KAMPALA INTERNATIONAL UNIVERSITY WESTERN CAMPUS

SCHOOL OF PHARMACY

ANTIOXIDANT ACTIVITY OF THE ETHANOLIC EXTRACTS OF LEAVES OF *AMARANTH* SPP AND *CUCURBITA* SPP IN *DROSOPHILA MELANOGASTER*

BY:

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DECEMBER, 2018

DECLARATION

I, **Ndinawe Johnmark**, solemnly declare that this is my original work and it has not been done or submitted by any other person elsewhere.

..... 12/12/2018

Signature Date

APPROVAL

I certify that all the work produced in this research by this student is an original work and has been scrutinized fully for submission.

..... 12/12/2018....

Signature Date

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Thank you my dearest **LORD** for this journey of research that I have finished and I pray you continue to grant unto me favor and knowledge as I continue with the rest of my studies. **AMEN**

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ABBREVIATIONS

DPPH	2, 2-diphenyl-1-picrylhydrazyl
ROS	Reactive oxygen species
HIV	Human immunodeficiency virus
BHT	Butylatedhydroxytoluene
BHA	Butylatedhydroxyanisole
SOD	superoxide Dismutase
IBR	Institute of Biomedical Research
SD	Standard Deviation
ANOVA	Analysis of Variance
TOR	Target of Rapamycin
MDA	Malondialdehyde
LPO	Lipid peroxidation
UV	Ultra violet

ABSTRACT

Background: Several plants show potent antioxidant and free radical scavenging properties, and their role in preventing oxidative stress-induced diseases such as Diabetes mellitus, Alzheimer's disease, Parkinson's disease, rheumatoid arthritis and many others.

Objective: In this study, the antioxidant activity of the ethanol extracts of leaves of *Amaranth* spp and *Cucurbita* spp and their ability to protect against oxidative stress in *Drosophila melanogaster* w^{1118}wild type flies was determined.

Materials and Methods: Flies cultured on food supplemented with 0.05 and 0.1mg/ml of *Amaranth* spp and *Cucurbita* spp leaf extracts were assayed for longevity, climbing activity, catalase activity, and oxidative stress resistance according to the established protocols. Scavenging activity of extracts on 2, 2-diphenyl-1-picrylhydrazyl free radical (DPPH) and reducing power activity were equally evaluated.

Results: There were significant effects of the extracts on DPPH free radical scavenging activity, catalase activity, climbing activity, longevity and oxidative stress resistance. *Cucurbita* extract showed no significant difference in climbing activity and catalase activity assay compared to the control.

Conclusion: This study shows that the ethanol extracts of leaves of *Amaranth* spp and *Cucurbita* spp exhibit varying degrees of protection against free radical induced oxidative stress in *Drosophila melanogaster.*

CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND

Humans live in the presence of various ubiquitous environmental stressors including UV radiation, microbes, allergens and various pollutants which amplify the generation of reactive oxygen species(ROS) in the body(Bouayed & Bohn, 2010).

Free radicals such as ROS are products of normal cellular metabolism but their overproduction results in oxidative stress that mediates several pathological processes, such as certain tumors(prostate and colon cancers) and coronary heart disease(Barros, Ferreira, Queirós, Ferreira, & Baptista, 2007). ROS are generated during normal aerobic metabolism, that is, endogenous sources primarily in mitochondria(Haenold, Wassef, Heinemann, & Hoshi, 2005), peroxisomes, endoplasmic reticulum and phagocytic cells. They are also induced by exogenous sources, including UV light radiation, environmental pollutants, and drugs such as paracetamol (Phaniendra, Jestadi, & Periyasamy, 2015).

Antioxidant molecules scavenge ROS and may serve as a possible preventive intervention for ROS-mediated diseases through reduction of oxidative stress which would result in injury to cells causing cell death(Anusuya, Anusuya, Manian, Siddhuraju, & Manian, 2009; Tandon, Gupta, & Tandon, 2005).Antioxidants may be enzymatic such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and peroxiredoxins or non-enzymatic such as ascorbic acid, glutathione, melatonin, vitamin E and uric acid(Ifeanyi, 2018; Mirończuk-Chodakowska, Witkowska, & Zujko, 2018).

Therefore the main dietary sources of antioxidants; grains, fruits and vegetables such as *Cucurbita* spp and *Amaranth* spp when consumed may be important for combating age-related diseases, such as some neurodegenerative diseases and cardiovascular diseases(Haenold et al., 2005; Kinyi, Byarugaba, & Vicente-Crespo, 2016).

1.2 PROBLEM STATEMENT

Environmental pollutants, UV radiation, and chemical exposure could lead to free radical induced damage causing diseases, such as diabetes mellitus, Parkinson's disease, Alzheimer's disease, atherosclerosis and hypertension, asthma, rheumatoid arthritis and various cancers such as prostate, breast and lung cancers due to cell function impairment as a result of oxidative stress damage(Devasagayam et al., 2004; Phaniendra et al., 2015).

Many of the Pumpkin(*Cucurbita* spp) and Dodo (*Amaranth* spp) farmers in the districts of Uganda appreciated the crops nutritional and medicinal values since they said that pumpkins provided vitamins, minerals, starch and proteins but they have not considered these plants as priority dietary options as they are mostly grown by low income members of the community who mainly utilize the leaves as vegetables and occasionally consume the fruit when cooked (Ondigi, Toili, Ijani, & Omuterema, 2008).

In-vitro antioxidant studies have revealed potent antioxidant and free radical scavenging activity of parts or whole plants of *Cucurbita* and *Amaranth* species and their role in preventing the development of diseases such as diabetes mellitus, hypertension and cancer as well as slow down the progress of malaria, tuberculosis and HIV (Conforti et al., 2005; de Carvalho et al., 2012; Kinyi et al., 2016; Kumar et al., 2010).

Therefore it is important that the potential to protect against oxidative stress of *Amaranth* spp and *Cucurbita* spp be tested in the Wild-type W^{1118}*Drosophila melanogaster* flies as the *in-vivo* experimental model as this would draw attention as a nutritional boost toward combating free radical generated disease conditions as well as supply practical diet guidance for the public (Liu et al., 2014).

1.3 RESEARCH QUESTIONS

i) Do these ethanol extracts of the leaves of *Amaranth* spp and *Cucurbita* spp possess *in vitro* antioxidant activity?

ii) Do these extracts increase the antioxidant activity *in vivo* after their consumption in *Drosophila melanogaster*?

iii) Do these extracts have the ability to protect against oxidative stress?

1.4 PURPOSE OF THE STUDY

To determine the *in vivo* antioxidant activity of the ethanol extracts of leaves of *Amaranth* spp and *Cucurbita* spp in *Drosophila melanogaster.*

1.5 SPECIFIC OBJECTIVES

i) To measure the *in-vitro* antioxidant activity of the ethanol extracts of leaves of *Amaranth* spp and *Cucurbita* spp.

ii) To determine the *in-vivo* antioxidant activity of the ethanol extracts of leaves of *Amaranth* spp and *Cucurbita* spp in *Drosophila melanogaster*.

iii) To determine the protective activity of the ethanol extracts of leaves of *Amaranth* spp and *Cucurbita* spp against oxidative stress in *Drosophila melanogaster*.

1.6 JUSTIFICATION OF THE STUDY

A great number of plants possess antioxidant properties and are more effective antioxidants *in vitro* than butylatedhydroxyanisole (BHA) or butylatedhydroxytoluene (BHT)(Mondal, Hossain, & Islam, 2017).Thus attention is now increasingly paid to the non-toxic antioxidants of natural origin that have a therapeutic value(Amarowicz, Pegg, Rahimi-Moghaddam, Barl, & Weil, 2004; Borah, Yadav, & Unni, 2011).

Many disease states such as prostate cancer have been shown to be a result of an imbalance between free radicals and antioxidants(Barros, Baptista, & Ferreira, 2007; Phaniendra et al., 2015). Prostate cancer, the most common cancer among men in Africa and the third most common cancer overall with 59,500 incident cases per year(16.4% of all cancer in men)is an increasingly important cancer in Africa with respect to morbidity and mortality because incidence rates are increasing, particularly within Uganda(Okuku et al., 2016).

Therefore, the ability to determine the antioxidant activity in vegetables such as *Amaranth* spp and *Cucurbita* spp provides a basis to evaluate the hypothesis that consumption of these plant species offers protection against free-radical induced oxidative damage and diseases due to oxidative stress thus improving our health(Amarowicz et al., 2004; Prior & Cao, 2000).

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1INTRODUCTION

The role of plants in medicine and healthcare is highly recognized(Dissanayake, Deraniyagala, Hettiarachchi, & Thiripuranathar, 2018). Many plants contain substantial amounts of antioxidants that can be utilized to scavenge the excess free radicals and can offer a source of new drugs(Gutiérrez-Rebolledo, Drier-Jonas, & Jiménez-Arellanes, 2017; Ifeanyi, 2018).

2.2 IN-VITRO ANTIOXIDANT ACTIVITY OF PLANTEXTRACTS

Natural antioxidants from plants are increasingly gaining interest among researchers, food manufacturers, and consumers(Pise, Jena, Maharana, & Sabale, 2010). Vegetables have been recognized as important sources of these antioxidants(Skotti, Anastasaki, Kanellou, Polissiou, & Tarantilis, 2014).

Natural antioxidants found in the diet of grains, vegetables and fruits such as vitamin C, vitamin D, melatonin, and polyphenols in the form of raw extracts or chemical constituents are considered to combat destructive processes due to oxidative stress and have received considerable attention for their potential applications in the prevention of numerous diseases (Dissanayake et al., 2018; Valenzuela et al., 2014).

A study conducted in Kamuli and Gulu Districts in Uganda by Andabati Brain and Muyonga John (2014) showed that traditional food plants used medicinally by these communities had a very high total phenolic content, flavonoid content as well as antioxidant activity(Andabati & Muyonga, 2016).

2.2.1 Antioxidant Activity of Cucurbita spp

The Cucurbitaceae family includes *Cucurbita moschata, Cucurbita pepo, Cucurbita argyrosperma, Cucurbita maxima* and *Cucurbita ficifolia*(Perez Gutierrez, 2016).

Pumpkin, a highly nutritious and antioxidant rich vegetable is widely grown all over the world(Muzzaffar et al., 2016)and is being used in nutraceutical industry(Peiretti, Meineri, Gai, Longato, & Amarowicz, 2017).

In vitro antioxidant assays have revealed potent antioxidant and free radical scavenging activity of superoxide anion, hydroxyl radical and DPPH free radicals of the aerial part of

C.maxima comparable with that of the standard drug ascorbic acid due to the high amounts of phenolic and flavonoid content(Bhavani Yenda, 2015; Saha, Mazumder, & Haldar, 2011).

2.2.2 Antioxidant Activity of Amaranth Spp

Amaranth plants produce grains and leafy edible vegetables with no significant differences between the species(Conforti et al., 2005).*Amaranth* seeds and grains are rich in several phytonutrients that act as powerful dietary antioxidants(Peiretti et al., 2017; Yawadio Nsimba, Kikuzaki, & Konishi, 2008)

Analysis of biochemical composition of the water extracts of leaves of *Amaranthus tricolor* and *Amaranthus spinosus* Linn. revealed the presence of low-molecular antioxidant metabolites: betacyanin (amaranthine), ascorbic acid, soluble monosaccharide and disaccharides, and organic acids that protect against diseases such as diabetes, skin diseases, dysentery, gonorrhea, and acute bronchitis(Chen, Wu, Shieh, Kuo, & Hsieh, 2006; Gins, Gins, Pivovarov, & Kononkov, 2016).

2.3 IN-VIVO ANTIOXIDANT ACTIVITY OF PLANT EXTRACTS

2.3.1 Enzymatic and Non-Enzymatic Effects of Exogenous Antioxidants

The modulatory effect observed in fruits and vegetables could be as a result of the presence of vitamins and minerals which act as cofactors for antioxidant enzymes contributing to their modulation of oxidative stress and enhancement of endogenous antioxidant enzyme activities(Ajagun-ogunleye, 2018).

There is a strong correlation between the intake of exogenous compounds containing antioxidants and the modulation of the endogenous enzymatic and non-enzymatic effects within living micro-organisms (Sarangarajan, Meera, Rukkumani, Sankar, & Anuradha, 2017).

Ananas sativa, Pineapple fruit and pulp, has exhibited the ability to protect against oxidative stress through enhancement of endogenous cellular antioxidant enzyme activity in *Drosophila melanogaster*(Ajagun-ogunleye, 2018).

2.3.2 Antioxidant Activity in *Drosophila Melanogaster*

Drosophila as a model is widely used in free radical research and aging(Niveditha, Deepashree, Ramesh, & Shivanandappa, 2017). Its genome is well characterized, is highly homologous to the genome of vertebrates (yet much less redundant), and has yielded valuable insights into many complex biologic problems(Cross et al., 2002).

Drosophila melanogaster has been found to have antioxidants such as superoxide dismutase, catalase, melatonin and many others that play a role in the fly's circadian rhythm regulation, free radical scavenging and protection against oxidative stress(Tinkerhess, Ginzberg, Piazza, & Wessells, 2012).

Dietary antioxidants, such as vitamin C, vitamin D, melatonin, and polyphenols when fed to *Drosophila melanogaster* ameliorate the effects of oxidative stress-inducing chemicals, such as hydrogen peroxide on *Drosophila* lifespan as they decrease the levels of SOD and catalase contrary to the increased levels of ROS over time(Jordan et al., 2012; Le Bourg, 2001; Niveditha, Deepashree, et al., 2017; Sadowska-Bartosz & Bartosz, 2014).

2.3.3Antioxidants against Oxidative Stress

Oxidative stress is an important factor in the pathogenesis of many diseases including atherosclerosis, ischemic heart disease, ageing, diabetes mellitus, cancer, neurodegenerative diseases among others (Lee, Hwang, Ha, Jeong, & Kim, 2003).

The protection offered by different edible plants against oxidative stress in several diseases has been attributed to various antioxidants (Saha et al., 2011).

Ananas sativa, Pineapple fruit and pulp, one of the most common plants in the tropical regions of Africa with high antioxidant properties has exhibited the ability to protect against oxidative stress through enhancement of endogenous cellular antioxidant enzyme activity in *Drosophila melanogaster*(Ajagun-ogunleye, 2018)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 INTRODUCTION

This chapter described the materials and methods that were used while carrying out the research. It included study design, area of research, ethical considerations, chemicals, reagents and equipment.

3.2 MATERIALS

2,2-diphenyl-1-picrylhydrazyl (DPPH), Hydrogen peroxide, 95% ethanol, L-Ascorbic acid (as positive control), Potassium ferricyanide (1% w/v), phosphate buffer (0.2 M, pH 6.6), trichloro acetic acid (10%), ferric chloride (0.1%) and ascorbic acid (1%), Vials, Test tubes, conical flasks, measuring cylinders, Beakers, micropipette, Thermometer, Vortex agitator, UV spectrophotometer, Electronic balance, Digital biochemical incubator(SPX- 150A), mortar and pestle, whatman filter paper grade 1, sieve, rotatory evaporator, drying trays.

3.3 METHODS

3.3.1 Study Design

The research was carried out using a controlled experimental study design.

3.3.2Area of Research

The research was done at the Institute of Biomedical Research (IBR) at Kampala International University-Western Campus in Bushenyi District.

3.3.3Plant Collection and Extraction

The leaves of *Cucurbita* spp and *Amaranth* spp were bought from the vendors in the markets within Ishaka town in Bushenyi District in the Western region of Uganda and air dried for two weeks. Air-dried samples (50g) were ground into a fine powder in a mortar using a pestle and extracted with 250ml Ethanol (95%). The solvent was removed using a rotatory evaporator at 37^0C to obtain a dry extract. The extracts were stored at 4^0C until experimental use(Salazar et al., 2008).

3.3.4Drosophila Culture and Fly Stock Husbandry

The standard medium for the flies constituted Nipagin (methyl-p-hydroxybenzoate), glucose, yeast, propionic acid, corn meal, agar, and distilled water.

Drosophila melanogaster (fruit flies) were put in vials that contain the standard culture cornmeal medium for growth and multiplication. The flies were maintained at 25^0C under a 12/12 hour light and dark cycle in a digital fly incubator (SPX- 150A).

3.3.4.1 Protocol of Preparing Food for the Flies

One liter of fly food was made for approximately 70 vials and these were the ingredients for the *Drosophila* culture; Distilled water(1.03L), Nipagin (23.3ml),Cornmeal(70gm), Glucose/dextrose(75gm), Yeast (15gm), Agar (10.5gm), Propionic acid(3.7ml).

The paste was made by mixing some distilled water, corn meal and agar, before boiling for 10 minutes. Nipagin, glucose, and yeast were then mixed separately and added to the mixture above. The mixture was brought to boil while stirring thoroughly with the stirrer. The food was allowed to cool to about 60°C, after which propionic acid was added and mixed thoroughly. The paste was allowed to cool for a while then distributed into vials or bottles.

3.3.5 *IN VITRO* ANTIOXIDANT ACTIVITY OF SAMPLE EXTRACTS

The antioxidant potential of the ethanol extract of leaves of *Cucurbita* spp and *Amaranth* spp was determined from their scavenging activity by the DPPH Radical Scavenging Assay and Reducing power assay.

3.3.5.1DPPH Radical Scavenging Assay

3.3.5.1.1 Principle

DPPH(2,2-diphenyl-1-picrylhydrazyl) is characterized as a stable free radical which on mixing with a substance that can donate a hydrogen atom, it gives rise to the reduced form with the loss of purple color to become yellow.

3.3.5.1.2 Protocol for DPPH assay

DPPH radical-scavenging assay of plant extracts against stable DPPH (2,2-diphenyl-1-picrylhydrazyl radical) was determined spectrophotometrically by a slight modification of the method described in (Samarth et al., 2008). Stock solutions of crude extracts were prepared as 1 mg/ml in ethanol. 1ml of different concentration samples was added to 5ml of 0.004% methanol solution of DPPH. After 30 min of incubation in the dark at room temperature, the absorbance was read against a blank at 517 nm. The assay was carried out in triplicate and

percentage of inhibition was calculated using the following formula: %Inhibition = ((AB-AA)/AB) × 100, where AB = Absorbance of blank; AA = Absorbance of sample.

3.3.5.2 Reducing Power Assay

3.3.5.2.1 Principle

The reducing power of ethanol extract of leaves of *Curcubita* spp and *Amaranth* spp was determined by a slight modification of the method adopted by(Jayanthi & Lalitha, 2011). Antioxidants react with potassium ferricyanide (Fe3+) to form potassium ferrocyanide (Fe2+), which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700 nm.

Potassium ferricyanide + Ferric chloride $\xrightarrow{\text{antioxidant}}$ Potassiumferrocyanide + Ferrous chloride

3.3.5.2.2 Phosphate buffer preparation

3.48g dibasic potassium phosphate was mixed with 2.72g monobasic potassium phosphate in 100 ml with water.

3.3.5.2.3 Protocol for reducing power assay

1ml of various concentrations of the plant extracts was mixed with phosphate buffer (2.5ml) and potassium ferricyanide (2.5ml). This mixture was kept at 50°C in a water bath for 20 minutes. After cooling, 2.5ml of 10% trichloroacetic acid was added and centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5ml) was mixed with distilled water (2.5ml) and a freshly prepared ferric chloride solution (0.5ml). The absorbance was measured at 700 nm. The blank was prepared in similar manner excluding samples. Ascorbic acid at various concentrations was used as a standard. Increased absorbance of the reaction mixture indicated an increase in reducing power. Reducing power was measured by varying the concentration of the extract and the contact time.

3.3.6 *IN VIVO* ANTIOXIDANT ACTIVITY OF SAMPLE EXTRACTS

These experiments were performed using *Drosophila melanogaster* that was fed on standard cornmeal medium containing the leaf extract for each of the plant species, that is, *Cucurbita* spp and *Amaranth* spp at different concentrations as was pre-determined from the preliminary studies. These flies were then be subjected to climbing (negative geotaxis), enzyme catalase, lifespan and DPPH and oxidative stress induction assays.

The control group was the fruit flies that were given the standard cornmeal medium without the ethanol leaf extracts.

3.3.6.1 DPPH Radical Scavenging Assay

In this *in vivo* experiment, the same procedure as described above was used and the experiment was carried out after pre-treatment for five days and ten days.

3.3.6.2 Climbing (Negative Geotaxis) Assay

The traditional negative geotaxis assay relies on measuring how many flies climb above a predetermined height in 10 seconds(Nichols & Pandey, 2014) and it was done by a slight modification of the method described in (Ali, Escala, Ruan, & Zhai, 2011). Ten flies from control group and ten flies from the treatment group which had been fed on the various food concentrations for thirty days were separated after placing them under anesthesia, using the *Drosophila* carbon dioxide (CO_2) anesthesia apparatus and each group was placed in a separate vial. The flies were allowed to recover from anesthesia before proceeding with the assay.

The climbing apparatus was prepared for the control and treatment groups. Two empty polystyrene vials were vertically joined by tape facing each other. For the lower vial, a vertical distance of 8 cm was measured above the bottom surface and each vial was marked by drawing a circle around the entire circumference of the vial.

A group of ten flies were transferred into the lower vial carefully preventing the escape of any fly. Immediately the lower vial was covered with the top vial and the contacting openings were taped securely. The flies were allowed to acclimatize to the new setting for 1 minute before conducting the assay. The flies were tapped down gently to the bottom of the vial and the number of flies that climbed above the 8-cm mark by 10 seconds after the tap was measured. The assay was repeated for the same group ten times, allowing for 1 minute rest period between each trial. The number of flies per group that pass the 8-cm mark was recorded as a percentage of total flies(Ali et al., 2011).

3.3.6.3 Lifespan (Longevity) Assay

Ten male flies from both the control and treatment group were separated after placing them under anesthesia using the *Drosophila* carbon dioxide (CO_2) anesthesia apparatus and the control and treatment groups were placed in a separate vial. The flies were allowed to recover from anesthesia before proceeding with the assay. Rate of survival was evaluated by a daily counting of live flies until all the flies had died(Ajagun-ogunleye, 2018).

3.3.6.4 Oxidative Stress Induction Assay

In the second *in vivo* experiment, ten fruit flies both from the control and treatment groups that had been starved for about 3 hours were added into fresh vials. Hydrogen peroxide (1%), one of the common agents for oxidative stress induction(Jordan et al., 2012)was added on the filter paper, impregnated with 10% sucrose which will be inserted into the fresh vials containing the flies. The flies were left in the vials for the observation of the effect of hydrogen peroxide on the flies. The flies in the treatment group had undergone pre-treatment with the ethanol leaf extracts for about 14 days before induction of oxidative stress(Ajagun-ogunleye, 2018).

The response of the flies to hydrogen peroxide-induced oxidative stress in both treatment and control groups was obtained by counting the number of live flies until all of them had died.

3.3.6.5 Enzyme Catalase Assay

The assay was carried out after five days and ten days both for the control group and the treatment group. The flies per group were rendered motionless by chilling them on ice, they were manually grounded and homogenized in ice cold phosphate buffer saline (pH 7.4), centrifuged at 2,500rpm for 10 minutes at 4^0C, supernatant was filtered, and the resulting homogenate kept at 4^0C for the biochemical catalase assay.

Catalase activity unit is defined as the number of micromoles of hydrogen peroxide decomposed per unit time. The *in vivo* catalase activity was determined as previously done by Iwase et al. (2013). Each solution from the control group and treatment group (100-mL) was added in a Pyrex tube (13 mm diameter × 100 mm height, borosilicate glass; Corning, USA). Subsequently, 100 ml of 1% Triton X-100 and 100 ml of undiluted hydrogen peroxide (30%) were added to the solutions and mixed thoroughly and were then incubated at room temperature.

Following completion of the reaction, the height of O_2-forming foam in the test tube that remained constant for 15 minutes was measured using a ruler(Iwase et al., 2013).

3.4 DATA INTERPRETATION, STATISTICAL ANALYSIS, AND DATA PRESENTATION

All experimental data was expressed as mean ± standard deviation (SD), with the aid of Graph Pad Prism version 6 Statistical software. Significant differences were analyzed using a one-way ANOVA and two-way ANOVA. The results obtained were considered as statistically significant if $P < 0.05$.

The raw data that was generated from the climbing assay represented the number of flies crossing the 8-cm mark in 10 seconds in each group. This was converted to percentage and the average pass rate computed for each group over 10 sessions.

Survival analysis data for lifespan (longevity) assay was presented as cumulative survival curves and analyzed using log rank (Mantel-Cox) test. The other tools that were used included Microsoft Excel, Microsoft Power point, tables and graphs.

3.5ETHICAL CONSIDERATIONS

Authentication from the school of pharmacy was obtained to continue with my research.

CHAPTER FOUR

4.0 RESULTS

4.1 *IN VITRO* ANTIOXIDANT ACTIVITY OF THE ETHANOLIC EXTRACTS OF LEAVES OF *AMARANTH* SPP AND *CUCURBITA* SPP.

4.1.1 DPPH Radical Scavenging Assay

Amaranth spp at 0.5mg/ml had the highest % DPPH activity than the other concentrations of the same extract (10.5%). DPPH scavenging activity decreased as the concentration of the *Amaranth* extract increased i.e. 8.2% and 7.4% for 0.75 and 1mg/ml respectively. The percent DPPH scavenging activity for *Cucurbita* spp increased with increasing extract concentrations i.e. 8.4%, 14.9%, 21.2, 28.2% at 0.25, 0.5, 0.75 and 1 mg/ml respectively. There were no significant effects within the 0.25mg/ml of extracts of *Cucurbita* spp and *Amaranth* spp (P =0.582) as shown in **figure 1**.

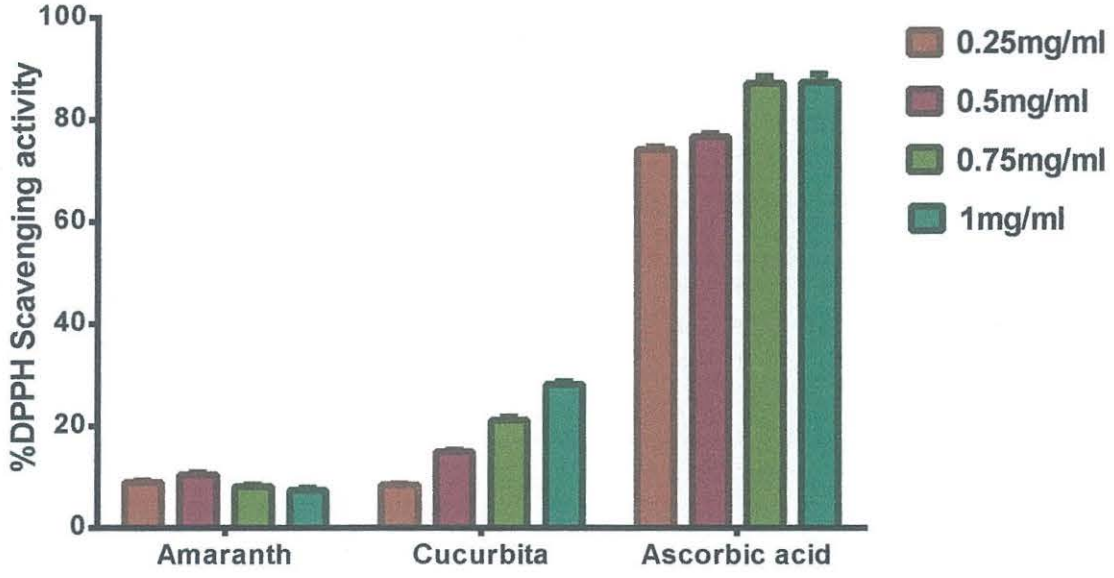

Figure 1: *In vitro* DPPH radical scavenging activity of *Amaranth* spp and *Cucurbita* spp

4.1.2 Reducing Power Assay

Amaranth spp and *Cucurbita* spp showed significant differences in the reducing power activity compared to the blank ($P < 0.05$). Reducing power activity of *Amaranth* spp increased with increasing concentrations as shown by increasing absorbencies at 700nm i.e. 0.071, 0.098, 0.1123, 0.141 at 0.25, 0.5, 0.75 and 1mg/ml respectively. *Cucurbita* spp showed increasing reductive power at increasing extract concentrations i.e. 0.1363, 0.1767, 0.242 and 0.2453 at 0.25, 0.5, 0.75 and 1mg/ml respectively as shown in **figure 2.**

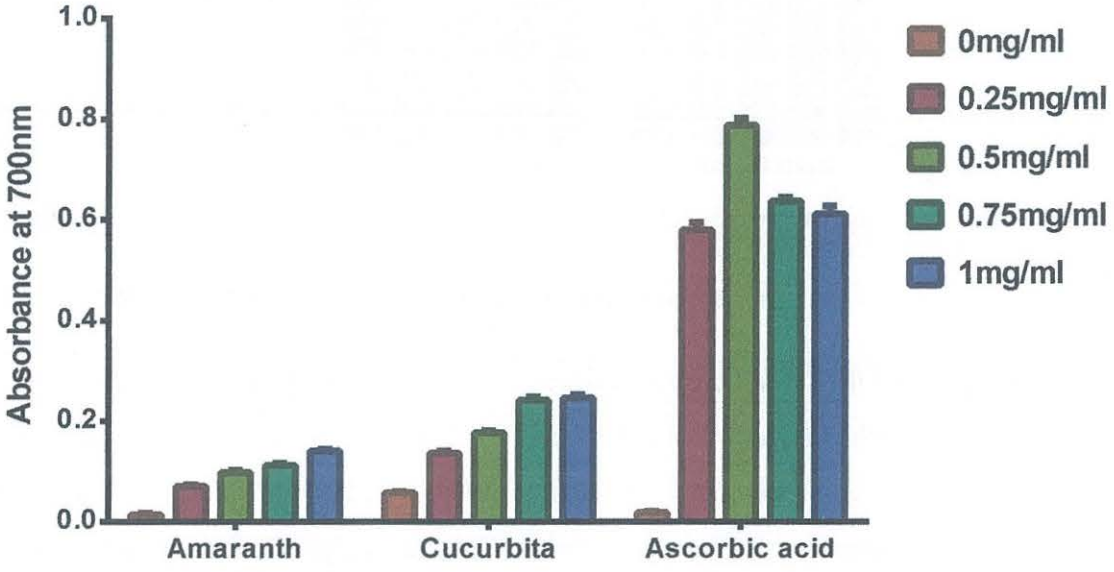

Figure 2: Reducing power activity of *Amaranth* spp and *Cucurbita* spp

4.2 *IN VIVO* ANTIOXIDANT ACTIVITY OF THE ETHANOLIC EXTRACTS OF LEAVES OF *AMARANTH* SPP AND *CUCURBITA* SPP

4.2.1 DPPH free radical scavenging activity

4.2.1.1 DPPH free radical scavenging activity of *Amaranth* spp

The results obtained after 5 days of pretreatment showed more percent DPPH free radical scavenging activity than after 10 days of pre-treatment with similar extract concentrations i.e. 47.9% and 39.7% at 0.05 and 0.1mg/ml respectively after 5 days pretreatment, and 47.5% and 35.8% at 0.05 and 0.1 mg/ml respectively after 10 days pre-treatment. There was a significant increase in the DPPH free radical scavenging activity of the 0.05mg/ml treatment group relative to control group ($P < 0.05$). There were no significant differences within the 0.1 mg/ml treatment group compared to the control after 5 days ($P = 0.6459$) and after10 days ($P = 0.9889$) pretreatment as shown in **figure 3.**

Rahmatullah et al., 2013; Sreelatha, Dinesh, & Uma, 2012). The decrease in scavenging activity of the *Amaranth* extract could be due to the pro-oxidant effect of the plant phytochemicals such as flavonoids, carotenoids and polyphenols which bring about gene down regulation of cytochrome enzyme system (Rahal et al., 2014).

The reducing power activity of the extracts has been shown to increase in the presence of phytochemicals which act as antioxidants. Antioxidants react with potassium ferricyanide (Fe3+) to form potassium ferrocyanide (Fe2+), which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700 nm. The reducing power activity seen in *Amaranth* spp and *Cucurbita* spp could be because of polyphenols that potentially quench free radicals by forming resonance-stabilized phenoxyl radicals (Pramanik, Bhattacharjee, & Bhattacharyya, 2014).

A strong correlation between the intake of exogenous compounds containing antioxidants and the modulation of the endogenous enzymatic effects has been shown within living micro-organisms (Sarangarajan et al., 2017). The increase in the *in vivo* DPPH free radical scavenging activity seen in *Amaranth* spp and *Cucurbita* spp could be because of the enhancement of the endogenous antioxidant defenses by the exogenous phytochemicals in the plant extracts (Ajagun-ogunleye, 2018; Sarangarajan et al., 2017). The decrease in scavenging activity seen with increasing extract concentration could be attributed to the fact that cumulative administration of exogenous antioxidants results in inhibition of the synthesis of endogenous antioxidants (Sadowska-Bartosz & Bartosz, 2014).

Catalase is a tetrahedrical protein, constituted by four heme groups which catalyze the dismutation of hydrogen peroxide in water and oxygen (Umesh et al., 2012). The significant increase in catalase activity seen in the *Amaranth* extract could be because of phytochemicals such as tocopherol, flavonoids and polyphenols which act as cofactors for antioxidant enzymes enhancing their endogenous antioxidant enzyme activities (Ajagun-ogunleye, 2018).

Dietary supplementation with exogenous antioxidants such as flavonoids has been shown to improve on the life span of *D. melanogaster flies* in some studies (Ajagun-ogunleye, 2018). The lifespan extending effect of the extracts of *Amaranth* spp and *Cucurbita* spp could be due to their antioxidant value which may regulate the metabolic pathways as has been shown in the Rapamycin (mTOR) pathway and enhancement of the endogenous defenses due to the presence of low-molecular antioxidant metabolites such as amaranthine, ascorbic acid,

soluble monosaccharide and disaccharides, and organic acids (Gins et al., 2016; Ivana Bjedov, Janne M. Toivonen, Fiona Kerr, Cathy Slack, Jake Jacobson, Andrea Foley, 2010; Pallauf, Duckstein, & Rimbach, 2017; Partridge, 2016).

An inverse relationship between aging and climbing activity has been shown (Jo & Imm, 2017). The increase in climbing performance of *Drosophila melanogaster* could be attributed to the phytochemicals such as vitamins and flavonoids which have been linked to neuroprotective activity through neutralization of the excessive free radicals and/or by enhancing the antioxidant defenses (Niveditha, Shivanandappa, & Ramesh, 2017).

Oxidative stress is an important factor in the pathogenesis of many diseases and the protection offered by plants against oxidative stress in several diseases has been attributed to various antioxidants (Saha et al., 2011). The extracts increased the flies resistance against oxidative stress and this could be because of the enhancement of endogenous cellular antioxidant enzyme activity and decreasing the levels of malondialdehyde (MDA) an oxidative stress marker of lipid peroxidation (LPO) as previously shown in studies done on plants such as *Ananas sativa* (pineapple) and *Punica granatum* (pomegranate) juice which also showed to protect against oxidative stress through in model organisms in *Drosophila melanogaster* and mice (Ajagun-ogunleye, 2018; Al-olayan, El-khadragy, Metwally, & Moneim, 2014).

5.2 CONCLUSION

In summary, the ethanol extracts of leaves of *Amaranth* spp (dodo) and *Cucurbita* spp (pumpkin), exhibited *in vitro* antioxidant activity. The extracts also have *in vivo* antioxidant activity in the model organism; *Drosophila melanogaster* as shown, by increasing the scavenging activity and reducing power activity.

The extracts increased the health span of the flies as shown by the increase in climbing performance which points out the effect of the extracts on the locomotor activity and also increased the longevity of the model organism; *Drosophila melanogaster* by increasing the flies ability to resist against oxidative stress and by enhancing the activities of cellular endogenous antioxidant enzyme activity for example catalase enzyme.

5.3 RECOMMENDATIONS

Based on the results, it is important to note that *Amaranth* spp (Dodo) and *Cucurbita* spp (pumpkin) are important nutritional supplements in human diet when consumed in the

recommended dietary ranges and therefore the population should be informed of these benefits.

Further work should be carried out to isolate the active compounds and appropriate elucidation of other mechanisms or nutritional pathways by which the extracts exhibit showed benefits.

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APPENDICES

1.0 TUKEY'S MULTIPLE COMPARISONS TEST FOR IN-VITRO EXPERIMENTS.

1.1 DPPH Radical scavenging assay

Tukey's multiple comparisons test	Adjusted P Value			
Concentration (mg/ml)	0.25mg/ml	0.5mg/ml	0.75mg/ml	1mg/ml
Amaranth vs. Cucurbita	0.582	< 0.0001	< 0.0001	< 0.0001
Amaranth vs. Ascorbic acid	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Cucurbita vs. Ascorbic acid	< 0.0001	< 0.0001	< 0.0001	< 0.0001

1.2 Reducing power assay

Tukey's multiple comparisons test	Adjusted P Value				
Concentration (mg/ml)	0 mg/ml	0.25mg/ml	0.5mg/ml	0.75mg/ml	1mg/ml
Amaranth vs. Cucurbita	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Amaranth vs. Ascorbic acid	0.8026	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Cucurbita vs. Ascorbic acid	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

2.0TUKEY'S MULTIPLE COMPARISONS TEST FOR IN-VIVO EXPERIMENTS.

2.1 DPPH Radical scavenging assay for Cucurbita extract

Tukey's multiple comparisons test	Adjusted P Value	
Concentration (mg/ml)	5 Days	10 Days
Control vs. Cucurbita(0.05mg/ml)	< 0.0001	< 0.0001
Control vs. Cucurbita(0.1mg/ml)	< 0.0001	< 0.0001
Control vs. Ascorbic acid(0.05mg/ml)	< 0.0001	< 0.0001
Control vs. Ascorbic acid(0.1mg/ml)	< 0.0001	0.0746
Cucurbita(0.05mg/ml) vs. Cucurbita(0.1mg/ml)	0.7897	0.7061
Cucurbita(0.05mg/ml) vs. Ascorbic acid(0.05mg/ml)	0.9866	0.1213
Cucurbita(0.05mg/ml) vs. Ascorbic acid(0.1mg/ml)	0.0281	< 0.0001
Cucurbita(0.1mg/ml) vs. Ascorbic acid(0.05mg/ml)	0.4982	0.7177
Cucurbita(0.1mg/ml) vs. Ascorbic acid(0.1mg/ml)	0.2429	< 0.0001
Ascorbic acid(0.05mg/ml) vs. Ascorbic acid(0.1mg/ml)	0.0095	< 0.0001

2.2 DPPH Radical scavenging assay for Amaranth extract

Tukey's multiple comparisons test	Adjusted P Value	
Concentration (mg/ml)	**5 days**	**10 days**
Control vs. Amaranth (0.05mg/ml)	< 0.0001	< 0.0001
Control vs. Amaranth (0.1mg/ml)	0.6459	0.9889
Control vs. Ascorbic acid (0.05mg/ml)	< 0.0001	< 0.0001
Control vs. Ascorbic acid (0.1mg/ml)	< 0.0001	0.0259
Amaranth (0.05mg/ml) vs. Amaranth (0.1mg/ml)	0.0004	< 0.0001
Amaranth (0.05mg/ml) vs. Ascorbic acid (0.05mg/ml)	> 0.9999	0.6104
Amaranth (0.05mg/ml) vs. Ascorbic acid (0.1mg/ml)	0.0164	< 0.0001
Amaranth (0.1mg/ml) vs. Ascorbic acid (0.05mg/ml)	0.0005	< 0.0001
Amaranth (0.1mg/ml) vs. Ascorbic acid (0.1mg/ml)	< 0.0001	0.0692
Ascorbic acid (0.05mg/ml) vs. Ascorbic acid (0.1mg/ml)	0.0131	< 0.0001

2.3 Catalase activity for Amaranth extract

Tukey's multiple comparisons test	Adjusted P Value	
Concentration (mg/ml)	**5 days**	**10 days**
Control vs. Amaranth (0.05mg/ml)	0.2433	0.018
Control vs. Amaranth(0.1mg/ml)	0.9826	0.3638
Control vs. Ascorbic acid(0.05mg/ml)	0.018	0.8199
Control vs. Ascorbic acid(0.1mg/ml)	0.0009	0.0556
Amaranth(0.05mg/ml) vs. Amaranth (0.1mg/ml)	0.5121	0.0003
Amaranth (0.05(mg/ml) vs. Ascorbic acid(0.05mg/ml)	0.6728	0.1547
Amaranth (0.05mg/ml) vs. Ascorbic acid(0.1mg/ml)	0.0943	0.9826
Amaranth(0.1mg/ml) vs. Ascorbic acid(0.05mg/ml)	0.0556	0.0556
Amaranth (0.1mg/ml) vs. Ascorbic acid(0.1mg/ml)	0.003	0.0009
Ascorbic acid(0.05mg/ml) vs. Ascorbic acid(0.1mg/ml)	0.6728	0.3638

2.4 Catalase activity for Cucurbita extract

Tukey's multiple comparisons test	Adjusted P Value	
Concentration (mg/ml)	**5 days**	**10 days**
Control vs. Cucurbita (0.05mg/ml)	0.7418	0.0451
Control vs. Cucurbita (0.1mg/ml)	0.7418	0.0237
Control vs. Ascorbic acid(0.05mg/ml)	0.0062	0.7418
Control vs. Ascorbic acid(0.1mg/ml)	0.0002	0.0237
Cucurbita (0.05mg/ml) vs. Cucurbita (0.1mg/ml)	> 0.9999	0.998
Cucurbita (0.05mg/ml) vs. Ascorbicacid (0.05mg/ml)	0.083	0.0031
Cucurbita (0.05mg/ml) vs. Ascorbic acid (0.1mg/ml)	0.0031	< 0.0001
Cucurbita (0.1mg/ml) vs. Ascorbic acid (0.05mg/ml)	0.083	0.0016
Cucurbita (0.1mg/ml) vs. Ascorbic acid (0.1mg/ml)	0.0031	< 0.0001
Ascorbic acid (0.05mg/ml) vs. Ascorbic acid (0.1mg/ml)	0.5612	0.2465

2.5 Lifespan (longevity) assay

Tukey's multiple comparisons test	Adjusted P Value
control vs. Amaranth (0.05mg/ml)	0.6782
control vs. Amaranth (0.1mg/ml)	0.9979
Control vs. Cucurbita (0.05mg/ml)	0.9994
Control vs. Cucurbita (0.1mg/ml)	0.9888
control vs. Ascorbic acid (0.05mg/ml)	0.9987
control vs. Ascorbic acid (0.1mg/ml)	0.9292
Amaranth (0.05mg/ml) vs. Amaranth (0.1mg/ml)	0.9349
Amaranth (0.05mg/ml) vs. Cucurbita (0.05mg/ml)	0.9053
Amaranth (0.05mg/ml) vs. Cucurbita (0.1mg/ml)	0.9788
Amaranth (0.05mg/ml) vs. Ascorbic acid (0.05mg/ml)	0.9218
Amaranth (0.05mg/ml) vs. Ascorbic acid (0.1mg/ml)	0.9983
Amaranth (0.1mg/ml) vs. Cucurbita (0.05mg/ml)	> 0.9999
Amaranth (0.1mg/ml) vs. Cucurbita (0.1mg/ml)	> 0.9999
Amaranth (0.1mg/ml) vs. Ascorbic acid (0.05mg/ml)	> 0.9999
Amaranth (0.1mg/ml) vs. Ascorbic acid (0.1mg/ml)	0.9981
Cucurbita (0.05mg/ml) vs. Cucurbita (0.1mg/ml)	> 0.9999
Cucurbita (0.05mg/ml) vs. Ascorbicacid (0.05mg/ml)	> 0.9999
Cucurbita (0.05mg/ml) vs. Ascorbic acid (0.1mg/ml)	0.995
Cucurbita (0.1mg/ml) vs. Ascorbic acid (0.05mg/ml)	> 0.9999
Cucurbita (0.1mg/ml) vs. Ascorbic acid (0.1mg/ml)	0.9999
Ascorbic acid (0.05mg/ml) vs. Ascorbic acid (0.1mg/ml)	0.997

2.6 Climbing activity assay

Tukey's multiple comparisons test	
Concentration (mg/ml)	**Adjusted P Value**
Control vs. Amaranth (0.05mg/ml)	0.0203
Control vs. Amaranth (0.1mg/ml)	0.0009
Control vs. Cucurbita (0.05mg/ml)	0.9926
Control vs. Cucurbita (0.1mg/ml)	0.2723
Control vs. Ascorbic acid (0.05mg/ml)	0.0046
Control vs. Ascorbic acid (0.1mg/ml)	0.0015
Amaranth (0.05mg/ml) vs. Amaranth (0.1mg/ml)	0.591
Amaranth (0.05mg/ml) vs. Cucurbita (0.05mg/ml)	0.0671
Amaranth (0.05mg/ml) vs. Cucurbita (0.1mg/ml)	0.7263
Amaranth (0.05mg/ml) vs. Ascorbic acid (0.05mg/ml)	0.9784
Amaranth (0.05mg/ml) vs. Ascorbic acid (0.1mg/ml)	0.7686
Amaranth (0.1mg/ml) vs. Cucurbita (0.05mg/ml)	0.0028
Amaranth (0.1mg/ml) vs. Cucurbita (0.1mg/ml)	0.0626
Amaranth (0.1mg/ml) vs. Ascorbic acid (0.05mg/ml)	0.9592
Amaranth (0.1mg/ml) vs. Ascorbic acid (0.1mg/ml)	0.9999
Cucurbita (0.05mg/ml) vs. Cucurbita (0.1mg/ml)	0.6139
Cucurbita (0.05mg/ml) vs. Ascorbic acid (0.05mg/ml)	0.0153
Cucurbita (0.05mg/ml) vs. Ascorbic acid (0.1mg/ml)	0.0049
Cucurbita (0.1mg/ml) vs. Ascorbic acid (0.05mg/ml)	0.288
Cucurbita (0.1mg/ml) vs. Ascorbic acid (0.1mg/ml)	0.1075
Ascorbic acid (0.05mg/ml) vs. Ascorbic acid (0.1mg/ml)	0.9947

2.7 Oxidative stress assay

Tukey's multiple comparisons test	Adjusted P Value			
Concentration (mg/ml)	**Day 1**	**Day 5**	**Day 10**	**Day 15**
Control vs. H202 (1%)	> 0.9999	0.217	0.01	0.0131
Control vs. Amaranth (0.05mg/ml) +H202 (1%)	> 0.9999	0.2654	0.01	0.0131
Control vs. Amaranth (0.1mg/ml) +H202 (1%)	> 0.9999	0.8681	0.0454	0.0131
Control vs. Cucurbita (0.05mg/ml)+H202 (1%)	> 0.9999	0.321	0.01	0.0131
Control vs. Cucurbita (0.1mg/ml)+H202 (1%)	> 0.9999	0.4056	0.0246	0.0131
Control vs. Ascorbic acid (0.05mg/ml) +H202 (1%)	> 0.9999	0.723	0.0246	0.0131
Control vs. Ascorbic acid (0.1mg/ml) +H202 (1%)	> 0.9999	0.9483	0.0749	0.0131
H202 (1%) vs. Amaranth (0.05mg/ml) +H202 (1%)	> 0.9999	> 0.9999	> 0.9999	> 0.9999
H202 (1%) vs. Amaranth (0.1mg/ml) +H202 (1%)	> 0.9999	0.9138	0.9962	> 0.9999
H202 (1%) vs. Cucurbita (0.05mg/ml)+H202 (1%)	> 0.9999	> 0.9999	> 0.9999	> 0.9999
H202 (1%) vs. Cucurbita (0.1mg/ml)+H202 (1%)	> 0.9999	0.9999	0.9999	> 0.9999
H202 (1%) vs. Ascorbic acid (0.05mg/ml) +H202 (1%)	> 0.9999	0.9778	0.9999	> 0.9999
H202 (1%) vs. Ascorbic acid (0.1mg/ml) +H202 (1%)	> 0.9999	0.8117	0.9778	> 0.9999
Amaranth (0.05mg/ml) +H202 (1%) vs. Amaranth (0.1mg/ml) +H202 (1%)	> 0.9999	0.9483	0.9962	> 0.9999
Amaranth (0.05mg/ml)+H202 (1%) vs. Cucurbita (0.05mg/ml)+H202 (1%)	> 0.9999	> 0.9999	> 0.9999	> 0.9999

Amaranth (0.05mg/ml)+H202 (1%) vs. Cucurbita (0.1mg/ml)+H202 (1%)	> 0.9999	> 0.9999	0.9999	> 0.9999
Amaranth (0.05mg/ml) +H202 (1%) vs. Ascorbic acid (0.05mg/ml) +H202 (1%)	> 0.9999	0.9899	0.9999	> 0.9999
Amaranth (0.05mg/ml) +H202 (1%) vs. Ascorbic acid (0.1mg/ml) +H202 (1%)	> 0.9999	0.8681	0.9778	> 0.9999
Amaranth (0.1mg/ml)+H202 (1%) vs. Cucurbita (0.05mg/ml)+H202 (1%)	> 0.9999	0.972	0.9962	> 0.9999
Amaranth (0.1mg/ml)+H202 (1%) vs. Cucurbita (0.1mg/ml)+H202 (1%)	> 0.9999	0.9899	> 0.9999	> 0.9999
Amaranth (0.1mg/ml) +H202 (1%) vs. Ascorbic acid (0.05mg/ml) +H202 (1%)	> 0.9999	> 0.9999	> 0.9999	> 0.9999
Amaranth (0.1mg/ml) +H202 (1%) vs. Ascorbic acid (0.1mg/ml) +H202 (1%)	> 0.9999	> 0.9999	> 0.9999	> 0.9999
Cucurbita (0.05mg/ml)+H202 (1%) vs. Cucurbita (0.1mg/ml)+H202 (1%)	> 0.9999	> 0.9999	0.9999	> 0.9999
Cucurbita (0.05mg/ml) +H202 (1%) vs. Ascorbic acid (0.05mg/ml) +H202 (1%)	> 0.9999	0.9962	0.9999	> 0.9999
Cucurbita (0.05mg/ml) +H202 (1%) vs. Ascorbic acid (0.1mg/ml) +H202 (1%)	> 0.9999	0.9138	0.9778	> 0.9999
Cucurbita (0.1mg/ml) +H202 (1%) vs. Ascorbic acid (0.05mg/ml) +H202 (1%)	> 0.9999	0.9993	> 0.9999	> 0.9999
Cucurbita (0.1mg/ml) +H202 (1%) vs. Ascorbic acid (0.1mg/ml) +H202 (1%)	> 0.9999	0.9573	0.9993	> 0.9999
Ascorbic acid (0.05mg/ml) +H202 (1%) vs. Ascorbic acid (0.1mg/ml) +H202 (1%)	> 0.9999	0.9993	0.9993	> 0.9999