Pharmacological Screening of Substituted 1, 4 Dihydropyrimidines

Submitted in partial 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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CO-SUPERVISOR : **Dr KN Venugopala**

REFERENCE DECLARATION

I, Reshme Govender – 19500281, Prof Bharti Odhav and Dr KN Venugopala do hereby declare that in respect of the following dissertation:
Title: Pharmacological Screening of Substituted 1, 4 Dihydropyrimidines.

1. As far as we ascertain:

a) no other similar dissertation exists;

2. All references as detailed in the dissertation are complete in terms of all personal communication engaged in and published works consulted.

Signature of student	**Date**
Signature of supervisor	**Date**
Signature of co-supervisor	**Date**

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** and **Dr K.N. Venugopala**.

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Student's signature

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ACKNOWLEDGEMENTS

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ABBREVIATIONS

Å	Angstrom
Ala	Alanine
Apaf 1	Apoptotic protease activating factor 1
Asp	Aspartate
CO_2	Carbon dioxide
ddH_2O	Double distilled water
DHPM	Dihydropyrimidine
DHPM 1	Methyl-2-(3-bromophenylamino)-4-(4-chlorophenyl)-6-methyl-1,4-dihydropyrimidine-5-carboxylate
DHPM 2	Methyl-4-(4-chlorophenyl)-2-(2-hydroxy-4-nitrophenylamino)-6-methyl-1,4-dihydropyrimidine-5-carboxylate
DHPM 3	Methyl-2-(3-bromo-4-fluorophenylamino)-4-(4-chlorophenyl)-6-methyl-1,4-dihydropyrimidine-5-carboxylate
DHPM 4	Methyl-4-(4-chlorophenyl)-2-(4-cyanophenylamino)-6-methyl-1,4-dihydropyrimidine-5-carboxylate
DHPM 5	Methyl- 4-(4-chlorophenyl)-6-methyl-2-(3-(trifluoromethylthio) phenylamino) -1, 4-dihydropyrimidine-5-carboxylate
DHPM 6	Methyl- 2-(4-bromophenylamino)-4-(4-chlorophenyl)-6-methyl-1,4-dihydropyrimidine-5-carboxylate
DHPM 7	Methyl-2-(3-chloro-5-hydroxyphenylamino)-4-(4-chlorophenyl)-6-methyl-1,4-dihydropyrimidine-5-carboxylate
DHPM 8	Methyl-4-(4-chlorophenyl)-2-(2,4-dimethoxybenzylamino)-6-methyl-1,4-dihydropyrimidine-5-carboxylate
DHPMs	Dihydropyrimidines
DISC	Death-inducing signaling complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2, 2-diphenyl-1-picrylhydrazyl
EtOH	Ethyl alcohol
FADD	Fas-Associated protein with death domain
FCS	Foetal calf serum
Gln	Glutamine
Glu	Glutamate

H_2O	Water
HCl	Hydrochloric Acid
His	Histidine
HTS	High throughput screening
IC	Inhibitory Concentration
Ile	Isoleucine
IUPAC	International Union of Pure and Applied Chemistry
Leu	Leucine
LO	Lipoxygenase
MCF – 7	Breast adencarcinoma
MCR	Multi-Component Reaction
MIC	Minimum Inhibitory Concentration
min	minutes
mm	millimeters
MTT	3-(4, 5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
NSAID	Non-Steroidal Antiinflammatory Drugs
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PDB	Protein Data Bank
Phe	Phenylalanine
PI	Propidium Iodide
$POCl_3$	Phosphorus oxychloride
PS	Phosphatidylserine
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
TLC	Thin layer chromatography
TNFR 1	Tumor Necrosis Factor Receptor 1
UACC – 62	Melanoma cell line
Val	Valine
WS	Working Solution

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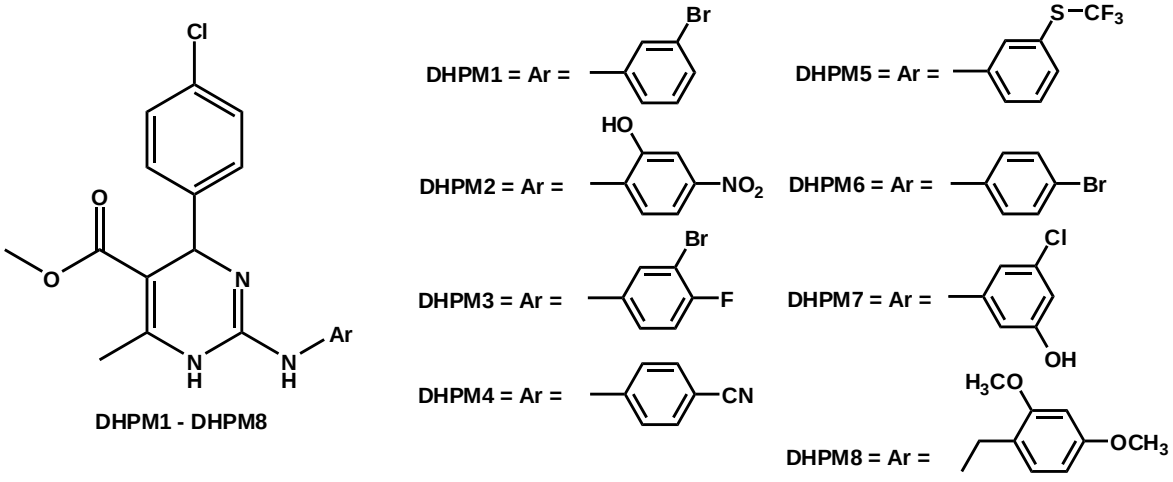
ABSTRACT

Pharmacological research is essential for the advancement of treatment therapies to combat diseases that plague mankind. Pyrimidines have been a subject under investigation by medicinal chemists for many years due to their interesting pharmacological properties. In previous studies, pyrimidines and their derivatives have been reported to have antimicrobial, anti-inflammatory, antimalarial, analgesic, and antitumour activities amongst other biological activities.

Although there has been a significant amount of research carried out on these heterocycles, there will always be a continuous need for the discovery of novel synthetic drugs which have a higher degree of potency and fewer side effects.
Hence, this study was undertaken to determine the pharmacological activities of eight novel 1, 4 dihydropyrimidine analogues (**DHPM 1 – 8**), that have been synthesized in our laboratory. The dihydropyrimidines were synthesized and characterized and thereafter evaluated for *in vitro* antimicrobial, antioxidant, anti-inflammatory, cytotoxicity and apoptotic activities. The compounds also underwent a safety study.



Antimicrobial activity was evaluated using the disk diffusion assay; compounds displaying superior activity were subjected to further analysis to establish the minimum inhibitory concentration. Overall compounds **DHPM 7** and **8** showed the best antibacterial activity against Gram positive bacteria.

The minimum inhibitory concentration (MIC) for **DHPM 7** against the Gram positive organisms (*B.cereus, S.aureus and B.coagulans*) was 0.75 µg/mL; however **DHPM 7** had a MIC of 0.37 µg/mL against *M. luteus.* **DHPM 8** *displayed* an MIC of 0.75 µg/mL against *B.cereus, S.aureus, M.luteus, S.faecalis and B.coagulans*.

Antioxidant activity was assessed using the DPPH method. **DHPM 2** showed outstanding free radical scavenging capacity of 90.63% at a concentration of 1 mg/mL. The **DHPM 1** - **8** were analysed for their lipoxygenase inhibitory activity. Excellent inhibition ranging from 59.37 ± 0.6 to 81.19 ± 0.94% was demonstrated. The inhibitory activity was elucidated by a molecular docking study against the lipoxygenase enzyme (PDB code = 3V99) using the MOE 2013.08 and Leadit 2.1.2 software and high affinities were demonstrated.

DHPM 1 - **8** were tested for cytotoxic activity against two human cancer cell lines, MCF-7 and UACC-62 by means of the MTT assay. It was observed for the MCF-7 cell line, **DHPM 1**, **4**, **6**, **7** and **8** displayed cytotoxicity above 89% at 50 µg/mL. The **DHPMs** at 50 µg/mL were noted to be very effective against the Melanoma cell line with **DHPM 2** having a cytotoxicity value of 82.62% and **DHPM 1**, **4**, **5**, **6**, **7** and **8** exhibiting cytotoxicity greater than 96%. Only slight inhibition of the proliferation of PBMC's was noted. IC_{50} values of **DHPM 1-8** were determined and the best activity overall was displayed by **DHPM 8**. The IC_{50} of **DHPM 8** was 0.92 ± 0.09 and 1.97 ± 0.08 µM against MCF - 7 and UACC - 62 cell lines, respectively. The compounds that displayed toxicity towards the UACC - 62 cell line were investigated for their apoptotic inducing potential. The apoptotic studies were performed by flow cytometry using the following assays; Annexin V, JC-1 and Caspase -3 assays. The effect of these compounds was compared to a known anti-cancer drug, Camptothecin. On evaluation of the mechanism of action of the compounds, it was found that most compounds are using apoptotic pathways for cell death.

Our studies have identified antimicrobial activity (**DHPM 1-8**) against Gram positive organisms, high antioxidant activity **(DHPM 2),** anti-inflammatory activity **(DHPM 1-8)** and anticancer activity **(DHPM 1-8)** against UACC-62 and MCF-7 cells.

DHPM 1-8 were found to have no toxicity at 100 µg/mL in the brine shrimp assay and hence are probably safe as therapeutic agents. Furthermore molecular docking studies confirmed the activity of DHPM **1-8** as potential lipoxygenase inhibitors.

DHPM 1-8 are novel compounds with great potential to be developed into chemotherapeutic agents.

1. INTRODUCTION

Medicinal chemistry is a science that involves the design, synthesis and characterization of pharmacologically active compounds. It also includes the study of the metabolism of the compounds, the structure activity relationships and the elucidation of their action at a molecular level (Thiel, 2013). The objective of medicinal chemistry is to discover and develop new drugs to combat and prevent disease.

Approximately ninety percent of pharmaceutical drugs and agrochemicals contain a heterocyclic ring structure, this confirms that heterocyclic chemistry is a cornerstone in the drug discovery process (Saini *et al*., 2013). Important classes of heterocycles in drug discovery are pyrimidines and their derivatives. Pyrimidines have a six membered ring structure, which contains two nitrogen atoms located at position one and three (Rao *et al*., 2013). This class of heterocycles are found extensively in nature and they are often the core structure of pharmacologically active compounds as the pyrimidine ring is a component of all living organisms (Thiel, 2013).

Dihydropyrimidines **(DHPMs)** which are derivatives of pyrimidines were first synthesized in 1893, by Pietro Biginelli (Biginelli and Gazz, 1893). Dihydropyrimidines are known as Biginelli compounds. They were overlooked as potential synthetic drugs and were not investigated until the 1980's when the interest in the compounds escalated (Kappe, 2000c). Combinatorial chemistry is a very constructive discipline of chemistry as it allows for the creation of various libraries of **DHPMs** which can undergo high throughput screening (HTS). HTS techniques have led to the unearthing of several remarkable biological properties of dihydropyrimidines (Kappe, 2000a).

Dihydropyrimidines have remarkable pharmacological efficiency and are known to possess a wide spectrum of biological activities such as anti-cancer (Kumar *et al*., 2009), anti-bacterial (Gößnitzer *et al*., 2002, Sedaghati *et al*., 2012), anti-fungal (Gößnitzer *et al*., 2002, Hussein *et al*., 2011, Sedaghati *et al*., 2012), antihypertensive (Chikhale *et al*., 2009), anti-tubercular (Trivedi *et al*., 2010, Narayanaswamy *et al*., 2013), and anti-inflammatory activities (Devi *et al*., 2009).

The above pharmacological properties have prompted further research into the synthesis of various derivatives of dihydropyrimdines by creating structural modifications of the side chains, with a common dihydropyrimidine pharmacophore.

The aim of this research was to investigate the biological properties of the eight synthesized novel dihydropyrimidines (**DHPM 1 – 8**) which was accomplished through the following objectives:

Objectives

1. To determine the antimicrobial (antibacterial and antifungal) activity of **DHPM 1 – 8**.
2. To determine the antioxidant activity of the eight **DHPM 1-8**.
3. To determine the anti-inflammatory activity of the eight **DHPM 1-8**.
4. To perform molecular modelling studies to determine the mode of binding of **DHPM 1-8** as lipoxygenase inhibitors.
5. To evaluate the safety of **DHPM 1-8** using the Brine Shrimp Lethality Assay and the Ames Mutagenicity Assay.
6. To determine the anticancer activity of **DHPM 1-8** using the MTT assay 3- [4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide assay on human breast adenocarcinoma (MCF - 7) and human melanoma (UACC - 62) cell lines.
7. To determine the apoptotic activity of **DHPM 1-8** that show potent cytotoxic activity using assays for the following:
    - Annexin V
    - JC - 1
    - Caspase - 3

An overview of the research and the structure of the thesis are presented in Figure 1.

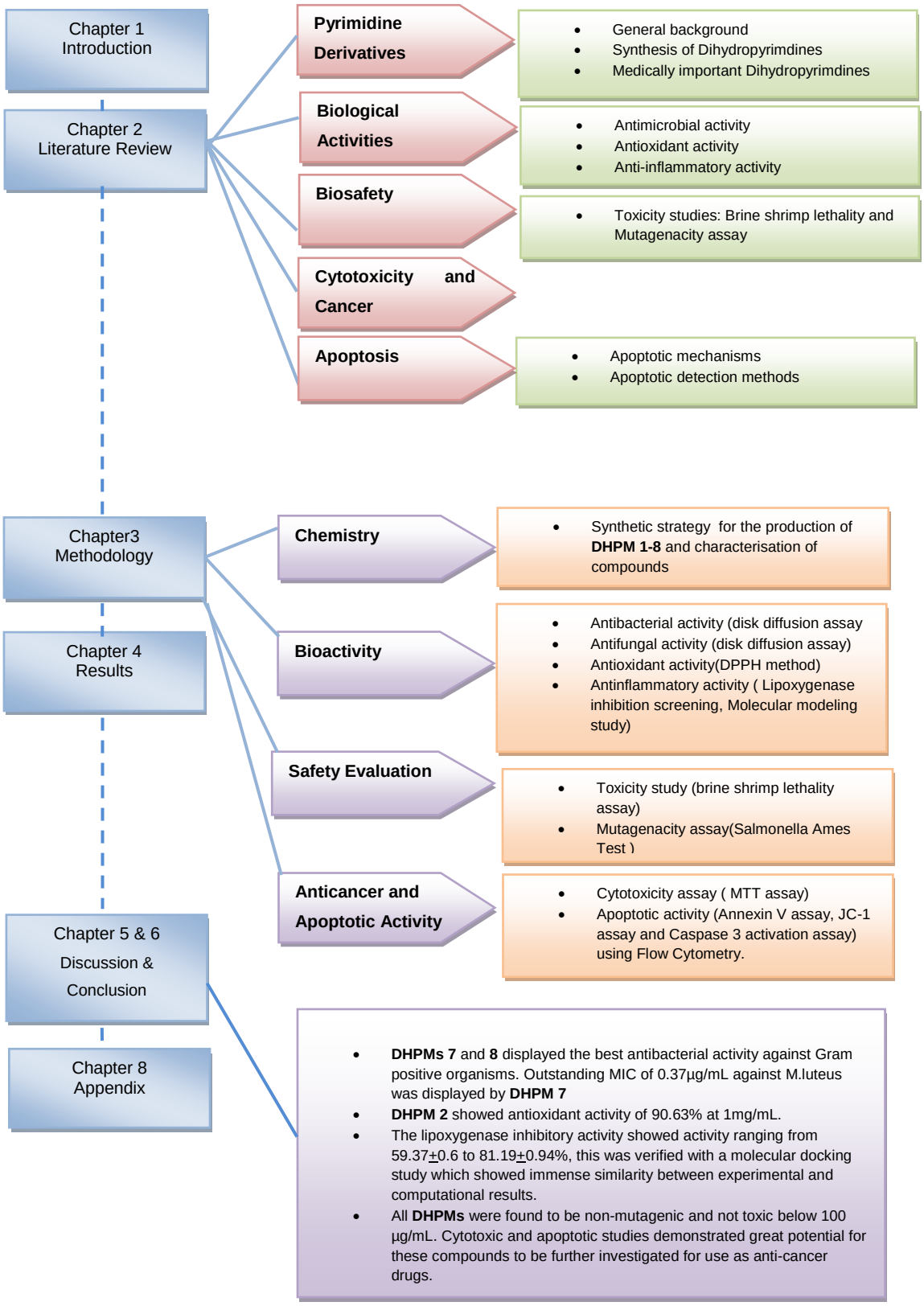
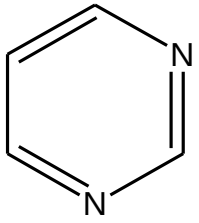


Figure 1. Overview of the thesis.

2. LITERATURE REVIEW

2.1. Pyrimidine derivatives

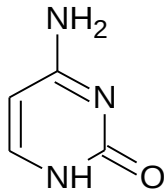
Pyrimidines are an important class of aromatic heterocyclic compounds containing two nitrogen atoms **(1)**. Pyrimidine derivatives are important lead compounds for the development of biologically active compounds (Mishra and Tomar, 2011).



(1)

The molecular formula of pyrimidine is $C_4H_4N_2$ and its molecular weight is 80 daltons. Pyrimidine is colourless in nature and has a boiling point of 124°C and a melting point of 22.5°C. X-ray diffraction studies performed to determine the dimensions of the pyrimidines at -2°C found that the dimensions are similar to that of their bioisosteres pyridines (Verma *et al*., 2012).

Pyrimidines have been known for their exceptional biological worth. Their biological activity can be attributed to the fact that there are pyrimidine bases in cytosine **(2),** uracil **(3)** and thymine **(4)**, which are integral components of nucleic acids; DNA and RNA.



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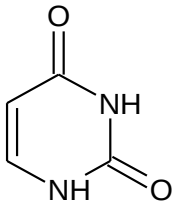

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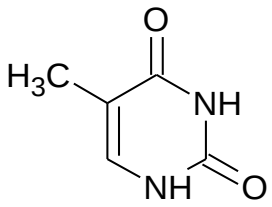
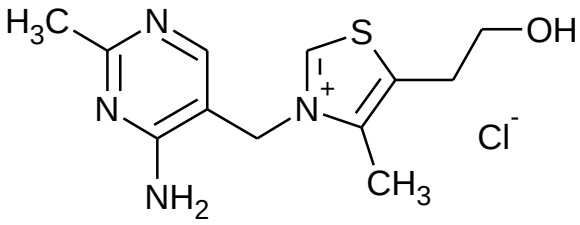


(4)

It is also significant to note that the pyrimidine pharmacophore is also present in various B vitamins such as Vitamin B1 **(5),** Vitamin B2 **(6)** and Vitamin B9 **(7)**.The pyrimidine nucleus is also found in certain hypnotics e.g. barbituric acid and its analogue Veranal.



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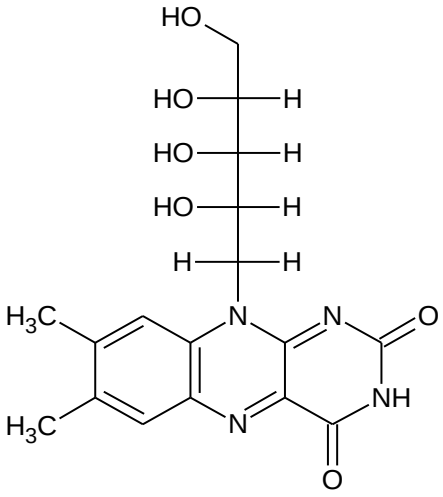

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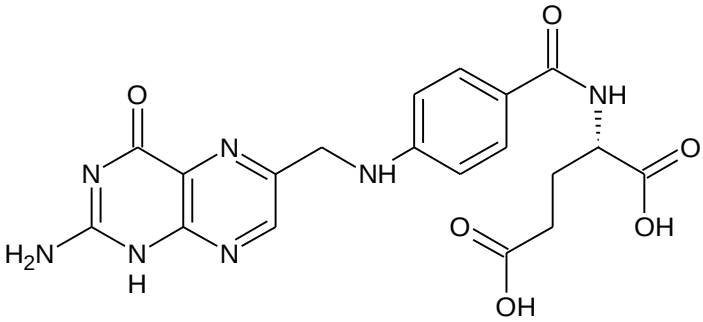

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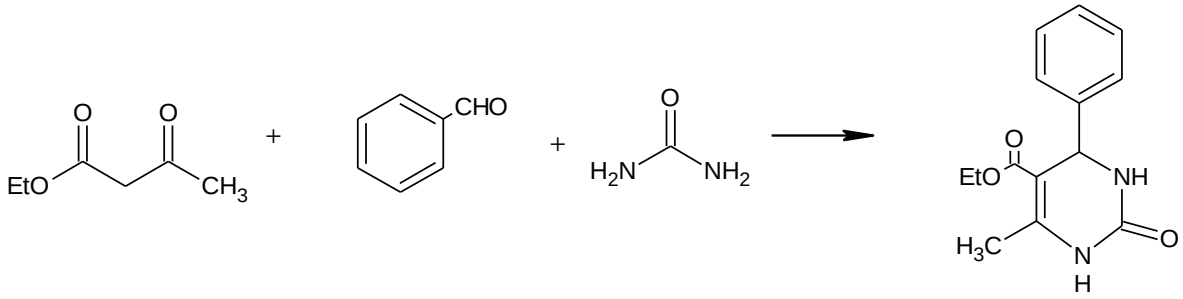
2.2. General synthesis of dihydropyrimidines

In medicinal chemistry, multicomponent reactions (MCR's) are of escalating importance. MCR's offer important advantages, they are used to produce libraries of drugs which are screened for biological activities. By the use of combinatorial chemistry, MCR's are capable of producing new lead compounds for drug discovery. Drugs with diverse applications are a result of reactions in which there is a combination of different reactants in a single reaction, this is also known as combinatorial efficiency (Thakur and Trivedi, 2011).

MCR's are described as reactions in which there are three or more reactants. These reactants are brought together in a reaction vessel and they form novel products which are composed of a fraction of all the reactants (Dallinger and Kappe, 2005).

An important MCR was reported in 1893 by Piettro Biginelli, this was the synthesis of 3, 4- dihydropyrimidin-2(1H)-ones **(8)** otherwise known as **DHPMs**. This cyclocondensation reaction illustrated in Figure 2, involved the heating of an ethyl acetoacetate **(9)**, aromatic aldehyde **(10)** and urea **(11)** which was dissolved in ethanol, using HCl as a catalyst. This synthetic reaction is known as the Biginelli Synthesis. The product of this novel reaction precipitated when cool and was positively classified as a 3,4-dihydropyrimidin-2(1H)-one (Kappe, 2000a). The Biginelli synthesis did have a disadvantage of low yield production however research has found reactions that improved this drawback, these include the Lewis-acid catalysis, and processes that are solvent free and microwave synthesis.



(9) **(10)** **(11)** **(8)**

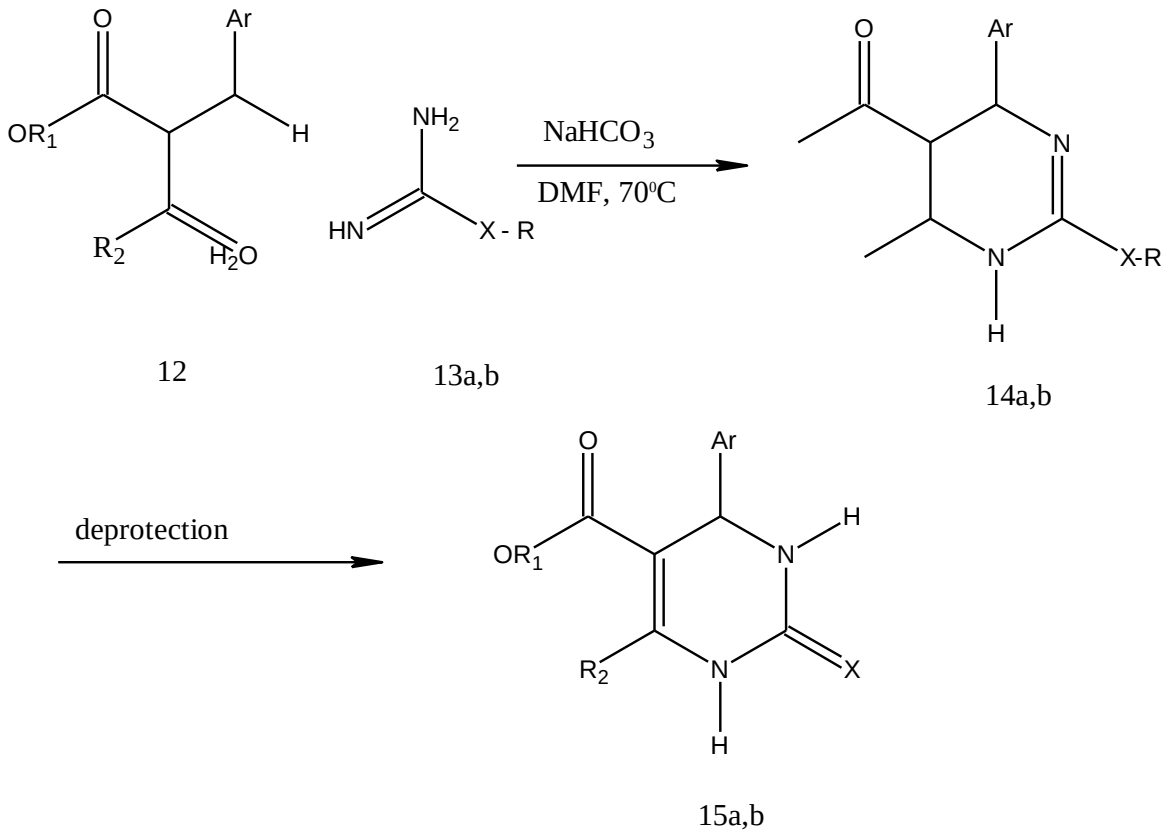
Figure 2. The Biginelli Dihydropyrimidine synthesis.

The Biginelli reaction depicting an acid catalyzed cyclocondensation of a 1, 3 dicarbonyl component (9), an aromatic aldehyde (10) and urea (11) or a urea analogue.

There are alternate synthetic strategies for the synthesis of **DHPMs**. However, most of the alternate methods are complex when compared to the Biginelli one-pot one step process. In chemistry today most of the alternate methods cannot measure up to the fundamental approach of the Biginelli MCR, with the exception of the the 'Atwal modification' of the Biginelli condensation (Figure 3: **12**, **13**, **14** and **15**)**.**

The Atwal modification involves the condensation of an enone with a thiourea /urea in basic conditions, finally there is deprotection of the resultant 1, 4 dihydropyrimidine with HCl and the product is the preferred **DHPM**. This method requires one to have enones prepared in advance, however it is known to be a reliable and widely applicable method. Therefore, the Atwal Modification is a worthy alternative synthetic procedure compared to the conventional Biginelli synthesis.

Since the discovery of the Biginelli dihydropyrimidine MCR in 1893, the process has advanced from the synthesis of straight forward pyrimidine heterocycles to the creation of targeted libraries of compounds with various biological functionalities. A survey of the literature has revealed that the above two methods have been most frequently used for the synthesis of **DHPMs** (Kappe, 2000b, Kappe, 2000c, Kappe, 2003, Kappe and Stadler, 2004, Yadav *et al*., 2007, de Fátima *et al*., 2014)



a: X = O, R = Me
b: X = S,R = 4 methoxybenzyl

Figure 3. The Atwal-modification of the Biginelli condensation reaction (O'Reilly and Atwal, 1987).

2.3. Medically important dihydropyrimidines

There are various dihydropyrimidines that possess important medical properties and exhibit superior pharmacological activities. The synthetic flexibility of the pyrimidine ring structures has allowed the production of derivatives with varying biological activities. The derivatives are achieved by substitution of the aryl ring or substitution at the different carbon positions (Selvam *et al*., 2012).

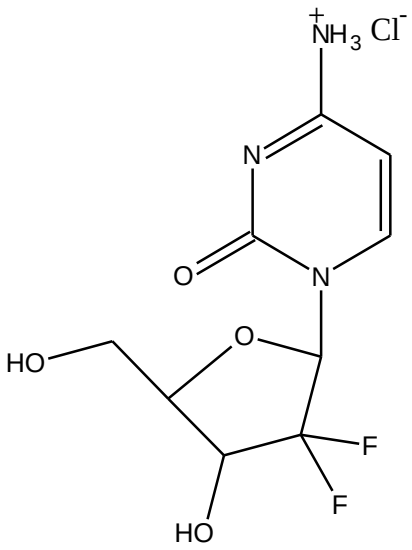
To understand the pharmacological properties of these derivatives, we need to understand the relationship of the chemical compound with the human body and how the compound relates to the body. It is also essential to understand the mechanism of action of the compounds on the body.

A few of the current pyrimidine based drugs available in the commercial market:

2.3.1. Gemcitabine

Gemcitabine HCl **(16)** is identified as a pyrimidine antimetabolite /nucleoside drug. It has a IUPAC name of 4-amino-1-[(2R, 4R, 5R)-3, 3-difluoro-4- hydroxy-5-(hydroxymethyl)-oxolan-2-yl] pyrimidin-2-one mono-hydrochloride (Menon *et al*., 2012).

Gemcitabine is the preferred treatment for cancer of the pancreas and its use is progressively increasing to treat other cancers such as breast, bladder, and non-small cell lung cancer. Although the drug has an extensive range of use, there is still the dilemma of understanding resistance and sensitivity of the drug (de Sousa and Monteiro, 2014).



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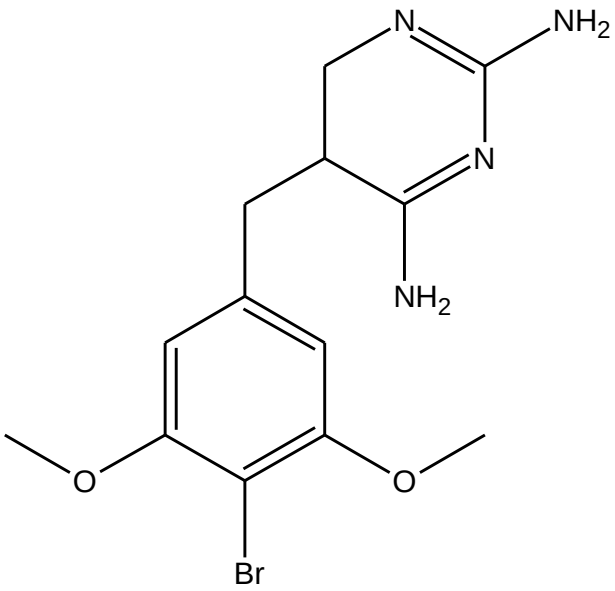
2.3.2. Brodimoprim

Brodimoprim **(17)** is a 2,4-diaminobenzylpyrimidine based anti-bacterial agent with the IUPAC name of 5-[(4- bromo- 3,5- dimethoxyphenyl) methyl] pyrimidine- 2,4- diamine] (Mishra and Tomar, 2011). This compound is known for its efficacy in the treatment of respiratory infections, typhoid fever and bacterial gastroenteritis (Braunsteiner and Finsinger, 1993). There are various other pyrimidine based antibacterial drugs such as Trimethoprim, Tetroxoprim, Metioprim and Piromidic acid (Selvam *et al*., 2012)



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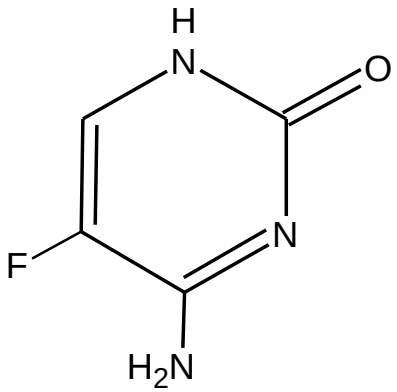
2.3.3. Flucytosine

Flucytosine **(18)** is the only anti-fungal drug approved by the FDA that falls into the classification of being pyrimidine based (Dismukes, 2000). The IUPAC name of Flucytosine is 5-Fluorocytosine / 4-Amino-5-fluoropyrimidine-2(1H)-one (Selvam *et al*., 2012). Flucytosine has a narrow range of activity limited to the following organisms, the *Candida* species, *Cryptococcus neoformans* and a few moulds (Dismukes, 2000).



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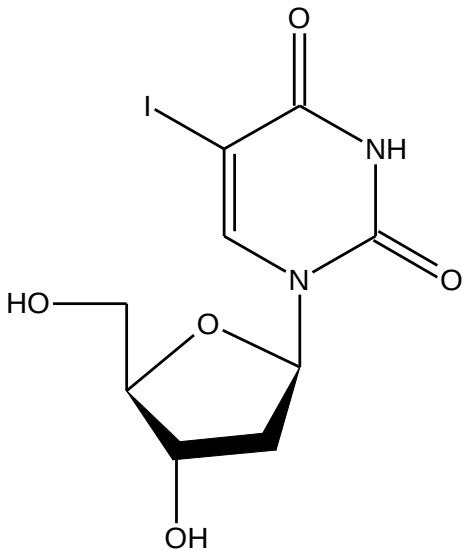
2.3.4. Idoxuridine

Pyrimidines are also known to exhibit antiviral properties. Idoxuridine **(19)**, [IUPAC name 5-Iododeoxyuridine] is an antiviral agent that demonstrates highly selective activity and has been used widely to treat viral infectivity. In addition to Idoxuridine, Broxuridine is another available pyrimidine antiviral agent (Jain *et al*., 2006).



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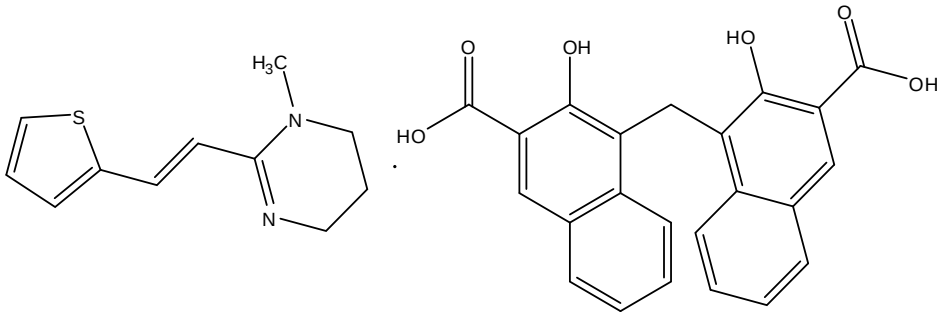
2.3.5. Pyrantel embonate (Pyrantel Pamoate)

There are pyrimidine analogues which are used as anthelmintics and their function is to eliminate parasites from the body. Pyrantel Pamoate **(20)** is a pyrimidine based anthelmintic and its IUPAC name is 1, 4, 5, 6-tetrahydro -1-methyl - 2 - (trans-2 - (2-thienyl) - vinyl) – pyrimidine embonic acid salt. This drug is quite efficient in treating infestations caused by pinworms and roundworms (Pandey *et al*., 1971).



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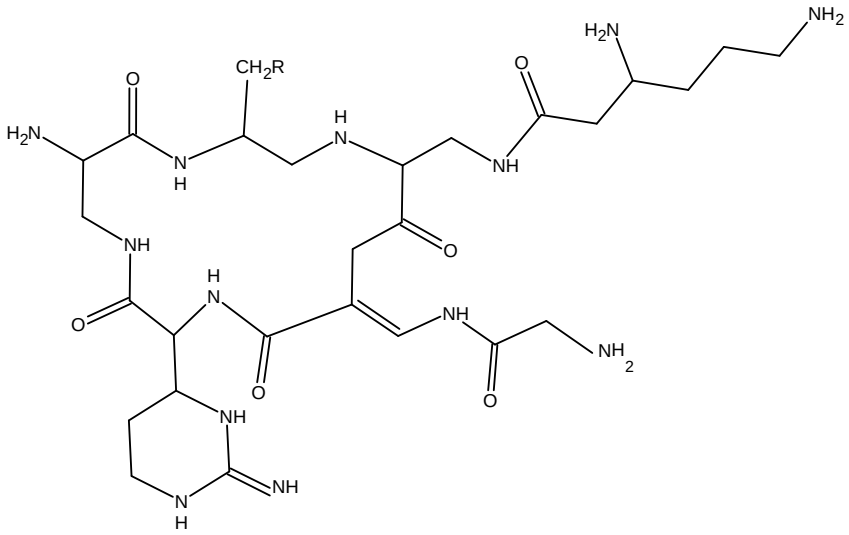
2.3.6. Capreomycin

Capreomycin **(21)** is a pyrimidine containing drug that is used to treat multi-resistant drug tuberculosis. It is produced by a bacterium called *Streptomyces capreolus* (Herr and Redstone, 1966). Another related pyrimidine based antitubercular drug is Viomycin.



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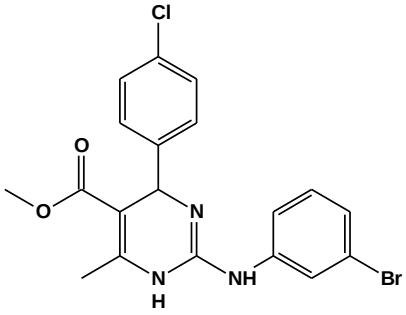
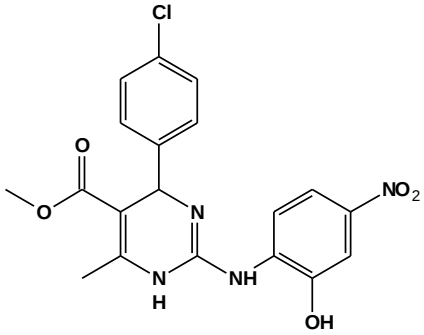
2.4. Synthesis of Dihydropyrimidine compounds (DHPM 1 – 8)

The pharmacological significance of dihydropyrimidine derivatives used for medicinal purposes are well documented in sections 2.3 of this thesis. Their pharmacological activities were taken into consideration when conducting the research and development of these heterocyclic compounds [**DHPM 1-8**].

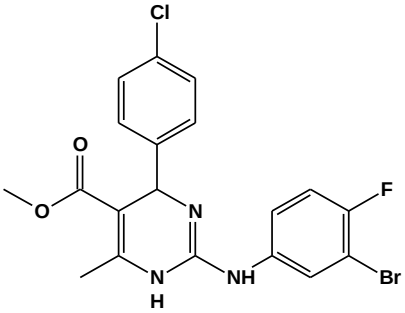
DHPM 1-8 were synthesized and characterized at Durban University of Technology using Lipinski's rule of five (Lipinski *et al*., 2012). All of the eight analogues that have been synthesized have the common 1, 4 dihydropyrimidine nucleus with different side chains. These structures are depicted in Figure 4.



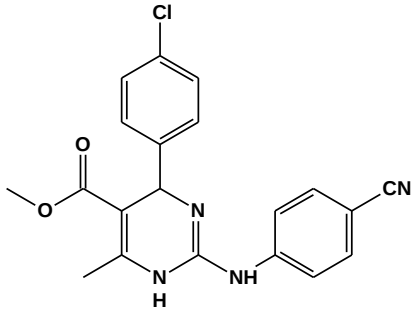
Methyl 2-((3-bromophenyl)amino)-4-(4-chlorophenyl)-6-methyl-1,4-dihydropyrimidine-5-carboxylate (**DHPM 1**)



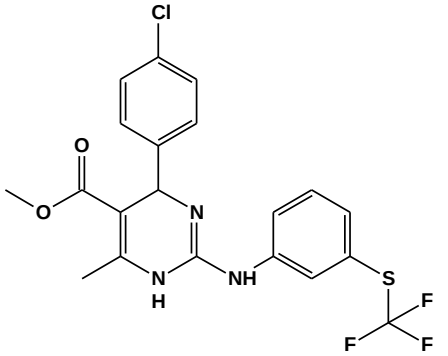
Methyl 4-(4-chlorophenyl)-2-((2-hydroxy-4-nitrophenyl)amino)-6-methyl-1,4-dihydropyrimidine-5-carboxylate (**DHPM2**)



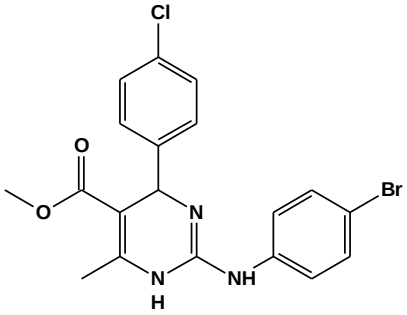
Methyl 2-((3-bromo-4-fluorophenyl)amino)-4-(4-chlorophenyl)-6-methyl-1,4-dihydropyrimidine-5-carboxylate (**DHPM3**)



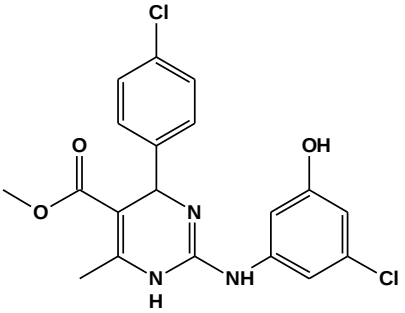
Methyl 4-(4-chlorophenyl)-2-((4-cyanophenyl)amino)-6-methyl-1,4-dihydropyrimidine-5-carboxylate (**DHPM4**)



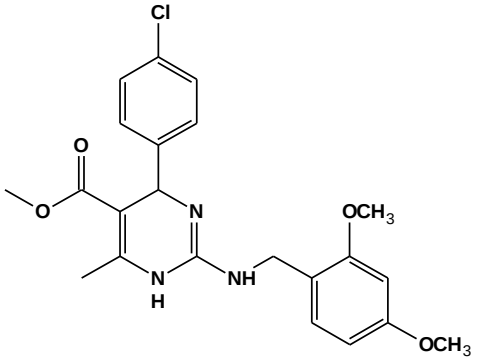
Methyl 4-(4-chlorophenyl)-6-methyl-2-((3-((trifluoromethyl) thio)phenyl)amino)-1,4-dihydropyrimidine-5-carboxylate (**DHPM5**)



Methyl 2-((4-bromophenyl)amino)-4-(4-chlorophenyl)-6-methyl-1,4-dihydropyrimidine-5-carboxylate (**DHPM6**)



Methyl 2-((3-chloro-5-hydroxyphenyl)amino)-4-(4-chlorophenyl)-6-methyl-1,4-dihydropyrimidine-5-carboxylate (**DHPM7**)



Methyl 4-(4-chlorophenyl)-2-((2,4-dimethoxybenzyl)amino)-6-methyl-1,4-dihydropyrimidine-5-carboxylate (**DHPM8**)

Figure 4. Structures and IUPAC names of DHPM 1-8 synthesized.

2.5. Biological activities of dihydropyrimidines

2.5.1. Anti-microbial activity

Despite the progress in medicinal chemistry, the emergence of new infectious diseases and the increase in resistance to anti-microbial agents is a threat to public health (Cos *et al*., 2006). Therefore there is a great need for research to be conducted into the development of novel anti-microbial agents.

Currently, the screening methods to detect antibacterial and anti-fungal activity are categorized into three groups, which include diffusion, bioautography and dilution methods. Bioautographic and diffusion methods are qualitative methods as they only give an indication into the presence or absence of anti-microbial activity while the dilution method is quantitative because it is used to determine the minimum inhibitory concentration (Valgas *et al*., 2007).

It is well documented in the literature that modified Biginelli product scaffolds (dihydropyrimidines) possess a broad range of biological activities which include both anti-bacterial and anti-fungal activities (Jadhav *et al*., 2012). Many dihydropyrimidine derivatives that have been synthesized were analyzed for anti-microbial activity(Gößnitzer *et al*., 2002, Singh *et al*., 2008, Rajanarendar *et al*., 2010, Hussein *et al*., 2011) and it was observed that certain DHPM derivatives demonstrated higher activity than the standard reference drugs, Ciprofloxacin (Chitra *et al*., 2010) and Amphotericin B (Rajanarendar *et al*., 2010).

2.5.2. Anti-oxidant activity

Anti-oxidants are classified by their mode of action as free radical scavengers, chelators of metal ions and as oxygen scavengers. Reactive oxygen species (ROS) and free radicals are the contributing agents for many human diseases (Jadhav *et al*., 2012). Free radicals are unstable and are able to react with biological substrates such as DNA, proteins and lipids, thus causing cellular damage (Gressler *et al*., 2010). This type of cell / tissue damage has been linked to the following medical conditions: atherosclerosis, ageing, cancer, cardiovascular and Alzheimer’s disease.

Anti-oxidants have always interested researchers because they play a vital role in the prevention of degenerative diseases. The development and identification of novel anti-oxidants that are capable of scavenging and eradicating reactive oxygen species such as the superoxide-(O_2), hydroxyl-(OH), peroxyl-(ROO) and nitric oxide radicals (NO) are essential to prevent oxidation related diseases (Albano *et al*., 2012).

There are several methods to measure free radical scavenging capacity, but it has been reported that the most reliable method measures the disappearance of coloured free radicals such as the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) by means of a spectrophotometer (Choi *et al*., 2007).

There are many dihydropyrimidine derivatives that have been found to exhibit moderate to good antioxidant activity. It was previously reported that dihydropyrimidine derivatives synthesized with a 3-nitrophenyl moiety at position four on the dihydropyrimidine ring showed good anti-oxidant activity (Kumar *et al*., 2009); it was also found that DHPM derivatives with hydroxyl groups on the benzene rings of the DHPMs contributed to high antioxidant activity (Gangwar and Kasana, 2012).

2.5.3. Anti-inflammatory activity

Inflammation is the normal response of the body's immune system when it is invaded by pathogens, stimulated by antigens or when the body experiences physical, chemical or traumatic injury (Baylac and Racine, 2003). Pro-inflammatory cytokines and chemokines are produced during the interaction of endogenous or exogenous antigens within the immune system. This interaction is as a result of the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) within the cellular immune system. The continual production of ROS from inflamed cells can bring about cell damage and lead to various conditions such as arthritis, asthma and psoriasis (Rashmi *et al*., 2011, Abbas *et al*., 2012)

Inflammation is a complex process which involves chemical messengers, of which an essential class is pro-inflammatory leukotrienes. Leukotrienes are a product of arachidonic acid metabolism, which starts with its oxidation by the lipoxygenase enzyme (5-LOX) (Baylac and Racine, 2003). The Lipoxygeanse enzyme catalyses the

incorporation of oxygen into arachidonic acid and linoleic acid, which are poly-unsaturated fatty acids (PUFAs) (Figure 5).

Currently non-steroidal anti-inflammatory drugs (NSAID's) such as aspirin, phenylbutazone, ibuprofen and indomethacin are used for the treatment of inflammatory conditions (Sondhi *et al*., 2012). However, NSAID's are associated with several side effects that are an important health concern when used continuously. These side effects include adverse reactions in the gastrointestinal tract, kidney, liver, central nervous system and dermis (Brooks, 1998). Therefore, there is a constant need to develop new and safer anti-inflammatory drugs with fewer health risks.

Many synthetic dihydropyrimidines were reported to have satisfactory anti-inflammatory activity (Tozkoparan *et al*., 1999, Chikhale *et al*., 2009, Mishra *et al*., 2010, Abbas *et al*., 2012, Bhalgat *et al*., 2014).

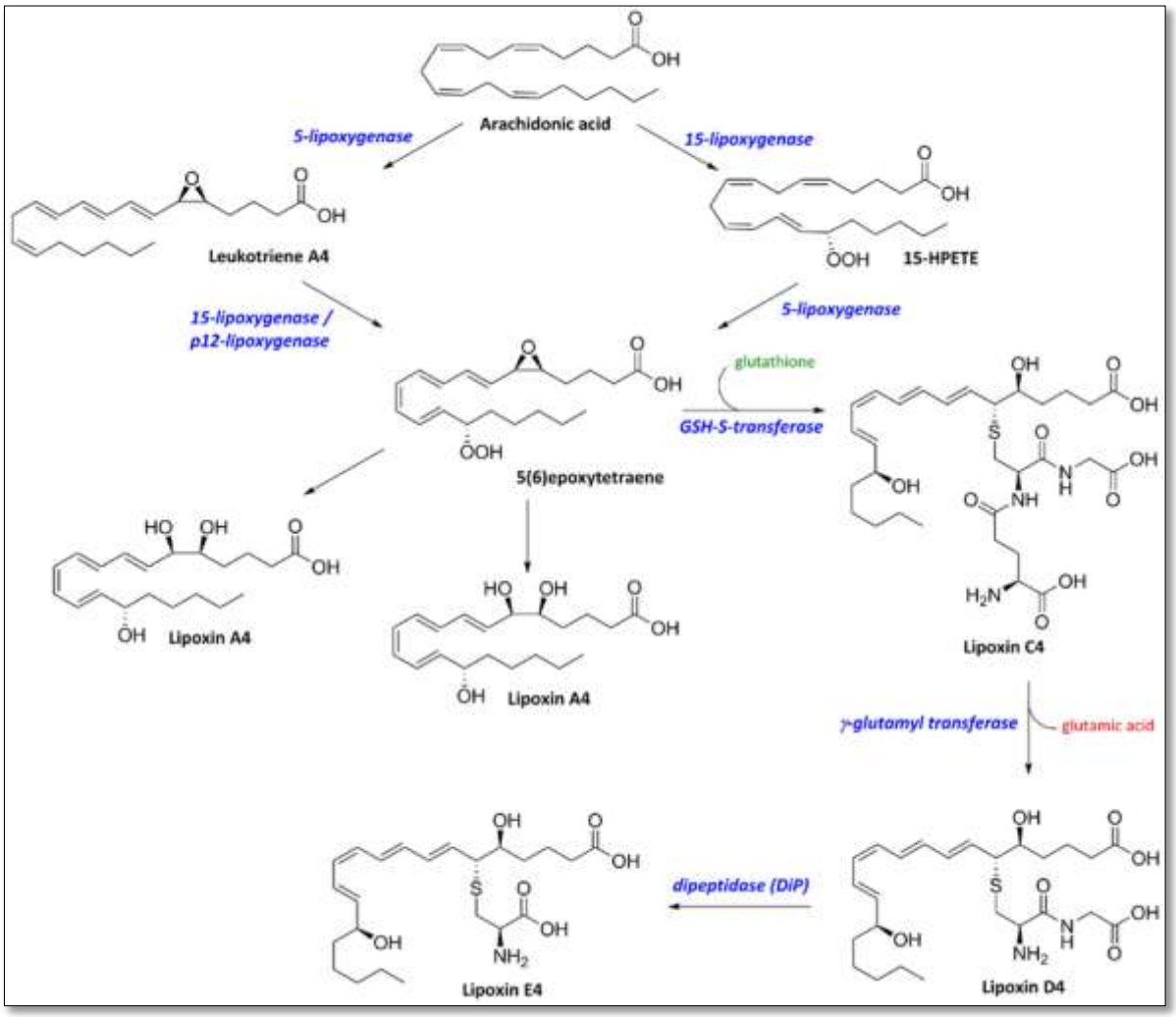


Figure 5. Oxidation reactions of lipoxygeanses (Wisastra and Dekker, 2014).

2.6. Biosafety of compounds

All drugs are synthesized with the aim of the drug possessing pharmacological properties. However, it is known that there can be traces of impurities in the synthetic drugs. These impurities arise during synthesis or storage of the drugs. Impurities can be present in the drug as a result of the use of reagents used to produce the drug and also the presence of by- products and intermediates of the synthetic reaction (Kenyon *et al*., 2007).

Impurities in drugs carry the risk of toxicity in terms of producing a mutagenic and carcinogenic effect (Müller *et al*., 2006).To ensure effective control of impurities in drugs there are various guidelines and regulations that need to be adhered to. If the guidelines are followed this ensures that there is minimal contamination in the drugs produced (Jacobson-Kram and McGovern, 2007).

It was therefore necessary to evaluate the toxicity and mutagenicity of the compounds in this research. There are various methods to assess the biosafety of compounds. The biosafety tests used in this study included the brine shrimp lethality assay for toxicity and the Salmonella Ames assay for determining the mutagenic effect.

2.6.1. Toxicity

Bioactive compounds have been discovered to be toxic at elevated concentrations (McLaughlin *et al*., 1993). Synthetic bioactive compounds need to be monitored for toxicity to determine the levels at which they are considered safe. In this instance the brine shrimp lethality assay was used to examine the toxicity of the **DHPM** compounds. The brine shrimp lethality test was reported to be an effective means of determining toxicity (Carballo *et al*., 2002). In this assay the eggs of *Artemia salina* are hatched in a brine solution and the nauplii (Figure 6) are exposed to different concentrations of compounds. The brine shrimp lethality assay is an attractive method as it is considered to be a reliable, quick, economical and a simple benchtop test. *Artemia salina* are used as a test organism as it is known to be a suitable tool to determine whether the pharmacologic activities produced by compounds are also toxic to the nauplii of the organism (Meyer *et al*., 1982).

Figure 6. The life cycle of the *Artemia salina* (Gajardo and Beardmore, 2012).

2.6.2. Mutagenicity

When conducting safety studies it is important to be able to identify mutation inducing substances. Mutagenic compounds are hazardous to mankind and it has been discovered that when compounds can cause mutagenesis in bacteria they may cause cancer in animals (Déciga-Campos *et al*., 2007).

The Ames Salmonella / microsome mutagenicity bioassay (Maron and Ames, 1983) is capable of identifying drugs or chemicals that are able to bring about genetic damage which in turn results in genetic mutations (Tejs, 2008). The assay uses Salmonella strains with different mutations in genes in the histidine operon. Salmonella strains are grown on minimal agar with traces of histidine and bacteria that can revert to histidine independence form colonies. Spontaneous revertant colonies are normally constant but on exposure to a mutagen the revertant colonies increase in a dose-dependent manner.

This short-term bacterial assay is utilized when new drugs are presented to regulatory bodies for registration (Mortelmans and Zeiger, 2000). In this research the following standard Salmonella tester strains were used, TA 98 and TA 100, (Figure 7 A and Figure 7 B) both these strains contain the R-factor plasmid named pKM101. Maron and Ames, (1983) recommend these two strains amongst others for general mutagenicity assays. TA 98 is sensitive to frame shift mutations whilst TA 100 is sensitive to base pair mutations (Chandrasekhar *et al*., 2013).

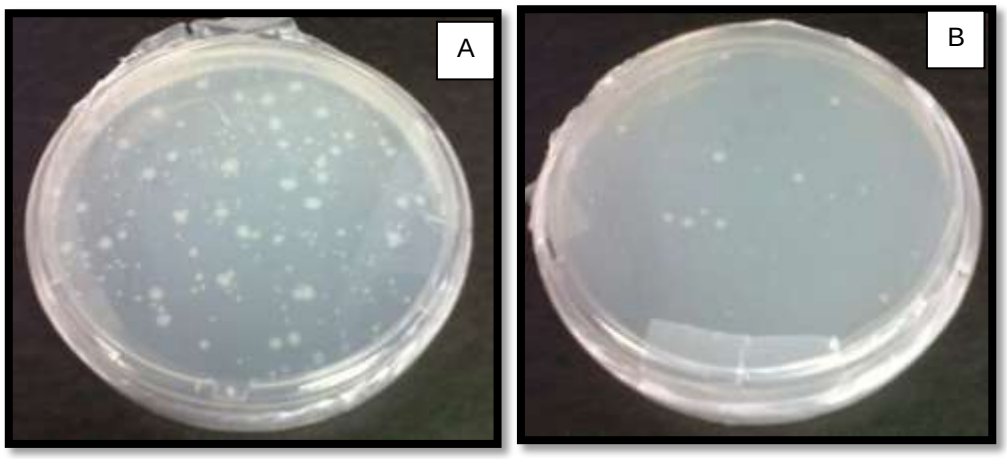


Figure 7. A - *Salmonella typhimurium* TA 100 and B – *Salmonella typhimurium* TA 98

2.7. Molecular modeling

Molecular modeling is a tool that is used for the discovery, design and prediction of the mechanisms of action and activity of active compounds. Molecular modeling is easy to carry out with the software that is available; however the difficulty lies in utilizing the correct model and in interpretation.

Molecular modeling uses computers to construct molecules and to conduct calculations to determine the chemical characteristics of various molecules (Mukesh and Rakesh, 2011). Molecular docking is a technique that predicts the orientation or pose of all the possible conformations of molecules within the enzymes active site and it can also be used to determine the binding affinity between different molecules by use of scoring functions (Eweas *et al*., 2014). Therefore molecular docking is an important tool for the rational design and studying of structure activity relationships in lead compounds.

2.8. Cancer

Cancer is a devastating and life threatening disease that affects more than 10 million people worldwide every year (Bariwal *et al*., 2012). Cancer is accountable for 25% of the deaths in developed countries and 15% of the deaths worldwide**.** It is a disease that affects approximately 200 different types of cells (de Mesquita *et al*., 2009). Cancer displays the following characteristics (Figure 8):

a) Evasion of growth suppressors
b) Avoids immune destruction
c) Programmed cell death (apoptosis) is evaded
d) The replicative potential is immortal
e) Induction of angiogenesis
f) Activation of metastasis and invasion
g) Tumour promoting inflammation
h) Genome instability and mutation
i) Deregulation of cellular energetics
j) Sustains proliferative signalling

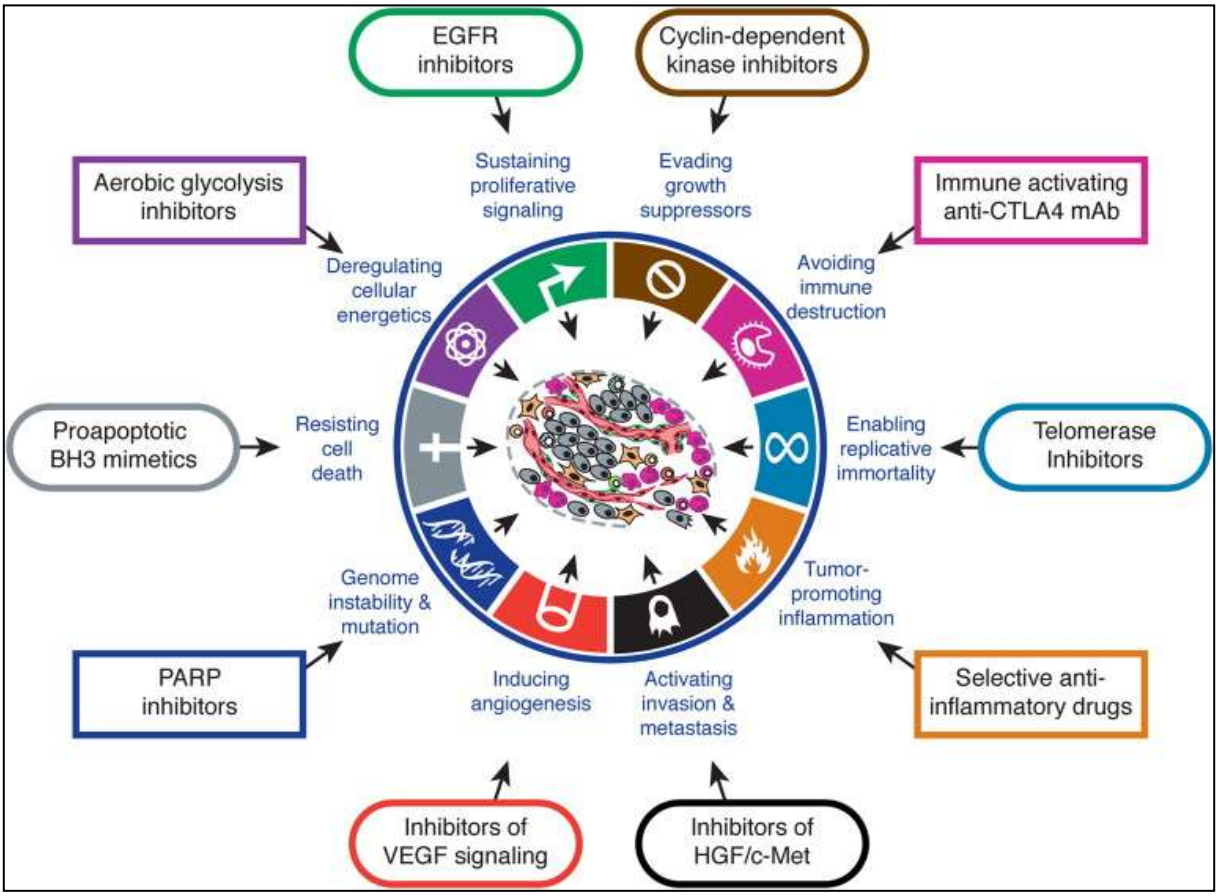


Figure 8. Characteristics of cancer cells (Hanahan and Weinberg, 2011).

The above are the malignant properties of cancer, which distinguish them from tumours that are benign. Benign tumours are self-limiting, non-invasive and do not metastasize (Sukhramani *et al*., 2011). In this study, cancer cells were exposed to **DHPM 1-8** and their cytotoxic effect was determined using the MTT assay. Some compounds maybe cytotoxic to cancer cells and yet be without effect to normal cells.

Although, there have been numerous developments in medicine, cancer is still a life threatening disease. Current treatment modalities present many hurdles, which include drug resistance, toxicity and low specificity. Therefore it is essential to discover new compounds with cytotoxic activities and reduced side effects (de Mesquita *et al*., 2009). In cancer many genetic changes and mutations occur. These transform a normal cell into a malignant cell and this in turn leads to increased proliferation and decreased apoptosis. The deregulation of apoptosis can lead to various diseases, while abnormal apoptosis may lead to cancer (Jayakiran, 2015). Dihydropyrimidines have been reported to have anticancer properties and are new leads in anticancer therapy (Klimova *et al*., 2012, Tawfik *et al*., 2009). Some of the current anticancer drugs belonging to this class include Gemcitabine, Fluorouracil and Cytarabine.

2.9. Apoptosis

Cell death occurs by two different modes, which are apoptosis and necrosis. Apoptosis is an active process in which there is a programmed and managed form of cell death (Schutte *et al*., 1998). Necrosis is a passive mode of cell death, which is disorderly and accidental and is regarded to be a toxic process (Saraste and Pulkki, 2000).

During apoptosis there are specific biochemical and physical changes (Figure 9) that occur within the cytoplasm, nucleus and plasma membrane. These characteristic changes include chromatin condensation and fragmentation, cell shrinkage, plasma membrane blebbing and the development of apoptotic bodies that contain cytoplasmic or nuclear material (Lawen, 2003).

Necrotic cells exhibit the following characteristics:

- cytoplasmic swelling
- vacuolation
- plasma membrane disruption
- mitochondrial dilation
- presence of swollen endoplasmic reticulum and Golgi apparatus
- condensation of the chromatin network.

The swollen cells burst and discharge their contents over neighboring cells, which results in an anti-inflammatory response (Wyllie, 1997).

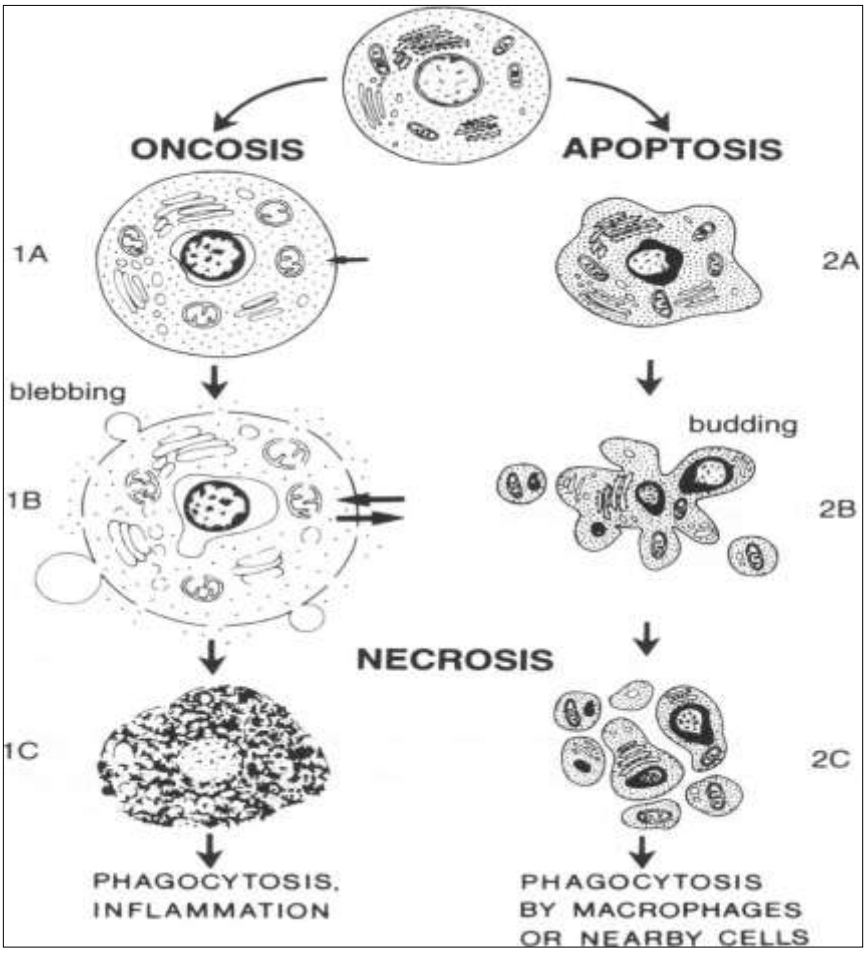


Figure 9. Pathways of cell death that lead to necrosis.

A typical cell is at the top, 1A- Cell swelling. 1B-Vacuolisation occurs with blebbing thus increasing permeability. 1C - Necrotic changes occurs, coagulation, shrinkage and karyolysis. 2A – Shrinkage. 2B - Budding. 2C - Formation of apoptotic bodies (Majno and Joris, 1995).

2.9.1 Apoptotic mechanisms

Apoptotic mechanisms are very intricate and they involve a cascade of molecular events. It is important to understand the mechanisms of apoptosis as it helps with the understanding of the pathogenesis of disordered apoptosis. In turn this assists with the developing of drugs that have the potential to target particular genes or pathways. There are two major pathways of apoptosis (Figure 10); the intrinsic and extrinsic pathways trigger its initiator caspase which activate caspase -3. This pathway, whereby caspase-3 is activated is also known as the execution pathway. The results of the execution pathway include cell shrinkage, chromatin condensation, cytoplasmic blebbing, apoptotic body formation and phagocytosis of these apoptotic bodies by neighbouring parenchymal / neoplastic cells or macrophages (Wong, 2011).

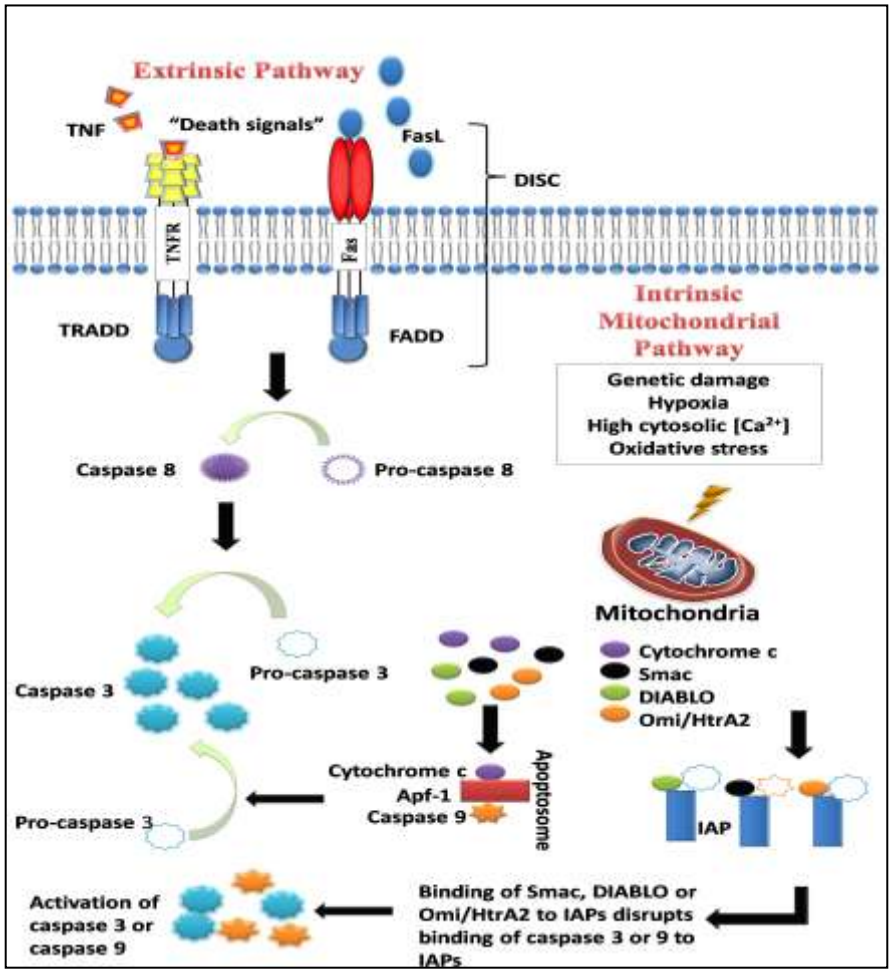


Figure 10. Diagrammatic representation of the apoptotic pathways: extrinsic, intrinsic and perforin/granzyme pathway (Wong, 2011).

2.9.1.1. The extrinsic pathway

The extrinsic pathway is also known as the death receptor pathway. This pathway begins when the death receptors are activated and transmit apoptotic signals after it binds with certain ligands. The death receptors which are well known are TNFR1 (Tumor Necrosis Factor Receptor 1), a related protein called Fas and their ligands, TNF and Fas ligand (FasL). The death receptors have a death domain which recruits adaptor proteins called Fas associated death domain (FADD). This interacts with an initiator protein, caspase 8 and the complex formed is known as the death inducing signal complex (DISC) (Vermeulen *et al*., 2005, Sankari *et al*., 2012). The recruitment of caspase 8 to DISC activates downstream caspases such as caspase 3 which will initiate apoptosis of the target cell and result in the death of the cell (Bridgham *et al*., 2003).

2.9.1.2. The intrinsic pathway

The intrinsic or mitochondrial pathway is induced by intracellular and extracellular stresses. These stresses include hypoxia, genetic damage, and an increased concentration of cytosolic calcium (Lawen, 2003). This results in the increased permeability of the mitochondrial membrane which is a crucial step of the intrinsic pathway leading to apoptosis (Kroemer, 2003). The disruption of the mitochondrial membrane triggers the release of apoptogenic factors such as cytochrome C to the cytosol. This release of cytochrome C results in the activation of an apoptotic protease activating factor 1 (Apaf- 1). The binding of Apaf – 1 to an initiator protein caspase 9 results in the formation of an apoptosome. The activation of the apoptosome bound caspase 9 results in the activation of the effector caspase (caspase – 3) which will initiate the degradation of the cells (Hausmann *et al*., 2000).

2.9.2. Apoptosis detection

Apoptosis takes place through complex events that are regulated at different points and there are various technical methods used for detecting apoptotic cells. Some of the methods include light microscopy, electron microscopy, gel electrophoresis, flow cytometry, *in situ* – end labelling method and immunohistochemistry. Although there are an extensive range of methods available, it is important to understand the application, the advantages and the disadvantages of each assay based on the above methodologies.

The assays available can be categorized into six main groups as follows:

- Cytomorphological modifications
- DNA fragmentation
- Detection of caspases, cleaved substrates and regulators / inhibitors
- Membrane modification
- Apoptotic detection in whole mounts
- Detection of mitochondrial changes (Elmore, 2007)

For the accurate quantification of apoptotic cells flow cytometry is used. Flow cytometry is a method that uses DNA staining to differentiate between apoptotic and non-apoptotic cells. This method allows the enumeration, examination and the sorting of particles in fluid. It allows for the multi parametric testing of physical or chemical characteristics of cells passing through a flow cytometer. Flow cytometry is based on the principle whereby apoptotic cells are stained with a fluorescent dye and these cells are passed through a light beam of a single wavelength. Cells moving through cause the light to scatter, these scatters are distinguished into a forward scatter and a side scatter. The scatters facilitate the differentiation of cells into apoptotic and into non apoptotic cells (Archana *et al*., 2013).

The following assays were used to detect apoptosis:

2.9.2.1. Plasma membrane modification

The loss of plasma membrane integrity (Figure 11) is an early event of apoptosis which exposes phosphatidylserine (PS) residues on the outer plasma membrane of cells undergoing apoptosis (van Engeland *et al*., 1998). This is detected by Annexin V that binds to PS preferentially. This Annexin-FITC assay is also performed by propidium iodide staining to distinguish between necrotic and apoptotic cells. The Annexin V assay is said to be a very simple and rapid method to detect apoptosis (Sgonc and Gruber, 1998).

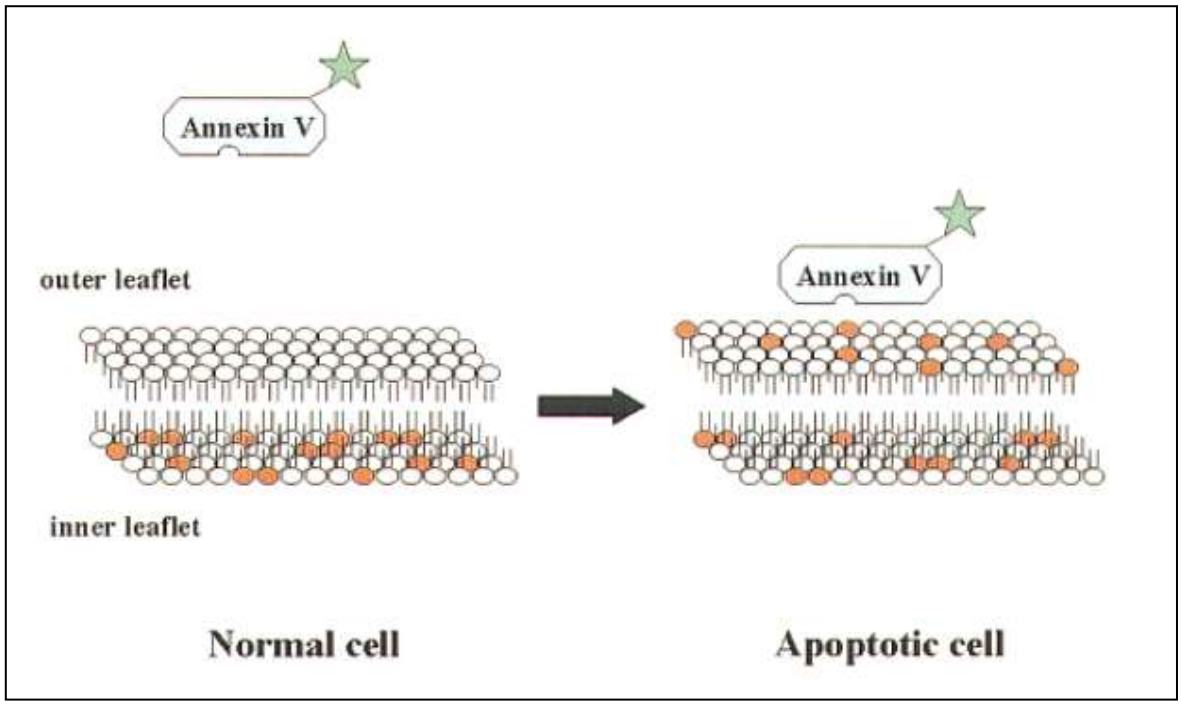


Figure 11. Schematic illustration of the loss of membrane lipid asymmetry of the membrane during apoptosis (van Engeland *et al*., 1998).

2.9.2.2. Mitochondrial changes

Mitochondrial changes occur in the early stages of apoptosis. These changes are associated with a decline in membrane potential and the release of cytochrome c that activates caspases. Mitochondria play a central role in programmed cell death through the release of cytochrome c and other features that are proapoptotic. To measure the mitochondrial potential one can use flow cytometry (Gottlieb and Granville, 2002).

The JC-1 assay was used to determine changes in mitochondrial potential. JC-1 is a lipophilic cationic fluorochrome, which accrues in the mitochondria of healthy cells and forms aggregates, however in apoptotic cells, JC -1 remains as a monomer in the cytoplasm. These monomers and aggregates are differentiated by their emission spectra that differ.

2.9.2.3. Detection of Caspase- 3 activity

There are various caspase activity assays to detect the more than 13 recognized caspases (Elmore, 2007). Caspase-3 is essential in apoptosis since its role is proteolytic cleavage of significant proteins such as poly (ADP-ribose) polymerase (PARP) (Cohen, 1997). The detection of caspase 3 *in situ* has been reported to be a unique, direct and sensitive method to detect apoptosis when comparing detection by secondary methods such as DNA fragmentation or cleavage of caspase (Archana *et al*., 2013). Caspase assays are used to detect early to late stages of apoptosis.

3. METHODOLOGY

3.1. Introduction

Figure 12 is a synopsis of the methodology used in this study. Dihydropyrimidine derivatives (**DHPM 1 -8**) were synthesized and characterized at the Durban University of Technology by Dr K.N. Venugopala.

The **DHPM 1-8** were screened for their biological activities, which were antimicrobial, antioxidant, anti-inflammatory activities and molecular docking studies were undertaken. Cytotoxicity studies were performed on all compounds using two cell lines (MCF - 7 and UACC -62).

All compounds which were found to be active against the UACC - 62 cell were further investigated for apoptotic activity. The Annexin V assay was conducted to determine the plasma membrane potential, the JC-1 assay was used to detect mitochondrial damage and the detection of caspase-3 activity was undertaken to demonstrate apoptosis. The **DHPM 1** - **8** underwent a safety evaluation that included the brine shrimp lethality assay and the Ames mutagenicity assay.

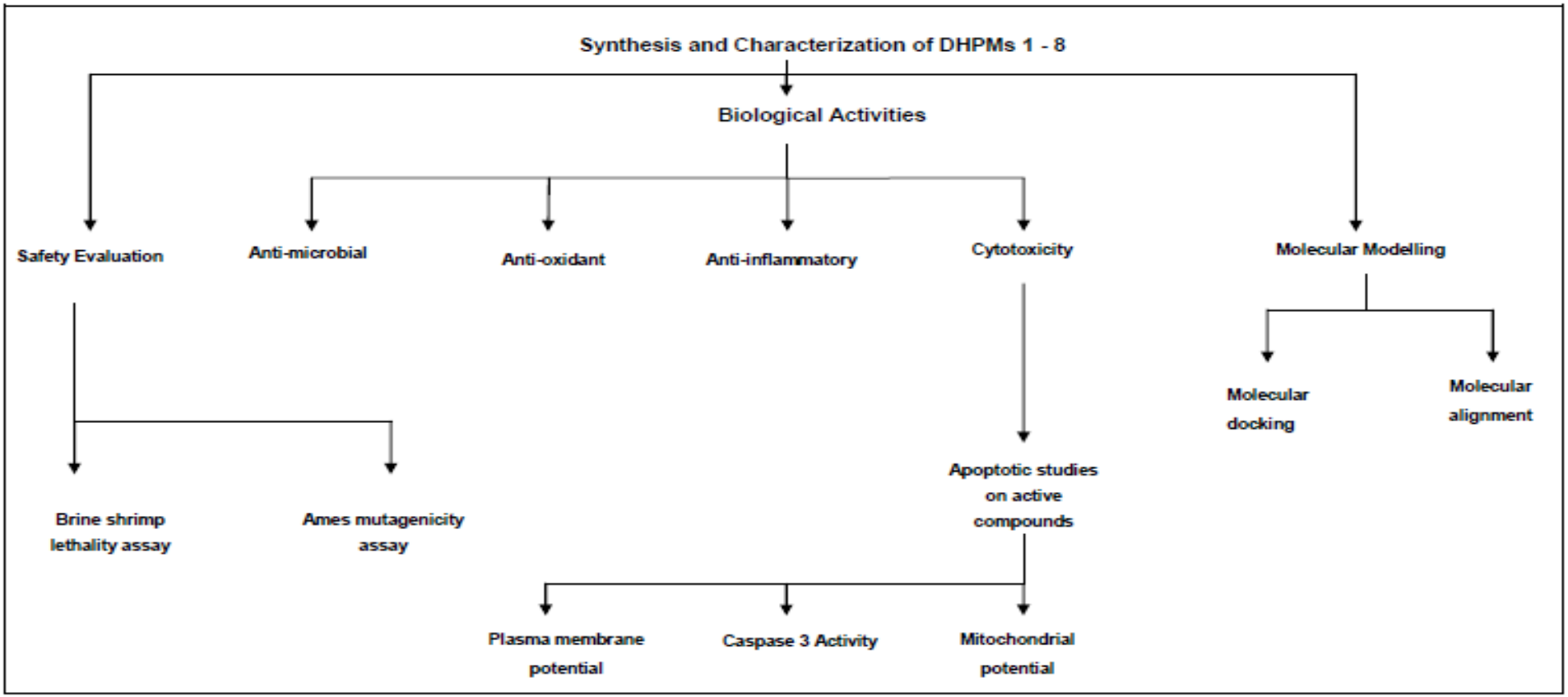


Figure 12. Methodology overview of pharmacological screening of DHPM 1 – 8.

3.2. Synthesis of DHPM 1-8

3.2.1. Synthetic strategy

Chemicals used in this study were acquired from Sigma Aldrich and Merck. The synthetic strategy employed to produce **DHPM 1-8** is illustrated in Figure 13. **Intermediates 1** and **2** were synthesized as per procedures reported by (Narayanaswamy *et al*., 2013),(Nayak *et al*., 2010).

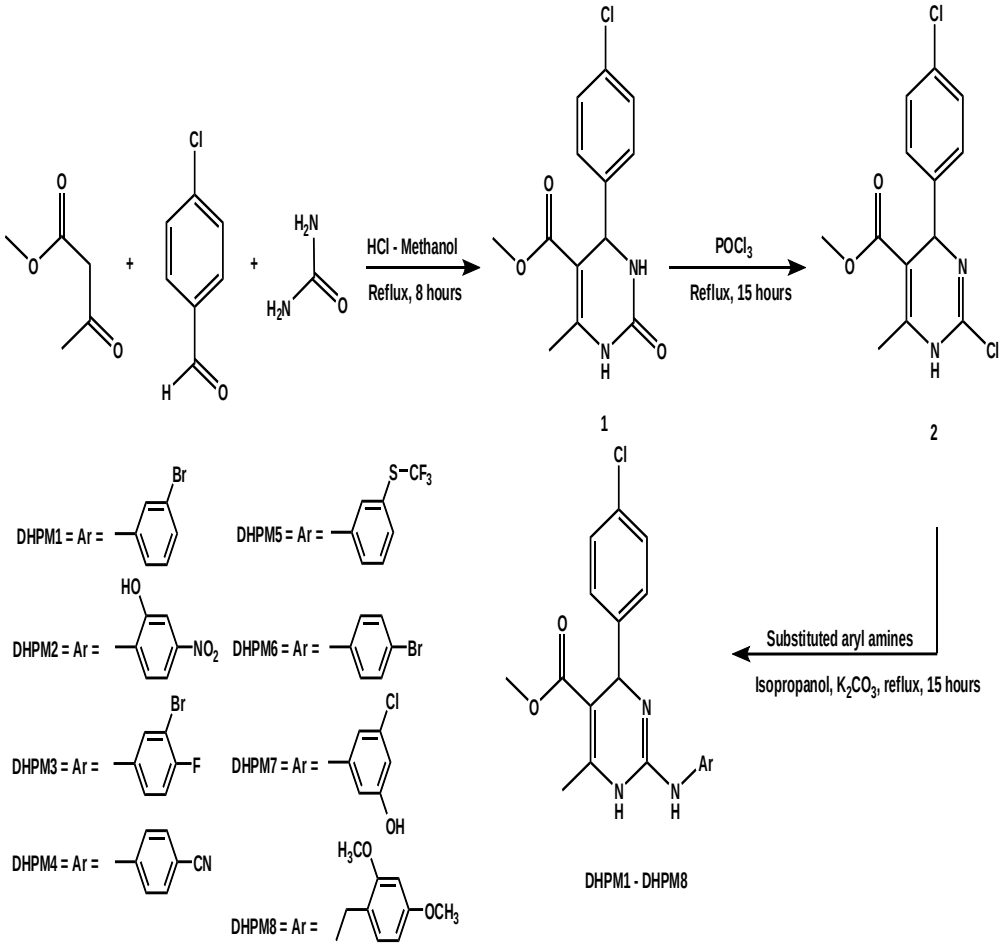


Figure 13. Synthetic route to 1, 4-dihydropyrimidine analogues DHPM 1 - 8.

3.2.1.1. Synthesis of Intermediate 1

Intermediate 1 is methyl 4-(4-chlorophenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate. A solution of methyl acetoacetate (0.12 mol), 4-chlorobenzaldehyde (0.1 mol) and urea (0.1 mol) was refluxed for 8 hours in the presence of concentrated hydrochloric acid (0.05 mol) (Figure 13) in 5 mL of ethanol (EtOH). The completion of reaction was monitored by TLC. The reaction mixture was then cooled to room temperature and the pure precipitate was collected by filtration. The solid obtained was washed with cold EtOH, dried and recrystallized using EtOH as the solvent.

3.2.1.2. Synthesis of Intermediate 2

Intermediate 2 is methyl 2-chloro-4-(4-chlorophenyl)-6-methyl-1,4-dihydropyrimidine-5-carboxylate. A solution of Intermediate 1 (1 mol) in $POCl_3$ (5 mol) was refluxed for 15 hours (Figure 13). The reaction was monitored by TLC. Unreacted $POCl_3$ was evaporated completely and the remaining residue was taken in ethyl acetate and washed with 10% sodium bicarbonate solution followed by water and finally a brine solution. The ethyl acetate layer was dried over sodium sulphate and evaporated to obtain a solid which was recrystallized using EtOH as a solvent.

3.2.1.3. Synthetic procedure for the synthesis of methyl-2-(3-bromophenylamino)-4-(4-chlorophenyl)-6-methyl-1,4-dihydropyrimidine-5-carboxylate [DHPM 1].

A mixture of **Intermediate 2** (1 mmol), 3-bromo aniline (1 mmol) and potassium carbonate (1 mmol) in isopropanol (10 mL) was refluxed for 15 hours as depicted in Figure 13. TLC was used to monitor the completion of the reaction. The end product obtained was filtered, washed with isopropanol and dried. Column chromatography was used to purify the crude product, *n*-hexane and ethyl acetate (4:6) was used as an eluent to obtain the title compound **DHPM 1.**

3.2.1.4. Synthetic procedure for the synthesis methyl-4-(4-chlorophenyl)-2-(2-hydroxy-4-nitrophenylamino)-6-methyl-1,4-dihydropyrimidine-5-carboxylate [DHPM 2].

A mixture of **Intermediate 2** (1 mmol), 2-hydroxy-4-nitro aniline (1 mmol) and potassium carbonate (1 mmol) in isopropanol (10 mL) was refluxed for 15 hours as depicted in Figure 13. TLC was used to monitor the completion of the reaction. The end product obtained was filtered, washed with isopropanol and dried. Column chromatography was used to purify the crude product, *n*-hexane and ethyl acetate (4:6) was used as an eluent to obtain the title compound **DHPM 2**.

3.2.1.5. Synthetic procedure for the synthesis methyl-2-(3-bromo-4fluoro-phenylamino)-4-(4-chlorophenyl)-6-methyl-1,4-dihydropyrimidine-5-carboxylate [DHPM 3].

A mixture of **Intermediate 2** (1 mmol), 3-bromo-4-fluoro aniline (1 mmol) and potassium carbonate (1 mmol) in isopropanol (10 mL) was refluxed for 15 hours as depicted in Figure 13. TLC was used to monitor the completion of the reaction. The end product obtained was filtered, washed with isopropanol and dried. Column chromatography was used to purify the crude product, *n*-hexane and ethyl acetate (4:6) was used as an eluent to obtain the title compound **DHPM 3**.

3.2.1.6. Synthetic procedure for the synthesis of methyl-4-(4-chlorophenyl)-2-(4-cyanophenylamino)-6-methyl-1,4-dihydropyrimidine-5-carboxylate[DHPM 4]

A mixture of **Intermediate 2** (1 mmol), 4-aminobenzonitrile (1 mmol) and potassium carbonate (1 mmol) in isopropanol (10 mL) was refluxed for 15 hours as depicted in Figure 13. TLC was used to monitor the completion of the reaction. The end product obtained was filtered, washed with isopropanol and dried. Column chromatography was used to purify the crude product, *n*-hexane and ethyl acetate (4:6) was used as an eluent to obtain the title compound **DHPM 4**.

3.2.1.7. Synthetic procedure for the synthesis of methyl 4-(4-chlorophenyl)-6-methyl-2-(3-(trifluoromethylthio)phenylamino) -1,4-dihydropyrimidine-5-carboxylate [DHPM 5]

A mixture of **Intermediate 2** (1 mmol), 3-((trifluoromethyl)thio)aniline (1 mmol) and potassium carbonate (1 mmol) in isopropanol (10 mL) was refluxed for 15 hours as depicted in Figure 13. TLC was used to monitor the completion of the reaction. The end product obtained was filtered, washed with isopropanol and dried. Column chromatography was used to purify the crude product, *n*-hexane and ethyl acetate (4:6) was used as an eluent to obtain the title compound **DHPM 5**.

3.2.1.8. Synthetic procedure for the synthesis of methyl 2-(4-bromophenylamino)-4-(4-chlorophenyl)-6-methyl-1,4-dihydropyrimidine-5-carboxylate [DHPM 6]

A mixture of **Intermediate 2** (1 mmol), 4-bromoaniline (1 mmol) and potassium carbonate (1 mmol) in isopropanol (10 mL) was refluxed for 15 hours as depicted in Figure 13. TLC was used to monitor the completion of the reaction. The end product obtained was filtered, washed with isopropanol and dried. Column chromatography was used to purify the crude product, *n*-hexane and ethyl acetate (4:6) was used as an eluent to obtain the title compound **DHPM 6**.

3.2.1.9. Synthetic procedure for the synthesis of methyl2-(3-chloro-5-hydroxyphenylamino)-4-(4-chlorophenyl)-6-methyl-1,4-dihydropyrimidine-5-carboxylate [DHPM 7].

A mixture of **Intermediate 2** (1 mmol), 3-chloro-5-hydroxy aniline (1 mmol) and potassium carbonate (1 mmol) in isopropanol (10 mL) was refluxed for 15 hours as depicted in Figure 13. TLC was used to monitor the completion of the reaction. The end product obtained was filtered, washed with isopropanol and dried. Column chromatography was used to purify the crude product, *n*-hexane and ethyl acetate (4:6) was used as an eluent to obtain the title compound **DHPM 7**.

3.2.1.10. Synthetic procedure for the synthesis of methyl4-(4-chlorophenyl)-2-(2,4-dimethoxybenzylamino)-6-methyl-1,4-dihydropyrimidine-5-carboxylate [DHPM 8]

A mixture of **Intermediate 2** (1 mmol), 2,4-dimethoxy aniline (1 mmol) and potassium carbonate (1 mmol) in isopropanol (10 mL) was refluxed for 15 hours as depicted in Figure 13. TLC was used to monitor the reaction completion. The end product obtained was filtered, washed with isopropanol and dried. Column chromatography was used to purify the crude product, *n*-hexane and ethyl acetate (4:6) was used as an eluent to obtain the title compound **DHPM 8**.

3.3. Characterisation of DHPM 1-8

3.3.1. Thin layer chromatography

The chemical reactions were monitored by analytical thin layer chromatography with silica gel plates (Merck 60 F-53 254). 5 μL of **DHPM 1-8** at a concentration 500 μg/mL was applied on the TLC plate. The chromatogram was developed with a solvent system which was *n*-hexane and ethyl acetate [4:6] in a pre-saturated TLC chamber. The TLC plate was dried and visualized under UV lighting. The shape and colour of the resolved components on TLC were recorded. The *Rf* of each compound, which is equal to the distance migrated over the total distance covered by the solvent was calculated using the formula below:

Rf = distance travelled by sample / distance travelled by solvent

3.3.2. Melting point

The melting points of **DHPM 1-8** were determined using the open capillary tube method, with the aid of a Büchi Melting Point B-545 apparatus, at a temperature range between 110-250 °C. The capillary tube was filled with **DHPM 1-8.** The capillary tube was inserted into a Büchi melting point apparatus. The melting point was determined by gradient temperature method at 5 °C / min.

3.3.3. NMR

NMR was conducted with a 400MHz Bruker spectrophotometer. 12 mg of purified test compound was dissolved in 400 μL dimethyl sulphoxide (DMSO)-*d6* solvent in an NMR tube. This was placed in the spinner turbine. The NMR spectra (^{1}H and ^{13}C) were conducted using the internal standard tetramethylsilane (TMS). The data was collected and the phase correction was performed manually. Chemical shifts are shown in ppm downfield from TMS. The splitting pattern is abbreviated as s: singlet; m: multiplet and d: doublet.

3.3.4. High resolution mass spectrometry

A Bruker MicroTOF QII mass spectrophotometer was used to perform HRMS analysis. This was used in a positive mode with a Waters Xbridge C18 column (150 mm x 4.6 mm x 5 microns) and UV/VIS detector (215 nm) at a flow rate of 250 μL min^{-1}. A two-buffer system was employed, utilizing formic acid as the ion-pairing agent. Buffer A consisted of 0.1% formic acid / H_2O (v/v) and buffer B consisted of 0.1% formic acid / ACN (v/v), with a linear gradient from 5 to 95% B in 30 min. This was used in a positive mode, with an internal calibration with tune mix.

3.4. Biological activities

3.4.1. Antimicrobial activity

The antimicrobial activity [antibacterial, antifungal] and the Minimum Inhibitory Concentration [MIC] of the Dihydropyrimidines [**DHPM 1-8**] were carried out using the agar disk diffusion assay (Cos *et al*., 2006)

3.4.1.1. Antibacterial activity

Ten bacterial strains were used as test organisms and these were obtained from the stock culture collection at the Department of Biotechnology and Food Technology, Durban University of Technology. **DHPM 1-8** were tested for their *in vitro* anti-bacterial activity against six Gram positive microorganisms (*Bacillus cereus, Staphylococcus aureus, Bacillus coagulans, Bacillus stearothermophilus, Micrococcus luteus* and *Streptococcus faecalis*) and four Gram negative microorganisms (*Citrobacter freundii, Escherichia coli, Serratia marcescens* and *Klebsiella pneumonia*).

Stock cultures of the microorganisms were prepared on Nutrient Agar (NA) plates (Oxoid). The cultures were also stored in micro banks (Davies Diagnostics, South Africa) at -70°C. For the assay, the cultures were grown in Mueller Hinton Broth (MHB) (Biolab) at 37°C for 24h. The absorbance of the broth cultures were adjusted to the McFarland standard of 0.5 which is equivalent to 1.5 x 10^8 CFU/mL (Matasyoh *et al*., 2008).

DHPM 1-8 was tested at a concentration of 3 mg/mL using dimethylsulfoxide [DMSO] as a solvent. Ten microlitres of the drug (**DHPM 1-8**) was transferred into sterile 5 mm disks that were made of Whatman No.1 filter paper. The disks were air dried in a biological safety cabinet [Labtec Bioflow II, South Africa]. The treated disks were placed on the surface of Mueller-Hinton agar plates that were inoculated with 100 µL of bacterial culture; these were incubated at 37°C for 24 h. DMSO served as the negative control. Ciprofloxacin was used as the positive control. All tests were carried out in triplicate. The inhibition was determined by measuring the diameter (mm) of the clearing around the treated disks.

3.4.1.2. Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration was determined by testing the **DHPM 1-8** that showed inhibition in the antibacterial assay. All compounds that produced zones over 9 mm were analysed for MIC. The **DHPM 1** - **8** were analysed for antibacterial activity at descending concentrations (3, 1.5, 0.75, 0.37, 0.18 and 0.09 mg/mL). The lowest concentration of test sample that inhibited growth after incubation was recorded as the minimum inhibitory concentration (MIC).

3.4.2 Antifungal activity

The three yeast and two fungi used as test organisms were as follows: *Candida albicans, Saccharomyces cerevisiae, Candida utilis, Aspergillus niger and Aspergillus flavus*. The fungi were grown on Sabouraud Dextrose Agar (SDA) for 4 to 7 days at 30°C until sporulation.

The yeast cultures were grown in Sabouraud Dextrose Broth for 24 h at 37°C. The absorbance of the yeast broth cultures were adjusted to the McFarland standard of 0.5 which is equivalent to 10^8 CFU/mL (Matasyoh *et al*., 2008). The fungal spores were collected in 10 mL sterile distilled water and a Nebauer counting chamber was used to count the spores. The concentration of fungal spores was adjusted to 10^6 spores/mL.

Sterile 5 mm Whatman No.1 filter paper disks were inoculated with 10 µL of **DHPM 1-8** at concentration of 3 mg/mL. The inoculated disks were air dried in a biological safety cabinet [Labtec Bioflow II, South Africa]. These disks were placed on SDA plates that had 100 µL of spore / yeast suspension swabbed onto them. The SDA plates were incubated at 30°C. DMSO served as the negative control, whilst Amphotericin B (Fluka, Biochemika), was used as a positive control. The inhibition was determined by measuring the diameter (mm) of the clearing around the impregnated disks. All tests were carried out in triplicate.

3.4.3. Antioxidant activity

The antioxidant activity of **DHPM 1-8** was tested using the DPPH(1,1-diphenyl-2-picrylhydrazyl radical) photometric assay (Choi *et al*., 2007). **DHPM 1-8** were made up to final concentrations of 1000 µg/mL, 500 µg/mL, 250 µg/mL, 100 µg/mL, 80 µg/mL, 60 µg/mL, 40 µg/mL, 20 µg/mL and 1 µg/mL in methanol. The comparative positive control used was Rutin (Sigma-Aldrich R5143) which is found in the Buckwheat plant *Fagopyrum* family, *Polygonaceae* (Zielinska *et al*., 2010).

For the bioassay, 400 µL of 0.3 mM DPPH in methanol, was added to 1 mL of **DHPM 1-8** at different concentrations [1000 µg/mL, 500 µg/mL, 250 µg/mL, 100 µg/mL, 80 µg/mL, 60 µg/mL, 40 µg/mL, 20 µg/mL, 1 µg/mL] and these were allowed to react at room temperature, in the dark for 30 min. The negative control was 1 mL of methanol and 400 µL DPPH. The positive control was DPPH solution and 610 µg/mL Rutin solution. All tests were carried out in triplicate. The absorbance values were measured in a Varian Cary 1E UV-visible spectrophotometer at 518 nm.

The average absorbance values were converted into the percentage antioxidant activity, using the formula below:

% Inhibition = 100 – [(Abs Sample – Abs Blank)/Abs Negative Control x 100]

Statistical analysis of the results of the Antioxidant assay was conducted using one way ANOVA followed by the Bonferroni's multiple comparison test; p = 0.05. The data was analyzed using GraphPad Prism©.

3.4.4. Anti-inflammatory activity

Briefly, lipoxygenases (LO) are dioxygenases that catalyse the addition of oxygen to unsaturated fatty acids that contain a cis, cis-1,4 pentadiene system (Baylac and Racine, 2003).

The anti-inflammatory activity was determined using the lipoxygenase inhibitor screening assay kit (Catalogue no. 760700, Cayman Chemicals) and following the manufacturer's protocol. The **DHPM 1-8** were screened at 1 mg/mL for anti-inflammatory activity.

The assay was performed in a 96 well plate for colorimetric assays. The assay consisted of blank wells, to which assay buffer was added, 100% initial activity wells (90 µL lipoxygenase enzyme, 10 µL DMSO), inhibitor wells (90 µl lipoxygenase enzyme and 10 µL of **DHPM 1-8** at 1 mg/mL) and the positive control (90 µL 15-LO-Standard and 10 µL assay buffer). The reaction was initiated by adding 10 µL of Arachidonic Acid (substrate) to all wells. A plate shaker was used to shake the plates for 5 minutes. Chromogen (100 µL) was added to each well to stop enzyme catalysis and develop the reaction. After covering the plate with a cover sheet and shaking for 5 minutes, the product of the enzymatic reaction was determined spectrophotometrically at $A_{490 - 500nm}$.

The percentage inhibition was determined using the following equation:

% Inhibition = [Initial Activity – Inhibitor/Initial Activity] x 100

3.4.5. Molecular modeling study

The molecular modeling study was conducted as a result of the excellent lipoxygenase activity of the **DHPMs**. The approach of molecular modeling is used extensively in the design and discovery of drugs. Molecular modeling assists with predicting the mechanism of activity of compounds. It is also used to determine the binding mode of the compounds which allow inhibition of the lipoxygenase enzyme.

The molecular modeling study consisted of:

3.4.5.1. Molecular alignment

The molecular alignment of 5-lipoxygenase and 15-lipoxygenase was done using MOE 2013.08. The following protein data bank codes were used for the alignment: 5-lipoxygenase (PDB) 3V99 and 15-lipoxygenase (4NRE).

3.4.5.2. Molecular docking studies with Leadit 2.1.2

DHPM 1-8 were constructed and saved as MOE files. A rigid receptor was used as a docking protocol. The placement method used was a triangle matcher. Two rescoring was computed, where rescoring one was London dG and rescoring two was affinity. A force field was used as refinement.

3.4.5.3. Molecular docking studies with MOE 2013.08

DHPM 1-8 were constructed and stored as mol2 files. The lipoxygenase enzyme crystal structure combined with arachidonic acid was retrieved from PDB code 3V99.

The above protein was loaded into Leadit 2.1.2 and the receptor components were chosen by using chain A as a main chain which was combined with arachidonic acid. The binding site used arachidonic acid as a reference ligand to which all coordinates were computed. Amino acids within radius 6.5 A^{o} were selected in the binding site. All chemical ambiguities of residues were left as default. Ligand binding was driven by enthalpy (classic Triangle matching). For scoring, all default settings were restored. Intra-ligand clashes were computed by using clash factor = 0.6. The maximum number of solutions per iteration was 200. The maximum solution per fragmentation was 200. The base placement method was used as a docking strategy.

3.4.6. Safety evaluation

3.4.6.1. Brine shrimp lethality assay

The toxicity of **DHPM 1-8** was determined using the brine shrimp lethality assay (McLaughlin *et al*., 1993, Meyer *et al*., 1982), with minor modifications.

3.4.6.1.1. Hatching the shrimp

Ten milligrams of *Artemia salina* eggs (Natures Petland, Durban, South Africa) were added to a sterile chamber containing sterile artificial 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]. The pH of the artificial seawater was adjusted to 9.0 with 0.1 M Na_2CO_3 to prevent the death of the shrimp due to the decrease in pH once they hatch. The eggs were incubated at room temperature for 24 h, after that 15 mL of yeast solution was added to the chamber to feed the hatched larvae (nauplii). The larvae were used for the assay after a 48 h incubation period.

3.4.6.1.2. Bioassay

The assay was conducted in 6 well plates. Each well contained 5 mL artificial sea water, 10 brine shrimps and a drop of yeast suspension (3 mg yeast extract in 5 mL artificial sea water). 100 µL of **DHPM 1-8** at different concentrations [1000 µg/mL, 500 µg/mL, 100 µg/mL, 10 µg/mL] were added to the wells. The negative control was the solvent, DMSO and the positive control was potassium dichromate (Sahgal *et al*., 2010).

Each concentration was tested in triplicate. The plates were incubated at room temperature for 24 h. Dead larvae were counted at 1, 2, 3, 4, 20 and 24 h and the percentage death was determined.

3.4.6.2. Salmonella Ames mutagenicity assay

The Ames mutagenicity assay was conducted, with minor modifications according the method described by Maron and Ames (1983) and Mortelmans and Zeiger, (2000). The assay was performed to determine if any of the **DHPM's 1-8** displayed mutagenic potential.

Salmonella typhimurium strains TA 98 and TA 100 were obtained from the South African Medical Research Council (MRC), Durban. Single colonies were aseptically removed from the pure culture and these were inoculated into sterile Erlenmeyer flasks containing 25 mL of nutrient broth (Oxoid No.2) and 78 µL of Ampicillin (8 mg/mL). The flasks were then incubated at 37°C for 16 h, on a shaker (150 rpm). After 16 h an optical density between 1.2 - 1.4 was achieved at 660 nm.

In a sterile test tube, 100 µL of the 16 h Salmonella culture was added to 2 mL of 0.05 mM histidine/0.05 mM biotin top agar (See Appendix 4), this was vortexed and overlayed onto glucose minimal agar plates (See Appendix 5). The plates were incubated at 37°C for 24 h. Well separated colonies from these master plates were used to make initial broth cultures for the assay.

The initial broth cultures were made by inoculating nutrient broth with *Salmonella typhimurium* strains TA 98 and TA 100 from the master plates and incubating these cultures in a shaking incubator (150 rpm) at 37°C for 16 h.

DHPM 1-8 were dissolved in DMSO to obtain concentrations of 10 µg/mL, 100 µg/mL and 1000 µg/mL. DMSO was used as a negative control and sodium azide was used as a positive control. Sodium azide was dissolved in DMSO to obtain concentrations of 5 µg/mL, 10 µg/mL and 20 µg/mL. All tests were carried out in triplicate. For the assay, 100 µL of bacterial culture, 100 µL of test compound and 2.9 mL of soft agar were added to a sterile test tube and gently vortexed and poured over glucose minimal plates. The plates were incubated at 37°C for 48 h. The revertant colonies (histidine dependent) were counted and the mutant frequency determined using the following formula:

Mutant frequency = Revertant number of colonies/Negative control

Statistical analysis of the results of the safety evaluation was conducted using one way ANOVA followed by the Bonferroni's multiple comparison test; $p = 0.05$. The data was analyzed using GraphPad Prism.

3.4.7. Determination of anticancer activity

3.4.7.1. Cell lines

Breast cancer cells (MCF - 7) and melanoma cancer cells (UACC - 62) were used in this study. The cell lines were provided by Biosciences, CSIR (Pretoria). The cells were received in 25 cm^2 tissue culture flasks and were incubated at 37°C in a humidified incubator (Snjiders Hepa, United Scientific group, Cape Town, South Africa) with 5% CO_2. After three days, the cells were 80% confluent and they were transferred to a 75 cm^2 tissue culture flask (Greiner, Germany).

PBMC's were also used in this study. The PBMC's were used to determine whether the compounds displayed any toxicity towards normal cells. These were isolated from Reagent Buffy coats that were donated by the South African Blood Bank (East Coast Region, Pinetown).

3.4.7.2. Cell maintenance

The maintenance of cells was performed according by protocols obtained from Freshney (2005). Cell culture procedures were carried out in a laminar flow cabinet (Scientific Engineering INC) which was sterilized by UV-light and swabbing with 70% ethanol (Merck, South Africa) before each use. MCF 7 (Figure 14 A and 14 B) and UACC-62 (Figure 14 C and 14 D) cells were grown in a monolayer with Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich) containing glucose (4,5 $g.l^{-1}$), 1 mM L-glutamine and 1 mM sodium pyruvate. The medium was supplemented with 10% heat inactivated foetal calf serum (FCS) and 1% antibiotic - antimycotic solution (Sigma-Aldrich, Inc). The cells were subcultured every 2 - 3 days after the flasks were 80% confluent, this allowed the cells to be maintained in an exponential growth phase.

The cells were subcultured using the following protocol:

The medium was removed from the flasks and the monolayer of cells was washed twice with 5 mL phosphate buffer saline (PBS). The cells were trypsinized by adding Trypsin (1 mL) and incubated at 37°C in a humidified incubator for three minutes. The flasks were then lightly tapped to assist in the detachment of the monolayer of cells. After detaching the cells, 10 mL of filtered medium was added to the flask and 5 mL of the cells were transferred to a new flask containing 20 mL of medium, these flasks were then incubated at 37°C in a humidified incubator with 5% CO_2. The cell culture

flasks were examined daily for colour changes and turbidity of the media. Cell growth was also monitored daily under an inverted microscope (Nikon).

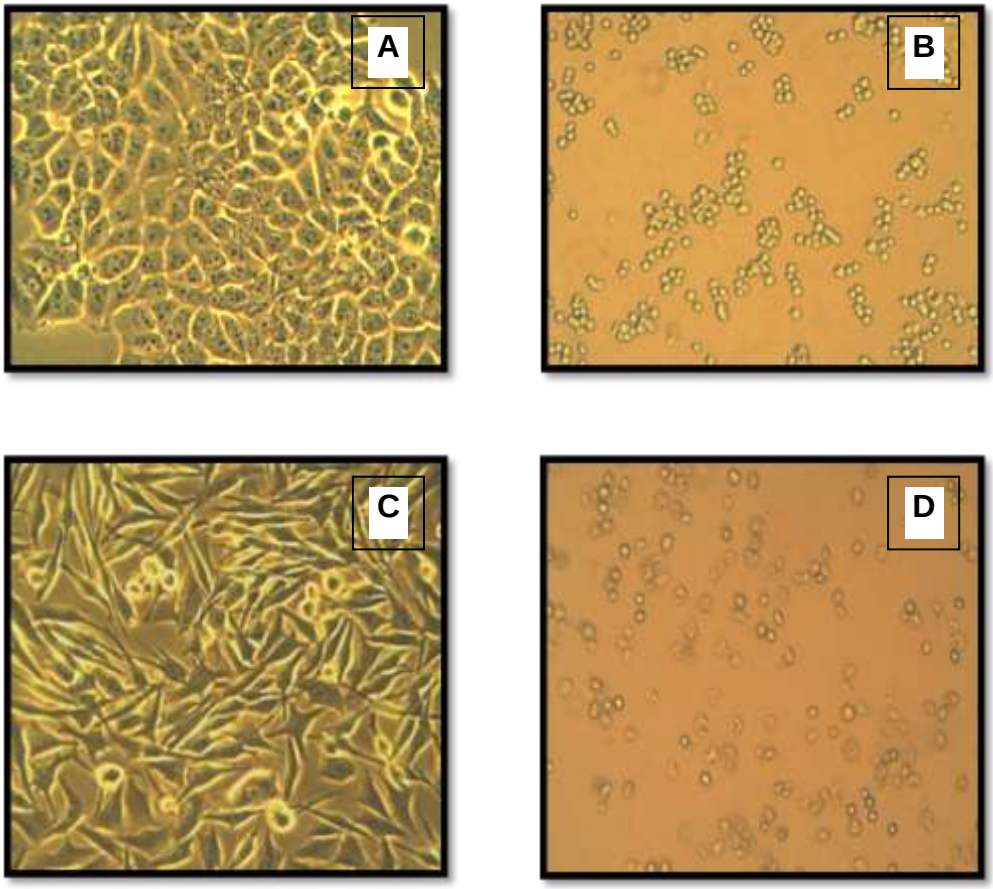


Figure 14. Microscopic observation of the morphology of MCF-7 and UACC-62 cells.

The MCF-7 monolayer showed spindle shaped cells at 100x (A) and detached cells at 100x magnification (B). The monolayer of UACC-62 cells at 100x (C) and detached cells at 100x (D) magnification.

3.4.7.3. Storage of cells

Cells were stored according to protocols described by Freshney (2005). After trypsinization as described in 3.4.7.2., 10 mL of DMEM was added to the flasks and the cells were centrifuged at 1200 rpm for 3 minutes to form a pellet. The cells were then resuspended in 2 mL of cryo-protective medium (10% DMSO, 20% FCS and 70% DMEM). Equal aliquots (1 mL) of cryoprotective medium and cell suspension were added to cryotubes (Corning, South Africa). The cryotubes were placed on ice for slow cooling. The tubes were transferred to a thermos flask and kept overnight at -20°C. The cells were then transferred to a - 70°C bio-freezer (Snijders Scientific, Holland) and stored until required.

3.4.7.4. Regeneration of cells

Cells were regenerated according to protocols of Freshney (2005). Cells were removed from the - 70°C biofreezer, the cryovial was swabbed with ethanol and rapidly thawed in a water bath at 37°C. The cells were then transferred to 20 mL of pre-warmed DMEM (supplemented with 10% FCS and 1% antibiotic - antimycotic solution) in a 75 cm^2 tissue culture flask. The flasks were incubated at 37°C in a humidified incubator with CO_2.

3.4.7.5. Enumeration of cells

The number of viable cells were determined with the Trypan Blue dye exclusion assay (Freshney, 2005). This assay is based on the inability of viable cells to be permeable to the Trypan Blue dye. Uptake of dye occurs if the cell membrane integrity is compromised. After staining, viable cells remain unstained and have a refractile ring around them and the cells that are non-viable become blue and the refractile ring is absent (Longo-Sorbello *et al*., 2005).

Cells were enumerated using a haemocytometer. Equal aliquots (100 µL) of cell suspension and Trypan Blue [Biowhittaker, Wakersville (USA)] were mixed in a centrifuge tube. A 10 µL aliquot of this mixture was loaded into the two chambers of the haemocytometer. The cells in the centre square and the four 1 mm corner squares of both the chambers were counted. The volume of cell suspension available in one primary square is 0.1 mm^3 (1.0 mm^2, 0.1 mm/1.0,10^4 mL). Viable cells which lay within, or touched the left or top boundaries were enumerated.

The following equation was used to determine the number of viable cells per mL in the suspension:

Cells / mL = Average number of cells per chamber x 2 (dilution factor) x 10^4

3.4.7.6. Isolation of PBMC

PBMC's were isolated from Reagent Buffy coats donated by the South African Blood Bank (East C**o**ast Region, Pinetown). PBMC's were isolated according to Boyum (1967) with minor modifications. The isolation was performed under sterile conditions in a class II biological safety cabinet (Lab Aire, South Africa). All equipment and reagents were disinfected with 70% ethanol.

The Buffy coats were mixed gently with an equal aliquot of PBS containing 1% Penicillin/Streptomycin. This mixture was deposited very carefully into a centrifuge tube containing an equal volume of Ficoll Histopaque (Sigma-Aldrich, South Africa). The ratio of blood, PBS and Ficoll were as follows 1:1:1 (v/v/v). The tubes were handled with care to prevent the mixing of the different solutions as this would hinder the separation of the different types of cells. The tubes were centrifuged at 1500 rpm for 30 min at RT and this resulted in four distinct layers being formed.

The upper layer (plasma) was removed with a pipette and discarded. The next layer was the peripheral blood mononuclear cell layer (PBMC) (Figure 15) and it is composed of lymphocytes. This layer was removed using a sterile pipette and transferred to a sterile centrifuge tube. The PBMC layer was checked for the presence of red blood cells and if any were present they were lysed by adding sterile double distilled water. Immediately after this PBS was added to prevent the rest of the cells from lysing. The PBMC's were washed three times with 45 mL PBS and 5 mL Penicillin / Streptomycin. The washing was done by centrifugation at 1500 rpm for 10 min at RT. After the washing process, the pellet of cells was suspended in RPMI – 1640.

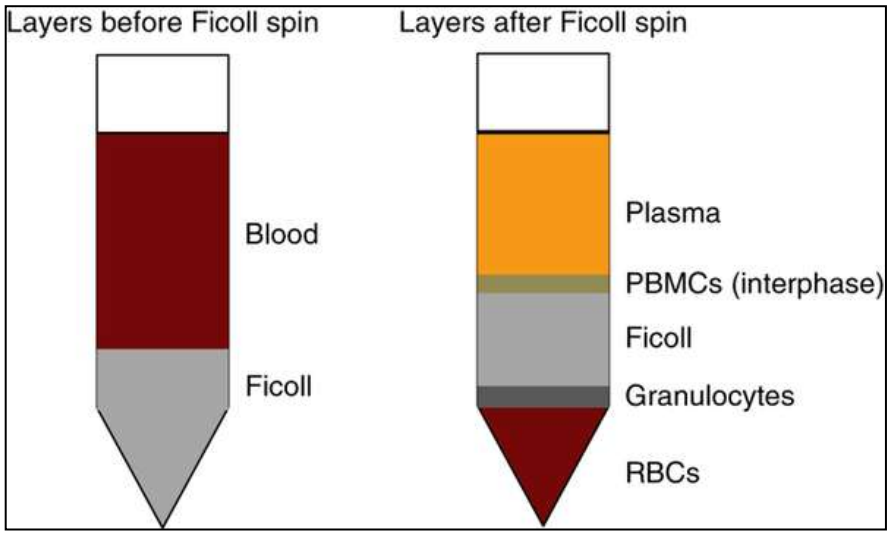


Figure 15. Illustration of isolation of PBMC from Buffy coats (Lin *et al*., 2014).

3.4.7.7. Sub-culturing and maintenance of PBMC's (Freshney, 2005)

PBMC's were cultured in tissue culture flasks (T 25 cm^2) using filtered medium that consisted of RPMI 1640, heat inactivated Foetal Bovine Serum and Penicillin/Streptomycin. The PBMC cultures were grown in a humidified incubator with 5% CO_2 at 37°C. The cultures were examined daily for turbidity and colour changes in the media. Cell growth was monitored under a microscope (Nikon). Cells were enumerated using a haemocytometer as per 3.4.7.5.

3.4.7.8. Cytotoxicity assay on MCF – 7 and UACC – 62 cells and PBMC's

The cytotoxic effect of the **DHPM 1-8** on the MCF - 7, Melanoma (UACC - 62) and PBMC cell lines was determined using the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay as described by Mossman (1983) with minor modifications.

The MTT assay is a colorimetric assay in which the reduction of MTT is determined by mitochondrial succinate dehydrogenase. The MTT reagent is reduced to insoluble, purple formazan crystals once it enters the cell. The purple crystals can be dissolved with organic solvents such as a 100% DMSO or isopropanol.

Once solubilised the formazan is measured spectrophotometrically. This assay is used to measure the viability of cells as the reduction of MTT can only occur in cells that are metabolically active.

The MTT assay was carried out in 96 well, flat bottom microtiter plates (Costar, Corning, USA). An aliquot of 90 µL (1 x 104 cells) of the MCF - 7 and Melanoma cell suspension was transferred to the microtiter plates. Sterile distilled water was added to the outer wells to prevent evaporation of the medium. The plates were incubated in a 37°C humidified incubator for 24 h to allow the cells to attach to the wells. An aliquot of 10 uL of the **DHPM 1-8** test compounds was added to the wells at two concentrations (100 µg/mL and 50 µg/mL). In control wells, 10 µL of DMSO (solvent control) and 10 µL of DMEM (medium control) were added. The positive control used was 10 µL Camptothecin.

The plates were incubated at 37°C for 48 h in a humidified incubator with 5% CO_2. After incubation, 10 µL of MTT reagent (Sigma, St Louis, USA) was added to the plates and they were incubated for a further 4 h at 37°C in a humidified incubator with 5% CO_2. After incubation, 100 µL of DMSO was added to the wells and the plates were incubated for an additional 1 h. DMSO was added to dissolve the insoluble formazan crystals that were formed by the MTT reagent once it enters the cells. The absorbance of the plates was read at a wavelength of 590 nm on an ELISA microplate reader (Digital Analogue Systems, Italy).

The percentage cytotoxicity was determined using the following equations:

$$\% \text{ Cell Viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100$$

$$\% \text{ Cytotoxicity} = 100 - \% \text{ Cell Viability}$$

Statistical analysis of the results of the cytotoxicity assay was conducted using one way ANOVA followed by the Bonferroni's multiple comparison test; $p = 0.05$. The data was analyzed using GraphPad Prism.

3.5. IC_{50} of active DHPMs on MCF – 7 and UACC – 62 cells

All compounds which inhibited growth of the two cell lines (MCF – 7 and UACC – 62) by 80% were further evaluated to determine their IC_{50} using the MTT assay as described by Mossman (1983). The active **DHPMs** were analysed at ten different concentrations to determine their IC_{50} values. The procedure for analysis is described in section 3.4.7.8.

3.6. Apoptotic studies

The active **DHPMs** were tested to determine which **DHPM** induced apoptosis in the Melanoma cell line. The following apoptotic activities were investigated: the plasma membrane changes (using Annexin V), mitochondrial changes (using JC-1) and caspase 3 activation (using caspase 3). Due to financial constraints, apoptotic kits had to be shared and single samples were assayed. These assays are described below:

3.6.1. Plasma membrane changes

The Annexin V-FITC Apoptosis detection kit (BD Biosciences) was used as per manufacturer's protocol. The melanoma cells (5 x 10^5 /mL) were seeded in T 25 tissue culture flasks and were incubated overnight. The cells were then treated with 1 mL of **DHPM 1-8** at 50 µg/mL and incubated for 24 h. The controls used were DMSO (negative control), Camptothecin (positive control), medium control, unstained cells, stained cells (Annexin – FITC) and stained cells (Propidium Iodide (PI) only).

After the incubation the medium was removed and saved and the cells were dissociated as follows:

The cells were washed with 5 mL of PBS twice and 1 mL of dissociation buffer (Life Technologies) was added to the flasks. The flasks were incubated for 2 minutes and then they were lightly tapped to detach cells. After detachment, 1 mL of medium was added to the flask. The cells were removed and added to the centrifuge tubes with the medium that was saved earlier. The cells were centrifuged in the Eppendorf 5180 centrifuge at 1200 rpm for 3 minutes.

For the staining procedure, the cells were washed twice with cold PBS. The cells were resuspended in 500 µl of Annexin V binding buffer. An aliquot of 100 µl of the cell suspension was added to a centrifuge tube to which 5 µl of FITC Annexin V and 5 µl of PI were added. The cells were then gently vortexed and incubated for 15 minutes at 25°C in the dark. Binding buffer (200 µl) was added to each tube and the samples were then analysed in the Flow Cytometer (BD Accuri C6, BD Biosciences, USA) within an hour.

3.6.2. Mitochondrial changes

The BD Mito Screen (JC-1) kit (BD Biosciences) was used as per manufacturer's protocol. The melanoma cells (5 x 10^5 /4 mL) were seeded in T 25 tissue culture flasks and were incubated overnight. The cells were then treated with 1 mL of **DHPM 1-8** at 50 µg/mL and incubated for 24 h. The controls used were DMSO (negative control), Camptothecin (positive control), untreated / unstained cells, and untreated / stained cells. After incubation the cells were dissociated as described in 3.6.1.

For the assay, the cells were washed twice in cold PBS. An aliquot of 500 µL of JC-1 working solution (WS) was added to each pellet except the unstained control. The cells were gently vortexed to resuspend cells in the JC-1 working solution. The cells in the JC-1 WS were incubated for 15 min at 37°C in a 5% CO_2 incubator. After the incubation the cells were centrifuged in the Eppendorf 5180 centrifuge at 1200 rpm for 3 minutes and the supernatant discarded. The cells were then washed twice with assay buffer. After washing and centrifugation, the pellet was resuspended in 250 µL of Assay buffer. The cells were then analysed by flow cytometry.

3.6.3. Caspase - 3 activation

The PE Active Caspase – 3 Apoptosis Kit (BD Biosciences) was used as per manufacturer's protocol. The melanoma cells (5 x 10^5 /4 mL) were seeded in T 25 tissue culture flasks and were incubated overnight. The cells were then treated with 1 mL of **DHPM 1-8** at 50 µg/mL and incubated for 24 h. The controls used were DMSO (negative control), Camptothecin (positive control), untreated/unstained cells, and untreated/stained cells. After incubation, the cells were dissociated as described in 3.6.1.

The assay was performed as follows; the cells were washed twice with cold PBS and then resuspended in 500 µL of BD Cytofix / Cytoperm. The cells were then incubated on ice for 20 min; and then were pelleted and washed twice with 500 µL of BD Perm/Wash buffer. After resuspending the cells in 500 µL of BD Perm/Wash buffer and 20 µL of antibody, they were incubated for 30 min at room temperature. Cells were then pelleted and washed with 1 mL of BD Perm/Wash buffer. The cells were then resuspended in 250 µL of BD Perm/Wash buffer and analyzed by flow cytometry.

4. RESULTS

4.1. Synthesis of DHPM 1-8

Figure 13 depicts the synthesis of 2, 4, 5-trisubstituted pyrimidine analogues **DHPM 1-8**. Synthesis of **Intermediate 1** has been achieved by Biginelli reaction by retaining Lewis acid as a catalyst as described in the literature (Nayak *et al*., 2010). Purification of the intermediate was achieved by using methanol as a solvent for recrystallization and the yield obtained was found to be 66%. **Intermediate 2** was synthesized by reacting Intermediate **1** with phosphorous oxychloride for 15 h. Intermediate **2** was obtained at 75% yield after recrystallization using methanol. Compounds **DHPM 1-8** were obtained by refluxing mono/disubstituted aromatic amines with **Intermediate 2** at equimolar proportion with potassium carbonate in isopropanol medium. The crude compounds were purified by column chromatography. The yield of the final compounds was in the range of 62-74%. The physicochemical constants of **DHPM 1-8** are tabulated in Table-1. Characterisation details are listed in Appendices 8.5.

Table 1. Physicochemical constants of DHPM 1-8

Compound	Ar	M. F (Molecular Weight)	Yield (%)[ab]	m.p. (°C)	cLogP[c]
DHPM 1	3-Br-C_6H_4	$C_{19}H_{17}BrClN_3O_2$ (433)	66	218-220	6.0560
DHPM 2	2-OH,4-NO_2-C_6H_3	$C_{19}H_{17}ClN_4O_5$ (416)	69	174-176	4.8054
DHPM 3	3-Br,4-F-C_6H_3	$C_{19}H_{16}BrClFN_3O_2$ (451)	73	238-240	6.3049
DHPM 4	4-CN-C_6H_4	$C_{20}H_{17}ClN_4O_2$ (380)	62	116-118	5.0256
DHPM 5	3-SCF_3-C_6H_4	$C_{20}H_{17}ClF_3N_3O_2S$ (455)	68	222-224	6.5586
DHPM 6	4-Br-C_6H_4	$C_{19}H_{17}BrClN_3O_2$ (433)	68	228-230	6.0560
DHPM 7	3-Cl,5-OH-C_6H_3	$C_{19}H_{17}Cl_2N_3O_3$ (405)	66	200-202	4.3300
DHPM 8	2,4-OCH_3-benzyl	$C_{22}H_{24}ClN_3O_4$ (429)	74	222-224	5.3218

[a] All of the products were characterized by spectral and physical data.

[b] Yields after purification by column chromatography.

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

4.2. Characterisation of DHPM 1-8

Finally, the chemical structure of 1, 4-dihydropyrimidine analogues **DHPM 1-8** was confirmed by H-NMR (Tron GC, 2011), C-NMR (Devi *et al*., 2009) and HRMS. In ^{1}H-NMR spectra of **DHPM 1-8**, the ester methoxy and methyl group on heteroaryl ring exhibited chemical shift in the range of δ = 3.59-3.63 and δ = 2.08-2.42 ppm, respectively. A single heterocyclic proton is noticed in the range of δ = 5.34-5.43 ppm. In ^{13}C-NMR spectra, carbonyl carbon of ester functional group is observed in the range of δ = 165.10-165.5 ppm. In HRMS, molecular ion peaks were in good agreement with proposed molecular weight. *c*Log*P* of the compounds, **DHPM 1-8** was calculated by ChemBioDraw Ultra 13.0v program and the values were in the range of 4.3300-6.5586. Characterisation details are listed in Appendices 8.6.

4.3. Anti-microbial activity of DHPM 1-8

4.3.1. Anti-bacterial activity of DHPM 1-8

Table 2 provides a summary of the results of the disk diffusion antibacterial assay. DMSO was used as a negative control and displayed no activity against all the bacteria. Ciprofloxacin displayed antibacterial activity against all bacteria used. Some of the **DHPM** compounds exhibited activity against Gram positive organisms and no inhibition against Gram negative organisms used. Ciprofloxacin produced greater activity against all bacteria on comparison to the synthetic compounds **DHPM 1-8**.

DHPM 1 and **DHPM 3** showed poor activity against *B.cereus*, *M.luteus* and *S.aureus*.
DHPM 3 and **DHPM 4** displayed weak activity only against *B.cereus* (6.00 ± 0 mm).
DHPM 5 showed activity against *B.cereus, M.luteus, S.aureus* and *B.stearothermophilus*. The lowest activity was against *B.cereus* and *B.stearothermophilus* producing zones of inhibitions of 6.00 ± 0 mm. Higher activity was displayed against *M.luteus* and *S.aureus* which produced zones of inhibition of 8.00 ± 0 mm and 7.00 ± 0 mm, respectively.
DHPM 6 displayed moderate activity against *B.cereus, M.luteus, S.aureus* and *C*. Zones of inhibition of 7.00 ± 0 mm were produced against *B.cereus, B.coagulans, S.aureus* whilst a zone of inhibition 8.33 ± 0.88 mm was found against *M.luteus* (Figure 16. G).
DHPM 7 shows moderate activity against the following Gram positive organisms *B.cereus, M.luteus, S.aureus, B.coagulans* and *B.stearothermophilus.* The highest

activity was against *M.luteus* which showed a zone of inhibition of 13 ± 0 mm, this was followed by *B.cereus* which had a zone of inhibition of 10 ± 0 mm. The lowest activity of **DHPM 7** was shown by *S.aureus, B.coagulans* and *B.stearothermophilus* with 9.00 ± 0 mm, 7.00 ± 0 mm and 7.00 ± 0 mm zones of inhibition respectively (Figure 16. A, E, H).

DHPM 8 showed excellent antibacterial activity against all Gram positive organisms used in this assay. The highest zone of inhibition produced was 15 ± 0 mm against *M. luteus* by **DHPM 8** and the lowest activity was against *S. faecalis* (9.00 ± 0 mm) (Figure 16. B, C, D, F).

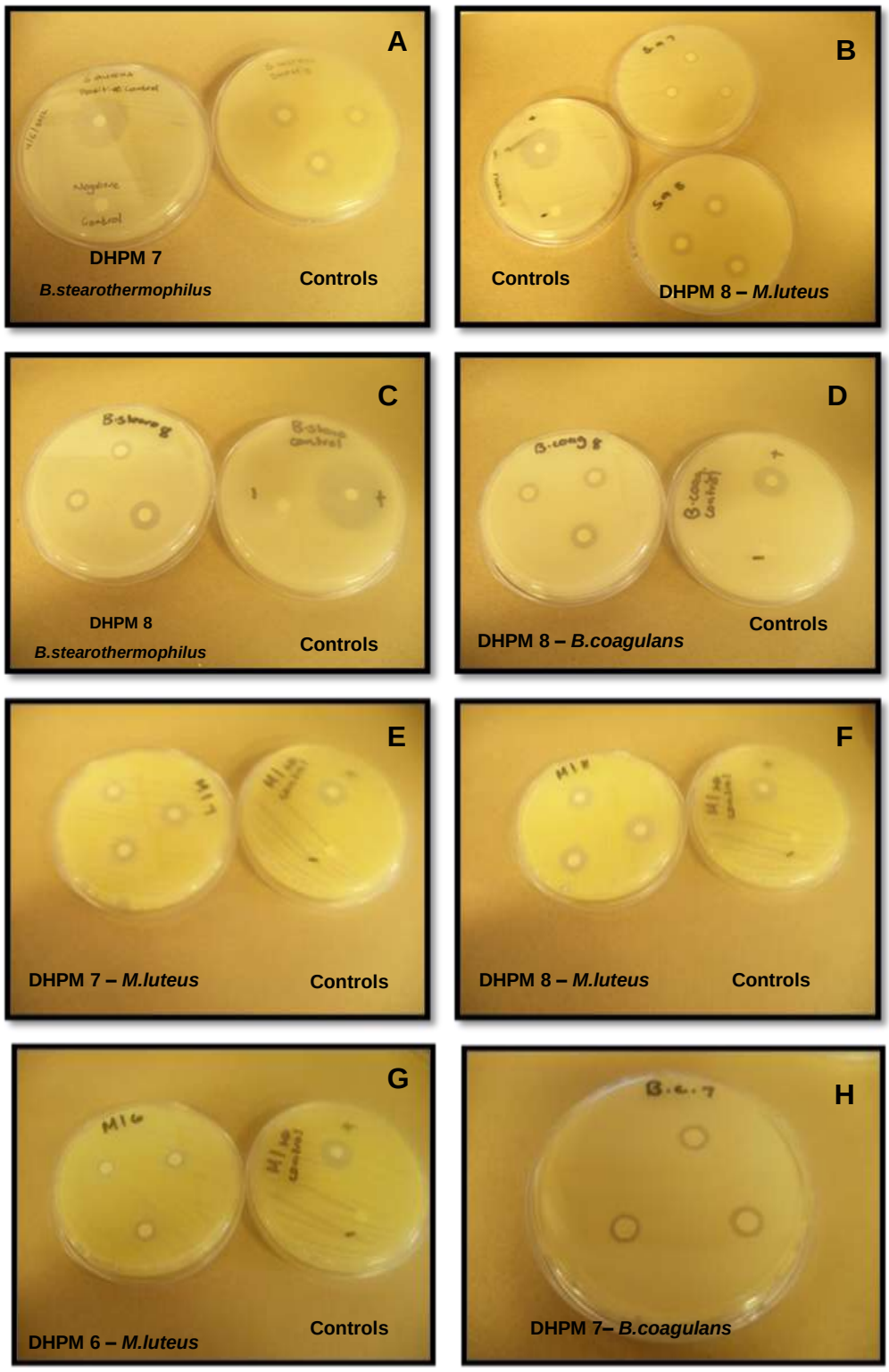


Figure 16. Antibacterial disk diffusion assay of DHPM 1-8.

Table 2. Antibacterial activity of DHPM 1-8 at concentration of 3 mg/mL

Organism	Zone of Inhibition (mm) [a]								
	DHPM 1	DHPM 2	DHPM 3	DHPM 4	DHPM 5	DHPM 6	DHPM 7	DHPM 8	Ciprofloxacin
B. cereus	6±0	7±0	6±0	6±0	6±0	7±0	**10±0**	**11.33 ±2.08**	19±0.71
M.luteus	6±0	6±0	na	na	8±0	8.33±0.58	**13±0**	**15±0**	17±1.41
S.aureus	6.67±0.58	6±0	na	na	7±0	7±0	**9±0**	**12±0**	26±1.41
B.coagulans	na	na	na	na	na	7±0	7±0	**12 ±1.73**	18±0.71
B.stearothermophilus	na	na	na	na	6±0	na	7±0	**13±1.73**	31±0.71
S.faecalis	na	na	na	na	na	na	na	**9±0**	22±0.71
E.coli	na	na	na	na	na	na	na	na	22±0.71
S.marcesens	na	na	na	na	na	na	na	na	33±0.71
C.freundii	na	na	na	na	na	na	na	na	21±0.71
K.pneumoniae	na	na	na	na	na	na	na	na	32±0.71

Data are mean± S D (n=3); na: no activity

a Diameter of zone of inhibition including disc diameter of 5 mm

4.3.2. Minimum inhibitory concentration (MIC)

Table 3 provides the results of the average results of the disk diffusion assay that was used to determine the minimum inhibitory concentration. All compounds which inhibited bacteria and produced zones of inhibition of over 9 mm were analysed further for MIC. DMSO was used as the negative control and Ciprofloxacin was used as the positive control.

Table 3. Minimum Inhibitory Concentration of DHPM 1-8

Bacteria	MIC (µg/mL)	
	DHPM 7	**DHPM 8**
Bacillus cereus	0.75 ± 0	0.75 ± 0
Staphylococcus aureus	0.75 ± 0	0.75 ± 0
Bacillus coagulans	0.75 ± 0	0.75 ± 0
Bacillus stearothermophilus	na	0.75 ± 0
Micrococcus luteus	0.37 ± 0	0.75 ± 0
Streptococcus faecalis	na	0.75 ± 0

na: not applicable

From the antibacterial activity it was determined that **DHPM 7** and **DHPM 8** were the most effective, active compounds producing zones of inhibition of 9 mm and greater against some of the Gram positive organisms tested.

DHPM 7 showed MIC value of 0.75 µg/mL against the following bacteria: *B. cereus, S. aureus* and *B. coagulans.* **DHPM 7** produced the best MIC of 0.37 µg/mL against *M. luteus* (Figure 17 A).

DHPM 8 displayed a MIC value of 0.75 µg/mL against all Gram positive organisms [*B.cereus*, *S. aureus*, *B*. *coagulans* (Figure 17 B), *B*. *stearothermophilus*, *M*. *luteus* and *S. faecalis*.

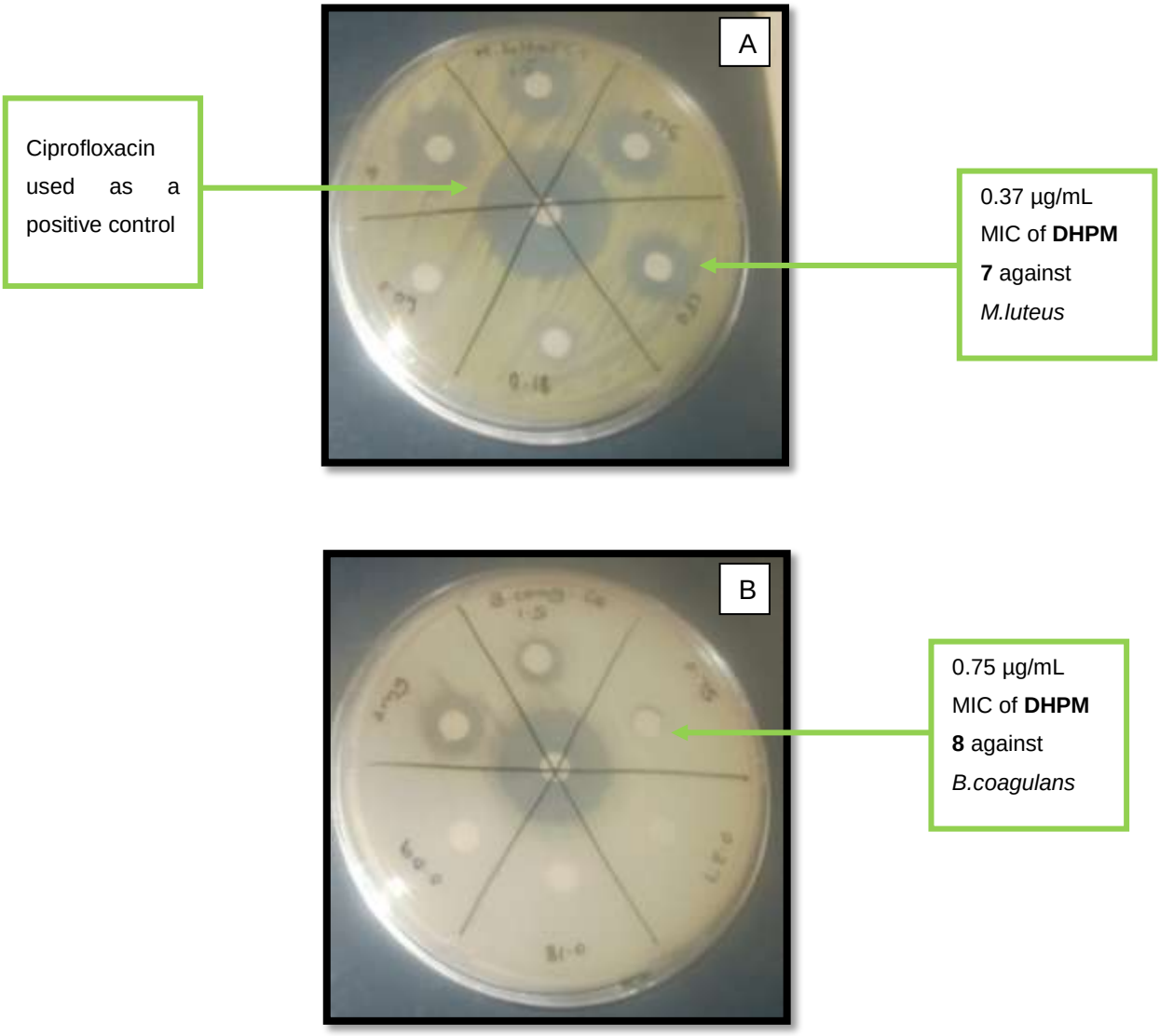


Figure 17. Disk diffusion assay to determine MIC.

A – DHPM 7 against *M.luteus*. B – DHPM 8 against *B.coagulans*.

4.3.2. Anti-fungal activity

All **DHPMs** were tested for anti-fungal activity against three yeasts [*S.cerevisiae, C.utilis* and *C.albicans*] and two fungi [*A.niger* and *A.flavus*]. The disk diffusion assay was performed using DMSO as the negative control and Amphotericin B as the positive control. **DHPM 1-8** were tested at a concentration of 3 mg/mL; however the compounds showed no anti-fungal activity against the organisms used for this assay.

4.4. Anti-oxidant activity of DHPM 1-8

In this investigation, **DHPM 1-8** was evaluated for their free radical scavenging capacity by using the DPPH assay. Rutin, a flavonoid was used as the positive control. The free radical scavenging capacity of Rutin was 95.2 ± 0.7%.

DHPM 1-8 showed increasing free radical scavenging capacity with the increase in the concentration of the compounds (See Figure 18, 19 and Table 4). Overall, the best antioxidant activity of 90.63 ± 1.1 was displayed by **DHPM 2**. That was similar to the activity of Rutin. Therefore **DHPM 2** can be considered to have good antioxidant activity.

All other **DHPMs** displayed very poor activity, which was less than 32% at a concentration of 1000 µg/mL of compound. The lowest concentration at which **DHPMs 2-7** displayed activity was 1 µg/mL while **DHPM 1** and **8** showed activity at the minimum concentration of 20 µg/mL.

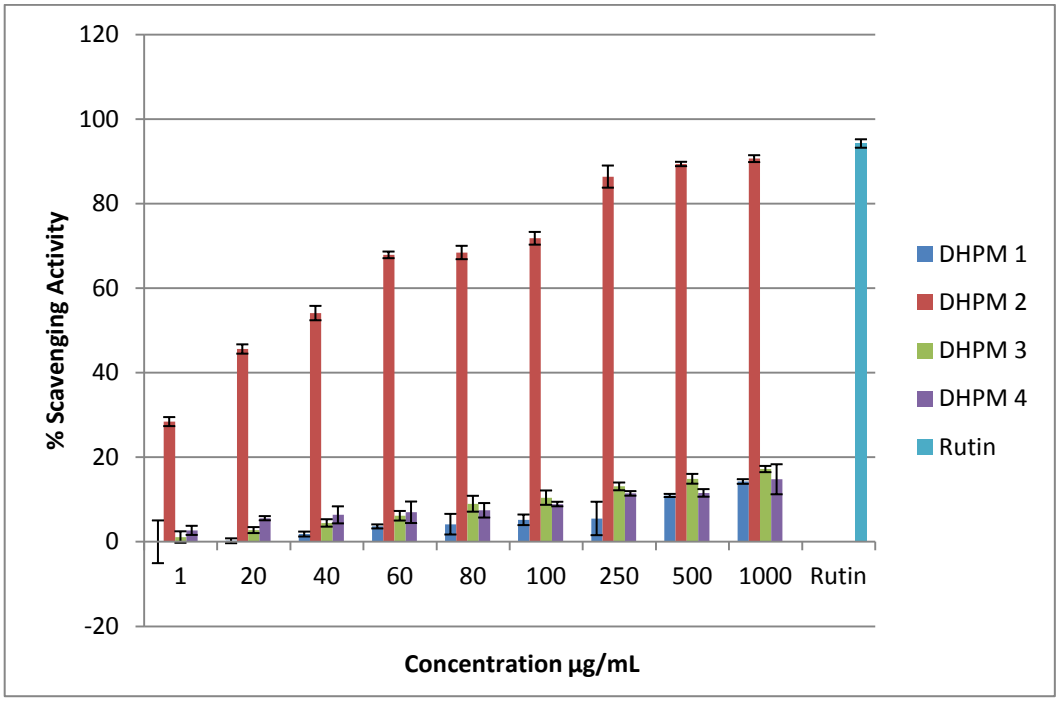


Figure 18. Anti-oxidant activity of DHPM 1-4.

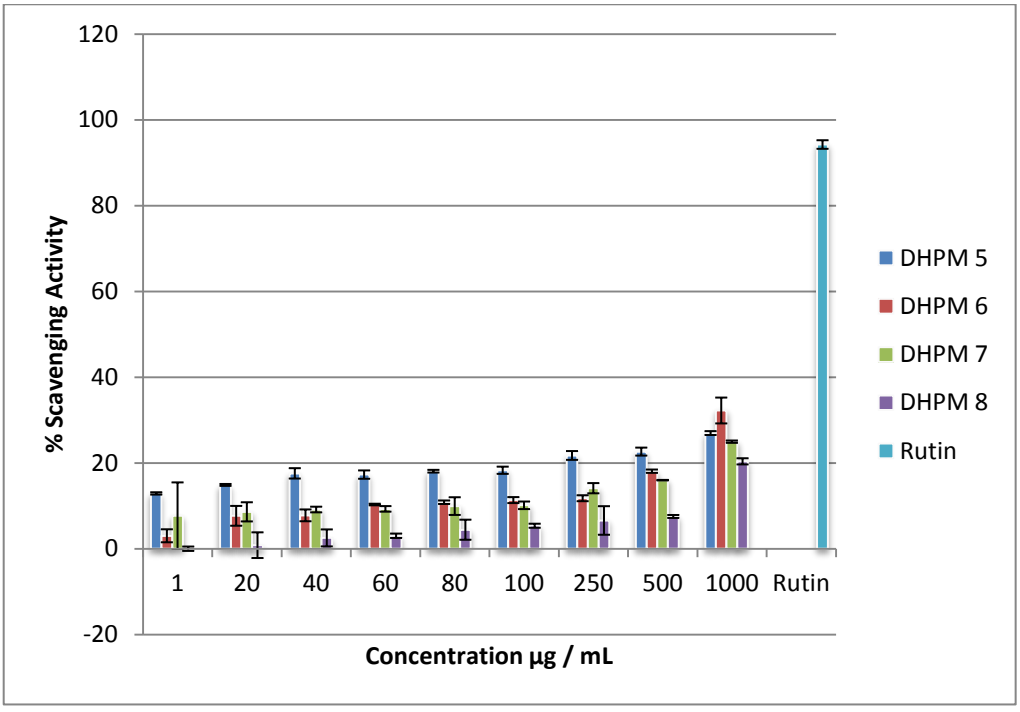


Figure 19. Anti-oxidant activity of DHPM 5-8.

Table 4. Statistical analysis of antioxidant activity at 1000 µg/ml

Bonferroni's multiple comparison test	
Sample	**Free radical scavenging capacity**
DHPM1 vs. Rutin	****
DHPM2 vs. Rutin	ns
DHPM3 vs. Rutin	****
DHPM4 vs. Rutin	****
DHPM5 vs. Rutin	****
DHPM6 vs. Rutin	****
DHPM7 vs. Rutin	****
DHPM8 vs. Rutin	****

ns - P > 0.05, * - P ≤ 0.05, ** - P ≤ 0.01, *** - P ≤ 0.001, **** - P ≤ 0.0001

4.5. Anti-inflammatory/ lipoxygenase inhibitory activity of DHPM 1-8

The anti-inflammatory effect of **DHPM 1-8** was determined at a concentration of 1 mg/mL (Figure 20 and Table 5). The assay was conducted using the lipoxygenase assay inhibitor screening kit (Cat No. 760700) from Cayman Chemicals. All compounds displayed promising anti-inflammatory activity above 59.37%. The best anti-inflammatory activity of 80.94% was shown by **DHPM 7**. Arachidonic acid was used as the substrate in this investigation and the positive control used was 15 – lipoxygenase standard. This promising activity was further investigated by a molecular docking study.

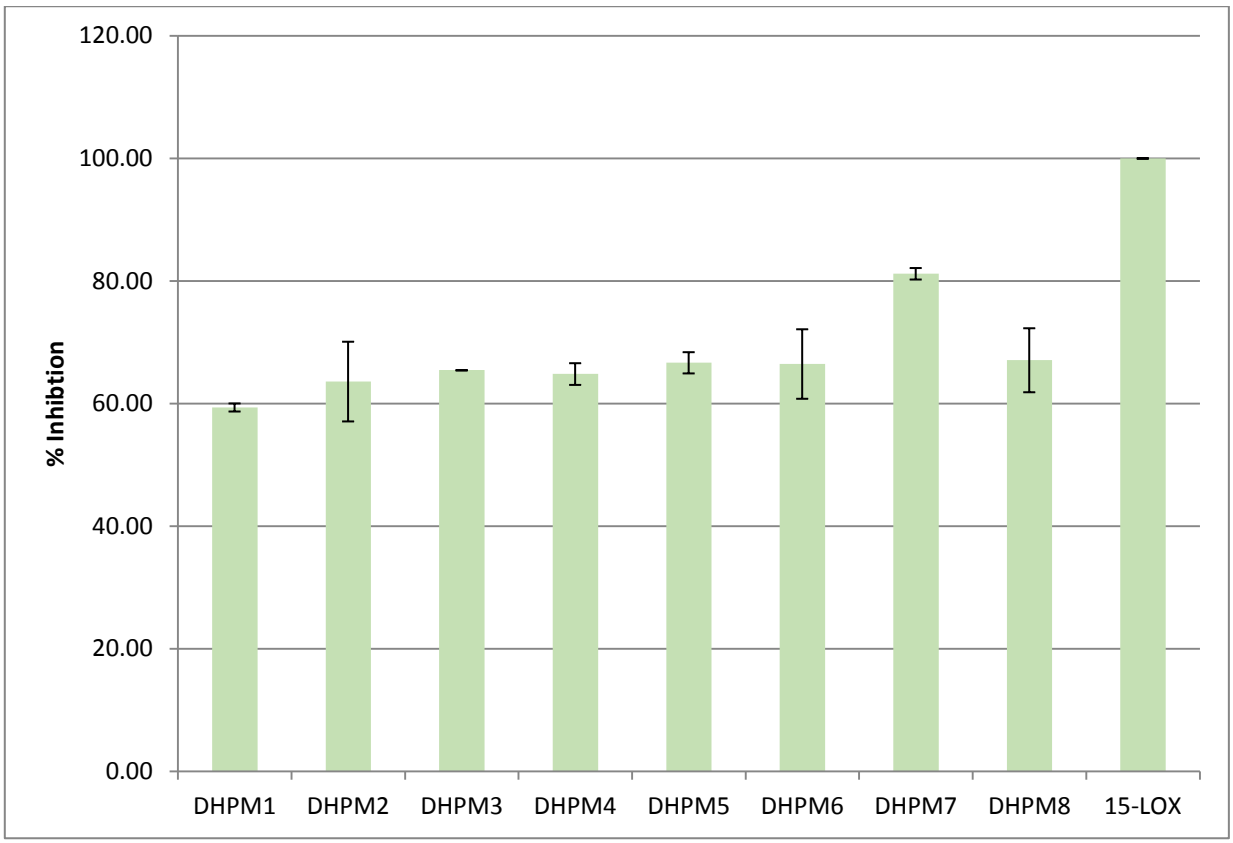


Figure 20. The 15-lipoxygenase inhibitory activity of DHPM 1-8.

Table 5. Statistical analysis of antiinflammatory activity at 1000 µg/ml

Bonferroni's multiple comparison test	
Sample	**Antiinflammatory Activity**
DHPM 1 vs. 15 - LOX	****
DHPM 2 vs. 15 - LOX	****
DHPM 3 vs. 15 - LOX	****
DHPM4 vs. 15 - LOX	****
DHPM 5 vs. 15 - LOX	****
DHPM 6 vs. 15 - LOX	****
DHPM 7 vs. 15 - LOX	****
DHPM 8 vs. 15 - LOX	****

ns - P > 0.05, * - P ≤ 0.05, ** - P ≤ 0.01, *** - P ≤ 0.001, **** - P ≤ 0.0001

4.6. Molecular modelling study

The lipoxygenase inhibitory activity was explained computationally by studying the crystal structure of the human lipoxygenase enzyme. The crystal structures of 5-lipoxygenase and 15-lipoxygenase have been previously reported in a protein data bank (Gilbert *et al*., 2012, Kobe *et al*., 2014). To help with the identification of the main binding site of the human 5-lipoxygenase, the conjugation of the enzyme with arachidonic acid was performed. Human 15-lipoxygenase was conjugated with a substrate mimic whilst the **DHPMs** tested against 15-lipoxygenase were not conjugated with the arachidonic acid. The molecular alignment of the crystal structures of 5-lipoxygenase (PDB code = 3V99) and 15-lipoxygenase (PDB code = 4NRE) was performed to identify their main binding site and confirm that the binding site is at the same point (Figure 21).

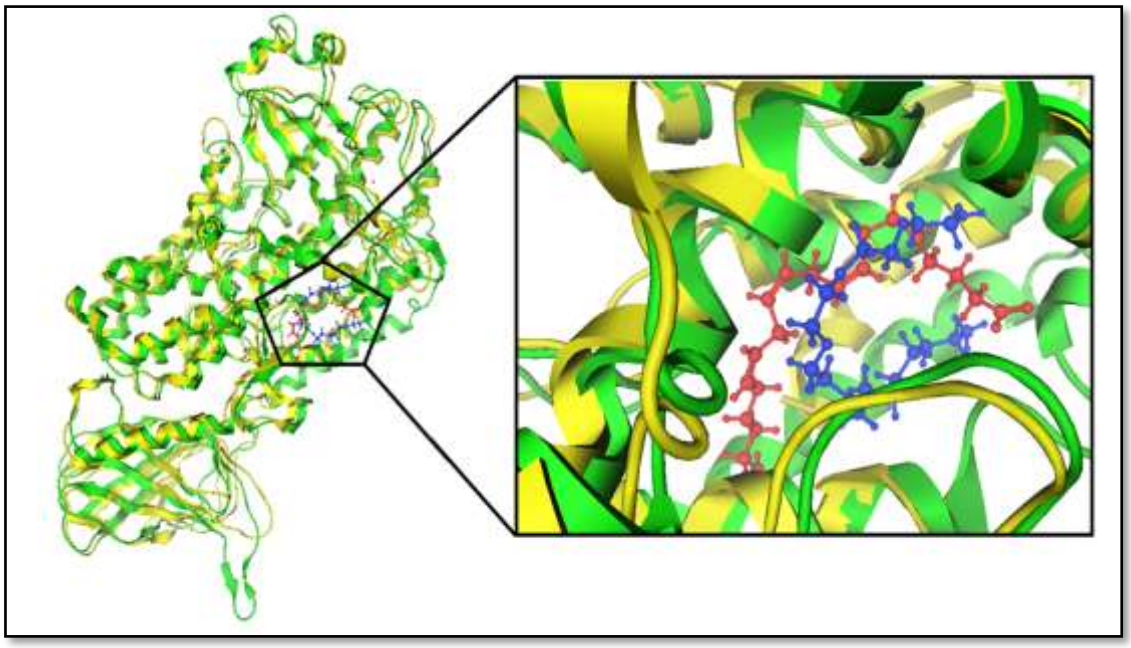


Figure 21. Sequence alignment between A) 5-lipoxygenase (Yellow) and complexed with arachidonic acid (red), B) 15-lipoxygenase (green) and complexed with a substrate mimic (blue).

The results of the alignment exhibited that the both structures have a homologous sequence. It was also found that both arachidonic acid and the substrate mimic had the identical binding cavity. The following amino acids were found in the main site: His 373, Ile 676, Ala 416, Asp 602, Asp 602, Ala 672, Glu 369, Val 426, Val 671, Ile 406, Leu 415, Phe 177, Gln 557, and Gln 413 in addition to the following water molecules H_2O 995 and H_2O 922 (Figure 22).

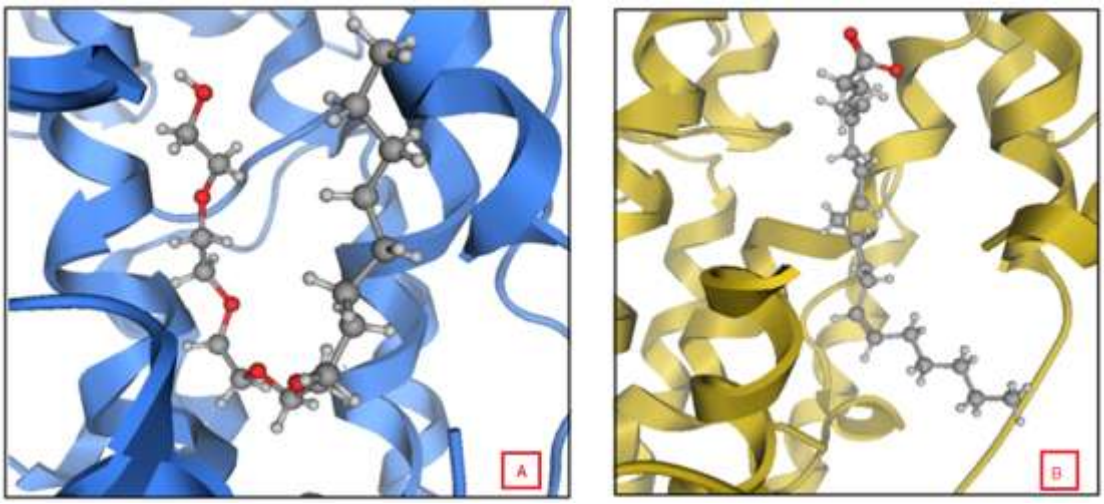


Figure 22. A) The substrate that mimics arachidonic acid and its binding mode in 15-lipoxygenase, B) Binding mode of arachidonic acid in human lipoxygenase. It shows the main interactions with the solvent (HOH) found in the binding site.

Note: Two dimensional figures were obtained by computing the ligand interactions with MOE 2013.08.

The molecular docking study was carried out using MOE 2013.08 (Chhillar *et al*., 2006). This resulted in a number of docking scores which is listed in Table 6 and these were parallel to the biological results as displayed in Figure 23. An example of this is seen in the docking score for **DHPM 7**, this compound had the highest docking score of -6.42 kcal/mol and **DHPM 7** also demonstrated the highest percentage of lipoxygenase inhibition, 81.19 ± 0.94%. It must be noted that the similarity between the experimental and computational results was very high.

Table 6. Docking results of the synthesized compounds DHPM 1-8 against human lipoxygenase in complex with arachidonic acid

Compound	Docking score using MOE 2013.08	Main residue that interacts	Main pharmacophore	Distance (A^o)	Binding Free Energy (Kcal/mol)
DHPM 1	-5.29	• Phe 177 • HOH 922	• -C=O of ester • N=C of pyrimidine ring	• 3.09 • 3.20	• -11.5 • -2.5
DHPM 2	-5.40	• Gln 363 • HOH 922	• -NH of pyrimidine • -C=O of ester	• 3.5 • 2.83	• -1 • -1.3
DHPM 3	-5.8	• Phe 177 • HOH 931	• N=C of pyrimidine ring • -C=O of ester	• 3.02 • 3.17	• -14.04 • -1.1
DHPM 4	-5.7	• Leu 368 • Phe 177	• N atom of cyano group • HN-Phenyl	• 3.71 • 3.15	• -0.8 • -13.6
DHPM 5	-5.9	• Phe 177	• -C=O of ester	• 3.34	• -7.3
DHPM 6	-6.12	• Ile 406 • Phe 177	• HN- Phenyl • N=C	• 3.58 • 3.02	• -0.7 • -14.9
DHPM 7	-6.42	• Gln 363 • HOH 955	• NH of pyrimidine ring • HN- Phenyl	• 3.37 • 3.42	• -11.8 • -2.0
DHPM 8	-6.20	• Phe 177 • Gln 413	• C=O of ester • OCH_3 group	• 2.96 • 3.77	• -10.1 • -1.1

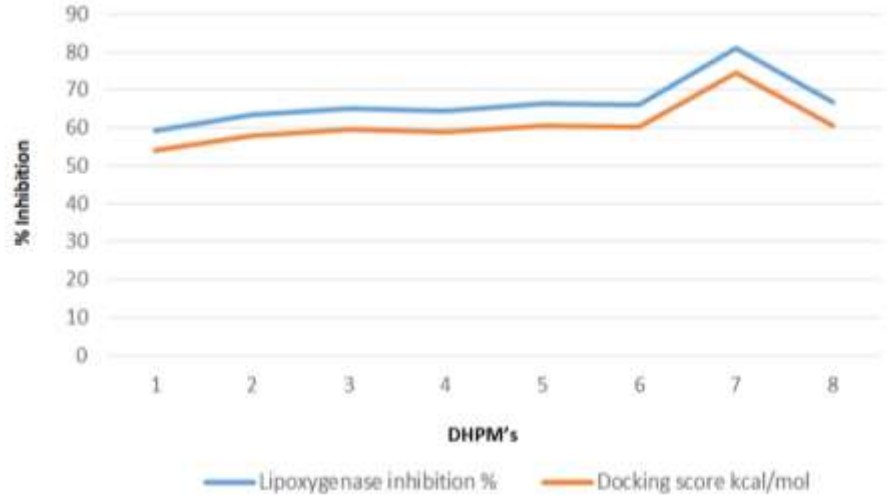


Figure 23. A correlation between the molecular docking and biological results.

Molecular docking of **DHPM 8** performed with Leadit 2.1.2 (Rarey et al., 1996) software illustrated different binding modes with different residues at the active site of the enzyme. The residues found were His 367, Val 671, as well as Fe^{2+} ion (Figure 24 A). In Figure 24 B it is noted that at another position a hydrogen bond with Phe 177 and Gln 413 was displayed.

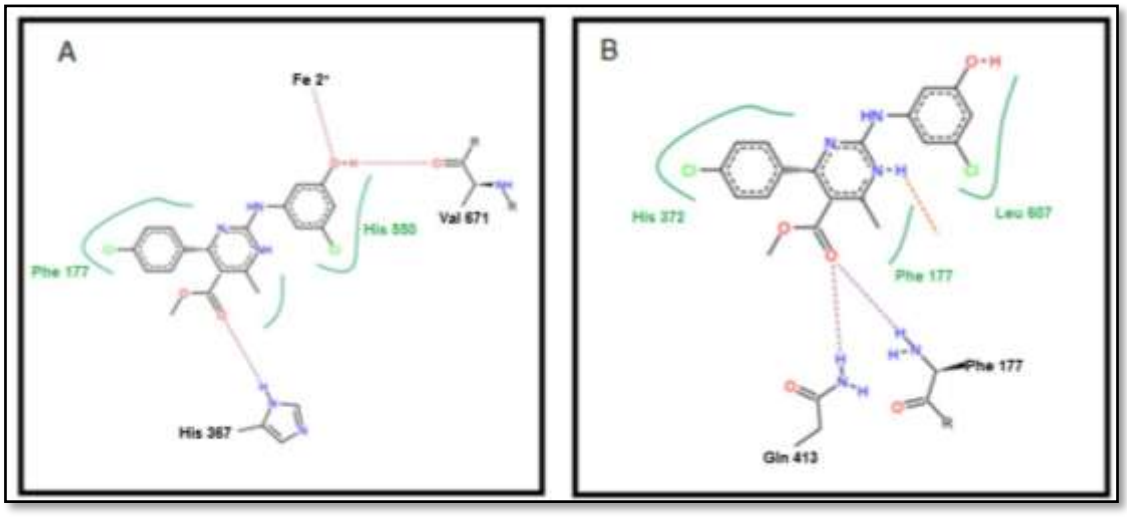


Figure 24. Two different binding modes resulted from Leadit 2.1.2 docking.

Note: Two dimensional representations of the docking interactions were obtained by Leadit 2.1.2.

DHPM 8 with the second best activity in lipoxygenase inhibition (67.08 ± 5.22%) had the second rank in MOE 2013.08 docking, with an affinity of -6.20 kcal/mol. **DHPM 8**

displayed a hydrogen bond with Phe 177 and with Gln 413 (Figure 25). Docking of **DHPM 8** with Leadit 2.1.2 exposed three binding interactions with Phe 177, Ala 672 and with Fe^{2+} (Figure 25). Two hydrogen bonds were formed with Phe 177 and Ala 672 in addition to this ligand-Fe^{2+} interaction was also observed.

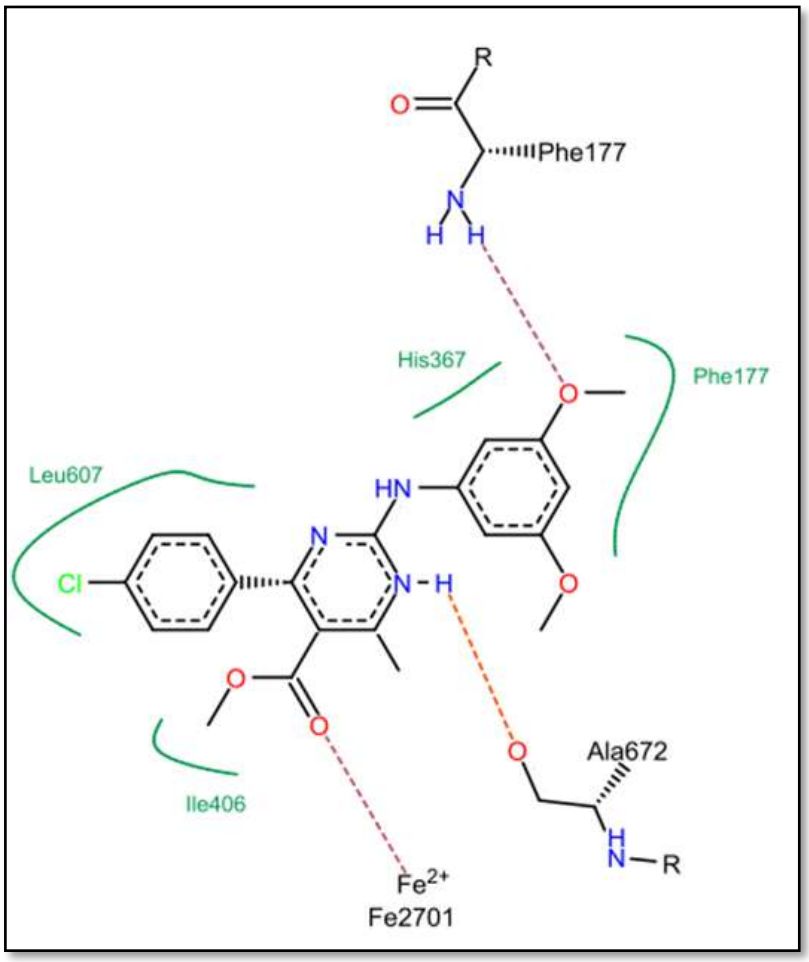


Figure 25. Binding mode of compound DHPM 8 in its active site.

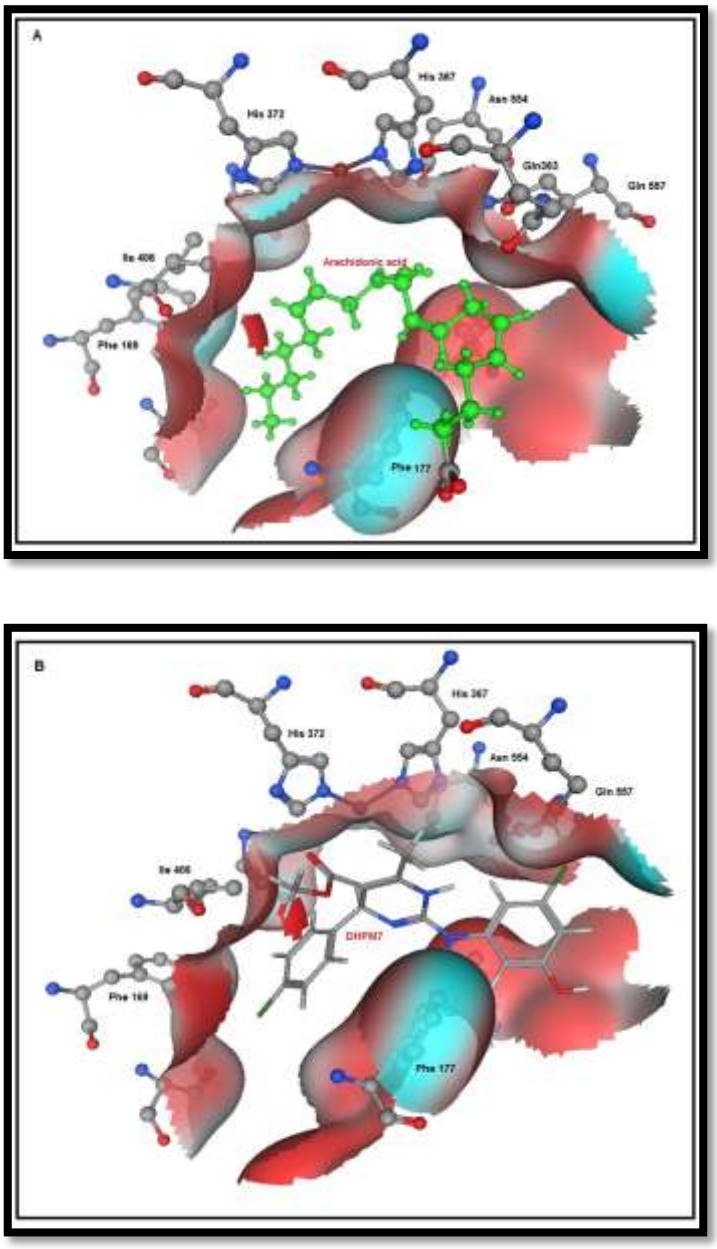


Figure 26. A comparative binding mode between A) Arachidonic acid and B) DHPM 7 inside the active site of lipoxygenase. The blue parts represent the mild polar parts and the red colored parts represent the non-polar hydrophobic parts.

The placement of the best position of the most active compound **DHPM 7** inside the active site was compared to that of arachidonic acid (Figure 26). This figure provides good insights into the hydrophilic and hydrophobic regions of the pocket. It also depicts the best conformation for **DHPM 7** to fill in the total space of the pocket in addition to excellent orientation of its groups towards the best interactions.

4.7. Safety evaluation

4.7.1. Brine shrimp lethality assay

The brine shrimp lethality assay was conducted on **DHPM 1-8** at the following concentrations ranging from 10 µg/mL, 100 µg/mL, 500 µg/mL and 1000 µg/mL. The activity of the shrimp was determined at 1, 2, 3, 4, 20 and 24 h. According to the literature available, a compound is considered to be toxic if it produces ≥ 50% shrimp death.

All compounds were toxic at 1 mg/mL apart from **DHPM 7** which caused 10% shrimp death (Table 7 highlighted in yellow). **DHPM 2**, **5** and **6** were found to be toxic at 500 µg/mL (Table 7 – highlighted in red), while all other compounds were found to be safe at this concentration. During the first 4 h of testing the compounds did not display highly toxic activity; it was after 20 h that most of the toxicity was displayed. All compounds were found to have no toxicity ≤ 100 µg / mL. Statistical analysis is depicted in Table 9.

Table 7. Toxicity of DHPM 1-8 against *Artemia salina* over 24 h

Compound	Concentration (µg/mL)	% Death					
		1 h	2 h	3 h	4 h	20 h	24 h
DHPM 1	10	0	0	0	0	0	0
	100	0	0	0	0	0	0
	500	3	3	3	3	10	10
	1000	17	17	37	37	70	83
DHPM 2	10	0	0	0	0	0	0
	100	0	0	0	0	0	0
	500	0	7	7	10	50	77
	1000	13	13	13	13	77	83
DHPM 3	10	0	0	0	0	0	0
	100	0	0	0	0	0	0
	500	17	20	20	20	20	20
	1000	20	40	40	43	50	50
DHPM 4	10	0	0	0	0	0	0
	100	0	0	0	0	0	0
	500	0	0	0	0	17	17
	1000	0	0	0	13	17	57
DHPM 5	10	0	0	0	0	0	0
	100	0	0	0	0	0	0
	500	0	20	20	20	50	70
	1000	13	23	23	33	80	90
DHPM 6	10	0	0	0	0	0	0
	100	0	0	0	0	0	0
	500	0	23	30	30	80	100
	1000	3	43	47	47	87	100
DHPM 7	10	0	0	0	0	0	0
	100	0	0	0	0	0	0
	500	0	0	0	0	10	10
	1000	10	10	10	10	10	10
DHPM 8	10	0	0	0	0	0	0
	100	0	0	0	0	0	0
	500	0	0	0	0	27	40
	1000	0	40	40	40	50	70

4.7.2. Salmonella Ames mutagenicity assay

Table 8 displays the mutant frequency of **DHPM 1-8** tested against the *Salmonella typhimurium* strains, TA 98 and TA 100. The mutant frequency is the quotient of the number of revertant colonies over the colonies in the negative control. In this assay DMSO, the solvent was used as the negative control (Figure. 27 B) while sodium azide, a known mutagen was the positive control (Figure. 27 A). Maron and Ames (1983) reported that a mutagenic potential is assumed if the mutant frequency of a compound is greater than 2; a compound is a possible mutagen if its mutant frequency ranges between 1.7 and 1.9; and if the mutant frequency is below 1.6 then no mutagenic potential is assumed. **DHPM 1-8** displayed no mutagenic potential against TA 98 and TA 100 up to a concentration of 1000 µg/mL. Sodium azide is a known mutagen and it did demonstrate mutagenicity by producing increasing mutagenic potential with the increase in concentration of the mutagen. Statistical analysis is illustrated in Table 9.

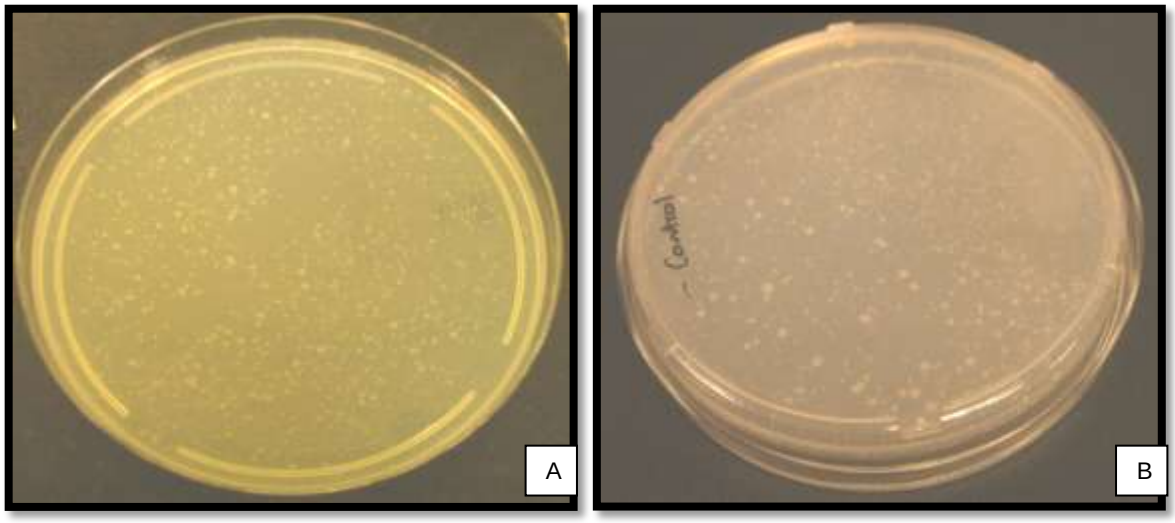


Figure 27. A – *Salmonella typhirium* TA 100 treated with 5 µg/mL sodium azide. B - *Salmonella typhirium* TA100 treated with DMSO (negative control)

Table 8. Mutant frequency of S. typhimurium TA 100 and TA 98 against DHPM 1-8

DHPM	Concentration(µg/mL)	Mutant Frequency	
		TA 100	TA 98
1	1000	0.81 ± 0.13	0.24±0.06
	100	0.8 ± 0.15	0.22±0.03
	10	0.61 ± 0.32	0.04±0.06
2	1000	0.08 ± 0.01	0.29±0.03
	100	0 ± 0	0.2±0.06
	10	0 ± 0	0.14±0.03
3	1000	0 ± 0	0.35±0.06
	100	0 ± 0	0.27±0.13
	10	0 ± 0	0.14±0.09
4	1000	0 ± 0	0.25±0.25
	100	0 ± 0	0.24±0
	10	0 ± 0	0.14±0.09
5	1000	0.78 ± 0.15	0.41±0.09
	100	0.5 ± 0.33	0.02±0
	10	0.49± 0.18	0.06±0.09
6	1000	0.77 ± 0.06	0.53±0.06
	100	0.71 ± 0.09	0.18±0.06
	10	0.57 ± 0.05	0.1±0.09
7	1000	0.65 ± 0.56	0.18±0.03
	100	0.43 ± 0.18	0.18±0.03
	10	0.18 ± 0.16	0.1±0.03
8	1000	1.02 ± 0.04	0.1±0.09
	100	0.93 ± 0.06	0.08±0.03
	10	0.77 ± 0.21	0.02±0.03
NaN_3	20	6.67±0.13	0.88 ± 0.02
	10	3.67±0.16	0.76 ± 0.61
	5	2.67±0.19	0.53 0.18

Table 9. Statistical analysis of brine shrimp lethality (500 µg/mL) and Salmonella Ames test (1000 µg/mL)

Bonferroni's multiple comparison test			
Comparison	**Brine shrimp lethality** (+ Control Potassium dichromate)	**Salmonella Ames test** (+ Control NaN_3)	
		TA 98	**TA 100**
DHPM 1 vs. control	****	ns	ns
DHPM 2 vs. control	ns	ns	****
DHPM 3 vs. control	****	ns	****
DHPM 4 vs. control	****	ns	****
DHPM 5 vs. control	*	ns	*
DHPM 6 vs. control	ns	ns	ns
DHPM 7 vs. control	****	ns	ns
DHPM 8 vs. control	***	ns	ns

ns - P > 0.05, * - P ≤ 0.05, ** - P ≤ 0.01, *** - P ≤ 0.001, **** - P ≤ 0.0001

4.8. Determination of the cytotoxicity of DHPM 1-8

4.8.1. Cytotoxicity against MCF -7 and UACC – 62 cells

The cytotoxic effect of **DHPM 1-8** was determined on the MCF - 7 and UACC – 62 cell lines at 50 µg/mL and 100 µg/mL. The purpose of the cell viability assay was to determine the cytotoxic effect against the above cell lines and to establish whether **DHPM 1-8** would be effective anticancer agents. These results are illustrated in Figure 28, Figure 29 and Table 10**.** The DHPMs showed an inhibitory effect against both the cell lines in a concentration dependent manner at a concentration of 50 µg/mL to 100 µg/mL. The effect of the **DHPMs** was also compared to the effect produced by Doxorubicin and Camptothecin, known anti-cancer agents.

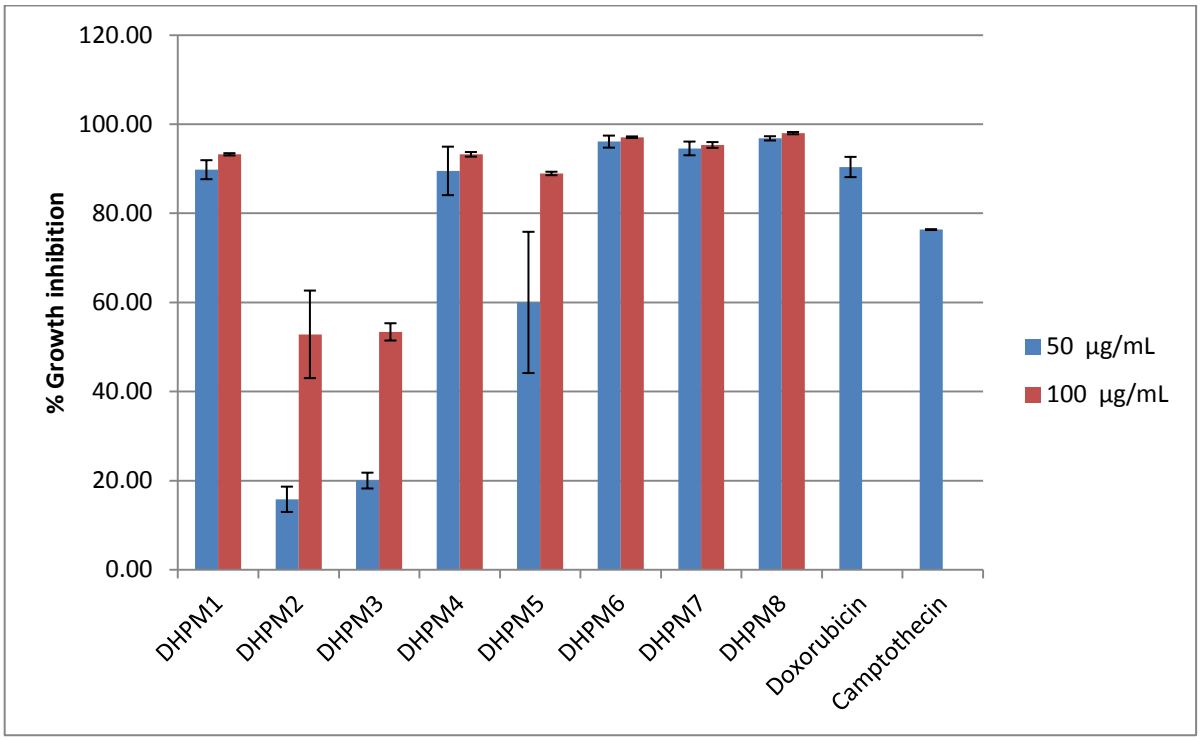


Figure 28. Growth inhibition produced by DHPMs against MCF-7 cells.

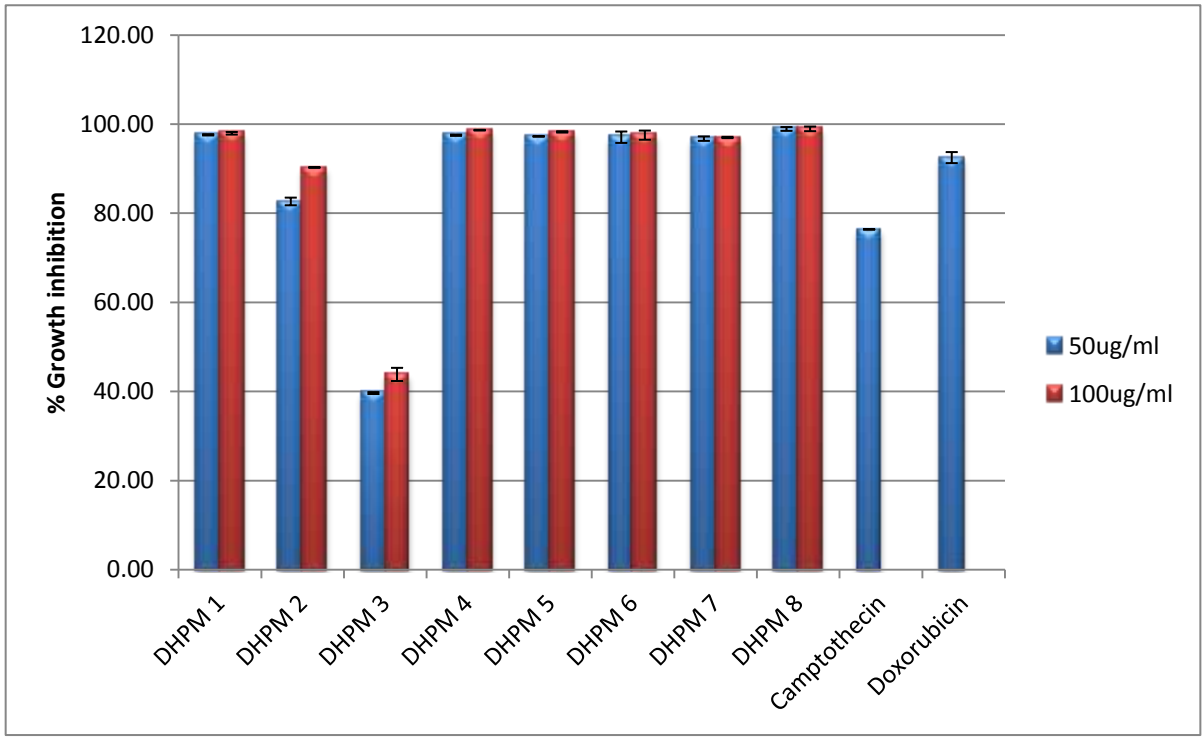


Figure 29. Growth inhibition produced by DHPMs against UACC-62 cells.

4.8.2. Cytotoxicity against PBMC's

The PBMC's were treated with **DHPM 1-8** at concentrations of 50 µg/mL and 100 µg/mL. The treated PBMC's displayed cell viability above 75% as shown in Figure 30 and the statistical analysis is illustrated in Table 10. The PBMC's were also treated with the standard Camptothecin. **DHPM 2** and **4** had a proliferative effect on the PBMC's, whilst other **DHPMs** displayed less than 21% toxicity.

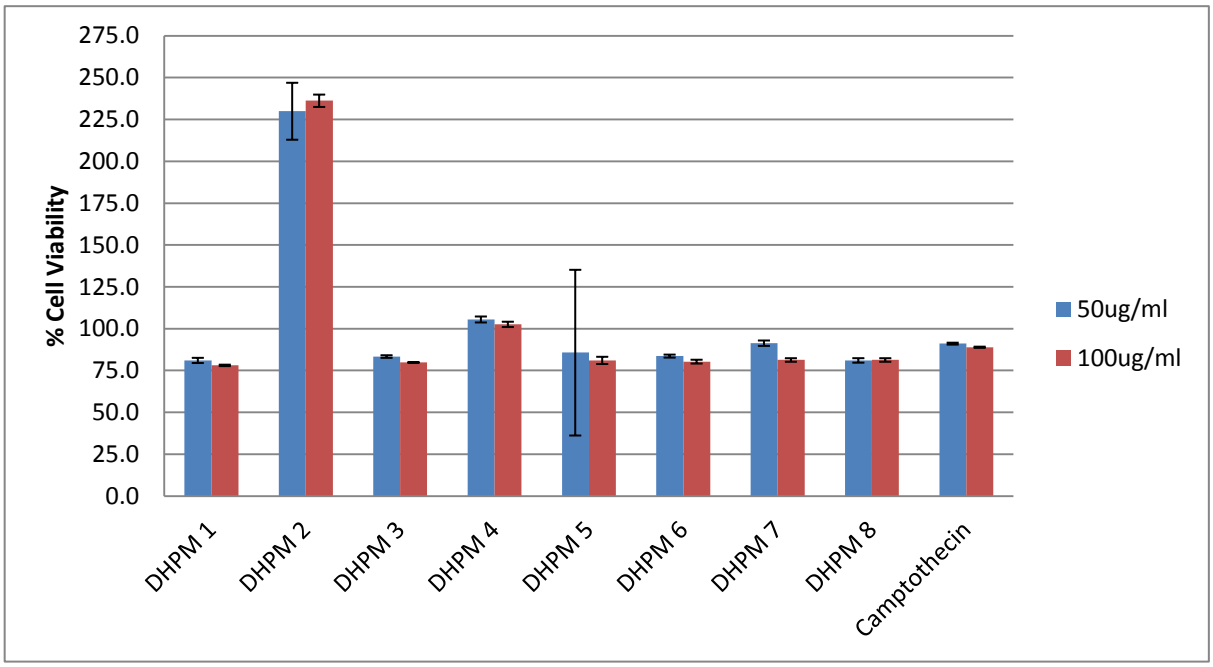


Figure 30. Cell viability of PBMC's treated with DHPM 1 – 8

On comparison of cell viability of **DHPM 1-8** against the MCF – 7, UACC – 62 and PBMC's, it is evident that the **DHPMs** are highly toxic to the MCF – 7 and UACC-62 cell lines, as the growth of the cell lines was significantly inhibited. Cancer cells exposed to **DHPM 1** and **DHPM 4-8** at 50 µg/mL and 100 µg/mL displayed < 5% viability, while under the same conditions PBMC's exhibited > 75% viability.

Table 10. Statistical analysis of growth inhibitory activity of MCF-7, UACC-62 and PBMC cell lines at 100 µg/ml

Bonferroni's multiple comparison test			
Sample	**MCF-7**	**Melanoma(UACC-62)**	**PBMC**
DHPM 1 vs. Camptothecin	ns	ns	ns
DHPM 2 vs. Camptothecin	ns	ns	****
DHPM 3 vs. Camptothecin	**	**	ns
DHPM 4 vs. Camptothecin	**	**	ns
DHPM 5 vs. Camptothecin	*	*	ns
DHPM 6 vs. Camptothecin	ns	ns	ns
DHPM 7 vs. Camptothecin	ns	ns	ns
DHPM 8 vs. Camptothecin	ns	ns	ns

ns - P > 0.05, * - P ≤ 0.05, ** - P ≤ 0.01, *** - P ≤ 0.001, **** - P ≤ 0.0001

4.9. IC $_{50}$ values

4.9.1. MCF – 7 IC $_{50}$

All **DHPMs** which inhibited 80% or more growth of MCF – 7 were further evaluated to determine their IC_{50} values. These results are depicted in Figure 35 in Appendix 7. and Table 11.

Table 11. IC 50 values against MCF-7

Compound	IC $_{50}$ (µg/mL)	R Square value (Goodness of fit)
DHPM 1	22.22	0.9980
DHPM 4	17.45	0.9872
DHPM 6	7.02	0.9975
DHPM 7	35.33	0.9874
DHPM 8	0.92	0.9921
Camptothecin (standard)	1.61	0.9882

4.9.2. IC_{50} values against UACC-62 cells

The **DHPMs** which displayed growth inhibition greater than 80% against the UACC – 62 cell line were analysed further to determine their IC_{50} values. These results are displayed in Table 12 and Figure 36 (Appendix 7).

Table 12: IC 50 values against UACC – 62

Compound	IC_{50} (µg/mL)	R Square value (Goodness of fit)
DHPM 1	6.63	0.9922
DHPM 2	21.40	0.9825
DHPM 4	6.21	0.9983
DHPM 5	7.22	0.9848
DHPM 6	12.77	0.9998
DHPM 7	19.55	0.9901
DHPM 8	1.97	0.9938
Camptothecin (standard)	0.09	0.9816

4.10. Apoptotic activity of active DHPMs against UACC-62 cells

4.10.1. Morphology of UACC-62

Cells that were treated with **DHPM 1, 2, 4-8** were also studied for morphological changes microscopically. After treatment, characteristic changes of apoptosis were observed in the morphology of the treated cells. The untreated cells remained intact and displayed no morphological changes.

In Figure 31 A (the untreated cells) the normal morphology of UACC-62 is depicted. There is no apparent difference in morphology between the solvent control cells (Figure 31 B) and untreated cells. The positive control (Camptothecin) in Figure 31 C, shows changes in cell morphology where cells have rounded up and separated from each other. In Figure 31 D, E and F which are **DHPM 2**, **5**, **7** treated cells respectively, there are various cell morphology changes taking place. In Figure 31 D, **DHPM 2** has brought about the effect of cell separation, some cells have rounded up and others are shrinking. **DHPM 5** (Figure 31 E) effect on the cells has brought about the rounding of cells and **DHPM 7** (Figure 31 F) has made the cells round up, there is shrinking of cells and many of the cells have undergone fragmentation.

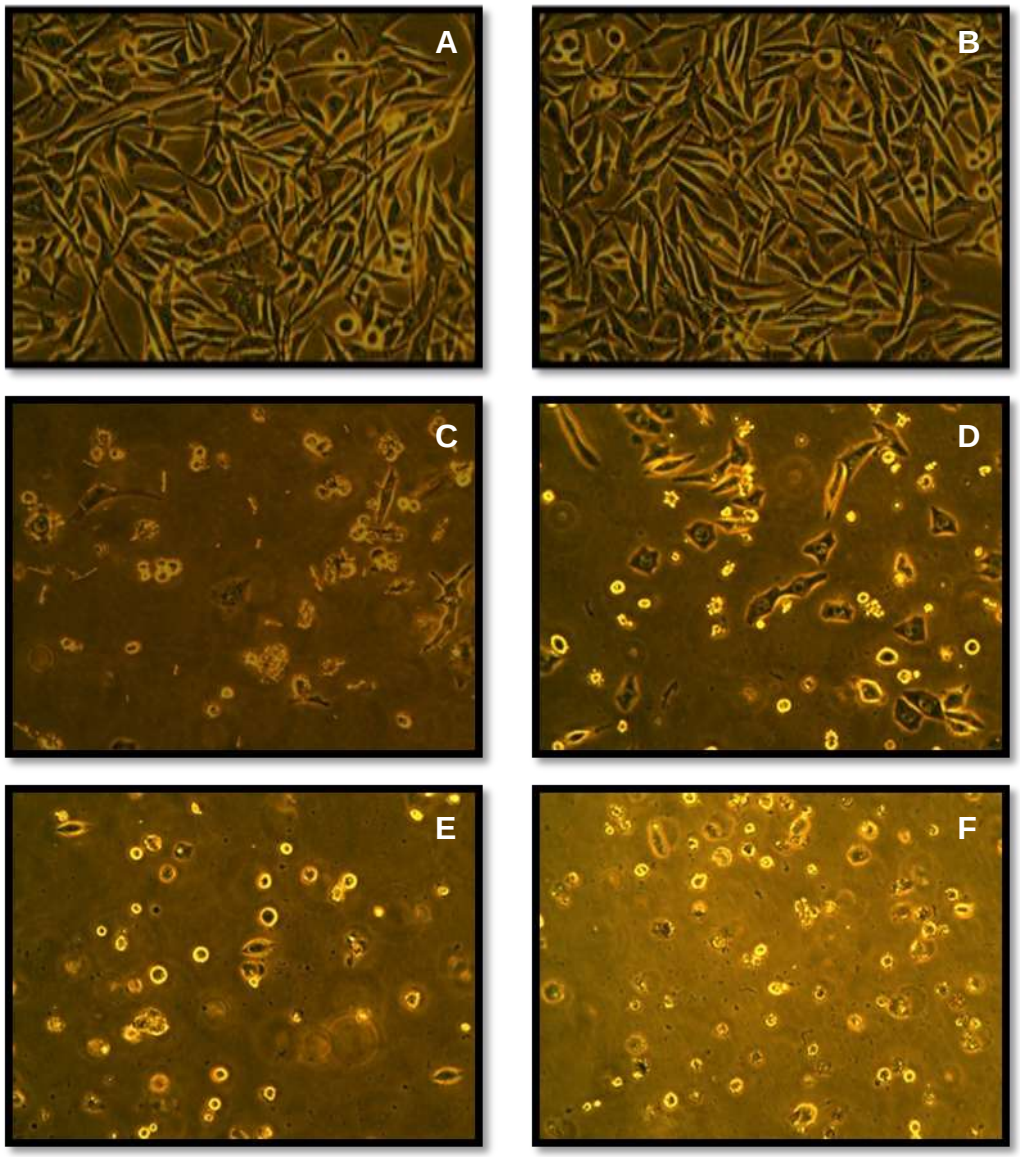


Figure 31. Microscopic examination of UACC-62 cells displaying morphological changes of apoptosis. A - untreated cells, B- DMSO control, C- positive control, Camptothecin, D- DHPM 2, E – DHPM 5 and F – DHPM 7 (100x magnification).

4.10.2. Detection of plasma membrane changes using the Annexin V assay

The Annexin V- FITC assay was performed on UACC – 62 cell line treated with active **DHPMs** to detect apoptosis. Early apoptosis is demonstrated by the loss of symmetry of the phospholipid membrane of cells. As a result, phosphatidylserine is exposed on the plasma membranes outer surface. Annexin V is used to detect the alteration in the asymmetry of the cell membrane (O'Brien *et al*., 1997).

According to this assay viable cells are $FITC^-/PI^-$, cells undergoing apoptosis are $FITC^+/PI^-$ and cell undergoing necrosis are $FITC^+/PI^+$ (Vermes *et al*., 1995). The results are obtained in a scatter plot with four quadrants: the upper right quadrant denotes necrotic/dead cells, the upper left quadrant denotes late apoptotic cells, viable cells are found in the lower left and apoptotic cells are found in the lower right quadrant. The cells treated with **DHPMs** (50 µg/mL) were exposed to Annexin V-FITC. The various controls are presented in frames I-L in Figure 32, with Camptothecin being the positive control, Camptothecin stained with annexin only, Camptothecin stained with PI only, the untreated cells and negative control (DMSO).

All compounds were able to induce apoptosis with **DHPM 8** and **DHPM 7** producing 78% and 36.60%, respectively (Table 13). The compounds also generated late apoptosis and necrosis in the cells; this is possibly due to their elevated cytotoxic nature or the incubation period of the cells with the **DHPMs**. The high percentage seen in the quadrant depicting late apoptosis indicates that apoptosis could have occurred early into the incubation of 24 h, thus it could have been detected early if there had been a shorter incubation period, this is also supported by the low percentage of viable cells amongst cells treated with **DHPM 1, 2** and **6**.

Table 13.UACC-62 treated with DHPM 1-8 and stained with Annexin V-FITC/PI

Treatment(50 µg/mL)	Viable (%)	Apoptotic (%)	Late Apoptotic (%)	Necrotic (%)
DHPM 1	21.40	0.57	16.30	61.70
DHPM 2	9.59	14.80	27.70	9.59
DHPM 4	58.90	0.08	0.94	40.10
DHPM 5	45.90	6.73	7.79	39.50
DHPM 6	24.50	36.60	35.3	3.56
DHPM 7	44.60	6.29	14.30	34.90
DHPM 8	12.20	78.00	8.27	1.56
Camptothecin (annexin)	58.3	41.20	0.079	0.45
Camptothecin (PI only)	15.50	0	0	84.5
Camptothecin (A + PI)	51.90	2.11	34.30	11.60
Untreated /Unstained	100	0	0	0
Negative Control	80.80	6.40	10.60	2.22
Positive Control	73.60	2.84	4.26	19.30

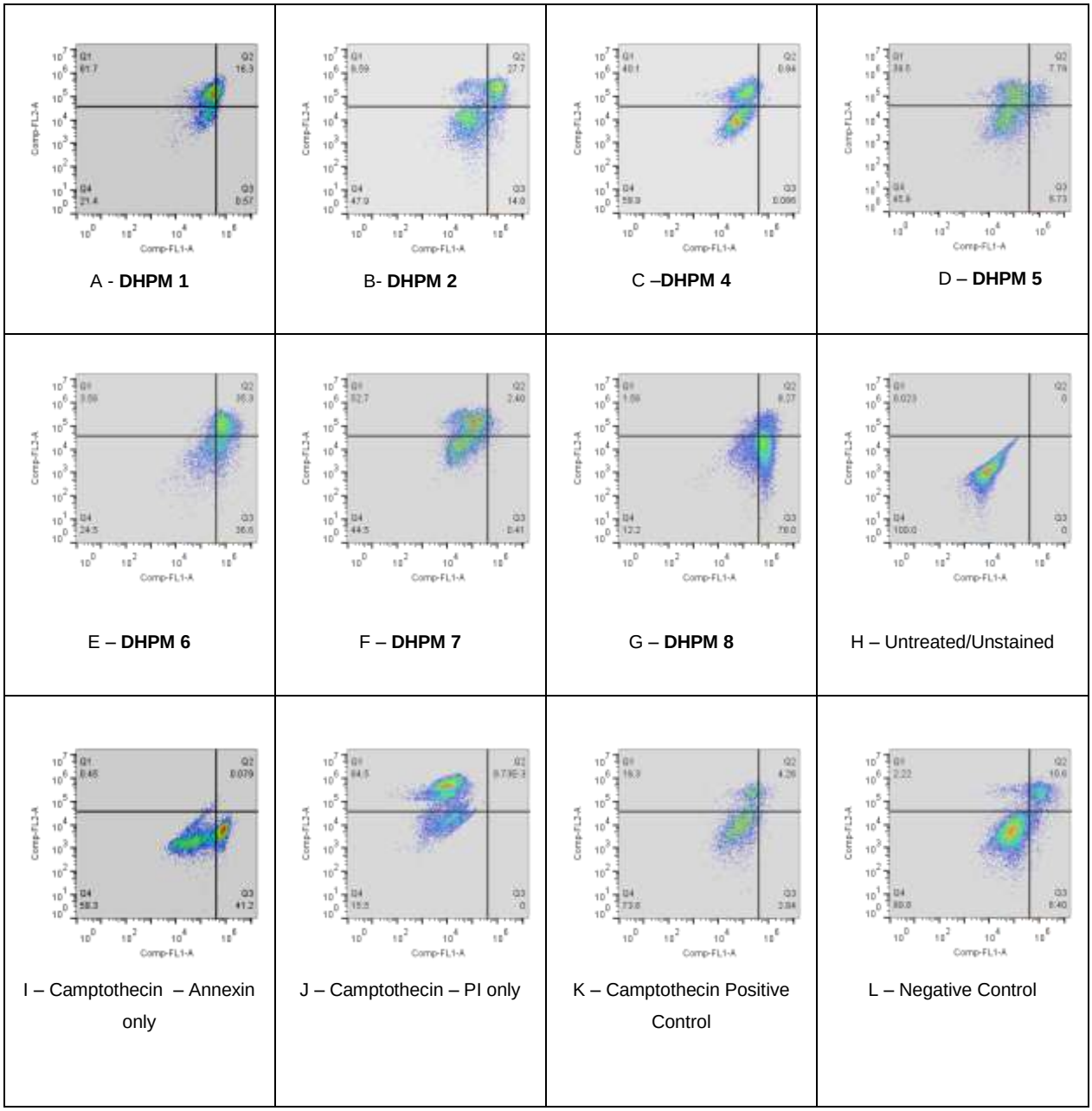


Figure 32. Flow cytometry analysis demonstrating the externalization of phosphatidyl-serine in UACC-62 cells treated for 24 h with DHPM 1-8.

Annexin V-FITC Apoptosis Detection assay results are presented in a scatter plot form, denoting the percentage of cells that are necrotic (upper left), viable (lower left), apoptotic (lower right) and late apoptotic (upper right).

4.10.3. Caspase 3 activity

Caspase 3 activity was detected using the Caspase -3 activity kit (BD Bioscience). Caspase enzymes are from a family of cysteine proteases and they play an important role in cell death (Wang *et al*., 2005). Caspases are also known as the executioners of apoptosis. The active PE caspase 3 detection assay allows for the detection and quantification of active caspase 3 by FACS analysis (BD Bioscience). This assay is based on the principle that active caspase 3 is recognized by monoclonal antibodies that were specifically made against the cleaved form of caspase 3. The monoclonal antibodies are conjugated with the fluorochrome PE (Bunpo *et al.*, 2004). Consequently, the apoptotic cells that contain active caspase-3 will fluoresce.

All compounds displayed caspase-3 activity (Table 14 & Figure 33). **DHPM 1**, **2**, **4** - **8** displayed caspase activity that was superior to the positive control, Camptothecin. **DHPM 8** produced the greatest caspase-3 activity of 51% and the lowest activity of 24.40% was displayed by **DHPM 4**. It is evident that the **DHPMs** are inducing apoptosis through the caspase -3 pathways.

Table 14. UACC-62 cell line treated with DHPM 1-8 and stained with Caspase 3- PE

Treatment	Caspase-3 Negative	Caspase-3 Positive
DHPM 1	52.60	47.40
DHPM 2	49.70	50.30
DHPM 4	75.60	24.40
DHPM 5	54.10	45.90
DHPM 6	64.10	35.90
DHPM 7	46.40	53.60
DHPM 8	49.00	51.00
Untreated/Unstained	96.60	3.45
Stained	90.6	9.36
Negative Control	98.50	1.46
Positive Control	66.30	33.70

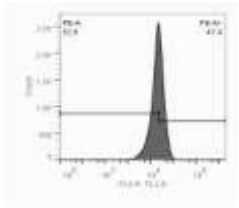
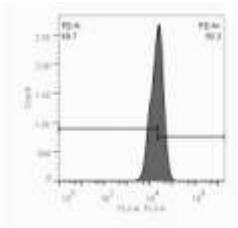
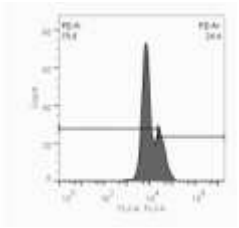
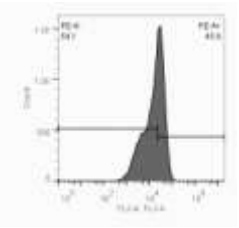
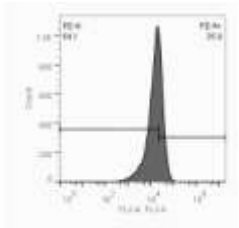
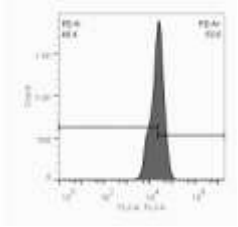
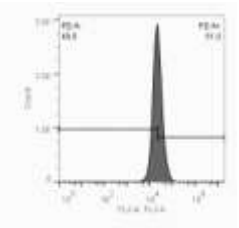
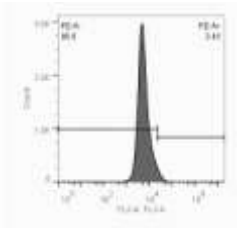
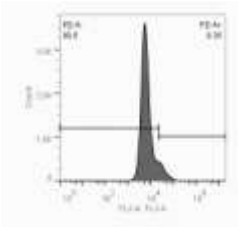
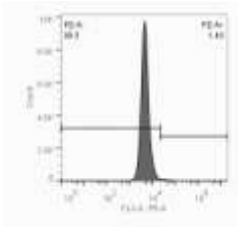
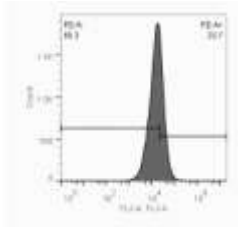
 A - **DHPM 1**	 B- **DHPM 2**	 C –**DHPM 4**	 D – **DHPM 5**
 E – **DHPM 6**	 F – **DHPM 7**	 G – **DHPM 8**	 H- Untreated/Unstained
 I - Stained	 J - DMSO	 K – Camptothecin	

Figure 33. Flow cytometry analysis demonstrating the release of active caspase 3 in UUAC-62 cells treated for 24 h with DHPMs. PE caspase 3 assay results are presented in a histogram.

4.10.4. Mitochondrial potential

The JC-1 assay was used to detect change in the mitochondrial membrane potential and it also enables the detection of early apoptosis. JC-1 collects in the mitochondria of healthy cells and forms aggregates whilst it does not accumulate in the mitochondria of apoptotic cells; it remains as monomers in the cytoplasm. The monomers and aggregates have differing emission spectra thus allowing them to be detected (Moghimi *et al*., 2005). The analysis of the **DHPMs** showed a high percentage of monomers (Table 15), thus proving that the mitochondrial membrane was altered. The JC – 1 activity ranged between 47.30% and 87.40%. All **DHPMs** other than **DHPM 2** [47.30% JC-1 activity] had greater activity than the positive control, Camptothecin which had JC -1 activity of 56.50%. The scatter plots are depicted in Figure 34.

Table 15. UACC-62 cell line treated with DHPMs and stained with JC-1

Treatment	**JC-1 Monomers (Apoptotic cells)**	**JC-1 Aggregates (Healthy cells)**
DHPM 1	66.20	33.70
DHPM 2	47.30	52.00
DHPM 4	77.30	22.10
DHPM 5	70.70	29.2
DHPM 6	83.10	16.6
DHPM 7	74.40	25.40
DHPM 8	87.40	12.50
Stained	20.10	75.10
Negative Control	11.60	84.80
Positive control (Camptothecin)	56.50	27.60

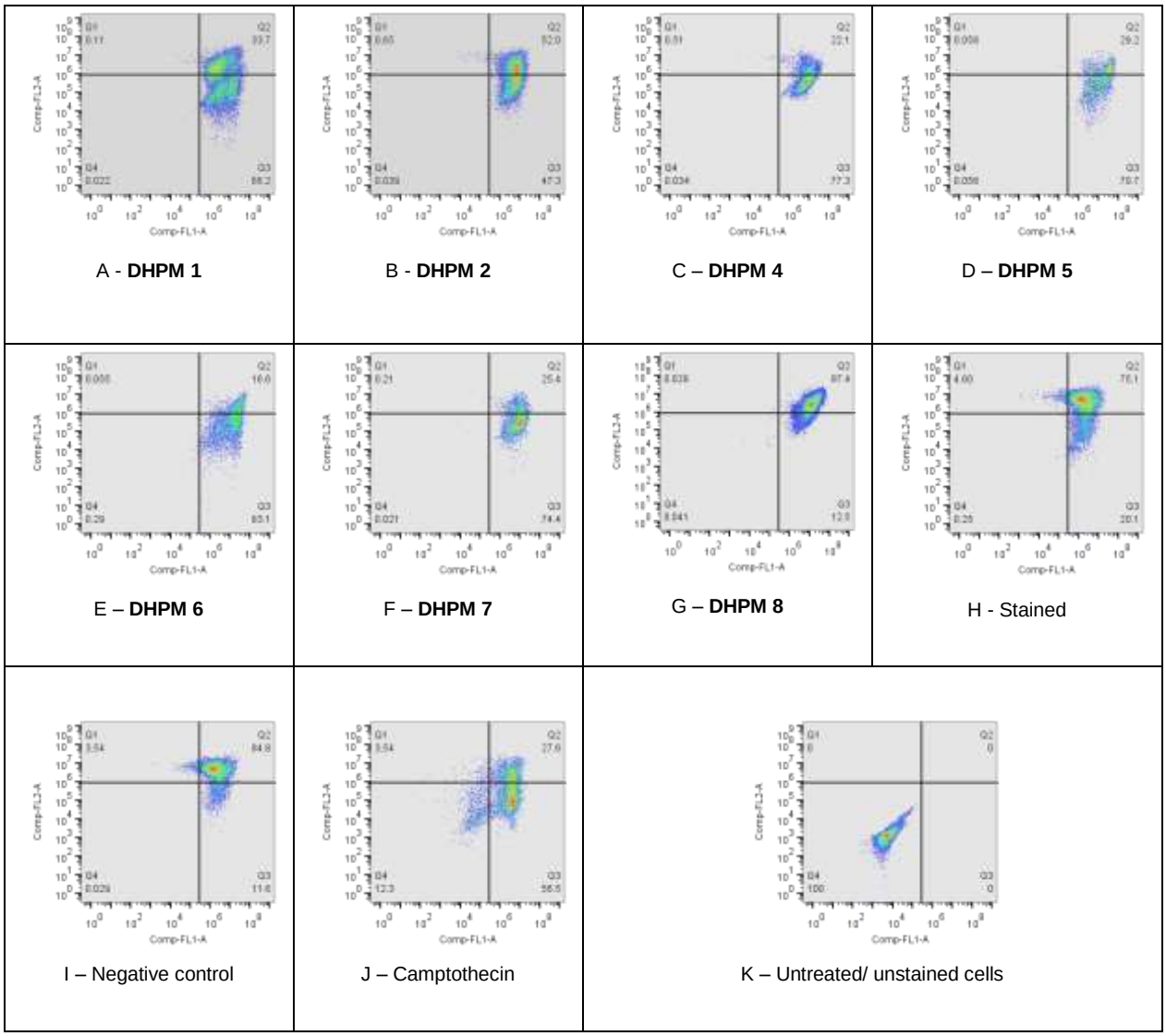


Figure 34. Flow cytometry analysis demonstrating the mitochondrial potential in UACC-62 cells treated for 24 h with DHPMs.

5. DISCUSSION

5.1. Antimicrobial activity

5.1.1. Antibacterial activity

DHPM 1-8 was tested at a concentration of 3 mg/mL. Ciprofloxacin was used as the standard drug (positive control). The **DHPMs** were found to display activity against the Gram positive organisms (*B.cereus, M.luteus, S.aureus*, *B.coagulans, B. stearothermophilus and S. faecalis*). No inhibitory effect was displayed by the **DHPM 1 – 8** against the Gram negative organisms (Table 2).

DHPM 1-5 showed weak activity against *B.cereus*, due to electronegative atoms at different positions of the phenylamino ring, while **DHPM 6** having bromine as electronegative atom at fourth position exhibited moderate activity at 7 mm.

DHPM 7 having two electron withdrawing groups which are chlorine at third and hydroxy at fifth position on phenylamino ring and **DHPM 8** having dimethoxy functional groups at second and fourth position as electron releasing groups on benzylamino ring displayed promising activity with zones of inhibition of 10 mm and 11.33 mm $\pm$2.08,respectively.

Overall the best inhibitory effect was displayed by **DHPM 7** having two electron withdrawing groups at third and fifth position on phenylamino ring and **DHPM 8** which has electron donating 2, 4-dimethoxy functional groups on the benzylamino ring system which is attached to the dihydropyrimidine pharmacophore.

According to literature, substitution of pyrimidine derivatives with an electron donation moiety, increases antimicrobial activity, whilst moderate activity is displayed by derivatives which are substituted with electron drawing moieties. Electron drawing moieties are known to decrease antimicrobial activity (Fayed and Ghanem, 2013).

All compounds that displayed a significant inhibitory effect, that is, compounds that produced zones of inhibition greater than 9 mm were further analysed to determine the Minimum Inhibitory Concentration (Table 3).

5.1.2. Antifungal activity

In this study, **DHPM 1-8** was tested for antifungal activity against 2 fungi (*A.flavus* and *A.niger*) and 3 yeasts (*C.utilis, S.cerevisiae* and *C.albicans*). **DHPM 1-8** were found to be inactive against the organisms used. Although Biginelli compounds have not been explored extensively as antifungal agents, it was found in literature that many synthetic dihydropyrimidines do possess excellent antifungal activity. It was reported that compounds possessing a phenyl ring with a substituted with an electron withdrawing substituent as opposed to an electron releasing group at the C-4 position display high antifungal activity (Jalali *et al*., 2012).

In a previous study, it was established that excellent antifungal activity was displayed by pyrimidine compounds which carry a dimethylated adduct and a benzimidazole substituted derivative (Bhalgat *et al*., 2014). Thus, it can be stated that the nature of the substituents on the phenylamino ring of the pyrimidine molecules will have an influence on the antifungal activity. This can be taken into consideration when synthesizing compounds for future studies.

5.1.3. Minimum inhibitory concentration

Minimum inhibitory concentration (Table. 3) was determined for compounds which showed the activity ≥ 9 mm. These compounds were **DHPM 7** and **8**. The MIC for **DHPM 7** and **8** against the Gram positive organisms was 0.75 µg/mL; however **DHPM 7** had a remarkable MIC of 0.37 ug/mL against *M.luteus*.

This activity can be attributed to **DHPM 7** containing two electron withdrawing substitutions, chlorine at position C - 3 and a hydroxy at position C - 5 on the phenylamino ring. **DHPM 8** has an electron donating 2, 4-dimethoxy substitution at position C-2 and position C-4 on the phenylamino ring.

From literature surveyed, it has been observed that the antimicrobial activity of compounds is dependent on the type (electron withdrawing or electron donating group) and position of substitutions made on the main pharmacophore. This may result in an increased activity of a compound (Gopalakrishnan *et al*., 2009, Sharma *et al*., 2014).

5.2. Antioxidant activity

Free radicals are the causative agents for many diseases; hence there is always a need for the development of new antioxidants to prevent damage that is brought about by these free radicals. It is known that several diseases in humans are induced by the increased production of reactive oxygen species (ROS) (Jadhav *et al*., 2012).

In many of the diseases, it was also found that natural antioxidant defences are deficient. Therefore it has been suggested that the advancement of disease can be impeded by supplementation with antioxidants. Antioxidant therapy is of use in diseases such as diabetes, inflammatory and degenerative diseases (Maxwell, 1995).

In this study the potential antioxidant effect of **DHPM 1-8** was evaluated at concentrations ranging from 1 µg/mL to 1000 µg/mL. The free radical scavenging capacity results of **DHPM 1 - 8** and Rutin is expressed as percentage inhibition (Figure 26 and 27). **DHPM 1**, **3 - 8** demonstrated reduced scavenging capacity in comparison to the positive control Rutin.

DHPM 2 displayed excellent scavenging capacity which was 90.63 ± 1.1% in comparison to the standard Rutin which exhibited a scavenging capacity at 94.81 ± 0.3%. From statistical analysis (Table 4), there is no significant difference in the free radical scavenging activity between the positive control and **DHPM 2**. **DHPM 2** has a hydroxy group at second position and nitro group at the fourth position on phenyl ring, which is attached to the dihydropyrimidine pharmacophore by a secondary amine bridge. It was reported that compounds which have a OH group are likely to have antioxidant properties (Gangwar and Kasana, 2012). In a previous study, it was observed that the antioxidant activity of a compound is dependent on the number of active groups (OH or NH_2) present in the compound. The position of the active compound also plays a major role in the antioxidant activity (Bendary *et al*., 2013).

5.3. Anti-inflammatory activity

The anti-inflammatory activity of **DHPM 1-8** was determined at a concentration of 1 mg/mL. All **DHPMs** displayed moderate anti-inflammatory activity (59.37 – 81.19%). **DHPM 7** showed excellent anti-inflammatory activity of 81.19% (Figure 20), this may be due to **DHPM 7** having two electron withdrawing groups which are chlorine at third and hydroxy at fifth position on phenylamino ring. Literature revealed that modifying

the structure of a compound can result in changes that affect the activity of the compound qualitatively and quantitatively (Mishra *et al*., 2010).

In a previous study (Tozkoparan *et al*., 1999), a novel series of thiazolo [3, 2] pyrimidine having 4-bromo, 4-methoxy, 4-methyl, 2-fluorophenyl at position 5 and 4-methyl, 4-methoxy, 4-chloro and non-substituted benzylidene was synthesized. Compounds exhibiting good antiinflammatory activity where found to bear a 4-bromo- and 2-fluorophenyl at position 5 of the condensed ring. Novel dihydropyrimidine carbonitrile and triazole fused derivatives were synthesized by Bhalgat *et al*., 2014. A compound with 3-OCH_3 groups present in its structure were found to have good antiinflammatory activity.

As a result of the promising anti-inflammatory activity of the **DHPM's**, the crystal structure of the human lipoxygenase was studied to elucidate the inhibitory activity computationally by means of a molecular modeling study.

5.4. Molecular modeling

Studying the crystal structure of the human 5-lipoxygenase and 15-lipoxygenase enzyme revealed that the main binding site for both enzymes is at the same point. It was found that the structures of both enzymes have identical conserved cavities with a similar capacity and the same amino acids were found at the binding site. This was also found in a previous study of the two structures (Kobe *et al*; 2014).

The docking scores, displayed by **DHPM 7** using MOE 2013.08 (Chhillar *et al*., 2006), was similar to the biological activity presented by **DHPM 7** when the lipoxygenase Inhibitory Assay was performed. **DHPM 7** exhibited a hydrogen bond with Gln 363 with its –NH group of the pyrimidine ring at a distance = 3.37 A^o. A high binding free energy of this compound was noticed on comparison to other compounds, a direct contact with H_2O 955 water molecule which was close to arachidonic acid with the aniline (-NH) group.

The molecular docking of **DHPM 8** with Leadit 2.1.2 (Rarey *et al*., 1996) software showed different binding modes and residues on the enzymes active site. These residues are depicted in Figure. 24 A and Figure. 24 B. Thus the great inhibitory effect could be as a result of the different residues on the enzymes active site

Both the inhibitory activity results of lipoxygenase and the molecular docking results confirmed the activity of these compounds as potential lipoxygenase inhibitors. These inhibitors may be of great importance in cancer inhibition and this was confirmed by the use of two cancer cell lines: human breast cancer (MCF-7) and human melanoma (UACC-62) cells.

5.5. Safety evaluation

5.5.1 Brine shrimp lethality

The **DHPMs** were tested for toxicity using the brine shrimp assay. **DHPM 7** exhibited no toxicity to the brine shrimp. However, **DHPM 1-6** and **8** were toxic at a concentration of 1 mg/mL. **DHPM 1**, **5** and **6** also displayed toxicity at 500 µg/mL. All compounds were found not to demonstrate any toxicity at 100 µg/mL. The brine shrimp assay is a bench top assay that is quick and inexpensive to conduct (Meyer *et al*., 1982). The assay was also reported to correlate well to cytotoxic and antitumour activities (McLaughlin *et al*., 1993). The compounds were initially analysed at 1 mg/mL and it was found after 24 h all compounds apart from **DHPM 7** led to shrimp mortality ≥ 50% (Table 7). The compounds were then evaluated at lower doses (10 µg/mL, 100 µg/mL and 500 µg/mL) and counted after 1, 2, 3, 4, 20 and 24 h incubation. It was noticed after 20 hour incubation only **DHPM 6** (500 µg/mL) had produced 80% shrimp death, while the percentage death for the remaining compounds was ≤ 50%. After 24 hours of incubation the most potent compounds at 500 µg/mL were **DHPM 2**, **5** and **6**. **DHPM 2** and **DHPM 6** were statistically not significantly different from the positive control, potassium dichromate, whereby $P > 0.05$. It was noted that after 24 hours of exposure to the compounds [**DHPM 1-8**] at a concentration of 10 µg/mL and 100 µg/mL there was no shrimp death. These compounds are probably safe to use at concentrations ≤ 100 µg/mL.

5.5.2. Salmonella Ames mutagenicity assay

The detection of substances able to induce mutations is very important in safety studies as mutagenic substances can potentially induce cancer. The Salmonella Ames test is used widely to screen new drugs to determine their mutagenic potential (Mortelmans and Zeiger, 2000). In this study, the Salmonella Ames mutagenicity assay was carried out on **DHPM 1-8** using the two tester strains TA 98 and TA 100.

DHPM 1-8 did not display a mutagenic effect on the tester strains. The mutant frequency of the control (NaN_3), did increase with the increase in concentration, however this increase did not produce a mutagenic effect.

5.6. Cytotoxicity assay

In this study, the MTT assay was used to determine the toxicity of **DHPM 1-8** on the MCF-7, UACC – 62 cell lines. The effects of the **DHPMs** were also evaluated against PBMC's (normal cells).

The MTT assay uses a tetrazolium salt, in a quantitative colorimetric assay which is used to determine the survival and proliferation of mammalian cells. This assay allows for the detection of living cells and not dead cells, therefore the method can be used to determine a cytotoxicity, proliferation and activation of the cells (Mosmann, 1983).

DHPM 1-8 were screened at 50 µg/mL and 100 µg/mL. After 48 hours of incubation it was demonstrated that all **DHPMs** were able to inhibit the growth of the MCF- 7 cell line, with the lowest percentage inhibition being 52.83% at 100 µg/mL. **DHPM 8** produced the best growth inhibition of the MCF – 7 cell line (97.99%). The overall growth inhibition against MCF - 7, was exceptional with **DHPM 1** and **DHPM 4-8** displaying a percentage growth inhibition greater than 88%. On comparison to the controls used (Doxorubicin and Camptothecin), the **DHPMs** produced similar and in some instances better growth inhibition.

However, compound **DHPM 2** bearing a hydroxy group at second and a group nitro at fourth position of phenyl ring exhibited 15.82 ± 2.84% inhibition against MCF-7 whereas compound **DHPM 3** having bromine and fluorine atoms at third and fourth positions respectively, on phenyl ring exhibited 20.02 ± 1.78% inhibition against MCF-7.

DHPM 1, 2, 4-8 illustrated exceptional growth inhibition against the UACC – 62 cell line. **DHPM 1, 2, 4-8** had growth inhibition values greater than 96%, which was greater activity than that produced by the controls used (Doxorubicin and Camptothecin). However, compound **DHPM 3** having bromine and fluorine atoms at third and fourth positions respectively, on the phenyl ring, exhibited 39.62 ± 0.21% inhibition against UACC-62 cells.

DHPM 1, **3**, **5-8** was found to be least toxic to the PBMC's with toxicities less than 21%. **DHPM 2** ($P \leq 0.0001$) and **DHPM 4** ($P \leq 0.05$) produced a proliferative effect on the PBMC's. When comparing the inhibitory effect of the **DHPMs** against the MCF – 7 and UACC – 62 cell lines and the inhibitory effect of the **DHPMs** against the PBMC, it must be noted that the toxicity towards PBMC's is significantly lower.

Currently, there are many pyrimidine based drugs which are used as anticancer agents (5-Fluorouracil (5-FU), Tegafur and Thioguanine). A few novel pyrimidine compounds were synthesized (Salman *et al*., 2013), 5,6-diamino-2- mercapto pyrimidine- 4-ol hydrochloride and 2-mercapto-6-methyl pyrimidin-4(3H)-one showed moderate anticancer activity against the MCF - 7 cell lines. These compounds contained a nitrogen heterocyclic ring in the molecule. The compound, 6-diaminouracil- 2, 4-diol hydrochloride, illustrated high anticancer activity against the HEPG2 and MCF - 7 cell lines. Based on the structural activity relationship from the results obtained it was concluded that anticancer activity was due to the presence of nitrogen heterocyclic rings and the 2-thiouracil moiety.

A novel series of 2,4,5-substituted pyrimidine derivatives were synthesized and evaluated in vitro for inhibition against human hepato cellular carcinoma (Xie *et al*., 2009). Many compounds displayed potent inhibition with an IC_{50} values less than 0.10 µM. From the structure–activity relationships of these compounds, it was concluded that electron donating groups at the second position of pyrimidine nucleus will determine the anticancer activity and the para-substitution of aromatic ring with electron-donating group will increase the anticancer activity. It is evident from literature that structural activity relationship plays an important role in the biological activities of compounds.

In the present study, it may also be concluded that the anticancer activity obtained is due to the different substitutions present on the phenyl ring of the **DHPM** derivatives.

5.7. IC_{50} of DHPM 1-8 against UACC-62 and MCF-7 cells

The IC_{50} values were determined for all compounds that inhibited more than 80% cell growth of MCF-7 and UACC-62.

All **DHPMs** analysed against MCF -7 displayed IC_{50} values less than 35.33 µg/mL. The best IC_{50} was displayed by **DHPM 8** which was 0.92 µg/mL and this was a lower concentration than the IC_{50} achieved for the control Camptothecin that was used.
The IC_{50} values for **DHPM 1**, **2** and **4-8** were determined in the UACC – 62 cell line. All compounds displayed IC_{50} values of ≤ 21.40 µg/mL. **DHPM 8** displayed the best IC_{50} of 1.97 µg/mL. The control, Camptothecin's IC_{50} was 0.09 µg/mL.

5.8. Apoptotic studies

5.8.1. Plasma membrane changes to detect apoptosis

Changes in the plasma membrane were detected using the Annexin – V FITC Kit (BD Biosciences) which allows for detection of early apoptosis. This assay is easy to carry out and is very sensitive (Vermes *et al*., 1995). The plasma membrane is a site where structural or functional damage can lead to cell death (Kanduc *et al*., 2002). In this study, **DHPM 1, 2, 4-8** were subjected to the Annexin – V assay and tested on UACC-62 cells at a concentration of 50 µg/mL. The untreated cells were found to be viable (100%) after exposure to Annexin V. UACC-62 treated with the **DHPM 1, 2, 4-8** showed results that were Annexin positive.

The synthetic compounds **DHPM 1, 2, 4-8** produced cell death using the early apoptotic pathway (0.08 – 78%) and cell death by late apoptotic pathway (0.94 - 35.33%). **DHPM 8** produced a high population of apoptotic cells, with 78% undergoing early apoptosis. This was greater than the effect produced by the positive control Camptothecin. The negative control displayed 6.40% of cells undergoing early apoptosis whilst 80.80% of the cells were still viable.

5.8.2. Caspase – 3 activity

Caspases are enzymes which play a vital function in cell death. Caspases are engaged in both the initiation and execution of apoptosis (Salvesen and Dixit, 1999). Caspase activity is present when cells are undergoing late apoptosis.

UACC – 62 treated with the **DHPM 1, 2, 4-8** showed considerable release (24.40 - 53.60%) of caspase when exposed to the **DHPMs** at 50 µg/mL. The positive control demonstrated caspase activity of 33.70% whilst **DHPM 1**, **2**, **5 - 8** exhibited activity

which was significantly greater than the control. Overall, **DHPM 7** activated the highest release of caspase -3 (53.60%) compared to 1.46% by the negative control (dmso). Caspase 3 activity was detected using the Caspase -3 activity kit (BD Bioscience).

5.8.3. Mitochondrial potential

During the early stages of apoptosis there are changes in the mitochondrial membrane potential. In this study the JC-1 assay was used for the detection of early apoptosis by detecting changes in mitochondrial membrane potential by using flow cytometry. **DHPM 1, 2, 4-8** showed a high percentage of the formation of monomers in the cytoplasm, which in turn depicts that there, was a change in mitochondrial potential and apoptosis occurred. The apoptotic activity for **DHPM 1, 2, 4-8** was between 47.30 – 87.40%. The activity of the synthetic **DHPMs** was greater than the activity produced by the positive control, Camptothecin (56.50%) with the exception of DHPM 4 which had an apoptotic cell population of 47.30%.

6. CONCLUSIONS

In this study a novel series of substituted 1, 4-dihydropyrimidine analogues [**DHPM 1-8**] were synthesized, characterized and screened for their pharmacological activities. The final compounds **DHPM 1 -8** had good yields of 62 – 74%. **DHPM 1 – 8** were characterized using HRMS, H-NMR and C-NMR. The pharmacological screening performed included antimicrobial, antioxidant and antiinflammatory (lipoxygenase) activity. A molecular docking study, safety study (brine shrimp lethality and mutagenicity) and anticancer activity were also determined. A summary of results is found in Table 16.

DHPM 1 - 8 displayed moderate antimicrobial activity. DHPM 2 displayed antioxidant activity of 90.63% that was comparable to the positive control, Rutin. It was noted that, compounds **DHPM 7** and **8** exhibited potential lipoxygenase enzyme inhibition activity. The activity of the compounds against the lipoxygenase enzyme was also confirmed by a molecular docking study. In terms of safety, DHPM 1 - 8 were found to be non-mutagenic against *S.typhimurium* (TA 98 and TA 100), all **DHPMs** were also found to be not toxic to *Artemia salina* at a concentration ≤ 100 µg/mL. The most significant activity displayed was the cytotoxicity against the Melanoma and MCF-7 cell lines. **DHPM 1**, **4 - 8** had greater cytotoxic activity (>96%) than the standards, Doxorubicin and Camptothecin, used against the Melanoma cell line. **DHPMs 1**, **4**, **6** - **8** displayed high activity against MCF-7 with cytotoxicity values ranging between 89 - 98% toxicity. **DHPM 1-8** were found to display negligible toxicity towards normal PBMCs. These compounds have the potential to be developed into anti-cancer drugs.

Apoptotic studies were performed to determine the cell death mechanism. The Annexin V –FITC assay demonstrated that the mode of cell death is induced foremost by apoptosis and there was also a loss of mitochondrial potential as detected in the JC-1 assay. However it must be noted that significant activity was detected in the Caspase-3 pathway. In 24 h most compounds targeted the late apoptotic cycle by activating caspase-3. This research provides confirmation of the prospective anti-cancer activity of the **DHPMs**. These **DHPMs** are able to suppress cell proliferation in UUAC - 62 and MCF - 7 and induce apoptosis in UUAC - 62 *in vitro*.

Out of all the pharmacological screenings, test compounds exhibited significant anticancer properties. **DHPM 1-8** have similar pharmacophore to commercially available anticancer drugs (Gemcitabine and Fluorouracil) and have the potential to be developed into anti-cancer drugs. Further studies may be needed for the modification of the 1, 4 Dihydropyrimidine scaffolds to obtain potent lipoxygenase and cancer chemoprotective agents.

Table 16. Summary of results of DHPM 1-8.

Activity	DHPM 1	DHPM 2	DHPM 3	DHPM 4	DHPM 5	DHPM 6	DHPM 7	DHPM 8
Antibacterial	Moderate activity (*B.cereus*, *M.luteus, S.aureus)*	Moderate activity (*B.cereus*, *M.luteus* and *S.aureus)*	Moderate activity (*B.cereus*)	Moderate activity (*B.cereus*)	Moderate activity (*B.cereus*, *M.luteus*, *S.aureus & B.stearothermophilus.*	Moderate activity (*B.cereus*, *M.luteus*, *S.aureus & B.coagulans).*	Good activity(*B.cereus*, *M.luteus*, *S.aureus B.stearothermophilus)*	Highest activity against *B.cereus*, *M.luteus*, *S.aureus, B.coagulans, S.faecalis and B.stearothermophilus.*
MIC (µg/mL)	NA	NA	NA	NA	NA	NA	*B.coagulans and S.aureus (0.75*). *M.luteus (0.37*)	*B.cereus, M.luteus S.aureus, B.coagulans S.faecalis,, B.stearothermophilus.*
Antifungal	-	-	-	-	-	-	-	-
Antioxidant	Weak activity <20%	Highest activity of 94.21%	Weak activity <20%	Weak activity <20%	Moderate activity >20%	Moderate activity >20%	Moderate activity >20%	Moderate activity >20%
Antiinflammatory (% Inhibition)	59.37	63.60	65.44	64.83	66.66	66.46	81.19	67.08
Anticancer								
MCF-7 (% Inhibition at 50 µg/mL)	89.79	15.82	20.02	89.51	60.00	96.09	94.56	96.82
MCF-7 IC_{50} (µg/mL)	22.22	NA	NA	17.45	NA	7.02	35.33	0.92
UACC-62 (% Inhibition 50 µg/mL)	91.57	82.62	39.62	97.47	97.22	97.04	96.72	98.89

UACC-62 IC_{50} (µg/mL)	6.63	21.40	NA	6.21	7.22	12.77	19.55	1.97
Apoptotic	Apoptotic studies revealed that **DHPM 1, 2, 4-8** induced apoptosis in UACC-62.							
PBMC'S (% Cell viability)	78.10	236.20	79.90	102.60	81.10	80.30	81.30	81.30
Safety evaluation								
Brine shrimp lethality - Toxicity	≤500 µg/mL	≤100 µg/mL	≤500 µg/mL	≤500 µg/mL	≤100 µg/mL	≤100 µg/mL	≤1000 µg/mL	≤500 µg/mL
Mutagenicity	All compounds found to be non mutagenic							
Molecular modeling	Molecular docking studies resulted in docking scores parallel to biological results. The similarity between experimental and computational results was very high.							

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APPENDIX

1. 0.5mM histidine/0.5mM biotin solution for the top agar in mutagenicity test

Ingredients to make 250 mL

- 30.5 mg D-Biotin
- 26.2 mg L-Histidine
- Double distilled water (ddH_2O)

The D-Biotin was dissolved in warm double distilled water, then the L-Histidine was added and dissolved. This solution was filter sterilized and stored at 4°C.

2. Vogel – Bonner medium E [50x strength stock solution]

Ingredients to make 1000 mL

- 670 mL Warm ddH_2O (45 °C)
- g $MgSO_4$. 7 H_2O
- 100 g Citric acid monohydrate
- 500 g K_2HPO_4
- 175 g $NaHNH_4PO_4$. 4 H_2O

Warm water and add the salts to the water in a 2 L flask. Place on a magnetic stirrer. Each salt must be dissolved completely before adding the next salt. Once dissolved adjust the volume to 1 L. Autoclave the solution at 121 ° C for 20 min.

3. 40 % Glucose Solution

Ingredients to make up 1000 mL

- 400 g Glucose
- 1000 mL double distilled water

Dissolve glucose in the double distilled water and autoclave at 121°C for 20 min.

4. **Top Agar**

Ingredients to make up 1000 mL

- 6g Bacteriological Agar
- 5g NaCl
- 1000 mL double distilled water (ddH_2O)

Dissolve agar and NaCl in 1000 mL of ddH_2O. Distribute 100 mL aliquots into Schott bottles and autoclave at 121°C for 20 minutes. Once the agar is warm add 10 mL of 0.5 mM histidine/ 0.5 mM biotin solution and mix gently.

5. **Minimal Glucose Agar**

Ingredients to make up 1000 mL

- 15 g Bacteriological Agar
- 20 mL Vogel-Bonner medium E
- 50 mL 40% glucose solution
- 930 mL ddH_2O

Add agar to the Schott bottle with the double distilled H_2O and a magnetic stirrer bar. Autoclave at 121°C for 20 minutes. After the agar has cooled add the Vogel-Bonner medium and glucose solution. Mix thoroughly and pour into petri dishes (30 mL).

6. **Molecular modeling software**

The Molecular Operating Environment (MOE) 2013.08 package license was purchased from Chemical Computing Group Inc, Canada. The Leadit 2.1.2 was obtained from BiosolveIT GmbH, Germany.

7. Dose Responsive Curves of MCF-7 and UACC-62.

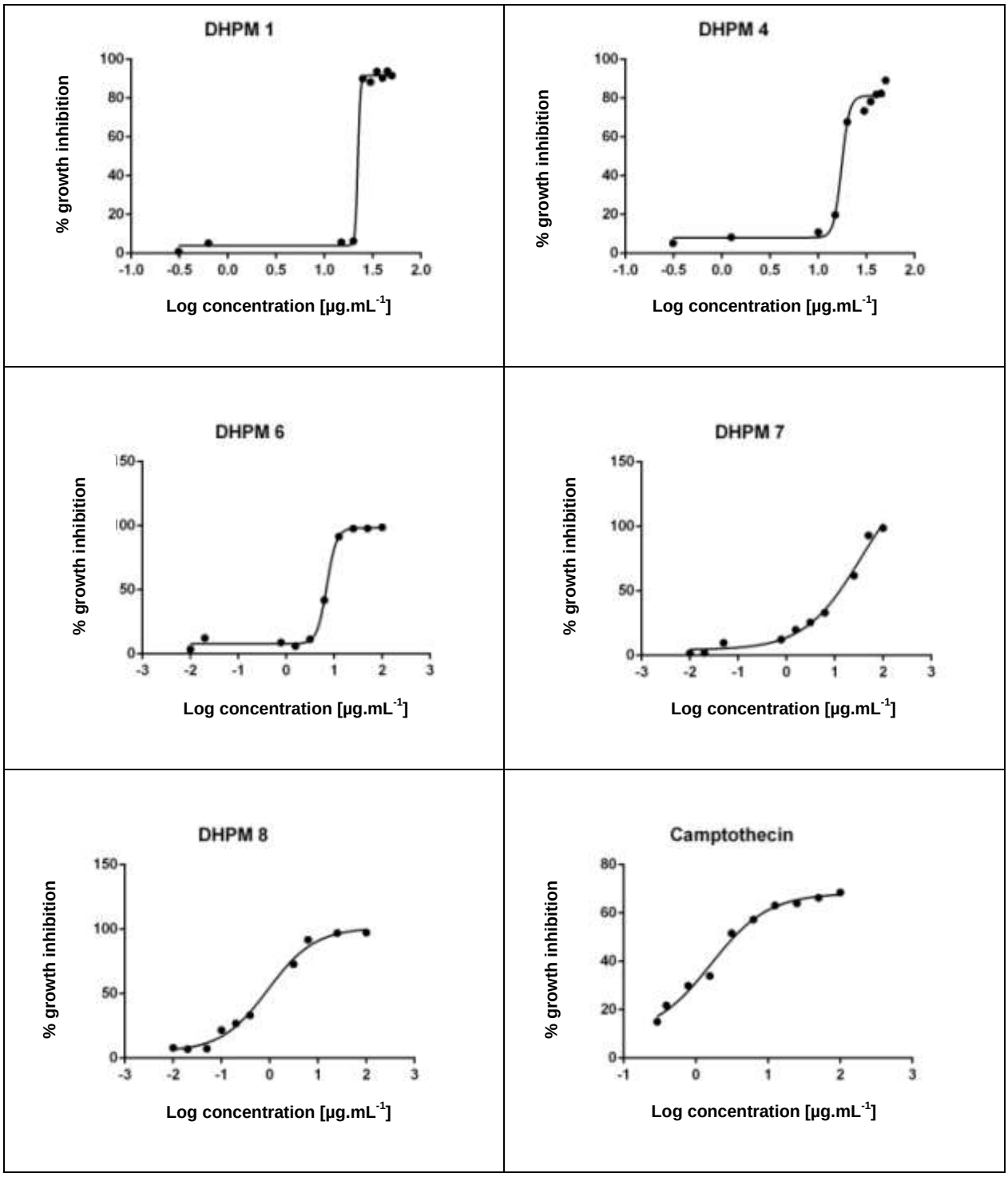

Figure 35. Dose responsive curves of the MCF - 7 cells treated with DHPM 1, 4, 6, 7, 8 and Camptothecin.

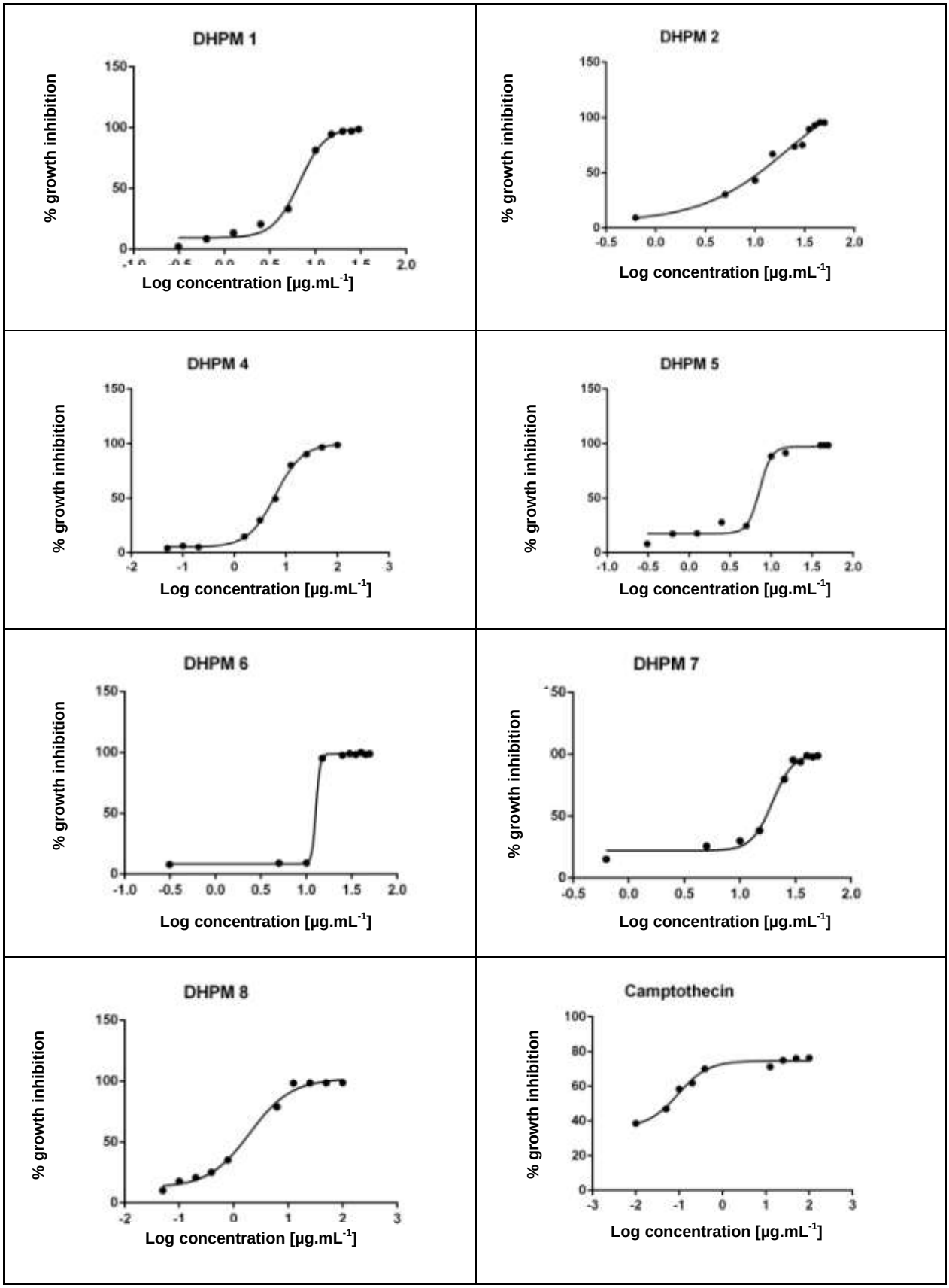

Figure 36. Dose responsive curves of the UACC - 62 cell line treated with DHPM 1, 2, 4-8 and Camptothecin.

8. Characterisation of Intermediate 1 and 2

8.1. Methyl 4-(4-chlorophenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate **(Intermediate 1)**

Appearance: yellow solid; yield 66%. mp 205–206 °C. IR (KBr) ν/cm 3240, 3110, 1724, 1704, 1649, 1490, 1461, 781; ^{1}H-NMR (400 MHz, DMSO-*d*6) δ 2.30 (s, 3H), 3.92 (s, 3H), 5.43 (s, 1H), 7.13 (d, 2H, *J* = 9.05 Hz), 7.51 (s, 1H), 7.86 (d, 2H, *J* = 9.05 Hz), 9.01 (s, 1H). ^{13}C-NMR (400 MHz, DMSO-*d*6) δ 18.66, 52.05, 54.35, 109.58, 113.20, 128.23, 136.26, 148.25, 153.40, 159.18, 167.76. MS: m/z 281 $[M + H]^{+}$.

8.2. Methyl 2-chloro-4-(4-chlorophenyl)-6-methyl-1,4-dihydropyrimidine-5-carboxylate **(Intermediate 2)**

Appearance: brown solid; yield 72%. mp 218–219 °C. IR (KBr) ν/cm 3244, 3108, 1708, 1641, 1491, 1466, 786. ^{1}H-NMR (400 MHz, DMSO-*d*6) δ 2.32 (s, 3H), 3.91 (s, 3H), 5.44 (s, 1H), 7.13–7.86 (m, 4H), 9.02 (s, 1H). ^{13}C-NMR (400 MHz, DMSO-*d*6) δ 18.72, 53.67, 54.35, 105.41, 113.20, 128.54, 135.29, 141.20, 145,67, 158.39, 167.76. MS m/z 299 $[M + H]^{+}$.

9. Characterisation of DHPM 1-8

Methyl-2-(3-bromophenylamino)-4-(4-chlorophenyl)-6-methyl-1,4-dihydropyrimidine-5-carboxylate (DHPM 1)

Appearance: white solid. ^{1}H-NMR (DMSO-d_6) δ 2.08 (s, 3H, -CH_3), 3.63 (s, 3H, -OCH_3), 5.43 (s, 1H), 7.21-7.53 (m, 8H), 9.99 (s, 1H), 11.1 (s, 1H). ^{13}C-NMR (DMSO-d_6) δ 18.13, 52.20, 103.82, 122.75, 123.66, 127.35, 129.05, 129.48, 130.27, 132.23, 133.57, 136.70, 140.57, 145.43, 149.33, 165.18. HRMS calculated for $C_{19}H_{17}BrClN_3O_2$ 433.0193, found 434.0302 $(M+H)^+$.

Methyl-4-(4-chlorophenyl)-2-(2-hydroxy-4-nitrophenylamino)-6-methyl-1,4-dihydropyrimidine-5-carboxylate **(DHPM 2**)

Appearance: yellow solid. ^{1}H-NMR (DMSO-d_6) δ 2.41 (s, 3H, -CH_3), 3.58 (s, 3H, -OCH_3), 5.40 (s, 1H), 6.82-7.53 (m, 4H), 8.1-8.16 (m, 2H), 9.80 (s, 1H), 10.28 (s, 1H), 11.08 (s, 1H), 11.99 (s, 1H). ^{13}C-NMR (DMSO-d_6). δ 18.00, 52.06, 52.26, 103.62, 114.54, 117.31, 121.62, 124.46, 125.64, 129.08, 133.36, 139.75, 140.52, 145.12, 149.96, 159.59, 165.14. HRMS calculated for $C_{19}H_{17}ClN_4O_5$ 416.0887, found 417.0990 $(M+H)^+$.

Methyl-2-(3-bromo-4-fluorophenylamino)-4-(4-chlorophenyl)-6-methyl-1,4-dihydropyrimidine-5-carboxylate **(DHPM 3)**

Appearance: white solid. ^{1}H-NMR (DMSO-d_6) δ 2.42 (s, 3H, -CH_3), 3.60 (s, 3H, -OCH_3), 5.35 (s, 1H), 7.32-7.52 (m, 6H), 7.77-7.80 (d, 1H), 9.55 (s, 1H), 10.52 (s, 1H). ^{13}C-NMR (DMSO-d_6) δ 18.07, 52.04, 52.41, 103.30, 116.71, 116.94, 120.99, 121.25, 123.06, 123.16, 129.18, 129.30, 129.75, 131.84, 131.93, 133.37, 140.63, 145.05, 150.13, 160.60, 163.09, 165.10. HRMS calculated for $C_{19}H_{16}BrClFN_3O_2$ 451.0098, found 452.0144 $(M+H)^+$.

Methyl-4-(4-chlorophenyl)-2-(4-cyanophenylamino)-6-methyl-1,4-dihydropyrimidine-5-carboxylate **(DHPM 4)**

Appearance: yellow solid. ^{1}H-NMR (DMSO-*d*$_6$) δ 2.38 (s, 3H, -CH_3), 3.60 (s, 3H, -OCH_3), 5.39 (s, 1H), 7.32 (d, 4H), 7.45 (d, 2H), 7.8 (d, 2H), 10.77 (s, 2H). ^{13}C-NMR (DMSO-*d*$_6$) δ 18.76, 51.84, 52.65, 102.77, 106.84, 119.29, 122.87, 128.59, 129.27, 133.08, 134.17, 141.74, 143.04, 147.56, 148.26, 165.55. HRMS calculated for $C_{20}H_{17}ClN_4O_2$ 380.1040, found 381.1126 $(M+H)^+$.

Methyl 4-(4-chlorophenyl)-6-methyl-2-(3-(trifluoromethylthio)phenylamino) -1,4-dihydropyrimidine-5-carboxylate **(DHPM 5)**

Appearance: pale yellow solid. ^{1}H-NMR (DMSO-*d*$_6$) δ 2.42 (s, 3H, -CH_3), 3.63 (s, 3H, -OCH_3), 5.43 (s, 1H), 7.34-7.62 (m, 8H), 9.99 (s, 1H), 10.98 (s, 1H). ^{13}C-NMR (DMSO-*d*$_6$) δ 18.01, 51.82, 52.62, 119.25, 123.07, 128.82, 129.26, 133.07, 134.16, 148.18, 165.50. HRMS calculated for $C_{20}H_{17}ClF_3N_3O_2S$ 455.0682, found 456.0792 $(M+H)^+$.

Methyl 2-(4-bromophenylamino)-4-(4-chlorophenyl)-6-methyl-1,4-dihydropyrimidine-5-carboxylate **(DHPM 6)**

Appearance: pale yellow solid. ^{1}H-NMR (DMSO-*d*$_6$) δ 2.08 (s, 3H, -CH_3), 3.63 (s, 3H, -OCH_3), 5.41 (s, 1H), 7.17 (d, 2H), 7.18 (d, 2H), 7.47 (d, 2H), 7.6 (d, 2H), 9.93 (s, 1H), 10.83 (s, 1H). ^{13}C-NMR (DMSO-*d*$_6$) δ 18.02, 52.11, 52.14, 103.72, 120.29, 127.07, 128.07, 128.98, 129.40, 132.13, 133.24, 133.44, 134.09, 140.58, 145.24, 149.29, 165.09. HRMS calculated for $C_{19}H_{17}BrClN_3O_2$ 433.0193, found 434.0294 $(M+H)^+$.

Methyl2-(3-chloro-5-hydroxyphenylamino)-4-(4-chlorophenyl)-6-methyl-1,4-dihydropyrimidine-5-carboxylate (**DHPM 7**)

Appearance: white solid. ^{1}H-NMR (DMSO-*d*$_6$) δ 2.40 (s, 3H, -CH_3), 3.59 (s, 3H, -OCH_3), 5.33 (s, 1H), 6.77 (s, 2H), 7.29-7.45 (m, 5H), 10.18 (s, 2H). ^{13}C-NMR (DMSO-*d*$_6$) δ 18.17, 51.93, 52.53, 102.85, 115.62, 116.80, 119.39, 129.13, 129.16, 131.14, 133.21, 141.07, 145.75, 149.26, 157.86, 165.24. HRMS calculated for $C_{19}H_{17}Cl_2N_3O_3$ 405.0647, found 406.0726 $(M+H)^+$.

Methyl4-(4-chlorophenyl)-2-(2,4-dimethoxybenzylamino)-6-methyl-1,4-dihydropyrimidine-5-carboxylate **(DHPM 8)**

Appearance: pale yellow solid. ^{1}H-NMR (DMSO-d_6) δ 2.37 (s, 3H, -CH_3), 3.60 (s, 3H, -OCH_3), 3.75 (s, 6H), 4.27 (m, 2H), 5.43 (s, 1H), 6.42 (d, 1H), 6.56 (s, 1H), 7.11 (d, 1H), 7.28 (d, 2H), 7.42 (d, 2H), 9.95 (s, 1H), 10.77 (s, 1H). ^{13}C-NMR (DMSO-d_6) δ 18.14, 51.72, 51.93, 55.72, 55.92, 98.97, 102.01, 104.77, 116.39, 128.92, 129.17, 130.16, 133.20, 141.01, 145.78, 150.20, 158.54, 161.16, 165.27. HRMS calculated for $C_{22}H_{24}ClN_3O_4$ 429.1455, found 430.1552 $(M+H)^+$.

Drug Design, Development and Therapy

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ORIGINAL RESEARCH

Design, synthesis, and computational studies on dihydropyrimidine scaffolds as potential lipoxygenase inhibitors and cancer chemopreventive agents

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Abstract: Dihydropyrimidine scaffold has a wide range of potential pharmacological activities such as antiviral, antitubercular, antimalarial, anti-inflammatory, and anticancer properties. 5-Lipoxygenase enzyme is an enzyme responsible for the metabolism of arachidonic acid to leukotrienes. The elevated levels of this enzyme and its metabolites in cancer cells have a direct relation on the development of cancer when compared to normal cells. The development of novel lipoxygenase inhibitors can have a major role in cancer therapy. A series of substituted 1,4-dihydropyrimidine analogues were synthesized and characterized by ^{1}H-NMR, ^{13}C-NMR, and HRMS. Molecular docking against lipoxygenase enzyme (protein data bank code =3V99) was done using Molecular Operating Environment 2013.08 and Leadit 2.1.2 softwares and showed high affinities. The synthesized compounds were tested for their lipoxygenase inhibitory activity and showed inhibition ranging from 59.37%±0.66% to 81.19%±0.94%. The activity was explained by a molecular docking study. The title compounds were also tested for cytotoxic activity against two human cancer cell lines Michigan Cancer Foundation-7 and human melanoma cells and a normal peripheral blood mononuclear cell line.

Keywords: 1,4-dihydropyrimidines, synthesis and characterization, molecular docking study

Introduction

Dihydropyrimidines (DHPMs) are well-known scaffolds that are easily prepared through condensation reaction of urea/thiourea, β-ketoester, and aryl aldehyde.[1] DHPM derivatives have a significant role in medicinal chemistry for various pharmacological activities,[2,3] such as anticancer,[4] antibacterial,[5–7] antifungal,[8] antihypertensive, antitubercular,[9,10] antimalarial,[11] antiviral,[12,13] and anti-inflammatory[14] activities. 5-Lipoxygenase is an enzyme responsible for the metabolism of arachidonic acid. It is found in plants and animals with high percentage of sequence identity especially in the catalytic site.[15] 5-Lipoxygenase catalyzes the metabolism of arachidonic acid into hydroperoxyeicosatetraenoic acid and leukotrienes. The elevated levels of this enzyme have a direct relation to the development of cancer.[16] The presence of high levels of lipoxygenase metabolites in many cancers, such as lung, prostate, breast, colon, skin, and brain cancers, has been reported.[17] Inhibition of lipoxygenase may be an important target for the prevention of cancer and can occur through interfering with signaling cascade needed for cancer cell growth. Molecular modeling approach is widely used for the discovery, design, and prediction of the activity and mechanisms by which the active compounds act. Molecular docking is the

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process that predicts the orientation of all possible conformations of the tested compounds within the active site of the enzyme. It allows deriving all the features that are required for best interactions with the active enzyme site and for the optimization of any discovered lead compound. In addition, it can assist in studying the structure–activity relationship of the tested compounds. In continuation of our research on the development of novel heterocyclic compounds for various pharmacological activities[18–20] and screening of pharmacologically active heterocyclic compounds for polymorphism property,[21,22] the main aim of this work was to design, synthesize, and characterize some of the 1,4-dihydropyrimidine derivatives and to screen these compounds for their lipoxygenase inhibitory activity and cytotoxic activity against two cancer cell lines, such as Michigan Cancer Foundation-7 (MCF-7) and human melanoma cells (UACC-62), and a normal cell line, peripheral blood mononuclear cells (PBMCs), and to look for any promising activity. Molecular modeling study was performed to predict the mode of binding by which these compounds may inhibit lipoxygenase enzymes.

Materials and methods

Chemicals were procured from Merck & Co., Inc. (Whitehouse Station, NJ, USA) and Sigma-Aldrich Co. (St Louis, MO, USA). Monitoring of chemical reactions was done on analytical thin-layer chromatography with Merck 60 F-254 silica-gel plates using solvent system (*n*-hexane and ethylacetate [4:6]), and visualization was done with ultraviolet light. Büchi Melting Point B-545 apparatus was used to check melting points. NMR spectra (^{1}H and ^{13}C) were recorded in dimethyl sulfoxide (DMSO)-d_6 solvent using tetramethylsilane as an internal standard with a 400-MHz Bruker spectrometer. Chemical shifts were showed in parts per million downfield from tetramethylsilane, and the splitting pattern is abbreviated as s, singlet; m, multiplet; and d, doublet. High-resolution mass spectrometry (HRMS) analysis was performed on a BrukerMicroTOF QII mass spectrometer in positive mode with internal calibration.

Molecular Operating Environment (MOE) 2013.08 package license was purchased from Chemical Computing Group Inc (Montreal, QC, Canada),[23] and Leadit 2.1.2 software license was purchased from BioSolveIT GmbH (Sankt Augustin, Germany).[24,25]

The synthetic strategy employed to produce DHPM1–DHPM8 is illustrated in Figure 1. Synthesis of 1 and 2 was performed as per the reported procedures.[9,26]

General procedure employed in the preparation of DHPM1–DHPM8

A mixture of compound 2 (1 mmol), substituted phenyl/benzyl amines (1 mmol), and potassium carbonate (1 mmol) in isopropanol (10 mL) was refluxed for 15 hours as depicted in Figure 1. Thin-layer chromatography was used to monitor

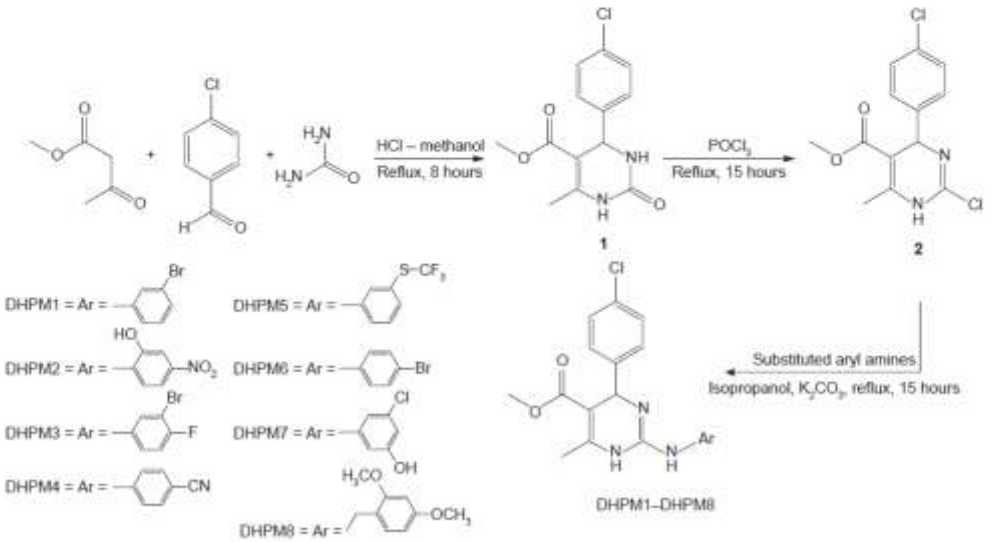

Figure 1 General route for the synthesis of 1,4-dihydropyrimidine analogues DHPM1–DHPM8.
Abbreviation: DHPM, dihydropyrimidine.

reaction completion. The product obtained was filtered, washed with isopropanol, and dried. Column chromatography was used to purify the crude product using *n*-hexane and ethylacetate (4:6) as eluent to obtain title compounds DHPM1–DHPM8 at 62%–74% yield. The physicochemical constants of the title compounds are tabulated in Table 1.

Methyl-2-(3-bromophenylamino)-4-(4-chlorophenyl)-6-methyl-1,4-dihydropyrimidine-5-carboxylate (compound DHPM1)

Appearance: white solid. ^{1}H-NMR (DMSO-d_6) δ 2.08 (s, 3H, –CH_3), 3.63 (s, 3H, –OCH_3), 5.43 (s, 1H), 7.21–7.53 (m, 8H), 9.99 (s, 1H), 11.1 (s, 1H). ^{13}C-NMR (DMSO-d_6) δ 18.13, 52.20, 103.82, 122.75, 123.66, 127.35, 129.05, 129.48, 130.27, 132.23, 133.57, 136.70, 140.57, 145.43, 149.33, 165.18. HRMS calculated for $C_{19}H_{17}BrClN_3O_2$ 433.0193, found 434.0302 (M+H)$^+$.

Methyl-4-(4-chlorophenyl)-2-(2-hydroxy-4-nitrophenylamino)-6-methyl-1,4-dihydropyrimidine-5-carboxylate (compound DHPM2)

Appearance: yellow solid. ^{1}H-NMR (DMSO-d_6) δ 2.41 (s, 3H, –CH_3), 3.58 (s, 3H, –OCH_3), 5.40 (s, 1H), 6.82–7.53 (m, 4H), 8.1–8.16 (m, 2H), 9.80 (s, 1H), 10.28 (s, 1H), 11.08 (s, 1H), 11.99 (s, 1H). ^{13}C-NMR (DMSO-d_6). δ 18.00, 52.06, 52.26, 103.62, 114.54, 117.31, 121.62, 124.46, 125.64, 129.08, 133.36, 139.75, 140.52, 145.12, 149.96, 159.59, 165.14. HRMS calculated for $C_{19}H_{17}ClN_4O_5$ 416.0887, found 417.0990 (M+H)$^+$.

Methyl-2-(3-bromo-4-fluorophenylamino)-4-(4-chlorophenyl)-6-methyl-1,4-dihydropyrimidine-5-carboxylate (compound DHPM3)

Appearance: white solid. ^{1}H-NMR (DMSO-d_6) δ2.42 (s, 3H, –CH_3), 3.60 (s, 3H, –OCH_3), 5.35 (s, 1H), 7.32–7.52 (m, 6H), 7.77–7.80 (d, 1H), 9.55 (s, 1H), 10.52 (s, 1H). ^{13}C-NMR (DMSO-d_6) δ 18.07, 52.04, 52.41, 103.30, 116.71, 116.94, 120.99, 121.25, 123.06, 123.16, 129.18, 129.30, 129.75, 131.84, 131.93, 133.37, 140.63, 145.05, 150.13, 160.60, 163.09, 165.10. HRMS calculated for $C_{19}H_{16}BrClFN_3O_2$ 451.0098, found 452.0144 (M+H)$^+$.

Methyl-4-(4-chlorophenyl)-2-(4-cyanophenylamino)-6-methyl-1,4-dihydropyrimidine-5-carboxylate (compound DHPM4)

Appearance: yellow solid. ^{1}H-NMR (DMSO-d_6) δ 2.38 (s, 3H, –CH_3), 3.60 (s, 3H, –OCH_3), 5.39 (s, 1H), 7.32 (d, 4H), 7.45 (d, 2H), 7.8 (d, 2H), 10.77 (s, 2H). ^{13}C-NMR (DMSO-d_6) δ 18.76, 51.84, 52.65, 102.77, 106.84, 119.29, 122.87, 128.59, 129.27, 133.08, 134.17, 141.74, 143.04, 147.56, 148.26, 165.55. HRMS calculated for $C_{20}H_{17}ClN_4O_2$ 380.1040, found 381.1126 (M+H)$^+$.

Methyl 4-(4-chlorophenyl)-6-methyl-2-(3-(trifluoromethylthio)phenylamino)-1,4-dihydropyrimidine-5-carboxylate (compound DHPM5)

Appearance: pale yellow solid. ^{1}H-NMR (DMSO-d_6) δ 2.42 (s, 3H, –CH_3), 3.63 (s, 3H, –OCH_3), 5.43 (s, 1H), 7.34–7.62 (m, 8H), 9.99 (s, 1H), 10.98 (s, 1H). ^{13}C-NMR (DMSO-d_6) δ 18.01, 51.82, 52.62, 119.25, 123.07, 128.82, 129.26, 133.07, 134.16, 148.18, 165.50. HRMS calculated for $C_{20}H_{17}ClF_3N_3O_2S$ 455.0682, found 456.0792 (M+H)$^+$.

Methyl 2-(4-bromophenylamino)-4-(4-chlorophenyl)-6-methyl-1,4-dihydropyrimidine-5-carboxylate (compound DHPM6)

Appearance: pale yellow solid. ^{1}H-NMR (DMSO-d_6) δ2.08 (s, 3H, –CH_3), 3.63 (s, 3H, –OCH_3), 5.41 (s, 1H), 7.17 (d, 2H), 7.18 (d, 2H), 7.47 (d, 2H), 7.6 (d, 2H), 9.93 (s, 1H),

Table 1 Physicochemical characteristics of the compounds DHPM1–DHPM8

Compound	Ar	Molecular formula (molecular weight)	Yield (%)[a,b]	Melting point (°C)	cLogP[c]
DHPM1	3-Br–C_6H_4	$C_{19}H_{17}BrClN_3O_2$ (433)	66	218–220	6.0560
DHPM2	2-OH, 4-NO_2–C_6H_3	$C_{19}H_{17}ClN_4O_5$ (416)	69	174–176	4.8054
DHPM3	3-Br, 4-F–C_6H_3	$C_{19}H_{16}BrClFN_3O_2$ (451)	73	238–240	6.3049
DHPM4	4-CN–C_6H_4	$C_{20}H_{17}ClN_4O_2$ (380)	62	116–118	5.0256
DHPM5	3-SCF_3–C_6H_4	$C_{20}H_{17}ClF_3N_3O_2S$ (455)	68	222–224	6.5586
DHPM6	4-Br–C_6H_4	$C_{19}H_{17}BrClN_3O_2$ (433)	68	228–230	6.0560
DHPM7	3-Cl, 5-OH–C_6H_3	$C_{19}H_{17}Cl_2N_3O_2$ (405)	66	200–202	4.3300
DHPM8	2,4-OCH_3-benzyl	$C_{22}H_{24}ClN_3O_4$ (429)	74	222–224	5.3218

Notes: [a]All the products were characterized by spectral and physical data. [b]Yields after purification by column chromatography. [c]cLogP was calculated using ChemBioDraw Ultra 13.0v.

Abbreviation: DHPM, dihydropyrimidine.

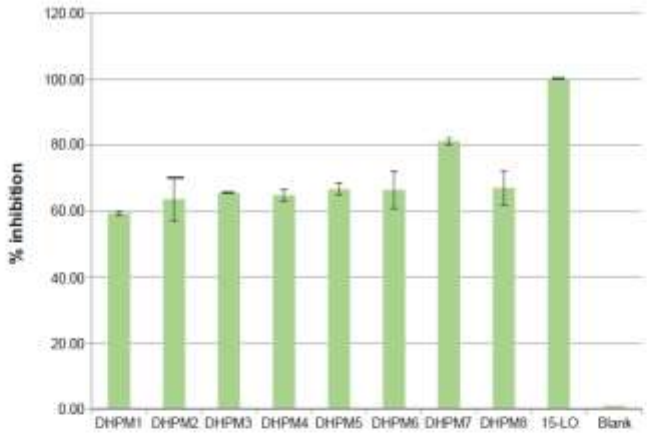

Figure 2 15-Lipoxygenase inhibitory rate of DHPM1–DHPM8.
Notes: Data are presented as mean ± standard deviation of three replicates from three independent experiments. Blank is assay with buffer alone without test compounds.
Abbreviations: 15-LO, 15-lipoxygenase inhibitor; DHPM, dihydropyrimidine.

10.83 (s, 1H). ^{13}C-NMR (DMSO-d_6) δ 18.02, 52.11, 52.14, 103.72, 120.29, 127.07, 128.07, 128.98, 129.40, 132.13, 133.24, 133.44, 134.09, 140.58, 145.24, 149.29, 165.09. HRMS calculated for $C_{19}H_{17}BrClN_3O_2$ 433.0193, found 434.0294 $(M+H)^+$.

Methyl 2-(3-chloro-5-hydroxyphenylamino)-4-(4-chlorophenyl)-6-methyl-1,4-dihydropyrimidine-5-carboxylate (compound DHPM7)

Appearance: white solid. ^{1}H-NMR (DMSO-d_6) δ 2.40 (s, 3H, $-CH_3$), 3.59 (s, 3H, $-OCH_3$), 5.33 (s, 1H), 6.77 (s, 2H), 7.29–7.45 (m, 5H), 10.18 (s, 2H). ^{13}C-NMR (DMSO-d_6) δ 18.17, 51.93, 52.53, 102.85, 115.62, 116.80, 119.39, 129.13, 129.16, 131.14, 133.21, 141.07, 145.75, 149.26, 157.86, 165.24. HRMS calculated for $C_{19}H_{17}Cl_2N_3O_3$ 405.0647, found 406.0726 $(M+H)^+$.

Methyl 4-(4-chlorophenyl)-2-(2,4-dimethoxybenzylamino)-6-methyl-1,4-dihydropyrimidine-5-carboxylate (compound DHPM8)

Appearance: pale yellow solid. ^{1}H-NMR (DMSO-d_6) δ 2.37 (s, 3H, $-CH_3$), 3.60 (s, 3H, $-OCH_3$), 3.75 (s, 6H), 4.27 (m, 2H), 5.43 (s, 1H), 6.42 (d, 1H), 6.56 (s, 1H), 7.11 (d, 1H), 7.28 (d, 2H), 7.42 (d, 2H), 9.95 (s, 1H), 10.77 (s, 1H). ^{13}C-NMR (DMSO-d_6) δ 18.14, 51.72, 51.93, 55.72, 55.92, 98.97, 102.01, 104.77, 116.39, 128.92, 129.17, 130.16, 133.20, 141.01, 145.78, 150.20, 158.54, 161.16, 165.27. HRMS calculated for $C_{22}H_{24}ClN_3O_4$ 429.1455, found 430.1552 $(M+H)^+$.

Molecular modeling

Molecular alignment

The molecular alignment was done using MOE 2013.08 for the alignment of both 5-lipoxygenase and 15-lipoxygenase with protein data bank (PDB) codes 3V99 and 4NRE, respectively.

Molecular docking studies with MOE 2013.08

All the compounds were built and saved as MOE files. (4*S*)-2-(Substituted-phenyl/or benzyl)-4-(4-chlorophenyl)-6-methyl-5-methyl carboxylate-1,4-dihydropyrimidine enantiomer was used for docking process. Rigid receptor was used as a docking protocol. Both the receptor and the solvent were kept as a "receptor". Triangle matcher was used as a placement method. Two rescoring were computed, rescoring 1 was selected as London dG, rescoring 2 was selected as affinity. Force field was used as a refinement.

Molecular docking studies with Leadit 2.1.2

All compounds were built and saved as mol2 files. (4*S*)-2-(substituted-phenyl/or benzyl)-4-(4-chlorophenyl)-6-methyl-5-methyl carboxylate-1,4-dihydropyrimidine enantiomer was used for docking process. The crystal structure of lipoxygenase enzyme complexed with arachidonic acid was downloaded from PDB (code =3V99). The protein was loaded into Leadit 2.1.2, and the receptor components were chosen by selection of chain A as a main chain, which is complexed with arachidonic acid. Binding site was defined by choosing arachidonic acid as a reference ligand,

to which all coordinates were computed. Amino acids within radius 6.5 Å were selected in the binding site. All chemical ambiguities of residues were left as default. Ligand binding was driven by enthalpy (classic Triangle matching). For scoring, all default settings were restored. Intraligand clashes were computed by using clash factor =0.6. Maximum number of solutions per iteration =200. Maximum solution per fragmentation =200. The base placement method was used as a docking strategy. Docking results are tabulated in Table 2.

Pharmacology

Lipoxygenase inhibitory assay

The assay was done using the lipoxygenase Inhibitor Screening Assay Kit (Cayman Chemicals, Ann Arbor, MI, USA). The assay was performed following the manufacturer's protocol. Briefly, lipoxygenases are dioxygenases that catalyze the addition of oxygen to unsaturated fatty acids, which contain a *cis*,*cis*-1,4-pentadiene system.[27] The activity of DHPM1–DHPM8 was determined at 1 mg/mL using 15-lipoxygenase as standard positive control. The 100% initial inhibitor activity was determined using the lipoxygenase enzyme and solvent DMSO, which was used to dissolve the inhibitor. The inhibitor (DHPM1–DHPM8) activity was determined by adding lipoxygenase enzyme to the inhibitor. The reaction was initiated by the addition of the substrate, arachidonic acid, to all wells. Chromogen was added to all wells to stop enzyme catalysis and to develop the reaction. The plate was covered with a plate cover and the product of the enzymatic reaction was determined spectrophotometrically at 490–500 nm. Lipoxygenase results are presented in Table 3, the percentage inhibition is depicted in Figure 2 and it was determined using the following equation:

$$\%\ \text{inhibition} = \frac{\text{Initial activity} - \text{Inhibitor}}{\text{Initial activity}} \times 100$$

Cell culture

Two human cancer cell lines such as MCF-7 and UACC-62 (human melanoma cell) were provided by The Council for Scientific and Industrial Research Biosciences, Pretoria, South Africa. The cells were cultured in Dulbecco's Modified Eagle's Medium, supplemented with 10% (v/v) fetal calf serum and 100 µg/mL streptomycin and 100 units/mL penicillin in humidified 5% CO_2 at 37°C. The PBMCs were separated, stored according to reported protocol,[28] and grown aseptically in tissue culture flasks (T 75 cm^2) using medium that comprised RPMI-1640, heat-inactivated filtered fetal calf serum (50 mL), 100 µg/mL streptomycin, and 100 units/mL penicillin. The cultures were incubated in humidified 5% CO_2 incubator at 37°C.

Cytotoxic activity

The in vitro cytotoxicity of the compounds DHPM1–DHPM8 was screened against UACC-62 and MCF-7 tumor cell lines and peripheral blood mononuclear cell line using the MTT assay.[29] The assay was carried out in 96-well, flat-bottomed microtiter plates. A volume of 90 µL of $\pm 1\times10^4$ cells was added to each well, and 10 µL of the test compounds (50 µg/mL) were added to the respective wells. In the control

Table 2 Docking results of the synthesized compounds DHPM1–DHPM8 against human lipoxygenase in complex with arachidonic acid

Compound	Docking score using MOE 2013.08	Main residue that interacts	Main pharmacophore	Distance (Å)	Binding free energy (kcal/mol)
DHPM1	−5.29	Phe 177	–C=O of ester	3.09	−11.5
		HOH 922	N=C of pyrimidine ring	3.20	−2.5
DHPM2	−5.40	Gln 363	–NH of pyrimidine	3.5	−1
		HOH 922	–C=O of ester	2.83	−1.3
DHPM3	−5.8	Phe 177	N=C of pyrimidine ring	3.02	−14.04
		HOH 931	–C=O of ester	3.17	−1.1
DHPM4	−5.7	Leu 368	N atom of cyano group	3.71	−0.8
		Phe 177	HN-Phenyl	3.15	−13.6
DHPM5	−5.9	Phe 177	–C=O of ester	3.34	−7.3
DHPM6	−6.12	Ile 406	HN-Phenyl	3.58	−0.7
		Phe 177	N=C	3.02	−14.9
DHPM7	−6.42	Gln 363	NH of pyrimidine ring	3.37	−11.8
		HOH 955	HN-Phenyl	3.42	−2.0
DHPM8	−6.32	Phe 177	C=O of ester	2.89	−10.55

Abbreviations: MOE, Molecular Operating Environment; DHPM, dihydropyrimidine.

Table 3 15-Lipoxygenase inhibitor assay of compounds DHPM1–DHPM8

Compound	Concentration of test compounds (μM)	Lipoxygenase inhibition (%)
DHPM1	2.30	59.37±0.66
DHPM2	2.40	63.60±6.50
DHPM3	2.21	65.44±0.00
DHPM4	2.63	64.83±1.77
DHPM5	2.19	66.66±1.73
DHPM6	2.30	66.46±5.67
DHPM7	2.46	81.19±0.94
DHPM8	2.33	67.08±5.22
15-LO	3.19	100.00±0.0

Abbreviations: DHPM, dihydropyrimidine; 15-LO, 15-lipoxygenase inhibitor.

wells, DMSO and media were added. The positive controls used were doxorubicin and camptothecin at 50 μg/mL. The plates were incubated at 37°C with 5% CO_2 for 48 hours. After incubation, 20 μL of MTT reagent was added and the plates were then incubated for a further 4 hours at 37°C in a humidified incubator with 5% CO_2. After incubation, 100 μL of DMSO was added to each well and the plate was incubated for 1 hour at 37°C in a humidified incubator with 5% CO_2. The absorbance was read at 590 nm on an enzyme-linked immunosorbent assay plate reader (Digital Analogue Systems, Italy), and the results are tabulated in Table 4. The percentage of cell viability and percentage of growth inhibition were determined using the following formulas:

$$\%\ \text{cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100$$

$$\%\ \text{growth inhibition} = 100 - \%\ \text{cell viability}$$

Further test compounds that exhibited over 80% cell growth inhibition on UACC-62 and MCF-7 tumor cell lines were used to determine half-maximal inhibitory concentration, which was determined from the chart of cell viability percentage against test compounds concentration (μM) (Table 5).

Statistical analysis

Each experiment was performed in triplicate, and the data are presented as mean ± standard deviation. Using analysis of variance, statistical significance was determined and mean values with probability values of $P<0.05$ were taken as statistically significant.

Results and discussion

Chemistry

Figure 1 depicts the synthesis of 2,4,5-trisubstituted pyrimidine analogues DHPM1–DHPM8. Synthesis of intermediate 1 has been achieved by Biginelli reaction by retaining Lewis acid as a catalyst as described in the literature.[26] Purification of the intermediate was achieved using methanol as a solvent for recrystallization method and the yield obtained was found to be 66%. Intermediate 2 was synthesized by reacting the intermediate 1 with phosphorous oxychloride for 15 hours. Compound 2 was obtained at 75% yield after recrystallization using methanol. Compounds DHPM1–DHPM8 were obtained by refluxing mono/disubstituted aromatic amines with intermediate 2 at equimolar proportion with potassium carbonate in isopropanol medium. The crude compounds were purified by column chromatography. The yield of the final compounds was in the range of 62%–74%. Finally, the chemical structure of 1,4-dihydropyrimidine analogues DHPM1–DHPM8 was confirmed by ^{1}H-NMR, ^{13}C-NMR, and HRMS. In ^{1}H-NMR of DHPM1–DHPM8, the ester methoxy and methyl group on heteroaryl ring exhibited chemical shift in the range of δ =3.59–3.63 and δ=2.08–2.42 ppm, respectively. Single heterocyclic proton

Table 4 In vitro cytotoxicity of DHPM1–DHPM7 against MCF-7, UACC-62, and PBMC cell lines

Compound	Cell growth inhibition (%) at 50 μg/mL		
	MCF-7	UACC-62	PBMC
DHPM1	89.79±2.13	97.57±0.14	18.92±1.54
DHPM2	15.82±2.84	82.62±0.86	0.00±0.00
DHPM3	20.02±1.78	39.62±0.21	16.60±0.77
DHPM4	89.51±5.44	97.47±0.14	0.00±0.00
DHPM5	60.00±15.85	97.22±0.07	14.29±1.10
DHPM6	96.09±1.35	97.04±1.28	16.34±0.89
DHPM7	94.56±1.54	96.72±0.50	8.62±1.61
DHPM8	96.82±0.47	98.89±0.43	18.92±1.34
Camptothecin	76.34±0.10	92.46±1.24	8.90±0.57

Note: Percentage of cell growth inhibition by the MTT assay after 48 hours of test compounds exposure at 50 μg/mL concentration.
Abbreviations: DHPM, dihydropyrimidine; MCF-7, Michigan Cancer Foundation-7; PBMC, peripheral blood mononuclear cell; UACC-62, human melanoma cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Table 5 In vitro cytotoxicity (IC_{50}) of the test compounds DHPM1–DHPM8 against MCF-7 and UACC-62

Compound	IC_{50} (μM)[a,b]	
	MCF-7	UACC-62
DHPM1	22.22±0.01	6.63±0.03
DHPM2	NT	21.4±0.31
DHPM3	NT	NT
DHPM4	17.45±0.01	6.21±0.02
DHPM5	NT	0.85±0.04
DHPM6	7.02±0.01	12.77±0.01
DHPM7	35.33±0.37	19.55±0.02
DHPM8	0.92±0.09	1.97±0.08

Note: [a]IC_{50} is the test samples (DHPM1–DHPM8) concentration effective in inhibiting 50% of the cell growth measured by the MTT assay after 48 hours of exposure to test compounds. [b]Only compounds that exhibited over 80% of growth inhibition were taken for IC_{50} determination.

Abbreviations: DHPM, dihydropyrimidine; IC_{50}, half-maximal inhibitory concentration; MCF-7, Michigan Cancer Foundation-7; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NT, not tested; UACC-62, human melanoma cells.

is noticed in the range of δ=5.34–5.43 ppm. In ^{13}C-NMR, carbonyl carbon of ester functional group is observed in the range of δ=165.10–165.5 ppm. In HRMS, molecular ion peaks were in good agreement with the proposed molecular weight. *c*Log*P* of the title compounds DHPM1–DHPM8 was calculated by ChemBioDraw Ultra 13.0v program, and the values were in the range of 4.3300–6.5586.

Molecular modeling study

In order to explain the lipoxygenase inhibitory activity computationally, the crystal structure of human lipoxygenase was studied. Two crystal structures of both 5-lipoxygenase and 15-lipoxygenase were reported in PDB.[30,31] The structure of human 5-lipoxygenase was complexed with arachidonic acid, which can help more in the identification of the main binding site of that enzyme. While, the human 15-lipoxygenase was found complexed with a substrate mimic, our compounds which were tested against 15-lipoxygenase were not complexed with arachidonic acid. A previous study confirmed that the two structures are similar,[31] and both of them have the same conserved cavity with the same conserved amino acids at the binding site. They also have similar volume of the cavity. A molecular alignment of the crystal structures of 5-lipoxygenase with PDB code =3V99 and 15-lipoxygenase with PDB code =4NRE was done. The aim of this alignment was to identify the main binding site of both and to prove that they are the same (Figure 3).

According to the alignment results, both the structures have a homologous sequence. The same binding cavity for both arachidonic acid and substrate mimic was identified. The amino acids that are found in the main site are His 373, Ile 676, Ala 416, Asp 602, Asp 602, Ala 672, Glu 369, Val 426, Val 671, Ile 406, Leu 415, Phe 177, Gln 557, and Gln 413 in addition to water molecules H_2O 995 and H_2O 922 (Figure 4).

Molecular docking was done using MOE 2013.08 in which the (4*S*)-2-(substituted-phenyl/or benzyl)-4-(4-chlorophenyl)-6-methyl-5-methyl carboxylate-1,4-dihydropyrimidine enantiomer was used for docking as it showed better results than the (4*R*)-2-(substituted-phenyl/or-benzyl)-4-(4-chlorophenyl)-6-methyl-5-methyl carboxylate-1,4-dihydropyrimidine enantiomer. Docking resulted in a number of docking scores (Table 2), which were parallel to the biological results (Figure 5). For example; the docking score

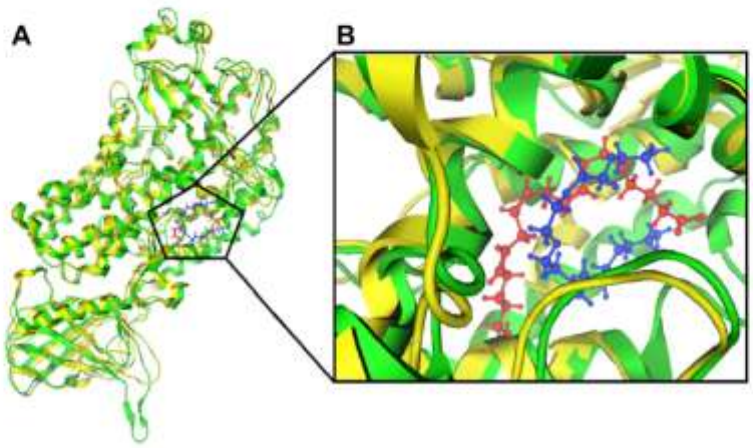

Figure 3 Sequence alignment between (**A**) 5-lipoxygenase (yellow color) complexed with arachidonic acid (red color) and (**B**) 15-lipoxygenase (green color) complexed with a substrate mimic (blue color).

Note: The two structures are represented as sketches.

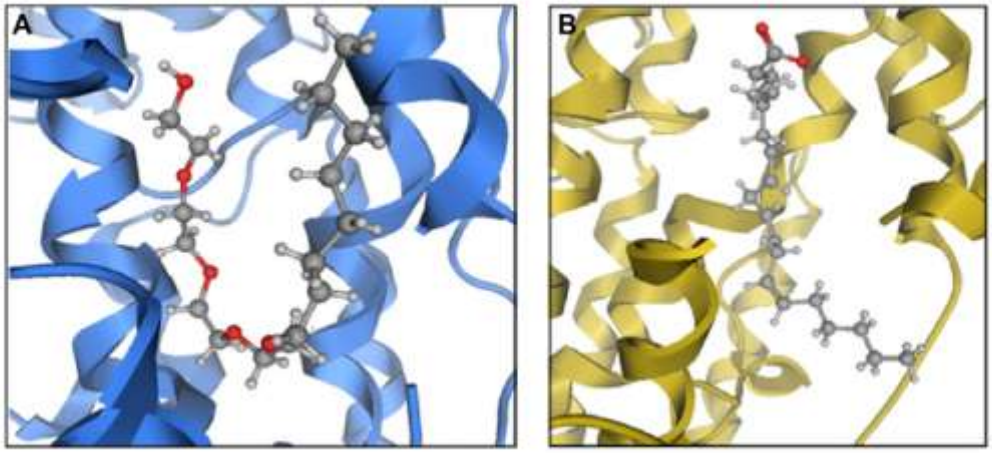

Figure 4 (**A**) The substrate that mimics arachidonic acid and its binding mode in 15-lipoxygenase. (**B**) Binding mode of arachidonic acid in human lipoxygenase.
Notes: Compounds are represented as element color (red for oxygen, gray for carbon and white for hydrogen). Backbone is represented as cartoon.

for compound DHPM7 was the highest =–6.42 kcal/mol and also had the highest percent of lipoxygenase inhibition =81.19%±0.94%. This compound showed a hydrogen bond with Gln 363 with its –NH group of the pyrimidine ring with a distance =3.37 Å. The binding free energy of this conformation was high compared to the other compounds and a direct contact with a water molecule H_2O 955 that is close to arachidonic acid with the aniline –NH group. Molecular docking of compound DHPM7 with Leadit 2.1.2[25] showed two poses; the first one revealed a hydrogen bond between His 367 with the C=O group of the carboxylate side chain, in addition to the interaction of hydroxyl group with both Fe^{2+} and Val 671 (Figure 6A). The second pose of DHPM7 showed two hydrogen bonds between C=O of carboxylate side chain and two $–NH_2$ groups of both Phe 177 and Gln 413 (Figure 6B).

Molecular docking of the compound DHPM8 with Leadit 2.1.2[25] software showed two binding modes with different residues in the active site of the enzyme such as Fe^{2+} ion and Ala 672 (Figure 7A). In another pose, it showed a hydrogen bond with both Val 671 and Ala 672 and Asn 554 (Figure 7B).

The placement of the best pose of the most active compound DHPM7 inside the active site was compared to that of arachidonic acid (Figure 8). This figure provides good insights of the hydrophilic and hydrophobic regions of the pocket. Also, it showed how the best conformation of DHPM7 can fill in the total space of the pocket in

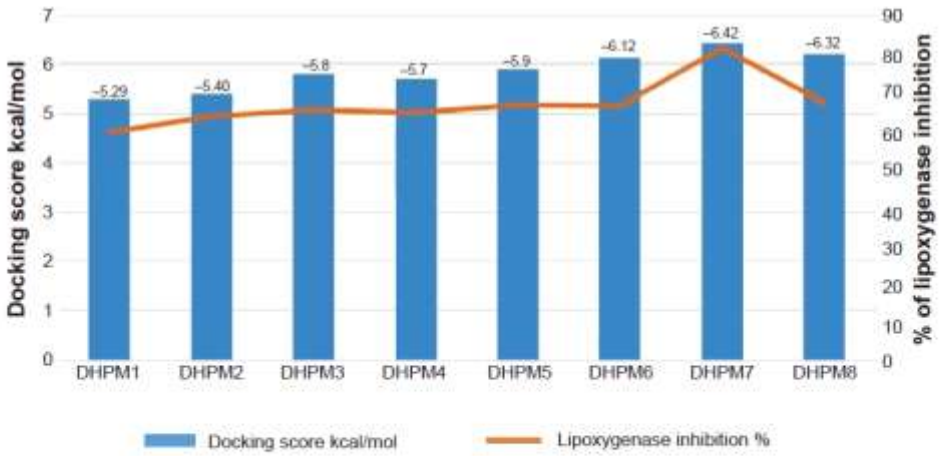

Figure 5 A correlation between the docking and biological results showing a high similarity between the experimental and computational results was observed.

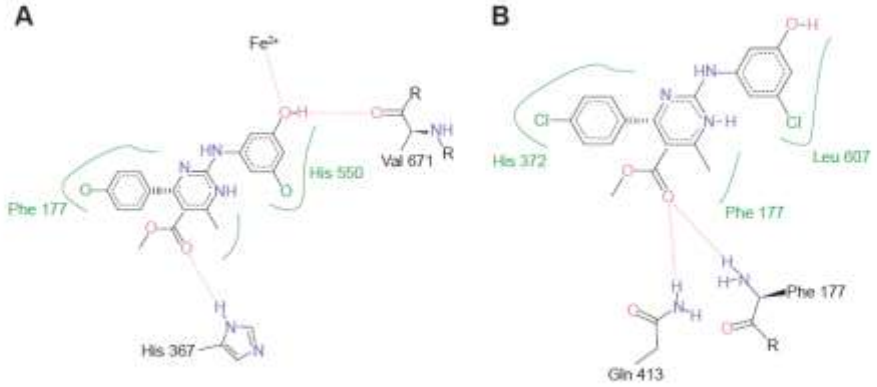

Figure 6 Two different binding modes for the compound dihydropyrimidines-7 resulting from Leadit 2.1.2 docking.

addition to excellent orientation of its groups toward the best interactions.

Both the inhibitory activity results of lipoxygenase and the molecular docking results confirmed the activity of these compounds as potential lipoxygenase inhibitors. These inhibitors may be of great importance in cancer inhibition, and this was confirmed by the use of two cancer cell lines such as breast cancer (MCF-7) and melanoma (UACC-62).

Identification of the cytotoxic activity

The title compounds were screened at 50 µg/mL concentration against MCF-7, UACC-62, and PBMC cell lines for cytotoxicity after 48 hours of treatment. From the percentage of cell growth inhibition values (Table 4), it was apparent that most of the test compounds exhibited promising anticancer activities compared to standard drugs (Figure 9). It was observed for the MCF-7 cell line that DHPM1, DHPM4, and DHPM6–8 displayed cytotoxicity over 89.51%±5.44% after 48 hours. However, the compound DHPM2 bearing 2-hydroxy at second and nitro at fourth position of phenyl ring exhibited 15.82%±2.84% against MCF-7 cell lines, whereas the compound DHPM3 having bromine and fluorine atoms at the third and fourth positions, respectively, on the phenyl ring exhibited 20.02%±1.78% against MCF-7 cell lines. The title compounds at 50 µg/mL were noted to be very effective against the UACC-62 cell line with DHPM2 having a cytotoxicity value of 82.62%±0.86% and DHPM1 and DHPM4–8 having cytotoxicity above 96.72%±0.50%. However, the compound DHPM3 having bromine and fluorine atoms at the third and fourth positions, respectively, on the phenyl ring exhibited 39.62%±0.21% against UACC-62 cell lines when compared to other analogues in the series (Figure 9). The DHPM analogues were not toxic to PBMCs on comparing the activity of the DHPM to MCF-7 and UACC-62.

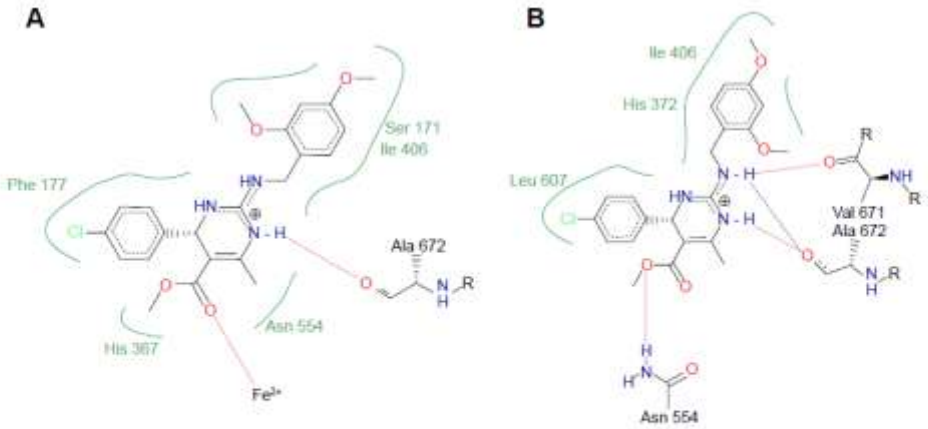

Figure 7 Binding modes of compound dihydropyrimidine-8 in the active site.

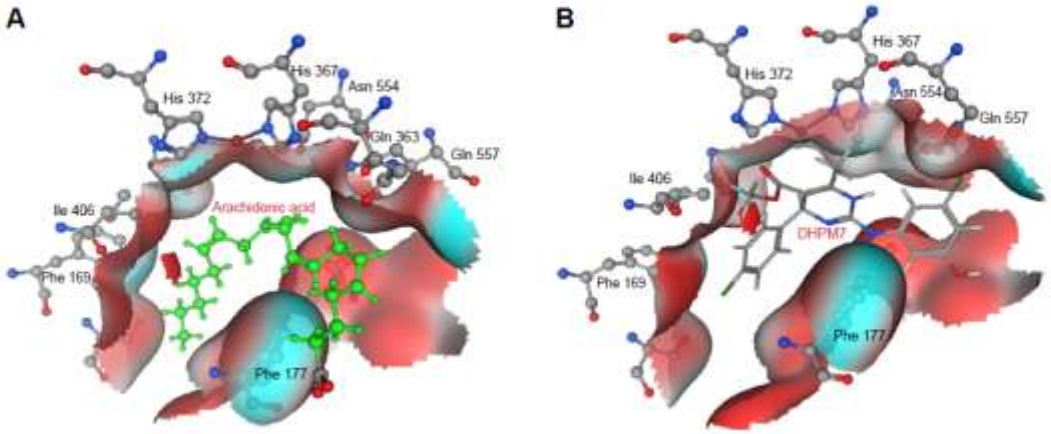

Figure 8 A comparative binding mode between (**A**) arachidonic acid and (**B**) compound 7 inside the active site of the lipoxygenase.
Note: The blue parts represent the mild polar parts and the red colored parts represent the nonpolar hydrophobic parts.
Abbreviation: DHPM, dihydropyrimidine.

The compound DHPM8 has the methoxy group at the second and fourth positions on the benzyl ring and exhibited half-maximal inhibitory concentration values of 0.92±0.09 µM and 1.97±0.08 µM against MCF-7 and UACC-62 cell lines, respectively, according to Table 5. According to our findings, compound DHPM7 and DHPM8 exhibited promising lipoxygenase enzyme inhibition activity and anticancer activity as predicted.

Conclusion

In the present investigation, the synthesis and characterization of 1,4-dihydropyrimidine analogues DHPM1–DHPM8 resulted in good yields. Purity of the compounds was confirmed by HPLC, and it was more than 99%. Structural elucidation was completed by NMR (^{1}H and ^{13}C) and HRMS studies. It was noted that the compound DHPM7 exhibited potential lipoxygenase enzyme inhibition activity of 81.19%±0.94% at a concentration 2.46 µM. The activity of the compounds against lipoxygenase enzyme was interpreted by a molecular docking study. The cytotoxic activity of compound DHPM8 in terms of half-maximal inhibitory concentration was 0.92±0.09 µM and 1.97±0.08 µM versus human cancer cell lines MCF-7 and UACC-62, respectively. Further studies may be needed for more modification of the compound DHPM7 to obtain potent lipoxygenase inhibitor and cancer chemoprotective agents.

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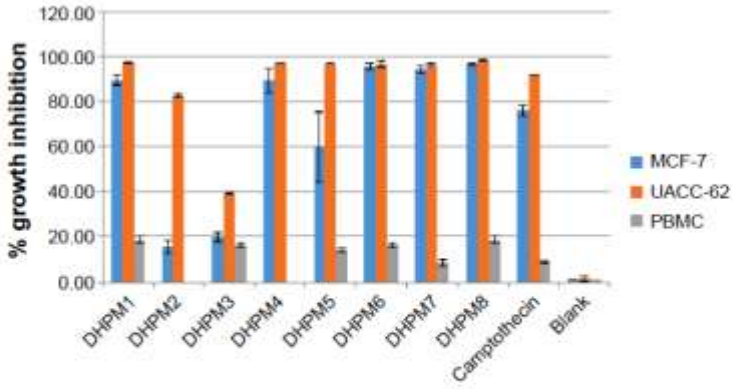

Figure 9 Percentage of growth inhibition of DHPM1–DHPM8 against MCF-7, UACC-62, and PBMC cell lines.
Note: Data are presented as mean ± standard deviation at three replicates from three independent experiments. Blank, culture medium alone without test compounds.
Abbreviations: MCF-7, Michigan Cancer Foundation-7; UACC-62, human melanoma cells; PBMC, peripheral blood mononuclear cell; DHPM, dihydropyrimidine.

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Disclosure

The authors report no conflicts of interest in this work.

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