The impact of aromatic ring count on the bioavailability of chemical compounds

by

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PREFACE

The research contained in this dissertation was completed by the candidate while based in the Department of Biochemistry, School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg, South Africa. The research was financially supported by the National Research Foundation (NRF).

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

Signed:

Date: 21/08/2019

DECLARATION: PLAGIARISM

I, Michael P Mcoyi, declare that:

(i) the research reported in this dissertation, except where otherwise indicated or acknowledged, is my original work;

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ABSTRACT

Aromatic rings are a negative contributing factor in the bioavailability of chemical compounds in drug discovery. Due to the inevitable effect the aromatic rings possess on the bioavailability of chemical compounds, methods to continuously evaluate their effect should be ceaseless. As such, this study aimed at investigating the impact of aromatic ring count on the bioavailability of chemical compounds by screening a small collection compound library comprising 13 compounds. The permeability of chemical compounds was evaluated using the PAMPA assay; the results further analysed through spectrophotometry. The PAMPA assay is an automated system and was used to allow for rapid screening of compounds and the screening was successful. Overall, only four chemical compounds showed poor permeability: ammonium bromide, gibberellic acid, salicylic acid, and PMSF. Osiris Property Explorer was used to screen all 13 compounds for cLogP, LogS, TPSA, Toxicity risks, drug-likeness and drug score. Most of these compounds produced suitable logP (< 5) and were therefore predicted to possess good absorption and permeability properties. Percentage protein binding was assessed separately; all chemical compounds were screened for percentage protein binding using Amicon Ultra-15 Centrifugal Filter method. The results indicated a broad range of protein binding for all chemical compounds tested (70.29 – 98.23%). Lipinski's Rule of Five was applied to all chemical compounds and compounds were scored against the four rules. Most compounds adhered to all four rules with only two compounds violating one or two rules. A relationship between parameters: permeability, drug-score, and percentage protein binding, against the aromatic ring count of chemical compounds, was explored. Overall, no real relationship existed between any of these parameters and aromatic ring count, as was indicated by low correlation coefficients (R^2) (< 0.95). Overall, this study resulted in the successful screening of 13 chemical compounds and the establishment of effective permeability, percentage protein binding assays for future studies.

CONFERENCE PROCEEDINGS

Poster presentations of this study:

1. **Mcoyi, M.P**., Hewer, R. Evaluation of permeability of natural compounds for early-stage drug discovery. Presented at the *25th SASBMB Congress 2016*, 10 - 14 July 2016, East London, South Africa.

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ABBREVIATIONS

ºC	Degrees Celsius
µM	Micromolar
µL	Microlitre
µg / µl	Micrograms/ microlitre
ABC	ATP binding cassette
ADMET	Absorption, distribution, metabolism, excretion and toxicity
AIDS	Acquired immunodeficiency syndrome
ACD	Advanced chemical development
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BCS	Biopharmaceutical classification system
BSA	Bovine serum albumin
CADD	Computer-aided drug discovery
CC_{50}	Concentration required to reduce cell viability by 50%
Cat#	Catalogue number
CNS	Central nervous system
Da	Dalton
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GI	Gastrointestinal
g/Mol	Grams per mole

HIV	Human immunodeficiency virus
HTS	High throughput screening
H2L	Head to lead
IC_{50}	Inhibitory concentration 50
$LogP_e$	Log effective permeability
LY	Lucifer yellow
MTT	(3-(4, 5- dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide
MW	Molecular weight
MWCO	Molecular weight cut-off
mg /ml	Milligram/ millilitre
mM	Milimolar
NCE	New chemical entity
nm	Nanometer
MS	Microsoft
NMR	Nuclear magnetic resonance
PAMPA	Parallel artificial membrane permeability assay
PBS	Phosphate buffered saline
Pe	Effective permeability
PPB	Percentage protein-binding
PI	Protease inhibitor
PMSF	Phenylmethylsulfonyl flouride
RO5	Rule of Five
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute Medium

SD	Standard deviations
SISA	Simple interactive statistical analysis
SMILES	Simplified molecular-input line-entry system
SLC	Solute carrier
TPSA	Topological polar surface area
USA	United States of America
UV/ VIS	Ultraviolet / visible
VS	Virtual screening

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CHAPTER 1: INTRODUCTION

1.1 Brief history of natural compounds

Natural products are described as compounds that are found occurring naturally (plants) or from living organisms, are bioactive and a group of compounds with particular molecular properties (Croteau *et al*., 2000). Plants are therefore a reach source of natural products and a few of the earliest bioactive compounds extracted from plants include colchicine, morphine, and atropine, to name a few (Balunas and Kinghorn, 2005 & Veeresham, 2012). One of the notable breakthroughs in pharmaceutical industries emanated in the year 1806 when Friedrich Serturner isolated a pure natural compound, morphine from the opium poppy. Later in 1897, Bayer and colleague isolated aspirin as the first semi-synthetic pure drug which was synthesized from a natural compound, salicylic acid, which was isolated from the willow tree. Moreover, the isolation of morphine laid an important foundation for the isolation of more other natural compounds like strychnine, colchicine, and codeine and, oxycodone which is still useful today for modulating analgesic activities. Isolated in the same period was quinine which is one of the famous drugs of natural origin which, and for more than 300 years, was the only cure used for malaria. A further contribution of natural products in drug discovery continued in the year 1942 when penicillin began to commercialize as the first natural product originate antibacterial commercial drug. The success of penicillin as a natural product changed the way pharmaceutical society view natural products as it expanded the knowledge of antibacterial therapies and the discovery of more antibiotics such as streptomycin. This historical importance of natural products provided new inspiration in the development of new drugs for different therapeutic targets and more natural product drug derivatives are still introduced on the market today as FDA approved (Jantan *et al*., 2015). In 2000 and 2006 alone, an estimated 26 natural products were approved by the FDA for the drug market and a further increase of natural product approval for the market is expected in the coming years as interest on natural products keeps growing (Lam, 2007).

Half of all the drugs in the market today originated from natural products and the success of natural products over the years was facilitated by, mostly the development of synthetic chemistry which was a very important factor as it enabled the development of synthetic methods, isolation techniques, and structure exposition methods (Dias *et al*., 2012). Natural

products possess different chemical structural moieties thus a plea for structure elucidation methods, and this structure difference in natural products serves as an advantage for binding to protein receptors or enzymes and also for further discovery of new drug candidates.

1.2 Natural products properties

As mentioned above in the description of natural products that they are a group of compounds with particular properties and these properties are helpful in differentiating between natural products and other compounds like synthetic compounds. Chemical informatics, shortly, cheminformatics analysis is used to identify the molecular properties that distinguish natural products from other available compounds (Brielmann *et al*., 1999). Through cheminformatics analysis, which was performed by Henkel *et al*., (1999) natural products appeared to have much more complex chemical structures, with an average of three stereogenic centers. Further analysis revealed that bridgehead atoms in natural products were more than there are in other compounds and more oxygen-containing but less nitrogen-containing moieties than other compounds. These properties define and separate natural products from the rest of the available compounds.

1.3 Comparison between natural compounds and synthetic compounds

A comparison of synthetic compounds to natural compounds reveals that synthetic compounds usually adhere to parameters which are mostly compiled in the Lipinski's rule of five (RO5). Synthetic compounds have lower molecular weight than natural compounds as the rule of five states that; an orally active drug should have a molecular weight of less than 500 Da, whereas natural compounds break this rule as some have a molecular weight greater than 500 Da but are still orally active drug potential candidates. This does not mean natural products are all greater in structure size than synthetic compounds since they also incorporate small molecules. The analyses by Lee and Schneider, on natural products based on the Rule-of-Five, revealed that natural products are slightly more lipophilic with a logP of 2.9 rather than 2.1 for most synthetic compounds. Natural products contain half the number of nitrogen atoms as synthetic compounds, 0.84 against 2.69, but contained twice as many oxygen atoms. This analysis was presented through the work of Feher and Schmidt (2003), where they calculated more than 40 different molecular properties for natural products and synthetic compounds. Compared to synthetic compounds, natural compounds contain 10 times less sulfur and halogens. In addition,

through the Feher and Schmidt analysis, natural product structures displayed two or more rings than synthetic compounds, this, in turn, makes natural products to have more unsaturated moieties than synthetic compounds. In addition, the binding of natural compounds is greater than that observed in synthetic compounds. The distribution of the parameters from the rule of five is actually based on the calculated potential drugs' properties and by definition, most natural compounds lie outside of these parameter's cut-offs. Fascinatingly, most of these natural compounds are orally active compounds and potential therapeutics. Thus, the search for new therapeutics has produced tens of thousands of natural compounds. Natural compounds are typically identified through activity assays and advanced further along the drug discovery and development pipeline based on their modulation of a specific target.

1.4 Toxicity

1.4.1 Toxicity screening in drug discovery and development

Most drug candidates fail due to toxicity at preclinical stages of drug discovery. Kramer *et al* reported attrition due to the toxicity of 70% occurring in the preclinical stage, and 23% of compound failure during registration due to attrition caused by toxicity and safety. To provide early estimates of the drug safety and to determine whether drug candidates is likely to proceed or fall out in further development stages, early *in vivo* preclinical toxicity testing is applied. Preclinical toxicity studies are important in providing an early assessment of a compound or drug's risk in humans to avoid late-stage failures or withdrawals because drug failures and withdrawals in late-stage development due to toxicity are actually considered the main factor contributing to increased drug development costs and has gained considerable attention from the pharmaceutical industries. Accordingly, this has inspired the development and introduction of early preclinical toxicity testing models (Fielden and Kolaja 2008). There are different types of early preclinical assays applied to assess drug safety and these assays comprise of both high and low-to-intermediate throughput *in vitro* assays and *in vivo* toxicity studies (Kramer *et al*., 2007). Toxicity screening models and their applications are preferably required in as early as the lead optimization step and this is a demand that most pharmaceutical industries are fighting to meet and thus the development of several approaches. The proposed models include *in silico* methods, a series of *in vitro* screening assays and limited *in vivo* animal tests and for the best rational approach, all these strategies need to be combined when evaluating toxicity.

1.4.2 Toxicity screening assays

There are different toxicity screening assays that are commonly used in preclinical development each having a different contribution in predicting drug toxicity. The toxicity screening assays include the screening through MTT assay and screening through ATP measurement, to name a few. The MTT (3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) in its original form is a yellow tetrazolium salt and is reduced to a blue precipitate formazan dye through a mitochondrion of metabolically active cells. The blue precipitate is extracted by an organic solvent. MTT assay is mostly used in cytotoxicity experiments where cell viability is measured through the quantification of the blue precipitate by fluorescence. This is the most famous method of testing for compound toxicity on all cell types where the HTS approach is applied with 96-well or more plates. The limitation of this assay is that it measures cell viability based on the activity of mitochondria, as live cells will have active mitochondria but cells affected by the compound's toxicity will die due to inactive mitochondria thus selecting for compounds that directly affect mitochondria might result in a toxicity overestimation. ATP measurement is easy and simple, it measures the amount of ATP released by live cells through chemiluminescence and is quantified through luciferin-luciferase assay. This assay can use 96-well or up to 384-well plate treated live cells with different compounds at different concentrations or a specific concentration.

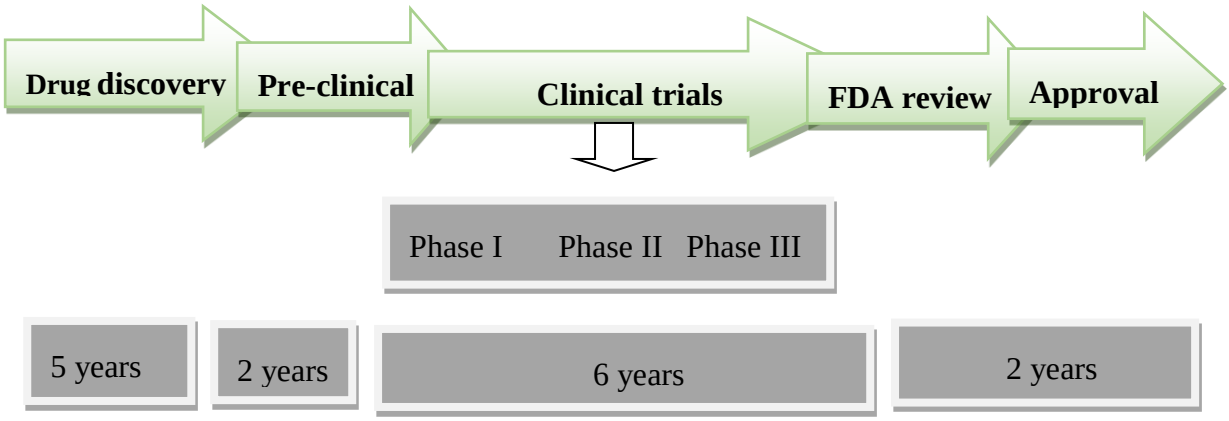
1.5 Drug discovery and development

The development of new drug molecules requires vast resources in intellect, as this is a complex field of research that basically incorporates multidisciplinary scientific systems such as medicine, chemistry, biotechnology, and pharmacology. Drug discovery is described as a process where new therapeutics which are against a specific target is discovered. The drug discovery process is a very lengthy and costly process that takes about 10-15 years of drug candidates' development at an estimated cost of up to $1.5 billion per successful drug. It is estimated that for every successful drug candidate reaching the market, an initial 5000-10000 entities are evaluated for therapeutic activity. From the initial compound entities, approximately 250 entities go into the clinical stage and it is reported that only one in these candidates might

be FDA approved. The drug discovery and development consist of different stages starting from the discovery of drugs which is the initial stage. The development stage includes; pre-clinical, clinical, FDA review and post-FDA approval.

The design of new drugs needs to consider the effect the drugs have on their targets, this is called target validation, but more prominently, the design needs to address the drug's ability to elicit the effect against the human arrangement complications. These newly designed drug molecules are referred to as "lead" compounds, and these are basically chemical entities possessing pharmacological activity or bioactivity against specific set target with a potential to become drug candidate (Han and Amidon, 2000 & Wohnsland and Faller, 2001). Visual screening of lead compounds is impossible but the starting point for the development of a drug candidate is the compound's chemical structure. This is well described by Lipinski using the rule-of-five defined above (Section 1.3). When identified, through the discovery process, lead compounds proceed further into a development program where it is processed for a long period of time before FDA approval and market. Whilst the design of lead compound is important, equal importance must be employed to both target validation and compound bioavailability for attaining positive therapeutic effect. Lead compounds are derived and designed from HIT compounds, from what is normally known as Heat to Lead (H2L) stage where HITS compounds are vigorously screened through high throughput screening models and optimized in order to identify lead compounds. The early discovery stage of the identification of HIT compounds is the selection of compounds from the compound library through either random or rational screening methods. This discovery takes approximately 5 years with approximate cost contribution of 25% as demonstrated in Figure 1.1. This signifies how difficult it is to screen compounds on compound libraries, and even then, only about 5000 compounds go through as potential HIT compounds. In definition, HIT compounds are compounds possessing specific inhibition against a specific target. Screening of HIT compounds involves random screening approach and rational design approach.

Identification of HIT compounds	Test the safety and biological activity in animal studies	Assess the safety and dosage in human	Determine the efficacy and assess side effects	Determine the long term effect; Validate efficacy	The review process for approval	Postmarketing testing
5000-10000 compounds	250 compounds		5 compounds		1 compound	



Approximate cost contribution:

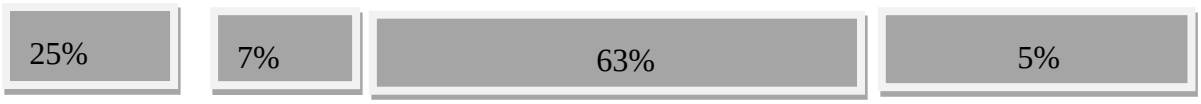


Figure 1.1: Diagram representation of the drug discovery and development in different stages and approximate years and cost contributing to each stage of the process. Clinical stage is the final valuation stage before reaching the FDA and thus drug assessment in this stage is longer and more costly than the rest of the stages. The drug discovery stage involves multiple evaluation models and assays to identify potential HITS compounds and thus takes approximately 5 years with a 25% cost contribution. Pre-clinical and FDA approval are two of the least time consuming and low-cost stages as pre-clinical is actually a median stage leading to the actual clinical testing and FDA approval is at the final post-marketing testing.

1.5.1 Random screening approach

Random screening approach screens compound libraries without any previous knowledge of the compounds that are screened against a specific target. The combination of natural libraries, parallel synthesis, combinatorial chemistry and drug repositioning all form part of the compound libraries.

Table 1.1: Different random screening methods contributing to the compound libraries.

Random screening methods	**Contribution to the compound libraries**
Combinatorial chemistry	Compiles molecules related to an active particular scaffold against a target and synthesis of compounds not limited to a single target.
Drug repositioning	Redistribution of FDA approved drugs into new disease models.
Natural library	Natural products derived directly from plants.
Parallel synthesis	Synthesis of compounds in parallel through spatially separated compartments.

All of these screening techniques are normally screened through High Throughput Screening (HTS) automated systems. The evidence of random screening using natural library was documented through the screening of the famous South African plant used for a traditional medicine called *Sutherlandia frutescens*, derived from a natural product (Stokes, 2002). This natural product derivative had reached phase II in clinical trials as it was proposed to possess HIV/AIDS possible treatment activity (Hewer *et al*., 2012). Random screening using HTS increases failure rate as there will be thousands of compounds randomly screened without any prior knowledge and thus high running cost is associated with HTS. HITS compounds are normally identified in early discovery stages as these are compounds with no specific target screened against but due to this, High Throughput Screening of HITS compounds is not favourable to cost-effectiveness.

1.5.2 Rational design approach

This approach selects a group of compounds with potential activity against a specific target from the available compound libraries. Unlike the random screening approach, the selection of compounds in this approach is due to prior compound knowledge against their target.

Table 1.2: Structure-based and ligand-based design both contributing to the rational design approach.

Structure-based design	Ligand-based design
Homology modelling	Pharmacophore modelling
Threading	3D QSAR
De Novo design	(VS)Virtual screening
NMR	
X-ray crystallography	
Virtual screening	

Both structure-based design and ligand-based design are used in early stage of discovery and they share virtual screening which, through computational methods, predicts ligand interaction with a specific target. The incorporation of computational methods through rational drug design results in the rapid and low-cost evaluation of compound interactions with their specific target and thus HIT identification. The evidence of the success of rational drug design was documented through the discovery of ritonavir and indinavir, both are PIs. Rational drug design is also useful in prediction of the adsorption, distribution, metabolism, excretion, and toxicity (ADMET) properties due to the fact that all of these properties can be tested *in silico*, which is computer modeling. ADMET properties are important in the discovery of drugs more specifically in bioavailability prediction.

1.6 Bioavailability

Bioavailability is the key factor in drug discovery and development and it is well defined through the five major properties, which are adsorption, distribution, metabolism, excretion, and toxicity. Bioavailability is defined as the degree at which a drug molecule reaches the

systematic circulation. By definition, the degree at which a drug, after being administered, reaches the target is 100% bioavailable, but unfortunately, this is not true for orally administered drugs. Due to the oral administration route, drug bioavailability is affected, mainly by the ADMET properties. With absorption, a drug molecule is required to cross through a physical and selective barrier of both the small and large intestines and also endure through enterocytes and first phase metabolism in the liver. When a drug molecule has passed through the absorption barriers, it is distributed throughout the body to stimulate response to the target. The distribution of a drug molecule must be attained while the drug defends itself from the body's defence system because the body develops a defensive approach against xenobiotics. This is achieved through metabolism in the liver and excretion of compounds into bile, gut lumen, and urine.

Absorption is one of the important properties from the ADMET properties and in order to clearly understand absorption, an understanding of a compound's permeability is crucial as it plays a central role in compound's oral absorption. Different techniques have been used in an attempt to study permeability of potential drug candidates and most of these techniques are *in vitro*, *in vivo*, *in situ* and *ex vivo* methods. The most effective methods thus far have been the *in vivo* methods which better investigate drug permeability. Considering the time and cost effectiveness of discovery of new drugs, *in vitro* methods are most favourable as they provide rapid screening option whilst minimizing the costs. The main advantage of the cell-mediated system is the ability to represent the human intestinal barrier and thus the more accurate prediction of the drug behaviour in actual human intestinal barrier rather than using *in vivo* assay (Orsi and Essex, 2010).

1.7 Permeability

1.7.1 Transporter- mediated permeability

Studies reveal that there are 1022 membrane transporters encoded by the human genome but in our study, we only focused on the two types of drug transporters namely active and passive transported. In drug pharmacokinetics, it is essential to test the permeation of drugs that cross through a biological membrane thus it is important to know the types of transporters involved in the permeation of drugs to their target (Kell *et al*., 2014).

There are two super-families in which transporters fall under, these are the solute carrier (SLC) and ATP binding cassette (ABC) transporters. The major difference between the two is that ABC transporters are primary active transporters whereas SLC transporters depend on secondary transporters. This means that when ABC transporters need to pump their substrates across membranes they depend only on the hydrolysis of ATP. SLC transporters, on the other hand, consist of genes that encode ion-coupled secondary active transporters and are thus assisted transporters. The active efflux of environmental toxins, xenobiotics, cellular waste as well as the influx of nutrients that are essential, is also driven by these two membrane transporters and this activity may or may not depend on energy.

The membrane permeation of both organic and inorganic compounds is completed by both ABC and SLC transporters and further assistance from channels, although channels allow permeability in their open state. Briefly, a complex between a substrate and transporter is formed and it results in a change in the conformation of the transporter and that causes the translocation of the substrate to the opposite side or target site of the biological membrane. The two types of mediated transporter mechanisms are passive diffusion and active transport and are both involved in the transport of mostly, solutes across the membranes (Kell *et al*., 2011). Active transport falls under ABC super-family because it involves the transport of solutes against their electrochemical gradients, this means that solutes will be concentrated on just one side of the plasma membrane and therefore cellular energy is required. This mode of transportation is one of the few energy-dependent mechanisms (Giacomini and Sugiyama, 2006). By definition, active transport is the concentrative uptake of drugs which moves drugs from low drug concentration to a high drug concentration, while conversely, passive transport is naturally not concentrative and moves drugs from high concentration region to a low concentration region. One other difference between these two types of drug transporter is that passive diffusion does not involve carrier proteins that cross the membrane whereas active transport usually has carrier proteins as a mode of drug entry (Lentz *et al*., 2000). The most famous example of the active transport carrier protein is the P-glycoprotein, and is the most studied and understood transporter in drug discovery (Amin, 2013 & Lin and Yamazaki, 2003). This blood-brain barrier protein serves as an efflux transporter and depends mainly on ATP for its function and is famous for granting multi-drug resistance to tumour cells (Bode, 2009, Carpenter *et al*., 2014 & Pardridge, 2012).

The second type of transporter, passive transport involves the diffusion of solute or drugs from a high concentration gradient, across a membrane, to a low concentration gradient (Doppenschmitt *et al*., 1999 & Leung *et al*., 2012). The most famous example of the passive transport system is the parallel artificial membrane plate assay (PAMPA) which involves the movement of drugs or compounds from a high concentration region, the donor, into a low concentration region, the acceptor and does not depend on cellular energy for transport but rather diffusion of drugs or solutes. No energy is required in facilitated diffusion and basically involves the diffusion of both organic compounds and ions across the plasma membrane (Giacomini and Sugiyama, 2006) and so this type of transport falls under SLC super-family. Passive transport uses two means of drug or compound transport through membrane namely, paracellular and transcellular methods (Doan *et al*., 2002). One other example of active transport, which is a derivative of endocytosis is called transcytosis (Figure 1.2) which basically captures surface substances of the enterocyte layer and passes them through a membrane layer while they remain bound within a vesicle.

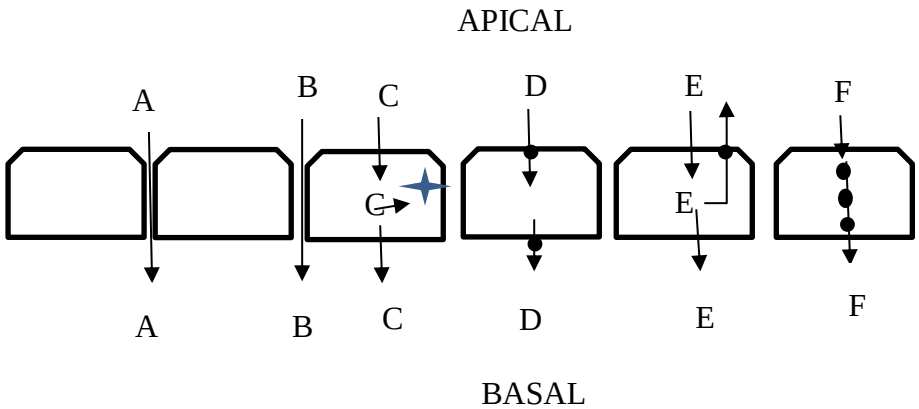


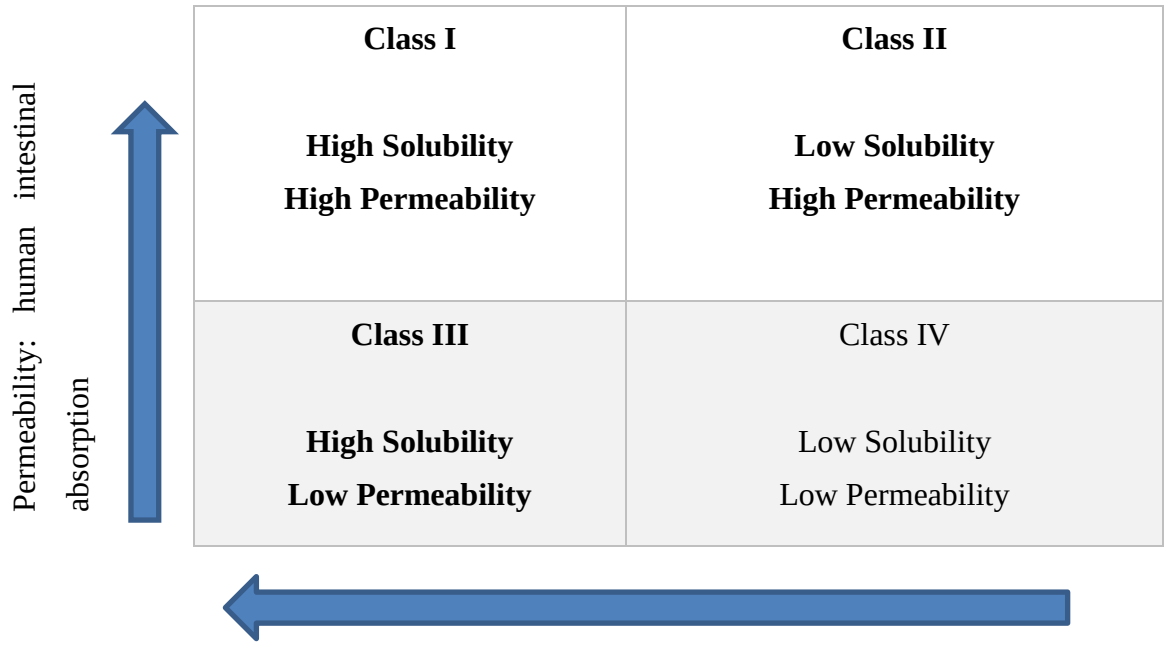
Figure 1.2 Representation of absorption from the intestinal barrier. [A] paracellular diffusion, [B] paracellular enhanced through modulation of tight joints, [C] transcellular passive diffusion, [ ] intracellular metabolism, [D] carrier-mediated transcellular transport, [E] polarized apically modified transcellular, [F] transcellular vesicular transcytosis. Figure reproduced from Ramlucken, 2014.

1.7.2 Solubility and dissolution

In the discussion of absorption and permeability, compound solubility and dissolution rate are the first most important factors that need to be addressed on the early stages of drug discovery.

The rate at which a compound completely breaks down into a solvent state is called dissolution and the ability of a compound to turn into a liquid solution is called solubility. Solubility is key in bioavailability as it accounts for more than 40 % NCEs insoluble in water which in turn leads to inadequate bioavailability. After oral administration, a drug normally stays in intestinal fluid for some time and is in, later transported to the permeable barrier and therefore it is very important for a drug to maintain its dissolution rate at minimal.

There is a direct relationship between solubility and membrane permeability of drugs through drug absorption, this relationship was introduced in the year 1995 by the Biopharmaceutical classification system (BCS). The system provides a systematic outline to understand the model of drug absorption in terms of solubility and permeability (Sachan *et al*., 2014). The behavior of drugs absorbed inside the human intestines differs according to their solubility strength and permeability ability. The BCS system separates these drugs into four categories or classes *in vivo/in vitro* (I, II, III, and IV) as indicated in Figure 1.2. This system is a guideline for formulating strategies in improving the success of drugs in bioavailability and improving efficiency in drug development stages.



Solubility: volume of water required to dissolve the highest dose strength across the physiological pH range

Figure 1.3: The representation of the Biopharmaceutics classification system. The direct relationship between solubility and permeability, drugs absorbed in the human intestine against its solubility. Adapted from Amidon *et al*, 1995.

Table 1.3: The chemical compounds reported to fall into each Class based on their dissolution rate and their absorption ability.

Solubility	Permeability	
	High	Low
High	Class I • Propranolol • Metoprolol • Diltiazem • Verapamil	Class III • Acyclovir • Neomycin B • Enalaprilat • Alendronate
Low	Class II • Ketoconazole • Mefenamic acid • Nisoldipine • Nicardipine	Class V • Chlorothiazide • Furosemide • Tobramycin • Cefuroxime

Class I drugs are highly soluble and highly permeable drugs, which are most successful when it comes to bioavailability. Exceedingly successful in bioavailability means that these drugs possess at least 100% expectancy in intestinal absorption and 85% of these drugs completely disintegrate with 30 minutes at optimum pH. Class II drugs are highly permeable meaning they are absorbed easily but however possess low solubility meaning the rate of dissolution is low. Drugs in this Class display flexible bioavailability and thus need improvements in solubility for better bioavailability. Meanwhile, Class III drugs are the opposite of Class II, these are drugs possessing high solubility and low permeability. Finally, Class IV drugs and these are very poorly absorbed drugs with low solubility and this category contains drugs that are mostly poor drug candidates and are not appropriate for oral drug discovery. The BCS system groups all the highly permeable drugs in Class I and II to be having a high *in vivo/in-vitro* correlation and low permeable drugs in Class III and IV demonstration a low *in vivo/in-vitro* correlation (Le Ferrec *et al*., 2001).

Compounds in Class I which include propranolol, metoprolol, diltiazem, and verapamil, are all good potential drug candidates but this does not mean these drugs go straight to the market, they would still have to qualify for pharmacokinetics and pharmacodynamics for their specific target. Propranolol, for example, is known to be highly soluble in water and ethanol but however it suffers a high first-pass metabolism by the liver and only about 25%, on average, reaches systematic circulation. Compounds in Class II include ketoconazole and these can also be used for controlled drug delivery but however will require enhancements. Permeability tends to vary over a narrower range than solubility, meaning the difference between a highly permeable and a low permeable compound can be 50-fold whilst the difference between highly soluble and a low soluble compound might be a million-fold. Thus, the Class II drugs will still be a good candidate and possess merely good bioavailability. However, compounds in Class III and IV are generally problematic in controlled drug release development as they both display fairly low permeability. The solubility of drugs eventually affects its absorption, but there are quiet notable factors or properties that directly affect solubility, properties like ionization, particle size, and lipophilicity. To precisely explain the relationship each of these properties has with drug solubility and in turn effects on permeability, Di and Kerns (2015) used a schematic diagram linking each property to the other, presented in Figure 1.4 below.

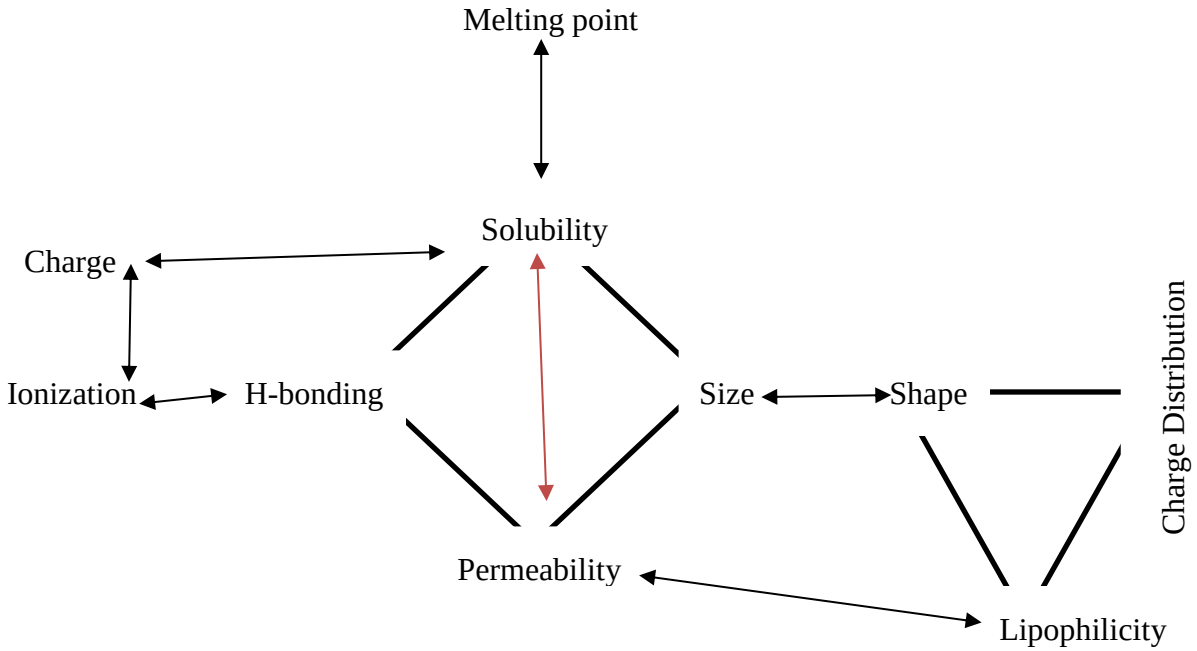


Figure 1.4: Schematic representation of the effect each property has on solubility and permeability. All of these properties should be considered when addressing the issues of solubility and/or permeability, but as stated as above, permeability varies in a much narrow range than solubility. Reproduced from Di and Kerns, 2015.

As helpful as the Lipinski's rule of five is, there are other properties or criterions that should be incorporated with, to increase a compound's potential to become more drug-like. Drug-like meaning the compound possessing functional groups and physiochemical properties that are similar to those of the known drugs. The above properties are all important contributors to a compound's drug-likeness and careful evaluation of each one should be ceaseless, as they are contributing to the drug discovery database. Nonetheless, Lipinski's rule of five is useful in excluding non-drug like compounds. Bemis and Murcko (1996) described the framework of specific drugs as one of an alternative model of addressing the compound's drug-likeness and according to their study, there are about 33 frameworks used in drugs. These framework systems are structural filters that eliminate less "drug-like" compounds whilst promoting the progress of more "drug-like" compounds. All the available methods that address the issue of compounds and their drug-likeness properties are proof of how the field of drug discovery branches into different multidisciplinary (Ursu *et al.*, 2011), this makes it inconceivable that a single study could address the success of potential drug candidates or natural compounds in general (Di *et al.*, 2003).

1.7.3 *In vitro* tools for predicting drug permeability

Absorption of drugs is one of the significant properties in drug discovery and development and thus it is imperative to find the most accurate and suitable screening assays or methods in evaluating intestinal permeability (Milanetti *et al.*, 2015). The use of standardized *in vitro* assays in high throughput screening of potential drug candidates and their application *in vitro* have been more successful than the *in vivo* uses. A few advantages of using the *in vitro* methods rather than *in vivo* is that it requires a much lesser number of compounds to be tested, experimental evaluations of test compounds are much easier as they exclude live animals, involves much quicker turn-around time, permits a perfect assessment of the mechanisms that are involved in membrane transport, provides data or information on the compound's metabolism that occur during transport, structure-transport relationships are easily designed

and developed and lastly, it delivers the free from contaminating plasma protein samples for analysis.

1.7.3.1 Cell-based assays in permeability

In substitution of live animal experiments, different *in vitro* screening assays that actually use cell monolayers are employed (Volpe, 2011). In this type of screening assay, cell types are grown on permeable membranes which resembles the gastral epithelial layer after being differentiated. This permeable membrane system allows for the measurement of permeability through the test compound's passive diffusion and it assesses the absorption of these compounds in correlation to the GI tract absorption model.

1.8 Aromatic rings

A potential drug candidate is normally categorized by its adherence to the Lipinski's rule of five and one of the reasons this rule is so widely used is because medicinal chemists can consciously apply it to both small and large compounds. The principle of RO5 is the simplistic analysis of drug properties one of which is the number of aromatic rings contained in a chemical compound. Aromatic ring count generally involves benzenoid and heteroaromatics, heteroaromatics include pyridine and imidazole. Each ring in a chemical compound is counted individually and this means that groups such as indole and naphthalene each contain two aromatic rings. The aromatic rings are an important structural factor in drug discovery as most currently marketed drugs contain at least one aromatic ring (Ward and Beswick, 2014).

1.9 Rationale for the study

The bioavailability of a promising candidate can now be assessed within the early-stage drug discovery phase through adherence to Lipinski "Rule of Five" (RO5) and numerous *in silico* computer-aided drug discovery programs which are formulated from the experimental assessment of the ADME qualities of successful drugs. Due to their complexity, natural compounds cannot be accurately assessed by existing ADME models and theoretical evaluations. This project seeks to determine whether aromatic rings influence the bioavailability of natural products. To this end, the plasma protein-binding, permeability and metabolism of a collection of natural compounds were experimentally determined and carefully correlated to total aromatic ring count.

1.10 Aims

The aim of this study is the experimental evaluation of the ADME properties of a small collection of natural compounds in order to determine the influence of aromatic ring count on the bioavailability.

1.11 Objectives

The objectives include the design, development, and validation of relevant biological assays; the evaluation of the compound collection within these assays.

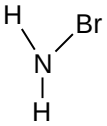
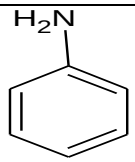
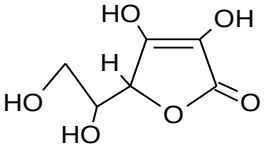
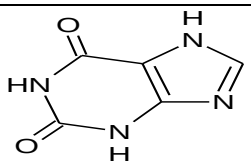
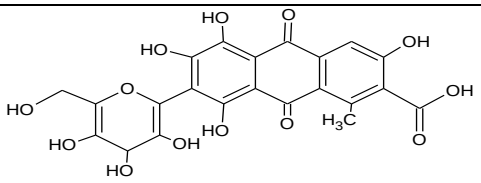
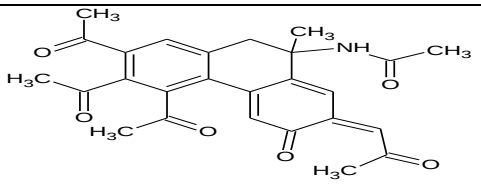
- Specifically, plasma protein-binding, permeability and cytotoxicity of a collection of natural compounds experimentally determined and carefully delineated.
- Description of the Lipinski's rule of five involvement in guiding early-stage drug discovery from natural compounds.

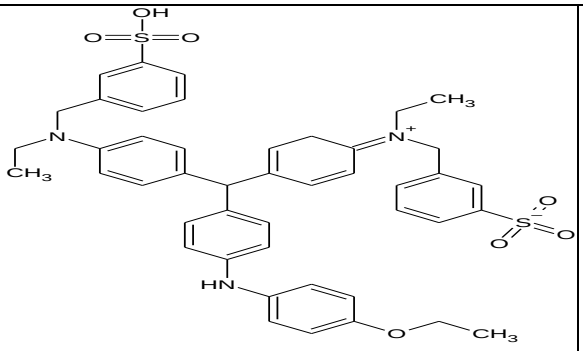
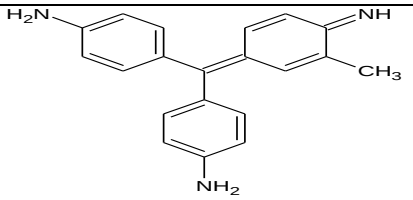
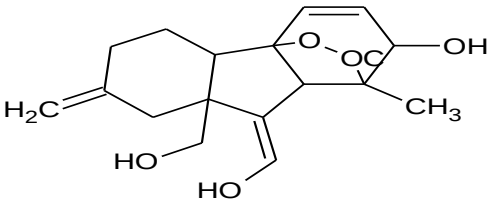
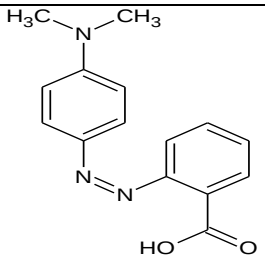
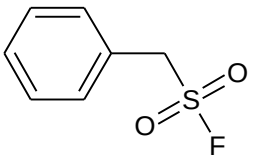
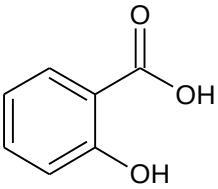
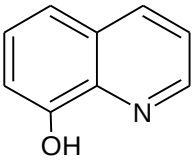
CHAPTER 2: MATERIALS AND METHODS

2.1. Chemical compounds

Different chemical compounds; namely, aniline (cat. 242284, Sigma- Aldrich, USA), ascorbic acid (cat. A92902, Sigma-Aldrich, USA), ammonium bromide (cat. 121249797, Sigma-Aldrich, USA), caffeine (cat. C0750, Sigma-Aldrich, USA), carminic acid (cat. C1022, Sigma-Aldrich, USA), coomassie blue (cat. 20279, Thermo-Fisher Scientific, USA), fuchsine (cat. 215597, Sigma-Aldrich, USA), gibberellic acid (cat. 77065, Sigma-Aldrich, USA), salicylic acid (cat. 247588, Sigma-Aldrich, USA), methyl red (cat. 08714, Sigma-Aldrich, USA), 8-hydroquinone (cat. H9003, Sigma-Aldrich, USA) and colchicine (cat. C9754, Sigma-Aldrich, USA) were dissolved in dimethyl sulfoxide (DMSO) and stored in stocks (10 mg/ml) at 4 °C. Table 2.1 illustrates these compounds with their chemical structures and corresponding molecular weight in alphabetical order. The chemical structures were all constructed using the Advanced Chemical Development (ACD) / ChemSketch program (available at https://webstore.acdlabs.com/software-solutions/acd-chemsketch/ and last accessed on December 2018).

Table 2.1: The chemical structure and corresponding molecular weight (g/mole) of the chemical compounds investigated within this study.

	Compound name	Chemical structure	Molecular weight (g/mole)	Source
1	Ammonium bromide		94.91	Synthetically derived
2	Aniline		93.13	Synthetic compound
3	Ascorbic acid		176.12	Natural origin
4	Caffeine		194.19	Natural origin
5	Carminic acid		492.38	Synthetic compound
6	Colchicine		399.43	Natural origin

7	Coomassie blue		825.97	Synthetically derived
8	Fuchsine		337.86	Synthetically derived
9	Gibberellic acid		346.37	Natural origin
10	Methyl red		269.30	Synthetic origin
11	Phenylmethylsulfonyl fluoride (PMSF)		174.20	Synthetic origin
12	Salicylic acid		138.12	Natural origin
13	8-hydroxyquinoline		145.16	Synthetically derived

2.2 Lipinski's rule of five and Osiris property explorer

Lipinski's rule of five analyses orally active compounds with their adherence to the four rules described by Lipinski (Lipinski, 2004). For potential drug candidates, the 13 compounds of this study were assessed to determine the number of hydrogen bond donors, the number of hydrogen bond acceptors, the molecular weight and logP. According to the Lipinski Rule of Five (RO5), a drug candidate is more likely to be orally bioavailable if it possesses a molecular weight of less than 500 Da, log P values not greater than 5, no more than 5 hydrogen donors and no more than 10 hydrogen acceptors. Experimental compounds were scored according to these four rules (i.e. compounds adhering to all four rules were assigned a score of four out of 4 (4/4) while compounds violating one of these rules scored three out of three (3/3) and so forth. For further assessment, the Osiris property explorer online program was used (available at https://www.organic-chemistry.org/prog/peo/ and last accessed on December 2018). This program was used to predict solubility (log S), cLogP, Toxicity Risk, Molecular weights, TPSA, Drug-likeness, and the overall drug-likeness score of each compound. In order to do this, chemical structures of each compound were generated in the program window either through use of the chemical drawing tool provided or through entry of the simplified molecular-input line-entry system (SMILES) code into the SMILES translator provided.

2.3 Effective permeability assay

2.3.1 Standard curve construction

The standard curves for all thirteen compounds were constructed by a two-fold serial dilution of a compound solution in 5% DMSO in phosphate buffered saline (PBS) beginning at an initial concentration of 500 µM for each compound. Ultraviolet / visible (UV/VIS) spectroscopy via the NanoDrop 2000 (Thermo-Fischer Scientific, USA) was used to determine the absorbance of each compound at eight different concentrations (500 – 12.5 µM) at a fixed wavelength

determined individually for each compound. Absorbance as a factor of concentration was then plotted for each compound. A linear regression line was fitted using Excel 2010 (Microsoft Corporation, USA). All equations were produced with a regression coefficient (R^2 value) of greater than 0.95.

2.3.2 Parallel artificial membrane permeability assay

The parallel artificial membrane permeability assay was conducted as per manufacturer's instructions and as previously described (Faller *et al*., 2001). Briefly, the stock solution of each compound was diluted in PBS to order to obtain the donor solution at a final compound concentration of 500 µM (< 5% final DMSO concentration). Each donor well of the 96-well multiscreen filter plate (cat. ELIIP10SSP, Merck-Millipore, USA) was coated with 5% hexane in hexadecane solution and allowed to evaporate for 1 hour. A solution comprising 5% DMSO in PBS (300 µL) was pipetted into the acceptor plate which was covered by the donor plate to create a PAMPA sandwich. The above-prepared solutions of the experimented compounds (150 µL) were then transferred into the wells of the donor plate. The donor plate was covered to prevent evaporation and the completed PAMPA sandwich was incubated at 37° C. After incubation periods ranging from 4 to 72 hours, the donor plate and the acceptor plate were separated and absorbance of both the donor and the acceptor plate solutions were measured using the NanoDrop 2000 (Thermo-Fischer Scientific, USA). Lucifer yellow (LY; Sigma-Aldrich, USA) was used as a non-permeant control and integrity marker for assay validation. Compound and LY permeability was conducted in experimental and biological triplicates (n=3). The following formulae **[1 & 2]** (Faller *et al*., 2001) was then employed to calculate the log effective permeability ($LogP_e$) of the chemical compounds and LY percentage transmittance:

$$LogP_e = \log [C^*-\ln (1-[\text{drug}]\ \text{acceptor} / [\text{drug}]\ \text{equilibrium})] \quad \textbf{[1]}$$

Where: $C = (V_d * V_a / (V_d + V_a)\ \text{Area}^* \text{ time}$,

V_d = Volume of the donor plate (cm^3)

V_a = Volume of the acceptor plate (cm^3)

Area = Membrane surface area (cm^2)

Time = Incubation time for plate (s)

% T = Acceptor Abs / Abs Equilibrium * 100 **[2]**

Where: Acceptor Abs = Absorbance value from the acceptor plate

Abs Equilibrium = Absorbance of the acceptor + Absorbance of the donor

2.4 Evaluation of compound toxicity

2.4.1 Cultivation of mammalian cells

The HEK293T cell-line (Cat, 103, The reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: HEK-293 Cells from Dr. Andrew Rice), was cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 4500 mg/L glucose (Sigma-Aldrich, USA), 10% heat-inactivated fetal bovine serum (FBS; Thermo-Fisher Scientific, USA), 20 U /ml penicillin-streptomycin (Sigma- Aldrich, USA) and 20 µg/µl gentamicin selective antibiotic (Gibco, USA) (10 % DMEM). The cells were sub-cultured every 3 days through centrifugation at 200 *x* g for 5 minutes and resuspension of the cell pellet in 10 % DMEM. Cell counting was completed through the dilution of cell suspension at 1: 10 with 0.4% Trypan Blue stain and determined manually or by means of the T20 Automated Cell Counter (Bio-Rad, USA). Cells were cultured at a concentration of 1 x 10^5 cells/ml in a controlled environment incubator (SHEL LAB, USA) at 37°C and 5% CO_2.

2.4.2 Cytotoxicity bioassay

The cytotoxicity bioassay was conducted as previously described (Harrison *et al*, 2015). Briefly, HEK293T cells were added to a 96 well culture plate (TPP, Switzerland) at a concentration of 2 x 10^4 cells per well (100 µl) and left to equilibrate at 37 °C and 5% CO_2 for four hours. Thereafter, 100 µl of each test compound [Warfarin (Sigma-Aldrich, USA) or Auranofin (Biomol International, USA)] at 8 different concentrations (serially diluted from 200 to 1.56 µM in 10% DMEM media) was added to the plate. Following an incubation of 72 hours at 37°C with 5% CO_2, 20 µl of a 5mg/ ml MTT solution (VWR Life Sciences, USA) was added to each well and mixed. The plate was then incubated under previously described conditions for 4 hours. The waste media was removed by inversion of the plate and replaced with 150 µl MTT solvent (4 mM HCl, 0.1% Triton X100 in isopropanol). Absorbance readings were

obtained at a fixed wavelength of 590 nm (VersaMax microplate reader; Molecular Devices, USA) and the concentration required to kill 50% of cells (CC_{50}) was calculated for each compound using Origin 8.1 (OriginLab, USA). Standard deviations (SD) were determined using Excel 2010 (Microsoft Corporation, USA).

2.5 Percentage protein-binding

2.5.1 Percentage protein binding by filtration

Amicon Ultra-15 Centrifugal Filter devices (Merck-Millipore, USA) with a molecular weight cut-off (MWCO) of 50K were used to separate bound proteins from the unbound proteins as per a previously described method (Barre *et al.*, 1984). Briefly, into the 30K filter device, a maximum of 15 mL of each compound (500 µM) was mixed with bovine serum albumin (BSA) (Sigma-Aldrich, USA) at molar equivalents. The capped centrifugal filter tube was centrifuged using a swinging-bucket rotor centrifuge (Beckman Coulter, USA) at 4000 *x* g for 20 minutes. The bound protein and compound fraction were measured using the NanoDrop 2000 (Thermo-Fischer Scientific, USA) at optimal wavelengths determined for each compound. Compound concentration was calculated from a standard curve constructed for each compound. To complete the percentage protein-binding (PPB) calculations, the following formulae **[3 & 4]** were applied:

$$PPB = ((T-F)/T) * 100 \quad \textbf{[3]}$$

Where: T= Fraction bound

F = Fraction unbound

$$F_u = C_u / C_{initial} \quad \textbf{[4]}$$

Where: F_u = Fraction unbound

C_u = Concentration of the unbound compound

$C_{initial}$ = Initial concentration of the compound

2.5.2 Percentage protein binding by permeation

The 96-well multiscreen filter plate (cat. ELIIP10SSP, Merck-Millipore, USA) was used to assess percentage protein binding of Warfarin (Sigma- Aldrich, USA) to BSA (Sigma- Aldrich, USA). Briefly, the donor compartment of the plate was filled with 150 µl Warfarin at 100 µM

while the acceptor compartment was filled with 300 µL BSA at a molar concentration equivalent. The plate was allowed to incubate at 37º C for 5 hours after which the data was captured and analyzed as above (Section 2.4.1).

2.6 Statistical Analysis

All experiments were carried out in at least triplicates and Microsoft (MS) Office Excel ™ 2010 was used to calculate all averages and standard deviations, as well as to construct line graphs and tables. The Origin 6.1 software was used in calculation of CC_{50}. Statistical analysis through *p*-values was calculated to assess statistical differences within parameters and assay conditions. This was done using the online simple interactive statistical analysis (SISA) pairwise t-test (www.quantitativeskills.com/sisa/). The value of $p < 0.05$ were considered statistically significant at a 95% confident interval (CI). The correlation coefficient (r^2) was used to measure the fit of the equation.

CHAPTER 3: RESULTS

3.1 Theoretical characterization and assessment

3.1.1 Lipinski's Rule of Five

The Lipinski RO5 evaluates potential drug candidates according to 4 criteria; namely, the number of hydrogen acceptors, the number of hydrogen donors, the logP and the molecular weight. Compounds that adhere to the Lipinski RO5 are more likely to be orally bioavailable than those with properties that violate the four RO5 criteria. Typically, compounds that violate more than 2 of the rules are considered to have poor bioavailability and unlikely to be pursued further as candidates to be administered orally (without structural changes made to the compound to address the specific violation). As shown in Table 3.1, the compounds assessed within this study adhered well to the Lipinski RO5 with 11 of the 13 compounds producing scores of 4/4. The only exemption to this was the pigment, coomassie blue, which, as a large biomolecule, greatly exceeded the 500 kDa cut-off imposed by the RO5. In addition, lower bioavailability of carminic acid was predicted as it also exceeded the < 5 hydrogen bond donor criteria and therefore obtained a Lipinski RO5 score of 2/4. As such, the number of aromatic rings within a compound may play a direct role in determining the bioavailability of the compound as each ring increases the molecular weight of the compound by approximately 78 kDa. As displayed in Table 3.2, the chemical compounds assessed within this study contained at least two or more aromatic rings.

Table 3.1: The scoring of chemical compounds using the Lipinski's rule of five

Compound name	**Rule of five scores (/4)**[a]
Ammonium bromide	4
Aniline	4
Ascorbic acid	4
Caffeine	4
Carminic acid	2
Colchicine	4
Coomassie blue	3
Fuchsine	4
Gibberellic acid	4
Methyl red	4
Phenylmethylsulfonyl fluoride	4
Salicylic acid	4
8-hydroxyquinoline	4

[a] The above values are scores obtained by each compound when assessed through the four criteria that constitute the Lipinski Rule of 5.

Table 3.2: The number of aromatic rings in each experimental compound

Compound name	**Aromatic ring count**
Ammonium bromide	0
Aniline	1
Ascorbic acid	1
Caffeine	2
Carminic acid	4
Colchicine	3
Coomassie blue	6
Fuchsine	3
Gibberellic acid	3
Methyl red	2
Phenylmethylsulfonyl fluoride	1
Salicylic acid	1
8- hydroxyquinoline	2

3.1.2 Osiris Property Explorer

To further explore the physio-chemical and drug-like properties of the test compounds, each compound was screened through the Osiris Property Explorer program. The compounds screened all produced suitable clogP (< 5.00) and only two compounds were identified with solubility values > - 4 (Table 3.3). Therefore, 11 chemical compounds were predicted to possess good absorption and permeability properties (Escobedo-González *et al*., 2017). Good absorption ability of these compounds is complemented by their high TPSA scores. This notwithstanding, many of the compounds were predicted to have toxicity issues and were flagged as potentially mutagenic, tumorigenic and/or irritant (Table 3.3). In addition, the majority of the compounds produced low drug-likeness scores with only four of the compounds (ascorbic acid, caffeine, colchicine, and gibberellic acid) producing positive drug-likeness values. A drug-like score ranges from 0 to 1, where 1 indicates a good drug candidate while indicates a poor drug candidate. Each of the properties shown in Table 3.3 and the molecular weight of each compound (Table 2.1) combined produce the compound's overall drug score. As shown in Table 3.2, the compounds generally produced low overall drug scores (ranging: 0.07 – 0.63) with the exception of ascorbic acid which produced an acceptable score of 0.74.

Table 3.3: Prediction of molecular properties of the test compounds using the Osiris Property Explorer Program

Compound name	**cLogP[a]**	**Solubility[b] (Log S)**	**Drug–-likeness[c]**	**Drug score[d]**	**TPSA[f]**	**Toxicity Risks[e]**
Ammonium bromide	0.0	-0.53	-1.0	0.63	0.0	None
Aniline	0.98	-1.69	-1.98	0.07	26.02	Mutagenic, tumorigenic, Irritant, reproductive effective
Ascorbic acid	-2.46	-0.35	0.02	0.74	107.2	None
Caffeine	-0.18	-1.14	2.59	0.20	58.44	Mutagenic, tumorigenic,

						reproductive effective
Carminic acid	-0.93	-3.32	-3.02	0.14	242.5	Irritant, reproductive effective, mutagenic
Colchicine	1.86	-3.05	1.02	0.42	83.09	Reproductive effective
Coomassie blue	2.28	-6.34	-16.7	0.1	89.85	Reproductive effective
Fuchsine	1.78	-3.40	-2.29	0.18	75.89	Mutagenic, tumorigenic, reproductive effective
Gibberellic acid	-1.25	-0.15	0.47	0.39	125.03	None
Methyl red	3.24	-4.16	-21.42	0.08	65.26	Mutagenic, tumorigenic, reproductive effective
Phenylmethylsulfonly fluoride	1.97	-1.37	-12.22	0.29	42.52	Irritant
Salicylic acid	0.8	-1.33	-1.44	0.13	57.53	Mutagenic, Irritant, reproductive effective
8-hydroxyquinoline	1.63	-2.03	-1.55	0.12	33.12	Mutagenic, tumorigenic, Irritant,

[a]Logarithm of compound's partition of coefficient between n-octanol and water, hydrophilicity measure.
[b]Measure of compound's aqueous solubility

[c]Score of compounds containing predominantly fragments which are frequently present in commercial drugs
[d]Combination of drug-likeness, cLogP, LogS, molecular weight, and toxicity risks.
[e]Toxicity risk predictor of a chemical structure.
[f] Topological polar surface area

The above parameters assist in predicting potential drug candidates from experimental compounds. The chemical structure of a compound effects its physiochemical properties and in turn influences the compound's bioavailability (Mao *et al*., 2016). This prompted the evaluation of a correlation between the aromatic ring number and the drug score produced from the Osiris Property Explorer program. However, as displayed in Figure 3.1, there is no direct correlation to be drawn from the number of aromatic rings possessed by the compound and the drug-score produced by Osiris Property Explorer. It can be concluded from this that, beyond increasing the size of the molecule, the number of aromatic rings does not impact the predicted drug-like score of the compound (at least within this small set of representative compounds).

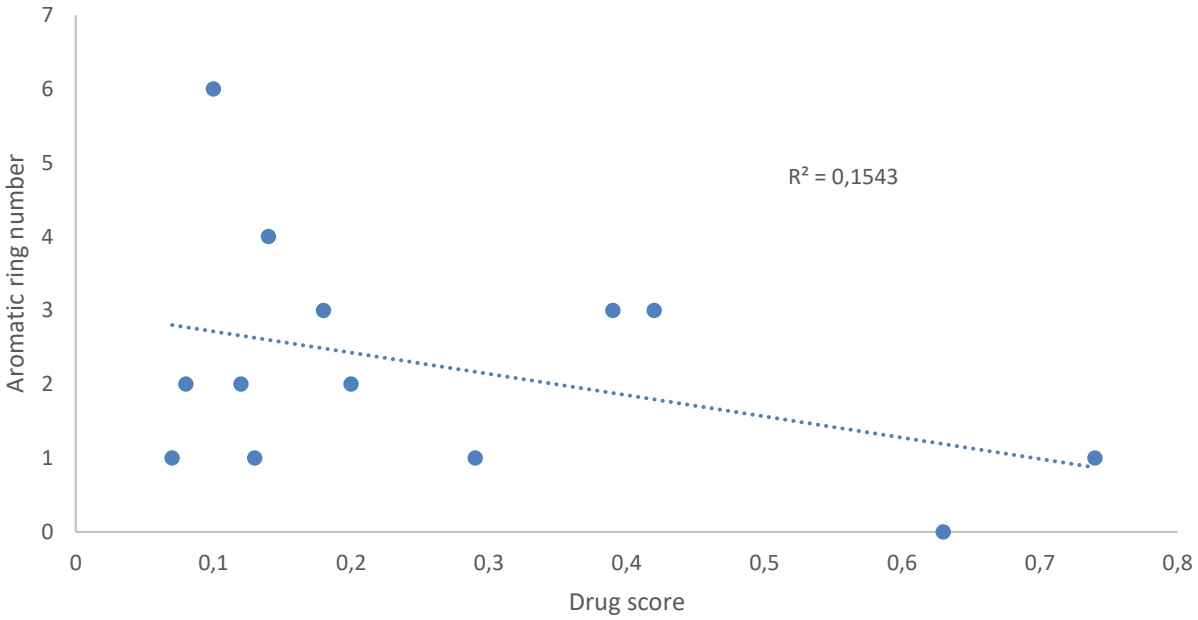


Figure 3.1: The relationship between the aromatic ring number of each compound and their corresponding drug score generated through Osiris Property Explorer. The correlation coefficient value of 0.1543 represents a weak relationship between the aromatic ring count and drug score. There is a significant difference between the aromatic ring count and the chemical compound's drug score (p = 0.05).

Ritchie and Macdonald (2009) pronounced the correlation between aromatic ring count and the lipophilicity of compounds. This thus prompted the evaluation of a correlation of lipophilicity values of chemical compounds, generated through the Osiris Property Explorer program, and aromatic ring count. However, as can be observed from the Figure 3.2 below, there is no direct relationship to be drawn between the aromatic ring counts possessed by chemical compounds and their lipophilicity values as produced by the Osiris Property Explorer program. It can thus be concluded that despite all chemical compounds possessing lipophilicity values less than 5.00, the number of aromatic rings does not directly impact the predicted lipophilicity of the chemical compounds.

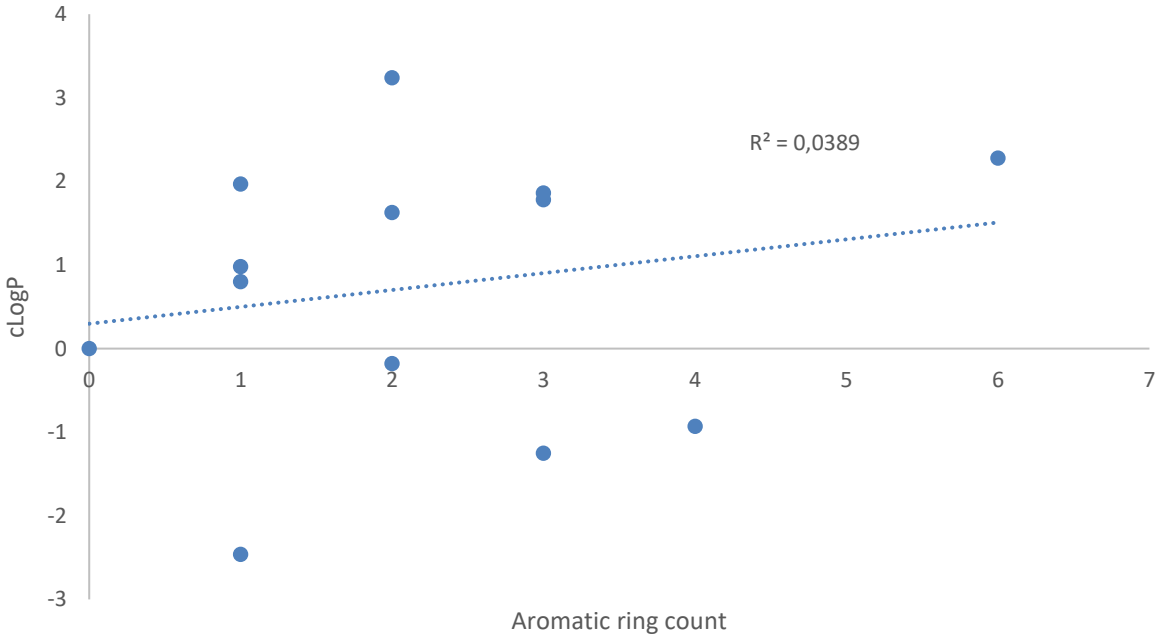


Figure 3.2: Relationship between the lipophilicity of chemical compounds and the aromatic ring count. A weak relationship was observed between lipophilicity and aromatic ring count indicated by the low correlation coefficient value of 0.0389. According to Lipinski's Rule of Five, lipophilicity values of compounds intended for oral administrative should be less than 5.00. There is a significant difference between the aromatic ring count and lipophilicity of chemical compounds (p = 0.05).

Lipophilicity is inversely proportional to solubility, this means that as lipophilicity increases, solubility decrease or vice versa. The above figure displayed a low regression coefficient

value for lipophilicity and aromatic ring count while Figure 3.3 below displayed a positive regression coefficient value of 0.5986 from the correlation between solubility and aromatic ring count. The solubility values were generated through the Osiris Property Explorer program. From the figure below, it can be concluded that increasing the number of aromatic rings in chemical compound structure results in an increased compound solubility. However, the correlation coefficient is lower than 0.95 and thus the relationship between the aromatic ring count and solubility, in this study, is weak.

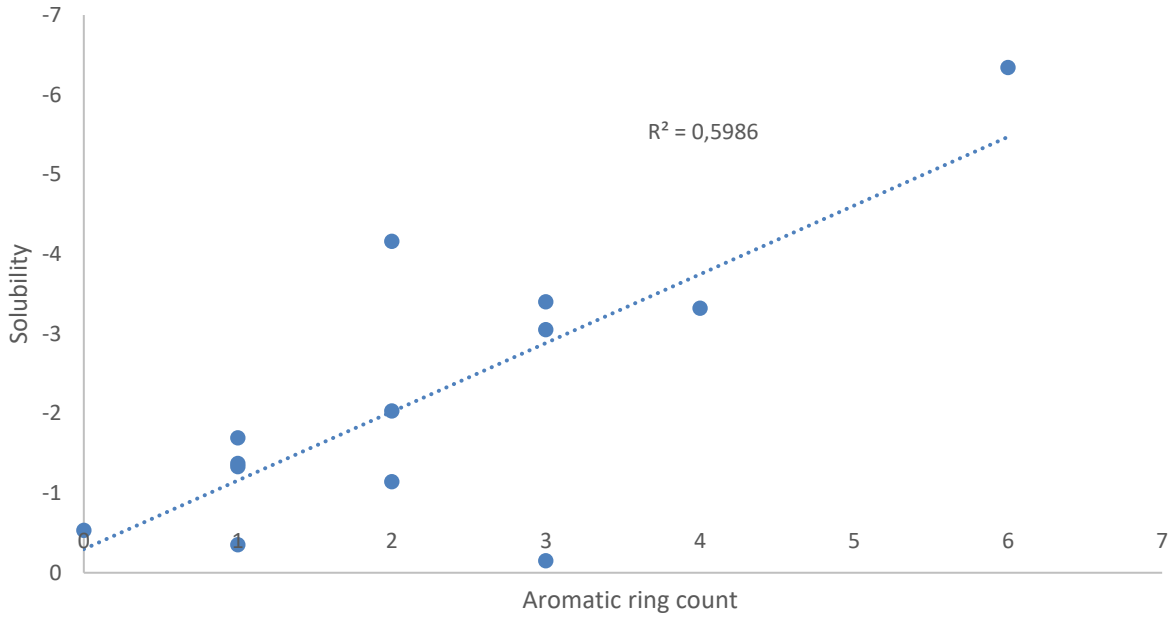


Figure 3.3: Relationship between the aromatic ring count and solubility of chemical compounds. The regression coefficient value of 0.5986 is an indicative of a positive relationship between aromatic ring count and solubility. However, there is a significant difference between the aromatic ring count and solubility of chemical compounds ($p = 0.05$) < 0.95.

Drug-likeness scores were generated from the Osiris Property Explorer program. Drug-likeness, as described above, score compounds against fragments that are similar to those in commercial drugs, and aromatic rings, amongst other properties, is well described for commercial drugs. However, on the contrary, there was no significant correlation between the drug-likeness and aromatic ring count, ($p=0.05$). The correlation coefficient was 0.0908 and this is a clear indication of a lack of a relationship between aromatic ring count and drug-likeness.

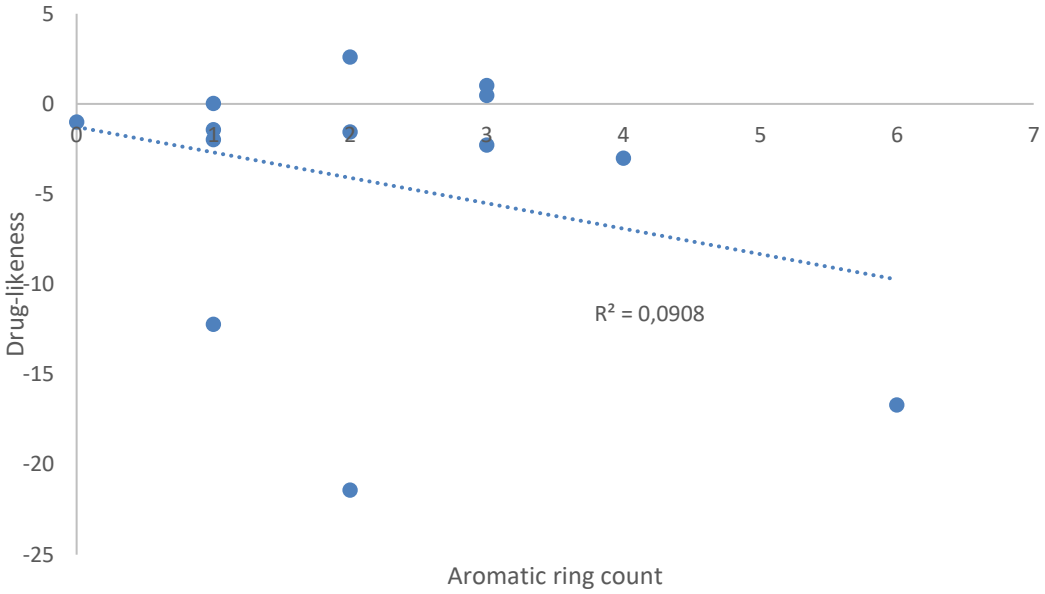


Figure 3.4: Relationship between the aromatic ring count and the drug-likeness score of chemical compounds. The regression coefficient value, 0.0908 indicate a positive relationship between the aromatic ring count and drug-likeness but however, the relationship is weak as it is closer to 0.0 and thus the correlation between the two parameters is not significant. Most points fall below zero and only three points were greater than zero, this means that out 13 compounds, only 3 compounds possess predominantly fragments that are frequently present in commercial drugs.

Topological polar surface area values were generated through the Osiris Property Explorer program and it assist in predicting the compound's drug likeness. Chemical compounds with high TPSA accounts for a poor penetration of compounds in a hydrophobic environment while those with low TPSA are not transported (Fernandes and Gattass, 2009). Chemical compounds possessing high TPSA have values greater than 140 while compounds with low TPSA have values less than or equal to 60. The relationship between the aromatic ring count and the TPSA was explored and the resulting regression coefficient values was 0.3347.

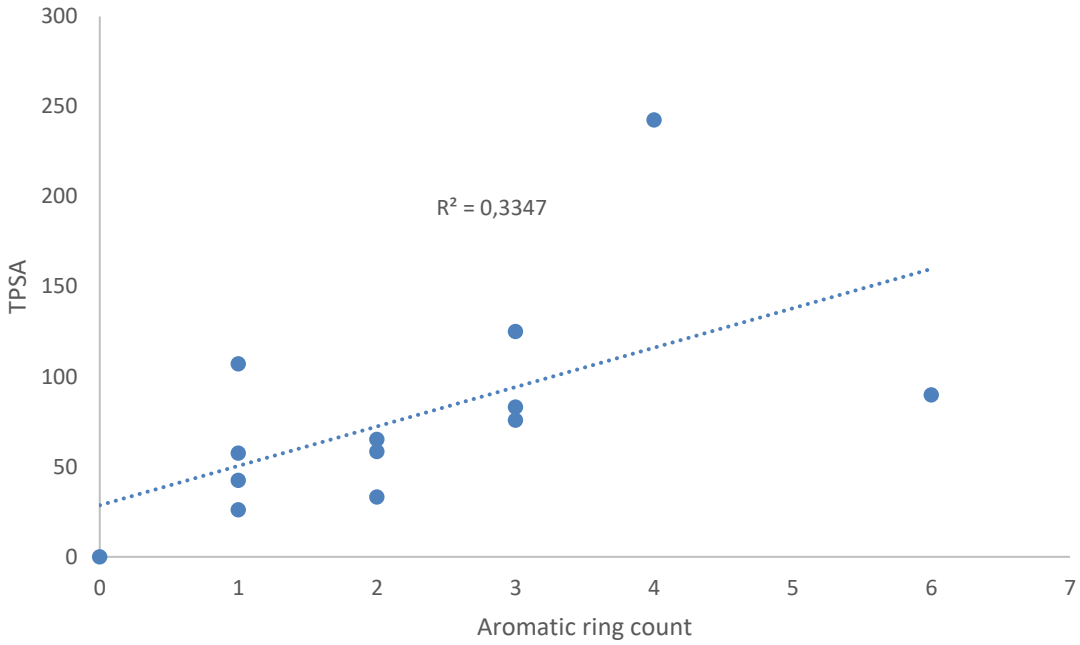


Figure 3.5: Relationship between the aromatic ring count and TPSA score generated through the Osiris Property Explorer. The regression coefficient value of 0.3347 is an indicative of a positive but weak and insignificant correlation between the aromatic ring count and TPSA (p= 0.05) <0.95. Overall TPSA scores were low, with most points falling below 150, ranging from 150 to 45, implying the ease of absorption of the chemical compounds.

3.2 Evaluation of compound permeability

The PAMPA assay is used to predict *in vivo* drug permeability and specifically assess the passive permeability ability of a chemical compound. In the PAMPA assay, a sandwich is formed which consists of a 96 - well donor compartment filled with test compounds dissolved in a buffer solution and a 96 - well acceptor compartment which is filled with a buffer solution only (free of test compounds). The donor compartment is impregnated with an alkane (hexadecane) which forms a rate-limiting artificial membrane barrier where compounds migrate by diffusion. Test compounds are collected through the acceptor compartment, which is infused with the donor compartment and both are separated by a lipophilic microfilter and were analysed through UV / Vis spectrophotometry (Mason, 2009 & Ramlucken, 2014).

Lucifer Yellow was utilized within this study as a membrane integrity marker. To this end, LY was assessed within the PAMPA assay and absorbance values of the donor compartment and acceptor compartment solutions were obtained and utilized to calculate the percentage transmittance (Table 3.4). Similar to a previously published report by Ramlucken (2014), a transmittance value approximating 0 was obtained for LY following replicate analysis (Table 3.4) indicating LY was not able to penetrate through the membrane. This finding was anticipated and in line with numerous other reports that establish LY to be a compound achieving low-to-no permeability across biological and artificial membranes (Stewart, 1978). This finding served to confirm the rigidity of the micro-filter donor compartment and validate the assay for further use.

Table 3.4: Calculated percentage transmittance of Lucifer Yellow across the artificial membrane of the permeability assay.

Donor plate (Absorbance)[a,b]	Acceptor plate (Absorbance)[a,b]	Equilibrium (Absorbance)	% Transmittance[c] ± SD[d]
0.546	-0.001	0.545	-0.002 ± 0.234

[a] The above average values are obtained through replicate analysis (n = 3)
[b] Absorbance readings determined at a wavelength of 427 nm
[c] % Transmittance = Acceptor Abs / Abs Equilibrium * 100
[d] SD = standard deviation

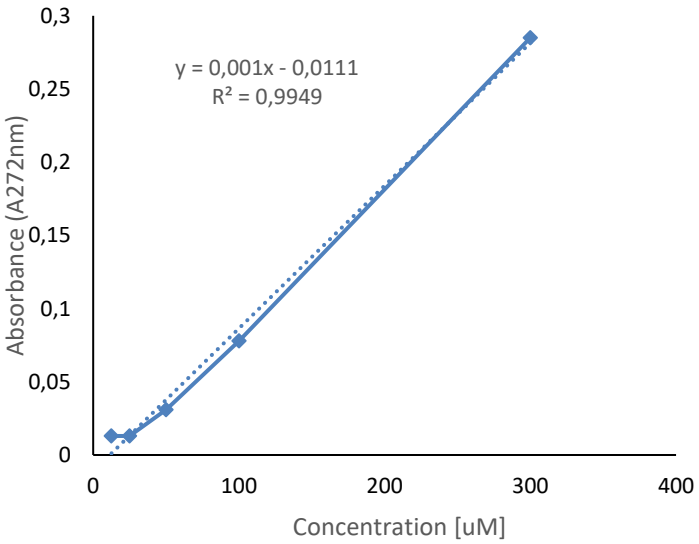
Following validation of the membrane integrity and assay procedure, the thirteen experimental chemical compounds (as described in Table 2.1) were successfully assessed for effective permeability in PAMPA assay. Initially, standard curves were generated for each of the test compounds. Wavelengths specific to each of the test compounds (Table 3.5) were obtained by identifying the absorbance maxima of the compound over a full UV/ VIS spectrum. Using this wavelength, a standard curve for each compound was constructed. Representative curves for caffeine, aniline, colchicine and salicylic are shown in Figure 3.3. Here, the standard curves for each compound were constructed after the determination of their specific wavelengths. With a regression line applied, the straight-line equation was determined and the R^2 values of these curves were determined. Similarly, standard curves were produced for all compounds and, in each case, the R^2 values of ≥ 0.95 were observed.

Table 3.5: The absorbance maxima wavelengths of the compounds used within this study.

Compound name	**Absorbance maxima wavelength (nm)**
Ammonium bromide	269
Aniline	330
Ascorbic acid	264
Caffeine	272
Carminic acid	275
Colchicine	248
Coomassie blue	465
Fuchsine	540
Gibberellic acid	254
Methyl red	450
Phenylmethylsulfonyl fluoride	174
Salicylic acid	295
8-hydroxyquinoline	240

The standard curves were constructed using the compound's specific wavelengths. For caffeine (A) the absorbance maxima wavelength was 272 nm, aniline (B) absorbance maxima wavelength was 330 nm, colchicine (C) absorbance maxima wavelength was 248 nm and salicylic acid (D) absorbance maxima wavelength was 295 nm. The absorbance values were plotted against the compound concentrations produced through serial dilutions. The correlation coefficient (r^2) values were well above 0.95. The standard curves equation of the straight line was used to calculate the unknown chemical compound concentrations.

A



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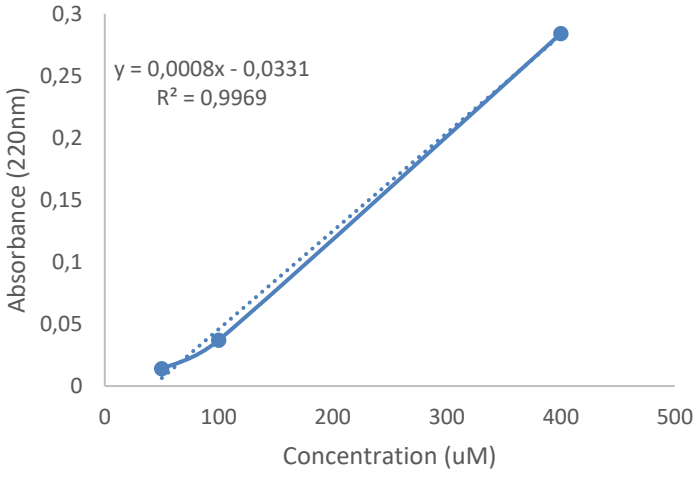
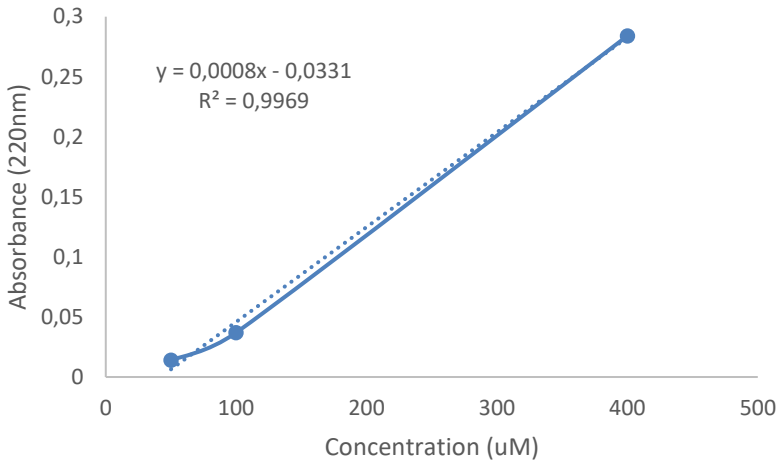


C



D

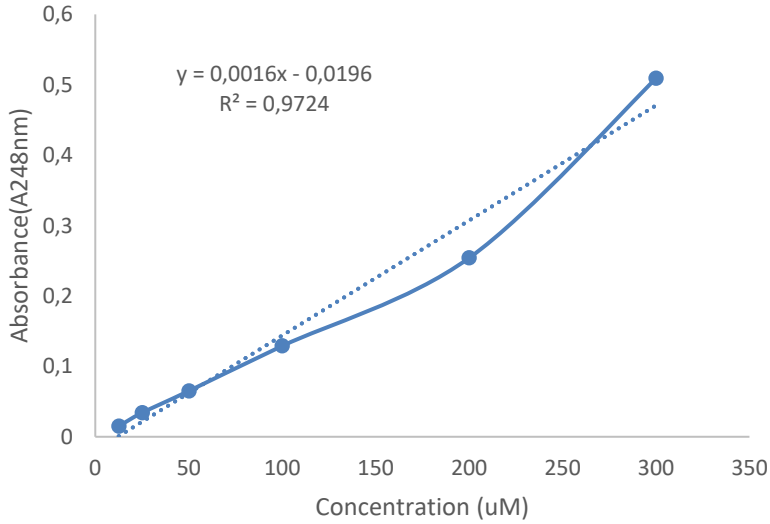


Figure 3.6: Caffeine (A), aniline (B), colchicine (C) and salicylic acid (D) standard curve constructed at different concentrations at specific wavelengths.

The standard curves were constructed through a two-fold serial dilution starting from 10 to 300 µM concentrations against the absorbance of each concentration at their specific wavelengths. The figures were constructed using Microsoft Excel, 2010 and the equation of a straight line, with a regression coefficient greater than 0.95, was used to calculate the unknown concentration of the chemical compounds below for effective permeability calculations.

Adhering to an established protocol (Faller *et al*, 2001), the permeability of each compound was then determined within the PAMPA assay following a 5-hour incubation. The absorbance of each compound in the donor and acceptor compartments was measured and the concentration determined from the standard curves previously constructed. Using the appropriate formulae (described in Section 2.3.2), the LogPe and effective permeability (P_e) values of each compound were calculated and reported in Table 3.6 below.

Table 3.6: The determined LogP_e and effective permeability (P_e) of each chemical compound.

	Compound name	**Log P_e** [a]	**P_e (cm s^{-1})**
1	Ammonium bromide	-4.20	6.31E-05
2	Aniline	-5.30	5.01E-06
3	Ascorbic acid	-6.32	4.79E-07
4	Caffeine	-5.64	2.29E-06
5	Carminic acid	-6.14	7.24E-07
6	Colchicine	-7.85	1.41E-08
7	Coomassie blue	-9.87	1.35E-10
8	Fuchsine	-8.93	1.17E-09
9	Gibberellic acid	-3.87	1.35E-04
10	Methyl red	-9.25	5.62E-10
11	Phenylmethylsulfonyl fluoride	-3.25	5.62E-04
12	Salicylic acid	-4.32	4.78E-05
13	8-hydroxyquinoline	-7.35	4.47E-08

[a] Each value represents the average concentration calculated from replicate experiments (n = 3).

The PAMPA assay is capable of distinguishing between compounds of high, low or moderate permeability through the calculated effective permeability values. Compounds with $LogP_e$ values less than -5.00 are considered highly permeable compounds while compounds with $LogP_e$ values greater than -5.00 are considered to be compounds with lower permeability (Di Li *et al*., 2003). Ammonium bromide, gibberellic acid, salicylic acid, and PMSF yielded $LogP_e$ values higher than -5.00 while the remainder of the compounds produced $LogP_e$ values less than -5.00. Chemical compounds with $LogP_e$ values less than -5.00 possess high permeability while compounds with $LogP_e$ values greater than -5.00 possess low permeability, as reported by Di Li *et al* (2003).

A distinction was observed between natural compounds and synthetic compounds based on the calculated average permeability of each grouping**.** The average permeability of natural compounds combines; ascorbic acid, caffeine, colchicine, gibberellic acid, and salicylic acid, while the average permeability of synthetic compounds combines; aniline, methyl red, fuchsine, PMSF, ammonium bromide, carminic acid. 8-hydroxyquinoline and coomassie blue. The average permeability ($LogP_e$) of natural compounds was -5.60 while the average permeability of synthetic compound group was -6.78. This means that, on average, natural compounds produced an effective permeability which was higher, not significantly ($p = 0.05$), than the effective permeability of the synthetic compounds. On average, both natural and synthetic compounds show reasonably high effective permeability and with natural compounds showing a higher $LogP_e$. A possible relationship between the number of aromatic rings in each chemical structure and the effective permeability was explored and is shown graphically in Figure 3.4. As shown, a positive relationship exists, an upward trend is observed but however, as evidenced through the correlation coefficient value of 0.2268.

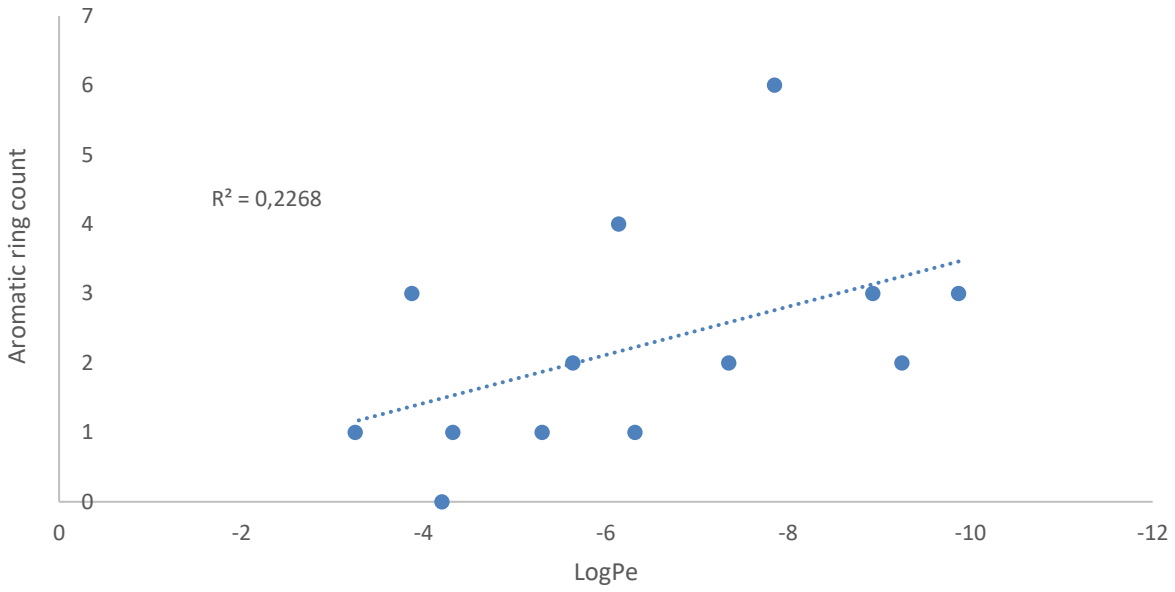
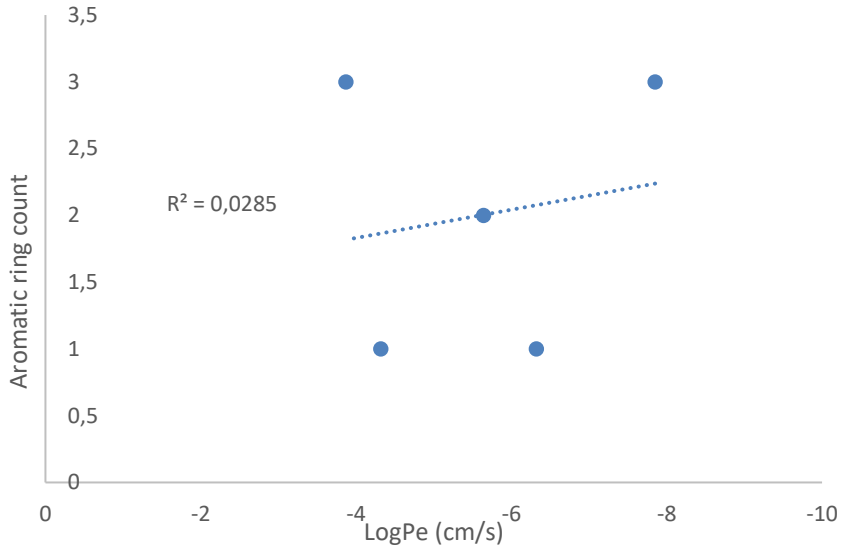


Figure 3.7: The relationship between the aromatic ring count for each test compound and effective permeability. There is a weak relationship between the effective permeability and the number of aromatic rings in each compound. There is no significant correlation between the aromatic ring count and the effective permeability ($p = 0.05$), < 0.95.

The above figure indicates a weak relationship between the number of aromatic rings in each compound and their effective permeability. In this instance, the effective permeability and aromatic ring count were represented for all thirteen chemical compounds (i.e. both natural and synthetic compounds). However, there are noticeable differences between natural compounds and synthetic compounds, the most obvious is their structural make-up. Natural compound structures are more complex than synthetic compound structures, possessing two or more rings than synthetic compounds (Feher and Schmidt, 2003). There is therefore, differences between the aromatic ring count of natural compounds and those of synthetic compounds hence, a relationship between the number of aromatic rings on natural compounds against their effective permeability was explored (Figure 3.8A). Also, a relationship between the synthetic compound effective permeability and the number of aromatic ring count was explored (Figure 3.8B).

A



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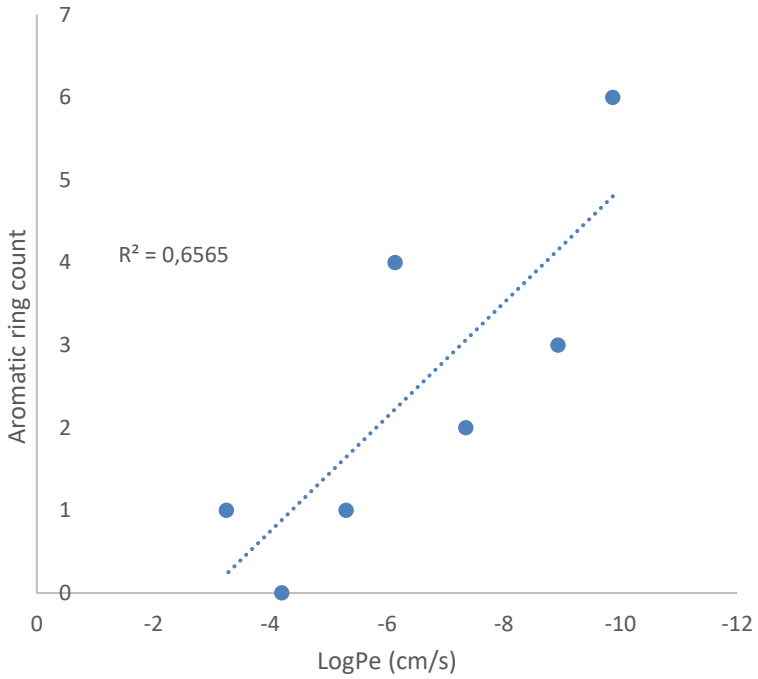


Figure 3.8: Relationship between the effective permeability and the number of aromatic rings for natural compounds (A) and synthetic compounds (B) structure. There is no true relationship between the effective permeability of natural compounds and the number of aromatic rings as indicated by the correlation coefficient of 0.0285 (A). A relationship exists between the effective permeability of synthetic compounds and the number of aromatic rings displaying a correlation coefficient of 0.6565 (B) as indicative of a positive correlation between the two parameters.

The relationship between effective permeability of the chemical compounds and molecular weight was also explored. From the figure below, it was clearly evident that coomassie blue, the compound with the highest molecular weight of the compounds tested, produced the lowest $LogP_e$ value while ammonium bromide, the compound with the lowest molecular weight of the compounds tested, produced one of the highest $LogP_e$ values. However, after plotting the molecular weight of each compound as a factor of its corresponding LogPe, there was no true relationship between these two parameters (Figure 3.9) the correlation coefficient was 0.3983.

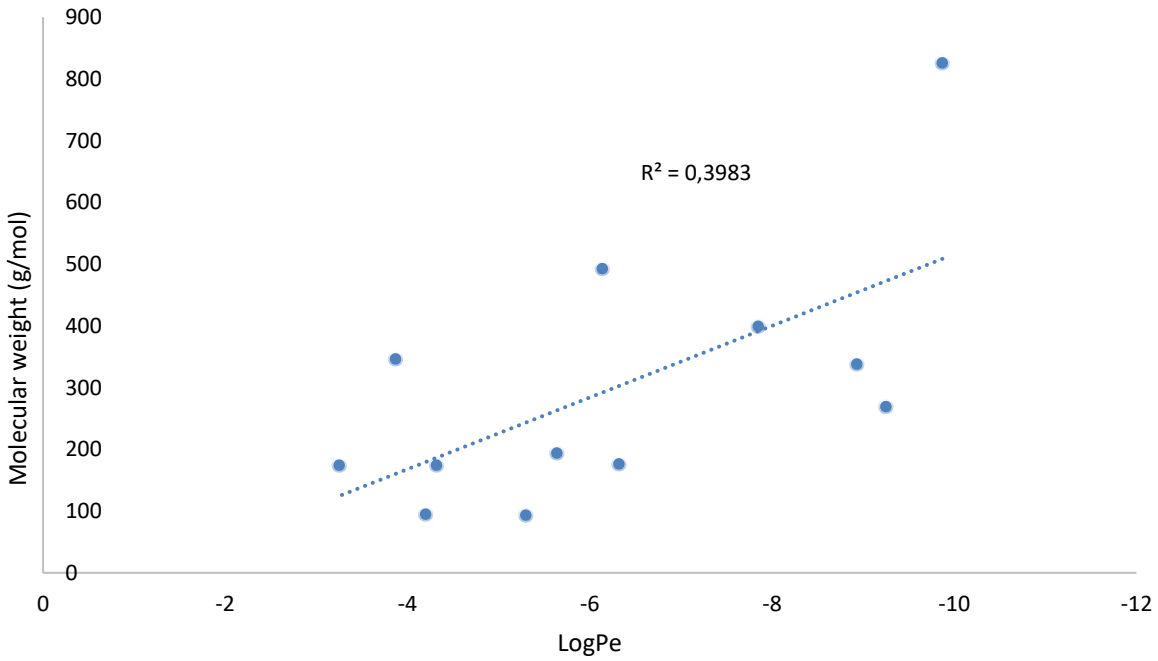
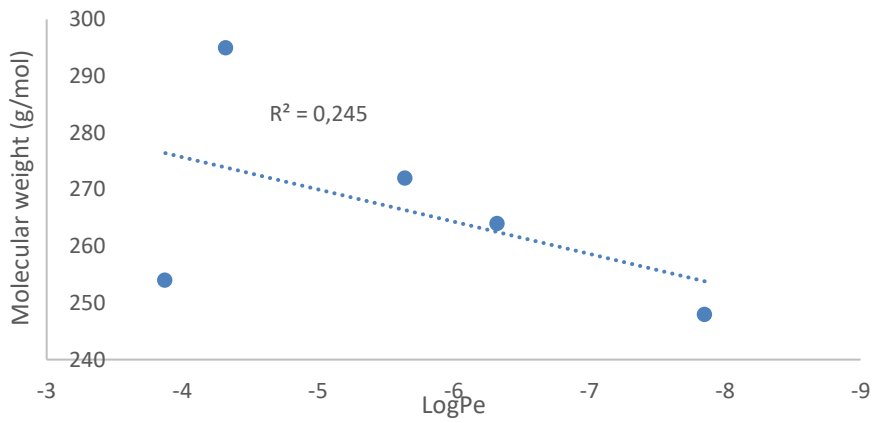


Figure 3.9: Relationship between molecular weight and $LogP_e$ of the experimental compounds. The values in the x-axis represent effective permeability from the test compounds. There is a weak relationship between the molecular weight of compounds and the effective permeability, indicated by a low regression coefficient (R^2 = 0.3983). The effective permeability of compounds is independent of their molecular weights.

The relationship between the molecular weight of natural compounds and the effective permeability (A) was explored. There was a positive correlation between the molecular weight and the effective permeability of natural compounds with the correlation value of 0.245. Also, the relationship between the molecular weight and the effective permeability of synthetic

compounds was explored (B). There was a positive correlation between the molecular weight and the effective permeability of synthetic compounds indicated by the correlation value of 0.7108. The regression value of synthetic compounds was higher than that of natural compounds. The regression value of synthetic compounds suggests that increasing the size of a compound influences its effective permeability. However, the regression value of natural compounds is low, indicating the complexity in natural compounds structures, which is unrelated to the effective permeability.

A



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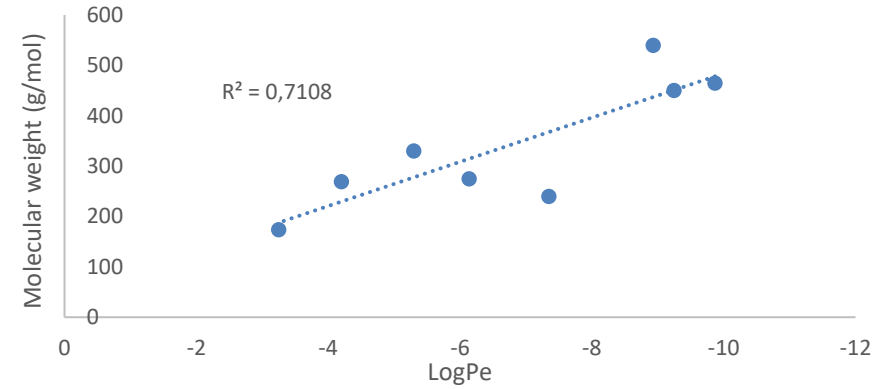


Figure 3.10: The relationship between the effective permeability and molecular weight of natural compounds (A) and synthetic compounds (B). There is a positive correlation between the molecular weight and the $LogP_e$ of both, the natural compounds and synthetic compound group. A relationship between the molecular weight and $LogP_e$ of natural compounds produced a correlation coefficient value of 0.245 which was significantly ($p > 0.05$) lower than the correlation coefficient value of 0.7108 produced from the relationship between the molecular weight and $LogP_e$ of synthetic compounds.

3.2.1 Effective permeability with increased incubation times

To determine whether increased incubation periods would alter the effective permeability of the compounds, the assay optimized and described above was adapted to include five distinct incubation periods of 4, 5, 11, 24 and 72 hours. For the purpose of this experiment, the compounds salicylic acid, caffeine, and colchicine were utilized to represent chemical compounds with 1, 2 and multiple aromatic rings respectively. The $LogP_e$ was calculated for each of the three compounds following replicate analysis at the five different time points. As depicted in Table 3.7, altering the incubation time did not increase the effective permeability of the three test compounds in any noticeable or significant ($p > 0.05$). These findings contradict studies by Faller *et al*., 2001 and Sugano *et al*., 2010 which suggest that increased incubation periods may increase the determined effective permeability of a test compound. Furthermore, these results clearly demonstrate that altered incubation times did not offer any selective advantage to the compounds based on the number of rings present in the structure.

Table 3.7: Evaluated average $LogP_e$ for varying incubation time of three different chemical compounds.

Time (hour)	**Average $LogP_e$ (cm/s)[a]**		
	Caffeine	Salicylic acid	Colchicine
4	-5.82	-4.45	-7.85
5	-5.64	-4.32	-7.85
11	-5.96	-4.01	-7.85
24	-6.13	-4.89	-7.91
72	-5.75	-4.21	-7.73
Average	-5,86	-4,376	-7,838
SD[b]	0,190	0,330	0,066

[a] Values determined as the average of replicate experiments (n=3)
[b] SD = Standard deviation

3.3 Percentage plasm-protein binding of experimental compounds

Percentage protein binding typically refers to the degree at which the test compound binds to a serum protein (typically serum albumin) and is calculated by deducing the amount of compound bound to protein in relation to the unbound fraction of the compound (Barre *et al.,* 1984). In this experiment, PPB was determined using a micro spin filter cartridge with a MWCO of 30 kDa. As such, samples less than 30 kDa were sieved through the filter unit and those larger than 30 kDa were retained above the membrane. The protein used in this experiment, which was BSA with a MW of 66.5 kDa, was unable to sieve through the filter unit. Compounds which bound to the protein were trapped together with protein in the upper compartment of the filter unit which enabled the percentage protein-binding of the compound to be determined. Using this method, the percentage protein binding of the 13 compounds was determined and reported in Table 3.8. The results indicate a broad range of protein binding for the 13 chemical compounds tested (70.29 – 98.23%). The results displayed for percentage protein –binding are, overall, high values and thus small fractions of compounds will reach the target for therapeutic activity, in the framework of this study.

Table 3.8: Evaluation of the percentage protein binding of the test chemical compounds.

Chemical compound	**Protein-binding (%)[a]**
Ammonium bromide	75.35
Aniline	92.68
Ascorbic acid	85.25
Caffeine	80.42
Carminic acid	73.25
Colchicine	73.28
Coomassie blue	71.57
Fuchsine	98.23
Gibberellic acid	78.32
Methyl red	70.29

Phenylmethylsulfonyl fluoride	90.15
Salicylic acid	90.62
8-hydroxyquinone	93.84

[a] Percentage protein-binding calculated from replicate experiments (n≥3)

As indicated in Table 3.8 above, the percentage protein-binding for all thirteen compounds was, in overall, high. An average percentage protein-binding was calculated for both natural compounds and synthetic compounds and the results are displayed in the below figure. The average percentage protein-binding for natural compounds was 67.98% while the average percentage protein-binding for synthetic compounds was calculate at 85.01%. Natural compounds have 32.02 % on average, of compound fraction unbound while synthetic compounds have 16.99% on average, of compound fraction unbound. This means there will be more of natural compounds available for therapeutic activity than there is of synthetic compounds.

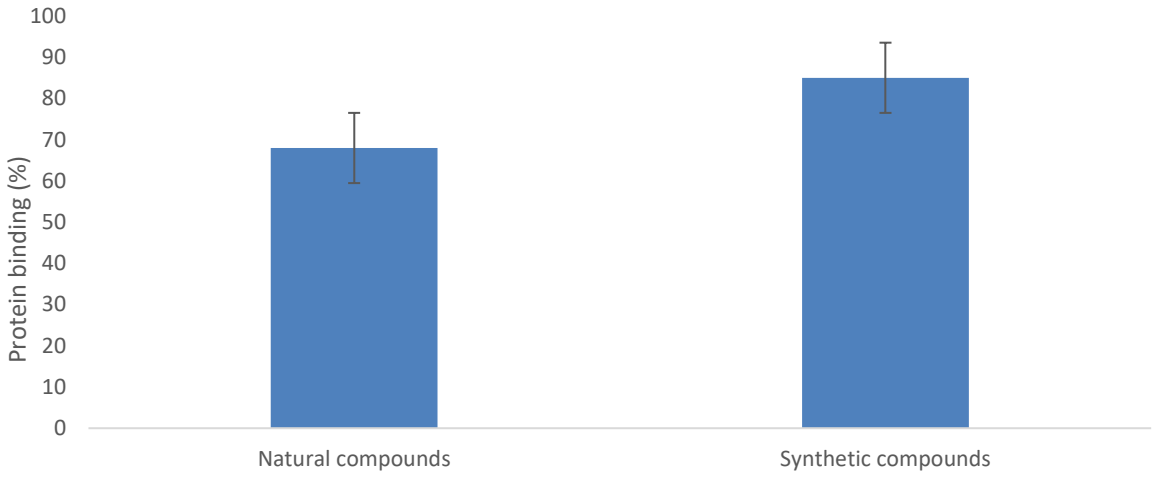


Figure 3.11: The difference between natural compounds and synthetic compounds based on their percentage protein binding. The above is the calculated average percentage protein-binding for a group of natural compounds and a group of synthetic compounds. There was a difference between natural compounds and synthetic compounds in terms of their average percentage protein-binding. Natural compounds displayed a lower average percentage protein-binding (67.98%) whilst the synthetic compounds displayed a higher average percentage protein-binding (85.01%).

The relationship between the aromatic ring count and percentage protein-binding was explored and the results displayed in Figure 3.12 below. There was a positive correlation between the aromatic ring count and percentage protein-binding with the regression value of 0.1628. There was no true relationship between the percentage protein-binding of chemical compounds and their aromatic ring count, suggesting that an increased in aromatic rings of a chemical compound structure does not affect its protein binding ability.

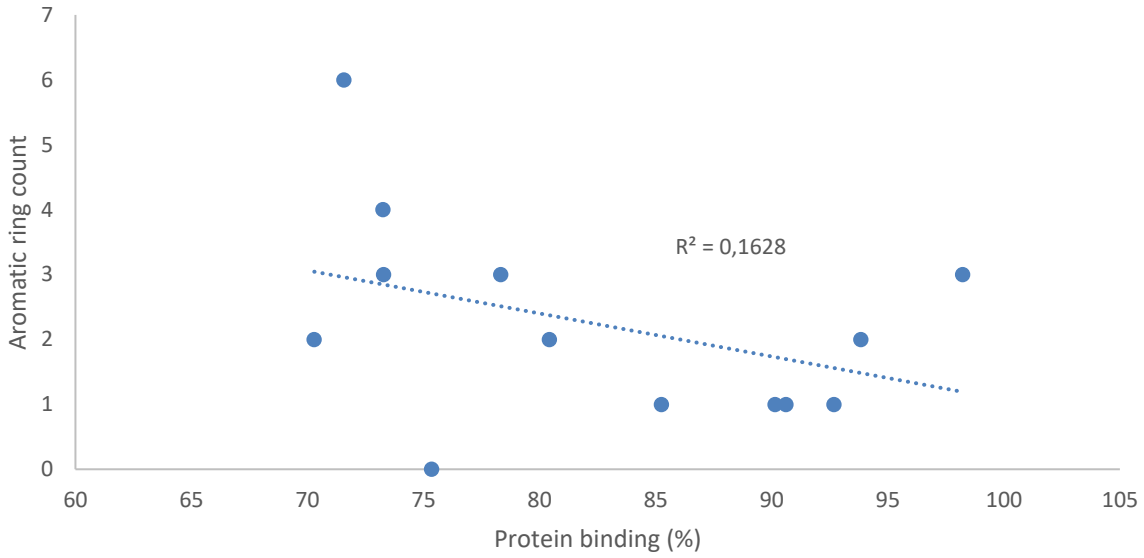
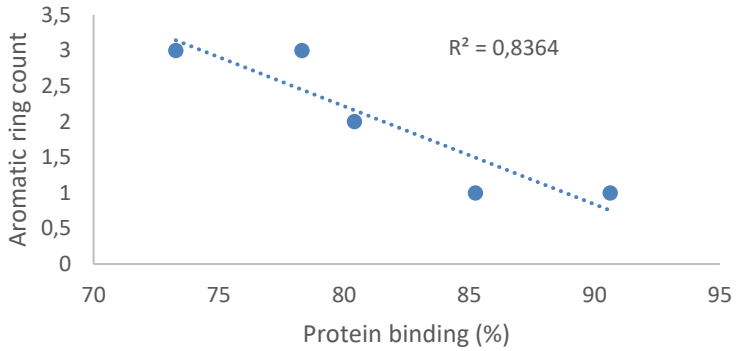


Figure 3.12: The percentage protein binding of the test compounds against the number of aromatic rings in each chemical compound structure. The low regression coefficient (R^2 = 0.1628) above, is indicative of a weak relationship between the protein binding of compounds and the number of aromatic rings in each chemical compound. The weak relationship implies that each parameter is independent of each other.

As observed in Figure 3.11, there is a difference between natural compounds and synthetic compounds in terms of their contribution in percentage protein-binding. The average percentage protein-binding values are 67.98% and 85.01% for a set of natural compounds and synthetic compounds (ascorbic acid, caffeine, colchicine, gibberellic acid, and salicylic acid) and (ammonium bromide, aniline, carminic acid, methyl red, fuchsine, PMSF and 8-hydroxyquinoline) respectively. A relationship between aromatic ring number and the percentage protein-binding of natural compounds (Figure 3.13A), and a relationship between

the aromatic ring number and percentage protein-binding of synthetic compounds was explored and the findings are displayed below (Figure 3.13B). There were noticeable and significant ($p > 0.05$) differences between the correlation coefficient of the natural compounds and that of the synthetic compounds; natural compounds displayed a high regression coefficient value of 0.8364 whereas synthetic compounds displayed a low regression coefficient value of 0.1717.

A



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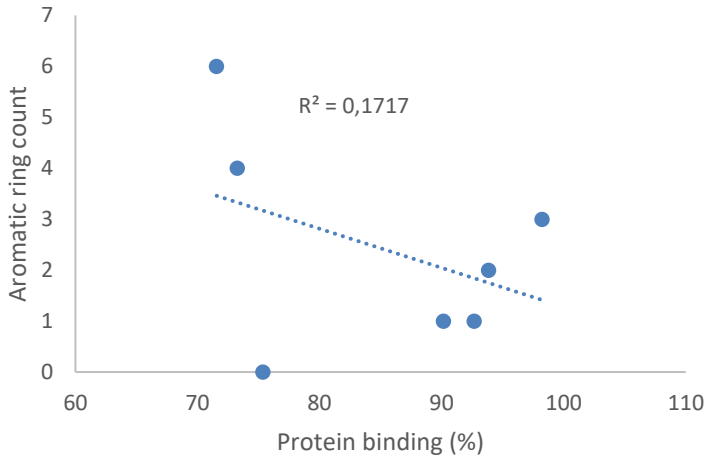

Figure 3.13: Relationship between the percentage protein-binding and the number of aromatic rings for natural compounds (A) and synthetic compounds (B). A true relationship between percentage protein-binding and aromatic ring number on a group of natural compounds, with a correlation coefficient of 0.8364 whilst synthetic compounds displayed a weak relationship between the aromatic ring count and percentage protein-binding.

The relationship between the chemical compound molecular weight and percentage protein-binding was explored and results displayed in Figure 3.14 below. The correlation coefficient value displayed on the figure indicates a positive relationship but however, a relationship between the two parameters is weak. The findings in this study suggest that increasing the size of a molecule does not possess major effect on its binding ability.

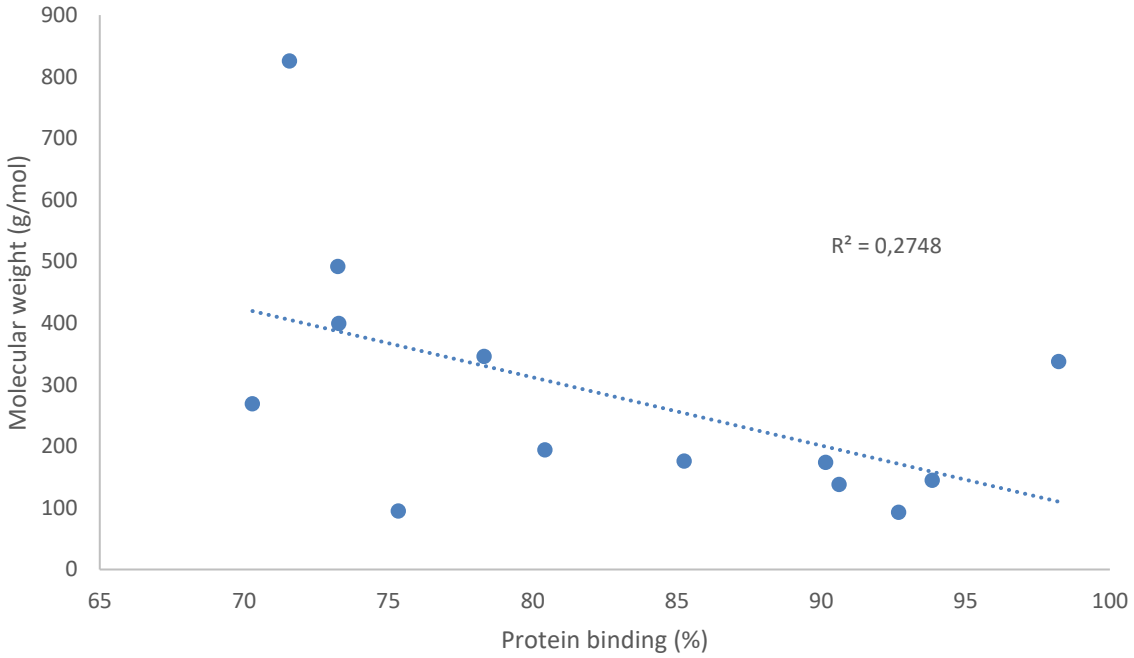

Figure 3.14: Relationship between the percentage protein-binding and molecular weight. Percentage protein-binding for all test compounds was above 60% as indicated here hence all points congested in a range 60-100 %. Even so, there was a weak relationship between molecular weight and percentage protein binding observed, this is indicated by the correlation coefficient value 0.2748. Each of the two parameters is independent of the other. There is an upward trend but this is just a trend rather than a statistically validated correlation.

For further evaluation, the effect of protein binding on the compounds effective permeability was evaluated by incubating each compound with BSA prior to application to the PAMPA assay. The introduction of BSA reduced the amount of compound available for diffusion through the membrane and, in all cases, significantly ($p < 0.05$) reduced the effective permeability values as indicated by the concentration values below.

Table 3.9: Evaluated percentage protein-binding and the effective permeability through the usage of PAMPA plate.

Compound	Donor Concentration (µM)	Acceptor Concentration (µM)	Percentage protein binding (%)	$LogP_e$	P_e
[1]Warfarin	122.12	59.42	49	-5.03	9.33×10^{-6}
[2]Warfarin + BSA	287.22	108.02	38	-5.13	7.41×10^{-6}
[3]BSA + Warfarin	330.52	79.82	24	-5.30	5.01×10^{-6}

As can be observed above, the percentage protein-binding of [1]Warfarin was 49%, and the complex of both the [3]BSA and Warfarin was half, 24%.

[2]Warfarin + BSA, was Warfarin on the Donor compartment and BSA on the Acceptor compartment as a protein bound Warfarin, at equilibrium, should be equal the amount of free Warfarin, the unbound. The results above suggest that this was true as Warfarin bound to BSA was 108.02 µM which was close to the amount of free Warfarin, 122.12 µM.

3.4 Evaluation of cytotoxicity of caffeine, warfarin and auranofin

The cytotoxicity of caffeine, warfarin and auranofin in HEK293-T cells was tested and CC_{50} values were determined. Overall, these three compounds (caffeine, warfarin and auranofin) did demonstrate cytotoxicity ($CC_{50} \leq 10$ µM) within the range evaluated. The dose-response sigmoidal curves of caffeine, warfarin and auranofin was generated and is demonstrated in Figure 3.15. The CC_{50} values were 1.619µM ± 0.195, 1.679µM ± 0.238 and 0.218µM ± 0.135

for caffeine, warfarin and auranofin respectively. The CC_{50} of caffeine and warfarin was approximately equal; and auranofin CC_{50} was approximately 2-fold more cytotoxic than both caffeine and warfarin. The CC_{50} values vary according to the values on the Y- axis, in instances where Y- values just decrease or increase, the curve may appear to fit well but the CC_{50} values can be wildly wrong.

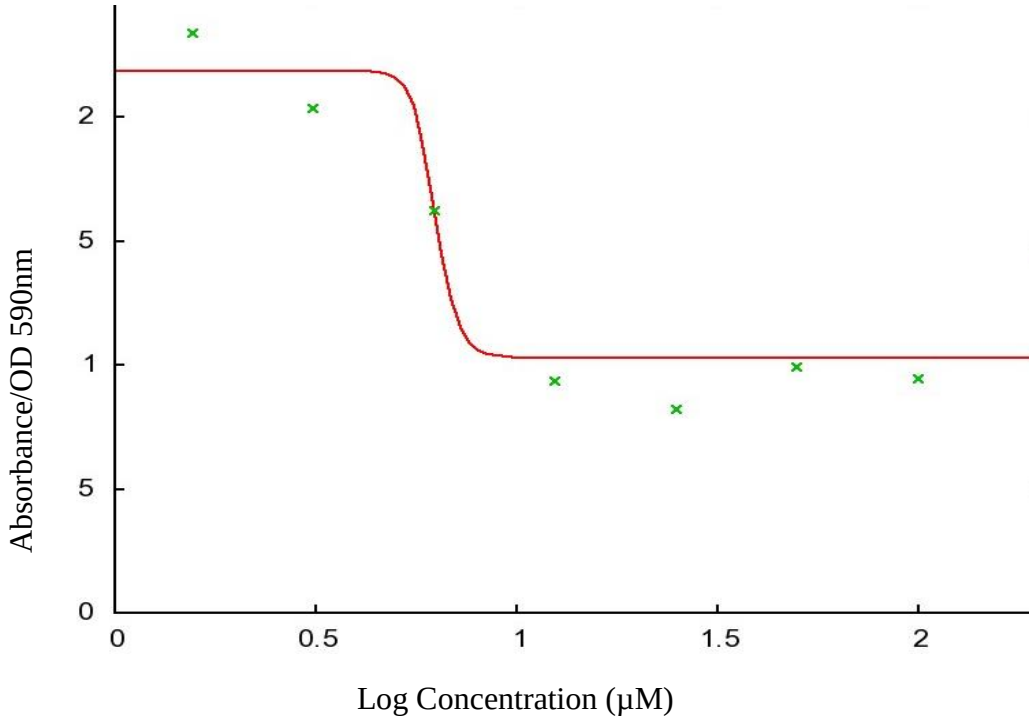

Figure 3.15: A representative graph that demonstrate the relationship between HEK-293T cell viability and warfarin concentration. The viability of cells was quantified through and MTT assay where the absorbance of the reduced formazan product was read at 590nm for all three compounds. The figure was generated through the online Origin 8.1 program. The program generates a dose-response plot and fit it to a curve to give the mid-point ligand concentration or the CC_{50}. The sigmoidal curve displays the concentration at which cell viability is reduced by 50%.

To determine whether the aromatic ring number have an effect on the chemical cytotoxicity ability, a relationship between the aromatic ring count and cytotoxicity concentrations of caffeine, warfarin and auranofin was explored. The concentration of a chemical compound is directly proportional to their cytotoxicity and the bigger the molecule/ compound the higher the cytotoxicity effect. The correlation coefficient of a relationship between the aromatic ring

number and cytotoxicity was 0.7807 implicating a positive relationship, though the R^2 value was below 0.95, there is a true relationship between the aromatic ring number and cytotoxicity.

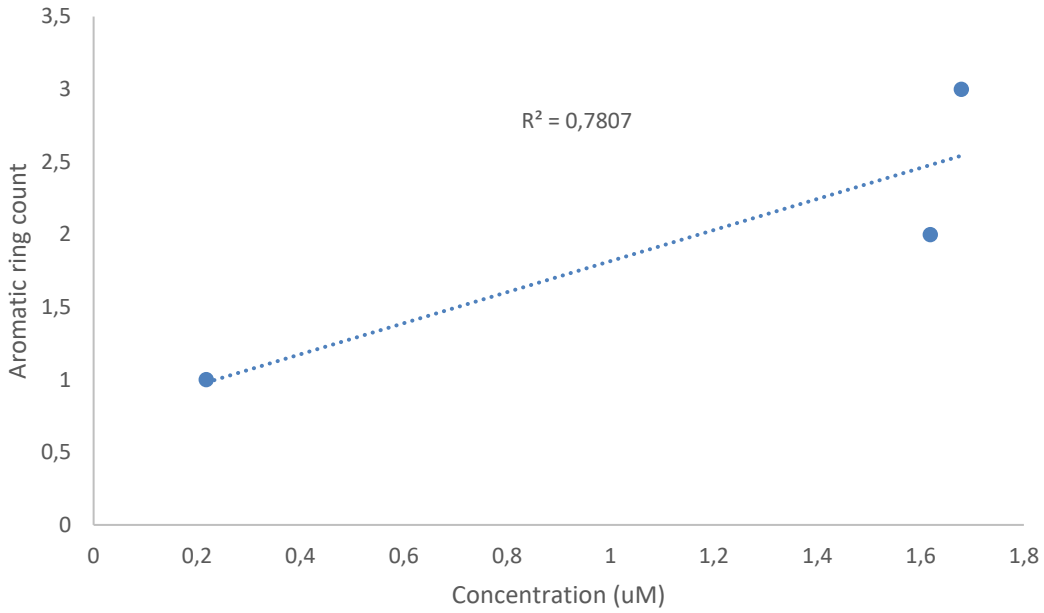

Figure 3.16: The relationship between the aromatic ring number and the caffeine, warfarin and auranofin cytotoxicity concentration- response. Caffeine cytotoxicity was 1.619, warfarin was 1.679 and auranofin was 0.218, and as indicated in the figure; the higher the aromatic ring number the higher the cytotoxicity concentration. This correlation implies that there might be a negative effect aromatic ring number has on the chemical compound cytotoxicity ability.

CHAPTER 4: DISCUSSION

The increasing need for the production of novel drugs against new and existing diseases has propagated investigations to identify novel therapeutics from chemical compounds of different nature. This study focuses on assessing properties that affect the bioavailability of natural and synthetic compounds, more specifically the effect of aromatic ring number on the bioavailability of these compounds. Thirteen compounds were screened for a potential correlation between the aromatic ring count and bioavailability. The chemical compound wavelengths and standard curves were constructed using a spectrophotometer, and NanoDrop confirmed that the wavelengths were in correlation with the wavelengths described in the literature. Overall, 13 chemical compounds were identified and screened for permeability, protein binding and the aromatic ring count. The ability of a compound to reach its target (permeability) and the degree at which the compound reaches the target (bioavailability) is important because it explains the compound's bioactive ability. The PAMPA assay was used in predicting the chemical compound's passive permeability. Transcellular passive permeability is the most commonly used pathway for orally administered drugs (Pappenheimer and Reiss, 1987). Protein binding experiments were undertaken through ultrafiltration using the Amicon Ultra- 15 Centrifugal filter unit as described by Barre *et al.*, 1984. The PAMP technique was applied once again for percentage protein binding further analysis. The toxicity studies were performed through the cell culture of HEK293-T mammalian cells. The rest of the properties were generated through the Osiris Property Explorer program.

4.1 *In silico* prediction of chemical compounds drug-likeness

Through the analysis of the 13 chemical compounds according to the Lipinski RO5, carminic acid and coomassie blue do not entirely adhere to the rule-of-five, since the hydrogen bond donors of carminic acid was < 5, and coomassie blue with molecular weight > 500 Da. This was not expected as both the carminic acid and coomassie blue are synthetic compounds and therefore are expected to stringently compile to the Lipinski RO5. Natural products, on the other hand, do not usually adhere to Lipinski RO5, but in this study they all scored 4/4 meaning they did comply with the Lipinski RO5. The cLogP values of all 13 chemical compounds predicted were ideal as they complied with Lipinski RO5 which states that the cLogP must be less than

5. As stated before, high solubility of a compound is usually an indicative of good absorption. According to the Osiris Property Explorer predictions (Table 3.3), all 13 chemical compounds were highly soluble and therefore are well absorbed. However, the overall drugs-core (includes cLogP, aqueous solubility, MW and overall drug-likeness) predicted from the Osiris Property Explorer was low, with 10 chemical compounds displaying different toxicity effects. Furthermore, Lipinski RO5 demonstrated that 11 of 13 experimental compounds are potential drugs as they adhered to all four rules. The disparity between the solubility property and drug-likeness of the chemical compounds obtained from Osiris Property Explorer and the Lipinski RO5 may be due to the prediction models used. Osiris Property Explorer uses the increment system that is based solely on atom type contributions and it includes compounds that are within the 99% and 95% confidence ellipse (Abrahams, 2014). Well-absorbed compounds decrease outside the 95% ellipse and so the Osiris Property Explorer excludes these compounds.

4.2 The influence of aromatic ring count on predicted bioavailability

The chemical structure of a compound influences its physiochemical properties which, in turn, influences its bioavailability (Di and Kerns, 2015). There are different physiochemical properties that influence the compound's bioavailability and these include ADME/T properties and MW, lipophilicity, aqueous solubility, acid-based ionization constant (pKa), number of hydrogen bond donors and acceptors, rotatable bonds (ROT), number of aromatic rings, polar surface area (PSA) and acid/base properties. Recent studies have demonstrated that the number of aromatic rings influences chemical compound's developability and, in turn, bioavailability (Mao *et al*., 2016 & Ritchie *et al*., 2011). On average, the number of aromatic rings of compounds in preclinical trials is reported to be 3.3 but the aromatic ring number decreases as compounds lure closer to the market (Lipinski, 2000). In 2009, Ritchie and Macdonald, analysed the influence of aromatic ring number in chemical compounds against aqueous solubility, lipophilicity and serum albumin binding and concluded that chemical compounds containing fewer aromatic rings are better potential drug candidates than compounds containing more than three aromatic rings (Ward *et al*., 2014). Ritchie and colleagues demonstrated that an addition of aromatic heterocycles have less effect on the compound's lipophilicity than the addition of aromatic carbon-containing rings does, and that the aromatic heterocycles increases

the TPSA of a compound and potentially decrease the oral absorption and cell penetration (permeability).

4.2.1 Osiris Property Explorer properties

The relationship between the aromatic ring number and lipophilicity was explored and the results presented in Figure 3.2 suggest that there is no true relationship between the aromatic ring count and lipophilicity. There was a significant difference ($p > 0.005$) between the aromatic ring count and lipophilicity with a regression coefficient value of 0.0389 which clearly indicate a lack of correlation. The aqueous solubility of a compound plays a crucial role in its bioavailability (Jorgensen and Duffy, 2002) and therefore an investigation of the influence of aromatic ring number on solubility was incorporated. Solubility is inversely proportional to lipophilicity (Ran and Yalkowsky, 2001), and therefore an expectation would be that the relationship between the aromatic ring number and solubility will show an increased positive relationship, in correlation with a lipophilicity regression value. As observed in Figure 3.3, a positive correlation between the aromatic ring number and solubility displayed a regression coefficient value of 0.5986 which is approximately 50% greater than the regression coefficient value of the correlation between the lipophilicity and aromatic ring count. This means that increasing the aromatic ring number leads to an increased solubility but does not influence lipophilicity. Two chemical compounds, coomassie blue and carminic acid, contain aromatic rings greater than 3 (Table 3.2). Coomassie blue contains 6 aromatic rings, and this is above the aromatic ring count threshold (< 3), and on the contrary it displayed a solubility value greater than -4, which is an indicative of a poorly soluble compound. However, looking at carminic acid which contains 4 aromatic rings, one ring extra to the threshold and it displayed solubility value (-3.32) close to -4. It may be resolved that adding one extra aromatic ring does not have a major effect on the solubility of a chemical compound, and in support of this, Ritchie and Macdonald stated that adding two or more aromatic rings decreases the solubility. Coomassie blue has 3 more (< 3) aromatic rings and that is why it displayed a poor solubility.

As high as the aromatic ring number of coomassie blue was, it did not influence its lipophilicity (2.28) as it fell well below 5.00. Thus, increasing the aromatic ring number by 2 or more appears to be affecting aqueous solubility, and the lipophilicity remains relatively low or constant. In support of this statement, ammonium bromide does not have any aromatic rings and it displayed a lipophilicity value of 0 and is well absorbed. A similar trend was described by Lamanna and

co-workers, where they discarded insoluble compounds based on the compound's molecular weight and increased aromatic ring number (Lamanna *et al*., 2008). Aqueous solubility is important on the permeability and oral absorption of a potential drug candidate (Lipinski, 2004), and if a compound in lead optimization stage possesses poor solubility, decreasing the number of aromatic rings may be beneficiary (Ritchie and Macdonald, 2009). As a structural feature, adding an aromatic ring increases ligand-binding energy and consequently increases a compound's potency and so in development, medicinal and pharmaceutical chemist will seek to add an aromatic ring in an attempt to increase compound's potency. However, in the context of orally administered drugs, adding a single aromatic ring increases the molecular weight by 78 and in turn increases the lipophilicity by 2.14 units (Ritchie and Macdonald, 2009) and thus makes a compound toxic.

Recent analysis has demonstrated that the mean values of lipophilicity, H-bond donors and total polar surface area (TPSA) between older and newer oral drugs are not changing (Leeson and Davis 2004), and this proves that these parameters (lipophilicity, H-bond donors and TPSA) are fundamental drug discovery functions and alteration of these parameters has been reported to caused an increase in the rate of attrition owing to safety findings (Reitchert, 2003). Chemical compounds with TPSA scores greater than 140 A^2 are not easily absorbed while those with TPSA scores $\leq$ 60 A^2 are easily absorbed (Palm *et al*., 1997). The findings in Table 3.3 demonstrate that 12 out of 13 chemical compounds in this study have TPSA scores of less than 140 A^2, with aniline, caffeine, PMSF, salicylic acid, 8- hydroxyquinoline and ammonium bromide scoring less than 60 A^2 as an indication of a high capacity of penetrating cell membranes. Carminic acid however, displayed a TPSA score of 242.5 A^2 which is greater than 140 A^2, demonstrating a low capacity of penetrating cell membranes and may lead to high attrition rate. The reason for high TPSA score of carminic acid might be explained through Ritchie and colleagues' study which states that an addition of an aromatic heterocycle leads to an increased TPSA scores and carminic acid possesses 4 aromatic rings which is greater than the threshold. When interrogating the relationship between the aromatic ring number and the TPSA scores (Figure 3.5), an opposite was observed, the correlation coefficient was 0.3347 indicating a weak and insignificant ($p= 0.05$) correlation between the TPSA scores and aromatic ring number. This means that, regardless of increasing or decreasing the aromatic ring number of a compound, polar compounds may still be able to cross through the lipid bilayer.

4.2.2 Permeability

The chemical compounds used in this study all have different physiochemical properties, for some compounds, their physiochemical properties are well described in the literature. The effective permeability, as a physiochemical property, of different synthetic compounds is described in literature and that includes some of the synthetic compounds in this study. The effective permeability of most natural compounds, on the hand, is not well defined in literature.

Control compounds used in this study were caffeine and lucifer yellow, and this was because these two compounds fall into the 20 model drugs suggested by the FDA to use in evaluating the permeability assay's appropriateness (Benet *et al*., 2008). These compounds have been used in different studies as positive and negative controls to validate the permeability system. The advantage in using these two compounds is that they are commercially available, and are widely used because of their merit in passive paracellular and transcellular permeability characteristics. Both control compounds displayed expected behaviour thereby validating the PAMPA system. The results obtained from caffeine were conclusive, the effective permeability was -5.64, and a similar value was obtained from the study by Li Di *et al*, 2003 and Mensch *et al*, 2010. The overall effective permeability results obtained from 13 chemical compounds in this study, indicated that most of these chemical compounds did exhibit promising transcellular passive diffusion properties, with an exception of phenylmethylsulfonyl, salicylic acid, gibberellic acid and ammonium bromide, which did not display encouraging transcellular passive diffusion properties. The $LogP_e$ values calculated for most of the chemical compounds were well above the threshold (-5.00), which is an indication of compounds with high effective permeability. Interestingly, it must be noted that salicylic acid, a hormone found in a plant and widely used as a medication for skin infections, exhibited a slightly poor permeability (-4.32). It must be noted, however, that salicylic acid is a natural compound, and as described in the above section (Section 1.3), most natural compounds violate most rules of potential drug candidates but do possess pharmacological activity. This is supported by a second natural compound, gibberellic acid, which is also a hormone found in plants but exhibited a poor permeability (-3.87).

The permeability of a drug is usually influenced by multiple factors, one of which is the number of aromatic rings on a chemical compound structure. Thus, a relationship between the effective permeability and aromatic ring count was investigated, and the results in Figure 3.4 indicates a weak correlation between the two parameters, with R^2 of 0.22, ($R^2 < 0.5$). Coomassie blue is widely used in biochemical studies, mostly for protein staining, and methyl red in microbiological studies for bacterial staining and as expected, both displayed a relatively high effective permeability. Coomassie blue has the highest number of aromatic rings (6) and is a synthetic compound and this prompted an investigation of a possible correlation between the aromatic ring numbers of synthetic compounds against their specific effective permeability. It is not surprising that there was a positive relationship between synthetic compounds aromatic ring count and the effective permeability ($R^2 = 0.66$). This was expected as synthetic compounds are likely to adhere to Lipinski RO5 for potential drug candidates. Natural compounds, on the other hand, displayed a weak correlation ($R^2 = 0.0285$) between the aromatic ring count and their effective permeability. However natural compounds do not require stringent compliance to the Lipinski RO5. Natural compounds have been deprioritized or in some cases eliminated from the drug discovery process due to the fact that some natural compounds do not comply to Lipinski RO5 yet they can be excellent drug candidates (Ganesan, 2008).

Amidon *et al* (1995) described an association between the permeability and the aqueous solubility of chemical compounds (Figure 1.3). Different classes of compounds are described in their study, and each class has different levels of permeability and solubility. The overall permeability results in this study indicate that there was generally high chemical compound permeability and apparent low solubility. In the context of Amidon and colleagues' study in Figure 1.3, our chemical compounds fall in Class II. Class II compounds are highly permeable and low solubility class of compounds which, amongst many, includes compounds such as ketoconazole, mefenamic acid, nisoldipine and nicardipine, to name a few.

4.2.3 Protein binding

Protein binding is one of many physiochemical factors that influence the compound's ADME and in turn bioavailability (Zhang *et al*., 2012). There is a high concentration of protein in plasma and when drugs are absorbed, most of them bind to these proteins and thus decreasing

the drug concentration intended for pharmacological target activity. Free drug theory is commonly used to explain the pharmacokinetics/ pharmacodynamic correlation, the theory state that in an absence of energy-dependent processes, after equilibrium, the amount of free drug in plasma is equal to the amount of free drug in tissues, and only the free drug in the tissues is available for pharmacological activity (Bohnert and Gan, 2013). Percentage protein-binding was used to measure the amount of free compound in plasma (unbound) and the amount of compound bound to plasma, specifically serum albumin. The results displayed in Table 3.8 demonstrate a relatively high percentage protein-binding (PPB), with all 13 chemical compounds showing PPB > 70%, and salicylic acid displayed 90.62% PPB which was similar to a PPB presented by Kratochwil *et al*., 2002. This means that the compound fraction that will be unbound is, on average, less than 30% and this is a rather low free unbound fraction. Bovine serum albumin was used as a plasma protein, because of its low cost and easy availability (Alam *et al*., 2009) and it is apparent that all of these compounds have a high affinity for bovine serum album. Similar results were presented by Banis *et al* 2017, where their findings demonstrated a mean percentage protein binding of 72.7 % for BSA. Natural compounds displayed a low average percentage protein binding (67.98%) while synthetic compounds displayed a higher average percentage protein binding (85.01%). The difference in average PPB implies that natural compounds have 32.02%, on average, of free or unbound fraction for pharmacological activity, while synthetic compounds only have 14.99 % of unbound fraction for pharmacological activity. The interaction of compounds with BSA is electrostatic, hydrophobic, satiable and reversable, and BSA binds mostly to organic anions, basic and neutral drugs (Zhang *et al*., 2012) and therefore natural compounds are expected to possess a high affinity for binding to BSA than synthetic compounds but the average PPB demonstrate the opposite.

The percentage protein-binding of compounds to BSA is influenced by the confirmation changes of compounds (Zeitlinger *et al*., 2011), and the type of method used to measure PPB. The influence of aromatic ring number, as a structural feature, was interrogated and the results presented in Figure 3.12 indicates an upward trend but statistically, there is a weak correlation between the aromatic ring number and PPB indicated by a correlation coefficient of 0.1628. The low regression coefficient value of 0.1628 suggests that the overall percentage protein binding is independent of the aromatic ring number, meaning the PPB is not affected by the

number of aromatic ring number within a chemical compound. However, when the influence of aromatic ring number on a group of natural compounds was analysed, a surprising correlation coefficient value of 0.8364 was observed. This means that there is a significant ($p > 0.05$) correlation between the aromatic ring number and natural compounds PPB, meaning that increasing the number of aromatic rings of natural compounds do actually results in increased PPB. On the hand, synthetic compounds aromatic rings and PPB displayed a low correlation coefficient value of 0.1717, implying a lack of correlation. The correlation coefficient value between the aromatic ring number of synthetic compounds and PPB was not expected as literature states that the number of aromatic ring number do influence compound's developability (Mao *et al*., 2016 & Ritchie *et al*., 2011), and most synthetic compounds in this study are FDA approved, meaning they have passed through preclinical development. The reason for this unexpected output might have been due to the MW effect or the method used to analyse percentage protein binding, which in this study was ultrafiltration. The results displayed in Figure 3.14 demonstrated a correlation coefficient value of 0.2745, which is an indicative of a weak relationship between the MW and PPB. This means that PPB was independent of the MW and thus increasing the size of a compound did not influence or affect the percentage protein binding, in this study.

Ultrafiltration is a simple and a rapid technique for unstable compounds, it is a plastic device with a filter membrane made up of cellulose acetate. The filter membrane however, causes nonspecific binding of compounds and the nonspecific binding is thought to account for 20-30% effect on tested compounds (Zhang *et al*., 2012). There are two models of ultrafiltration, one is the use of individual vials (Amicon Ultra-15 Centrifugal Filter device) or a 96-well ultrafiltration device (substituted by the PAMPA plate in this study) (Peng *et al*., 2001).
The results displayed in Table 3.9 were obtained through the 96-well ultrafiltration device and after 24hour incubation, warfarin PPB calculated value of 49% was observed. This PPB value was different from what Kratochwil and colleagues obtained, they presented a warfarin PPB value of 99% which is almost 50% higher than the PPB value obtained in this study. However, Kratochwil and co-workers used equilibrium dialysis to measure warfarin PPB, which is a method frequently used for PPB calculation and is labelled as the 'gold standard' because it is believed to be more accurate than ultrafiltration (Son *et al*., 1996).

The introduction of BSA to warfarin, reduced the PPB and significantly reduced effective permeability of warfarin, as displayed in Table 3.9. The compound- BSA protein complex reduced the PPB by half (from 49% to 24%), and this is because the compound-BSA protein complex cannot permeate through cell membranes by passive transcellular or paracellular permeation (Zhang *et al*., 2012). The drug-plasma protein complexes exist as drug reservoirs for the unbound drugs, because when different drug elimination processes remove drugs from the body, the complex delay the duration of a drug action (Zhange et al., 2012). Moreover, the drug-plasma protein complex is usually pharmacologically inactive (Lin et al., 2003). There is abundant plasma protein binding data, but it is not easy to classify plasma protein binding of compounds and there are no suggestions in designing lead-like compounds through the examination of the existing drug properties (Zhang *et al*., 2012).

4.3 Cytotoxicity of auranofin, caffeine and warfarin

The activity of caffeine, warfarin and auranofin was investigated in HEK-293T mammalian cells (Figure 3.15). An overall high cytotoxicity was observed for all three compounds, caffeine, warfarin and auranofin. Auranofin yielded a high cytotoxicity, CC_{50} value of 0.218 µM which correlated with previously reported CC_{50} values for auranofin (CC_{50} < 1.652 µM) (Abrahams, 2014; Mphahlele, 2012 & Lasagna-Reeves *et al*., 2010). Auranofin is a gold salt, and like other gold-containing compounds, it can cause toxicity to cells. Gold-containing compounds are well known by inducing the increased levels of reactive oxygen species (ROS) (Kudrin, 2000), and these ROS results in auto-oxidative stress (Omata *et al*., 2006) through the destruction of the biological molecule interactions (Thannickal and Fanburg, 2000). Gold-containing compounds tend to have varying oxidation states and these changing oxidation states may lead to toxic redox properties in the media used for cell culture (Shaw, 1999). Caffeine exhibited cytotoxicity, with CC_{50} value of 1.619µM, and this was an unexpected output because caffeine is an FDA approved psychoactive drug and a widely used stimulant. Acosta and Anuforo reported a possible caffeine cytotoxicity due to the duration treatment of cells with caffeine longer than 24 hours and concentrations greater than 20mM. Caffeine cytotoxicity in this study was possibly due to the treatment duration of cells which was 72 hours rather than 24 hours or less. Other reported caffeine cytotoxicity effects include cell hypertrophy (Boyd, 1965) and cell chromosomal breakage (Thayer *et al*., 1975). To produce these caffeine cytotoxicity effects, the concentration had to be greater than 20mM, and Jang *et al*., 2002, used a similar assay (MTT

assay) as was used in this study, and reported caffeine cytotoxicity at 10mM concentrations, and established a cytotoxicity effect increasing in a time-dependent manner (Jang *et al*., 2002). Though the concentrations used in this study were relatively low, the duration of treatment might have been the critical contributing factor. Jang and co-workers displayed an, approximately, 50% increase in cytotoxicity after 12 hours of caffeine treatment. Warfarin yielded cytotoxicity in HEK-293T mammalian cells, displaying a CC_{50} value of 1.679µM. Warfarin is an FDA approved oral anticoagulant drug and thus the observed cytotoxicity in HEK-293T cells was unexpected. The reason for the cytotoxicity effect displayed by warfarin might possibly be time-dependent effects, as reported by Kubat *et al*., 2018, warfarin displayed significantly high cytotoxicity from a 24-hour to 48-hour incubation (Kubat *et al*., 2018).

4.4 Conclusion

Overall results of this study demonstrated the value of predictive screening coupled with molecular experimental analysis of natural and synthetic compounds. The predictive and theoretical techniques predicted an overall low drug score for all thirteen chemical compounds. The drug score was further validated through molecular modelling studies, where synthetic compounds outperformed natural compounds, possessing relatively high effective permeability, and plasma protein binding. In overall assessment, aromatic ring count did not have a significant correlation with physiochemical properties discussed in this study, with an exception of natural compounds and plasma protein binding. This study could not validate whether caffeine and warfarin were cytotoxic against mammalian cells, however, our results corroborate the findings from previous studies showing that treatment durations longer than 24 hours on HEK-293T cells result in cytotoxicity. In conclusion, the aromatic ring number within chemical compounds, both synthetic and natural compounds, does not influence the bioavailability of compounds based on the physiochemical properties discussed in this study.

4.5 Future studies

The results from this study can be advanced by conducting the following experiments:

1) Increase the number of both natural and synthetic compound to be screened.
2) Molecular modelling of aqueous solubility and metabolism for both synthetic and natural compounds to guide the early-stage assessment of compound bioavailability.
3) Evaluation of cytotoxicity using a different cell line and reduced treatment duration.
4) Identifying the structural moieties of natural compounds involved in plasma protein binding as well as the residues to which these compounds bind and the subsequent mechanism of action, to guide preclinical lead-like compound selection.

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