IN VITRO ASSESSMENT OF *KALANCHOE PINNATA* AND METFORMIN PREPARATION

A Thesis

by

PEDRO RAMON

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This thesis meets the standards for scope and quality of
Texas A&M University-Corpus Christi and is hereby approved.

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ABSTRACT

Metformin is the most common oral medication prescribed by doctors for the management of type II diabetes. As healthcare costs continue to rise, many affected by the disease are turning to alternative medicine for treatment and are combining them with modern medicine. *Kalanchoe pinnata* (*K. pinnata*) is currently being used as a "cure-all" by herbal practitioners in various countries. However, there is a shortage of scientific literature that supports the use of *K. pinnata* as a treatment for diabetes, the safety of the plant extract, and possible drug interactions for those concurrently taking metformin. In this study, combined preparations of *K. pinnata* and metformin were investigated to determine cytotoxicity, anti-oxidative, and anti-inflammatory activity in human skeletal muscle myoblasts (HSMM) and human diabetic skeletal muscle myoblasts (DHSMM). The effects of the combined preparations on the cytotoxicity of stress induced hydrogen peroxide-challenge in HSMM cell cultures were determined.

Results showed that combinatorial preparations sustained cell viability for three days in both HSMM and DHSMM cells. However, a significant decrease in cellular viability occurred for both cell lines 72 hours post-treatment with a combinatorial preparation. They also showed that a *Kalanchoe pinnata* treatment (400 μg/mL) significantly increased catalase (CAT) activity for non-diabetic and diabetic human skeletal muscle myoblasts and an H2O2 stress-induced human skeletal muscle myoblast cell line, and significantly lowered malondialdehyde (MDA) levels. Diabetic and non-diabetic human skeletal muscle cells showed increased superoxide dismutase (SOD) activity when treated with metformin only. Stress induced HSMM cells were observed to have significantly higher SOD activity when treated with a combinatorial preparation of metformin and *K. pinnata* (100 μM metformin with 100 μg/mL *K. pinnata*). Combined metformin and *K. pinnata* at concentrations of 50 μM and 150 μg/mL respectively resulted in a

significant increase in reduced glutathione levels in non-diabetic and diabetic human skeletal muscle myoblasts, and H2O2 stress-induced human skeletal muscle myoblasts. Combined preparations of metformin and *K. pinnata* may modulate immune responses by significantly elevating concentrations of interleukin (IL) 2, tumor necrosis factor-alpha (TNF-α), and interleukin-10 in HSMM and DHSMM cells. Treatment with 400 μg/mL of *K. pinnata* only reduces oxidative damage to human skeletal muscle. However, the combined preparation of metformin and *K. pinnata*, at concentrations of 50 μM and 150 μg/mL respectively was noted to be an effective treatment for lowering cellular oxidation by increasing the level of reduced glutathione in the cell.

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Finally, I dedicate this work to my family and friends who have shown constant support, encouragement, and unconditional love. This research project was an intense undertaking, but they made it possible to complete.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

1.1 Diabetes Mellitus

Diabetes Mellitus, more simply known as "diabetes," is a chronic disease identified by abnormally high levels of glucose in the bloodstream [1]. Diabetes has been confirmed to be caused by one of two mechanisms [2]. The first mechanism leads to type I diabetes, which is the inadequate or non-insulin production by beta cells within the pancreas [2]. Patients diagnosed with type I diabetes must inject insulin into their bloodstream to control blood glucose concentrations. The second mechanism leads to type II diabetes, which is a decreased sensitivity of cells to insulin or a defect in insulin secretion by the beta cells of the pancreas [2]. Type II diabetic patients do not respond to the increasing concentration of glucose in the blood after a meal [1]. In either type, when glucose levels become significantly high, the risk of health complications significantly increases [1,3]. Diabetes has the potential to damage organs such as the eyes, kidneys, and nerves [1]. Diabetes has also been linked to heart disease, stroke, and the loss of extremities [3,4]. In 2017, 30.3 million people in the U.S. alone were diagnosed with diabetes, an increase from 26 million in 2010 [5]. Another 86 million adults in the U.S. have prediabetes, a condition in which blood glucose levels are higher than normal, yet not high enough to be diagnosed with the disease [5]. The American Diabetes Association (ADA) defines pre-diabetes as a range of blood glucose concentration from 100 mg/dL to 125 mg/dL [6]. Normal blood glucose levels for non-diabetic patients range from 70 to 100 mg/dL [7]. Those commonly affected by diabetes are Blacks, Hispanics, and American Indians; these ethnic groups are twice as likely to be diagnosed compared to Caucasians [8]. To help better manage type II diabetes, a combination of lifestyle changes with pharmacological medication is necessary [7]. For example the Centers for Disease Control and Prevention (CDC) reports that maintaining a

sedentary lifestyle, will result in a 15 to 30 percent increased chance that prediabetic patients will advance to type II diabetes [9]. Metformin is a prescription drug commonly prescribed to those with type II diabetes [9,10]. Metformin, along with physical activity, is usually the first combinatorial treatment offered by physicians to those with type II diabetes [11].

1.2 Treatment options for type II diabetes

There are various classes of drugs that lower blood glucose levels in type II diabetes. One class, the sulfonylureas, has been used since the 1950s to stimulate the beta cells of the pancreas to release more insulin [12]. There are two generations of sulfonylureas, although chlorpropamide is the only first-generation sulfonylurea still used today [12]. Three second-generation drugs used to treat hyperglycemia in type II diabetic patients, glipizide, glyburide, and glimepiride, are administered in smaller doses than the first-generation sulfonylureas. All sulfonylurea drugs have similar effects on blood glucose levels, but differ in their side effects, frequency taken, and their interactions with other drugs [12].

Another class of drug used in the treatment of type II diabetes is the meglitinides. The meglitinides are similar to sulfonylureas, in that they also stimulate beta cells to release insulin [13]. Repaglinide and nateglinide are examples of meglitinides that are taken before meals. Since both sulfonylureas and meglitinides stimulate the release of insulin, it is possible for users to experience hypoglycemia.

Rosiglitazone and pioglitazone are in a group of drugs known as thiazolidinediones. These drugs improve insulin sensitivity in muscle and adipose tissues while reducing glucose production in the liver [12]. Patients using thiazolidinediones are closely monitored for liver problems as a precaution since thiazolidinediones are hepatotoxic. Thiazolidinediones may also increase the

risk of heart failure in some individuals, and there is a debate about whether rosiglitazone contributes to an increased risk for heart attacks [14].

Metformin is another type II diabetes oral medication marketed under the generic trade name Glucophage. Metformin is usually well tolerated. Despite being commonly prescribed, metformin is contraindicated in people with any condition that increases the risk of lactic acidosis, for example, those with kidney disorders, lung or liver disease. According to the Food and Drug Administration (FDA), users who experienced heart failure are at an increased risk of lactic acidosis if given metformin. Despite the adverse effects of metformin, it is efficient at lowering both basal and postprandial plasma glucose [15]. It can significantly reduce the rate of glucose production in patients through a reduction in glucogenesis [16]. Metformin works by decreasing hepatic glucose production, decreasing intestinal absorption of glucose, and improving insulin sensitivity within cells [17]. Metformin has an oral bioavailability of up to 60% under fasting conditions and is absorbed slowly [17]. Studies using intravenous single-dose metformin have demonstrated that it does not undergo hepatic metabolism nor biliary excretion [17]. Metformin is excreted unchanged in the urine [17]. Renal clearance is approximately three and a half times greater than creatinine clearance, indicating that tubular secretion is the primary route of elimination [17]. According to the FDA, 90% of the drug is eliminated through the renal route within the first 24 hours, with a plasma elimination half-life of about 6 hours [17].

Metformin is a member of the biguanide class of antidiabetic medications, which originates from *Galega officinalis*, a French lilac commonly used for centuries in folk medicine. French diabetologist Jean Sterne first studied the antihyperglycemic properties of *galegine*, an alkaloid isolated from *Galega officinalis*, that has a chemical structure similar to metformin [18]. The medicinal study of local plants may be beneficial to the scientific community by elucidating

useful chemical compounds that have therapeutic properties. Another plant that may have anti-hyperglycemic properties and needs further study is *Kalanchoe pinnata*.

1.3 Herbal treatment of type II diabetes with *K. pinnata* preparation

Kalanchoe pinnata is a plant native to the Caribbean, Africa, and South America [19]. The leaves and the stems of the plant have been used for the treatment of various diseases and ailments [20]. The chemical compounds contained within the leaves of *K. pinnata* include alkaloids, glycosides, and steroids, among many others [21]. Crude extracts of *K. pinnata* (crushed leaves and roots) have shown antiviral activity [22]. Common compounds in *K. pinnata* that have been investigated to reduce inflammation, and produce antibacterial and antiviral properties, include flavonoids and phenols [23]. Recent scientific research into *K. pinnata* has explored only the antibacterial and antiviral properties of the plant. However, *K. pinnata* is being used as a "master herb" or "cure-all" by communities of tribal and herbal practitioners in various countries [20]. Despite *K. pinnata's* use for a variety of medicinal purposes, research into the use of *K. pinnata* for the treatment of diabetes and diabetic inflammation is limited [19]. Little is known regarding the use of *K. pinnata* as a treatment for diabetes, including the safety of the plant extract, and its possible interference with metformin. As healthcare costs continue to rise, many are turning to alternative medicines for treatment and are combining them with modern medicine.

1.4 Type II diabetic inflammation

Inflammation is a common risk factor with diabetes and is caused by pro-inflammatory mediators [24]. Pickup et al. revealed that pro-inflammatory mediators such as IL-2 and tumor necrosis factor-alpha are statistically higher in diabetic patients [24]. As high glucose

concentration builds up in the blood of diabetic patients, the rate of oxidative stress in cells increases, forcing them to secrete pro-inflammatory mediators in response [25]. Hyperglycemia also reduces the functionality of neutrophils by diminishing chemotaxis, phagocytic activity, and bactericidal ability [25]. According to Furnary et al., the neutrophilic function is reduced in proportion to the increase in blood glucose level, where 200 mg/dL is the threshold of neutrophilic dysfunction [26]. Oxidative stress is also created as adenosine triphosphate (ATP) is produced by the mitochondrial electron transfer system. Water molecules are generated as oxygen is reduced by the systems, but reactive oxygen species (ROS) can be produced as a byproduct. The oxygen used during ATP synthesis can lead to the production of superoxide anions. In a hyperglycemic state, superoxide anion production increases [27]. If cellular oxidation occurs at a fast rate, free radicals will begin to accumulate in the bloodstream. The free radicals are capable of damaging proteins and nucleic acids, vital to cellular function. Matough et al. demonstrated that type II diabetes induces the generation of free radicals while suppressing cellular antioxidant defenses [28]. Kawahito et al. reported that oxidative damage leads to further diabetic complications in those affected by the disease [25]. Since diabetes has complex pathophysiology, effective diabetic treatment may require a multi-target, multi-effect approach to prevent complications. For example, a preliminary study done by Menon et al. demonstrated that diabetic rats administered aqueous *K. pinnata* showed a decrease in body weight [29].

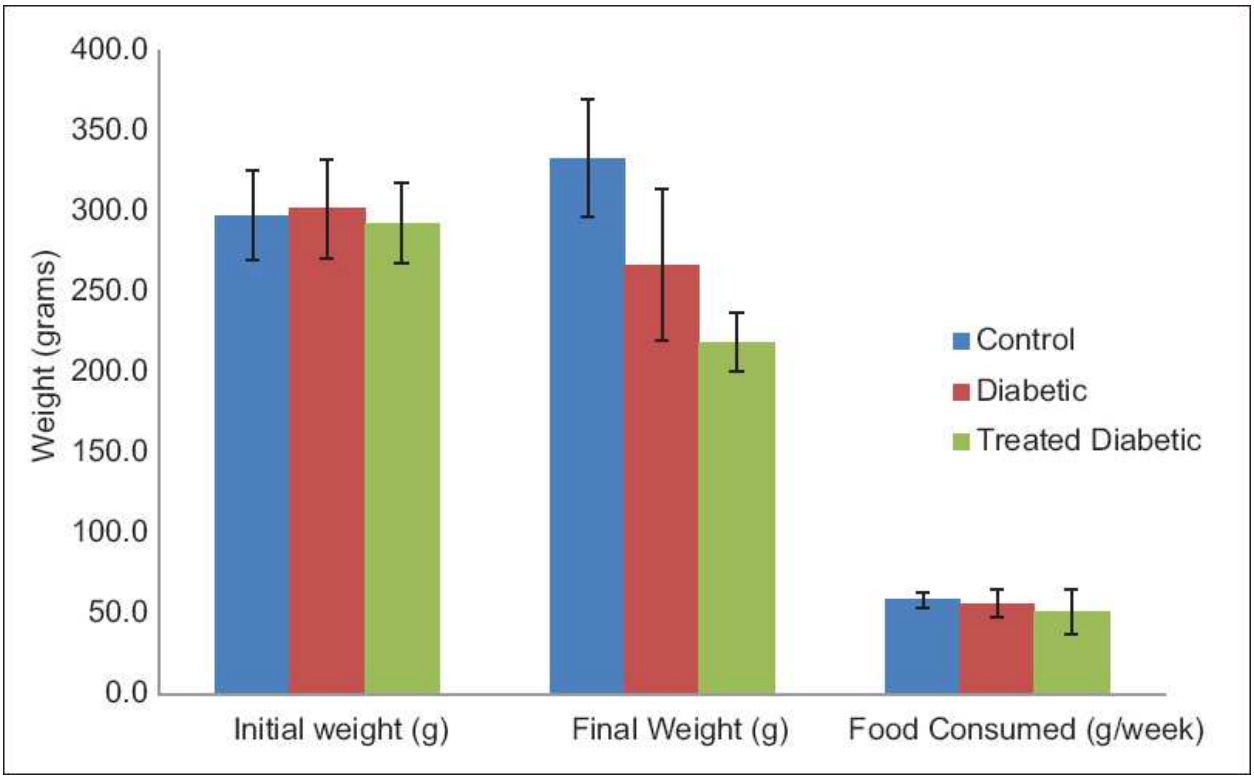

Figure 1: Trends regarding the bodyweight changes and food intake of diabetic rats administered aqueous preparation of *Kalanchoe pinnata*. Means ± standard error of the mean, values were not significantly different among the groups ($P > 0.05$) Taken from Menon et al.

The study also concluded that *K. pinnata* administration reduced fasting blood glucose in diabetic rats [29]. However, in another study, *K. pinnata* consumption increased alkaline phosphatase (ALP) and alanine aminotransferase (ALT) activities, along with uric acid levels in type II diabetic rats [30]. Higher than normal levels of ALP in the blood may indicate problems with either liver or gallbladder function, while elevated uric acid levels in the blood are associated with kidney failure [31,32]. High levels of ALP combined with an increasing concentration of ALT is indicative of liver inflammation [31]. Many hypoglycemic drugs administered to diabetic patients have undesirable side effects. Therefore, it is imperative to develop a combined multi-approach treatment option that is both safe and effective. Unlike metformin, research into the clinical application of *K pinnata* has been limited. The effect of an

aqueous preparation of *K. pinnata* on diabetic cells is not understood. This study is aimed at determining the effect of K. *pinnata* and metformin combinatorial preparations on the viability and biochemistry of diabetic cells in an in-vitro model of the disease.

It will determine the cytotoxic effect of the preparation on HSMM and DHSMM cells, the effect of the preparation on antioxidants, as measured by superoxide dismutase activity, catalase activity, reduced glutathione and malondialdehyde (MDA) levels, and the inflammatory effect of the preparation, as measured by the concentrations of cytokines (IL-2 and TNF-α) and anti-inflammatory mediators (IL-6 and IL-10).

1.5 Objectives

Objective 1:

Determine the cytotoxicity of a combined *K. pinnata* and metformin preparation on human skeletal muscle myoblasts and human diabetic skeletal muscle myoblast cell cultures. It was hypothesized that combinatorial treatments of *K. pinnata* and metformin will result in increased cellular viability.

Objective 2:

Determine if a combined *K. pinnata* and metformin preparation will prevent oxidative stress in HSMM and DHSMM *in vitro* cells. It was predicted that combinatorial treatment of *K. pinnata* and metformin will reduce oxidative damage to both diabetic and non-diabetic human skeletal muscle myoblasts via an increase in antioxidative activities

Objective 3:

Determine if a combined treatment of *K. pinnata* and metformin preparation will lower pro-inflammatory cytokine concentrations in cultured cells. It was theorized that oxidative stress stimulates pro-inflammatory mediators causing the development of a disease state. Therefore, it is anticipated that improved antioxidant activities would lead to a reduction in pro-inflammatory markers expressed by human skeletal muscle myoblasts.

2. MATERIALS AND METHODS

2.1 Extract and sample preparation

Preparation of *K. pinnata* extract

The concentrations for *K. pinnata* in combination with metformin ranged from 0-400 μg/mL, due to similar ranges used in other studies [33–35]. A relatively high dose of 400 ug/mL of *K. pinnata* only was chosen as a treatment option since higher concentrations of phytochemicals are present in larger concentrations of crude *K. pinnata* extracts [36]. The components of *K. pinnata* were extracted by the hot water extraction method [37]. Ten leaves of *K. pinnata* were ground up using a pestle and mortar. The crushed leaves were then mixed with 100 mL of distilled water and stirred at 71°C for 12 hours. The solution was then filtered by gravity filtration so that any insoluble materials were removed. The supernatant was freeze-dried. For subsequent cell culture experiments, the *K. pinnata* leaf extract was weighed and then dissolved in dimethyl sulphoxide (DMSO) (Corning Cellgro, Virginia, USA) and then passed through a 0.20 μm filter. Working concentrations of the plant extract at 0, 25, 50, 100, 150, 200, 300, and 400 μg/mL were prepared by diluting the stock solution in DMSO.

Preparation of metformin solutions

Metformin tablets (500 mg) were crushed up with a pestle and mortar and suspended in distilled water to create 0, 10, 30, 50, 100, 150, and 200 μM, and 5mM solutions. A 5 mM metformin concentration only treatment option was examined as it as an effective dose for diabetics [38–40]. Micromolar concentrations potentially have protective properties against cell death and senescence [41]. According to He et al. low metformin concentrations (< 50μM) are achieved in the portal vein as well as in tissue circulation [42]. He et al. also suggested that metformin concentrations greater than 80 μM are unachievable in the portal vein and are therefore clinically irrelevant or even toxic [42]. Therefore, a range of 0-200 μM for the combination preparations, and one 5 mM concentration of metformin were investigated.

Preparation of cultured human skeletal muscle cells

Human skeletal muscle myoblast and human diabetic muscle myoblast cells (Lonza, Maryland, USA) were cultured at the concentration of 5 x 10^5 cells/mL in skeletal muscle growth media-2 (SkGM™-2 Medium) containing human Epidermal Growth Factor [hEGF], Dexamethasone, L-glutamine, Fetal Bovine Serum [FBS], Gentamicin/Amphotericin-B [GA], 50 U/mL of penicillin, and 50 mg/mL streptomycin. The cell suspension was incubated at 37°C in a humidified atmosphere containing 5% CO_2. When cell growth reached 10^6 cells/mL, cells were harvested according to the manufacturer's instructions (Lonza, Maryland, USA).

Determination of cell concentration

The number of viable cells was assessed by a 0.4% trypan blue solution. A volume of 100 μL of 0.4% trypan blue solution was transferred to a 0.5 mL microcentrifuge tube followed by adding 100 μL of cell suspension and vortexed thoroughly. The dilution factor used throughout this study was 2. Ten microliters of the cellular solution were pipetted into a hemocytometer chamber

and covered with a glass coverslip. The number of cells in the one-millimeter-center square and four one millimeter-corner squares were counted. A factor of 10^4 was used in cell counting to take into account that each square has a volume of 0.0001 mL (1 mm x 1 mm x 0.1 mm = 0.1 mm^3). Non-viable cells were stained blue while viable cells remained transparent. The total number of cells per unit volume was determined by the following formula:

Cells/mL= average viable cells counted/ number of squares counted x dilution factor x 10^4.

2.2 Cytotoxicity

Assessment of cytotoxicity

CCK-8 cytotoxicity assay was used to determine cell viability and cell proliferation of in- vitro cell cultures [43]. The CCK-8 assay utilized the enzymatic activity of dehydrogenase within living cells. A water-soluble tetrazolium salt (WST-8) was reduced by dehydrogenases in cells to give an orange color indicating dye (formazan product) [43]. The amount of formazan generated was proportional to the number of viable living cells [43].

One hundred µL of cell suspension (10^6 cells/mL) were pipetted into each well of a 96 well plate. The plate was pre-incubated for 24 hours in a humidified incubator at 37°C with 5% CO_2 [43]. Afterward, 10 µL of varying concentrations of combined metformin and *K. pinnata* preparation were added to the well plate to test for cytotoxicity (ratio of treatment and the cell suspension was 1:10 v/v). The well plate was then incubated again for 48 hours. At the end of the time interval, 10 µL of CCK-8 solution was added to each well of the plate and re-incubated for 4 hours at 37°C [43]. The absorbance of the formazan dye was measured at 450 nm with a spectrophotometric plate reader [43]. The cell viability was calculated by the following formula:

$$\text{Cell Viability (\%)} = \frac{A_{substance} - A_{blank}}{A_{0\ substance} - A_{blank}} \times 100$$

$A_{substance}$: absorbance of a well with cells, CCK-8 solution, and substance solution.

$A_{blank:}$ absorbance of a well with medium and CCK-8 solution, without cells.

$A_{0\ substance}$: absorbance of a well with cells, CCK-8 solution, without substance solution.

2.3 Antioxidant activity

Induction of oxidative stress in cell lines

Three separate sets of cultured cells (control, HSMM under H_2O_2 -induced oxidative stress, DHSMM) were used to determine how combined preparations of *K. pinnata* and metformin affected human muscle cell lines under various oxidative situations. To induce oxidative stress in HSMM cultured cells, a concentration of 0.1 M of H_2O_2 was added to fresh cell culture medium. The final working concentration of hydrogen peroxide in the cell culture was 50μM. After the cell cultures were suspended in the prepared medium containing 50μmol of H_2O_2 and the cells adjusted to a concentration of 10^6 cells/mL, the combined experimental preparation was added. Following treatment, cell pellets were harvested for the assays of lipid peroxidation and reduced glutathione levels, along with superoxide dismutase and catalase activities.

Measurement of superoxide dismutase activity

Cell pellets (10^6 cells/mL) were lysed by sonication in a cold 20 mM Hepes buffer (pH=7.2) containing 1mM EGTA, 210 mM mannitol, and 70 mM sucrose. The lysate was then centrifuged for 5 minutes [33,34]. Cellular supernatant was used for the measurement of superoxide dismutase activity. A one-milliliter reaction mixture contained 500 μL of 0.1 M sodium phosphate buffer, 32 μL of 3.3 mM ethylenediaminetetraacetic acid (EDTA), 60 μL of 8.1 mM pyrogallol, and an appropriate amount of cellular supernatant that contained 11.51mg/dL of protein. The enzymatic activity of superoxide dismutase was determined by measuring a change

in absorbance at 420 nm against a blank for 2 minutes that contained all the ingredients except cellular supernatant. One unit of enzyme was defined as the amount of enzyme that caused half-maximal inhibition of pyrogallol autoxidation [44].

Measurement of catalase activity

Cells were collected by centrifugation at 2,000 x g for 10 min. at 4°C. The cell pellet was sonicated on ice in 1-2 ml of cold buffer (50 mM potassium phosphate, pH 7.0 containing 1 mM EDTA), and then centrifuged at 10,000 x g for 15 minutes at 4°C. The supernatant was removed for the catalase assay. The catalase assay was conducted according to the method of Sinha [45]. Briefly, 200 µL of extract sample was added with 400 µL 0.1 M phosphate buffer (pH=7.5) and 400 µL of 0.2 M H_2O_2 to create a 1 mL sample solution. Two milliliters of 5% potassium dichromate and glacial acetic acid (1:3 v/v) were added to the sample. The mixture was then heated in a water bath for 10 minutes and allowed to cool. The absorbance was read at 570 nm against a reagent blank using a spectrophotometric plate reader.

Determination of reduced glutathione

The reduced GSH levels were measured by following Elman's method [46]. The harvested pellets were lysed in a hypotonic solution for 45 minutes at 37°C and then processed for the assay [47]. One hundred microliters of the lysate were mixed with 10% trichloroacetic acid (TCA) and centrifuged at 2000 x g for 15 minutes. One milliliter of supernatant was treated with Ellman's reagent [19.8 mg of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) in 100 mL of 0.2 phosphate buffer (pH 8)]. The absorbance was read at 412 nm.

Determination of Malondialdehyde (MDA)

This study utilized the thiobarbituric acid reacting substances test (TBARS) method for investigating lipid peroxidation in cells after the treatment of a combined *K. pinnata* and metformin preparation. Cells were lysed in ice-cold physiological saline, followed by centrifugation at 28,000 x g for 5 minutes at 4°C [48]. The cellular supernatant was used for the measurement of MDA [48]. The supernatant was mixed with Thiobarbituric Acid (TBA). Samples and MDA standards (16.7, 8.35, 4.18, 2.09, 1.04, 0.52, 0.26, and 0 μM) were acid treated with 10% (w/v) trichloroacetic acid (TCA) [49]. A volume of 150 μL of MDA standards and cellular supernatant was added to the wells of a 96 well plate. Afterward, 75 μL of TBA was added to each well. The absorbance of each well was pre-read using a microplate reader set to 532 nm. Once the pre-read absorbance data was collected, the well plate was covered with a plate cover and incubated for 3 hours at 50°C. Absorbance was collected again after incubation using a microplate reader set to 532 nm. The pre-read absorbance was subtracted from the final absorbance reading to correct for the sample's contribution to the final absorption at 532 nm. A standard curve was constructed from the standards and the concentrations of MDA within the samples were extrapolated from the standard curve. The total protein in the cellular supernatant was determined using a Stanbio kit [50].

2.4 Cytokine Quantitation

The cytokines of interest included IL-2, IL-6, IL-10, and TNF-α, and the concentrations were monitored using enzyme-linked immunosorbent assay (ELISA) kits. Each ELISA kit used a specific monoclonal antibody that specifically binds to the respective cytokine [51]. ELISA experiments were performed according to their respective antibody as instructed in the BD OptEIA human ELSA kit [52]. It should be noted that for all ELISA studies, interference by drug metabolites, soluble receptors, or other binding proteins in specimens has not been thoroughly

investigated in the use of ELISA [52]. Therefore, the possibility of interference cannot be excluded.

Fifty μL of diluted ELISA reagent was added to each well of an antibody-coated well plate. Then, 100 μL of each standard and diluted sample were added to the well plate. The plate was covered with a plate sealer and incubated for 2 hours at room temperature. Afterward, the wells were aspirated and washed with a wash buffer solution. The washing step was repeated 5 times using 300 μL/well for a total of 5 washes. Any residual buffer was removed by blotting the plate onto absorbent paper to drain excess solution. Next, 100 μL of a detection solution was pipetted to each well followed by further incubation in the dark for 1 hour at room temperature. The wells were aspirated and washed again but for a total of 7 times. Afterward, 100 μL of 3,3',5,5'-tetramethylbenzidine (TMB) was added to each well and the plate was incubated for 30 minutes at room temperature [51]. The TMB was used as an indicator dye which turned blue in proportion to the amount of cytokine present [51]. Afterward, a 50 μL stop solution was added, which turned the blue dye to yellow [51]. The color intensity of each well was measured with a spectrophotometric plate reader at 450 nm within 30 minutes after the addition of the stop solution [51]. All reagents and samples were brought to room temperature before the ELISA procedure was performed. A standard curve was used for each assay to determine cytokine concentration levels of samples.

2.5 Statistical Analysis

All data were obtained from three separate experiments, and each experiment included three controls (metformin only, *K. pinnata* extract only, and untreated) and combined *K. pinnata* and metformin preparation performed in triplicate. The data is presented as the mean ± standard error of the mean. The results among different concentrations were evaluated by one-way ANOVA

($P<0.05$). Post hoc analysis was performed using Duncan's multiple range test significance level ($P<0.05$) to test for a significant difference among the means.

3. RESULTS

3.1 Cytotoxicity of combined metformin and *K. pinnata* preparations on human skeletal muscle myoblasts

The cytotoxicity of a combined *K. pinnata* and metformin preparation at various concentrations were investigated in both HSMM and DHSMM cell lines. Several combinatorial preparations were examined. Figure 2 shows the change in the cell viability of human skeletal muscle myoblasts over seven days of incubation after the addition of either metformin, *K. pinnata*, or combinatorial preparations.

On day 1, the cellular viability of HSMM cells treated with either combinatorial preparations or 5 mM metformin only were not significantly different. The *K. pinnata* only treatment showed a significant decrease in cellular viability. By day 3, HSMM cells treated with metformin only, *K. pinnata* only, and two of the combinatorial treatments (150 μM metformin/50 μg/mL *K. pinnata*; 10 μM metformin/300 μg/mL *K. pinnata*) showed a decrease in cell viability. The other combinatorial treatments showed a significant increase in HSMM cell growth. Interestingly, all but one combined preparation (10 μM metformin/300 μg/mL *K. pinnata*) showed higher HSMM cell viability compared to metformin or *K. pinnata* alone.

Human skeletal muscle myoblast cells treated with *K. pinnata* only showed complete cell death by day 5 (Figure 2). However, HSMM cells remained viable with most other treatment options but with low cellular confluency. Human skeletal muscle myoblasts treated with combined 10

μM of metformin and 300 μg/mL of *K. pinnata* exhibited a decrease in cell viability on day 5 but increased by day 7.

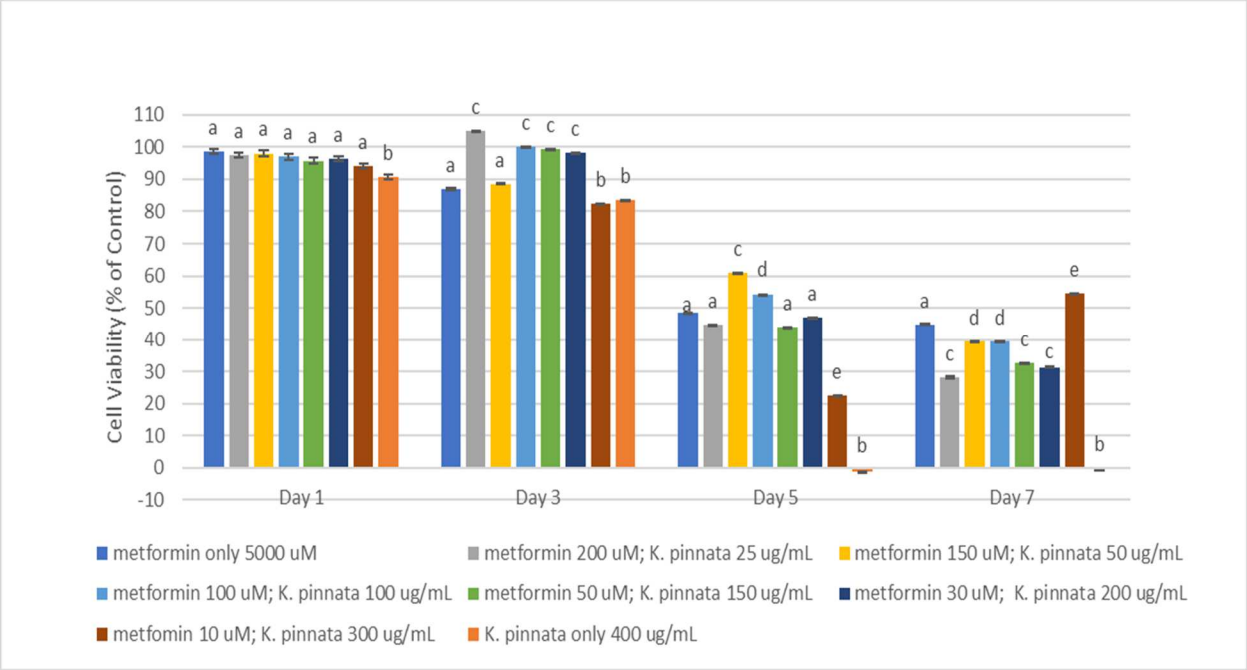

Figure 2: **Change in the Viability of HSMM Treated with Metformin, *K. pinnata*, and Different Combinations of Metformin and *K. pinnata*.** One-way ANOVA and Duncan's multiple range test were used to statistically compare the difference in cell viability of HSMM after treatment. Columns with different letters are significantly different from one another ($P<0.05$).

Figure 3 illustrates the change in cell viability of human diabetic skeletal muscle myoblasts over a period of 7 days of incubation after the addition of metformin, *K. pinnata*, and various combinations of metformin and *K. pinnata*. Twenty-four hours post-treatment, DHSMM myoblasts showed sustained viability for all treatments except those with high *K. pinnata* concentrations (Figure 3). By day 3, there were increases in cell viability across all treatment options. By day 5, viability had decreased significantly across all treatments, especially for *K. pinnata* only, a trend that continued through day 7. Overall, the data indicate that a combinatorial preparation of *K. pinnata* and metformin promoted higher cellular viability in both HSMM and DHSMM cells compared to a *K. pinnata* alone.

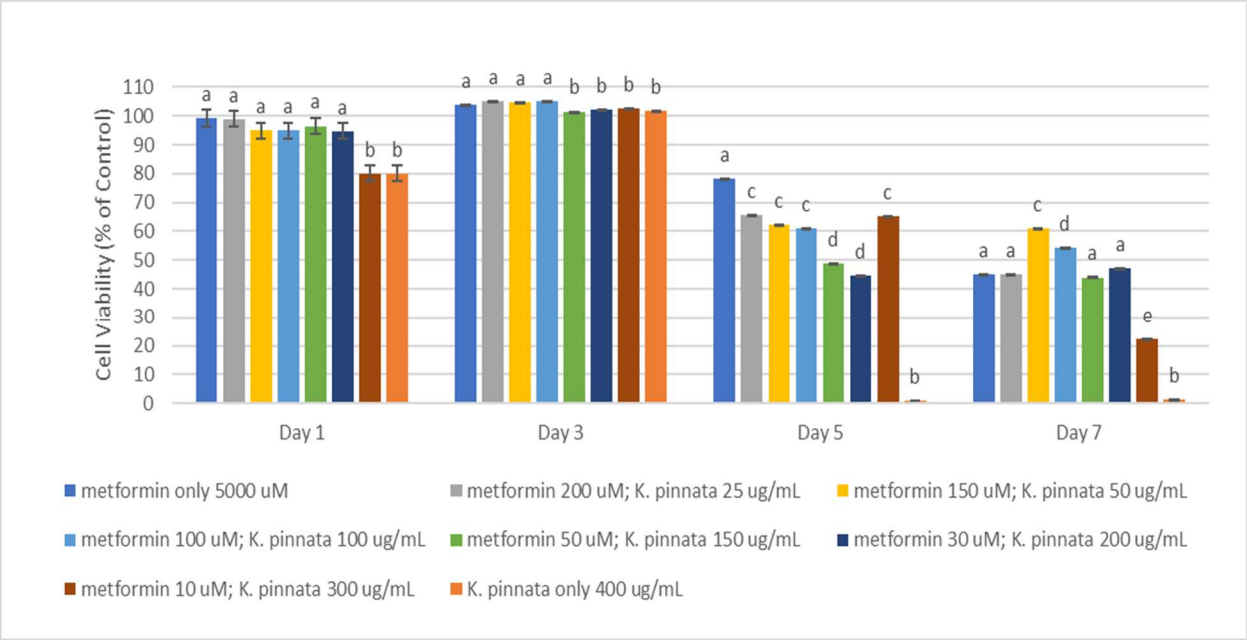

Figure 3: **Change in the Viability of DHSMM Treated with Metformin, *K. pinnata*, and Different Combinations of Metformin and *K. pinnata*.** One-way ANOVA and Duncan's multiple range test were used to statistically compare the difference in cell viability of DHSMM after treatment. Columns with different letters are significantly different from one another ($P<0.05$).

3.2 Effects of combined metformin and *K. pinnata* preparations on H_2O_2 stress-induced human skeletal muscle myoblasts

Figure 4 shows the cellular viability over a 48-hour period of H_2O_2 stress-induced human skeletal muscle myoblasts after applied treatments with metformin only, *K. pinnata* only, or a combined metformin/*K. pinnata* preparation. Oxidative stress by H_2O_2 significantly reduced the cellular viability of HSMM cells. HSMM cells treated with hydrogen peroxide (OX) demonstrated cellular viability levels five times lower than non-oxidatively stressed HSMM cells 24 hours post-treatment. By day 3 cell viability levels for all treated cells had decreased drastically. A downward trend in viability was observed as K. pinnata concentrations increased.

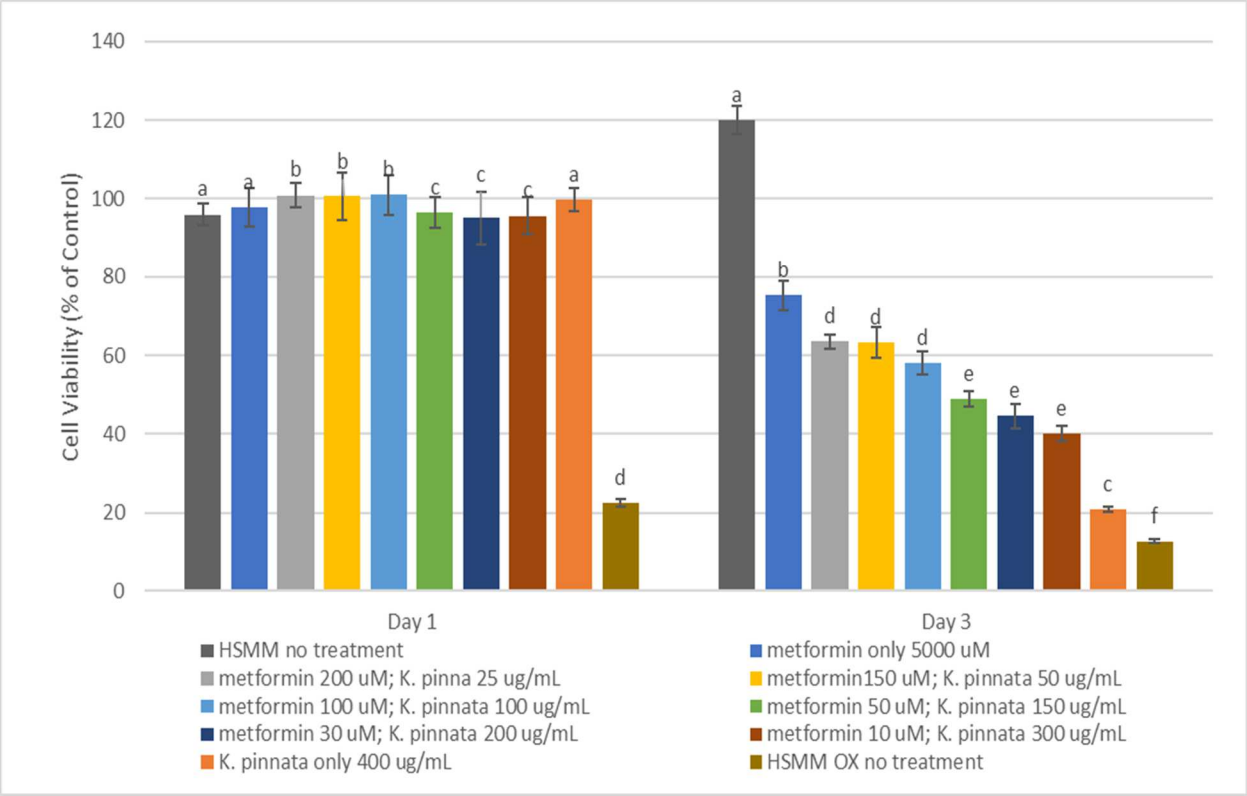

Figure 4: **Cell Viability of Stress-Induced HSMM Treated with Metformin, *K. pinnata*, and Different Combinations of Metformin and *K. pinnata*.** One-way ANOVA and Duncan's multiple range test were used to statistically compare the difference in cell viability of stressed-induced HSMM cells after treatment. Columns with different letters are significantly different from one another ($P<0.05$).

3.3 Effects of combined metformin and *K. pinnata* preparations on the activity of antioxidant enzymes Superoxide Dismutase and Catalase

Table 1 shows superoxide dismutase activity in treated and non-treated HSMM cells. Superoxide dismutase activity in HSMM cells was not significantly altered among the untreated, and two of the combinatorial treatment groups (150 µM metformin and 50 µg/mL of *K. pinnata*; 50 µM metformin and 150 µg/mL of *K. pinnata*). Other treatments showed a significant increase in SOD

activity compared to the untreated control group. However, the group treated with metformin only was more effective in promoting SOD activity in HSMM cells.

In H_2O_2 stressed induced HSMM cells, the combinatorial preparation containing 100 μM of metformin and 100 μg/mL of *K. pinnata* showed a significant increase in superoxide dismutase activity compared to all other treatment options (Table 2). In human diabetic skeletal muscle cells, a metformin only treatment significantly increased the superoxide dismutase activity compared to all other treatments.

Catalase is an enzyme that catalyzes the dismutation of H_2O_2 which leads to the formation of water and oxygen. The decline of CAT activity is associated with elevated concentrations of hydrogen peroxide. Tables 4, 5, and 6 illustrate the CAT activities in HSMM cells, H_2O_2 stress-induced HSMM cell line, and DHSMM cells respectively. Catalase activity was significantly elevated in HSMM cells when treated with 400 μg/mL of *K. pinnata*. Human skeletal muscle cells treated with metformin alone or a combination of 200 μM metformin and 25 μg/mL of *K. pinnata* did not significantly increase catalase activity compared to non-treated HSMM cells. However, all other combinations of metformin and *K. pinnata* were found to have significantly higher catalase activity compared to the control (Table 4).

In H_2O_2 stress induced human skeletal muscle myoblasts, 400 μg/mL *K. pinnata* significantly increased catalase activity compared to stress-induced myoblasts that received no treatment (Table 5). A preparation of 50 μM of metformin combined with 150 μg/mL of *K. pinnata* was the only combinatorial preparation that did not significantly increase catalase activity compared to all other treatments. Table 6 shows the catalase activity in human diabetic skeletal muscle myoblasts. A treatment of *K. pinnata* only increased catalase activity significantly compared to

all other treatment groups. All experimental treatments offered significant increases in catalase activity over the control.

Table 1: **Observed Cellular Superoxide Dismutase (SOD) Activity in HSMM Cell Line.** The table shows the SOD activity of HSMM cells. One-way ANOVA and Duncan's multiple range test were used to statistically compare the difference in SOD activity among the groups. Data that share different letters are significantly different ($P<0.05$).

[metformin] (μM)	[*K.pinnata*] (μg/mL)	SOD activity (U/mg of protein/min)	(%) Increase in SOD activity
0	0	60.48 ± 4.68 [a]	
5000	0	137.81 ±15.80 [b]	127.86
200	25	68.91 ± 3.34 [c]	13.94
150	50	59.92 ± 6.40 [a]	-0.93
100	100	86.88 ± 10.91 [d]	43.65
50	150	62.91 ± 5.57 [a]	4.02
30	200	68.91 ± 9.12 [c]	13.94
10	300	71.9 ± 6.97 [c]	18.88
0	400	71.90 ± 12.94 [c]	18.88

Table 2: **Observed Cellular Superoxide Dismutase (SOD) Activity in Stressed Induced Cell Line.** The table shows the SOD activity of H_2O_2 Stressed Induced cells. One-way ANOVA and Duncan's multiple range test were used to statistically compare the difference in SOD activity among the groups. Data that share different letters are significantly different ($P<0.05$).

[metformin] (μM)	[*K.pinnata*] (μg/mL)	SOD activity (U/mg of protein/min)	(%) Increase in SOD activity
0	0	57.68 ± 7.63 [a]	
5000	0	77.89 ± 12.31 [b]	35.04
200	25	59.92 ± 17.30 [a]	3.88
150	50	56.92 ± 11.83 [a]	-1.32
100	100	107.85 ± 24.48 [c]	86.97
50	150	56.92 ± 8.04 [a]	-1.32
30	200	59.92 ± 13.68 [a]	3.88
10	300	59.92 ± 12.06 [a]	3.88
0	400	83.89 ± 17.68 [b]	45.44

Table 3: **Observed Cellular Superoxide Dismutase (SOD) Activity in DHSMM Cell Line.** The table shows the SOD activity of DHSMM cells. One-way ANOVA and Duncan's multiple range test were used to statistically compare the difference in SOD activity among the groups. Data that share different letters are significantly different (P<0.05).

[metformin] (μM)	[*K.pinnata*] (μg/mL)	SOD activity (U/mg of protein/min)	(%) Increase in SOD activity
0	0	40.35 ± 10.95 [a]	
5000	0	65.91 ± 11.48 [b]	63.35
200	25	44.94 ± 14.31 [a]	11.38
150	50	59.92 ± 12.06 [c]	48.50
100	100	59.92 ± 14.35 [c]	48.50
50	150	56.92 ± 13.16 [c]	41.07
30	200	47.93 ± 6.61 [a]	18.79
10	300	56.92 ± 10.20 [c]	41.07
0	400	59.92 ± 14.63 [c]	48.50

Table 4: **Observed Cellular Catalase (CAT) Activity in HSMM Cell Line**. The table shows the CAT activity of HSMM cells. One-way ANOVA and Duncan's multiple range test were used to statistically compare the difference in CAT activity among the groups. Data that share different letters are significantly different (P<0.05).

[metformin] (μM)	[*K.pinnata*] (μg/mL)	CAT activity (U/mg of protein/min)	(%) Increase in catalase activity
0	0	50.32 ± 3.36 [a]	
5000	0	53.86 ±3.37 [a]	7.03
200	25	55.22 ± 3.49 [a]	9.74
150	50	64.72 ± 0.30 [c]	28.62
100	100	79.48 ± 7.61 [b]	57.95
50	150	64.48 ± 4.41 [c]	28.14
30	200	64.60 ± 1.67 [c]	28.38
10	300	80.34 ± 1.17 [b]	59.66
0	400	86.88 ± 2.16 b	72.66

Table 5: **Observed Cellular Catalase (CAT) Activity in Stressed Induced Cell Line.** The table shows the CAT activity of H_2O_2 Stressed Induced cells. One-way ANOVA and Duncan's multiple range test were used to statistically compare the difference in CAT activity among the groups. Data that share different letters are significantly different ($P<0.05$).

[metformin] (μM)	[*K.pinnata*] (μg/mL)	CAT activity (U/mg of protein/min)	(%) Increase in catalase activity
0	0	10.15 ± 4.12 [a]	
5000	0	22.13 ± 0.79 [b]	118.03
200	25	17.74 ± 2.35 [b]	74.78
150	50	44.35 ± 9.22 [d]	336.95
100	100	30.83 ± 0.65 [e]	203.75
50	150	13.11 ± 2.43 [a]	29.16
30	200	42.93 ± 5.55 [d]	322.96
10	300	22.93 ± 2.28 [b]	125.91
0	400	50.83 ± 9.23 c	400.79

Table 6: **Observed Cellular Catalase (CAT) Activity in DHSMM Cell Line.** The table shows the CAT activity of DHSMM cells. One-way ANOVA and Duncan's multiple range test were used to statistically compare the difference in CAT activity among the groups. Data that share different letters are significantly different ($P<0.05$).

[metformin] (μM)	[*K.pinnata*] (μg/mL)	CAT activity (U/mg of protein/min)	(%) Increase in catalase activity
0	0	30.26 ± 3.36 [a]	
5000	0	38.06 ± 6.56 [b]	25.78
200	25	45.65 ± 0.22 [d]	50.86
150	50	62.5 ± 3.58 [e]	106.54
100	100	58.36 ± 0.16 [e]	92.86
50	150	41.94 ± 0.97 [d]	38.6
30	200	53.24 ± 1.36 [f]	75.94
10	300	44.91 ± 1.68 [d]	48.41
0	400	66.27 ± 0.12 c	119

3.4 Effects of combined metformin and *K. pinnata* preparations on reduced glutathione levels

Diabetic, non-diabetic, and stress-induced HSMM cells treated with combined metformin and *K. pinnata* preparation (50 μM metformin; 150 μg/mL *K. pinnata*) had significantly higher concentrations of reduced glutathione than any other treatment option. (Tables 7- 9). The results

from this study indicate that a combined preparation of 50 μM metformin with 150 μg/mL of *K. pinnata* significantly increases reduced glutathione concentrations in human skeletal myoblasts.

Table 7: **Reduced Glutathione Content in HSMM Cell Line**. The table shows the cellular concentration of reduced glutathione (GSH) in micromolar for HSMMM cell line. One-way ANOVA and Duncan's multiple range test were used to statistically compare the difference in reduced glutathione content among the groups. Data that share different letters are significantly different ($P<0.05$).

[metformin] (μM)	[*K.pinnata*] (μg/mL)	[GSH] (μM)	(%) Increase in reduced glutathione
0	0	2.12 ± 0.04 [a]	
5000	0	13.33 ± 0 [b]	528.77
200	25	6.11 ± 0.05 [c]	188.21
150	50	6.66 ± 0.06 [c]	214.15
100	100	17.22 ± 0.25 [d]	712.26
50	150	17.77 ± 0.06 [d]	738.21
30	200	9.44 ± 0.02 [e]	345.28
10	300	8.89 ± 0.02 [e]	319.34
0	400	2.22 ± 0.02 a	4.72

Table 8: **Reduced Glutathione Content in H_2O_2 Stressed Induced Cell Line**. The table shows the cellular concentration of reduced glutathione (GSH) in micromolar for H_2O_2 stressed cell line. One-way ANOVA and Duncan's multiple range test were used to statistically compare the difference in reduced glutathione content among the groups. Data that share different letters are significantly different ($P<0.05$).

[metformin] (μM)	[*K.pinnata*] (μg/mL)	[GSH] (μM)	(%) Increase in reduced glutathione
0	0	3.11 ± 0.03 [a]	
5000	0	10.00 ± 0.10 [b]	221.54
200	25	5.00 ± 0.06 [c]	60.77
150	50	10.00 ± 0.03 [b]	221.54
100	100	9.44 ± 0.07 [d]	203.54
50	150	22.22 ± 0.03 [e]	614.47
30	200	11.67 ± 0.03 [f]	275.24
10	300	11.67 ± 0.05 [f]	275.24
0	400	3.89 ± 0.02 a	25.08

Table 9: **Reduced Glutathione Content in DHSMM Cell Line**. The table shows the cellular concentration of reduced glutathione (GSH) in micromolar for DHSMMM cell line. One-way ANOVA and Duncan's multiple range test were used to statistically compare the difference in reduced glutathione content among the groups. Data that share different letters are significantly different ($P<0.05$).

[metformin] (μM)	[*K.pinnata*] (μg/mL)	[GSH] (μM)	(%) Increase in reduced glutathione
0	0	5.25 ± 0.03 [a]	
5000	0	13.89 ± 0.03 [b]	164.57
200	25	11.67 ± 0 [b]	122.29
150	50	15.00 ± 0.03 [b]	185.71
100	100	11.11 ± 0.05 [b]	111.62
50	150	23.89 ± .07 [c]	355.05
30	200	14.44 ± 0.03 [b]	175.05
10	300	10.56 ± 0.07 [b]	101.14
0	400	21.67 ± 0 c	312.76

3.5 Effects of combined metformin and *K. pinnata* preparations on lipid peroxidation

It was determined that all treatment options significantly decreased MDA concentrations in all HSMM cell lines (Tables 10-12). However, the most effective treatment for decreasing MDA concentrations was 400 μg/mL of *K. pinnata*.

Table 10: **HSMM Cellular MDA Concentrations.** This table illustrates the concentration of MDA extracted from HSMM cells after treatment of a combined preparation. One-way ANOVA and Duncan's multiple range test were used to statistically compare the difference in MDA content among the groups. Data that share different letters are significantly different ($P<0.05$).

[metformin] (μM)	[*K.pinnata*] (μg/mL)	[MDA] (uM)	(%) Decrease in MDA
0	0	52.35 ± 1.25 [a]	
5000	0	25.15 ±0.11 [b]	52
200	25	22.63 ± 0.47 [c]	56.77
150	50	21.55 ± 0.11 [c]	58.83
100	100	24.32 ± 0.61 [b]	55.54
50	150	22.42 ± 0.28 [c]	57.17
30	200	22.25 ± 0.13 [c]	57.5

10	300	21.38 ± 0.16 [c]	59.16
0	400	21.51 ± 0.64 c	58.91

Table 11: **MDA Content in H_2O_2 Stressed Induced Cell Line**. The table shows the cellular concentration of MDA in micromolar for H_2O_2 stressed cell line. One-way ANOVA and Duncan's multiple range test were used to statistically compare the difference in MDA content among the groups. Data that share different letters are significantly different ($P<0.05$).

[metformin] (μM)	[*K.pinnata*] (μg/mL)	[MDA] (uM)	(%) Decrease in MDA
0	0	62.32 ± 1.1 [a]	
5000	0	23.63 ± 0.24 [b]	62.08
200	25	21.21 ± 0.69 [e]	65.97
150	50	20.39 ± 0.30 [e]	67.28
100	100	22.07 ± 0.49 [d]	64.59
50	150	20.77 ± 0.24 [e]	66.67
30	200	20.47 ± 0.46 [e]	67.15
10	300	19.91 ± 0.30 [e]	68.05
0	400	18.26 ± 0.88 c	70.70

Table 12: **DHSMM Cellular MDA Concentrations.** This figure illustrates the concentration of MDA extracted from DHSMM cells after treatment of a combined preparation. One-way ANOVA and Duncan's multiple range test were used to statistically compare the difference in MDA content among the groups. Data that share different letters are significantly different ($P<0.05$).

[metformin] (μM)	[*K.pinnata*] (μg/mL)	[MDA] (uM)	(%) Decrease in MDA
0	0	63.45 ± 0.98 [a]	
5000	0	21.42 ± 0.30 [b]	66.24
200	25	21.55 ± 1.15 [b]	66.04
150	50	19.35 ± 0.22 [c]	69.50
100	100	20.73 ± 0.24 [d]	67.33
50	150	19.26 ± 0.13 [c]	69.65
30	200	18.87 ± 0.27 [c]	70.26
10	300	18.31 ± 0.50 [c]	71.14
0	400	15.71 ± 0.50 e	75.24

3.6 Effects of combined *K. pinnata* and metformin preparations on inflammation

Considering two combinatorial preparations (100 μM metformin with 100 μg/mL *K. pinnata;* 50 μM metformin with 150 μg/mL *K. pinnata*) significantly increased cell viability and antioxidant levels, these treatments were used to investigate cytokine production. Figures 5 through 7 represent the cellular concentrations of IL-2 in normal, stress-induced, and diabetic human skeletal muscle myoblasts. All investigated treatments significantly raised IL-2 concentrations compared to untreated cells. In both HSMM and DHSMM cells, a metformin only treatment significantly lowered IL-2 levels compared to all other treatment options (Figures 5 and 7). In H_2O_2 stress-induced HSMM cells, a *K. pinnata* only treatment significantly lowered the concentration of IL-2 compared to all other treatments (Figure 6).

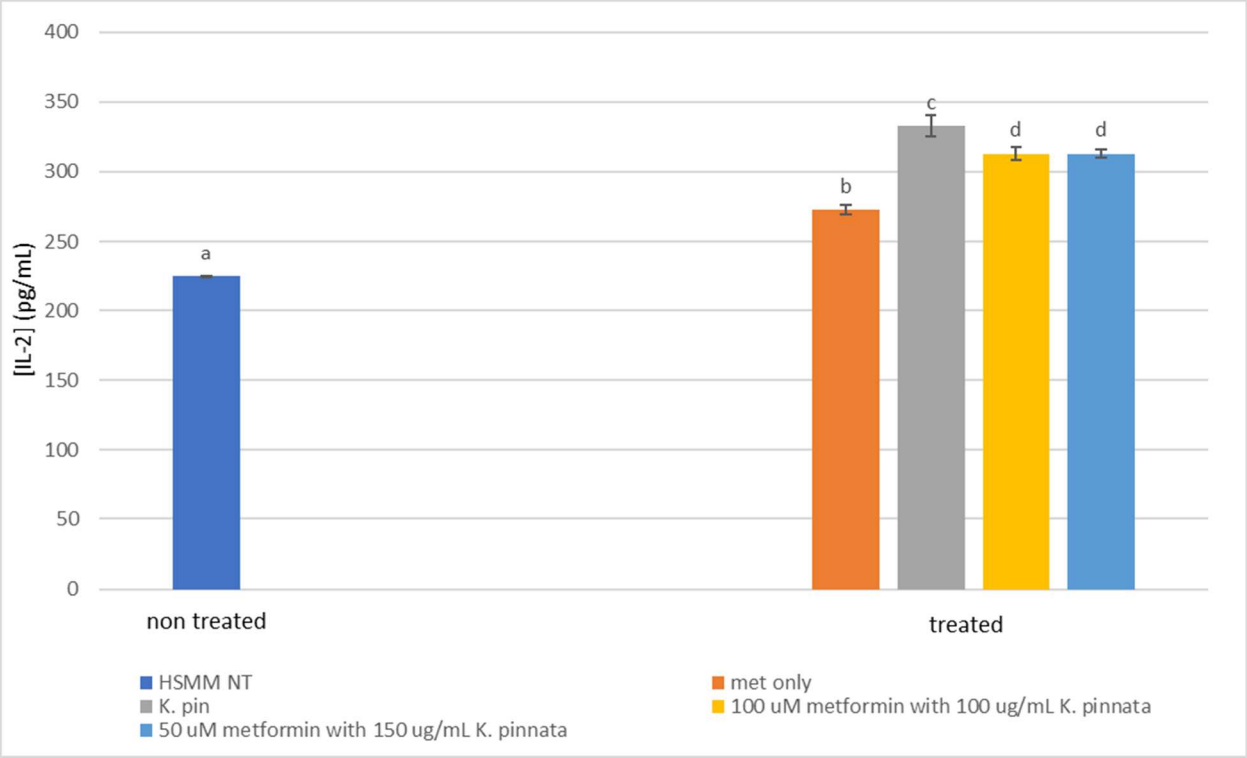

Figure 5: **Concentration of IL-2 in HSMM Cell Line.** The figure illustrates the concentration of IL-2 from HSMM cell line comparing various treatments. One-way ANOVA and Duncan's multiple range test were used to statistically compare the difference in IL-2 of HSMM after treatment. Columns with different letters are significantly different from one another ($P<0.05$).

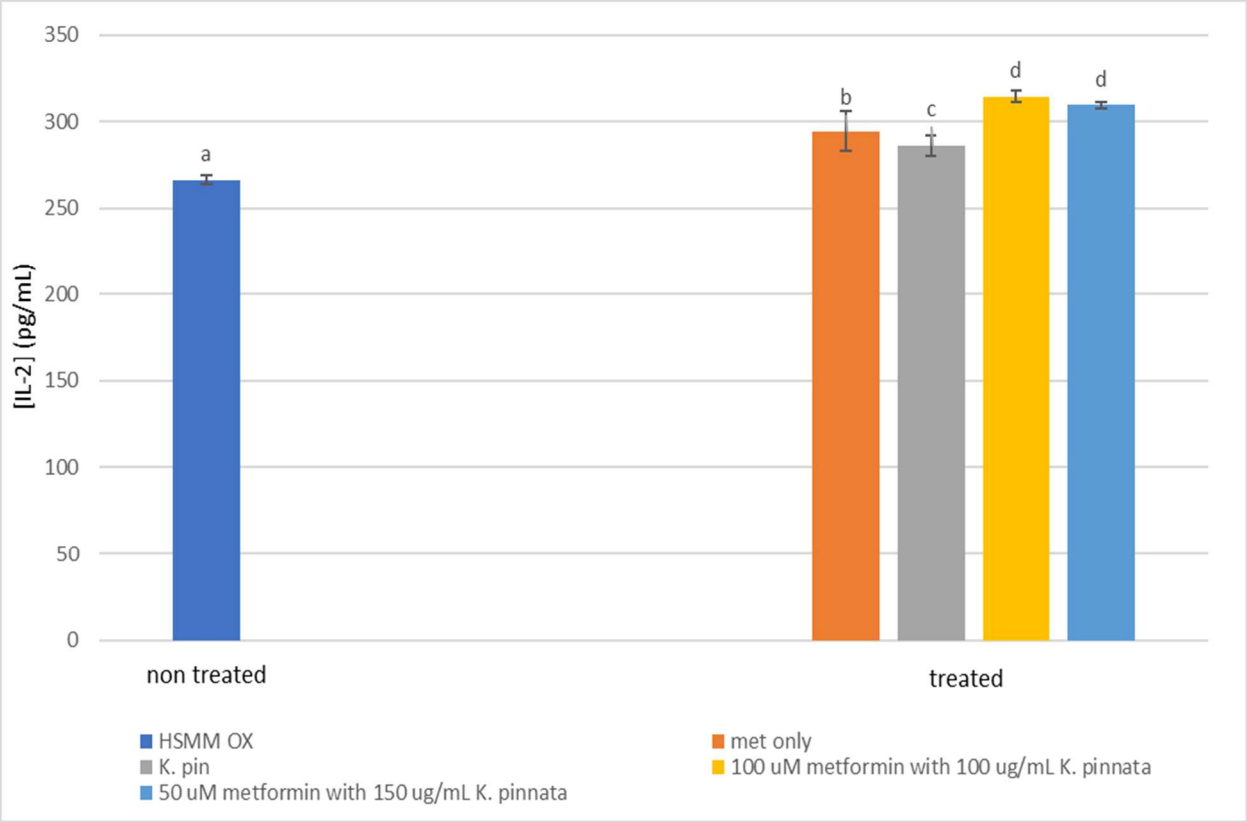

Figure 6: **Concentration of IL-2 in Stress Induced HSMM Cell Line.** The figure illustrates the concentration of IL-2 from stressed induced HSMM cell line after treatment of combined preparation. One-way ANOVA and Duncan's multiple range test were used to statistically compare the difference in the production of IL-2 among the groups. Columns with different letters are significantly different from one another ($P<0.05$).

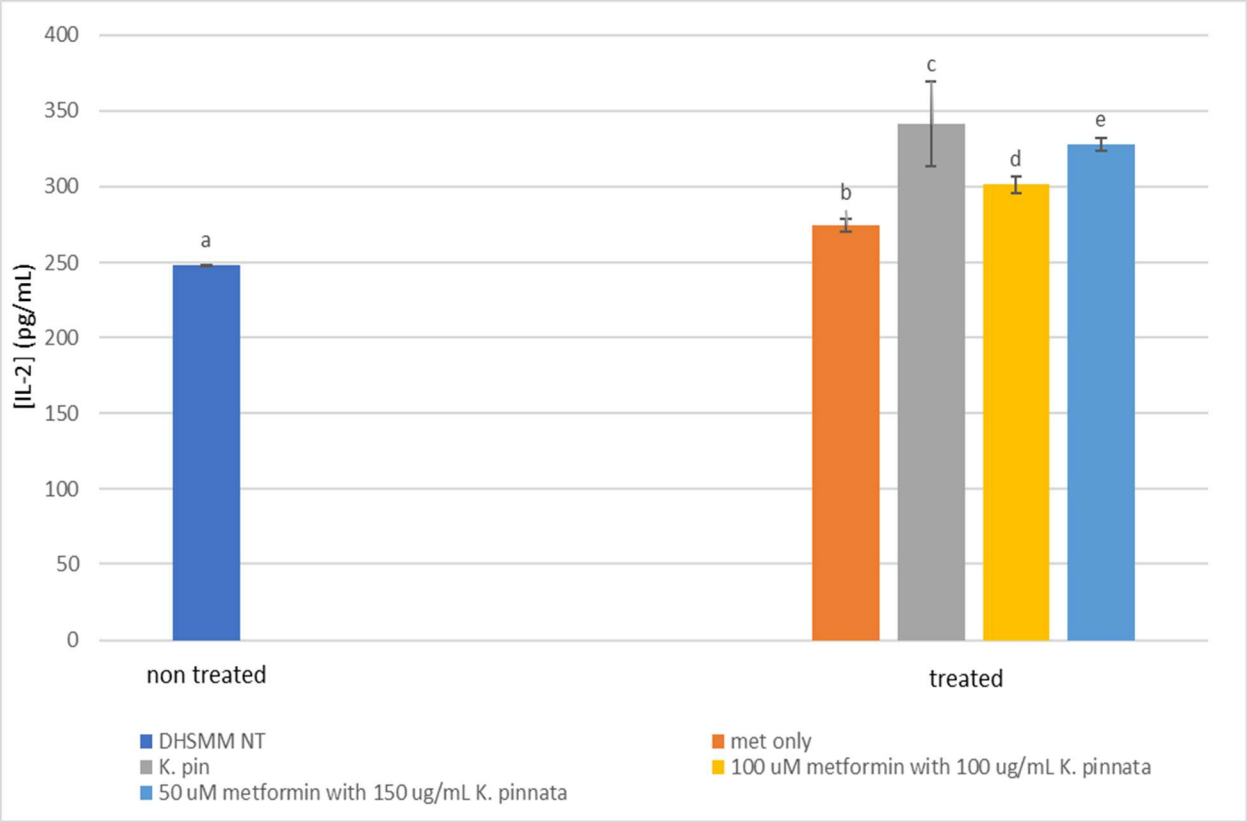

Figure 7: **Concentration of IL-2 in DHSMM Cell Line**. This figure represents the concentrations of IL-2 obtained from DHSMM cell line after treatment of combined preparation. One-way ANOVA and Duncan's multiple range test were used to statistically compare the difference in the production of IL-2 among the groups. Columns with different letters are significantly different from one another ($P<0.05$).

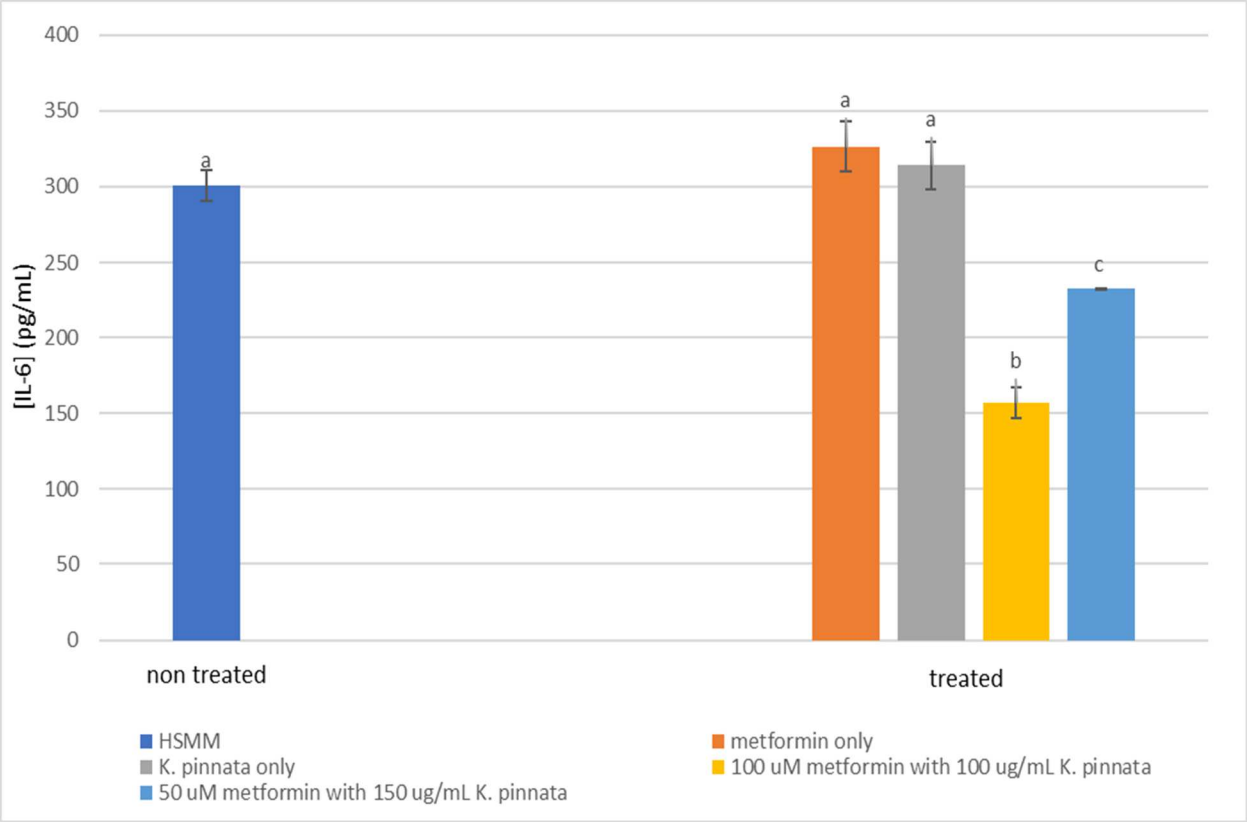

Figure 8: **Concentration of IL-6 in HSMM Cell Line.** The figure illustrates the concentration of IL-6 from HSMM cell line comparing various treatments. One-way ANOVA and Duncan's multiple range test were used to statistically compare the difference in the production of IL-6 among the groups. Columns with different letters are significantly different from one another ($P<0.05$).

Concentrations of cellular IL-6 for all investigated cell lines are shown in Figures 8 through 10. Combinatorial preparations significantly lowered IL-6 concentrations in human skeletal muscle cells (Figure 8). The metformin and *K. pinnata* only treatments were insignificant compared to untreated HSMM cells. All treatment options significantly raised the concentrations of IL-6 in H_2O_2 stress-induced HSMM cells (Figure 9). A metformin only treatment was observed to have significantly higher concentrations of IL-6 relative to all other treatments.

Figure 10 shows the IL-6 concentrations observed in the DHSMM cell line. Interleukin 6 levels were significantly higher in non-treated diabetic cells. Combinatorial preparations had significantly lower IL-6 concentrations compared to other treatments.

This study concluded that either a metformin or *K. pinnata* only treatment promotes an increase in IL-6 concentrations in HSMM cells, while in stress-induced HSMM cells, the most effective treatment for significantly raising IL-6 concentrations was found to be metformin only treatment. All treatment options significantly reduced IL-6 concentrations in DHSMM cells. This study also concluded that combinatorial preparations significantly reduce IL-6 concentrations in both HSMM and DHSMM cells compared to either a metformin or *K. pinnata* only treatment.

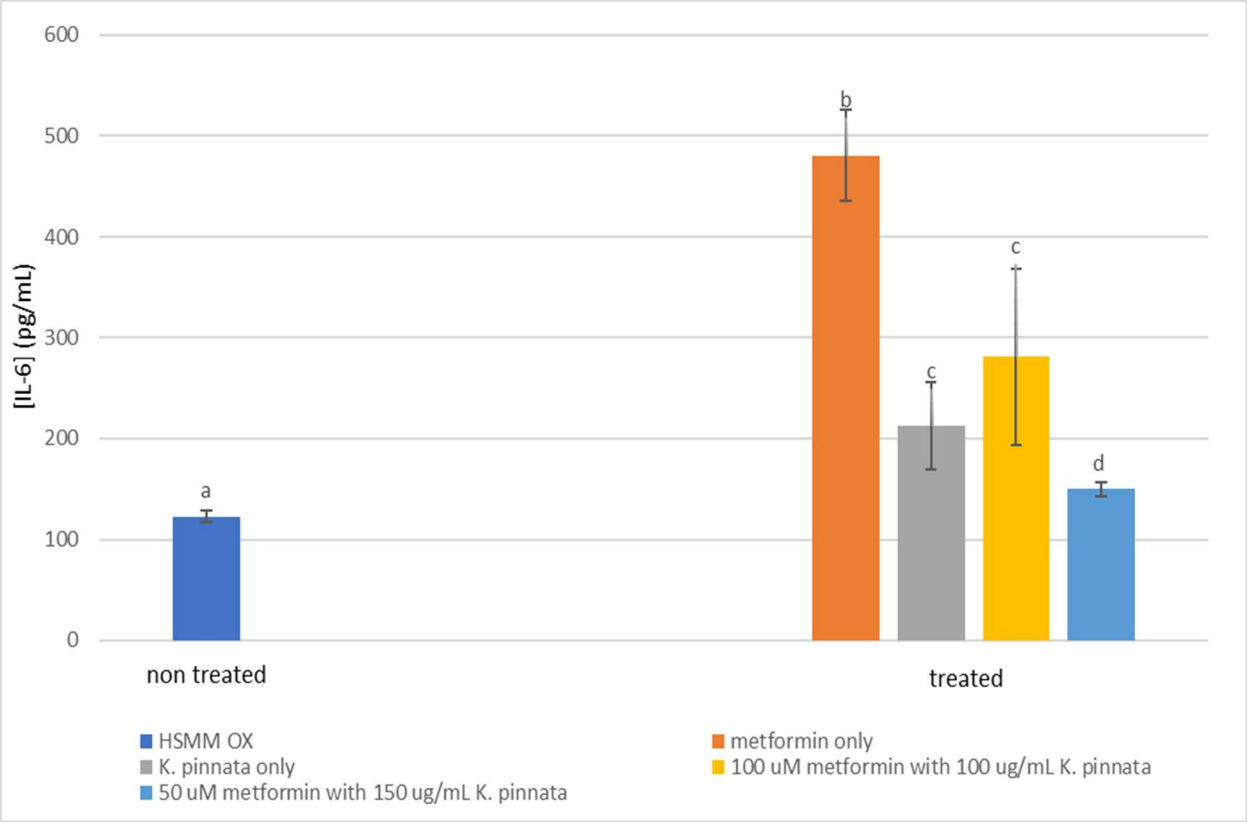

Figure 9: **Concentration of IL-6 in Stress Induced HSMM Cell Line.** The figure illustrates the concentration of IL-6 from stressed induced HSMM cell line after treatment of combined

preparation. One-way ANOVA and Duncan's multiple range test were used to statistically compare the difference in the production of IL-6 among the groups. Columns with different letters are significantly different from one another (P<0.05).

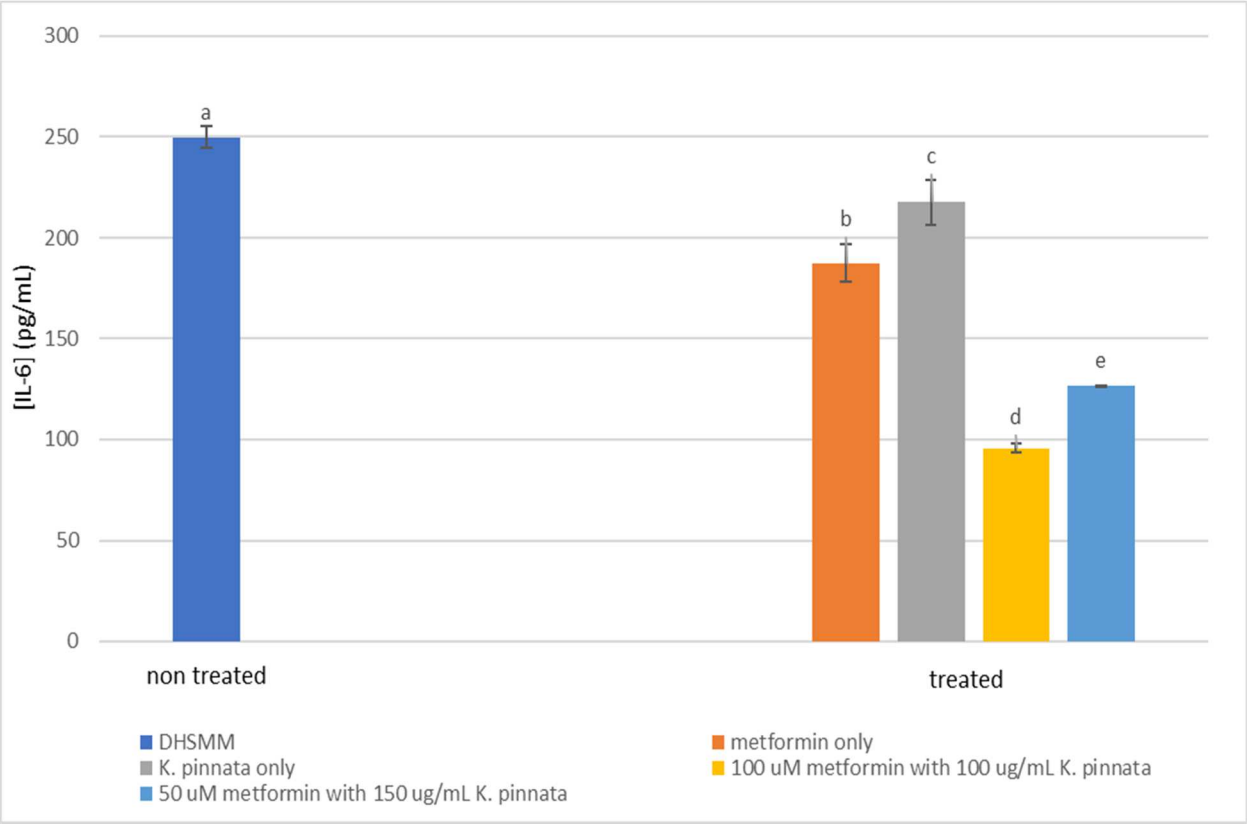

Figure 10: **Concentration of IL-6 in DHSMM Cell Line.** The figure illustrates the concentration of IL-6 from DHSMM cell line comparing various treatments. One-way ANOVA and Duncan's multiple range test were used to statistically compare the difference in the production of IL-6 among the groups. Columns with different letters are significantly different from one another (P<0.05).

All treatment options significantly raised IL-10 levels in HSMM cells (Figure 11). Both the *K. pinnata* only and the combinatorial preparation with 50 µM of metformin and 150 µg/mL of *K. pinnata* significantly increased concentrations of IL-10 compared to the other treatment options. In H_2O_2 stress-induced HSMM cells both combinatorial treatments significantly increased concentrations of IL-10 (Figure 12). However, a metformin only treatment significantly lowered IL-10 concentrations in stress-induced HSMM cells compared to all other treatments. The

combinatorial preparation with 50 µM of metformin and 150 µg/mL of *K. pinnata* significantly raised the concentration of IL-10 in DHSMM cells compared to other treatments (Figure 13). This study concluded that a combinatorial preparation of metformin and *K. pinnata* (50 µM, 150 µg/mL) significantly increases concentrations of IL-10 in both DHSMM and stress-induced HSMM myoblasts.

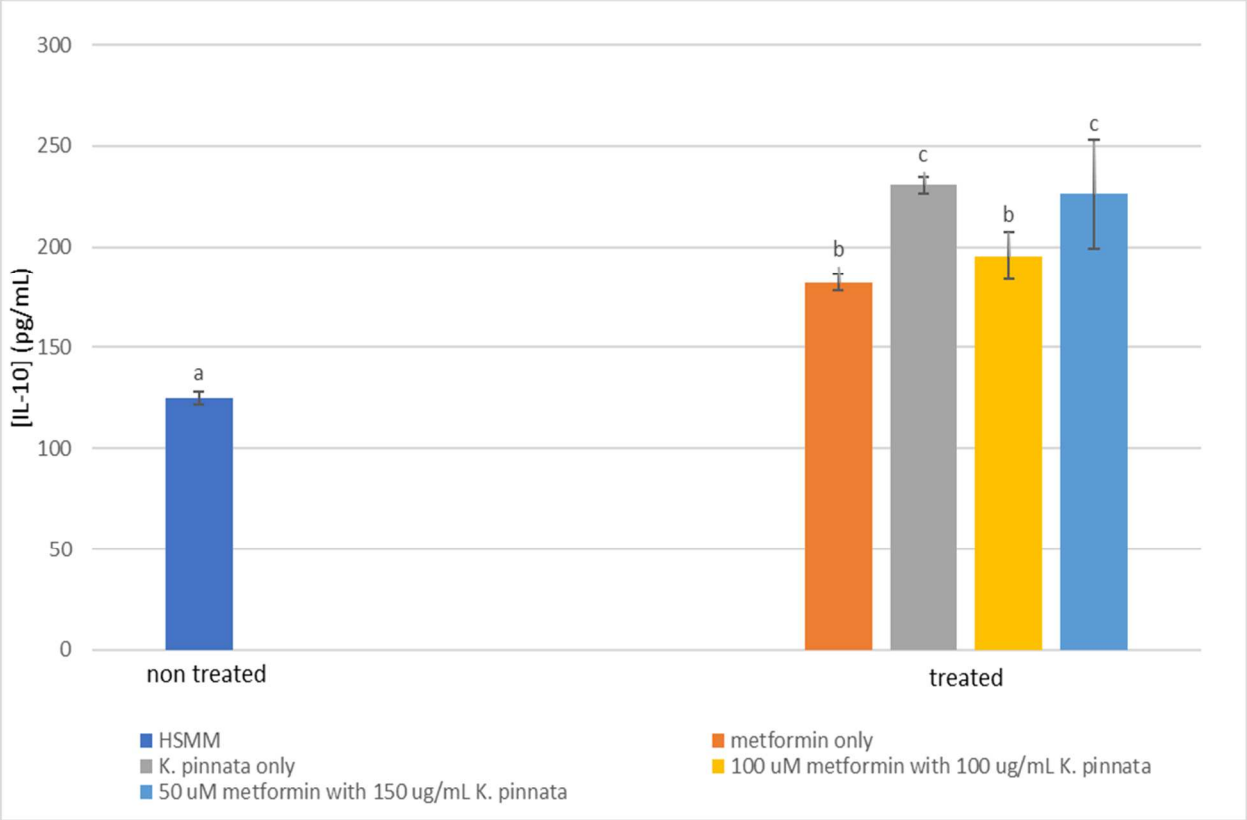

Figure 11: **Concentration of IL-10 in HSMM Cell Line.** The figure illustrates the concentration of IL-10 from HSMM cell line comparing various treatments. One-way ANOVA and Duncan's multiple range test were used to statistically compare the difference in the production of IL-10 among the groups. Columns with different letters are significantly different from one another ($P<0.05$).

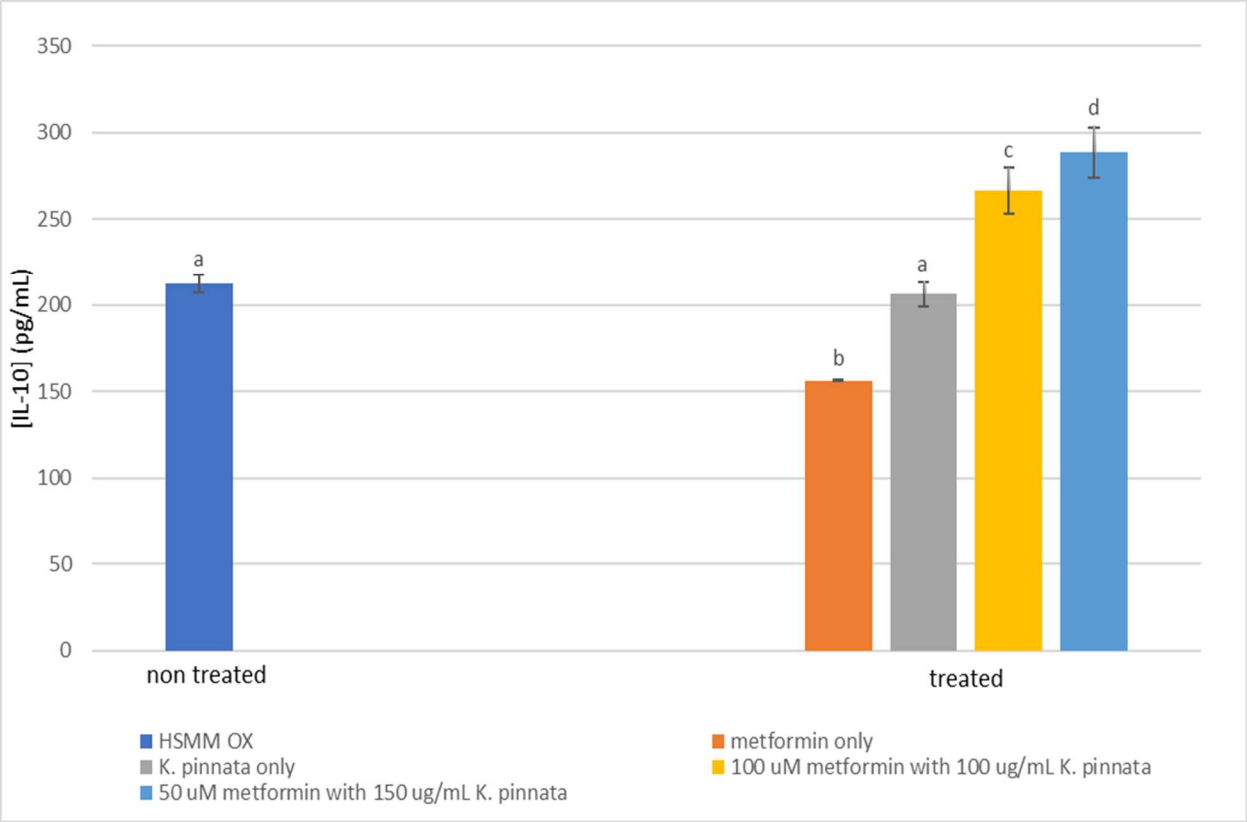

Figure 12: **Concentration of IL-10 in Stress Induced HSMM Cell Line.** The figure illustrates the concentration of IL-10 from stressed induced HSMM cell line after treatment of combined preparation. One-way ANOVA and Duncan's multiple range test were used to statistically compare the difference in the production of IL-6 among the groups. Columns with different letters are significantly different from one another ($P<0.05$).

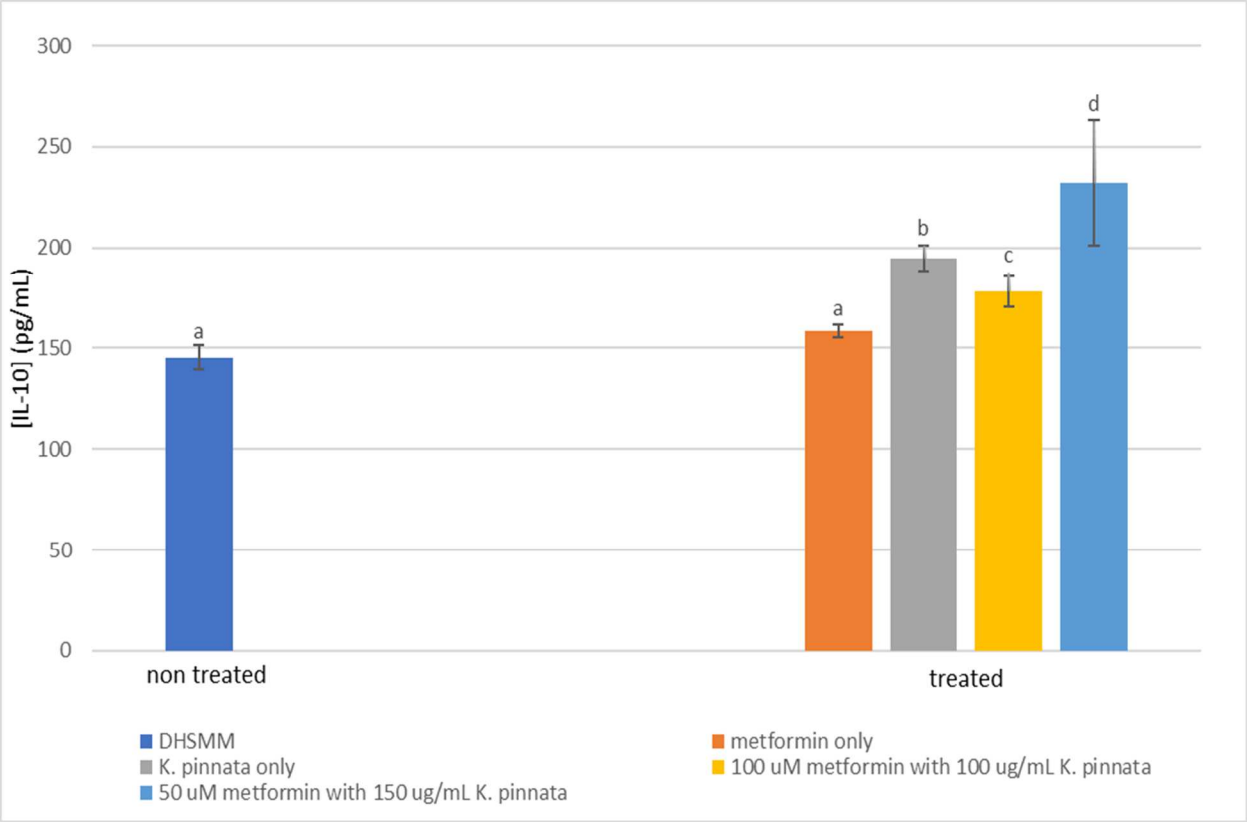

Figure 13: **Concentration of IL-10 in DHSMM Cell Line.** The figure illustrates the concentration of IL-10 from DHSMM cell line comparing various treatments. One-way ANOVA and Duncan's multiple range test were used to statistically compare the difference in the production of IL-6 among the groups. Columns with different letters are significantly different from one another ($P<0.05$).

All treatment options significantly elevated tumor necrosis factor-alpha concentrations in HSMM cells compared to nontreated HSMM cells (Figure 14). A combination of 100 µM metformin and 100 µg/mL of *K. pinnata* significantly increased TNF-α compared to the other treatments. Both metformin alone and *K. pinnata* alone significantly increased TNF-α concentrations in stress-induced HSMM cells compared to combinatorial treatments (Figure 15). Concentrations of tumor necrosis factor-alpha were insignificant in both combinatorial preparations compared to nontreated stress-induced HSMM cells. The concentrations of tumor necrosis factor-alpha were significantly higher in all treatment options compared to nontreated DHSMM cells (Figure 16).

Overall, it was observed that nontreated cells had significantly lower concentrations of TNF-α compared to cells that were given a treatment option (Figures 14-16). It was also observed that a metformin only treatment significantly increased TNF-α concentrations in both the stress-induced and diabetic skeletal muscle cells compared to untreated cells (Figures 15 and 16).

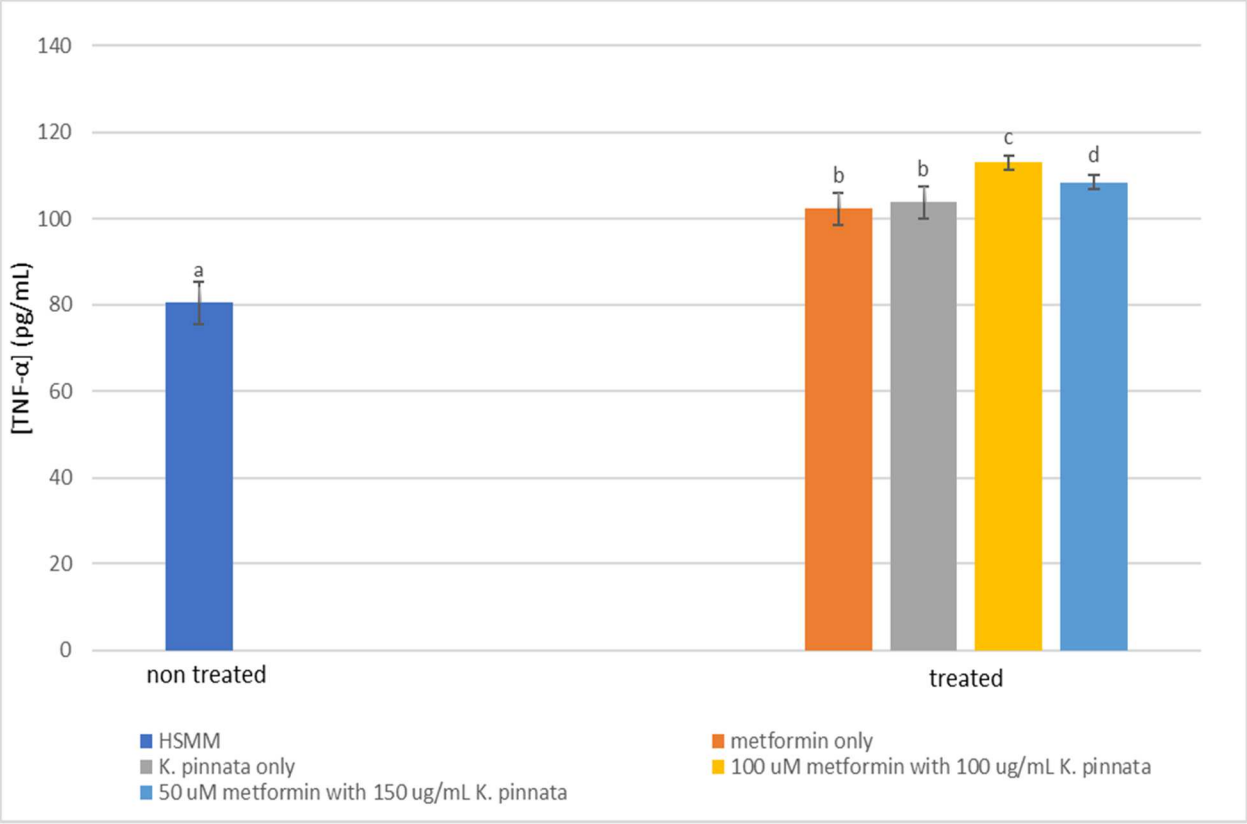

Figure 14: **Concentration of TNF-α in HSMM Cell Line.** The figure illustrates the concentration of TNF-α from HSMM cell line comparing various treatments. One-way ANOVA and Duncan's multiple range test were used to statistically compare the difference in the production of TNF-α among the groups. Columns with different letters are significantly different from one another ($P<0.05$).

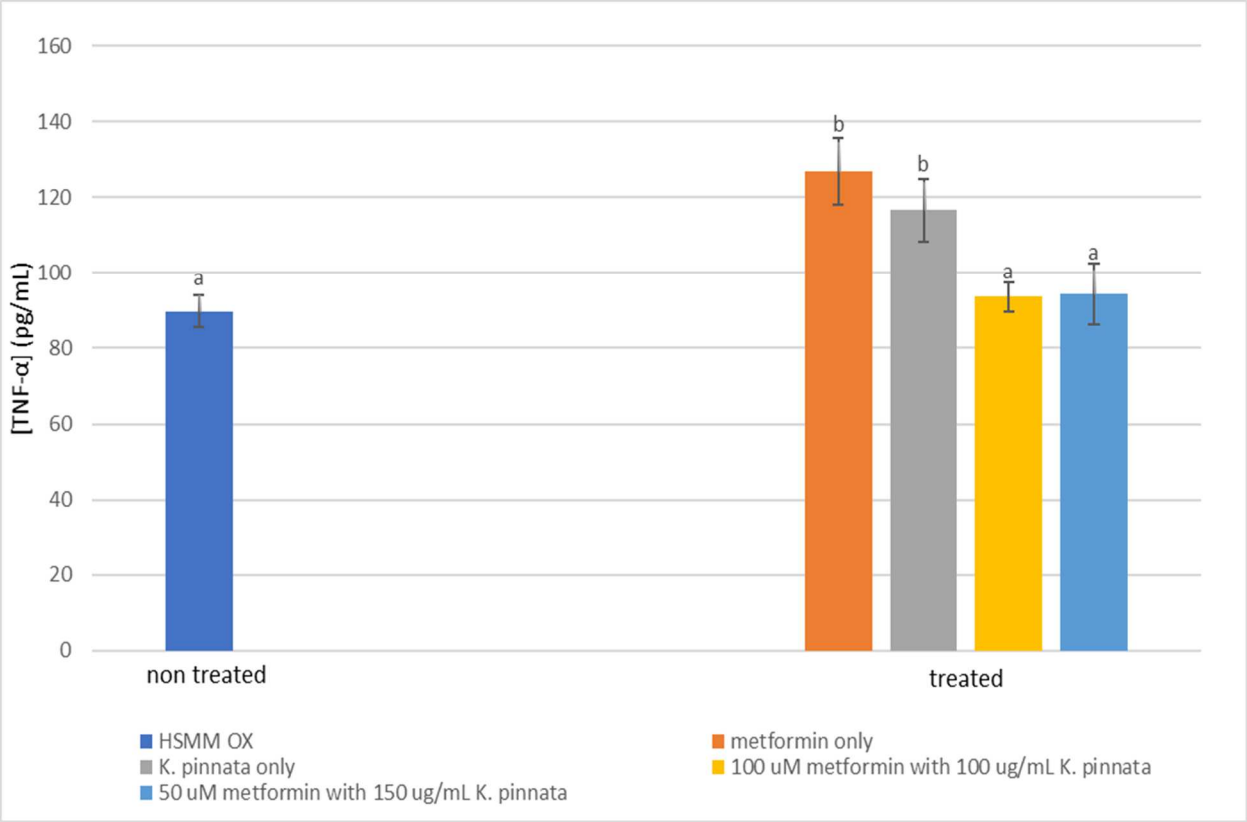

Figure 15: **Concentration of TNF-α in stress induced HSMM Cell Line.** The figure illustrates the concentration of TNF-α from stress induced HSMM cell line comparing various treatments. One-way ANOVA and Duncan's multiple range test were used to statistically compare the difference in the production of TNF-α among the groups. Columns with different letters are significantly different from one another ($P<0.05$).

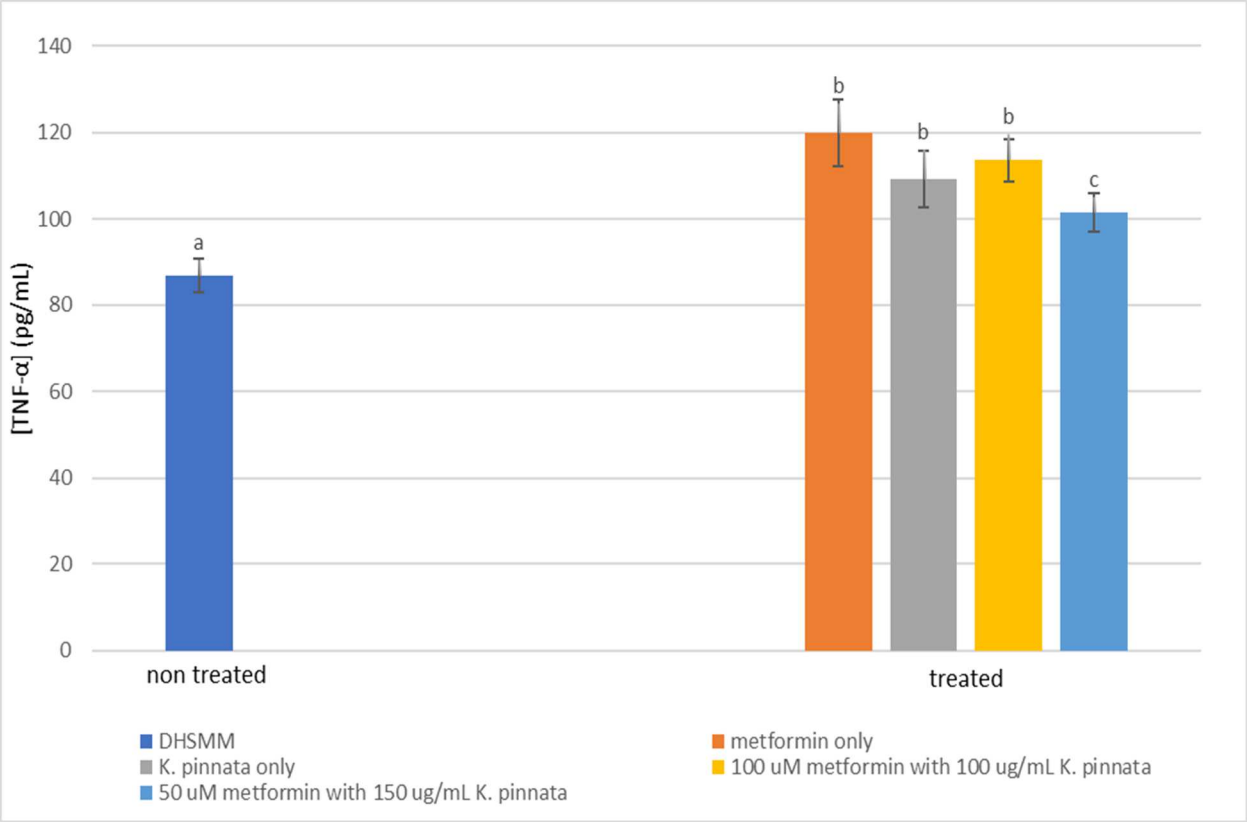

Figure 16: **Concentration of TNF-α in DHSMM Cell Line.** The figure illustrates the concentration of TNF-α from DHSMM cell line comparing various treatments. One-way ANOVA and Duncan's multiple range test were used to statistically compare the difference in the production of TNF-α among the groups. Columns with different letters are significantly different from one another ($P<0.05$).

4. DISCUSSION

Due to the prevalence of type II diabetes and the emergence of alternative medicinal practices, it is vital to develop a combined multi-approach treatment option that is both safe and effective to treat type II diabetes. This study aimed at determining the effectiveness of metformin and *K. pinnata* combinatorial preparation for the treatment of type II diabetes in an in-vitro model of the disease.

Data from this study indicate that a combinatorial preparation of *K. pinnata* and metformin promotes the proliferation of HSMM and DHSMM up to 72 hours post-treatment. Diabetic human skeletal muscle myoblast cells benefited more from a combinatorial preparation treatment than that of HSMM cells. However, since cellular viability dropped significantly around days 5-7, exposure to a combinatorial treatment option indicated cytotoxic properties and further study on long term use is needed. The observed decrease in cell viability in combinatorial treatments at the end of the 7 days may be due to the downregulation of oxidative phosphorylation, energy storage, and transfer systems over time [53]. In addition, it is conceivable that the individual components in the combinatorial preparation may act upon different cellular signaling pathways that result in a decrease in cellular viability. In a review study by Wang et al., metformin has the ability to act intracellularly and decrease insulin-like growth factor 1 (IGF-1) in cells, thereby reducing the growth of the cell. In the same review, it is explained that metformin also has the potential to activate the AMPK signaling pathway [54]. Activation of the AMPK pathway allows cells to preserve energy needed for cell survival by modulating cell metabolism, however, this is at the expense of cellular growth and proliferation [55].

Aside from metformin having the ability to affect cell viability, *K. pinnata* also has the potential to modulate cell growth and viability. However, *K. pinnata* interferes with a cell's viability differently than metformin. Alcoholic extractions of *K. pinnata* at concentrations around 100 μg/mL have been shown to cause genetic damage to cellular DNA [56]. Although this study utilized aqueous *K. pinnata* extracts at similar concentrations, it is logical to assume that the possibility of aqueous *K. pinnata* can also damage cellular DNA, thereby, lowering cellular viability. This study indicated that cell viability decreases over time when exposed to combinatorial preparations of metformin and *K. pinnata*. In response to cell death being

observed in this study, cellular oxidation was questioned to determine if cell stress played a role in cell viability.

Hydrogen peroxide is a relatively weak oxidizing agent but at high levels is cytotoxic [57]. However, hydrogen peroxide is also an important regulator of cellular redox status and signaling pathways. When produced in excess, it can trigger oxidative damage, which can only be counteracted by antioxidant systems [58]. Oxidative stress plays an important role in the development of diabetic vascular complications, particularly type II diabetes [59,60]. Evidence has accumulated that reactive oxygen species (ROS) affect human skeletal muscle cells by influencing cellular processes that lead to increased expression of antioxidants [61,62]. Antioxidants can neutralize free radicals produced by ROS by accepting unpaired electrons and inhibiting the oxidation of other cellular molecules that are vital to cellular survival. Depending on the oxygen consumption rate, cells constitutively express different levels of antioxidants, including mitochondrial antioxidant manganese superoxide dismutase (Mn-SOD, SOD2), cytosolic copper-zinc superoxide dismutase (Cu,Zn-SOD, SOD1), glutathione peroxidase (GPX) and catalase (CAT), and the nonenzymatic antioxidant glutathione (GSH) [61].

A significant contrast in cellular viability between HSMM cells and stress-induced HSMM cells was observed. There was also a significant difference in cell viability between HSMM and DHSMM cells. This difference in cellular viability is believed to be caused by oxidative damage induced by a type II diabetes disease state. It was noted in this study that after 72 hrs. post-treatment, DHSMM cells were 5 times more likely to survive than HSMM cells under H_2O_2 stress. It suggests that a combinatorial *K. pinnata* and metformin preparation may protect human diabetic skeletal muscle myoblasts from oxidative stress caused by H_2O_2.

Regarding superoxide dismutase activity, a high dose of metformin (5 mM) significantly increased SOD activity in both HSMM and DHSMM cells. However, a combinatorial preparation of 100 μM metformin and 100 μg/mL of *K. pinnata* significantly increased SOD activity in H_2O_2 stressed induced human skeletal muscle myoblasts. This study demonstrated that a 100 μM metformin and 100 μg/mL of *K. pinnata* combinatorial preparation assists human skeletal muscle myoblasts challenged with oxidative stress by promoting an increase in SOD activity compared to any other treatment option.

A combined treatment of metformin and *K. pinnata* may prevent hydrogen peroxide-induced oxidative damage by increasing superoxide dismutase activity. The metformin only treatment was most effective at increasing SOD activity in both HSMM and DHSMM cell lines. This study reinforces that a metformin only treatment significantly increases superoxide dismutase in a diabetic disease state [63].

On the other hand, significant increases in catalase activity for all cell lines occurred when cells were treated with 400 μg/mL of *K. pinnata*. This study suggests that 400 μg/mL of *K. pinnata* may reduce oxidative damage by increasing catalase activity in human skeletal muscle cells compared to other treatment groups. This study confirms that *K. pinnata* increases catalase activity and helps in combating oxidative stress in type II diabetes [29]. It should be noted, however, that catalase has a relatively low affinity for H_2O_2 when compared with other peroxidases [64]. The rate of H_2O_2 detoxification increases with the intracellular concentration of catalase, preventing H_2O_2 accumulation and subsequent oxidative injury under conditions where other peroxidases are saturated [65,66]. Catalase content of skeletal muscle tissue has been identified to be relatively low, being only 1.4% of that found in the liver [64].

Combinatorial treatment of 50 μM metformin and 150 μg/mL *K. pinnata* significantly increased reduced glutathione concentrations in diabetic, non-diabetic, and H_2O_2 stress-induced human skeletal muscle myoblasts. This study suggests that a combinatorial preparation is more effective at increasing reduced glutathione levels compared to either metformin or *K. pinnata* alone. A combined preparation of metformin and *K. pinnata* can protect skeletal myoblasts from H_2O_2 oxidative damage by utilizing reduced glutathione and maintain cellular redox homeostasis. Reduced glutathione could also assist patients with type II diabetes by improving sugar metabolism [67]. Researchers from the University of Rome found that increasing concentrations of reduced glutathione in type II diabetic cells significantly increased glucose uptake and sensitivity to insulin [68]. In addition, impaired fibroblast growth was prevented by increasing reduced glutathione concentrations [68]. This suggests that oxidative stress is an important contributor to cellular dysfunction in a type II diabetic disease state and that GSH supplementation can protect the body against oxidative stress and improve cell metabolism [69].

All treatment options helped DHSMM cells reduce lipid peroxidation, preventing oxidative damage from occurring in the cell. A *K. pinnata* only treatment significantly reduced MDA concentrations for all cell lines and outperformed every other treatment option when it came to reducing lipid peroxidation. This study indicates that *K. pinnata* alone is better suited for protecting human skeletal muscle myoblasts from oxidative damage caused by lipid peroxidation. In another study, concentrations of *K. pinnata* extract at 125 μg/mL produced an anti-lipid peroxidation effect on in vitro cells and anti-oxidation increases as *K. pinnata* concentrations increase [68]. This study in combination with the aforementioned study, reveals that *K. pinnata* possesses significant oxidative radical scavenging activities.

Based on in vitro studies, interleukin -2 has been considered a key growth and death factor for antigen-activated T lymphocytes [70]. New findings redefine the pivotal role for IL-2 as the major inducer for the developmental production of suppressive T-regulatory cells [70]. By adjusting the number of T-regulatory cells, IL-2 becomes essential for tolerance and immunity in cells. Adaptive T-reg cells are generated in the periphery, require IL-2 for their survival and function, and are believed to suppress immune responses by releasing anti-inflammatory cytokines such as IL-10 and TGF-β [71]. Combined preparations of metformin and *K. pinnata* significantly elevated concentrations of IL-2 and TNF-α. Despite using TNF alpha concentrations as an indicator of inflammation in this study, emerging evidence suggests that tumor necrosis factor-alpha may also play a role in muscle repair [72]. A study conducted by Li showed that TNF-α activates satellite cells to enter the cell cycle and accelerates G_1 – to – S phase transition, and these actions may involve activation of early response genes via TNF alpha stimulated serum response factor (SRF) [72]. Inflammation is a key response to muscle injury and is essential for muscle regeneration [73]. Tumor necrosis factor-alpha levels indicated in this study are hypothesized to contribute to muscle cell growth and cellular myoblast regeneration.

In this study, all treatment options raised IL-10 levels in both the HSMM and DHSMM cell lines. In HSMM cells the highest concentration of IL-10 occurred in cells treated with *K. pinnata* only. The combinatorial preparation with 50 μM of metformin and 150 μg/mL of *K. pinnata* was observed to have the highest concentrations of IL-10 in both the stress-induced HSMM cells and DHSMM cells. Interleukin-10 regulates proinflammatory cytokines by downregulating the expression of Th1 cytokines [74]. Treatment options raising concentrations of IL-10 may help reduce the severity of tissue damage through the reduction of Th1 cytokines. Human skeletal muscle cells under oxidative stress or struggling in type II diabetic disease state may benefit

from a combined metformin and *K. pinnata* preparation. In addition, DHSMM cells may benefit further from IL-10 as it may also prevent insulin resistance in skeletal muscle [75].

Combined preparations significantly lowered concentrations of the anti-inflammatory glycoprotein IL-6 in both diabetic and non-diabetic human skeletal muscle cells. High concentrations of IL-6 were observed when HSMM and stress-induced HSMM cells were given 5 mM of metformin. Higher levels of IL-6 were observed when DHSMM cells were treated with 400 μg/mL of *K. pinnata* compared to other treatment options. Interleukin 6 is considered a myokine and has anti-inflammatory properties. Data suggests that IL-6 inhibits TNF-α and suppresses inflammation [76]. Therefore, treatments increasing concentrations of IL-6 in cells may offer higher cellular viability by reducing inflammation. Moreover, IL-6 contributes to insulin resistance through STAT 3 and SOCS 3 pathways [77]. These pathways inhibit IRS-1 of the insulin receptor and decrease cellular insulin sensitivity. This study showed that combinatorial treatments lowered IL-6 concentrations in both HSMM and DHSMM cell lines. By decreasing concentrations of IL-6, it is probable that HSMM and DHSMM cell might be more sensitive to insulin.

However, a high concentration of metformin (5mM) is suggested to be ineffective at improving insulin resistance in all variations of HSMM cell lines based on the reduced glutathione and IL-6 data. The reduced glutathione concentrations of the high dose of metformin alone were lower than the reduced glutathione concentrations of a combinatorial treatment (50 μM of metformin; 150 μg/mL of *K. pinnata*) (Tables 7-9). This study makes the argument that by increasing reduced glutathione concentrations there is an improvement in a cell's ability to effectively regulate glucose metabolism. The study conducted by researchers at the University of Rome found that increasing concentrations of reduced glutathione correlated to significant increases in

cells' sensitivity to insulin [68]. However, according to this study, a 5 mM concentration of metformin did not fare as well compared to other treatment options suggesting that a 5mM metformin dose does not improve insulin sensitivity by increasing reduced glutathione concentrations in cells. This study also implies that increases in IL-6 concentrations decreases cellular insulin sensitivity through the inhibition of IRS-1 of the insulin receptor. A 5 mM treatment of metformin significantly increased IL-6 concentrations in HSMM and stress-induced HSMM cells. If cells increase insulin sensitivity through IL-6 regulation, a decrease in IL-6 would indicate an improvement in insulin sensitivity. This study supports that high doses of metformin, particularly at a concentration of 5mM, may not improve insulin resistance compared to a combinatorial treatment.

Overall, this study showed that a *K. pinnata* only treatment was effective at significantly increasing CAT activity and significantly lowering concentrations of MDA in diabetic and nondiabetic human skeletal muscle cells. However, a combinatorial treatment was shown to promote higher concentrations of reduced glutathione and may prevent oxidative damage in HSMM and DHSMM cells. A metformin only treatment was most effective at significantly increasing SOD activity in both HSMM and DHSMM cells.

This study also indicated that combined preparations of metformin and *K. pinnata* may modulate immune responses by significantly elevating concentrations of Interleukin (IL) 2, tumor necrosis factor-alpha (TNF-α), and interleukin-10 in HSMM and DHSMM cells. This study concluded that combined preparations significantly lower the concentration of IL-6 in diabetic and non-diabetic human skeletal muscle cells.

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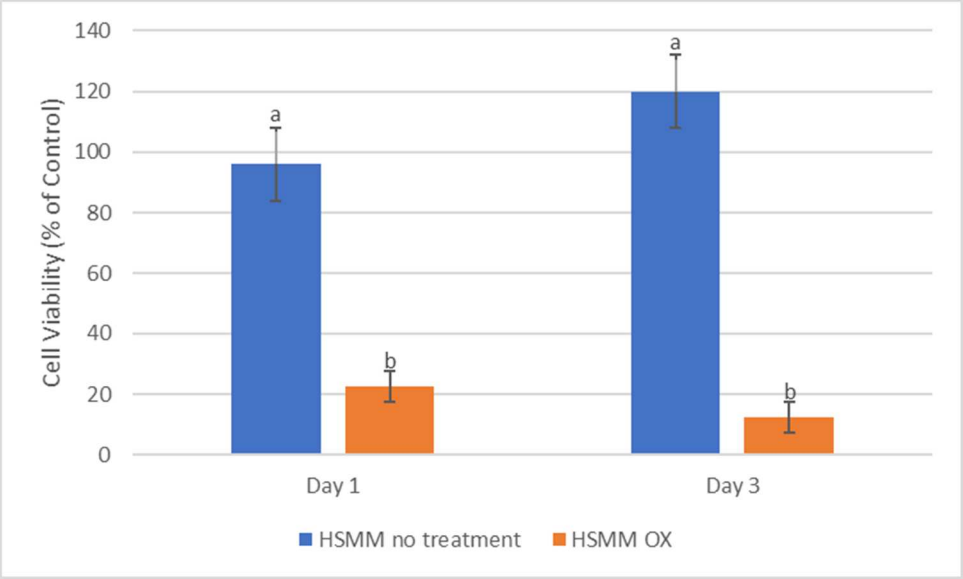
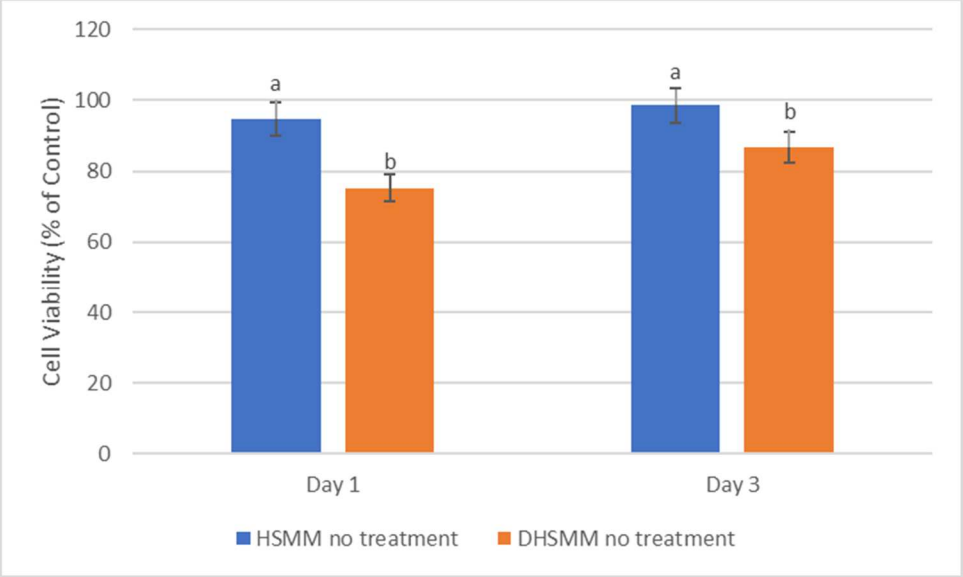
LIST OF APPENDICES

APPENDIX PAGE



1: **Cell viability of untreated HSMM cells compared to H_2O_2 stressed induced HSMM cells.** One-way ANOVA and Duncan's multiple range test were used to statistically compare the difference in cell viability among the groups. Data that share different letters are significantly different ($P<0.05$).



2: **Cell viability of untreated HSMM cells compared to untreated DHSMM cells.** One-way ANOVA and Duncan's multiple range test were used to statistically compare the difference in cell viability among the groups. Data that share different letters are significantly different ($P<0.05$).