SMALL LACCASES AS CATALYSTS FOR THE SYNTHESIS OF ANTIOXIDANTS

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Blessing Nemadziva

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SUPERVISOR : **Prof Tukayi Kudanga**

CO-SUPERVISOR : **Dr Marilize Le Roes-Hill**

REFERENCE DECLARATION

I, Mr. B. Nemadziva – 21143322 and Prof Tukayi Kudanga (Supervisor) do hereby declare that in respect of the following dissertation:

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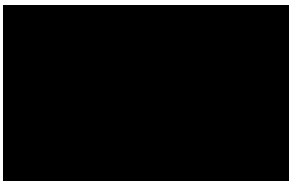

17/08/2018

Signature of student **Date**

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Signature of supervisor **Date**

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AUTHORS DECLARATION

This study presents original work by the author. It has not been submitted in any form to another academic institution. Where use was made of the work of others, it has been duly acknowledged in the text. The research described in this dissertation was carried out in the Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, South Africa, under the supervision of **Prof Tukayi Kudanga** and **Dr Marilize Le Roes-Hill**.

Student's signature

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ABSTRACT

The rise in antioxidant demand for industrial applications has necessitated the need to investigate new methods for antioxidant production. Conventionally, antioxidants have been used in the food industry. However, newer applications in industries such as pharmaceuticals, cosmetics, medicine, nano-bioscience, as well as in chemical industries, have contributed to the increase in antioxidant demand. The market for antioxidants has been forecasted to increase by 6.42% compound annual growth rate (CAGR) between 2015 and 2022. Therefore, there is now a need to develop new processes for antioxidant synthesis to meet this rising demand. Biocatalysis has gained notable attention as a viable approach for antioxidant synthesis. Laccases are the preferred enzymes since their reaction mechanism involves the use of molecular oxygen to oxidise phenolic compounds to corresponding radicals, with water as the only by-product. Most laccase antioxidant synthesis research has employed fungal and plant laccases. However, bacterial laccases may be promising biocatalysts, considering the advances in molecular technology which make expression in bacterial hosts easier. This study focused on the biotransformation of natural phenolic compounds using small laccase (SLAC), a two-domain bacterial laccase native to *Streptomyces coelicolor*. Because of the low redox potential of the enzyme, a preliminary substrate screening process was conducted to identify phenolics oxidisable by the SLAC. Caffeic acid, 2,6-dimethoxyphenol, catechol, gallic acid, guaiacol, ferulic acid, and pyrogallol were identified as SLAC substrates and further coupling reaction studies were conducted using caffeic acid and gallic acid. Coupling reactions were carried out either in biphasic systems consisting of water-immiscible organic solvents and a buffer system or monophasic systems consisting of miscible organic solvents that form a homogenous phase with the buffer system. Coupling products were monitored using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC), purified using preparative TLC and column chromatography, and characterised by liquid chromatography-mass spectrometry (LCMS) and nuclear magnetic resonance spectroscopy (NMR). Antioxidant capacity of the oxidation products were investigated by using the 2,2’-diphenyl-1-picrylhydrazyl (DPPH) and Trolox equivalence antioxidant capacity (TEAC) assays.

Two oxidation products (one from caffeic acid and another from gallic acid) were successfully produced, purified and characterised. The oxidation product obtained from the SLAC-catalysed oxidation of caffeic acid was identified as a *β-β* dimer using LC-MS and NMR. When the reaction was carried out at a large-scale, a 32.8% yield of the dimer was achieved. Results showed that optimum yield of the dimer was achieved when the reaction was carried out for 6 h in a biphasic system consisting of 80% ethyl acetate and sodium acetate buffer pH 7.5. The dimer demonstrated superior antioxidant capacity, showing a 1.5-fold increase in DPPH radical scavenging capacity and a 1.8-fold improvement in TEAC. The dimer exhibited several positive physicochemical attributes, including improved solubility properties in aqueous media and remarkable stability in acidic pH (pH 2.2 and pH 5.5).

One oxidation product from the SLAC-catalysed oxidation of gallic acid was successfully produced, purified and partially characterised. Optimum yield of gallic acid oxidation product was achieved when the reaction was conducted in a biphasic system consisting of 80% ethyl acetate and Tris-HCl buffer pH 8.0, using 0.5 U SLAC and a reaction time of 4 h. However, the oxidation product showed a lower antioxidant capacity than the substrate, as demonstrated by standard antioxidant assays (DPPH and TEAC).

In conclusion, two antioxidant products were successfully produced, purified and characterised. Furthermore, selected physicochemical and antioxidant activities were determined. Overall, this study has highlighted the potential of the small laccase as a catalyst for the synthesis of antioxidants.

1. INTRODUCTION

The incidence of chronic or non-communicable diseases (NCDs) caused by oxidative stress is increasing (Bloom et al., 2012). For example, diabetes and cancer accounted for 42% of all deaths recorded in 2014 in the United States of America (Kochanek et al., 2016). In South Africa, Statistics South Africa reports of 2005 revealed that chronic diseases accounted for 20% deaths of people between the age of 35 and 64 (Van Zyl et al., 2012). On a global scale, NCDs account for 60% of all deaths (Habib and Saha, 2010). This burden of NCDs, coupled with an increase in nutritional health awareness, has led to intensive research activities on antioxidants, especially during the past two decades. Studies such as the Carotene and Retinol Efficacy Trial (CARET) and the Physicians' Health Study I (PHS I) are examples of extensive research performed on this topic (Hennekens et al., 1996, Lippman et al., 2009). Oxidative stress is a condition that arises from the excessive accumulation of reactive oxygen species (ROS) more than cellular antioxidants can handle (Dalle-Donne et al., 2006). ROS are the most common form of free radicals formed during most biochemical reactions (Mohajeri and Asemani, 2009). Free radicals are known for their highly reactive properties which can result in cell damage and homeostatic destabilisation through destruction of biological molecules such as nucleic acids, proteins and lipids (Lobo et al., 2010). Evidence from several studies has indicated the contribution of oxidative stress in several human diseases, among them, ischemic diseases, cancer, diabetes, neurological disorders and aging (Dalle-Donne et al., 2006, Jenner, 2003, Sayre et al., 2001, Lobo et al., 2010)). Normally, the body produces enough cellular antioxidants which neutralizes and thwarts the destructive effect of ROS. However, under some pathophysiological conditions, there is an imbalance between ROS and cellular antioxidant production, resulting in the aforementioned health conditions.

The significant role of antioxidants in combating oxidative stress has made them a subject of much interest in human health and nutrition research. Antioxidants are molecules with electron donating capacity, which delay or prevent the destructive effect of ROS by being oxidised themselves (Halliwell et al., 1995, Shahidi and Zhong, 2015). The global market for antioxidants has been forecasted to increase by a CAGR of 6.42% between 2015 and 2022 (Allied Market Research, 2016). Among the reasons for this rise includes a general increase in health awareness thus creating a need for healthy dietary supplements. Antioxidants are now being incorporated into food products to improve shelf life as well as in pharmaceutical products and cosmetics as

antiaging therapy (Transparency Market Research, 2015, Sathya and Siddhuraju, 2012). This increased demand for antioxidants may have influenced the recent increase in research activities focusing on the synthesis of new antioxidants.

Natural foods, particularly fruits and vegetables, are considered a good source of natural antioxidants (Cerón et al., 2014). However, their efficacy is not convincing, mainly due to the poor radical scavenging properties of most natural monomeric antioxidants (Adelakun et al., 2012b, Sánchez-Moreno et al., 1999). In addition, the antioxidant content may vary with the type of the plant and therefore many plants are not a dependable source of good quality antioxidants. There are also synthetic antioxidants available on the market as food preservatives. The most common include butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (Shahidi, 2000). However, a regulatory Acceptable Daily Intake has been established on the use of these synthetic products because of their reported side effects (Soubra et al., 2007). Synthetic antioxidants have been reported to promote carcinogenic activity, and many affect function of organs such as lungs, kidneys and the liver (Hocman, 1988, Babich, 1982). Consequently, attention is now being shifted towards improving the bioactive efficacy of natural antioxidants using enzyme-catalysed reactions (Adelakun et al., 2012a, Gavezzotti et al., 2014). Much research has focused on the use of oxidases in the synthesis of antioxidants with enhanced bioefficacy. Enzymes such as polyphenol oxidases, peroxidases and laccases have been considered in these studies (Burton et al., 1998, Tuck and Hayball, 2002, Burton, 2003b).

Laccase has been the preferred biocatalyst for organic synthesis because it is regarded as a ‘green catalyst’ requiring only oxygen for catalysis and producing water as the sole by-product (Riva, 2006). It belongs to the blue copper oxidase family, and contains a cluster of four copper atoms which are vital for the catalytic functions of the enzyme. Its mechanism involves the monoelectronic oxidation of a substrate to the corresponding radical. Aromatic compounds, phenols and aliphatic amines can all be oxidised by laccase action. The production of radicals is useful since it opens avenues for industrial application. Radicals can be involved in coupling reactions to produce oligomeric units of commercial value (Jeon and Chang, 2013). β-5 dimers of ferulic acid and hydrocerulignone (from the modification of 2,6-dimethoxyphenol) are examples of oligomeric antioxidants with enhanced bioactivity that have been produced through laccase-catalysed coupling reactions (Adelakun et al., 2012a,b). Interestingly, most research on laccase has focused on fungal laccases. However, laccases are ubiquitous and are also present in prokaryotes. The well-advanced tools for genetic manipulation in bacteria has opened up vast

possibilities for heterologous expression in hosts such as *Escherichia coli* (Sharma et al., 2007, Santhanam et al., 2011). Machczynski and colleagues successfully expressed bacterial laccase from *Streptomyces coelicolor* in *E. coli* and upon characterisation, they discovered that unlike eukaryotic laccases which had three domains, it had two domains (Machczynski et al., 2004). The term small laccase (SLAC) has then been used to describe this laccase. Characterisation of the enzyme showed promising potential for industrial application, especially its thermostability, activity over a broad pH range and resistance to detergents.

Regardless of their robust nature, to date, no work has been published concerning the exploitation of SLAC for the synthesis of antioxidants. This study seeks to explore the potential of SLAC as biocatalysts for enhancing the bioefficacy of natural antioxidants.

Research questions:

- Can new antioxidants be produced using SLAC as biocatalyst?
- Do the synthesised antioxidants have better properties than the monomeric units?

Hypotheses

1. *SLAC catalysed oxidation of monomeric phenolic compounds produces new antioxidants.* Many researchers have used peroxidases (Zhang et al., 2012, Yu et al., 2007, El Agha et al., 2008) and fungal laccases (Mustafa et al., 2005) in the synthesis of new bioactive compounds. Since SLAC has the similar mode of action to fungal laccases, new antioxidants can also be expected to be produced using SLAC as the catalyst.

2. *Oligomeric antioxidants will have better properties than the monomeric antioxidants.* Adelakun et al. (2012b) showed that β-5 dimers had better antioxidant properties than their monomeric form (ferulic acid). The antioxidant capacity of the oligomeric products is expected to be better than their monomeric units since dimerisation may result in more sites to react with the free radicals (Matsuura and Ohkatsu, 2000), as well as an increase in electron donating groups (Srinivasan et al., 2007).

Aim: To produce new antioxidants using a SLAC-catalysed process

Objectives:

- To produce, purify and characterise the small laccase from *Streptomyces coelicolor* A3(2).
- To synthesise phenolic antioxidants through coupling reactions catalysed by SLAC.
- To optimise reaction conditions for coupling reactions through reaction engineering so as to maximise substrate conversion and product formation.
- To purify the coupling products and characterise their structure and antioxidant properties

2. LITERATURE REVIEW

2.1. INTRODUCTION

This chapter presents a review of literature relevant to this study. Some of the areas covered in this review include: the general trends and incidences that have motivated the rise in antioxidant research; the importance of antioxidants as therapeutic compounds; approaches to antioxidant production; laccase catalysis as an approach to antioxidant synthesis; a survey of previous studies on laccase-catalysed antioxidant production; methods used in analysing antioxidant activity; reaction systems used in the production of antioxidants, and methods used in the characterisation of antioxidants. The literature review concludes with a scope of this study as a way of linking the literature reviewed and the current study.

2.2. OXIDATIVE STRESS: GLOBAL TRENDS

The prevalence of chronic diseases has been reported to be on the rise, particularly in developing countries. Chronic diseases are generally described as health conditions that lasts for more than 12 months and requiring constant medical attention (Goodman et al., 2013). Unlike other diseases which can be cured, chronic diseases are characterised as non-curable when contracted (National Center for Health Statistics, 2011). Some of the most common chronic diseases include; cardiovascular conditions, hypertensions, stroke, cancers, chronic respiratory diseases, diabetes, arthritis and asthma. There are several risk factors that have been linked with chronic diseases such as alcohol harm, tobacco use, physical inactivity and high cholesterol (Van Zyl et al., 2012). These risk factors are related to lifestyle, thus chronic diseases are sometimes referred to as lifestyle diseases (Galobardes et al., 2003). Low to middle income nations such as Afghanistan, Algeria, Bangladeshi and South Africa have been adversely affected by the 'burden of chronic disease' which has exerted strain on health services (Yach et al., 2004) as well as the economy because of the need for constant medical attention required by patients with chronic conditions. Chronic diseases accounted for 29 million deaths worldwide in 2002 (Yach et al., 2004), the toll rose to 36 million by 2008 (Van Zyl et al., 2012). With such adverse effects, world governing bodies such as the World Health Organisation (WHO) are setting intervention strategies to try and mitigate the impact on chronic diseases. As an example, WHO has set goals to reduce death rates caused by chronic diseases by 2% every year (Ezzati et al., 2004, Asaria et

al., 2007). One of the strategies involves encouraging healthy diets. This is because research findings have linked the occurrences of chronic diseases to oxidative stress (Bloom et al., 2012).

2.3. OXIDATIVE STRESS

Oxidative stress is a condition where the equilibrium between reactive oxygen species (ROS) accumulation and antioxidant availability shifts in favour of ROS (Sies, 1991). ROS are naturally produced during metabolic processes such as respiration. However, under some pathophysiological conditions, there are more ROS being produced than could be contained by the available antioxidants. There are two defense mechanisms in which the body maintains the levels of ROS, a balance commonly referred to as 'redox homeostasis' (Marengo et al., 2016), vis; enzymatic and nonenzymatic scavenging mechanisms.

2.3.1. Enzymatic approach to ROS mitigation

During metabolic processes occurring in the mitochondria and peroxisomes, highly reactive oxygen species such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet-}$) and hydroxy radicals ($OH^{\bullet}$) are generated (Dröge, 2002). Superoxide dismutase (SOD) was the first enzyme discovered to play a role in the elimination of ROSs (McCord and Fridovich, 1969). SOD catalyses a biochemical reaction that dismutates two $O_2^{\bullet-}$ to produce H_2O_2 and O_2 as byproducts (Figure 1). There are other enzymes involved in the elimination of ROSs; catalase catalyses the conversion of H_2O_2 to O_2 and H_2O; peroxiredoxins also catalyses the conversion of H_2O_2 to H_2O in a reaction that involves the reduction of alkyl hydroperoxides to alcohols (Marengo et al., 2016); thioredoxins help eliminate ROSs by means of a 2-cystein active site that reacts with free radicals.

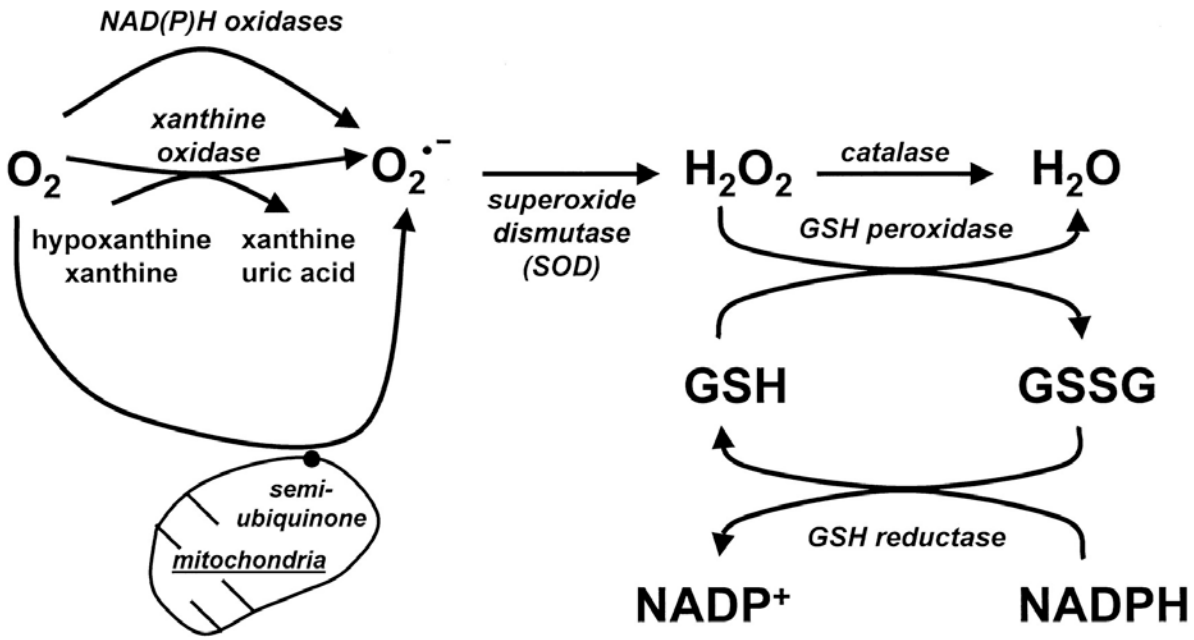


Figure 1: Pathways for ROS production and clearance. SOD and catalase are some of the enzymes pivotal for the elimination of ROS in mammalian cells (Dröge, 2002). Catalase action on H_2O_2 involves the change of state of Glutathione from reduced (GSH) to its oxidised (GSSG) form.

2.3.2. Non-enzymatic approach to ROS mitigation

Antioxidants is a general term referring to substances that neutralizes free radical activity. This includes both enzymatic and non-enzymatic antioxidants. Non-enzymatic antioxidants are mostly plant based compounds such as α-tocopherol, ascorbic acid, carotenoids, flavonoids and polyphenols (Devasagayam et al., 2004). Non-enzymatic antioxidants have an ability to donate electrons to ROS thus reducing free radicals to a more stable state. The presence of adequate antioxidants in the body is essential to maintain redox homeostasis as well as thwarting the deleterious effects of oxidative stress.

2.4. OXIDATIVE STRESS AND CHRONIC DISEASES

Oxidative stress has been linked with several chronic conditions. A free radical-antioxidant imbalance that is skewed towards free radicals can result in the destruction of biological molecules. This destruction of biological molecules like lipids, proteins and DNA can affect cell function resulting in physiological conditions.

2.4.1. Physiological implications of lipid peroxidation

The free radical oxidation of lipids involves a series of steps starting with the transfer of a hydrogen atom from the lipid to the free radical until the cyclisation of the resulting lipid peroxyl radical (Porter et al., 1995). It is reported that polyunsaturated lipids are more prone to oxidation by free radicals than saturated lipids (Niki et al., 2005). This may be because unsaturated lipids contain double bonds which are electron rich and can offer attacking sites to the free radicals. The lipid peroxidation products have been implicated with pathophysiological conditions such as inflammation, atherosclerosis, neurodegenerative disorders as well as cancers (Guéraud et al., 2010). According to Singer and Nicolson's fluid mosaic model (Singer and Nicolson, 1972), lipids are integral components of cell membranes. They form the lipid bilayer which basically defines cells and their functions. Lipid peroxidation therefore can alter membrane structure which can result in pathophysiological conditions.

2.4.2. Physiological implications of protein alteration

ROS have been reported to react with proteins in three ways; (i) ROS can react directly with individual amino acids. Amino acids such as methionine, cysteine, arginine, and histidine are reported to be the most prone to attack by free radicals; (ii) ROS can cause the cleavage of the polypeptide chain; (iii) ROS reaction with lipids results in several lipid peroxidation products, which subsequently react with proteins to form protein cross linkages (Lobo et al., 2010). Because of the significant role of proteins as biocatalysts, transmembrane proteins involved in movement of molecules across the cell membrane and as receptors, any alteration of protein structure can affect cell function. ROS mediated protein oxidation has been implicated to aging (Stadtman, 2006, Lobo et al., 2010). It has been observed that the amount of oxidised protein in animals increased with age, highlighting the direct link between protein oxidation and aging (Stadtman, 2006).

2.4.3. Effects of DNA modification

Reactive oxygen species have been reported to contribute towards carcinogenesis through the alteration of the genetic material and also preventing normal DNA replication as a result of chromosomal damage (Valko et al., 2004). Free radicals are involved in the modification of the DNA strand through deletion, reaction with nitrogenous bases, oxidation of deoxyribose, DNA-protein crosslinking, frame shifts, chromosomal arrangements, strand breakage and creation of base free sites (Dexheimer, 2013). The body has developed mechanisms of repairing damaged

DNA which includes base excision repair, mismatch repair, nucleotide excision repair and double strand break repair (Dexheimer, 2013). It is estimated that a human cell is exposed to ROS at a frequency of 100 000/day. Any subsequent DNA lesions that escapes repair mechanism may lead to mutagenesis. Therefore, it is vital to enhance antioxidant availability in the body by increased intake of plant-based phenolics.

2.5. PLANT-BASED PHENOLICS AS NON-ENZYMATIC ANTIOXIDANTS

Plant derived phenolic compounds such as vitamins E and flavonoids are involved in the non-enzymatic defense mechanism against free radical activity (Marengo et al., 2016). The involvement of plant derived antioxidants in thwarting free radical activity has opened a research niche exploring the capacity of various plant derived compounds as antioxidants (Dai and Mumper, 2010, Prior and Cao, 2000, Serafini et al., 2011). Plant based diets are being promoted because of the abundance of bioactive phenolic phytochemicals present in plants which can help in fortifying the antioxidant network in living systems (Sies et al., 2005). This has seen the adoption of Mediterranean diets which are characteristic of high vegetable content (Saura-Calixto and Goñi, 2006), in place of western based diets which generally consists of high calorie foods (Morabito et al., 2014).

2.6. APPLICATIONS OF ANTIOXIDANTS

Phenolic compounds have become a subject of interest because of their antioxidant properties. Research on antioxidants is already being transferred from the labs to various industrial applications. The global market for antioxidants is predicted to increase as a result of antioxidant applications in industries such as food, medical, and cosmetics industries (Guaadaoui et al., 2014).

2.6.1. Food industry

Lipid oxidation during storage is often a challenge in the food industry. It causes deterioration of food quality including a decrease in nutritional value, repugnant flavours, alteration of food texture and colour, which results in economic losses to food producers (Sardarodiyan and Sani, 2016, Thorat et al., 2013). Traditionally, antioxidants have been applied in the food industry as preservatives to retard the rate of oxidative degradation especially in foods with high lipid content (Ngo et al., 2011). Natural antioxidants such as tocopherols, vitamins, flavonoids,

lycopenes and synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been consequently incorporated into food products to reduce the rate of lipid oxidation (Thorat et al., 2013). Naturally derived antioxidants are widely preferred because they are generally considered safe (Thorat et al., 2013) and in some countries regulatory measures have been instated to minimize the application of synthetic antioxidants in food (Soubra et al., 2007). There is also currently an increased interest in the development of nutraceuticals and functional foods (Shui and Leong, 2006) which has motivated many researchers to explore various approaches for producing plant-derived antioxidants with enhanced bioactivity.

2.6.2. Application in cosmetic formulations

The action of free radicals on biological molecules such as proteins has been implicated in aging (Stadtman, 2006). Consequently, antioxidants have been applied in topical products to prevent free radical action. Antioxidant compounds such as vitamin E, coenzyme Q10, lycopene and silymarin have been added to cosmetic formulations because of their antioxidative properties (Allemann and Baumann, 2008).

2.6.3. Medical applications

Several epidemiological studies have shown that diets high in fruits and vegetables minimise the risk of chronic diseases, thereby supporting the widely accepted hypothesis that antioxidants have therapeutic properties against oxidative stress-induced diseases (Rodrigo et al., 2007). Consequently, antioxidants have been applied as medical supplements in the treatment of pathophysiological conditions associated with oxidative stress. Antioxidant molecules such as polyphenols, vitamins A, C and E and coenzyme Q10 have been used as antioxidant supplements. There have been some promising results from clinical studies investigating the suitability of antioxidants as therapeutic drugs. For example; (i) curcumin, a polyphenol compound, has been used as a therapeutic drug for the treatment of chronic kidney disease due to its superior antioxidant activity against ROS in the mitochondria (Saso and Firuzi, 2014); (ii) N-acetyl-L-cysteine has been effectively used in the treatment of chronic obstructive pulmonary disease (Hillas et al., 2013), and (iii) Polyphenolic antioxidants are effective agents in the treatment of osteoarthritis (Firuzi et al., 2011). However, the efficacy of antioxidants as therapeutic drugs for the treatment of chronic diseases remains a widely debated subject because of several unconvincing results obtained from other clinical trials (such as the Physician Health

Study and the *beta*-Carotene and Retinol Efficacy Trial (Goodman et al., 2011) and meta-analyses (Myung et al., 2010). Some researchers argue that the lack of success in the trials are as a result of factors such as flaws in the methodologies employed, lack of good biological rationale for selecting antioxidant compounds, and insufficient knowledge on the bioavailability and pharmacokinetic properties of selected antioxidant compounds (Goodman et al., 2011, Rodrigo et al., 2007). Since there is compelling evidence from *in vitro* tests and epidemiological studies on the role of antioxidants in thwarting ROS chain reactions, several researchers have shifted focus to enhancing antioxidants properties for improved bioavailability (Saso and Firuzi, 2014), improved antiradical capacity (Adelakun et al., 2012b), enhanced solubility (Ghoul and Chebil, 2012) among other bioactive and physicochemical properties.

2.7. APPROACHES TO ANTIOXIDANT PRODUCTION

In addition to advocating for the adoption of Mediterranean diets, researchers as well as the pharmaceutical industry have explored various approaches of producing antioxidants with enhanced bioactive capacity. This is mostly because diet-based antioxidants are an inconsistent source of good quality antioxidants due to variation in quality of plants. Some of the presently used methods for extraction and production of antioxidant compounds include the heat reflux extraction method, accelerated solvent method, supercritical fluids, employing high pressure protocols, use of microwave and ultrasound extraction processes, and chemical synthesis (Martins et al., 2011). Most of these processes are energy intensive, depending heavily on solvents thus making the purification of products difficult and expensive (Martins et al., 2010). Conventional chemical processes employed in the production of bioactive compounds are generally long, energy intensive, low yielding and associated with excessive amounts of wastes which have a negative impact on the environment. Metrics such as the E–factor, have highlighted the inefficiencies of chemical synthesis; the amount of waste generated per kilogram of any fine chemical or pharmaceutical product manufactured was 5–100 times higher than the product (Sheldon, 2017, Li and Trost, 2008). Such concerns have prompted the formation of bodies such as the American Chemical Society Green Chemistry Institute Pharmaceutical Roundtable (ACS GCIPR) to promote the adoption of green technologies in pharmaceutical industries (Constable et al., 2007). Thus newer, economically feasible and environmentally benign processes have become a priority in a bid to meet the rising demand for bioactive compounds. Biocatalysis has been identified as an alternative to antioxidant production.

2.8. BIOCATALYSIS AS AN ALTERNATIVE FOR THE SYNTHESIS OF BIOACTIVE COMPOUNDS

Biocatalysis is gaining notable attention in organic synthesis. This is because biocatalysts offer environmentally benign and shorter processes for the synthesis of valuable compounds. Unlike conventional means, enzymes are generally selective, a trait which is of importance when producing compounds of therapeutic value (Maugh, 1984). The biological synthesis of bioactive phenolic compounds has been achieved using oxidoreductase enzymes such as tyrosinases, peroxidases and laccases (Burton, 2003b).

2.8.1. Peroxidases

Peroxidases (EC 1.11.1.7) are ferric enzymes. They usually contain ferriprotoporphyrin IX haem group at the active site which consists of four pyrrole nitrogens bound to a Fe(III) ion (O'Brien, 2000). In biological systems, peroxidases are involved in synthesis and degradation of lignin, oxidation of toxic compounds, peroxidases are involved in the defense mechanism against pathogens and stress, elimination of toxic substances such as H_2O_2 and other compounds (O'Brien, 2000). Peroxidases' mechanism of action is initiated by the reaction of the enzyme with H_2O_2 resulting in the formation of an enzyme intermediate as well as the subsequent reduction of H_2O_2 to water. The unstable enzyme intermediate has to undergo a two cycled one-electron oxidation of phenolic substrates to return to the stable Fe(III) state (Hamid and Khalil ur, 2009). The oxidation of phenolic substrates results in the generation of radicals. Because peroxidase catalyses the generation of radicals, it has been employed in organic synthesis for oxidative polymerisation of phenols (Kobayashi and Higashimura, 2003).

2.8.2. Tyrosinases

Tyrosinases (phenol: oxidoreductase, E.C. 1.14.18.1) are multicopper oxidases involved in the one-electron oxidation of substrates in the presence of molecular oxygen yielding water as byproduct (Burton, 2003a). Tyrosinase consists of two copper ions in its active site, these metal ions are responsible for binding both oxygen and the substrate. Tyrosinase substrates are phenols, hence its commonly known as polyphenol oxidase. In nature, tyrosinases are involved in wound healing through browning reactions. Tyrosinases are also involved in melanin and pigment

synthesis (Kim and Uyama, 2005). Its mechanism of action involves the hydroxylation of phenolic compounds into catechols which are subsequently oxidised to produce quinones (Decker and Tuczek, 2000, Burton, 2003b). Tyrosinases have been successfully used in the biotransformation of phenolic compounds into potential commercially valuable compounds such as antioxidants (Espín et al., 2001, Guazzaroni et al., 2012) and biopolymers (Halaouli et al., 2006).

2.8.3. Laccases

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) also belong to the multicopper oxidase family of enzymes. Their role in nature involves both anabolic and catabolic processes such as cell wall formation, lignification, sclerotisation and delignification (Riva, 2006). Laccases are generally regarded as "green catalysts" because of their ability to oxidise a diverse range of compounds (including phenols, diphenols, methoxy-substituted phenols, phenolic and alkyl amines) to corresponding radicals in the presence of molecular oxygen and subsequently producing water as the only by-product (Kudanga et al., 2011a, Polak and Jarosz-Wilkolazka, 2012). The laccase active site consists of a cluster of four copper atoms. The copper atoms are separately identified based on their different electronic paramagnetic resonance (EPR) signals (Dwivedi et al., 2011); Type 1 is where substrate oxidation occurs, Type 2 and two Type 3 copper atoms form the trinuclear cluster where oxygen is reduced to water (Figure 2) (Dwivedi et al., 2011, Riva, 2006). Laccase catalytic mechanism generally involves the abstraction of a single electron from substrates to produce reactive free radicals (Kudanga and Le Roes-Hill, 2014). These free radicals are vital intermediates, which undergo coupling reactions to produce dimeric, oligomeric, polymeric or cross-coupling products (Figure 3).

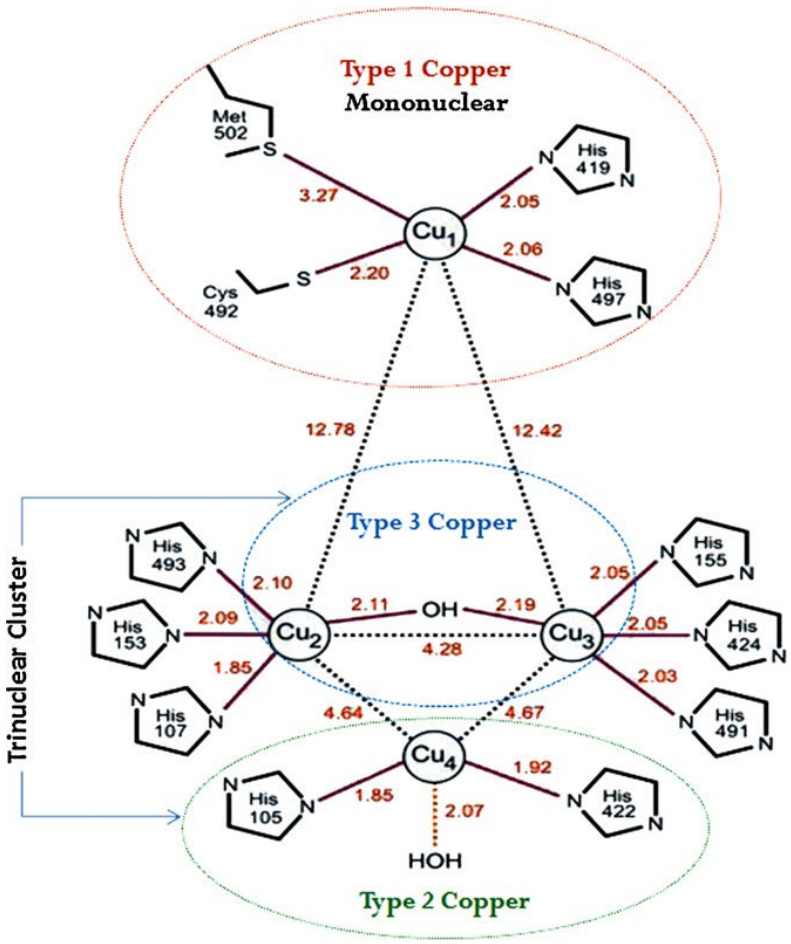


Figure 2: Schematic representation of laccase active site (Dwivedi et al., 2011).

Of the enzymes employed in the biotransformation of phenolic compounds, laccase has been much preferred over tyrosinase and peroxidase. This may be because the tyrosinase mechanism of action involves the formation of a quinone, which can inhibit the enzyme through formation of an inactive complex as a result of quinone-enzyme covalent bonding (Burton, 2003b), whereas the dependence of peroxidase on H_2O_2 makes it a health hazard as well as expensive. Many researchers have investigated the potential of laccase in catalysing the production of bioactive compounds including antioxidants, alkaloids and antibiotics.

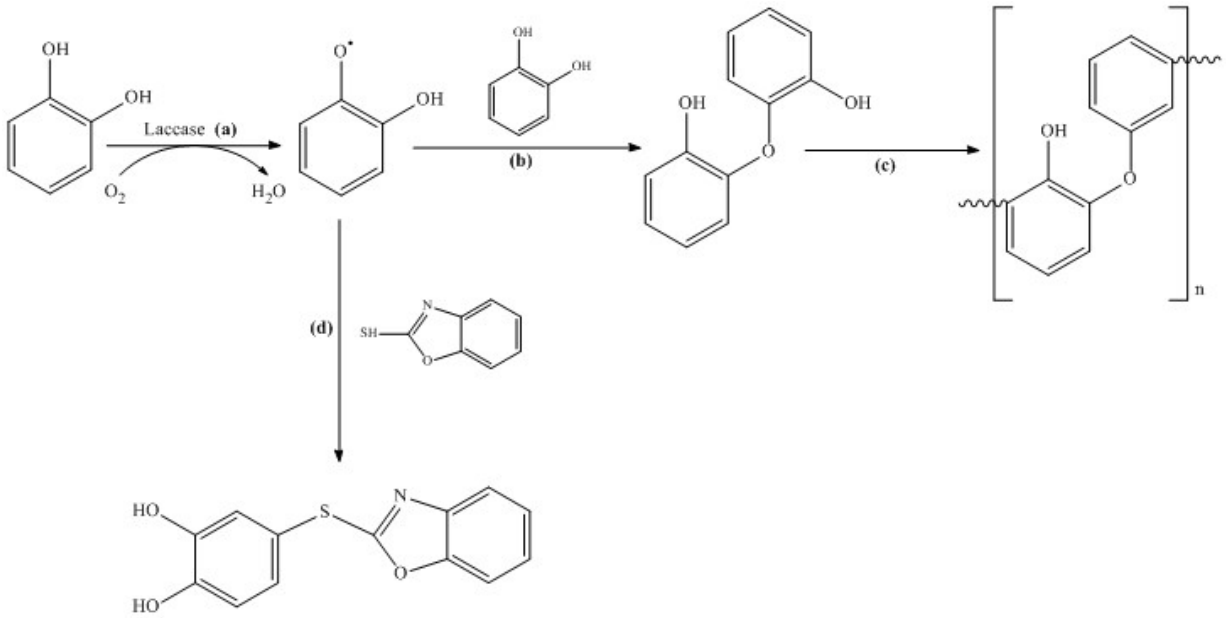


Figure 3: Laccase mechanism of action involves (a) the generation of phenoxy radical intermediates. The radicals undergo coupling reactions to form (b) dimers, (c) polymers and (d) cross coupling products when phenoxy radicals are reacted with non-laccase substrates (Kudanga et al., 2017).

2.9. LACCASE CATALYSED SYNTHESIS OF BIOACTIVE COMPOUNDS

Laccase applications in organic synthesis have been increasing in recent years mainly because the enzyme has a broad substrate specificity. Phenolic compounds, amino-phenols, polyamines, anilines, aromatic and alkyl amines and benzenethiols all fall under the laccase substrate range (Kunamneni et al., 2008a, Madhavi and Lele, 2009). Compounds carrying these functional groups have therefore become targets for biocatalytic reactions using laccases. The product range is further widened by coupling reactions involving a laccase substrate and a non-laccase substrate (variable reaction partner) to create new heteromolecular hybrid molecules (Mikolasch and Schauer, 2009). The most frequently investigated compounds are phenolics.

2.9.1. Laccase-catalysed synthesis of antioxidant phenolic compounds

Laccase oxidation of substrates to their respective radicals is a pre-requisite for the production of dimeric, oligomeric or polymeric compounds (through homomolecular coupling reactions) or cross-coupling products (through heteromolecular coupling of the radicals) (Kudanga et al., 2011b, Pezzella et al., 2015). Phenolic compounds have been modified mainly through homomolecular coupling (Table 1). Several studies have focused on producing novel antioxidant compounds through laccase-mediated dimerisation of phenolic compounds. Adelakun and coworkers (2012a,b) used monomeric natural phenolic compounds as laccase substrates for the

production of new antioxidants. Using ferulic acid as starting material, two derivatives, *β*-5 and *β*-*β* dimers, were successfully produced (Adelakun et al., 2012b). The *β*-5 dimers showed enhanced antioxidant activity while *β*-*β* dimers had lower activity compared to ferulic acid. The enhanced activity of the *β*-5 dimer was attributed to the increase in electron donating groups on the compound and the carboxylic acid group with an adjacent unsaturated C–C double bond which can provide additional attack sites for free radicals (Srinivasan et al., 2007). 2,6-Dimethoxyphenol (2,6-DMP) was also used in a laccase-oxidised reaction that resulted in the formation of a symmetrical C–C linked 2,6-DMP dimer, 3,3′,5,5′-tetramethoxy biphenyl-4,4′-diol, with approximately twice the antioxidant activity of 2,6-DMP (Adelakun et al., 2012a). During laccase catalysis, 2,6-DMP is oxidised to phenoxy radical species which form *para*-radical species through resonance stabilisation; the dimer is subsequently formed through radical coupling of two *para*-radical species (Figure 4). The superior antioxidant activity of the dimer was attributed to the increased functional groups with electron donating capacity (Matsuura and Ohkatsu, 2000), the reduction in the O-H bond dissociation energy and increased stability of radical due to resonance delocalisation (Sánchez-Moreno et al., 1998).

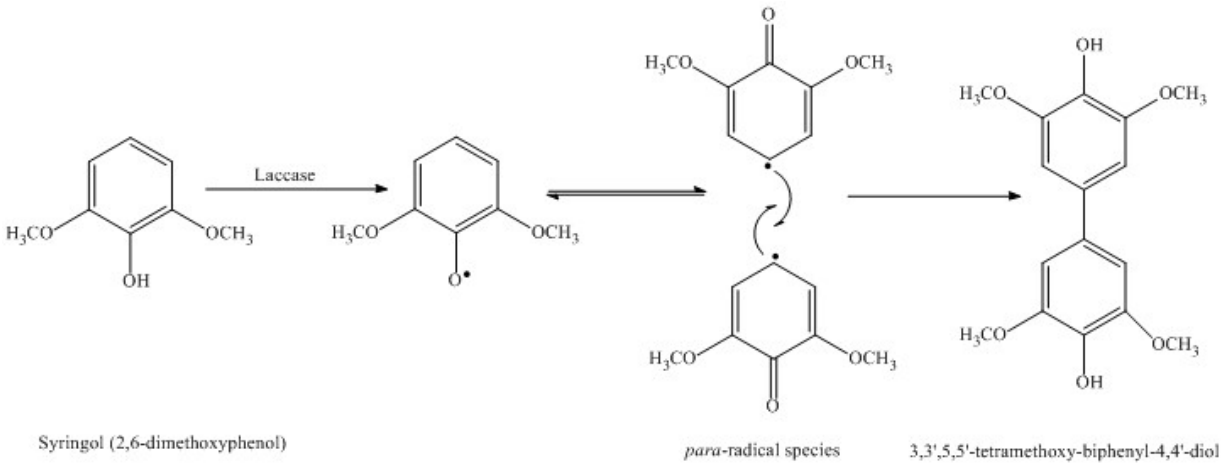


Figure 4: Proposed mechanism of a laccase catalysed homomolecular coupling reaction of 2,6-dimethoxyphenol to produce a C – C dimer (3,3′,5,5′-tetramethoxy biphenyl-4,4′-diol) (Adelakun et al., 2012a).

Osman et al. (2007) also demonstrated the ability of laccases to catalyse the coupling of monomeric natural phenolics into macromolecular compounds. Using (+)-catechin as substrates in a laccase/ABTS mediated system, dehydrocatechin type A (hydrophobic) and type B (hydrophilic) dimers were identified, along with trimers and tetramers whose identity was not elucidated (Osman et al., 2007). Laccase has also been successfully used as catalyst for improving the properties of natural phenolic compound rutin. Rutin is naturally a hardly water

soluble flavonoid glycoside. *Myceliophthora* laccase was used as the catalyst to synthesise polymerised rutin (poly(rutin)), which showed significantly improved solubility and radical scavenging properties (Kurisawa et al., 2003a). Rutin is commonly found on the market as a dietary supplement for its remarkable antioxidant activity. Recent research has revealed rutin as an effective antithrombotic agent (Jasuja et al., 2012). Rutin acts as an excellent inhibitor of protein disulfide isomerase (PDI), the enzyme which, when secreted rapidly from platelets and endothelial cells, is responsible for thrombosis (blood clotting). The production of poly(rutin), which has already proved to have enhanced properties such as improved solubility, may potentially enhance its biological properties.

Lignans are dimeric forms of phenylpropanoid units that have been identified as one of the primary active groups of *Eucommia ulmoides*, a Chinese traditional medicine that is recognised for its anticancer activities (Li and Zhang, 2008, Zhou et al., 2009), antioxidant activity (Xu et al., 2010, Zhang et al., 2013), antibiotic properties (Ji and Su, 2008), blood pressure reduction (Greenway et al., 2011), and antihypertensive activity (Luo et al., 2004). Wan et al. (2007) used crude *Rhus* laccases (CRL) and purified *Rhus* laccases (PRL) derived from the *Rhus vernicifera* plant in a domino oxidation of phenylpropanoids to produce bioactive compounds. Even though *Rhus* laccases are often marginalised for their low activity, the investigation resulted in the formation of several compounds of therapeutic importance. Two compounds that were identifiable include pinoresinol (8% and 23.5% yield using CRL and PRL, respectively) and dehydrodiisoeugenol (24.5% and 25% yield using CRL and PRL, respectively) (Wan et al., 2007). Pinoresinol has proven to be an effective anti-inflammatory drug (During et al., 2012, Jung et al., 2010). Research also showed that pinoresinol-rich olive oil had chemopreventive properties (Fini et al., 2008). Dehydrodiisoeugenol is popularly used in treating gastrointestinal disorders (Li and Yang, 2012) and can be applied as an antioxidant or anti-inflammatory agent (Murakami et al., 2005b).

Myceliophthora thermophila laccase was used as an oxidant in the synthesis of aminonaphthoquinones (Wellington and Kolesnikova, 2012). The enzyme catalysed the amination of 1,4-dihydroxy-2-naphthoic acid with primary aromatic amines by facilitating C–N bond formation. Aminonaphthoquinones are a class of phenolic compounds that are known to have anticancer activity. The process resulted in the synthesis of eleven compounds with varying physiological properties. Some of the compounds exhibited high potency when tested against TK10 (renal), UACC62 (melanoma), and MCF7 (breast) cancer cell lines. The compounds also

recorded a weak cytotoxicity on HeLa cell lines, highlighting their importance as potential anticancer drugs (Wellington and Kolesnikova, 2012).

Catechol thioethers have been produced by reacting laccase-oxidised catechol with thiols. Laccase oxidation of catechol produces o-benzoquinone, which subsequently reacts with a thiol by nucleophilic conjugate addition to produce a catechol thioether (Abdel-Mohsen et al., 2014) (Figure 5). Using 2-mercaptobenzoxazole and 2-mercaptobenzothiazole as thiols, thioester yields in the range of 74-96% were produced at room temperature, atmospheric pressure, and a pH of 6.0 (Abdel-Mohsen et al., 2014). Catechol thioethers find application as antimicrobial and antioxidant agents (Adibi et al., 2011).

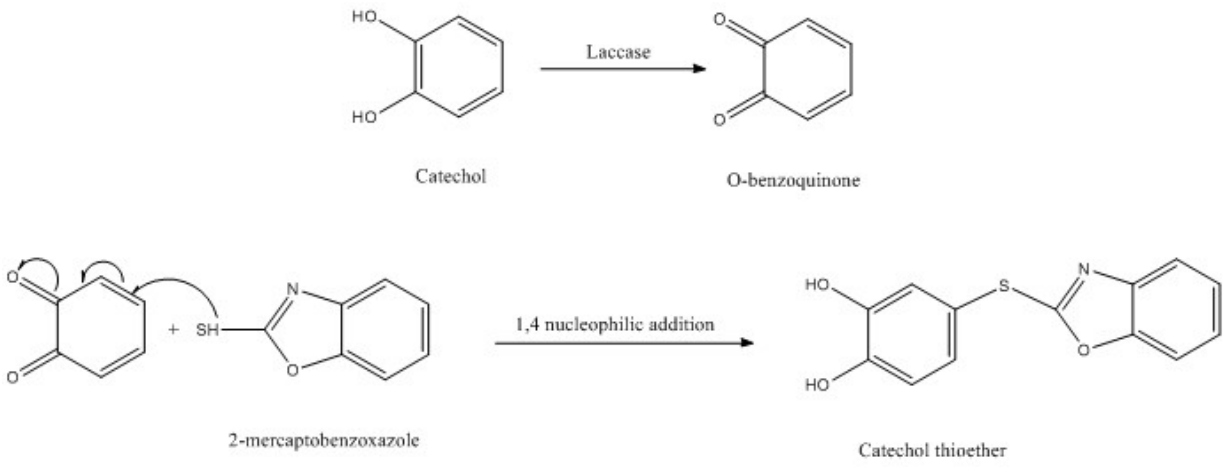


Figure 5: Reaction mechanism of the heteromolecular coupling of catechol and 2-mercaptobenzoxazole to produce catechol thioethers (Abdel-Mohsen et al., 2014, Kudanga et al., 2017).

Laccase has also been used in the synthesis of 2,3-ethylenedithio-1,4-quinones by cross-coupling 1,2-ethanedithiol with substituted hydroquinones (Cannatelli and Ragauskas, 2015a). The reaction proceeds via sequential oxidation and addition reactions initiated by laccase-catalysed oxidation of a hydroquinone into the corresponding 1,4-quinone derivative. The highly reactive 1,4-quinones then undergo nucleophilic addition by 1,2-ethanedithiol followed by further oxidation and addition steps to produce the respective 2,3-ethylenedithio-1,4-quinone products (Figure 6). It was argued that the products are similar to several quinone-containing derivatives of natural compounds which have exhibited antitumor and antimicrobial activities (Abraham et al., 2011, Bozic et al., 2010). In related studies, *Trametes villosa* laccase was employed in the α-arylation of benzoylacetonitrile by hydroquinones to produce benzylic nitriles (Cannatelli and Ragauskas, 2015b). Benzylic nitriles are primary ingredients in the production of several

pharmaceutical products such as antihelmintic drugs and analgesics (Kermanshai et al., 2001, Vardanyan and Hruby, 2006).

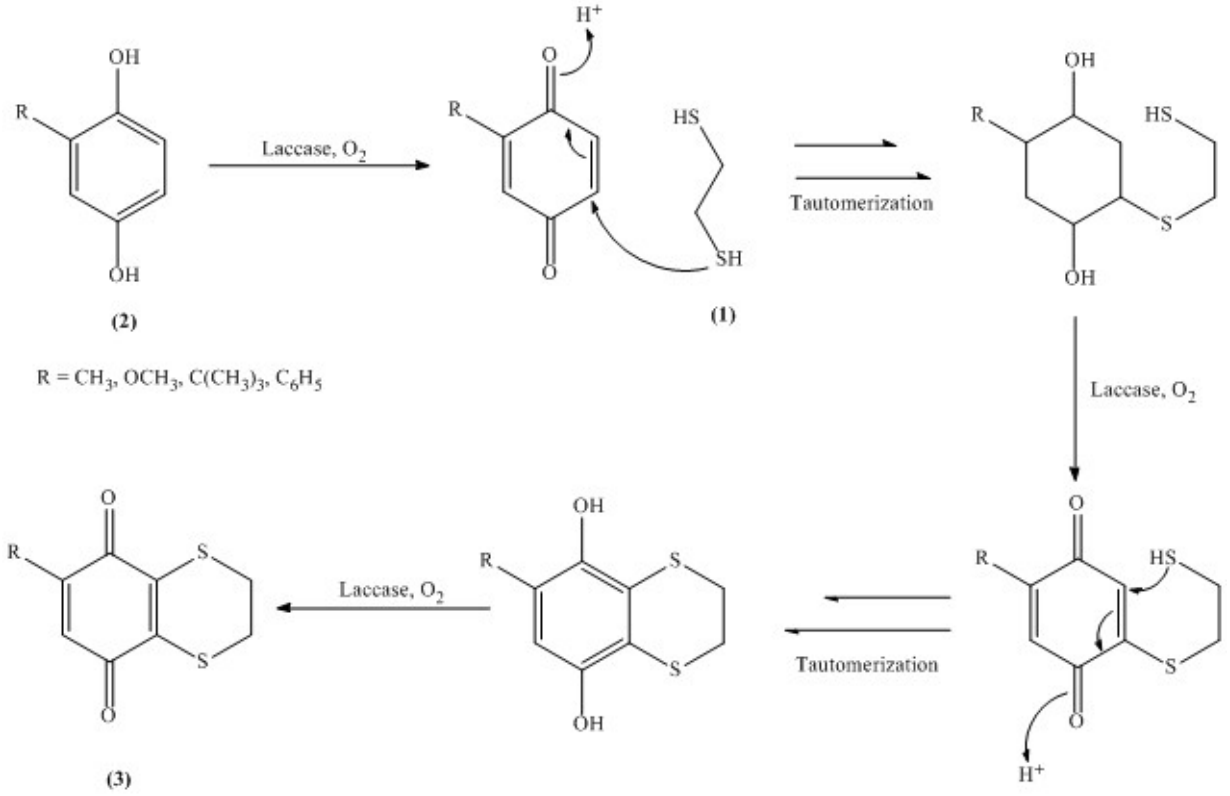


Figure 6: Proposed reaction mechanism for the laccase-catalysed reaction of 1,2-ethanedithiol (1) with substituted hydroquinones (2) to produce 2,3-ethylenedithio-1,4-quinones (3) (Kudanga et al., 2017, Cannatelli and Ragauskas, 2015a).

Table 1: Laccase catalysed oxidation of phenolics for the synthesis of bioactive compounds

Substrate	Substrate category	Source of laccase	Positive effects observed	Potential applications	References
2,6 - Dimethoxyphenol (2,6-DMP)	Phenolic compound	*T. pubescens*	A 2,6-DMP dimer produced (20.91% yield) showed 100% increase in antioxidant activity compared to 2,6-DMP	Potential use in the development of nutraceuticals and as components for cosmetic products.	(Adelakun et al., 2012a)
3-hydroxyanthranilic acid (3-HAA)	Phenolic compound (aminophenol)	*Pycnoporus cinnabarinus* and *Cytisus lirsutus*	Cinnabarinic acid was produced and expressed antibacterial activity against all bacterial strains it was tested against.	Antibacterial compounds	(Eggert et al., 1995, Eggert, 1997)
4-methyl-3-hydroxyanthranilic acid	Phenolic compound (aminophenol)	*T. versicolor*	Actinocin was produced (74% yield). Actinocin has antimicrobial properties.	Antibiotics	(Osiadacz et al., 1999)
Catechin	Phenolic compound	*T. versicolor*	Hydrophilic linear oligomers with high antioxidant activity were produced.	Useful as pharmaceutical drugs with antioxidant, antimutagenic, anti-carcinogenic, antiviral, and anti-inflammatory properties	(Jadhav and Singhal, 2014)
(+)-Catechin	Phenolic compound	*Myceliophthora* sp.	Poly(catechin) was produced which showed improved radical scavenging ability and no pro-oxidant	Therapeutic agent against oxidative stress	(Kurisawa et al., 2003b)

			properties. It also showed xanthine oxidase inhibitory activity which was hardly measurable in catechin.		
Esculin	Phenolic compound	*T. versicolor*	Oligomeric esculin compounds which registered a 189-times increase in solubility compared to the natural esculin.	Antioxidant additives for cosmetics, food and beverages	(Anthoni et al., 2010)
Ferulic acid	Phenolic compounds	*T. pubescens*	*β*-5 dimer produced displayed higher antioxidant activity than ferulic acid.	Antioxidant additives for cosmetic and pharmaceutical industries	(Adelakun et al., 2012b)
Ferulic acid	Phenolic compound	*T. versicolor*	Ferulic acid dilactones were produced. However, Adelakun et al. (2012b) reported lower activities compared to substrate.	Antioxidant additives for food and pharmaceutical products	(Constantin et al., 2012a)
Hydroxytyrosol	Phenolic compound	*T. pubescens*	Dimer, oligomers and polymers of hydroxytyrosol with superior antioxidant properties. Dimer had 87.6% radical scavenging ability compared to hydroxytyrosol with 33%	Antioxidant additives, also a potential ingredient in skin care products and nutraceuticals.	(Burton and Davids, 2012, Zwane et al., 2012)
Phenylpropanoids	Phenolic compound	*R. vernicifera*	Dehydrodiisoeugenol and pinoresinol were produced (yield 8-25%).	Anticancer and antioxidant drugs	(Wan et al., 2007)
Penicillin X	Antibiotics	*T. versicolor*	No positive effect. Dimers of penicillin X produced had lower activity than penicillin X	Antibiotics	(Agematu et al., 1993)
Rutin (quercetin-3-rutinoside)	Phenolic compound	*M. thermophila*	Poly(rutin) with improved water solubility and improved radical scavenging ability.	Antithrombotic agent and antioxidant	(Kurisawa et al., 2003a)

Sesamol	Phenolic compound	*T. versicolor*	A novel sesamol trimer (yield 61%).	Antioxidant additive for food products	(Constantin et al., 2012b)
Silybin A	Phenolic compound	*T. versicolor*	Symmetric dimer of silybin A produced (yield 87%) had significantly improved radical scavenging activity compared to monomeric silybin A.	Antioxidant additives	(Gavezzotti et al., 2014)
Silybin	Phenolic compound	*T. pubescens*	C-21-C-21' and C-20-O-C-21' dimers produced showed antiradical activity.	Antioxidant additive	(Gažák et al., 2008)
Trans-resveratrol	Phenolic compound	*M. thermophila*	A trans-dehydrodimer produced had improved polarity properties as well as antioxidant activity.	Antioxidant additive	(Nicotra et al., 2004)
Tyrosol	Phenolic compound	*Trametes trogii*	Tyrosol dimer produced showed higher antimicrobial activities compared to tyrosol. The dimer also expressed insecticide activity against *Tuta absoluta.*	Antibiotic or insecticide	(Chakroun et al., 2013)

2.9.2. Laccase-mediated development of phenolic based food colourants

The food industry has, over the years, been infiltrated by synthetic compounds. Synthetic dyes, as an example, have been incorporated into food as colourants. Health concerns have been raised over the use of dyes such as Sudan red, tartrazine, carmoisine, amaranth, allura red and *para* red, because of their reported adverse effects, particularly carcinogenicity (Dar et al., 2013, Kobylewski and Jacobson, 2012), genotoxicity (Hassan, 2010, Sasaki et al., 2002), adverse effect on human organs (Amin et al., 2010, Axon et al., 2012, Gao et al., 2011), and in general their implication in the contraction of several chronic diseases. Immense pressure from modern day consumers (Amin et al., 2013, Bearth et al., 2014, Dickson-Spillmann et al., 2011, Shim et al., 2011), health organisations such as the World Health Organisation (Ng, 2011), and the science community, has prompted the food industry to search for ways to replace these synthetic colourants with naturally derived pigments (Chattopadhyay et al., 2008, Jain and Mathur, 2015, Suh and Choi, 2012, Wissgott and Bortlik, 1996). Recently, there has been a rise in popularity of nutraceuticals; which have been defined by Bull et al. (2000) as "food that has a component incorporated into it to give it a specific medical or physiological benefit, other than purely nutritional benefit." Components such as dietary fibers, probiotics, prebiotics, polyunsaturated fatty acids and polyphenols are being incorporated into food to develop nutraceuticals (Das et al., 2012). As a result, several laccase researches have reported the production of polyphenols with potential application as nutraceutical components.

Laccases frequently catalyse the oxidation of various substrates to products that are colourful. Therefore, a number of papers have already highlighted the potential of laccases as a biocatalyst for the production of food colourants, some of which also have bioactivity such as antioxidant activity. A *M. thermophila* laccase which optimally function in water miscible conditions, at room temperature and in a neutral pH range was employed in the oxidation of ferulic acid (FA) and ethyl ferulate (FE) (Aljawish et al., 2014a). Major subsequent products were β-5' dimeric units of both FA and FE. The reactions resulted in a colour change from colourless to white and dark brown for FE and FA, respectively. It was claimed that the FA reaction products retained their brown colour for two months, under light, and under room temperature conditions (Aljawish et al., 2014a), though no records accompanied these claims. In addition to colour, the reaction products also exhibited antioxidant activity. The results affirmed the preceding work in which a biphasic system was used for the oxidation of FA to produce brown coloured oxidation products in the aqueous phase and yellow coloured products in the organic phase (Mustafa et al., 2005). The yellow coloured product remained colour-stable under room temperature and light

for more than 45 days (Mustafa et al., 2005) though no information was provided to support the claim. In related studies, Aljawish et al. (2014b) reported the appearance of a yellow-orange colour from an FA engrafted chitosan. The colour was reportedly stable and remained even after washing with organic solvents. Such FA-chitosan derivatives can therefore be further developed and find useful application as food colourants. Coincidentally, food developers have for a long time considered the potential of chitosan in the development of nutraceuticals (Je and Kim, 2012, Klinkesorn, 2013). The production of chitosan derivatives that impart colour and with a proven colour stability could be quite significant for the food industry. Rutin, morin, and quercetin are natural plant-derived flavonoids that also produced a dark brown colour after being subjected to a laccase-catalysed oxidation process (Kim et al., 2007, Kim et al., 2008). A laccase/ABTS system was used for the oxidation of (+)-catechin resulting in the production of dehydrodicatechin A (Osman et al., 2007), which had previously been identified as the yellow oxidation product of catechin (Guyot et al., 1996, Osman et al., 2007).

However, there are still a number of challenges related to laccase-catalysed production of colourants. The pigments produced must be highly stable and retain their colour over a long period of time. One of the reasons for prevalence of synthetic colourants as well as cases of food adulteration across the globe by illegal azo dyes such as Sudan red is because of their high stability and also their ability to impart an intense colour on the food products (Li et al., 2010, Oluwaniyi et al., 2009). The stability of colourants, particularly of natural origin, is reported to be affected by parameters such as pH, light and temperature (Mapari et al., 2010, Wissgott and Bortlik, 1996, Woo et al., 2011). Thus, the production of naturally derived colourants with a highly stable colour is a huge step towards introducing and promotion of naturally derived colourants in the food industry.

2.10. NONCONVENTIONAL MEDIA IN LACCASE-CATALYSED SYNTHESIS OF BIOACTIVE COMPOUNDS

Laccase biocatalysis is mostly done in nonconventional media (organic solvents). The use of organic solvents in biocatalysis has been adopted in synthetic chemistry because of several reasons: (i) most phenolic substrates are sparsely soluble in aqueous media; (ii) enhanced stability of enzymes; (iii) minimisation of undesired side reactions which are mostly caused by aqueous media; (iv) organic media provides kinetic and thermodynamic conditions that promote synthetic reactions to occur; (v) ease of reaction product recovery; (vi) general insolubility of

enzymes in organic solvents can make it possible to recover them for reuse (Adelakun et al., 2012b, Kvittingen, 1994). The reaction systems used for laccase catalysis includes monophasic and biphasic systems (Adelakun et al., 2012b). Monophasic systems involve the use of water miscible solvents such as methanol and ethanol. The solvent forms a homogenous phase with the enzyme-containing aqueous phase. Biphasic systems consist of water immiscible solvents such as ethyl acetate and hexane, forming two polarity-based layers where the enzyme is usually in the aqueous phase. While there are many advantages to employing nonconventional media in enzyme catalysed organic synthesis, there are also shortcomings. For instance, enzyme-solvent interactions have been reported to affect enzyme stability and activity through alteration of thermodynamic water activity (Rodakiewicz-Nowak, 2000). Enzymes can be affected more in water-miscible solvents where there is a direct interaction between enzyme and solvent (Riva, 2006). Because of the effect of nonconventional media on enzyme activity, it is common practice among researchers to add the enzyme at intervals to ensure reaction progress (Wellington and Kolesnikova, 2012). Reaction progress can also be aided by the use of mediator systems which act as redox shuttles (Riva, 2006).

2.11. STRUCTURE-ACTIVITY RELATIONSHIPS OF ENZYMATICALLY SYNTHESISED ANTIOXIDANTS

In laccase-catalysed synthesis of bioactive compounds, the main aim is to produce coupling products exhibiting improved bioactive properties compared to the initial starting materials. In most cases, the structure of the products determines their efficacy. Depending on the intended purpose of the bioactive compound, several factors can determine the efficacy of the coupling products. In this section, some of these factors are discussed, with reference to antioxidant capacity.

Some of the factors that determine the bioefficacy of antioxidants include (i) the structure of the compound, and (ii) the stability of the compound (Adelakun et al., 2012b). Several researchers have analysed the structure-activity relationship (SAR) of antioxidants (Bendary et al., 2013, Rice-Evans et al., 1996). The structure of the antioxidant is pivotal in determining its activity (Table 2). Firstly, the antioxidant must have active groups (e.g. hydroxyl, alkyl or aniline) (Bendary et al., 2013) attached to the aromatic ring and the more active groups present, the more bioactive the antioxidant can be (Bendary et al., 2013, Lien et al., 1999). For example, hydroxytyrosol consist of two hydroxyl groups attached to its aromatic ring, however, after a

laccase-catalysed oxidation process, a hydroxytyrosol dimer with four hydroxyl groups is produced (Zwane et al., 2012) (Table 2). This dimer showed a 3-fold increase in antioxidant activity when tested using the ferric-reducing antioxidant power (FRAP) assay. Adelakun et al. (2012b) also attributed the enhanced activity of the dimeric form of ferulic acid (*β*-5) to increased electron donating groups. Functional groups such as alkyl, aniline or hydroxyl groups enhance antioxidant activity (Bendary et al., 2013) while bulky alkyl groups contribute towards the stability of phenoxy radicals (Decker, 2008, Eskin and Przybylski, 2000). However, Rakesh and colleagues observed that compounds containing moieties such as nitro groups or halogens, which are electron withdrawing groups, have poor antioxidant activity (Rakesh et al., 2015). The position of the active groups on the aromatic ring also determines the activity of the product. Enhanced activity of a phenolic antioxidant can be achieved when active groups occupy the *ortho* or *para* position to the hydroxyl group (Decker, 2008). Recently, Najafi (2014) investigated the relationship of the active substituents' position on the daidzein aromatic ring and the compound's antioxidant activity. It was concluded that the *ortho* position can result in production of useful bioactive compounds.

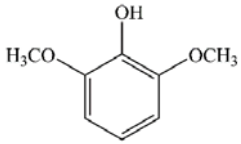
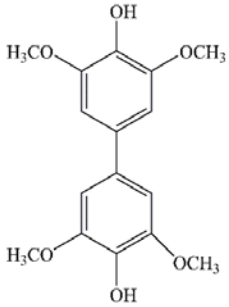
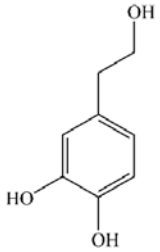
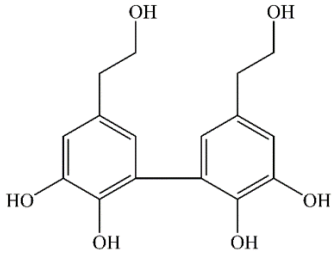
The bond dissociation enthalpies (BDE) of the active groups will also determine the inertia of the antioxidant in releasing the electron. The antioxidants containing active groups with lower BDE are better antioxidants because they readily release electrons to radical species (Szymusiak and Zielinski, 2003). Adelakun et al. (2012b) showed that the *β*-*β* dimers of ferulic acid had a lower antioxidant activity than the monomeric ferulic acid. This is consistent with earlier findings which showed that bis-ferulic acid (*β*-*β* dimers) had a higher BDE (85.76 kcal/mol) than ferulic acid (84.70 kcal/mol) (Murakami et al., 2005a). The determination of BDE varies with compounds and also experimental conditions, thus many researchers focusing on the BDE of phenolic compounds have published contrasting results (dos Santos and Simoes, 1998, Klein and Lukeš, 2006, Szymusiak and Zielinski, 2003). However, in general, hydroxyl moieties have lower BDE than other active groups such as alkyl and aniline groups (Bendary et al., 2013) which probably explains why phenolics are frequently used as antioxidants.

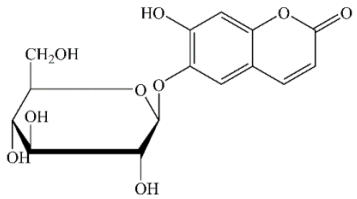
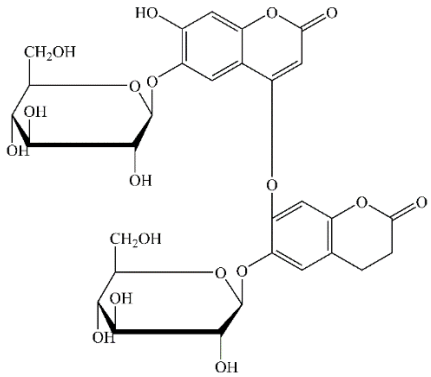
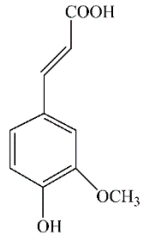
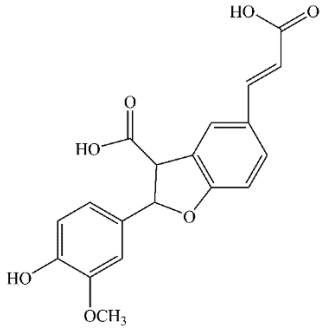
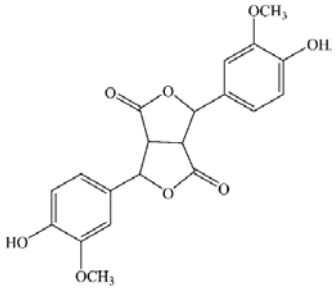
An ideal antioxidant must also produce a stable radical, which discourages the propagation of the oxidation chain bubble (Alov et al., 2015). The stability results from the resonance delocalisation of lone electrons into the aromatic ring and absence of groups prone to attack by oxygen (Flora, 2009, Shahidi and Naczk, 2004). Synthetic antioxidants such as butylated hydroxyanisole (BHA) are effective antioxidants which forms stable radicals because they

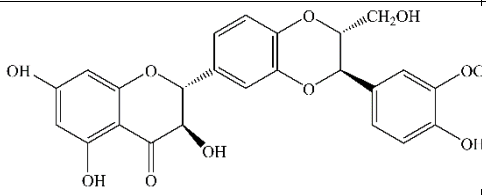
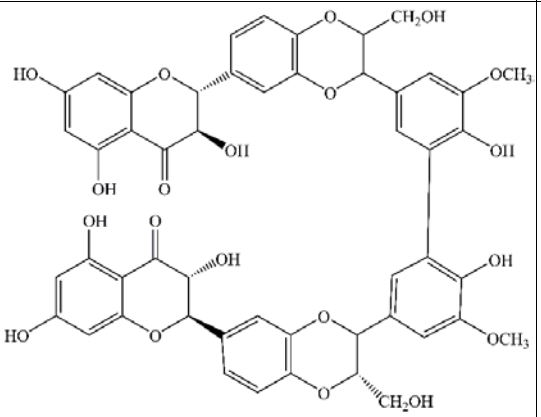
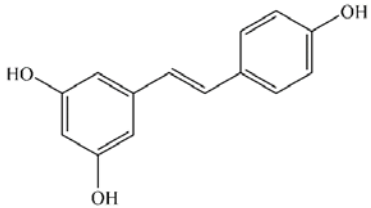
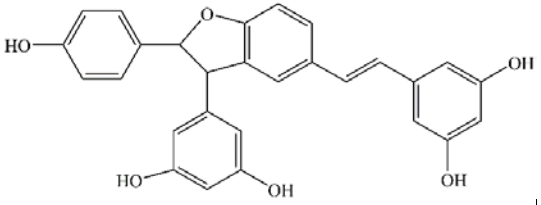
contain bulky groups on the *ortho* positions of the aromatic ring (Shahidi and Naczk, 2004). However, these bulky groups may also reduce antioxidant activity by steric masking of the phenolic hydroxyl group (Murakami et al., 2005a).

Hydrophobicity is also another attribute for an effective antioxidant especially in a multicellular environment. Hydrophobicity enables the bioavailability of the antioxidant at sites where free radicals are generated (Ishige et al., 2001), for instance, free radicals can be generated from lipid peroxidation on the lipid bilayer. In this case, hydrophobic antioxidants will be effective free radical scavengers because of their lipophilic properties (Lu et al., 2006). An ideal antioxidant would thus consist of a balance of electron donating groups such as hydroxyl groups, which will ensure the free radical scavenging ability of the antioxidant, and also hydrophobic moieties which will enable the bioavailability of the antioxidant in multicellular systems. Research that focused on improving the hydrophilicity of silybin resulted in its compromised antioxidant activity in lipophilic environments (Gažák et al., 2010, Gažák et al., 2004), highlighting hydrophobicity as an important factor in the function of antioxidants in cell medium.

Table 2: Structure activity relationship of laccase-catalysed phenolic coupling products

Substrate	Product	Effect	Structure – activity relationship	References
 2,6-DMP	 3,3′,5,5′-tetramethoxy biphenyl-4,4′-diol	Increased antioxidant activity	Increased electron donating groups on the dimer reduces the bond dissociation enthalpies (BDE) of the hydroxyl moieties and facilitated the subsequent formation of a stable radical due to the ease of electron delocalisation into the benzene ring.	(Adelakun et al., 2012a, Wan et al., 2008a, Wan et al., 2008b)
 3-hydroxytyrosol	 3-hydroxytyrosol dimer	Improved antioxidant activity	The increased number of hydroxyl groups on the dimer offers more attacking sites for free radicals, thus improving the free radical scavenging ability of the dimer.	(Burton and Davids, 2012, Zwane et al., 2012)

 Esculin	 Di-esculin	Improved solubility	Molecular modelling studies showed that the esculin monomer could form only 9 hydrogen bond interactions with water, while after dimerisation, the number of hydrogen bonds increased to 22, thus the dimer had improved solubility.	(Anthoni et al., 2010)
 Ferulic acid	 *β*–5 ferulic acid dimer	Improved antioxidant activity	Coupling of ferulic acid resulted in an increase in number of electron donating groups on the product. The unsaturated C=C bond along with its adjacent carboxylic acid group were preserved during oxidation process and offers extra attacking sites to free radicals.	(Adelakun et al., 2012b, Constantin et al., 2012a)
		Lower antioxidant activity compared to ferulic acid	The *β*–*β* dimer has lost the unsaturated C=C bond as well as the carboxylic acid group during the coupling process, which may explain its low antioxidant potential.	

	β–β ferulic acid dimer			
 Silybin A	 Silybin A dimer	Increased antioxidant activity	The coupling of two silybin A monomers leads to higher resonance. High resonance allows the antioxidant to form stable radicals when it has been oxidised by free radicals.	(Gavezzotti et al., 2014)
 Trans-resveratrol	 Trans-resveratrol dehydrodimer	Increased antioxidant activity and increased solubility	Increased number of hydroxyl groups increases the antioxidant activity. The increased hydroxyl groups also enable the formation of more hydrogen bond interactions with water molecules thus increasing solubility.	(Nicotra et al., 2004)

2.12. MEASURING ANTIOXIDANT CAPACITY

Increased interest in antioxidants has caused many researchers to devise methods of evaluating the efficacy of phenolic compounds as antioxidants. According to literature, as of 2013, a record 29 methods are reported to have been used to determine antioxidant capacity of plant extracts, food, nutraceuticals and phenolic compounds (Alam et al., 2013). However, Prior et al. (2005) argues that there is need for the standardisation of methods for analysing antioxidant capacity. Prior and co highlights several reasons why standardisation is important for antioxidant capacity assays: chiefly, having standard assays will be beneficial for regulatory issues and can also be helpful for comparative purposes (Prior et al., 2005). Antioxidant assays can be broadly categorised into two major groups based on the interaction between the antioxidant and the free radicals. These groups are Hydrogen Atom Transfer (HAT) and the Single Electron Transfer (SET) reaction mechanisms (MacDonald-Wicks et al., 2006). HAT methods measure antioxidant activity based on the antioxidant's capacity to donate hydrogen to the free radical while SET are based on the ability of an antioxidant to transfer an electron to the free radical. Some of the popular assays that have been used to determine antioxidant capacity includes Trolox equivalent antioxidant capacity (SET); Folin-Ciocalteu method (SET); Ferric Reducing Antioxidant Power (SET) and 2,2-Diphenyl-1-picrylhydrazyl Assay (utilises both HAT and SET).

2.12.1. Trolox equivalent antioxidant capacity (TEAC)

The TEAC assay determines the amount of antioxidant required for the reduction of the 2,2'-azinobis(3-ethylbenzothiazolline-6-sulfonic acid) radical ($ABTS^{\bullet+}$) in comparison to a Trolox standard. The assay can be followed spectrophotometrically at 734 nm since the reduction of the intense blue-green $ABTS^{\bullet+}$ results in the loss of colour. The method was developed by Miller et al. (1993) and initially involved the generation of the $ABTS^{\bullet+}$ radical through reacting metmyoglobin and H_2O_2 to produce ferrylmyoglobin. Ferrylmyoglobin subsequently reacts with ABTS to produce the ABTS radical. Inaccuracy concerns were raised about the protocol because the antioxidant was added before the free radical was generated. In a modified method by Re et al. (1999), the $ABTS^{\bullet+}$ was generated by reacting ABTS with potassium persulfate in the dark for 16 h prior to the addition of the test compound. Many researchers have reported different approaches of generating the $ABTS^{\bullet+}$ ranging from chemical to enzymatic oxidation. Chemicals such as manganese dioxide, ABAP have been employed while enzymes such as horseradish peroxidase, hemoglobin, metmyoglobin and also laccase have been used (Prior et al., 2005).

After the generation of the ABTS radical, it is quantified spectrophotometrically at 734 nm. The test compound is now added. Following the reaction using the spectrophotometer, the Trolox equivalent antioxidant capacity of a compound will be the concentration in mM of Trolox required to give the same $ABTS^{\bullet+}$ reduction as 1 mM of the test compound (Adelakun et al., 2012a).

2.12.2. Folin-Ciocalteu method

The Folin-Ciocalteu (F-C) method is also referred as the Total Phenolics assay. It is usually used to quantify phenolic content of plants and foods. Under alkaline conditions, electrons are transferred from phenolics to the phosphomolybdic/phosphotungstic acid complexes within the F-C reagent to form blue complexes that can be measured colorimetrically using a spectrophotometer at 765 nm (Ainsworth and Gillespie, 2007). Generally, a test sample is mixed with the F-C reagent, afterwards, 700 mM Na_2CO_3 will be added and the mixture is incubated at room temperature in the dark for about 2 h. Spectrophotometric readings will then be taken. Total phenolic content is usually expressed as gallic acid equivalents (GAE).

2.12.3. Ferric Reducing Antioxidant Power (FRAP)

The FRAP assay was first reported by Benzie and Strain (1996) as a method to determine the antioxidant capacity of plasma. It has now found wide application in determining antioxidant capacity of food samples. The FRAP mechanism is based on the ability of an antioxidant to reduce ferric(III) ions in acidic conditions (pH 3.6) (López-Alarcón and Denicola, 2013). In the assay, Fe(III) forms a complex with 2,4,6-tripyridyl-s-triazine (TPTZ) as $[Fe(III)\text{-}(TPTZ)_2]^{3+}$ and is reduced by antioxidants to $[Fe(II)\text{-}(TPTZ)_2]^{2+}$, a deep blue complex that can be quantified by spectrophotometry at 593 nm (Magalhães et al., 2008). A FRAP reagent is prepared by mixing acetate buffer (pH 3.6), TPTZ and $FeCl_3$ at ratio 10:1:1. The assay is done at 37°C by mixing the antioxidant with the FRAP reagent. Antioxidant capacity from the FRAP assay is expressed as FRAP values, which is defined as the concentration of standard antioxidant solution (usually ascorbic acid) with the capacity to reduce Fe(III) to Fe(II) equivalent to 1 mM of the antioxidant compound being tested (Adelakun et al., 2012a).

2.12.4. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Assay

The DPPH assay was first reported by Blois (1958). The DPPH assay determines the antioxidant capacity of a compound by measuring its ability to reduce DPPH$^{\bullet}$, a commercially available purple stable free radical (Kedare and Singh, 2011). The ability of an antioxidant to reduce DPPH$^{\bullet}$ can be analysed by either electron spin resonance (EPR) or though taking spectrophotometric readings of the DPPH$^{\bullet}$/antioxidant solution at 517 nm (Prior et al., 2005). The DPPH assay has been widely used in the food and pharmaceutical industries because of its simplicity. Since DPPH$^{\bullet}$ is available as a radical, there is no need for the generation step. The procedure involves preparing a certain concentration of DPPH$^{\bullet}$ solution in an organic solvent such as methanol. Various research groups have used different concentrations of DPPH ranging from 22.5 µM to 250 µM (Sharma and Bhat, 2009). The DPPH solution will be mixed with different concentrations of the test compound and absorbance of the DPPH/antioxidant mixture will be analysed at 517 nm using a spectrophotometer. By plotting a curve of absorbance (at 517 nm) vs antioxidant concentration, the percentage remaining DPPH can be calculated. DPPH assay results are usually expressed as EC_{50} values. The EC_{50} value is the concentration of an antioxidant compound required to have a 50% reduction in DPPH$^{\bullet}$ concentration (Adelakun et al., 2012a).

2.13. THE POTENTIAL OF BACTERIAL LACCASES AS INDUSTRIAL BIOCATALYSTS

As highlighted by this literature review, laccases have been demonstrated as useful biocatalysts in the synthesis of bioactive compounds with potential value in areas such as the food and pharmaceutical industries. The review has also indicated that a majority of the laccases employed are derived mostly from fungi as well as plants. Laccases are most widely distributed in fungi with over 60 strains reported to produce this enzyme (Mogharabi and Faramarzi, 2014), and Mayer and Staples (2002) claiming that “laccase has been documented in virtually every fungus examined for it”. This may be because laccase’s role in the delignification process makes it pivotal in fungus’ access to nutrients (Mayer and Staples, 2002, Riva, 2006).

Laccases are ubiquitous and have also been discovered in bacteria. Bacterial laccases may present useful industrial biocatalysts. The emergence and subsequent advances in the field of molecular biology, particularly the ease of expression in bacterial hosts, has opened more opportunities in developing biocatalysts better equipped for industrial application (Kudanga et

al., 2017). This makes bioprospecting for laccases in bacteria important because it may lead to the isolation of robust laccases.

Recently, a laccase was isolated from *Streptomyces coelicolor*, a bacteria resident in soil (Machczynski et al., 2004). Machczynski et al. (2004) overexpressed and characterised the laccase in *Escherichia coli*. The successful expression of *S. coelicolor* laccase in *E. coli* enabled the extensive characterisation of the enzyme which would have been difficult due to the complex growth cycle of *S. coelicolor* that is similar to fungi (Figure 7). The laccase demonstrated some attributes that makes it ideal for industrial application. Unlike most laccases which consist of 3 domains, *S. coelicolor* laccase is a two-domain laccase, making it smaller and thus it has been referred to as Small LACcase (SLAC) (Machczynski et al., 2004). SLAC has demonstrated characteristics of extremophiles, registering optimum activity at a broad pH range between 4 and 8 and a temperature optimum activity at 60-70°C. SLAC demonstrated a high thermostability with a half-life of at least 10 h at 80°C (Sherif et al., 2013). SLAC was also reported to be resistant to denaturation by detergents (Machczynski et al., 2004), making it an ideal robust enzyme with potential value as an industrial catalyst.

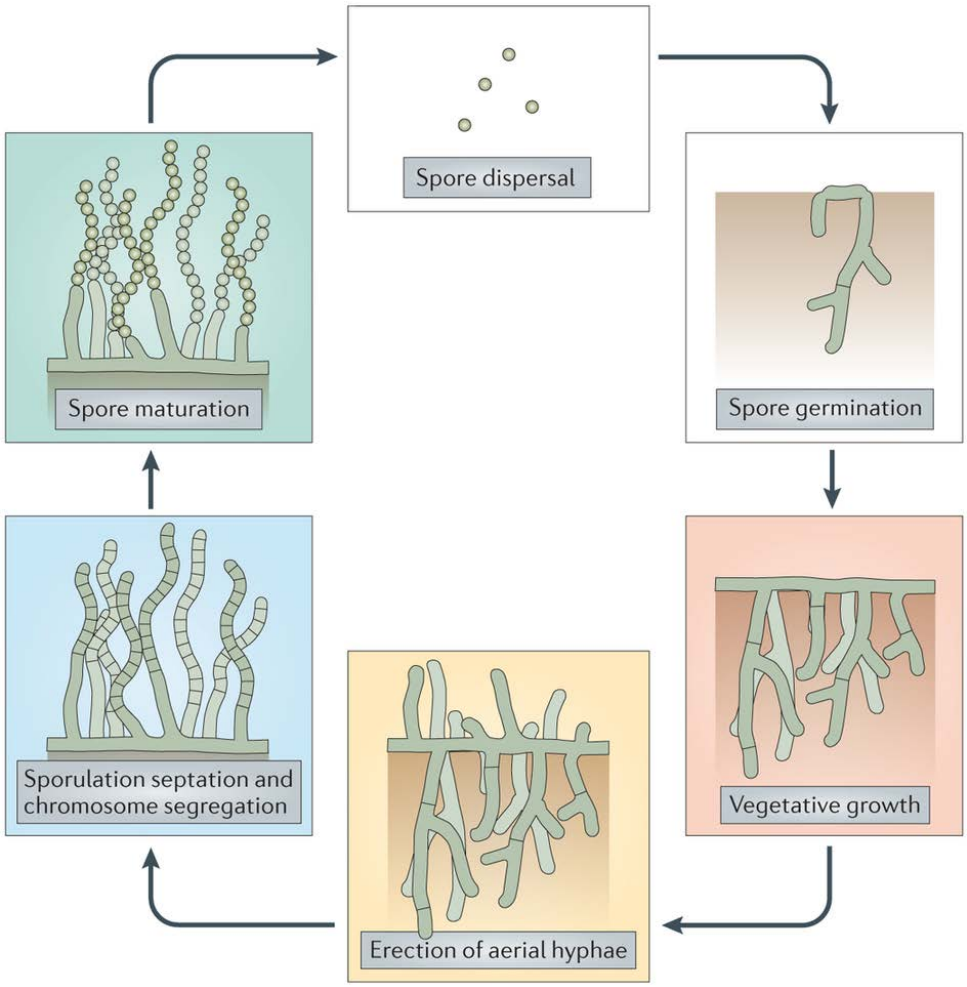


Figure 7: The life cycle of streptomycetes (Bush et al., 2015).

2.14. SCOPE OF THE STUDY

This study investigated the potential of SLAC as a biocatalyst for the synthesis of antioxidants. SLAC was produced from *E. coli* BL21(DE) cloned with a pET-20b(+) vector (Novagen) containing the gene coding for SLAC (pSLAC). The enzyme was purified and used in phenolic coupling reactions. A preliminary screening process was done to determine phenolic compounds oxidisable by SLAC. Caffeic acid, 2,6-dimethoxyphenol, catechol, gallic acid, guaiacol, ferulic acid, and pyrogallol were identified as SLAC substrates and coupling reactions were conducted using caffeic acid and gallic acid. Coupling products were identified and successfully separated by thin layer chromatography (TLC). The products were purified using preparative TLC (caffeic acid) and column chromatography (gallic acid). The coupling products were characterised by liquid chromatography-mass spectrometry (LCMS) and nuclear magnetic resonance (NMR). With the aid of high performance liquid chromatography (HPLC), reaction conditions for the oxidation of caffeic acid and gallic acid were optimised for the enhanced conversion of substrate

into product. The antioxidant capacity of the coupling products was determined using the DPPH and TEAC assays. Stability tests were carried out on the caffeic acid coupling product to determine its potential for application as a commercial antioxidant.

3. ENZYME PRODUCTION AND PRELIMINARY SLAC-CATALYSED COUPLING REACTIONS

3.1. INTRODUCTION

The search for cost effective methods for producing antioxidant molecules is a rapidly widening research niche with the market value for antioxidants predicted to increase by 6.42% CAGR between 2015 and 2022 (Allied Market Research, 2016). Antioxidants are compounds that have the ability to delay or prevent the free radical-initiated oxidation reactions when present in low concentrations (Shahidi and Zhong, 2015). Applications of antioxidant compounds are increasing. They are used in food industries (as additives especially in lipid-containing foods to prevent oxidative spoilage), in nutraceuticals, in pharmaceutical products (such as antiaging creams) and in medicine, as therapeutic drugs (Sindhi et al., 2013).

Most antioxidants have been recovered from plant sources through processes such as the heat reflux extraction method, accelerated solvent method, supercritical fluids extraction, employing high pressure protocols, use of microwave and ultrasound extraction processes (Martins et al., 2011). Chemical synthesis has also been used to produce antioxidants (Li and Trost, 2008). Most of these processes are markedly long, energy intensive and low yielding, resulting in excessive amounts of chemical wastes which poses environmental and health hazards (Li and Trost, 2008). It has also been reported that the development of new bioactive compounds is generally expensive and time-consuming (Gavezzotti et al., 2014). Such concerns have prompted the search for newer, economically feasible and environmentally benign processes for the production of antioxidants.

Biocatalysis is gaining notable attention in organic synthesis. This is because biocatalysts offer environmentally benign protocols and involve less process steps for the synthesis of valuable compounds. Laccases are one group of enzymes that have shown encouraging potential as biocatalysts in organic synthesis. Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) belong to the multicopper oxidase family of enzymes, and their role in nature involves both synthetic and degradative reactions (Riva, 2006). Laccases are generally regarded as ‘green catalysts’ because of their ability to oxidise a diverse range of compounds (including phenols, diphenols, methoxy-substituted phenols, phenolic and alkyl amines) to corresponding radicals in

the presence of molecular oxygen, concomitantly producing water as the only by-product (Kudanga et al., 2011a). Their catalytic mechanism generally involves the abstraction of a single electron from substrates to produce reactive free radicals (Kudanga and Le Roes-Hill, 2014). These free radicals are vital intermediates which undergo coupling reactions to produce dimeric, oligomeric, polymeric or cross-coupling products. Therefore, the ability of laccases to catalyse oxidative coupling reactions make them relevant in organic synthesis. Coupling of natural monomeric antioxidant units can result in novel compounds with enhanced properties. As a result, in the past two decades, there has been an increase in research activity exploiting laccases in the synthesis of antioxidants.

Although laccases are ubiquitous in nature and have been discovered in plants, fungi and in prokaryotes (Riva, 2006), most researchers have employed plant and fungal laccases (Kurisawa et al., 2003a, Kurisawa et al., 2003b, Kudanga et al., 2011b, Adelakun et al., 2012a,b, Gavezzotti et al., 2014, Zwane et al., 2012). However, plant and fungal laccases may not be ideal industrial catalysts for the production of antioxidants considering the following: (i) Plant laccases have been discovered in higher plants such as *Rhus vernicifera* (Kurisawa et al., 2003a,b) which makes it cost-intensive, as well as an environmental concern to harvest sufficient enzyme for industrial processes; (ii) Plant laccases generally have low activities (Kurisawa et al., 2003a); (iii) Most fungal laccases that have been characterised to function in mild conditions (mesophilic temperatures ranging from 30-55°C and mild acidic pH) which may not be suitable for most industrial processes that are usually characterised by high temperatures, pH, pressure and salt concentration (Hildén et al., 2009); (iv) Most fungi are generally slow growers, requiring complex growth conditions which makes production of adequate enzyme difficult; (v) Limited knowledge of heterologous expression in eukaryotic hosts (Santhanam et al., 2011).

Therefore, bioprospecting prokaryotic cells for laccases may result in the identification of robust enzymes with ideal industrial attributes. For example, a two domain, bacterial Small LACcase (SLAC) native to *Streptomyces coelicolor* was overexpressed in *Escherichia coli* (Machczynski et al., 2004). Extensive charaterisation studies showed that the enzyme has several attributes that make it potentially useful for industrial application such as thermo-stability, maintaining activity at high alkali conditions (pH 9.5) and resistance to denaturation by detergents (Machczynski et al., 2004; Sherif et al., 2013). Despite its positive attributes, SLAC has not been employed as a catalyst for the synthesis of antioxidants. The aim of this study was to produce and purify SLAC and to determine its potential for application in coupling reactions.

3.2. MATERIALS AND METHODS

3.2.1. Chemicals and enzymes

Chemicals were purchased from Sigma Aldrich (South Africa). The *E. coli* clone used for the expression of SLAC (wild type) was kindly provided by Prof. Gerard Canters, Leiden Institute of Chemistry, University of Leiden (Netherlands).

3.2.2. Production of SLAC

The production of SLAC was performed according to the protocol described by Prins et al. (2015) with a few modifications. *E. coli* BL21(DE) cloned with a pET-20b(+) vector (Novagen) containing the gene coding for SLAC (pSLAC) was cultured in 2xYT media (16.0 g/L tryptone, 10.0 g/L yeast extract and 5.0 g/L NaCl) overnight. Overnight culture (1%, v/v) was inoculated in 2xYT media containing 100 µg/mL ampicillin and incubated at 37°C, 180 rpm until the OD_{600} reached 0.8. Induction for the overexpression of SLAC was achieved through the addition of isopropyl *β*-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM. The culture was further incubated at room temperature (22±3°C), 160 rpm for 20 h. Cells were harvested by centrifugation (5000 x g, 10 min, 4°C) and resuspended in sodium phosphate buffer (pH 7.3). Cells were sonicated to release SLAC, which is produced intracellularly. The soluble fractions were collected by centrifuging (13,500 x g, 40 min, 4°C) and incubated in 1 mM $CuSO_4$ for 20 h at 4°C. The fractions were then dialysed for four 1 h sessions at room temperature (22 ± 3°C) with sodium phosphate buffer (10 mM, pH 7.3); 1 mM EDTA was added for the second session to chelate excess copper. The enzyme was further purified using ammonium sulphate precipitation, ion exchange chromatography and size exclusion chromatography.

3.2.3. Determination of enzyme activity

SLAC activity was determined using the 2,2´-Azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) activity assay (Ander and Messner, 1998). SLAC oxidation of ABTS was monitored by spectrophotometry at 420 nm (ABTS, ε_{420} = 36 $mM^{-1}cm^{-1}$). The reaction mixture consisted of citrate buffer (833 µL, 50 mM, pH 4.5), ABTS (110 µL, 5 mM) and SLAC (57 µL). The reaction was conducted at room temperature (22±3°C). One unit of laccase activity was defined as the amount of enzyme required to oxidise 1 µmol of ABTS in 1 minute at room temperature (22±3°C).

3.2.4. Effect of inhibitors on SLAC activity

The effect of five (5) putative laccase inhibitors namely; dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), sodium azide (NaN_3), kojic acid and *p*-coumaric acid on SLAC activity was investigated according to a method described by Kudanga et al. (2009) with slight modifications. Inhibitors were preincubated with SLAC in 50 mM citrate buffer (pH 4.5, 25°C, 60 min) at various concentration levels (0.05 - 5 mM) and the residual SLAC activity was determined using the ABTS activity assay as described above (Section 3.2.3).

3.2.5. Effect of metal ions on SLAC activity

The effect of metal ions; Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Na^{+} and Zn^{2+} on SLAC activity was studied. The metal ions were preincubated with SLAC in 50 mM citrate buffer (pH 4.5, 25°C, 60 min) at various concentrations (1 - 20 mM) and the residual SLAC activity was determined using the ABTS activity assay as described in Section 3.2.3.

3.2.6. Preliminary phenolic coupling reactions

3.2.6.1. Substrate screening for the identification of SLAC oxidisable phenolic compounds

Phenolic substrates were screened to identify compounds that are oxidisable by SLAC. SLAC oxidation of substrates was monitored by HPLC. The reactions were carried out at pH levels ranging from pH 3.5-9.0 (50 mM citrate buffer, pH range 3.5-6.0; 50 mM phosphate buffer, pH range 6.5-7.5; 50 mM Tris-HCl, pH range 8.0-9.0) to determine the optimum pH for substrate oxidation. The reaction mixture contained buffer (833 µL, 50 mM), phenolic substrate (110 µL, 10 mM) and SLAC (0.64 U). The reaction was conducted at room temperature (22±3°C) for 5 min.

3.2.6.2. Oxidation of gallic acid by SLAC

Oxidation reactions were carried out in a buffer-organic solvent mixture. The reaction mixture contained gallic acid (10 mM final concentration), SLAC (0.64 U) in 50 mM Tris-HCl buffer (pH 8.0). Product yield was improved by investigating four organic solvents as co-solvents: the water miscible solvents, methanol and ethanol, as well as the water immiscible solvents, ethyl

acetate and hexane at 50, 70, 80 and 90% organic solvent concentration (v/v) using 50 mM Tris-HCl (pH 8.0) as a buffer. The reaction was conducted for 3 h (25°C, 250 rpm).

3.2.7. Analysis of products

3.2.7.1. Thin layer chromatography (TLC)

Product formation was monitored by thin layer chromatography using the aluminum backed silica gel 60 F_{254} TLC plates (Sigma, South Africa). A solvent system containing ethyl acetate, methanol, water and formic acid (8:0.5:0.5:0.01 v/v) was used as the mobile phase for the chromatographic separation of products. Product formation was detected by spotting 5 µL of sample on TLC. TLC plates were viewed under UV light at 254 nm.

3.2.7.2. High Performance Liquid Chromatography (HPLC)

HPLC was used for the analysis of oxidation products. For the preparation of samples, an equal volume of ice cold methanol was added to stop enzyme activity. The samples were kept on ice for 20 min, followed by centrifugation (13,500 x g, 10 min, 4°C) to precipitate out the enzyme, as well as removing polymeric precipitates. Samples were filtered through 0.22 µm microfilters, transferred to clean HPLC vials and analysed using the Shimadzu HPLC system (Shimadzu, Japan). Separation of oxidation products was performed using the Sunfire C18 reverse phase column using an elution gradient consisting of 0.1% formic acid (A) and acetonitrile (B) as solvent at a flow rate of 1mL min^{-1}, an injection volume of 10 µL and an oven temperature of 25ºC. The gradient was set up as follows: 98% A to 0% A (20 min); 0% A to 98% A (20–21 min); 98% A (21–23 min). Peaks were analysed using the Shimadzu Labsolutions software.

3.2.8. Purification of products

Purification of the coupling product was performed by column chromatography. Following the coupling reaction, the organic phase was separated using a separating funnel. The organic phase was then evaporated by a rotary evaporator (Heidolph, Germany) and the crude residue was purified by column chromatography using ethyl acetate, methanol, water and formic acid (8:0.5:0.5:0.01 v/v) as the mobile phase. Pure fractions of the coupling product were monitored by TLC, pooled together and dried using a rotary evaporator. The product was washed with acetone, water and acetone again before being dried in clean glass vials.

3.2.9. Characterisation of products

3.2.9.1. Liquid Chromatography-Mass Spectrometry (LCMS)

LCMS was performed on a Shimadzu HPLC system connected to a Shimadzu LCMS-2020 mass spectrometer (Shimadzu, Japan). The products were separated using the same linear gradient of acetonitrile (solvent B) and 0.1% formic acid (solvent A) as described in Section 3.2.7.2, at a flow rate of 1 mL min^{-1}, using an injection volume of 10 μL and an oven temperature of 25 °C. MS spectra were acquired in negative mode and electrospray voltage was set to +3500 V. Dry gas flow was set to 9 L min^{-1} with a temperature of 350°C and the nebulizer gas pressure was set to 35 psi.

3.2.10. Determination of antioxidant properties

Antioxidant activities of gallic acid and the oxidation product were determined using the TEAC and DPPH assays.

3.2.10.1. Trolox equivalent antioxidant capacity (TEAC) assay

The ABTS radical scavenging activity of gallic acid and the reaction product was determined according to the protocol by Re et al. (1999). $ABTS^{\bullet+}$ solution was prepared 12–16 h before use by reacting 7 mM of ABTS salt with 2.45 mM potassium persulfate and then storing in the dark until the assay was performed. The $ABTS^{\bullet+}$ solution was diluted with methanol to give an absorbance of 0.70 ± 0.002 at 734 nm. Each sample (100 μL) prepared at different concentrations was mixed with 1100 μL $ABTS^{\bullet+}$ solution and the absorbance was read after 30 min incubation at 25°C.

3.2.10.2. 2,2'-diphenyl-1-picrylhydrazyl (DPPH) scavenging effect

DPPH working solution was prepared by dissolving DPPH in methanol to a final concentration of 0.025 mg mL^{-1}. Test samples for both gallic acid and the reaction product were prepared in methanol at various concentrations. To conduct the assay, 0.1 mL of test sample was mixed with 3.9 mL of DPPH. The mixture was shaken vigorously and incubated in the dark at 25°C for 60 min. After incubation, the absorbance was measured at 517 nm. The EC_{50} of the test samples were determined by plotting the percentage remaining DPPH against initial concentration.

3.3. RESULTS AND DISCUSSION

3.3.1. Production and purification of SLAC

The possibility of heterologous expression in bacterial hosts makes the large-scale production of valuable biological molecules cheaper and easier (Kudanga et al., 2017). *S. coelicolor* is a soil bacterium which has been reported to produce several valuable molecules, including SLAC. However, *S. coelicolor* has a complex growth cycle (Figure 7) and its culture under laboratory conditions takes roughly 5 days (Kieser et al., 2000), which is longer than most common bacteria. Despite its ability to produce valuable biomolecules, the culture of *S. coelicolor* for commercial purposes may not be economically feasible considering its growth requirements, growth cycle and culture maintenance requirements. Advances in genetic manipulation, particularly in bacterial hosts has made it possible to exploit bacteria such as *S. coelicolor* for the production of valuable biological molecules such as SLAC. The successful expression of SLAC in *E. coli* host by Machczynski et al. (2004) proved to be a remarkable achievement since it paved way for extensive studies on bacterial laccases.

In this study, expression of SLAC was performed in shake flasks at 37°C. IPTG is commonly used as an inducer for protein expression in *E. coli*. The genetic manipulation of *E. coli* for expression of foreign proteins usually occurs by inserting the protein gene into the lactose operon region; in the case of SLAC, pSLAC1 was inserted. IPTG is an effective inducer of protein expression because it is an analogue of allolactose, which binds and inactivates the lactose repressor site, thus triggering protein expression (Hansen et al., 1998). IPTG cannot be metabolised by β-galactosidase, which results in high expression of foreign protein.

After enzyme production, purification of SLAC involved a 3-step purification protocol, which included $(NH_4)_2SO_4$ precipitation, ion exchange chromatography and size-exclusion chromatography, resulting in a 2.2 purification fold (Table 3).

Table 3: Purification table for SLAC

Purification step	Total protein (mg/mL)	Total activity (U/mL)	Specific activity (U/mg)	Purification fold
Crude	4.64	15.3	3.3	1
NH_4SO_4 precipitation	3.55	10.55	2.97	1.1
Ion exchange chromatography	1.39	7.06	5.1	1.5
Size exclusion chromatography	0.12	0.85	7.33	2.2

SDS-PAGE analysis showed a pure SLAC protein achieved after size-exclusion chromatography (Figure 8). The single band corresponded to the size of SLAC reported by Machczynski et al. (2004) which was 32 kDa.

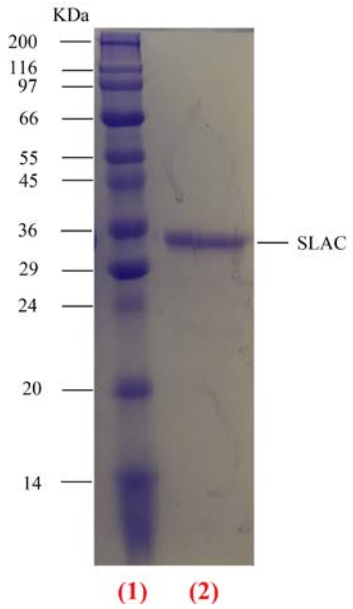


Figure 8: SDS-PAGE pictogram showing the SLAC single band (lane 2) and the molecular weight marker (lane 1).

3.3.2. Characterisation of SLAC

The characterisation of SLAC was performed in order to determine optimum reaction conditions. Since extensive characterisation studies on SLAC have already been reported (Machczynski et al., 2004, Sherif et al., 2013, Prins et al., 2015), in this study, focus was on pH which significantly impacts enzyme function during coupling reactions. The effect of common inhibitors was also studied because this has not been extensively investigated; in addition to the effect of DTT, EDTA, NaN_3 which have been investigated by Prins et al. (2015), this study also investigated the effect of kojic acid and *p*-coumaric on SLAC activity. The effect of possible metal contaminants on SLAC activity was also studied because this had not been investigated before.

3.3.2.1. Reaction conditions

Optimum pH for SLAC activity was investigated over a broad pH range of pH 3.5 – 9.0, using ABTS as substrate. Results showed that the optimum pH for SLAC activity on ABTS was at 4.5 (Figure 9). The optimum pH for SLAC is comparable to most fungal laccases (Atalla et al., 2013).

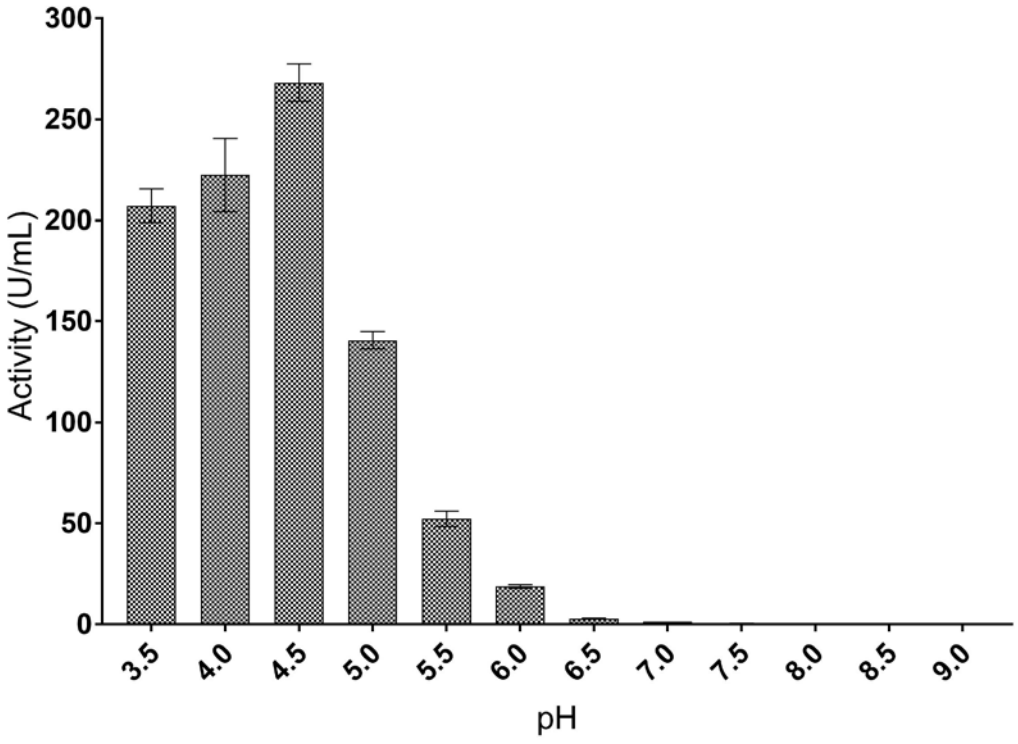


Figure 9: Effect of pH on the activity of SLAC as determined using the ABTS activity assay. All results are means ± standard deviation of three replicate determinations.

It has been reported that oxidation of phenolic compounds by laccases is pH-dependent (Xu, 1997). Therefore, it was also important to determine the pH at which SLAC reacted optimally with the substrates selected for further studies, caffeic acid and gallic acid. The pH optimum for caffeic acid was pH 7.5 (Figure 10) while the optimum for gallic acid was at pH 8.0 (Figure 11).

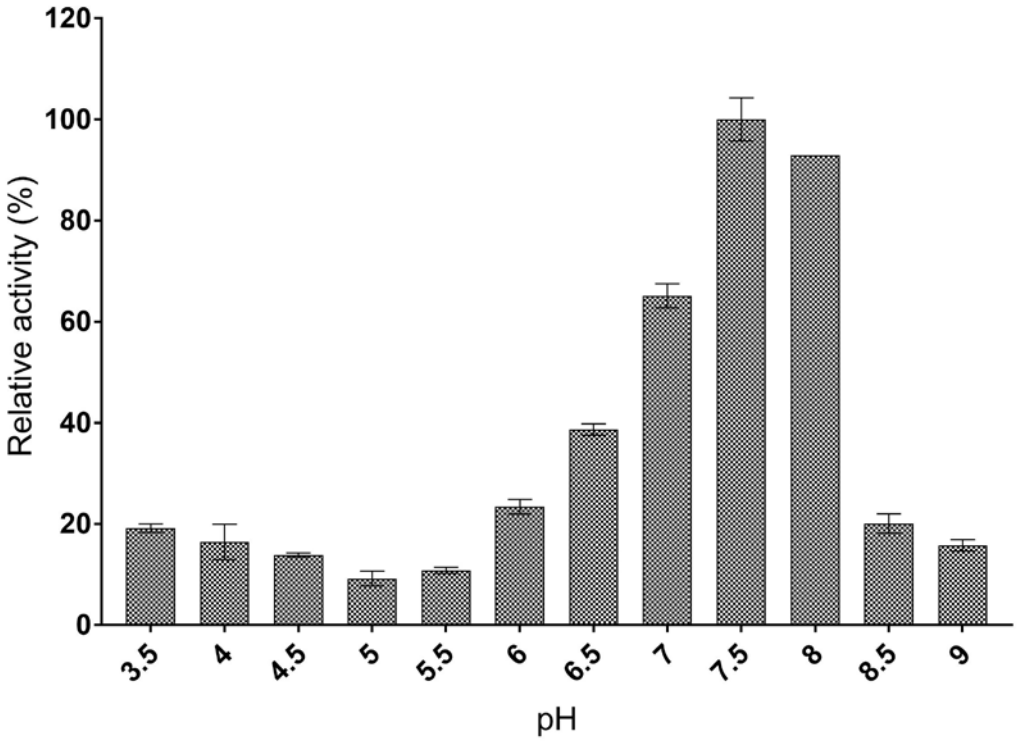


Figure 10: Effect of pH on SLAC-catalysed oxidation of caffeic acid. All results are means ± standard deviation of three replicate determinations.

It was observed that the optimum pH for SLAC oxidation of phenolic compounds was generally in the alkaline region. This suggests that the interaction of SLAC with a substrate is influenced by the structure and ionic properties of the compound. Previous studies showed that as pH increases, the redox potential (E^0) of phenolic compounds decreases, thus making the transfer of electrons from the phenolic compound to the laccase T1 site easier (Xu, 1997).

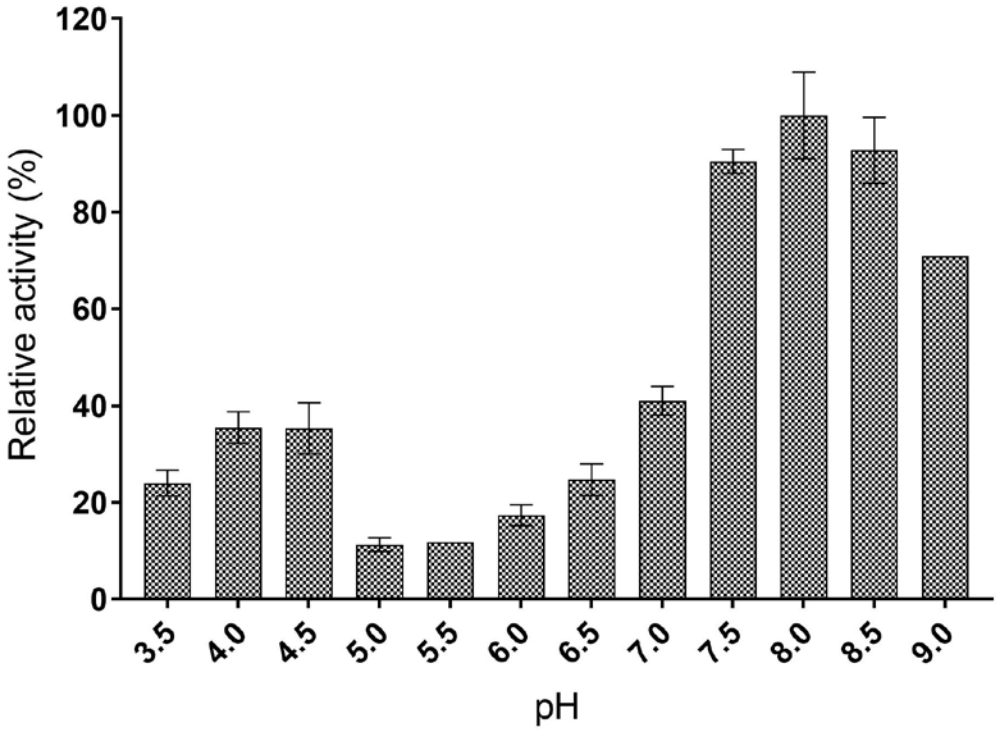


Figure 11: Effect of pH on SLAC-catalysed oxidation of gallic acid. All results are means ± standard deviation of three replicate determinations.

The pH-activity profiles of laccase-phenolics reactions generally follow a bell-shaped curve because after reaching optimum pH, further increase in hydroxide ion concentration results in the inhibition of enzyme activity due to hydroxide anions binding to the trinuclear cluster (Xu, 1997). It was observed that there was a relatively low activity for both caffeic acid and gallic acid at mild acidic pH (from pH 3.5 to pH 5.0), after which the SLAC activity increased until it reached optimum (Figures 10 and 11). Similarly low substrate affinity at mild acidic pH has been previously observed during laccase-catalysed oxidation of phenolic substrates such as acetosyringone and syringaldehyde (Xu, 1997).

3.3.2.2. Effect of selected inhibitors on SLAC activity

The effect of selected common enzyme inhibitors on SLAC activity was investigated. The order of inhibition was as follows: DTT > EDTA > *p*-coumaric acid > NaN_3 > Kojic acid. DTT was the most potent inhibitor of SLAC, causing 50% reduction in activity at 0.389 mM (Table 4). DTT is a sulfhydryl organic compound that is capable of forming stable copper complexes thus preventing substrate access to laccase active site (Johannes and Majcherczyk, 2000). DTT is also known to inhibit enzyme activity by reducing disulphide bonds on the protein molecules (Wu et

al., 2010). EDTA was also a potent inhibitor (I_{50}=5.65 mM). EDTA is a known metal chelator and may affect enzyme activity by chelating the copper ions found in the SLAC catalytic core. NaN_3 had an I_{50} value of 19.9 mM (Table 4). This indicates that SLAC is more resistant to NaN_3 inhibition compared to fungal laccases which have been reported to be completely inhibited by NaN_3 at 10 mM (Ben Younes and Sayadi, 2011). NaN_3 affects laccase activity by binding to the trinuclear cluster, thereby affecting electron transfer (Ben Younes and Sayadi, 2011). Kojic acid and *p*-coumaric acid are putative laccase inhibitors (Eggert et al., 1996) which did not significantly inhibit SLAC activity (Table 4).

Table 4: The I_{50} values of selected inhibitors against SLAC. All results are means ± standard deviation of three replicate determinations

Inhibitor	I_{50} value* (mM)
Ethylenediaminetetraacetic acid (EDTA)	5.650 ± 0.359
Dithiothreitol (DTT)	0.389 ± 0.049
Sodium azide (NaN_3)	19.914 ± 0.419
Kojic acid	23.894 ± 0.580
p-Coumaric acid	16.546 ± 0.388

* I_{50} value is defined as the concentration of compound that results in 50% loss of enzyme activity

3.3.2.3. Effect of metal ions on SLAC activity

The effect of metal ions on the activity of SLAC was investigated over a concentration range of 0 to 20 mM. Metal ions are common contaminants in laboratories working on biocatalysis studies as well as in medium to large-scale applications of enzymes. Among the metal ions tested, the effect of Cu^{2+} and Fe^{2+} were noteworthy. The increase in Cu^{2+} ions resulted in increased SLAC activity. The activity-concentration profile of Cu^{2+} followed a bell-curved trend, with the peak at 5 mM corresponding to a 188% increase in SLAC activity (Figure 12). Further increase in Cu^{2+} concentration resulted in a decline in activity. These findings are comparable to literature; Forootanfar et al. (2011) reported an enhanced activity of a *Paraconiothyrium variabile* laccase after incubation in Cu^{2+} ions. It has also been reported that copper content of laccases is usually incomplete and is determined by cultivation factors such as temperature, oxygen and media type (Sherif et al., 2013). Therefore, preincubation with 5 mM Cu^{2+} may have contributed to the full incorporation of Cu^{2+} in SLAC resulting in enhanced activity. Fe^{2+} on the other hand was observed to have a significant inhibitory effect on SLAC activity. Preincubation in 1 mM

concentration of Fe^{2+} resulted in a 95% loss of SLAC activity (Figure 12). This again is comparable to results by Forootanfar et al. (2011), who reported a 91.5% loss of activity at 10 mM ferric ion concentration.

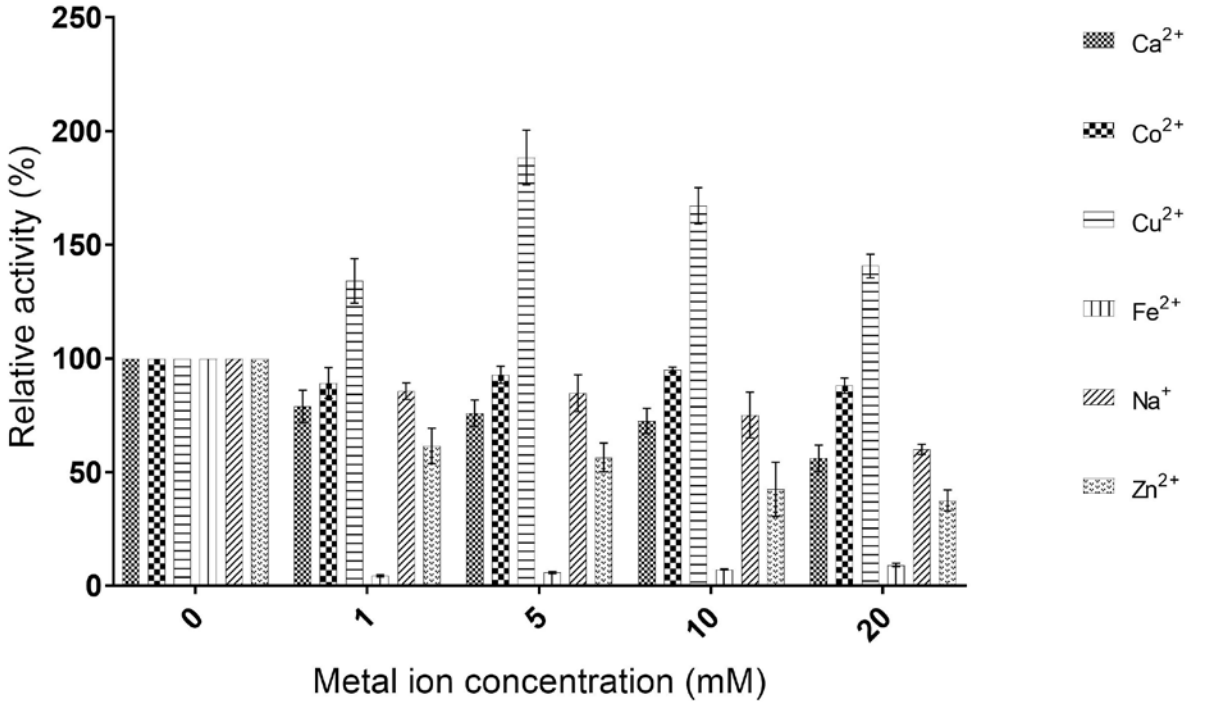


Figure 12: Effect of metal ions on the activity of SLAC. All results are means ± standard deviation of three replicate determinations.

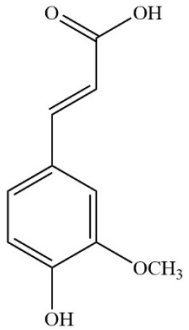
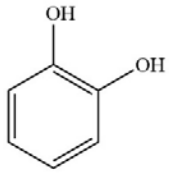
3.3.3. Preliminary coupling reactions

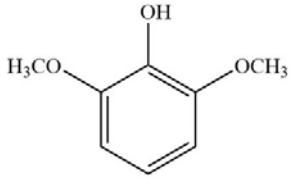
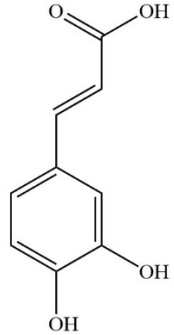
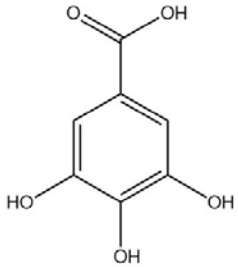
3.3.3.1. Substrate screening and determination of reaction conditions for product formation

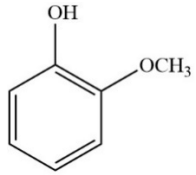
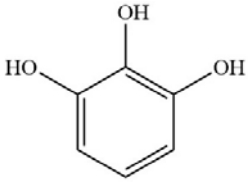
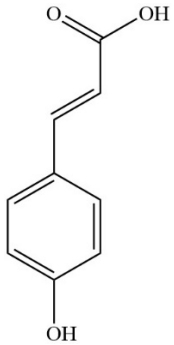
The stability of the SLAC at extreme conditions, coupled with the generally clean reaction characteristic of laccases, presents an ideal, green and robust enzyme for synthetic applications. Despite all these attributes, SLAC also has its shortcomings. Compared to plant and fungal laccases, SLAC has a lower redox potential of 0.5 V (Machczynski et al., 2004). The feasibility of a reaction between SLAC and a substrate is determined by the redox potential difference (ΔE^0) between the enzyme and the substrate; substrates with redox potential lower than 0.5 V are expected to be readily oxidised by SLAC (Frasconi et al., 2010, Xu et al., 2000). This explains why some phenolic substrates of high redox potential could not be oxidised by SLAC. Therefore, it was necessary to screen potential substrates for oxidation with SLAC. SLAC showed oxidase

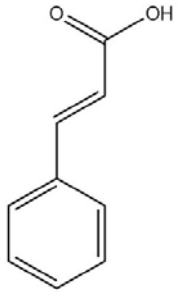
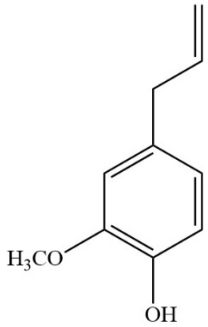
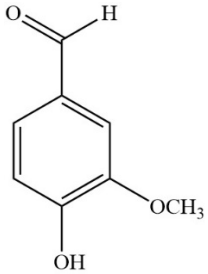
activity against 7 of 14 substrates tested. Table 5 summarises the reaction of SLAC with phenolic substrates and the redox potential of the substrates screened. The results are consistent with previous studies which reported that compounds such as *p*-coumaric acid (E^0=0.66 V) could not be oxidised by SLAC while ferulic acid (E^0=0.50 V) and caffeic acid (E^0=0.36 V) were oxidisable (Jørgensen and Skibsted, 1998, Sherif et al., 2013). It has also been reported that reactivity of substrates decreases with increase in molecular size due to substrate restricted accessibility to enzyme active site (d'Acunzo et al., 2002). This possibly explains why some phenolic substrates such as quercetin (302.236 g/mol) and rutin (610.52 g/mol) could not be oxidised by SLAC even though their redox potential was within SLAC oxidation range (Table 5).

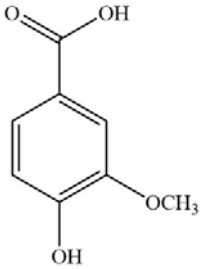
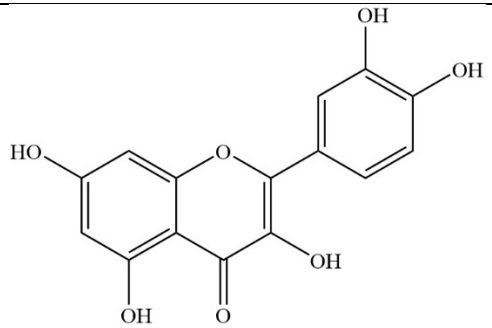
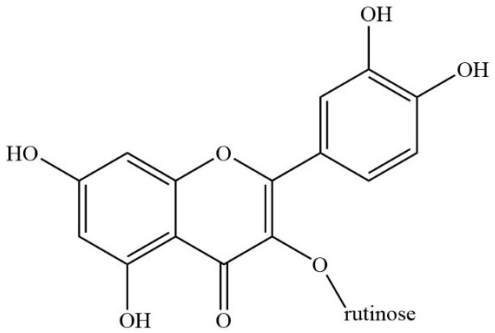
Table 5: Phenolic substrate screened for oxidation by SLAC

Substrate	Reaction outcome	Redox potential	Remarks	Reference*
 Ferulic acid	(+)	0.50 V	Substrate oxidised since its redox potential is within SLAC oxidation range.	(Jørgensen and Skibsted, 1998)
 Catechol	(+)	0.20 V	Positive reaction may be attributed to the low redox potential and low molecular weight of catechol (110.1 g/mol). Catechol contains two hydroxyl groups which are electron donating moieties.	(Enache and Oliveira-Brett, 2011)

 2,6-dimethoxyphenol	(+)	No relevant literature available	Positive reaction may be attributed to the low molecular weight of 2,6-dimethoxyphenol (154.16 g/mol). The electron donating moieties, particularly the methoxyl groups, make the compound easily oxidisable	No relevant literature available
 Caffeic acid	(+)	0.36 V	Substrate oxidised. Redox potential within SLAC oxidation range.	(Jørgensen and Skibsted, 1998)
 Gallic acid	(+)	No relevant literature available	Positive reaction may be due to the low molecular weight of gallic acid (170.12 g/mol) and the presence of three hydroxyl moieties which are electron donating.	No relevant literature available.

 Guaiacol	(+)	0.32 V; 0.39 V	Redox potential is within SLAC oxidation range.	(Samet et al., 2002)
 Pyrogallol	(+)	No electrochemical reports in literature	Substrate oxidised probably because pyrogallol is a low molecular weight compound (126.11 g/mol). The compound also contains three hydroxyl groups which are electron donating.	No relevant literature available
 *p*-coumaric acid	(-)	0.74 V	Substrate has a higher redox potential than that of SLAC.	(Teixeira et al., 2013)

 Cinnamic acid	(-)	No electrochemical reports in literature	Lack of electron donating moieties (eg. hydroxyl groups) on the aromatic ring makes compound difficult to oxidise.	No relevant literature available
 Eugenol	(-)	0.45 V	It is known that the rate of laccase-oxidised reactions depend on the redox potential difference (ΔE^0) between the enzyme and the substrate (Frasconi et al., 2010). The low ΔE^0 of 0.05 V between SLAC and eugenol may mean that the reaction proceeds at a very slow rate.	(Vasiliadou, 2017)
	(-)	0.96 V	Substrate not oxidised because of a higher redox potential than that of SLAC.	(Deng et al., 2015)

Vanillin				
 Vanillic acid	(-)	0.73 V	Substrate not oxidised because of a higher redox potential than that of SLAC.	(Simić et al., 2007)
 Quercetin	(-)	0.1 V	Substrate not oxidised. This may be owing to the large molecular size of quercetin which may restrict substrate accessibility to the enzyme active site.	(Simić et al., 2007)
	(-)	0.23 V	Substrate not oxidised by SLAC. This may be owing to the large molecular size of rutin which may restrict substrate accessibility to the enzyme active site.	(Simić et al., 2007)

Rutin				

(+) Positive reaction – Substrate oxidised by SLAC

(-) Negative reaction – Substrate not oxidised by SLAC

*Literature cited in the “Reference” column of table is for the quoted redox potential values only.

Positive reactions were identified by analysing oxidation products using HPLC (Tables 6). Among the phenolic substrates that had positive reaction with SLAC (Table 6), only caffeic acid and gallic acid were further studied. The rest of the substrates were not further investigated because several researchers have already reported laccase coupling products using ferulic acid (Adelakun et al., 2012b, Mustafa et al., 2005, Carunchio et al., 2001, Tranchimand et al., 2006) and 2,6-dimethoxyphenol (Adelakun et al., 2012a), while no solvent system could be developed to separate reaction products from the SLAC-catalysed oxidation of catechol, guaiacol, and pyrogallol.

Table 6: Oxidation of phenolic molecules by SLAC

Phenolic substrate	Reaction pH	λMax of SLAC oxidation products (nm)
Caffeic acid	7.5	336; 366
Catechol	8.0	386
2,6-dimethoxyphenol	8.0	469
Ferulic acid	8.0	286; 589
Gallic acid	8.0	231; 306,399
Pyrogallol	7.5	286; 442; 582
Guaiacol	9.0	497

3.3.3.2. Purification and analysis of reaction products

Thin Layer Chromatography (TLC) is an effective and economic separation method that remains popular in organic research. TLC involves the separation of compounds based on their distribution between 2 phases (mobile phase and stationary phase). For this study, Silica gel 60 F_{254} TLC plates were used. The SiO_2 matrix on the F_{254} plates makes the stationary phase polar. The separation of compounds on the TLC can be determined by three factors; (i) polarity and size of molecule; (ii) polarity of stationary phase; (iii) polarity of solvent (mobile phase). In this study, only the polarity of the mobile phase could be controlled because the compounds being separated were novel and of unknown polarity characteristics and molecular weight. A total of 32 solvent systems with varying polarities were used in an attempt to separate coupling products

obtained from the SLAC-catalysed oxidation of caffeic acid and gallic acid. Interaction of coupling products with stationary phase suggested that products were generally more polar than the substrates. Phenolics are considered polar compounds, owing to their hydroxyl functional groups. After coupling reactions, it is expected that the reaction products will be more polar since the coupling products may theoretically have more polar functional groups (Sherma and Fried, 2003). Therefore, to separate the coupling products, polar solvent systems were employed. Solvent systems for the separation of caffeic and gallic acid oxidation products were successfully developed (ethyl acetate/ methanol/ water/ formic acid in the ratio 8:1:1:0.02 (v/v) for caffeic acid and 8:0.5:0.5:0.01 (v/v) for gallic acid). The latter sections of this chapter will discuss the results on the gallic acid oxidative coupling reactions. Results on the oxidation of caffeic acid by SLAC will be reported in Chapter 4.

3.3.3.3. Characterisation of gallic acid oxidation product

Oxidation of gallic acid by SLAC resulted in the formation of one major product designated as G1 (Rf = 0.36), (Figure 13), (t_R = 11.725), (Figure 14a).

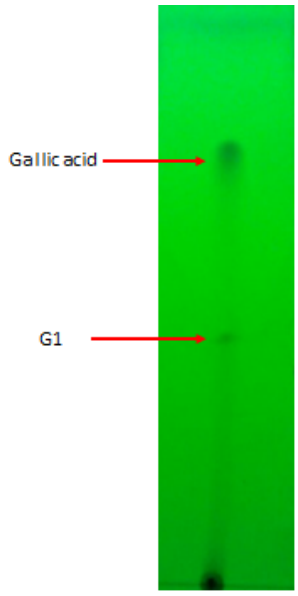


Figure 13: Separation of gallic acid oxidation product (Rf = 0.36) and gallic acid (Rf = 0.70) by TLC.

LC-MS spectra of the gallic acid oxidation product showed intensities at *m/z* 263 (Figure 14b).

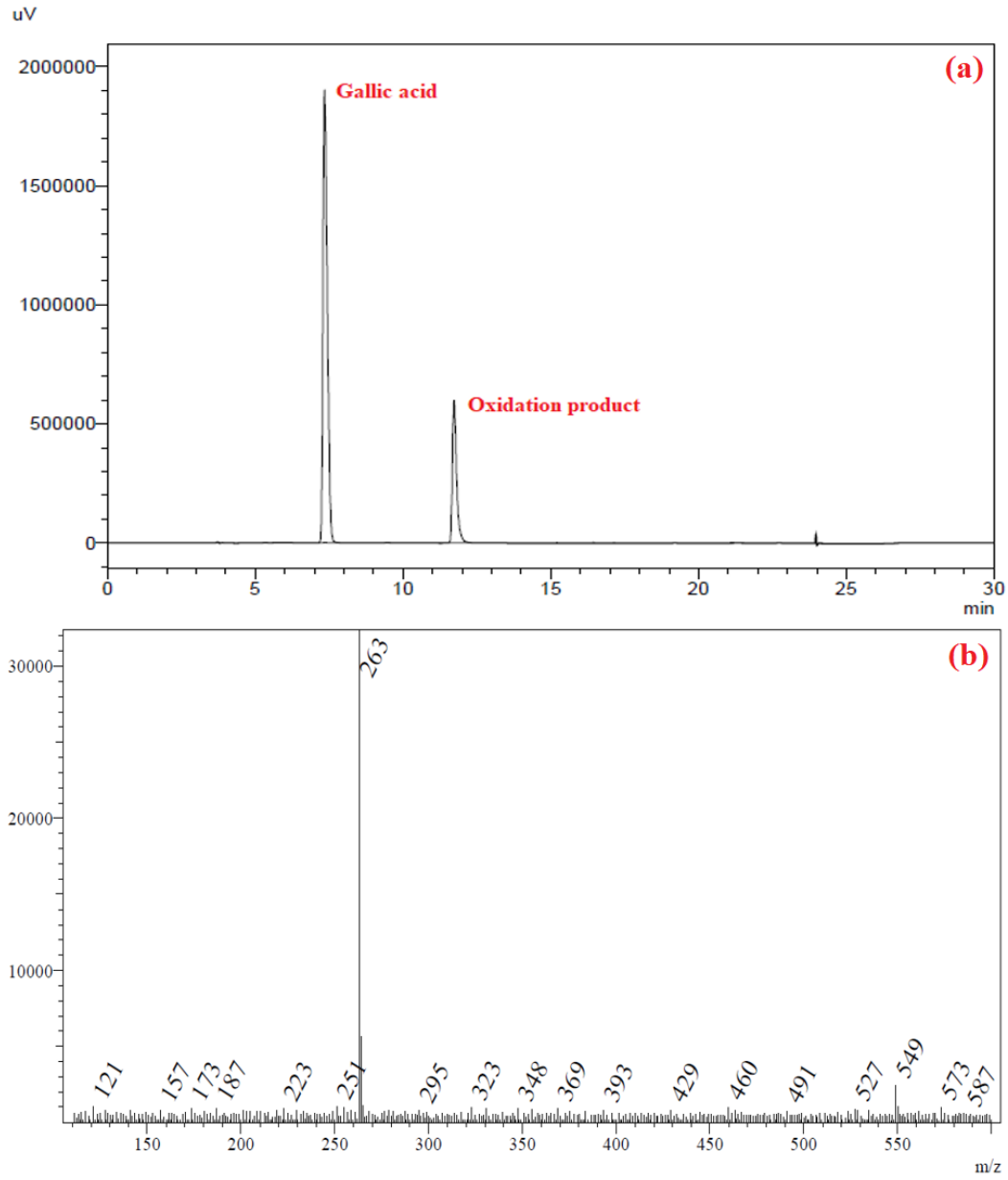


Figure 14: HPLC chromatogram of: (a) gallic acid (t_R = 7.342) and oxidation product (t_R = 11.725) of SLAC-catalysed oxidation of gallic acid, (b) mass spectra of SLAC oxidation product (G1).

Based on elementary analysis (Appendix 1), the structure ($C_{12}H_8O_7$) shown in Figure 15 was predicted. Usually laccase mechanism of action involves the generation of radicals which undergo coupling to form oligomeric products. However, due to steric hinderance at positions 2 and 6, it is plausible that gallic acid underwent oxidative decarboxylation followed by coupling reaction to form the predicted product, Dibenzo[*b*,*e*][1,4]dioxine-1,2,3,8,9-pentaol, designated as G1 (Figure 15). Due to the observed low antioxidant capacity of G1 (reported in Section 3.3.3.7 below), it was decided not to further characterise the compound by NMR.

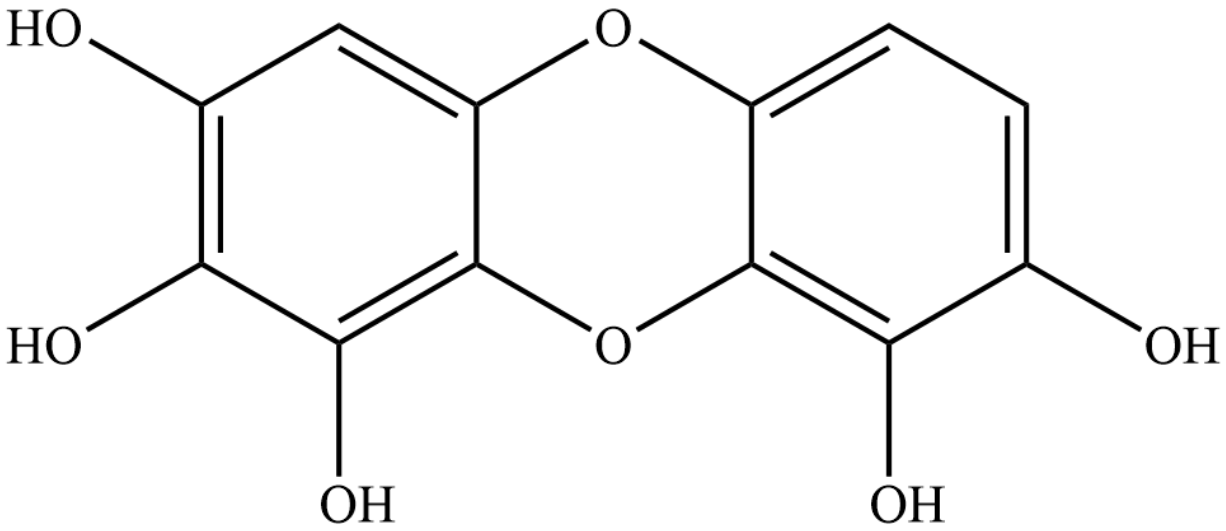


Figure 15: Proposed structure of product, Dibenzo[*b,e*][1,4]dioxine-1,2,3,8,9-pentaol (G1) formed during SLAC-catalysed oxidation of gallic acid.

3.3.3.4. Optimisation of reaction conditions for production of G1

Organic solvents are commonly employed in synthetic chemistry because they provide kinetic and thermodynamic conditions that promote synthetic reactions to occur (Kvittingen, 1994). In biphasic systems, organic solvents have also been reported to prevent polymerisation by facilitating separation of oxidation products which are usually soluble in organic phase (Witayakran and Ragauskas, 2009). The effect of the nature and concentration of two water-miscible (ethanol and methanol) and two water-immiscible (ethyl acetate and hexane) solvents were investigated to determine the best conditions for G1 formation. Product formation was monitored using HPLC standard curves plotted for both gallic acid and the oxidation product (Appendix 2).

For water-miscible solvents, there was a decline in G1 formation as the solvent concentration increased from 50 – 90% (v/v) (Figure 16). In contrast, production of G1 in water-immiscible solvents exhibited a bell-shaped trend; there was an increase in the formation of G1 as the concentration of solvent (both ethyl acetate and hexane) was increased from 50–80%, afterwards a decline in product formation was observed. Ethyl acetate (80%, v/v) was identified as the best organic solvent for product formation. In general, production of G1 decreased in the order of ethyl acetate > hexane> ethanol> methanol. This trend shows that water-miscible solvents have a negative impact on the formation of G1. This may be explained by two factors: (i) water-miscible solvents form homogenous phases with enzyme which affects the stability and activity

of laccase (Riva, 2006); (ii) there is no separation of oxidation products which may be further oxidised into polymers by radical and laccase action (Witayakran and Ragauskas, 2009).

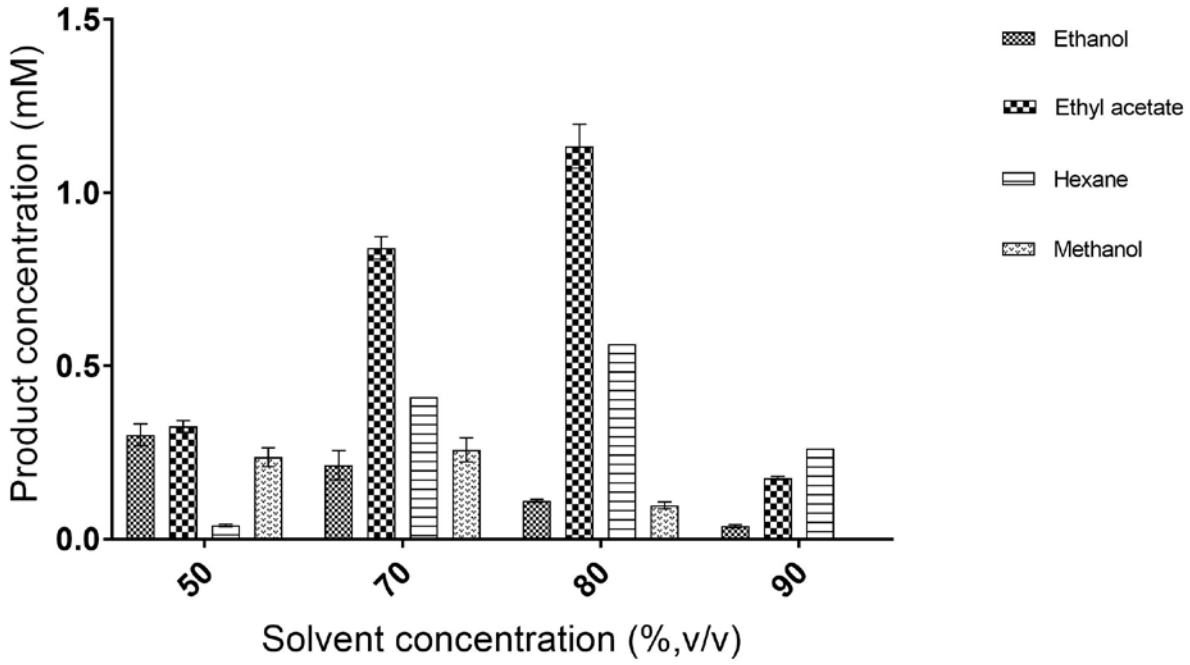


Figure 16: The effect of solvent concentration on the formation of G1. All results are means ± standard deviation of three replicate determinations.

Enzyme activity was monitored at the optimum solvent concentration conditions (80%, v/v ethyl acetate: buffer system, pH 8.0) during the oxidation reaction. It was observed that as the oxidation reaction progressed, 68.4% of enzyme activity was lost in the first 30 min (Figure 17). By 90 min, 94.7% of activity was lost. Therefore, the enzyme was added at 90-minute intervals for the reaction to proceed. Organic solvents have previously been reported to affect laccase activity (Riva, 2006, Rodakiewicz-Nowak, 2000), thus the loss of enzyme activity was not surprising.

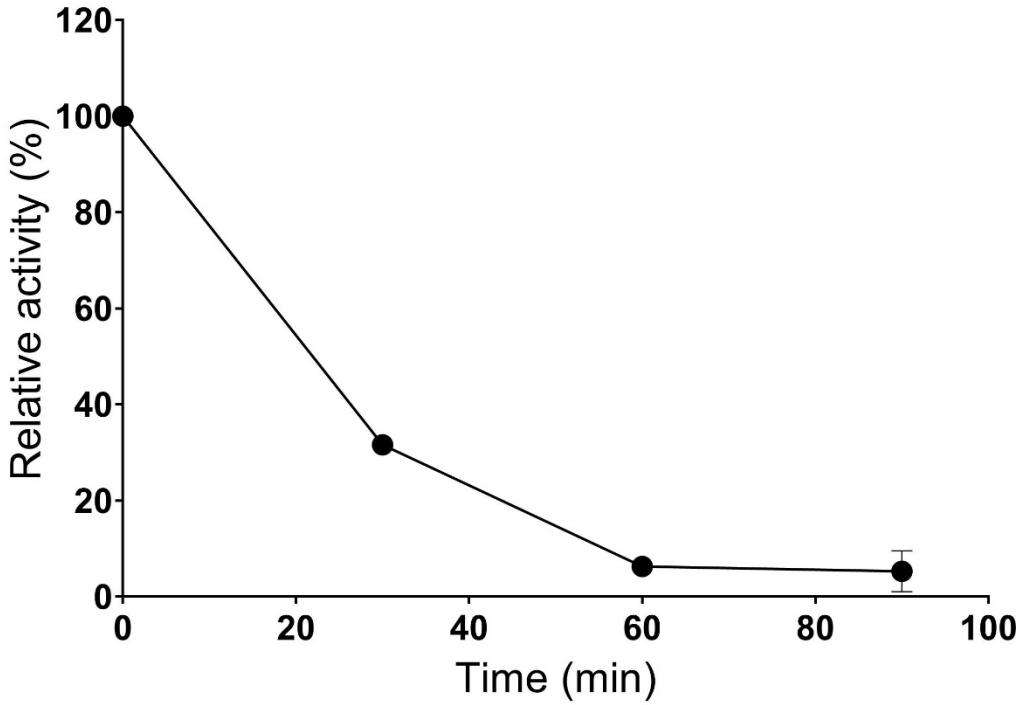


Figure 17: Effect of organic ethyl acetate on the activity of SLAC. Activity was monitored using 5mM ABTS as substrate. All results are means ± standard deviation of three replicate determinations.

3.3.3.5. Effect of enzyme activity on G1 formation

It is important to determine the minimum activity of enzyme required to achieve the highest product formation, especially in commercial applications where any possible losses and wastages need to be prevented. High enzyme activities may also cause rapid proliferation of radicals which results in polymerisation reactions. In this study, the effect of enzyme activity on the formation of G1 was investigated in the range 0.25–1.25 U. The production of G1 at varying enzyme activities followed a bell-shaped trend which showed a peak at 0.5 U (Figure 18). A related study (Aktaş and Tanyolaç, 2003) showed a negatively skewed trend reaching a plateau where further increase in enzyme concentration had no effect on product formation. The decline in G1 formation as enzyme activity was increased beyond 0.5 U may indicate product loss due to polymerisation reactions.

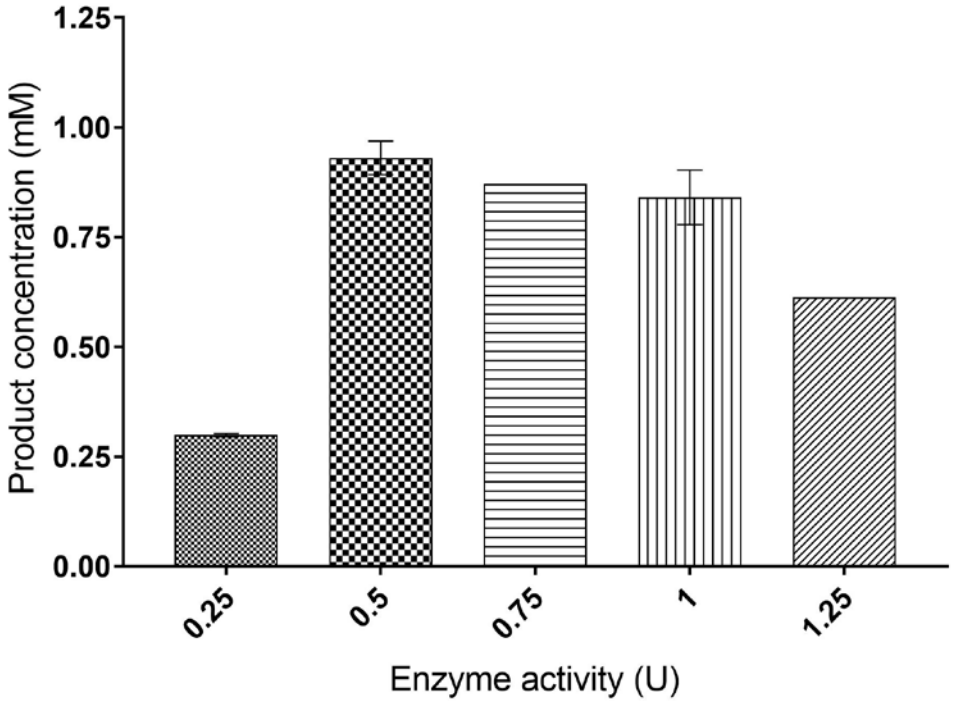


Figure 18: Effect of SLAC activity on the formation of G1. All results are means ± standard deviation of three replicate determinations.

3.3.3.6. Effect of reaction time on formation of product G1

The effect of reaction time on the formation of G1 was monitored by HPLC over a 12 h period. Results showed that maximum yield of G1 was achieved at 4 h, after which the concentration of product started decreasing. A 15% product yield (relative to starting material) was the highest possible substrate conversion into G1 achieved when reaction was conducted for 4 h (Figure 19). This low product yield attained may be attributed to the non-specificity of laccases which is known to result in a mixture of products ranging from oligomeric to polymeric products (Claus, 2004, Kudanga et al., 2017). The generation of reactive free radicals makes it difficult to control undesirable side-reactions in subsequent coupling reactions.

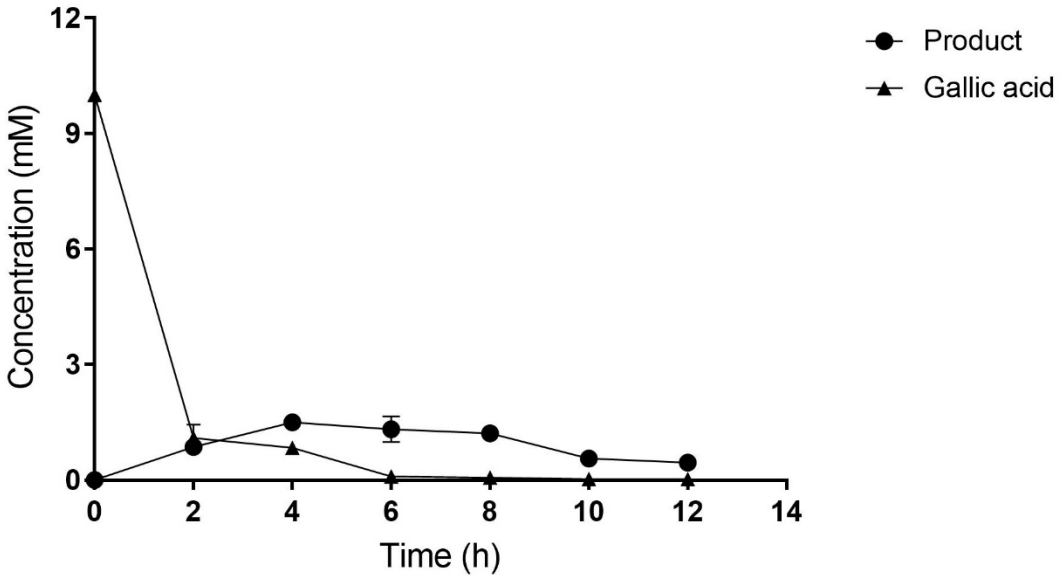


Figure 19: Effect of reaction time on product G1 formation. All results are means ± standard deviation of three replicate determinations.

3.3.3.7. Determination of antioxidant capacity

Antioxidant capacity of G1 was compared to gallic acid using the DPPH and TEAC assays. The DPPH and TEAC are SET based assays which measure the ability of an antioxidant to reduce $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals to their more stable states (1,1-diphenyl-2-picryl hydrazine and 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid, respectively) (Floegel et al., 2011). Both assays showed a lower antioxidant capacity of G1 compared to gallic acid (Table 7). The TEAC assay showed a 3-fold decline while DPPH showed a 2-fold decrease in antioxidant capacity. Structure-activity relationships studies have shown that the antioxidant potential of phenols is dependent on the following factors: (i) the number of active groups such as hydroxyl, alkyl or aniline moieties attached to the aromatic ring (Kudanga et al., 2017); (ii) the ability of an antioxidant to form stable phenoxy radicals (Kudanga et al., 2017, Rice-Evans et al., 1997) (iii) the position of active groups on the aromatic ring; enhanced antioxidant activity has been observed when active groups occupy the *ortho* and *para* positions of the aromatic ring (Kudanga et al., 2017). The antioxidant capacity of gallic acid may be attributed to its structure, which consists of three hydroxyl moieties occupying the *ortho* and *para* positions of the aromatic ring. As shown in Figure 20, the C2 and C6 are sterically hindered because of the bulky carboxyl group at C1, therefore any possible SLAC-catalysed coupling of gallic acid is likely to occur at the expense of either of the hydroxyl groups occupying C3, C4 or C5, which negatively affects

the antioxidant capacity of the resultant oxidation product. The loss of a hydroxyl group may have contributed to reduced antioxidant capacity of G1.

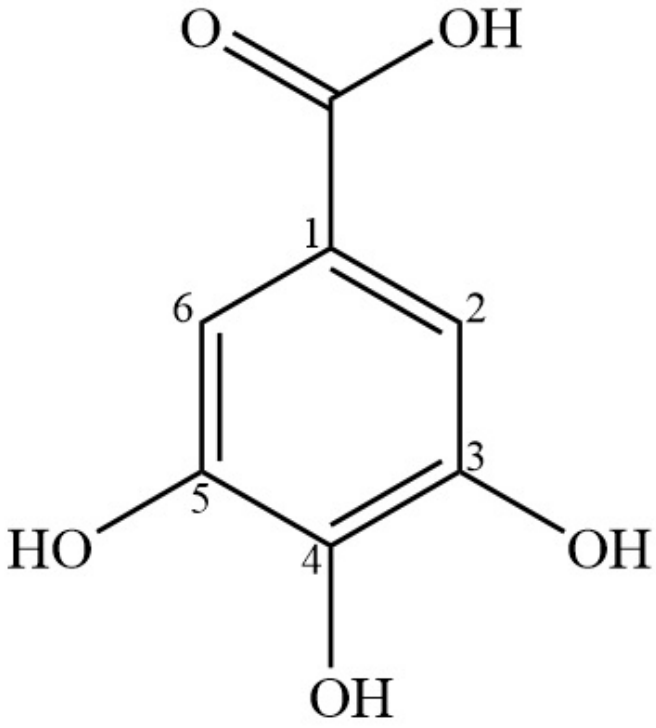


Figure 20: Chemical structure of gallic acid.

The results obtained from the elementary analysis (Appendix 1) of G1 suggest that the SLAC catalysed oxidation of gallic acid underwent oxidative decarboxylation followed by coupling reaction as outlined earlier (Section 3.3.3.3). The plausible resultant oxidation product (Figure 15) is a diaryl ether which consists of two galloyl monomers joined by two C-O-C linkages. Aromatic compounds with ether linkages occur at the expense of hydroxyl groups, making the resultant compounds less reactive; ether linkages such as arylglycerol-β-aryl ethers (β-O-4), noncyclic benzyl aryl ethers (α-O-4) and diaryl ethers (4-O-5) are common linkages in naturally occurring inert materials such as lignin and coal (Dorrestijn et al., 2000). Since the main aim of the study was to produce new oligomeric antioxidants with improved capacity, elucidation of the product's chemical structure by NMR was not further pursued.

Table 7: Antioxidant activity of gallic acid and its oxidation product (G1) determined using the DPPH and TEAC assays. All results are means ± standard deviation (SD) of three replicate determinations

Molecule	Molecular weight	EC_{50} DPPH[a] (mM)	TEAC value[b] (mM)
Gallic acid	170.12	0.334 ± 0.001	1.369 ± 0.100
G1	263.00	0.744 ± 0.04	4.252 ± 0.005

[a]EC_{50} value is defined as the concentration (mM) of substrate that results in 50% loss of $DPPH^{\bullet}$ (Adelakun et al., 2012a)

[b]TEAC value is defined as the concentration of Trolox (mM) with an antioxidant potential equivalent to 1 mM of compound under investigation (Adelakun et al., 2012a).

3.4. CONCLUSION

This study reported the production, characterisation and application of SLAC in oxidation reactions with potential for organic synthesis. The substrate screening process identified 2,6-dimethoxyphenol, caffeic acid, catechol, gallic acid, guaiacol, ferulic acid, and pyrogallol as SLAC-oxidisable substrates. The study has also shown that SLAC catalytic potential is affected by its low E^0 which (in the absence of mediators) limits its application to substrates with a redox potential less than 0.5 V. A gallic acid oxidation product was successfully produced and purified. However, the product showed a lower antioxidant capacity when compared to gallic acid. Although the product has a lower antioxidant capacity, overall, the study has shown the potential of the SLAC as a catalyst in organic synthesis.

4. SMALL LACCASE-CATALYSED SYNTHESIS OF A CAFFEIC ACID DIMER WITH HIGH ANTIOXIDANT CAPACITY

4.1. ABSTRACT

The increase in antioxidant demand for application in industries such as food and pharmaceuticals, has necessitated the need to investigate new production methods. The application of laccase for antioxidant synthesis is gaining notable attention as a viable approach to address this need. In this study, a *β-β* caffeic acid dimer, phellinsin A, was synthesised using the small laccase, SLAC, a two-domain bacterial laccase native to *Streptomyces coelicolor* A3(2). Phellinsin A showed a 1.5-fold increase in 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity and a 1.8-fold improvement in trolox equivalence antioxidant capacity (TEAC) when compared to caffeic acid. Phellinsin A also showed improved solubility properties in aqueous media and remarkable stability at acidic pH (pH 2.2 and pH 5.5). The study demonstrates the potential of bacterial laccases as catalysts for the biotransformation of natural phenolics into value-added compounds.

4.2. INTRODUCTION

Mediterranean plant based diets have gained popularity among consumers because they contain high levels of bioactive compounds such as plant phenolics. Scientific research has linked plant phenolics with therapeutic properties such as improvement of plasma antioxidant capacity (Morabito et al., 2014), anticarcinogenicity (El Gharras, 2009) and minimising oxidative stress in general (Manna et al., 2002). Plant phenolics are a group of phenol based bioactive compounds which exist in plants as secondary metabolites. Caffeic acid is one of the major hydroxycinnamic acids found in plants. In particular, it is one of the major phenolic acids found in potatoes, wines and coffee.

The use of phenolic compounds for their antioxidant properties has been limited because of their low solubility in aqueous media (Ghoul and Chebil, 2012) and susceptibility to heat, oxygen and light. Simple monomeric phenolics were reported to have a short half-life in the body (Kurisawa et al., 2003a) and may be metabolised to less active metabolites (Heleno et al., 2015). Caffeic

acid, for example, is converted into conjugates such as glucuronide, sulphated or methylated derivatives during *in vivo* metabolic processes (Heleno et al., 2015). Piazzon et al. (2012) showed that some of these structural alterations have a significant impact on antioxidant properties of caffeic acid; close to 20-fold decrease in antioxidant capacity of a caffeic acid metabolite (caffeic acid 4'-*O*-glucuronide) was reported in comparison to the parent compound. Caffeic acid is a simple phenolic compound consisting of only two hydroxyl groups on its aromatic ring and a C=C sidechain which are pivotal for its antioxidant properties. This may explain why metabolic conjugation significantly affects its bioactive properties. Modification processes that increase the number of hydroxyl groups could mitigate the reduction in activity resulting from these conjugation reactions.

Consequently, interest in biocatalytic approaches of improving the properties of phenolic compounds is increasing. The enzymatic modification of phenolic compounds has also been motivated by various other factors. These include the poor antioxidant capacity of some natural phenolics (Adelakun et al., 2012b); some monomeric forms acting as pro-oxidants (Uyama, 2007); the high cost of extraction of natural phenolics (Kudanga et al., 2017); inefficiencies of chemical synthesis; and the need to enhance bioavailability of natural phenolics, which is usually reduced due to poor solubility properties (Gavezzotti et al., 2014). On the other hand, high molecular weight phenolics have been shown to have better bioactive properties compared to low molecular weight phenols (Ma et al., 2009). Of the biocatalytic approaches, laccases have generated the most interest in improving antioxidant capacity through dimerisation (Ncanana et al., 2007, Adelakun et al., 2012a,b, Zwane et al., 2012, Gavezzotti et al., 2014) or polymerisation (Kurisawa et al., 2003a,b) to higher molecular weight compounds.

Laccase (E.C. 1.10.13.2) is a multi-copper oxidase that use oxygen to abstract a single electron from a phenolic substrate to produce phenoxy radicals with water as a by-product (Kudanga et al., 2017). Its ability to produce phenoxy radical intermediates has been applied in the formation of coupling products of therapeutic value.

Although several researchers have reported the synthesis of compounds with enhanced bioactive properties using laccases (as reviewed in Kudanga et al. (2017)), the work has employed only plant and fungal laccases. However bacterial laccases would be attractive industrial catalysts due to the ease of expression in hosts such as *Escherichia coli* (Sharma et al., 2007) and some favourable biochemical properties. For example, Machczynski et al. (2004) characterised and

overexpressed in *E. coli* a two domain (small) laccase (SLAC) that is native to *S. coelicolor*. This enzyme has several attributes that makes it potentially valuable for industrial application. It is thermally stable, recorded optimal activity at pH as high as 9.5 and is resistant to denaturation by detergents (Machczynski et al., 2004). Despite these favourable properties, there is no record of its application in biocatalysis. This is the first report of the application of SLAC in the synthesis of a dimeric antioxidant using caffeic acid as substrate. The bioactive and physicochemical properties of the synthesised compound were also investigated.

4.3. MATERIALS AND METHODS

4.3.1. Chemicals and enzymes

Chemicals were purchased from Sigma Aldrich (South Africa). *E. coli* cloned for the expression of SLAC (wild type) was kindly provided by Prof. Gerard Canters, Leiden Institute of Chemistry, University of Leiden (Netherlands).

4.3.2. Production of SLAC

The production of SLAC was done according to the protocol described by Prins et al. (2015) with a few modifications (see Section 3.2.2).

4.3.3. Enzyme activity

SLAC activity was determined using the 2,2´-Azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) activity assay (see Section 3.2.3).

4.3.4. Oxidation of caffeic acid by SLAC

Oxidation reactions were carried out in a buffer-organic solvent mixture. The reaction mixture contained caffeic acid (10 mM final concentration), SLAC (0.64 U) in 50 mM sodium phosphate buffer (pH 7.5) (conditions determined from preliminary experiments). Optimum product yield was determined by comparing four organic solvents: the water miscible solvents, methanol and ethanol, and the water immiscible solvents, ethyl acetate and hexane at 50, 70, 80 and 90% organic solvent concentration (v/v) using 50 mM sodium phosphate (pH 7.5) as a buffer. The reaction was conducted for 3 h (25°C, 250 rpm). Since it was previously observed that SLAC

was affected by organic solvents, 0.64 U/ mL enzyme was added at 90-min intervals to maintain enzyme activity.

4.3.5. Analysis of products

4.3.5.1. Thin layer chromatography (TLC)

Product formation was monitored by thin layer chromatography using the aluminum backed Silica gel 60 F_{254} TLC plates (Sigma, South Africa). A solvent system containing ethyl acetate, methanol, water and formic acid (8:1:1:0.02 v/v) was used as the mobile phase for the chromatographic separation of products. Plates were viewed under UV light at 254 nm.

4.3.5.2. High Performance Liquid Chromatography (HPLC)

HPLC was used for the analysis of oxidation products. Sample preparations were done as previously described in Section 3.2.7.2.

4.3.6. Purification of products

Purification of the oxidation product was done by preparatory thin layer chromatography glass plates (500 µm, silica gel 60 F_{254}, Sigma, South Africa) using ethyl acetate, methanol, water and formic acid (8:1:1:0.02 v/v) as the mobile phase. Pure bands were observed under UV light at 254 nm and were marked with a pencil. The product bands were scrapped off the plate and re-dissolved in methanol. After filtering off the silica using 0.22µm syringe filters, the product was washed with acetone, methanol and acetone again before being dried in clean glass vials.

4.3.7. Characterisation of products

4.3.7.1. Liquid Chromatography-Mass Spectrometry (LCMS)

LC-MS was performed on a Shimadzu HPLC system connected to a Shimadzu LCMS-2020 mass spectrometer (Shimadzu, Japan). The products were separated using the same linear gradient of acetonitrile (solvent B) and 0.1% formic acid (solvent A) as described in Section 3.2.7.2, at a flow rate of 1 mL/min, using an injection volume of 10 µL and an oven temperature of 25°C. MS spectra were acquired in negative mode and electrospray voltage was set to +3500 V. Dry gas flow was set to 9 L min^{-1} with a temperature of 350 °C and the nebulizer gas pressure was set to 35 psi.

4.3.7.2. Nuclear Magnetic Resonance (NMR)

For NMR analysis, 5-10 mg samples were dissolved in 0.5 mL CD_3OD. ^1H, ^{13}C and 2D NMR spectra were recorded on a Bruker Avance instrument operating at 400 MHz. Chemical shifts were reported as δ values (ppm) relative to the solvent line of CD_3OD (3.34 ppm for ^1H; 49.0 ppm for ^{13}C).

4-(3,4-dihydroxybenzylidene)-2-(3,4-dihydroxyphenyl)-5-oxotetrahydrofuran-3-carboxylic acid **P1**, ^1H NMR (CD_3OD, 400 MHz): δ_H 7.50 (d, *J* = 1.9 Hz, H-7'), 7.22 (d, *J* = 2.0 Hz, H-2'), 7.09 (dd, *J* = 8.2, 2.0 Hz, H-6'), 6.83 (d, *J* = 8.2 Hz, H-5'), 6.76 (d, *J* = 8.1 Hz, H-5), 6.75 (d, *J* = 2.1 Hz, H-2), 6.69 (dd, *J* = 8.1, 2.1 Hz, H-6), 5.60 (d, *J* = 2.5 Hz, H-7), 3.94 (d, *J* = 2.3 Hz, H-8); ^{13}C NMR (CD_3OD, 100 MHz): δ_C 178.2 (C-9), 175.5 (C-9'), 149.4 (C-4'), 146.8, 146.7, 146.6 (C-3', C-3 and C-4); 139.9 (C-7'), 133.9 (C-1), 127.6 (C-1'), 125.7 (C-6'), 123.3 (C-8'), 118.3 (C-6), 117.8 (C-2'), 116.6 (C-5), 116.4 (C-5'), 113.5 (C-2), 84.8 (C-7), 58.7 (C-8).

4.3.8. Determination of antioxidant properties

Antioxidant activities of caffeic acid and the oxidation product were determined using methods: TEAC and DPPH assay.

4.3.8.1. Trolox equivalent antioxidant capacity (TEAC) assay

The ABTS radical scavenging activity of caffeic acid and the reaction product was determined according to the protocol by Re et al. (1999) with slight modifications. $ABTS^{\bullet+}$ solution was prepared 12–16 h before use by reacting 7 mM of ABTS salt with 5.95 U SLAC and then storing in the dark until the assay was performed. The $ABTS^{\bullet+}$ solution was diluted with methanol to give an absorbance (Libra S21 spectrophotometer, Biochrom, Cambridge, United Kingdom) of 0.70 ± 0.002 at 734 nm. Each sample (100 μL) prepared at different concentrations was mixed with 1100 μL $ABTS^{\bullet+}$ solution and the absorbance was read after 30 min incubation at 25°C.

4.3.8.2. 2,2'-diphenyl-1-picrylhydrazyl (DPPH) scavenging effect

DPPH was prepared by dissolving DPPH in methanol to a final concentration of 0.025 mg/mL. Test samples for both caffeic acid and the reaction product were prepared in methanol at various concentrations. To conduct the assay, 0.1 mL of test sample was mixed with 3.9 mL of DPPH. The mixture was shaken vigorously and incubated in the dark at 25°C for 60 min. After

incubation, the absorbance was measured by spectrometry at 517 nm. The EC_{50} of the test samples were determined by plotting the percentage remaining DPPH against sample concentration. Percentage remaining DPPH was calculated by calculating the percentage of the concentration of DPPH at T_{60} against DPPH concentration at T_0.

4.3.9. Physicochemical properties

The physicochemical properties of caffeic acid and the oxidation product were compared by investigating pH stability, temperature stability and photo-stability. The physicochemical properties were studied in hydro-alcohol solutions due to the solubility characteristics of caffeic acid, which is not soluble in aqueous medium. Quantification of both caffeic acid and the oxidation product was achieved by HPLC using standard curves plotted for both caffeic acid and oxidation product (Appendix 3).

4.3.9.1. pH stability

pH stability was investigated at pH 2.2, 5.5 and 7.5 following the protocol described by Amendola et al. (2010). The test samples were dissolved in buffer-alcohol solution (85:15 v/v) using 50 mM citrate-phosphate buffer at pH 2.2, 5.5 and 7.5 to a final concentration of 0.5 mM. The test flasks were covered with aluminum foil to eliminate light interference and were kept at 4°C for 30 days. Samples were taken for analysis at 5-day intervals.

4.3.9.2. Temperature stability

Storage temperature stability was analysed for 30 days at 4°C, 25°C and 37°C according to the method adopted and modified from Amendola et al. (2010). The test samples were dissolved in a hydro-alcohol solution of water and methanol (85:15 v/v) to a final concentration of 0.5 mM. The test flasks were covered with aluminum foil and kept at 4°C, 25°C and 37°C for 30 days. Samples were taken for analysis at 5-day intervals.

4.3.9.3. Photo-stability

Photo-stability was investigated using fluorescent light. The test samples were prepared by dissolving the compounds in a hydro-alcohol solution of water and methanol (85:15 v/v) to a final concentration of 0.5 mM. The test flasks were exposed to fluorescent light (Osram

L18W/640 with a luminous flux of 1200 lm, 18 W and luminance of 508 542 lx) for 30 days. Samples were taken for analysis at 5-day intervals.

4.4. RESULTS AND DISCUSSION

4.4.1. Structural characterisation of SLAC oxidation product

The SLAC catalysed oxidation of caffeic acid resulted in the formation of one major product (yield 32.8%) designated as P1 (Rf = 0.47), (Figure 21), (t_R = 8.886), (Figure 22a).

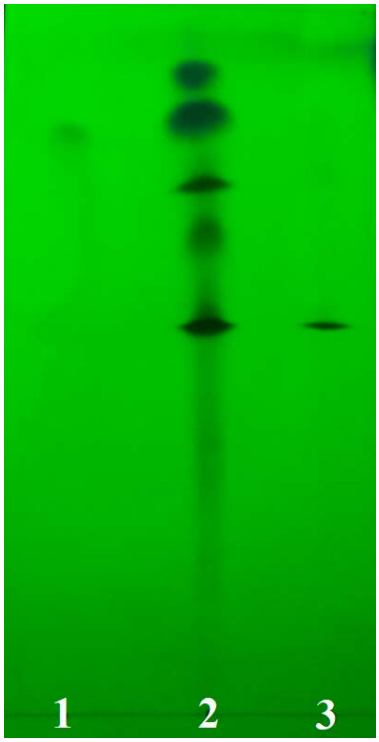


Figure 21: Separation of coupling products using TLC. Lane (1) is caffeic acid standard, (2) caffeic acid oxidation products (3) purified P1.

LCMS analysis conducted in the negative mode indicated that the product was a dimer *m/z* 357, [M] 358.07 (Figure 22b). The *in-situ* fragmentation pattern showed dominant signals at *m/z* 313; – 1 carboxyl group and *m/z* 269; – 2 carboxyl groups.

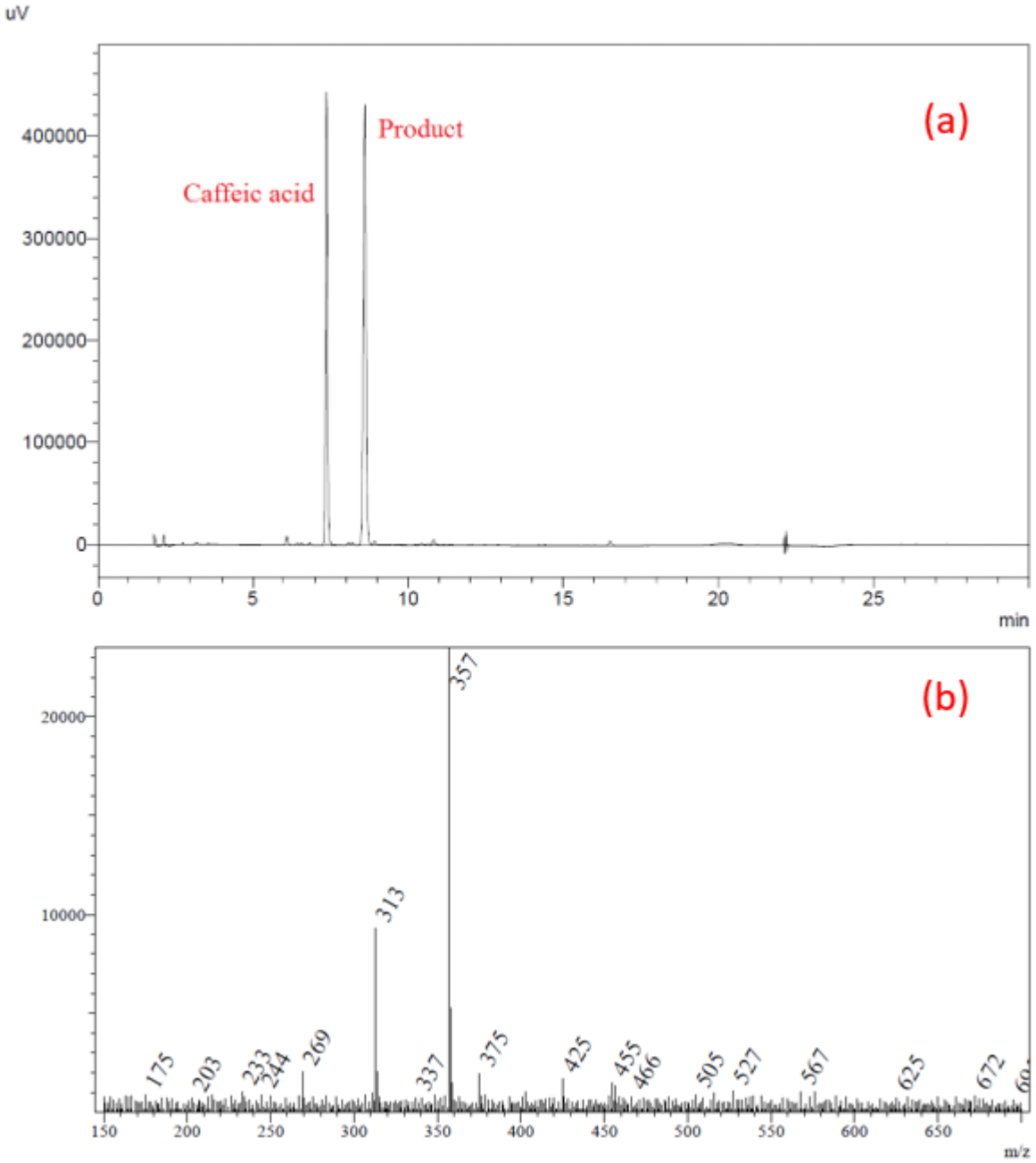


Figure 22: HPLC chromatogram of: (a) caffeic acid (t_R = 7.373) and product (t_R = 8.886) of SLAC-catalysed oxidation of caffeic acid, (b) mass spectra of SLAC oxidation product (P1).

The NMR results of P1 indicated that it was a dimer ((Z)-4-(3,4-dihydroxybenzylidene)-2-(3,4-dihydroxyphenyl)-5-oxotetrahydrofuran-3-carboxylic acid) of caffeic acid through a *β-β* linkage (Figure 23). Since the molecule was not symmetrical, the resonances for each of the aromatic protons in the rings could be seen separately. *Ortho* coupling was seen for H-5 and H-6 at δ_H 6.76 (d, J = 8.1 Hz) and δ_H 6.69 (dd, J = 8.1, 2.1 Hz) and H-5' and H-6' at δ_H 6.83 (d, J = 8.2 Hz) and δ_H 7.09 (dd, J = 8.2, 2.0 Hz). H-6 and H-6' were *meta* coupled to H-2 and H-2' respectively at δ_H 6.75 (d, J = 2.1 Hz) and 7.22 (d, J = 2.0 Hz). The olefinic H-7' resonance was

deshielded at δ_H 7.50 appearing as a doublet with J = 1.9 Hz due to long range coupling with H-8, which appeared as a triplet at δ_H 3.94 (J = 2.3 Hz) due to the double doublet coalescing. H-7 seen at δ_H 5.60 as a doublet with J = 2.5 Hz. The small J value of H-7 and H-8 indicated that the dihedral angle was approaching 90°. The carbon resonances for the two carbonyl groups could be seen at δ_C 178.2 (C-9) and 175.5 (C-9'), assigned due to HMBC correlations with H-7 and H-8 (for C-9) and H-7' (for C-9'). C-4' was distinctly separated from the other aromatic oxygenated resonances at δ_C 149.4, and assigned due to HMBC correlations with H-2', H-6' and H-5'. The other three aromatic C-O resonances, C-3', C-3 and C-4 appeared at δ_C 146.8, 146.7 and 146.6 and can be interchanged. Both C-1 and C-1' at δ_C 134.0 and 127.6 were identified by HMBC correlations with H-5 and H-5' respectively. The remaining singlet carbon resonance at δ_C 123.3 was assigned to C-8'. For details of ^{1}H and ^{13}CNMR, COSY, HSQC and HMBC spectrum, please refer to Appendix 4a-h.

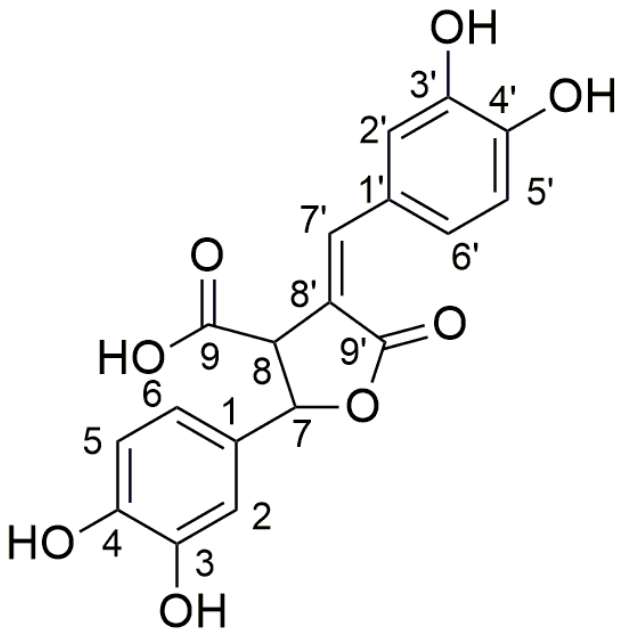


Figure 23: The *β-β* linked dimer (phellinsin A) formed during SLAC-catalysed oxidation of caffeic acid. Oxidation reactions were carried out in buffer-organic solvent systems using 50 mM sodium phosphate buffer (pH 7.5) and varying concentrations of selected organic solvents. Dimer was purified using preparatory thin layer chromatography.

The dimer has been reported in culture broth of *Phellinus* sp. and was identified as pellinsin A (Hwang et al., 2006) and has also been previously synthesised from *Lycopus europaeus* catechol oxidase (Rompel et al., 1999) and the cis form from *Momordica charantia* peroxidase-catalysed processes (Wan et al., 2008c). However, the SLAC-catalysed process may potentially be the

cheapest method compared to previously described protocols (Rompel et al., 1999, Wan et al., 2008c) which employed plant-derived biocatalysts thus making the processes potentially cost-intensive. In addition, the dependence of peroxidases on H_2O_2 makes the synthetic process expensive as well as a health hazard (Burton, 2003b).

Caffeic acid is one of the putative lignin monomers. Although *β*-O-4 linkages are the predominantly formed linkages in polymer formation in nature (Kandanarachchi et al., 2002), it has been observed that dimerisation usually results in *β*-5 and *β*-*β* linkages (Vanholme et al., 2010). The suggested mechanism for the formation of the *β*-*β* dimer is shown in Figure 24 (Rompel et al., 1999, Wan et al., 2008c). A radical is formed from the SLAC oxidation of the hydroxyl group occupying the *para* position of caffeic acid. The lone electron on the subsequent radical can occupy different positions through resonance stabilisation. C–C linkage of two phenoxy radicals with unpaired electrons at *β* positions results in the formation of the *β*-*β* caffeic acid dimer, phellinsin A. A similar reaction pathway was proposed by Adelakun et al. (2012b) for the formation of a laccase catalysed *β*-*β* ferulic acid dimer, suggesting that the *β*-*β* linkage is one of the most preferred radical coupling position for phenolic acids.

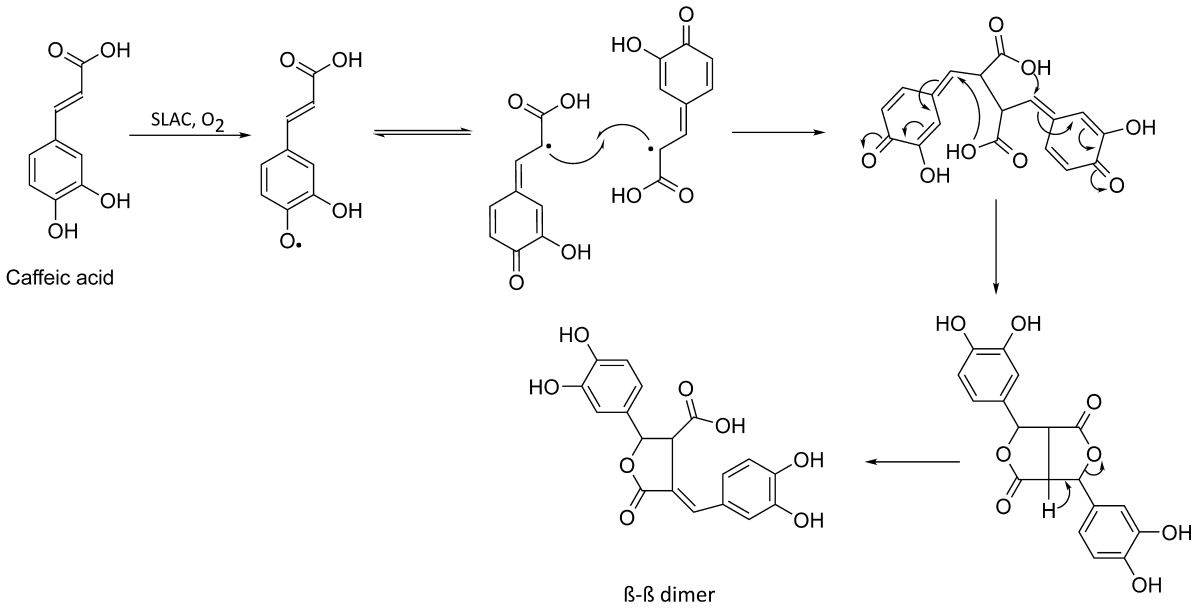


Figure 24: Proposed reaction pathway for the SLAC-catalysed synthesis of the *β*-*β* caffeic acid dimer. A C–C bond is formed between two *β*-positioned caffeic acid radicals. The intermediate compound undergoes rearrangement reactions to form the *β*-*β* dimer, phellinsin A.

4.4.2. Effect of solvent on the formation of P1

Laccase catalysis of phenolic compounds is usually done in buffer-organic reaction systems. This is because of the sparse solubility of most phenolic compounds (including caffeic acid) in aqueous media. It has also been observed that increasing solvent concentrations help minimise polymerisation (Adelakun et al., 2012a,b). For this study, four solvents; methanol, ethanol, ethyl acetate and hexane were investigated at four different solvent concentrations (50, 70, 80 and 90%) to determine the best solvent conditions for the production of phellinsin A.

Results indicated that there was an increase in product formation as the polarity of the solvent decreased; hexane> ethyl acetate > methanol > ethanol (Figure 25). There was a general increase in phellinsin A formation as the solvent concentration increased from 50% to 80%, after which a sharp decline in product formation at 90% co-solvent, was observed. Although 80% hexane resulted in the highest yield of phellinsin A, ethyl acetate which yielded the second highest concentration of phellinsin A, offered a cleaner reaction with less side reactions, thus making it easier for purification. Therefore, the scale up reactions were carried out using 80% ethyl acetate as co-solvent. It was observed that there was a significant loss of enzyme activity as the oxidation reaction progressed; by 90 min, 94.7% of activity was lost. Therefore, 0.64 U enzyme was periodically added at 90-minute intervals for the reaction to proceed. Organic solvents have been reported to affect laccase activity by direct interaction with the enzyme molecule or through modifying the thermodynamic water activity of the enzyme (Riva, 2006, Rodakiewicz-Nowak, 2000).

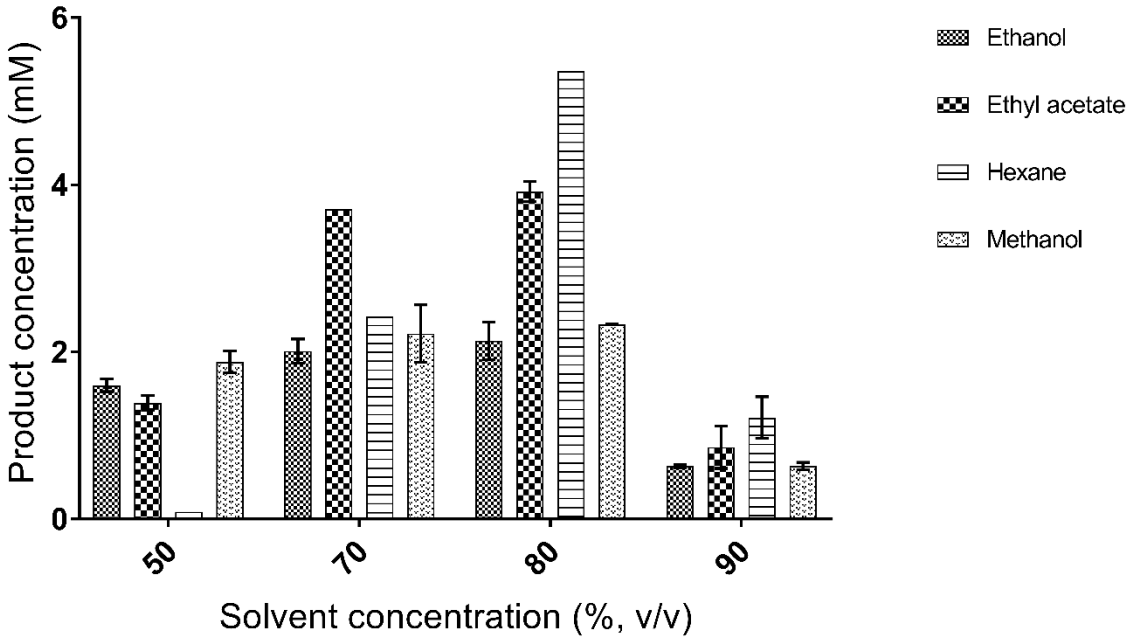


Figure 25: Effect of organic solvents on the synthesis of phellinsin A. Caffeic acid was oxidised by SLAC in either biphasic or monophasic reaction systems at varying concentrations of the test organic solvent (ethanol, ethyl acetate, hexane or methanol). Phellinsin A formation was monitored by HPLC analysis. All results are means ± standard deviation of three replicate determinations.

4.4.3. Effect of reaction time on phellinsin A yield

Substrate conversion to the dimer was monitored by HPLC over a 12 h oxidation period. Results showed that maximum dimer production was achieved at 6 h after which the yield started decreasing (Figure 26). There was a further decrease in the yield when the reaction was checked after 24 h. This may be due to a combination of non-specific side reactions as well as polymerisation reactions which are usually associated with laccase-mediated radical reactions (Kudanga et al., 2017).

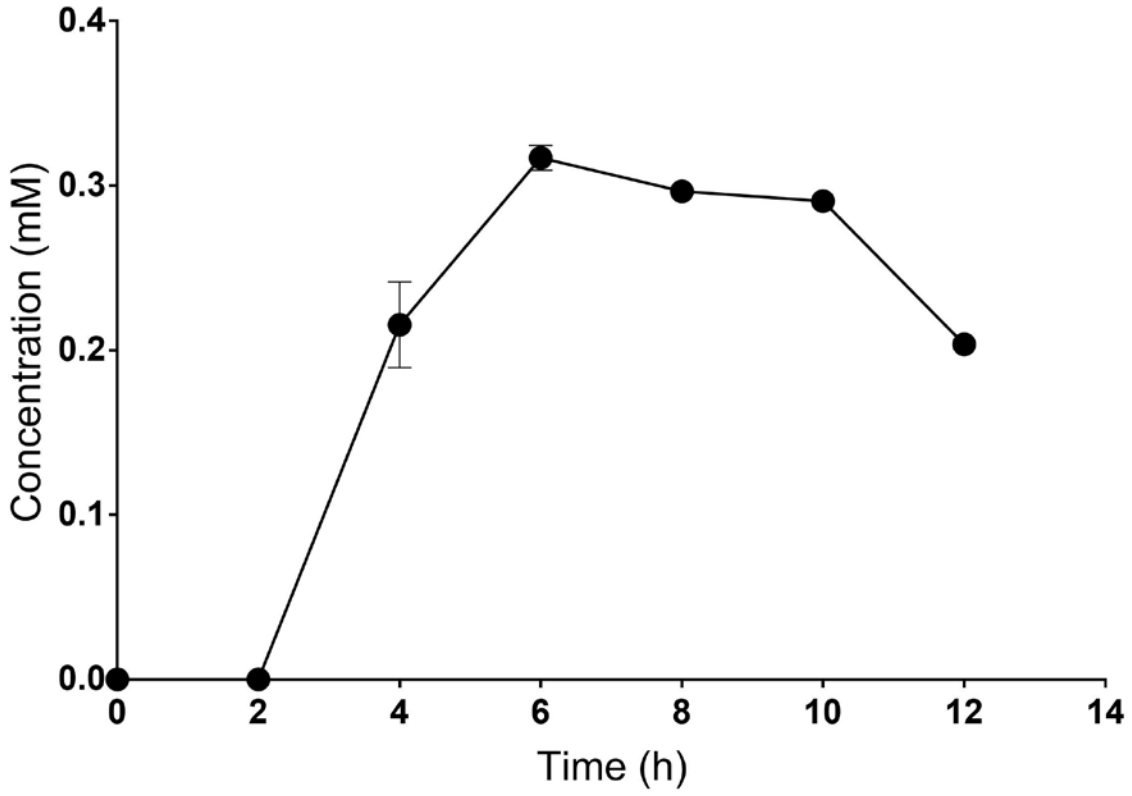


Figure 26: Effect of incubation time on dimer concentration during SLAC-catalysed oxidation of caffeic acid. All results are means ± standard deviation of three replicate determinations.

4.4.4. Antioxidant properties

Antioxidant capacity of phellinsin A was compared to caffeic acid using DPPH and TEAC assays. The DPPH and TEAC assays are based on the radical scavenging ability of an antioxidant to reduce $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ to their more stable states (1,1-diphenyl-2-picryl hydrazine and 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid, respectively) (Floegel et al., 2011). Both assays showed an improved antioxidant capacity of P1; 1.5-fold and 1.8-fold increase in antioxidant capacity for DPPH and TEAC assays, respectively compared to caffeic acid (Table 8). Studies of structure-activity relationships (SAR) of antioxidants have attributed the antioxidant capacity of a compound to factors such as the availability of electron donating groups attached to the aromatic ring (Bendary et al., 2013, Kudanga et al., 2017) as well as ability to form stable radicals. Caffeic acid consists of two hydroxyl groups, as well as an unsaturated C=C bond in the side chain. Phellinsin A, on the other hand has four hydroxyl groups as well as the unsaturated C=C bond, which explains its improved antioxidant capacity. It has been reported that effective antioxidants are able to form stable phenoxy radicals through either hydrogen bonding (Amić et al., 2003) or delocalisation of lone electrons into the aromatic ring (Kudanga

et al., 2017, Marinova and Yanishlieva, 2003); since the structure of phellinsin A does not consist of adjacent aromatic rings which enable expanded electron delocalisation (Bendary et al., 2013), enhanced antioxidant activity may be attributed to increased number of hydroxyl groups which facilitates radical stability through hydrogen bonding.

Table 8: Antioxidant activity of caffeic acid and phellinsin A determined using DPPH and TEAC assays. All results are means ± standard deviation of three replicate determinations

Compound	Molecular weight (g/mol)	EC_{50} DPPH[a] (mM)	TEAC value[b] (mM)
Caffeic acid	180.16	0.44 ± 0.008	2.17 ± 0.060
P1	358.07	0.29 ± 0.004	3.88 ± 0.004

[a]EC_{50} value is defined as the concentration (mM) of substrate that results in 50% loss of $DPPH^{\bullet}$ (Adelakun et al., 2012a)

[b]TEAC value is defined as the concentration of Trolox (mM) with an antioxidant potential equivalent to 1 mM of compound under investigation (Adelakun et al., 2012a)

4.4.5. Physicochemical properties

Besides enhanced bioactivity, physicochemical profiles also qualify a compound for commercial development (Tietgen and Walden, 2013). Conditions such as pH, temperature, air and light were reported to affect stability of phenolic compounds (Pavlovska and Tanevska, 2013). Despite significant progress being made in laccase synthesis of bioactive compounds, comprehensive characterisation of products has been neglected. A literature survey of all laccase mediated coupling products synthesised since 2000 showed that only one article investigated physicochemical properties of the coupling products (Aljawish et al., 2014b). The extent of product characterisation has narrowly focused mostly on bioactivity (Table 9). However, an extensive analysis of coupling products is required for the constructive progression of laccase biotransformation technology. Therefore, the physicochemical properties of phellinsin A were investigated.

Table 9: A literature survey of the characterisation tests conducted on laccase coupling products reported since year 2000. The table only contains compounds synthesised as potential antioxidants

Substrate	Source of laccase	Product formed	Characterisation tests conducted	References
2,6 dimethoxyphenol	*Trametes pubescens*	2,6 DMP dimer	Trolox equivalent antioxidant capacity (TEAC); 1,1-diphenyl-2-picrylhydrazyl (DPPH) and the Ferric Reducing Antioxidant Power(FRAP) antioxidant assays	(Adelakun et al., 2012a)
Catechin	*Trametes versicolor*	Hydrophilic linear oligomers	DPPH and reducing power antioxidant assays	(Jadhav and Singhal, 2014)
(+)-Catechin	*Myceliophthora* sp.	Poly(catechin)	Xanthine oxidase inhibitory activity; Superoxide scavenging activity	(Kurisawa et al., 2003b)
Chitosan + caffeic acid/ gallic acid/ quercetin/ tannic acid	*T. versicolor*	Caffeic acid/ gallic acid/ quercetin/ tannic acid - functionalized chitosan	TEAC antioxidant assay; Antimicrobial activities	(Božič et al., 2012b, Božič et al., 2013, Božič et al., 2012a)
Chitosan + ferulic acid/ethyl ferulate	*Myceliophthora thermophila*	Ferulic acid/ethyl ferulate - functionalized chitosan derivatives	TEAC antioxidant assay; antimicrobial analysis; Rheological analysis; thermal stability; grafting stability; colour stability (CIELab system); cytotoxicity tests	(Aljawish et al., 2014b, Aljawish et al., 2014a)

Esculin	*T. versicolor*	Oligoesculin	DPPH antioxidant assay; xanthine antioxidant assay; solubility tests by computer modelling	(Anthoni et al., 2010)
Ferulic acid	*T. pubescens*	*β*-5 and *β-β* ferulic acid dimers	DPPH and TEAC antioxidant assays	(Adelakun et al., 2012b)
Hydroxytyrosol	*T. pubescens*	Dimer, oligomers and polymers of hydroxytyrosol	DPPH, FRAP and Low-density lipoprotein antioxidant (LDL) assays; cytotoxicity tests;	(Zwane et al., 2012)
Phenylpropanoids	*Rhus vernicifera*	Dehydrodiisoeugenol and pinoresinol	Only structural characterisation reported	(Wan et al., 2007)
Rutin	*M. thermophila*	Poly(rutin)	LDL antioxidant assay; solubility tests	(Kurisawa et al., 2003a)
Sesamol	*T. versicolor*	Sesamol trimer	Only structural characterisation reported	(Constantin et al., 2012b)
Silybin A	*T. versicolor*	Silybin A dimer	DPPH antioxidant assay	(Gavezzotti et al., 2014)
Silybin	*T. pubescens*	C-21-C-21’ and C-20-O-C-21’ dimers	Only structural characterisation reported	(Gažák et al., 2008)
Trans-resveratrol	*M. thermophila*	trans-dehydrodimer	DPPH antioxidant assay	(Nicotra et al., 2004)

Tyrosol	*Trametes trogii*	Tyrosol dimer	Antimicrobial tests, toxicity bioassays, phytotoxicity tests, insecticide activity tests	(Chakroun et al., 2013)

4.4.5.1. Phellinsin A solubility

Caffeic acid is not soluble in aqueous media; therefore, it was dissolved in at least 50% methanol for complete solvation at room temperature. However, phellinsin A was completely soluble in aqueous media at room temperature (Figure 27). This may be attributed to the increased number of hydroxyl groups, which improves polarity and enhances the compound's interactions with aqueous media. These observations agree with previous molecular modelling studies by Anthoni et al. (2010) who attributed the improved solubility of laccase-synthesised oligoesculin to increased hydrogen bond interactions. Solubility is an important factor in drug development since it can give a theoretical indication of the bioavailability of a compound *in vivo* (Khadka et al., 2014).

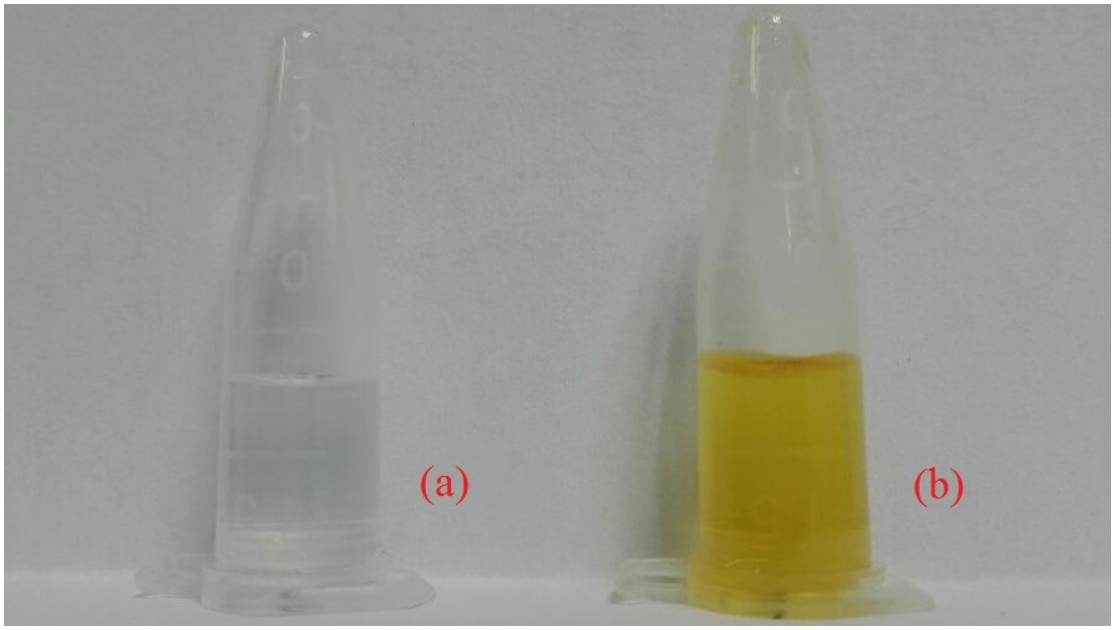


Figure 27: Caffeic acid (a) dissolved in 50% methanol solvent; the dimer (b) dissolved in water.

4.4.5.2. Temperature stability

Phellinsin A showed significant instability when incubated at human body temperature (37°C) in a hydro-alcohol solution of water and methanol (85:15 v/v). In the first 5 days of incubation, there was 63.6% loss of phellinsin A and by day 20, it was completely degraded (Figure 28). At storage temperatures (4°C and 25°C), phellinsin A exhibited relative stability, recovering 85.5% and 39.3% of the compound, respectively after 30 days of incubation. These results have shown that the concentration of phellinsin A decreased with increasing temperature. Despite phellinsin A exhibiting significant instability at body temperature over prolonged periods, it can still be an

effective antioxidant. Pharmacokinetic studies have shown that most phenolic compounds are metabolised within 24 h, after which the plasma concentration reaches basal levels (Karakaya, 2004, Bourne and Rice-Evans, 1998). At body temperature, about 60 – 80% of phellinsin A was retained during the first 24 h (Figure 28 and Appendix 6), when the compound is expected to be metabolised. Therefore, phellinsin A can be a useful antioxidant since it has shown relative stability at storage temperature (4°C) while exhibiting significant stability within the metabolism period. On the other hand, caffeic acid showed a contrasting trend; increasing stability as temperature increased. Caffeic acid exhibited the highest stability at 37°C and retained 76.5% of the molecule by day 30 (Figure 28). Previous studies on temperature–antioxidant activity of phenolic acids determined in triacylglycerols of sunflower oil showed that cinnamic acid derivatives including caffeic acid were more effective antioxidants at 90°C than at 22°C, owing to their higher stability factor at high temperatures (Marinova and Yanishlieva, 2003, Réblová, 2012).

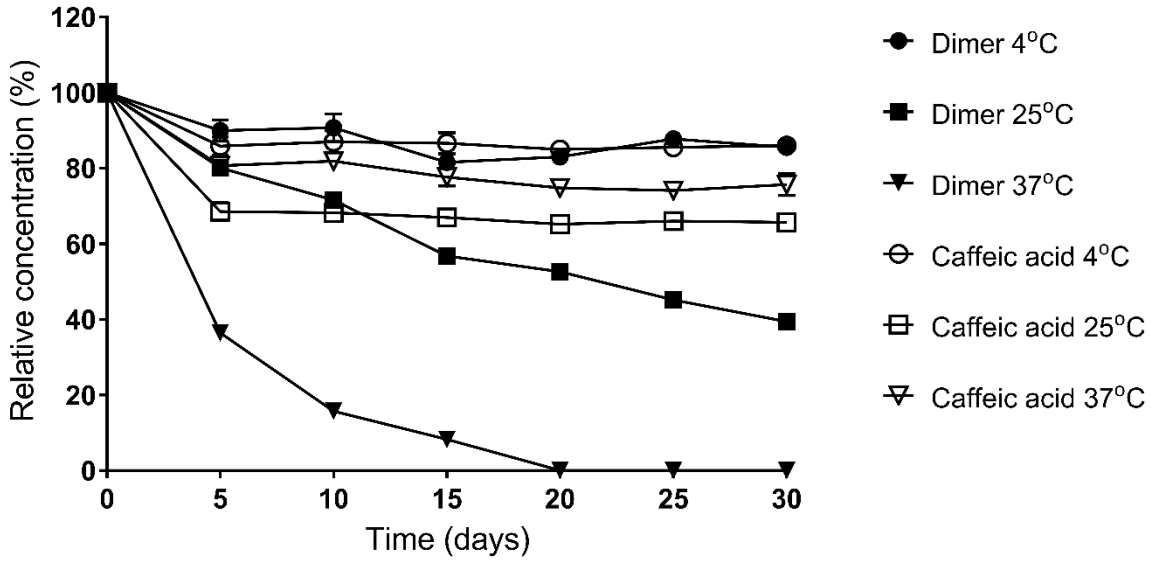


Figure 28: Stability profile of caffeic acid and phellinsin A at 4°C, 25°C and 37°C. Test samples were prepared in hydro-alcohol solutions and incubated in the dark at the investigated temperature for 30 days. All results are means ± standard deviation of three replicate determinations.

4.4.5.3. pH Stability

pH stability was investigated considering potential applications and the related environments. For instance, if phellinsin A is considered for the development of a nutraceutical, then it could be interesting to observe the effect of gastrointestinal pH (pH 2.2 for the stomach and pH 7.5 for alkaline intestinal conditions) (Evans et al., 1988), or if phellinsin A could be an ingredient in

development of topical products, then its stability at skin pH (pH 5.5) (Schmid-Wendtner and Korting, 2006) is important. pH stability tests were done at 4°C to reduce any microbial contamination which is common especially at acidic buffer conditions (Amendola et al., 2010). Results showed that the stability of the SLAC synthesised phellinsin A is pH-dependent. It showed remarkable stability in acidic conditions but was extremely unstable in alkaline conditions. At pH 5.5, it was completely stable; no compound loss observed after monitoring for 30 days. Therefore, the product may be considered for application in the development of skincare products. At pH 7.5 phellinsin A was completely degraded within 5 days of incubation (Figure 29). Similarly, previous studies on stability profiles of phenolic compounds have demonstrated that phenolic compounds such as caffeic acid, gallic acid, flavonoids, chlorogenic acid and green tea catechins are pH-sensitive; exhibiting greater stability in acidic pH and are unstable in alkali conditions (Chang et al., 2006, Friedman and Jürgens, 2000, Zhu et al., 1997). It has been suggested that the effect of pH on stability of a phenolic compound is related to the resonance stabilisation of its subsequent phenoxide ions which are mostly stable in acidic conditions than in alkaline medium (Friedman and Jürgens, 2000). It was therefore not surprising that the dimer with a higher electron density was more stable than the monomeric unit.

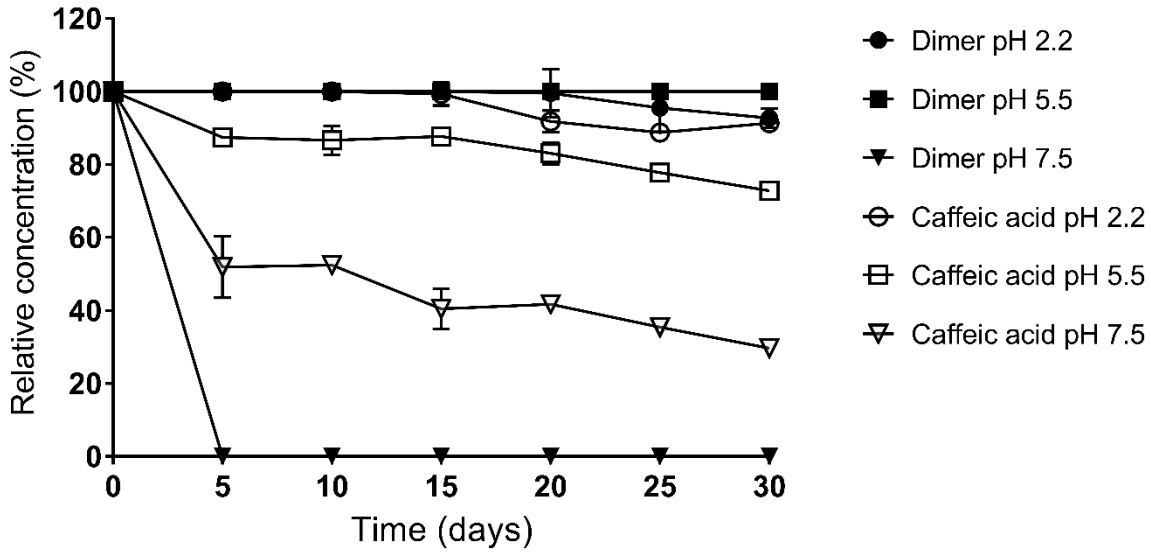


Figure 29: Stability profile of caffeic acid and phellinsin A incubated in citrate-phosphate buffer at pH 2.2, 5.5 and 7.5. Test samples were prepared in buffer-alcohol solutions at varying pH and were incubated in the dark at 4 °C for 30 days. All results are means ± standard deviation of three replicate determinations.

4.4.5.4. Light stability

Light significantly impacted the stability of phellinsin A with only 11.5% of the compound recovered after 30 days of incubation (Figure 30). Photo-stability tests were carried out in conditions that, to a large extent, simulated normal storage environments at room temperature, oxygen and under normal fluorescent light. Photodegradation of phenolic compounds is induced when valence band electrons are excited in the presence of oxidants such as molecular oxygen causing a compound to be more susceptible to oxidative degradation (Ahmed et al., 2010). The susceptibility of the phellinsin A to degradation was not surprising since previous investigations have shown that polyphenols are generally light sensitive (Volf et al., 2014, Munin and Edwards-Levy, 2011). Therefore, the compound may be most suitable for application in topical product formulations since it has shown the highest stability at pH 5.5 and significant light stability (approximately 80% retained within 24 h, Appendix 6) during the period in which it is expected to be metabolised. Due to the susceptibility of phellinsin A to light as well as high temperatures, the best storage conditions will be at 4°C in packaging material that excludes light (such as amber bottles).

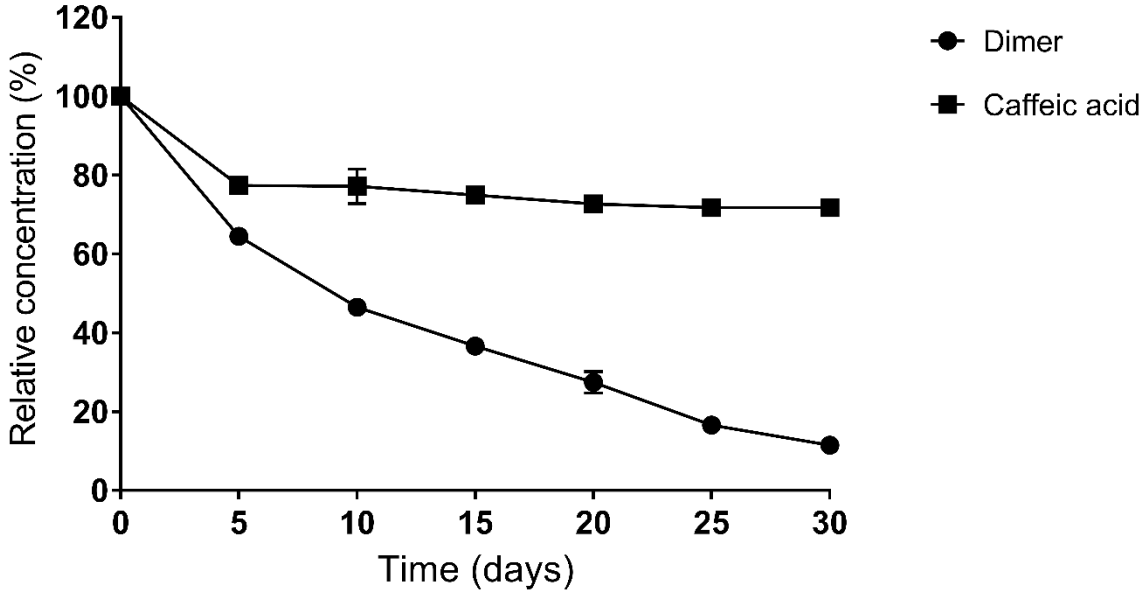


Figure 30: The photo-stability profile of caffeic acid and phellinsin A. Test samples were prepared in hydro-alcohol solutions and were exposed to fluorescent light for 30 days. All results are means ± standard deviation of three replicate determinations.

4.5. CONCLUSION

This study has reported, for the first time, the application of SLAC as a biocatalyst in the synthesis of antioxidants. A *β*-*β* caffeic acid dimer, phellinsin A was successfully synthesised and its bioactive and physicochemical properties elucidated. The dimer exhibits improved solubility properties and enhanced antioxidant properties compared to the parent compound, caffeic acid. This study indicates that SLAC could be a potential biocatalyst for the production of value added antioxidant products.

5. GENERAL DISCUSSION

The ability of laccases to oxidise a wide range of compounds make them important catalysts both in nature and for industrial applications. The biochemical and catalytic properties of laccases are based on their physiological roles in nature. For example, fungal laccases are important in fungi's ability to access nutrients. They generally have high redox potential which enables the enzyme to depolymerise complex natural polymers such as lignin. On the other hand, bacterial laccases are involved in anabolic processes such as sporulation, pigmentation and metal homeostasis (Sherif et al., 2013). Although Bugg et al. (2011) reported that laccases in *S. coelicolor* are involved in lignin depolymerisation, studies by Majumdar et al. (2014) showed that small laccases from *S. coelicor* were inefficient lignin degraders, and suggests that the depolymerisation of lignin may be possible with the aid of mediators.

Consistent with previous results by Machczynski et al. (2004) and Sherif et al. (2013), this study showed that SLAC has oxidase activity against a range of phenolic substrates including caffeic acid, 2,6-dimethoxyphenol, catechol, gallic acid, guaiacol, ferulic acid, and pyrogallol. However, in contrast to reports by Sherif et al. (2013), SLAC could not oxidise quercetin. These contrasting results may be due to the differences in reaction conditions; in this study, oxidation reactions were conducted at 25°C, pH 7.3 while Sherif et al. (2013) conducted their reactions at 60°C, pH 7.0. Related studies have shown that laccase reactivity on phenolic substrates can be enhanced by conducting reactions at enzyme optimum temperature; up to 96.3% substrate consumption was reported when phenols were oxidised by a *Paraconiothyrium variabile* laccase at its optimum temperature of 50°C (Asadgol et al., 2014). Sherif et al. (2013) indicated that at pH 7.0–10.0, there is 40-50% enzyme loss after 5 h of preincubation. Therefore, while employing high reaction temperatures may enhance enzyme reactivity, it may result in loss of enzyme activity due to heat denaturation. However, conducting laccase oxidation reactions at ambient temperatures will make the process more economically feasible than at higher temperatures.

Dimeric oxidation products for caffeic acid and gallic acid were successfully produced, purified and characterised. There was low substrate conversion into product, which resulted in 32.8% and 15% product yield for caffeic acid and gallic acid, respectively. It has also been observed from previous reports that the product yield from laccase coupling reactions are generally in the range of 10% - 40% (relative to starting material) (Adelakun et al., 2012a,b, Chirivì et al., 2012,

Gavezzotti et al., 2014, Zwane et al., 2012). This low product yield is caused by the non-specificity of laccase reactions. Although the generation of free radicals is vital for enabling coupling reactions, their subsequent coupling is difficult to control and usually leads to formation of a mixture of racemic products (Kudanga et al., 2017). Some researchers have reported the selective dimerisation of phenolic compounds achieved through protecting some reactive groups by either benzylation or methylation (Gavezzotti et al., 2014, Gažák et al., 2008). However, this adds process steps to the synthesis protocol, which may make the process complex and expensive. Therefore, other means of reaction engineering for optimum product formation may be required.

Reaction conditions for the maximum production of G1 and P1 were optimised by investigating the effect of solvent systems, reaction time and enzyme concentration. It was observed that product formation was higher in biphasic systems than in monophasic systems. Maximum product formation for G1 was achieved in 80% ethyl acetate while for P1, maximum yield was achieved in 80% hexane. Although hexane was the best solvent system for P1 formation, it was observed that ethyl acetate which produced the second highest yield, offered a cleaner reaction with less side reactions. These findings suggest that solvents play a pivotal role in laccase-catalysed coupling reactions. It has been observed in previous studies that different solvents may influence the formation of different coupling products. For example, Adelakun et al. (2012b) reported that the formation of a β-β ferulic acid dimer was favoured when ethanol was used as solvent in a monophasic system while a β-5 ferulic acid dimer was the primary product when ethyl acetate was used in a biphasic system. Organic solvents have been reported to contribute towards the selectivity of enzymes (Carrea et al., 1995). However, it is well known that laccases are non-selective enzymes with a wide range of substrates. Therefore, the influence of solvents in reaction selectivity may occur during the non-enzymatic phase of the reaction which includes resonance stabilisation of subsequently formed radicals, as well as radical-radical coupling (Adelakun et al., 2012b). This study has shown that ethyl acetate offers the best conditions for the conversion of caffeic acid and gallic acid into P1 and G1, respectively. Emerging reports are indicating that selective coupling in laccase-catalysed reactions may also be dependent on substrate structure (Llevot et al., 2016, Constantin et al., 2012a). For example, selective dimer formation was achieved when *ortho*-methoxy-*para*-substituted phenol substrates containing bulky/electron withdrawing moieties (such as nitrile, ketone or alkylene) on the *para* position were used; symmetrical dimers for 4-methylguaiacol, acetovanillone, eugenol, methylvanillate, vanillin and vanillonitrile were selectively produced with yields over 85% (Llevot et al., 2016).

Therefore, reaction engineering for selective product formation remains a subject of enquiry in organic synthesis.

Results from this study have confirmed reports from literature that organic solvents significantly affect enzyme activity (Riva, 2006). It was observed that the residual activity of SLAC was reduced by 94.7% after 90 min in 80% ethyl acetate. Theoretically, biphasic systems are expected to preserve the enzyme since there will be no direct interaction between the enzyme and the solvent. However, the significant decline in residual activity observed in this study indicated the negative impact of ethyl acetate on SLAC activity. Enzyme denaturation in organic solvents is caused by the destruction of the hydration shell which surrounds the protein molecule in aqueous medium, and as a result affecting the protein conformation (van Erp et al., 1991). Laccase stability, structure and activity can also be affected by organic solvents through the modification of thermodynamic water activity (Riva, 2006). Enzyme loss may be a concern on a commercial-scale where it would be ideal to retain the enzyme for recycling.

Laccase-catalysed coupling of monomeric phenolic units is aimed at enhancing the properties of the compounds, particularly antioxidant capacity. The antioxidant capacity of the coupling products (G1 and P1) were determined using standard antioxidant assays, DPPH and TEAC. The product P1 showed a 1.5-1.8 - fold increase in antioxidant capacity when compared to the substrate, caffeic acid. Extensive structural characterisation studies carried out through LCMS and NMR showed that the coupling reaction resulted in the doubling of hydroxyl groups which are known to increase the radical scavenging capacity of a compound (Kudanga et al., 2017). The increase in molecular size of P1 (358.07 g/mol) and the double phenolic ring system also facilitates the formation of stable phenoxy radicals by resonance stabilisation (Kudanga et al., 2017). On the other hand, the oxidation product of gallic acid resulted in a 50% decrease in antioxidant capacity. Results from the elementary analysis of G1 (Appendix 1) could suggest that the gallic acid oxidation process involved oxidative decarboxylation followed by a coupling reaction. The resulting oxidation product was most likely a diaryl ether joined by two C-O-C linkages; ethers are generally known as relatively inert compounds which explains why they are widely used as solvents (Macomber, 1996). From a commercial perspective, the production of a compound with poor properties is pointless. This may be avoided by the use of predictive approaches which may involve the screening of substrates for the identification of those that are likely to result in compounds with enhanced bioactivity. Usually, the structure of the parental compound may indicate the possible outcome of the coupling reaction. For example, based on

the structures of caffeic acid and gallic acid, predictions could be made and possible coupling products determined. Caffeic acid contains two hydroxyl groups; at the *meta* and *para* position relative to the C=C–COOH group. According to SAR studies, SLAC oxidation of caffeic acid will result in a non-reversible reaction to form a *p*-phenoxy radical. The lone pair on the subsequent radical may occupy different positions due to resonance stabilisation (Figure 31). Therefore, based on the position of the lone electrons, predictions can be made on the possible resultant coupling products; for example, *β*-*β* dimers, *β*-5 dimers, 5-5 dimers or *β*-O-4 dimers. From these possible products, it can be theoretically deduced if antioxidant properties will be enhanced. *β*-*β*, *β*-5, and 5-5 dimers are expected to have enhanced antioxidant capacity because of the doubling of hydroxyl groups. On the other hand, a decrease in antioxidant capacity is expected from the *β*-O-4 dimer since the coupling reaction proceeds at the expense of one hydroxyl group.

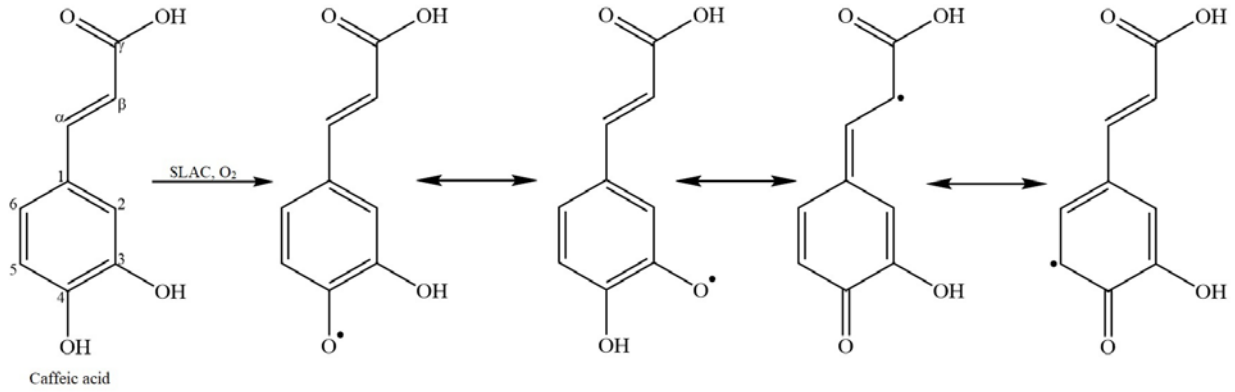


Figure 31: SLAC-catalysed oxidation of caffeic acid and the possible phenoxy radicals produced. Adapted from Adelakun et al. (2012b).

Similar predictions can also be made based on the structure of gallic acid and the possible coupling combinations that may occur (Figure 32). For instance, the presence of a COOH group at C1 may make it difficult for any bond formation at C2 and C6 due to stearic hindrance. Therefore, any possible radical-radical coupling is likely to result in the loss of a hydroxyl group, which may affect the antioxidant capacity of the resultant coupling product, as was observed in this study. Such prediction approaches (which may be aided by computer models) may be helpful in preventing the synthesis of a compound with less favourable properties.

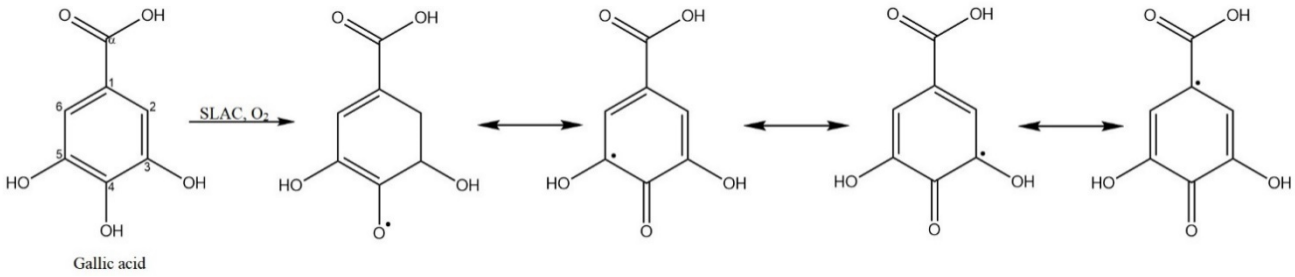


Figure 32: SLAC-catalysed oxidation of gallic acid and the possible phenoxy radicals produced. Adapted from Abdel-Hamid and Newair (2011).

Generally, the research has highlighted the potential of small laccase as a green biocatalyst for the synthesis of antioxidant compounds with enhanced properties. The ease of expression in prokaryotic model organisms such as *E. coli* is a major advantage for prokaryotic laccases since it enables the production of adequate enzyme for industrial application. The effect of organic solvents on laccases and low product yields have been common challenges in laccase-catalysed reactions. Future studies could possibly further elucidate kinetic and thermodynamic mechanisms involved in laccase interaction with organic solvents, and also give a better understanding on reaction engineering processes for improved yields.

6. CONCLUSION AND RECOMMENDATIONS

6.1. CONCLUSIONS

Laccase catalysis remains a promising and viable approach for the synthesis of antioxidants. Laccase is an ideal green catalyst for industrial application because of its clean reaction that utilises molecular oxygen, producing water as the only by-product. Most of the laccase antioxidant synthesis protocols have utilised fungal laccases, however, laccases are ubiquitous and are also present in bacteria. Bacterial laccases are interesting because of the possibility of heterologous expression in hosts such as *E. coli*. The aim of this study was to employ small laccase, a bacterial laccase native in *S. coelicolor* as a biocatalyst for the synthesis of new antioxidants. The objectives of the study included:

- To produce, purify and characterise the small laccase from *Streptomyces coelicolor* A3(2).
- To synthesise phenolic antioxidants through coupling reactions catalysed by SLAC.
- To optimise reaction conditions for coupling reactions through reaction engineering so as to maximise substrate conversion and product formation.
- To purify the coupling products and characterise their structure and antioxidant properties

These objectives were successfully accomplished. The major findings from the study can be summmarised thus:

- SLAC was successfully produced from *E. coli* BL21(DE) cloned with a pET-20b(+) vector containing the gene coding for SLAC (pSLAC). The enzyme was purified using $(NH_4)_2SO_4$ precipitation, ion exchange chromatography and size exclusion chromatography. The purified enzyme corresponded with enzyme reported by Machczynski et al. (2004) with a size of 32 kDa. The enzyme was successfully characterised, focussing on parameters that had not been exhaustively studied before (such as effect of metal ions)
- A substrate screening process was conducted on a range of phenolic compounds that were available. Caffeic acid, 2,6-dimethoxyphenol, catechol, gallic acid, guaiacol, ferulic acid, and pyrogallol were identified as SLAC substrates. SLAC's low redox potential of 0.5 V presents a major limitation in the application of the enzyme. This means SLAC can only oxidise phenolics with redox potential less than 0.5 V.

- Coupling reactions were carried out in monophasic and biphasic systems, using caffeic acid and gallic acid as substrates. Coupling products were successfully purified.
- The caffeic acid oxidation product was extensively characterised by LCMS and NMR. The oxidation product was determined by LCMS to be a dimer (*m/z* 357.07) and the structure of the dimer was elucidated by NMR as a *β-β* dimer. The dimer exhibited a 1.5-1.8-fold increase in antioxidant activity when tested using standard antioxidant assays (DPPH and TEAC assays). Extensive characterisation of physicochemical properties of the dimer showed that the dimer had improved solubility in aqueous medium and was stable in acidic conditions.
- The product of SLAC-catalysed oxidation of gallic acid was successfully produced, purified and partially characterised. Based on elementary analysis, the product suggested a diaryl ether joined by two C-O-C linkages. However, the oxidation product showed a 50% decline in antioxidant capacity as demonstrated by standard antioxidant assays (DPPH and TEAC), therefore no further structural and physicochemical characterisation were investigated.

Overall, the study has demonstrated the potential of bacterial laccases as biocatalysts for antioxidant synthesis. Two antioxidant compounds, a *β-β* caffeic acid dimer and a gallic acid diaryl ether were successfully produced. The study also explored the physicochemical properties of the synthesised *β-β* caffeic acid dimer, thus assessing its potential applicability as a commercial antioxidant. The study of physicochemical properties of coupling products has not been a popular research area, yet it is significant since it indicates the potential for industrial application. This study has shown the feasibility of using SLAC as a biocatalyst for the synthesis of new antioxidants.

6.2. RECOMMENDATIONS

6.2.1. General observations from the study and directions for future research

The use of laccases as biocatalysts offer economically viable domino processes for the synthesis of bioactive compounds. However, the translation of this green technology into a feasible industrial process requires several factors to be considered. For example, there is a need to develop a robust enzyme with properties that are ideal for industrial application. Specific research areas could include heterologous expression so as to produce enough enzyme with improved activity, thermostability and ability to withstand organic solvents and inhibitors which are frequently encountered in industrial applications (Mate and Alcalde, 2015, Kudanga and Le Roes-Hill, 2014, Kunamneni et al., 2008b). This study has demonstrated that high concentrations of organic solvents that are used to increase product yield affect enzyme activity. Therefore, reaction engineering to increase product yield remains a major challenge in laccase-mediated synthesis of bioactive compounds. However, other key research areas that need particular attention could include: (i) the search for cheap substrate sources, (ii) bioprospecting for laccase mediator systems to aid laccase functionality, (iii) production of enantiomerically pure products and (iv) enhancing the stability of resultant antioxidant molecules.

6.2.2. The search for cheap substrate source

Researchers pursuing the laccase-mediated biotransformation of phenolics with the aim of producing compounds of enhanced properties have mainly focused on phenolic compounds of natural origin. This is strategic since potential substrates can be easily found from the environment, thus making the technology economically feasible. For instance, since caffeic acid has been reported as a major phenolic acid found in potato peels (Chen and Ho, 1997), investigations can be made to determine whether the *β-β* caffeic acid dimer produced in this study can also be produced if potato peels were used as substrate source. Potato peels are considered a zero-value waste and are reported to present a waste management problem for European potato industries (Arapoglou et al., 2010). Therefore, laccase technology can in this case contribute towards the recycling of waste into value added commercial products.

6.2.3. Bioprospecting for natural laccase mediator systems (LMS) to aid laccase functionality

One of SLAC's major setbacks is its low redox potential. The study showed that SLAC can only oxidise phenolic compounds with a redox potential lower than 0.5 V. The study also showed that SLAC is incapable of oxidising high molecular weight phenolics such as quercetin and rutin possibly because their size restricts active site accessibility. These are major limitations since larger compounds cannot be oxidised by the enzyme. The challenges can be averted by employing laccase mediator systems (LMS). The potential of laccases can be extended beyond the oxidation of its natural substrates through LMS (Riva, 2006). This involves the generation of radicals from small compounds within laccase's redox potential range (Witayakran and Ragauskas, 2009). The generated radicals can then act as redox shuttles, oxidising substrates with higher redox potentials and those too large to fit the enzyme active site (Zhu et al., 2014). The LMS technology has been extensively used in the textile industry, pulp and paper industry, alcohol oxidation, and lignin degradation (D'Alfonso et al., 2014, Morozova et al., 2007, Fabbrini et al., 2002). Some researchers are of the opinion that the use of LMS presents an opportunity to mine the plethora of low molecular weight phenolics and other bioactive compounds entrapped within biopolymers such as lignin (Christopher et al., 2014, Rich et al., 2016). The degradation of lignin, which is the second most abundant biopolymer, and is laden with bioactive functional groups such as phenolic hydroxyls, benzyl alcohols, carbonyls and methoxyls (Boeriu et al., 2004, El Mansouri and Salvadó, 2007), can present a wealthy source of substrates for the synthesis of valuable bioactive compounds (Barclay et al., 1997, Božič et al., 2012a). The widely available artificial LMSs such as ABTS and 1-hydroxybenzotriazole (HBT) remain expensive and are potential contaminants when applied in the synthesis of compounds of therapeutic value such as antioxidants (Cañas and Camarero, 2010). Therefore, bioprospecting for more efficient natural mediator systems for the degradation of biopolymers also remains a key research area.

6.2.4. Towards the production of enantiomerically pure compounds

To date, much of the research on the exploitation of laccase for biotransformation has only produced racemic mixtures of oligomeric and cross-coupling products (which can explain the low product yield achieved in this study; 15% and 32.8% for gallic acid and caffeic acid respectively). This is a limitation especially in the synthesis of therapeutic drugs, which in most cases requires enantiomerically pure compounds. It has also been observed that enantiomers can

have significantly different bioactivities (Gavezzotti et al., 2014, Lee and Lui, 2003). Therefore, research is now also focusing on synthesising enantiomerically pure compounds (Gil Girol et al., 2012, Kim et al., 2012). Strikingly, *in vivo* laccase-catalysed coupling reactions are highly stereospecific, leading to the formation of compounds such as lignans, lignins and suberins (Orlandi et al., 2001, Zoia et al., 2008). Development of a protocol that can mimic the same specificity *in vitro* will be valuable in industrial processes. Research towards production of pure final products thus represents a primary focus area for future research. A number of studies have laid a foundation for future studies in this respect as explained below.

Davin and colleagues demonstrated the role played by a 78-kDa protein (dubbed "dirigent" protein) isolated from *Forsythia intermedia* in the *in vivo* synthesis of stereospecific dimers of E-coniferyl alcohol, which are building units for lignin polymers in plants (Davin et al., 1997). Coupling reactions carried out in the absence of the dirigent protein resulted in the racemic dimers (±)-dehydrodiconiferyl alcohols, (±)-pinoresinols, and (±)-guaiacylglycerol 8-O-4-(coniferyl alcohol) ethers (Davin and Lewis, 2005). However, in the presence of the dirigent protein, stereospecific coupling reaction occurred resulting in (+)-pinoresinol as the only product (Figure 33) (Davin et al., 1997, Davin and Lewis, 2000, Halls et al., 2004). This trend was reproducible when either laccase, flavin mononucleotide (FMN), flavin adenine dinucleotide (FDN), ammonium peroxydisulfate or an oxidase native to *F. intermedia* was used as oxidant, proving that stereoselectivity in the reaction was not promoted by the oxidant employed. The substrate specificity of the dirigent protein from *F. intermedia* restricts it only to the production of (+)-pinoresinol. This knowledge has already opened fresh avenues of enquiry, allowing scientists to bioprospect for their homologous proteins in nature (Präg et al., 2014, Pickel and Schaller, 2013, Gil Girol et al., 2012, Umezawa, 2003) as well as taking advantage of the modern-day tools such as molecular technology (Kazenwadel et al., 2013, Kim et al., 2012) to design modified proteins of such ilk that can control directed coupling to produce desired bioactive products.

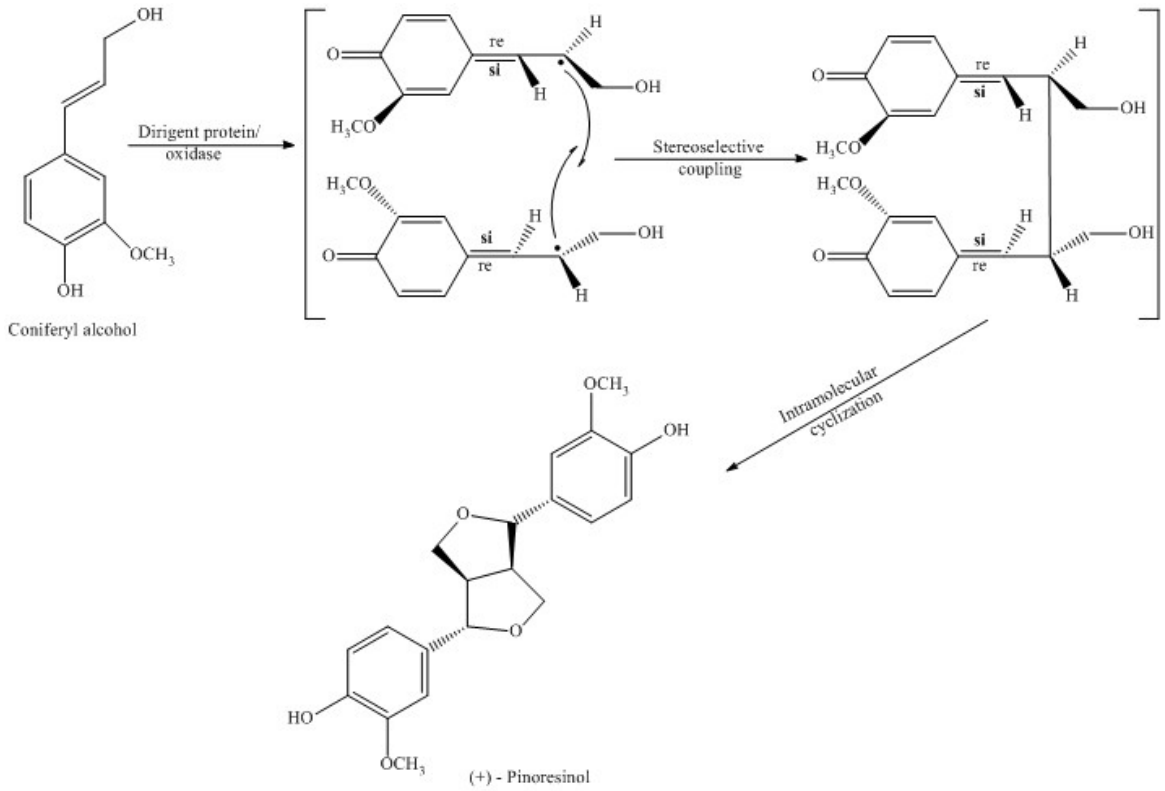


Figure 33: Dirigent proteins are responsible for the in vivo stereospecific synthesis of (+)-pinoresinol.

In related studies, stereospecific bioactive lignans were synthesised by attaching chiral auxiliary compounds to the substrates (Orlandi et al., 2001). Riva and coworkers have also carried out extensive research on protecting functional groups of laccase substrates as a strategy for reducing the diversity of products formed in the oxidation reactions (Gavezzotti et al., 2014, Gažák et al., 2008). A benzyl group was added to protect the OH group on the C´7 of silybin A resulting in the 87% yield of its symmetric dimer (Gavezzotti et al., 2014) (Figure 34). As shown by earlier studies, benzylation of functional groups seemed to be better than adding methyl groups which made deprotection impossible (Gažák et al., 2008).

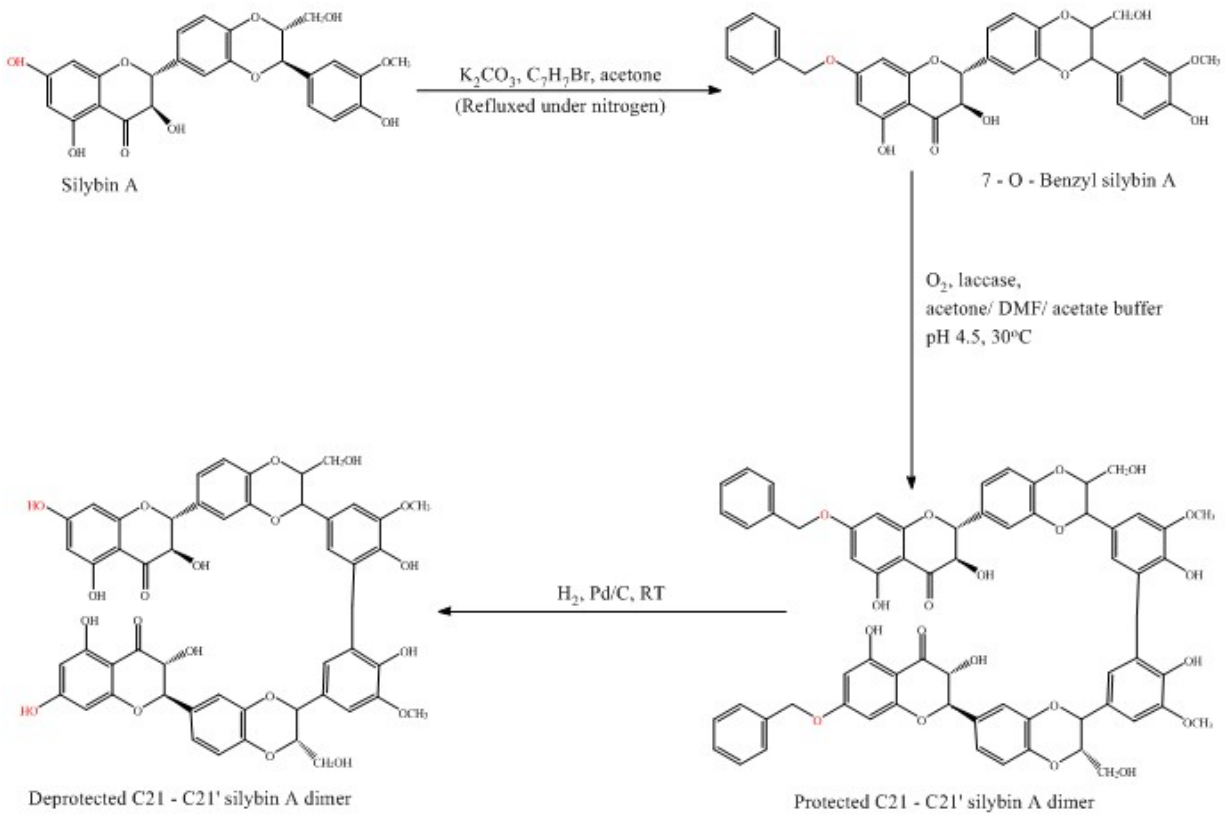


Figure 34: Protection of functional groups by either benzylation or methylation has been used to promote stereospecificity in laccase coupling reactions.

It has also been reported that regioselectivity can be influenced by the reaction conditions such as pH and solvents (Chioccara et al., 1993, Orlandi et al., 2001). Horseradish peroxidase-catalysed coupling of lignans (isoeugenol, methyl ferulate or coniferyl alcohol) under acidic pH resulted in dimer formation, neutral pH promoted the formation of oligomers while a racemic *β*–O–4 product was formed in the presence of methanol solvent (Chioccara et al., 1993).

The emergence and subsequent advances in the field of molecular biology has opened a host of opportunities in developing biocatalysts better equipped for industrial application. Besides the improved expression of proteins in heterologous hosts, molecular techniques also allow bioprospecting in unculturable microorganisms as well as database mining. Using bioinformatics databases, it is now possible to profile the sequence of the polypeptide chain. With this information, predictions can be made on how alteration of the amino acid sequence can affect the characteristics of the enzyme. Usually, these alterations are performed at or near the enzyme's catalytic core (Mate and Alcalde, 2015, Prins et al., 2015, Turner, 2009). However, in addition to carrying out these modifications with the goal of improving enzyme activity and/or

robustness, genetic manipulation could also focus on improving stereoselectivity and facilitating the production of pure compounds (Robert et al., 2011).

6.2.5. Towards the improvement of antioxidant stability

Although the study has shown that it is possible to enhance the bioactivity of natural phenolics through SLAC-catalysed coupling reactions, the purified oxidation products were unstable and susceptible to degradation by temperature, pH and light. One approach to enhance the stability of antioxidants is the application of nanotechnology through the entrapment of antioxidant molecules in nanoparticles (Shim et al., 2016). Mukherjee et al. (2009) generally defines nanoparticles as colloidal particles with the size of 10 – 100 nm. Nanoparticles have been lauded as effective carriers of entrapped bioactive compounds in dermal products (Dingler et al., 1999) as well as drug delivery systems *in vivo* (Genç et al., 2015). It has been shown that nanoencapsulation of antioxidant compounds in solid matrices such as chitosan nanoparticles or solid lipid nanoparticles (SLN) help protect antioxidants from chemical degradation by surrounding mediums as well as facilitating a controlled drug release at the target site (Bala et al., 2006, Osorio et al., 2010, Genç et al., 2015, Dingler et al., 1999). Nanoencapsulation can also help improve the shelf life, solubility and bioavailability of the laccase-synthesised antioxidant compounds (Shim et al., 2016). Natural polymers such as chitosan have been used as encapsulation material which can make the nanocapsules safe and the process economically feasible. Researchers who have investigated antioxidant nanoencapsulation have mostly worked on monomeric antioxidant compounds, however, the same technology can also be employed to improve the stability of laccase-synthesised antioxidant compounds such as the *β-β* caffeic acid dimer which has already shown improved bioactivity.

Such holistic future researches that focus on the synthetic properties of the enzyme, reaction engineering processes to optimise synthesis of specifically desired products with desirable physicochemical properties could possibly facilitate transfer of laccase catalysis technology from bench scale to industrial application processes.

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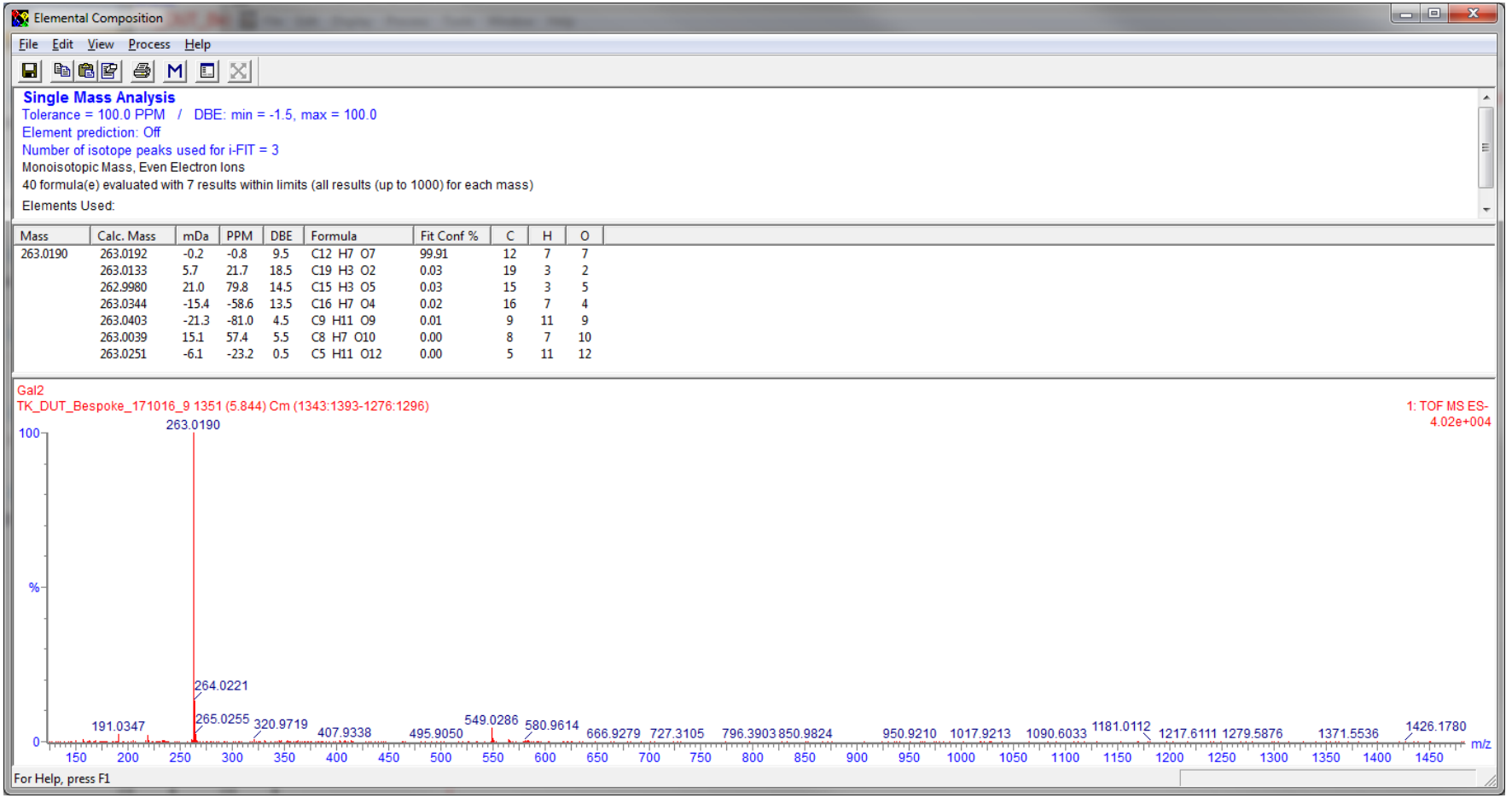
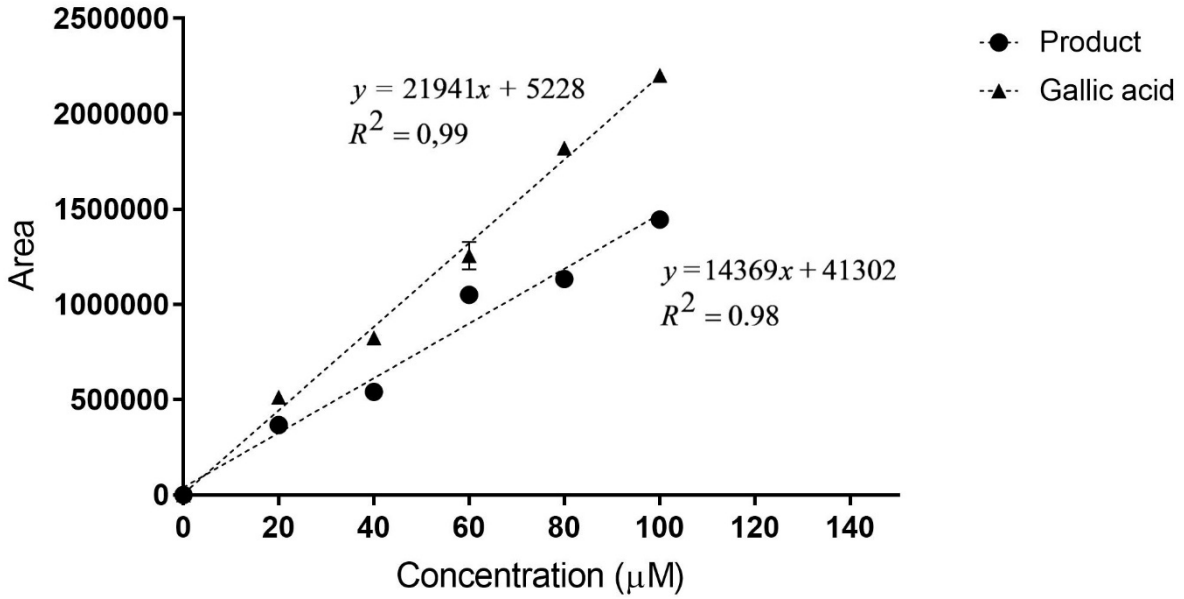
8. APPENDICES

APPENDIX 1. ELEMENTARY ANALYSIS OF GALLIC ACID OXIDATION PRODUCT (G1)

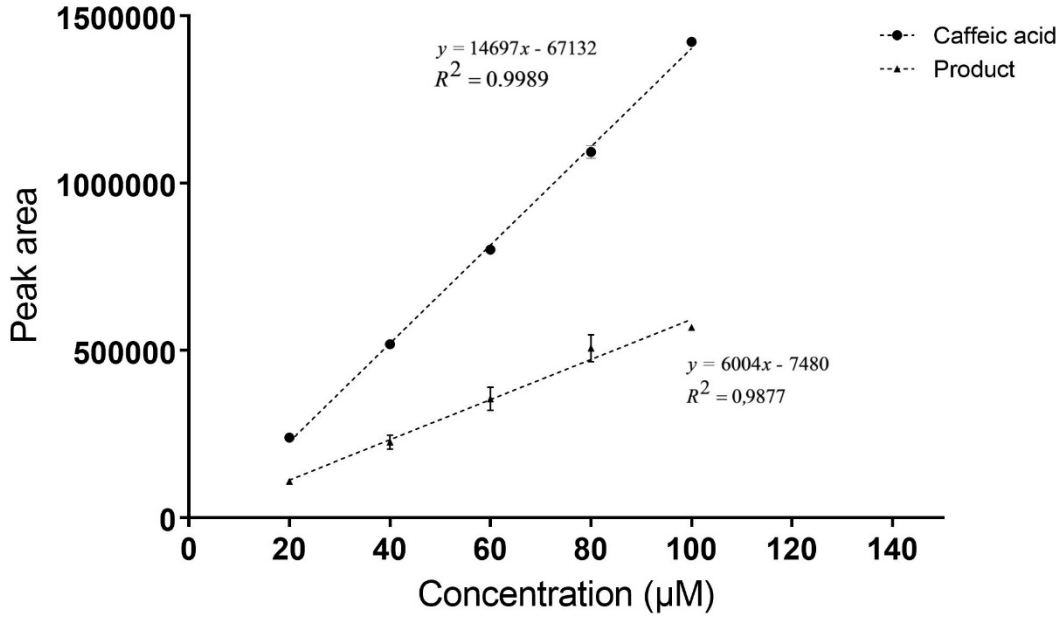


APPENDIX 2. STANDARD CURVE USED FOR THE QUANTIFICATION OF GALLIC ACID AND ITS COUPLING PRODUCT



Standard curve used for the quantification of gallic acid and its coupling product. All results are means ± standard deviation of three replicate determinations.

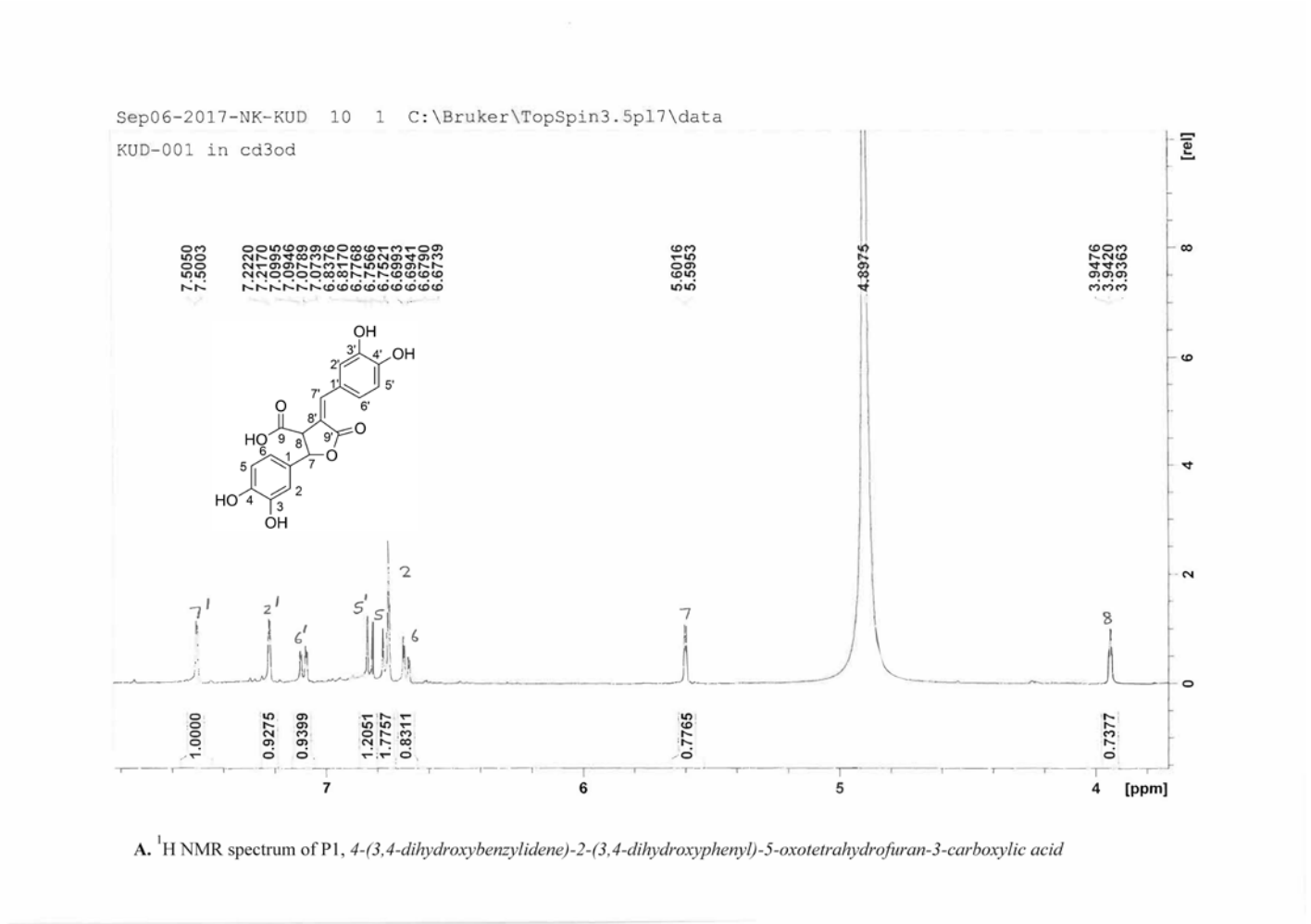
APPENDIX 3. STANDARD CURVE USED FOR THE QUANTIFICATION OF CAFFEIC ACID AND ITS COUPLING PRODUCT



Standard curve used for the quantification of caffeic acid and its coupling product All results are means ± standard deviation of three replicate determinations.

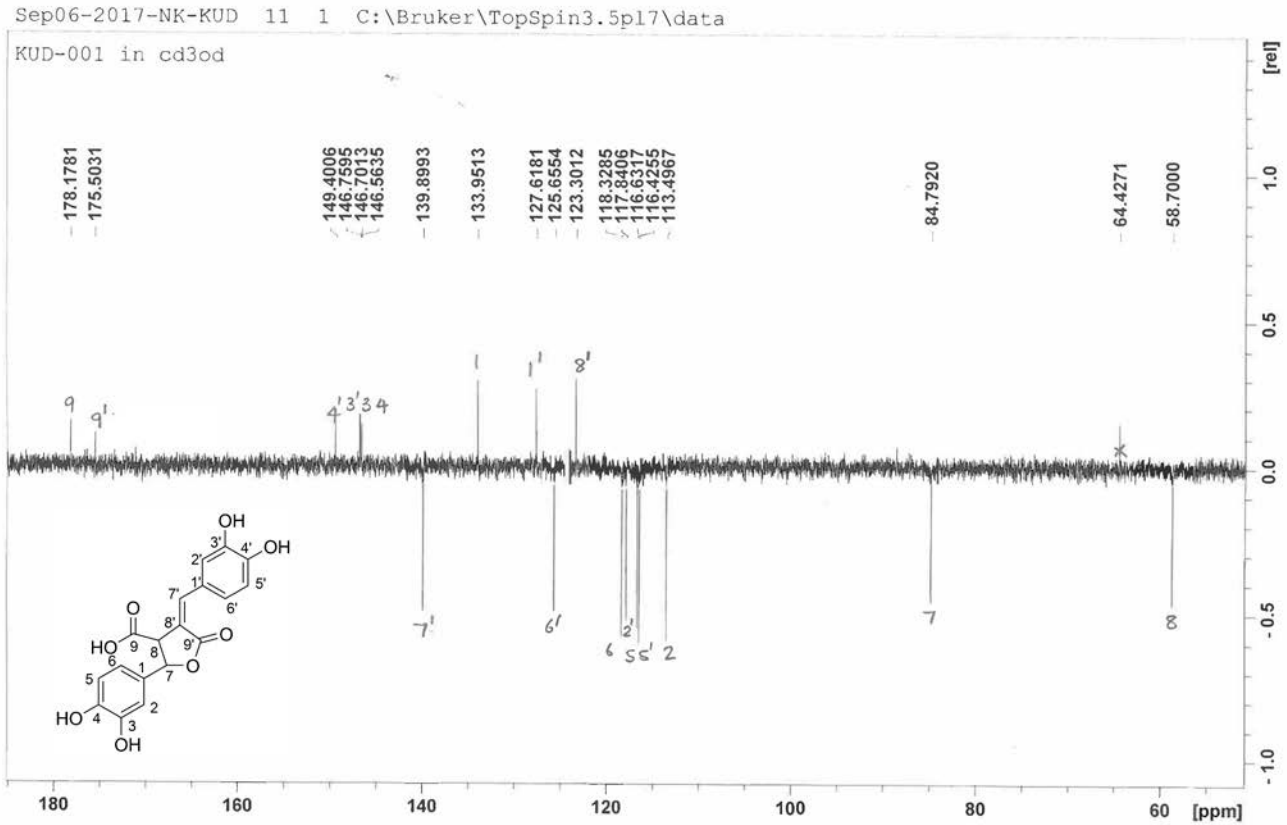
APPENDIX 4. NMR DATA FOR *β-β* CAFFEIC ACID DIMER

4a. ^{1}H NMR spectrum of *β-β* caffeic acid dimer



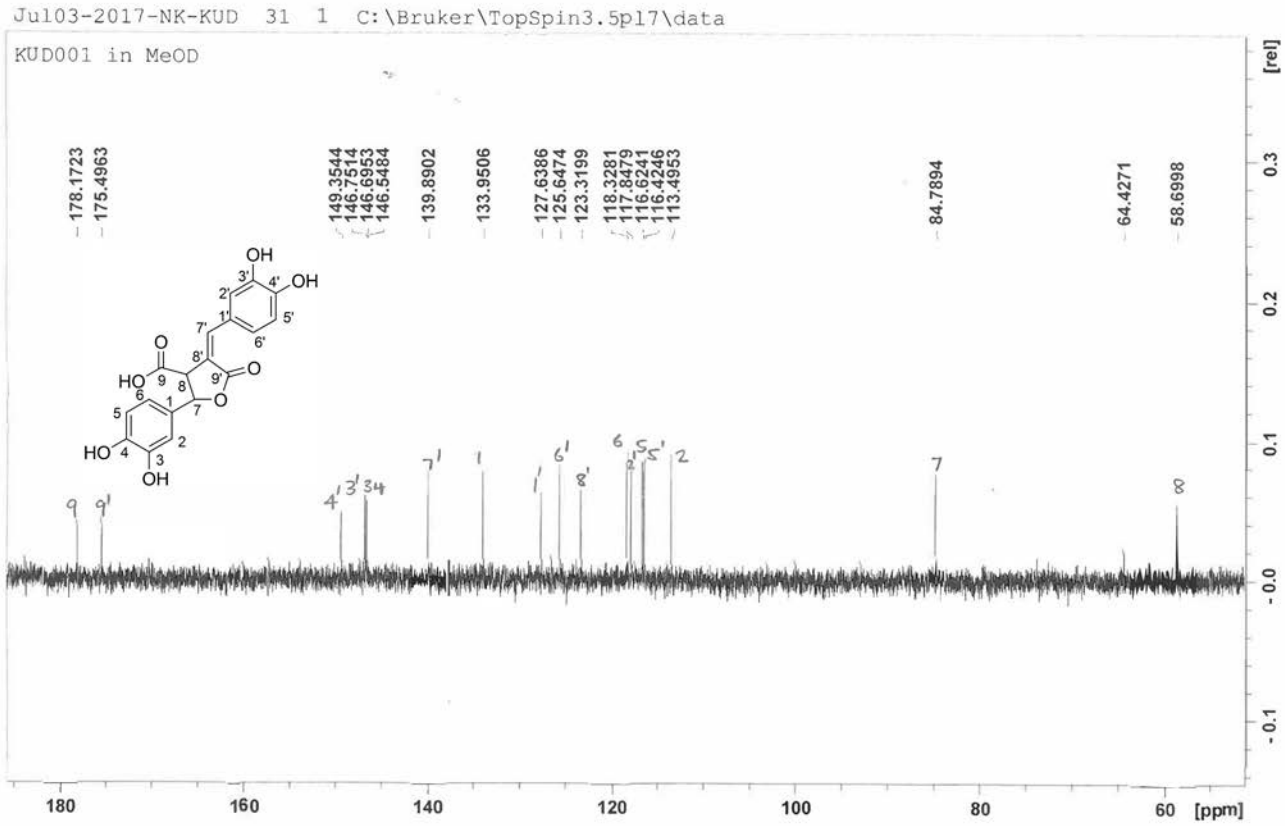
A. ^{1}H NMR spectrum of P1, *4-(3,4-dihydroxybenzylidene)-2-(3,4-dihydroxyphenyl)-5-oxotetrahydrofuran-3-carboxylic acid*

4b. ^{13}C NMR (APT) spectrum of *β-β* caffeic acid dimer



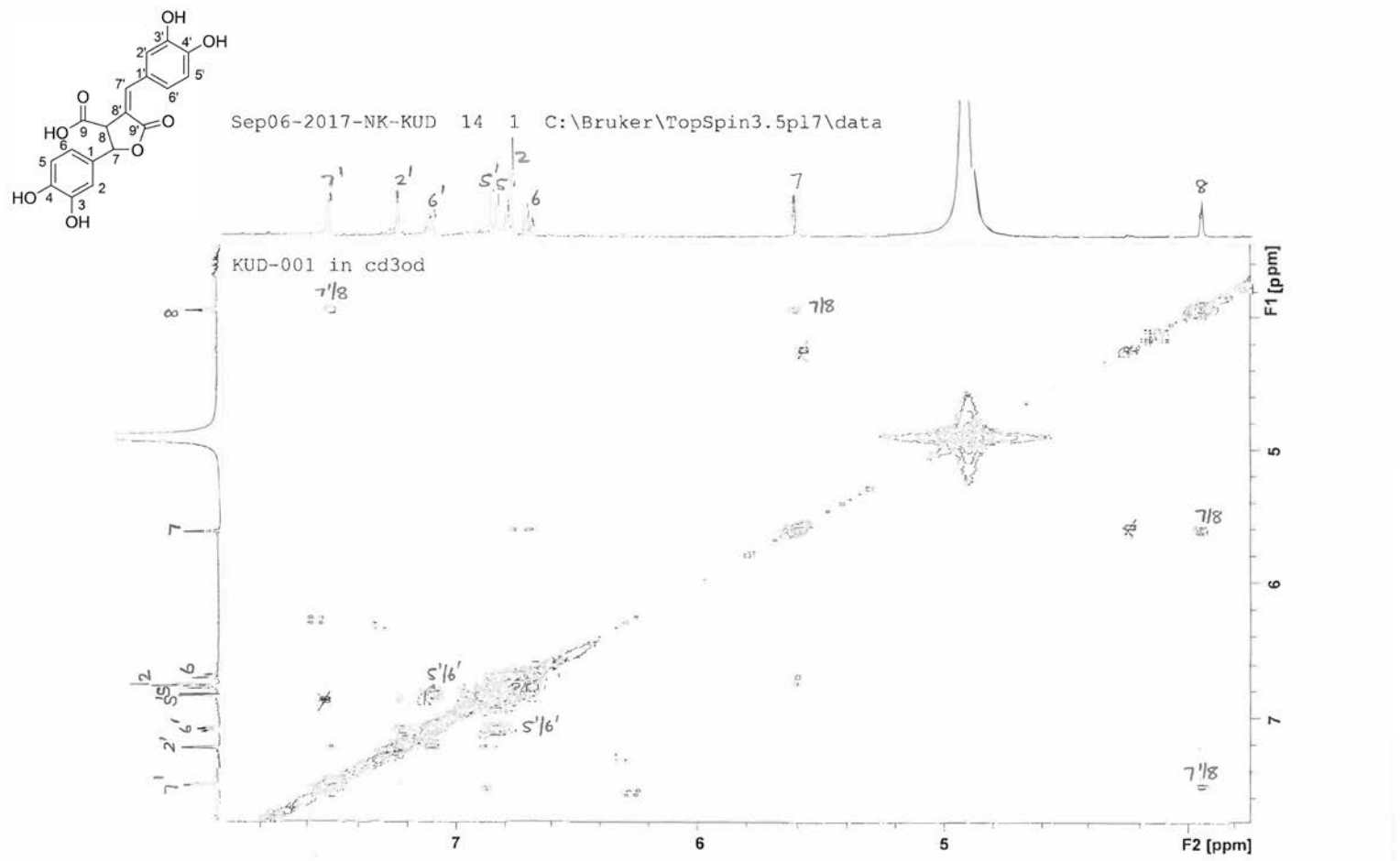
B. ^{13}C NMR (APT) spectrum of P1, *4-(3,4-dihydroxybenzylidene)-2-(3,4-dihydroxyphenyl)-5-oxotetrahydrofuran-3-carboxylic acid*

4c. ^{13}C NMR spectrum of *β-β* caffeic acid dimer



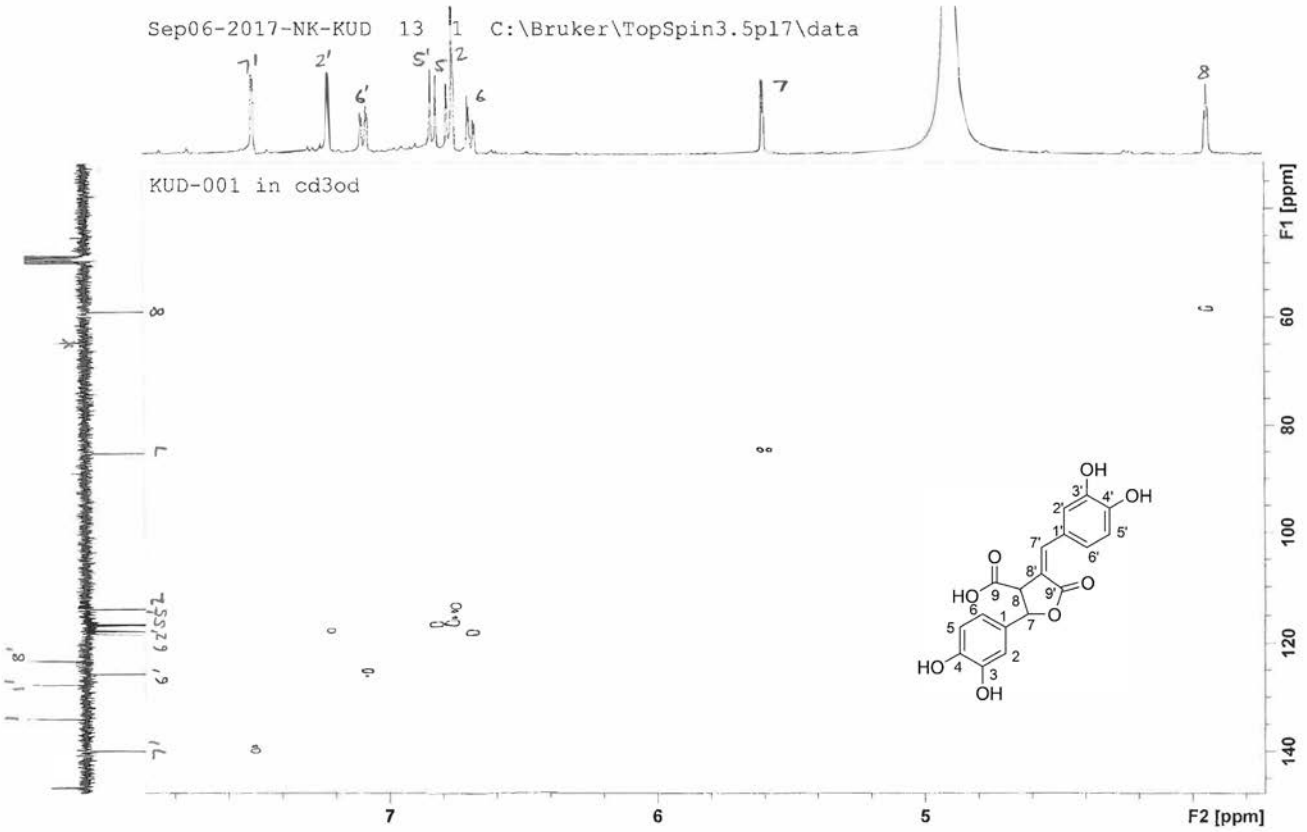
C. ^{13}C NMR spectrum of P1, *4-(3,4-dihydroxybenzylidene)-2-(3,4-dihydroxyphenyl)-5-oxotetrahydrofuran-3-carboxylic acid*

4d. COSY spectrum of *β-β* caffeic acid dimer



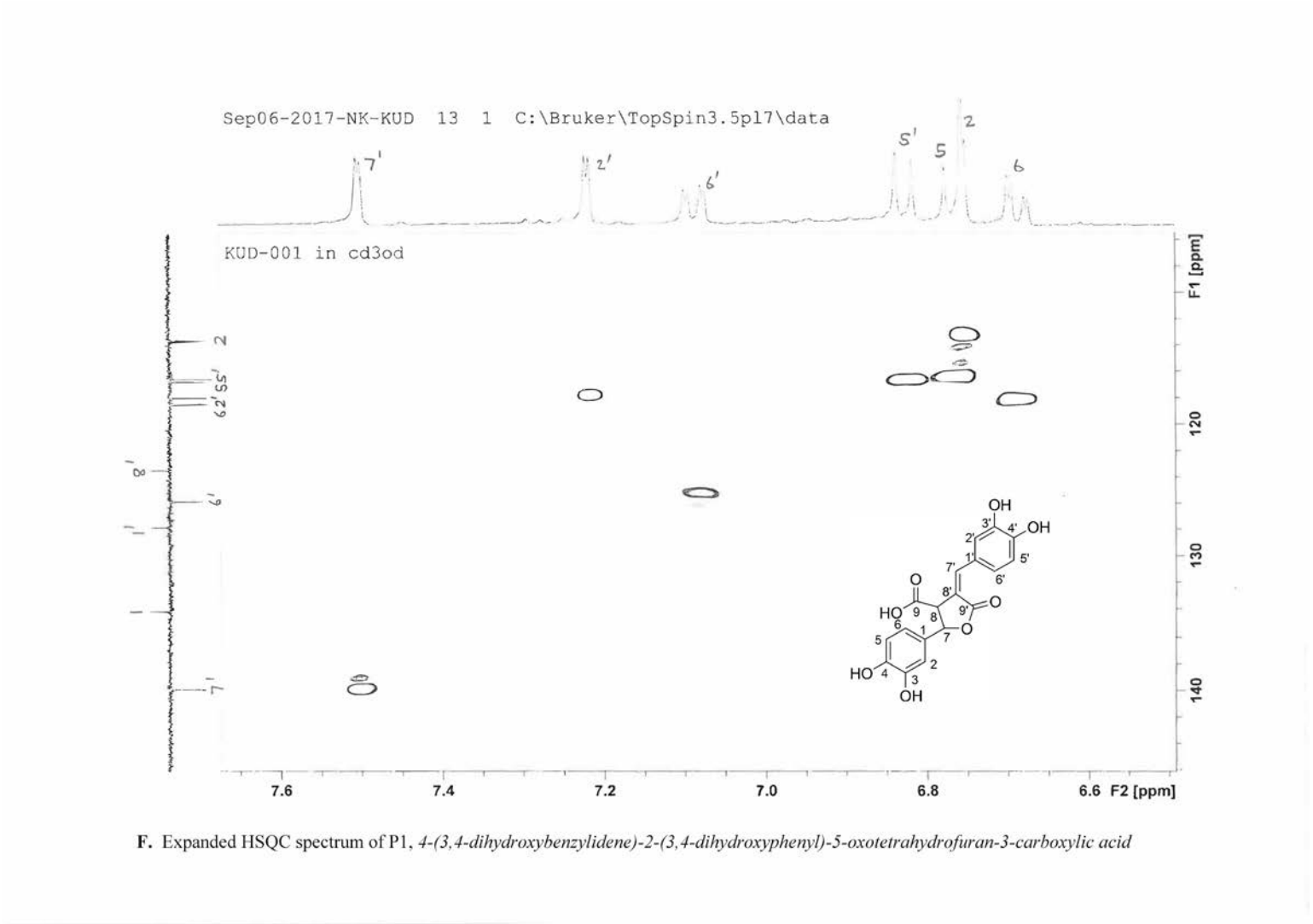
D. COSY spectrum of P1, *4-(3,4-dihydroxybenzylidene)-2-(3,4-dihydroxyphenyl)-5-oxotetrahydrofuran-3-carboxylic acid*

4e. HSQC spectrum of *β-β* caffeic acid dimer



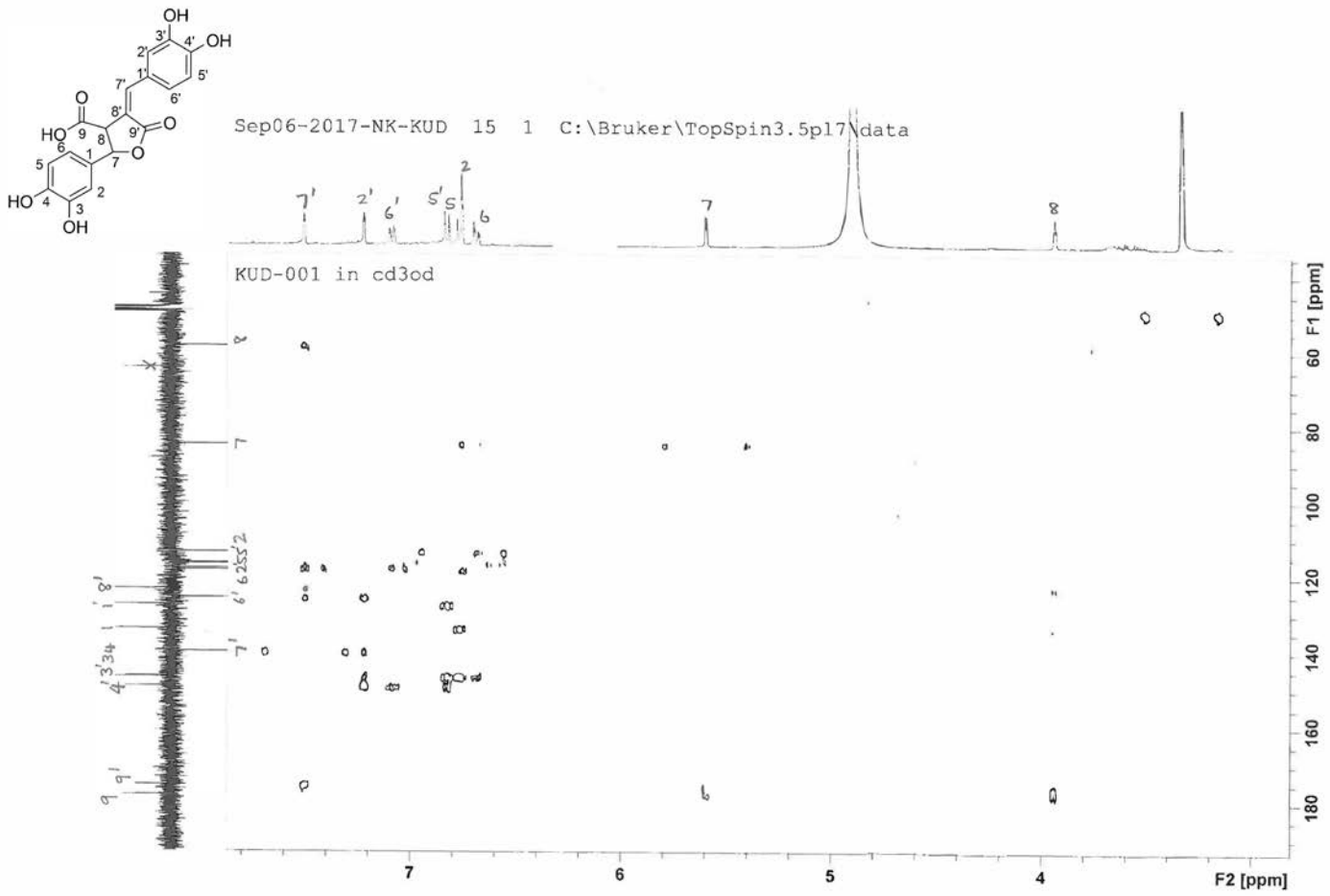
E. HSQC spectrum of P1, *4-(3,4-dihydroxybenzylidene)-2-(3,4-dihydroxyphenyl)-5-oxotetrahydrofuran-3-carboxylic acid*

4f. Expanded HSQC spectrum of *β-β* caffeic acid dimer



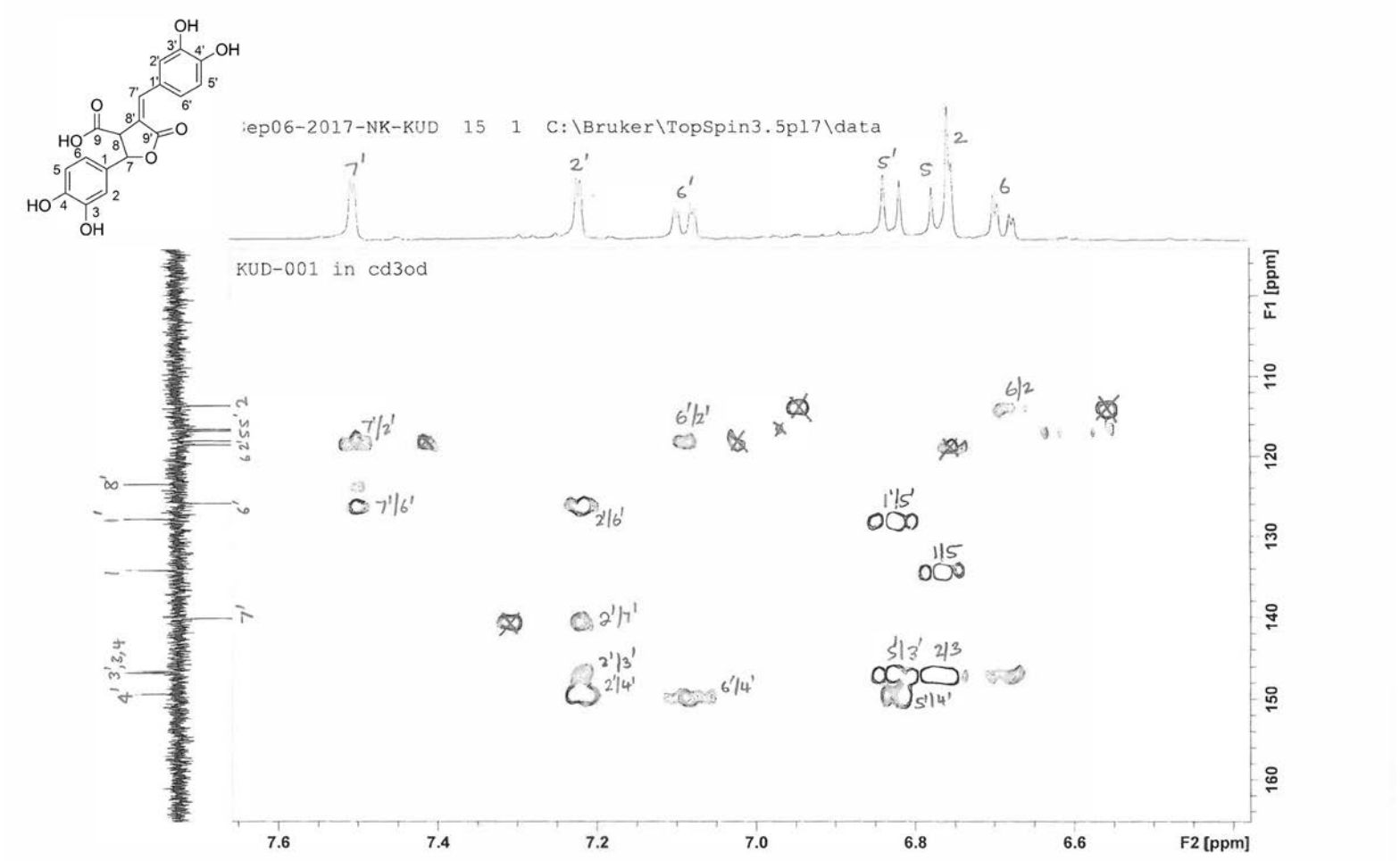
F. Expanded HSQC spectrum of P1, *4-(3,4-dihydroxybenzylidene)-2-(3,4-dihydroxyphenyl)-5-oxotetrahydrofuran-3-carboxylic acid*

4g. HMBC spectrum of *β-β* caffeic acid dimer



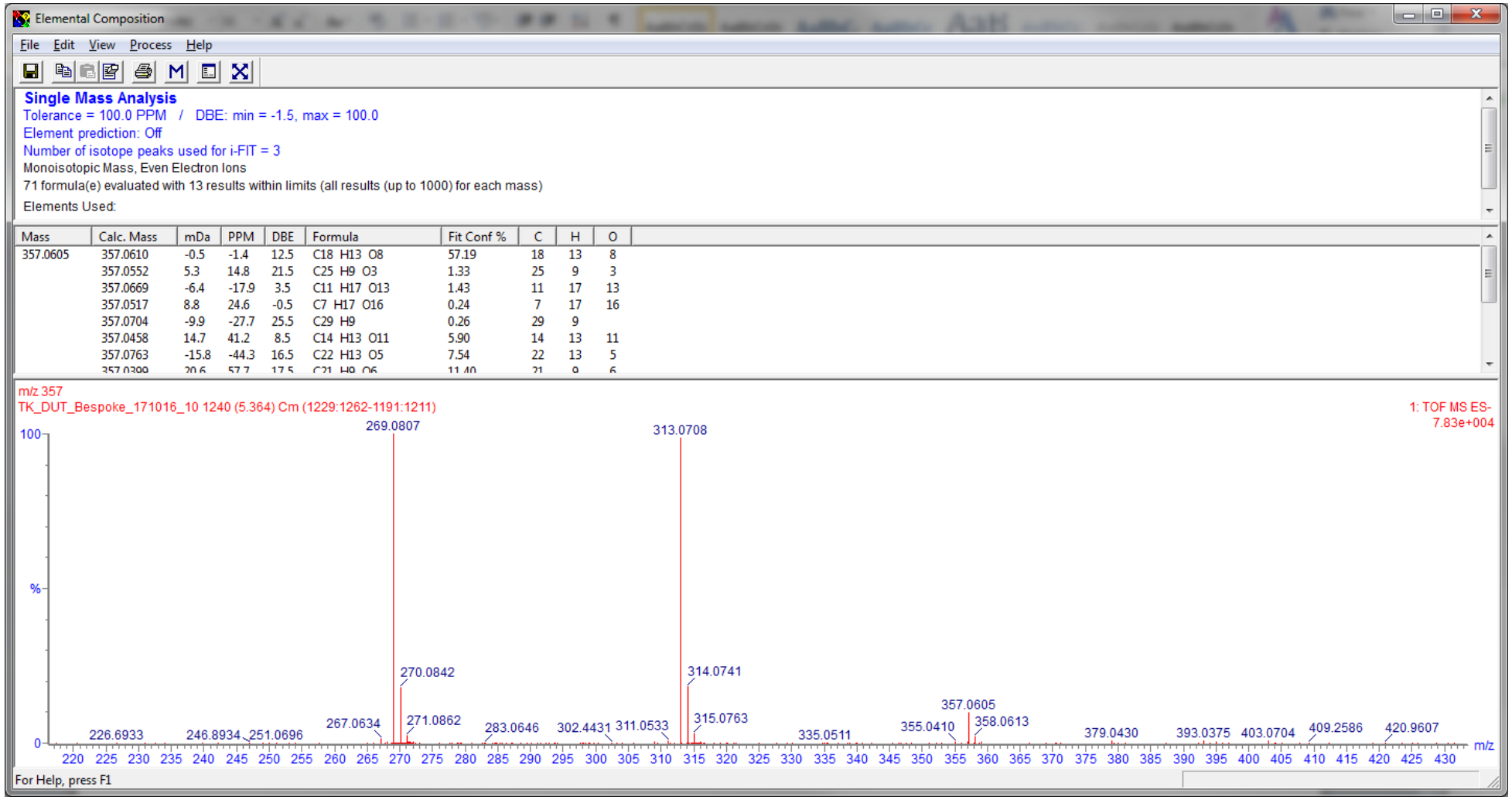
G. HMBC spectrum of P1, *4-(3,4-dihydroxybenzylidene)-2-(3,4-dihydroxyphenyl)-5-oxotetrahydrofuran-3-carboxylic acid*

4h. Expanded HMBC spectrum of *β-β* caffeic acid dimer

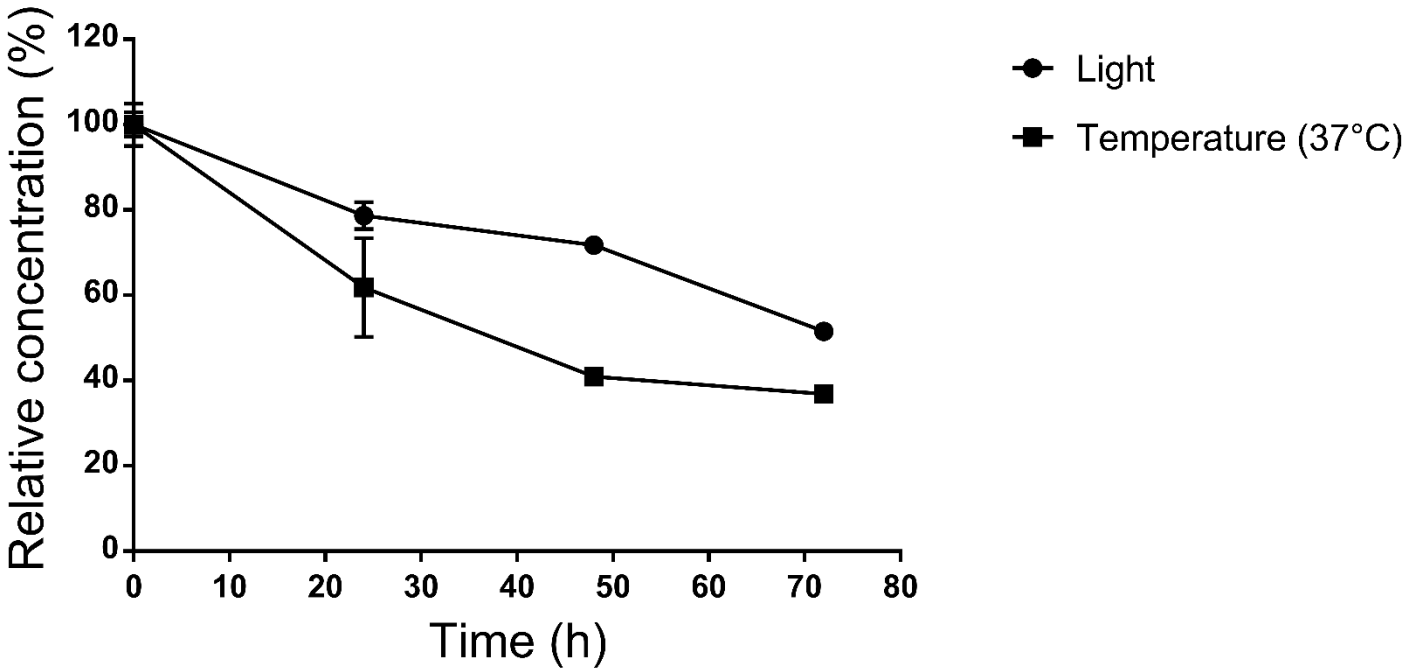


H. Expanded HMBC spectrum of P1, *4-(3,4-dihydroxybenzylidene)-2-(3,4-dihydroxyphenyl)-5-oxotetrahydrofuran-3-carboxylic acid*

APPENDIX 5. ELEMENTARY ANALYSIS OF CAFFEIC ACID OXIDATION PRODUCT (PHELLINSIN A)



APPENDIX 6. STABILITY PROFILES OF PHELLINSIN A INCUBATED AT 37°C OR UNDER FLUORESCENT LIGHT OVER A 72 H PERIOD.



Stability profiles of phellinsin A incubated at 37°C or under fluorescent light over a 72 h period. For temperature stability, test samples were prepared in hydro-alcohol solution and incubated in the dark at 37°C for 72 h. For light stability, test samples were prepared in hydro-alcohol solution and were exposed to fluorescent light for 72 h. All results are means ± standard deviation (SD) of three replicate determinations.