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Protective Effects of Aqueous Extract of Terminalia arjuna bark Against Doxorubicin-induced Cardiotoxicity

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SENIOR THESIS APPROVAL

This Honors thesis entitled

"Protective effects of aqueous extract of *Terminalia arjuna* bark against doxorubicin-induced cardiotoxicity"

written by

Sarah Elisabeth Bishop

and submitted in partial fulfillment of
the requirements for completion of
the Carl Goodson Honors Program
meets the criteria for acceptance
and has been approved by the undersigned readers.

Dr. Timothy Hayes, thesis director

Dr. Ruth Plymale, second reader

Dr. Kent Faught, third reader

Dr. Barbara Pemberton, Honors Program director

April 14, 2014

My name is Sarah Bishop and I am a pursuing a Bachelor of Science degree in both chemistry and biology at Ouachita Baptist University. Part of my degree is obtaining research hours for both chemistry and biology. In the summer of 2013, I participated in research at the University of Arkansas for Medical Sciences (UAMS) College of Pharmacy Department of Pharmaceutical Sciences under the INBRE Summer Research Fellowship. As a result of my research experience I produced this thesis is addressed to future and current science students at OBU and science students in general. This thesis addresses how my idea of research has changed before and after this experience and how I have changed as a student and as a person. Please enjoy.

Pre-research ideas:

I thought I would be wearing a lab coat and goggles, sitting at a clean white lab bench and be in a chemistry setting with bunsen burners. I thought I would report to my mentor the results of the tests I ran and then go back to work. I thought I would have privacy and be a professional . . . a chemist. I know it sounds as if I viewed myself as a very capable woman. After all, I did just come out of the class Experimental Techniques, where we used practically every instrument available in the chemistry department to analyze compounds we synthesized. I thought I knew everything about working in a lab. And oh, how I was wrong. Just because I would make A's on my paper and knew how to work the instruments at OBU (well, at least I thought I knew how to run the instruments) did not mean I knew how to work the instruments at UAMS, or would even be allowed to. Something I learned at UAMS was that I would actually have to understand, *truly understand,* and *truly grasp,* the concept of my research, not be able to just put words on a paper and get fluorescence results back, but *truly* understand what the fluorescence meant.

When asked a question, there were no other students I could hide behind. No more "sit quietly, head down and wait until someone else says something," because I didn't want to look like a fool. No. It was me and Dr. Liu. It was me answering questions, or at least giving an answer, and hoping it was right. Me feeling dumb at times for not remembering anatomy and for feeling like I was embarrassing my school and professors for passing me in the class when I could not remember the material now. Me feeling embarrassed and ashamed telling Dr. Liu I wanted to become a pharmacist when I could not remember some important equilibriums in the blood stream. I felt like crying some times when asked a math question for dilutions and I felt it was an eternity before I answered. Where was my pen and paper and, for goodness sakes, my calculator?! No, Dr. Liu made me give an answer on the spot and made me toughen my skin on the spot.

Please don't get me wrong, Dr. Liu was a very kind and patient man. He encouraged questions and understood I could not know everything. He only had one rule: a student is not to be blamed, until they are taught the lesson. Once they are told the correct answer, it is then their responsibility to remember it.

The INBRE Fellowship was a 10-week program which consisted of research, a final paper, and a presentation. Dr. Liu and I spent the first two days discussing the background in the research and how to fit a project into ten weeks. I know that may sound like some time, but ten weeks was broken down into one week to plan, six weeks to run the experiment and collect data,

one week to prepare a powerpoint for presentation, one week to practice and present, and then the final week to clean up in lab. Practically, only six weeks for lab work.

As time went on, I began to grasp the concepts. I began to be not as afraid to give the answers to the questions, and started to get answers right! I started to form a trusting relationship with my mentor. I felt more on his level, not professionally or academically, but where I could have a conversation *with* him about the research instead of being *told* about the research.

For the most of the lab time, Dr. Liu would be watching over my shoulder. I would be able to do most of the work myself such as changing the water for the mice, cleaning their cages and making solutions, but he would do the ultrasound and termination of the mice at the end. I was never alone and at first felt insulted, because I thought he assumed I couldn't do anything, but that was just rude of me. It was his lab and he wanted to make sure I didn't waste any of his materials. He wanted to ensure that the solutions were properly made and to be honest, I am glad he was there, because I would probably have messed up if he wasn't. Dr. Liu also showed me some tricks to help better my lab technique.

Over the six weeks of research, I ran tests, collected data and made conclusions. The following describes my lab procedures and contains the results of the experiments. Preceding this paragraph was what I thought of research before I started work. Following my research, my views have changed and my appreciation has greatly increased of research and researchers.

Protective effects of aqueous extract of *Terminalia arjuna* bark against doxorubicin-induced cardiotoxicity

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Abstract

The bark of Terminalia arjuna (*TA*), a tropical tree, has been used in Ayurvedic medicine for treatment of cardiovascular disease. *TA* bark is known to contain various antioxidants, and recently it has been suggested to enhance function of the normal heart as an over-the-counter supplement in the USA. The mechanism underlying cardiac actions of *TA* bark are unknown. Doxorubicin (DOX), a commonly-used anticancer drug, is known to cause cardiotoxicity, a major concern in chemotherapy. The aim of this study is to investigate whether aqueous extracts of *TA* bark (TA_{aq}) protect the heart from DOX treatment by counteracting the oxidative stress caused by DOX. H9c2 cells, a cell line derived from the rat heart, were used for the *in vitro* study to examine cellular mechanis(s) of actions of TA_{aq}. Echocardiography was used to monitor cardiac function of mice with and without co-treatment of DOX and TA_{aq}. Our results showed that treatments of H9c2 cells with DOX (1 μM) for 24 hr caused an increase in superoxide production and damage to the growth network which were attenuated by co-treatment with TA_{aq} (100 μg/ml). Our *in vivo* data showed that TA_{aq} (50-100 μg/ml in drinking water) prevented the decrease in left ventricle function caused by multiple weekly treatments with DOX. These preliminary data suggest that TA_{aq} protects the heart in part from oxidative stress caused by DOX.

Introduction

Doxorubicin (DOX), a commonly-used anticancer drug, is known to cause cardiotoxicity likely via increased oxidative stress of cardiac myocytes (1). The cardiotoxicity of DOX is a major concern in cancer chemotherapy. The bark of *Terminalia arjuna (TA),* a tropical tree, has been used in Ayurvedic medicine for treatment of cardiovascular disease for centuries. *TA* bark is known to contain active organic constituents such as tannins, triterpenoid saponins, flavonoids, gallic acid, ellagic acid, oligomeric proanthocyanidins, and phytosterols, and recently it has been suggested to enhance function of the normal heart as an over-the counter supplement in the USA. However, the mechanism underlying cardiac actions of *TA* bark are unknown. The aim of this study is to investigate whether aqueous extracts of *TA* bark (TA_{aq}) protect the heart from DOX treatment by counteracting the oxidative stress caused by DOX.

Experimental Procedures

TA_{aq} bark preparations

The aqueous extracts of *TA* bark were prepared as described previously (2). Briefly, 1.005g of pulverized crude *TA* bark was added to 50 mL of boiling milli-Q water and continued to boil for 15-20 minutes. After centrifugation at 6000 x *g* for 2 minutes, the supernatant (decoction) was collected and filtered through a 0.22 μm filter. The concentration and percent yield for the TA_{aq} stock solution was 4.5 mg/ml and 20%, respectively.

Growth medium

H9c2 cells (American Type Culture Collection® CRL-1446™), a commonly used cell model derived from rat heart, were cultured in Dulbecco's Modified Eagle's Medium (DMEM) following ATCC instructions. Briefly, cells were thawed and maintained in high glucose DMEM medium supplemented with 10% FBS growth medium in a T-25 culture flask incubated at 37°C. After growing to approximately 70% confluence, cells were distributed to 24-well plates for study.

Fluorescence microscopy

Two fluorescent dyes were used to detect the oxidative stress of H9c2 cells; one superoxide-sensitive dihydroethidin (DHE) and mitochondria-sensitive MitoTracker®, both of whose oxidized forms emit fluorescence detected at 580 nm (excitation at 535 nm). H9c2 cells were treated with TA_{aq} (100 μg/mL) 20-30 min before DOX (1 μM) treatment and maintained in a 5% CO_2 incubator at 37°C for 2-24 hours. At 30-45 min before the termination of DOX treatments cells were incubated with DHE (3 μM) or MitoTracker® (0.1 μM) in the incubator at 37°C. At the end of experiments, dyes were washed off with Hepes-buffered salt solution. The fluorescence images of DHE and MitoTracker were acquired with AxioVision software using the same exposure duration to yield sub-maximal intensity at the same magnification within each individual experiment, and analyzed using NIH ImageJ.

Daily drinking water and DOX treatment

Four C57/BL6 mice were used for the in vivo experiment. Two of four C57/BL6 mice received drinking water containing TA_{aq} (50 or 100 μg/mL) for 4 days before DOX injection. Water intake of each mouse was measured and recorded daily. Water was daily replaced with freshly-made *TA* bark drinking water. One of the two C57/BL6 mice receiving drinking water containing TA and one of the two C57/BL6 mice receiving drinking water not containing TA_{aq} received a weekly injection of DOX (10 mg/kg body weight, i.p.) for two weeks. The other two mice, one of which received drinking water containing TA_{aq} and one receiving drinking water not containing TA_{aq}, received a weekly injection of the same volume of saline.

Echocardiography

Echocardiograms were obtained using a Vevo® 2100 high-resolution imaging system (VisualSonics) specifically for the analysis of small animals. Briefly, the mouse was weighed and anesthetized with isoflurane at 3% at 1 liter per minute (lpm) medical-grade air. Once the mouse was unconscious, the mouse was quickly transferred and taped to a heated platform in a

dorsal recumbent position. The isoflurane was then reduced to 1%. Vital signs (heart rate, respiration rate and body temperature) were monitored throughout the imaging.
The hair of the chest was removed using a chemical hair remover (Nair). Prewarmed ultrasound gel (Aquasonic 100) was spread over the clean chest. A MS-550D probe was used to acquire ultrasound images of the heart. The ultrasound examination for each mouse took less than 1 hour. After the examination, the mouse regained consciousness and was returned to its cage. Echocardiography was performed weekly to assess the left ventricular function before, during, and 2 weeks after 2-week treatments with DOX.

Histological examination

After termination of the mice, the heart was removed from the body and flushed through the aorta with control HTBSS solution followed by 10% neutral buffered formalin. After being blotted dry, the heart was weighed to determine the ratio of heart weight to body weight. The heart of each mouse was then returned to the buffered formalin solution for 24 hours before being embedded in paraffin. Each heart was cut into sections of 5-μm thickness which were mounted on glass microscope slides. Histological analysis of the sections of each heart was performed after a Gomori Trichome stain (performed by pathology core facility).

Results and Discussion

Attenuation of DOX-induced superoxide production and cell damages by TA_{aq}

Oxidative stress of H9c2 cells was assessed using superoxide-sensitive DHE staining of cells treated with DOX and co-treated with TA_{aq} for 24 hours. Figure 1 shows after 24 hours, DOX (1 μM) enhanced superoxide production (Figure 1C) compared with the control (Figure 1A) and TA_{aq} alone (Figure 1B). DOX also reduced cell growth and connection and reduced the cell number (Figure 1C). Cotreatment of DOX with TA_{aq} significantly reduced the production of superoxide and cell damage (Figure 1D). The quantitative analysis of relative superoxide production induced by DOX and inhibition by TA_{aq} is shown in Figure 2.

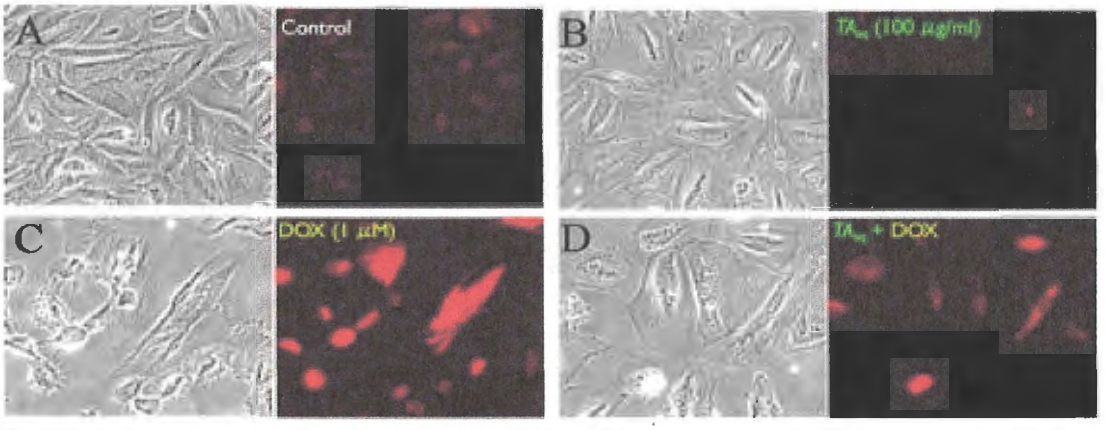

Figure 1. Superoxide production was measured by DHE fluorescence in H9c2 cells treated with or without DOX and co-treatment with or without TA_{aq} for 24 hours.

The increase in superoxide production induced by DOX and the inhibitory effect of TA_{aq} on the DOX-induced superoxide production could be observed as early as 2 hours treatment of DOX (Figure 3). The results also showed concentration-dependent inhibitory action of TA_{aq} on the superoxide production induced by DOX (1 μM). Therefore, TA_{aq} reduced the superoxide production in both DOX-treated and non-treated H9c2 cells; TA_{aq} at 100 ug/mL yielded the most significant effect.

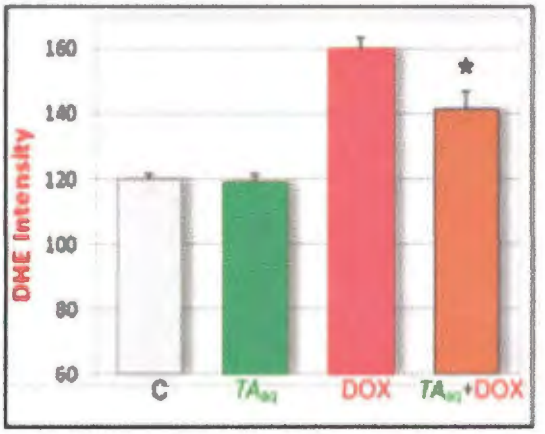

Figure 2. Summary of inhibitory effect of TA_{aq} on DOX-induced superoxide production.

Mitochondria are the most abundant organelle in heart cells, and it is the most likely location for production of superoxide free radicals. Therefore, we investigated if such oxidative stress was localized in mitochondria. We used MitoTracker®, a mitochondrion-specific dye, to detect the oxidative stress in mitochondria of H9c2 cells treated with DOX with or without co-treatment of TA_{aq} for 24 hours. Figure 4 shows that there was oxidative stress in mitochondria of untreated H9c2 cells (A). DOX (1 μM) caused a dramatic increase in mitochondrial oxidative stress of H9c2 cells after 24-hours (Figure 4B). Similarly, co-treatment of DOX with TA_{aq} (100 μg/ml) significantly reduced the mitochondrial oxidative stress caused by DOX (Figure 4C). Figure 5 shows the concentration-dependent inhibition of DOX-induced mitochondria by TA_{aq}, consistent with the result shown in Figure 3.

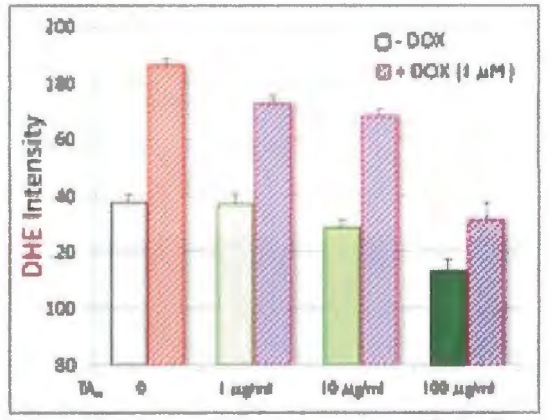

Figure 3. Concentration-dependent effect of *TA*aq on DOX-induced superoxide production

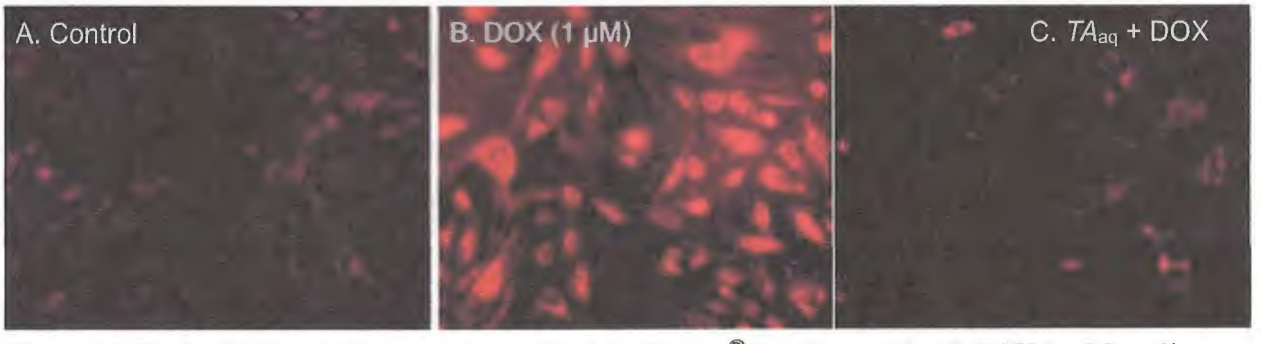

Figure 4. Mitochondrial oxidative stress detected by MitoTracker® in cells treated with DOX (1 μM) and/or co-treated with TA_{aq} (10 mg/ml) + DOX (1 μM)

DOX is known to induce oxidative stress in heart cells, a primary mechanism underlying its side effect of cardiotoxicity. This study used H9c2, a commonly-used cell model derived from embryonic rat heart to investigate the protective effect of TA_{aq} on DOX-induced cardiotoxicity. First, our results demonstrated a DOX-induced increase in superoxide production and cell damage in H9c2 cells, consistent with those described in the literature. Second, our results showed that the increased oxidative stress was specifically localized in the mitochondria of H9c2 cells, suggesting that DOX causes damage to mitochondria in these cells. Third, our data showed that TA_{aq}, a known cardiotonic, reduced DOX-induced oxidative stress in a concentration-dependent manner, suggesting antagonizing action on DOX-induced cardiotoxity.

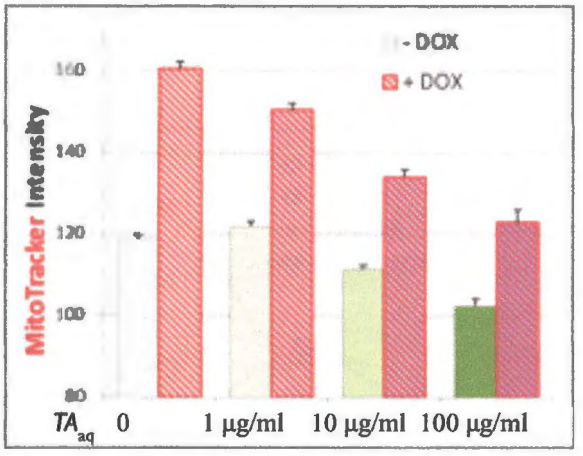

Figure 5. Concentration-dependent inhibition of DOX-induced mitochondrial oxidative stress by TA_{aq}.

DOX-induced decline in left ventricular (LV) function of the mouse heart

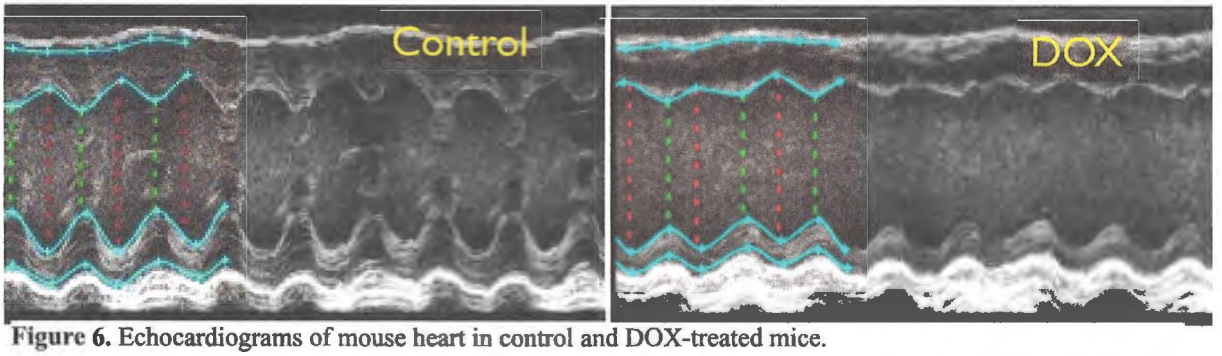

Figure 6. Echocardiograms of mouse heart in control and DOX-treated mice.

Weekly echocardiography enabled the assessment of LV function before, during, and after treatment of mice with DOX. In this study, two groups of mice were given drinking water containing freshly-made TA_{aq} four days before DOX treatments. Figure 6 shows representative echocardiograms of the heart in an untreated and DOX-treated mouse, respectively. The measured parameters are shown in Table 1 and Figure 7. The cardiac function of individual hearts at the 4th week was compared to that before pharmacological intervention (or the baseline). During the period of 4 weeks, cardiac function did not significantly change in the control and TA_{aq}-treated mice. In contrast, there was more than a 20% decline in cardiac function of the DOX-treated heart 2 weeks after termination of DOX injection. The DOX-induced decline in cardiac function was not observed in the mouse treated with TA_{aq} prior to DOX injections.

Ratio (4th week to the baseline)

	Control	TA	DOX	TA+DOX
LVID;d	0.94	1.00	1.20	0.85
LVID;s	0.89	1.08	1.50	0.70
Stroke Volume	1.05	0.93	0.74	0.98
Ejection Fraction	1.25	0.90	0.79	1.45
Cardiac Output	1.04	0.93	0.74	0.89

Table 1. Relative change in parameters of cardiac function in TA_{aq}, DOX and TA_{aq}+DOX mice. LVID: LV internal dimension

Figure 7 shows that the cardiac function in control and TA_{aq}-treated hearts remained relatively constant during this 4-week experiment. One week after the 1st injection of DOX, the ejection fraction of the heart was transiently increased, possibly due to adaptive response of cardiac function to DOX. However, the ejection fraction declined more than 20% 2 weeks after termination of DOX administration.

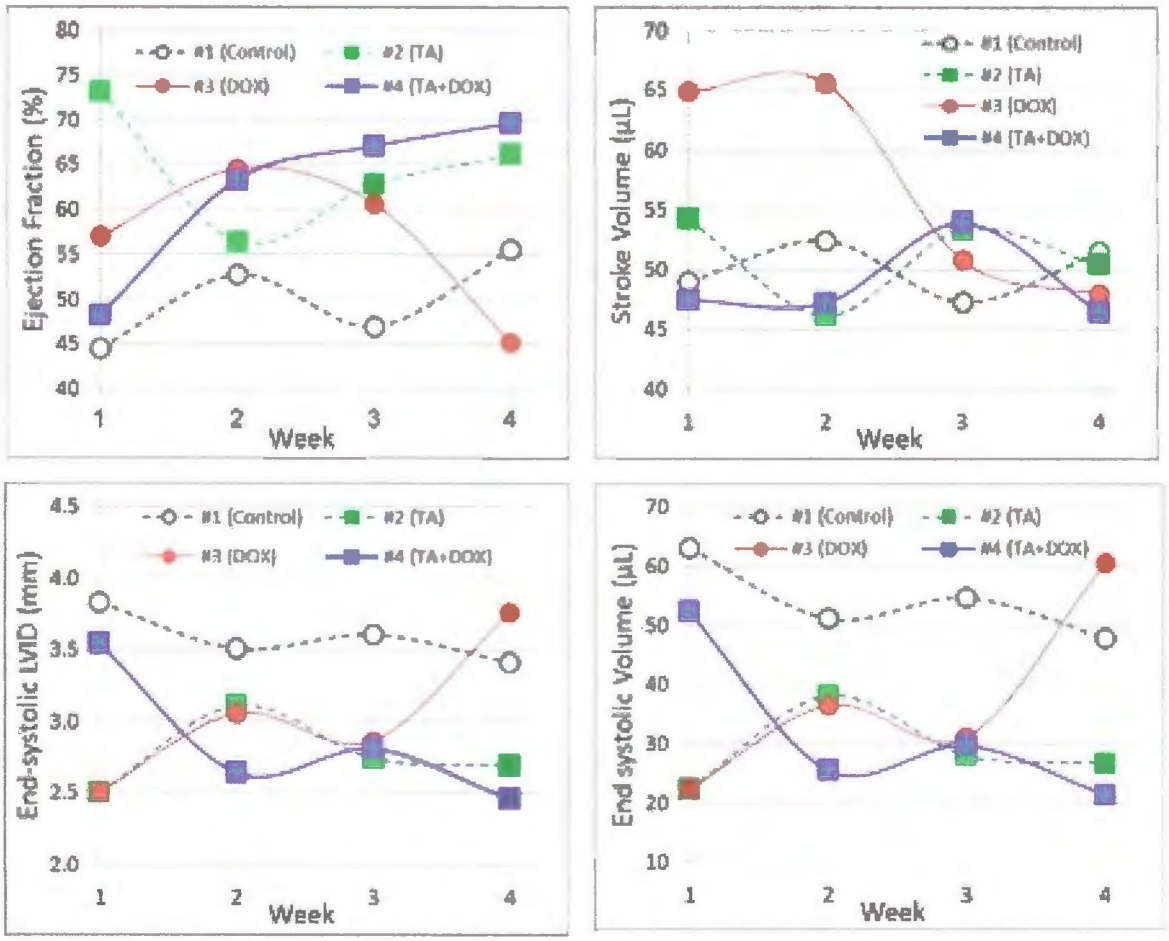

Figure 7. Chronological monitor of function of the mouse heart in these 4 groups

This result was consistent with the clinical observation that cardiotoxicity is often observed a certain period of time after termination of chemotherapy (1,3). In summary, the echocardiographic (*in vivo*) data are consistent with the *in vitro* data, i.e., TA_{aq} prevented the decline in cardiac function induced by DOX treatment.

Conclusion

Our data suggest that TA_{aq} exerts protective and therapeutic effects on DOX-induced cardiotoxicity, likely resulting in part from its capability to reduce the mitochondrial oxidative stress caused by DOX. Thus, TA_{aq} is of potential value in the clinic for cancer patients receiving chemotherapy. Future studies will investigate detailed cellular mechanism(s) underlying the cardiac protective action of TA_{aq} and identify correspondent active components in TA_{aq}.

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National Institute of General Medical Sciences- NIGMS (P20GM103429)

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Post-research ideas:

What I considered, at the beginning of my research, annoying and added pressure with Dr. Liu standing behind me, became enjoyable and needed. When I was alone in the lab, there were times it was hard not to mess up and at times it could be lonely. At other times, I became more comfortable in the lab and enjoyed the relaxation.

I thought people did research for their own personal gain. I know the reason for doing research is to help others and to find a cure, but with the great discoveries come great fame and great fortune . . . Nobel Prize fortune. I mean, this is your career after all. You go through years of school, so why don't you want recognition for your intellectual abilities and break-through ideas? I thought you would want recognition, and I was right to a point. It wasn't so much wanting to be published for personal gain, but for professional gain-professional gain to be able to earn more grants, maintain and expand your lab space, hire lab technicians, and maintain your job. I did not realize there are many research institutions with the motto "publish or perish." Researchers need grants to provide the funding to buy the expensive antibodies, the expensive chemicals, and pay for the expensive instruments to be used. Researchers need to be published to show their research had meaningful results and to prove to organizations which provide grants that the money they give will be well spent and will be furthering research.

My mentor, Dr. Liu, does research because it interests him and he wants to find a cure to a problem facing patients. He wants to help people. While I am sure there are some exceptions, as there seem to be in every situation in life, the people at UAMS all seemed to have heart, drive, charisma, a purpose and goal for their work. Their research is their baby.

One day in lab, I was talking to Dr. Liu and we came to talk about one of his friends who just found out she had breast cancer. One of the downfalls about being in science and cancer research is that you know the statistics, the downsides of chemotherapy, and the long term effects of treatment. Our project was examining how to lower the cardiotoxic effects of doxorubicin, a commonly used anticancer drug. We were examining how the aqueous extracts of *Terminalia arjuna* bark can protect the heart from doxorubicin-induced oxidative stress, which would cause heart problems or heart failure 5-15 years after treatment with doxorubicin.

Dr. Liu knows his friend could be treated with doxorubicin and knows the resulting effects doxorubicin would have on her heart. One cannot be ignorant. However, there is a bright side. Dr. Liu is researching a cure for this. Adding the aqueous extracts of the *Terminalia arjuna* bark could prevent or delay the damage the doxorubicin causes on the heart. He is researching something that could prevent people from having as many heart conditions due to treatment of doxorubicin. His research could helping limit the worry cancer patients have about the side effects the chemotherapy has on their heart and allow them to enjoy life.

The answer can lie in a few tests. It could be a simple fix: just consume TA_{aq} and DOX at the same time during chemotherapy. No worries, no problems, no fear of the future. A solution was there. I was looking at it, working with it. Why was this not on the market? Why was funding hard to get for this? Why? The answers facing so many problems with current medicine, cancer and cardiovascular issues was right there. It is amazing how he could come up with this hypothesis, how he could put these pieces together.

So, what was a main reason he had trouble finding funding? He didn't know what the active compounds were in the TA_{aq}. Before funding, organizations want to know what is in TA_{aq}.

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Dr. Liu said he would need funding in order to be able to carry out the tests. He needed someone to have faith in him that even though he didn't know what the TA_{aq} was composed of, the investment of money into this research could lead to promising results to the investigation of TA_{aq} and its interactions with DOX. However, the money from funding comes from government dollars, tax dollars, your dollars. Therefore, just "giving away" the money on a leap of faith is not encouraged. Furthermore, most analytical techniques and compounds are organic- our solution is an aqueous solution. Organic and aqueous solutions separate out from one another and many solvents used when analyzing organic compounds cannot be used to analyze aqueous compounds. This was the problem facing Dr. Liu.

When I began this fellowship, I thought I understood research and I thought I gave research its proper respect. I was wrong. My view of research has expanded and my appreciation has elevated. We are surrounded by hundreds of people who stay in a lab half the size of many classrooms, sometimes crammed with supplies and instruments and run a single test multiple times in order to make sure results are conclusive. Sometimes the results of the tests are not what one expected to see; sometimes you think you might have found a solution, but the tests say you didn't so you have to try again.

Researchers do not give up. Researchers read countless scientific journals, articles and papers which other researchers have written to try to use the information they found and combine with their own to find an answer or make a discovery. Researchers are the ultimate treasure hunters. The treasure they seek is a solution to the problem, whether that is fixing the side effects of certain cancers, turning receptors back on to prevent cancer or whatever the solution may be. They obtain their clues by reading, testing and taking chances. There is so much not known in science. There are clues not found yet. Researchers make the connections no one has made yet.

My mentor has been working on our project in bits and pieces for over seven years. When he participates in undergraduate fellowship research he has the funds to work on the project. It takes time and motivation to not give up. Research also takes creativity. Some people think it is easy to classify someone as an artsy or scientific, type A or type B personality, cluttered or organized; however, a scientist needs both. Scientists must be organized and meticulous, but they also have to be creative. Research is not about reproducing someone else's work. It is about taking someone else's findings, combining them with your current knowledge and creating a unique experiment to demonstrate your ideas or hypothesis of what may happen. Researchers also have to be aware of their audience, as in the organizations which fund the projects and give the grants. Researchers have to make sure they are researching something which will be of interest to the organizations and appeal to them, because researchers need their projects to be selected in order to have money to support their research.

I conducted research the summer of 2013 at UAMS in Dr. Liu's lab. In the fall of 2014, I will be attending UAMS College of Pharmacy. My whole thesis has been fueled by my respect for research and appreciation for my experience, but I myself am not pursuing a career in research. Please do not think in any way that my thesis is hypocritical or insincere because I am not pursuing that career path myself. That summer has shown me a new insight into research, allowed me to make new friends, expand my network, go on field trips to places I would not have been able to attend before, present at conferences, and travel. I am thankful. I am honored

and truly enjoy presenting my work and get excited to share the good news of my results. I would consider research as a summer job because research can be relaxing at times when the work and tests conducted become a habit; however, they can also become very repetitive and I desire some variety in my future career.

I am still in contact with Dr. Liu and send him updates in relation to pharmacy school and presentations. I will see Dr. Liu again- many times, actually. He will be one of my pharmacy teachers at UAMS. I may not learn anything about the tree bark of *Terminalia arjuna* in his class, but I know I will be learning. That's what life is: we learn, we grow, we make mistakes, we want redos, we practice learning, we practice speaking, we practice new skills and we will eventually make progress. So, no matter what specific area of pharmacy, science or life I find myself in, as long as I keep moving forward and keep learning I am making progress and making a difference. That is what research taught to me to do with my life: make a difference. If we can each do that with our own lives, we will each be a success.