Pharmacological screening of synthetic piperidine derivatives

Submitted in complete fulfillment for the Degree of Master of Applied Sciences in Biotechnology in the Department of Biotechnology and Food Technology, Durban University of Technology, Durban, South Africa

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REFERENCE DECLARATION

I, Miss. L. Naicker – 20905484 and Prof Bharti Odhav (full name of promoter/ supervisor) do hereby declare that in respect of the following dissertation

Title: Pharmacological screening of synthetic Piperidine Derivatives

1. As far as we ascertain:

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AUTHOR'S DECLARATION

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

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Student's signature

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LIST OF ABBREVIATIONS

4-DAMP	4-Diphenyl acetoxy-N-methylpiperidine methiodide
ATR	Attenuated total reflectance
CFU /mL	Colony Forming Units per mililitre
DMSO	Dimethyl Sulfoxide
DPPH	(2, 2 – Diphenyl-1-Picrylhydra-Zyl)
INT	*p*-Iodinitetrazolium Violet
IUPAC	International Union of Pure and Applied Chemistry
MIC	Minimum Inhibitory Concentration
MTT	(4,5-Dimethylthiazol-2-Yl-) -2,5- diphenyltetrazolium Bromide
NDGA	Nordihydroguaiaretic acid
NSAID	Non-Steroidal Anti Inflammatory Drugs
PBMC	Peripheral Blood Mononuclear Cell
PDA	Potato Dextrose Agar
PM1	Methyl 2,6-diphenyl-1-*p*-tolyl-4-(*p*-tolylamino)-1,2,5,6-tetrahydropyridine-3-carboxylate
PM2	Methyl 2,6-bis(4-cyanophenyl)-1-*p*-tolyl-4-(*p*-tolylamino)-1,2,5,6-tetrahydropyridine-3-carboxylate
PM3	Ethyl 1-(4-bromophenyl)-4-(4-bromophenylamino)-2,6-diphenyl-1,2,5,6-tetrahydropyridine-3-carboxylate
PM4	Ethyl 1-(4-bromo-3-methoxyphenyl)-4-(4-bromo-3-methoxyphenylamino)-2,6-di(pyridin-3-yl)-1,2,5,6-tetrahydropyridine-3-carboxylate
PM5	Ethyl 1-(4-methyl-3-(trifluoromethyl)phenyl)-4-(4-methyl-3-(trifluoromethyl)phenylamino)-2,6-di(pyridin-3-yl)-1,2,5,6-tetrahydropyridine-3-carboxylate
PM6	Ethyl 1-(4-chloro-2-fluoro-6-iodophenyl)-4-(4-chloro-2-fluoro-6-iodophenylamino)-2,6-di(pyridin-3-yl)-1,2,5,6 tetrahydro pyridine-3-carboxylate
SDA	Sabouraud Dextrose Agar
TB	Tuberculosis

TDH	Threonine Dehydrogenase
THP	Tetrahydropyridine

CONFERENCE PRESENTATION

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ABSTRACT

Piperidine derivatives are essential heterocyclic compounds that have beneficial roles in the medical and commercial sector. They can be isolated from plant material and can be chemically synthesised using simple cost efficient methods. Piperidines and their derivatives are clinically used to prevent postoperative vomiting, facilitate radiological evaluation, correct gastrointestinal function as well as speed up gastric emptying before anaesthesia. Piperidine derivatives also demonstrate a wide spectrum of biological activities which include; antimicrobial, anticancer, anti- TB, anti-HIV, anti-inflammatory, analgesic, anti-influenza, anti-inflammatory and antitumor activity. The properties of piperidine derivatives depend on the nature of the side chains and their orientation.

Based on the promising data that demonstrated the synergistic effects of biological agents with piperidine derivatives, the aim of our research is to determine the pharmacological activities, i.e. (i) antimicrobial activity, (ii) anti-inflammatory, (iii) anti-oxidant activity, (iv) cytotoxicity, and (v) biosafety of six piperidine derivatives, **PM1** to **PM6**.

All six piperidine derivatives (**PM1**-**PM6**) screened for antimicrobial activity exhibit characteristics of varying degrees of microbial inhibition against some Gram-positive and Gram-negative bacteria (*B. cereus, B. subtilis, E. coli, S. aureus, Kl. Pneumonia, M. liuteus and P. aurenginosa*) with the exception of *B. polymixa, S. marcescens* and *S. faecalis.* Certain piperidine derivatives did not demonstrate high inhibition activity towards the fungal strains, with inhibition only shown against four fungal species; *A. niger, A. flavus, C. albicans and S. cerevisiae.* Thus it is proposed that minor changes could be made to the structure of the compounds so that they can alter the effect that the compounds have on the specific fungi strains. With regard to antioxidant activity it is noted that the concentrations of the test compounds are directly proportional to the percentage of scavenging capacity. In comparison of the piperidine derivatives (**PM1**-**PM6**) to Rutin (reference standard), it was illustrated that Rutin displayed the best antioxidant activity. All six piperidine derivatives (**PM1-PM6)** showed greater than 50% anti-inflammatory activity, whilst the anti-inflammatory reference standard NCGA displayed the greatest activity in

comparison to the piperidine derivatives tested. The safety of the piperidine derivatives was tested by assaying cytotoxicity, against melanoma, MCF7 cancer cells and normal fibroblasts as well as Brine shrimp lethality assay. All piperidine derivatives demonstrated high cytotoxicity activity against both cancer cell lines (melanoma and MCF7) and around 50 – 52% cytotoxicity against healthy cells. Chloro substitution of the phenyl ring increases cytotoxicity of compounds (Aerluri *et al.*, 2012). This compound can be used in the treatment of cancer cells while inhibiting 50% of normal cells. All six piperidine derivatives (**PM1-PM6)** were also tested for toxicity against *Artemia salina* in a brine shrimp lethality assay. Piperidine derivatives exhibited varying degree of toxic activity towards the shrimp, with all derivatives displaying ± 50% toxic activity at 1000 µg/mL. These results reveal a directly proportional relationship between concentration of drug and toxicity.

It remains a future research objective to modify these piperidine compounds (**PM1-PM6)** chemically to produce more derivatives for further biological evaluation. All the studied piperidine compounds have possible leads for optimization to carry out pre-clinical trials. We can conclude that the substitution of different side chains on the piperidine nucleus results in varying degree of pharmacological activity. Also, compounds containing the substitution of a chloro group at position 4 and a fluoro group at position 2 on the phenyl ring attached to carbon 2 and 6 on the piperidine nucleus resulted in high pharmacological activity. This good pharmacological activity was also exhibited by compounds containing substitutions of a methoxy group at position 3 on the phenyl ring attached to carbon 1 and 6 on the piperidine nucleus. Compounds containing a methoxy group positioned at carbon 4 on the phenyl ring which is attached to carbon 1 and 4 on the piperidine nuleus presented low pharmacological activity. Low activity was also exhibited by compounds containing substitution of a cyano group at position 4 on the phenyl ring which is attached to carbon 2 and 6 on the piperidine ring and a methyl group at position 4 on the phenyl group attached to a nitrogen at position 1 on the piperidine nucleus.

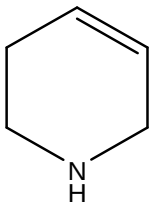
CHAPTER 1: INTRODUCTION, AIMS AND OBJECTIVES

1.0 Introduction

Diseases and infections are recognized as a major medical challenge in most healthcare systems (Hogberg *et al*., 2010). Resistance and multidrug-resistant pathogens are spreading with extraordinary speed globally (Hogberg *et al*., 2010) leading to increased mortality rates amongst patients infected (Freire-Moran *et al*., 2011). Microorganisms are known to frequently form a resistance against pharmaceutically available drugs (Padmavathi *et al*., 2005). These drugs frequently lack selectivity and easy availability as well as unfavorable side effects (Padmavathi *et al*., 2005). The key to efficient and effective treatment of emerging diseases lies in the early stages of identification, diagnosis and treatment of these infections. Thus, there is a constant need for novel therapeutic agents. There has been a vast interest in the synthesis of heterocyclic compounds especially alkaloids due to their diverse reactivity, physiological and pharmacological activity (Padmavathi *et al*., 2005, Aeluri *et al.*, 2012, Perumal *et al*., 2014). Among these compounds, tetrahydropyridine (THP) **(1)** derivatives have recently generated intense attention in the field of organic, bio-molecular chemistry and biotechnology due to their useful biological properties and their simple synthetic mechanism (Blackburne *et al*., 1975).

1.1 An overview of the piperidine compounds

Tetrahydropyridine **(1)** (Maxime, 2004) is the core of piperidine derivatives. It is found in numerous natural and synthetic pharmaceuticals and a wide variety of biologically active compounds (Mishra and Ghosh, 2011, Sumati *et al*., 2013).



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Piperidine scaffolding has found beneficial roles in numerous pharmaceutical drugs that are currently available in the market (Perumal *et al*., 2014, Das and Brahmachari, 2013, Sumati *et al*., 2013). Piperidine derivatives can be isolated from plant materials and synthesized using one or more of the many chemical reactions that has been established for the synthesis of piperidine derivatives (Anthal *et al.,* 2013). The synthesis of piperidines and their derivatives have attracted the attention of organic and medicinal chemists, as these are commonly used in numerous natural products, pharmaceuticals and agrochemicals (Pizzuti *et al*., 2008). Alogliptin, Ritalin, and Risperidone are pharmaceutically available drugs containing the piperidine nucleus that are used for the treatment of diabetes, improve concentration in children and reduce schizophrenia. The drug CP-690550 also known as Janus kinase 3 (JAK3), inhibits autoimmune diseases and is used in transplant patients (Aeluri *et al*., 2012).

Derivatives containing the tetrahydropyridine nucleus **(1)** demonstrate a comprehensive variety of pharmacological activities including antimicrobial (Yuefen Zhou *et al*., 2007), antimalarial (Safaei-Ghomi and Ziarati, 2013), anticonvulsant, anti-parasitic, cytotoxic, anti-inflammatory, pesticidal and anti-HIV-1 properties (Prachayasittikul *et al*., 2009, Sumati Anthal *et al*., 2013). The nucleus is present in various therapeutic agents including numerous antihistamines, antiseptics, anti-arrhythmic, anti-rheumatic and many other pharmaceutical and natural products (Ravindernath and Reddy, 2013).

Numerous methods have been developed for the synthesis of piperidine derivatives (Chang *et al*., 2010). These methods include, cyclization techniques such as imino Diels-Alder reactions, aza-Prins cyclizations, intramolecular Michael reactions and intramolecular Mannich reaction (Das and Brahmachari, 2013) .

Piperidines are synthesized because they are not available naturally in the large qualities which are needed in pharmaceutical industry to produce drugs. Although plants are known to contain many derivatives and other compounds (Reshmi *et al*., 2010) the extraction and purification of piperidine compounds is a lengthy and expensive process. Therefore, syntheses of piperidine compounds are necessary in order to obtain pure compounds in reduced time.

1.2 Research Aim and Objectives

The research aim of this project was to evaluate the pharmacological properties of piperidine derivatives (Figure 1) and to determine their suitability for the development of new pharmaceutical drugs.

The objectives of this study were to:

(i) Ascertain the purity and structure of the compounds (**PM1**-**PM6**),

(ii) Evaluate the antimicrobial, antioxidant, anti-inflammatory and cytotoxicity activities of the piperidine derivatives,

(iii) Determine the bio-safety properties of the study compounds using, *Salmonella* mutagenicity assay (mutagenic potential), Brine shrimp lethality assay (toxicity) and *in vitro* toxicity by assaying the toxicity in vitro using cell cultures.

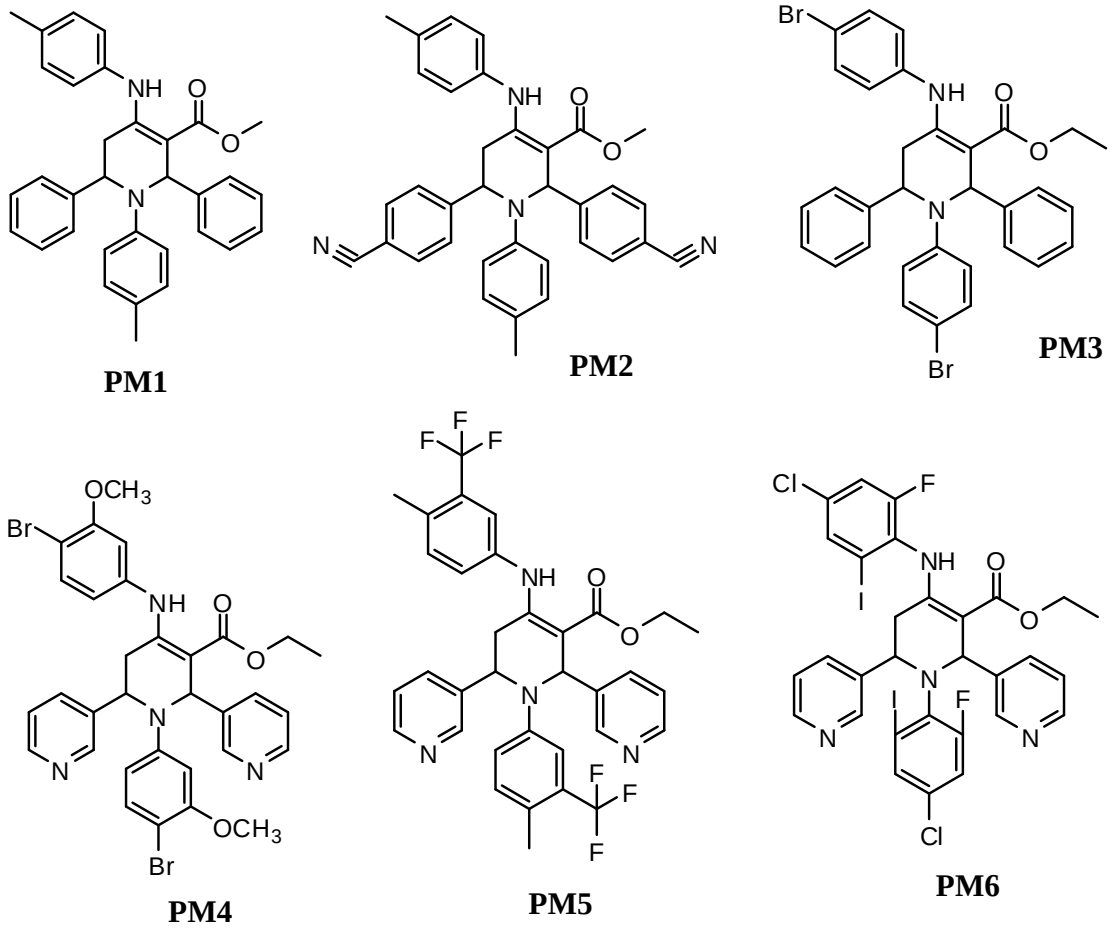


Figure 1 Chemical structures of piperidine derivatives **PM1**-**PM6**.

Compound code	International Union of Pure and Applied Chemistry (IUPAC) name of compounds
PM1	Methyl 2,6-diphenyl-1-*p*-tolyl-4-(*p*-tolylamino)-1,2,5,6-tetrahydro pyridine-3 carboxylate
PM2	Methyl 2,6-bis(4-cyanophenyl)-1-*p*-tolyl-4-(*p*-tolylamino)-1,2,5,6- tetrahydropyridine-3-carboxylate
PM3	Ethyl 1-(4-bromophenyl)-4-(4 bromophenyl amino)-2,6-diphenyl-1,2,5,6-tetrahydropyridine-3-carboxylate,
PM4	Ethyl 1-(4-bromo-3-methoxy phenyl)-4-(4-bromo-3-methoxyphenylamino)-2,6-di(pyridin-3-yl)-1,2,5,6-tetrahydropyridine-3-carboxylate

PM5	Ethyl 1-(4-methyl-3-(trifluoromethyl)phenyl)-4-(4-methyl-3-(trifluoromethyl) phenyl amino)-2,6-di(pyridin-3-yl)-1,2,5,6 tetrahydropyridine-3-carboxylate
PM6	Ethyl 1-(4-chloro-2-fluoro-6-iodophenyl)-4-(4-chloro-2-fluoro-6-iodophenylamino)-2,6-di(pyridin-3-yl)-1,2,5,6-tetrahydropyridine-3-carboxylate)

1.3 Structure of the Thesis

This thesis is divided into eight chapters.

Chapter 1 covers the introduction of the thesis, an overview of the piperidine compounds and the research aims and objectives.

Chapter 2 covers the literature review of piperidine derivatives including their pharmacological activities, difference between synthetic and isolated piperidine derivatives, mechanism of action and their role in the medical and chemical industries.

Chapter 3 focuses on the material and methodology utilized and applied to perform the various pharmacological activities.

Chapter 4 presents the results achieved from the various pharmacological assays conducted.

Chapter 5 focuses on discussing the results obtained in chapter 4 and that of other piperidine derivatives researched and a possible explanation for the results obtained for the piperidine derivatives screened.

Chapter 6 focuses on a conclusion of the thesis and what future work can be conducted on these six piperidine derivatives. The references used in this thesis are listed and then followed by the appendixes.

CHAPTER 2: LITERATURE REVIEW

2.0 Piperidines

Piperidines are organic compounds consisting of a six membered ring with a molecular formula $(CH_2)_5NH$.This heterocyclic amine consists of a six-membered ring containing five methylene bridges (-CH_2-) and one amine bridge (-NH-) (Maxime, 2004). Heterocyclic compounds are referred to as compounds that have one or more atoms in the ring other than carbon. Amines containing nitrogen as part of a complex ring system commonly occur in nature. Piperidine is a simple nitrogen heterocyclic compound referred to as a secondary amine (Quellette, 1984). Piperidine is identified as a colorless fuming liquid with an odor described as ammoniacal, which is like pepper (Hall, 1957). Therefore piperidine obtained its name from the genus name *Piper*, which is the Latin word for pepper (Hall, 1957).

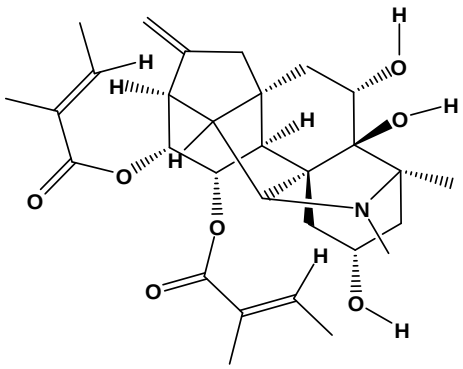
The properties of piperidines are dependent on conformation and arrangement of side chains (Blackburne *et al.*, 1975).

The equatorial conformation of piperidine derivatives of the was found to be much more stable by 0.72 kcal/mol in the gas phase than axial conformation (Blackburne *et al.,* 1975). It was acknowledged that piperidine compounds in an equatorial conformation are more stable in nonpolar solvents displaying between 0.2 and 0.6 kcal/mol, but when piperidine compounds are present in polar solvents the axial conformation may be more stable. Both the equatorial and axial conformers interconvert rapidly through nitrogen inversion; the free energy activation barrier for this process, estimated at 6.1 kcal/mol, is substantially lower than the 10.4 kcal/mol for ring inversion (Blackburne *et al.*, 1975).

Along with obtaining the correct conformation the characteristics of piperidine compounds are of vital importance. Piperidine properties consist of, a molecular formula which is $C_5H_{11}N$, molar mass which is 85.15 g/mol, appearance that is colourless liquid, density of 0.862 g/mL, boiling point is 106 °C, solubility in water which is miscible and acidity (pKa) which is 11.22 (Irvine and Saxy, 1969).

The piperidine conformation and its characteristics play a vital role in the compound's ability to function and produce biological activity.

The piperidine moiety is not only an important starting material for biologically active compounds but are also an important building blocks for many natural products, chemical products and pharmaceuticals (for example, anopterine **(2)**, scopolamine **(3),** and morphine **(4)** (Chang *et al*., 2010). The nucleus also possesses analgesic, anesthetic as well as ganglionic blocking properties.The modified side chains are responsible for the antibiotic, antifungal, anti-oxidant, anti-inflammatory and anticancer properties. (Sergeant and May, 1970).



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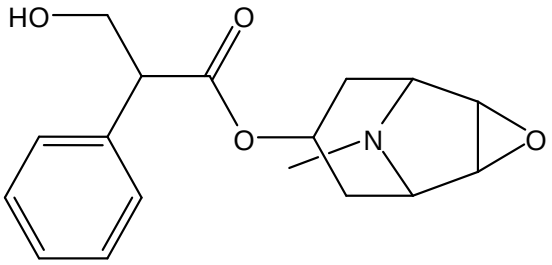

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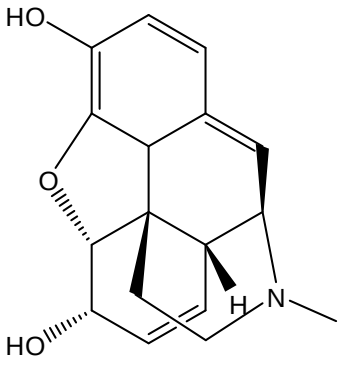

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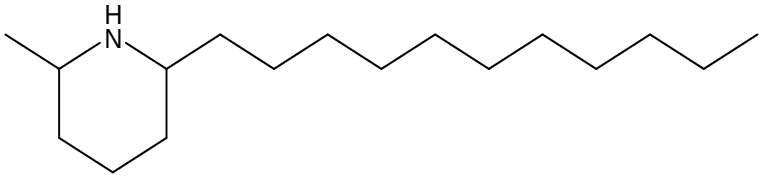
The piperidine structural moiety is widespread throughout nature in numerous derivatives and their synthesis usually involves multi-step processes. Piperidine derivatives such as tetrahydropyridine are synthetically produced (Przedborski *et al*., 2000). This is not as abundant as their saturated counterparts and therefore its serves as a beneficial piperidine derivative (Overman *et al.*, 1993).

2.1 Natural sources of piperidines and their derivatives

The piperidine moiety (Bailey *et al*., 1998) occurs within nature (Amat *et al*., 2003) in natural products. Some examples of natural piperidine derivatives **(5, 6)** are, solenpsin and scoulerine. These natural compounds demonstrate a comprehensive range of beneficial biological activities (Asano *et al*., 2000). Many naturally occurring piperidine derivatives have been experimentally proven as outstanding antibacterial agents.



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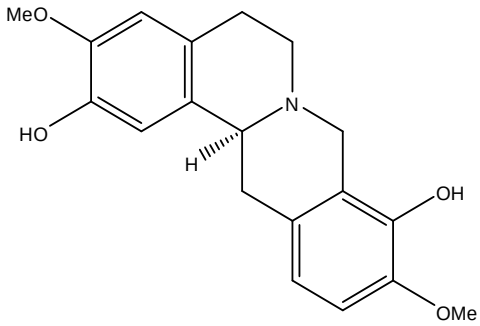

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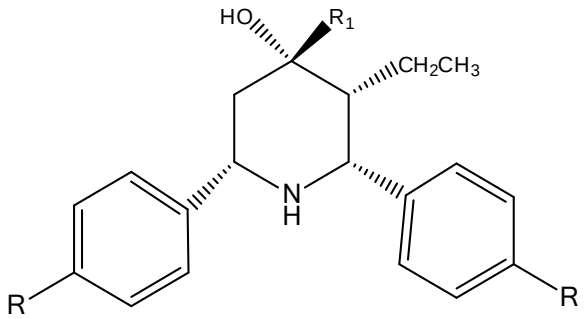
Solenopsin **(5)** is a compound that is closely correlated to the family of alkaloids which have been isolated from ants therefore they are referred to as the fire ant toxin (Senning, 2006). The fire ant toxin demonstrates hemolytic activity, vesicant activity and well provokes the release of histamine from mast cells (Douglass *et al*., 1988).

Scoulerine **(6)** occurs in the Opium poppy (Frick *et al*., 2004), *Croton flavens* (Eisenreich *et al.*, 2003) and in certain plants in the Erythrina genus (Ito, 1999). Scoulerine is an antagonist at the α2-adrenoceptor, α1D-adrenoceptor and 5-HT receptor (Ko *et al.*, 1993, Ko *et al*., 1994). Scoulerine **(5)** has also been found to be a gamma-aminobutyric acid (GABA) agonist receptor (Eisenreich *et al*., 2003).

Piperidine derivatives occurs in various plants from Africa and in their roots (Uhegbu and Maduagwu, 1995). They are natural constituents in white pepper (Henry, 1949), tobacco leaves (Irvine and Saxy, 1969) and black pepper (Hawley, 1981). Piperidine derivatives also occur in the root of the kava shrub (*Piper methysticum*) with the stem peelings being used currently as a source of kavalactones in the kava diet (Dragull *et al.*, 2003). Piperidine derivatives are also found in hemlock which was used to put Socrates to death.

The fruit, stems and leaves of the flowering plant *Senna spectabilis* is known to contain piperidine analogue, 3-hydroxy-2,6-dialkyl-piperidine **(7)** (Maruthavanan and Venkatesan, 2013). *Senna spectabilis* (*sin. Cassia excelsa, C. spectabilis*) is an endemic tree of South America and Africa. It is also common in Brazil, where it is known as “canafistula-de-besouro” and “cassia-do-nordeste”. In folk medicine this plant is used for the treatment of insomnia, constipation, epilepsy, anxiety, malaria, dysentery and headache (Melo *et al*., 2014).



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Piperidine derivatives are commonly detected in food products (Singer and Lijinsky, 1976). The piperidine ring is also found in compounds derived from pomegranate, sedum, labella, pine species, papaya and the scent gland of musk deer and in betel nut (Harold and Banks, 1993). Piperidine occurs naturally in the body from the degradation of lysine, cadaverine, and pipecolic acid. Piperidine derivatives have been found in brain, skin, urine of animals and cerebrospinal fluid (Kataoka, 1997). Exogenous piperidine is absorbed from the respiratory tract, gastrointestinal tract

and skin. It is found in most tissues in the body including the brain where this is excreted as unchanged piperidine or its metabolites (Butt, 2003).

2.2 Biological, chemical and medicinal properties of piperidine and its derivatives

Piperidine and its derivatives have a major impact in the medical field due to their wide range of pharmacological activities. The initial application of piperidines in the medical field goes back many years when they were recognised as a structural component of morphine. It is due to these structural components that makes piperidine and its derivatives essential in the synthesis of many significant pharmaceuticals and fine chemicals (Rafiq *et al.*, 2013). During synthetic reactions the addition of piperidine fragments which develops a moiety which as enhanced potential against infection (Wong *et al*., 2011).

The piperidine moiety is an essential pharmacophore which is found in numerous alkaloids, pharmaceuticals, agrochemicals and as synthetic and biological intermediates (Misra *et al.*, 2009). They are biologically active piperidine derivatives which are targeted for their total or partial synthesis (Ramalingan *et al.*, 2004).

Piperidines are known to have central nervous system (CNS) depressant action at low dosage levels and stimulatory activity with increased doses. In addition, the nucleus also possesses; analgesic, ganglionic blocking and anesthetic properties (Sergeant and May, 1970). The subunit has great chemotherapeutic effect as numerous lead molecules with prominent biological activities used as clinical candidates for several diseases (Misra *et al*., 2009). Piperidines are believed to cure illness such as constipation, diarrhoea, earache, gangrene, heart disease, hernia, hoarseness, indigestion, insect bites, insomnia, joint pain, liver problems, lung disease, oral abscesses, sunburn, tooth decay, and toothaches (Lokhande *et al*., 2007). It has been shown that piperidine can dramatically increase absorption of selenium, vitamin B, beta-carotene and curcumin as well as other nutrients (Lokhande *et al*., 2007). Many plant-derived molecules have shown a promising effect in therapeutics (Lokhande *et al*., 2007). Piperidine derivatives are used clinically to prevent postoperative vomiting, speed up gastric emptying before

anaesthesia or to facilitate radiological evaluation, and to correct a variety of disturbances of gastrointestinal function (Lavanya *et al*., 2009). Piperidine derivatives are in demand as they provide benefits for both humans and livestock through decreased associated medical costs, improved livestock production systems, and associated economic gains to agricultural producers (Brown *et al*., 2012). Piperidine is used to treat gonorrhea, dysentery, syphilis, abdominal pain and asthma and has also inhibitory effect on Hepatitis C virus protease (Reshmi *et al*., 2010).There are various uses of piperidine including the relief of pain, rheumatism, chills, flu, colds, muscular aches and fever. Externally it is used for its rubefacient properties and as a local application for relaxed sore throat and some skin disorders.

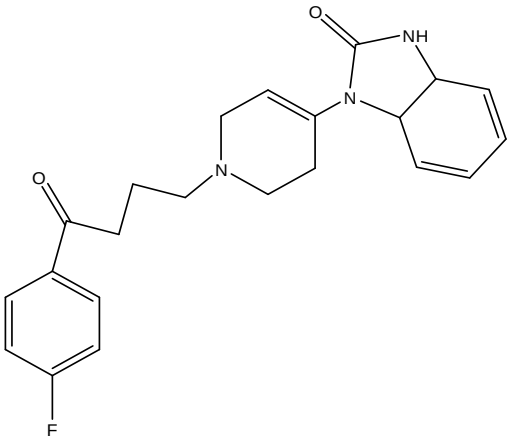
Piperidine and its derivatives are ubiquitous building blocks in the synthesis of pharmaceuticals and fine chemicals. The piperidine structure occurs in many pharmaceuticals such as paroxetine, risperidone, methylphenidate, raloxifene, minoxidil, thioridazine, haloperidol, droperidol, mesoridazine, meperidine, melperone the psychochemical agents Ditran-B (JB-329), *N*-methyl-3-piperidyl benzilate (JB-336) and in many others (Reshmi *et al*., 2010).

Piperidine is also commonly used in chemical degradation reactions, such as the sequencing of DNA in the cleavage of particular modified nucleotides. Piperidine is also commonly used as a base for the deprotection of amino acids used in solid-phase peptide synthesis (George *et al*., 1988).

Piperidine derivatives are utilized for several pharmacological activities, much of these includes; antibacterial (Gram positive and Gram negative) (Pizzuti *et al*., 2008, Balasubramaian *et al*., 2005), antifungal (Pizzuti *et al*., 2008, Venugopala *et al*., 2012, Balasubramaian *et al.*, 2005), anti-inflammatory, antioxidant, anti-HIV (Pizzuti *et al*., 2008) and anticancer activities also known to exhibit insecticidal activity against mosquitoes and flies (Atal *et al*., 1985).

Piperidines are used commercially as solvents in curing agent and epoxy resins. They form intermediates; in organic synthesis, in foods as food additives, and as constituents in the manufacturing of pharmaceuticals (Butt, 2003).

Piperidine derivatives such as tetrahydropyridines are derivatives that are useful against several metabolic disorders and human ailments. The prominent biological activities associated with these pharmacophores are antiparasitic, antimicrobial, anticancer and antiviral (Gwaltney *et al*., 2003). The addition of a tetrahydropyridine moiety in a single structure can enhance the biological and pharmacological activities of the compound (Ravindernath and Reddy, 2013). Tetrahydropyridine compounds have potent biological, pharmacological and therapeutic properties (Ravindernath and Reddy, 2013) such as antimicrobial, anti- inflammatory, anti-rheumatic, anti- arrhythmic, analgesic, antimalarial, anticonvulsant and they are used to treat neuropsychiatric disorders (Das and Brahmachari, 2013). Tetrahydropyridine derivatives, droperidol **(8)** and tazomeline **(9)** are beneficial anti-emetic and antipsychotic medication used to treat cognitive dysfunctional schizophrenia (Das and Brahmachari, 2013).



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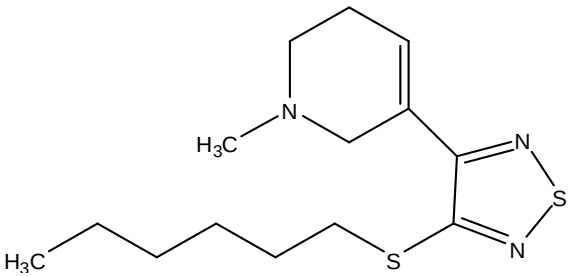

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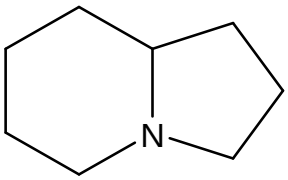
Tetrahydropyridine compounds possess a pleasant roasty, cracker-like and popcorn-like odour. These compounds are responsible for the flavour present in foods such as rice, bread, popcorn, tao shells, and tortilla chips. Tetrahydropyridines are potent odorants with odour thresholds of 0.1 and 1ppb in water (Buttery *et al.*, 1997).

Disubstituted 2,6 piperidine derivatives have been utilized as intermediates in the synthesis of complex ring systems such as indolizidine **(10)** and quinolizdine **(11)** systems (Pizzuti *et al*., 2008).1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has recently been found to be neurotoxic, causing persistent parkinsonism in humans and other animal species (Ravindernath and Reddy, 2013).



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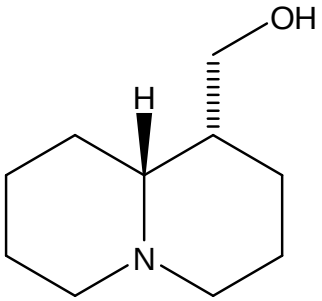

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Piperidine derivatives are also used as a solvent and as a base. Piperidine derivatives; *N*-formylpiperidine is a polar aprotic solvent with better hydrocarbon solubility than other amide solvents and 2,2,6,6-tetramethylpiperidine is a highly sterically hindered base, useful because of its low nucleophilicity and high solubility in organic solvents. A significant industrial application of piperidine is for the production of dipiperidinyl dithiuram tetrasulfide, which is used as a rubber vulcanization accelerator (Hawley, 1981).

Piperidines are used widely as secondary amines as they convert ketones to enamines (Vinayak and Maitland Jones, 1990). These enamines are used in the stork enamine alkylation reaction. Chloramine is formed by the addition of calcium hypochlorite to piperidine. This chloramine undergoes dehydrohalogenation to develop the cyclic imine (George *et al*., 1988).

2.3 The need to synthesize piperidines and their derivatives

Piperidine compounds are very expensive to purchase whereas it is much cheaper to synthesize piperidine derivatives (Pandey and Chawla, 2012). The goal in synthesising piperidine compounds is to create multi-substituted piperidines (Das and Brahmachari, 2013, Maxime, 2004).

Advantages of synthesising piperidine and its derivatives include; a mild reaction condition, simplicity in operation, inexpensive catalyst, and good to high yields, which makes it a useful and attractive process for synthesis of piperidine compounds (Balasubramaian *et al*., 2005).

Compounds are also synthesized as they are not available naturally in large qualities which are needed in the pharmaceutical industry to produce medicines. Plants and animals contain many compounds therefore the synthesis of piperidine compounds is necessary in order to obtain a pure product. The piperidine parent molecule is flexible in nature and hence various derivatives can be easily prepared by altering its substitute (Colapret *et al*., 1989). Compounds are synthesized to better understand their structures and function. Synthesis aids in the production of compounds that do not form naturally from research purpose.

Due to piperidines being frequently required in the pharmaceutical industry and in research, functionalized piperidines are synthesized and used for preparation of drugs that are in demand to treat illnesses, for example; heart disease. Piperidines are widely distributed throughout nature and represent very important scaffolds for drug discovery, featuring as the core of many pharmaceuticals (Risi *et al.*, 2008).

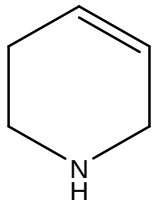
2.4 Applications of piperidine derivatives

The piperidine ring is one of the most common structural features found in biologically active agents and is also the most common building block in natural and synthetic products (Harrit and Provoost, 2005, Maxime, 2004). Over the past few years piperidine and its derivatives have been used in clinical and pre-clinical (Watson *et al*., 2000). It is therefore convenient to arrange this large family of

compounds into different subclasses based on the varying structural patterns amongst them (Fodor and Colasanti, 1985).

Fluoroquinolone is an antibacterial agent having substituted piperidine rings at the C-7 position. This compound is the most attractive drugs in the anti-infective chemotherapy field. These antibiotics exert their effect by inhibition of two type II bacterial topoisomerase enzymes, DNA gyrase and Topoisomerase IV (Chai *et al*., 2010).

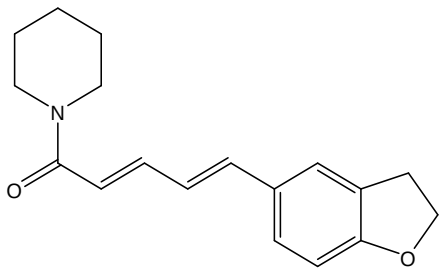
Several compounds consisting of reduced pyridine, for example the 1,2,3,6-tetrahydropyridine 1 ring **(1)** are known to exhibit a variety of biological activities (Risi *et al.*, 2008).



1

2.4.1 *N*-acyl derivatives of piperidine

N-piperyl piperidine also known as piperine **(12)**, was the first of the piperidine class to be discovered in 1938 and since then many other *N*-acyl derivatives have been isolated (McGeoch, 2009).

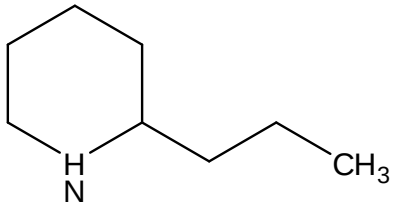


12

Many *N*-acyl or piperdine derivative exhibit antimicrobial activity and are also used in the treatment of asthma and bronchitis. It is acknowledged that by comparing piperidine drugs the bioavailability of the drug increases. An example is the increase of the Sparteine an anti-arrhythmia drug by 100% when the drug was in the presence of piperidine. Another piperidine derivative known as antiepilepsinine, is commonly used in China to aid in the prevention of epileptic attacks since 1975. However, *N*-acyl piperidines in general demonstrate behaviour similar to that of classic central nervous system (CNS) depressants, only differing in the fact that high doses of *N*-acyl piperidine alkaloid do not lead to anaesthesia (McGeoch, 2009).

2.4.2 Alkyl piperidines

Alkyl piperidines include coniine **(13)** and coniceines **(14)**. These are extremely toxic piperidine derivatives that occur naturally in hemlock. The extracts from this hemlock plant was used to cause the death of Socrates around 400 B.C (McGeoch, 2009).



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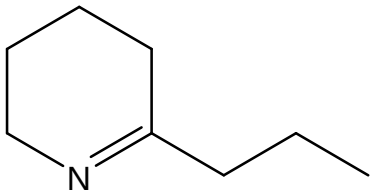


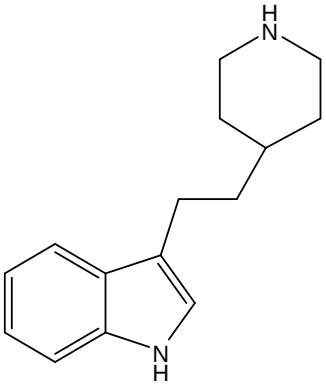
14

The pharmacological properties of alkyl piperidine have been studied for many years. This piperidine derivative shows neuromuscular blocking effects and it is believed that alkyl piperidines are the sources of the sedative, narcotic, anodyne, antispasmodic and anaphrodisiac properties of the poison hemlock. (McGeoch, 2009).

An example of a alkyl piperidine that was first to achieve marketing approval and commercial availability is discussed below.

2.4.2.1 4-Alkyl-piperidine

An example of 4-alkyl-piperidine derivative is Indalpine **(15)** (Mc Geoch, 2009)



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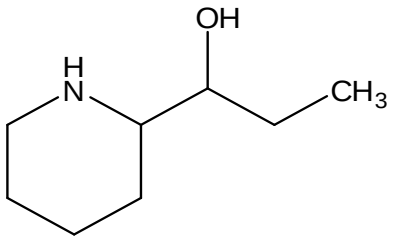
Indalpine was initially discovered by a pharmaceutical company in Paris in the nineteen hundreds as an antidepressant drug (Ahmed *et al*., 2012). Indalpine gained marketing approval and commercial availability around the year 1982. Indalpine was developed by the pharmacologists Le Fur and Uzdan at Pharmuka based on the findings by Shopsin and Ahmed in 2012.

2.4.3 Piperidine alcohol and ketone alkaloids

The commonly known piperidine alcohol is 2-(1-hydroxyalkyl) **(16)** piperidine conhydrine (McGeoch, 2009).



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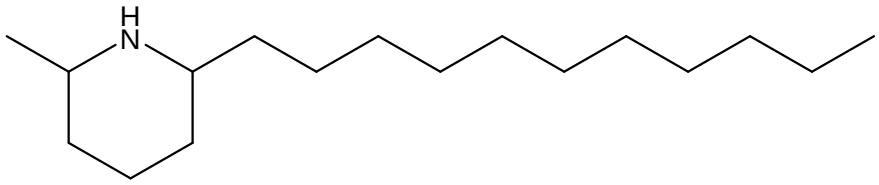
Little is known about the pharmacological applications of piperidine alcohols and ketones. The common piperidine ketone analogues studied are the pelleterines.

Pellerterines are effective as anthelmintic agents. *N*-methylpelleterine acts within the gastrointestinal tract to eliminate tapeworms. Pellerterines are associated with the treatment of the discomfort of muscle cramps and convulsions (McGeoch, 2009).

2.4.4 Piperidine alkaloids with long aliphatic side chains

Piperidine alkaloids containing aliphatic side chains commonly occurs in the animal (insects) and plant kingdoms (McGeoch, 2009). An example of these alkaloids is solenopsin, which constituent largely of fire ants venom of **(17)** (McGeoch, 2009).



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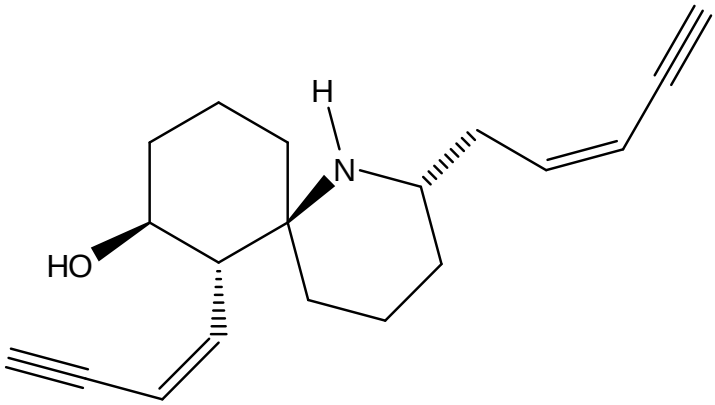
2.4.5 Other piperidine derivatives

There are various piperidine derivatives available which vary in their arrangements and side chains. Of the many piperidine derivatives available, the fused piperidines and spiro-piperidines rings which are known to occur in the animal kingdom exhibit a broad spectrum of pharmacological activity. An example of a spiropiperidine is histrionicotoxin **(18)**, a local anaesthetic isolated from the Colombian frog. An example of a fused piperidine ring is adaline **(19)**. Adaline contains an azabicyclononane skeleton that is present in the European ladybug *Adalia Bipunctata,* which functions as a defence mechanism (McGeoch, 2009).



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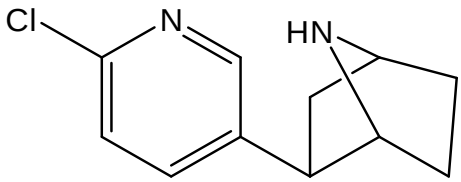

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2.5 Synthesis of piperidine derivatives

There has been much interest in the synthesis of heterocyclic systems due to their pharmacological and physiological activity. The synthesis of piperidines is a process that produces multi-substituted piperidines (Maxime, 2004). Early researchers focused their research on the development of efficient methods to explore the reactivity of different heterocyclic compounds (Bhaskar Reddy *et al*., 1999). Piperidines are synthesized from pyridines which are important synthetic tools used in the preparation of various piperidine derivatives. Access to piperidine derivatives containing a tetravalent carbon via the reduction of pyridine is not the best strategy to use. However pyridines can be reduced to produce piperidine and its derivatives through a one or two step process. The most common method used for the reduction of pyridines is the treatment the reaction with an electrophile such as phenyl chloroformate and a reducing agent such as $NaBH_4$ (Maxime, 2004). Pyridines can be reduced to piperidine through a modified Birch reduction using sodium in ethanol (Marvel and Lazier, 1941). Industrial piperidine is manufactured by the

hydrogenation of pyridine, usually using a molybdenum disulfide catalyst (Figure 2) (Eller *et al*., 2002).

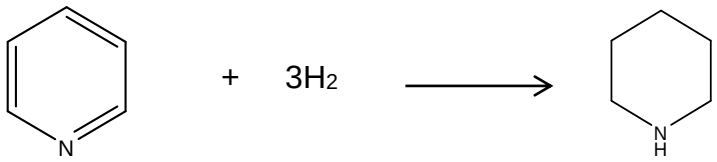


Figure 2 Chemical equation for the synthesis of piperidine by pyridine hydrogenation (Eller *et al.*, 2002).

Classical procedures namely microwave irradiation and ultrasound techniques are utilized in industry to intensify organic reactions. The approach of using coupling microwave, ultrasound and conventional methods have proved as viable routes for various types of reactions as there are many advantages, such as; less reaction time, reduction of side reactions, better yield and simplicity of products. Design and synthesis of restricted molecules is an important approach towards improving potency and selectivity (Bhaskar Reddy *et al*., 2005).

Many different synthetic methods have been used to construct piperidine analogues and these have been reviewed by Maxime in 2004. Popular synthesis techniques involving crystallization include; aza-Prins-cyclization, imino Diels-Alder reaction, tandem cyclopropane ring-opening cyclization, intramolecular Mannich reaction with iminium ions and intramolecular Michael reactions (Das and Brahmachari, 2013). These reactions are multistep processes that utilize expensive catalysts. The synthetic technique commonly utilized is multi component reaction (MCR) due to its one pot atom and step economical procedures.(Aeluri *et al.*, 2012).

According to literature, Multi Component Reactions (MCRs) (Figure 3) was used for the construction of piperidine derivatives starting from aromatic aldehydes, substituted anilines, β-keto esters and zirconium chloride ($ZnCl_2$) as the catalyst (Aeluri *et al*., 2012, Venugopala *et al*., 2012). Piperidine derivatives are also formed

by the reaction between acetone, ammonium acetate and substituted aromatic aldehyde in the presence of ethanol (Pandey and Chawla, 2012).

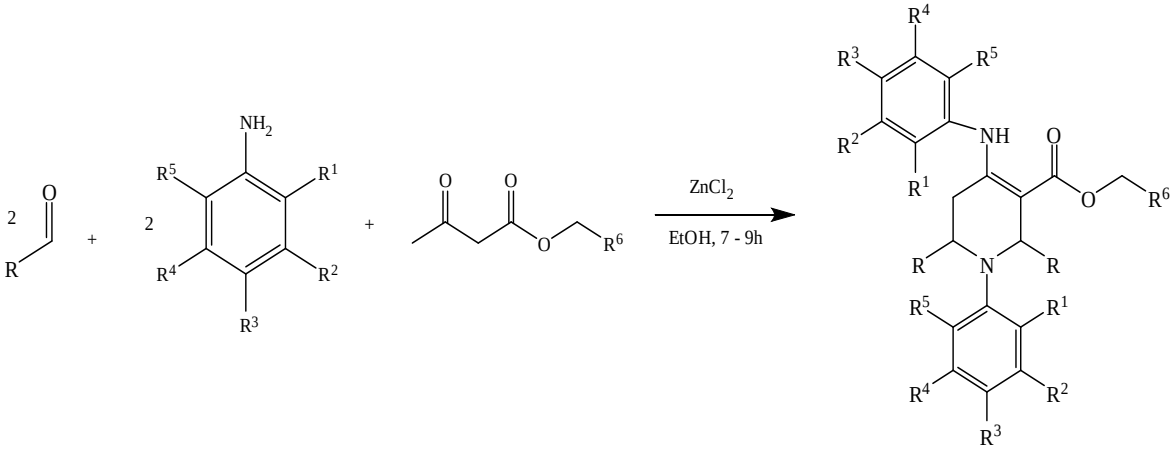
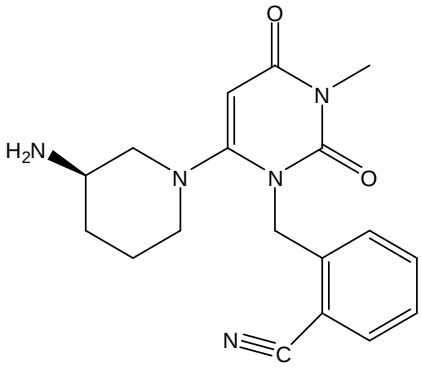


Figure 3 A general multicomponent reaction utilised for the synthesis of piperidine derivatives with zinc chloride as the catalyst (Das and Brahmachari, 2013).

2.6 Current pharmaceutical drugs with a piperidine nucleus

Alogliptin **(20)** and Risperidone **(21)** are pharmaceutically available drugs containing the piperidine nucleus. These are utilized for many functions including; the treatment of diabetes, improvement in children's concentration and in the treatment of schizophrenia. Compound CP-690550 **(22)** containing a piperidine nucleus that serves as a Janus kinase 3 (JAK3) which inhibits autoimmune disease and is used for transplant patients. Piperidine derivatives; Ritalin **(23)** and Risperidone are used in the treatment of neuropsychiatric disorders. GTS-21 is used for the treatment of Alzheimer's disease and Ro10-5824 is used to treat central nervous system disorders. Piperidine derivatives possess enzyme inhibitory activity against farnesyl transferase which is known to be involved in the development of progeria and various forms of cancers (Aeluri *et al*., 2012, Das and Brahmachari, 2013).



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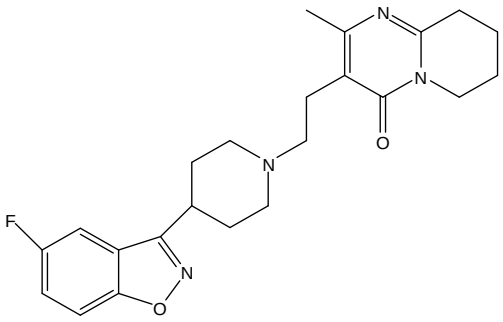

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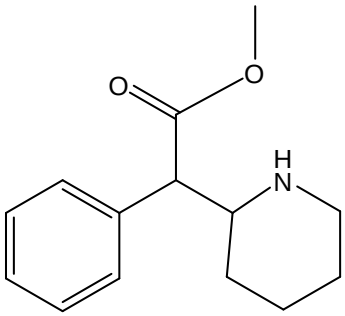

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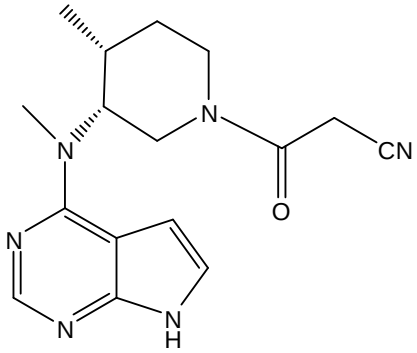


23

The first antibacterial agent containing nitrogen was nalidixic acid (Kiran *et al*., 2013). Fluoroquinolones are well recognized antibacterial compounds which are used in the medicinal industry (Huang *et al.*, 2010). Piperidine ring containing compounds that are used for the treatment of *Mycobacterium tuberculosis* are antihistamine drugs, Bamipine and Diphenylpyraline. These antihistamine drugs also exhibit H_1- receptor antagonistic activity (Thanusu *et al.*, 2010). The presences of the piperidine ring in compounds is of vital importance in the health care system as it is used for the treatment of kidney stones, peptic ulcers and pyelonephritis. Piperidine derivatives were found to oppose urease inhibition activity (Khan *et al*., 2006).

2.7 Some side effects associated with piperidine derivatives

Piperidine alkaloids are acutely toxic to adult livestock species and produce musculoskeletal deformities in neonatal animals. These teratogenic effects include multiple congenital contracture (MCC) deformities and cleft palate in cattle, pigs,

sheep, and goats. Poisonous plants containing teratogenic piperidine alkaloids include poison hemlock (*Conium maculatum*), lupine (*Lupinus spp.),* and tobacco (*Nicotiana tabacum*) and the wild tree tobacco (*Nicotiana glauca*). There is abundant epidemiological evidence in humans that link maternal tobacco use with a high incidence of oral clefting in new-borns, this association may be partly attributable to the presence of piperidine alkaloids in tobacco products. Piperidine alkaloids from poisonous plants have provided insights into the mechanisms underlying multiple congenital (MC) defects and cerebral palsy (CP) in livestock and humans. They accumulate in foetal blood and act at foetal nicotinic acetylcholine receptors, which may be more susceptible to them than adult receptors. Their teratogenic actions are hypothesized to involve persistent foetal nicotinic acetylcholine receptor desensitization; leading to an inhibition of foetal movements. Through this proposed mechanism, livestock teratogens such as anabasine could also carry teratogenic risks in humans due to potential exposure from tobacco consumption and/or tobacco replacement therapy (Abate *et al*., 1998).

2.8 Biological activity of piperidine derivatives

The piperidine ring is often used as a template for biologically active compounds (Alexidis *et al*., 1996) as it demonstrates a wide range of pharmacological activities (Venugopala *et al.*, 2012). Pharmacological activities include; anti-acetylcholinesterase activity (Sorrentino and Capasso, 1971), anti-inflammatory (Sugimoto *et al.*, 1990), Histamine H1 Antagonists (Fonquerna *et al*., 2005), antidiabetic and for the treatment of obesity (Haider *et al*., 2014)**.** Piperidine derivatives have been reported to have antipyretic, analgesic, insecticidal, anti-inflammatory, immune-modulatory, antitumor and antidepressant activities (Venugopala *et al*., 2012). Some piperidines have been used clinically as an effective anticonvulsant, exhibiting more potent effects than other piperidine compounds such as piperine (Balamurgan, 2012). Piperidines have shown significant pharmacological results against HIV and in anti-cancer therapy. Two deoxynojirimycin derivatives have already found clinical applications in treatment of type-II diabetes and Gaucher's disease. Alkaloids containing the piperidine nucleus exhibited a promising wide range of biological activities which include antimicrobial (Dorman and Deans, 2000), anti-parasitic, antimutagenic, antioxidant, radical

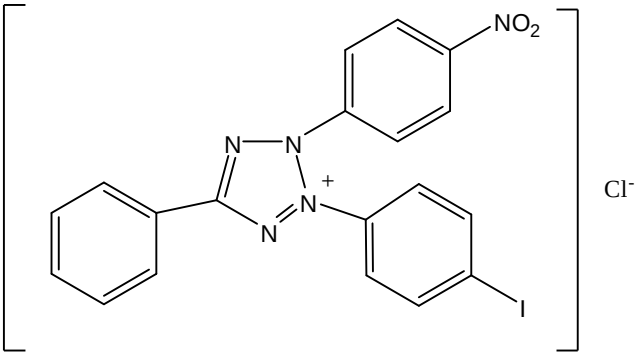
scavenging property (Gulcin, 2005) cytotoxicity, anti-inflammatory, pesticidal and anti-HIV-1 properties (Venugopala *et al*., 2012). The inhalation of black pepper oil containing natural piperidine compounds increase the involuntary swallowing movement (Vijayakumar *et al*., 2004). Piperidine alkaloids occur in both plants and humans. The black pepper fruit contains 1% volatile oil, resin, a waxy alkaloid. It is used for several medicinal properties. The piperidine heterocyclic ring occupies a place in the annals of human (Benedict, *et al*., 2012). The pharmacological action includes cytotoxic (Balamurgan, 2012), antifungal, anti-inflammatory, antioxidant and anticancer effect and it is known to have insecticidal activity against mosquitoes and flies (Atal *et al*., 1985).

2.8.1 Antimicrobial activity

Antibiotic resistance is a serious medical predicament affecting the healthcare industry globally. This situation has resulted due to the major decline in the research and development of new drugs retaining pharmacological properties (Theuretzbacher, 2013, Ashbolt, 2004). The increased development of resistant and multi-resistant bacterial strains worldwide is placing a significant burden on healthcare and society (Hawkey, 2008). Therefore the development of novel, efficient and inexpensive drugs is of vital importance.

The protocols used for the detection of antimicrobial activity are classified into two basic groups, namely, diffusion and dilution (Eloff, 1998). The third more defined method utilized is the Minimum Inhibition Concentration (MIC) assay technique. It detects microbial growth as a change in the electrical conductivity of the growth medium. The Minimum Inhibition Concentration (MIC) was one assay that is conducted using micro plate broth dilution technique. MIC is defined as the lowest concentration of an antimicrobial drug that kills all organisms. In broth dilution assay the microorganisms are tested for their ability to produce visible growth on a series of suitable agar plates or broth dilutions containing microbial agent being inoculated into micro plates (Langfield *et al.*, 2004). The MIC values are determined using growth indicators; white-yellow colour change in wells of the micro-plate is an indication of microorganism inhibition and pink-red colour change indicates growth of microorganism. The colour change was made possible by the addition of *p*-iodonitrotetrazolium violet (INT) reagent **(24)** to the micro-plate after 16 hours of

incubation. The INT mechanism is based on the transfer of electrons from NADH, a product of threonine dehydrogenase (TDH) catalyzed reaction, to the tetrazolium dye (INT). Threonine dehydrogenase (TDH) from microorganisms catalyzes the NAD-dependent oxidation of threonine to form 2-amino-3-ketobutyrate and NADH. During growth of microorganisms an electron is transferred from NADH to INT resulting in formazan dye which is purple in colour. Therefore clearing zones on the micro plates wells are an indication of microbial inhibition (Sumthong, 2000).



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Antimicrobial potency is demonstrated by many synthesised compounds. Amongst these compounds are the piperidine derivatives. Piperidine derivatives have been reported in the literature as antibacterial and antifungal active agents (Girgis, 2009). Piperidine exhibited significant activities against Gram-positive and Gram negative micro-organisms (Berggren *et al*., 2012, Zhou *et al.*, 2008). The activities of piperidine compounds were more potent than those of gemifloxacin, linezolid, and vancomycin *(*Rafiq *et al*., 2013*)*. Piperidine derivatives which bear anoxime substituent and a substituted amino substituent in the piperidine ring have been synthesized and coupled with naphthyridine acid to produce a series of novel fluoroquinolone derivatives (Chai *et al.*, 2010).

Piperidine derivatives, in combination with ciprofloxacin significantly reduced the MICs and mutation prevention concentration of ciprofloxacin for *Staphylococcus aureus,* including methicillin-resistant *S. aureus* (Dorman and Deans, 2000). The enhanced accumulation and decreased efflux of ethidium bromide in the wild-type

and mutant (CIPr-1) strains in the presence of piperidine analogues suggest its involvement in the inhibition of bacterial efflux pumps (Dorman and Deans, 2000).

Piperidines act as aminoglycoside mimetics that inhibit bacterial growth. *Pseudomonas aeruginosa* has been identified as a particularly problematic pathogen. It is an invasive, gram-negative bacterium that causes a wide range of severe infections including; acquired pneumonias, bloodstream infections, surgical wound infections and infections of immune compromised patients. In cystic fibrosis patients the *Pseudomonas aeruginosa* incites inflammation that destroys lung tissue and leads to respiratory failure. Compounding the problem of causing life-threatening infections, *P. aeruginosa* has a strong propensity to develop resistance to virtually any antibiotic. Some researchers believe that there is a decrease in antibacterial activity of piperidine in the presence of serum (Reshmi *et al*., 2010).

Carrie and Kozyrskyj (2006) have identified that numerous piperidine derivatives that were synthesized by substitutions on the phenyl group of the piperidine ring structure have higher antibacterial activity than commercial available antibiotics such as gemifloxcin and vancomycin. Piperidine derivatives comprising a flouro group as a substitution on the piperidine nucleus demonstrate pronounced antibacterial activity (Haider *et al*., 2014). Antibacterial inhibition activity is increased if the electron donating groups as well as the electron withdrawing groups present on the piperidine ring (Premalatha *et al*., 2013). The positioning of methyl and ethyl groups at position 3 of the piperidine ring structure is an influencing factor of great antibacterial potential (Premalatha *et al*., 2013). Piperidine compounds containing hydroxy, methyl, and nitro substitutions on the phenyl ring revealed significant inhibition against the bacterial species tested resulting in tremendous antibacterial potential (Ravindernath and Reddy, 2013). It has been shown that piperidine derivatives containing a fluoro substituent on phenyl group attached to a piperidine ring structure exhibit great inhibition activity against *Candida albicans (*Rafiq *et al*., 2013*)*. Fluoroquinolone is an antibacterial agent having substituted piperidine rings at the C-7 position. This compound is the most attractive drug in the anti-infective chemotherapy field. These antibiotics exert their effect by inhibition of two type II bacterial topoisomerase enzymes, DNA gyrase and topoisomerase IV (Chai *et al*., 2010).

2.8.2 Anticancer activity

Cancer has become a predominant killer worldwide, thus the search for newer treatment methods continues to grow. Although drugs can be beneficial in some aspects of health care, they may also possess toxic or mutagenic properties (Abate *et al.*, 1998). The (4,5-Dimethylthiazol-2-yl-) -2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay was used to evaluate the cell proliferation inhibition in the presence of the test sample (Hanelt *et al*., 1994). This technique may be considered as a quick and inexpensive method for the cytotoxic evaluation of the compounds on cancer cell lines.

2.8.2.1 Principle of Cell viability and Cytotoxicity Assay

Cell viability and cytotoxicity assays are used for the screening of drugs and testing their toxic effects. Various reagents are utilized for the detection of viable cells. Cell viability is based on various cell functions such as enzyme activity, cell membrane permeability, cell adherence, ATP production, co-enzyme production, and nucleotide uptake activity. Many methods have been established for counting and standardizing the number of live healthy cells such as Colony Formation, Crystal Violet, Tritium-Labeled Thymidine and Trypan Blue method (Aerluri *et al*., 2012).

MTT is regarded as a reductive coloring reagent and dehydrogenase to determine cell viability and cytotoxicity with a colorimetric method. This method is regarded as a better method when compared to the previously mentioned methods. This is because it is easy-to-use, safe, has a high reproducibility, and is widely used in both cell viability and cytotoxicity studies. Amongst the enzyme-based assays, the MTT assay is acknowledged as the best known method for determining mitochondrial dehydrogenase activities in the living cells. MTT assay depends on mitochondrial activity and not the cell itself. In the method, MTT is reduced to a purple formazan by NADH. However, MTT formazan is insoluble in water, and it forms purple needle-shaped crystals in the cells (Aerluri *et al.*, 2012). Therefore prior to measuring the absorbance, an organic solvent (DMSO) is required to solubilize the crystals.

Piperidine derivatives containing chloro substitutions of the phenyl group, which increases cytotoxic levels of the compound (Aerluri *et al.*, 2012). According to

literature (Aerluri *et al*., 2012) piperidine 1,2,5,6,tetrahydropyridine-3- carboxylate displays effects of toxicity against A549 (human epithelial lung carcinoma), DU145 (human prostate cancer), HeLa (human epithelial cervical cancer and SK-N-SH (human neuroblastoma) cell line and this may have resulted from the chloro substitution of phenyl group. 3-hydroxy-2,6-dialkyl-piperidine displays cytotoxic effects and inhibitory activity towards 2,2-diphenyl-1-picrylhydrazyl (DPPH), 5-lipoperoxidase and cycloxiygenase-1 and 2 (Melo *et al*., 2014).

The presence of a methoxy and chloro substituents on the phenyl group in piperidine derivatives is believed to increases the cytotoxic properties (Aerluri *et al*., 2012). The presence of a fluoro group on positions C2 and C6 of the piperidine ring structure increases toxicity activity (Jahan *et al*., 2013). Piperidine derivatives, 2-hydroxymethyl-1-[(4-bromo-phenyl)-2-oxoethyl]-piperidinium bromide and 2-hydroxymethyl-1-[(4-flouro-phenyl)-2-oxoethyl]-piperidinium bromide exhibited high toxicity levels due to the compounds existing as bromo and fluoro derivatives respectively (Jahan *et al*., 2013). Studies show that piperidine structures have proved to be potentially valuable lead compounds for the design of potent and selective P450 inhibitors and for non-toxic cytoprotective agents as well. Piperidine was found to be a most cytotoxic compound causing extensive lactate dehydrogenase (LDH) leakage and glutathione (GSH) depletion after four hours of incubation in a carbon dioxide incubator. None of the piperidine compounds increased lipid peroxidation levels (Abate *et al.*, 1998). Piperidine ring caused a significant change on the biological activity profile. Some piperidine compounds remained negative in the cytotoxicity tests and are proved to be inhibitors of P450 isoforms.

Studies have shown that piperidine structures have proven to be valuable lead compounds for the design of selective and extremely potent P450 inhibitors and for non-toxic cytoprotective agents as well (Abate *et al.*, 1998).

Piperidines have been tested as growth inhibitors against Ehrlich ascites tumor cells (EAC) in Swiss albino mice and were found to exhibit remarkable growth inhibitor activities. The *in vitro* anticancer activity of piperidine was tested against the serous ovarian cancer ascites, OV 90 cell in comparison to *cis*-platin. The complex

exhibited the highest growth inhibitor activity with mean IC_{50} value of 43.13 µM. It is generally accepted that binding of *cis*-platin to genomic DNA (gDNA) in the cell nucleus is the main event responsible for its antitumor properties. Piperidine compounds reveal considerable anti-tumor properties against colon (HCT-116), breast (T-47D), leukemia [HL-60 (TB), MOLT-4, RPMI-8226] and prostate (PC-3) cancers (El-Deen *et al*., 2013).

2.8.3 Anti-TB activity

Piperine, an analogue of piperidine is used as an inhibitor of Rv1258c of *Mycobacterium tuberculosis*. Rifampicin in combination with piperine, a trans-trans isomer of 1-piperoyl-piperidine, reduced the MIC and mutation prevention concentration (MPC) of rifampicin for *M. tuberculosis* H37Rv, including multidrug-resistant *M. tuberculosis* and clinical isolates. Moreover, piperine effectively enhanced the bactericidal activity of rifampicin in time-kill studies and also significantly extended its post-antibiotic effect. In the presence of rifampicin, *M. tuberculosis* rifampicin-resistant mutant showed a 3.6-fold overexpression of the Rv1258c gene. The 3D structure of Rv1258c protein , using in silicon modelling, was analysed to elucidate the binding of piperine to the active site. Piperine's involvement in the inhibition of clinically over expressed mycobacterial putative efflux protein (Rv1258c) was beneficial. Piperine may be useful in augmenting the anti-mycobacterial activity of rifampicin in a clinical setting. Previous work, demonstrated piperine and its analogues to be inhibitors of the NorA efflux pump of *S. aureus* (Sharma *et al.*, 2010).

2.8.4 Analgesic activity

Analgesic activity is observed in a compound having methyl function at the para-position with the dosage level of 50, 75 and 100 mg/kg. The incorporation of methyl group could be proven to increase the efficacy of the methyl function, thus the activity of which can be compared with that of N-methyl function on the piperidine nucleus. However when the methyl group is displaced from the nitrogen atom of the piperidine to that of phenacyl function is resulted in decrease in analgesic activity. Difference in activity can be attributed to the electron added or removed from the

aromatic ring or the side chains, which is expected to produce hindrance to interact with the receptor at the electronic level (Ahmed, 2012).

2.8.5 Antioxidant activity

Numerous compounds are known to display antioxidant properties for the scavenging of free radicals. The latter introduces a series of chain reactions causing oxidative tissue damage and a comprehensive range of degenerative diseases for example premature aging, diabetes, cancer and some cardiovascular diseases (Lindsey *et al*., 2002).

Recently piperidine derivatives were also shown to possess strong antioxidant properties measured as inhibition of lipid peroxidation, hydroxyl radical scavenging activity, and interaction with the stable free radical, diphenyl-/3-picrylhydrazyl (DPPH) (Alexidis *et al.*, 1996). The use of DPPH in antioxidant assays of piperidines are well documented probably because of their rapid reaction, reliability and independence of sample polarity (Prashanth *et al*., 2012). Antioxidant activity of piperidine compounds is related to their electron or hydrogen radical releasing ability to DPPH so that they become stable diamagnetic molecules. This might be the reason for the higher antioxidant activity of these compounds. It is noted that the concentration of the piperidine derivatives and the percentage of scavenging capacities are directly proportional as shown by Ravindernath and Reddy (2013). The coupling of 2,6-dimethylpiperidine showed much better activity than the 4-methylpiperidine. According to literature piperidine derivative thiotetrahydropyridine exhibited 1.70% - 22.39% (Prachayasittikul *et al*., 2009) antioxidant activity, which is extremely low. This difference of results could have originated from the nucleus of thiotetrahydropyridine containing 1,2,3,4 tetrahydropyridine. Compounds; methyl6-(2-hydroxyphenyl)-2-phenyl-1-(2-sulfanyl -1*H*-benzo[d]imidazol-5-yl)-4-[(2-sulfanyl-1*H*-benzo[d]imidazol-6-yl)amino]-1,2,5,6-tetrahydro-3-pyridinecarboxylate, methyl 6-(2-methoxyphenyl)-2-phenyl-1-(2-sulfanyl-1*H*-benzo[d]imidazol-5-yl)-4-[(2-sulfanyl-1*H*-benzo[d]imidazol-6-yl)amino]-1,2,5,6-tetrahydro-3-pyridinecarboxylate and methyl 6-(4-nitrophenyl) -2-phenyl-1-(2-sulfanyl-1*H*-benzo[d]imidazol-5-yl)-4-[(2-sulfanyl-1*H*-benzo[d]imidazol-6-yl)amino]-1,2,5,6-tetrahydro-3-pyridinecarboxylate displays high antioxidant activity due to the

substitution of hydroxyl, methoxy and nitro moieties on piperidine ring or benzene ring respectively (Ravindernath and Reddy, 2013).

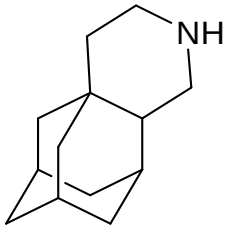
Piperidine derivative, methyl 6-(2-methoxyphenyl)-2-phenyl-1-(2-sulfanyl-1*H*-benzo[d]imidazol-5-yl)-4-[(2-sulfanyl-1*H*-benzo[d]imidazol-6-yl)amino]-1,2,5,6-tetra hydro-3-pyridinecarboxylate compound containing the methoxy substitution on the piperidine ring exhibited IC_{50} values between 4.2 µM (Ravindernath and Reddy, 2013). In 2013 a group of scientists also stated that compounds containing the piperidine skeleton, for example, 2,6-dipheny-l-3-alkylpiperidin-4-one-O-[2,4,6-tritertiarybutyl-cyclohexa-2,5-dienon-4-yl]oximes which comprises of a high content of alkyl groups, which demonstrates good antioxidant activity (Premalatha *et al*., 2013).

The wide variations in free radical scavenging activities may be due to the variations in the proton-electron transfer by the compounds due to difference in their structures and stability. An insight in to the structure–activity relationship gives an idea that activity generally increases with number and strength of electron donating groups (Prashanth *et al*., 2012).

2.8.6 Anti-influenza activity

In the face of the persistent threat of human influenza A (H3N2, H1N1) and B infections, the outbreaks of avian influenza (H5N1) in Southeast Asia, and the potential of a new human or avian influenza A variant to unleash a pandemic, there is much concern about the shortage in both the number and supply of effective anti-influenza virus agents. It is noted that piperidine compounds that bear the pharmacophore group of rimantadine and a six-membered ring carbon skeleton in the vicinity of the adamantane moiety, for example, piperidine derivative, 4-azatetracyclo[7.3.1.17,11.02,7]tetradecane **(25)** demonstrates an increased anti-influenza A virus activity and excellent selectivity. The rigid carbon framework in the piperidines fits better than a free rotating group into a lipophilic pocket in the M2 receptor. Piperidine compounds are inactive against influenza B virus, which is in accordance with their putative mode of action, namely interaction with the influenza A virus M2 protein, which is different in influenza B virus. Piperidines were also tested for their trypanocidal activity, but none had significant activity against

Trypanosoma brucei. Piperidine compounds retain their potency against influenza A virus. A study indicates that large and extended lipophilic moieties in the vicinity of adamantane skeleton are compatible with biological activity and suggests that there is a complementary acceptor group/site within the lumen of the M2 channel pore. It was also noted that by moving the amine nitrogen atom from the 2-adamantyl carbon enhances activity. Non-substituted piperidine compounds are more active, with N-alkylation causing a clear reduction in potency and selectivity. The N-substitution of the parent amines with other alkyl substituents has been reported and it is evident that the small size of the N-alkyl group enhances the activity of the respective compounds against influenza A/H3N2 strains (Zoidis *et al*., 2009).



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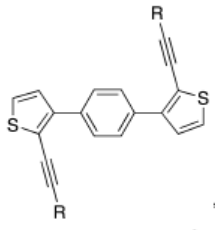
2.8.7 Anti-tumor activity

Piperidine compounds reveal considerable anti-tumor properties against colon (HCT-116), breast (T-47D), leukemia [HL-60 (TB), MOLT-4, RPMI-8226] and prostate (PC-3) cancers. Piperidine compounds, at a dose of 10 µM were tested on human tumor cell lines, representing leukemia, melanoma and cancers of the lung, colon, brain, ovary, breast, prostate and kidney according to the previously reported standard procedure (Girgis, 2009). Piperidine compounds reflect mild activity against most of the tested human tumor cells. However, all the tested compounds reveal considerable anti-tumor properties against colon (HCT-116), breast (T-47D), leukemia [HL-60(TB), MOLT-4, RPMI-8226] and prostate (PC-3) cancers, considering 50% inhibition of cell growth at 10µM of drug. Structure activity relationships based on previous data explains that the substituent attached to the phenyl group oriented at the 40-position of the synthesized heterocyclic compounds plays an important role in developing the observed anti-tumor properties. It has been

noticed that, adoption of a methoxy function (an electron-donating group) as a substituent attached to the phenyl group at this position seems more favourable for constructing an anti-tumor active agent than in the case of using a chlorine residue (deactivating moiety). Also, adoption of a thienyl function **(26)** (which could be recognized as a bio-isostere of the phenyl group) at the 4-position of the constructed heterocycle seems to be a successful choice for designing an anti-tumor active agent (Girgis, 2009).



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2.8.8 Anti-inflammatory activity

Many synthesized compounds inhibit the development of pro-inflammatory signal molecule, for example, prostaglandins. Pro-inflammatory molecules are responsible for inflammation and the cause of pain. To evaluate the efficiency of a compound to relieve the effects of inflammation and pain the compound can be tested in an *in vitro* assay (White and Glass, 1994). This assay measures the degree of inhibition of the cyclooxygenase enzyme active in prostaglandin synthesis. Cyclooxygenase exist in two isoforms, namely. COX-1 and COX-2. Compounds that display positive results towards COX-1 and COX-2 are therefore considered beneficial and used therapeutically (Mantri and Witiak, 1994).

The 5-lipoxgenase activities of the novel compounds were determined using a common technique (Evan, 1987, Baylac and Kline, 1972, Alitonou *et al*., 2006) . The 5-lipoxgenase assay kit was purchased from Sigma chemical company. Buffer used was 0.1 M potassium phosphate at pH 9. Linoleic acid was used as the substrate for the 5-lipoxgenase enzyme. Linoleic acid was enzymatically converted to conjugated dienes resulting in an increase in the absorbance at 490-500 nm. Absorbance was plotted graphically against the different concentrations used. The slopes of the straight line of the tested samples and the control curves were used to

determine the percentage activity of the enzyme. Nordihydroguaiaretic acid (NDGA), a known inhibitor of soybean lipoxgenase was used as the positive control (Lourens *et al*., 2004).

It was reported by Girgis (2009) that the anti-inflammatory properties of the prepared piperidine compounds (at a dose of 50 mg/kg body weight) and assessed using *in vivo* acute carrageenan-induced paw oedema in rats. Exhibited considerable anti-inflammatory activity which was exhibited similar to indomethacin (reference standard) (at a dose of 10 mg/kg body weight). Piperidine compounds exhibit anti-inflammatory properties greater than indomethacin. It has also been noticed, that the anti-inflammatory activity for many of the constructed heterocyclic compounds is extended after twenty four hours of inflammation induction (Girgis, 2009).

The study of the piperidine structural activity relationships based on the observed results indicated, that the type of substituent attached to the phenyl group at the 4-position of the constructed heterocycles plays an important role in developing the total observed pharmacological properties. Generally, it has been found that by substituting the phenyl group with a halogen atom (either bromine, chlorine or fluorine) is associated with a decrease in the observed anti-inflammatory properties However, substitution of the phenyl group with a methoxy function ‘‘as a representative example of electron-donating residue’’ is associated with enhancement in the observed anti-inflammatory activity. However, no precise rule could be attained concerning the effect of methyl group attachment to the oxindolyl nitrogen on the observed anti-inflammatory properties (Girgis, 2009).

Compound 4-(4-Bromophenyl)-5-[(4-bromophenyl)methylene]-1,1-dimethyl-dispiro [3H-indole-3,2-pyrrolidine-3,3-piperidine]-2(1H),4-dione has been recorded in literature to have decreased anti-inflammatory activity due to the bromo substitution of the phenyl group (Girgis, 2009). Girgis (2009) also stated that compound 4-(4-chlorophenyl)-5-[(4-chlorophenyl) methylene] -1,1-dimethyl-dispiro[3H-indole-3,2-pyrrolidine-3,3-piperidine]-2(1H),4-dione displays reduced anti-inflammatory activity, which arises from chloro on the phenyl ring attached to the piperidine ring. Girgis in 2009 stated that the substitution of phenyl group with a methoxy group, in which the methoxy functions is a representative example of an electron-donating residue is associated with improvement in the observed anti-inflammatory activity.

2.9 Compounds used in this study

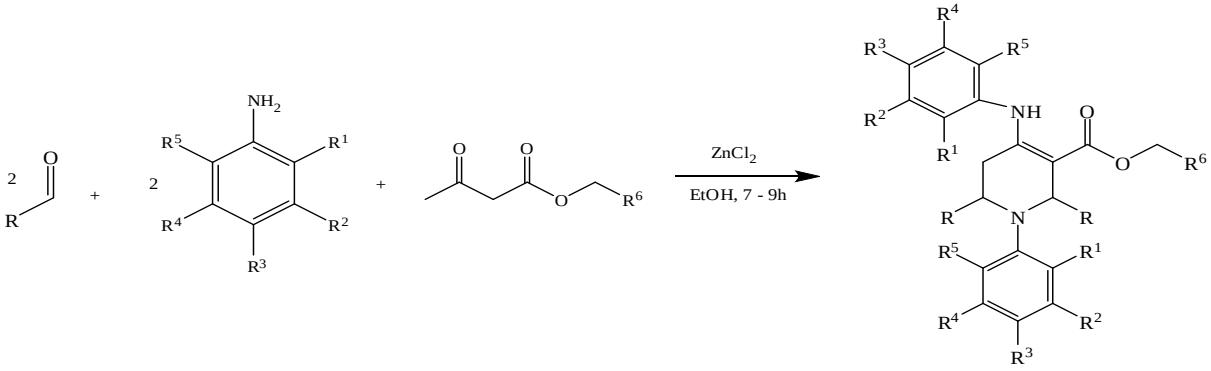
The six piperidine compounds in this study were synthesized according to a method reported by Venugopala *et al.*, (2012). Structures of compounds were confirmed by Infrared spectroscopy and melting points were in agreement with those reported in literature (Venugopala *et al.*, 2012*)*. Compound (**PM1**) Methyl 2,6-diphenyl-1-p-tolyl-4-(p-tolylamino)-1,2,5,6-tetrahydropyridine-3-carboxylate was previously synthesized by Venugopala *et al.*, (2012) which revealed a similar melting point of 495 K.

In this title compound, $C_{33}H_{32}N_2O_2$, the tetrahydropyridine ring adopts a boat conformation with the carbonyl group in an *s-cis* conformation with respect to the C=C bond of the six-membered tetrahydropyridine ring. The molecular conformation is stabilized by intramolecular N—H···O, C—H···O and C—H···π interactions. Formation of centrosymmetric head to- head dimers is observed through pairwise intermolecular N—H···O hydrogen bonds. Additional weak C—H···O and C—H··· interactions stabilize the three-dimensional molecular assembly (Venugopala *et al.*, 2012).

All chemicals used were obtained from Sigma and Merck chemical companies and used without further purification. The process of synthesis involved organic (basic, level 1) chemistry in which substituted aromatic amine (0.01 mol), substituted aromatic aldehyde (0.01 mol), methyl acetoacetate / ethyl acetoacetate (0.005 mmol), $ZnCl_2$ (0.01 mol) and ethanol (10 mL) were added to a 50 mL round bottom flask. This sample was stirred in a reflux condenser at room temperature to allow mixing of the sample for 7-9 hours. Reactions were monitored by TLC, TLC which was performed on Merck 60F-254 silica gel plates with ethyl acetate and *n*-hexane (3:2) as the solvent system and visualized using ultra violet (UV) light at 245 nm. The solid precipitate obtained was vacuum filtered and washed with aqueous ethanol to obtain a crude product. Suitable crystals for x-ray analysis were grown from a mixture of the compound in ACN: THF (V/V; 1:1) solvent by slow evaporation at room temperature. The percentage yield of the compounds was calculated by the actual yield divided by the expected yield multiplied by 100. The molar mass of compounds was determined by the sum of the atomic mass of the constituent

atoms, for example NaCl molar mass is calculated to the sum of the atomic mass of Na and atomic mass of Cl. Melting points of the compounds was determined by Thiele apparatus. Formation of the compounds was confirmed by LCMS (liquid chromatography mass spectrometry), an Agilent Technology 1200 series instrument. This synthetic methodology has been performed previously by Venugopala *et al*., (2012).

The synthesis process used for the development of the title compounds **PM1**-**PM6** is illustrated in Figure 4.



Substituents	**R**	**R^1**	**R^2**	**R^3**	**R^4**	**R^5**	**R^6**
Compound							
PM1	Phenyl	H	H	Me	H	H	H
PM2	4-Cyanophenyl	H	H	Me	H	H	H
PM3	Phenyl	H	H	Br	H	H	CH_3
PM4	Pyridyl	H	OCH_3	Br	H	H	CH_3
PM5	Pyridyl	H	CF_3	CH_3	H	H	CH_3
PM6	Pyridyl	F	H	Cl	H	I	CH_3

Figure 4 Schematic diagram illustrating the synthesis of piperidine derivatives (**PM1-PM6**).

CHAPTER 3: METHODOLOGY

3.1 Pharmacological activity

Due to the vast increase in drug resistance by many microorganisms of the treatment of biological infections the need for new compounds that are economically feasible and possess broad spectrum of activities have placed huge demands in the healthcare and pharmaceutical industries (Moges *et al*., 2014)

Six piperidine derivatives (**PM1-PM6)** which were previously synthesised and characterised by a post-doctoral fellow, Dr Venugopala (Venugopala *et al*., 2012) were investigated in this study for their pharmacological activities i.e. antibacterial, antifungal, antioxidant, anti-inflammatory, anticancer activities and for their brine shrimp lethality assays. All assays were conducted in triplicate.

3.1.1 Antimicrobial Activity

The antimicrobial potential (antibacterial and antifungal) of six piperidine derivatives was assessed using selected microbial strains, of which ten were bacterial species and seven fungal species. The bactericidal activity, fungicidal activity and the MIC (minimum inhibitory concentration) of the six piperidine derivatives using the agar disc diffusion method were evaluated (Cos *et al*., 2006, Eloff, 1998). All cultures were obtained from the Stock Collection Center at the Durban University of Technology which is housed in the Department of Biotechnology and Food Technology.

3.1.1.1 Antibacterial assay

The ten bacterial strains utilized were based on the standard recommendations and were obtained from the stock collection center situated at the Department of Biotechnology and Food Technology in Durban University of Technology. The bacterial species used were: *Bacillus cereus* (Figure 5a), *Bacillus subtilis* (Figure 5b), *Bacillus polymixa* (Figure 5c)*, Escherichia coli* (Figure 5d)*, Klebsiella pneumonia* (Figure 5e), *Micrococcus luteus* (Figure 5f), *Pseudomonas aeruginosa* (Figure 5g), *Staphylococcus aureus* (Figure 5h)*, Streptococcus faecalis* (Figure 5i)

and Serratia marcescens (Figure 5j). The microbial cultures were verified by their Gram reactions. Stock cultures were prepared from Culture Collection Centre and stored in micro banks vials in 50% glycerol. When required, the bacterial cultures were plated out on Nutrient Agar (Sigma) plates and grown in Nutrient Broth (Sigma) for 24 hours at 37°C. The concentration of the bacterial cells was adjusted to MacFarland Standard of 0.5 nm absorbance which corresponded to 10^8 CFU/mL. Below are some images of bacterial cultures grown at room temperature for 24 hours (Figure 5) (Eloff,1998).

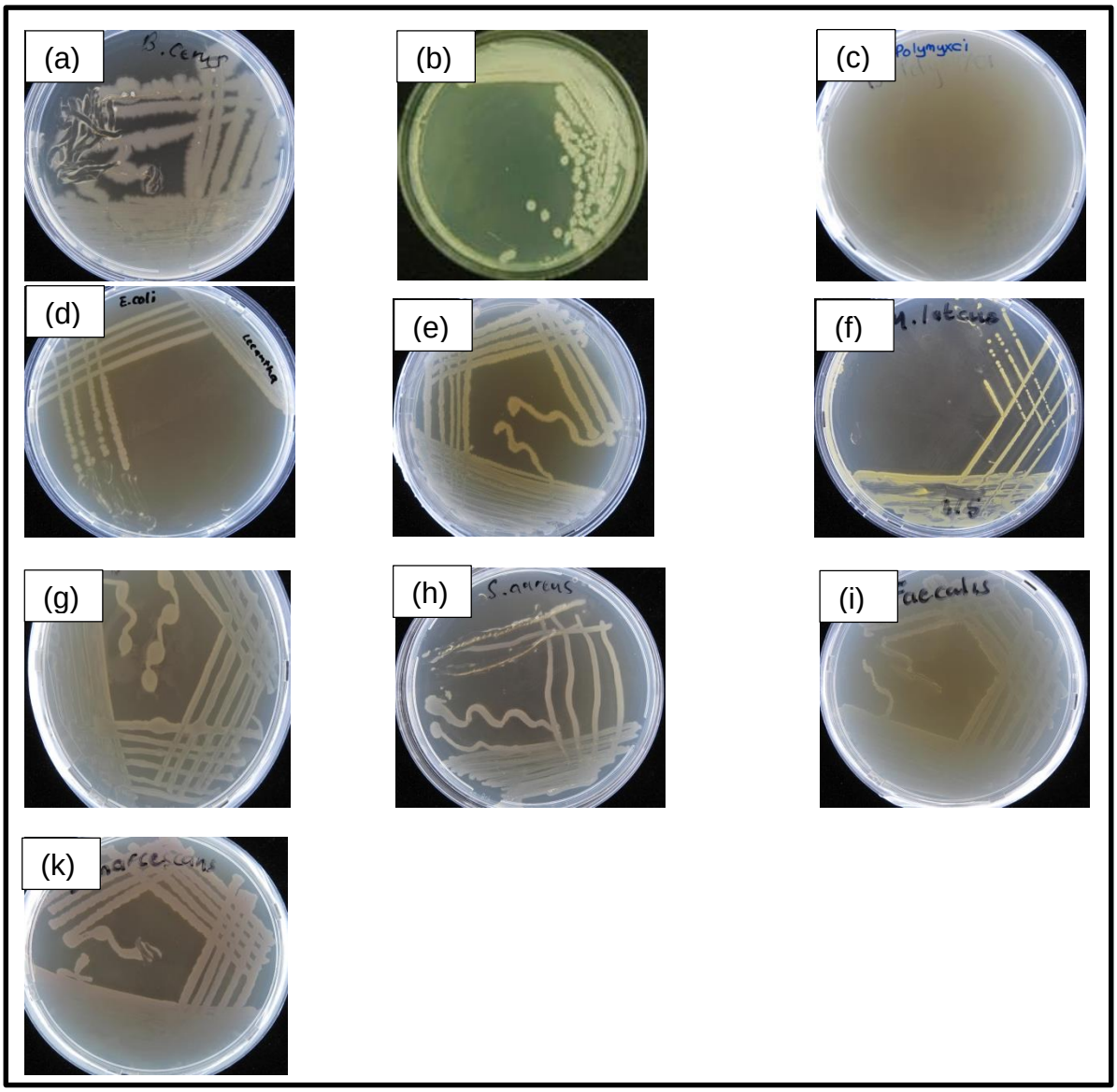


Figure 5 Images of bacterial cultures (a) *Bacillus cereus,* (b) *Bacillus subtilis,* (c) *Bacillus polymixa,* (d) *Escherichia coli,* (e) *Klebsiella pneumonia*, (f) *Micrococcus luteus*, (g) *Pseudomonas aeruginosa*, (h) *Staphylococcus aureus,* (i)

Streptococcus faecalis and (j) *Serratia marcescens*) grown at 37°C on Sabouraud dextrose agar plates (nutrient agar) after 1 day.

In a 250 mL volumetric flask 10 mL of molten sterile nutrient agar kept at 45°C was inoculated with 0.1 mL of 10^8 CFU/mL of each of the respective bacterial species. A suspension (1 mL of 10^8 CFU/mL) of each test bacteria was poured over the base of a 9 cm petri dish and spreaded using sterile stabs. The filter disks were prepared by cutting 5 mm disks from Whatman No. 1 filter paper which was further autoclaved to obtain sterility. These sterile discs were inoculated with 10 µL of 3 mg/mL of the test compound and then air dried in open sterile petri dishes in a biological safety laminar cabinet (Labtec Bioflow II, South Africa). The dried discs were then positioned onto the inoculated surface of bacterial or fungal agar plates and incubated at 37°C for 24 hours. Hundred percent Dimethyl sulphoxide (DMSO) at a concentration of 10 µL was used as the negative control and Ciprofloxacin (10 µL of 3 mg/mL) was used as the positive control. The antibacterial effects of the compounds were determined by measuring the diameter of clearing around the disks in mm. Each test compound was tested in triplicate.

3.1.1.1.1 Minimum inhibitory concentration assay

The Minimum Inhibition Concentration (MIC) is an assay that is conducted using micro plate broth dilution technique (Eloff, 1998). MIC is defined as the lowest concentration of an antimicrobial drug that kills all organisms. In broth dilution assay the microorganisms are tested for their ability to produce visible growth on a series of suitable agar plates or broth dilutions containing microbial agent being inoculated into micro plates (Langfield *et al.*, 2004). The MIC values were determined using growth indicators; white-yellow colour change in wells of the micro-plate is an indication of microorganism inhibition and pink-red colour change indicates growth of microorganism. The colour change was made possible by the addition of *p*-iodonitrotetrazolium violet (INT) reagent to the micro-plate after 16 hours of incubation (Sumthong, 2000).

The minimum inhibitory concentration (MIC) assay was carried out using a broth dilution technique (Eloff, 1998), which was monitored to determine the level at which the tested sample produced no inhibition towards the test organism. The test

compounds (six piperidine derivatives) were dissolved in DMSO and serially diluted 2 fold to obtain the following concentrations in mg/mL; 6, 3, 1.5, 0.75, 0.375, 0.187, 0.09 and 0.05. This experiment required the use of 96 well microtiter plates which contained twelve columns and eight rows. These wells were inoculated with 50 µL of sterile Mueller Hinton broth. In row A, 50 µL of 6mg/mL of test compound (PM1-PM6) which was dissolved in DMSO was introduced. After adequate mixing of the media and test compound (taking up and releasing three times) in row A, 50 µL were transferred into row B. This process of transferring was repeated until all rows were completed and in row H the 50 µL were discarded. Two columns were utilized as sterility controls (Mueller Hinton broth (MHB) and growth controls (MHB and test organism) respectively. After the additions of 50 µL of the respective test organism to each row excluding the sterility control the microtiter was sealed with parafilm and incubated at 37°C at 100% relative humidity for 24 hours. Post incubation; 50 µL of 0.2 mg/mL of INT (*p*-iodonitrotetrazolium violet) was added to all wells in the microtiter which was then returned to the incubator for a further 30 minutes to ensure the occurrence of adequate colour development. The *p*-iodonitrotetrazolium violet is a dehydrogenase detecting reagent which is converted into an intensely coloured formazan by metabolically active organisms (Navarro *et al.*, 1998). Positive MIC results were indicated by clear solution or a definite decrease in colour reaction. Ciprofloxacin (positive control) was made up of to a concentration of 1000 µg/mL.

3.1.1.2 Antifungal Activity

The antifungal assay was carried out using seven fungal cultures of which four were molds, namely, *Aspergillus flavus* (Figure 6a), *Aspergillus niger* (Figure 6b), *Pencillium digitatium* (Figure 6c) and *Fusarium verticilloides* (Figure 6d) and three yeast, namely, *Candida albicans* (Figure 6e), *Candida utilis* (Figure 6f) and *Saccharomyces cerevisiae* (Figure 6g).

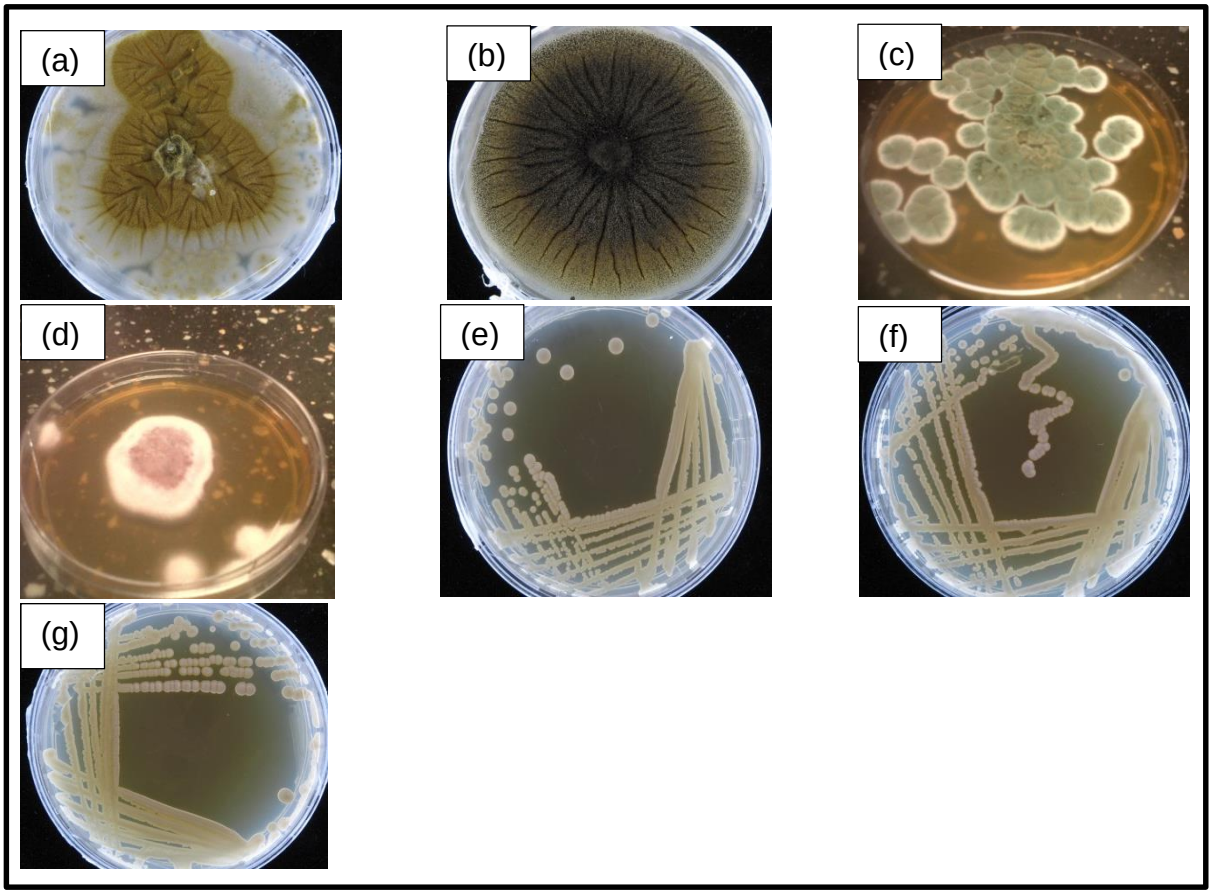


Figure 6 Images of fungal cultures (a) *Aspergillus flavus*, (b) *Aspergillus niger,* (c) *Pencillium digitatium, (*d) *Fusarium verticilloides, (*e) *Candida albicans*, (f) *Candida utilis* and (g) *Saccharomyces cerevisiae*.) grown at 27°C on Sabouraud dextrose agar plates (SDA) after 7 days.

The fungal strains were grown in Sabouraud dextrose broth at 28°C for 4-7 days. These fungi were inoculated onto Sabouraud Dextrose Agar (SDA) and incubated at 28°C for 4 to 7 days until sporulation occurred. The spores were collected in 10 mL sterile distilled water, counted in a Neubauer counting chamber and the concentration was adjusted to 10^6 spores/ mL. Ten microliters of distilled water containing the fungal spores (10^6 spores/ mL) was poured over the Sabouraud dextrose agar base plates (Biolab, Merck, South Africa). The filter disks were prepared by cutting 5 mm disks from Whatman No. 1 filter paper, autoclaved, inoculated with 10 µL of sample at a concentration of 3 mg/mL and dried in open sterile petri dishes in a biological safety cabinet (Labtec Bioflow II, South Africa). These were then inoculated onto SDA plates and incubated at 25°C for 24 hours.

DMSO (100%, 10 µL) was used as the negative control whilst Amphotericin B at 3 mg/mL (Sigma) was used as a positive control. Each test compound concentration was tested in triplicate and the results were determined by measuring the zones of inhibition in mm.

3.1.2 Antioxidant activity

The antioxidant properties of the six piperidine derivatives were tested using the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) photometric assay described by (*Choi et al.,* 2002*)*.

3.1.2.1 Preparation of the test compounds

The six test piperidine compounds were prepared in stock solutions (2000 µg/mL) by dissolving the compound in 100% DMSO and thereafter diluted to make up final concentrations of 1000, 800, 600, 400, 200 and 1 µg/mL in methanol. Solvent was prepared by the combination of methanol and 100% DMSO at a ratio 1:1. Rutin (Sigma-Aldrich R5143) which is located in the buckwheat plant *Fagopyrum esculentum* was used as the positive control (Yu *et al.*, 2005).

3.1.2.2 DPPH photometric Assay

The assay was performed by one millilitre of 0.3 mM DPPH (2, 2 diphenyl-2-picryl hydrate) in the solvent was added to 2.5 mL of different concentrations of the test compounds and this was left to react at room temperature for 30 minutes. The blank consisted of 1 mL of solvent and 1 mL of DPPH solution whilst the negative control consisted of 1 mL solvent plus. The positive control consisted of 1 mL of DPPH solution plus 2.5 mL of 1mM Quercitin-3-rutinoside (Rutin) (Sigma) which is a potent anti-oxidant. The radical scavenging activity was measured as the decolourization percentage of each test each compound. Each test was carried out in triplicate. The absorbance values were measured in a Varian Cary 1E UV-visible spectrophotometer at 518 nm and the average absorbance values were converted into the percentage antioxidant activity using the appropriate equation **(i)**.

$$\% \text{ Inhibition} = 100 - \frac{(\text{Abs of test sample} - \text{Abs of blank})}{(\text{Abs of negative control}} \times 100 \quad \text{---------- (i)}$$

3.1.3 5-Lipoxgenase activity

In order to conduct a lipoxygenase inhibitor screening assay one needs to understand how the assay works. It detects and measures the hydroperoxides produced in the lipoxygenase reaction using purified lipoxygenase. The detection reaction is equally sensitive to hydroperoxides at various positions with the fatty acids and will work with fatty acids of carbon length. It is therefore regarded as the general detection methods for lipoxygenase and can be used to screen test compounds to determine those that inhibit lipoxygenase enzymes.

The anti-inflammatory activities of the six piperidine derivatives were determined using a 5-Lipoxgenase assay technique (Evan, 1987, Baylac and Kline, 1972, Alitonou *et al*., 2006) . The 5-Lipoxgenase assay kit was purchased from Sigma chemical company. Linoleic acid was used as the substrate for the 5-Lipoxgenase enzyme whilst 0.1 M potassium phosphate at pH 9 was used as the buffer. Linoleic acid was enzymatically converted to conjugated dienes resulting in an increase in the absorbance at 490-500 nm. Absorbance was plotted graphically against the different concentrations used. The slopes of the straight line of the tested samples and the control curves were used to determine the percentage activity of the enzyme. Nordihydroguaiaretic acid (NDGA), a known inhibitor of soybean Lipoxgenase was used as the positive control (Lourens *et al*., 2004).

3.1.3.1 5- Lipoxgenase Assay

The assay protocol was performed according to a 5-Lipoxygenase inhibitory kit (Sigma). All chemicals in the kit was provided in liquid form. The 5- lipoxygenase assay was performed by; adding 100 µL of assay buffer to blank wells, followed by the addition of 90 µL 15-Lipoxgenase and 10 µL of assay buffer to the positive control wells, then adding 90 µL of Lipoxygenase enzyme and 10 µL of solvent to 100% initial activity wells and add 90 µl of Lipoxygenase enzyme and 10 µL of inhibitor (drug) to inhibitor wells. This addition of chemicals was followed by the addition of 10 µL of Linoleic acid to all wells. The 96 well plates was then placed on

a shaker for five minutes and thereafter the lid was removed and the absorbance was read at 490 nm using an Elisa plate reader.

3.1.4 *In vitro* Anticancer / Cytotoxicity assay

The cytotoxicity inhibition assay was determined using the MTT assay technique (Hanelt *et al.*, 1994). The principle of the MTT assay is to test the metabolic competence based upon the assessment of mitochondrial performance. The MTT assay is a colorimetric assay which relies on the conversion of yellow tetrazolium bromide (MTT) to the purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells (Mosmann, 1983).

3.1.4.1 Cell lines

In this study two cancer cell lines and one healthy cell line were used. The cancer cell lines utilized in this study were MCF7 (Figure 7a) and UCAA-62 (melanoma) (Figure 7b) which are breast cancer cells and skin cancer cells respectively. The normal cells (Figure 7c) utilized were mice fibroblasts, which was obtained from mice. The cells were received in an active state from the stock collection center at The Durban University of Technology in the Department of Biotechnology and Food technology. These cells were immediately incubated at 37°C in a humidified incubator (Snjiders Hepa, United Scientific group, Cape Town, South Africa) with 5% CO_2. When cells were 80% confluent, the cells were sub-cultured and stock cultures were stored at -70°C in a bio-freezer (Snijders Scientific, Holland) until required.

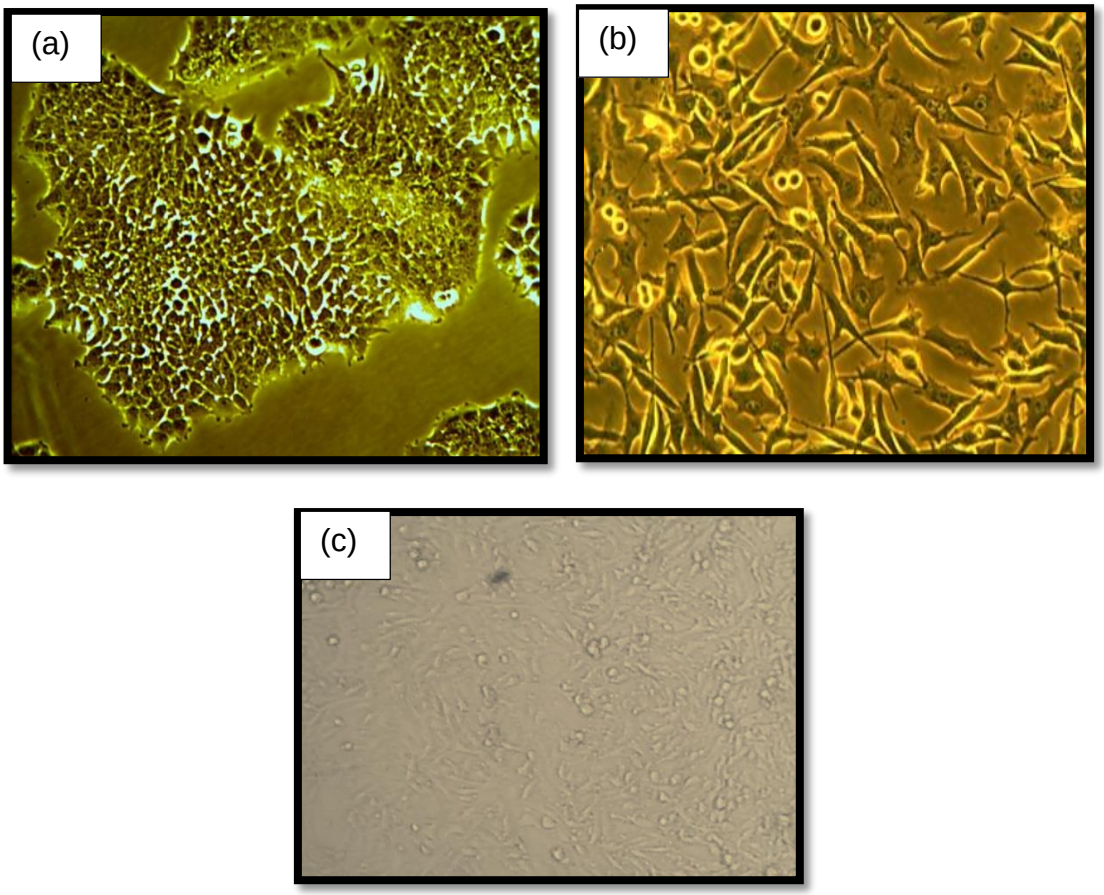


Figure 7 Microscopic observation of the morphology of MCF7 (a), UCAA-62 (Melanoma) (b) and Fibroblast (c) cells lines respectively. The monolayer shows spindle-shaped cells at 100X magnification using a light microscope.

3.1.4.2 Regeneration of cells

Regeneration of cells was accomplished by protocols obtained from literature (Freshney, 2005). The cells were removed from the bio-freezer (-70°C) and left to slowly thaw at 37°C. After thawing, the cells were transferred to 75 cm^2 tissue culture flasks containing 15 mL of fresh prepared 10% complete culture medium. Flasks containing the cells were then incubated at 37°C in a humidified incubator with 5% CO_2 atmosphere for 24 – 48 hours.

3.1.4.3 Storage of cells

Cells are very delicate and expensive to purchase. Cells may also become contaminated unintentionally therefore storage of cells is vital. Cells were stored

according to standard protocols (Freshney, 2005). After trypsinization the cells were pelleted and washed thrice with room temperature Phosphate Buffered Saline (PBS) (Sigma). The cells were then re-suspended in 0.5 mL foetal calf serum (FCS) and cooled on ice. A cryoprotective agent comprised of 20% dimethyl sulphoxide (DMSO) and Dulbecco's modified Eagle's medium (DMEM) (v/v 1:4 respectively) were prepared and stored on ice. The cryoprotective agent and cell suspension were added in equal amounts (0.5 mL) to a cyrotube (Corning, South Africa) and sealed with para-filmed. The tubes were transferred to the thermo flask and preserved overnight at -20°C. Thereafter cells were subsequently transferred to a -70°C bio-freezer and stored until required.

3.1.4.4 Cell Maintenance

Maintenance of cancer cells were accomplished according to standard protocols obtained from Freshney (2005). All cell culture procedures were carried out in a laminar flow hood. The laminar flow hood was sterilised by swabbing with 70% ethanol: 30% water (v/v) (Sigma) before and after use. This hood was fitted with a UV (245 nm) lamp (Scientific Engineering INC) used for sterilisation of experimental instruments before use. The cells were grown aseptically in 75 cm^2 tissue culture flasks using 0.22 mm sterile filters to filter sterilise Complete Culture Media which were Dulbecco's modified Eagle's medium (DMEM) or RPMI-1640 medium. DMEM and RPMI culture media were prepared in a 50 mL sterile centrifuge tube containing 10 mL foetal calf serum (FBS) and 1 mL Pen/strep. Cells were incubated in a humidified incubator at 5% CO_2 at 37°C. The culture flasks were monitored for colour changes and turbidity in media on a daily basis and observed under an inverted microscope (Nikon) for cell growth. All chemicals, media and flasks were purchased from Sigma and Merck chemical companies. When culture flasks were 80% confluent the cells were harvested and divided into two new flasks, then medium was added to the new flasks and incubated at 37°C in a humidified incubator.

3.1.4.5 Enumeration of cells

The cells were enumerated using a haemocytometer chamber. The cell suspension was mixed with equal volume aliquots of 0.2% Trypan Blue (v/v 1:1) to enable

stress-free observation and counting of cells without killing them. This mixture was spread across the grid by capillary action. The volume of cell suspension that occupied one primary square is 0.1 mm^3 (1.0 mm^2, 1.0 mm/ 1.0, 10^4 mL). Only the viable cells that lay within or touched the left and right boundary of the haemocytometer were counted. The number of viable cells per mL in a sample was calculated by a simple equation **(ii)** (Freshney, 2005).

Cells per mL = Average number of cells per primary square X 10^4 dilution factor **(ii)**

3.1.4.6 Preparation of compounds

All six piperidine compounds were dissolved in 2% DMSO to produce a stock concentration of 1 mg/mL. The stock solutions were further diluted to; 1000, 500, 250, 100, 50, 10 and 0 µg/mL.

3.1.4.7 Protocol for MTT Assay and % cell viability

The MTT cytotoxicity assay was performed according to a standard methodology (Mosamann, 1983) with minor modifications. This assay was carried out in 96 well flat bottomed microtiter plates (Sigma). Aliquots of 100 µL of 10 000 cells per mL of media were be added to 96 well flat bottomed microtiter plates. The plates containing the cells were incubated overnight ±17 hours at 37°C in a 5% CO_2 humidified incubator with to allow the cells to attach to the bottom of the wells. Post incubation treatment entailed removing 50 µL of cultured media from the wells and inoculating the wells with 50 µL of each concentration of the respective test compounds. Plates were returned to the humidified incubator for 20 hours. Thereafter 100 µL of 5 mg/mL MTT reagent was added to all wells and plates were incubated for a further 4 hours (Maduray, 2010). Post incubation, medium was removed and the insoluble formazan was extracted into DMSO (100%). Optical densities at 490 nm were measured on an ELISA plate reader (Digital Analogue System, Italy) with a reference wavelength of 630 nm. The negative control comprised of untreated cells in wells containing complete culture medium and DMSO. The percentage of cell viability **(iii)** and cytotoxicity **(iv)** of each test compound were calculated using the equation listed on the next page.

$$\% \text{ Cell viabilty} = \frac{\text{Abs of treated cells}}{\text{Abs of untreated cells}} \times 100 \quad \text{---------- (iii)}$$

$$\% \text{ Cytotoxcity} = 100 - \% \text{ cell viabilty} \times \frac{\text{Average abs of treated cells}}{\text{Average abs of untreated cells}} \quad \text{---------- (iv)}$$

3.1.5 Brine shrimp lethality assay

The toxicity of the piperidine derivatives was evaluated using brine shrimp lethality assay (Meyer *et al*., 1982) with minor modifications. This is a simple toxicity technique utilised to determine the toxic levels as well as the lethal concentrations of test sample in a medium containing brine shrimp.

3.1.5.1 Hatching the shrimp

Twenty five milligrams of *Artemia salina* (brine shrimp) eggs from Natures Petland in Durban South Africa were added to artificially prepared sea water (23 g NaCl, 11 g $MgCl_2.6H_2O$, 4 g Na_2SO_4, 1.3 g $CaCl_2.2H_2O$, 0.7 g KCl in 1000 mL distilled water) which was then stored at room temperature (27°C). The pH of artificial sea water was adjusted to 9.0 using 0.1M sodium carbonate (Na_2CO_3) to avoid risk of killing the *Artemia* larvae during incubation due to the decreased pH at room temperature. The *Artemia salina* eggs were incubated in a hatching chamber at room temperature for 24 hours. After 24 hours of incubation 15 mL of yeast solution was added to the chamber to feed the larva, 48 hours later the moving larvae were individually counted using counting chamber and placed in sterile petri plates.

3.1.5.2 Preparation of test compounds

The six piperidine derivatives were prepared in nine different dilutions; for example, 0, 10, 100, 200, 300, 400, 500, 800 and 1000 µg/mL in 100% DMSO. DMSO (100%) was used as a control. The test involved hatching the shrimp and then exposing the larvae to the test compounds.

3.1.5.3 Bioassay

This assay was conducted using 6 well microtiter plates. In each well ten brine shrimp larvae were added which was followed by the addition of 5 mL of artificial sea water and finally one drop of yeast solution (3 mg in 5 mL sea water). Each of six test compounds at different dilutions (0, 10, 100, 200, 300, 400, 500, 800 and 1000 µL) were inoculated into the respective wells. The 6 well microtitre plates were incubated at 27°C for 24 hours. 100% DMSO was used as a negative control. Post incubation, dead larvae were counted and the percentage death determined. The percentage death caused by the compound at respective doses was determined by a simple equation **(v)** (Meyer *et al*., 1982). Results obtained were expressed as mean ± standard deviation values and IC_{50} values.

% Death = Number of dead shrimp /10 X 100 **(v)**

CHAPTER 4: RESULTS

The current study focused on the pharmacological activities i.e. antimicrobial, anti-oxidant, anti-inflammatory, anti-cancer and brine shrimp lethality activity of piperidine derivatives (**PM1**-**PM6**). No pharmacological work has been conducted on these six piperidine derivatives prior to this study. All experiments were carried out in triplicate, with the results analyzed accordingly. The physiochemical characteristics of the six piperidine derivatives (**PM1**-**PM6**) are depicted in Table 1.

Table 1 Physicochemical characteristics of piperidine derivatives **PM1**-**PM6**

Compound code	**Compound IUPAC name**	**Molecular formula**	**Molar mass**	**Yield (%)** [a, b]	**Melting point (°C)**		***c*Log*P*** [c]
					Reported	**Observed**	
PM1	Methyl 2,6-diphenyl-1-p-tolyl-4-(p-tolylamino)-1,2,5,6-tetrahydropyridine-3-carboxylate	$C_{33}H_{32}N_2O_2$	488	80	220-222 (Venugopala *et al.*, 2012)	220	8.4666
PM2	Methyl 2,6-bis(4-cyanophenyl)-1-p-tolyl-4-(p-tolylamino)-1,2,5,6-tetrahydropyridine-3-carboxylate	$C_{35}H_{30}N_4O_2$	538	75	214 (Venugopala *et al.*, 2012)	215	7.3326

PM3	Ethyl 1-(4-bromophenyl)-4-(4-bromophenylamino)-2,6-diphenyl-1,2,5,6-tetrahydropyridine-3-carboxylate	$C_{32}H_{28}Br_2N_2O_2$	632	85	NR	185	10.1968
PM4	Ethyl 1-(4-bromo-3-methoxyphenyl)-4-(4-bromo-3-methoxyphenylamino)-2,6-di(pyridin-3-yl)-1,2,5,6-tetrahydropyridine-3-carboxylate	$C_{32}H_{30}Br_2N_4O_4$	692	81	NR	200	6.7653
PM5	Ethyl 1-(4-methyl-3-(trifluoromethyl)phenyl)-4-(4-methyl-3-(trifluoromethyl)phenylamino)-2,6-di(pyridin-3-yl)-1,2,5,6-tetrahydropyridine-3-carboxylate	$C_{34}H_{30}F_6N_4O_2$	640	79	NR	208	8.5957

PM6	Ethyl 1-(4-chloro-2-fluoro-6-iodophenyl)-4-(4-chloro-2-fluoro-6-iodophenylamino)-2,6-di(pyridin-3-yl)-1,2,5,6-tetrahydropyridine-3-carboxylate	$C_{30}H_{22}Cl_2F_2I_2N_4O_2$	831	65	NR	226	9.6583

Notes:

[a] All the products were characterized by IR spectra, and physical constants (melting point and cLog*P*),

[b] Yields after purification by column chromatography,

[c] *c*Log*P* was calculated using ChemBioDraw Ultra 13.0v

Abbreviation: PM, piperidine derivative; NR not reported

This table shows the physicochemical characteristic of **PM1**-**PM6**. Each of the compounds differs from their substituted aromatic amine, substituted aromatic aldehyde and methyl acetoacetate/ ethyl acetoacetate. The melting points of **PM1** and **PM6** were similar to those previously reported literature. Compounds PM3-PM6 melting points were not previously reported in literature. The melting points for **PM1**- **PM6** ranged from 220°C to 185°C. The yields achieved for the compounds were satisfactory. I.R. peaks (Appendix C) for, N-H ranged from a high of 3257 cm^{-1} to a low of 3234 cm^{-1}, ArC-H ranged from a 3082 – 3026 cm^{-1}, C=O ranged from 1662 – 1650 cm^{-1}, C=C ranged from 1444 – 1488 cm^{-1} and C-N ranged from 1336 – 1311 cm^{-1}.

4.1 Antimicrobial Activity

4.1.1 Antibacterial Activity

The piperidine derivatives were tested for *in vitro* antibacterial activity against ten bacterial species which included Gram positive and Gram negative species. All data collected were expressed as mean of three samples and are expressed as zones of inhibition in mm of the average of three replicates ± standard deviation (Table 2). The piperidine derivatives were tested at a concentration of 3 mg/mL on bacteria at a concentration of 10^8 CFU (colony forming units) using the agar disk diffusion technique (Eloff, 1998). Of the compounds screened those compounds revealing ≥5 mm inhibition zones and againstspecific bacterial species were further tested for minimum inhibition concentration (MIC) utilizing a standard broth dilution technique (Eloff, 1996). The minimum inhibition concentrations of each compound was evaluated at eight different concentrations; 6, 3, 1.5, 0.75, 0.375, 0.1875, 0.0093, and 0.0045 mg/mL. All results obtained were compared to Ciprofloxacin, a standard antibacterial drug. The antibacterial activity of piperidine derivatives and Ciprofloxacin is shown in Table 2.

Table 2 Antibacterial activity of piperidine derivatives (**PM1**-**PM6**) at 3 mg/mL

Compounds code	Zones of inhibition (mm) [a, b, c]									
	Bacterial species									
	B.c	***E.c***	***Kl***	***S.a***	***S.m***	***B.p***	***B.s***	***M.l***	***P.a***	***S.f***
PM1	5	5	5	5	n/a	n/a	5	6	n/a	n/a
PM2	6	6	4	6	n/a	n/a	7	6	6	n/a
PM3	5	5	2	5	n/a	n/a	5	3	1	n/a
PM4	4	3	4	5	n/a	n/a	3	5	5	n/a
PM5	5	5	4	5	n/a	n/a	6	5	5.5	n/a
PM6	5	5	4	5	n/a	n/a	5	5	6	n/a
Control [d]	11	11	11	11	11	11	11	11	11	11

The data collected for **PM1**-**PM6** is represented as mean values, standard deviation =0, number of sample (n=3), n/a – (No activity),

[a] Negative control (DMSO) – No activity,

[b] Concentration 3 mg/mL,

[c] Abbreviations for the bacterial species (Bacterial species are abbreviated as follows; *B.cereus (B.c), E.coli (E.c), Kl.pneumoniae (Kl), S.aureus (S.a), S.marcescens (S.m), B.polymixa (B.p), B.subtilis (B.s), M.luteus (M.l), P.aurenginosa (P.a)* and *S.faecalis, (S.f),*

[d] Ciprofloxacin (positive control).

The results obtained are summarized as zones of inhibition in Table 2. Ciprofloxacin (positive control) displayed 11 mm inhibition zones against all bacterial species tested. The six piperidine derivatives inhibited the four Gram positive bacteria (*B.cereus, B.subtilis, M.luteus* and *S.aureus*) and three Gram negative bacteria (*E.coli, Kl.pneumoniae* and *P.aurenginosa*). None of the piperidine derivatives demonstrated any inhibitory activity towards *S.faecalis and B.polymixa* (Gram positive bacteria) and *S.marcescens* (Gram negative bacteria).

B.cereus, S.aureus, and *B.subtilis* were inhibited by all piperidine derivatives (**PM1, PM2, PM3, PM4, PM5** and **PM6**). *E.coli* was inhibited by **PM1, PM2, PM3, PM5** and **PM6** while *Kl. pneumonia* was inhibited by **PM2, PM4, PM5** and **PM6**. *M.luteus* was inhibited by piperidine derivatives **PM1, PM2, PM4, PM5** and **PM6**.

The least effective piperidine derivative was **PM4** which displayed moderate zones of inhibition (5 mm) towards *B.subtilis, S.aureus* and *P.aurenginosa.* The strongest inhibitory activity was demonstrated by compound **PM2** which inhibited *B.cereus, E.coli, S.aureus, B.subtilis, P.aurenginosa, Kl.pneumoniae and M.luteus* with ≥6 mm zones of inhibition. Piperidine derivative PM1 demonstrated antibacterial inhibitory activity towards *B.cereus, S.aureus, B.subtilus M.luteus* (Gram positive) and. *E.coli* (Gram negative) with inhibition zones ranging from 5 mm towards *B.cereus, S.aureus, B.subtilis* and 5mm and 6mm towards *E.coli* respectively. **PM1** exhibits better antibacterial potency towards Gram positive than Gram negative bacteria.

Piperidine derivative **PM3** revealed antibacterial activity towards three Gram positive bacteria species, i.e. *B.cereus, S.aureus, B.subtilis* and only one Gram negative bacterial species *E.coli*. Bacterial species *B.cereus*, *S.aureus, B.subtilis* and *E.coli* were inhibited by **PM3** by 5 mm. This shows that **PM3** exhibits better antibacterial potency towards Gram positive than Gram negative bacteria and as **PM3** inhibits two more Gram positive bacteria than Gram negative bacteria. Piperidine derivative **PM5** inhibited four Gram positives bacteria species, i.e. *B.cereus, B.subtilis, M.luteus, S.aureus* and two Gram negative bacterial species *E.coli,* and *P.aurenginosa*. **PM5** revealed inhibition zones of 5 mm *B.cereus* and *S.aureus* but *B.subtilis* revealed 6 mm inhibition zones towards Gram positive bacterial species whereas Gram negative bacterial species i.e. *E.coli, M.luteus and P.aurenginosa* revealed a constant zone of inhibition of 5 mm. Piperidine compound

PM6 presented similar antibacterial activity as **PM5** towards four Gram positives bacteria species, namely, *B.cereus, S.aureus, B.subtilis, M.luteus* and three Gram negative bacterial species *E.coli* and *P.aurenginosa*. **PM6** revealed inhibition zones of 5 mm towards *B.cereus, S.aureus, B.subtilis, E.coli and M.luteus* while *P.aurenginosa* revealed a 6 mm inhibition zones. Overall this indicates that **PM5** and **PM6** presented greater antibacterial potency towards Gram positive than Gram negative bacteria.

Piperidine derivatives displaying positive inhibitory effects of 5 mm and greater were evaluated for MIC (lowest concentrations of the tested compounds that exhibited inhibition towards bacterial growth) using the broth dilution microplate technique against the respective bacterial species as explained in the methodology. The MIC's results are presented in Table 3. Amongst the six compounds, compound **PM2** exhibited the lowest MIC results against the seven bacteria tested in comparison to the other piperidine derivatives (**PM1**-**PM6**). **PM2** displayed a MIC of 750 µg/mL against *B.subtilis.* Inhibition activity of 1500 µg/mL was shown against *B.cereus, E.coli, S.aureus, P.aurenginosa, Kl.pneumoniae and M.luteus.* Piperidine derivative **PM1** revealed a MIC of 1500 µg/mL against *E.coli, M.luteus, S.aureus, B.cereus* and *B.subtilis* (Table 3). Compound **PM3** displayed a MIC at 1500 µg/mL (Table 3) against *E.coli, B.cereus, S.aureus and B.subtilis*. Compound **PM4** displayed a MIC at 1500 µg/mL towards *B.subtilis, S.aureus* and *M.luteus*. **PM5** produced a MIC at 1500 µg/mL against *E.coli*, *M.luteus, S.aureus, B.subtilis, P.aeruginosa* and *B.cereus.* PM6 (Table 3) was 1500 µg/mL against *B.cereus, M.luteus, S.aureus, B.subtilis, E.coli and P.aeruginosa* whilst Ciprofloxacin displayed the best MIC at 7 µg/mL against all bacteria tested.

Table 3 Minimum Inhibition Concentration **(**MIC) of piperidine derivatives (**PM1**-**PM6**) using broth dilution technique

	Minimum Inhibition Concentration (µg/mL) [a]						
	Bacterial species						
Compounds code	***B.cereus***	***E.coli***	***Kl.pneumoniae***	***S.aureus***	***B.subtilis***	***M.luteus***	***P.aurenginosa***
PM1	1500	1500	n/a	1500	1500	1500	n/a
PM2	1500	1500	1500	1500	750	1500	1500
PM3	1500	1500	n/a	1500	1500	n/a	n/a
PM4	n/a	n/a	n/a	1500	1500	1500	n/a
PM5	1500	1500	n/a	1500	1500	1500	1500
PM6	1500	1500	n/a	1500	1500	1500	1500
Control [b]	7	7	7	7	7	7	7

The data collected for **PM1**-**PM6** is represented as mean values, standard deviation =0, number of sample (n=3), n/a (No activity),

[a] Negative control (DMSO) – No activity,

[b] Ciprofloxacin (Positive control).

4.1.2 Antifungal Activity

The *in vitro* antifungal activities of the six piperidine derivatives, **PM1**-**PM6** were evaluated against seven fungal species, namely, *Candida albicans*, *Candida utilis*, *Saccharomyces cerevisiae*, *Penicillum digitatium, Fursarium verticilliodes*, *Aspergillus flavus* and *Aspergillus niger*. The results are summarised in Table 4.

Table 4 Antifungal activity of piperidine derivatives (**PM1**-**PM6**) at 3 mg/mL

Compounds code	Zones of inhibition (mm) [a, b]						
	Fungal species						
	A.niger	***A.flavus***	***F.verticilliodes***	***P.digitatium***	***C.utilis***	***C.albicans***	***S.cerevisiae***
PM1	5	5	n/a	n/a	n/a	5	2
PM2	5	5.5	n/a	n/a	n/a	5	2
PM3	n/a	n/a	n/a	n/a	n/a	n/a	n/a
PM4	n/a	n/a	n/a	n/a	n/a	n/a	n/a
PM5	5	n/a	n/a	n/a	n/a	6	5
PM6	5	n/a	n/a	n/a	n/a	6	5
Control [c]	7.5	7.5	7.5	7.5	7.5	7.5	7.5

The data collected for **PM1**-**PM6** is represented as mean values, standard deviation =0, number of sample (n=3), n/a – (No activity),

[a] Negative control (DMSO) – No activity,

[b] Concentration 3 mg/mL,

[c] Amphotericin B (positive control).

All six piperidine derivatives exhibited no inhibitory activity towards *Candida utilis*, *Penicillum digitatium and Fursarium verticilliodes*. Four of the six piperidine derivatives displayed varying degree of inhibition towards the rest of the fungal species (Table 4). Compounds **PM3** and **PM4** displayed no inhibitory activity towards all seven fungal strains. However compound **PM1** presented a moderate inhibition activity of 5 mm towards *A.niger, A.flavus, Saccharomyces cerevisiae* and *C. albicans*. **PM2** showed high antifungal activity of ≥5 mm inhibitory zones towards *A.niger, A.flavus, Saccharomyces cerevisiae and C.albicans.* Compounds **PM5** and **PM6** displayed ≥5 mm inhibition activities towards *A.niger and C.albicans*.

The antifungal activity results of the piperidine derivatives (Table 4) show that compounds **PM3** and **PM4** had no inhibition activity towards all the fungi under investigation. All compounds display no antifungal activity towards *Fursarium verticilliodes, Candida utilis* and *Penicillum digitatium.* **PM1** and **PM2** showed high antifungal activity against *A. niger, A flavus* and *C. albicans* whereas **PM5** and **PM6** showed high antifungal activity against *A. niger*, *C. albicans and S. cerevisiae.* Amphotericin B displayed the best antifungal activity against all the fungi tested.

4.2 Antioxidant activity

The antioxidant activities of six piperidine derivatives, **PM1**-**PM6** were determined using the stable DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging activity technique (Akula and Odhav, 2008) with the results depicted in Table 5. The results are displayed as mean ± standard deviation values with the IC_{50} values expressed in µg/mL. The significant difference between the control and the six compounds (**PM1**-**PM6**) tested was $p < 0.01$ which was determined by the use of One way Anova programme.

The results indicate that all of the piperidine derivatives had significant free radical-scavenging activities. These results demonstrate that the concentration of the piperidine derivatives tested and the percentage of scavenging capacity are directly proportional to each other.

Compound **PM4** showed the highest DPPH free radical scavenging capacity of 78% at 1000 µg/mL and **PM2** had the lowest percentage of scavenging capacity of 49% at 1000 µg/mL.

Overall, all the six piperidine derivatives (**PM1**-**PM6**) tested revealed antioxidant potentials greater than 50% at 1 mg/mL in comparison to the control Rutin which displayed a higher scavenging capacity of 97% at the same concentration. The IC_{50} values of all test compounds (**PM1**-**PM6**) ranged between 584.80 µg/mL and 959.69 µg/mL antioxidant activity. The IC_{50} value of Rutin was 464.68 µg/mL.

Piperidine derivative **PM4** revealed the highest antioxidant free radical potential of 78% and an IC_{50} value of 584.80 µg/mL. The lowest DPPH free radical scavenging activity of 49% was shown by **PM2,** which had the highest IC_{50} value of 959.68 µg/mL.

Compounds **PM1** and **PM3** displayed DPPH free radicals activities of 58 and 61% respectively. **PM1** exhibited an IC_{50} value of 838.93 µg/mL with **PM3** presenting an IC_{50} value of 730.99 µg/mL. Piperidine derivatives **PM5** and **PM6** displayed high DPPH free radical scavenging activities of 65% (IC_{50} value of 689.66 µg/mL) and 70% (IC_{50} value of 667.56 µg/mL) respectively.

Overall, all the six piperidine derivatives (**PM1**-**PM6**) displayed ≥49% antioxidant activity. This however was low in comparison to the reference standard Rutin, which exhibited an antioxidant activity of 97% at 1000 µg/mL.

This demonstrates that IC_{50} values are inversely proportional to the DPPH free radicals scavenging capacity. All IC_{50} values displayed by the piperidine derivatives (**PM1**-**PM6**) were much higher than Rutin, which exhibited an IC_{50} value of 464.68 µg/mL.

Table 5 DPPH free radical scavenging activity of piperidine compounds (**PM1**-**PM6**)

Compounds code	DPPH free radical scavenging activity (%) [a]							
	Concentration (µg/mL)							
	0	**200**	**400**	**600**	**800**	**1000**	**IC_{50} value**	**p. value**
PM1	0 ± 0.01	7 ± 0.01	29 ± 0	35 ± 0.01	49 ± 0	58 ± 0.01	838.93	0.0002***
PM2	0 ± 0.02	13 ± 0.02	26 ± 0.01	33 ± 0	41 ± 0	49 ± 0	959.69	0.0001***
PM3	0 ± 0.01	22 ± 0.02	36 ± 0	47 ± 0	53 ± 0	61 ± 0	730.99	0.0001***
PM4	0 ± 0.01	25 ± 0.01	48 ± 0	55 ± 0	66 ± 0.01	78 ± 0	584.8	0.0009***
PM5	0 ± 0.04	19 ± 0	38 ± 0	48 ± 0	56 ± 0	67 ± 0	689.66	0.0004***
PM6	0 ± 0.01	15 ± 0.01	35 ± 0.01	51 ± 0	59 ± 0	70 ± 0	667.56	0.0002***
CONTROL [b]	0 ± 0	22 ± 0	48 ± 0	77 ± 0	85 ± 0	97 ± 0	464.68	0.0003***

The data collected for **PM1**-**PM6** is represented as mean ± standard deviation values, number of samples (n=3),

[a] Negative control (DMSO) = 0,

[b] Rutin (positive control),

All values are significant with p value (two tailed)***= $p < 0.01$ (appendix).

4.3 Anti-inflammatory activity

The anti-inflammatory activities of the six piperidine derivatives (**PM1**-**PM6**) were evaluated utilizing a 5-lipoxgenase Inhibition assay kit (Sigma). The anti-inflammatory activities of the piperidine compounds were recorded at various different concentrations of 125, 250, 500, 1000, 2000 and 3000 µg/mL and then compared to the standard nordihydroguaiaretic acid (NDGA).

All results were expressed as mean ± standard deviation values with the IC_{50} values expressed in µg/mL. The significant difference between the control and the six compounds tested was determined by the one way Anova programme which displayed a significant p values < 0.05.

All six piperidine derivatives presented a certain degree of anti-inflammatory activity, ranging from 55% (**PM6**) to 76% (**PM4**) at a concentration of 3000 µg/mL (Table 6). In comparison to the six piperidine compounds tested; the highest anti-inflammatory activity was displayed by **PM4** whilst **PM6** displayed the lowest anti-inflammatory activity. The anti-inflammatory activity of 70 and 68% at 3000 µg/mL was revealed by piperidine derivatives **PM1** and **PM2** respectively. The other piperidine derivatives, namely, **PM3** and **PM5** displayed anti-inflammatory potency of 60 and 65% at 3000 µg/mL respectively. Overall all the six piperidine derivatives screened for anti-inflammatory activity exhibited good anti-inflammatory potential resulting in effective anti-inflammatory drugs. However, the test compounds revealed much lower anti-inflammatory activity at 3000 µg/mL than that of the NDGA which presented an anti-inflammatory activity of 88%.

These piperidine derivatives revealed anti-inflammatory activities with IC_{50} ranging from the highest (2251.62 µg/mL) for **PM6** to the lowest (1805.05 µg/mL) for **PM4**. Piperidine compound **PM1, PM2, PM3** and **PM5** exhibited anti-inflammatory activities of 68, 74, 60, and 65% at 3000 µg/mL respectively. These compounds showed IC_{50} values of 2049.18, 1824.82, 2358.49 and 2173.91 µg/mL respectively. The piperidine derivatives tested demonstrated low anti-inflammatory activity and high IC_{50} values in comparison to the reference standard nordihydroguaiaretic acid (NDGA) which displayed an IC_{50} value of 1547.99 µg/mL and 88% anti-inflammatory activity at 3000 µg/mL.

Table 6 Anti-inflammatory activity of six piperidine derivatives (**PM1**-**PM6**)

Compounds code	Anti-inflammatory activity (%) [a] Concentration (µg/mL)								
	0	**125**	**250**	**500**	**1000**	**2000**	**3000**	**IC $_{50}$ value**	**p. value**
PM1	0 ± 0.01	2 ± 0.11	22 ± 0	28 ± 0.02	34 ± 0.04	46 ± 0.02	68 ± 0.05	2049.18	0.0147**
PM2	0 ± 0.02	3 ± 0	15 ± 0.18	26 ± 0	47 ± 0.03	53 ± 0	74 ± 0.03	1824.82	0.0198**
PM3	0 ± 0.01	3 ± 0	16 ± 0.2	26 ± 0.02	29 ± 0.01	39 ± 0.04	60 ± 0	2358.49	0.0154**
PM4	0 ± 0	3 ± 0.01	16 ± 0.2	24 ± 0.02	44 ± 0.04	54 ± 0.04	76 ± 0.03	1805.05	0.0217**
PM5	0 ± 0.04	2 ± 0.07	14 ± 0.16	19 ± 0.02	34 ± 0.02	44 ± 0.07	65 ± 0.016	2173.91	0.0243**
PM6	0 ± 0.01	2 ± 0.01	19 ± 0.25	23 ± 0.01	32 ± 0.013	34 ± 0.011	55 ± 0.011	2551.02	0.0125**
CONTROL [b]	0 ± 0	12.5 ± 1	25.5 ± 1	35.4 ± 1	49.2 ± 1	61.5 ± 1	88.2 ± 1	1547.99	0.0094**

The data collected for **PM1**-**PM6** is presented as mean ± standard derivation values, number of sample (n=3),

[a] Negative control (DMSO) = 0.

[b] NDGA (Positive control),

All values are significant with p value (two tailed) **= $p < 0.05$ (appendix).

4.4 *In vitro* Anticancer Activity

The piperidine derivatives (**PM1**-**PM6**) were evaluated for their *in vitro* cytotoxicity using MTT assay technique (Hanelt *et al*., 1994). The derivatives were evaluated for their cytotoxic activity at 0, 10, 50, 100, 250, 500 and 1000 µg/mL on two cancer cell types and one normal untransformed mice cell type. The two cancer cell lines are, MCF7 and melanoma and the normal cells fibroblast. The cytotoxic activities of all six compounds (**PM1**-**PM6**) against MCF7, melanoma and fibroblast are summarized in Tables 7a, 7b and 7c respectively. Results are expressed as mean ± standard deviation values. The significant difference between the control and the six piperidine derivatives tested, were determined by one way Anova programme and the level of significance revealed was $p < 0.0001$. The cytotoxicity activities are expressed as percentage cell death and inhibition concentration (IC_{50}) is expressed in µg/mL units.

Table 7a and Table 7b demonstrates the cytotoxic activities of all six the piperidine derivatives tested. According to literature it test as not been carried out on these compounds. As it is evident in these tables, compounds **PM1, PM2, PM3, PM4, PM5** and **PM6** demonstrated high levels of toxicity against both cancer cell lines with cytotoxic activity ranging between 87 – 91% against MCF 7 cancer cells and between 71% to 95% against melanoma cancer cells at a concentration of 1000 µg/mL.

Analysis of the MTT assay suggests that high cytotoxic levels of the piperidine compounds were demonstrated by low IC_{50} values ranging between 415.63 and 488.28 µg/mL on MCF7 cancer cells (Table 7a) and between 438.60 and 612 µg/mL on melanoma cancer cells (Table 7b). Table 7c demonstrates that all the six piperidine derivatives (**PM1**-**PM6**) revealed between 50 – 51% cytotoxic activities at a concentration of 1000 µg/mL against the normal cells with high IC_{50} values ranging from 720.46 to 789.89 µg/mL.

Piperidine derivative **PM6** exhibited the highest cytotoxicity activity on both cancer cells with cytotoxic effect displayed of 91% at 1000 µg/mL with a low IC_{50} value of 415.63 µg/mL on MCF7 cancer cell lines and 95% at 1000 µg/mL with a low IC_{50} value of 438.60 µg/mL melanoma cancer cells. The least cytotoxic compound screened was **PM3** with a cytotoxic activity of 87 % at 1000 µg/mL with a high IC_{50}

value of 488.28 µg/mL on MCF7 cancer cells and 71% at 1000 µg/mL with an IC_{50} value of 612 µg/mL melanoma cancer cells. Piperidine derivative **PM1** exhibited a cytotoxic effect of 88% at 1000 µg/mL with an IC_{50} value of 477.10 µg/mL on MCF7 cancer cells and 88% at 1000 µg/mL with IC_{50} value of 494.10 µg/mL melanoma cancer cells. Compound **PM2** displayed cytotoxic activity of 88% at 1000 µg/mL with an IC_{50} value of 455.79 µg/mL on the MCF7 cancer cells and 89% at 1000 µg/mL with IC_{50} value of 491.20 µg/mL on melanoma cancer cells. **PM4** which displayed a moderate cytotoxicity activity of 90% at 1000 µg/mL with an IC_{50} value of 475.29 µg/mL on MCF7 cancer cells and 94% at 1000 µg/mL with IC_{50} of value 452.50 µg/mL on these melanoma cancer cell lines. Piperidine derivative **PM5** displayed a cytotoxic activity of 89% at 1000 µg/mL with an IC_{50} value of 466.15 µg/mL on MCF7 cancer cells and 92% at 1000 µg/mL with an IC_{50} value of 465.60 µg/mL on melanoma cancer cells.

Since all the six piperidine derivatives displayed high cytotoxic activities on both MCF7 and melanoma cancer cell lines, the toxicities of the derivatives were tested on fibroblast cells. All the piperidine derivatives demonstrated a moderate inhibition of ±50% cytotoxic effect on healthy cells with IC_{50} values ranging from 720.46 – 782.89 µg/mL at 1000 µg/mL. Compounds **PM4** and **PM6** revealed the highest cytotoxic effect of 52% at a concentration of 1000 µg/mL on fibroblast (normal) cells with **PM4** displaying the lowest IC_{50} value of 720.46 µg/mL at 1000 µg/mL and **PM6** revealed a slightly higher IC_{50} value of 736 µg/mL at 1000 µg/mL. **PM1** displayed 51% cytotoxicity activity at 1000 µg/mL with an IC_{50} value of 757.58 µg/mL. Piperidine derivative **PM5** also revealed a 50% cytotoxic effect against the healthy cells with an IC_{50} values of 753.01 µg/mL. Fifty percent cytotoxic activity was displayed by **PM2** and **PM3** at a concentration of 1000 µg/mL. The highest IC_{50} value of 789.89 µg/mL was revealed by piperidine compound **PM3** with **PM2** displaying the second highest IC_{50} value of 782.25 µg/mL.

Table 7a Cytotoxicity studies of piperidine derivatives (**PM1**-**PM6**) against MCF7 cancer cell line

Cytotoxicity activity (%) against MCF7 cancer cell line									
Compounds code	**Concentration (µg/mL)**								
	0	**10**	**50**	**100**	**250**	**500**	**1000**	**IC $_{50}$ value**	**p. value**
PM1	7 ± 0.06	6 ± 0	19 ± 0	33 ± 0.02	48 ± 0	69 ± 0	88 ± 0	477.10	< 0.0001
PM2	1 ± 0.02	2 ± 0	22 ± 0	45 ± 0	49 ± 0	79 ± 0	88 ± 0	455.79	< 0.0001
PM3	5 ± 0.06	1 ± 0	39 ± 0	45 ± 0.02	49 ± 0	60 ± 0	87 ± 0	488.28	< 0.0001
PM4	0 ± 0.11	1 ± 0	12 ± 0	21 ± 0.01	49 ± 0	69 ± 0	90 ± 0	475.29	< 0.0001
PM5	0 ± 0.02	11 ± 0	22 ± 0	34 ± 0.01	43 ± 0	75 ± 0	89 ± 0	466.85	< 0.0001
PM6	1 ± 0.02	1 ± 0	45 ± 0	67 ± 0.02	74 ± 0	82 ± 0	91 ± 0	415.63	< 0.0001
CONTROL [a]	1 ± 0.02	1 ± 0	0 ± 0	0 ± 0.3	0 ± 0	0 ± 0	0 ± 0	-	-

The data collected from **PM1**-**PM6** is presented as mean ± standard derivation values, number of sample (n=3),

[a] Positive control (DMSO) = 0,

All values are significant with p value (two tailed) ****= p < 0.0001 (appendix).

Table 7b Cytotoxicity studies of piperidine derivatives (**PM1**-**PM6**) against melanoma cancer cell line

Compounds code	Cytotoxicity activity (%) against melanoma cancer cell line								
	Concentration (µg/mL)								
	0	**10**	**50**	**100**	**250**	**500**	**1000**	**IC_{50} value**	**p. value**
PM1	0 ± 0	19 ± 0	25 ± 0	37 ± 0	42 ± 0	61 ± 0	88 ± 0	494.1	< 0.0001
PM2	0 ± 0	15 ± 0	25 ± 0	38 ± 0	43 ± 0	60 ± 0	89 ± 0	491.2	< 0.0001
PM3	0 ± 0	21 ± 0	28 ± 0	34 ± 0	41 ± 0	44 ± 0	71 ± 0	612	< 0.0001
PM4	0 ± 0	19 ± 0	31 ± 0	39 ± 0	45 ± 0	71 ± 0	94 ± 0	452.5	< 0.0001
PM5	0 ± 0	10 ± 0	28 ± 0	38 ± 0	44 ± 0	68 ± 0	92 ± 0	465.6	< 0.0001
PM6	0 ± 0	12 ± 0	36 ± 0	42 ± 0	48 ± 0	76 ± 0	95 ± 0	438.6	< 0.0001
CONTROL [a]	1 ± 0	1 ± 0	0 ± 0	0 ± 0.3	0 ± 0	0 ± 0	0 ± 0	-	-

The data collected from **PM1**-**PM6** is presented as mean ± standard derivation values, number of sample (n=3),

[a] Positive control (DMSO) = 0,

All values are significant with p value (two tailed) ****= p < 0.0001 (appendix).

Table 7c Cytotoxicity studies of six piperidine derivatives (**PM1**-**PM6**) against Fibroblast healthy cell line

Cytotoxicity activity (%) against fibroblast normal cell line									
Compounds code	**Concentration (µg/mL)**								
	0	**10**	**50**	**100**	**250**	**500**	**1000**	**IC $_{50}$ value**	**p. value**
PM1	0 ± 0.06	11 ± 0	20 ± 0	28 ± 0.02	38 ± 0	46 ± 0	51 ± 0	757.58	< 0.0001
PM2	0 ± 0.02	8 ± 0	18 ± 0	27 ± 0	36 ± 0	44 ± 0	50 ± 0	782.25	< 0.0001
PM3	0 ± 0.06	5 ± 0	15 ± 0	23 ± 0.02	35 ± 0	44 ± 0	50 ± 0	789.89	< 0.0001
PM4	0 ± 0.11	16 ± 0	22 ± 0	32 ± 0.01	44 ± 0	49 ± 0	52 ± 0	720.46	< 0.0001
PM5	0 ± 0.02	6 ± 0	17 ± 0	28 ± 0.01	37 ± 0	48 ± 0	51 ± 0	753.01	< 0.0001
PM6	0 ± 0.02	10 ± 0	20 ± 0	29 ± 0.02	40 ± 0	48 ± 0	52 ± 0	736.38	< 0.0001
CONTROL [a]	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0

The data collected from **PM1**-**PM6** is presented as mean values, ± standard derivation values, number of sample (n=3). [a] Positive control (DMSO) = 0. All values are significant with p value (two tailed) ****= p < 0.0001 and ***= p < 0.001 (appendix).

4.5 Brine shrimp lethality assay

The toxicity of the six piperidine derivatives was evaluated using brine shrimp lethality assay technique which is convenient for testing new compounds (Anna *et al.*, 2011, Meyer *et al.*, 1982). The compounds toxicity was evaluated at 0, 10, 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 µg/mL. The Brine shrimp lethality activity is expressed as percentage means ± standard deviation values with IC_{50} values expressed in µg/mL. The significant difference between the control and six compounds tested, were determined by the one way Anova programme and revealed a significance $p < 0.05$. These results are represented in Table 8. The six piperidine derivatives (**PM1**-**PM6**) were not toxic at the concentrations tested however low levels of toxicity were displayed at 300 µg/mL

Table 8 shows that of all piperidine derivatives (**PM1**-**PM6**) tested **PM5** and **PM6** demonstrated highest toxicity of 50% against the brine shrimp larvae at 1000 µg/mL. **PM1** and **PM2** showed the least toxic effect of 30% was. **PM3** and **PM4** displayed moderate toxicity of 40% against the brine shrimp larvae.

The IC_{50} values for **PM1, PM2, PM3, PM4, PM5** and **PM6** were; 1440.922, 1886.72, 1351.35, 1231.53, 1106.19 and 1106.19 µg/mL respectively.

Table 8 Toxicity of piperidine derivatives (**PM1**-**PM6**) against *Artemia salina*

Compounds code	Toxicity activity (%) against *Artemia salina*										
	Concentration (µg/mL)										
	0	**10**	**100**	**200**	**300**	**4000**	**500**	**800**	**1000**	**IC_{50} value**	**p. value**
PM1	0	0	0	0	10	10	10	20	30	1440.922	0.0353*
PM2	0	0	0	0	10	10	10	20	30	1886.72	0.353*
PM3	0	0	0	0	10	10	20	30	40	1351.35	0.0384*
PM4	0	0	0	0	10	10	20	40	40	1231.53	0.0424*
PM5	0	0	0	0	10	10	20	40	50	1106.19	0.0499*
PM6	0	0	0	0	10	10	20	40	50	1106.19	0.0499*
CONTROL [a]	0	0	0	0	0	0	0	0	0	-	-

The data collected from **PM1**-**PM6** is presented as mean values, standard deviation =0, number of sample (n=3),

[a] Positive control (DMSO) = 0,

All values are significant with p value (two tailed) *= $p < 0.05$.

CHAPTER 5: DISCUSSION

The discussion of results now follows.

5.1 Antimicrobial Activity

The six piperidine derivatives (**PM1**-**PM6**) were screened for *in vitro* antibacterial activity against ten bacterial species which include Gram positives and Gram negatives (Table 2). All results collected are expressed as mean ± standard deviation values with the results of the antibacterial and antifungal activities displayed as zones of inhibition (mm) which are presented in Table 2. It is more attractive to hypothesize that the observed results (Table 2) show that the six piperidine derivatives appear to be related to the nature of the substitutions on the piperidine moiety. These results indicate that all six piperidine derivatives (**PM1**-**PM6**) exhibited varying degree of potent inhibitory effect towards the four Gram positive bacterial species (*B.cereus, B.subtilis*, *M.luteus* and *S.aureus)* and three Gram negative bacterial species *(E.coli, Kl.pneumoniae and P.aurenginosa),* with exceptions to *S. faecalis* (Gram positive)*, S.marcescens* (Gram positive) *and B.polymixa* (Gram negative)*.*

The piperidine derivative that displayed the least antibacterial activity was **PM4**, which showed moderate inhibitory potential of 5 mm inhibition zones towards *B.subtilis, S.aureus* and *P.aurenginosa.* However piperidine derivative **PM2** exhibited the highest inhibition activity in comparison to the other piperidine derivatives (**PM1, PM3, PM4, PM5** and **PM6**). **PM2** revealed high inhibition effects towards seven bacterial species with ≥6 mm zones of inhibition activity towards *B.cereus, E.coli, S.aureus, B.subtilis, P.aurenginosa, Kl.pneumoniae and M.luteus*. Among the six piperidine derivatives screened, **PM2** demonstrated the lowest MIC against seven bacterial species tested in comparison to the other piperidine derivatives (**PM1, PM3, PM4, PM5** and **PM6**). **PM2** revealed an MIC of 1.5 mg/mL against *B.cereus, E.coli, S.aureus, P.aurenginosa, Kl.pneumoniae and M.luteus.* High inhibition was presented by **PM2** against *B.subtilis* with an MIC of 0.75 mg/mL. Piperidine derivative **PM1** displayed an MIC of 1.5 mg/mL against *E.coli, M.luteus, S.aureus, B.cereus* and *B.subtilis*. Piperidine derivative **PM3** presented an MIC of 1.5 mg/mL against *E.coli, B.cereus, S.aureus and B.subtilis*. Another piperidine

derivative tested was **PM4**, which produced an MIC of 1.5 mg/mL towards *B.subtilis, S.aureus* and *M.luteus*. **PM5** produced an MIC of 1.5 mg/mL against *E.coli*, *M.luteus, S.aureus, B.subtilis, P.aeruginosa* and *B.cereus.* The MIC of compound **PM6** was 1.5 mg/mL against *B.cereus, M.luteus, S.aureus, B.subtilis, E.coli and P.aeruginosa.* Piperidine derivatives comprising of, phenyl (**PM1** and **PM3**), 4-cyano phenyl (**PM2**), pyridyl (**PM4**, **PM5** and **PM6**) groups as substitutions on carbon 2 and carbon 6 of the piperidine ring and a methyl or ethyl group on carbon 3 of the piperidine ring displayed moderate growth inhibition. Both the electron donating as well as electron withdrawing groups present as substitutions on the piperidine ring enhanced the antibacterial activity.

These results indicate that all piperidine derivatives tested (**PM1**-**PM6**) display bactericidal properties, which affect specific Gram positive and Gram negative microorganisms. This statement is supported by previous studies which show that piperidine derivatives are known to respond well to a range of bacterial species including gram positive and gram negative and these piperidine derivatives are proven in science to be regarded as potent antibiotics (Berggren *et al*., 2012, Zhou *et al.*, 2008).

Our study indicates that the substitution of a cyano group at carbon 4 of the phenyl ring that's attached to carbons 2 and 6 on the piperidine ring (Table 1) demonstrated high antibacterial activity, but not to the same level obtained by Prachayasittikul *et al*., (2009) who claimed to obtain MIC's at 32 and 128 µg/mL for piperidine derivatives tested against selected bacterial species.

According to previous studies conducted by Ravindernath and Reddy (2013) piperidine derivatives containing hydroxy, methyl, and nitro substitutions on the phenyl ring demonstrates significant inhibition against the bacterial species tested resulting in potent antibacterial activity. This could be one of the reasons for piperidine derivative **PM2** displaying the greatest antibacterial activity in comparison to the other piperidine derivatives.

The electron donating groups as well as the electron withdrawing groups present on the piperidine ring enhance antibacterial inhibitory activity (Premalatha *et al.*, 2013).

Piperidine derivatives containing a flouro group substituted at position 2 on phenyl group that is attached to carbons 1 and 4 on the piperidine nucleus demonstrates good antibacterial activity. This could be the rationale behind piperidine derivatives **PM6** demonstrating good antibacterial activity. **PM5** also contains a flouro group substituted at position 3 of the phenyl group attached to carbon 1 and 4 of the piperidine ring.

PM1, PM2, PM5 and **PM6** displayed inhibitory activity towards specific bacterial species due to the methyl or ethyl group positioned at carbon3 of the piperidine ring. The positioning of methyl and ethyl groups at carbon 3 of the piperidine ring structure has has an influencing factor of high antibacterial activity (Premalatha *et al*., 2013).

Piperidine derivative **PM3** and **PM4** showed low inhibition activity in comparison to the other piperidine compounds tested. This could have resulted from the substitution of a bromo group at position 4 of a phenyl group attached to carbon 1 and 4 of the piperidine nucleus.

Carrie and Kozyrskyj (2006) have stated that numerous piperidine compounds have been synthesized with various substitutions on the phenyl group of the piperidine nucleus which have resulted in greater inhibition activity than antibacterial standards gemifloxcin and vancomycin.

A 2,6 di-substituted piperidine 4-one derivative demonstrated antimicrobial minimum inhibition concentration at 200 µg/mL towards *B.cereus, S.aureus, E.coli* and 100 µg/mL towards *A.niger* (Perumal *et al*., 2014). This thesis research achieved antibacterial inhibition towards the same and additional Gram positive and Gram negative bacterial strains and fungal strain but at a much high MIC of 1500 µg/mL. The reason behind this huge on C2 and C6 of the piperidine ring.

The *in vitro* antifungal activity of the six piperidine derivatives were evaluated against seven fungal species, namely, *Candida albicans*, *Candida utilis*, *Saccharomyces cerevisiae*, *Penicillum digitatium, Fursarium verticilliodes*, *Aspergillus flavus* and *Aspergillus niger*. It was observed (Table 4) that all six piperidine derivatives (**PM1**-**PM6**) displayed no inhibition activities towards *Candida utilis*, *Penicillum digitatium and Fursarium verticilliodes* and only four of the six piperidine derivatives (**PM1, PM2, PM5** and **PM6**) had shown varying degree of inhibition towards *Candida*

albicans, *Saccharomyces cerevisiae*, *Aspergillus flavus* and *Aspergillus niger* fungal species. Piperidine derivatives **PM3** and **PM4** demonstrated no inhibitory potential towards all seven fungal strains. However compound **PM1** presented moderate inhibition activity of 5 mm towards *A. niger, A. flavus, S. cerevisiae* and *C. albicans*. **PM2** displayed pronounced antifungal activity of ≥5 mm inhibition zones towards *A. niger, A. flavus, S cerevisiae and C. albicans.* Piperidine derivative **PM5** and **PM6** displayed ≥5 mm inhibition activity towards *A. niger and C. albicans*.

The antifungal activity results (Table 4) indicate that compounds **PM3** and **PM4** are significantly toxic toward all the fungi under investigation. This could have arisen from the substitution of a bromine group at position 4 on the phenyl group which is attached to carbon 1 and 4 of the piperidine ring. All piperidine derivatives tested were highly toxic displaying no inhibition activity towards *Fursarium verticilliodes, Candida utilis* and *Penicillum digitatium.* Piperidine derivatives **PM5** and **PM6** with the electron donating pyridyl group substituted at carbon 2 and carbon 6 on the piperidine ring are relatively good antifungal agents. However the degree of spore germination inhibition varied with the test compounds as well as with the fungi under investigation. The antifungal activities demonstrated by piperidine derivatives **PM1** and **PM2** were the best activity in comparison to the other piperidine derivatives tested. Overall the control Amphotericin B showed the greatest growth inhibition towards all fungi.

High antifungal activity shown by **PM1, PM2, PM5** and **PM6** could have resulted from the ethyl or methyl group being substituted at carbon 3 of the piperidine ring which is stated in literature to increase the antifungal activity of the compound (Premalatha *et al.,* 2013).

Rafiq *et al*., (2013) have proven that a derivative containing a fluoro substitution on amino (NH) group of a piperidine ring structure like piperidine compound **PM5** and **PM6** exhibit great inhibition activity against fungal specie *Candida albicans*.

Piperidine derivatives **PM3** and **PM4** did not exhibit any inhibitory potential which could have been influenced by the substitution of a bromine group at position 4 of the phenyl group, which is attached carbon 1 and carbon 4 of the piperidine ring.

The antimicrobial activities of the six piperidine derivatives (**PM1**-**PM6**) are depicted in Table 2, indicate that compounds **PM3** and **PM4** comprise of no inhibitory activity towards all seven fungal strains. The reason behind this ineffectiveness of piperidine derivatives **PM3** and **PM4** may be the inability of these compounds to weaken the mechanism of action in the fungi to allow for the compounds to exhibit antifungal activity.

5.2 Antioxidant activity

The antioxidant activities of the six piperidine derivatives were determined by the stable DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging activity technique (Akula and Odhav, 2008) with the results depicted in Table 5. The results of the percentage of antioxidant activity was expressed as mean ± standard deviation values with the significant difference between the values of same test ranging between $p < 0.001$ and $p < 0.01$ utilizing a One way Anova programme.

The results show that all six piperidine derivatives tested exhibited significant free radical-scavenging activities. This test indicates that the concentration of the piperidine derivatives and the percentage of scavenging capacities are directly proportional as shown by Ravindernath and Reddy in 2013.

Piperidine derivative **PM4** demonstrated the highest scavenging capacity of 78% at 1000 µg/mL and **PM2** demonstrated the least percentage of scavenging potential of 49% at 1000 µg/mL. Overall all piperidine derivatives tested revealed antioxidant potentials greater than 49% at 1 mg/mL whilst the control Rutin which displayed 97% scavenging capacity at the same concentration. The IC_{50} values of all piperidine derivatives, **PM1**-**PM6** ranged between 584.8 µg/mL to 959.69 µg/mL antioxidant activity. This states that a minimum of 584.8 µg of drug is required per mL to achieve 50% antioxidant activity.

Piperidine derivative **PM4** presented the highest antioxidant free radical scavenging potential, which may have resulted from the presence of a pyridyl substituent at positions 2 and 6 on the piperidine ring and a methoxy group at position 3 on the phenyl group that is attached at position 4 on the piperidine ring.

DPPH free radical scavenging activity was presented by **PM2** (50%). The low antioxidant activity of **PM2** may have developed from the substitution of a cyano

group at position 4 on the phenyl group attached to carbons 2 and 6 on the piperidine ring structure and a methyl group at position 4 on the phenyl group attached to nitrogen 1 on the piperidine ring. The low activity could not have resulted from the methyl group substituted at position 4 on the phenyl group attached to nitrogen 1 on the piperidine ring because compound **PM1** demonstrated high antioxidant activity with a methyl group substituted at position 4 on the phenyl group attached to nitrogen 1 on the piperidine ring.

The piperidine derivatives containing specific substitutions groups, namely, phenyl (**PM1** and **PM3**), cyano (**PM2**), pyridyl (**PM5** and **PM6**), which are substituted at positions 2 and 6 of the piperidine ring resulted in different levels of DPPH free radicals activity. The substitutions on the phenyl group with 4-Methyl-3-trifluoromethylphenyl and 4-chloro-2-flouro-4-iodophenyl respectively resulted in a decrease in activity (**PM5** and **PM6**).

Due to the other piperidine derivatives containing specific substitutions groups, namely, phenyl (**PM1** and **PM3**) substituted on C2 and C6 of the piperidine ring resulted in a low DPPH free radicals activity of 58 and 61% respectively. Due to the phenyl groups of piperidine derivatives **PM5** and **PM6** being substituted with 4-methyl-3-trifluoromethyl phenyl and 4-chloro-2-flouro-4-iodophenyl respectively has stemmed in a decrease in DPPH free radical scavenging potency of 65 and 70% respectively. Although **PM1, PM3, PM5** and **PM6** have not revealed the highest antioxidant activities it has demonstrated activities over 50% which can be used as antioxidant drugs. Overall all piperidine derivatives (**PM1**-**PM6**) screened displayed good antioxidant potencies but were extremely low in comparison to the reference standard Rutin, which presented an antioxidant activity of 99% at 1000 µg/mL.

Piperidine derivative **PM4** which displayed the highest DPPH free radical scavenging capacity revealed the lowest IC_{50} of 545.80 µg/mL while the lowest antioxidant activity piperidine derivative **PM2** displayed the highest IC_{50} of 959.69 µg/mL. Piperidine derivatives **PM1, PM3, PM5** and **PM6** revealed IC_{50} values of 838.93, 730.99, 689.66 and 667.56 µg/mL respectively. It may be concluded that IC_{50} values are inversely proportional to the DPPH free radicals scavenging capacity. All IC_{50} values displayed by the piperidine derivatives (**PM1**-**PM6**) were

much higher than reference standard Rutin, which exhibited an IC_{50} value of 464.68 µg/mL.

When electrons are added or removed from a compound side chain or nucleus (substitutions) this will result in the compound displaying enhanced or reduced biological activities, in this assay the addition or removal of side chains form the piperidine nucleus resulted in enhanced antioxidant activity. These results suggest that all the six piperidine derivatives tested exhibited significant antioxidant activity but not greater than the standard Rutin.

According to a previous report thiotetrahydropyridine exhibited 1.70-22.39% (Prachayasittikul *et al*., 2009) antioxidant activity which is extremely low in comparison to the six piperidine derivatives tested. This difference in results could have originated from the nucleus of thiotetrahydropyridine containing 1,2,3,4 tetrahydropyridine and the six piperidine compounds containing 1,2,5,6 tetrahydropyridine.

Compounds; methyl 6-(2-hydroxyphenyl)-2-phenyl-1-(2-sulfanyl-1*H*-benzo [d] imidazol-5-yl)-4-[(2-sulfanyl-1*H*-benzo[d]imidazol-6-yl)amino]-1,2,5,6-tetrahydro-3-pyridinecarboxylate, methyl 6-(2-methoxyphenyl)-2-phenyl-1-(2-sulfanyl-1*H*-benzo [d]imidazol-5-yl)-4-[(2-sulfanyl-1*H*-benzo[d]imidazol-6-yl)amino]-1,2,5,6-tetrahydro-3-pyridinecarboxylate and methyl 6-(4-nitrophenyl) -2-phenyl-1-(2-sulfanyl-1*H*-benzo[d]imidazol-5-yl)-4-[(2-sulfanyl-1*H*-benzo[d]imidazol-6-yl)amino]-1,2,5,6 -tetrahydro-3-pyridinecarboxylate display great antioxidant potential due to the substitution of hydroxyl, methoxy and nitro on piperidine ring/benzene ring respectively (Ravindernath and Reddy, 2013). This maybe the reason that piperidine derivative **PM4** reveal the highest antioxidant activity in comparison to the other compounds (**PM1, PM2, PM3, PM5** and **PM6**) as it contains a methoxy group at position 3 of the phenyl group which is attached to carbons 1and 4 on the piperidine ring structure.

Methyl 6-(2-methoxyphenyl)-2-phenyl-1-(2-sulfanyl-1*H*-benzo[d]imidazol-5-yl)-4-[(2-sulfanyl-1H-benzo[d] imidazol-6-yl)amino]-1,2,5,6-tetrahydro-3-pyridine carboxylate compound containing the methoxy substitution on the piperidine ring like PM4 exhibited IC_{50} values between 4.2 µM (Ravindernath and Reddy, 2013)

which is similar to 1.78 µM IC_{50} displayed by **PM4**. It may be concluded that substitution of methoxy group attached to the phenyl group on piperidine ring influences high antioxidant activity.

Premalatha and coworkers (2013) stated that compounds containing the piperidine skeleton, i.e. 2,6-dipheny-l-3-alkylpiperidin-4-one-O-[2,4,6-tritertiarybutyl-cyclo hexa-2,5-dienon-4-yl]oximes comprised of a high content of alkyl substituents that lead to good antioxidant activity.

5.3 Anti-inflammatory activity

The anti-inflammatory activity of the six piperidine derivatives (**PM1**-**PM6**) was determined by the use of a 5-lipoxgenase Inhibition assay kit (Sigma). Results are presented Table 6. The anti-inflammatory activities were recorded at different concentrations of 125, 250, 500, 1000, 2000 and 3000 µg/mL and then compared to the standard nordihydroguaiaretic acid (NDGA).

The percentage of anti-inflammatory activity was expressed as mean ± standard deviation values with IC_{50} values represented in µg/mL units (Table 6).

The significant difference between the six piperidine derivatives tested, were determined by the one way Anova programme and the level of significance was $p < 0.05$ which was also achieved by Girgis (2009) who tested dispiro[3H-indole-3-2'-pyrrolind-3' .3'-piperiinde]- 2(1H),4''-dones, compound containing the piperidine ring. The anti-inflammatory activity displayed by reference standard nordihydroguaiaretic acid (NDGA) of 88% at 3000 µg/mL with an IC_{50} of 1549.99 µg/mL which was similar to the results obtained by (Girgis, 2009) who achieved similar results in an anti-inflammatory assay.

The results (Table 6) show the six piperidine compounds tested demonstrated a degree of anti-inflammatory activity, ranging from 55% (**PM6**) to 76% (**PM4**) at a concentration of 3000 µg/mL. In comparison to all piperidine derivatives tested, **PM4** demonstrated the highest anti-inflammatory activity while **PM6** demonstrated the lowest anti-inflammatory activity. The results obtained for compound **PM6** is known to be correct as literature states, "substituting a phenyl group with a halogen atom, i.e. chloro, bromo or flouro is associated with low anti-inflammatory activity" and **PM6** contains a chloro and flouro groups substituted at positions 2 and 6 on the

phenyl group attached to carbons 1 and 6 on the piperidine structure. This resulted in **PM6** displaying low anti-inflammatory activity in comparison to the other five piperidine derivatives tested. The highest anti-inflammatory activity of **PM4** resulted from the substitution of a methoxy group at position 3 on the phenyl group attached to carbons 1 and 6 on the piperidine ring, which according to research states, that methoxy functions as a representative example of an electron-donating residue is associated with improvement in the observed anti-inflammatory activity (Girgis, 2009).

Although piperidine derivatives **PM3** and **PM4** contain a bromo group substituted at position 4 on the phenyl group attached at carbons 2 and 6 of the piperidine ring it also has an ethyl ester substituted at position 3 on the piperidine ring which may have resulted in the high anti-inflammatory potency of these two derivatives and the reasons for **PM4** displaying the highest activity.

The anti-inflammatory activities of 70 and 68% at 3000 µg/mL was revealed by piperidine derivatives **PM1** and **PM2**. This high activity could have developed from the methoxy group substituted at position 3 on the phenyl group attached to carbons 2 and 6 of the piperidine ring structure and the substitution of a methyl group on carbon 3 of the piperidine ring. The other piperidine derivatives **PM3** and **PM5** displayed anti-inflammatory potency of 60 and 65% at 3000 µg/mL respectively. These good results could have developed from the ethyl ester being substituted on carbon 3 of the piperidine ring. Overall all piperidine derivatives screened for anti-inflammatory activity exhibited good potencies which could be effective as anti-inflammatory drugs but their activities displayed at 3000 µg/mL are less than that of the reference standard which presented an anti-inflammatory activity of 88%.

These piperidine derivatives revealed anti-inflammatory activities with IC_{50} ranging from, 2251.62 µg/mL for **PM6** to 1805.05 µg/mL for **PM4**. Piperidine derivatives **PM1, PM2, PM3** and **PM5** exhibited anti-inflammatory activities of 68, 74, 60, and 65% at 3000 µg/mL respectively. These compounds exhibited IC_{50} at 2049.18, 1824.82, 2358.49 and 2173.91 µg/mL respectively.

It is observed that the six piperidine derivatives tested demonstrated lower activity in comparison to the reference standard nordihydroguaiaretic acid (NDGA) which

exhibits an IC_{50} value of 1547.99 µg/mL and 88% anti-inflammatory potency at 3000 µg/mL. It has been observed that the percentage of anti-inflammatory activity of **PM1-PM6** is presented in Table 6. This table indicates that all compounds **(PM1-PM6)** tested displayed high anti-inflammatory activities.

Compound 4-(4-bromophenyl)-5-[(4-bromophenyl)methylene]-1,1-dimethyl–dispiro [3*H*-indole-3,2-pyrrolidine-3,3-piperidine]-2(1*H*),4-dione has been reported in literature to have decreased anti-inflammatory potency due to bromo substitution on the phenyl ring (Girgis, 2009). Girgis (2009) also stated that compound 4-(4-Chlorophenyl)-5-[(4-chlorophenyl)methylene]-1,1-dimethyl-dispiro[3H-indole-3,2-pyrrolidine-3,3-piperidine]-2(1H),4-dione displays reduced anti-inflammatory activity which have developed through chloro substitution on phenyl ring attached to the piperidine ring.

5.4 *In vitro* Anticancer Activity

The *in vitro* cytotoxicity activities of six piperidine derivatives were determined by the MTT assay technique (Hanelt *et al*., 1994). The compounds were evaluated for their cytotoxic effects at 0, 10, 50, 100, 250, 500 and 1000 µg/mL on two cancer cell lines and one untransformed cell line namely, MCF7 (breast cancer cells), melanoma (skin cancer cells) and fibroblast (normal cells). The *in vitro* cytotoxic activity revealed (Table 7a, 7b and 7c) the significance between the results obtained was $p < 0.001$.

Table 7 in chapter 4 reveals the cytotoxicity potencies of all six piperidine derivatives tested, prior to this test no prevous test had been conducted. It is evident in these tables that piperidine derivatives **PM1, PM2, PM3, PM4, PM5** and **PM6** demonstrated high levels of cytotoxicity against both cancer cell line and 50% toxicity on the healthy cell. Analysis of the MTT assay reveals that the cytotoxicity levels of the screened compounds ranged between 415.63 – 488.28 µg/mL on MCF7 cancer cells, between 438.60 - 612 µg/mL on melanoma cancer cells and between 720.46 – 789.89 µg/mL on fibroblast cells.

Of the piperidine drugs screened, **PM6** exhibited the highest cytotoxicity activity of 91% at 1000 µg/mL with the lowest IC_{50} of 415.63 µg/mL on MCF7 cancer cells. **PM6** also revealed the highest cytotoxicity activity of 95% at 1000 µg/mL with the

lowest IC_{50} of 438.6 µg/mL melanoma cancer cell line. These results may have developed from the substitution of a chloro group with at position 4 flouro group at position 2 on the phenyl group attached to carbons 2 and 6 on the piperidine ring, which according to research states increases cytotoxic levels of the compound (Aerluri *et al.*, 2012). This compound however revealed 52% cytotoxic effect against fibroblast cells at 1000 µg/mL. This however states that compound **PM6** is killing 91 and 95% of the cancer cell respectively but also killing 50% of healthy cells at same concentration of 1000 µg/mL.

The least cytotoxic piperidine derivative screened was **PM3**, with cytotoxic effects of 87 % at 1000 µg/mL (IC_{50} of 488.28 µg/mL) on MCF7 cancer cells and 71% at 1000 µg/mL (IC_{50} of 612 µg/mL) on melanoma cancer cells. **PM3** is 50% toxic against healthy cells (fibroblast) with a good IC_{50} value of 789.89 µg/mL. This concludes that **PM3** kills MCF7, melanoma and fibroblast cells. This low cytotoxic potency displayed by **PM3** may have developed from the substitution of a bromo group at positions 4 on the phenyl group attached to carbons 2 and 6 on the piperidine ring.

Piperidine derivative **PM1** exhibited a cytotoxic effect of 88% at 1000 µg/mL with an IC_{50} of 477.10 µg/mL on MCF7 cancer cells and 88% at 1000 µg/mL with IC_{50} of 494.10 µg/mL on melanoma cancer cells. **PM1** also revealed a cytotoxic effect of 51% on fibroblast healthy cells at 1000 µg/mL with an IC_{50} value of 757.58 µg/mL. These results could have resulted from the methoxy in **PM1** and phenyl rings are at positions 2 and 6 with p.tolylamino at positions 4 on the piperidine ring.

This shows that the substitution of a phenyl group at positions 2 and 6 of the piperidine ring results in lower cytotoxic activity in comparison to the other groups substituted at these positions on a piperidine ring.

Piperidine derivative **PM2** revealed a cytotoxic effect of 88% at 1000 µg/mL with an IC_{50} of 455.79 µg/mL on MCF7 cancer cells and 89% at 1000 µg/mL with IC_{50} of 491.20 µg/mL melanoma cancer cells. **PM2** also revealed a cytotoxic effect of 50% on fibroblast healthy cells at 1000 µg/mL with an IC_{50} value of 782.25 µg/mL. These results could have resulted from a cyano group being substituted at position 4 on the phenyl group which is attached to carbons 2 and 6 on piperidine ring.

Another piperidine derivative tested was **PM4**, which displayed moderate cytotoxicity activity of 90% at 1000 µg/mL with an IC_{50} of 475.29 µg/mL on MCF7 cancer cells and 94% at 1000 µg/mL with an IC_{50} of 452.5 µg/mL on melanoma cancer cells. **PM4** also revealed a cytotoxic effect of 52% on fibroblast healthy cells at 1000 µg/mL with an IC_{50} value of 720.46 µg/mL. These results could have resulted from the substitution of a pyridyl group on carbons 2 and 6 of the piperidine nucleus as well as the substitution of a bromo group at position 4 on the phenyl group attached at positions 1 and 4 on piperidine ring.

Finally, piperidine derivative **PM5** has presented a cytotoxic effect of 89% at 1000 µg/mL with an IC_{50} of 466.85 µg/mL on MCF7 cancer cells and 92% at 1000 µg/mL with IC_{50} of 465.60 µg/mL melanoma cancer cells. **PM5** also revealed a cytotoxic activity of 51% against fibroblast cells with an IC_{50} value of 753.01 µg/mL. These results could have developed from the substitution tri-fluoro substituted at position 3 on the phenyl group and a pyridyl group substituted on carbons 2 and 6 of the piperidine ring.

All piperidine derivatives are highly toxic towards MCF7 and melanoma cancer cells. However these compounds are only 50% cytotoxic towards healthy cells. Therefore it these piperidine derivatives may be used in clinical trials to treat MCF7 and melanoma cancer cells but keeping in mind approximately 50% of healthy cells were inhibited. Compounds that contained the substitution of a methoxyl and chloro group on the phenyl ring of the piperidine nucleus displayed increased cytotoxic activities. The substitution of a chloro group at position 4 and fluoro group at position 2 on the phenyl ring which is attached to carbon 2 and 6 on the piperidine nucleus presented the highest cytotoxic activity.

Aerluri *et al*., (2012) specified that piperidine derivative 1,2,5,6,tetrahydropyridine-3- carboxylate displays effects of toxicity against A549 (human epithelial lung carcinoma), DU145 (human prostate cancer), HeLa (human epithelial cervical cancer and SK-N-SH (human neuroblastoma) and this may have resulted from the chloro substitution on the phenyl ring. Aerluri *et al*., (2012) also suggests that the presence of a methoxyl and chloro as substitution on the phenyl group on the piperidiine ring increases the cytotoxic properties.

5.5 Brine shrimp lethality assay

The toxicity of the six piperidine derivatives (**PM1**-**PM6)** was evaluated using brine shrimp lethality assay technique (Anna *et al*., 2011, Meyer *et al.*, 1982). The toxicity of the compounds was evaluated at 0, 10, 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 µg/mL with the results of cell death percentage expressed as mean ± standard deviation values in Table 8. The significant difference between the six piperidine compounds tested revealed a p value < 0.05 which was determined by one way Anova programme. The IC_{50} values were expressed in µg/mL.

These results are represented in Table 8. This table state, of all six piperidine derivatives tested, the derivative with the highest toxicity at 1000 µg/mL was **PM5** and **PM6** with 50% toxicity and the piperidine derivatives revealing the minimum toxicity of 30% is **PM1** and **PM2**. Piperidine derivatives, **PM3** and **PM4** displayed moderate toxicity of 40%. The IC_{50} values for **PM1, PM2, PM3, PM4, PM5** and **PM6** were; 1440.922, 1886.72, 1351.35, 1231.53, 1106.19 and 1106.19 µg/mL respectively. These results portray to directly proportional relationship between concentration of drug and toxicity.

Piperidine derivatives **PM5** and **PM6** displayed the highest toxicity results due to the a fluoro group substituted on the phenyl group attached of the piperidine ring structure which increases toxicity activity (Jahan *et al*., 2013). With reference to the toxicity levels of 40% revealed by **PM3** and **PM4**, these may have contributed from the bromo group substituted at position 4 on the phenyl group attached to carbons 2 and 6 of the piperidine ring. It has been report that bromo as a derivative increases toxicity of a compound (Jahan *et al*., 2013). It indeed increased toxicity of the compounds but not higher than the compounds containing fluoro.

PM1 and **PM2** demonstrated the least toxic potency and this may have developed from bromo and flouro group not being present. It shows that the presence of methoxy group positioned at carbon 4 on the phenyl group attached at carbons 1 and 4 of the piperidine ring decreases compound toxicity.

It has been reported that piperidine derivatives, 2-hydroxymethyl-1-[(4-bromo-phenyl) -2-oxoethyl]-piperidinium bromide and 2-hydroxymethyl-1-[(4-flouro-

phenyl)-2-oxoethyl]-piperidinium bromide exhibited high toxicity levels due to both compounds existing as bromo and fluoro derivatives (Jahan *et al*., 2013).

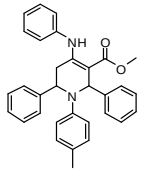
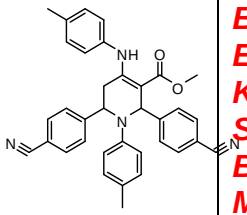
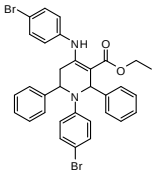
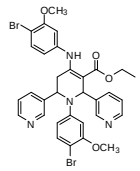
CHAPTER 6: CONCLUSION

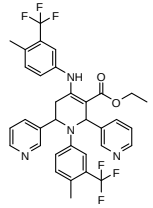
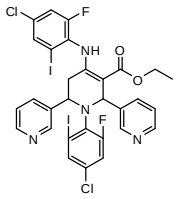
A summary of the pharmacological activities of six piperidine derivatives (**PM1-PM6)** is presented in Table 9.

All six piperidine derivatives (**PM1-PM6)** screened for antimicrobial activity exhibit characteristics of varying degree of microbial inhibition against some Gram-positive and Gram-negative bacteria (*B. cereus, B. subtilis, E. coli, S. aureus, Kl. Pneumonia, M. luteus and P. aurenginosa*) with exceptions to *B. polymixa, S. marcescens* and *S. facealis.* Piperidine derivative **PM4** displayed the lowest antibacterial activity whereas **PM2** showed the highest bacterial inhibition activity. Furthermore, **PM2** exhibited the lowest MIC results against the seven bacterial species tested in comparison to the other piperidine derivatives. All six piperidine compounds displayed no activity towards *F. verticilliodes, C. utilus* and *P. digitatium* fungal cultures. Piperidine compounds **PM3** and **PM4** revealed no activity against all seven fungal cultures. With reference to the antioxidant activity of piperidine derivatives, **PM4** demonstrated the highest scavenging capacity of 78% at 1000 µg/mL and **PM2** demonstrated the least percentage of scavenging potential of 49% at 1000 µg/mL. All piperidine derivatives tested revealed antioxidant activities greater than 49% at 1 mg/mL as compared to the control Rutin, which displayed 97% scavenging capacity at 1 mg/mL. The least anti-inflammatory activity was showed by **PM6** and the highest anti-inflammatory activity was displayed by **PM4.** However, NDGA presented the highest anti-inflammatory activity in comparison to all six piperidine derivatives tested (**PM1**-**PM6**). **PM6** demonstrated the highest cytotoxic activity on both cancer cell lines. **PM3** was the least cytotoxic compound on MCF7 cancer and melanoma cancer cell lines. All piperidine derivatives demonstrated a moderate inhibition of ±50% cytotoxic effect on fibroblast cells. **PM4** and **PM6** revealing the highest cytotoxic effect (52%) on fibroblast cells whereas **PM2** and **PM3** showed the lowest cytotoxic activity of 50% against fibroblast cells. **PM5** and **PM6** gave the highest toxicity against brine shrimp larvae in comparison to **PM3** and **PM4**, which gave the least toxic effect against the brine shrimp larvae. These results reveal a directly proportional relationship between concentration of drug and toxicity.

Table 9 Summary of the pharmacological activities (MIC - Minimum inhibition concentration (µg/mL), IC_{50} – 50% Inhibition concentration (µg/mL), MEL – Melanoma cancer cells, FIB – Fibroblast healthy cells) displayed by the tested piperidine derivatives (**PM1-PM6)**

	Antimicrobial (MIC)		**Antioxidant** [c]	**Anti-inflammatory** [d]	**Cytotoxicity** [e]			**Brine shrimp** [f]
					IC_{50} (µg/mL)			
	Antibacterial [a]	**Antifungal** [b]	**IC_{50} (µg/mL)**	**IC_{50} (µg/mL)**	**MCF7**	**MEL**	**FIB**	**IC_{50} (µg/mL)**
POSITIVE CONTROLS	*B. cereus* ++++ *E. coli* ++++ *Kl. pneumoniae* ++++ *S. aureus* ++++ *S. marcescens* ++++ *B. polymixa* ++++ *B. subtilus* ++++ *M. luteus* ++++ *P. aurenginosa* ++++ *S. facealis* ++++	*A. niger* +++++ *A. flavus* +++++ *F. verticilliodes* +++++ *P. digitatium* +++++ *C. utilis* +++++ *C. albicans* +++++ *S. cerevisiae* +++++	464.68	1547.99	n/a	n/a	n/a	n/a

	Antimicrobial (MIC)		Antioxidant [c]	Anti-inflammatory [d]	Cytotoxicity			Brine shrimp [f]
					IC_{50} (µg/mL)			
	Antibacterial [a]	Antifungal [b]	IC_{50} (µg/mL)	IC_{50} (µg/mL)	MCF7	MEL	FIB	IC_{50} (µg/mL)
PM1 	*B. cereus* ++ *E. coli* ++ *S. aureus* ++ *B. subtilus* ++ *M. luteus* ++	***A. niger*** + ***A. flauvs*** + ***C. albican*** +	838.93	2049.18	477.10	494.10	747.58	1440.92
PM2 	***B. cereus*** ++ ***E. coli*** ++ ***Kl. pneumonia*** ++ ***S. aureus*** ++ ***B. subtilus*** +++ ***M. luteus*** ++ ***P. aurenginosa*** ++	***A. niger*** + ***A. flauvs*** + ***C. albican*** +	959.69	1824.82	455.79	491.20	782.25	1886.72
PM3 	*B. cereus* ++ *E. coli* ++ *S. aureus* ++ *B. subtilus* ++ *Kl. pneumoniae* ++	n/a	730.99	2358.49	488.28	612	789.89	1351.35
PM4 	*S. aureus* ++ *M. luteus* ++ *P. aurenginosa* ++	n/a	**584.80**	**1805.05**	475.29	452.50	**720.46**	1231.53

	Antimicrobial (MIC)		Antioxidant [c]	Anti-inflammatory [d]	Cytotoxicity IC_{50} (µg/mL)			Brine shrimp [f]
	Antibacterial [a]	**Antifungal [b]**	**IC_{50} (µg/mL)**	**IC_{50} (µg/mL)**	**MCF7**	**MEL**	**FIB**	**IC_{50} (µg/mL)**
PM5 	*B. cereus* ++ *E. coli* ++ *S. aureus* ++ *B. subtilus* ++ *M. luteus* ++ *P. aurenginosa* ++	***A. niger* +** ***S.cerevisiae* +** ***C. albicans* +**	689.66	2173.91	466.85	465.60	753.01	**1106.19**
PM6 	*B. cereus* ++ *E. coli* ++ *S. aureus* ++ *B. subtilus* +++ *M. luteus* ++ *P. aurenginosa* ++	***A. niger* +** ***S.cerevisiae* +** ***C. albicans* +**	667.56	2551.02	**415.63**	**438.60**	736.38	**1106.19**

KEY: 0.003 µg/mL : +, 0.0015 µg/mL: ++, 0.00075 µg/mL : +++, 7 µg/mL : ++++, 0.15 µg/mL : +++++, n/a – no activity, [a] Positive control (Ciprofloxacin), [b] Positive control (Amphotericin B), [c] Positive control (Rutin), [d] Positive control (NDGA), [e] Positive control (DMSO), [f] Positive control (DMSO), the red colour indicates the best activity.

Future work should involve clinical trials on these piperidine derivatives (**PM1-PM6)** as they demonstrate good antibacterial, antifungal, antioxidant, anti-inflammatory and low toxicity levels against *Artemia salina* in a brine shrimp lethality assay**.**

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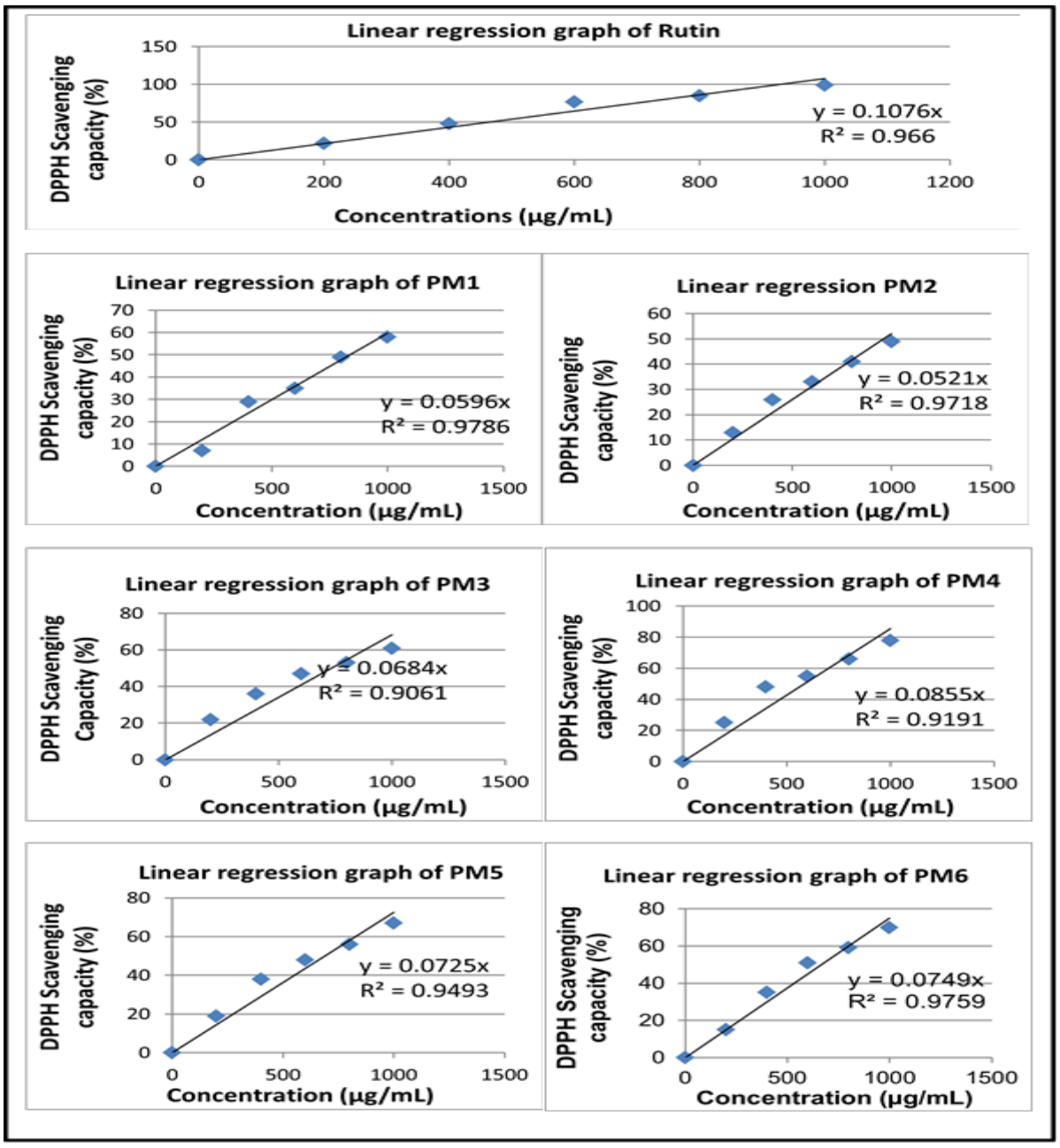
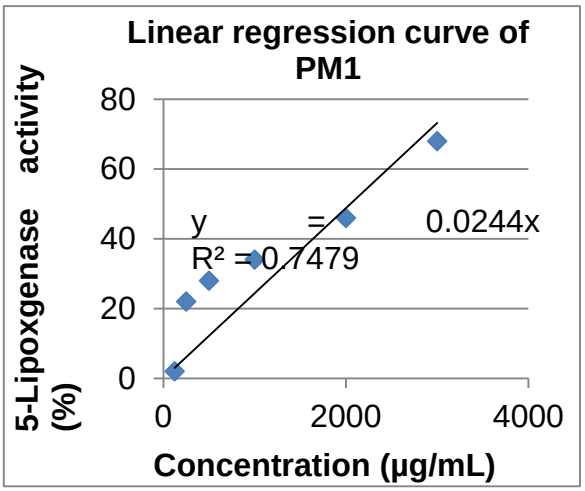
APPENDIX

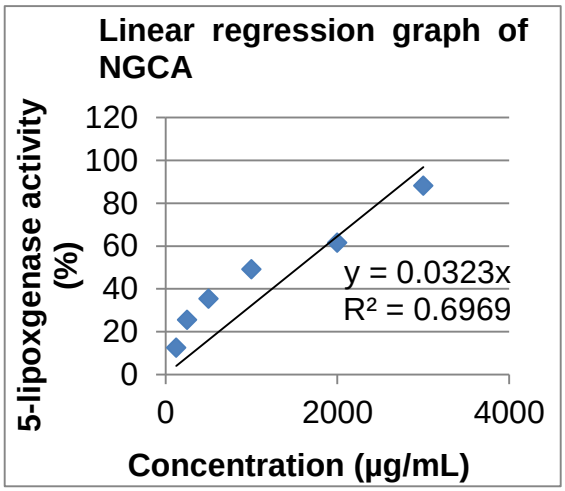
APPENDIX A: LINEAR REGRESSIONS GRAPHS OF THE SIX PIPERIDINE DERIVATIVES

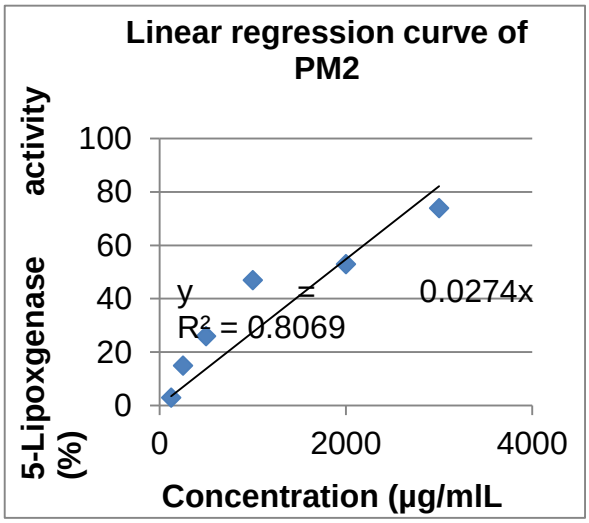
A.1, 8.1.1 Linear regression graphs of the six piperidine derivatives used to determine the 50% antioxidant activity at 0, 200, 400, 600, 800 and 1000 µg/mL.

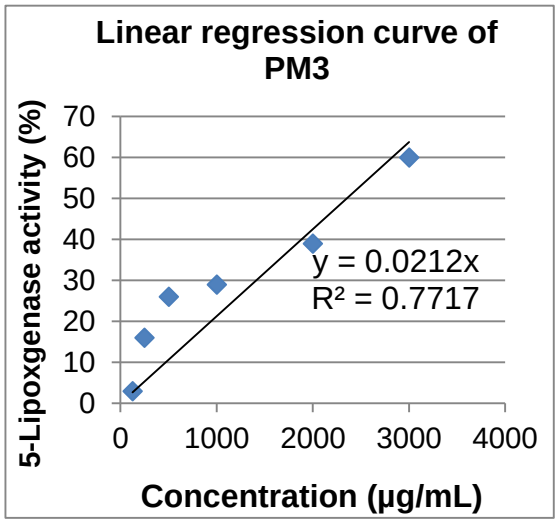


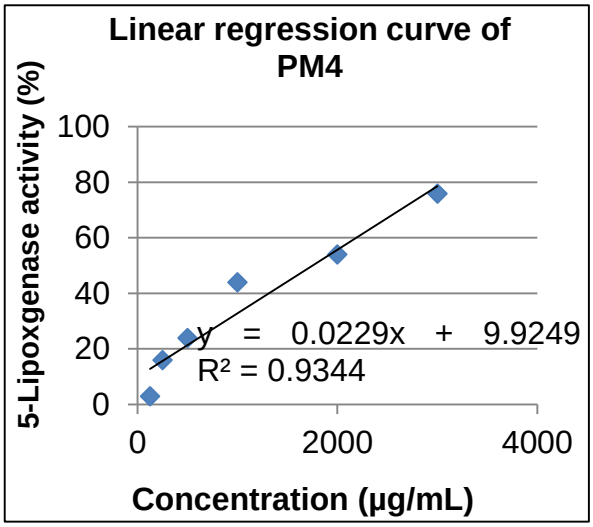
A.1, 8.1.2 Linear regression graphs of six piperidine compounds used to determine 50% 5-Lipoxgenase activity

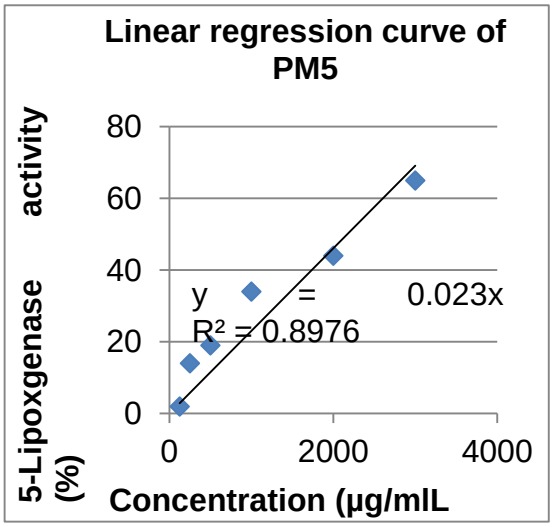


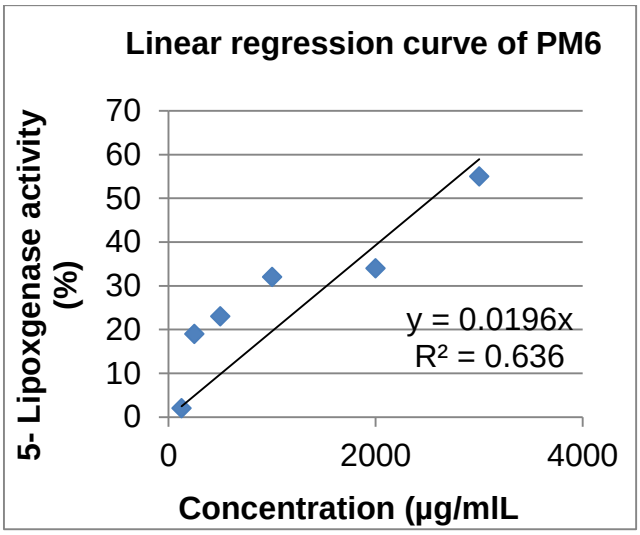




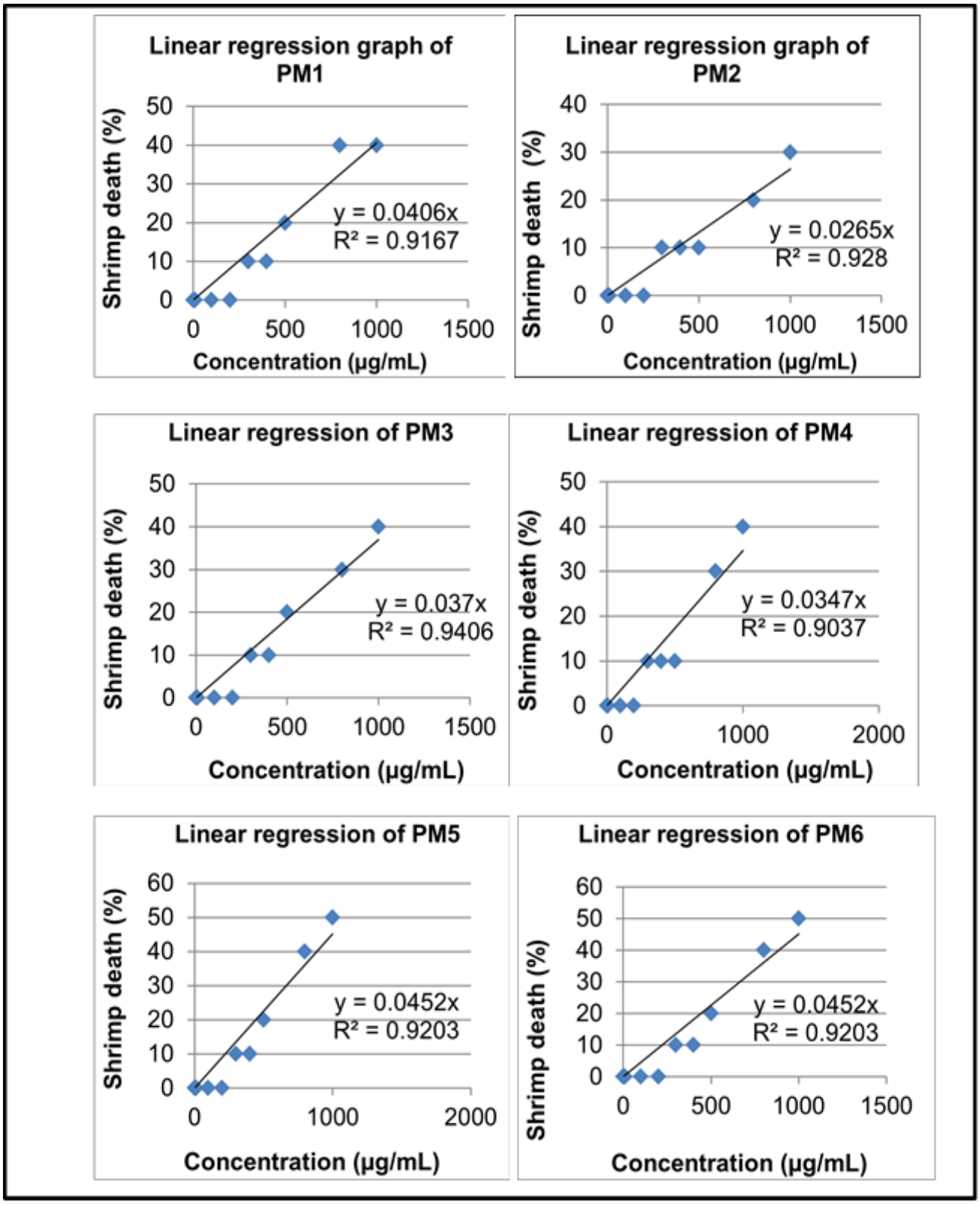




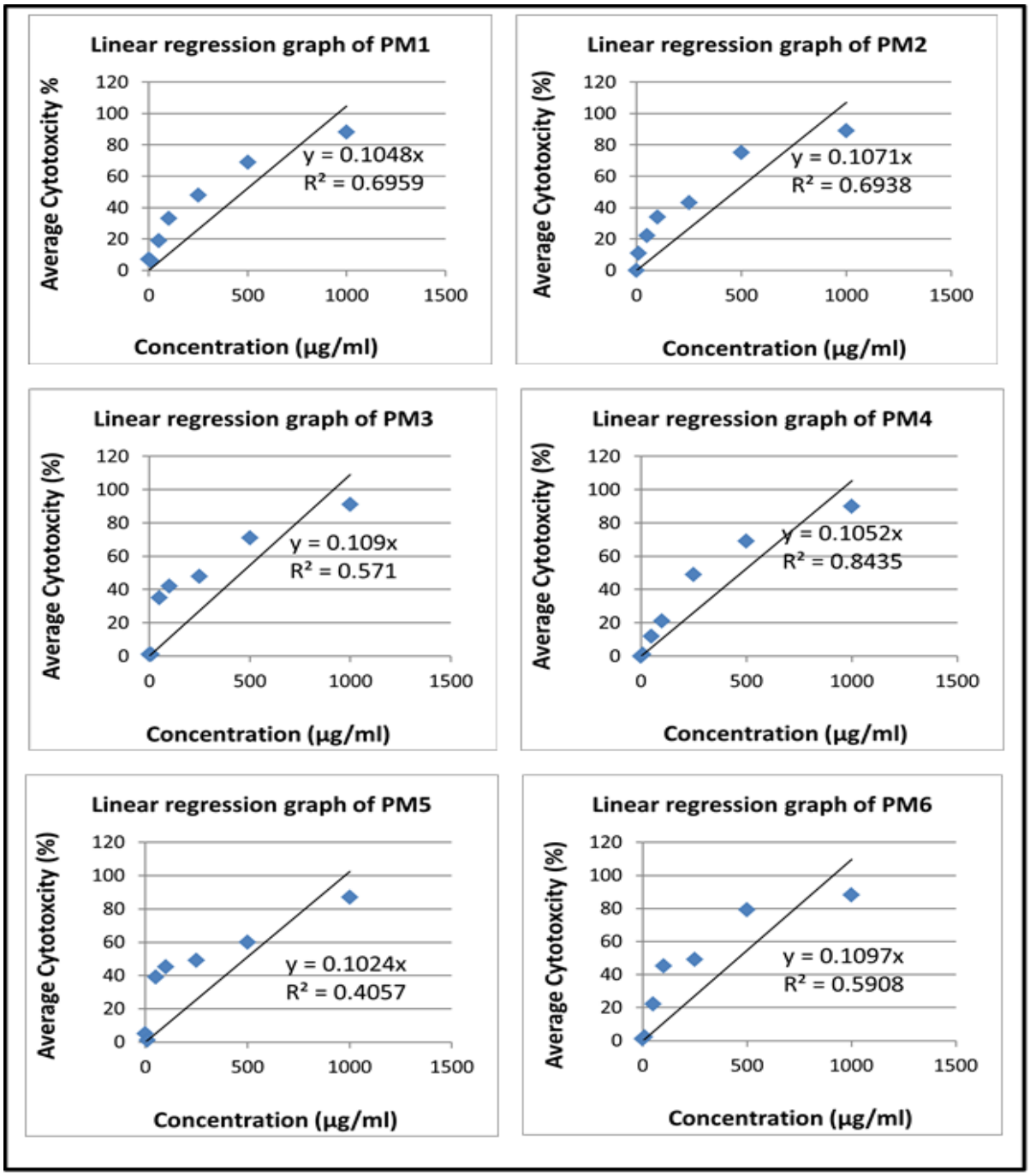



Linear regression curve of PM6
5- Lipoxgenase activity (%)
70
60
50
40
30
20
10
0
0
2000
4000
Concentration (µg/mlL
y = 0.0196x
R² = 0.636

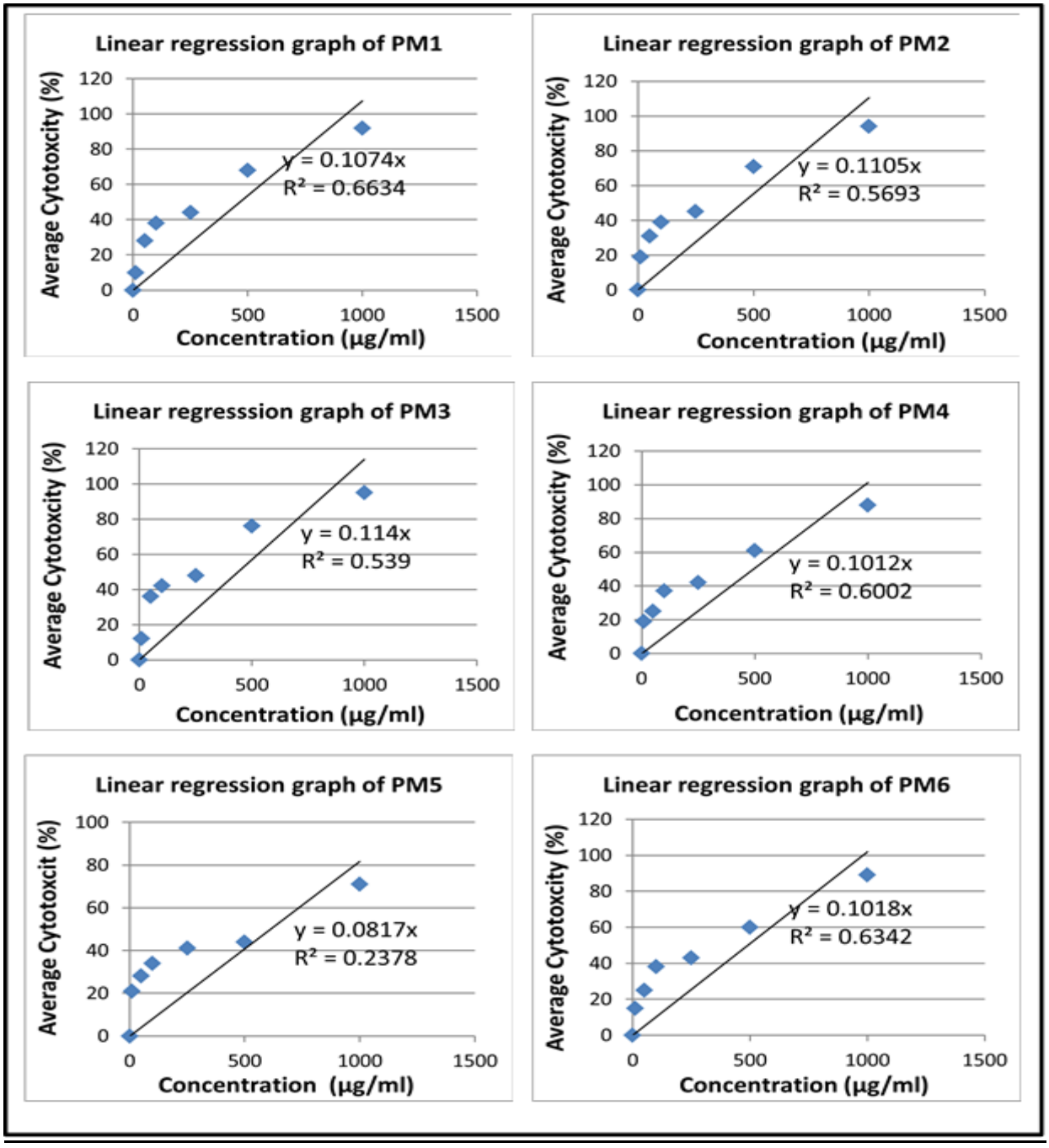
A.1, 8.1.3 Linear regression graphs of six piperidine derivatives used to determine brine shrimp lethality activity



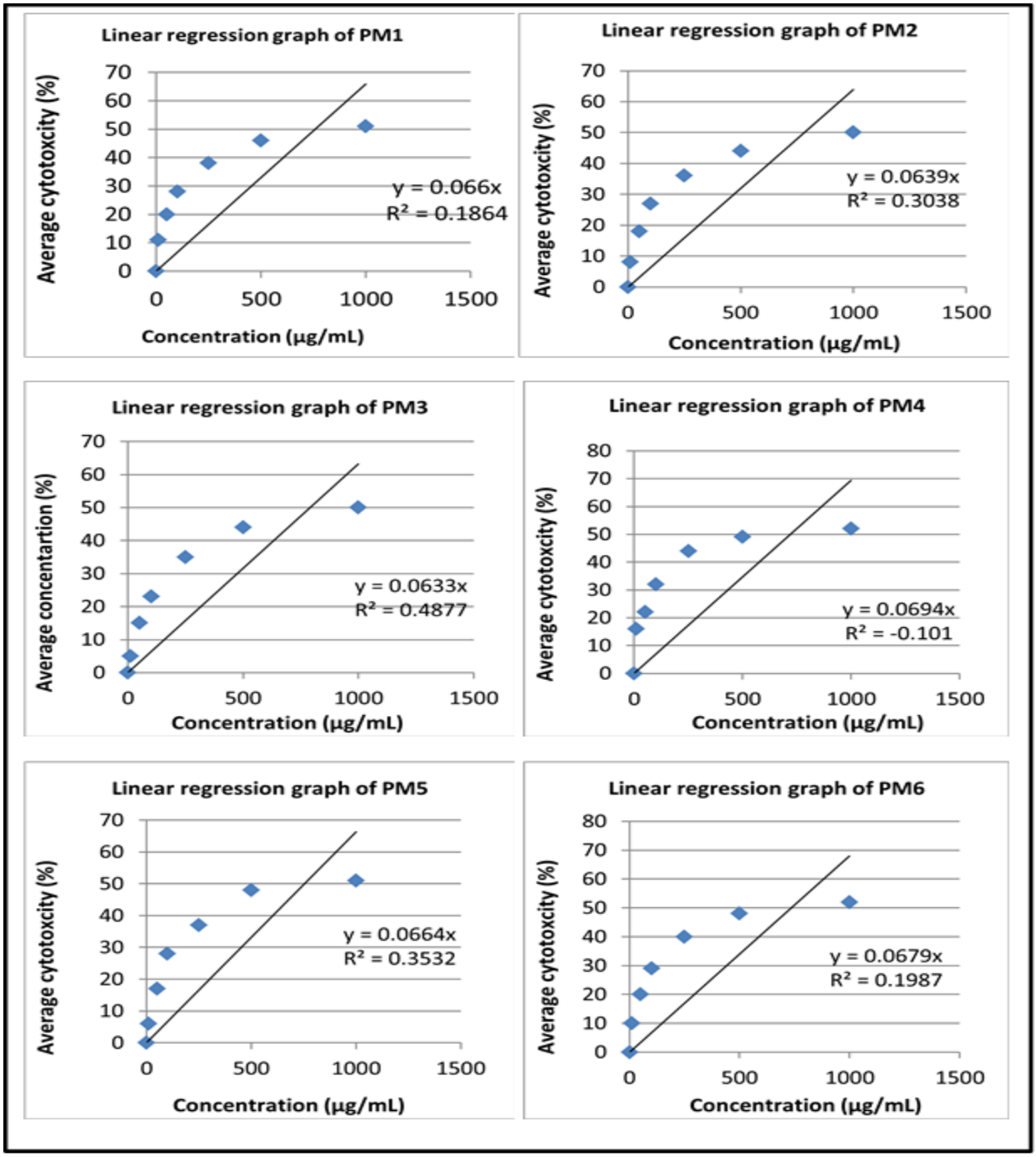
A.1, 8.1.4 Linear regression graphs of six piperidine derivatives at nine concentrations (0, 10, 100, 200, 300, 400, 500, 800 and 1000 µg/mL) used to determine their cytotoxic activity against MCF7 cancer cells



A.1, 8.1.5 Linear regression graphs of six piperidine derivatives at nine concentrations (0, 10, 100, 200, 300, 400, 500, 800 and 1000 µg/mL) used to determine their cytotoxicity activity against Melanoma cancer cells.



A.1, 8.1.6 Linear regression graphs of six piperidine derivatives at nine concentrations (0, 10, 100, 200, 300, 400, 500, 800 and 1000 µg/mL) used to determine their cytotoxicity activity against Fibroblast healthy cells



APPENDIX B: MATERIALS, CHEMICALS, SOLUTIONS AND EQUIPMENTS

A.2, 8.2.1 Culturing media for bacterial cultures

A.2, 8.2.1.1 Mueller-Hinton Broth (MHB)

Beef dehydrated infusion 300.0 g/L, casein hydrolysate 17.5 g/L and starch 1.5 g/L at pH 7.3 ± 0.1 at 25 °C.

A.2, 8.2.1.2 Mueller-Hinton Agar (MHA)

Beef infusion solid 4 g/L, starch 1.5 g/L, casein hyrolysate 17.5 g/L and agar 15 g/L at pH 7.4 ± 0.2 at 37 °C.

A.2, 8.2.2 Culturing media for fungal cultures

A.2, 8.2.2.1 Sabouraud dextrose Agar (SDA)

Mycological peptone 10 g/L, dextrose 40 g/L and agar 10 g/L at pH 5.6 ± 0.2.

A.2, 8.2.3 Media for Brine Shrimp Lethality assay

A.2, 8.2.3.1 Tryptone Soy Agar

Tryptone 15.0 g/L, Soy peptone 5.0 g/L, Sodium Chloride 5.0 g/L and Agar 13.0 g/L at pH 7.3 ± 0.2

A.2, 8.2.3.2 Artificial Sea water

23 g/L Sodium chloride (Merck), 11 g/L magnesium chloride (Merck), 4g/L sodium sulphate (Merck), 1.3 g/L calcium chloride (Merck) and 0.7g/L potassium chloride (Merck) dissolving pH was adjusted to 9.0 using 1 M sodium carbonate.

A.2, 8.2.3.3 Yeast Extract

3 mg of yeast extract (Biolab) dissolved in 1 mL of artificial sea water.

A.2, 8.2.4 Reagents and Standards

A.2, 8.2.4.1 0.3 mM DPPH Reagent

0.0058 g DPPH (sigma) dissolved in 50 mL methanol (Merck).

A.2, 8.2.4.2 1 mM Rutin standard

0.015 g of Rutin (Sigma 95% HPLC grade) dissolved in 25 mL of methanol (Merck).

A.2, 8.2.4.3 MTT reagent

5 mg of MTT powder added to 1 mL of phosphate buffer Saline (Sigma).

A.2, 8.2.5 Assay kits

A.2, 8.2.5.1 5-Lipoxgenase assay screening kit

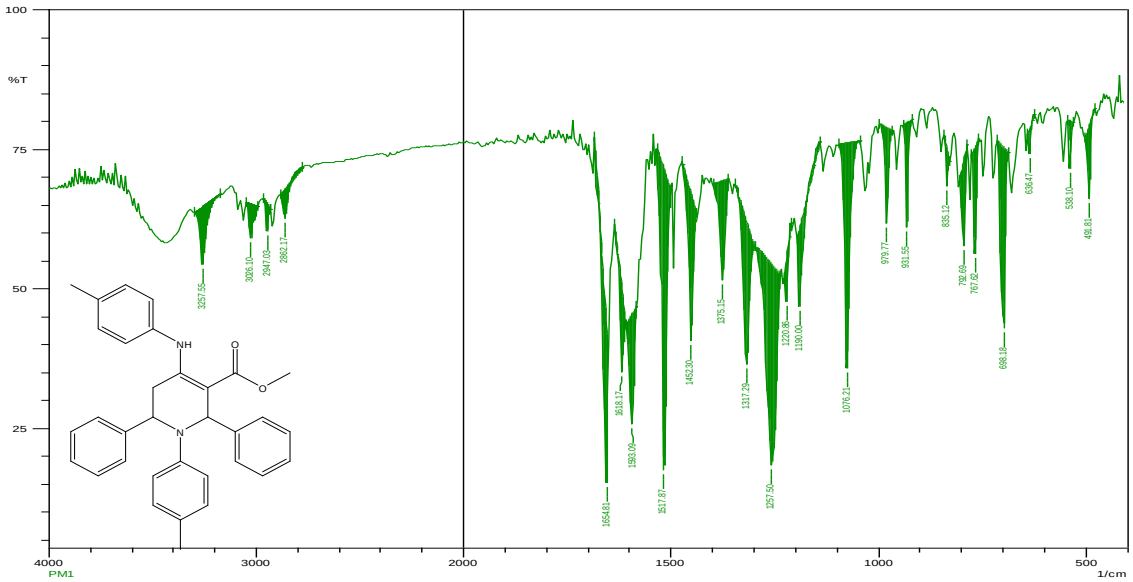
A.2, 8.2.6 Equipment

A.2, 8.2.6.1 Elisa plate reader

APPENDIX C: CHARACTERISATION OF THE SIX PIPERIIDNE DERIVATIVES.

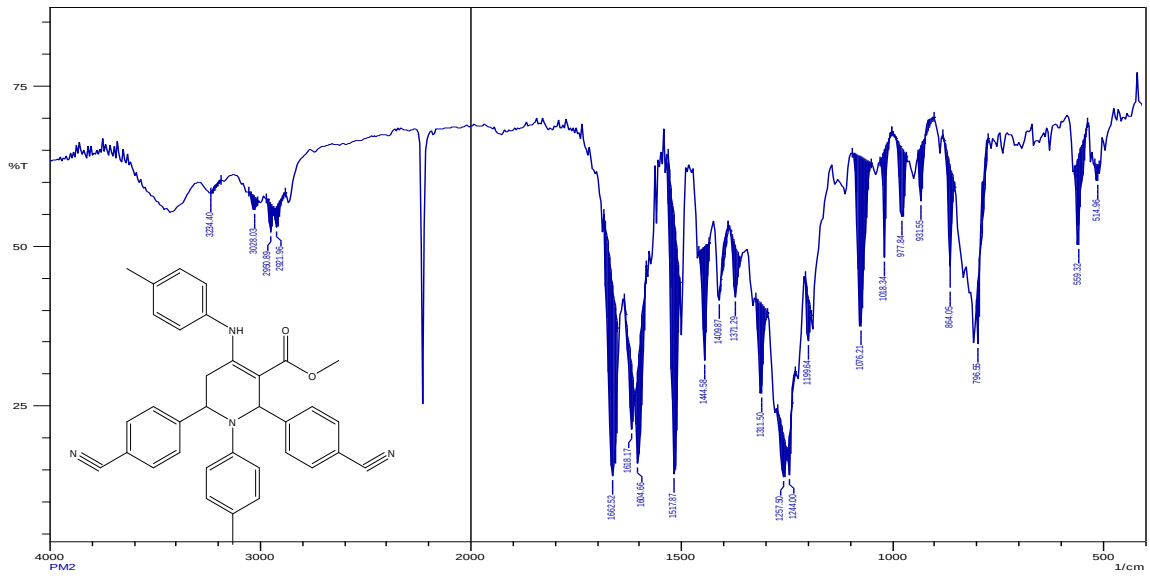
A.3, 8.3 Infrared Spectroscopy

A.3, 8.3.1 Infrared Spectra of PM1



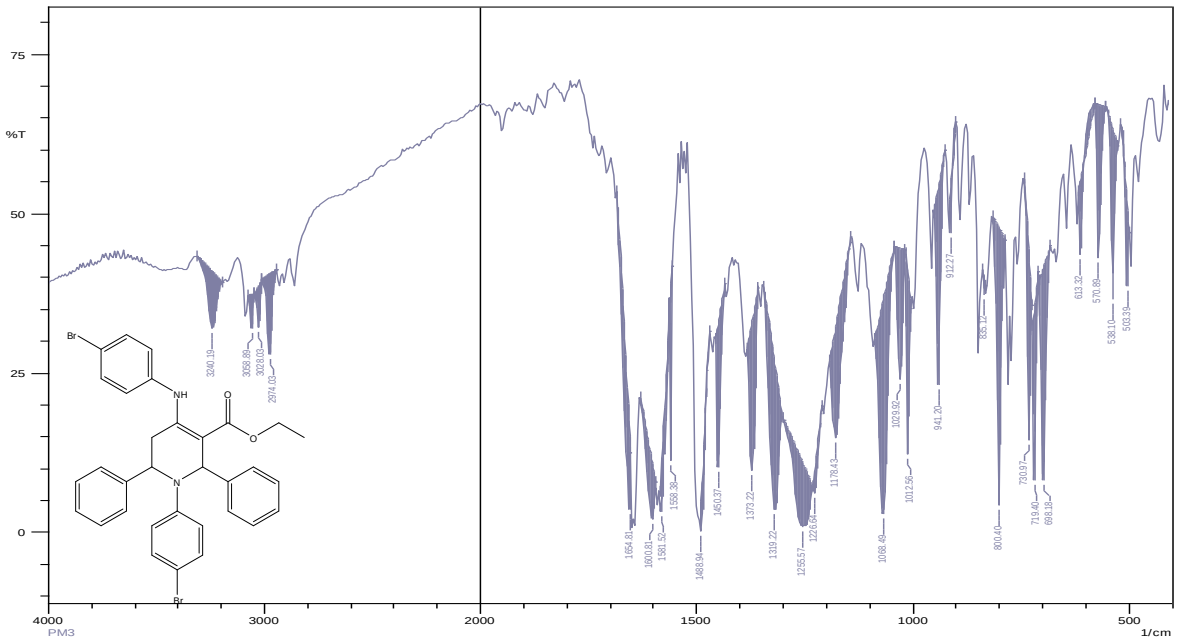
IR (KBr) cm^{-1}: 3257 (N-H str), 3026 (ArC-H str), 2947, (AlpC-H str), 1654 (C=O str), 1618, 1517, 1452 (C=C str), 1317 (C-N str).

A.3, 8.3.2 Infrared Spectra of PM2



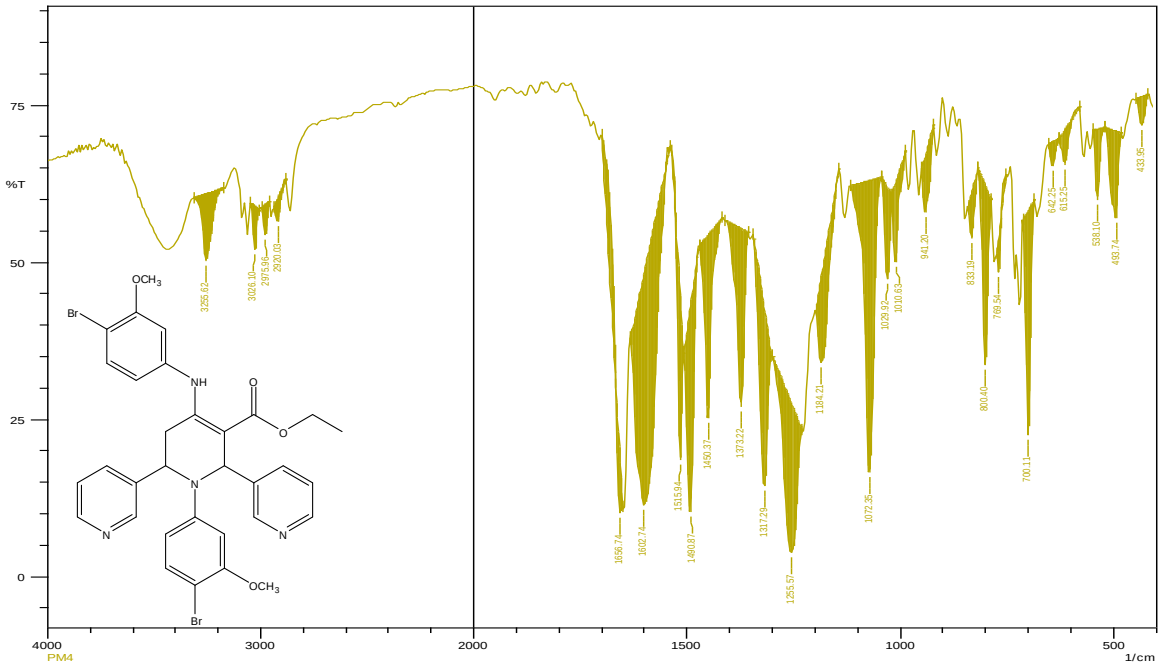
IR (KBr) cm^{-1}: 3234 (N-H str), 3028 (ArC-H str), 2950, (AlpC-H str), 1662 (C=O str), 1618, 1517, 1444 (C=C str), 1311 (C-N str).

A.3, 8.3.3 Infrared Spectra of PM3



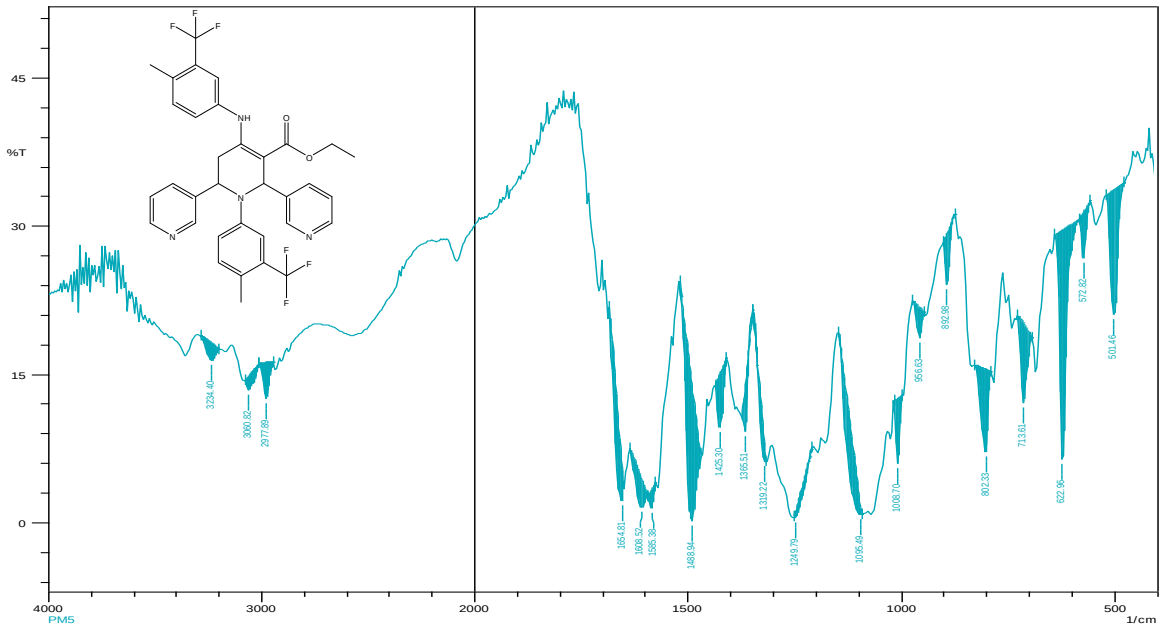
IR (KBr) cm^{-1}: 3240 (N-H str), 3058 (ArC-H str), 2974, (AlpC-H str), 1654 (C=O str), 1600, 1558, 1488 (C=C str), 1319 (C-N str).

A.3, 8.3.4 Infrared Spectra of PM4



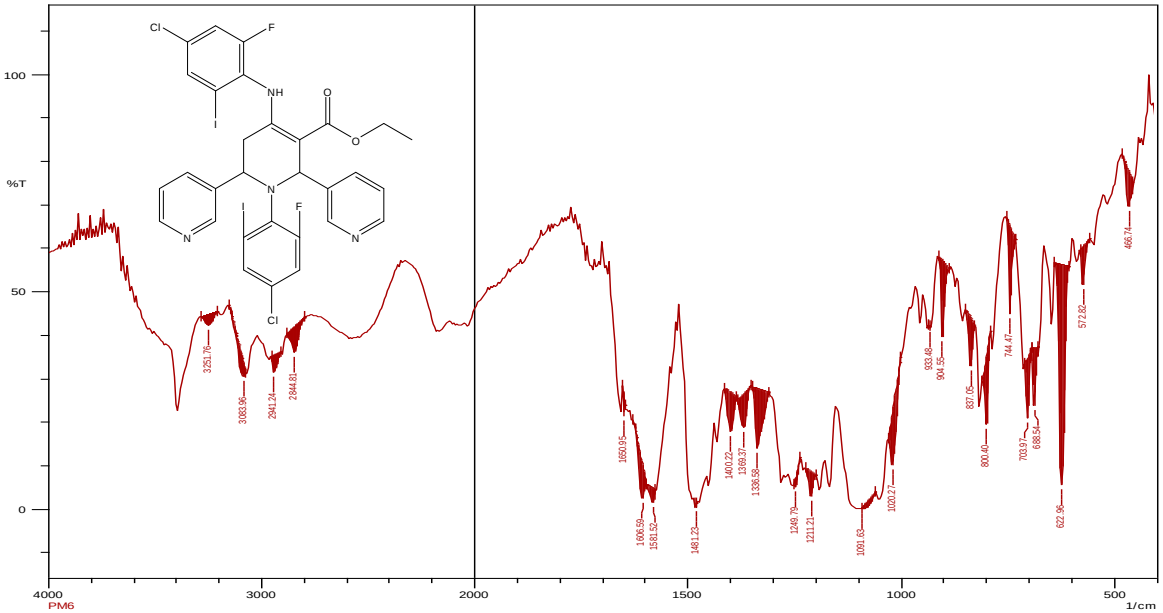
IR (KBr) cm^{-1}: 3255 (N-H str), 3026 (ArC-H str), 2975, (AlpC-H str), 1656 (C=O str), 1602, 1515, 1450 (C=C str), 1317 (C-N str).

A.3, 8.3.5 Infrared Spectra of PM5



IR (KBr) cm^{-1}: 3234 (N-H str), 3060 (ArC-H str), 2977, (AlpC-H str), 1654 (C=O str), 1608, 1585, 1425 (C=C str), 1319 (C-N str).

A.3, 8.3.6 Infrared Spectra of PM6



IR (KBr) cm^{-1}: 3251 (N-H str), 3083 (ArC-H str), 2941, (AlpC-H str), 1650 (C=O str), 1608, 1581, 1481 (C=C str), 1336 (C-N str).