OPEN ACCESS

African Journal of
Pharmacy and Pharmacology

8 April, 2018
ISSN 1996-0816
DOI: 10.5897/AJPP
www.academicjournals.org

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African Journal of Pharmacy and Pharmacology

Table of Contents: Volume 12 Number 13 8 April, 2018

ARTICLES

academicJournals

Vol. 12(13), pp. 159-167, 8 April, 2018
DOI: 10.5897/AJPP2018.4904
Article Number: C72C07656648
ISSN 1996-0816

http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Review

A systematic review: Application of *in silico* models for antimalarial drug discovery

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Received 14 February, 2018; Accepted 26 March, 2018

Malaria remains the global public health problem due to the reemergence of drug resistance. There is an urgent need for development of new antimalarial candidates which are effective against resistant malaria parasite. This systematic review evaluates the published research studies that applied *in silico* modeling during the discovery process of antimalarial drugs. Literature searches were conducted using PubMed, EBSCO, EMBASE, and Web of Science to identify the relevant articles using the search terms "Malaria" "*In silico* model", "Computer-based drug design", "Antimalarial drug", and "Drug discovery". Only the articles published in English between 2008 and May 2015 were included in the analysis. A total of 17 relevant articles met the search criteria. Most articles are studies specific to *Plasmodium falciparum* targets; 3 and 1 articles, respectively involve target for *P. vivax* and liver stage of *Plasmodium*. Both structure-based and ligand-based approaches were applied to obtain lead antimalarial candidates. Two articles also assessed absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties. Confirmation of activity of the candidate leads by *in vitro* and/or *in vivo* assays were reported in some studies. Homology modelling, molecular docking, 2D- or 3D-QSAR and pharmacophore modeling are commonly applied methods. One study used *de novo* synthesis for lead identification and one study applied phylogenetic analysis for target identification/validation.

Key words: *Plasmodium*, malaria, antimalarial drug, drug discovery, *in silico* modeling, computer-based drug design, systematic review.

INTRODUCTION

Malaria is one of the most important global infectious diseases affecting hundreds of millions of people each year and is the main cause of socio-economic loss in developing countries (WHO, 2015). The major problem in malaria treatment and control is the emergence and spread of multidrug resistance *Plasmodium falciparum*

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including artemisinin drugs. It is therefore an urgent need to search for alternative effective drug candidates. Nevertheless, the process of drug discovery and development is both time-consuming (approximately 10-15 years) and resource-consuming (approximately $1.5 billion per successful drug) (Ren et al., 2016).

Rational drug design is an intensive process of finding new medications based on knowledge of a biological target. Drug design involves the design of small molecules that are complementary in shape and charge to the biomolecular target with which they interact. Advances in genomics and computational methods over the past decades present new challenges and opportunities in drug discovery and development process. The convergence of these technological trends has been a great benefit to computational science and informatics. Information on gene expression, drug-target interactions (DTI), including protein networks, which are rapidly accumulating are becoming increasingly accessible and standardized (Margolis et al., 2014). Computational-based or *in silico* approach can facilitate the discovery process for antimalarial drugs through utilizing a number of available databases of chemical compounds and *Plasmodium* proteins. The costs are minimal, humans are rarely at risk, and biosafety facilities are not required. Most drugs in development fail during clinical trials due to poor pharmacokinetics properties and toxicity. These properties such as absorption, distribution, metabolism, excretion including toxicity (ADMET) play an important role in drug discovery and development. ADMET prediction can help eliminate compounds with unfavorable drug ability properties. *In silico* modeling can be applied for prediction of either pharmacodynamics (targets of drug action and toxicity) or pharmacokinetic properties of the candidate molecules (Mendez et al., 2016). Both properties involve interactions with multiple biological systems (Hodos et al., 2016; Lounnas et al., 2013; Peltenburg et al., 2013).

Two approaches have been successfully applied for rational drug design, that is ligand-based and structure-based drug design (Lounnas et al., 2013; March-Vila et al., 2017). Ligand-based drug design or indirect drug design relies on knowledge of other molecules that bind to the biological target of interest. These molecules are used to drive a “pharmacophore” model that defines the minimum necessary characteristics a molecule must possess in order to bind to the target. A model of the biological target may also be used to design new molecular entities that interact with the target. The structure-based drug design or direct drug design relies on knowledge of the 3-dimensional structure of the biological target obtained through X-ray crystallography or NMR spectroscopy. 3D-QSAR refers to the application of force field calculations requiring three-dimensional structures, for example based on protein crystallography or molecule superimposition. Using the structure of the biological target, candidate drugs that are predicted to bind with high affinity and selectivity to the target may be designed using interactive graphics and the intuition of a medicinal chemist or various automated computational procedures to suggest new drug candidates (Lounnas et al., 2013; March-Vila et al., 2017). This systematic review focused on the analysis of research articles which applied computational approaches for antimalarial drug discovery. The information is useful for further development of effective antimalarial drugs.

MATERIALS AND METHODS

Literature searches

The following electronic databases were searched for research articles published during 2013 and 2017: PubMed, EBSCO, EMBASE, and Web of Science and Google Scholar. Thesaurus and free-text searches were also performed across each database to combine the terms “Malaria” “*In silico* model”, “Computer-based drug design”, “Antimalarial drug”, and “Drug discovery”. Duplicate articles were recorded and excluded from the analysis.

Eligibility criteria

Research articles published in English between 2008 and 2017 on the application of *in silico* (computer-based) modeling approach for discovery of antimalarial targets and lead candidates were included in the analysis. The number of papers referenced was likely an under-representation of the overall body of literature on *in silico* modeling due to the strict search criteria and the search terms used.

Study selection process

The searched articles from all databases were downloaded into Endnote and merged to remove duplicates. The titles and abstracts of all search articles were initially screened for potential relevance. The articles for which there was uncertainty about relevance were retained and the full texts were further reviewed. Articles that did not meet the inclusion criteria were excluded. Full-texts of all articles that met the eligibility criteria were downloaded into Endnote database.

Data extraction

The following information were extracted from each of the included article: the names of the authors, article title, journal and volume/issue published, publication year, software packages and algorithms applied, approach applied (structure-based design, ligand-based design), computational methods (homology modeling, molecular docking, pharmacophore modeling, 2- or 3-D quantitative structure activity relationship: QSAR), experimental methods (*in vitro, in vivo* assays), targets, hit or lead compounds, sets of database used, and server used.

RESULTS

Study selection

A comprehensive search of databases provided 129

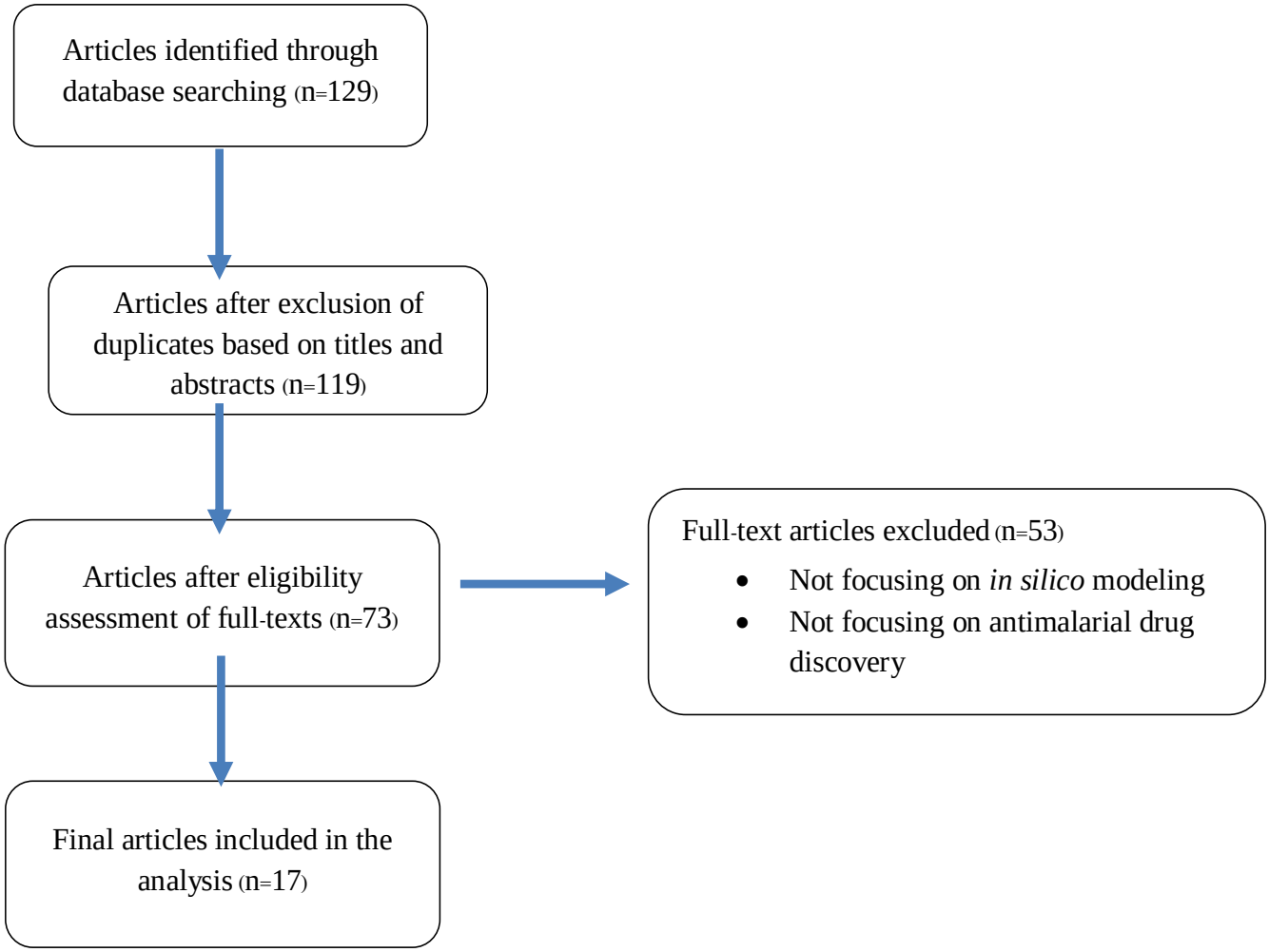

Figure 1. Flow diagram of the systemic analysis process.

potentially related articles based on the search terms described above which were published since 2008 (Figure 1). Of these articles, 10 duplicate articles were initially excluded based on the screening of titles and abstracts. Forty-six out of the 119 full-text articles were excluded from the analysis based on the eligibility criteria. Fifty-three articles were further excluded due to unrelated topics and finally 17 articles were included in the analysis.

Study characteristics

Table 1 summarizes the approaches and methodologies employed including potential targets and lead candidates from the 17 research articles included in the current systematic review. The published *in silico* models were mostly applied for lead identification/optimization (13 articles) of the known *Plasmodium* targets (13 targets). Most articles are specific for *P. falciparum* targets (Campbell et al., 2014; Kumari et al., 2016; LaMonte et al., 2017; Lhouvum et al., 2013; MacDonald and Boyd, 2015; Raza et al., 2017; Ren et al., 2016; Sharma et al., 2016); 3 articles involved target for *P. vivax* (MacDonald et al., 2015; Rout et al., 2016; Bouillon et al., 2013) and 1 article involved target for liver stage of *Plasmodium* (Sullivan et al., 2015). Five articles were applied *in silico* modeling for target identification/validation (Lhouvum et al., 2013; Mehra et al., 2015; Paul et al., 2015; Rout et al., 2015; Pandey et al., 2014). Both structure-based (Bouillon et al., 2013; LaMonte et al., 2017; MacDonald and Boyd, 2015; Raza et al., 2017; Ren et al., 2016; Rout and Mahapatra, 2016; Sullivan et al., 2015) and ligand-based (Villalobos et al., 2013; Kumari et al., 2016; Ren et al., 2016; Sharma et al., 2016) approaches were applied to obtain lead antimalarial candidates. Two articles also assessed ADMET properties (Ren et al., 2016; Rout and Mahapatra, 2016). Confirmation of activity of the candidate leads by *in vitro* and/or *in vivo* assays were reported in some studies (Bouillon et al., 2013;

Table 1. Software and methods applied for *in silico* modeling during antimalarial drug discovery process.

Reference	Application	Software applied	Methods applied	Potential drug targets	Lead candidates
Villalobos et al. (2013)	Lead identification /optimization	Marvin 5.12 / MOE v 2009.10	Molecular docking / 2D &3D QSAR	*Plasmodium* Cytochrome bc1	A series of 4(1H)-quinolone acting as Qo site inhibitors
Bouillon et al. (2013)	Lead identification /optimization	FlexX,/ FlexX-Pharm	Homology modeling / Molecular docking + Experimental (in vitro, in vivo)	Subtilisin-like serine protease SUB1 of P. vivax merozoites (PvSUB1)	5 Candidate compounds with 1 most potential (compound 2)
Lhouvum et al. (2013)	Target identification /Validation	MODELLER 9v9 / Patchdock / Fire-Dock / GROMACS	Homology modeling / Molecular docking / Molecular dynamic (MD) simulation	*P. falciparum* metalloprotease (PFI1625c)	-
Ren et al. (2016)	Lead identification /optimization+	GOLD 5.1 / Discovery Studio 3.1	Homology modeling / Molecular docking /pharmacophore modeling/ ADMET modeling	*P. falciparum* lipid kinase PI(4)K (phosphatidylinositol-4-OH kinase) = PfPI(4)K	KAI715 and 2 potential compounds
Pandey et al. (2014)	Target identification /Validation	MEGA 5.2 /	Phylogenetic analysis.	*P. falciparum* phosphatome: phosphatases:	67 Candidate phosphatases
Campbell et al. (2014)	Lead identification /optimization	MEGA5 / Ligsite algorithm / GLIDE Schrodinger Glide Lead identification	Molecular Modeling / homology model / Molecular docking + *in vivo* experiments	*P. falciparum* phosphatome	4 Candidate compounds
Verma et al. (2015)	Lead identification /optimization	Modeler 9.10 / VMD 1.91) / VEGAZZ / Video match / Surflex-Dock in Sybyl X 13/AUTO DOCK	Structural Modeling / Molecular Dynamics (MD) Simulations / Virtual Screening + *in vitro* assay	Cytidine triphosphate synthase (CTPS), choline kinase (CK), and glutathione-D-trabsferase (GST)	Arylsulfonyloxy acetimidamides as their consensus inhibitors
MacDonald and Boyd (2015)	Lead identification /optimization	FRED	Molecular docking	*P. vivax* peptidyl-prolyl cis/trans isomerase (PPIase)	Rapamycin and derivatives
Sullivan et al. (2015)	Lead identification /optimization	Quantum-based computational process	Molecular modeling + *in vivo* experiments	*Plasmodium* liver stage	Cethromycin and derivatives
Mehra et al. (2015)	Target identification	FireDock / GROMACS program suite 4.6.2	Molecular docking / Molecular dynamic (MD) simulation	Target = between EC (ICAM-1) and RBC(PfEMP1) cytoadherence complex	-
Paul et al. (2015)	Target identification /Validation	MODELLER 9.0 / Gromacs 5.0	Homology modeling / Molecular dynamics simulation Validation: PROCHECK, RAMPAGE, CHIMERA Stability: Gromacs 5.0	N-myristoyltransferase (NMT) = PfMNT	-
Rout et al. (2015)	Target identification /Validation	OrthoMCL database / MODELLER v9.13 / Dock Blaster software / AutoDock 4	Homology modeling / virtual screening / Molecular docking Db = Interpro, ZINC Server = BiS	11 Potential non-homologous proteins	-
Sharma et al. (2016)	Lead identification /optimization	MODELLER 9.13 / Schrodinger Maestro 9.8 / GROMACS 4.5.6	Homology modeling/3 D QSAR /Molecular docking / Molecular dynamic (MD) simulation	DegP protein of *P. falciparum*, also known as HtrA (heat shock regulated)	7 Potential lead compounds

Table 1. Contd.

Kumari et al. (2016)	Lead Identification /optimization	Schrodinger Maestro / GOLD v5.2 / Schrodinger Glide /Corina 2.64v/VLifeMDS	Pharmacophore modeling /3 D QSAR / Molecular docking	*P. falciparum* M18 Aspartyl Aminopeptidase (PfM18AAP)	10 novel PfM18AAP inhibitors from ChEMBL antimalarial library, 2 novel inhibitors from each derivative of quinine, chloroquine, 8-aminoquinoline, and 10 novel inhibitors from WHO antimalarial drugs. Additionally, high throughput virtual screening identified top 10 compounds as antimalarial leads.
Rout and Mahapatra (2016)	Lead identification /optimization	MODELLER v9.15 / LigandScout v3.0 / ZINCPharmer database / AutoDock v4.2.6 / FlexX docking / ROMACS v5.0 / MedChem Designer	Homology modeling / Virtual screening / Molecular docking / Molecular dynamic (MD) simulation /ADMET	*P. vivax* M17 LAP (Leucine Amino Peptidase) -	Bestatin and other 9 related compounds
LaMonte et al. (2017)	Lead identification /optimization	Docktite application / Molecular Operating Environment (MOE)	Molecular modeling experiments / Docking experiments + *In vitro* assays	*Plasmodium* Proteasome (asexual and sexual blood stage)	Carmaphycin B scaffold
Raza et al. (2017)	Lead identification /optimization	The PROCHECK / ProSA online server / ProSA2003 / Chem-T Autodock Vina / i-GEMDOCK v2.1	3 D Virtual screening /homology modeling/pharmacophore modeling /Molecular docking	*P. falciparum* Inosine monophosphate dehydrogenase (PfIMPDH)	5 Ribavirin derivatives

Campbell et al., 2014; LaMonte et al., 2017; Sullivan et al., 2015). Homology modelling, molecular docking, 2D- or 3D-QSAR and pharmacophore modeling were commonly applied methods. One study used *de novo* synthesis for lead identification (Rout and Mahapatra, 2016) and one study applied phylogenetic analysis for target identification/ validation (Pandey et al., 2014).

The articles included in the analysis relied on diverse sets of software tools and web servers. Several software tools, servers, and frameworks were explicitly applied (Maier and Labute, 2014; Webb and Sali, 2014). The most commonly used software were MODELLER and GROMACS. In general, authors reported computational tools directly employed in the analysis (for example, bioinformatic web servers, molecular dynamics suites, and visualization programs) and did not explicitly report indirectly employed computational tools (for example, scripting languages, server operating systems, and clusters). Lead identification/optimization involved identification of potent antimalarial candidate molecules which are inhibitors of known specific *Plasmodium* targets (13 targets) encoded by the malaria parasite genome. These included: inosine monophosphate dehydrogenase (IMPDH) (Raza et al., 2017), proteasome (LaMonte et al., 2017), lipid kinase PI(4)K (phosphatidylinositol-4-OH kinase) (Ren et al., 2016), heat shock regulated A (HrA or DegP) (Sharma et al., 2016), *P. falciparum* M18 (Kumari et al., 2016), *P. falciparum* aspartyl aminopeptidase (PfM18AAP) (Kumari et al., 2016), leucine amino peptidase (M17 LAP) (Rout and Mahapatra, 2016), *P. falciparum* glycogen synthase kinase-3 (PfGSK-3), *P. falciparum* CTP synthetase (PfCTPS), choline kinase (PfCK), and glutathione S-transferase (PfGST) (Verma et al., 2015), *Plasmodium* peptidyl-prolyl cis/trans isomerase (MacDonald and Boyd, 2015), *Plasmodium* phosphatases, cytochrome bc1 (Jimenez et al., 2013), and *P. vivax* subtilin-like serine protease (PvSUB1) (Bouillon et al., 2013). Application of the modeling for target identification/validation included *N*-myristoyltransferase (NMT) (Paul et al., 2015), *Plasmodium* phosphatases (Campbell et al., 2014; Pandey et al., 2014), and PFI1625c metalloprotease (Lhouvum et al., 2013). One articles involving ICAM-1 and PfEMP1

cytoadherence complex applied the computer-based modeling for both target identification/validation and lead identification/optimization (Mehra et al., 2015).

Various commercial and public databases of proteins and compounds (ligands) were used as sources of computer-based modeling. A multitude of databases of compounds were used as sources of chemical structures in one or more of the highlighted studies including ZINC (Rout et al., 2015; Rout and Mahapatra, 2016; Verma et al., 2015), ChEMBL-NTD (Sullivan et al., 2015), DrugBank (Ren et al., 2016; Rout et al., 2015; Rout and Mahapatra, 2016), PubChem (Kumari et al., 2016; Rout and Mahapatra, 2016), and ChemSpider (Rout and Mahapatra, 2016). For antimalarial protein targets, the databases applied included PlasmoDB 9.2 (Lhouvum et al., 2013; Pandey et al., 2014; Rout et al., 2015; Sharma et al., 2016), PFAM (Pandey et al., 2014; Rout et al., 2015), CDD [29], ZINC (Lhouvum et al., 2013; Pandey et al., 2014), and InterPro (Campbell et al., 2014; Rout et al., 2015). Protein structures were downloaded from these databases and validated using RAMPAGE (Paul et al., 2015; Sharma et al., 2016), PROCHECK (Bouillon et al., 2013; Campbell et al., 2014; Kruggel and Lemcke, 2009; Lhouvum et al., 2013; Mehra et al., 2015; Paul et al., 2015; Raza et al., 2017; Rout et al., 2015; Rout and Mahapatra, 2016), Verify-3D (Campbell et al., 2014; Lhouvum et al., 2013; Rout et al., 2015; Rout and Mahapatra, 2016; Sharma et al., 2016), ERRAT (Campbell et al., 2014; Lhouvum et al., 2013; Rout et al., 2015; Rout and Mahapatra, 2016; Sharma et al., 2016), CHIMERA (Paul et al., 2015), and PROSA (Rout et al., 2015; Rout and Mahapatra, 2016; Sharma et al., 2016; Verma et al., 2015). Stability check was performed using Gromacs (Lhouvum et al., 2013; Mehra et al., 2015; Paul et al., 2015; Rout and Mahapatra, 2016; Sharma et al., 2016).

DISCUSSION

Lead identification/optimization

Most studies applied *in silico* modeling for structure-based or ligand-based design for potential antimalarial candidates using known targets specific to *Plasmodium*. Inosine monophosphate dehydrogenase (IMPDH) is an important enzyme in the purine biosynthesis of *P. falciparum*. A 3D homology model for this parasite enzyme was made using human IMPDH (PDB code 1NF7) as a template (Raza et al., 2017). The *in silico* combinatorial library of 1,347 ribavirin derivatives was designed and virtually screened. Finally, 5 ligands were shown to be more specific to *P. falciparum* IMPDH than human IMPDH II.

Proteasome inhibitors have been demonstrated as potential antimalarial compounds with action on asexual and sexual *Plasmodium* blood stages. Various proteasome inhibitors with potent antimalarial activity and low host cytotoxicity were designed and evaluated based on the carmaphycin B scaffold (LaMonte et al., 2017). Cell-based and *Plasmodium* proteasome assays revealed one promising compound (compound 18) active against both asexual blood stages and gametocytes. *In vitro* evolution in *S. cerevisiae*, biochemical assays, and molecular modeling studies confirmed that this activity is due to specific inhibition of the β5 subunit of the proteasome.

Plasmodium lipid kinase PI(4)K (phosphatidylinositol-4-OH kinase) is a ubiquitous eukaryotic enzyme that phosphorylates lipids to regulate intracellular signaling and trafficking. Virtual screening for inhibitors of this enzyme as potential antimalarial drugs was performed (Ren et al., 2017). A homology modeling of PI(4)K from *P. falciparum* was initially built. The compound KAI715 which showed high potent activity and selectivity for PI(4)K was docked into the enzyme ATP-binding site. The optimized pharmacophore modeling of this target-ligand complex (HyopA) was then used to search a large chemical database. All drug-like hit compounds with satisfactory ADMET properties that passed the pharmacophore-based virtual screening were identified using Lipinski's rule of five (Lipinski et al., 2016) and ADMET (aqueous solubility, human intestinal absorption, penetration across blood-brain barrier (BBB), cytochrome P450 2D6 inhibition, hepatotoxicity, and plasma-protein binding) prediction. The molecular docking method was then further carried out to re-filter these screened compounds.Two more potent compounds in addition to KAI715 were designed.

DegP protein has been shown to be involved in regulation of thermo-oxidative stress generated during asexual life cycle of *Plasmodium*. The protein is probably required for survival of parasite in host. A 3D structure of PfDegP was generated using MODELLER based on PlasmoDB and protein database bank (PDB). The model was validated using RAMPAGE and ERRAT. An *in silico* screening of small molecule database against PfDegP was performed using Glide and molecular dynamics simulation of protein and protein-ligand complex was carried out using GROMACS. Seven lead compounds which were inhibitors of PfDegP were finally generated (Sharma et al., 2016).

P. falciparum M18 aspartyl aminopeptidase (PfM18AAP) is only aspartyl aminopeptidase which is found in the *Plasmodium* genome and is essential for its survival. This enzyme performs various functions in the parasite and the erythrocytic host such as hemoglobin digestion, erythrocyte invasion, parasite growth, and parasite escape from the host cell. The 3D-QSAR modeling, pharmacophore modeling, and molecular docking were employed to identify novel potent inhibitors of PfM18AAP (Kamuri et al., 2016). Ten novel PfM18AAP inhibitors from ChEMBL antimalarial library, 2 novel inhibitors from each derivative of quinine, chloroquine, 8-

aminoquinoline, and 10 novel inhibitors from WHO antimalarial drugs were used.

P. vivax leucine amino peptidase (M17 PvLAP) belonging to the metallo-aminoeptidase family, plays a significant role in the catalysis of the terminal stage of hemoglobin degradation which is essential for growth and development of *P. vivax* (Lee et al., 2010). A homology model was generated using MODELLER and applied various *in silico* methods such as structure based, ligand based and *de novo* drug designing to design potential compounds that are inhibitors of these enzymes (Rout and Mahapatra, 2016). Out of the ten potential candidates, 2-[(3-azaniumyl-2-hydroxy-4-phenylbutanoyl) amino]-4-methylpentanoate was identified as the best inhibitor in terms of docking score and pharmacophoric features. The reliability of the binding mode of the inhibitor was confirmed by molecular dynamics (MD) simulation study with GROMACS software. Finally, *in silico* ADMET properties were evaluated.

P. falciparum glycogen synthase kinase-3 (PfGSK-3) is one of the eukaryotic protein kinases that have been identified as essential for *Plasmodium*. Although the physiological functions of PfGSK-3 are still unknown, it has been suggested as a putative target for novel antimalarial drugs. Homology modeling and molecular docking and 10 different HsGSK-3b templates were applied for the model building of PfGSK-3 (Kruggel and Lemcke, 2009). The evaluated top models were used to compile an ensemble of PfGSK-3 models and for the structure-based design of potential ATP-binding site inhibitors of PfGSK-3. Thieno[2,3-b]pyridines were identified as a new class of PfGSK-3 inhibitors.

P. falciparum CTP synthetase (CTPS), choline kinase (CK), and glutathione S-transferase (GST) selected based on their connectedness and functional importance in biochemical/metabolic pathways of *P. falciparum*, were used in virtual screening of ZINC database entries which led to the design and synthesis of arylsulfonyloxy acetimidamides as their inhibitors (Verma et al., 2015).

Peptidyl-prolyl cis/trans isomerases (PPIases) constitute an enzyme superfamily that converts *cis* and *trans* amide bonds in proteins and peptides, and in addition, cellular processes such as apoptosis or protein synthesis in almost all living cells. Fk506-binding proteins (FKBPs) are the largest and most varied of the PPIases. Through application of molecular docking, it was demonstrated that the substrates ILS-920 and WYE-592 bind less-favorably with human FKBP12 (hFKBP12) and *P. falciparum* FKBP35 (PfFKBP35) compared to a competing substrate rapamycin (MacDonald and Boyd, 2015).

Quantum-similarity approach was applied for discovery of novel liver-stage antimalarials (Sullivan et al., 2015). Testing of only five of the model-predicted compounds *in vitro* and *in vivo* hepatic stage drug inhibition assays with *P. berghei* identified four novel chemical structures. All inhibited liver stage *Plasmodium* at a single oral dose in the quantitative PCR mouse liver-stage sporozoites-challenge model. Cethromycin (ABT-773), a macrolide-quinoline hybrid, was also identified as a potential candidate for further development due to its extensive safety profile.

P. falciparum phosphatases are important elements of intraerythrocytic development expressed throughout the life cycle. *P. falciparum* Mitogen-Activated Protein Kinase (MAPK) phosphatase (MKP) subgroup signaling cascades are critical components of sexual stage proliferation. Interaction of the MKP with its phosphoprotein substrate depends on three conserved residues in the consensus atypical dual-specificity phosphatase (DUSP) domain binding pocket.

A homology model of the atypical dual-specificity phosphatase (DUSP) domain was developed for use in high-throughput *in silico* screening of the available library of antimalarial compounds from ChEMBL-NTD (Campbell et al., 2014). Seven compounds were selected for further evaluation of antimalarial activity *in vitro.* Out of these, 4 compounds (390097, 524725, 525841, and 585222) showed promising activity against C9 parasites.

The cytochrome bc_1 complex (ubiquinol: cytochrome *c* oxidoreductase, respiratory Complex III) is a key enzyme of the mitochondrial electron-transfer chain in all metazoa and protozoa including *Plasmodium.* It catalyzes the transfer of electrons from ubiquinol to cytochrome *c*. The inhibition of cytochrome bc1 blocks the mitochondrial respiratory chain and the consequent arrest of pyrimidine biosynthesis, which is essential for parasite development.

The 2D- or 3D-SAR was developed and a docking analysis was conducted for a series of 4(1H)-quinolones as cytochrome bc1 inhibitors (Villalobos et al., 2013). The substituents R1 and R4 in 4(1H)-quinolones analogues were key modulators to enhance the antimalarial activity. The appropriate binding conformations and orientations of these compounds interacting with cytochrome bc1 were also revealed by molecular docking. Eight promising compounds were designed and presented as reference compounds for synthesis and antimalarial evaluation.

Subtilisin-like serine protease SUB1 of *Plasmodium* merozoites plays a dual role in egress from and invasion into host erythrocytes.

The *P. vivax*-SUB1 (*Pv*SUB1) was characterized and shown to display similar cellular location, auto-processing, and enzymatic properties as its *Pf*SUB1 ortholog (Bouillon et al., 2013). Homology modeling and molecular docking were applied to search for potential *Pv*SUB1 inhibitors using 3D models of *Pv*SUB1 as targets. The 306 best predicted hits were selected and tested for their inhibitory potency on the *Pv*SUB1 recombinant enzyme. Active compounds were then tested for their antimalarial activity *in vitro*. Five most promising compounds particularly compound 2 were shown to exhibit specific activity against *P. falciparum* merozoite egress and invasion in *P. berghei*-infected mice.

Target identification/validation

A number of *Plasmodium* targets were identified and validated by application of *in silico* modeling approach.

interstitial cell adhesion molecule- (1ICAM-1) belongs to the immunoglobulin-like superfamily and plays an important role in cell recognition, cell adhesion and cell aggregation. ICAM-1 has been identified as an endothelial receptor for *P. falciparum* infected red blood cells (IRBCs). Cytoadherence of parasitized red cells is mediated by PfEMP1 (*P. falciparum* erythrocyte membrane protein 1) expressed on the surface of IRBCs. Cytoadherence of ICAM-1 to surface expressed antigen on IRBCs leads to cascade of consequences that contribute to the development of cerebral malaria. IRBC-endothelial cells cytoadherence therefore represents a promising target to attenuate or eliminate down-stream pathophysiological events. Target identification/validation was applied to understand the forces operating between ICAM-1 and PfEMP1 cytoadherence complex and designing better cytoadherence peptides which could be useful for development of potential anti-adhesion therapeutics (Mehra et al., 2015).

P. falciparum NMT (PfNMT) is responsible for the sexual blood stages of the parasite and is essential for transmission. The 3D-structure of PfNMT was modeled using Modeler (v.9.0) taking *P. vivax* NMT (PvNMT) as the template (Paul et al., 2015). The generated structure was then validated using various programs such as PROCHECK, RAMPAGE server, and CHIMERA and the stability of the model was checked by Gromacs 5.0. The enzyme was shown to be a vital target and the modeled structure could be further applied in molecular docking studies for novel drug design.

A search for unique proteins was conducted by a comparative genomics study (Rout et al., 2015). Eleven proteins (phosphoenolpyruvate carboxykinase, pyridoxine/pyridoxal 5-phosphate biosynthesis enzyme, beta-hydroxyacyl-ACP dehydratase, NADH dehydrogenase, fumarate hydratase, phosphoenolpyruvate carboxylase, putative uncharacterized protein, uncharacterized protein involved in lipopolysaccharide biosynthesis, RNA pseudouridylate synthase, pseudouridine synthase, and lipoate–protein ligase) were prioritized as antimalarial drug targets. The homology models of two uncharacterized proteins were built using MODELLER (v9.13) software from possible templates. Functional annotation of these proteins was done by the InterPro databases and ProBiS server by comparison of predicted binding site residues. The model was subjected to *in silico* docking study to search for potent lead compounds from the ZINC database by Dock Blaster software using AutoDock 4. Results from this study would facilitate the selection of proteins and putative inhibitors for entry into drug design production pipelines.

Six *Plasmodium* specific phosphatases and 33 putative phosphatases with absence of human orthologs were identified (Pandey et al., 2014). Using phylogenetic analysis of the blood-stage asexual cycle, the enzymes were identified as atypical MAPK phosphatases. This has led to the identification of the putative phosphatase from PF3D7_1305500 as an important element of intraerythrocytic development expressed throughout the life cycle (35). Additional bioinformatics analysis delineated a conserved signature motif and three residues with potential importance to functional activity of the atypical dual-specificity phosphatase (DUSP) domain.

PFI1625c was identified as a putative metalloprotease present in *Plasmodium* genome (Lhouvum et al., 2013). Probing PFI1625c active site with 199 different peptides from a combinatorial peptide library indicated preference of PFI1626c toward hydrophobic residue substituted peptides. The peptide P550 (LVIVAKRA) was shown to exhibit significantly better interaction within the active site than a template peptide (LSRVAKRA). The molecular dynamic's simulation studies confirmed integrity of the complex. The structural and biochemical differences between PFI1625c with human metalloprotease suggest that the enzyme could be exploited as drug targets for future antimalarial development.

Conclusions

In silico modeling is a useful tool that plays important role in identification/validation of potential antimalarial drug targets as well as identification of potential lead candidates during drug discovery phase. In addition, it is also useful in elucidating their mechanisms of action and their potential clinical efficacy. Exploring potential drugs *via* computational modeling is a safe, frugal, and effective method to discover, develop, or repurpose potential treatments. Nevertheless, the antimalarial activity of these potential candidate compounds need to be further confirmed by nonclinical and clinical validation using a series of *in vitro* and *in vivo* assays. Multi-targeting approaches have the best potential to be the most effective. In addition, the *in silico* approach should also be applied in parallel for the prediction of drug-like and ADME properties of the candidate molecules.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Vol. 12(13), pp. 168-175, 8 April, 2018
DOI: 10.5897/AJPP2018.4894
Article Number: 43B4EF556650
ISSN 1996-0816

http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

In vitro anti-inflammatory activity of *Vangueria infausta*: An edible wild fruit from Zimbabwe

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Received 24 January, 2018; Accepted 14 February 2018

This study set out to investigate the anti-inflammatory activity of *Vangueria infausta*, an edible wild fruit from Zimbabwe. The importance lies in the fact that this plant species could be developed as a low cost and effective therapeutic agent, with little or no side effects from natural sources. The fruit pulp of *V. infausta* was subjected to cold ethanol extraction to get crude extract. Flavonoids were isolated by thin layer chromatography (TLC) and unsaponifiable matter by liquid-liquid extraction using petroleum ether. The three were assayed by egg albumin denaturation and Nitric Oxide radical scavenging assays. Unsaponifiable fraction, crude ethanol extract and flavonoid fraction exhibited potent anti-inflammatory activity with a high of 93.12±0.03% (at 400 mg/L), 79.91±0.042% (at 800 mg/L) and 54.40±0.061% (at 800 mg/L), respectively in the egg albumin denaturation assay, whereas, in the nitric oxide (NO) radical scavenging assay it was respectively 68.99±0.058% (800 mg/L), 82.85±0.047% (at 800 mg/L) and 33.46±0.036% (at 800 mg/L). *V. infausta* crude extract and unsaponifiable fraction were superior to indomethacin and quercetin standards at lower concentrations in the egg albumin inhibition assay. This study shows that *V. infausta* possess potent anti-inflammatory phytochemicals that could be developed into anti-inflammatory drugs.

Key words: *Vangueria infausta*, unsaponifiable fraction, crude ethanol extract, flavonoids, anti-inflammatory activity, egg albumin assay, NO radical scavenging assay.

INTRODUCTION

Inflammation is a pathophysiological response of living tissues to injury that leads to the local accumulation of plasmatic fluids and blood cells (Huang et al., 2011; Vazquez et al., 2011; Kandati et al., 2012). It involves a complex series of biochemical events closely related to pathogenesis of various ailments such as osteoarthritis, rheumatoid arthritis, ankylosing spondylitis, migraine and acute gout (Huang et al., 2011; Vazquez et al., 2011; Kandati et al., 2012). It is usually characterized by redness, swollen joints and joint pain, stiffness and loss of joint function and can be acute or chronic inflammation (Kumar et al., 2013). The pathogenesis of many diseases and conditions, including several types of cancers, has been implicated in chronic inflammation (Moro et al., 2012).

Currently, inflammation is being treated using non-

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steroidal anti-inflammatory drugs. These drugs, despite being used to treat this condition, are associated with undesirable side effects, including renal damage, hyperglycemia, hypertension, gastrointestinal ulceration and bleeding among others. In addition to the side effects, the greatest shortcomings of currently available potent synthetic drugs are their toxicity and resurfacing of symptoms after discontinuation (Bhagyasri et al., 2015). These modern pharmaceuticals are also out of reach of a large proportion of population in developing countries because they are expensive.

It is against this background that the use of other sources of human knowledge be explored to provide common health benefits. Natural remedies to diseases are generally considered safe with little or no side effects. The screening of bioactive phytochemicals from plants has led to the discovery of new medicinal drugs with high efficacy in treatment and protection against diseases (Kumar et al., 2004; Sheeja and Kuttan, 2007).

Vangueria infausta is an edible fruit that grows in the wild in Zimbabwe. It is commonly consumed among the rural folk who are normally marginalised from modern health delivery system. The tree flowers from September to November and fruits in the November to April period where the fruit is abundant. The aim of this project was to explore the potential of *V. infausta*, an edible wild fruit from Zimbabwe, as a possible source of anti-inflammatory agents and hence recommend its use for nutraceutical purposes.

MATERIALS AND METHODS

Species collection

Ripe *V. infausta* fruits were collected from the Tsotsi Forest, in Insiza District of Bulawayo, Zimbabwe in January 2017. The fruits were identified by a worker at the National Herbarium of Zimbabwe, at the Harare Botanic gardens. The fruits are recorded under flora of Zimbabwe: individual record number 4211: *V. infausta*. The fruits were shade-dried for four weeks until constant weight was obtained. Pestle and mortar was used to grind the fruits to powder. Further grinding was done to reduce particle size using a grinding machine (Model: SM-45°C). The powder was stored in an air-tight plastic container until required for use.

Chemicals and standards

The standards used were indomethacin, an anti-inflammatory drug that was purchased from a local pharmacy and the flavonoid quercetin from Sigma Aldrich, South Africa. All other chemicals used were of analytical reagent grade and were also purchased from Sigma Aldrich, (South Africa) and Skylabs (South Africa).

Preparation of crude extract

15 g of powdered *V. infausta* sample were weighed using a Mettle Toledo digital analytical balance (model AB204-S, Ohio, USA) and mixed with 50 mL of analytical grade absolute ethanol in a 250 mL conical flask. This was done in triplicate and the samples were shaken for 30 min on a Labotec horizontal shaker (Midrand, South Africa). The samples were then filtered using Whatman No. 1 filter paper and placed in reagent bottles. The solvent maceration protocols were repeated three times and the collected filtrates were combined and concentrated under reduced pressure on a rotor vapour set at 40°C. This *V. infausta* sample is referred to as the crude ethanol extract in this study.

Extraction of the unsaponifiable fraction

The unsaponifiable fraction was extracted according to the method of Kovacs et al. (1979). 10 g of homogenized fruit samples were directly saponified in a round-bottom flask which had 25 mL of 50% KOH and 100 mL 95% ethanol. The mixture was refluxed for an hour with moderate stirring using a heating mantle and magnetic stirrer. The mixture was then cooled to room temperature and transferred to a separating funnel with the aid of 30 mL of 95% ethanol, 50 mL warm water and 50 mL cold water. The unsaponifiable fraction was extracted exhaustively 6 times with 150 mL portions of petroleum ether. The portions were then combined and washed with distilled water until soap-free and evaporated to dryness using a rotary evaporator at 40°C. The weight of concentrate was recorded as total unsaponifiable fraction (Jeong and Lachance, 2001). This sample of *V. infausta* is referred to as the unsaponifiable fraction in this study.

TLC isolation of flavonoids

Analytical thin-layer chromatography

This was done according to the method of Lihua et al. (2009) with some minor modifications. Thin layer chromatography (TLC) plates (10 x 1.5 cm) were activated by heating them at 100°C for about 10 min, and allowing them to cool to room temperature. Using a pencil and a ruler, pencil lines were drawn 1.5 cm from one edge of the plates. Extracts of samples were spotted on the pencil line using very thin capillary tubes. The plates were developed in a development chamber with a trial solvent. The solvent front was allowed to migrate up the TLC plate until it is about 1 cm from the top. The TLC plates were removed from the development chamber and the solvent front quickly marked with a pencil. They were air dried and then sprayed with 1% aluminium chloride solution, left to dry and then visualized under UV light at 365 nm. The positions of the flavonoids on the chromatograms were marked and captured on camera. The chloroform-methanol (10:1.25, v/v) gave the best separation of the spots.

Preparative thin-layer chromatography

Thick pre-coated silica gel plates measuring 20 cm x 20 cm were used. The solvent system used for the separation of phytochemicals was chloroform:methanol (10:1.25, v/v). Ethanol extracts of the fruit samples were deposited as a concentrated band 1.5 cm from the edge of the TLC plate and allowed to dry. The plates with dried samples were gently lowered into the development chamber, closed and left to develop. The plates were removed when the solvent had moved three quarters of the plates' length and the position of the solvent front immediately marked with a pencil. The retention factor (R_f) values of the different bands were calculated using the equation:

$$R_f = \frac{\text{Distance travelled by spot from origin}}{\text{Distance travelled by solvent from origin}}$$

Using a previously reported method (Mittal, 2013) with some modifications the bands that tested positive for flavonoids in the analytical TLC were scratched off, combined together mixed with 5 ml of absolute ethanol, allowed to stand for 10 minutes, filtered with Whatman No.1 filter paper and filtrate collected in glass vials. This *V. infausta* sample is referred to as the flavonoid fraction in this study.

Preparation of standard solutions

Precisely 36.7 mg of quercetin were dissolved in 25 cm^3 of methanol to form 1468 mg/L stock solution. This stock solution was serially diluted to give solutions of 800, 600, 400 and 200 mg/L. Similarly, 285.5 mg of indomethacin were also dissolved in 25 cm^3 of methanol to make a stock solution of 11420 mg/L which was serially diluted to give solutions of 200, 150, 100 and 50 mg/L as well as the 800, 600, 400 and 200 mg/L solutions.

Preparation of sample solutions for anti-inflammatory assays

The recovered solutions of crude ethanol extracts, unsaponifiable fraction and flavonoids fraction were serially diluted to produce solutions of concentrations 800, 600, 400 and 200 mg/L of extract and assayed for anti-inflammatory activity.

Anti-inflammatory activity assays

Preparation of phosphate buffer saline

2.725 g of anhydrous sodium dihydrogen orthophosphate, 0.800 g disodium hydrogen orthophosphate and 22.500 g sodium chloride were weighed on a Mettler Toledo digital analytical balance (AB204-S, Ohio, USA) and dissolved in distilled water. The solution was diluted to the mark with distilled water in a 250 mL volumetric flask. The pH was adjusted to 7.4 using 0.1 N HCl or NaOH.

In vitro inhibition of egg albumin denaturation

The anti-inflammatory activity of *V. infausta* crude ethanol extract, unsaponifiable fraction and flavonoids fraction were determined *in vitro* for inhibition of denaturation of egg albumin (protein) according to the method of Mizushima and Kobayashi (1968) with some modifications. 0.2 mL of 1% egg albumin solution, 2 mL of sample extract or standard and 2.8 mL of phosphate buffered saline (pH 7.4) were mixed together to form a reaction mixture of total volume 5 mL. The control was made by mixing 2 mL of triple distilled water, 0.2 mL 1% egg albumin solution and 2.8 mL of phosphate buffered saline to make a total volume of 5 mL. The reaction mixtures were then incubated at 37±2°C for 30 min and heated in a water bath at 70±2°C for 15 min. After cooling, the absorbance was measured at 280 nm by UV/Vis spectrophotometer (Genesys10S, ThermoFisher Scientific Inc., USA) using triple distilled water as the blank. The percentage inhibition was calculated using the relationship:

$$\text{Percentage Inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance control}} \text{x}100$$

Nitric oxide radical scavenging assay

This assay was done according to the method of Panda et al. (2009). The extracts were prepared and these were then serially diluted with distilled water to make concentrations from 200 to 800 mg/L. The freshly prepared solutions were refrigerated at 4°C for later use. Griess reagent was prepared by mixing equal amounts of 1% sulphanilamide in 2.5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid immediately before use. 0.5 mL of 10 mM sodium nitroprusside in phosphate buffered saline was mixed with 1 mL of the sample or standard in ethanol and incubated at 25°C for 180 min. The extract was mixed with an equal volume of freshly prepared Griess reagent. Control samples without the extracts or standard but with an equal volume of buffer were prepared in a similar manner as done in the test samples. The absorbance was measured at 546 nm using a Ultraviolet–visible (UV/Vis) spectrophotometer (Genesys10S, ThermoFisher Scientific Inc., USA) by using triple distilled water as blank. The percentage inhibition of the extract and standard was calculated and recorded. The percentage nitrite radical scavenging activity of the sample extracts or standard were calculated using the formula:

$$\%\text{NO scavenged} = \frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance ofcontrol}} \times 100$$

Statistical analysis

The results are expressed as mean ± standard deviation of three replicate measurements.

RESULTS

Table 1 shows the yield of crude ethanol extract, flavonoids fraction and unsaponifiable fraction of the *V. infausta* fruit.

Inhibition of egg albumin denaturation

The results in Figure 1 and Table 2 show that percent protein (albumin) denaturation inhibition generally increases in a dose-dependent manner, rising gradually for the crude and flavonoid fractions of *V. infausta* as well as for the quercetin standard. However, for the unsaponifiable fraction percent inhibition rises steadily with concentration from 200 to 400 mg/mL, decrease steadily and becomes constant (Figure 1). The unsaponifiable fraction had the highest inhibition of heat induced protein (albumin) denaturation that varied from 79.14±0.027% at 200 mg/L to 93.12±0.03% at 400 mg/L, crude ethanol extract, had least percent inhibition of 63.03±0.013% at 200 mg/L and highest inhibition of 79.91±0.042% at 800 mg/L, flavonoids fraction had least percent inhibition of 31.14±0.032% at 200 mg/L and highest inhibition of 54.40±0.061% at 800 mg/L, standard quercetin had the least percent inhibition of 30.41±0.036% at 200 mg/L and highest inhibition of 94.04±0.052% at 800 mg/L and that of the standard indomethacin drug (not shown in Figure 1 since its concentration range was 50 to 200 mg/L) was 76.70±0.073% at 200 mg/L. Quercetin standard protein denaturation inhibitory activity surpasses that of the

Table 1. Yields of crude ethanol extract, flavonoids and unsaponifiable fraction of *V. infausta.*

Name of fruit	Crude extract yield / g	Flavonoids yield / mg per g sample	Unsaponifiable fraction mg /g sample
V. infausta	3.16±0.02	1.89±0.04	294.15±0.24

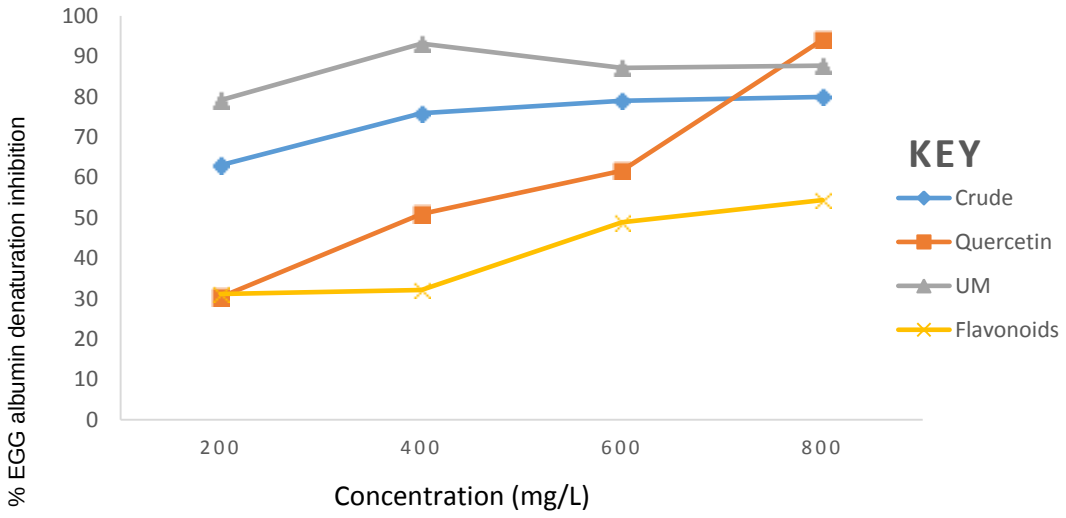

Figure 1. Variation of percent protein denaturation inhibition of crude ethanol extract, unsaponifiable and flavonoids fractions of *V. infausta* and quercetin standard.

Table 2. Inhibition of egg albumin denaturation by *V. infausta*, absorbance measured at 280 nm.

Concentration (mg/L)	% Inhibition of egg albumin denaturation				
	Crude ethanol extract	Flavonoids	Unsaponifiable fraction	Indomethacin	quercetin
200	63.03±0.013	31.14±0.032	79.14±0.027	76.70±0.073	30.41±0.036
400	75.89±0.018	32.14±0.033	93.12±0.030	-	50.95±0.039
600	78.99±0.032	48.90±0.045	87.12±0.041	-	61.67±0.043
800	79.91±0.042	54.40±0.061	87.66±0.044	-	94.07±0.052

unsaponifiable fraction at high concentration, close to 800 mg/L (Figure 1). Crude ethanol extract had the second highest inhibition of egg albumin denaturation, which was, however, surpassed by that of standard quercetin around 700 mg/L. The flavonoids had the least percentage inhibition of egg albumin denaturation throughout the concentration range studied (200-800 mg/L).

NO radical scavenging activity

Table 3 illustrates that, crude ethanol extract of *V. infausta* had NO radical scavenging activity, varying from 78.45±0.058% at 200 mg/L to 82.85±0.047% at 800 mg/L, flavonoids fraction had mild NO radical scavenging activity varying from 29.14±0.038% at 200 mg/L to 33.46±0.036% at 800 mg/L, whereas, unsaponifiable fraction varied from 28.82±0.046% at 200 mg/L to 68.99±0.058% at 800 mg/L. Indomethacin (a drug used to treat inflammation) had NO radical scavenging activity that varied from 48.90±0.037% at 200 mg/L to 90.29±0.074% at 800 mg/L and quercetin standard (a typical flavonoid) ranged from 38.58±0.057% at 200 mg/L to 94.15±0.076% at 800 mg/L. Figure 2 shows that between 200 and 600mg/L, crude ethanol extract of *V. infausta* had the highest NO radical scavenging activity. Indomethacin has the second highest NO radical scavenging activity between 200 and about 500 mg/L, but surpasses that of crude ethanol extract of *V. infausta* at concentrations greater than 700 mg/L. However, above 500 mg/L quercetin standard NO radical

Table 3. NO radical scavenging activity of *V. infausta*, absorbance measured at 280 nm.

Concentration (mg/L)	% NO radical scavenging activity				
	Crude ethanol extract	Flavonoids	Unsaponifiable fraction	Indomethacin	quercetin
200	78.45±0.058	29.14±0.038	28.82±0.046	48.90±0.037	38.58±0.057
400	81.73±0.038	30.45±0.040	32.90±0.037	60.90±0.041	45.64±0.046
600	81.87±0.044	32.42±0.046	38.96±0.044	73.55±0.056	83.17±0.053
800	82.85±0.047	33.46±0.036	68.99±0.058	90.29±0.074	94.15±0.076

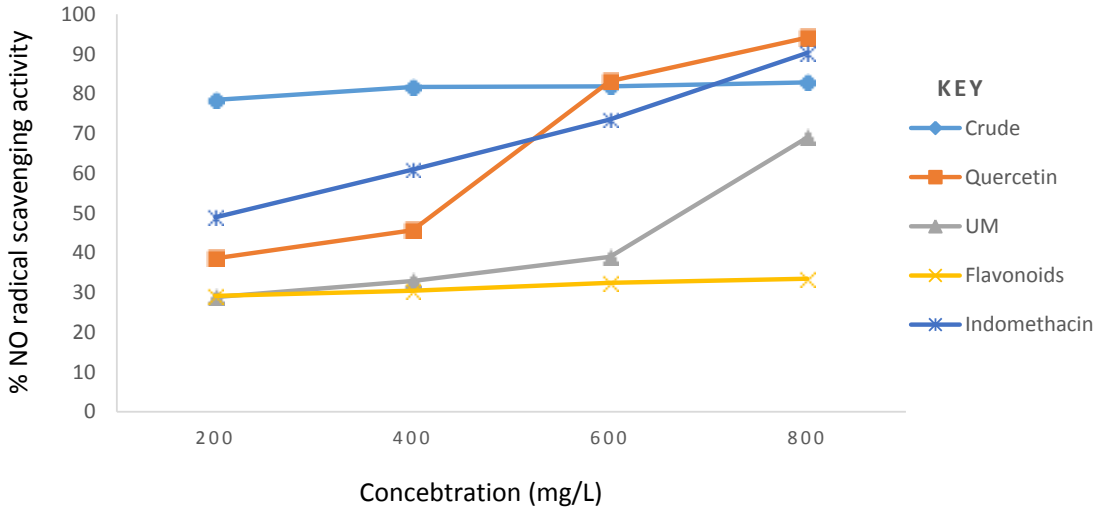

Figure 2. Variation of percent NO scavenging activity of crude ethanol extract, unsaponifiable and flavonoids fraction of *V. infausta* as well as quercetin and indomethacin standards

scavenging activity surpasses that of indomethacin and at 600 mg/L it has almost the same radical scavenging activity as crude ethanol extract of *V. infausta* and surpasses it beyond this concentration. Interestingly, the unsaponifiable fraction of *V. infausta*, which had highest inhibition of protein (albumin) denaturation, has low nitric oxide (NO) radical scavenging activity which suddenly rises at high concentration beyond 600 mg/L. The flavonoids fraction of *V. infausta* has consistently low NO scavenging activity which seems to be constant throughout the concentration range studied (Figure 2).

DISCUSSION

Protein denaturation is the loss of biological functional properties of protein biomolecules. This protein denaturation is a well recorded cause of inflammatory and arthritic conditions such as osteoarthritis, spondylitis, cancer, rheumatoid arthritis, to name a few (Chandra et al., 2012; Stevens et al., 2005; Sangeetha and Vidhya, 2016). Auto-antigens produced in certain arthritic conditions are due to denaturation of proteins *in vivo* (Opie, 1962; Umapathy et al., 2010). Consequently, inhibiting protein denaturation may be helpful in preventing inflammatory conditions. Phytocompounds that prevent protein denaturation are therefore suitable targets for the development of anti-inflammatory drugs.

The present study showed the *in vitro* anti-inflammatory activity (inhibition of protein denaturation) of *V. infausta* crude ethanol extract,as well as unsaponifiable and flavonoid fractions which were compared to quercetin and indomethacin standards. Figure 1 and Table 2 show that the crude and especially unsaponifiable fraction has very high inhibition of heat induced protein (albumin) denaturation over the range of concentrations studied (200 to 800 mg/L). The *V. infausta* flavonoids fraction also had significant anti-inflammatory activity (54.40±0.061%) but at high concentration (800 mg/L).

The unsaponifiable fraction at 200 mg/L had inhibition (79.14±0.027%) that was comparable to indomethacin (76.70±0.073%). Inhibition of protein denaturation by quercetin standard was comparable to that of *V. infausta* unsaponifiable fraction and crude ethanol extract at concentrations above 700 mg/L (Figure 1). It is evident from this data that *V. infausta* contains phytocompounds

that are suitable candidates for anti-inflammatory drug development. The unsaponifiable fraction is known to contain phytosterols, beta-carotenoids, tocopherols, hydrocarbons and terpenoids.

Terpenoids and steroids, the major constituents of the unsaponifiable fraction, are reported to possess anti-inflammatory activity (Perez, 2001). Terpenoids are reported to inhibit the development of chronic joint swelling (Bhagyasri et al., 2015). This could be the reason for the high protein denaturation inhibition of the unsaponifiable fraction of *V. infausta*. The likely constituents of phytocompounds in fruit of *V. infausta* are polyphenolic compounds, alkaloids, saponins, flavonoids, steroids and tannins. All these compounds are reported to have anti-inflammatory activity (Manach et al., 1996; Latha et al., 1998; Liu, 2003; Akindele and Adeyemi, 2007; Ilkay Orhan et al., 2007).

Alkaloids containing pyridine ring system have striking anti-inflammatory activity, for example, berberine from berberis is used to treat rheumatisms (Bhagyasri et al., 2015). The *in vitro* anti-inflammatory activity of crude ethanol extract of *V. infausta* could be attributed to the synergistic effect of these compounds. Mbukwa et al. (2007) have isolated a number of flavonoids including quercetin from aerial parts of *V. infausta*. Flavonoids are believed to be the major anti-inflammatory agents in plant sources (Bhagyasri et al., 2015). Some are reported to act as phospholipase inhibitors and some act as TNF-α inhibitors in inflammatory conditions (Bhagyasri 2015).

In addition, flavonoids are also known to inhibit cyclooxygenase and lipoxygenase pathways of arachidonic metabolism but this depends on their structure (Bhagyasri et al., 2015). In this study, although standard quercetin showed a steady increase in anti-inflammatory activity with concentration, the cocktail of flavonoid fraction of *V. infausta* did not exhibit the same trend (Figure 1). This could be attributed to the fact that the flavonoids could be working synergistically against each other in anti-inflammatory activity or other factors which could be their chemical structures since mechanism of inhibition is dependent on structure as suggested by Bhagyasri et al. (2015).

NO radical is a powerful pleiotropic link in physiological processes and a diffusible free radical in pathological conditions (Rintu et al., 2015). Nitric Oxide is produced in mammalian cells, and is responsible for various physiological processes, including fighting viruses and bacteria. However, excessive production of NO is associated with a number of ailments such as airway inflammation in asthma patients (Rao et al., 2016). The NO radical is known to react with superoxide radical anion (O_2^-) to form peroxynitrite ($ONOO^-$), which is a cytotoxic molecule (Rintu et al., 2015).

The protonated form of peroxynitrite, known as peroxynitrous acid (ONOOH) is a powerful oxidant (Malinski, 2007; Saumya et al., 2011). The damage caused by this powerful oxidant is through nitration or hydroxylation of aromatic compounds such as the amino acid tyrosine (Rintu et al., 2015). Peroxynitrite is reported to form an adduct with carbon dioxide dissolved in body fluids under physiologic conditions which causes oxidative damage to proteins in living systems (Sbazó et al., 2007).

In the Greiss assay, spontaneous decomposition of sodium nitroprusside in phosphate buffer generates NO radical which reacts with oxygen to form nitrite ions which are then estimated by UV-Vis after reaction with Greiss reagent. In the present study nitrite produced in the reaction mixture was reduced by *V. infausta* crude ethanol extract, the unsaponifiable fraction and the flavonoid fraction. This is due to anti-inflammatory phytocompounds which compete with oxygen to react with nitric oxide (Lalenti et al., 1993). The anti-inflammatory potency of *V. infausta* was evaluated for its NO radical scavenging activity. The NO radical scavenging activity of *V. infausta* crude ethanol extract was consistently high and constant varying from 78.45±0.058% at 200 mg/L to 82.85±0.047% at 800 mg/L (Figure 2).

The crude ethanol extract is a cocktail of different phytocompounds, which are likely working synergistically to scavenge for the NO radical. The unsaponifiable fraction of *V. infausta* had low NO radical scavenging activity at concentrations varying from 28.82±0.046% at 200 mg/L to 38.96±0.059% at 600 mg/L but rising sharply to 68.99 ± 0.058% at 800 mg/L (Figure 2). This could be due to the fact that phytocompounds that act by NO radical scavenging mechanism increase with increase in extract concentration.

The NO radical scavenging assay of flavonoid fraction of *V. infausta* was consistently low and constant throughout the concentration range studied, varying from 29.14±0.038% at 200 mg/L to 33.46±0.036% at 800 mg/L. Both the quercetin standard and the indomethacin standard showed a gradual increase in NO radical scavenging activity with increase in standard concentration. The radical scavenging activity of *V. infausta* crude ethanol extract is superior to each of the standards over a wide concentration range studied (200 to 600 mg/L for quercetin, and 200 to 700mg/L for indomethacin). This signifies that *V. infausta* has anti-inflammatory phytocompounds that could be drug targets for further development to anti-inflammatory drugs.

Although quercetin (a flavonoid standard) exhibits potent anti-inflammatory activity in both assays (Figures 1 and 2), it would be expected that the flavonoid extract of *V. infausta* exhibit the same or even better anti-inflammatory activity. It could be important to isolate and test anti-inflammatory activity of individual flavonoids. The result shown by flavonoids might imply that the anti-inflammatory activity exhibited by *V. infausta* is not due to one type of phytochemical only but a combination of them working in synergy. The result of the *V. infausta* unsaponifiable fraction in both assays reveals that the

phytocompounds mostly exhibit inhibition of protein denaturation mechanism rather than NO radical scavenging activity mechanism. The crude ethanol extracts likely exhibit both mechanisms.

Conclusion

The study has shown that the crude ethanol extract and unsaponifiable fraction of *V. infausta* has significant anti-inflammatory activity as assessed by two assays, inhibition of heat induced protein denaturation and NO radical scavenging activity. Isolated flavonoids also show acceptable anti-inflammatory activity especially with the inhibition of protein denaturation assay at high concentrations. A comparison of anti-inflammatory activity of *V. infausta* and the standards quercetin (a flavonoid) and indomethacin (drug used to relieve inflammation), in the inhibition of protein denaturation, shows that unsaponifiable fraction and the crude ethanol extracts has potent anti-inflammatory phytochemicals that could be developed to anti-inflammatory drugs. The phytochemicals are effective especially at lower concentrations. The yields of unsaponifiable fraction, crude ethanol extracts and flavonoids (Table 1) show that it could be economically viable to extract anti-inflammatory phytocompounds from *V. infausta.*

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors would like to express their sincere gratitude to the Bindura University of Science Education Research Board for providing funds for purchasing chemicals.

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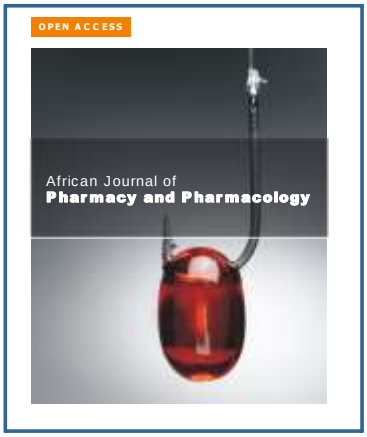

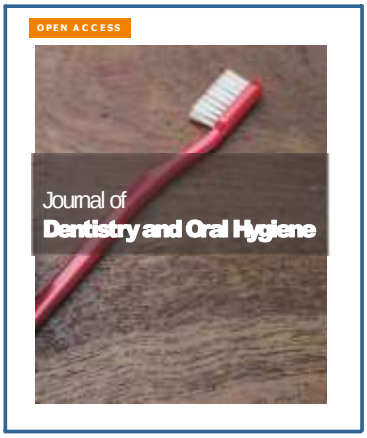

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