ANTIOXIDANT AND INSULIN-LIKE PROPERTIES OF EXTRACTS FROM WHEAT GRAINS FERMENTED BY SELECTED INDIGENOUS *GANODERMA* SPP.

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FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
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ABSTRACT

Ganoderma are cosmopolitan wood decaying white rot basidiomycete fungi, which have been used for centuries for medicinal purposes, particularly in China, Japan and Korea. It is believed to possess many health benefits and has been reported to be effective against various diseases by virtue of their antioxidant potential. In the present study, solid-substrate fermentation of wheat grains with indigenous *Ganoderma* spp. selected based on ethnomycological knowledge was carried out. The species included *Ganoderma australe* (Fr.) Pat. (KUM60813), *Ganoderma neo-japonicum* Imazeki (KUM61076) and *Ganoderma lucidum* (Fr.) Karst. (*VITA GL*) (a commercial strain). Antioxidant activities of the crude aqueous and ethanol extracts of the unfermented and fermented wheat grains were investigated by the ferric reducing antioxidant power (FRAP), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity, diphenyl-1-picryl-hydrazyl (DPPH) free radical scavenging ability and lipid peroxidation assays. Among the six mycelia extracts tested, the ethanol extract from wheat fermented with KUM61076 mycelia showed the most potent antioxidant activities whereas, the ethanol extract of wheat grains fermented with KUM60813 mycelia had good potential to protect palm oil (food grade) against oxidation. Total phenol content (TPC) in the ethanol extracts were higher than that in the aqueous extracts. The antioxidant activities of the mycelia extracts had a positive correlation with their polyphenol contents. These phenolic compounds may play a vital role in the antioxidant activities of the selected *Ganoderma* spp. The present study also evaluated the insulin-like properties of extracts of unfermented and fermented wheat grains through lipogenic, lipolytic and adipokine expression. 3T3-L1 adipocytes treated with ethanol extract of wheat grains fermented with KUM61076 mycelia, significantly stimulated lipogenesis (in the absence of insulin) and exerted relatively mild anti-

adrenaline induced lipolytic activities. In general, the expressions of target genes such as adiponectin, peroxisome proliferator-activated receptor gamma (PPARγ), glucose transporter 4 (GLUT4) and hormone sensitive lipase (HSL) were up-regulated by the ethanol extract of wheat grains fermented with *Ganoderma* spp. mycelia. However, both the aqueous and ethanol extracts of unfermented and fermented wheat grains demonstrated a dose-dependent inhibition of preadipocyte differentiation and reduced the expression level of adiponectin in adipocytes when the cells were subjected to oxidative stress. In conclusion, these results suggest that wheat grains fermented with *Ganoderma* spp. especially KUM60813 and KUM61076 have good antioxidant and insulin-like properties which may potentially serve as a therapeutic agent in the management of diabetes. To our knowledge this is the first report on antioxidant and insulin-like properties of extracts from wheat grains fermented by *Ganoderma* spp. mycelia.

ABSTRAK

Ganoderma merupakan sejenis kulat basidiomiset reput putih yang mereputkan kayu dan digunakan selama berabad-abad dalam perubatan tradisional, terutamanya di Negara Cina, Jepun dan Korea. Ia dipercayai mempunyai banyak manfaat kesihatan dan telah dilaporkan berkesan terhadap pelbagai penyakit kerana potensi antioksidannya. Dalam kajian ini, penapaian substrat pepejal telah dijalankan menggunakan bijirin gandum oleh spesies *Ganoderma* pribumi yang dipilih berdasarkan pengetahuan etnomikologi. Spesies *Ganoderma* yang dikaji adalah *Ganoderma australe* (Fr.) Pat. (KUM60813), *Ganoderma neo-japonicum* Imazeki (KUM61076) dan *Ganoderma lucidum* (Fr.) Karst. (*VITA GL*) (baka komersial). Aktiviti antioksidan ekstrak mentah air dan etanol bijirin gandum yang tidak ditapai dan ditapai dengan spesies *Ganoderma* telah dikaji dengan menggunakan kaedah-kaedah berikut: kuasa antioksidan penurunan ferrik (FRAP), aktiviti penyah-radikal '2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)', aktiviti penyah-radikal 'diphenyl-1-picryl-hydrazyl (DPPH)' dan peroksidaan lipid. Antara enam ekstrak miselia yang diuji, ekstrak etanol bijirin gandum yang ditapai dengan miselia KUM61076 telah menunjukkan aktiviti antioksidan yang paling tinggi sedangkan, ekstrak etanol bijirin gandum yang ditapai dengan miselia KUM60813 mempunyai potensi yang baik untuk melindungi minyak sawit (gred makanan) daripada pengoksidaan. Kandungan jumlah fenol (TPC) dalam ekstrak etanol adalah lebih tinggi daripada ekstrak air mentah. Aktiviti antioksidan ekstrak miselia mempunyai hubungkait positif dengan kandungan polifenolnya. Oleh itu, sebatian fenolik boleh memainkan peranan penting dalam menentukan aktiviti antioksidan spesies *Ganoderma* terpilih. Kajian ini telah menilai ciri menyamai insulin bagi ekstrak bijirin gandum yang tidak ditapai dan yang ditapai dengan spesies *Ganoderma* melalui aktiviti lipogenik, anti-lipolitik dan ekspressi adipokin. Apabila 3T3-L1 adiposit dirawat

dengan ekstrak etanol bijirin gandum ditapai dengan miselia KUM61076, lipogenesis ketara (dalam ketiadaan insulin) dan aktiviti lipolitik anti-adrenalin sederhana dirangsang. Secara umum, tahap ekspressi gen-gen sasaran seperti adiponektin, ‘peroxisome proliferator-activated receptor gamma (PPARγ)’, ‘glucose transporter 4 (GLUT4)’ dan ‘hormone sensitive lipase (HSL)’ telah dinaik-kawal oleh ekstrak etanol bijirin gandum yang ditapai. Walau bagaimanapun, kedua-dua ekstrak air dan etanol mentah bijirin gandum yang tidak ditapai dan yang ditapai menunjukkan perencatan terhadap pembezaan preadiposit dan mengurangkan tahap ekpressi adiponektin dalam adiposit apabila sel-sel terdedah kepada tekanan oksidatif. Sebagai kesimpulan, keputusan ini menunjukkan bahawa bijirin gandum yang ditapai dengan miselia spesies *Ganoderma* terutamanya KUM60813 dan KUM61076 mempunyai aktiviti antioksidan yang baik dan sifat-sifat menyamai insulin dan berpotensi sebagai agen terapeutik dalam pengurusan penyakit kencing manis. Sepanjang pengetahuan kami, ini adalah laporan pertama mengenai sifat antioksidan dan aktiviti menyamai insulin daripada bijirin gandum yang ditapai oleh miselia spesies *Ganoderma*.

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Thank You.

Sarasvathy Subramaniam

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4-AAP	4-aminoantipyrine
AA	Ascorbic acid
ABTS	2,2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
ADP	Adenosine-5’-diphosphate
AMP	Adenosine-5’-monophosphate
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
aP2	adipocyte Protein 2
ATP	Adenosine-5-triphosphate
Bax	Bcl-2-associated X protein
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BMI	Body mass index
C/EBPα	Ccaat enhancer binding protein-α
cAMP	Cyclic 3’-5’-adenosine monophosphate
CASP3	Caspase 3
CD36	Cluster of Differentiation 36
cDNA	Complementary deoxyribonucleic acid
CoA	Coenzyme A
CT	Cycle threshold
DAP	Dihydroxyacetone phosphate
DM	Diabetes mellitus
DM1	Part-1 differentiation
DM2	Part-2 differentiation

DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide triphosphate
DPPH	Diphenyl-1-picryl-hydrazyl
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESPA	Sodium N-ethyl-N-(3-sulphopropyl) m-anisidine
FBS	Fetal Bovine Serum
FE	$FeSO_4.7H_2O$ equivalent
$FeSO_4.7H_2O$	Ferrous Sulfate Heptahydrate
FFA	Free fatty acids
FRAP	Ferric reducing antioxidant power
g	Gram
g	Gravity
G-1-P	Glycerol-1-phosphate
GA	Gallic acid
GAE	Gallic acid equivalent
GK	Glycerol kinase
GLUT4	Glucose transporter 4
GOx	Glucose oxidase
GPO	Glycerol phosphate oxidase
GPx3	Glutathione peroxidase 3
GYMP	Glucose-yeast-malt-peptone
H_2O_2	Hydrogen peroxide
HSL	Hormone sensitive lipase

IBMX	1-methyl-3-isobutylxanthine
KH_2PO_4	Potassium phosphate monobasic
K_2HPO_4	Potassium phosphate dibasic
LPL	Lipoprotein lipase
MAPK	Mitogen-activated protein kinase
MDI	1-methyl-3-isobutylxanthine-dexamethasone-insulin
$MgSO_4.7H_2O$	Magnesium sulphate heptahydrate
mM	Millimolar
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
Na_2CO_3	Sodium carbonate
NADH	Nicotinamide adenine dinucleotide
NH_4Cl	Ammonium chloride
NHMS	National Health and Morbidity Survey
$O_2\cdot$	Superoxide anion radical
OD	Optical density
$OH\cdot$	Hydroxyl radical
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
POD	Peroxidases
polyA	Poly Adenine
polyT	Poly Thymine
PPARγ	Peroxisome proliferator-activated receptor gamma

RNA	Ribonucleic acid
RNase	Ribonuclease
RNS	Reactive nitrogen species
ROO·	Peroxyl radical
ROS	Reactive oxygen species
ROX™	6-carboxy-X-rhodamine
rRNA	Ribosomal ribonucleic acid
RT	Reverse transcription
RT buffer	Reverse transcriptase buffer
RT-PCR	Reverse transcription polymerase chain reaction
SD	Standard deviation
spp.	Species
SSF	Solid substrate fermentation
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
TEAC	Trolox equivalent antioxidant capacity
THBQ	Tert-butylhydroquinone
TNF-α	Tumor necrosis factor-alpha
TPC	Total phenolic content
TPTZ	Tripyridyltriazine
TZD	Thiazolidinedione
VDAC	Voltage-dependent anion channel
VLDL	Very low density lipoproteins
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

Diabetes mellitus (DM) has become one of the most common public health disorders, throughout the world. Diabetes mellitus can be described as a complex metabolic disorder caused by an inherited or acquired deficiency in insulin secretion, insulin action or both (Nathan *et al*., 2009). Diabetes mellitus is characterized by elevated blood glucose level (hyperglycemia), insulin resistance, autoimmune destruction of pancreatic β-cell and the symptoms include thirst, polyuria, blurring of vision, and weight loss (American Diabetes Association, 2013). There are three major forms of DM: Type 1 DM (Insulin Dependent Diabetes Mellitus), Type 2 DM (Non Insulin Dependent Diabetes Mellitus) and Gestational DM. Among the three major types of DM, Type 2 DM is the most common form of diabetes which contributes to more than 90-95 % of all cases of diabetes worldwide (American Diabetes Association, 2013) and this form of DM is closely associated with adipocyte dysfunction and obesity.

This phenomenon is worrying because Type 2 DM usually occurs in conjunction with other metabolic syndrome including obesity, hypertension and dyslipidemia. Obesity has a strong correlation with Type 2 DM and high prevalence of obesity had been proven to cause epidemic levels of DM. The adipocytes in obese individuals which are enlarged with high concentration of lipid develop resistance to insulin. Thus, understanding the mechanisms involved in the regulation of preadipocyte proliferation, differentiation (lipogenesis), lipolysis as well as uptake of glucose into adipocyte are essential for the treatment of DM as well as obesity (Lim *et al*., 2008).

It is generally agreed that oxidative stress is involved in the pathogenesis of DM, cardiovascular diseases and cancer (Dierckx *et. al.*, 2003). Insulin resistance or lack of insulin secretion diminishes glucose uptake by cells, contributing to high levels of blood glucose (hyperglycemia). Subsequently, hyperglycemia leads to glucose auto-oxidation, non-enzymatic glycation and monocyte dysfunction, which can result in increased reactive oxygen species (ROS) and free radical generation (Dierckx *et. al.*, 2003). The free radicals are able to attack and deteriorate biologically active molecules; thus leading to tissue damage and cell death (Daker *et al*., 2008; Kalyoncu *et al*., 2010). As a result, numerous scientific efforts have been dedicated to discover effective antioxidants to scavenge free radicals (Mohammadi and Yazdanparast, 2009). However, naturally occurring antioxidants may provide many advantages over the synthetic ones because they are assumed to be safer for human consumption. Fruits and vegetables are therefore highly recommended in daily dietary intake as they are rich in antioxidants (Lee *et al*., 2008; Kalyoncu *et al*., 2010). Mushrooms are also known to contain several bioactive compounds with antioxidant properties (Cheung *et al*., 2003). Hence, in this study the extracts of selected *Ganoderma* spp. were screened for their potential antioxidant activities.

Ganoderma is a white rot basidiomycete fungus, which has long been prescribed in the Pacific Rim areas, such as China, Korea, Japan, and other Asian countries for their pharmaceutical, nutraceutical and spiritual properties (Wasser, 2005; Paterson, 2006). Over 250 genus of Ganoderma have been identified and described in scientific literature. However, *Ganoderma lucidum* (Lingzhi or Reishi) and *Ganoderma tsugae* Murrill (Ling Chih) are the most reported on the aspects of cultivation, chemical analysis, pharmacology, and therapeutic effects (Chen *et al*., 2008; Saltarelli *et al*., 2009). In folk medicine, Ganoderma was reported to have numerous therapeutic effects

which include anti-inflammatory, antitumor, antiviral (e.g., anti-HIV), antibacterial and antiparasitic, antidiabetic, blood pressure regulation, reduction in the risk of cardiovascular disorders, immunomodulation, hepato-protection and regulation of chronic bronchitis (Wasser & Weis, 1999; Paterson, 2006).

Ganoderma australe and *Ganoderma neo-japonicum* are also considered as varieties of Ling Zhi, have not been studied extensively, neither for their biochemical composition nor biological activity, such as antioxidant activity and potential insulin-like therapeutic value in the management of hyperglycemia or diabetes. So far, there is only one report (Lin *et al*., 1995) available on the free radical scavenging and antihepatotoxic activity of *G. neo-japonicum* fruiting bodies. *G. australe* has only been explored for its properties of delignification (Elissetche *et al*., 2007), bioremediation of contaminated soil (Rigas *et al*., 2007) and investigation on its genetic diversity (Kaliyaperumal & Kalaichelvan, 2008). This may possibly be due to the difficulty in collecting the tropical wild fruiting bodies as they are rare mushrooms. However, *G. australe* and *G. neo-japonicum* are used by the indigeneous communities in Malaysia for a variety of illness as well as for general wellness (Lee *et al*., 2007). Currently, there is much interest to assess and validate scientifically the medicinal mushrooms based on ethno-mycological knowledge.

The 3T3-L1 preadipocyte cell line is a Swiss albino 3T3 cells derived from a mouse embryo, developed through clonal isolation by Green and Kehinde (1974), was selected for the current study. 3T3-L1 preadipocyte cell line is proven to be an excellent and cost effective model for preliminary screening of various bioactive compounds as potential anti-diabetic and anti-obesity agents; particularly glucose metabolism, as these cells can differentiate from preadipocyte fibroblastic form to adipocyte under appropriate culture conditions (Kohn *et al.*, 1996). 3T3-L1 cells have a fibroblast-like

morphology, but, under appropriate conditions, the cells differentiate into a mature adipocyte-like phenotype (Ito *et al.*, 2009). The uses of adipocyte cellular model also help to minimize the usage of laboratory animals especially for research purpose. Since dietary management is a starting point for the treatment of DM and obesity, it is significant to study the effects of *Ganoderma* spp. extracts on proliferation and differentiation of 3T3-L1 preadipocyte into adipocyte for prevention and reduce the severity of those metabolic syndromes.

1.1 Objective of this study

The aim of this study was to assess the antioxidant and insulin-like properties of extracts of the mycelia biomass of *G. australe* (KUM60813), *G. neo-japonicum* (KUM61076) and *G. lucidum* (*VITA GL)* using the 3T3-L1 preadipocyte cell lines.

The specific objectives were to:

(a) produce mycelia of selected *Ganoderma* spp. via solid substrate fermentation (SSF) and prepare crude aqueous and solvent extracts of unfermented wheat and wheat fermented with *G. australe* (KUM60813), *G. neo-japonicum* (KUM61076) and *G. lucidum* (*VITA GL*).

(b) study the *in vitro* antioxidant potential of the extracts.

(c) evaluate the toxicity and insulin-like activity of the extracts in the absence and presence of oxidative stress using preadipocyte 3T3-L1 cell lines.

CHAPTER 2

LITERATURE REVIEW

2.1 Natural Products

A natural product is a chemical compound or substance produced by a living organism - found in nature that usually has a pharmacological or biological activity for use in pharmaceutical drug discovery and drug design. Not all natural products can be fully synthesized and many natural products have very complex structures that are too difficult and expensive to synthesize on an industrial scale. Such compounds can only be harvested from their natural source - a process which can be tedious, time consuming, and expensive, as well as being wasteful on the natural resource. However, these compounds are important in the treatment of life-threatening conditions (Newman and Cragg, 2007).

Natural products may be extracted from tissues of terrestrial plants, marine organisms, venoms and toxins, or microorganism fermentation broths. A crude (untreated) extract from any one of these sources typically contains a novel, structurally diverse chemical compounds, which the natural environment is a rich source of. Chemical diversity in nature is based on biological and geographical diversity, so researchers travel around the world obtaining samples to analyze and evaluate drug discovery screens or bioassays. This effort to search for natural products is known as bioprospecting.

Pharmacognosy provides the tools to identify, select and process natural products destined for medicinal use. Usually, the natural product compound has some form of biological activity and that compound is known as the active principle - such a structure can act as a lead compound (not to be confused with compounds containing the lead element). Many of today's medicines are obtained *directly* from a natural source. On the other hand, some medicines are developed from a lead compound originally obtained from a natural source. This means the lead compound:

- can be produced by total synthesis, or
- can be a starting point (precursor) for a semi-synthetic compound, or
- can act as a template for a structurally different total synthetic compound.

This is because most biologically active natural product compounds are secondary metabolites with very complex structures. This has an advantage in that they are extremely novel compounds but this complexity also makes many lead compounds' synthesis difficult and the compound usually has to be extracted from its natural source - a slow, expensive and inefficient process. As a result, there is usually an advantage in designing simpler analogues (http://en.wikipedia.org/wiki/Natural product, 28 November 2009).

It can be challenging to obtain information from practitioners of traditional medicine unless a genuine long term relationship is made. Ethno-botanist Richard Schultes had the good sense to approach the Amazonian shamans with respect, dealing with them on their terms and not as a latter-day conquistador. He became a "depswa" - medicine man - sharing their rituals while gaining knowledge. They responded to his enquiries in kind, leading to countless new medicines for treating our "civilized" illnesses. On the other hand Cherokee herbalist David Winston recounts how his uncle,

a medicine priest, would habitually give misinformation to the visiting ethnobotanists. The acupuncturists who investigated Mayan medicine recounted in the book 'Wind in the Blood' (Garcia *et al.,* 1999) had something to share with the native healers and thus were able to find information not available to anthropologists. The issue of rights to medicine derived from native plants used and frequently cultivated by native healers complicates the issue.

2.2 The genus *Ganoderma*

Ganoderma is a cosmopolitan polypore mushroom involved in the fundamental process of lignin, cellulose, and related polysaccharide decomposition of hardwoods such as oak, maple, sycamore and ash. The genus was named by Karsten in 1881 and includes almost 80 species. It is distinct polypore fungi from other families in producing spores with a complex wall structure; the spore wall is double-layered, with ornamentations on the inner layer penetrating a colourless outer layer. The members of the genus are primarily saprophytic, medium to large in size and usually found on living or dead woods. The identification of these fungi in those days was mainly based on host specificity, geographical distribution, and macro-morphological features of the fruiting bodies. However, the great variability in macroscopic and microscopic characters of basidiocarps has resulted in a large number of synonyms and a confused taxonomy (Seo and Kirk, 2000). They have been classified based on the colour and shapes of their fruiting bodies, each with different uses (Table 2.1). The fruiting bodies are in bracket-like shape and has thick texture. The upper surface of the pileus is usually hard, crusty, wavy and sometimes varnished or dusted with spores, zoned, ridged, or grooved, coriaceous.

Several species of *Ganoderma* have been used in traditional Asian medicines for thousands of years. Collectively, the *Ganoderma* have been investigated for a variety of potential therapeutic benefits such as anticancer, immunoregulatory, liver protecting, hypoglycemic, antibacterial, antiviral, antifungal and antifibrotic effects, antioxidant activities, reduction of blood cholesterol, inhibition of blood vessel regeneration (angiogenesis), protection against radiation-induced damage, reduction of lower urinary tract symptoms and increase of endurance for vigorous exercise (Wasser, 2005; Paterson, 2006). The general bioactive compounds isolated from *Ganoderma* and their respective medicinal function is listed in Table 2.2. They are important economically because of their extensive and potential use in traditional Asian medicines and bioremediation.

2.2.1 Scientific classification

Kingdom : Fungi

Phylum : Basidiomycota

Class : Basidiomycetes

Order : Polyporales

Family : Ganodermataceae

Genus : *Ganoderma*

Table 2.1: Classification of *Ganoderma* spp. based on the characteristics and uses of their fruiting bodies

Colour	**Taste**	**Use**
Blue	Sour	Improves eyesight and liver function; calms nerves
Purple	Sweet	Enhances function of ears, joints, muscles; helps complexion
Red	Bitter	Aids internal organs; improves memory; enhances vitality
White	Hot (or pungent)	Improves lung function; gives courage and strong will
Black	Salty	Protect kidneys
Yellow	Sweet	Strengthens spleen function; calms the ‘‘spirit’’ (shen)

(Wasser, 2005).

Table 2.2: Isolated compounds and effects of *Ganoderma* spp.

Compound	Effect	Reference
Adenosine	Antiplatelet aggregation	Kawagishi *et al*. (1997), Shimizu *et al*. (1985)
Lectins	Mitogenic	Ngai and Ng (2004)
Polysaccharides	Antifibriotic	Park *et al*. (1997)
	Antiherpetic	Eo *et al*. (1999a,b, 2000), Kim *et al*. (2000), Oh *et al*. (2000)
	Anti-inflammatory	Ukai *et al*. (1983)
	Hepatoprotective	Zhang *et al*. (2002)
	Hypoglycaemic	Hikino *et al*. (1985, 1989), Hikino and Mizuno (1989), Tomoda *et al*. (1986), Zhang and Lin (2004)
	Immuno-modulatory – anti-tumour	Gao *et al*. (2000a,b), Li *et al*. (2000), Li and Zhang (2000), Ooi *et al*. (2002), Sasaki *et al*. (1971), Sone *et al*. (1985)
	Miscellaneous (radiation protection, DNA damage, anti-oxidant)	Kim and Kim (1999b), Lee *et al*. (2001)
Protein (‘‘LZ-8’’)	Immunodulatory & Immunosuppressive	van der Hem *et al*. (1995)
Terpenoids and related compounds	Anti-bacterial	Smania *et al*. (1999)
	‘‘Anti-complement’’	Min *et al*. (2001)
	Anti-inflammatory	Kleinwa¨chter *et al*. (2001)
	Antioxidant	Zhu *et al*. (1999)
	Antiplatelet aggregation	Shiao (1992)

'**Table 2.2**, continued'

Compound	Effect	Reference
Terpenoids and related compounds	Antiviral	El-Mekkawy *et al*. (1998), Mothana *et al*. (2003)
	Cytotoxicity	Gao *et al*. (2002), Gonzalez *et al*. (2002), Kimura *et al*. (2002), Lin *et al*. (1991), Su *et al*. (2000), Wu *et al*. (2001)
	Enzyme inhibitors	Lee *et al*. (1998)
	Hepatoprotective	Chen and Yu (1999), Kim *et al*. (1999)
	Hypolipidemic (chloresterol inhibitors)	Komoda *et al*. (1989), Shiao (1992)
	Hypotensive	Morigiwa *et al*. (1986)

(Paterson, 2006)

2.2.2 *Ganoderma australe* (Fr.) Pat. (KUM60813)

Ganoderma australe (Figure 2.1 (A)) is a white-rot fungus that causes selective wood biodelignification in some hardwoods. The fruiting bodies are usually brown or grey in colour and can grow up to 600 mm across on living trees causing the white rot to kill and decay the tree. It can live for few more years on the dead stump. It is usually found near the base of trees, often at the site of old wound. A new fertile layer is added each year. Messages written on the white pore layer become permanent when the fungus dries, consequently it is sometimes referred to as 'Artist's Conk' (Martínez *et al*., 1994). The spore print is brown in colour. This wood-decaying fungus is found in wet sclerophyll forest and rainforest on native or exotic trees. The shelf-like fruit bodies are generally fan or hoof-shaped, dry, brown, and often cracked. The brown bracket often has a white rounded margin (Martínez *et al*., 1991). *G. australe* which is also considered as varieties of Ling Zhi, have not been studied extensively, neither for its biochemical composition nor biological activity. So far, *G. australe* has only been explored for its properties of delignification (Elissetche *et al*., 2007), bioremediation of contaminated soil (Rigas *et al*., 2007) and investigation on its genetic diversity (Kaliyaperumal and Kalaichelvan, 2008).

2.2.3 *Ganoderma neo-japonicum Imazeki* (KUM61076)

Ganoderma neojaponicum (identified by Prof. Yao Yi-Jian, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China) is a fungal species described by Imazeki in the year 1939. It is a polypore mushroom that is hard in texture with a conspicuous red-brown-varnished, kidney-shaped cap and white to dull brown pores underneath depending on specimen age (Figure 2.1 (B)).

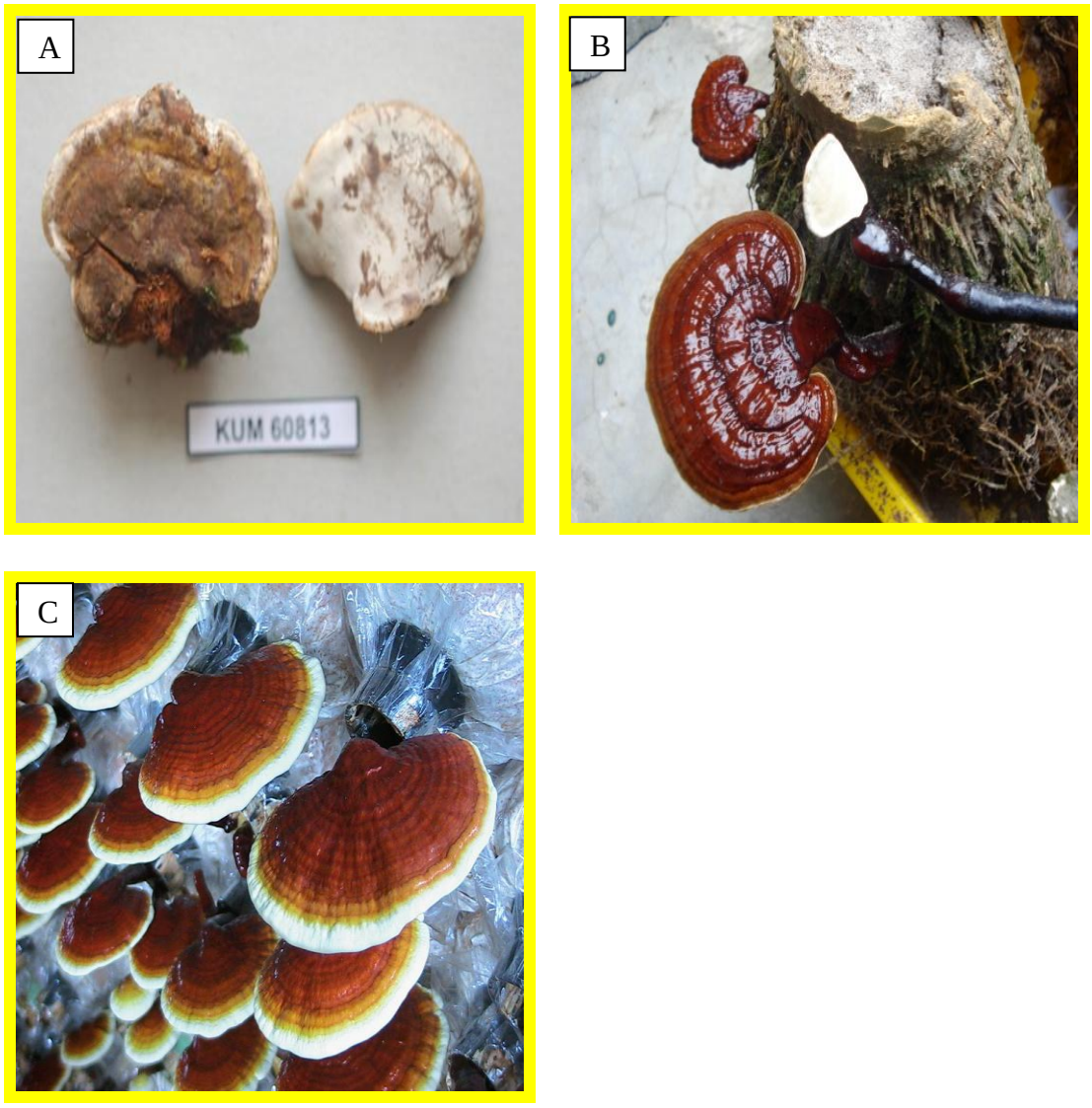


Figure 2.1: (A) ***Ganoderma australe*** **(Fr.) Pat. (KUM60813)**

(B) ***Ganoderma neo-japonicum Imazeki*** **(KUM61076)**

(C) ***Ganoderma lucidum*** **(Fr.) Karst. (*VITA GL*)**

It lacks gills on its underside and releases its spores through fine pores, leading to its morphological classification as a polypore. This mushroom is frequently used by indigenous communities in Malaysia for a variety of illness as well as for general wellness (Lee *et al*., 2007). The boiled mushroom decoction is consumed to treat fever, epilepsy and as health tonic (Sabaratnam *et al*., 2013). *Ganoderma neo-japonicum* which is also considered as varieties of Ling Zhi, have not been studied extensively, neither for its biochemical composition nor biological activity. So far, there are only two reports available describing the free radical scavenging and antihepatotoxic activities (Lin *et al*., 1995) as well as neurohealth activity (Vikineswary *et al*., 2013) of its fruiting bodies. Currently, there is much interest to assess and validate scientifically the medicinal properties of this mushroom based on ethno-mycological knowledge.

2.2.4 *Ganoderma lucidum* (Fr.) Karst. (*VITA GL*)

Ganoderma lucidum (Figure 2.3 (C)) is one of the world's oldest and economically important medicinal mushroom which prescribed in the Pacific Rim areas, such as China, Korea, Japan and other Asian countries for their pharmaceutical, nutraceutical and spiritual properties (Wasser, 2005; Paterson, 2006). In Latin, *lucidum* means shiny or brilliant and aptly describes the fruiting body of this mushroom, which has a modeled, sculptured, varnished appearance. *Ganoderma lucidum* (Lingzhi or Reishi) is the most cited species on the aspects of cultivation, chemical analysis, pharmacology, and therapeutic effects (Chen *et al*., 2008; Saltarelli *et al*., 2009). In folk medicine, *Ganoderma lucidum* was reported to have numerous therapeutic effects which include anti-inflammatory, antitumor, antiviral (e.g., anti-HIV), antibacterial and antiparasitic, antidiabetic, blood pressure regulation, reduction in the risk of

cardiovascular disorders, immunomodulation, hepato-protection and regulation of chronic bronchitis (Wasser and Weis, 1999; Paterson, 2006).

2.3 Solid-substrate Fermentation (SSF)

In nature, many wild mushrooms grow based on seasons after which it may cease to grow at similar location or may appear at another inaccessible location (Kalyoncu *et al*., 2010). The domestication of a wild medicinal mushroom to a commercial scale may take a very long time as traditional methods of cultivating the fruiting bodies of the mushrooms takes several months. Moreover, it is also at such cultivation period, quality of the product would not be within a control (Fang and Zhong, 2002; Chien *et al*., 2011). Some fungal spores may have constitutive dormancy and fail to germinate even when the environmental conditions are suitable for growth (Deacon, 1980). Hence, the solid substrate fermentation (SSF) of the fungal mycelia is viewed as a promising alternative technique to yield enhanced production of valuable metabolites (Fang and Zhong, 2002).

SSF is a process whereby non-soluble substrate is fermented by microorganisms with sufficient moisture but in the absence of free flowing-water. The insoluble substrate used provides both physical support and a source of nutrients. SSF, unlike that of slurry state, requires no complex fermentation controls and has many advantages over submerged liquid fermentation (SLF). The use of solid cultures provides a number of potential advantages. For instance, SSF needs low-cost substrates and results in greater production of mycelium in a shorter time and more compact space with a lower chance of contamination (Pandey *et al*., 2000).

Current trends on SSF have focused on the application of SSF for the development of bioprocess such as bioremediation and biodegradation of hazardous

compounds, biological detoxification of agro-industrial residues, biotransformation of crops and crops-residues for nutritional enrichment, biopulping, and production of value-added products such as biologically active secondary metabolites, including antibiotics, alkaloids, enzymes, etc.

In the present study, whole wheat kernels were used since it has been shown that these are an excellent substrate for use in mixed solid-state bioreactors (Nagel *et al.* 2000). The carbon compounds from the wheat grains serve to supply energy for fungal mycelium metabolism and provide the carbon for building carbohydrates, lipids, nucleic acids, and proteins. Without a carbon source, the mycelium growth will be poor or ceased.

2.4 Antioxidants

Antioxidant is defined as 'any substance that when present at low concentration compared with that of an oxidizable substrate, significantly delays or inhibits oxidization of that substrate by neutralizing free radicals' (Devare *et al*., 2012). The major characteristic of an antioxidant is its ability to trap free radicals. Basically, antioxidant system includes both endogenous (antioxidants made in the body) and exogenous (antioxidants obtained from the diet or supplements).

Numerous studies have reported that antioxidants reduce the risk of chronic diseases including cancer and heart disease. Plant-source food antioxidants such as vitamin C, vitamin E, carotenes, phenolic acids, phytate and phytoestrogens have been recognized as having the potential to reduce disease risk. Most of the antioxidant compounds in a typical diet are derived from plant sources and belong to various classes of compounds with a wide variety of physical and chemical properties.

However, artificial antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are widely used (Chan and Coxon, 1987). However, their use in food products has been declining due to their instability, strict legislation on the use of synthetic food additives, as well as due to a suspected action as promoters of carcinogenesis (Madavi and Salunkhe, 1995).

The production of reactive oxygen and nitrogen species can be balanced by the antioxidant action of endogenous as well as exogenous enzymes. This antioxidant defense system has significant importance as they represent the direct removal of free radicals and therefore providing maximal protection for biological sites (Valko *et al*., 2006).

Antioxidants exert their effects through simple or complex mechanisms including prevention of chain reaction initiation, binding of transition metal ion catalysts, decomposition of peroxidases, prevention of continued hydrogen abstraction, and radical scavenging. Hence, prevent the overproduction of free radicals and protect the biological systems in the human body from oxidative damage (Yazdanparast and Ardestani, 2007).

The free radical scavenging activity of antioxidants encompassed by a redox transition involving the donation of a single electron (or H atom, equivalent to the donation of an electron and a H^+ ion) to a free radical species. During the course of this electron transfer, the radical character is transferred to the antioxidant, yielding an antioxidant-derived radical (Halliwell, 2012). Thus, antioxidants neutralize the unpaired electrons and prevent them from taking electrons from other molecules.

2.5 Diabetes mellitus, obesity and oxidative stress

At present, DM has become one of the most common public health disorders, worldwide. Diabetes mellitus can be defined as a complex metabolic disease caused by an inherited or acquired deficiency in insulin secretion, insulin action or both (Nathan *et al*., 2009). Diabetes mellitus is characterized by elevated blood glucose level, insulin resistance and pancreatic β-cell dysfunction (American Diabetes Association, 2013).

There are three major types of DM: Type 1 DM (Insulin Dependent Diabetes Mellitus), Type 2 DM (Non Insulin Dependent Diabetes Mellitus), and Gestational DM. Type 1 DM, also known as Juvenile onset DM, is an auto-immune disease caused by destruction of the β-cells in the islets of Langerhans, leading to an absence or nearly no insulin production. Type 1 DM has an early onset during childhood or adolescence and the patients usually need insulin medications throughout their life (La Greca and Mackey, 2009). Type 2 DM is a chronic, lifelong disease, which generally develops later in life due to insulin resistance or insufficient insulin production by β-cells in the islets of Langerhans (Kaufman, 2011). Type 2 DM is a 7^{th} leading cause of death worldwide.

Gestational DM usually occurs in non-diabetic pregnant women but normally resolves after delivery of the baby. During pregnancy, an adaptive change occurs to preserve carbohydrates for the growing fetus. The concentration of lipolytic hormone elevates and thus, the disposal of free fatty acids into blood stream increases. Increased free fatty acids will reduce glucose oxidation, and cause insulin resistance in the liver and skeletal muscle, which will eventually lead to glucose intolerance (Golay and Ybarra, 2005).

Worldwide the prevalence of DM has increased tremendously due to population growth, aging, urbanization, and the increasing prevalence of obesity (Hutchinson *et al*., 2013). The number of adults with DM worldwide is expected to rise from 171 million in 2000 to 366 million in the year 2030 (Wild *et. al.*, 2004). However, a more recent study had estimated that by the year 2030, the number of people diagnosed with DM could increase up to 552 million, compared to 366 million in the year 2011 (Diabetes Atlas, 2012).

In Malaysia, the reported prevalence (First National Health and Morbidity Survey; NHMS 1) of DM was 6.3% in 1986, 8.3% in 1996 (NHMS 2), 14.9% in 2006 (NHMS 3) and WHO estimated that by 2030, Malaysia would have a total number of 2.48 million diabetics (prevalence of 10.8%), compared to 0.94 million in 2000, which represent 164% increased. However, according to the NHMS 4 report, about 2.6million Malaysians have diabetes as of the year 2011.

Among the three major types of DM, Type 2 DM is the most common form of diabetes which contributes to more than 90-95 % of all cases of diabetes worldwide (American Diabetes Association, 2013). In Malaysia, almost 96 % of diabetic patients are Type 2 DM and the prevalence of Type 2 DM in the ethnic Indians had the highest (Rampal *et al.*, 2012). This phenomenon should not be neglected because Type 2 DM usually occurs in conjunction with other metabolic syndrome (also known as insulin resistance syndrome) such as obesity, hypertension and dyslipidemia. (Nguyen *et al.*, 2008).

Diabetes mellitus is a complex, multi-factorial disease associated with considerable mortality, morbidity, and long-term complications that threaten the quality of life. The adverse outcomes of DM can be classified into macrovascular and microvascular complications. The most common macrovascular complications are

cardiovascular diseases, atherosclerosis, strokes and peripheral vascular disease while microvascular complications are retinopathy, nephropathy and neuropathy (Wu *et al*., 2012). According to the WHO in the year 2000, 30-45 % of diabetic patients worldwide had retinopathy, 10-20 % had nephropathy, 20-35 % had neuropathy and 10-25 % had cardiovascular diseases (Narayan *et. al.*, 2000).

Today, DM had become the 7th leading cause of global deaths worldwide (Diabetes Atlas, 2012). Etiology of DM may vary depending on the types of diabetes but generally involves a complex integration between genetic, metabolic framework, physical activities, eating habits and social-cultural factors (Narayan *et. al.*, 2000).

Genes (heredity) play an important role in the manifestation of all types of diabetes. Those who have a family history of DM are genetically susceptible to DM compared to others (Jungtrakoon *et al*., 2012). Besides genetics, obesity is also an important risk factor for diabetes, especially Type 2 DM because excessive fat stored will interfere with the body's ability to utilize insulin effectively and thus lead to high blood glucose level (Kaufman, 2011). Therefore, a recent increase in the incidence of Type 2 DM among children is not surprising because obesity is becoming more common among children nowadays due to unhealthy eating habits and lack of physical activities (Nguyen *et al*., 2008).

Table 2.3: Comparison between Type 1 DM, Type 2 DM and Gestational DM

Type of DM	Type 1 DM	Type 2 DM	Gestational DM
Usual age at onset	Usually under 20 years old	Usually over 40 years old	During Pregnancy
Development of symptoms	Rapid	Rare	Rapid
% of diabetic population	About 9 %	About 90 %	About 1 %
Association with obesity	Rare	Common	Common
β-cell islets	Destroyed	Not destroyed	Not destroyed
Insulin secretion	Decreased	Normal or increased	Normal or increased
Treatment	Insulin injection	Diet or exercise; oral drug stimulator to increase insulin sensitivity.	Insulin injection and diet

Obesity refers to an excess accumulation of body fat which develops slowly as a result of chronic imbalance between caloric intake and energy expenditure over a prolonged period of time (Puhl and Heuer, 2012). This results in increased number of adipocyte due to rapid differentiation of adipose precursor cells (*i*.e. preadipocyte) into mature adipocyte as well as increased lipid accumulation in adipocyte and non-adipose tissues such as muscle, liver and pancreas (James *et al.*, 2012). A person with a body mass index (BMI) of more than 30 is considered obese (Boo *et al*., 2010).

Obesity is a leading metabolic disease and rapidly approaching an epidemic state worldwide especially in developing countries. According to the International Obesity Task Force, in the year 2010, one billion adults of the world's population were overweight (BMI 25-29.9 Kg/m²) and 475 million people were obese (with BMI $\geq$ 30 kg/m^2) (Swinburn *et al*., 2011). When Asian-specific cutoff points for the definition of obesity (body mass index >28 kg/m2) are taken into account, the number of adults considered obese globally is over 600 million (Swinburn *et al*., 2011).

Genetic factor plays a significant role in causing obesity. An obese individual usually has a lower basal metabolic rate and tends to store more energy in the form of fat than a normal person (Chung and Leibel, 2012). Besides that, mutation in any gene related to the regulation of lipid and glucose metabolism will increase the risk of developing obesity. For example, mutation of the melanocortin-4-receptor gene significantly impairs the activation of cyclic AMP (cAMP) which subsequently leads to the defects in fat breakdown mechanism and results in obesity (Larsen *et. al.*, 2005).

In addition to genetic predisposition, environment also strongly influences obesity. Over the past decade, major economic and social changes with sedentary lifestyles and increasing availability of calorie rich foods have triggered a tremendous increase in the prevalence of obesity (Gunter and Leitzmann, 2006). Psychological

factors, including binge eating and dieting, had been proven to cause excessive energy storage and weight gain (Tanofsky-Kraff *et. al.*, 2006). Besides psychological factors, certain illnesses can also lead to obesity. Diseases such as Cushing's syndrome (excessive glucocorticoid) results in increased adiposity which inevitably leads to obesity (Wake *et. al.*, 2006). Obesity can also be caused by prolonged drug intake such as steroids and anti-depressants. For example, thiazolidinedione (TZD) which is an insulin sensitizer in the treatment of Type 2 DM causes deposition of fats in peripheral subcutaneous tissues and eventually lead to inappropriate weight gain (Greenfield and Campbell, 2004).

The human body has various complex antioxidant systems to defend it against free radical induced damage on tissues and cellular systems. The imbalanced condition between the production of free radicals such as ROS and reactive nitrogen species (RNS), and a biological system's ability to readily detoxify the reactive intermediates or to repair is defined as oxidative stress (Halliwell, 2012). Free radical induced damage and oxidative stress has been suggested to be a common pathway linking diverse mechanisms for the pathogenesis [including microvascular (retinopathy, nephropathy and neuropathy) and macrovascular (coronary heart disease, cerebrovascular diseases and peripheral vascular disease)] of complications of diabetes (Rahimi *et al.*, 2005).

2.5.1 Correlation between DM, obesity and oxidative stress

Obesity constitutes a health risk as it is usually associated with many metabolic health disorders for example arterial hypertension, osteoarthritis, dyslipidemia, cardiovascular diseases, Type 2 DM and depression (James *et al*., 2012). Obesity has a strong correlation with Type 2 DM and adipocyte are the plausible common relation between Type 2 DM and obesity (Golay and Ybarra, 2005). Adipocyte are the major

storage depot of excess energy in the form of triglycerides. When accumulation of triglyceride exceeds the capability of adipocyte to accommodate, adipocyte will begin to enlarge. In overweight people where their adipocyte are enlarged with elevated concentration of lipid, the cells will undergo desensitization towards insulin (insulin resistance) and progress into Type 2 DM (Lofgren *et. al.,* 2005). Development of insulin resistance in adipocyte is one of the major typical features in the development of Type 2 DM (Greenfield and Campbell, 2004). Therefore, obesity may be a strong predisposing factor of Type 2 DM.

On the other hand, studies have shown that Type 2 DM may lead to the development of obesity. Insulin resistance and hyperinsulinemia in Type 2 DM will cause a diversion of free fatty acids and glucose uptake in non-adipocyte (muscle cells and liver), resulting in increased number of cells with excessive fat accumulation (Pausova, 2006).

2.5.2 Insulin resistance

Insulin resistance is a condition whereby the cells are not sensitive to insulin stimulation and cause defect in the normal glucose uptake, metabolism and storage (Greenfield and Campbell, 2004). When the sensitivity of glucose transporters (GLUT4) and/or insulin receptors at the cell membrane are decreased (Zorzano *et. al.,* 2005), glucose uptake by these cells will also decrease, resulting in hyperglycemia and DM. Besides that, the pancreas will be triggered to produce more insulin as an attempt to maintain the effects of insulin on the cells but this leads to hyperinsulinemia. Prolonged hyperinsulinemia will then lead to β-cell dysfunction and damage (Greenfield and Campbell, 2004).

2.5.3 Current treatment for Type 2 DM

Insulin and some oral hypoglycemic agents such as metformin and thiazolidinedione (TZD) or "glitazones" are currently used in the treatment of Type 2 DM (Greenfield and Campbell, 2004). Metformin (oral antihyperglycemic biguanide) improves insulin sensitivity by enhancing activation of AMP-activated protein kinase to stimulate glucose uptake as well as to reduce glucose production in the liver (Hawley *et. al.*, 2002). Metformin is the most frequently used anti-diabetic drug since it has been rarely associated with risk of lactic acidosis except in patients with severe kidney impairment (Bailey and Turner, 1996). Biguanides decrease gluconeogenesis from pyruvate, alanine, and lactate; thus increases blood lactate level and leads to lactic acidosis. Usually the development of lactic acidosis coexists with substantial tissue hypoperfusion and hypoxic conditions (Stang *et al.*, 1999).

Another group of "insulin-sensitizing agents", TZDs are suggested to improve insulin sensitivity via their action on PPARγ (Gurnell *et. al.*, 2003). However, increased expression of PPARγ activity will give rise to some side effects including increased differentiation of preadipocyte followed by deposition of fat in peripheral subcutaneous tissues, leading to weight gain (Gurnell *et. al.*, 2003).

Besides that, sulfonylureas for example chlorpropamide was also used to treat Type 2 DM. Sulfonylureas increase insulin production by stimulating the pancreas to release more insulin into the blood stream (Ketz, 2001). Nevertheless, the usage of sulfonylureas must be monitored closely since it can induce hypoglycemia during overproduction of insulin. In addition, sulfonylureas can also cause weight gain, mainly as a result of edema and reduction of the osmotic diuresis. Although the second generation sulfonylureas with decreased side effects were discovered, the price of these second generation sulfonylureas is generally quite high (Geng *et. al.*, 2007).

Despite the increasing availability of drugs for the treatment of Type 2 DM, successful management of this disease requires improvement. Furthermore, the occurrence of Type 2 DM is also rising very rapidly. Most of the modern oral medications have a tendency to cause side effects. Therefore, some Type 2 DM patients prefer to use traditional remedies as an alternative to cure diabetes. Hence, investigations for a better treatment regime or supplements to control Type 2 DM are required.

2.5.4 Adipocyte

Adipocyte are mainly distributed in retroperitoneal, perirenal, orbital, subcutaneous and visceral parts of the body and are predominantly found in the visceral and subcutaneous part of the body. Patients with visceral obesity will usually accumulate fats in the visceral part of the body (apple-shaped fat distribution) and they are more predisposed to developing metabolic and cardiovascular complications (Arner, 1997).

Adipocyte are specialized cells which play a critical role in maintaining energy balance and energy metabolism by serving as both storage depot and endocrine organ. As the largest energy storage depot, almost 95 % of the body's fat is stored in adipocyte in the form of triglyceride-containing vesicles (Large *et. al.*, 2004). Adipocyte are capable of changing their diameter and volumes in order to accumulate lipid. The nucleus of an adipocyte filled with lipid will be pushed to the periphery to create a bigger storage space for lipid. Small amounts of triglycerides are also stored in the liver and muscle tissues (Coppack *et. al.*, 1994).

Adipocyte regulate energy balance whereby it 'buffers' the daily influx of dietary fat that enters into the bloodstream. In energy deprivation, triglycerides from adipocyte will be mobilized to other organs like muscle tissues or liver for gluconeogenesis (Large *et. al.*, 2004). This function is controlled by a complex system involving hormonal and neuronal signals (Jazet *et. al.*, 2003).

Generally, two types of adipose tissues are found in most species including brown adipose tissues and white adipose tissues. Both types of adipose tissues have distinct morphological and functional characteristic. Brown adipose tissues are composed of multilocular cells with highly invaginated mitochondria and they are commonly found in newborns (Feve, 2005). Brown adipose tissue do not provide energy for the body to carry out body tasks but they have heat-releasing function to help keep the body warm (Jazet *et. al.*, 2003). In contrast, white adipose tissues are composed of highly anabolic unilocular cells and they are usually found in normal adult human (Feve, 2005). The main function of white adipose tissue is to act as an inert storage repository of triglycerides and the triglycerides will be utilized when the body lacks energy (Jazet *et. al.*, 2003).

2.5.4.1 Lipogenesis and lipolysis

Lipogenesis is a process of increasing adipocyte size (hyperplasia) and number (hypertrophy) through the differentiation of preadipocyte from precursor cells into mature fat cells to provide more space for triglyceride storage (Rosen and Spiegelman, 2000). *In vitro*, lipogenesis occurs in a well organized sequence that involves proliferation of mesenchymal cell (clonal expansion) into preadipocyte. Next, appropriate pro-differentiative hormonal agents like insulin will induce differentiation of preadipocyte. Two days after the initiation of differentiaton, a permanent growth

arrest takes place and preadipocyte will receive an appropriate combination of mitogenic and adipogenic signals to continue through the subsequent differentiation steps, whereby growth arrested cells will gradually develop into spherical, lipid-filled mature adipocyte (MacDougald and Mandrup, 2002).

Lipogenesis and preadipocyte differentiation is tightly regulated by insulin. As a potent adipogenic hormone, insulin triggers the intrinsic cascade of adipocyte differentiation through binding to the insulin receptor (Klemm *et. al.*, 2001). High levels of insulin receptors are usually found in differentiated adipocyte whereas undifferentiated preadipocyte expressed only low levels of insulin receptors (Accili and Taylor, 1991). Therefore, during the induction of differentiation in preadipocyte, insulin signal was expressed through the insulin-like growth factor-I receptors, which are a homologous to the insulin receptor (Smith *et. al.*, 1988).

In the cytosol of liver, adipose tissue and intestinal mucosa, *de novo* lipogenesis occurs. *De novo* lipogenesis refers to the synthesis of fatty acids followed by synthesis of triglycerides. Insulin and glucose are known to stimulate hepatic *de novo* lipogenesis whereas glucagon, polyunsaturated fatty acids and fasting will inhibit hepatic *de novo* lipogenesis (Large and Arner, 1998). Regulations of *de novo* lipogenesis in human adipocyte still remain unclear. However, some studies showed that insulin is able to increase fatty acid synthase expression and activity in primary rodent adipocyte cell cultures (Moustaid *et. al.*, 1996).

2.5.4.2 Regulation of glucose and lipid metabolism in adipocyte

Adipose tissue plays a vital role in the regulation of energy homeostasis, insulin sensitivity, and carbohydrate/lipid metabolism. These actions are mediated by both the actions of a number of non-secreted proteins and hormones produced in the adipocyte.

Adipocyte produce a number of hormones that have wide-ranging effects on energy intake, energy expenditure, and carbohydrate and lipid metabolism, including nutrient partitioning and fuel selection.

Foods which contain carbohydrate and fat will be metabolized into glucose and fatty acids respectively. The excess amount of glucose and fatty acids in the circulation will be brought to the liver to carry out anabolic processes such as lipogenesis and glycogen synthesis (Jequier, 1994). Regulation of glucose and lipid metabolism is controlled by various transporters, enzymes and hormones. For example, glucose is transported by GLUT4 whereas fatty acid is taken into cells by specific receptor (CD36) and binding protein (aP2) (Memon *et. al.*, 2000).

Metabolism refers to physical and chemical processes within the cells in the body. Lipid metabolism consists of hydrolysis of triacylglycerols into free fatty acids (FFA) and glycerol whereas glucose will be converted into acetyl CoA in glucose metabolism. The fatty acids will undergo β-oxidation for adenosine-5-triphosphate (ATP) production while acetyl-CoA will be used in the Krebs cycle to generate energy (Hoppel, 2003).

Lipogenesis, β-oxidation and lipolysis are the three main pathways of lipid metabolism and are highly controlled by hormonal agents such as insulin and adrenaline (Arner and Ostman, 1974) as well as environmental factors such as physical activity and food intake (Large *et. al.*, 2004). As for glucose metabolism, the main pathways involved are glycolysis and gluconeogenesis which are also controlled by insulin.

Table 2.4: *In vitro* models of adipogenesis

Cell lines	**Organ / Stage of development**	**Inducing agents used for differentiation**
ES cells	Mouse blastocyst	Retinoic acid
CH3 10T1/2	Mouse embryo	Demethylating agent 5'-azacytidine
TA1	Derived from 5'-azacytidine-treated 10 T1/2	10% FBS, insulin, and Dex
3T3-L1	17- to 19-day disaggregated mouse embryo	10% FBS, Dex and MIX, insulin (high concentration)
3T3-F442A	Same as above	10% FBS, insulin
Ob17	Epididymal fat pads of adult *ob/ob* mouse	10% FBS, insulin, and T_3
Primary cultures	**Source/age**	**Inducing agents used for differentiation**
Rat	Subcutaneous epididymal, retroperitoneal/newborn (48h), 4 weeks old, or adult	Insulin (low concentration in 10% FBS, high concentration in serum free, accelerated)
Mouse	Subcutaneous/8-12 day old	Serum free; insulin, HDL, Dex
Rabbit	Perirenal/4 week old	Serum free; insulin, Dex
Pig	Perirenal/subcutaneous/fetal, newborn (1-7 day old)	Serum free; insulin, with or without glucocorcoids
Human	Subcutaneous (abdominal) /variable age	Serum free; insulin (high cencentration) and glucocorticoids

(Gregoire *et al*., 1998)

When nutrient intake exceeds the energy expenditure, the substrate-induced increase in citric acid cycle activity generates an excess of mitochondrial NADH (mNADH) and ROS. In turn, cells will prevent mNADH and ROS formation by inhibiting insulin stimulated glucose uptake and preventing the entry of energetic substrates (pyruvate and fatty acids) into the mitochondria; to protect themselves against ROS mediated cellular damage. When the adipose tissues are overnourished, a controversy exists as to whether FFA or glucose is the primary fuel supply. In such situations, an influx of substrates into the citric acid cycle produces excessive mitochondrial acetyl-CoA and NADH which may be prevented in several ways, one of which is the inhibition of beta-oxidation of FFA. The increased level of intracellular FFA, in turn, directs to reduced GLUT4 translocation to the plasma membrane, resulting in resistance to insulin stimulated glucose disposal to adipose tissue. In this case, insulin resistance may be considered a compensatory mechanism that defends the cells against further insulin stimulated glucose and fatty acid uptake and therefore oxidative damage (Gummersbach *et al*,. 2009).

2.5.4.3 Insulin and epinephrine pathway

Insulin is a metabolic hormone produced by the β-cells of the pancreas. Insulin plays a critical role in virtually all aspects of adipocyte biology since adipocyte are highly responsive to insulin (Kahn and Flier, 2000). Insulin has a predominant role in regulating blood sugar level, maintaining homeostasis of energy metabolism and coordinates the storage and utilization of fuel molecules in adipose tissue, liver and skeletal muscle. Besides enhancing the storage of triglycerides in adipocyte by increasing fatty acid uptake and stimulating differentiation of preadipocyte to adipocyte, insulin also promotes glucose transport through GLUT4 as well as inhibits lipolysis

(Kahn and Flier, 2000). In addition, insulin also stimulates lipogenesis through the induction of key lipogenic enzymes such as fatty acid synthase and glycerophosphate dehydrogenase (Kersten, 2001).

Insulin action is initiated through the binding and activation of its cell surface receptor, which consists of two α- and two β-subunits. Insulin binds to the extracellular α-subunits and transmits a signal across the plasma membrane to activate the intracellular tyrosine kinase domain of the β-subunit which leads to the phosphorylation of insulin receptor substrate-1. Phosphorylated insulin receptor substrate-1 will initiate the expression of transcription factors such as enhancer binding protein-α (C/EBPα) and PPARγ. These factors in turn will stimulate expression of adipocyte specific proteins (lipoprotein lipase, fatty acid synthase) which are involved in the regulation of lipid metabolism, adipocyte differentiation and insulin sensitivity (Pessin and Saltiel, 2000). However, the activities of insulin in adipocyte may be counteracted by other hormones such as adrenaline, isoproterenol and several other catecholamines.

2.6 Determination of antioxidant and insulin-like potential of *Ganoderma* spp.

2.6.1 Antioxidant assays

Many methods have been employed generally to measure and compare the antioxidant activities of food. Recently, oxygen radical absorbance capacity assays and enhanced chemiluminescence assays have been used to assess the antioxidant activity of foods, serum and other biological fluids. Methods used require special equipment and analysis in order for effective determination. Normally, these analytical methods measure free radicals like 1,1-diphenyl-2-picrylhydrzyl (DPPH) radical, superoxide anion radical (O_2·), the hydroxyl radical (OH·) or the peroxyl radical (ROO·). The

various methods can give rise to different results and these depend on the specific radical that is being used as the reactant. Moreover, there are several other methods used to determine resistance of lipid or lipid emulsions to oxidation in the presence of a certain antioxidant that is being tested (Prakash *et al.*, 2001).

There are several types of antioxidant bioassays commonly used recently. These include DPPH free radical scavenging system, autoxidation of linoleic acid in water-alcohol system, Trolox equivalent antioxidant capacity (TEAC) assay system, xanthine/xanthine oxidase superoxide scavenging system, tyrosinase inhibitory assay system, ferric reducing/antioxidant power (FRAP) assay system, thiobarbituric acid (TBA) assay system, Beta-carotene-linoleate model system and so forth. Each system relates to respective radicals being tested, different mechanism, and the point of measurement.

The DPPH assay is a fast method to determine the free radical scavenging activity of the mushroom extracts based on the reduction of DPPH radical. In the presence of antioxidant, the DPPH free radical will donate its hydrogen which subsequently be reduced. The scavenging activity of unfermented and fermented wheat grains extracts were tested using an ethanol solution of the stable free radical, DPPH. Unlike laboratory-generated free radicals such as the hydroxyl radical and superoxide anion, DPPH has the advantage of being unaffected by certain side-reactions, such as metal ion chelating and enzyme inhibition (Amarowicz *et al.*, 2004). A freshly prepared DPPH solution exhibits a deep violet colour with maximum absorption at 515 nm. This violet colour becomes colorless or pale yellow when neutralized in the presence of an antioxidant. Therefore, the more rapidly the absorbance decreases, the more potent is the antioxidant activity of the test sample.

The $[ABTS]^+$ assay is a fast and easier method to determine free radical scavenging activity and it is based on the reduction of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) $[ABTS]^+$ radical monocation. Because ABTS and potassium persulfate react stoichiometrically at a ratio of 1:0.5, this will result in incomplete oxidation of the ABTS. In the presence of hydrogen donating antioxidant the $[ABTS]^+$ free radical will subsequently be reduced.

FRAP assay is an economical and simple assay whereby the reagents are easy to prepare; results are highly reproducible and rapid. Unlike other antioxidant assays which apply the inhibition principle, the FRAP assay is simple and measures 'antioxidant power' directly. In the presence of antioxidants at low pH, the ferric-tripyridyltriazine [Fe(III)-TPTZ] complex is reduced to a ferrous-tripyridyltriazine [Fe(II)-TPTZ] complex. This complex gives rise to deep blue colour which can be detected spectrophotometrically at 593nm. The development of blue colour from the reduction reaction occurs due to the ability of the antioxidant to donate an electron to reduce Fe (III) to Fe (II). This reaction can be summarized as follows:

$$\text{Fe (III)-TPTZ} + \text{Antioxidant} \longrightarrow \text{Fe (II)-TPTZ} + \text{Antioxidant}^+$$

Folin Ciocalteu's method (Singleton and Rossi, 1965) was typically used to measure the total phenolic content of foods or plant extracts. Phenolic content of a compound contributes to free radical chain breaking properties and the ability to neutralize free radicals by donating hydroxyl group. The Folin-Ciocalteu reagent is a solution of complex polymeric ions formed from the combination of phosphomolybdic and phosphotungtic acids. The reagent oxidizes phenolates (ionized phenolics) present in the sample and reduces the acids to form a blue complex that absorbs at 750nm. The

color develops in a slightly alkaline environment which is provided by the sodium carbonate.

2.6.2 3T3-L1 Preadipocyte viability and proliferation

The morphology of murine preadipocyte was fibroblastic with huge nucleus in the middle of each cell. 3T3-L1 preadipocyte grow in loose cluster or individually and the proliferation is regulated by several hormones for instance growth factors, insulin, glucocorticoids and sex steroid hormones. Preadipocyte were cultured in DMEM medium supplemented with 10% FBS to proliferate until confluent state. The confluent preadipocyte were then subjected to differentiation. Trypan blue staining method was used to estimate the number of viable cells based on the principle that only dead / damaged cells will take up trypan blue dye while viable cells do not get stained. Trypsin-EDTA dissolves fibronectin on the cell surface and therefore promotes cell detachment from the bottom of flask. However, prolonged trypsinization was avoided to prevent cellular damage and death.

2.6.3 *In vitro* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

MTT assay was carried out based on the method described by Mossmann (1983) which detect only viable cells. MTT is a yellow coloured tetrazolium salt which will be reduced by viable cells to form insoluble purple formazan crystals. Formazan crystals can be solubilized by 100% dimethyl sulfoxide (DMSO) and the amount of formazan formed is directly proportional to the number of viable cells. The colour formation can then be quantified using a spectrophotometer. This is an approach to measure the number of viable cells in each culture well. Thus, the inhibition or proliferation of the

cells can be estimated. MTT assay is usually used to test cell viability, proliferation, cell population doubling time and cytotoxicity of extracts/compounds (Mosmann, 1983).

2.6.4 Oil Red O quantitative assay (Lipogenesis)

Oil Red O is a red dye which stains neutral lipids in cells. The amount of Oil Red O staining is directly proportional to the amount of lipid accumulated in adipocyte which can be visualized miroscopically (qualitative measurement) or measured spectrophotometrically (quantitative measurement) at 510 nm. Therefore, lipogenic activity and differentiation rate in adipocyte could be easily assessed using Oil Red O staining. (Akerblad *et. al.*, 2002).

2.6.5 Glycerol quantitative assay (Lipolysis)

Lipolysis is the process in which triglyceride are hydrolysed into free fatty acids and glycerol. The amount of glycerol found in adipocyte can be quantified using a colorimetric method of Trinder, 1969. This assay was designed to measure the lipolytic activity indirectly, by measuring the glycerol concentration which released into the culture media.

Glycerol is first phosphorylated by ATP to form glycerol-1-phosphate (G-1-P) and adenosine-5'-diphosphate (ADP) in the reaction catalyzed by glycerol kinase (GK). Then G-1-P is oxidized by glycerol phosphate oxidase (GPO) to form dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H_2O_2). After that, peroxidase (POD) catalyzes the coupling of H_2O_2 with 4-aminoantipyrine (4-AAP) and sodium N-ethyl-N-(3-sulphopropyl) m-anisidine (ESPA) to form quinoneimine dye (purple coloured substance). Quinoneimine dye absorbs at 570 nm and the increase in absorbance is directly proportional to the amount of glycerol formed in the sample. Increased amount

of glycerol when compared to the basal conditions signify lipolysis whereas declined glycerol level indicate anti-lipolysis.

$$\text{Glycerol + ATP} \xrightarrow{\text{GK}} \text{G-1-P + ADP}$$

$$\text{G-1-P} \xrightarrow{\text{GPO}} \text{DAP + } H_2O_2$$

$$H_2O_2 + \text{4-AAP + ESPA} \xrightarrow{\text{POD}} \text{Quinoneimine dye + } H_2O$$

2.6.6 RNA extraction

RNA extraction is the course of purifying RNA material from a biological source. Standard phenol-chloroform extraction is commonly carried out using Trizol® reagent provided with the kit. The process is usually hampered with the presence of ribonuclease enzymes. Chaotropic agents, such as guanidium isothiocyanate, were used to protect RNA from endogenous RNases. The sample is then transferred into a spin column which binds nucleic acids. The column is washed with washing buffer (provided in the kit) and purified RNA material is eluted with elution solution.

2.6.7 Reverse transcription (RT)

In reverse transcription, RNA is transcribed to double stranded DNA, called complementary DNA (cDNA). The process require recognized functional primers (*i.e.*, for specific target enrichment), random primers or in some cases polyT primers due to the presence of polyA tail in most transcribed RNAs and most importantly the enzyme reverse transcriptase which is a RNA-dependant DNA polymerase.

In this study, relative expression of the target genes was normalized with endogenous control (18S rRNA). Variance of starting genetic material (*i.e.*, total cDNA templates) between samples was normalized by referencing the cycle threshold (CT) value with the endogenous control's CT value (ΔCT). Hence, the quantity of the cDNA was not quantified prior to RT-PCR.

Reverse transcription PCR based assays are currently the most common method for characterization and confirming gene expression levels of different sample populations. Early attempts to simultaneously amplify specific DNA sequences and detect the product of the amplification used ethidium bromide (Kroh *et al.*, 2010) With the advent of fluorogenic DNA binding chemicals (SyberDyes®) and fluorogenic probes (TaqMan®), high sensitivity and rapid real time quantification of fluorescence amplicon is made possible. This also eliminated the tedious need for post-PCR processing. Dissimilar to SyberDyes® which intercalates with any double-stranded DNA, TaqMan® probes only binds to the specific complementary sequence within the target gene. However, in both cases, fluorescence signal increases with each amplification cycle.

Although the real time PCR method with TaqMan® probes allows the determination of any selected gene expression with high specificity and broad magnitude coverage, there are a couple of assumptions which must be taken into consideration. Firstly, the chosen reference gene (*i.e.*, endogenous control) does not vary in number of copies or expression level under different experimental conditions. Secondly, the amplification efficiency of the genes is assumed 100 % if the ΔΔCT method is used to evaluate relative expression otherwise it is adjusted in the calculation by determining the amplification efficiency earlier (Kroh *et al.*, 2010).

2.6.8 Relative expression

The relative expression of each gene was compared to endogenous control housekeeping genes. The basis of relative expression comparison has been revealed previously (Kroh *et al*., 2010). Briefly, by using a real time PCR system and appropriate detection probe (in this study: TaqMan®), amplification of a genetic template can be monitored in real time PCR. Differences in the amount of starting template are reflected in the number of PCR cycles required to produce an equal intensity of signal (*i.e.*, threshold).

In a real time-PCR reaction TaqMan® probes are used, the probe is designed with a reporter dye and a quencher moiety at the respective 5' and 3' ends. Due to the proximity of the reporter and quencher, the fluorescence of the reporter is quenched. The probes are designed to anneal to target sequence within the forward and reverse PCR primers. During the extension cycle, the Taq polymerase with an intrinsic 5' to 3' nuclease activity cleaves the reporter dye from the probe. Once released, the reporter emits the fluorescence signal which is proportional to the amount of amplicon in each cycle of amplification; therefore the relative amount of template can be determined (Wang *et. al.*, 2006).

Prior to comparing the levels of a selected gene between samples, the expression of the selected gene is usually normalized with a biological normalizer (*i.e.*, housekeeping gene; endogenous control). This normalization is used to compensate for variations in sample mass and/or metabolism (*e.g.*, cell number, total mass DNA/RNA, mass of tissue, volume *etc.*) An endogenous control used in this study was eukaryotic18S rRNA. The following table (Table 2.5) shows the list of genes investigated in this study and their physiological role.

The passive reference dye, 6-carboxy-X-rhodamine (ROX™) was used in all real time PCR reactions in this study to normalize fluorescent fluctuations and to compensate wellto-well variations including volume and concentration differences. It was incorporated in the Assay Master Mix. Rn value is defined as the fluorescent emission intensity of the reporter dye divided by the fluorescent emission intensity of the passive reference dye (ROX™). The PCR cycle number at which fluorescence reaches a threshold value above the baseline fluorescence emission was used for quantitative measurement. This cycle number is called the cycle threshold number (CT) and is inversely proportional to the template of genetic material.

Table 2.5: List of genes investigated in this study and their physiological role

Gene	Description / Function
Adiponectin	• enhances insulin sensitivity through activation of AMPK • regulates hepatic gluconeogenesis by decreasing the mRNA expression of phosphoenolpyruvate carboxykinase and glucose-6-phosphatase • reduces TNF-α induced inflammatory responses
Bcl-2-associated X protein (BAX)	• pro-apoptotic protein and involved in p53-mediated apoptosis • interacts and induces opening of mitochondrial voltage-dependent anion channel, VDAC • under stress, triggers the release of cytochrome c that leads to programmed cell death • promotes CASP3 activation
Glucose transporter 4 (GLUT4)	• facilitates insulin stimulated glucose transportation across cell membranes, mainly in muscle and fat cells • GLUT4 depletion causes insulin resistance and related metabolic disorders
Glutathione peroxidase 3 (GPx3)	• catalyzes reduction of hydrogen peroxide, lipid peroxides, and organic hydroperoxides, by glutathione
Hormone sensitive lipase (HSL)	• is an intracellular neutral lipase that catalyses epinephrine stimulated lipid mobilization/hydrolysis • affects adipocyte lipolysis, spermatogenesis, steroidogenesis
Lipoprotein lipase (LPL)	• mainly hydrolyses triglycerides in circulating chylomicrons and very low density lipoproteins (VLDL) • serves as ligand factor for receptor-mediated lipoprotein uptake
PPARγ	• plays crucial role in cellular differentiation, regulation of carbohydrate, lipid and protein metabolism, and tumorigenesis of higher organism

CHAPTER 3

MATERIALS & METHODS

3.1 Materials requirements

3.1.1 Mushroom sample

The mycelium cultures of *G. australe* (KUM60813) and *G. neo-japonicum* (KUM61076) were obtained from Mushroom Research Center Fungal Collection, University of Malaya. The *G. lucidum* (*VITA GL*) culture was obtained from Ganofarm Sdn. Bhd., Tanjung Sepat, Selangor. The master stock cultures maintained at 4˚C in sterile Bijou-bottles containing 3ml of sterile tap water were kept in Mycology Laboratory, Institute of Post-Graduate Studies, University of Malaya.

3.1.2 *Ganoderma* spp. mycelia biomass production via SSF

The axenic mycelia cultures from stock cultures were grown on glucose-yeast-malt-peptone, GYMP agar ($MgSO_4.7H_2O$, 1 g/L; KH_2PO_4, 1 g/L; K_2HPO_4, 1 g/L; NH_4Cl, 1 g/L; Glucose, 15 g/L; yeast extract, 8 g/L; malt extract, 8 g/L; peptone, 8 g/L; agar, 17.5 g/L; distilled water, 1 L) at 25 ± 2 ºC for seven days (modified method from Tang and Zhong, 2002). Wheat grains purchased from a local supermarket were soaked in distilled water and kept overnight at room temperature. After the water was drained, 50 g of the grains were distributed into each of several 250 mL Erlenmeyer flasks and sterilized at 121 °C, 15 psi for 20 minutes. The flasks were cooled overnight and then inoculated with five 7 mm diameter plugs each of mycelia with three replicates for each strain and one control flask which only contained the solid substrate. The inoculated

flasks were incubated at room temperature (25 ± 2 ºC) for 14 days in an incubator under dark and static condition. Wheat grains without the mycelium plugs served as control. On day 14, the unfermented and fermented wheat grains were lyophilized for 2-3 days using freeze-dryer. All dried samples were weighed and stored in an airtight container before extraction. Percentage yield of fermented substrate was determined by the following equation:

$$\text{Percentage yield of fermented substrate} = \frac{W_b}{W_0} \times 100$$

W_b refers to the fresh weight of biomass after 14 days cultivation period and W_0 is the weight of unfermented substrate on day 0 of the cultivation period.

3.1.3 Extraction of total solubles from *Ganoderma* spp. grown on solid substrate

Biologically active substances from the unfermented and fermented wheat grain were extracted with two different solvents, i.e. ethanol and water. The crude ethanol extracts were prepared by shaking the freeze-dried and broken up wheat grain samples with 95 % ethanol (1:20 w/v) at 25 ºC on a rotary shaker for seven days (Zaidman *et al*., 2008). The ethanol extracts were filtered through Whatman No. 1 filter paper and vacuum concentrated (178 kPa, at 50 ˚C) using Büchi Rotavapor R-114 (Switzerland) before freeze drying. To prepare the aqueous extracts, the samples were boiled in water (100 ºC, 1 hour) and centrifuged at 5000 × *g* for 15 min (Lin *et al*., 1995). The decoctions were filtered through Whatman No.1 filter paper placed on a Buchner® funnel. The filtrates were then frozen overnight before freeze-drying.

The dried crude ethanol and aqueous extracts were weighed and stored in glass vials and placed in dark containers at -20 ºC until used for further tests. All crude extracts used for antioxidant assays, lipogenesis assay, lipolysis assay and gene expression studies were freshly prepared. The crude extracts were dissolved completely in ultra pure water (for aqueous extracts) or 10 % (v/v) DMSO (for ethanol extract); and filter-sterilized using 0.2 µm sterile filter before diluting to the desired concentrations, depending on the requirement for each assay.

3.2 Determination of antioxidant potential and polyphenol content in extracts of unfermented and fermented wheat grains

3.2.1 DPPH free radical scavenging activity assay

The DPPH assay was performed by a method adapted from Daker *et al*., (2008). In this assay butylated hydroxytoluene (BHT, 25 µg/mL) was used as positive control. Briefly, five microlitre of 25 µg/mL of BHT and various concentrations of extracts of unfermented and fermented wheat grains were added in a 96 well plate. Then, one hundred and ninety five microlitres of DPPH were added into the wells containing extracts. The absorbance of the incubation mixture was measured every 20 minutes for three hours at a wavelength of 515nm. The percentage of DPPH scavenged by the test samples was calculated according to the following formula:

$$\text{DPPH scavenged (\%)} = \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \times 100\%$$

3.2.2 ABTS radical scavenging assay

The scavenging capacity of the aqueous and ethanol extracts of unfermented and fermented wheat grains on $ABTS^{\cdot+}$ was measured according to the method outlined by Ng *et al*., (2011). ABTS was dissolved in 5 mL of distilled water to yield a concentration of 7 mM. ABTS radical monocation $[ABTS]^+$ was produced by reacting ABTS stock solution with 104 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12–16 hours before use. Then the $[ABTS]^+$ solution was diluted with ethanol to an absorbance of 0.70 (±0.02) at 734 nm and equilibrated at 30°C. Ten microlitre of various concentrations of extracts of unfermented and fermented wheat grains were added in a 96 well plate. Then one hundred microlitres of $[ABTS]^+$ was added to the wells containing test sample. The absorbance of the incubation mixture was measured after four minutes at a wavelength of 734 nm. In this assay BHT (91 µg/mL) was used as positive control. The percentage of $[ABTS]^+$ scavenged by the test samples was calculated according to the following formula:

$$[ABTS]^+ \text{ scavenged (\%)} = \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \times 100\%$$

3.2.3 Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was conducted according to the method modified by Ng *et al*., (2011). Freshly prepared FRAP reagent was warmed to 37 ˚C. A series of standard was prepared by adding 10 µL of various concentrations of $FeSO_4.7H_2O$ (concentration range of 0 – 1000 µmol/mL) to 300 µL of FRAP reagent. As for the extracts, 10 µL of extracts was used to substitute the $FeSO_4.7H_2O$. An ELISA micro plate reader was then

used to read the absorbance of the mixture at 593 nm. The plate was monitored spectrophotometrically using the kinetic mode between 0 to 4 minutes. Absorbance was read against water as the reagent blank. In this assay, BHT (3.33 µg/mL) was used as positive control. The FRAP value for the extracts were quantified using the calibration plot (Appendix A (I)) and were expressed as µmol $FeSO_4.7H_2O$ equivalent / 100 g of extract.

3.2.4 Total phenol content (TPC) in extracts of unfermented and fermented wheat grains

TPC was determined by the Folin-Ciocalteu's method modified by Ng *et al*., (2011). Folin Ciocalteu's phenol reagent (0.1 v/v) was prepared and 50 µL of it was mixed with 50 µL of extracts of unfermented and fermented wheat grains. After 3 minutes incubation at ambient temperature, 100 µL of 10 % (w/v) sodium carbonate (Na_2CO_3) was added and incubated again in the dark for one hour. The colour change was measured spectrophotometrically at 750 nm. In this assay BHT (250 µg/mL) was used as a positive control and gallic acid (GA) (0 – 25 µg/mL) was used as a standard. The results were calculated using the GA calibration plot (Appendix A (II)) and expressed as milligrams GA equivalent over 1 g lyophilized extract (mg GA / 100 g of extract).

3.2.5 Inhibition of lipid peroxidation of cooking oils by extracts of unfermented and fermented wheat grains

This assay was carried out according to Daker *et al*., (2008) with slight modification. The crude extracts were dissolved in analytical grade DMSO to obtain a stock solution of 50 mg/mL. The extracts were diluted accordingly with DMSO then

mixed with 19.8 ml of palm cooking oil to yield final extract concentrations of 250 µg/mL. Erlenmeyer flasks containing oil added with 1% (v/v) DMSO (blank) and BHT, 250 µg/mL (positive control) were prepared. In order to simulate frying conditions, the flasks were heated over hot plate to allow the contents to boil (at about 180 ºC) for 10 minutes.

Lipid peroxidation assay was carried out in microcentrifuge tubes, which contained 125 µL of TCA (15% w/v), 250 µL of TBA (1% w/v) and 250 µL of cooking oils heated with DMSO, BHT, or extracts. The reaction tubes were then incubated in a boiling water bath for ten minutes. Upon cooling, the tubes were centrifuged at 3500 x *g* for ten minutes to separate the aqueous and oil phase. The formation of thiobarbituric acid reactive substances (TBARS) was measured at 532 nm. Finally the oil mixtures were sealed with parafilm and stored in the dark. The assay was carried out to determine the storage effect of used oil over 50 days (days 0, 10, 30 and 50). The results were expressed as absorbance at 532nm against incubation period (days).

3.3 3T3-L1 preadipocyte viability in the absence and presence of oxidative stress

3.3.1 Cell number estimation with trypan blue stain

This assay was carried out according to Tolnai (1975). Confluent preadipocyte were harvested from 25 cm^2 culture flasks using 1 mL of 0.25 % Trypsin-EDTA. A 10 µL 3T3-L1 preadipocyte suspension obtained through trypsinization was mixed well with 10 µL of trypan blue dye to obtain a 1:1 dilution. The mixture was then loaded onto a Neubour improved haemocytometer and observed under an inverted microscope.

The number of viable cells present in preadipocyte suspension was calculated using the following formula:

Cell concentration (cells/ml)	= mean of viable cells × dilution factor x 10^4
	= mean of viable cells × 2 x 10^4

3.3.2 Colorimetric MTT assay

The MTT reagent was prepared by dissolving 5 mg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) in 1 mL of phosphate buffer saline (PBS). Then the MTT reagent was filter-sterilized with 0.2 µm filter and stored at -20ºC. MTT reagent is stable at -20˚C in the dark for up to six months, provided there is no contamination.

Preadipocyte were seeded at density, 10 000 cells / well in 96-well tissue culture plate were allowed to attach for 24 hours prior to the cell viability assay. To study the significance of oxidative stress on 3T3-L1 cell viability, endogenous cellular production of ROS was induced by incubating cells for 2 hours with 2 mU/mL of glucose oxidase (GOx) before incubating the cells with different doses of extracts. Concentration of GOx used was determined based on a preliminary dose-response study (Appendix B (V)).

Subsequently, the preadipocyte were treated with various extracts at different concentrations and incubated in the absence (48 hours) and presence (24, 48 and 72 hours) of oxidative stress before MTT reagent was added into each well. In this MTT assay, ultra pure water (control for aqueous extract) and 1 % (v/v) DMSO (control for ethanol extract) was used to replace the various extracts of unfermented and fermented wheat grains. After 4 hours, the culture medium containing MTT was carefully removed

using a small-bore needle syringe (27G) to ensure the formazan formed was not aspirated along with the medium. One hundred microlitres of absolute DMSO was then added into each well and left on plate shaker at room temperature for 5 minutes to make sure all formazan crystals were dissolved completely in DMSO. The absorbance of formazan was measured at 560 nm. The cell viability was expressed in percentage of viability over the control cells.

$$\text{Percentage of viability (\%)} = \frac{\text{Cells treated with extract}}{\text{Control cells}} \times 100\ \%$$

3.4 Preadipocyte differentiation

Confluent preadipocyte (day 0) were induced to differentiate into adipocyte according to a standard MDI (1-methyl-3-isobutylxanthine (IBMX), dexamethasone, and insulin) protocol but with some modifications (Takenouchi et *al.*, 2004). For the first 24 hours, confluent preadipocyte were incubated in humidified air jacketed incubator with Part-1 differentiation media (DM1) which consisted of DMEM supplemented with 10 % FBS, 0.5 mM IBMX, 1.0 µM dexamethasone and 10 µg/mL insulin.

Subsequently, the cells were cultured in Part-2 differentiation (DM2) media which consisted of DMEM supplemented with 10 % FBS and 10 µg/mL insulin only. The cells were maintained in DM2 until they were fully differentiated into mature adipocyte. Optimum period for any experiment to be carried out on adipocyte was nine to twelve days after the initiation of differentiation.

3.4.1 Lipogenesis in the absence and presence of oxidative stress (Oil Red O assay)

The lipogenic activity and differentiation level in preadipocyte could be easily assessed using Oil Red O staining (Lim *et al.*, 2008). The Oil Red O stock solution was prepared by dissolving 500 mg Oil Red O with 100 ml isopropanol. Next, the stock solution was diluted to 60 % (v/v) with distilled water to form Oil Red O working solution. The working solution was stable for 3 hours and had to be filtered prior to staining process.

Various concentrations of aqueous and ethanol extracts of unfermented and wheat fermented with *Ganoderma* spp. were used to replace insulin in DM2 to assess their lipogenic activity. To investigate the oxidative stress effect on 3T3-L1 preadipocyte differentiation, endogenous cellular production of ROS was induced by incubating cells for 2 hours with 2 mU/mL of GOx prior to treating the cells with different doses of extracts on day 2. Insulin (1µM) was used as the positive control and cells treated with ultra pure water / 1 % DMSO was used as the experimental control in this assay. Concentration of insulin and GOx used was determined based on a preliminary dose-response study (Appendix B (II) and (VI)).

Twelve days after the initiation of differentiation, culture plates containing differentiated adipocyte were washed twice with PBS followed by fixation in 10 % formaldehyde for an hour. Fixed cells were then rinsed with 60% isopropanol and stained by immersion in Oil Red O working solution at room temperature for 10 minutes. After staining, the cells were washed with distilled water again to remove excess Oil Red O dye which did not incorporate into the adipocyte. Next, the stained adipocytes were allowed to dry at room temperature.

The amount of lipid accumulated in adipocyte could be visualized microscopically after the Oil Red O stain had dried. To measure the lipogenesis quantitatively, isopropanol was added into each well to dissolve the Oil Red O dye incorporated into the adipocyte and the absorbance of Oil Red O staining was measured spectrophotometrically at 510 nm.The quantity of lipogenesis was expressed in percent of difference as compared to untreated cells.

$$\text{Percentage of differences (\%)} = \frac{\text{(Stimulated – Control)}}{\text{Control}} \times 100\ \%$$

3.4.2 Lipolysis (Glycerol quantitative assay)

The spent media of completely differentiated adipocyte (section 3.4) was aspirated and replaced with fresh growth media. Subsequently, the cells were incubated with 100 µg/mL freshly prepared unfermented and fermented wheat grain extracts or positive control (epinephrine, 1 µM) separately for 24 hours at 37 ˚C (Lim *et al*., 2008). Concentration of epinephrine used was determined based on a preliminary dose-response study (Appendix B (IV)).

The release of glycerol was quantified by using a commercialized lipolysis assay kit by Enzychrom^{TM} (Cat. no.: EAPL-200). The assay was performed as per the instruction in the protocol. Briefly, at the end of incubation, the culture supernatants from each well were collected into microcentrifuge tubes and 10 µL of samples were transferred into separate wells of the 96-well assay plate. One hundred microliters of working reagent was added into each well and incubated for 20 minutes at room temperature. Optical density of quinoneimine dye formed (indication of glycerol release) was measured at 570 nm. The concentration of glycerol released in the medium

were quantified using the glycerol calibration plot (Appendix B (III)) and were expressed as µg/mL of glycerol.

$$[\text{Glycerol}] = \frac{(OD_{Sample} - OD_{Medium})}{\text{Slope}} \quad (\mu g/ml)$$

OD_{Sample} and OD_{Medium} are optical density values of the sample and medium. The slope was obtained from the glycerol calibration plot (Appendix B (III)).

3.4.3 Gene expression assessed by real time reverse transcription polymerase chain reaction (RT-PCR)

3.4.3.1 Cell culture treatments

3T3-L1 preadipocyte cells were harvested and differentiated in 25 cm^2 flasks as described earlier (Section 3.4). On day-2 after the initiation of differentiation, the spent media was aspirated and replaced with media containing 100 µg/mL of fermented wheat grain extracts. The cells were allowed to differentiate and fully mature into adipocyte before total RNA was extracted.

3.4.3.2 RNA extraction

Cellular total RNA was extracted and purified by using a commercialized spin cartridge kit, RNAqueous® - 4PCR kit, AmBion (Part no.: AM1914). The extraction and purification processes were performed as per the protocol provided. In brief, spent media was removed and the cells were washed with PBS thoroughly. Next, the cells were lysed and homogenized in the lysis buffer provided. The lysate was then

transferred into a microcentrifuge tube. The homogenization was performed by draining it through a small-bore needle syringe (27G) several times. An equal volume of 64% (v/v) ethanol was added into the homogenate and the mixture was centrifuged in a RNA filter cartridge. RNA binds to the silica base membrane in the cartridge. Impurities were washed out with the wash buffers provided in the kit. Finally, the RNA was eluted out with elution solution and collected in a recovery tube. Homogenates were kept ice-cold to prevent RNase activity. Purified RNA was used immediately for reverse transcription.

Purity of the RNA extracted was checked spectrophotometrically (A_{260}: A_{280} = 1.8 - 2.1) and the concentration of the RNA was calculated by the following formula:

Total RNA (µg) = A260nm × 40 µg/ml × Dilution factor × Volume (ml)

3.4.3.3 Reverse transcription (RT)

The total RNA extracted was used immediately for cDNA synthesis. Reverse transcription was performed by using a commercialized kit (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Product no.: 4368814). Two micrograms of total RNA was used in every 20 µL reactions as instructed in the user's manual. For every 20 µL reaction, the total RNA sample was mixed with an equal volume of the 2X reaction master mix. The content of the master mix used was as stated in Table 3.1. The mixture was mixed carefully and was briefly centrifuged to spin down the contents and eliminate air bubbles. Reverse transcription was performed on a thermalcycler under the conditions shown in the following table. The cDNA product was stored at -80˚ C.

Table 3.1: Composition of 2X concentrated reaction master mix for reverse transcription.

Reaction Master Mix (2X)	
Component	**Volume (µl)**
10X RT buffer	2.0
25X dNTP mix (100 mM)	0.8
10X RT Random primers	2.0
Multiscribe reverse ™ transcriptase	1.0
Nuclease-free water	4.2
Total volume per reaction	10

Table 3.2: Thermalcycler conditions for RT

Step	1	2	3	4
Temperature (ºC)	25	37	85	4
Time	10min	120min	5sec	Hold

3.4.3.4 Gene expression assay

Reaction setup for all TaqMan® Gene Expression assay was performed according to the reaction setup instructions generated by the StepOne software (Ver. 2.0, Applied Biosystems). Briefly, reaction mix which consisted of the TaqMan® Gene Expression Master Mix (Product no.: 4374657) and assay mix was prepared separately, as each assay mix contained corresponding primers and probe for each gene assessed. All reagents were kept on ice, once thawed. Each reaction was run in triplicate. The reaction mix was mixed with either sterile ultra-pure water (for no template control reactions) or isolated cDNA. The mixture was transferred into fluorescence-compatible fast reaction tubes/strips (MicroAmp™; Product no.: 4358293) and capped with optical caps (Product no.: 4323032). The strips were centrifuged briefly and loaded into the real time PCR thermalcycler (StepOnePlus™ Real Time PCR system).

In this study, all fluorescent emissions were referenced to ROX™ to increase precision of the intensity by normalizing each value to a steady internal normalizer. The relative expression of the investigated genes was normalized with the endogenous control (*e.g.*, 18s). CT values are means of triplicate measurements. Relative quantification was determined by the ΔΔCT method (Kroh *et al.*, 2010). The calculations and formulas involved were as follows. In the respective experiments and if necessary, eukaryotic 18S rRNA was used as the inter-plate calibrator.

$$\text{CT Target} - \text{CT Endogenous control} = \Delta CT$$

$$\Delta CT \text{ Sample} - \Delta CT \text{ Calibrator} = \Delta\Delta CT$$

$$\text{Relative fold change} = 2^{-\Delta\Delta CT}$$

Results were expressed as n-fold difference over respective controls. Fold variation values lower than 0 were expressed as negative values (*e.g.,* a n-fold variation of 0.50 is expressed as -2.00) and values lesser 1 indicates down-regulations the gene. For statistical analyses of real time RT-PCR experiments, results for a given gene were expressed as difference from the ΔCT value obtained between treated versus un-treated.

3.4.3.5 Genes investigated in this study

The genes used in this study were PPARγ, adiponectin, LPL, GLUT4, HSL, Bax and GPx3. They were purchased from Applied Biosystems. All TaqMan® probes used in this study were labelled with FAM™ reporter dye at the 5'end and a MGB quencher at the 3' end. The following table (Table 3.3) shows the list of genes and corresponding accession numbers investigated in this study.

3.5 Statistical analysis and software

3.5.1 Analyses

All experiments were performed in triplicate, unless stated otherwise and the data were calculated as mean ± standard error mean (SEM). One-way analysis of variance with Duncan's multiple range tests (DMRT) was used to determine any significant differences between means. Statistical significance was accepted at $p<0.05$. Pearson's correlation test was used to determine the correlation between the biochemical assays.

Table 3.3: Selected genes for gene expression study in 3T3-L1 adipocyte

NO.	Gene name and abbreviation	Assay ID	Accession number
1	Glucose transporter 4 (GLUT4)	Mm00436615_m1	NM_009204.2
2	Hormone sensitive lipase (HSL)	Mm00495359_m1	NM_010719.5
3	Lipoprotein lipase (LPl)	Mm00434770_m1	NM_008509.2
4	Glutathione peroxidise 3 (GPx3)	Mm00492427_m1	NM_008161.2
5	bcl-2-Associated X Protein (Bax)	Mm00432051_m1	NM_007527.3
6	Peroxisome proliferator-activated receptor gamma (PPARγ)	Mm01184322_m1	NM_011146.3
7	Adiponectin	Mm00456425_m1	NM_009605.4

General abbreviation of genes selected for this study and corresponding assay ID and accession information available on Applied Biosystems website. Information on the functions of these genes is available in the literature review section (Sec.2.6.8). Assay ID refers to the Applied Biosystems Gene Expression Assays inventoried kits with proprietary primer and TaqMan® probe mix. Assay ID with "Mm" prefix is defined as "Mus musculus". All Gene Expression Assay kits indicated here is FAM/MGB probed.

3.5.2 Software

Analyses and graphs were prepared with Microsoft Office 2003 EXCEL, STATGRAPHICS Plus software (version 3.0, Statistical Graphics Corp., Princeton, NJ) and GraphPad Prism version 5.02 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

CHAPTER 4

RESULTS

4.1 Solid substrate fermentation and extraction yield

The white mycelia inoculum grown in GYMP agar medium in the sealed Petri dish spread out and fully colonized the agar plate in seven days (Figure 4.1). All the mycelia of three different *Ganoderma* spp. used in this study had identical appearance and similar growth rate. The 7 mm mycelia plugs were then transferred into Erlenmeyer flasks containing autoclaved wheat grains for SSF (Figure 4.2). A preliminary study was done on four different solid substrates; wheat, green beans, corn, and white polished rice. All the *Ganoderma* spp. mycelia studied showed good growth on wheat grains (Figure 4.2).

The percentage of moisture content, yield of fermented wheat, and total solubles yield from extracts of unfermented and fermented wheat grains are shown in Table 4.1. Generally, the yields of aqueous extracts were significantly higher ($p<0.05$) than those of ethanol extracts for all the fermented wheat grain compared to the unfermented wheat grain. This indicated that the fermentation process improved the contents of total soluble polysaccharides which could be precipitated from the aqueous extract by the use of absolute ethanol (Lee *et al.*, 2007).

A resinum dark brown crude ethanol extract was obtained. Each of the ethanol extracts obtained were dissolved in absolute DMSO to give 10 mg/mL stock solutions and further dilution was done from that. All the *in vitro* assays were carried out immediately after the preparation of samples using DMSO.

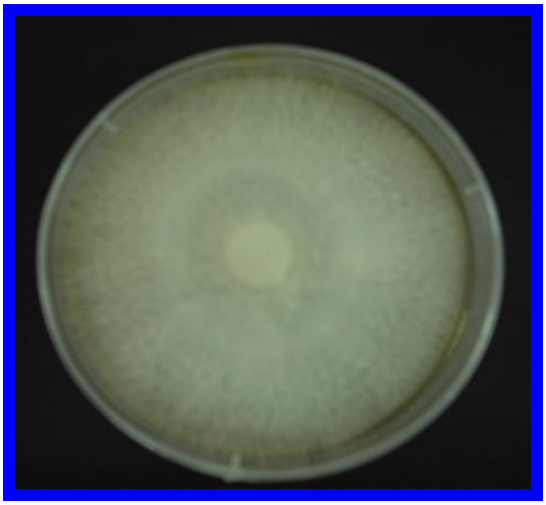


Figure 4.1: *G.australe* mycelia grown on GYPM agar

(A) Unfermented wheat grains

(B) Fermented wheat grains

Figure 4.2: (A) Unfermented wheat grains and (B) wheat grains fermented with *G. australe* mycelia

Sterilized wheat grains were inoculated with five 7 mm diameter plugs of G. *australe*. mycelia and incubated at room temperature (25 ± 2ºC) for 14 days in an incubator under dark and static condition. All the mycelia of three different *Ganoderma* spp. used in this study had identical appearance and similar growth rate.

Table 4.1: Percentage of moisture content, yield of fermented wheat, and yield of solubles extracted from unfermented and fermented wheat grains

Sample	% moisture content (mean ± SD)	Yield of fermented substrate (%) (Wet weight)	Total solubles (%)	
			Hot water extract (mean ± SD)	Ethanol extract (mean ± SD)
Unfermented wheat	41.55 ± 1.27	93.34	0.51 ± 0.20^{a}	0.85 ± 0.16^{a}
KUM60813	44.00 ± 1.47	93.27	1.45 ± 0.10^{c}	1.14 ± 0.13^{d}
KUM61076	40.76 ± 0.99	93.83	1.39 ± 0.39^{b}	1.06 ± 0.13^{c}
VITA GL	44.32 ± 1.46	91.32	1.73 ± 0.25^{d}	0.96 ± 0.43^{b}

Values are expressed as mean ± standard deviation (SD) of triplicate measurements. Values with different alphabets within a column are significantly different ($p<0.05$) based on Duncan's multiple-range test.

Similarly, the aqueous extracts dissolved completely in ultra pure water instead of DMSO and were filter-sterilized with 0.2 µm sterile filter before diluting to the appropriate concentrations.

4.2 Antioxidant activity of extracts of unfermented and fermented wheat grains and correlation analysis between TPC levels and the antioxidant indices

The effects of unfermented and fermented wheat extracts on the DPPH· scavenging activity is depicted in Figure 4.3 (A & B). The scavenging activity of both the aqueous extracts and ethanol extracts of fermented wheat grain varied depending on the *Ganoderma* spp. used for fermentation. As for the extracts, generally the ethanol extracts of all the samples tested had a significantly ($p < 0.05$) higher DPPH$^{\cdot}$ scavenging abilities compared to their respective aqueous extracts.

The scavenging effects on DPPH$^{\cdot}$ by ethanol extracts of each sample was concentration dependent, with stronger inhibition occurring at higher concentrations. However, the scavenging ability of aqueous extract of unfermented wheat grain decreased with an increase of extract concentration from 100 µg/mL to 250 µg/mL. The ethanol extracts of the unfermented and wheat grain fermented with KUM60813, KUM61076 and *VITA GL* mycelium showed scavenging potencies of 36.04 %, 43.18 %, 61.02 % and 27.43 %, respectively at a final concentration of 250 µg/mL.

Among the fermented wheat grain, ethanol extract of KUM61076 fermented wheat grain demonstrated the most efficient radical scavenging ability (Figure 4.3 A & B). Whereas wheat grain fermented with *VITA GL* was less effective in scavenging DPPH radicals compared to the two wild *Ganoderma* spp. used in this study. The

experimental positive control, BHT (25 µg/mL) showed higher scavenging effects compared to the wheat grain extracts tested in this study.

Figure 4.4 (A & B) portrays $[ABTS]^+$ scavenging capacity by the aqueous and ethanol extracts of unfermented and *Ganoderma* spp. fermented wheat grain. In this assay, BHT, 91 µg/mL was used as a positive standard reference for comparison with each crude extract. The crude extracts were able to reduce the green coloured stable $[ABTS]^+$ to the colourless neutral form. Based on the results obtained, the $[ABTS]^+$ scavenging ability of all the extracts tested increased in a dose-dependent manner. The ethanol extracts of fermented wheat grain were more potent in scavenging the ABTS radical with regards to their respective aqueous extracts (Figure 4.4 (A&B)). In addition, the aqueous extract of unfermented wheat was consistently higher compared to the aqueous extracts of fermented wheat grain at all concentrations tested. Ethanol extract of KUM60813 fermented wheat grain showed the highest percentage (45.7 %) of $[ABTS]^+$quenching capacity, at the extract concentration of 10 mg/mL; followed by KUM61076 and *VITA GL* fermented wheat grain which showed 34.57 % and 21.64 %, quenching respectively. This was possibly due to aggregation of the extract at higher concentration which in turn could enhance the quenching potency.

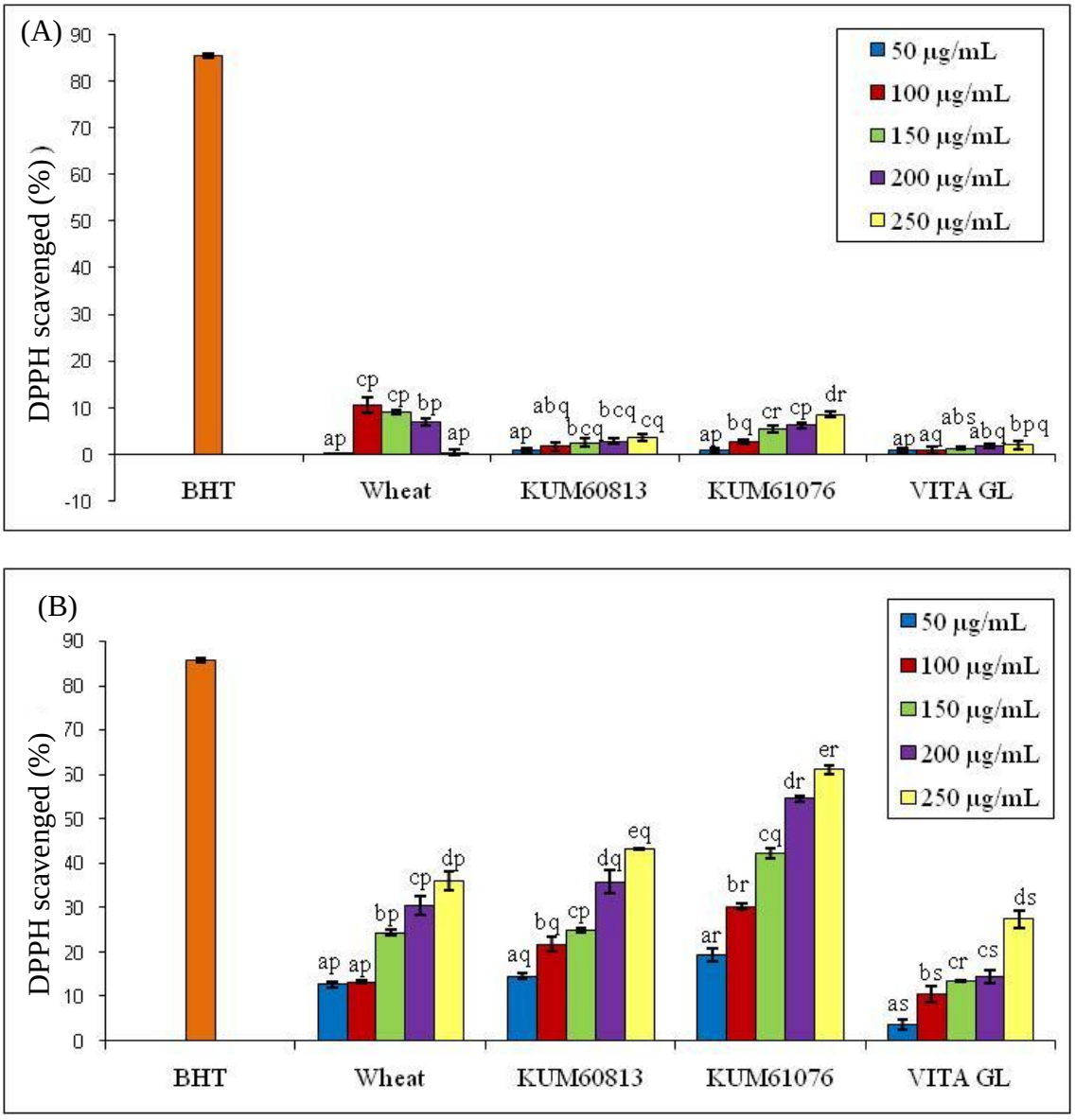


Figure 4.3: DPPH radical scavenging ability of aqueous (A) and ethanol (B) extracts of unfermented wheat and wheat grains fermented with KUM60813, KUM61076 and *VITA GL*.

Each value is expressed as mean ± SD (n=3). For the same extract with different concentrations, means with different alphabets (a-e) are significantly different ($p<0.05$, ANOVA). For different extracts with the same concentration, means with different alphabets (p-t) are significantly different ($p<0.05$, ANOVA).

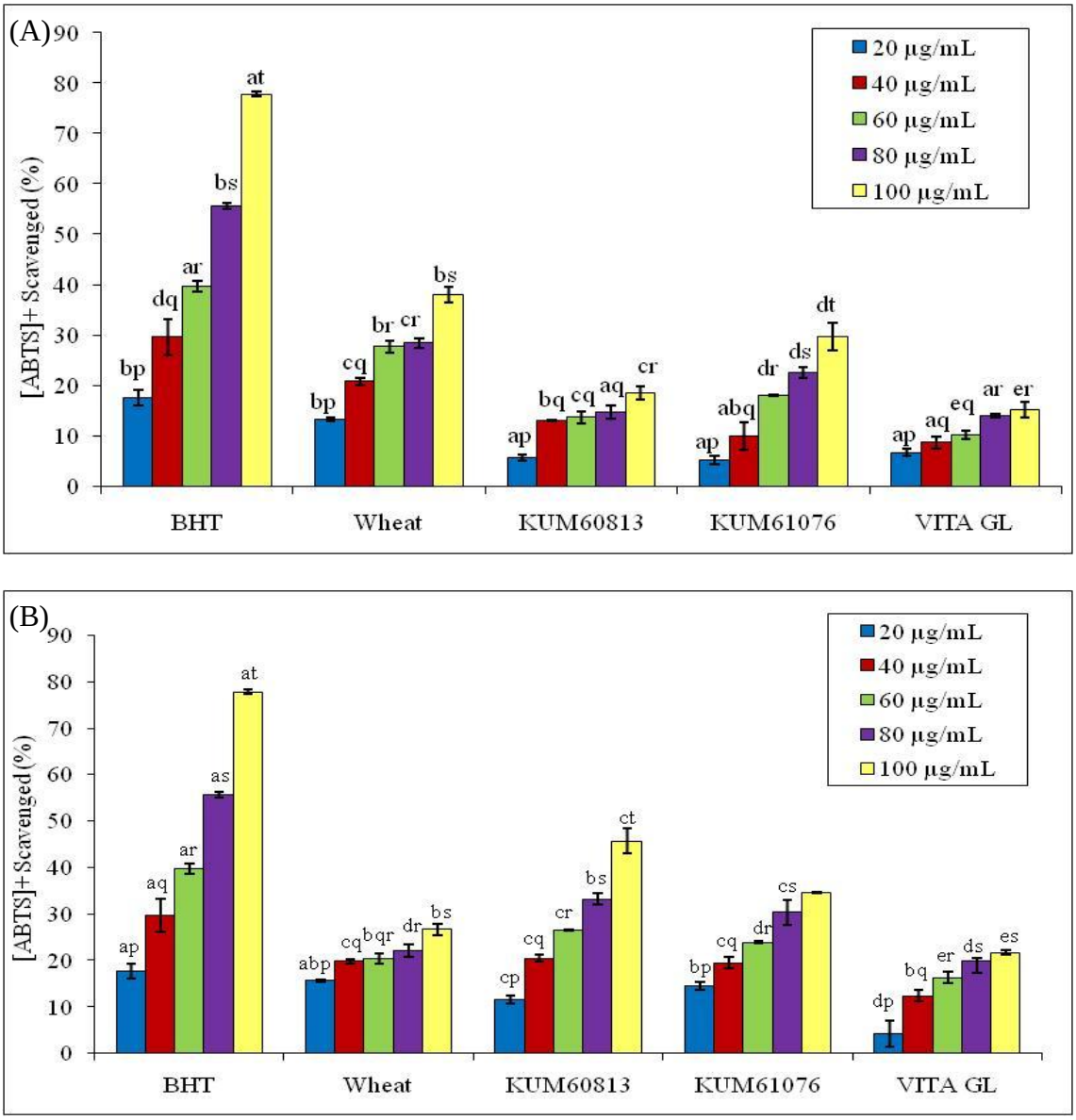


Figure 4.4: $[ABTS]^+$ radical scavenging ability of aqueous (A) and ethanol (B) extracts of unfermented wheat and wheat grains fermented with KUM60813, KUM61076 and *VITA GL*.

Each value is expressed as mean ± SD (n=3). For different extracts with the same concentration, means with different alphabets (a-e) are significantly different ($p<0.05$, ANOVA). For the same extract with different concentrations, means with different alphabets (p-t) are significantly different ($p<0.05$, ANOVA).

Similar to the ABTS and DPPH free radical scavenging pattern, FRAP levels of the aqueous extracts from unfermented and fermented wheat grain were significantly lower ($p<0.05$) than those of ethanol extracts (Table 4.2). The order of potency of ferric reducing capacity per 100 grams of crude ethanol extracts was KUM61076 > unfermented wheat grain > KUM60813 > *VITA GL*. The ethanol extract of wheat fermented with KUM61076 exhibited the highest ferric reducing capacity with FRAP value, 41.36 ± 0.91 mmol FE / 100 g of extract. With regards to the aqueous extracts, the control showed better ferric reducing activity (25.16 ± 1.28 mmol FE / 100 g of extract) which was also significantly higher compared to the fermented wheat grain. Meanwhile BHT showed a FRAP value of 163.84 ± 9.44 mmol FE / 100 g of BHT.

In this study, total phenol content in both aqueous and ethanol extracts of unfermented and fermented wheat grain was measured. In general, the phenol content in the ethanol extracts was twofold higher ($p<0.05$) compared to their respective aqueous extracts. The total phenol content in wheat grain fermented with KUM61076 mycelia was the highest in both ethanol and aqueous extracts, while the wheat fermented with *VITA GL* mycelia yielded lowest phenol content, 365.32 ± 10.93 mg of GAE / 100 g of extract (from aqueous extract) and 655.99 ± 23.31 mg of GAE / 100g of extract (from ethanol extract). Total phenol content in BHT was 1832.36 ± 105.56 mg of GAE / 100 g of BHT.

Pearson correlation analysis was carried out to determine the relationship between TPC levels and the antioxidant indices such as FRAP levels, ABTS and DPPH scavenging ability of the extracts prepared (Appendix A(III)). Strong correlations existed between all the parameters compared (Table 4.3). Cheung *et al.* (2003) reported that the strong antioxidant properties of water extracts of *Lentinus edodes* and *Volvariella volvacea* could be attributed by their polyphenol content. Therefore, higher

Table 4.2: Total phenol content and ferric reducing antioxidant power of the aqueous and ethanol extracts of unfermented and fermented wheat grains

Sample	Total phenol Content (mg of GAE / 100g extract)	FRAP value (mmol FE / 100 g of extract)
Control		
Aqueous	530.90 ± 23.89^{bc}	25.16 ± 1.28^{e}
Ethanol	1172.69 ± 65.65^{de}	35.03 ± 1.40^{f}
KUM60813		
Aqueous	488.67 ± 22.83^{ab}	15.02 ± 0.84^{b}
Ethanol	965.48 ± 16.38^{d}	21.11 ± 0.42^{d}
KUM61076		
Aqueous	616.44 ± 25.68^{c}	14.30 ± 1.04^{b}
Ethanol	1258.67 ± 102.64^{e}	41.36 ± 0.91^{g}
VITA GL		
Aqueous	365.32 ± 10.93^{a}	12.68 ± 0.95^{a}
Ethanol	655.99 ± 23.31^{c}	17.13 ± 1.18^{c}
BHT	1832.36 ± 105.56	163.84 ± 9.44

GAE, gallic acid equivalents; FRAP, ferric reducing antioxidant power

Values expressed are means ± SD of three measurements. Means with different alphabets within a column are significantly different ($p<0.05$, ANOVA).

phenol content is an important factor in contributing to the high antioxidant capacities of these fermented and unfermented wheat grain extracts.

The protective effect of extracts of unfermented and fermented wheat grains against peroxidation of palm cooking oil (occurred during food preparation) was investigated and compared with the synthetic antioxidants. The palm oil (sold in plastic bags) was chosen for this study to investigate the quality of the locally produced palm oil compared to other more expensive oil varieties available in the market.

The 50-day storage effect of unheated and heated oil was assessed to determine the storage effect on lipid peroxidation. The unheated oil by itself showed signs of auto-oxidation to a certain degree. On Day 0, all the heated oils with or without the presence of extracts showed no significant difference in their absorbance value as compared to the unheated oil; except for the oil treated with aqueous extract of KUM60813 showed a mild increase in the absorbance reading (Figure 4.5 (a)). On Day 10, Day 30 and Day 50, TBARS were observed in oil supplemented with DMSO, BHT and aqueous extracts of unfermented and fermented wheat grains indicating the occurrence of lipid peroxidation. Oils supplemented with ethanol extracts of wheat grains fermented with KUM61076 and *VITA GL* mycelia showed no significant differences in their absorbance value from Day 0 to Day 50. This proofs that lipid peroxidation in palm cooking oil was not affected by the presence of both the dry extracts.

Table 4.3: Correlation between total phenol content and antioxidant activities.

Parameters	Pearson correlation value (r^2)
TPC/DPPH	+0.957[a]
TPC/ABTS	+0.763[a]
TPC/FRAP	+0.888[a]

The correlation between TPC, FRAP, ABTS and DPPH status of all crude extracts were assessed using Pearson correlation analysis (GraphPad Prism for Windows, Version 5.01). Positive (+) value denotes positive correlation.

[a] Significant correlations are indicated, $p<0.01$ level (2-tailed).

On the other hand, lipid peroxidation in palm cooking oil was extensively minimized, when heating oil was done in the presence of ethanol extract of wheat grains fermented with KUM60813 mycelia. Statistical analysis of the results indicated that even after fifty days, the oil heated with ethanol extract of wheat grain fermented with KUM60813 mycelia, did not deteriorate; the absorbance were always almost similar to the absorbance reading of unheated oil and there were no pink TBARS observed (Figure 4.5 (b)). When compared to BHT, 250 µg/ml the ethanol extract of wheat grains fermented with KUM60813 mycelia showed a far better inhibitory effect against oil peroxidation, even though their concentrations were similar. This indicated that the ethanol extract of wheat grain fermented with KUM60813 mycelia has a good potential as a natural antioxidant for food preservative to replace synthetic chemicals.

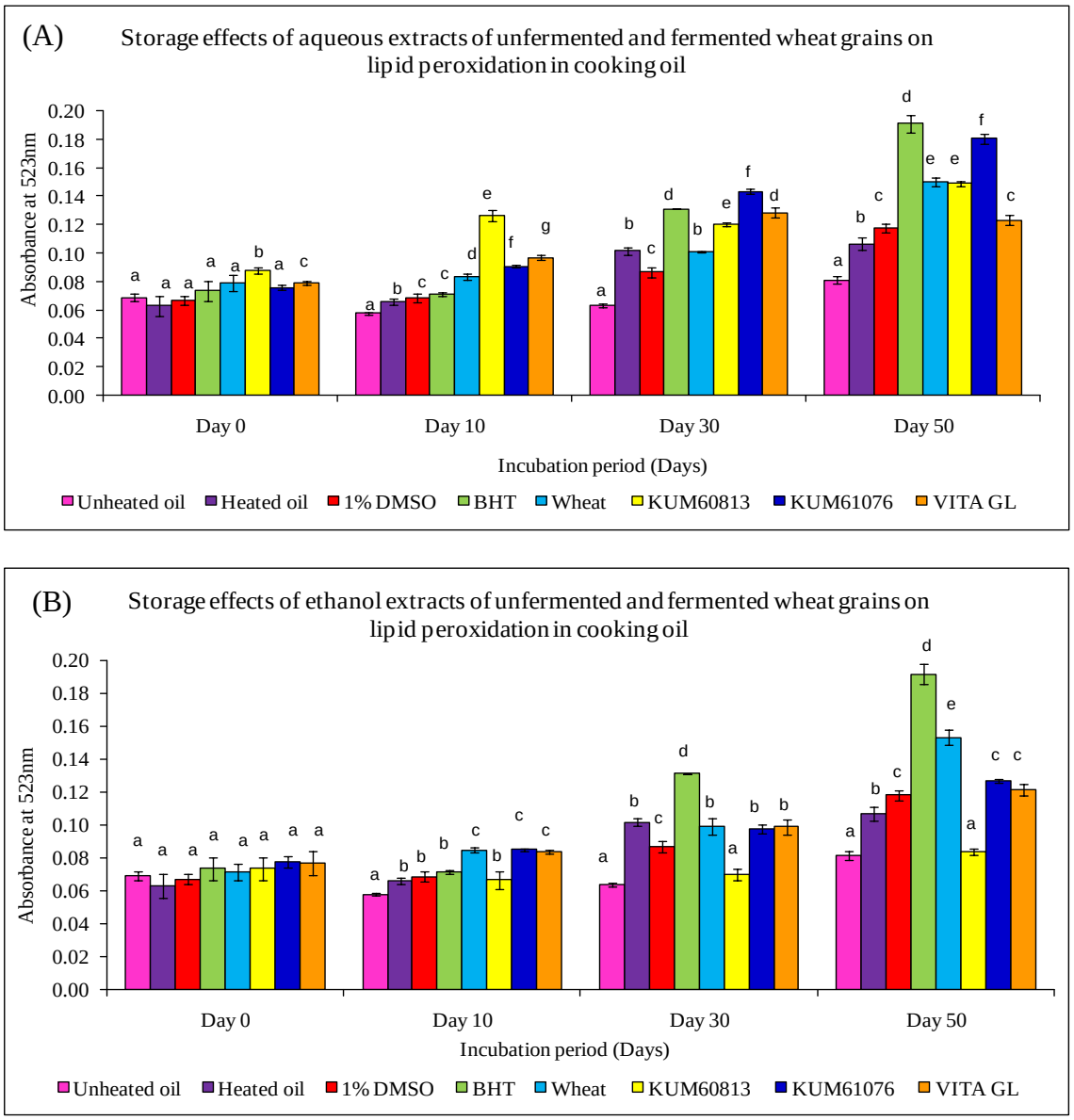


Figure 4.5: Storage effects of aqueous (A) and ethanol (B) extracts of unfermented and fermented wheat grains on lipid peroxidation in palm cooking oil

Absorbance values of different reaction mixtures to demonstrate inhibition of lipid peroxidation in cooking oil by BHT, aqueous and ethanol extracts of unfermented wheat and wheat grains fermented with KUM60813, KUM61076 and *VITA GL* mycelia. Concentration of each sample is 250 µg/mL. Each value is expressed as mean ± SD (n=3). Means with different alphabets within a day are significantly different ($p<0.05$, ANOVA).

4.3 Effect of extracts of unfermented and fermented wheat grains on viability of 3T3-L1 preadipocyte in the absence and presence of oxidative stress

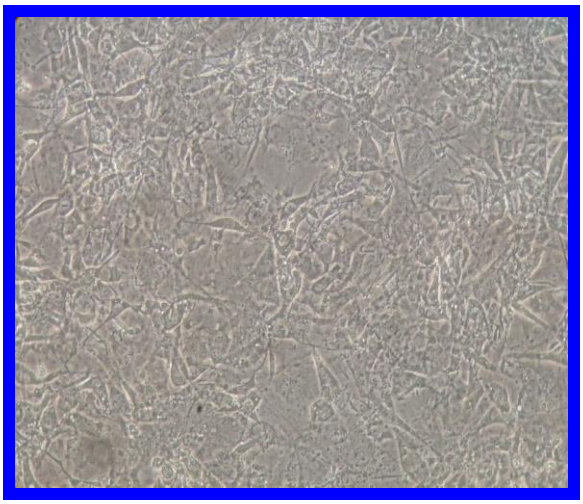


Figure 4.6 : Confluent preadipocyte (200X)

The morphology of preadipocyte was fibroblastic-like (Figure 4.6) which adhere onto the surface of culture plates in clusters due to the presence of fibronectin and extracellular matrix which interconnect the preadipocyte (Gregoire *et. al.*, 1998). In this study, the 3T3-L1 pre-adipocyte viability was screened using MTT assay to determine the optimal cell density for further test. The best cell density was selected based on the appropriate absorbance reading (±0.500) (Appendix B (I)). From the standard graph, a cell density of 10,000 cells per well were chosen as the best cell concentration as it showed an average range of absorbance reading.

To determine the cytotoxicity of both the aqueous and ethanol extracts of unfermented and fermented wheat grain 3T3-L1 preadipocyte was incubated with the extracts at various concentrations (20 – 100 µg/mL) and the cell viability was measured using the MTT assay. No significant cytotoxicity was observed when the cells were incubated with unfermented and fermented wheat grain extracts up to 100 µg/mL, the

cell viability was > 90% when compared to the control cells (Figure 4.7 & Figure 4.8). The fermented wheat grain extracts significantly ($p<0.05$) increased the viability of 3T3-L1 preadipocyte (7 – 37 % stimulation of proliferation) compared to the control cells (cells treated with ultra pure water, 100 % viability).

In the first series of experiment the effect of extracts of unfermented and fermented wheat grains on 3T3-L1 preadipocyte viability was evaluated. Next, the protective effects of these extracts on oxidative stress induced 3T3-L1 preadipocyte cells and its underlying mechanism was investigated. Endogenous cellular production of ROS was induced by incubating cells for 2 hours with 2 mU/mL of glucose oxidase (GOx).

The GOx treated cells (without treatment of extracts) constantly showed an 80 – 85 % inhibition of the preadipocyte viability after 24, 48 and 72 hours incubation (Figures 4.9 A-H). After the 24 hours treatments, none of the aqueous and ethanol extracts provided a repair/protective effect towards the 3T3-L1 cells, upon exposure to oxidative stress. However, when the treatment time was prolonged to 48 and 72 hours, these extracts were able to raise the cell viability up to 50 % (48 hours) and 75 % (72 hours), regardless of the extract types. Interestingly, incubation with ethanol extracts of KUM61076 mycelia fermented wheat grains for 48 and 72 hours considerably enhanced viability of cells against GOx induced cell death as compared to the other extracts tested.

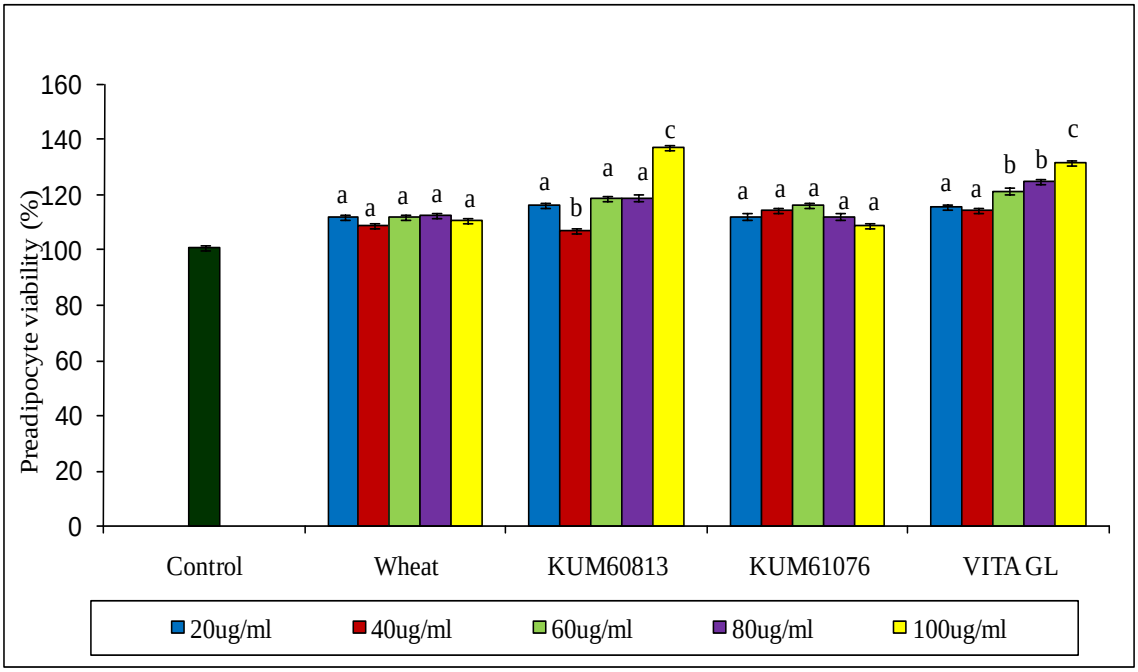


Figure 4.7: Effect of aqueous extracts of unfermented and fermented wheat grains on 3T3-L1 preadipocyte viability

Y-axis indicates the mean percentages of preadipocyte viability ± SD of triplicate assays compared to control value (cells treated with ultra pure water). Values above 100% indicates preadipocyte proliferation. Preadipocyte (10 000 cells / well) were incubated with various concentrations of aqueous extracts of unfermented and *Ganoderma* spp. mycelia fermented wheat grain (20, 40, 60, 80, and 100 µg/mL) for 48 hours prior to MTT assay. Means with different alphabets within a extract are significantly different ($p<0.05$, ANOVA).

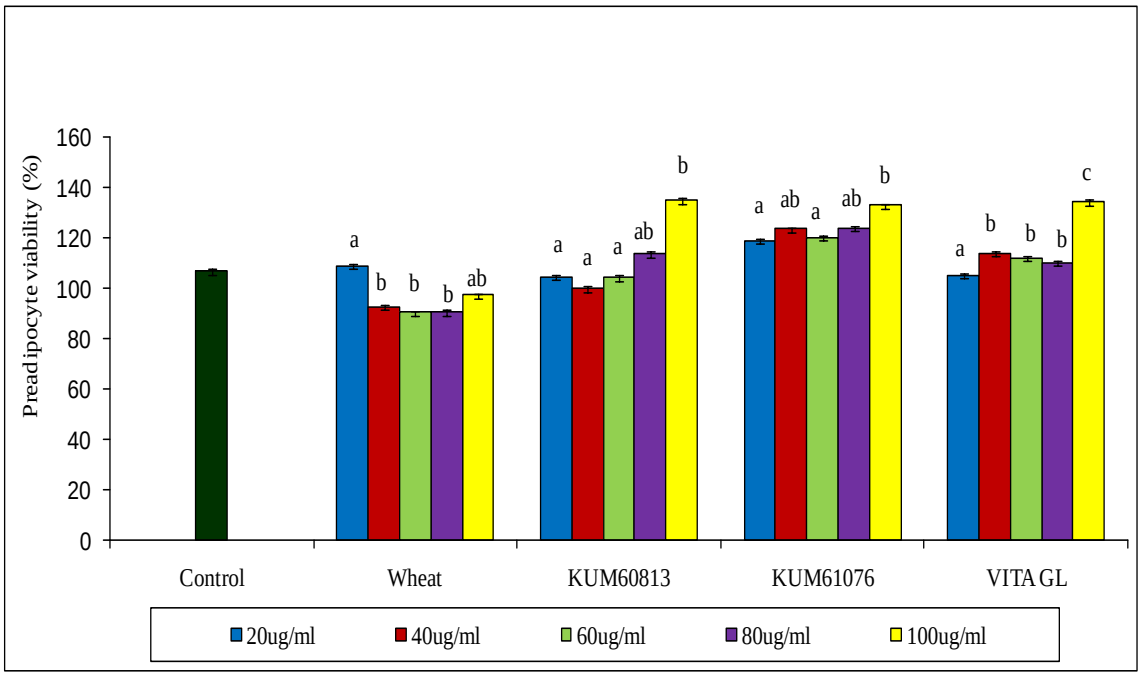


Figure 4.8: Effect of ethanol extracts of unfermented and fermented wheat grains on 3T3-L1 preadipocyte viability

Y-axis indicates the mean percentages of preadipocyte viability ± SD of triplicate assays compared to control value (cells treated with 1% DMSO). Values above 100% indicates preadipocyte proliferation. Preadipocyte (10 000 cells / well) were incubated with various concentrations of aqueous extracts of unfermented and *Ganoderma* spp. mycelia fermented wheat grain (20, 40, 60, 80, and 100 µg/mL) for 48 hours prior to MTT assay. Means with different alphabets within a extract are significantly different ($p<0.05$, ANOVA).

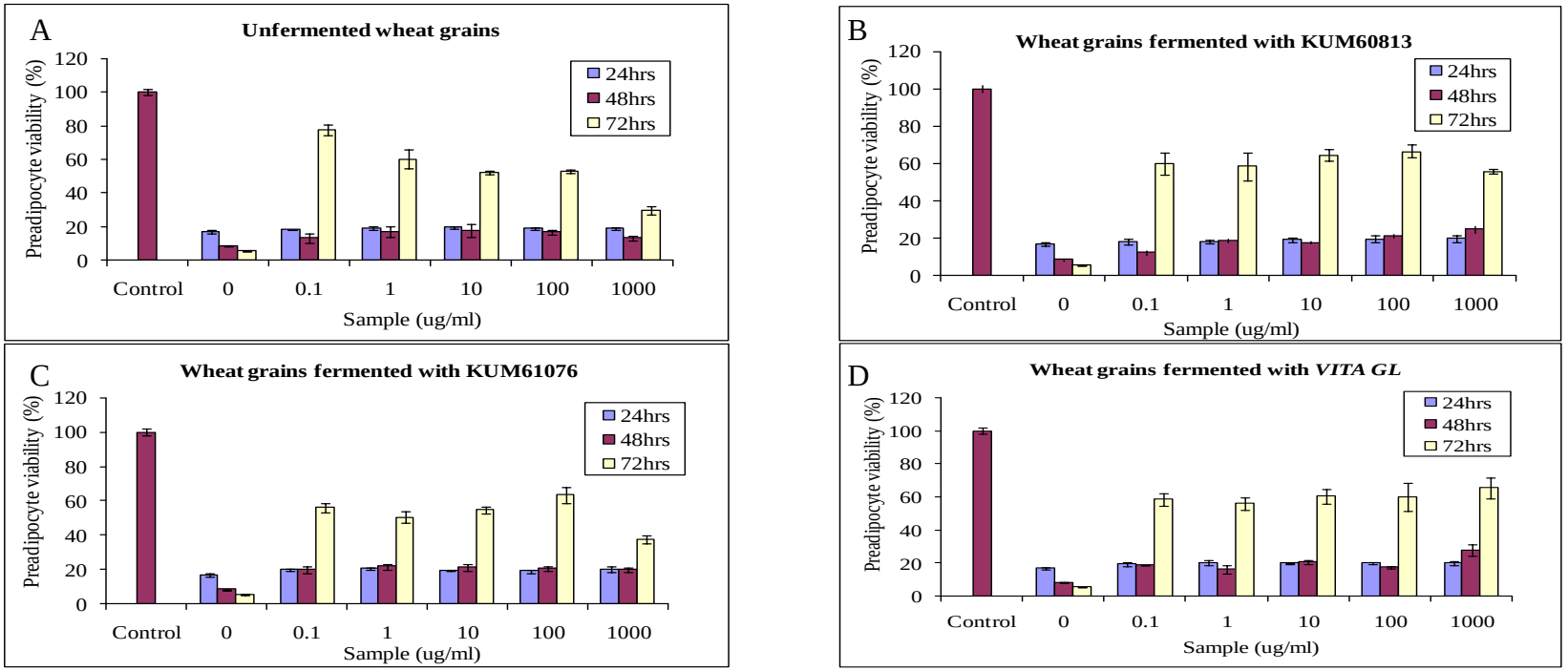


Figure 4.9 (A-D): Effect of aqueous extracts of unfermented and fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress

The aqueous extracts of unfermented and fermented wheat grains were assessed for their cytoprotective effects on 3T3-L1 preadipocytes. The cells were treated with various concentrations (0.1 – 1000 µg/mL) of aqueous extracts after inducing oxidative stress with 2 mU/mL of GOx and followed by MTT assay to determine the cell viability. The values were calculated against control (without GOx treatment) which contained ultra pure water instead of aqueous extracts. Values are expressed as mean ± SD of triplicate measurements.

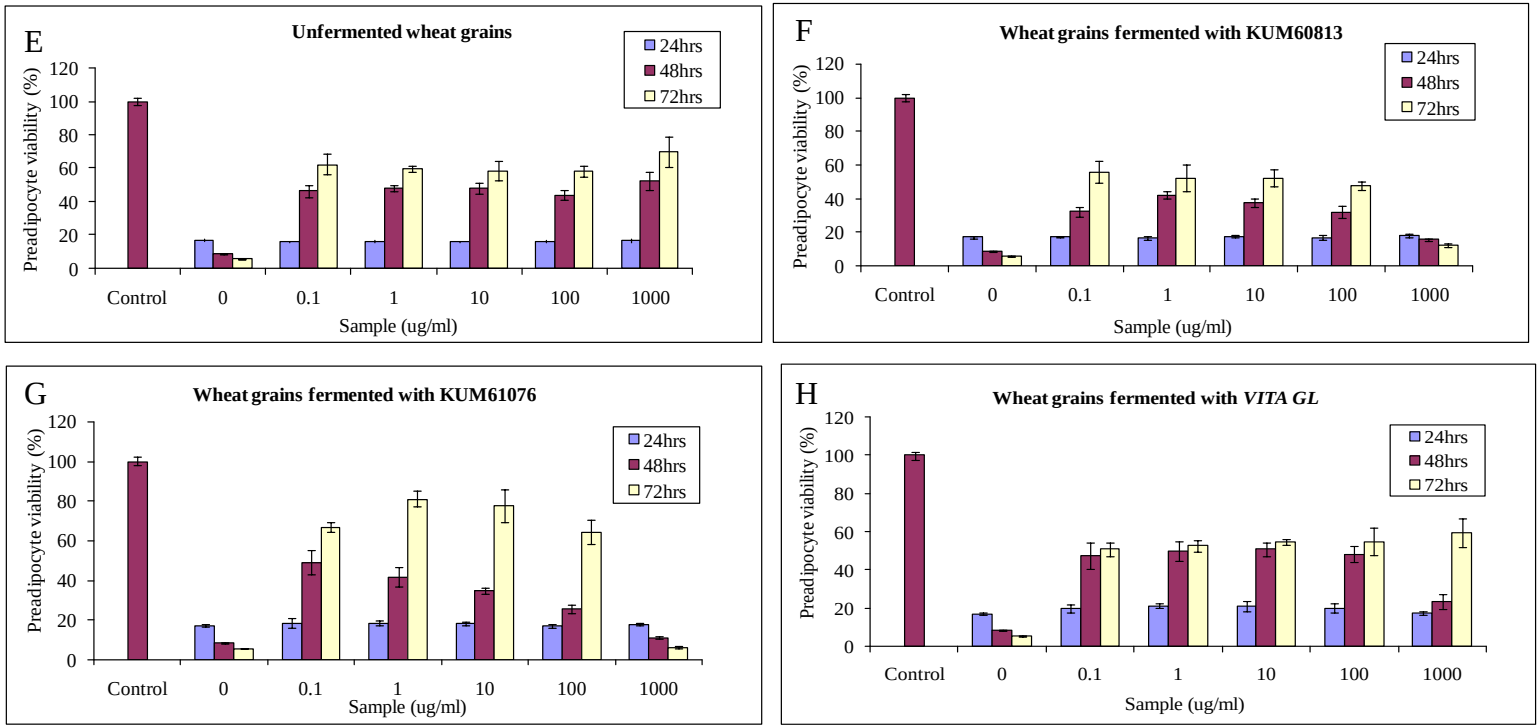


Figure 4.9 (E-H): Effect of ethanol extracts of unfermented and fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress

The ethanol extracts of unfermented and fermented wheat grains were assessed for their cytoprotective effects on 3T3-L1 preadipocytes. The cells were treated with various concentrations (0.1 – 1000 µg/mL) of ethanol extracts after inducing oxidative stress with 2 mU/mL of GOx and followed by MTT assay to determine the cell viability. The values were calculated against control (without GOx treatment) which contained 1 % DMSO instead of ethanol extracts. Values are expressed as mean ± SD of triplicate measurements.

4.4 Effect of extracts of unfermented and fermented wheat grains on lipogenesis in 3T3-L1 preadipocyte in the absence and presence of oxidative stress

Following the screening of both the aqueous and ethanol extracts of unfermented and *Ganoderma* spp. fermented wheat grain for proliferative activity, Oil Red O quantification assay was carried out on all the extracts to identify the lipogenic effect of each *Ganoderma* spp. mycelia extracts on 3T3-L1 preadipocyte. The confluent preadipocyte (Figure 4.6) were induced to differentiate with DM 1 (as mentioned in section 3.4) whereby the cells accumulated lipid droplets and the nucleus was pushed to the peripheral (Figure 4.10). Fully differentiated adipocyte were filled with large lipid droplets which appeared yellowish under the inverted microscope (Figure 4.11).

Highly differentiated adipocyte had more lipid droplets in the cytoplasm compared to the undifferentiated adipocyte. Lipid globules accumulated in adipocyte were stained with Oil Red O dye. Oil Red O dye taken up by the lipids could be clearly observed as red globules under an inverted microscope (Figure 4.12). Since insulin was known to stimulate adipogenesis in adipocyte, 1 μM of insulin was used as positive control in adipogenesis study. The concentration of insulin was determined based on a preliminary dose-response study (Appendix F). Insulin (1 μM) stimulated lipogenesis by 39% when compared to the experimental control cells (ultra pure water).

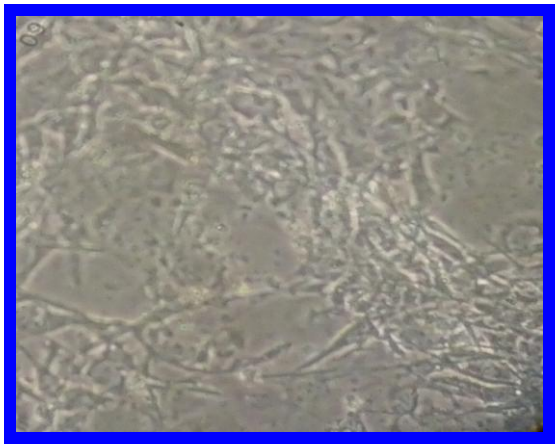


Figure 4.10: Differentiating preadipocyte beginning to accumulate lipid (200X)

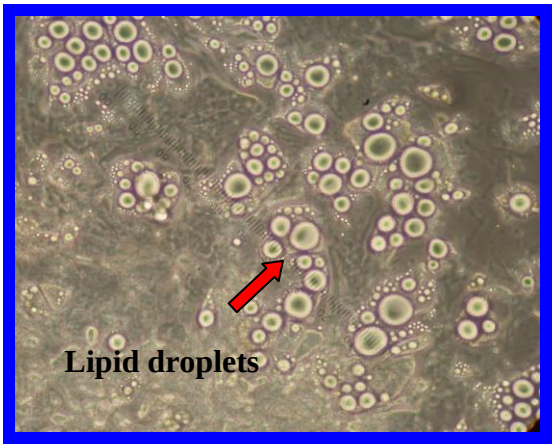
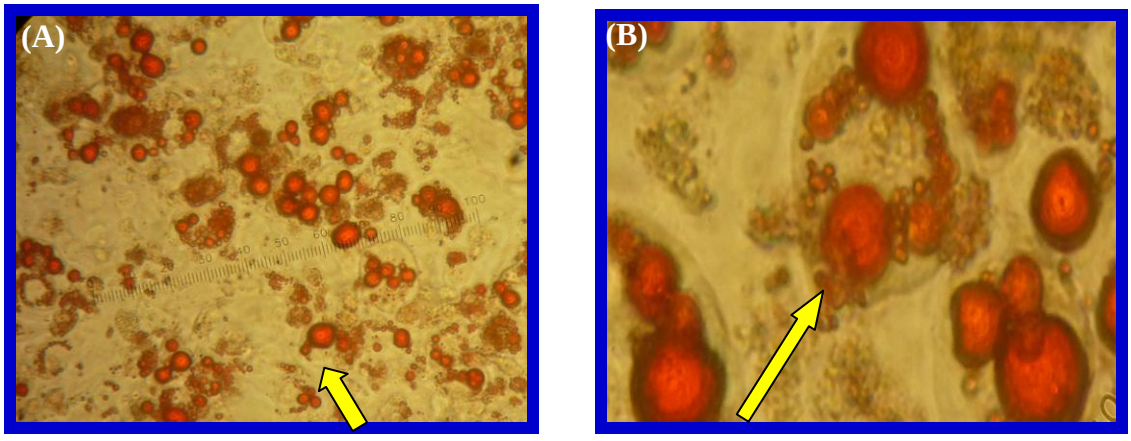


Figure 4.11: Fully differentiated adipocytes (full of lipid) which appear purplish yellow (400X)



Lipid droplets stained with Oil Red O dye

Figure 4.12 : (A) and (B) Fully differentiated adipocyte (indicated by yellow arrows) stained with Oil Red O dye [(A) is 200X and (B) is 400X]

With regard to the Oil Red O quantification assay, the ethanol extracts of KUM60813 and KUM61076 fermented wheat grains showed a dose-dependent increase in lipogenesis which was comparable to that of the experimental positive control (Figure 4.15). Wheat grain fermented with KUM61076 exerted highest lipogenic activity which was approximately 70 % increase in lipogenesis compared to experimental control cells at a concentration of 100 µg/mL; whereas insulin only stimulated 39 % lipogenesis (Figure 4.15). In addition, the lipogenic activity of these fermented wheat grains was significantly higher as compared to the unfermented wheat grain, where the maximum lipogenesis achieved was 13 % only (at extract concentration of 40 µg/mL); and the activity dropped as the concentration was increased (Figure 4.15). These results suggest that fermentation with *Ganoderma* spp. mycelia especially KUM61076 enhanced the insulin-like properties.

In contrast, the aqueous extracts of both the unfermented and fermented wheat grains did not cause significant stimulation of lipogenic activity in adipocyte (Figure 4.13). Moreover, co-incubation of these aqueous extracts with insulin (1 µM) reduced insulin induced lipogenic activity and lipid accumulation in 3T3-L1 adipocyte (Figure 4.14). Similarly, supplementation of the ethanol extracts of fermented wheat grains to the media in the presence of insulin (1 µM) significantly decreased lipid accumulation in adipocyte compared to the control cells (Figure 4.16). In addition, the ethanol extracts of fermented wheat grains demonstrated similar lipogenic activity regardless of the *Ganoderma* spp. mycelia used for fermentation.

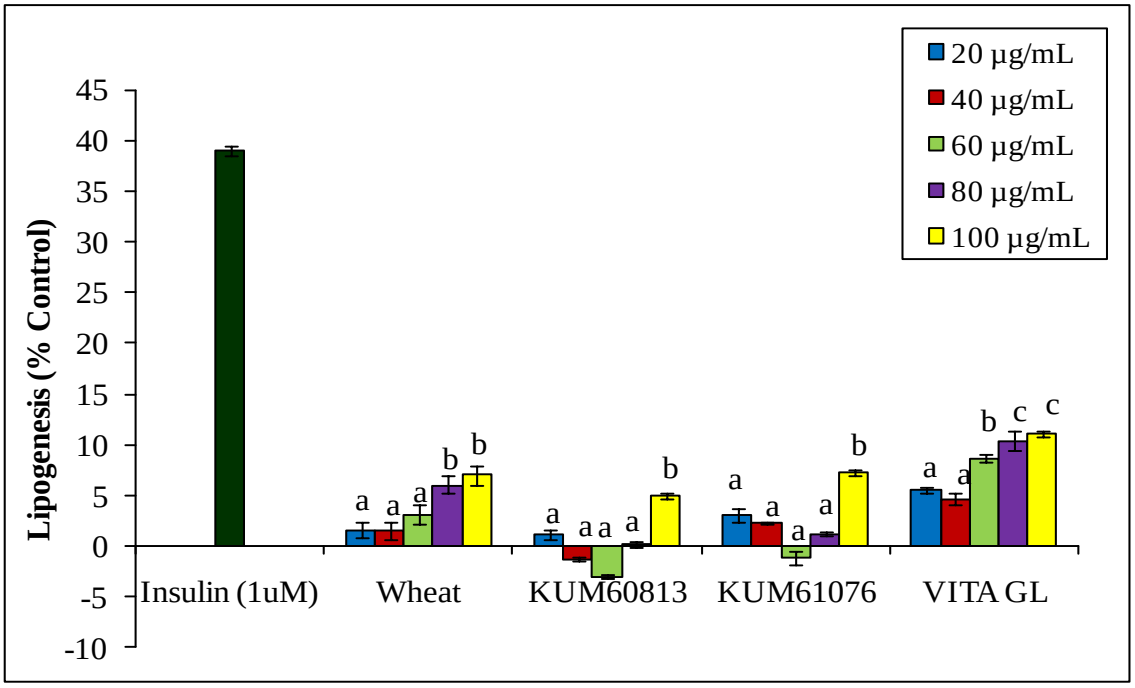


Figure 4.13: Effects of aqueous extracts of unfermented and *Ganoderma* spp. mycelia fermented wheat grains on lipogenesis in 3T3-L1 preadipocyte in the absence of insulin

Y-axis indicates the mean percentages of the lipogenesis ± SD of quadruplicate assays compared to control values (cells treated with ultra pure water). Preadipocyte (80 000 cells / well, in a 24 well plate) were induced to differentiate with various concentrations of aqueous extracts of unfermented and fermented wheat grains prior to Oil Red O quantification assay. Means with different alphabets within an extract are significantly different ($p<0.05$, ANOVA).

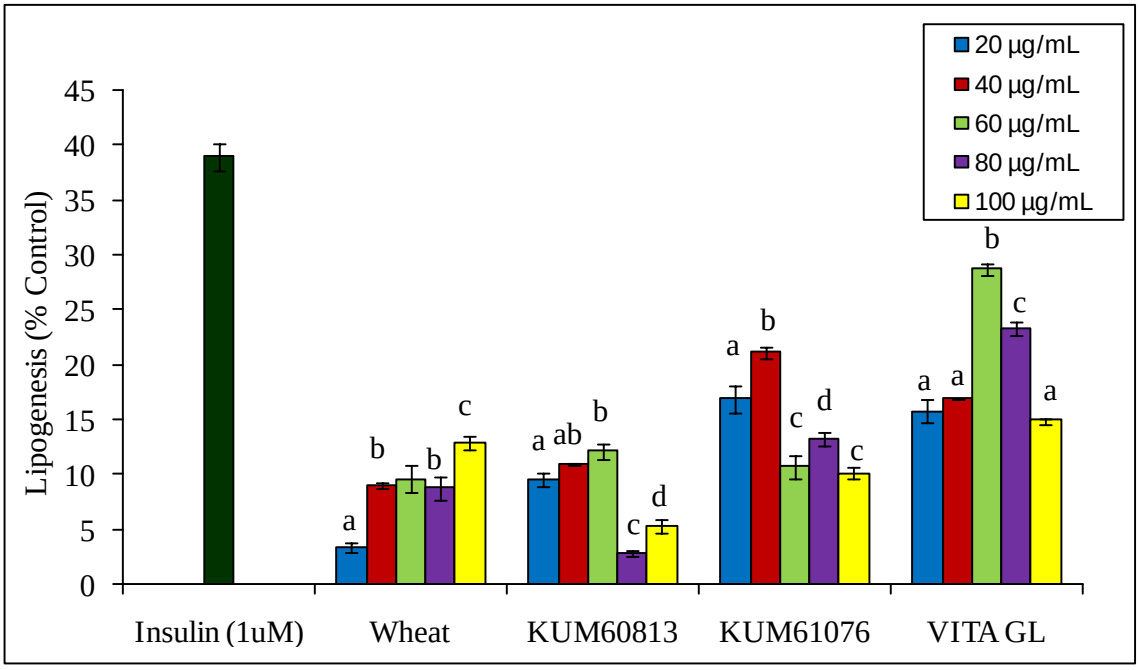


Figure 4.14: Effects of aqueous extract of unfermented and *Ganoderma* spp. mycelia fermented wheat grains on lipogenesis in 3T3-L1 preadipocyte in the presence of insulin

Y-axis indicates the mean percentages of the lipogenesis ± SD of quadruplicate assays compared to control values (cells treated with ultra pure water). Preadipocyte (80 000 cells / well, in a 24 well plate) were induced to differentiate with various concentrations of aqueous extracts of unfermented and fermented wheat grains in the presence of insulin, 1 µM prior to Oil Red O quantification assay. Means with different alphabets within an extract are significantly different ($p<0.05$, ANOVA).

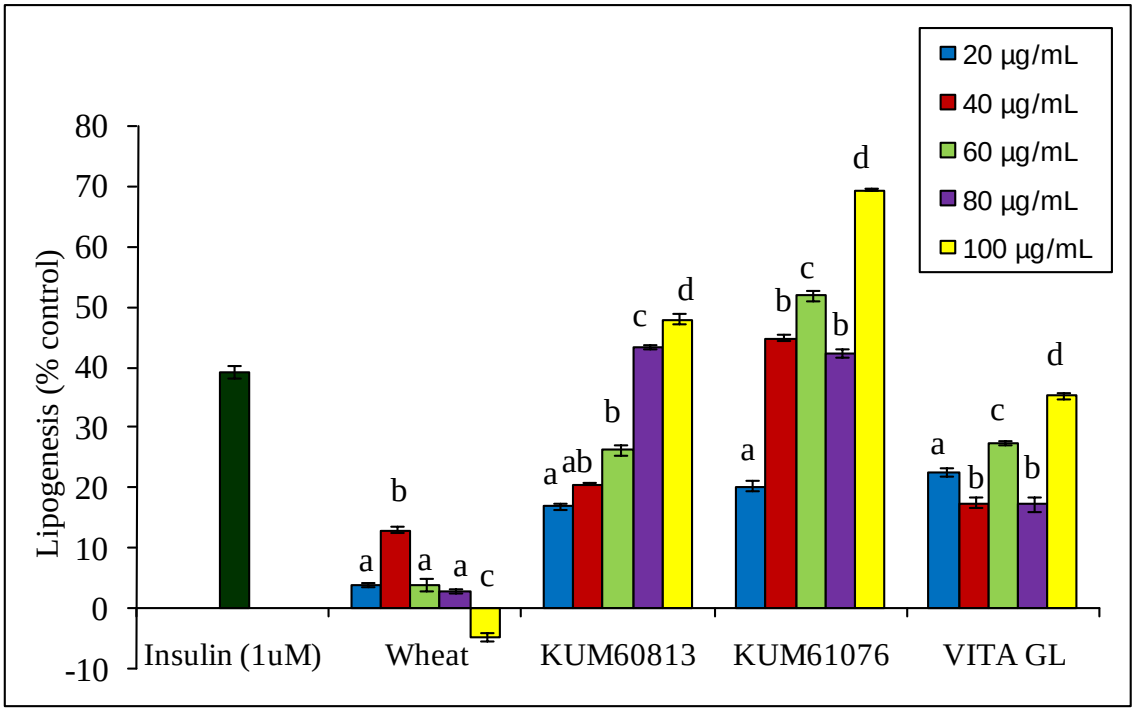


Figure 4.15: Effects of ethanol extracts of unfermented and *Ganoderma* spp. mycelia fermented wheat grains on lipogenesis in 3T3-L1 preadipocyte in the absence of insulin

Y-axis indicates the mean percentages of the lipogenesis ± SD of quadruplicate assays compared to control values (cells treated with 1% DMSO). Preadipocyte (80 000 cells / well, in a 24 well plate) were induced to differentiate with various concentrations of ethanol extracts of unfermented and fermented wheat grains prior to Oil Red O quantification assay. Means with different alphabets within an extract are significantly different ($p<0.05$, ANOVA).

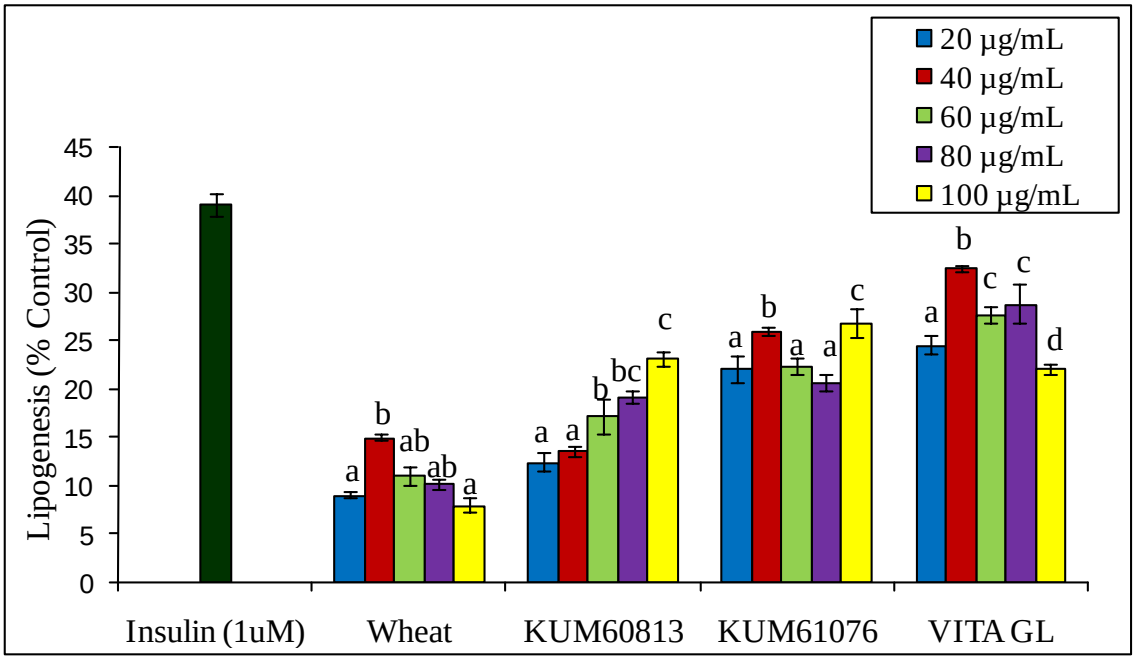


Figure 4.16: Effects of ethanol extracts of unfermented and *Ganoderma* spp. mycelia fermented wheat grain on lipogenesis in 3T3-L1 preadipocyte in the presence of insulin

Y-axis indicates the mean percentages of the lipogenesis ± SD of quadruplicate assays compared to control values (cells treated with 1% DMSO). Preadipocyte (80 000 cells / well, in a 24 well plate) were induced to differentiate with various concentrations of ethanol extracts of unfermented and fermented wheat grains in the presence of insulin, 1 µM prior to Oil Red O quantification assay. Means with different alphabets within an extract are significantly different ($p<0.05$, ANOVA).

The impact of oxidative stress on 3T3-L1 preadipocyte differentiation into mature adipocyte and accumulation of lipid was also assessed in this study. Kojima *et al*., 2010 reported that differentiated 3T3-L1 cells (adipocyte) acquired resistance to GOx-induced oxidative stress. However, this will be the first study on the effect of GOx-induced oxidative stress on 3T3-L1 preadipocyte differentiation and their treatment with various unfermented and *Ganoderma* spp. mycelia fermented wheat grain extracts.

Both the aqueous and ethanol extracts of unfermented and fermented wheat grains (except aqueous extract of wheat grains fermented with KUM60813) demonstrated a dose-dependent stimulation on 3T3-L1 preadipocyte differentiation against GOx-induced oxidative stress (Figure 4.17 and 4.18). The stimulation level on preadipocyte differentiation was significantly higher ($p<0.05$) in the cells treated with ethanol extracts (especially fermented wheat grains) as compared to their respective aqueous extracts. At a lower extract concentration (10 – 50 µg / mL), the aqueous extracts inhibited stress induced preadipocyte differentiation at various magnitudes (Figure 4.17). However when the extract concentration increased from 100 to 1000 µg/mL, the aqueous extracts significantly promoted preadipocyte differentiation and lipid accumulation (about 20 – 30 % increase in lipogenesis). Among the extracts tested, both the aqueous and ethanol extract of wheat grains fermented with *VITA GL* mycelia demonstrated the highest level of 3T3-L1 differentiation and lipid accumulation at all the extract concentrations tested.

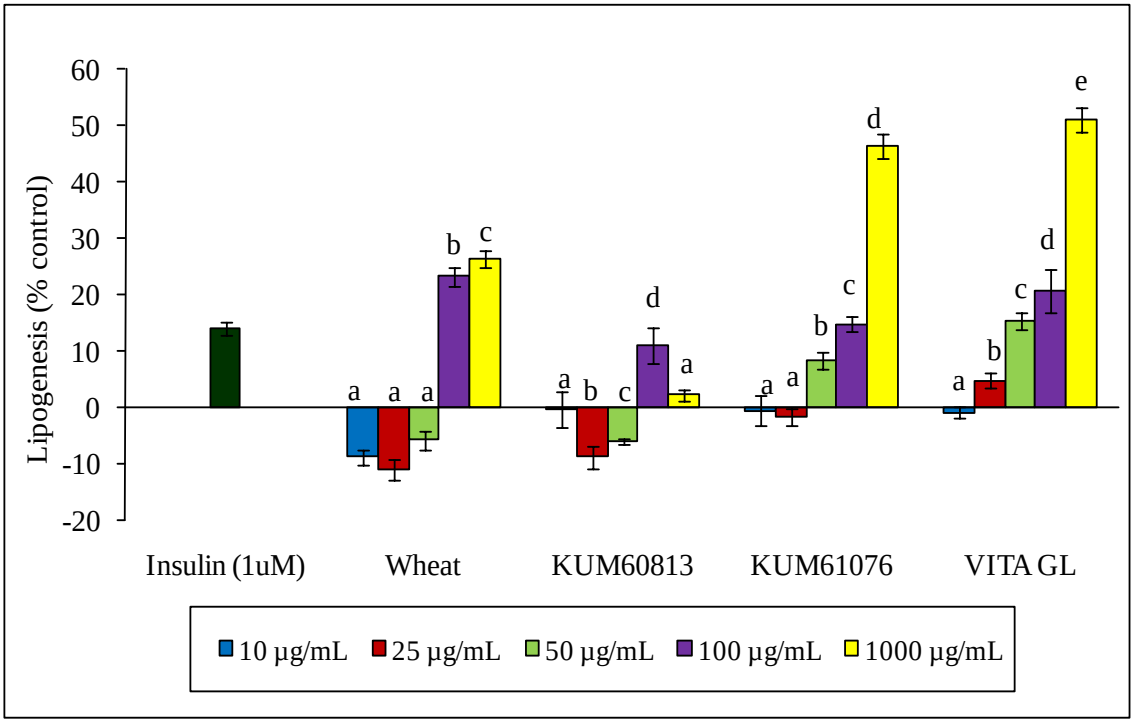


Figure 4.17: Effects of aqueous extracts of unfermented and fermented wheat grains on lipogenesis in oxidative stress induced 3T3-L1 preadipocyte

Y-axis indicates the mean percentages of the lipogenesis ± SD of quadruplicate assays compared to control values (cells treated with ultra pure water). A negative value indicates inhibition of lipogenesis. Oxidative stress in preadipocyte (80 000 cells / well, in a 24 well plate) was induced with 2 mU/mL of GOx and these cells were incubated with various concentrations of aqueous extracts of unfermented and *Ganoderma* spp. mycelia fermented wheat grains (10, 25, 50, 100, and 1000 µg/mL) for 48 hours. Means with different alphabets within an extract are significantly different ($p<0.05$, ANOVA).

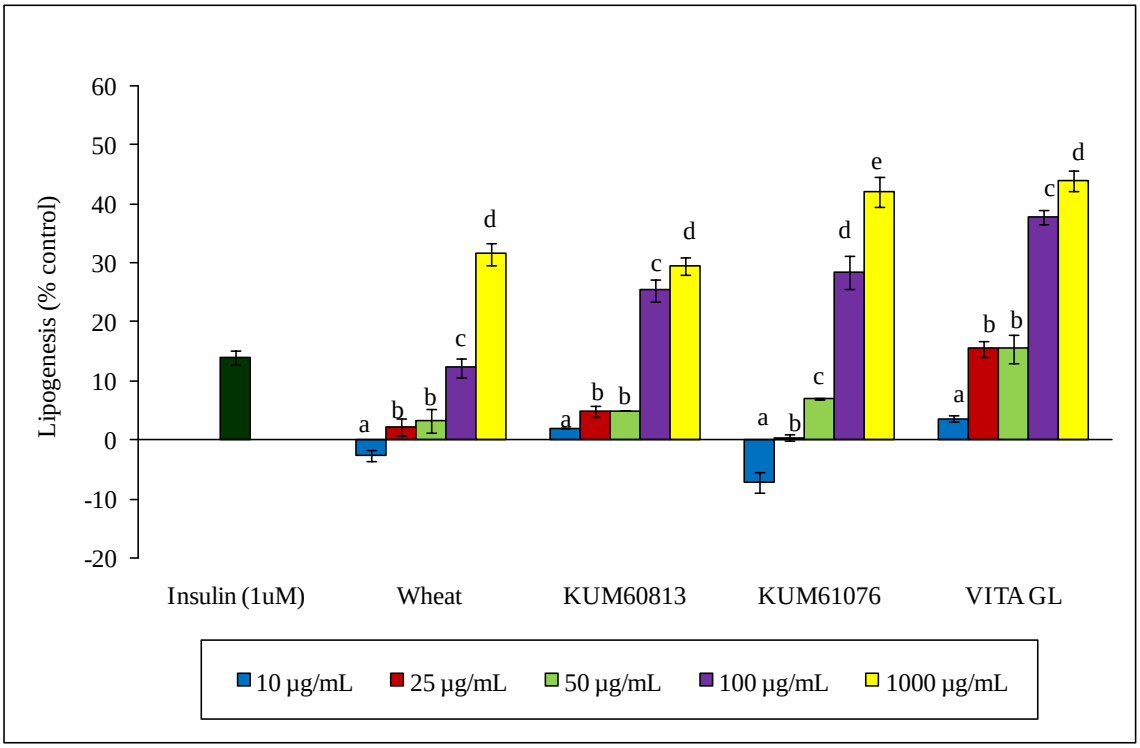


Figure 4.18: Effects of ethanol extracts of unfermented and fermented wheat grain on lipogenesis in oxidative stress induced 3T3-L1 preadipocyte

Y-axis indicates the mean percentages of the lipogenesis ± SD of quadruplicate assays compared to control values (cells treated with 1 % DMSO). A negative value indicates inhibition of lipogenesis. Oxidative stress in preadipocyte (80 000 cells / well, in a 24 well plate) was induced with 2 mU/mL of GOx and these cells were incubated with various concentrations of ethanol extracts of unfermented and *Ganoderma* spp. mycelia fermented wheat grains (10, 25, 50, 100, and 1000 µg/mL) for 48 hours. Means with different alphabets within an extract are significantly different ($p<0.05$, ANOVA).

4.5 Effect of extracts of unfermented and fermented wheat grains on lipolysis in 3T3-L1 adipocyte

After screening for proliferative and adipogenic activities, both the crude aqueous and ethanol extracts were tested for lipolytic activity in 3T3-L1 preadipocyte. Epinephrine, 1 µM was used as an experimental positive control to stimulate lipolytic activity in the adipocyte. Concentration of epinephrine used was determined based on a preliminary dose-response study. Lipolytic activity exerted by the extracts of unfermented and fermented wheat grains were assessed by comparing the amount of glycerol released to that induced by epinephrine. When adipocyte were incubated with epinephrine, the release of glycerol increased significantly by 78.19 % as compared to the experimental blank (Figure 4.19). Epinephrine induced lipolysis in adipocyte (Appendix G), whereas insulin inhibited lipolysis (Appendix H).

Based on the results obtained in this study, the aqueous extracts of unfermented and fermented wheat grains were found to stimulate lipolysis in a dose-dependent manner when compared to the experimental control (Figure 4.19). Similarly, the ethanol extracts of unfermented wheat grain and wheat grain fermented with *VITA GL* enhanced lipolysis in the mature adipocyte in a dose-dependent manner. On the other hand, the ethanol extracts of wheat grain fermented with KUM60813 and KUM61076 demonstrated a dose-dependent decrease in the lipolytic activity (Figure 4.20). Nevertheless, the amount of glycerol released by both the crude extracts of unfermented and fermented wheat grain were 1.5 – 4 fold significantly lower (20.17 – 54.53 %) as compared to the amount of glycerol released by epinephrine (78 %).

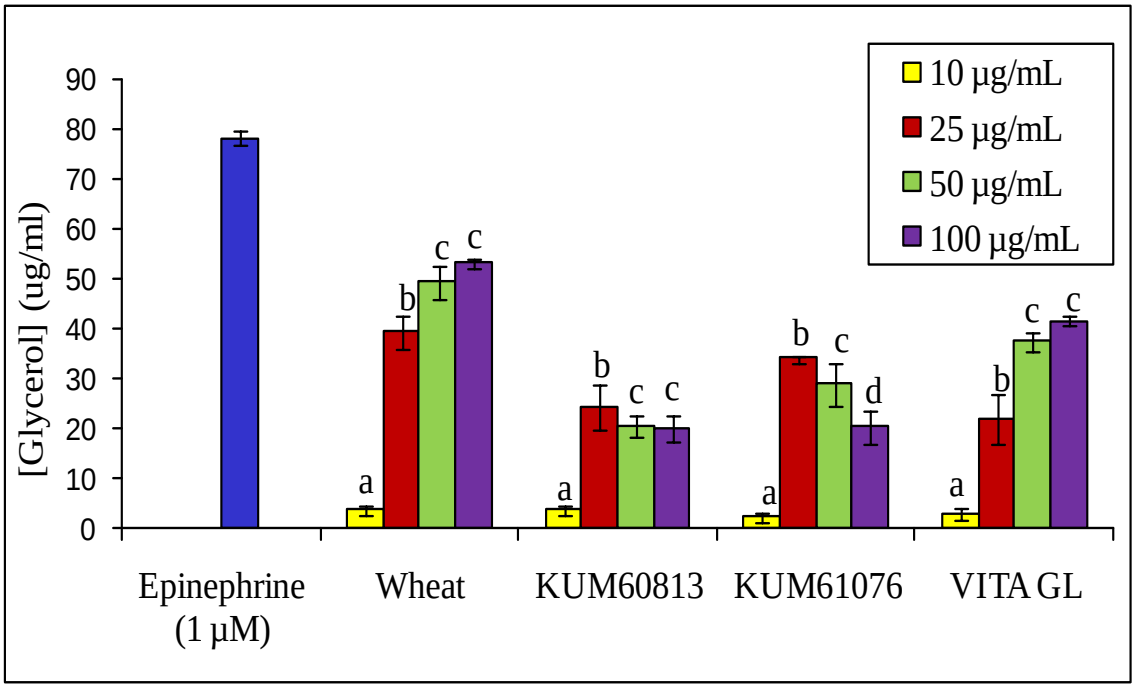


Figure 4.19: Effects of aqueous extracts of unfermented and fermented wheat grains on lipolysis in 3T3-L1 adipocyte in the absence of epinephrine

Y-axis indicates the mean amount of glycerol released ± SD of quadruplicate assays compared to control (cells treated with ultra pure water). Adipocyte (80 000 cells / well, in a 24 well plate) were treated with various concentrations of aqueous extracts of unfermented and *Ganoderma* spp. mycelia fermented wheat grains (10, 25, 50 and 100 µg/mL) or epinephrine (1 µM) prior to glycerol quantification. Means with different alphabets within an extract are significantly different ($p<0.05$, ANOVA).

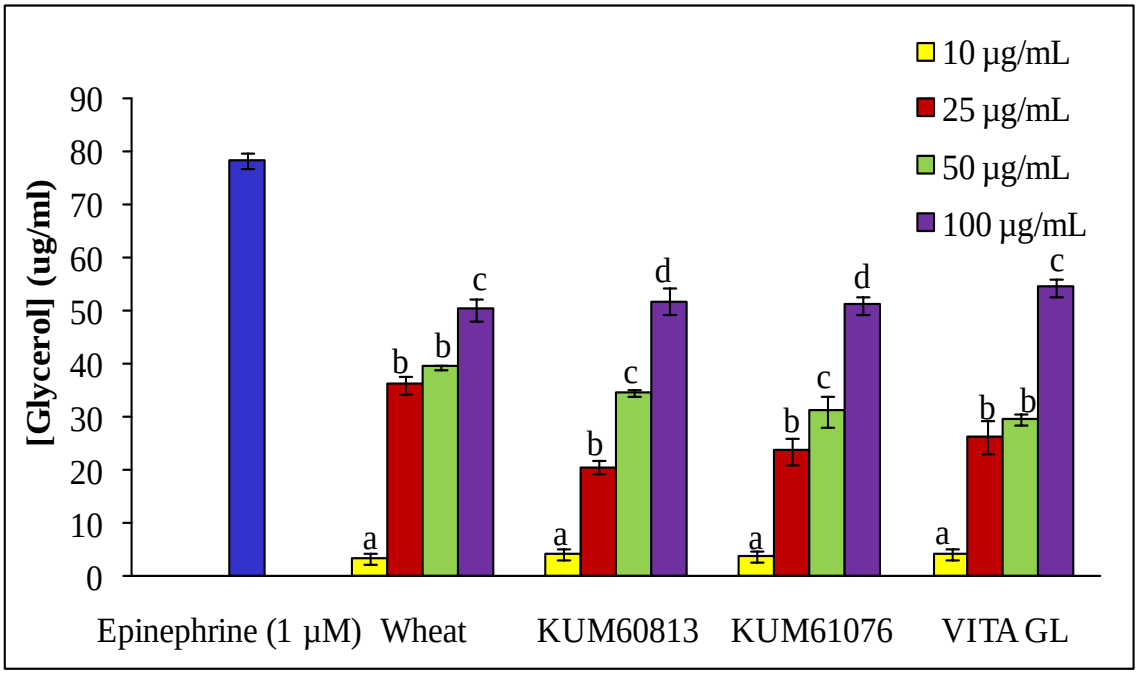


Figure 4.20: Effects of ethanol extracts of unfermented and fermented wheat grains on lipolysis in 3T3-L1 adipocyte in the absence of epinephrine

Y-axis indicates the mean amount of glycerol released ± SD of quadruplicate assays compared to control (cells treated with 1 % DMSO). Adipocyte (80 000 cells / well, in a 24 well plate) were treated with various concentrations of ethanol extracts of unfermented and *Ganoderma* spp. mycelia fermented wheat grains (10, 25, 50 and 100 µg/mL) or epinephrine (1 µM) prior to glycerol quantification. Means with different alphabets within an extract are significantly different ($p<0.05$, ANOVA).

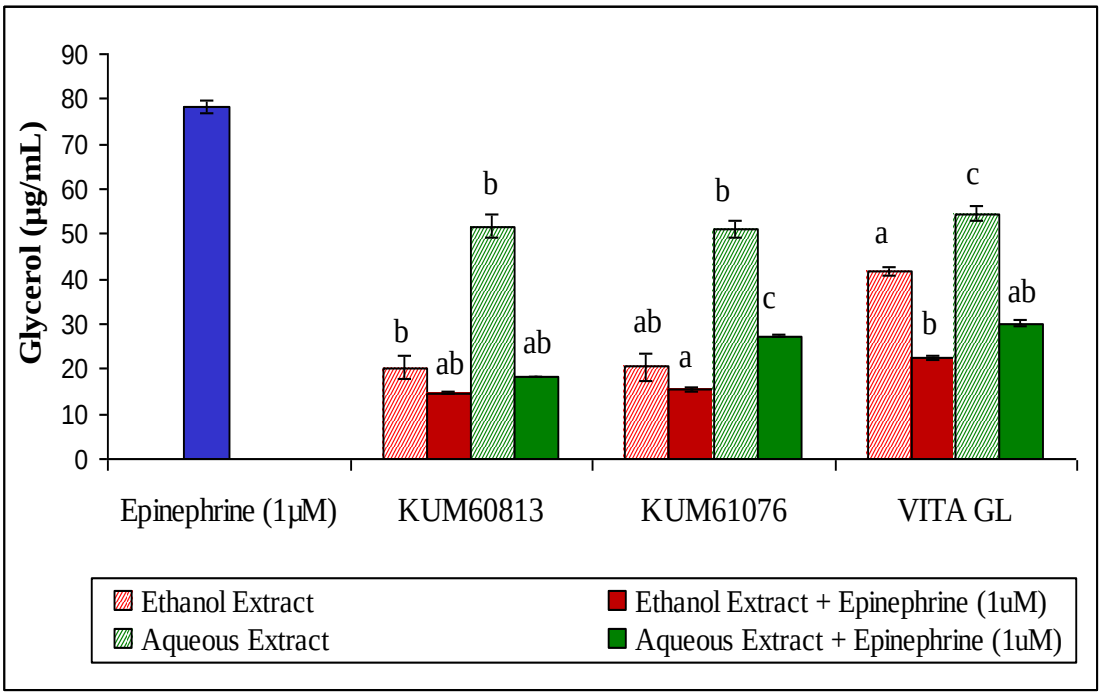


Figure 4.21: Effects of extracts of unfermented and fermented wheat grains on lipolysis in 3T3-L1 cells in the presence of epinephrine (1 µM)

Y-axis indicates the mean amount of glycerol released ± SD of quadruplicate assays compared to control (cells treated with ultra pure water). Adipocyte (80 000 cells / well, in a 24 well plate) were treated with various concentrations of both ethanol and aqueous extracts of *Ganoderma* spp. mycelia fermented wheat grains (50 µg/mL) or epinephrine (1 µM) for 24 hours prior to glycerol quantification assay. Means with different alphabets within an extract are significantly different ($p<0.05$, ANOVA).

The extracts were also screened for anti-epinephrine induced lipolysis in mature adipocyte. When co-incubated with epinephrine (1 µM), the extracts significantly inhibited 60 – 80 % of epinephrine induced lipolysis in adipocyte (Figure 4.21). The data obtained clearly showed that the ethanol extracts of wheat grain fermented with KUM60813 and KUM61076 have insulin-like effect in inhibiting epinephrine induced lipolysis in spite of being moderately lipolytic in itself.

4.6 Extracts of fermented wheat grains modulated the expression of PPARγ targeting genes in adipocyte in the absence and presence of oxidative stress

The results from the oil red o and glycerol quantification assays demonstrated that the ethanol extracts of fermented wheat grain stimulated lipogenesis dose-dependently (Figure 4.13 – 4.16), while inhibiting lipogenesis in oxidative stress induced 3T3-L1 preadipocyte (Figure 4.17 and 4.18) and epinephrine induced lipolysis in adipocyte (Figure 4.21). To investigate this insulin-like properties of the ethanol extracts further, the relative expression of five selected genes, representing selective key positions in the insulin and epinephrine pathways, were examined in adipocyte treated with extracts of fermented wheat grains. Figure 4.22 shows that PPARγ expression was significantly modulated in adipocyte treated with ethanol extracts of fermented wheat grain as compared to the control cells. Adipocyte treated with ethanol extract of KUM60813 (100 µg/mL) fermented wheat grain showed elevated expression of PPARγ by 12.24 ± 0.56-fold (p<0.05) and this was followed by the ethanol extracts of KUM61076 and *VITA GL* (100 µg/mL) fermented wheat grain, 10.21 ± 0.05-fold and 3.73 ± 0.13-fold (p<0.05) respectively.LPL, GLUT4 and adiponectin were examined, here, in order to demonstrate that metabolic events occur concurrently with PPARγ switch. In the

presence of ethanol extract of KUM60813 fermented wheat grain, the mRNA level of LPL, GLUT4 and adiponectin were significantly elevated [3.22 ± 0.02-fold ($p<0.05$), 12.39 ± 0.38-fold ($p<0.05$), and 9.23 ± 0.35-fold ($p<0.05$), respectively], compared to incubations with other ethanol extracts (Figure 4.22). These data correlate well with the microscopic examination that revealed robust accumulation of lipid bodies.

In order to examine the effects of oxidative stress on adipokine gene expressions during the course of differentiation, 3T3-L1 preadipocyte were incubated with 2 mU/mL of GOx for 2 hours under serum free conditions and subsequently treated with 100 µg/mL ethanol extracts of fermented wheat grain for 48 hours. Total RNA was extracted from the cells and mRNA levels of PPARγ, adiponectin, bax and GPx3 were measured using quantitative RT-PCR. Based on the results obtained, the glucose oxidase treatment reduced lipogenesis (Figure 4.18), as well as suppressed the PPARγ and adiponectin expression levels in 3T3-L1 preadipocyte compared to the cells treated with the extracts alone (in the absence of oxidative stress).

In cells treated with ethanol extract of KUM60813 fermented wheat grain after exposed to oxidative stress, a significant decrease in expressions of adiponectin (74%) [from 9.23 ± 0.54-fold to 2.39 ± 0.22-fold ($p<0.05$)] and PPARγ (96%) [from 12.24 ± 0.42-fold to 1.05 ± 0.16-fold ($p<0.05$)] was observed. Similarly, a 12 % [from 3.40 ± 0.06-fold to 3.00 ± 0.05-fold ($p<0.05$)] decrease in adiponectin expression and 90 % decrease in PPARγ expression [from 3.73 ± 0.13-fold to 0.38 ± 0.04-fold ($p<0.05$)] were detectable in the cells treated with ethanol extract of *VITA GL* fermented wheat grains after oxidative stress induction by GOx. The RT-PCR results also revealed a 68 % [from 5.58 ± 0.31-fold to 1.78 ± 0.13-fold ($p<0.05$) and 10.21 ± 0.42-fold to 2.39 ± 0.03-fold ($p<0.05$), respectively] reduction in expression of adiponectin and PPARγ in

the cells treated with KUM61076 fermented wheat ethanol extract, compared to the cells without oxidative stress induction (Figure 4.23).

3T3-L1 preadipocyte induced with oxidative stress and treated with ethanol extracts of wheat fermented with KUM60813, KUM61076 and *VITA GL* showed increased GPx3 expression (2.02 ± 0.03-fold ($p<0.05$), 1.74 ± 0.19-fold($p<0.05$) and 1.14 ± 0.02-fold respectively). These results suggest that 3T3-L1 preadipocyte treated with ethanol extracts of KUM60813 and KUM61076 fermented wheat grain acquire resistance against oxidative stress by increasing the expression of anti-oxidative enzyme gene (*i.e*,. GPx3).

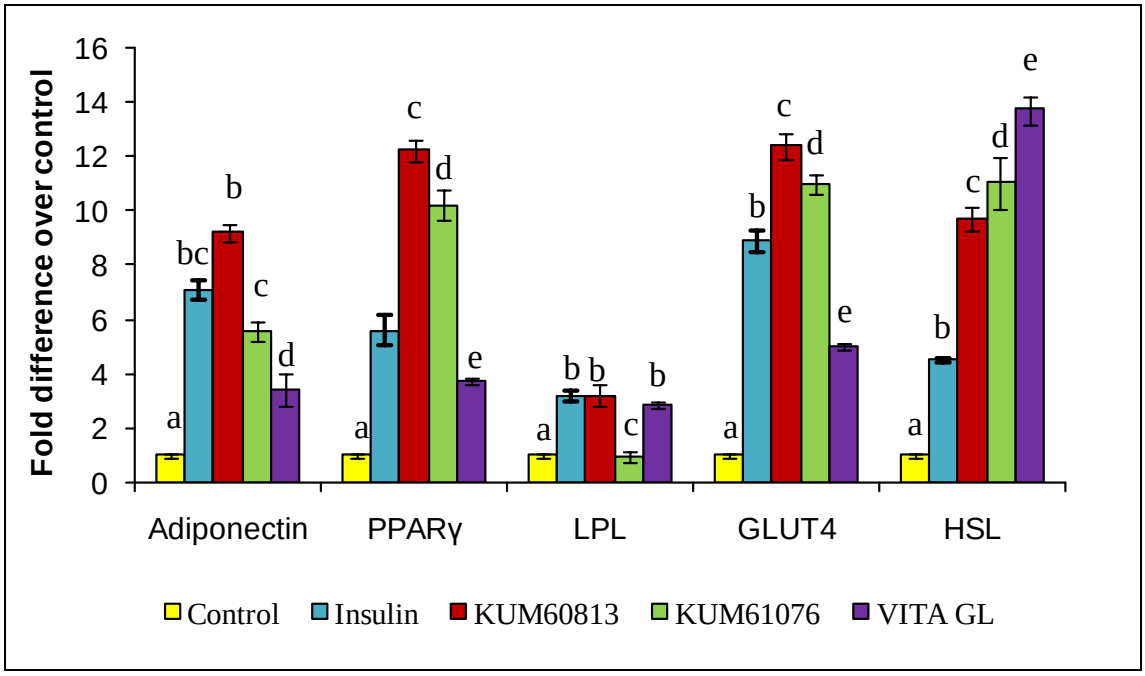


Figure 4.22: Effect of ethanol extracts of fermented wheat grains on gene expression in adipocyte

Cells were treated with 100 µg/mL of ethanol extracts of fermented wheat grains for 48 hours (Sec 3.4.3.1). Total RNA was extracted and cDNA were synthesized (Sec. 3.4.3.2). RT-PCR was performed and the relative expression of the genes was calculated using the delta-delta CT method (Sec. 3.4.3.4.3). They were normalized with 18S (control) eukaryotic rRNA. Results were expressed as n-fold difference over control. Fold variation values lower than 1 indicates downregulation. The results presented are mean ± SD. Statistical significance was calculated based on the mean ΔΔCT values by one way ANOVA; ($p < 0.05$). Means with different alphabets within a gene are significantly different ($p<0.05$, ANOVA).

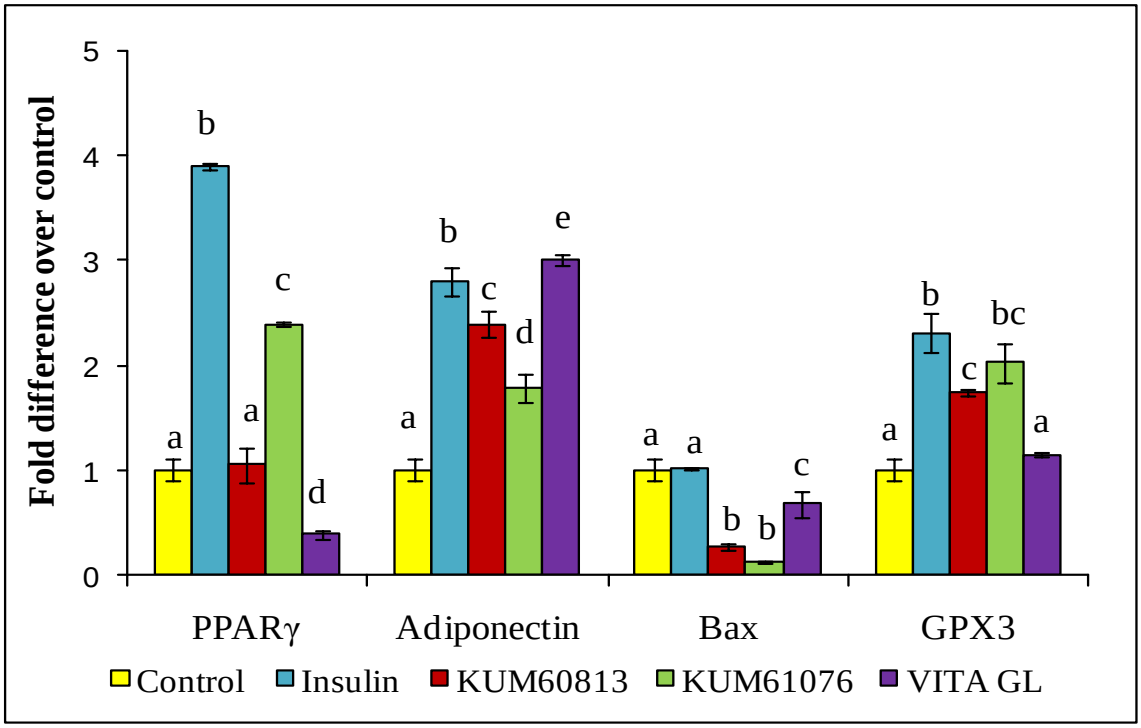


Figure 4.23: Effect of ethanol extracts of fermented wheat grains on gene expression in adipocyte under oxidative stress

Cells were induced with oxidative stress (2 mU/mL, GOx) and treated with 100 µg/mL of ethanol extracts of fermented wheat grains for 48 hours (Sec 3.4.1). Total RNA was extracted and cDNA were synthesized (Sec. 3.4.3.2 and 3.4.3.3). RT-PCR was performed and the relative expression of the genes was calculated using the delta-delta CT method (Sec. 3.4.3.4). They were normalized with 18S (control) eukaryotic rRNA. Results were expressed as n-fold difference over control. Fold variation values lower than 1 indicates downregulation. The results presented are mean ± SD. Statistical significance was calculated based on the mean ΔΔCT values by one way ANOVA; ($p < 0.05$).

CHAPTER 5

DISCUSSION

5.1 SSF, yield of mycelia biomass and extracts

Mushrooms spent 90 % of their life-span as mycelia, take up nutrients from their environment and shield themselves from invaders. Many valuable bioactive compounds are secreted as extracellular compounds by the mushroom during this critical life process. Thus, the current study focused on growing the *Ganoderma* spp. mycelia via SSF and investigated the antioxidant and insulin-like properties of the mycelia extracts. *G. australe* and *G. neo-japonicum* used in this study are not commercially cultivated and so, the amount of these wild mushrooms is not sufficient for scientific validation of the beneficial effects and commercial exploitation.

As it normally takes 6-12 months to complete a fruiting body culture in solid-state fermentation, many attempts are also being made to obtain cellular materials or to produce bioactive substances from a submerged mycelia culture. Although the technique of submerged culture for mycelia growth of *G. lucidum* has been well developed, the authors have demonstrated that the components of the mycelia obtained from different type cultures have significant variations which affect the medicinal properties (Yang *et al*., 2003). Moreover, solid substrate fermentation is now being used as an alternative to improve feed values of waste cellulosic materials, for enzyme production and improving the nutritional values of existing foods; especially the oriental food preparation in Asian countries, where products like *tempe* (fermented soya bean) are produced using SSF (Pandey *et al*., 2000).

In the present study, whole wheat kernels were used since it has been shown that these are an excellent substrate for use in mixed solid-state bioreactors (Nagel *et al.* 2000). The carbon compounds from the wheat grain serve to supply energy for fungal mycelium metabolism and to provide the carbon for building carbohydrates, lipids, nucleic acids, and proteins. Without a carbon source, the mycelium growth will be poor or absent.

Factors such as heat and the type of particular solvent used also play a significant role in bioactivity of the end product. In this study, the unfermented and fermented wheat grains were boiled in distilled water for an hour to obtain hot aqueous extract. The hot water extraction has been a popular method for mushroom extraction, especially to have a higher yield of water soluble polysaccharides (Zhang *et al*., 2007). In addition, Lee *et al.* (2007) reported that the yields of water extracts of *P. citrinopileatus* were significantly higher than those of ethanol extracts for three types of samples; fruiting bodies, mycelia and culture filtrate. Besides that, more often then not, traditional medicines were prepared using either hot water or cold water extraction method. The difference of the yield might be due to the fact that water extracts contained certain amount of soluble polysaccharides that could be precipitated from aqueous suspension of fruiting bodies or mycelia (Lee *et al*., 2007). In this study, the similar finding on the yield of aqueous extracts was obtained.

On the other hand, ethanol was chosen for solvent extraction to extract out bioactive compounds with lower polarity, *i.e.* triterpenes/triterpenoids and sterol compounds; it is easier to evaporate since it has high melting point; and noticeably, it is cheap and easier to obtain. Rotary evaporator was used for efficient and gentle removal of solvents from samples by evaporation under as low temperature as possible and

reduced pressure to preserve the freshness of extracts and prevent the possible degradation of antioxidant and insulin-like properties of the extract.

5.2 Antioxidant activity of extracts of unfermented and fermented wheat grains and correlation analysis between TPC levels and the antioxidant indices

Mushrooms accumulate a variety of secondary metabolites, including phenolic compounds, polyketides, terpenes and steroids. Also, a mushroom phenolic compound has been found to be an excellent antioxidant and synergist that is not mutagenic (Cheung *et al*., 2003). General antioxidant activities of the crude aqueous and ethanolic unfermented and fermented wheat grain extracts were determined to assess its potential as a source of antioxidant compounds. Antioxidant insufficiency has been associated with the pathogenesis of many diseases, whereas the antioxidant potential of mushroom extracts has been linked to many beneficial activities against chronic diseases and cancer (Barros *et. al.*, 2008; Paterson, 2006). To the best of our knowledge, the antioxidant activities of wheat grain fermented with mycelia of *G. australe* (KUM60813), *G. neo-japonicum* (KUM61076) and *G. lucidum* (*VITA GL*) has not been reported previously. The data reported here could be the first comprehensive antioxidant assessment on wheat grain fermented with *Ganoderma* spp. mycelia.

In general, all the ethanol extracts tested depicted higher antioxidant activity as compared to their respective aqueous extracts. This could be due to the variability in the extraction method and the use of different solvent systems which might play a significant role in the free radical scavenging activity. In this study, the ethanol extraction was conducted at room temperature, whereas the hot water extraction was done by boiling the unfermented and fermented wheat grains for an hour at 100˚C.

Hence, there could be a possibility that the antioxidant components could be present in the ethanol extracts than the hot water extracts. This is because the heat treatment would have degraded the bioactive compounds which are responsible for antioxidant activity of the mushroom mycelia. Zhang and Hamauzu (2004) reported that heat treatment by cooking could reduce antioxidant component (i.e., phenolics, ascorbic acid and carotenoids) and activity.

Among the extracts tested, ethanol extract of KUM61076 mycelia fermented wheat grain demonstrated the highest antioxidant activity. Enhanced production of antioxidant compounds *i.e.* total phenolic content (Table 4.2) during fermentation of wheat grains by KUM61076, may have contributed to its higher free radical scavenging and reducing activity (Shahidi and Wanasundara, 1992; Shimada *et al.*, 1992). Conversely, the wheat fermented with KUM60813 and *VITA GL* was found to have lower total phenolic content which contributed to their lower antioxidant activity compared to KUM61076 fermented wheat grains. Phenolic compounds are one of the major groups of non-essential dietary components that have been associated with the inhibition of atherosclerosis and cancer; and to treat diabetes (Teissedre and Landrault, 2000; Anderson *et al*., 2004). The bioactivity of phenolics may be related to their ability to chelate metals, inhibit lipoxygenase activity and scavenge free radicals (Decker, 1997). Mushrooms contain a variety of secondary metabolites, including phenolic compounds, polyketides, terpenes and steroids. Izlina *et al*., (2010) reported that there is a correlation between the antioxidant activities of plant extracts with the content of their phenolic compounds. In addition, Cheung *et al*. (2003) reported that the strong antioxidant properties of edible mushrooms such as *L. edodes* and *V. volvacea* could be attributed to their high polyphenolic content.

Interestingly, the ethanol extract of KUM61076 mycelia fermented wheat grain showed a higher antioxidant activity and total phenol content compared to the unfermented wheat grains. During the SSF process, different hydrolytic enzymes produced by the mycelia released the phenolic compounds from their conjugates (Bhanja *et al.*, 2009). Wheat kernels contain a number of phenolic compounds that typically found conjugated with sugars, fatty acids or proteins (Robbins, 1980; Naczk and Shahidi, 2006). Therefore, it is essential to conduct hydrolysis process to maximize the yield of the phenolics of cereal grains (Wojdyło and Oszmain´ ski, 2007). Generally, acidic or alkaline hydrolysis is used for extraction and determination of polyphenolic compounds (Kim et al., 2006). However these techniques were found to degrade some of the phenolic compounds (Robbins, 2003). Hence, it is necessary to use an improved method for extraction of polyphenols. Enzymatic reactions have been reported to release phenolic acids (mainly ferulic and p-coumaric acids). The enzymes (e.g. pectinases, cellulases, a-amylases, xylanase, b-glucosidase, bxylosidase, b-galactosidase and b-hesperidinase etc.) are employed for the degradation of the carbohydrate linkages. The process, however, is not considered as economically feasible because the commercial enzymes used for this process are expensive (Bhanja *et al.*, 2009). Thus, solid-state fermentation process with *in situ* hydrolysis is viewed as an alternative method to improve the phenolic content and antioxidant potential in fermented foods (wheat grains has been employed as the substrate in the present study).

In this study, the wheat grain fermented with *VITA GL* (commercial *G. lucidum*) had lower antioxidant activities compared to the two wild *Ganoderma* spp. This is because the *VITA GL* has been continuously cultivated by farmers over years and this has led it to lose its antioxidant properties. Conversely, the wild *G.australe* and *G.neo-japonicum* were collected from forest, hence retaining their antioxidant activity.

Besides, the experimental positive control, BHT showed higher antioxidant activity compared to the wheat grain extracts tested in this study. Similar findings have been reported previously (Elmastas et al., 2007; Mau et al., 2004), where the phenols (pure compounds) such as BHT, BHA and gallic acid were good in antioxidant activity as compared to the crude extracts tested in their studies.

Pearson correlation analysis was carried out to determine the relationship between TPC levels and the antioxidant indices such as FRAP levels, ABTS scavenging activity and DPPH scavenging ability of the extracts prepared. Strong correlations existed between all the parameters compared (Table 4). Several other reports also concurred with the present finding. Yen and Wu (1993) reported that the antioxidant activity of the methanolic extract from peanut hulls correlated with its content of total phenols. Therefore, the high phenolic content is an important factor in contributing to the high antioxidant capacities of these fermented and unfermented wheat grain extracts.

In this study, the lipid peroxidation assay using palm cooking oil was designed to investigate if the extracts of unfermented and fermented wheat grain were able to protect the oil against peroxidation. The assay was based on the formation of thiobarbituric reactive substance (TBARS) which absorbs at 532 nm. The results obtained (Section 4.2) indicated that lipid peroxidation in palm cooking oil was extensively minimized, when heating oil was done in the presence of ethanol extract of wheat grains fermented with KUM60813 mycelia. Even after fifty days, the oil heated with ethanol extract of wheat grain fermented with KUM60813 mycelia, did not deteriorate. This indicates that ethanol extract of wheat grains fermented with KUM60813 mycelia has a good potential as a natural antioxidant and could be exploited as a natural food preservative. Shahidi and Wanasundra, (1992) reported that tert-butylhydroquinone (THBQ) is deemed to be the best antioxidant for protecting cooking

oils against oxidation. Despite being efficient in withstanding auto-oxidation, only few synthetic compounds have been currently permitted for use in food industry due to their potential toxicity and carcinogenicity. Hence, there is an increasing awareness among the public in the consumption of natural antioxidant that could lower the risk of health problems.

5.3 Effect of extracts of unfermented and fermented wheat grain on 3T3-L1 preadipocyte viability in the absence and presence of oxidative stress

The effect of both the aqueous and ethanol extracts of unfermented and fermented wheat grains on preadipocyte viability and proliferation was assessed. All the extracts (except ethanol extract of unfermented wheat grains) tested in this study demonstrated mild proliferative effect on 3T3-L1 preadipocyte (7 – 37 % stimulation of proliferation) compared to the control cells (cells treated with ultra pure water / 1 % DMSO). Next the 3T3-L1 preadipocyte were subjected to oxidative stress and then treated with extracts of unfermented and fermented wheat grains; subsequently cell viability was analyzed. This was done to assess the ability of the extracts to repair/recover or protect the preadipocyte from oxidative stress induced cell death. Glucose oxidase (GOx) was used as a source of oxidative stress for continuous H_2O_2 supply. The GOx enzyme is as oxido-reductase which catalyses the glucose in culture media to H_2O_2 and D-glucono-δ-lactone.

The MTT assay revealed that the ethanol extracts of wheat fermented with KUM61076 mycelia significantly reduced the stress induced cell death after 48 and 72 hours treatment; suggesting that 3T3-L1 cells acquired resistance to oxidative stress following the treatment. The repair mechanism and/or cytoprotective effect possibly

involves antioxidant properties of the corresponding extracts. Antioxidants (higher phenolic content, Table 4.2) from ethanol extracts of wheat fermented with KUM61076 mycelia may readily be available to cells and protect cellular lipids, proteins, and DNA from oxidative damage by neutralizing cellular ROS. Moreover, the up-regulation of GPx3 mRNA (Figure 4.23) when the stress induced cells were treated with KUM61076 mycelia confirmed that the repair mechanism was due to the antioxidant capacity of the extract.

5.4 Effect of extracts of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence and presence of oxidative stress

Preadipocyte differentiation is a very complex process that is activated and promoted by coordinated signals of growth factors, cytokines, and hormones (Spiegelman and Farmer, 1982). Subsequent to growth arrest, DNA replication and cell doubling occur in preadipocyte and this requires continuous and appropriate supplementation of an inducing agent such as insulin. The preadipocyte will steadily perform *de novo* lipogenesis and at the same time acquire sensitivity to insulin by expression of insulin receptors and glucose transporters (Gregoire *et. al.*, 1998). Mature adipocytes were lipid-filled with increased buoyancy. Therefore, adipocyte have higher tendency to lift from the plate and thus the cells must be handled gently. Based on the present optimized culture method, the duration needed for preadipocyte proliferation and differentiation was approximately 15 days. Therefore adipocyte culture experiments were carefully planned and staggered to ensure availability of continuous supply of adipocyte for subsequent experiments.

Although the defined stage at which preadipocyte can be considered as fully differentiated is not clearly known, differentiated adipocyte can be evidenced by visualizing the lipid accumulation in adipocyte cytoplasm (Gregoire *et. al.*, 1998). Hence, in this study, the lipid accumulated in adipocyte was stained with Oil Red O dye and the amount of lipid accumulation could be visualized microscopically (Figure 4.12) and quantified spectrophotometrically at 510 nm since the amount of Oil Red O staining is directly proportional to the amount of lipid accumulated in adipocyte.

Insulin is capable of inducing lipogenesis, inhibiting lipolysis, and stimulating uptake of glucose and free fatty acids by peripheral cells such as liver, muscle and adipose tissues (Greenfield and Campbell, 2004). These actions of insulin on lipogenesis and anti-lipolysis in human adipose tissue *in vivo* could be physiologically counteracted by β-adrenoceptor agonists such as epinephrine and isoproterenol (Hagström-Toft *et al.*, 1992). Therefore, crude extracts or compounds which are able to stimulate lipogenesis, inhibit adrenaline induced lipolysis as well as enhance uptake of glucose and free fatty acids are known to have 'insulin-like' potential and may have the potential as anti-diabetic agents. Besides that, anti-diabetic drugs such as troglitazone and rosiglitazone have also been reported to enhance preadipocyte differentiation and glucose uptake in adipocyte (Tafuri, 1996; Håkan *et. al.*, 2005).

The use of insulin or the synthetic anti-diabetic drugs for treatment of Type 2 DM may give rise to side effects such as weight gain, obesity or hypoglycemia due to increased lipogenic and anti-lipolytic activities. TZDs are suggested to improve insulin sensitivity via their action on PPARγ (Gurnell *et. al.*, 2003). However, increased expression of PPARγ activity will give rise to some side effects including increased differentiation of preadipocyte followed by deposition of fat in peripheral subcutaneous tissues, leading to weight gain (Gurnell et. al., 2003). Besides that, sulfonylureas for

example chlorpropamide was also used to treat Type 2 DM. Sulfonylureas increase insulin production by stimulating the pancreas to release more insulin into the blood stream (Ketz, 2001). Nevertheless, the usage of sulfonylureas must be monitored closely since it can induce hypoglycemia during overproduction of insulin. In addition, sulfonylureas can also cause weight gain, mainly as a result of edema and reduction of the osmotic diuresis. Thus, this study attempted to identify 'insulin-like' extracts of wheat grain fermented with *Ganoderma* spp. mycelia which exert minimal lipogenic and anti-epinephrine induced lipolytic effects.

Oil Red O quantification assay indicated that the ethanol extracts of KUM60813 and KUM61076 fermented wheat grains demonstrated a dose-dependent increase in lipogenesis which was comparable to insulin. Moreover, the lipogenic activity of the ethanol extracts was 2 – 43 folds higher as compared to the corresponding aqueous extracts. Lipogenesis in preadipocyte indicates fat accumulation. Although lipogenesis is one of the 'insulin-like' characteristic, strong lipogenic activity may give rise to problems such as obesity. Therefore, moderate lipogenic extracts (ethanol extracts of wheat grain fermented with KUM60813 and KUM61076 which had equivalent lipogenic activity as insulin, 1 μM) may be preferred since these extracts are less likely to contribute to the development of obesity.

Current findings indicate that besides hormonal stimuli ROS and free radicals may also play an important role in preadipocyte differentiation. Usually oxidative stress is accompanied by increased visceral fat accumulation and reduced insulin-stimulated glucose transportation in muscles. If ROS production is augmented by extra- or intracellular stimuli, they can chronically affect biological systems: increased levels of oxidative stress are associated with impairment of glucose utilization and lactate production (Gummersbach *et al.*, 2009). In this study, we showed that both the aqueous

and ethanol extracts of unfermented and fermented wheat grain protect against the impairment in 3-isobutyl-1-methylxanthine, dexamethasone, and insulin stimulated preadipocyte differentiation and lipid accumulation induced by directly exposing 3T3-L1 cells to oxidative stress. The protective effect of these extracts may be due to their ability to induce GPx activity which enables the extracts to neutralize the hydrogen peroxide generated by GOx treatment and stimulate preadipocyte differentiation (Kuppusamy *et al.*, 2005).

5.5 Effect of extracts of unfermented and fermented wheat grain on lipolysis in 3T3-L1 adipocyte

Increasing lipolysis in adipocyte may be a potentially useful therapeutic target for treating obesity. However, chronically high levels of fatty acids in the blood, typically observed in obesity, are correlated with many detrimental metabolic consequences such as insulin resistance, cardiovascular disease and hypertension (Ahmadian *et al*., 2010). Although anti-adrenaline induced lipolysis is one of the insulin's characteristic, strong anti-adrenaline induced lipolytic activity is not favorable since it inhibits lipid mobilization that might pose a problem for obesity. Thus, based on the basal and anti-adrenaline induced lipolysis studies, ethanol extracts of wheat fermented with KUM60813 mycelia are more suitable for the development of anti-diabetic agent since they possessed mild lipolytic activity while stimulating lipogenesis moderately.

In contrast, extracts with high lipolytic effect are desirable for the development of anti-obesity drugs. Among the eight different extracts screened, aqueous extracts of unfermented and fermented wheat grain have a great potential to be used in anti-obesity treatment since it showed good lipolytic activity; this is followed by the ethanol extract

of *VITA GL* fermented wheat grain (Figure 4.19 and 4.20). However, strong lipolysis of adipocyte in the long run may lead to adverse effects such as accumulation of fatty acids in other organ besides adipocyte and increased blood viscosity (Voet *et al*., 1999). Therefore, usage of strong lipolytic extracts in treating obesity should be closely monitored. Since the present study does not focus on the development of anti-obesity agent, further investigations to identify the mechanism of action involved were not carried out. Nevertheless, studies on the anti-obesity potential of these extracts may be carried out in future.

5.6 Modulation of PPARγ targeting genes expression by extracts of fermented wheat grains in adipocyte in the absence and presence of oxidative stress

The highly complex genetic reprogramming that occurs during lipogenesis is under the tight control of hormones, cytokines, nutrients, and signaling molecules that change the expression and/or activity of a variety of transcription factors, which in turn finely regulate the sense and the magnitude of the adipose conversion process. Today many pro- and anti-lipogenic transcription factors are known to regulate lipogenesis (Fève, 2005). PPARγ has been identified as a central lipogenic regulator/switch and its agonists are transcription factors that regulate expression of genes involved in glucose and lipid metabolism. For example, PPARγ agonists like rosiglitazone and troglitazone are used widely in the treatment of Type 2 DM.

Ethanol extract of KUM60813 fermented wheat grain significantly stimulated lipogenic differentiation in 3T3-L1 cells and also up-regulated the gene expression of PPARγ, adiponectin, GLUT4, HSL and LPL. Previous studies have shown that PPARγ and C/EBPα cross-regulate each other to maintain their gene expression and also

modulate the expression of other lipogenic marker genes such as aP2, GLUT4, and LPL, during preadipocyte differentiation (Gregoire *et al.*, 1998; Rosen and Spiegelman, 2006). Therefore, ethanol extract of KUM60813 fermented wheat grain stimulated 3T3-L1 cells differentiation via the modulation of PPARγ.

Among the lipogenic marker genes shown in Figure 4.22, GLUT4 expression was dramatically elevated (24%) in response to the treatment with ethanol extract of KUM60813 fermented wheat grain. GLUT4 expression is regulated by PPARγ (Yang *et al.*, 1992; Gregoire et al.1998), and thus this extract may alter GLUT4 expression through the up-regulation of PPARγ. GLUT4 has a central role in the regulation of insulin-induced glucose uptake by adipocyte.

On the other hand, lipolytic activity in adipocyte is highly dependent on hormone sensitive lipase (HSL), which is regulated by insulin and catecholamine. Epinephrine is well known to stimulate lipolysis by promoting adenylate cyclase activity to increase cAMP level leading to the activation of HSL. However, the activation of cAMP phosphodiesterase (PDE) and stimulation of protein phosphatase-1 by insulin may inhibit the HSL activation (Chaves *et al*., 2011). The ethanol extract of *VITA GL* fermented wheat grain (100 μg/mL) showed an elevated HSL expression and this was followed by the ethanol extracts of KUM60813 and KUM61076 (100 μg/mL) fermented wheat grain. This data correlates well with the data from other experiments which showed that ethanol extract of *VITA GL* stimulated lipogenesis moderately while strongly stimulating basal and epinephrine-induced lipolysis in adipocyte.

H_2O_2 is one of the products of free radical dismutation (Valko *et. al.*, 2006). With regards to diabetes mellitus, H_2O_2 has been linked to insulin resistance via the inhibition of signaling pathways, including nuclear factor-κB, p38 MAPK and NH2-terminal Jun kinases / stress activated protein kinases (Gardner *et. al.*, 2003). It also

causes alteration to mitochondrial activity and production of cytosolic ATP, thus disrupting signal transduction of insulin secretion along the way (Cumaoğlu *et. al*., 2011).

Alteration in antioxidant defense system has been detected in patients with chronic diseases, including diabetes mellitus (Kuppusamy *et. al.,* 2005, Gupta & Chari, 2006). Endogenous enzymes as well as exogenous antioxidant molecules act as important markers in antioxidant analysis. Interestingly, in the present study the expression of GPx3 mRNA was elevated up to 2-fold (Figure 4.23), indicating that 3T3-L1 cells treated with ethanol extract of fermented wheat grain acquired resistance against oxidative stress stimulus. GPx is an enzyme that can effectively convert hydrogen peroxide to water, utilizing glutathione as oxygen donor.

The results of RT-PCR revealed that expression of PPARγ and adiponectin declined dramatically in oxidative stress induced 3T3-L1 adipocyte as compared to cells treated with ethanol extracts of fermented wheat grain alone. This observation is in agreement with reports of Soares *et al*., 2005 and Kamigaki *et al*., 2006, which demonstrated that 3T3-L1 cells are highly sensitive to oxidative stress, with subsequent reduction in adiponectin secretion in the presence of GOx and this effect was dose-dependent. Moreover, these findings suggest that GOx-mediated down regulation of PPARγ contribute to the alteration of lipogenic gene expression in adipocyte, and ROS appears to be involved as a mediator in this process. A similar finding was reported by Furukawa *et al*., 2004, where oxidative stress dysregulated mRNA expressions of adipocytokines and PPARγ white adipose tissue of KKAγ mice.

Generally, when cell damage is irretrievable under excessive stress conditions, p53 provokes the expression of apoptosis-inducible genes such as Bax, NADH oxidase (Noxa), and Bcl2 niding component 3 (Puma) and leads to apoptosis via the activation

of the mitochondria-mediated signalling pathway (Kojima *et al*., 2010). In this study, the expression of Bax gene remains unaffected after oxidative stress stimulation in the cells. This result indicates that the crude extracts of unfermented and fermented wheat grains were able to protect the adipocyte from cellular damage or cell death due to oxidative stress though there was a significant drop in lipogenic activity and PPARγ expression in the adipocyte.

5.7 Future investigation

The following studies are required to validate the findings of the present study:

I. Comprehensive chemical and physiological investigations are needed to identify the lead compounds present in the crude extracts of *Ganoderma* spp. fermented wheat grains that contributed to their antioxidative and insulin-like properties.

II. This study may be repeated with fruiting bodies of the same *Ganoderma* spp. as a starting material for comparison of antioxidant and insulin-like activities with their respective mycelia.

III. Protein expression assay can be performed to provide further understanding on the antioxidant and insulin-like activities of crude extracts of *Ganoderma* spp. fermented wheat grains.

IV. *In vivo* experiments can also be carried out to confirm the insulin-like potential of the crude extracts of *Ganoderma* spp. fermented wheat grains and further identify safety and therapeutic effects of the crude extracts on diabetic patients.

CONCLUSION

The total antioxidant capacity of both the aqueous and ethanol extracts of unfermented and fermented wheat grain and their insulin-like potential were investigated in this study. The key findings of this study are as follows:

1. Ethanol extracts of both the unfermented and fermented wheat grain possess better antioxidant activity compared to their corresponding aqueous extracts.

2. The antioxidant activity of wheat grain can be enhanced via fermentation with *G. neo-japonicum* (KUM61076).

3. Total phenol content in the ethanol extracts were significantly higher than that in the aqueous extract. This observation suggested that antioxidant activities of the mycelia extracts had positive correlation with their polyphenol contents. Thus, the phenolic compounds may play an important role in the antioxidant activities of the selected *Ganoderma* spp.

4. The wheat grains fermented with *G. australe* (KUM60813) and *G. neo-japonicum* (KUM61076) have greater antioxidant potential compared to the commercially available *G.lucidum* (*VITA GL*), suggesting that the *Ganoderma* spp. have nutraceutical potential and can be used as an easily accessible source of natural antioxidant.

5. Among the six mycelia extracts tested, ethanol extract of KUM60813 fermented wheat grains showed a unique combination of mild proliferative, lipogenic and anti-adrenaline induced lipolytic activities but at the same time up-regulated the gene expression of PPARγ, adiponectin, Glut-4, LPL and HSL.

6. The protective effect against oxidative stress induced predipocyte differentiation may be partially enhanced by maintaining intracellular redox state by antioxidants from ethanol extract of KUM61076 mycelia fermented wheat grains.

PUBLICATIONS / PRESENTED ABSRACTS

List of manuscripts

1. **Sarasvathy S**, Vikineswary S and Kuppusamy UR. Solid-substrate fermentation of wheat grains by mycelia of indigenous *Ganoderma* spp. to enhance the antioxidant activities. (*International Journal of Medicinal Mushroom* - submitted revised manuscript)

2. **Sarasvathy S**, Vikineswary S and Kuppusamy UR. Mycelia extracts of indigenous *Ganoderma* spp. stimulate lipogenesis, lipolysis and adiponectin expression in 3T3-L1 adipocytes. (Under review)

Presented abstracts in conferences

1. **Sarasvathy S**, Vikineswary S and Kuppusamy UR (2012). Evaluation of *Ganoderma* spp. mycelia extracts for insulin-like properties in 3T3-L1 mouse preadipocytes. National Postgraduate Conference, University of Malaya.

2. **Sarasvathy S**, Vikineswary S and Kuppusamy UR (2011). Antioxidant properties of mycelium extracts of selected indigenous *Ganoderma* spp. The 6th International Medicinal Mushroom Conference, Zagreb, Croatia.

The 6th International Medicinal Mushroom Conference

Antioxidant Properties of Mycelium Extracts of Selected Indigenous Ganoderma spp.

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Ganoderma is a cosmopolitan genus of wood decaying higher Basidiomycetes white rot fungi, which has been used for medicinal purposes for centuries, particularly in China, Japan, and Korea. It has been believed to possess many health benefits and reported to be affective against various diseases by virtue of their antioxidant potential.

In the present study, solid-state fermentation of wheat grains with selected indigenous *Ganoderma* spp., including KUM60813, KUM61076 and VITA GL (a commercial strain), was carried out. Antioxidant activities of the crude ethanol and hot water extracts from the fermented and unfermented grains were investigated by the ferric reducing antioxidant power (FRAP), Trolox Equivalent Antioxidant Capacity (TEAC) and 1-diphenyl-2-picrylhydrazyl (DPPH) free-radical scavenging ability. Among the six mycelium extracts tested, the ethanol extract of KUM61076 was the most potent radical scavenging activity in each assay, 89.00 ± 1.13 μmol of $FeSO_4.7H_2O$ equivalents/g of extract in the FRAP assay, 30.26 ± 0.33 % (at 100μg/mL) in the DPPH free-radical scavenging assay and 9.48 ± 1.79 mg TE/g of extract in TEAC assay. Total phenolic content in the ethanol extracts were higher than that in the hot aqueous extracts. The antioxidant activities of the mycelium extracts had positive correlation with their polyphenol contents, suggesting that the phenolic compounds may play an important role in the antioxidation activities of the *Ganoderma* spp. studied.

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EVALUATION OF GANODERMA SPP. MYCELIA EXTRACTS FOR INSULIN-LIKE PROPERTIES IN 3T3-L1 MOUSE PREADIPOCYTES

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Adipocyte dysfunction is strongly associated with the progression of cardiovascular risk factors and diabetes. It is accepted that the regulation of lipogenesis and adipokine expression, notably adiponectin, is able to prevent these disorders. The present study evaluates the *in vitro* insulin-like properties of indigenous *Ganoderma australe* (KUM60813) and *Ganoderma neo-japonicum* (KUM61076) ethanolic extracts through lipogenic, anti-lipolytic and adipokine expression. *Ganoderma lucidum* (VITA GL) (a commercial strain) was included in the study as a reference. Among the mycelia extracts tested, *G.neo-japonicum* induced significant lipogenesis in the dose range of 20 – 100 μg/mL with the highest activity (69%) at 100 μg/mL. This extract (100 μg/mL) also inhibited approximately 48% of epinephrine (1μM) induced lipolysis. At the molecular level, treatment with *Ganoderma* spp. mycelia extracts up-regulated the key adipocyte differentiation regulator PPARγ and the downstream effectors such as adiponectin and GLUT-4. Taken together, these results suggest that Ganoderma especially *G.neo-japonicum* has insulin-like properties and may be useful as potential therapeutic agent in the management of diabetes.

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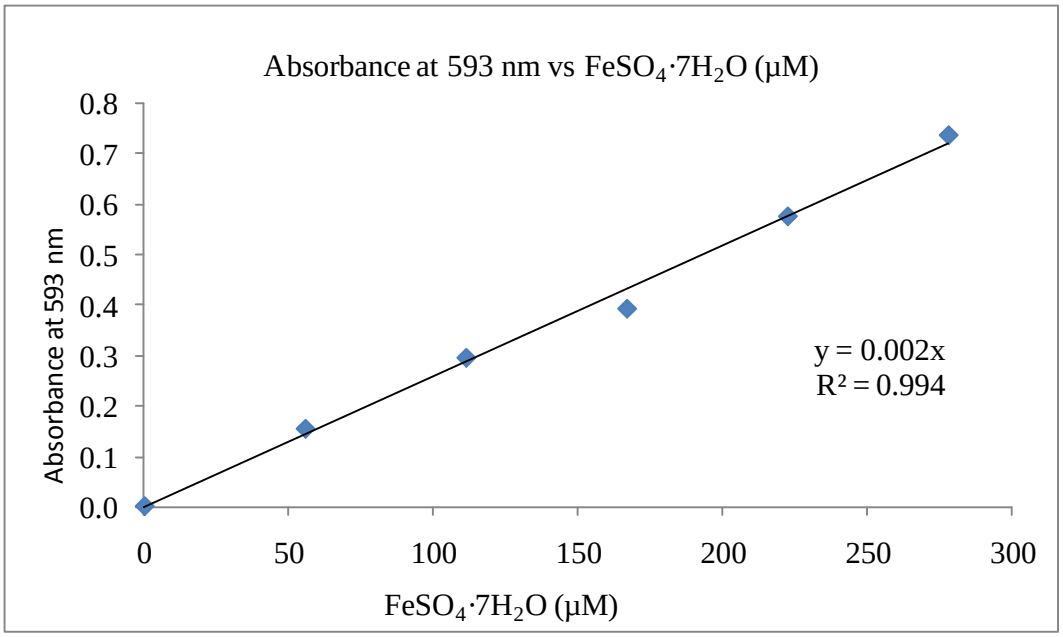
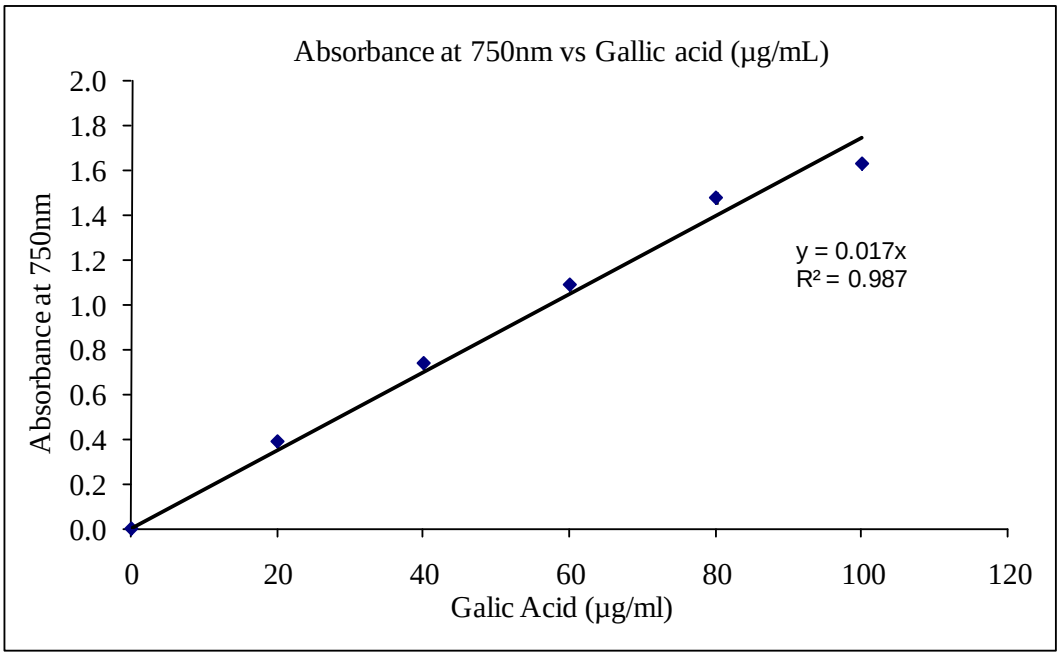
APPENDIX A: STANDARD CURVES OF ANTIOXIDANT ASSAYS

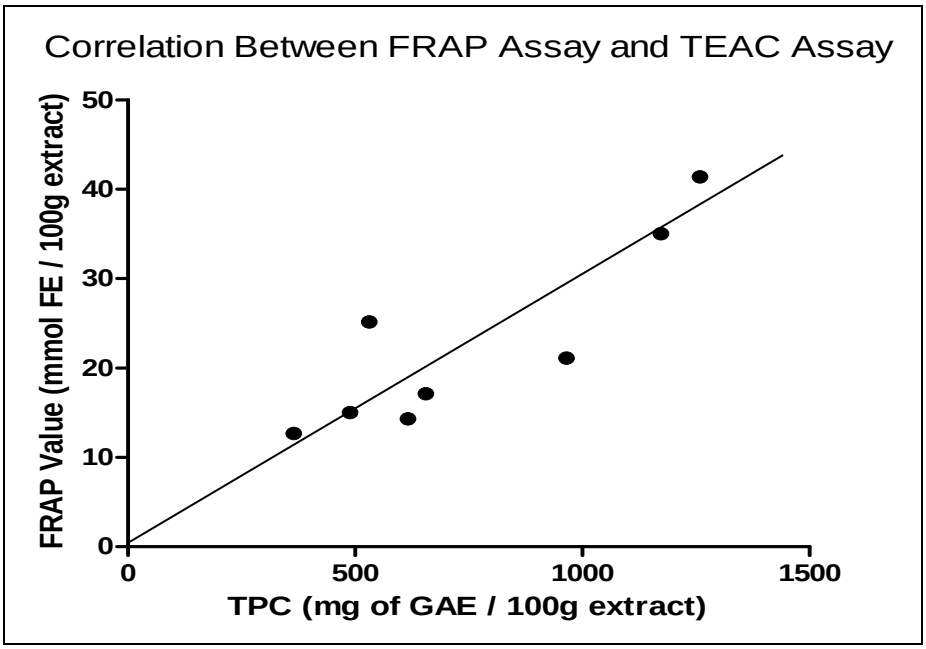
I) $FeSO_4 \cdot 7H_2O$ calibration plot

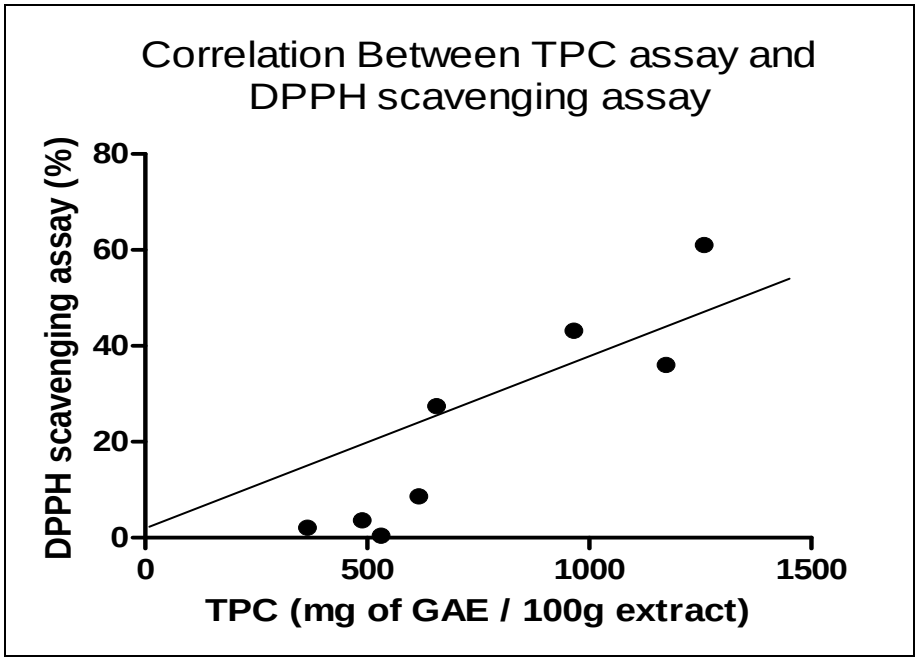


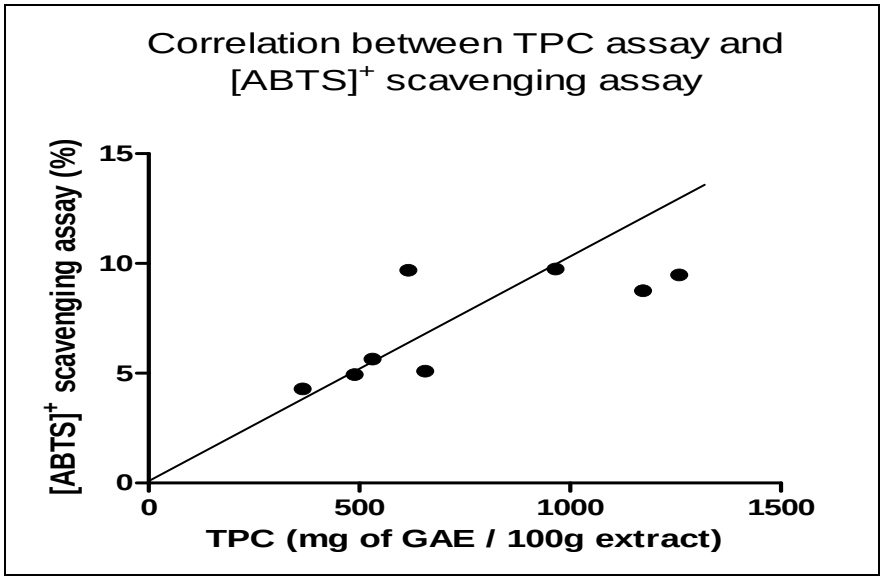
II) Gallic acid calibration plot



III) Correlation graphs

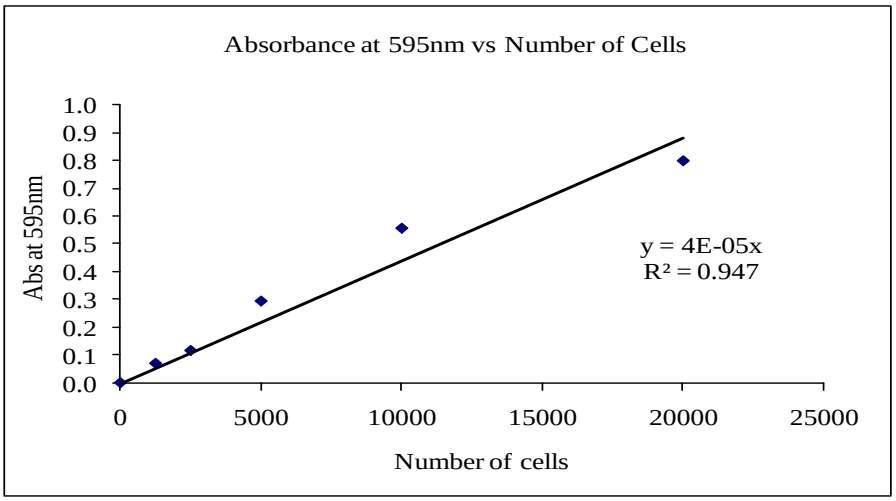






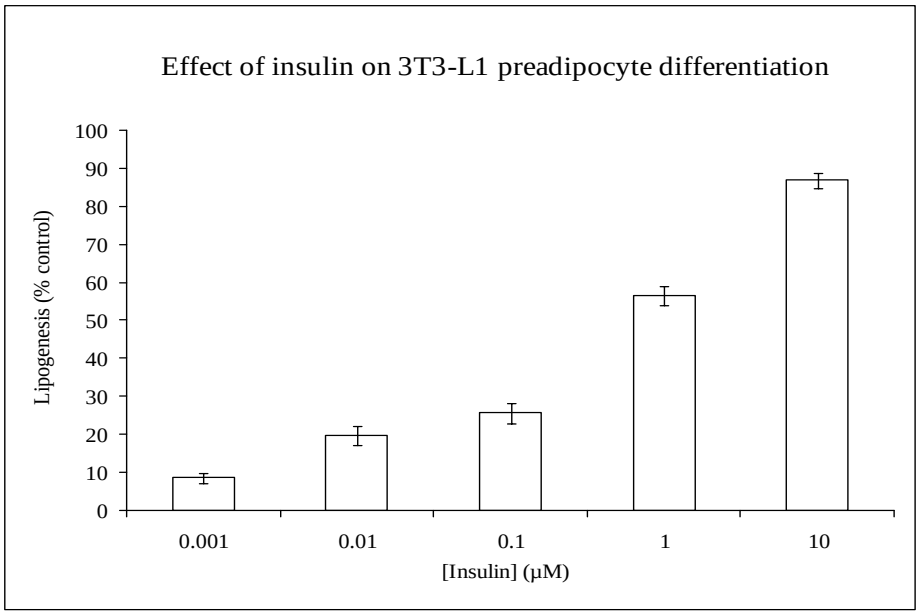
APPENDIX B: STANDARD CURVES OF CELL BASED ASSAYS

I) Determination of 3T3-L1 preadipocyte seeding density for MTT assay



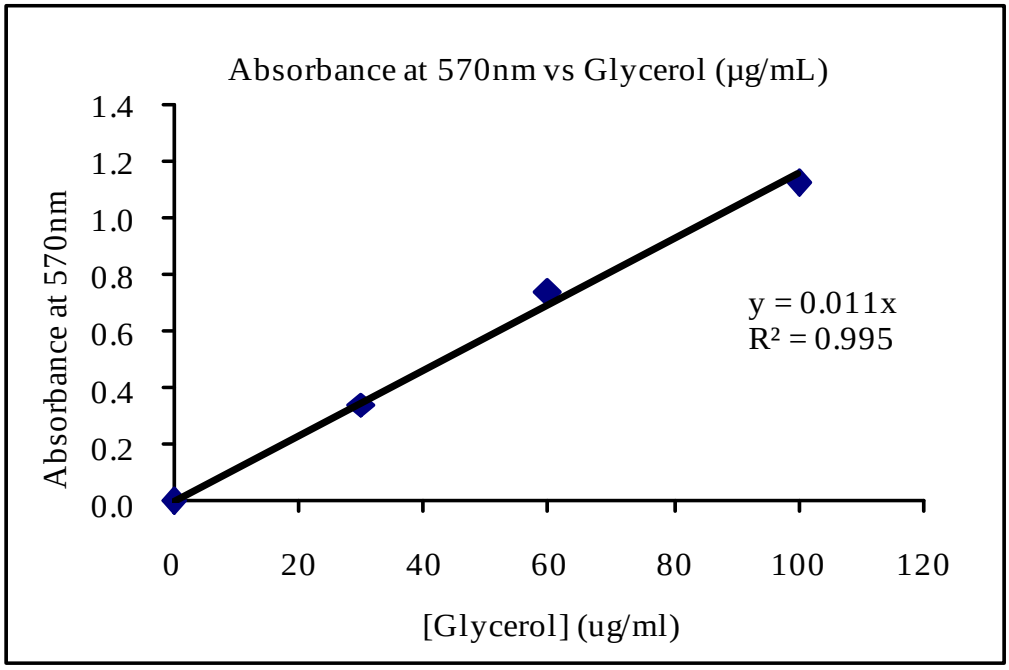
Cells seeded at different density to obtain optimal number of cells for MTT assay in the presence of extracts. Based on the result obtained, 10 000 cells was chosen for MTT assay since its absorbance reading was 0.5 at 595nm.

II) Insulin standard curve (Lipogenesis Assay)

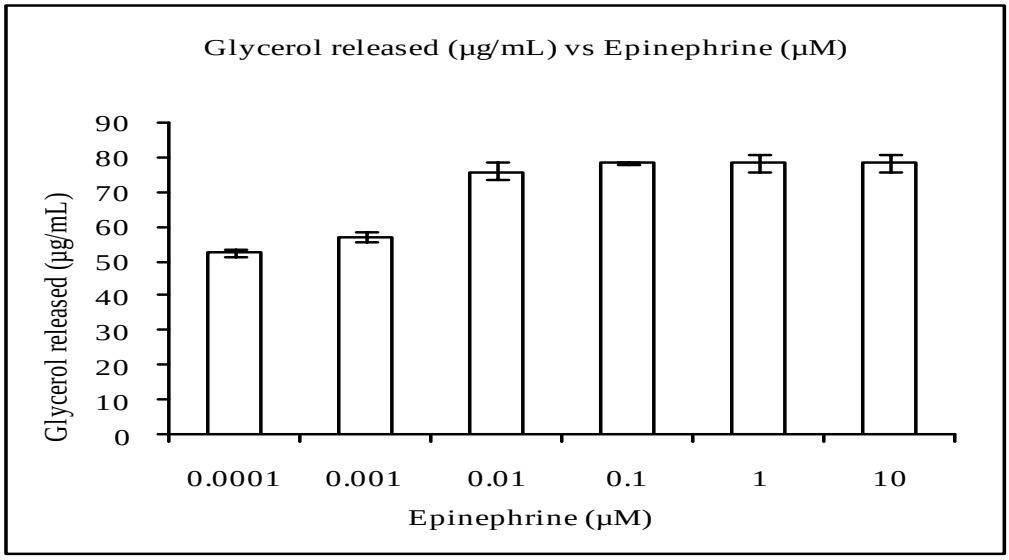


Insulin at the concentration range of 0.1, 1, 10, 100 and 1000 µM was used to determine the effect of insulin on 3T3-L1 preadipocyte lipogenesis with Oil Red O assay. Y-axis indicates the mean percentages of lipogenesis ± SEM of quadruplicate assays compared to control value (untreated cells). Based on the result obtained, 1 µM of insulin was chosen as the positive control for Oil Red O assay since it is exerting medial lipogenic activity in 3T3-L1 preadipocyte.

III) Glycerol calibration plot (Lipolysis Assay)

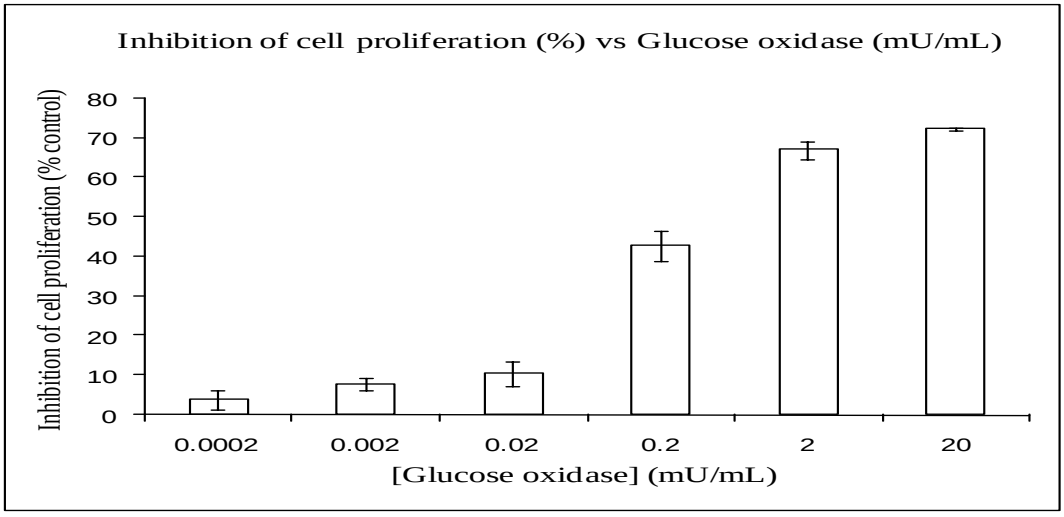


IV)Epinephrine standard curve (Lipolysis Assay)



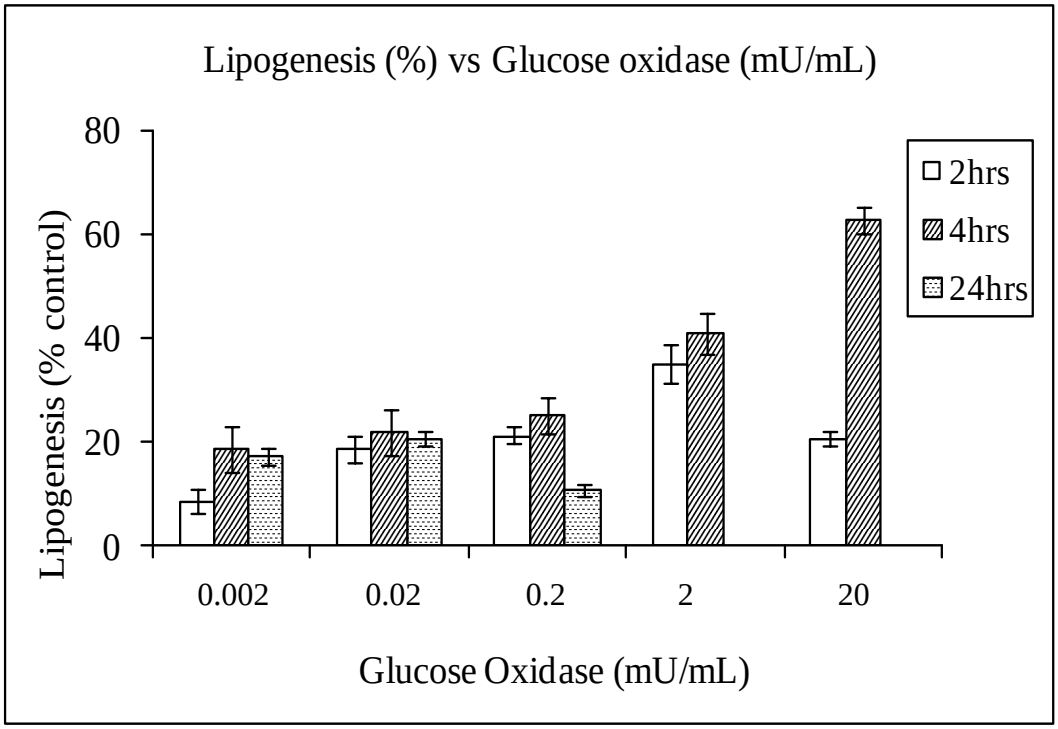
Epinephrine at the concentration range of 0.0001, 0.001, 0.01, 0.1, 1 and 10 µM was used to determine the effect of adrenaline on adipocyte lipolysis with glycerol quantification assay. Y-axis indicates the mean glycerol released ± SEM of quadruplicate assays compared to control value (untreated cells). Based on the result obtained, 1 µM of adrenaline was identified as the optimum concentration for glycerol quantification assay.

V) Glucose oxidase standard plot (MTT assay)



Glucose oxidase concentrations ranging from 0.0002 – 20 mU/mL were incubated for two hours with the concentration of 10,000 cells/well. The absorbance of the mixture was read after 24, 48 and 72 hours of incubation (with 10µl MTT solution) at 37ºC and 5% of carbon dioxide (CO_2). The data presented as mean (n=3) ± S.E.M as a single representation of three separate experiments.

VI) Glucose oxidase standard plot (Lipogenesis assay)



Glucose oxidase at the concentration range of 0.002 – 20 mU/mL was used to determine the effect of oxidative stress on 3T3-L1 preadipocyte lipogenesis with Oil Red O assay. Y-axis indicates the mean percentages of lipogenesis inhibition ± SEM of trplicate assays compared to control value (untreated cells). Based on the result obtained, 2 mU/mL of GOD was chosen to induce oxidative stress for Oil Red O assay.

APPENDIX C: STATISTICAL TABLES

1) DPPH free radical scavenging effect of aqueous extracts of unfermented and fermented wheat grains

ANOVA: Effect of different concentrations of aqueous extract of unfermented wheat grains on antioxidant activity measured by DPPH radical scavenging method (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	285.286	4	71.3216	95.31	0.0000
Within groups	7.48348	10	0.748348		
Total (Corr.)	292.77	14			

Multiple range test: Effect of different concentrations of aqueous extract of unfermented wheat grains on antioxidant activity measured by DPPH radical scavenging method (between concentrations)

Concentration (µg/mL)	Difference	+/- Limits
50 - 100	*-10.3233	1.5738
50 - 150	*-9.018	1.5738
50 - 200	*-6.761	1.5738
50 - 250	-0.218667	1.5738
100 - 150	1.30533	1.5738
100 - 200	*3.56233	1.5738
100 - 250	*10.1047	1.5738
150 - 200	*2.257	1.5738
150 - 250	*8.79933	1.5738
200 - 250	*6.54233	1.5738

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of aqueous extract of KUM60813 fermented wheat grains on antioxidant activity measured by DPPH radical scavenging method (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	12.2088	4	3.0522	5.24	0.0155
Within groups	5.82966	10	0.582966		
Total (Corr.)	18.0385	14			

Multiple range test: Effect of different concentrations of aqueous extract of KUM60813 fermented wheat grains on antioxidant activity measured by DPPH radical scavenging method (between concentrations)

Concentration (µg/mL)	Difference	+/- Limits
50 - 100	-0.723667	1.38905
50 - 150	*-1.525	1.38905
50 - 200	*-1.817	1.38905
50 - 250	*-2.61767	1.38905
100 - 150	-0.801333	1.38905
100 - 200	-1.09333	1.38905
100 - 250	*-1.894	1.38905
150 - 200	-0.292	1.38905

150 - 250	-1.09267	1.38905
200 - 250	-0.800667	1.38905

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of aqueous extract of KUM61076 fermented wheat grains on antioxidant activity measured by DPPH radical scavenging method (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	110.216	4	27.5541	75.60	0.0000
Within groups	3.64462	10	0.364462		
Total (Corr.)	113.861	14			

Multiple range test: Effect of different concentrations of aqueous extract of KUM61076 fermented wheat grains on antioxidant activity measured by DPPH radical scavenging method (between concentrations)

Concentration (µg/mL)	Difference	+/- Limits
50 - 100	*-1.818	1.09831
50 - 150	*-4.58	1.09831
50 - 200	*-5.38333	1.09831
50 - 250	*-7.712	1.09831
100 - 150	*-2.762	1.09831
100 - 200	*-3.56533	1.09831
100 - 250	*-5.894	1.09831
150 - 200	-0.803333	1.09831
150 - 250	*-3.132	1.09831
200 - 250	*-2.32867	1.09831

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of aqueous extract of *VITA GL* fermented wheat grains on antioxidant activity measured by DPPH radical scavenging method (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	3.23434	4	0.808584	2.50	0.1091
Within groups	3.23103	10	0.323103		
Total (Corr.)	6.46536	14			

Multiple range test: Effect of different concentrations of aqueous extract of *VITA GL* fermented wheat grains on antioxidant activity measured by DPPH radical scavenging method (between concentrations)

Concentration (µg/mL)	Difference	+/- Limits
50 - 100	-0.0703333	1.03411
50 - 150	-0.434333	1.03411
50 - 200	-0.945333	1.03411
50 - 250	*-1.166	1.03411
100 - 150	-0.364	1.03411
100 - 200	-0.875	1.03411
100 - 250	*-1.09567	1.03411
150 - 200	-0.511	1.03411
150 - 250	-0.731667	1.03411

200 - 250	-0.220667	1.03411

* denotes a statistically significant difference.

2) DPPH free radical scavenging effect of ethanol extracts of unfermented and fermented wheat grains

ANOVA: Effect of different concentrations of ethanol extract of unfermented wheat grains on antioxidant activity measured by DPPH radical scavenging method (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	1274.96	4	318.741	157.45	0.0000
Within groups	20.2437	10	2.02437		
Total (Corr.)	1295.21	14			

Multiple range test: Effect of different concentrations of ethanol extract of unfermented wheat grains on antioxidant activity measured by DPPH radical scavenging method (between concentrations)

Concentration (µg/mL)	Difference	+/- Limits
50 - 100	-0.646333	2.58847
50 - 150	*-11.7127	2.58847
50 - 200	*-17.6873	2.58847
50 - 250	*-23.3323	2.58847
100 - 150	*-11.0663	2.58847
100 - 200	*-17.041	2.58847
100 - 250	*-22.686	2.58847
150 - 200	*-5.97467	2.58847
150 - 250	*-11.6197	2.58847
200 - 250	*-5.645	2.58847

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of ethanol extract of KUM60813 fermented wheat grains on antioxidant activity measured by DPPH radical scavenging method (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	1554.39	4	388.598	188.25	0.0000
Within groups	20.643	10	2.0643		
Total (Corr.)	1575.04	14			

Multiple range test: Effect of different concentrations of ethanol extract of KUM60813 fermented wheat grains on antioxidant activity measured by DPPH radical scavenging method (between concentrations)

Concentration (µg/mL)	Difference	+/- Limits
50 - 100	*-7.194	2.61387
50 - 150	*-10.2763	2.61387
50 - 200	*-21.192	2.61387
50 - 250	*-28.5523	2.61387
100 - 150	*-3.08233	2.61387
100 - 200	*-13.998	2.61387
100 - 250	*-21.3583	2.61387

150 - 200	*-10.9157	2.61387
150 - 250	*-18.276	2.61387
200 - 250	*-7.36033	2.61387

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of ethanol extract of KUM61076 fermented wheat grains on antioxidant activity measured by DPPH radical scavenging method (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	3492.21	4	873.051	785.47	0.0000
Within groups	11.115	10	1.1115		
Total (Corr.)	3503.32	14			

Multiple range test: Effect of different concentrations of ethanol extract of KUM61076 fermented wheat grains on antioxidant activity measured by DPPH radical scavenging method (between concentrations)

Concentration (µg/mL)	Difference	+/- Limits
50 - 100	*-10.8577	1.91802
50 - 150	*-22.8577	1.91802
50 - 200	*-35.0627	1.91802
50 - 250	*-41.6107	1.91802
100 - 150	*-12.0	1.91802
100 - 200	*-24.205	1.91802
100 - 250	*-30.753	1.91802
150 - 200	*-12.205	1.91802
150 - 250	*-18.753	1.91802
200 - 250	*-6.548	1.91802

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of ethanol extract of *VITA GL* fermented wheat grains on antioxidant activity measured by DPPH radical scavenging method (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	889.099	4	222.275	108.19	0.0000
Within groups	20.545	10	2.0545		
Total (Corr.)	909.644	14			

Multiple range test: Effect of different concentrations of ethanol extract of *VITA GL* fermented wheat grains on antioxidant activity measured by DPPH radical scavenging method (between concentrations)

Concentration (µg/mL)	Difference	+/- Limits
50 - 100	*-6.63133	2.60766
50 - 150	*-9.57333	2.60766
50 - 200	*-10.573	2.60766
50 - 250	*-23.5843	2.60766
100 - 150	*-2.942	2.60766
100 - 200	*-3.94167	2.60766
100 - 250	*-16.953	2.60766
150 - 200	-0.999667	2.60766

150 - 250	*-14.011	2.60766
200 - 250	*-13.0113	2.60766

* denotes a statistically significant difference.

ANOVA: Effect of 50 µg/mL of extract on antioxidant activity measured by DPPH radical scavenging method (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	1227.11	7	175.301	271.15	0.0000
Within groups	10.3441	16	0.646509		
Total (Corr.)	1237.45	23			

Multiple range test: Effect of 50 µg/mL of extract on antioxidant activity measured by DPPH radical scavenging method (between extracts)

Extract	Difference	+/- Limits
AW - BW	-0.800333	1.39174
AW - CW	-0.726333	1.39174
AW – DW	-0.728	1.39174
AW - AE	*-12.4857	1.39174
AW - BE	*-14.4097	1.39174
AW - CE	*-19.1883	1.39174
AW - DE	*-3.62533	1.39174
BW - CW	0.074	1.39174
BW - DW	0.0723333	1.39174
BW - AE	*-11.6853	1.39174
BW - BE	*-13.6093	1.39174
BW - CE	*-18.388	1.39174
BW - DE	*-2.825	1.39174
CW - DW	-0.00166667	1.39174
CW - AE	*-11.7593	1.39174
CW - BE	*-13.6833	1.39174
CW - CE	*-18.462	1.39174
CW - DE	*-2.899	1.39174
DW - AE	*-11.7577	1.39174
DW - BE	*-13.6817	1.39174
DW - CE	*-18.4603	1.39174
DW - DE	*-2.89733	1.39174
AE - BE	*-1.924	1.39174
AE - CE	*-6.70267	1.39174
AE - DE	*8.86033	1.39174
BE - CE	*-4.77867	1.39174
BE - DE	*10.7843	1.39174
CE - DE	*15.563	1.39174

* denotes a statistically significant difference.

ANOVA: Effect of 100 µg/mL of extract on antioxidant activity measured by DPPH radical scavenging method (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	2236.47	7	319.496	240.41	0.0000
Within groups	21.2635	16	1.32897		
Total (Corr.)	2257.73	23			

Multiple range test: Effect of 100 µg/mL of extract on antioxidant activity measured by DPPH radical scavenging method (between extracts)

Extract	Difference	+/- Limits
AW - BW	*8.79933	1.9954
AW - CW	*7.779	1.9954
AW - DW	*9.525	1.9954
AW - AE	*-2.80867	1.9954
AW - BE	*-11.2803	1.9954
AW - CE	*-19.7227	1.9954
AW - DE	0.0666667	1.9954
BW - CW	-1.02033	1.9954
BW - DW	0.725667	1.9954
BW - AE	*-11.608	1.9954
BW - BE	*-20.0797	1.9954
BW - CE	*-28.522	1.9954
BW - DE	*-8.73267	1.9954
CW - DW	1.746	1.9954
CW - AE	*-10.5877	1.9954
CW - BE	*-19.0593	1.9954
CW - CE	*-27.5017	1.9954
CW - DE	*-7.71233	1.9954
DW - AE	*-12.3337	1.9954
DW - BE	*-20.8053	1.9954
DW - CE	*-29.2477	1.9954
DW - DE	*-9.45833	1.9954
AE - BE	*-8.47167	1.9954
AE - CE	*-16.914	1.9954
AE - DE	*2.87533	1.9954
BE - CE	*-8.44233	1.9954
BE - DE	*11.347	1.9954
CE - DE	*19.7893	1.9954

* denotes a statistically significant difference.

ANOVA: Effect of 150 µg/mL of extract on antioxidant activity measured by DPPH radical scavenging method (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	183.7	7	597.672	1333.04	0.0000
Within groups	7.17366	16	0.448354		
Total (Corr.)	4190.88	23			

Multiple range test: Effect of 150 µg/mL of extract on antioxidant activity measured by DPPH radical scavenging method (between extracts)

Extract	Difference	+/- Limits
AW - BW	*6.69267	1.159
AW - CW	*3.71167	1.159
AW - DW	*7.85567	1.159
AW - AE	*-15.1803	1.159
AW - BE	*-15.668	1.159
AW - CE	*-33.028	1.159
AW - DE	*-4.18067	1.159
BW - CW	*-2.981	1.159
BW - DW	*1.163	1.159

BW - AE	*-21.873	1.159
BW - BE	*-22.3607	1.159
BW - CE	*-39.7207	1.159
BW - DE	*-10.8733	1.159
CW - DW	*4.144	1.159
CW - AE	*-18.892	1.159
CW - BE	*-19.3797	1.159
CW - CE	*-36.7397	1.159
CW - DE	*-7.89233	1.159
DW - AE	*-23.036	1.159
DW - BE	*-23.5237	1.159
DW - CE	*-40.8837	1.159
DW - DE	*-12.0363	1.159
AE - BE	-0.487667	1.159
AE - CE	*-17.8477	1.159
AE - DE	*10.9997	1.159
BE - CE	*-17.36	1.159
BE - DE	*11.4873	1.159
CE - DE	*28.8473	1.159

* denotes a statistically significant difference.

ANOVA: Effect of 200 µg/mL of extract on antioxidant activity measured by DPPH radical scavenging method (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	7651.96	7	1093.14	546.96	0.0000
Within groups	31.977	16	1.99856		
Total (Corr.)	7683.93	23			

Multiple range test: Effect of 200 µg/mL of extract on antioxidant activity measured by DPPH radical scavenging method (between extracts)

Extract	Difference	+/- Limits
AW - BW	*4.14367	2.44698
AW - CW	0.651333	2.44698
AW - DW	*5.08767	2.44698
AW - AE	*-23.412	2.44698
AW - BE	*-28.8407	2.44698
AW - CE	*-47.49	2.44698
AW - DE	*-7.43733	2.44698
BW - CW	*-3.49233	2.44698
BW - DW	0.944	2.44698
BW - AE	*-27.5557	2.44698
BW - BE	*-32.9843	2.44698
BW - CE	*-51.6337	2.44698
BW - DE	*-11.581	2.44698
CW - DW	*4.43633	2.44698
CW - AE	*-24.0633	2.44698
CW - BE	*-29.492	2.44698
CW - CE	*-48.1413	2.44698
CW - DE	*-8.08867	2.44698
DW - AE	*-28.4997	2.44698
DW - BE	*-33.9283	2.44698
DW - CE	*-52.5777	2.44698
DW - DE	*-12.525	2.44698
AE - BE	*-5.42867	2.44698

AE - CE	*-24.078	2.44698
AE - DE	*15.9747	2.44698
BE - CE	*-18.6493	2.44698
BE - DE	*21.4033	2.44698
CE - DE	*40.0527	2.44698

* denotes a statistically significant difference.

ANOVA: Effect of 250 µg/mL of extract on antioxidant activity measured by DPPH radical scavenging method (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	10703.7	7	1529.1	1113.22	0.0000
Within groups	21.9773	16	1.37358		
Total (Corr.)	10725.7	23			

Multiple range test: Effect of 250 µg/mL of extract on antioxidant activity measured by DPPH radical scavenging method (between extracts)

Extract	Difference	+/- Limits
AW - BW	*-3.19933	2.02861
AW - CW	*-8.21967	2.02861
AW - DW	-1.67533	2.02861
AW - AE	*-35.5993	2.02861
AW - BE	*-42.7433	2.02861
AW - CE	*-60.5803	2.02861
AW - DE	*-26.991	2.02861
BW - CW	*-5.02033	2.02861
BW - DW	1.524	2.02861
BW - AE	*-32.4	2.02861
BW - BE	*-39.544	2.02861
BW - CE	*-57.381	2.02861
BW - DE	*-23.7917	2.02861
CW - DW	*6.54433	2.02861
CW - AE	*-27.3797	2.02861
CW - BE	*-34.5237	2.02861
CW - CE	*-52.3607	2.02861
CW - DE	*-18.7713	2.02861
DW - AE	*-33.924	2.02861
DW - BE	*-41.068	2.02861
DW - CE	*-58.905	2.02861
DW - DE	*-25.3157	2.02861
AE - BE	*-7.144	2.02861
AE - CE	*-24.981	2.02861
AE - DE	*8.60833	2.02861
BE - CE	*-17.837	2.02861
BE - DE	*15.7523	2.02861
CE - DE	*33.5893	2.02861

* denotes a statistically significant difference.

3) ABTS radical scavenging effect of aqueous extracts of unfermented and fermented wheat grains

ANOVA: Effect of 20 µg/mL of extract on antioxidant activity measured by ABTS radical scavenging method (between aqueous extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	359.636	4	89.9089	100.91	0.0000
Within groups	8.91007	10	0.891007		
Total (Corr.)	368.546	14			

Multiple range test: Effect of 20 µg/mL of extract on antioxidant activity measured by ABTS radical scavenging method (between aqueous extracts)

Extracts	Difference	+/- Limits
Wheat - KUM60813	*7.52667	1.71727
Wheat - KUM61076	*8.08	1.71727
Wheat - *VITA GL*	*6.50667	1.71727
Wheat - BHT	*-4.33667	1.71727
KUM60813 - KUM61076	0.553333	1.71727
KUM60813 - *VITA GL*	-1.02	1.71727
KUM60813 - BHT	*-11.8633	1.71727
KUM61076 - *VITA GL*	-1.57333	1.71727
KUM61076 - BHT	*-12.4167	1.71727
VITA GL- BHT	*-10.8433	1.71727

* denotes a statistically significant difference.

ANOVA: Effect of 40 µg/mL of extract on antioxidant activity measured by ABTS radical scavenging method (between aqueous extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	915.909	4	228.977	52.82	0.0000
Within groups	43.3503	10	4.33503		
Total (Corr.)	959.259	14			

Multiple range test: Effect of 40 µg/mL of extract on antioxidant activity measured by ABTS radical scavenging method (between aqueous extracts)

Extracts	Difference	+/- Limits
Wheat - KUM60813	*7.64667	3.78786
Wheat - KUM61076	*10.88	3.78786
Wheat - *VITA GL*	*12.07	3.78786
Wheat - BHT	*-8.81333	3.78786
KUM60813 - KUM61076	3.23333	3.78786
KUM60813 - *VITA GL*	*4.42333	3.78786
KUM60813 - BHT	*-16.46	3.78786
KUM61076 - *VITA GL*	1.19	3.78786
KUM61076 - BHT	*-19.6933	3.78786
VITA GL - BHT	*-20.8833	3.78786

* denotes a statistically significant difference.

ANOVA: Effect of 60 µg/mL of extract on antioxidant activity measured by ABTS radical scavenging method (between aqueous extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	1699.62	4	424.906	410.43	0.0000
Within groups	10.3527	10	1.03527		
Total (Corr.)	1709.98	14			

Multiple range test: Effect of 60 µg/mL of extract on antioxidant activity measured by ABTS radical scavenging method (between aqueous extracts)

Extracts	Difference	+/- Limits
Wheat - KUM60813	*14.0267	1.85108
Wheat - KUM61076	*9.60333	1.85108
Wheat - *VITA GL*	*17.51	1.85108
Wheat - BHT	*-11.91	1.85108
KUM60813 - KUM61076	*-4.42333	1.85108
KUM60813 - *VITA GL*	*3.48333	1.85108
KUM60813 - BHT	*-25.9367	1.85108
KUM61076 - *VITA GL*	*7.90667	1.85108
KUM61076 - BHT	*-21.5133	1.85108
VITA GL - BHT	*-29.42	1.85108

* denotes a statistically significant difference.

ANOVA: Effect of 80 µg/mL of extract on antioxidant activity measured by ABTS radical scavenging method (between aqueous extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	3493.5	4	873.375	969.10	0.0000
Within groups	9.01227	10	0.901227		
Total (Corr.)	3502.51	14			

Multiple range test: Effect of 80 µg/mL of extract on antioxidant activity measured by ABTS radical scavenging method (between aqueous extracts)

Extracts	Difference	+/- Limits
Wheat - KUM60813	*13.68	1.72709
Wheat - KUM61076	*5.91	1.72709
Wheat - *VITA GL*	*14.49	1.72709
Wheat - BHT	*-27.2367	1.72709
KUM60813 - KUM61076	*-7.77	1.72709
KUM60813 - *VITA GL*	0.81	1.72709
KUM60813 - BHT	*-40.9167	1.72709
KUM61076 - *VITA GL*	*8.58	1.72709
KUM61076 - BHT	*-33.1467	1.72709
VITA GL - BHT	*-41.7267	1.72709

* denotes a statistically significant difference.

ANOVA: Effect of 100 µg/mL of extract on antioxidant activity measured by ABTS radical scavenging method (between aqueous extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	7555.19	4	1888.8	693.59	0.0000
Within groups	27.2323	10	2.72323		
Total (Corr.)	7582.43	14			

Multiple range test: Effect of 100 µg/mL of extract on antioxidant activity measured by ABTS radical scavenging method (between aqueous extracts)

Extracts	Difference	+/- Limits
Wheat - KUM60813	*19.55	3.0022
Wheat - KUM61076	*8.33	3.0022
Wheat - *VITA GL*	*22.78	3.0022
Wheat - BHT	*-39.65	3.0022
KUM60813 - KUM61076	*-11.22	3.0022
KUM60813 - *VITA GL*	*3.23	3.0022
KUM60813 - BHT	*-59.2	3.0022
KUM61076 - *VITA GL*	*14.45	3.0022
KUM61076 - BHT	*-47.98	3.0022
VITA GL - BHT	*-62.43	3.0022

* denotes a statistically significant difference.

4) ABTS radical scavenging effect of ethanol extracts of unfermented and fermented wheat grains

ANOVA: Effect of 20 µg/mL of extract on antioxidant activity measured by ABTS radical scavenging method (between ethanol extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	326.586	4	81.6464	34.85	0.0000
Within groups	23.4265	10	2.34265		
Total (Corr.)	350.012	14			

Multiple range test: Effect of 20 µg/mL of extract on antioxidant activity measured by ABTS radical scavenging method (between ethanol extracts)

Extracts	Difference	+/- Limits
Wheat - KUM60813	*4.08667	2.78452
Wheat - KUM61076	1.14	2.78452
Wheat - *VITA GL*	*11.37	2.78452
Wheat - BHT	-2.02	2.78452
KUM60813 - KUM61076	*-2.94667	2.78452
KUM60813 - *VITA GL*	*7.28333	2.78452
KUM60813 - BHT	*-6.10667	2.78452
KUM61076 - *VITA GL*	*10.23	2.78452
KUM61076 - BHT	*-3.16	2.78452
VITA GL - BHT	*-13.39	2.78452

* denotes a statistically significant difference.

ANOVA: Effect of 40 µg/mL of extract on antioxidant activity measured by ABTS radical scavenging method (between ethanol extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	454.883	4	113.721	34.81	0.0000
Within groups	32.6734	10	3.26734		
Total (Corr.)	487.557	14			

Multiple range test: Effect of 40 µg/mL of extract on antioxidant activity measured by ABTS radical scavenging method (between ethanol extracts)

Extracts	Difference	+/- Limits
Wheat - KUM60813	-0.583333	3.28848
Wheat - KUM61076	0.34	3.28848
Wheat - *VITA GL*	*7.45667	3.28848
Wheat - BHT	*-9.84667	3.28848
KUM60813 - KUM61076	0.923333	3.28848
KUM60813 - *VITA GL*	*8.04	3.28848
KUM60813 - BHT	*-9.26333	3.28848
KUM61076 - *VITA GL*	*7.11667	3.28848
KUM61076 - BHT	*-10.1867	3.28848
VITA GL - BHT	*-17.3033	3.28848

* denotes a statistically significant difference.

ANOVA: Effect of 60 µg/mL of extract on antioxidant activity measured by ABTS radical scavenging method (between ethanol extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	945.87	4	236.467	280.13	0.0000
Within groups	8.4412	10	0.84412		
Total (Corr.)	954.311	14			

Multiple range test: Effect of 60 µg/mL of extract on antioxidant activity measured by ABTS radical scavenging method (between ethanol extracts)

Extracts	Difference	+/- Limits
Wheat - KUM60813	*-6.14667	1.67147
Wheat - KUM61076	*-3.57333	1.67147
Wheat - *VITA GL*	*4.04333	1.67147
Wheat - BHT	*-19.3233	1.67147
KUM60813 - KUM61076	*2.57333	1.67147
KUM60813 - *VITA GL*	*10.19	1.67147
KUM60813 - BHT	*-13.1767	1.67147
KUM61076 - *VITA GL*	*7.61667	1.67147
KUM61076 - BHT	*-15.75	1.67147
VITA GL - BHT	*-23.3667	1.67147

* denotes a statistically significant difference.

ANOVA: Effect of 80 µg/mL of extract on antioxidant activity measured by ABTS radical scavenging method (between ethanol extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	2444.17	4	611.043	273.44	0.0000
Within groups	22.3463	10	2.23463		
Total (Corr.)	2466.52	14			

Multiple range test: Effect of 80 µg/mL of extract on antioxidant activity measured by ABTS radical scavenging method (between ethanol extracts)

Extracts	Difference	+/- Limits
Wheat - KUM60813	*-11.2433	2.71957
Wheat - KUM61076	*-8.38	2.71957
Wheat - *VITA GL*	2.23	2.71957
Wheat - BHT	*-33.69	2.71957
KUM60813 - KUM61076	*2.86333	2.71957
KUM60813 - *VITA GL*	*13.4733	2.71957
KUM60813 - BHT	*-22.4467	2.71957
KUM61076 - *VITA GL*	*10.61	2.71957
KUM61076 - BHT	*-25.31	2.71957
VITA GL - BHT	*-35.92	2.71957

* denotes a statistically significant difference.

ANOVA: Effect of 100 µg/mL of extract on antioxidant activity measured by ABTS radical scavenging method (between ethanol extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	5981.03	4	1495.26	784.79	0.0000
Within groups	19.0531	10	1.90531		
Total (Corr.)	6000.08	14			

Multiple range test: Effect of 100 µg/mL of extract on antioxidant activity measured by ABTS radical scavenging method (between ethanol extracts)

Extracts	Difference	+/- Limits
Wheat - KUM60813	*-19.07	2.51119
Wheat - KUM61076	*-7.96	2.51119
Wheat - *VITA GL*	*4.97	2.51119
Wheat - BHT	*-51.1167	2.51119
KUM60813 - KUM61076	*11.11	2.51119
KUM60813 - *VITA GL*	*24.04	2.51119
KUM60813 - BHT	*-32.0467	2.51119
KUM61076 - *VITA GL*	*12.93	2.51119
KUM61076 - BHT	*-43.1567	2.51119
VITA GL - BHT	*-56.0867	2.51119

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of aqueous extract of unfermented wheat grains on antioxidant activity measured by ABTS radical scavenging method (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	1029.36	4	257.34	237.85	0.0000
Within groups	10.8194	10	1.08194		
Total (Corr.)	1040.18	14			

Multiple range test: Effect of different concentrations of aqueous extract of unfermented wheat grains on antioxidant activity measured by ABTS radical scavenging method (between concentrations)

Concentration (µg/mL)	Difference	+/- Limits
20 - 40	*-7.56	1.89234
20 - 60	*-14.4467	1.89234
20 - 80	*-15.1667	1.89234
20 - 100	*-24.8133	1.89234
40 - 60	*-6.88667	1.89234
40 - 80	*-7.60667	1.89234
40 - 100	*-17.2533	1.89234
60 - 80	-0.72	1.89234
60 - 100	*-10.3667	1.89234
80 - 100	*-9.64667	1.89234

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of aqueous extract of KUM60813 fermented wheat grains on antioxidant activity measured by ABTS radical scavenging method (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	260.125	4	65.0311	59.97	0.0000
Within groups	10.8441	10	1.08441		
Total (Corr.)	270.969	14			

Multiple range test: Effect of different concentrations of aqueous extract of KUM60813 fermented wheat grains on antioxidant activity measured by ABTS radical scavenging method (between concentrations)

Concentration (µg/mL)	Difference	+/- Limits
20 - 40	*-7.44	1.8945
20 - 60	*-7.94667	1.8945
20 - 80	*-9.01333	1.8945
20 - 100	*-12.79	1.8945
40 - 60	-0.506667	1.8945
40 - 80	-1.57333	1.8945
40 - 100	*-5.35	1.8945
60 - 80	-1.06667	1.8945
60 - 100	*-4.84333	1.8945
80 - 100	*-3.77667	1.8945

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of aqueous extract of KUM61076 fermented wheat grains on antioxidant activity measured by ABTS radical scavenging method (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	1150.66	4	287.665	88.02	0.0000
Within groups	32.6807	10	3.26807		
Total (Corr.)	1183.34	14			

Multiple range test: Effect of different concentrations of aqueous extract of KUM61076 fermented wheat grains on antioxidant activity measured by ABTS radical scavenging method (between concentrations)

Concentration (µg/mL)	Difference	+/- Limits
20 - 40	*-4.76	3.28884
20 - 60	*-12.9233	3.28884
20 - 80	*-17.3367	3.28884
20 - 100	*-24.5633	3.28884
40 - 60	*-8.16333	3.28884
40 - 80	*-12.5767	3.28884
40 - 100	*-19.8033	3.28884
60 - 80	*-4.41333	3.28884
60 - 100	*-11.64	3.28884
80 - 100	*-7.22667	3.28884

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of aqueous extract of *VITA GL* fermented wheat grains on antioxidant activity measured by ABTS radical scavenging method (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	152.393	4	38.0983	36.91	0.0000
Within groups	10.3232	10	1.03232		
Total (Corr.)	162.716	14			

Multiple range test: Effect of different concentrations of aqueous extract of *VITA GL* fermented wheat grains on antioxidant activity measured by ABTS radical scavenging method (between concentrations)

Concentration (µg/mL)	Difference	+/- Limits
20 - 40	*-1.99667	1.84844
20 - 60	*-3.44333	1.84844
20 - 80	*-7.18333	1.84844
20 - 100	*-8.54	1.84844
40 - 60	-1.44667	1.84844
40 - 80	*-5.18667	1.84844
40 - 100	*-6.54333	1.84844
60 - 80	*-3.74	1.84844
60 - 100	*-5.09667	1.84844
80 - 100	-1.35667	1.84844

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of BHT on antioxidant activity measured by ABTS radical scavenging method (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	6588.11	4	1647.03	481.72	0.0000
Within groups	34.1904	10	3.41904		
Total (Corr.)	6622.3	14			

Multiple range test: Effect of different concentrations of BHT on antioxidant activity measured by ABTS radical scavenging method (between concentrations)

Concentration (µg/mL)	Difference	+/- Limits
20 - 40	*-12.0367	3.36395
20 - 60	*-22.02	3.36395
20 - 80	*-38.0667	3.36395
20 - 100	*-60.1267	3.36395
40 - 60	*-9.98333	3.36395
40 - 80	*-26.03	3.36395
40 - 100	*-48.09	3.36395
60 - 80	*-16.0467	3.36395
60 - 100	*-38.1067	3.36395
80 - 100	*-22.06	3.36395

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of ethanol extract of unfermented wheat grains on antioxidant activity measured by ABTS radical scavenging method (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	190.949	4	47.7372	46.81	0.0000
Within groups	10.1985	10	1.01985		
Total (Corr.)	201.147	14			

Multiple range test: Effect of different concentrations of ethanol extract of unfermented wheat grains on antioxidant activity measured by ABTS radical scavenging method (between concentrations)

Concentration (µg/mL)	Difference	+/- Limits
20 - 40	*-4.21	1.83724
20 - 60	*-4.71667	1.83724
20 - 80	*-6.39667	1.83724
20 - 100	*-11.03	1.83724
40 - 60	-0.506667	1.83724
40 - 80	*-2.18667	1.83724
40 - 100	*-6.82	1.83724
60 - 80	-1.68	1.83724
60 - 100	*-6.31333	1.83724
80 - 100	*-4.63333	1.83724

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of ethanol extract of KUM60813 fermented wheat grains on antioxidant activity measured by ABTS radical scavenging method (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	2014.0	4	503.5	250.34	0.0000
Within groups	20.1126	10	2.01126		
Total (Corr.)	2034.11	14			

Multiple range test: Effect of different concentrations of ethanol extract of KUM60813 fermented wheat grains on antioxidant activity measured by ABTS radical scavenging method (between concentrations)

Concentration (µg/mL)	Difference	+/- Limits
20 - 40	*-8.88	2.58007
20 - 60	*-14.95	2.58007
20 - 80	*-21.7267	2.58007
20 - 100	*-34.1867	2.58007
40 - 60	*-6.07	2.58007
40 - 80	*-12.8467	2.58007
40 - 100	*-25.3067	2.58007
60 - 80	*-6.77667	2.58007
60 - 100	*-19.2367	2.58007
80 - 100	*-12.46	2.58007

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of ethanol extract of KUM61076 fermented wheat grains on antioxidant activity measured by ABTS radical scavenging method (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	788.404	4	197.101	108.46	0.0000
Within groups	18.1733	10	1.81733		
Total (Corr.)	806.578	14			

Multiple range test: Effect of different concentrations of ethanol extract of KUM61076 fermented wheat grains on antioxidant activity measured by ABTS radical scavenging method (between concentrations)

Concentration (µg/mL)	Difference	+/- Limits
20 - 40	*-5.01	2.45253
20 - 60	*-9.43	2.45253
20 - 80	*-15.9167	2.45253
20 - 100	*-20.13	2.45253
40 - 60	*-4.42	2.45253
40 - 80	*-10.9067	2.45253
40 - 100	*-15.12	2.45253
60 - 80	*-6.48667	2.45253
60 - 100	*-10.7	2.45253
80 - 100	*-4.21333	2.45253

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of ethanol extract of *VITA GL* fermented wheat grains on antioxidant activity measured by ABTS radical scavenging method (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	574.779	4	143.695	61.76	0.0000
Within groups	23.2656	10	2.32656		
Total (Corr.)	598.045	14			

Multiple range test: Effect of different concentrations of ethanol extract of *VITA GL* fermented wheat grains on antioxidant activity measured by ABTS radical scavenging method (between concentrations)

Concentration (µg/mL)	Difference	+/- Limits
20 - 40	*-8.12333	2.77495
20 - 60	*-12.0433	2.77495
20 - 80	*-15.5367	2.77495
20 - 100	*-17.43	2.77495
40 - 60	*-3.92	2.77495
40 - 80	*-7.41333	2.77495
40 - 100	*-9.30667	2.77495
60 - 80	*-3.49333	2.77495
60 - 100	*-5.38667	2.77495
80 - 100	-1.89333	2.77495

* denotes a statistically significant difference.

5) Determination of FRAP values for aqueous and ethanol extracts of unfermented and fermented wheat grains

ANOVA: Effect of 1 mg/mL of extract on antioxidant activity measured by FRAP method (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	927987.0	8	115998.0	145.13	0.0000
Within groups	28774.7	36	799.297		
Total (Corr.)	956762.0	44			

Multiple range test: Effect of 1 mg/mL of extract on antioxidant activity measured by FRAP method (between extracts)

Extracts	Difference	+/- Limits
BHT - AW	*441.95	36.2638
BHT - BW	*470.648	36.2638
BHT - CW	*472.48	36.2638
BHT - DW	*477.718	36.2638
BHT - AE	*413.734	36.2638
BHT - BE	*455.838	36.2638
BHT - CE	*405.62	36.2638
BHT - DE	*464.93	36.2638
AW - BW	28.698	36.2638
AW - CW	30.53	36.2638
AW - DW	35.768	36.2638
AW - AE	-28.216	36.2638
AW - BE	13.888	36.2638
AW - CE	*-36.33	36.2638

AW - DE	22.98	36.2638
BW - CW	1.832	36.2638
BW - DW	7.07	36.2638
BW - AE	*-56.914	36.2638
BW - BE	-14.81	36.2638
BW - CE	*-65.028	36.2638
BW - DE	-5.718	36.2638
CW - DW	5.238	36.2638
CW - AE	*-58.746	36.2638
CW - BE	-16.642	36.2638
CW - CE	*-66.86	36.2638
CW - DE	-7.55	36.2638
DW - AE	*-63.984	36.2638
DW - BE	-21.88	36.2638
DW - CE	*-72.098	36.2638
DW - DE	-12.788	36.2638
AE - BE	*42.104	36.2638
AE - CE	-8.114	36.2638
AE - DE	*51.196	36.2638
BE - CE	*-50.218	36.2638
BE - DE	9.092	36.2638
CE - DE	*59.31	36.2638

* denotes a statistically significant difference.

BHT	Butylated hydroxytoluene
AW	Wheat (Water extract)
BW	KUM60813 (Water extract)
CW	KUM61076 (Water extract)
DW	*VITA GL* (Water extract)
AE	Wheat (Ethanol extract)
BE	KUM60813 (Ethanol extract)
CE	KUM61076 (Ethanol extract)
DE	*VITA GL* (Ethanol extract)

6) Determination of total phenolic content in aqueous and ethanol extracts of unfermented and fermented wheat grains

ANOVA: Effect of 1 mg/mL of extract on antioxidant activity measured by TPC method (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	1120.67	8	140.083	47.56	0.0000
Within groups	106.045	36	2.94569		
Total (Corr.)	1226.71	44			

Multiple range test: Effect of 1 mg/mL of extract on antioxidant activity measured by TPC method (between extracts)

Contrast	Difference	+/- Limits
BHT - AW	*15.094	2.20147
BHT - BW	*15.906	2.20147
BHT - CW	*14.05	2.20147
BHT - DW	*17.022	2.20147
BHT - AE	*9.364	2.20147

BHT - BE	*10.3	2.20147
BHT - CE	*8.282	2.20147
BHT - DE	*13.996	2.20147
AW - BW	0.812	2.20147
AW - CW	-1.044	2.20147
AW - DW	1.928	2.20147
AW - AE	*-5.73	2.20147
AW - BE	*-4.794	2.20147
AW - CE	*-6.812	2.20147
AW - DE	-1.098	2.20147
BW - CW	-1.856	2.20147
BW - DW	1.116	2.20147
BW - AE	*-6.542	2.20147
BW - BE	*-5.606	2.20147
BW - CE	*-7.624	2.20147
BW - DE	-1.91	2.20147
CW - DW	*2.972	2.20147
CW - AE	*-4.686	2.20147
CW - BE	*-3.75	2.20147
CW - CE	*-5.768	2.20147
CW - DE	-0.054	2.20147
DW - AE	*-7.658	2.20147
DW - BE	*-6.722	2.20147
DW - CE	*-8.74	2.20147
DW - DE	*-3.026	2.20147
AE - BE	0.936	2.20147
AE - CE	-1.082	2.20147
AE - DE	*4.632	2.20147
BE - CE	-2.018	2.20147
BE - DE	*3.696	2.20147
CE - DE	*5.714	2.20147

* denotes a statistically significant difference.

7) Effect of aqueous and ethanol extracts of unfermented and fermented wheat grains on 3T3-L1 preadipocyte viability

ANOVA: Effect of different concentrations of aqueous extract of unfermented wheat grains on 3T3-L1 preadipocyte viability measured by MTT assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	4.27409	4	1.06852	0.09	0.9822
Within groups	113.879	10	11.3879		
Total (Corr.)	118.153	14			

Multiple range test: Effect of different concentrations of aqueous extract of unfermented wheat grains on 3T3-L1 preadipocyte viability measured by MTT assay (between concentrations)

Concentration (µg/mL)	Difference	+/- Limits
20 - 40	-0.796667	6.1393
20 - 60	0.453333	6.1393
20 - 80	0.746667	6.1393
20 - 100	-0.19	6.1393
40 - 60	1.25	6.1393
40 - 80	1.54333	6.1393
40 - 100	0.606667	6.1393
60 - 80	0.293333	6.1393
60 - 100	-0.643333	6.1393

80 - 100	-0.936667	6.1393

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of aqueous extract of KUM60813 fermented wheat grains on 3T3-L1 preadipocyte viability measured by MTT assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	876.701	4	219.175	9.52	0.0019
Within groups	230.157	10	23.0157		
Total (Corr.)	1106.86	14			

Multiple range test: Effect of different concentrations of aqueous extract of KUM60813 fermented wheat grains on 3T3-L1 preadipocyte viability measured by MTT assay (between concentrations)

Concentration (µg/mL)	Difference	+/- Limits
20 - 40	6.64	8.72789
20 - 60	6.26667	8.72789
20 - 80	8.46	8.72789
20 - 100	*-12.3867	8.72789
40 - 60	-0.373333	8.72789
40 - 80	1.82	8.72789
40 - 100	*-19.0267	8.72789
60 - 80	2.19333	8.72789
60 - 100	*-18.6533	8.72789
80 - 100	*-20.8467	8.72789

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of aqueous extract of KUM61076 fermented wheat grains on 3T3-L1 preadipocyte viability measured by MTT assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	76.7574	4	19.1893	1.65	0.2363
Within groups	116.06	10	11.606		
Total (Corr.)	192.817	14			

Multiple range test: Effect of different concentrations of aqueous extract of KUM61076 fermented wheat grains on 3T3-L1 preadipocyte viability measured by MTT assay (between concentrations)

Concentration (µg/mL)	Difference	+/- Limits
20 - 40	0.0133333	6.19782
20 - 60	-1.22333	6.19782
20 - 80	0.736667	6.19782
20 - 100	5.31333	6.19782
40 - 60	-1.23667	6.19782
40 - 80	0.723333	6.19782
40 - 100	5.3	6.19782
60 - 80	1.96	6.19782
60 - 100	*6.53667	6.19782
80 - 100	4.57667	6.19782

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of aqueous extract of *VITA GL* fermented wheat grains on 3T3-L1 preadipocyte viability measured by MTT assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	846.865	4	211.716	27.82	0.0000
Within groups	76.0983	10	7.60983		
Total (Corr.)	922.963	14			

Multiple range test: Effect of different concentrations of aqueous extract of *VITA GL* fermented wheat grains on 3T3-L1 preadipocyte viability measured by MTT assay (between concentrations)

Concentration (µg/mL)	Difference	+/- Limits
20 - 40	0.826667	5.01863
20 - 60	*-7.49	5.01863
20 - 80	*-6.18667	5.01863
20 - 100	*-20.1133	5.01863
40 - 60	*-8.31667	5.01863
40 - 80	*-7.01333	5.01863
40 - 100	*-20.94	5.01863
60 - 80	1.30333	5.01863
60 - 100	*-12.6233	5.01863
80 - 100	*-13.9267	5.01863

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of ethanol extract of unfermented wheat grains on 3T3-L1 preadipocyte viability measured by MTT assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	2038.55	4	509.637**********		0.0000
Within groups	0.0	10	0.0		
Total (Corr.)	2038.55	14			

Multiple range test: Effect of different concentrations of ethanol extract of unfermented wheat grains on 3T3-L1 preadipocyte viability measured by MTT assay (between concentrations)

Concentration (µg/mL)	Difference	+/- Limits
20 - 40	*-21.34	0.0
20 - 60	*-26.98	0.0
20 - 80	*-16.21	0.0
20 - 100	*2.39	0.0
40 - 60	*-5.64	0.0
40 - 80	*5.13	0.0
40 - 100	*23.73	0.0
60 - 80	*10.77	0.0
60 - 100	*29.37	0.0
80 - 100	*18.6	0.0

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of ethanol extract of KUM60813 fermented wheat grains on 3T3-L1 preadipocyte viability measured by MTT assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	1031.74	4	257.935	31.94	0.0000
Within groups	80.7679	10	8.07679		
Total (Corr.)	1112.51	14			

Multiple range test: Effect of different concentrations of ethanol extract of KUM60813 fermented wheat grains on 3T3-L1 preadipocyte viability measured by MTT assay (between concentrations)

Concentration (µg/mL)	Difference	+/- Limits
20 - 40	4.59	5.17031
20 - 60	-5.11	5.17031
20 - 80	-2.44	5.17031
20 - 100	*-19.8767	5.17031
40 - 60	*-9.7	5.17031
40 - 80	*-7.03	5.17031
40 - 100	*-24.4667	5.17031
60 - 80	2.67	5.17031
60 - 100	*-14.7667	5.17031
80 - 100	*-17.4367	5.17031

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of ethanol extract of KUM61076 fermented wheat grains on 3T3-L1 preadipocyte viability measured by MTT assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	359.207	4	89.8018	2.82	0.0840
Within groups	318.986	10	31.8986		
Total (Corr.)	678.193	14			

Multiple range test: Effect of different concentrations of ethanol extract of KUM61076 fermented wheat grains on 3T3-L1 preadipocyte viability measured by MTT assay (between concentrations)

Concentration (µg/mL)	Difference	+/- Limits
20 - 40	0.916667	10.275
20 - 60	6.75333	10.275
20 - 80	-4.04333	10.275
20 - 100	-7.77	10.275
40 - 60	5.83667	10.275
40 - 80	-4.96	10.275
40 - 100	-8.68667	10.275
60 - 80	*-10.7967	10.275
60 - 100	*-14.5233	10.275
80 - 100	-3.72667	10.275

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of ethanol extract of *VITA GL* fermented wheat grains on 3T3-L1 preadipocyte viability measured by MTT assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	625.178	4	156.295	9.67	0.0018
Within groups	161.697	10	16.1697		
Total (Corr.)	786.875	14			

Multiple range test: Effect of different concentrations of ethanol extract of *VITA GL* fermented wheat grains on 3T3-L1 preadipocyte viability measured by MTT assay (between concentrations)

Concentration (µg/mL)	Difference	+/- Limits
20 - 40	-3.56667	7.31557
20 - 60	*-11.24	7.31557
20 - 80	*-8.85333	7.31557
20 - 100	*-18.7067	7.31557
40 - 60	*-7.67333	7.31557
40 - 80	-5.28667	7.31557
40 - 100	*-15.14	7.31557
60 - 80	2.38667	7.31557
60 - 100	*-7.46667	7.31557
80 - 100	*-9.85333	7.31557

* denotes a statistically significant difference.

8) Effect of aqueous and ethanol extracts of unfermented and fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress (24, 48 and 72 hours incubation).

ANOVA: Effect of 0.1 µg/mL of aqueous extract of unfermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	7344.62	2	3672.31	1032.77	0.0000
Within groups	21.3348	6	3.5558		
Total (Corr.)	7365.96	8			

Multiple range test: Effect of 0.1 µg/mL of aqueous extract of unfermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Incubation days	Difference	+/- Limits
24 - 48	-3.23	3.76741
24 - 72	*58.92	3.76741
48 - 72	*62.15	3.76741

* denotes a statistically significant difference.

ANOVA: Effect of 1 µg/mL of aqueous extract of unfermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	3308.39	2	1654.19	159.71	0.0000
Within groups	62.1455	6	10.3576		
Total (Corr.)	3370.53	8			

Multiple range test: Effect of 1 µg/mL of aqueous extract of unfermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Incubation days	Difference	+/- Limits
24 - 48	-0.11	6.42988
24 - 72	*40.6167	6.42988
48 - 72	*40.7267	6.42988

* denotes a statistically significant difference.

ANOVA: Effect of 10 µg/mL of aqueous extract of unfermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	2001.05	2	1000.52	417.20	0.0000
Within groups	14.3893	6	2.39821		
Total (Corr.)	2015.44	8			

Multiple range test: Effect of 10 µg/mL of aqueous extract of unfermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Incubation days	Difference	+/- Limits
24 - 48	0.89	3.09398
24 - 72	*32.0667	3.09398
48 - 72	*31.1767	3.09398

* denotes a statistically significant difference.

ANOVA: Effect of 100 µg/mL of aqueous extract of unfermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	2303.08	2	1151.54	8045.23	0.0000
Within groups	0.8588	6	0.143133		
Total (Corr.)	2303.94	8			

Multiple range test: Effect of 100 µg/mL of aqueous extract of unfermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Incubation days	Difference	+/- Limits
24 - 48	*-1.14	0.755865
24 - 72	*33.35	0.755865
48 - 72	*34.49	0.755865

* denotes a statistically significant difference.

ANOVA: Effect of 1000 µg/mL of aqueous extract of unfermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	155.296	2	77.6478	3.67	0.0911
Within groups	127.035	6	21.1725		
Total (Corr.)	282.331	8			

Multiple range test: Effect of 1000 µg/mL of aqueous extract of unfermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Incubation days	Difference	+/- Limits
24 - 48	-4.66	9.19307
24 - 72	5.50333	9.19307
48 - 72	*10.1633	9.19307

* denotes a statistically significant difference.

ANOVA: Effect of 0.1 µg/mL of aqueous extract of KUM60813 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	3911.23	2	1955.61	331.43	0.0000
Within groups	35.4029	6	5.90049		
Total (Corr.)	3946.63	8			

Multiple range test: Effect of 0.1 µg/mL of aqueous extract of KUM60813 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Incubation days	Difference	+/- Limits
24 - 48	-4.30333	4.85308
24 - 72	*41.9133	4.85308
48 - 72	*46.2167	4.85308

* denotes a statistically significant difference.

ANOVA: Effect of 1 µg/mL of aqueous extract of KUM60813 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	5598.44	2	2799.22	150.27	0.0000
Within groups	111.77	6	18.6283		
Total (Corr.)	5710.21	8			

Multiple range test: Effect of 1 µg/mL of aqueous extract of KUM60813 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Incubation days	Difference	+/- Limits
24 - 48	1.65	8.62305
24 - 72	*53.7133	8.62305
48 - 72	*52.0633	8.62305

* denotes a statistically significant difference.

ANOVA: Effect of 10 µg/mL of aqueous extract of KUM60813 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	4132.46	2	2066.23	533.66	0.0000
Within groups	23.2309	6	3.87182		
Total (Corr.)	4155.7	8			

Multiple range test: Effect of 10 µg/mL of aqueous extract of KUM60813 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Incubation days	Difference	+/- Limits
24 - 48	-0.316667	3.93126
24 - 72	*45.2967	3.93126
48 - 72	*45.6133	3.93126

* denotes a statistically significant difference.

ANOVA: Effect of 100 µg/mL of aqueous extract of KUM60813 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	4487.02	2	2243.51	1585.01	0.0000
Within groups	8.49273	6	1.41546		
Total (Corr.)	4495.51	8			

Multiple range test: Effect of 100 µg/mL of aqueous extract of KUM60813 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Incubation days	Difference	+/- Limits
24 - 48	*3.25667	2.37696
24 - 72	*48.91	2.37696
48 - 72	*45.6533	2.37696

* denotes a statistically significant difference.

ANOVA: Effect of 1000 µg/mL of aqueous extract of KUM60813 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	2252.68	2	1126.34	444.48	0.0000
Within groups	15.2043	6	2.53404		
Total (Corr.)	2267.88	8			

Multiple range test: Effect of 1000 µg/mL of aqueous extract of KUM60813 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Incubation days	Difference	+/- Limits
24 - 48	*5.49667	3.18039
24 - 72	*35.97	3.18039
48 - 72	*30.4733	3.18039

* denotes a statistically significant difference.

ANOVA: Effect of 0.1 µg/mL of aqueous extract of KUM61076 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	2490.07	2	1245.04	300.51	0.0000
Within groups	24.8587	6	4.14311		
Total (Corr.)	2514.93	8			

Multiple range test: Effect of 0.1 µg/mL of aqueous extract of KUM61076 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Incubation days	Difference	+/- Limits
24 - 48	0.796667	4.06665
24 - 72	*35.6767	4.06665
48 - 72	*34.88	4.06665

* denotes a statistically significant difference.

ANOVA: Effect of 1 µg/mL of aqueous extract of KUM61076 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	1601.04	2	800.519	171.63	0.0000
Within groups	27.9859	6	4.66432		
Total (Corr.)	1629.02	8			

Multiple range test: Effect of 1 µg/mL of aqueous extract of KUM61076 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Incubation days	Difference	+/- Limits
24 - 48	2.18667	4.31487
24 - 72	*29.3233	4.31487
48 - 72	*27.1367	4.31487

* denotes a statistically significant difference.

ANOVA: Effect of 10 µg/mL of aqueous extract of KUM61076 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	2301.82	2	1150.91	444.03	0.0000
Within groups	15.5519	6	2.59198		
Total (Corr.)	2317.38	8			

Multiple range test: Effect of 10 µg/mL of aqueous extract of KUM61076 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Incubation days	Difference	+/- Limits
24 - 48	2.54667	3.21654
24 - 72	*35.1267	3.21654
48 - 72	*32.58	3.21654

* denotes a statistically significant difference.

ANOVA: Effect of 100 µg/mL of aqueous extract of KUM61076 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	3932.4	2	1966.2	267.16	0.0000
Within groups	44.1571	6	7.35952		
Total (Corr.)	3976.55	8			

Multiple range test: Effect of 100 µg/mL of aqueous extract of KUM61076 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Incubation days	Difference	+/- Limits
24 - 48	1.99	5.41999
24 - 72	*45.3033	5.41999
48 - 72	*43.3133	5.41999

* denotes a statistically significant difference.

ANOVA: Effect of 1000 µg/mL of aqueous extract of KUM61076 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	525.148	2	262.574	112.98	0.0000
Within groups	13.9439	6	2.32398		
Total (Corr.)	539.092	8			

Multiple range test: Effect of 1000 µg/mL of aqueous extract of KUM61076 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Incubation days	Difference	+/- Limits
24 - 48	-0.186667	3.04572
24 - 72	*16.11	3.04572
48 - 72	*16.2967	3.04572

* denotes a statistically significant difference.

ANOVA: Effect of 0.1 µg/mL of aqueous extract of *VITA GL* fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	2953.3	2	1476.65	303.63	0.0000
Within groups	29.1801	6	4.86336		
Total (Corr.)	2982.48	8			

Multiple range test: Effect of 0.1 µg/mL of aqueous extract of *VITA GL* fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Incubation days	Difference	+/- Limits
24 - 48	-0.24	4.40597
24 - 72	*38.3067	4.40597
48 - 72	*38.5467	4.40597

* denotes a statistically significant difference.

ANOVA: Effect of 1 µg/mL of aqueous extract of *VITA GL* fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	2402.26	2	1201.13	70.63	0.0001
Within groups	102.043	6	17.0072		
Total (Corr.)	2504.3	8			

Multiple range test: Effect of 1 µg/mL of aqueous extract of *VITA GL* fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Incubation days	Difference	+/- Limits
24 - 48	0.723333	8.23929
24 - 72	*35.0133	8.23929
48 - 72	*34.29	8.23929

* denotes a statistically significant difference.

ANOVA: Effect of 10 µg/mL of aqueous extract of *VITA GL* fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	3093.29	2	1546.65	204.71	0.0000
Within groups	45.3319	6	7.55532		
Total (Corr.)	3138.62	8			

Multiple range test: Effect of 10 µg/mL of aqueous extract of *VITA GL* fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Incubation days	Difference	+/- Limits
24 - 48	1.84333	5.49162
24 - 72	*40.2167	5.49162
48 - 72	*38.3733	5.49162

* denotes a statistically significant difference.

ANOVA: Effect of 100 µg/mL of aqueous extract of *VITA GL* fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	3339.98	2	1669.99	70.59	0.0001
Within groups	141.938	6	23.6564		
Total (Corr.)	3481.92	8			

Multiple range test: Effect of 100 µg/mL of aqueous extract of *VITA GL* fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Incubation days	Difference	+/- Limits
24 - 48	-2.30667	9.71736
24 - 72	*39.6633	9.71736
48 - 72	*41.97	9.71736

* denotes a statistically significant difference.

ANOVA: Effect of 1000 µg/mL of aqueous extract of *VITA GL* fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	3313.66	2	1656.83	150.90	0.0000
Within groups	65.8759	6	10.9793		
Total (Corr.)	3379.54	8			

Multiple range test: Effect of 1000 µg/mL of aqueous extract of *VITA GL* fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Incubation days	Difference	+/- Limits
24 - 48	*8.92667	6.62005
24 - 72	*44.4267	6.62005
48 - 72	*35.5	6.62005

* denotes a statistically significant difference.

ANOVA: Effect of 0.1 µg/mL of ethanol extract of unfermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	3490.97	2	1745.49	49.43	0.0002
Within groups	211.869	6	35.3115		
Total (Corr.)	3702.84	8			

Multiple range test: Effect of 0.1 µg/mL of ethanol extract of unfermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Contrast	Difference	+/- Limits
24 - 48	*24.7533	11.8722
24 - 72	*48.2367	11.8722
48 - 72	*23.4833	11.8722

* denotes a statistically significant difference.

ANOVA: Effect of 1 µg/mL of ethanol extract of unfermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	2933.82	2	1466.91	1595.64	0.0000
Within groups	5.51593	6	0.919322		
Total (Corr.)	2939.33	8			

Multiple range test: Effect of 1 µg/mL of ethanol extract of unfermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Contrast	Difference	+/- Limits
24 - 48	*30.1567	1.91561
24 - 72	*43.0933	1.91561
48 - 72	*12.9367	1.91561

* denotes a statistically significant difference.

ANOVA: Effect of 10 µg/mL of ethanol extract of unfermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	2456.73	2	1228.36	281.46	0.0000
Within groups	26.1859	6	4.36432		
Total (Corr.)	2482.91	8			

Multiple range test: Effect of 10 µg/mL of ethanol extract of unfermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Contrast	Difference	+/- Limits
24 - 48	*31.2767	4.17381
24 - 72	*37.88	4.17381
48 - 72	*6.60333	4.17381

* denotes a statistically significant difference.

ANOVA: Effect of 100 µg/mL of ethanol extract of unfermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	2658.89	2	1329.44	208.90	0.0000
Within groups	38.1845	6	6.36409		
Total (Corr.)	2697.07	8			

Multiple range test: Effect of 100 µg/mL of ethanol extract of unfermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Contrast	Difference	+/- Limits
24 - 48	*27.3033	5.04013
24 - 72	*41.4067	5.04013
48 - 72	*14.1033	5.04013

* denotes a statistically significant difference.

ANOVA: Effect of 1000 µg/mL of ethanol extract of unfermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	4353.55	2	2176.77	87.47	0.0000
Within groups	149.313	6	24.8855		
Total (Corr.)	4502.86	8			

Multiple range test: Effect of 1000 µg/mL of ethanol extract of unfermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Contrast	Difference	+/- Limits
24 - 48	*35.4767	9.9666
24 - 72	*52.85	9.9666
48 - 72	*17.3733	9.9666

* denotes a statistically significant difference.

ANOVA: Effect of 0.1 µg/mL of ethanol extract of KUM60813 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	2254.87	2	1127.43	61.60	0.0001
Within groups	109.815	6	18.3025		
Total (Corr.)	2364.68	8			

Multiple range test: Effect of 0.1 µg/mL of ethanol extract of KUM60813 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Contrast	Difference	+/- Limits
24 - 48	*12.9467	8.5473
24 - 72	*38.1233	8.5473
48 - 72	*25.1767	8.5473

* denotes a statistically significant difference.

ANOVA: Effect of 1 μg/mL of ethanol extract of KUM60813 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	1534.3	2	767.149	109.86	0.0000
Within groups	41.8974	6	6.9829		
Total (Corr.)	1576.2	8			

Multiple range test: Effect of 1 μg/mL of ethanol extract of KUM60813 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Contrast	Difference	+/- Limits
24 - 48	*26.2467	5.27949
24 - 72	*28.95	5.27949
48 - 72	2.70333	5.27949

* denotes a statistically significant difference.

ANOVA: Effect of 10 μg/mL of ethanol extract of KUM60813 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	1755.93	2	877.964	101.86	0.0000
Within groups	51.7163	6	8.61938		
Total (Corr.)	1807.64	8			

Multiple range test: Effect of 10 μg/mL of ethanol extract of KUM60813 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Contrast	Difference	+/- Limits
24 - 48	*18.4167	5.86559
24 - 72	*34.18	5.86559
48 - 72	*15.7633	5.86559

* denotes a statistically significant difference.

ANOVA: Effect of 100 μg/mL of ethanol extract of KUM60813 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	1476.11	2	738.056	151.49	0.0000
Within groups	29.2321	6	4.87201		
Total (Corr.)	1505.35	8			

Multiple range test: Effect of 100 μg/mL of ethanol extract of KUM60813 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Contrast	Difference	+/- Limits
24 - 48	*11.97	4.40989
24 - 72	*31.0967	4.40989
48 - 72	*19.1267	4.40989

* denotes a statistically significant difference.

ANOVA: Effect of 1000 µg/mL of ethanol extract of KUM60813 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	55.0641	2	27.532	28.35	0.0009
Within groups	5.82673	6	0.971122		
Total (Corr.)	60.8908	8			

Multiple range test: Effect of 1000 µg/mL of ethanol extract of KUM60813 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Contrast	Difference	+/- Limits
24 - 48	*-2.64667	1.96884
24 - 72	*-6.04333	1.96884
48 - 72	*-3.39667	1.96884

* denotes a statistically significant difference.

ANOVA: Effect of 0.1 µg/mL of ethanol extract of KUM61076 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	2770.6	2	1385.3	198.41	0.0000
Within groups	41.8916	6	6.98193		
Total (Corr.)	2812.5	8			

Multiple range test: Effect of 0.1 µg/mL of ethanol extract of KUM61076 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Contrast	Difference	+/- Limits
24 - 48	*20.4167	5.27912
24 - 72	*42.96	5.27912
48 - 72	*22.5433	5.27912

* denotes a statistically significant difference.

ANOVA: Effect of 1 µg/mL of ethanol extract of KUM61076 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	4234.12	2	2117.06	262.23	0.0000
Within groups	48.439	6	8.07317		
Total (Corr.)	4282.56	8			

Multiple range test: Effect of 1 µg/mL of ethanol extract of KUM61076 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Contrast	Difference	+/- Limits
24 - 48	*17.33	5.6767
24 - 72	*52.16	5.6767
48 - 72	*34.83	5.6767

* denotes a statistically significant difference.

ANOVA: Effect of 10 µg/mL of ethanol extract of KUM61076 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	4090.61	2	2045.31	469.42	0.0000
Within groups	26.1424	6	4.35707		
Total (Corr.)	4116.76	8			

Multiple range test: Effect of 10 µg/mL of ethanol extract of KUM61076 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Contrast	Difference	+/- Limits
24 - 48	*10.84	4.17033
24 - 72	*49.66	4.17033
48 - 72	*38.82	4.17033

* denotes a statistically significant difference.

ANOVA: Effect of 100 µg/mL of ethanol extract of KUM61076 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	3571.34	2	1785.67	941.23	0.0000
Within groups	11.383	6	1.89717		
Total (Corr.)	3582.72	8			

Multiple range test: Effect of 100 µg/mL of ethanol extract of KUM61076 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Contrast	Difference	+/- Limits
24 - 48	*6.06	2.75186
24 - 72	*44.96	2.75186
48 - 72	*38.9	2.75186

* denotes a statistically significant difference.

ANOVA: Effect of 1000 µg/mL of ethanol extract of KUM61076 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	128.29	2	64.1452	142.34	0.0000
Within groups	2.70393	6	0.450656		
Total (Corr.)	130.994	8			

Multiple range test: Effect of 1000 µg/mL of ethanol extract of KUM61076 fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Contrast	Difference	+/- Limits
24 - 48	*-6.04667	1.34121
24 - 72	*-9.08333	1.34121
48 - 72	*-3.03667	1.34121

* denotes a statistically significant difference.

ANOVA: Effect of 0.1 µg/mL of ethanol extract of *VITA GL* fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	1881.73	2	940.864	78.74	0.0000
Within groups	71.6947	6	11.9491		
Total (Corr.)	1953.42	8			

Multiple range test: Effect of 0.1 µg/mL of ethanol extract of *VITA GL* fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Contrast	Difference	+/- Limits
24 - 48	*30.6133	6.90624
24 - 72	*30.7333	6.90624
48 - 72	0.12	6.90624

* denotes a statistically significant difference.

ANOVA: Effect of 1 µg/mL of ethanol extract of *VITA GL* fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	1887.48	2	943.741	152.97	0.0000
Within groups	37.0175	6	6.16959		
Total (Corr.)	1924.5	8			

Multiple range test: Effect of 1 µg/mL of ethanol extract of *VITA GL* fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Contrast	Difference	+/- Limits
24 - 48	*30.3833	4.96252
24 - 72	*31.0467	4.96252
48 - 72	0.663333	4.96252

* denotes a statistically significant difference.

ANOVA: Effect of 10 µg/mL of ethanol extract of *VITA GL* fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	1926.14	2	963.072	145.88	0.0000
Within groups	39.6113	6	6.60189		
Total (Corr.)	1965.76	8			

Multiple range test: Effect of 10 µg/mL of ethanol extract of *VITA GL* fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Contrast	Difference	+/- Limits
24 - 48	*28.6067	5.13343
24 - 72	*32.9933	5.13343
48 - 72	4.38667	5.13343

* denotes a statistically significant difference.

ANOVA: Effect of 100 µg/mL of ethanol extract of *VITA GL* fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	2308.26	2	1154.13	123.15	0.0000
Within groups	56.2313	6	9.37189		
Total (Corr.)	2364.49	8			

Multiple range test: Effect of 100 µg/mL of ethanol extract of *VITA GL* fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Contrast	Difference	+/- Limits
24 - 48	*28.8067	6.11628
24 - 72	*37.4633	6.11628
48 - 72	*8.65667	6.11628

* denotes a statistically significant difference.

ANOVA: Effect of 1000 µg/mL of ethanol extract of *VITA GL* fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	2975.77	2	1487.88	77.89	0.0001
Within groups	114.616	6	19.1026		
Total (Corr.)	3090.39	8			

Multiple range test: Effect of 1000 µg/mL of ethanol extract of *VITA GL* fermented wheat grains on 3T3-L1 preadipocyte viability in the presence of oxidative stress measured by MTT assay (between incubation days)

Contrast	Difference	+/- Limits
24 - 48	7.14667	8.73214
24 - 72	*41.6467	8.73214
48 - 72	*34.5	8.73214

* denotes a statistically significant difference.

9) Effect of aqueous extracts of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin

ANOVA: Effect of different concentrations of aqueous extract of unfermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin measured by Oil Red O assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	3161.68	5	632.336	449.45	0.0000
Within groups	16.8829	12	1.40691		
Total (Corr.)	3178.56	17			

Multiple range test: Effect of different concentrations of aqueous extract of unfermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin measured by Oil Red O assay (between concentrations)

Contrast	Difference	+/- Limits
0.1 - 20	*37.3953	2.11013
0.1 - 40	*37.4663	2.11013
0.1 - 60	*35.8423	2.11013
0.1 - 80	*32.948	2.11013
0.1 - 100	*31.96	2.11013

20 - 40	0.071	2.11013
20 - 60	-1.553	2.11013
20 - 80	*-4.44733	2.11013
20 - 100	*-5.43533	2.11013
40 - 60	-1.624	2.11013
40 - 80	*-4.51833	2.11013
40 - 100	*-5.50633	2.11013
60 - 80	*-2.89433	2.11013
60 - 100	*-3.88233	2.11013
80 - 100	-0.988	2.11013

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of aqueous extract of KUM60813 fermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin measured by Oil Red O assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	3831.6	5	766.32	964.77	0.0000
Within groups	9.53162	12	0.794301		
Total (Corr.)	3841.13	17			

Multiple range test: Effect of different concentrations of aqueous extract of KUM60813 fermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin measured by Oil Red O assay (between concentrations)

Contrast	Difference	+/- Limits
0.1 - 20	*37.8887	1.58551
0.1 - 40	*40.288	1.58551
0.1 - 60	*41.982	1.58551
0.1 - 80	*38.806	1.58551
0.1 - 100	*34.007	1.58551
20 - 40	*2.39933	1.58551
20 - 60	*4.09333	1.58551
20 - 80	0.917333	1.58551
20 - 100	*-3.88167	1.58551
40 - 60	*1.694	1.58551
40 - 80	-1.482	1.58551
40 - 100	*-6.281	1.58551
60 - 80	*-3.176	1.58551
60 - 100	*-7.975	1.58551
80 - 100	*-4.799	1.58551

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of aqueous extract of KUM61076 fermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin measured by Oil Red O assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	3437.41	5	687.483	752.97	0.0000
Within groups	10.9564	12	0.913032		
Total (Corr.)	3448.37	17			

Multiple range test: Effect of different concentrations of aqueous extract of KUM61076 fermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin measured by Oil Red O assay (between concentrations)

Contrast	Difference	+/- Limits
0.1 - 20	*35.9123	1.69988
0.1 - 40	*36.7593	1.69988
0.1 - 60	*40.1473	1.69988
0.1 - 80	*37.7477	1.69988
0.1 - 100	*31.749	1.69988
20 - 40	0.847	1.69988
20 - 60	*4.235	1.69988
20 - 80	*1.83533	1.69988
20 - 100	*-4.16333	1.69988
40 - 60	*3.388	1.69988
40 - 80	0.988333	1.69988
40 - 100	*-5.01033	1.69988
60 - 80	*-2.39967	1.69988
60 - 100	*-8.39833	1.69988
80 - 100	*-5.99867	1.69988

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of aqueous extract of *VITA GL* fermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin measured by Oil Red O assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	2487.33	5	497.467	506.21	0.0000
Within groups	11.7928	12	0.982736		
Total (Corr.)	2499.13	17			

Multiple range test: Effect of different concentrations of aqueous extract of *VITA GL* fermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin measured by Oil Red O assay (between concentrations)

Contrast	Difference	+/- Limits
0.1 - 20	*33.5133	1.76357
0.1 - 40	*34.2893	1.76357
0.1 - 60	*30.267	1.76357
0.1 - 80	*28.5737	1.76357
0.1 - 100	*27.8673	1.76357
20 - 40	0.776	1.76357
20 - 60	*-3.24633	1.76357
20 - 80	*-4.93967	1.76357
20 - 100	*-5.646	1.76357
40 - 60	*-4.02233	1.76357
40 - 80	*-5.71567	1.76357
40 - 100	*-6.422	1.76357
60 - 80	-1.69333	1.76357
60 - 100	*-2.39967	1.76357
80 - 100	-0.706333	1.76357

* denotes a statistically significant difference.

ANOVA: Effect of 20 µg/mL of aqueous extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin measured by Oil Red O assay (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	34.7379	3	11.5793	35.49	0.0001
Within groups	2.61025	8	0.326281		
Total (Corr.)	37.3482	11			

Multiple range test: Effect of 20 µg/mL of aqueous extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin measured by Oil Red O assay (between extracts)

Contrast	Difference	+/- Limits
1 - 2	0.493333	1.0755
1 - 3	*-1.483	1.0755
1 - 4	*-3.882	1.0755
2 - 3	*-1.97633	1.0755
2 - 4	*-4.37533	1.0755
3 - 4	*-2.399	1.0755

* denotes a statistically significant difference.

ANOVA: Effect of 40 µg/mL of aqueous extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin measured by Oil Red O assay (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	54.8185	3	18.2728	61.08	0.0000
Within groups	2.39316	8	0.299145		
Total (Corr.)	57.2117	11			

Multiple range test: Effect of 40 µg/mL of aqueous extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin measured by Oil Red O assay (between extracts)

Contrast	Difference	+/- Limits
1 - 2	*2.82167	1.02981
1 - 3	-0.707	1.02981
1 - 4	*-3.177	1.02981
2 - 3	*-3.52867	1.02981
2 - 4	*-5.99867	1.02981
3 - 4	*-2.47	1.02981

* denotes a statistically significant difference.

ANOVA: Effect of 60 µg/mL of aqueous extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin measured by Oil Red O assay (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	244.156	3	81.3853	227.93	0.0000
Within groups	2.85653	8	0.357066		

Total (Corr.)	247.012	11

Multiple range test: Effect of 60 µg/mL of aqueous extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin measured by Oil Red O assay (between extracts)

Contrast	Difference	+/- Limits
1 - 2	*6.13967	1.1251
1 - 3	*4.305	1.1251
1 - 4	*-5.57533	1.1251
2 - 3	*-1.83467	1.1251
2 - 4	*-11.715	1.1251
3 - 4	*-9.88033	1.1251

* denotes a statistically significant difference.

ANOVA: Effect of 80 µg/mL of aqueous extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin measured by Oil Red O assay (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	199.853	3	66.6177	156.13	0.0000
Within groups	3.4135	8	0.426687		
Total (Corr.)	203.267	11			

Multiple range test: Effect of 80 µg/mL of aqueous extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin measured by Oil Red O assay (between extracts)

Contrast	Difference	+/- Limits
1 - 2	*5.858	1.2299
1 - 3	*4.79967	1.2299
1 - 4	*-4.37433	1.2299
2 - 3	-1.05833	1.2299
2 - 4	*-10.2323	1.2299
3 - 4	*-9.174	1.2299

* denotes a statistically significant difference.

ANOVA: Effect of 100 µg/mL of aqueous extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin measured by Oil Red O assay (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	59.1345	3	19.7115	65.02	0.0000
Within groups	2.42527	8	0.303158		
Total (Corr.)	61.5598	11			

Multiple range test: Effect of 100 µg/mL of aqueous extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin measured by Oil Red O assay (between extracts)

Contrast	Difference	+/- Limits
1 - 2	*2.047	1.03669
1 - 3	-0.211	1.03669
1 - 4	*-4.09267	1.03669
2 - 3	*-2.258	1.03669
2 - 4	*-6.13967	1.03669
3 - 4	*-3.88167	1.03669

* denotes a statistically significant difference.

10) Effect of aqueous extracts of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin

ANOVA: Effect of different concentrations of aqueous extract of unfermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin measured by Oil Red O assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	2424.06	5	484.813	203.21	0.0000
Within groups	28.6287	12	2.38573		
Total (Corr.)	2452.69	17			

Multiple range test: Effect of different concentrations of aqueous extract of unfermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin measured by Oil Red O assay (between concentrations)

Contrast	Difference	+/- Limits
0.1 - 20	*35.5693	2.7478
0.1 - 40	*29.9337	2.7478
0.1 - 60	*29.3643	2.7478
0.1 - 80	*30.2093	2.7478
0.1 - 100	*25.9863	2.7478
20 - 40	*-5.63567	2.7478
20 - 60	*-6.205	2.7478
20 - 80	*-5.36	2.7478
20 - 100	*-9.583	2.7478
40 - 60	-0.569333	2.7478
40 - 80	0.275667	2.7478
40 - 100	*-3.94733	2.7478
60 - 80	0.845	2.7478
60 - 100	*-3.378	2.7478
80 - 100	*-4.223	2.7478

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of aqueous extract of KUM60813 fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin measured by Oil Red O assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	2554.13	5	510.826	360.48	0.0000
Within groups	17.0046	12	1.41705		

Total (Corr.)	2571.13	17

Multiple range test: Effect of different concentrations of aqueous extract of KUM60813 fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin measured by Oil Red O assay (between concentrations)

Contrast	Difference	+/- Limits
0.1 - 20	*29.367	2.11772
0.1 - 40	*27.9613	2.11772
0.1 - 60	*26.8317	2.11772
0.1 - 80	*36.1297	2.11772
0.1 - 100	*33.592	2.11772
20 - 40	-1.40567	2.11772
20 - 60	*-2.53533	2.11772
20 - 80	*6.76267	2.11772
20 - 100	*4.225	2.11772
40 - 60	-1.12967	2.11772
40 - 80	*8.16833	2.11772
40 - 100	*5.63067	2.11772
60 - 80	*9.298	2.11772
60 - 100	*6.76033	2.11772
80 - 100	*-2.53767	2.11772

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of aqueous extract of KUM61076 fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin measured by Oil Red O assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	1757.28	5	351.457	140.92	0.0000
Within groups	29.9287	12	2.49406		
Total (Corr.)	1787.21	17			

Multiple range test: Effect of different concentrations of aqueous extract of KUM61076 fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin measured by Oil Red O assay (between concentrations)

Contrast	Difference	+/- Limits
0.1 - 20	*22.0503	2.8095
0.1 - 40	*17.8203	2.8095
0.1 - 60	*28.239	2.8095
0.1 - 80	*25.706	2.8095
0.1 - 100	*28.804	2.8095
20 - 40	*-4.23	2.8095
20 - 60	*6.18867	2.8095
20 - 80	*3.65567	2.8095
20 - 100	*6.75367	2.8095
40 - 60	*10.4187	2.8095
40 - 80	*7.88567	2.8095
40 - 100	*10.9837	2.8095
60 - 80	-2.533	2.8095
60 - 100	0.565	2.8095
80 - 100	*3.098	2.8095

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of aqueous extract of *VITA GL* fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin measured by Oil Red O assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	1325.27	5	265.053	159.81	0.0000
Within groups	19.9022	12	1.65852		
Total (Corr.)	1345.17	17			

Multiple range test: Effect of different concentrations of aqueous extract of *VITA GL* fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin measured by Oil Red O assay (between concentrations)

Contrast	Difference	+/- Limits
0.1 - 20	*23.1683	2.29106
0.1 - 40	*22.0457	2.29106
0.1 - 60	*10.212	2.29106
0.1 - 80	*15.5653	2.29106
0.1 - 100	*24.0183	2.29106
20 - 40	-1.12267	2.29106
20 - 60	*-12.9563	2.29106
20 - 80	*-7.603	2.29106
20 - 100	0.85	2.29106
40 - 60	*-11.8337	2.29106
40 - 80	*-6.48033	2.29106
40 - 100	1.97267	2.29106
60 - 80	*5.35333	2.29106
60 - 100	*13.8063	2.29106
80 - 100	*8.453	2.29106

* denotes a statistically significant difference.

ANOVA: Effect of 20 µg/mL of aqueous extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin measured by Oil Red O assay (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	351.168	3	117.056	47.85	0.0000
Within groups	19.5724	8	2.44655		
Total (Corr.)	370.74	11			

Multiple range test: Effect of 20 µg/mL of aqueous extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin measured by Oil Red O assay (between extracts)

Contrast	Difference	+/- Limits
1 - 2	*-6.20233	2.94505
1 - 3	*-13.519	2.94505
1 - 4	*-12.401	2.94505
2 - 3	*-7.31667	2.94505
2 - 4	*-6.19867	2.94505
3 - 4	1.118	2.94505

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* denotes a statistically significant difference.

ANOVA: Effect of 40 µg/mL of aqueous extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin measured by Oil Red O assay (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	276.399	3	92.133	384.84	0.0000
Within groups	1.91525	8	0.239406		
Total (Corr.)	278.314	11			

Multiple range test: Effect of 40 µg/mL of aqueous extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin measured by Oil Red O assay (between extracts)

Contrast	Difference	+/- Limits
1 - 2	*-1.97233	0.921262
1 - 3	*-12.1133	0.921262
1 - 4	*-7.888	0.921262
2 - 3	*-10.141	0.921262
2 - 4	*-5.91567	0.921262
3 - 4	*4.22533	0.921262

* denotes a statistically significant difference.

ANOVA: Effect of 60 µg/mL of aqueous extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin measured by Oil Red O assay (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	733.244	3	244.415	92.06	0.0000
Within groups	21.2397	8	2.65496		
Total (Corr.)	754.484	11			

Multiple range test: Effect of 60 µg/mL of aqueous extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin measured by Oil Red O assay (between extracts)

Contrast	Difference	+/- Limits
1 - 2	-2.53267	3.06792
1 - 3	-1.12533	3.06792
1 - 4	*-19.1523	3.06792
2 - 3	1.40733	3.06792
2 - 4	*-16.6197	3.06792
3 - 4	*-18.027	3.06792

* denotes a statistically significant difference.

ANOVA: Effect of 80 µg/mL of aqueous extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin measured by Oil Red O assay (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	678.116	3	226.039	162.31	0.0000
Within groups	11.1414	8	1.39267		
Total (Corr.)	689.258	11			

Multiple range test: Effect of 80 µg/mL of aqueous extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin measured by Oil Red O assay (between extracts)

Contrast	Difference	+/- Limits
1 - 2	*5.92033	2.22198
1 - 3	*-4.50333	2.22198
1 - 4	*-14.644	2.22198
2 - 3	*-10.4237	2.22198
2 - 4	*-20.5643	2.22198
3 - 4	*-10.1407	2.22198

* denotes a statistically significant difference.

ANOVA: Effect of 100 µg/mL of aqueous extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin measured by Oil Red O assay (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	155.356	3	51.7853	67.58	0.0000
Within groups	6.13056	8	0.76632		
Total (Corr.)	161.486	11			

Multiple range test: Effect of 100 µg/mL of aqueous extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin measured by Oil Red O assay (between extracts)

Contrast	Difference	+/- Limits
1 - 2	*7.60567	1.64824
1 - 3	*2.81767	1.64824
1 - 4	*-1.968	1.64824
2 - 3	*-4.788	1.64824
2 - 4	*-9.57367	1.64824
3 - 4	*-4.78567	1.64824

* denotes a statistically significant difference.

11) Effect of ethanol extracts of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin

ANOVA: Effect of different concentrations of ethanol extract of unfermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin measured by Oil Red O assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	3568.42	5	713.683	427.69	0.0000
Within groups	20.0244	12	1.6687		
Total (Corr.)	3588.44	17			

Multiple range test: Effect of different concentrations of ethanol extract of unfermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin measured by Oil Red O assay (between concentrations)

Contrast	Difference	+/- Limits
0.1 - 20	*35.0043	2.29808
0.1 - 40	*25.9863	2.29808
0.1 - 60	*35.0023	2.29808
0.1 - 80	*36.1297	2.29808
0.1 - 100	*43.7327	2.29808
20 - 40	*-9.018	2.29808
20 - 60	-0.002	2.29808
20 - 80	1.12533	2.29808
20 - 100	*8.72833	2.29808
40 - 60	*9.016	2.29808
40 - 80	*10.1433	2.29808
40 - 100	*17.7463	2.29808
60 - 80	1.12733	2.29808
60 - 100	*8.73033	2.29808
80 - 100	*7.603	2.29808

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of ethanol extract of KUM60813 fermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin measured by Oil Red O assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	2466.38	5	493.276	283.49	0.0000
Within groups	20.8804	12	1.74003		
Total (Corr.)	2487.26	17			

Multiple range test: Effect of different concentrations of ethanol extract of KUM60813 fermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin measured by Oil Red O assay (between concentrations)

Contrast	Difference	+/- Limits
0.1 - 20	*22.0433	2.34668
0.1 - 40	*18.3853	2.34668
0.1 - 60	*12.7473	2.34668
0.1 - 80	*-4.43433	2.34668
0.1 - 100	*-8.94467	2.34668
20 - 40	*-3.658	2.34668

20 - 60	*-9.296	2.34668
20 - 80	*-26.4777	2.34668
20 - 100	*-30.988	2.34668
40 - 60	*-5.638	2.34668
40 - 80	*-22.8197	2.34668
40 - 100	*-27.33	2.34668
60 - 80	*-17.1817	2.34668
60 - 100	*-21.692	2.34668
80 - 100	*-4.51033	2.34668

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of ethanol extract of KUM61076 fermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin measured by Oil Red O assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	3873.04	5	774.609	426.82	0.0000
Within groups	21.7781	12	1.81484		
Total (Corr.)	3894.82	17			

Multiple range test: Effect of different concentrations of ethanol extract of KUM61076 fermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin measured by Oil Red O assay (between concentrations)

Contrast	Difference	+/- Limits
0.1 - 20	*18.6627	2.39659
0.1 - 40	*-5.84233	2.39659
0.1 - 60	*-12.8877	2.39659
0.1 - 80	*-3.309	2.39659
0.1 - 100	*-30.35	2.39659
20 - 40	*-24.505	2.39659
20 - 60	*-31.5503	2.39659
20 - 80	*-21.9717	2.39659
20 - 100	*-49.0127	2.39659
40 - 60	*-7.04533	2.39659
40 - 80	*2.53333	2.39659
40 - 100	*-24.5077	2.39659
60 - 80	*9.57867	2.39659
60 - 100	*-17.4623	2.39659
80 - 100	*-27.041	2.39659

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of ethanol extract of *VITA GL* fermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin measured by Oil Red O assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	1246.4	5	249.281	108.34	0.0000
Within groups	27.6104	12	2.30087		
Total (Corr.)	1274.01	17			

Multiple range test: Effect of different concentrations of ethanol extract of *VITA GL* fermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin measured by Oil Red O assay (between concentrations)

Contrast	Difference	+/- Limits
0.1 - 20	*16.408	2.69849
0.1 - 40	*21.4783	2.69849
0.1 - 60	*11.622	2.69849
0.1 - 80	*21.763	2.69849
0.1 - 100	*3.73867	2.69849
20 - 40	*5.07033	2.69849
20 - 60	*-4.786	2.69849
20 - 80	*5.355	2.69849
20 - 100	*-12.6693	2.69849
40 - 60	*-9.85633	2.69849
40 - 80	0.284667	2.69849
40 - 100	*-17.7397	2.69849
60 - 80	*10.141	2.69849
60 - 100	*-7.88333	2.69849
80 - 100	*-18.0243	2.69849

* denotes a statistically significant difference.

ANOVA: Effect of 20 µg/mL of ethanol extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin measured by Oil Red O assay (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	1894.72	4	473.679	243.09	0.0000
Within groups	19.4856	10	1.94856		
Total (Corr.)	1914.2	14			

Multiple range test: Effect of 20 µg/mL of ethanol extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin measured by Oil Red O assay (between extracts)

Contrast	Difference	+/- Limits
1 - 2	*35.0043	2.53954
1 - 3	*22.0433	2.53954
1 - 4	*18.6627	2.53954
1 - 5	*16.408	2.53954
2 - 3	*-12.961	2.53954
2 - 4	*-16.3417	2.53954
2 - 5	*-18.5963	2.53954
3 - 4	*-3.38067	2.53954
3 - 5	*-5.63533	2.53954
4 - 5	-2.25467	2.53954

* denotes a statistically significant difference.

ANOVA: Effect of 40 µg/mL of ethanol extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin measured by Oil Red O assay (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	2365.73	4	591.433	356.52	0.0000
Within groups	16.589	10	1.6589		
Total (Corr.)	2382.32	14			

Multiple range test: Effect of 40 µg/mL of ethanol extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin measured by Oil Red O assay (between extracts)

Contrast	Difference	+/- Limits
1 - 2	*25.9863	2.34319
1 - 3	*18.3853	2.34319
1 - 4	*-5.84233	2.34319
1 - 5	*21.4783	2.34319
2 - 3	*-7.601	2.34319
2 - 4	*-31.8287	2.34319
2 - 5	*-4.508	2.34319
3 - 4	*-24.2277	2.34319
3 - 5	*3.093	2.34319
4 - 5	*27.3207	2.34319

* denotes a statistically significant difference.

ANOVA: Effect of 60 µg/mL of ethanol extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin measured by Oil Red O assay (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	3770.0	4	942.501	378.42	0.0000
Within groups	24.9064	10	2.49064		
Total (Corr.)	3794.91	14			

Multiple range test: Effect of 60 µg/mL of ethanol extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin measured by Oil Red O assay (between extracts)

Contrast	Difference	+/- Limits
1 - 2	*35.0023	2.87113
1 - 3	*12.7473	2.87113
1 - 4	*-12.8877	2.87113
1 - 5	*11.622	2.87113
2 - 3	*-22.255	2.87113
2 - 4	*-47.89	2.87113
2 - 5	*-23.3803	2.87113
3 - 4	*-25.635	2.87113
3 - 5	-1.12533	2.87113
4 - 5	*24.5097	2.87113

* denotes a statistically significant difference.

ANOVA: Effect of 80 µg/mL of ethanol extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin measured by Oil Red O assay (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	3919.81	4	979.952	457.20	0.0000
Within groups	21.4336	10	2.14336		
Total (Corr.)	3941.24	14			

Multiple range test: Effect of 80 µg/mL of ethanol extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin measured by Oil Red O assay (between extracts)

Contrast	Difference	+/- Limits
1 - 2	*36.1297	2.66345
1 - 3	*-4.43433	2.66345
1 - 4	*-3.309	2.66345
1 - 5	*21.763	2.66345
2 - 3	*-40.564	2.66345
2 - 4	*-39.4387	2.66345
2 - 5	*-14.3667	2.66345
3 - 4	1.12533	2.66345
3 - 5	*26.1973	2.66345
4 - 5	*25.072	2.66345

* denotes a statistically significant difference.

ANOVA: Effect of 100 µg/mL of ethanol extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin measured by Oil Red O assay (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	8742.85	4	2185.71	1305.29	0.0000
Within groups	16.745	10	1.6745		
Total (Corr.)	8759.59	14			

Multiple range test: Effect of 100 µg/mL of ethanol extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the absence of insulin measured by Oil Red O assay (between extracts)

Contrast	Difference	+/- Limits
1 - 2	*43.7327	2.35418
1 - 3	*-8.94467	2.35418
1 - 4	*-30.35	2.35418
1 - 5	*3.73867	2.35418
2 - 3	*-52.6773	2.35418
2 - 4	*-74.0827	2.35418
2 - 5	*-39.994	2.35418
3 - 4	*-21.4053	2.35418
3 - 5	*12.6833	2.35418
4 - 5	*34.0887	2.35418

* denotes a statistically significant difference.

12) Effect of ethanol extracts of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin

ANOVA: Effect of different concentrations of ethanol extract of unfermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin measured by Oil Red O assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	2096.72	5	419.344	242.04	0.0000
Within groups	20.7904	12	1.73254		
Total (Corr.)	2117.51	17			

Multiple range test: Effect of different concentrations of ethanol extract of unfermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin measured by Oil Red O assay (between concentrations)

Contrast	Difference	+/- Limits
0.1 - 20	*29.9337	2.34162
0.1 - 40	*24.0183	2.34162
0.1 - 60	*27.9563	2.34162
0.1 - 80	*28.804	2.34162
0.1 - 100	*31.0567	2.34162
20 - 40	*-5.91533	2.34162
20 - 60	-1.97733	2.34162
20 - 80	-1.12967	2.34162
20 - 100	1.123	2.34162
40 - 60	*3.938	2.34162
40 - 80	*4.78567	2.34162
40 - 100	*7.03833	2.34162
60 - 80	0.847667	2.34162
60 - 100	*3.10033	2.34162
80 - 100	2.25267	2.34162

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of ethanol extract of KUM60813 fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin measured by Oil Red O assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	1421.8	5	284.36	80.39	0.0000
Within groups	42.4475	12	3.53729		
Total (Corr.)	1464.25	17			

Multiple range test: Effect of different concentrations of ethanol extract of KUM60813 fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin measured by Oil Red O assay (between concentrations)

Contrast	Difference	+/- Limits
0.1 - 20	*26.549	3.34588
0.1 - 40	*25.4237	3.34588
0.1 - 60	*21.7703	3.34588
0.1 - 80	*19.7907	3.34588
0.1 - 100	*15.8477	3.34588

20 - 40	-1.12533	3.34588
20 - 60	*-4.77867	3.34588
20 - 80	*-6.75833	3.34588
20 - 100	*-10.7013	3.34588
40 - 60	*-3.65333	3.34588
40 - 80	*-5.633	3.34588
40 - 100	*-9.576	3.34588
60 - 80	-1.97967	3.34588
60 - 100	*-5.92267	3.34588
80 - 100	*-3.943	3.34588

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of ethanol extract of KUM61076 fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin measured by Oil Red O assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	683.785	5	136.757	40.91	0.0000
Within groups	40.1124	12	3.3427		
Total (Corr.)	723.898	17			

Multiple range test: Effect of different concentrations of ethanol extract of KUM61076 fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin measured by Oil Red O assay (between concentrations)

Contrast	Difference	+/- Limits
0.1 - 20	*16.973	3.25255
0.1 - 40	*13.03	3.25255
0.1 - 60	*16.6903	3.25255
0.1 - 80	*18.3803	3.25255
0.1 - 100	*12.1823	3.25255
20 - 40	*-3.943	3.25255
20 - 60	-0.282667	3.25255
20 - 80	1.40733	3.25255
20 - 100	*-4.79067	3.25255
40 - 60	*3.66033	3.25255
40 - 80	*5.35033	3.25255
40 - 100	-0.847667	3.25255
60 - 80	1.69	3.25255
60 - 100	*-4.508	3.25255
80 - 100	*-6.198	3.25255

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of ethanol extract of *VITA GL* fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin measured by Oil Red O assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	546.186	5	109.237	29.91	0.0000
Within groups	43.8247	12	3.65206		
Total (Corr.)	590.011	17			

Multiple range test: Effect of different concentrations of ethanol extract of *VITA GL* fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin measured by Oil Red O assay (between concentrations)

Contrast	Difference	+/- Limits
0.1 - 20	*14.4377	3.39973
0.1 - 40	*6.55167	3.39973
0.1 - 60	*11.3373	3.39973
0.1 - 80	*10.205	3.39973
0.1 - 100	*16.9777	3.39973
20 - 40	*-7.886	3.39973
20 - 60	-3.10033	3.39973
20 - 80	*-4.23267	3.39973
20 - 100	2.54	3.39973
40 - 60	*4.78567	3.39973
40 - 80	*3.65333	3.39973
40 - 100	*10.426	3.39973
60 - 80	-1.13233	3.39973
60 - 100	*5.64033	3.39973
80 - 100	*6.77267	3.39973

* denotes a statistically significant difference.

ANOVA: Effect of 20 µg/mL of ethanol extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin measured by Oil Red O assay (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	498.28	3	166.093	60.45	0.0000
Within groups	21.9824	8	2.7478		
Total (Corr.)	520.262	11			

Multiple range test: Effect of 20 µg/mL of ethanol extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin measured by Oil Red O assay (between extracts)

Contrast	Difference	+/- Limits
1 - 2	*-3.38467	3.1211
1 - 3	*-12.9607	3.1211
1 - 4	*-15.496	3.1211
2 - 3	*-9.576	3.1211
2 - 4	*-12.1113	3.1211
3 - 4	-2.53533	3.1211

* denotes a statistically significant difference.

ANOVA: Effect of 40 µg/mL of ethanol extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin measured by Oil Red O assay (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	734.645	3	244.882	564.00	0.0000
Within groups	3.47352	8	0.43419		
Total (Corr.)	738.119	11			

Multiple range test: Effect of 40 µg/mL of ethanol extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin measured by Oil Red O assay (between extracts)

Contrast	Difference	+/- Limits
1 - 2	*1.40533	1.24067
1 - 3	*-10.9883	1.24067
1 - 4	*-17.4667	1.24067
2 - 3	*-12.3937	1.24067
2 - 4	*-18.872	1.24067
3 - 4	*-6.47833	1.24067

* denotes a statistically significant difference.

ANOVA: Effect of 60 µg/mL of ethanol extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin measured by Oil Red O assay (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	453.517	3	151.172	35.92	0.0001
Within groups	33.6698	8	4.20873		
Total (Corr.)	487.187	11			

Multiple range test: Effect of 60 µg/mL of ethanol extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin measured by Oil Red O assay (between extracts)

Contrast	Difference	+/- Limits
1 - 2	*-6.186	3.8627
1 - 3	*-11.266	3.8627
1 - 4	*-16.619	3.8627
2 - 3	*-5.08	3.8627
2 - 4	*-10.433	3.8627
3 - 4	*-5.353	3.8627

* denotes a statistically significant difference.

ANOVA: Effect of 80 µg/mL of ethanol extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin measured by Oil Red O assay (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	522.394	3	174.131	43.70	0.0000
Within groups	31.8799	8	3.98498		
Total (Corr.)	554.274	11			

Multiple range test: Effect of 80 µg/mL of ethanol extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin measured by Oil Red O assay (between extracts)

Contrast	Difference	+/- Limits
1 - 2	*-9.01333	3.75862
1 - 3	*-10.4237	3.75862

1 - 4	*-18.599	3.75862
2 - 3	-1.41033	3.75862
2 - 4	*-9.58567	3.75862
3 - 4	*-8.17533	3.75862

* denotes a statistically significant difference.

ANOVA: Effect of 100 µg/mL of ethanol extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin measured by Oil Red O assay (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	617.609	3	205.87	79.55	0.0000
Within groups	20.7044	8	2.58805		
Total (Corr.)	638.314	11			

Multiple range test: Effect of 100 µg/mL of ethanol extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of insulin measured by Oil Red O assay (between extracts)

Contrast	Difference	+/- Limits
1 - 2	*-15.209	3.02902
1 - 3	*-18.8743	3.02902
1 - 4	*-14.079	3.02902
2 - 3	*-3.66533	3.02902
2 - 4	1.13	3.02902
3 - 4	*4.79533	3.02902

* denotes a statistically significant difference.

13) Effect of aqueous extracts of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of oxidative stress

ANOVA: Effect of different concentrations of water extract of unfermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of oxidative stress measured by Oil Red O assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	2209.07	4	552.267	203.17	0.0000
Within groups	27.1828	10	2.71828		
Total (Corr.)	2236.25	14			

Multiple range test: Effect of different concentrations of water extract of unfermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of oxidative stress measured by Oil Red O assay (between concentrations)

Contrast	Difference	+/- Limits
10 - 25	*-4.96433	2.99947
10 - 50	*-5.95667	2.99947
10 - 100	*-14.9713	2.99947
10 - 1000	*-34.305	2.99947
25 - 50	-0.992333	2.99947
25 - 100	*-10.007	2.99947
25 - 1000	*-29.3407	2.99947

50 - 100	*-9.01467	2.99947
50 - 1000	*-28.3483	2.99947
100 - 1000	*-19.3337	2.99947

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of water extract of KUM60813 fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of oxidative stress measured by Oil Red O assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	2033.32	4	508.33	361.61	0.0000
Within groups	14.0575	10	1.40575		
Total (Corr.)	2047.38	14			

Multiple range test: Effect of different concentrations of water extract of KUM60813 fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of oxidative stress measured by Oil Red O assay (between concentrations)

Contrast	Difference	+/- Limits
10 - 25	*-2.997	2.15701
10 - 50	*-2.997	2.15701
10 - 100	*-23.494	2.15701
10 - 1000	*-27.5347	2.15701
25 - 50	0.0	2.15701
25 - 100	*-20.497	2.15701
25 - 1000	*-24.5377	2.15701
50 - 100	*-20.497	2.15701
50 - 1000	*-24.5377	2.15701
100 - 1000	*-4.04067	2.15701

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of water extract of KUM61076 fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of oxidative stress measured by Oil Red O assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	5058.18	4	1264.54	376.34	0.0000
Within groups	33.6008	10	3.36008		
Total (Corr.)	5091.78	14			

Multiple range test: Effect of different concentrations of water extract of KUM61076 fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of oxidative stress measured by Oil Red O assay (between concentrations)

Contrast	Difference	+/- Limits
10 - 25	*-7.63233	3.33482
10 - 50	*-14.3187	3.33482
10 - 100	*-35.744	3.33482
10 - 1000	*-49.376	3.33482
25 - 50	*-6.68633	3.33482
25 - 100	*-28.1117	3.33482
25 - 1000	*-41.7437	3.33482

50 - 100	*-21.4253	3.33482
50 - 1000	*-35.0573	3.33482
100 - 1000	*-13.632	3.33482

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of water extract of *VITA GL* fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of oxidative stress measured by Oil Red O assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	3444.76	4	861.19	344.81	0.0000
Within groups	24.976	10	2.4976		
Total (Corr.)	3469.74	14			

Multiple range test: Effect of different concentrations of water extract of *VITA GL* fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of oxidative stress measured by Oil Red O assay (between concentrations)

Contrast	Difference	+/- Limits
10 - 25	*-11.9163	2.87514
10 - 50	*-11.9383	2.87514
10 - 100	*-34.2233	2.87514
10 - 1000	*-40.398	2.87514
25 - 50	-0.022	2.87514
25 - 100	*-22.307	2.87514
25 - 1000	*-28.4817	2.87514
50 - 100	*-22.285	2.87514
50 - 1000	*-28.4597	2.87514
100 - 1000	*-6.17467	2.87514

* denotes a statistically significant difference.

ANOVA: Effect of 10 µg/mL of water extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of oxidative stress measured by Oil Red O assay (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	215.975	3	71.9917	106.49	0.0000
Within groups	5.40849	8	0.676061		
Total (Corr.)	221.384	11			

Multiple range test: Effect of 10 µg/mL of water extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of oxidative stress measured by Oil Red O assay (between extracts)

Contrast	Difference	+/- Limits
1 - 2	*-4.632	1.54813
1 - 3	*4.591	1.54813
1 - 4	*-6.281	1.54813
2 - 3	*9.223	1.54813
2 - 4	*-1.649	1.54813
3 - 4	*-10.872	1.54813

* denotes a statistically significant difference.

ANOVA: Effect of 25 µg/mL of water extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of oxidative stress measured by Oil Red O assay (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	411.264	3	137.088	107.19	0.0000
Within groups	10.2314	8	1.27893		
Total (Corr.)	421.495	11			

Multiple range test: Effect of 25 µg/mL of water extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of oxidative stress measured by Oil Red O assay (between extracts)

Contrast	Difference	+/- Limits
1 - 2	*-2.66467	2.12931
1 - 3	1.923	2.12931
1 - 4	*-13.233	2.12931
2 - 3	*4.58767	2.12931
2 - 4	*-10.5683	2.12931
3 - 4	*-15.156	2.12931

* denotes a statistically significant difference.

ANOVA: Effect of 50 µg/mL of water extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of oxidative stress measured by Oil Red O assay (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	267.044	3	89.0145	34.78	0.0001
Within groups	20.4724	8	2.55905		
Total (Corr.)	287.516	11			

Multiple range test: Effect of 50 µg/mL of water extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of oxidative stress measured by Oil Red O assay (between extracts)

Contrast	Difference	+/- Limits
1 - 2	-1.67233	3.012
1 - 3	*-3.771	3.012
1 - 4	*-12.2627	3.012
2 - 3	-2.09867	3.012
2 - 4	*-10.5903	3.012
3 - 4	*-8.49167	3.012

* denotes a statistically significant difference.

ANOVA: Effect of 100 µg/mL of water extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of oxidative stress measured by Oil Red O assay (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	1002.49	3	334.165	82.11	0.0000
Within groups	32.5558	8	4.06947		
Total (Corr.)	1035.05	11			

Multiple range test: Effect of 100 µg/mL of water extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of oxidative stress measured by Oil Red O assay (between extracts)

Contrast	Difference	+/- Limits
1 - 2	*-13.1547	3.79826
1 - 3	*-16.1817	3.79826
1 - 4	*-25.533	3.79826
2 - 3	-3.027	3.79826
2 - 4	*-12.3783	3.79826
3 - 4	*-9.35133	3.79826

* denotes a statistically significant difference.

ANOVA: Effect of 1000 µg/mL of water extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of oxidative stress measured by Oil Red O assay (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	480.702	3	160.234	41.15	0.0000
Within groups	31.1489	8	3.89361		
Total (Corr.)	511.851	11.			

Multiple range test: Effect of 1000 µg/mL of water extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of oxidative stress measured by Oil Red O assay (between extracts)

Contrast	Difference	+/- Limits
1 - 2	2.13833	3.71528
1 - 3	*-10.48	3.71528
1 - 4	*-12.374	3.71528
2 - 3	*-12.6183	3.71528
2 - 4	*-14.5123	3.71528
3 - 4	-1.894	3.71528

* denotes a statistically significant difference.

14) Effect of ethanol extracts of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of oxidative stress

ANOVA: Effect of different concentrations of ethanol extract of unfermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of oxidative stress measured by Oil Red O assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	4084.72	4	1021.18	418.38	0.0000
Within groups	24.4077	10	2.44077		
Total (Corr.)	4109.13	14			

Multiple range test: Effect of different concentrations of ethanol extract of unfermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of oxidative stress measured by Oil Red O assay (between concentrations)

Contrast	Difference	+/- Limits
10 - 25	2.24967	2.84224
10 - 50	*-2.991	2.84224
10 - 100	*-32.0703	2.84224
10 - 1000	*-35.3187	2.84224
25 - 50	*-5.24067	2.84224
25 - 100	*-34.32	2.84224
25 - 1000	*-37.5683	2.84224
50 - 100	*-29.0793	2.84224
50 - 1000	*-32.3277	2.84224
100 - 1000	*-3.24833	2.84224

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of ethanol extract of KUM60813 fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of oxidative stress measured by Oil Red O assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	715.986	4	178.997	34.41	0.0000
Within groups	52.0118	10	5.20118		
Total (Corr.)	767.998	14			

Multiple range test: Effect of different concentrations of ethanol extract of KUM60813 fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of oxidative stress measured by Oil Red O assay (between concentrations)

Contrast	Difference	+/- Limits
10 - 25	*8.58433	4.14905
10 - 50	*5.92433	4.14905
10 - 100	*-11.144	4.14905
10 - 1000	-2.42133	4.14905
25 - 50	-2.66	4.14905
25 - 100	*-19.7283	4.14905
25 - 1000	*-11.0057	4.14905
50 - 100	*-17.0683	4.14905
50 - 1000	*-8.34567	4.14905
100 - 1000	*8.72267	4.14905

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of ethanol extract of KUM61076 fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of oxidative stress measured by Oil Red O assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	4633.71	4	1158.43	320.10	0.0000
Within groups	36.1891	10	3.61891		
Total (Corr.)	4669.9	14			

Multiple range test: Effect of different concentrations of ethanol extract of KUM61076 fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of oxidative stress measured by Oil Red O assay (between concentrations)

Contrast	Difference	+/- Limits
10 - 25	1.04233	3.46088
10 - 50	*-8.96567	3.46088
10 - 100	*-15.455	3.46088
10 - 1000	*-47.089	3.46088
25 - 50	*-10.008	3.46088
25 - 100	*-16.4973	3.46088
25 - 1000	*-48.1313	3.46088
50 - 100	*-6.48933	3.46088
50 - 1000	*-38.1233	3.46088
100 - 1000	*-31.634	3.46088

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of ethanol extract of *VITA GL* fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of oxidative stress measured by Oil Red O assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	4935.04	4	1233.76	252.79	0.0000
Within groups	48.8062	10	4.88062		
Total (Corr.)	4983.85	14			

Multiple range test: Effect of different concentrations of ethanol extract of *VITA GL* fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of oxidative stress measured by Oil Red O assay (between concentrations)

Contrast	Difference	+/- Limits
10 - 25	*-5.783	4.01916
10 - 50	*-16.2963	4.01916
10 - 100	*-21.677	4.01916
10 - 1000	*-52.0907	4.01916
25 - 50	*-10.5133	4.01916
25 - 100	*-15.894	4.01916
25 - 1000	*-46.3077	4.01916
50 - 100	*-5.38067	4.01916
50 - 1000	*-35.7943	4.01916
100 - 1000	*-30.4137	4.01916

* denotes a statistically significant difference.

ANOVA: Effect of 10 µg/mL of ethanol extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of oxidative stress measured by Oil Red O assay (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	153.176	3	51.0585	10.26	0.0041
Within groups	39.8013	8	4.97516		
Total (Corr.)	192.977	11			

Multiple range test: Effect of 10 µg/mL of ethanol extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of oxidative stress measured by Oil Red O assay (between extracts)

Contrast	Difference	+/- Limits
1 - 2	*-8.598	4.19971
1 - 3	*-8.232	4.19971
1 - 4	*-7.856	4.19971
2 - 3	0.366	4.19971
2 - 4	0.742	4.19971
3 - 4	0.376	4.19971

* denotes a statistically significant difference.

ANOVA: Effect of 25 µg/mL of ethanol extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of oxidative stress measured by Oil Red O assay (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	469.059	3	156.353	55.86	0.0000
Within groups	22.3908	8	2.79885		
Total (Corr.)	491.45	11			

Multiple range test: Effect of 25 µg/mL of ethanol extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of oxidative stress measured by Oil Red O assay (between extracts)

Contrast	Difference	+/- Limits
1 - 2	-2.26333	3.14996
1 - 3	*-9.43933	3.14996
1 - 4	*-15.8887	3.14996
2 - 3	*-7.176	3.14996
2 - 4	*-13.6253	3.14996
3 - 4	*-6.44933	3.14996

* denotes a statistically significant difference.

ANOVA: Effect of 50 µg/mL of ethanol extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of oxidative stress measured by Oil Red O assay (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	1027.78	3	342.595	185.38	0.0000
Within groups	14.7845	8	1.84806		

Total (Corr.)	1042.57	11

Multiple range test: Effect of 50 µg/mL of ethanol extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of oxidative stress measured by Oil Red O assay (between extracts)

Contrast	Difference	+/- Limits
1 - 2	0.317333	2.55961
1 - 3	*-14.2067	2.55961
1 - 4	*-21.1613	2.55961
2 - 3	*-14.524	2.55961
2 - 4	*-21.4787	2.55961
3 - 4	*-6.95467	2.55961

* denotes a statistically significant difference.

ANOVA: Effect of 100 µg/mL of ethanol extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of oxidative stress measured by Oil Red O assay (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	280.731	3	93.5771	12.95	0.0019
Within groups	57.8236	8	7.22795		
Total (Corr.)	338.555	11			

Multiple range test: Effect of 100 µg/mL of ethanol extract of unfermented and fermented wheat grains on 3T3-L1 preadipocyte differentiation in the presence of oxidative stress measured by Oil Red O assay (between extracts)

Contrast	Difference	+/- Limits
1 - 2	*12.3283	5.06201
1 - 3	*8.38333	5.06201
1 - 4	2.53733	5.06201
2 - 3	-3.945	5.06201
2 - 4	*-9.791	5.06201
3 - 4	*-5.846	5.06201

* denotes a statistically significant difference.

23. Effect of ethanol extracts of unfermented and fermented wheat grains on lipolysis in 3T3-L1 adipocytes in the absence of epinephrine

ANOVA: Effect of different concentrations of ethanol extract of unfermented wheat grains on lipolysis in 3T3-L1 adipocyte measured by glycerol quantification assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	4632.38	3	1544.13	270.09	0.0000
Within groups	45.7365	8	5.71706		
Total (Corr.)	4678.12	11			

Multiple range test: Effect of different concentrations of ethanol extract of unfermented wheat grains on lipolysis in 3T3-L1 adipocyte measured by glycerol quantification assay (between concentrations)

Contrast	Difference	+/- Limits
10 - 25	*-35.9167	4.50196
10 - 50	*-45.8887	4.50196
10 - 100	*-49.7497	4.50196
25 - 50	*-9.972	4.50196
25 - 100	*-13.833	4.50196
50 - 100	-3.861	4.50196

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of ethanol extract of KUM60813 fermented wheat grains on lipolysis in 3T3-L1 adipocyte measured by glycerol quantification assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	759.872	3	253.291	31.28	0.0001
Within groups	64.7903	8	8.09879		
Total (Corr.)	824.662	11			

Multiple range test: Effect of different concentrations of ethanol extract of KUM60813 fermented wheat grains on lipolysis in 3T3-L1 adipocyte measured by glycerol quantification assay (between concentrations)

Contrast	Difference	+/- Limits
10 - 25	*-20.722	5.35828
10 - 50	*-16.722	5.35828
10 - 100	*-16.4167	5.35828
25 - 50	4.0	5.35828
25 - 100	4.30533	5.35828
50 - 100	0.305333	5.35828

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of ethanol extract of KUM61076 fermented wheat grains on lipolysis in 3T3-L1 adipocyte measured by glycerol quantification assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	1754.99	3	584.996	74.83	0.0000
Within groups	62.5423	8	7.81778		
Total (Corr.)	1817.53	11			

Multiple range test: Effect of different concentrations of ethanol extract of KUM61076 fermented wheat grains on lipolysis in 3T3-L1 adipocyte measured by glycerol quantification assay (between concentrations)

Contrast	Difference	+/- Limits
10 - 25	*-31.8333	5.2645
10 - 50	*-26.6663	5.2645

10 - 100	*-18.1943	5.2645
25 - 50	5.167	5.2645
25 - 100	*13.639	5.2645
50 - 100	*8.472	5.2645

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of ethanol extract of *VITA GL* fermented wheat grains on lipolysis in 3T3-L1 adipocyte measured by glycerol quantification assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	2769.22	3	923.072	118.32	0.0000
Within groups	62.4118	8	7.80148		
Total (Corr.)	2831.63	11			

Multiple range test: Effect of different concentrations of ethanol extract of *VITA GL* fermented wheat grains on lipolysis in 3T3-L1 adipocyte measured by glycerol quantification assay (between concentrations)

Contrast	Difference	+/- Limits
10 - 25	*-19.1113	5.25901
10 - 50	*-34.5557	5.25901
10 - 100	*-38.6667	5.25901
25 - 50	*-15.4443	5.25901
25 - 100	*-19.5553	5.25901
50 - 100	-4.111	5.25901

* denotes a statistically significant difference.

ANOVA: Effect of 10 µg/mL of ethanol extract of unfermented and fermented wheat grains on lipolysis in 3T3-L1 adipocyte measured by glycerol quantification assay (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	4.49284	3	1.49761	1.42	0.3065
Within groups	8.43206	8	1.05401		
Total (Corr.)	12.9249	11			

Multiple range test: Effect of 10 µg/mL of ethanol extract of unfermented and fermented wheat grains on lipolysis in 3T3-L1 adipocyte measured by glycerol quantification assay (between extracts)

Contrast	Difference	+/- Limits
1 - 2	-0.138667	1.93302
1 - 3	1.389	1.93302
1 - 4	0.722667	1.93302
2 - 3	1.52767	1.93302
2 - 4	0.861333	1.93302
3 - 4	-0.666333	1.93302

* denotes a statistically significant difference.

ANOVA: Effect of 25 µg/mL of ethanol extract of unfermented and fermented wheat grains on lipolysis in 3T3-L1 adipocyte measured by glycerol quantification assay (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	605.368	3	201.789	14.31	0.0014
Within groups	112.787	8	14.0983		
Total (Corr.)	718.154	11			

Multiple range test: Effect of 25 µg/mL of ethanol extract of unfermented and fermented wheat grains on lipolysis in 3T3-L1 adipocyte measured by glycerol quantification assay (between extracts)

Contrast	Difference	+/- Limits
1 - 2	*15.056	7.06967
1 - 3	5.47233	7.06967
1 - 4	*17.528	7.06967
2 - 3	*-9.58367	7.06967
2 - 4	2.472	7.06967
3 - 4	*12.0557	7.06967

* denotes a statistically significant difference.

ANOVA: Effect of 50 µg/mL of ethanol extract of unfermented and fermented wheat grains on lipolysis in 3T3-L1 adipocyte measured by glycerol quantification assay (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	1383.67	3	461.223	47.78	0.0000
Within groups	77.2247	8	9.65308		
Total (Corr.)	1460.89	11			

Multiple range test: Effect of 50 µg/mL of ethanol extract of unfermented and fermented wheat grains on lipolysis in 3T3-L1 adipocyte measured by glycerol quantification assay (between extracts)

Contrast	Difference	+/- Limits
1 - 2	*29.028	5.8499
1 - 3	*20.6113	5.8499
1 - 4	*12.0557	5.8499
2 - 3	*-8.41667	5.8499
2 - 4	*-16.9723	5.8499
3 - 4	*-8.55567	5.8499

* denotes a statistically significant difference.

ANOVA: Effect of 100 µg/mL of ethanol extract of unfermented and fermented wheat grains on lipolysis in 3T3-L1 adipocyte measured by glycerol quantification assay (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	2423.21	3	807.737	174.47	0.0000
Within groups	37.0377	8	4.62971		
Total (Corr.)	2460.25	11			

Multiple range test: Effect of 100 µg/mL of ethanol extract of unfermented and fermented wheat grains on lipolysis in 3T3-L1 adipocyte measured by glycerol quantification assay (between extracts)

Contrast	Difference	+/- Limits
1 - 2	*33.1943	4.05128
1 - 3	*32.9443	4.05128
1 - 4	*11.8057	4.05128
2 - 3	-0.25	4.05128
2 - 4	*-21.3887	4.05128
3 - 4	*-21.1387	4.05128

* denotes a statistically significant difference.

24. Effect of aqueous extracts of unfermented and fermented wheat grains on lipolysis in 3T3-L1 adipocytes in the absence of epinephrine

ANOVA: Effect of different concentrations of aqueous extract of unfermented wheat grains on lipolysis in 3T3-L1 adipocyte measured by glycerol quantification assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	3693.31	3	1231.1	557.40	0.0000
Within groups	17.6692	8	2.20865		
Total (Corr.)	3710.97	11			

Multiple range test: Effect of different concentrations of aqueous extract of unfermented wheat grains on lipolysis in 3T3-L1 adipocyte measured by glycerol quantification assay (between concentrations)

Contrast	Difference	+/- Limits
10 - 25	*-32.9167	2.7982
10 - 50	*-36.1943	2.7982
10 - 100	*-46.9723	2.7982
25 - 50	*-3.27767	2.7982
25 - 100	*-14.0557	2.7982
50 - 100	*-10.778	2.7982

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of aqueous extract of KUM60813 fermented wheat grains on lipolysis in 3T3-L1 adipocyte measured by glycerol quantification assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	3713.41	3	1237.8	521.03	0.0000
Within groups	19.0053	8	2.37567		
Total (Corr.)	3732.41	11			

Multiple range test: Effect of different concentrations of aqueous extract of KUM60813 fermented wheat grains on lipolysis in 3T3-L1 adipocyte measured by glycerol quantification assay (between concentrations)

Contrast	Difference	+/- Limits
10 - 25	*-16.4723	2.90207
10 - 50	*-30.6387	2.90207
10 - 100	*-47.6943	2.90207
25 - 50	*-14.1663	2.90207
25 - 100	*-31.222	2.90207
50 - 100	*-17.0557	2.90207

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of aqueous extract of KUM61076 fermented wheat grains on lipolysis in 3T3-L1 adipocyte measured by glycerol quantification assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	3449.08	3	1149.69	237.58	0.0000
Within groups	38.7133	8	4.83916		
Total (Corr.)	3487.79	11			

Multiple range test: Effect of different concentrations of aqueous extract of KUM61076 fermented wheat grains on lipolysis in 3T3-L1 adipocyte measured by glycerol quantification assay (between concentrations)

Contrast	Difference	+/- Limits
10 - 25	*-20.0003	4.14191
10 - 50	*-27.5003	4.14191
10 - 100	*-47.3617	4.14191
25 - 50	*-7.5	4.14191
25 - 100	*-27.3613	4.14191
50 - 100	*-19.8613	4.14191

* denotes a statistically significant difference.

ANOVA: Effect of different concentrations of aqueous extract of *VITA GL* fermented wheat grains on lipolysis in 3T3-L1 adipocyte measured by glycerol quantification assay (between concentrations)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	3812.98	3	1270.99	328.52	0.0000
Within groups	30.9508	8	3.86885		
Total (Corr.)	3843.93	11			

Multiple range test: Effect of different concentrations of aqueous extract of *VITA GL* fermented wheat grains on lipolysis in 3T3-L1 adipocyte measured by glycerol quantification assay (between concentrations)

Contrast	Difference	+/- Limits
10 - 25	*-22.111	3.70345
10 - 50	*-25.2503	3.70345
10 - 100	*-50.278	3.70345

25 - 50	-3.13933	3.70345
25 - 100	*-28.167	3.70345
50 - 100	*-25.0277	3.70345

* denotes a statistically significant difference.

ANOVA: Effect of 10 µg/mL of aqueous extract of unfermented and fermented wheat grains on lipolysis in 3T3-L1 adipocyte measured by glycerol quantification assay (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	1.65507	3	0.55169	0.55	0.6643
Within groups	8.07698	8	1.00962		
Total (Corr.)	9.73205	11			

Multiple range test: Effect of 10 µg/mL of aqueous extract of unfermented and fermented wheat grains on lipolysis in 3T3-L1 adipocyte measured by glycerol quantification assay (between extracts)

Contrast	Difference	+/- Limits
1 - 2	-0.805667	1.89189
1 - 3	-0.472	1.89189
1 - 4	-0.972333	1.89189
2 - 3	0.333667	1.89189
2 - 4	-0.166667	1.89189
3 - 4	-0.500333	1.89189

* denotes a statistically significant difference.

ANOVA: Effect of 25 µg/mL of aqueous extract of unfermented and fermented wheat grains on lipolysis in 3T3-L1 adipocyte measured by glycerol quantification assay (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	410.135	3	136.712	26.09	0.0002
Within groups	41.9262	8	5.24077		
Total (Corr.)	452.061	11			

Multiple range test: Effect of 25 µg/mL of aqueous extract of unfermented and fermented wheat grains on lipolysis in 3T3-L1 adipocyte measured by glycerol quantification assay (between extracts)

Contrast	Difference	+/- Limits
1 - 2	*15.6387	4.31036
1 - 3	*12.4443	4.31036
1 - 4	*9.83333	4.31036
2 - 3	-3.19433	4.31036
2 - 4	*-5.80533	4.31036
3 - 4	-2.611	4.31036

* denotes a statistically significant difference.

ANOVA: Effect of 50 µg/mL of aqueous extract of unfermented and fermented wheat grains on lipolysis in 3T3-L1 adipocyte measured by glycerol quantification assay (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	173.985	3	57.995	21.37	0.0004
Within groups	21.7078	8	2.71347		
Total (Corr.)	195.693	11			

Multiple range test: Effect of 50 µg/mL of aqueous extract of unfermented and fermented wheat grains on lipolysis in 3T3-L1 adipocyte measured by glycerol quantification assay (between extracts)

Contrast	Difference	+/- Limits
1 - 2	*4.75	3.10155
1 - 3	*8.222	3.10155
1 - 4	*9.97167	3.10155
2 - 3	*3.472	3.10155
2 - 4	*5.22167	3.10155
3 - 4	1.74967	3.10155

* denotes a statistically significant difference.

ANOVA: Effect of 100 µg/mL of aqueous extract of unfermented and fermented wheat grains on lipolysis in 3T3-L1 adipocyte measured by glycerol quantification assay (between extracts)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	30.7942	3	10.2647	2.37	0.1463
Within groups	34.6277	8	4.32846		
Total (Corr.)	65.4218	11			

Multiple range test: Effect of 100 µg/mL of aqueous extract of unfermented and fermented wheat grains on lipolysis in 3T3-L1 adipocyte measured by glycerol quantification assay (between extracts)

Contrast	Difference	+/- Limits
1 - 2	-1.52767	3.91726
1 - 3	-0.861333	3.91726
1 - 4	*-4.278	3.91726
2 - 3	0.666333	3.91726
2 - 4	-2.75033	3.91726
3 - 4	-3.41667	3.91726

* denotes a statistically significant difference.