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THE ANTI-FUNGAL AND ANTI-OXIDANT PROPERTIES OF POLYPHENOLS EXTRACTED FROM THE RESURRECTION PLANT, *Myrothamnus flabellifolia*

Dissertation submitted for the degree of Master of Science

in the Department of Molecular and Cell Biology, Faculty of Science

UNIVERSITY OF CAPE TOWN

University of Cape Town

By

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LIST OF ABBREVIATIONS

γ	Gamma
~	approximately
μ	micro (10^{-6})
Δ	delta, indicates gene mutation
λ	lambda
°C	Degree Celsius
μg	Microgram
μl	Microlitre
A_{600}	Absorbance reading at 600 nm
A_{488}	Excitation wavelength at 488 nm
A_{520}	Emission wavelength at 520 nm
A_{765}	Absorbance reading at 765 nm
BSA	bovine serum albumin
C	carbon
CSM	complete synthetic medium
CTA1	catalase A
CTT1	catalase T
Cu	copper
Cu^{2+}	copper ions
CUP1	copper binding protein
CRS5	copper-resistant suppressor
CuZnSoD	copper/zinc superoxide dismutase
DCF	2',7'-dichlorofluorescein
DCHF-DA	2',7'-dichlorodihydrofluorescein diacetate
DCHF	2',7'-dichlorodihydrofluorescein
dH_2O	distilled water
DNA	Deoxyribonucleic acid
EtBr	ethidium bromide
EGCG	epigallocatechin gallate

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Fe^{3+}	Ferric acid
Fe^{2+}	ferrous ion
g	gram
GSH	glutathione
GSH1	γ-glutamylcysteine synthetase
GSH2	glutathione synthetase
GRL1	glutathione reductase
GSSG	glutathione disulphide
h	hours
HIV	human immunodeficiency virus
H_2O_2	hydrogen peroxide
HSP12p	heat shock protein 12
INT	Iodonitrotetrazolium chloride
M. flabellifolia	*Myrothamnus flabellifolia*
mM	Millimolar (10^{-3})
mg	Milligram (10^{-3})
mgGAE/g DW	milligrams gallic acid equivalent per gram dry weight
MnSoD	manganese superoxide dismutase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
n	number (of)
NADPH	nicotinamide adenine dinucleotide phosphate
NADP+	nicotinamide adenine dinucleotide phosphate
nM	nanomole (10^{-9})
O_2	Oxygen
$^1O_2$	singlet oxygen
$^{\cdot}OH$	Hydroxyl radical
$^{\cdot}O_2^-$	superoxide anion
PBS	Phosphate buffer saline
PCR	polymerase chain reaction
PI	Propidium iodide
RNA	ribonucleic acid

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RSA	Republic of Southern Africa
ROS	Reactive oxygen species
$RO_2^{\cdot}$	Lipid peroxide radical
s^{-1}	per second
S. cerevisiae	*Saccharomyces cerevisiae*
SD	Standard deviation
SOD	Superoxide dismutase
UV	ultraviolet light
URA	uracil
USA	United State of America
% (v/v)	percentage volume per volume (1ml/ 100ml)
% (w/v)	percentage gram (s) solute per 100ml
%	Percentage
YEPD	yeast extract peptone dextrose (glucose) growth medium
YNB	yeast nitrogen based medium
QH_2	Ubiquinol

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ABSTRACT

In this study the effects of *M. flabellifolia* polyphenols on growth of *S. cerevisiae* yeast strains was investigated. This study showed that *M. flabellifolia* polyphenols inhibited growth of both the wild type and the Δ*hsp12* yeast strains largely by binding protein in the growth medium. A decreased specific growth rate, reduced maximum biomass, and prolonged lag phase were observed for both strains. The specific growth rate of both strains decreased by over 60 % when 650 µg/ml polyphenols were present in YEPD medium, compared to a decrease of 16 % when the same concentration of polyphenols was present in a nitrogen based medium (YNB). Furthermore, the lag phase and the maximum biomass of both strains were largely affected by the presence of polyphenols in YEPD medium compared with the presence of polyphenols in YNB medium. Further studies showed that growth in in the presence of a combination of polyphenols and Fe^{3+} or Zn^{2+} metal ions in YEPD medium improved the specific growth rate of only the wild type strain, with iron improving the specific growth rate far more than zinc. Growth of yeast in the presence of up to 680 µg/ml gallic acid had no effect on the growth of either strain, suggesting that only high molecular weight polyphenols have the ability to bind proteins and chelate metal ions. Pre-treatment of both the wild type and the Δ*hsp12* strains with polyphenols did not affect the viability of either strain.

In the second part of this study the anti-oxidant properties of *M. flabellifolia* polyphenols were investigated using the wild type and rho^0 mutant strains. The results showed that the wild type strain but not the rho^0 mutant strain was able to adapt to lethal H_2O_2 concentrations following 24 h starvation. This study also showed that prolonged pre-

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treatment of the wild type and the rho^0 mutant strains with polyphenols was toxic to both strains. The wild type strain lost 35 % viability and the rho^0 mutant strain lost 20 % viability after pre-treatment with 650 µg/ml polyphenols for 24 h. The present study showed that polyphenols can either act as anti-oxidants or as pro-oxidants under certain conditions by producing ROS. Pre-treatment with polyphenols did not protect the wild type strain, but instead promoted cell death. However, 24 h pre-treatment with polyphenols protected the rho^0 mutant strains. The results showed that *M. flabellifolia* polyphenols were producing small amounts of ROS *in vivo* and *in vitro*. In addition, polyphenols were able to scavenge considerable amounts of H_2O_2. These results showed that incubation of wild type cells with H_2O_2 in the absence of polyphenols resulted in significantly reduced DCHF-DA oxidation compared when DCHF-DA was incubated with rho^0 mutant cells in the presence of H_2O_2 or when DCHF-DA was incubated with H_2O_2 in the absence of cells. These results suggested that the anti-oxidant defence systems of the wild type strain were able to neutralize H_2O_2. However, these anti-oxidant defence systems were also contributing to the oxidation of polyphenols. This oxidation resulted in polyphenols having pro-oxidant activities in the wild type strain and anti-oxidant activities in the rho^0 mutant strain. The results showed that *M. flabellifolia* polyphenols might offer protection of cells when used in moderate concentrations.

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CHAPTER ONE

LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Studies of stress responses of the budding yeast *Saccharomyces cerevisiae* have been of particular interest to many researchers. This yeast is used in the baking and fermentation industries as well as in the biotechnological industries to produce nutritional supplements such as proteins and B group vitamins such as niacin and folic acid. It is also used as a model organism to study different aspects of stress biochemistry. Since it was the first eukaryotic organism to have its genome completely sequenced, it has opened up many possibilities for biotechnological and biomedical research. *S. cerevisiae* also offers the advantage of having genes which are homologous to those of higher plants, mammals and humans. Therefore, the information obtained from studies of this yeast may serve as a model of stress responses in other eukaryotic organisms (Ruis, 1997; Kolkman et al., 2005; Karreman, 2006).

Desiccated plants and yeasts cells are continuously exposed to oxidative stress, since loss of water has been shown to aid the formation of reactive oxygen species (ROS) (Pereira et al., 2003). Oxidative stress occurs when cellular defences or repair mechanisms are unable to deal adequately with the ROS generated (Pereira et al., 2003; Dickinson and Schweizer, 2004).

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1.2 OXIDATIVE STRESS

Yeasts growing aerobically are constantly exposed to oxidative stress caused by partially reduced forms of molecular oxygen (O_2). These are collectively known as reactive oxygen species (ROS) and include the hydroxyl radical ($OH^{\cdot}$), the superoxide anion ($^{\cdot}O_2^-$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). ROS are generated during normal cellular metabolism, for example by electron leakage from the mitochondrial respiratory chain and by H_2O_2 generating reactions catalysed by oxidases. ROS can also originate from exposure to pro-oxidants such as H_2O_2, menadione or paraquat, and to ionizing radiation (Moradas-Ferreira et al., 1996; Jamieson, 1998).

Singlet oxygen ($^1O_2$) is a more reactive form of O_2 generated by the movement of one of the unpaired electrons to complete a shell, leaving the other orbital empty (Halliwell and Gutteridge, 1984, Dickinson and Schweizer, 2004). It is generated by interaction with photo-excited chlorophyll molecules in the triplet state, air pollution, and pathogenic fungi. One of the most common and abundant ROS is the ($^{\cdot}O_2^-$), a moderately reactive radical formed by the one-electron reduction of O_2. The primary source of the $^{\cdot}O_2^-$ is the leakage of electrons from complex I (NADH: ubiquinone segments) and complex III (QH_2: cytochrome 2) during respiration in the mitochondria. Superoxide anions give rise to other ROS, which are highly reactive and thereby cause biological damage. Figure 1.1 details the formation of all ROS and the mechanisms that cells use to neutralise and remove the products of oxidative damage to DNA, proteins and lipids (Dickinson and Schweizer, 2004).

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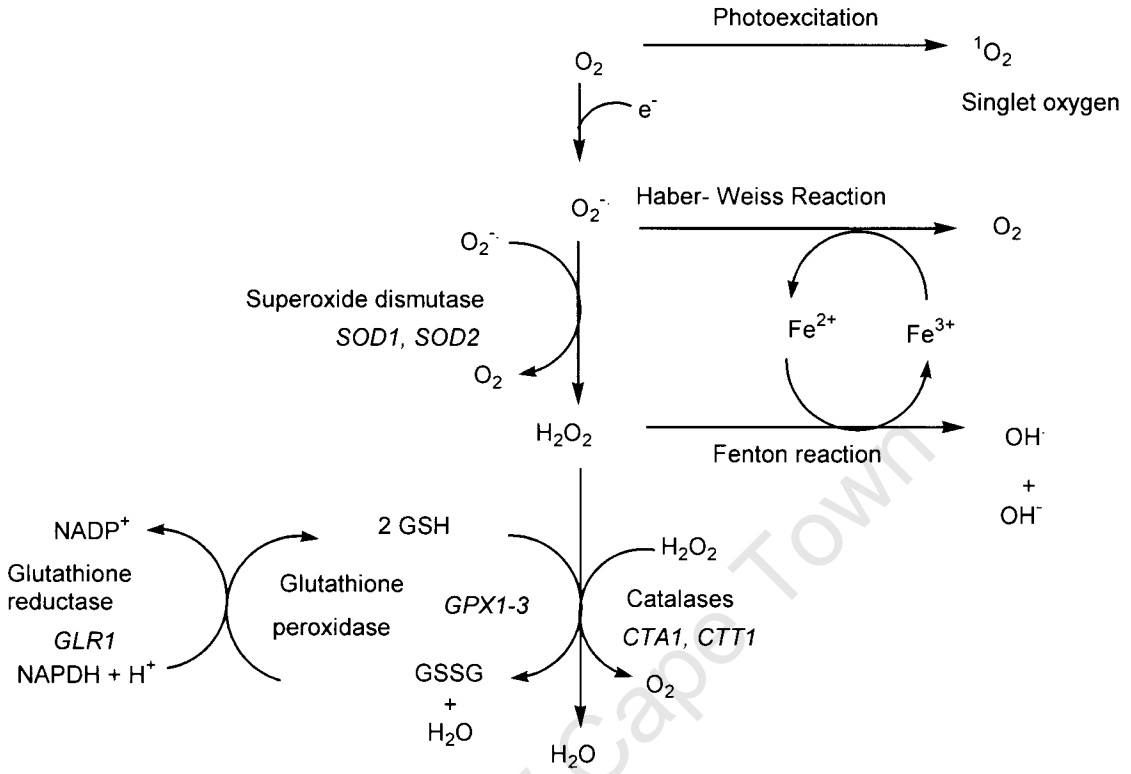

Figure 1.1: Formation of ROS and defence systems that are responsible for neutralizing and removing them from cells. *SOD1* and *SOD2* encode copper/zinc superoxide dismutases and manganese superoxide dismutases respectively, *CTA1* and *CTT1* encode catalase A and catalase T respectively, GSH is glutathione, *GRL1* encode glutathione reductase (Dickinson and Schweizer, 2004).

ROS are highly damaging to cellular constituents, including DNA, lipids and proteins. DNA damage in yeasts is not caused by $^{\cdot}O_2^-$ and H_2O_2, but by the generation of hydroxyl radicals. Reactions that generate the hydroxyl radical are catalysed by metal ions through the Haber-Weiss and Fenton reactions, initially between the oxidised form of metal ions such as Fe^{3+}or Cu^{2+} reacting with superoxide:

Haber-Weiss reaction $\quad Fe^{3+} + {}^{\bullet}O_2^- \longrightarrow Fe^{2+} + O_2$

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The reduced form of the metal ions can be converted to the oxidised metal ions and hydroxyl radicals in the presence of H_2O_2 using the Fenton reaction (Kehrer, 2000; Dickinson and Schweizer, 2004):

Fenton reaction $Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$

The hydroxyl radical reacts in a diffusion-limited manner with sugars, amino acids, phospholipids, nucleotides and organic acids. It can participate in hydrogen abstraction, addition and electron transfer reactions to generate other radicals that are generally less reactive than the hydroxyl radical (Dickinson and Schweizer, 2004).

Oxidative DNA damage can cause base and sugar damage, single-strand breaks, abasic sites and DNA-protein cross-links. The major product of base damage is 8-hydroxyguanine, which is detectable in yeast exposed to lethal concentrations of H_2O_2 but not detectable in yeast exposed to sub-lethal concentrations of H_2O_2. Sub-lethal concentrations of H_2O_2 induce resistance to oxidative stress. Since H_2O_2 increases the frequency of intra-chromosomal recombination in *S. cerevisiae*, it is likely that oxidative DNA damage plays a role in DNA recombination in yeast (Moradas-Ferreira et al., 1996).

Lipids have a critical structural and functional role in membranes, and thus a disruption of lipid structure can lead to cell death (Kehrer, 2000). Lipid peroxidation impairs the structural integrity of membranes by generating shorter fatty acyl chains and increasing membrane fluidity (Moradas-Ferreira et al., 1996). Lipid peroxidation occurs when a double bond in a polyunsaturated fatty acid is attacked by a free radical, yielding a new radical that can readily react with molecular oxygen to yield a di-radical. The di-radical can abstract a hydrogen atom from another fatty acid yielding another radical and a lipid

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hydroperoxide, as shown in Figure 1.2 (Wikipedia. 2007). The lipid hydroperoxide is very unstable and decomposes to various species including malondialdehyde (Kehrer, 2000). The end products of lipid peroxidation, such as epoxides and aldehydes, are able to interact with DNA and proteins, and inactivating the latter (Moradas-Ferreira et al., 1996).

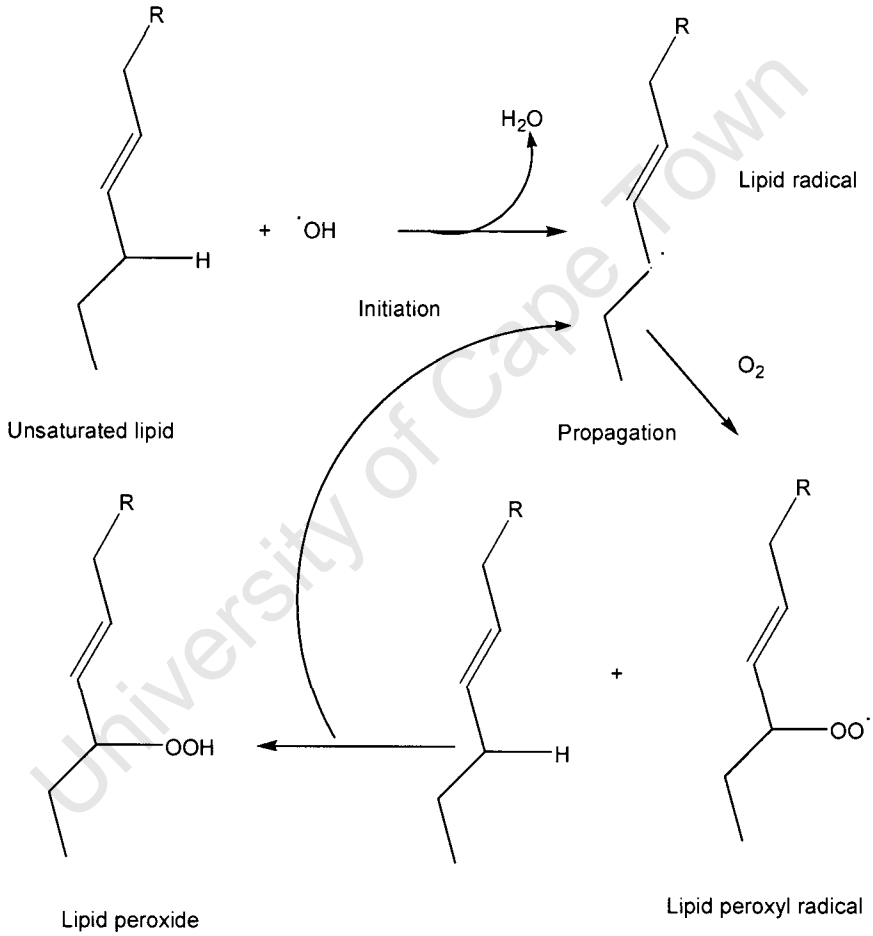

Figure 1.2: Overview of Lipid peroxidation (Wikipedia. 2007).

Oxidative damage to proteins can lead to increased proteolytic susceptibility and decreased biological activity, brought about by oxidation of certain amino acids such as histidine, arginine, lysine, proline, methionine and cysteine, and cross-linking to other proteins. Cysteine disulphide bonds formed by oxidation can be reduced by glutathione or

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thioredoxin, whilst oxidised methionine residues can be reduced by methionine sulphoxide reductase (Moradas-Ferreira et al., 1996). Although most of the oxidised proteins that are functionally inactive are rapidly removed, some gradually accumulate and contribute to the damage associated with aging (Kehrer, 2000).

1.2.1. Oxidative defence systems

Following chemical and physical damage elicited by stress, the cell initiates repair, recovery and defence mechanisms to protect itself against further damage, (Dickinson and Schweizer, 2004). Yeast cells use enzymatic and non-enzymatic defence systems to protect their cellular constituents and maintain cellular ROS at physiological level (Moradas-Ferreira et al., 1996; Jamieson, 1998).

1.2.1.1. Non-enzymatic defence systems

Non-enzymatic defence systems consist of small molecules which are soluble in either an aqueous or, in some instances, a lipid environment and these include glutathione, phytochelatins, polyamines, ascorbic acid, lipid-soluble antioxidants, and thioredoxin. They act generally as radical scavengers, being oxidized by ROS and thereby removing oxidants from solution (Jamieson, 1998; See Figure 1.1)

Glutathione (GSH) also known as γ-L-glutamyl-L-cystinylglycine is a tri-peptide that acts as a free radical scavenger with the redox active sulphyldryl group reacting with oxidants to produce oxidised glutathione (GSSG). GSH synthesis is a two step process catalysed by γ-glutamylcysteine synthetase, encoded by *GSH1,* and glutathione

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synthetase, encoded by *GSH2*. *GSH1* catalyses the condensation of cysteine onto the γ-carbon of glutamate in the rate limiting step of this synthetic pathway, and *GSH2* forms GSH by the addition of glycine (Toledano et al., 2003). The reduced form (GSH) is regenerated by the reaction of GSSG with NADPH catalysed by glutathione reductase, encoded by *GRL1*, (see Figure 1.1) (Jamieson, 1998; Dickinson and Schweizer, 2004). Glutathione reductase and glutathione peroxidase are crucial for the antioxidant property of glutathione to maintain high reduced GSH:GSSG ratio inside the cells (Dickinson and Schweizer, 2004). *S. cerevisiae* strains deficient in *GSH1* and *GSH2* are viable, but grow at a slower rate and have a longer lag phase than wild types strains. They also show a defect in sporulation. Δ*gsh1* strains cannot grow by respiration *i.e.* on non-fermentable carbon sources such as glycerol and acetate, and are dependent upon exogenous GSH for growth in minimal medium (Moradas-Ferreira et al., 1996; Dickinson and Schweizer, 2004)

Phytochelatins are structurally related to GSH and play an analogous role (Jamieson, 1998). Phytochelatins consist of three amino acids, glutamic acid, cysteine, and glycine with glutamic acid, and cysteine residues linked through a γ-carboxylamide bond. Phytochelatins form a family of structures with increasing repetitions of the γ-glutamic-cysteine dipeptide followed by a terminal glycine: $((\gamma\text{-glutamic-cysteine})_n\text{-glycine})$; n has been reported to be as high as 11, but is generally in the range between 2 and 5. Phytochelatins have been identified in a variety of plants species and some micro-organisms (Cobbett, 2000).

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Metallothioneins are small cysteine-rich proteins with antioxidant properties, which have the capacity to bind a number of different metals ions, especially copper (Cu) (Jamieson, 1998; Dickinson and Schweizer, 2004). In yeast there are two metallothioneins genes, *CUP1* and *CRS5*. *CUP1* protect cells against Cu toxicity (Dickinson and Schweizer, 2004).

Ascorbic acid is an antioxidant present in eukaryotes, especially in plants (Jamieson, 1998). It has strong *in vitro* antioxidant activity with the ability to scavenge a range of free radical species including lipid peroxyl radicals ($RO_2^{\cdot}$), superoxide ions and hydroxyl radicals. Ascorbic acid also plays an important role in recycling vitamin E after its reaction with lipid radicals. It can, however, reduce ferric ions to the ferrous form and, in the presence of H_2O_2, can stimulate hydroxyl radical formation via the Fenton reaction making it a pro-oxidant under certain conditions (Dickinson and Schweizer, 2004).

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1.2.1.2. Enzymatic defence systems

Cellular antioxidant defences also include several enzymes capable of removing oxygen radicals and their products and/or repairing the damage caused by oxidative stress. The synthesis of catalase, superoxide dismutases, glutathione peroxidases and other small proteins of unknown function are induced in response to oxidative stress (Jamieson, 1998).

S. cerevisiae has two haemoprotein **catalases**, namely catalase A and catalase T, which are encoded by the *CTA1* and *CTT1* genes respectively, and these can catalyse the breakdown of H_2O_2 into O_2 and H_2O (Jamieson, 1998). Catalase A is located in the

peroxisome, whereas catalase T is located in the cystosol (Jamieson, 1998; Dickinson and Schweizer, 2004). Unlike glutathione peroxidase, the two catalases lack the ability to react with larger hydroperoxides such as *tetra*-butyl hydroperoxide (Dickinson and Schweizer, 2004). Yeast strains that are deficient in *CTA1* and *CTT1* are hypersensitive to H_2O_2 and elevated temperature in stationary phase. Single and double catalase mutants are unable to mount an adaptive stress response to H_2O_2, suggesting that both catalases are important in the resistance of yeast to H_2O_2 (Jamieson, 1998).

Superoxide dismutases (SODs) are a group of metallo-enzymes that catalyse the conversion of O_2^- to O_2 and H_2O_2. Yeasts cells and other eukaryotes possess two intracellular SODs, namely the mitochondrially-located manganese superoxide dismutase (MnSoD) and the cytoplasmically-located copper/zinc superoxide dismutase (Cu/ZnSoD). The Cu/ZnSoD is the major enzyme involved in removing superoxide anions from the cytoplasm and the peroxisome, while the MnSoD enzyme protects the mitochondria against superoxides generated during respiration and exposure to ethanol (Jamieson, 1998). *S. cerevisiae* cells that lack both *SOD* genes are unable to synthesise methionine and lysine, are defective in sporulation and show an increased mutation rate. Both Δ*sod1* and Δ*sod2* mutants are hypersensitive to oxygen (Santoro and Thiele, 1997; Jamieson, 1998; Dickinson and Schweizer, 2004).

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1.3. RESURRECTION PLANTS

Desiccation tolerance is the ability of an organism to survive following a loss of about 80 – 95 % of its cellular water content (Bewley, 1979; Illing et al., 2005; Moore, 2006). Alternatively, it has been proposed that desiccation tolerance is the ability of an organism to dry to equilibrium with 'moderately dry' air between 50- 70 % relative humidity at 20-30 °C, followed by the resumption of normal function upon rehydration (Alpert and Oliver, 2002; Moore, 2006). Although the vegetative tissues of most higher plants are unable to survive desiccation to an air-dried state, seeds and pollen grains from such plants often can withstand air dryness for prolonged periods (Farrant and Kruger, 2001). Interestingly, a small group of vascular angiosperm plants known as ''resurrection plants" have the ability to revive from an air-dry state (Kranner et al., 2002; Bartels, 2005).

The resurrection plants are able to withstand severe water loss that is, anhydrobiosis, a state characterised by little intracellular water content and almost no metabolic activity (Kranner et al., 2002). Some resurrection plants are even able to equilibrate the leaves with air to 0 % (v/v) relative humidity (Bartels, 2005). Desiccation tolerance is more common among lower order plants such as lichens, algae, and bryophytes. There are no reports of desiccation tolerant representatives among the gymnosperms (Farrant and Kruger, 2001).

The resurrection plants are found in places where rainfall is seasonal and unpredictable. They grow on rocky outcrops (Figure 1.3), at low to moderate elevations in tropical and subtropical climates. The majority of the resurrection plants are found to occur in the

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southern hemisphere of Africa and Australia. The most common resurrection plant in Southern Africa is the dicotyledonous plant from the family *Myrothamnaceae* (Bartels, 2005). The resurrection capacity is normally extended to all tissues of the plant. The resurrection plants are able to survive in the desiccated state until water becomes available and allowing them to rehydrate and resume full physiological activities. The leaves of these plants shrink and curl up during the dehydration process. Mature tissue such as leaves and roots of such plant are able to remain in the air-dried state for months by reaching a quiescent state, which is comparable with dormancy in seeds (Bartels, 2005).

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Figure 1.3: Resurrection plants growing on rocky outcrops of Namibia (W Brandt)

Resurrection plants are classified into poikilochlorophyllous and homoiochlorophyllous plants. The former lose their chlorophyll and their membrane thylakoids become partially degraded during water loss. Homoiochlorophyllous plants, such as *Craterostigma*

plantagineum and M. flabellifolia, retain their chlorophyll and their photosynthetic structures remains intact, although there are changes in the photosynthetic pigments. Homoiochlorophyllous plants take a shorter period of time to recover and restore photosynthetic activities (within 24 h) than poikilochlorophyllous plants which take somewhat longer to recover these activities (Bartels, 2005).

1.4. THE RESURRECTION PLANT *Myrothamnus flabellifolia*

M. flabellifolia, a small woody shrub, is Southern Africa's most widely distributed resurrection plant (Moore, 2006). The multi-stemmed shrub can grow between 0.5 and 1.5 meters tall on the rocky inselbergs of southern Africa (Sherwin et al., 1998; Moore et al., 2007). *M. flabellifolia* earned the English vernacular name 'resurrection plant' because of the remarkable feature that a seemingly dead plant can unfold its leaves and turn bright green when placed in water (Figure 1.4) (Van Wyk et al., 1997).

Figure 1.4: The ressurection plant *M. flabellifolia* folds its leaves againt the stem when dessicated, but unfolds and returns to its bright green colour when water is provided (W. Brandt).

Friedrich Welwitsch first reported the presence of the plant in 1859, naming it *Myrothamnus* (*myron*: aromatic, *thamnos*: bush and *flabellifolia*: fan-like leaves). The

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leaves have a camphor/balsamic-like odour (Van Wyk and Gericke, 2000; Moore et al., 2007). The plant is a geophyte and possesses an extensive root system which extends into the crevices and cracks of the sandstone and granite slopes upon which it grows (Moore, 2006). The soil becomes saturated as a result of rain but also dries very rapidly. During dehydration the plant curls its leaves against the stem segments and changes from green to a dull brown colour (Figure 1.4). The plants revive when water is provided, resulting in a slow uncurling of leaves whilst returning to their previous green colour (Sherwin et al., 1998).

M. flabellifolia is widely used as a medicinal plant by the indigenous people. It is known as bergboegoe and uvukwabafile in Afrikaans and Zulu, respectively. Uvukwabafile means wakes from the dead (Van Wyk and Gericke, 2000). It is this reviving ability which is seen as a symbol of hope and is believed to be passed on to the ill person during treatment (Van Wyk et al, 1997). *M. flabellifolia* is used to treat conditions including depression and a variety of ailments. The Pedi people smoke the leaves in pipes to alleviate chest pains, and the Karanga people chew the aromatic leaves for oral ailments (Viljoen et al., 2002). The plant is also used by inhaling the smoke from burning leaves to treat chest pains and asthma (Van Wyk et al, 1997).

The medicinal properties of this plant have been attributed to antimicrobial properties of essential oils present (Viljoen et al., 2002). Different studies have reported different compounds, probably as the plants were sourced from different regional populations. Major essential oils reported are carvone perillic acid, 1, 8-cineole, diosphenol, trans-pinocarveol, and pinocarvone (Viljoen et al., 2002).

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Recently high polyphenol contents have also been reported to be present in the leaves of *M. flabellifolia* (Moore, 2006). The major polyphenol was determined to be 3, 4, 5-tri-*O*-galloyquinic acid (Figure 1.5). This compound was found to be able to protect artificial membranes (liposomes) against desiccation and free radical induced oxidation (Moore et al., 2005). Medicinally, gallotannins such as 3, 4, 5-tri-*O*-galloyquinic acid are known for their wound healing properties. Galloyquinic acid derivatives have been identified as compounds possessing high activity against HIV reverse transcriptase (Bokesch et al., 1996) and DNA polymerase (Koonjul et al., 1999).

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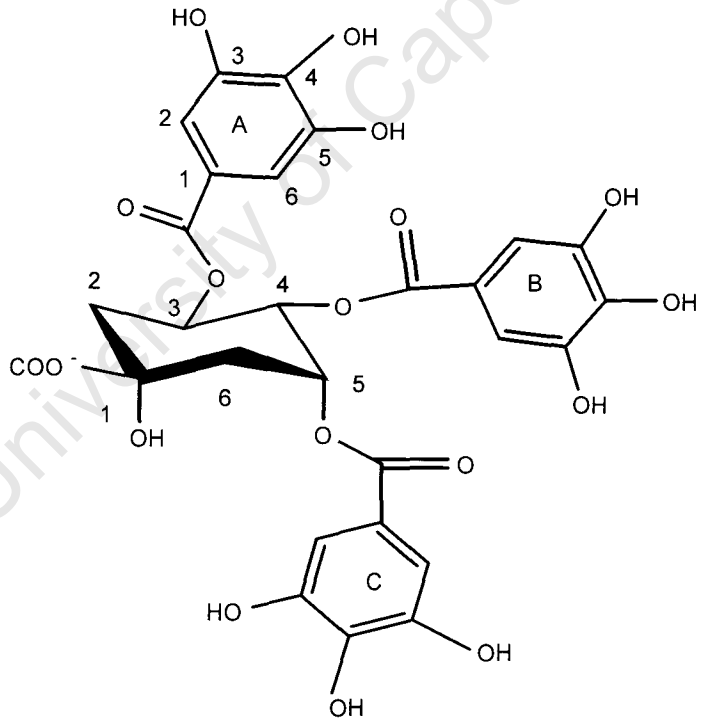

Figure 1.5: The structure of 3,4,5 tri-*O*-galloyquinic acid , the predominant polyphenol isolated from leaves of *M. flabellifolia*.

1.5. POLYPHENOLS

Phenolics are a group of phytochemicals that are widely distributed throughout the plant kingdom and accumulate as secondary metabolites (Hättenschiwiler and Vitousek, 2000; Balasundram et al, 2006; Crozier et al., 2006). They are derived from compounds of the pentose phosphate, shikimate, and phenylpropanoid pathways (Balasundram et al, 2006; Crozier et al., 2006). A variety of roles for polyphenols have been proposed including protecting plants from herbivores and microbial infection, attracting pollinators and seed-dispersing animals, and acting as allelopathic agents, UV protectants and signaling molecules (Crozier et al., 2006). Polyphenols also exhibit a wide range of physiological properties, including anti-allergenic, anti-artherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic, cardioprotective and vasodilatory effects (Balasundram et al, 2006).

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1.5.1. Classification of phenolic compounds

Structurally, phenolics are defined by the presence of an aromatic ring bearing one or more hydroxyl groups which maybe substituted with esters and glucosides (Hättenschiwiler and Vitousek, 2000; Balasundram et al, 2006). Over 800 phenolic structures have been reported, ranging from simple, low molecular-weight phenols (hydroxybenzoic acid derivatives) to large complex tannins of relatively high molecular weight (Hättenschiwiler and Vitousek, 2000; Balasundram et al, 2006; Crozier et al., 2006). The hydroxybenzoic acids include gallic, p-hydroxybenzoic, protocatechuic, vanillic and syringic acids, which have the benzoic acid structure in common (Figure

1.6a). In contrast, hydroxycinnamic acids, are aromatic compounds with a three-carbon side chain attached to the benzoic ring, with caffeic, ferulic, p-coumaric and sinapic acids being the most common examples (Figure 1.6b; Balasundram et al, 2006).

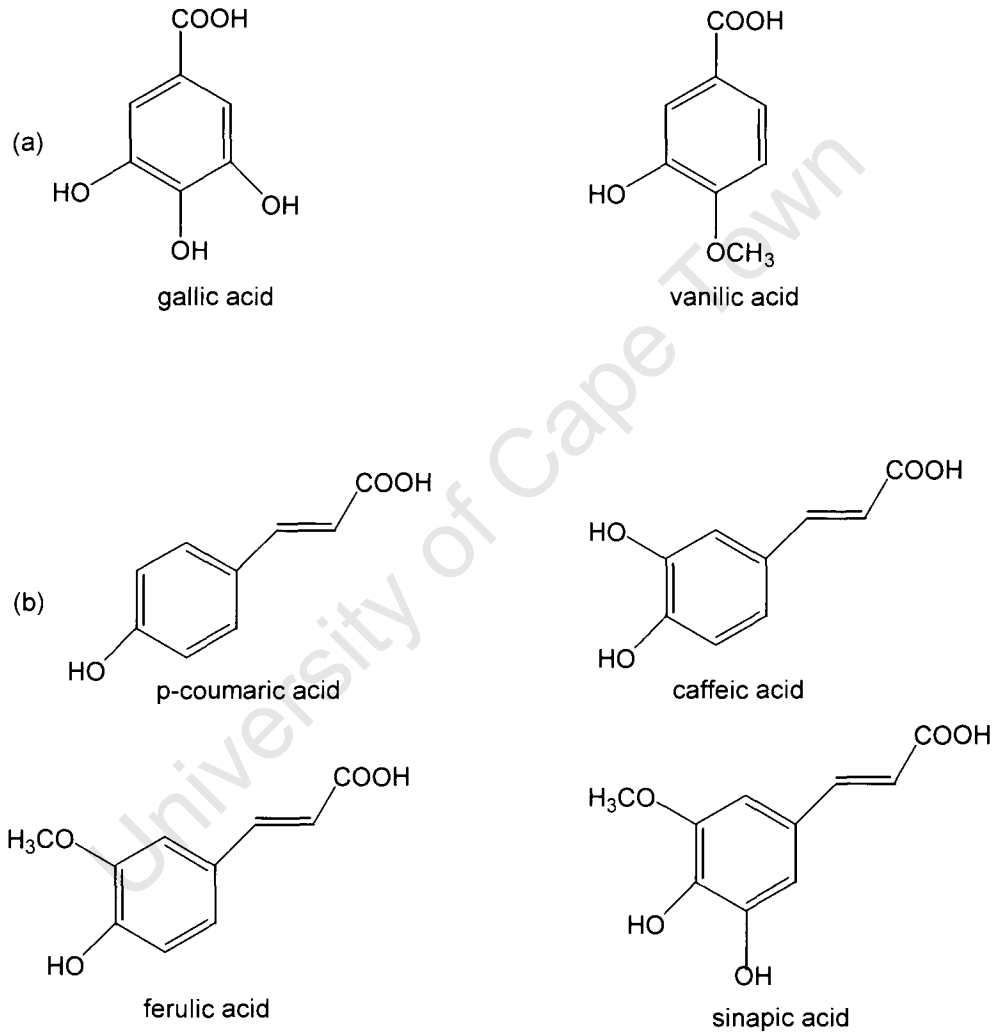

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Figure 1.6: Examples of (a) hydrobenzoic acid and (b) hydroxycinnamic acid (Balasundram et al, 2006).

1.5.1.1. FLAVONOIDS

Flavonoids constitute the largest and most diverse family of polyphenols throughout the plant kingdom. Their common structure consists of phenylbenzopyrone, a C15 compound, with two aromatic rings connected by a three-carbon bridge (Figure 1.7). Flavonoids are present in high concentrations in the epidermis of leaves and the skins of fruits, and have anti-oxidant and free-radical scavenging properties. As a result of their high extinction coefficients in the UV range, they may acts as UV protectants.

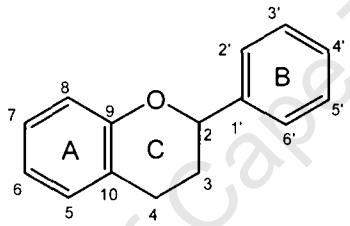

Figure 1.7: Generic structure of flavonoids molecules

Variation in the substitution patterns to the 'C' ring of the basic flavonoid structure give rise to different flavonoids such flavones, flavonols, flavan-3-ols, isoflavones, flavones and anthocyanidins (Figure 1.8). These substitutions may include glycosylation, acylation, oxygenation, and alkylation (Balasundram et al, 2006; Crozier et al., 2006). The presence of both sugar and hydroxyl groups increases the solubility of flavonoids in water, whereas the attachment of methyl groups and isopentyl units makes the flavonoids lipophilic (Crozier et al., 2006).

Of the flavonoids, flavonols are the most widespread. They occur throughout the plant kingdom but are not found in fungi and algae. They usually occur as co-pigments with anthocyanins in the petals and leaves of higher plants. Flavonols such as myricetin,

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quercetin, isorhamnetin and kaempferol occurs as *O*-glycosides / aglycones and absorb in the 330-380 nm region (Harborne, 1967).

Flavones have a similar structure to flavonols but lack the 3-hydroxyl substitution. Flavones such as luteolin and apigenin, have A- and C- ring substitutions but lack oxygenation at C3. These compounds generally have a wide variety of substituents such as hydroxylation, methylation, O- and C- alkylation, and glycosylation.

Flavan-3-ols are the most complex sub-class of flavonoids ranging from monomers such as catechin and its isomer epicatechin, to condensed tannins such as proanthocyanidins. Flavan-3-ols, proanthocyanidins and flavanones have a saturated C2 carbon bond in the heterocyclic 'C' ring. Consequently, these compounds are non-planar unlike other flavonoids (Crozier et al., 2006).

Anthocyanidins are widely distributed throughout the plant kingdom, and are found in the fruits and flowers tissues where they are responsible for red, blue, and purple pigments. They are also found in leaves, stems, seeds and root tissues. Their functions include the protection of plants against excess light by shading leaf mesophyll cells, and attracting pollinating insects. The common anthocyanidins include pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin, which occur as sugar conjugates in some plant tissues. In addition, they also form conjugates with hydroxycinnamates and organic acids such as malic and acetic acid.

Isoflavones are characterised by having the B-ring attached at the C3 rather than at the C2 position. They are found almost exclusively in leguminous plants with the highest

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concentrations occurring in soya beans. The isoflavones, namely genistein, daidzein and coumestrol, which are present in lucerne and clovers have sufficient oestrogen activity to affect the reproduction of grazing animals and are referred to as phyto-oestrogens (Crozier et al., 2006).

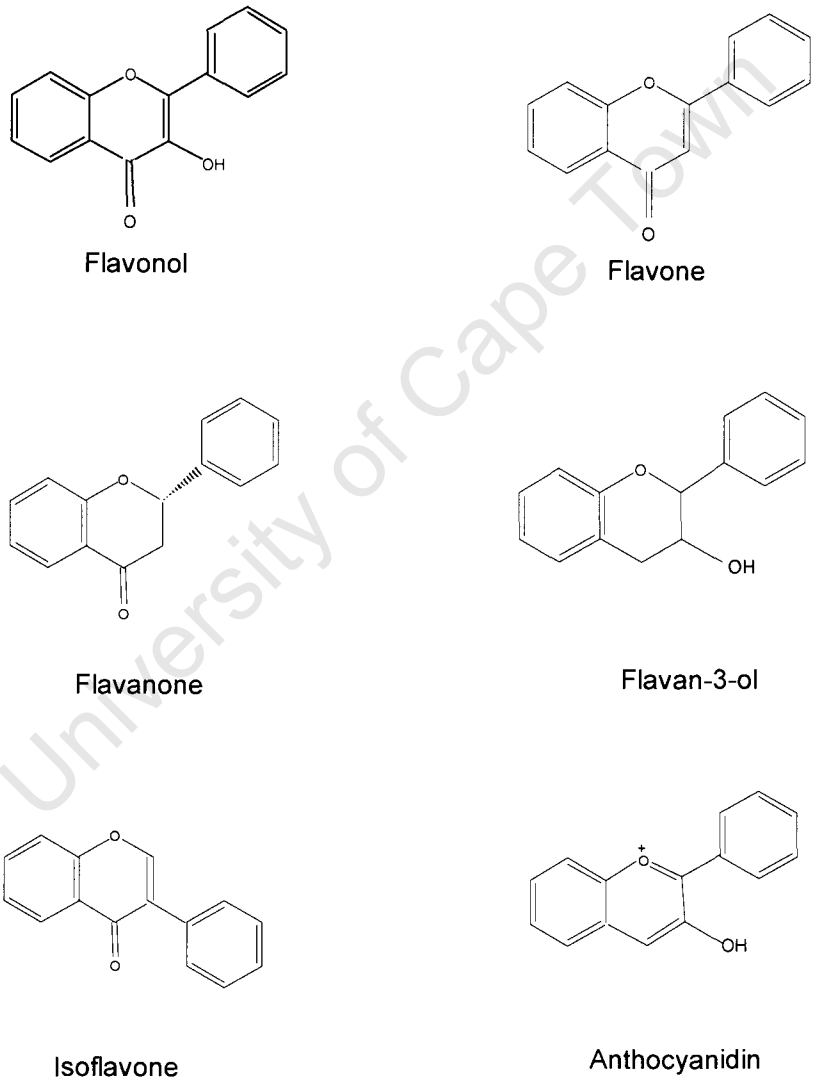

Figure 1.8: Generic structures of major classes of flavonoids

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1.5.1.2. Non-flavonoids

The main non-flavonoids are the phenolics, the hydrocinammates and their conjugated derivatives, and the stilbenes. Phenolics are known as hydroxybenzoates, the principal component being gallic acid. Gallic acid is the base unit of the gallotannins whereas gallic acid and hexahydroxydiphenoyl moieties are both subunits of ellagitannins (Crozier et al., 2006).

Hydroxycinnamates have been shown to possess high antioxidant activity compared to hydroxybenzoic acids, possibly due to the presence of the -CH=CH-COOH group which ensures greater H-donating ability and radical stabilisation than the –COOH group in the hydroxybenzoic acids (Balasundram et al, 2006). Cinnamic acids are the most widespread group of phenylpropanoids, and include the ferulic acids, sinapic, caffeic and coumaric acids (Crozier et al., 2006).

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Figure 1.9: Generic structures of non flavonoids

Stilbenes or phytoalexins are produced by plants in response to attack by fungal, bacterial and viral pathogens. The most common stilbene is resveratrol, which occurs as both the *cis*- and the *trans*-isomers. Resveratrol is mainly present as *trans*-resveratrol-3-*O*-

glucosides which are known as piceid and polydatin. The major source of stilbenes includes grapes, wines, soya and peanut products (Crozier et al., 2006).

Tannins constitute a complex group of naturally occurring polymers. The term tannin was originally used to describe vegetable components responsible for converting animal hides into leather by forming stable complexes with skin collagen. Tannins are considered to be the polyphenolic metabolites of plants. These polymers have molecular weights larger than 500 Daltons and have the ability to precipitate gelatine and other proteins from solution. Tannins are divided in to two groups based on their structure, namely the hydrolysable and condensed tannins (Figure 1.10) (Bennick, 2002).

Hydrolysable tannins are synthesised by a wide variety of plants of which pentagalloylglucose is very common (Figure 1.10) (Mueller-Harvey, 2001; Hagerman, 2002). Hydrolysable tannins such as gallotannins and ellagitannins are readily hydrolysed with dilute acid, releasing gallic acid and/or ellagic acid by treatment whereas, condensed tannins are not readily hydrolysed (Bennick, 2002; Crozier et al., 2006). Gallotannins consists of a polyol surrounded by gallic acid units. These units can be joined together with other gallic acid moieties through depside bonds. In contrast, ellagitannins are more complex than gallotannins and are formed as a result of oxidative reactions between gallic acids units (Mueller-Harvey, 2001). Condensed tannins (proanthocyanidin) are polyhydroxy flavanol oligomers and polymers linked by C-C bonds between flavanols subunits. An example of a proanthocyanidin is sorghum procyanidin where n = 15 (Figure 1.10). Proanthocyanidins yield anthocyanidin pigments upon oxidative cleavage in hot alcohol (Hagerman, 2002).

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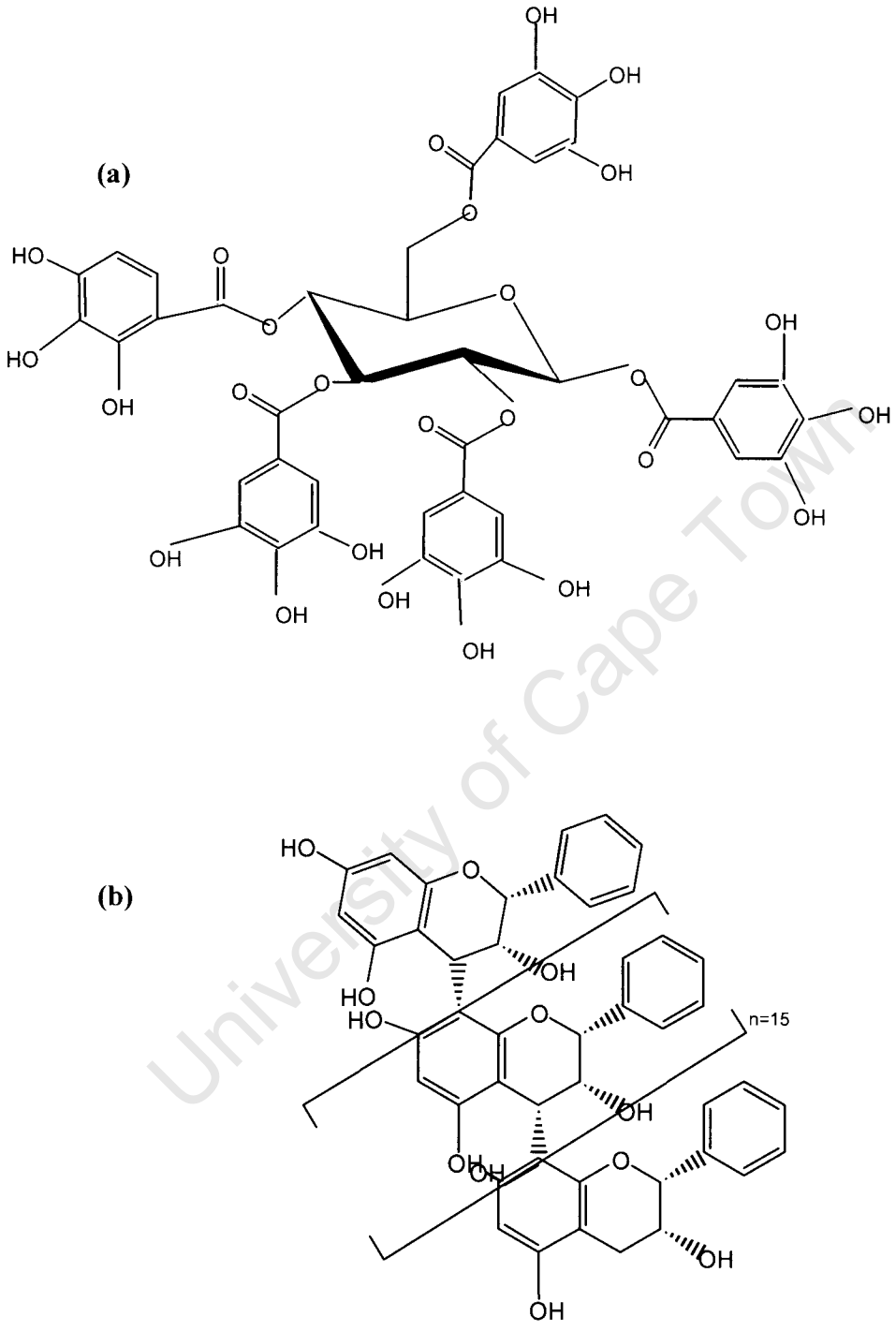

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Figure 1.10: Examples of structure for (a) hydrolysable and (b) condensed tannins

BIOLOGICAL ROLES OF POLYPHENOLS

1.6.1. Polyphenols as anti-nutrients

The selection of food by animals mostly depends on its nutrients (fibre, proteins, polysaccharides, and soluble sugars) composition including polyphenol content, possibly to minimize any unpalatable astringent properties of tannins. Tannins can also interfere with animal metabolism and normal growth (Bennick, 2002). It has been previously reported that the presence of tannins in the feed of animals leads to decreased growth, body weight gain and impaired calcium absorption, which could potentially affect bone metabolism. Tannins also interfere with iron absorption and this could be due to the presence galloyl groups as previously reported (Bennick, 2002).

Polyphenols have been traditionally considered anti-nutrients since they have the ability to reduce the digestibility of proteins and thereby increasing fecal nitrogen excretion. Polyphenols also allosterically inhibit digestive enzymes such as hydrolases, isomerases, oxygenases, oxidoreductases, polymerases, phosphatases, proteins phosphokinases and amino acid oxidases. Other polyphenols are also able to form complexes with metal cations, and therefore interfere with the intestinal absorption of minerals such as iron and copper (Ferguson, 2001).

The ability of tannins to precipitate proteins varies considerably. It is known that proteins that lack secondary or tertiary structure and have high proline content are readily precipitated by tannins (Bennick, 2002). The interaction of polyphenols and proteins can occur by covalent, ionic, hydrophobic interactions, and hydrogen bonding. Polyphenols

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are prone to oxidation and this gives rise to the highly reactive ortho-quinones which could potentially cause protein-polyphenol cross-links. Proline has been showed to bind to polyphenols by hydrogen bonding since the carbonyl oxygen adjacent to the secondary amine is a strong hydrogen bond acceptor. The formation of the tannin-protein complex is pH-dependent, with the greatest precipitation occurring near the iso-electric point of the protein. This has been ascribed to the decreased electrostatic repulsion of protein molecules at this pH (Bennick, 2002).

Studies on the interaction of bovine serum albumin (BSA) with polyphenols have showed that polyphenols are multivalent ligands that can form cross-links between two or more protein molecules. Thus, the stoichiometry and size of the polyphenol-protein complex depends on the concentration of the reactants and the protein:polyphenol ratio (Bennick, 2002).

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1.6.2. Mechanism of action of anti-oxidants

Anti-oxidants are a group of molecules that are able to significantly inhibit or delay oxidative processes, while being oxidized themselves. Anti-oxidants are used industrially to prevent polymers from oxidative degradation and as additives to cosmetics, food, beverages and baking products. Polyphenols are low to medium sized molecular weight antioxidants and are generally water-soluble (Vaya and Aviram, 2007).

The antioxidant activity of polyphenols and their derivatives depends on the number and positions of the hydroxyl groups bound to the aromatic ring. Three principle mechanisms by which polyphenols can act as antioxidants have been proposed. These include (1)

suppressing reactive oxygen species formation by inhibiting enzymes or chelating trace metal elements involved in radical formation, (2) scavenging reactive oxygen species and (3) singlet oxygen quenching (Andjelkovic et al., 2006).

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1.7. OBJECTIVES OF THIS STUDY

Polyphenols clearly have significant biological properties. They are widely used industrially as food supplements and in cosmetics to prevent oxidation. Due to the cost and the side effects of synthetic antioxidants, interests in natural antioxidants have increased considerably in recent years (Akagawa et al., 2003). There is also growing evidence that suggest that moderate polyphenols consumption may provide protection against microbial infections, oxidation, and also promote the life span of certain organisms. However, high levels of polyphenols may produce adverse effects. These include decreased nutrient utilization, and the inhibition of intestinal bacteria and fermentation in the animal gut (Becker and Makkar, 1999).

The objectives of this study can be divided into 2 parts. Firstly, to investigate the effects of *M. flabellifolia* polyphenols on growth of *S. cerevisiae* yeast strains. A simple and effective method to determine growth of yeast was developed. Secondly, to investigate the anti-oxidant properties of *M. flabellifolia* polyphenols using *S. cerevisiae* strains. The viability of cells was analysed using flow cytometry and propidium iodide staining.

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CHAPTER 2

THE EFFECT OF *M. flabellifolia* POLYPHENOLS ON GROWTH OF THE WILD TYPE AND THE Δ*HSP12* STRAINS

2.1. INTRODUCTION

The toxicity of polyphenols from different sources towards a variety of micro-orgasms is well documented, and this affects food science, wood science, soil science, plant pathology, pharmacology, and human and animal nutrition (Scalbert, 1991). In particular, tannins have been shown to inhibit growth of intestinal bacteria such as *Bacteroides fragilis*, *Clostridium perfringens* and *Escherichia coli* (Chung et al., 1998). The mechanisms by which tannins inhibit growth of micro-organisms have not yet been fully elucidated although some ideas have been proposed (Scalbert, 1991). These ideas include iron depletion, since complex formation between metal ions and tannins affects microbial growth by inhibiting the activities of metallo-enzymes (Bossi et al., 2007). Secondly, tannins may inhibit growth of micro-organisms by forming complexes with cell wall proteins and also by forming complexes with proteins in growth media (Bossi et al., 2007).

The toxicity of polyphenols is usually estimated by using one or more of the following methods: plate counts, disk inhibition, and nephelometry or respirometry for bacteria and yeasts (Scalbert, 1991). Plate counts involve plating serial dilutions of a culture on solid medium and determining the time required for visible colonies to form or the linear radial growth rate of single colonies (Salvesen and Vadstein, 2000). These methods require

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large amount of reagents, are time consuming and are prone to errors. Another common method used to analyse the viability status of cells involves the use of vital stains. Viable cells do not stain with these vital stains whereas non-viable cells are stained. Several hundred cells are then counted under a microscope and the ratio of stained cells to the total number counted is determined. These methods have limitations, for instance stains such a trypan blue and methylene blue tend to overestimate cell viability, whereas 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and iodonitrotetrazolium chloride (INT) are affected by the metabolism of micro-organisms and are also reduced by polyphenols. This makes it difficult to compare results from different laboratories and also even more complex to determine the toxicity of the compound to the micro-organisms. Since *S. cerevisiae* is widely used in the fermentation industry and in stress research, its sensitivity towards polyphenols is of economic interest.

The aim of this study was to investigate the inhibitory effect of polyphenols from *M. flabellifolia* and its hydrolytic product, gallic acid on the growth of *S. cerevisiae* yeast strains and to identify possible mechanisms of inhibition.

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2.2. MATERIALS AND METHODS

2.2.1. EXTRACTION OF POLYPHENOLS

Dry leaves of M. *flabellifolia* plants were collected near Outjo, Namibia and maintained at room temperature in the laboratory until used. These leaves were ground to a fine powder using a pestle and mortar in the presence of liquid nitrogen, and the powder was sieved through a 2 mm mesh sieve. The sieved powder (2.5 g) was extracted twice with 25 ml of 50 % (v/v) hexane-chloroform to remove the lipids and pigments such as chlorophyll. Extraction was achieved by sonicating the mixture in an ultrasonic water bath for 30 min at room temperature, followed by centrifugation at 10000 g for 10 min at 4 °C. The pellets (powder) from both extracts were pooled and air dried to remove any residual hexane-chloroform. Polyphenols from this powder were extracted twice with 70 % acetone. The supernatants were pooled and rotary evaporated at 40 °C for 3 h to remove the acetone. The remaining aqueous solution was lypholized and stored at -20 °C.

2.2.2. QUANTIFICATION OF POLYPHENOLS

Total polyphenols in the *M. flabellifolia* 70% acetone extract were determined spectrophotometrically using the Folin Ciocalteau method (Slinkard and Singleton, 1977), using gallic acid as the standard (Figure 2.1). Ten µl of the extract at a concentration of 1 mg/ml in H_2O were mixed with 0.49 ml of H_2O, 0.25 ml of 1 N Folin reagent and then the mixture was vortexed vigorously. After vortexing, 1.25 ml of sodium carbonate (20 % (w/v)) was added to the mixture, after which the mixture was again vortexed vigorously and allowed to stand for 2 h at room temperature. The absorbance

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was measured at 765 nm against the blank. The results were expressed as milligrams gallic acid equivalent per milligram dry weight (mg GAE/mg DW) (Makkar, 2003). The extract was found to contain 0.392 ± 0.03 mg GAE/mg DW total phenolics.

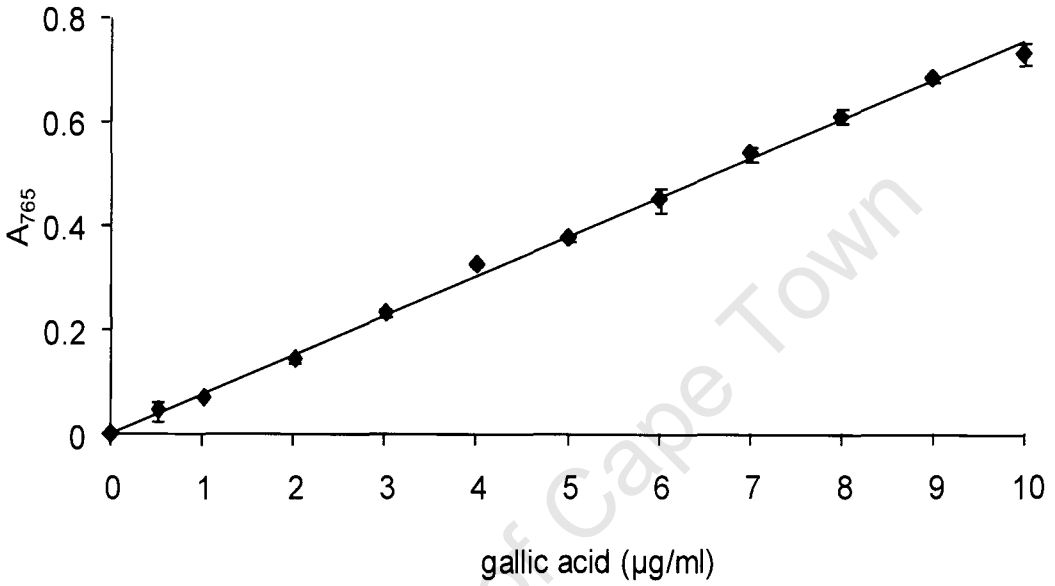

Figure 2.1: Gallic acid determination using the Folin Ciocalteau method (Slinkard and Singleton, 1977). The result shows the mean ± SD of triplicate samples. Error bars not visible are within data points.

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2.2.3. ORGANISMS AND CULTURE CONDITIONS

The *S. cerevisiae* yeast strains used were of the W303 background (*a/α, ade2-1/ade2-1, trp1-1/trp1-1, leu2-3/leu2-112, his3-11/his3-15, ura3/ura3, canr1-100/CAN*) and were used in the haploid form. These strains were kind gifts from Dr P. Meacock, University of Leicester, Leicester, U.K. The Δ*hsp12* strain was constructed and tested as previously described (Praekelt and Meacock, 1990). Yeast cells were routinely grown in YEPD medium (1 % (w/v) yeast extract, 2 % (w/v) peptone and 2 % (w/v) glucose) or grown in synthetic YNB medium (0.67 % (w/v) yeast nitrogen base medium without amino acids,

2 % (w/v) glucose and 0.077 % (w/v) yeast synthetic drop-out medium without tryptophan. Tryptophan was added as a supplement as described by the manufacturer). For solid medium, 2 % (w/v) agar was added. Yeast growth was routinely monitored by measuring the turbidity at 600 nm (A_{600}). Cells were counted using a hemocytometer slide. 10^7 cells/ml was found to be equivalent to A_{600} = 0.072. The yeast cells were maintained in 25 % glycerol stock solution and stored in the freezer at -20 °C.

2.2.4. GROWTH OF YEAST ON YEPD AGAR MEDIUM

A 1 ml aliquot of an overnight culture of the wild type or the *Δhsp12* yeast cells were grown to mid-log phase in 50 ml fresh YEPD medium. The mid-log phase culture was diluted to A_{600} = 0.8 with dH_2O, after which it was serially 10-fold diluted. Ten µl of each dilution was spotted on YEPD agar plates containing 0, 16.3, or 650 µg/ml polyphenols. The plates were incubated for 48 h at 30 °C.

2.2.4. GROWTH OF YEAST IN LIQUID YEPD AND YNB MEDIA

1 ml of mid-log phase yeast cultures was diluted to an A_{600} = 0.4, and inoculated into YEPD or YNB media with and without polyphenols in the concentration range 0 to 650 µg/ml. Two hundred µl of the culture in either YEPD or YNB medium were added to each well of a 96-well plate. The plates were incubated at 30 °C inside a plate reader (Bandelin electronic D-1000, Germany) for up to 36 h. The A_{600} was automatically recorded for each well every 1 or 2 h. The changes in the A_{600} as a function of time were used to generate growth curves, which were analyzed using mathematical growth models (Section 2.2.5). To investigate the effect of a combination of polyphenols and metals in

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media, $FeCl_3$ up to 60 µM or $ZnSO_4$ up to 40 µM was added to YEPD medium, followed by addition of the polyphenols. The effect of gallic acid on yeast growth was investigated by adding up to 680 µg/ml gallic acid in YEPD medium.

2.2.5. MODELLING OF GROWTH CURVES OF THE WILD TYPE AND THE Δ*HSP12* YEAST STRAINS

Growth curves of the wild type and the Δ*hsp12* yeast strains were fitted to modified Logistic and Gompertz models respectively (Zwietering et al., 1990) using LAB FIT software (Silva et al., 2007). The forms of the Logistic model (1) and the Gompertz model (2) used are shown below:

$$Y = \frac{A}{\left\{1 + \exp\left[\frac{4\mu}{A}(\lambda - t) + 2\right]\right\}} \quad (1)$$

$$Y = Aexp\left\{-\exp\left[\frac{\mu.e}{A}(\lambda - t) + 1\right]\right\} \quad (2)$$

In both cases Y is the relative population density at time t, A is the maximal asymptote value reached (the maximum biomass of the population), µ is the maximum specific growth rate given by the slope of the linear part of the curve, and λ is the lag phase of the curve (the x-axis intercept tangent). A growth curve of the wild type strain in YEPD medium analysed using the modified Logistic model is shown in Figure 2.2. The growth parameters generated showed a lag phase of 9.2 ± 0.04 h, a specific growth rate of 0.19 ± 0.05 h^{-1}, and a maximum biomass with A_{600} = 1.12 ± 0.06.

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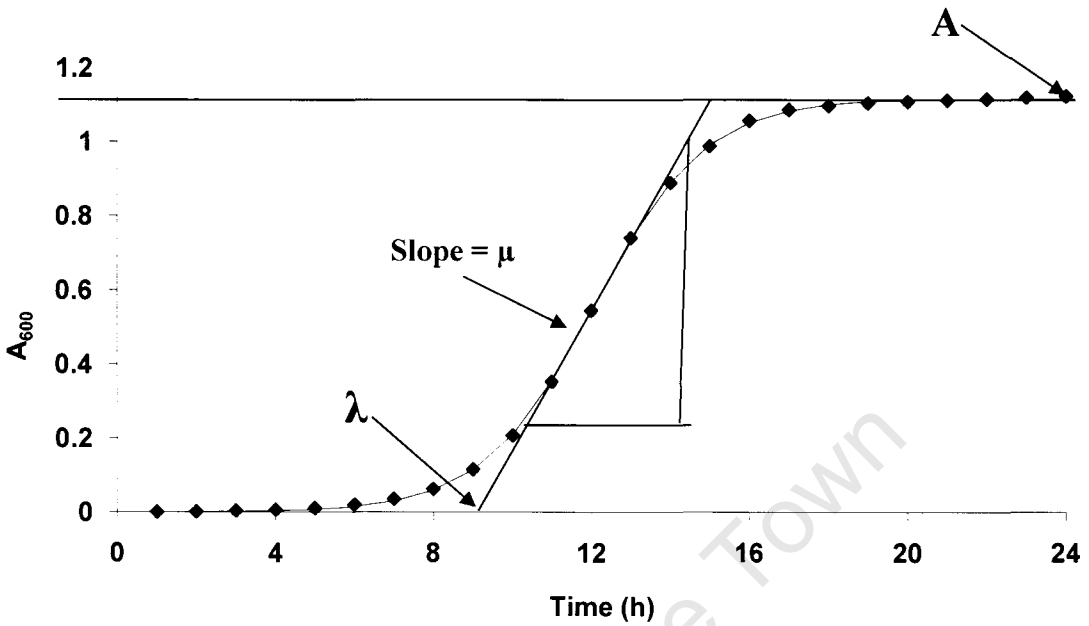

Figure 2.2: Growth curve of the wild type strain in YEPD medium monitored at 30 °C for 24 h using an automatic plate reader. The data generated was fitted to the Logistic model using LAB FIT software. "A" shows the upper asymptote value (maximum biomass), λ represents the lag phase and μ is the maximum specific growth rate. Legend: actual data: ♦, fitted data: —. The result shows the mean of triplicate growth curves.

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2.2.6. VIABILITY OF YEAST

A 1 ml aliquot of a glycerol stock culture was grown to mid-log phase in 50 ml YEPD medium at 30 °C. The mid-log phase culture was re-suspended in PBS buffer pH 7.4 containing up to 650 μg/ml polyphenol to approximately 0.5 x 10^7 cells/ml, after which it was incubated for 24 h at 30 °C. The cells were washed with PBS buffer to remove any unbound polyphenols and the suspension was then spotted onto YEPD agar plates to determine the viability of yeast cell. The plates were incubated at 30 °C for 48 h (Sections 2.3.1 and 2.3.5).

2.3. RESULTS

2.3.1. EFFECT OF POLYPHENOLS ON GROWTH OF YEASTS IN YEPD MEDIUM

2.3.1.1. Growth of the wild type and the Δ*hsp12* yeast cells on solid YEPD medium

To determine the sensitivity of yeast cells to *M. flabellifolia* polyphenols, serial dilutions of the wild type and the Δ*hsp12* cells were spotted onto solid YEPD agar plates supplemented with increasing concentration of polyphenols. The inoculated plates were incubated for 48 h at 30°C. The results (Figure 2.3) showed that polyphenols inhibited growth of both strains. The wild type strain grew marginally better than the Δ*hsp12* strain in the absence of polyphenols. Although the wild type strain showed sensitivity to low concentrations of polyphenols, growth of the Δ*hsp12* strain was promoted by low concentrations (16.3 µg/ml) of polyphenols. A higher concentration of polyphenols (650 µg/ml) inhibited the growth of both strains although the Δ*hsp12* strain was more tolerant of polyphenols. Prolonged incubation (72 h) resulted in uniform growth in all concentrations of polyphenols (results not shown), indicating that polyphenols only delayed growth of the strains.

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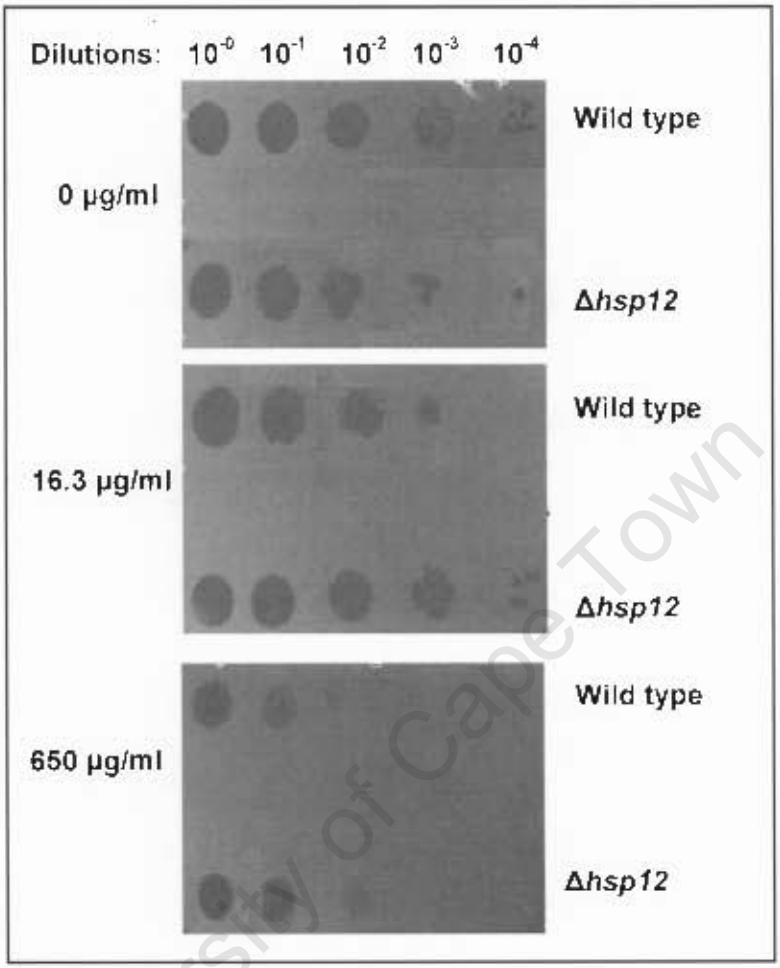

Figure 2.3: Effect of polyphenols on growth of the wild type and the Δ*hsp12* strains on YEPD agar plates. Cells were serially diluted 10-fold from a mid-log phase culture and 10 µl of the diluted cultures were spotted onto plates containing 0, 16.3 or 650 µg/ml polyphenols before the plates were incubated at 30 °C for 48 h.

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2.3.1.2. Growth of the wild type and the Δ*hsp12* strains in liquid YEPD medium.

To test for sensitivity of polyphenols in liquid medium, both the wild type and the Δ*hsp12* strains were grown in YEPD medium containing various concentrations of polyphenols. Growth was monitored at 30 °C for 24 h using an automatic plate reader. The results (Figure 2.4A and 2.5A) showed that polyphenols inhibited growth of both strains in a concentration dependent manner. These results also showed that the Δ*hsp12* cells tolerated polyphenols better than the wild type cells. The growth parameters generated from mathematically modelling growth showed that the lag phase of both strains was prolonged as the concentration of the *M. flabellifolia* polyphenols in the medium increased. The growth of the wild type strain in YEPD medium containing 650 µg/ml polyphenols had a lag phase of 16.2 ± 0.4 h, compared to a lag phase of 9.2 ± 0.03 h when polyphenols were absent in the same medium (Figure 2.4B). Similarly Δ*hsp12* cells had a lag phase of 7.3 ± 0.2 h in the absence of polyphenols but this increased to 13.3 ± 0.3 h in the presence of 650 µg/ml polyphenols (Figure 2.5B).

The specific growth rate of the Δ*hsp12* strain increased when 6.5 µg/ml polyphenols were present in the medium by an increase of 0.02 h^{-1}. However, the specific growth rate of both strains was also reduced in the presence of higher polyphenols concentrations in the medium. The specific growth rate of the wild type strain decreased from 0.19 ± 0.04 h^{-1} to 0.05 ± 0.02 h^{-1} in the presence of 650 µg/ml polyphenols. Similarly the specific growth rate of the Δ*hsp12* strain decreased from 0.12 ± 0.01 h^{-1} to 0.060 ± 0.01 h^{-1}. The biomass of both strains also decreased when polyphenols were present in the medium. The

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biomass of the wild type strain decreased by 58 % when 650 µg/ml polyphenols were present in the medium whereas the biomass of Δ*hsp12* strain decreased by 50 %. The biomass of the Δ*hsp12* strain was slightly increased when 6.5 µg/ml polyphenols were present in the medium.

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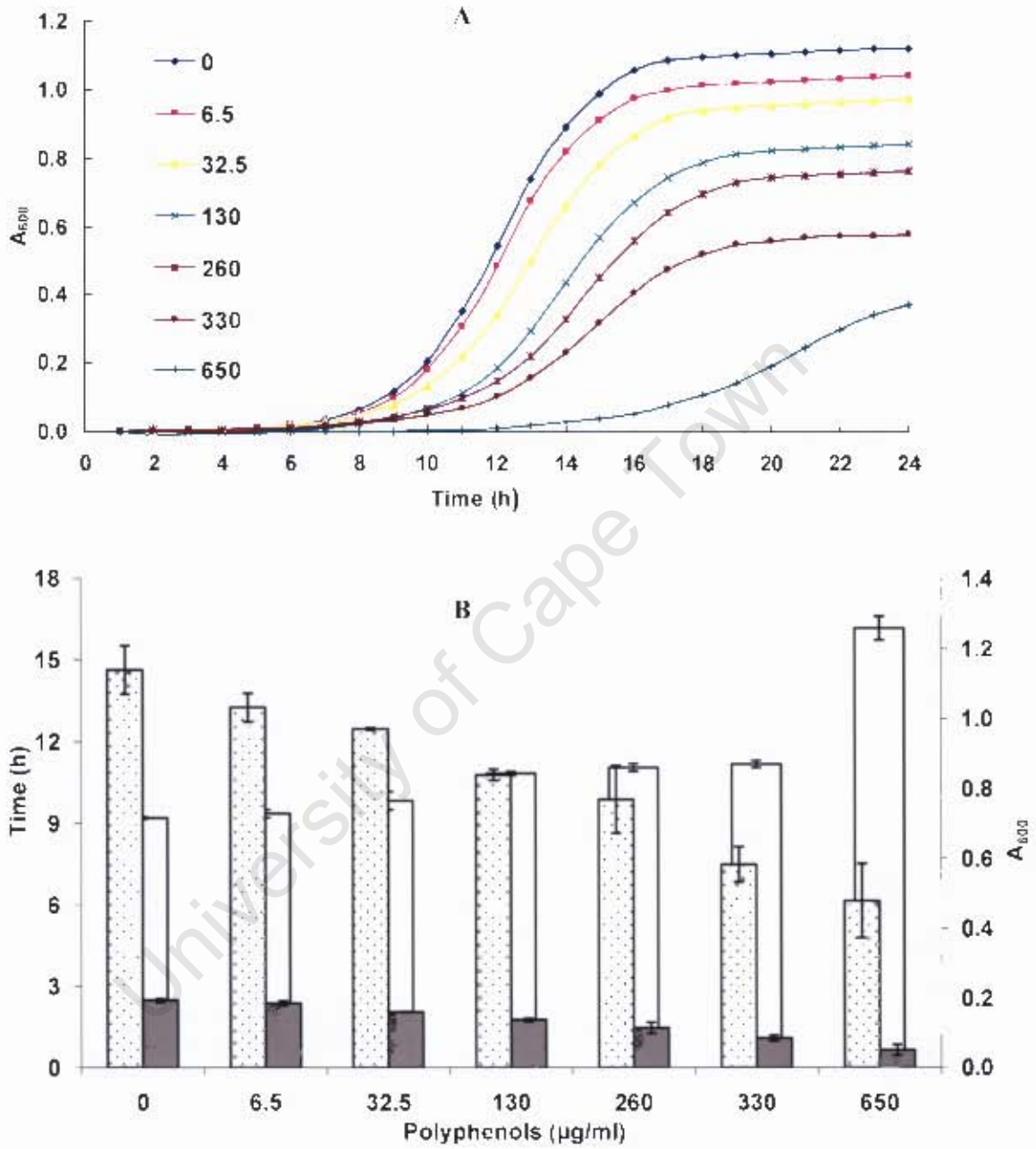

Figure 2.4: Growth curves of the wild type strain in YEPD medium supplemented with increasing concentration of *M. flabellifolia* polyphenols. Polyphenols were added to YEPD medium to final concentrations of 0, 6.5, 32.5, 130, 260, 330, or 650 µg/ml. Growth curves were generated using an automatic plate reader with the temperature set at 30 °C with data collected every 1 h for 24 h. **A:** Growth curves generated from the actual data **B:** Growth parameters generated after fitting the data in "A" above to the Logistic model. Legend: gray bars: specific growth rate; unfilled bars: lag phase; dotted bars: maximum biomass. The results shows the mean ± SD of triplicate samples.

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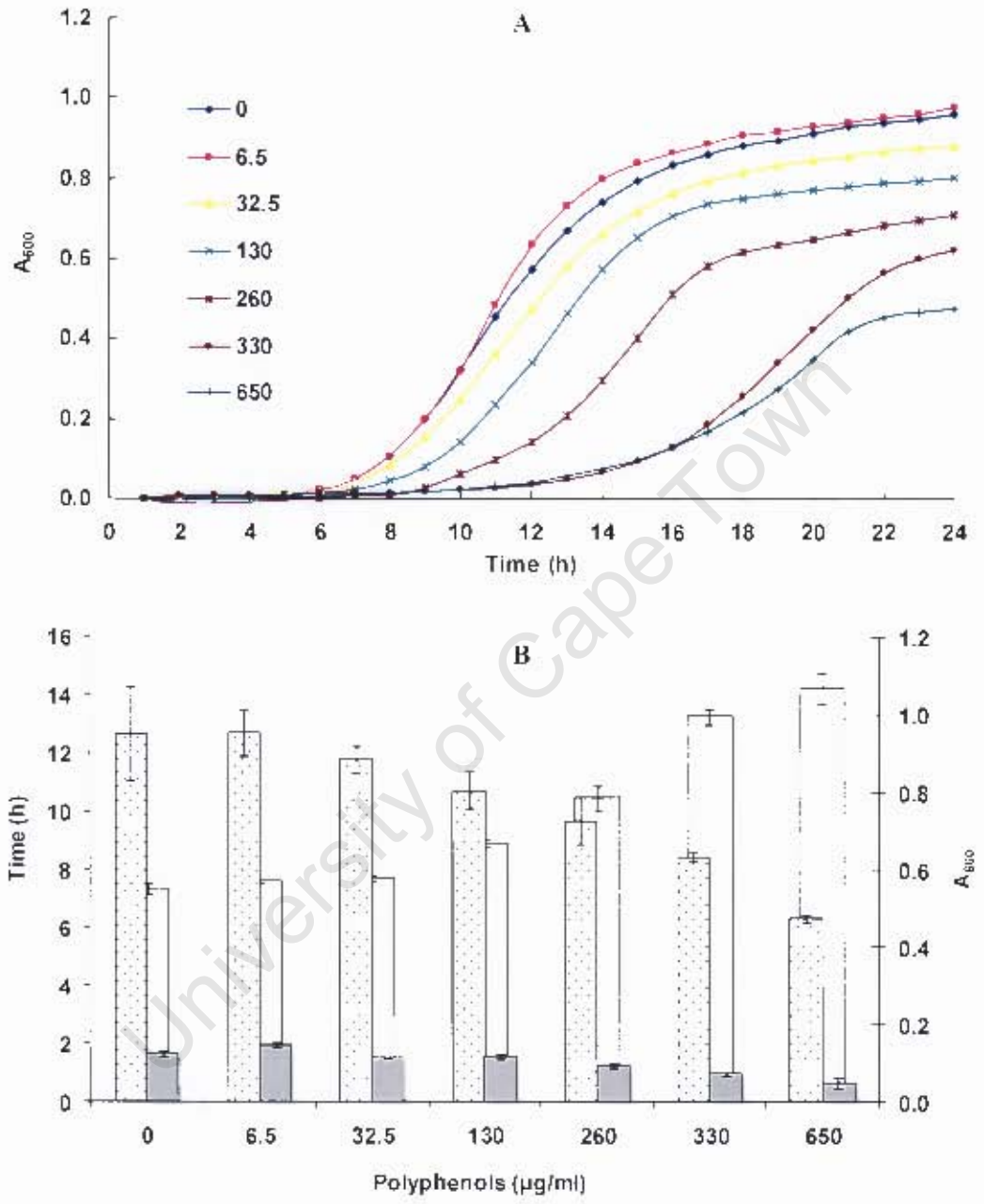

Figure 2.5: Growth curves of the *Δhsp12* strain in YEPD medium supplemented with increasing concentration of *M. flabellifolius* polyphenols. Polyphenols were added to YEPD medium to final concentrations of 0, 6.5, 32.5, 130, 260, 330, or 650 µg/ml. Growth curves were generated using an automatic plate reader with the temperature set at 30 °C with data collected every 1 h for 24 h. **A:** Growth curves generated from the actual **B:** Growth parameters generated after fitting the data in "A" above to the Gompertz model. Legend: gray bars: specific growth rate; unfilled bars: lag phase; dotted bars: maximum biomass. The results shows the mean ± SD of triplicate samples.

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2.3.2. EFFECT OF POLYPHENOLS ON GROWTH OF YEAST IN YNB MEDIUM

The inhibitory effects of *M. flabellifolia* polyphenols on yeast growth in YEPD medium were suspected to be due to protein precipitation, making these proteins unavailable as a source of amino acids. The experiment to determine the sensitivity of *M. flabellifolia* polyphenols on growth of the wild type and the Δ*hsp12* strains was therefore repeated with a nitrogen base medium (YNB) that contains ammonium sulphate and amino acids rather than peptides and proteins.

The results (Figures 2.6A and 2.7A) showed that polyphenols were less toxic to both yeast strains in this medium. The growth parameters generated showed a linear increase in the lag phase of both strains as the concentration of polyphenols increased (Figures 2.5B and 2.6 B). The growth of the wild type strain in YNB medium containing 650 ug/ml polyphenols had a lag phase of 10.8 ± 0.2 h, compared to a lag phase of 7.9 ± 0.3 h when polyphenols were absent. Similarly, the Δ*hsp12* strain had a lag phase of 8.9 ± 0.5 h in the presence of 650 ug/ml polyphenols, compared to a lag phase of 5.6 ± 0.3 h when polyphenols were absent. The lag phase of the wild type and the Δ*hsp12* strains were delayed by 42 % and 60 %, respectively when 650 ug/ml polyphenols were present in YNB medium compared to a delay of 76 % and 80 % for the wild type and the Δ*hsp12* strains, respectively, in YEPD medium containing the same polyphenol concentrations.

The specific growth rate of both strains remained relatively constant when up to 260 µg/ml polyphenols were present in the medium. However, this decreased in the presence

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of 650 µg/ml polyphenols by 16 % in both strains (Figures 2.6B and 2.7B), compared to a decrease of over 60 % when the same polyphenol concentration was present in YEPD medium. The maximum biomass production also remained constant at up to 260 µg/ml polyphenol concentrations but decreased above these concentrations. The maximum biomass decreased by less than 10 % for both strains in YNB medium in the presence of 650 µg/ml polyphenols, as compared to a decrease of 42 % and 50 % for wild type and Δ*hsp12* respectively in YEPD medium. These results suggested that the inhibitory effects of *M. flabellifolia* polyphenols were largely due to protein precipitation, however, other factors might also contribute resulting in the 10 % decreased biomass obtained.

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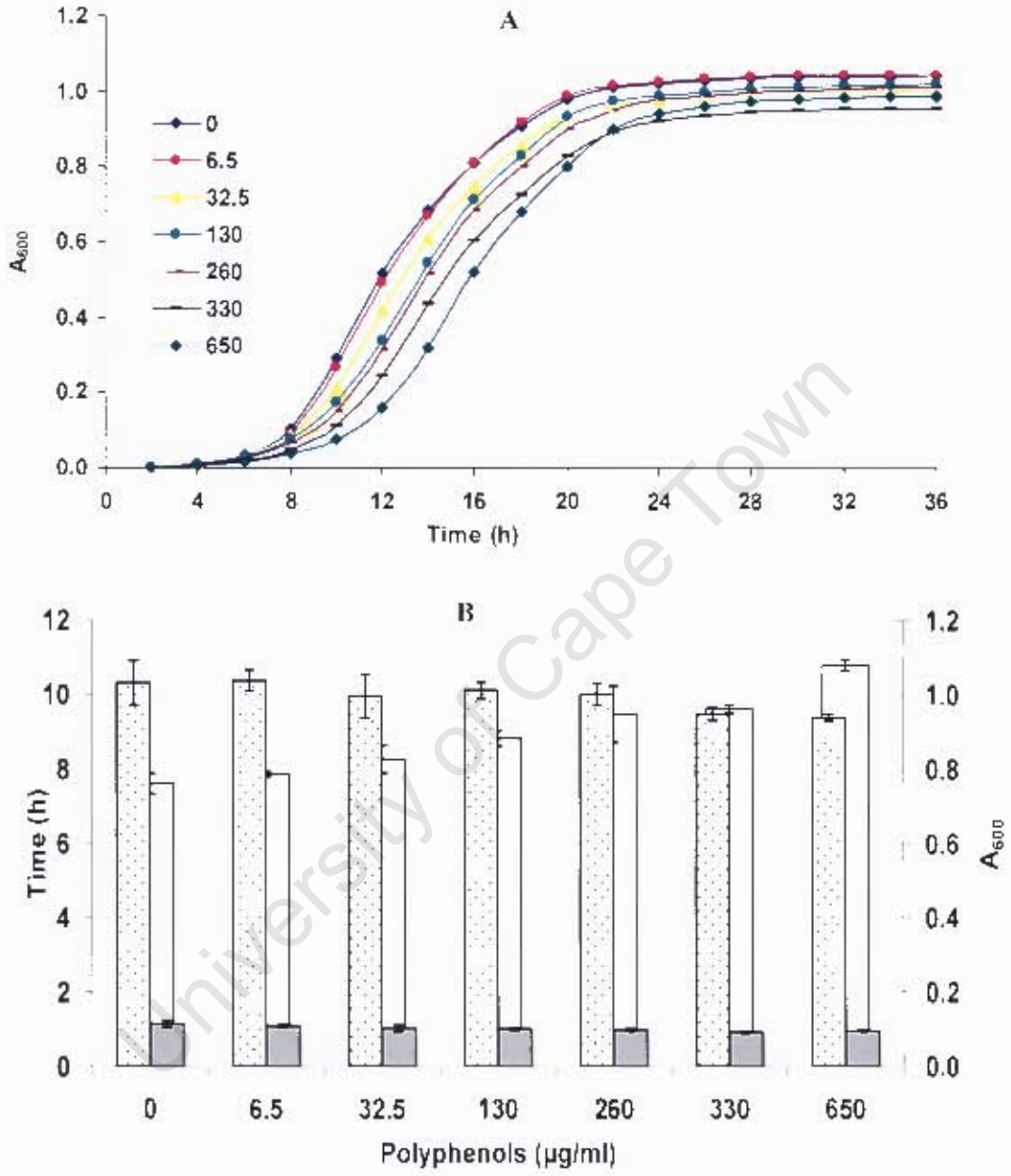

Figure 2.6: Growth curves of the wild type strain in YNB medium supplemented with increasing concentration of *M. flabellifolia* polyphenols. Polyphenols were added to YNB medium to final concentrations of 0, 6.5, 32.5, 130, 260, 330, or 650 µg/ml. Growth curves were generated using an automatic plate reader with the temperature set at 30 °C with data collected every 1 h for 24 h. **A:** Growth curves generated from the actual data **B:** Growth parameters generated after fitting the data in "A" above to the Logistic model. Legend: gray bars: specific growth rate, unfilled bars: lag phase and dotted bars: maximum biomass. The results shows the mean ± SD of triplicate samples.

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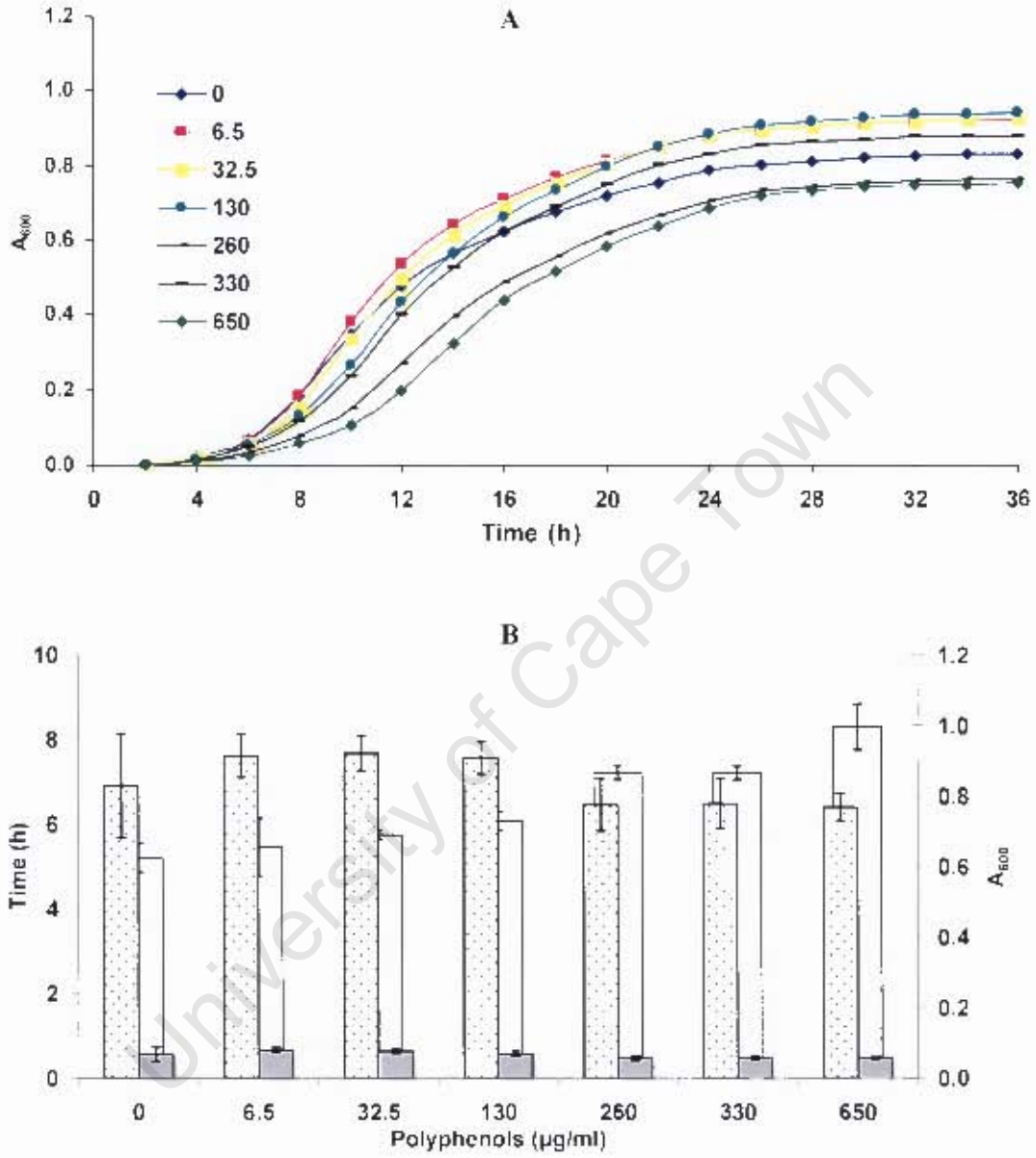

Figure 2.7: Growth curves of the Δ*hsp12* strain in YNB medium supplemented with increasing concentration of *M. flabellifolia* polyphenols. Polyphenols were added to YNB medium to final concentrations of 0, 6.5, 32.5, 130, 260, 330, or 650 µg/ml. Growth curves were generated using an automatic plate reader with the temperature set at 30 °C with data collected every 1 h for 24 h. **A:** Growth curves generated from the actual data **B:** Growth parameters generated after fitting the data "A" above to the Gompertz model. Legend: gray bars: the specific growth rate; unfilled bars: lag phase; dotted bars: maximum biomass. The results shows the mean ± SD of triplicate samples.

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2.3.3. GROWTH OF THE WILD TYPE AND THE Δ*HSP12* STRAINS IN YEPD MEDIUM SUPPLEMENTED WITH A COMBINATION OF METAL IONS AND *M. flabellifolia* POLYPHENOLS

Polyphenols are also known to form complexes with metal cations, and therefore may interfere with the absorption of minerals such as iron, zinc and copper (Ferguson, 2001). Wauters et al., (2001) showed that the sensitivity of *S. cerevisiae* to tannic acid in YEPD medium was solely due to iron deprivation and not due to deprivation of either protein or other metal ions. To investigate whether a similar effect was brought about by *M. flabellifolia* polyphenols, growth of the wild type and the Δ*hsp12* yeast strains was therefore repeated in YEPD medium supplemented with a combination of metals ions and polyphenols. 60 μM $FeCl_3$ or 40 μM $ZnSO_4$ were added to YEPD medium together with up to 650 μg/ml polyphenols, and the growth experiments repeated. The concentrations of the metal ions used were similar to those used by Wauters et al., (2001).

The results showed that supplementing metal ions in YEPD medium in the absence of polyphenols did not affect the growth of both strains at all metal ions concentrations used (results not shown). The growth parameters generated showed that the specific growth rate of both the wild type and the Δ*hsp12* strains remained constant at even the highest metal ion concentration used (Figures 2.8A and 2.9A). However a combination of metal ions and polyphenols in YEPD medium resulted in a slight improvement in the specific growth rate of the wild type strain (Figure 2.8A), which increased by 11.4 % when 130 μg/ml polyphenols were present in the medium supplemented with 60 μM $FeCl_3$. However, the specific growth rate of the wild type strain was only increased by less than

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5 % when 40 μM $ZnSO_4$ was present in the medium containing 130 μg/ml polyphenols. In contrast, the specific growth rates of the Δ*hsp12* strain decreased by 22.5 % when 130 μg/ml polyphenols was present in YEPD medium supplemented with 60 μM $FeCl_3$ and by 16 % when 130 μg/ml polyphenols was present in YEPD medium supplemented with 20 μM $ZnSO_4$.

The lag phase of both wild type and Δ*hsp12* strains remained constant when up to 650 μg/ml polyphenols was added in YEPD medium containing both the $FeCl_3$ and $ZnSO_4$ metal ions (Figures 2.8B and 2.9B). The maximum biomass for both strains also remained constant when the same polyphenol concentrations were added in YEPD medium containing both $FeCl_3$ and $ZnSO_4$ metal ions (Figures 2.8C and 2.9C).

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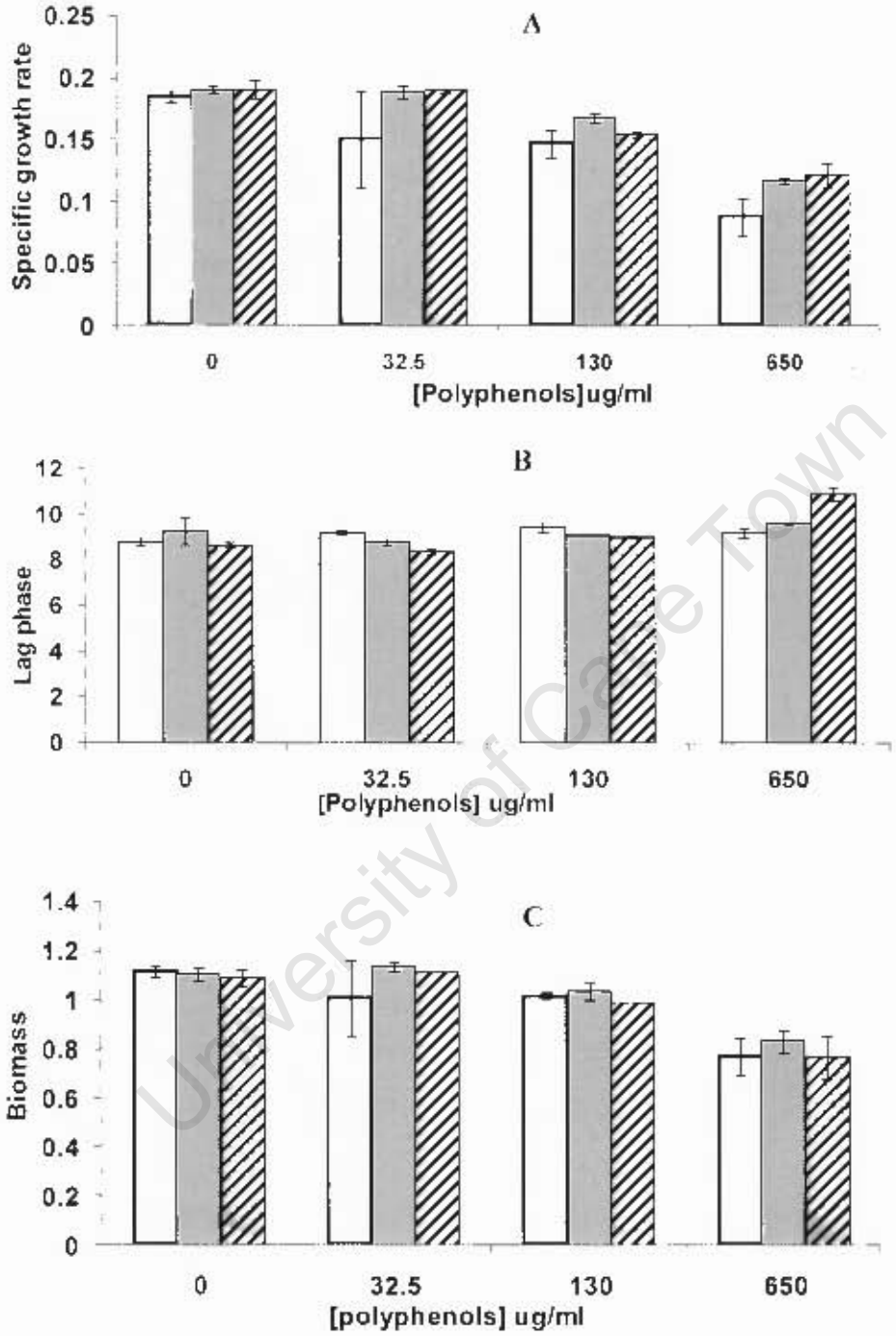

Figure 2.8: Growth parameters of the wild type strain in YEPD medium containing a combination of metal ions and *M. flabellifolia* polyphenols. $FeCl_3$ and $ZnSO_4$ were added to final concentrations of up to 60 µM in YEPD medium, after which M. *flabellifolia* polyphenols were added to final concentrations up to 650 µg/ml. The growth parameters were generated using a Logistic model fitted with LABFIT software. Legend: white bars: polyphenols alone, gray bars: 60 µM $FeCl_3$ plus polyphenols, bars with diagonal lines: 40 µM $ZnSO_4$ plus polyphenols. A: specific growth rate, B: lag phase, and C: maximum biomass. The results shows the mean ± SD of triplicate samples.

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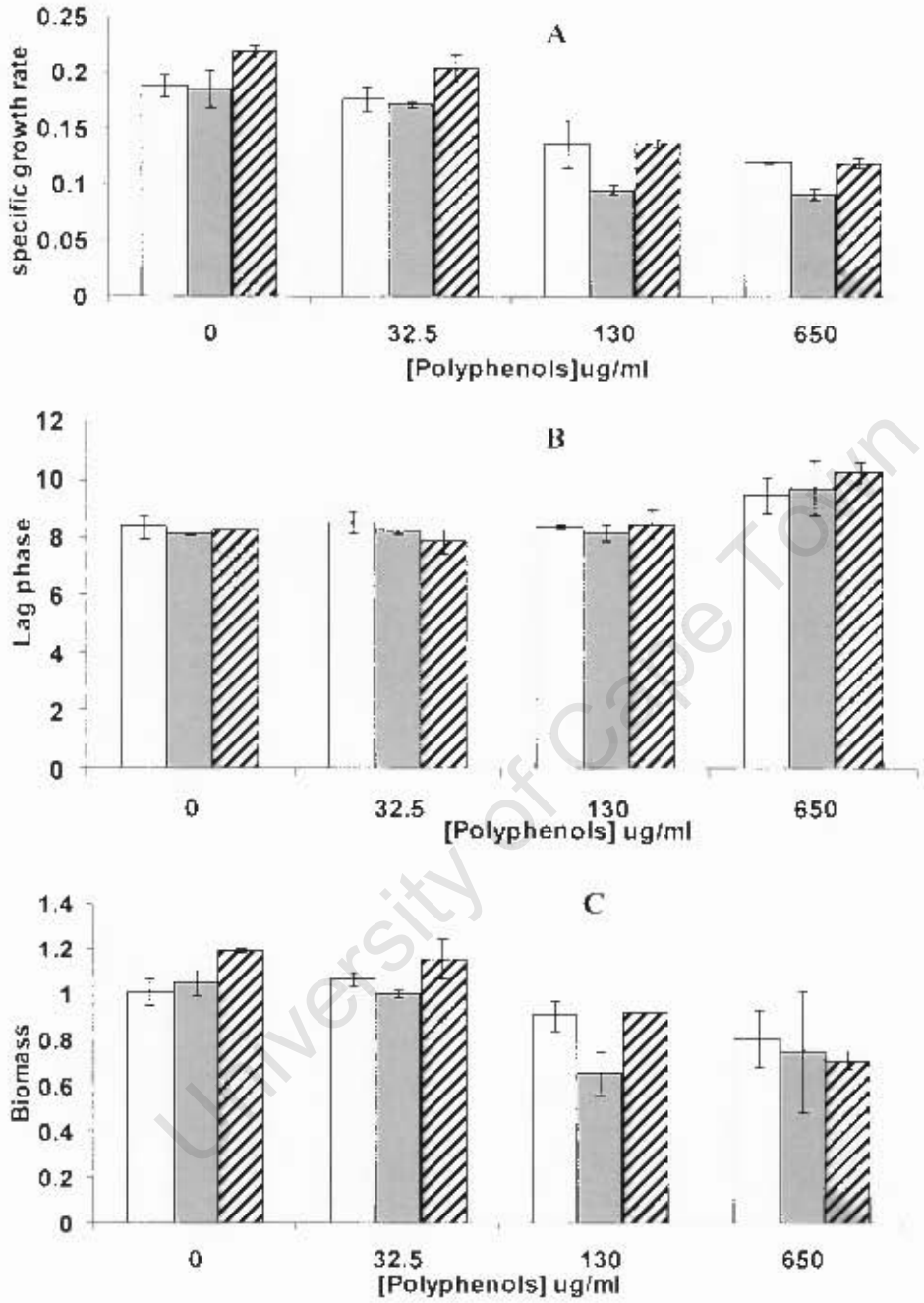

Figure 2.9: Growth parameters of the *Δhsp12* strain in YEPD medium containing a combination of metal ions and *M. flabellifolia* polyphenols. $FeCl_3$ and $ZnSO_4$ were added to final concentrations of up to 60 µM in YEPD medium, after which M. *flabellifolia* polyphenols were added to the final concentrations up to 650µg/ml. The growth parameters were generated using a Gompertz model fitted with LABFIT software Legend: white bars: polyphenols alone, gray bars: 60 µM $FeCl_3$ plus polyphenols, bars with diagonal lines: 40 µM $ZnSO_4$ plus polyphenols. A: specific growth rate, B: lag phase, and C: maximum biomass. The results shows the mean + SD of triplicate samples.

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2.3.4. GROWTH OF THE WILD TYPE AND THE Δ*HSP12* STRAINS IN YEPD MEDIUM SUPPLEMENTED WITH GALLIC ACID

The results thus far suggested that the inhibition of yeast growth by *M. flabellifolia* polyphenols was mostly due to protein precipitation and slightly metal ion binding in the growth medium. If this were the case, then the use of gallic acid rather than *M. flabellifolia* polyphenols might not result in reduced yeast growth. High molecular weight tannins such as 3,4,5 tri-*O*-galloylquinic acid differ from simple phenols in that simple phenolics do not cross link or precipitate proteins or metal ions. To ascertain if the ability of *M. flabellifolia* polyphenols to inhibit growth of yeast was due to protein precipitation, yeast cells were grown in YEPD medium containing up to 680 ug/ml gallic acid. The highest concentration of gallic acid used (680 µg/ml) was 4 x higher in molar terms than the concentration of polyphenols used. The results (Figures 2.10A and 2.11A) showed that the presence of up to 680 µg/ml gallic acid in YEPD medium had no effect on growth of both strains with constant growth parameters observed at all concentrations of gallic acid used (Figures 2.10B and 2.11B).

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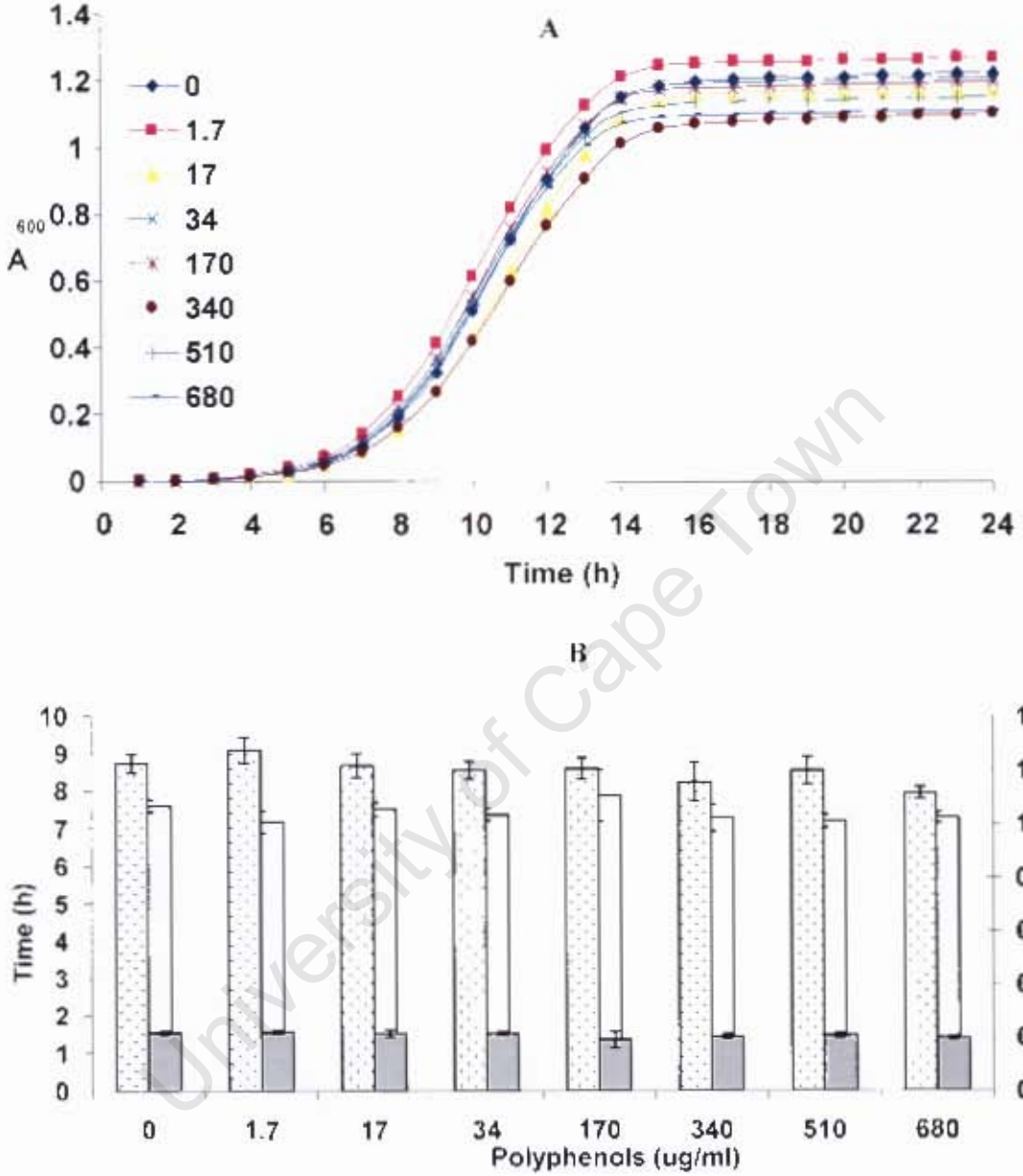

Figure 2.10: Growth of the wild type strain in YEPD medium supplemented with increasing concentrations of gallic acid. Gallic acid was added to final concentrations of 0, 1.7, 17, 34, 170, 340, 510, and 680 μg/ml. Growth curves were generated using an automatic plate reader with the temperature set at 30 °C with data collected every 1 h for 24 h. **A:** Growth curves generated from the actual data **B:** Growth parameters generated after fitting the data in "A" above to the Logistic model. Legend: gray bars: the specific growth rate, unfilled bars: the lag phase and dotted bars: maximum biomass. The results shows the mean + SD of triplicate samples.

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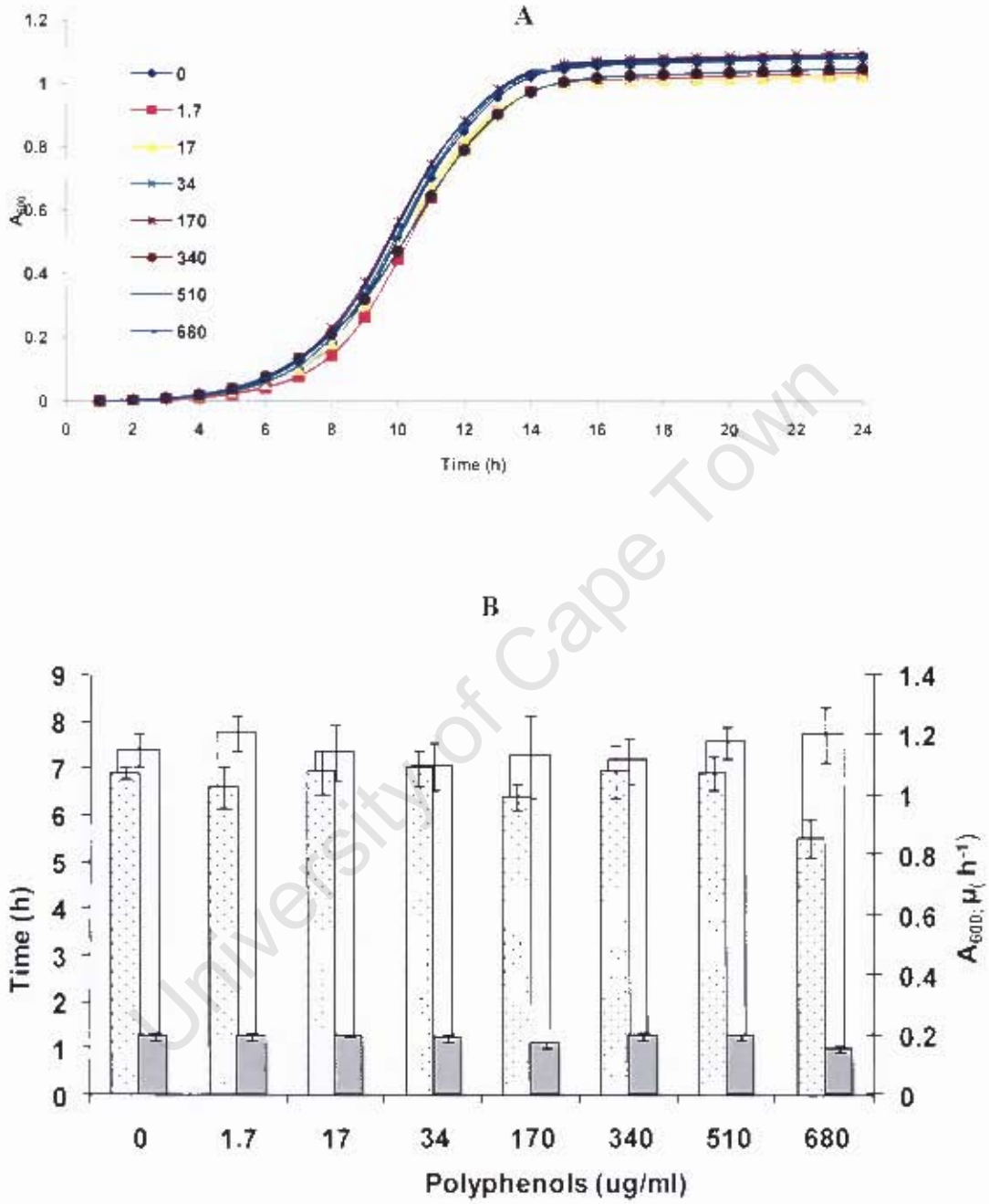

Figure 2.11: Growth of the *Δhsp12* strain in YEPD medium supplemented with increasing concentrations of gallic acid. Gallic acid was added to final concentrations of 0, 1.7, 17, 34, 170, 340, 510, and 680 µg/ml. Growth curves were generated using an automatic plate reader with the temperature set at 30 °C with data collected every 1 h for 24 h. **A:** Growth curves generated from the actual data **B:** Growth parameters generated after fitting the data in "A" above to the Gompertz model. Legend: gray bars: the specific growth rate, unfilled bars: the lag phase and dotted bars: maximum biomass. The result shows the mean ± SD of triplicate samples.

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2.3.5. VIABILITY OF YEAST STRAINS AFTER TREATMENT WITH POLYPHENOLS

Since polyphenols are known to precipitate proteins, it is possible that inhibition of yeast growth was due to polyphenols binding to cell wall proteins and thus affecting the budding process. To investigate if the inhibitory effects of *M. flabellifolia* polyphenol extracts on growth of yeast was due to interaction with the cell wall proteins, both strains were pre-treated with increasing concentrations of polyphenols for 24 h in PBS buffer, after which the cells were washed to remove unbound polyphenols and subsequently spotted onto YEPD agar plates. The results (Figure 2.12) showed that even after prolonged treatment with polyphenols, both strains were able to resume growth at approximately the same rate as untreated cells. This result clearly showed that *M. flabellifolia* polyphenols did not affect cells directly but did so by binding to some components in the medium.

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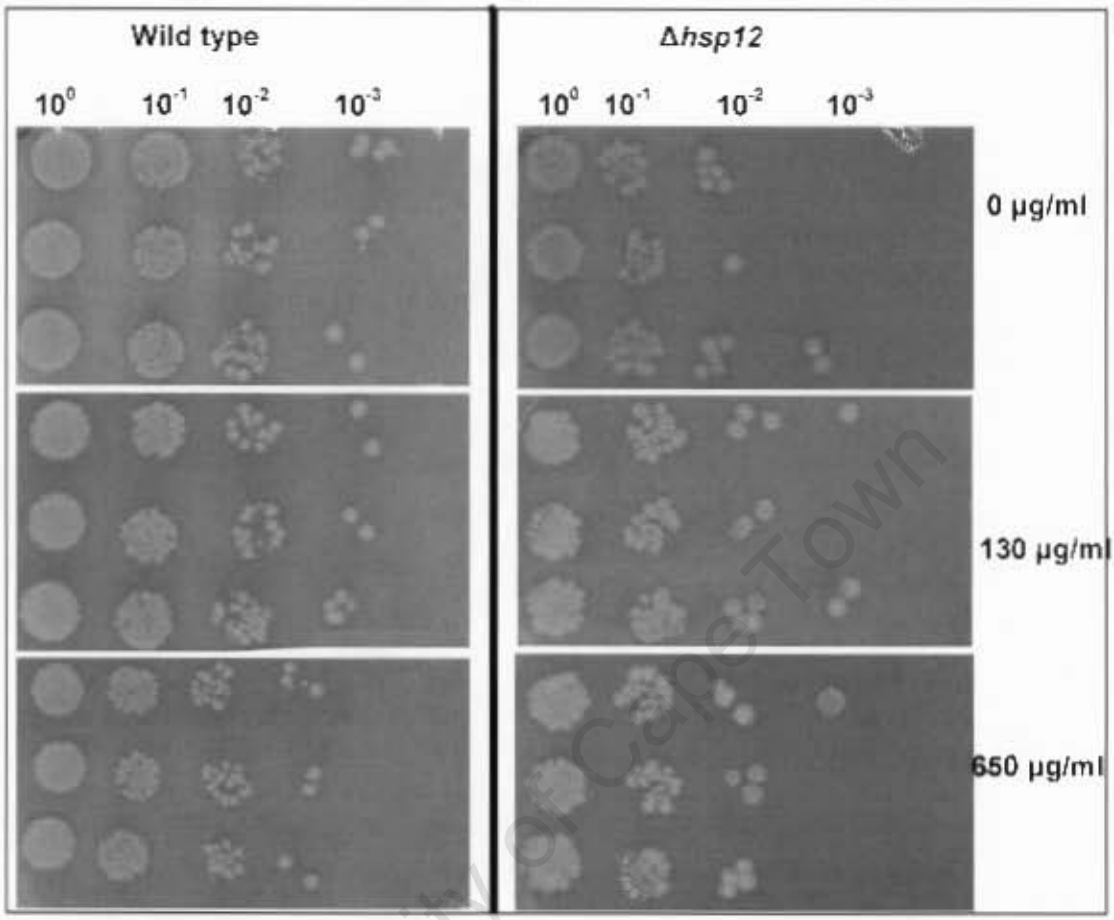

Figure 2.12: Viability of the wild type and the Δ*hsp12* strains after treatment with increasing concentrations of polyphenols for 24 h. After treatment with 0, 130, or 650 µg/ml polyphenols, the cells were washed free of polyphenols before they were spotted onto YEPD agar plates, which were then incubated at 30 °C for 48 h.

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2.4. DISCUSSION

In the present study, *M. flabellifolia* polyphenols were found to inhibit growth of both the wild type and the *Δhsp12* yeast strains. This was shown to be due to polyphenols binding proteins and metal ions in the growth media rather than binding to the yeast cells walls. However, growth inhibition was largely due to protein binding rather than binding of metal ions in the growth medium. The specific growth rate of both strains decreased by over 60 % when 650 μg/ml polyphenols were present in protein based medium (YEPD), compared to a decrease of 16 % when the same polyphenols concentration was present in a nitrogen based medium (YNB). Furthermore, the lag phase and the maximum biomass of both strains were largely affected by the presence of polyphenols in YEPD medium compared with the presence of polyphenols in YNB medium. A combination of polyphenols and metal ions (Fe^{3+} or Zn^{2+}) in YEPD medium improved the specific growth rate of only the wild type strain, with iron improving the specific growth rate far more than zinc. A previous study had shown that growth of *S. cerevisiae* in YEPD medium with tannic acid could be restored by addition of iron but not other metal ions or ammonium sulphate (Wauters et al., 2001). A possible explanation of these effects on growth of yeast by polyphenols is that polyphenols chelate iron and thus limit its availability to yeast cells. Furthermore, addition of polyphenols to an iron solution resulted in the formation of a blue black colour (results not shown), suggesting that polyphenols might also be oxidizing iron in the growth medium. Growth of yeast in the presence of gallic acid, a 'low molecular weight' polyphenol, had no effect on growth of both strains. A previous study (Chung et al., 1993) showed that gallic acid and ellagic

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acid did not inhibit the growth of all 15 tested food-borne bacteria. They concluded that the ester linkage between the gallic acid and the glucose moieties (to form high molecular weight polyphenols such as tannins) is important for the antimicrobial potential of gallic acid.

Pre-treatment of both the wild type and the *Δhsp12* strains with polyphenols for 24 h followed by plating on YEPD medium did not affect the viability of either strain. Polyphenols are known to inhibit growth of micro-organisms by affecting cell membrane integrity. A previous study (Kim and Fung, 2004) showed that bacterial cells treated with polyphenols had ruptured cell walls and membranes together with irregular disruption of the intracellular matrix, and which the authors proposed that polyphenols killed bacteria by affecting cell membrane integrity. In prokaryotic organisms, the cell membrane is a site of electron transport and ATP generation. Since polyphenols have the ability to quench electrons from free radicals and to delocalize them within the phenolic ring, they can easily accept electrons from the electron transport chain in the bacterial membrane, thereby disrupting oxidative phosphorylation and inhibiting bacterial growth (Vattem and Shetty, 2005).

The present study showed that low concentrations of polyphenols were able to promote yeast growth. Other polyphenols such as resveratrol have been shown to extend the life span of yeast by stimulating Sir2p and thereby increasing DNA stability. The ability of Sir2p to extend yeast lifespan is thought to stem from its role in stabilizing repetitive DNA (Howitz et al., 2003). Sir2p proteins are a class of sirtuin enzymes.

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These enzymes are a phylogenetically conserved familiy of enzymes found in diverse organisms, from archaea to human. The sirtuin enzymes have highly conserved catalytic domains that catalyze NAD^+ -dependent protein deacetylation and/or ADP-ribosyltranfer (Yang and Sauve, 2006). The results presented showed that growth of the Δ*hsp12* strain was promoted by the presence of 6.5 µg/ml and 16.3 µg/ml polyphenols in liquid and solid YEPD medium, respectively. However, the method by which this occurred was not investigated. In particular, it was found that Δ*hsp12* strain could tolerate the presence of polyphenols in growth medium better compared with the wild type. These results agreed with those previously reported (Shamrock, 2007) and showed that the Δ*hsp12* strain grew slower and was able to tolerate stress better than the wild type strain.

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CHAPTER THREE

THE ANTI-OXIDANT PROPERTIES OF POLYPHENOLS FROM THE RESURRECTION PLANT *M. flabellifolia* IN YEAST CELLS

3.1. INTRODUCTION

Polyphenols are a large group of anti-oxidants naturally present in fruits, vegetables, and certain beverages, including wine, coffee, and tea. Epidemiological studies have shown an inverse association between intake of some beverages rich in polyphenols and the risk of carcinogenesis and cardiovascular diseases (Akagawa et al., 2003). These protective effects have been attributed to the ability of polyphenols to act as free radical scavengers, which quench hydroxyl radicals ($^{\cdot}OH$), superoxide anion radicals ($^{\cdot}O_2^-$), and chelate metal ions such as iron and copper. The metal-chelating properties of polyphenols are considered to play a role in all oxidative stress conditions involving transition metal ions (Akagawa et al., 2003).

In contrast to the beneficial effects, it has been reported that some polyphenols promote oxidative damage in the presence of metal ions under certain conditions *in vitro*. These harmful effects are suspected to result from the pro-oxidant action of polyphenols and have been suggested to induce mutagenesis and carcinogenesis. These pro-oxidant properties arise from the auto-oxidation of polyphenols (Akagawa et al., 2003). *M. flabellifolia* is a desiccation tolerant plant containing large amounts of polyphenols, which may play a role in protecting the plant against oxidative damage caused by desiccation and dehydration. The predominant (>90 %) low molecular mass polyphenol has been identified as 3,4,5 tri-

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O-galloyquinic acid (Moore at al., 2005). This polyphenol has been reported to protect linoleic acid against free radical-induced oxidation with 15 μM 3,4,5 tri-*O*-galloyquinic acid reducing the rate of linoleic acid oxidation by approximately 95 % (Moore at al., 2005).

Oxidative stress has been implicated in a large variety of biological processes and diseases, including cancer, tumor promotion, heart disease, ageing, apoptosis and drug toxicity, since it causes damage to cellular components such as DNA, proteins, and lipids (Jamieson, 1992; Unlu and Koc, 2007). Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), $^{\cdot}OH$, and $^{\cdot}O_2^-$, are constantly being produced as by-products of aerobic metabolism and by the immune system in response to pathogens (Jamieson, 1992). Since mitochondrial respiratory chains present on the inner mitochondrial membranes are a major source of intracellular ROS, mitochondria are oxidatively damaged more rapidly than other components of the cell. As a result, all aerobically growing organisms have developed mechanisms to protect their cellular components against ROS (Unlu and Koc, 2007). Yeast possess both enzymatic and non-enzymatic antioxidant defences against ROS that maintain the concentration of ROS at physiologically benign levels (Moradas-Ferreia et al., 1996). A list of such defence systems is shown (Table 3.1).

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Table 3.1: Primary antioxidant defences in yeast (Moradas-Ferreia et al., 1996; Jamieson, 1998; Unlu and Koc, 2007)

(ORF) gene	Gene product	Sub-cellular location	Functions
SOD1	CuZnSOD	cytoplasmic	Dismutation of O_2^-
SOD2	MnSOD	mitochondrial	Decomposition of H_2O_2
CTA1	catalase A	peroxisomal	Reduction of H_2O_2
CTT1	catalase T	cytoplasmic	Reduction of protein Disulphides; scavenging of free radicals
CCP	cytocrome C	mitochondrial intermembrane space	Reduction of H_2O_2
GSH1[a]	glutathione	mitochondrial	Reduction of protein disulphides; scavenging of free radicals
GLR1	glutathione reductase		Reduction of oxidized glutathione
ZWF	glucose-6-phosphate dehydrogenase		Reduction of $NADP^+$ to NADPH
CUP1	metallothionein		Binding of Cu, preventing the Fenton reaction; scavenging O_2^-, and $^{\cdot}OH$
TRX2	thioredoxin	mitochondrial	Reduction of protein disulphodes
TRX	thioredoxin peroxidase		Reduction of H_2O_2, and alkyl
SPE2[a]	polyamines		protection of lipids from oxidation

a. *GSH1* and *SPE2* encode enzymes involved in glutathione and polyamine biosynthesis (γ-glutamyl-cysteine synthetase and S-adenosylmethione decarboxylase respectively. SOD: superoxide dismutase.

Certain genes, such as those for catalases, SODs, glutathione peroxidases and cytochrome c peroxidase are repressed by glucose. De-repression occurs with respiratory adaptation that follows glucose exhaustion during aerobic batch fermentation. This helps the yeast tolerate higher levels of endogenous production of ROS during respiratory, as compared to fermentative growth. Thus yeast cells growing on non-fermentable substrates such as ethanol and glycerol express higher levels of these antioxidant defences enzymes and display higher oxidative stress tolerances (Moradas-Ferreia et al., 1996).

H_2O_2 belongs to the non-radical group of ROS. It has an intermediate oxidation number and can be converted into other more reactive ROS such as $^{\cdot}OH$ by various means such as catalase, peroxidase and glutathione. H_2O_2 is a broad-range chemical catalyst having both reducing and oxidizing properties (Bienert et al., 2006). It is known that exposure of cells

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to sub-lethal concentrations of H_2O_2 renders cells resistant to subsequent treatment with higher concentrations. This phenomenon has been observed in both prokaryotes and eukaryotes including *S. cerevisiae*, and was termed adaptation (Izawa et al., 1995). The increased antioxidant status after respiratory adaptation is probably a factor contributing to the increased oxidative stress tolerance of stationary phase yeast cells (Moradas-Ferreia et al., 1996).

In this chapter the antioxidant properties of *M. flabellifolia* polyphenols have been investigated using the wild type and the petite mutant (rho^0) strains.

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3.2. MATERIALS AND METHODS

3.2.1. ORGANISMS AND CULTURE CONDITIONS

The *S. cerevisiae* yeast strains used were of the W303 background (*a/α, ade2-1/ade2-1, trp1-1/trp1-1, leu2-3/leu2-112, his3-11/his3-15, ura3/ura3, canr1-100/CAN*) and were used in haploid form. The rho^0 mutants were generated by growing the wild type strain in 50 ml 0.077 % (w/v) complete synthetic media without uracil (CSM-URA, BIO-101, USA) containing 2 % (w/v) glucose, 25 µg/ml ethidium bromide (EtBr) and 0.1 mg/ml Ampicilin. The rho^0 mutants were initially tested by their inability to grow in a medium containing 1 % (v/v) yeast extract, 2 % (w/v) peptone and 3 % (v/v) glycerol as a carbon source for 48 h at 30 °C. Their rho^0 status was confirmed by failure to amplify the *RPMI* gene using PCR. The *HSP12* gene was used as a positive control for the PCR. Both strains were routinely cultured as described in Section 2.2.3.

3.2.2. EXTRACTION OF POLYPHENOLS

M. flabellifolia polyphenols were extracted as described previously (Section 2.2.1.) using 70 % acetone. Polyphenols were lyophilized and stored at -20 °C and were quantified using the Folin Ciocalteau reagent using gallic acid as the standard.

3.2.3. DETERMINATION OF YEAST VIABILITY USING FLOW CYTOMETRY

Membrane permeability was determined by propidium iodide (PI) fluorescence of the cells (Chen et al., 2003). PI is a nucleic acid probe that is used to assess the viability of micro-organisms. PI does not cross intact membranes of micro-organisms, but can pass through

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compromised membranes. Once inside the cell, it intercalates into RNA and DNA (Chitarra et al., 2006). The fluorescence is then increased 20- to 30-fold, the fluorescence excitation maximum is shifted by 30-40 nm to the red and the fluorescence emission maximum is shifted by ~ 15 nm to the blue (Molecular probes, 1999). PI stained cells are assumed to be non viable (Chitarra et al., 2006). Flow cytometry uses the principle of hydrodynamic focusing by presenting single cells or particles in suspension to a laser light source where the characteristics of each cell or particle are determined using an optical-to-electronic coupling system that records the scattering of incident laser and the emission of fluorescence. The data is then displayed as a histogram of fluorescence intensity versus cell number with that intensity (Bioscience, 2000; Robinson, 2004; Marion, 2007).

Yeast cells were suspended in PBS buffer pH 7.4 to approximately 0.5 x 10^7 cells/ml, after which PI was added to final concentration of 3 µg/ml. Stained cells were analyzed using flow cytometry within 15 minutes of staining using a Cytomics FC 500 flow cytometer (Beckman Coulter, USA), with an excitation wavelength set at 488 ± 10 nm and emitted from a 15 mW argon laser. The flow rate was adjusted to keep the total events below 100 per second. PI was detected as a red signal using a 620 nm bypass filter. A maximum of 10 000 events were counted for each sample. Data was analyzed using CPX Analysis software version 2.2 (Beckman Coulter, USA).

3.2.4. TREATMENT OF CELLS WITH POLYPHENOLS AND H_2O_2

Cells were grown in YEPD medium to a mid-log phase culture at 30 °C. The mid-log phase culture was diluted to 0.5 x 10^7 cells/ml in PBS pH 7.4. The diluted cells were pre-treated

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with increasing concentrations of polyphenols up to 650 µg/ml, after which the cells were incubated for 1 or 24 h at 30 °C. The cells were then washed with PBS buffer and incubated in H_2O_2 up to 750 mM concentration for 1 h at 30 °C to induce oxidation. Cells were then washed with PBS buffer and stained with PI as described in Section 3.3.3.

3.2.5. ESTIMATION OF ROS PRODUCTION USING 2',7'-DICHLORODIHYDROFLUORESCEIN DIACETATE (DCHF-DA)

The production of ROS by polyphenols was estimated using the non-fluorescent probe, DCHF-DA. DCHF-DA is able to diffuse through intact plasma membrane of the cells, and once inside the cells it is hydrolyzed by non-specific esterases to form the non-fluorescent 2', 7'-dichlorodihydrofluorescein (DCHF). The DCHF formed is then oxidized by ROS to the fluorescent 2',7'-dichlorofuorescein (DCF) (Figure 3.1). DCHF-DA was dissolved in 50 mM DMSO to a final concentration of 1 mM and stored at 4 °C (Loetchutinat et al., 2005; Labieniec and Gabryelak, 2007). Cell suspensions were diluted to 3×10^5 cells per ml in PBS Buffer pH 7.4, after which they were washed and incubated with increasing concentration of polyphenols up to 650 µg/ml alone, and/or with H_2O_2 up to 10 mM at 30 °C for 1 h. Treated cells were stained with 10 µM DCHF-DA for a further 1 h at 37 °C. The fluorescence intensity of DCF was measured using an Aminco SPF-500 fluorimeter (American instrument company, Silver Spring, MD) with the excitation and emission wavelengths set at 488 and 520 nm, respectively.

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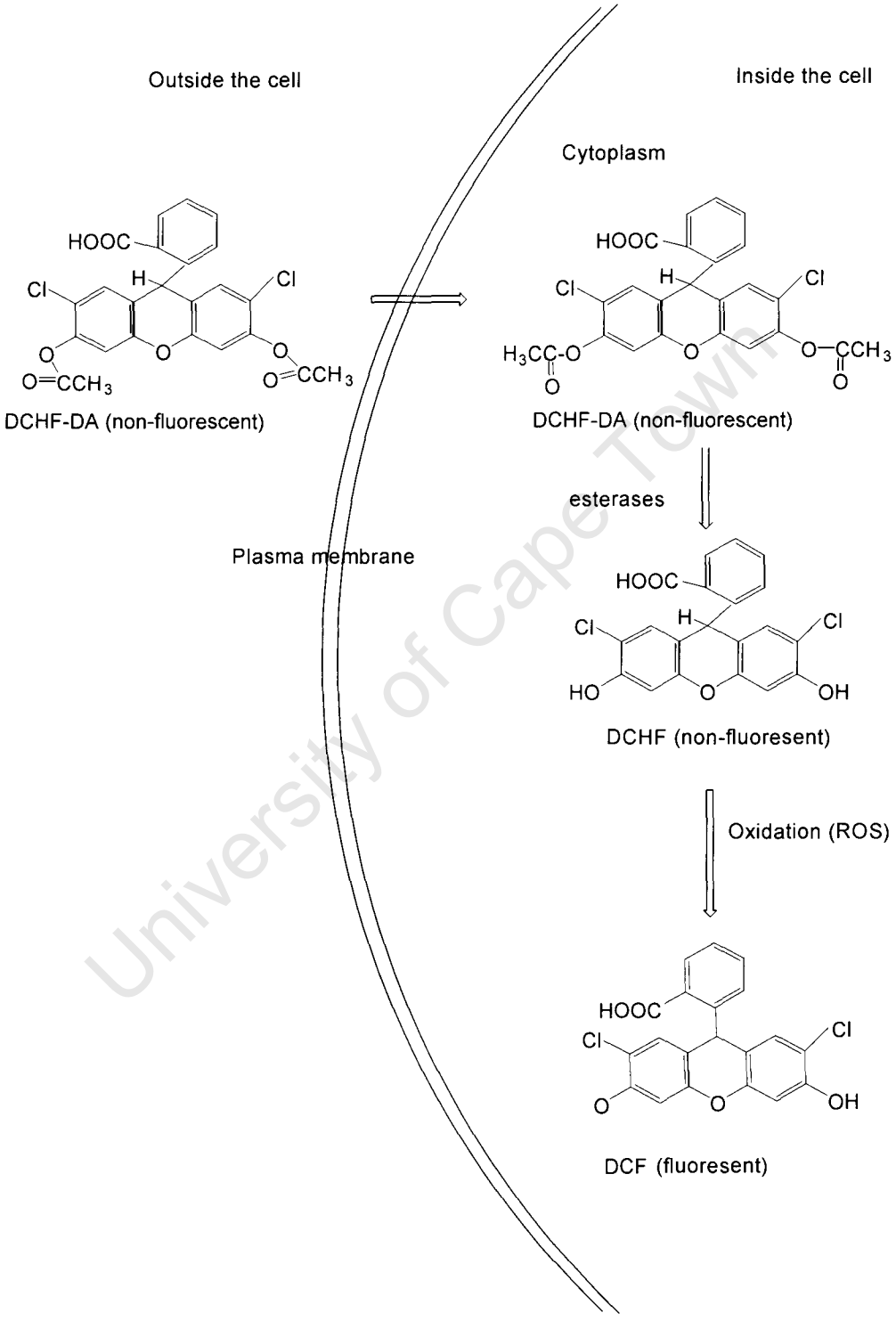

Figure 3.1: Metabolism of DCHF-DA in cells (Jakubowski and Bartosz, 1997; Loetchutinat et al., 2005)

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3.3. RESULTS

3.3.1. VIABILITY OF THE WILD TYPE STRAIN AFTER PROPIDIUM IODIDE STAINING

Flow cytometry was used to measure the viability of yeast cells by monitoring the permeability of plasma membranes using PI as a marker for dead cells. Wild type cells (0.5 x 10^7) in PBS buffer were treated with 300 mM H_2O_2 for 1 h at 30 °C, or heated at 80 °C for 20 min, after which they were washed with PBS and the viability determined. Untreated cells were used as the control for live cells. 98 % of these cells were counted in gate A and showed the lowest PI fluorescence (Figure 3.2: panel 1). 80 °C heat killed cells were used as the control for dead cells and 100 % of these cells were counted in gate B (Figure 3.2: panel 2). The fluorescence of these cells increased by approximately 80-fold compared to the fluorescence of live cells. These results showed that PI was able to discriminate between a population of dead and live cells with high resolution. The fluorescence of cells treated with 300 mM H_2O_2 showed two populations, with 28 % of these cells counted in gate A and 72 % counted in gate B and corresponding to live and dead cells, respectively (Figure 3.2: panel 3). This showed that PI staining was also able to discriminate dead and live cells in a population.

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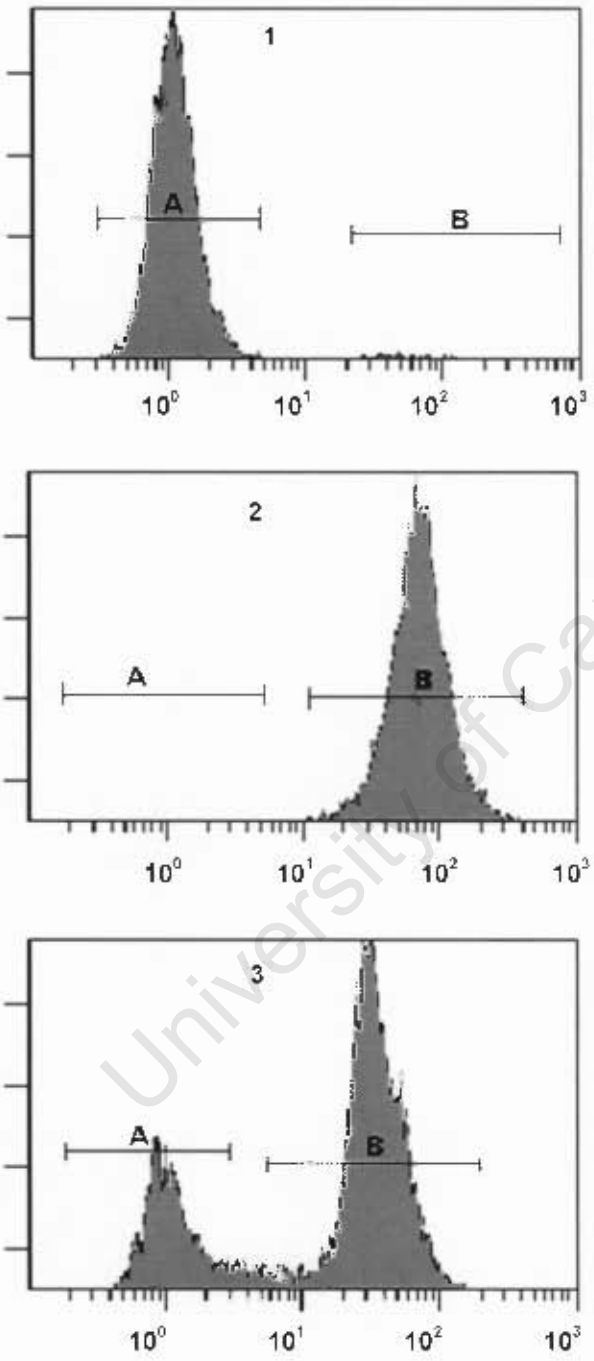

Untreated cells

Gate A: 98.4 ± 1.0 %

Heated cells

Gate B:

99.7 ± 0.1 %

H_2O_2 treated cells

Gate A: 27.8 ± 2.3 %

Gate B: 72.2 ± 2.0 %

Figure 3.2: Viability of wild type cells after PI staining and analysis with flow cytometry. Gates A and B in panel 1-3 represents live and dead cells respectively. Panel 1: untreated stained cells; panel 2: heated cells; panel 3: cells treated with 300 mM H_2O_2. The result shows the mean ± SD of triplicate samples.

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3.3.2. EFFECT OF H_2O_2 ON THE SURVIVAL OF WILD TYPE CELLS

Since H_2O_2 is relatively stable *in vivo* compared to other ROS and is produced by most aerobic organisms in the mitochondria, it was used as the oxidant in this study. The effect of H_2O_2 on the viability of wild type cells was investigated following starvation of a mid-log phase culture for 1 or 24 h in PBS buffer at 30 °C. The starved cells were then treated with up to 750 mM H_2O_2 for 1 h at 30 °C, after which the cells were washed with PBS, and the percentage survival of cells determined. The results (Table 3. 2) indicated that whereas starvation of a mid-log phase culture for 1 h resulted in considerable loss of viability, cells that had been starved for 24 h become resistant to 750 mM H_2O_2. The wild type strain lost 85 % viability after 1 h starvation followed by treatment with 250 mM H_2O_2 for 1 h, compared to a viability loss of only 1.2 % when they were treated with the same concentration of H_2O_2 after 24 h starvation. These results suggested that the wild type strain was able to adapt to lethal oxidation following starvation.

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Table 3.2: Percentage survival of wild type cells after treatment with H_2O_2 for 1 h at 30 °C. The cells were starved for 1 or 24 h in PBS pH 7.4 at 30 °C before being treated with H_2O_2. The percentage survival was analyzed using flow cytometry.

$[H_2O_2]$ mM	% survival of wildtype cells	
	1 h (starvation)	24 h (starvation)
0	100 ± 0.2	100 ± 0.5
100	42.4 ± 7.4	98.9 ± 2.0
250	15.2 ± 1.2	98.8 ± 0.9
750	4.4 ± 1.7	96.6 ± 0.8

The data show the mean ± SD from triplicate experiments

3.3.3. ANTI-OXIDANT PROPERTIES OF *M. flabellifolia* POLYPHENOLS IN THE WILD TYPE STRAIN

To investigate the effect of *M. flabellifolia* polyphenols on the viability of wild type yeast cells, cells were incubated with increasing concentrations of polyphenols at 30 °C for 1 or 24 h in PBS after which the cells were washed with PBS buffer and the percentage survival determined. Treatment of wild type cells with up to 650 µg/ml polyphenols for 1 h had no effect on the viability (Figure 3.3A), whereas treatment for 24 h resulted in decreased viability. The wild type strain lost 35 % viability after treatment with 650 µg/ml polyphenols concentration for 24 h (Figure 3.3B). These results suggested that prolonged incubation of the wild type strain with a high concentration of polyphenols might be toxic to the cells.

The anti-oxidant properties of *M. flabellifolia* polyphenols were then investigated by pre-treating wild type cells with up to 650 µg/ml polyphenols for 1or 24 h at 30 °C, after which the cells were washed and then treated with H_2O_2 for 1 h at 30 °C and the viability of cells determined as before. The results (Figure 3.4.3A and B) indicated that pre-treatment of wild type cells with *M. flabellifolia* polyphenols for either 1 or 24 h did not protect the cells against H_2O_2 induced oxidation, but instead resulted in an increased number of cells losing viability. Pre-treatment of cells with high polyphenol concentrations followed by treatment with a high H_2O_2 concentration resulted in more cells losing viability. However, 24 h pre-treatment with polyphenols resulted in cells becoming slightly resistant to treatment with H_2O_2 compared to cells that were treated for 1 h.

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The wild type strain lost 66 % viability after pre-treatment with 16.3 µg/ml polyphenols for 24 h followed by treatment with 250 mM H_2O_2. However, this strain lost 83 % viability after pre-treatment with 650 µg/ml polyphenols and then treatment with 250 mM H_2O_2 under the same conditions. Even though pre-treatment of the wild type strain with up to 650 µg/ml polyphenols for 1 h had no effect on the viability, the strain lost up to 90 % viability after treatment with 16.3 µg/ml polyphenols followed by treatment with 250 mM H_2O_2. These results showed that the harmful effects of polyphenols and H_2O_2 are cumulative and might have resulted from the pro-oxidant properties of the polyphenols as previously reported (Akagawa et al., 2003).

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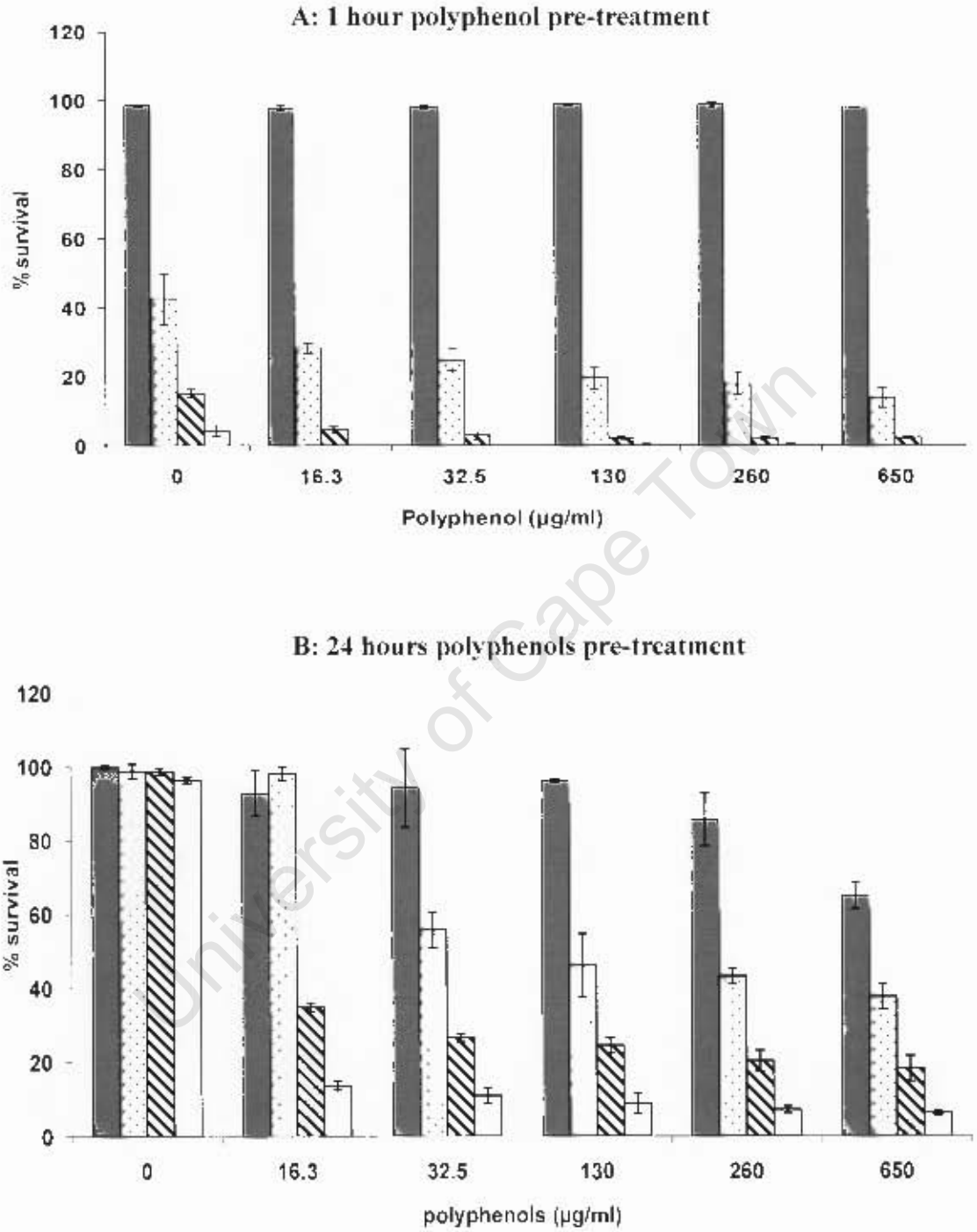

Figure 3.3: Percentage survival of the wild type strain after pre-treatment with polyphenols for 1 h (A) and 24 h (B) at 30 °C, followed by treatment with H_2O_2 1 h at 30 °C. Polyphenols were added to 0.5 x 10^7 cells per ml in PBS buffer pH 7.4 at the indicated concentrations and incubated at 30°C for 1 h (A) or 24 h (B). After pre-treatment with polyphenols, cells were washed, and then treated with H_2O_2. The percentage survival was analyzed using flow cytometry. Legend: gray bars: 0 mM H_2O_2; dotted bars: 100 mM H_2O_2; diagonal bars: 250 mM H_2O_2; white bars: 750 mM H_2O_2. The data show the mean ± SD from triplicate experiments

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3.3.4. EFFECTS OF H_2O_2 ON SURVIVAL OF PETITE (RHO^0) MUTANTS

Since mitochondria are the primary site for ROS generation, the effect of H_2O_2 on the viability of rho^0 mutants was investigated. Rho^0 yeast mutants contain no mitochondrial DNA, and therefore results in the mitochondria having no cytochrome *b* and cytochrome oxidase subunits (Davidson and Schiesti, 2001). Rho^0 mutants were generated by growth of wild type cells in the presence of ethidium bromide. The effect of H_2O_2 on the viability of rho^0 mutant cells was investigated following starvation of a mid-log phase culture for 1 or 24 h in PBS buffer at 30 °C. The starved cells were then treated with up to 250 mM H_2O_2 for 1 h at 30 °C, after which the cells were washed with PBS buffer. The percentage survival of cells was determined using PI staining and flow cytometry. Low concentrations of H_2O_2 were used in this experiment since the rho^0 cells were more sensitive to H_2O_2. The results (Table 3.3) showed that rho^0 mutants were hypersensitive to H_2O_2 compared with wild type cells and were unable to adapt to become more resistant after starvation in PBS for 24 h. The rho^0 mutants strain lost 89 % viability after 1 h starvation followed by treatment with 100 mM H_2O_2 for 1 h, compared to a viability loss of 87 % when they were treated with the same concentration of H_2O_2 after 24 h starvation. These results showed that rho^0 mutants were not able to adapt to lethal oxidation following exposure to starvation as compared to the wild type.

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Table 3. 3:

Percentage survival of rho mutants cells after treatment with H_2O_2 for 1 h at 30 °C. The cells were starved for 1 or 24 h in PBS pH 7.4 at 30 °C before being treated with H_2O_2. The percentage survival was analyzed using flow cytometry.

$[H_2O_2]$ mM	% survival of rho^0 mutants cells	
	1 h (Starvation)	24 h (Starvation)
0	100 ± 0.3	100 ± 0.1
50	67.1 ± 1.1	24.1 ± 1.9
100	10.7 ± 3.7	13.2 ± 0.1
150	2.9 ± 1.7	2.6 ± 0.1

The data show the mean ± SD from triplicate experiments.

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3.3.5. ANTI-OXIDANT PROPERTIES OF *M. flabellifolia* POLYPHENOLS ON PETITE MUTANTS OF YEAST CELLS

The anti-oxidant properties of *M. flabellifolia* polyphenols were next investigated using the rho^0 mutant strain. Firstly, the effect of *M. flabellifolia* polyphenols on the viability of rho^0 cells were determined by incubation with increasing concentrations of polyphenols at 30 °C for 1 or 24 h, after which the cells were washed and the viability determined. Treatment of rho^0 mutants with up to 650 µg/ml polyphenols for either 1 or 24 h resulted in approximately 20 % decreased viability (Figure 3.4A and B). These results are similar to those obtained using the wild type strain, in which treatment with 650 µg/ml polyphenols for 24 h resulted in a 35 % loss of viability.

Secondly, the antioxidant properties of *M. flabellifolia* polyphenols were also investigated by pre-treating the rho^0 mutants cells with up to 650 µg/ml polyphenols for 1 or 24 h at 30 °C, after which the cells were washed and then treated with H_2O_2 for 1 h at 30 °C and the viability of cells determined as before. The results (Figure 3.4 A and B) showed that pre-treatment of rho^0 mutants with up to 650 µg/ml polyphenols for 1 or 24 h resulted in slight protection of the cells against subsequent treatment with H_2O_2. Pre-treatment with polyphenol for 24 h, followed by oxidation with H_2O_2 protected the cells in all the concentrations of polyphenols used, however, pre-treatment for 1 h only protected the cells at low polyphenols concentration of 16.3 ug/ml. These results suggested that a deleted mitochondrial gene might be responsible for the auto-oxidation of polyphenols which results in the production of H_2O_2.

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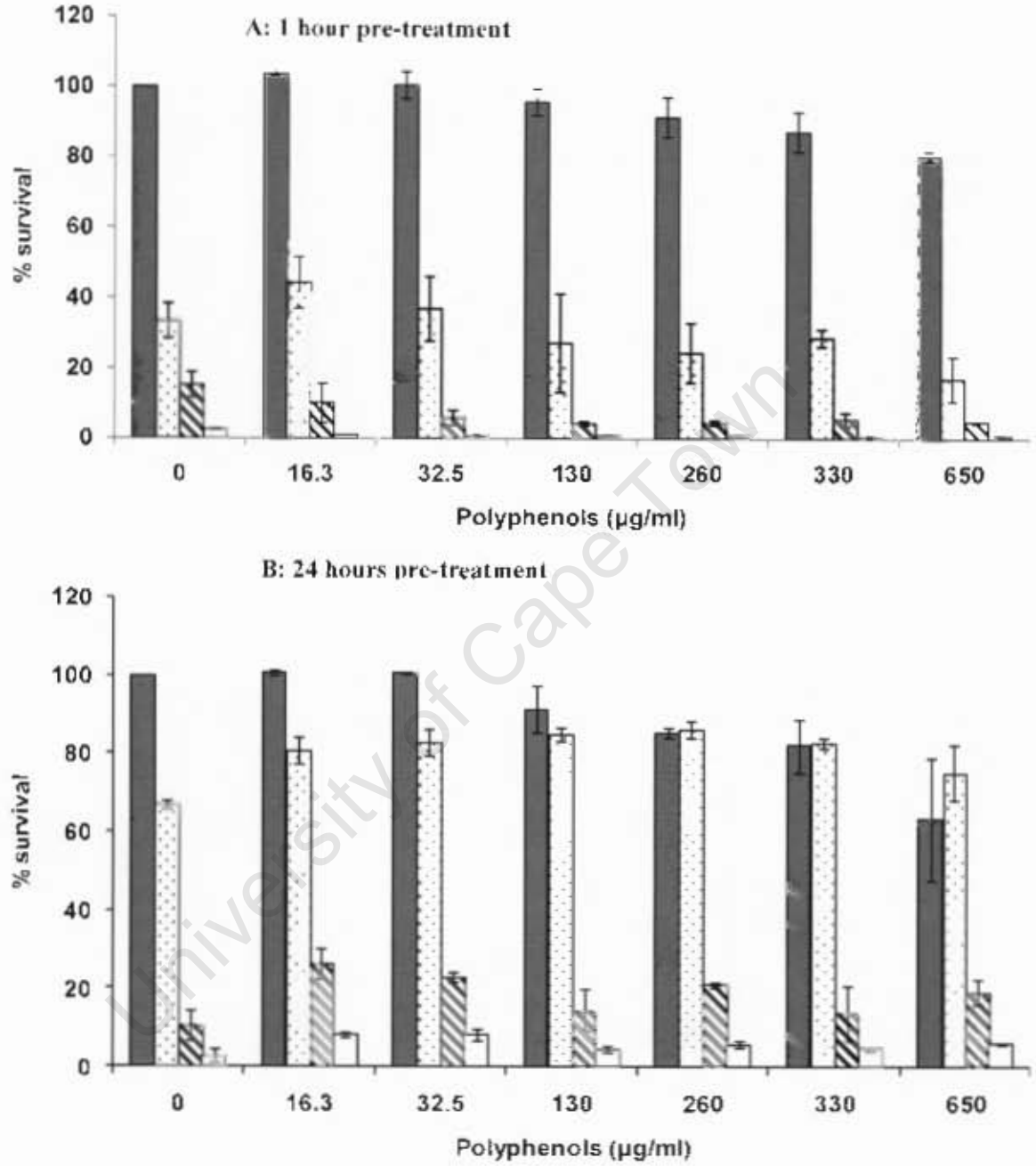

Figure 3.4: Percentage survival of the rho^0 mutant strain after pre-treatment with polyphenols for 1 h (A) and 24 h (B) at 30 °C, followed by treatment with H_2O_2 for 1 h at 30 °C. Polyphenols were added to 0.5 x 10^7 cell/ml in PBS buffer pH 7.4 at the indicated concentrations and incubated at 30 °C for 1(A) and 24 h (B). After pre-treatment with polyphenols, cells were washed and then treated with H_2O_2. The percentage survival was analyzed using flow cytometry. Legend: gray bars: 0 mM H_2O_2, dotted bars: 50 Mm H_2O_2, diagonal bars: 100 Mm H_2O_2, white bars: 150 mM H_2O_2. The data shows the mean ± SD from triplicate experiments

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3.3.6. PRODUCTION OF ROS BY *M. flabellifolia* POLYPHENOLS USING THE PROBE DCHF-DA

In contrast to their anti-oxidant properties, polyphenols are known to produce ROS under certain conditions, thus acting as pro-oxidants. In the previous sections, it was shown that *M. flabellifolia* polyphenols did not protect wild type cells against oxidative damage in the presence of H_2O_2 as expected, but instead promoted cell death which might have resulted from the production of ROS by these polyphenols. The production of ROS by *M. flabellifolia* polyphenols was therefore investigated using the probe DCHF-DA (Figure 3.1) in PBS buffer. This method measures the formation of the fluorescent DCF.

To investigate whether *M. flabellifolia* polyphenols showed pro-oxidant activities, up to 650 µg/ml polyphenols and/or up to 10 mM H_2O_2 were incubated for 1 h at 30 °C in PBS buffer, followed by incubation with DCHF-DA for 1 h at 37 °C. The results (Figure 3.5 gray bars) showed that incubation of polyphenols and DCHF-DA in the absence of H_2O_2 and yeast cells resulted in increased formation of DCF. The amount of DCF produced increased with an increase in the polyphenol concentration and suggesting that polyphenols produced small amounts of ROS at high concentrations. Incubation of 650 µg/ml polyphenols and DCHF-DA in PBS resulted in an approximate 21-fold increase in the amount of DCF formed. Incubation of H_2O_2 with DCHF-DA alone in the absence of polyphenols and yeast cells resulted in significant DCHF-DA oxidation. However, the addition of polyphenols together with the H_2O_2 clearly reduced the oxidation of DCHF-DA compared when DCHF-DA was incubated with H_2O_2 alone. The results also showed that

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addition of polyphenols together with H_2O_2 resulted in protection when low polyphenols concentrations (up to 16.3 µg/ml) were present and in pro-oxidant activity when higher polyphenols concentrations (from 32.5 µg/ml up to 650 µg/ml) were used. These results suggested that although polyphenols were producing small amounts of ROS, they were able to scavenge considerable amounts of H_2O_2 at all concentrations used.

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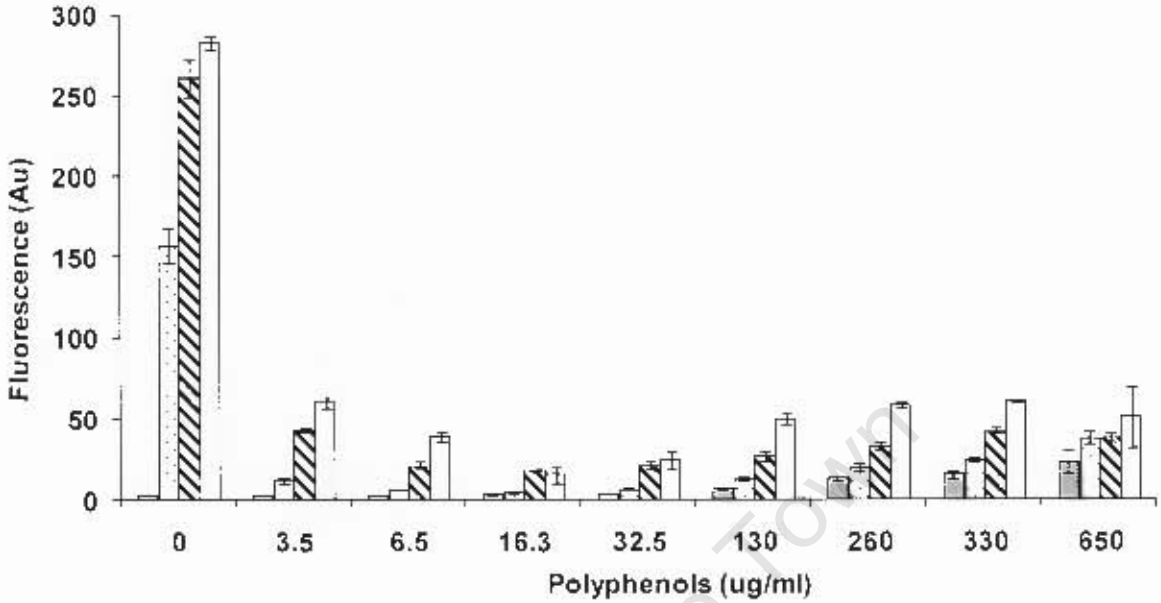

Figure 3.5: The fluorescence intensity of DCF in the presence of increasing concentrations *M. flabellifolia* polyphenols with up to 10 mM H_2O_2. Polyphenols were incubated with and without H_2O_2 for 1 h and then incubated with DCHF-DA. Legend: gray bars: 0 mM H_2O_2; dotted bars: 1 mM H_2O_2; diagonal bars: 4 mM H_2O_2; unfilled bars: 10 mM H_2O_2. Data show the mean ± SD from triplicate experiments.

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3.3.7. PRODUCTION OF ROS BY *M. flabellifolia* POLYPHENOLS IN YEAST STRAINS

The ability of *M. flabellifolia* polyphenols to act as pro-oxidant in wild type and rho^0 mutant cells was suspected to be due to polyphenols producing ROS in PBS buffer. In order to determine the production of ROS by *M. flabellifolia* polyphenols in wild type and rho^0 cells, both strains were treated with up to 650 µg/ml polyphenols and/or up to 10 mM H_2O_2, after which they were incubated with DCHF-DA.

The result (Figure 3.6A) showed that incubation of wild type cells with polyphenols in the absence of H_2O_2 resulted in the production of small amounts of ROS. These results where similar to the results (Figure 3.5) in which incubation of polyphenols and DCHF-DA in the absence of cells showed a significant DCHF-DA oxidation. However, the incubation of wild type cells with H_2O_2 in the absence of polyphenols resulted in significantly reduced DCHF-DA oxidation. These results are contrary to the results (Figure 3.5 and 3.6B) in which incubation of H_2O_2 with either rho^0 cells or no cells resulted in significant DCHF-DA oxidation. Addition of polyphenols together with H_2O_2 resulted in reduced DCHF-DA oxidation.

The results (Figure 3.6B) showed that incubation of rho^0 cells with polyphenols in the absence of H_2O_2 resulted in small amounts of ROS being produced. These results are similar to the results (Figure 3.5 and 3.6A) in which incubation of polyphenols and DCHF-DA in the absence of cells and/or in the presence of wild type cells showed an in increase in DCF formation indicating production of ROS by polyphenols. Similarly, incubation of rho^0

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cells with H_2O_2 showed a significant DCHF-DA oxidation. In addition, incubation of rho^0 cells with DCHF-DA in the presence H_2O_2 and polyphenols resulted in significantly reduced DCHF-DA oxidation. These results are also similar to the results (Figure 3.5 and 3.6A) in which incubation of DCHF-DA with polyphenols in the presence of H_2O_2 with either in the presence or in the absence of cells resulted in significantly reduced DCHF-DA oxidation. These results suggested that although polyphenols can act as anti-oxidants, they can also act as pro-oxidants by producing ROS that contribute to damaging the cells under certain conditions. Since the anti-oxidant properties of these polyphenols were only seen in cells that lacked mitochondrial DNA, it is possible that the electron transport chain contribute to the oxidation of polyphenols.

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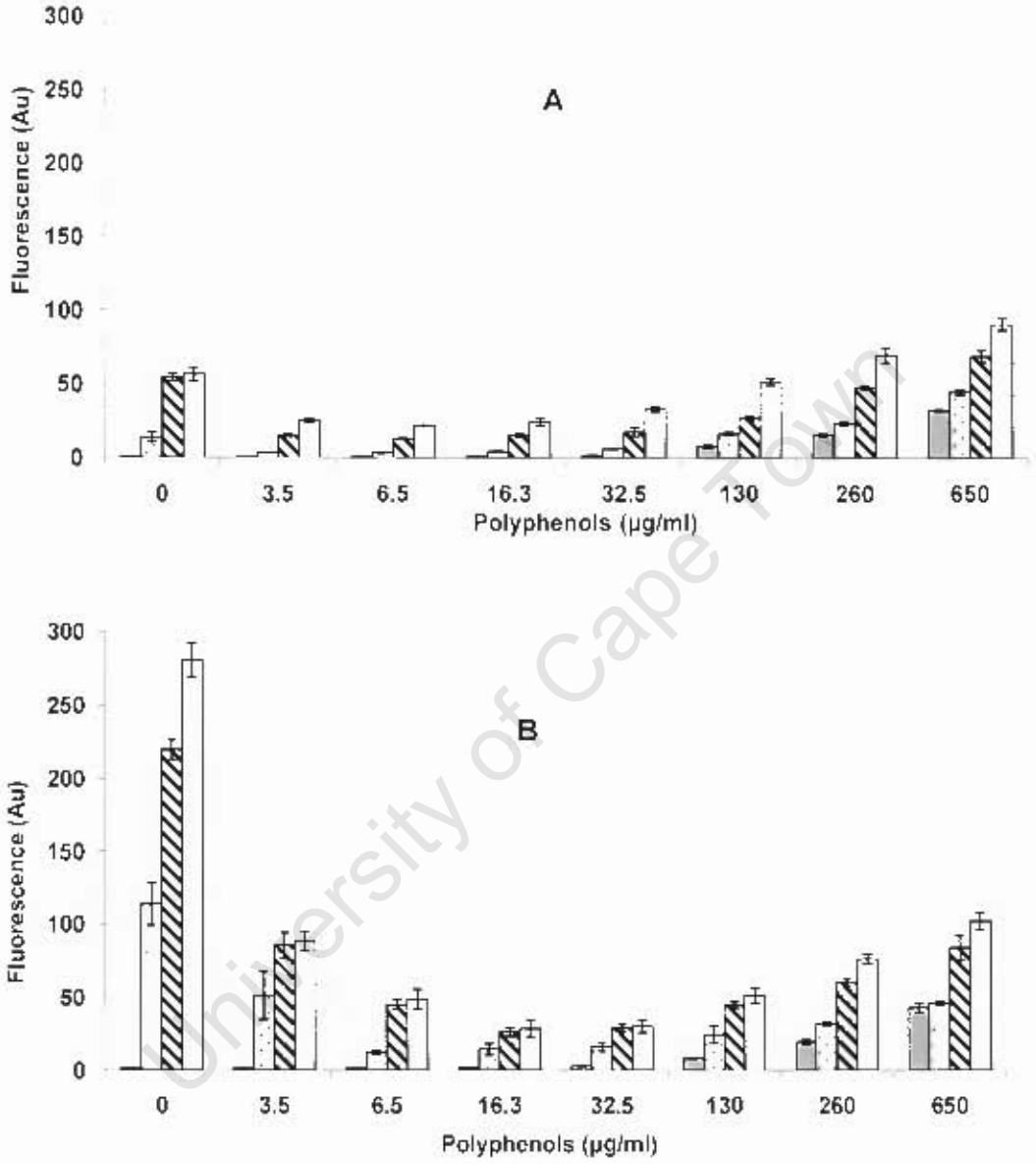

Figure 3.6: The fluorescence intensity of DCF in the presence of increasing concentrations of *M. flabellifolia* polyphenols and up to 10 mM H_2O_2. Polyphenols were incubated with and without H_2O_2 for 1 h and then incubated with DCHF-DA. **A**: wild type cells; **B**: rho^0 mutant cells. Legend: gray bars: 0 mM H_2O_2; dotted bars: 1 mM H_2O_2; diagonal bars: 4 mM H_2O_2; unfilled bars: 10 mM H_2O_2. The data shows the mean ± SD from triplicate experiments.

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3.4. DISCUSSION

The results showed that the wild type strain and not the rho^0 mutant strain was able to adapt to lethal H_2O_2 concentrations following 24 h starvation. In fact, the rho^0 mutant strain was hyper-sensitive to H_2O_2. Previous studies (Grant et al., 1997, Jamieson, 1992) also showed that both the wild type and the rho^0 mutant strains were able to adapt to lethal H_2O_2 concentrations following pre-treatment with sub-lethal concentrations of H_2O_2. In addition, the rho^0 mutant strain was hyper-sensitive to H_2O_2 compared with the wild type strain. Grant et al.,1997) suggested that the oxidation sensitivity of rho^0 mutant strain was due to a defect in an energy requiring process that is needed for detoxification of ROS or for the repair of oxidatively damaged molecules. The ability of the wild type strain to adapt to lethal concentration of oxidants could also be due to increased synthesis of anti-oxidants in the cells. A previous study (Sousa-Lopes et al., 2004) had suggested that the ability of the wild type strain to adapt to oxidation could also be due to the decrease in the permeability and the uptake of oxidants at the plasma membrane. They showed that the permeability constant for H_2O_2 is 5 times lower in the cells grown to stationary phase than in exponentially growing cells, and also that the consumption of H_2O_2 was higher in exponentially growing cells, despite low amount of scavenging enzymes. Starved yeast cells have a thick, less porous cell wall and, under certain conditions, contain mitochondria that are more rounded and numerous than those observed in exponentially growing cells (Werner-Washburne, 1993).

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The pre-treatment of both the wild type and the rho^0 mutant strains with *M. flabellifolia* polyphenols showed that polyphenols were toxic to the cells only after prolonged incubation. The wild type strain lost 35 % viability after pre-treatment with 650 µg/ml polyphenols for 24 h while the rho^0 mutant strains lost 20 % viability after 24 h pre-treatment with the same polyphenol concentration. These results were contrary to the results in previous section in which viability was determined by plating the yeast cells on agar plates and then evaluating the difference in the number of the colonies with the un-treated cells.

The present study showed that polyphenols can act as anti-oxidants and pro-oxidants in certain conditions by producing ROS. Pre-treatment of the wild type strain with polyphenols did not protect the cells, but instead promoted cell death. However, 24 h pre-treatment with polyphenols protected the rho^0 mutants. The pro-oxidant effects seen in the wild type strain may be brought about by the production of H_2O_2 due to auto-oxidation of polyphenols as seen with other polyphenols such as epigallocatechin gallate (EGCG). The mechanism for the generation of reactive species by auto-oxidation of EGCG has already been proposed (Sang et al., 2005). They proposed that under neutral or slightly alkaline pH in cell culture medium, EGCG, cathechins and other polyphenols can be oxidized to form phenolic radicals, superoxide radicals ($^{\cdot}O_2^-$), and H_2O_2 in a reaction catalyzed by metal ions such as Cu^{2+} and Fe^{3+}. The $^{\cdot}O_2^-$ can react with another EGCG molecule to form $^{\cdot}$EGCG which can then collide with an equivalent molecule of $^{\cdot}$EGCG to form an EGCG dimer. More likely, EGCG may react with EGCG to form EGCG dimer radical, which has the potential to react with molecular O_2 to generate EGCG dimers and $^{\cdot}O_2^-$.

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The O_2^- generated can be converted to H_2O_2 in the presence of superoxide dismutase (SOD) (Sang et al., 2005).

Furthermore, this study has shown that *M. flabellifolia* polyphenols can produce small amounts of ROS both *in vivo* and *in vitro*. In addition, polyphenols were able to scavenge considerable amounts of H_2O_2. These results showed that incubation of wild type cells with H_2O_2 in the absence of polyphenols resulted in significantly reduced DCHF-DA oxidation. A possible explanation for these effects on the wild type strain by H_2O_2 is that the anti-oxidant defence systems of these cells reduced the oxidation of H_2O_2. Since the anti-oxidants properties of these polyphenols were only seen in cells that lack the mitochondrial DNA, it is possible that the electron transport chain contribute to the oxidation of polyphenols.

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CHAPTER FOUR

CONCLUSIONS

The aims of this study were divided into 2 parts. Firstly the effects of *M. flabellifolia* polyphenols on growth of *S. cerevisiae* yeast strains were investigated. Growth of both strains was monitored using an automatic plate reader, after which the growth curves generated were fitted with either the Gompertz or Logistic models. This method provided a rapid and efficient way to generate and analyse growth curves of micro-organisms. The results showed that polyphenols inhibited growth of both the wild type and the *Δhsp12* yeast strains by mostly binding proteins rather than chelating metal ions nor binding to the yeast cell walls. The present findings suggested that *M. flabellifolia* plant extracts should be used with caution when treating ailments as these might affect the gastrointestinal microbial population.

The second part of this work involved the investigation of the anti-oxidant properties of *M. flabellifolia* using wild type and rho^0 mutant yeast strains. Flow cytometry using propidium iodide was used to analyse the viability of cells after pre-treatment with polyphenols and /or H_2O_2. Pre-treatment of the wild type strain with polyphenols followed by treatment with H_2O_2 did not protect cells against oxidation but instead promoted cell death. However, rho^0 mutant cells were protected by polyphenols against oxidation. These studies have provided a rapid and simple method to investigate the effect of antimicrobial compound on the growth of micro-organisms. This method may also be used industrially to assess the stress status of strains used in fermentation or other applications.

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