Chemical Genetic Analysis of *Alepidea amatymbica*, a traditional medicine in Africa

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Abstract

Natural products are a reliable source of drug leads, with plant natural products contributing some of modern medicine's most important pharmaceuticals. Africa has a rich source of medicinal plants that have not yet been heavily investigated for their therapeutic potential. One such plant is *Alepidea amatymbica*. The traditional use of *A. amatymbica* suggests a broad range of bioactivity that has yet to be fully explored. In this thesis, the mechanism of action of *A. amatymbica* extract and semi-purified compounds were explored using chemical genetics. Using the genetic model *Saccharomyces cerevisiae* (Baker's yeast), initial phenotypic screening of the crude extract and the semi-purified compound B showed that *A. amatymbica* is a potential substrate of the pleiotropic drug response system. A genome-wide analysis, using the haploid deletion collection in the *pdr1*Δ*pdr3*Δ background, revealed five genes that when deleted showed significantly reduced growth in the presence of *A. amatymbica*. These were Din7, Ura5, Eft2, Glo2 and Get5. The functions of the first four genes are mitochondrial genome stability, *de novo* pyrimidine biosynthesis, ribosomal translocation and a function relating to glyoxalase system, respectively. The most sensitive gene, Get5, is a member of the guided-entry tail anchored protein (Get) complex involved in tail anchor (TA) protein biosynthesis. Upon further investigation, we found the entire Get family, as well as their interacting chaperones, to be bioactive in *A. amatymbica.* Further evaluation of the Get pathway was conducted by overexpressing Get3 in *pdr1*Δ*pdr3*Δ*get1*Δ and *pdr1*Δ*pdr3*Δ*get2*Δ coincidently with *A. amatymbica* extract treatment, where we found that Get3 overexpression confers sensitivity to *A. amatymbica*. A proteomic analysis using a GFP library was conducted to investigate the mislocalization of TA protein when the cells were treated with *A. amatymbica* extract, whereby changes in localization of ER proteins Erg9 and Cyb5 were detected. Together, these results identified genes, proteins and pathways involved in buffering the activity of *A. amatymbica* extract.

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Abbreviations

ABC	ATP-Binding Cassette
CM	Complete Minimum
DMA	Deletion Mutant Array
DMSO	Dimethyl Sulfoxide
ER	Endoplasmic Reticulum
GET	Guided entry tail anchored protein
GFP	Green Fluorescent Protein
GO	Gene Ontology
HTS	High-Throughput Screen
NP	Natural product
ORF	Open Reading Frame
PDR	Pleiotropic Drug Resistance
SC	Synthetic Complete
SD	Synthetic Dropout
TA	Tail anchored (protein)
WT	Wild Type
NCE	New chemical entities
TOR	Target of rapamycin
YPD	Yeast extract peptone dextrose

Chapter 1

Literature review

1.1 Drug discovery

New drugs are continually being needed to address unmet medical needs in our healthcare systems. For this reason, there is a perpetual need for drug discovery and development (Sinha & Vohora, 2018). Drug discovery is both a resource and time-consuming process. Generally, the process involves target identification, hit identification, lead generation and optimization and finally the identification of a candidate for further development (Mohs & Greig, 2017).

Drug discovery of natural products is broadly distinguished based on three different approaches. In a bioactive-guided screening approach, a known synthetic or semi-purified compound or a crude extract is screened for biological activity using cell-based assays where the cellular drug target is unknown. This is done early in the experimental process and often involves screening a large number of compounds using defined biochemical assays on biologically relevant surrogates to predict the response of the organism (Clemons, 2004). A bioactive hit prompts further study to determine the cellular target and mechanism of action (Lee et al., 2012).

In contrast, a chemical screening approach does not begin with investigating biological activity. Instead, the extract is screened for chemical novelty before a biological assay is done. For this, structural elucidation is done using sophisticated analytical methods like nuclear magnetic resonance (Bracegirdle et al., 2020) and mass spectrometry (Wohlleben et al., 2016). Presently, large databases exist where mass spectra data can be compared to determine if what is obtained is already known. In the third approach, target-oriented screening, a known molecular target is used to predict compounds that are able to interact with the target. In this

case, the researcher has a pathology of interest in mind and the drug is meant to hit the molecular target involved in that pathology (Wohlleben et al., 2016).

1.2 Natural products

Natural products are chemical entities formed by living organisms, with pharmacological properties that can be used in drug discovery and design (Mathur & Hoskins, 2017). The molecules are not essential for growth and reproduction but generally function in assisting and improving the survival of the organism such as chemical defence against predators (Baker et al., 2007). Many of these molecules are intermediates derived from basic biosynthetic pathways that have undergone modifications in response to abiotic and biotic stress (Baker et al., 2007). As a result, many secondary metabolites possess drug-like properties that have contributed to drug discovery (Clardy & Walsh, 2004).

The testament of this is seen in today's pharmacopeia. The discovery of penicillin in 1928 began an era of modern drug discovery programmes. From 1940 onwards, many microorganisms were being screened for bacterial metabolites (Baltz, 2008). Many of these have become the mainstay of drug prescriptions in antibacterial therapeutics where they account for the majority of antibiotics used in clinics. These drugs include erythromycin, streptomycin, tetracycline and vancomycin (Katz & Baltz, 2016). Most successful new drugs, drug leads and new chemical entities (NCE) were sourced from natural products. It is estimated that prior to the era of genomics and high-throughput screening, more than 80% of drugs were derived or inspired by natural products (Harvey, 2008). More recently, in the period spanning 1981 to 2014, 38.1% of all drugs approved by the US Food and Drug Administration were either unaltered natural products, natural products derivatives or they were synthetics with natural products pharmacophore (Newman & Cragg, 2016). In 2019, 28% of NCE's were derived from natural products while of all small molecules approved between 1981 and 2019,

natural products accounted for 36.3% (Newman & Cragg, 2020). This shows that natural products are a reliable source of new drugs or drug leads.

1.3 Natural products research over the years

Towards the end of the 1990s, approximately 80% of drugs were either natural products or natural product analogues (Li & Vederas, 2009). However, natural product research began to decline for several reasons. Regulatory concerns regarding access to biodiversity (based on the UN convention on biodiversity) thereby causing difficulty in patenting natural products-based drugs (Harrison, 2014), incompatibility with high through-put drug screening and discovery processes (Harvey, 2008), the high rediscovery rate of known compounds (Zarins-Tutt et al., 2016), and complex natural product structures leading to difficult syntheses (Harvey et al., 2015) have contributed to the waning interest, particularly in the pharmaceutical industry.

Consequently, focus began to shift towards high-throughput synthetic drug discovery utilizing man-made chemicals instead of natural ingredients (Li et al., 2019; Thomford et al., 2018). Unfortunately, this strategic shift gave an overall reduction in novel lead compounds, leading to a substantial decline in new drug approval (Li & Vederas, 2009). Emphasis was subsequently made for more random screening, with assumptions that the low hanging fruits have all been picked and only through larger more rigorous screening can the few remaining be harvested (Monciardini et al., 2014). But as Newman and Cragg (2007) observed, this was also met with limited success with very few lead structures and drug candidates discovered over a two decade period (Newman & Cragg, 2007). The lack of success is attributable to synthetic compounds occupying a limited chemical space unlike natural products that have higher chemical diversity and are evolutionary optimized for biochemical interactions such as binding to specific target proteins or biomolecules that are pertinent to a biological function (Rosén et al., 2009). This outcome suggested the diversity within biologically relevant

'chemical space' might be more important than library size, prompting a re-shift towards diversity-oriented syntheses in order to improve the hit rates (Lenci et al., 2018). This inevitably brings natural products back to the forefront of drug discovery since they provide good models for chemically-diverse syntheses.

Many of the concerns associated with natural products research have been circumvented with technological advancements in analytical techniques leading to improvements in natural products compatibility in high-throughput screening, isolation, dereplication and lead optimisation, in addition to improvements in chemical syntheses (Atanasov et al., 2021; Li & Vederas, 2009; Thomford et al., 2018). Furthermore, the emergence of genome mining and engineering have reinvigorated the field (Atanasov et al., 2021).Thus, there has been a resurgence in the use of natural products for new drug discovery and natural products research is yet again gaining traction in the field (Li et al., 2019).

1.4 Traditional medicine

The use of natural products in medicine dates back to antiquity, with the earliest use of medicinal plant recorded in the Paleolithic age some 60,000 years ago (Fabricant & Farnsworth, 2001). The World Health Organization defines traditional medicine as any non-Western medical practice (Karunamoorthi et al., 2013). The Unani and Ayurvedic medicine existed 2,500 years ago in Greece and India (Ravishankar & Shukla, 2007; Yuan et al., 2016). Traditional medicines in Asia have flourished since 221BC (Wang et al., 2020; Yuan et al., 2016). These, and other ethno-medicines employ various natural products in the treatment of diseases. Examples of these are *Cupressus sempevirens* (cypress oil), cedar oils (*Cedrus* sp.), licorice oils (*Glycyrrhiza glabra*), myrrh (*Commiphora sp.*), poppy juice (*Papaver somniferum*), *Spiraea ulmaria* and cinchona (Patridge et al., 2016; Permin et al., 2016).

Of these, the opium plant, *Papaver somniferum,* is known for the pharmacologically-active compound – morphine. The drug was first isolated in 1805 by Friedrich Sertuner, setting the stage for the beginning of drug discovery (Joo, 2014). Subsequently, other active compounds were uncovered including for instance in 1820, the antimalarial quinine alkaloids which interestingly followed from the traditional use of the cinchona plant from which it was isolated (Dvorkin-Camiel & Whelan, 2008). Other drugs were later isolated from their medicinal plant sources. Most notable of these is the antimalarial drug artemisinin, which was derived from the Chinese medicinal plant *Artemisia annua* in 1972, and whose discovery led to the 2015 Nobel Prize in Medicine (Su, 2015).

1.5 Plant natural products

Plants have been valuable sources of new drugs. It is known that plants have been used as medicine for millennia. Indeed many of the drugs in use today have been derived from plant natural products (Newman & Cragg, 2020). However, the scientific breakthrough in plant based natural products discovery perhaps occurred with the 1785 discovery of digoxin from *Digitalis purpurea* by William Withering (Krikler, 1985). Discovery of chemical compounds from other *Digitalis* spp. followed with the isolation of acetyldigoxin, digitalin, digitoxin, deslanoside, and lanatosides A, B and C from D. *lanata* (Lahlou, 2013).

This initiated research interests into other medicinal plants, leading to the discovery of many other plant based natural products. As mentioned earlier, this includes: morphine, quinine alkaloids and artemisinin. Initially, efforts were limited to identifying and isolating natural products from their plant sources, and later synthesis was employed to increase production and ease down costs of producing these natural products. The first of such compounds to have been synthesized was salicylic acid in 1853 (Wood, 2015). Plant-derived natural compounds have since become indispensable for modern pharmacotherapy, with prominent examples in the field

of anticancer agents. This includes paclitaxel that was derived from the Pacific yew (Taxus) tree, vincristine and vinblastine that came from periwinkle, and camptothecin isolated from *Camptotheca acuminata Decne* (Kingston & Newman, 2002).

1.6 African medicinal plants

The African continent is home to a rich source of plant diversity containing about 25% of the world's higher plants (van Wyk, 2008). Of these, around 5,400 medicinal plant taxa have been recorded with over 16,300 medicinal uses (Neuwinger, 2000). With high regular usage of traditional medicine in 80% of the continental population and rather costly western medicine option (Arnold et al., 2002; Okoli & Mtunzi, 2017; Wyk & Gericke, 2000), Africa has an informal medicine market that is worth potentially millions of dollars (Cunningham et al., 1988). Despite its popularity, there is little scientific research being done on these traditional medicines leading to a generally poor understanding of the science behind these traditional medicine. Over the years, the recognition of the importance of natural resources towards socio-economic development (Geldenhuys & Wyk, 2002) has reignited scientific interests in these medicinal plants.

This has led to ethnobotanical and ethnomedical surveys on commercially important plants of Africa documenting not only their traditional uses but also their developmental challenges to commercialization. Other studies have sought to investigate the bioactivities specific to medicinal plants of interest. A subset of these plants are shown in the table below.

Medicinal plant	**Bioactivity or traditional use**	**Constituents**	**Countries**	**Reference**
Prunus africana	Prostate gland hypertrophy	Sterols, triterpenes, n-docosanol	Cameroon, Kenya, Madagascar	(Abdullahi, 2011)
Acacia ataxacantha	Cough , yellow fever	-	Nigeria	(Dambatta & Aliyu, 2012)
Enantia chlorantha	Hepatitis	Protobeberine	Cameroon	(Fokunang et al., 2011)
Equisetum arvense	Inflammation	-	South America	(Madikizela et al., 2017)
Artemisia afra	Sedative, CNS related ailments	Monoterpenes	South Africa	(Stafford et al., 2005)
Agathosma betulina	antispasmodic, antipyretic, cough remedy, diuretic	limonene, menthone, diosphenol,l-pulegone	South Africa	(Street & Prinsloo, 2013)
Dalbergia melanoxylon	Rashes	-	South Africa	(Tshikalange et al., 2016)
Alepidea amatymbica	Antimicrobial, anti-fungal	Diterpenes	South Africa	(Afolayan & Lewu, 2009)
Gunnera perpensa	Anti-bacterial, anti-fungal	-	South Africa	(Buwa & van Staden, 2006)
Combretum micranthum	Anti-bacterial, antifungal	Tannins, flavonoids, terpenoids, stilbenoids	South Africa	(Eloff et al., 2008)
Gladiolus quartinianus	Anti-cancer	-	Cameroon	(Kuete et al., 2013)
Elephantorrhiza burkei	Anti-bacterial	-	South Africa	(Madikizela et al., 2017)

Table 1.1: Medicinal plants of Africa**.** The bioactivity or traditional use, the bioactive components, and geographic location indicated for each plant. -, unknown**.**

The use of African plants in traditional medicine can be traced as far back as 2000BC (Busia, 2005). In South Africa, like in many parts of Africa, traditional medicine represents a hidden informal economy that is less supported than its western counterpart (Cunningham et

al., 1988). Nevertheless, South Africa has similarities with Japan in its dual system of 'western' and indigenous medicine (Busia, 2005).

It has been observed that many medicinal plants in Africa exhibit bioactivities relating to anti-inflammation and immune modulation (Gqaleni et al., 2012; Madikizela et al., 2017; Patel et al., 2018), antimicrobial and antifungal (Mulaudzi et al., 2009; Ngouana et al., 2011; Okoli & Mtunzi, 2017; Sunday Uko et al., 2019), anti-cancer (Kuete et al., 2013; Mbaveng et al., 2017; Otang et al., 2014) as well as anti-diabetic (Ocloo & Dongdem, 2011; Odeyemi & Bradley, 2018).

1.7 ***Alepidea amatymbica*** (Apiaceae)

The plant in this study *Alepidea amatymbica* Eckl. & Zeyh (also known as *Alepidea aquatica Kuntze, Eryngium amatymbica, kalmoes; Iqwili; ikhathazo (Zulu)* and tinsel flower), is a herbaceous plant found throughout the African continent but more commonly in the grasslands of the Eastern and South African region (Thayer & Austin, 1992). It is one of 27 species of the genus *Alepidea*, and one of six in the genus known to be used as traditional medicine (Lahlou, 2013). The plant is robust and erect with dark green leaves arising from a single or branched rhizome that has a toothed and bristle margin. It grows up to two metres in height and carries a flowering stalk holding numerous small flowers arranged in dense, rounded heads (**Fig. 1.1**) (Afolayan & Lewu, 2009).

Its traditional medicinal use covers a range of general treatments such as cold, chest pain, asthma, influenza, diarrhoea, abdominal cramp, sore throat and rheumatism (Wintola & Afolayan, 2014). An infusion or decoction of the bark is taken as cure to these mentioned conditions (Maroyi, 2008). Alternatively, the rhizomes and roots are chewed or smoked (Afolayan & Lewu, 2009). Its use has been recorded in six countries across Southern and

Eastern Africa, including South Africa, Swaziland, Lesotho, Zimbabwe Kenya and Ethiopia. It is particularly popular in South Africa where it is commercially traded under the Zulu name 'Ikhathazo' (Afolayan & Lewu, 2009). Preliminary studies on the extract of *A. amatymbica* indicate bioactivity relating to anti-microbial, anti-fungal, anti-helminthic, anti-plasmodial, anti-hypertensive, anti-inflammatory and anti-viral activities (Wintola & Afolayan, 2014). In traditional medicine, the extract is highly regarded as a remedy for respiratory tract infections, asthma, sore throat, gastrointestinal complaints, fever, rheumatism, bleeding wounds, and headache as well as activity against HIV (Louvel et al., 2013). However, there is a dearth in scientific studies investigating the underlying bioactivity for drug development beyond these folklore uses (Wintola & Afolayan, 2014). For instance, antifungal studies conducted on *Candida albicans*, *Aspergillus flavus* and, *Aspergillus niger* yielded inconclusive results (Muleya et al., 2017).

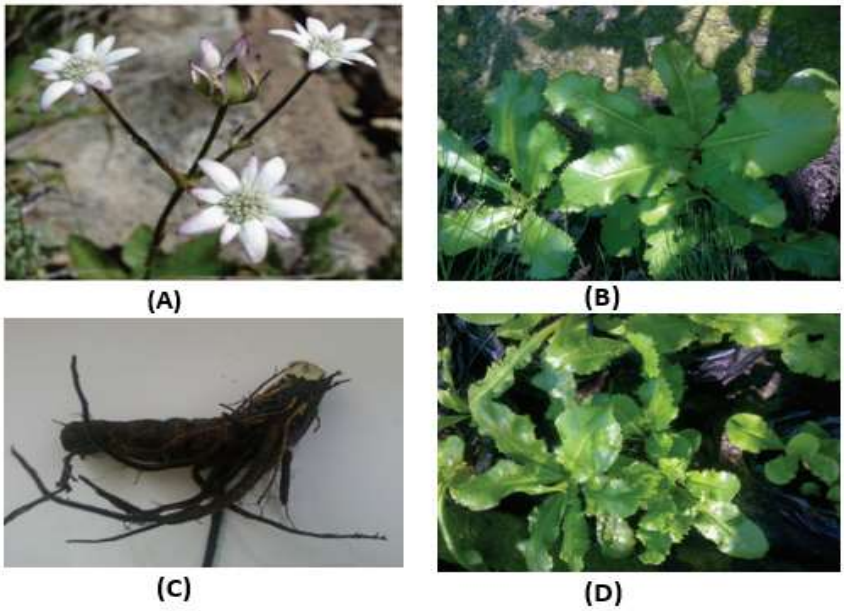


Figure 1.1: Images of *Alepidea amatymbica* (A-B) In its natural habit, (C) as a dried rhizome (D) growing in the nursery (Afolayan & Lewu, 2009)**.**

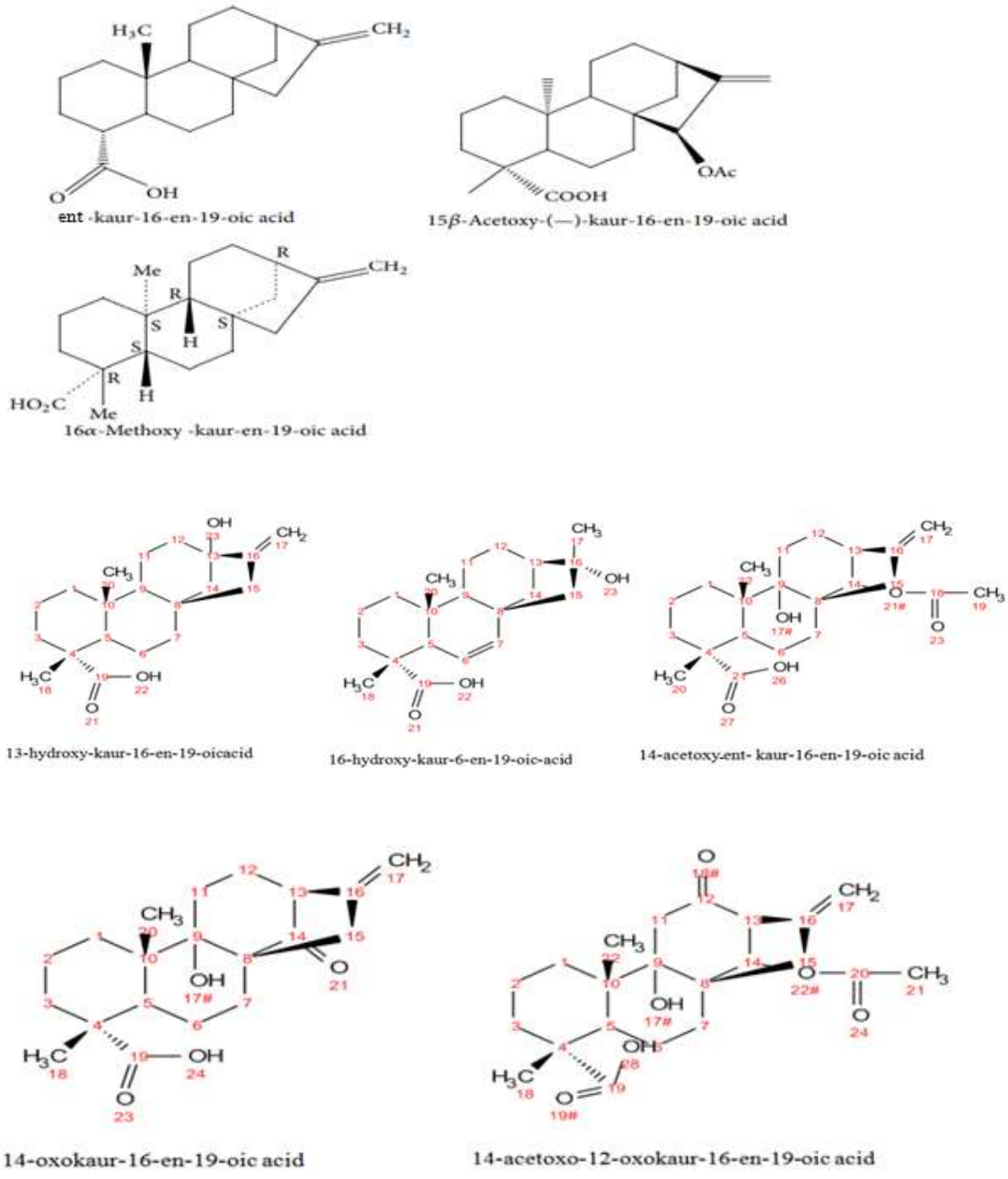


Figure 1.2: Chemical structures of kaurene-type diterpenoids in *Alepidea amatymbica* (Afolayan & Lewu, 2009; Muleya et al., 2017).

Phytochemicals	Compound	Plant source	Reference
Terpenes (kaurene-type diterpenes)	ent-9, (11)-dehydro-16-kauren-19-oic acid	Rhizomes, root	(Rustaiyan & Sadjadi, 1987)
	ent-16-kauren-19-oic acid	Dried rhizomes	(Rustaiyan & Sadjadi, 1987)
	Wedelia seco-kaurenolide	Dried rhizomes	(Rustaiyan & Sadjadi, 1987)
	13-acetoxy	Dried rhizomes	(Rustaiyan & Sadjadi, 1987)
	13-hydroxy-kaur-16-en-oicacid	Dried roots	(Muleya et al., 2017)
	16-hydroxy-kaur-6-en-19-oic-acid	Dried roots	(Muleya et al., 2017)
	14-acetoxy ent-kaur-16-en-19-oic acid	Dried roots	(Muleya et al., 2017)
	14-oxo-16-en-19-oic acid	Dried roots	(Muleya et al., 2017)
	14-acetoxo-12-oxokaur-16-en-19-oic acid	Dried roots	(Muleya et al., 2017)
Phenolic acid	Phenolic acid	Rhizomes	(Louvel et al., 2013)
Rosmarinic acid	3’ -O-β-d-Glucopyranosyl rosmarinic acid	Rhizomes	(Louvel et al., 2013)

Table 1.2: Compounds isolated from *A. amatymbica*

1.8 Kaurene diterpenes

It has been hypothesized that the bioactivities of *A. amatymbica* can be attributed to kaurene-type diterpenoids and their derivatives (**Fig. 1.2; Table 1.2**) which constitute 11.8% of rhizome and root dry mass (Wintola & Afolayan, 2014). However, most of these compounds have not been tested individually (Afolayan & Lewu, 2009).

A study of five diterpenic acids isolated from *A. amatymbica* showed all were antimicrobial (Muleya et al., 2017). These diterpenoids (13-hydroxy-16-kauren-19-oic acid, 16-hydroxy-kaur-6-en-19-oic acid, 14-acetoxy ent-kaur-16-en-19-oic acid, 14-oxokaur-16-en-19-oic acid, and 14-acetoxo-12-oxokaur-16-en-19-oic acid), inhibited the growth of *Psuedomonas aeruginosa* and *Enterococcus faecalis.* One of them, 14-acetoxo-12-oxokaur-16-en19-oic acid, showed potency against *Staphylococcus aureus*, *E. faecalis* and *Escherichia coli*, and a relatively high lipoxygenase inhibition activity. The isolated diterpenoids were also shown to be toxic to cancer cell lines. This suggests that the diterpenic acids might be responsible for the biological activities of *A. amatybica* extract in traditional medicine.

1.9 Chemical genetics

In drug discovery, understanding the mechanism of action of a bioactive compound or extract is important (Parsons et al., 2004). Chemical genetics is an approach used to study genes involved in extract/compound bioactivity (Cong et al., 2012).

In combination with a tractable model organism and molecular toolkit, chemical genetics is a powerful technique in deducing cellular drug targets (Cong et al., 2012). One such model organism is the unicellular eukaryote *Saccharomyces cerevisiae* (Baker's yeast), where a genome-wide collection of defined gene deletion mutants is exposed to a compound and the fitness of individual mutants is measured to determine the contribution of each gene to

bioactivity. Gene deletions that render cells hypersensitive to a drug/extract identify the importance of that gene and associated pathways to the mode of action (Giaever & Nislow, 2014). This is based on the concept that the primary effect of a drug is to bind a gene product in a manner that inhibits its function (O'Connor et al., 2011). Hence by deleting the corresponding gene target, the primary effect of the drug can be mimicked (O' Connor et al., 2011). Since it is a genome-wide analysis, the technique provides an unbiased view of the cellular response (Piotrowski et al., 2017). Overall, the mode of action of thousands of compounds and extracts has been elucidated using chemical genetic analyses in yeast (Piotrowski et al., 2017)

The use of yeast as a chemical genetic tool has had a profound impact in the field due to its known genome and available genomic reagents and tools (Piotrowski et al., 2017). Libraries comprised of deletions of non-essential genes, knockdowns of essential genes, overexpressions of non-essential and essential genes, and fluorescently tagged non-essential and essential proteins are all vital tools to genome-wide analyses in yeast. Not only is the organism amenable to genetic manipulations, it also has a relatively short doubling time and is cheap and can be easily cultured. Also, it has been observed that 23% of yeast genes have homology to human genes (Liu et al., 2017). This conservation between human and yeast genes makes yeast an ideal organism for study of human conditions.

Chemical genetic analysis identifies a chemical genetic interaction, whereby a chemical probe (a bioactive compound) results in the growth defect of a gene deletion mutant compared to growth in the absence of the chemical probe. Further, a gene deletion collection can be utilized for elucidation of compound mechanism of action by subjecting it to the chemical probe. A chemical genetic profile can be created from gene deletion mutants exhibiting a growth defect or sensitivity as a result of exposure to the chemical perturbation to predict mode of action for a compound/extract (Boone et al., 2007). Further, when an array of mutants is

challenged with a compound and monitored for fitness defects, chemical-genetic interaction profile can be generated that provides a quantitative, unbiased description of the cellular function(s) perturbed by the compound (Simpkins et al., 2018).

1.10 Aim

Since little is known about the chemistry and biology mediating the traditional use of *A. amatymbica*, this thesis aims to characterise the chemical biology of the *A. amatymbica* extract to further understand its traditional use. The specific aims are the following:

Aim 1: To identify the most potent bioactive compound in the *A. amatymbica* extract.

Aim 2: To determine the potential mechanism of action of the *A. amatymbica* extract using chemical genetic analysis in yeast.

Chapter 2:

2.1 Introduction

The *A. amatymbica* extract was prepared by Drs Edwina Muleya and Joe Bracegirdle and Robert Keyzers in a collaboration between Victoria University of Wellington and Midlands State University in Zimbabwe. Here we use chemical genetics to investigate the genes, and pathways underlying the molecular mechanism of the extract. The use of gene deletions, gene overexpressions and GFP-tagged proteins allows us to investigate mechanism of action at the gene and protein level. The yeast deletion collection consists of ~4,300 strains each having a different gene deletion was used to identify genes required to buffer the bioactivity of the extract. A GFP library consisting of ~400 strains each having a uniquely tagged protein, was used to identify proteins that altered in response to the addition of the extract. Together, these were used to deduce the mechanism of action of the extract.

2.2 Materials and methods

2.2.1 Yeast strains

All *S. cerevisiae* strains were derived from the wild-type (WT) Y7092 strain (**Table 2.1**)

Strain	Genotype	Reference
Y7092	*MATα can1Δ::STE2pr-Sp_HIS5; lyp1Δ; his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2*	(Tong & Boone, 2007)
pdr1Δpdr3Δ (Y7092 derived)	*MATα pdr1Δ::natR pdr3Δ::URA3 can1Δ::STE2pr-Sp_HIS5; lyp1Δ; his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2*	(Coorey et al., 2015)
pdr1Δpdr3Δ deletion collection *(*Y7092 derived)	*pdr1::natR pdr3::URA3 xxx::kanR his3Δ1 ura3Δ0 met15Δ1 leu2Δ0 can1::STE2_pr-His5 lyp1Δ*	(Coorey et al., 2015)
BY4741	*MAT*a, *his3Δ1*, *leu2Δ0*,*met15Δ0*, *ura3Δ0*	(Baker Brachmann et al., 1998)
SWAT-NOP1pr-GFP	*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hphΔn::URA3::SpNOP1pr-sfGFP-XXX (or hphΔn::URA3::SpNOP1pr-MTS-sfGFP-XXX or hphΔn::URA3::SpNOP1pr-SPkar2-sfGFP-XXX)*	(Ast et al., 2013)

Table 2.1: Yeast strains used in this thesis**.**

2.2.2 Media

Amino acid composition for Synthetic complete (SC) media:

3 g adenine, 2 g alanine, 2 g asparagine, 2 g aspartic acid, 2 g cysteine, 2 g glutamic acid, 2 g glutamine, 2 g glycine, 2 g histidine, 2 g inositol, 2 g isoleucine, 10 g leucine, 2 g lysine, 2 g methionine, 0.2 g para-aminobenzoic acid, 2 g phenylalanine, 2 g proline, 2 g serine, 2 g threonine, 2 g tryptophan, 2 g tyrosine, 2 g uracil and 2 g valine.

SC agar media composition:

0.1% (w/v) monosodium glutamate, without acids or ammonium sulphate (Sigma-Aldrich), 0.17% (w/v) Yeast Nitrogen Base (Formedium), 0.2% (w/v) amino acid mixture to suit

(Formedium), 2% (w/v) glucose (Sigma-Aldrich) and 2% (w/v) granulated bacteriological grade agar (Formedium).

Synthetic dropout (SD) agar:

SC agar with the absence of specific amino acid(s).

Synthetic dropout (SD) broth:

SC broth with the absence of specific amino acid(s).

Complete Minimum (CM) media composition:

0.2% (w/v) amino acid mixture (Sigma-Life Sciences) at pH 5.8 and 2% (w/v) glucose (Sigma-Aldrich).

CM agar media composition:

0.2% (w/v) amino acid mixture (Sigma-Life Sciences) at pH 5.8, 2% (w/v) glucose (Sigma-Aldrich) and 2% (w/v) granulated bacteriological grade agar (Formedium).

Yeast peptone dextrose (YPD) media composition:

2% (w/v) yeast extract (Formedium), 2% (w/v) peptone (Formedium), 0.012% (w/v) adenine (Formedium) and 2% glucose (Sigma-Aldrich).

YPD agar media composition:

2% (w/v) yeast extract (Formedium), 2% (w/v) peptone (Formedium), 0.012% (w/v) adenine (Formedium), 2% (w/v) agar granulated bacteriological grade (Formedium) and 2% glucose (Sigma-Aldrich).

LB agar:

0.5% (w/v) yeast extract (Formedium), 1% (w/v) tryptone (Formedium), 0.5% (w/v) sodium chloride (Thermo Fisher Scientific) and 2% (w/v) agar granulated bacteriological grade (Formedium).

LB broth:

0.5% (w/v) yeast extract (Formedium), 1% (w/v) tryptone (Formedium) and 0.5% (w/v) sodium chloride (Thermo Fisher Scientific).

2.2.3 Compound preparation

The *A. amatymbica* extract and all semi-purified compounds (including compound B) were prepared by Drs. Edwina Muleya, Joe Bracegirdle and Rob Keyzers at Victoria University of Wellington. The extracts and compounds were dissolved in DMSO.

2.2.4 Liquid-based bioactivity assays

Bioactivity was assessed by quantifying the growth of yeast as described previously (Treco & Winston, 2008). Yeast strains were freshly streaked from frozen stocks onto YPD agar and incubated at 30°C for 48 h. A single colony was inoculated into 2 mL of relevant media and incubated at 30°C for 16-18 h with constant rotation. Optical density (OD) of the overnight culture was measured at 660 nm using a spectrophotometer (Jenway Genova MK3), and then diluted in 1 mL of relevant media to give a final OD of 0.1. In a 96-well tissue culture plate (Biofill), 99 µL of CM media containing the yeast cells was added, followed by 1 µL of either the *A. amatymbica* extract, a semi purified compound or DMSO vehicle control in triplicates. The plates were shaken on a MixMate plate shaker (Eppendorf) for 30 sec at 1,000 rpm. An initial absorbance reading was taken at time zero (T0) using an Envision 2102 Multilabel plate reader (Perkin Elmer) at 590 nm. The plates were then incubated at 30°C, and OD readings were later taken after 18 h and every hour thereafter until OD 0.4 (mid-log phase) was reached in the control wells. OD readings were normalized by subtracting the T0 OD readings from each time point. Residual growth of the yeast was calculated based on the growth

of the cells in the treated wells in comparison to untreated wells. A two-tailed student t-test was performed to assess significant differences in growth.

2.2.5 Agar-based bioactivity assays

Growth in agar was quantified as previously described (Wagih et al., 2013). In 24-well tissue culture plates (Biofill), 1 µL of extract or compound B was mixed with 1 mL of molten agar (~60^0C) via gentle pipetting. The plates were left to set at room temperature for 1 h. Cells of overnight cultures were then diluted to three different concentrations (1 x 10^7 cells/mL, 1 x 10^5 cells/mL and 1 x 10^3 cells/mL), and 2 µL of each concentration was spotted onto each well. The plates were left to dry for approximately 1 hour before incubation at 30^0C. Photographs were taken at 24 h and 48 h using a digital camera (Canon EOS 600D) and differences in growth were based on visual observation.

2.2.6 Genome-wide analysis

Sensitivity of gene deletion strains in a genome-wide library was measured as previously described (Parsons et al., 2006) . The 4,300 strains in the homozygous haploid deletion library were replicated using an automated replicator (Singer Rotor, HDA) onto 40 mL of CM agar with *A. amatymbica* extract or DMSO vehicle control. Plates were incubated at 30°C for 24 h, photographs were taken using a digital camera (Canon EOS 600D), and growth was quantified using SGA tools (Wagih et al., 2013). All deletion mutants with Z-score greater than 1 or less than -1 were chosen for validations using liquid growth assays as previously described.

2.2.7 Genomic DNA extraction

Genomic DNA extractions were performed as described previously (Sobanski & Dickinson, 1995). First, 1 mL of overnight grown yeast culture was centrifuged in a 1.5 mL tube. Then 200 µL of DNA breaking buffer and 100 µL of beads were added onto the pellets, followed by the addition of 200 µL of phenol:chloroform:isoamyl alcohol (25:24:1), and the tubes were vortexed for 5 min, and centrifuged for 5 min. The aqueous phase was added to a tube with 1 mL 95% ethanol and then centrifuged for 5 min before the supernatant was discarded and the pellets were dried, resuspended in 40 µL TE, and stored in -20^{0}C.

2.2.8 Proteome-wide analysis

Using a green fluorescent protein (GFP) library of *S. cerevisiae* strains (Weill et al., 2018), localization of 320 proteins in response to the *A. amatymbica* extract was monitored using confocal fluorescent microscopy. The 320 strains were pinned in 384 colony format on SC agar, grown for 16 h at 30°C, and then pinned into a Cell Carrier optically clear bottom 384 well plate (Perkin Elmer) treated with 0.003% SDS, and incubated at 30^{0}C for 4 h. The plates were then treated with either vehicle control (0.5% DMSO) or three extract treatments (15 µg/mL, 20 µg/mL or 25 µg/mL), shaken for 15 sec at 2,000 rpm with a MixMate plate shaker (Eppendorf), and incubated at 30^{0}C for 2 h. GFP fluorescence was imaged using an IN Cell Analyzer 7.3, 6500 HS high-throughput confocal microscope (General Electric), with the following specifications: Objective: Nikon 60X/0.95, Imaging mode: 2D, green excitation: 488 nm, emission: 524 nm, exposure: 1000 ms, laser Power: 100%, brightfield: default values using, DIC filter, and images analysed by eye (specifically two independent persons).

2.2.9 PCR amplification:

All PCRs were performed using BIO RAD T100™ Thermal Cycler (Bio-Rad Laboratories) with reagents and volume given in **Table 2.2.** The reaction conditions are given in **Table 2.4** and **Table 2.5.** Specifically 5 µL of PCR products were electrophoresed on a 1% agarose gel stained with ethidium bromide and visualised under UV to ensure the PCR products were of the correct size when compared to 1 kb Plus DNA Ladder (Thermo Scientific™).

Reagent	**Reaction volume (µL)**
H_20	18.875
10X Buffer	2.5
dNTPs	2
Deletion primer (Forward) or confirmation primer forward external	0.5
Deletion primer (Reverse) or confirmation primer reverse external	0.5
rTaq	0.125
Plasmid DNA	0.5

Table 2.2: PCR reagents and reaction volumes

2.2.10 Construction of deletion cassette

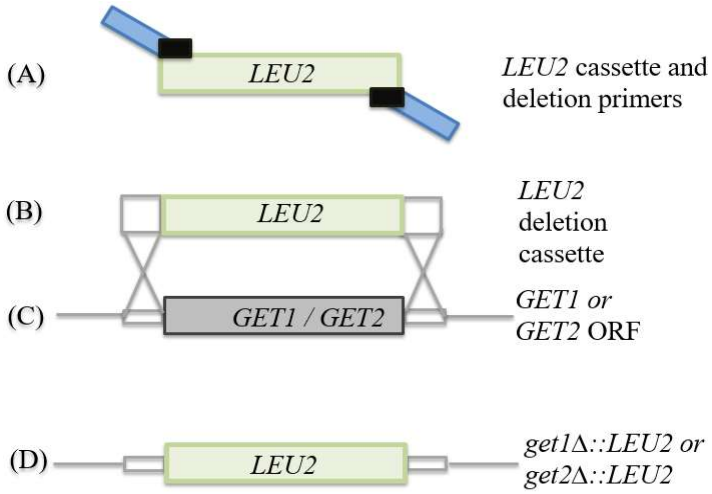
The *LEU2* deletion cassette was constructed by amplifying the gene from *pUG73* plasmid with deletion primers listed in **Table 2.3**. Each 68 base pair deletion primer consists of an 18 bp region with homology to the 5' or 3' ends of the *LEU2* cassette and the remaining 50 bp region with homology to the 5' or 3' flanking genomic regions of the *GET2* or *GET2* locus. The *GET1* and *GET2* locus were replaced by *LEU2* cassette by homologous recombination resulting in *get1::LEU2* and *get2::LEU2* mutants (**Fig. 2.1**). The *LEU2* gene in the mutants permits selection in media lacking the amino acid leucine.

Primer	Sequence 5' to 3'
GET1 deletion primer forward	TTGCAATCCTTGAACTACGTCTAGTTGATTGA
	AATAGGAGAAATGAGCTGAAGCTTCGTACGC
GET1 deletion primer reverse	AATACATAAACATATTATATATACGTACATAA
	TGTAATAACACTACATAGGCCACTAGTGGATCTG
GET2 deletion primer forward	AGCGCAGATTACTAAGAGAAAGGAGACAGAAGAAGT
	TTAGCAATGAGCTGATGAAGCTTCGTACGC
GET2 deletion primer reverse	TCAGGTTGGCGACGGGAATTACTCCCTCGGGCACCAGTG
	AAACTACATAGGCCACTAGTGGATCTG

Table 2.3: Deletion PCR primers**.** The deletion primers were designed with 50 bp of homology to the 5' or 3' flanking regions of Get1 or Get2 loci and 18 bp with homology to *Leu2* cassette.

PCR phase	Temperature (^{0}C)	Time	Number of cycles
Initial denaturation	94^{0}C	5 minutes	1
Denaturation	94 ^{0}C	30 seconds	36
Annealing	58 ^{0}C	1 minute	36
Extension	68 ^{0}C	3 minutes	36
Final extension	72 ^{0}C	10 minutes	1

Table 2.4: PCR conditions for the deletion of *GET1* and *GET2*



1Figure 2.1 : Construction of *get1::LEU2* and *get2::LEU2*. (A) Blue bar represents 50 bp sequence of the deletion primer homologous to the 5' or 3' flanking region of the *GET1* or *GET2* locus. Black bar represents 18 bp sequence of the deletion primer homologous to the *LEU2* cassette. (B) *LEU2* deletion cassette from the PCR reaction using the deletion primers and *LEU2* cassette. (B-C) Schematic diagram of recombination event between *LEU2* gene deletion cassette and *GET1* or *GET2* locus leading to (D) *get1::LEU2* or *get2::LEU2* mutant.

2.2.11 Construct confirmation by PCR

Genomic DNA was harvested from a single colony of *get1::LEU2* and *get2::LEU2,* and 5' and 3' genomic flanking regions of the deletion cassette were amplified using PCR-based strategy illustrated in **Fig. 2.2**. Confirmation primers are listed in **Table 2.5** and PCR conditions given in **Table 2.6**.

Primer	Sequence (5' to 3')
Confirmation primer reverse external *get1::LEU2*	CACCAATTTACAGATTCGGATTAAC
Confirmation primer forward external *get1::LEU2*	TTACTAAAGTTTGGTGACAGGAAGC
Confirmation primer reverse external *get2::LEU2*	GAGCATTACTCTCTTTGTGAAAAGG
Confirmation primer forward external *get2::LEU2*	TATACATCGATTTTTCACGAACTCA

Table 2.5: Confirmation PCR primers**.** All primers were constructed according to a few references (Baudin et al., 1993; Wach et al., 1994)**.**

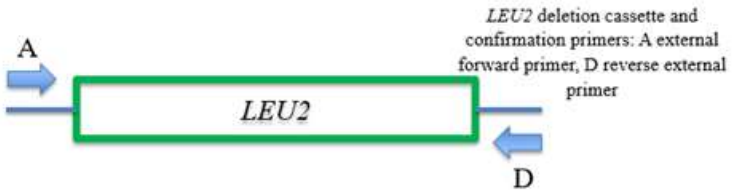


Figure 2.2: Construction of confirmation primers. Strategy used to confirm the correct insertion of *LEU2* deletion cassettes into respective *GET1* and *GET2* loci. 25 bp confirmation primers were used to amplify sequences overlapping the 5' and 3' flanking regions of the deletion cassettes. Direction of the arrows designates the direction of primer extension. Primers denoted as A external forward primer and D reverse external primer (sequences given in **Table 2.5**)**.**

PCR phase	Temperature (^{0}C)	Time	Number of cycles
Initial denaturation	92^{0}C	5 minutes	1
Denaturation	94 ^{0}C	30 seconds	36
Annealing	58 ^{0}C	30 seconds	36
Extension	72 ^{0}C	3 minutes 30 seconds	36
Final extension	72 ^{0}C	10 minutes	1

Table 2.6: PCR conditions for the confirmation of get1::LEU2 and get2::LEU2

2.2.12 Yeast transformation

All transformations were performed as previously described (Gietz & Schiestl, 2007) (**Table 2.7**). Overnight cultures of *get1*Δ and *get2*Δ was sub-cultured in YPD broth to obtain an OD of 0.2, and the culture was grown with shaking at 250 rpm at 30^{0}C until cells were harvested when an OD of 0.8 was achieved. Cells were first washed with 25 mL of dd H_2O and then 10 mL of 1M LiOAc-TE, and then resuspended in 1 mL of LiOAc-TE. 360 µL of the transformation mixture was added to 100 µL of cells, with 4 µL of pBY011 plasmid or water control. Cells were then heat-shocked at 42^{0}C for 40 min, harvested by centrifugation, and resuspended in 1 mL water, of which 300µL was then plated onto SD-Ura agar using a cell spreader and incubated at 30^{0}C for two days.

Transformation mixture components	Volume (µL)
PEG 50% w/v	240
LiAc	36
SS-carrier DNA	50
Plasmid DNA	4
H_2O	30
Total volume	360

Table 2.7: Components of transformation mixture.

2.2.13 Bacterial plasmid DNA extraction

The overexpression bacterial plasmid in the pBY011 vector was extracted from *Escherichia coli* (Birnboim & Doly, 1979). *E. coli* was streaked onto LB+ampicillin agar and incubated at 37^{0}C for 24 h. A single colony was transferred into 2 mL LB+ampicillin and

incubated for 24h at 37^0C with vigorous shaking. The culture was centrifuged, pellets were dried and, resuspended in 100 µL ice-cold solution I, then in 200 µL of freshly prepared ice-cold Solution II, followed by 150 µL of ice-cold solution III. The mixture was centrifuged, the supernatant precipitated in absolute alcohol, allowed to stand at 4^0C for 5 min, then centrifuged, and the supernatant was removed, pellets rinsed in 70% ethanol, centrifuged, air dried, resuspended in 1x TE and stored in -20^0C. Solutions I, II and III were prepared as previously described (Birnboim & Doly, 1979) (**Table 2.8**).

Solution I	**Solution II**	**Solution III**
1 M Glucose – 5 mL 1 M Tris-Cl (pH 8.0) – 25 mL 0.5 M EDTA (pH 8.0)- 2 mL ddH_2O – 68 mL	0.4 M Sodium hydroxide – 1 mL 2 % SDS- 1 mL	5 M Potassium acetate– 60 mL Glacial acetic acid - 11.5 mL ddH_2O – 28.5 mL

Table 2.8: Solutions for bacterial plasmid DNA extraction.

2.2.14 Overexpression assay

Overexpression of genes under the regulation of a galactose promoter was investigated as previously described (Reynolds et al., 1997). *pdr1*Δ*pdr3*Δ*et1*Δ and *pdr1*Δ*pdr3*Δ*get2*Δ transformed with pBY011 overexpression plasmid were grown overnight at 30^0C in synthetic drop out media in 2% raffinose, and then: (1) plated on agar containing 2% glucose (repression) or 2% galactose (overexpression) with appropriate concentrations of *A. amatymbica* extract or compound B, incubated at 30^0C for 24 h, and imaged using a digital camera (Canon EOS 600D); (2) grown in SD-URA and 2% galactose (overexpression) in the presence of *A. amatymbica*.

2.2.15 Dose response of compound B and semi-purified compounds

Bioactivity of semi-purified fractions was measured on agar or in liquid as previously described (**2.2.4** and **2.2.5**).

2.2.16 Gene annotation

Genes found to be sensitive to *A. amatymbica* were analysed using online tools to better understand the mechanism of action of the extract. Spatial Analysis of Functional Enrichment (SAFE) (https://thecellmap.org/) was used to determine processes and pathways critical within those sensitive gene set (Usaj et al., 2017). The YEASTRACT+ information system (http://www.yeastract.com/) was used to predict transcription regulators associated with the sensitive input gene set (Monteiro et al., 2019). GENEONTOLOGY (http://geneontology.org/) was used to map the gene ontology (GO) categories that were statistically over represented within the sensitive input genes (Ashburner et al., 2000).

2.3 Results

2.3.1 The *A. amatymbica* extract is a PDR substrate

The pleiotropic drug resistance (PDR) network in yeast is comprised of drug efflux transporters encoded by the ATP binding cassette (ABC) and major facilitator superfamily (MFS) genes (Kolaczkowska & Goffeau, 1999). The system functions to remove xenobiotics from the cells, leading to less compound accumulating in the cell (DeRisi et al., 2000). The ABC transporters are controlled by the transcription factors Pdr1 and Pdr3. Hence, in PDR-attenuated yeast strains lacking the Pdr1 and Pdr3, the expression of ABC transporters is reduced leading to greater drug sensitivity (Coorey et al., 2015).

To determine if the *A. amatymbica* extract was a substrate of the PDR network, the growth of the PDR-attenuated strain *pdr1*Δ*pdr3*Δ was compared to wild type (BY4741) in the presence and absence of the extract (**Fig. 2.3**). The cells were inoculated in SC media treated with the extract in triplicates at concentrations ranging from 2.4 x 10^{-3} µg/mL to 10 µg/mL. A vehicle control (DMSO) was included as a baseline for normal growth. Growth was measured based on absorbance and residual growth was calculated as the mean percentage of growth in the treated compared to the untreated controls. Based on 40% growth inhibition (60% residual growth) as a cut-off for bioactivity in *pdr1*Δ*pdr3*Δ the extract exhibited bioactivity at 2.5 µg/mL while in WT bioactivity was observed at 10 µg/mL. Overall, the extract was bioactive in both WT and *pdr1*Δ*pdr3*Δ, with more potency observed in *pdr1*Δ*pdr3*Δ. Based on this growth defect, the extract is a likely substrate of the PDR drug efflux pumps. Hence further experiments were conducted using the *pdr1*Δ*pdr3*Δ background.

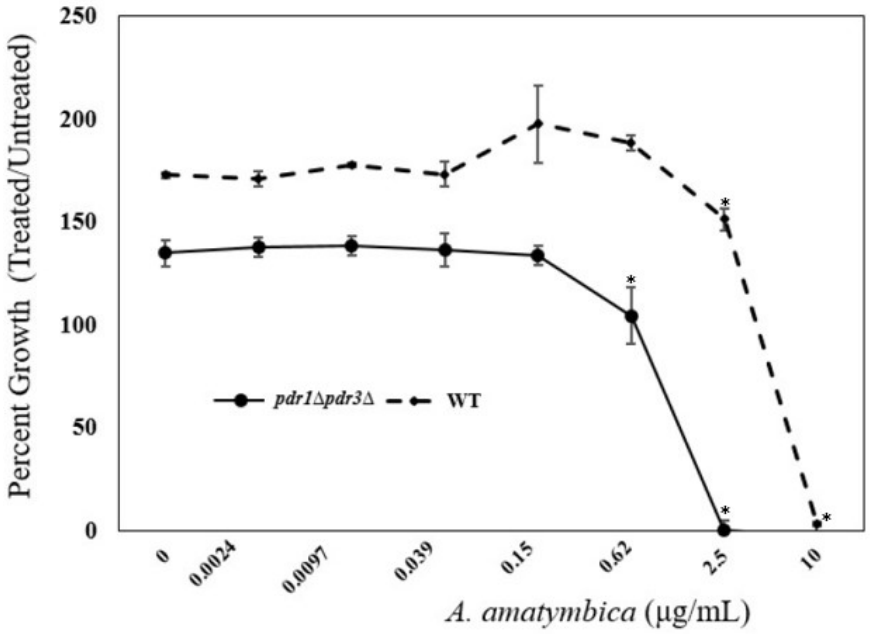


Figure 2.3: Liquid-based assay of WT (BY4741) and *prd1*Δ*pdr3*Δ grown in *A. amatymbica* extract. Cells were inoculated in SC media treated with increasing concentrations of extract. The growth was measured based on OD_{590} absorbance in treated compared to vehicle control cells. Error bars represent mean ±SD. *, $p \leq 0.05$, student t- test relative to control.

2.3.2 The *A. amatymbica* extract was more bioactive in CM media

To determine in which liquid media the *A. amatymbica* extract was most bioactive, the growth of *pdr1Δpdr3Δ* was evaluated in SC media and CM media (**Fig. 2.4**). The CM media has fewer nutrients compared to SC media, and ensures that the physiological condition is buffered at pH 5.8, since bioactivity can be influenced by many factors. The residual growth of *pdr1Δpdr3Δ* inoculated in either SC or CM was calculated at various concentrations of extract. Bioactivity in CM was observed at 0.44 µg/mL of extract with ~60% residual growth, while bioactivity in SC media was observed around 10 times that concentration at 4 µg/mL

(**Fig.2.4**). These results indicate that the extract was more bioactive in CM media, thus further experiments were conducted in CM media.

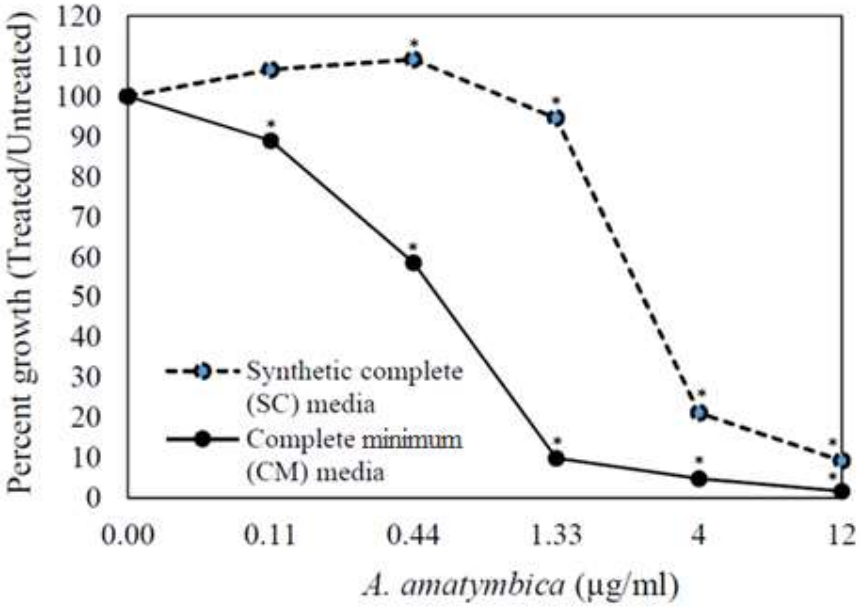


Figure 2.4: Liquid-based bioactivity assay of *pdr1*Δ*pdr3*Δ yeast strain in different media. Cells were inoculated in SC or CM media treated with increasing concentrations of the *A. amatymbica* extract. The growth was measured based on OD_{590} absorbance in treated compared to vehicle control cells (DMSO). Error bars represent mean ±SD. * $p \leq 0.05$, student t- test relative to control.

2.3.3 Compound B is potent in liquid media

To determine which of the compounds in the *A. amatymbica* extract were bioactive, the growth of *pdr1*Δ*pdr3*Δ in CM media was evaluated in the presence of five different semi-purified fractions generated by chemistry student Joe Bracegirdle (**Fig. 2.5**). These compounds were used here at concentrations ranging from 0.004 µg/mL to 10 µg/mL. Relative to the 0.5% DMSO vehicle control, growth in fraction-treated cells was measured at mid-log growth phase.

Based on the criterion of 40% growth inhibition for bioactivity, compound B was potently bioactive at 3.33 µg/mL and 10 µg/mL where these concentrations were nearly lethal. In contrast, compounds C, E, F and G were not bioactive at any concentration. Hence, compound B is the most potent of the semi-purified compounds, suggesting a role of compound B in the bioactivity of the *A. amatymbica* extract.

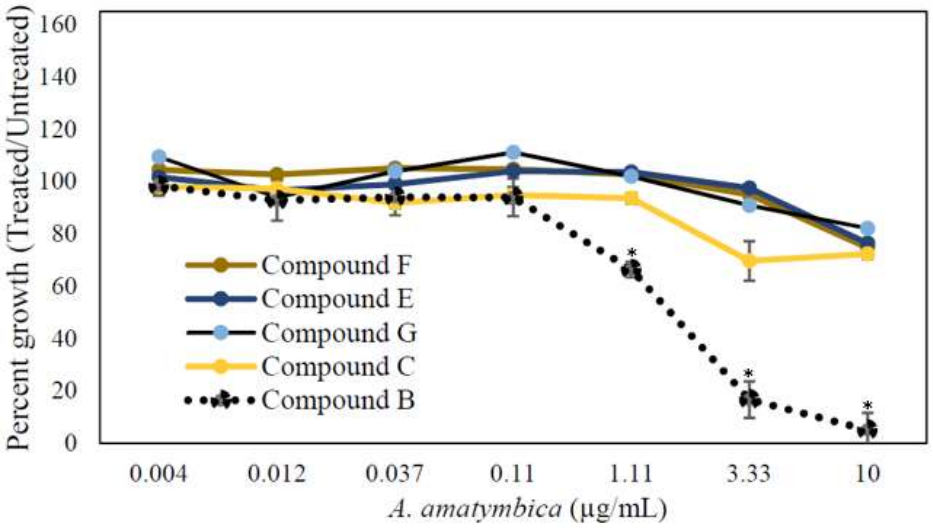


Figure 2.5: Liquid-based bioactivity assay of *pdr1*Δ*pdr3*Δ in response to different semi-purified fractions derived from the *A. amatymbica* extract. Liquid-based bioactivity assay of *pdr1*Δ*pdr3*Δ yeast strain in different media. Cells were inoculated in CM media treated with increasing concentrations of the semi-purified fractions. The growth was measured based on OD_{590} absorbance in treated compared to vehicle control cells. Error bars represent mean ±SD. *, $p \leq 0.05$, student t test relative to control.

2.3.4 The extract and compound B are bioactive in agar

Genome-wide analyses using mutant libraries of yeast have been used to successfully identify mechanism of action of extracts and compounds in agar (Giaever et al., 2004; Parsons et al., 2006). Compounds that are bioactive in liquid media may not be bioactive in agar, or

alternatively, these compounds may be less potent in agar. Therefore, to determine the bioactivity of the *A. amatymbica* extract and compound B in agar, growth of serial dilutions of *pdr1*Δ*pdr3*Δ was evaluated on CM agar treated with the extract and compound B at concentrations ranging from 0.375 to 6 mg/mL (**Fig. 2.6**). Relative to control, the crude extract and compound B were both bioactive with very little growth at 6 mg/mL. At 1.5 and 3 mg/mL, the growth in extract-treated cells was comparable to control while there was very little growth in compound B-treated cells compared to control. These results established that the *A. amatymbica* extract and compound B were both bioactive in agar, albeit with the latter being more potent than the former.

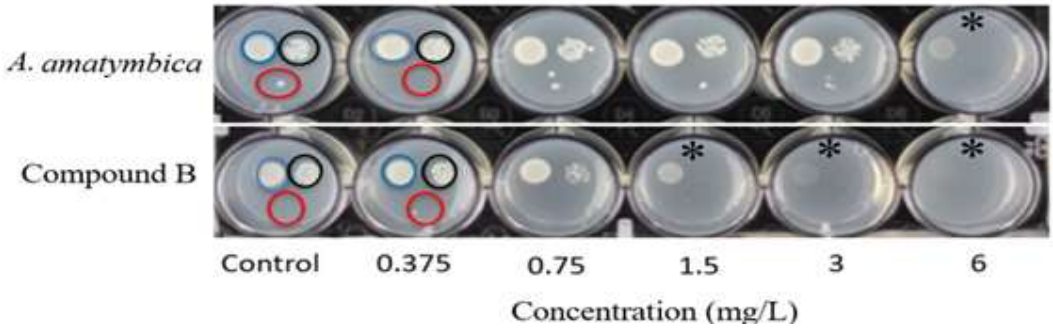


Figure 2.6: Agar-based dose response assay of the *A. amatymbica* extract and semi-purified compound B. Serial dilutions (1 x 10^3 (red), 1 x 10^5 (blue) or 1 x 10^7 cells/mL (Fischli et al.)) of *pdr1Δpdr3Δ* were spotted on agar treated with varying concentrations of extract or semi-purified compound, incubated for 24 hours and imaged using a digital camera. *, Visibly distinct growth inhibition.

2.3.5 Optimisation of *A. amatymbica* extract for genome-wide analysis in agar

To conduct a viability-based genome-wide analysis of the gene deletion library in the *pdr1Δpdr3Δ* background, a concentration was first identified that yielded 10-20% growth inhibition in the *pdr1*Δ*3*Δ strain in the same format used in the genome-wide analysis. This

concentration ensures there is a large window (80-90%) to detect additional growth inhibition due to the gene deletion. Using a representative library plate in 1,536 colony format, growth was quantified using SGA tools (Wagih et al., 2013). At 38 µg/mL of the aqueous extract, approximately 20% of growth was inhibited across all 1,536 colonies in the entire treated plate compared to the vehicle control (**Fig. 2.7**). Unfortunately, there was not enough compound B to conduct a genome-wide analysis.

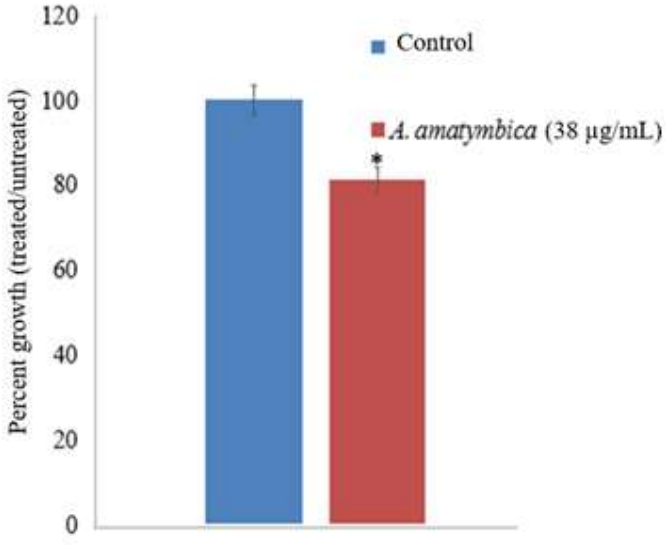


Figure 2.7: Optimisation of *A. amatymbica* extract treatment for genome-wide analysis of gene deletion library. A random plate from the gene deletion library (comprised of 1,536 colonies) was grown on agar containing 38 µg/mL of extract or vehicle control, incubated for 24 hours, and imaged using a digital camera. SGAtools was used to calculate average colony size and standard deviation across the plate for each media. Error bars represent mean ± SD, *, $p \leq 0.05$, student t- test relative to control.

2.3.6 The *A. amatymbica* extract affects various biological processes

Hypersensitivity of gene deletion strains to an extract or compound indicates that gene and its associated pathway or process is involved in the bioactivity (Boone et al., 2007). Out of

4,300 gene deletion strains in the *pdr1*Δ*pdr3*Δ background, it was determined that 98 gene deletion mutants had significantly altered growth in 38 µg/mL *A. amatymbica* extracts compared to the vehicle control (**Fig. 2.8**). There were 54 and 44 mutants where growth was increased and decreased with the extract, respectively. As false positive results may exist in high-throughput assays, sensitivity was then validated using a low-throughput liquid growth assay. Each deletion mutant was treated with 25 µg/mL in liquid CM media, as this concentration gave approximately 20% growth inhibition in *pdr1*Δ*pdr3*Δ in liquid, and residual growth was calculated compared to untreated controls of each strain. Of the 61 mutants that exhibited reduced growth in the high-throughput assay, five were confirmed to have significant growth defects when treated with *A. amatymbica* extract in the low-throughput assay (**Fig. 2.9**).

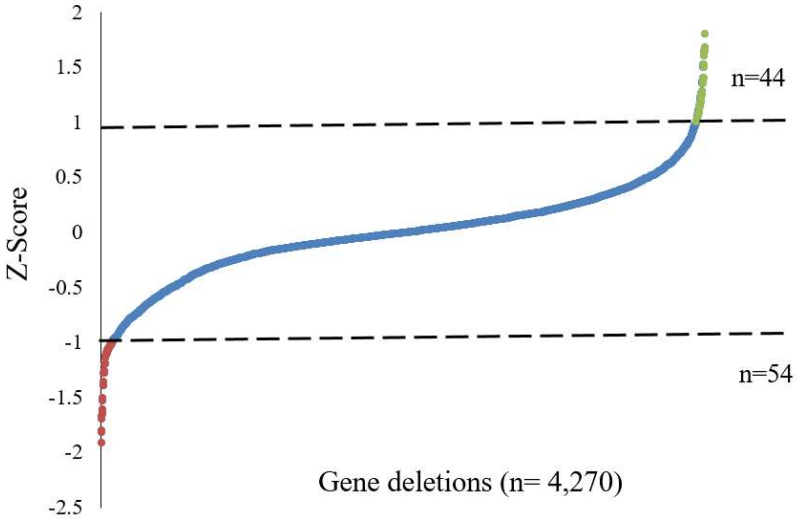


Figure 2.8: Sensitivity of 4,270 haploid gene deletion strains to the *A. amatymbica* extract. Each strain was quantified in quadruplicate where the growth ratio of treated to untreated cells is represented in the Z-score; $Z < -1$ reflects reduced growth in the extract (red) and $Z > 1$ reflects increased growth in the extract (green). Growth not significantly different in treated and untreated media were indicated in blue.

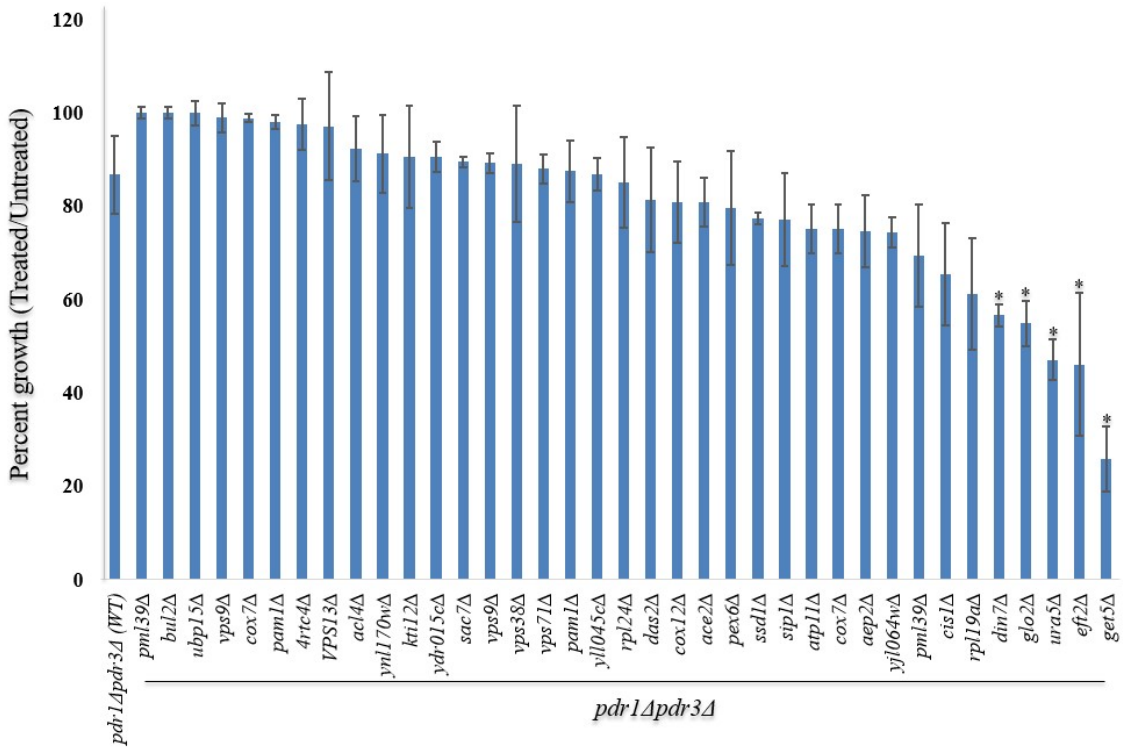


Figure 2.9: Low-throughput growth analysis confirms 5 gene deletion strains as sensitive to the *A. amatymbica* extract. Cells were inoculated in CM media treated with 25µg/mL of the A. amatymbica extract. The growth was measured based on OD_{590} absorbance in treated compared to untreated cells. Error bars represent mean ±SD. *, $p \leq 0.05$, student t- test relative to control.

2.3.7 Functional annotation of genes altered by *A. amatymbica* extract

The five gene deletion mutants sensitive to the *A. amatymbica* extract were processed through several enrichment analysis programs to annotate and understand the mechanism of action of the *A. amatymbica* extract. First, we searched for transcriptional regulation networks based on GO terms that are over-represented in the five genes relative to a random set of genes (Monteiro et al., 2019). The system classifies a list of input genes based on their regulatory associations with known transcription factors. 57, 26, 25, 24 and 42 transcription factors were associated with Get5, Din7, Glo2, Ura5 and Eft2 respectively. Nine of these regulators (Ino2,

Yap1, Pdr3, Rpn4, Met32, Gcn4, Pdr1, Ndt80 and Cbf1) interact with all five sensitive genes (**Fig. 2.10**).

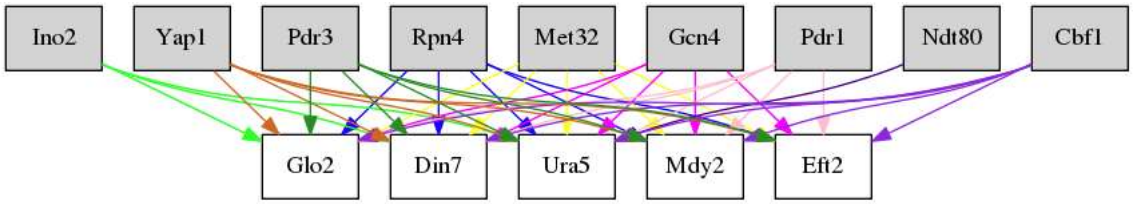


Figure 2.10: Transcriptional network regulating the five gene deletions sensitive to the *A. amatymbica* extract. Nine transcription factors (grey) either positively or negatively interact with the five sensitive genes.

A Spatial Analysis of Functional Enrichment (SAFE) analysis was also conducted on the five sensitive genes in the context of the yeast genetic interaction network. SAFE places a gene set into major functional domains (Baryshnikova et al., 2010; Usaj et al., 2017). Genetic interactions of the *A. amatymbica* extract gene set occurred within the domains of cytokinesis, tRNA wobble modification, peroxisome, mitochondria, vesicle trafficking and ribosome biogenesis (**Fig. 2.11**). Of which, vesicle traffic, peroxisome and mitochondria were significant ($P < 0.05$)

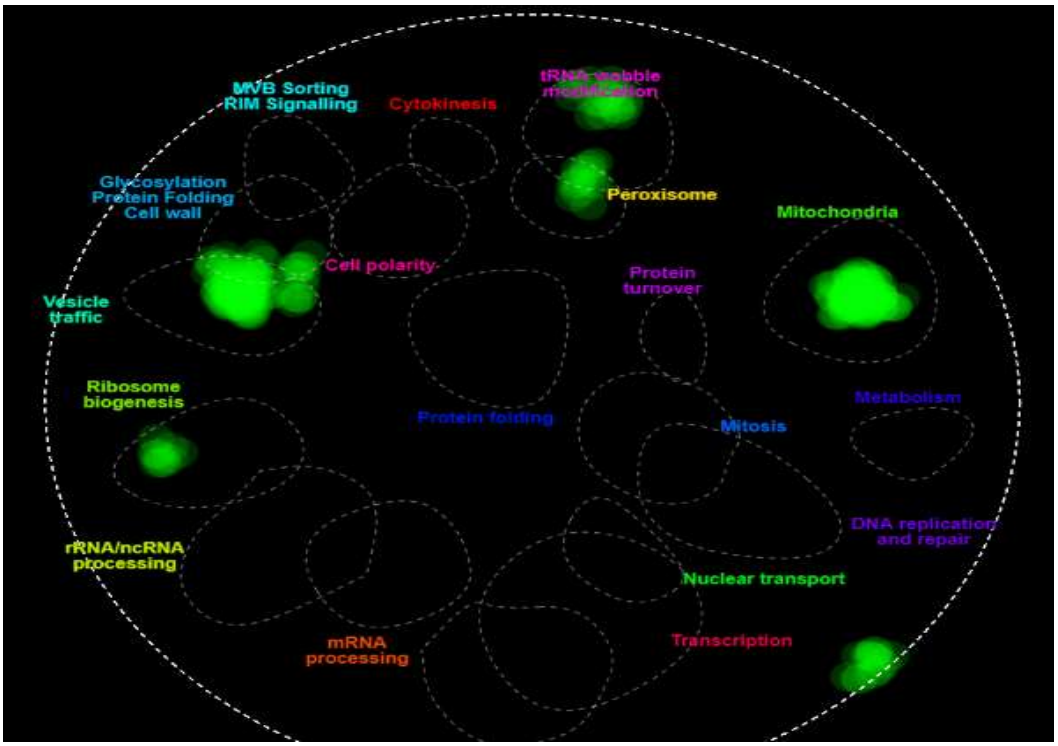


Figure 2.11: Spatial analysis for functional enrichment (SAFE) reveals enrichment for vesicle trafficking and mitochondria. The five sensitive genes were mapped to an annotated network. The increase in size and density of the green colour indicates association of the extract-sensitive genes and their interactions within the indicated functional domain in the network.

Gene	Description
GET5	Protein with a role in insertion of tail-anchored proteins into the ER membrane; forms a complex with Get4p; required for efficient mating; involved in shmoo formation and nuclear migration in the pre-zygote; associates with ribosomes
EFT2	Elongation factor 2 (EF-2), also encoded by EFT1; catalyzes ribosomal translocation during protein synthesis; contains diphthamide, the unique posttranslationally modified histidine residue specifically ADP-ribosylated by diphtheria toxin
GLO2	Cytoplasmic glyoxalase II, catalyzes the hydrolysis of S-D-lactoylglutathione into glutathione and D-lactate
URA5	Major orotate phosphoribosyltransferase (OPRTase) isozyme that catalyzes the fifth enzymatic step in de novo biosynthesis of pyrimidines, converting orotate into orotidine-5'-phosphate; minor OPRTase encoded by URA10
DIN7	Mitochondrial nuclease functioning in DNA repair and replication, modulates the stability of the mitochondrial genome, induced by exposure to mutagens, also induced during meiosis at a time nearly coincident with commitment to recombination

Table 2.9: Annotation of the genes that were found to be sensitive to *A. amatymbica* extract. Descriptions for each gene was obtained from Saccharomyces Genome Database (SGD).

The five gene deletion strains that showed significant reduced growth in the presence of the extract were analysed using gene ontology at three levels of classification (molecular function, biological process and cellular component). At the biological process level, there was significant enrichment for metabolic process (**Fig. 2.12A**). For the cellular component category, enrichment was significant for unclassified and mitochondrion (**Fig. 2.12B**), while there were significant enrichments for hydrolase activity and ion binding in the molecular function category (**Fig. 2.12C**).

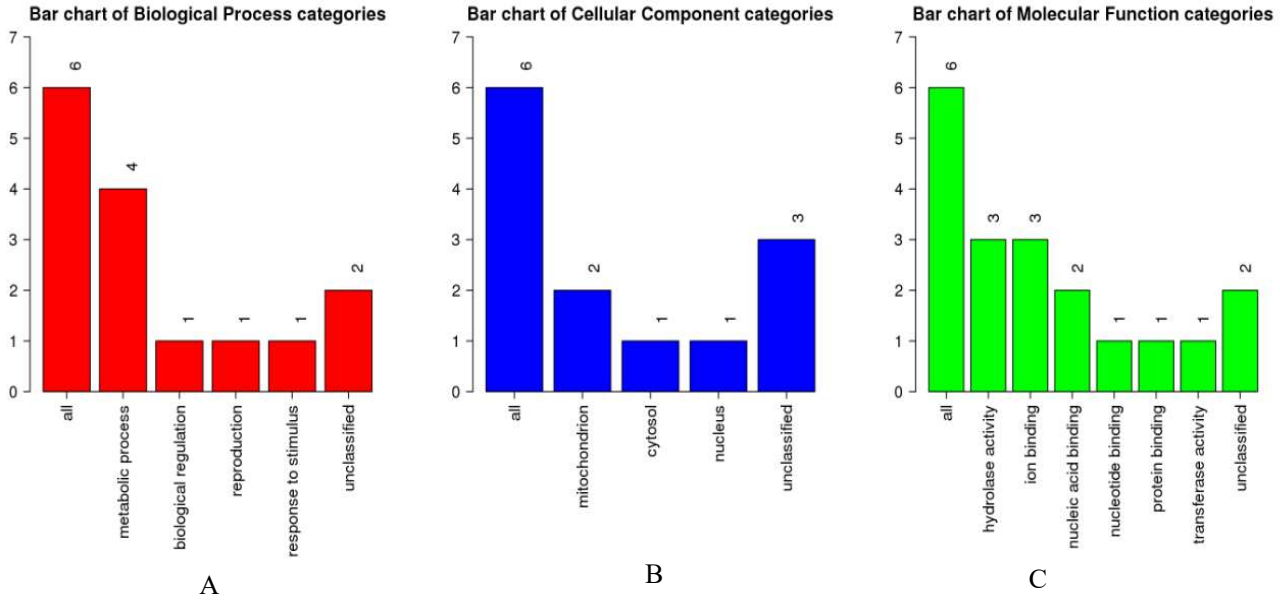


Figure 2.12: GO enrichment analysis of 5 genes sensitive to *A. amatymbica* extract reveals several processes to be enriched for (A) molecular functions, (B) biological processes and (C) cellular components. Numbers above each bar indicate the number of genes associated with that category.

2.3.8 Extract sensitivity in all members of the guided-entry tail anchored protein (GET) family

The genome-wide analysis revealed one of the genes sensitive to *A. amatymbica* to be Get5 (Mdy2) (**Fig. 2.9**). Interestingly, Get5 has been implicated in tail anchored (TA) protein synthesis (Jonikas et al., 2009; Schuldiner et al., 2008). The guided entry tail anchored protein (GET) pathway uses several proteins (Get 1-5) to transport tail anchored proteins to the endoplasmic reticulum (Simpson et al., 2010). To determine if the GET complex was affected by *A. amatymbica,* a liquid assay was conducted on each member of the GET complex (Get 1-5) as well the chaperones Sgt2, Ssa1 and Ydp1 known to be involved in the GET pathway

(Schuldiner et al., 2008). Relative to 80% residual growth in *pdr1*Δ*3*Δ, the *pdr1*Δ*pdr3*Δ*get5*Δ strain was most sensitive at ~30% residual growth while *pdr1*Δ*pdr3*Δ*get1*Δ was less sensitive, albeit still significantly sensitive, at around ~50% residual growth (**Fig. 2.13**). All chaperones were also sensitive where *pdr1*Δ*pdr3*Δ*sgt2*Δ, *pdr1*Δ*pdr3*Δ*ssa1*Δ and *pdr1*Δ*pdr3*Δ*ydp1*Δ were each significantly sensitive with only 25-34% residual growth (**Fig. 2.13**). These results confirm the importance of the GET pathway and its associated chaperones in buffering the bioactivity of the *A. amatymbica* extract.

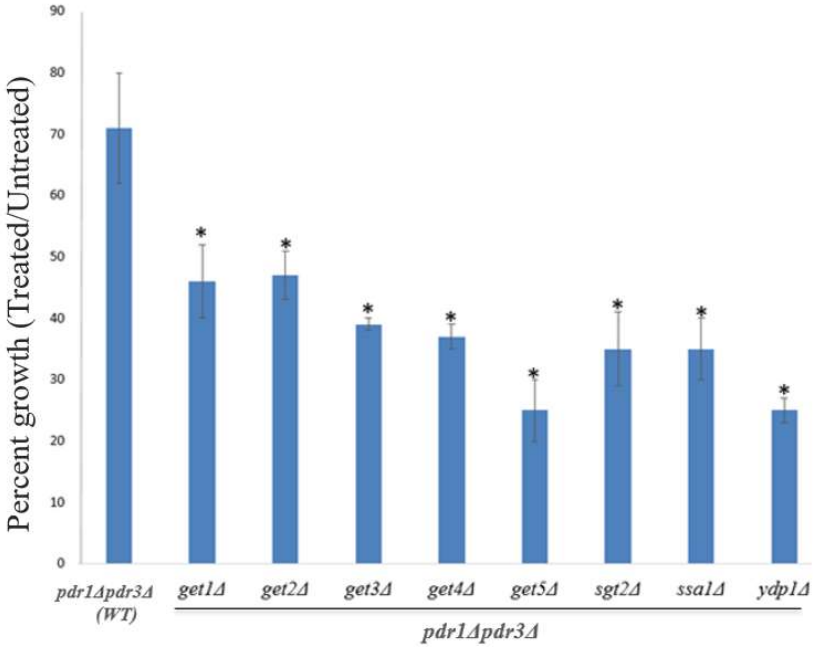


Figure 2.13: Sensitivity of genes involved in the guided entry tail anchored protein (GET) pathway to the *A. amatymbica* extract. Cells were inoculated in CM media treated with 25 µg/mL of the *A. amatymbica* extract. The growth was measured based on OD_{590} absorbance in treated compared to untreated cells. Error bars represent mean ±SD. *, $p \leq 0.05$, student t-test relative to control.

2.3.9 Compound B is less potent against guided-entry tail anchored protein (GET) deletion strains

Since compound B was the most bioactive semi-purified fraction of the *A. amatymbica* extract (**Fig. 2.5**), it is plausible that compound B is the major bioactive specifically the compound mediating the sensitivity of the GET gene deletion strains. To determine if compound B was potent against the five GET gene deletions, a liquid growth assay was conducted. Relative to approximately 80% residual growth of *pdr1*Δ*pdr3*Δ, *pdr1*Δ*pdr3*Δ*get1*Δ, *pdr1*Δ*pdr3*Δ*get2*Δ, *pdr1*Δ*pdr3*Δ*get3*Δ and *pdr1*Δ*pdr3*Δ*get4*Δ exhibited residual growths at

approximately 60%, 60%, 45% and 30% respectively (**Fig. 2.14**). *pdr1*Δ*pdr3*Δ*get5*Δ was sensitive at around 30% residual growth. These results reveal that the GET complex buffers the effect of compound B, albeit compound B is not as potent as the *A. amatymbica* extract.

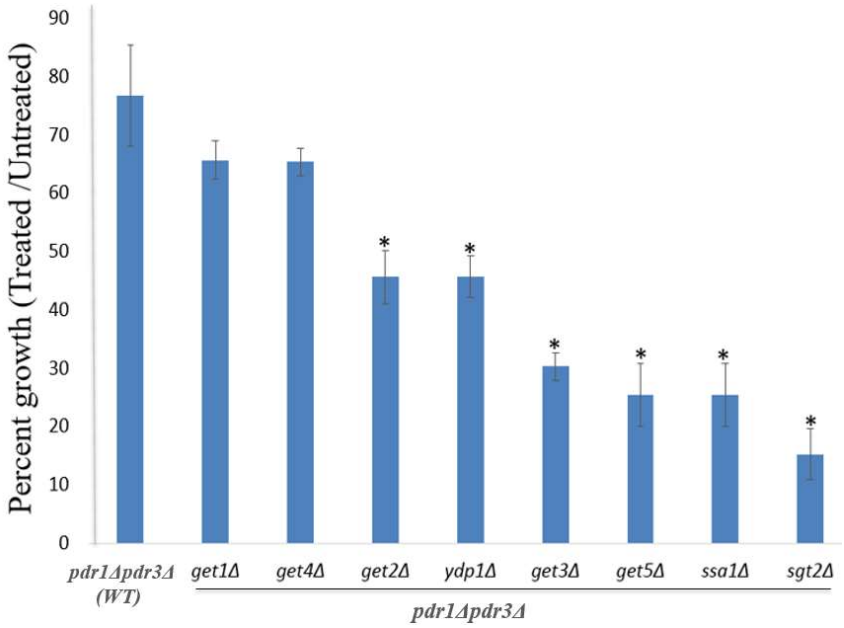


Figure 2.14: Sensitivity of genes involved in the guided entry tail anchored protein (GET) pathway to Compound B. Cells were inoculated in CM media treated with 25 µg/mL of compound B . The growth was measured based on OD_{590} absorbance in treated compared to untreated cells. Error bars represent mean ±SD. *, $p \leq 0.05$, student t-test relative to control.

2.4.0 Overexpression of GET3 in get1Δ or get2Δ increases extract bioactivity

Overexpression of Get3 in the *get1*Δ background has been observed to be lethal in the presence of GET substrates (Schuldiner et al., 2008). Get3 recognition is a key step in the biosynthesis of tail anchored (TA) proteins whose loss can lead to defective insertion of TA proteins into mitochondria (Schuldiner et al., 2008). In the absence of Get1 and Get2, Get3-TA complexes aggregate in the cytosol leading defects in TA protein biogenesis (Schuldiner et al., 2008). To overexpress Get3 in *pdr1Δpdr3Δget1Δ* and *pdr1Δpdr3Δget2Δ* backgrounds, the gene

deletions and overexpression had to be engineered, particularly since the *pdr1Δpdr3Δget1Δ* and *pdr1Δpdr3Δget2Δ* strains used above could not be transformed with the Get3 overexpression plasmid containing uracil selection.

First, Get1 and Get2 genes were deleted using PCR-based gene deletion strategy (Baudin et al., 1993). GET1::LEU2 and GET2::LEU2 deletion cassettes were constructed using the *pUG73* plasmid as template and confirmed via electrophoresis on agarose gel where an expected 2,428bp was observed (**Fig. 2.15)**. The PCR products were then transformed in *pdr1*Δ*pdr3*Δ. The genomic DNA of transformants was extracted, and PCR products specific for the gene deletions were amplified and electrophoresed on agarose gel where 2,949 bp expected bands confirmed the Get1 deletion (**Fig. 2.16A**) and 3,005 bp confirmed the Get2 deletion (**Fig. 2.16B**).

We determined if Get3 overexpressed in *pdr1Δpdr3Δget1*Δ and *pdr1Δpdr3Δget2*Δ background was lethal in the presence of the *A. amatymbica* extract using an overexpression plasmid under the control of the GAL1 promoter. The *pdr1Δpdr3Δget1Δ* and *pdr1Δpdr3Δget2Δ* strains were grown overnight in raffinose where the GAL inducible GET3 was repressed, and then grown in either galactose (to induce GET3 overexpression) or glucose (to repress GET3 overexpression). Using agar medium that is more conventionally used in yeast with the GAL1 promoter, we determined if GET3 overexpression enhanced bioactivity of the *A. amatymbica* extract in *pdr1Δpdr3Δget1Δ* and *pdr1Δpdr3Δget2Δ* strains (**Fig. 2.17**). Overnight cultures grown in raffinose were inoculated on agar media containing glucose (to repress GET3 overexpression) or galactose (to induce GET3 overexpression). Relative to the empty vector where there was visible growth of *pdr1Δpdr3Δget1Δ* and *pdr1Δpdr3Δget2Δ* strains in galactose media with the *A. amatymbica* extract, there was no visible growth in these strains overexpressing GET3 in galactose media with the *A. amatymbica* extract (**Fig. 2.17**). This

distinction was not seen in glucose media, thus these results reinforce the importance of the GET pathway as a mechanism buffering the bioactivity of the *A. amatymbica* extract.

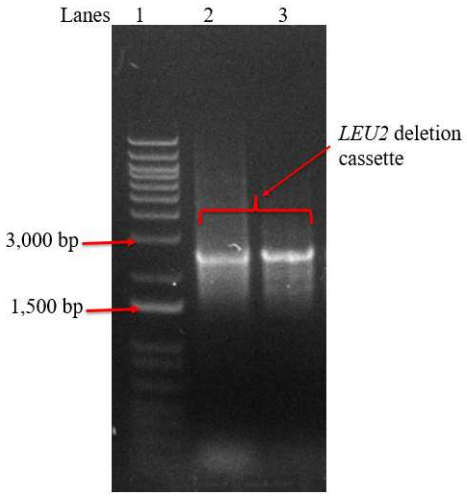


Figure 2.15: Construction of the GET1::LEU2 and GET2::LEU2 deletion cassettes. The deletion cassettes were amplified using the *pUG73* plasmid as template and PCR products were electrophoresed on 1% agarose gel. Lane 1:1 -kb plus DNA ladder; Lane 2: GET1::LEU2 deletion cassette; Lane 3: GET2::LEU2 deletion cassette.

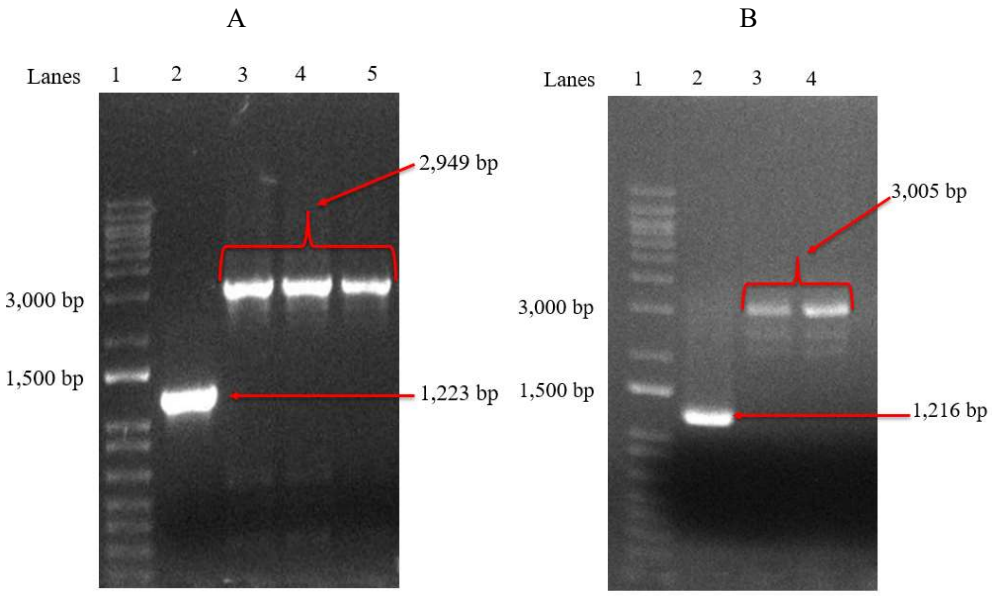


Figure 2.16: Confirmation of transformation of (A) *get1::LEU2* and (B) *get2::LEU2* cassettes upon integration in the genome. The PCR products were amplified using primers specific for each locus in the genome and electrophoresed on 1% agarose gel. (A) Lane 1: 1 kb plus DNA ladder; Lane 2. Wild type (no integration control); Lanes 3-5: Three independent transformants. (B) Lane 1: 1 kb plus DNA ladder; Lane 2. Wild type (no integration control); Lanes 3-4: Two independent transformants.

2.4.1 Get3 overexpressed in *pdr1Δpdr3Δget1*Δ and *pdr1Δpdr3Δget2*Δ background is sensitive to *A. amatymbica* on agar

To see if overexpression of Get3 in *get1*Δ and *get2*Δ background would be potent in a different media, the assay was conducted on agar. At 38 µg/mL of *A. amatymbica* the growth of Get3 overexpressed in *get1*Δ background is visibly inhibited compared to glucose treatment and DMSO (**Fig. 2.17**). Growth inhibition is also seen when Get3 is overexpressed in *get2*Δ background.

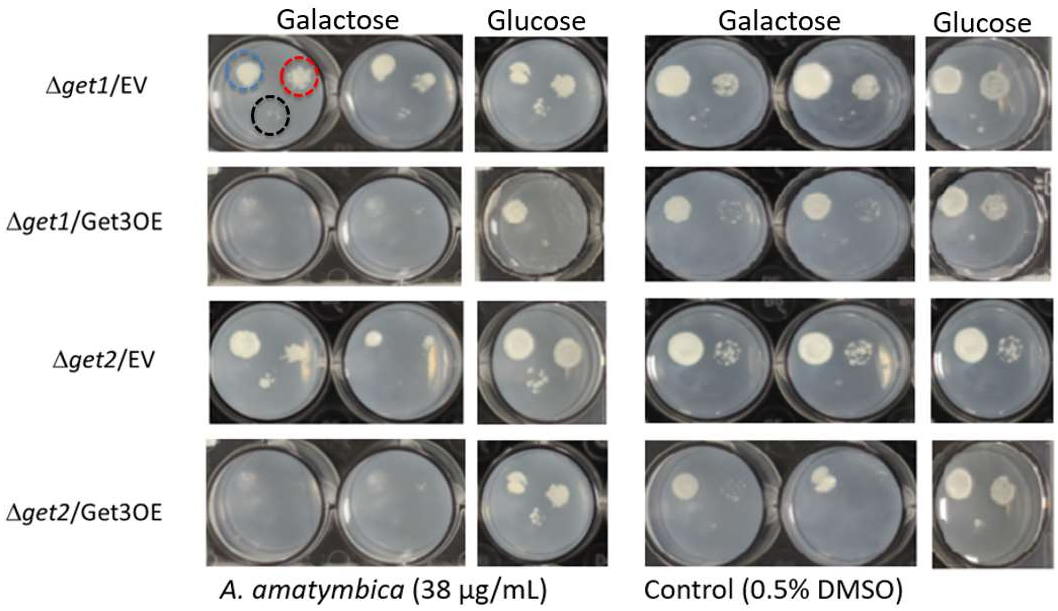


Figure 2.17: Over expressing Get3 in the presence of *A. amatymbica* on agar. Cells were spotted on agar treated with *A. amatymbica* or DMSO in either galactose or glucose. Spots represent 3 concentrations : 1 x 10^7 (blue), 1 x 10^5 (red) and 1 x 10^3 (Fischli et al.). Images were taken at 24 h. EV, cells with empty vector plasmid. OE, cells with overexpression plasmid.

2.4.2 SDS mimics *pdr1Δpdr3Δ* response

With the results above confirming the importance of the GET pathway, we next sought to monitor localisation of the proteins specifically transported via the GET pathway. A libray of such GFP-tagged proteins exists (Weill et al., 2018); however, this library does not exist in the *pdr1Δpdr3Δ* background. We thus attempted to mimic the PDR-attentuated response by treating wild-type (BY4741) cells with sodium dodecyl sulfate (SDS). The addition of a small amount of SDS along with L-proline in the growth medium leads to a transient opening of the cell wall/membrane, thus increasing yeast cell permeability (Liu et al., 2007). Growth of wild-type cells treated with SDS was compared to *pdr1Δpdr3Δ* in CM media, whereby the 0.003%

SDS treatment in BY4741 elicited a response similar to PDR-attentuated strain (**Fig 2.18**). Hence 0.003% SDS was used to investigate the impact of the *A. amatymbica* extract in the GFP-tagged strains in the BY4741 background.

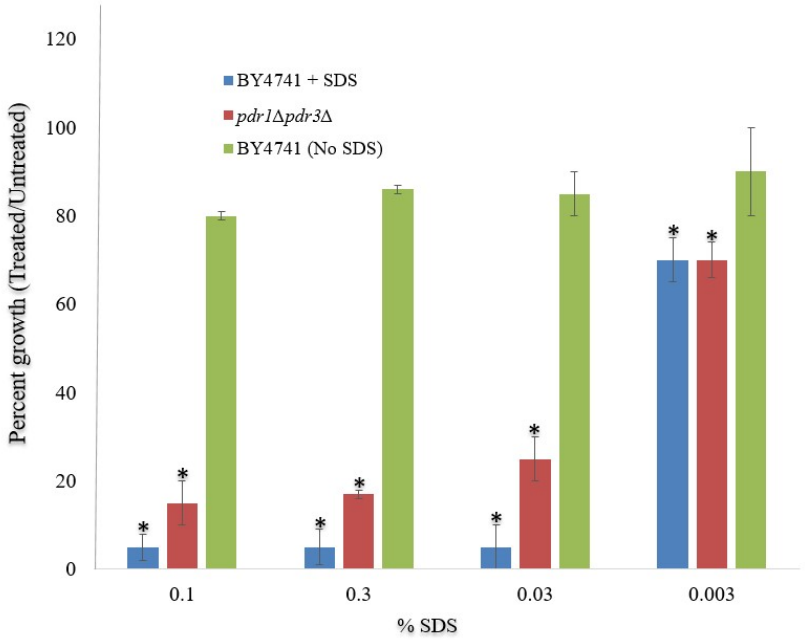


Figure 2.18: Wild-type (BY4741) yeast cells treated with 0.003% SDS mimics *pdr1*Δ*pdr3*Δ response in CM media. BY4741 yeast cells were grown in SDS. *pdr1Δpdr3Δ* was grown without SDS. Error bars represent mean ± SD. *p ≤ 0.5, student t test compared to no SDS treatment.

2.4.3 *A. amatymbica* extract does not affect GET mutants

To investigate proteomic changes induced by *A. amatymbica*, a high throughput microscopy screen monitoring protein localisation was performed. Changes in protein localisation in response to chemical perturbation has been used to understand mechanisms of action (Tkach et al., 2012). Since the Get complex is important in mediating efficient targeting of TA proteins into the ER (Schuldiner et al., 2008), it is important to consider whether a defect

in a Get gene in the presence of *A. amatymbica* leads to off-targeting of the TA protein. Using a SWAT *NOP1promoter-GFP* library (Weill et al., 2018), with GFP proteins tagged at the amine (N) terminal, Get strains were observed under *A. amatymbica* treatment (**Fig. 2.19**). The images were analysed by eye to determine if there was an obvious change in localisation and were crossed checked by two different individuals for validation. Get3 and Get4 showed no obvious change in cytosolic localization of GFP between control and treatment. Likewise, no change in the expected ER localisation was observed in Get1 and Get2.

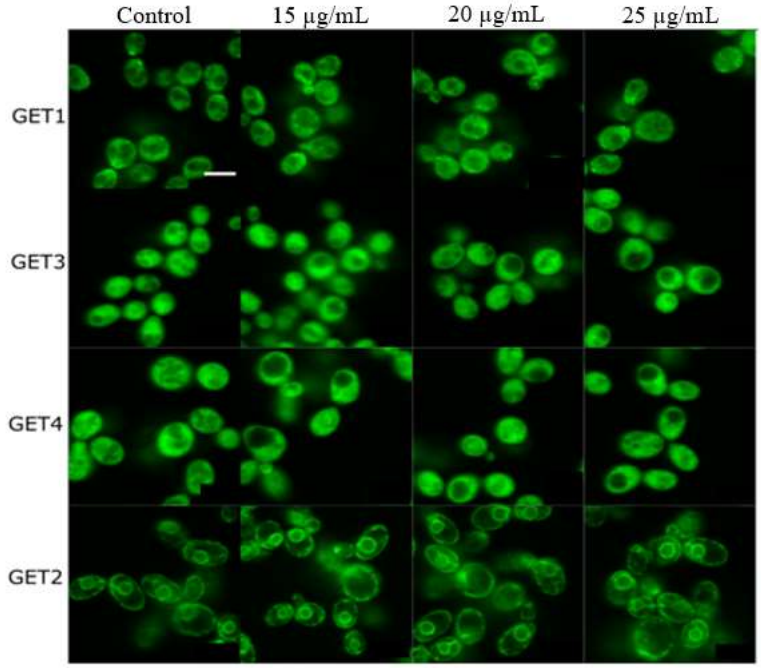


Figure 2.19: GET pathway is not affected by *A. amatymbica* treatment. Cells were treated with SDS for 2 h in CM media prior to *A. amatymbica* treatments at 15 µg/mL, 20 µg/mL and 25 µg/mL and DMSO control for 4 h. Images were visualised using a fluorescent microscope. Scale bar = 5 µm.

2.4.5 *A. amatymbica* affects ERG9 and CYB5 localisation

Since targeting of TA proteins into the ER is mediated by the Get pathway (Schuldiner et al., 2008), it is important to consider whether TA proteins are mislocalized when treated with the *A. amatymbica* extract. Using the SWAT *NOP-1promoter-GFP* library (Weill et al., 2018), I screened 315 predicted TA proteins. This library is designed so that TA proteins are tagged with a GFP at the amine (N) terminal instead of the carboxyl (C) terminal end. This allows for more accurate targeting signal of TA proteins since C-tagged TA proteins are known to being wrongly localized or degraded (Weill et al., 2018). A change in predominant localisation from perinuclear ER to cortical ER was observed for both ERG9 and CYB5 at 20 µg/mL and 25 µg/mL concentrations of *A. amatymbica*, compared to control (**Fig. 2.20**), while no change in punctate localization was observed in ERG6. Interestingly, ERG9 and CYB5 are both involved in lipid metabolism (Karst & Lacroute, 1977; Truan et al., 1994).

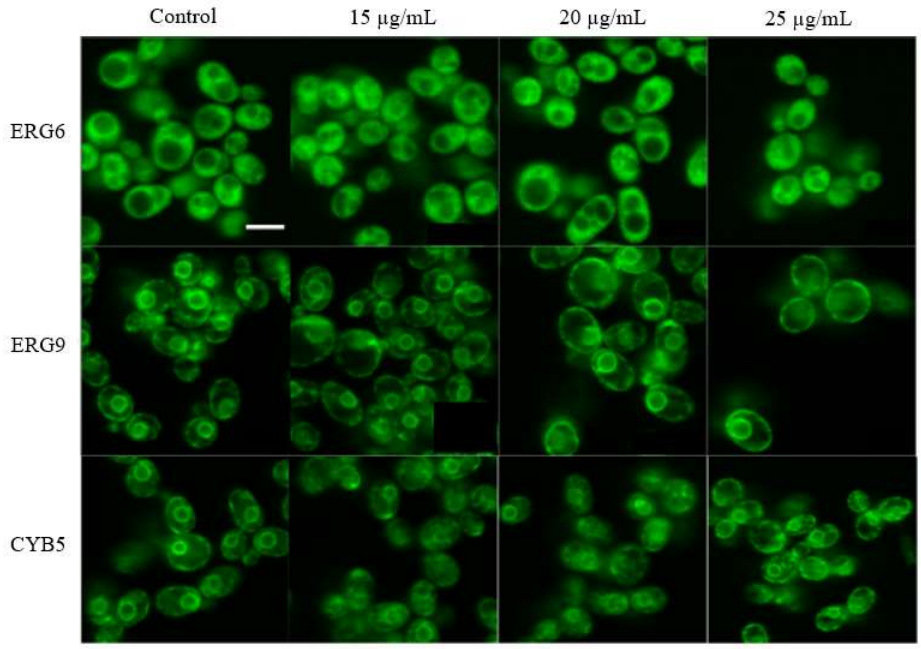


Figure 2.20: ERG9 and CYB5 are affected by *A. amatymbica* treatment. Cells were treated with SDS for 2 h in CM media prior to *A. amatymbica* treatments at 15 µg/mL, 20 µg/mL and 25 µg/mL and DMSO control for 4 h. Images were visualised using a fluorescent microscope. Scale bar = 5 µm.

2.4 Discussion

2.4.1 Summary

The aim of the thesis was to characterise the mechanism of action of the *A. amatymbica* extract and determine the most potent component of the extract. The *A. amatymbica* extract inhibited the growth of the PDR-attenuated yeast strain *pdr1*Δ*pdr3*Δ, showing that the extract is a potential substrate of the PDR drug efflux system. The unbiased approach of genome-wide analysis led to further analysis revealing mode of action for *A. amatymbica* involving the guided entry tail anchored (GET) protein pathway in yeast, orthologous transmembrane to the recognition complex (TRC) in humans. Gene deletions sensitive to *A. amatymbica* were also implicated in protein synthesis and pyrimidine synthesis. Furthermore, localisation of GFP-tagged proteins regulated by the GET pathway showed a potential function of *A. amatymbica* in antioxidant activity and lipid metabolism. In addition, it was noted that compound B, a semi-purified component of the *A. amatymbica* extract inhibited yeast growth similar to the crude extract of *A. amatymbica*, indicating that this compound is potentially the most potent component of bioactivity of *A. amatymbica* and potentially the major bioactive compound in the extract.

2.4.2 *A. amatymbica* as a PDR substrate

Increased xenobiotic sensitivity due to loss of function mutations in either the drug efflux transporters or their transcriptional regulators is a well-studied phenomenon (Coorey et al., 2015). The PDR network is comprised of an array of highly inducible drug efflux transporters encoded by the ATP binding cassette (ABC) superfamily and major facilitator superfamily (MFS) genes (Kolaczkowska & Goffeau, 1999). The network is mainly controlled by two transcription factors: the binuclear Zn_2Cys_6 zinc finger protein Pdr1p and its homolog

Pdr3p and to a lesser extent by the bZIP family regulators Yrr1p and Yap1p. The PDR attenuated response allows for mechanism of action of xenobiotics that are PDR substrates, to be studied at a lower concentration (Coorey et al., 2015). Consistent with established PDR substrates including cycloheximide, fluconazole, ketoconazole, quercetin, amphotericin B and the natural products latrunculin A and plakortolide X (Coorey et al., 2015), the inhibition of *pdr1Δpdr3Δ* at a lower *A. amatymbica* concentration (**Fig. 2.3**) compared to BY4741 suggests that *A. amatymbica* is potentially a substrate of the PDR. This is supported by the analysis showing Pdr1p and Pdr3p both interacting with five genes that are sensitive to *A. amatymbica* (**Fig. 2.10**). Compound B also inhibits the growth of *pdr1Δpdr3Δ* more so than BY4741 (**Fig. 2.5**) suggesting that compound B is also a potential substrate of the PDR network. Taken together, this suggests that both the crude extract of *A. amatymbica* and compound B induce a xenobiotic stress response where the cells lacking the PDR1 and PDR3 master regulators of the PDR system, are not able to successfully pump out xenobiotics.

The involvement of the PDR system in the bioactivity of *A. amatymbica* has not been previously reported. *A. amatymbica* is known to inhibit the growth of *Aspergillus flavus*, *Aspergillus niger*, *Penicillium notatum* and *Candida albicans* (Afolayan & Lewu, 2009). In these studies, the role of the drug efflux system was not reported where concentrations of 0.1-5 mg/mL inhibited 50-80% growth of WT strains of these species presumably with functional PDR systems (Afolayan & Lewu, 2009; Mulaudzi et al., 2009). For studies done on agar, Mulaudzi et al (2009) found 50% growth inhibition growth on potato dextrose agar for *Aspergillus flavus*, *Aspergillus niger* and *Penicillium notatum* in 0.1-5 mg/mL *A. amatymbica* (Mulaudzi et al., 2009). 50-80% growth inhibition observed previously at 5 mg/mL, albeit in different fungal species, seem comparable with observations in this thesis where visible inhibition was observed at 6 mg/mL *A. amatymbica* (**Fig.2.6**). Difference in fungal species aside, the comparability in *A. amatymbica* potency between presumably PDR-replete and PDR-

attenuated strains could be explained by possible differences in sourcing of plant material since extracts made from wild plants are more potent than extracts made from cultivated plants (Rustaiyan & Sadjadi, 1987). Hence, the down-regulation of the PDR system does not necessarily negate the traditional use of *A. amatymbica* in treating antifungal infections (Afolayan & Lewu, 2009), or alternatively raises concern about concentrations used in traditional medicine compared to yeast cells in this thesis. The bioactivity of *A. amatymbica* in both the presence and absence of the drug efflux pump suggests a broad range of cellular targets underlying its traditional use. Further laboratory assays of the crude extract and active compounds will be required in order to better understand the involvement of the PDR system in the bioactivity of *A. amatymbica,* relative to its traditional use.

2.4.3 *A. amatymbica* potentially affects guided entry for tail-anchored protein (GET)

In the presence of *A. amatymbica*, all five GET mutant strains (*pdr1Δpdr3Δget1Δ*, *pdr1Δpdr3Δget2Δ*, *pdr1Δpdr3Δget3Δ*, *pdr1Δpdr3Δget4Δ*, *pdr1Δpdr3Δget5Δ*) showed reduced growth (**Fig. 2.13**). The guided entry of tail anchored protein (GET) pathway uses these 5 genes to post-translationally conduct tail-anchored (TA) proteins to the endoplasmic reticulum (ER) where they are inserted into the lipid bilayer before they are directed to their respective destinations in the secretory pathway (Simpson et al., 2010). Hence, reduced growth when these genes are absent, suggests these gene deletions buffer the effect of *A. amatymbica* extract.

Briefly, the GET pathway begins with Sgt2 recognizing a TA protein emerging from the ribosome, forming Sgt2-TA complex (Chang et al., 2012; Zhang et al., 2021). Next, Get4 and Get5 form a 'scaffolding complex' where Sgt2–TA complex is recruited at the Get5 domain, while Get4 recruits Get3 (Chang et al., 2012). A "hand-off" reaction follows resulting in TA protein transfer from Sgt2 to Get3 (Mateja & Keenan, 2018). Get3 then dissociates from

Get4 and is directed to the Get1/Get receptor complex located at the ER. At the ER, Get1/2 disrupts the TA protein binding site in Get3, releases the TA protein for insertion into the ER and Get3 is recycled to the cytosol to initiate a new round of targeting (Rabu et al., 2009).

Consistent with this model, *pdr1Δpdr3Δget1*Δ and *pdr1Δpdr3Δget2*Δ show similar sensitivity in the presence of the crude extract of *A. amatymbica* (**Fig. 2.13**), although this appears not to be the case with compound B (**Fig. 2.14**). This suggests the functions of Get1/Get2 are potentially required to buffer the effects of *A. amatymbica*. However, the Get1/Get2 genes seem less affected by compound B where *pdr1Δpdr3Δget1*Δ showed less growth inhibition in comparison to crude *A. amatymbica* extract (**Fig. 2.14**). It is plausible that due to redundancy in TA insertion pathway, other pathways are recruited in the presence of compound B but not in crude *A. amatymbica* extract. One such pathway is the chaperone-dependent pathway (Chang et al., 2010). This is supported by lower sensitivity of the chaperone mutant strain *ydp1*Δ in compound B (**Fig. 2.14**) compared to crude *A. amatymbica* extract (**Fig. 2.13**). Another pathway known to serve as back up to GET in yeast, is the signal recognition independent targeting pathway, which involves Snd1, Snd2 and Snd3. These genes were found to synthetically compensate for the loss of both the signal recognition particle and GET pathways, and act as a backup targeting system (Aviram et al., 2016). Hence, in the presence of these alternative pathways, there is a possibility of TA targeting occurring independently of GET leading to differences in sensitivities in the GET genes to *A. amatymbica* in this study.

Since Get1/Get2 receptor is required for recruiting Get3-TA protein complexes, and the loss of either Get1 or Get2 causes Get3-TA complexes to aggregate in the cytosol (Schuldiner et al., 2008), we sought to investigate whether overexpressing Get3 when either Get1 or Get2 is absent, would increase sensitivity to the *A. amatymbica* extract. As predicted, mutants overexpressing Get3 in both *pdr1Δpdr3Δget1*Δ and *pdr1Δpdr3Δget2*Δ background were more sensitive to *A. amatymbica* compared to mutants lacking the Get3 overexpression plasmid

(**Fig.2.17**). This suggests Get1/Get2 is potentially involved in buffering the activity of *A. amatymbica.*

Furthermore, the sensitivity of *pdr1Δpdr3Δget5*Δ to both the crude *A. amatymbica* extract (**Fig. 2.13**) and compound B (**Fig. 2.14**), and that of *pdr1Δpdr3Δsgt2*Δ (**Fig. 2.13**) suggests the activity of *A. amatymbica* is buffered by the functions of Get5 and Sgt2 which occurs further upstream in the GET pathway. With chaperones also sensitive (**Fig. 2.13**), and none of the deletion strains showing significant resistance, this supports the earlier mentioned possibility that other pathways are simultaneously involved in buffering the activity of *A. amatymibca* with regards to TA biosynthesis. The redundancy in pathways is common in yeast, where genes performing related functions are distributed on alternate pathways to compensate for the blockage of one pathway (Li et al., 2010). For *A. amatymbica*, this study has shown one of the pathways buffering its effect, potentially with regards to TA biosynthesis, to be the GET pathway.

2.4.4 Potential function in the TRC40 pathway

As mentioned earlier, Get3 is an important component of the GET pathway (Schuldiner et al., 2008), whose sensitivity to *A. amatymbica* (**Fig. 2.13; Fig. 2.14**) has not been previously studied. In mammalian cells, the orthologue of Get3, known as TRC40 or Asna1, is a component of transmembrane recognition complex (Bozkurt et al., 2009; Colombo et al., 2016), known to be inhibited by a number of compounds (Van Puyenbroeck & Vermeire, 2018).

Since *A. amatymbica* extract and compound B both led to sensitivity in the GET mutants (**Fig. 2.13; Fig. 2.14**), it is important to consider the potential effects the extract and compound may have in the transmembrane recognition complex pathway. Interestingly, Sec61,

a gene encoding membrane proteins that mediate ER protein translocation (Potenza et al., 1992), is a known drug target in the transmembrane recognition complex pathway (McKenna et al., 2016). Mycolactone, a natural product produced by *Mycobacterium ulcerans,* is known to inhibit the transmembrane complex by non-selectively inhibiting Sec61-dependent translocation across ER (McKenna et al., 2016), while suppressing the inflammatory cytokine (Grotzke et al., 2017). Similarly, the *Pseudomonas aeruginosa* protein, exotoxin A, is known to indirectly target the Sec61 channel, keeping it in a closed state by competing with the cytosolic protein calmodium for binding site on Sec61α (Wirth et al., 2003). The fungal macrocycle, cotransin, prevents access of proteins to the ER lumen by inhibiting signal peptide-dependent gating of the Sce61 (Klein et al., 2015). Apratoxin A, a secondary metabolite isolated from a marine cyanobacterium, has been hypothesized to inhibit co-translational translocation by down-regulating a subset of membrane proteins (Liu et al., 2009). Sec61 is also a known target of eeyarestatin I, which prevents nascent chain transfer to Sec61 in the signal recognition pathway (Cross et al., 2009).

Since drugs often share molecular targets (AY et al., 2007), and pathways in yeast are conserved in higher eukaryotes (Aviram et al., 2016), future studies could compare mechanisms of action for *A. amatymbica* with these other compounds that work through the TRC40 pathway. These experiments could occur in yeast or mammalian cells.

2.4.5 Potential function in antioxidant activity

Din7 is a mitochondrial nuclease involved in DNA repair and replication and modulates the stability of the mitochondrial genome (**Table 2.9**). This activity is supported by functional annotation analysis showing significant enrichment for mitochondria (**Fig. 2.11**; **Fig. 2.12B**). A possible factor relating to this activity is the defective insertion of the TA proteins into

mitochondrial membrane in the absence of a functional Get1/Get2 receptor at the ER (Vitali et al., 2018). Since this was not confirmed by protein localization assay (**Fig. 2.19**), it is plausible that our treatment (concentration or time) was not potent enough to mimic genetic deletions in that publication, or alternatively, that the off-targeting did not occur as predicted.

Considering that *din7*Δ is sensitive to *A. amatymbica* (**Fig. 2.9**), it is plausible that *A. amatymbica* elicits an antioxidant response. Though antioxidant activity of *A. amatymbica* has not been well studied, the antioxidant properties of ent-kaurene diterpenes, the type of compound that is compound B, have been suspected (Thirugnanasampandan et al., 2008). Ent kaurene diterpenoid melissoidesin, isolated from the acetone extract of the leaves of *Isodon wightii*, exhibited promising antiacetylcholinesterase and antioxidant properties that can be used in food and drug preparations (Thirugnanasampandan et al., 2008). Thus the potential antioxidant properties of *A. amatymbica* could be attributed to Compound B- a kaurene diterpene.

Furthermore, the anti-oxidant potential of *A. amatymbica* is consistent with previous studies examining the anti-inflammatory activity of the extract (Mulaudzi et al., 2009), since the antioxidant activities of phenolic and flavonoid compounds are known to also exhibit anti-inflammatory activity (Arulselvan et al., 2016). Taken together, this provides preliminary evidence to validate the traditional use of *A. amatymbica* as an anti-inflammatory agent (Afolayan & Lewu, 2009).

2.4.6 Potential anti-cancer activity of *A. amatymbica*

Glo2 is part of the glyoxalase system, which detoxifies methylglyoxal and other endogenous harmful metabolites into non-toxic *d*-lactate, protecting the cell from damage and apoptosis (Antognelli et al., 2017). Yeast cells lacking Glo2 gene were sensitive to 25 µg/mL

of *A. amatymbica* (**Fig. 2.9**). Since Glo2 gene has been implicated in various types of cancers (Antognelli et al., 2017; de Bari et al., 2021; Hosoda et al., 2015) and the gene is under the regulatory control of Yap1p (**Fig. 2.10**), a potent oncogene that is amplified in these cancerous cells (Huang et al., 2005), it is important to consider the potential anti-cancer activity of *A. amatymbica*.

The glyoxalase system is an enzymatic network found in the cytosol of all mammalian cells consisting of Glo2, Glo1 and reduced glutathione (He et al., 2020). The system has been associated with various conditions including neurodegenerative disorders and cancer (He et al., 2020). Dysregulation of Glo2 is known to cause prostate tumorigenesis (Xu & Chen, 2006) and lung cancer (Antognelli et al., 2017), and has been found to be mediated by the natural product oleuropein, a polyphenolic compound enriched in olive oil (de Bari et al., 2021).

The anti-cancer activity of *A. amatymbica* has previously been studied in various cancer cell lines where anti-cancer activity was suspected to be attributed to the inhibition of lipoxygenase activities (Muleya et al., 2017) and the antioxidant activity of *A. amatymbica* (Wintola & Afolayan, 2014). The known interplay between anti-cancer and antioxidant activities (de Bari et al., 2021) complements the results of this study which suggests a potential function of *A. amatymbica* in both of these activities. However, the anti-cancer activity of *A. amatymbica* has not previously been studied in the context of glyoxalase activity. Future studies could investigate the glyoxalase system as a potential pathway for anti-cancer activity of *A. amatymbica*. This will further support the traditional use of the plant as a cancer prevention treatment (Wintola & Afolayan, 2014).

2.4.7 Potential function for *A. amatymbica* in lipid metabolism

Lipids have gained much attention recently due to their involvement in health and disease (Klug & Daum, 2014). Lipid metabolism involves a number of organelles mainly the ER, the Golgi apparatus, and the mitochondria (Fagone & Jackowski, 2009). A class of membrane lipids, the sterols, are primarily synthesized in the ER prior to transport (Peretti et al., 2008). In yeast, the major sterol is ergosterol (Erg), which is the end product of the yeast sterol biosynthetic pathway, the equivalent of mammalian cholesterol (Henneberry & Sturley, 2005). The ergosterol pathway involves a number of ERG genes interacting with different pathways (Jordá & Puig, 2020) including, interestingly, cytochrome b_5 (Cyb5), a known electron donor (Lamb et al., 1999).

When exposed to *A. amatymbica* extract, GFP tagged- Erg9 and Cyb5 both showed to a slight decrease in protein abundance and a slight change in localisation from perinuclear ER to cortical ER for Erg9 (**Fig. 2.20**). Eukaryotic cells are known to rapidly adjust the size, shape, and composition of organelles when met with changing physiological demands (Schmidt et al., 2019). Although such phenotypes possibly relate to ER membrane expansion associated with unfolded-protein response (UPR) (Schuck et al., 2009), UPR was not investigated in this study. However, the involvement of Erg9 and Cyb5 with this phenotype, genes which are involved in lipid metabolism and ER membrane biogenesis (Papagiannidis et al., 2021), suggests *A. amatymbica* potentially buffers the processes in these pathways.

Since Erg9 and Cyb5 are each involved in sterol and lipid biosynthesis (Karst & Lacroute, 1977; Truan et al., 1994), it is important to consider the potential activity of *A. amatymbica* extract in lipid metabolism. Using the HIP-HOP chemogenomics database (Lee et al., 2014), Get5-deficiency was most sensitive to the delta (9) fatty acid desaturase (OLE1) pathway. Delta (9) fatty acid desaturase (OLE1) is required for monounsaturated fatty acid synthesis and for normal distribution of mitochondria by catalysing the double bond formation

between carbons 9 and 10 of palmitoyl and stearoyl coenzyme A (Stukey et al., 1989). The involvement of Get5 in OLEI pathway complements the phenotype observed with Erg9 and Cyb5 (**Fig. 2.20**) in relation to a possible function of *A. amatymbica* extract in sterol and lipid biosynthesis

Furthermore, since the diterpene, compound B, elicits similar sensitivity in *pdr1Δpdr3Δget5*Δ (**Fig. 2.14**), it is plausible that compound B is involved in lipid metabolism. Although no lipid metabolism studies have been done on *A. amatymbica* to date, a study of diterpenes, albeit diterpene alkaloids, isolated from the medicinal plant *Nigella sativa*, found potent lipid metabolism activity in primary mouse hepatocytes (Morikawa et al., 2004). These activities mirror that of clofibrate (Morikawa et al., 2004), a peroxisome proliferator-activated receptor alpha (PPAR-α) agonist (Pan et al., 2018) known to be neuroprotective against neuroinflammation, neurotoxicity, neuronal damage and axonal injury (Moreno & Cerù, 2015; Oyagbemi et al., 2020). Interestingly, these neuroprotective effects are associated with antioxidant and anti-inflammatory properties (Gray et al., 2012), reinforcing the antioxidant potential of *A. amatymbica* extract as discussed earlier. Hence, this possibly validates the medicinal use of *A. amatymbica* (Afolayan & Lewu, 2009). Further, since kaurene-type diterpenes are a major bioactive component of *A. amatymbica* (Afolayan & Lewu, 2009), including compound B, this suggests a potential role of these compounds in lipid metabolism.

2.4.8 Potential function in protein synthesis

The sensitivity of yeast cells lacking the Eft2 gene when treated with *A. amatymbica* suggests a potential function of the extract in protein synthesis (**Fig. 2.9**). Protein synthesis as performed by ribosome, is well conserved in eukaryotes (von der Haar, 2008). Yeast cells synthesize around 13,000 proteins per second (Shah et al., 2013). In this process, the elongation

factor 2 (EF2), plays an essential role by catalysing ribosomal translocation (Justice et al., 1998).

The elongation factor 2 is a known target of antifungal drugs (Domínguez & Martín, 1998). The sordarin family of compounds, is known to block ribosomal translocation by stabilizing the fungal EF2-ribosome (Chiba et al., 2006). Sodarins are diterpene metabolites isolated from the fungus *Sordaria araneosa* (Pongcharoen et al., 2008). The antifungal drug fusidic acid, a triterpene, isolated from the fungus *Fusidium coccineum* (Brahmachari, 2019), also targets the elongation factor and inhibits the ribosomal peptide elongation and ribosomal recycling (Borg et al., 2015). With *pdr1Δpdr3Δeft2*Δ being sensitive to *A. amatymbica* (**Fig. 2.9**), analyses revealing significant enrichment for ribosome biogenesis and tRNA wobble modification (**Fig. 2.11**), and the involvement of the transcriptional activator- Gcn4p (**Fig.2.10**), this suggests a potential function of Eft2 in buffering the effects of *A. amatymbica* extract in protein synthesis. Further, since diterpenes are known to target the Eft2 protein, albeit fungal diterpenes, there is potentially a role of compound B in this bioactivity as structurally similar compounds often share biological activities (Martin et al., 2002).

Taken together, the results provide some validation of the traditional use of *A. amatymbica* as an antifungal medicine (Wintola & Afolayan, 2014), with the mechanism of action targeting protein synthesis, potentially attributable to the diterpenic components such as compound B. This could be the subject of future investigations.

2.4.9 Potential anti-viral activity of *A. amatymbica*

The traditional use of *A. amatymbica* extract as an antiviral medicine has not been fully studied (Louvel et al., 2013; Otang et al., 2012). For common respiratory RNA viruses, a suspected mechanism of action for antiviral drugs involves the inhibition of the viral

pyrimidine biosynthesis pathway (Cheung et al., 2017). This is because to replicate efficiently, viruses not only depend on their hosts for an adequate supply of pyrimidine nucleotides, but also up-regulate pyrimidine nucleotide biosynthesis in infected cells (Okesli et al., 2017). A compound screen, done by Kao et al (2010), revealed FA-613, an inhibitor of de novo pyrimidine biosynthesis, to be potent against various virus strain including SARS and MERS coronavirus (Cheung et al., 2017; Kao et al., 2010). This compound was found to interfere with the de novo pyrimidine synthesis by targeting the dihydroorate dehydrogenase (Kao et al., 2010).

In yeast cells lacking the Ura5 gene, 25 µg/mL of crude *A. amatymbica* extract led to a 60% growth inhibition, relative to 20% in wild-type (**Fig. 2.9**). Interestingly, Ura5 gene is involved in de novo biosynthesis of pyrimidines (**Table 2.9**). Ura5 encodes the enzyme orotate phosphoribosyltransferase (OPRTase) which catalyses the fifth enzymatic step of the process. The sensitivity of *pdr1Δpdr3Δura5Δ* to *A. amatymbica* suggests the Ura5 might be involved in buffering the effects of *A. amatymbica*. This in turn potentially infers bioactivity in de novo pyrimidine biosynthesis. Altogether, this may support the antiviral claims of *A. amatymbica* in traditional medicine. Further, pyrimidine synthesis inhibition is known to induce host innate immunity (Kao et al., 2010), which might help in potential clinical development of *A. amatymbica* as a potential antiviral drug. However future studies could further investigate the activity *A. amatymbica* is having on Ura5.

2.5.0 Potential variation in the bioactivity of *A. amatymbica*

The bioactivity of *A. amatymbica* could have varied throughout the course of the thesis for several reasons. This may have been due to factors that are known to affect the bioactivity of natural products (Yilmaz & Karadeniz, 2014). One of these is to do with storage. Studies

conducted on African medicinal plants have shown noticeable differences in phytochemical properties between fresh and stored samples (Laher et al., 2013; Yilmaz & Karadeniz, 2014). These differences might be due to stability of compounds. It is plausible that storage of *A. amatymbica* extract at -20^{0}C and continuous thawing and freezing may have had an effect on the bioactivity of the extract with each use. Shelf-life is especially important for natural products with pharmaceutical potential since the potency of these compounds needs to be guaranteed (Cheong et al., 2018). Although the effect of storage on bioactivity of *A. amatymbica* was not specifically monitored in this study, crude extract of *A. amatymbica* and compound B remained more potent to *pdr1*Δ*pdr3*Δ (**Fig. 2.3**) compared to BY4741, suggesting there was not a significant change in bioactivity.

Another aspect to consider is the plant part from which the extract was obtained and whether the plant is cultivated or grown in the wild (Louvel et al., 2013). It has been observed that *A. amatymbica* grown in the wild were more potent compared to plants that were cultivated (Mangoale & Afolayan, 2020), while there was little difference in potency between the part of the plant used and the type of extract prepared (Mangoale & Afolayan, 2020).

3.0 Conclusion

To conclude, this thesis investigated the bioactivity of *A. amatymbica,* a traditional medicine of Eastern and South Africa. Using yeast as a model organism, the project aimed to determine the potential mechanism of action of the *A. amatymbica* extract. Initial phenotypic screening of the crude extract and the semi purified compound B showed that *A. amatymbica* extract is a potential substrate of the PDR system in yeast. A genome-wide analysis, using the haploid deletion collection in the *pdr1*Δ*pdr3*Δ background, revealed significant enrichment for mitochondrial function, tRNA wobble modification and ribosome biogenesis. We revealed five

genes that when deleted showed significantly reduced growth in the presence of *A. amatymbica* extract compared to the untreated control. Of these, *pdr1*Δ*pdr3*Δ*get5*Δ was the most sensitive. Since Get5 is a member of the Get complex, the sensitivity of rest of the Get family was evaluated in low-throughput liquid assay. The entire Get family, as well as their interacting the chaperones were found to buffer *A. amatymbica* extract bioactivity. Further, evaluation of the Get pathway was conducted by overexpressing Get3 in *pdr1*Δ*pdr3*Δ*get1*Δ and *pdr1*Δ*pdr3*Δ*get2*Δ coincident with *A. amatymbica* extract treatment. This was meant to cause cytosolic aggregation of Get3-TA proteins leading to added stress and hence increased sensitivity to *A. amatymbica* extract. We further conducted a proteomic analysis using an N-terminally tagged GFP library, to investigate the mislocalization of TA protein when the cells are treated with *A. amatymbica* extract. We showed changes in localization of ER proteins (Erg9 and Cyb5), suggesting a potential function in lipid metabolism. This coincides with a potential antioxidant activity given the sensitivity of *din7*Δ to *A. amatymbica* extract whereby Din7 is a mitochondrial nuclease which modulates the stability of the mitochondrial genome. Furthermore, this activity may relate to the anti-cancer activity of *A. amatymbica* extract with *pdr1Δpdr3Δglo2Δ* also being sensitive to the extract. Finally two other genes, Eft2 and Ura5, found to be sensitive to *A. amatymbica* extract suggests potential functions of *A. amatymbica* extract in protein synthesis and anti-viral activity. Taken together, the activity of *A. amatymbica* extract encompasses interaction with the Get complex, with potential functions in antioxidant and anti-cancer activity, lipid metabolism, protein synthesis and pyrimidine biogenesis.

4.0 Future directions

Unfortunately, time did not allow for further characterization of the bioactivity of *A. amatymbica* extract. At the end of the thesis, there are questions that remain unanswered. The following assays could be carried out in the future to further study the role of GET complex in the bioactivity of *A. amatymbica* extract. Additionally, the potential antioxidant and lipid metabolism functions of *A. amatymbica* extract could be explored.

4.1 Genome-wide screen of gene deletion in a PDR-positive background

Although the study was based on *pdr1*Δ*pdr3*Δ strain due to limited amount of *A. amatymbica* extract and compound B, future study could explore the bioactivity in wild-type (BY4741) strain, albeit this will require more extract and compound. Using a yeast deletion library (Giaever & Nislow, 2014), a genome wide screen could be conducted to determine strains that are sensitive in the presence of the PDR system. Sensitivity of yeast to a functioning PDR system could be compared to sensitivity when the PDR system has been down regulated. This would help understand the antifungal traditional use of *A. amatymbica* in medicinal applications where the PDR system is functional when the *A. amatymbica* extract is consumed orally.

4.2 Localization of TA proteins

Since SDS was used to mimic the PDR response in the part of the study that monitored localization of TA proteins, it is not known whether the SDS treatment is having a similar effect to a PDR-attenuated strain or whether the master regulators, Pdr1p and Pdr3p, are still functional and actively contributing in drug efflux. The latter would have contributed to less potency in *A. amatymbica*. A GFP screen done in the *pdr1*Δ*pdr3*Δ background, would

determine the changes in TA protein localization in response to *A. amatymbica* when the PDR system is not functional. If all things equal, the same result will be obtained, which would then inform a change in an experiment condition specifically treatment with *A. amatymbica* for more than four hours. Also, since yeast membranes permeabilised with SDS are known to affect cell viability by allowing entry of various toxins and leakage of cellular content (Pannunzio et al., 2004), it is unknown how much of an effect this has had on the experiment.

Furthermore, since the study was done using a subset of GFP library where proteins are tagged to the N terminal, it is not known what the effect of *A. amatymbica* is in a C-terminally tagged library, suggesting a possible future experiment that monitors localisation of all proteins, not just TA proteins.

4.3 Characterisation of lipid metabolism

Further study could be done to investigate the potential lipid metabolism activity of *A. amatymbica*. The Erg9p and Cyb5p proteins are both involved in sterol metabolism of which lipid droplets serve a critical function in sterol esterification. Lipids can be measured in cells treated with *A. amatymbica* (Patel et al., 2019). If *A. amatymbica* affects lipid metabolism, changes in the number of lipid droplet in treatment compared to control will be observed. The number of lipid droplets can be compared to the *dga1*Δ*lro1*Δ strain that is only able to produce one lipid per cell (Choudhary et al., 2011).

Further, the function of *A. amatymbica* in ergosterol biosynthesis could be studied in the context of ERG9, which encodes squalene synthase Erg9p, responsible for converting diphosphate to squalene (Jennings et al., 1991). In mutant yeast lacking the ERG9 gene, the amount of squalene could be observed when treated with the extract to confirm if the extract is a substrate at squalene synthase step of ergosterol biosynthesis.

4.4 Reactive oxygen species assay

To confirm the antioxidant activity of *A. amatymbica*, an analysis of reactive oxygen species (ROS) could be done. ROS are highly reactive metabolites of oxygen, such as superoxide, hydrogen peroxide (H_2O_2), and peroxynitrite (Dikalov & Harrison, 2014). Since antioxidants can react with ROS to produce stable products, the level of ROS could be used to determine if *A. amatymbica* exhibits an antioxidant effect by reducing the level of ROS. ROS can be measured in the absence of the Tpk3p gene in yeast, which is known to regulate mitochondrial biogenesis and when absent, causes the mitochondria to produce large amounts of ROS (Chevtzoff et al., 2010). Alternatively, studies could be done with yeast cells lacking the Sod1 gene that detoxifies superoxide radicals produced during aerobic respiration and its deletion renders yeast hypersensitive to oxygen (Martins & English, 2014). If in the presence of *A. amatymbica*, the growth of *sod1Δ* is rescued, this will support the antioxidant activity of the extract.

4.5 Identifying the target of *A. amatymbica*

To better understand the bioactivity of *A. amatymbica* extract, other approaches could be taken. One approach is by using a heterozygous deletion library which identifies genes that require one copy to be sensitive to a treatment, i.e. haploinsufficiency. This method was successfully used to identify the target of rapamycin (Gibbons et al., 2009). A proteomic profiling could also be done (Tashiro & Imoto, 2012). For this, various expression profiles of cells under compound treatment are compared against expression profiles of other known compounds. Since compounds with similar expression profiles have similar modes of action (Cong et al., 2012), a profile that is comparable to *A. amatymbica* extract would determine potential targets of *A. amatymbica* extract.

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