ABSTRACT

Title of Dissertation: INHIBITORY ACTIVITIES OF POLYPHENOL-RICH SORGHUM BRAN EXTRACTS ON OBESITY-RELATED DISEASES; BREAST CANCER, DIABETES, AND MUSCLE DAMAGES

Wu-Joo Daniel Lee, Doctor of Philosophy, 2026

Dissertation directed by: Professor Seong-Ho Lee
Department of Nutrition and Food Science

Obesity remains a leading global cause of mortality, with its prevalence continuing to rise. Functional food products represent a promising strategy for mitigating obesity and its associated disorders, and sorghum has emerged as a potential candidate due to its high polyphenol content and reported anti-obesity properties. Despite this potential, sorghum remains underutilized in human diets, and its biological activities related to obesity are not well characterized. This study investigates the effects of polyphenol-rich sorghum bran extracts (SBE) on obesity-related diseases. The findings demonstrate that SBE significantly inhibited the proliferation of MDA-MB-231 human breast cancer cells by inducing apoptosis, arresting the cell cycle at the G2/M phase,

enhancing endoplasmic reticulum (ER) stress, and promoting mitochondrial dysfunction and autophagy. Additionally, SBE exhibited anti-diabetic activity by improving insulin tolerance in high-fat diet (HFD)-fed mice and enhancing glucose uptake in murine myoblast cells. SBE also conferred antioxidant protection in myoblasts exposed to oxidative stress by attenuating apoptosis and S-phase arrest, reducing reactive oxygen species (ROS) production, and suppressing ER stress and autophagy. Collectively, these results suggest that polyphenol-rich sorghum products may serve as an effective dietary approach for addressing obesity and its related metabolic disorders.

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by

Wu-Joo Daniel Lee

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Advisory committee:
Professor Seong-Ho Lee, Chair
Special Graduate Faculty Dmitriy Smolensky
Professor Shaik O. Rahaman
Assistant Professor Yuanyuan Li
Professor Debabrata Biswas

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List of abbreviations

AAPH	2,2'-Azobis(2-amidinopropane) dihydrochloride
AGE	advanced glycation end-products
Akt	protein kinase B
AMPK	5' adenosine monophosphate-activated protein kinase
ATF3	activating transcription factor 3
Bak	Bcl-2 homologous antagonist/killer
Bax	bcl-2-like protein 4
Bcl-2	B-cell lymphoma 2
BMI	body mass index
Brk	breast tumor kinase
caspase	cysteine-aspartic proteases, cysteine aspartases or cysteine-dependent aspartate-directed proteases
CD4+ cells	T helper cells
CDK	cyclin dependent kinase
CDKN1α	cyclin dependent kinase inhibitor 1 alpha
cIAP-2	cellular inhibitor of apoptosis 2
CO_2	carbon dioxide
COX-2	cyclooxygenase-2
DMEM	Dulbecco's modified Eagle's media
DPPH	2,2-diphenyl-1-picrylhydrazyl
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinases
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FRAP	ferric reducing ability of plasma
GLP-1	glucagon-like peptide 1
GLUT4	glucose transporter type 4

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GPDH	glycerol-3-phosphate dehydrogenase
H2AX	H2A histone family member X
H_2O_2	hydrogen peroxide
HIF-1α	hypoxia-inducible factor 1-alpha
HO1	heme oxygenase 1
ICAM-1	intercellular adhesion molecules 1
IGF	insulin-like growth factor
IGF-1R	insulin-like growth factor 1 receptor
IL	interleukin
iNOS	inducible nitric oxide synthase
IRS	insulin receptor substrate
JAK	Janus kinase
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide
JNK	c-Jun N-terminal kinases
LC3	Microtubule-associated proteins 1A/1B light chain 3B
LPS	lipopolysaccharide
MHC	major histocompatibility complex
MAPK	mitogen-activated protein kinase
MCP-1	macrophage inflammatory protein-1
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NOS2	nitric oxide synthase, inducible
p21	cyclin-dependent kinase inhibitor 1
p53	cellular tumor antigen p53
PARP	poly (ADP-ribose) polymerase
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PGE	prostaglandin E synthase
PI3K	phosphoinositide 3-kinase
PMN	polymorphonuclear

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PPAR-α	peroxisome proliferator-activated receptor alpha
pRb	retinoblastoma protein
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SMAC	second mitochondria-derived activator of caspases
SREBP-1c	sterol regulatory element-binding protein 1c
STAT	signal transducer and activator of transcription
T2D	type 2 diabetes
TBS	tris buffered saline
TG	triglyceride
TNF	tumor necrosis factor
VEGF	vascular endothelial growth factor
XIAP	X-linked inhibitor of apoptosis protein

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Chapter 1. Literature review

1.1. Introduction

Since 1980, the occurrence of obesity is increased by 2 times over the world and being overweight or obese is connected with health problems affecting more than 2 million people. (Friedrich, 2017). In the United States, it is expected that nearly a quarter of the population will have morbid obesity in 2030 (Ward et al., 2019). With the trend of increasing obesity, the occurrence of related diseases such as type 2 diabetes and cancers. These suggest that body weight control can effectively prevent and attenuate the diseases (Sarma et al., 2021). Among being physically active and medical treatments, maintaining healthy diets is considered a convenient method to reduce weight for obese and overweight people, and mostly plant-based food sources are encouraged.

While it is the fifth-most important crop product worldwide, the primary uses of sorghum in the United States are feeding livestock and producing biofuel. It is widely known that sorghum contains high amounts of essential nutrients including starch, dietary fiber, and proteins, and it is rich in micronutrients such as vitamins and minerals. In addition, there are plenty of phytochemicals from sorghum and these materials are beneficial to human health and contribute to body weight control.

Polyphenols are most abundant bioactive phytochemicals in plants and have diverse structures. They have been found to have strong antioxidant capacity (Stagos, 2019) and antimicrobial activity (Daglia, 2012) and therefore play roles in protective responses in plants. Especially, many researchers have used the compounds in attenuating progression of chronic diseases such as obesity due to their strong antioxidative activities and preventive properties against oxidative stress (Mao et al., 2017).. In fact, it has been widely discovered that oxidative

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stress has a strong connection with several chronic diseases, including cancer and diabetes. Therefore, polyphenol-rich sorghum sources can potentially be utilized not only to prevent body weight increase but also attenuate obesity-caused symptoms.

The purpose of this research is to identify the protective activities of a specific polyphenol-rich sorghum bran extracts on metabolic disorders highly associated with obesity.

1.2. Obesity and its prevalence

Obesity, a disease defined by excessive body fat accumulation, is one of the leading causes of death across the world and linked to various chronic diseases, such as cardiovascular diseases, type 2 diabetes, cancer, fatty liver etc. To determine obesity is, several methods have been utilized. Primarily diagnosed using several anthropometric methods including body mass index (BMI), and other factors are also evaluated; waist circumference, thickness of subcutaneous fat (fat under the skin) and bioelectrical impedance analysis. Recent statistics indicated that more than 1 billion people were living with obesity in 2022, and the prevalence for adults was doubled from 1990 (Anderer, 2024; Taylor, 2024).. In the United States, the prevalence of obesity has continuously increased since the 1960s (May et al., 2013). It is anticipated that around half of the population will be obese in 2030, and about quarter will be considered severely obese (Ward et al., 2019). With the increase of obese people, the cost of medical expenses also increased. Between 2001 to 2016, it was estimated that obese people spent about $ 2,500 more on medical expenses compared to non-obese people annually, and the comprehensive medical cost expense was more than $ 200 million in the United States (Cawley et al., 2021a). In addition, obese people were expected to have significantly decreased economic productivity compared to non-obese people. It was reported that obesity caused annual productivity loss per employee from

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$ 271 to $ 542 in the United States in 2016 (Cawley et al., 2021b). This is linked to increased job absenteeism due to injury or illness.

1.3. Etiologies of obesity

Obesity is a multifactorial disease characterized by excessive body fat accumulation. Its development is influenced by a combination of environmental, behavioral, metabolic, and genetic factors, with genetic predisposition contributing to variations in obesity risk among individuals. First, mutations in specific single genes can cause obesity. One example is mutated leptin and the receptor. Leptin is an adipokine required for long term regulation of body weight through regulating appetite and satiety in the hypothalamus of brain. When energy balance is positive and body accumulates more fat, leptin is released from body fat and binds to leptin receptor (LEPR) and induces decreased appetite and energy expenditure. If leptin level becomes low due to genetic mutation of leptin gene in adipose tissues, this will cause failure of reduced energy intake and induced energy consumption (Blüher et al., 2009). Mutations in LEPR also causes leptin resistance through impairment of leptin-melanocortin signaling in hypothalamus and causes obesity (Chaves et al., 2022). Besides leptin-melanocortin axis components, mutations in multiple other genes are highly associated with genetic obesity. One example is the fat mass and obesity-associated (FTO) genes, such as 2-oxoglutarate-dependent dioxygenases whose variants are strongly associated with obesity (Hess & Brüning, 2014). Since FTO can affect appetite and fat oxidation in adults (Ponce-Gonzalez et al., 2023), altercations in these genes can negatively affect fat metabolism and increases the chance of obesity. Syndromic disorders also can be connected to obesity. Prader-Willi's syndrome, a genetic disorder affecting many parts of human body, causes extreme hunger and this can be led to overeating, obesity and even type 2 diabetes (Khan et al., 2018). There are also epigenetic issues contributing to obesity.

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Epigenetic regulation has become a critical lens for understanding the biological mechanisms that contribute to obesity. Among these regulatory layers, DNA methylation has been widely implicated, with studies showing that aberrant methylation of genes involved in insulin signaling can disrupt glucose homeostasis and promote metabolic dysfunction. Histone modifications further contribute to obesity-related phenotypes; changes in histone acetylation and methylation have been linked to altered chromatin accessibility and the progression of adiposity. In addition, non-coding RNAs, including microRNAs and long non-coding RNAs, play essential roles in post-transcriptional control of metabolic pathways, and dysregulation of these molecules has been associated with impaired glucose and lipid metabolism. Together, these epigenetic mechanisms highlight the complex regulatory networks that underlie obesity and offer promising avenues for mechanistic insight and therapeutic development (Mahmoud, 2022).

Non-genetic factors also play a significant role in the occurrence of obesity. This pattern is generally defined by consuming more refined grains, red meats and processed meats, high-sugary snacks such as drinks and cookies, fried foods, and insufficiently eating vegetable, fruits, and nuts This diet is rich in high calories and lacks dietary fiber and probiotics. Especially, western diet is considered a major factor of obesity in the United States (Rakhra et al., 2020). Change in lifestyle also became a factor of obesity. Since the industrial revolution, people work sitting more and many workplaces do not need much physical activity, while ninety percent of the world population lived in agricultural societies which required a lot of physical exertion. This major change in lifestyle resulted in increased cases of several diseases including type 2 diabetes (Levine, 2015). Drugs are also a contributing factor in obesity. For example, atenolol, a chemical used for treating heart diseases, has been known to increase obesity by increasing serum glucose level (Masood & Moorthy, 2023). Some medications for mental health can also lead to obesity.

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One example is antiseizure drugs. While side-effects vary by drugs, it was reported that some antiseizure drugs are strongly connected increased appetite and weight gain in children (Buraniqi et al., 2022). Moreover, It was discovered that some medications for treating depression can cause increased appetite and weight gain (Lee et al., 2016).

1.4. Obesity and breast cancer

Obesity has been identified as a significant risk factor for both cancer development and increased mortality.. While mortality rates over decades have been declining with earlier detection and advanced treatments, breast cancer is the most frequently diagnosed cancer in the United States and the second leading cause of cancer death in female (Giaquinto et al., 2024). In particular, the breast cancer risk increased with higher body mass index and weight gain in postmenopausal women (Naaman et al., 2022).

Diet and physical activity are key factors affecting obesity and breast cancer risk. While consuming more dietary fiber through whole grains, fruits, and vegetables significantly decreases breast cancer risk, consuming more red and processed meats have been found to be strongly associated with higher breast cancer incidence (Farvid et al., 2018). Researchers identified the increased breast cancer risk connected with higher consumption of saturated fat and iron (Xia et al., 2015) in red meat and high content of additive in process meats. Many micronutrients also can affect the occurrence of breast cancer. For example, it has been discovered that sufficiently consuming vitamin D and calcium had protective activities on breast cancer risk and decrease the risk of obesity and overweight (Cui & Rohan, 2006). Avoiding alcohol beverages can potentially reduce the risk of not only obesity but also breast cancer (Shield et al., 2016). In addition to dietary patterns, increasing physical activity is also considered as an effective way to reduce

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breast cancer risk and enhance rehabilitation activity in breast cancer patients (Volaklis et al., 2013).

Obesity increases the risk of breast cancer through modulating metabolic pathways. Adipocytes represent a major component of breast tissue and are therefore an important factor in breast cancer risk. While adipocytes primarily store energy in tissues, they also play roles in metabolism and immune response. During these processes, the pre-adipocytes differentiate and mature into adipocytes. Since breast cancer cells have been found to be locally permeated into neighboring tissues, excessive adipogenesis can result in the development of breast cancer. This phenomenon is known as cancer-associated adipogenesis (CAA). The key proteins regarding CAA are peroxisome proliferator activated receptor gamma (PPAR-γ) and CCAAT enhancer binding protein alpha (C/EBP-α).

These proteins are involved in adipogenesis and reflect the degree of tumor development in breast tissues. CAA also induces the elevated secretions of proinflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-alpha) (Kakkat et al., 2024). In addition, alterations in gut microbiota induced by diet-caused obesity are additional risk factors in breast cancer. For example, Studies have revealed that obesity-caused alteration in gut microbiota can possibly increase the breast cancer risk through dysbiosis. In female 3-week-old BALB/c mice, the group with induced obesity showed shifted fecal microbiome population and increased mammary gland macrophage infiltration (Arnone et al., 2024). This suggests that properly managing gut health would be critical in lowering the risk of breast cancer.

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1.5. Obesity and diabetes

Diabetes Mellitus (DM), commonly known as diabetes, is a disease defined by abnormally high blood sugar level. Over 800 million people worldwide are estimated to have diabetes in 2022, increasing by more than 600 million compared to 1990 (Zhou et al., 2024). Furthermore, diabetes was the eighth leading cause of death in the United States in 2020, becoming the seventh excluding COVID-19 (Ahmad & Anderson, 2021). While type 1 diabetes account for about 5-10% of total diabetes and is caused by insufficient production and secretion of insulin from pancreas with early onset, type 2 diabetes (T2D) account for 90-95% of diabetes and is associated with insulin resistance without affecting insulin secretion and shows late onset (E. Ahmad et al., 2022).

Excessive accumulation of body fat in obese people has been found to be a factor in selective insulin resistance leading to impaired glucose uptake and metabolic dysfunctions (Hagberg & Spalding, 2024). In addition to hyperglycemia, elevated level of free fatty acid (FFA) have been linked to type 2 diabetes. Researchers evaluated that an increase of serum FFA impaire insulin-stimulated glucose uptake and glycogen synthesis (Boden, 2001).
Moreover, obesity induces sustained stress on pancreatic β cells, ultimately leading to β-cell dysfunction. (Cerf, 2013). As a result, insulin secretion is impaired, and this negatively affects glucose homeostasis.

T2D is strongly associated with lifestyle. While T1D is hard to prevent and requires lifetime insulin medication, T2D is both preventable and manageable with changes in lifestyle including diet and physical activity. It was reported that reducing weight by lifestyle modification focusing on changing diet and increasing physical activity can significantly reduce

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the risk of T2D (Steyn et al., 2004) and help glycemic control in T2D patients (Johansen et al., 2017).

1.6. Obesity, inflammation, and oxidative stress

Excessive body fat accumulation is connected to chronic systemic inflammation in adipose tissues. This is caused by pro-inflammatory cytokines infiltrated from macrophages. Related proteins include TNF, IL-1, IL-6, and IL-8 (Moghbeli et al., 2021). Elevated inflammatory responses trigger increased oxidative stress and lead to various diseases such as cardiovascular diseases.

One factor of obesity-induced inflammation is increased intestinal antigens. It was discovered that obesity significantly increases intestinal permeability and therefore induces higher levels of antigen absorption, including LPS in mice (Wang et al., 2024). As LPS is widely known to increase release of inflammatory cytokines, obesity associated with physiological changes in intestines can be a risk factor in elevated inflammatory responses. In addition, dietary factors themselves can also trigger increased inflammation. Specifically, free fatty acids were found to be indirectly bind to toll-like receptors and trigger kappa-light-chain-enhancer of activated B cells (NF-κB) in rat liver. As a result, inflammatory responses are activated, potentially inducing the development of steatohepatitis (Boden et al., 2005). Additionally, obesity promotes inflammation-driven adipocyte death. Researchers have found that hypertrophic adipocytes have increased inflammatory responses compared to normal adipocytes. As a result, impaired and damaged adipocytes produce increased level of cytokines such as TNF- and IL-6 (Hildebrandt et al., 2023). Another factor related to obesity-induced inflammation is hypoxia due to elevated oxygen consumption. It was observed that elevated level of hypoxia-

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inducible factor 1 (HIF-1) as a hallmark of initial inflammation, was remarkably increased in obese rodent tissues and resulting elevated cytotoxic T-cell invasion (Rausch et al., 2008). Thus, repression of HIF-1 alpha release (downregulation of HIF-1) would be effective target in preventing obesity-induced inflammation (Lee et al., 2014). These results suggest that obese people have higher risks of diseases related to inflammation, such as cancer and diabetes.

Oxygen itself is essential in live tissues for mitochondrial oxidative phosphorylation and cellular respiration pathway and subsequent energy (ATP) production. During these physiological metabolisms, oxygen is converted to incomplete and unstable derivates referred to as reactive oxygen species (ROS). Excessive amount of ROS give damages to DNA, RNA, proteins and lipids, and leads to oxidative damage and oxidative stress (Cichoż-Lach & Michalak, 2014). In addition, ROS production is induced by inflammatory pathways. For example, TNF-α is linked to elevated oxidative stress by binding to several receptors and initiating NF-κB signaling pathway (Suematsu et al., 2003). As serum TNF-α level is inappropriately high in obese populations, obesity is promoting increased oxidative stress in tissues and causes various diseases as TNF-α is connected with increased mitochondrial ROS (Chen et al., 2008).

1.7. Sorghum

Sorghum bicolor, commonly called sorghum, is the fifth most important crop following rice, wheat, maize, and barley. It first originated in 8000 B.C. and became domesticated primarily in northeast Africa, probably in Ethiopia (Amarakoon et al., 2021). It spread from Africa to Asia and Europe, especially Spain, in the Middle Ages during the Arab Agricultural Revolution (Watson, 1974). The characteristics such as heat-tolerance, drought-resistance, and nitrogen-

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efficiency (Hadebe et al., 2017) led sorghum to be widely cultivated across the world and utilized food sources in Asia and Africa. It was reported that the annual produce of sorghum around the world was 60 millions of tons and one sixth of the production was from the United States (Amarakoon et al., 2021). While the USA was the top sorghum exporter in the world from 2015 to 2021 (Charyulu et al., 2024), the crop has been primarily used in livestock feeding, forage, and biofuel production in the country. Sorghum is an underutilized human food source in the USA, and it can potentially be a beneficial and effective material for human food production in the future. In addition to the high accessibility, the nutritional characteristics of sorghum can make the crop to be a health-beneficial food source.

Sorghum grain contains various nutrients such as carbohydrates, lipids, and proteins, and is rich in diverse micronutrients including minerals and vitamins (Rooney & Pflugfelder, 1986). Specifically, many researchers have found that there are many kinds of plant-source materials, also known as phytochemicals. One example is phenolic compounds including polyphenols. Polyphenols are secondary metabolites in plants and show protective activities against ultraviolet, microbial infections, and herbivore attacks (Hättenschwiler & Vitousek, 2000). Moreover, polyphenols have been found to be as one of the strong antioxidants. Based on the abundance and high antioxidant capacity, polyphenols have been studied as an effective method to treat and attenuate several human diseases known to be strongly connected with oxidative stress. Therefore, the whole grain sorghum can be an efficient source of energy and other essential nutrients. In addition to the nutrient factors, protein in sorghum were found to be less digestible compared to those from other cereals (Wong et al., 2009). In this research, brans from four different species were used and the characteristics are indicated in Table 1 (Lee et al., 2022). While SC84 sorghum brans contain high amount of phenolic compounds compared to PI570481

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species, it was determined that PI570481 sorghum brans were rich in 3-deoxyanthocyanidins (3-DA) than SC84. 3-DA are a subclass flavonoid family and used for nature food colorants stable under pH change. In addition, it was discovered that 3-DA played diverse roles in health including healing wounds, attenuating allergic responses, relieving cardiovascular risks by lowering serum low-density lipoprotein (LDL) in humans, and inhibiting parasite proliferation (Xiong et al., 2019). Therefore, it was anticipated that PI570481 sorghum brans can yield more biological activities despite its lower content of total phenols compared to SC84 brans rich in tannins.

Nutritional factors of sorghum have been effective in various kinds of diseases. Especially, it has been found that the crop can potentially be utilized to obesity and related diseases and symptoms.

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Table 1. Amounts of phenolic compounds in different species of sorghum bran extracts

	PI570481	SC84	Sumac	White
Total polyphenols mg GAE/g *	38.11 ± 1.38	55.57 ± 4.78	12.4 ± 0.67	0.67 ± 0.88
Condensed Tannins mg CE/g #	179.01 ± 20.99	415.83 ± 89.61	13.03 ± 0.27	ND

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1.8. Sorghum and obesity

Both in vitro and in vivo research, and clinical trials have evaluated the anti-obesity activities of sorghum bran and the extracts. When treated to 3T3-L1 preadipocyte cells, sorghum water extract remarkably inhibited the differentiation of preadipocytes and accumulation of triglyceride in the cells. These results were induced by suppresses of lipid metabolism indicated with inhibited activities of glycerol 3-phosphate dehydrogenase (GPDH) and the pancreatic lipase enzymes (Park et al., 2011). In addition, flavonoids, polyphenols rich in sorghum species, induced suppressed activities of pancreatic lipase and this suggests that sorghum component can be effective in preventing lipid absorption in human body (Ofosu et al., 2020).

There were some experiments which evaluated the inhibitory activities of sorghum on obesity. Primarily, lower level of triglyceride in high-fat diet induced obese rats was decreased by feeding the animals with sorghum diet (Chung et al., 2011). Tannin-rich sorghum, yielded anti-obesity activities in rabbits. The animals fed with normal corn-based diet with tannins showed decreased body gain weight, and feed conversion ratio compared to the control group provided with only normal diet (Al-Mamary et al., 2001). Tannins also showed anti-obesity effects in rats. Obese rats induced by high fat diet showed lower body mass index, liver weight, fatty liver, and sterol regulatory element-binding protein 1c (*SREBP-1c*) when fed with extruded sorghum flour containing tannin. On the other hand, expressions of PPAR*α* and *adiponectin receptor 2* (*AdipoR2)* were remarkably enhanced by sorghum diet, indicating that sorghum can strengthen lipid oxidation (de Sousa et al., 2018), and this suggests that sorghum is beneficial in lowering the amount of body fat.

Clinical trials to humans revealed the inhibitory activities of sorghum on obesity. For example, extrude sorghum diet induced higher weight loss, and decreased waist circumstance,

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body fat percentage in overweight and obese people (Anunciação et al., 2019). Moreover, healthy people provided with whole grain sorghum showed higher satiety and it was indicated by promoted expressions of postprandial glucagon-like peptide 1 (GLP-1) and gastric inhibitory polypeptide (GIP) (Stefoska-Needham et al., 2016).

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Table 2. Inhibitory activities of sorghum on obesity

Sorghum component	Research model	Anti-obesity activities	References
Sorghum water extract	3T3-L1 preadipocytes	↓ Differentiation, GPDH, triglyceride accumulation	(Park et al., 2011)
Flavonoids	Enzyme model	↓ Pancreatic lipase	(Ofosu et al., 2020)
Sorghum diet	Rats with high-fat induced obesity	↓ Triglyceride level	(Chung et al., 2011)
Tannins	Rabbits	↓ Body weight gain, feed conversion ratio	(Al-Mamary et al., 2001)
	Rats with high-fat induced obesity	↓ BMI, liver weight, fatty liver, SREBP-1c ↑ PPARα, AdipoR2	(de Sousa et al., 2018)
Extrude sorghum diet	Overweight and obese people	↑ Weight loss ↓ Waist circumstance	(Anunciação et al., 2019)
Whole sorghum grain	Healthy people	↑ Satiety, GLP-1, GIP	(Stefoska-Needham et al., 2016)

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1.9. Sorghum and cancer

It has been widely discovered that obesity and cancer occurrence are strongly connected, and dietary intervention can be an effective method to prevent and attenuate the risk of cancer. And \anti-cancer effects of sorghum and the components in many cancers have been identified. The models include breast, colon, liver, lung cancer, and leukemia. Since cancer cells have abnormal cell growth pathways compared to normal cells, downregulating these pathways and inhibiting the cell proliferation is critical in managing cancer. The target pathways include apoptosis, cell cycle arrest, angiogenesis, and metastasis. Mostly phenolic compounds from sorghum have been known to be anti-cancer chemicals among other extracts.

Apoptosis is a programmed cell death and occurred by intrinsic and extrinsic factors, such as DNA damage, accumulation of endoplasmic stress, and oxidative stress. When fed with *Hwanggeumchal* sorghum extracts, the expression of tumor suppressing factor p53 was significantly enhanced in xenograft animals. On the other hand, they showed inhibited activation of STAT5b/IGF-1R signaling pathways, and downregulated HIF-1α, Bcl-2, and breast tumor kinase (Brk) expressions (Park et al., 2012). In addition, 3-deoxyanthocyanidins (3-DA) extracted from red sorghum induced apoptotic pathways in breast cancer cells by upregulating p53 expression and inhibiting Bcl-2 activity (Suganyadevi et al., 2013). Similarly, 3-DA activated apoptosis in colon cancer cancers. p53-independent apoptotic pathway was strengthened in colon cancers by 3-DXA compared to the control (Massey et al., 2014). Anthocyanin extracted from sorghum also induced apoptosis in colon cancer cells by suppressing the activations of cIAP-2, survivin, XIAP, and insulin-like growth factor binding proteins (Mazewski et al., 2018). In addition to these specific compounds, phenol-rich crude sorghum extracts and the grains upregulated apoptotic pathways in colon cancer cells. Phenol-rich

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sorghum extracts enhanced the expressions of cleaved PARP and caspase-3, and the activities of pH2AX, pERK, pJNK and ATF3 (Lee et al., 2020). Moreover, in mice with colon cancer, consumption of a mixed cereal grain diet containing sorghum was associated with improved biomarkers, indicating potential chemopreventive effects mediated through enhanced apoptosis.. p53 and caspase 3 protein expressions were enhanced, and gene expression of CDKN1α was upregulated (Song et al., 2020). Phenol-rich bran extracts also stimulated apoptotic pathways in lung cancer cells by producing more ROS, increasing activities of caspase 3, caspase 8, cleaved PARP1, cleaved caspase 3, and XIAP, and inhibiting IGF-1, IGF-2, survivin, and SMAC activities (Smolensky et al., 2018). In leukemia cells, a 3-DA family chemical apigeninidin induced apoptosis. When the cells were treated with 3-DA, Bcl-2 expression was inhibited, and BAK, BAX, caspase-9, caspase-3, cleaved PARP, lamin B activities were upregulated. Moreover, mitochondrial cytochrome C and apoptosis-inducing factor levels were increased indicating that the programmed cell death signal was enhanced by apigeninidin (Woo et al., 2012).

Cell cycle is a cellular process for DNA duplication and cell division, which consists of G1, S, G2 and M phases. There are checkpoints controlled by different protein expressions, and cancer cells have abnormal regulation so that the proliferation is improperly maintained. Sorghum and the extracts executed the inhibition of cancer cell growth by affecting the checkpoints. *Hwanggeumchal* sorghum extracts downregulated cyclin D, cyclin E, and pRb expressions and therefore induced G1 phase arrest in breast cancer cells (Park et al., 2012). In colon cancer cells, G1 phase arrest was induced by anthocyanin-rich plant extracts (Mazewski et al., 2018), and S phase arrest also occurred with upregulated p21 expression and suppressed CDK 6 activity when treated with phenol-rich sorghum extracts (Lee et al., 2020). With an in

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vivo model, both gene and protein expressions of cyclin D1 were observed by mixed cereal grain diet containing sorghum (Song et al., 2020). Crude high-polyphenol sorghum extracts enhanced the activities of p21 and Checkpoint kinase 2 (Chk2) in HepG2 cells and therefore inhibited the cell growth (Smolensky et al., 2018).

During cellular growth, new blood vessels are formed from the existing vessels, and this is referred to as angiogenesis. In cancer cells, this process remarkably contributes to the transition of benign tumors to malignant ones. Researchers have evaluated that sorghum extracts inhibited this transition step and therefore the cereal can be used in chemotherapy. In breast xenograft mice with breast cancer, *Hwanggeumchal* sorghum extracts significantly downregulated vascular endothelial growth factor (VEGF) and VEGF-R2 by suppressing hypoxia induction factor (HIF-1) activity (Park et al., 2012). Procyanidin rich sorghum extract also inhibited VEGF expression in C57 mice with lung cancer (Wu et al., 2011).

Metastasis is a crucial process for cancer spread from original sites to their target organs. This occurs when the tumor cells lose their adhesive property and acquire invasive capacity. Not much research evaluated the anti-migration activity of sorghum extracts on cancer cells. Park et al (2012) reported that *Hwanggeumchal* sorghum extracts remarkably inhibited breast cancer cell metastasis to lung in xenografts. This effect was accompanied by downregulated Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway (Park et al., 2012). Another phenol-rich sorghum extracts also significantly inhibited colon cancer cell migration and invasion by suppressed expressions of β-catenin, VEGF and MMP-9 proteins (Lee et al., 2020).

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Table 1. Inhibitory activities of sorghum on cancer

Sorghum component	Pathway	Anti-cancer activities	Type	References
Hwanggeumchal sorghum extracts	Apoptosis	↑ p53 ↓ STAT5b/IGF-1R, HIF-1α, Bcl-2, Brk (in vivo)	Breast	(Park et al., 2012)
3-deoxyanthocyanidins		↑ p53 ↓ Bcl-2		(Suganyadevi et al., 2013)
		↑ p53-independent pro-apoptotic activity	Colon	(Massey et al., 2014)
Anthocyanin		↓ cIAP-2, survivin, XIAP, insulin-like growth factor binding proteins		(Mazewski et al., 2018)
Phenol-rich crude sorghum extract		↑ PARP, caspase-3, pH2AX, pERK, pJNK, ATF3		(Lee et al., 2020)
Diet with mixed cereal grain containing sorghum		↑ p53, caspase-3, CDKN1α (in vivo)		(Song et al., 2020)
Phenol-rich bran extract		↑ ROS, caspase-3 and -8, cleaved PARP1, cleaved caspase 3, XIAP ↓ IGF-1, IGF-2, survivin, SMAC activities	Lung	(Smolensky et al., 2018)
Apigeninidien		↓ Bcl-2 ↑ BAK, BAX, caspase-9, caspase-3, cleaved PARP, lamin B, mitochondrial cytochrome C, apoptosis-inducing factor levels	Leukemia	(Woo et al., 2012)
Hwanggeumchal sorghum extracts	Cell cycle arrest	↓ Cyclin D and E, pRb ↑ G1 arrest	Breast	(Park et al., 2012)
Anthocyanin-rich plan extract		↑ G1 arrest	Colon	(Mazewski et al., 2018)
Phenol-rich sorghum extracts		↑ p21 ↓ CDK6 ↑ S arrest		(Lee et al., 2020)

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Mixed cereal grain		↑ p53, CDKN1α ↓ Cyclin D1, NOS2, COX-2 (in vivo)		(Song et al., 2020)
High-polyphenol extracts		↑ p21, Chk2	Leukemia	(Smolensky et al., 2018)
Hwanggeumchal sorghum extracts	Angionenesis	↓ VEGF, VEGF-R2, HIF-1α (in vivo)	Breast	(Park et al., 2012)
Procyanidin-rich extract		↓ VEGF (in vivo)	Lung	(Wu et al., 2011)
Hwanggeumchal sorghum extracts	Metastasis	↓ Metastasis to lung, JAK/STAT	Breast	(Park et al., 2012)
Phenol-rich sorghum extract		↓ Migration, *β*-catenin	Colon	(Lee et al., 2020)

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1.10. Sorghum and diabetes

Studies indicate that majority of individuals with type 2 diabetes are obese, and obesity represents the most impactful modifiable risk factor for disease development. Thus, treating obesity can both directly and indirectly prevent diabetes and improve disease prognosis. Researchers have evaluated the beneficial effects of sorghum and the components on diabetes. For example, activities of intestinal enzymes involved in starch digestion were suppressed by consumption of flavonoids. α-amylase and α-glucosidase showed significantly decreased activity by the phenolic compounds, and this resulted in the decreased level of Advanced glycation end-products (AGEs) (Farrar et al., 2008; Nguyen et al., 2014). This suggests that flavonoids can be utilized to suppress the digestion of polysaccharides such as amylose and amylopectin and therefore reduce blood sugar level. In addition, crude ethanol sorghum extracts inhibited the activities of these two enzymes (Kim et al., 2014). Moreover, sorghum containing food products were known to be effective in slowing down starch digestion. Grain sorghum muffin induced higher amount of slowly-digestible and resistant starch, and reduced amount of readily digestive starch (Poquette et al., 2014). This suggests that consuming sorghum products can prevent and attenuate diabetes by inhibiting starch digestion and regulating blood sugar level.

The anti-diabetic activities of sorghum were also evaluated by *in vivo* works. Diabetic rats induced by streptozotocin (STZ) showed enhanced expression of phosphorylated AMPK to total AMPK ratio when fed with sorghum ethanol extract diet, indicating increased energy homeostasis, and glucose and fatty acid uptake. On the other hand, the sorghum diet remarkably suppressed the activities of phosphorylated p38, and expressions of phosphoenolpyruvate carboxykinase (PEPCK) (Kim & Park, 2012). These proteins are responsible for gluconeogenesis in hepatic tissues, and the inhibition is a marker of homeostasis indicating

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increased glucose uptake. Blood glucose level also was significantly decreased by sorghum diet compared to the control one. Accompanied by the increased AMPK activity, macrophage infiltration was also significantly suppressed by sorghum extract diet in STZ-induced diabetic rats (Mukai et al., 2020). This suggests that sorghum can contribute to promoting insulin activity.

Human clinical studies showed that sorghum-containing food products were found to have anti-diabetic activities. For example, healthy adults provided with sorghum-containing muffins showed decreased serum glucose and insulin levels (Poquette et al., 2014). Connected with the enzyme activities written above, this result suggests that sorghum can be included in food products containing wheat to be consumed by people with obesity and/or diabetes. Especially, white wheat is known to be its high glycemic index while adding sorghum to wheat products can be effective in inhibiting blood glucose increase. Moreover, sorghum showed preventive effects on postprandial glycemia. Healthy adults who consumed extruded sorghum drink recorded lower glycemic response compared to the control group (Anunciação et al., 2018).

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Table 4. Inhibitory activities of sorghum on diabetes

Sorghum component	Research model	Anti-diabetic pathways	References
Flavonoids	Enzyme model	↓ α-amylase, α-glucosidase, AGEs	(Farrar et al., 2008) (Nguyen et al., 2014)
Crude ethanol extracts	Enzyme model	↓ α-amylase, α-glucosidase	(Kim et al., 2014)
Grain sorghum muffin	Enzyme model	↑ Slowly-digestible and resistant starch ↓ Readily digestive starch	(Poquette et al., 2014)
Sorghum ethanol extract diet	STZ-induced diabetic rats	↑ pAMPK/AMPK, glucose uptake ↓ pp38/p38, PEPCK	(Kim & Park, 2012)
Sorghum extract diet	STZ-induced diabetic rats	↑ pAMPK ↓ Microphage infiltration	(Mukai et al., 2020)
Sorghum-containing muffin	Healthy adults	↓ Serum glucose and insulin	(Poquette et al., 2014)
Extruded sorghum drink	Healthy adults	↓ Glycemic response	(Anunciação et al., 2018)

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1.11. Sorghum and inflammation

Obesity induces chronic and low-grade systemic inflammation because excessive accumulation of adipose tissue acts as an active endocrine organ, releasing inflammatory mediators. Although proinflammatory signaling in the adipose tissues is required for proper adipose tissue remodeling and expansion (Asterholm et al., 2014), inflammation leads to various negative symptoms in body. Therefore, It is effective target for prevention of obesity-related metabolic disorders. It has been evaluated that sorghum extracts showed anti-inflammatory activities with in vitro experiments. Treatment with apigenin- and apigeninidin-containing sorghum extracts downregulated cyclooxygenase-2 (COX-2) activity, and decreased prostaglandin E2 (PGE2) production in LPS-induced peripheral blood mononuclear cells (PBMCs) (Makanjuola et al., 2018). Phenolic compounds are known to suppress inflammation in several types of cells through modulating different pathways. Treatment of phenols remarkably inhibited AAPH-induced ROS production and oxidative damage in, and decreased migration in polymorphonuclear (PMN) cells. PBMC treated with polyphenol-rich sorghum leaf sheath extract yielded enhanced expressions interleukin-6 (IL-6), MCP-1, macrophage inflammatory protein-1 (MIP-1α, and MIP-1β), suggesting that sorghum extracts can induce immune response and therefore prevent unexpected inflammatory symptoms (Benson et al., 2013). In HUVEC cells, phenols upregulated HO1 and eNOS activity, and downregulated NOX4, MCP-1 and ICAM1 (Francis et al., 2019). Non-phenolic compounds extracted from sorghum also conducted anti-inflammatory activities. In lipopolysaccharide (LPS)-induced RAW 264.7 cells, triacylglycerols, tocopherols, carotenoids, and unsaturated fatty acids from sorghum induced suppressed inflammatory responses which is associated with inhibition of IL-1β, IL-6, and COX-2 expression (Zhang et al., 2019). In addition, kafirin (an alcohol-soluble protein) extracted from sorghum suppressed

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expressions of IL-1β, IL-6 and TNF-α, pERK and pJNK, and decreased ROS production in LPS-induced THP-1 cells (Sullivan et al., 2018).

The inhibitory activities of sorghum extracts on inflammation were also demonstrated using *in vivo* models. When fed with sorghum-containing diet, mice with ear edema showed decreased ear thickness compared to the control group. The expressions of iNOS and COX-2 were also downregulated (Shim et al., 2013). In addition, male Sprague–Dawley rats were used to evaluate the anti-inflammatory effects of sorghum on inflammatory bowel disease. After the animals were treated with dextran sulfate sodium (DSS), the group with sorghum diet showed remarkably higher diversity and richness in gut microbiota. This result suggests that sorghum can be an effective method in treating impaired gut microbiota and utilized in treating the related chronic diseases, such as Crohn's disease or ulcerative colitis (Ritchie et al., 2015).

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Table 5. Inhibitory activities of sorghum on inflammation

Sorghum component	Research model	Mechanisms	References
Apigenin Apigeninidin	LPS-induced PBMC cells	↓ COX-2, PGE2	(Makanjuola et al., 2018)
Phenols	Red blood cells	↓ AAPH-induced oxidative damage	(Benson et al., 2013)
	PMN cells	↓ ROS, cell migration	
	PBMC cells	↑ IL-6, MCP-1, MIP-1α, MIP-1β	
	HUVEC cells	↑ HO1, eNOS ↓ NOX4, MCP-1, ICAM1	(Francis et al., 2019)
Triacylglycerols Tocopherols Cartenoids Unsaturated fatty acids	LPS-induced RAW 264.7 cells	↓ IL-1β, IL-6, COX-2	(Zhang et al., 2019)
Kafirin	LPS-induced THP-1 cells	↓ IL-1β, IL-6, TNF-α, pERK, pJNK, ROS	(Sullivan et al., 2018)
Sorghum-containing diet	Mice with ear edema	↓ Ear thickness, iNOS, COX-2	(Shim et al., 2013)
Sorghum diet	DSS-treated SD rats	↑ Diversity and richness in gut microbiota	(Ritchie et al., 2015)

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1.12. Sorghum and antioxidant activity

It has been demonstrated that sorghum components, notably phenolic compounds, possess strong antioxidant activities.. Rao et al. demonstrated that brans of a black sorghum species showed higher phenolic compound concentrations compared to other samples and yielded higher antioxidant capacities. The characteristics were 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity, higher ferric reducing ability of plasma (FRAP) (Rao et al., 2018). In addition, black sorghum-containing hybrids were found to be more antioxidant compared to black sorghum, due to their high-content of condensed tannins (Dykes et al., 2013). It was also identified that irrigation treatments also affected the antioxidant characteristics of sorghum. Deficit irrigation treatment of sorghum remarkably led to the increased amount of phenolic compounds and higher antioxidant activity in the grains (Wu et al., 2017). In addition, sorghum as a food component showed improved antioxidant activity. Wu et al. evaluated that Chinese steamed bread containing sorghum during processing showed higher antioxidant capacity compared to non-sorghum containing bread (Wu et al., 2018).

Several in vitro experiments evaluated the antioxidant capacity of phenol-rich sorghum extracts. In Caco-2 human colorectal cancer cells, black sorghum extracts showed higher cellular antioxidant activity compared to brown sorghum extracts, while brown sorghum extracts yielded elevated cell membrane and intracellular antioxidant effects (Xiong et al., 2021). Phenol-rich sorghum containing bread significantly enhanced cellular antioxidant activity in HepG2 cells (Wu et al., 2018).

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1.13. Research objectives

Hypothesis 1.

Will polyphenol-rich sorghum bran extracts reduce the proliferation of triple-negative breast cancer cells?

Hypothesis 2.

Will polyphenol-rich sorghum brans affect body weight, fat accumulation and glucose tolerance in high-fat diet-induced mice?

Hypothesis 3.

Will polyphenol-rich sorghum-bran extracts prevent hydrogen-peroxide induced oxidative stress in myoblast cells?

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Chapter 2. Mechanistic studies of anti-proliferative activity of a polyphenol-rich sorghum bran extract in triple-negative MDA-MB-231 breast cancer cells

Abstract

The occurrence of breast cancer has been increasing since mid-2000s in the United States. Chemoprevention using food components and phytochemicals has been receiving great attention to prevent breast cancer. Sorghum bran contains a substantial amount of bioactive compounds including polyphenols. In the current study, we investigated an extract of a polyphenol-rich sorghum bran PI570481 using breast cancer cell line to evaluate the anti-cancer activities and elucidate proposed anti-cancer mechanisms. Treatment of PI570481 to MDA-MB-231 breast cancer cells extract showed increased numbers of both early and late apoptotic cells with increased cleavage of poly (ADP-ribose) polymerase (PARP) and cysteine-aspartic proteases (caspase) 3. Treatment of PI570481 extract induced G2/M phase growth arrest by suppressing expression of cyclin B1. In addition, PI570481 extract promoted mitochondrial dysfunction, and endoplasmic reticulum (ER) stress and increased phosphorylation of eukaryotic initiation factor 2 (eIF2) α and extracellular signal-regulated kinases (ERKs) mitogen-activated protein kinases (MAPK) in MDA-MB-231 breast cancer cells. The ratio of 1A/1B light chain 3B (LC3) II to LC3 I was also increased by PI570481 extract. Our data demonstrate that polyphenol-rich sorghum (PI570481) bran possesses anti-cancer activity through modulating cancer hallmarks including apoptosis, cell cycle arrest, mitochondrial dysfunction, ER stress and autophagy in human triple-negative breast cancer cells.

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2.1. Introduction

Cancer is one of the most prevalent chronic diseases around the world and it was the second leading cause of death in the United States in 2021 (F. B. Ahmad et al., 2022). It was predicted that around 2 million of people would be diagnosed with the disease and 600,000 people have died due to cancer in the U.S. in 2023. Among cancers, the estimated new cases of breast cancer were 31 % of the total cases for women in the same country. While cancer incident trends are getting improved for men, the rate of breast cancer have been increasing since the mid-2000s (Siegel et al., 2023). Cancer is mostly treated with medical methods such as medications and radiation, and in addition, food products are also used to decrease the risk. One of the well-known food components which contribute to decrease of cancer risk is polyphenols. Many polyphenolic compounds have been known to be used in treating cancers (Hazafa et al., 2020).

Sorghum is the fifth mostly cultivated crop in the world and used widely for diverse purposes including food, feed, and bioenergy production (Zheng et al., 2023). Especially, it is well-known for containing high yield of polyphenols including tannin (de Morais Cardoso et al., 2017). As polyphenols and other phenolic compounds have been known for their effects including antibacterial (Coppo & Marchese, 2014), anti-inflammatory , and antioxidant activities (Bors & Michel, 2002), sorghum itself and sorghum products can be used to treat some chronic diseases. Especially, many researchers have evaluated the anti-cancer activities of phenolic compounds on different cancer cell lines (P. B. Bhosale et al., 2020) .

In this research, a specific species of sorghum bran extract containing high amount of polyphenols was used to evaluate its inhibitive activities on one of the breast cancer cell lines.

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2.2. Materials and methods

2.2.1. Sorghum extract

An extract from a sorghum (PI570481) bran cultivated in Puerto Vallarta, Mexico was provided by Kansas State University. The polyphenol-rich sample was prepared based on previous research (Lee et al., 2020). 70 % ethanol containing 5 % of citric acid was added to the bran with 10 % w/w ratio to collect the extract. The mixture was incubated under room temperature for 2 hours and stored at -20 °C for overnight. On the following day, the sample was centrifuged at 1000 rpm for 10 minutes and the collected supernatant was used for the research. For the comparison, white sorghum bran extract containing low amount of polyphenols was used as the control.

2.2.2. Chemicals

Antibodies for poly (ADP-ribose) polymerase (PARP), cyclin B1, microtubule-associated proteins 1A/1B light chain 3B (LC3), phosphorylated and non-phosphorylated eukaryotic initiation factor 2 (eIF2) α, phosphorylated and non-phosphorylated extracellular signal-regulated kinases (ERKs), and β-actin were purchased from Cell Signaling Technology (Danvers, MA, USA). All the other chemical materials were purchased from Fisher Scientific (Waltham, MA, USA) unless indicated.

2.2.3. Cell culture and treatment

Human breast cancer cell lines, MDA-MB-231 (triple-negative) and MCF-7 (triple-positive) were purchased from American Type Culture Collection (Manassas, VA, USA). These cells

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were cultured with Dulbecco's modified Eagle's media (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin, and 1% streptomycin at 37 °C with 5 % CO_2 and humid atmosphere. 70 % ethanol containing 5 % citric acid was a vehicle used to dilute the sorghum extract sample.

2.2.4. Cell proliferation

MDA-MB-231 and MCF-7 cells were plated in 96-well plates at a density of 1 x 10^4 and 2 x 10^4 cells/well. After being incubated overnight, different concentrations of PI570481 sorghum bran extract dissolved in the vehicle were diluted with the complete growth medium being added to the cell for the treatment. The cells were treated for 24 hours at 37 °C. Subsequently, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to the cells in each well and incubated at 37 °C for 2 hours. Afterwards, dimethyl sulfoxide (DMSO) was added to dissolve the dyes and the absorbance was measured at 540 nm with an ELISA microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA).

2.2.5. Apoptosis

The number of apoptotic cells were measured using PI/Annexin V-FITC kit based on the manufacturer's instruction. MDA-MB-231 cells were plated into 6-well plates by the density of 3 x 10^5 cells/well. On the following day, the cells were treated with the complete growth medium containing 0, 5, and 10 mg/mL of PI570481 sorghum bran extract. After 24-hour treatment, all the cells in each well were harvested by trypsinization and washed with phosphate-buffered saline (PBS). The washed cells were collected by centrifugation at 3000 rpm for 5 minutes, and the pellets were stained with Annexin V-FITC. The percentage of early and late apoptosis was

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assessed by flow cytometry using a fluorescence-activated cell sorting (FACS) facility at the University of Maryland.

2.2.6. Cell cycle analysis and mitochondrial membrane potential

MDA-MB-231 cells were seeded, incubated, and treated as described above. After being treated, all the cells were harvested by trypsinization and fixed with 70 % ethanol in PBS buffer at -80 °C. The fixed cells were washed with PBS and stained with 70 µM of propidium iodide (PI) containing 0.5 mg/mL of RNAse for 15 minutes. After the staining, the proportion of the cells by different stages of the cycle was analyzed using a FACS facility. To measure the mitochondrial membrane potential, the cells were washed with PBS buffer after the treatment. 1 µM of 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) in the growth medium was added to the cells for 1 hour. Then the cells were trypsinized and suspended in PBS buffer. The mitochondrial membrane voltage indicating the potential was assessed by flow cytometry, by identifying the percentage of green and red stained cells.

2.2.7. Western blotting

MDA-MB-231 cells were seeded and treated the same as described above. After the treatment, the cells were washed with cold PBS buffer twice, and radio-immunoprecipitation assay (RIPA) buffer containing 1 % of combined protease and phosphatase inhibitor was used to collect the cell lysates. The lysate samples were centrifuged at 10,000 rpm for 30 minutes at 4 ℃, and the supernatants were collected. To quantify the protein concentration in the lysates, bicinchoninic acid (BCA) assays were executed based on the manufacturer's instruction. Following the quantification, the lysate samples were mixed with the loading buffer and heat denatured. The

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protein samples were separated by size using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Tris-buffered saline with 0.1% Tween 20 (TBST) containing 5 % skim milk (w/v) was used to block the membranes, followed by washing with TBST. Primary antibodies were added to the membranes for overnight saturation at 4 °C, and saturation with secondary antibodies were conducted consequently. To detect protein expressions, Pierce ECL western blotting substrate was used to generate chemiluminescent, and the visible images were created with Chemidoc MP Imaging System (Bio-Rad, Hercules, CA, USA).

2.2.8. Statistical analysis

One-way analysis of variance (ANOVA) followed by Duncan's multiple tests was used to analyze the results with SPSS (IBM Inc.). All the experiments were executed in triplicate. Data are presented as means ± standard deviations, and *p*-values less than 0.05 were considered significant differences.

2.3. Results

2.3.1. Polyphenol-rich sorghum bran extract inhibited MDA-MB-231 cell growth.

We treated different types of sorghum bran extract to MDA-MB-231 and MCF-7 cell lines for 24 h and performed MTT assay to measure viability of cells. The result indicates that that sorghum bran extract containing high amount of polyphenols significantly inhibited the growth of MDA-MB-231 cell proliferation, compared to vehicle-treated control group in a dose dependent manner while white sorghum extract did not change the viability (Fig. 4.1A). Especially,

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PI570481 bran extract showed dose-dependent inhibitory effects on the cell growth, and therefore, the proper dose could be determined by 5 and 10 mg/mL respectively. On the other hand, viability of MCF-7 cells was not changed with treatment of polyphenol-rich extract (Fig 4.1B).

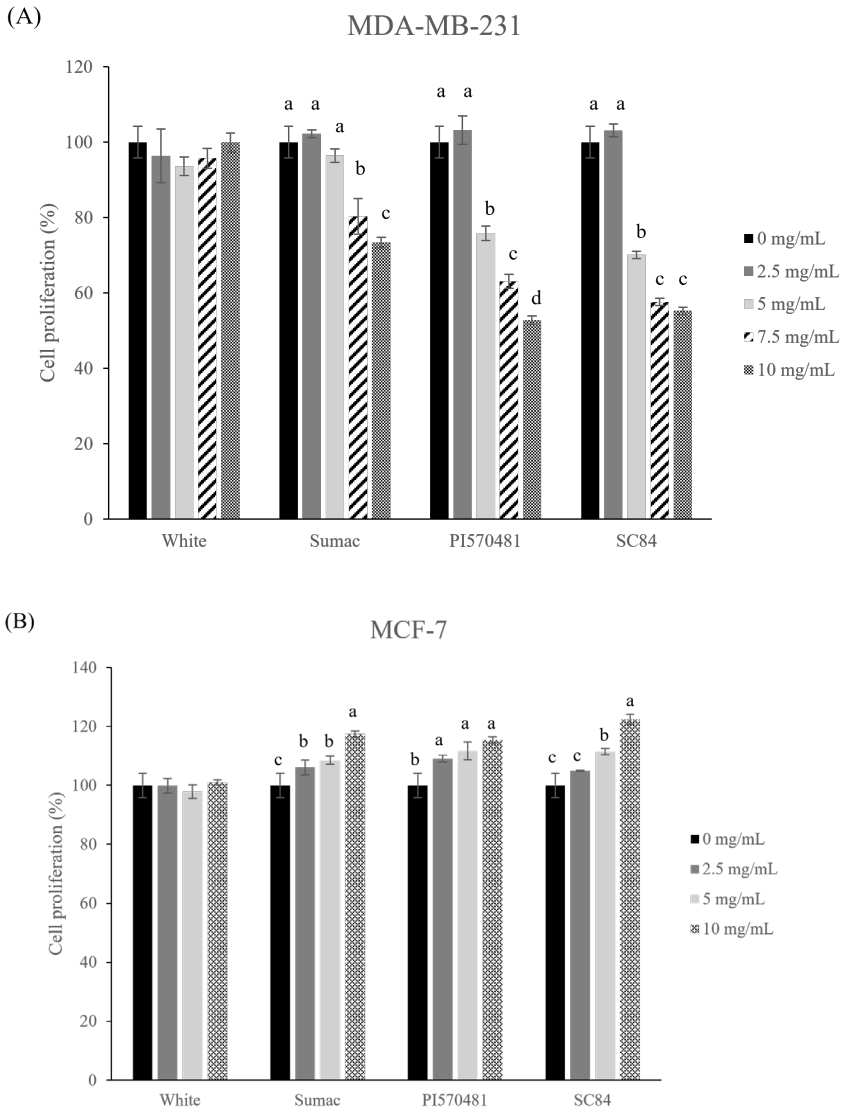

Figure 2.1. Polyphenol-rich sorghum bran extract inhibited MDA-MB-231 cell growth.

The cells were treated with different types of sorghum bran extracts at a dose of 0, 5, and 10 mg/mL for 24 h. After the treatment, MTT assay was performed to measure the cell proliferation. (A) MDA-MB-231 (B) MCF-7. Statistical differences are indicated with different letters ($p < 0.05$).

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2.3.2. PI570481 extract induced apoptosis in MDA-MB-231 cells.

Apoptosis is a progress of programmed cell death required for maintaining homeostasis in living organisms. To evaluate the effects of PI570481 extract on MDA-MB-231 cell apoptosis, we treated the cells with 0, 5, and 10 mg/mL of the polyphenol-rich extract. The percentage of live cells was 90.63 %, 70.13 % and 63.97 % in the cells treated with 0, 5 and 10 mg/mL of the extract, respectively. The rate of total apoptosis was 8.17 %, 26.17 % and 31.47 % in the cells treated with 0, 5 and 10 mg/mL of PI570481 extract (Fig 4.2A-B). Next, we performed WB to measure molecular marker protein of apoptosis. As shown in Fig. 4.2C left, treatment of PI570481 significantly induced cleavage of poly (ADP-ribose) polymerase (PARP) that is required to repair damaged DNA. In addition, there was 2.92 and 4.03-fold induction of cleaved caspase-3 in the cells treated with 5 and 10 mg/mL of PI570481 respectively (Fig. 4.2C right). These results suggest that PI570481 polyphenols stimulate apoptotic pathways in MDA-MB-231.

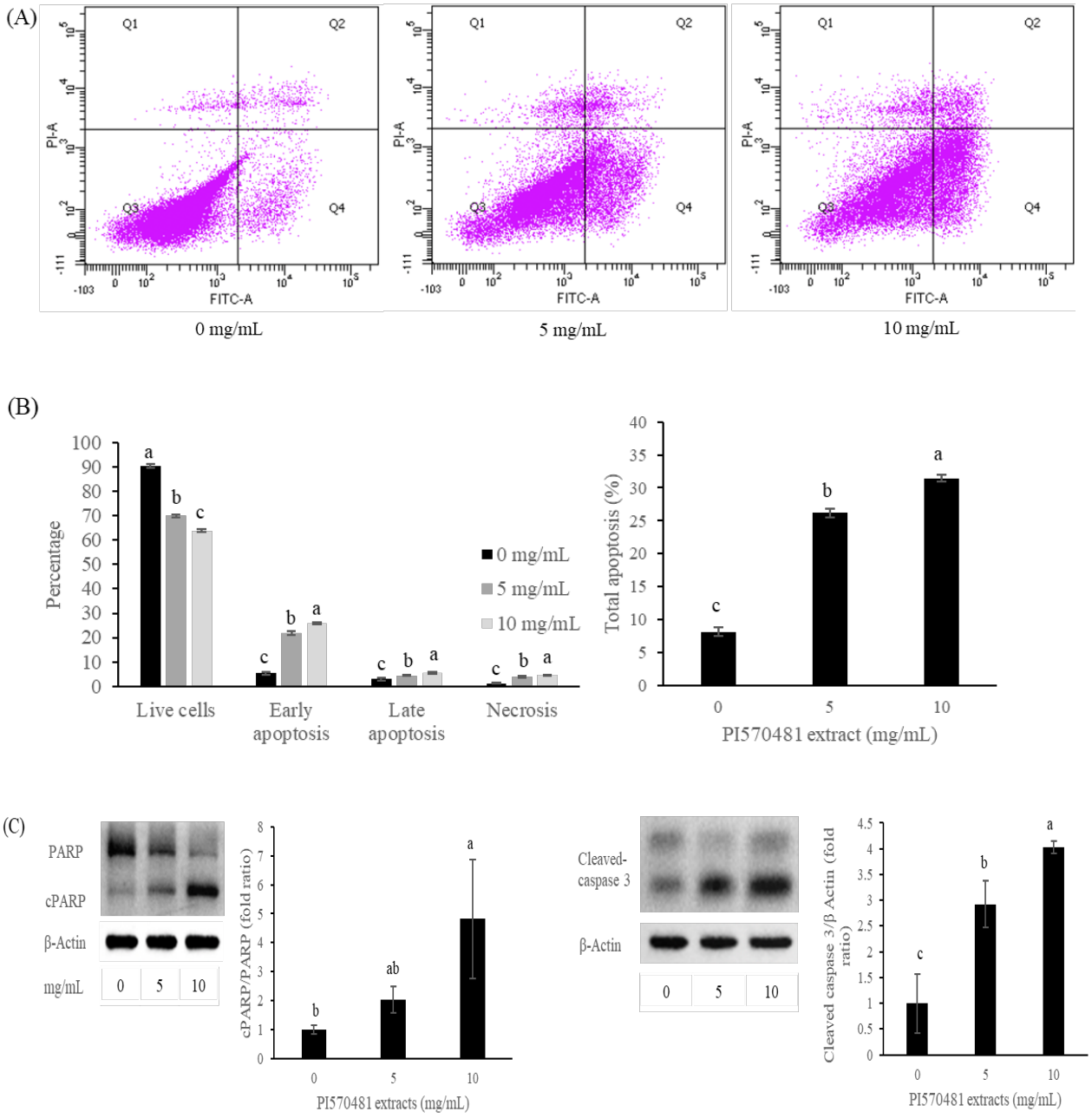

Figure 2.2. PI570481 extract induced apoptosis in MDA-MB-231 cells.I570481 extract induced apoptosis in MDA-MB-231 cells.

The cells were treated with 0, 5, and 10 mg/mL of PI5704810 extract for 24 h. The cells were stained with Annexin V and analyzed by FACS to calculate the number of apoptotic cells. Western blotting was conducted to identify key proteins. The extract induced both early (Q4) and late (Q2) apoptosis, and necrosis (Q1) in MDA-MB-231 human breast cancer cell. Statistical differences are indicated with different letters ($p < 0.05$).

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2.3.3. PI570481 extract induced cell cycle arrest in MDA-MB-231 cell.

Cell cycle arrest is one of the anti-cancer mechanisms to affect cancer cell proliferation and tightly regulated by cyclin-dependent kinase (CDK) and cyclins depending on cell cycle phage. We investigated if PI570481 affects distribution of cell cycle using FACS analysis. The results indicate that the ratio of combined G2 and M phase were significantly increased in MDA-MB-231 cells treated with 10 mg/mL of PI570481 extract treatment (Fig. 4.3A-B). Since cyclin B1 is a major cyclin to transit the cell cycle from S to G2 phase, we measured expression of cyclin B1. As shown in Fig. 4.3C, PI570481 significantly decreased expression of cyclin B1.

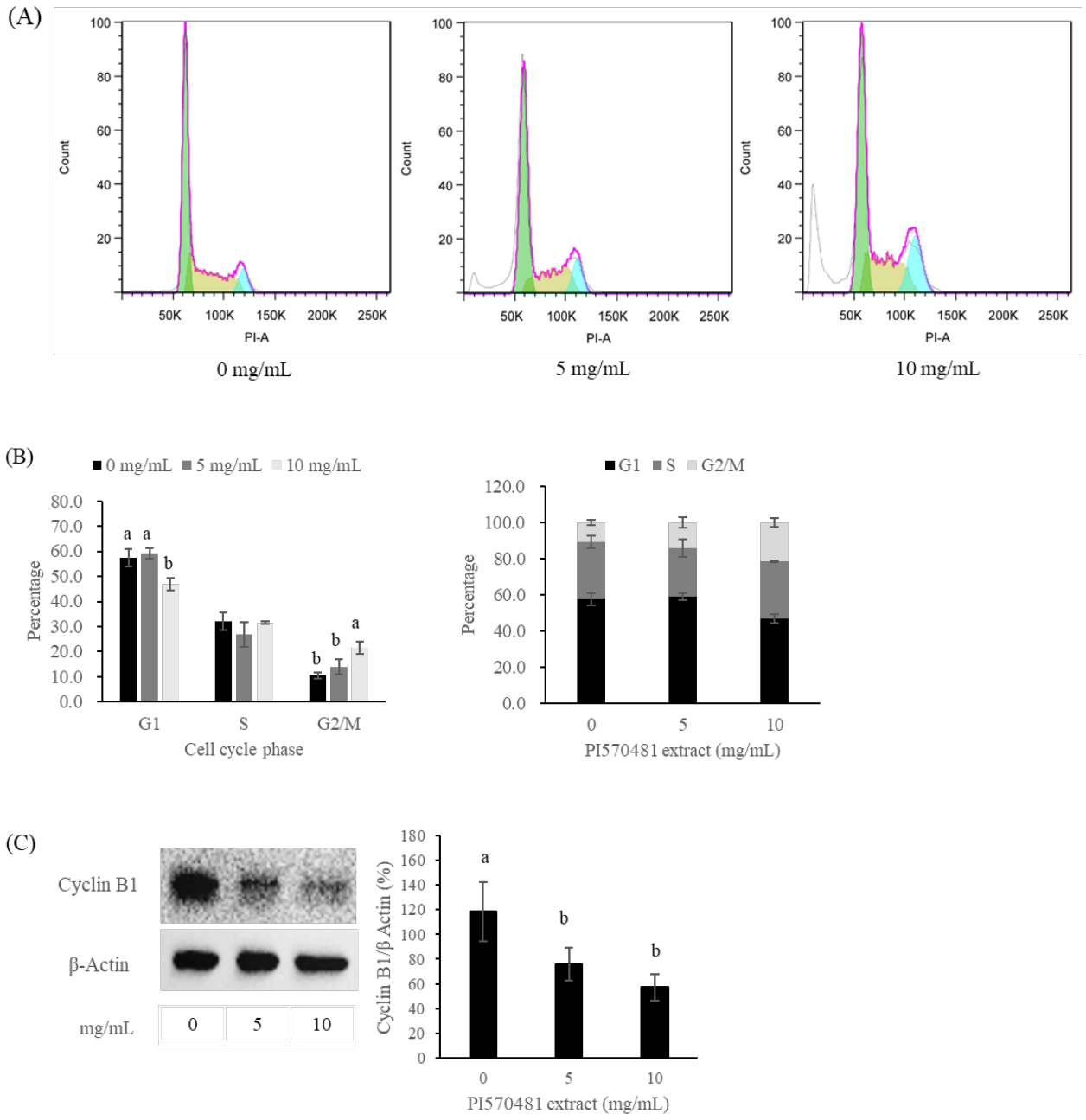

Figure 2.3. PI570481 extract induced cell cycle arrest in MDA-MB-231 cell.

The cells were treated with 0, 5, and 10 mg/mL of PI570481 extract for 24 h. The cells were fixed, stained with PI and analyzed by FACS to calculate the cell cycle distribution. Western blotting was conducted to identify key proteins. Statistical differences are indicated with different letters ($p < 0.05$).

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2.3.4. PI570481 extract disrupted mitochondrial membrane potential in MDA-MB-231 cells.

Loss or compromised mitochondrial membrane potential by internal or external factors is broadly accepted as one of mechanisms to induce apoptosis and repress cell viability (Zorova et al., 2018). To investigate if PI570481 influences mitochondrial membrane potential, we measured JC-1 staining in the cells treated with PI570481. The results indicate that the loss of the mitochondrial membrane potential, calculated by the ratio of unhealthy (green) cells to healthy (red) ones, was 0.60 % for the control group while it increased by 4.97 % and 17.51 % in the cells treated with 5 and 10 mg/mL of PI570481 in a dose-dependent manner (Fig 4.4).

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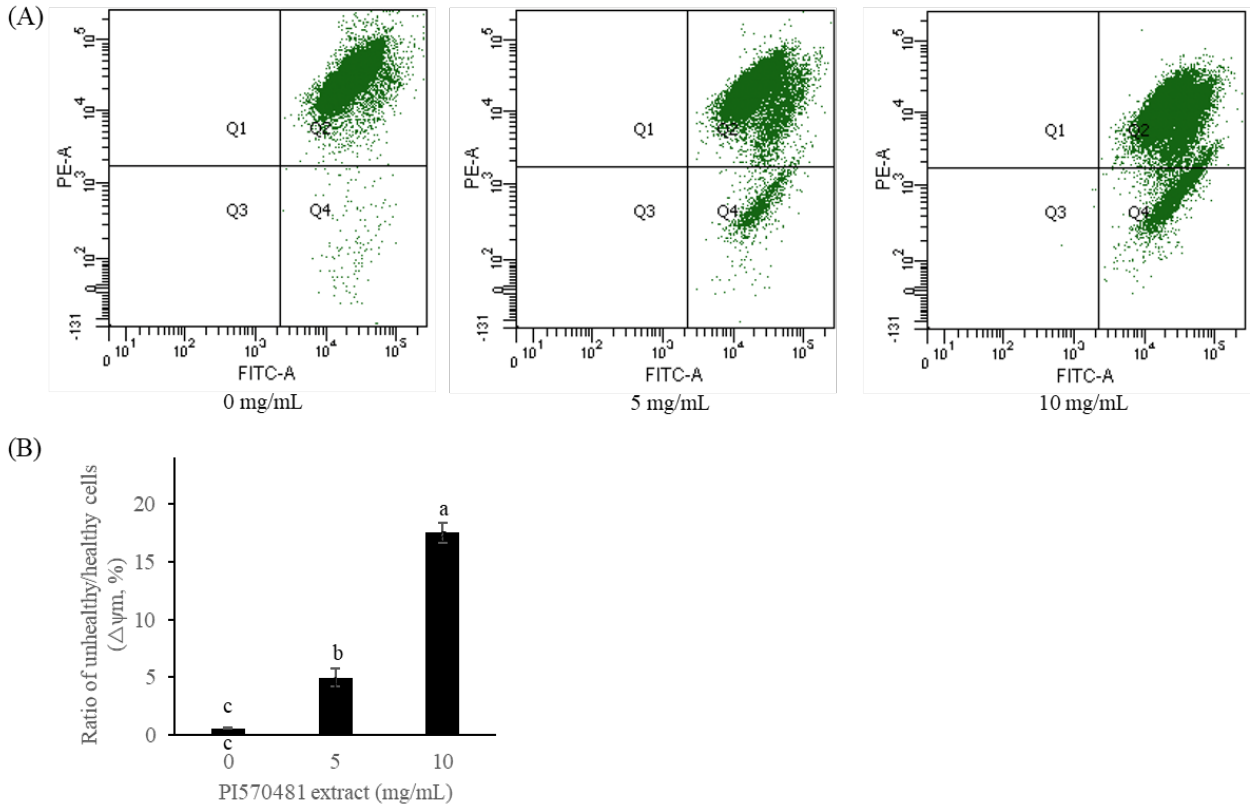

Figure 2.4. PI570481 extract disrupted mitochondrial membrane potential in MDA-MB-231 cells.

The cells were treated with 0, 5, and 10 mg/mL of PI570481 extract for 24 h and followed by JC-1 staining. The results were analyzed by FACS depending on the color. The extract induced mitochondrial dysfunction in MDA-MB-231 human breast cancer cell line by depolarization. Healthy cells (Q2) showed red color by JC-1 staining while unhealthy cells (Q4) were stained green due to depolarization. Statistical differences are indicated with different letters ($p < 0.05$).

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2.3.5. PI570481 extract induced ER stress and autophagy in MDA-MB-231 cells.

ER stress is a common cellular event when cancer cells are treated with diverse form of anticancer compounds and induce apoptosis. On the other hand, ER stress and mitogen-activated protein kinases (MAPK) pathways are strongly connected in cell death (Darling & Cook, 2014). As shown in Fig. 4.5A, PI570481 treatment induced phosphorylation of eIF2α (peIF2α) and extracellular signal-regulated kinases (pERKs) significantly.

Autophagy is another form of cell death process mediated by lysosome. With proper autophagy, living organisms can not only recycle cell components for the growth (Mizushima & Komatsu, 2011), but misfolded proteins also can be degraded to maintain the cellular health (Wirawan et al., 2012). One marker indicating the progress of autophagy is microtubule-associated proteins 1A/1B light chain 3B (LC3). This protein is composed of subunits I and II, and the higher ratio of LC3 II compared to LC I means that cells are undergoing autophagic progress compared to the lower expression ratio. To prove our hypothesis that autophagy is involved in PI570481-induced cell death, we measured LC3 proteins. As a result, the ratio of LC3 II to LC3 I ratio was significantly increased in MDA-MB-231 cells treated with PI570481 extract compared to the control group (Fig. 4.5B).

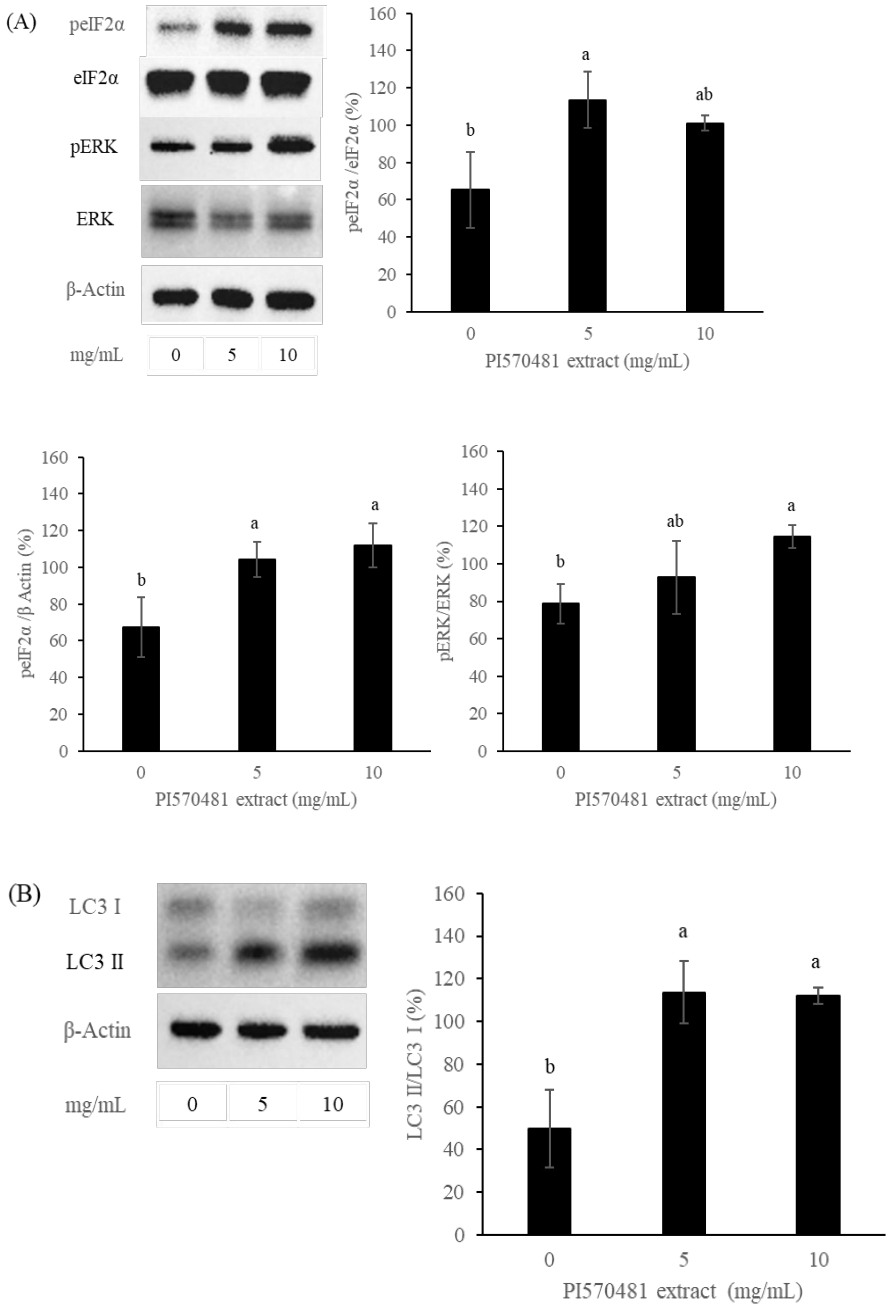

Figure 2.5. PI570481 extract induced ER stress and autophagy in MDA-MB-231 cells.

The cells were treated with 0, 5, and 10 mg/mL of PI570481 extract for 24 h. Western blotting was conducted to identify key proteins. The extracts led to increased ER stress and MAPK activation in MDA-MB-231 human breast cancer cell line. No dose-dependency was observed. Statistical differences are indicated with different letters ($p < 0.05$).

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2.4. Discussion

Sorghum has been widely used in food products due to its high amount of vitamins and minerals, and low digestibility (Khoddami et al., 2023). Especially, carbohydrates composing sorghum are polysaccharides and this shows lower digestibility compared to corn starch. Based on these characteristics and high content of dietary fiber, sorghum can be used in calorie-reduced diet. Especially, Western diets have been known to be high-caloric and are one of the causes of obesity (Rakhra et al., 2020), sorghum can be an effective food component and utilized more widely as current global usage of sorghum is limited (Stefoska-Needham, 2024). Specifically, since obesity has been known to increase the risk of cancer (Vucenik & Stains, 2012), sorghum can be a method of attenuating and preventing cancer. Recently we reviewed health benefit of sorghum in diverse types of human chronic diseases (Amarakoon et al., 2021), and this research focused on the anti-cancer activities of a specific polyphenol-rich sorghum species.

Our data show that PI570481 sorghum bran extract containing high amount of polyphenols significantly inhibited the proliferation of MDA-MB-231 cells with dose-dependency. Antiproliferative activity of the sorghum bran extract was observed from the dose of 5 mg/mL, and the inhibitory efficiency became around 50 % at 10 mg/mL concentration. However, no suppressed growth was observed from MCF-7 cells. This suggests that triple-positive breast cancer cells possibly can show different pathways from triple-negative breast cancer cells, indicating that different treatments should be conducted between different types of breast cancer diagnoses.

One of prominent observations in the cells treated with PI570481 extract was remarkable induction of apoptosis. With Annexin staining followed by FACS analysis, it was evaluated that the number (percentage) of both early and late apoptotic cells was significantly increased by

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PI570481 extract treatment compared to the control group. One of the factors showing different apoptotic activities was PARP cleavage. PARP protein participates in repairing damaged DNA and is a main target of apoptotic processes. Therefore, the ratio of cPARP to PARP is good indication of DNA damage-mediated apoptosis. In our results, the ratio increased by treating the cells with 10 mg/mL extract treatment. Our results also show that PI570582 significantly enhanced the activation of caspase 3, by cleaving the protein. Therefore, it can be concluded that the polyphenol-rich sorghum bran extract promoted programmed cell death in MDA-MB-231 cells. In addition, PI570481 led to a dose dependent increase of necrosis although the percentage of necrotic cells is relatively lower than late and early apoptotic cells.

A considerable difference in MDA-MB-231 cell cycle change was observed by PI570481 extract treatments. With the polyphenol-rich compound treatments, the cells showed changes in G2/M phase. Significant arrest in G2/M phase was induced by 10 mg/mL of PI570481 extract treatments, while others showed no change. Specifically, changes in protein expressions support this result. Cyclin B1, a checkpoint marker protein for G2/M phase, showed suppressed expression by treating the cells with the extract. Therefore, these data suggest that polyphenol-rich sorghum bran extract inhibits MDA-MB-231 cell proliferation by altering the cell mitosis process.

In addition to direct inhibitory activities, polyphenol-rich sorghum bran extract was found to be effective in compromising MDA-MB-231 cell health. It was observed that the cells treated with PI570481 extract showed increased loss of mitochondrial membrane potential. This induced mitochondrial dysfunction negatively affects cell health and growth, and can be one of the targets for inhibiting cancer progression (Hsu et al., 2016). And we suggest that polyphenol-rich sorghum bran can be effective in both treating and preventing breast cancer.

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Abnormality in protein synthesis and folding induces ER stress in cells and this is one of the factors contributing to inhibited cell proliferation. Our data indicates that PI570481 extract enhanced the activation of eIF2α and ERK protein, showing that the polyphenol-rich compound caused accumulation of misfolded or unfolded proteins in MDA-MBA-231 cells and therefore inhibited the cell proliferation. In addition, this induced ER stress response can strengthen apoptosis in the cells.

Autophagy plays a critical role in suppressing uncontrolled cell proliferation and has emerged as an important therapeutic target in cancer. Notably, autophagy contributes to the delivery of endogenous cytosolic antigens to MHC class II–containing compartments, thereby facilitating MHC-II presentation to $CD4^+$ T cells and potentially enhancing antitumor immune activation (Crotzer & Blum, 2009). Phytochemicals have been widely recognized as modulators of autophagy and show promising anticancer potential (Moosavi et al., 2018). Consistent with this concept, our findings demonstrate that PI570481 treatment markedly increased LC3 II expression in MDA-MB-231 breast cancer cells, accompanied by a significant elevation in the LC3 II/LC3 I ratio. These results support the notion that polyphenol-rich sorghum bran extracts can effectively induce autophagy and may serve as a valuable strategy for breast cancer therapy.

2.5. Conclusion

Our data indicate that polyphenol-rich sorghum bran PI570481 extract is effective in inhibiting proliferation of MDA-MB-231 cells. The mechanism includes activation of apoptosis, cell cycle arrest, mitochondrial dysfunction, ER stress, and autophagy (Figure 4.6).

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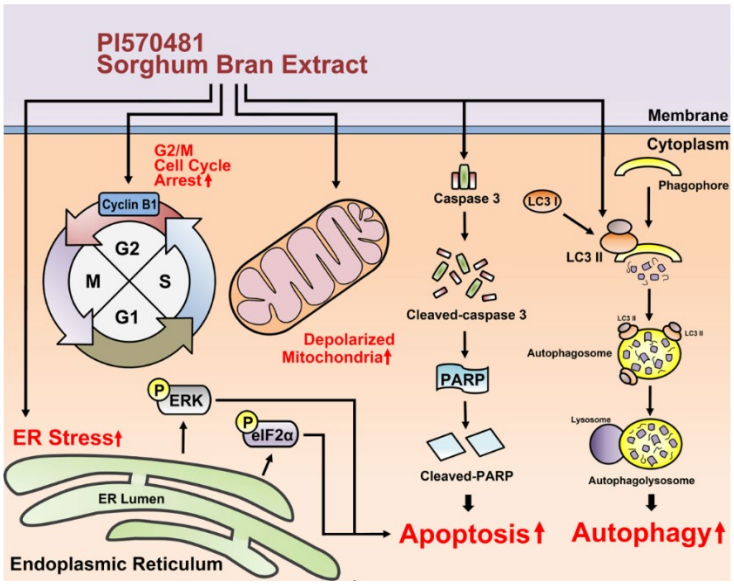

Figure 2.6. The proposed anti-cancer mechanisms of PI570481 sorghum bran extract in MDA-MB-231 cells.

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Chapter 3. Effect of high phenolic sorghum on body weight, fat accumulation and glucose tolerance in a high-fat diet-induced mice

Abstract

Type 2 diabetes mellitus is characterized by impaired insulin sensitivity and is strongly associated with obesity. Dietary bioactive compounds offer potential complementary strategies to reduce obesity-related metabolic disorders. In this study, the anti-diabetic effects of polyphenol-rich sorghum bran extracts were investigated using C57BL/6J mice and C2C12 murine myotube cells. Although the PI570481 sorghum bran extract did not significantly mitigate body weight gain in mice fed a high-fat diet, it notably improved insulin tolerance. Furthermore, treatment with the extract enhanced glucose uptake in C2C12 myotubes, which was accompanied by the upregulation of key insulin signaling pathways. Collectively, these findings suggest that polyphenol-rich sorghum bran extracts may serve as a promising nutritional intervention for preventing or attenuating type 2 diabetes in individuals with obesity.

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3.1. Introduction

Obesity is one of the most widespread abnormal body conditions across the world and is a metabolic disorder and primary cause of many types of chronic diseases such as type 2 diabetes, fatty liver, dyslipidemia, hypertension, cardiovascular and cerebrovascular diseases and cancer (Jung & Choi, 2014; Koliaki et al., 2019; Krupa-Kotara & Dakowska, 2021). It was reported that the number of people with obesity in the world in 2016 tripled compared to the prevalence in 1975 (Ahmed & Konje, 2023). Obesity is generally characterized by excessive accumulation of fat pad and elevated levels of circulating triglyceride, cholesterols, cholesterol esters, inflammatory cytokines and adipokines. Obesity is primarily caused by prolonged positive energy balance resulting from excessive consumption of energy, lack of physical activity, sedentary lifestyle, socio-economic factors and genetic factors (Apovian, 2016; Hill et al., 2012; Hopkins & Blundell, 2016).

Type II diabetes is a disease caused by impaired insulin sensitivity and glucose intolerance and resultant increase of blood sugar (dysglycemia). Higher rate of body fat was strongly correlated with increased insulin resistance compared to body weight (Boden et al., 1993). Therefore, it can be predicted that attenuating obesity, especially managing body fat percentage is an effective method to prevent or relieve diabetes. Along with medication and increasing body activity, diet can significantly decrease blood glucose levels. Mostly, plant-based food ingredients are widely known in treating diabetes.

Sorghum, the fifth most used cultivated crop in the world, is widely used for various purposes including food (Zheng et al., 2023). In addition to its dietary fiber-rich characteristic (Espitia-Hernández et al., 2022), sorghum is well-known for containing a high content of polyphenols (de Morais Cardoso et al., 2017) which possess diverse health benefit effects such as

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anti-inflammatory (Yahfoufi et al., 2018), anti-cancer (Pritam Bhagwan Bhosale et al., 2020), antibacterial (Coppo & Marchese, 2014), and other activities such as anti-obese and anti-diabetic effects (Ikarashi et al., 2011). For example, polyphenols showed inhibitive activities on starch digestion (Forester et al., 2012), enhanced glucose uptake in adipose and muscle tissues (Dzah et al., 2023). Regarding cellular anti-diabetic mechanism, polyphenols enhanced the phosphorylation of insulin receptor substrate (IRS) 1 and activated phosphoinositide 3-kinase (PI3K) and protein kinase B (Akt) pathways and translocation of glucose transporter type 4 (GLUT4) and subsequently promotes glucose uptake into cells (Cao et al., 2023). Enhanced glucose uptake into body tissue leads to lowered blood glucose level, and this is linked to inhibition of body fat accumulation. These studies propose the possibility that high phenolic sorghum may prevent obesity and diabetes through modulation of these cellular pathways.

Current study is designed to examine if a specific species of sorghum bran containing a high number of polyphenols possess anti-obesity and anti-diabetic activities in high-fat diet-induced murine model.

3.2. Material and method

3.2.1. Materials

The insulin, glucose, and 2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl) amino]-D-glucose (2-NBDG) were purchased from Sigma-Aldrich (USA). The primary antibodies for Akt (cat. #4691), p-Akt (cat. #4060), and β-actin (cat. #5125) were purchased from Cell Signaling Technology (USA). Carbenicillin and ampicillin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Infinity^{TM} triglyceride reagent used for triglyceride measurement, and Pierce BCA protein assay kit for protein measurement were purchased from Thermo Fischer Scientific

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(Waltham, MA, USA). All other chemicals were purchased from Fisher Scientific (Pittsburg, PA, USA).

3.2.2. Animal diet preparation

Different sorghum diets were specified by USDA. The customized diets were made by Bioserve (Flemington, NJ) following rodent sterilized high-fat diet (# S3282) and control diet (#S 4031). All the diets were stored in refrigerator for usage.

3.2.3. Animal experiment

C57BL/6J male mice (aged 5 weeks) were purchased from Charles River Laboratories (Maryland, USA). All experimental procedures were approved by the University of Maryland Institutional Animal Care and Use Committee (Approval No. R-NOV-20-59). All the animals were fed with a normal diet to adapt to the facility (25 ± 2 °C, 60 ± 10% relative humidity, and with 12 h light–dark cycle). After acclimation, mice were divided into one of five groups: standard diet (SD), 60% high-fat diet (HFD), HFD with PI570481 (PI), HFD with sumac sorghum bran, and HFD with white sorghum (Figure 1A). The mice were fed with different diets by different groups for 12 weeks. Food intake was monitored daily, and the body weight of the mice was measured at week intervals. The mice were sacrificed 18 weeks after an initial special diet or water feeding. Blood samples from the mice were collected by cardiac puncture.

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3.2.4. Glucose and insulin tolerance test (GTT and ITT)

Five mice per group were selected for GTT and ITT at week 16 and week 17, respectively. For GTT, one g of glucose/kg body weight was injected intraperitoneally after 6hr fasting. Mouse blood samples were collected from lateral tail vein using a lancet. Blood glucose level was measured 0, 15, 30, 60, and 120 min after glucose injection. For ITT, 0.75 unit of insulin/kg body weight was injected intraperitoneally after 6-hour fasting. Blood glucose level was measured with the same method.

3.2.5. Triglyceride of blood serum

The mouse blood serum was obtained by centrifugation of blood at 8000g for 15min. The levels of Triglyceride (TG) were measured by Triglyceride colorimetric assay kit (Cayman chemical, Michigan, USA)

3.2.6. Preparation of PI570481 sorghum bran extract

Crude sorghum bran extract of PI570481 was prepared as chapter 4.2.1 with no citric acid in the solvent.

3.2.7. Cell culture

Mouse myoblast C2C12 cell was obtained from American Type Culture Collection (ATCC, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), and 1% penicillin and streptomycin solution in a humidified atmosphere of 5% CO_2 at 37°C. For differentiation into myotube, C2C12 cells (1×10^5 cells/well) were seeded in the 6 well plates

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for 48 hr. After reaching 80% confluence, myocytes were cultured in DMEM supplemented with 2% horse serum for 5 days. The medium was replaced after 2, 4 days of culture.

3.2.8. Cell viability

For short cell viability, C2C12 cells (1×10^4 cells/well) were seeded in 96-well plates for overnight and treated with or without PI extract (0 to 10 mg/mL) for 24 hr. Cell viability was determined using MTT assay according to the manufacturer's protocol.

3.2.9. Glucose uptake

C2C12 cells (5×10^3 cells/well) were seeded in 24 well plates. After differentiation into myotube, cells were starved for 2 hr in serum free media and treated with PI at concentrations of 2.5 and 5 mg/mL, and 20 μM 2-NBDG and fluorescently labeled deoxyglucose analog in glucose-free media without or with 500nM insulin for 24 hr. After incubation, cells were washed twice with cold phosphate buffered saline (PBS) and then 2-NBDG taken up by cells was detected by a spectrophotometer (excitation/emission = 485/528)

3.2.10. Western blot

The differentiated C2C12 myotube was pre-treated with or without PI sorghum extract (2.5 and 5 mg/mL) for 24 hr. The cells were starved in serum-free DMEM for 2 hr and then treated with PI at concentrations of 2.5 and 5 mg/mL and without or with 500 nM insulin in serum free media for 30 min. After incubation. Cells were washed twice with cold PBS twice. After that, radioimmunoprecipitation assay (RIPA) buffer containing 1% protease and phosphatase inhibitor

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was added each well. The cells were harvested with cell scraper on the ice and vortexed in 10 min term for 1 hr. The lysates were centrifuged at 13,000g for 30 min at 4 °C. Protein concentration measurements were performed by BCA assay (Thermo Fisher Scientific, USA). Equal amounts of protein were subjected to 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose blotting membranes (Millipore Sigma, cat. # 10600003, USA). The membranes were blocked with 5% bovine serum albumin (BSA) for 30 min in Tris-buffered saline (TBS) containing 0.1% Tween-20, then incubated with 1/1000 diluted primary antibody solution at 4 ℃ during overnight. The membranes were washed three times by TBST and then incubated with secondary antibody for 2 hr. After washing, the membrane was treated with enhanced chemiluminescence using electrochemiluminescence (ECL) solution. Protein bands were detected by ChemiDoc System (BioRad, USA). Bands on the Western blot was quantified image J.

3.2.11. Statistical analysis

Data are expressed as means ± SE indicated in figure legends, and differences were considered significant at $p < 0.05$. Statistical analysis was performed with the student's t-test by using Microsoft Excel.

3.3. Results

3.3.1. Effects of sorghum bran diet on change of body weight and food intake

To investigate anti-obesity effects of sorghum bran, mice were fed HFD with or without sorghum bran for 12 weeks. HFD-fed mice showed significant increase of body weight compared to those

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fed control diet (SD). The treatment of sorghum bran-containing diets including PI, sumac and white did not change the body weight compared with HFD-fed group. The average body weight during the experimental period in mice were 31.62 ± 0.97 g for SD, 38.77 ± 1.34 g for HFD, 39.13 ± 1.23 g for PI, 39.52 ± 1.26 g for sumac, 40.34 ± 1.36 g for white-fed group, respectively (Figure 1A). The average daily food intake of all groups was not different among all groups. (Figure 1B). The body weight gain (Figure 1C) and food efficiency ratio (Figure 2D) were significantly higher in the HFD group than that in the SD group. However, it was not significantly different in sorghum bran diet groups.

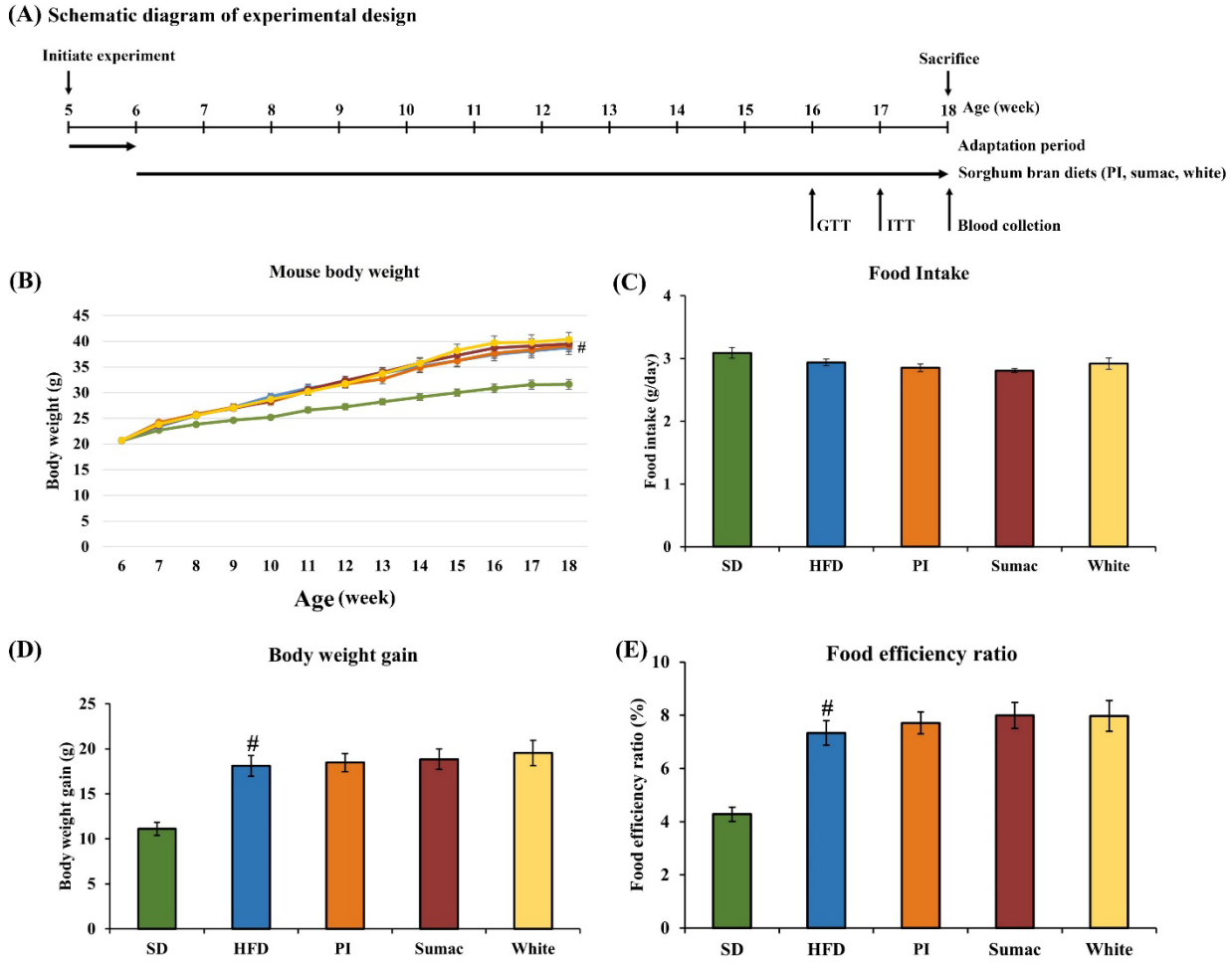

Figure 3.1. Effects of sorghum bran diets on bodyweight and change of food intake and body weight in HFD-induced obese mouse model

(A) Schematic diagram of experimental design. (B) body weight of total mice (C) Daily food intake, (D) Body weight gain, (=12th week body weight – 0^{th} week body weight), (E) Food efficiency ratio (=body weight gain/food intake for total experimental periods × 100), Data are represented as mean ± SE (SD = 17, HFD = 18, PI = 18, Sumac = 18, White = 15) Significance is indicated as # $p < 0.05$ vs SD group

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3.3.2. Effects of sorghum bran diet on change of visceral fat weight and organ weight

The weights of epididymal (Figure 2A) and retroperitoneal (Figure 2B) adipose tissue in the HFD group were higher than those of the SD group. However, the weight of adipose tissue in the sorghum bran diet group was not significantly different. The average organ weight for the liver and heart were not statistically different among all groups (Figure 2C and D). However, the average weight of kidney was higher in white sorghum group than that of the other groups (Figure 2E). And the average weight of spleen was lower in PI-fed group than that of the other groups (Figure 2F).

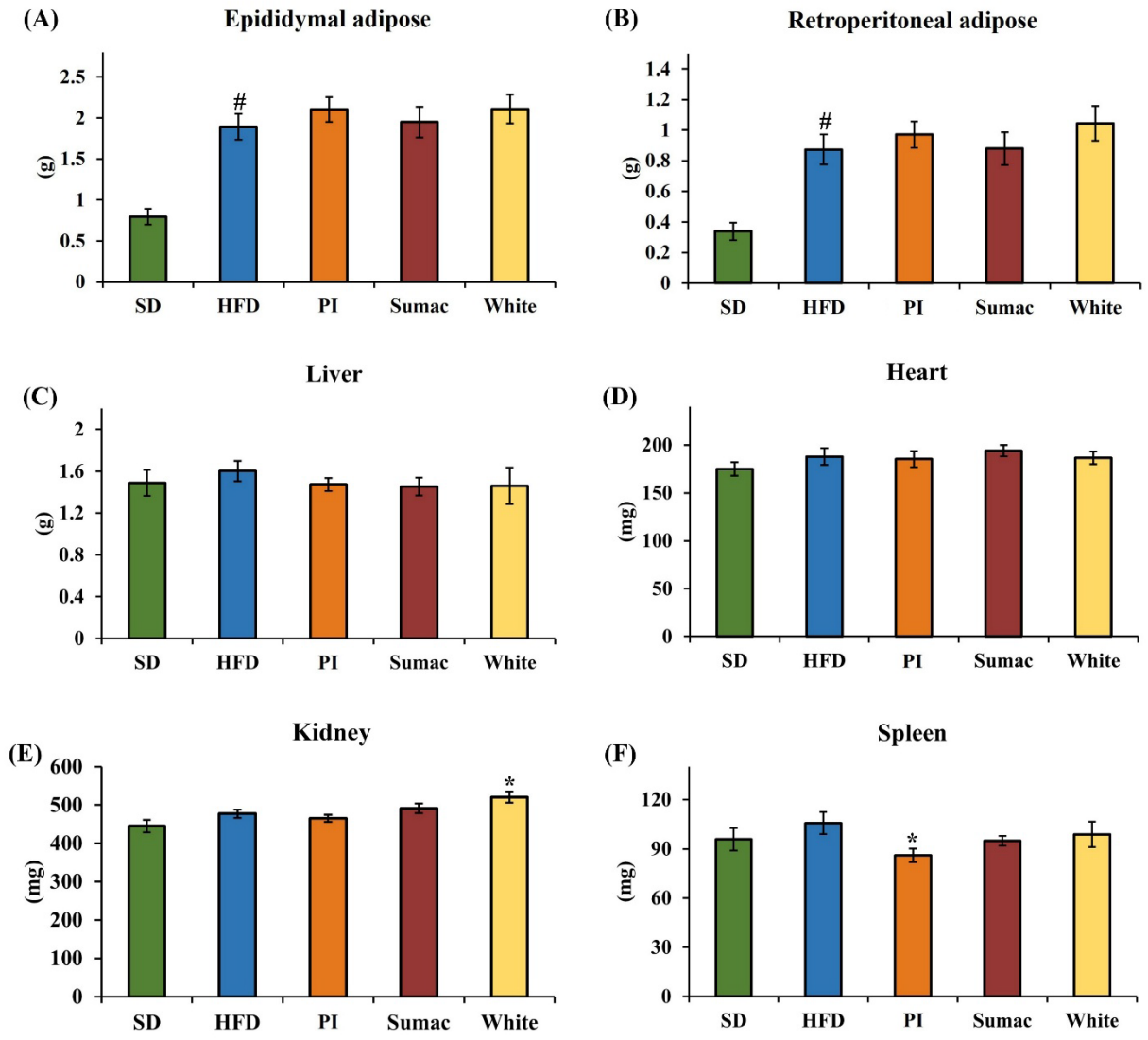

Figure 3.2. Effect of sorghum bran diets on the ratios of visceral body fat and the extents of organ weight in HFD-induced obese mouse model.

(A) Epididymal adipose, (B) Retroperitoneal adipose. (C) Liver (D) Heart (E) Kidney (F) Spleen. Data are represented as mean ± SE (SD = 17, HFD = 18, PI = 18, Sumac = 18, White = 15) Significance is indicated as # and *. # $p < 0.05$ vs SD group. * $p < 0.05$ vs HFD group.

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3.3.3. Effects of sorghum bran diet on change of triglyceride level, glucose and insulin tolerance

The triglyceride levels in the blood serum were higher in the HFD group than those of the SD group. The serum triglyceride level of the sorghum bran diet group was slightly decreased, but it was not significantly different (Figure 3A).

To examine if treatment of different sorghum bran diets affects blood glucose, an intraperitoneal GTT was performed after a glucose overload. Drastic increase of blood glucose levels was observed at 15 minutes after intraperitoneal (IP) injection of glucose to mice and time-dependent decrease afterwards. While HFD group increased the glucose level compared to SD group, blood glucose the sumac and white group did not show significant difference compared with the HFD group. However, the PI-fed group showed a tendency ($p = 0.089$) to reduce blood glucose level compared to HFD group at 15 min after IP injection. (Figure 3B). We also performed an ITT to see if treatment of sorghum brans affects insulin tolerance. IP injection of insulin remarkably lowered blood glucose level for the SD group compared to HFD group. Furthermore, PI group significantly ($p < 0.05$) reduced blood glucose compared to HFD group at 15 min after IP injection (Figure 3C). These data propose a potential that PI sorghum may influence glucose tolerance and attenuate insulin resistance in HFD-fed mice.

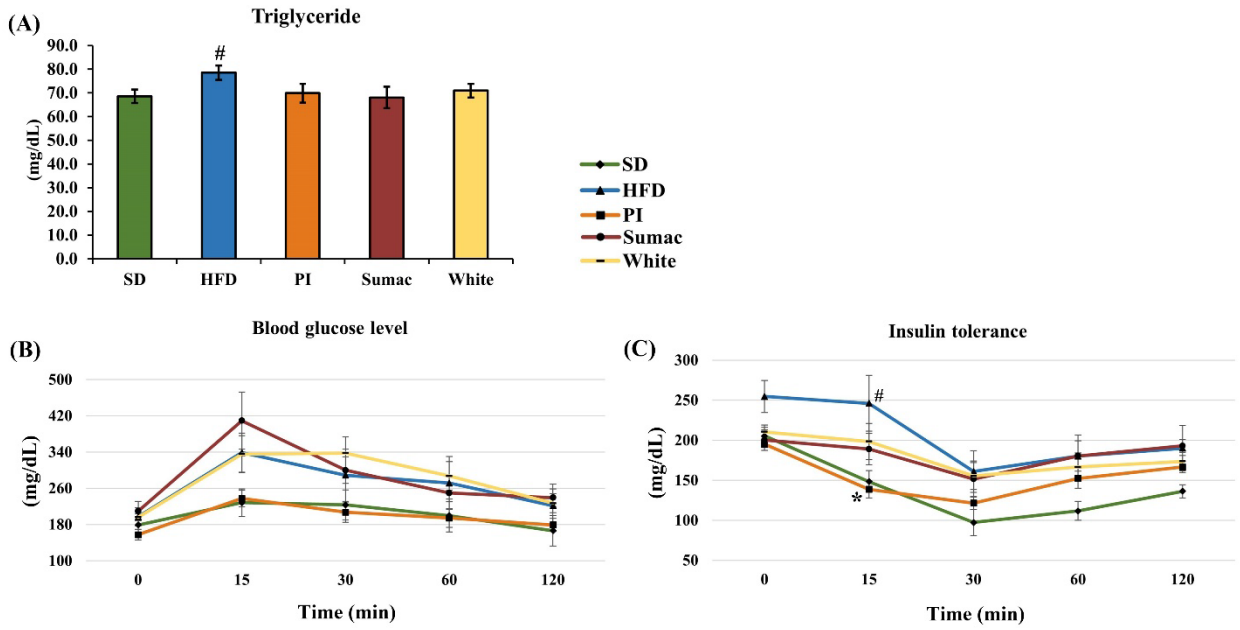

Figure 3.3. Effects of sorghum bran diets on glucose and insulin tolerance in HFD-induced obese mouse model.

Data are represented as mean ± SE (n = 5) Significance is indicated as # and *. # $p < 0.05$ vs SD group. * $p < 0.05$ vs HFD group.

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3.3.4 Effects of PI extract on change of glucose uptake and insulin signaling pathway in C2C12 myotube

To see if treatment of PI bran extracts affects glucose uptake in muscle cells, we performed *in vitro* study using differentiated C2C12 cells. First, to examine any potential toxicity of PI extracts, cell viability was measured using MTT assay. The results indicate that no significant toxicity was detected in the C2C12 cells treated with 0, 1.25, 2.5, 5, and 10 mg/mL of PI extracts for 24 hr (Figure 4A).

And glucose uptake was measured from differentiated C2C12 cells treated with 500 nM insulin, 2.5 and 5 mg/mL of PI. Insulin, which we employed as a positive control, also potently stimulated the uptake of 2-NBDG. The results indicate that the glucose uptake was significantly increased in a dose-dependent manner by treatment of PI extract (100 %, 124 %, 159 % and 120 % in 0, 2.5, 5 mg/mL and 500 nM insulin, respectively).

To investigate the molecular mechanisms by which PI extract increase glucose uptake, western blot was performed to measure expression of insulin pathway marker proteins from the cells treated with 0, 500nM insulin, 5 and 10 mg/mL of PI sorghum extract (Figure 4C). The results indicate that the phosphorylated Akt was dramatically expressed by treatment of PI extract in 5mg/mL group (100 %, 115 %, 383 %, and 325 % in 0, 2.5, 5 mg/mL and 500 nM insulin, respectively). These results suggest that insulin pathway was significantly activated by PI extract.

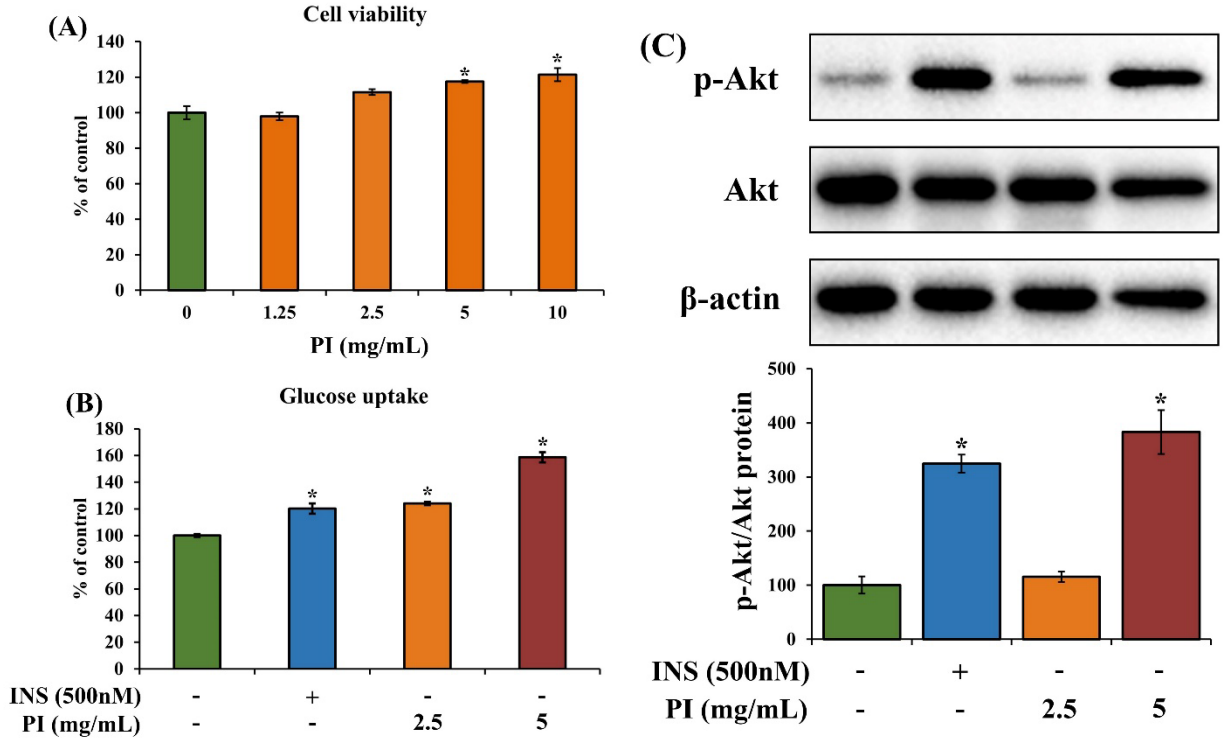

Figure 3.4. Effects of PI sorghum bran extract on change of glucose uptake and insulin signaling pathway in C2C12 myotube.

(A) Cell viability (B) Glucose uptake (C) p-Akt/Akt protein. C2C12 cells were treated with 2.5 and 5 mg/mL PI in differentiation media for 24 hr. After serum starvation for 2 hr, the cells were treated with PI at concentrations of 2.5 and 5 mg/mL without or with 500nM insulin in serum free media for 30 min. The cells were harvested for western blotting. Data are represented as mean ± SE (n = 3). Significance is indicated as *. $p < 0.05$ vs control (untreated cells).

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3.4. Discussion

Since the Industrial Revolution, Western lifestyle has become common in the modern age. Specifically, Western diet, combined with lack of physical activity, has been found to be a critical factor in the increase of various diseases related to obesity (Rakhra et al., 2020). Since Western diet is rich in sugar and saturated fat, and lacks vegetables and whole grains, high intake of the diet can be connected to higher chance of obesity (Clemente-Suárez et al., 2023). Along with other diseases such as cancer and cardiovascular diseases, diabetes is one of the major concerns linked with obesity. High body weight, especially high body fat rate strongly leads to insulin resistance and inhibited insulin activity alters physiological metabolism (Zegarra-Lizana et al., 2019). This compromised body homeostasis is chronic and becomes one of the leading causes of death across the world (Zheng et al., 2018). To prevent and attenuate the disease, plant-based food sources have been widely regarded as a solution with their nutrients including dietary fiber and phenolic compounds. One of the plant-source ingredients which can widely be used to improve human health is sorghum. It has been found that sorghum contains a high number of phenolic compounds, and the crop has low digestibility (Khoddami et al., 2023). Although these species-specific characteristics are common, the worldwide usage of sorghum in modern society is limited (Stefoska-Needham, 2024). As the population of obese people is rapidly growing and obesity is known to increase the risk of various diseases such as diabetes, many researchers are discovering methods to attenuate the disease to decrease the mortality rate and improve social welfare. Along with medication, proper food intake can be an effective method to prevent and treat diabetes and as polyphenols have been known to have anti-diabetic activities (Shahwan et al., 2022), phenol-rich food sources can be used more widely to decrease the frequency. And brans from sorghum species are possible food components to develop specific diets for diabetes patients and obese

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people. Therefore, the objective of this research is to evaluate the anti-obesity and anti-diabetic effects of phenol-rich extracts from specific sorghum brans.

In our previous study, we demonstrated the anti-adipogenic activity of high-phenolic sorghum brans in 3T3-L1 cells (Lee et al., 2022). Sorghum bran extracts (PI570481, SC84, and Sumac) reduced intracellular lipid accumulation and expression of adipogenic and lipogenic proteins in a dose-dependent manner in differentiated 3T3-L1 cells. These data propose a potential use of high-phenolic sorghum bran for the prevention of obesity. Based on these results, we hypothesized that different sorghum brains also have an anti-obesity effect in mouse experiments.

Obesity is closely related to the body and adipose tissue weight gain (Koenen et al., 2021). Thus, the anti-obesity efficacy of sorghum bran diet was evaluated by a change in body and adipose tissue weight. The body weight gain, food efficiency ratio, and adipose tissue weight gain of mice in all of sorghum bran diet groups were not reduced compared to the HFD group. This result shows that all sorghum bran diet groups did not have a potent anti-obesity effect.

Metabolic tolerance tests have been the mainstay for diagnosing diabetes for decades. These tests represent simple but powerful screening methods to diagnose impaired carbohydrate metabolism, glucose intolerance and early insulin resistance (IR) (Benedé-Ubieto et al., 2020). In this study, we observed that PI contributes to *in vivo* glucose tolerance and attenuates insulin resistance. Although, sorghum bran diet did not have an anti-obesity effect, we demonstrated that the potential of anti-diabetic effects of PI sorghum bran diet. To confirm the possibility of anti-diabetic effects, we conducted an experiment that measured the glucose uptake and confirmed the insulin signaling pathway in differentiated C2C12 myotube.

It is known that insulin stimulates IRS/PI3K/Akt signaling pathway (Petersen & Shulman, 2018). The insulin binds to insulin receptor in the membrane of muscle cells, which stimulates

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phosphorylation of IRS protein. And then phosphorylated IRS1 protein triggers downstream PI3K pathway that subsequently phosphorylates Akt. Akt plays a key role in glucose metabolism downstream of the insulin signaling pathways and exhibits altered responses among insulin-sensitive targets such as skeletal muscle cells. The activation of Akt is directly linked to the increased translocation of GLUT4 from cytoplasm to the cell membrane in muscle cells (Wong et al., 2020). The result shows that PI extract stimulated an increase in cellular concentrations of phosphorylated Akt. Finally, glucose consumption was increased by PI extract in C2C12 cell.

AMP-activated protein kinase (AMPK) is another important mediator of glucose homeostasis by insulin-dependent/insulin-independent mechanisms. In the insulin-independent pathway, the activated AMPK stimulates glucose uptake by promoting GLUT4 translocation into the plasma membrane (Lemieux et al., 2003; Musi & Goodyear, 2003). In addition, the Ras/mitogen-activated kinases (MAPK) pathway acts in the signaling pathway of insulin toward glucose uptake (Boucher et al., 2014; Tunduguru & Thurmond, 2017). However, our result indicates that phosphor-AMPK and phosphor-extracellular signal-regulated kinase (ERK) were not changed by treatment of PI (data was not shown). Thus, we speculate that enhanced glucose uptake by treatment of PI extract is AMPK and MAPK-independent.

5.5. Conclusion

The research data suggests that polyphenol-rich PI570481 sorghum bran extracts can be possibly effective in preventing and attenuating T2D by enhancing insulin tolerance and glucose uptake into skeletal muscle tissues, and potentially lowering serum TG and glucose level.

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Chapter 4. Protective activities of polyphenol-rich sorghum bran extracts against hydrogen-peroxide induced oxidative stress in C2C12 myoblast cells

Abstract

Oxidative stress is a critical contributor to the development of numerous diseases and pathological conditions and is closely associated with obesity. Excess adiposity has been shown to promote excessive production of reactive oxygen species (ROS), leading to cellular damage, particularly in skeletal muscle tissue. Dietary polyphenols have gained attention for their protective effects against oxidative stress, with sorghum recognized as a rich source of these bioactive compounds. In this study, the protective effects of polyphenol-rich PI570481 sorghum bran extracts were evaluated in murine myoblast cells subjected to hydrogen peroxide–induced oxidative stress. Treatment with sorghum bran extracts markedly attenuated oxidative stress–induced morphological alterations, apoptosis, cell cycle arrest, ROS accumulation, endoplasmic reticulum stress, and autophagy. These findings suggest that polyphenol-rich sorghum bran extracts possess significant cytoprotective properties and may serve as a promising nutritional strategy to preserve skeletal muscle health in individuals with obesity.

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4.1. Introduction

Excessive oxidative stress has been implicated in the development and progression of numerous diseases. In particular, elevated oxidative stress has been strongly associated with several chronic conditions. For example, oxidative stress has been shown to stimulate cancer progression (Nourazarian et al., 2014) and cellular transformation (Hayes et al., 2020). Patients with cancer also exhibit significantly increased oxidative stress levels accompanied by suppressed antioxidant enzyme activity (Jelic et al., 2021). Diabetes is similarly linked to oxidative stress, both as a contributing cause (Maiese, 2015) and as a consequence of the disease (Chen et al., 2025). Because obesity is a major risk factor for many of these chronic diseases, it is reasonable to predict that individuals with obesity may face heightened risks associated with elevated oxidative stress.

Preventing obesity and its related complications requires maintaining a healthy body weight, typically achieved through reducing excess body fat. Increasing skeletal muscle mass is also essential, as it supports optimal body fat ratios and enhances basal metabolic rate. Greater muscle mass improves glucose utilization (Merz & Thurmond, 2020), which can help prevent hyperglycemia. Thus, preserving an appropriate muscle-to-fat ratio is a key factor in mitigating obesity and its associated diseases.

Oxidative stress has also been closely linked to skeletal muscle dysfunction. Elevated levels of reactive oxygen species (ROS) can impair biological function and disrupt cellular homeostasis, ultimately contributing to muscle damage (Lian et al., 2022). Maintaining adequate antioxidant activity is therefore critical for protecting muscle tissue from oxidative injury. Obesity further exacerbates this issue, as it is a known contributor to abnormal oxidative stress and is strongly associated with numerous oxidative stress–related diseases (Marseglia et al.,

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2014). Beyond chronic conditions such as diabetes and cancer, oxidative stress itself can directly induce muscle disease (Lian et al., 2022). Because maintaining healthy muscle mass is essential for reducing obesity risk, protecting muscle tissue from oxidative stress is a vital component of preventing obesity-related disorders.

Sorghum, one of the world's most important cereal crops, has gained attention for its potential health benefits. Certain sorghum varieties contain high levels of phenolic compounds, which are strongly associated with antioxidant capacity and the reduction of excessive oxidative stress in the human body. These phenolic compounds can scavenge free radicals, potentially mitigating oxidative stress elevated by obesity.

In this chapter, C2C12 mouse myoblast cells were used to evaluate the protective effects of PI570481 sorghum bran extract (SBE) against hydrogen peroxide–induced oxidative stress in muscle cells.

4.2. Materials and methods

4.2.1. Sorghum bran extracts

PI570481 sorghum variety was cultivated in Puerto Vallarta, Mexico, and phenolic-rich bran samples were provided by Kansas State University. Bran extracts were prepared following previously established methods with minor modifications. Briefly, the brans were mixed with 70% ethanol at a 10% w/w ratio and incubated at room temperature for 2 hours. The mixture was then stored at −20 °C overnight before being centrifuged at 1,000 rpm for 10 minutes. The resulting supernatant was collected and used for subsequent experiments

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4.2.2. Cell culture

Mouse myoblast cells were purchased and used as described in chapter 5.2.2. The cells were obtained from American Type Culture Collection (ATCC, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), and 1% penicillin and streptomycin solution in a humidified atmosphere of 5 % CO_2 at 37 °C. In this chapter, non-differentiated myoblasts were used for the experiments. 70 % ethanol was used as a vehicle during experiments.

4.2.3. Chemicals

Antibodies for poly (ADP-ribose) polymerase (PARP), microtubule-associated proteins 1A/1B light chain 3B (LC3), phosphorylated and non-phosphorylated p38 mitogen-activated protein kinases (p38s) (MAPKs), and β-actin were purchased from Cell Signaling Technology (Danvers, MA, USA). All the other chemical materials were purchased from Fisher Scientific (Waltham, MA, USA) unless indicated.

4.2.4. Cell proliferation

C2C12 murine myoblast cells were plated in 96-well plates at the density of 5 * 1000^3 cells/well. After being incubated overnight, the cells were treated with different concentrations of PI570481 (0. 5, 7.5, and 10 mg/mL) extract with 800 μM of hydrogen peroxide dissolved in the complete growth medium. After 24-hour treatment under 37 ℃, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to the cells followed by 2-hour incubation. Do dissolve the colored compounds formed by MTT solution, dimethyl sulfoxide

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dissolve and the optical density was measured at 540 nm with an ELISA microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA).

4.2.5. Cell morphology

C2C12 cells were seeded in 96-plate wells at the density of 5 * 1000^3 cells/well. On the following day, the complete growth medium containing 800 μM of hydrogen peroxide with different concentrations of PI570481 extract (0, 5, 7.5, and 10 mg/mL) was added to the cells. After 6- and 24-hour treatment respectively, the cell density and morphology were observed with an optical microscope (ECHO REB-100, ECHO company, San Diego, CA). Microscopic examination was performed at 100× magnification.

4.2.6. Apoptosis

To calculate the amounts of apoptotic cells, PI/Annexin V-FITC kit was used based on the manufacturer's instruction. C2C12 cells were plated in 6-well plates at the density of 1 * 10^5 cells/well and incubated at 37 ℃. On the following day, 800 μM of hydrogen peroxide with different concentrations of PI570481 sorghum bran extract dissolved in the complete growth medium was added to the cells. After 24-hour treatment, the cells were harvested by trypsinization and washed with phosphate-buffered saline (PBS) twice. The washed cells were centrifugated at 3000 rpm for 5 minutes for collecting the pellets, and the pellets were stained with Annexin V for 15 minutes. Flow cytometry method using a fluorescence-activated cell sorting (FACS) facility at the University of Maryland was used to assess the percentage of both early and late apoptotic cells.

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4.2.7. Cell cycle analysis

As described above, C2C12 cells were plated, incubated, and treated by the same methods. Following 24-hour treatment, the cells were trypsinized for harvesting and fixed with 70 % (v/v) ethanol in PBS at -80 ℃. The fixed cells were then wished with PBS buffer to remove ethanol, and the pellets were stained with 70 μM propidium iodide (PI) containing 05 mg/mL of RNAse. After 15-minute staining, FACS flow cytometry was used to measure the proportion of the cells by different cycle stages.

4.2.8. ROS measurement

C2C12 cells were seeded in 24-well plates at $5 * 10^4$ cells/well density. After overnight incubation, the cells were treated with different concentrations (0. 5, 7.5, and 10 mg/mL) of PI570481 sorghum bran extract dissolved in the complete growth medium. After 24-hour treatment, the cells were rinsed with PBS and stained with 50 μM of 2',7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) dissolved in serum free DMEM for 30 minutes. After staining process, the cells were rinsed with Hanks' Balanced Salt Solution (HBSS) and 800 μM of H_2O_2 dissolved in HBSS was added. After 1-hour ROS inducing process, dissolved ROS was measured with an ELISA microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA) with 485 nm/20 excitation, 530 nm/25 emission.

4.2.9. Western blotting

C2C12 cells were plated in 100 mm plates at $5 * 10^5$ cells/plate. Following one-day incubation, the cells were treated with 800 μM H_2O_2 with different concentrations of (0, 5, 7.5, and 10

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mg/mL) PI570481 sorghum bran extract dissolved in the complete growth medium for 6 hours at 37 ℃. After the treatment, the cells were rinsed with ice-cold PBS buffer twice, and radio-immunoprecipitation assay (RIPA) buffer containing 1 % of combined protease and phosphatase inhibitor was added to collect the lysates. The lysates were centrifuged at 10000 rpm for 30 minutes and the supernatants were collected for the experiment. To evaluate the protein concentration in the lysate samples, bicinchoninic acid (BCA) assay was conducted based on the manufacturer's instruction. All loading samples were made with equal amount of total protein and heat denatured. The protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Membranes with transferred protein samples were blocked with bovine serum albumin (BSA) dissolved in Tris-buffered saline containing 0.1 % (v/v) of Tween-20 (TBST) for 30 minutes. Primary antibody was diluted in TBST containing 3 % BSA and added to the membranes for overnight at 4 ℃. Following the saturation, the membranes were rinsed with TBST three times and secondary antibody was added in the same way. After 1-hour saturation, the membranes were rinsed with TBST three times and Pierce ECL western blotting substrate was added. The protein detection was measured by generated chemiluminescent and visible images were created with Chemidoc MP Imaging System (Bio-Rad, Hercules, CA, USA).

4.2.10. Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey's range test was used to analyze the results with GraphPad Prism (GraphPad Software Inc.). All the data were indicated with mean ± standard deviation of three independent results.

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4.3. Results

4.3.1. Effect of PI570481 sorghum bran extract on C2C12 cells exposed to ROS

Because hydrogen peroxide (H_2O_2) is a widely used and well-established inducer of oxidative stress, C2C12 cells were first exposed to varying concentrations of H_2O_2 to determine an appropriate dose for inducing measurable oxidative damage. After confirming that the PI570481 sorghum bran extract (SBE) exhibited no cytotoxicity on its own, C2C12 cells were co-treated with SBE under oxidative stress conditions. The results (Fig 4.1) showed that SBE significantly attenuated the H_2O_2-induced inhibition of cell proliferation, with protective effects increasing in a dose-dependent manner compared to cells treated with H_2O_2 alone.

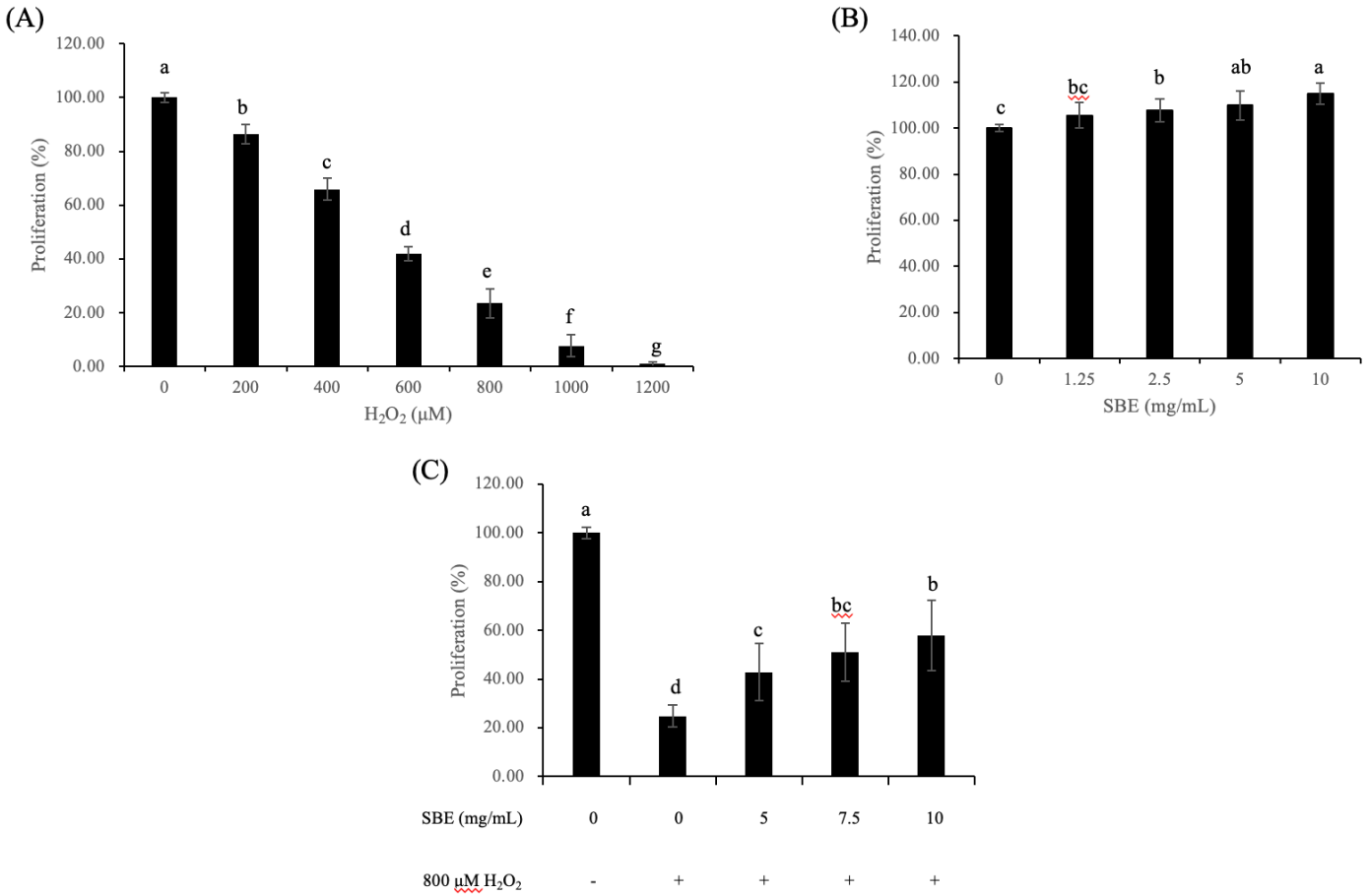

Figure 4.1. Effects of PI570481 sorghum bran extract on the proliferation of C2C12 murine myoblast cells exposed to H_2O_2-induced oxidative stress

Cell viability of C2C12 cells under different concentrations of H_2O_2 (A), PI570481 sorghum bran extract (B), and co-treatment of H_2O_2 and sorghum bran extract (C). Statistical differences are indicated with different letters ($p < 0.05$).

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4.3.2. Effect of PI570481 sorghum bran extract on H_2O_2-induced morphological change in C2C12 cells

As described above, H_2O_2-induced reactive oxygen species (ROS) significantly inhibited the proliferation of C2C12 cells, and this effect was attenuated by PI570481 sorghum bran extract (SBE). However, after 24 hours of treatment, no measurable changes were observed in several cellular indicators, including apoptosis markers and mitochondrial dysfunction (data not shown). In addition, cell morphology remained consistent across treatment groups, with all surviving cells exhibiting a typical adherent phenotype regardless of cell density (Figure 4.2-2). To further investigate potential early-stage morphological alterations, cell morphology was examined at earlier time points, specifically at 6 and 24 hours following treatment. Cells exposed to H_2O_2 alone for 6 hours displayed pronounced shrinkage, consistent with previous findings (Duan et al., 2017), whereas co-treatment with SBE mitigated these morphological changes without dose-dependency (Figure 4.2-1). These observations suggest that H_2O_2-induced physiological alterations occur within a relatively short time frame—less than one overnight period—and that surviving cells do not exhibit substantial morphological differences at 24 hours, regardless of SBE concentration.

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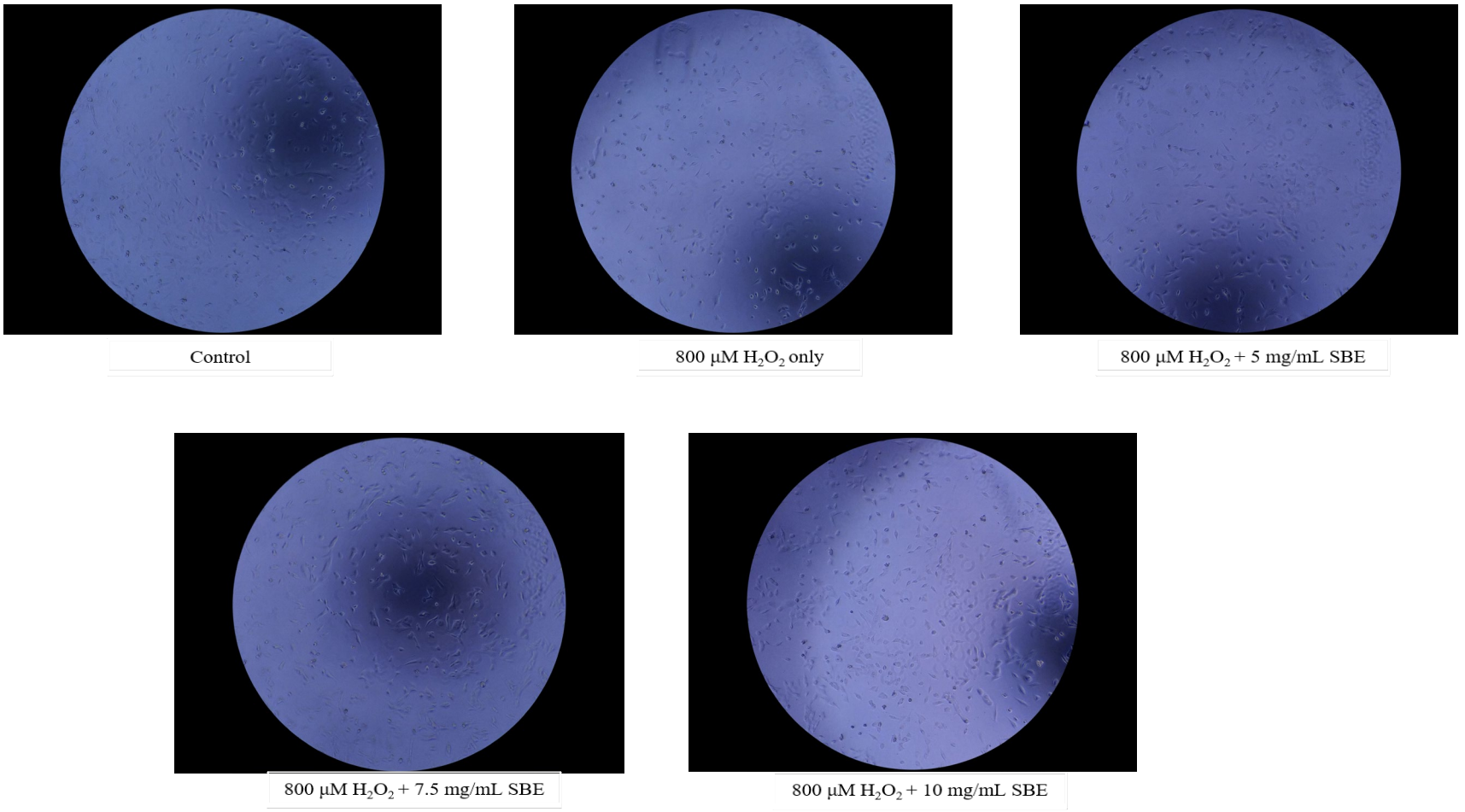

Figure 4.2-1. Effects of PI570481 extract on the morphology of C2C12 murine myoblast cells under H_2O_2-induced oxidative stress after 6-hour treatment.

Microscopic analysis revealed that C2C12 myoblasts exhibited a shrunken morphology following 6-h exposure to hydrogen peroxide, and the protective effect of SBE did not demonstrate a clear dose-dependent response.

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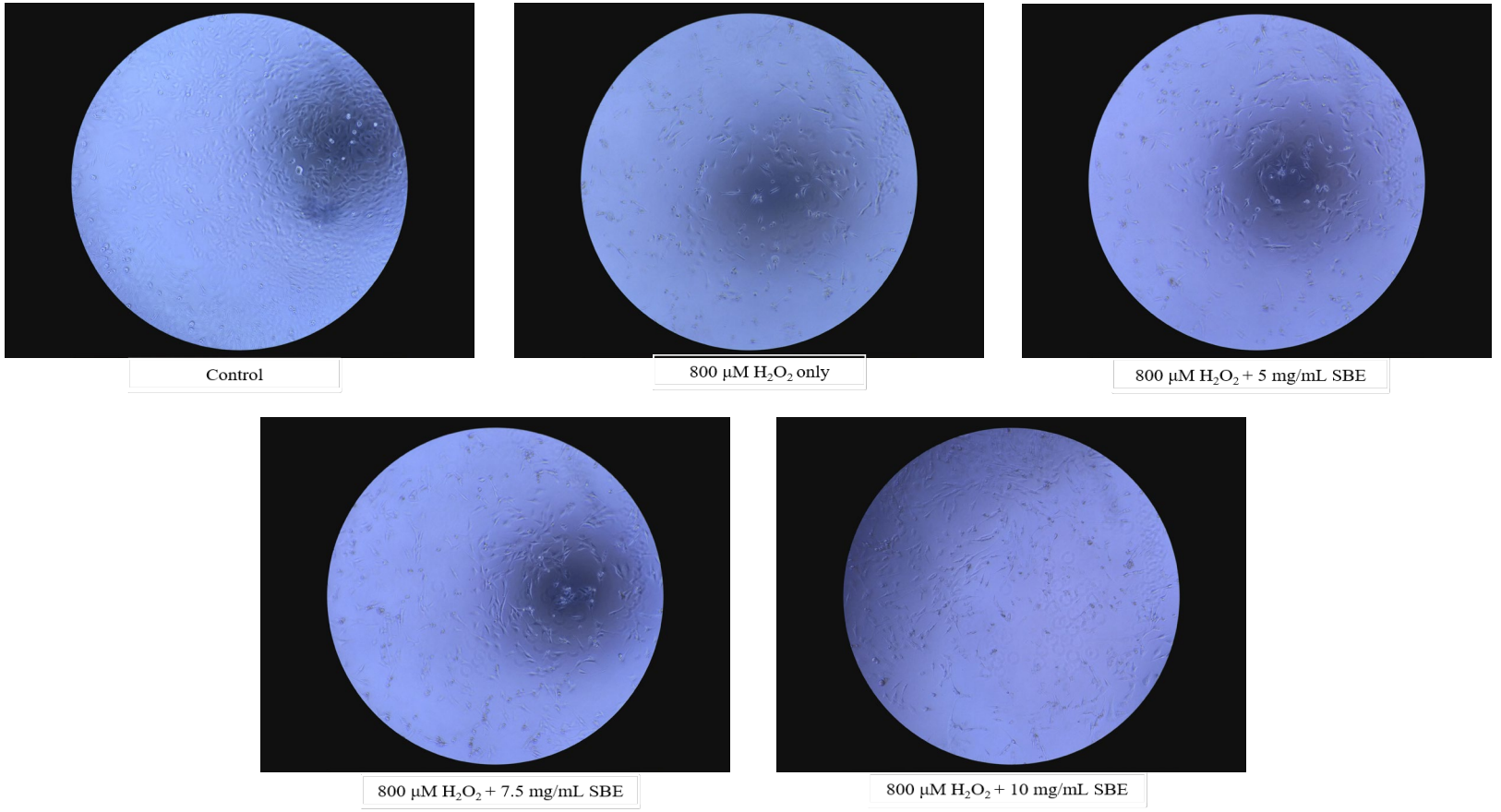

Figure 4.2-2. Effects of PI570481 extract on the morphology of C2C12 murine myoblast cells under H_2O_2-induced oxidative stress after 24-hour treatment.

The microscopic images indicate that C2C12 myoblast cell shape change was not significant between groups for 24-hour treatment.

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4.3.3. Effect of PI571481 sorghum bran extract on H_2O_2-induced apoptosis in C2C12 cells

Apoptosis is a programmed form of cell death essential for maintaining tissue homeostasis in multicellular organisms. This process can be activated by various external stressors, including oxidative stress, which is known to increase apoptotic activity in numerous tissues. To evaluate the effects of PI570481 sorghum bran extract (SBE) on oxidative stress–induced apoptosis, we quantified apoptotic C2C12 cells following H_2O_2 exposure. Annexin V staining, which detects early apoptotic cells by binding to phosphatidylserine on altered cell membranes, revealed that H_2O_2 significantly increased the proportion of early-stage apoptotic cells. Co-treatment with SBE markedly reduced this early apoptotic population, although the effect did not exhibit dose dependency. In contrast, late-stage apoptosis showed a dose-dependent increase in response to H_2O_2 when cells were treated with SBE (Fig. 4.3-1). To further assess apoptotic signaling, we examined the expression of poly(ADP-ribose) polymerase (PARP), measuring the ratio of cleaved PARP (cPARP) to its full-length form (Fig. 4.3-2). PARP cleavage was highest under oxidative stress alone, whereas SBE co-treatment reduced PARP activation, with a modest dose-dependent effect observed beginning at 7.5 mg/mL. Collectively, these findings indicate that the phenolic-rich PI570481 SBE effectively suppresses ROS-induced apoptotic activity in C2C12 myoblasts.

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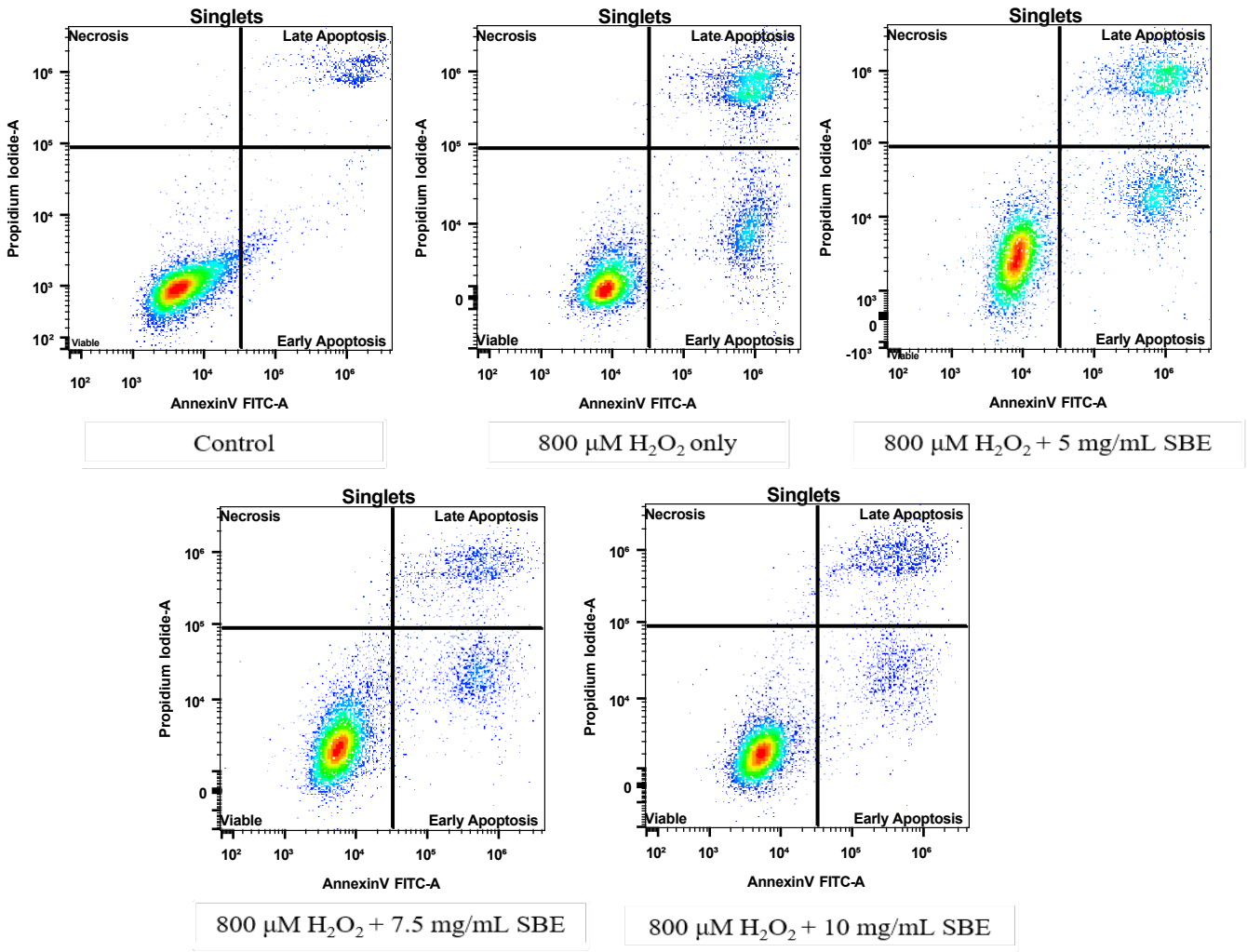

Figure 4.3-1. Effects of PI570481 extract on the apoptosis of C2C12 murine myoblast cells under H_2O_2-induced oxidative stress after 6-hour treatment.

H2O2 remarkably induced both early (lower right) and late (upper right) apoptosis, and both were attenuated by the sorghum bran extract.

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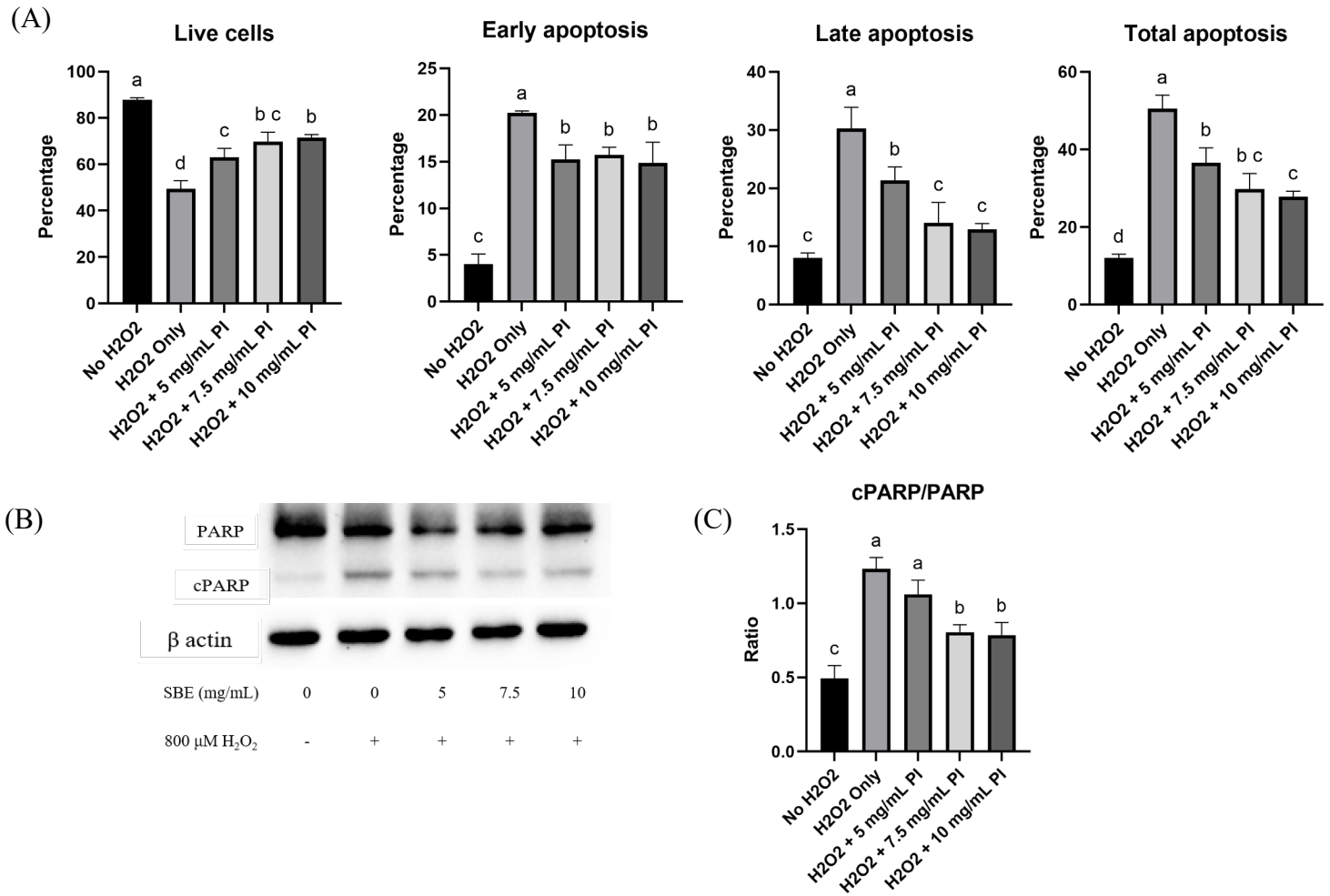

Figure 4.3-2. Effects of PI570481 extract on the apoptosis of C2C12 murine myoblast cells under H_2O_2-induced oxidative stress after 6-hour treatment

The sorghum bran extract attenuated the oxidative stress-induced apoptosis by inhibiting the amount of both early and late apoptotic cells (A) and suppressing the activity of PARP protein (B) (C). Statistical differences are indicated with different letters ($p < 0.05$).

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4.3.4. Effect of PI571481 sorghum bran extract on H_2O_2-induced growth arrest in C2C12 cells

Cell cycle arrest is a regulatory mechanism in which cells halt proliferation and progression through the cell cycle, typically in response to stressors such as DNA damage or oxidative imbalance. To assess whether oxidative stress induced such arrest in C2C12 myoblasts, cells were stained with propidium iodide (PI) to quantify DNA content and determine cell cycle distribution. The results showed that H_2O_2-induced oxidative stress significantly inhibited myoblast growth by blocking progression from the S phase to the G2/M phase. Co-treatment with PI570481 sorghum bran extract (SBE) markedly attenuated this growth arrest in a dose-dependent manner (Fig. 4.4). These findings indicate that SBE mitigates ROS-induced disruption of normal cell cycle progression in C2C12 cells.

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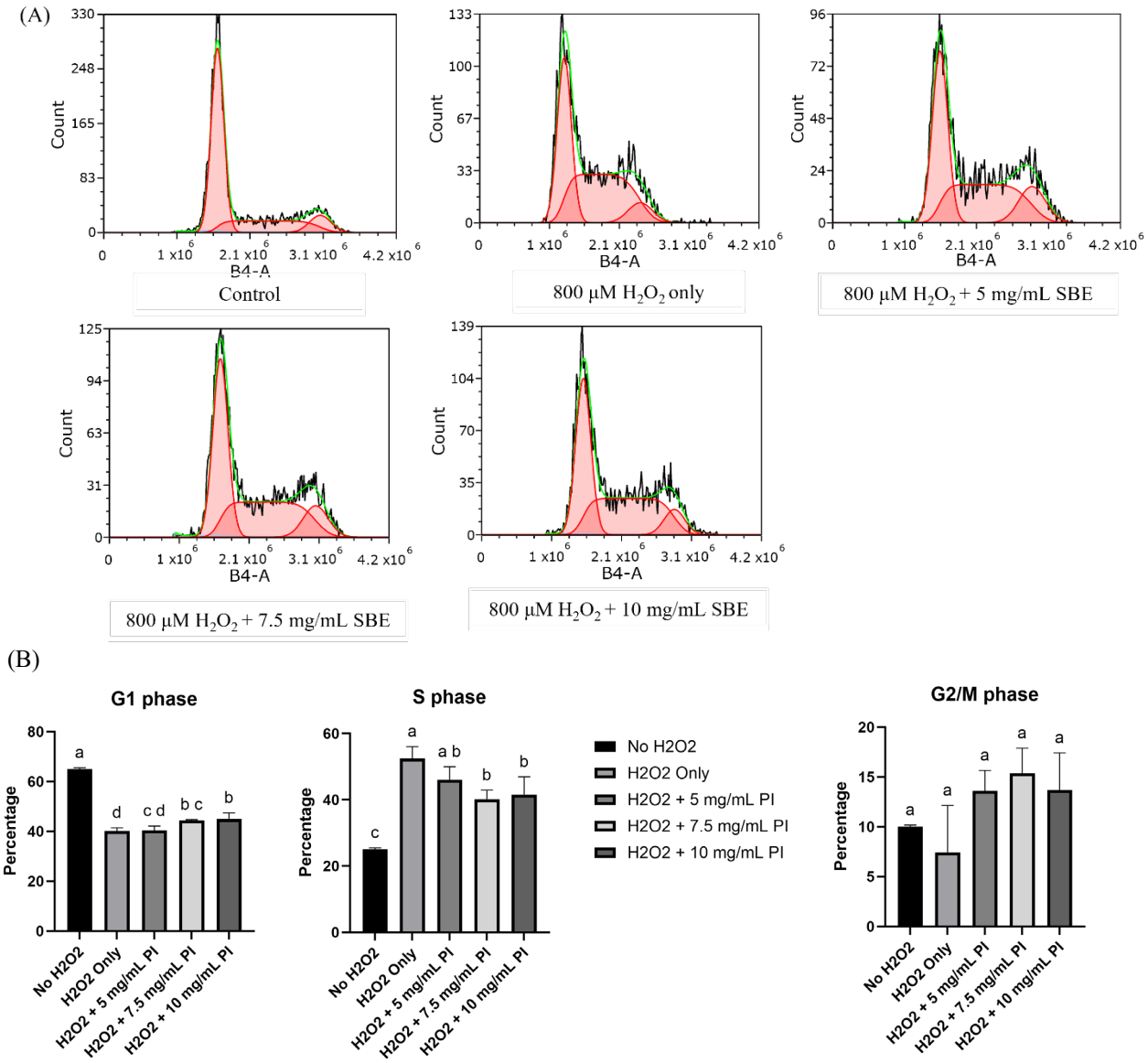

Figure 4.4. Effects of PI570481 extract on the cell cycle of C2C12 murine myoblast cells under H_2O_2-induced oxidative stress after 6-hour treatment

The oxidative stress significantly induced S-phase growth arrest in the cells and this was remarkably attenuated by the sorghum bran extract. Statistical differences are indicated with different letters ($p < 0.05$).

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4.3.5. Effects of PI570481 sorghum bran extract on H_2O_2-induced ROS generation and activation of MAPK pathway in C2C12 cells

Hydrogen peroxide is widely used as an inducer of oxidative stress due to its ability to elevate intracellular reactive oxygen species (ROS) levels. To assess the protective effects of PI570481 sorghum bran extract (SBE) against H_2O_2-induced ROS generation, intracellular fluorescence intensity was measured in C2C12 cells as an indicator of ROS accumulation. SBE treatment at concentrations of 7.5 mg/mL and above significantly suppressed ROS production compared with the H_2O_2-treated control group (Fig. 4.5A). In addition to its effects on ROS levels, the influence of SBE on mitogen-activated protein kinase (MAPK) signaling was examined. Phosphorylated p38 MAPK was markedly upregulated in response to H_2O_2-induced oxidative stress, whereas co-treatment with SBE substantially attenuated this activation (Fig. 4.5B–C). No clear dose-dependent trend was observed. These findings indicate that PI570481 SBE effectively reduces ROS accumulation and mitigates p38 MAPK activation under oxidative stress conditions in C2C12 myoblasts.

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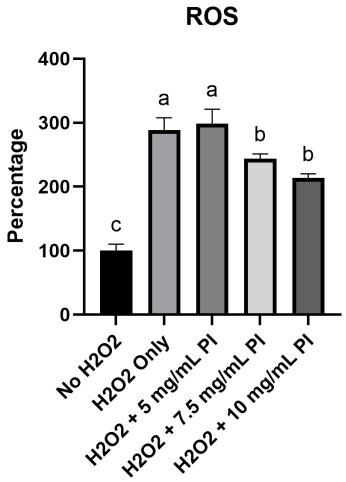
(A)



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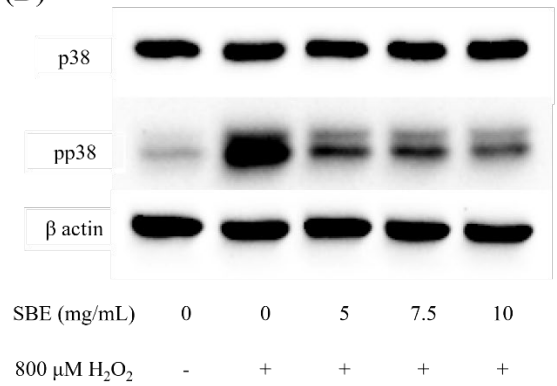

(C)

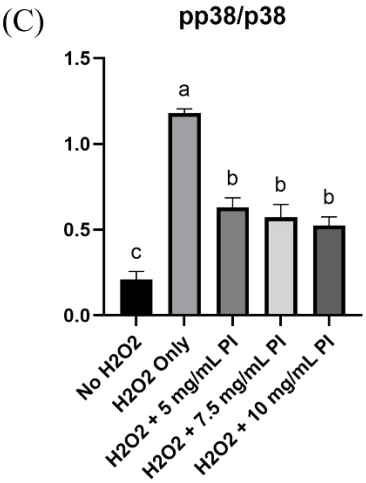

Figure 4.5. Effects of PI570481 extract on reactive oxygen species production and MAPK pathway in C2C12 murine myoblast cells under 6-hour exposure to H_2O_2

The sorghum bran extract significantly reduced the oxidative stress-induced production of ROS in the cells (A). In addition, the extract inhibited the expression of phosphorylated p38, one of the MAPK markers (B) (C). Statistical differences are indicated with different letters ($p < 0.05$).

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4.3.6. Effects of PI570481 sorghum bran extract against H_2O_2-induced autophagy

To assess the protective effects of the phenolic-rich PI570481 sorghum bran extract (SBE) on autophagy in C2C12 myoblasts, the expression of microtubule-associated protein 1A/1B light chain 3B (LC3) was examined by western blot analysis. LC3 exists in two forms, LC3-I and LC3-II, with the conversion of LC3-I to LC3-II serving as a key indicator of autophagosome formation and autophagic activation. In our study, H_2O_2-induced oxidative stress significantly increased the LC3-II/LC3-I ratio, indicating enhanced autophagy. Co-treatment with SBE markedly reduced this ratio, although no clear dose-dependent trend was observed (Fig. 4.6). These findings suggest that PI570481 SBE effectively suppresses oxidative stress–induced autophagic activation in C2C12 myoblast cells.

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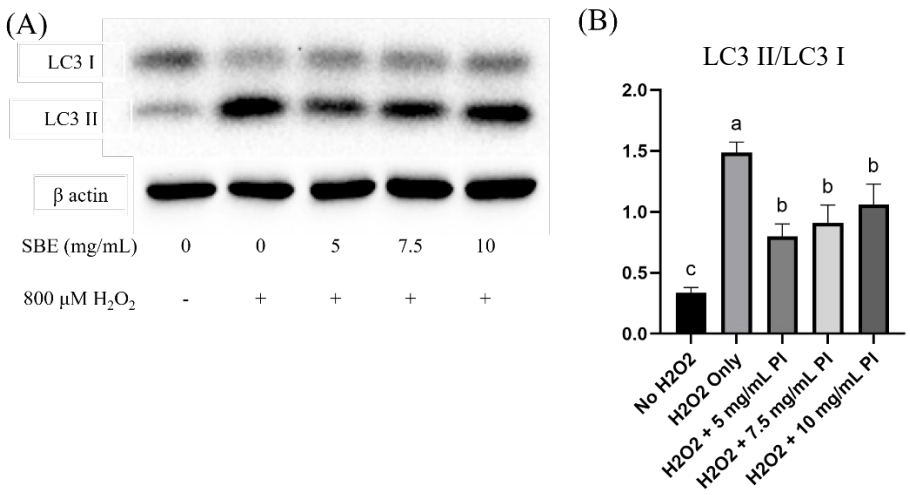

Figure 4.6. Effects of PI570481 extract on H_2O_2-induced autophagy in C2C12 murine myoblast cells

The ratio of LC3 II to LC3 I was remarkably increased by oxidative stress and significantly decreased by the extract. Statistical differences are indicated with different letters ($p < 0.05$)

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4.4. Discussion

The findings of this study demonstrate that phenolic-rich PI570481 sorghum bran extract exerts significant protective effects against oxidative stress–induced damage in C2C12 myoblasts, highlighting its potential as a functional food ingredient for mitigating obesity-associated skeletal muscle dysfunction. Obesity is strongly linked to chronic oxidative stress, which disrupts cellular homeostasis, impairs muscle regeneration, and increases susceptibility to metabolic diseases. The ability of sorghum bran extract to counteract these effects underscores the relevance of plant-derived polyphenols in addressing obesity-related pathophysiology.

The marked reduction in cell viability following exposure to 800 μM H_2O_2 reflects the severity of oxidative stress that skeletal muscle cells may experience under obesity-related metabolic imbalance. Co-treatment with sorghum bran extract significantly improved cell survival, suggesting that the phenolic constituents effectively neutralize reactive oxygen species or enhance endogenous antioxidant defenses. This protective effect is consistent with the known bioactivity of cereal-derived polyphenols, which have been reported to modulate redox balance and support cellular resilience under stress.

Morphological observations further support the protective role of the extract. Early alterations in cell adherence at 6 hours indicate that oxidative stress rapidly compromises cytoskeletal integrity and cell–matrix interactions. The absence of morphological differences at 24 hours, despite reduced cell density, suggests that structural deterioration occurs early and that intervention must precede the peak of ROS-induced cytotoxicity. This temporal pattern implies that sorghum bran extract is most effective when present before or during the initial stages of oxidative insult, aligning with the preventive rather than restorative nature of dietary antioxidants.

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The extract's ability to attenuate apoptosis is particularly noteworthy. Excessive H_2O_2 exposure led to substantial cleavage of PARP, a hallmark of irreversible DNA damage and apoptotic commitment. Sorghum bran extract significantly reduced PARP cleavage, indicating preservation of DNA repair capacity and suppression of apoptotic signaling. Given that chronic oxidative stress in obesity contributes to muscle atrophy partly through apoptosis, the inhibition of PARP cleavage by sorghum polyphenols suggests a mechanism by which sorghum consumption may help maintain muscle mass in obese individuals.

Cell cycle analysis revealed that oxidative stress induced S-phase arrest, likely due to impaired DNA replication or checkpoint activation in response to genotoxic stress. The extract alleviated this arrest, suggesting that it supports DNA synthesis and genomic stability under oxidative conditions. This finding is important because impaired cell cycle progression can hinder muscle regeneration, particularly in obesity where satellite cell function is already compromised.

The reduction in intracellular ROS levels in pre-treated cells further confirms the antioxidant capacity of the extract. This is relevant in the context of obesity, where individuals exhibit exaggerated oxidative responses to physiological challenges such as exercise. The observed modulation of MAPK signaling, particularly the activation of p38 by H_2O_2, suggests that oxidative stress triggers pathways associated with the unfolded protein response and endoplasmic reticulum stress. The ability of sorghum bran extract to counteract these effects implies that it may help prevent the accumulation of misfolded proteins, a process that contributes to muscle dysfunction and metabolic decline in obesity.

Autophagy regulation represents another critical aspect of muscle health. While basal autophagy is essential for removing damaged organelles and maintaining cellular quality control,

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excessive or dysregulated autophagy contributes to muscle wasting. The increase in LC3-II/LC3-I ratio following H_2O_2 exposure indicates heightened autophagic activity, likely as a compensatory response to oxidative damage. Sorghum bran extract significantly reduced this response, suggesting that it helps maintain autophagy at physiological levels and prevents excessive degradation of cellular components. This regulatory effect may support healthier muscle regeneration and protect against obesity-related muscle atrophy.

Collectively, these findings indicate that phenolic-rich PI570481 sorghum bran extract provides multifaceted protection against oxidative stress–induced damage in skeletal muscle cells. By reducing ROS accumulation, preserving DNA integrity, modulating apoptotic and stress-response pathways, and maintaining balanced autophagy, the extract demonstrates strong potential as a dietary strategy for mitigating obesity-associated muscle dysfunction. Given the underutilization of sorghum as a human food source in the United States, these results highlight an opportunity to incorporate sorghum-based ingredients into functional foods aimed at improving metabolic and muscular health in populations at risk for obesity-related complications.

4.5. Conclusion

The research data suggest that PI570481 sorghum bran extract effectively protected C2C12 myoblast cells from H_2O_2-induced oxidative stress. The SBE minimized anti-proliferative activities by suppressing apoptosis, repairing DNA replication during cell division, inhibiting ROS generation and MAPK pathway, and attenuating autophagy.

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