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Oxidative stress is the imbalance between the production of reactive oxygen species (ROS) relative to the antioxidant defense and plays a critical role in the pathogenesis of various diseases. The body naturally produces antioxidants such as glutathione (GSH) to counteract ROS under oxidative stress. Phytochemicals from certain plants such as tef (*Eragrostis tef)* have been shown to have antioxidant activities. Tef, an annual grass native to Ethiopia and Eritrea, is believed to be one of the earliest plants domesticated by humans. It is grown for human consumption in East Africa, and also serves as a valuable forage grass in other countries, including the United States. Tef has higher nutrient content as compared to other grains. Although in vitro studies have shown the antioxidant properties of tef grain extracts, studies using relevant cell models are lacking. Moreover, the molecular mechanisms involved in tef-induced antioxidant activity remained unknown. In this study, we analyzed the antioxidant properties of brown and ivory tef seeds and biomass using THP-1 (human leukemia monocytic cell line) due to their capability to differentiate into macrophages and induce GSH response. Our findings showed that tef extracts do not have cytotoxic effects. Grain extracts, particularly for the brown organic fractions increased cellular GSH levels in THP-1 monocytes when treated at 50 µg/mL for 24 hours. However, tef biomass extracts showed no increase in GSH levels in THP-1 monocytes. Separation (HPLC) and purification (ISCO) of the brown organic fraction further increased GSH levels in treated THP-1 cells. To understand the mechanism of tef extract-induced glutathione levels, we studied the modulation of the Nrf2 and NF-κB luciferase activity using a Luciferase reporter cell line. Our findings showed that the tef extracts increased the Nrf2-regulated luciferase activity by 20-fold while the NF-κB-regulated luciferase activity was only

slightly increased in the presence of the inflammatory cytokine TNF-α. Furthermore, tef extracts enhance the transcription of Nrf2-regulated genes including Keap-1, IKKβ, NQO-1, HO-1, GR, GSS, GCLC, and GCLM. Gene set enrichment analysis (GSEA) of the transcriptome data revealed the enrichment of genes involved in various pathways including the TNF-α signaling via NF-κB, the inflammatory response, and the ROS signaling pathways in THP-1 cells treated with tef extracts compared to the control group. Furthermore, heatmap of differentially expressed genes (DEGs) showed increased expressions of over 90 genes involved in various pathways including iron storage protein-ferritin pseudo genes *FTH1P10, FTH1P7, FTH1P11, FTH1P20, SLC11A1, SLC45A3, SLC12A7, SLC7A7, and SLC43A2, ZNF, GSR* and *TNF* in cells treated with the tef extracts compared to control. Taken together, our findings indicate that tef grains possess phytochemicals that increase antioxidant levels. The increase in antioxidant levels from tef extracts is primarily attributed to the Nrf2-signaling pathways. This is the first investigation of the cellular mechanisms of increased antioxidant levels in human leukemia monocytic cell lines by tef grain phytochemicals.

STUDYING THE MOLECULAR MECHANISMS OF TEF (*ERAGROSTIS TEF*) ANTIOXIDANT PROPERTIES IN HUMAN THP-1 MONOCYTES

by

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APPROVAL PAGE

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CHAPTER I: INTRODUCTION

Oxidative Stress: Impact on Human Health

Oxidative stress is defined as the excess of reactive oxygen species (ROS) in relative to antioxidant defense (Sharma et al., 1999). Maintaining a balance between ROS and antioxidants is crucial for physiological function and metabolic processes. ROS, also known as free radicals, are highly reactive compounds formed from diatomic oxygen, water, and hydrogen peroxide. They are normally generated as by-products of cellular aerobic metabolism. Hydrogen peroxide (H2O2), hydroxyl radical (•OH), and superoxide radicals (O2•−) are the commonly known ROS. Several stress conditions including metabolic disorders and diets can induce oxidative stress (Migliore & Coppede, 2009). Environmental stressors such as ionizing and UV radiation, pollutants, xenobiotics, and drugs can greatly increase ROS production (Pizzino et al., 2017). Acrolein has been shown to cause oxidative stress and generate ROS (Sun et al., 2014). The induction of oxidative stress can cause damage to lipids, proteins, and deoxyribonucleic acid (DNA) through oxidative chain reactions (Ozcan et al., 2015). This damage can also result in inflammation. A large number of studies demonstrate that oxidative stress is a key driver of many pathological diseases, both chronic and degenerative (Pizzino et al., 2017). It has also been shown to accelerate the aging process and contribute to the development of acute diseases such as stroke (Liguori et al., 2018). Although the issue revolves around low antioxidant levels, supplementing antioxidants is not a comprehensive solution. That is why oxidative stress represents a multifaceted overall health concern.

The negative effects of reactive oxygen (ROS) and nitrogen species (NOS) are neutralized by our body's antioxidant defenses (Sachdev et al., 2021). Cells possess two complex mechanisms to cope with stresses, enzymatic and non-enzymatic. Enzymatic mechanisms are

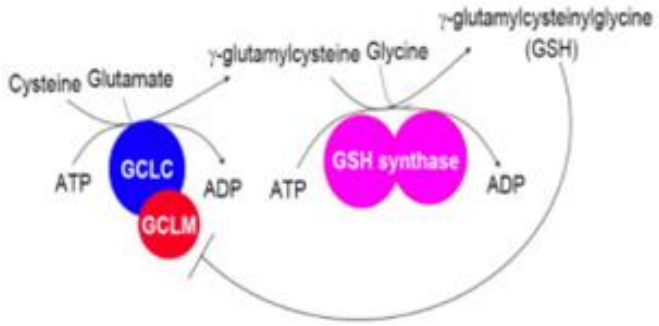
composed of enzymes such as superoxide dismutase (SOD), peroxidase, catalase, and reductase. Nonenzymatic mechanisms include antioxidant molecules such as GSH, free radical scavengers, albumins, and ascorbic acid (Kleczkowski, 2003). Sources of naturally occurring antioxidants include fruits and vegetables, seeds, and whole grains. Assessing the levels of oxidants and antioxidants can provide insights into how these levels affect diseases.

Glutathione is a tripeptide present in most cells, consisting of three amino acids: glutamate, cysteine, and glycine (Aoyama et al., 2012). Glutathione is predominantly present in its reduced form (GSH), with minimal amount in its fully oxidized form (GSSG) (Pisoschi et al., 2021). GSH is a potent non-enzymatic antioxidant capable of counteracting oxidative stress and mitigating its effects, through hydrophilic scavenging of radicals and electrophiles. It is regarded as the most abundant endogenous antioxidant protecting cells against oxidative stress. GSH is a key determinant of redox signaling, involving detoxification, apoptosis, and immune function (Lu, 2013). Depletion of GSH has been shown to initiate apoptosis, inducing ROS production (Deponte, 2017; Teskey et al., 2018). Supplementation of glutathione has been shown to prevent ROS-induced apoptosis (Purohit et al., 2007).

The synthesis of GSH is regulated by two enzymatic steps. First is the formation of γ-glutamylcysteine from glutamate and cysteine and second is the formation of GSH from γ-glutamylcysteine and glycine (Lu et al., 2013). GSH is accumulated in the cells in three ways. The first is through a 2-step process catalyzed by the enzymes glutamate cysteine ligase (GCL), composed of a catalytic (GCLC) and a modifier (GCLM), and glutathione synthase. The second is through the regeneration of oxidized GSSG to reduced GSH, by glutathione reductase and NADPH. The third is through recycling of cysteine by GGTP. The antioxidant function of GSH is largely attributed to GSH peroxidase (GPx)-catalyzed reactions. These reactions reduce

hydrogen peroxide and lipid peroxide, as GSH is oxidized to GSSG. GSSG is in turn reduced back to GSH by GSSG reductase, forming a redox cycle. Glutathione peroxidase has the capability to scavenge free radicals and detoxify hydrogen peroxide (Pizzorno, 2014).

Figure 1. GSH Biosynthesis Pathway (Lu et al., 2013)



GSH Biosynthesis Pathway (Lu et al., 2013). The biosynthesis of GSH occurs in a series of enzymatic steps within cells.

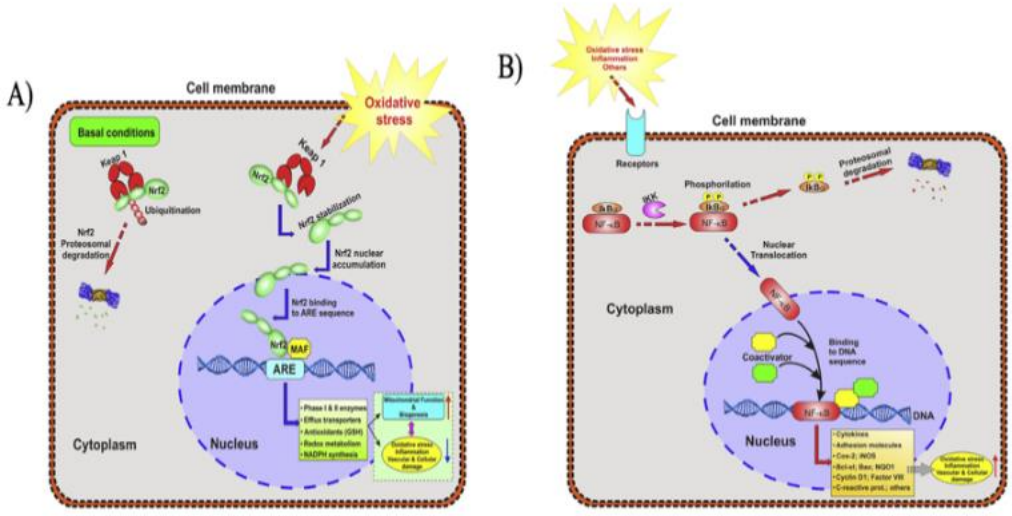
The signaling mechanism for increased GSH is known. The protective effect of GSH is mediated through the activation of the Nrf2-HO dependent signaling pathway (Kwon et al., 2019). Constitutive NF-κB activity has also been shown to promote GSH biosynthesis. (Meng et al., 2010).

The Role of Nuclear Factor Erythroid 2 (Nrf2) in ROS Detoxification

Comprehensive studies have indicated a connection between Nrf2 and the nuclear factor kappa-light-chain-enhancer of activated B (NF-κB) pathway. NF-κB induces the expression of pro-inflammatory genes involved in inflammation, cancer, and stress responses (Meng et al., 2010). Multiple studies have shown that NF-κB suppresses the activity of Nrf2 (Yerra et al., 2013). NF-κB activation mechanism is similar to Nrf2. Under typical conditions, NF-κB is bound to its inhibitor, I-κB, in the cytoplasm. The p50/65 heterodimer of NF-κB cannot enter the

cell's nucleus. When triggered by growth and inflammatory factors, the inhibitor is then phosphorylated and ubiquitinated, allowing the NF-κB p50/65 heterodimer to translocate to the nucleus and bind to the promoter region (Hoesel & Schmid, 2013). Upon activation, NF-κB induces the production of proinflammatory cytokines such as TNF-α, resulting in inflammatory response (Yu et al., 2020). TNF-α is a known activator of NF-κB. A study showed that the NF-κB pathway is essential in maintaining steady levels of redox scavenger GSH. Inactivation of IκκB resulted in low cellular GSH level (Peng et al., 2010).

Figure 2. Nrf2 and NF- κB Signaling Pathways



Nrf2 and NF- κB Signaling Pathways. A) Nrf2 antioxidant signaling pathway, and B) NF-κB proinflammatory signaling pathway (Sivandzade et al., 2019).

Antioxidant Properties of Phytochemicals

Numerous studies offer compelling evidence supporting the use of plants as dietary supplements for promoting human health. Consumption of fruits and vegetables, as well as grains, has been linked to reduced risk of chronic diseases (Boeing et al., 2012). Phytochemicals from different plant species have been shown to have antioxidant activities based on in vitro

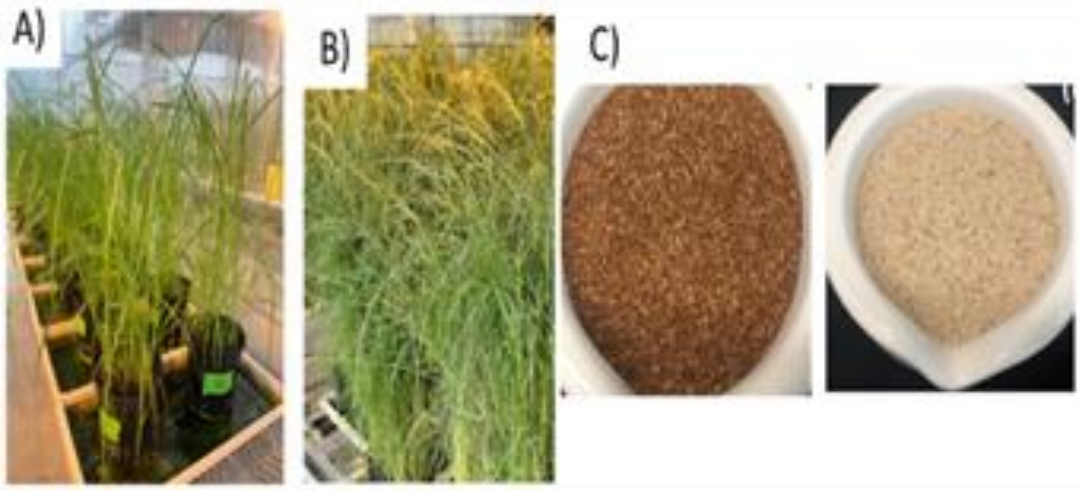
studies. For example, extract from *Lactuca indica,* belonging to the *Compositae* family, was found to have significant free radical scavenging activity, reducing oxidative stress, and chemopreventive therapeutic properties, using human promyelocytic leukemia HL-60 cells (Wang et al., 2003). Wheat has been suggested as a dietary source of natural antioxidants. Antioxidants including lutein, zeaxanthin, and β-carotene were detected in ethanol extracts of seven wheat varieties (Zhou et al., 2004). Phytochemicals analysis of methanolic extract of *Cymbopogon citratus* leaves revealed the presence of tannins, flavonoids, and other phenolic compounds. *Cymbopogon citratus* extract also exhibited antioxidant activity (Unuigbe et al., 2019). Antioxidant activity was observed in phytochemicals derived from 13 medicinal grasses. *Cymbopogon spp., Cymbopogon nardus* and *Cenchrus ciliaris* methanolic root extracts contained high concentrations of phenolics and flavonoids (Gebashe et al., 2020). The observed antioxidant activity in various plants may be due to the abundance of phytochemicals. Plants phytochemicals can be explored for therapeutic purposes.

Antioxidant Properties of Tef (*Eragrostis tef)*

Tef (*Eragrostis tef)* is an annual grass (Figure 3) native to Ethiopia and Eritrea. It is the only cultivated species out of 350 *Eragrostis* species, with over 6,000 years of cultivation (Tadele & Hibistu, 2021); Habtegebrial et al., 2007). Tef is traditionally used to produce the bread 'injera' and is one of the most important staple crops in Ethiopia. Tef accounts for the greatest land use for cereal cultivation in Ethiopia (Ligaba-Osena et al., 2021). Tef is also economically superior in Ethiopia relative to other cereal crops (Lee, 2018). It is grown on a global scale primarily as a forage crop, in countries such as the United States, Canada, Australia, and Switzerland (Flynn, 2019). Tef grains are the smallest of all cereal grains, measuring around 1.0 mm in length and 0.60 mm in width (Gebru et al., 2020). The grains' color ranges from light

ivory to reddish brown. Tef grains have high mineral and crude fiber content and low glycemic index. Compared to other grains, tef grains have elevated levels of essential amino acids and longer shelf life (Gebru et al., 2020). Tef grains are also gluten free (Spaenij-Dekking et al., 2005). In recent years, there has been growing interest in tef grains due to their perceived nutritional and health benefits.

Figure 3. The Morphology of Tef Grains and Biomass



The Morphology of Tef. A) Tef plants before flowering, B) tef inflorescence, and C) brown and ivory tef seeds used for extraction.

Despite the nutritional and health benefits, tef cultivation lags behind other cereal crops. For centuries, tef has received less attention in terms of research, development, and promotion. With persistent global temperature increase and drought occurrences, there is great interest in alternative forages. Tef is a C4, early maturing crop that grows wells under different soil conditions (Tefera and Ketema, 2001; Ketema 1983, 1991). It is drought tolerant and pest resistant (Tadese, 1993; Jones, 1988). Tef cultivation is well-suited for the United States, particular in the Midwest region. Tef can be grown in the summer months and requires minimal irrigation (Flynn, 2019). Previous studies indicated that tef produces higher biomass yield and

superior quality feed as compared to other annual grasses (Habtegebrial, et al., 2019). The widespread practice of feeding dairy cows poor-quality feed has been shown to decrease animal productivity and increase methane emissions. Increased milk yield, nutrient digestibility, and nitrogen utilization efficiency were observed in cows fed with tef straw (Mekuriaw et al., 2020). Tef has also been proposed as a high-quality feed for beef cattle. The nutritive value depends on the maturity stage at when the tef is harvested. With advancing maturity, forage quality (crude protein content) and yield decreases (Vinyard et al., 2018).

Numerous studies have explored both the perceived and actual benefits of antioxidants against oxidative stress. Efforts to find natural compounds with effective antioxidative properties have significantly increased throughout the year. The potential of *Eragrostis* species as a source of natural health supplements, antioxidants and neuroprotectants has been demonstrated in several studies. In vitro studies have shown that tef biomass has antioxidant properties. Compelling evidence indicated that varieties of tef grains have phenolic and flavonoid content and antioxidant potential, using methanol extraction method (Reta et al., 2022). Brown tef is considered a super grain, exhibiting higher antioxidant properties compared to the ivory variety. Fermentation and thermal processing have been shown to further increase the antioxidant potential (Shumoy, 2017; Kataria, 2021). Methanol extracts from E. tef varieties exhibited radical scavenging activity (Boka et al., 2013). Similarly, the aerial part of seven *Eragrostis* species showed antioxidant and neuroprotective activities (Na et al., 2017). Phenolic extracted from aerial part of E. ferruginea provided neuroprotective effect against amyloid beta peptide, a major cause in pathology of Alzheimer's disease. Compounds found in cancer and diabetic treatment drugs, triterpenoids and diterpenoids, were extracted from E. ferruginea (Na et

al., 2010). Tef grains have the potential for the treatment and prevention of various diseases such as diabetes, anemia, and malaria (Gebremariam et al., 2014; Salawu et al., 2014).

Nonetheless, the antioxidant properties of tef extracts have not been evaluated using a mammalian cell model, until recently. Previous in vitro studies investigating the antioxidant properties of *Eragrostis* tef primarily relied on methanolic extracts. The phytochemical contents may have been underestimated in the literature because bound phytochemicals were not included. Cotter et al. (2023) reported the antioxidant properties of tef grain extracts in THP-1 monocytes. Though the molecular mechanism behind these antioxidant properties remains unclear, the increase in GSH levels is thought to be related to antioxidant target genes (Cotter et al., 2023). In the present study, we have tested the hypothesis that tef phytochemicals increase the antioxidant GSH level, which is regulated by antioxidant (Nrf2) and pro-inflammatory (NF-κB) pathways-mediated signaling. This study was designed to investigate phytochemical properties of the organic, hexane, and aqueous fractions obtained from the methanol-chloroform extraction protocol. Specifically, THP-1 cells have been chosen for this study due to their ability to differentiate into macrophages and induce GSH response (Zhang et al., 2017). The first aim is to study the antioxidant (GSH) property of tef grain phytochemicals in THP-1 monocytes. Because the mechanism for increased GSH is unknown, the second aim is to uncover the molecular and signaling mechanisms regulating increased GSH levels in THP-1 monocytes treated with tef extracts. The antioxidant properties of tef extracts are attributed in part to the antioxidant regulated genes, therefore, the third aim is to analyze the expression of Nrf2 regulated genes in THP-1 monocytes treated with tef extracts. The analysis of proinflammatory genes regulated by NF-κB can offer information into the link between the NF-κB and the Nrf2 pathways. Comparative transcriptome analysis provides insight into the molecular pathways and

genes associated with GSH response. The fourth aim is to study the antioxidant (GSH) property of tef biomass phytochemicals as a potential high-quality forage. The identification of novel antioxidant pathway-dependent anti-inflammatory phytochemicals has become a crucial aspect in drug discovery.

CHAPTER II: METHODS AND MATERIALS

Chemicals and Materials

Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Gibco, Invitrogen. 2-Cyano-3,12-dioxooleana-1,9-dien-28-imidazolide (CDDO-Im) was purchased from Toronto Research Chemicals, Inc. GSH, o-phthalaldehyde (OPT), NADPH, 3 -(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and bovine serum albumin (BSA) were purchased from Calbiochem. Coomassie protein assay reagent was purchased from Sigma. Tissue culture flasks and 24-well plates were purchased from Corning. DNase I amplification grade, high-capacity cDNA reverse transcription kit, and PowerUp® SYBR™ Green master mix were purchased from Thermo Fischer Scientific. Primers were purchased from IDT.

Tef Grains and Biomass

Brown and ivory tef seeds were purchased from Maskal Teff. For preparing biomass extracts, brown and ivory tef seeds were grown at the UNCG Biology research greenhouse in June 2022. The biomass was harvested in July 2022, approximately six weeks (before flowering), and then freeze dried immediately. The plants were allowed to regrow for a second round of biomass harvest. Freeze-dried biomass and seeds were ground to fine powder using a spice grinder.

Extraction

Brown and ivory tef seeds (2 kg) were ground over several cycles using a blender. The ground material was suspended in 1.5 L of $CHCl_3$: MeOH and allowed to stand overnight at room temperature. The solution was then filtered and dried using a vacuum rotary evaporator. The remaining tef marc (insoluble residue) was resuspended in 1.0 L of $CHCl_3$ and allowed to

soak for an hour before filtering and adding to the first extraction solution to resuspend it. The solution was transferred to a separatory funnel and 250 mL of methanol and 1250 mL of H2O were added. The organic bottom layer was drawn off into a round-bottom flask and evaporated to dryness. The dried organic extract was then reconstituted in 2 L of hexane:MeOH:MeCN (2:1:1). The biphasic solution was shaken vigorously and then transferred to a separatory funnel. The MeOH:MeCN layer (organic) and hexane layer were drawn off into a round bottom flask and evaporated to dryness under vacuum. The extracts were left to dry under nitrogen for 24 hours. The dried fractions were dissolved in DMSO at 50 mg/mL stock concentration prior to using for treating the THP-1 monocytes. The same extraction protocol was followed for preparing the biomass extracts by adjusting the volumes for lower starting materials.

High-Performance Liquid Chromatography (HPLC) and Teledyne ISCO Chromatography

Tef extracts were dissolved in chloroform and absorbed onto Celite 545 (Acros Organics). It was then subjected to chromatographic separation via normal phase flash chromatography on a Teledyne ISCO Combiflash Rf 200 which was monitored by an evaporative light scattering detector (ELSD) and photodiode array (PDA) detector. A gradient of hexane-chloroform-methanol at a 35 mL/min flow rate and RediSep Silica 24g Gold column.

Cell Culture

THP-1 monocytes, human leukemia monocytic cell line, were purchased from ATCC. The monocytes were kept in 25 cm2 tissue culture flasks and maintained routinely in RPMI 1640 with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, under 5% CO2 humidified atmosphere and 37°C. Cells were subcultured at 70-80% confluence, about twice a week.

Cell Viability with Trypan Blue

Cell viability was assessed using 0.4 % Trypan blue dye staining assay. One-to-one ratio of cell suspensions and Trypan blue dye were transferred to a microcentrifuge tube and mixed by vortexing. After 1 minute, the solution was transferred to a hemacytometer well. The number of viable (trypan blue negative) and dead (trypan blue positive) cells were counted using a light microscope. Cell viability was determined by dividing the numbers of unstained cells by the total number of cells. For all assays, cell confluency of 90% or higher was required.

Cell Extraction Preparation

THP-1 monocytes were treated with 50 ug/mL tef extracts in 10 mL RPMI media for 24 hours. Three biological and nine technical replicates were used to ensure reproducibility for all assays. After treatment, cells were collected and resuspended in 1 mL 1X phosphate-buffered saline (PBS), and sonicated. Sonicated cells were centrifuged at 13,000 rpm at 4℃ for 10 min. Supernatants were collected and kept on ice for quantification of the total protein and GSH.

Assay for Protein Content

Total protein was assayed using the Coomassie protein assay kit (Thermo Fisher Sicnetific). The standard contained 790 uL of Coomassie protein assay reagent and 10 uL of 1.48 mg/mL BSA standard. Then 3 uL of each sample was added to microcentrifuge containing 790 uL of Coomassie protein assay reagent. The tubes were vortexed immediately after the addition of the extracted protein, followed by incubation at room temperature for 10 minutes. The sample (200 uL) was then transferred to 96-well plates and the absorbance was measured at 595 nm using the microplate reader.

Assay for Glutathione (GSH) Content

Hissin and Hilf's (1976) fluorometric method was used to measure the total cellular GSH content, using GSH standard curve (Hissin & Hilf, 1976). Triterpenoid compound 2-cyano-3,12-dioxooleana-1,9-dien-28-imidazolide (CDDO-Im) (CDDO) was used as a positive control (data not shown). CDDO is known to induce GSH. Extracted protein, 10 uL, was incubated with 37 uL of 0.1 mol/L sodium phosphate buffer containing 5 mmol/L EDTA and 12.5 uL of 25% metaphosphoric acid (HPO3). After 10 minutes of incubation on ice, the samples were centrifuged at 13,000 rpm at 4°C for 5 minutes. The resulting supernatant, 45 uL, was incubated with 1.89 mL of 0.1 mol/L sodium phosphate buffer containing 5 mmol/L EDTA and 100 uL o-phthalaldehyde (OPT) solution, in glass tubes. The standard sample contained 100 uL of 5 μg/mL GSH standard, 1.8 mL of 0.1 mol/L sodium phosphate buffer containing 5 mmol/L EDTA and 100 uL o-phthalaldehyde (OPT) solution. After 15 minutes of incubation in the dark at room temperature, 200 uL of the solution was transferred to 96-well plates and the fluorescence was measured at excitation 360 nm emission 460 nm, using the microplate reader.

qPCR Analysis

To study the effects of tef extracts on the expression of oxidative stress pathway genes, THP-1 monocytes were treated with 50 ug/mL active brown tef fraction B 25-4. After 24 hours, the total RNA was isolated using Trizol reagent. The RNA was treated with DNase I, and 1 mg RNA of each sample was reverse transcribed into cDNA using the high-capacity cDNA reverse transcription kit (Thermo Fischer Scientific, USA), according to the manufacture's protocol. The forward and reverse primers used in qPCR for Nrf2, NF-κB, IKKβ, NQO-1, HO-1, glutathione reductase, glutathione synthase, glutathione peroxidase, GCLC, and GCLM genes are listed in Table 1. Relative expression as fold change in expression in the treated samples as compared to

the vehicle control were calculated using the ΔΔCT method (Livak & Schmittgen, 2001) available with the QuantStudio3 software (Applied Biosystems) using GAPDH gene as an internal control.

Luciferase Assay

Nrf2/ARE Luciferase Reporter HEK293 Stable Cell Line (P/N SL-0042) and NF-κB Luciferase Reporter HEK293 Stable Cell Line (P/N SL-0012) were purchased from Signosis, Inc. HEK293 human embryonic kidney cells were cultured in complete growth medium. To test whether tef extracts activate the Nrf2 antioxidant pathway, Nrf2/ARE reporter cells were treated with active tef extracts for 24 hours in 96-wells plate. To test whether tef extracts activate the NF-κB proinflammatory pathway, NFκB reporter cells were treated with tef extracts, liposaccharide (LPS), and tumor necrosis factor alpha (TNF-α) for 24 hours in 96-wells plate. Co-treatment was also carried out. After 24 hours, the media was removed using a multichannel pipette, and 40 µL lysis buffer was added to each well. The plate was shaken for 15 minutes and 20 µL of solution from each well was transferred to wells on an opaque 96-well solid bottom plate. Immediately, 100 µL of luciferase substrate was added to each well. The plate was read immediately using Bio-Tek Synergy™ plate reader. The level of luminescence measured is positively correlated with the level of transcription factor activation.

RNA-Sequencing

To determine the effect of tef extract on cellular transcriptome, RNA sequencing was performed. THP-1 monocytes were treated with or without active brown tef fraction B 25-4. After 24 hours, the RNA was extracted using Trizol reagent and treated with Amplification grade DNase I (Invitrogen) to remove traces of genomic DNA. RNA quantity and purity were determined spectrophotometrically. DNA library preparation was carried out following the

NEBNext® Ultra™ II RNA Library Prep Kit of Illumina® (E7770, E7775) protocol. The DNA band was cut and purified after gel electrophoresis. The DNA was quantified using Qubit (Thermo Fisher Scientific). Samples were pooled and sent for sequencing.

RNA-Seq Data Analysis

Data analysis was performed in consultation with Bioinformatics Core at NCSU Center for Human Health and the Environment. An average of ~25.3 million paired-end raw RNAseq data were generated for each replicate. The quality of sequenced data was assessed using the fastqc application, and 12 poor-quality bases were trimmed from the 5'-end. The remaining good-quality reads were aligned to the Human reference genome (hg39 version 100) downloaded from the Ensembl database using STAR (Dobin et al., 2013) aligner. Per-gene counts of uniquely mapped reads for each replicate were calculated using the htseq-count script from the HTSeq (Anders et al., 2015) python package. The count matrix was imported to R statistical computing environment for further analysis. Initially, genes that had no count in most replicate samples were discarded. The remaining count data were normalized for sequencing depth and distortion, and dispersion was estimated using DESeq2 (Love et al., 2014)Bioconductor package in the R statistical computing environment (R Core Team (2022). We fitted a leaner model using the treatment levels, and differentially expressed genes were identified after applying multiple testing corrections using the Benjamini-Hochberg procedure (Benjamini & Hochberg, 1995). The final significant genes were generated using padj<0.05.

Geneset Enrichment Analysis

The Human Hallmark gene list was downloaded from the Gene Set Enrichment Analysis website (https://www.gsea-msigdb.org/gsea/index.jsp, version 2022). The rank was generated for all genes passing the filter by multiplying the log2 Fold Change from the DESeq2 with 1/p-

value. Then, fgsea algorism with 1000 permutations to identify a significant mouse Hallmark pathway by comparing it with a ranked gene list (Korotkevichn et al., 2019). The top 20 pathways were plotted using a bar plot after sorting the output using a Normalized Enrichment Score.

Statistical Analysis

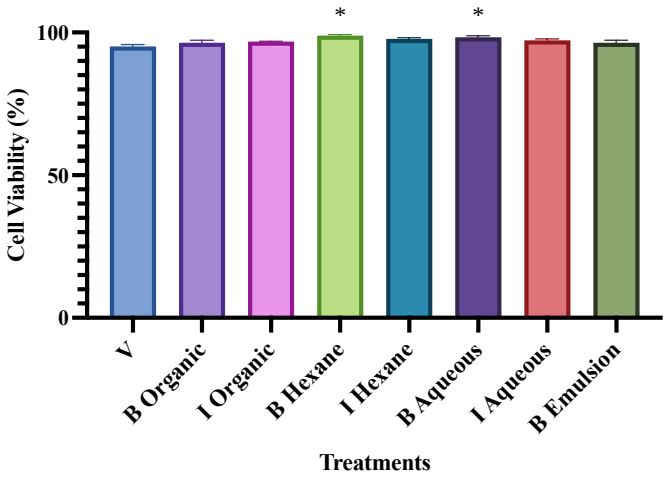
All data were subjected to the analysis of variance (ANOVA) using the PROC GLM procedure available in SAS statistical package (Lee, 1987). After significant F-tests, the Tukey's multiple comparisons were used to separate the means ($P < 0.05$). Data represents mean + SD of at least three independent experiments (Keselman and Rogan, 1977). A value of $p<0.05$ was considered significant amongst all the treatments.

CHAPTER III: RESULTS

The Effect of Tef Grain Extract on Cell Viability

Methanol-chloroform extraction of tef grains yielded three fractions: organic, hexane, and aqueous. The emulsion fraction was presented solely in the brown tef. THP-1 monocytes were incubated with 50ug/mL brown and ivory crude fractions for 24 hours in RPMI media. Tef extracts were dissolved in DMSO at 50 mg/mL stock concentration and Trypan blue was used to determine cell viability. We previously tested the effect of tef extracts on cell viability at different incubation times, and the result showed that 24 hours was optimum to study antioxidant activity (Cotter et al., 2023). As shown in Figure 4, only a slight difference in cell viability was observed among the treatments: Brown (B) organic (96.5%), ivory (I) organic (96.9%), B hexane (99.0%), I hexane (97.7%), B aqueous (98.4%), I aqueous (97.3%), and B emulsion (96.5%), as compared to the V (95.1%) (Figure 4).

Figure 4. Cell Viability of THP-1 Monocytes Treated with Crude Tef Grain Extracts: 24 Hours



Cell Viability Using Trypan Blue for THP-1 monocytes treated with 50 ug/mL brown and ivory tef grain crude extracts for 24 hours. The relative cell viability was calculated by diving

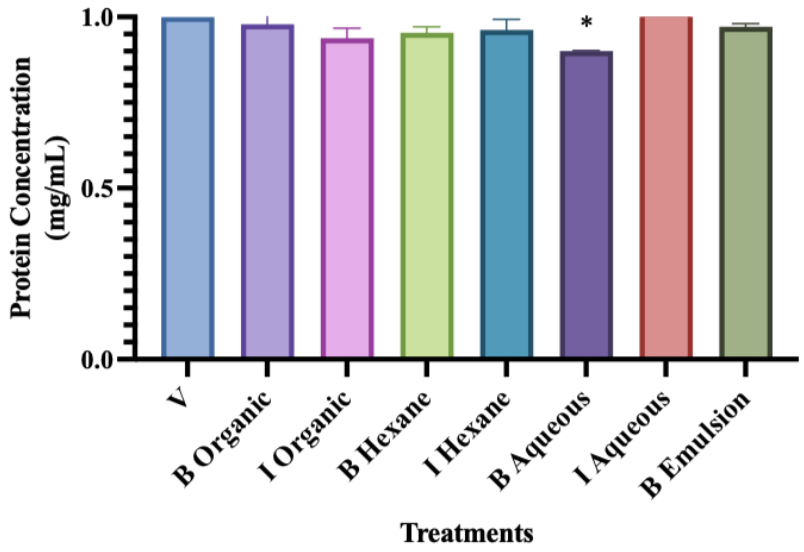
the numbers of unstained cells by the total cells. V, vehicle DMSO treated (negative control), B, brown, I, ivory. Bars represent the mean and SD of three independent experiments. *$p<0.05$.

Tef Grain Extract Increased GSH Levels in THP-1 Monocytes

Immortalized THP-1 monocytes have been extensively used in immunology and toxicology research due to their homogeneous background, which minimizes the degree of variability in cell phenotype. These monocytes are an ideal model system to study biological processes under control and reproducible conditions. The THP-1 monocytes are shown to have normal cell viability characteristics expected under standard in vitro conditions. Previous studies reported that 50 ug/mL tef extract dissolved in DMSO was not toxic (Cotter et al., 2023). Hereafter, DMSO was used as negative control. Six million cells were plated per treatment.

As shown in Figure 5, after 24 hours of treatment, protein quantification was carried out using the Bradford assay. Treatments with tef extract showed minor changes in protein concentration, with no significant difference among the different fractions ((B organic (0.979 mg), I organic (0.938 mg), B hexane (0.954 mg), I hexane (0.962 mg), I aqueous (1.021 mg), and B emulsion (0.971 mg)), except for the B aqueous. A 10% decrease in protein concentration was observed in the B aqueous (0.900 mg) fraction (Figure 5). To normalize the samples, we divided the GSH concentrations by mg of protein. Samples are expressed as nmol GSH/mg protein, and the protein concentration was quantified for all the GSH assays.

Figure 5. Protein Concentration of THP-1 Monocytes Treated with Crude Tef Grain Extracts: 24 Hours

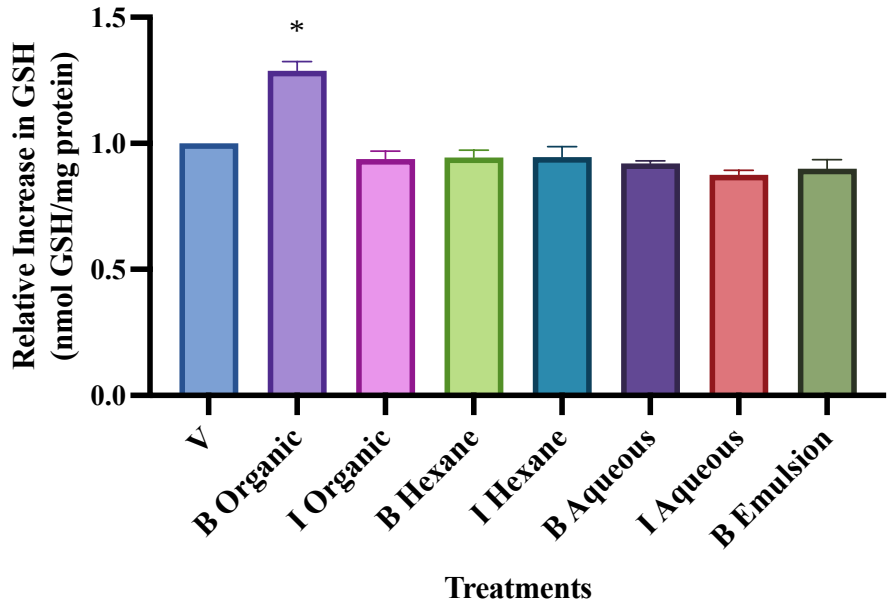


Total Protein Quantification. THP-1 monocytes were treated with 50 ug/mL brown and ivory crude tef grain extracts for 24 hours. After 24 hours, the total protein was extracted and measured. V, vehicle DMSO treated (negative control), B, brown, I, ivory. Bars represent the mean and SD of three independent experiments. *$p<0.05$.

Macrophages, through the differentiation of monocytes, produce reactive oxygen species (ROS), which trigger oxidative GSH response to counter rising ROS. Quantification of GSH level can be a useful measure of antioxidant response. GSH deficiency or low GSH/GSSG ratio is associated with several chronic illnesses. In vitro assays have shown that tef grains have antioxidant property. To analyze the influence of tef phytochemicals on antioxidant level, we treated THP-1 monocytes for 24 hours with the 50 µg/mL crude extract fractions obtained from 1 lb seed and quantified the relative increased in GSH (Figure 6). Triterpenoid compound 2-cyano-3,12-dioxooleana-1,9-dien-28-imidazolide (CDDO-Im) (CDDO) was used for the induction of

GSH (data not shown). We followed the Hissin and Hilf (1976) GSH protocol to quantify GSH level. After treatment with tef extracts for 24 hours, a significant increase in GSH levels was observed in the B organic fraction as compared to the V and other treatments, reaching 28.8% increase while no marked effect observed for the other fractions (Figure 6).

Figure 6. Relative Increase in GSH of THP-1 Monocytes Treated with Crude Tef Grain Extracts: 24 Hours

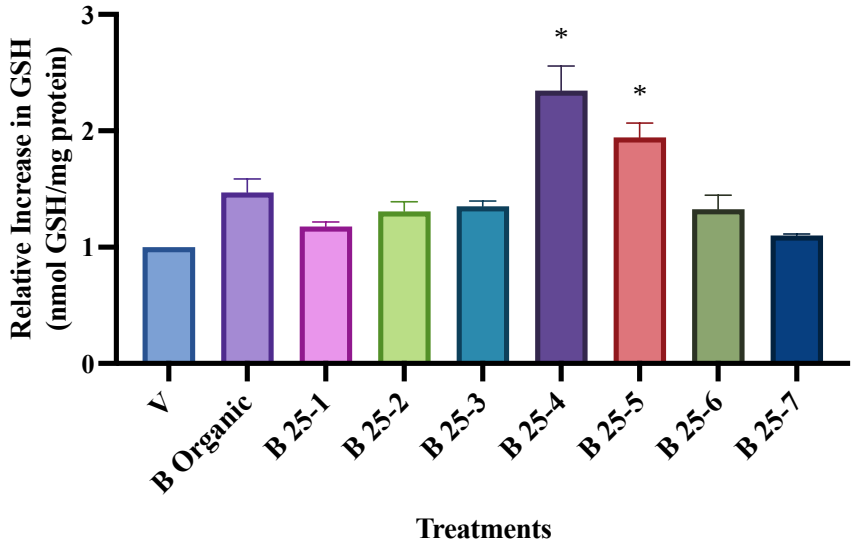


Relative Increase in GSH. THP-1 monocytes were treated with 50 ug/mL brown and ivory crude tef grain extracts for 24 hours. After 24 hours, the total protein was extracted to determine the level of GSH. V, vehicle DMSO treated (negative control), B, brown, I, ivory. Bars represent the mean and SD of three independent experiments. *$p<0.05$.

We performed flash or column chromatography of the crude B organic fraction for separation (HPLC) and purification (ISCO) of components of the extract. Seven ISCO fractions were obtained, designated as B 25-1 to B 25-7. After 24 hours treatment of fractions B 25-1 to B 25-7, a significant increase in GSH levels was observed for the B 25-4 and B 25-5 fractions, as

compared to the V and other treatments. B 25-4 and B 25-5 fractions increased GSH levels by 134.6% and 94.3%, respectively. Crude B organic fraction also exhibited an increased GSH levels, but not as high as the forementioned fractions. The other fractions induced a smaller increase in GSH levels (Figure 7).

Figure 7. Relative Increase in GSH of THP-1 Monocytes Treated with Purified Brown Tef Grain Fractions B 25-1 to B 25-7: 24 Hours

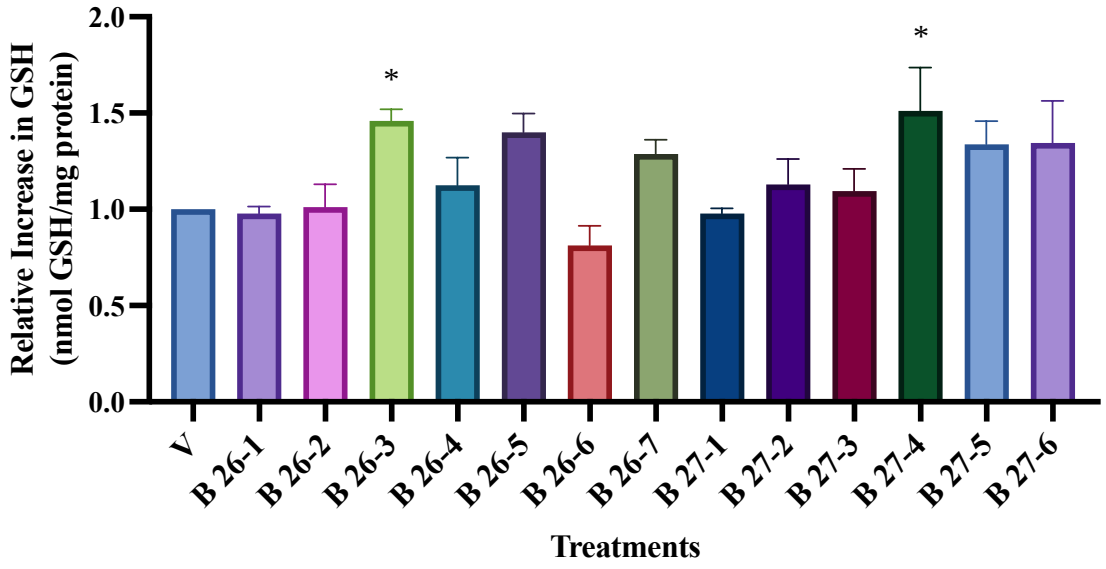


Relative Increase in GSH. THP-1 monocytes were treated with 50 ug/mL purified brown tef extract fractions B 25-1 to B 25-7 for 24 hours. After 24 hours, the total protein was extracted to determine the level of GSH. V, vehicle DMSO treated (negative control), B, brown, I, ivory. Bars represent the mean and SD of three independent experiments. *$p<0.05$.

The active fractions, B 25-4 and B 25-5, were analyzed by mass spectrometry. Mass spectrometry data revealed that the two fractions were not pure compounds. Flash chromatography was repeated to further separate and purify fractions B 25-4 and B 25-5. Seven fractions were obtained from B 25-4, designated as B 26-1 to B 26-7 while six fractions were obtained from B 25-5, designated as B 27-1 to B 27-6. After 24 hours of treatments, fractions B

26-3 and B 27-4 showed a significant increase in GSH levels (46.0% and 51.0%, respectively). Fractions B 26-5 (39.9%), B 26-7 (28.7%), B 27-5 (33.8%), and B 27-6 (28.7%) also showed an increase in GSH levels. A decrease in GSH levels was observed in fractions B 26-1 (-2.3%), B 26-6 (-18.7%) and B 27-1 (-2.2%). Other fractions including B 26-2, B 26-4, B 27-2, and B 27-3 showed little to no change in GSH levels (Figure 8). Overall, the relative increase in GSH levels was lower than the level observed in the first round of HPLC/ISCO (I.e., B 25-4 and B 25-5) (Figure 7), suggesting that further purification of the active fractions B 25-4 and B 25-5 resulted in the spreading of the activity to multiple fractions.

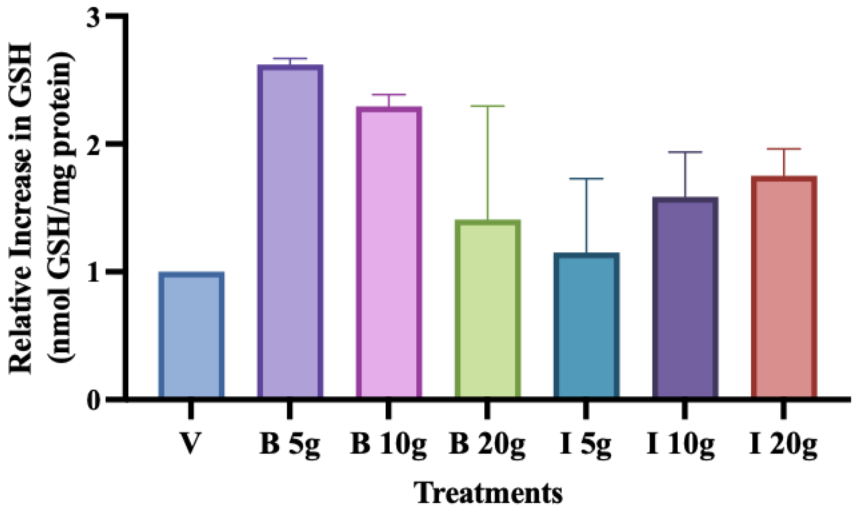
Figure 8. Relative Increase in GSH of THP-1 Monocytes Treated with Purified Brown Tef Grain Fractions B 26-1 to B 26-7 and B 27-1 to B 27-6: 24 Hours



Relative Increase in GSH. THP-1 monocytes were treated with 50 ug/mL purified brown tef extract fractions B 26-1 to B 26-7 and B 27-1 to B 27-6 for 24 hours. After 24 hours, the total protein was extracted to determine the level of GSH. V, vehicle DMSO treated (negative control), B, brown, I, ivory. Bars represent the mean and SD of three independent experiments. *$p<0.05$.

To determine the minimum amount of tef seeds used for extraction, we prepared extracts from 5, 10, and 20 g brown and ivory seeds. The THP-2 cells were incubated with 50 ug/mL brown and ivory crude organic fractions. After 24 hours, the protein concentration and GSH level were quantified. We observed increased GSH levels with decreased starting materials for the brown seeds (Figure 9). Conversely, increasing the amount of starting material for the ivory tef increases the GSH levels. This data provides evidence that the brown seed extracts have more antioxidant property than the ivory seeds.

Figure 9. Relative Increase in GSH of THP-1 Monocytes Treated with 50 ug/mL Brown and Ivory Tef Crude Extracts from Varying Grain Weight: 24 Hours



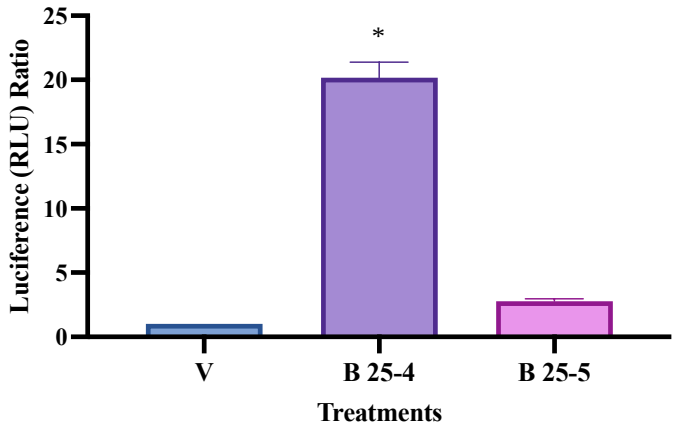
Relative Increase in GSH. THP-1 monocytes were treated with 50 ug/mL brown and ivory crude tef extracts for 24 hours. Brown and ivory tef grain were extracted from 20, 10, and 5 g. After 24 hours, the total protein was extracted to determine the level of GSH. V, vehicle DMSO treated (negative control), B, brown, I, ivory. Bars represent the mean and SD of three independent experiments.

Activation of Nrf2 and NF-κB Signaling Pathways by Tex Grain Extract

The signaling mechanism of the observed increase in GSH level after treatment within the THP-1 monocytes is known. As presented in earlier section, our studies showed that tef grain extracts increased GSH level in THP-1 monocytes (Figures 6 and 7), suggesting that tef grains have phytochemical that have antioxidant properties. To investigate which signaling pathways are involved in the increase GSH levels, we treated Nrf2/ARE and NF-κB reporter cell lines with 50 ug/mL active brown tef fractions B 25-4 and B 25-5 for 24 hours, and the luciferase activity was ratio was quantified.

As shown in Figure 10, we observed significant activation (20-fold) of the Nrf2/ARE signaling pathway after 24 hours of incubation with fraction B 25-4. Whereas fraction B 25-5 showed a slight increase in activation (Figure 10). Increased activation of the Nrf2/ARE antioxidant signaling pathway by tef extracts may explain the relative increase in GSH levels from the GSH assays.

Figure 10. Nrf2 Luciferase Reporter Cell Line

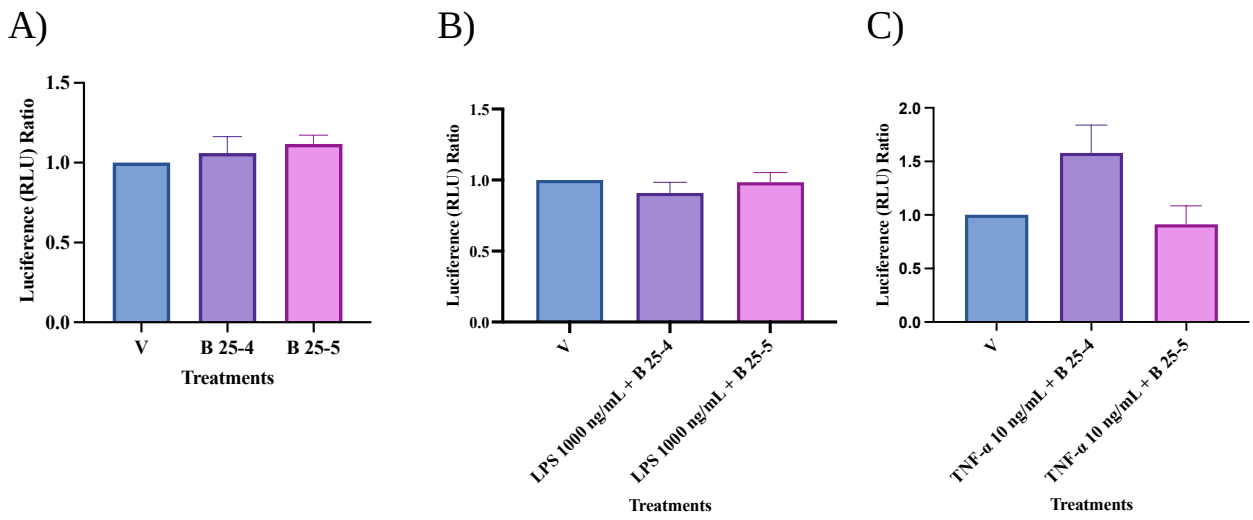


Nrf2 Luciferase Reporter Cell Line. Nrf2 cells were treated with 50 ug/mL brown tef grain fractions B 25-4 and B 25-5 for 24 hours. Lysis buffer and luciferase substrate were added.

The luciferase assay was conducted to measure RLU of tef extract on Nrf2-induced activation. Bars represents the mean and SE of three independent experiments. *p<0.05.

Tef extracts alone did not activate the NF-κB proinflammatory signaling pathway (Figure 11A). In the presence of the lipopolysaccharides (LPS) which is a known activators of NF-κB translocation to the nucleus, tef extracts did not activate the NF-κB signaling pathway, even at high concentrations (Figure 11B). In the presence of the tumor necrosis factor alpha (TNF-α), which is an inflammatory cytokine and an activator of the NF-κB signaling pathway, no significant increase in NF-κB activity were observed when co-treated with tef extracts (Figure 11C).

Figure 11. NF-κB Luciferase Reporter Cell Line



NF-κB Luciferase Reporter Cell Line. A) NF-κB reporter cells were treated with 50 ug/mL B 25-4 and B 25-5, B) 1,000 ng/mL LPS with or without 50 ug/mL brown tef grain fractions B 25-4 and B 25-5 and C) 10 ng/mL TNF-α with or without 50 ug/mL brown tef fractions B 25-4 and B 25-5, for 24 hours. Lysis buffer and luciferase substrate were added. The

luciferase assay was conducted to measure RLU of tef extract on TNF-α-induced inflammation. Bars represents the mean and SE of three independent experiments.

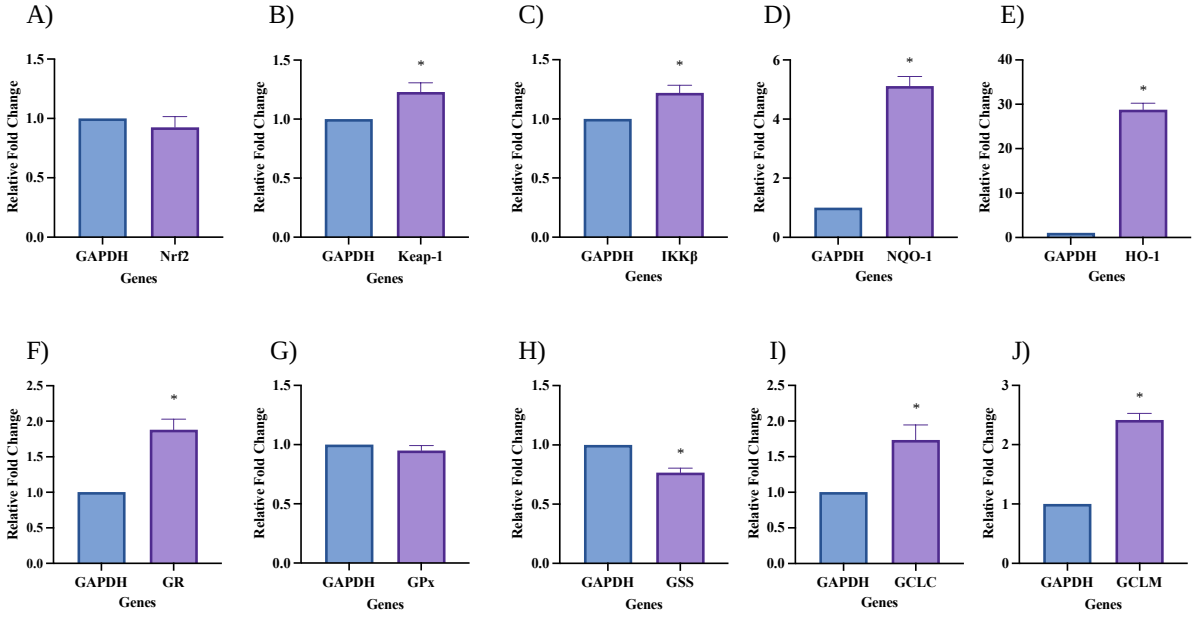
Expression Analysis of Nrf2 and NF-κB Regulated Genes

As mentioned previously, GSH is accumulated in the cells in three ways. The first involves two enzymes GCL (composed of two subunits GCLC and GCLM) and glutathione synthase, the second involves glutathione reductase and NADPH, and the third is through recycling of cysteine by GGTP. The Nrf2 via the antioxidant response element (ARE) and NF-κB are key transcription factors that regulates the expression of these genes. In this study, we analyzed the expression of selected Nrf2 (Nrf2, NQO-1, HO-1, glutathione reductase, glutathione synthase, glutathione peroxidase, GCLC, GCLM) and NF-κB (Keap1, IKKβ) regulated genes.

We observed that tef grain extracts increase the activation of the Nrf2 signaling pathway based on the luciferase activity in the Nrf2/ARE reporter cell line. Similarly, the extracts mildly activate the NF-κB signaling pathway in the NF-κB reporter cell line in the presence of cytokine TNF-α. We treated THP-1 monocytes with the brown fraction B 25-4 for 24 hours to determine whether the extract modulates the expression of genes involved in the Nrf2 and NF-κB signaling pathways. The THP-1 monocytes were incubated with 50 ug/mL active brown tef fraction B 25-4 for 24 hours and total RNAs were extracted, and gene expression was analyzed using qPCR. The qPCR data was analyzed following the delta delta Ct method (Livak & Schmittgen, 2001), ANOVA, and the Tukey's multiple comparisons was performed to assess the significance differences between the control and the extract treated groups.

As shown in Figure 12, active tef extract fraction B 25-4 significantly increased the level of Keap-1, IKKβ, NQO-1, HO-1, GR, GSS, GCLC, and GCLM, as compared to the control V. Tef extract increased the NQO-1 transcript level by over 5.12-folds, and HO-1 transcript level by over 28.79-fold (Figures 12D& 12E). The transcript of Nrf2 involved in Nrf2 antioxidant pathway showed a slight decrease by 0.07-fold when treated with B 25-4 (Figure 12A). The transcript of its inhibitor, Keap-1, showed a slight increase, by 1.23-fold (Figure 12B). Gene involved in the NF-κB proinflammatory pathway, IKKβ, inhibitor of NF- κB, showed a slight increase, by 1.22-fold (Figure 12C). The transcript of glutathione reductase (GR) increased by 1.88-fold (Figure 12F). Transcripts of GSH peroxidase (GSS) showed a slight decrease (0.95-fold) (Figure 12G). Transcripts of genes involved in GSH biosynthesis including GCLC and GCLM showed an increase by 1.74- and 2.42-fold, respectively (Figures 12I & 12J). Transcript levels of GSH synthetase (GSS) which is another gene also involved in GSH biosynthesis showed a decrease by 0.77-fold (Figure 12H).

Figure 12. Relative Fold Change of Nrf2 and NF-κB Regulated Genes

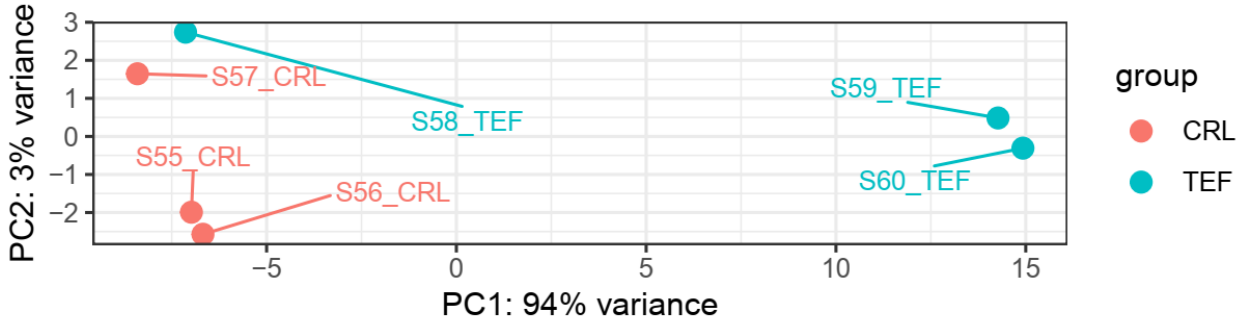


Relative Fold Change of Nrf2 and NF-κB Regulated Genes. THP-1 monocytes were treated with 50 ug/mL brown tef grain fraction B 25-4 for 24 hours. After 24 hours, the RNA was isolated, treated with DNase, synthesized into cDNA, and amplified using qPCR. GAPDH was used as the housekeeping gene. A) Nrf2, B) Keap-1, C) IKKβ, D) NQO-1, E) HO-1, F) GR (glutathione reductase), G) GPx (glutathione peroxidase), H) GSS (glutathione synthetase), I) GCLC, and J) GCLM. Bars represent the mean and SE of three independent experiments. *p<0.05.

Transcriptome Analysis of THP-1 Monocytes Treated with Tef Grain Extract

We performed transcriptome analysis of control THP-1 and those treated with tef extracts. Principal component analysis (PCA) was conducted to determine the uniformity among the samples. As shown in Figure 13, PCA1 explains about 94% of the variation between libraries of the two treatment groups while PCA2 explained only about 3% of the variation. Libraries from cells treated with the active brown tef fraction B 25-4 have a different transcriptome profile as compared to the control (V), except for one of the treatments, S58_TEF (Figure 13), which could be due to experimental artifacts. There is not much variation between libraries prepared from control untreated cells.

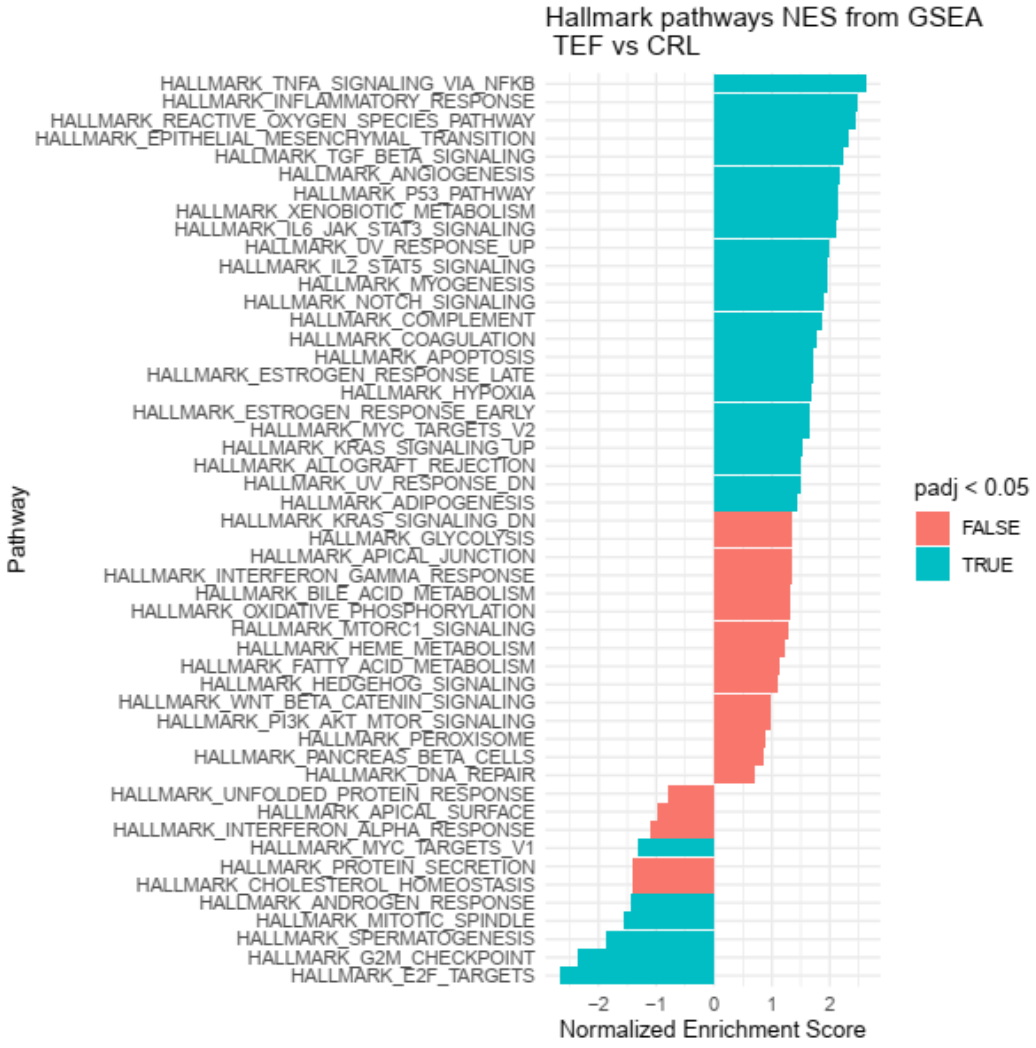
Figure 13. Principal Component Analysis (PCA) Plot



Principal Component Analysis (PCA) plot. PCA illustrating variations between libraries from control and brown tef fraction B 25-4 treated THP-1 monocytes. THP-1 monocytes were treated with 50 ug/mL brown tef fraction B 25-4 for 24 hours. After 24 hours, the RNA was isolated, treated with DNase, and a DNA library was generated. The samples were pooled and sent for sequencing.

To determine the enrichment of different signaling pathways by tef extracts, we subsequently implemented the GSEA analysis to normalized counts of all genes expressed in the 50 ug/mL B 25-4 tef extract treatment compared to those expressed in the controls using the Hallmark database. The analysis showed 24 gene sets that were significantly enriched in the THP-1 monocytes treated with B 25-4 and 6 gene sets that were significantly enriched in the control. According to the GSEA, the TNF-α signaling via NF-κB, the inflammatory response, and the reactive oxygen species (ROS) pathways are the top three enriched pathways. Our luciferase assay data support the activation of the NF-κB proinflammatory signaling pathway. Inflammatory response signaling pathways includes the NF-κB signaling pathway. Other notable enriched pathways include the epithelial mesenchymal transition pathway (EMT), the TGFβ, the angiogenesis, and the p53 signaling pathways.

Figure 14. Hallmark Pathways NES from Gene Set Enrichment Analysis (GSEA)

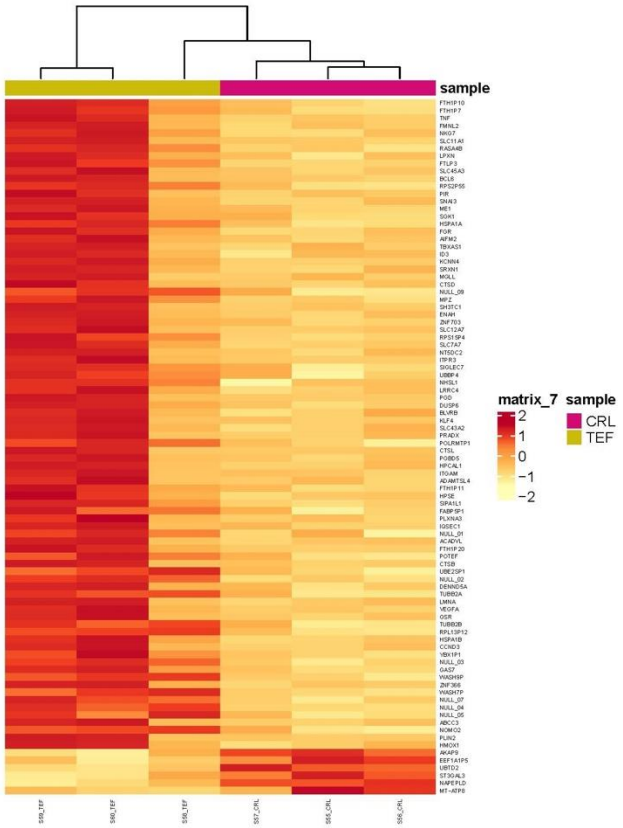


Gene Set Enrichment Analysis (GSEA) results of 50 ug/mL brown tef B 25-4 vs. V comparisons. RNA-Seq was performed on samples collected after 24 hours incubation of THP-1 monocytes. Results of GSEA Hallmark analysis showing enriched gene sets. Bars in blue indicate significant enrichment at p-value<0.05, bars in orange represent gene sets with p-value>0.05.

Figure 15 shows a heat map of 92 differentially regulated genes. Expression of several gene families were upregulated by tef B 25-4 treatment including TNF, GSR, ferritin heavy chain pseudogenes (FTH1P), solute carrier (SLC) gene family, zinc finger proteins (ZNF), and

heat shock proteins (HSP). TNF-α is a key regulator of inflammatory response. GSR maintain the reduced form of GSH. Ferritins are iron storage proteins that play a role in the delivery of irons to the cells and mediate iron uptakes. FTH1 pseudogenes (FTH1P10, FTH1P7, FTH1P11, FTH1P20) may have regulatory effect on the synthesis of the ferritin. Solute carrier genes include SLC11A1, SLC45A3, SLC12A7, SLC7A7, and SLC43A2. Overexpression of SLC22A10 and SLC22A15 has been shown to increase GSH transport (Nayak et al., 2022). ZNF703 and ZNF has been implicated in promoting tumor growth and other cancer related processes (Jen & Wang, 2016). HSPs are essential in maintain cellular homeostasis and ensuring the proper functioning of proteins. The primary role of HSP is to protect cells from environmental and physiological stresses (Stahelin, 2022). Another notable gene highly expressed is heme oxygenase 1 (HMOX1). HMOX1 encodes the HO-1 enzyme. GSR gene encodes the GR enzyme, which could also play a role in the increased GSH levels observed from the GSH assays (Figure 15). Gene families MT-ATP8, NAPEPLD, AKAP91P5, EEF1A, UBTD2, and ST3GAL3 were downregulated by tef extracts.

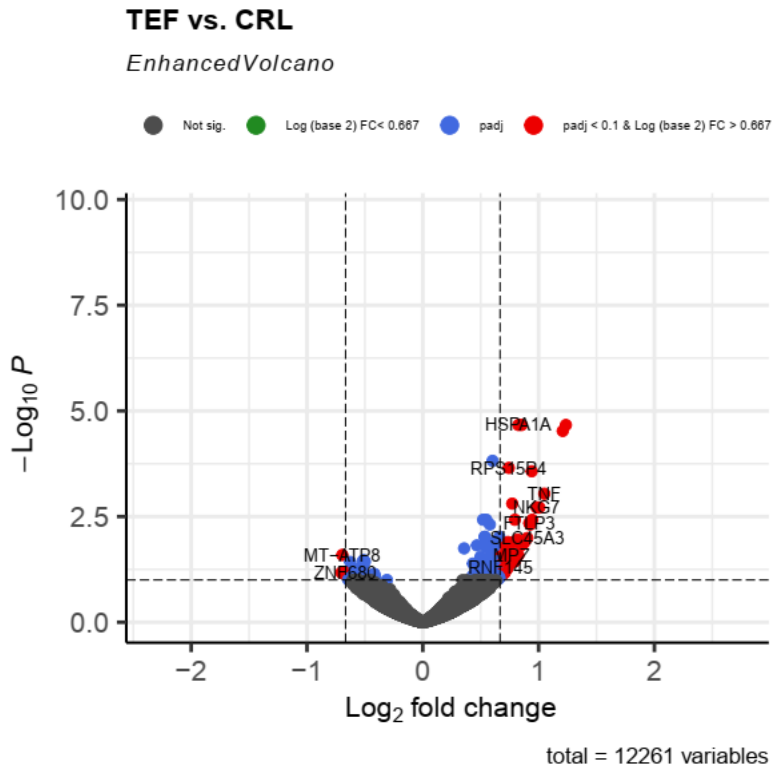
Figure 15. Heatmap of Differentially Regulated Genes



Heatmap of Differentially Regulated Genes. Heat map of the top 92 marker genes for the comparison of THP-1 monocytes treated with brown tef fraction B 25-4 (left column) and the V (right column). Expression values are represented as colors and range from red (high expression) to orange (moderate), and yellow (lowest expression), on sample level.

The enhanced volcano plot examines the magnitude of change (fold change) in gene expressions of all genes, 12,261. The top three genes that had the most significant fold change are heat shock protein A1A (HSPA1A), ribosomal protein S15 pseudogene 4 (RPS15P4), and tumor necrosis factor (TNF) (Figure 16).

Figure 16. Enhanced Volcano Plot of Differentially Expressed Genes (DEGs)

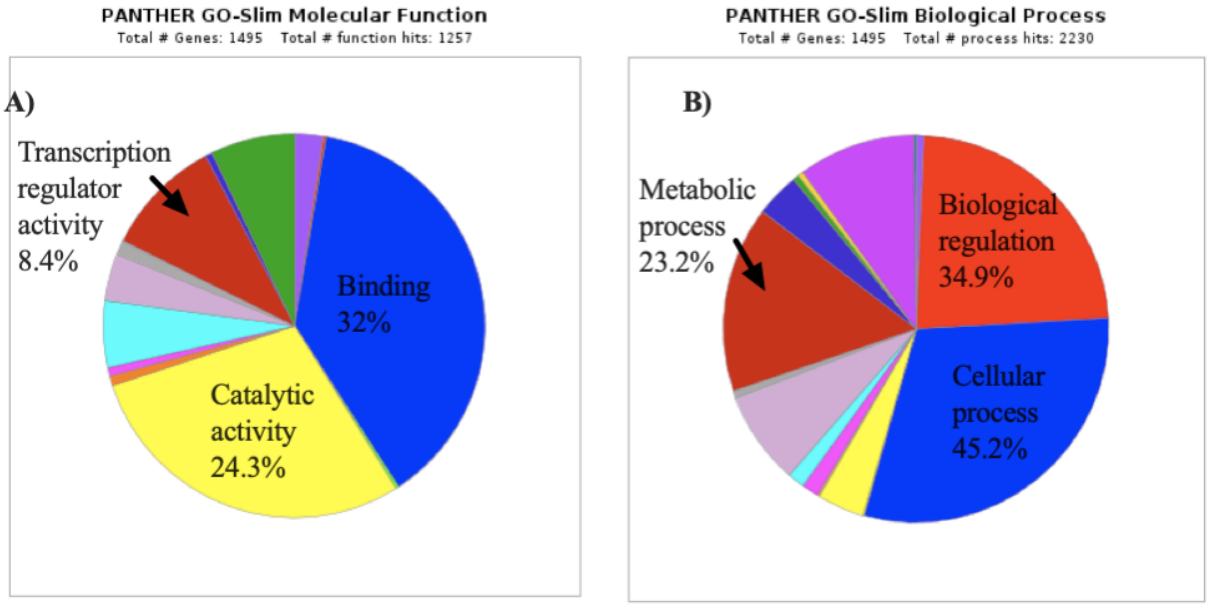


Enhanced Volcano Plot of Differentially Expressed Genes (DEGs). Fold changes of gene expression were plotted. Significant DEGs were identified by >four-fold change (log2 FC > 2) and <0.05 FDR, and are shown in red.

Statistically significant ($p<0.05$) upregulated genes were subjected to the PANTHER classification system using the RNA-Seq data. Functional enrichment analysis was classified into two categories, molecular function, and biological process. The analysis using the PANTHER classification system identified 13 molecular functions positively enriched in the active brown tef fraction B 25-4 treated THP-1 monocytes. Most of the processes were related to binding (32.0%), catalytic activity (24.3%), and transcription regulator activity (8.4%). Binding includes NF-κB binding and upregulation of the I-kappaB kinase/NF-κB signaling pathway, oxidative stress response, regulation of protein phosphorylation, and regulation of lymphocyte activation.

As shown in Figure 17, the top 3 components of biological processes are cellular process (45.2%), biological regulation (34.9%), and metabolic process (23.2%). Detoxification accounts for 0.10%. Tef extracts may mediate various regulatory processes of post-transcription, which then affect other biological functions.

Figure 17. PANTHER Classification System of Functional Enrichment Analysis



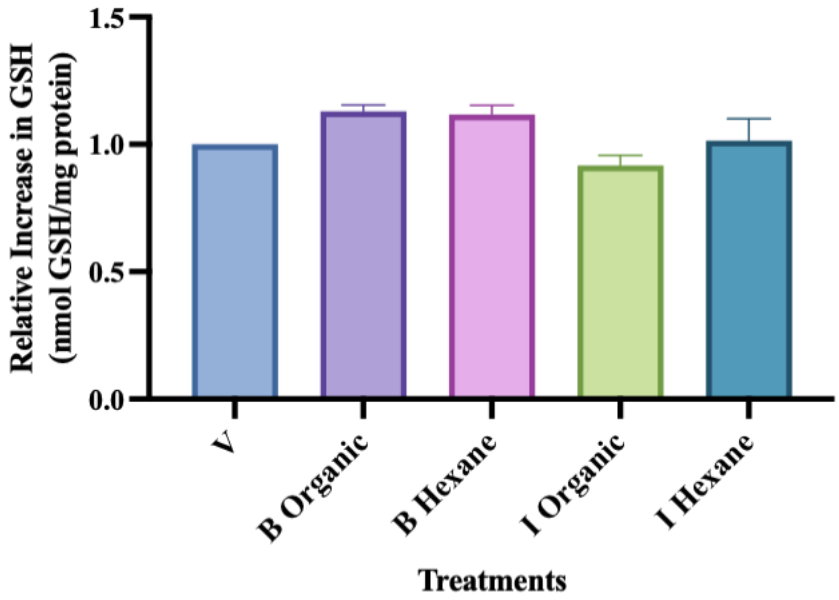
PANTHER Classification System of Functional Enrichment Analysis. Functional enrichment analysis of 1782 statistically significant, $p<0.05$, upregulated genes in THP-1 monocytes between the brown tef fraction B 25-4 treated and the V. The analysis was performed using RNA-Seq data and the PANTHER classification system. A) Molecular functions, and B) biological process enriched in the comparison of tef extract B 25-4 treatment and the V.

Tef Biomass Extract Have No Antioxidant Properties

Studies have shown that extracts from aerial parts of tef possess antioxidant activity using free radical assays. To evaluate the antioxidant properties of tef biomass, we treated THP-1 monocytes with 50 ug/mL brown and ivory crude fractions, organic and hexane. In previous

GSH assays, the organic and hexane fractions displayed the greatest increase in GSH levels. After 24 hours, the protein concentration and GSH levels were quantified. Cell viability showed no change across all treatments. The I organic fraction showed a slight decrease in GSH levels. Overall, we observed no significant increase in GSH levels in THP-1 monocytes treated with brown and ivory biomass extracts (Figure 18).

Figure 18. Relative Increase in GSH of THP-1 Monocytes Treated with Crude Tef Biomass Extracts: 24 Hours



Relative Increase in GSH. THP-1 monocytes were treated with 50 ug/mL brown and ivory crude tef biomass extracts for 24 hours. After 24 hours, the total protein was extracted to determine the level of GSH. V, vehicle DMSO treated (negative control), B, brown, I, ivory. Bars represent the mean and SD of three independent experiments.

CHAPTER IV: DISCUSSION

This study examined the antioxidant properties of tef extracts in THP-1 monocytes. THP-1 cell line is widely used for studying macrophage-related physiological processes. Several studies on the antioxidant properties of tef extracts reported the antioxidant and neuroprotective properties of the extracts (Na et al., 2017). Nonetheless, the mechanism is not fully understood. Moreover, human cell models are needed to evaluate the effectiveness of such claims. The present study provided several lines of evidence on the antioxidant properties of tef phytochemicals in THP-1 monocytes. All performed studies indicate that tef extracts, particularly the brown organic fraction, contain phytochemicals that have antioxidative properties.

A balance between ROS production and the antioxidant defense is needed for proper physiological function. Excessive ROS can induce oxidative stress which has been linked to several pathologies. It is linked to the formation of many degenerative diseases (Poljsak et la., 2013). Glutathione (GSH) is a powerful antioxidant and often referred to as the master antioxidant. GSH is the most abundant thiol that defends against oxidative stress. Cells have antioxidant and anti-inflammatory defense mechanisms.

We demonstrated the antioxidant properties of tef phytochemicals based the increase GSH levels in THP-1 monocytes after 24 hours through activation of the antioxidant Nrf2/ARE signaling pathway and the proinflammatory NF-κB signaling pathway. All cell treatments were performed with 90% confluency or higher. Luciferase assays were conducted using Nrf2/ARE and NF-κB stably transfected cell line. The response of stably transfected reporter cell line is highly specific. (Lai et al., 2006). Nrf2 and NF-κB activation were evaluated assessing their nuclear translocation. These data indicated both the antioxidant and proinflammatory signaling pathways are involved in the observed increase antioxidant levels. This study may provide

understanding on the molecular action of tef phytochemicals, which is important in the treatment and prevention of certain diseases. In addition to activating the Nrf2 signaling pathway, other pathways might be involved. Antioxidant signaling pathways involving GSH will need to be analyzed. Further separation and purification of the active extract showed reduced antioxidant levels (Figure 8), possibly due to the fact that it is not a pure compound, or that the actives might have been separated. The increase in GSH levels can be attributed to more than one compound working together. The active fraction needed more investigation to identify the major active compounds and to demonstrate their mode of action.

The cellular GSH level in THP-1 monocytes did not increase when treated with tef biomass extracts (Figure 18). It is possible that the antioxidant activity of the biomass extracts is variable among cell lines. Further study will need to analyze GSH levels in relevant cell lines, such as the BAEC cell line. Tef biomasses were collected before flowering. It has been established that the plant's mineral and digestible constituents are at highest levels during the succulent growth stage and decline after flowering. However, secondary metabolites such as phenols which have antioxidant activities are accumulated at later developmental stages, and their concentrations might be lower at flowering. Therefore, there is a need to harvest the biomass at different developmental stages and determine the antioxidant properties.

Our study using the Nrf2 and NF-kB luciferase reporter cell lines revealed that tef extracts activate the Nrf2 and NF-kB signaling (Figure 10 & 11), suggesting that the extracts primarily affect the antioxidant pathway. Activation of the NF-kB pathway was observed only in the presence of the pro-inflammatory TNF-a, not the LPS even at high concentrations. A recent study has shown that NF-κB activation can have anti and pro-oxidant effects (Lingappan, 2018). Low doses of ROS have been shown to activate cell survival signaling pathways, including the

Nrf2 antioxidant signaling pathway. The activation of the ROS signaling pathway might explain the downstream activation of the Nrf2 signaling pathway, and ultimately the increase in GSH levels observed. Although activation of the NF-κB increases TNFα-induced ROS production, cancer research studies have shown that increased intracellular ROS can promote cancer cell cycle arrest (Liou & Storzz, 2014). The EMT pathway is a hallmark for cancer, and the TGFβ, the angiogenesis, and the p53 signaling pathways are involved in cancer progression. Recent findings suggest the importance of zinc finger protein in cancer onset and progression. The GSEA enrichment analysis showed that the E2F targets, G2M checkpoint, and the spermatogenesis signaling pathways are the top 3 signaling pathways significantly enriched in the control (Figure 14). Through these results, it was suggested that tef extracts increase GSH levels through their role as up-regulator of the Nrf2 and NF-κB pathways.

To further investigate the expression of genes in the THP-1 monocytes treated with active brown tef fraction B 25-4, we performed qPCR and RNA-seq. Based on the qPCR result, we did not observe an increase in Nrf2 and Keap-1 gene expression by the tef extracts, suggesting that activation of both genes may not depend on transcript abundance, but rather on posttranscriptional protein activation such as protein phosphorylation. It is known that one of the ways Nrf2 is activated is through dissociation from Keap-1. The expression of IKKB gene was also not increase by the active brown tef fraction B 25-4. IKKβ is the inhibitor of NF-κB.

It has been shown that Nrf2 activation induces gene expression of GCLC and GCLM (Zhang et al., 2017). We observed increase in gene expressions of GCLC and GCLM. Other genes related to GSH biosynthesis such as cysteine and sulfur amino acid precursor might be involved. Cryoprotective genes, HO-1 and NQO-1, showed significant increase in transcript levels. Nrf2 activation is known to induce HO-1 and NQO-1 (Zou et al., 2012). Gene expression

of glutathione reductase also increased slightly. Overall, our data support the enhancement and induction of Nrf2 target genes (GCLC, GCLM, HO-1, NQO-1) and reduction of GSH from GSSG through glutathione reductase.

Our transcriptome analysis using RNA-seq showed that controls and two of the tef treated samples have different transcriptome profiles, however, the transcriptome profile of one of the treated samples S58_TEF was similar with the control samples which could be due to experimental artifacts. Tef extracts enriched several pathways including the TNF-α signaling via NF-κB, the inflammatory response, and the reactive oxygen species (ROS) pathways. The ferritin heavy chain pseudogenes FTH1P10, FTH1P7, and TNF were top in the list of genes upregulated by the tef extracts. Pseudogenes are usually non-coding genes but may have a regulatory role in ferritin biosynthesis and may play a role in the delivery of iron to the cells. Tef has been shown to increase cellular ferritin, which is an established marker of iron bioavailability, when compared to other cereals wheat and rice (Ligaba-Osena et al. 2021) and has a potential to treat iron-deficient conditions (Habte al., 2022). High iron in tef has been shown to prevent and treat iron-deficient anemia (Irge, 2017).

Heme oxygenase 1 (HO-1) is critical in heme catabolism. It also functions in immune regulation and protection of cells and tissues from various stressors. Increased expression of HMOX1 might explain the significant increase in the transcript level of HO-1 and slight increase in GR in the qPCR data. Based on the enhanced volcano plot, heat shock protein A1A (HSPA1A), ribosomal protein S15 pseudogene 4 (RPS15P4), and tumor necrosis factor (TNF), showed the greatest fold change in gene expression when treated with tef extract. It has been reported that heat shock proteins regulate cellular redox status. The GSH is an indicator of the cellular redox environment. Upregulation of HSPA1A might contribute to the increased GSH

levels observed in the GSH assay. The RPS15P4 is a protein-coding gene. Activation of (RPS15P4) has been linked to various tumors and cancers. It is not clear why the tef extract increase the RPS15P4, but because the tef extracts contain many compounds, we cannot rule out that some compounds may increase the expression of tumor-related genes. The TNF encodes proinflammatory cytokines. Cytokines are mainly secreted by macrophages and can induce ROS (Chen et al., 2017; Lo et al., 1996). Increased levels of ROS might possibly contribute to the activation of the Nrf2 signaling pathway. Upregulation of TNF further supports the activation of the NF-κB luciferase assay data. High expression of TNF might explain why co-treatment of TNF-α and tef extract B 25-4 further increase activation of the NF-κB signaling pathway, as compared to NTF-α alone, in the luciferase assay. An increase in gene expression levels of GCLC and GCLM from qPCR data might explain the increase in GSH levels observed in the GSH assays. PANTHER classification analysis revealed that tef extracts may mediate various regulatory processes of post-transcription, which then affect other biological functions. Further study is needed to fully understand the connection between antioxidant properties of the tef extract and on antioxidant and proinflammatory genes.

CHAPTER V: SUMMARY

In this study, we found that tef extracts do not have cytotoxic effects. The grain extracts, especially from a brown variety increase cellular GSH levels in THP-1 monocytes. Using cell lines stably expressing a luciferase gene, our findings provided an insight into the molecular mechanisms that regulate increased GSH levels in THP-1 monocytes treated with tef grain extracts. Tef extracts primarily trigger the Nrf2 signaling, and mildly the NF-κB signaling pathways only in the presence of the proinflammatory cytokine TNF-α. This suggests that tef phytochemicals primarily regulate the antioxidant pathway. Gene expression analysis using qPCR and RNA-seq revealed that genes belonging to several families were upregulated by the tef extracts including TNF, GSR, ferritin heavy chain pseudogenes (FTH1P), solute carrier (SLC) gene family, zinc finger proteins (ZNF), and heat shock proteins (HSP), while the MT-ATP8, NAPEPLD, AKAP91P5, EEF1A, UBTD2, and ST3GAL3 were downregulated by tef extracts. However, further study is needed to understand the role of these gene families in the observed increase in GSH level. We found that the extracts used in our study contain a large number of compounds, and there is a need to further purify the extracts to identify the specific phytochemicals that showed antioxidant activity in the THP-1 monocytes.

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APPENDIX A: TABLES

Table A1. Primers Used in qPCR for Gene Expression Analysis

Target Human Gene	Forward Primer	Reverse Primer
GAPDH	5’- CGACCACTTTGTCAAGCTCA-3’	5’-AGGGGTCTACATGGCAACTG-3’
Nrf2	5’- CCCAGTGTGGCATCACCAGAAC-3’	5’- GCATCATGCACGTGAGTGCTC-3’
NQO-1	5’- TTACTATGGGATGGGGTCCA-3’	5’- TCTCCCATTTTTCAGGCAAC-3’
HO-1	5’- TCCGATGGGTCCTTACACTC-3’	5’- TAAGGAAGCCAGCCAAGAGA-3’
GR	5’- CAGTGGGACTCACGGAAGAT-3’	5’- AAACCCTGCAGCATTTCATC-3’
GSS	5’- AGCCAATGCTCTGGTGCTAC-3’	5’- ACCTTCGACGGATTACATGG-3’
GPx	5’- GTGCTCGGCTTCCCGTGCAAC-3’	5’- CTCGAAGAGCATGAAGTTGGGC-3’
GCLC	5’- ACCATCATCAATGGGAAGGA-3’	5’- GCGATAAACTCCCTCATCCA-3’
GCLM	5’- CTCCCTCTCGGGTCTCTCTC-3’	5’- ATCATGAAGCTCCTCGCTGT-3’
Keap-1	5’- CTGCACAACTGTATCTATGCTGCTGG-3’	5’- CGTAGATTCTCCCCTGGTGGACA-3’
IKKβ	5’- AACCAGCATCCAGATTGACC-3’	5’- CTCTAGGTCGTCCAGCGTTC-3’