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Manfreda Maculosa Leaf Extracts And Their Effects On Whole And Selected Myotoxic Components In Crotalid Venom

Charles Steven Gilbert
University of Texas at El Paso

MANFREDA MACULOSA LEAF EXTRACTS AND THEIR EFFECT ON WHOLE AND SELECTED MYOTOXIC COMPONENTS IN CROTALID VENOM

CHARLES STEVEN GILBERT

Master's Program in Environmental Science

APPROVED:

Carl S. Lieb, Ph.D., Chair

Robert A. Kirken, Ph.D.

Chuan Xiao, Ph.D.

Stephen L. Crites., Ph.D.
Dean of the Graduate School

Dedication

As I have become older, I have come to realize that none of us have really achieved everything we have without having learned something from other people. It is said we stand on the shoulders of giants. I want to thank the "giants" that have helped me get to where I am today:

To my wife, Debbie for lessons about loyalty, stubbornness, and especially love.
To my boys: Michael, Jared, and Jorel for patience.
To my mother, 'Joni' (of Blessed Memory) for teaching me responsibility, 'social decorum', and especially how to accept 'no' as an answer.
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To my step-father, Chuck who led by example and taught me how to listen to my children and to be aware of what's going on around me.
To my sister, Lisa.
To my 'Uncle Dick' (of Blessed Memory) and his wife Pat who helped me learn the bonds of family.
To Stanley and Myra who also helped me learn the bonds of family.
To my cousins Sally, Marty, and Marsha who have kept me in the family.
To my friend, 'Ron' for a long-term friendship and early lessons about dancing, what to wear, and that loyalty is part of friendship and that forgiveness isn't about changing the past but deciding the path for the future.
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To my friends and family friends:
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Everyone here, and many, many more, have taught me things not because it was necessarily their intention. I think it was because it was who they are. They taught me invaluable lessons about family, community, and our obligations to one another. These people are my heroes and the giants on whose shoulder's I stand.

In deepest gratitude, I give thanks, and I offer the only thing worthy of such legacy: I promise to pay it forward.

Shalom,

Charles 'Chuck' Gilbert

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by

CHARLES STEVEN GILBERT, B.S.

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Abstract

Manfreda spp have been used for the treatment of snakebite by several cultures in North and Central America (Austin & Honychurch, 2004, Johnson, 1999, Moerman, 2008, Verhoek, 1978a). There has been no systematic study of the genus *Manfreda* for its usefulness in the treatment of snakebite.

While the annual incidence of snakebite in the United States is relatively low (approximately 8,000 - 10,000), with a low mortality rate of 5 – 6 cases per year, there is still significant morbidity associated with snakebite (CDC - NIOSH, 2018, Springhouse Corp (eds), 2005). Worldwide numbers of the cases of snakebite can only be estimated and run as high as 1,841,000 cases with fatalities as high as 94,000 (Kasturiratne *et al.*, 2008). Currently the only treatment for snakebite consists of antivenom administration in a hospital setting due to the possibility of an anaphylactic response. A plant-based treatment could have fewer reactions and provide onsite treatment for snakebite injuries thus possibly lowering the morbidity of the local tissue effects (Houghton, 1994).

An aqueous extract of *Manfreda maculosa* leaves was examined to see if it could mitigate the effects of whole venom obtained from *Crotalus viridus viridus* in an *in vitro* study using human skeletal muscle cells. Experiments measuring cell death using differential nuclear staining and experiments examining cell proliferation using human skeletal myoblasts or differentiated myocytes (myotubes) were performed. While the clinical picture of envenomation shows tissue necrosis, this study found no cell death, neither necrosis nor apoptosis, occurring in venom-treated HuSKM myoblasts and myocytes or necrosis with the myotoxins in the same cells using the methods employed.

The aqueous extract of *Manfreda maculosa* leaves was not reliably found to offer any protection from rattlesnake evenomation. However, previous research had not used human skeletal muscle cells or a cell proliferation assay within the setting of venom toxicity studies. The further use of these technologies may prove useful in elucidating venom toxicity.

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Introduction

In the United States there are approximately 8,000 – 10,000 snakebites annually resulting in significant morbidity even if only 5 - 6 bites result in death (Blackman & Dillon, 1992, Kurecki, III & Brownlee, Jr., 1987, O'Neil *et al.*, 2007, Springhouse Corp (eds), 2005). However, the worldwide estimates are much higher and range from 421,000 to 1,841,000 venomous snakebites annually with fatalities in the range between 20,000 to 94,000 (Chippaux, 2008, Kasturiratne *et al.*, 2008). High morbidity (Blackman & Dillon, 1992, Gold *et al.*, 2004, Gutiérrez *et al.*, 2006, Juckett & Hancox, 2002, Springhouse Corp (eds), 2005, Warrell, 2007) is generally associated with local tissue effects, such as myonecrosis, which was one focus of the present study.

Snake venom is a complex mix of proteins and peptides (Harvey, 1991, Markland, 1998) that affect multiple systems, i.e., coagulation (Markland, 1998), proteases (Wagner & Prescott, 1966), neurotoxins (Juckett & Hancox, 2002), and others (Camey *et al.*, 2002). Snake venom differs between species and even the venom from two different snakes of the same species can differ significantly from each other. The treatment of snakebite and the development of antivenoms [also cited as 'antivenins' (Blackman & Dillon, 1992, Castro *et al.*, 2003, Gold *et al.*, 2004, Juckett & Hancox, 2002)] is thus very difficult (Galan *et al.*, 2004).

Currently the only treatment for snakebite consists of the administration of antivenom, administered in the hospital setting due to the possibility of anaphylactic responses. Antivenom is developed now in sheep (Ovine sources) because there are fewer anaphylactic reactions than from equine sources (Juckett & Hancox, 2002). Since the lag time between the envenomation event and the administration of antivenom is always greater than zero, serious local effects can occur in proportion to the lag time (Ownby *et al.*, 1985). In addition, antivenom preservatives, such as phenol, cause additional undesired side-effects (Zychar *et al.*, 2008). A plant-based treatment could have fewer reactions and provide for onsite treatment of snakebite injuries (Houghton, 1994).

Choosing plants that have an ethnobotanical application in the treatment of various diseases and injuries has proven a useful starting point for finding bioactive substances (Castro *et al.*, 2003, Gurib-Fakim, 2006, Pereda-Miranda, 1995). The genus *Manfreda* has a list of cultural uses that span more than four centuries of recorded history. This finding provides an ethnobotanical basis for studying *Manfreda spp* for their effects in snakebite (Cox & Balick, 1994). *Manfreda spp* have been used for the treatment of snakebite by several cultures in North and Central America (Austin & Honychurch, 2004, Johnson, 1999, Moerman, 2008, Verhoek, 1978a). Aqueous extractions of leaf and root pulp have been used externally and internally for the treatment of snakebite (Moerman, 2008, Verhoek, 1978a). Some *Manfreda spp* bear the common name of "rattlesnake master" indicative of their use in the treatment of snakebite (Austin & Honychurch, 2004). Several *Manfreda spp*, as well as the species in general, have been listed in ethnobotanic literature to include *M. brunnea*, *M. maculata*, *M. variegata*, and *M. virginica* (Johnson, 1999). It is reported that several Native American groups (Catawba, Cherokee, Creek, and Seminole) used *M. virginica* for snakebite (Moerman, 2008). Verheok (1978a) reported on the usage of *Manfreda spp* for snakebite in Mexico. At present, there has been no scientific evaluation of the genus *Manfreda* for its usefulness in the treatment of snakebite (Gurib-Fakim, 2006, Heinrich *et al.*, 1998, Houghton, 1994, Houghton & Osibogun, 1993, Martz, 1992, Soares *et al.*, 2005).

The actual phytochemical components responsible for any anti-ophidian venom effects are not known. Saponins, which fall in the class of glycosides, are prevalent in the genus *Manfreda* (Verhoek, 1978a). There was an early attempt to classify steroidal sapogenins in *Manfreda* (Marker & Lopez, 1947). "Catequines, flavones, anthocyanines and condensated tannins" have been identified in other plant species as active ingredients in plant extracts with antivenom properties (Castro *et al.*, 1999). In more recent work, steroidal sapogenins in *Agave utahensis* have been shown to have cytotoxic effects (Yokosuka & Mimaki, 2009); however, no study has been made of the genus *Manfreda* (Alonso-Castro *et al.*, 2011).

For this study, an aqueous extract of *Manfreda maculosa* leaves was examined to see if it could mitigate the effects of rattlesnake whole venom obtained from *Crotalus viridus viridus*. Experiments measuring cell death using differential nuclear staining and experiments examining cell proliferation using human skeletal myoblasts or differentiated myocytes (myotubes) were performed. Additionally, two myotoxic polypeptides from *Crotalus viridus concolor* were examined for toxicity both in cell death and cell proliferation, unfortunately the supply of these agents become unavailable commercially during the course of experimentation and these components could not be evaluated with the plant extract.

The Cultural Uses of Manfreda Species

The Taxonomic History of *Manfreda*

Plants of the genus *Manfreda* are of New World origin and range from Virginia to Florida and Texas down through Mexico to Honduras (Verhoek-Williams, 1975) and currently number about 33 species (Cruz, 2009, The Plant List, 2013), They are placed in the family Asparageceae (The Plant List, 2013). This modern classification is based on a phylogenetic plant systematic analysis by the Angiosperm Phylogeny Group (APG III) system (The Angiosperm Phylogeny Group, 2009, The Plant List, 2013). The older taxonomy was to place the genus *Manfreda* in the family Agavaceae, a family-level taxon no longer recognized in the APG III system (The Angiosperm Phylogeny Group, 2009).

The first species in the current genus *Manfreda* was originally described by Carolus Linnaeus (aka, Carl von Linné) as *Agave virginica* L. (Linnaeus, 1753); it would remain under this name for over a century until allocated to *Manfreda virginica* (L.) Salisb. by Richard Salisbury (Salisbury, 1866). Several decades later J. N. Rose, rearranging the suborder Agaveae, supported using the genus *Manfreda* for *M. virginica* (L.) Salisb. ex Rose (Rose, 1899). Later work by Rose allocated the genus *Manfreda* in the family Amarillidaceae (Rose, 1903). In 1966 work by Lloyd Shinners, based on specimens derived from Texas and a view that emphasized vegetative over floral characters, placed *Manfreda* entirely into the genus *Polianthes* (Shinners, 1966). Later work by Susan Verhoek reviewed specimens of all species within *Polianthes* (Verhoek-Williams, 1975); she concluded that there was sufficient reason not to ignore floral characteristics and revived the recognition of the genus *Manfreda* as a separate lineage (Verhoek, 1978b). The relevant comparisons based on Verhoek's description are summarized in the Table 1. While there is still some remaining controversy over whether the genus *Manfreda* should be recognized (Wynn Anderson, personal communication) the APG III classification does so (The Plant List, 2013).

Based on the botanic literature, allocation of this genus is to three possible families: Agavaceae, Amarillidaceae, and Asparagaceae. There are also potentially three genera in use: *Agave*, *Manfreda*, and *Polianthes* depending on whose classification is used. Furthermore, knowledge of the common names of plants is necessary in the ethnographic literature and the genus *Manfreda* is know by many names, such as, aloe, American aloe, false aloe, rattlesnake master, huaco, amole, and barbasco (see Table 2). Also, these common names can and do refer to more than one plant species. The references and meanings to these names will be cited as I explore the specific uses of these plants of the genus *Manfreda*.

While there are 33 accepted species of Manfreda in the New World (Cruz, 2009, The Plant List, 2013) there are five listed as growing in the United States (USDA & NRCS, 2019). These are:

- *Manfreda longiflora* (Rose) Verh-Will.
- *Manfreda maculosa* (Hook.) Rose
- *Manfreda sileri* Verh-Will.
- *Manfreda variegata* (Jacobi) Rose
- *Manfreda virginica* (L.) Salisb. ex Rose

Endemic to the eastern United States, the principal species is *Manfreda virginica* (L.) Salisb. ex Rose and ranges from east Texas to northern Florida and north to North Carolina and the western tip of Virginia and then west as far as the Ohio river (Verhoek-Williams, 1975).

The Uses of the Manfreda sp. Plants

The use by Native Americans of the genus *Manfreda*, while principally medicinal, did include several other types of uses. For the purposes of simplifying this study of *Manfreda* and it's cultural uses, I have categorized the usages of *Manfreda* into three basic groups: medicinal, soap, and other. I have also sorted the uses by the people or culture that is using the plant and the geographic location where this use was determined. Using this schema, I have created a table to summarize the uses of *Manfreda* spp. (see Table 2).

T. N. Campbell's work in 1951 was compiled from the medical herbarium and notes of Gideon Lincecum who studied the Choctaw, Chickasaw, and Creek Indians of Mississippi and Georgia. Lincecum's work was amassed in the early 19th century and reported to be at The University of Texas at Austin's Library; not seen by author (Campbell, 1951). John Witthoft (Witthoft, 1947) quoted from the previously published notes and herbarium samples made by Anna Rosina Gambold on the Cherokee Indians in the area of the Conasauga River (now in northwestern Georgia) in the early 19th century. These plant lists reported by both Campbell and Witthoft are important because they predate the removal of the Indian populations to reservations (aka, "Trail of Tears") which occurred around 1830-1850 (Campbell, 1951). Reports on medicinal plant use from the Native Americans in Oklahoma may be from the period after the "Trail of Tears" relocation of native peoples.

The reports on the use of *Manfreda* for snakebite show that either the leaves or the roots were ingested or used as an external wash (see Table 2). In some instances, the medicine-man would chew the plant then suck the poison out of the wound (no incision was made) while singing a medicine song (Sturtevant, 1954, Swanton, 2000). They would extract some venom and sing, then extract a little more venom and sing some more, etc. An example of a song of the Creek medicine-man is offered here (Swanton, 2000):

"O, spirit of the white fox, come
O, spirit of the white fox, come
O, spirit of the white fox, come
O, hater of snakes, come
Snakes [who] have hurt this man, come
Come, O white fox, and kill this snake.
O, spirit of the red fox, come, etc.
O, spirit of the black fox, come, etc."

A note was included to indicate that a verse regarding a blue or yellow fox was lost (Swanton, 2000). There was one comment on the Creek Indian usage of *Manfreda* in "sweet milk;" this comment was likely made after the introduction of the Old World bovines (Campbell, 1951).

In Mexico, the plants of the genus *Manfreda* commonly were called “amole” and used for soap. According to Rose (Rose, 1903), “the root is always to be found in great quantities in every Mexican marketplace.” Verhoek (Verhoek, 1978a) quotes from a mid-16th century Aztec document translated by the Spanish chronicler Sahagún (in the Florentine Codex):

> “It is long and narrow like reeds. It has a shoot; its flower is white. It is a cleanser. The large, the thick roots remove one's hair, make one bald; the small, the slender ones are cleansers, a soap, they wash, they cleanse, they remove the filth.
>
> I use amolli; I soap myself.
>
> The animals which are not strong, such as small fish, die from it; for therewith are the small fish taken. He who swallows a leech, this one drinks [an infusion of] amolli to kill the leech which is within.”

The rise of modern soaps and detergent has decreased the use of plants as a source of soap (Verhoek, 1978a). Outside of Mexico, Taylor reports that the Creek Indians used *Manfreda* as a soap (Taylor, 1940).

Other medicinal usage regards a host of digestive disorders, i.e., colic, dyspepsia, and diarrhea, and act as a carminative, antispasmodic, and a stomachic (Austin & Honychurch, 2004, Hamel & Chiltoskey, 1975, Taylor, 1940, Verhoek, 1978a). *Manfreda* was also called for at those unfortunate times when the patient had swallowed a leech or had worms (Hamel & Chiltoskey, 1975, Verhoek, 1978a). There are reports of *Manfreda* spp being used as a liver tonic (Hamel & Chiltoskey, 1975), for dropsy (Taylor, 1940, Verhoek, 1978a), and for headaches (Hernández-Sandoval *et al.*, 2008, Verhoek, 1978a).

Miscellaneous or other uses include the plants being used as fish poisons (Hernández-Sandoval *et al.*, 2008, Verhoek, 1978a, Verhoek-Williams, 1975). *Manfreda* plants were also prized as ornamentals because of their striking floral displays (Rose, 1903, Verhoek, 1978a, Verhoek-Williams, 1975). The dried flower stalks were also used as arrow shafts (Verhoek, 1978a).

Table 1: Verhoek's summary of genus *Manfreda* characteristics

Characteristic	***Manfreda***	***Polianthes***
Flowers	Green or Brown; solitary at nodes	White or Red; usually paired
Stamens	Long-exserted	Included
Styles	Long-exserted	Included or shortly-exserted
Stigma	Clavate	Lobed

Table 1: Comparison between two genera that continues to be a question today; should the plants all be classified under the older name of *Polianthes*?

Table 2: Summary of the uses of the plants of the genus *Manfreda*.

(see Table 2 next two pages).

Not Spec. = Not Specified

General Use	People	Geographic Area	Common Names	Plant spp.	Part of Plant Used	Preparation	Method of Use	Notes	References
Soap/shampoo	Mexico	Mexico and Texas	Amole	*M. brachystachya; M. guttata; M. maculosa; M. variegata; M. brunnea; M. spp.*	Rhizome	Cut, grated or mashed; dried for storage	Soaked in water	Soap/shampoo "Played a significant" economic role	(Rose, 1899;Verhoek, 1978a)
Soap	Creek	Not spec.	Not spec.	*M. virginica*	"root"	Not spec.	Not spec.		(Taylor, 1940)
Medicinal	Mexico	Mexico	Huaco	*M. maculosa; M. variegata;*	Leaves; "root"	Leaves chewed or mashed	Taken internally	Snakebite	(Rose, 1903;Verhoek, 1978a)
Medicinal	Mexico	Mexico	Huaco	*M. brunnea*	Leaves	Bruised and put into aguardiente Alt/ precipitate powder from extract and use as a plaster	Applied externally	Snakebite	(Verhoek, 1978a)
Medicinal	Catawba	North and South Carolina	"Virginian agave"; "American aloe"; "false aloe"	*M. virginica*	Rhizome	In water	Taken internally Applied externally	Snakebite	(Taylor, 1940;Verhoek, 1978a)
Medicinal	Creek	Florida	"Rattle snake's master"	*M. virginica*	"root"	Not spec.	Taken internally Applied externally	Snakebite	(Sturtevant, 1954)
Medicinal	Creek	Mississippi and Georgia	False aloe	*M. virginica*	"root"	"boiled in sweet milk"	"taken freely, or chewed and swallowed"	Snakebite	(Campbell, 1951)
Medicinal	Blacks	South Carolina	"Negroes Rattle snake's master"	*M. spp.*	Not Spec.	Not Spec.	Not Spec.	Snakebite	(Austin and Honychurch, 2004)

General Use	People	Geographic Area	Common Names	Plant spp.	Part of Plant Used	Preparation	Method of Use	Notes	References
Medicinal	Creek	Oklahoma	“abi-tcåpko” (“long stem” in Creek)	*M. virginica*	“root”	Decoction	Applied externally	Snake or centipede bite	(Swanton, 2000) [reprinted, originally published in 1928.]
Medicinal	Mexico	Mexico	Amole	*M. spp.*	“root”	Infusion	Taken internally	Swallowed leeches	(Verhoek, 1978a)
Medicinal	Catawba	North and South Carolina	Not spec.	*M. virginica*	Rhizome	In water and a tsp. of whiskey	Taken internally	Dropsy	(Taylor, 1940;Verhoek, 1978a)
Medicinal	Not Spec.	Southern US	False aloe; “rattlesnake master”	*M. virginica*	“root”	In water	Taken internally	Colic, dyspepsia, stomachic, carminative, antispasmodic	(Taylor, 1940;Verhoek, 1978a;Austin and Honychurch, 2004)
Medicinal	Mexico	Yucatan		*M. variegata; M. paniculata; M. petskinil*	Leaves	Leaves applied to forehead	Applied externally	Headache	(Verhoek, 1978a;Hernández-Sandoval et al., 2008)
Medicinal	Cherokee	Northwestern Georgia	False aloe	*M. virginica*	“root”	Chewed	Taken internally	“Diarrhoea” [sic] “Strong medicine”	(Witthoft, 1947)
Medicinal	Cherokee	North Carolina	False aloe; aloe	*M. virginica*	“root”	Chewed	Taken internally	“Diarrhea”; liver tonic; worms “Strong medicine”	(Hamel and Chiltoskey, 1975)
Other:	Mexico	Mexico	Amole	*M. spp.*	“root”	Not spec.		Fish poison (“barbasco”); “aporaka” (Tarahumara)	(Verhoek-Williams, 1975;Verhoek, 1978a;Hernández-Sandoval et al., 2008)
Other:	Mexico	Mexico	Not spec.	*M. maculosa; M. longiflora; M. variegata*	Plant / flowers			Ornamentals	(Rose, 1903;Verhoek-Williams, 1975;Verhoek, 1978a)
Other:	Mexico	Honduras	“cebolla de cerro”	*M. brachystachya*	Flower stalk			Arrows	(Verhoek, 1978a)

Overview of Methods

Envenomation yields two kinds of effects, systemic and local. Systemic effects are well-controlled through the administration of antivenom. Local effects on tissue post-envenomation include hemorrhagic, neurotoxic, and myotoxic effects. This research focused on the *Manfreda* extract effects in neutralizing whole venom myotoxic components. These experiments employed both whole rattlesnake venom (CvvV) from *Crotalus viridus viridus* (Sigma-Aldrich, St Louis, MO), commonly known as the Prairie rattlesnake, and Myotoxin I (MTI) and Myotoxin II (MTII) from *Crotalus viridis concolor* (Sigma-Aldrich, St Louis, MO), the Midget faded rattlesnake. Myotoxin I and II are basic polypeptides with no enzymatic activity and are one of three classes of myotoxic components found in snake venoms (Ownby, 1998).

The leaves of *Manfreda maculosa* were extracted and tested. The plants acquired were grown in garden and greenhouse settings. The original plant extract (designated MMPE for *Manfreda maculosa* Plant Extract) was prepared after the method of Biondo, et al (2003). In brief, this method calls for the leaves to be clipped and washed using deionized water, patted dry, and weighed. Leaves were homogenized using de-ionized water (500 g/L) in a blender at room temperature. After maceration they were filtered through a fine filter. The aqueous extracts were centrifuged at 10,000 × g for 10 minutes. Supernatants were freeze-dried and stored at –20 °C. Leaf extracts were weighed and dissolved in phosphate buffered saline. Stock solutions were stored at –20°C. They used a 1:50 w/w ratio of toxin to extract for their testing (Biondo *et al.*, 2003). During the processing, extreme foaming became problematic and the changes to this procedure are noted in the subsequent "specific methods" that follow. Also, the procedure was modified to substitute a liquid nitrogen step and to grind the frozen material with mortar and pestle (courtesy of Dr. Elizabeth Walsh, Biology).

I obtained commercially available primary human myoblasts, and differentiated them into myotubes, herein called myocytes. Differentiated and undifferentiated cells were tested against the commercially available purified venom components Myotoxin I (MTI) and Myotoxin II

(MTII) and whole venom (Sigma-Aldrich, St Louis, MO). Several concentrations of *Manfreda* extract were tested. Suggested guidelines for the standardization of venom assays (Gutiérrez *et al.*, 1990, Ownby, 1990, Theakston, 1990) stress the importance of controls and a systematic approach. Tween 20 was used as a positive control for 100% inactivation and as a proxy for necrosis. The venom component alone was used as the basic control for venom activity. *Manfreda* extract alone was used as a control for effects from the plant extract itself. Growth media with cells by itself was used as a background blank.

Cell death, using fluorescent microscopy, was initially considered a reasonable endpoint. A literature review indicated that Myotoxin II (*C. v. concolor*) would be the best agent (Engle *et al.*, 1983). The myoblast (uninucleate) cells can be differentiated into myocytes (a fusion of cells that are multinucleate). Myocytes have been indicated to be more sensitive to myotoxins, at least of the PLA2 variety, than undifferentiated myoblasts (Lomonte *et al.*, 1999). Human myocytes have also been shown to have more of the muscle proteins present (Serena *et al.*, 2010). Therefore, I indirectly modelled cellular death as a percentage of dead nuclei.

I used a Promega Cell Proliferation Assay which is a colorimetric test of cell metabolism. It measures the reduction of tetrazolium salts by mitochondrial metabolism; the abbreviation MTS is used for this assay in this study. This protocol was used with both myoblast and myocyte cells. Our knowledge of the toxin effects on mitochondria is limited to a note on MTI that indicated mild swelling and MTII that showed normal mitochondria (Engle *et al.*, 1983). Cell data from cell proliferation assays was used to examine the responses to the various toxins and controls.

Solution preparations have been included in the Appendix.

Pseudoreplication was an issue here because the cell samples are considered auto-correlated, that is, not completely independent. Also, statistical analysis was problematic since the sample sizes are small. Treated and untreated undifferentiated myoblasts, and differentiated myotubes, were graphically compared looking at trends in the distribution of responses, effects, and effect sizes. Morphological comparisons were made to make sure that the myoblasts and

myocytes actually differentiated, and to observe the effects of the toxin treatments at the level of light microscopy.

Specific Methods

Cell Lines

HeLa-Green Flourescent Protein (HeLa-GFP) cells (Aguilera *et al.*, 2006) were initially tested using cell death as the tested outcome. This cell line was obtained from the Cytometry, Screening and Imaging (CSI) Core Facility.

In addition, three other cell lines: HaCaT is a Human keratinocyte cell line; Hs27 is a Human foreskin fibroblast cell line; and Jurkat is an Acute T Cell Leukemia cell line that were observed using the MTS protocol, courtesy of C. Lema.

Cryopreserved human skeletal muscle myoblasts (HuSKM) were obtained commercially (Zenbio, Inc., Research Triangle Park, NC) and were cultured and/or differentiated according to vendor guidelines. A description of how the cells were handled is below.

Cell Counts

Cell counts were performed using an Improved Neubauer Hemacytometer. Cells were diluted 1:10 with trypan blue (see Appendix). The cells were counted in 5 square millimeters on each side of the hemacytometer, the 4 corners and one more centrally located. The cell counts from each side of the hemacytometer were then averaged and the average used to determine the number of cells/mm^3 which is equivalent to cells/µL.

Crotalid Toxin Preparation

Myotoxin I & II (Sigma-Aldrich, St Louis, MO) derived from *Crotalus viridus concolor* (MTI and MTII, respectively) were obtained commercially. Whole venom (Sigma-Aldrich, St Louis, MO) obtained from *Crotalus viridus viridus* (CvvV) was also obtained commercially.

Contents of a vial were diluted in 1 mL of Dulbecco's Phosphate Buffered Saline (DPBS); vial sizes were 1 g, 5 g, or 10 g. Aliquots of 50 µL of the stock solution were pipetted into microcentrifuge tubes for storage at -20°C. On use, the sufficient aliquots for the experiment were thawed in a 37°C water bath, mixed, and centrifuged in a microcentrifuge for 1

minute. The stock evaluated for cloudiness or precipitates. These stock solutions were diluted in cell media to a working solution of 2x the final dose in a 100 µL volume for the MTS1 procedure with a final reaction volume is 200 µL. For the modified MTS2 procedure the stock would be diluted to a working dilution of 10x the final dilution in media.

HeLa-Green Fluorescent Protein (HeLa-GFP)

HeLa-GFP Cell Expansion

The cell expansion protocol used was the laboratory standard protocol which is summarized here. An aliquot of HeLa-GFP cells was obtained from cell storage and thawed in a 37°C water bath. The cells were then added 10-15 mL of the HeLa-GFP growth media (see Formulary in the Appendix for the formulation in a standard lab stocked 15 mL conical centrifuge tube. Cells were mixed and centrifuged at 1200 rpm for 7 minutes. Media was decanted and a fresh 10-15 mL of cell growth media was added. Cells were then transferred to a T-75 flask (TPP 90076) and incubated at 37°C with a 5% CO_2 concentration. Cells were checked at regular intervals and the growth media was exchanged as needed until adequate growth was achieved.

HeLa-GFP Cell Passaging

Cells in the T-75 culture flask were checked under the fluorescent scope for fluorescence; a check on this cell lines viability. The media was aspirated and then 2 mL of 0.25% Trypsin working solution (see Appendix) was added. The cells were then incubated for 5 minutes until cell release. After cell release, 10 mL of cell growth media was added. One mL of the cell solution was kept for cell counts.

HeLa-GFP Plate Preparation

In order to prepare the Falcon 96-well plates (BD Biosciences, Bedford, MA) cell death bioassay, the cells in the culture flask were checked for proper growth on an inverted cell culture microscope. The grown cells were trypsinized in the cell flask to release the cells from the flask surface. A 5 mL aliquot cells were transferred to a 15 mL conical centrifuge tube and centrifuged at 1200 rpm for 7 minutes. The growth media was decanted and resuspended in 5 mL of fresh cell growth media. An aliquot was loaded into the hemocytometer and the cell concentration was determined. Calculations were made and the cells were seeded in the 96-well

plate at 20000 cells/well in a final volume of 100 µL of cell growth media. The cell suspension was mixed as it was loaded into the plate using 2 aliquots of 50 µL each to insure homogeneity. Each treatment was allocated a minimum of 3 replicates each. Plate diagrams were drawn in the lab notebook to facilitate plate set-up during the experiments to include: Test wells, positive controls, negative control of media only, and media blanks with no cells. Initial pilot tests used one replicate to extend the time variable if necessary. In the HeLa-GFP study one well was extended to 96 hours. Cells were incubated at 37°C with a 5% CO_2 concentration during the experiment. Cells are checked daily and media is replaced as needed. Experimental incubations were performed at 24, 28, 72, and 96 hours.

Cryopreserved Human Skeletal Muscle Cells (HuSKM)

HuSKM Cell Expansion

A cell vial was retrieved from storage and thawed in a 37°C water bath. The cells were transferred and rinsed from the cryotube into a 15 ml conical tube using 10 mL of cell growth media (SKM-M). The cells were centrifuged at 280 x g at 20°C for 5 minutes and the media decanted. Cells were transferred to a 75 cm^2 tissue culture flask (Corning) using 20 mL of media. Cells were incubated at 37°C with a 5% CO_2 concentration. Cells were ready for passage at a confluence of 60-70%.

HuSKM Passaging

The cells were rinses two times using 10 mL of Dulbecco's Phosphate Buffered Saline (DPBS). Two mL of Trypsin-EDTA solution was added to the culture flask for 5 minutes until the cells released from the flask. This is the point at which we considered that the cells were passaged and we added 10 mL of the cell growth media (SKM-M). The cells were transferred to a 15 mL conical tube and centrifuged at 280 x g at 20°C for 5 minutes and the media decanted. A fresh 10 mL of SKM-M was added and a cell aliquot was withdrawn for a cell count. Cells were mixed to a cell density of 5000 – 15000 cells/cm^2 for continued growth in the T-75 cm^2 and the previous sets repeated as necessary.

HuSKM Plate Preparation

In order to prepare the Falcon 96-well plates (BD Biosciences, Bedford, MA) for the MTS bioassay, the cells in the culture flask were checked for proper growth on an inverted cell culture microscope. Cells were counted and seeded at 10000 cells/well into a Falcon 96-well plate. The outer row of wells is filled with 200 µL of DPBS to slow desiccation. Plate diagrams were drawn in the lab notebook to facilitate plate set-up during the experiments to include: Test wells, positive controls, negative control of media only, and media blanks with no cells. The cell suspension was mixed as it was loaded into the plate using 2 aliquots of 50 µL each to insure

homogeneity. Each treatment was allocated a minimum of 3 replicates each. Cells were incubated at 37°C with a 5% CO_2 concentration during the experiment. Cells are checked daily and media is replaced as needed. Experimental incubations were performed at 24, 28, 72, and 96 hours.

Assay Test Controls

Tween 20 was used as a positive control in selected experiments at concentrations of 0.1%, 0.05%, and 0.025%. Tween 20 at 0.1% was the final choice as the positive control. H_2O_2 (see Appendix) was also utilized as a positive control with a 1mM (~ 0.003%) final concentration. The H_2O_2 positive control was increased in later experiments to 10 mM because of the wide variability of action that was observed in an effort to finalize a positive control for general use in this assay.

Cell Death Endpoint Studies

Stock solutions of Hoechst 33342 (Hoechst; Invitrogen, Eugene, OR); and Propidium iodide (PI; MP Biomedicals, Solon, OH) were diluted in cell media and added to each well for a final concentration of the fluorophores was 20 µg/mL for this part of the study. Cells were incubated at 37°C with a 5% CO_2 concentration during the experiment. Experimental incubations were performed at 24, 28, 72, and 96 hours.

The cells in the plate wells were analyzed for fluorophore activity using a BD Pathway 855 BioImager system and its AttoVision v.1.6.2.02 Image Analysis Software (BD Biosciences, Rockville, MD).

Differential Nuclear Staining – Annexin (DNS-A) Assay

This study was performed with the Annexin V-FITC Kit (Beckman Coulter). The wells were seeded with 10000 cells/well. The 96-well plates were setup to include: Test replicates, positive control (Tween20 - 0.1%), and negative controls (DMSO and untreated). Added compounds so that the final DMSO concentration did not to exceed 1% of final concentration. Cells were incubated at 37°C with a 5% CO_2 concentration during the experiment. Myoblasts were exposed to *Crotalus viridus viridus* whole venom (CvvV) at 100 µg/mL for 24, 48 and 96 hours using a reaction volume of 100 µL. At the end of the incubation period 100 µL of staining solution was added per well in darkness. Stock solutions of Hoechst 33342 (Hoechst; Invitrogen, Eugene, OR); and Propidium iodide (pI; MP Biomedicals, Solon, OH) were diluted in cell media and added to each well for a final concentration of Hoechst at 2 µg/mL and pI at 1 µg/mL. The plate was then incubated at 37°C with 5% CO_2 for one hour and read in the BioImager. Cells were analyzed using a BD Pathway 855 Bioimager system and its AttoVision v.1.6.2 software (BD Biosciences, Rockville, MD).

When setting up the BioImager, the bottom of the plate was first cleaned with ethanol and then placed on the BioImager and oriented per standard protocol. The 10x objective was used. The BioImager was setup per protocol and each dye was focused, the exposure was adjusted to

dark background, and an autofocus test was performed to insure minimal to no movement. The imager settings were recorded per session and differed between sessions

Cell Proliferation (MTS) Assay

The CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) (Promega, Madison, WI) was used and the optical density obtained by the use of a SpectraMax Plate Reader with SoftMax Pro v5.4.1 software (Molecular Devices, LLC., Sunnyvale, CA) at 490 nm with a reference wavelength of 650 nm.

Myoblasts and/or myocytes were exposed to whole venom (in a range from 5 – 1000 µg/mL) at 24, 48, 72, or 96 hours using a reaction volume of depending on the MTS procedure.

Method One (MTS1) – Working solutions were made from the stock solution in a 2x of the final concentration using the appropriate cell medium. For the final reaction volume of 200 µL, 100 µL of the 2x toxin was added to 100 µL of medium in the 96-well plate.

To perform the MTS1 assay 100 µL of media was removed from the well without disrupting the cells and 20 µL of the MTS reagent was added. The plate was incubated at 37°C, 5% CO_2 for 2 hours. The plate was read in the plate reader mentioned above with the lid removed.

Method Two (MTS2) – Working solutions were made from the stock solution in a 10x of the final concentration using the appropriate cell medium. For a final reaction volume of approximately 100 µL, 10 µL of the 10x toxin was added to 100 µL of medium in the 96-well plate.

To perform the MTS1 assay 20 µL of the MTS reagent was added. The plate was incubated at 37°C, 5% CO_2 for 2 hours. The plates were read in the plate reader mentioned above with the lid removed.

Plant Extract I (MMPE: *Manfreda maculosa* plant extract)

The plant, *Manfreda maculosa* (Mountain States Wholesale Nursery, Phoenix, AZ), was obtained and leaf material was collected. The process called for a ratio of 2 mL of de-ionized water (dI-H_2O) per gram of plant material. Approximately 169.3 grams of plant material was

collected and added about 350 mL dI-H_2O. The leaf material was added to a counter-top blender (Sears) for one minute on high. The liquid was filtered through a GF/C (Whatman) filter using a Millipore Vacuum filter apparatus. The blended material was very foamy and that made further processing quite problematic. Before continued filtering the extract was centrifuged at 3000 rpm, 1348 x g for 10 minutes. The extract was filtered using a 0.45 µm filter which was too slow so it was changed back to a GF/C filter. The filter was again changed half way through. Approximately 250 mL of liquid extract was collected and then frozen and stored at -20°C. The plant extract material was lyophilized in a LabConco Free Dry System/Freezone 45 for 5 days at 133 x 10-3 mbar at -48°C. The free-dried residue was mixed as a 30 mg/mL stock in DPBS.

Plant Extract II (PEII: *Manfreda maculosa* plant extract)

Manfreda maculosa was processed and 53 g of leaf material obtained. The leaves were rinsed in de-ionized water and frozen at -20°C. The frozen leaves were placed in a mortar with liquid nitrogen and ground with a pestle. The powder and bits were placed in a 250 mL beaker. The plant material was extracted with 200 mL of dI-H2O by placing the material on ice and extracted over-night. The extract was divided into four 50 mL centrifuge tubes and centrifuged for 5.5 minutes at 300 x g at room temperature. The clear fluid middle layer was aspirated out and 126 mL of clear yellow solution was recovered. The extract was placed on ice and then filtered through a Whatman GF/C filter in a Millipore Vacuum filter apparatus. The collected fluid was split into two T-75 flasks (Corning). The flasks were laid flat and frozen at -20°C and lyophilized as previously described. The resulting powder was stored at -20°C. A quantity of 40.2 mg was recovered was made into a 40000 µg/mL stock solution and designated PEII.

Data analysis

This document was produced using Microsoft Word. Data processing and visualization were done using Microsoft Excel, R version 3.6.1 (2019-07-05) (R Core Team, 2019), the Tidyverse 1.2.1 package (Wickham, 2017), and the R readxl package (Wickham & Bryan, 2019).

A 2x2 factorial ANOVA was used to analyze the DNS data (in Figures 4 & 5). An ANOVA followed by a Tukey's test was used with the MTS data. Statistics were calculated using SAS v 9.3.

Results

Figure 1 shows a pilot test analysis of Myotoxin I and Myotoxin II with the HeLa-GFP human cancer cell line (Aguilera, et al., 2006). This test involved a 24 hr toxin incubation. A cell in the third column was held initially unfinalized to continue an incubation to test if more time would have aided the finding of cell death. That well showed continued growth and no toxicity after 5 days, suggesting that time does not appear to be a factor for the lack of cell death found here. Our positive control for cell death was Tween 20 at 0.1%; this result of 0% live cells confirmed the use of this concentration. The 10x objective on the BioImager was used for this analysis.

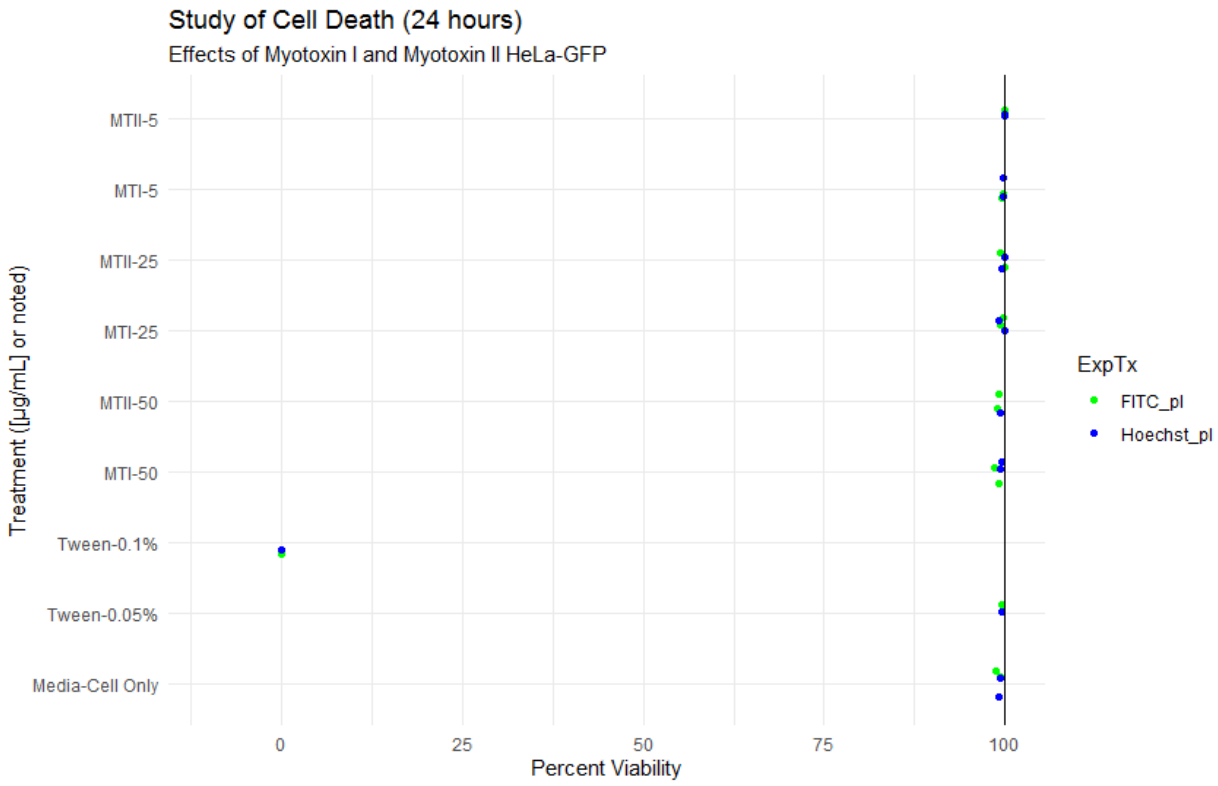


Figure 1: Myotoxins - Hela-GFP - Cellular Death

MTI = Myotoxin I; MTII = Myotoxin II

Figure 2 describes the results of experiment to examine cell death using the differential nuclear staining approach with the Annexin (DNS-A) fluorophore. I used a concentration range for the whole venom that included a range from non-toxic to toxic for the cells as shown in the cell proliferation experiments. This profile shows very low level of cell death, both apoptotic and necrotic. The Tween 20 control showed the highest level of cell death, as was expected, but also showed high variability in the results. This particular problem plagued many of the experiments. The Camptothecin is an agent to promote apoptosis; however, it didn't seem to have that effect here with this cell line. This experiment was also analyzed in terms of cell count (see Figure 3). This experiment was repeated for extended time and at the highest whole venom concentration of 100 µg/mL (see Figures 4 & 5).

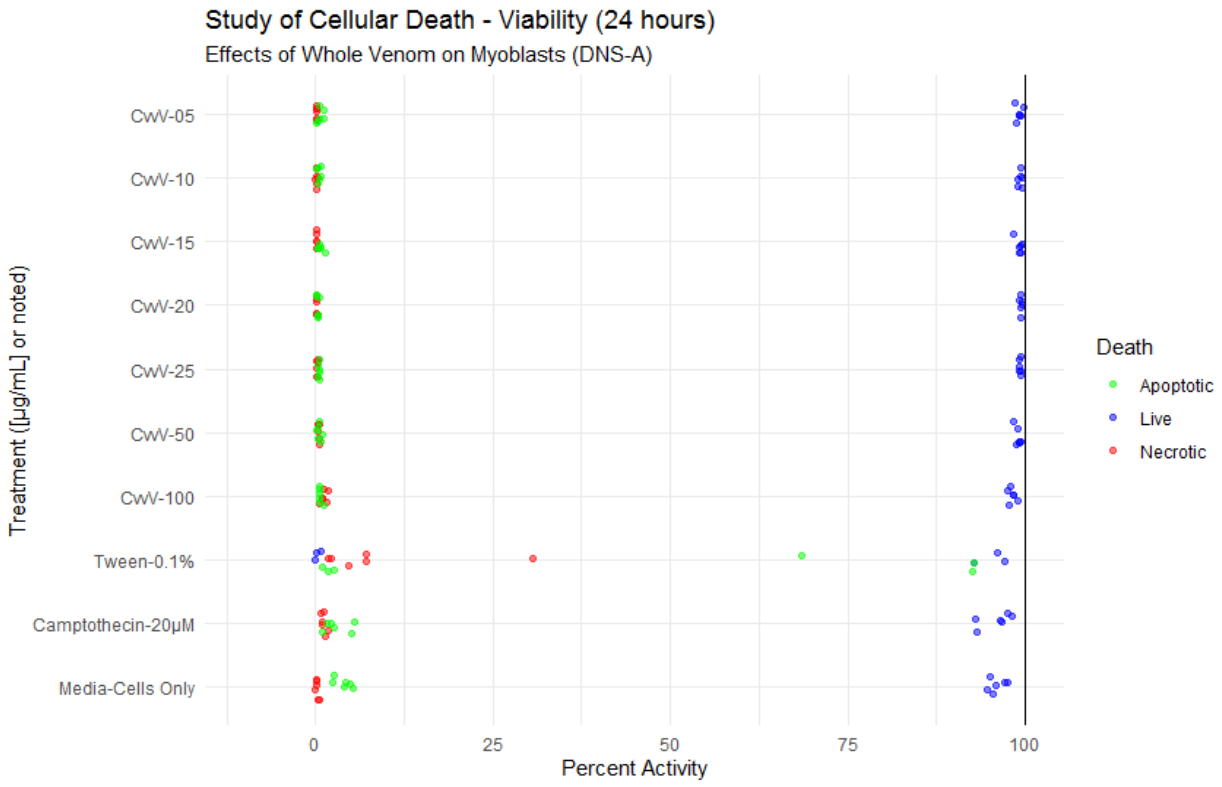


Figure 2: Whole Venom - HuSKM - Myoblasts - Cell Death - Viability

CvvV = Crotalus viridus viridus whole venom

The graph in Figure 3 examines the cell count of the previous DNS-A experiment with whole venom on myoblasts for 24 hours (see Figure 2). There were decreased cell counts for the Tween 20 and the 100 µg/mL whole venom. Observed here that there was some crystallization of the Tween 20. Moreover, the cells treated with venom detached and aggregated together (see Figure 20: D - H on both myoblast and myocyte panels). Camptothecin shows increased variability compared to the other ranges. There appeared to be some effect in terms of cell reproduction with venom concentrations above 50 µg/mL.

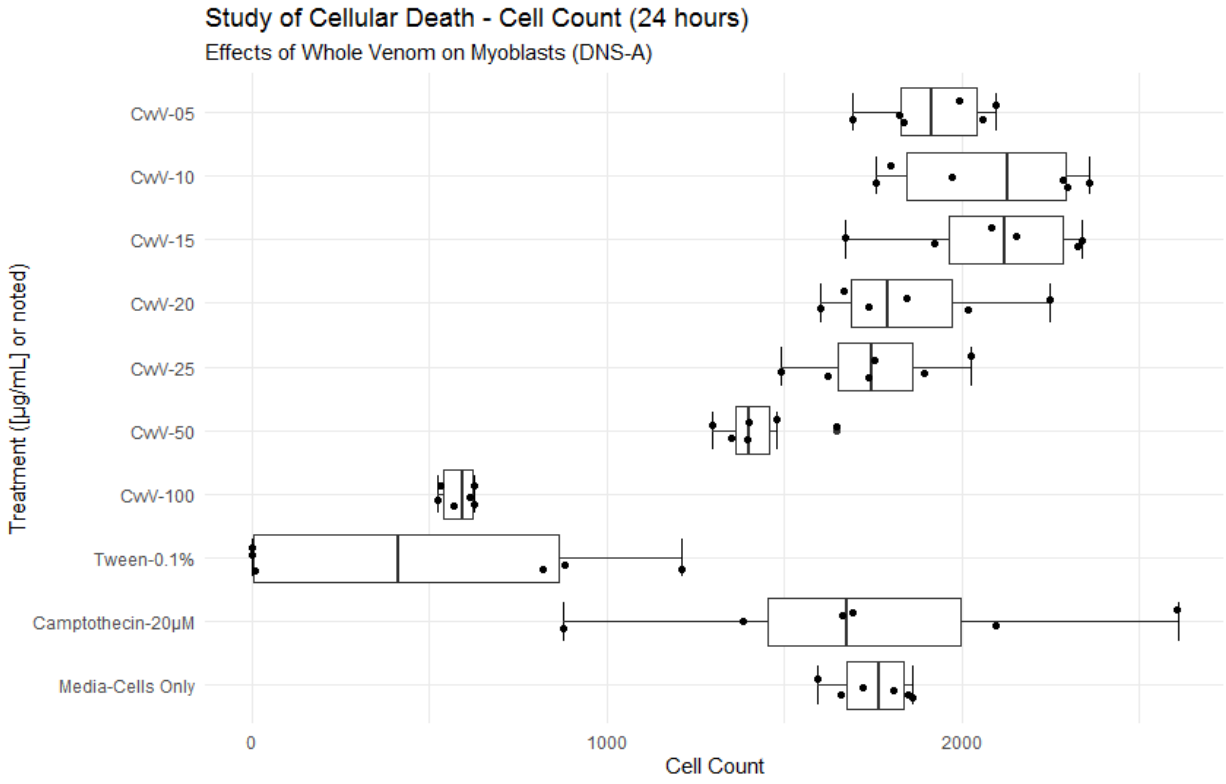


Figure 3: Whole Venom - HuSKM - Myoblasts - Cell Death - Cell Count

The DNS-A experiment (Figure 4) was a time extension of the previous experiment (Figures 2 & 3). At 48 and 96 hours there was no major decrease in the level of live cells with the exception of the positive control (where I expected to see more dead cells). The camptothecin control doesn't seem to promote apoptosis in this system. The whole venom doesn't seem to cause cell death, either. The Mean % Cell Viability values for the treatments (0 and 100 µg/mL) ranged from 92 – 98%. There was a statistical difference between the 96-hour whole venom treatment and the other treatments [$p < 0.01$], however, as noted, the difference between the means was about 6%. There was an interaction reported between Treatment and Time ($p < 0.01$). Tween 20 was used as a positive control for necrosis. Camptothecin was used as a positive control for apoptosis; the DMSO was a vehicle control for camptothecin.

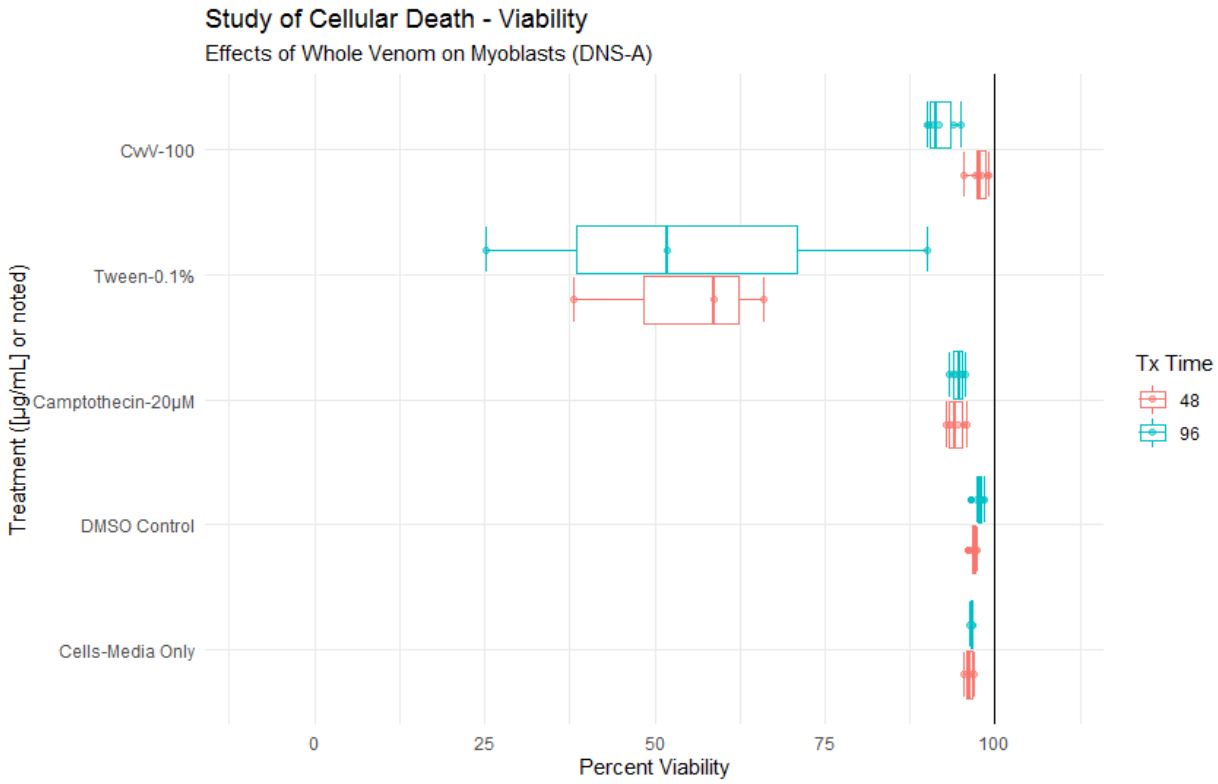


Figure 4: Whole Venom - HuSKM - Myoblasts - Cell Death - Viability

Figure 5 presents cell count data in the previous DNA-A myoblast experiment (see Figure 4 for the cell death viability). Between 48 and 96 hours there was a major decrease in the level of live cells in our Tween control. The camptothecin control also show slightly higher activity in the 96-hour sample wells. The whole venom did not show a decrease outside the range of the controls (as seen in Figures 2 & 3 for the 24 hour DNS-A study). There was a statistical difference in Mean % Cell Viability between treatments (0 and 100 µg/mL) [columns CvvV-100 and Cells-Media only; $p < 0.015$]. There were no significant interactions reported between Treatment and Time. Camptothecin and DMSO treatments are included in the graph for completeness.

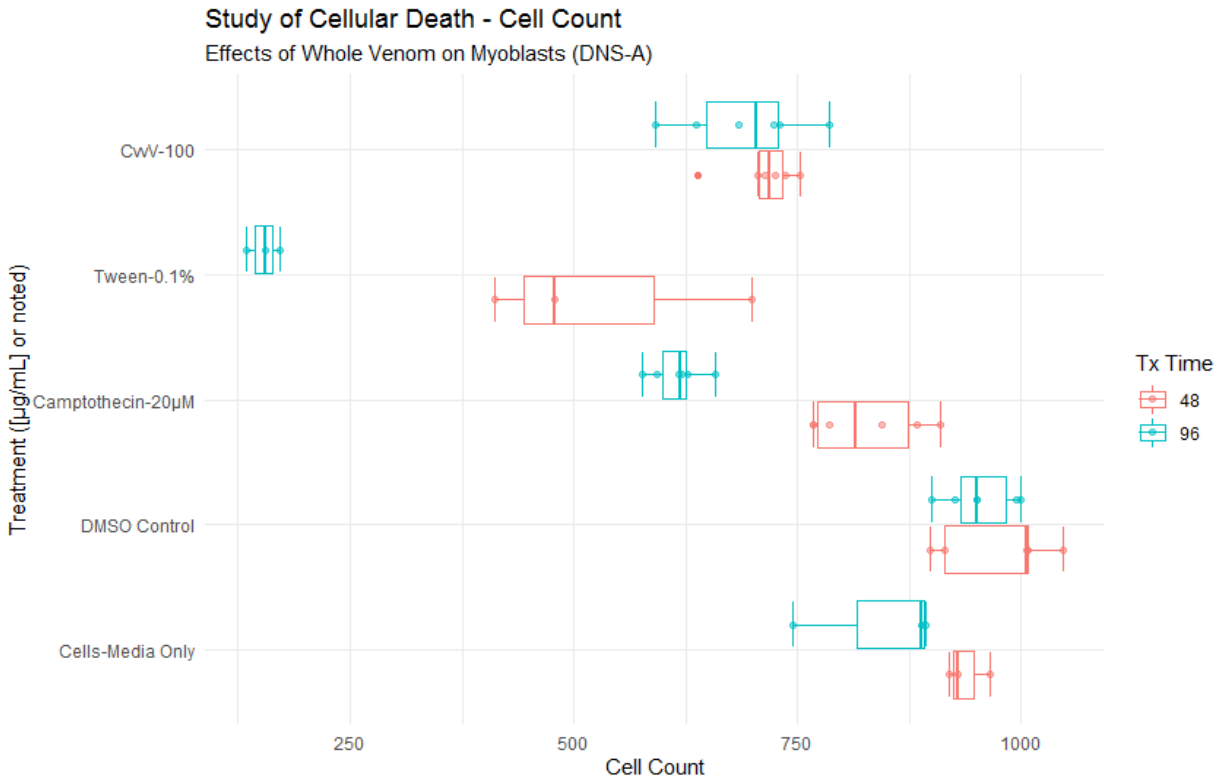


Figure 5: Whole Venom - HuSKM - Myoblasts - Cell Death - Cell Count

The results in Figure 6 depict an experiment to compare myoblasts and myocytes treated with the myotoxins and stained with propidium iodide (pI) and Hoechst. This treatment indicates no necrotic effect of the myotoxins on the myoblast cells. The myocytes show the same finding if you compare the level of the media control with all the toxin concentrations; they appear broadly equivalent. Some failure of the hydrogen peroxide positive control with the myocytes is notable. This experiment can be paired with the MTS1 study in Figure 8 which is of similar design.

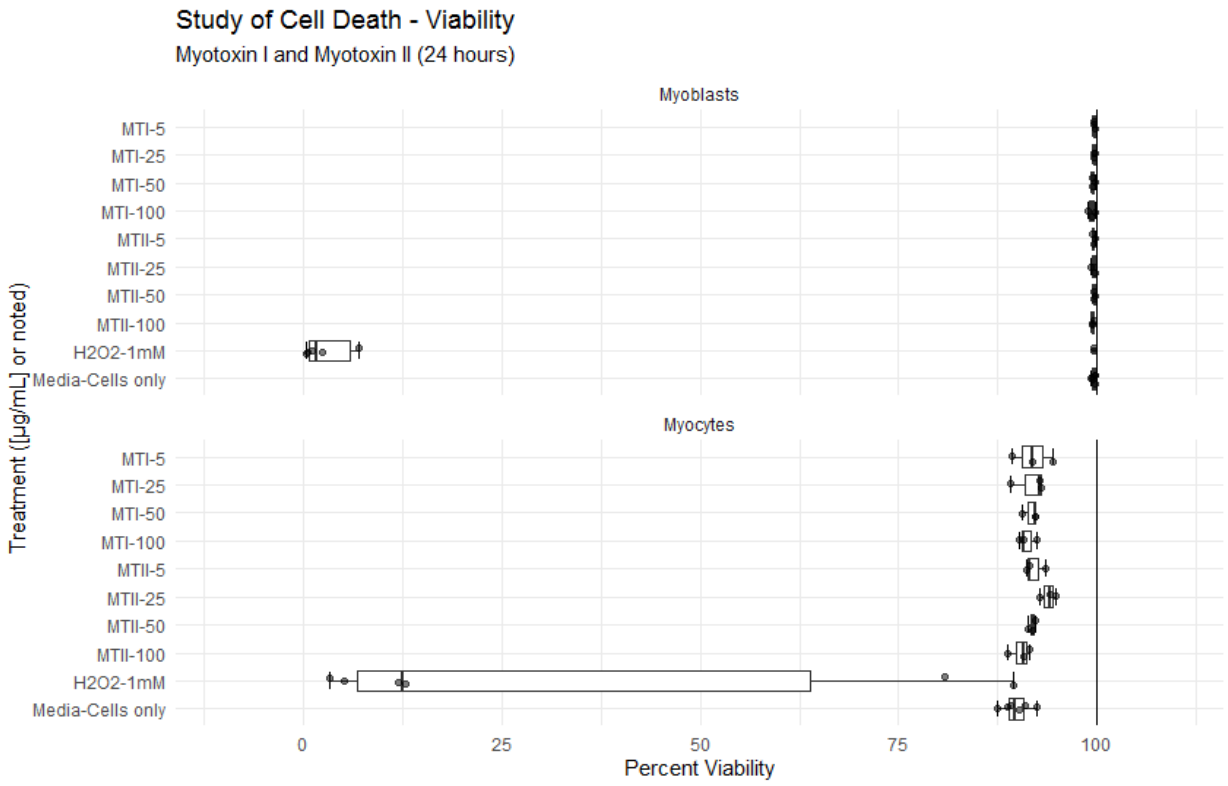


Figure 6: Myotoxins - HuSKM - Myoblasts/Myocytes - Cell Death - Viability

Additional cell lines were tested as possible candidates because they were readily available (Figure 7). HaCaT is a Human keratinocyte cell line; Hs27 is a Human foreskin fibroblast cell line; and Jurkat is an Acute T Cell Leukemia cell line. None of these additional cell lines appeared to show any sensitivity to the tested myotoxins at these concentrations. This line of inquiry was subsequently dropped. (Courtesy of C. Lema).

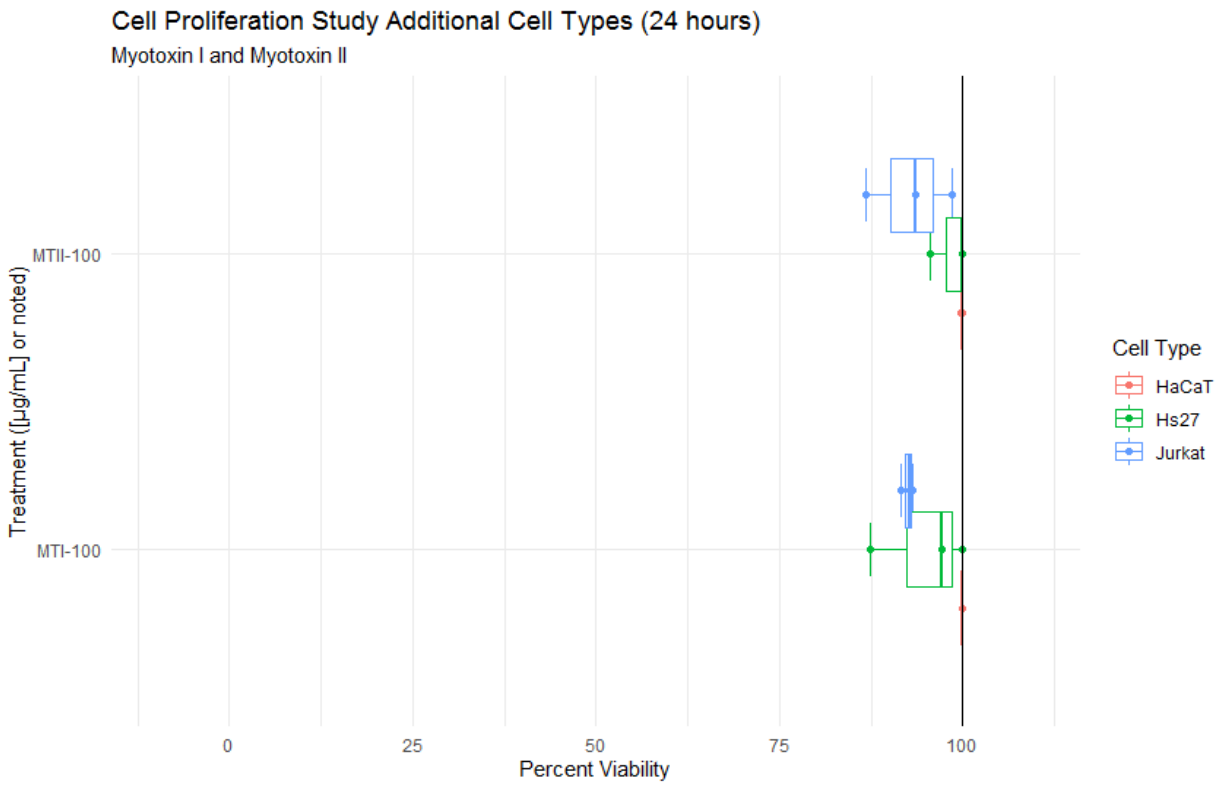


Figure 7: Myotoxins - Additional Cell Type - Cell Proliferation

The 24-hour experiment shown in Figure 8 can be paired the cell death experiment shown previously in Figure 6. From these data, it seems reasonable to continue to infer that myocyte cells are a little more sensitive to the myotoxins than the myoblasts. The same patterns in the positive control are seen here: more variability occurs with the myocytes. Additionally there was a slight trend showing a dose response to the myotoxins. This trend prompted a change in time to retest for 48 hours, and to increase concentrations. This was the first iteration of the MTS cell proliferation study (MTS1). Contamination on myocyte plate was noted.

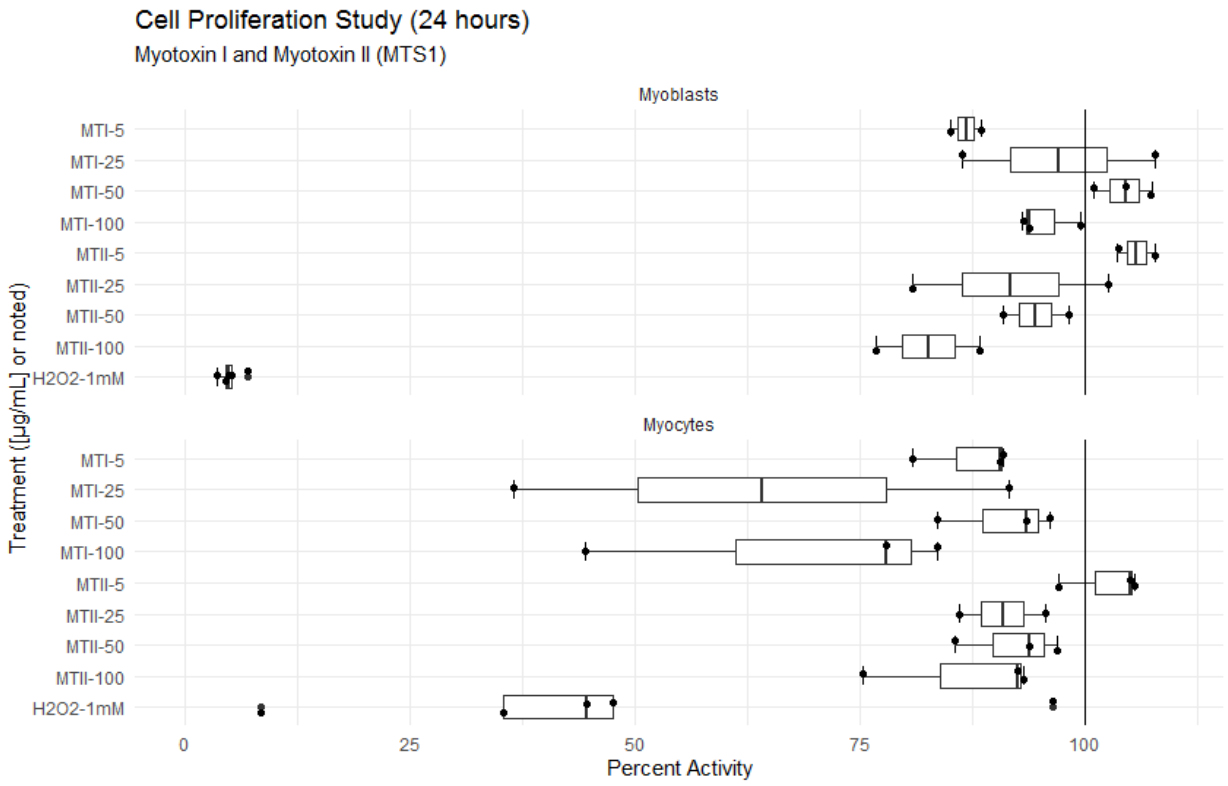


Figure 8: Myotoxins - HuSKM - Myoblasts/Myocytes - Cell Proliferation MTS1

Figure 9 plots the data from a 48-hour experiment that can be paired with Figure 8, a cell proliferation experiment shown previously. This 48-hour test (50% - 100%) shows a decrease in the Percent Activity compared to the 24-hour test (75% - 100%). It is not as clear here that myocyte cells are more sensitive to the myotoxins than the myoblasts. The same pattern appears in the positive control here, as noted previously, i.e., it is more variable with the myocytes. The myoblast data here represents two experimental plates combined together. 500 µg/mL dose of myotoxins gave us a near LC50.

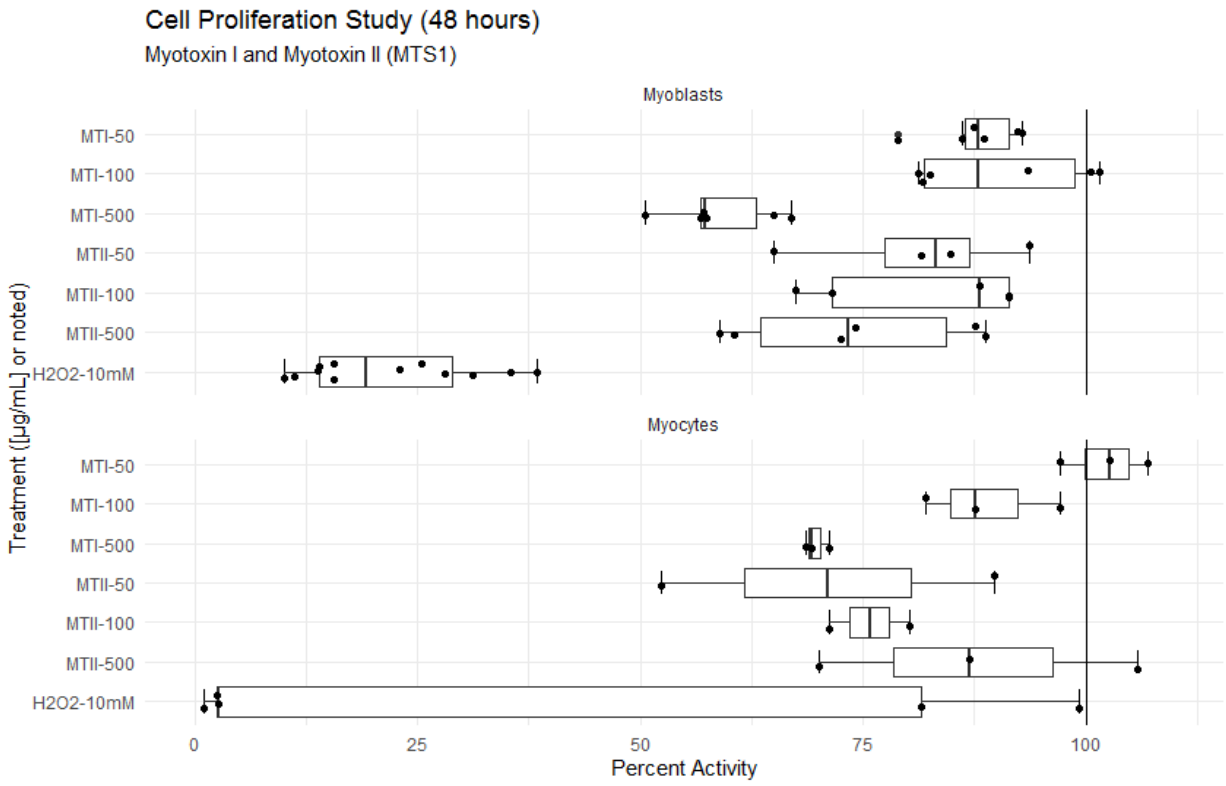


Figure 9: Myotoxins - HuSKM - Myoblasts/Myocytes - Cell Proliferation MTS1

In Figure 10 are the results of the experiment testing the effects of the two myotoxins at 48 and 72 hours in the concentration range of 100 µg/mL to 1000 µg/mL. It appeared that the time of incubation may have been a factor for decreased activity; however, comparison of equivalent concentrations with the findings in Figure 8 for 48 hours appear contradictory. The concentration of 500 µg/mL appeared to be optimal in this series. Here the positive control functioned as expected; it showed low cell activity. Due to unavailability of further myotoxins from SIGMA and limited stock the MYS cell proliferation protocol was changed to MTS2. LC50 shows up at 72 hours at 1000 µg/mL (MTI at 50.6% and MTII at 49.0%, mean values), however, due to unavailability of further myotoxins from SIGMA and limited stock this could not be pursued further.

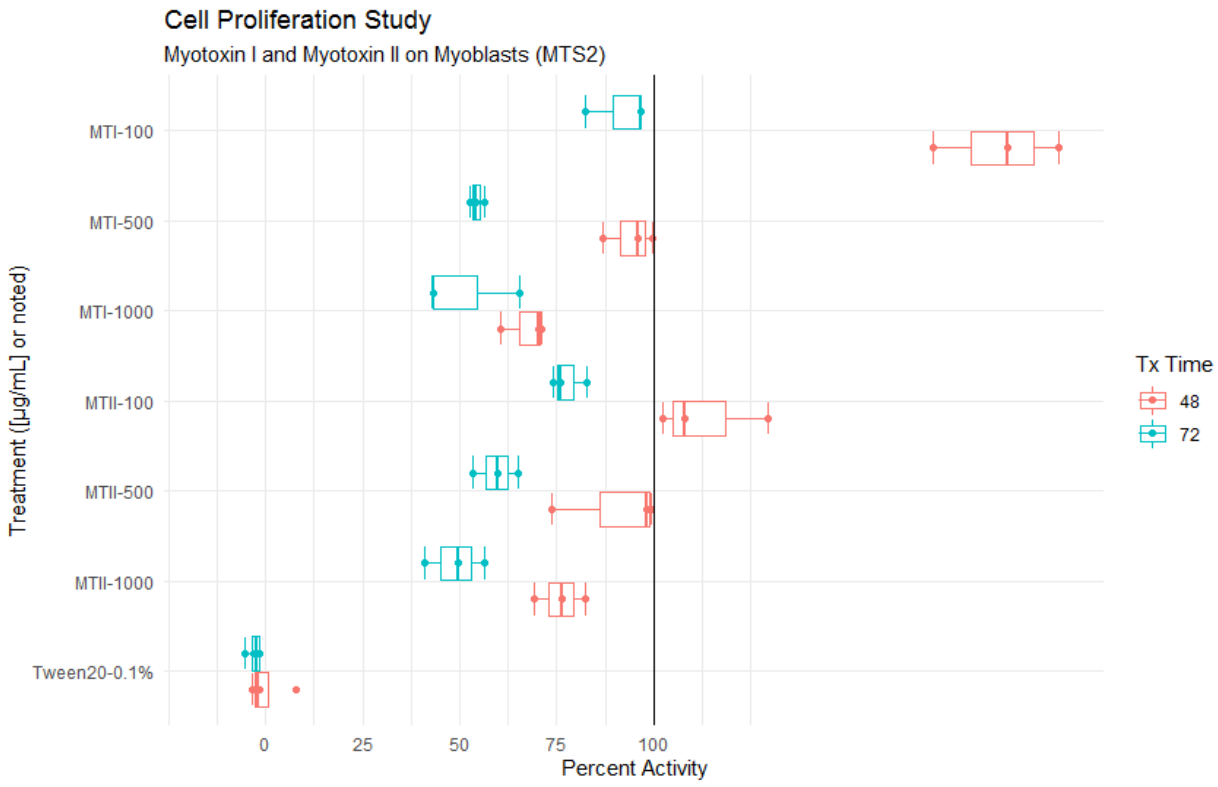


Figure 10: Myotoxins - HuSKM - Myoblasts/Myocytes - Cell Proliferation MTS2

Figure 11 shows the results of an experiment with whole venom. The effect of whole venom on myoblasts over time appears to show that whole venom has a greater decrease in activity over 24 hours than over 72 hours: a graded response over time for concentrations over 25 µg/mL. At 5 µg/mL of whole venom the response starts at about 85% for 24 hours, but longer exposure at 48 and 72 hours yields less activity (just below 60% for both). The effect size of this system is between 50% - 75%. The hydrogen peroxide control didn't function as expected in this system, as it completely failed to inactivate the cells.

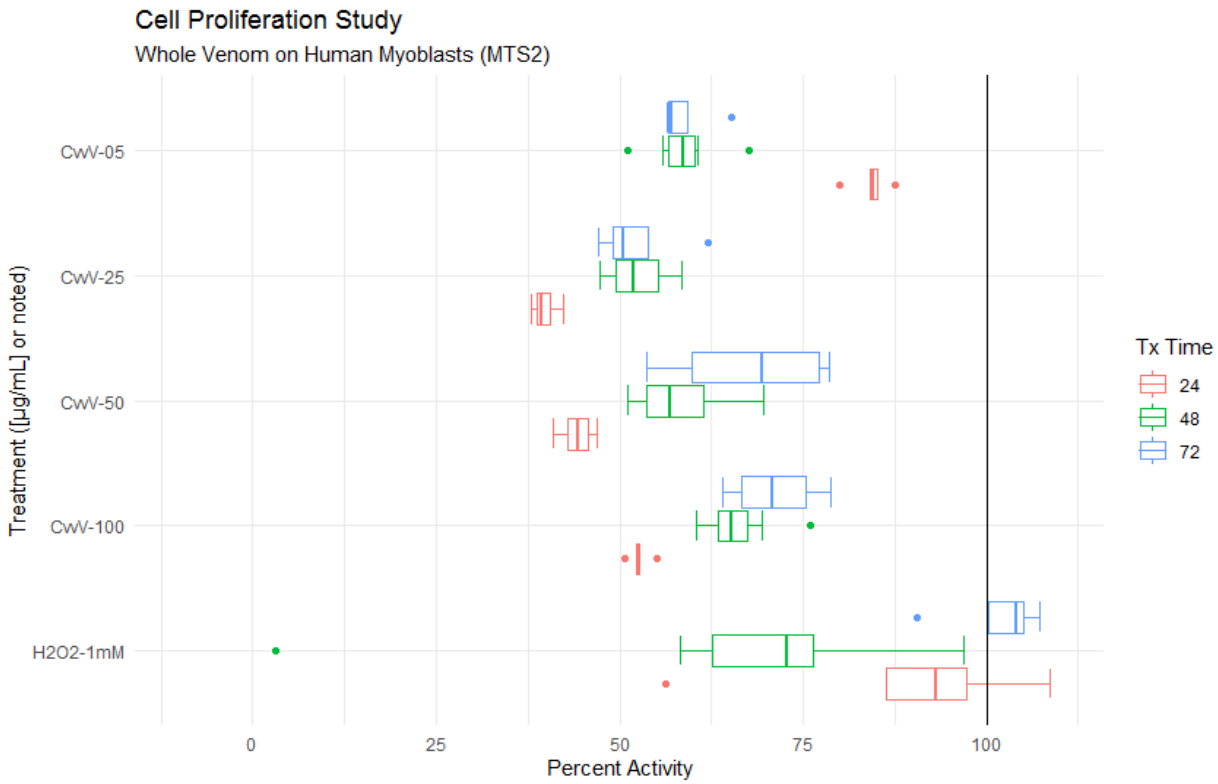


Figure 11: Whole Venom - HuSKM - Myoblasts - Cell Proliferation MTS2

The preceeding graphs of experimental result laid the groundwork for tests that underly the purpose of the research, to examine extracts from the *Manfreda* plant for their effects on snake envenomation. Figure 12 shows the experimental effects of *Manfreda maculosa* plant extract on human myoblasts over 48 hours. The dose response for the whole venom appears to follow the dose range in the literature (i.e., 5 – 100 µg/mL). Microscopic examination showed that the cells were lysed above 100 µg/mL. This set our concentration level for Manfreda extract treatment at 100 µg/mL.

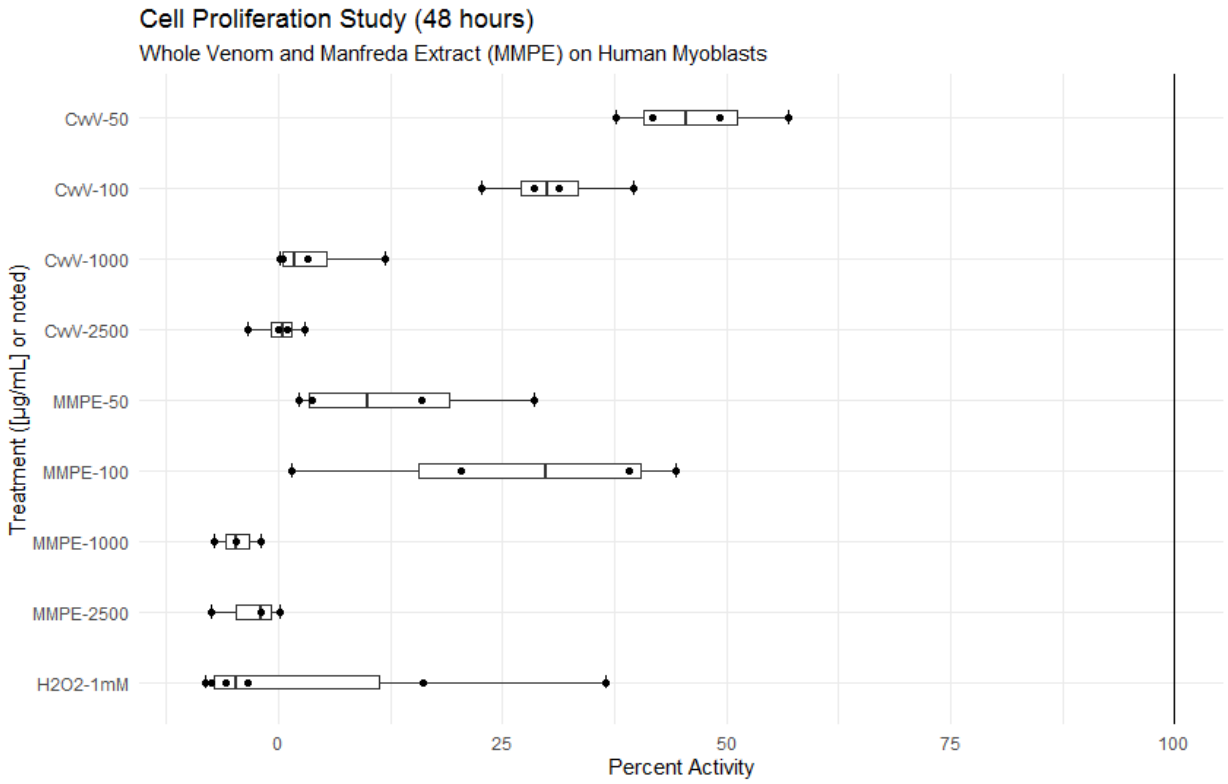


Figure 12: CvvV and MMPE - HuSKM - Myoblasts - Cell Proliferation MTS1

MMPE = *Manfreda maculosa* plant extract, this is the first extract.

In Figure 13 are the results of an experiment replicating whole venom and the *Manfreda* extract effects on myoblasts for 48 and 72 hours. The whole venom effect size is showing activity in the range of 50% - 75%. The effect size of plant extract lies in the range of 60% - 100%. The positive control concentration was increased in this experiment and still shows wide variability and didn't work consistently. The dose response for whole venom noted previously (see Figure 13) has disappeared.

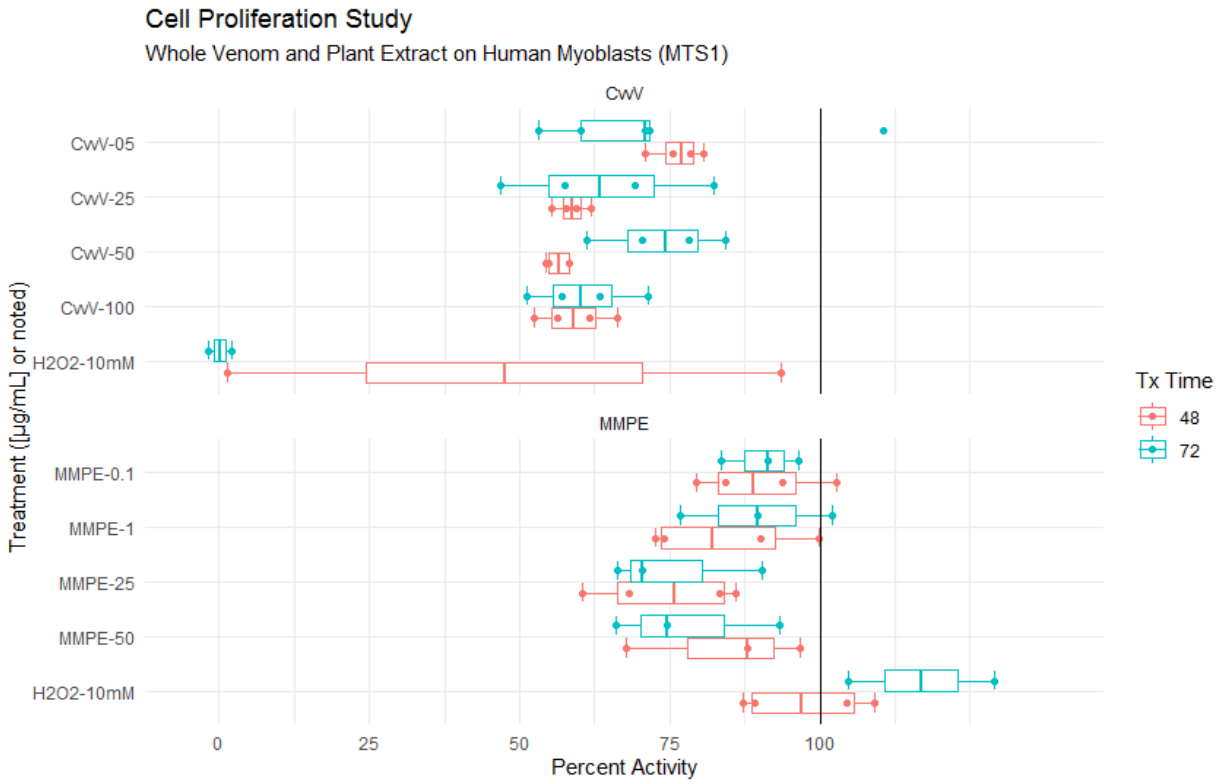


Figure 13: CvvV and MMPE - HuSKM - Myoblasts - Cell Proliferation MTS1

This experiment (Figure 14) looked at the effects of whole venom and plant extract combined in myoblasts. The treatment line shows the whole venom concentration, then "ra" (removed venom added media and plant extract to appropriate volume), or "a" (added plant extract), followed by the time that elapsed before the plant extract was added (at 1 hr or 3 hr), then the incubation continued on to 48 hours whereupon the activity was measured. PEII was

dosed at 100 µg/mL. The concentration groupings from 5 µg/mL to 100 µg/mL appeared generally (with two exceptions one at 25 µg/mL and another at 50 µg/mL that were outliers to this observation) at the same levels, especially at the 100 µg/mL group. The approximate equivalent levels indicate that the plant extract had no effect. The pattern changes above 500 µg/mL where it looks like when the whole venom remains with the plant extract the activity levels are higher but in these grouping the whole venom with no plant extract matched with the higher activity levels. When the venom remains with the cells, plant extract or not, the cells have a higher activity.

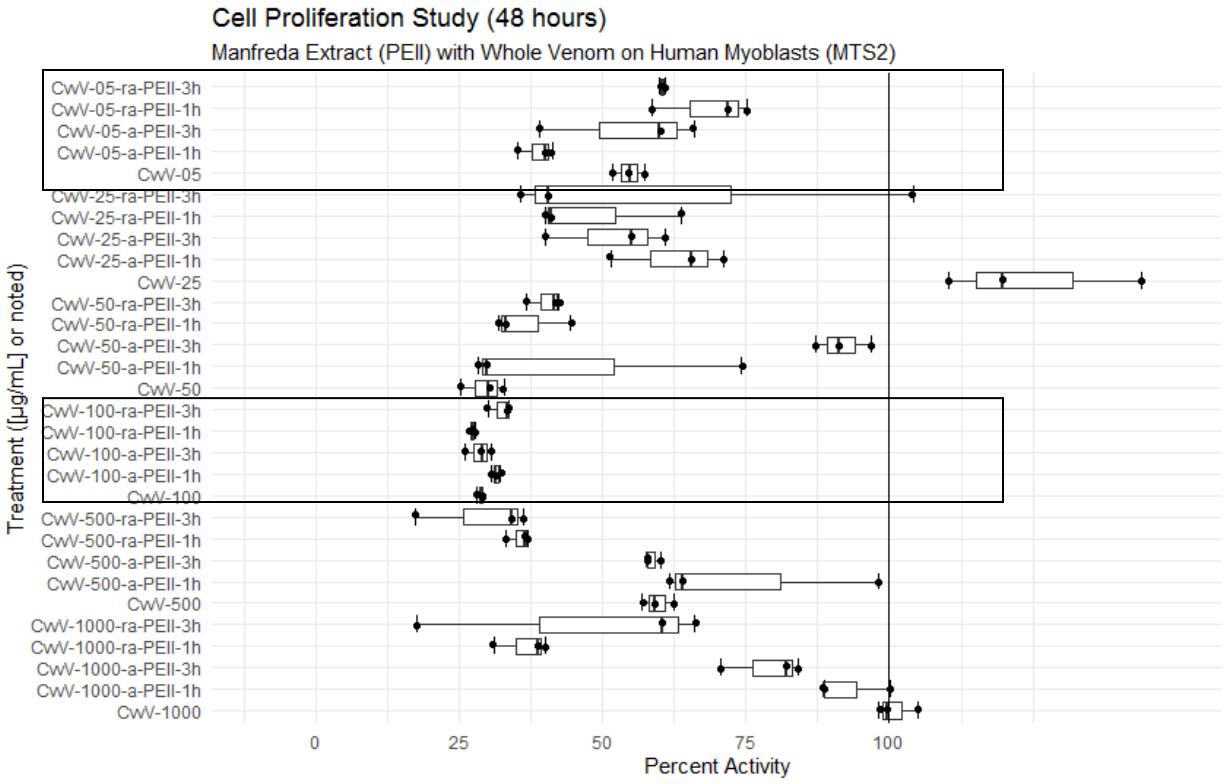


Figure 14: CvvV with PEII - HuSKM - Myoblasts - Cell Proliferation MTS2

Two groups have been outlined on the Figure 14 graph: the 5 µg/mL and the 100 µg/mL groups. If the plant extract had on effect, I expected that the venom only sample would have lower activity than the samples treated with the plant extract. PEII = Plant Extract II

The experiment displayed in Figure 15 looked at the effects of whole venom and plant extract combined in myocytes. As in Figure 14, the treatment line shows the whole venom concentration, then “ra” (removed venom added media and plant extract to appropriate volume), or “a” (added plant extract) then the time that elapsed before the plant extract was added (at 1 hr or 3 hr), then the incubation continued on to 48 hours when the activity was measured. PEII was dosed at 100 µg/mL. The concentration groupings appear generally at the same levels. There is still variability in one to two observations in some groups. The approximate equivalent levels within groupings indicate that the plant extract had no effect. The pattern changes at 100 µg/mL where, when the whole venom is not removed, the activity levels are higher. However, the whole venom with no plant extract matched with the higher activity levels, suggesting that it may be an artifact. When the venom remains with the cells, plant extract or not, the cells have a higher activity.

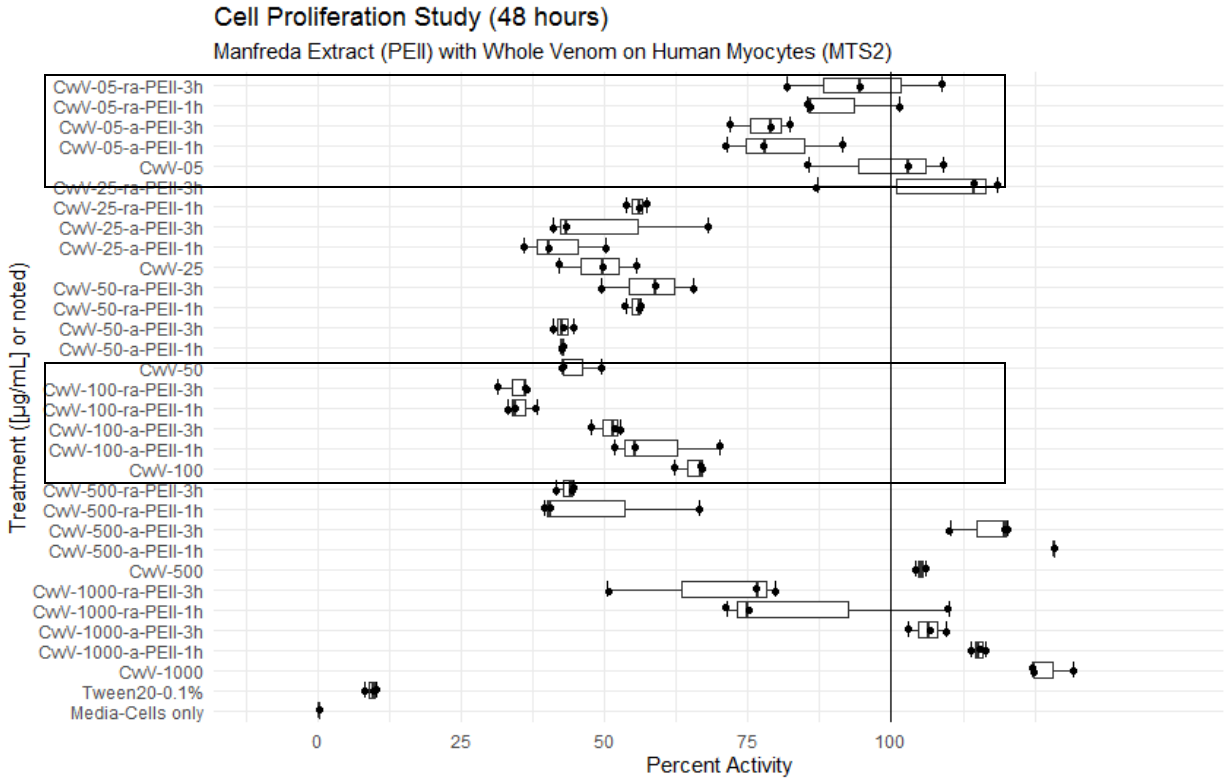


Figure 15: CvvV with PEII - HuSKM - Myocytes - Cell Proliferation MTS2

The same two groups have been outlined on the Figure 15 graph: the 5 µg/mL and the 100 µg/mL groups. If the plant extract had on affect, I would have expected that the venom only sample would have lower activity than the samples treated with the plant extract, instead in this figure (15) in the 100 µg/mL group some of the plant extract tests showed lower activity.

This experiment (Figure 16) involved a 1-hour pre-treatment incubation with plant extract II. Adjacent measures show that there appeared to be no effect of the plant extract on the whole venom. PEII by itself did not decrease activity. The Tween 20 positive control worked in this experiment, but the camptothecin treatment didn't give consistent results. The DMSO negative control produced the expected results in that it didn't decrease activity.

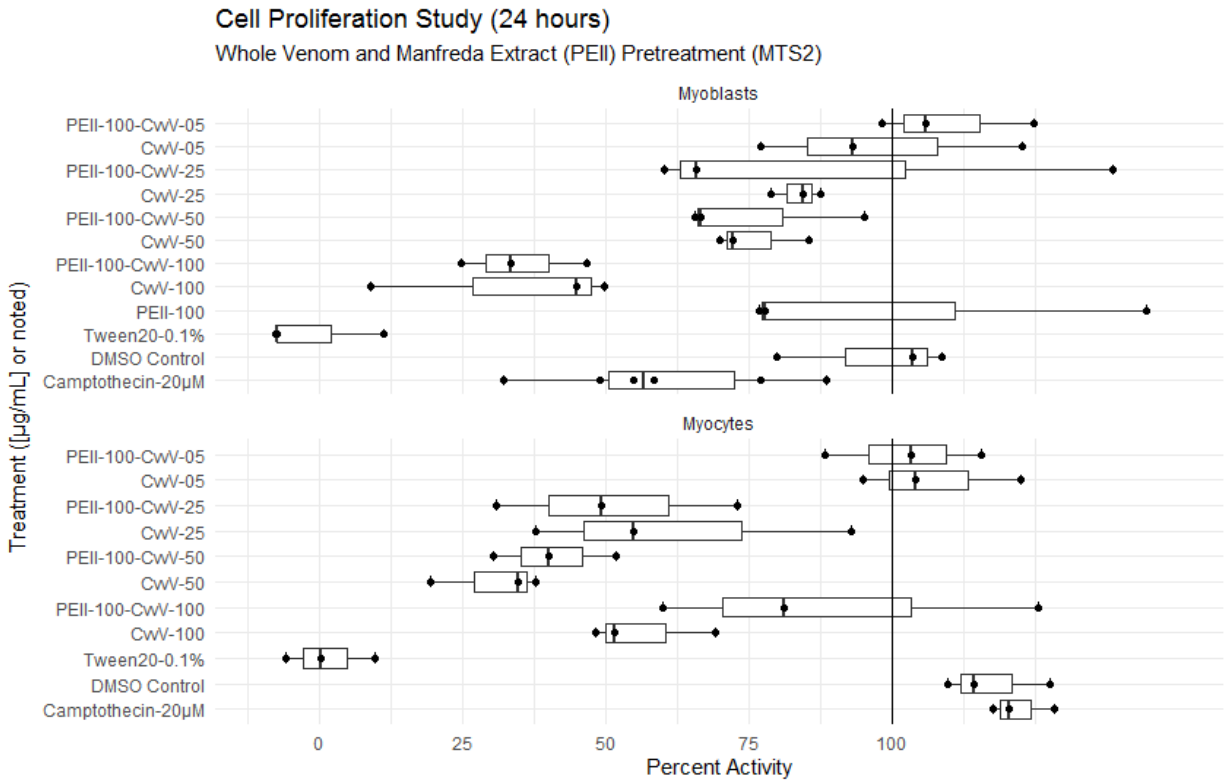


Figure 16: CvvV with PEII - HuSKM - Myoblasts/Myocytes - Cell Proliferation MTS2

Figure 17 shows the results of a repeat experiment to examine the effects of whole venom human skeletal muscle cells. There is not a linear dose response to activity levels but there does appear to be some indirectly proportional relationship between time and activity. Myocytes appeared to be more sensitive to the toxin than the myoblasts. Tween 20 was a good positive control. Hydrogen peroxide, again, was not a successful positive control, and camptothecin and etoposide did not induce high levels of apoptosis in these cells.

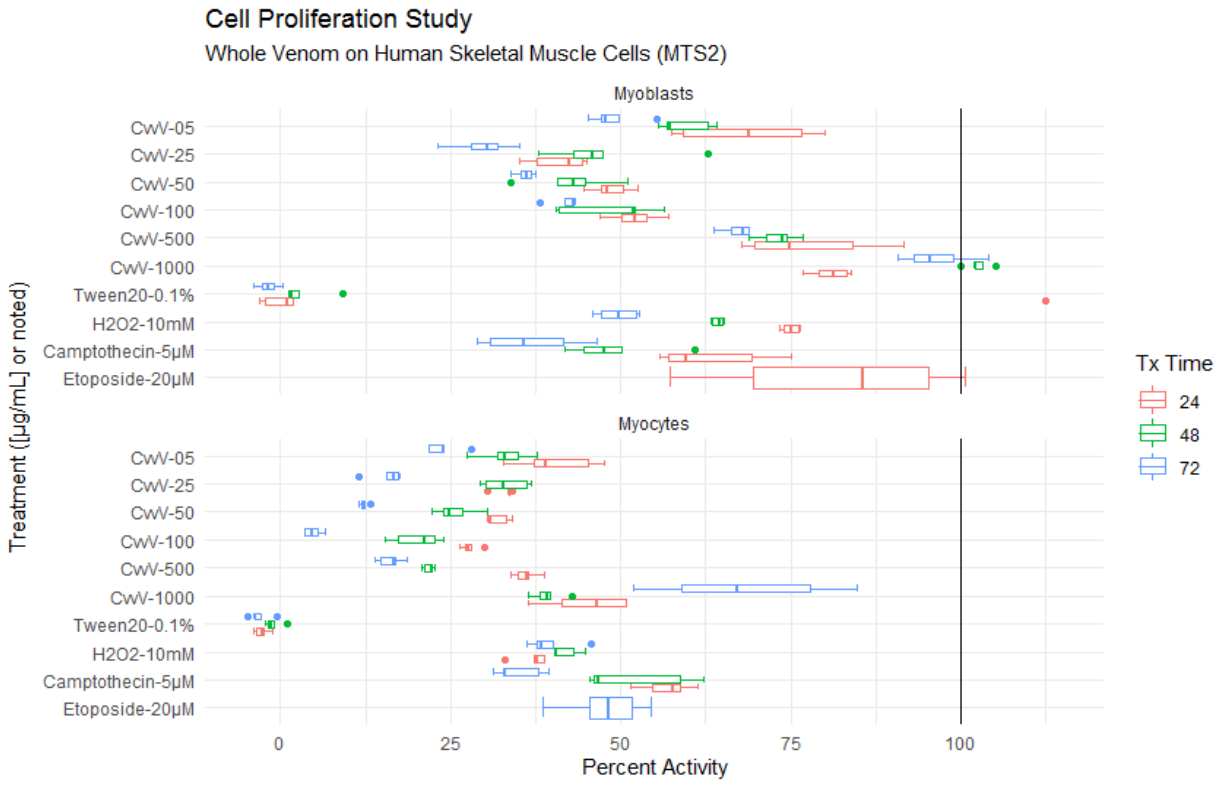


Figure 17: CvvV - HuSKM - Myoblasts/Myocytes - Cell Proliferation MTS2

Figure 18 is a repeat experiment examining the effects of plant extract II on human skeletal muscle cells. There is decreased activity of cells when plant extract II is at levels above 100 µg/mL. However, it did not appear to have a dose response or time dependent relationship to activity levels. Tween 20 was a good positive control. Hydrogen peroxide was not a successful control, and camptothecin and etoposide do not induce high levels of apoptosis in these cells.

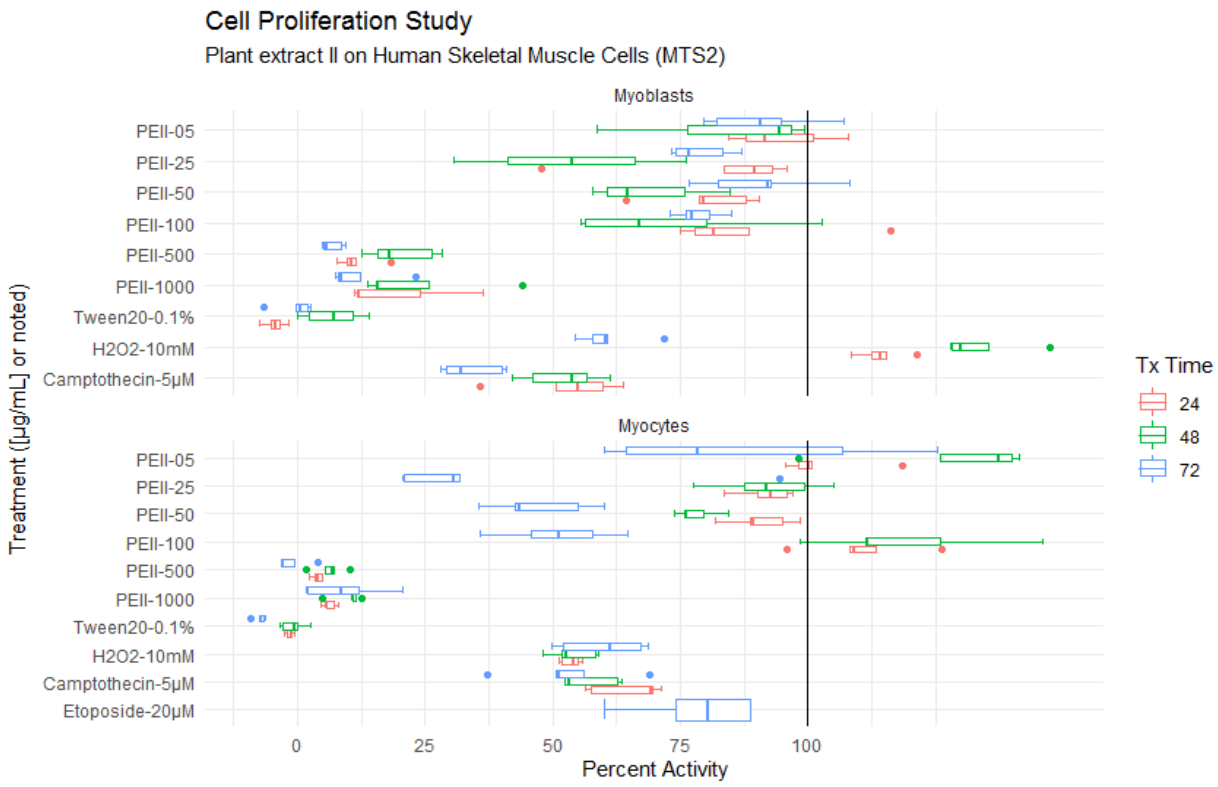


Figure 18: PEII - HuSKM - Myoblasts/Myocytes - Cell Proliferation MTS2

Figure 19 represents a non-specific background experiment that served as a check of background levels caused by the reagents in the bioassay system used for the MTS assay in these experiments. These were incubated without cells to examine the effects of the CvvV and PEII alone on the MTS methodology outcomes. No levels were above 50%. Background values with the whole venom appear consistent with concentration and time and do not vary overmuch. The values of background with the plant extract II appear to vary with concentration and time; they appear to decrease as length of incubation increases and are higher at lower concentrations.

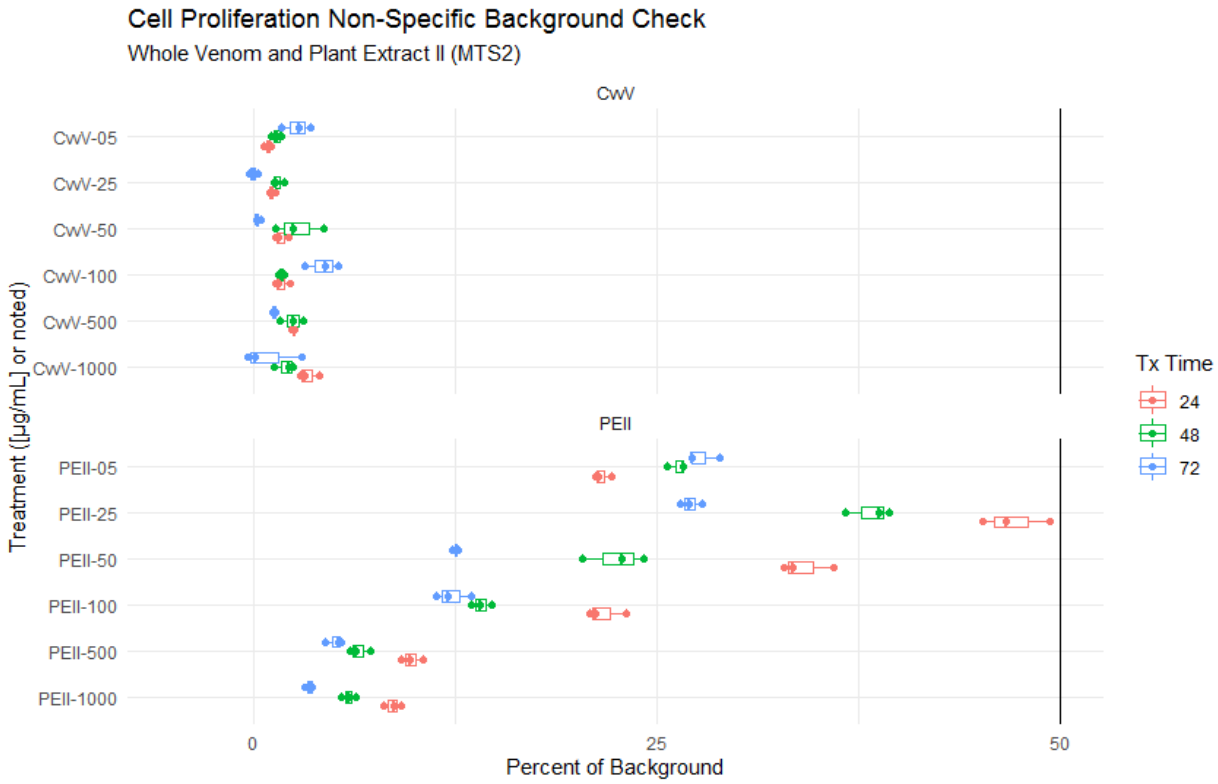
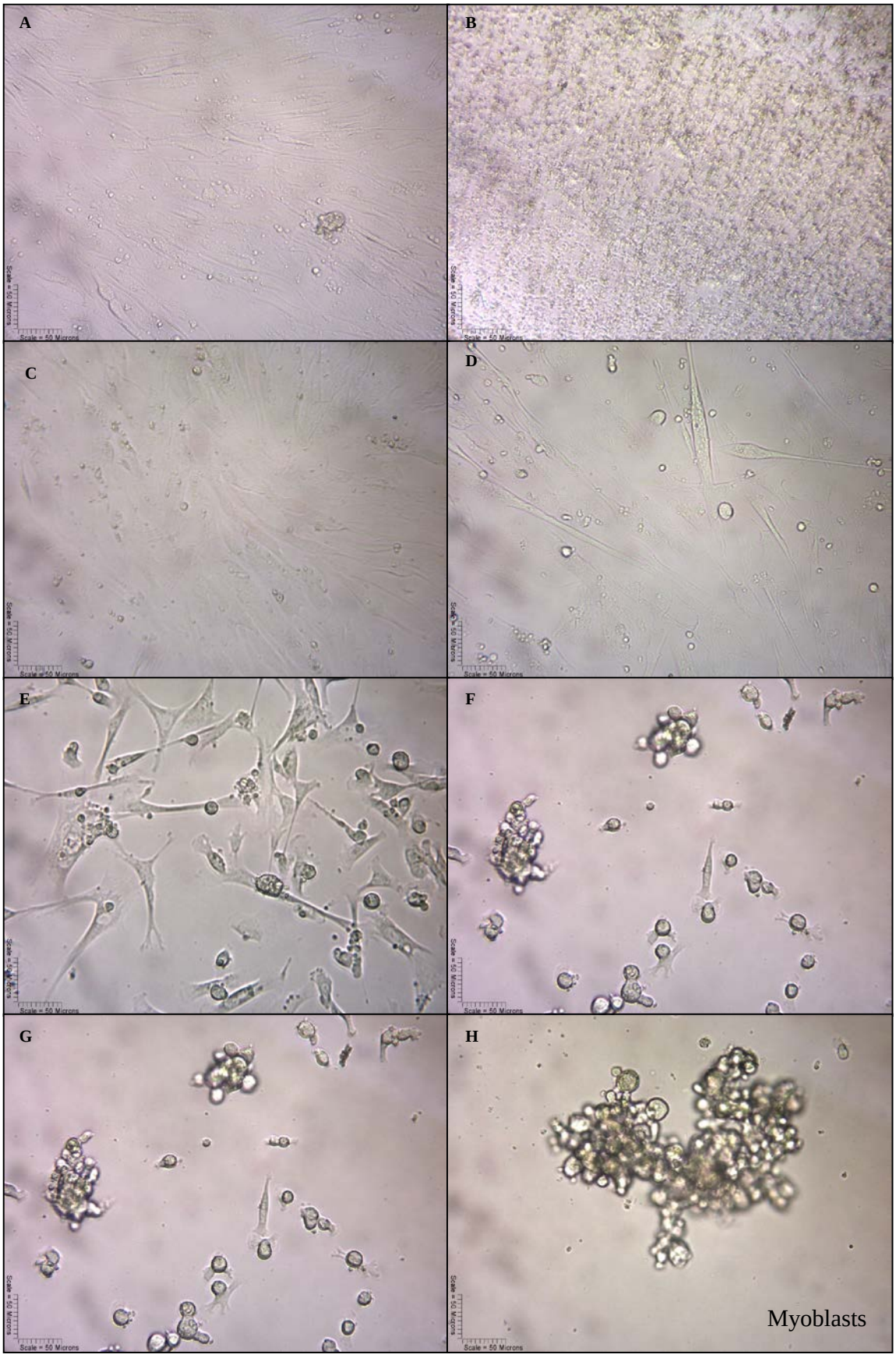


Figure 19: CvvV/PEII - Non-Specific Background - Cell Proliferation MTS2

Figure 20 is a light microscopy morphological comparison of HuSKM myoblasts and myocytes. Cell detachment and aggregation were seen at a slightly higher whole venom concentration in myoblasts (Figure 20F, 100 µg/mL) than myocytes (Figure 20D, 25 µg/mL). Some "crystallization" appeared with Tween 20 at 0.1% (Figure 20, Myocytes 20B) and this was a likely cause of cell count errors.


A
B
C
D
E
F
G
H
Myoblasts

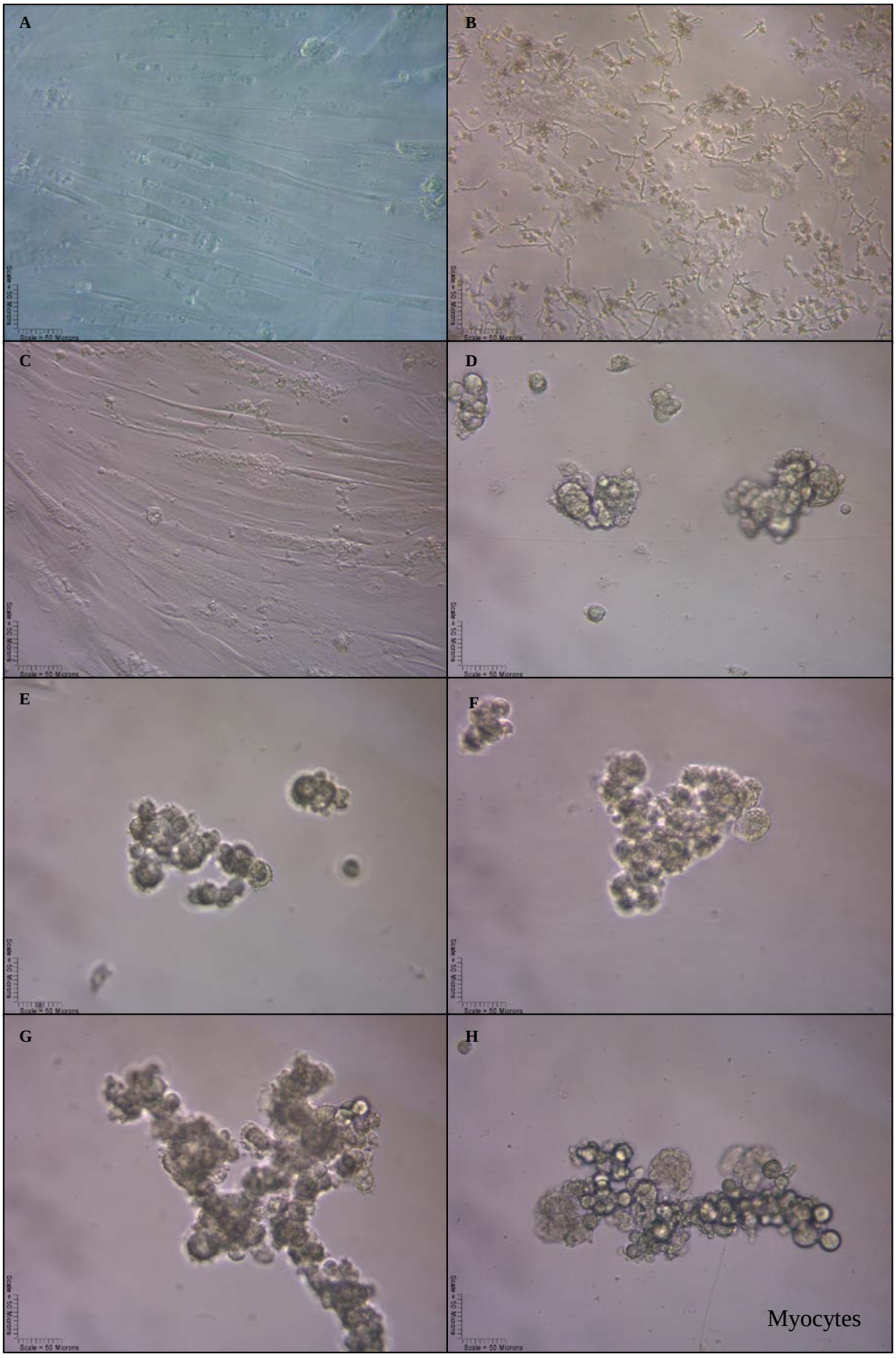

A
B
C
D
E
F
G
H
Myocytes

Figure 20. MTS Assay Morphology; Whole Venom (Cvv) for 48 h: Myoblasts and Myocytes.

Set up as two panels: Myoblasts and Myocytes.

A. No Treatment Control; **B.** Tween 20 - 0.1% (Positive Control); **C.** 5 µg/mL CvvV; **D.** 25 µg/mL CvvV; **E.** 50 µg/mL CvvV; **F.** 100 µg/mL CvvV; **G.** 500 µg/mL CvvV; **H.** 1000 µg/mL CvvV.

The micrograph in Figure 21 is from the DNS-A experiment shown in the graph in Figure 4. Myoblasts and myocytes are adherent to the surface and treatment with venom detaches and aggregates the cells (Figure 20); although apparently they still remain viable as indicated by not being permeable to Propidium Iodide (pI). Viable cells are shown in blue (Hoechst) while pI-permeable necrotic cells are shown in magenta. Image capture and analysis were performed with the BD Pathway Bioimager and Attovision v.1.6.2 software.

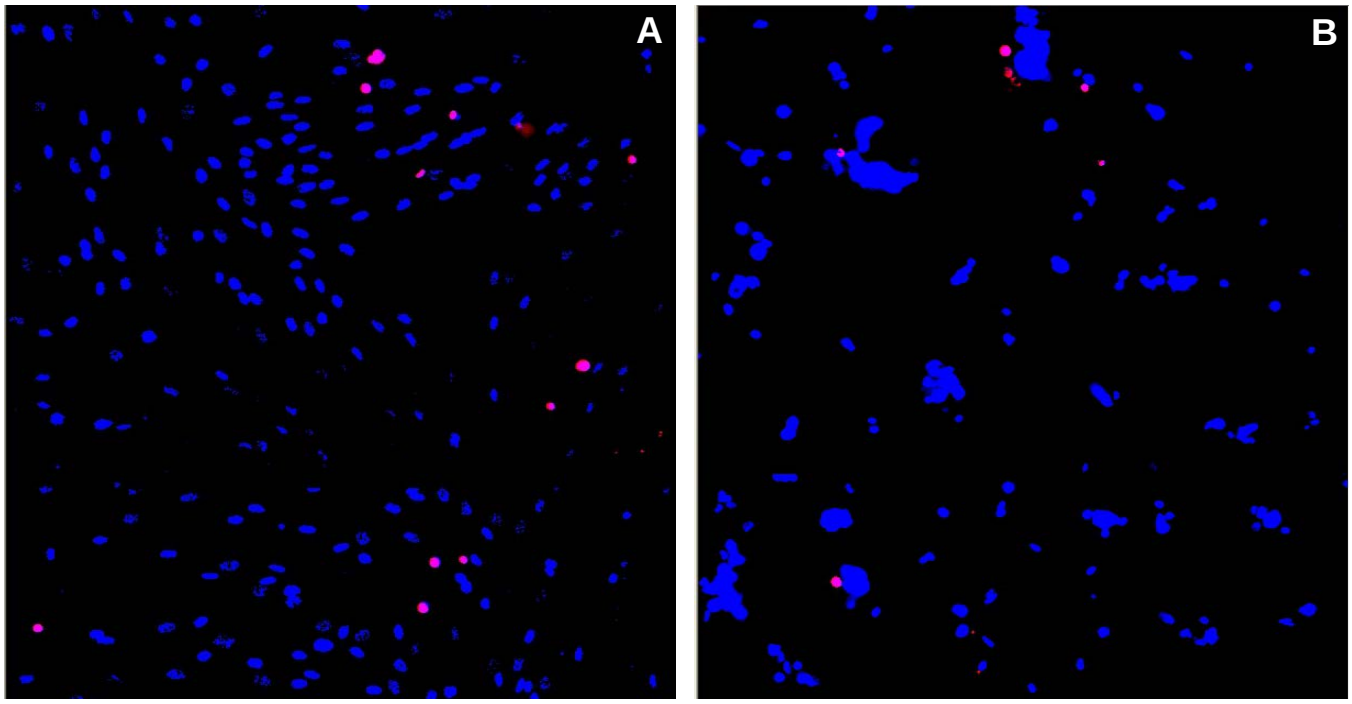


Figure 21. DNS-A Myoblasts with 48-hour treatment of whole venom.

A. Untreated control. **B.** Whole Venom at 100 µg/mL.

Discussion

Problems with the positive controls continued and were not completely resolved throughout this study. The myocytes appeared to be more resistant to the hydrogen peroxide that the myoblasts. Since the myocytes are multinucleated and much larger than the myoblasts, it may be that their different metabolisms are the source of this difference. The problem of resistance was also seen with the Tween 20, but the crystallization formed prevented employing higher concentrations of Tween.

Another feature of the MTS testing was that it presents several values for cell activity above 100%. This finding is problematic in two ways: 1), it is not possible that there could be values above 100% theoretically and 2), the baseline is set with an average of the media blanks included in the testing. Increasing the number of blanks did not improve the problem. These values remain un-interpretable in terms of the results and made the combining of results across the experiments questionable.

The results of this in vitro study showed that venom-induced necrosis of human skeletal myoblasts and myocytes (see Figures 2-6, 21) was limited. The clinical picture of snakebite shows that myonecrosis is a local symptom of envenomation. The results seen here of no cell death are different from what is seen in the clinical picture. These results indicate that the mechanism of whole venom myotoxicity may be more complicated than originally appreciated, especially since cellular necrosis was not observed. It may be that the cell death seen at the tissue level *in vivo* is an emergent property that doesn't exist at the *in vitro* cell level studied here. Another possible explanation is that there is binding occurring with the serum proteins that are in the cell media and that these proteins are inactivating venom components. In the case of the myotoxins, higher concentrations need to be checked since there were some dosage issues noted and the myotoxins became unavailable. In the initial study in Figure 6, the concentration may have been too low to elicit a cell death response. More experiments are needed to elucidate the

mechanisms underlying the myotoxic effects that occur during snakebite envenomation. Myoblasts exposed to whole venom up to 96 hours showed negligible cytotoxic effect as assessed by the DNS assay at 100 µg/mL of CvvV concentration. Cell aggregation associated with venom treatment and Tween 20 background crystal formation, were issues that may be responsible for cell count differences seen (Figures 3 & 5) and for results seen in the DNS assay (Figures 1 - 6). Further experimentation is required to resolve these issues.

The HuSKM myoblasts and myocytes were not as resistant to venom treatment as measured by the loss of cell membrane permeability in the MTS experiments. In fact, this bioassay showed that differences due to envenomation between venom-treated cells and non-treated cells could be measured. Nevertheless, there is not any clear picture of a dose-response in cell activity observed using the MTS assay over any time period in either myoblasts or myocytes.

Information derived from the whole venom treated cells with plant extract in Figures 14 - 16 do not show any patterns in the data visualization suggestive of interactions indicative of extract effects that actually mitigate the affects of whole snake venom. A much larger set of repeat experiments would be needed to have the power to resolve and quantify the small effect size, if any, that exists in a treatment group defined by the venom concentration.

In summary, this study found no necrosis or apoptosis occurring in venom-treated HuSKM myoblasts and myocytes (Figures 2 - 5), or necrosis with the myotoxins in the same cells (Figure 6) using the methods employed. Previous research had not used HuSKM cells or the MTS cell proliferation assay within the setting of venom toxicity studies. The further use of these technologies may prove useful in elucidating venom toxicity.

References

Aguilera RJ, Montoya J, Primm TP, and Varela-Ramirez A. 2006. Green Fluorescent Protein as a Biosensor for Toxic Compounds. *Reviews in Fluorescence* 3: 463-476.

Alonso-Castro AJ, Villarreal ML, Salazar-Olivo LA, Gomez-Sanchez M, Dominguez F, and Garcia-Carranca A. 2011. Mexican medicinal plants used for cancer treatment: Pharmacological, phytochemical and ethnobotanical studies. *Journal of Ethnopharmacology* 133 (3): 945-972.

Austin DF, and Honychurch PN. 2004. *Florida Ethnobotany*.: CRC Press, Boca Raton, FL.

Biondo R, Pereira AM, Marcussi S, Pereira PS, França SC, and Soares AM. 2003. Inhibition of enzymatic and pharmacological activities of some snake venoms and toxins by *Mandevilla velutina* (Apocynaceae) aqueous extract. *Biochimie* 85 (10): 1017-1025.

Blackman JR, and Dillon S. 1992. Venomous snakebite: past, present, and future treatment options. *Journal of the American Board of Family Practice* 5 (4): 399-405.

Camey KU, Velarde DT, and Sanchez EF. 2002. Pharmacological characterization and neutralization of the venoms used in the production of Bothropic antivenom in Brazil. *Toxicon* 40 (5): 501-509.

Campbell TN. 1951. Medicinal Plants Used by Choctaw, Chickasaw, and Creek Indians in the Early Nineteenth Century. *Journal of the Washington Academy of Sciences* 41 (9): 285-290.

Castro KNC, Carvalho ALO, Almeida AP, Oliveira DB, Borba HR, Costa SS, and Zingali RB. 2003. Preliminary in vitro studies on the *Marsypianthes chamaedrys* (boia-caá) extracts at fibrinoclotting induced by snake venoms. *Toxicon* 41 (7): 929-932.

Castro O, Gutiérrez JM, and Barrios M. 1999. Neutralización del efecto hemorrágico inducido por veneno de *Bothrops asper* (Serpentes: Viperidae) por extractos de plantas tropicales. *Revista de Biología Tropical* 47 (3): 605-616.

CDC - NIOSH. 2018. "Venemous Snakes." 2019. Available from https://www.cdc.gov/niosh/topics/snakes/default.html.

Chippaux J-P. 2008. Estimating the Global Burden of Snakebite Can Help To Improve Management. *PLoS Medicine* 5 (11): 1-2.

Cox PA, and Balick MJ. 1994. The ethnobotanical approach to drug discovery. *Scientific American* 270 (6): 82-87.

Cruz CC. 2009. Systemática del Genéro *Manfreda* Salisb. (Agavaceae). D.Sc. Tesis, Colegio de Postgraduados, Montecillo, Mexico.

Engle CM, Becker RR, Bailey T, and Bieber AL. 1983. Characterization of Two Myotoxic Proteins from Venom of *Crotalus viridis concolor*. *Toxin Reviews* 2 (2): 267-283.

Galan JA, Sanchez EE, Rodriguez-Acosta A, and Perez JC. 2004. Neutralization of venoms from two Southern Pacific Rattlesnakes (*Crotalus helleri*) with commercial antivenoms and endothermic animal sera. *Toxicon* 43 (7): 791-799.

Gold BS, Barish RA, and Dart RC. 2004. North American snake envenomation: diagnosis, treatment, and management. *Emergency Medical Clinics of North America* 22 (2): 423-43, ix.

Gurib-Fakim A. 2006. Medicinal Plants: Traditions of Yesterday and Drugs of Tomorrow. *Molecular Aspects of Medicine* 27: 1-93.

Gutiérrez JM, Rojas G, Lomonte B, Gené JA, Chaves F, Alvarado J, and Rojas E. 1990. Standardization of assays for testing the neutralizing ability of antivenoms. *Toxicon* 28 (10): 1127-1129.

Gutiérrez JM, Theakston RDG, and Warrell DA. 2006. Confronting the Neglected Problem of Snake Bite Envenoming: The Need for a Global Partnership. *PLoS Medicine* 3 (6): 727-731.

Hamel PB, and Chiltoskey MU. 1975. *Cherokee plants and their uses: a 400 year history*.: Herald Pub. Co., Sylva, NC.

Harvey AL, editor. 1991. *Snake Toxins*. Pergamon Press, Inc., New York.

Heinrich M, Ankli A, Frei B, Weimann C, and Sticher O. 1998. Medicinal plants in Mexico: healers' consensus and cultural importance. *Social Science & Medicine* 47 (11): 1859-1871.

Hernández-Sandoval L, Orellana R, and Carnevali G. 2008. Two new species of Manfreda Salisb. (Agavaceae) from the Yucatán Peninsula, Mexico. *The Journal of the Torrey Botanical Society* 135 (2): 168-177.

Houghton PJ. 1994. Treatment of Snakebite with Plants. *Journal of Wilderness Medicine* 5 (4): 451-452.

Houghton PJ, and Osibogun IM. 1993. Flowering plants used against snakebite. *Journal of Ethnopharmacology* 39 (1): 1-29.

Johnson T. 1999. *CRC Ethnobotany Desk Reference*. CRC Press, Boca Raton, FL.

Juckett G, and Hancox JG. 2002. Venomous snakebites in the United States: management review and update. *American Family Physician* 65 (7): 1367-1374.

Kasturiratne A, Wickremasinghe AR, de Silva N, Gunawardena NK, Pathmeswaran A, Premaratna R, Savioli L, Lalloo DG, and de Silva HJ. 2008. The Global Burden of Snakebite: A Literature Analysis and Modelling Based on Regional Estimates of Envenoming and Deaths. *PLoS Medicine* 5 (11): 1-14.

Kurecki BA, III, and Brownlee HJ, Jr. 1987. Venomous snakebites in the United States. *Journal of Family Practice* 25 (4): 386-392.

Linnaeus C. 1753. *Species Plantarum*. Laurentius Salvius, Stockholm.

Lomonte B, Angulo Y, Rufini S, Cho W, Giglio JR, Ohno M, Daniele JJ, Geoghegan P, and Gutiérrez JM. 1999. Comparative study of the cytolytic activity of myotoxic phospholipases A2 on mouse endothelial (tEnd) and skeletal muscle (C2C12) cells in vitro. *Toxicon* 37 (1): 145-158.

Marker RE, and Lopez J. 1947. Steroidal Sapogenins. No. 171. Biogenesis of the Steroidal Sapogenins in Agaves, Manfreda and Hesperaloe. *Journal of the American Chemical Society* 69 (10): 2403-2404.

Markland FS. 1998. Snake venoms and the hemostatic system. *Toxicon* 36 (12): 1749-1800.

Martz W. 1992. Plants with a reputation against snakebite. *Toxicon* 30 (10): 1131-1142.

Moerman DE. 2008. *Native American Ethnobotany*.: Timber Press, Portland, OR.

O'Neil ME, Mack KA, Gilchrist J, and Wozniak EJ. 2007. Snakebite injuries treated in United States emergency departments, 2001-2004. *Wilderness Environmental Medicine* 18 (4): 281-287.

Ownby CL. 1998. Structure, Function and Biophysical Aspects of the Myotoxins from Snake Venoms. *Toxin Reviews* 17 (2): 213-238.

Ownby CL. 1990. Comments on letter to the editor--Standardization of assays for testing the neutralizing ability of antivenoms. *Toxicon* 28 (10): 1129-1130.

Ownby CL, Colberg TR, and Odell GV. 1985. Response to letter to the editor by Russell, Ruzic and Gonzalez. *Toxicon* 23 (2): 205-208.

Pereda-Miranda R. 1995. Bioactive Natural Products from Traditionally Used Mexican Plants. In *Phytochemistry of Medicinal Plants*, eds JT Arnason, R Mata and JT Romeo, 83-112. Plenum Press, New York.

R Core Team. 2019. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Rose JN. 1899. A Proposed Rearrangement of the Suborder Agaveae. *Contributions of the United States National Herbarium* 5: 151-157.

-----. 1903. Amarillidaceae. *Contributions of the United States National Herbarium* 8: 8-23.

Salisbury RA. 1866. *The Genera of Plants*. J. Van Voorst, London.

Serena E, Zatti S, Reghelin E, Pasut A, Cimetta E, and Elvassore N. 2010. Soft substrates drive optimal differentiation of human healthy and dystrophic myotubes. *Integrative Biology* 24(10): 193-201.

Shinners LH. 1966. Texas *Polianthes*, Including *Manfreda* (*Agave* Subgenus *Manfreda*) and *Runyonia* (Agavaceae). *SIDA* 2 (3): 333-338.

Soares AM, Ticli FK, Marcussi S, Lourenço MV, Januário AH, Sampaio SV, Giglio JR, Lomonte B, and Pereira PS. 2005. Medicinal plants with inhibitory properties against snake venoms. *Current Medicinal Chemistry* 12 (22): 2625-2641.

Springhouse Corp (eds). 2005. 8th ed. *Professional Guide to Diseases*.: Lippincott Williams & Wilkins, Philadelphia, PA.

Sturtevant W. 1954. The Mikasuki Seminole: Medical Beliefs and Practices. PhD Thesis, Yale University, New Haven, CT.

Swanton JR. 2000. *Creek Religion and Medicine*.: University of Nebraska Press.

Taylor LA. 1940. *Plants Used as Curatives by Certain Southeastern Tribes*.: Botanical Museum of Harvard University, Cambridge, MA.

The Angiosperm Phylogeny Group. 2009. An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG III. *Botanical Journal of the Linnean Society* 161: 105-121.

The Plant List. 2013. "The Plant List." 2019. Available from http://www.theplantlist.org/.

Theakston RDG. 1990. Comments on letter of Gutiérrez et al. on standardization of assays for testing the neutralizing activity of antivenoms. *Toxicon* 28 (10): 1131-1132.

USDA, and NRCS. 2019. "The PLANTS Database." 2019. Available from http://plants.usda.gov.

Verhoek S. 1978a. Huaco and Amole: A Survey of the Uses of Manfreda and Prochnyanthes. *Economic Botany* 32 (2): 124-130.

-----. 1978b. Two New Species and a New Combination in Manfreda (Agavaceae). *Britonnia* 30 (2): 165-171.

Verhoek-Williams SE. 1975. A Study of the Tribe Poliantheae (including *Manfreda*) and Revisions of *Manfreda* and *Prochnyanthes* (Agavaceae). Ph. D. Thesis, Cornell University, Ithaca, NY.

Wagner FW, and Prescott JM. 1966. A comparative study of proteolytic activities in the venoms of some North American snakes. *Comparative Biochemistry and Physiology* 17 (1): 191-201.

Warrell DA. 2007. Venomous Animals. *Medicine* 35 (12): 659-662.

Wickham H. 2017. tidyverse: Easily Install and Load the 'Tidyverse'. R package version 1.2.1. https://CRAN.R-project.org/package=tidyverse

Wickham H and Bryan J. 2019. readxl: Read Excel Files. R package version 1.3.1.

Witthoft J. 1947. An Early Cherokee Ethnobotanical Note. *Journal of the Washington Academy of Sciences* 37(3): 73-75.

Yokosuka A, and Mimaki Y. 2009. Steroidal saponins from the whole plants of Agave utahensis and their cytotoxic activity. *Phytochemistry* 70 (6): 807-815.

Zychar BC, Castro Jr NC, Marcelino JR, and Gonçalves LR. 2008. Phenol used as a preservative in *Bothrops* antivenom induces impairment in leukocyte-endothelial interactions. *Toxicon* 51 (7): 1151-1157.

Glossary

CvvV = Crotalus viridus viridus whole venom

Hela-GFP = HeLa- Green Flourescent Protein cells

HuSKM = Human Skeletal Muscle cells

MMPE = *Manfreda maculosa* plant extract

MTI = Myotoxin I

MTII = Myotoxin II

Not Spec. = Not Specified

PEII = Plant Extract II (*Manfreda maculosa*)

Appendix

Formulary:

Table 3: Growth Media for HeLa-GFP Cells

Dulbecco's Modified Eagle's Medium (DMEM)	
10% Cosmic Calf Serum (Hyclone)	
1x Penicillin-Streptomycin-Amphotericin B	Antibiotic-Antimycotic Solution (Sigma Chemical Co.) – 100x Stock [Penicillin; 10 mg Streptomycin; 25 µg Amphotericin B]
1x Kanamycin Sulfate (GIBCO/BRL)	Diluted from original 100x

Table 4: Human Skeletal Muscle Cell Growth Medium (HuSKM-M)

Skeletal Muscle Cell Growth Medium (cat # SKM-M)
DMEM
Fetal bovine serum
Bovine Serum Albumin
Fetuin
Human Epidermal Growth Factor
Dexamethasone
Human Insulin
Penicillin
Streptomycin
Amphotericin B
1.0 g/L D-glucose

Table 5: Human Skeletal Muscle Cell Differentiation Medium (HuSKM-D)

Skeletal Muscle Cell Differentiation Medium (cat # SKM-D)
DMEM
Horse serum
Bovine Serum Albumin
Fetuin
Penicillin
Streptomycin
Amphotericin B
1.0 g/L D-glucose

Table 6: Hoechst and Propidium iodide

240 µL	Cell medium
5 µL	Hoechst (stock 1 mg/mL = 1 µg/µL)
5 µL	Propidium iodide (PI) (stock 1 mg/mL)

Table 7: Fluorophore Colors

Fluorophore	**Color**	**Stains Target**
FITC	Green	All cells
Hoechst	Blue	All cells
Propidium iodide	Red	Dead cells
Annexin		

Table 8: 0.25% Trypsin Working Solution [0.25% Trypsin; 0.7 mM EDTA]

5 mL	Trypsin-Versene (GIBCO/BRL) 2.5% Trypsin (10x)
45 mL	DMEM (without Sodium pyruvate) without Calf serum and supplements.

Table 9: Trypan Blue for cell counts

50 µL	Trypan Blue (Cell laboratory stock)
400 µL	Hank's Balanced Salt Solution
50 µL	Cell solution to count

Table 10: Positive control H2O2

30% H2O2 (8.82 M)	10 µL : 990 µL = 0.3% (0.0882 M)
0.3%	2 µL into 200 µL ~ 0.003% final concentration (0.000882 M) ~1 mM

Table 11: DNS-A Procedure staining solution/well

100 µL	2x binding buffer (from the Annexin Kit)
10 µL	Hoechst for a final concentration of 2 µg/mL
10 µL	Propidium iodide for a final concentration of 1 µg/mL
10 µL	Annexin for a final concentration of 0.25 µg/mL

Sample R-Code:

```
## Set libraries ----
library(tidyverse)
library(readxl)
library(ggplot2)
##Import data ----
## Cell Lines ----
# 20110520_HelaGFP Myotoxin FITC_pI  Hoeckst_pI ----
pI_HelaGFP <- read_xls("C:/Users/Owner/Documents/20180415_ThesisReGraphReImage/2. Prepared Data/20110520_HelaGFP.xls", sheet = 1)
# 20111028_MTS_HaCaT_Hs27_Jurkat_Myotoxins Cell Screen ----
MTS_20111028_MyotoxinCellScreen <- read_xls("C:/Users/Owner/Documents/20180415_ThesisReGraphReImage/2. Prepared Data/20111028_MTS_HaCaT_Hs27_Jurkat_Myotoxins.xls", sheet = 1)
# 20111018_Myob_pIH_ManfredaProjLabs  MyoB MyoC  ----
pIH_MyoB_MyoC <- read_xls("C:/Users/Owner/Documents/20180415_ThesisReGraphReImage/2. Prepared Data/20111018_Myob_pIH_ManfredaProjLabs.xls", sheet = 2)


## Data Processing ----
## Cell Lines
# 20110520_HelaGFP Myotoxin FITC_pI  Hoeckst_pI ----
pI_HelaGFP <- pI_HelaGFP %>%
  mutate(Treatment = Treatment %>% as.factor())
#  levels(pI_HelaGFP$Treatment)
# [1] "Media-Cell Only" "MTI-25"         "MTI-5"          "MTI-50"         "MTII-25"
# [6] "MTII-5"          "MTII-50"        "Tween-0.05%"    "Tween-0.1%"
## Graphic analysis
pI_HelaGFP %>%
  filter(TxClass != "NoTx Control") %>%
  mutate(TxSort = fct_reorder(Treatment, Sort, .desc = TRUE)) %>%
  mutate(NegativePercent = NegativePercent * 100) %>%
```

```
ggplot(aes(y = NegativePercent, x = TxSort)) +
geom_jitter(aes(color = ExpTx), width = 0.2, na.rm = TRUE) +
scale_color_manual(breaks = c("FITC_pI", "Hoechst_pI"),
                   values=c("green", "blue")) +
scale_y_continuous(breaks = c(0, 25, 50, 75, 100), limits = c(-10, 100)) +
geom_hline(yintercept = 100) +
theme_minimal() +
labs(title = "Study of Cell Death (24 hours)",
    subtitle = "Effects of Myotoxin I and Myotoxin II HeLa-GFP",
    x = "Treatment ([µg/mL] or noted)",
    y = "Percent Viability") +
coord_flip()
```

Curriculum Vita

Charles Steven Gilbert

Upon graduation from Montebello Sr. High School in 1971, I attended The University of California at Los Angeles for one year before enlisting in the US Army. After basic training I was assigned to the 82nd Airborne Division, Ft. Bragg, North Carolina. After two year I changed my military occupational specialty to the medical laboratory. During the training, I obtained credit hours through the Baylor University (Waco, Texas) Extension at the Academy of Health Sciences, Ft. Sam Houston, Texas. The next four years I worked in the clinical laboratory and medical research at the US Army Institute of Surgical Research, Brook Army Medical Center, Ft. Sam Houston, Texas co-authoring my first research paper. After leaving the military I worked various jobs and went back to school to finish a degree in biology obtaining credit hours at San Antonio Community College and transferring to The University of Texas at San Antonio to graduate with my Bachelor of Science degree in biology in 1982. I continued another two years at The University of Texas at San Antonio obtaining a Composite Science Secondary Teaching Certification.

In 1984, I continued at The University of Texas Health Science Center at San Antonio, Department of Pediatrics, Division of Hematology/Oncology in medical research. During this time, I supervised a laboratory and trained post-doctoral physicians in ultrastructural immuno- and cyto-histochemistry. Additional research papers were co-authored while I was at the Health Science Center. After ten years, I moved with my mentor to Carolinas Medical Center, Charlotte, North Carolina where I published my second first author paper. During my time in medical research, I expanded my training to include computer science, data management, and statistics.

My family moved to El Paso, Texas in 2002. It was at this time that I worked as a contract computer technician. In 2003, I attended the Texas Master Naturalist training and embarked on what was to be my fourth career in the environmental sciences. In 2006, I began working as a groundskeeper at the Centennial Museum on The University of Texas At El Paso campus. I started working to be a native plant specialist for the desert southwest. In 2008 I entered into the Master's Program in Environmental Science. In this program I leveraged my medical research experience to study a plant that had reported ethnobotanic effects for the treatment of snakebite.

Publications as first author:

Gilbert CS, Parmley RT. 1998. The morphology of human neutrophils: A comparison of cryofixation, routine glutaraldehyde fixation, and the effects of dimethylsulfoxide. *Anat Rec* 252: 254-263.

Gilbert CS, Parmley RT, Rice WG, Kinkade JM,Jr. 1993. Heterogeneity of peroxidase-positive granules in normal human and Chédiak-Higashi neutrophils. *J Histochem Cytochem* 41 (6): 837-849.

Additional publications available on request.

Email address: chasgilbert@sbcglobal.net

This thesis/dissertation was typed by Charles Gilbert