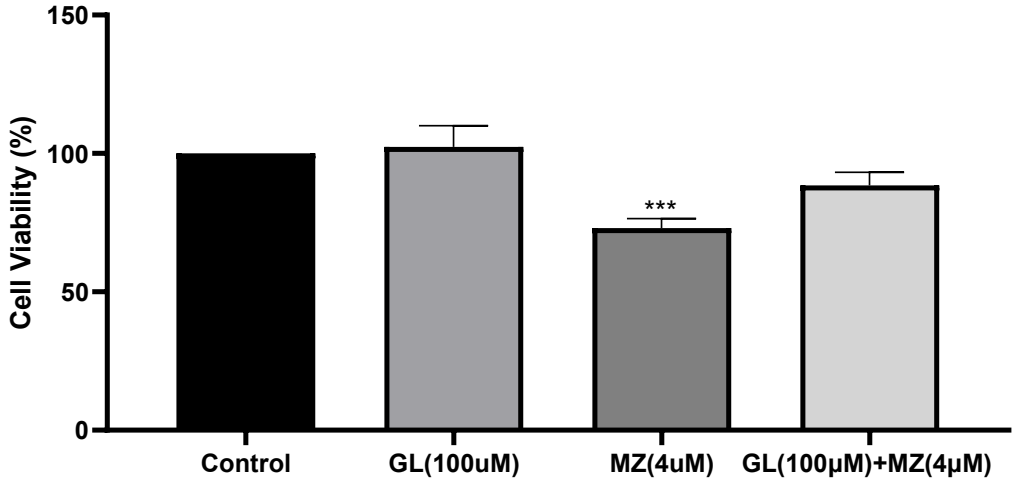

Figure 4. Cytotoxicity of glyphosate (100 µM) plus mancozeb (4 µM) mixture on Neuro-2a cells as determined by Tryban blue exclusion assay. To determine GL and MZ concentrations to be used in combination treatments, a mixture of 100 µM GL + 4 µM MZ was also assessed. Neuro-2a cells were treated for 24 hours with GL at a concentration of 100 µM, MZ at a concentration of 4 µM or mixture of 100 µM GL + 4 µM MZ. No significant changes in cell viability were observed when treatment groups were compared to control. Significant decrease in cell viability was observed in MZ (4 µM) treatment group when compared to control. No significant changes in cell viability were observed when comparing GL (100 µM) and GL (100 µM) + MZ (2 µM) mixture to control. No significant changes in cell viability when comparing GL (100 µM) + MZ (4 µM) mixture to GL (100 µM) or MZ (2 µM). All values are expressed as percentage of control group. The experiment was repeated in triplicate, n=3. Data were analyzed using one-way ANOVA followed by Tukey's post-hoc test. ***$p<0.001$.

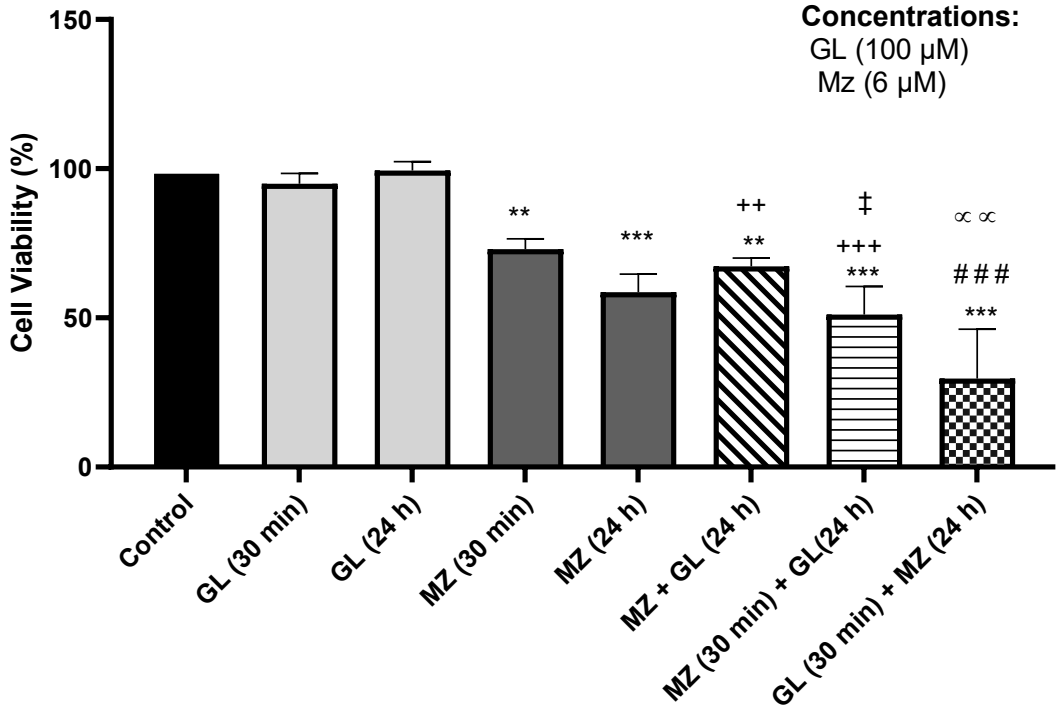

Figure 5. Cytotoxicity of glyphosate (100 µM) and mancozeb (6 µM) combinations on Neuro-2a cells as determined by Tryban blue exclusion assay. Neuro-2a cells were treated with 100 µM of GL for 30 minutes, GL (30 min) or for 24 hours, GL (24 h), 6 µM of MZ for 30 minutes, MZ (30 min) or for 24 hours, MZ (24 h), mixture of 100 µM GL plus 6 µM MZ for 24 hours, GL + MZ (24 h), combination of 6 µM MZ for 30 minutes followed by 100 µM GL for 24 hours, MZ (30 min) + GL (24 h) or combination of 100 µM GL for 30 minutes followed by 6 µM MZ for 24 hours, GL (30 min) + MZ (24 h). Significant decreases in cell viability were observed with MZ treatment groups, MZ (30 min) and MZ (24 h) and all three forms of combinations, MZ + GL (24 h), MZ (30 min) + GL (24 h) and GL (30 min) + MZ (24 h) when compared to control group. Significant decreases in cell viability were observed in mixture of MZ + GL (24 h) treatment group when compared to GL (24 h) treatment. Significant decreases in cell viability were observed in MZ (30 min) + GL (24 h) treatment group when compared to MZ (30 min) or GL (24 h) treatment groups. Significant decreases in cell viability were observed in GL (30 min) + MZ (24 h) treatment group when compared to GL (30 min) or MZ (24 h). All values are expressed as percentage of control group. The experiment was repeated in triplicate, n=3. Data were analyzed using one-way ANOVA followed by Tukey's post-hoc test. **$p<0.01$, ***$p<0.001$ compared to control, ++$p<0.01$, +++$p<0.001$ compared to GL (24 h), ‡$p<0.05$ compared to MZ (30 min), ###$p<0.001$ compared to GL (30 min), ααp<0.01 compared to MZ (24 h).

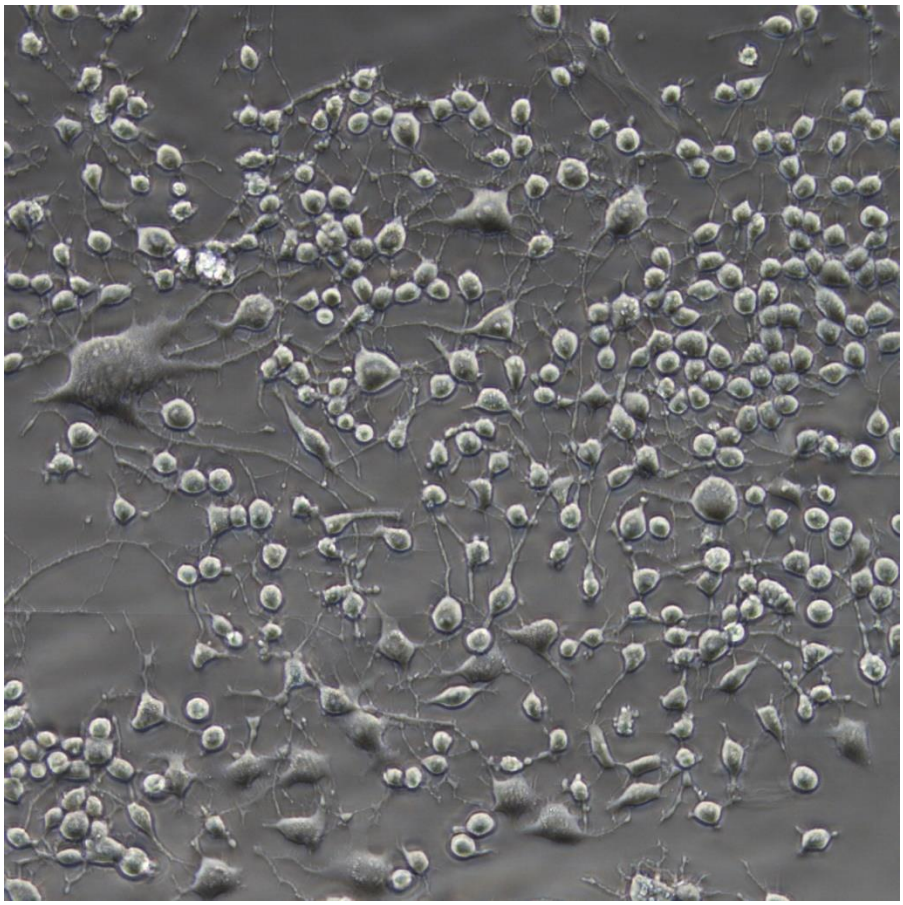

Figure 6. Phase contrast light micrograph of control Neuro-2a cells. Control Neuro-2a cells appear polymorphic with fusiform and stellate shaped cells. Control Neuro-2a cells show long neuretic processes characteristic of this cell line. x200

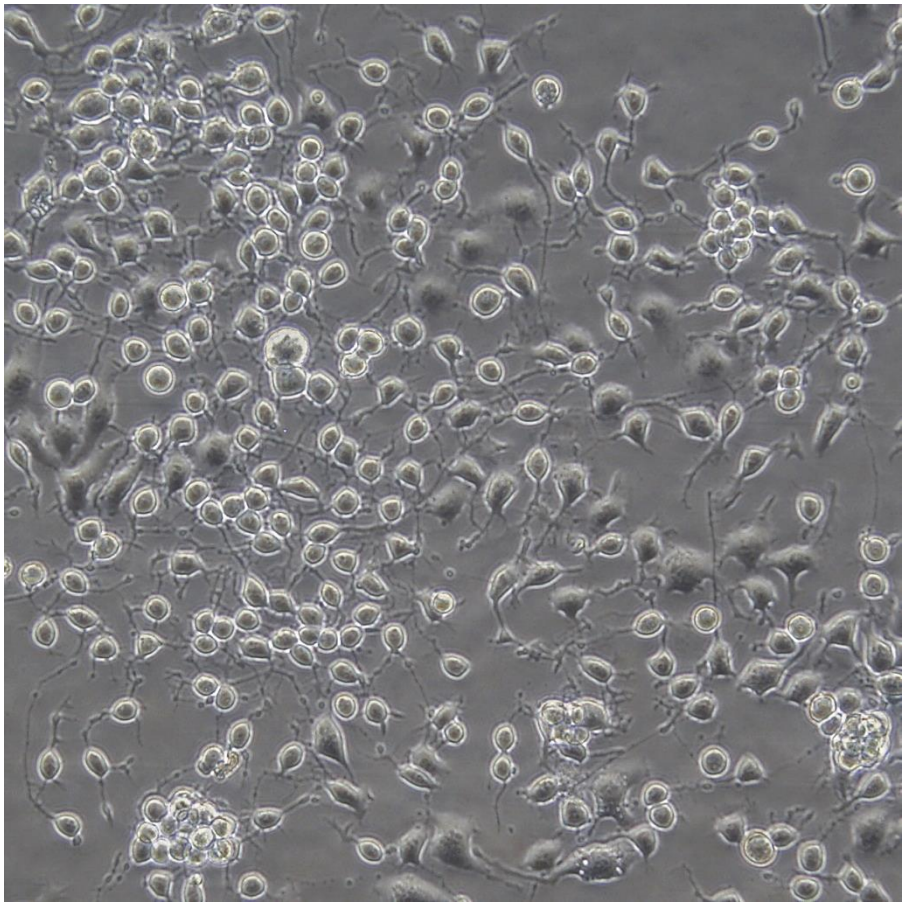

Figure 7. Phase contrast light micrograph of Neuro-2a cells treated with 100 µM glyphosate for 30 minutes. Neuro-2a cells were treated for 30 minutes with 100 µM of glyphosate and then were refed with media for 24 hours. Mainly cells appear translucent as if they are lifting off the substrate but still show a complex pattern of neuretic processes. x200

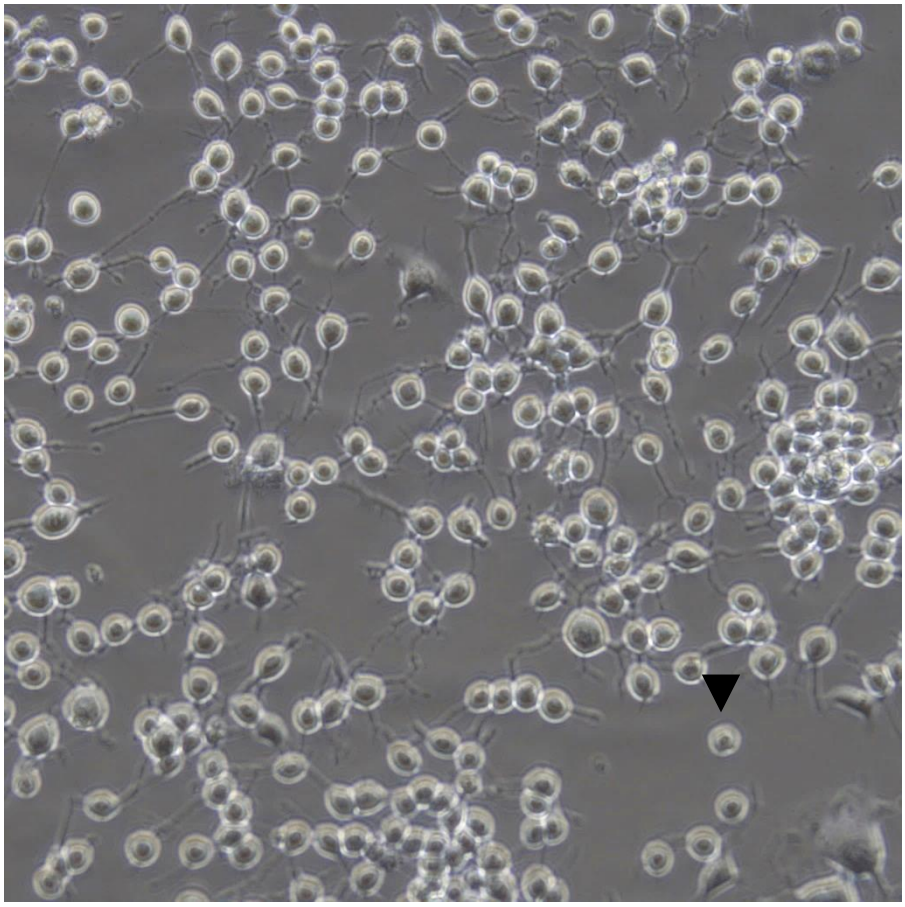

Figure 8. Phase contrast light micrograph of Neuro-2a cells treated with 100 µM glyphosate for 24 hours. Neuro-2a cells were treated for 24 hours with 100 µM of glyphosate. GL (24 h) treated cells appear somewhat similar to GL (30 min) treated cells except that many cells are more rounded (arrow heads) with loss of neuretic processes. x200

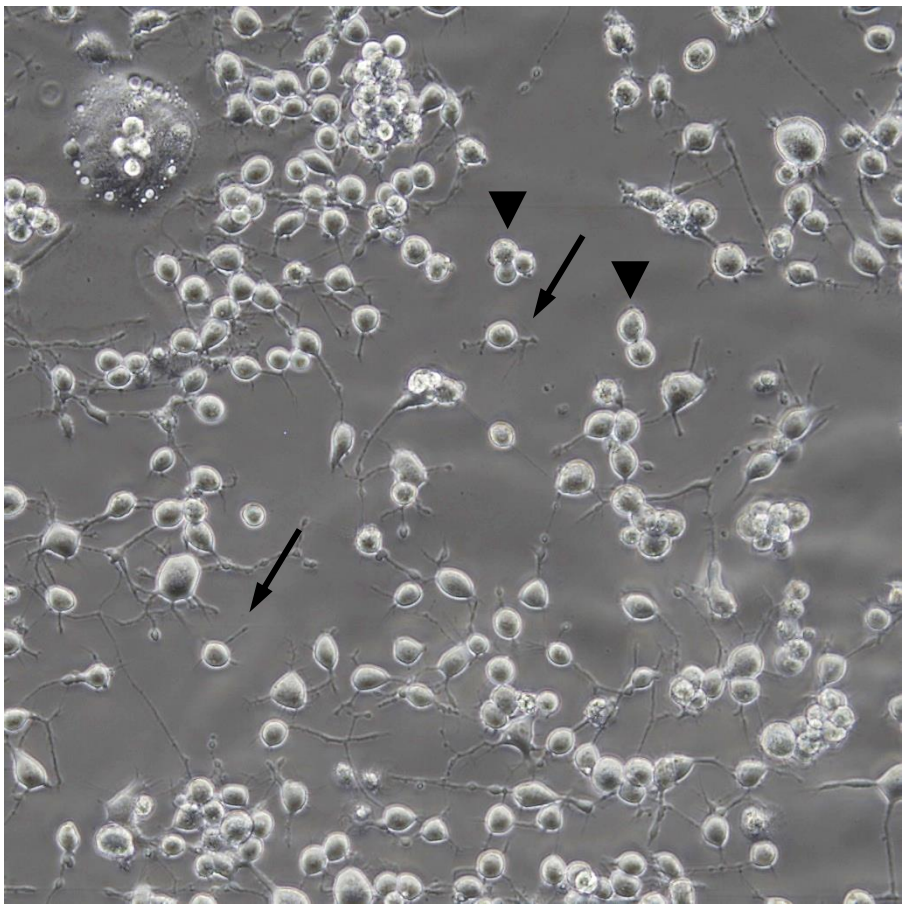

Figure 9. Phase contrast light micrograph of Neuro-2a cells treated with 6 µM mancozeb for 30 minutes. Neuro-2a cells were treated for 30 minutes with 6 µM of mancozeb and then were refed with media for 24 hours. Cells appear less confluent with round floating cells (arrow heads) and altered neuretic processes (arrows). x200

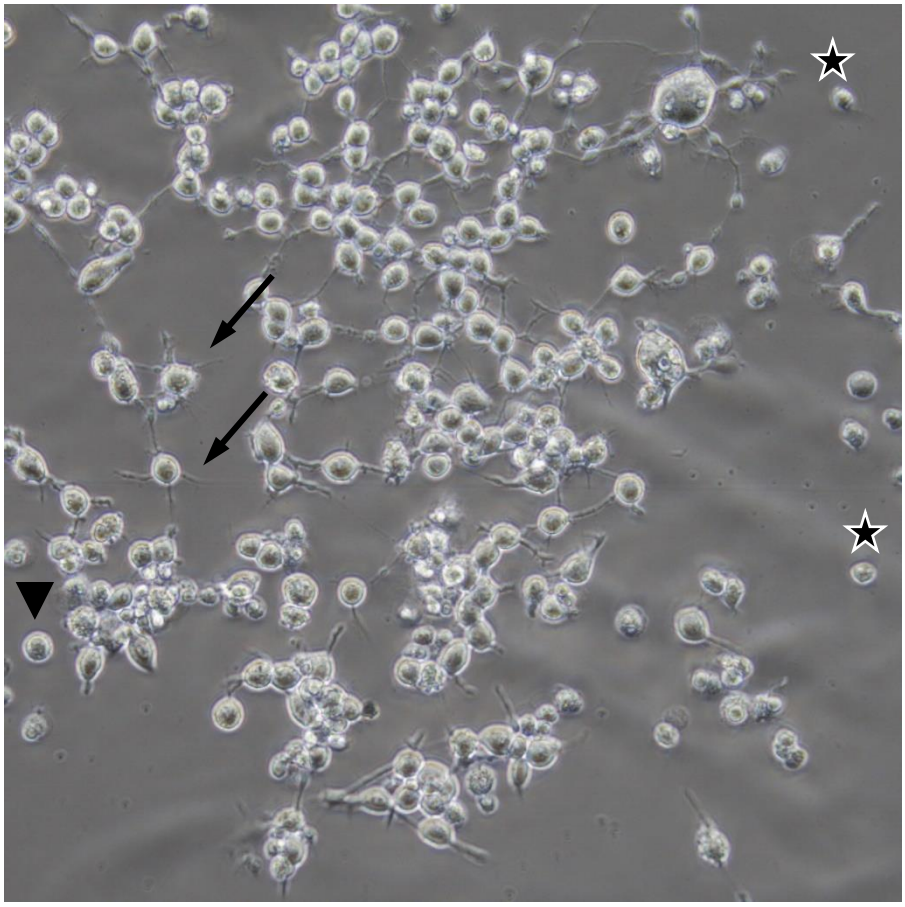

Figure 10. Phase contrast light micrograph of Neuro-2a cells treated with 6 µM mancozeb for 24 hours. Neuro-2a cells were treated for 24 hours with 6 µM of mancozeb. Cells appear to have altered neuretic processes (arrows) with round floating cells (arrow heads) and cellular debris (star). x200

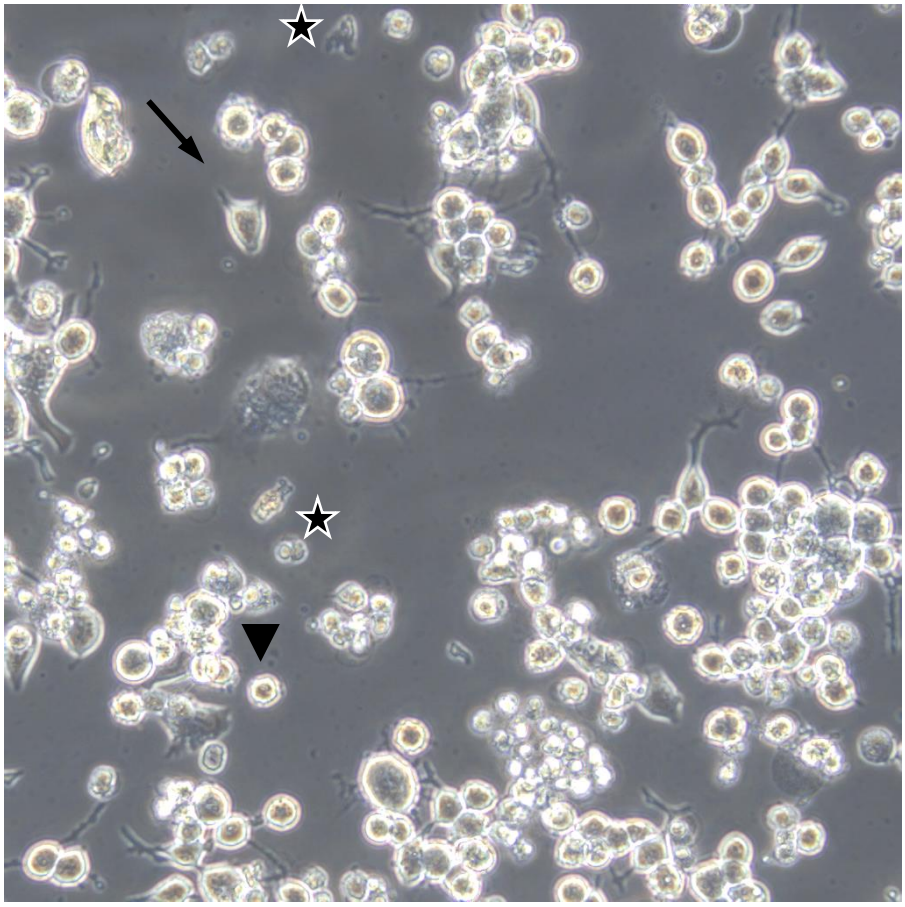

Figure 11. Phase contrast light micrograph of Neuro-2a cells treated with mixture of 100 µM glyphosate plus 6 µM mancozeb for 24 hours. Neuro-2a cells were treated for 24 hours with MZ + GL mixture. Cells showed altered neuretic processes (arrows) with cellular debris (star) and round floating cells (arrow heads). x200

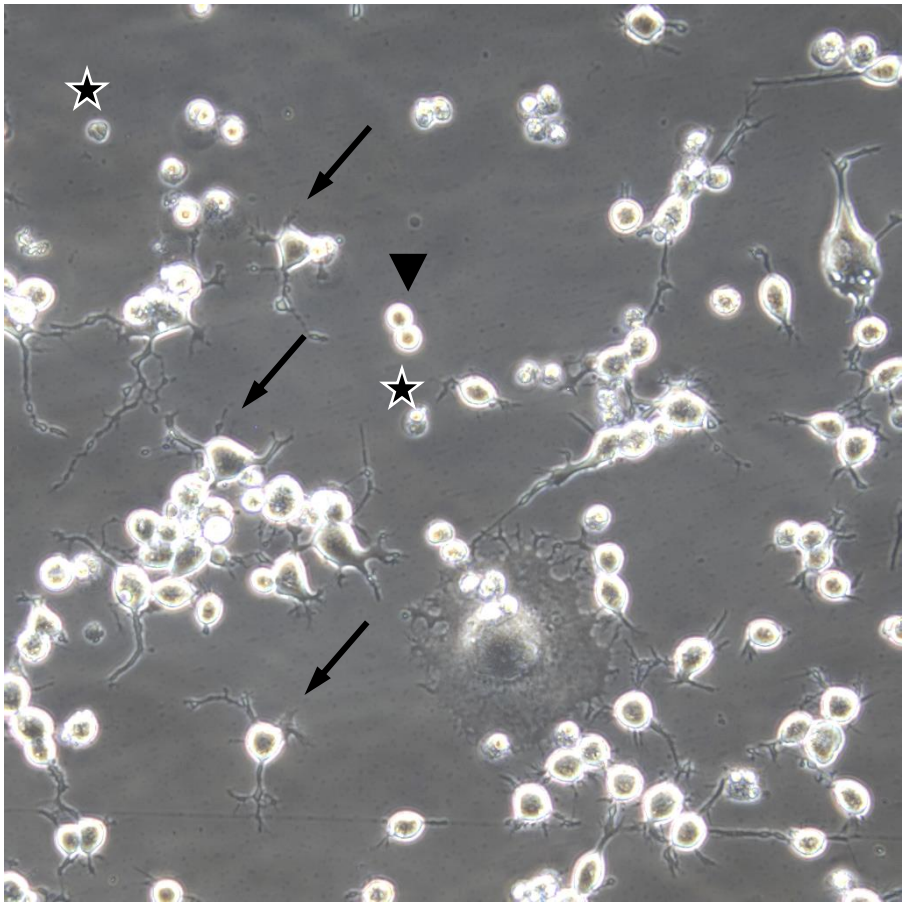

Figure 12. Phase contrast light micrograph of Neuro-2a cells treated with 6 µM mancozeb for 30 minutes followed by 100 µM glyphosate for 24 hours. Cells showed altered neuretic processes (arrows) with numerous cellular debris (star) and round floating cells (arrow heads). x200

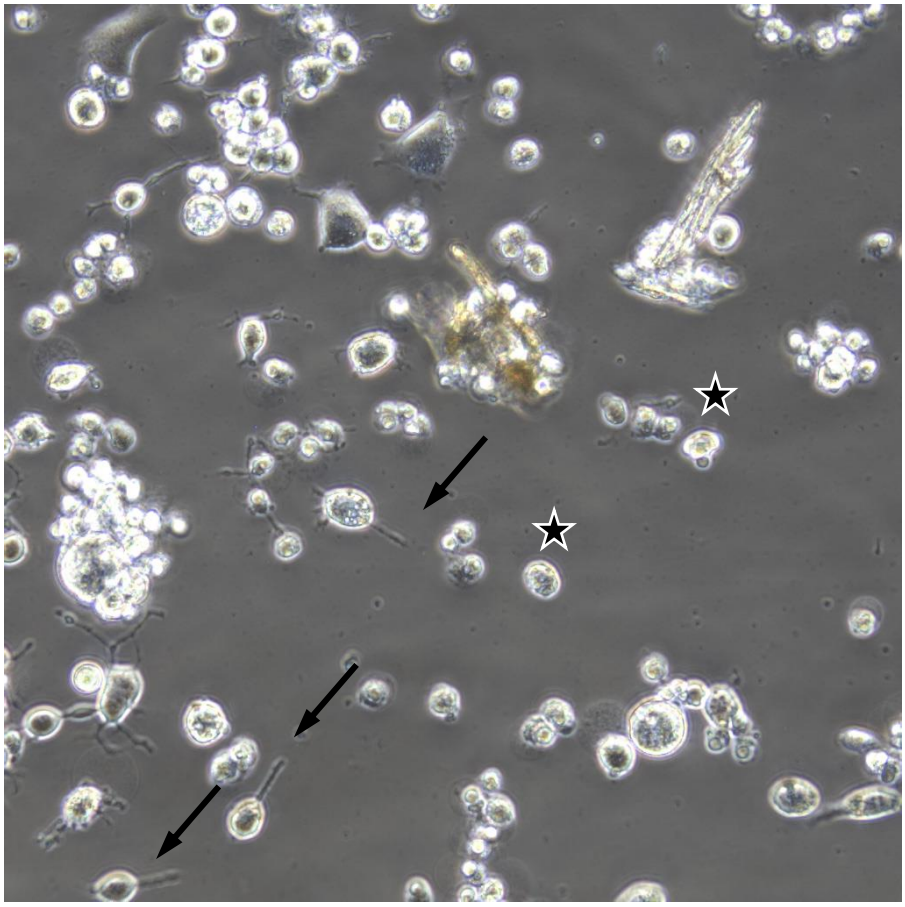

Figure 13. Phase contrast light micrograph of Neuro-2a cells treated with 100 µM glyphosate for 30 minutes followed by 6 µM mancozeb for 24 hours. Cells showed disrupted neuretic processes (arrows) with numerous cellular debris (star). x200

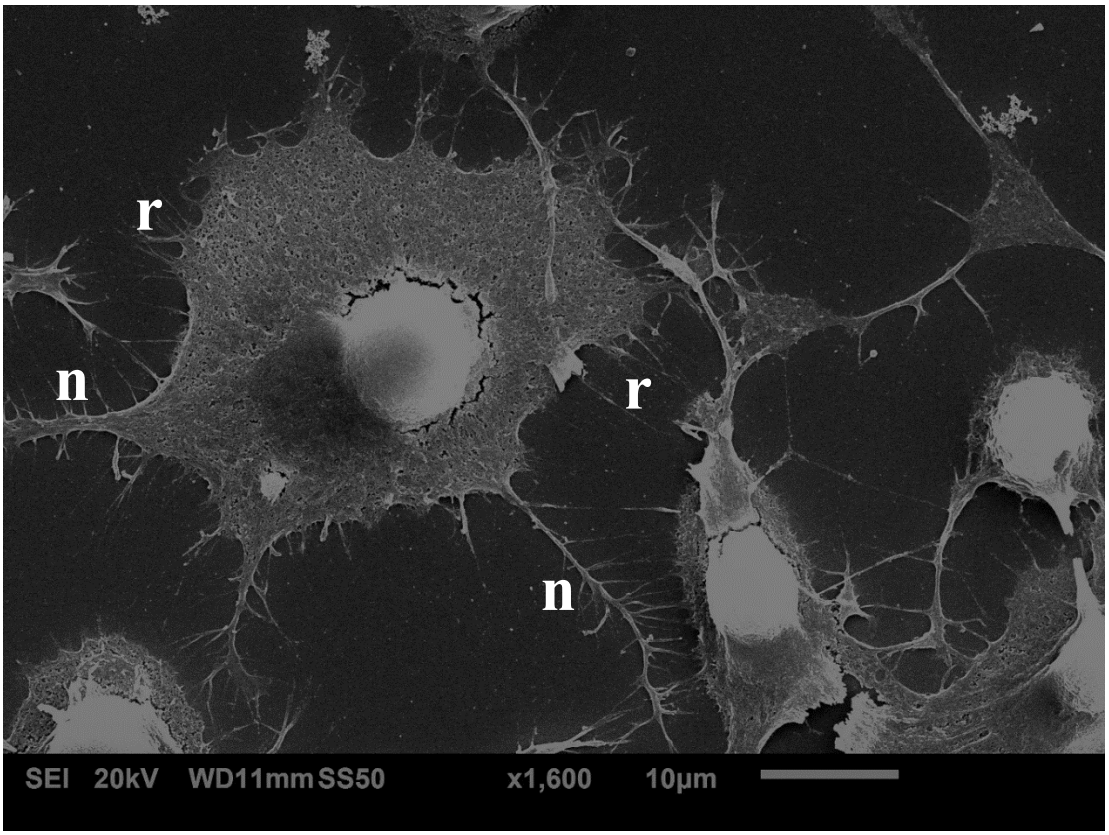

Figure 14. Scanning electron micrograph of control Neuro-2a cells. This micrograph is a representative of control Neuro-2a cells. Control Neuro-2a cells demonstrate long neuretic processes which appear as flattened projections or as thin tubular structures (n). Control Neuro-2a cells also show numerous retraction fibers (r). Total magnification, x1600.

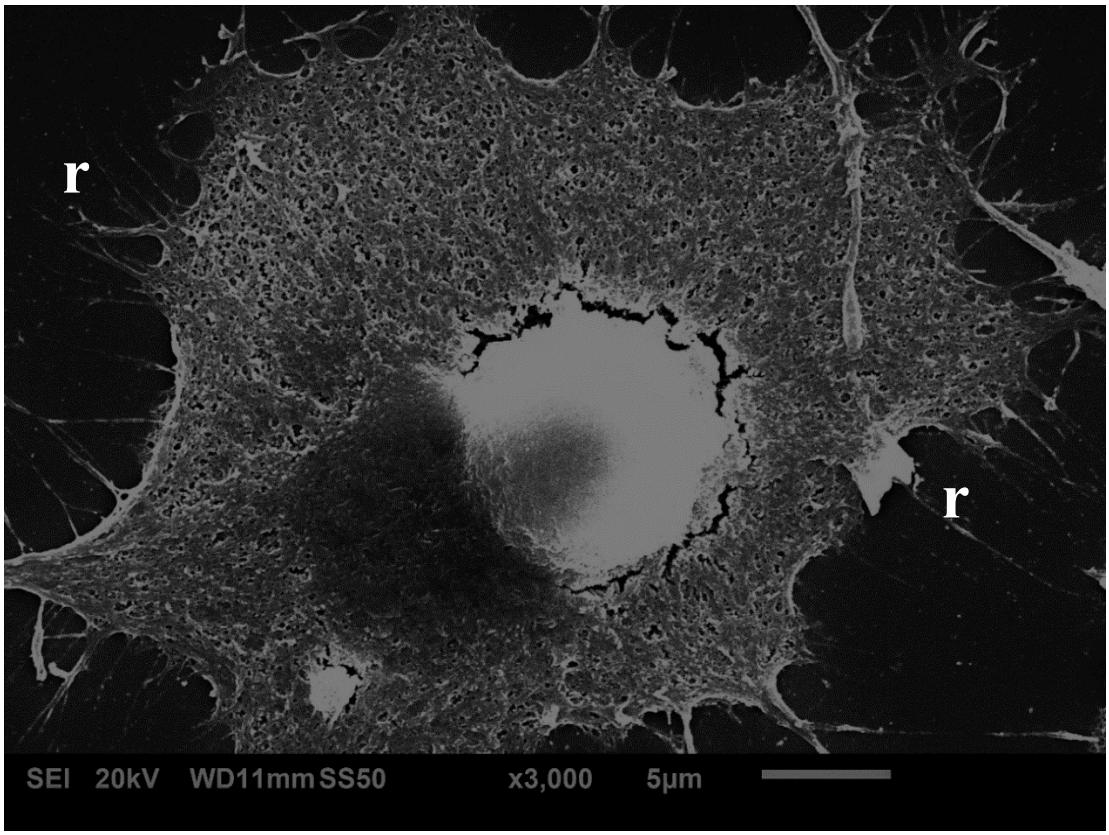

Figure 15. Higher magnification Scanning electron micrograph of a control Neuro-2a cell. Representative micrograph of control Neuro-2a cell with numerous retraction fibers (r). Total magnification, x3,000.

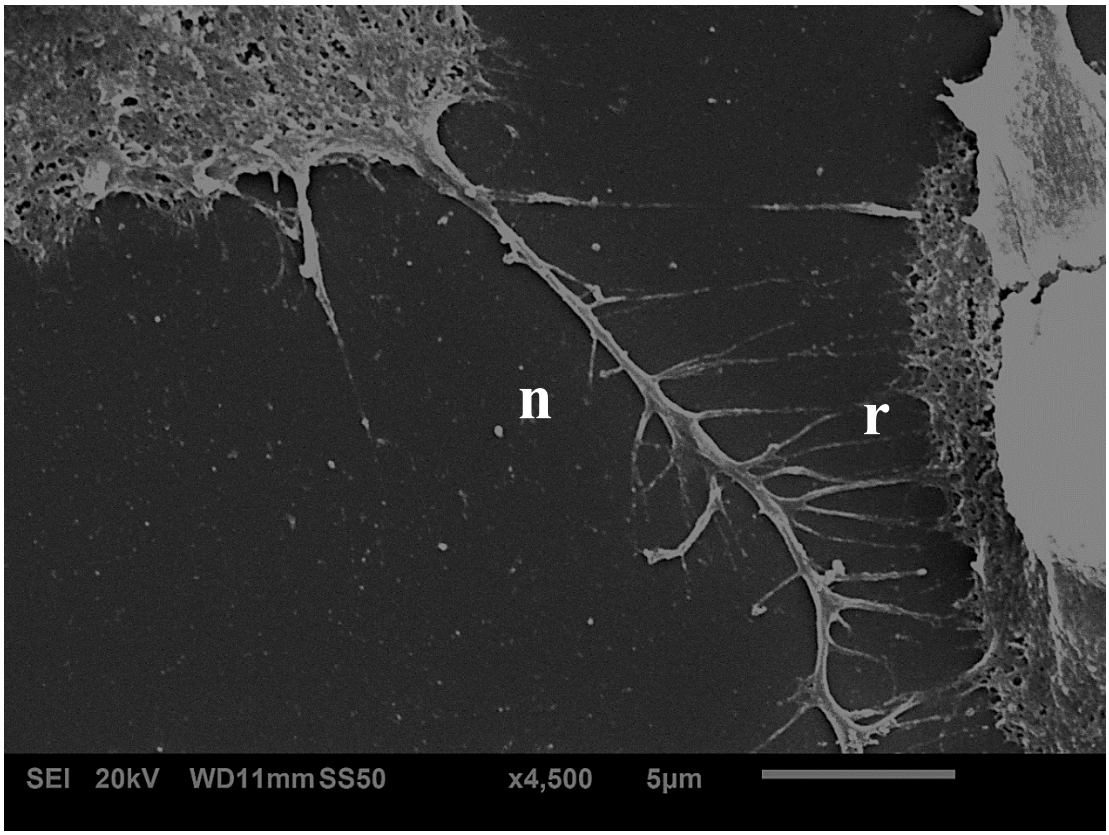

Figure 16. Scanning electron micrograph of a control Neuro-2a cell showing a neuretic process. Representative micrograph of control Neuro-2a cell showing long neuretic process (n) with numerous retraction fibers (r). Total magnification, x4,500.

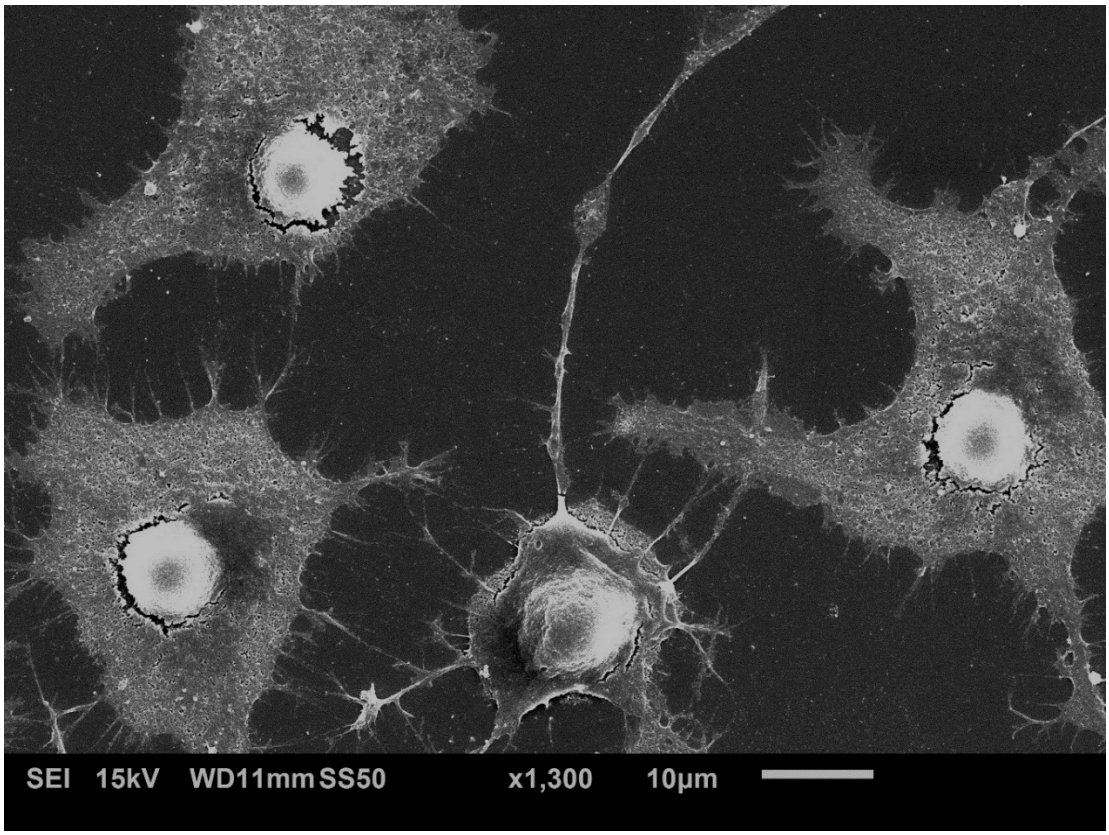

Figure 17. Scanning electron micrograph of Neuro-2a cells treated with 100 µM of glyphosate for 30 minutes. This micrograph is a representative of Neuro-2a cells treated for 30 minutes with 100 µM of glyphosate. Some cells have rounded appearance. Total magnification, x1,300.

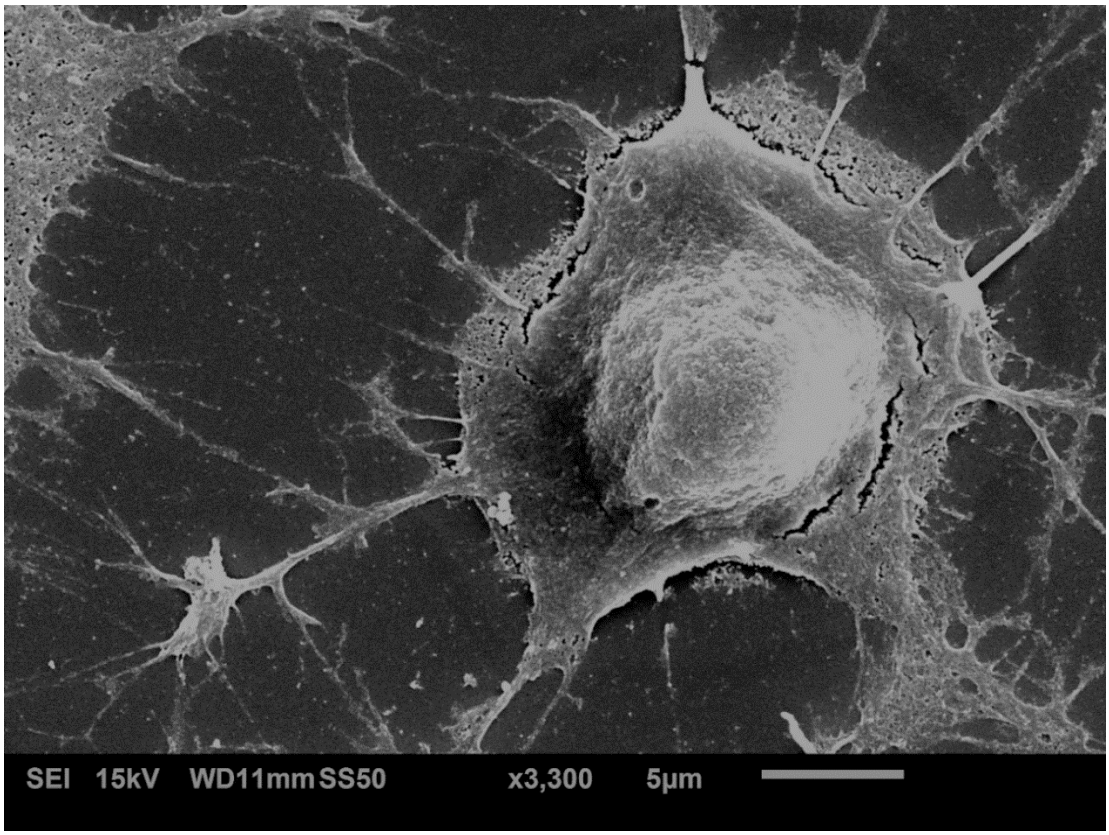

Figure 18. Higher magnification Scanning electron micrograph of Neuro-2a cells treated with 100 µM of glyphosate for 30 minutes. Representative micrograph of Neuro-2a cell treated for 30 minutes with 100 µM of glyphosate. Cells have a more rounded appearance. Total magnification, x3,300.

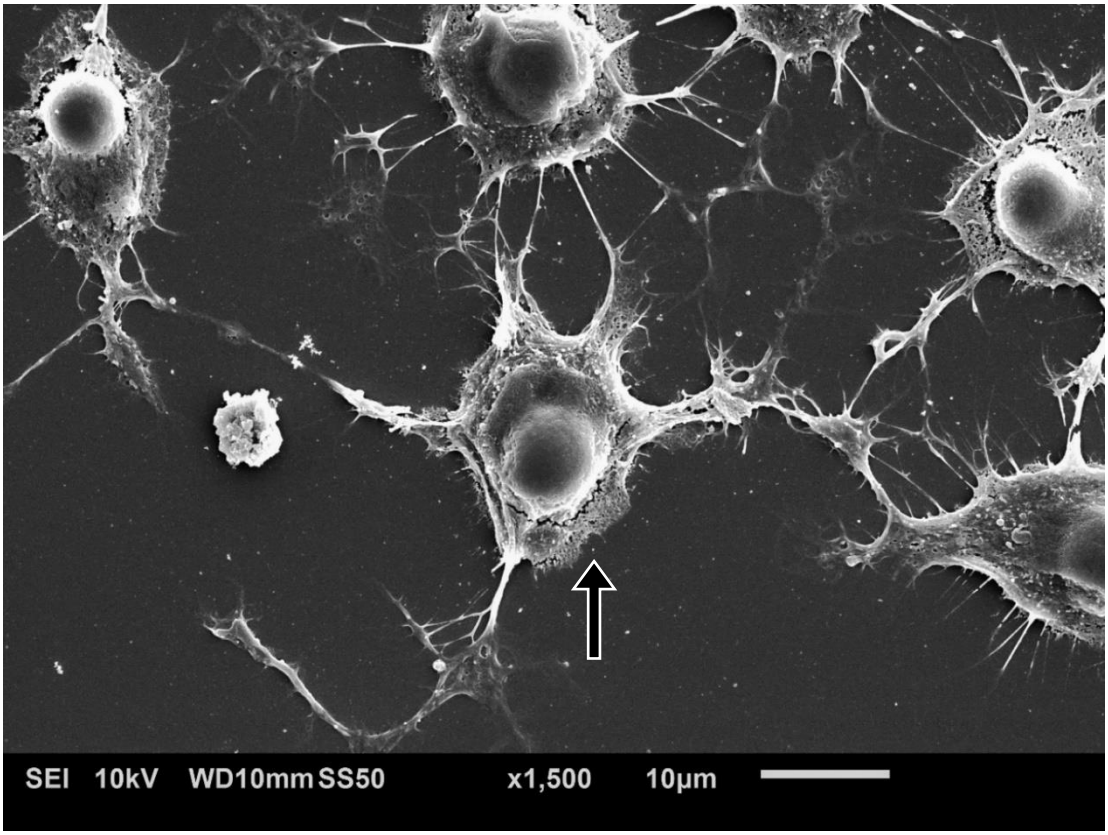

Figure 19. Scanning electron micrograph of Neuro-2a cells treated with 100 µM of glyphosate for 24 hours. This micrograph is a representative of Neuro-2a cells treated for 24 hours with 100 µM of glyphosate. Cells appear rounded with fewer retraction fibers (black arrow). Total magnification, x1,500.

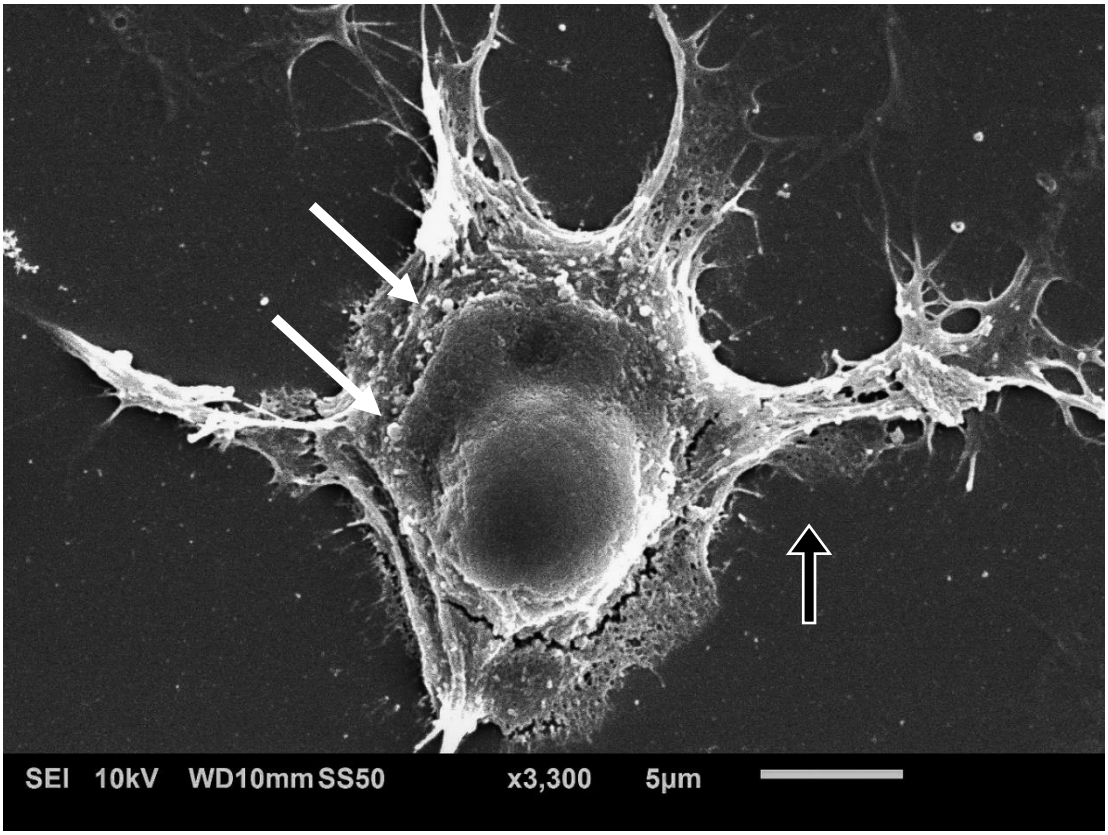

Figure 20. Higher magnification Scanning electron micrograph of Neuro-2a cells treated with 100 µM of glyphosate for 24 hours. Representative micrograph of Neuro-2a cell treated for 24 hours with 100 µM of glyphosate. Cell surface blebbing (white arrow) and fewer retraction fibers are noted (black arrow). Total magnification, x3,300.

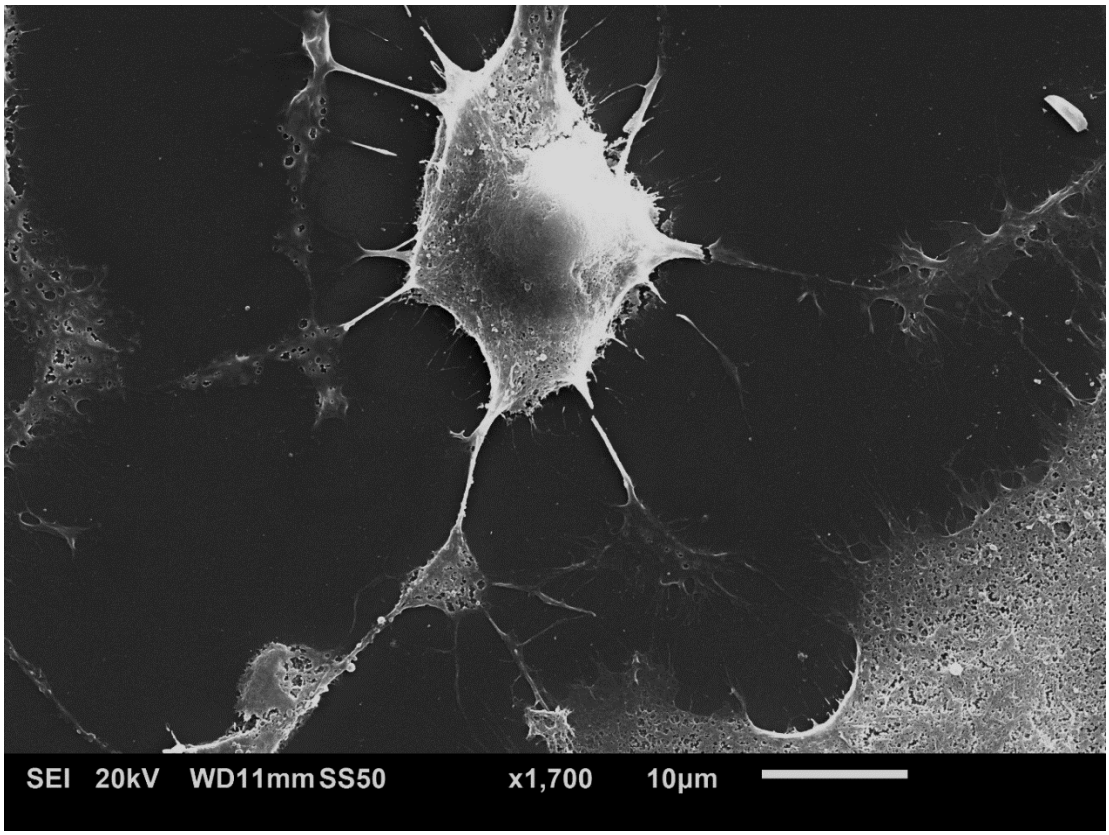

Figure 21. Scanning electron micrograph of Neuro-2a cells treated with 6 µM of mancozeb for 30 minutes. This micrograph is a representative of Neuro-2a cells treated for 30 minutes with 6 µM of mancozeb. Cells have a rounded appearance. Total magnification, x1,700.

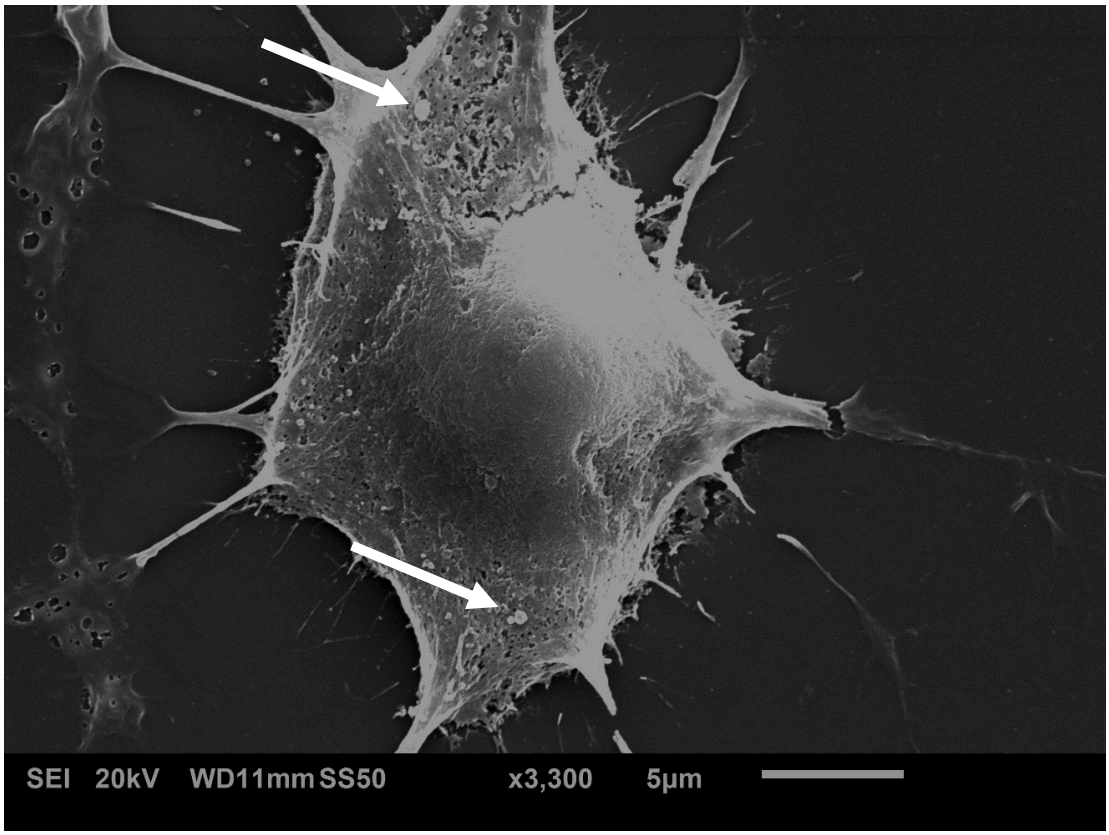

Figure 22. Higher magnification Scanning electron micrograph of Neuro-2a cells treated with 6 µM of mancozeb for 30 minutes. Representative micrograph of Neuro-2a cell treated for 30 minutes with 6 µM of mancozeb. Cell surface blebbing (white arrow) and fewer retraction fibers are noted. Total magnification, x3,300.

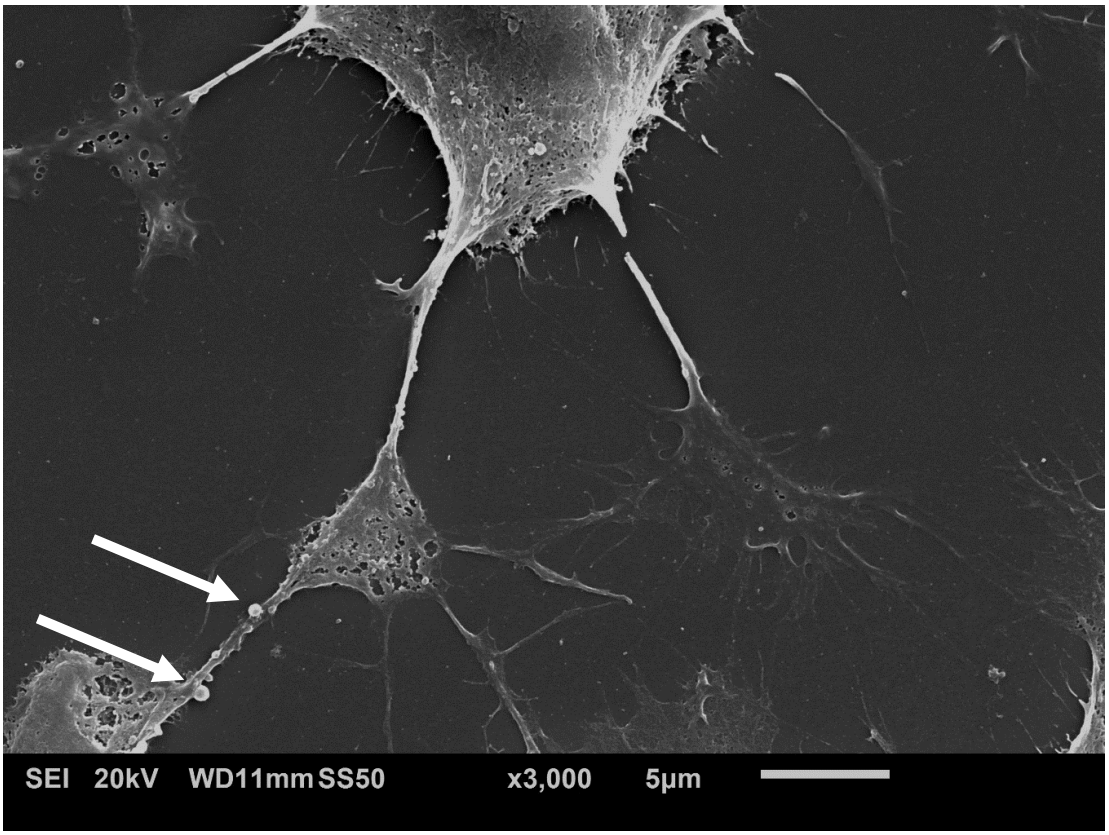

Figure 23. Scanning electron micrograph of a Neuro-2a cell neuretic process treated with 6 µM of mancozeb for 30 minutes. Representative micrograph of Neuro-2a cell process treated for 30 minutes with 6 µM of mancozeb. Blebs are noted along the neuretic process (white arrows). Total magnification, x3,000.

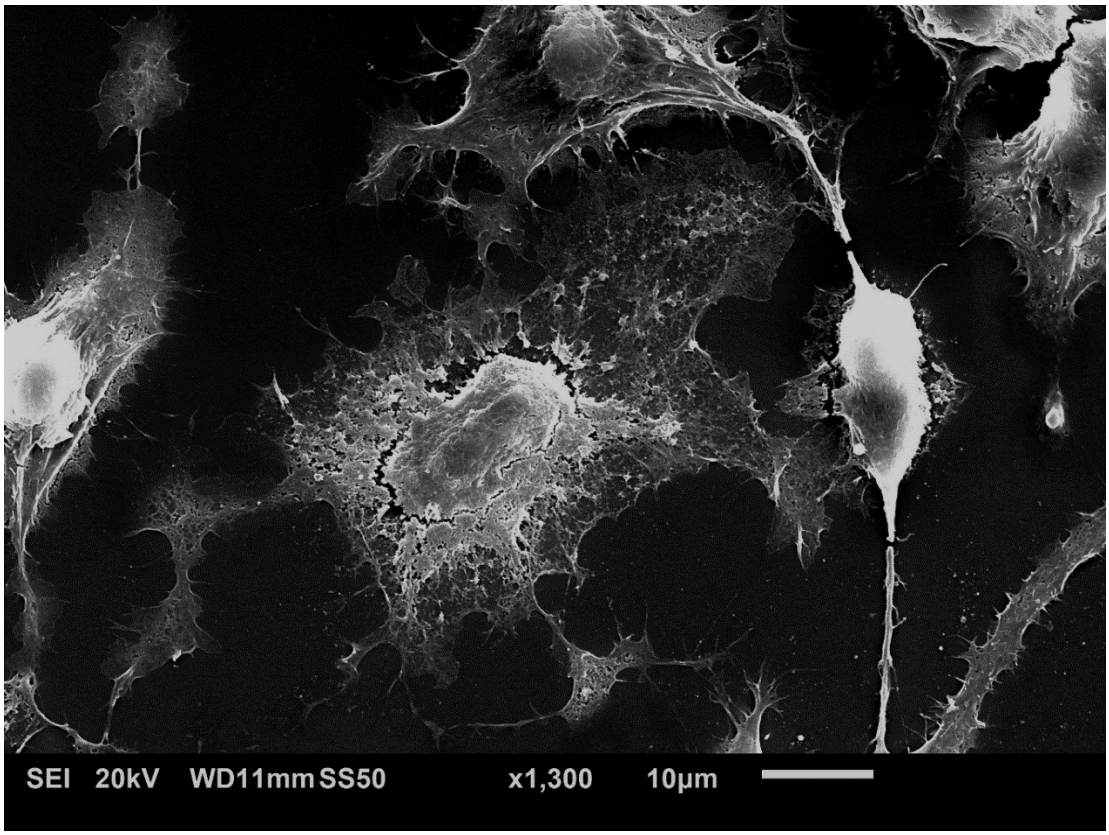

Figure 24. Scanning electron micrograph of Neuro-2a cells treated with 6 µM of mancozeb for 24 hours. This micrograph is a representative of Neuro-2a cells treated for 24 hours with 6 µM of mancozeb showing disrupted neuretic processes. Total magnification, x1,300.

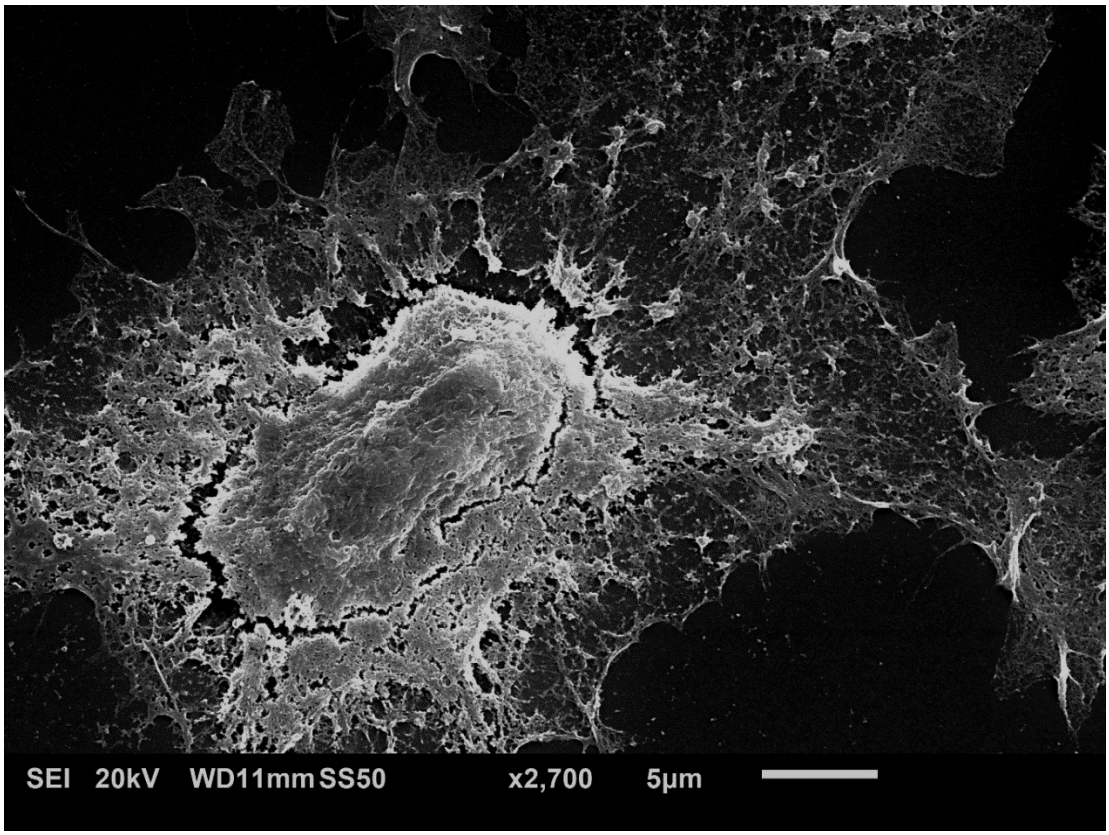

Figure 25. Higher magnification Scanning electron micrograph of Neuro-2a cells treated with 6 µM of mancozeb for 24 hours. Representative micrograph of Neuro-2a cell treated for 24 hours with 6 µM of mancozeb. Loss of retraction fibers and disrupted neuretic processes are noted. Total magnification, x2,700.

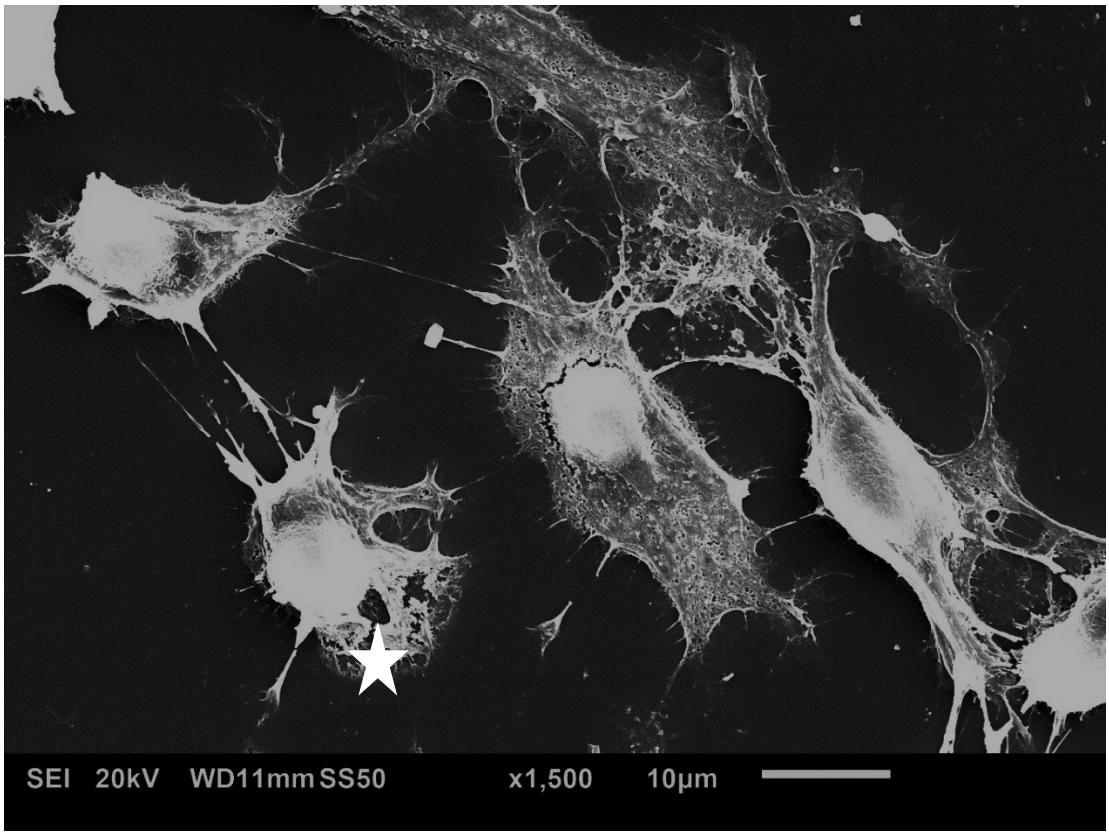

Figure 26. Scanning electron micrograph of Neuro-2a cells treated with a mixture of 100 µM glyphosate plus 6 µM of mancozeb for 24 hours. This micrograph is a representative of Neuro-2a cells treated for 24 hours with mixture of glyphosate plus mancozeb. Cells show disrupted neuretic processes and plasmalemmal toroids (white star). Some cells demonstrate a rounded appearance. Total magnification, x1,500.

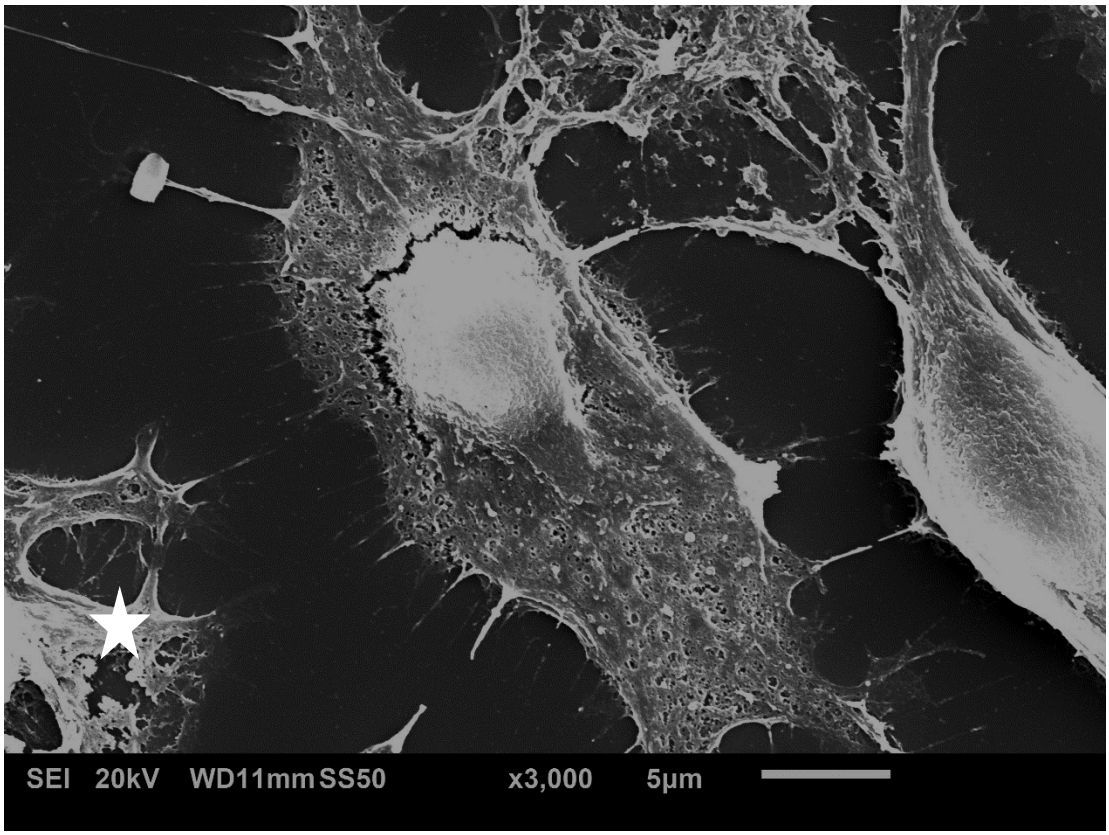

Figure 27. Higher magnification Scanning electron micrograph of Neuro-2a cells treated with mixture of 100 µM glyphosate plus 6 µM of mancozeb for 24 hours. Representative micrograph of Neuro-2a cells treated for 24 hours with mixture of glyphosate plus mancozeb showing plasmalemmal toroids (white star). Total magnification, x3,000.

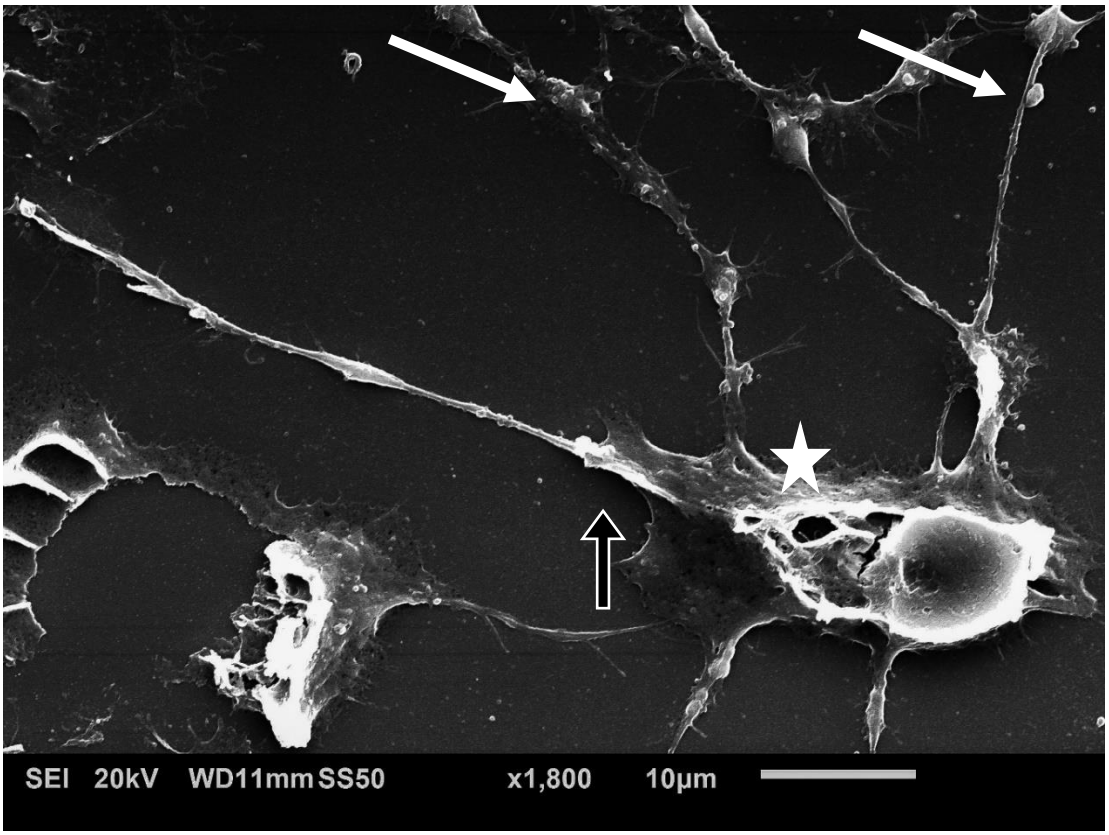

Figure 28. Scanning electron micrograph of Neuro-2a cells treated with 6 µM of mancozeb for 30 minutes followed by 100 µM glyphosate for 24 hours. This micrograph is a representative of Neuro-2a cells treated with mancozeb for 30 minutes followed by glyphosate for 24 hours showing loss of retraction fibers (black arrow), blebbing (white arrow) and plasmalemmal toroids (white star). Total magnification, x1,800.

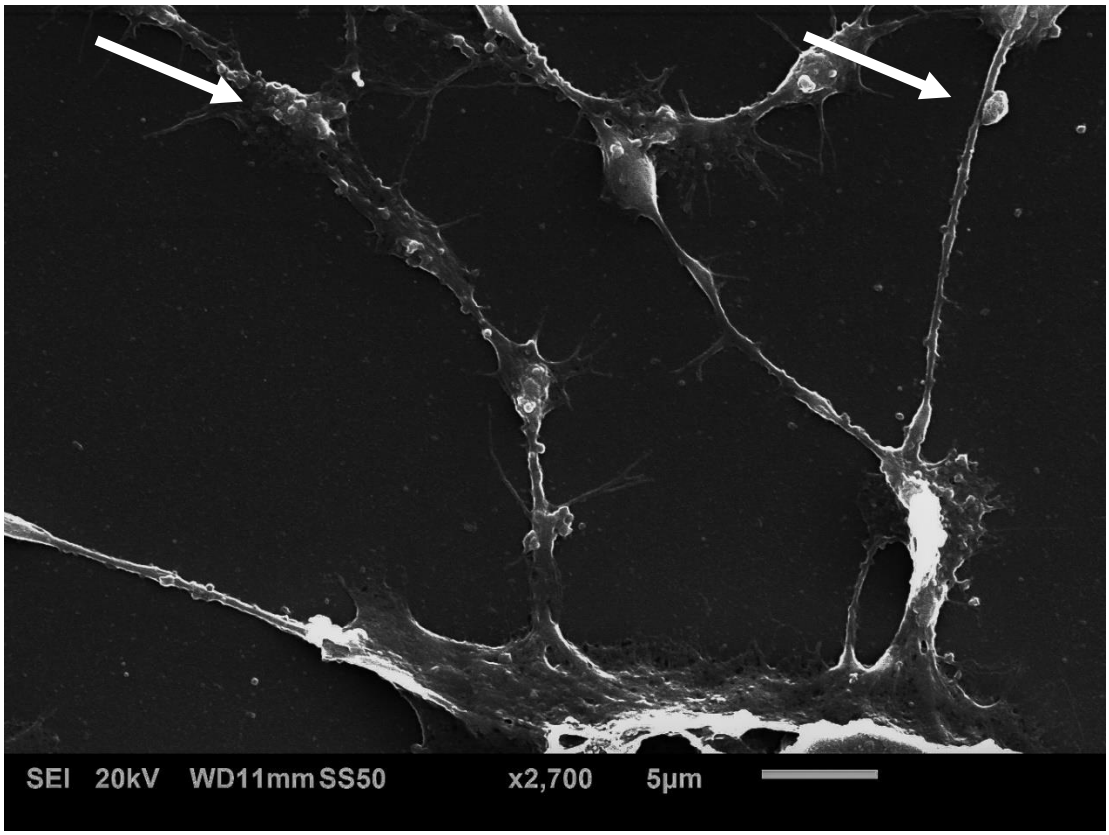

Figure 29. Higher magnification Scanning electron micrograph of Neuro-2a cells treated with 6 µM of mancozeb for 30 minutes followed by 100 µM glyphosate for 24 hours. Representative micrograph of Neuro-2a cells treated with mancozeb for 30 minutes followed by glyphosate for 24 hours. Multiple blebs are noted along the neuretic processes (white arrow). Total magnification, x2,700.

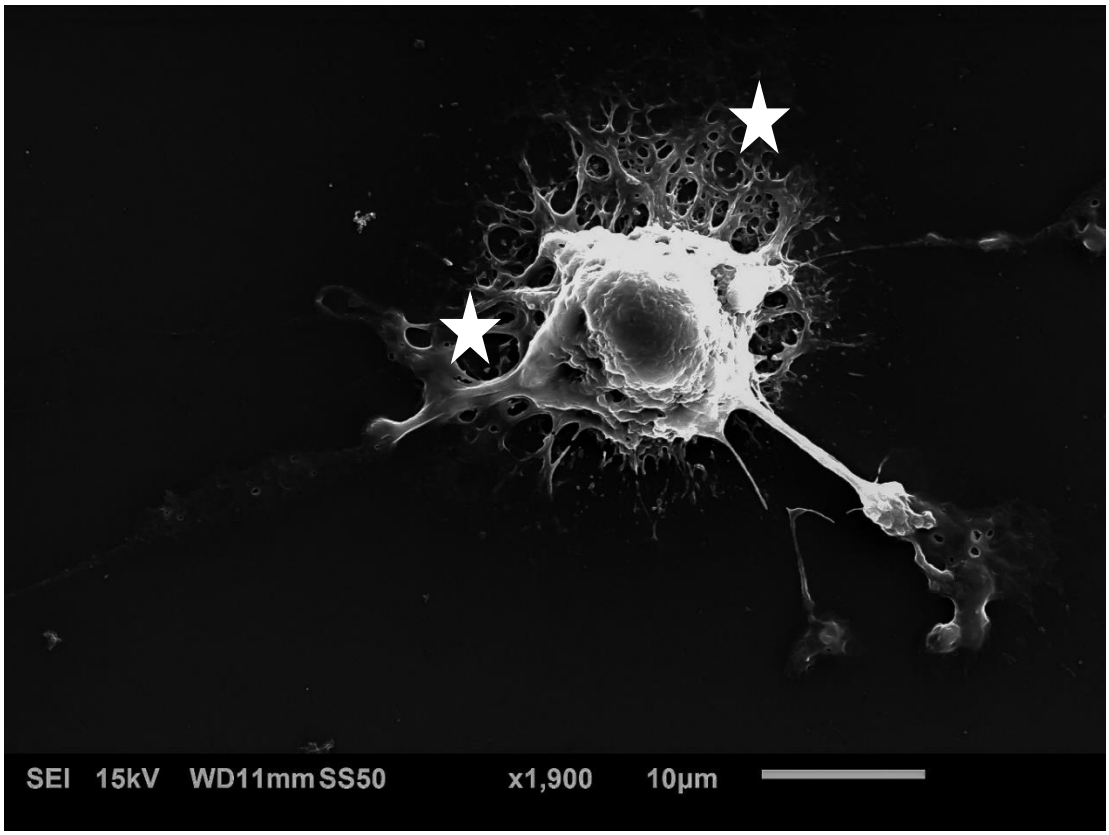

Figure 30. Scanning electron micrograph of Neuro-2a cells treated with 100 µM glyphosate for 30 minutes followed by 6 µM of mancozeb for 24 hours. This micrograph is a representative of Neuro-2a cells treated with glyphosate for 30 minutes followed by mancozeb for 24 hours showing disruption of the neuretic processes and plasmalemmal toroids (white star). Total magnification, x1,900.

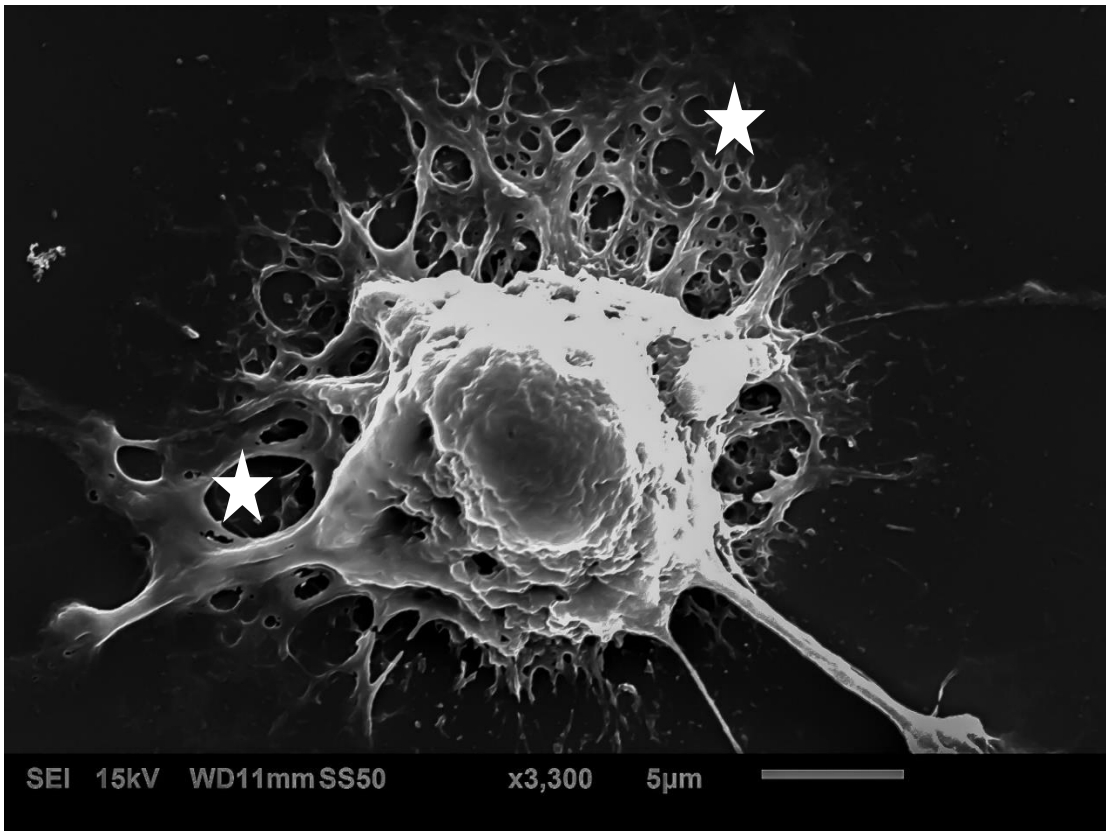

Figure 31. Higher magnification Scanning electron micrograph of Neuro-2a cells treated with 100 µM glyphosate for 30 minutes followed by 6 µM of mancozeb for 24 hours. This micrograph is a representative of Neuro-2a cells treated with glyphosate for 30 minutes followed by mancozeb for 24 hours showing plasmalemmal toroids (white star). Total magnification, x3,300.

	Media	100 µM GL	6 µM MZ
Copper (ppb/mg)	0.066±0.024	0.071±0.020	0.070±0.022
Magnesium (ppb/mg)	85.95±21.16	88.49±9.28	123.3±16.30
Manganese (ppb/mg)	0.000±0.06	0.000±0.05	**1.696±0.180 ***
Iron (ppb/mg)	1.044±0.013	1.021±0.041	0.962±0.018
Zinc (ppb/mg)	0.653±0.148	0.669±0.012	1.608±0.033

Table 1. ICP-OES: Metal levels in Media, 100 µM glyphosate and 6 µM mancozeb. Levels of copper, magnesium, manganese, iron and zinc were measured in media, 100 µM glyphosate (GL) and in 6 µM of mancozeb (MZ) using Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES). Both the 100 µM of GL and the 6 µM of MZ were prepared by diluting stock solutions of GL and MZ, respectively using media. No significant difference was observed in levels of copper, magnesium, iron and zinc between the treatment compounds and the media. Levels of manganese were significantly higher in 6 µM of MZ compared to the media. Metal levels are expressed as ppb/mg of dry weight. Values represent mean +/- SEM of three experiments, n=3. Data were analyzed using a one-way ANOVA followed by Dunnett's post-hoc test. *$p<0.05$.

	Copper in Neuro-2a cells (ppb/mg)	Copper in Media (ppb/mg)
Control	0.119 ±0.010	0.145±0.002
GL (30min)	0.153 ±0.019	0.121±0.040
GL (24 h)	0.129 ±0.012	0.081±0.016
MZ (30 min)	0.124 ±0.014	0.095±0.024
MZ (24 h)	**0.257±0.013** **	**0.048±0.003** *
GL + MZ (24 h)	0.202 ±0.042	0.105±0.03
MZ (30 min) + GL (24 h)	**0.238 ±0.027** *, +, ‡	0.073±0.02
GL (30 min) + MZ (24 h)	**0.265 ±0.032** **, #	**0.046±0.003** *

Table 2. ICP-OES: Copper levels in Neuro-2a cells and in media after treatment with glyphosate, mancozeb or their combinations. Neuro-2a cells were treated with 100 µM of GL, 6 µM of MZ or their combinations. Copper concentrations were assessed in the cells and in the media collected from the flasks following the treatment period using ICP-OES. Copper levels are expressed as ppb/mg of dry weight. The experiment was repeated in triplicate, n=3 and each n number represent a pooled sample from 6 to 12 flasks. Significant increases in intracellular copper levels were observed in MZ (24 h) treatment and in combination treatments MZ (30 min) + GL (24 h) and GL (30 min) + MZ (24 h) when compared to control Neuro-2a cells. Increases in intracellular copper levels were significantly higher in combination treatment MZ (30 min) + GL (24 h) compared to MZ (30 min) and GL (24 h) treatment groups. Increases in intracellular copper levels were also significantly higher in combination treatment GL (30 min) + MZ (24 h) compared to GL (30 min) treatment group. In the media collected after treatments, significant decreases in copper levels were observed in MZ (24 h) treatment and in combination treatment of GL (30 min) + MZ (24 h) when compared to media collected from control group. Data were analyzed using one–way ANOVA followed by Dunnett's post-hoc test, *$p<0.05$, **$p<0.01$ compared to control group. Data were analyzed using Student's t-test when comparing between two treatment groups, +$p<0.05$ compared to GL (24hr), #$P<0.05$ compared to GL (30min), ‡$p<0.05$ compared to MZ (30min).

	Zinc in Neuro-2a cells (ppb/mg)	Zinc in Media (ppb/mg)
Control	5.403±0.267	2.389±0.122
GL (30min)	4.614±0.260	1.410±0.088
GL (24 h)	5.416±0.166	1.121 ±0.106
MZ (30 min)	5.992±0.216	1.430±0.049
MZ (24 h)	**6.992±0.403** **	0.973±0.622
GL + MZ (24 h)	**6.717±0.073** *, + +	1.049±0.535
MZ (30 min) + GL (24 h)	**7.862±0.354** ***,++, ‡	**0.417±0.048** *
GL (30 min) + MZ (24 h)	**7.719±0.151** ***,#	**0.653±0.039** *

Table 3. ICP-OES: Zinc levels in Neuro-2a cells and in media after treatment with glyphosate, mancozeb or their combinations. Neuro-2a cells were treated with 100 μM of GL, 6 μM of MZ or their combinations. Zinc concentrations were assessed in the cells and in the media collected from the flasks following the treatment period using ICP-OES. Copper levels are expressed as ppb/mg of dry weight. The experiment was repeated in triplicate, n=3 and each n number represent a pooled sample from 6 to 12 flasks. Significant increases in intracellular zinc levels were observed in MZ (24 h) treatment and in all combination treatments MZ + GL (24 h), MZ (30 min) + GL (24 h) and GL (30 min) + MZ (24 h) when compared to control Neuro-2a cells. Increases in intracellular zinc levels were significantly higher in mixture of MZ + GL (24 h) compared to GL (24 h) treatment group. Increases in intracellular zinc levels were significantly higher in combination treatment MZ (30 min) + GL (24 h) compared to MZ (30 min) and GL (24 h) treatment groups. Increases in intracellular zinc levels were also significantly higher in combination treatment GL (30 min) + MZ (24 h) compared to GL (30 min) treatment group. In the media collected after treatments, significant decreases in zinc levels were observed in MZ (30 min) + GL (24 h) and in GL (30 min) + MZ (24 h) combination treatments when compared to media collected from control group. Data were analyzed using one–way ANOVA followed by Dunnett's post-hoc test, *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to control group. Data were analyzed using Student's t-test when comparing between two treatment groups, ++$p<0.01$ compared to GL (24hr), #$P<0.05$ compared to GL (30min), ‡$p<0.05$ compared to MZ (30min).

	Manganese in Neuro-2a cells (ppb/mg)	Manganese in Media (ppb/mg)
Control	0.000±0.000	0.000±0.001
GL (30min)	0.000±0.000	0.000±0.008
GL (24 h)	0.000±0.000	0.000 ±0.038
MZ (30 min)	0.000±0.000	0.0156±0.193
MZ (24 h)	**0.178±0.012 ****	**1.604±0.100 *****
GL + MZ (24 h)	**0.152±0.010 *,++**	**1.177±0.199 **,++**
MZ (30 min) + GL (24 h)	0.016±0.005	0.003±0.002
GL (30 min) + MZ (24 h)	**0.248±0.021 ***,#**	**1.545±0.133 ***,#**

Table 4. ICP-OES: Manganese levels in Neuro-2a cells and in media after treatment with glyphosate, mancozeb or their combinations. Neuro-2a cells were treated with 100 μM of GL, 6 μM of MZ or their combinations. Manganese concentrations were assessed in the cells and in the media collected from the flasks following the treatment period using ICP-OES. Manganese levels are expressed as ppb/mg of dry weight. The experiment was repeated in triplicate, n=3 and each n number represent a pooled sample from 6 to 12 flasks. Significant increases in intracellular manganese levels were observed in MZ (24 h) treatment, in GL + MZ (24 h) mixture treatment and GL (30 min) + MZ (24 h) combination treatment when compared to control Neuro-2a cells. Increases in intracellular manganese levels were significantly higher in mixture of GL + MZ (24 h) compared to GL (24 h) treatment group. Increases in intracellular manganese levels were also significantly higher in combination treatment GL (30 min) + MZ (24 h) compared to GL (30 min) treatment group. In the media collected after treatments, significant increases in manganese levels were observed in MZ (24 h), GL + MZ (24 h) mixture and in GL (30 min) + MZ (24 h) combination treatment groups when compared to media collected from control group. Data were analyzed using one–way ANOVA followed by Dunnett's post-hoc test, *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to control group. Data were analyzed using Student's t-test when comparing between two treatment groups, ++$p<0.01$ compared to GL (24hr), #$P<0.05$ compared to GL (30min).

	Magnesium in Neuro-2a cells (ppb/mg)	Magnesium in Media (ppb/mg)
Control	151.4±9.774	107.7±17.69
GL (30min)	152.6±5.070	100.5±16.00
GL (24 h)	157.4±9.531	82.08±13.71
MZ (30 min)	155.1±7.646	102.0±4.930
MZ (24 h)	158.5±8.647	103.5±13.17
GL + MZ (24 h)	159.8±9.282	102.0±9.614
MZ (30 min) + GL (24 h)	162.5±12.276	102.7±5.870
GL (30 min) + MZ (24 h)	159.4±12.046	85.92±16.02

Table 5. ICP-OES: Magnesium levels in Neuro-2a cells and in media after treatment with glyphosate, mancozeb or their combinations. Neuro-2a cells were treated with 100 μM of GL, 6 μM of MZ or their combinations. Magnesium concentrations were assessed in the cells and in the media collected from the flasks following the treatment period using ICP-OES. Magnesium levels are expressed as ppb/mg of dry weight. The experiment was repeated in triplicate, n=3 and each n number represent a pooled sample from 6 to 12 flasks. No significant changes in magnesium levels were observed with any of the treatment groups whether in the cells or in the media collected after treatments from the flasks. Data were analyzed using one–way ANOVA followed by Dunnett's post-hoc test, *$p<0.05$.

	Iron in Neuro-2a cells (ppb/mg)	Iron in Media (ppb/mg)
Control	2.412±0.220	1.511 ±0.136
GL (30min)	2.240±0.198	1.702±0.011
GL (24 h)	2.582±0.167	1.165 ±0.015
MZ (30 min)	2.728±0.122	1.646±0.012
MZ (24 h)	2.801±0.128	0.928±0.392
GL + MZ (24 h)	3.104±0.216	1.134±0.013
MZ (30 min) + GL (24 h)	3.282±0.564	1.330±0.103
GL (30 min) + MZ (24 h)	3.076±0.473	1.021±0.427

Table 6. ICP-OES: Iron levels in Neuro-2a cells and in media after treatment with glyphosate, mancozeb or their combinations. Neuro-2a cells were treated with 100 µM of GL, 6 µM of MZ or their combinations. Iron concentrations were assessed in the cells and in the media collected from the flasks following the treatment period using ICP-OES. Iron levels are expressed as ppb/mg of dry weight. The experiment was repeated in triplicate, n=3 and each n number represent a pooled sample from 6 to 12 flasks. No significant changes in iron levels were observed with any of the treatment groups whether in the cells or in the media collected after treatments from the flasks. Data were analyzed using one–way ANOVA followed by Dunnett's post-hoc test, *$p<0.05$.

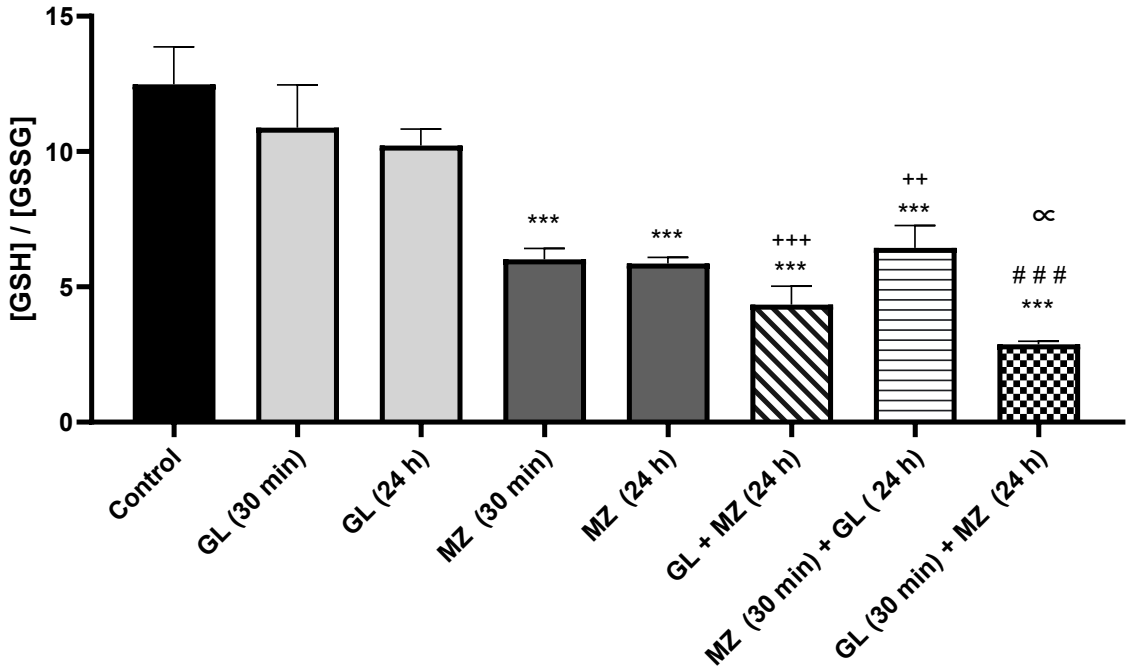

Figure 32. GSH/GSSG ratio in Neuro-2a cells treated with glyphosate, mancozeb or their combinations. Neuro-2a cells were treated with GL (100 μM), MZ (6 μM) or their combinations. Reduced glutathione (GSH) to oxidized glutathione (GSSG) ratio (GSH/GSSG) was assessed by dividing the RLU of reduced glutathione by the RLU of oxidized glutathione. No significant changes in GSH/GSSG ratio were observed with GL treatment groups. Significant decreases in GSH/GSSG ratios were observed with MZ treatment groups and all three forms of GL and MZ combinations. Combination treatments of MZ + GL (24 h) and MZ (30 min) + GL (24 h) resulted in a significant decrease in GSH/GSSG ratio compared to GL (24 h) treatment. Combination treatment of GL (30min) + MZ (24hr) resulted in a significant decrease in GSH/GSSG ratio when compared to both GL (30 min) and MZ (24 h). The experiment was repeated in triplicate, n=3. Data were analyzed using one-way ANOVA followed by Tukey's post-hoc test. ***$p<0.001$ compared to control, ++$p<0.01$, +++$p<0.001$ compared to GL (24 h), ###$p<0.001$ compared to GL (30 min), α$p<0.05$ compared to MZ (24 h).

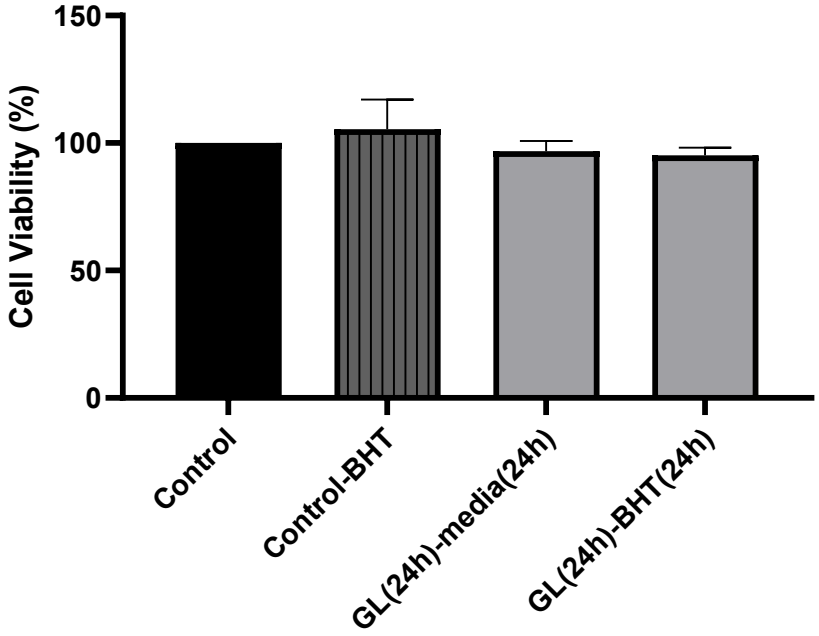

Figure 33. Glyphosate exposed Neuro-2a cells post-treated with butylated hydroxytoluene. Neuro-2a cells were treated for 24 hours with 100 μM of GL. Following the 24 hours, GL was removed and cells were post-treated for 24 hours with 50 μM butylated hydroxytoluene (BHT), GL (24 h) - BHT (24 h) or were left to recover in media, GL (24 h) - media (24 h). No significant changes in cell viability were observed with GL groups whether cells were post-treated with BHT, GL (24 h) - BHT (24 h), or were left to recover in media, GL (24 h) - media (24 h) compared to control group. No significant changes in cell viability were also observed with GL (24 h) - BHT (24 h) compared to control BHT group. All values are expressed as percentage of control group. The experiment was repeated in triplicate, n=3. Data were analyzed using one-way ANOVA followed by Tukey's post-hoc test. *$p<0.05$

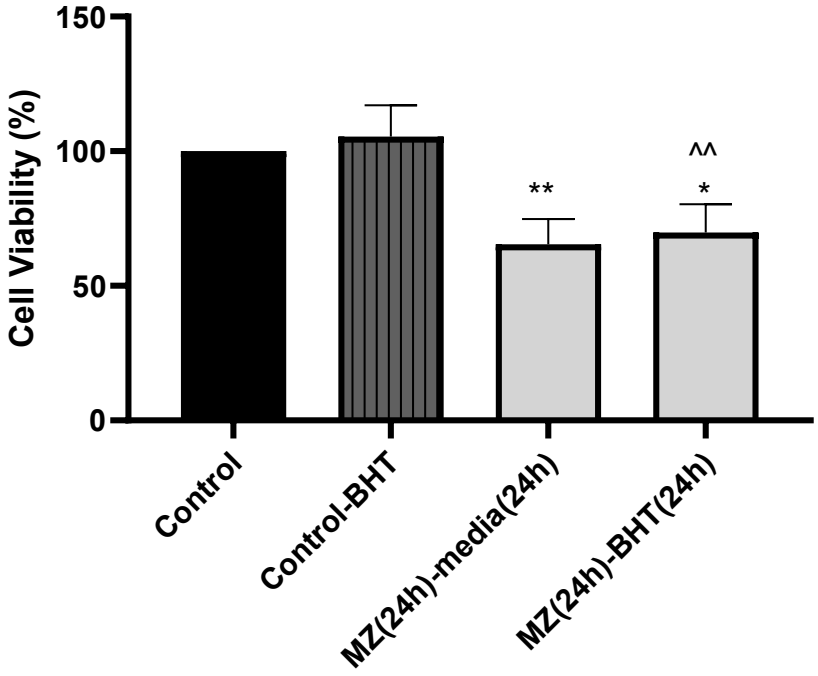

Figure 34. Mancozeb exposed Neuro-2a cells post-treated with butylated hydroxytoluene. Neuro-2a cells were treated for 24 hours with 6 µM of MZ. Following the 24 hours, MZ was removed and cells were post-treated for 24 hours with 50 µM butylated hydroxytoluene (BHT), MZ (24 h) - BHT (24 h) or were left to recover in media, MZ (24 h) - media (24 h). Significant decreases in cell viability were observed with MZ groups whether cells were post-treated with BHT, MZ (24 h) - BHT (24 h), or were left to recover in media, MZ (24 h) - media (24 h) compared to control group. Significant decreases in cell viability were also observed with MZ (24 h) - BHT (24 h) compared to control BHT group. All values are expressed as percentage of control group. The experiment was repeated in triplicate, n=3. Data were analyzed using one-way ANOVA followed by Tukey's post-hoc test. *$p<0.05$, **$p<0.01$ compared to control, ^^$p<0.01$ compared to control-BHT.

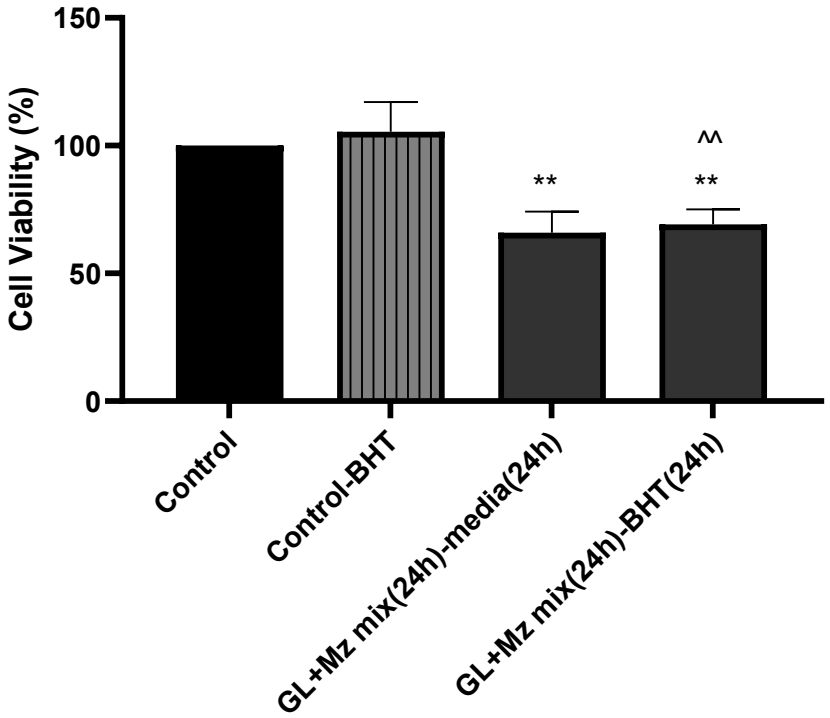

Figure 35. Mixture exposed Neuro-2a cells post-treated with butylated hydroxytoluene. Neuro-2a cells were treated for 24 hours with a mixture of 100 µM GL plus 6 µM of MZ, GL + MZ. Following the 24 hours, the mixture was removed and cells were post-treated for 24 hours with 50 µM butylated hydroxytoluene (BHT), GL + MZ mix (24 h) - BHT (24 h) or were left to recover in media, GL + MZ mix (24 h) - media (24 h). Significant decreases in cell viability were observed with mixture groups whether cells were post-treated with BHT, GL + MZ mix (24 h) - BHT (24 h), or were left to recover in media, GL + MZ (24 h) mix - media (24 h) compared to control group. Significant decreases in cell viability were also observed with GL + MZ (24 h) mix - BHT (24 h) compared to control BHT group. All values are expressed as percentage of control group. The experiment was repeated in triplicate, n=3. Data were analyzed using one-way ANOVA followed by Tukey's post-hoc test. **$p<0.01$ compared to control, ^^$p<0.01$ compared to control-BHT.

CHAPTER 4. DISCUSSION

Excessive application of pesticides has increased the risk of environmental contamination and hence the possibility of combined exposure to multiple pesticides. Toxicological effects of pesticides in combination are often different from those reported when pesticides are tested separately and therefore toxicological assessments of single pesticides may rarely reflect their combined adverse outcomes (Hernández et al., 2017). The herbicide glyphosate and the fungicide mancozeb are pesticides with massive global application. In Europe, traces of both compounds have been identified in fruits such as kiwi, oranges and pears, in vegetables such as potatoes and in dry beans, rye and rice (EFSA, 2022). In France, glyphosate metabolite, aminomethyl phosphonic acid (AMPA) and mancozeb metabolites, ethylene thiourea (ETU) and ethylene urea (EU) were among pesticides that are frequently found in urine samples of residents living in proximity to vineyards (Dereumeaux et al., 2022). In Brazil, both glyphosate and mancozeb have been detected in levels exceeding their maximum limits in drinking water (Panis et al., 2022). Combined exposure to glyphosate and mancozeb whether simultaneously or sequentially is therefore likely to occur in occupational or industrial settings or through food or water consumption. Toxicities associated with glyphosate and mancozeb have been investigated separately in several studies. Glyphosate and glyphosate-based formulations have been linked to adverse effects on liver, kidneys, thyroid and nervous system as well as teratogenic and carcinogenic effects (Benedetti et al., 2004; Cattani et al., 2014; Dallegrave et al., 2003; De Liz Oliveira Cavalli et al., 2013; Dedeke et al., 2018; Gasnier et al., 2009; Gui et al., 2012a; Marrie, 2004; Myers et al., 2016; Peixoto, 2005; Romano et al., 2012a; Tizhe et al., 2014; Wunnapuk et al., 2014; L. Zhang et al., 2019). Mancozeb

has also been linked to hepatotoxicity, nephrotoxicity, GI toxicity, neurotoxicity, reproductive toxicity and endocrine disruption (Akthar et al., 2020; Domico et al., 2006; Hoffman et al., 2016; Hoffman and Hardej, 2012; Kistinger and Hardej, 2022; Ksheerasagar and Kaliwal, 2003; Pirozzi et al., 2016). In the current investigation, mouse neuroblastoma Neuro-2a cell line is used as an *in vitro* model to assess neurotoxicity of different glyphosate and mancozeb combinations and to compare it to that of glyphosate and mancozeb when examined separately.

In this study, cell viability was assessed using trypan blue exclusion assay in Neuro-2a cells treated with glyphosate, mancozeb or their combinations. Concentrations of glyphosate and mancozeb evaluated in the study are comparable to reference doses (RfD) of either compound as set by the EPA (USEPA, 1993, 2005). Data from trypan blue exclusion assay demonstrate that 24 hours treatment with glyphosate from 500 to 10,000 µM and mancozeb from 4 to 10 µM cause a concentration dependent reduction in Neuro-2a cells viability. This is consistent with previous *in vitro* studies where glyphosate at concentrations from 5,000 to 40,000 µM caused a concentration dependent reduction in PC-12 cells viability following 24 to 72 hours of treatment (Gui et al., 2012). Martinez et al. (2020) observed similar results where glyphosate 100 to 20,000 µM induced reduction in SH-SY5Y cells viability following 48 hours of treatment (Martínez et al., 2020). Similarly, mancozeb at 5 to 20 µM resulted in concentration dependent reduction in rat hippocampal astrocytes cells viability following one hour treatment (Tsang and Trombetta, 2007). Domico et al. (2006) have also reported a reduction in mesencephalic cells viability following 24 hours treatment with mancozeb from 10 to 120 µM (Domico et al., 2006).

Cell viability of Neuro-2a cells were also assessed after treatment with various combinations of glyphosate and mancozeb. A mixture of non-lethal concentrations of glyphosate (100 μM) and mancozeb (2 μM) was initially tested. This mixture of 100 μM glyphosate plus 2 μM mancozeb did not induce significant changes in Neuro-2a cells viability following 24 hours of treatment when compared to control Neuro-2a cells and when compared to either of the compounds alone. Next a mixture of 100 μM glyphosate plus 4 μM of mancozeb was tested and this mixture also did not result in significant changes in cells viability after 24 hours of treatment when compared to control Neuro-2a cells and when compared to either of the compounds alone. Mixture of 100 μM glyphosate plus 6 μM mancozeb was then tested and it induced a reduction in Neuro-2a cells viability following 24 hours of treatment when compared to control Neuro-2a cells and when compared to glyphosate treated cells. This mixture however did not induce greater reduction in Neuro-2a cells viability when compared to mancozeb treated cells. In addition to mixtures, sequential forms of glyphosate and mancozeb combinations were also assessed. Combination of 6 μM mancozeb for 30 minutes followed by 100 μM glyphosate for 24 hours induced a reduction in cells viability when compared to control Neuro-2a cells and it also induced a greater reduction in cells viability than that caused by either glyphosate or mancozeb alone. Similarly, combination of 100 μM glyphosate for 30 minutes followed by 6 μM mancozeb for 24 hours induced a reduction in cells viability when compared to control Neuro-2a cells and it also induced a greater reduction in cells viability than that caused by either glyphosate or mancozeb alone. This is in agreement with observations of Astiz, Alaniz and Marra (2009) reporting a greater reduction in liver and brain cells survival upon treatment with combinations of

glyphosate, zineb (zinc ethylene bis-dithiocarbamate) and the insecticide dimethoate compared to any of the pesticides when tested separately (Astiz, Alaniz, et al., 2009).

Light micrographs of Neuro-2a cells were taken following treatment with glyphosate, mancozeb and their combinations. Neuro-2a cells treated with 100 µM glyphosate had a rounded appearance with loss of neuretic processes in the 24 hours treatment. Neuro-2a cells treated with 6 µM of mancozeb for 30 minutes and for 24 hours demonstrated altered neuretic processes and round floating cells with cellular debris noted in the 24 hours treatment. This is in agreement with light microscopy observations of Tsang and Trombetta (2007) reporting morphological changes in rat hippocampal astrocytes after 1 hour treatment with mancozeb at concentration of 7.5 µM (Tsang and Trombetta, 2007). Light micrographs of Neuro-2a cells treated with mixture of 100 µM glyphosate plus 6 µM mancozeb for 24 hours, combination of 6 µM mancozeb for 30 minutes followed by 100 µM glyphosate for 24 hours and combination of 100 µM glyphosate for 30 minutes followed by 6 µM mancozeb for 24 hours confirmed cell viability results where all combination groups showed less confluent cells with severely altered neuretic processes, cellular debris and numerous round floating cells.

Morphological changes in Neuro-2a cells were further observed using scanning electron microscopy following treatment with glyphosate, mancozeb and their combinations. Glyphosate (100 µM) treated cells had rounded appearance with fewer retraction fibers. Similar observations were seen by Coullery et al. (2016) who observed shorter and fewer retraction fibers in axons of primary hippocampal pyramidal cells treated with 4 mg/ml of glyphosate from 2 to 4 days (Coullery et al., 2016). In the current study, cellular blebbing was also noted with 24 hour 100 µM glyphosate treatment that is

a sign of cells undergoing apoptosis (Poon et al., 2010). Studies by Gui et al (2012) and Martinez et al. (2020) showed induction of apoptotic pathways in PC-12 cells and in SH-SY5Y cells as a result of glyphosate treatment as indicated by increases in caspase-3/7 activity, over expression of BAX and down expression of BCL2 proteins (Gui et al., 2012b; Martínez et al., 2020).

Scanning electron micrographs of the 6 μM mancozeb treated Neuro-2a cells demonstrated cytoplasmic fragmentation and cellular blebbing. This is indicative of cellular injury and apoptosis might be undergoing. Evidence of apoptosis activation in response to mancozeb exposure has been reported in number of *in vitro* studies (Calviello et al., 2006; Hoffman and Hardej, 2012). Calviello et al (2006) showed pro-oxidant effects and altered expression of the apoptotic proteins BCL2 and cMYC in mancozeb treated rat fibroblasts and peripheral blood mononucleated cells (Calviello et al., 2006). Likewise, Hoffman and Hardej (2012) reported activation of intrinsic and extrinsic apoptotic pathways in mancozeb treated HT-29 cells as indicated by increased caspase-3/7, 8 and 9 activities (Hoffman and Hardej, 2012).

Scanning electron micrographs of mancozeb (6 μM) treated Neuro-2a cells for 24 hours as well as glyphosate (100 μM) and mancozeb (6 μM) combinations including mixture of glyphosate plus mancozeb for 24 hours, mancozeb for 30 minutes followed by glyphosate for 24 hours and glyphosate for 30 minutes followed by mancozeb for 24 hours demonstrated disrupted neuretic processes and plasmalemmal toroids indicating damage to the plasma membrane. Cellular blebbing was also noted in mancozeb for 30 minutes followed by glyphosate for 24 hours combination treated Neuro-2a cells. In addition, disruptions in the neuretic processes and plasmalemmal toroids were noted to be

more severe in Neuro-2a cells treated with glyphosate for 30 minutes followed by mancozeb for 24 hours. Previous unpublished scanning electron microscopy work from our lab showed the presence of holes in the myocyte surface in the Tibialis cranialis of Long Evans rats treated with dithiocarbamates, mancozeb and disulfiram. Similarly, Scanning electron microscopic observations of Dhaneshwar and Hardej (2021) showed cellular blebbing and deterioration of the plasmalemma in mancozeb treated HT-29 cells (Dhaneshwar and Hardej, 2021). Disruptions in neuretic processes observed in this study could be a sign of oxidative damage. Previous studies have demonstrated inhibitory effects of oxidative stress on neuritic processes outgrowth. Neely et al. (1999) observed neuritic outgrowth inhibition in Neuro-2a cells exposed to the lipid peroxidation product, 4-hydroxynonenal (HNE) (Neely et al., 1999). In addition, Ghaffari et al. (2014) showed Neuro-2a cells with altered neuretic processes outgrowth following H_2O_2-induced oxidative challenge (Ghaffari et al., 2014). Cellular blebbing, neuretic processes disruptions and plasmalemmal toroids observed in our scanning electron microscopy data are suggestive of cytotoxicity of mancozeb and glyphosate and mancozeb combinations to Neuro-2a cells and that cytotoxicity might be related to undergoing oxidative injury.

Glyphosate and dithiocarbamate compounds such as mancozeb are potent metal chelators (Cheng and Trombetta, 2004; Delmaestro and Trombetta, 1995; Mertens et al., 2018; Yahfoufi et al., 2020). Alterations in levels of essential metals in response to exposure to glyphosate or mancozeb have been reported in previous studies (Kistinger and Hardej, 2022; Stephenson and Trombetta, 2020; Tsang and Trombetta, 2007; Yahfoufi et al., 2020). Essential metals are crucial for several cellular functions such as redox reactions, electron transport, cell proliferation and neurotransmitter synthesis and

accordingly alterations in their levels might lead to toxicity (Chen et al., 2016; Garza-Lombó et al., 2018). In the current investigation levels of copper, zinc, manganese, iron and magnesium were measured using ICP-OES in Neuro-2a cells treated with glyphosate, mancozeb or their combinations and also in the culture media before and after treating the cells.

Copper levels were measured in culture media and in treatment solutions prepared in culture media including 100 µM glyphosate and 6 µM mancozeb. Copper levels were also measured in Neuro-2a cells following treatment with 100 µM glyphosate, 6 µM mancozeb or their combinations and in the culture media collected from each treatment group after the incubation period. In the treatment solutions of 100 µM glyphosate and 6 µM mancozeb, levels of copper were not significantly different than copper levels found in the culture media.

Intracellular copper levels in Neuro-2a cells as well as copper levels in culture media collected after treatment were not changed in any of the 100 µM glyphosate treatment groups. Glyphosate's ability to chelate copper has been documented (Caetano et al., 2012; Undabeytia et al., 2002). Undabeytia et al. (2002) showed using infrared spectroscopy the formation of glyphosate-copper complexes mainly via participation of glyphosate's carboxylate and phosphonic moieties (Undabeytia et al., 2002). The formation of glyphosate-metal complexes has been reported to be largely dependent on glyphosate concentration and that might explain unaltered copper levels seen in this study in response to glyphosate treatments. Previous studies have reported the glyphosate-copper chelate formation happening at glyphosate concentrations higher than 500 µM (Glass, 1984; Undabeytia et al., 2002). In addition, a previous study by Glass (1984)

detected increase in the glyphosate-copper chelates formation at lower pH values (pH = 5) and with high glyphosate concentrations (600 μM) using polarograghy, ultraviolet and infrared spectrophotometric as well as liquid chromatographic techniques (Glass, 1984).

Intracellular copper levels were elevated in Neuro-2a cells following 24 hours treatment with 6 μM mancozeb. Several previous studies manifested the ability of mancozeb and other dithiocarbamate compounds to chelate copper and alter its levels (Hoffman et al., 2016; Kistinger and Hardej, 2022; Rose Stein and Trombetta, 1993; Trombetta et al., 1988). Hoffman et al. (2016) reported elevations in copper concentrations in human colon cells, HT-29 and Caco2 following treatment with EBDC compounds including, maneb, mancozeb, nabam and zineb (Hoffman et al., 2016). Kistinger and Hardej (2022) showed copper accumulation in the kidneys of rats exposed to EBDCs, mancozeb and nabam (Kistinger and Hardej, 2022). Akhtar and Trombetta (2023) observed copper accumulation in the hippocampus of rats treated with mancozeb (Akhtar and Trombetta, 2023). In addition, copper concentration elevations were reported in rat cerebral astrocytes and Neuro-2a cells treated with diethyldithiocarbamate (Rose Stein and Trombetta, 1993; Trombetta et al., 1988). Intracellular copper levels elevations seen in this study after 24 hours treatment with mancozeb were however associated with decrease in copper levels in the culture media collected from mancozeb treated cells. This suggests that intracellular copper levels elevations are due to transport of extracellular copper from the culture media into the cells. This is in agreement with previous *in vitro* studies suggesting intracellular copper levels elevations in rat thymocytes following treatment with dithiocarbamate compounds, diethydithiocarbamate and pyrrolidine

dithiocarbamate were a result of dithiocarbamates-mediated copper transport from culture media into the cells (Burkitt et al., 1998; Stefan et al., 1995).

Though intracellular copper levels were not changed with mancozeb 30 minutes, glyphosate 30 minutes and glyphosate 24 hours treatments, combinations of glyphosate and mancozeb including, mancozeb for 30 minutes followed by glyphosate for 24 hours and glyphosate for 30 minutes followed by mancozeb for 24 hours mancozeb induced elevations in intracellular copper levels in Neuro-2a cells. Intracellular copper levels elevations were higher in mancozeb 30 minutes followed by glyphosate 24 hours combination compared to either mancozeb 30 minutes or glyphosate 24 hours treatment. Intracellular copper levels elevations were higher in glyphosate 30 minutes followed by mancozeb 24 hours combination compared to glyphosate 30 minutes treatment and not when compared to mancozeb 24 hours treatment. This might indicate that intracellular copper elevations seen with glyphosate 30 minutes followed by mancozeb 24 hours are mainly induced by mancozeb. Similar to mancozeb 24 hours treatment, copper levels were reduced in culture media collected from glyphosate 30 minutes followed by mancozeb 24 hours combination treatment. Also suggesting the transport of extracellular copper from culture media into the cells causing intracellular copper levels elevations. Copper levels were also reduced in culture media collected from mancozeb 30 minutes followed by glyphosate 24 hours combination treatment the reduction in copper levels however was not statistically significant.

Zinc levels were measured in culture media and in treatment solutions prepared in culture media including, 100 µM glyphosate and 6 µM mancozeb. Zinc levels were also measured in Neuro-2a cells following treatment with 100 µM glyphosate, 6 µM

mancozeb or their combinations and in the culture media collected from each treatment group after the incubation period. In the treatment solutions of 100 μM glyphosate and 6 μM mancozeb, levels of zinc were not significantly different than zinc levels found in the culture media.

Intracellular zinc levels in Neuro-2a cells as well as zinc levels in culture media collected after treatment were not changed in any of the 100 μM glyphosate treatment groups. Glyphosate's ability to chelate zinc and alter its levels has been documented (Caetano et al., 2012; Tang et al., 2017). In a study by Caetano et al. (2012), glyphosate-zinc chelate was found to be the most thermodynamically stable among all other glyphosate-divalent metal complexes evaluated in the same study (Caetano et al., 2012). In addition, Tang et al. (2017) reported elevated zinc levels in liver of rats treated with glyphosate at doses 50 and 500 mg/kg which is approximately equivalent to 300 and 3000 μM, respectively (Tang et al., 2017). Lack of change in zinc levels seen in our study could be attributed to the lower concentration of glyphosate tested. In this study, alterations in zinc levels might have been observed if higher glyphosate concentrations were evaluated.

Intracellular zinc levels were elevated in Neuro-2a cells following 24 hours treatment with 6 μM mancozeb. Several previous studies manifested the ability of mancozeb and other dithiocarbamate compounds to chelate zinc and alter its levels (Hoffman et al., 2016; Kim et al., 1999; Stephenson and Trombetta, 2020). Hoffman et al. (2016) demonstrated elevated zinc levels in HT-29 and Caco2 colon cells after treatment with EBDCs, maneb, mancozeb and zineb (Hoffman et al., 2016). In addition, Stephenson and Trombetta (2020) reported elevated zinc levels in the right ventricular myocardium of

rats treated with mancozeb (Stephenson and Trombetta, 2020). In the current study, the intracellular elevations in zinc levels were associated with a decrease in zinc levels in the culture media collected from the 24 hours mancozeb treated Neuro-2a cells, the decrease however was not statistically significant.

Though intracellular zinc levels were not changed with mancozeb 30 minutes, glyphosate 30 minutes and glyphosate 24 hours treatments, mixture of glyphosate plus mancozeb for 24 hours as well as combinations of glyphosate and mancozeb including, mancozeb for 30 minutes followed by glyphosate for 24 hours and glyphosate for 30 minutes followed by mancozeb for 24 hours mancozeb induced elevations in intracellular zinc levels in Neuro-2a cells. Intracellular zinc levels elevations were higher in mancozeb 30 minutes followed by glyphosate 24 hours combination compared to either mancozeb 30 minutes or glyphosate 24 hours treatment. Intracellular zinc level elevations were higher in glyphosate 30 minutes followed by mancozeb 24 hours combination and in glyphosate plus mancozeb 24 hours mixture compared to glyphosate 30 minutes treatment and glyphosate 24 hours treatment, respectively but not when compared to mancozeb 24 hours treatment. This might indicate that intracellular zinc elevations seen with glyphosate 30 minutes followed by mancozeb 24 hours combination and with glyphosate plus mancozeb mixture are mainly induced by mancozeb. Zinc levels were reduced in culture media collected from all combination groups treatments, though the decrease in zinc levels was not statistically significant in the mixture treatment. This suggests that elevations in intracellular zinc levels seen in this study are due to the transport of extracellular zinc from culture media into the cells. This in agreement with a previous study by Kim et al. (1999) suggesting intracellular zinc levels elevations in

cerebral endothelial cells treated with the dithiocarbamate, pyrrolidine dithiocarbamate were due to transport of transport of extracellular zinc from the culture media into the cells (Kim et al., 1999).

Manganese levels were measured in culture media and in treatment solutions prepared in culture media including 100 μM glyphosate and 6 μM mancozeb. Manganese levels were also measured in Neuro-2a cells following treatment with 100 μM glyphosate, 6 μM mancozeb or their combinations and in the culture media collected from each treatment group after the incubation period. In the treatment solution of 100 μM glyphosate, levels of manganese were not significantly different than those found in the culture media. Whereas in the treatment solution of 6 μM mancozeb, manganese levels were significantly higher than levels found in the culture media.

Intracellular manganese levels in Neuro-2a cells as well as manganese levels in culture media collected after treatment were not changed in any of the 100 μM glyphosate treatment groups. The effect of glyphosate treatment on manganese levels was never reported in any previous *in vitro* study. Manganese levels however were assessed in Danish dairy cows that were exposed to glyphosate, low blood serum manganese and cobalt levels were associated with increased excretion of glyphosate in urine. In the same study serum levels of copper and zinc remained unchanged (Kruger et al., 2013). In addition, greenhouse experiments studying the effects of glyphosate on mineral concentrations in leaves and seeds of soybean plants showed decreased manganese levels with no change in copper or zinc levels (Cakmak et al., 2009).

Intracellular manganese levels were elevated in Neuro-2a cells following 24 hours treatment with 6 μM mancozeb. Several previous studies manifested the ability of mancozeb and other dithiocarbamate compounds to alter manganese levels (Hoffman et al., 2016; Stephenson and Trombetta, 2020; Tsang and Trombetta, 2007). Hoffman et al. (2016) demonstrated elevated intracellular manganese levels in HT-29 and Caco2 colon cells after treatment with EBDCs, maneb, mancozeb and zineb (Hoffman et al., 2016). Tsang and Trombetta (2007) also observed increase in manganese levels in mancozeb treated rat hippocampal astrocytes (Tsang and Trombetta, 2007). In addition, Stephenson and Trombetta (2020) reported increased manganese levels in the right ventricular myocardium of rats treated with mancozeb (Stephenson and Trombetta, 2020). In the present study, in addition to the intracellular manganese levels elevations, increases in manganese levels were observed in the culture media collected from the 24 hours mancozeb treated Neuro-2a cells. Also as mentioned earlier treatment solution of 6 μM mancozeb had significantly higher manganese levels than the culture media. Overall, these results suggest that mancozeb itself is the source of manganese elevations observed with mancozeb 24 hours treatment.

Even though intracellular manganese levels were not changed with glyphosate 30 minutes and glyphosate 24 hours treatments, mixture of glyphosate plus mancozeb for 24 hours as well as combination of glyphosate for 30 minutes followed by mancozeb for 24 hours induced elevations in intracellular manganese levels in Neuro-2a cells. Intracellular elevations in manganese were higher in glyphosate 30 minutes followed by mancozeb 24 hours combination and in glyphosate plus mancozeb 24 hours mixture compared to glyphosate 30 minutes treatment and glyphosate 24 hours treatment, respectively but not

when compared to mancozeb 24 hours treatment. This might indicate that intracellular manganese elevations seen with glyphosate 30 minutes followed by mancozeb 24 hours combination and with glyphosate plus mancozeb mixture are mainly induced by mancozeb. Similar to mancozeb 24 hours treatment, manganese levels were elevated in culture media collected from glyphosate 30 minutes followed by mancozeb 24 hours combination treatment as well as from glyphosate plus mancozeb 24 hours mixture treatment. These results are also suggestive of mancozeb being the source of manganese elevations seen with glyphosate plus mancozeb 24 hours mixture treatment and with glyphosate 30 minutes followed by mancozeb for 24 hours combination treatment.

Magnesium levels were measured in culture media and in treatment solutions prepared in culture media including, 100 μM glyphosate and 6 μM mancozeb. Magnesium levels were also measured in Neuro-2a cells following treatment with 100 μM glyphosate, 6 μM mancozeb or their combinations and in the culture media collected from each treatment group after the incubation period. In the treatment solutions of 100 μM glyphosate and 6 μM mancozeb, levels of magnesium were not significantly different than the levels found in the culture media.

Intracellular magnesium levels in Neuro-2a cells as well as magnesium levels in culture media collected after treatment were not changed in any of the 100 μM glyphosate treatment groups, in the 6 μM mancozeb treatment groups or in the combination treatment groups. Previous work by Caetano et al. (2012) documented the ability of glyphosate to chelate magnesium, though glyphosate-magnesium complex was the least thermodynamically stable among glyphosate-metal complexes tested in the study (Caetano et al., 2012). Glyphosate was reported to alter magnesium levels in leaves

and seeds of plants due to its chelating properties (Cakmak et al., 2009). In addition, rats orally treated with glyphosate at doses 50 to 500 mg/kg showed elevated magnesium levels in the brain (Tang et al., 2017). In the present study, lack of change in magnesium levels might be attributed to the low concentration of glyphosate tested. With regards to mancozeb, we believe this is the first *in vitro* study to assess magnesium levels following mancozeb treatment. The effect of mancozeb on magnesium levels also has never been reported in an *in vivo* study.

Iron levels were measured in culture media and in treatment solutions prepared in culture media including, 100 µM glyphosate and 6 µM mancozeb. Iron levels were also measured in Neuro-2a cells following treatment with 100 µM glyphosate, 6 µM mancozeb or their combinations and also in the culture media collected from each treatment group after the incubation period. In the treatment solutions of 100 µM glyphosate and 6 µM mancozeb, levels of iron were not significantly different than the levels found in the culture media.

Intracellular iron levels in Neuro-2a cells as well as levels in culture media collected after treatment were not changed in the 100 µM glyphosate treatment groups. Previous data have reported glyphosate's potential to chelate iron and to alter its levels in plants (Caetano et al., 2012; Cakmak et al., 2009). *In vivo* observations, documented iron accumulation in kidneys and of rats exposed to glyphosate (Tang et al., 2017). *In vitro* study by Liu et a. (2023) also showed increased intracellular iron levels in LO2 hepatocytes following treatment with glyphosate at concentrations from 500 to 2000 µM (Liu et al., 2023).In the present study, lower glyphosate concentration was assessed which could explain the unaltered iron levels noted.

Intracellular iron levels in Neuro-2a cells as well as levels in culture media collected after treatment were not changed in the 6 μM mancozeb treatment groups. Though the effect of mancozeb on iron levels has not been reported in any previous *in vitro* study, few *in vivo* studies have examined iron levels following mancozeb exposure (Kistinger and Hardej, 2022; Stephenson and Trombetta, 2020). Stephenson and Trombetta (2020) showed that levels of iron remained unaffected by mancozeb treatments in the myocardium and in the Vastus medialis of mancozeb treated rats (Stephenson and Trombetta, 2020). Kistinger and Hardej (2022) also observed no changes in iron levels in liver, kidneys, serum or clotted blood and a decrease in iron levels in feces of mancozeb treated rats (Kistinger and Hardej, 2022).

Iron levels in culture media collected after treatment were not changed in the glyphosate and mancozeb combination treatments. Intracellular iron levels in Neuro-2a cells were slightly elevated in all glyphosate and mancozeb combination treatments. The elevation however was not statistically significant compared to control Neuro-2a cells.

The present study demonstrated that mancozeb as well as glyphosate and mancozeb combinations can alter copper, zinc and manganese levels in Neuro-2a cells. The present study also showed that the type of metal altered, and the level of metal alteration differ from one glyphosate and mancozeb combination to another.

One of the aims of this study was to investigate redox balance disruptions in Neuro-2a cells exposed to glyphosate, mancozeb or their combinations. Intracellular metal overload has been associated with over-production of ROS through Fenton-like reactions (Bleackley and MacGillivray, 2011). Previous studies have documented the ability of

metal-containing DTC compounds such as mancozeb to generate ROS via Fenton-like reactions and to induce oxidative stress (Fitsanakis et al., 2002). In addition, glyphosate-based formulations such as Round-up® were reported to induce over-production of ROS and to diminish GSH levels (De Liz Oliveira Cavalli et al., 2013; El-Shenawy, 2009; Martínez et al., 2020). In the current study, ratio of reduced glutathione to oxidized glutathione, GSH/GSSG was evaluated as an indicator of redox balance disruptions in Neuro-2a cells exposed to glyphosate (100 μM), mancozeb (6 μM) or their combinations.

The GSH/GSSG ratio was significantly decreased in mancozeb treated Neuro-2a cells. This is in agreement with previous study by Hoffman et al. (2016) reporting reduction in GSH/GSSG ratio in mancozeb and maneb treated HT-29 and Caco2 colon cells (Hoffman et al., 2016). Similarly, Iorio et al. (2015) demonstrated a decrease in GSH/GSSG ratio in mancozeb treated mouse granulosa cells (Iorio et al., 2015).

The GSH/GSSG ratio was not changed in glyphosate treated Neuro-2a cells. A previous study by Cavalli et al. (2013) demonstrated a decrease in reduced glutathione (GSH) levels in Sertoli cells treated with glyphosate-based formulation (De Liz Oliveira Cavalli et al., 2013). Similarly, El-Shenawy (2009) reported decreased GSH levels in rats treated with a glyphosate-based formulation (El-Shenawy, 2009). In both aforementioned studies however Round-up®, a glyphosate-based herbicide was tested which in addition to glyphosate, it contains surfactants such as polyethyoxylated tallowamine (POEA) and other impurities (Cattani et al., 2017). Previous studies have demonstrated glyphosate-based formulations to induce more toxicity than the pure glyphosate (Benbrook, 2019). In the present study, we used the pure glyphosate for our investigation and also the concentration of glyphosate tested was lower than those used in the previously mentioned

studies which can explain the unchanged GSH/GSSG ratio in glyphosate treated Neuro-2a cells.

The GSH/GSSG ratio was significantly decreased in glyphosate and mancozeb combinations treated Neuro-2a cells including glyphosate plus mancozeb mixture treatment, mancozeb for 30 minutes followed by glyphosate for 24 hours combination treatment and in glyphosate for 30 minutes followed by mancozeb 24 hours combination treatment. This is in agreement with previous work by Astiz et al. (2009) showing a decrease in GSH/GSSG ratio in the plasma and the brains of rats treated with combination of glyphosate in addition to the insecticide dimethoate and the EBDC zineb and no change in GSH/GSSG ratio when rats were treated with glyphosate alone (Astiz, de Alaniz, et al., 2009).

In the present study, the decrease in GSH/GSSG ratio was greater in the glyphosate plus mancozeb 24 hours mixture and in the mancozeb 30 minutes followed by glyphosate for 24 hours combination treated Neuro-2a cells compared to glyphosate 24 hours treated cells and not when compared to mancozeb 24 hours or mancozeb 30 minutes treated cells, respectively. In contrast, the decrease in GSH/GSSG ratio was greater in the glyphosate 30 minutes followed by mancozeb 24 hours combination treated Neuro-2a cells compared to either glyphosate 30 minutes or mancozeb 24 hours treated cells. Overall, our results suggest that toxicity of mancozeb and combinations of glyphosate and mancozeb in Neuro-2a cells might be related to their ability to induce redox balance disruptions.

As mentioned earlier, mancozeb 24 hours treatment as well as glyphosate plus mancozeb for 24 hours mixture treatment were able to cause disruptions in the redox balance in Neuro-2a cells. Therefore, in the present study we aimed to investigate the ability of mancozeb and mancozeb plus glyphosate mixture treated Neuro-2a cells to recover when post-treated with the antioxidant, butylated hydroxytoluene (BHT). BHT as an antioxidant acts by capturing ROS and by terminating lipid peroxidation chain reactions (Hossain et al., 2020). Previous work by Tsang and Trombetta (2007) showed that post-treatment of rat hippocampal astrocytes for 24 hours with BHT improved cell viability in cells that were exposed to mancozeb for one hour (Tsang and Trombetta, 2007). In the current study, however no improvement in cell viability was noticed in Neuro-2a cells exposed to mancozeb for 24 hours then post-treated with BHT and hence mancozeb associated cytotoxicity was not alleviated by BHT post-treatment. Failure of BHT to alleviate mancozeb associated cytotoxicity in the current study might indicate that cellular antioxidant system has been too overwhelmed to be rescued at this timepoint and that this might be related to the duration of exposure of the cells to mancozeb. Similarly, unpublished work by Dhaneshwar and Hardej demonstrated failure of pre-treatment and co-treatment with BHT to increase cells viability in HT-29 colon cells exposed to mancozeb for 24 hours. In addition, a previous study by Eltayeb et al. (2023) demonstrated failure of antioxidants BHT and BHA (butylated hydroxy alanine) to ameliorate toxicity associated with potato by-products acrylamide and alpha-solanine and their combination in BEAS-2B cells also suggesting failure of antioxidant treatments to alleviate toxicity when the oxidative insult is too overwhelming (Eltayeb et al., 2023).

In the current study, no improvement in cell viability was noted in Neuro-2a cells exposed to mancozeb plus glyphosate mixture for 24 hours then post-treated with BHT for 24 hours. This also suggests failure of BHT to alleviate toxicity associated with glyphosate plus mancozeb mixture which might be an indication that the cellular antioxidant system is too overwhelmed to be recovered at this timepoint.

In conclusion, the current work demonstrates the ability of glyphosate, mancozeb and their combinations to induce cytotoxicity in Neuro-2a cells. The cytotoxicity exerted by combinations of glyphosate and mancozeb including mixture of glyphosate plus mancozeb, glyphosate followed by mancozeb or mancozeb followed by glyphosate was greater than that induced by the same concentration of glyphosate when tested alone. In addition, combination of glyphosate followed by mancozeb resulted in greater cytotoxicity than that induced by the same concentration of mancozeb or glyphosate when each of them was tested alone. Intracellular elevations in the levels of the metals, copper, zinc and manganese were observed in Neuro-2a cells exposed to mancozeb or combinations of glyphosate and mancozeb. The type of metal and the level of intracellular metal level elevations however differed from one form of combination to the other. This work also reported elevations in the intracellular metal levels in all forms of glyphosate and mancozeb combinations at a glyphosate concentration that did not induce any changes when glyphosate was tested alone. Redox balance disruptions as indicated by the decrease in GSH/GSSG ratio were observed in Neuro-2a cells exposed to mancozeb or combinations of glyphosate and mancozeb. The decrease in GSH/GSSG ratio reported with all forms of glyphosate and mancozeb combinations occurred at a glyphosate concentration that did not induce any changes in GSH/GSSG ratio when

glyphosate was tested alone. In addition, combination of glyphosate followed by mancozeb resulted in greater decrease in GSH/GSSG ratio in Neuro-2a cells than that seen in mancozeb alone treated cells. These findings suggest that the increase in intracellular metal levels that leads to disruptions in redox balance and oxidative injury are underlying mechanisms for toxicity associated with mancozeb as well as combinations of glyphosate and mancozeb in Neuro-2a cells. The findings in this study are significant since they add credence to the reports claiming the unpredictability of combined exposure to pesticides and hence more efforts should be directed towards studying and understanding the impacts of environmentally relevant pesticide combinations on human health.

REFERENCES

Adwas, A., Ibrahim Elsayed, A. S., Elsayed Azab, A., & Quwaydir, F. A. (2019). Oxidative stress and antioxidant mechanisms in human body. *Journal of Applied Biotechnology & Bioengineering*, *6*(1), 43–47. https://doi.org/10.15406/jabb.2019.06.00173

Adeyemi, J. O., & Onwudiwe, D. C. (2020). The mechanisms of action involving dithiocarbamate complexes in biological systems. *Inorganica Chimica Acta*, *511*(June), 119809. https://doi.org/10.1016/j.ica.2020.119809

Akhtar, M., & Trombetta, L. D. (2023). Low level mancozeb exposure causes copper bioaccumulation in the renal cortex of rats leading to tubular injury. *Environmental Toxicology and Pharmacology*, *100*(January). https://doi.org/10.1016/j.etap.2023.104148

Akthar, I., Wang, Z., Wijayagunawardane, M. P. B., Ratnayake, C. J., Siriweera, E. H., Lee, K. F., & Kodithuwakku, S. P. (2020). In vitro and in vivo impairment of embryo implantation by commonly used fungicide Mancozeb. *Biochemical and Biophysical Research Communications*, *527*(1), 42–48. https://doi.org/10.1016/j.bbrc.2020.04.051

Aoyama, K. (2021). Glutathione in the brain. *International Journal of Molecular Sciences*, *22*(9). https://doi.org/10.3390/ijms22095010

Aprioku, J. S., Amamina, A. M., & Nnabuenyi, P. A. (2023). Mancozeb-induced hepatotoxicity: protective role of curcumin in rat animal model. *Toxicology Research*, *12*(1), 107–116. https://doi.org/10.1093/toxres/tfac085

Astiz, M., Alaniz, M. J. T. d., & Marra, C. A. (2009). Effect of pesticides on cell survival in liver and brain rat tissues. *Ecotoxicology and Environmental Safety*, *72*(7), 2025–2032. https://doi.org/10.1016/j.ecoenv.2009.05.001

Astiz, M., de Alaniz, M. J. T., & Marra, C. A. (2009). Antioxidant defense system in rats simultaneously intoxicated with agrochemicals. *Environmental Toxicology and Pharmacology*, *28*(3), 465–473. https://doi.org/10.1016/j.etap.2009.07.009

Atwood, D., & Paisley-Jones, C. (2017). 2008-2012 Market Estimates. *Pesticides Industry Sales and Usage*.

Axelstad, M., Boberg, J., Nellemann, C., Kiersgaard, M., Jacobsen, P. R., Christiansen, S., Hougaard, K. S., & Hass, U. (2011). Exposure to the widely used fungicide mancozeb causes thyroid hormone disruption in rat dams but no behavioral effects in the offspring. *Toxicological Sciences*, *120*(2), 439–446. https://doi.org/10.1093/toxsci/kfr006

Baligar, P. N., & Kaliwal, B. B. (2001). Induction of gonadal toxicity to female rats after chronic exposure to mancozeb. *Industrial Health*, *39*(3), 235–243. https://doi.org/10.2486/indhealth.39.235

Beckhauser, T., Francis-Oliveira, J., & de Pasquale, R. (2016). Reactive oxygen species: Physiopathological effects on synaptic plasticity. *J Expr Neurosci*, *10*(1), 23–48. https://doi.org/10.4137/JEN.S39887.TYPE

Benbrook, C. M. (2016). Trends in glyphosate herbicide use in the United States and globally. *Environmental Sciences Europe*, *28*(1), 1–15. https://doi.org/10.1186/s12302-016-0070-0

Benbrook, C. M. (2019). How did the US EPA and IARC reach diametrically opposed conclusions on the genotoxicity of glyphosate-based herbicides? *Environmental Sciences Europe*, *31*(1). https://doi.org/10.1186/s12302-018-0184-7

Benedetti, A. L., Vituri, C. D. L., Trentin, A. G., Domingues, M. A. C., & Alvarez-Silva, M. (2004). The effects of sub-chronic exposure of Wistar rats to the herbicide Glyphosate-Biocarb®. *Toxicology Letters*, *153*(2), 227–232. https://doi.org/10.1016/j.toxlet.2004.04.008

Birben, E., Sahiner, U. M., Sackesen, C., Erzurum, S., & Kalayci, O. (2012). Oxidative stress and antioxidant defense. *World Allergy Organization Journal*, *5*(1), 9–19. https://doi.org/10.1097/WOX.0b013e3182439613

Bjorkhem, I., Henriksson-Freyschuss, A., Breuer, O., Diczfalusy, U., Berglund, L., & Henriksson, P. (1991). The antioxidant butylated hydroxytoluene protects against atherosclerosis. *Arteriosclerosis and Thrombosis*, *11*(1), 15–22. https://doi.org/10.1161/01.atv.11.1.15

Blair, A., & Zahm, S. H. (1995). Agricultural exposures and cancer. *Environmental Health Perspectives*, *103*(SUPPL. 8), 205–208. https://doi.org/10.1289/ehp.95103s8205

Bleackley, M. R., & MacGillivray, R. T. A. (2011). Transition metal homeostasis: From yeast to human disease. *BioMetals*, *24*(5), 785–809. https://doi.org/10.1007/s10534-011-9451-4

Boldt, D. H. (1999). New perspectives on iron: An introduction. *American Journal of the Medical Sciences*, *318*(4), 207–212. https://doi.org/10.1016/S0002-9629(15)40625-1

Burkitt, M. J., Bishop, H. S., Milne, L., Tsang, S. Y., Provan, G. J., Nobel, C. S. I., Orrenius, S., & Slater, A. F. G. (1998). Dithiocarbamate toxicity toward thymocytes involves their copper- catalyzed conversion to thiuram disulfides, which oxidize glutathione in a redox cycle without the release of reactive oxygen species. *Archives of Biochemistry and Biophysics*, *353*(1), 73–84. https://doi.org/10.1006/abbi.1998.0618

Caetano, M. S., Ramalho, T. C., Botrel, D. F., Da Cunha, E. F. F., & De Mello, W. C. (2012). Understanding the inactivation process of organophosphorus herbicides: A DFT study of glyphosate metallic complexes with Zn2+, Ca 2+, Mg2+, Cu2+, Co3+, Fe 3+, Cr3+, and Al3+. *International Journal of Quantum Chemistry*, *112*(15), 2752–2762. https://doi.org/10.1002/qua.23222

Cakmak, I., Yazici, A., Tutus, Y., & Ozturk, L. (2009). Glyphosate reduced seed and leaf concentrations of calcium, manganese, magnesium, and iron in non-glyphosate resistant soybean. *European Journal of Agronomy*, *31*(3), 114–119. https://doi.org/10.1016/j.eja.2009.07.001

Calviello, G., Piccioni, E., Boninsegna, A., Tedesco, B., Maggiano, N., Serini, S., Wolf, F. I., & Palozza, P. (2006). DNA damage and apoptosis induction by the pesticide Mancozeb in rat cells: Involvement of the oxidative mechanism. *Toxicology and Applied Pharmacology*, *211*(2), 87–96. https://doi.org/10.1016/j.taap.2005.06.001

Cattani, D., Cesconetto, P. A., Tavares, M. K., Parisotto, E. B., De Oliveira, P. A., Rieg, C. E. H., Leite, M. C., Prediger, R. D. S., Wendt, N. C., Razzera, G., Filho, D. W., & Zamoner, A. (2017). Developmental exposure to glyphosate-based herbicide and depressive-like behavior in adult offspring: Implication of glutamate excitotoxicity and oxidative stress. *Toxicology*, *387*(February), 67–80. https://doi.org/10.1016/j.tox.2017.06.001

Cattani, D., de Liz Oliveira Cavalli, V. L., Heinz Rieg, C. E., Domingues, J. T., Dal-Cim, T., Tasca, C. I., Mena Barreto Silva, F. R., & Zamoner, A. (2014). Mechanisms underlying the neurotoxicity induced by glyphosate-based herbicide in immature rat hippocampus: Involvement of glutamate excitotoxicity. *Toxicology*, *320*(1), 34–45. https://doi.org/10.1016/j.tox.2014.03.001

Chen, P., Miah, M. R., & Aschner, M. (2016). Metals and Neurodegeneration. *F1000Research*, *5*(0), 1–12. https://doi.org/10.12688/f1000research.7431.1

Cheng, S. Y., & Trombetta, L. D. (2004). The induction of amyloid precursor protein and α-synuclein in rat hippocampal astrocytes by diethyldithiocarbamate and copper with or without glutathione. *Toxicology Letters*, *146*(2), 139–149. https://doi.org/10.1016/j.toxlet.2003.09.009

Colosio, C., Fustinoni, S., Birindelli, S., Bonomi, I., De Paschale, G., Mammone, T., Tiramani, M., Vercelli, F., Visentin, S., & Maroni, M. (2002). Ethylenethiourea in urine as an indicator of exposure to mancozeb in vineyard workers. *Toxicology Letters*, *134*(1–3), 133–140. https://doi.org/10.1016/S0378-4274(02)00182-0

Coullery, R. P., Ferrari, M. E., & Rosso, S. B. (2016). Neuronal development and axon growth are altered by glyphosate through a WNT non-canonical signaling pathway. *NeuroToxicology*, *52*, 150–161. https://doi.org/10.1016/j.neuro.2015.12.004

Crépet, A., Héraud, F., Béchaux, C., Gouze, M. E., Pierlot, S., Fastier, A., Leblanc, J. C., Le Hégarat, L., Takakura, N., Fessard, V., Tressou, J., Maximilien, R., de Sousa, G.,

Nawaz, A., Zucchini-Pascal, N., Rahmani, R., Audebert, M., Graillot, V., & Cravedi, J. P. (2013). The PERICLES research program: An integrated approach to characterize the combined effects of mixtures of pesticide residues to which the French population is exposed. *Toxicology*, *313*(2–3), 83–93. https://doi.org/10.1016/j.tox.2013.04.005

Crews, F., Nixon, K., Kim, D., Joseph, J., Shukitt-Hale, B., Qin, L., & Zou, J. (2006). BHT blocks NF-κB activation and ethanol-induced brain damage. *Alcoholism: Clinical and Experimental Research*, *30*(11), 1938–1949. https://doi.org/10.1111/j.1530-0277.2006.00239.x

Dall'Agnol, J. C., Pezzini, M. F., Uribe, N. S., & Joveleviths, D. (2021). Systemic effects of the pesticide mancozeb – A literature review. *European Review for Medical and Pharmacological Sciences*, *25*(11), 4113–4120. https://doi.org/10.26355/eurrev_202106_26054

Dallegrave, E., Mantese, F. D. G., Coelho, R. S., Pereira, J. D., Dalsenter, P. R., & Langeloh, A. (2003). The teratogenic potential of the herbicide glyphosate-Roundup® in Wistar rats. *Toxicology Letters*, *142*(1–2), 45–52. https://doi.org/10.1016/S0378-4274(02)00483-6

De Liz Oliveira Cavalli, V. L., Cattani, D., Heinz Rieg, C. E., Pierozan, P., Zanatta, L., Benedetti Parisotto, E., Wilhelm Filho, D., Mena Barreto Silva, F. R., Pessoa-Pureur, R., & Zamoner, A. (2013a). Roundup disrupts male reproductive functions by triggering calcium-mediated cell death in rat testis and Sertoli cells. *Free Radical Biology and Medicine*, *65*, 335–346. https://doi.org/10.1016/j.freeradbiomed.2013.06.043

De Liz Oliveira Cavalli, V. L., Cattani, D., Heinz Rieg, C. E., Pierozan, P., Zanatta, L., Benedetti Parisotto, E., Wilhelm Filho, D., Mena Barreto Silva, F. R., Pessoa-Pureur, R., & Zamoner, A. (2013b). Roundup disrupts male reproductive functions by triggering calcium-mediated cell death in rat testis and Sertoli cells. *Free Radical Biology and Medicine*, *65*, 335–346. https://doi.org/10.1016/j.freeradbiomed.2013.06.043

Dedeke, G. A., Owagboriaye, F. O., Ademolu, K. O., Olujimi, O. O., & Aladesida, A. A. (2018). Comparative Assessment on Mechanism Underlying Renal Toxicity of Commercial Formulation of Roundup Herbicide and Glyphosate Alone in Male Albino Rat. *International Journal of Toxicology*, *37*(4), 285–295. https://doi.org/10.1177/1091581818779553

Delmaestro, E., & Trombetta, L. D. (1995). The effects of disulfiram on the hippocampus and cerebellum of the rat brain: a study on oxidative stress. *Toxicology Letters*, *75*(1–3), 235–243. https://doi.org/10.1016/0378-4274(94)03187-C

Dereumeaux, C., Mercier, F., Soulard, P., Hulin, M., Oleko, A., Pecheux, M., Fillol, C., Denys, S., & Quenel, P. (2022). Identification of pesticides exposure biomarkers for residents living close to vineyards in France. *Environment International*, *159*. https://doi.org/10.1016/j.envint.2021.107013

Dhaneshwar, A., & Hardej, D. (2021). Disruption of mitochondrial complexes, cytotoxicity, and apoptosis results from Mancozeb exposure in transformed human colon cells. *Environmental Toxicology and Pharmacology*, *84*(February), 103614. https://doi.org/10.1016/j.etap.2021.103614

Dill, Gerald M.; CaJacob, Claire; Padgette, S. (2008). Glyphosate-resitant crops: adoption, use and future considerations. *Pest Management Science*, *64*(11), 326–331. https://doi.org/10.1002/ps

Dill, G. M. (2005). Glyphosate-resistant crops: History, status and future. *Pest Management Science*, *61*(3), 219–224. https://doi.org/10.1002/ps.1008

Domico, L. M., Zeevalk, G. D., Bernard, L. P., & Cooper, K. R. (2006). Acute neurotoxic effects of mancozeb and maneb in mesencephalic neuronal cultures are associated with mitochondrial dysfunction. *NeuroToxicology*, *27*(5), 816–825. https://doi.org/10.1016/j.neuro.2006.07.009

Duke, S. O. (2005). Taking stock of herbicide-resistant crops ten years after introduction. *Pest Management Science*, *61*(3), 211–218. https://doi.org/10.1002/ps.1024

EFSA. (2022). The 2020 European Union report on pesticide residues in food. *EFSA Journal*, *20*(3). https://doi.org/10.2903/j.efsa.2022.7215

El-Shenawy, N. S. (2009). Oxidative stress responses of rats exposed to Roundup and its active ingredient glyphosate. *Environmental Toxicology and Pharmacology*, *28*(3), 379–385. https://doi.org/10.1016/j.etap.2009.06.001

Eltayeb, H. A., Stewart, L., Morgem, M., Johnson, T., Nguyen, M., Earl, K., Sodipe, A., Jackson, D., & Olufemi, S. E. (2023). Antioxidants Amelioration Is Insufficient to Prevent Acrylamide and Alpha-Solanine Synergistic Toxicity in BEAS-2B Cells. *International Journal of Molecular Sciences*, *24*(15). https://doi.org/10.3390/ijms241511956

Ercal, N., Gurrer-Orhan, H., & Aykin-Burns, N. (2001). Toxic Metals and Oxidative Stress Part I: Mechanisms Involved in Metal induced Oxidative Damage. *Current Topics in Medicinal Chemistry*, *1*(573), 529–539. https://doi.org/10.2174/1568026013394831

European Union. (2020). *(Text with EEA relevance) 21.6.2017*. 48–119. https://doi.org/10.2903/j.efsa.2019.5755

Fan, R., Zhang, W., Jia, L., Li, L., Zhao, J., Zhao, Z., Peng, S., Chen, Y., & Yuan, X. (2021). Combined developmental toxicity of the pesticides difenoconazole and dimethomorph on embryonic zebrafish. *Toxins*, *13*(12), 1–14. https://doi.org/10.3390/toxins13120854

Farina, M., Avila, D. S., Da Rocha, J. B. T., & Aschner, M. (2013). Metals, oxidative stress and neurodegeneration: A focus on iron, manganese and mercury. *Neurochemistry International*, *62*(5), 575–594. https://doi.org/10.1016/j.neuint.2012.12.006

Fitsanakis, V. A., Amarnath, V., Moore, J. T., Montine, K. S., Zhang, J., & Montine, T. (2002). *Catalysis of Catechol Oxidation By Metal-Dithiocarbamate*. *33*(12), 1714–1723.

Fraga, C. G. (2005a). Relevance, essentiality and toxicity of trace elements in human health. *Molecular Aspects of Medicine*, *26*(4-5 SPEC. ISS.), 235–244. https://doi.org/10.1016/j.mam.2005.07.013

Fraga, C. G. (2005b). Relevance, essentiality and toxicity of trace elements in human health. *Molecular Aspects of Medicine*, *26*(4-5 SPEC. ISS.), 235–244. https://doi.org/10.1016/j.mam.2005.07.013

Franco, R., Li, S., Rodriguez-Rocha, H., Burns, M., & Panayiotidis, M. I. (2010). Molecular mechanisms of pesticide-induced neurotoxicity: Relevance to Parkinson's disease. *Chemico-Biological Interactions*, *188*(2), 289–300. https://doi.org/10.1016/j.cbi.2010.06.003

Gandhi, S., & Abramov, A. Y. (2012). Mechanism of oxidative stress in neurodegeneration. *Oxidative Medicine and Cellular Longevity*, *2012*. https://doi.org/10.1155/2012/428010

Garza-Lombó, C., Posadas, Y., Quintanar, L., Gonsebatt, M. E., & Franco, R. (2018). Neurotoxicity Linked to Dysfunctional Metal Ion Homeostasis and Xenobiotic Metal Exposure: Redox Signaling and Oxidative Stress. *Antioxidants and Redox Signaling*, *28*(18), 1669–1703. https://doi.org/10.1089/ars.2017.7272

Gasnier, C., Dumont, C., Benachour, N., Clair, E., Chagnon, M. C., & Séralini, G. E. (2009). Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. *Toxicology*, *262*(3), 184–191. https://doi.org/10.1016/j.tox.2009.06.006

Ge, Y., Song, X., Chen, L., Hu, D., Hua, L., Cui, Y., Liu, J., An, Z., Yin, Z., & Ning, H. (2019). Cadmium induces actin cytoskeleton alterations and dysfunction in Neuro-2a cells. *Environmental Toxicology*, *34*(4), 469–475. https://doi.org/10.1002/tox.22700

Ghaffari, H., Venkataramana, M., Jalali Ghassam, B., Chandra Nayaka, S., Nataraju, A., Geetha, N. P., & Prakash, H. S. (2014). Rosmarinic acid mediated neuroprotective effects against H2O2-induced neuronal cell damage in N2A cells. *Life Sciences*, *113*(1–2), 7–13. https://doi.org/10.1016/j.lfs.2014.07.010

Glass, R. L. (1984). Metal Complex Formation by Glyphosate. *Journal of Agricultural and Food Chemistry*, *32*(6), 1249–1253. https://doi.org/10.1021/jf00126a010

Goldner, W. S., Sandler, D. P., Yu, F., Hoppin, J. A., Kamel, F., & Levan, T. D. (2010). Pesticide use and thyroid disease among women in the agricultural health study. *American Journal of Epidemiology*, *171*(4), 455–464. https://doi.org/10.1093/aje/kwp404

Gomes, M. P., Smedbol, E., Chalifour, A., Hénault-Ethier, L., Labrecque, M., Lepage, L., Lucotte, M., & Juneau, P. (2014). Alteration of plant physiology by glyphosate and its by-product aminomethylphosphonic acid: An overview. *Journal of Experimental Botany*, *65*(17), 4691–4703. https://doi.org/10.1093/jxb/eru269

Gómez-Giménez, B., Llansola, M., Cabrera-Pastor, A., Hernández-Rabaza, V., Agustí, A., & Felipo, V. (2018). Endosulfan and Cypermethrin Pesticide Mixture Induces Synergistic or Antagonistic Effects on Developmental Exposed Rats Depending on the Analyzed Behavioral or Neurochemical End Points. *ACS Chemical Neuroscience*, *9*(2), 369–380. https://doi.org/10.1021/acschemneuro.7b00364

Gonos ES, Kapetanou M, Sereikaite J, Bartosz G, Naparło K, Grzesik M, & Sadowska-Bartosz I. (2018). Originandpathophysiologyofproteincarbonylation,nitrationandchlorinationinage-relatedbraindiseasesandaging. *Aging (Albany NY)*, *10*(5), 868–901.

Gonzalez-Pinto, A., Martinez-Cengotitabengoa, M., Arango, C., Baeza, I., Otero-Cuesta, S., Graell-Berna, M., Soutullo, C., Leza, J. C., & Micó, J. A. (2012). Antioxidant defense system and family environment in adolescents with family history of psychosis. *BMC Psychiatry*, *12*. https://doi.org/10.1186/1471-244X-12-200

Gordon, C. J., Herr, D. W., Gennings, C., Graff, J. E., McMurray, M., Stork, L. A., Coffey, T., Hamm, A., & Mack, C. M. (2006). Thermoregulatory response to an organophosphate and carbamate insecticide mixture: Testing the assumption of dose-additivity. *Toxicology*, *217*(1), 1–13. https://doi.org/10.1016/j.tox.2005.08.014

Grau, D., Grau, N., Gascuel, Q., Paroissin, C., Stratonovitch, C., Lairon, D., Devault, D. A., & Di Cristofaro, J. (2022). Quantifiable urine glyphosate levels detected in 99% of the French population, with higher values in men, in younger people, and in farmers. *Environmental Science and Pollution Research*, *29*(22), 32882–32893. https://doi.org/10.1007/s11356-021-18110-0

Gui, Y. xing, Fan, X. ning, Wang, H. mei, Wang, G., & Chen, S. di. (2012a). Glyphosate induced cell death through apoptotic and autophagic mechanisms. *Neurotoxicology and Teratology*, *34*(3), 344–349. https://doi.org/10.1016/j.ntt.2012.03.005

Gui, Y. xing, Fan, X. ning, Wang, H. mei, Wang, G., & Chen, S. di. (2012b). Glyphosate induced cell death through apoptotic and autophagic mechanisms. *Neurotoxicology and Teratology*, *34*(3), 344–349. https://doi.org/10.1016/j.ntt.2012.03.005

Gullino, M. L., Tinivella, F., Garibaldi, A., Kemmitt, G. M., Bacci, L., & Sheppard, B. (2010). Mancozeb: Past, present, and future. *Plant Disease*, *94*(9), 1076–1087. https://doi.org/10.1094/PDIS-94-9-1076

Haffke, Susan C.; Seeds, N. W. (1975). Neuroblastoma: The E. COLI of Neurobiology? *Life Sciences*, *16*, 1649–1658.

Halliwell, B. (1992). Reactive Oxygen Species and the Central Nervous System. *Journal of Neurochemistry*, *59*(5), 1609–1623. https://doi.org/10.1111/j.1471-4159.1992.tb10990.x

Hernández, A. F., Gil, F., & Lacasaña, M. (2017). Toxicological interactions of pesticide mixtures: an update. *Archives of Toxicology*, *91*(10), 3211–3223. https://doi.org/10.1007/s00204-017-2043-5

Hoffman, L., & Hardej, D. (2012). Ethylene bisdithiocarbamate pesticides cause cytotoxicity in transformed and normal human colon cells. *Environmental Toxicology and Pharmacology*, *34*, 556–573. https://doi.org/10.1016/j.etap.2015.11.002

Hoffman, L., Trombetta, L., & Hardej, D. (2016). Ethylene bisdithiocarbamate pesticides Maneb and Mancozeb cause metal overload in human colon cells. *Environmental Toxicology and Pharmacology*, *41*, 78–88. https://doi.org/10.1016/j.etap.2015.11.002

Hossain, K. F. B., Hosokawa, T., Saito, T., & Kurasaki, M. (2020). Amelioration of butylated hydroxytoluene against inorganic mercury induced cytotoxicity and mitochondrial apoptosis in PC12 cells via antioxidant effects. In *Food and Chemical Toxicology* (Vol. 146). https://doi.org/10.1016/j.fct.2020.111819

Iorio, R., Castellucci, A., Rossi, G., Cinque, B., Cifone, M. G., Macchiarelli, G., & Cecconi, S. (2015). Mancozeb affects mitochondrial activity, redox status and ATP production in mouse granulosa cells. *Toxicology in Vitro*, *30*(1), 438–445. https://doi.org/10.1016/j.tiv.2015.09.018

Jaeger, J. W., Carlson, I. H., & Porter, W. P. (1999). Endocrine, immune, and behavioral effects of aldicarb (carbamate), atrazine (triazine) and nitrate (fertilizer) mixtures at groundwater concentrations. *Toxicology and Industrial Health*, *15*(2), 133–151. https://doi.org/10.1177/074823379901500111

Jelinek, M., Jurajda, M., & Duris, K. (2021). Oxidative stress in the brain: Basic concepts and treatment strategies in stroke. *Antioxidants*, *10*(12), 1–12. https://doi.org/10.3390/antiox10121886

Jellali, R., Gilard, F., Pandolfi, V., Legendre, A., Fleury, M. J., Paullier, P., Legallais, C., & Leclerc, E. (2018). Metabolomics-on-a-chip approach to study hepatotoxicity of DDT, permethrin and their mixtures. *Journal of Applied Toxicology*, *38*(8), 1121–1134. https://doi.org/10.1002/jat.3624

Johnson, V. J., Kim, S. H., & Sharma, R. P. (2005). Aluminum-maltolate induces apoptosis and necrosis in neuro-2a cells: Potential role for p53 signaling. *Toxicological Sciences*, *83*(2), 329–339. https://doi.org/10.1093/toxsci/kfi028

Kackar, R., Srivastava, M. K., & Raizada, R. B. (1997). Studies on rat thyroid after oral administration of mancozeb: Morphological and biochemical evaluations. *Journal of Applied Toxicology*, *17*(6), 369–375. https://doi.org/10.1002/(SICI)1099-1263(199711/12)17:6<369::AID-JAT449>3.0.CO;2-Q

Kamel, F., & Hoppin, J. A. (2004). Association of pesticide exposure with neurologic dysfunction and disease. *Environmental Health Perspectives*, *112*(9), 950–958. https://doi.org/10.1289/ehp.7135

Karihtala, P., & Soini, Y. (2007). Reactive oxygen species and antioxidant mechanisms in human tissues and their relation to malignancies. *Apmis*, *115*(2), 81–103. https://doi.org/10.1111/j.1600-0463.2007.apm_514.x

Kaul, L., Süss, R., Zannettino, A., & Richter, K. (2021). The revival of dithiocarbamates: from pesticides to innovative medical treatments. *IScience*, *24*(2), 1–14. https://doi.org/10.1016/j.isci.2021.102092

Kaye, E., Nyombi, A., Mutambuze, I. L., & Muwesa, R. (2015). Mancozeb Residue on Tomatoes in Central Uganda. *Journal of Health and Pollution*, *5*(8), 1–6. https://doi.org/10.5696/i2156-9614-5-8.1

Kim, C. H., Kim, J. H., Xu, J., Hsu, C. Y., & Ahn, Y. S. (1999). Pyrrolidine dithiocarbamate induces bovine cerebral endothelial cell death by increasing the intracellular zinc level. *Journal of Neurochemistry*, *72*(4), 1586–1592. https://doi.org/10.1046/j.1471-4159.1999.721586.x

Kistinger, B. R., & Hardej, D. (2022). The ethylene bisdithiocarbamate fungicides mancozeb and nabam alter essential metal levels in liver and kidney and glutathione enzyme activity in liver of Sprague-Dawley rats. *Environmental Toxicology and Pharmacology*, *92*(March), 103849. https://doi.org/10.1016/j.etap.2022.103849

Kőmíves, T., & Schröder, P. (2016a). On glyphosate. *Ecocycles*, *2*(2), 1–8. https://doi.org/10.19040/ecocycles.v2i2.60

Kőmíves, T., & Schröder, P. (2016b). On glyphosate. *Ecocycles*, *2*(2), 1–8. https://doi.org/10.19040/ecocycles.v2i2.60

Kruger, M., Schrodl, W., Neuhaus, J., & Shehata, A. A. (2013). Field Investigations of Glyphosate in Urine of Danish Dairy Cows. *Journal of Environmental & Analytical Toxicolog*, *03*(05). https://doi.org/10.4172/2161-0525.1000186

Ksheerasagar, R. L., & Kaliwal, B. B. (2003). Temporal effects of mancozeb on testes, accessory reproductive organs and biochemical constituents in albino mice. *Environmental Toxicology and Pharmacology*, *15*(1), 9–17. https://doi.org/10.1016/j.etap.2003.08.006

Laity, J. H., Lee, B. M., & Wright, P. E. (2001). Zinc finger proteins: New insights into structural and functional diversity. *Current Opinion in Structural Biology*, *11*(1), 39–46. https://doi.org/10.1016/S0959-440X(00)00167-6

Lajtha, A. (2009). *Handbook of Neurochemistry and Molecular Neurobiology: Brain and Spinal Cord Trauma*.

Lakomaa, E. L., Sato, S., Goldberg, A. M., & Frazier, J. M. (1982). The effect of sodium diethyldithiocarbamate treatment on copper and zinc concentrations in rat brain. *Toxicology and Applied Pharmacology*, *65*(2), 286–290. https://doi.org/10.1016/0041-008X(82)90011-4

Lee, G. H., & Choi, K. C. (2020). Adverse effects of pesticides on the functions of immune system. *Comparative Biochemistry and Physiology Part - C: Toxicology and Pharmacology*, *235*(January), 108789. https://doi.org/10.1016/j.cbpc.2020.108789

Lee, K. H., Cha, M., & Lee, B. H. (2020). Neuroprotective effect of antioxidants in the brain. *International Journal of Molecular Sciences*, *21*(19), 1–29. https://doi.org/10.3390/ijms21197152

Liesivuori, Jyrki; Savolainen, K. (1994). Dithiocarbamates. *Toxicology*, *91*, 37–42.

Liu, J., Yang, G., & Zhang, H. (2023). Glyphosate-triggered hepatocyte ferroptosis via suppressing Nrf2/GSH/GPX4 axis exacerbates hepatotoxicity. *Science of the Total Environment*, *862*, 3–9.

López-Fernández, O., Rial-Otero, R., & Simal-Gándara, J. (2013). Factors governing the removal of mancozeb residues from lettuces with washing solutions. *Food Control*, *34*(2), 530–538. https://doi.org/10.1016/j.foodcont.2013.05.022

Maggi, F., Tang, F. H. M., la Cecilia, D., & McBratney, A. (2019). PEST-CHEMGRIDS, global gridded maps of the top 20 crop-specific pesticide application rates from 2015 to 2025. *Scientific Data*, *6*(1), 1–20. https://doi.org/10.1038/s41597-019-0169-4

Maroni, M., Colosio, C., Ferioli, A., & Fait, A. (2000). Biological Monitoring of Pesticide Exposure: a review. Introduction. In *Toxicology* (Vol. 143, Issue 1). https://doi.org/10.1016/S0300-483X(99)00163-8

Marrie, R. A. (2004). *Reviews Environmental risk factors in multiple sclerosis aetiology*. *3*(December), 709–718.

Martínez, M. A., Ares, I., Rodríguez, J. L., Martínez, M., Martínez-Larrañaga, M. R., & Anadón, A. (2018). Neurotransmitter changes in rat brain regions following glyphosate exposure. *Environmental Research*, *161*(November 2017), 212–219. https://doi.org/10.1016/j.envres.2017.10.051

Martínez, M. A., Rodríguez, J. L., Lopez-Torres, B., Martínez, M., Martínez-Larrañaga, M. R., Maximiliano, J. E., Anadón, A., & Ares, I. (2020). Use of human neuroblastoma SH-SY5Y cells to evaluate glyphosate-induced effects on oxidative stress, neuronal development and cell death signaling pathways. *Environment International*, *135*(November 2019), 105414. https://doi.org/10.1016/j.envint.2019.105414

Merad-Boudia, M., Fideler, L., Nicole, A., Aral, B., Sinet, P. M., Briand, P., & Ceballos-Picot, I. (1994). Selective modulation of brain antioxidant defense capacity by genetic or metabolic manipulations. *Oxidative Stress, Cell Activation and Viral Infection*, 173–192. https://doi.org/10.1007/978-3-0348-7424-3_17

Mertens, M., Hoss, S., Gunter, N., Joshua, A., & Reichenbecher, W. (2018). GLYPHOSATE, A CHELATING AGENT - RELEVANT FOR ECOLOGICAL RISK ASSESSMENT? *Environmental Science and Pollution Research*, *25*, 5298–5317. https://doi.org/10.1117/12.477503

Miranda-Contreras, L., Dávila-Ovalles, R., Benítez-Díaz, P., Peña-Contreras, Z., & Palacios-Prü, E. (2005). Effects of prenatal paraquat and mancozeb exposure on amino acid synaptic transmission in developing mouse cerebellar cortex. *Developmental Brain Research*, *160*(1), 19–27. https://doi.org/10.1016/j.devbrainres.2005.08.001

Mnif, W., Hassine, A. I. H., Bouaziz, A., Bartegi, A., Thomas, O., & Roig, B. (2011). Effect of endocrine disruptor pesticides: A review. *International Journal of Environmental Research and Public Health*, *8*(6), 2265–2303. https://doi.org/10.3390/ijerph8062265

Momčilović, B., Prejac, J., Brundić, S., Morović, S., Skalny, A. V., Mimica, N., & Drmić, S. (2010). An essay on human and elements, multielement profiles, and depression. *Translational Neuroscience*, *1*(4), 322–334. https://doi.org/10.2478/v10134-010-0039-2

Myers, J. P., Antoniou, M. N., Blumberg, B., Carroll, L., Colborn, T., Everett, L. G., Hansen, M., Landrigan, P. J., Lanphear, B. P., Mesnage, R., Vandenberg, L. N., Vom Saal, F. S., Welshons, W. V., & Benbrook, C. M. (2016). Concerns over use of glyphosate-based herbicides and risks associated with exposures: A consensus statement. *Environmental Health: A Global Access Science Source*, *15*(1), 1–13. https://doi.org/10.1186/s12940-016-0117-0

Nawaz, A., Razpotnik, A., Rouimi, P., De Sousa, G., Cravedi, J. P., & Rahmani, R. (2014). Cellular impact of combinations of endosulfan, atrazine, and chlorpyrifos on human primary hepatocytes and HepaRG cells after short and chronic exposures. *Cell Biology and Toxicology*, *30*(1), 17–29. https://doi.org/10.1007/s10565-013-9266-x

Neely, M. D., Sidell, K. R., Graham, D. G., & Montine, T. J. (1999). The lipid peroxidation product 4-hydroxynonenal inhibits neurite outgrowth, disrupts neuronal microtubules, and modifies cellular tubulin. *Journal of Neurochemistry*, *72*(6), 2323–2333. https://doi.org/10.1046/j.1471-4159.1999.0722323.x

O Duke, S., & B Powles, S. (2008). Glyphosate: a once-in-a-century herbicide. *Pest Management Science*, *63*(11), 1100–1106. https://doi.org/10.1002/ps

Panis, C., Candiotto, L. Z. P., Gaboardi, S. C., Gurzenda, S., Cruz, J., Castro, M., & Lemos, B. (2022). Widespread pesticide contamination of drinking water and impact on cancer risk in Brazil. *Environment International*, *165*(February). https://doi.org/10.1016/j.envint.2022.107321

Peixoto, F. (2005). Comparative effects of the Roundup and glyphosate on mitochondrial oxidative phosphorylation. *Chemosphere*, *61*(8), 1115–1122. https://doi.org/10.1016/j.chemosphere.2005.03.044

Pirozzi, A. V. A., Stellavato, A., La Gatta, A., Lamberti, M., & Schiraldi, C. (2016). Mancozeb, a fungicide routinely used in agriculture, worsens nonalcoholic fatty liver disease in the human HepG2 cell model. *Toxicology Letters*, *249*, 1–4. https://doi.org/10.1016/j.toxlet.2016.03.004

Pisoschi, A. M., & Pop, A. (2015). The role of antioxidants in the chemistry of oxidative stress: A review. *European Journal of Medicinal Chemistry*, *97*, 55–74. https://doi.org/10.1016/j.ejmech.2015.04.040

Pizzorno, J. (2014). Glutatione! *Integrative Medicine*, *13*(1), 8–12.

Poon, I. K. H., Hulett, M. D., & Parish, C. R. (2010). Molecular mechanisms of late apoptotic/necrotic cell clearance. *Cell Death and Differentiation*, *17*(3), 381–397. https://doi.org/10.1038/cdd.2009.195

Romano, M. A., Wisniewski, P., Viau, P., Romano, R. M., Campos, D. A., Bernardi, M. M., Santos, L. D., De Souza, P. B., Nunes, M. T., & De Oliveira, C. A. (2012a). Glyphosate impairs male offspring reproductive development by disrupting gonadotropin expression. *Archives of Toxicology*, *86*(4), 663–673. https://doi.org/10.1007/s00204-011-0788-9

Romano, M. A., Wisniewski, P., Viau, P., Romano, R. M., Campos, D. A., Bernardi, M. M., Santos, L. D., De Souza, P. B., Nunes, M. T., & De Oliveira, C. A. (2012b). Glyphosate impairs male offspring reproductive development by disrupting gonadotropin expression. *Archives of Toxicology*, *86*(4), 663–673. https://doi.org/10.1007/s00204-011-0788-9

Rose Stein, M., & Trombetta, L. D. (1993). Effects of diethyldithiocarbamate on calmodulin in neuroblastoma cells. *Journal of Toxicology and Environmental Health*, *39*(4), 465–481. https://doi.org/10.1080/15287399309531765

Ross, J., Olmsted, J. B., & Rosenbaum, J. L. (1975). The ultrastructure of mouse neuroblastoma cells in tissue culture. *Tissue and Cell*, *7*(1), 107–135. https://doi.org/10.1016/S0040-8166(75)80010-3

Rossi, G., Buccione, R., Baldassarre, M., MacChiarelli, G., Palmerini, M. G., & Cecconi, S. (2006). Mancozeb exposure in vivo impairs mouse oocyte fertilizability. *Reproductive Toxicology*, *21*(2), 216–219. https://doi.org/10.1016/j.reprotox.2005.08.004

Rubio, F., Guo, E., & Kamp, L. (2014). Survey of Glyphosate Residues in Honey, Corn and Soy Products. *Journal of Environmental & Analytical Toxicology*, *05*(01), 1–8. https://doi.org/10.4172/2161-0525.1000249

Sharma, A., Kumar, V., Shahzad, B., Tanveer, M., Sidhu, G. P. S., Handa, N., Kohli, S. K., Yadav, P., Bali, A. S., Parihar, R. D., Dar, O. I., Singh, K., Jasrotia, S., Bakshi, P., Ramakrishnan, M., Kumar, S., Bhardwaj, R., & Thukral, A. K. (2019). Worldwide pesticide usage and its impacts on ecosystem. *SN Applied Sciences*, *1*(11), 1–16. https://doi.org/10.1007/s42452-019-1485-1

Shastry, A. Basu, A. Rajadhyaksha, M. (2001). Neuroblastoma cell lines - A versatile in vitro model in neurobiology. *Intern. J. Neuroscience*, *108*(020), 109–126.

Sies, H. (1999). Glutathione and its role in cellular functions. *Free Radical Biology and Medicine*, *27*(9–10), 916–921. https://doi.org/10.1016/S0891-5849(99)00177-X

Sreedhar, A. S., & Csermely, P. (2004). Heat shock proteins in the regulation of apoptosis: New strategies in tumor therapy - A comprehensive review. *Pharmacology and Therapeutics*, *101*(3), 227–257. https://doi.org/10.1016/j.pharmthera.2003.11.004

Stackpoole, S. M., Shoda, M. E., Medalie, L., & Stone, W. W. (2021). Pesticides in US Rivers: Regional differences in use, occurrence, and environmental toxicity, 2013 to 2017. *Science of the Total Environment*, *787*(xxxx), 147147. https://doi.org/10.1016/j.scitotenv.2021.147147

Stefan, C., Nobel, I., Kimland, M., Lind, B., Orrenius, S., & Slater, A. F. G. (1995). Dithiocarbamates induce apoptosis in thymocytes by raising the intracellular level of redox-active copper. *Journal of Biological Chemistry*, *270*(44), 26202–26208. https://doi.org/10.1074/jbc.270.44.26202

Stephenson, O. J., & Trombetta, L. D. (2020). Comparative effects of Mancozeb and Disulfiram-induced striated muscle myopathies in Long-Evans rats. *Environmental Toxicology and Pharmacology*, *74*(November 2019), 103300. https://doi.org/10.1016/j.etap.2019.103300

Sunderman, F. W., Schneider, H. P., & Lumb, G. (1984). Sodium diethyldithiocarbamate administration in nickel-induced malignant tumors. *Annals of Clinical and Laboratory Science*, *14*(1), 1–9.

Suzuki, Y. J., Forman, H. J., & Sevanian, A. (1996). Oxidants as stimulators of signal transduction. *Free Radical Biology and Medicine*, *22*(1–2), 269–285. https://doi.org/10.1016/S0891-5849(96)00275-4

Szolar, O. H. J. (2007). Environmental and pharmaceutical analysis of dithiocarbamates. *Analytica Chimica Acta*, *582*(2), 191–200. https://doi.org/10.1016/j.aca.2006.09.022

Tang, J., Hu, P., Li, Y., Win-Shwe, T. T., & Li, C. (2017). Ion imbalance is involved in the mechanisms of liver oxidative damage in rats exposed to glyphosate. *Frontiers in Physiology*, *8*(DEC), 1–12. https://doi.org/10.3389/fphys.2017.01083

Tizhe, E. V., Ibrahim, N. D. G., Fatihu, M. Y., Onyebuchi, I. I., George, B. D. J., Ambali, S. F., & Shallangwa, J. M. (2014). Influence of zinc supplementation on histopathological changes in the stomach, liver, kidney, brain, pancreas and spleen during subchronic exposure of Wistar rats to glyphosate. *Comparative Clinical Pathology*, *23*(5), 1535–1543. https://doi.org/10.1007/s00580-013-1818-1

Torretta, V., Katsoyiannis, I. A., Viotti, P., & Rada, E. C. (2018). Critical review of the effects of glyphosate exposure to the environment and humans through the food supply chain. *Sustainability (Switzerland)*, *10*(4), 1–20. https://doi.org/10.3390/su10040950

Trombetta, Louis D., Toulon, M., & Jamall, I. S. (1988). Protective effects of glutathione on diethyldithiocarbamate (DDC) cytotoxicity: A possible mechanism. *Toxicology and Applied Pharmacology*, *93*(1), 154–164. https://doi.org/10.1016/0041-008X(88)90035-X

Trombetta, Louis David, & Kromidas, L. (1992). A scanning electron-microscopic study of the effects of methylmercury on the neuronal cytoskeleton. *Toxicology Letters*, *60*(3), 329–341. https://doi.org/10.1016/0378-4274(92)90292-R

Tsang, M., & Trombetta, L. (2007). The protective role of chelators and antioxidants on mancozeb-induced toxicity in rat hippocampal astrocytes. *Toxicology and Industrial Health*, *23*(8), 459–470. https://doi.org/10.1177/0748233708089039

Tudi, M., Li, H., Li, H., Wang, L., Lyu, J., Yang, L., Tong, S., Yu, Q. J., Ruan, H. D., Atabila, A., Phung, D. T., Sadler, R., & Connell, D. (2022). Exposure Routes and Health Risks Associated with Pesticide Application. *Toxics*, *10*(6), 1–23. https://doi.org/10.3390/toxics10060335

Tudi, M., Ruan, H. D., Wang, L., Lyu, J., Sadler, R., Connell, D., Chu, C., & Phung, D. T. (2021). Agriculture development, pesticide application and its impact on the environment. *International Journal of Environmental Research and Public Health*, *18*(3), 1–24. https://doi.org/10.3390/ijerph18031112

Undabeytia, T., Morillo, E., & Maqueda, C. (2002). *FTIR Study of Glyphosate − Copper Complexes*. 1918–1921.

USDA. (2022). *Pesticide Data Program, Annual Summary, Calendar Year 2020*. https://www.ams.usda.gov/sites/default/files/media/2020PDPAnnualSummary.pdf

USEPA. (1993). United States Environmental Protection Agency - EPA R . E . D . Facts - Glyphosate. *Prevention, Pesticides and Toxic Substances*, 1–7.

USEPA. (2005). *Reregistration eligibility decision for Mancozeb*. *September*. http://archive.epa.gov/pesticides/reregistration/web/pdf/profenofos_red.pdf

USEPA. (2015). *Preliminary Ecological Risk Assesment In Support Of The Registration Review of Glyphosate and Its Salts*.

USEPA. (2019). Glyphosate. *Environmental Protection*.

Vellingiri, B., Chandrasekhar, M., Sri Sabari, S., Gopalakrishnan, A. V., Narayanasamy, A., Venkatesan, D., Iyer, M., Kesari, K., & Dey, A. (2022). Neurotoxicity of pesticides – A link to neurodegeneration. *Ecotoxicology and Environmental Safety*, *243*(August), 113972. https://doi.org/10.1016/j.ecoenv.2022.113972

Weisenburger, D. D. (2021). A Review and Update with Perspective of Evidence that the Herbicide Glyphosate (Roundup) is a Cause of Non-Hodgkin Lymphoma. *Clinical Lymphoma, Myeloma and Leukemia*, *21*(9), 621–630. https://doi.org/10.1016/j.clml.2021.04.009

Woodburn, A. T. (2000). Glyphosate: Production, pricing and use worldwide. *Pest Management Science*, *56*(4), 309–312. https://doi.org/10.1002/(SICI)1526-4998(200004)56:4<309::AID-PS143>3.0.CO;2-C

World Health Organization. (1988). Dithiocarbamate Pesticides, Ethylenethiourea, and Propylenethiourea: A General Introduction. *Environmental Health Criteria 78*.

Wunnapuk, K., Gobe, G., Endre, Z., Peake, P., Grice, J. E., Roberts, M. S., Buckley, N. A., & Liu, X. (2014). Use of a glyphosate-based herbicide-induced nephrotoxicity model to investigate a panel of kidney injury biomarkers. *Toxicology Letters*, *225*(1), 192–200. https://doi.org/10.1016/j.toxlet.2013.12.009

Yahfoufi, Z. A., Bai, D., Khan, S. N., Chatzicharalampous, C., Kohan-Ghadr, H. R., Morris, R. T., & Abu-Soud, H. M. (2020). Glyphosate Induces Metaphase II Oocyte Deterioration and Embryo Damage by Zinc Depletion and Overproduction of Reactive Oxygen Species. *Toxicology*, *439*(April). https://doi.org/10.1016/j.tox.2020.152466

Yehye, W. A., Rahman, N. A., Ariffin, A., Abd Hamid, S. B., Alhadi, A. A., Kadir, F. A., & Yaeghoobi, M. (2015). Understanding the chemistry behind the antioxidant activities of butylated hydroxytoluene (BHT): A review. *European Journal of Medicinal Chemistry*, *101*, 295–312. https://doi.org/10.1016/j.ejmech.2015.06.026

Zhang, L., Rana, I., Shaffer, R. M., Taioli, E., & Sheppard, L. (2019). Exposure to glyphosate-based herbicides and risk for non-Hodgkin lymphoma: A meta-analysis and supporting evidence. *Mutation Research - Reviews in Mutation Research*, *781*(February), 186–206. https://doi.org/10.1016/j.mrrev.2019.02.001

Zhang, Y., Wen, R., Bao, J., Gong, Y., & Wang, X. (2023). Mancozeb induces nephrotoxicity by impairing the oxidative phosphorylation pathway: A transcriptome study. *Ecotoxicology and Environmental Safety*, *249*(September 2022). https://doi.org/10.1016/j.ecoenv.2022.114471

Zhu, J., Lian, J., Wang, X., Wang, R., Pang, X., Xu, B., Wang, X., Li, C., Ji, S., & Lu, H. (2023). Role of endogenous and exogenous antioxidants in risk of six cancers: evidence from the Mendelian randomization study. *Frontiers in Pharmacology*, *14*(June), 1–11. https://doi.org/10.3389/fphar.2023.1185850

Vita

Name	*Heidi Ebid*
Baccalaureate Degree	*Bachelor of Science,* *Misr University for Science* *and Technology,* *Cairo, Egypt* *Major: Pharmaceutical Sciences*
Date Graduated	*May, 2010*
Other Degrees and Certificates	*Master of Science,* *St. John's University,* *Queens, NY* *Major: Toxicology*
Date Graduated	*May, 2017*