Faculty of Health and Life Sciences

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Phytochemical Investigation and Bioactivity Assessment of Medicinal Plant from Northern Nigeria

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy (Ph.D) in Natural Product Chemistry

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DEDICATION

I want to dedicate this work to HIM whose hands alone hold sustenance, blessings and mercy.

ABSTRACT

Boswellia dalzielii (Burseraceae) has ethnopharmacological importance and is claimed to have anti-infection and immunomodulatory effects. In the Northern part of Nigeria, a region with a tropical dry climate, an aqueous infusion of this plant is used in the treatment of infections and tumours.

The traditional formulation method was mimicked under laboratory conditions, and the effect of temperature and the impact of endophytic microbes present in aqueous infusion of *B. dalzielii* was also investigated. Activity-guided fractionation against *Staphylococcus aureus* and its methicillin-resistant strain resulted in the identification of two antibacterial compounds namely gallic acid and pyrogallol. The Minimum Inhibitory Concentration for pyrogallol and gallic acid against *S. aureus* growth are 508 and 753 µM, while against MRSA growth are 254 and 2032 µM, respectively. A growth Inhibition assay showed the activity of gallic acid as bacteriostatic, and pyrogallol as bacteriocidal against tested microorganisms. Interestingly, the bacteriocidal compound was found to arise by conversion of gallic acid by the endophyte *Enterobacter cloacae*. In addition, *Pantoea spp* was also isolated from the bark of *B. Dalzielii*. The sequences of both *E. cloacae* and *Pantoea spp* are deposited in the GenBank nucleotide database under the accession number MH764584 and MH764583, respectively.

Similarly, activity-guided fractionation of *B. Dalzielii* bark against breast cancer cell line (MCF7) using MTT cytotoxicity assay resulted in the identification of a cytotoxic compound, catechol, and the half maximal effective concentration (EC50) observed was

86µM. The growth inhibition effect of catechol was observed to be time- and concentration- dependent. Endophytic *Klebsiella pneumonia* species (strain A and B) were shown to be responsible for bioconversion of protocatechuic acid to catechol. In addition, *Pantoea agglomerans* was also isolated from the bark of *B. dalzielii*. The sequences of *Klebsiella pneumonia* A, *Klebsiella pneumonia* B and *Pantoea agglomerans* are deposited in the GenBank nucleotide database under the accession number MH762022, MH762023 and MH762024, respectively. All isolated compounds were identified using HPLC, TLC, NMR, FTIR and HRMS.

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1

INTRODUCTION

"Herbs and plants are medical jewels gracing the woods, fields and lanes which few eyes see, and few minds understand. Through this want of observation and knowledge the world suffers immense loss"

- Linnaeus 1707-1778

1.1 SYNOPSIS

The work described in this thesis is performed at Natural Product Research Laboratory, School of Pharmacy, Health and Life Sciences, De Montfort University since April 2015 as part of a research program. Presently, research on the use of combined drug therapy is consistently growing most especially in the fight against infection. Research effort in this era is to find solutions to life-threatening diseases and disorder and to achieve this, new theories, procedures and technologies are developed. The primary goal of this work is to obtain promising compounds from ethno-pharmaceutical plant and testing them alone and in synergy thus determining their bio-activity against micro-organisms and cancer cells.

1.2 BACKGROUND

In rural communities of the Northern part of Nigeria, traditional medicine is highly regarded primarily for its easy accessibility and affordability. The author grew up in a community where the *Boswellia* plants bark, *Boswellia odorata* and *Boswellia dalzielii*, were used as supplements for maintaining normal function of the body and the extraction is conducted via maceration and decoction. The theoretical point of view is that this plant helps the body in getting rid of nascent cells as well as promoting homeostasis. Growing up with this theoretical knowledge, the author wants to produce scientific results backing this ethno-pharmacological claims. *B. dalzielii* is also used alone or/and in combination with other plants for the treatment and management of cancer, commonly known as daji in Hausa language, a language commonly spoken in the Northern part of Nigeria. Example of such combination used for the treatment of cancer

that has boils as one of the symptoms include the use of the stem bark of *Spondias mombin* (Common name: Tsada), *Boswellia dalzielii* (Common name: Arrarabi), *Detarium microcarpum* (Common name: Taura), *Sclerocarya birrea* (Common name: Danya) and *Vitellaria paradoxa* (Common name: Kade). Two teaspoons of each of the plant are used measured and mixed. The combination is ingested three times a day using milk or water. Where water is used as a solvent, plant extraction is conducted either via maceration and decoction. This mixture is claimed to be used for the treatment of ulcer, liver cirrhosis, kidney disease and irregular menstrual period.

The purpose of this thesis is to provide scientific proof to some of the ethnopharmacological claims attributed to *Boswellia dalzielii*.

1.3 ETHNOPHARMACOLOGY

The appreciation of herbal medicines is associated with ethnicity, culture, geographical location and economic pressures (WHO, 2013). Currently, we can see a global spread of traditional health care systems and medicine through publications (Heinrich *et al.*, 1998; der Veen and Morales, 2015; Heinrich and Jäger, 2015). Available resources on the internet on the use of traditional medicines have also contributed to their globalisation (Sud and Sud, 2013). Also, immigration could impact and transform traditional medicine and evolve this knowledge, whereas urbanization could diminish the original value of such knowledge (Pirker *et al.*, 2012). Ethnopharmacology could be a stepping stone in validating ethnobotanical claims on the use of traditional medicines, where data collected via this route could be used for several bioassays to prove these claims.

The term Ethnopharmacology first appeared in 1967, in a symposium (Ethnopharmacologic search of psychoactive drugs) aimed at sharing and recording information on psychoactive plants used in indigenous societies (Efron, Holmstedt and Kline, 1967). The symposium led to the release of a document on psychoactive plants with abundant details on the plants` botany, extraction methods, pharmacological studies and efficacy obtained. This is very similar to the present bioactivity guided fractionation employed in present drug discovery (Weller, 2012). In this field, data are collected and studied (field studies) from different indigenous societies. These data are mostly historic, therefore it is paramount to put into account empirical underpinning to which these plants are evaluated at that time (de Montellano, 1975; Lardos, 2015).

Series of in-vitro test for activity is designed on the basis of the biochemistry and/or molecular biology of the disease to test extracts obtained from plant, resulting in the term Bioassay/Activity Guided Fractionation, as seen in the studies of (Phillipson *et al.*, 2002; Oldoni *et al.*, 2016; Erenler *et al.*, 2017). However, the bioassay designed for to verify the ethno-pharmacological claims might display an entirely different activity which could lead to an interesting finding. This was seen in the discovery of vinca alkaloids anticancer agents (vinblastine and vincristine) from *Catharanthus roseus*, a plant initially investigated for its reported use in Jamaica for the treatment of diabetes (reported in Gragg and Newman, 2010). In order to achieve a successful extraction of compounds from a plant, chemical examination (chemotaxonomic consideration) of the bioassay is encouraged. This would provide a possibility to the nature of active compounds present in the extract. Example, anti-inflammatory activity noted in a

member of *Asteraceae,* could be ascribed to the presence of sesquiterpene lactones present in many members of the family (Ivanescu, Miron and Corciova, 2015). These bioactive compounds can then be further subjected to isolation and structural elucidation using appropriate techniques (Cid and Bravo, 2015).

1.4 PLANT AS MEDICINE

For millennia, the plant kingdom has served as a vast reservoir of material used in the prevention and treatment of diseases (Grabley and Thiericke, 1999). Plants as a source of herbal medicine are used across the world and referred to as traditional medicine (TM) or complementary/alternative medicine in some countries (WHO, 2013). The term traditional medicine (TM) has been defined by the World Health Organization (WHO) as "the sum total of the knowledge, skills, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness". Whilst, Herbal medicines (HMs) were described to "include herbs, herbal materials, herbal preparations and finished herbal products, that contain as active ingredients parts of plants, or other plant materials, or combinations" (WHO, 2013). In 1985, WHO reported that about 80% of the world population relied on traditional medicine as the primary source of healthcare, especially in the rural communities. This dependence on traditional medicine is said to be mostly due to the easy accessibility and affordability of these medicines.To-date this percentile is regurgitated in the literature (Ramawat and Merillon, 2008; Salim, Chin and Kinghorn, 2008; Abdullahi, 2011; Mahomoodally and Fawzi, 2013).

The 19th century witnessed the birth of the reductionist approach to the treatment of ailments, where medicine is reduced to simple models of one drug-one target approach by extraction of active compounds from natural products. One of the famous discoveries is the isolation of morphine from opium of *Papaver Somniferum* in 1817 by Friedrich Sertürner (Krishnamurti and Rao, 2016). Another example of a famous compound from plants is quinine, a compound isolated from cinchona (quina-quina) tree by Pierre Joseph Pelletier and Joseph Caventou in 1820 (Achan *et al.*, 2011). In the 20th century, quinine was used as a template for the synthesis of other antimalarial drugs such as chloroquine and mefloquine (Jones, Panda and Hall, 2015). However by the end of the 20th century, the world witnessed the resistance of *Plasmodium falciparum* to quinine based drugs (Achan et al., 2011), this necessitated the development of artemisinin from *Artemisia annua*, as a novel scaffold for drug discovery. Artemisinin also serves as a lead compound used in deriving other semi-synthetic anti-malaria drugs such as dihydroartemisinin, artesunate and arteether (Balunas and Kinghorn, 2005; Cragg and Newman, 2013; Jones, Panda and Hall, 2015; MC, 2015). Artemisinin and its semi-synthetic derivatives such as artemether and artesunate are now used in combination therapy with drugs such as lumefantrine, mefloquine and amodiaquin having different modes of action against Plasmodium parasite, the causative agent of malaria (de Ridder, van der Kooy and Verpoorte, 2008). With this approach, the chance of development of resistance is believed to be greatly reduced (WHO, 2015).

Unlike the reductionist approach, the holistic approach to treating ailments is a multidimensional one, which was the initial form of most medicine and relates to the whole system rather than a specific part of the system. It puts into consideration the

physical, psychological, social and spiritual aspect of an individual. Here, an ailment is a consequence of patients` differential susceptibility in relation to the interaction between an individual and environmental factors. To achieve complete treatment of ailments, combined therapeutic approaches are used (Lyng, 1990; Bodeker, Kronenberg and Burford, 2008). This approach is seen in Ayurveda, Traditional Chinese medicines, traditional Europe and Middle American medicine, which are collections of natural remedies documented from centuries of experience, highly regarded and evolving in their own right (Mao and Wang, 2013). More so, both the Arabian and African heritage in folk medicine are in agreement with the eastern holistic approach toward disease treatment; whereas the Arabian folk medicine was believed to have developed for centuries due to commercial trading and networking, which led to the exchange of information and transferable skills. African traditional medicine has been orally transferred from generation to generation (WHO, 2013), where some herbal practitioners are self-taught while others inherited such knowledge and practice (which could be) through apprenticeship or family trade (Katoto, 2009; ACCD, 2012; eNCA, 2014; Health24, 2014; Saharatv, 2014). The lack of written/printed resources may explain the relatively minor role played by African herbal knowledge on a global scale when compared to Ayurveda and Traditional Chinese medicine. However, the first attempt to make an inventory on the use of African traditional medicine has been published, titled `Plant Resources of Tropical Africa 11` (Schmelzer *et al.*, 2008; Schmelzer, Gurib-Fakim and Arroo, 2013).

We currently have a dichotomy in health care approaches. On the one hand, we have evidence-based medicine, where carefully dosed active pharmaceutical ingredients (API) act on specific well-defined molecular targets. On the other hand, we have complementary and alternative medicine - where the efficacy of treatment or intervention is determined by the overall increase in wellbeing. Practitioners of evidence-based medicine may dismiss traditional medicine as 'old-fashioned', whereas practitioners of complementary and alternative medicine may refer to evidence-based health care as 'allopathic medicine' that is the treatment of symptoms but rarely ever the cause of any disease. However, complementary and alternative medicine is not without consequences.

The emphasis on using single compounds as active pharmaceutical ingredients (API) is due to the fact that the stability, dosing, mechanism of action, therapeutic efficacy and toxicity have been studied to a certain extent and is constantly updated (Siddiqui, AlOthman and Rahman, 2017). However, the use of herbs or mixture of herbs as drugs comes with numerous challenges, some of which include the methods of extraction: different extraction method result in different rate of extracting the active ingredients and in some circumstances result in production of artificial products thus changing the initial bioactivity or improving it (Albuquerque et al., 2017; Belwal et al., 2018; Zhang, Lin and Ye, 2018). Another challenge is toxicity and adverse effects: in most countries, there are no safety evaluation policies to regulate quality standard, proper prescription and potential hazards (Bandaranayake, 2006). Examples of plants reported for their adverse reactions are those belonging to the genus *Aristolochia* which are reported to

contain aristolochic acid and shown to have both nephrotoxic and genotoxic properties (Hwang et al., 2012; Sidorenko et al., 2012; Zhou et al., 2013; Koyama et al., 2016; Baudoux et al., 2018; Prinsloo, Nogemane and Street, 2018; Rietjens, 2018). More so, some medicinal herbs are associated with hepatotoxicity which could result to liver transplant or death, example include *Ephedra sinica*, *Actaea racemose*, *Aconitum carmichaelii* and *Piper methysticum* (Zheng and Navarro, 2016; de Boer and Sherker, 2017; Andrade *et al.*, 2018). In addition, assessing safety and efficacy of herbal product (s) is quite a challenge, since a single medicinal plant is expected to contain numerous amount of constituents, thus a mixture of medicinal herbal products might have several times that amount of constituents. Therefore, it will require tremendous resources and time to evaluate the safety of each active constituent present in a single medicinal plant, in this regard evaluating those present in a mixture of medicinal plants could be impossible (WHO, 2005). Despite these challenges, there are few plant mixtures approved as medicine and some are in different stages of clinical trials.

1.4.1 CLINICAL APPLICATIONS OF HERBAL MIXTURES

In ethnobotany, plant combinations and mixtures of phytochemicals derived from different plants are regularly reported, but none such mixtures have received FDA approval (Mukherjee and Houghton, 2009; Butassi *et al.*, 2015). The reason may be due to the fact that, if one drug could have multiple targets and one plant has numerous compounds, then the plant-target interaction and pharmacology will be too complex to elucidate, however not impossible.

Sativex, a mixture of delta-9-tetrahydrocannabinol and cannabidiol, extracts of *Cannabis sativa* has been approved in Canada for neuropathic pain relief in multiple sclerosis and may be used as an adjunctive analgesic in patients with advanced cancer (Bayer, 2007; Nurmikko *et al.*, 2007; Mishra and Tiwari, 2011). In addition, some of the herbal mixtures are now in various stages of clinical trials, examples include the completed trial on the use of *Ginkgo biloba* extract in the treatment of Alzheimer`s disease and *Viscum album* extract for cancer treatment (NIH, 2008, 2013). The double-blind randomised controlled trial on the effect of essential oil extract of three Cretan aromatic plants (*Coridothymus capitatus* L., *Origanum dictamnus* L. and *Salvia fruticosa* Mill.) on upper respiratory tract infection was reported to be statistically insignificant, however descriptive differences were reported among virus positive participants (Duijker *et al.*, 2015). The ethanol extract of *Solidago chilensis* was reported to alleviate pain and improve recovery in the external treatment of tendinitis of flexor and extensor tendons of the wrist and hand, in a placebo-controlled double-blind clinical study (Silva *et al.*, 2015). Another significant study is seen in the randomised phase III trial of *Viscum album* extract, where an increased effect on overall survival in patients with locally advanced or metastatic pancreatic cancer was reported (Tröger *et al.*, 2013).

1.4.2 PERCEIVED DRAWBACKS

Newman and Cragg (2016) revealed a varying percentile in the increase and decrease of approved drugs from natural product where the highest percentile was >45% in 1985, with an extreme decrease of >10% in 1997 (See Figure 1.1). Although, the percentile has increased to about >20% in 2014 (Newman and Cragg, 2016). This decline may have

resulted from phytochemical redundancy observed in plants especially among plants belonging to the same genus. Further contributing factors include inadequate documentation, lack of scientific data to back ethnobotanical claims and difficulties associated with securing authentic plant identity. In addition, extraction, purification, isolation and identification are considered time-consuming processes (Newman and Cragg, 2012). Also, variation in the availability of sufficient quantities of plant material for commercial purposes, due to seasonal and environmental factors, attributed to the decline in FDA approved drugs of plant origin. Furthermore, most natural product drug discovery is through phenotypic screens and activity guided fractionation, the properties of isolated compounds may not comply with Lipinski`s rule of fives (Table 1.1). However, Lipinski himself has stated that his rules do not seem to apply to natural products (Owens, 2003) which was suggested to be due to structural parameters including structural diversity, chiral centres and atomic composition (Keller, Pichota and Yin, 2006; Newman and Cragg, 2012; Barber and Rostron, 2013). Then, there is a lack of conventional criteria for evaluating quality, efficacy and safety combined with a reluctance to base decisions on ethnobotanical empiricism (Balunas and Kinghorn, 2005; McChesney, Venkataraman and Henri, 2007; Mukherjee and Houghton, 2009; Carmona and Pereira, 2013).

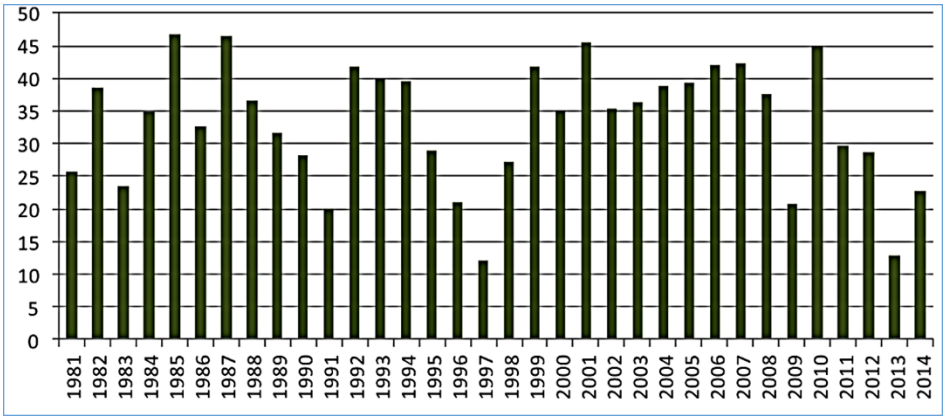


Figure 1. 1*: Percentage of approved drugs from natural product from 1981 – 2014.*

Lipinski`s rule of five
This empirical rule is used to distinguish between a drug like and non-drug like molecules, and it states that "poor absorption is more likely when: • The molecular weight is over 500 Dalton • Less lipophilicity (the logP is over 5) • There are more than five hydrogen bond donors (expressed as the sum of OHs and NHs) • There are more than ten hydrogen bond acceptors (expressed as the sum of Ns and Os)."

Table 1. 1: *The Lipinski`s rule of five (Barber and Rostron, 2013)*

New interdisciplinary technologies in the field of biotechnology, bioinformatics, medical chemistry and phytochemistry, have provided renewed interest in traditional remedies and a greater understanding of their effects at a molecular level (Balunas and Kinghorn, 2005; Verpoorte, Alfermann and Johnson, 2007; Cordell, 2011, 2014). These include: DNA barcoding for verification of plant genus and species (Cordell, 2014), the use of metabolomics for characterization of metabolite profiles rather than individual compounds in plants (Yan *et al.*, 2015), and biological evaluations aimed to address the potential risk in respect to dose specificity and side effects associated to the use of medicinal plants (Cordell, 2011; Cragg and Newman, 2013). Increased sensitivity of

spectroscopic methods and advances in information technology and computing power have eased the steps involved in product identification (Weckwerth and Fiehn, 2002; Dixon and Strack, 2003). In addition, genomic information from microbial source and their interaction with plant pave way to the discovery of known and unknown metabolites (Wilson *et al.*, 2014; Li *et al.*, 2015). These, combined with the techniques used in understanding systems biology such as the circulatory system via computational and mathematical modelling, allows the re-evaluation of traditional medical knowledge in the light of new insights into the signalling pathways that regulate cell physiology, and possibly to narrow the gap between the holistic and reductionist approach to health care.

1.5 *Boswellia dalzielii*

B. dalzielii Hutchinson (Bursearceae) is a tree species abundant in the West African regions of Ivory Coast, Northern Nigeria and Cameroon. It is a woodland savannah tree with a pale (peeling and ragged) papery bark, which forms leaves only after the flowering process. The blossoms are small white flowers on a leafless tree (Dalziel, 1910). The leaves are ovate-lanceolate (lowest) and elongated-lanceolate (higher) 5 – 9 cm long, 1 – 2 cm wide, acutely serrate, membranous and glabrous, the apices of the branches are about 1cm in diameter and the bark is described as thin and deciduous (Dalziel, 1910).

In northern Nigeria, *Boswellia dalzielii* is locally known as Ararabi or Hararabi, and the common name is frankincense tree (Dalziel, 1910; Kankara *et al.*, 2015). To the best of the author`s knowledge, the bark is most commonly used in the treatment of infections and tumours in Kaduna state in northern Nigeria, thus the bark is used in the present

study. Moreover, literature showed that the stem and leaves are used in maternal health care, especially in postpartum wound healing in Katsina state in northern Nigeria (Kankara et al., 2015), and in the treatment of diarrhoea, piles, heat rashes and umbilical cord complications in Kano state in northern Nigeria (Abubakar *et al.*, 2017). In the Republic of Benin, a decoction of the bark is used for the treatment of angina, dysentery and haemorrhage, and in combination with other plants, is used for the treatment of rheumatism, diarrhoea, leprosy and gastrointestinal disorder. In addition, the leaves are used for beverage flavour while the resins are used for fumigation and as incense (Kohoude *et al.*, 2017).

There is limited literature on *B. dalzielii*, evident from the search results obtained from ScienceDirect (21), Web of Science (no record found), PubMed (8) and Scopus (20). As such, other species belonging to the same genus will be discussed. *B. dalzielii* belongs to the family Burseraceae and genus *Boswellia* where *Boswellia* genus is reported to have thirty-three recorded accepted plant species by The Plant List (2013); African Plant Database and Tropicos documented thirty-two plant species. The most studied species of the *Boswellia* genus include *B. serrata*, *B. carterii*, *B. frereana* and *B. sacra*, this could be due to the economic importance in commercializing the resin (olibanum) collected from these plants. Resin extracts obtained from *Boswellia* trees have been used for centuries as a remedy for the treatment of diseases and disorders such as heart disease, inflammatory disease, diabetes, cancer and infection among others (Hamidpour *et al.*, 2013). Literature review on both phytochemistry and pharmacology will mention reported studies on other members of the *Boswellia* family.

1.5.1 PHYTOCHEMISTRY

Although, reports on the phytochemical analysis of *B. dalzielii* are very few, that of other species belonging to the genus *Boswellia* have been reported. There are different compounds present in various *Boswellia* species and the composition changes from species to species depending on climate and harvest conditions as well as geographical location (Hamidpour *et al.*, 2013). The frankincense of *Boswellia* species is reported to contain 60-85% resins (which constitute mostly pentacyclic triterpenes), 6-30% gums most of which are sugars (pentose and hexose sugar) and 5-9% essential oil (mainly mixture of monoterpenes, diterpenes and sesquiterpenes) (Rijkers *et al.*, 2006).

1.5.1.1 Terpenes

The most abundant terpenes are the boswellic acids and some identified terpenes (Figure 1.2) include α and β-boswellic acids, 3-acetyl-11-keto-β-boswellic acid, 3-acetyl-β-boswellic acid, 11- keto-β-boswellic acid, 3-keto-tirucallic acid, 3-*O*-acetyl-α-tirucallic acid, 9,11-dehydro-α-boswellic acid, acetyl-9,11-dehydro-α-boswellic acid, lupeolic acid, incensole, acetyl-lupeolic acid and 3-*O*-acetyl-β-tirucallic acid (Y. Liu *et al.*, 2014; Ammon, 2016; Mannino, Occhipinti and Maffei, 2016; Sharma *et al.*, 2016).

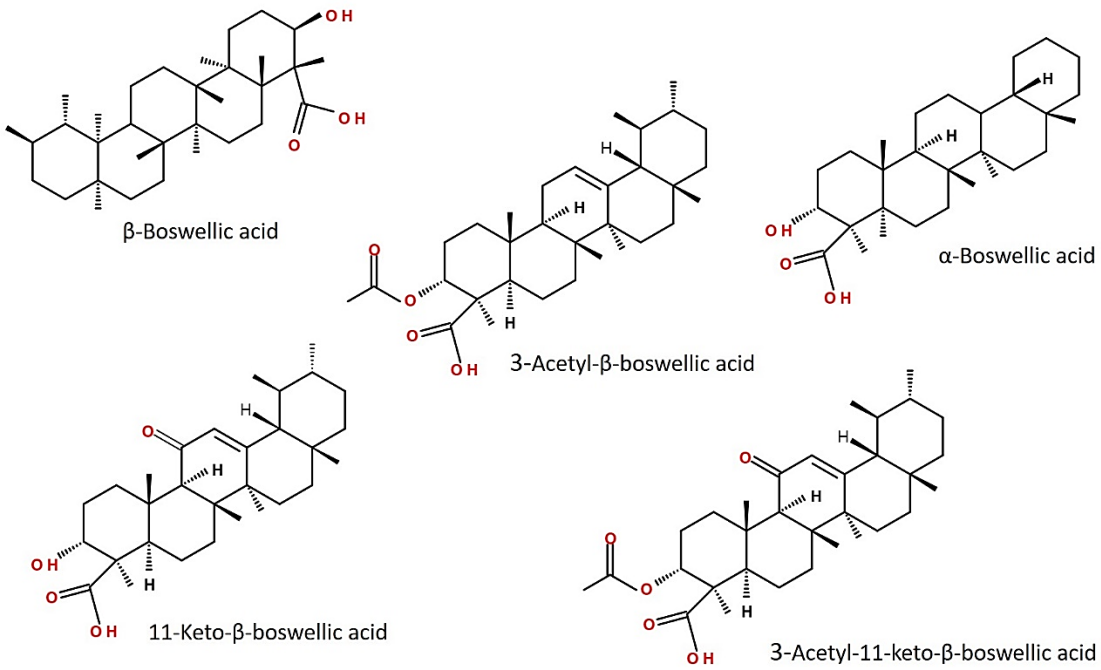


Figure 1. 2: *Chemical structures of some boswellic acids*

1.5.1.2 Essential oils

Essential oils in plant mostly occur as volatile aliphatic alcohols present in small concentrations. Literature reported the presence of the following essential oils in *B. dalzielii* which include α-terpinene, α-pinene, α-phellandrene, cryptone, p-isoprophyl benzyl alcohol, caryophyllene oxide, linalool, 3-carene, β-phellandrene, isolongifolene, myrcene, Z-β-farnesene, β-selinene, torreyol, 1,6-humulanedien-3-ol and terpinen-4-ol among others (Kubmarawa *et al.*, 2005; Kohoude *et al.*, 2017).

1.5.1.3 Others

Phenolic compounds isolated from the stem bark of *B. dalzielii* include protocatechuic acid, gallic acid and 4'-methoxy-(E)-resveratrol-3-O'rutinoside (Alemika, Onawunmi and Olugbade, 2005, 2006). In addition, Savithramma and colleagues identified phenols in both stem bark and leaves of *B. ovalifoliolata* including melilotic acid, salicylic acid,

coumarin, phloretic acid, gentisic acid and cinnamic acid, while flavonoids identified from stem bark include luteolin, rutin, petunidin, gorientin and vitexin, and those reported in the gum include rutin, vitexin and quercetin (Savithramma *et al.*, 2010).

1.5.2 PHARMACOLOGY

Boswellia dalzielii belongs to the genus *Boswellia*, and like the other genus members, it is claimed to have ethnopharmacological importance, some of which include anti-infection (stem), immunomodulatory (leaves) and anti-tumour effects (resins, leaves and stem) (Dalziel, 1910; Adamu *et al.*, 2005; Ibrahim *et al.*, 2006; Abubakar *et al.*, 2007; Younoussa, Nukenine and Esimone, 2016).

1.5.2.1 Anti-microbial activity

Aqueous, ethanol and methanol extracts of *B. dalzielii* stem bark exhibited broad spectrum activity against Gram positive bacteria (*Staphylococcus aureus* and *Bacillus cereus*), Gram negative bacteria (*Escherichia coli*, *Pseudomonas aeroginosa* and *Proteus mirabilis*) and fungi (*Candida albicans* and *Microsporum audouinii*) with MIC and MBC value between 2 – 22mg/mL and 4– 50mg/mL, respectively (Olukemi, Kandakai-Olukemi and Mawak, 2006; Adelakun *et al.* 2001). However, the studies conducted by Nwinyi *et al.* (2004) reported contrasting result showing that at a concentration of 2mg/mL, the aqueous extract of *B. dalzielii* stem bark has no antimicrobial activity against *S. aureus*, *B. subtilis*, *E. coli, P. mirabilis*, *P. aeruginosa*, *C. albicans* and *M. audouinii*. Also, the essential oil of *B. dalzielii* was shown to have antimicrobial activity against *B. subtilis*, *S. aureus* and *C. albicans* with a minimum inhibitory concentration (MIC) of 0.25mg/ml,

1mg/ml and 2mg/ml, respectively (Kubmarawa *et al.*, 2005). In addition, among four boswellic acids (β-boswellic acid, acetyl-11-keto-β-boswellic acid (AKBA), 11-keto-β-boswellic acid (KBA) and acetyl -β-boswellic acid (ABA)) tested for antimicrobial activity, AKBA was shown to exhibit better antimicrobial activity mostly against Gram-positive bacteria with a MIC of 2µg/ml for *S. aureus*, 2-4µg/ml for methicillin resistant strain of *S. aureus* (MRSA), 4-8µg/ml for *E. faecalis, E. faecium* and *S. epidermidis* and 2-8µg/ml for vancomycin resistant *E. faecalis* (Kubmarawa *et al.*, 2005).

1.5.2.2 Anti-cancer activity

Presently, there is very little literature on anticancer properties of extracts obtained from *Boswellia dalzielii*. One of the most recent literature is the study on the cyclohexane, dichloromethane, ethyl acetate and methanol extract of *B. dalzielii* which were shown to have cytotoxicity activity at 50mg/L against ovarian cancer cell line IGROV-1 using MTT assay and percentage inhibition reported were 92.60 ± 1.40%, 75.10 ± 2.80%, 64.50 ± 2.80% and 23.50 ± 2.50%, respectively (Kohoude *et al.*, 2017). Literature on the other member of *Boswellia* reported that the ethanol extract of *B. serrata* gum resin was used against five human leukemia cell lines (HL-60, K562, U937, MOLT-4 and THP-1) and growth inhibition reported was in the range of 57.0 - 124.1 µg/mL, moreover, the ethanol extract, containing AKBA among other constituents, was shown to have more potency against MOLT-4, K-562 and U-937 cell lines than AKBA (Hostanska, Daum and Saller, 2002). In addition, the cytotoxicity effect of ethanol extract of *B. ovalifoliolata* leaves was tested against two triple-negative breast cancer cells including

MDA-MB-231 and MDA-MB-453, and the IC_{50} values reported were 67.48±5.45 µg/mL and 70.03±4.76 µg/mL, respectively (Thummuri *et al.*, 2014).

The anticancer activity of plants belonging to genus *Boswellia* is mostly attributed to boswellic acids, examples can be seen in the studies of Hostanska, Daum and Saller (2002), Liu *et al.*, (2002),Park *et al.*, (2011), Shen *et al.*, (2012), Du *et al.*, (2015), Xue *et al.*, (2016), Conti *et al.*, (2018) and Li *et al.*, (2018). Triterpenes including 11-keto-β-boswellic acid (KBA), 3-*O*-acetyl-β-boswellic acid (ABA), β-boswellic acid (BA) and 3-*O*-acetyl-11-keto-β-boswellic acid (AKBA) were reported to inhibit the synthesis of protein, RNA and DNA in human leukemia HL-60 cells. These triterpenes were also shown to inhibit cell growth and AKBA was shown to be more potent with IC_{50} values of 0.6, 0.5 and 4.1 µM on DNA, RNA and protein synthesis, respectively (Shao *et al.*, 1998; Huang *et al.*, 2000). Four different concentrations of AKBA including 5µM, 10µM, 20µM and 40µM were used against three colorectal cancer cell (CRC) lines RKO, SW48, and SW480 and the result showed a dose dependent activity of AKBA with 40µM having the highest growth inhibition activity in a range of 89–98% in all treated cell lines after 72 hours treatment (Shen *et al.*, 2012). Furthermore, AKBA was shown to downregulate CXC chemokine receptor 4, a mediator in tumour metastasis, in pancreatic cell lines BxPC-3 and PANC-28 after treatment for 12 hours using 50µmol/L concentration (Park *et al.*, 2011).

1.5.2.3 Others

The extracts of *B. dalzielii* leaves were subjected to antioxidant assay against 1,1 – diphenyl-2-picrylhydrazyl free radical (DPPH) and results reported include 11.54 ± 0.20% at a concentration of 100 mg/L for essential oil extract while IC_{50} value of 6.10 ± 0.01 mg/L, 15.20 ± 0.01 mg/L, 78.10 ± 0.10 mg/L and IC_{50} > 100 mg/L was reported for methanol, ethyl acetate, dichloromethane and cyclohexane extracts (Kohoude *et al.*, 2017). The aqueous extract of *B. dalzielii* was shown to have no significant effect on total serum protein concentration, serum albumin component, bilirubin and serum alkaline phosphate activity, however, a significant decrease was observed on serum transaminases in female Wistar albino rats (Aliyu, Gatsing and Jaryum, 2007). Studies on other species in genus *Boswellia* showed that frankincense oils from *B. carterii* containing 1-octanol, linalool and α-pinene as active ingredients were shown to decrease oedema in a formalin-inflamed mouse hind paw model after 8hours treatment, and inhibited the development of ear oedema in xylene-induced ear oedema mouse model. Thus, frankincense oil was reported as an anti-inflammatory and analgesic agent and the activity was shown to be via the inhibition of nociceptive stimulus-induced inflammatory infiltrates and overexpression of COX-2 (Li *et al.*, 2016). More so, the essential oil of *B. carterii* was shown to have radical scavenging activity and IC_{50} value reported was 0.64µl/mL and the positive control used was butylated hydroxytoluene with a reported IC_{50} value of 7.4µg/mL. In addition, the essential oil of *B. carterii* was also reported to have a 51.68% inhibition of the oxidation of linoleic acid while the control had 69.60% inhibition (Prakash *et al.*, 2014).

1.6 ISOLATION OF MEDICINAL COMPOUNDS

1.6.1 EXTRACTIONS

Plant used as medicine could be directly chewed to exact desired effect, example is seen in the use of *Salvadora persica* stem for cleaning the teeth and gums, however, most plant materials undergo certain forms of extractions either with the aid of water, organic solvents and ointments, therefore hydrophilic components will sparingly dissolve or not dissolve in certain organic solvents such as hexane. The most fundamental aspect of every extraction process is the polarity of constituent compounds in a plant material. Other influencing factors include temperature, duration of extraction and pH (Hanson, 2005). The success in the extraction of bioactive compounds from natural products depends on the extraction procedure employed, and factors such as matrix properties of the plant part (leaves, stem, flower, fruit and bark), temperature, time and solvent contribute to this success (Hernández, Lobo and González, 2009). An example is seen in the extraction of alkaloids where stationary phase requires to be charged (Takla *et al.*, 2018).

For hundreds of years, extraction techniques have aimed at isolation of targeted bioactive compounds, and these techniques are either conventional or non-conventional. The conventional method includes three classic techniques namely soxhlet extraction, maceration and hydrodistillation. The Soxhlet extraction method employs the use of Soxhlet extractor invented by Franz Ritter von Soxhlet in 1879. In this technique, weighted plant sample is placed in a thimble which is then placed in the extraction chamber. The solvent of choice is heated to reflux to be recycled once the

Soxhlet chamber is full. The chamber is then emptied via the syphon. The advantage of this method is the recycling of a single batch of solvent and lack of required filtration step, however thermolabile bioactive compounds could decompose due to prolonged heating of solvents (Luque de Castro and Priego-Capote, 2010).

Maceration/steeping is as old as time and it is as simple as making a cup of tea. Although, this technique is the most common, simplest and inexpensive procedure for obtaining bioactive compounds, it involves several steps which include grinding of plant material to powdered or smaller particle to increase surface area expose to solvent (usually in a closed vessel with constant stirring), strain out solution, and filtration of solution which is then dried/concentrated on the rotary evaporator to yield crude extract (Seidel, 2006). Although maceration is less likely to result in the decomposition of thermolabile compounds, it is time-consuming and may result in ineffective extraction of compounds that have poor solubility at room temperature (Seidel, 2006). Hydrodistillation method is mostly used for the extraction of essential oil from plants. This method involves the use of hot water or steam as the influencing factor in the extraction of compounds from plant. However, it is time-consuming and could affect the integrity of thermolabile compounds (Pessoa *et al.*, 2007).

The limitations associated with conventional extraction techniques, especially for commercial purposes lead to the development of new techniques classified as non-conventional extraction methods, some of which are supercritical fluid extraction (SFE) and enzyme assisted extraction (EAE). Unlike the conventional method of extraction where a large volume of organic solvent waste is generated, the SFE technique is

environmentally friendly. Most widely used supercritical solvent for extraction is the supercritical CO_2 due to its low critical properties of 31.1^0C and 73.8 bar, other supercritical solvents include ethane, butane, nitrous oxide, trifluoromethane, water and ammonia. The critical point of a compound is paramount to its extraction and is defined as the highest temperature and pressure at which the compound can exist in a vapour-liquid equilibrium. A supercritical fluid is a single homogeneous fluid, as heavy as a liquid with a penetration power of gas is formed at temperatures and pressures above critical point (Nahar and Sarker, 2006).

Compounds of interest are sometimes trapped in a matrix such as polysaccharide-lignin network via chemical bonding, thus extraction of such compounds using extraction solvents could result in low yield. Therefore, the addition of specific enzymes such as cellulose and amylase during extraction results in better yield. The success of this technique relies on the enzyme composition and concentration, solid to water ratio, enzyme hydrolysis time and particle size of plant material (Azmir *et al.*, 2013; Vergara-Barberán *et al.*, 2015).

1.6.2 PURIFICATION

The most common form of purification is the use of separating funnel and chromatography. Chromatography is a method defined in the early 1900s by the work of a Russian botanist, Mikhail S. Tswett, whose work focused on separating compounds from plant pigment. Chromatography, a technique used in the separation of two or more compounds or ions from a mixture, relies on the differences in affinity or

interaction of compounds with a solid or stationary phase and the solvent system (mobile phase) (Ettre and Sakadynskii, 1993). Although different chromatographic techniques have been developed, thin layer chromatography (TLC), column chromatography and high performance chromatography (HPLC) were used in the present study.

Thin-layer chromatography is a microscale technique used for verifying the identity of a compound, monitoring the progress of a reaction, determining the number of components in a mixture, determine appropriate conditions for column chromatography and analysing the fractions obtained from column chromatography. This technique is a solid-liquid form of chromatography involving a polar absorbent which could be alumina ($Al_2O_3.xH_2O$) or silica ($SiO_2.xH_2O$) particles coated on a glass slide or plastic sheet creating a thin layer of the particular stationary phase, and a mobile phase which could be a single or combination of solvents. The affinity of the stationary phase for compounds depends on their polarity where the polar stationary phase has a stronger affinity for compounds with polar functional groups (Gorman and Jiang, 2004).

Although, all chromatographic methods have a principle similar to that of TLC, in the case of HPLC, column is used as stationary phase and contains a solid support over which the mobile phase flows with the sample in solution (See Figure 1.3). Chemical compounds in the solution migrate according to the non-covalent interactions of the compounds with the stationary phase. The high pressure pumps are needed to force the solvent through the stationary phase packed in the column. The interaction between the mobile phase, column and sample determine the degree of migration and

separation of components contained in the sample solution. Compounds with short retention time have a stronger interaction with the mobile phase than with the stationary phase thus elute from the column faster. This method is highly automated, sensitive and specific due to the use of specific wavelength, column type and flow rate. However, HPLC together with mass spectrometry is reported to be substantial in analytical studies of chemical compounds (Snyder, Kirkland and Dolan, 2010). The stationary phase column is the most important component in HPLC analysis and the following characteristics are considered when selecting a column, geometry-related properties responsible for column efficiency and flow resistance such as packing density and uniformity, surface area and particle shape, size and its distribution. Another characteristic considered is the surface chemistry related properties responsible for analyte retention and separation selectivity, such properties include ligand bonded to the surface of the base material (silica, polymeric, zirconia et cetera) and bonding density (Kazakevich & LoBrutto, 2007).

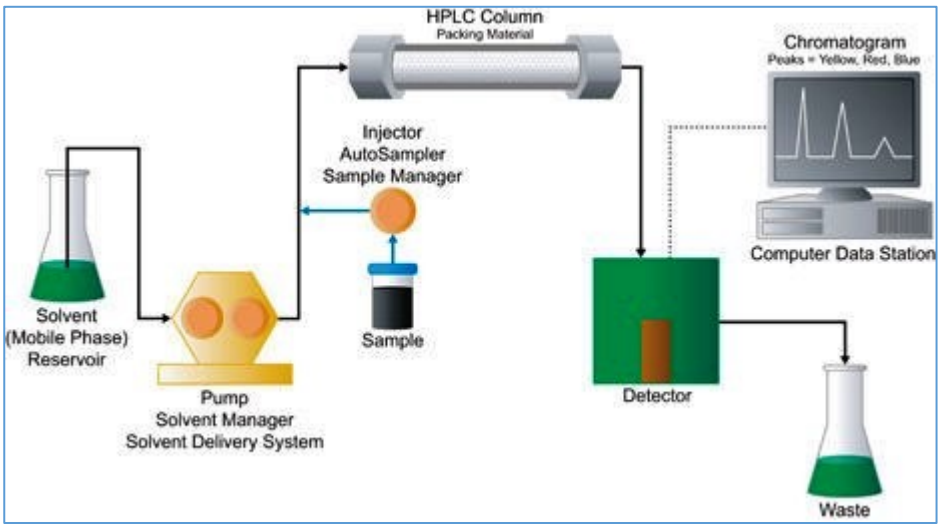


Figure 1. 3: *Schematic illustration of HPLC system (Waters, 2016).*

1.6.3 SPECTROSCOPY AND SPECTROMETRY

Spectroscopy involves the study of the interaction between light and compounds while spectrometry is used to obtain the quantitative measurement of compounds. This study employs the use of nuclear magnetic resonance (NMR) to determine the structure of an organic compound through the measurement of magnetic moments of hydrogen atom present in a compound. C-13 NMR observes the magnetic behaviour of carbon-13 nucleus; the sample is dissolved in a suitable inert solvent such as deuterated dimethylsulfoxide [$(CD_3)_2SO$], deuterated dichloromethane [CD_2Cl_2] and deuterated water [D_2O], peaks and their chemical shift values in part per million (ppm) are presented as a spectrum. The peaks presented in the spectrum provides information on unique carbon atoms environment in the structure and any symmetry in the structure affect the results by providing less number of peaks in the C-13 spectrum than the number of carbons present in the structure of the compound. For Hydrogen spectrum, spectral signals may appear as singlet, doublets or triplets which is due to the interaction between the protons attached to adjacent carbon atoms (Harborne, 1973; Williams and Fleming, 1995).

The spectrometric method used to obtain the actual molecular mass and compound composition of extracted compounds was the high-resolution mass spectrometry (HRMS). This method is used to obtain high resolution and high accuracy mass measurements due to its sensitivity, precision, accuracy and specificity for quantitative and qualitative analysis (Hamelin *et al.*, 2013; Stock, 2017). Just like other mass spectrometry, HRMS characterised compounds via the measurement of the m/z ratios

of ions and product ions after activation in tandem mass spectrometry (Figure 1.4) (Volmer, 2014).

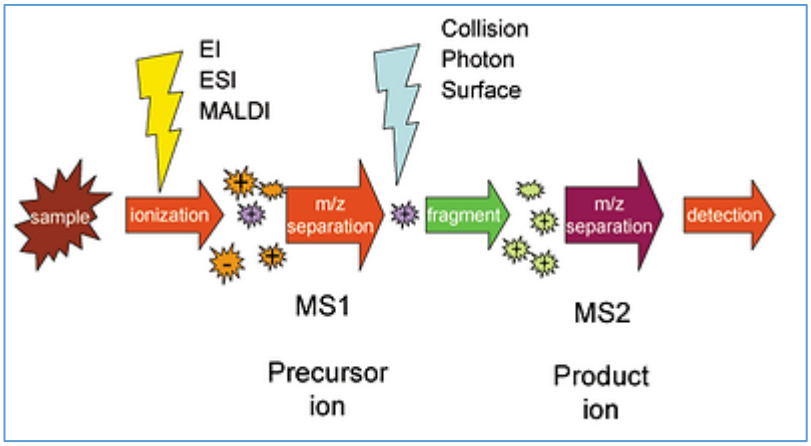


Figure 1. 4: *Illustration of the tandem mass spectrometry (Murray, 2016).*

1.7 BIOMOLECULAR INTERACTIONS

To understand synergy, it is important to understand polypharmacology. In drug discovery, the one gene, one drug, one disease approach involves the design of selective ligands that interact with particular target proteins, whereas polypharmacology involves the search for a drug that binds to multiple targets, or multiple drugs that bind to different targets within a network (Reddy and Zhang, 2013; Zhang *et al.*, 2016). These phenomena can only be appreciated through understanding drug –target interactions as well as the molecular networks involved. Polypharmacology, either as the therapeutic (healing) or adverse (harmful) effect, involves the binding of a drug, or drugs, to different multiple targets within networks. The polypharmacology approach evolved due to the lack of drug efficacy observed from one gene-one disease associated target drugs (Xie *et al.*, 2012). Therapeutic polypharmacology involves the simultaneous targeting of

multiple targets by one or more drugs in the treatment of diseases, where the targeted network or biochemical process may hypothetically be reset to its physiological state. A simple example is the use of β-lactamase inhibitors and β-lactam antibiotics to overcome antibiotic resistance. Another example is provided by the tyrosine kinase inhibitor Sorafenib, used for the treatment of some types of cancer, which not only inhibits Raf kinase isoforms, but also the platelet-derived and vascular endothelial growth factor receptor tyrosine kinases (Boran and Iyengar, 2010). Adverse polypharmacology occurs when drugs bind to targets other than the therapeutic target or drugs bind to the therapeutic target in non-target tissue resulting in negative effects. An example of adverse effects can be seen in the inhibition of both cyclooxygenase-1 and cyclooxygenase-2 by non-steroidal anti-inflammatory drugs. Cyclooxygenase-2 has a physiological role in many tissues and organs such as the kidney, where renal prostaglandins play a role in maintaining renal perfusion and glomerular filtration rate. Inhibition of cyclooxygenase-1 decreases the levels of prostaglandins responsible for pain and inflammation while inhibiting cyclooxygenase-2 decreases the level of prostaglandins results in tissue damage such as causing ulcer, acute renal failure and cardiovascular adverse effects (White, 2007; Boran and Iyengar, 2010; Xie *et al.*, 2012).

The function and expression level of a particular protein in a physiological process is controlled by the networks to which the protein belongs. Therefore, when one protein malfunctions or the process becomes aberrant, the whole signalling network which consists of the hormones, protein receptors, ligands, enzymes, transcription factors, ions and DNA/RNA that regulate this biochemical process, will be affected (Boran and

Iyengar, 2010). Understanding the pathophysiology of disease and disorder at molecular level coupled with –omics technologies (genomics, transcriptomics, proteomics, and metabolomics) may help us to understand the cross-link between biochemical networks and occurrence of diseases and disorders. This is especially true for multi-faceted diseases such as neurodegenerative diseases and cancer (Chandra and Padiadpu, 2013; Ellingson, Smith and Baudry, 2014; H. Liu *et al.*, 2014; Anighoro *et al.*, 2015).

1.7.1 EFFECT OF SINGLE CHEMICALS ON NETWORKS

Cancer is seen as a multifactorial disease, involving the reconstruction of the molecular networks essential for cell proliferation, differentiation or cell death (Sunil and Kamath, 2017). A commonly used chemotherapy drug is Paclitaxel, and the established, and widely accepted, mechanism of action of this drug is to prevent the depolymerization of microtubules by binding to the β-subunit of tubulin, thus inhibiting the metaphase anaphase transition and inducing apoptosis (Jordan and Wilson, 2004; McGrogan *et al.*, 2008; Gascoigne and Taylor, 2009). However, paclitaxel also activated the c-Jun N-terminal kinase/stress-activated protein kinase signalling pathway, via activation of the Ras and apoptosis signal-regulating kinase pathways in a dose-time-dependent manner (Wang *et al.*, 1998, 1999). The drug was further shown to induce p53 and p21 at low concentration, resulting in G1 and G2 arrest in a human alveolar adenocarcinoma cell line (Giannakakou *et al.*, 2001).

A holistic study of paclitaxel's mode of action showed a more complex chain of activities. Proteomic analysis of paclitaxel-treated HeLa cells identified several proteins that were

responsive to treatment. Some proteins were up-regulated and some were down-regulated (Lee *et al.*, 2005). The results were later confirmed by gene expression studies to include p53, MAPK, ErbB, Mtor, VEGF and T-cell receptor signalling pathway, focal adhesion and apoptosis (Qiao *et al.*, 2011). These findings correlate with those of proteomic findings, showing paclitaxel target-specific proteins to be involved in signal network, cellular processes and immune responses (Lee et al., 2005). More recently, paclitaxel was reported to induce apoptosis by reduction of p53, NFkB factors, pErk and BCL2 in NB4 leukaemia cells; and increase expression of Bax, caspase3 and 9 (Morales-Cano *et al.*, 2013). This implicates paclitaxel involvement in an array of multiple signalling pathways, some of which might not yet be understood and/nor elucidated. The above mentioned studies on paclitaxel show that the drug affects several pathways simultaneously, inferring that it did not act in isolation as it had been made out to be. It has become more widely accepted that not all drugs are selective, and at least some bind to several physiologic targets.

1.7.2 SYNERGY

Polypharmacology can either be synergistic - where the total effect of discrete agents is greater than the sum of the individual effects -, additive - where the more potent drug acts like the more concentrated form of the other -, or antagonistic - presenting a combined effect less than expected magnitude (Tallarida, 2006, 2012; Tallarida and Raffa, 2010). In ethnobotany, these concepts are frequently associated with the effect of herbal extracts due to the plethora of chemical compounds that are present in a plant. These chemicals may not necessarily act on the same receptors, and the presence of

one may not affect the biochemical interaction of the other but could have a similar effect. Therefore, in a situation where one metabolic pathway or signal network is blocked or impaired, other pathways might be enhanced.

We can see the interrelationship of metabolic pathways in tumour cells, where uncontrolled growth requires major changes in metabolism. Acceleration of aerobic glycolysis is used to sustain the demands for additional energy and biosynthetic precursors necessary for proliferation (the Warburg effect). The activity of the Krebs cycle is also enhanced and consequently anaplerotic pathways are activated to replenish the Krebs cycle intermediates which are also essential building blocks for lipid, protein, and nucleic acid biosynthesis. Notably, increased activity of pyruvate carboxylase is considered important to fulfil the high anabolic demands for growth in lung tumour tissues (Fan *et al.*, 2009).

Although synergistic/additive effects are regularly alluded to in phytochemistry/phytotherapy they are not usually accounted for. Quantifying this effect could serve as a guide for the overall combined effect, and for designing experiments to determine a mechanism of action. The most commonly used method to quantify combined drug effects for drugs with different relative potency is the isobologram, a graph of equally effective dose pairs (isoboles) for a single effect level (Tallarida, 2012). This method has been used in the study of paclitaxel (Photiou *et al.*, 1997) and β-Lapachone (Lamberti *et al.*, 2013) as well as a herbal combination used as as antitumor on prostate cancer cell lines (Chung, Tattersall and Cheung, 2004; Adams *et al.*, 2006). Further appropriate statistical analyses, e.g. regression analysis are

required to determine the nature of the interaction. Other quantitative approaches reported include the additive composite curve and the response surface analysis (Tallarida, 2001).

A recent study showed the potency of plant extracts compared to single approved constituents. Oral administration of *Taxus chinensis* taxane extracts improved the bioavailability and inhibitory activity of paclitaxel (Liu *et al.*, 2015). The anticancer effect of β-carboline alkaloids-enriched extract from *Poa pereira* was reported and this extract was also shown to enhance the cytotoxicity of carboplatin (Yu and Chen, 2014). Binary combinations of ginger (*Zingiber officinale*) phytochemicals synergistically inhibit proliferation of prostate cancer cells (Brahmbhatt *et al.*, 2013). Another example is the use of plant combinations in the treatment of diseases, which is a usual ethnobotanical practice. An example is an in-vitro antimicrobial synergy of plant combination used in the treatment of diarrhoea (van Vuuren, Nkwanyana and de Wet, 2015), however, the synergy was not quantified. The literature on the synergy between synthetic drugs and herbal medicines show several case studies evidencing interaction of herbal products and conventional drugs, where the herbal products either increase the action of conventional drugs or decrease its action (Izzo *et al.*, 2005; Ulrich-Merzenich, 2014).

1.8 ANTIMICROBIAL RESISTANCE

The first break in understanding infection was the Koch postulate in the 19th century where the cause of infective foci was attributed to microbial growth. Although Joseph Lister, a Professor of surgery, used carbolic acid for wound sterilization and the

prevention of sepsis in 1867, aseptic surgery was not a standard practice until the 1880s. The success of managing infections was observed with the discovery of penicillin by Alexander Fleming and the introduction of septic techniques, however, the emergence of antibiotic resistant bacterial strains due to indiscriminate use of antimicrobials is still eminent (Singhal and Kuar, 2018). In 1952, Selman Waksman was reported to have described antibiotic as a compound produced by a microorganism that kills or inhibits the growth of another microorganism. This description was said to define the activity of antibiotics but not the word, however, the term "antibiotic" is used to describe any class of molecule that inhibits or kills microbes by specific interactions with bacterial targets, without any consideration of the source of the particular compound or class, as such purely synthetic therapeutics are also considered as antibiotics examples include the sulfonamides, and trimethoprim (Davies and Davies, 2010). These compounds possess either bacteriostatic (prevention of bacterial growth) or bactericidal (causation of bacterial death) effect. However, the activity of certain bacteriostatic antibacterial agents may be bactericidal to certain susceptible organism at a higher concentration. Similarly, at lower concentrations, bactericidal agents could exhibit bacteriostatic activity. Chloramphenicol is reported as a bactericidal agent against *S. pneumonia* but bacteriostatic against *S. aureus*. More so, quinupristin-dalfopristin considered as a bactericidal agent against most strain of staphylococci and streptococci is reported as bacteriostatic against *Enterococcus faecium* (Pankey and Sabath, 2004).

1.8.1 ACTION OF ANTIBIOTICS

Most mono-therapeutic antibiotics are said to cripple an entire network rather than a single target, either by targeting multiple protein targets or the products of the action of multiple genes or the gene products themselves, thus low probability to drug resistance have been reported (Brötz-Oesterhelt and Brunner, 2008; Silver, 2012). The major targets of antibiotics include cell membrane (e.g mupirocin), structure or inhibition and/or regulation of enzymes involved in cell wall biosynthesis (e.g beta-lactams, vancomycin and bacitracin), nucleic acid metabolism/repair (rifampicin and quinolones) and protein synthesis (e.g tetracyclines, macrolides, and mupirocin) (Hancock *et al.*, 2005; Kapoor, Saigal and Elongavan, 2017) (See Figure 1.5).

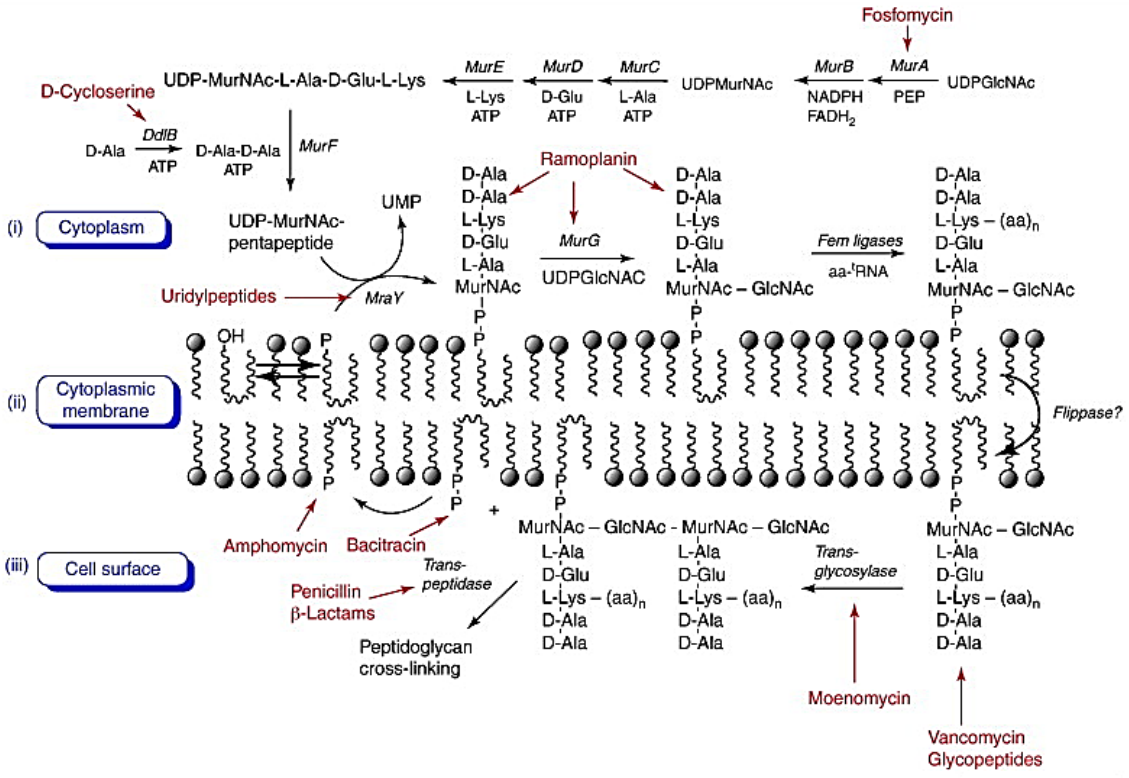


Figure 1. 4: *Major targets of antibiotics (Bugg* et al.*, 2011).*

Bacterial cell wall biosynthesis is catalysed by the enzymes MurA–F and MurG. Peptidoglycan units are then transported by bactoprenol-phosphate, a carrier lipid, across the cell membrane, generating Lipids I and II. In the cell wall, transglycosylation and pyrophosphorylation occur, where sugars and phosphates are added and a peptide bond between the peptide chains is formed (Walsh, 2003). Penicillin binding proteins (PBPs) catalyses the cross-linking of peptidoglycan (PGN) peptides in PGN biosynthesis, hence recognise the terminal alanine dipeptide (AD) unit (Scheffers and Pinho, 2005; Brötz-Oesterhelt and Brunner, 2008). β-Lactams antibiotics are known to interfere with the activity of more than one essential PBP due to their similar stereochemistry to AD, however, no β-Lactams antibiotics inhibit all the PBPs. Furthermore, they are reported to derange the activity of the PG biogenesis machinery, thus depleting cellular resources by inducing a substrate cycle of PGN synthesis and degradation (Cho, Uehara and Bernhardt, 2014). In most bacteria, the PBPs are reported to be encoded by more than a single gene (Brötz-Oesterhelt and Brunner, 2008), Scheffers and Pinho (2005) reported 12 PBPs for *Escherichia coli*, 14 PBPs for *Bacillus subtilis*, 5 PBPs for *Staphylococcus aureus* and 6 PBPs for *Streptococcus pneumonia*. Goffin and Ghuysen (1998) showed that all PBPs have three conserved motifs (SXXK, SXN AND KTG, where X is any amino acid) in common, occurring in the same order and the active site serine is in SXXK. Unlike the β-Lactams antibiotics, the glycopeptides vancomycin inhibits the process of transglycosylation by targeting the D-ala-D-ala terminus of lipid II peptidoglycan precursor, a substrate to multiple enzymes (Brötz-Oesterhelt and Brunner, 2008; Tortora, Funker and Case, 2010).

Certain antibiotics such as chloramphenicol inhibit protein biosynthesis by inhibiting the formation of peptide bonds in a growing peptide chain by reacting with the 50S subunit of the 70S prokaryotic ribosomal RNA (rRNA). Aminoglycosides group of antibiotics such as gentamicin act by changing the shape of the 30S of the 70S prokaryotic rRNA, resulting in misreading of mRNA and induction of nonsense protein. Tetracycline prevents the binding of aminoacyl-tRNA to A-site of the 30S of the 70S prokaryotic rRNA, thus preventing protein translation. Antibiotics that function by preventing DNA replication interfere with nucleic acid synthesis function by inhibiting DNA polymerase and DNA helicase as well as RNA polymerase, therefore preventing replication and transcription. Example of such antibiotics are the quinolones which are broad-spectrum antibiotic and act by inhibiting the bacterial DNA gyrase as a result preventing DNA replication (Tortora, Funke and Case, 2010).

1.8.2 RESISTANCE TO ANTIMICROBIAL AGENT

Presently, about 700 thousand individuals are reported to die of antimicrobial resistance per year, and by 2050 that number is estimated to increase to about 10 million. The resistance to antimicrobial agent is not a new phenomenon, it is used by microorganisms as a survival mechanism against other organisms. However, their ability to resist antimicrobial agents means we stand to lose more lives to life-threatening infectious diseases such as pneumonia, tuberculosis, human immunodeficiency virus (HIV) and malaria (O'Neill, 2016). Since the discovery of antibiotics in the 19th century, there has been a lack of new novel antibiotics (Figure 1.6), thus giving room to the emergence of resistance which is contributed by misuse of antimicrobial agents.

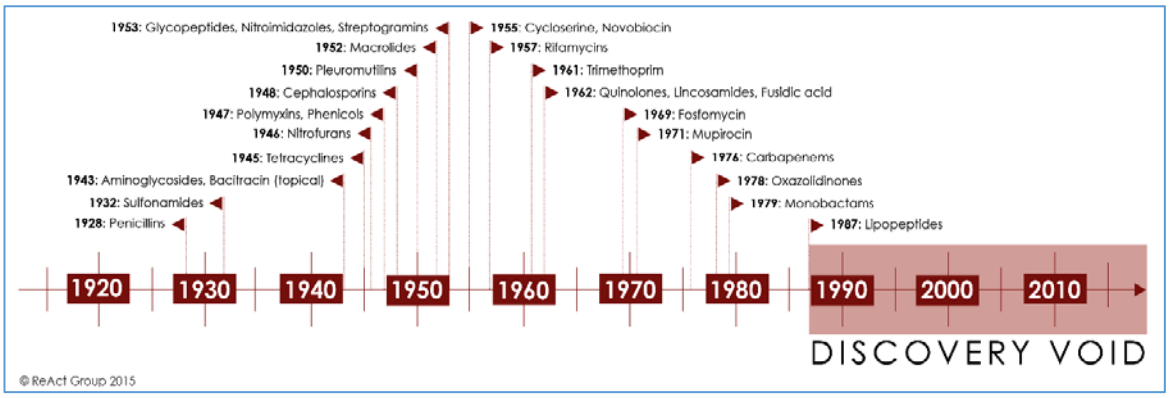


Figure 1. 5: *Time-line of antibiotic discovery (ReACT, 2018).*

1.8.3 *Staphylococcus aureus*

S. aureus is naturally susceptible to antibiotic, however, could develop resistance through horizontal transfer to genes from outside sources, chromosomal mutation or antibiotic selection. This gram-positive bacterium has the ability to colonize normal individuals without any symptoms, particularly in the nose and on the skin. Carriers of *S. aureus* are at high risk of infection when exposed to surgery, hemodialysis, chronic ambulatory peritoneal dialysis, or have an impaired immune system. This microorganism is easily spread among individuals via direct contact either through skin-to-skin contact with colonized or infected individuals or contaminated objects (Liu, 2009).

Methicillin-resistant *Staphylococcus aureus* (MRSA) are strains of S. aureus that are resistant to methicillin and other β-lactams such as oxacillin, nafcillin, carbapenems, penicillin, and amoxicillin (Hal, Lodise and Paterson, 2012). This intrinsic resistance is shown to be associated with the production of penicillin binding protein (PBP2a) encoded by the mecA gene unique to MRSA. This protein has a low binding affinity for

β-lactam antibiotics (Chambers, 1988). Infections due to MRSA are associated with increased morbidity, mortality, length of hospital stay and costs. MRSA infection is prevalent in both community and health care settings and is associated with numerous infections such as those of the bloodstream, lower respiratory tract, soft tissues, skin, endocarditis, osteomyelitis and urinary tract (Hassoun, Linden and Friedman, 2017; Emaneini *et al.*, 2018). In 2015, the presence of 16.8% MRSA was reported among invasive *S. aureus* isolates in Europe (ECDC, 2016) whereas, some years earlier in the USA, as much as 51.3% of *S. aureus* isolates were reported as MRSA (CDC, 2013). Also, <50% MRSA was reported for most African countries in 2013, however, an increase in prevalence was observed since 2000 (Falagas et al., 2013). In 2008, 44% of heath associated infection in hospitals of Iceland and Norway were associated with MRSA (Köck et al., 2010). Example of antibiotic used in the management of MRSA related infection include vancomycin, a glycopeptide antibiotic that targets the cell wall synthesis was reported to be less effective for the management of serious MRSA infections, as shown in the meta-analysis study conducted by Hal and colleagues, 2012 where the MIC (>2) of vancomycin was reported to be associated mortality irrespective of methodology (Hal, Lodise and Paterson, 2012). In order to predict the efficacy of vancomycin, the use of pharmacokinetic/pharmacodynamic parameter (the ratio of AUG/MIC) was suggested because is a time-dependent antibiotic. In addition, individualization of vancomycin therapy was suggested, using pharmacodynamics as the key to achieving successful disease management (Toutain, Bousquet-Mélou and Martinez, 2007). Other antibiotics used for managing MRSA related infection are listed in Table 1.2.

Site of Infection	Empirical therapy	Alternatives	Future Considerations	Other notes
Bacteremia/IE	Vancomycin	Daptomycin Teicoplanin Daptomycin + Ceftaroline (synergy)	Ceftaroline Ceftobiprole	Avoid: Clindamycin TMP-SMX Tigecycline
Mild SSTI with abscess <5 cm	Incision and drainage			
Moderate SSTI	TMP-SMX Clindamycin* Doxycycline/Minocycline Linezolid			* Limited due to increased resistance
Severe or complicated SSTI	Vancomycin	Daptomycin Linezolid Telavancin*	Ceftaroline Ceftobiprole Dalbavancin Oritavancin Tedizolid	* Only to be used when alternative treatments are not suitable due to safety concerns
Pneumonia	Vancomycin Linezolid	Telavancin*	Ceftobiprole	* Only to be used when alternative treatments are not suitable due to safety concernsAvoid: Daptomycin, Tigecycline
Bond and joint infections	Vancomycin	Daptomycin Vancomycin + Rifampin Linezolid Consider: TMP-SMX Clindamycin Fluroquinolone* Doxycycline/Minocycline	Tedizolid	*Not to be used as monotherapy

Table 1. 2*: Recommended antibiotic for MRSA related infection (VanEperen and Segreti, 2016).*

1.8.4 ANTIBACTERIAL SCREENING

Different methods have been developed for antibacterial screening and for plant extract, the recommended concentration to be used in testing is below 1 mg/mL for

crude extract and 100 µg/mL for isolated compounds. The most common methods used for antimicrobial testing so far include diffusion methods, dilution methods and bioautographic method (Mukherjee and Houghton, 2009).

The diffusion methods require the use of plant extract on an inoculated medium which could be either a hole, cylinder or disc, then the diameter around the medium is measured. The diffusion techniques are advised for polar compounds, because the use of an inappropriate solvent for non-polar compounds may result in false negative results, although the hole – plate technique is adequate for non-polar solvent. The diffusion method required the tested plant extract to be dissolved in an appropriate solvent as such both the cylinder and paper disc method is appropriate for water soluble extract. Although the diffusion methods are used for rapid preliminary screening, they are not appropriate for determining the minimum and maximum inhibitory concentrations of antimicrobial agents. However, with the standardized techniques and recommendations such as that provided by British Society for Antimicrobial Chemotherapy (BSAC) and Clinical and Laboratory Standard Institute (CLSI), the technique is simple, inexpensive, easy to interpret and can be used for enormous screening of antimicrobial agents and microorganism suspension of 0.5 McFarland (Balouiri, Sadiki and Ibnsouda, 2016).

The dilution techniques are usually used to determine the lowest concentration of the tested antimicrobial agent to inhibit visible growth of the microorganism tested. The methods required the use of known microbial and antimicrobial agent concentrations, the suspension is then incubated, and bacterial growth is determined by turbidimetry

or tetrazolium salts and values are a measure of mg/mL or µg/mL. Standard approaches are provided for both fastidious and non-fastidious microorganisms so to obtain replicable relevant clinical data, such guideline provided include that of CLSI and European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Balouiri, Sadiki and Ibnsouda, 2016; Mukherjee and Houghton, 2009). The bioautographic method requires the use of separated compounds on thin layer chromatography for the detection of growth inhibition directly on the TLC which can be achieved through direct spraying of broth culture on developed TLC; immersion bioautography which involved covering a developed TLC plate with molten seeded agar; and contact method where compounds developed on TLC are transferred on to agar medium previously inoculated with microorganism to be tested. Each of the mention bioautographic method have different incubation time, however, visualization is mostly achieved by the use of tetrazolium salts where the salts are converted into a coloured product by the enzyme dehydrogenases present in the cells. These techniques are used for both fungi and bacteria and compounds with antimicrobial activity can easily be identified, however, the concentration of a particular compound on TLC chromatogram could result in false negatives (Balouiri, Sadiki and Ibnsouda, 2016; Mukherjee and Houghton, 2009).

In order to combat antimicrobial resistance, combination therapies are exploited increasingly, an example is the use of WHO-approved combination therapies to prevent said resistance. Thus, combination therapies involving bioactive drugs and non-antibiotic plus bioactive drugs are exploited to tackle resistance to antibiotics and results obtained could be either synergistic interaction or reduced efficacy (Owen and Laird,

2018). The synergy between drugs is defined as “a greater effect in combination than the sum of the individual effects of the combined substances” (Efferth and Koch, 2011). There are three methods commonly used to screen the interaction of compounds against micro-organism and these include the E-test, microdilution checkerboard and Time-kill method (Orhan *et al.*, 2005; Sopirala *et al.*, 2010). The most popular method is the checkerboard method where screening is conducted in a microtiter plates and 2-fold dilution of one compound is done along the y-axis and the other compound on the x-axis and fractional inhibitory concentration index (FICI) can be analysed using a specific equation reported by Doern (2014) and the most common interpretation used are those reported by Odds (2003).

1.9 CANCER

Cancer as a disease involves the growth of abnormal cells, with the ability to ignore the rules of cell division, thus disrupting the balance between cell division and quiescence. This disease could further be described as a multi-genetic event involving different malfunctioned proteins, which result in changing the molecular network essential to cell proliferation, differentiation and cell death (Hassanpour and Dehghani, 2017). Gradually, these cells develop autonomy by decreasing their contact with normal cells thus destabilizing haemostasis; producing growth factors from oncogenes; overriding antigrowth signals from tumour suppression genes; inducing the growth of new blood vessel (Angiogenesis) for supply of oxygen and nutrient as well as providing a channel for transporting cancer cells (CCs) to other sites (metastasis); and finally by evading immune detection and destruction by the immune system (Boik, 2001). Cancer is

reported by WHO as the second leading cause of death globally and responsible for an estimated 8.8 million deaths in 2015. More so, Breast cancer is said to be the fifth cause of death with an estimate of 571,000 deaths in 2015, globally (WHO, 2018).

1.9.1 BREAST CANCER

Cancer is the second cause of death worldwide (Siegel, Naishadham and Jemal, 2013) and breast cancer is the most common cancer-related death among women with an estimated expected new cases of 252,710 in 2017 (Siegel, Naishadham and Jemal, 2013; Qu *et al.*, 2015; Klevos *et al.*, 2017). In Africa, breast cancer constitutes 25% of all diagnosed cancer and its prevalence varies across African nations. In Nigeria, 26% of breast cancer cases were categorised as triple-negative breast cancer and 27% as estrogen receptor negative (Lukong, Ogunbolude and Kamdem, 2017). The cause of breast cancer remains obscure however it begins with the development of a malignant tumour in breast tissue. Reported associated risk factor include obesity, estrogen exposure and the inheritance of susceptible genes such as BRCA1 and BRCA2 (Klevos *et al.*, 2017). Different options are available for the management of breast cancer depending on the type and stage, example include the use of surgery, chemotherapy such as anthracyclines, taxanes and 5-fluorouracil, hormone therapy for ER/PR positive cancer including selective estrogen receptor modulators, aromatase inhibitor and anastrozole, and HER2- targeted therapy (Klevos *et al.*, 2017).

Some of the therapeutic agents used for managing breast cancer are derived from plant example include taxol extracted from the bark of *Taxus brevifolia* and vinblastine from

Vinca rosea. The screening of plants for anti-cancer properties led to the report of commonly used plants believed to be cytotoxic against breast cancer cells due to their phytochemicals, some examples include *Echinacea purpurea*, *Allium sativum*, *Curcuma longa* and *Arctium lappa* (Shareef, Ashraf and Sarfraz, 2016). In Africa, breast cancer patients lack adequate access to social and economic resources and available treatment are expensive for most patient due to lack of funds and limited available financial options. As such, there is a prevalence in the use of alternative treatment for promoting homeostasis and preventing/suppressing certain type of cancer (Lukong, Ogunbolude and Kamdem, 2017). The anti-cancer effect of numerous natural products such as plant extracts have been tested both in in-vitro and in-vivo model, and phytochemicals belonging to different categories such as flavonoids, polyphenols, terpenes and alkaloids are believed to be responsible for these anticancer effects (Hosein Farzaei, Bahramsoltani and Rahimi, 2016).

1.9.1.1 GENUS *BOSWELLIA* AND BREAST CANCER

Plants belonging to genus *Boswellia* are extensively studied for their biological properties which is mostly attributed to compounds belonging to the pentacyclic triterpenoids class, in other words, boswellic acids (Hussain *et al.*, 2017). These boswellic acids, mainly boswellic acid (BA), acetyl-β-boswellic acid (ABA), 11-keto-β-boswellic acid (KBA) and acetyl-11-keto-β-boswellic acid (AKBA), are reported to be majorly obtained from the frankincense (resin) of plants belonging to genus *Boswellia* (Iram, Khan and Husain, 2017). 50µmol/L of AKBA was shown to downregulate CXC CHEMOKINE

RECEPTOR 4 in MDA-MB-231 and MCF7 breast cancer cell line, thus preventing cellular invasion (Park, Sung, *et al.*, 2011).

The essential oil of *Boswellia sacra* obtained via hydrodistillation was reported to induce specific cytotoxicity in MCF-7, MDA-MD-231 and T47D cell lines (Suhail *et al.*, 2011). Ethanol extract of *B. ovalifoliolata* leaves was reported to exert cytotoxic effect on breast cancer cell lines which include MDA-MB-231 and MDA-MB-453 and the reported IC_{50} are 67.48 ± 5.45 µg/mL and 70.03±4.76 µg/Ml, respectively. In addition, the ethanol extract was shown to have effects on both antiapoptotic (Bcl2) and proapoptotic (Bax) proteins by decreasing the level of Bcl2 and increasing the expression of Bax (Thummuri *et al.*, 2014).

1.9.1.2 OTHER PLANTS USED IN THIS STUDIES

1.9.1.3 Spondia mombin

The stem bark of *Spondia mombin* (local name: Tsada) was collected from the Northern part of Nigeria precisely from Kaduna State, Nigeria. The plant was authenticated as belonging to the Anacardiaceae family (Vouchers number: 2384) at the herbarium situated at Ahmadu Bello University, Zaria by Namadi Sunusi.

1.9.1.4 Detarium microcarpum

The stem bark of *Detarium microcarpum* (local name: Taura) was collected from the Northern part of Nigeria precisely from Kaduna State, Nigeria. The plant was

authenticated as belonging to the Fabaceae family (Vouchers number: 480) at the herbarium situated at Ahmadu Bello University, Zaria by Namadi Sunusi.

1.9.1.5 Sclerocarya birrea

The stem bark of *Sclerocarya birrea* (local name: Danya) was collected from the Northern part of Nigeria precisely from Kaduna State, Nigeria. The plant was authenticated as belonging to the Anacardiaceae family (Vouchers number: 6871) at the herbarium situated at Ahmadu Bello University, Zaria by Namadi Sunusi.

1.9.1.6 Vitellaria paradoxa

The stem bark of *Vitellaria paradoxa* (local name: Kade) was collected from the Northern part of Nigeria precisely from Kaduna State, Nigeria. The plant was authenticated as belonging to the Sapotaceae family (Vouchers number: 90072) at the herbarium situated at Ahmadu Bello University, Zaria by Namadi Sunusi.

1.9.2 CYTOTOXICITY SCREENING METHOD

Over the years, different anticancer agents of natural origin have been reported, for example Alvaradoin E from the leaves of *Alvaradoa haitiensis*, Palmerolide A from *Synoicum adareanum* and Silvestrol from the fruit and twigs of *Aglaia foveolata* (Kinghorn, Chin and Swanson, 2009). In the screening for compounds with cytotoxicity activity from natural products, different screening techniques have been used, most are colorimetric method used in determining cell viability.

1.9.2.1 In-vitro cytotoxicity screening

The first reported quantitative colorimetric method of screening cell proliferation, survival and cytotoxicity was reported in 1983. This assay was designed to avoid the use of radioisotope and washing steps to improve result precision. The method reduced (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl terazolium bromide (MTT) salt in active mitochondria in all metabolic active cells, producing a dark blue formazan product which can be spectrophotometrically measured at 570nm (Mosmann, 1983). The studies by Stockert *et al* (2012) disputed the claim that mitochondrial succinate dehydrogenase is responsible for the reduction of MTT salt to its formazan using two main properties of the mitochondria. The mitochondria have an oxidizing power rather than a reducing property and the presence of reduced nicotinamide adenine dinucleotide (NADH) coenzyme should not make MTT assay a direct method for the determination of mitochondrial activity, rather an indirect one (Stockert *et al.*, 2012). Studies suggested that tetrazolium salts can be reduced to formazan by plasma membrane-bound NADPH oxidase and the reduction of the salt is dependent on the rate at which glycolytic NADH is produced in the endoplasmic reticulum (Stockert *et al.*, 2012). Microscopic and colocalization studies revealed that formazan accumulates in lipid droplets rather than mitochondria and lysosomes (Diaz *et al.*, 2007; Stockert *et al.*, 2012). Although MTT assay is commonly used as a cytotoxicity screening technique, studies by Angius and Floris (2015) indicated that this assay is prone to error in cytotoxicity screening involving liposomes as drug vehicles. The error is said to be due to the lipophilic nature of MTT – formazan which can be stored in liposomes thus increasing cell viability (Angius and Floris, 2015). Other factors that can affect MTT assay result include metabolic

perturbations, effects of oxidoreductases and superoxidase (Berridge, Herst and Tan, 2005). Although MTT is widely used and in some cases could provide a false positive result, the assay is recommended to be accompanied by non-metabolic assay (Stepanenko and Dmitrenko, 2015). The zebrafish model was shown to complement MTT assay (Li *et al.*, 2012). Other tetrazolium salts used are the water soluble salts including 5-(3-(carboxymethoxy)phenyl)-3-(4,5-dimethyl-2-thiazolyl)-2-(4-sulfopheny)-2H-tetrazolium inner salt (MTS), sodium 5-(2,4-disulfophenyl)-2-(4-iodophenyl)-3-(4-nitrophenyl)-2H tetrazolium inner salt (WST) and sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-((phenylamino)-carbonyl)-2H tetrazolium inner salt (XTT) (Berridge, Herst and Tan, 2005).

Apart from tetrazolium salts, resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide) is also used for dye reduction assay. Resazurin is a blue dye that converts to a pink fluorescent resorufin in the presence of mitochondria enzymes. The reduction is measured with fluorescence spectroscopy using 560nm as excitation wavelength and the emission wavelength is 590nm, this setting is because peak absorption of resorufin is 570nm (Borra *et al.*, 2009). Samples are considered potentially cytotoxic if the mean viability is reduced to <70% when compared to blank control (ISO, 2017; Liu *et al.*, 2018).

Another screening technique used is the direct cell count/dye exclusion assay. The principle behind this assay is that the presence of intact cell membranes in living cells prevent the uptake of certain dyes such as trypan blue and eosin and reversal is the case in dead cells, consequently having a blue cytoplasm (Strober, 2001). This technique is considered as a simple and rapid means of determining cell viability because the

materials required are trypan blue dye and transmission microscope. However, the integrity of the cell membrane does not necessarily define the functionality of a cell and the subjectivity of the method in terms of dye uptake by injured cells put this method under scrutiny. Thus, dye exclusion assay is recommended to be performed with a fluorescent dye and observed under a fluorescence microscope (Strober, 2001). In addition, to overcome the limitations associated with dye exclusion assay, Avelar-Freitas *et al.* (2014) suggested the use of flow cytometry for cell viability assay. This suggestion was attributed to the formation of trypan blue complexes with either bovine serum or cytoplasmic proteins which emit fluorescence at 660nm and the complexes are said to be detectable with flow cytometry using 650nm low-pass band filter (Avelar-Freitas *et al.*, 2014).

The use of biomarkers for the measurement of cell viability and cytotoxicity is another in-vitro screening technique. The biomarkers used are reported to exhibit proteolytic activities associated with cell viability/death, for example, the activity of cellular protease reduces rapidly in dead cells. Thus, biomarker activities can be measured with the aid of a luminogenic substrate which can be combined with a fluorogenic substrate to increase assay sensitivity (Niles, Moravec and Riss, 2009).

Although extensive data can be generated from an in-vitro assay, results obtained are limited by the complexity of the human biology, such in-vitro results cannot be used to predict the outcomes of human clinical trial.

1.9.2.2 In-vivo cytotoxicity screening

In-vivo screening model is advantageous in anticancer drugs development in terms of cost, time and most importantly improve the prediction of drug efficacy. One of the common in-vivo model for screening anticancer drugs and validating their action is the zebrafish. The zebrafish (*Danio rerio*) models are considered a powerful tool for rapid drug discovery and biosafety studies in the screening of drugs efficacy for the nervous, digestive, reproductive and immune system among other (Huiting, Laroche and Feng, 2015). This consideration is because most organ development in zebrafish is similar to mammals (Wilkins and Pack, 2013). The zebrafish is used due to associated advantages including rapid development of its embryo, determination of drug bioavailability and drug toxicity at the tissue level (Huiting, Laroche and Feng, 2015). Zebrafish models use for cancer studies are generated using various methods which include the use of carcinogens such as N-nitrosodimethylamine, inactivation of tumour suppressor genes such as p53, transgenesis to generate transgenic zebrafish expressing mammalian oncogenes, and the use of xenograft to transplant human cancer cell into zebrafish (Terriente and Pujades, 2013; Wilkins and Pack, 2013; Huiting, Laroche and Feng, 2015; Wyatt, Trieu and Crawford, 2017).

Another widely used animal model is the mouse model (*Mus musculus*) and the advantages associated with this model include similarity in genetic material with human genetics, similar biological/organ system with human and their relatively small size. Certain limitations are associated with the mouse model, most importantly the mouse factor which is an umbrella term used to describe certain characteristics associated with

different strains, thus putting in consideration that the mouse factor contributes to results obtained, independently. These characteristics include the presence of different lesions in different mouse strains, strains and age-related diseases, variability among mouse models (Kohnken, Porcu and Mishra, 2017). Like the zebrafish model, mouse model used for cancer research are generated using carcinogens, xenograft and transgenesis (Gould, Junttila and de Sauvage, 2015; Kohnken, Porcu and Mishra, 2017).

1.10 ENDOPHYTES

Endophyte is a term derived from the Greek words “endo meaning within” and phyton meaning plant”. Endophytes are microorganisms (bacteria, archaea, fungi and protist) that are found in plant endosphere and have a symbiotic association which could be obligate or facultative without causing harm to host plant (Hardoim *et al.*, 2015). Obligate endophytes are those microorganisms that depend on plant metabolism for survival while facultative endophytes are those that survive outside the plant host and associate with the host via its neighbouring soil environment and the atmosphere (Hardoim, van Overbeek and Elsas, 2008; Abreu-Tarazi *et al.*, 2010). These microorganisms are said to be beneficial to host plant by promoting plant growth via synthesizing phytohormones such as indole-3-acetic acid and cytokinins (Ahmed and Holmström, 2014; Santoyo *et al.*, 2016); solubilize phosphorus in soil (Oteino *et al.*, 2015); improve plant host tolerance/resistance to stress by preventing ethylene synthesis in plants (Glick, 2014); control and prevent microbial pathogens by producing antimicrobial compounds (Esmaeel *et al.*, 2016; Larran *et al.*, 2016; Kandel *et al.*, 2017); increase plant biomass and height (Shi, Lou and Li, 2009; Barra *et al.*, 2016); produce

pigments (Qiu *et al.*, 2010) and enzymes(Bischoff *et al.*, 2009); and uptake of nutrients such as nitrogen (Robertson-Albertyn *et al.*, 2017), among others.

Colonization of host plant by endophytes was elucidated to occur via the soil-root interface i.e. the rhizosphere which was shown to involve two principle elements namely root exudates and microorganism quorum sensing compounds. In addition, geographical location, plant phenotype, plant developmental stage, soil type, plant nutritional status, climate changes and environmental factors such as level of carbon dioxide and oxygen and changes in temperature also contribute to the distribution and localization of endophytes in host plant (Kandel, Joubert and Doty, 2017; Liu *et al.*, 2017).

1.10.1 ISOLATION AND IDENTIFICATION

Endophytes are said to be present in different plant parts as shown in the studies conducted on the leaves of *Phaseolus vulgaris* (de Oliveira Costa *et al.*, 2012); the bark of *Populus euramericana* (de Oliveira Costa *et al.*, 2012); the seeds of *Urochloa ramose* (Verma and White, 2018); the buds of *Atractylodes lancea* (Zhou *et al.*, 2018); the root and seed of *Paullinia cupana* (Silva *et al.*, 2018); the roots, leaves, stems and flowers of *Rhizophora stylosa* and *Rhizophora mucronata* (Zhou *et al.*, 2018) and the soil-root interface (Liu *et al.*, 2017).

Prior to the use of molecular techniques, endophytes were isolated by a conventional approach which involves isolation of endophytes from sterile plant tissue. Nowadays, they are isolated via DNA sequencing of targeted regions such as 16S rRNA gene and the

internal transcribed spacer regions (ITS1 and ITS2) or via whole genome sequencing (Taghavi *et al.*, 2009; Sessitsch *et al.*, 2012).

1.10.2 APPLICATIONS OF ENDOPHYTES

Some applications of endophytes include their role as source of bioactive compounds; bioremediation, biotransformation/bioconversion and biodegradation; and enzyme production (Bartholdy, Berreck and Haselwandter, 2001; Lu and Shen, 2007; Bischoff *et al.*, 2009; Tomsheck *et al.*, 2010; Joseph and Priya, 2011; Kumar *et al.*, 2011; Russell *et al.*, 2011; Bezerra *et al.*, 2012). The review will focus on bioactive compounds from endophytes and bioconversion.

1.10.3 BIOACTIVE COMPOUNDS FROM ENDOPHYTES

Bioactive compounds produced by endophytes are shown to belong to have different bioactivity such as anticancer, antimicrobial and antioxidant among others. Example is paclitaxel, an alkaloid which is an anticancer agent produced by *Metarhizium anisopliae* (Liu et al., 2009), *Phoma medicaginis* (Zaiyou, Li and Xiqiao, 2017) and *Cladosporium cladosporioides* (Zhang, Zhou and Yu, 2009) which were all isolated from Taxus species namely *Taxus chinensis*, *Taxus wallichiana* and *Taxus media*, respectively. More so, 4'-hydroxy-deacetyl-18-deoxycytochalasin H, isolated from *Trichoderma harzianum*, an isolate of *Cola nitida* leaves was shown to have cytotoxic activity against cancer cell lines including murine lymphoma (L5178Y) and human ovarian cancer (A2780 sens) cell lines with IC_{50} values of 2.55 and 6.97 µM, respectively (H. Chen *et al.*, 2015). In addition, phenochalasin B obtained from endophyte *Eutypella scoparia* which was isolated from

Hevea brasiliensis leaf was shown to have potent cytotoxic effect against KH-oral cavity cancer cell lines (IC_{50} value of 2.46µM) (Kongprapan et al., 2015). 6-formamide-chetomin, an isolate of *Chaetomium species* which was obtained from the plant *Huperzia serrata*, was shown to have cytotoxic effect against human cervical (HeLa), human gastric (SGC-7901) and lung adenocarcinoma (A-549) cancer cell lines with IC_{50} values of 21.6 nM, 23.0 nM and 27.1 nM. Furthermore, 6-formamide-chetomin was shown to possess antibacterial activity against *Staphylococcus aureus*, *Enterococcus faecalis* and *Salmonella typhimurium* with a reported MIC of 0.78 µg/mL across the three microorganisms (Yu *et al.*, 2018). 5-butyl-6-(hydroxymethyl)-4-methoxy-2H-pyran-2-one and 4-methoxy-6-methyl-5-(3-oxobutyl)-2H-pyran-2-one were obtained from endophyte *Alternaria phragmospora*, an isolate of *Vinca rosea* and both have cytotoxic activity against myelogenous leukemia cell line K562 with reported IC_{50} values of 4.5 and 1.5 µM, respectively (Metwaly et al., 2014). Studies by Sang *et al.* (2017) showed that two α-pyrone derivatives were isolated from Endophytic *Phoma species* namely phomones D and phomones E, and both were subjected to cytotoxicity studies against human leukemia HL-60, human prostate PC-3 and human colon HCT-116 cancer cell lines with a reported range of 0.65-9.84 µM IC_{50} values. Rhytidchromones A and Rhytidchromones, oxygenated chromones obtained from, *Rhytidhysteron rufulum*, an endophytic fungus isolated from *Bruguiera gymnorrhiza*, were reported to have cytotoxicity against MCF-7 with reported IC_{50} value of 19.3 ± 2.5 and 17.7 ± 3.7 and against Kato-3 with reported IC_{50} value of 23.3 ± 1.1 and 16.0 ± 1.9 µM, respectively (Chokpaiboon *et al.*, 2016).

Examples of other bioactivity properties attributed to bioactive compounds from endophyte is seen in the studies of Leylaie and Zafari (2018), where trichodermin obtained from *Trichoderma koningiopsis*, an endophytic fungus isolated from *Vinca major*, was shown to have antifungal effect with MIC of 3.25 µg/mL across all tested microbes namely *Pyricularia oryzae*, *Botrytis cinerea* and *Aspergillus fumigatus*. 3-(4-nitrophenyl)-5-phenyl isoxazole, isolated from *Aspergillus niger*, was shown to inhibit the enzyme adenosine deaminase, and inhibit the growth of liver cancer cell lines HepG2 and SMCC-7721 cells with IC_{50} values of 0.347 and 0.380 mM, respectively (Zhang *et al.*, 2018). Actinoallolide A isolated from *Actinoallomurus fulvus* was shown to have antitrypanosomal activity against *Trypanosoma species* namely T. *b. brucei*, *T. b. rhodesiense* and *T. cruzi* with IC_{50} value of 0.0049, 0.086 and 0-226 µg/mL (Inahashi *et al.*, 2015). 3-methylcarbazoles obtained from endophytic *Streptomyces sp.*, an isolate of *Alpinia galangal* root, was reported to have a potent anti-inflammatory effect and suppressed the production of inflammatory mediators such as PGE2, NO, TNF-a, IL-6 and IL-10 in a dose-dependent manner which was studied in RAW264.7 macrophages (Taechowisan *et al.*, 2012). Antibiotics are also reported to be obtained from endophytes example include ecomycins, Pseudomycin A, Munumbicins, Kakadumycin A and Xiamycin-A isolated from *Pseudomonas viridiflava*, *Pseudomonas syringae*, *Streptomyces sp*. (NRRL 30562), *Streptomyces sp.* (NRRL 30566) and *Streptomyces sp.* strain GT2002/1503, respectively (Christina, Christapher and Bhore, 2013).

1.10.4 BIOCONVERSION

Bioconversion/biotransformation involves the use of micro-organisms as biocatalysts and is increasingly used in the preparation of pharmaceutical products (Hegazy *et al.*, 2015). In the field of natural product research, most compounds isolated are in minute quantity and in most cases the amount required for clinical trials are mostly chemically synthesized, thus resulting in the accumulation of chemical waste. However, this challenge can be tackled with the use of biotransformation where biocatalyst can result in the production of complex drugs and drugs intermediates (Hegazy *et al.*, 2015). Ultimately, removing the necessary step of chemical catalysis reaction, complicated separation and purification steps. In bioconversion, the catalytic activity of micro-organisms which involve several chemical reactions plays a vital role in the specific modification of a specific compound to a product with similar structure (Lilly, 1994). Bioconversion complies with green chemistry since it results in the pharmaceutical processes with minimal waste production and energy consumption (Guria, Guha and Bhattacharyya, 2014). Most chemical processes of drug production are conducted in less favourable conditions which are mostly less environmentally friendly and expensive, in contrast, bioconversion is enantiomer-specific and region-specific, environmentally friendly and less expensive (Collins and Kennedy, 1999; Rozenbaum *et al.*, 2006). The reactions involved in biotransformation include addition, elimination, substitution, pericyclic, rearrangement and redox reaction (Hegazy *et al.*, 2015).

The micro-organisms mostly used in bioconversion are endophyte species. The fungus *Glomerella cingulata* was reported to hydroxylate tetrahydrogeraniol and

tetrahydrolavandulol to hydroxycitronellol and 5-hydroxytetrahydrolavandulol, respectively (Hirokazu Nankai, Miyazawa and Kameoka, 1997). Hegaxzy and colleagues (2015) and Cao and colleagues (2015) published a comprehensive data on microbial transformation of certain phytochemicals such as monoterpenes by *Absidia gluaca* and sesquiterpenes by *Kluyveromyces marxianus*, *Aspergillus alliaceus*, and *Rhodotorula glutinous* among others (Cao *et al.*, 2015; Hegazy *et al.*, 2015). Some bioactive natural products were also reported to have undergone biotransformation example include artemisinin bioconversion by *Streptomyces griseus* to artemisitone-9, 9α-hydroxy-artemisinin, 9β-hydroxy-artemisinin and 3α-hydroxy-deoxyartemisinin (Liu *et al.*, 2006). Biotransformation of taxol/cephalomannine was also reported, this was achieved using *Streptomyces* sp. resulting in the products 10-hydroxyacetyl-10-deacetyltaxol, 3-(4-hydroxyphenyl)-3-dephenyltaxol and 4-hydroxycephalomannine (Chen *et al.*, 2001).

Recent literature reported the bioconversion of halimane to 2-oxo-derivative by *Fusarium oxysporum* whereas *Myrothecium verrucaria* transformed it to 18-19-dihydroxy, 18-formyl and 18-carboxyl products. Also, both *F. oxysporum* and *M. verrucaria* were shown to transform labdane to 7α-hydroxy and 7α/3β-hydroxy, respectively (Monteiro *et al.*, 2017). Other studies also reported the transformation of glycerol to polyhydroxyalkanoates by *Pseudomonas mediterranea* and *Pseudomonas corrugate* (Licciardello *et al.*, 2017); ginsenosid Rb1 to ginsenoside Rg3 by *Burkholderia sp.* (Fu, Yin and Yin, 2017); astragalosides to astragaloside IV by *Penicillium canescens* (Yao *et al.*, 2014) and ergopeptines to lysergic acid by *Rhodococcus erythropolis* (Thamhesl *et al.*, 2015).

Finally, it is important to point out that a recent literature showed that endophytes, isolated from three medicinal plant namely *Caralluma acutangula*, *Rhazya stricta* and *Moringa peregrine*, were shown to possess antioxidant properties and contain bioactive (flavonoid and phenolic) metabolites (Khan *et al.*, 2017). Thus, the question whether certain pharmacological attributes subscribed to a particular plant could be a result of the interaction between the host plant and endophytes is worth exploring.

1.11 AIM and OBJECTIVES OF THIS THESIS

The work presented in this thesis describes the antibacterial and cytotoxicity investigation of *Boswellia dalzielii* bark using the aqueous extract of *B. dalzielii* against microorganisms (*Staphylococcus aureus* and its methicillin-resistant strain) and cancer cell (breast cancer cell MCF7 and MCF10A as control).

1.12 STRUCTURE OF PROPOSED THESIS

This thesis has been divided into seven chapters:

Chapter 1 provides background information on ethnopharmacology, plant as medicine, phytochemistry and pharmacology of the plant of interest i.e. *B. dalzielii*, methods of isolation and identification of compounds, antimicrobial resistance, cancer and endophytes.

Chapter 2 entails detailed information of materials and apparatus used for phytochemistry, microbial and cytotoxicity assays, in addition, it provides information on microbial strains used and their storage.

Chapter 3 describes detailed antibacterial screening of aqueous and 50% methanol extracts of *B. dalzielii* against *S. aureus* and its methicillin-resistant strain. This chapter also provides detailed bioactivity guided screening of active extracts using Disc diffusion method to obtain compounds responsible for the antibacterial activity and said compounds were identified. Methods of minimum inhibitory concentration and synergy of said compounds were also provided.

Chapter 4 provides detail experimental procedure and results for the rationale on the role of microorganisms in aqueous extract of *B. dalzielii* and their capability to biotransform phytochemicals to other metabolites. Also, methods used in identifying these microorganisms was provided.

Chapter 5 includes detail method used in cytotoxicity screening of aqueous extracts of *B. dalzielii* (alone and in combination with four other plants) and the bioactivity guided assay that led to the isolation and identification of active compound.

Chapter 6 describes the identification of microorganisms responsible for the production of anticancer compound catechol, and the isolation and identification of the compound transformed in aqueous *B. dalzielii* bark fermented extract.

Chapter 7 gives detail method developments using vanillin sulphuric reagent, -anisaldehyde reagent, m-anisaldehyde using aqueous extracts of *B. dalzielii*.

Chapter 8 provides references used in this thesis.

2

MATERIALS

2.1 Plant material

The stem bark of *Boswellia dalzielii* was collected from the Northern part of Nigeria precisely from Kaduna State, Nigeria. The plant was authenticated as belonging to the Burseraceae family (Vouchers number: 2448) at the herbarium situated at Ahmadu Bello University, Zaria by Namadi Sunusi.

2.2 Reagents

2.2.1 Reagent used for microbial analysis

Mueller Hinton agar and broth (Oxoid, UK), purchased gallic acid (98% Agros Organic), pyrogallol (99% Alfa Aesar), cefotaxime and vancomycin (Sigma Aldrich, UK), cellulose acetate (Aldrich), MacCONKEY agar (consisting of bile salt, crystal violet, sodium chloride, lactose, peptone, protease peptone, neutral red and agar) (Oxiod), Lugol`s iodine solution (Fischer), 95% alcohol, safranin (Fisher), crystal violet (Fisher), oil immersion (Sigma), API®20E™ biochemical test kit and reagents (Biomerieux), sterile distilled water, ultrapure agarose (Invitrogen), 0.5 × TBE (Tris base,Boric acid and Ethylenediaminetetraacetic acid (EDTA)) (Invitrogen) and DNA ladder (Bioline).

2.2.2 Reagent used for phytochemical analysis

2.2.2.1

HPLC water and acetonitrile grade, distilled water, methanol, hexane, ethyl acetate, vanillin, sulphuric acid, m- anisaldehyde, p-anisaldehyde, formic acid, acetic acid, methanol and ethanol. Compounds used as references were gallic acid (98% Agros Organic), pyrogallol (99% Alfa Aesar), catechol (99% Alfa Aesar), protocatechuic acid

(97% Agros Organic) and compounds purchased from Sigma Aldrich include steroids (β-sitosterol and stigmasterol), flavonoids (kaempferol and nobiletin), and terpenes (farnesol and terpinene).

2.2.3 Reagent used for cytotoxicity analysis

MCF7 AND MCF10A were purchased from European Collection of Authenticated Cell Culture. Cell culture media (DMEM and DMEM/F12), Fetal bovine serum, Dulbecco`s phosphate buffered saline (DPBS), Trypsin-Ethylenediaminetetraacetic acid (EDTA), Trypan blue stain and Dimethyl sulfoxide (DMSO) were obtained from Fischer Scientific. MCF10A media supplement including Insulin, Hydrocortisone, Epithelial growth factor (EGF) were sourced from Fischer Scientific. Thiazolyl blue tetrazolium bromide (MTT) was purchased from Sigma Aldrich.

2.3 APPARATUS

General apparatus used include sterile pipettes and corresponding tips (1mL, 200µL, and 10µL).

2.3.1 Apparatus used for Microbial analysis

Apparatus used for microbial analysis include sterile cotton swab, sterile 96 well plate, sterile multichannel pipette, Eppendorf vials, sterile 1mL Pasteur pipettes,6mm whatmann glass filter paper, Innova incubator shaker, UV measuring device (SpectraMax Plus), LEICA ICC50 HD microsystem and glass slides, Oxidase strips, Bunsen burner, PCR machine (PIKOREAL 96), UV- cabinet, vortex, mini plate spinner (MPS

1000TM), Kinetex 5µm C18 (150 × 2.1mm), Bio-Rad illuminator, minisart sterile syringe filter (pore size of 0.2µm) sartorius, sterile loop, sterile spreader, cryopreservation beads, 50mL sample bottles and Innova incubator shaker.

2.3.2 Apparatus used for phytochemical analysis

Apparatus used for phytochemical analysis include *B. dalzielii* plant bark, measuring cylinder, 3mL disposable Pasteur pipettes, 2000W hot air gun, 100mL Duran bottle, foil paper, TLC silica plate (Macherey Nagel SIL-A UV-254) , TLC tank, 5µL spotters, spray bottle, ice box, mechanical shaker, fume cabinet, clean micro spatula, brush, FTIR 2 Bruker Alpha spectrometer, NMR tubes, Deuterated dimethyl sulfoxide and water, 400Bruker Avance Spectrometer, HPLC Agilent 1100 series and VisionHT C18 HL 5µ (250 × 4.6 mm) column, HPLC sample vial and 50mL sample bottles.

2.3.3 Apparatus used for cytotoxicity analysis

1.5mL microcentrifuge tubes, 10mL and 5mL serological pipettes, 96-well microtiter plates (flat-bottomed), 25 cm^2 and 75cm^2 culture flasks and 15mL centrifuge tubes were also purchased from Fischer Scientific. Cell culture work was conducted in HERA safe biosafety cabinet (Class II), haemocytometer was used to obtain cell number, samples were incubated in Hera cell 150 incubator and GloMax microplate reader was used for measuring absorbance. Other materials used include 1mL, 200µL and 10µL pipette and corresponding sterile pipette tips, multichannel pipette and serological pipette controller.

2.4 MICROORGANISM STRAINS

Methicillin-resistant *Staphylococcus aureus* (MRSA) NCTC 12497 and *Staphylococcus aureus* NCTC 6571 were used in this studies. These organisms were transferred from the master store by taking the wrong end of a sterile swab and transferring a bead into 10mL nutrient broth, and incubated at 37˚C at 100RPM using the incubator shaker series (innova®44), for 24hours. Other microorganisms used were those isolated from *B. dalzielii* bark namely *Enterobacter cloacae, Pantoea spp* and *Klebsiella pneumonia strain B* and *E*.

2.5 STORAGE OF MICROORGANISMS

From inoculums, microorganisms were plated using sterile loops, labelled and incubated in 37˚C incubation room for 18hours. From plated microorganisms, colonies were picked using a sterile loop and transferred to small tubes containing cryopreservation beads immersed in cryo-solution, purchased from technical services. Once organisms were transferred, the tube was closed and gently shake and allow to stand for 30 seconds, then the cryo-solution was gently removed using a pipette, then stored in the freezer at -20°C.

2.6 PREPARATION OF MEDIA AGAR AND BROTH

All agar and broth were prepared according to the manufacturer`s instructions. For instance, Mueller Hinton agar (CM0337) was measured as instructed (38g in 1 Litre of distilled water) and nutrient broth (CM0405) was measured as instructed (21g in I Litre of distilled water). Both broth/agar solutions were autoclaved at 121^{o}C for 15 minutes.

3

ANTIBACTERIAL SCREENING OF *Boswellia DALZIELII*

"All infections, of whatever type, with no exceptions, are products of parasitic beings; that is, by living organisms that enter in other living organisms, in which they find nourishment, that is, food that suits them, here they hatch, grow and reproduce themselves"

- Bassi 1773-1856

ABSTRACT

The activity-guided fractionation of aqueous fractions obtained from *Boswellia dalzielii* bark against *Staphylococcus aureus* and its methicillin-resistant strain resulted in the identification of two antibacterial compounds namely gallic acid and pyrogallol which were identified using HPLC, TLC, NMR, FTIR and HRMS. The Minimum Inhibitory Concentration for pyrogallol and gallic acid against *S. aureus* growth are 508 and 753 µM, while against MRSA growth are 254 and 2032 µM, respectively. A growth Inhibition assay showed the activity of gallic acid as bacteriostatic, while that of pyrogallol as bacteriocidal against tested microorganisms. Interestingly, pyrogallol was isolated from the 24 hours aqueous extract of *B. dalzielii* macerated for 24hours at 37°C.

3.1 INTRODUCTION

In most phytochemical investigations, plants that are reported to be used by specific ethnic groups and that are expected to have pharmacological effects, are screened using organic solvents such as methanol, ethyl acetate, acetone, dichloromethane and hexane (Iloki-Assanga *et al.*, 2015; Dhawan and Gupta, 2016; Thouri *et al.*, 2017; Hernández-Sánchez *et al.*, 2018). Some studies use 50% hydroalcoholic solvents, but only a few use water in studying the phytochemistry and pharmacological properties of plants with suspected ethnopharmacological properties (Ben Yakoub *et al.*, 2018; Ferreres *et al.*, 2018; Mishra *et al.*, 2018). Nevertheless, water is an abundantly available solvent and most commonly used in the preparation of herbal extracts in folk medicine. As such; it is advisable to use water as the solvent of extraction in ethnopharmacology (Hanson, 2005).

The rationale used in this study is to use water as the solvent used in preparing herbal extracts, in an effort to stay as close as possible to the traditional preparation methods. In addition, a 50% aqueous methanol was also used to address the possibility that the antibacterial effect of *B. dalzielii* could be attributed to non-polar compounds present in the plant. Although, the bioautographic method is an easy assay, the BSAC disc diffusion (BSAC, 2013), CLSI microdilution (CLSI, 2017) and microdilution checkerboard methods (Odds, 2003) were used in this study because these methods require the use of specific sample measurements while in the bioautographic method, the concentration of compounds present in sample are unknown.

This chapter will discuss the antibacterial activities of *B. dalzielii* bark via activity guided fractionation which was screened against *Staphylococcus aureus* and its Methicillin-resistant strain. *S. aureus* is one of the most common causes of infection in humans, some examples include bacteraemia, infective endocarditis and device-related infections (Bowler, Duerden and Armstrong, 2001; Tong *et al.*, 2015).

3.2 METHODS

3.2.1 DISC DIFFUSION ASSAY (British Society for Antimicrobial Chemotherapy (BSAC))

3.2.1.1 Sample preparation

100mg/ml of plant extracts and fractions of *B. dalzielii* bark was dissolved in sterile distilled water (5%DMSO) (DMSO: water = 95:5) or 100% sterile distilled water depending on the extraction procedure.

3.2.1.2 Method

Microorganisms were cultured in Mueller Hinton broth at 37°C overnight, the suspension was adjusted to 0.5 McFarland standard by diluting 1ml of inoculum with 9ml of broth and absorbance was read at 625nm which was between 0.08-0.13 (similar to the density of a 0.5 McFarland standard). Where absorbance reading obtained was below required range, the suspension was adjusted by adding 0.5mL-1mL of inoculum and if reading is above required range, 0.5mL-1mL of broth was added.

Petri dishes were placed in the 37°C incubation room to dry off water on the lids for about 15 minutes. The working environment was sterilized and the Bunsen burner was

on to provide a sterile environment. A sterilized cotton swab was used to spread micro-organisms on the plate and labelled accurately. Autoclaved discs obtained from 6mm Whatmann glass filter paper were placed immediately on each plated Petri dish. This was repeated for all Petri dishes and 10µL (equivalent to 1mg) of the test sample was placed on the 6mm disc. 5% DMSO in sterile water or 100% sterile water was used as negative control. Petri dishes were incubated in the 37°C incubation room for 18hours. The results were interpreted by measuring the diameter of the clear zone around the wells and experiments were conducted in triplicates. Selected fractions were tested on micro-organisms using Mueller Hinton agar to further select compounds with better activity. Samples with activity were purified and submitted for identification analysis.

3.2.2 IDENTIFICATION TECHNIQUES

3.2.2.1 Fourier-transform infrared spectroscopy (FTIR)

FTIR analysis was conducted using FTIR Bruker Alpha spectrometer having a platinum diamond ATR sampling accessory. Samples were loaded on FTIR device with the aid of a clean spatula and scanned 16 times over the range of 4000-360 cm^{-1} and the spectral resolution was 2 cm^{-1}. Data obtained as absorbance spectrum was processed on OPUS software 7.2.

3.2.2.2 Nuclear magnetic resonance (NMR)

20mg of all samples were transferred into labelled NMR tubes and accurately labelled in 700µL deuterated solvent (dimethyl sulfoxide for gallic acid and water for pyrogallol). The operating settings on the Bruker av400 spectrometer include 399.94MHz at 298 K

operating frequency, 64 scans, 6.5 µs pulse, 32, 768 data points and 4,800 Hz spectral width was conducted to acquire spectra which were further processed on Topspin 2.1 software.

3.2.3 MINIMUM INHIBITORY CONCENTRATION (Clinical and Laboratory Standard Institute (CLSI))

3.2.3.1 Sample preparation

A stock solution of 5000mg/L was prepared using sterile water for extracted compounds (gallic acid and pyrogallol), and positive control (cefotaxime for *S. aureus* and vancomycin for MRSA). And the following concentrations 1024, 512, 256, 128, 64, 32, 16 and 8 µg/mL were obtained for all compounds.

3.2.3.2 Method

Three well-isolated colonies were selected from a 24hrs culture on Mueller Hinton agar plate and transferred into 10mL of Mueller Hinton broth which was incubated at 37˚C overnight. The suspension turbidity was adjusted via measuring absorbance between 0.08-0.013 at 625nm, similar to that of a 0.5 McFarland standard. From this suspension, 0.1ml was transferred into a tube containing 9.9ml of broth, a 1:100 dilution which is said to be 1×10^6 CFU/mL, which when 50µL is added to an equal volume of antimicrobial agent solution should give a final cell number ranging from 2×10^5 CFU/mL to 8×10^5 CFU/mL. Control was prepared by adding 50µL of Mueller Hinton broth to 50µL of cell suspension, while blank was prepared by adding 50µL of sterile water to 50µL of Mueller Hinton broth. Plates were inoculated within 30 minutes of standardizing the inoculum

suspension at 37°C for 18 ± 2 hours. MIC was read as the lowest concentration without visible with turbidity similar to the negative control. The purity of the test suspension used was checked by transferring 10uL aliquot from the growth control well into 10mLs of Mueller Hinton broth, from which 100µL was spread over the surface of Mueller Hinton agar plate and incubated overnight. Accepted number of colonies recommended is between twenty to eighty colonies. The graph is a presentation of the subtraction of absorbance read before incubation from absorbance read after 24hours incubation at 37°C.

3.2.4 INHIBITION OF BACTERIAL GROWTH

3.2.4.1 Sample preparation

Samples were prepared as per the Minimum Inhibitory Concentration and 2-fold concentration of the MIC obtained for both micro-organisms was prepared. For *S. aureus*, concentrations of gallic acid used include 256, 128 and 64 µg/mL and that of pyrogallol used include 128, 64 and 32 µg/mL. While for MRSA, concentrations of gallic acid used include 512, 256 and 128 µg/mL and that of pyrogallol used include 64, 32 and 16 µg/mL.

3.2.4.2 Spectroscopic Method

Minimum Inhibitory Concentration method was employed and the 96-well plate was covered with sterile gas permeable moisture barrier seal. The 96 well plate containing samples was incubated in the spectrophotometer, which was set to have a temperature

of 37°C, a wavelength of 625nm and the absorbance was set to be taken at every hour interval for 18hours.

3.2.4.3 10µL Spot assay

Minimum Inhibitory Concentration method was employed and samples were prepared in sterile bottles and incubated at a temperature of 37°C.

3.2.4.3.1 Examining the effect of centrifuging step

Two sterile bottles were inoculated to 1×10^6 CFU/mL and labelled as "Sample **A**" and "Sample **B**". At zero hours, 100µL of the inoculum was transferred from bottle **A** into sterile Eppendorf tube and 900µL sterile phosphate buffered saline was added. Samples were centrifuged at 8000rpm for 10 minutes and the supernatant was discarded. This was repeated twice, before re-suspending pellet in 1mL of sterile water and serially diluted to 10^{-8}. Sample **B** was treated similar to sample **A** without the centrifuging step. 10µL of each diluent was spread over labelled Mueller Hinton agar plates in triplicate. Plates were incubated overnight at 37°C. Colony forming unit per millilitre (CFU/mL) was determined by multiplying the average number of colonies by the dilution factor.

3.2.4.3.2 Method

Sterile bottles containing relevant agents were inoculated to 1×10^6 CFU/mL. At different time interval of 0, 4, 8, 12 and 24 hr(s), samples were treated similarly to sample **A.** Colony forming unit per millilitre (CFU/mL) was determined by multiplying the average number of colonies by the dilution factor. Bactericidal activity is determined

when there is ≥3 $\log_{10}$ (99.9%) reduction in colony forming units per mL after 18- 24 hours incubation. The experiment was two independent studies.

3.2.5 CHECKERBOARD SCREENING METHOD

3.2.5.1 Sample preparation

Samples were prepared as per that of Minimum Inhibitory Concentration.

3.2.5.2 Method

Microorganisms were prepared as per the Minimum Inhibitory Concentration method. 50µL of cell suspension was added to the well and adjusted to 100µL with 25µL of each of the two antimicrobial agents. Absorbance was read at 625nm before incubation and plate were incubated at 37°C for 24hours, and after incubation, absorbance was read again. The difference between readings before and after incubation was calculated (i.e. absorbance in one well after incubation – absorbance in that well after incubation), giving an absorbance change value. Bacterial growth is shown as an absorbance change of greater than 0.05. The lowest combination of antimicrobials together to inhibit growth are thus determined and fractional inhibitory concentration index (FICI) is calculated in order to establish the antimicrobial relationships: synergy, addition, indifference or antagonism.

Thus, FICIs were calculated using the equation:

$FICI = FIC_{\text{antimicrobial A}} + FIC_{\text{antimicrobial B}} = (MIC_{\text{antimicrobial A}} \text{ in combination} \div MIC_{\text{antimicrobial A}} \text{ alone}) + (MIC_{\text{antimicrobial B}} \text{ in combination} \div MIC_{\text{antimicrobial B}} \text{ alone})$

Interactions are then determined from the following limits: Synergy (S): FICI < 0.5, addition (A): 0.5 ≤FIC≤1, indifferent (I): 1 < FICI ≤2, antagonism (An): FICI > 2.

3.3 RESULTS AND DISCUSSION

3.3.1 BIOACTIVITY GUIDED FRACTIONATION (BGF)

3.3.1.1 Extraction procedure one

200g of dried and powdered bark was macerated in 2L of 50% aqueous methanol at room temperature, in the dark on a rotary shaker. The extract was filtered using a Büchner funnel with Whatman Nr 1 filter paper and extracted with ethyl acetate (3 × 300mL). The ethyl acetate fraction was evaporated under reduced pressure to obtain a dry residue, which was labelled fraction 1 (1.08g). The water fraction was acidified by dropwise addition of fuming hydrochloric acid to a final concentration of 2M HCL. The mixture was left to hydrolyse by boiling for 30 minutes and, after cooling, washed with ethyl acetate (3 x 300 mL) to obtain fraction 2 (1.72g).

Fraction 1 was run through a flash column (silica 60Å particle, 35-70micron, column dimensions 30cm x 3cm ID) using dichloromethane: methanol: formic acid (72: 18: 0.1) Fractions of 10mL were collected and tested by TLC. Similar looking fractions were pooled, resulting in four fractions: i.e. fraction 1A (4-10), 1B (11-14), 1C (15-20) and 1D (21-50). Fraction 2 was also run through a flash column under similar conditions and six fractions were collected, i.e. fraction 2A (13-16), 2B (17-27), 2C (28-57), 2D (58-97), 2E (98-121) and 2F (122-145) (see Figure 3.1).

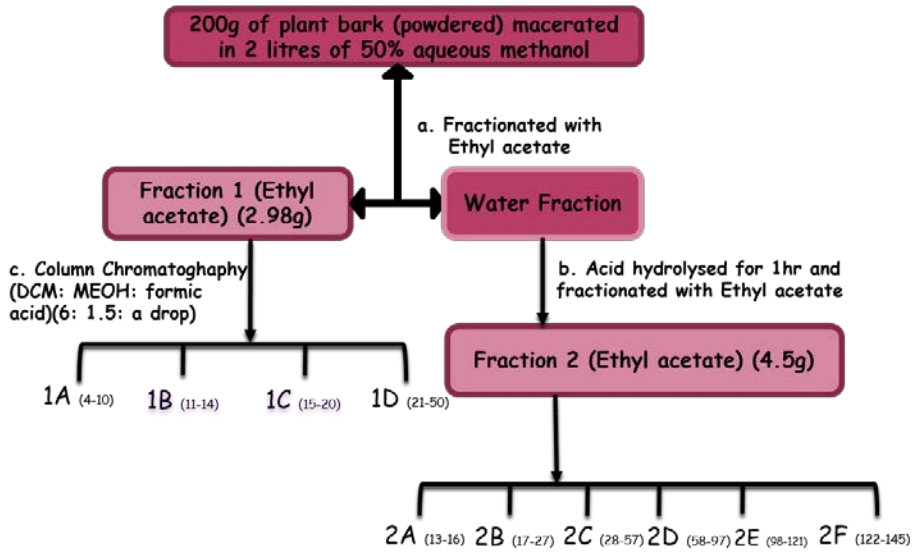


Figure 3. 1*: Extraction procedure using 50% aqueous methanol as solvent*

All column fractions obtained were prepared for antimicrobial disc diffusion assay. Absorbance recorded for adjusted suspensions of *Staphylococcus aureus* and MRSA was 0.112 and 0.108, respectively. Zone of inhibition obtained from the screening are shown in Table 3.1 and are recorded in millimetres ± standard deviation.

Fraction 1	*S. aureus* (mm)	MRSA (mm)	Fraction 2	*S. aureus* (mm)	MRSA (mm)
Fraction 1A	14.00 ± 0.00	8.50 ± 0.71	Fraction 2A	9 ± 1.00	
Fraction 1B	23.00 ± 2.83	18.50 ± 0.71	Fraction 2B	9.67 ±0.58	7.3 ± 0.58
Fraction 1C	19.67 ±0.58	14.67 ± 0.58	Fraction 2C	7.33 ± 0.58	
Fraction 1D	10.67 ± 0.58	8 ± 1.00	Fraction 2D	7.00 ± 0.58	
			Fraction 2E	7.67 ± 2.08	
			Fraction 2F		

Table 3. 1: *Zone of Inhibitions recorded for fractions obtained from 50% aqueous extract of B. dalzielii*

From the result obtained, 1B and 1C were selected for further purification, TLC of Fraction 1B and 1C revealed carryover of compounds from fraction 1B, due to this, the fractions were pooled together. Column chromatography was conducted using ethyl acetate: water: acetic acid (80: 10: 10) and four fractions were collected and named fraction 1Ba, 1Bb, 1Bc and 1Bd (see Figure 3.2). Fractions collected were further tested on both *S. aureus* and MRSA. Only fraction 1Bc had activity against both micro-organisms. From this fraction, 3.2mg compound was obtained, purified and labelled B1 via chromatography using ethyl acetate: water: acetic acid (86: 7: 7).

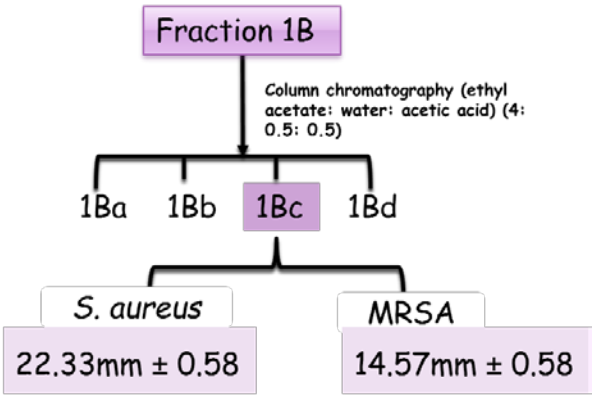


Figure 3. 2*: Fractions tested against* S. aureus *and MRSA*

3.3.1.2 Extraction procedure two

200g of plant material was macerated in 2L of 100% distilled water for six hours at room temperature, extract was filtered and vacuum-concentrated on freeze dryer. Crude extract was washed with ethyl acetate to obtained fraction 1. With the aid of 10-40µ silica gel, fraction 1 (0.76g) was column chromatographed with ethyl acetate: water: acetic acid (80: 10: 10) and 10 fractions were collected, namely, A (3-5), B (6-9), C (10-19), D (20-24), E (25-31), F (32-42), G (43-49), H (50-60), I (61-83) and J (84-92) (see Figure 3.3).

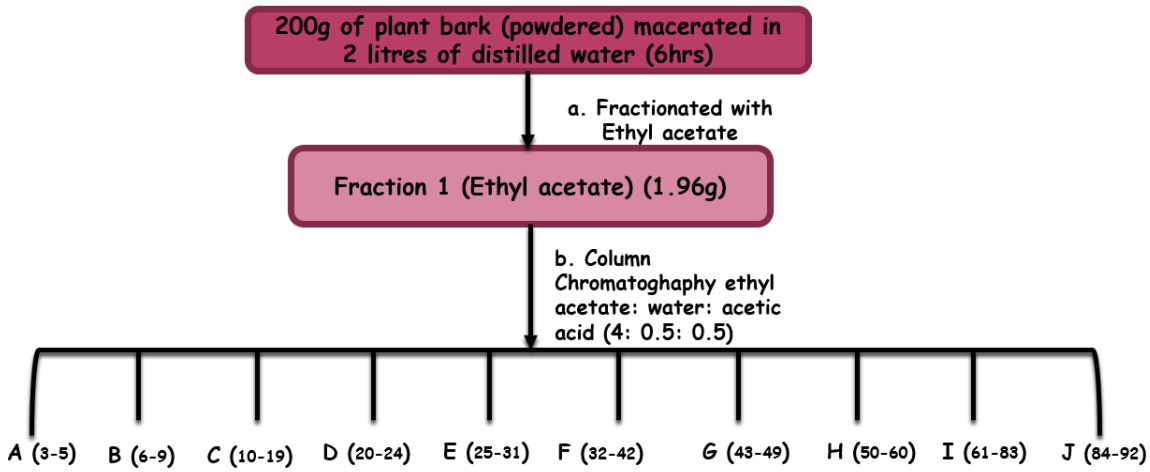


Figure 3. 3: *Extraction procedure using distilled water as solvent for six hours*

Equivalent of 100mg/mL of all fractions were prepared for antimicrobial disc diffusion assay using distilled water as solvent. Absorbance recorded for adjusted suspensions of *Staphylococcus aureus* and MRSA were 0.13 and 0.110, respectively. From these fractions, only fractions F and G had activity as shown in Table 3.2.

Fraction 1	*S. aureus* (mm)	MRSA (mm)
Fraction F	9.33 ± 0.58	
Fraction G	21.33 ± 1.15	14.33 ± 0.58

Table 3. 2: *Zone of inhibition recorded for fractions obtained from aqueous extract of* B. dalzielii

The TLC of both F and G reveal similar TLC chromatogram when viewed under the short wavelength, therefore both fractions were combined and further purified to obtain compound B2 (1.6mg) (see figure 3.4)

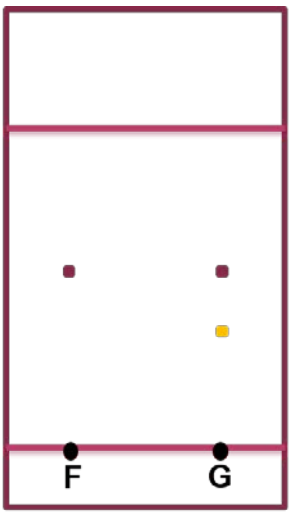


Figure 3. 4: *TLC illustration of fraction F and G*

3.3.1.3 Extraction procedure three

The extraction was conducted as used ethno-pharmacologically, the plant material is normally macerated in water for as long as the plant compounds dissolved in water with water being added when depleted, and the colour is an indication of when the plant cannot be extracted further. The plant extraction can be left for as long as a week, the visible change in the plant extract is the odour if left untouched.

200g of plant material was macerated in 2L of distilled water for 24hrs, the extract was filtered and vacuum-evaporated. The crude extract obtained was washed with ethyl acetate to obtained fraction 1. With the aid of 35-70µ silica gel, fraction 1 (1.26g) was column chromatographed with hexane: ethyl acetate: water: acetic acid (5: 15: 5: 5) and 5 fractions were collected, namely B1 (1-15) 206mg, B2 (16- 35) 197mg, B3 (40-55) 86mg, B4 (56-64) 62mg and B5 (70-89) 94mg (see Figure 3.5). All fractions (equivalent of 50mg/mL) collected were tested on *S. aureus* and MRSA. Absorbance recorded for

adjusted suspensions of *Staphylococcus aureus* and MRSA were 0.110 and 0.106, respectively.

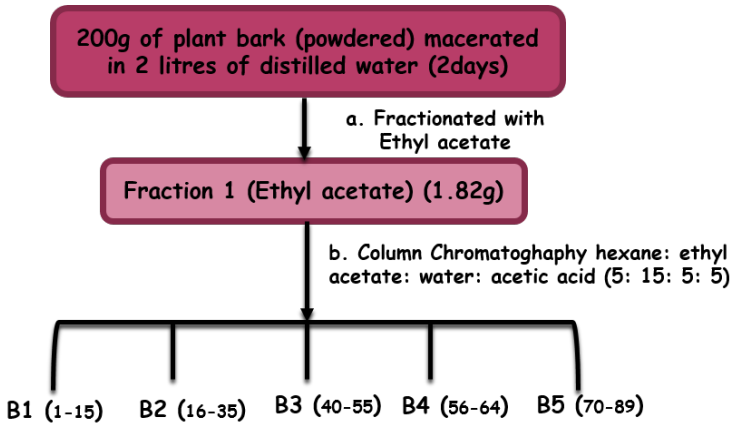


Figure 3. 5: *Extraction procedure using distilled water as solvent for 24hrs at 37˚C*

Of the five fractions, only two fractions had activity as seen in Table 3.3.

Fraction 1 (50mg/mL)	*S. aureus* (mm)	MRSA (mm)
B1	35.00 ± 1.00	38 ± 2.65
B2	24.00 ± 1.00	25.00 ± 1.73

Table 3. 3: *Zone of inhibition recorded for fractions obtained from 24hours maceration*

Fraction B1 and B2 were pooled together and further column chromatographed on 10-40µ silica gel to give six fractions labelled fraction 1 (1-20), fraction 2 (22-27), fraction 3 (38-46), fraction 4 (98-113), fraction 5 (116-135) and Fraction 6 (139-159). TLC of fractions was conducted using hexane: ethyl acetate (50:50 and a drop of formic acid). Fraction 2 and fraction 3 has similar chemical profile as seen in Figure 3.6 below, therefore the fractions were pooled together and labelled fraction 3.

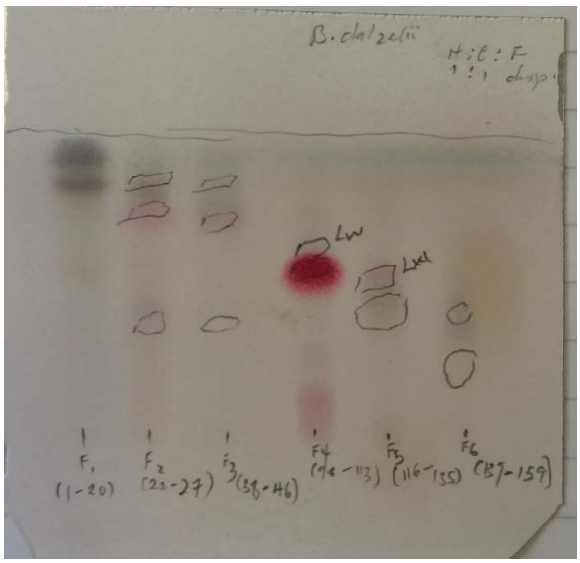


Figure 3. 6: *TLC chromatogram of fractions tested against S. aureus and MRSA*

50 mg/mL of all the five fractions was measured and tested on *S. aureus* and MRSA using the disc diffusion method and sterile distilled water was used as solvent. Absorbance recorded for adjusted suspensions of *S. aureus* and MRSA were 0.123 and 0.118 (See Figure 3.7), respectively. Zones of inhibition were measured for fraction 3, fraction 4 and fraction 6, see Table 3.4.

Fraction B1&2	*S. aureus* (mm)	MRSA (mm)
F3	24.67 ± 0.58	24.33 ± 0.58
F4	34.67 ± 0.58	31.00 ± 1.00
F6	23.67 ± 0.58	16.67 ± 1.53

Table 3. 4: *Zone of inhibition recorded for fractions obtained from pooled fraction B1 and B2*

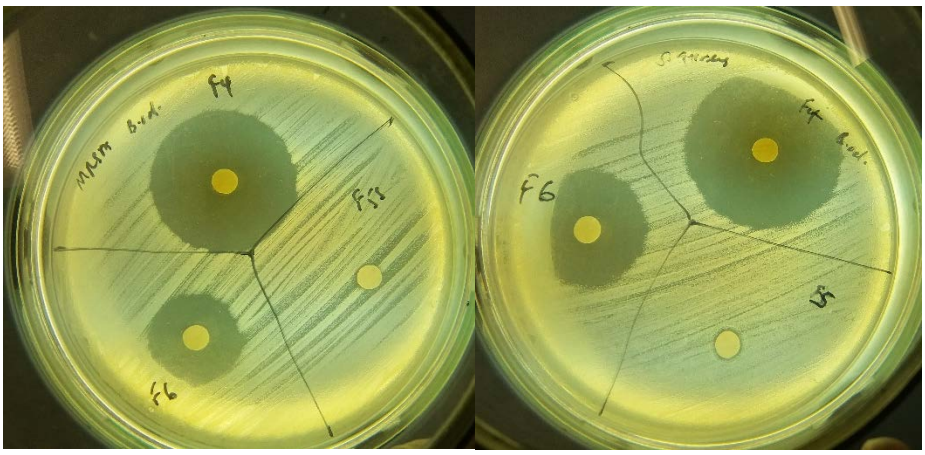


Figure 3. 7: *Image showing clear zone of inhibition obtained for* B. dalzielii *fractions against S. aureus and MRSA*

Major compounds of fraction 3 and fraction 4 were purified on 10-40µ silica gel using hexane: ethyl acetate (50:50 and a drop of formic acid) and labelled as B3 (8.6mg) and B4 (19mg). Major compound present in fraction 6 was not purified, this was due to the unstable nature of the compound in the extract.

3.3.1.4 Compounds obtained via BGF

TLC analysis of B1 obtained from procedure 1, B2 obtained from procedure 2, B3 obtained from procedure 3 and B4 obtained from procedure 3 was conducted using hexane: ethyl acetate (50:50 and a drop of formic acid) (see Figure 3.8). Result showed that compound B1, B2 and B3 have similar rf value of 0.20 while B4 has a rf value of 0.61. Purified compounds were then submitted for NMR, FTIR and HRMS analysis.

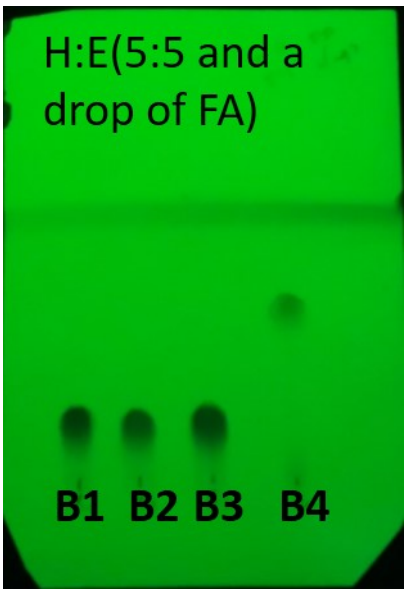


Figure 3. 8: *TLC chromatogram comparing all compounds collected from the three extraction procedures*

In the present study, manual fractionation was conducted using column chromatography, although this technique is tedious, it leads the researcher to specific compounds with biological activity i.e. antimicrobial activity. Different research studies have used the bioactivity guided fractionation in the search for active compounds in plants and natural products. Examples can be seen in the works of Michel *et al.*, (2013) seeking compounds with antihyperglycemic and anti-inflammatory activities from *Pterocarpus dalbergioides*; and that of Cunha *et al.*, (2017) in seeking compounds with antimicrobial and cytotoxic activities from *Cassia bakeriana*. However, the main challenges of this technique are obtaining a low yield of active compounds in most cases and the dereplication of known compounds with little/no special interest in the discovery of a lead compound in drug discovery (Weller, 2012). Bioactivity guided fractionation of the bark of *B. dalzielii* lead to the extraction of two active compounds which were purified and submitted for identification analysis.

3.3.2 IDENTIFICATION OF COMPOUNDS B1 AND B4

3.3.2.1 High resolution mass spectrometry (HRMS) results

5mg of compound B1 and B4 plus respective references were used for HRMS which was conducted at EPSRC National Facility Swansea. Results showed that observed mass of B1 was measured as MW 170.0179g/mol [$C_7H_6O_5$]; MS, m/z [E1-, %], 169.0142 (M-H, 100%) (See Figure 3.9) and predicted molecular composition was $C_7H_5O_5$. While that of B4 was predicted as MW 126.0280g/mol [$C_6H_6O_3$]; MS, m/z [E1-, %], 125.0247 (M-H, 100%) (see Figure 3.10) and predicted molecular composition is $C_6H_5O_3$.

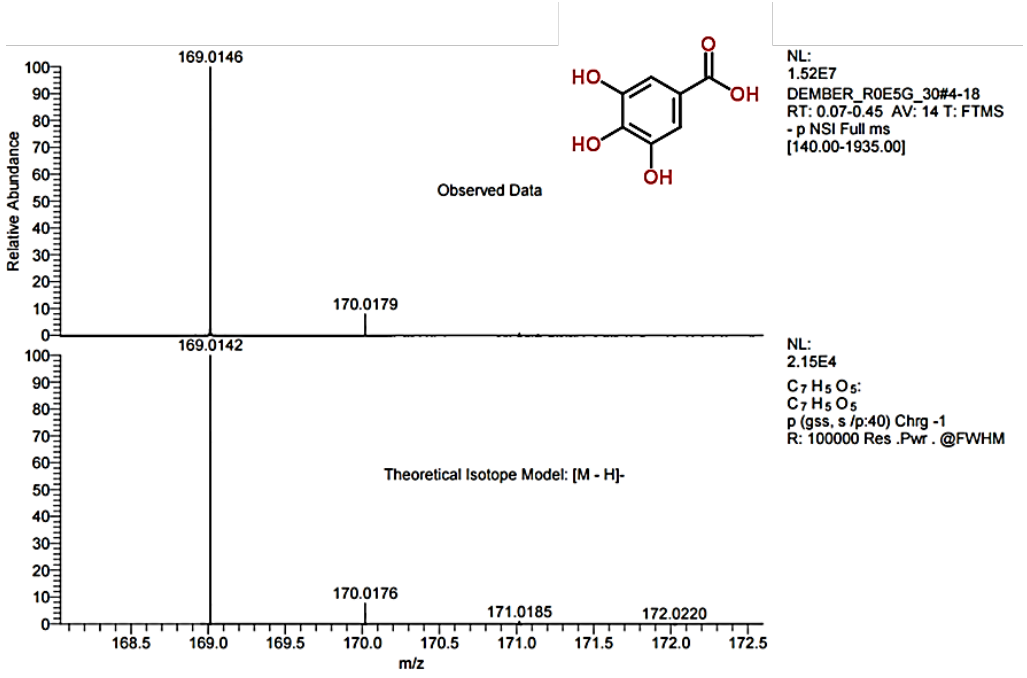


Figure 3. 9: *HRMS spectrum of compound B1 and purchased gallic acid suggesting that extracted compound B1 might be gallic acid.*

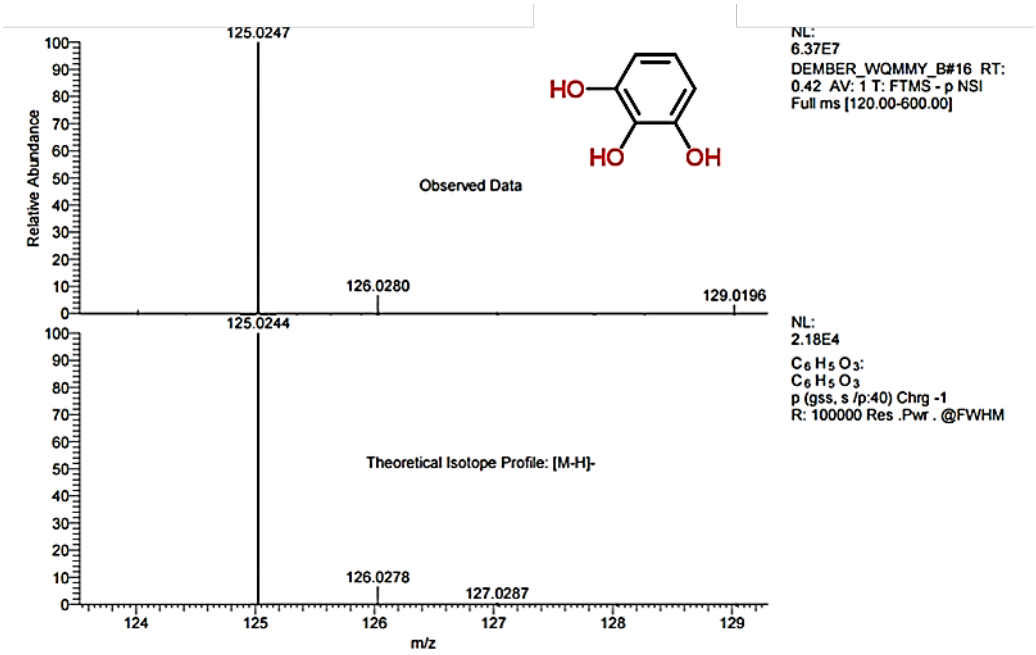


Figure 3. 10: *HRMS spectrum of Compound B4 and purchased pyrogallol suggesting that extracted compound B4 might be pyrogallol*

3.3.2.2 Fourier-transform infrared spectroscopy (FTIR) results

Samples analysed include extracted compound B1 (Figure 3.11) and B4 (Figure 3.12), and control compounds used are gallic acid and pyrogallol purchased from Sigma Aldrich. Figure 3.11 and Figure 3.12 show prominent peaks at 3267.5 cm^{-1} and 3210.32 cm^{-1}, which are indicative of compounds with hydrogen bonded alcohols/phenols in both extracted and purchased gallic acid and pyrogallol, respectively.

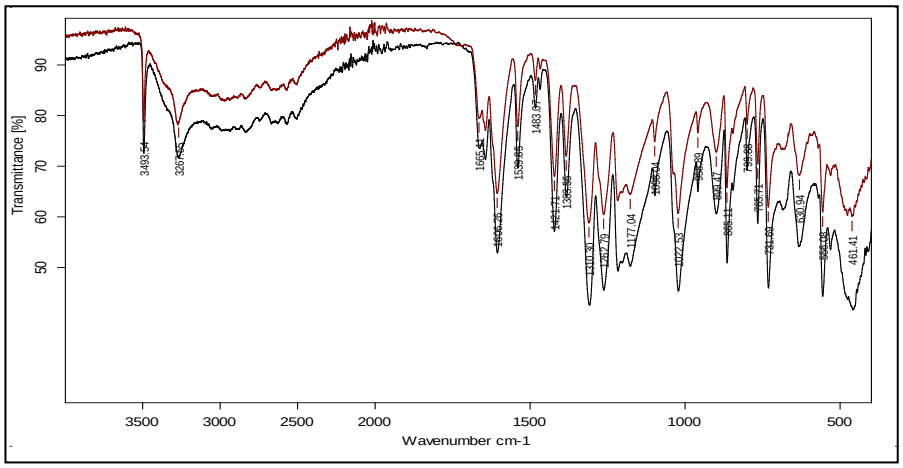


Figure 3. 11: *FTIR spectrum of gallic acid overlaying that purchased from Sigma Aldrich. The spectra of purchased gallic acid is consistent with that extracted from plant bark of* B. dalzielii.

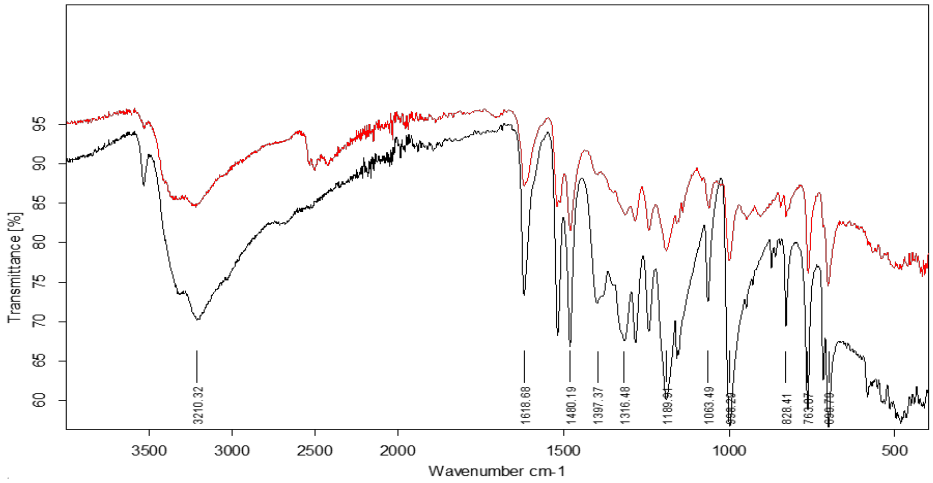


Figure 3. 12: *FTIR spectrum of pyrogallol overlaying that purchased from Sigma Aldrich. The spectra of purchased pyrogallol is consistent with that extracted in this study.*

3.3.2.3 Nuclear magnetic resonance (NMR) results

NMR analysis was conducted using a 20mg sample and reference gallic acid in 700µL deuterated dimethyl sulfoxide for both ^{1}H and ^{13}C analysis, and 20mg sample and reference pyrogallol in 700µL deuterated water for both ^{1}H and ^{13}C analysis. ^{1}H-NMR spectrum of gallic acid (sample and reference) showed a single signals at δ_H 6.92 ppm (2H, *s*), δ_H 8.82 ppm (H, *s*), δ_H 9.18 ppm (2H, *s*) and carbonyl proton at δ_H 12.23 ppm

(H, *s*) (Figure 3.13) while ^{13}C-NMR spectrum showed five signals at δ_C 145.4 ppm (C-3, C-1), δ_C 137.9 ppm (C-2), δ_C 120.4 ppm (C-5), δ_C 108.7 ppm (C-4, C-6), and δ_C 167.4 ppm for carbonyl group (Figure 3.14). ^{1}H-NMR spectrum of pyrogallol (sample and reference) showed two signals at δ_H 6.42-6.46 ppm (2H, *d*) and δ_H 6.60-6.67 ppm (1H, *t*) (Figure 3.15) while ^{13}C-NMR spectrum showed four signals at δ_C 145.37 ppm (C-3, C-1), δ_C 132.34 ppm (C-2), δ_C 120.50 ppm (C-5) and δ_C 108.38 ppm (C-4, C-6).

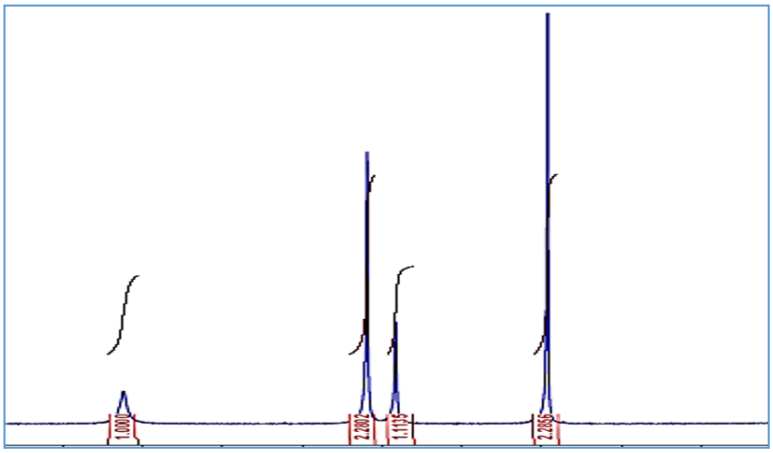


Figure 3. 13: *^{1}H-NMR spectrum of purchased gallic acid is consistent with that extracted from plant bark of* B. dalzielii. *^{1}H-NMR spectrum was obtained using bruker av400 spectrometer at 399.94 MHz using deuterated dimethyl sulfoxide.*

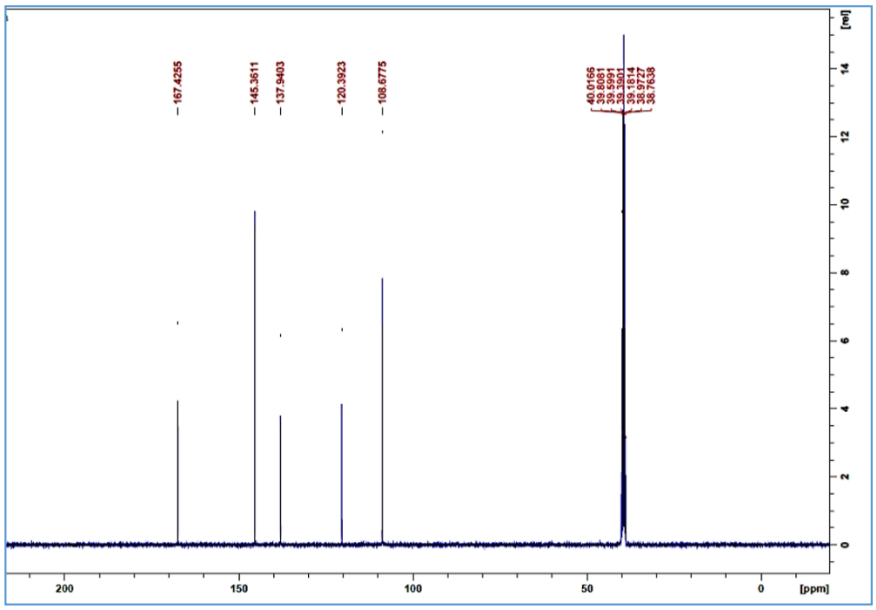


Figure 3. 14: *^{13}C-NMR spectrum of gallic acid is consistent with that extracted from plant bark of B. dalzielii. ^{13}C-NMR spectrum was obtained using bruker av400 spectrometer at 100.58 MHz using deuterated methanol.*

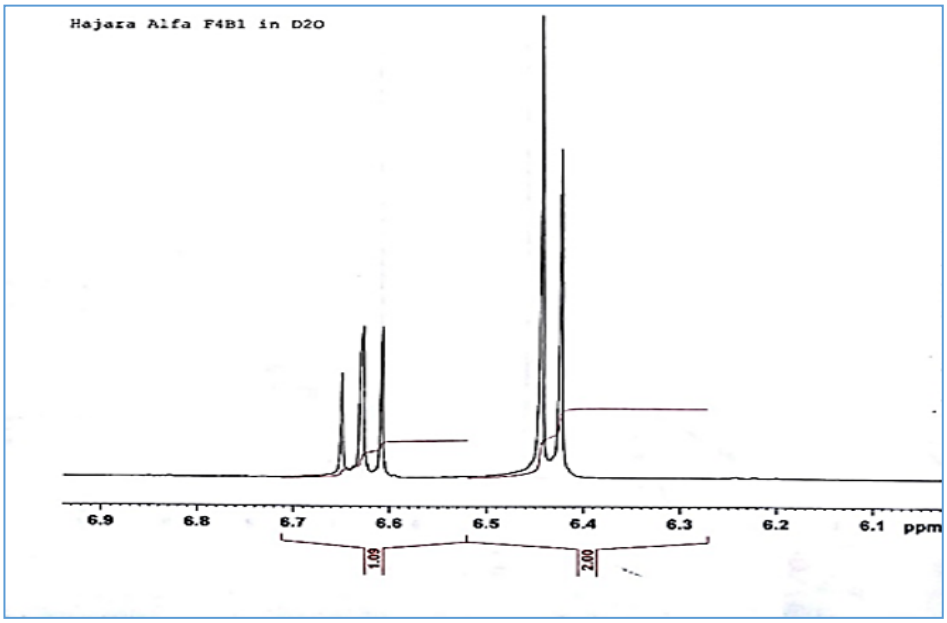


Figure 3. 15: *^{1}H-NMR spectrum of purchased pyrogallol is consistent with that extracted from plant bark of* B. dalzielii*. ^{1}H-NMR spectrum was obtained using bruker av400 spectrometer at 399.94 MHz using deuterated methanol.*

Although both extracted and purchased gallic acid (3,4,5-trihydroxybenzoic acid) have similar signals, the ^{13}C signals reported is identical to that reported in the studies

conducted on the oxidation of gallic acid (López *et al.*, 2013). H-NMR of gallic acid reported in the present study is similar to that reported in the studies on phenolic acids present in mango and papaya (López-Martínez *et al.*, 2015). Both the ^{13}C and ^{1}H signals of gallic acid reported in the present studies correspond with that extracted from *Elaeagnus angustifolia* (Abri, 2015). The studies conducted by Crisponi and colleagues (1990) reported an identical ^{13}C-NMR signal of pyrogallol (1,2,3-trihydroxybenzene) (Crisponi *et al.*, 1990).

3.3.3 MINIMUM INHIBITORY CONCENTRATION

The *Staphylococcus* strain used in the present studies is *S. aureus* NCTC 6571. This strain is susceptible to almost all antibiotics except colistin and polymyxin and is usually used as a control strain (PHE, 2017). MRSA NCTC 12497 was used as a test strain. MIC results presented were obtained from three independent studies and results are the mode of repeated concentrations. The graph shows data presented as the mean of absorbance and the error bars represent the standard deviation of the means.

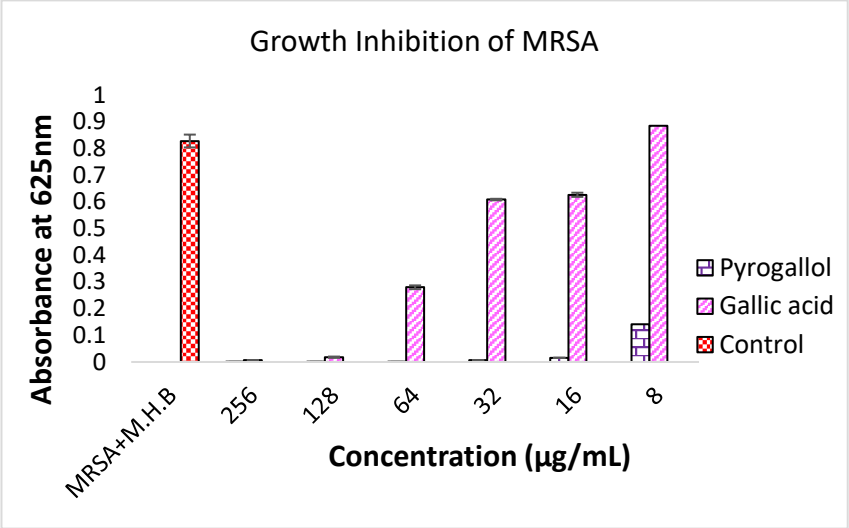


Figure 3. 16: *Growth inhibition graph of pyrogallol and gallic acid against MRSA*

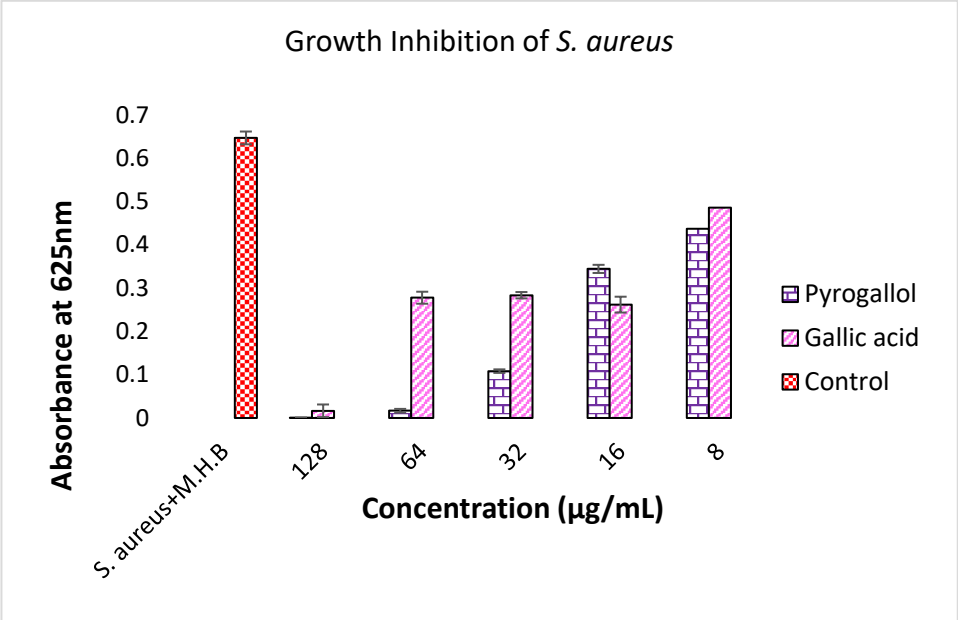


Figure 3. 17: *Growth inhibition graph of pyrogallol and gallic acid against S. aureus*

The MIC of pyrogallol against MRSA and *S. aureus* growth are 32 and 64 µg/mL, respectively, which correspond to 254 and 508 µM. While, that of gallic acid against MRSA and *S. aureus* growth are 256 and 128 µg/mL, respectively, which correspond to 2032 and 753 µM (Figure 3.16 and Figure 3.17). In addition, no visible growth was observed for all positive control. From the data obtained, pyrogallol was observed to have better antibacterial activity against MRSA compared to gallic acid and act poorly against the control strain. The studies of Borges *et al.*, (2013) showed that gallic acid has a MIC of 1750 µg/mL against *S. aureus* CECT 976. Gallic acid was shown to have antibacterial activity against *Pseudomonas* strains with MIC values above 2000 µg/mL (Sorrentino *et al.*, 2018). Pyrogallol was shown to have antibacterial activity with MIC value of 32 – 64 µg/mL against *V. parahaemolyticus* isolated from leg shrimp (*Litopenaeus vannamei*) (Tinh *et al.*, 2016).

3.3.4 INHIBITION OF BACTERIAL GROWTH

3.3.4.1 Spectroscopic method

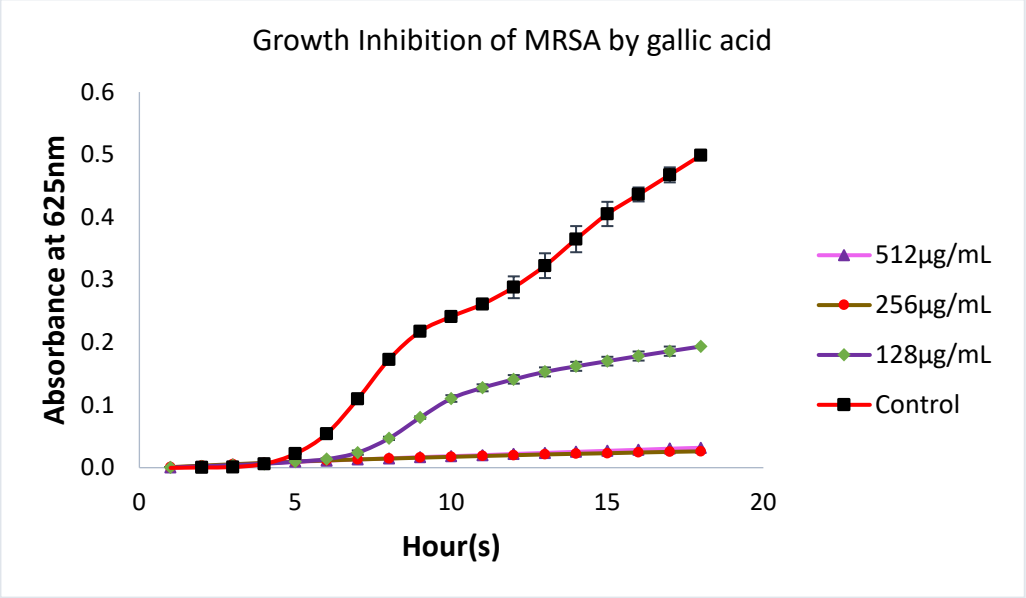


Figure 3. 18: *Spectrophotometric growth curve of gallic acid concentrations of 512, 256 and 128 µg/mL against MRSA*

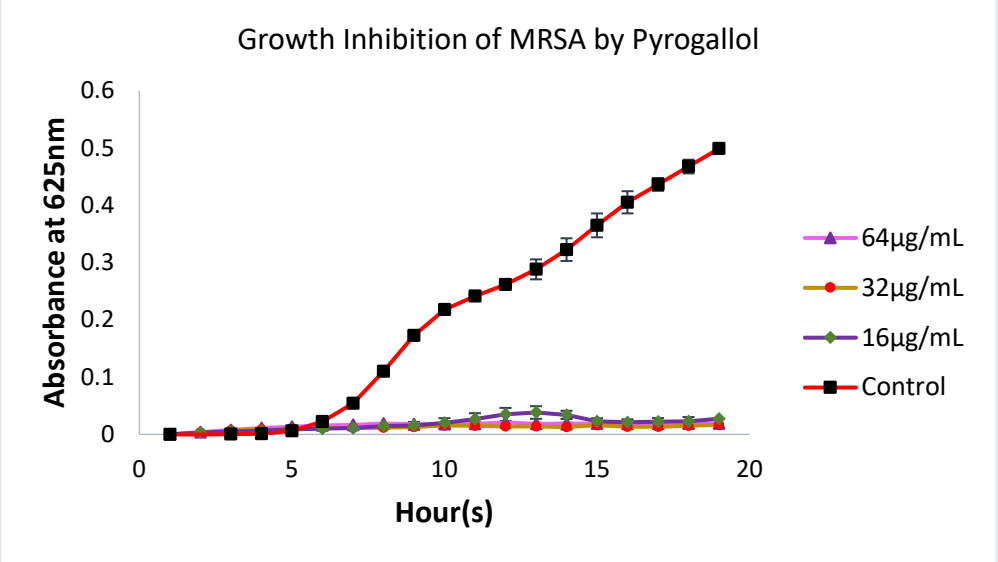


Figure 3. 19: *Spectrophotometric growth curve of pyrogallol concentrations of 64, 32 and 16 µg/mL against MRSA*

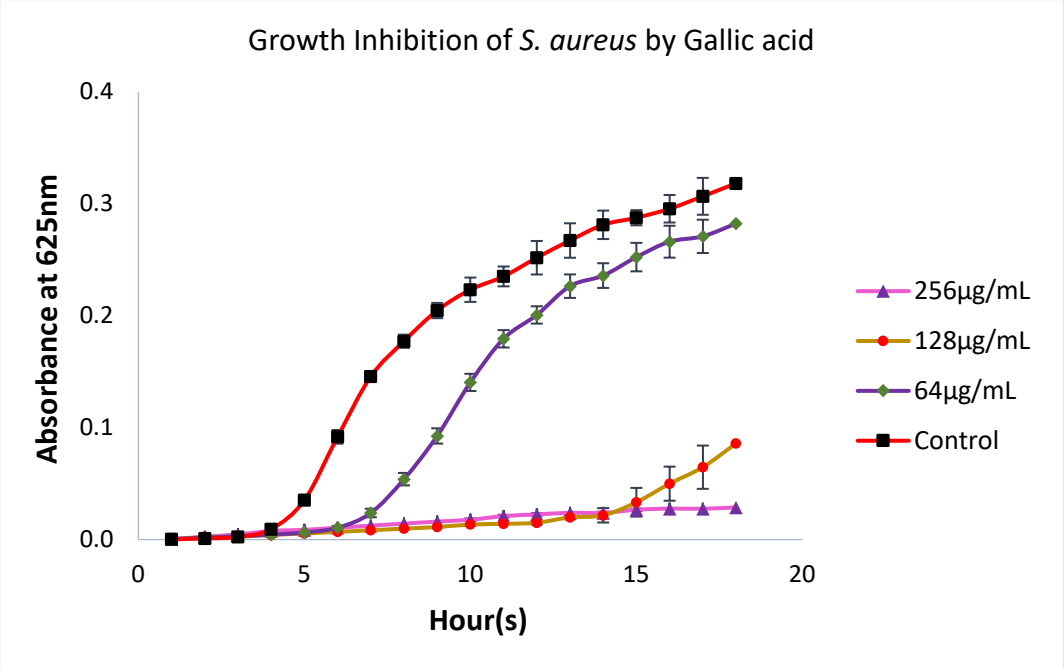


Figure 3. 20: *Spectrophotometric growth curve of gallic acid concentrations of 256, 128 and 64 µg/mL against S. aureus*

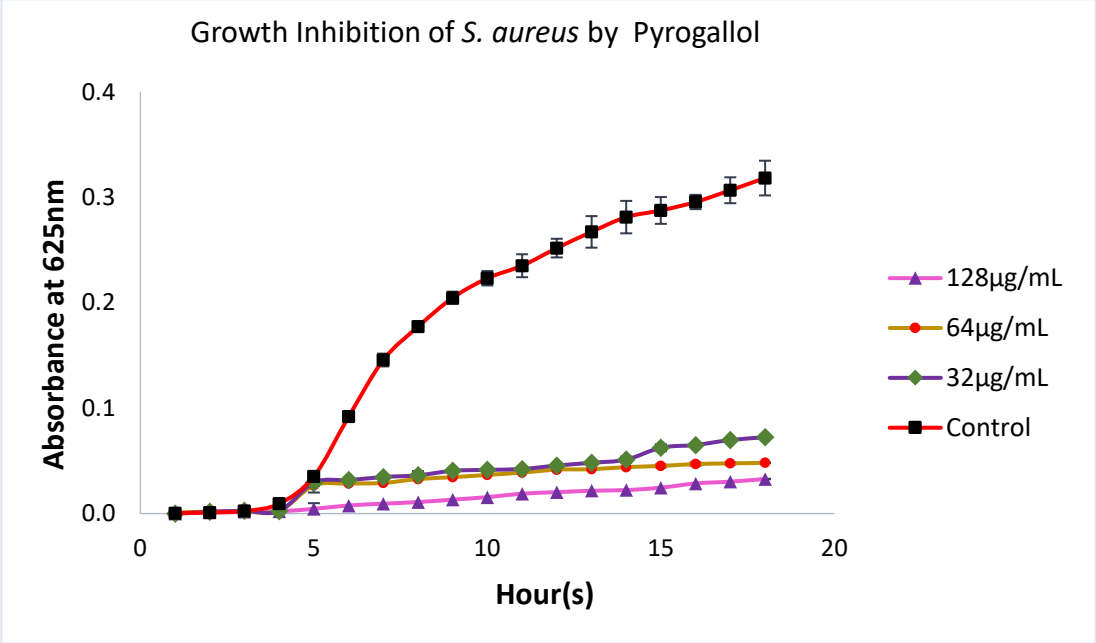


Figure 3. 21: *Spectrophotometric growth curve of pyrogallol concentrations of 128, 64 and 32 µg/mL against S. aureus*

Growth inhibition assay was conducted using spectrophotometer at 625nm. The data obtained showed a decrease in absorbance when both microbial strains were treated

with pyrogallol (as seen in Figures 3.19 and 3.21) when compared to gallic acid (as seen in Figures 3.18 and 3.20). It is easy to speculate that gallic acid could be bacteriostatic while pyrogallol could be bactericidal, however, this speculation could be wrong. Thus, the 10µL spot assay was conducted to determine the minimum bactericidal concentration via obtaining the number of viable bacteria cells after treatment with gallic acid and pyrogallol.

3.3.4.2 10µL Spot assay

The method of Hamoud *et al.*, (2014) was modified in determining the MBC values of both pyrogallol and gallic acid against MRSA and *S. aureus.* However, a neutralizer was not used in arresting the action of both gallic acid and pyrogallol on both bacteria strains used. Therefore, a centrifuging step was introduced. T-test analysis assuming unequal variance was used and p-value (0.35) obtained was greater than 0.05, indicating that there is no significant difference between the two data obtained for centrifuged and un-centrifuged samples.

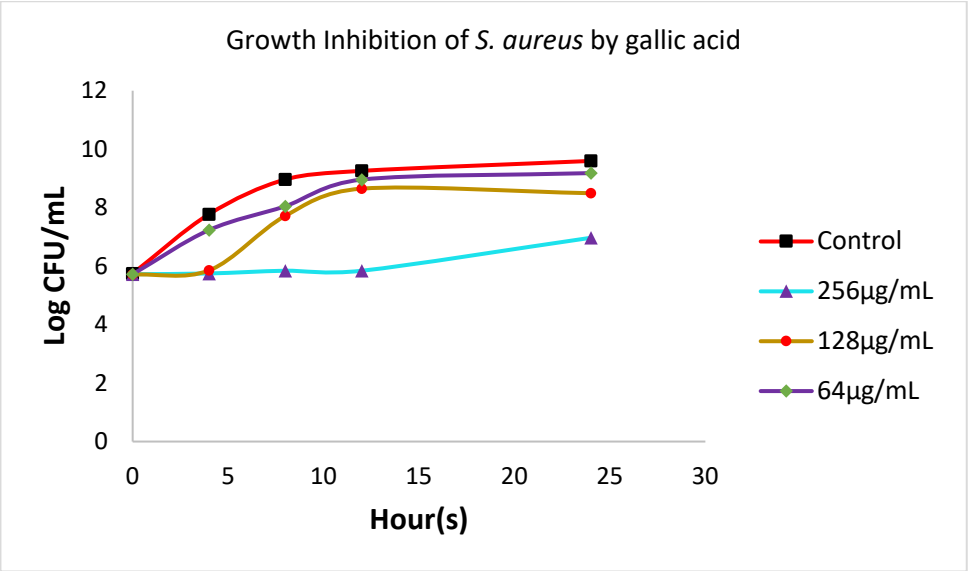


Figure 3. 22: *Growth Inhibition of S. aureus by gallic acid*

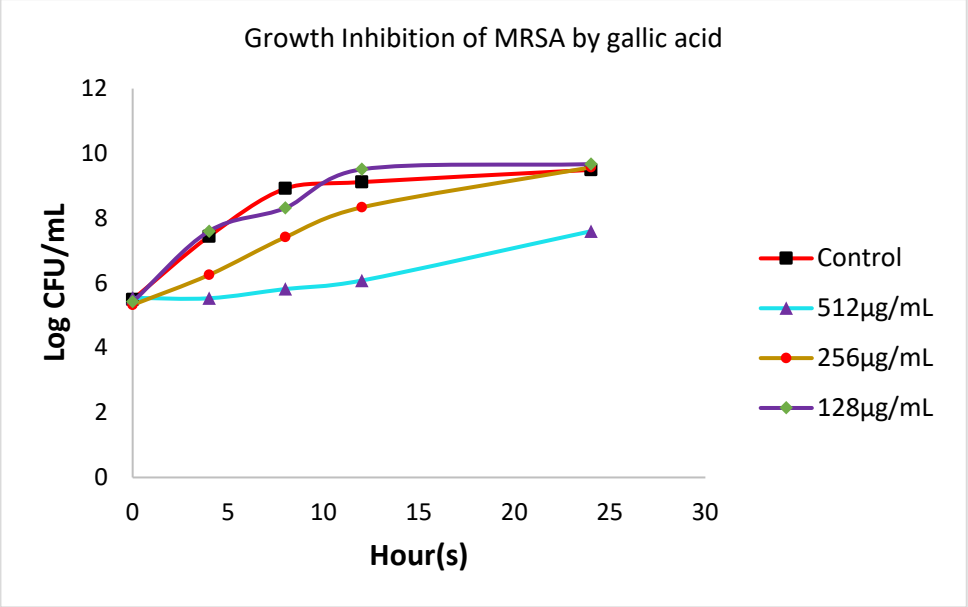


Figure 3. 23: *Growth Inhibition of MRSA by gallic acid*

At 24hours, gallic acid was shown to affect the growth of *S. aureus* with an average log reduction of 2.6 CFU/mL at a concentration of 256 µg/mL (1506µM), reducing bacterial growth to 6.9 CFU/mL from 9.5 CFU/ml (Figure 3.22) whilst its effect on the growth of MRSA was an average log reduction of 1.9 CFU/mL at a concentration of 512 µg/mL

(3012µM) (Figure 3.23), thus suggesting that gallic acid exhibit bacteriostatic function, preventing bacterial growth at a very high concentration.

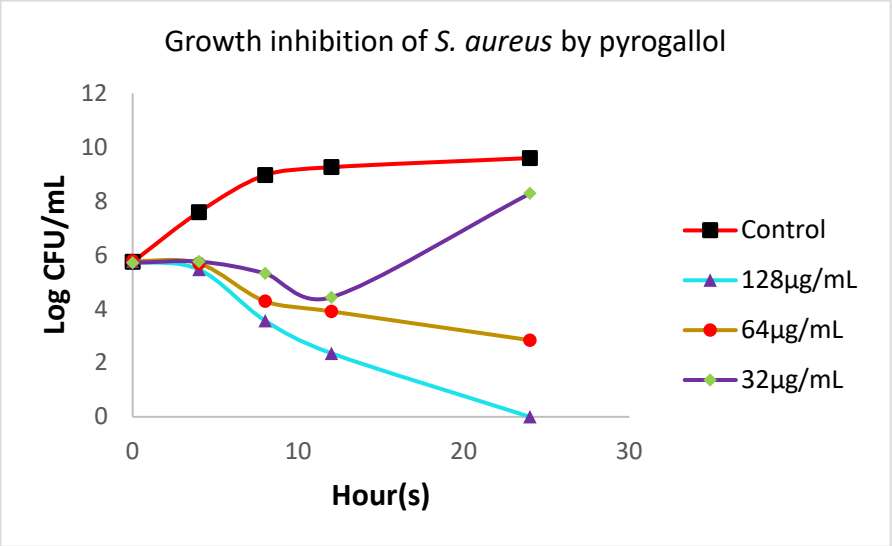


Figure 3. 24: *Growth inhibition of S. aureus by pyrogallol*

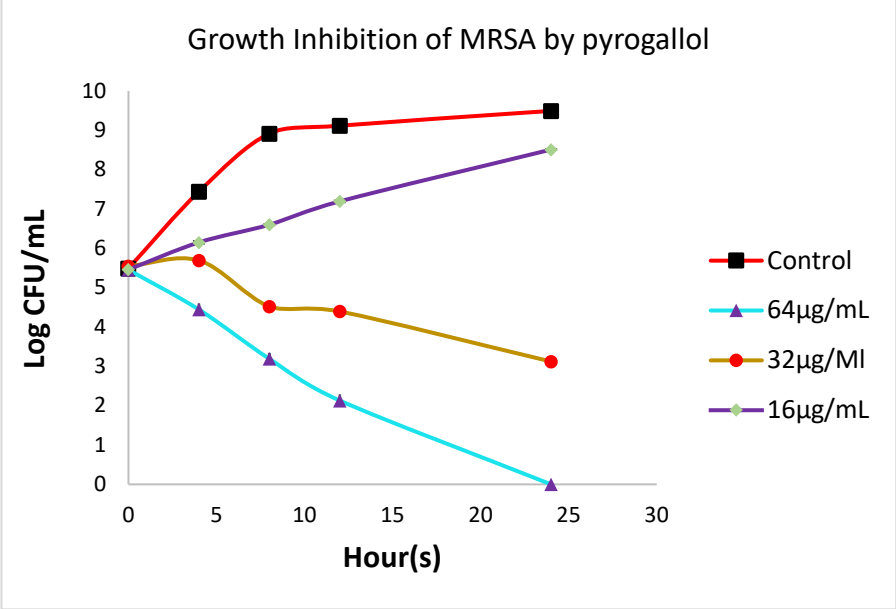


Figure 3. 25: *Growth Inhibition of MRSA by Pyrogallol*

At 24hours, pyrogallol completely inhibits the growth of both MRSA and *S. aureus* (Figure 3.24 and 3.25). At 12hours at a concentration of 128 µg/mL (1016µM), the compound affects the growth of *S. aureus* with an average log reduction of 7.1 CFU/mL,

reducing bacterial growth from 9.2 CFU/mL to 2.1 CFU/mL (Figure 3.24). Whilst its effect on the growth of MRSA was an average log reduction of 7.0 at a concentration of 64 µg/mL (508µM), reducing bacterial growth from 9.1 CFU/mL to 2.1 CFU/mL (Figure 3.25), thus indicating bactericidal function.

3.3.5 CHECKERBOARD SCREENING

The checkerboard screening method was used to determine potential interactions between pyrogallol and gallic acid against tested organisms (*S. aureus* and MRSA). The test was conducted in a three independent studies.

Gallic acid (µg/mL)					
512					
256					
128	1.625	2.011	2.794		
64	1.653	1.582	2.1209		
32	20.166	1.609	1.8179		
Pyrogallol (µg/mL)	**16**	**32**	**64**	**128**	**256**

Table 3. 5: *FICI obtained for S. aureus using equation provided. All combinations used are either indifferent or antagonistic.*

Gallic acid (µg/mL)					
512					
256	2.0180	1.9992	2.3653		
128	18.7909	18.9504	1.5956		
64	18.7909	5.4345	5.2093		
Pyrogallol (µg/mL)	**16**	**32**	**64**	**128**	**256**

Table 3. 6: *FICI obtained for MRSA using equation provided. All combinations used are either Indifferent or antagonistic.*

Absorbance obtained was used in calculating fractional inhibitory concentration index (FICI) and from the data obtained, no synergy interaction was observed for the combinations of pyrogallol and gallic acid used against *S. aureus* (Table 3.5) and MRSA (Table 3.6), in both cases, the FICI was above 0.5.

3.4 CONCLUSION

The checkerboard method was conducted as a screening method to evaluate the interaction between gallic acid and pyrogallol. The rationale behind the assay is due to the fact that *B. dalzielii* is used in the Northern part of Nigeria, a region that has a tropical dry climate. The country experiences a temperature range of 13 – 41°C (55.4 – 105.8°F) (Cooke and Mohammed, 2009). Micro-organisms are known to grow at different temperature in natural habitat example is the presence of *leucothrix mucor* on seaweeds (Hollants *et al.*, 2013). Plants and the soil on which they grow are natural habitats to micro-organisms, an example is the presence of *Arthrobacter globiformis* in soil (Casida and Jr., 1969). Maceration of plant materials in a tropical region could results in bioconversion by microorganisms present on the said plant. The presence of these microorganisms could be a result of contamination and/or habitat ecosystem. In the

end, ingesting plant extract obtained via cold maceration could contain both plant phytochemicals and metabolites obtained via bioconversion after a certain time interval. In the case of the present study, an individual could be ingesting both gallic acid and pyrogallol, where the latter was shown to have better antibacterial activity against *S. aureus* and its methicillin-resistant strain when compared to the former. Having more than one antibacterial compound in an extract could results in the possibility of synergetic interaction between the compounds, thus attributing the activity of said extract to the interaction. Therefore, it is important to evaluate their interaction. However, the checkboard screening assay showed that the interaction between gallic acid and pyrogallol is not synergistic rather antagonistic and indifferent for both MRSA and *S. aureus* depending on concentrations (see Table 3.5 and 3.6). The possibility of pyrogallol as a metabolic by-product of bioconversion was further investigated (See next Chapter). To the best of the author`s knowledge, this is the first work reporting the presence of pyrogallol in 24 hrs aqueous extract of *B. dalzielii* bark.

4

BIOCONVERSION OF GALLIC ACID BY *Enterobacter cloacae*

"The art of healing comes from nature and not from the physician. Therefore, the physician must start from nature with an open mind."

– Paracelsus 1493-1541

ABSTRACT

The role of endophyte was investigated on aqueous extract of *Boswellia dalzielii* bark, this led to the isolation and identification of *Enterobacter cloacae* and *Pantoea spp*. However, only *E. cloacae* bioconverted gallic acid to pyrogallol. The 16S rRNA sequences of both *E. cloacae* and *Pantoea spp* are deposited in the GenBank nucleotide database under the accession number MH764584 and MH764583, respectively.

4.1 INTRODUCTION

In folk medicine, maceration is one of the conventional method of extraction used and the matrix properties of the plant part, temperature and time contribute to the extract obtained. Ethnopharmacologically, this process is mostly a septic technique where the sterile conditions of materials used and that of the environment is not put into consideration. Thus, the source of contamination of plant macerated extract could be from the plant itself, the water and material used, and the environment.

The Hausa in the Northern part of Nigeria macerate the bark of *B. dalzielii* in cold water, and the resulting extract is used as a dietary supplement, or in the treatment of infections and tumours. Cold maceration of plants leads to the release of hydrophilic phytochemicals which may subsequently be metabolised by microorganisms residing in the herbal material, especially in temperate regions, resulting in a chemical and physical change (Young, 1974; Schaffner and Beuchat, 1986; Hilton and Cain, 1990). Almost all plants in nature are thought to live in symbiosis with endophytes which help fight pathogenic infection and environmental stress condition, thus promoting plant growth (Santoyo *et al.*, 2016). Specific microorganisms such as *Rhizobia* and endophytes have a symbiotic close relationship (agonism or mutualism) with their host plant; a further example is the interaction between some *Poaceae* grass family and endophytic fungi belonging to the genus *Epichloë* (Clavicipitaceae) (Gunatilaka, 2006; Saikkonen *et al.*, 2016). Therefore, microorganisms living on the surface or inside plants can be isolated, identified and their role can be defined (Hallmann *et al.*, 1997).

The previous chapter on the antimicrobial activity of *B. dalzielii* reported that pyrogallol was identified in an aqueous extract after maceration for 24h, whereas this compound was not detected in extracts either after six hours in water or after 24h in 50% aqueous methanol extracts. Therefore, it is important to further investigate the 24hours extract. This chapter covers the effect of temperature on aqueous extraction of *B. dalzielii*, and identification of microorganisms on the bark of *B. dalzielii.* The effect of microbial species on the phytochemical profile of aqueous extract of this plant will be characterised.

4.2 METHODS

4.2.1 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

4.2.1.1 Preparation of standard solution

Standard solutions were prepared by dissolving 1mg of purchased compounds (gallic acid and pyrogallol) in 2mL of methanol which is equivalent to 500ppm (500mg/L). Aliquots of the standard solution were used to prepare samples for a calibration curve. Six solutions were prepared from the standard working solution, this include: 250ppm (0.5mg/mL), 200ppm (0.4mg/mL), 150ppm (0.3mg/mL), 100ppm (0.2mg/mL), 50ppm (0.1mg/mL) and 25ppm (0.05mg/mL).

4.2.1.2 HPLC analysis

HPLC Agilent series 1100 system composed of autosampler and diode array detector was used qualitative and quantitative analysis. The separation was performed on VisionHT C18 HL 5µ (250 × 4.6 mm). A binary solvent system was used as mobile phase

consisting of 0.1% acetic acid in water and 0.1% acetic acid in acetonitrile in a ratio of 95/5 (v/v). The column temperature was set to 40°C with a solvent flow rate of 1mL/min and an injection volume of 5µL. The diode array detector was monitored at 280nm and run time was 30minutes. Chromatographic peaks were identified for both gallic acid and pyrogallol, and compared to the retention time of peaks present in *B. dalzielii* bark crude extracts in subsequent experiments. A linear calibration curve was plotted for the standard solutions for both compounds and equation generated was used to calculate the concentration of both compounds in prepared samples.

4.2.2 EFFECT OF TEMPERATURE ON AQUEOUS EXTRACT OF *B. dalzielii*

Three sterile bottles were labelled 4°C, 25°C and 37°C and one gram of plant bark was macerated in each of the sterile bottles using 10mL sterile distilled water and incubated for 24hrs at 4°C, 25°C and 37°C, respectively. Samples were filtered using minisart sterile syringe filter and 5µL was ran on the HPLC in triplicate using the method described in section 4.2.1.2. Compounds affected were selected as peaks on the HPLC chromatogram.

4.2.3 ISOLATION OF MICRO-ORGANISMS

10mL of sterile distilled water was added to three different 50mL sample bottles labelled A1, A2 and A3, and then one gram of plant material was added. Sample bottles were incubated at 37°C shaking at 100RPM for two hours. From each sample bottle, 10µL was plated on Mueller Hinton agar plate using a sterile spreader, this was conducted in triplicate and appropriately labelled. Single colonies of the most common visible micro-

organisms were selected and inoculated in 10mL sterile Mueller Hinton broth and incubated overnight at 37˚C shaking at 100RPM. Overnight cultures were plated on Mueller Hinton agar plate with the aid of a sterile loop and incubated overnight at 37˚C. Isolated colonies were then stored on cryopreservation beads and appropriately labelled.

4.2.4 EFFECT OF MICRO-ORGANISMS ON AQUEOUS EXTRACT OF *B. dalzielii*

4.2.4.1 Method I

One gram of the plant bark of *B. dalzielii* was macerated in 10mL of sterile distilled water in nine sterile bottles labelled 1, 6, 18, 24, 30, 42 and 48 hour(s) and samples were incubated at room temperature, placed on a mechanical shaker at 100RPM. At the specified time, samples were collected for thin layer chromatography (TLC) analysis was conducted on silica plate using hexane and ethyl acetate (50:50) plus a drop of formic acid as eluent. TLC analysis was also conducted on 6 and 24hours samples, together with the sample collected from one gram of plant bark of *B. dalzielii* macerated in 10mL 50% aqueous methanol overnight, using 0.5mg/mL of pyrogallol and gallic acid in methanol as standard references. Vanillin spray reagent was prepared using 2 grams of vanillin, 98mL ethanol and 2mL sulphuric acid. TLC plates were treated with vanillin spray reagent and heat with hot air blower for few seconds to visualise compounds. Also, at specific time, 1mL was collected from samples, filtered into HPLC sample vial using the minisart sterile syringe filter and appropriately labelled sterile (S) plus designated time. HPLC analysis was conducted using the method mentioned in section 4.2.1.2. and column used was VisionHT C18 HL 5µ.

4.2.4.2 Method II

One gram of plant bark was macerated in 10mLs of sterile distilled water. The sample was incubated for 24hours at 37˚C. 1.3g of cellulose acetate was measured as instructed by the manufacturer and loaded onto glass column. 10mL of 24hours aqueous extract at 37˚C was centrifuged and the supernatant was passed through cellulose acetate, then the cellulose acetate was washed with methanol to recover pyrogallol. Recovered pyrogallol was TLC analysed against pyrogallol standard using hexane and ethyl acetate plus a drop of formic acid as solvent.

In addition, 3mg pyrogallol was dissolved in 3mL of HPLC grade methanol. 1mL was transferred into HPLC sample vial and labelled “Not filtered”, 1mL was filtered with minisart sterile syringe filter into HPLC sample vial and labelled “Filtered”. 1mL of methanol was then used to rinse the syringe filtered to recover the pyrogallol bonded to the cellulose acetate in the filter and this was labelled “Methanol recovered”. Samples were HPLC analysed using the method mentioned in section 4.2.1.2.

4.2.4.3 Method III

From labelled sample bottles labelled 1, 6, 18, 24, 30, 42 and 48 hour (s) prepared in METHOD I, 3mL was collected from samples into labelled Eppendorf vials and centrifuged at 8000rpm for 10 minutes, then filtered with Whatman filter paper. 1mL of the filtrate was transferred into HPLC sample vial and appropriately labelled non-sterile (**NS**) plus designated duration of incubation. HPLC analysis was conducted using the

method mentioned in section 4.2.1.2. Samples were plated on Mueller Hinton agar plates to investigate the presence of microbial growth.

4.2.5 CONFIRMING THE EFFECT OF ISOLATED MICROORGANISMS ON AQUEOUS EXTRACT OF *B. dalzielii*

Three well-isolated colonies of both bacteria (colony X and Y) were transferred into three different 10mL sterile media including water, Mueller Hinton broth and six hours aqueous plant extract and incubated at 37˚C overnight. Samples were filtered using minisart sterile syringe filter and 1mL was transferred into HPLC vial for analysis. HPLC analysis was conducted using the method mentioned in section 4.2.1.2.

4.2.6 IDENTIFICATION OF MICROORGANISMS

4.2.6.1 Gram staining method

Isolated bacterial colonies were used to prepare smears for Gram staining. Fixed smears were stained with crystal violet for 60secs, excess stain was washed with water, then Lugol`s iodine solution was applied for 60 seconds which was also washed off with water. 95% alcohol was used to wash off the slides to remove the purple stain then water was used to wash the slide. The slides were stained with safranin for 60 seconds then washed with water and allowed to dry before examining under the oil immersion objective lens.

4.2.6.2 Oxidase strip method

Oxidase strip was further used to determine the identity of unknown microorganisms. This diagnostic test is for the detection of the cytochrome oxidase activity of microorganism within a minute. In the presence of some Gram-negative bacteria cytochrome oxidase enzyme, the *N,N*-dimethyl-*p*-phenylenediamine and α-naphthol react to indophenol. Smears of microorganisms to be tested was prepared on a slide and the paper zone saturated with a solution of *N,N*-dimethyl-*p*-phenylenediamine and α-naphthol was placed on the smear and allowed to stand for a minute. A positive result is obtained when the paper zone changes to a dark blue or black colour while a negative result is obtained when the paper remains pink.

4.2.6.3 MacCONKEY agar method

MacCONKEY agar plate test was further used to verify the identity of bacterial colonies isolated. Isolated colonies were plated on the said agar plate with the aid of a sterile loop and allowed to grow for 24 hours at 37°C.

4.2.7 API®20E BIOCHEMICAL ASSAY

The API®20E™ biochemical assay was conducted using the manufacturer's instruction and this test is specific to Enterobacteriaceae and/or non-fastidious Gram-negative rods. The inoculum of bacteria colonies isolated from *B. dalzielii* (white and yellow colonies) were prepared with sterile water and transferred into AP1 solutions on the strip. The strip box was sealed and incubated for 24hrs at 37°C. After the incubation period, the sections of tryptophan deaminase, indole test and Voges-Proskauer test were treated

with drops of appropriate reagent provided by the manufacturer and allowed to react for 10 minutes. Visual assessment of the strip was conducted as either positive or negative, using colour chart provided by manufacturer as control. Result obtained was entered into the online API identifier and identity of bacteria were obtained.

4.2.8 POLYMERASE CHAIN REACTION (PCR)

PCR analysis was conducted for both isolated bacteria (White and yellow colonies) from *B. dalzielii* using the following universal primers:

- 16S rRNA forward primer: AGAGTTTGATCCTGGCTCAG
- 16S rRNA reverse primer: ACGGCTACCTTGTTACGACTT

A 50µl master mix was prepared to contain 25µL of Bioline Mix (MyTaq^{TM} HS Red Mix), 1µL 10µM 16S rRNA forward primer, 1µl 10µM 16S rRNA reverse primer and 23µL of RNase-free water. A colony of the tested microorganisms was stabbed using a 10µL pipette tip and added into the PCR tube containing the master mix, the experiment was conducted in a UV- cabinet for PCR operation (UVT-B-AR). Samples were vortexed and spun in a mini plate spinner (MPS 1000TM) at 2500RPM (500×g), before placing it in the PCR machine (PIKOREAL 96). PCR reaction was allowed to run for 55mins using the following programme: Initial denaturation step at 95^{o}C for 3minutes, 30 cycles consisting of denaturation at 95^{o}C for 15seconds, annealing at 52^{o}C for 15seconds and extension at 72^{o}C for 20seconds, and a final extension period of 2minutes at 72^{o}C.

4.2.8.1 Gel electrophoresis

1% (w/v) ultrapure agarose was prepared by adding 0.5g of agarose to a 50mL solution containing SYBR safe DNA stain in 0.5 × TBE and dissolved by heating in the microwave for 1 minute, then poured into casting tray fitted with a comb and allowed to solidify. 3µL of DNA ladder (Bioline) and PCR products each were loaded and allowed to run at 60V for 90minutes with the aid of Bio-Rad. The resulting gel was visualized in a Bio-Rad illuminator with a chemiDocXRS camera, using Quantity One software. Band obtained were compared to the Bioline Easy Ladder.

4.2.9 MICROBIAL BIOCONVERSIONS

5g of plant material was macerated in 50mLs of distilled water for six hours at room temperature. The sample was filtered with a sterile microbial filter. 5ml filter-sterilised aqueous plant extracts were inoculated with 3 colonies each of isolated colonies (*E. cloacae* and *Pantoea spp*.) and incubated on a shaker (100RPM) at 37˚C for 24hours. Incubated samples were centrifuged and filtered then 1mL was transferred into HPLC vials and analysed on HPLC in triplicate after 24hours. Six hours sterile plant extract was also analysed on HPLC in triplicate. HPLC analysis was conducted using the method described in section 4.2.1.2.

4.2.10 CONVERSION OF GALLIC ACID TO PYROGALLOL

0.1% gallic acid was dissolved in sterile Mueller Hinton broth, which was then inoculated with *E. cloacae*. The inoculum was adjusted to ~10^8 using a wavelength setting of 625nm, before incubating at 37˚C for 24hours. 1mL of 0.1% gallic acid in sterile Mueller Hinton

broth and Mueller Hinton broth alone was collected as control. After 6, 12, 18 and 24 hour(s), 2mL of samples was transferred into a labelled Eppendorf vial and centrifuged at 8000rpm for 10 minutes, then filtered with Whatman filter paper. 1mL of the filtrate was transferred into HPLC sample vial for HPLC analysis using method detailed in section 4.1.1.2, however, HPLC column used was Kinetex 5µm C18 (150 × 2.1mm) and binary solvent system used as mobile phase consisted of 0.1% acetic acid in water and 0.1% acetic acid in acetonitrile (99/1, v/v).

4.3 RESULTS AND DISCUSSION

4.3.1 HPLC ANALYSIS

The 500PPM (1mg/2mL) stock solution prepared was used to prepare different concentrations and mean ± standard error of the compound peak was obtained. Using the area value of all working concentrations, a calibration curve for each of the standard compound (gallic acid and pyrogallol) was obtained.

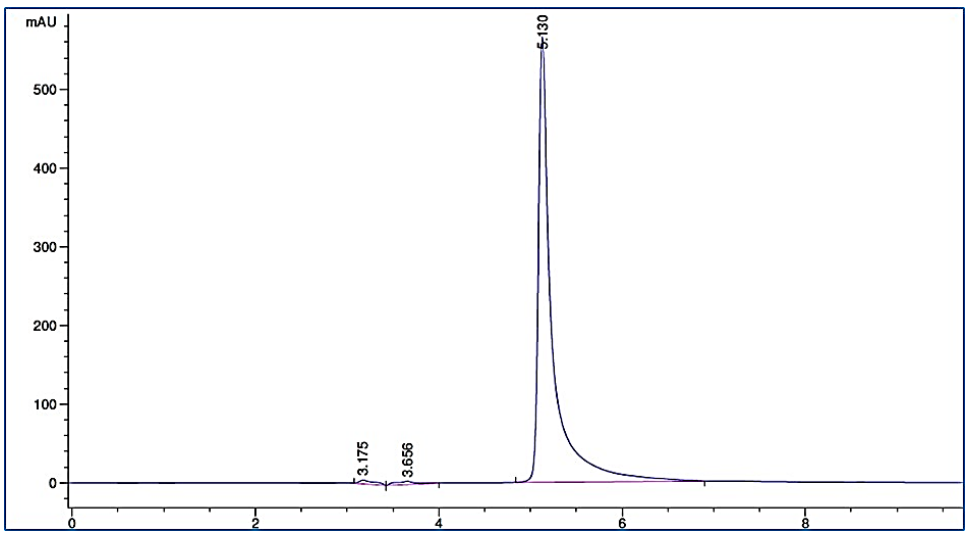


Figure 4. 1: *HPLC chromatogram of Gallic acid having a retention time of 5.1 minutes*

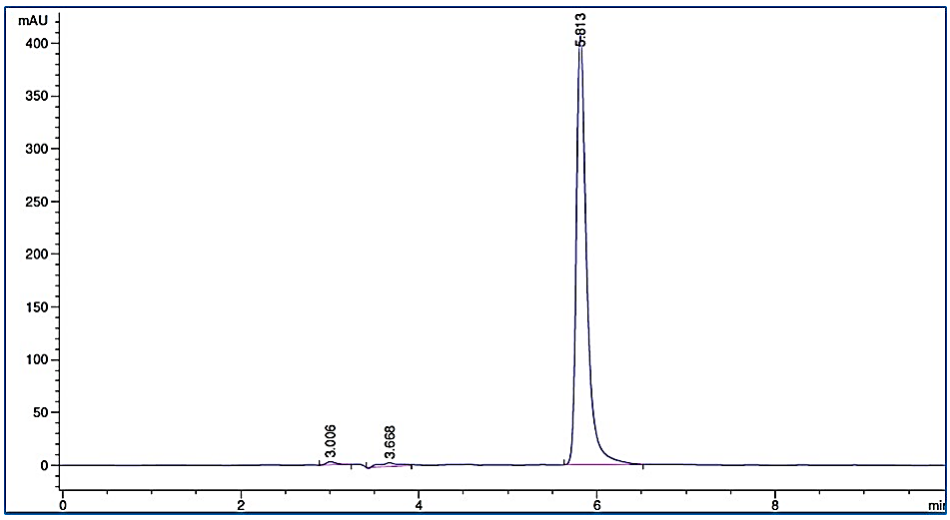


Figure 4. 2: *HPLC chromatogram of pyrogallol having a retention time of 5.8 minutes*

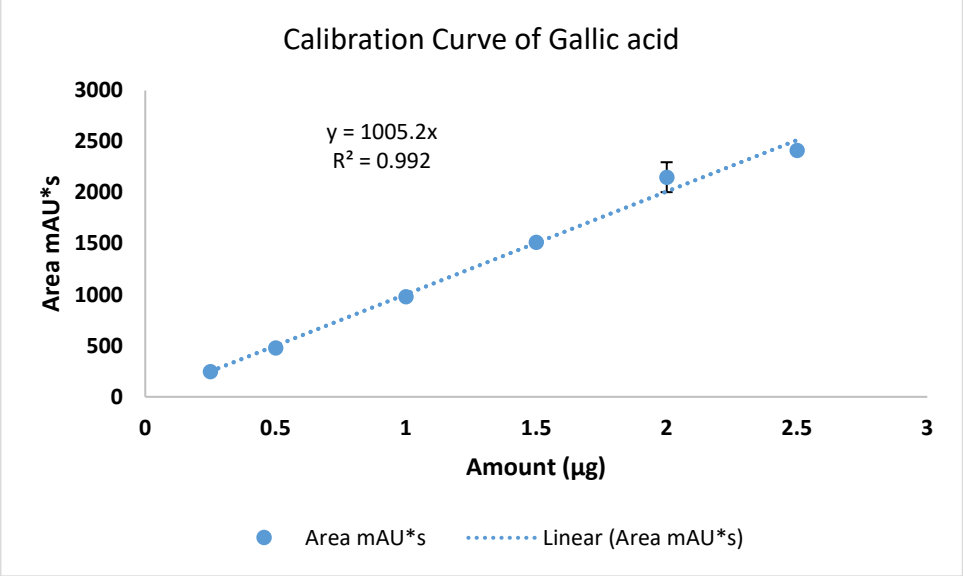


Figure 4. 3: *Calibration curve of gallic acid. Data represents mean ± standard error*

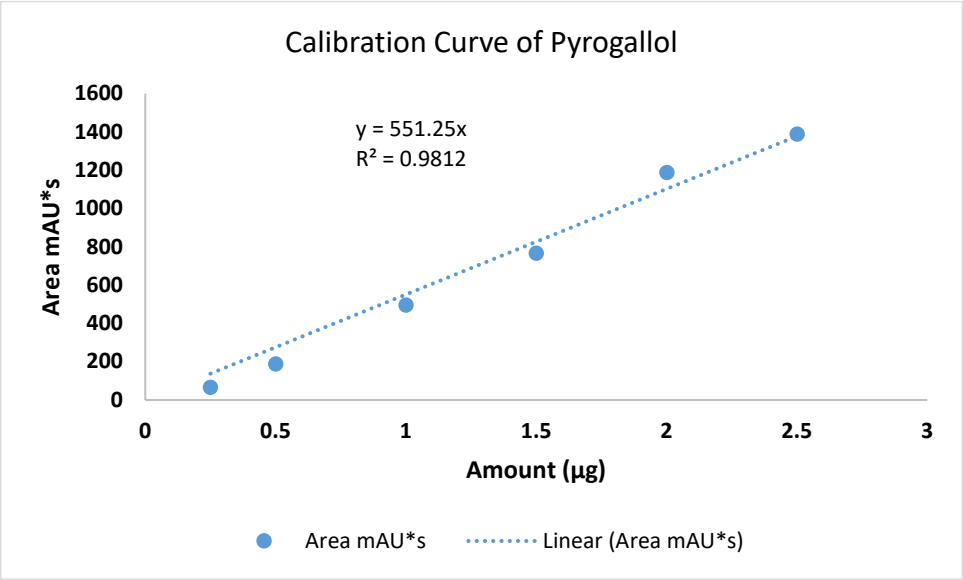


Figure 4. 4: *Calibration curve of pyrogallol. Data represents mean ± standard error*

4.3.2 EFFECT OF TEMPERATURE ON AQUEOUS EXTRACT OF *B. dalzielii*

Peaks selected for analysis had retention time 5.1 and 5.8, which are similar to gallic acid (Figure 4.1) and pyrogallol (Figure 4.2). The graph (Figure 4.5) showed the presence of pyrogallol in 25°C and 37°C samples, with a concentration of 86.3 and 140.8 mg/L, respectively. The peaks of compounds with the retention time of 4.1 and 9.5 present in 4°C sample was observed to be absent in 37°C sample (Figure 4.6), suggesting that other phytochemicals are vital to the micro-organisms present in the plant extract.

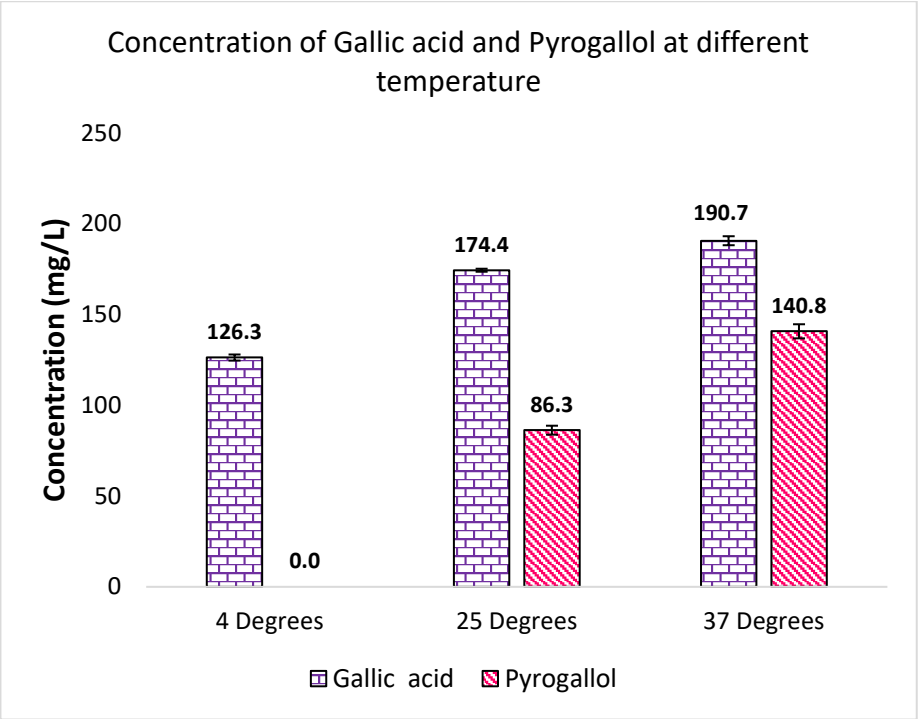


Figure 4. 5: *Effect of temperature on gallic acid and pyrogallol present in aqueous extract of* B. dalzielii*. Data represents mean ± standard error*

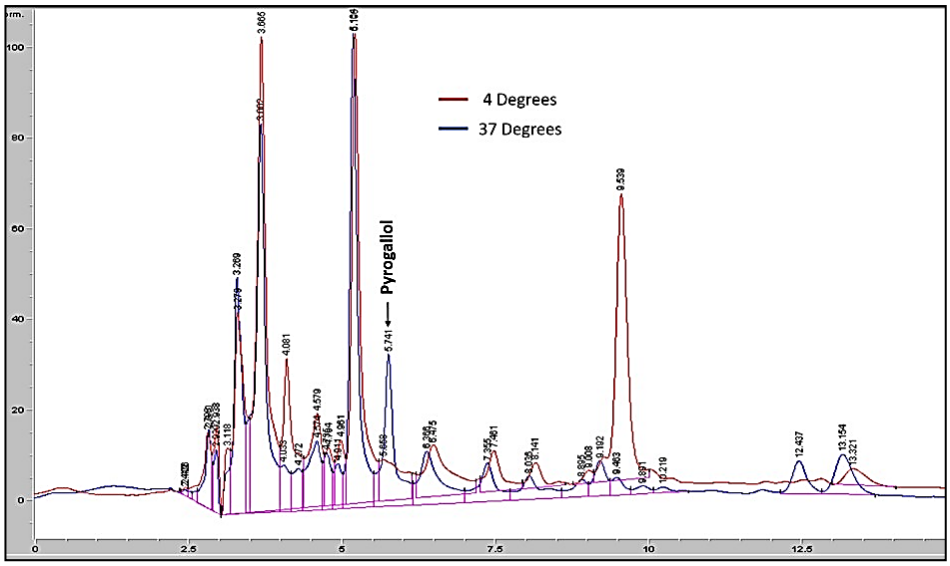


Figure 4. 6: *HPLC Chromatogram of aqueous extract of* B. dalzielii *incubated in 4˚C and 37˚C for 24hours*

4.3.3 ISOLATION OF MICROORGANISMS

Microbial growth was visible for all samples grown on all Mueller Hinton agar plate and two dominant colonies were observed on the plates rather than a wide range of different colonies. These organisms were labelled colony "**X**" which is white in colour and "**Y**" which is yellow in colour (Figure 4.7). Isolated colonies were then transferred into storage vials containing cryopreservation beads (cryobeads) immersed in cryo-solution and stored in the freezer at -20^{o}C.

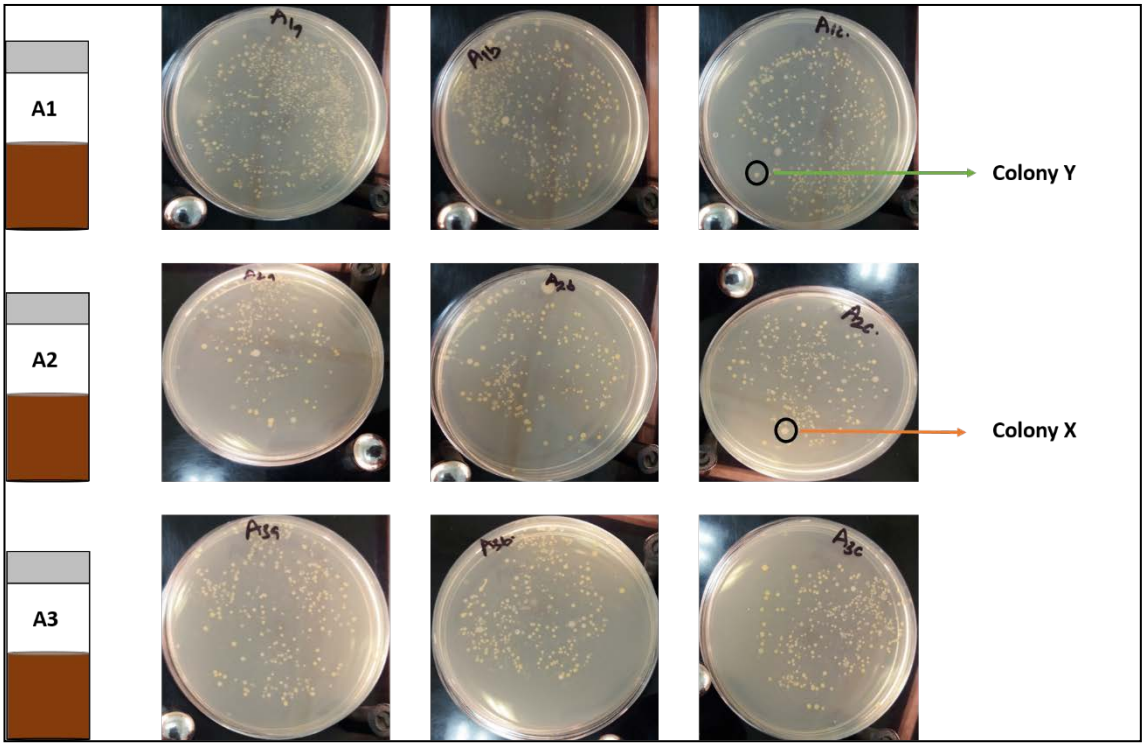


Figure 4. 7: *Microbial colonies observed in plant bark of B. dalzielii*

Furthermore, Mueller Hinton agar plates were exposed to working environment which include bench, laminar flow and shaker. The plates were then incubated overnight at 37°C and no microbial growth was observed. Also, plant material was spike on to Mueller

Hinton agar and incubated overnight at 37°C for 24hours and only two types of bacterial colonies were observed (Figure 4.8).

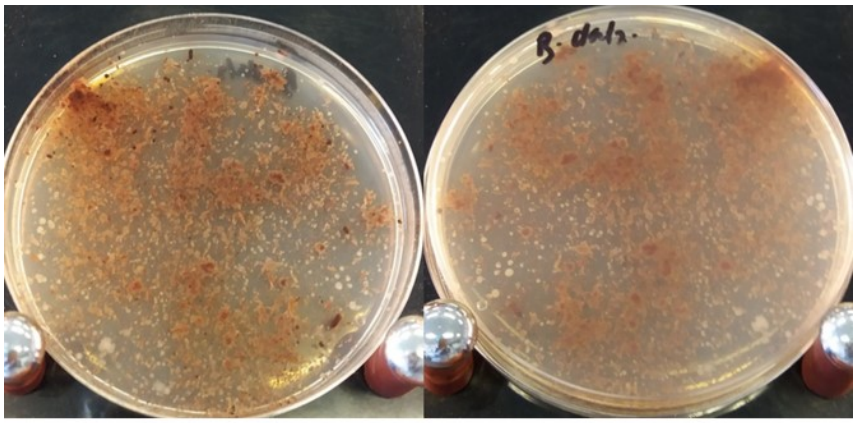


Figure 4. 8: *Plant material spiked on Mueller Hinton agar plate.*

4.3.4 EFFECT OF MICRO-ORGANISMS ON AQUEOUS EXTRACT OF *B. dalzielii*

Microbial growth was visible only in the non-sterile sample and two dominant colonies were observed on Mueller Hinton agar plates. These organisms coincide with those labelled colony "**X**" and "**Y**" as seen in (Figure 4.9).

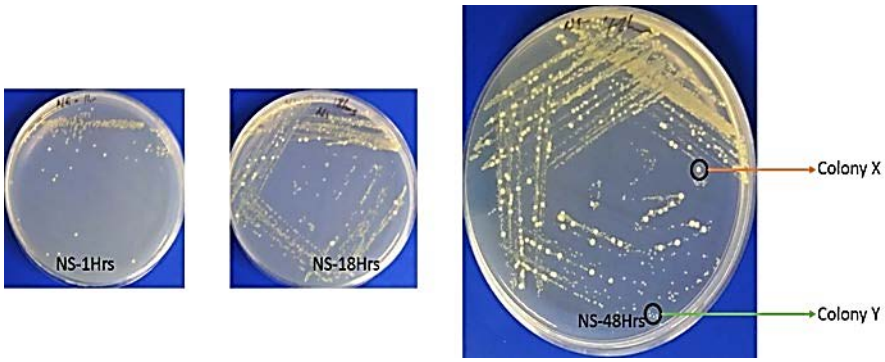


Figure 4. 9: *Microorganisms observed in extract of B. dalzielii*

TLC chromatogram of *B. dalzielii* bark conducted in 4.2.4.1 was sprayed with vanillin reagent to show different chemical profiling at different specified time showing the

presences of a compound labelled compound A at 12hrs (Figure 4.10). The chromatogram suggested that compound A is not a phytochemical present in the plant. This was verified by conducting a TLC with 1hr and 24hrs samples using purchased gallic acid and pyrogallol as standard references.

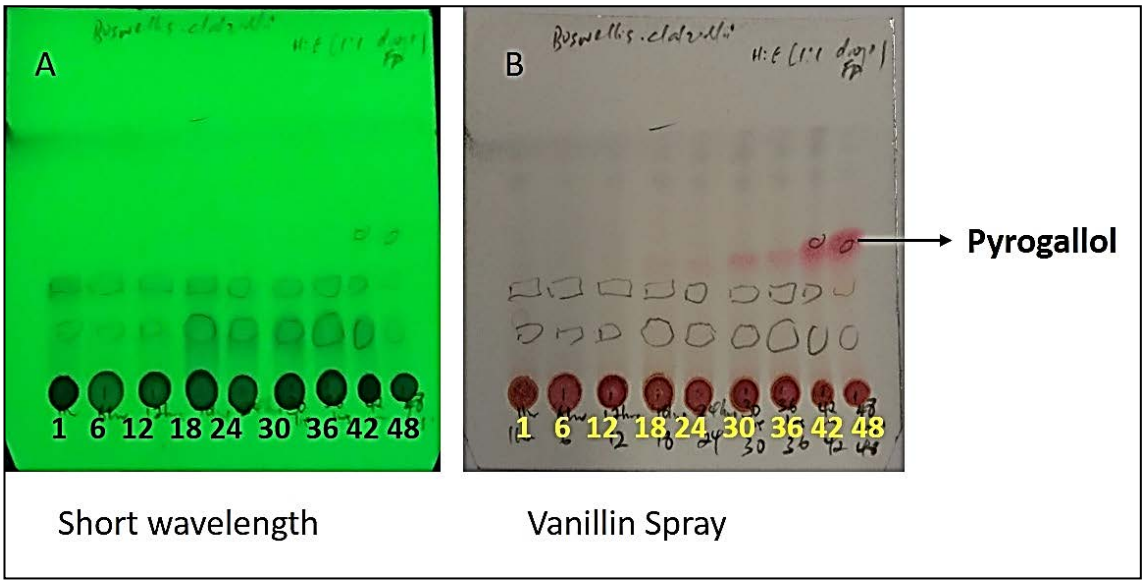


Figure 4. 10: *TLC chromatogram showing the presence of pyrogallol at 18hours when sprayed with vanillin reagent. Picture A was viewed at 254nm while picture B was viewed after vanillin spray treatment.*

Two compounds were previously identified in chapter 3 as gallic acid and pyrogallol, thus these compounds were purchased from Sigma Aldrich and used as references. TLC was conducted with standard compounds of gallic acid and pyrogallol, against 1hr and 24hrs samples. TLC chromatogram confirms that compound A in Figure 4.10 is pyrogallol, since purchased pyrogallol has similar R_f value of 0.69 with a compound present in 24hrs sample (Figure 4.11).

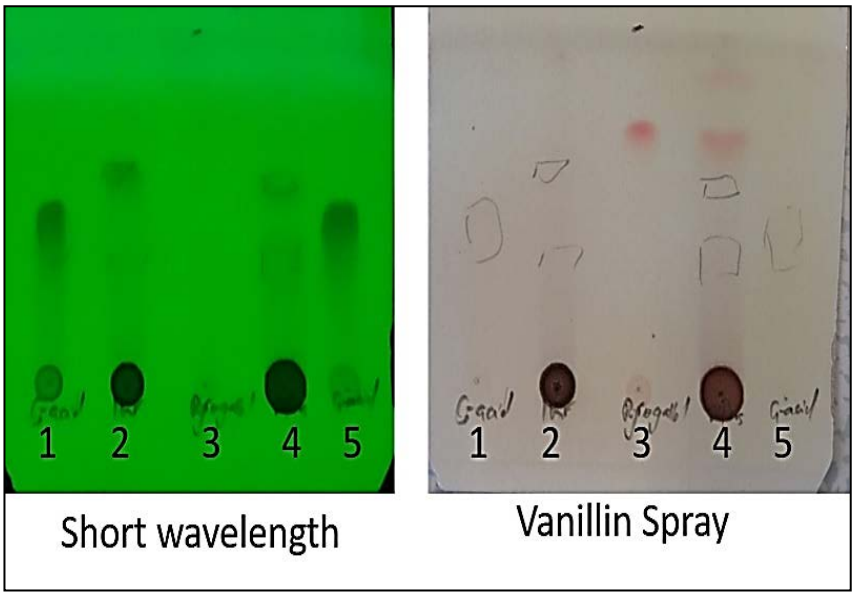


Figure 4. 11: *TLC chromatogram of gallic acid standard (1 and 5), pyrogallol standard (3), 1hr macerated plant bark (2) and 24hours macerated plant bark (4).*

Figure 4. 12: *TLC chromatogram showing the presence of gallic acid in both 50% aqueous methanol and six hours extract whereas pyrogallol was only present in 24hours extract. Where 1 = 24 hours 50% methanol aqueous extract, 2 = gallic acid, 3 = 6 hours aqueous extract, 4 = pyrogallol and 5 = 24 hours aqueous extract*

The 50% aqueous extraction was also conducted to check that pyrogallol was not a phytochemical already present in the plant (Figure 4.12). From the TLC chromatogram, pyrogallol standard was shown to have similar R_f value of 0.68 with a compound in 24hours extract and gallic acid was seen to have 0.24 R_f value with a compound in six

hours extract. HPLC chromatogram showed the appearance of pyrogallol after 24hours maceration at 37˚C.

HPLC analysis confirms the presence of gallic acid with a retention time of 5.1 in plant bark of B. dalzielii (non-sterile) macerated for 1hr, however, pyrogallol peak is not present in the sample. Unlike the 1hr non-sterile sample (Figure 4.13), the 24hrs non-sterile sample is seen to have both gallic acid and pyrogallol with a retention time of 5.1 and 5.8, respectively (Figure 4.14).

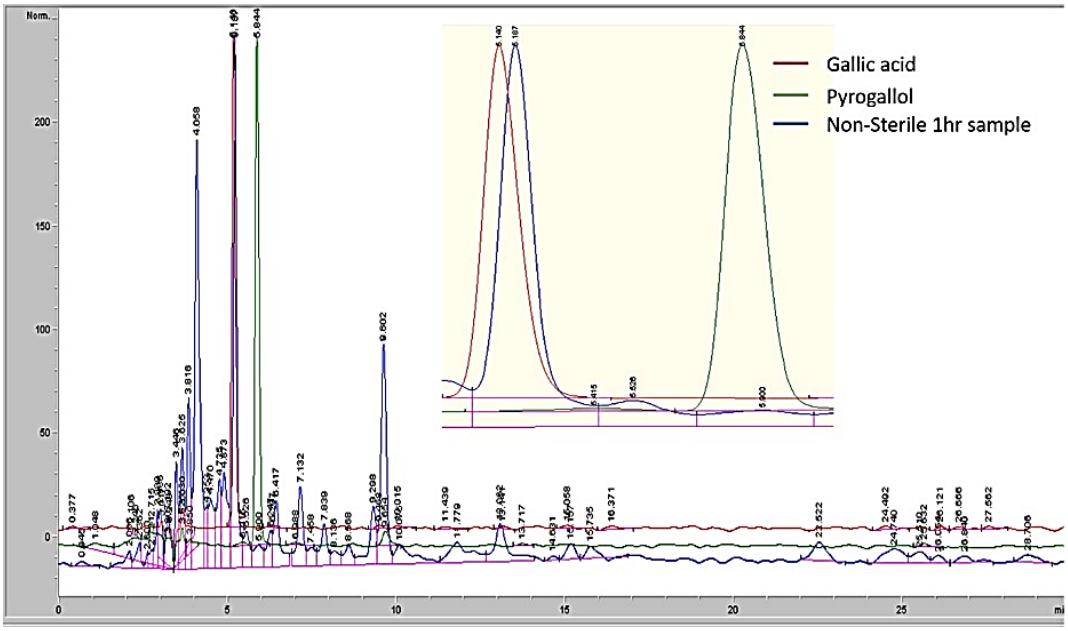


Figure 4. 13: *HPLC chromatogram showing the absence of pyrogallol peak in a one hour non-sterile sample.*

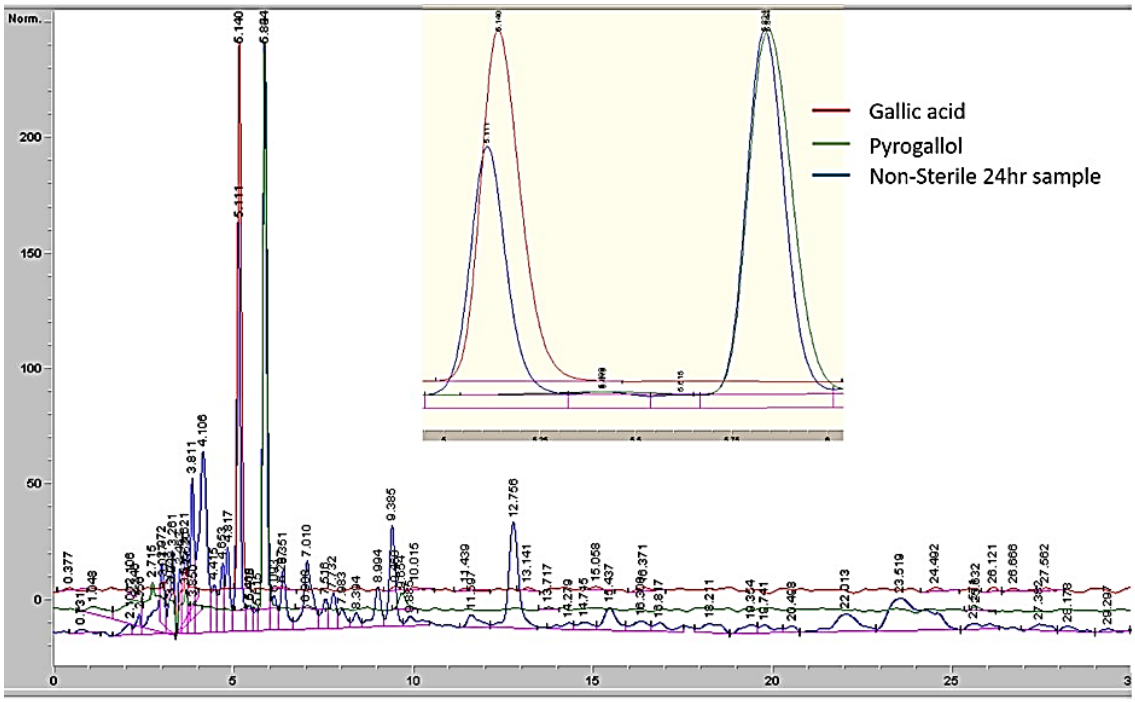


Figure 4. 14: *HPLC chromatogram showing the presence of pyrogallol peak in a 24hours non-sterile sample.*

Using the equation $y = 1005.2x$ (Figure 4.3) and $y = 551.25x$ (Figure 4.4) for gallic acid and pyrogallol, respectively, the concentration of both compounds in aqueous extracts were calculated. The HPLC analysis conducted on plant extract samples sterilized with minisart microbial filter 0.2µm showed that the optimal concentration of gallic acid was 689.0 mg/L in the 30 hours sample. Whilst, the concentration of pyrogallol increased gradually with the 48 hours sample having the highest concentration of 323.7 mg/L (Figure 4.15). It is noteworthy to mention that the concentration of both gallic acid and pyrogallol in all samples were obtained using equation generated from the linear calibration curve of both compounds.

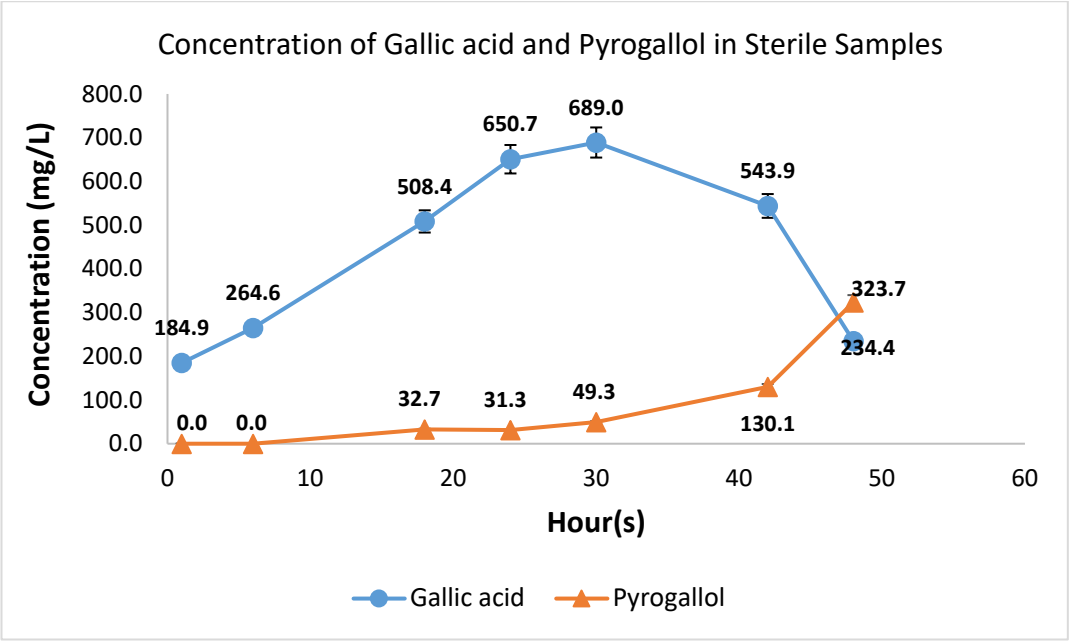


Figure 4. 15: *Concentrations of gallic acid and pyrogallol in sterile plant bark of* B. dalzielii *at different time interval. Data are presented as mean ± standard error for both compound.*

Column chromatography conducted using cellulose acetate as stationary phase, lead to the use of methanol in washing the cellulose acetate after treating with 24hours, thus, pyrogallol was recovered. Eluted pyrogallol was TLC analysed against pyrogallol standard and TLC chromatogram showed that pyrogallol was trapped on cellulose acetate. Both recovered and purchased pyrogallol have similar R_f value of 0.67 (Figure 4.16). In addition, the HPLC analysis conducted to confirm the effect of cellulose acetate on pyrogallol showed that of the 1.05mg/mL passed through the minisart syringe filter, about 0.34 mg/mL was bonded to the cellulose acetate and 0.53 mg/mL was recovered (Figure 4.17).

Figure 4. 16: *TLC chromatogram of pyrogallol (PYR) and recovered pyrogallol from 24hours extract.*

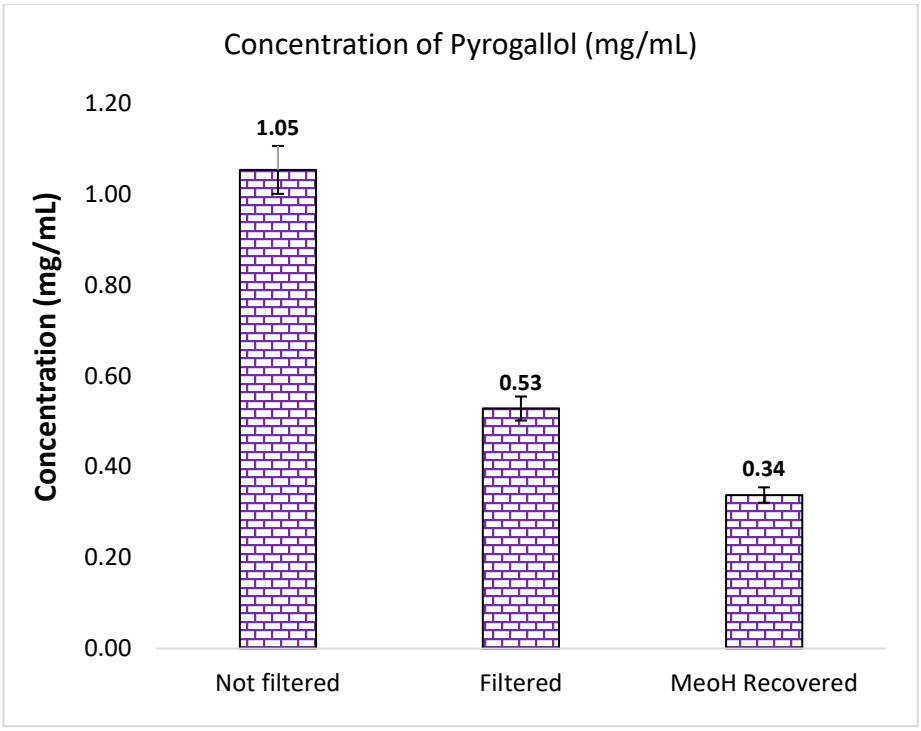


Figure 4. 17: *Effect of minisart syringe filter embedded with cellulose acetate. Data are presented as mean ± standard error.*

Furthermore, the HPLC analysis of the non-sterile samples was conducted and the amount of gallic acid and pyrogallol in plant bark extract was calculated for all samples. The concentration of gallic acid was seen to be optimal (565.5mg/L) in 18hours sample

while the concentration of pyrogallol was seen to be optimal (853.7mg/L) in 48hours sample (Figure 4.18).

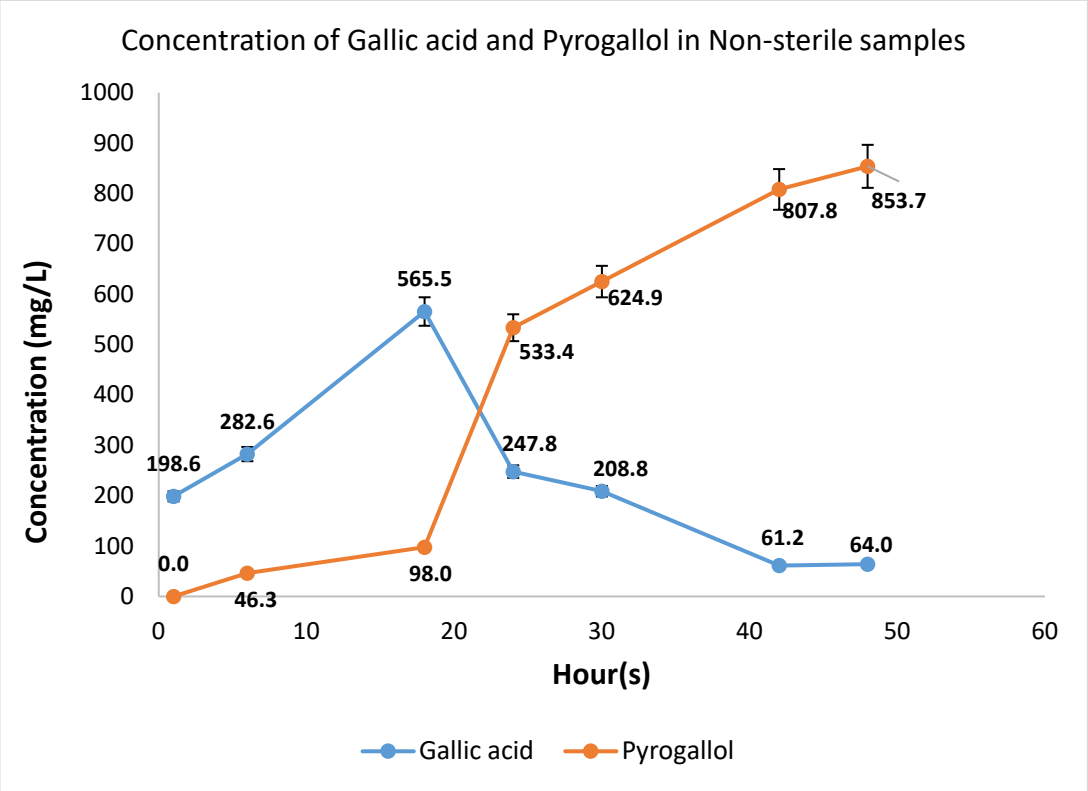


Figure 4. 18: *Concentrations of gallic acid and pyrogallol in non-sterile plant bark of* B. dalzielii *at different time interval. Data are presented as mean ± standard error for both compound.*

The concentration of pyrogallol in sterile samples was observed to be lower across all samples when compared to non-sterile samples. The highest concentration obtained in the non-sterile sample was calculated to be 853.7 mg/L while that obtained in the sterile sample was 323.7 mg/L in 48 hours (Figure 4.19).

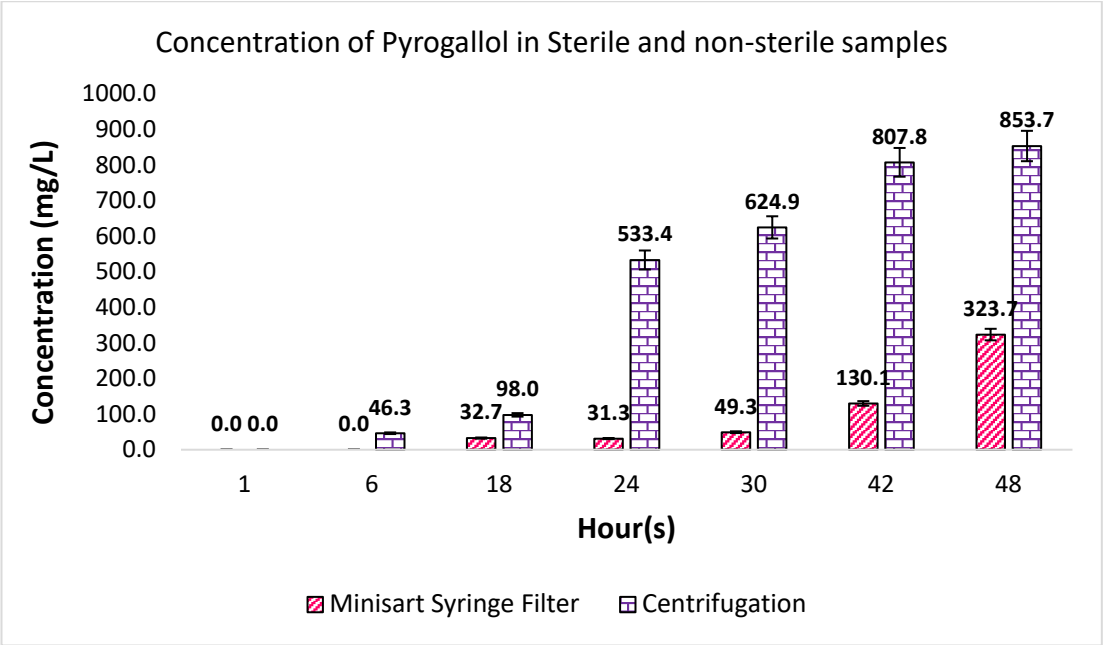


Figure 4. 19: *Concentration of pyrogallol in both sterile and non-sterile samples. Data are presented as mean ± standard error for both compound.*

4.3.5 CONFIRMING THE EFFECT OF ISOLATED MICROORGANISMS ON AQUEOUS EXTRACT OF *B. dalzielii*

HPLC chromatogram of sterile water inoculated with isolated bacteria from plant bark of *B. dalzielii* showed no peaks while that of Mueller Hinton broth give two new peaks at 4.329 and 10.165 (Figure 4.20) after inoculation and 24hours incubation. However, the peaks observed were not similar to that of pyrogallol.

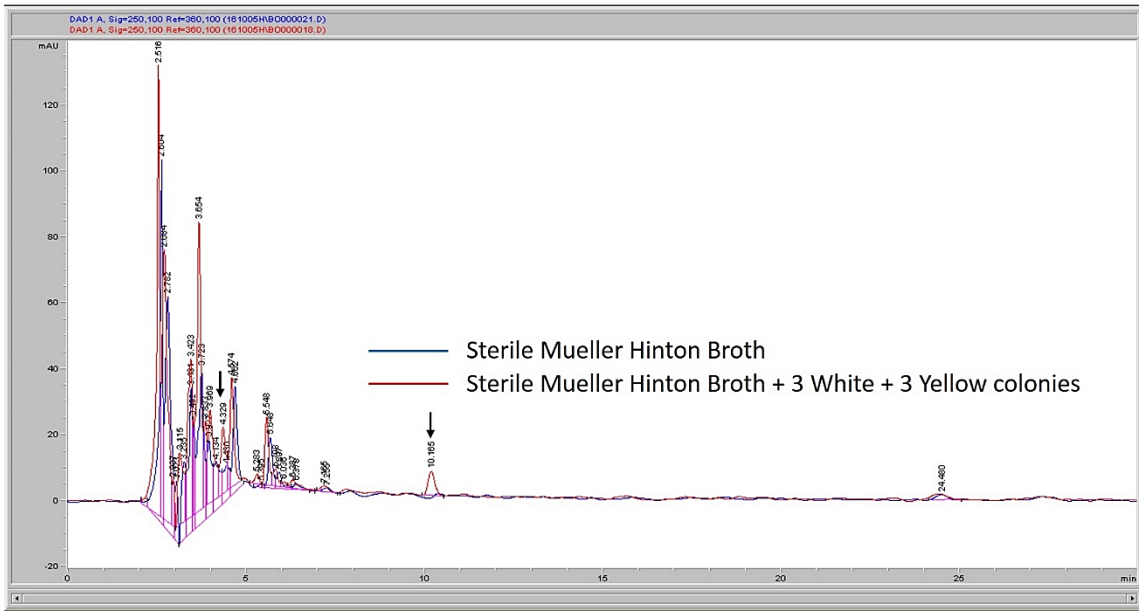


Figure 4. 20: *HPLC Chromatogram of sterile Mueller Hinton broth, before and after inoculation*

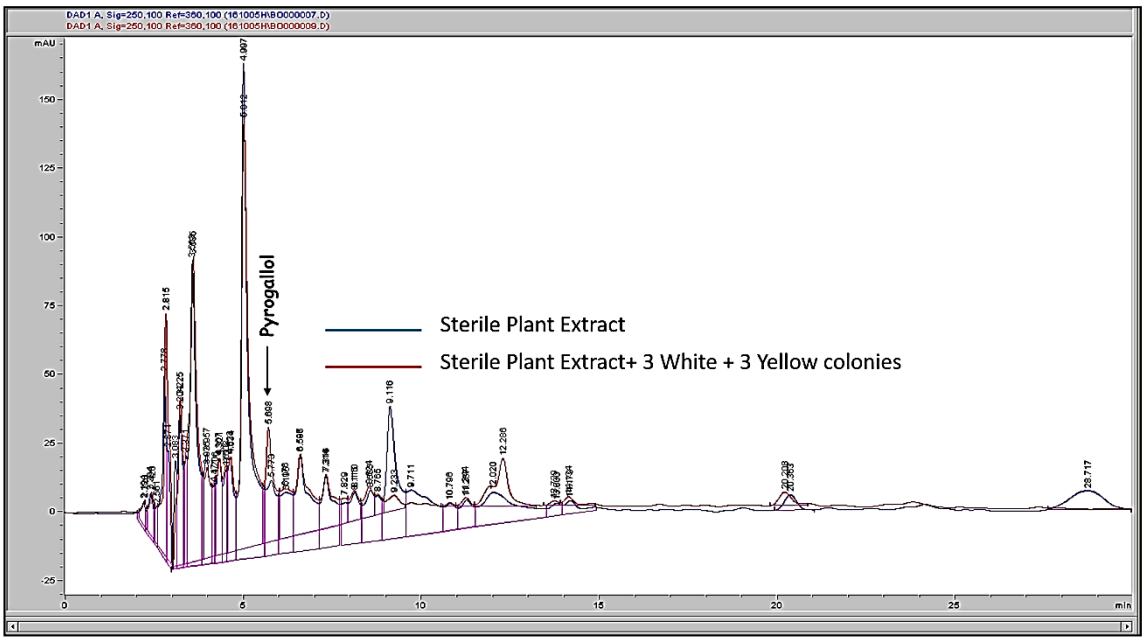


Figure 4. 21: *HPLC chromatogram of sterile plant extract before and after inoculation*

Figure 4.21 showed the presence of pyrogallol in the plant extract inoculated with bacteria isolated from the bark of *B. dalzielii*. This implicates pyrogallol as a metabolite of one of the two isolated bacteria.

4.3.6 IDENTIFICATION OF MICROORGANISMS

Gram-positive organisms stain purple because they resist decolourisation with alcohol and retain the primary stain (crystal violet) while Gram-negative organisms stain pink because they lose the primary stain during alcohol decolourisation stage, therefore they are free to take up the counterstain (safranin) (Coico, 2005). Both isolated colonies were Gram-negative rod bacteria and images were obtained using LEICA ICC50 HD microscopy at 1000 magnification using 1.25 oil lens (Figure 4.22)

Oxidase strip test is reported to be positive for all *Neisseria species* and most non-fermenting Gram-negative rods. Most fermenting Gram-negative rods are Oxidase negative except for some genus of Gram-negative bacteria such as Vibrios, Aeromonads and Pastuerella (Balows and Hausler, 1991). The Oxidase strip test conducted in the present study on both isolated bacteria was seen to be negative.

MacCONKEY agar test showed that the white isolated colony was a mucoid lactose fermenting (pink) bacteria while the yellow colony is lactose negative and had no colour (Figure 4. 23).

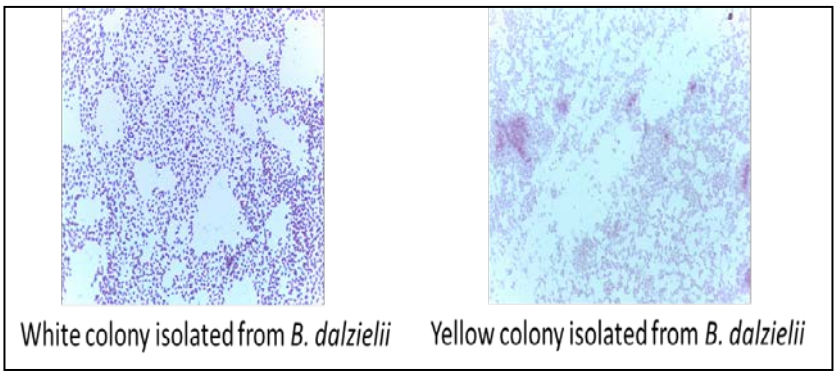


Figure 4. 22: *Gram staining of white and yellow isolated colonies from B. dalzielii*

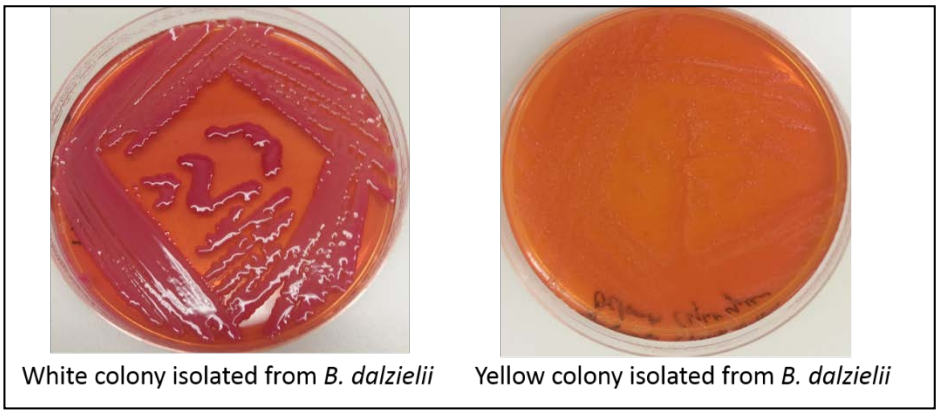


Figure 4. 23: *MacCONKEY agar test for both isolated bacteria from* B. dalzielii

4.3.7 API®20E BIOCHEMICAL ASSAY

The API®20E biochemical results obtained gave colourations, which were compared with control (Figure 4.24), results obtained from data entered into the API online database presented percentage identification of bacteria to be 98.9% for *Klebsiella pneumonia spp* 1 for white isolated colony "W" (Figure 4.25) and 99.1% for *Cronobacter spp*. for yellow isolated colony "Y" (Figure 4.26).

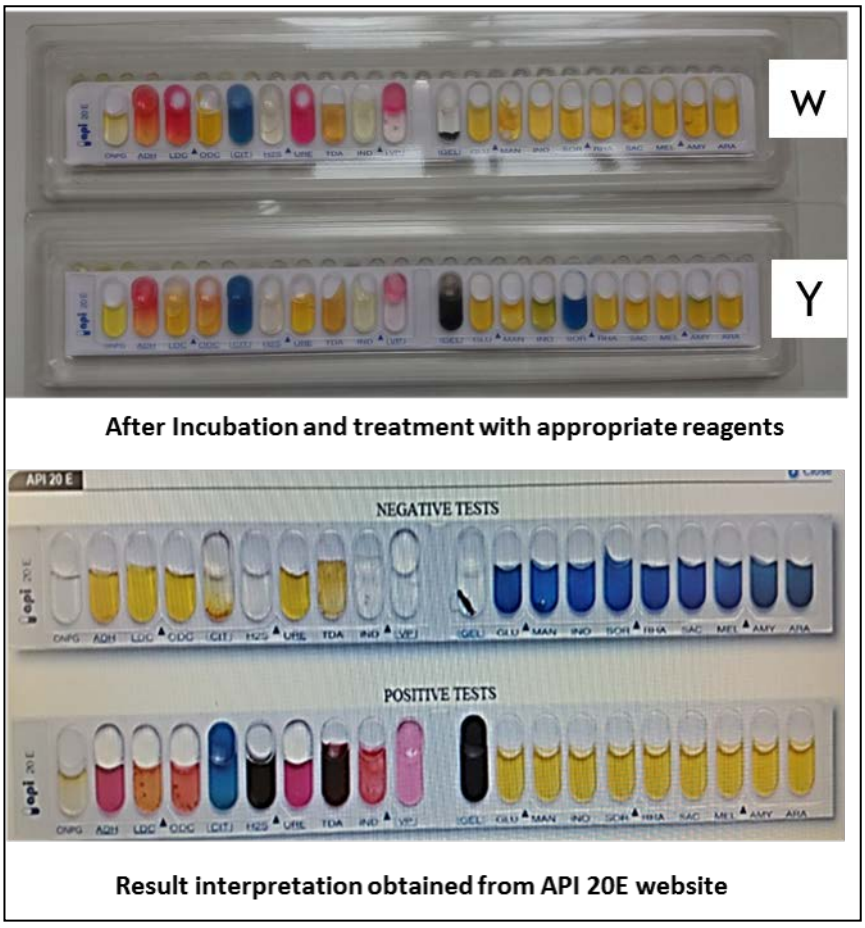


Figure 4. 24: *API biochemical analysis of bacteria isolates of B. dalzielii*

GOOD IDENTIFICATION

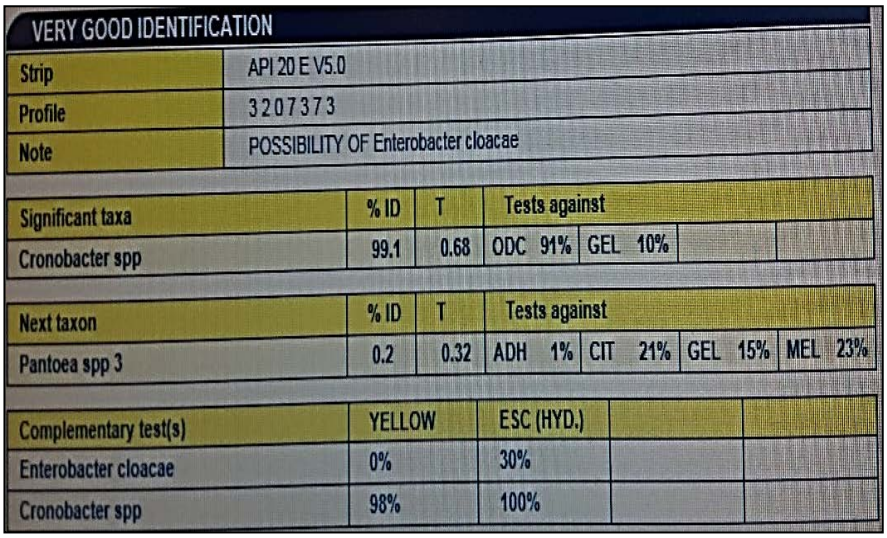
Strip	API 20 E V5.0
Profile	7215773
Note	POSSIBILITY OF Raoultella planticola

Significant taxa	% ID	T	Tests against
Klebsiella pneumoniae ssp pneumoniae 1	98.9	0.67	ADH 1%

Next taxon	% ID	T	Tests against
Klebsiella pneumoniae ssp pneumoniae 2	0.8	0.35	ADH 1% URE 1%

Complementary test(s)	5KG	METHYL RED
Klebsiella pneumoniae ssp pneumoniae	2%	9%
Raoultella terrigena	91%	60%
Raoultella planticola	98%	100%

Figure 4. 25: *Result on percentage identity obtained for white isolate colony "W" with a 98% identity for Klebsiella pneumonia spp 1.*



VERY GOOD IDENTIFICATION

Strip	API 20 E V5.0
Profile	3207373
Note	POSSIBILITY OF Enterobacter cloacae

Significant taxa	% ID	T	Tests against			
Cronobacter spp	99.1	0.68	ODC 91%	GEL 10%		

Next taxon	% ID	T	Tests against			
Pantoea spp 3	0.2	0.32	ADH 1%	CIT 21%	GEL 15%	MEL 23%

Complementary test(s)	YELLOW	ESC (HYD.)		
Enterobacter cloacae	0%	30%		
Cronobacter spp	98%	100%		

Figure 4. 26: *Result on percentage identity obtained for yellow isolate colony "Y" with 98% identity for Cronobacter spp.*

Research showed that use of API 20E is prone to misinterpretation since is subjective because it involves visual detection of colour changes before and after inoculation with bacteria species (Tomas *et al.*, 2018). Moreover, the diagnostic capability of this biochemical method in terms of accuracy was shown to be very low (Ferris *et al.*, 2017; Tomas *et al.*, 2018). For this reason, the use of molecular techniques involving specific markers such as 16S rRNA is encouraged to identify bacteria at a species level. 16S rRNA is a molecular marker having ~1500 base pair and it codes for catalytic RNA belonging to the 30S ribosomal subunit and is present in all prokaryotic cells. The 16S rRNA region consists of conserved sequences thus, the region is easily sequenced and use for accurate identification of bacteria species (Janda and Abbott, 2002; Srinivasan *et al.*, 2015). For this reason, 16S rRNA analysis was conducted.

4.3.8 PCR ANALYSIS

The 16S RNA size is ~1500bp which is above the highest base pair on the DNA ladder (Ikb). 40µL of each of the PCR products and 10 µL of both forward and reverse primer were submitted to Macrogen Europe Laboratory, Netherland for purification and sequencing. The gel results of 16S rRNA region for both PCR product of isolated bacteria gave a band above DNA ladder (Figure 4.27).

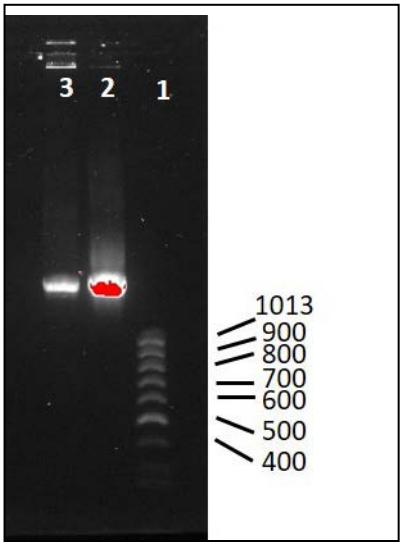


Figure 4. 27: *Gel electrophoresis image where lane 1 is DNA ladder, lane 2 is white isolated colony and lane 3 is yellow isolated colony.*

The PCR sequence results obtained for the isolated colonies for both forward and reverse primer were assembled on the CLC Main Workbench software and the consensus regions were obtained.

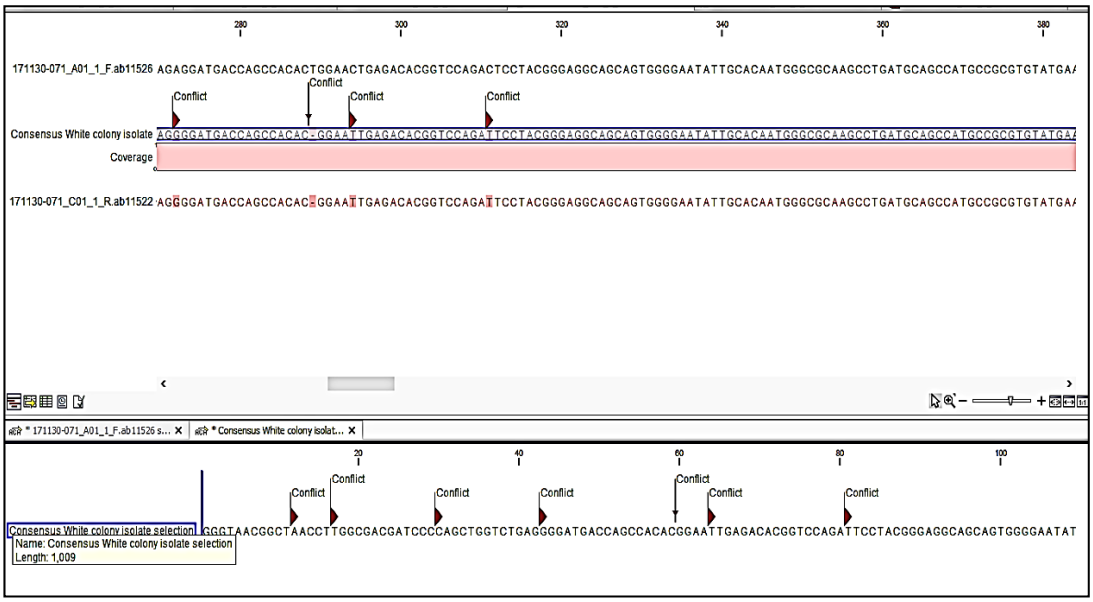


Figure 4. 28: *Consensus region obtained for the white isolated colony of B. dalzielii*

> Consensus White Colony Isolate

GGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAA
CTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAG
CCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGA
GGAAGGCGATGAGGTTAATAACCTTGTCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACT
CCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGC
GCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGA
AACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAG
AGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGA
AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGG
AGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACG
GCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTA
ATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTAGCAGAGATGCTTTG
GTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGG
GTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTAGGCCGGGAACTCAAAG
GAAACTGCCAGTGATAAACTGGAAGAAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGA
GTAGGGCTACCCACTTGCTACAATGGCGCAAACAAAGAGAGCGACCTCGC

Figure 4. 29: *Consensus sequence obtained for the white isolated colony of B. dalzielii with 1009 bases*

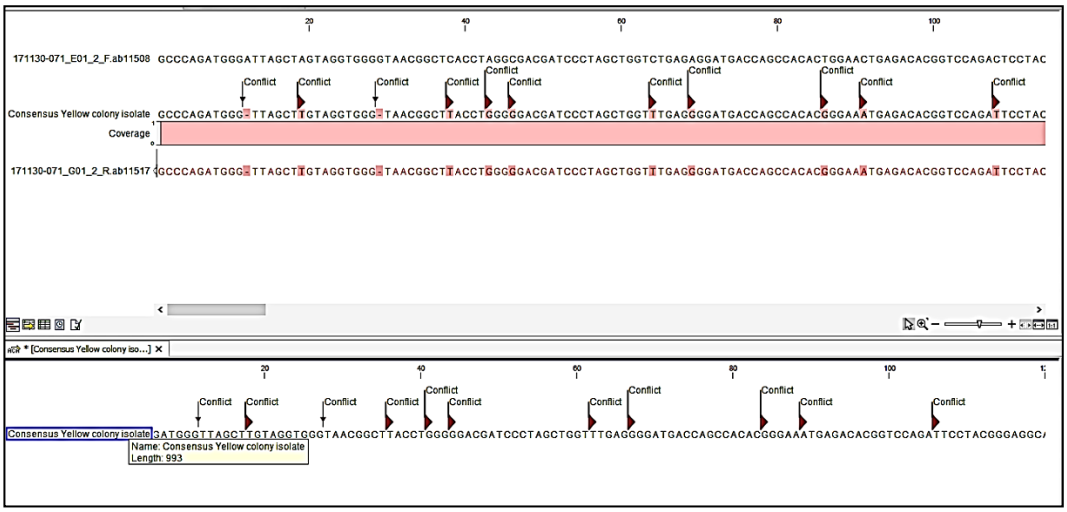


Figure 4. 30: *Consensus region obtained for the yellow isolated colony of B. dalzielii*

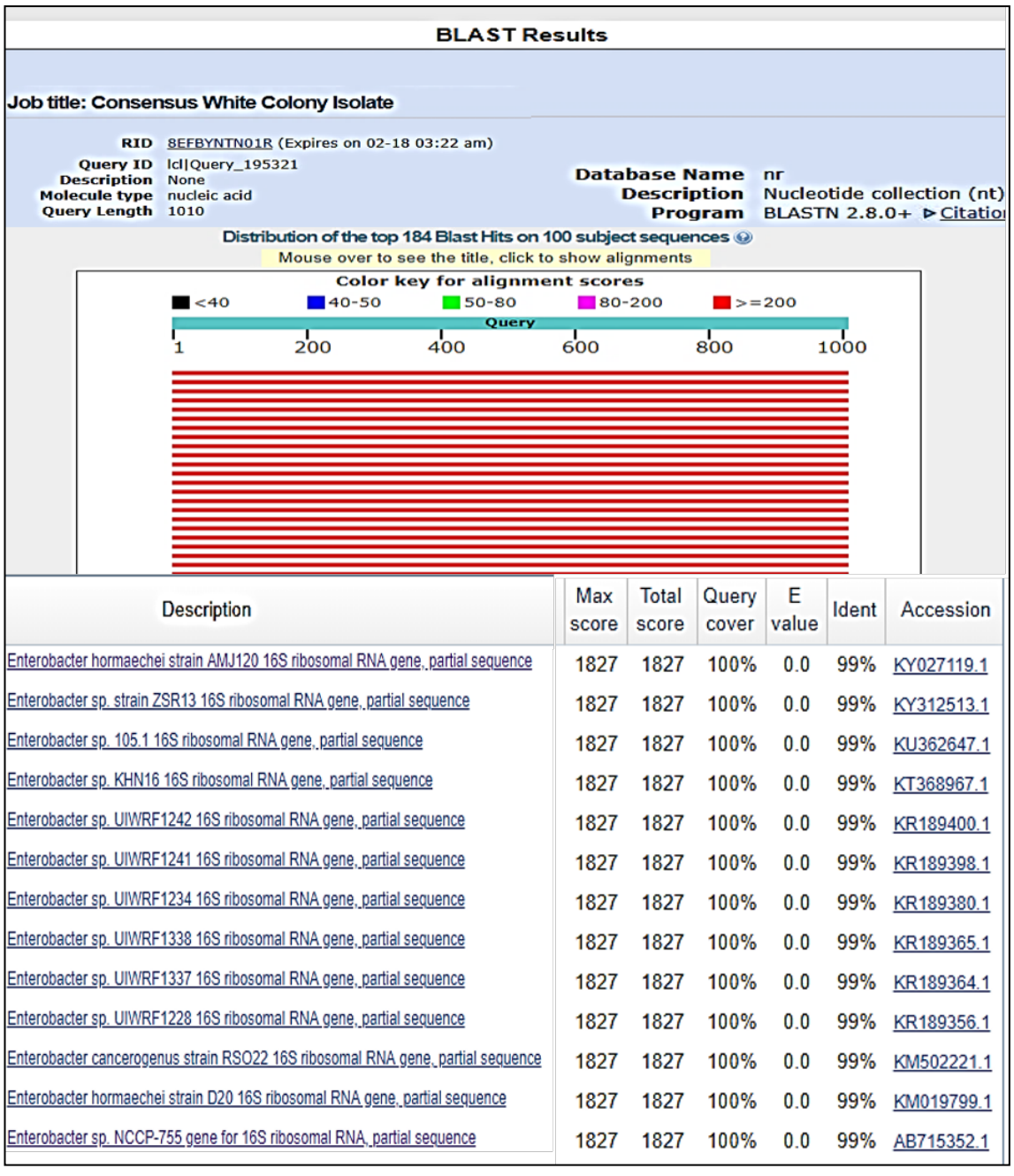
> Consensus Yellow Colony Isolate

GCCCAGATGGGTTAGCTTGTAGGTGGGTAACGGCTTACCTGGGGGACGATCCCTAGCTGGTTTG
AGGGGATGACCAGCCACACGGGAAATGAGACACGGTCCAGATTCCTACGGGAGGCAGCAGTGG
GGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGG
GTTGTAAAGTACTTTTAGCGGGGAGGAAGGCGATGCGGTTAATAACCGCGTCGATTGACGTTAC
CCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCCAGCGTT
AATTGGAATTACTGGGCGTAAAGCGCACGCCGGCGGTCTGTCAAGTTGGATGTGAAATCCCCGG
GCTTAACCTGGGAACTGCATTCGAAACTGGCCGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCC
AGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGA
CAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCA
CGCCGTAAACGATGTCGACTTGGAGGCTGTTTCCTTGAGAAGTGGCTTCCGGAGCTAACGCGTTA
AGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCAC
AAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAG
AGAACTTTCCAGAGATGCATTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTC
AGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAG
CGATTCGGTCGGGAACTCAAAGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGT
CAAGTCATCATGGCCCTTACGAGTAGGGCTAC

Figure 4. 31: *Consensus sequence obtained for the yellow isolated colony of B. dalzielii with 993 bases*

The consensus regions obtained for both the white (Figure 4.28 and 4.29) and yellow (Figure 4.30 and 4.31) isolated colonies of *B. dalzielii* were used for NCBI Blast Search. The blast search for white isolated colony consensus return with 100 hits, all having

100% query cover, 0.0 E value and 99% identity (Figure 4.32), mostly with *Enterobacter species.* No hit returned with a 100% similarity suggesting that the isolated sequence has not been submitted for this bacteria strain.



Description	Max score	Total score	Query cover	E value	Ident	Accession
Enterobacter hormaechei strain AMJ120 16S ribosomal RNA gene, partial sequence	1827	1827	100%	0.0	99%	KY027119.1
Enterobacter sp. strain ZSR13 16S ribosomal RNA gene, partial sequence	1827	1827	100%	0.0	99%	KY312513.1
Enterobacter sp. 105.1 16S ribosomal RNA gene, partial sequence	1827	1827	100%	0.0	99%	KU362647.1
Enterobacter sp. KHN16 16S ribosomal RNA gene, partial sequence	1827	1827	100%	0.0	99%	KT368967.1
Enterobacter sp. UIWRF1242 16S ribosomal RNA gene, partial sequence	1827	1827	100%	0.0	99%	KR189400.1
Enterobacter sp. UIWRF1241 16S ribosomal RNA gene, partial sequence	1827	1827	100%	0.0	99%	KR189398.1
Enterobacter sp. UIWRF1234 16S ribosomal RNA gene, partial sequence	1827	1827	100%	0.0	99%	KR189380.1
Enterobacter sp. UIWRF1338 16S ribosomal RNA gene, partial sequence	1827	1827	100%	0.0	99%	KR189365.1
Enterobacter sp. UIWRF1337 16S ribosomal RNA gene, partial sequence	1827	1827	100%	0.0	99%	KR189364.1
Enterobacter sp. UIWRF1228 16S ribosomal RNA gene, partial sequence	1827	1827	100%	0.0	99%	KR189356.1
Enterobacter cancerogenus strain RSO22 16S ribosomal RNA gene, partial sequence	1827	1827	100%	0.0	99%	KM502221.1
Enterobacter hormaechei strain D20 16S ribosomal RNA gene, partial sequence	1827	1827	100%	0.0	99%	KM019799.1
Enterobacter sp. NCCP-755 gene for 16S ribosomal RNA, partial sequence	1827	1827	100%	0.0	99%	AB715352.1

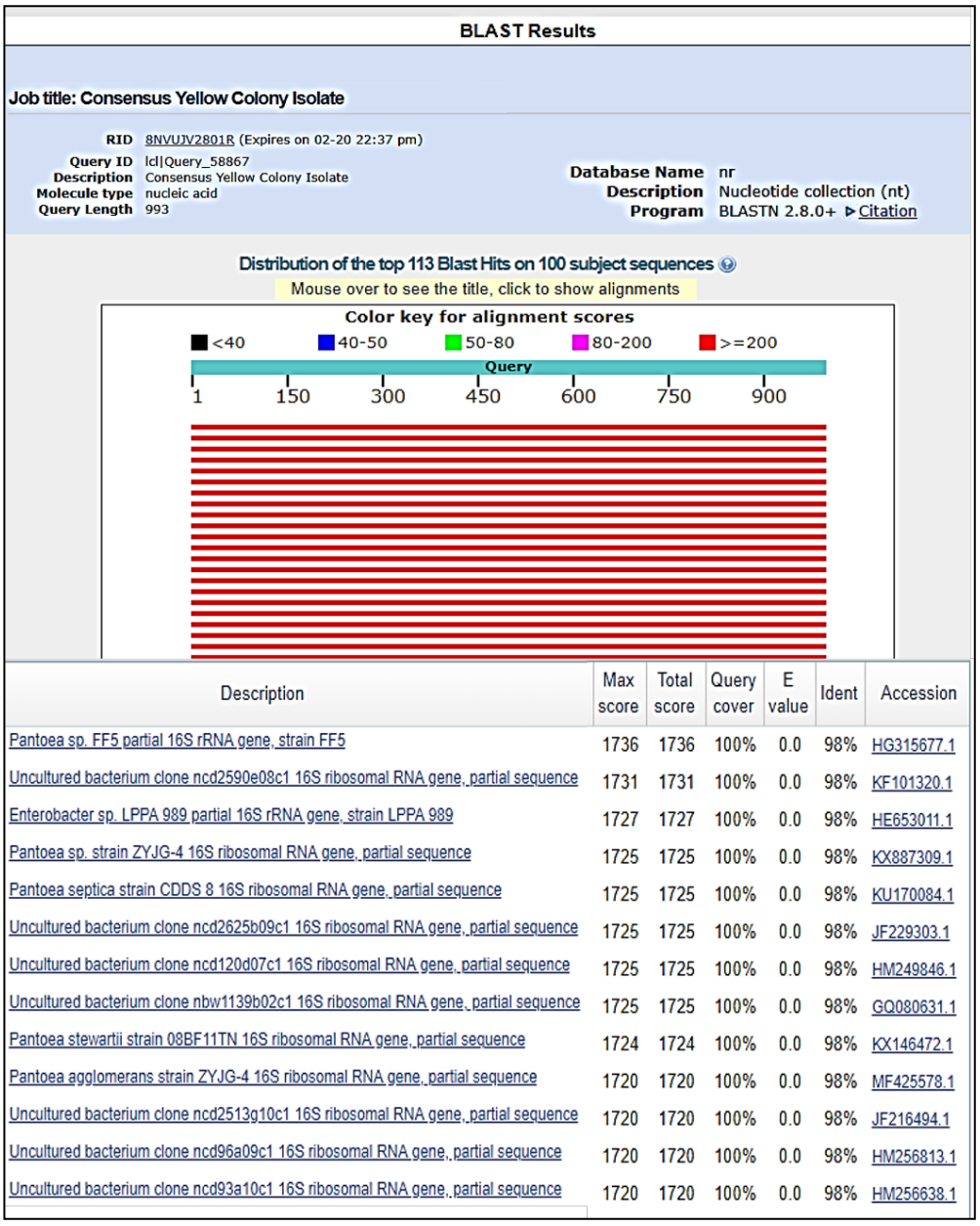
Figure 4. 32: *NCBI Blast Search for white isolated colony obtained from B. dalzielii*

Taxonomy and lineage report was generated to select the most frequent organism similar to the white isolated colony (Table 4.1). The report showed that white colony isolate of *B. dalzielii* is suspected to belong to the family Enterobacteriaceae, having 105 hits across 43 organisms; and belongs to the genus Enterobacter with 102 hits across 40 organisms. The white colony isolate is suspected to belong to the *Enterobacter cloacae* complex, having 45 hits across 6 organisms belonging to the *Enterobacter cloacae* complex, with predominant hits (24) to *Enterobacter cloacae*. Each of the 24 hits has different accession number and submitted by different authors, thus implicating the possibility of the white isolated colony obtained from *B. dalzielii* as *Enterobacter cloacae*, as it has a maximum score of 1827 (Table 4.1). Thus, the sequence was deposited in the GenBank database under the accession number MH764584 (https://www.ncbi.nlm.nih.gov/nuccore/mh764584).

	Taxonomy	Number of hits	Number of Organisms	Score
Kingdom	Bacteria	107	44	
Family	Enterobacteriaceae	105	43	
Genus	Enterobacter	102	40	
Species	*Enterobacter cloacae* complex	45	6	
Organisms	***Enterobacter cloacae***	**24**	**1**	**1827**
	Enterobacter hormaechei	12	3	1821
	Enterobacter cancerogenus	4	1	1821
	Enterobacter xiangfangensis	3	1	1821
	Enterobacter hormaechei subsp. Steigerwaltii	1	1	1827
	Enterobacter hormaechei subsp. Oharae	1	1	1823

Table 4. 1: *Taxonomy and lineage report of white colony isolated from B. dalzielii*

The blast search for the yellow isolated colony consensus return with 97 hits with 100% query cover and 0.0 E value, however, only 47 of these hits have 98% identity with the query submitted. And the hit with the maximum and total scores of 1736 is the *Pantoea spp* (Figure 4.33).



BLAST Results

Job title: Consensus Yellow Colony Isolate

RID 8NVUJV2801R (Expires on 02-20 22:37 pm)
Query ID lcl|Query_58867
Description Consensus Yellow Colony Isolate
Molecule type nucleic acid
Query Length 993

Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.8.0+ ▸Citation

Distribution of the top 113 Blast Hits on 100 subject sequences
Mouse over to see the title, click to show alignments

Description	Max score	Total score	Query cover	E value	Ident	Accession
Pantoea sp. FF5 partial 16S rRNA gene, strain FF5	1736	1736	100%	0.0	98%	HG315677.1
Uncultured bacterium clone ncd2590e08c1 16S ribosomal RNA gene, partial sequence	1731	1731	100%	0.0	98%	KF101320.1
Enterobacter sp. LPPA 989 partial 16S rRNA gene, strain LPPA 989	1727	1727	100%	0.0	98%	HE653011.1
Pantoea sp. strain ZYJG-4 16S ribosomal RNA gene, partial sequence	1725	1725	100%	0.0	98%	KX887309.1
Pantoea septica strain CDDS 8 16S ribosomal RNA gene, partial sequence	1725	1725	100%	0.0	98%	KU170084.1
Uncultured bacterium clone ncd2625b09c1 16S ribosomal RNA gene, partial sequence	1725	1725	100%	0.0	98%	JF229303.1
Uncultured bacterium clone ncd120d07c1 16S ribosomal RNA gene, partial sequence	1725	1725	100%	0.0	98%	HM249846.1
Uncultured bacterium clone nbw1139b02c1 16S ribosomal RNA gene, partial sequence	1725	1725	100%	0.0	98%	GQ080631.1
Pantoea stewartii strain 08BF11TN 16S ribosomal RNA gene, partial sequence	1724	1724	100%	0.0	98%	KX146472.1
Pantoea agglomerans strain ZYJG-4 16S ribosomal RNA gene, partial sequence	1720	1720	100%	0.0	98%	MF425578.1
Uncultured bacterium clone ncd2513g10c1 16S ribosomal RNA gene, partial sequence	1720	1720	100%	0.0	98%	JF216494.1
Uncultured bacterium clone ncd96a09c1 16S ribosomal RNA gene, partial sequence	1720	1720	100%	0.0	98%	HM256813.1
Uncultured bacterium clone ncd93a10c1 16S ribosomal RNA gene, partial sequence	1720	1720	100%	0.0	98%	HM256638.1

Figure 4. 33: *NCBI Blast Search for yellow isolated colony obtained from B. dalzielii*

Taxonomy and lineage report was generated to select the most frequent organism similar to the yellow isolated colony (Table 4.2). The report showed that the yellow colony isolate of *B. dalzielii* is suspected to belong to the class Gammaproteobacteria, having 48 hits across 26 organisms; and belonging to the genus *Pantoea* with 25 hits across 9 organisms. From the NCBI blast result, the specific identity of the yellow isolated colony of *B. dalzielii* was inconclusive, however, it is confirmed that it is suspected to be

a *Pantoea spp*. Thus, the sequence was deposited in the GenBank database under the accession number MH764583 (https://www.ncbi.nlm.nih.gov/nuccore/MH764583).

	Taxonomy	**Number of hits**	**Number of Organisms**	**Score**
Kingdom	Bacteria	109	33	
Class	Gammaproteobacteria	48	26	
Order	Enterobacterales	46	24	
Family	Erwiniaceae	26	10	
Genus	**Pantoea**	**25**	**9**	**1736**
Organisms	*Pantoea sp*. FF5	1	1	1725
	Pantoea sp.	6	1	1725
	Pantoea septica	8	1	1724
	Pantoea stewartii	1	2	1720
	Pantoea agglomerans	3	1	1720

Table 4. 2: *Taxonomy and lineage report of yellow colony isolated from B. dalzielii*

PCR analysis of the 16S RNA region showed that the AP1 20E biochemical analysis is not a sensitive test for identification micro-organisms. However, the test did show that the yellow isolate identified as *Pantoea spp*. via 16S RNA analysis had 0.2% identity with *Pantoea spp* 3 (Figure 4.26).

4.3.9 BACTERIUM RESPONSIBLE FOR THE PRODUCTION OF PYROGALLOL

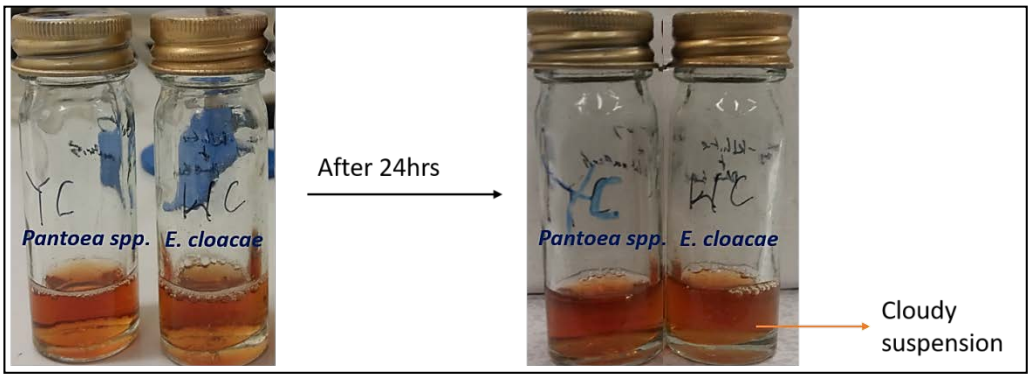


Figure 4. 34*: Inoculated sterile aqueous plant extract of B. dalzielii with isolated bacteria (Pantoea spp. and E. cloacae)*

HPLC chromatogram (Figure 4.35) showed that *Pantoea spp*. utilises the extract (Figure 4. 34) in producing a compound with a retention time of 12.78 while the compound with the retention time of 9.3 is seen to have decreased peak high in the presence of the bacteria.

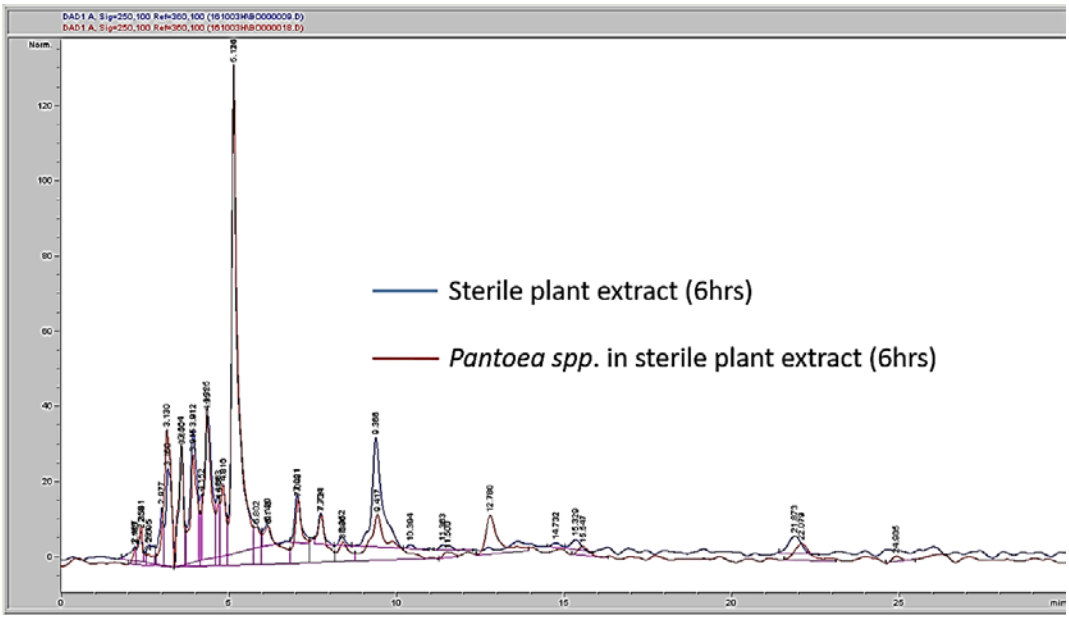


Figure 4. 35: *Sterile plant extract inoculated with Pantoea spp. isolated from B. dalzielii*

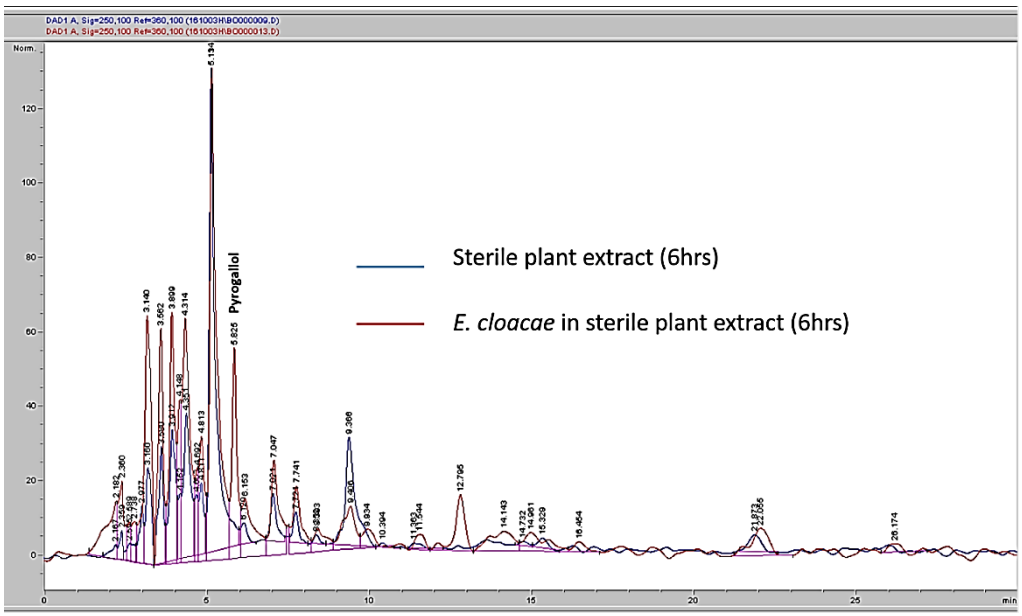


Figure 4. 36: *Sterile plant extract inoculated with E. cloacae isolated from B. dalzielii*

Figure 4.36 above showed that the *E. cloacae* utilises the extract in producing pyrogallol with a retention time of 5.8, which is similar to the retention time of standard pyrogallol purchased from Sigma Aldrich, see Figure 4.2.

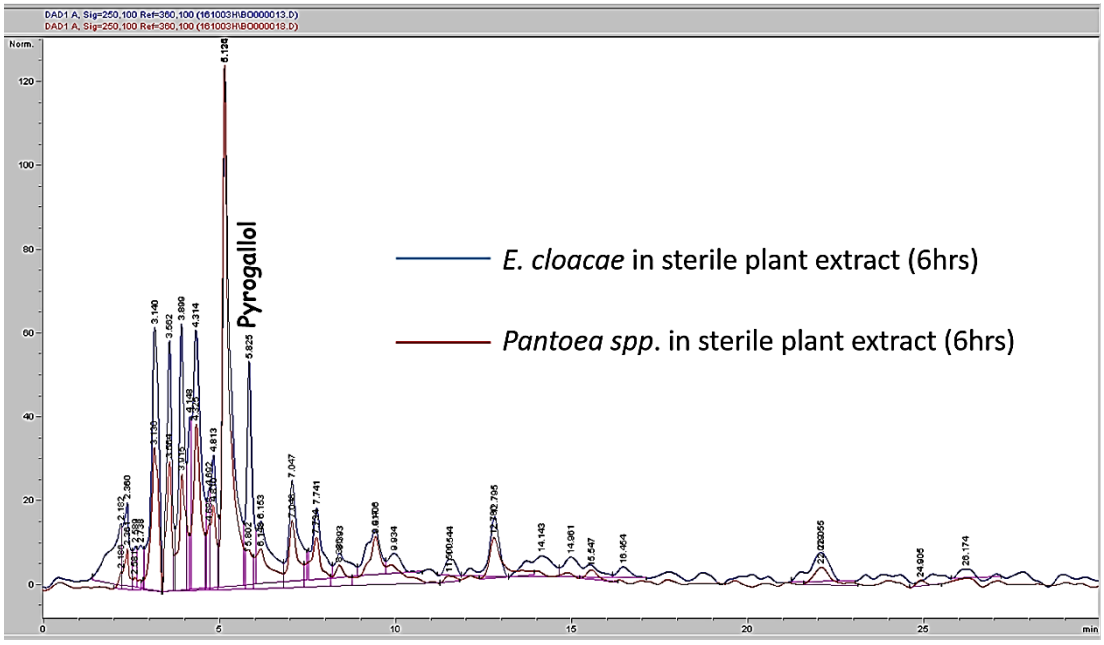


Figure 4. 37: *Comparing sterile plant extract inoculated with bacteria isolated from B. dalzielii*

HPLC analysis (Figure 4.37) showed that the difference between sterile plant extract inoculated with the *Pantoea spp*. with that inoculated with *E. cloacae* is the presence of pyrogallol peak in the HPLC chromatogram depicted blue. The bacteria *E. cloacae* was shown to be responsible for the conversion of gallic acid to pyrogallol.

4.3.10CONVERSION OF GALLIC ACID TO PYROGALLOL

The absorbance recorded for the suspensions used for the bioconversion are 0.100 and 0.099 Thin layer chromatography analysis detected the presence of pyrogallol in both samples. 2µg of gallic acid and pyrogallol was spotted as references while 2µl of sample was spotted for every specific hour samples (Figure 4.38). Figure 4.38A is an image of TLC chromatogram under short wavelength showing samples collected from inoculum with the absorbance of 0.099 at specific hour(s) of zero, six, 12, 18 and 24. Pyrogallol (R_f value = 0.54) was seen to be present in all sample inoculums with the absorbance of 0.099 except at zero hour after spraying with vanillin reagent (Figure 4.38B). The same is said for Figure 4.38C, showing the presence of pyrogallol (R_f value = 0.69) in inoculum with the absorbance of 0.100.

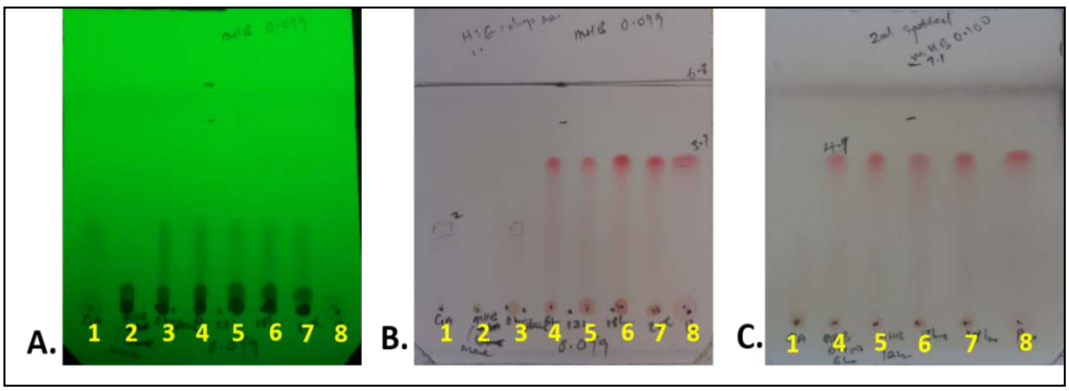


Figure 4. 38: *TLC analysis of gallic acid conversion to pyrogallol by E. cloacae where 1 = Gallic acid reference standard, 2 = Mueller Hinton broth, 3 = zero hour sample, 4 = 6hours sample, 5 =*

12 hours sample, 6 = 18hours sample, 7 = 24hours sample and 8 = pyrogallol reference standard.

HPLC analysis was conducted on all samples and the amount of pyrogallol produced and that of gallic acid spent was calculated. From the inoculated sample with the absorbance of 0.099, 0.36mg/mL of pyrogallol (Figure 4.39) was produced, while 0.45 mg/mL of pyrogallol (Figure 4.40) was produced in the inoculated sample with the absorbance of 0.100 after 24 hours. Thus, *E. cloacae* was shown to convert gallic acid to pyrogallol, and carbon dioxide is an addition expected product (Figure 4.41).

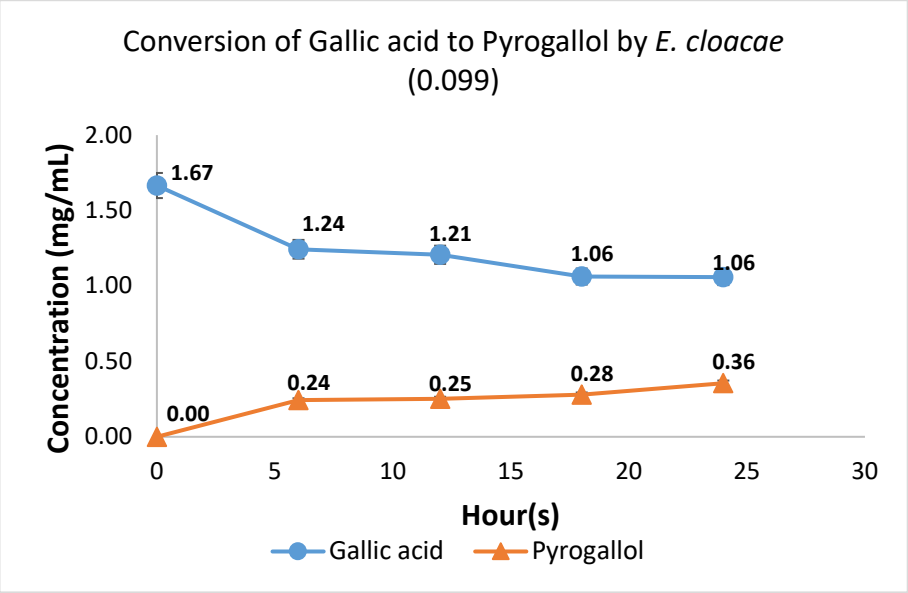


Figure 4. 39: *HPLC analysis of the bioconversion of gallic acid to pyrogallol from 0.099 sample. Data are presented as mean ± standard error for both compound.*

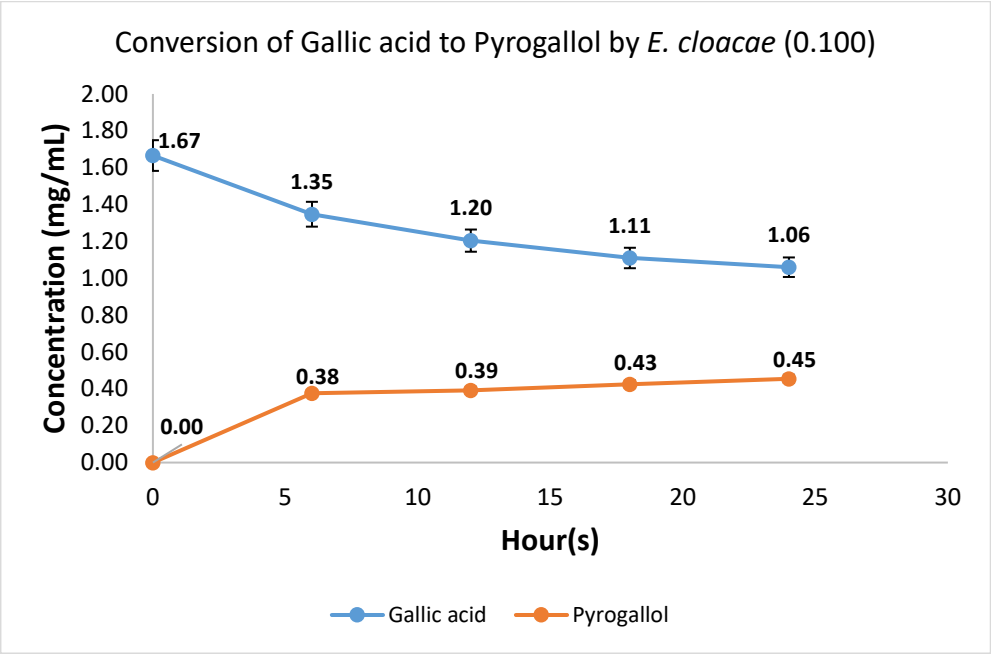


Figure 4. 40: *HPLC analysis of the bioconversion of gallic acid to pyrogallol from 0.099 sample. Data are presented as mean ± standard error for both compound.*

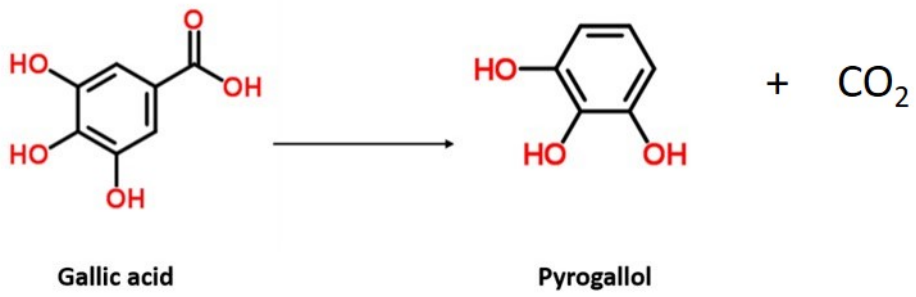


Figure 4. 41: *Conversion of gallic acid to pyrogallol and carbon dioxide by E. cloacae*

4.4 CONCLUSION

In the present studies, gallic acid was shown to be present in *B. dalzielii* which was later converted to pyrogallol by *Enterobacter cloacae* in 24hours water extract (Figure 4.41). This gave way to the possibility that in poor tropical regions where cooling facilities are rare, plant extract macerated for more than six hours could contain microbial metabolite

and the phytochemical profile may change dramatically over time, therefore, the ethnopharmacological claims of a plant could be misleading.

E. cloacae belongs to the genus *Enterobacter* and family *Enterobacteriaceae* which are facultative anaerobic Gram-negative bacteria. This micro-organism is found in terrestrial and aquatic environments (Hoffmann and Roggenkamp, 2003). The strains of *E. cloacae* are known as commensal microflora in human and animal intestinal tract. These strains also play a pathogenic role in plants, insects and humans. *E. cloacae* is reported as an important nosocomial pathogen and is responsible for lower respiratory tract, urinary tract, endocarditis, soft tissue and skin infection (Sanders and Sanders, 1997; Lee *et al.*, 2002). *Enterobacter* species were reported to bio-convert glycerol and 2,3-butanediol to ethanol and diacetyl by *E. aerogenes* and *E. cloacae*, respectively (Nwachukwu *et al.*, 2012; Zhang *et al.*, 2015). In 1985, Yoshia and Yamada reported the production of pyrogallol via the decarboxylation of gallic acid by *Citrobacter spp*. (Yoshida and Yamada, 1985). Similarly, pyrogallol production from gallic acid by *Enterobacter spp.* was also reported (Soni, Sharma and John, 2012), however, both studies were specifically designed to study said bioconversion. More so, *E. cloacae* was shown to biodegrade hexahydro-1,3,5-trinitro-1,3,5-s-triazine using a two-phase partitioning bioreactor (Pudge, Daugulis and Dubois, 2003).

In addition, another micro-organism isolated and identified in the bioburden of *B. dalzielii* aqueous extract is *Pantoea spp*. This strain belongs to the genus *Pantoea* which are rod shaped Gram-negative bacteria. *Pantoea spp* was reported to have been isolated from terrestrial and aquatic environment and also associate with animals, insects and

humans (Walterson and Stavrinides, 2015). Some of the species are plant pathogens, for example *P. agglomerans*, *P. stewartii* and *P. ananatis* (Roper, 2011; Nadha *et al.*, 2012). Presently, *Pantoea species* are considered problematic due to their pathogenic role in both immunocompetent and immunocompromised patients. These species are reported to cause osteomyelitis, bacteremia, septicemia and septic arthritis among others (Walterson and Stavrinides, 2015). The *Pantoea species* isolated was shown to lack the decarboxylation of gallic acid property associated with *E. cloacae*. However, another *Pantoea* species, *P. agglomerans,* was reported to decarboxylate gallic acid to pyrogallol (Zeida *et al.*, 1998).

In conclusion, the biotransformation of gallic acid to pyrogallol was studied first in the plant extract via inoculating sterile aqueous extract of B. dalzielii obtained from six hours maceration. This was further confirmed by using purchased gallic acid 0.15% in Mueller Hinton broth then observing its biotransformation by *E. cloacae* at time interval with the aid of HPLC analysis. To the best of the author`s knowledge, this is the first work reporting the presence bioconversion of gallic acid to pyrogallol by *E. cloacae* in aqueous extract of *B. dalzielii.*

5

CYTOTOXICITY SCREENING OF *Boswellia dalzielii*

"Cancer is the name given to a collection of related diseases. In all types of cancer, some of the body`s cells begin to divide without stopping and spread into surrounding tissues."

- National Cancer Institute, 2018

ABSTRACT

The activity-guided fractionation of aqueous *Boswellia dalzielii* bark extract against breast cancer cell line (MCF7) was conducted using MTT cytotoxicity assay and MCF10A was used as control. The crude extract of *B. dalzielii* was observed to have reduced cell viability to 87%, however, crude extract of five plant combination including *Spondias Mombin, Detarium microcarpum, Sclerocarya birrea, Vitellaria paradoxa* and *B. dalzielii* reduced cell viability to 68%. Moreover, 24 hours aqueous extract of *B. dalzielii* was shown to reduced cell viability to 45% and bioactivity-guided fractionation resulted in the identification of a cytotoxic compound, catechol, which was identified using HPLC, TLC, NMR, FTIR and HRMS. The half maximal effective concentration (EC50) of catechol against MCF7 cell line was 86µM. The growth inhibition effect of catechol was observed to be time- and concentration- dependent.

5.1 INTRODUCTION

This chapter will discuss the cytotoxicity screening of *B. dalzielii* bark on MCF-7 breast cancer cell using MCF-10A human breast epithelial cell line as control. This chapter will focus on the bioactivity guided fractionation of aqueous extract of *Boswellia dalzielii*. The rationale used in this chapter looks at how the plant material is used locally from the researcher`s knowledge. Water is used as solvent, mostly either by decoction or cold maceration. In this study, boiled and cold macerated samples were tested for cytotoxic effects. The plant is also used in combination with other plants and these combinations vary to different herbal practitioners and regions.

From the researcher's experience, there are different ways of preparing *B. dalzielii* which include using powdered stem bark in custard, cold maceration of whole stem bark in a container and preserving in the refrigerator and decoction i.e. boiling stem bark for about an hour to three hours. The plant bark is also used in combination with other plants including *Spondias Mombin, Detarium microcarpum, Sclerocarya birrea* and *Vitellaria paradoxa*. In this studies, MTT assay was used for the screening of plant bark of *B. dalzielii*, *S. Mombin, D. microcarpum, S. birrea* and *V. paradoxa* alone and in combination.

5.2 METHOD

5.2.1 HANDLINGS OF CELL LINES

MCF7 were maintained in DMEM containing 10% fetal bovine serum (FBS). MCF10A were maintained in DMEM/F12 containing the following supplements 10% FBS,

20ng/mL of EGF, 10µg/mL of insulin and 0.5mg/mL of Hydrocortisone. For both MCF7 and MCF10A, culturing conditions include 5% CO_2, 95% air and 37°C. Cultured cells are passaged at 70-80% confluence every 2-4 days depending on culture flasks. Trypan blue dye exclusion was used to determine the number of viable cells on haemocytometer with the aid of an inverted microscope.

In all assays conducted, the percentage final concentration of DMSO was < 0.2% v/v as recommended by the National Cancer Institute (NCI) (Selby *et al.*, 2017). In terms of sample concentration, although 15µg/mL is recommended by NCI for first single dose concentration (NCI, 2015), 10µg/mL was used in the present studies making the screening stringent.

5.2.2 SELECTION OF WAVELENGTH

200µL of different cell density of MCF7 including 5×10^3, 1×10^4, 1.5×10^5, 2×10^5 and 2.5×10^5, cells/mL were seeded in 96-well plates in triplicate. The plate was incubated for 12 hours to allow adherence of cells at 37°C in a humidified 5% CO_2/95% air incubator. After cells adherence, media was suctioned out and 200µL of complete media was added and the plate was incubated for 24 hours at 37°C in a humidified 5% CO_2/95% air incubator. After 24 hours, all media was suctioned out, 180µL of complete media was added to sample wells and 20µL of MTT 5mg/mL in PBS was added, then the plate was incubated for two hours. After two hours, media was suctioned out and 150µL DMSO was added to sample wells to solubilise generated formazan product. The plate was further incubated for 15 minutes and absorbance was measured with the aid of GloMax

microplate reader at 560nm and 600nm. Graph was plotted to select wavelength for subsequent experiments. Experiment was three independent studies and paired t-test was conducted.

5.2.3 MTT ASSAY

200µL of cell (1×10^4cells/mL) were seeded in 96-well plates in triplicate. The plates were incubated for 12 hours to allow adherence of cells at 37°C in a humidified 5% CO_2/95% air incubator. After cells adherence, media was suctioned out and 180µL of complete media was added, then 20uL of 10µg/mL samples in 0.1% DMSO was added to test wells, control wells were treated with 0.1% DMSO and blank with complete media. The procedure was continued as stated in section 5.2.2 and absorbance was measured at 560nm. % Cell viability was calculated using the formula:

$$\%\ cell\ viability = (A_T - A_B)/(A_C - A_B) \times 100$$

Where A_T represents the absorbance value of tested samples/compound, A_B represents the absorbance value of blank and A_C represents the absorbance value of control. Experiment was three independent studies. Tested sample(s) is/are considered potentially cytotoxic if the mean cell viability is reduced to <70% when compared to control. Thus, data obtained were only presented as mean ± standard error of mean (SEM).

5.2.4 EXTRACTION PROCEDURE WITH BETTER ACTIVITY

Two extraction procedure including Acid Based Extraction (ABE) and Acid Hydrolysis (AH) were employed to determine fractions with cytotoxicity activity against MCF7.

5.2.4.1 Acid hydrolysis

20 grams of plant bark of *B. dalzielii* was boiled in 200mL of distilled water for two hours. The sample was filtered using a Büchner funnel with Whatman Nr 1 filter paper and filtrate obtained was divided into two equal portions labelled portion A and portion B. Portion A was fractionated with ethyl acetate (3×20mL) with the aid of a separating funnel (Figure 5.1). The crude fraction obtained was dried on a rotary evaporator at 40°C and labelled non-hydrolysed, then stored in the refrigerator.

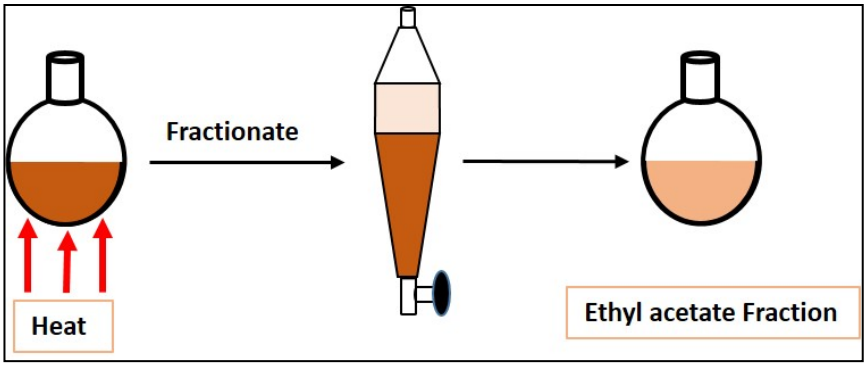


Figure 5. 1: *Non hydrolysed procedure*

Whilst portion B was acidified with concentrated hydrochloric acid (HCL) to obtain 2M solution and heat was applied for 30 minutes (Figure5 .2). With the aid of a separating funnel, ethyl acetate (3×20mL) was used in collecting organic compounds. The crude fraction obtained was dried on a rotary evaporator at 40°C and labelled as acid hydrolysed, then stored in the refrigerator.

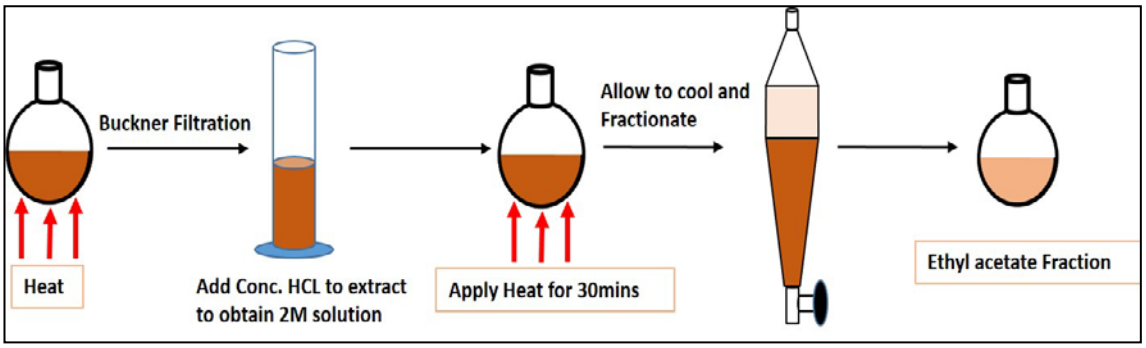


Figure 5. 2: *Acid hydrolysis procedure*

5.2.4.2 Acid base extraction

The method of Sharma *et al.*, (2016) was modified to collect organic acids present in plant bark of *B. dalzielii*. 10 grams of plant bark of *B. dalzielii* was boiled in 100mL of distilled water for two hours. The sample was filtered using a Büchner funnel with Whatman Nr 1 filter paper and adjusted to 3% sodium hydroxide (NaOH) solution, then the non-acidic part was collected by fractionating with ethyl acetate. The water fraction was acidified with 1N HCL and ethyl acetate and butanol were used to collect organic acid (3×20mL) (Figure 5.3). Samples collected were appropriately labelled and stored in the refrigerator.

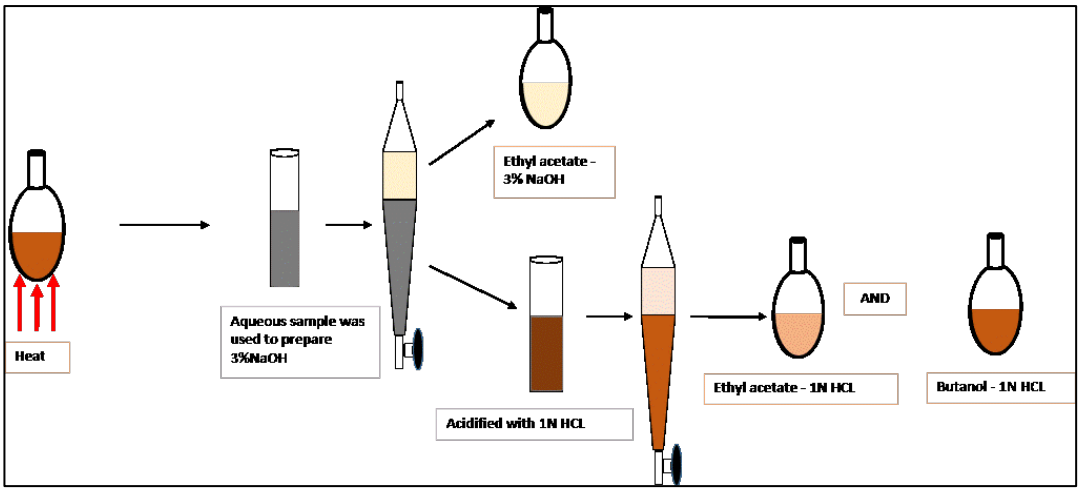


Figure 5. 3: *Acid base extraction procedure*

10mg in 1mL DMSO of samples collected from both extraction procedures were prepared as stock solutions. 100µg/mL of prepared samples were used as working solution and the final concentration of sample tested on MCF7 cells is 10µg/mL for 24 hours using the method outlined in section 5.2.3.

5.2.5 CYTOTOXICITY ACTIVITY OF FIVE PLANTS

Plant bark of *B. dalzielii* is ethno-pharmacologically used alone and also in combination with other plants, an example of such combination include *Spondias Mombin, Detarium microcarpum, Sclerocarya birrea* and *Vitellaria paradoxa* for treatment of cancer with rashes symptoms. Thus, the cytotoxicity of each of the five plants alone was conducted using MTT assay. Samples were prepared for each of the five plant barks using the method outlined in section 5.2.4.1. To test the activity of the plants in combination, two grams of each of the five plant barks was measured and combined to give a total of 10 grams, then the sample was also subjected to the procedure outlined in section 5.2.4.1. Also, the water fraction of the combined fraction was also prepared.

10mg in 1mL DMSO of samples collected from both non-hydrolysed and hydrolysed samples were prepared as stock solutions. 100µg/mL of prepared samples were used as working solution and the final concentration of sample tested on MCF7 cells is 10µg/mL for 24 hours using the method outlined in section 5.2.3.

5.2.6 BIOACTIVITY GUIDED FRACTIONATION OF ACID HYDROLYSED SAMPLE OF *B. dalzielii*

10 grams of plant bark of *B. dalzielii* was boiled in 100mL of distilled water for two hours and acid hydrolysed using procedure outlined in section 5.2.4.1. However, only the ethyl acetate fraction (0.56g) of the acid hydrolysed portion was used for column chromatography (CC). CC was performed using hexane and ethyl acetate in an equal ratio (1:1) and four fractions were collected. 10mg in 1mL DMSO of fractions obtained were prepared as stock solutions. 100µg/mL of prepared samples were used as working solution and the final concentration of sample tested on MCF7 cells is 10µg/mL for 24 hours using the method outlined in section 5.2.3.

5.2.7 CYTOTOXICITY ANALYSIS OF 24 HOURS AQUEOUS EXTRACT OF *B. dalzielii*

10 grams of plant bark of *B. dalzielii* was macerated in 100mL of sterile distilled water for 24 hours at 37°C. Separating funnel was used to collect organic compound using hexane and ethyl acetate. Samples collected were dried on a rotary evaporator at 40°C, labelled properly and stored in the refrigerator. Another 10 grams of plant bark of *B. dalzielii* was boiled in 100mL of distilled water for two hours and the sample was subjected to acid hydrolysis using the method outlined in section 5.2.4.1. However, the acidified sample was subjected to fractionation using hexane and ethyl acetate (3×20mL). 10mg in 1mL DMSO of samples obtained from both 24 hours maceration and acid hydrolysis were prepared as stock solutions. 100µg/mL of prepared samples were

used as working solution and the final concentration of sample tested on MCF7 cells is 10µg/mL for 48 hours using the method outlined in section 5.2.3.

5.2.8 CYTOTOXICITY ANALYSIS OF OLD AND NEW BATCHES OF *B. dalzielii*

10 grams of plant bark of *B. dalzielii* (both old and new batch) was macerated in 100mL of sterile distilled water for 24 hours at 37°C. Separating funnel was used to collect organic compound using hexane and ethyl acetate. The samples collected were dried on a rotary evaporator, labelled properly and stored in the refrigerator. 10mg in 1mL DMSO of samples obtained from both old and new batches of *B. dalzielii* were prepared as stock solutions. 100µg/mL of prepared samples were used as working solution and the final concentration of sample tested on MCF7 cells is 10µg/mL for 48 hours using the method outlined in section 5.2.3. In addition, thin layer chromatography was conducted using hexane and ethyl acetate (70:30) as the mobile phase to compare hexane fraction of both old and new batches of *B. dalzielii*.

5.2.9 BIOACTIVITY GUIDED FRACTIONATION OF 24 HOURS AQUEOUS SAMPLE OF *B. dalzielii*

10 grams of plant bark of *B. dalzielii* was macerated in 100mL of distilled water for 24 hours in 100mL of sterile distilled water for 24 hours at 37°C. Separating funnel was used to collect organic compound using hexane (3×20mL) and the sample collected was dried on a rotary evaporator at 40°C. 0.38g crude extract was collected and column chromatography was conducted using hexane and ethyl acetate (70:30) as solvent and five fractions were collected. 10mg in 1mL DMSO of fractions obtained were prepared

as stock solutions. 100µg/mL of prepared samples were used as working solution and the final concentration of sample tested on MCF7 cells is 10µg/mL for 48 hours using the method outlined in section 5.2.3. The fraction with activity was purified and the active compound was identified using the methods outlined in Chapter three, section 3.2.2.

5.2.10 HALF MAXIMAL EFFECTIVE CONCENTRATION (EC50)

Different concentration of identified compound was prepared including 12.5, 10, 7.5, 5 and 2.5µg/mL from a stock solution of 10mg in 1mL DMSO. Prepared samples were tested on MCF7 and MCF10A cells for 24, 48 and 72 hours. Control samples were treated with 0.125% DMSO and blank with complete media. Experiment was conducted using the method outlined in section 5.2.3.

5.3 RESULTS AND DISCUSSION

5.3.1 WAVELENGTH SELECTION

The device GloMax microplate reader lacks absorbance selection of 570nm, the wavelength recommended for spectrophotometric measurement of the dark blue formazan product of MTT (Mosmann, 1983). Thus, screening was conducted to select the wavelength for the measurement of MTT formazan product. Figure 5.4 shows that in the absence of 570nm, 560nm is a better wavelength than 600nm on the GloMax microplate reader and P-value obtained was 0.0006 predicting a difference between the two wavelengths. Thus, 560nm was used in subsequent assays. 560nm was also used in the MTT assay cytotoxicity screening of different leaves extracts of *Ricinus communis*

against human Caucasian skin fibroblast and macrophage cell lines (Nemudzivhadi and Masoko, 2014).

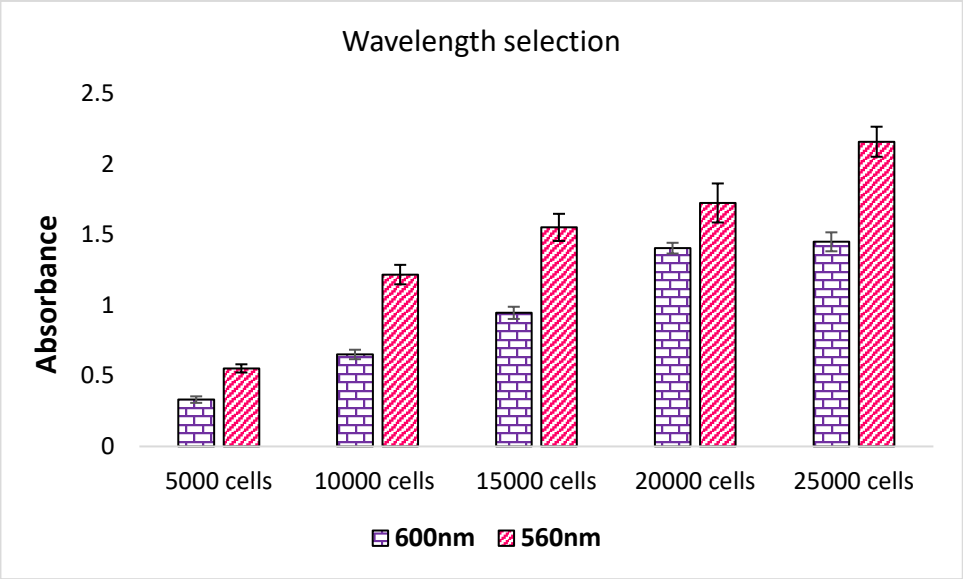


Figure 5. 4: *Wavelength selection for MTT assay ($p<0.05$) and data are mean ± SEM*

5.3.2 EXTRACTION PROCEDURE WITH BETTER ACTIVITY

In the selection of the extraction techniques that will lead to the extraction of potential cytotoxic compounds, crude extracts obtained via acid hydrolysis and acid base extraction techniques were used. Although 10µg/mL of extracts obtained via acid base extraction of B*. dalzielii* has no cytotoxic effect on MCF7 cells, 10µg/mL of the acid hydrolysed extract of B*. dalzielii* reduced viability to 85% (Figure 5.5). Thus, acid hydrolysis was selected as the extraction procedure used in subsequent screening assays.

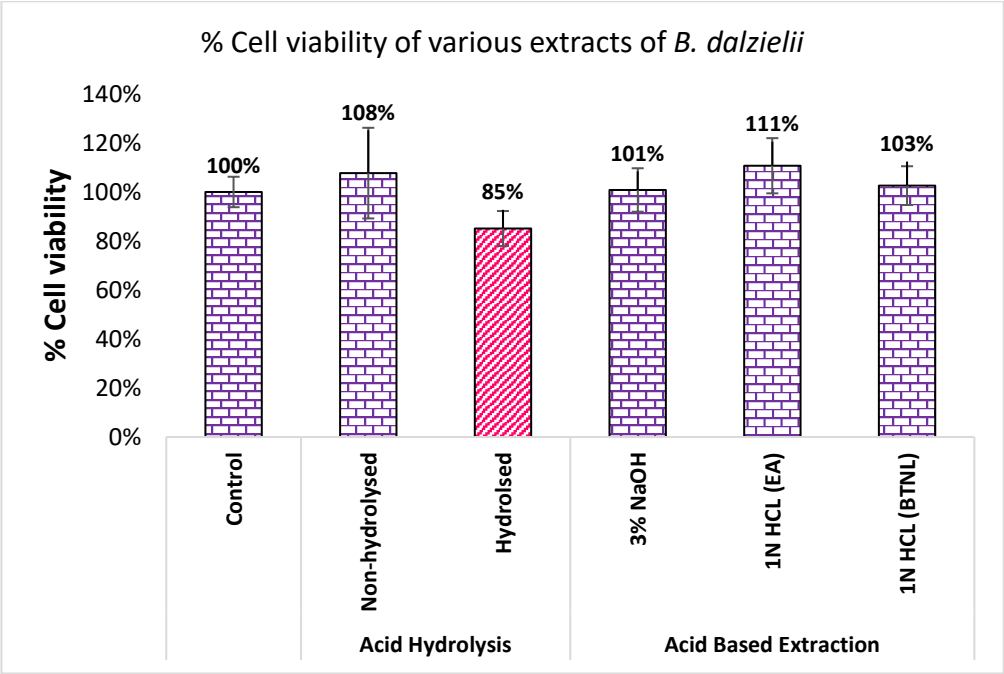


Figure 5. 5: *Percentage cell viability of acid hydrolysed and acid base extraction extracts of B. dalzielii tested against MCF7. Data are presented as mean ± SEM.*

5.3.3 CYTOTOXICITY ACTIVITY OF FIVE PLANTS

To test the cytotoxic effect of plant combination on MCF7 cells, individual plants were first acid hydrolysed and tested on MCF7 cells, before testing the cytotoxic effect of the acid hydrolysed extract of the combined plants. Result obtained showed that none of the plants reduced the viability of MCF7 to <70%, however, the crude extract of *B. dalzielii* was seen to reduce cell viability to 87% (Figure 5.6). An equal amount of the five plants was tested in combination and cell viability was reduced to 68% (Figure 5.7). Similarly, the dichloromethane extract of *S. mombin* was reported to exhibit poor antiproliferation activity against oral (KB), breast (MCF7), cervical (C-33A) and lung (A549) cancer cell lines at a concentration of 50 µg/mL (Akinmoladun *et al.*, 2015); and the extracts (hexane, dichloromethane, ethanol and water) of *D. microcarpum* was shown to have no cytotoxic activity against breast (MCF7), lung (A549), leukaemia (HL-

60), hepatocarcinoma (SMMC-7721) and colon (SW480) (Iweala *et al.*, 2015). Also, the water extract of *S. birrea* was shown to have no cytotoxic effect on not only MCF7 but also Hela and HT29 cancer cells (Tanih and Ndip, 2013).

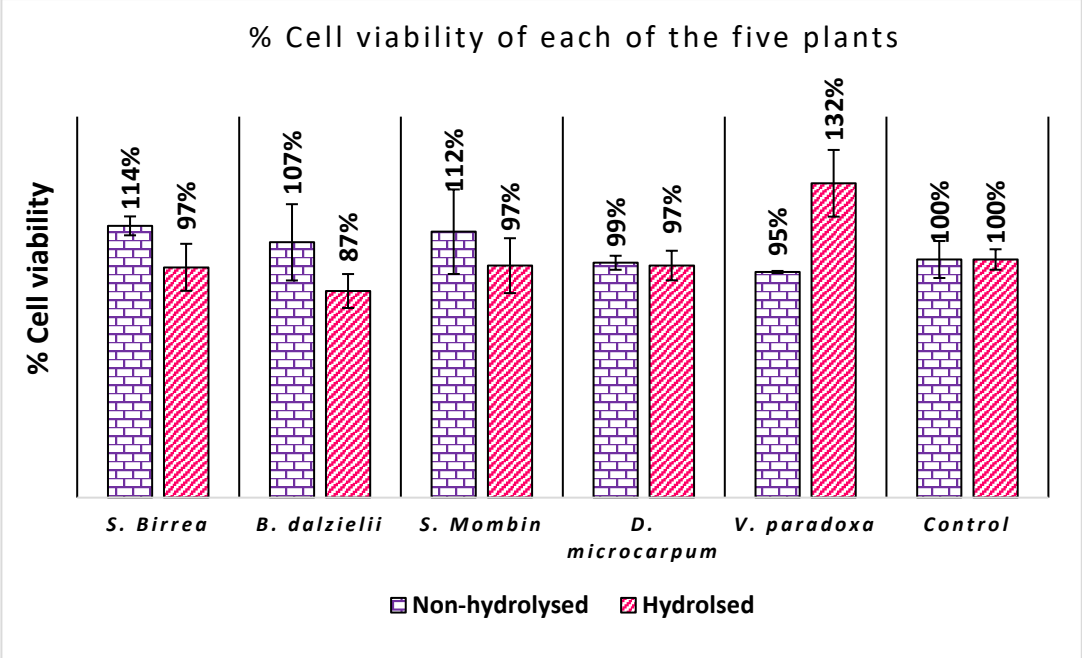


Figure 5. 6: *Percentage cell viability of each of the five plants, showing that the acid hydrolysed extract has better cytotoxicity activity than the non-hydrolysed extract except for V. paradoxa where the non-hydrolysed extract was seen to have better activity. Data are presented as mean ± SEM.*

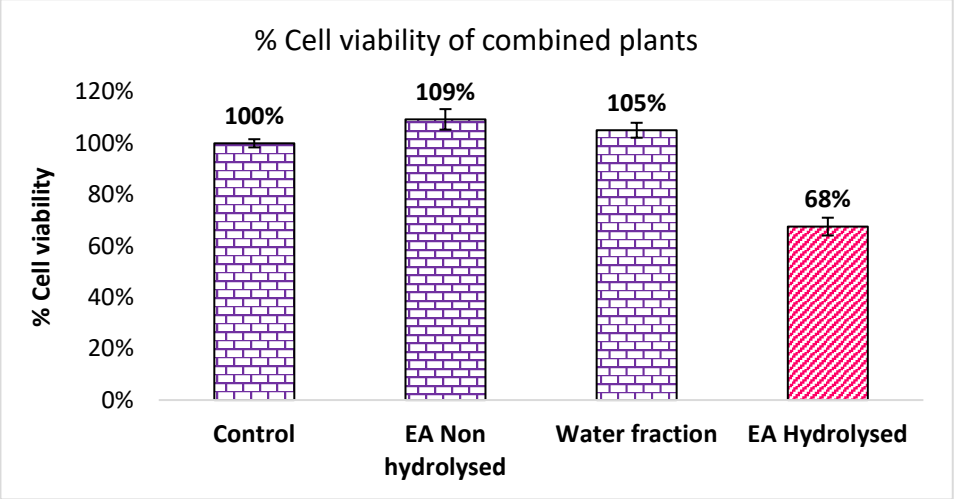


Figure 5. 7: *Percentage cell viability of the combined plant bark material, showing that the hydrolysed extract of the combined plants reduced cell viability to 68%. Data are presented as mean ± SEM.*

5.3.4 BIOACTIVITY GUIDED FRACTIONATION OF ACID HYDROLYSED SAMPLE OF *B. dalzielii*

The fractions obtained were dried on a rotary evaporator and extract yield obtained include 65.6mg, 27.8 mg, 25.2 mg and 31.5mg for fraction A, B, C and D, respectively. Fractions were tested, however, the cytotoxicity activity associated with the plant (Figure 5.6) was lost after fractionation (Figure 5.8). This loss in activity could be due to the minimum amount of the potential cytotoxic component in the extract, which could be remedied by increasing the concentration of tested extract in the initial screening. In addition, the activity observed could be due to the interaction(s) between compounds present in the plant crude extract.

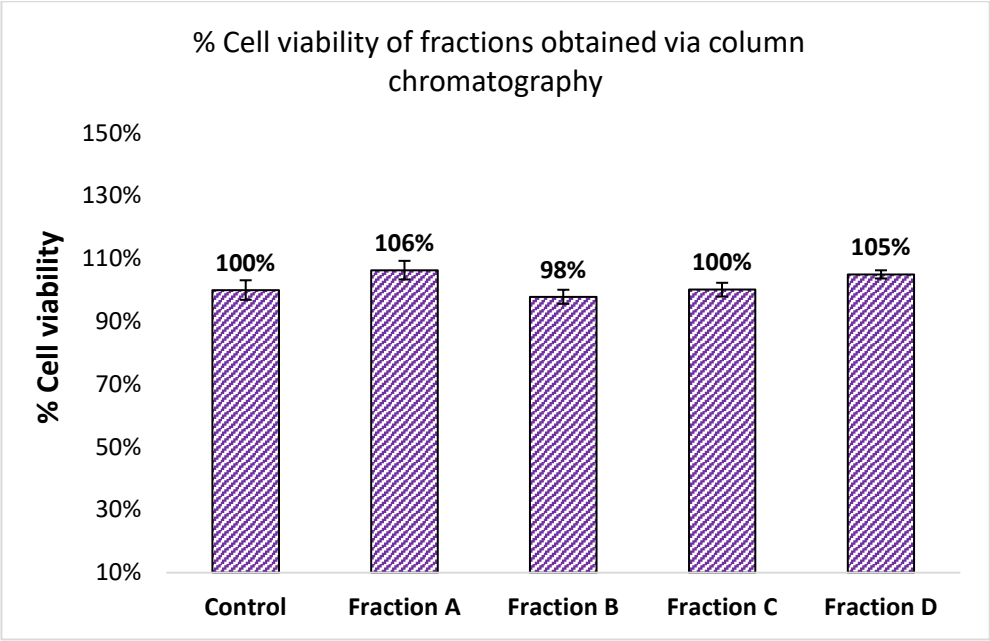


Figure 5. 8: *Percentage cell viability of fractions obtained after column chromatography. Data are presented as mean ± SEM.*

5.3.5 CYTOTOXICITY ANALYSIS OF 24 HOURS AQUEOUS EXTRACT OF *B. dalzielii*

The hexane fraction of 24 hours aqueous extract decreases cell viability to 62% after exposure for 48 hours (Figure 5.9). In comparison, the boiled sample was seen to lose activity, again this could be as a result of splitting the crude sample into hexane and ethyl acetate fraction. This result showed that the hexane extract obtained from 24 hours macerated aqueous extract has better activity than the boiled aqueous extract.

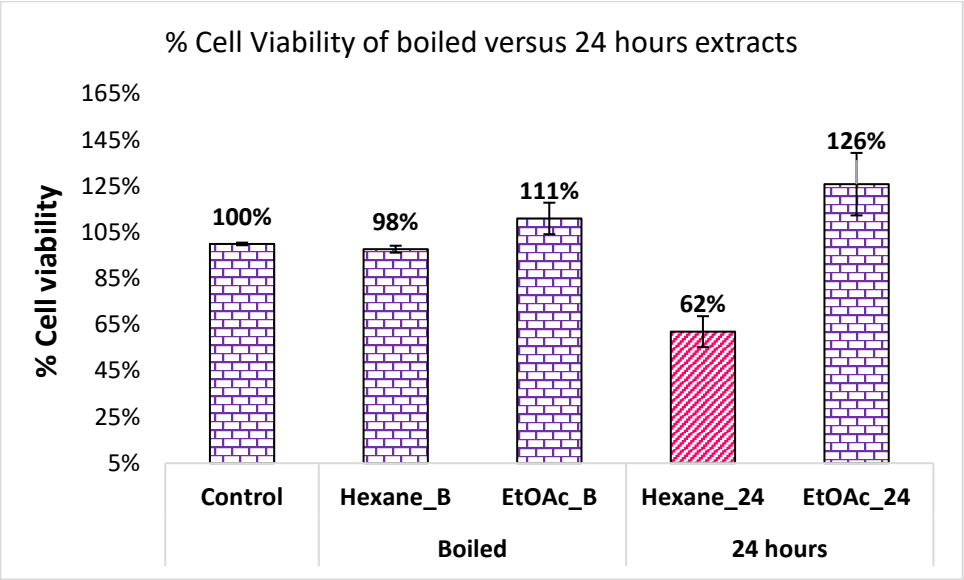


Figure 5. 9: *Percentage cell viability of fractions obtained from boiled aqueous and 24 hours cold macerated aqueous extracts. Data are presented as mean ± SEM.*

5.3.6 CYTOTOXICITY ANALYSIS OF OLD AND NEW BATCHES OF *B. dalzielii*

Due to shortage of the *B. dalzielii* plant batch initially used, a new batch was procured and also identified as *B. dalzielii*. The cytotoxicity assay was conducted to compare the old and new plant bark of *B. dalzielii*, although both hexane fractions of the new and old batches reduced MCF7 viability, the hexane fraction of the new batch was seen to have better activity reducing viability to 45% (Figure 5.11). TLC analysis showed that both old and new batches of *B. dalzielii* have similar phytochemicals labelled “a” (rf value = 0.57) and “b” (rf value = 0.41) which are visible under the short wavelength. However, the new batch of *B. dalzielii* was seen to have an additional compound labelled “c” (rf value = 0.32) (Figure 5.10).

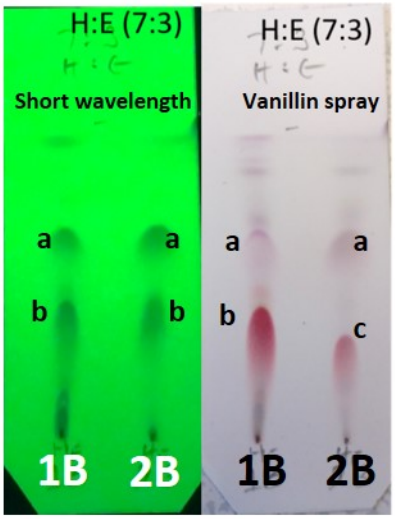


Figure 5. 10: *TLC chromatogram showing the presence compound "a" and "b" in both old (1B) and new (2B) batches of B. dalzielii*

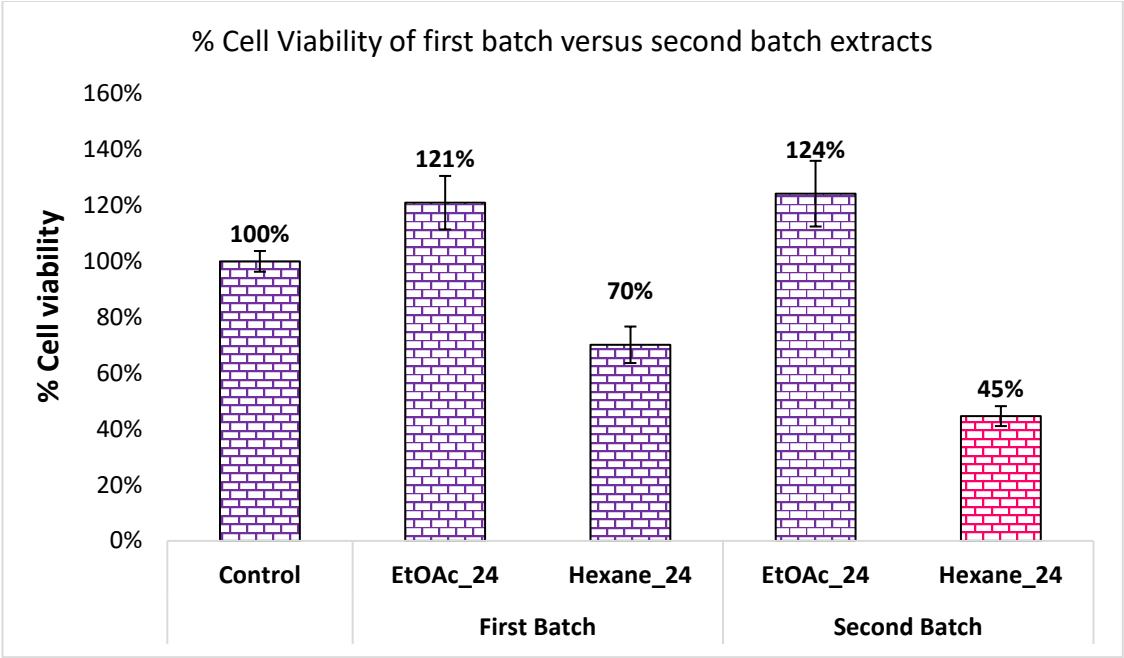


Figure 5. 11: *Percentage cell viability of fractions obtained from both old (first) and new (second) batches. Data are presented as mean ± SEM.*

5.3.7 BIOACTIVITY GUIDED FRACTIONATION OF 24 HOURS AQUEOUS SAMPLE OF *B. dalzielii*

The hexane fraction of 24hours aqueous extract of *B. dalzielii* was subjected to column chromatography. The fractions obtained were dried on a rotary evaporator and extract yield obtained include fraction A (3.6mg), B (1.7mg), C (0.98mg), D (12mg) and E (0.7mg). Of all the five fractions were tested on MCF7. The fractions with cytotoxicity activity are fraction D and E, reducing the viability of MCF7 to 50% and 74%, respectively after 48 hours (Figure 5.12). From the TLC chromatogram, it can be deduced that the activity of fraction E could be due to the carryover of compound "d" present in fraction D (Figure 5.13).

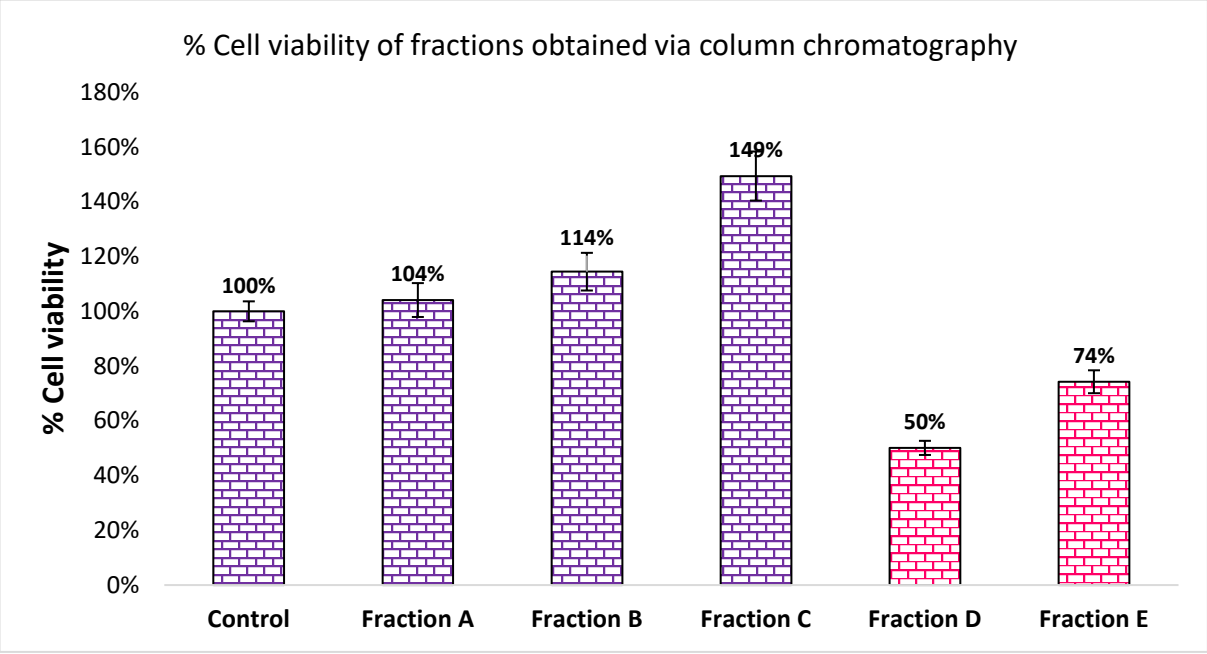


Figure 5. 12: *Percentage cell viability of fractions obtained from column chromatography of hexane fraction of 24 hours aqueous extract. Data are presented as mean ± SEM.*

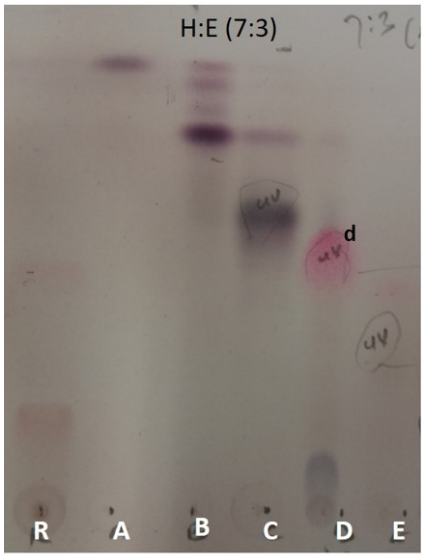


Figure 5. 13: *TLC chromatogram showing of fractions A-E*

Compound labelled "d" with the rf value of 0.62 in Fraction D was further purified and labelled as Compound D, then submitted for identification analysis.

5.3.8 IDENTIFICATION OF COMPOUNDS D

5.3.8.1 Fourier-transform infrared spectroscopy (FTIR) results

Compound D and reference compound (catechol) were analysed and the result obtained showed similar peaks between the extracted compound and catechol. The spectra of purchased catechol were consistent with that extracted from plant bark of *B. dalzielii*, suggesting that compound D is catechol (Figure 5.14). Figure 5.14 shows a prominent peak at 1260.54 cm^{-1} present in both extracted and purchased catechol. Although the whole spectrum was not provided, both extracted and purchased catechol were further analysed using NMR and HRMS analysis to further confirm the identity of compound D.

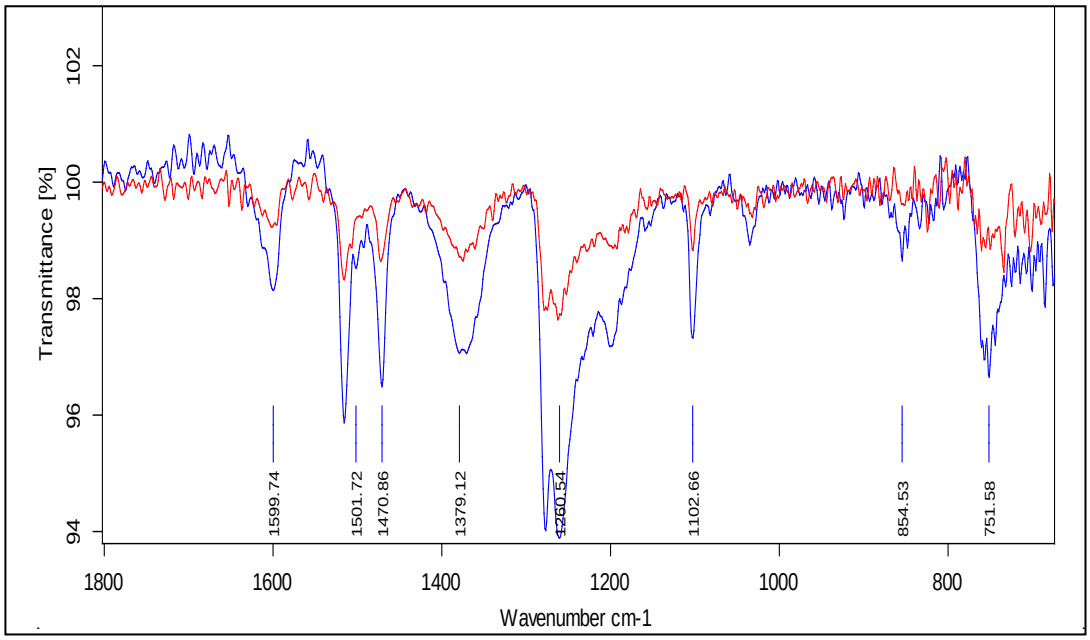


Figure 5. 14: *FTIR spectrum of catechol overlaying that purchased from Sigma Aldrich.*

5.3.8.2 Nuclear magnetic resonance (NMR) results

NMR analysis was conducted using deuterated methanol for both ^{1}H analyses using 20mg of extracted compound D and purchased catechol. Both compounds have similar ^{1}H-NMR spectrum showing signals at δ_H 6.67 ppm (2H, *ddd*) and δ_H 6.78 ppm (2H, *ddd*) (Figure 5.15). The spectrum provided had no expected hydroxyl signals, however, the result obtained is similar to that reported by (Choi *et al.*, 2018).

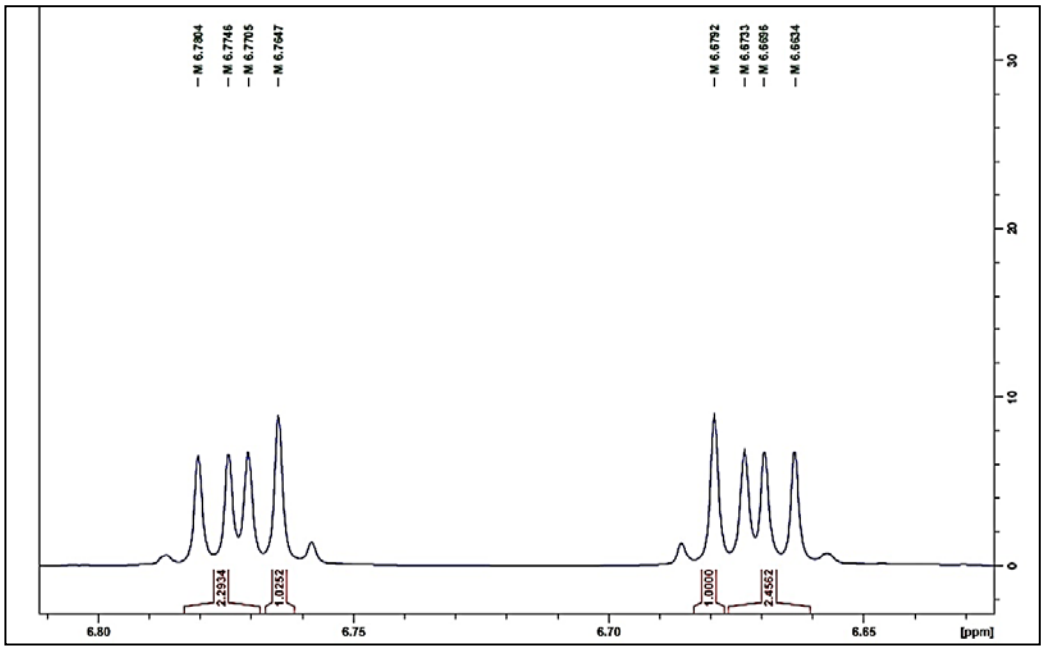


Figure 5. 15: *^{1}H-NMR spectrum of extracted catechol is consistent with that extracted from plant bark of B. dalzielii. ^{1}H-NMR spectrum was obtained using bruker av400 spectrometer at 399.94 MHz using deuterated methanol.*

5.3.8.3 High resolution mass spectrometry (HRMS) results

5mg of compound D plus respective reference (catechol) were used for HRMS which was conducted at EPSRC National Facility Swansea. Results showed that observed mass of compound D was measured as Mw 110 [$C_6H_6O_2$]; MS, m/z [E1-, %], 109.0295 (M-H, 100%) (See Figure 5.16) and predicted molecular composition was $C_6H_5O_2$.

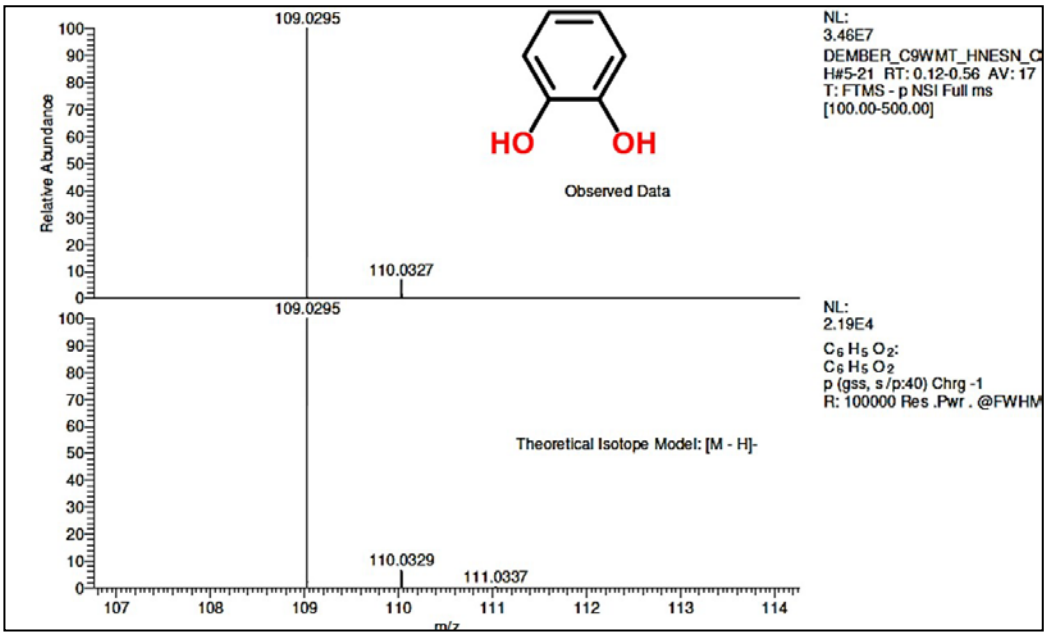


Figure 5. 16: *HRMS spectrum of Compound D and purchased catechol suggesting that extracted compound D might be catechol*

5.3.9 HALF MAXIMAL EFFECTIVE CONCENTRATION (EC50)

The cytotoxic effect of catechol was conducted on MCF7 at various hours of 24, 48 and 72 hours and MCF10A was used as control. A dose-response curve was generated via Hill equation using least squares regression to obtained EC50. Statistical analysis selected was non-linear regression, this is because, in ANOVA, grouping is not categorical thus the information that the same compound at different concentration was used is not recognised. And analysis of covariance was not used because it assumes a linear relationship between the response and covariate. However, t-test conducted between control containing growth media alone and control containing 0.125% DMSO indicated that there is no difference between the two controls (P= 0.48).

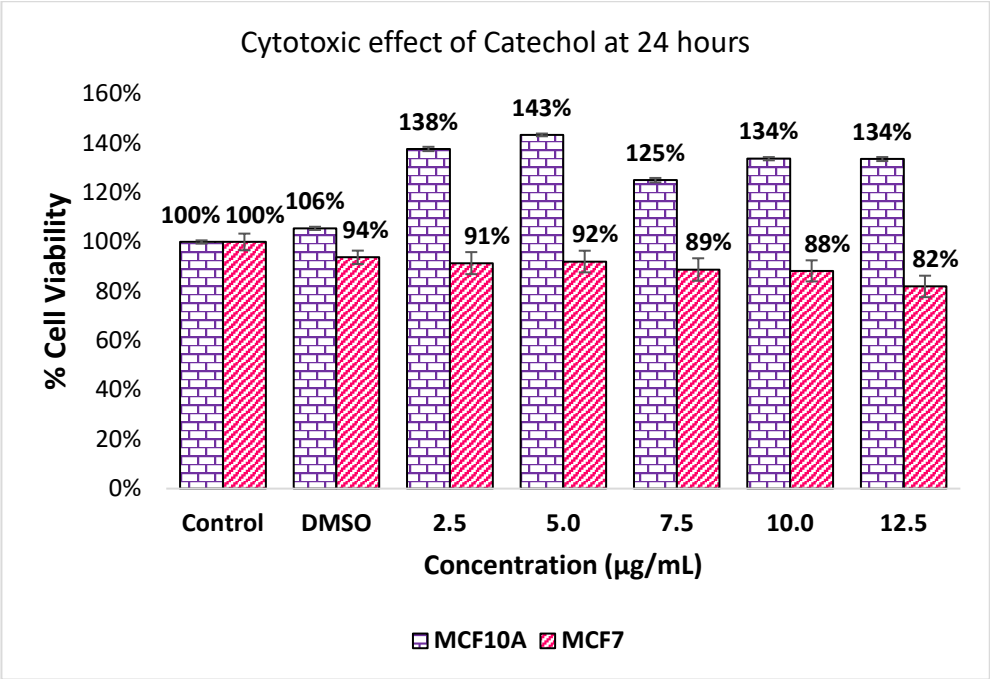


Figure 5. 17: *Cytotoxicity effect of catechol at different concentrations tested against MCF7 and MCF10A for 24 hours. Data are presented as mean ± SEM.*

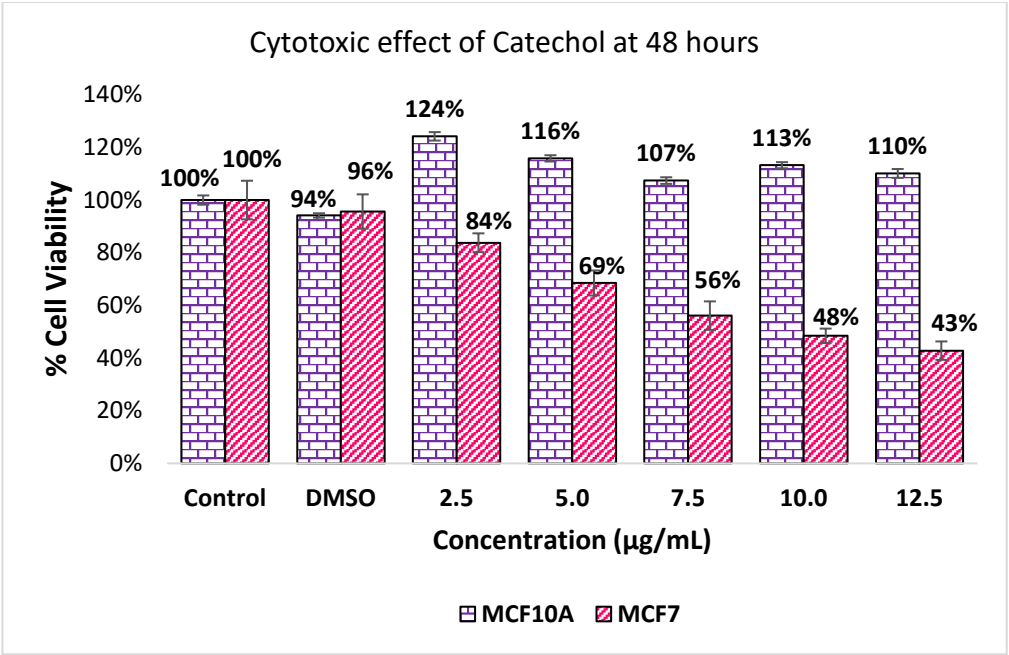


Figure 5. 18: *Cytotoxicity effect of catechol at different concentrations tested against MCF7 and MCF10A for 48 hours. Data are presented as mean ± SEM.*

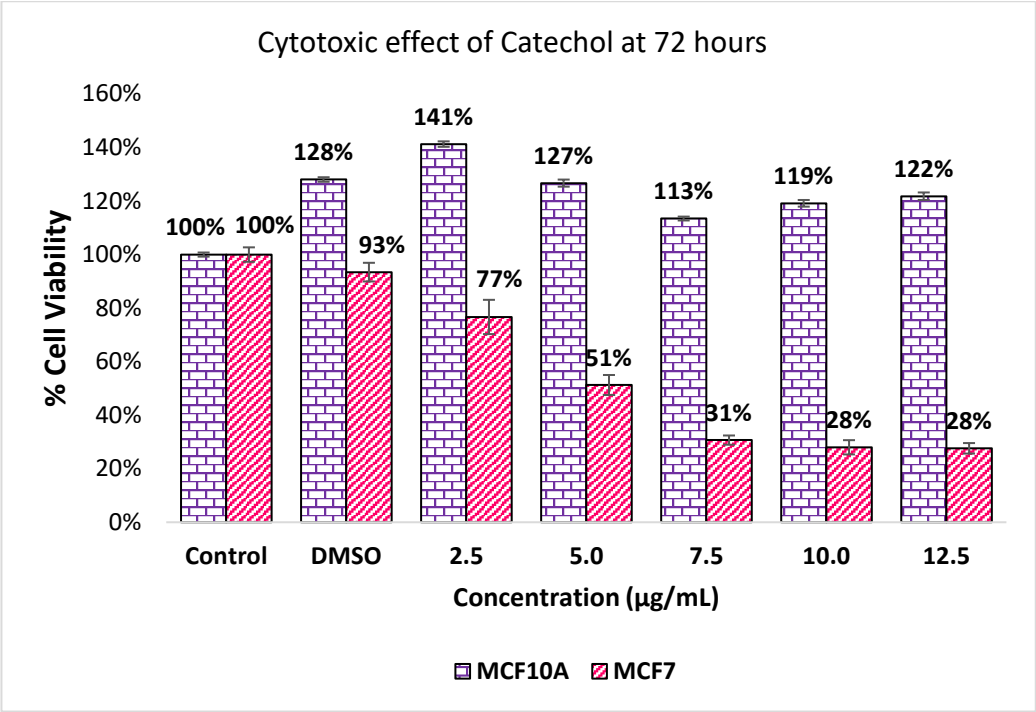


Figure 5. 19: *Cytotoxicity effect of catechol at different concentrations tested against MCF7 and MCF10A for 72 hours. Data are presented as mean ± SEM.*

12.5ug/mL highest concentration of catechol (114µM) used in the present study showed cytotoxicity against MCF 7, reducing cell viability to 82%, 43% and 24% after treatment for 24 hours (Figure 5.17), 48 hours (Figure 5.18) and 72 hours (Figure 5.19), respectively, while no cytotoxicity effect was observed against MCF10A.

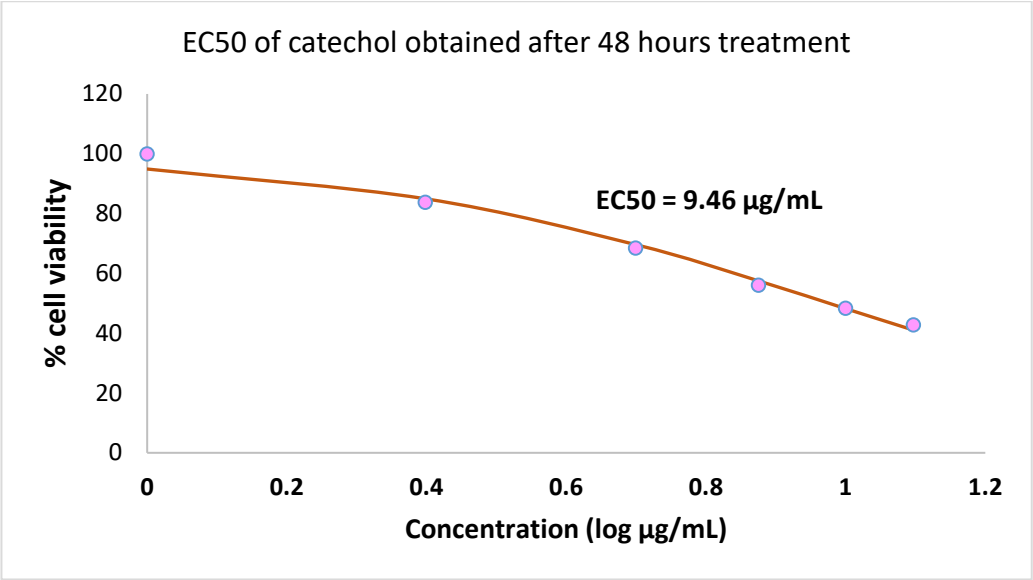


Figure 5. 20: *EC50 of catechol obtained after 48 hours treatment was calculated to be 9.46 µg/mL*

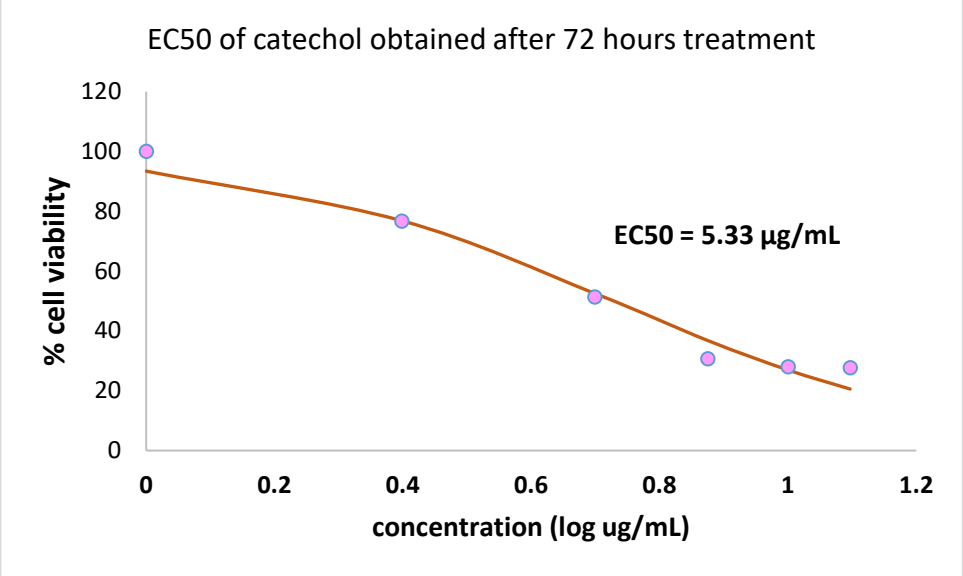


Figure 5. 21: *EC50 of catechol obtained after 72 hours treatment was calculated to be 5.33 µg/mL*

EC50 of catechol against MCF7 was calculated as 9.46µg/mL (86µM) after 48 hours treatment (Figure 5. 20) and 5.33 µg/mL (48.45 µM) after 72 hours (Figure 5. 21). In both 48 and 72 hours studies, concentrations >2.5 µg/mL where seen to reduce MCF7 cell viability to <70% with 12.5 µg/mL reducing viability to 43% and 28%. Overall, the analysis showed that the activity of catechol is time and concentration dependent. The study of

Wellington *et al.*, 2013 reported that catechol has a weak anticancer activity on MCF7 with GI50 of 52.41µM after 48 hours treatment whereas Madrid Villegas *et al.*, 2011 reported IC_{50} of 98.4µM for catechol against MCF7 after 72 hours treatment. In addition, catechol was shown to inhibit Breast cancer cells (MCF7 and MDA 231-MB) in a dose-dependent manner (Choi *et al.*, 2018).

5.4 CONCLUSION

The cytotoxicity of plants belonging to the genus *Boswellia* has been reported especially the plant, *Boswellia serrata*. *B. serrata* crude extracts and compounds mostly boswellic acids associated with cytotoxicity properties are collected using organic solvents (Bhushan *et al.*, 2007; Farajian Mashhadi *et al.*, 2016; Khan *et al.*, 2016; Rajabian *et al.*, 2016). In the present studies, water was used as the extraction solvent and crude extracts from different extraction procedures were tested. The acid based extraction method was used because it is one of the method used to collect total organic acids (Sharma *et al.*, 2016). Whilst, hydrolysis is used to separate aglycones from glycones (Nikolic, Stankovic and Markovic, 2005; Stalikas, 2007; Rivelli *et al.*, 2011). In the present study, *B. dalzielii* extract obtained via acid based extraction was shown to have no cytotoxicity effect on MCF7 cell line. However, that obtained via acid hydrolysis was shown to have reduced cell viability to 85%. Furthermore, using the four other plants in combination with *B. dalzielii* decreased cell viability to 68% suggesting that the mixture has potential cytotoxic effect compare to the individual plants, as seen in Figure 5.6 and 5.7.

The *B. dalzielii* crude extract with better cytotoxicity activity was that obtained from 24 hours maceration, reducing MCF7 viability to 45% after 48 hours treatment. Column chromatography of this extract resulted in the collection of five fractions (A-E), from which fraction D had the best cytotoxicity effect by reducing cell viability of MCF7 to 50%. The prominent compound present in this fraction was identified as catechol and EC50 obtained against MCF7 after 48 hours and 72 hours was 9.46µg/mL (86µM) and 5.33µg/mL (4.8µM), respectively. This result suggested that the cytotoxic effect of catechol on MCF7 is time-dependent. Figure 5.17, Figure 5.18 and Figure 5.19 suggested that the effect of catechol is also concentration dependent.

6

BIOCONVERSION OF PROTOCATECHUIC ACID

"Examining this water...I found floating therein divers earthy particles, and some green streaks, spirally wound serpent-wise...and I judge that some of these little creatures were above a thousand times smaller than the smallest ones I have ever yet seen, upon the rind of cheese, in wheaten flour, mould, and the like."

– Antonie van Leeuwenhoek 1632 – 1723

ABSTRACT

The role of endophyte was investigated on aqueous extract of *Boswellia dalzielii* bark used in cytotoxicity studies (Chapter five). Results obtained led to the isolation and identification of *Klebsiella pneumonia* B, *Klebsiella pneumonia* E and *Pantoea agglomerans*. However, only the isolated strains of *K. pneumonia* bioconverted protocatechuic acid to catechol and carbon dioxide. In addition, *E. cloacae* isolated in chapter four was also seen to bioconvert protocatechuic acid to catechol. Furthermore, *Klebsiella pneumonia* B and *Klebsiella pneumonia* E were also capable of bioconverting gallic acid to pyrogallol and carbon dioxide. The 16S rRNA sequences of *Klebsiella pneumonia* B, *Klebsiella pneumonia* E and *Pantoea agglomerans* are deposited in the GenBank nucleotide database under the accession number MH762022, MH762023 and MH762024, respectively.

6.1 INTRODUCTION

Cytotoxicity screening conducted in Chapter Five showed that 24hours aqueous extract of *B. dalzielii* bark has cytotoxic effect when compared to boiled aqueous extract. The compound with cytotoxic effect was isolated and identified as catechol. Thus, the source of catechol need to be determined and to achieve this, a series of analyses need to be conducted. From previous chapter (Chapter Four), results obtained showed that pyrogallol was a product of bioconversion of gallic acid by suspected bacteria *Enterobacter cloacae*, thus experiment will be designed to verify the implication of microorganisms in 24hours aqueous extract of *B. dalzielii* bark. This will commence with isolating microorganisms present on the bark of *B. dalzielii*, which will be followed by observing the effect of isolated microorganism on sterile aqueous extract of *B. dalzielii* bark. It is noteworthy to mention that the plant material used for cytotoxicity screening was a new batch of *B. dalzielii* bark obtained from the same region as the previous batch used for antimicrobial screening (Chapter Three) and it was also identified at the same herbarium at Ahmadu Bello University, Zaria, Kaduna State Nigeria.

6.2 METHODS

6.2.1 ISOLATION OF MICROORGANISMS

The method detailed in Chapter Four section 4.2.3 was used and samples were labelled B1, B2 and B3.

6.2.2 EFFECT OF ISOLATED MICROORGANISMS ON AQUEOUS EXTRACT OF *B. dalzielii*

10g of the plant bark of *B. dalzielii* was macerated in 100mL of sterile distilled water in a sterile 100mL bottle. The sample was placed on a mechanical shaker at 100RPM at room temperature for six hours. The sample was then sterilized using 0.2 micron Whatman sterile filter paper. 10mL of sterile aqueous extract was then transferred into six 50mL sterile sample bottles labelled control (reference), A, B, C, D, and E. And samples were inoculated with respective isolated microorganisms labelled A, B, C, D and E. Inoculated samples and control sample were incubated at 37°C overnight. TLC analysis was conducted on silica plate using hexane and ethyl acetate (50:50) plus a drop of formic acid and the analysis was repeated using hexane and ethyl acetate (70:30) as eluent and catechol as the reference compound. TLC chromatogram was treated with vanillin spray reagent to visualise compounds as mentioned in Chapter Four section 4.2.4.1. In addition, previously isolated and identified compounds (gallic acid and pyrogallol) in chapter three were also used as compound references. Affected samples and 1mg/ml of compound references and compound 3 were further analysed on HPLC using method outlined in Chapter Four section 4.2.1.2, however, HPLC column used was Kinetex C18 (250 ×4.6mm) and run time was 15minutes.

The main affected compound was obtained via column chromatography using hexane and ethyl acetate in a ratio of 50:50. Isolated compound was then identified using methods mention in Chapter Three section 3.2.2.

6.2.3 IDENTIFICATION OF BACTERIA SPECIES

Isolated microorganisms were used for gram staining, MacCONKEY agar plating and PCR analysis as detailed in Chapter Four sections 4.2.6.1, 4.2.6.3 and 4.2.8.

6.2.4 CONVERSION OF PROTOCATECHUIC ACID TO CATECHOL AND GALLIC ACID TO PYROGALLOL

The conversion was conducted using method detailed in Chapter Four section 4.2.10., 0.1% protocatechuic acid was prepared in sterile Mueller Hinton broth and three sterile bottles containing 10mL of 0.1% protocatechuic acid Mueller Hinton broth was inoculated with isolated *Klebsiella pneumonia* B, *Klebsiella pneumonia* E and *E. cloacae*, respectively. Similarly, 0.1% gallic acid prepared in sterile Mueller Hinton broth was also subjected to bioconversion by isolated *Klebsiella pneumonia* B and *Klebsiella pneumonia* E. All experiments were conducted in duplicate.

HPLC analysis was conducted as detailed in Chapter Four section 4.2.10., however, HPLC column used was Kinetex 5µm C18 (250 × 4.6mm), 15minutes run time and binary solvent system used as mobile phase consisted of 0.1% acetic acid in water and 0.1% acetic acid in acetonitrile (95/5, v/v).

6.3 RESULTS AND DISCUSSION

6.3.1 ISOLATION OF MICRO-ORGANISMS

Microbial growth was visible for all samples grown on all Mueller Hinton agar plate and five dominant colonies were observed on the plates (Figure 6.1). These organisms were

labelled colony A, B, C, D and E (Figure 6.2). Isolated colonies were further plated out to ensure that they are all single colonies, then transferred into storage vials containing cryopreservation beads (cryobeads) immersed in cryo-solution and stored in the freezer at -20^{o}C.

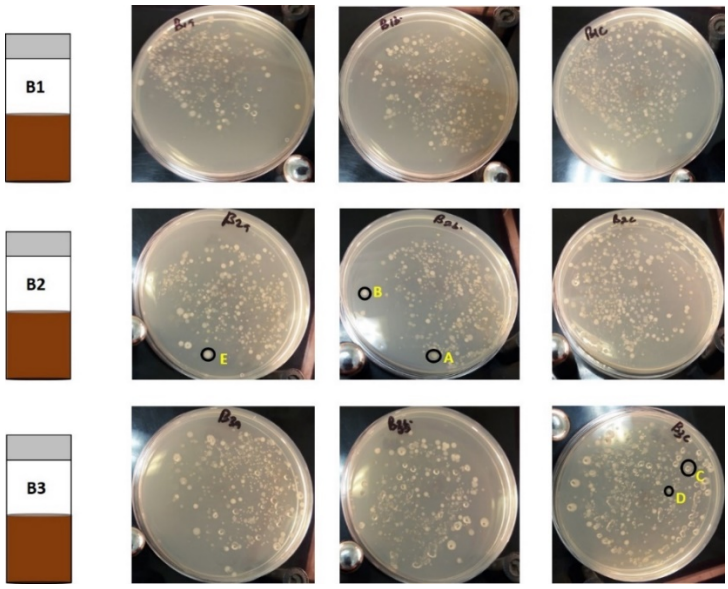


Figure 6. 1: *Microbial colonies observed in plant bark of B. dalzielii where labelled A-E*

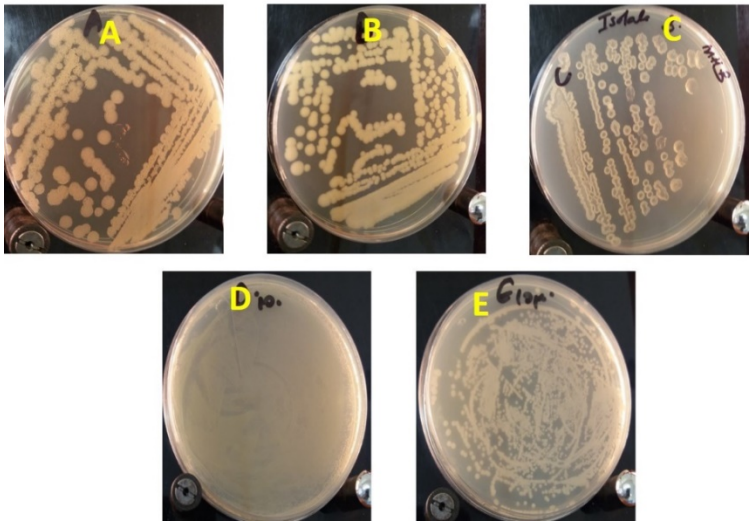


Figure 6. 2: *Isolated microorganisms labelled A – E*

6.3.2 EFFECT OF MICRO-ORGANISMS ON AQUEOUS EXTRACT OF *B. dalzielii*

6.3.2.1 TLC analysis

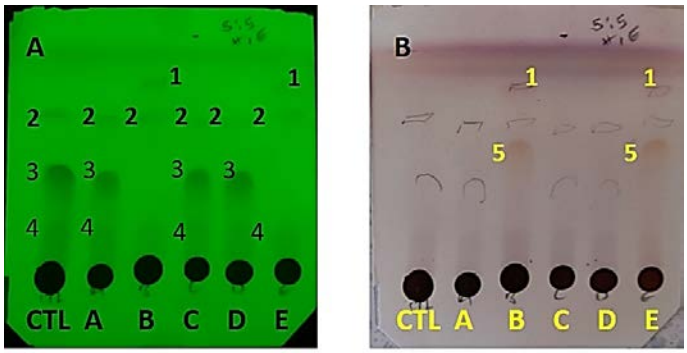


Figure 6. 3: *TLC chromatogram eluted with hexane and ethyl acetate (50:50) shows the presence of compounds in control and inoculated samples. Picture A viewed at 254nm shows the presence four prominent compounds labelled 2, 3 and 4 with R_f values of 0.79, 0.70, 0.42 and 0.18. While picture B was viewed after vanillin spray treatment and two main compounds labelled 1 and 5 were observed, R_f values of 0.79 and 0.58 were recorded, respectively.*

TLC analysis showed that samples inoculated with isolated microorganisms A, C and D have similar chemical profiles (compound 2, 3 and 4) with the control sample, as seen in Figure 6.3. However, samples inoculated with isolated microorganisms B and E have different chemical profiles consisting of two prominent compounds 1 and 5, while compound 3 is absent in both chemical profiles, as seen in Figure 6.3.

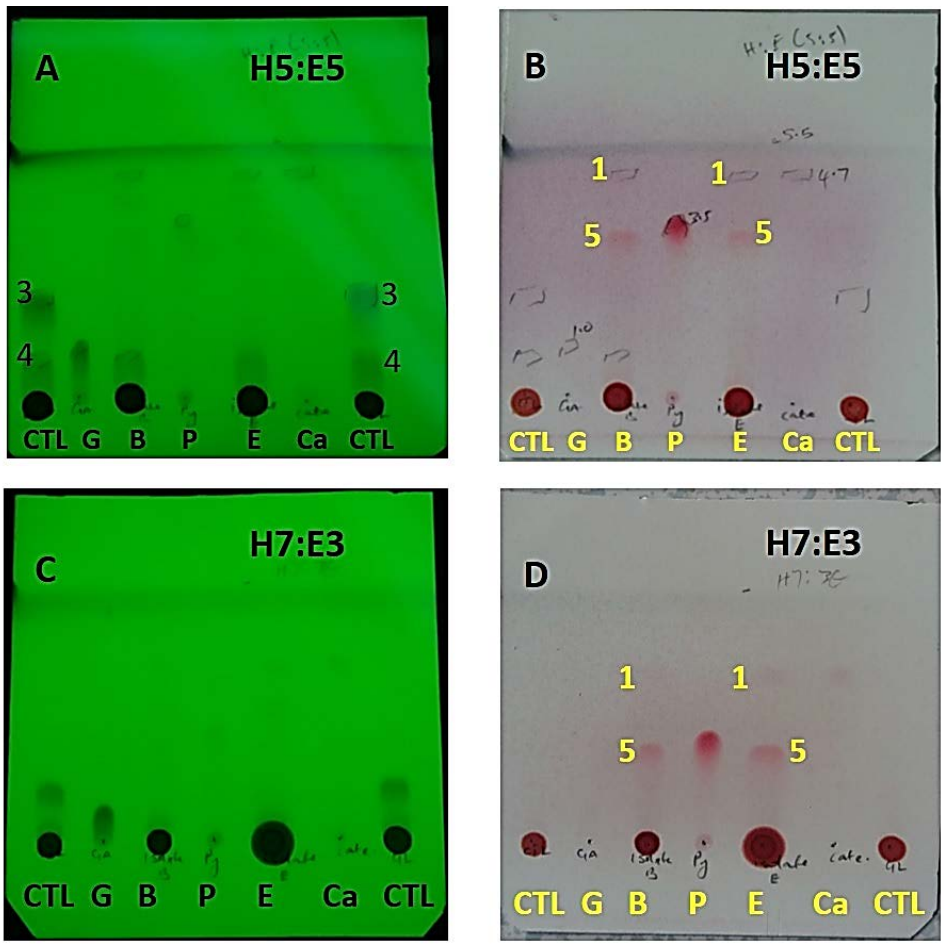


Figure 6. 4: *TLC chromatogram eluted with hexane and ethyl acetate (50:50 and 70:30), where CTL = sterile six hours aqueous B. dalzielii bark extract, G = gallic acid, B = sterile sample inoculated with Isolate B, P = pyrogallol, E = sterile sample inoculated with Isolate E, and Ca = catechol.*

Further TLC analysis was conducted with affected samples and compound references as seen in Figure 6.4B, where pyrogallol and catechol with R_f values of 0.64 and 0.85, respectively, were seen to be present in samples inoculated with Isolate B and Isolate E. Furthermore, gallic acid (R_f values of 0.18) was seen to be present in all samples as compound labelled 4, see Figure 6.4A. Figure 6.4 shows that catechol is present in samples inoculated with isolated microorganism B and E. Thus, TLC analysis suggested the involvement of microorganisms in obtaining catechol and HPLC analysis was

conducted to verify this claim. The affected compound in the control sample (compound labelled 3) was then isolated and identified.

6.3.3 ISOLATION OF COMPOUND 3

200g of plant material was macerated in 2L of 100% water for 6hours at room temperature, the extract was filtered and vacuum-concentrated. The crude extract was washed with ethyl acetate (3×300mL) to obtained fraction 1. With the aid of 10-40micron silica gel, fraction 1 (0.99g) was column chromatographed with Hexane: ethyl acetate (50:50) (Figure 6.5). Compound 3 was collected in fractions 17 – 24 and subjected to NMR, HRMS and FTIR analysis.

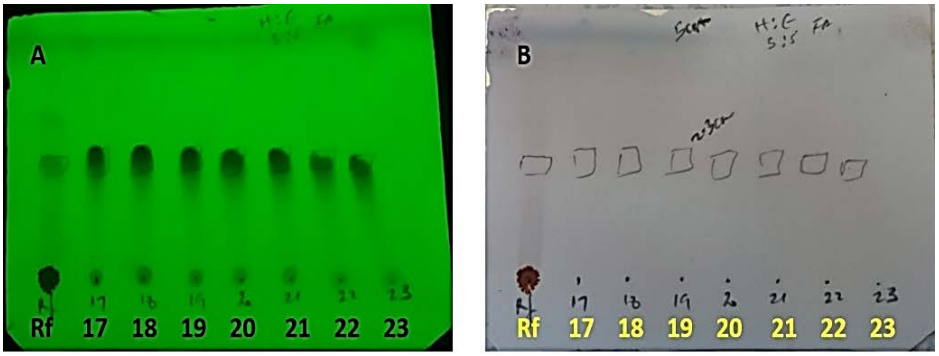


Figure 6. 5: *TLC chromatogram eluted with hexane and ethyl acetate (50:50) shows the isolated Compound 3. Compound 3 was visible (Picture A) at 254nm while picture B was viewed after vanillin spray treatment and R_f value of 0.46.*

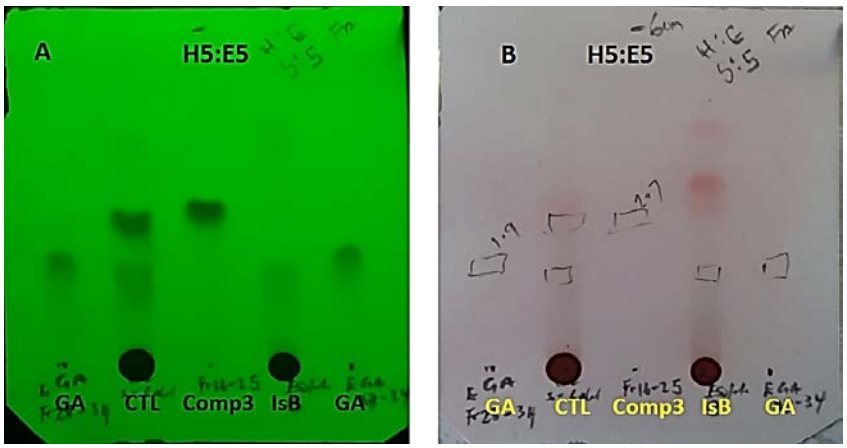


Figure 6. 6: *TLC chromatogram eluted with hexane and ethyl acetate (50:50), where GA = gallic acid, CTL = sterile six hours aqueous B. dalzielii bark extract, Comp3 = Isolated compound 3 and IsB = sterile sample inoculated with Isolate B.*

TLC analysis confirms the presences of compound 3 (R_f values of 0.45) in control samples and it absences in sample inoculated with Isolate B (see Figure 6.6), thus, suggesting that compound 3 might be crucial in the production of catechol.

6.3.3.1 HPLC analysis

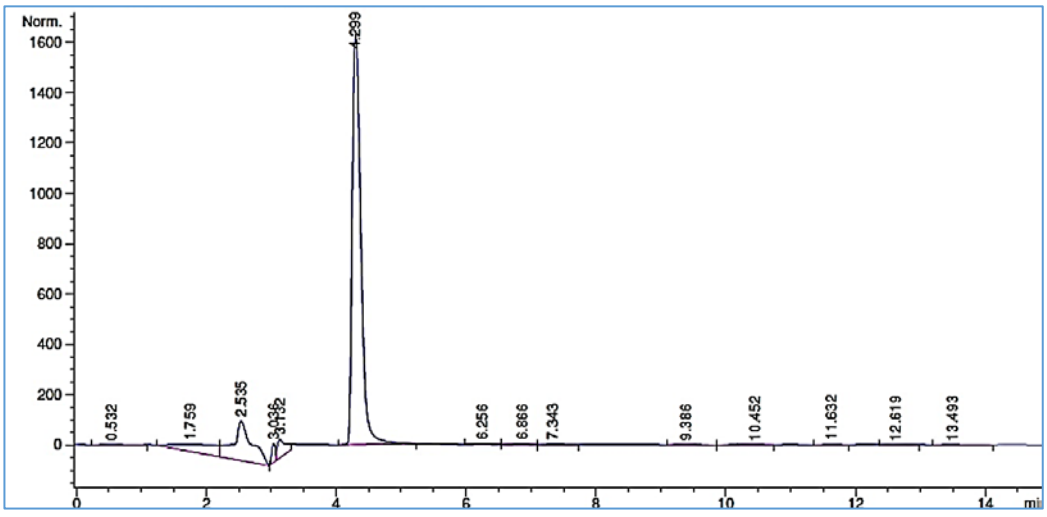


Figure 6. 7: *HPLC chromatogram of gallic acid with a retention time of 4.3*

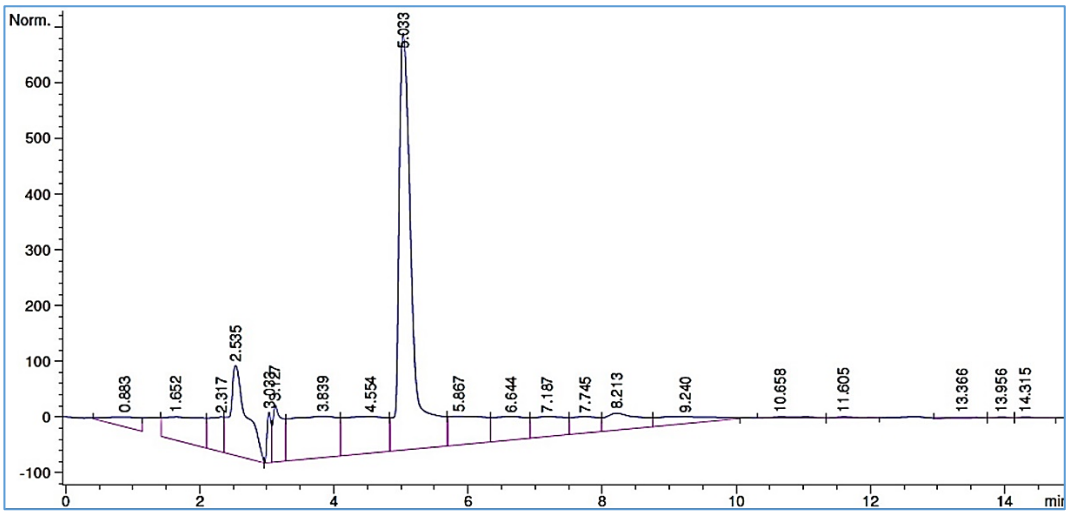


***Figure 6. 8**: HPLC chromatogram of pyrogallol with a retention time of 5.0*

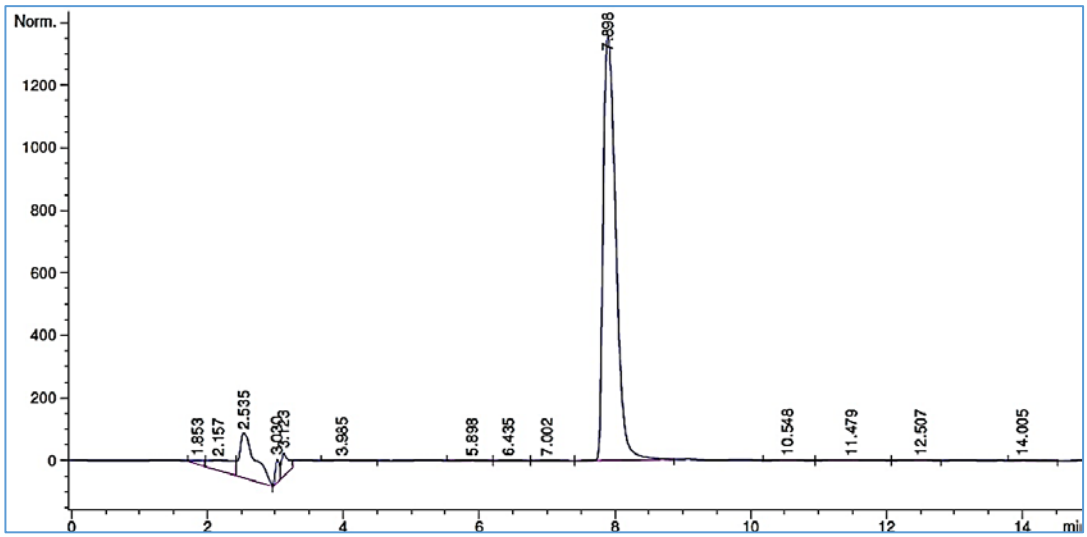


***Figure 6. 9:** HPLC chromatogram of compound 3 with a retention time of 7.9*

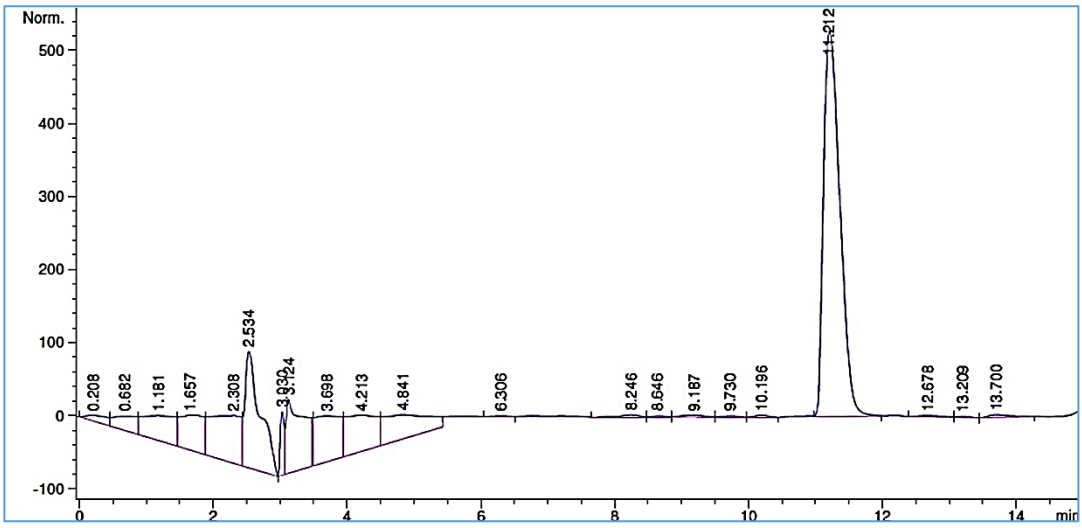


Figure 6. 10: *HPLC chromatogram of catechol with a retention time of 11.2*

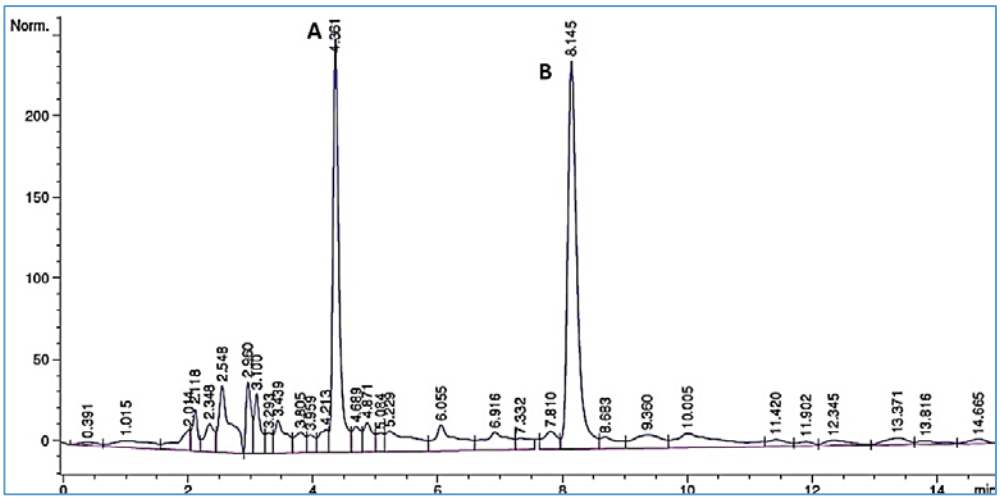


Figure 6. 11: *HPLC chromatogram of sterile aqueous extract of B. dalzielii obtained after six hours*

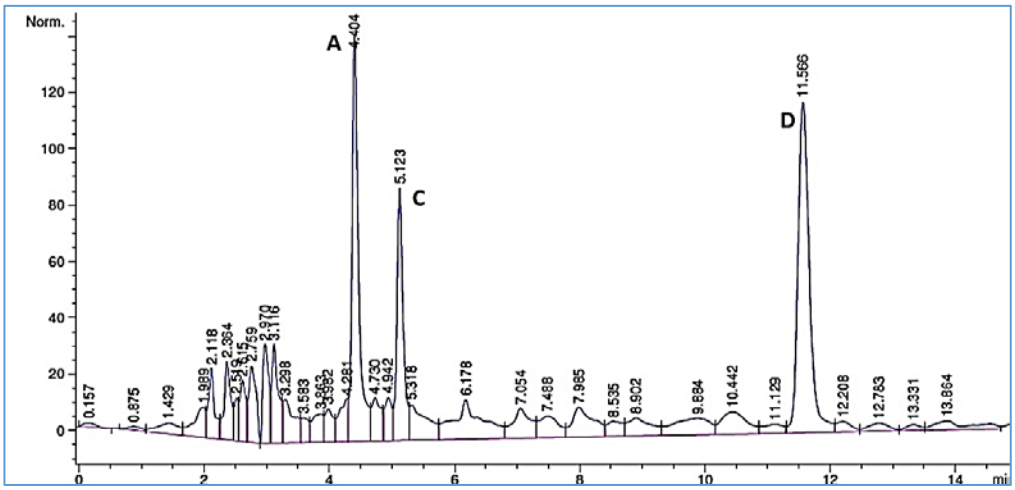


***Figure 6. 12**: HPLC chromatogram of sterile aqueous extract of B. dalzielii inoculated with Isolate B.*

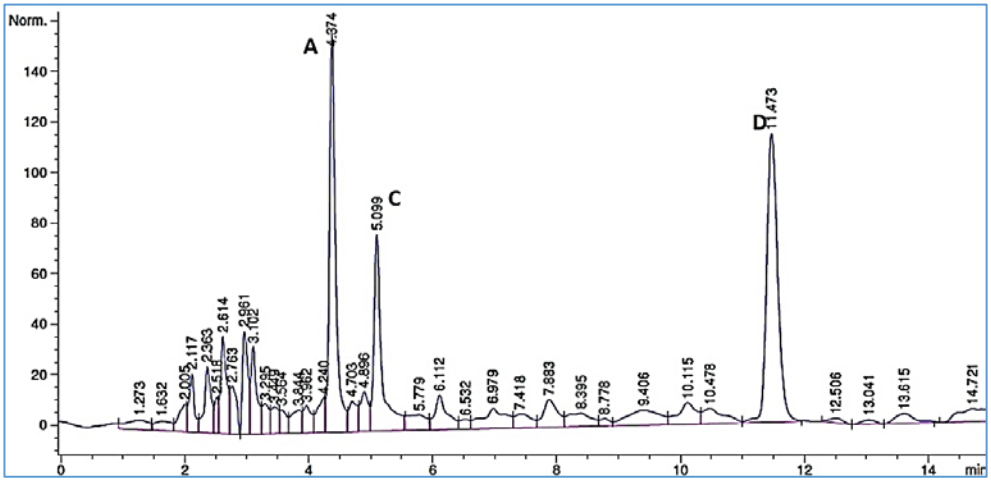


***Figure 6. 13:** HPLC chromatogram of sterile aqueous extract of B. dalzielii inoculated with Isolate E.*

HPLC analysis (Figure 6.12 and 6.13) showed the presence of three major compounds in both samples inoculated with isolated microorganisms (Isolate B and Isolate E) while only two major compounds were observed in the untreated sample (control) (Figure 6.11). In Figure 6.12 and 6.13, compound A has a similar retention time of 4.3 to that of gallic acid (see Figure 6.7), compound B has a similar retention time of 7.9 as isolated compound 3 (see Figure 6.9), compound C has similar retention time of 5.1 to that of pyrogallol (see Figure 6.8) and compound D has a similar retention time of 11.5 to that of catechol (See Figure 6.10).

HPLC analysis confirmed the presence of catechol in both samples inoculated with isolated microorganisms Isolate B and Isolate E as shown in Figure 6.12 and 6.13, respectively. Also, the absence of catechol was observed in the control sample, see Figure 6.11. Compound 3 labelled B in Figure 6.11 with a retention time of 8.1 was

observed in HPLC chromatogram of control sample which is sterile aqueous extract of B. dalzielii bark obtained after six hours. However, a decrease in the level of compound 3 was observed in samples inoculated with Isolate B (Figure 6.12) and Isolate E (Figure 6.13). Thus, HPLC analysis further confirmed the involvement of compound 3 in the production of catechol.

6.3.4 IDENTIFICATION OF COMPOUND 3

6.3.4.1 High Resolution Mass Spectrometry (HRMS) results

5mg of compound 3 and respective reference (Protocatechuic acid) were used for HRMS which was conducted at EPSRC National Facility Swansea. Results showed that observed mass of Compound 3 was measured as Mw 154 [$C_7H_6O_4$]; MS, m/z [E1-, %], 153.0197 (M-H, 100%) and predicted molecular composition was $C_7H_5O_4$ (Figure 6.14).

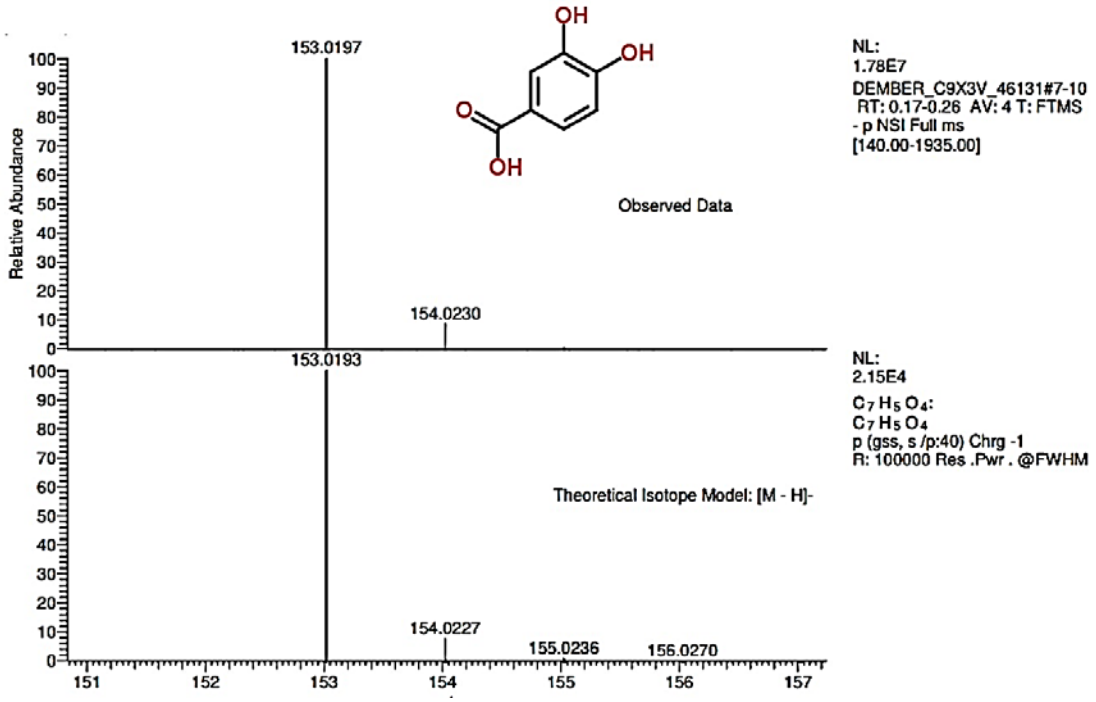


***Figure 6. 14**: HRMS spectrum of Compound 3 and purchased Protocatechuic acid suggesting that extract compound 3 might be Protocatechuic acid*

6.3.4.2 Fourier-Transform Infrared Spectroscopy (FTIR) results

Samples analysed include extracted compound 3 and purchased protocatechuic acid from Sigma Aldrich. The spectra of purchased protocatechuic acid is consistent with that extracted from plant bark of *B. dalzielii*, suggesting that compound 3 is protocatechuic acid. Figure 6.15 shows a prominent peak at 3293.45 cm^{-1} present for compounds with hydrogen bonded alcohols/phenols in both extracted and purchased protocatechuic acid.

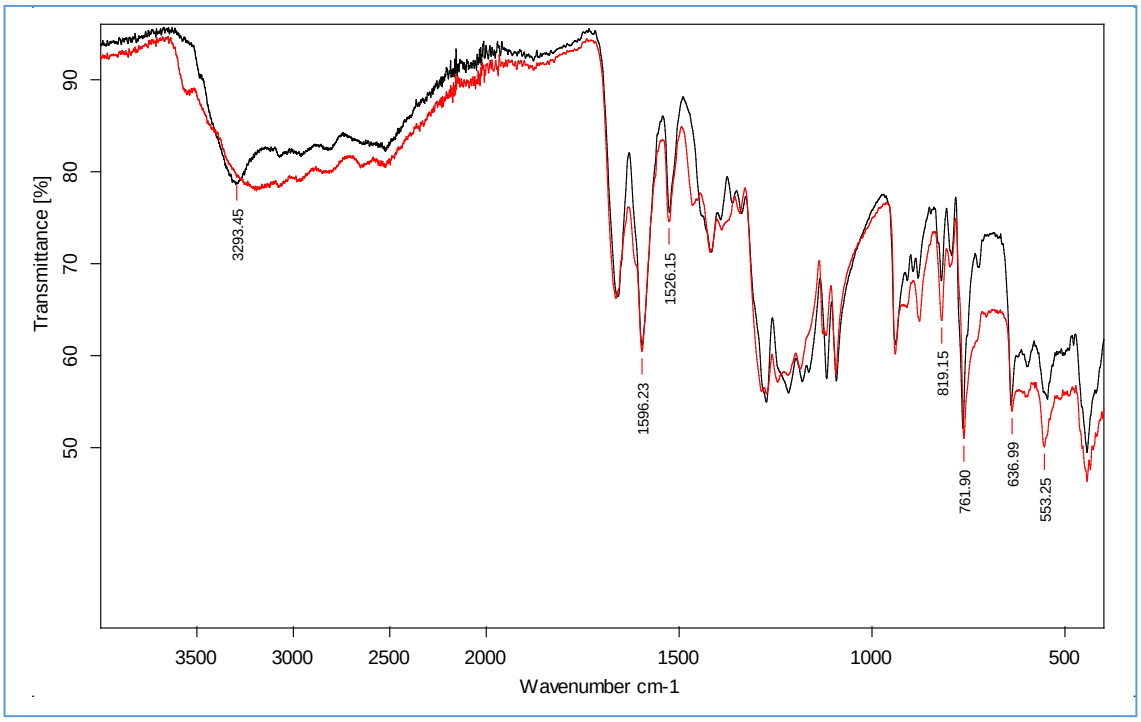


***Figure 6. 15**: FTIR spectrum of protocatechuic acid overlaying that purchased from Sigma Aldrich.*

6.3.4.3 Nuclear Magnetic Resonance (NMR) results

NMR analysis was conducted with deuterated methanol for ^{1}H analysis using 20mg of protocatechuic acid (extracted and purchased). ^{1}H-NMR spectrum of protocatechuic acid showed signals at δ_H 6.82 ppm (1H, *dd*), δ_H 7.44 ppm (1H, *dd*) and δ_H 7.46 ppm (1H, *dd*) (Figure 6.16). The ^{1}H-NMR spectrum obtained for both extracted and purchased protocatechuic acid is similar, suggesting that extracted compound 3 is protocatechuic acid. In addition, the study of Nguyen *et al.*, 2015 reported similar ^{1}H-NMR signals for protocatechuic acid.

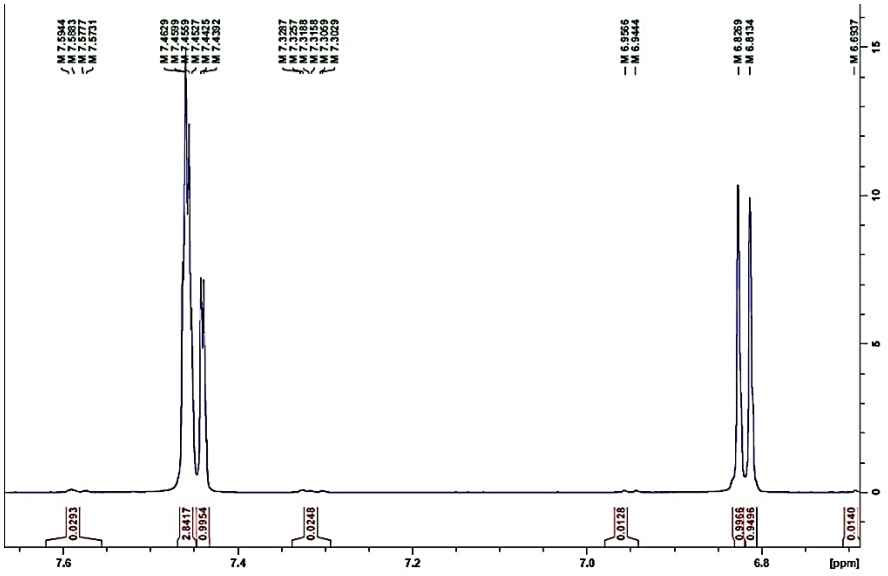


***Figure 6. 16**: ^{1}H-NMR spectrum of extracted protocatechuic acid is consistent with that extracted from plant bark of B. dalzielii. ^{1}H-NMR spectrum was obtained using bruker av400 spectrometer at 399.94 MHz using deuterated methanol.*

6.3.5 IDENTIFICATION OF MICROORGANISMS

Isolated microorganisms A and C were unfortunately not identified with the methods mentioned. However, Isolate B, D and E were observed to be Gram-negative rod

bacteria and images were obtained using LEICA ICC50 HD microscopy at 1000 magnification using 1.25 oil lens (Figure 6.17). MacCONKEY agar test showed that both isolate colony B and E were mucoid lactose fermenting (pink) bacteria while isolate colony D is lactose negative and had no colour (Figure 6.18).

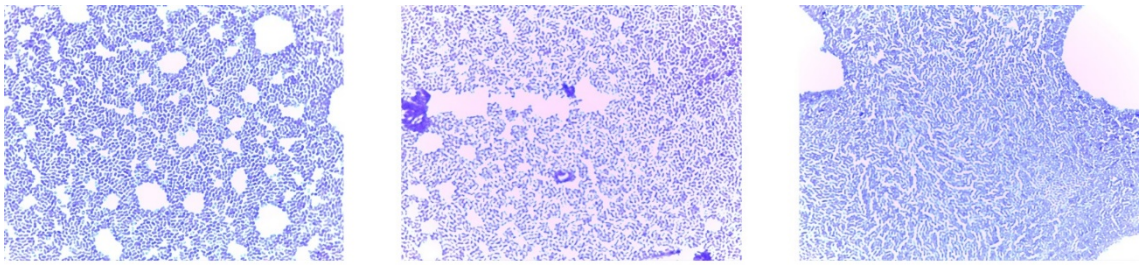


Figure 6. 17*: Gram staining image of isolate B, D and E.*

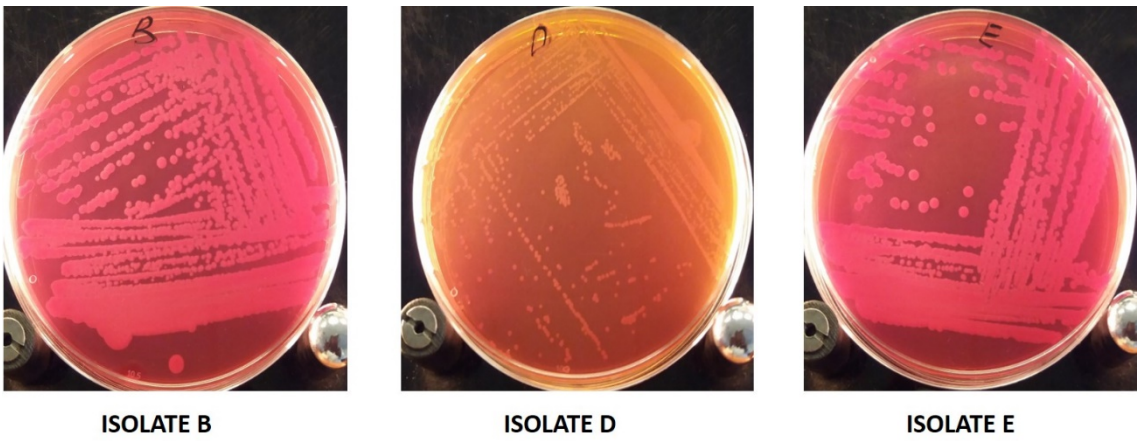


Figure 6. 18: *MacCONKEY agar test for Isolate B, D and E obtained from B. dalzielii bark, respectively.*

6.3.6 PCR ANALYSIS

40µL of each of the PCR products and 20 µL of both forward and reverse primer were submitted to Macrogen Europe Laboratory, Netherland for purification and sequencing. Gel results obtained was viewed in a Bio-Rad illuminator with a chemiDocXRS camera,

using Quantity One software. Band obtained were compared to the Bioline Easy Ladder. PCR products bands obtained were above DNA ladder (Figure 6.19).

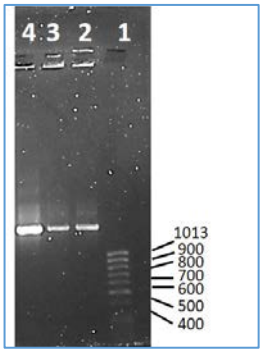


Figure 6. 19: *Gel electrophoresis image where lane 1 is DNA ladder, lane 2 is Isolate B, lane 3 is Isolate D and lane 4 is Isolate E.*

The sequence of the PCR products of isolated B (Figure 6.20 and 6.21), D (Figure 6.22 and 6.23) and E (Figure 6.24 and 6.25) bacteria were obtained for both forward and reverse primer were assembled on the CLC Main Workbench software and the consensus regions were obtained.

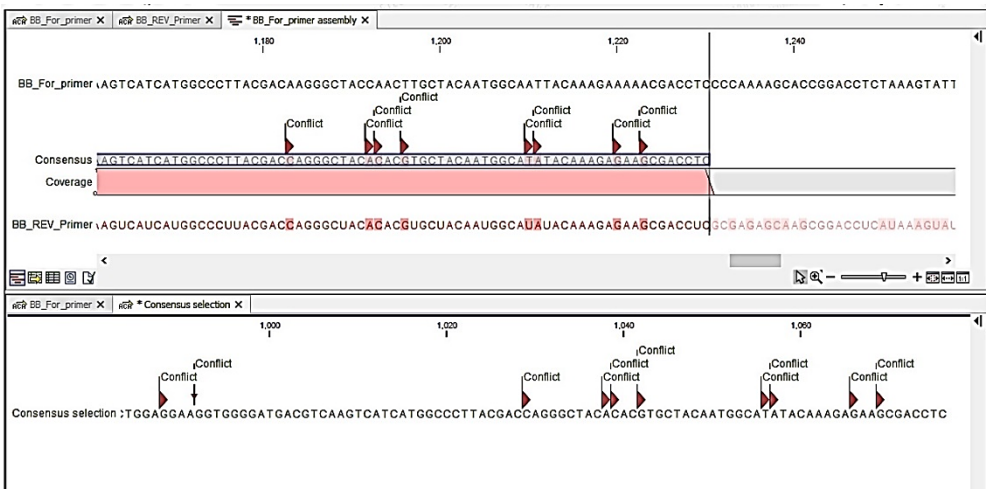


Figure 6. 20*:Consensus region obtained for Isolate B isolated from the bark B. dalzielii*

➢ **Consensus Isolate B**

CAAGACCAAAGGGGGGACCTTCGGGCCTCAAGCCTTAAGATGGCCCAGATGGGATTAGCTTGTAGGTG
GGGTAACGGCTTACCTGGGCGACGATCCCTAGCTGGTTTGAGAGGATGACCAGCCACACTGGAAATGA
GACACGGTCCAGATTCTTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCA
GCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGGCGTTAAG
GTTAATAACCTCATCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGG
TAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGT
CGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGG
GGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGG
CCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTA
GTCCACGCCGTAAACGATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTT
AAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAG
CGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACAGAACTTT
CCAGAGATGGATTGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGT
GAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTAGGCCGGGAAC
TCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACG
ACCAGGGCTACACACGTGCTACAATGGCATATACAAAGAGAAGCGACCTC

Figure 6. 21*: Consensus sequence obtained for Isolate B isolated from the bark of B. dalzielii with 1087 bases*

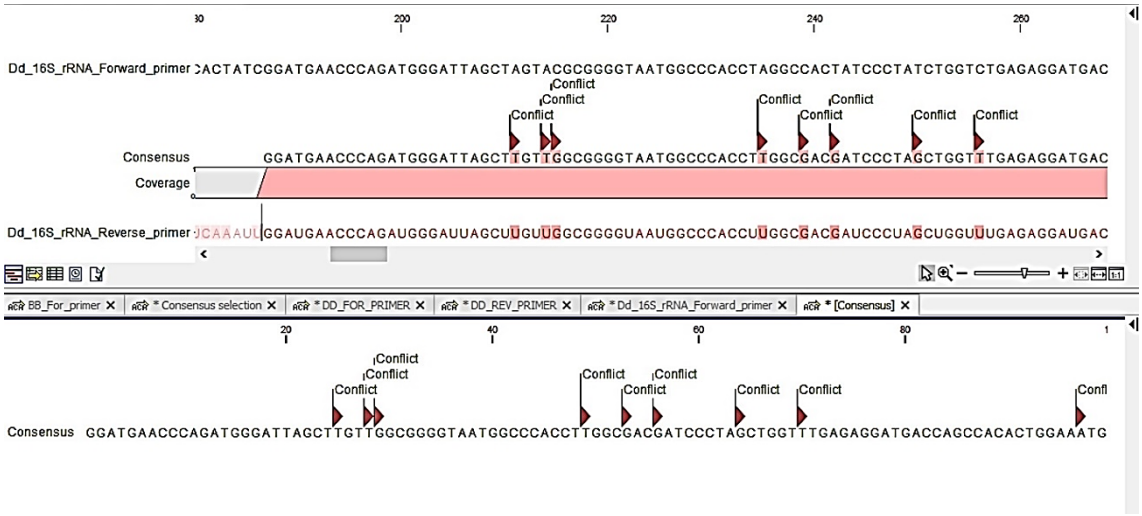


Figure 6. 22*: Consensus region obtained for Isolate D isolated from the bark B. dalzielii*

> Consensus Isolate D

GGATGAACCCAGATGGGATTAGCTTGTTGGCGGGGTAATGGCCCACCTTGGCGACGATCCCTAGCTGGTTT
GAGAGGATGACCAGCCACACTGGAAATGAGACACGGTCCAGATTCCTACGGGAGGCAGCAGTGGGGAAT
ATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTAC
TTTCAGCGGGGAGGAAGGCGGTGAGGTTAATAACCTTGCCGATTGACGTTACCCGCAGAAGAAGCACCGG
CTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGC
GCACGCAGGCGGTCTGTTAAGTCAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTTGAAACTGGC
AGGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAAT
ACCGGTGGC

Figure 6. 23*:Consensus sequence obtained for Isolate D isolated from the bark B. dalzielii with 501 bases*

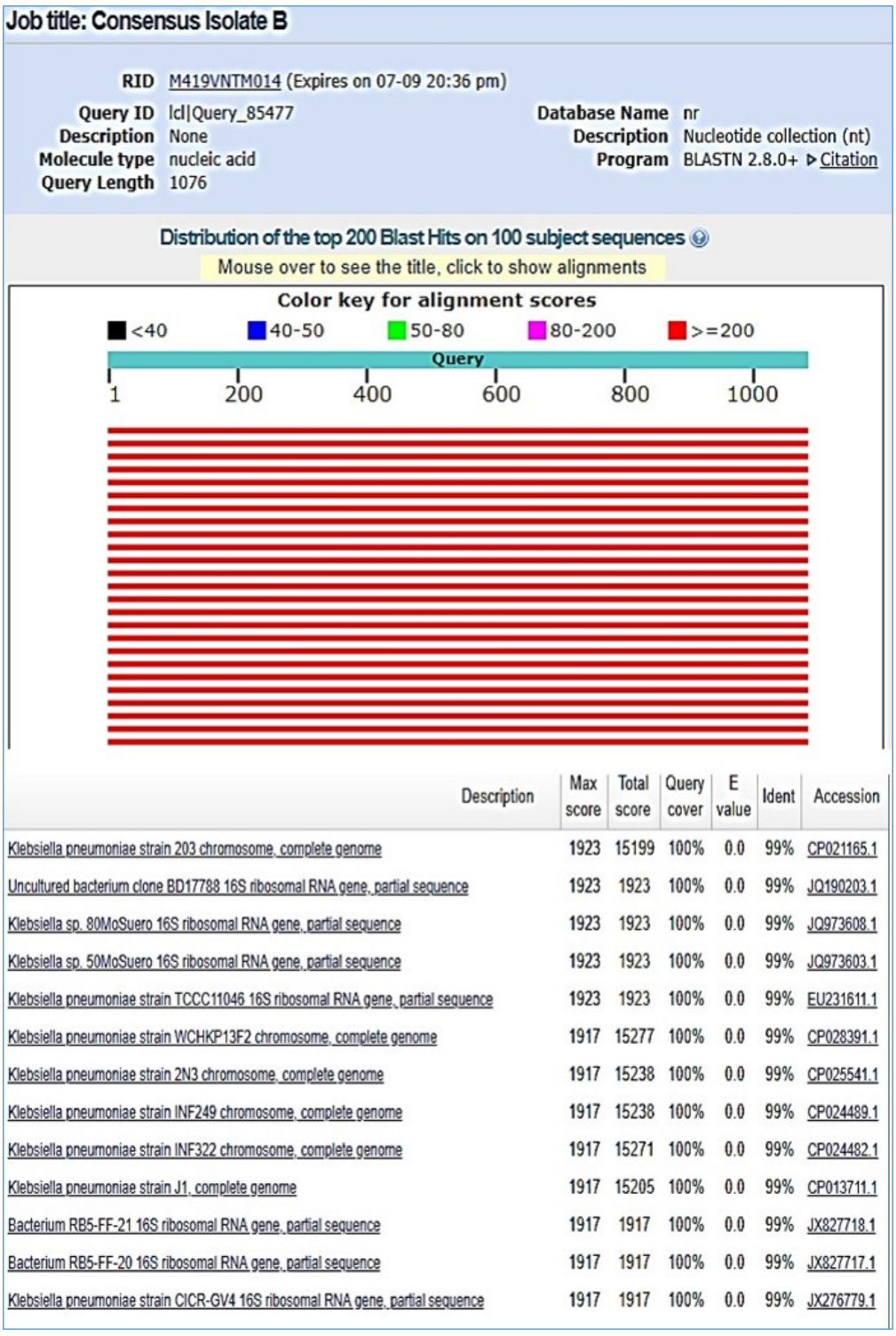
Figure 6. 24*: Consensus region obtained for Isolate E obtained from bark of B. dalzielii*

- **Consensus Isolate E**

GGATTAGCTTGTAGGTGGGTAACGGCTTACCTTGGGGACGATCCCTAGCTGGTTTGAGAGGATGACCAGCC
ACACGGGAAATGAGACACGGTCCAGATTCCTACGGGAGGCAGCAGTGGGAATATTGCACAATGGGCGCAA
GCCTGATGCAGCCATGCCGCGTGTTTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAG
GCGGTGAGGTTAATAACCTCATCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAG
CCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCCGGCGGTCTGTC
AAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGA
GGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGG
CCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGT
CCACGCCGTAAACGATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAAT
CGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGG
AGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACAGAACTTTCCAGAGAT
GGATTGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGG
GTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTCCGGCCGGGAACTCAAAGGAGACT
GCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACA
CGTGCTACAATGGCATATACAAAGAGAAGCGACCTC

Figure 6. 25*: Consensus sequence obtained for Isolate E isolated from bark of B. dalzielii with 1023 bases*

The consensus regions obtained for isolated colonies (Isolate B, D and E) from the bark of *B. dalzielii* were used for NCBI Blast Search. The blast search for Isolate B consensus return with 100 hits and all having 100% query cover, suggesting that Isolate B is *Klebsiella species*, with 99% similarity to *Klebsiella pneumonia* (Figure 6.26).



Description	Max score	Total score	Query cover	E value	Ident	Accession
Klebsiella pneumoniae strain 203 chromosome, complete genome	1923	15199	100%	0.0	99%	CP021165.1
Uncultured bacterium clone BD17788 16S ribosomal RNA gene, partial sequence	1923	1923	100%	0.0	99%	JQ190203.1
Klebsiella sp. 80MoSuero 16S ribosomal RNA gene, partial sequence	1923	1923	100%	0.0	99%	JQ973608.1
Klebsiella sp. 50MoSuero 16S ribosomal RNA gene, partial sequence	1923	1923	100%	0.0	99%	JQ973603.1
Klebsiella pneumoniae strain TCCC11046 16S ribosomal RNA gene, partial sequence	1923	1923	100%	0.0	99%	EU231611.1
Klebsiella pneumoniae strain WCHKP13F2 chromosome, complete genome	1917	15277	100%	0.0	99%	CP028391.1
Klebsiella pneumoniae strain 2N3 chromosome, complete genome	1917	15238	100%	0.0	99%	CP025541.1
Klebsiella pneumoniae strain INF249 chromosome, complete genome	1917	15238	100%	0.0	99%	CP024489.1
Klebsiella pneumoniae strain INF322 chromosome, complete genome	1917	15271	100%	0.0	99%	CP024482.1
Klebsiella pneumoniae strain J1, complete genome	1917	15205	100%	0.0	99%	CP013711.1
Bacterium RB5-FF-21 16S ribosomal RNA gene, partial sequence	1917	1917	100%	0.0	99%	JX827718.1
Bacterium RB5-FF-20 16S ribosomal RNA gene, partial sequence	1917	1917	100%	0.0	99%	JX827717.1
Klebsiella pneumoniae strain CICR-GV4 16S ribosomal RNA gene, partial sequence	1917	1917	100%	0.0	99%	JX276779.1

Figure 6. 26*: NCBI Blast Search for Isolate B obtained from B. dalzielii bark*

Taxonomy and lineage report generated showed that Isolate B belongs to the genus *Klebsiella* (Table 6.1) with a hit of 101 across 12 microorganisms. Taxonomy report showed that the 16S RNA sequence used to generate the taxonomy table is similar to

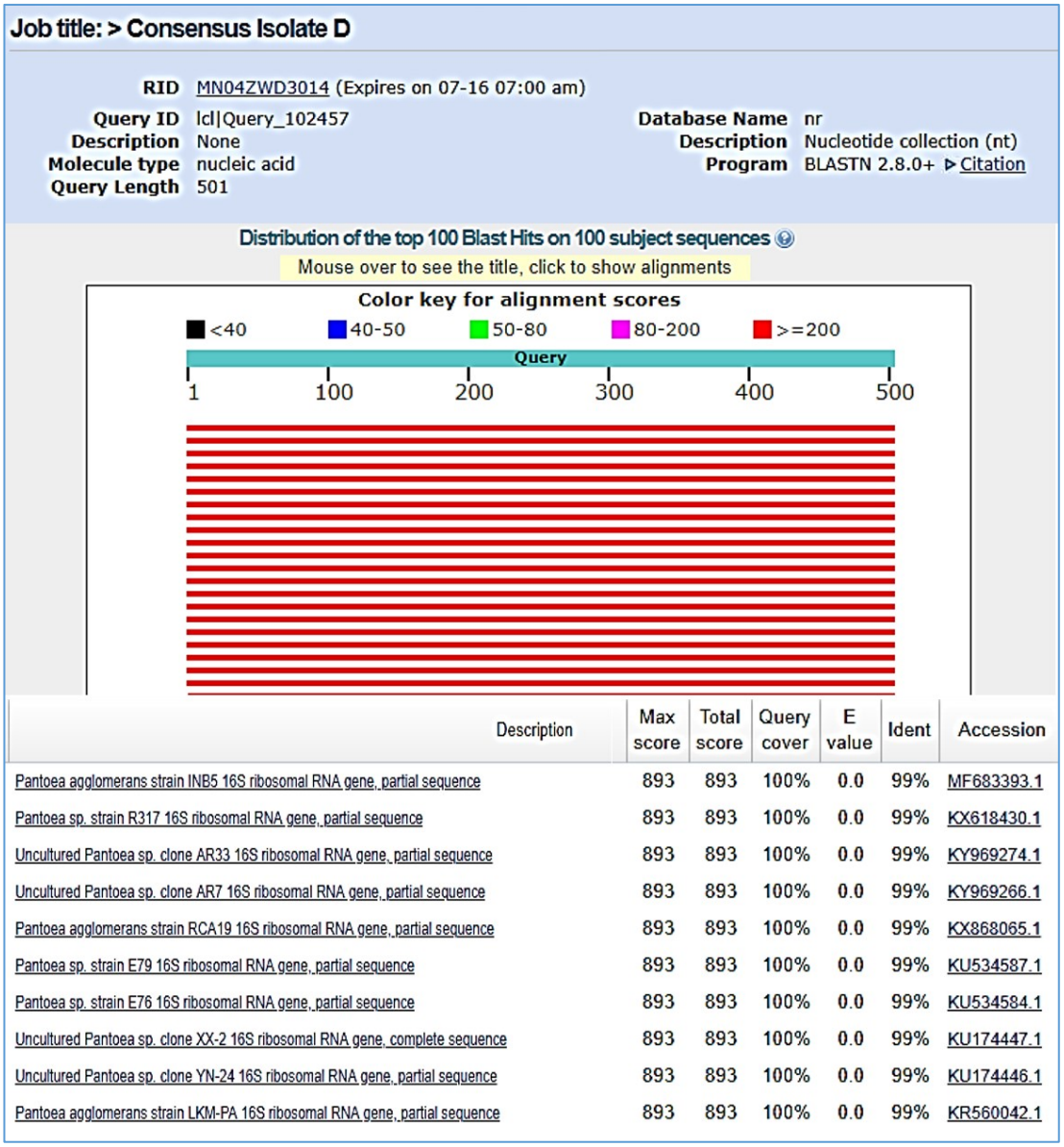
that of *Klebsiella pneumonia* with a hit of 73 across 4 microorganisms. However, all the 73 hits belong description details of *Klebsiella pneumonia*, thus, Isolate B is suggested to be a *Klebsiella* pneumonia. Thus, the sequence was deposited in the GenBank database under the accession number MH762022 (https://www.ncbi.nlm.nih.gov/nuccore/MH762022).

	Taxonomy	Number of hits	Number of Organisms	Score
Kingdom	Bacteria	106	16	
Genus	Klebsiella	101	12	
Organisms	*Klebsiella pneumonia*	72	4	1923
	Uncultured Klebsiella sp.	12	1	1917
	Klebsiella pneumonia subsp. Pneumonia	7	1	1912
	Klebsiella sp. 80MoSuero	1	1	1923
	Klebsiella sp. 50MoSuero	1	1	1923
	Klebsiella sp. 29MoSuero	1	1	1917

***Table 6. 1**: Taxonomy and lineage report generated for Isolate B suggesting that Isolate B could be Klebsiella pneumonia*

The consensus sequence of Isolate D was used for nucleotide blast search and result obtained provided 100 blast hits on 100 subject sequences (Figure 6.27). 78 of the blast hit have 99% identity with Isolate D query, while 21 blast hits have 893 score for both maximum and total scores, most of which are described as *Pantoea species*. From the NCBI result, taxonomy and lineage report generated suggested that Isolate D could be *Pantoea agglomerans* (Table 6.2). Thus, the sequence was deposited in the GenBank

database under the accession number MH762024 (https://www.ncbi.nlm.nih.gov/nuccore/MH762024).



Description	Max score	Total score	Query cover	E value	Ident	Accession
Pantoea agglomerans strain INB5 16S ribosomal RNA gene, partial sequence	893	893	100%	0.0	99%	MF683393.1
Pantoea sp. strain R317 16S ribosomal RNA gene, partial sequence	893	893	100%	0.0	99%	KX618430.1
Uncultured Pantoea sp. clone AR33 16S ribosomal RNA gene, partial sequence	893	893	100%	0.0	99%	KY969274.1
Uncultured Pantoea sp. clone AR7 16S ribosomal RNA gene, partial sequence	893	893	100%	0.0	99%	KY969266.1
Pantoea agglomerans strain RCA19 16S ribosomal RNA gene, partial sequence	893	893	100%	0.0	99%	KX868065.1
Pantoea sp. strain E79 16S ribosomal RNA gene, partial sequence	893	893	100%	0.0	99%	KU534587.1
Pantoea sp. strain E76 16S ribosomal RNA gene, partial sequence	893	893	100%	0.0	99%	KU534584.1
Uncultured Pantoea sp. clone XX-2 16S ribosomal RNA gene, complete sequence	893	893	100%	0.0	99%	KU174447.1
Uncultured Pantoea sp. clone YN-24 16S ribosomal RNA gene, partial sequence	893	893	100%	0.0	99%	KU174446.1
Pantoea agglomerans strain LKM-PA 16S ribosomal RNA gene, partial sequence	893	893	100%	0.0	99%	KR560042.1

***Figure 6. 27**: NCBI Blast Search for Isolate D obtained from B. dalzielii*

	Taxonomy	Number of hits	Number of Organisms	Score
Kingdom	Bacteria	111	23	
Class	Gammaproteobacteria	78	22	
Order	Enterobacterales	71	20	
Genus	Pantoea	69	18	
Organisms	*Pantoea agglomerans*	14	1	893
	Pantoea sp.	14	1	893
	Uncultured *Pantoea sp.*	7	1	893
	Pantoea anthophila	16	1	893
	Pantoea sp. ND12	1	1	889

***Table 6. 2**: Taxonomy and lineage report of Isolate D obtained from B. dalzielii*

The blast search for Isolate E consensus return with 100 hits and all having 100% query cover with 99% similarity to *Klebsiella pneumonia* suggesting that Isolate E is *Klebsiella species* (Figure 6.28). Taxonomy and lineage report was generated, the result obtained indicated that Isolate B belongs to the genus *Klebsiella* and all listed micro-organisms with similar 16S RNA sequences to that of Isolate E are *Klebsiella pneumonia* (Table 6.3). Thus, the sequence was deposited in the GenBank database under the accession number MH762023 (https://www.ncbi.nlm.nih.gov/nuccore/MH762023).

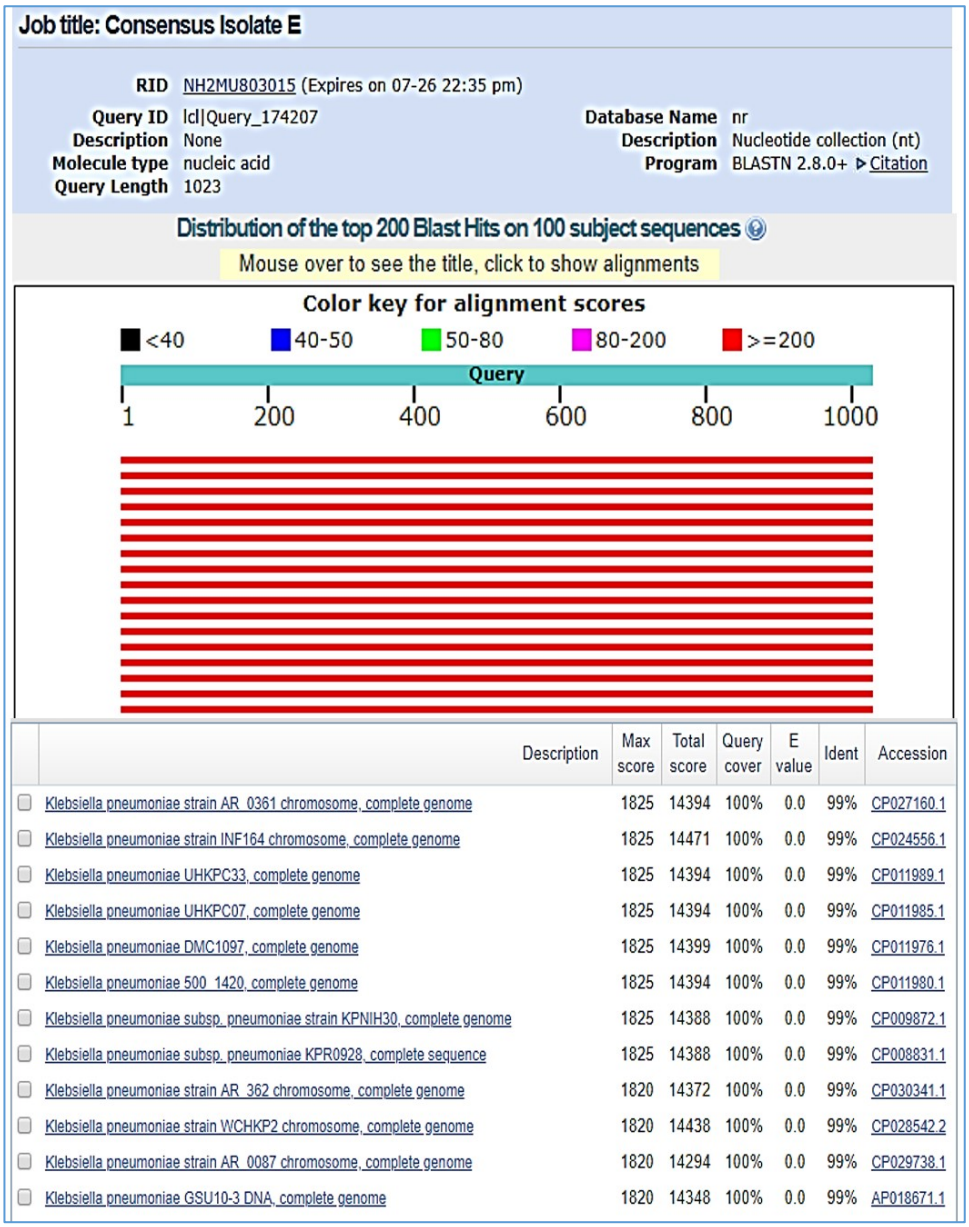


***Figure 6. 28**: NCBI Blast Search for Isolate E obtained from B. dalzielii*

	Taxonomy	Number of hits	Number of Organisms	Score
Genus	Klebsiella	100	8	
Organisms	*Klebsiella pneumonia*	84	7	1825
	Klebsiella pneumonia UHKPC33	1	1	1825
	Klebsiella pneumonia UHKPC07	1	1	1825
	Klebsiella pneumonia DMC1097	1	1	1825
	Klebsiella pneumonia 500 1420	1	1	1825
	Klebsiella pneumonia subsp. Pneumonia	10	2	1825
	Klebsiella pneumonia subsp. Pneumoniae KPRO928	1	1	1825

Table 6. 3*: Taxonomy and lineage report of Isolate E obtained from B. dalzielii*

Both Isolate B and Isolate E were suggested to be *Klebsiella pneumonia*, thus, pairwise alignment was conducted using EMBOSS Needle, an online sequence alignment tool. The consensus regions of both isolated microorganisms were used for this analysis. The pairwise alignment result returned for both identity and similarity between the two isolated microorganisms was 94.1%, suggesting that although they are similar but not 100% identical (Figure 6.29). Thus, the isolated microorganisms are labelled as *Klebsiella pneumonia* B and *Klebsiella pneumonia* E.

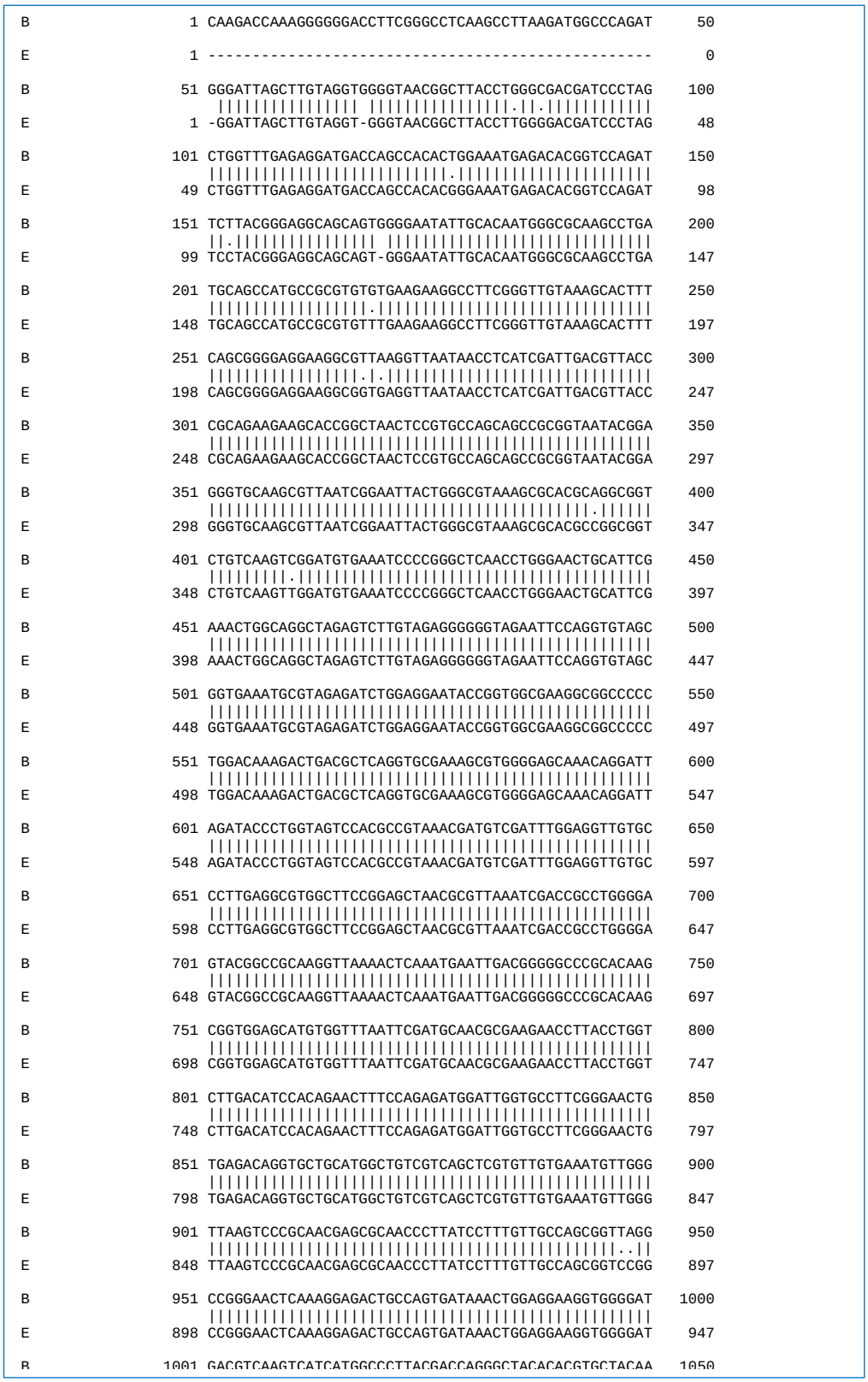


Figure 6. 29*: Pairwise alignment result obtained for K. pneumonia B and K. pneumonia E*

6.3.7 CONVERSION OF GALLIC ACID TO PYROGALLOL

Calibration curve of gallic acid (Figure 6.30) and pyrogallol (Figure 6.31) were used to calculate the amount of compound used and produced in bioconversion. HPLC analysis showed the presence of gallic acid with a retention time of 4.3 in 0.1% gallic acid in MHB (Figure 6.33) and its absence in Mueller Hinton broth (MHB) (Figure 6.32). And pyrogallol was seen in samples inoculated with isolated microorganisms (Figure 6.34).

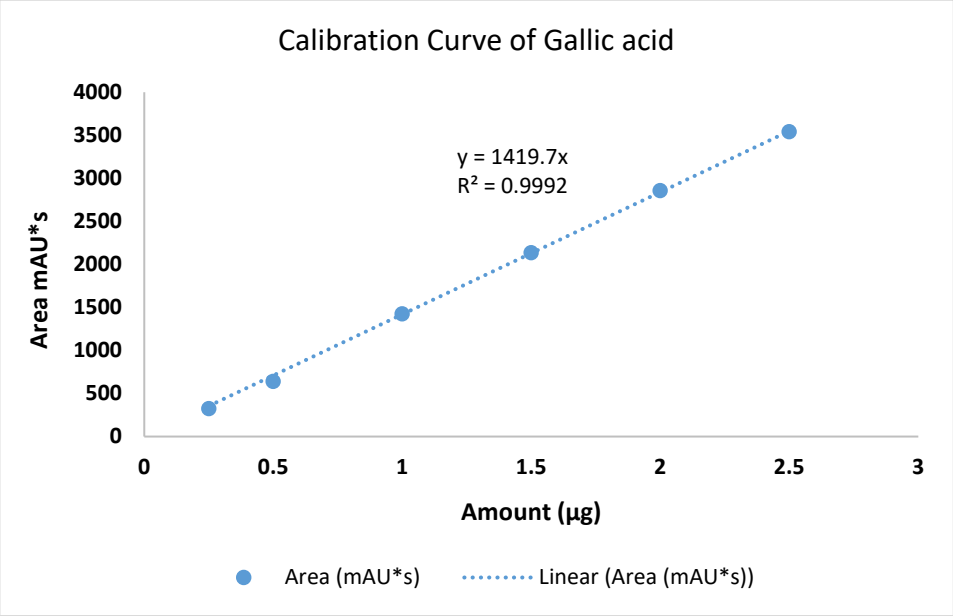


***Figure 6. 30**: Calibration curve of gallic acid. Data represents mean ± standard error*

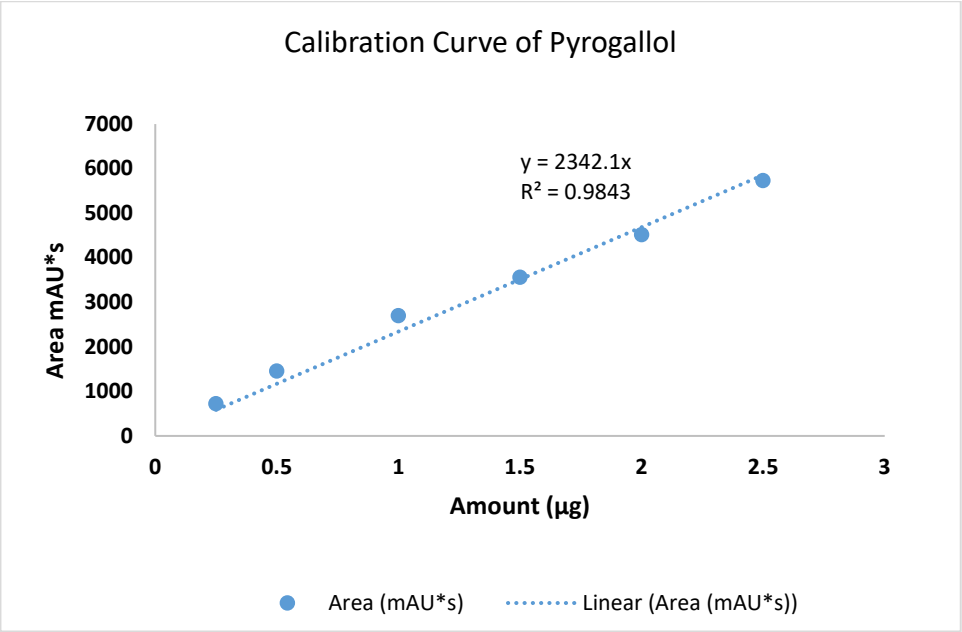


***Figure 6. 31**: Calibration curve of pyrogallol. Data represents mean ± standard error*

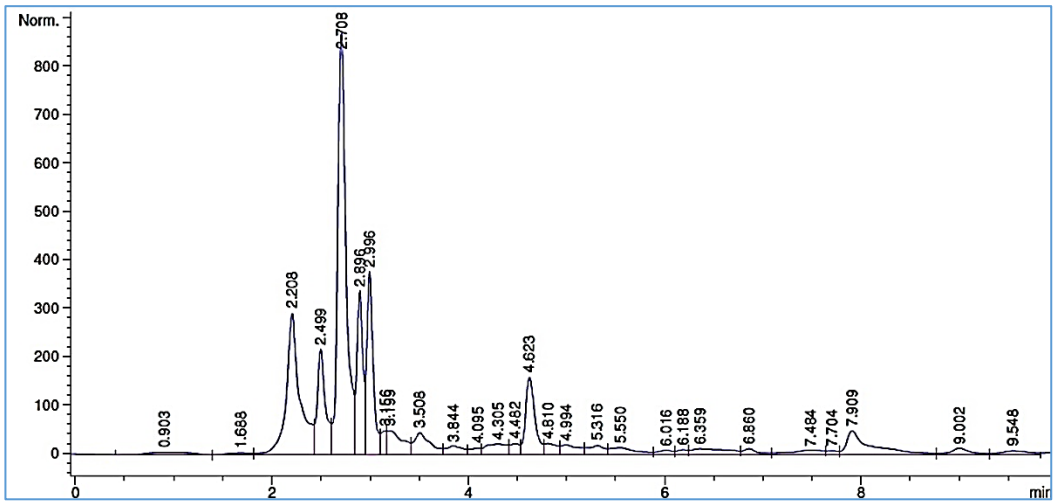


***Figure 6. 32**: HPLC chromatogram of sterile Mueller Hinton broth*

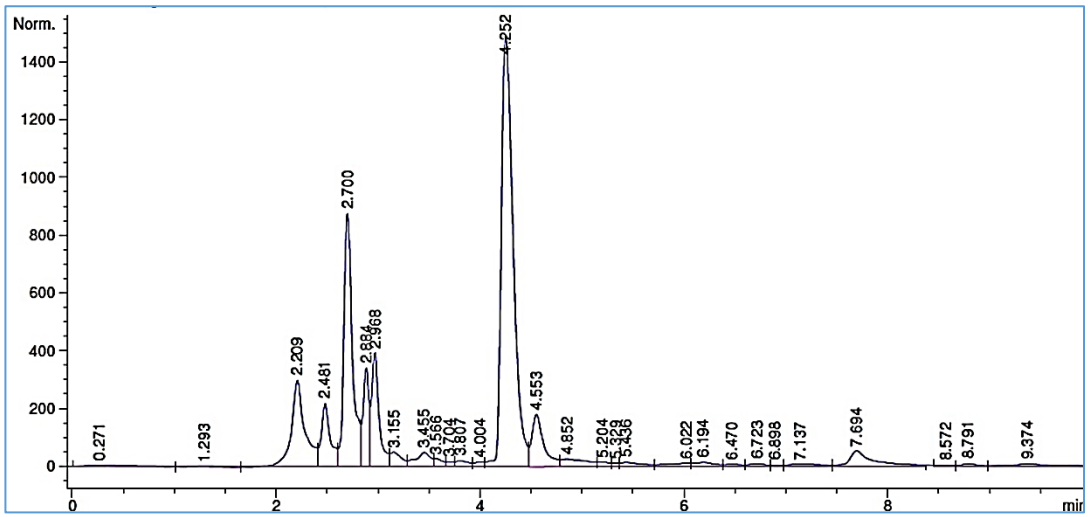


Figure 6. 33*: HPLC chromatogram of gallic acid (retention time of 4.3) in sterile Mueller Hinton broth*

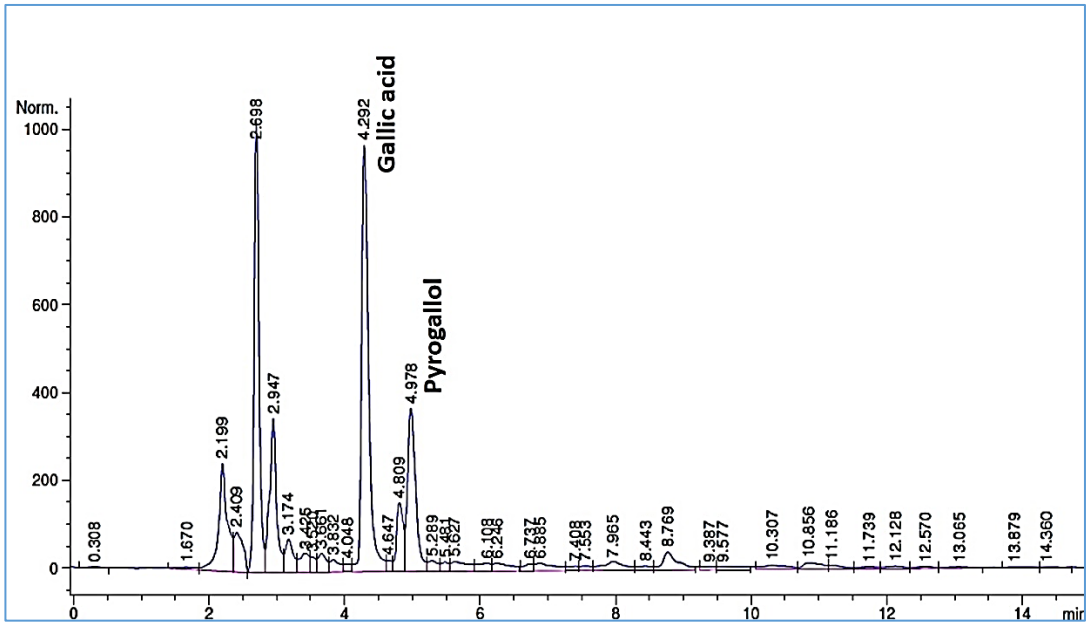


Figure 6. 34*: HPLC chromatogram of 24hours sample inoculated with isolated K. pneumonia B*

HPLC analysis was conducted on all samples and the amount of pyrogallol produced and that of gallic acid spent was calculated. Samples inoculated with *K. pneumonia* B with the absorbance of 0.108 and 0.125 were seen to produce 0.16mg/mL (Figure 6.35) and 0.27mg/mL (Figure 6.36), respectively, after 24hours.

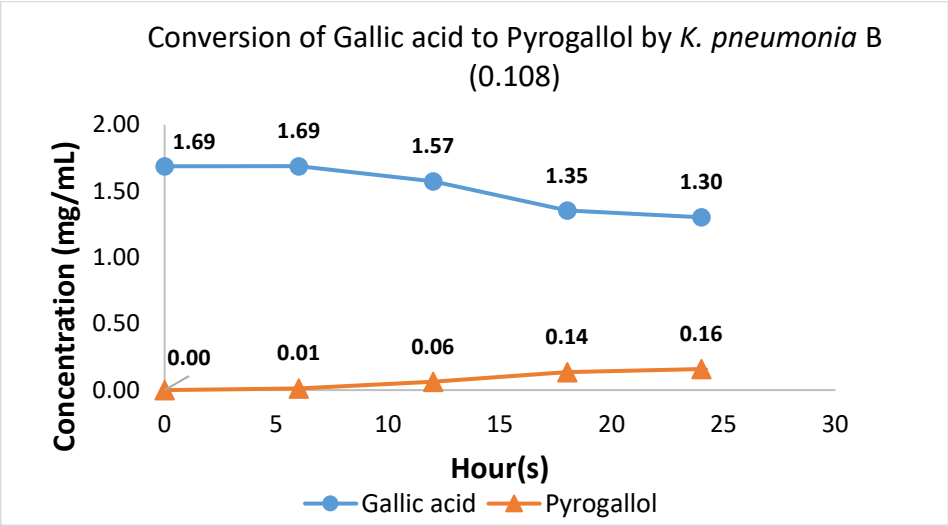


***Figure 6. 35**: HPLC analysis of the bioconversion of gallic acid to pyrogallol from samples inoculated with K. pneumonia B having 0.108 turbidity. Data are presented as mean ± standard error for both compound.*

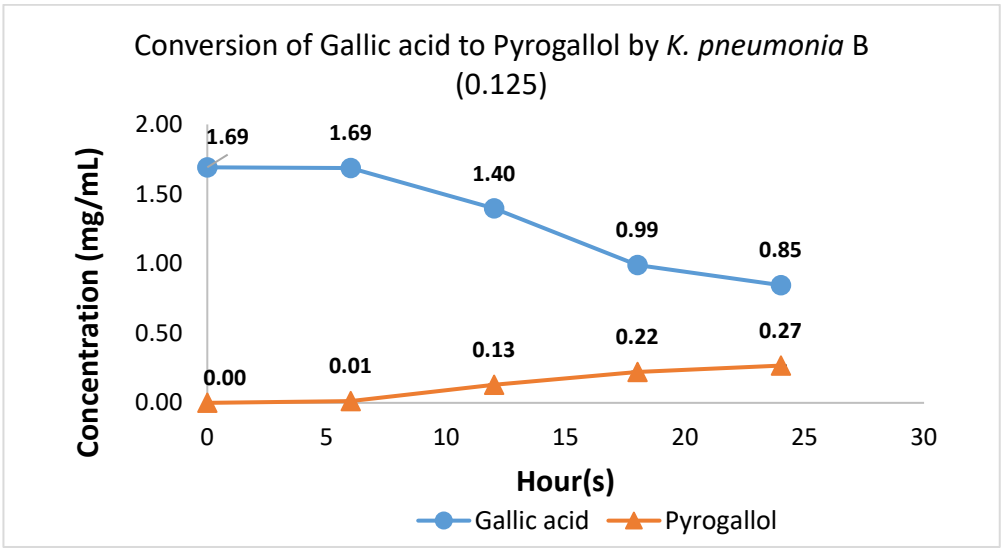


***Figure 6. 36**: HPLC analysis of the bioconversion of gallic acid to pyrogallol from samples inoculated with K. pneumonia B having 0.125 turbidity. Data are presented as mean ± standard error for both compound.*

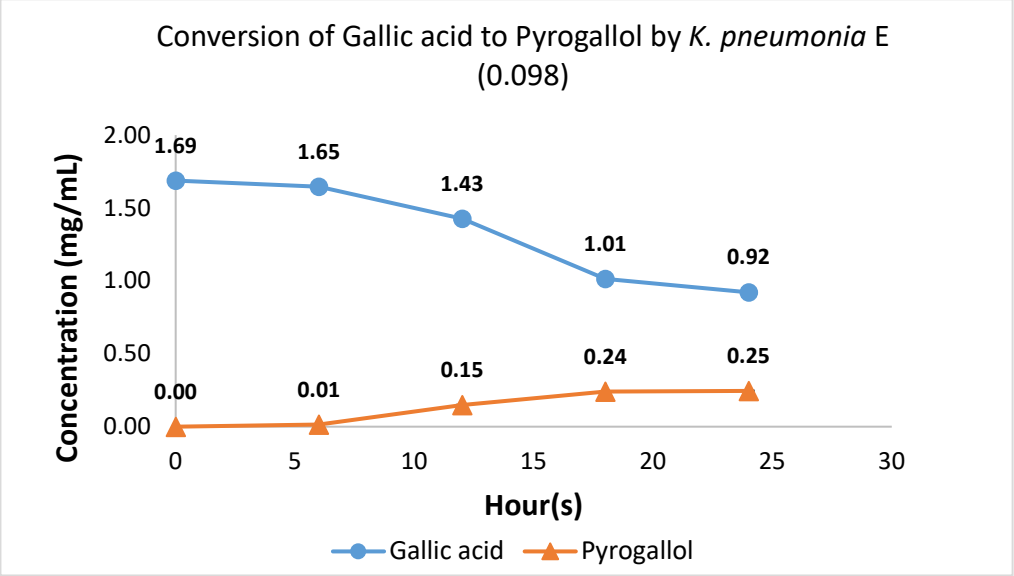


***Figure 6. 37**: HPLC analysis of the bioconversion of gallic acid to pyrogallol from samples inoculated with K. pneumonia E with 0.098 turbidity. Data are presented as mean ± standard error for both compound.*

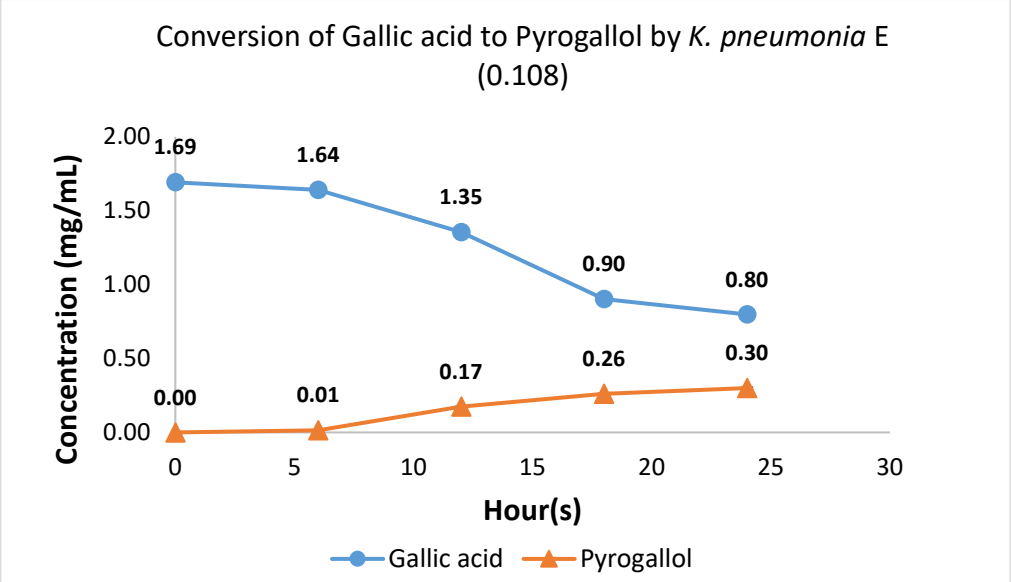


***Figure 6. 38**: HPLC analysis of the bioconversion of gallic acid to pyrogallol from samples inoculated with K. pneumonia E with 0.108 turbidity. Data are presented as mean ± standard error for both compound.*

Whilst, samples inoculated with *K. pneumonia* E with 0.098 and 0.108 turbidity were seen to produce 0.25mg/mL (Figure 6.37) and 0.30mg/mL (Figure 6.38), respectively after 24hours. Thus, both isolated *K. pneumonia* were seen to convert gallic acid to pyrogallol.

6.3.8 CONVERSION OF PROTOCHACEUIC ACID TO CATECHOL

Calibration curve of protochaceuic acid (Figure 6.39) and catechol (Figure 6.40) were used to calculate the amount of compound used and produced in bioconversion. HPLC analysis showed the presence of protocatechuic acid with a retention time of 8.0 in 0.1% protocatechuic acid in MHB (Figure 6.41) and its absence in Mueller Hinton broth (MHB). And catechol was seen in samples inoculated with isolated microorganisms (Figure 6.42). In chapter four, *E. cloacae* was isolated and shown to convert gallic acid to pyrogallol, thus it was tested for converting protocatechuic acid.

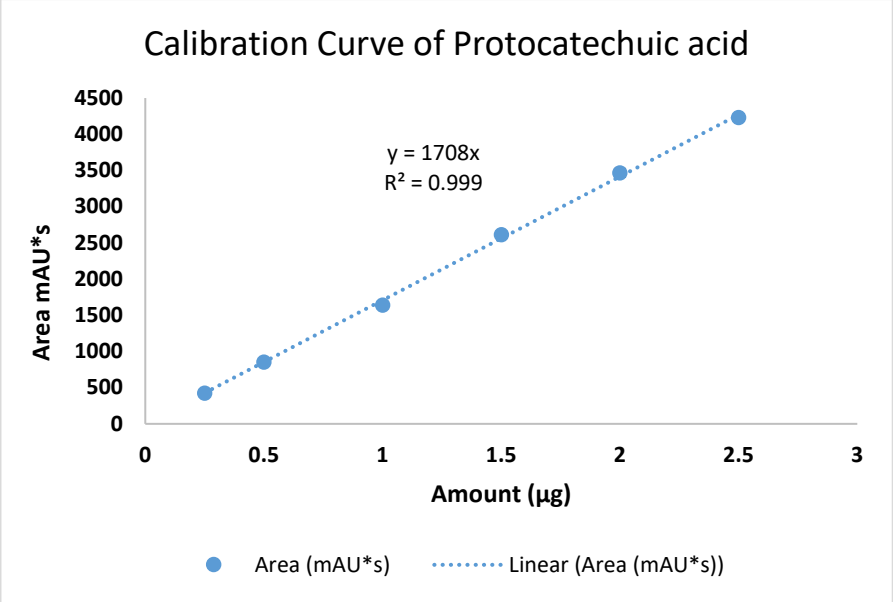


***Figure 6. 39**: Calibration curve of protocatechuic acid. Data represents mean ± standard error*

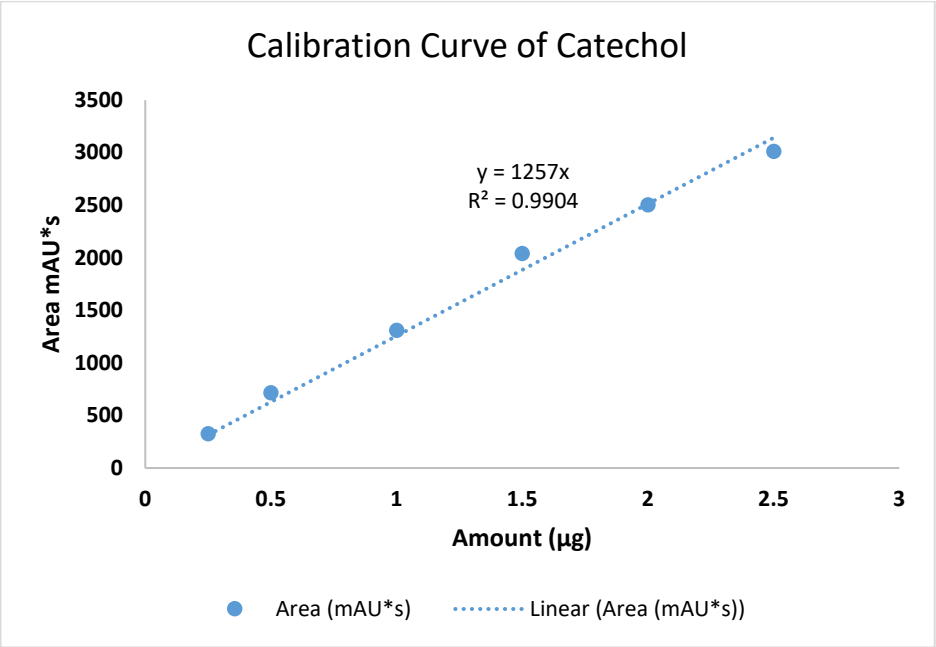


***Figure 6. 40**: Calibration curve of catechol. Data represents mean ± standard error*

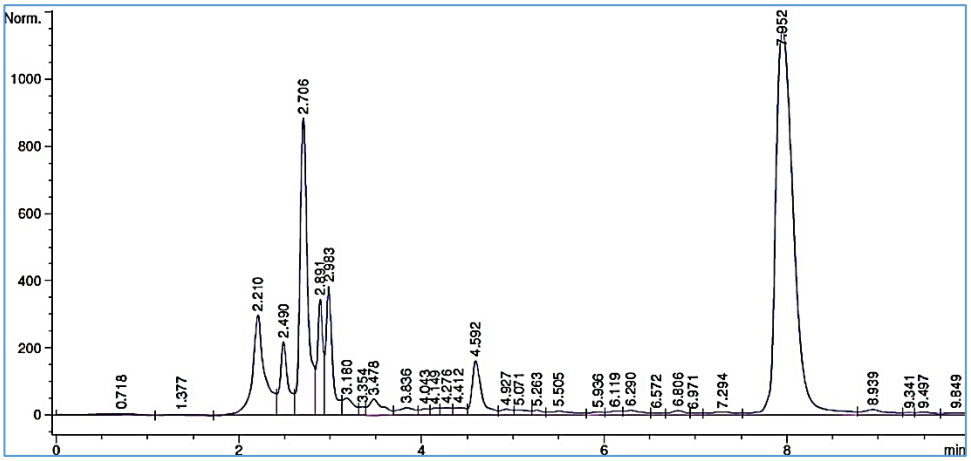


***Figure 6. 41**: HPLC chromatogram of protocatechuic acid (retention time of 8.0) in sterile Mueller Hinton broth*

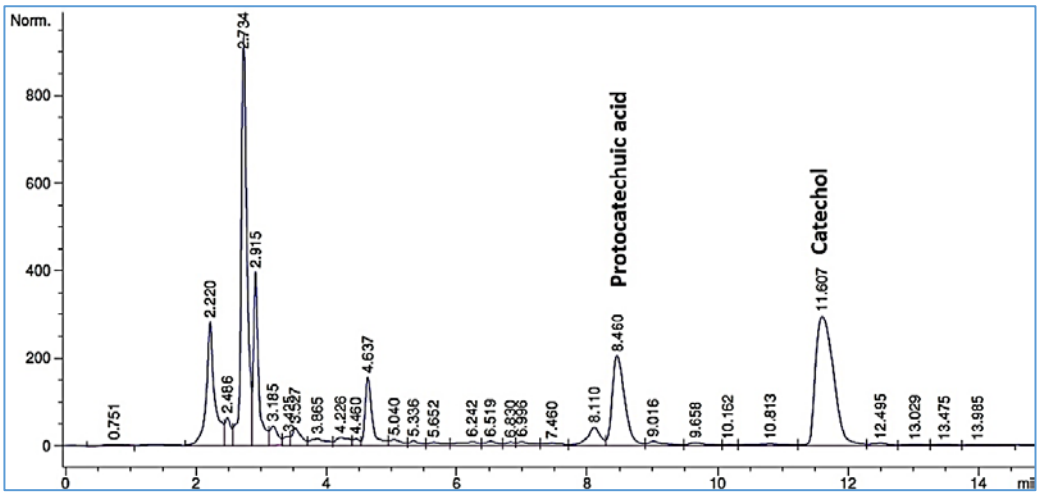


***Figure 6. 42**: HPLC chromatogram of 24hours sample inoculated with isolated K. pneumonia B, showing the presence of protocatechuic acid and catechol.*

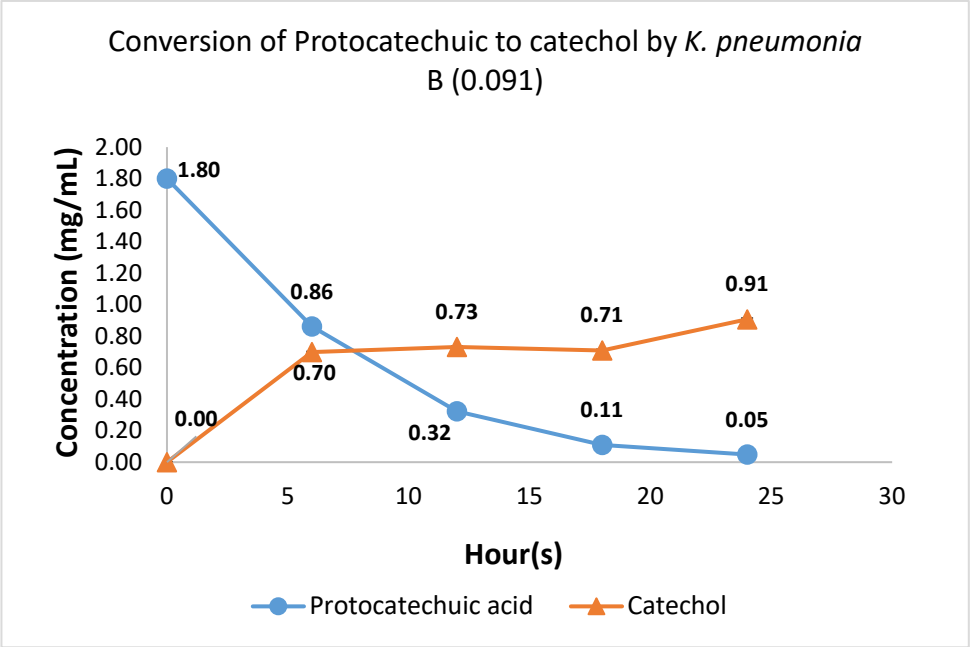


***Figure 6. 43**: HPLC analysis of the bioconversion of protocatechuic acid to catechol from samples inoculated with K. pneumonia B with 0.091 turbidity. Data are presented as mean ± standard error for both compound.*

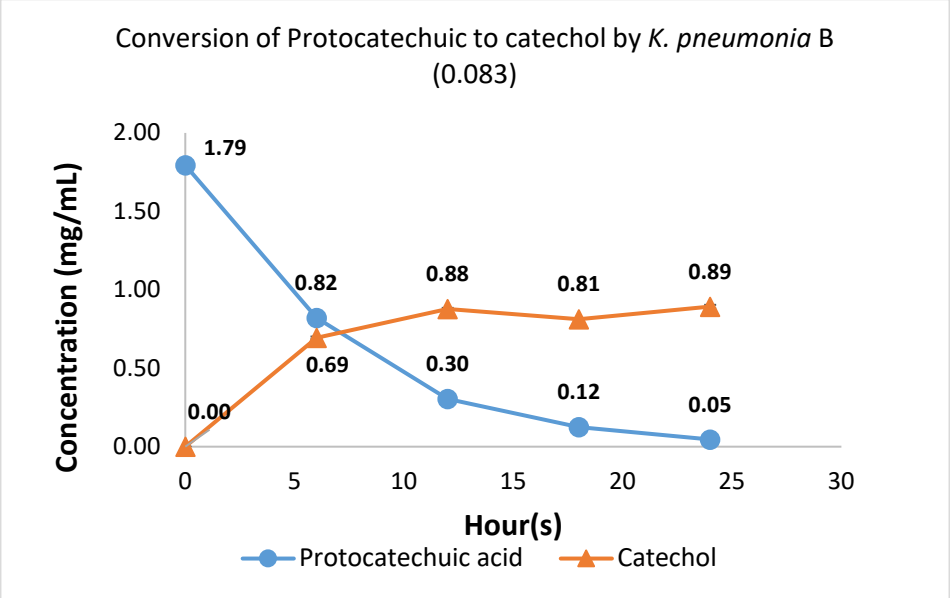


***Figure 6. 44**: HPLC analysis of the bioconversion of protocatechuic acid to catechol from samples inoculated with K. pneumonia B with 0.083 turbidity. Data are presented as mean ± standard error for both compound.*

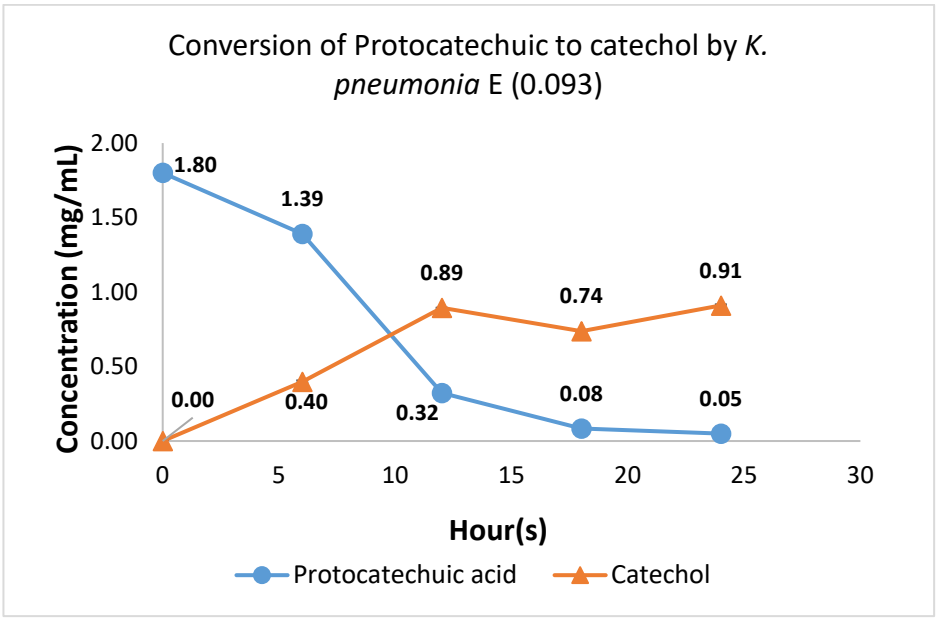


***Figure 6. 45**: HPLC analysis of the bioconversion of protocatechuic acid to catechol from samples inoculated with K. pneumonia E with 0.093 turbidity. Data are presented as mean ± standard error for both compound.*

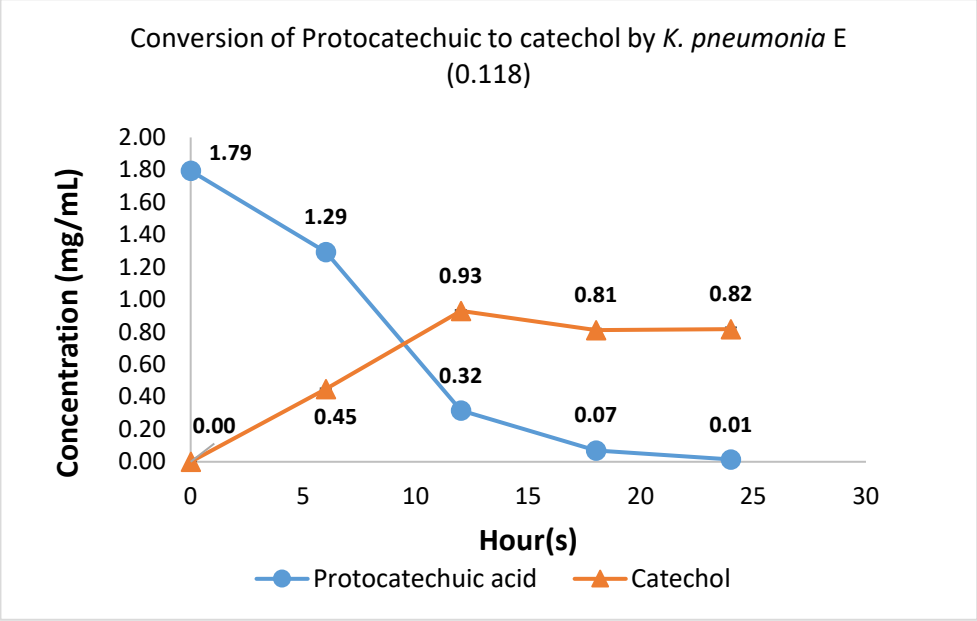


***Figure 6. 46**: HPLC analysis of the bioconversion of protocatechuic acid to catechol from samples inoculated with K. pneumonia E with 0.118 turbidity. Data are presented as mean ± standard error for both compound.*

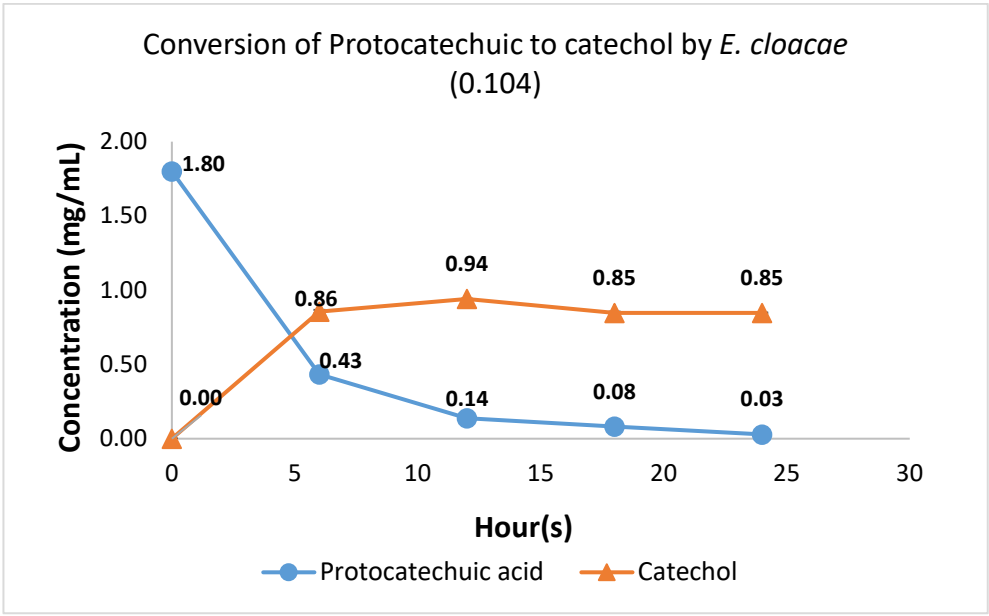


***Figure 6. 47**: HPLC analysis of the bioconversion of protocatechuic acid to catechol from samples inoculated with E. cloacae E with 0.104 turbidity. Data are presented as mean ± standard error for both compound.*

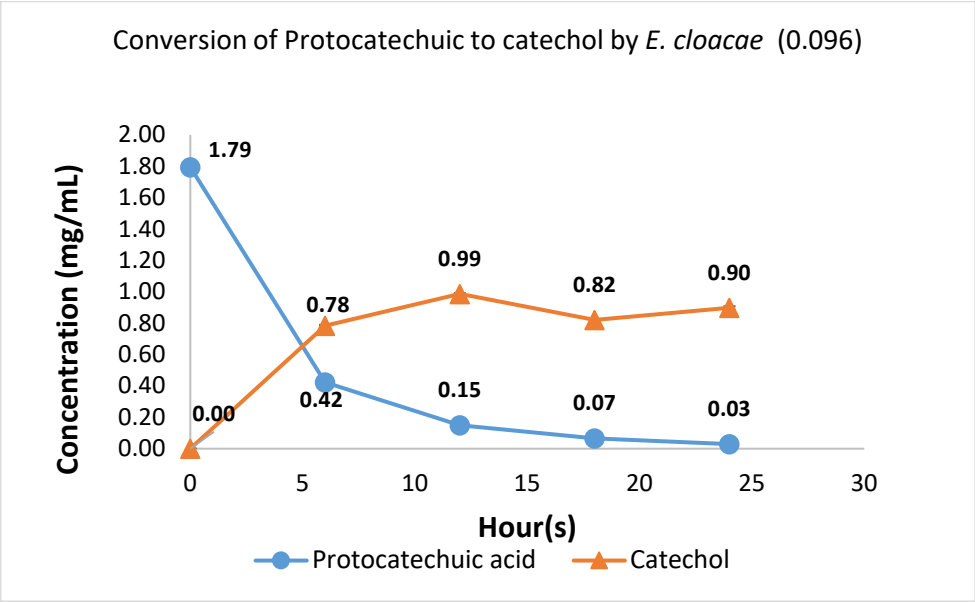


***Figure 6. 48**: HPLC analysis of the bioconversion of protocatechuic acid to catechol from samples inoculated with E. cloacae with 0.096 turbidity. Data are presented as mean ± standard error for both compound.*

HPLC analysis was conducted on all samples and the amount of catechol produced and that of protocatechuic acid spent was calculated. Samples inoculated with *K. pneumonia* B with the absorbance of 0.091 and 0.083 were seen to produce highest concentrations of 0.91mg/mL (Figure 6.43) and 0.88mg/mL (Figure 6.44) catechol after 12 and 24hours, respectively. However, samples inoculated with *K. pneumonia* E with 0.093 and 0.118 turbidities were seen to produce highest concentrations of 0.89mg/mL (Figure 6.45) and 0.93mg/mL (Figure 6.46) catechol after 12hours, respectively. Whilst, samples inoculated with *E. cloacae* with 0.104 and 0.096 turbidity were seen to produce 0.94mg/mL (Figure 6.47) and 0.99mg/mL (Figure 6.48) after 12hours, respectively. Thus, all three isolated microorganisms from *B. dalzielii* bark are seen to successfully convert protocatechuic acid to catechol.

6.4 CONCLUSION

In this chapter of the thesis, gallic acid and protocatechuic acid present in the aqueous extract of *B. dalzielii* bark were seen to be converted to pyrogallol and catechol, respectively, by isolated *K. pneumonia* B and *K. pneumonia* E. The study on the conversion of gallic acid to pyrogallol by *E. cloacae* was shown in chapter four of this thesis, and chapter six exploited the conversion of protocatechuic acid to catechol by *E. cloacae*. This further confirms the role of microorganisms in aqueous extract of natural products with ethnopharmacological claims.

Although, most strains of *K. pneumonia* are characterised as opportunistic human pathogens causing infections such as pneumonias and bacteremias, and rise in antibiotics resistance strains of *K. pneumonia* and the spread of virulent strains have been reported (Paczosa and Mecsas, 2016). *K. pneumonia* strains also play a role in plant growth, thus are characterised as endophyte (Dong, Chelius, *et al.*, 2003). In a recent study, *Enterobacteriaceae* including *K. pneumonia*. *E. cloacae* and *pantoea spp* were shown to be present on fresh iceberg lettuce pre and post-harvest (Osaili *et al.*, 2018). Another study showed that *K. pneumonia* as capable of infecting both plant and animal using maize and mice as test samples (HUANG *et al.*, 2016). All identified isolates (*K. pneumonia spp* and *pantoea spp*) in this chapter are endophytes and studies showed that endophytes play major roles in plant growth and survival (Kandel, Joubert and Doty, 2017). *K. pneumonia* was shown to enhance growth, increase chlorophyll content and contribute to nitrogen fixing in plants such as Alfalfa and maize (Dong, Iniguez, *et al.*, 2003; Iniguez, Dong and Triplett, 2004; Kifle and Laing, 2016). Also, *Pantoea spp* were

reported to partake in plant growth enhancement (Verma, Ladha and Tripathi, 2001; Verma *et al.*, 2004; Kifle and Laing, 2016). In terms of bioconversion, literature reported the production of 1,3-propanediol, 2,3-butanediol and 3-hydroxypropionic acid from glycerol using *k. pneumonia* (Li, Zhang and Dang, 2016; Y. Li *et al.*, 2016; Lee, Jung and Oh, 2018; Wang *et al.*, 2018). In addition, research showed that K. pneumonia strains are capable of degrading amino acids such as histidine, lysine and arginine and produce biogenic amines such as tyramine, spermidine and cadaverine (Pugin *et al.*, 2017).

In conclusion, the biotransformation of gallic acid to pyrogallol and protocatechuic acid to catechol was studied first in aqueous plant extract via inoculating sterile aqueous extract of *B. dalzielii* obtained from six hours maceration. This was further confirmed by using purchased gallic acid and protocatechuic acid (0.1%) in Mueller Hinton broth then observing their biotransformation by isolated microorganisms at time interval with the aid of HPLC analysis. To the best of the author`s knowledge, this is the first work reporting the bioconversion of these phytochemicals present in aqueous *B. dalzielii* bark by isolated *E. cloacae* and *K. pneumonia* strains B and E.

7

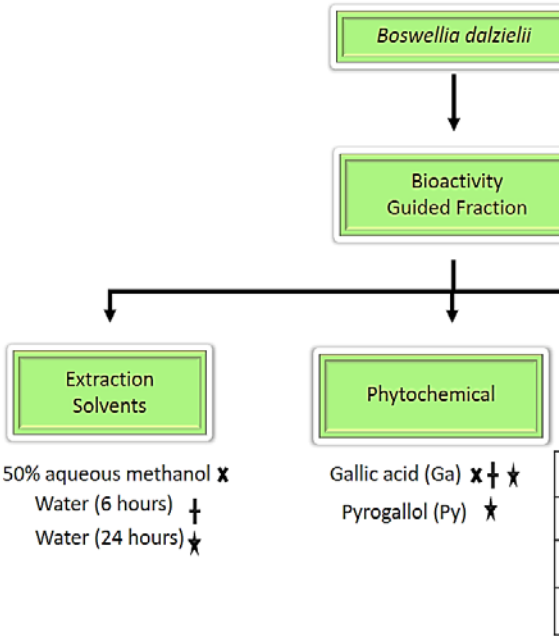
GENERAL DISCUSSION

In the introduction (chapter 1) the role of ethnopharmacology in the provision of health care and in modern drug development was highlighted. Of the non-western health care systems, Ayurveda or Traditional Chinese Medicine have attracted particular interest since they have a historical and written record of applications and efficacy. African Traditional Medicine is part of an oral tradition. As fewer records of use are available for study, this field of medicine and health care has remained relatively unexplored. The Hausa in the Northern part of Nigeria macerate the bark of *Boswellia dalzielii* in cold water, and the resulting extract is used in the treatment of infections and tumours. The aims and objectives of this thesis were to validate the traditional use of aqueous bark extracts of *B. dalzielli* by performing the extraction under carefully controlled conditions followed by chemical analysis and bioactivity screening of the aqueous extract.

The technical details of the extractions, analyses, and screening methods used in the present study are described in chapter 2.

The antibacterial screening of *Boswellia dalzielii* (chapter 3) initially resulted in the isolation and identification of gallic acid, a compound with poor antibacterial activity against both *S. aureus* and its methicillin-resistant strain (see Figure 7.1), this finding corroborated previous observations (Sanhueza et al., 2017). However, our results showed that aqueous maceration conducted for a longer period (24 hours) resulted in

better antibacterial activity. Thus, the length of maceration affects the composition and the efficacy of a plant extract, and this finding was correlated with a paper that reported that longer maceration of herbal material results in an increased number of phenolics in the extract (Kocabey, Yilmaztekin and Hayaloglu 2016). In the Boswellia extract, the increase in the amount of pyrogallol was particularly notable. Using the checkerboard method, no synergistic interaction between gallic acid and pyrogallol could be observed. Possibly, other synergy screening methods using both genomic and statistic based approach (Chen *et al.*, 2015; Roell, Reif and Motsinger-Reif, 2017), may still show synergistic interaction.



	Disc diffusion (mm)		MIC (μM)		MBC (log_{10} reduction) (2×MIC) CFU/ml	
	S. aureus	MRSA	*S. aureus*	MRSA	*S. aureus*	MRSA
Gallic acid	23±2	18±5	753	2,032	2.6 (24h)	1.9 (24h)
Pyrogallol	35±1	31±1	508	254	7.0 (12h)	7.1 (12h)

Figure 7. 1: *Bioactivity guided fractionation of B. dalzielii using different extraction solvents for maceration (50% aqueous methanol (×), water (6 hours) (+) and water (24 hours) (*)) resulted in the isolation of gallic acid and pyrogallol, however, pyrogallol was only present in the 24 hours aqueous macerated sample. Pyrogallol has better antibacterial activity (↑) when compared to gallic acid (↓).*

Chapter 4 of this thesis provides comprehensive data to understand the source of pyrogallol in the aqueous *B. dalzielii* extracts obtained after 24 hours maceration. The data collected showed that time and temperature affect the presence and concentration of phytochemicals in aqueous maceration as shown in figure 4.5, 4.15 and 4.18. Consequently, resulting in exploring the possibility of the involvement of microorganisms and their effect on the phytochemicals present in an aqueous plant extract. The results obtained showed that the bacterium *Enterobacter cloacae* acts as a biocatalyst in the conversion of gallic acid to pyrogallol (see Figure 7.2). This novel finding contributes to the growing notion that some phytochemicals believed to be plant-derived could actually be a product of the interaction of endophytic microbes with the plant as reported in other studies (e.g. El-Elimat *et al.*, 2014; Gutiérrez-García *et al.*, 2017; McMullin *et al.*, 2017). Thus, the possible explanation regarding the ethnopharmacological claims, i.e. the antibacterial activity of the plant bark of *B. dalzielii* in the Northern part of Nigeria, could be due to the temperature favouring the growth of the microorganisms capable of producing active compounds via bio-converting phytochemicals present in aqueous extracts. Also, *Pantoea sp.* is another bacterium isolated from the plant bark of *B. dalzielii*, but this bacterium cannot bio-convert gallic acid to pyrogallol. The 16S rRNA sequences of both isolated *E. cloacae* and *Pantoea species* are deposited in the GenBank database under the accession numbers MH764584 and MH764583, respectively.

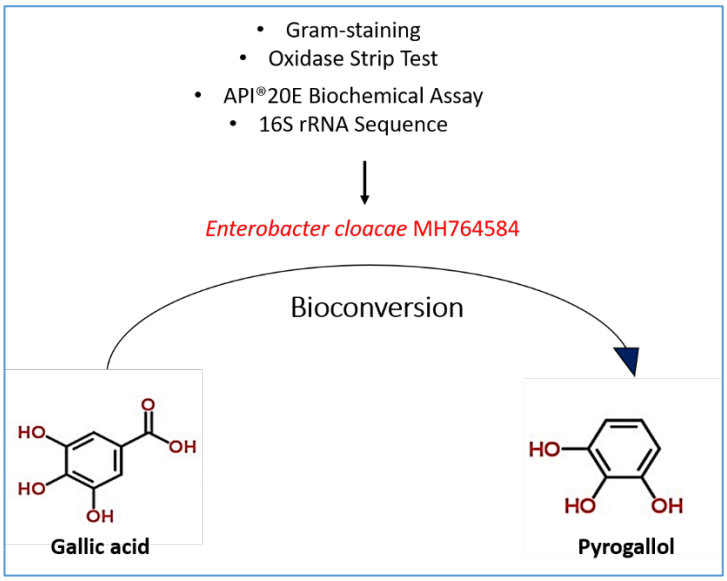


Figure 7. 2: *Bioconversion of gallic acid to pyrogallol.*

Chapter 5 of this thesis provides experimental data to support the ethnopharmacology claim of using *B. dalzielii* for cancer treatment alone and in combination. The in-vitro MTT assay was used to assess the cytotoxic property of aqueous extract of *B. dalzielii* plant bark against human breast cancer cell line MCF7, although this assay is recommended to be conducted with any of the in-vivo screening models as mentioned in section 1.9.2 of this thesis. However, only the MTT in-vitro assay was conducted in this thesis, thus is recommended that an in-vivo screening assay should be performed to corroborate the MTT assay results in the future. Two extraction methods were explored which include acid-based extraction and acid hydrolysis to test the cytotoxicity activity of the aqueous extract of *B. dalzielii*. The acid-based extraction was conducted because most literature on the cytotoxicity assay of *Boswellia species* pointed to the organic acids as being responsible, especially the boswellic acids as mentioned in section 1.9.1.1 of this thesis. However, the results obtained in this thesis showed that the

extracts obtained via the acid-based extraction method have no cytotoxic effect on MCF7 cells, therefore acid hydrolysis was used. The MTT cytotoxicity assay was conducted on MCF7 cells using MCF 1OA as control, and 10µg/mL extract sample was used. The cytotoxicity effect of aqueous extract of *B. dalzielii* plant bark obtained via acid hydrolysis was seen to have reduced MCF7 cell viability to 85%, however, when combined with extracts of other plants traditionally used in tumour treatment, i.e. *Spondias mombin*, *Detarium microcarpum*, *Sclerocarya birrea* and *Vitellaria paradoxa* the viability decreased to 68%. Thus, it is recommended to study the synergistic effect of the combination used for future study. Interestingly, the aqueous extract of *B. dalzielii* obtained after 24 hours maceration was seen to reduce the viability of MCF7 cells to 62%. In this study, only one combination was used to compare cytotoxicity activity of plant extract alone and in conjunction and the results obtained showed better cytotoxicity activity when plants are combined (see Figure 5.6 and 5.7). However, the aim of this thesis was on the cytotoxicity effect of *B. dalzielii*. Moreover, a better result was obtained for the aqueous extract of *B. dalzielii* obtained via 24 hours maceration (see Figure 5.9) thus, the used of five combined plants for treatment might not necessarily be required.

The bioactivity guided fractionation of the aqueous extract of *B. dalzielii* plant bark obtained via 24 hours maceration resulted in the extraction, isolation and identification of catechol as the main compound responsible for the cytotoxic effect. The concentration of catechol causing 50% maximum effect (EC_{50}) was calculated to be 9.46µg/mL (86µM) after 48 hours treatment (Figure 5. 20) and 5.33 µg/mL (48.45 µM)

after 72 hours (Figure 5. 21). The highest concentration of catechol 12.5ug/mL (114µM) used in the present study showed cytotoxicity against MCF 7, reducing cell viability to 82%, 43% and 24% after treatment for 24 hours (Figure 5.17), 48 hours (Figure 5.18) and 72 hours (Figure 5.19), respectively, while no cytotoxicity effect was observed against MCF10A. Thus, the cytotoxicity activity of catechol was observed to be time and concentration dependent, corroborating the findings reported by Choi *et al.*, (2018). This chapter also contributes to the growing literature on the effect of time and temperature on the phytochemicals present in aqueous plant extract. Also, it contributes to the use of natural products with ethnopharmacological claims by providing scientific data as seen in the case of the cytotoxic effect of *B. dalzielii* on breast cancer cells (See Figure 7.3).

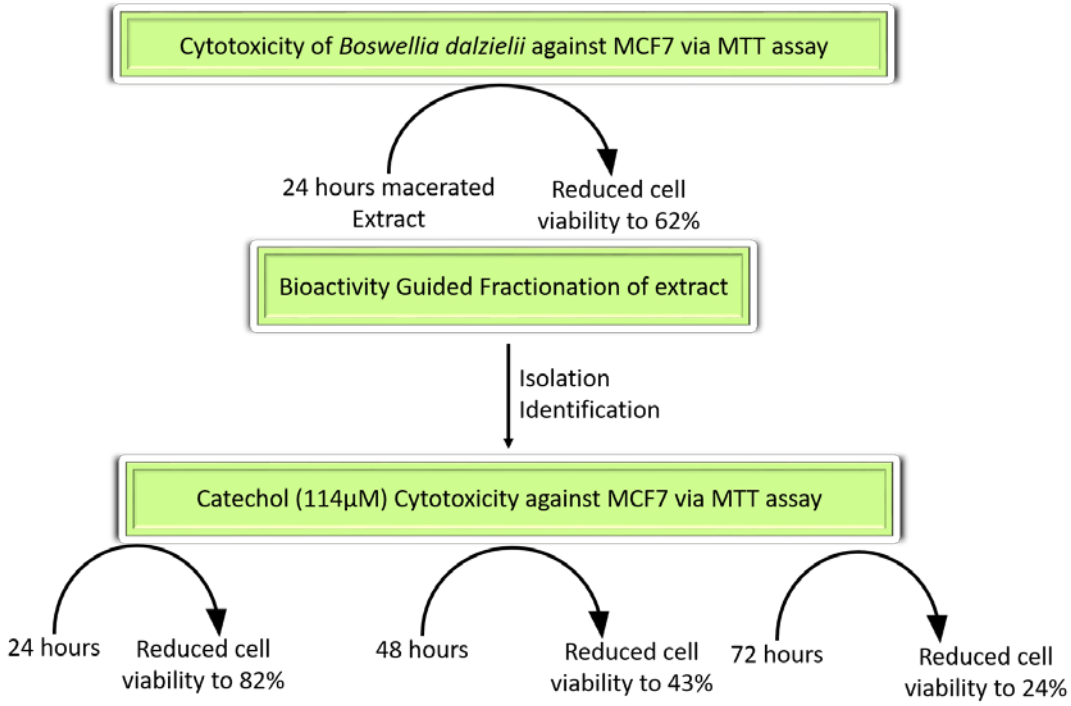


Figure 7. 3: *Illustration of the cytotoxicity assay conducted on the 24 hours extract of B. dalzielii plant bark obtained via maceration.*

Furthermore, it is important to mention that a new batch *B. dalzielii* was procured due to a shortage in the first batch used at the beginning of the study presented in this thesis. Although some variability was observed using TLC analysis (Figure 5. 10), for future research it is recommended to explore in detail the variability in terms of microorganisms, phytochemicals and bioactivity of *B. dalzielii* plant parts collected throughout a year. This will contribute to tackling the issues regarding the variability in plant samples for therapeutic applications.

Finally, chapter 6 of the study presented in this thesis discusses the involvement of microorganisms in the production of catechol. Protochaceuic acid was identified as the compound that is bio-converted to catechol and the bacteria responsible for this bio-conversion are *Klebsiella pneumonia* B and *Klebsiella pneumonia* E. These two strains of *Klebsiella pneumonia* where also shown to bio-convert gallic acid to pyrogallol. Furthermore, *E. cloacae* was also shown to be capable of bio-converting protochaceuic acid to catechol. Another microorganism isolated from the plant bark of *B. dalzielii*, *Pantoea agglomerans*, cannot bio-convert protochaceuic acid to catechol (See Figure 7.4). The 16S rRNA sequences of *Klebsiella pneumonia* B, *Klebsiella pneumonia* E and *Pantoea agglomerans* are deposited in the GenBank nucleotide database under the accession number MH762022, MH762023 and MH762024, respectively.

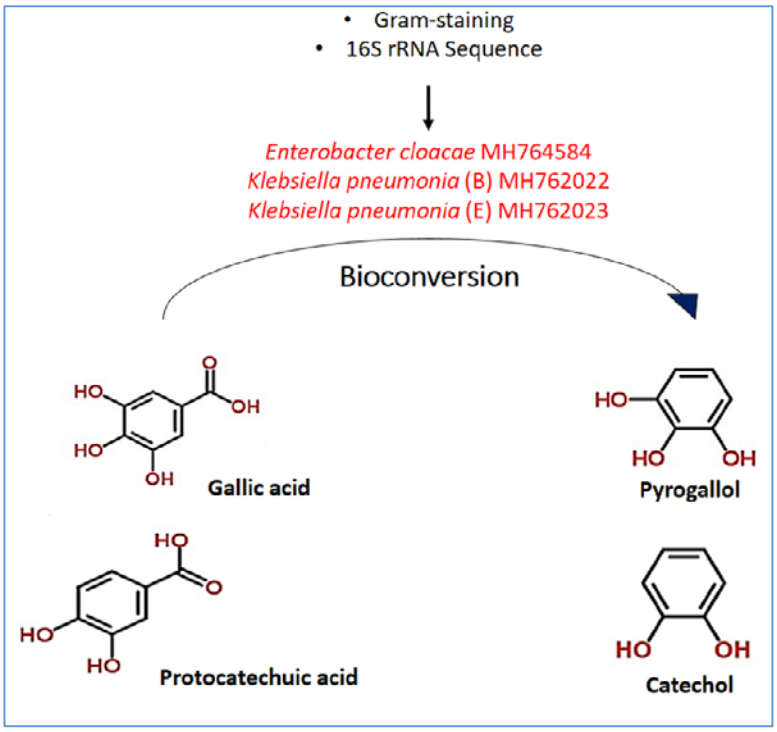


Figure 7. 4: *Bioconversion of gallic acid to pyrogallol and protocatechuic acid to catechol*

In conclusion, the study presented in this thesis contributes to the growing notion that some phytochemicals believed to be plant-derived could be a product of the interaction of endophytic microbes with plant exudates. Also, this study also contributes to the growing emphasis on the use of ethnopharmacology in search of active compounds with bioactivity from traditional medicine. The traditional use of aqueous extract of the bark of B. dalzielli in the treatment of infections and tumours was validated in part. Antimicrobial activity of the extracts was sufficient to treat topical infections, some weak to moderate anticancer activity was found after in vitro screening.

8

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9

APPENDIX I

THIN LAYER METHOD DEVELOPMENT

ABSTRACT

In phytochemistry, spray reagents are used for visualizing compounds present in natural products. In this chapter, m-anisaldehyde vanillin spray reagent was used to visualized compounds present in the aqueous extract of *B. dalzielii* bark. This was due to lack of p-anisaldehyde reagent. Although, vanillin also produced good visualization, the combination of vanillin and m-anisaldehyde reagents produced better colouration for phenols and sterols.

9.1 INTRODUCTION

Thin-layer chromatography (TLC) is a microscale method which involve the use of a stationary phase made up of either glass slide or plastic sheet thin layer coated with a polar absorbent which could be either (Al2O3.xH2O)n or silica (SiO2.xH2O)n particles and a mobile phase which could be a single or combination of solvents (Gorman and Jiang, 2004). This technique has been evolved for use in the screening of bioactive compounds and regarded as an effective-directed analysis and regarded as bioautographic assays (Marston, 2011).

Over the years, different TLC visualization reagents have been reported, some are used for the detection of specific phytochemicals such as aluminium chloride and ethanolamine diphenylborate for flavonoid, Emerson reagent, p-anisaldehyde-sulfuric acid and chloranil reagent for phenols and Dragendorff`s reagent for alkaloids (Jork *et al.*, 1994; Wagner and Bladt, 1996). In these studies, non-specific vanillin-sulfuric and anisaldehyde reagents were used.

9.2 METHODS

9.2.1 Preparation of vanillin reagent

2g of vanillin was measured and transferred into a 100mL Duran bottle. 98mL of ethanol was measured in a measuring cylinder then added to the vanillin and mixed with the aid of a magnetic stirrer. Duran bottle containing vanillin solution was transferred on ice

and 2mL of sulfuric acid was added dropwise. Vanillin reagent was wrapped with a foil paper and stored in the refrigerator.

9.2.2 Preparation of m-anisaldehyde reagent

0.5mL of m-anisaldehyde was measured and transferred into a 100mL Duran bottle containing 85mL of methanol, then 10mL of acetic acid and 5mL of sulfuric acid was added on ice. The prepared reagent was wrapped with a foil paper and stored in the refrigerator.

9.2.3 Preparation of m-anisaldehyde vanillin reagent

1g of vanillin was measured and transferred into a 100mL Duran bottle containing 49mL of ethanol, 42.5 of methanol, 0.25mL of m-anisaldehyde then 5mL of acetic acid, and 2.5mL of sulfuric acid was added on ice. The prepared reagent was wrapped with a foil paper and stored in the refrigerator.

9.2.4 Preparation of p-anisaldehyde vanillin reagent

0.5mL of p-anisaldehyde was measured and transferred into a 100mL Duran bottle containing 85mL of methanol, then 10mL of acetic acid and 5mL of sulfuric acid was added on ice. The prepared reagent was wrapped with a foil paper and stored in the refrigerator.

9.2.5 TLC analysis of boiled plant bark

10g of *B. dalzielii* plant bark was measured and added to a 250mL round bottom flask containing 100mL of distilled water. The sample was boiled for an hour and allowed to cool. 40µL of the aqueous sample was spotted on four TLC plates with an approximate dimension of 10cm×3cm. Four TLC tanks were cleaned and three of those tanks had a different ratio of hexane and ethyl acetate was added as follows: 90:10, 80:20 and 50:50 plus a drop of acetic acid. The fourth tank contains ethyl acetate, acetic acid and water in the ratio of 100: 1.0: 1.0, respectively. TLC plates were allowed to run in the four prepared tanks, afterwards dried in the fume cupboard before viewing TLC plate under the short wavelength then spraying with vanillin reagent. The whole procedure was repeated and TLC plate was sprayed with m-anisaldehyde reagent and m-anisaldehyde vanillin reagent.

9.2.6 TLC analysis of known compounds

1mg/mL of the following compounds gallic acid, pyrogallol, β-sitosterol, stigmasterol, kaempferol, nobiletin, farnesol and terpinene was prepared. 5µL of each was spotted on three TLC plate with an approximate dimension of 10cm×3cm and each of this plate was labelled vanillin, m-anisaldehyde and p-anisaldehyde.

9.3 RESULTS AND DISCUSSION

9.3.1 Vanillin sprayed cromatogram

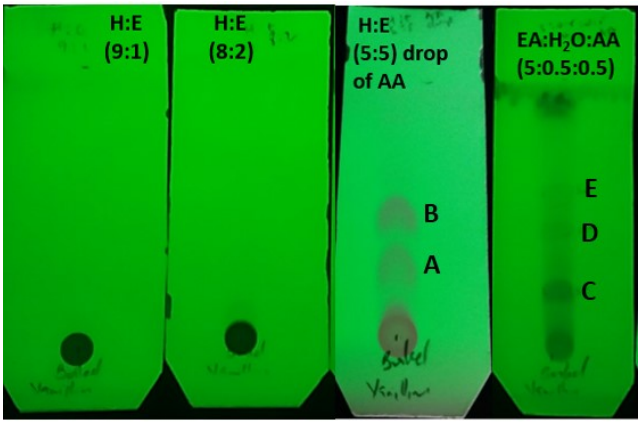

Figure 9. 1: *Analysed TLC plates viewed under short wavelength*

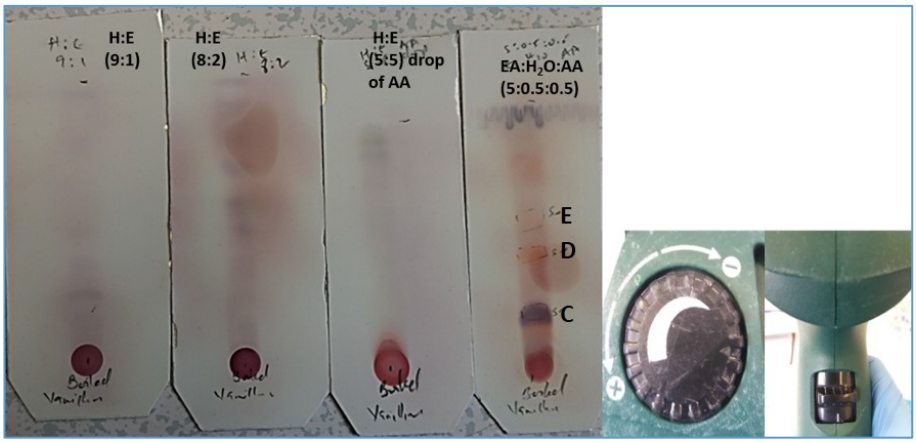

Figure 9. 2: *Analysed TLC plates sprayed with vanillin reagent and heat source used was 2000W hot air gun set at position I (40-450˚C).*

Compounds labelled A and B obtained after running with 5mL of hexane and 5mL of ethyl acetate plus a drop of acetic acid were visible under the short wavelength, however, their visibility was lost after spraying with vanillin reagent (see Figure 7.1 and 7.2).

9.3.2 m-Anisaldehye sprayed chromatogram

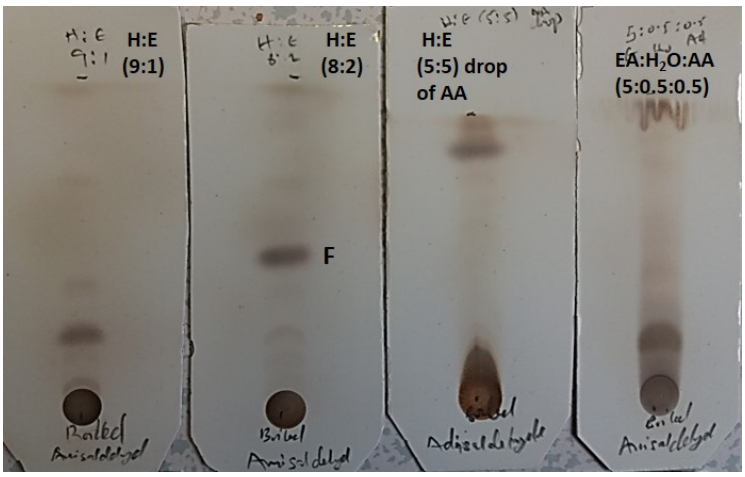

Figure 9. 3: *Analysed TLC plates sprayed with m-anisaldehyde reagent and heat source used was 2000W hot air gun set at position II (90-600˚C).*

Compound labelled F was only visible after spraying with m-anisaldehyde (Figure 7.3) but wasn`t visible with vanillin reagent (Figure 7.2) nor under the short wavelength (Figure 7.1). m-Anisaldehyde was used due to lack of p-Anisaldehyde. Therefore, a different reagent containing both m-anisaldehyde and vanillin was made.

9.3.3 m-Anisaldehye vanillin sprayed chromatogram

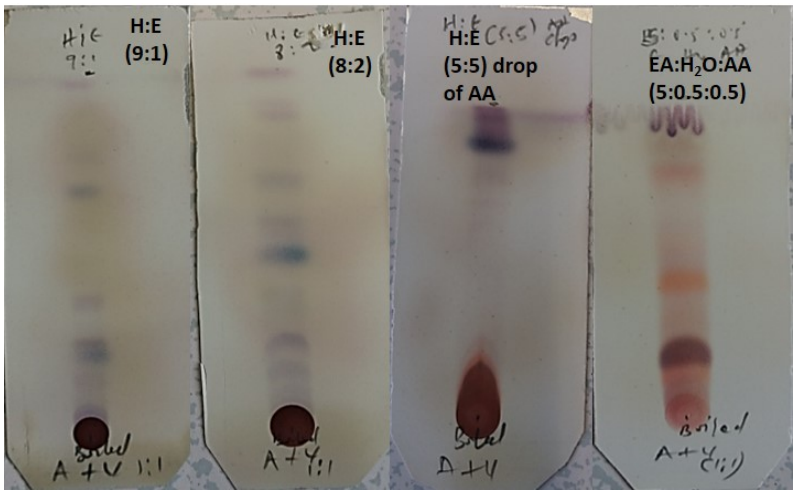

Figure 9. 4: *Analysed TLC plates sprayed with m-anisaldehyde vanillin reagent and heat source used was 2000W hot air gun set at position I (40-450˚C).*

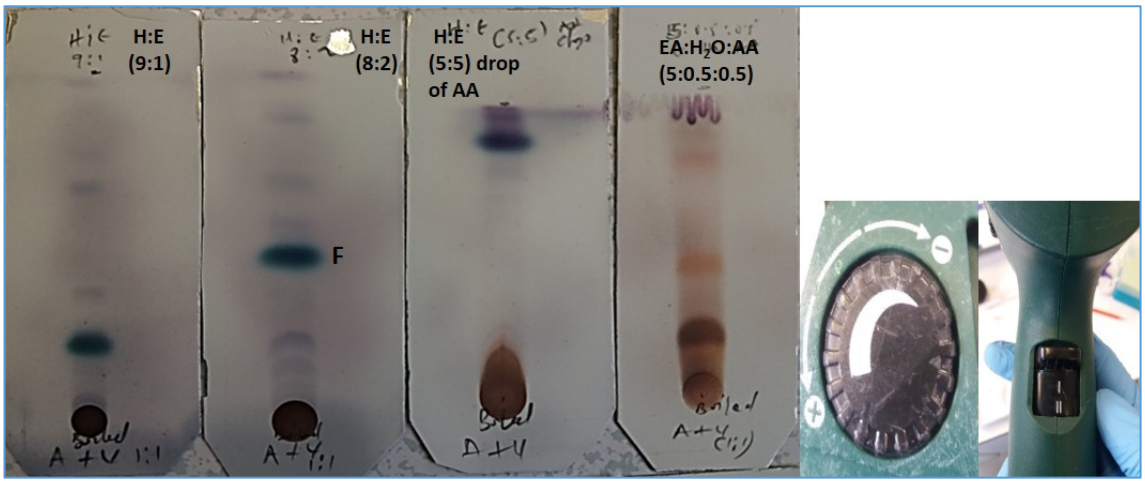

Figure 9. 5: *Analysed TLC plates sprayed with m-anisaldehyde vanillin reagent and heat source used was 2000W hot air gun set at position II (90-600˚C).*

TLC chromatogram analysed with m-anisaldehyde vanillin reagent (Figure 7.5) after running with the 80mL of hexane and 20mL of ethyl acetate provided better visibility when compared to TLC plate viewed under the short wavelength (Figure 7.1) and TLC chromatograms obtained after spraying with vanillin (Figure 7.2) and m-anisaldehyde reagent alone (Figure 7.4).

9.3.4 TLC analysis of known compounds

TLC analysis of known compounds was conducted to show that where p-anisaldehyde reagent is available, the use of m-anisaldehyde vanillin is not necessary. However, m-anisaldehyde vanillin provided distinct colouration compared to the individual reagent sprays used (Figure 7.6)

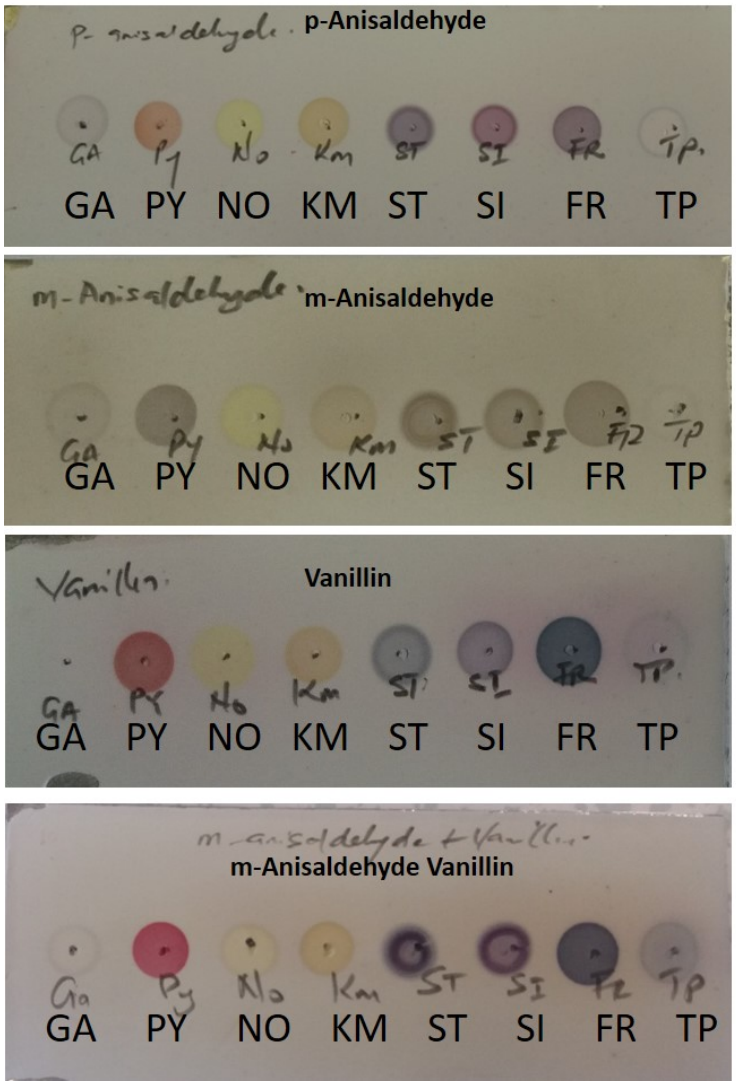

Figure 9. 6: *Analysed TLC plates sprayed with m-anisaldehyde vanillin, vanillin, p-anisaldehyde and m-anisaldehyde reagents and heat source used was 2000W hot air gun set at position II (90-600˚C). Where GA = gallic acid, PY = pyrogallol, NO = nobiletin, KM = kaempferol, ST = stigmasterol, SI = sitosterol, FR = farnesol and TP = terpinene.*

From Figure 7.6, gallic acid and terpinene were seen to have a more intense colour when treated with m-anisaldehyde vanillin spray when compared to other reagents used. Also, the spots for stigmasterol and sitosterol are more prominent when compared to the other spray reagent used.

9.4 CONCLUSION

In the absence of p-anisaldehyde reagent, m-anisaldehyde was used. However, there was no distinct difference obtained between compounds because the colouration obtained after heating is between light brown to intense brown. Vanillin reagent was used and some compounds visible under the short wavelength were not reacting with the vanillin reagent. Thus, a reagent containing both vanillin and m-anisaldehyde was made and better visibility was obtained. To confirm the reactivity of m-anisaldehyde vanillin spray, known compounds purchased from Sigma Aldrich were used. These compounds belong to the class of phenol (gallic acid), flavonoids (kaempferol and nobiletin), terpenes (farnesol and terpinene) and sterols (stigmasterol and β-sitosterol). The TLC analysis of these compounds was conducted and all the four spray reagents were used. From the TLC chromatogram, m-anisaldehyde vanillin spray reagent produced better colouration especially for gallic acid, sitosterol and stigmasterol.