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EVALUATION OF THE INHIBITORY EFFECT OF A CANNABIDIOL DERIVATIVE AGAINST THE PLASMODIUM FALCIPARUM DIHYDROFOLATE REDUCTASE

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Abstract:

Introduction: *The alarming rate of drug failure against malaria necessitates the development and deployment of novel and highly efficacious antimalarial drugs. The use of natural plant remedies in curtailing the malaria catastrophe remains uncertain, as only a small fraction of plants has been evaluated and developed for their medicinal potentials. However, many communities in malaria-affected regions still rely on natural plants and herbs, despite documented concerns about efficacy and adverse effects. One plant of continuous controversy is* Cannabis sativa, *known mainly for its psychoactive effects but possesses other activities. Cannabidiol, an isolated constituent of cannabis has been reported to possess moderate antimalarial activity* in vitro.

Materials and Methods: *The Autodock Vina software was used in carrying out a molecular docking study on cycloguanil, cannabidiol and its C_2H_5 analogue against the Plasmodium falciparum dihydrofolate reductase. The physiochemical analysis, lipophilicity, solubility, pharmacokinetics and Lipinski drug likeness of these molecules were also evaluated through extensive structure-activity relationship study.*

Results: *The free binding energies of cycloguanil, cannabidiol and its modified analogue were -8.0, -8.9 and -8.9Kcal/mol respectively. The low binding score (more negative value) exhibited by cannabidiol and its C_2H_5 analogue shows they are respectively the better antimalarial agents. The inability of the C_2H_5 analogue of cannabidiol to cross the blood brain barrier as displayed in the structure-activity relationship results shows it poses no threat to normal brain function which makes it the most ideal antimalarial agent against the P. falciparum DHFR. All the experimental ligands exhibited high gastrointestinal absorption rates and are also moderately soluble in water.*

Conclusion: *The results showed that the C_2H_5 analogue of gedunin might be the most ideal antimalarial agent haven exhibited a low binding score and no interference with the CNS through BBB permeation. The laboratory synthesis and preclinical study on this compound is therefore recommended in order to confirm itsantimalarial potentials.*

Keywords: *Docking; Cannabidiol; Plasmodium falciparum; Blood Brain Barrier; Dihydrofolate reductase.*

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INTRODUCTION:

Malaria is a potentially fatal condition caused by the *Plasmodium sp*. carried by a specific type of mosquito. It is prevalent in tropical areas including Africa, Asia, Central and South America, some Caribbean and Pacific islands, and areas of the Middle East [1]. When an infected mosquito bites a human, the parasites are carried to the liver where they grow and multiply. They then enter the bloodstream, growing inside red blood cells and destroying them. This produces what is known as 'daughter parasites,' and the process continues, causing a variety of symptoms. When a mosquito bites and feeds on an infected person's blood, they take up these parasites along with their meal, allowing the disease to be spread from person to person [2].

According to statistics published on the World Health Organization website, there were 216 million cases of malaria in 2016 alone, with as many as 445,000 people dying from the disease. A shocking 80% of these deaths occurred in just 15 countries, all of which were in sub-Saharan Africa with the exception of India [3]. The majority of these countries are poor and do not have the budget to develop effective malaria prophylaxis programs or treatment strategies. The most at risk are the very young, pregnant women, and the elderly. These vulnerable people are more likely to suffer severe symptoms and complications as a result of malaria infection [4].

Malaria can be usually prevented with the use of certain prophylactic medications including chloroquine and mefloquine. However, some of the people most at risk of infection do not have easy access to these drugs [5]. Another worrying issue is that in many countries where malaria is prevalent, these drugs are becoming ineffective. Chloroquine resistance is now widespread in most malarial regions, and mefloquine resistance is rapidly spreading across South-East Asia, an area popular with travelers from all over the world [6].

The rapid spread of *Plasmodium falciparum* strains that are resistant to chloroquine and pyrimethamine/sulfadoxine (Fansidar) underscores the need for pharmacological initiatives to counter the resulting increases in malaria mortality and morbidity rates [7]. New antimalarial agents in such initiatives may be derived as novel compounds or modifications of existing drugs. One prophylactic drug that has undergone a resurgence of interest for use against malaria is the biguanide proguanil. This compound is cyclized by hepatic cytochrome P450 isoenzymes to the active metabolite cycloguanil, which has been reported to act on *P. falciparum* DHFR [8]. This enzyme, which in *P. falciparum* is fused with thymidylate synthase as a homodimeric bifunctional protein, catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate, thus providing a source of one-carbon donors for methyl transfer reactions and dTMP synthesis required for parasite survival [9].

One of the most significant problems with malaria is that many strains are now becoming drug-resistant, making it harder to prevent and treat this life-threatening disease. This issue has led scientists to begin investigating alternative treatments for malaria, including a number of plant-based medicines. One such medicine is cannabis, a plant which has been used as a traditional remedy for malaria for centuries. Cannabis in the twenty-first century is perceived primarily as the most widely used illegal recreational drug, but this relatively recent notoriety obscures its extensive utilization as a medicine throughout the world for several thousand years. Evidence of its use in treating malaria, constipation, pain, and dysmenorrhoea appear from oral tradition as early as 2600 B.C.E. in China and in following centuries it crops up in pharmacopoeias from Asia, the Middle East, Southern Africa and South America [10, 11].

The emergence of a multidrug-resistant strain of *Plasmodium falciparum* raises concern about malaria control strategies. Unfortunately, the role(s) of natural plants/remedies in curtailing malaria catastrophe remains uncertain. The claims of potential antimalarial activity of *Cannabis sativa in vivo* have not been well established nor the consequences defined. This study was therefore, designed to evaluate the inhibitory effects of the C_2H_5 cannabidiol derivative on the *P. falciparum* dihydrofolate reductase where cycloguanil serves as the control drug.

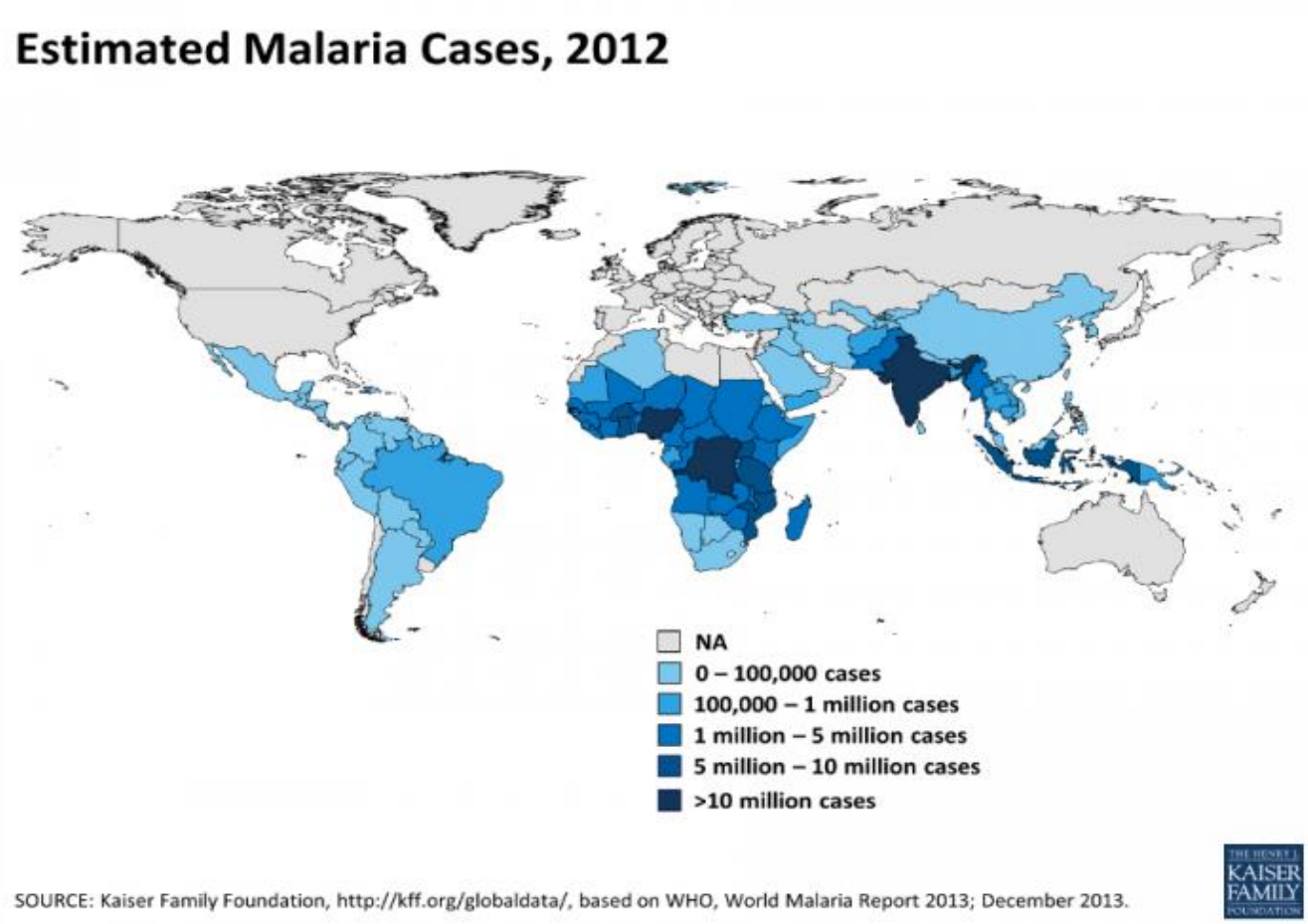

Figure 1: Estimated cases of malaria, 2012

MATERIALS AND METHODS:

Protein 3D Structure:

The crystallized 3D structure of the *Plasmodium falciparum* dihydrofolate reductase was downloaded from the Protein Data Bank (PDB) repository [12]. The Protein Data Bank (PDB) is a 3D (three-dimensional) database for structural proteins and nucleic acids which are large biological molecules [13]. Biologists and biochemists from all over the world submit these data which were obtained through NMR spectroscopy, X-ray crystallography and cryo-electron microscopy [14].

Ligand Preparation:

The 2D structure of cannabidiol and its C_2H_5 analogue were designed using the MarvinSketch software [15]. The designed structures were downloaded and saved as mrv files in preparation for docking.

Physiochemical Properties:

The *Plasmodium falciparum*physicochemical properties, i.e. molecular weight (MW), hydrophobicity, aromaticity, isoelectric point (pI), instability index, aliphatic index, grand average of hydropathicity (GRAVY) and amino acid composition were calculated by the ExpasyProtparam server [16].

Calculation of pharmacokinetic parameters and toxicity potential:

Chemical structures and SMILES notations ofcannabidiol, its C_2H_5 analogue and cycloguanil were obtained by using the OpenBabel software [17]. SMILES notations of these compounds were then fed into the online SwissADME server [18] to calculate various molecular properties and to predict bioactivity score for the drug target. The SwissADME server was also used in constructing the boiled-egg plot.

Molecular Docking:

The AutoDock module was used for molecular docking [19]. The crystal structure of *P. falciparum* DHFR was downloaded from the Protein Data Bank (PDB ID: 3UM8) [12]. All water molecules in 3UM8were deleted and polar hydrogen atoms added. An automatic docking protocol was applied and the 3UM8structure was utilized in the subsequent docking

experiments against the experimental ligands without energy minimization, with other parameters established by default in the software. Total scores were expressed in Kcal/mol to present binding affinities.

Secondary Structure Prediction:

The *P. falciparum* DHFR secondary structures were predicted using the Chou and Fasman secondary structure prediction server (CFSSP) [20]. The FASTA format of the amino acid sequence of interest was uploaded on the server which automatically predicts the secondary structure of the protein.

RESULTS:

Protein Secondary Structure

The secondary structures making up the *P. falciparum* DHFR were shown in Figure 2. Here, regions of the α-helices "H", β-sheets "E", turns "T" and coils "C" were shown in red, green, blue and yellow respectively. It also revealed the individual amino acids constituents of each secondary structure, showing the total number of residues alongside estimation in percentage.

Figure 2: Predicted secondary structures of *P. falciparum* DHFR

2D Structure of Cannabidiol:

Figure 3 shows the 2D structure of cannabidiol and its C_2H_5 derivative as designed by the MarvinSketch software. The modification that gave rise to this derivative was made through the substitution of the methyl group attachment to the carbon-14 (C_{14}) of cannabidiol with an ethyl (C_2H_5) group. The position of each atoms making of the cannabidiol structure were depicted in the 3D structure of the compound shown in figure 4

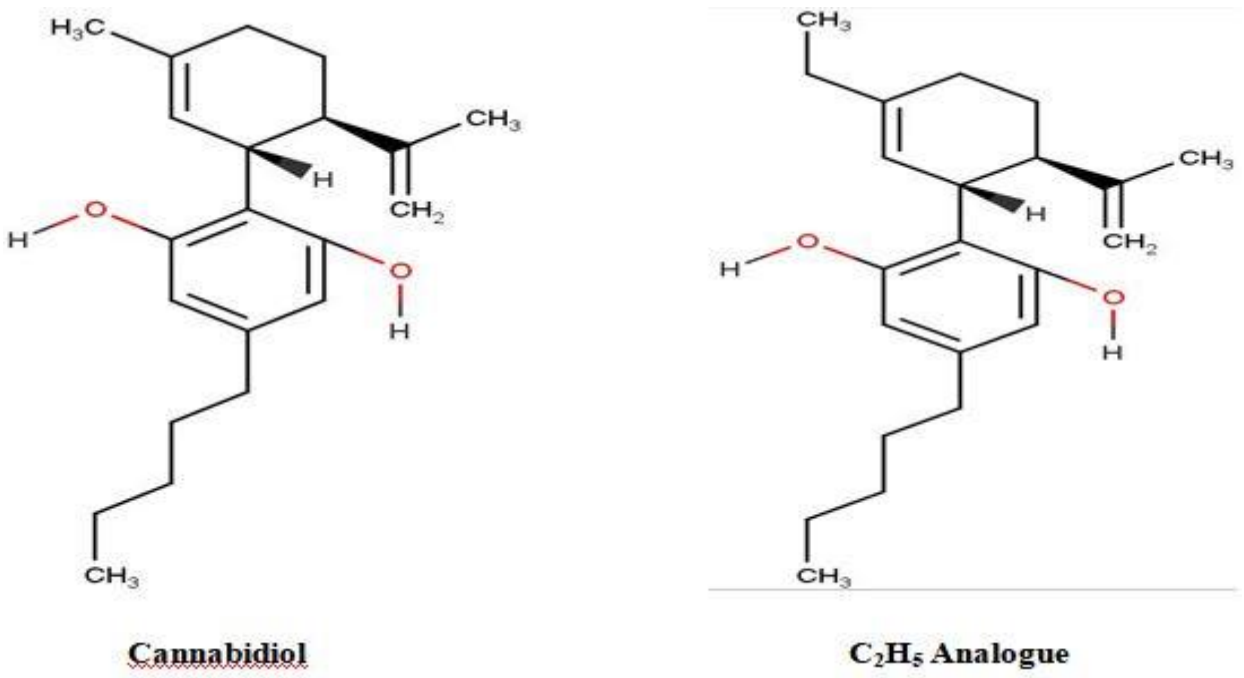

Figure 3. The two dimensional (2D) structure of cannabidiol and its modified derivative as designed using the MarvinSketch software.

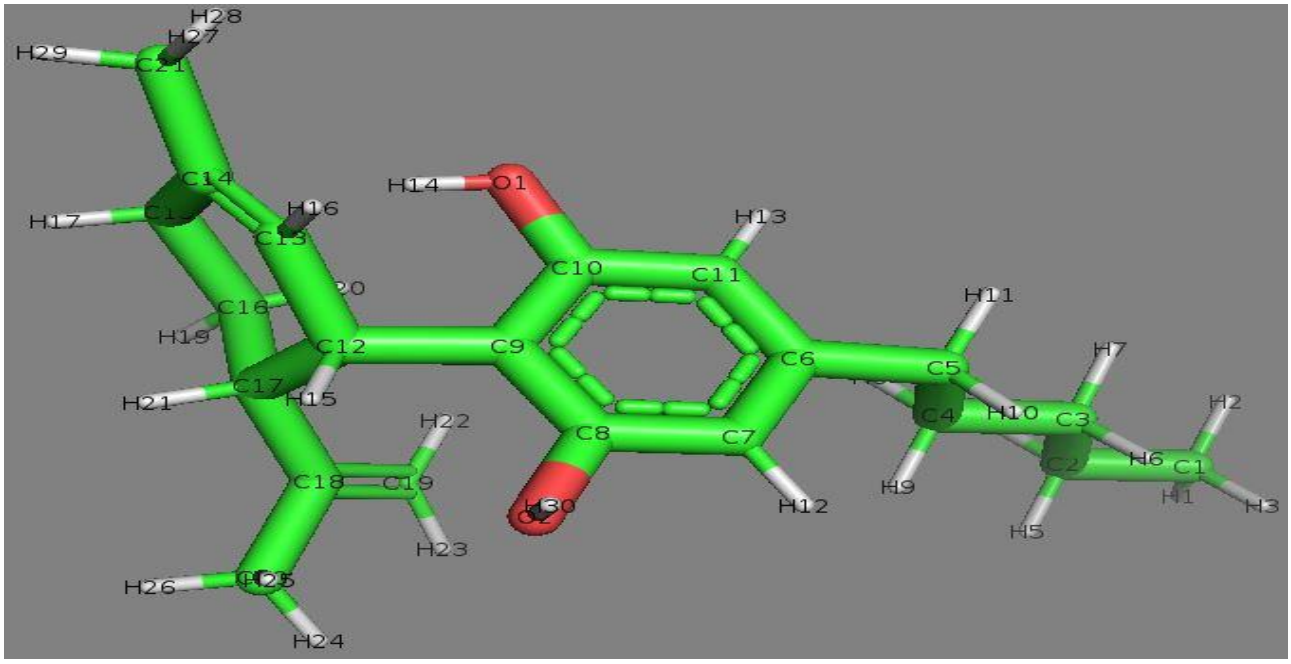

Figure 4: 3D structure of cannabidiol, showing all atoms making up the structure

Boiled-egg Plot:
The boiled-egg plot in figure 5 revealed the measure of gastrointestinal retention and cerebral entrance of the experimental ligands used for the purpose of this study. Cycloguanil, cannabidiol and its C_2H_5 analogue were designated molecule 1, 2 and 3 respectively as shown in the figure below.

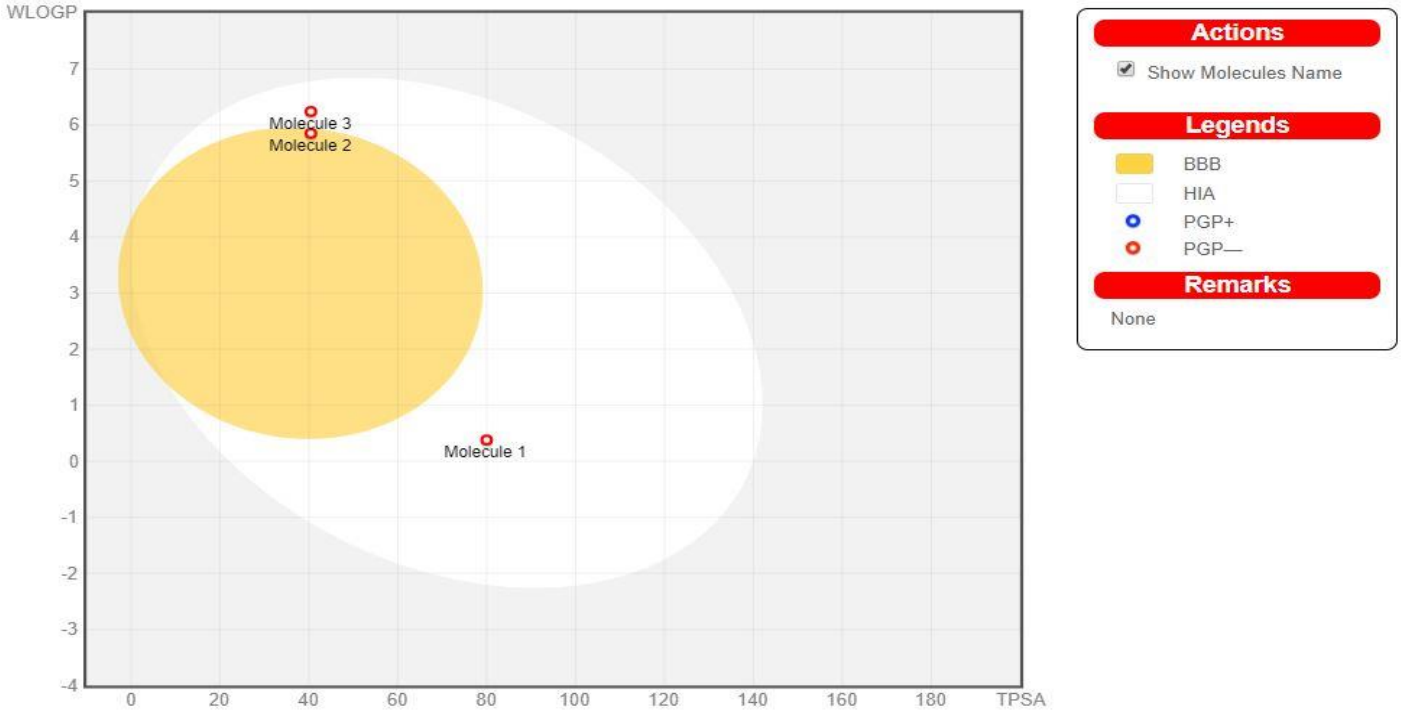

Figure 5: The boiled egg plot showing the rate of gastrointestinal absorption and blood brain barrier permeation.

Binding Energy Prediction and *In Silico* Pharmacokinetics:
Table 1 illustrates the specific pharmacokinetics and drug likeness parameters of each experimental compound. The binding energy scores which are pointers to the binding affinity of compounds to enzymes were also illustrated in the table.

Table 1: Drug likeness parameters of cycloguanil, cannabidiol and its modified analogue

Parameters	**Cycloguanil**	**Cannabidiol**	**C_2H_5 analogue**
Formula	$C_{11}H_{14}ClN_5$	$C_{21}H_{30}O_2$	$C_{22}H_{32}O_2$
Molecular weight g/mol	251.72	314.46	328.49
Docking score Kcal/mol	-8.0	-8.9	-8.9
Num. H-Bond acceptors	2	2	2
Num. H-Bond donors	2	2	2
TPSA Å²	80.00	40.46	40.46
Lipophilicity WLOGP (Log $P_{o/w}$)	1.35	5.85	6.24
Water Solubility Log S	Soluble	Moderately Soluble	Moderately Soluble
GI absorption	High	High	High

BBB permeant	No	Yes	No
P-gp substrate	No	No	No
CYP2C9 inhibitor	No	Yes	Yes
Lipinski Drug likeness	Yes; 0 Violation	Yes; 1 Violation	Yes; 1 Violation
Synthetic accessibility	3.27	4.05	4.16

Molecular Docking:

The binding energies which were predicted through the molecular docking experiment are shown in figure 6 and 7. The docking protocol also revealed the binding orientation and poses exhibited by the experimental compounds. The figures show the molecular docking score of cycloguanil and the C_2H_5 analogue of cannabidiol against the *P. falciparum* DHFR active site.

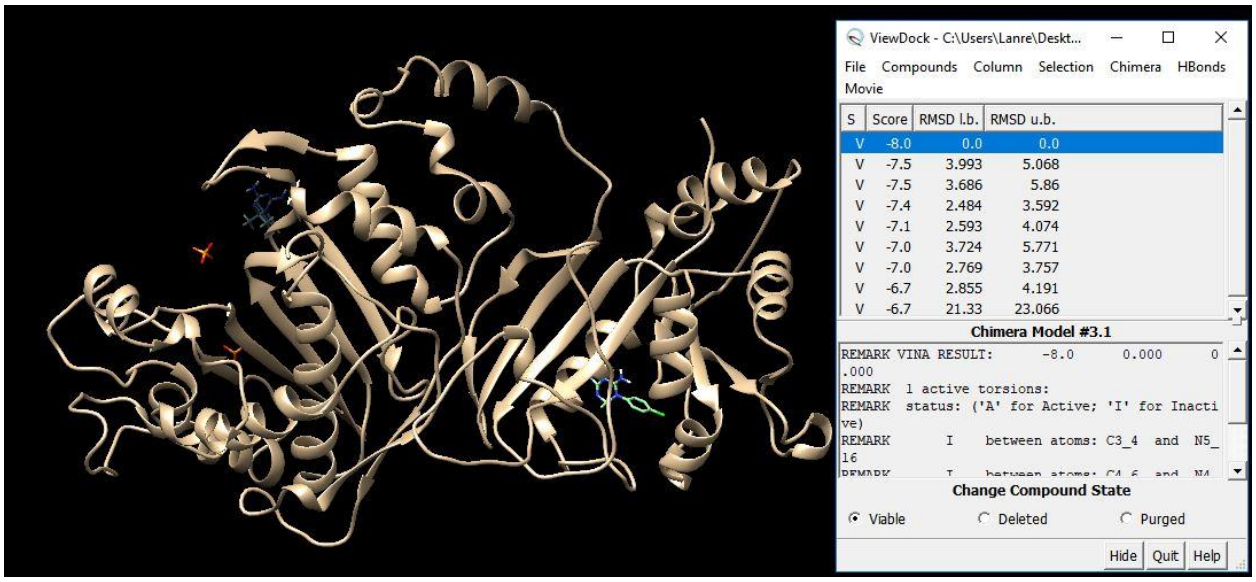

Figure 6: Cycloguanil in complex with *P. falciparum* dihydrofolatereductase

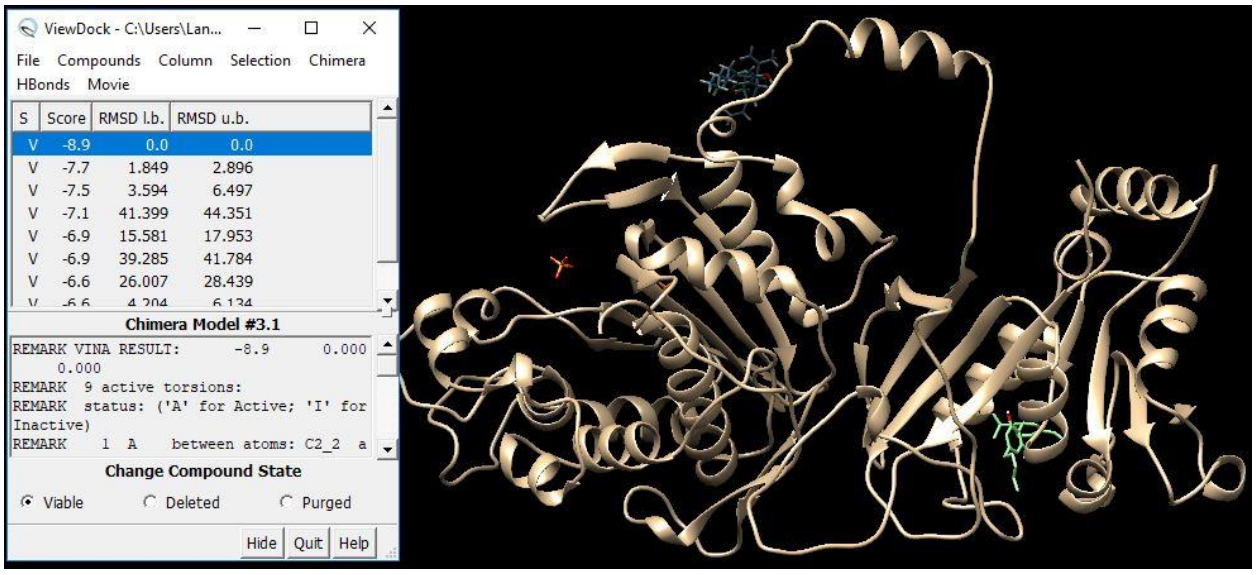

Figure 7: C_2H_5 analogue of cannabidiol in complex with*P. falciparum* dihydrofolate reductase

DISCUSSION:

The physicochemical property of proteins is critical for sustainability, efficiency, and stability in a biological system. Various physiochemical parameters of proteins such as amino acid composition, extinction coefficient**,** instability index, grand average of hydropathicity (GRAVY)**,** aliphatic index, theoretical *pI*, atomic composition and molecular weight allows us to understand the stability, activity and nature of the protein [21]. The result from the physiochemical property analysis of the *P. falciparum* DHFR showed that the enzyme is a slightly acidic enzyme with a theoretical *pI*value in the acidic range of 6.86. The protein is made up of a total of 608 amino acids as shown in table 1 with an estimated molecular weight of 71737.20daltons.The hydrophobicity scale produces a descriptive value for the relative hydrophobicity of residues of amino acids. The more positive or negative the value, the more hydrophobicity or hydrophilicity of the sequences located in that protein region [22]. The GRAVY calculator used in predicting the hydrophobicity assigned to the protein a value of -0.506.

The instability index describes the estimated level of protein stability in a test tube. A protein is said to be stable if its instability index is smaller than 40 and unstable if its instability index is greater than 40 [22]. The *P. falciparum* DHFR is therefore classified as a stable protein with an instability value of 35.23. The time taken for half the quantity of a cellular protein to disappear after it has been synthesized in the cell gives definition to the protein's half-life. ProtParam server uses the "N-end rule". This rule finds the relationship between the protein's half-life and its N-terminal residue [23]. The N-end rule started with the observation that the determination of the stability in vivo of any protein depends greatly on the N-terminal residue of the protein from [24]. Methionine (Met) is the N-terminal amino acid of the amino acid sequence hence, its estimated half-life in the mammalian reticulocytes in vitro is 1 hour. The extinction coefficient is the quantity of light absorbed by a protein at a specific wavelength. Studies have shown the possibility of estimating the molar extinction coefficient of a protein by the application of the knowledge if the composition of its amino acid [25]. The extinction coefficient of the enzyme was predicted to be 90620 M^{-1} cm^{-}.

The two most common secondary structural elements are the alpha helices and beta sheets, though beta turns and omega loops occur as well. Secondary structure elements typically spontaneously form as an intermediate before the protein folds into its three dimensional tertiary structure [33]. It has been shown that α-helices are more stable, robust to mutations and designable than β-strands in natural proteins [34], thus designing functional all-α proteins is likely to be easier that designing proteins with both helices and strands; this has been recently confirmed experimentally [35]. A total of 437 amino acid residues from the *P. falciparum* DHFR formed the secondary structure alpha helix while a total of 306 and 61 residues formed the beta pleated sheets and beta turns respectively. The high percentage helix (71.9%) exhibited by the alpha helix secondary structures of the phosphodiesterase-5 protein showed that the protein is a stable protein. This is also confirmed in theinstability index result from the ExPASy ProtParam analysis which predicted the*P. falciparum* DHFR as a stable protein.

Lipinski's rule of five which can also be referred to as the Pfizer's rule of five is a rule described for the evaluation of drug likeness or for the determination of biological and pharmacological activities in specific compounds for the purpose of evaluating physical and chemical properties in determining likely orally active drugs for administration [26]. The rule states that, orally active drugs in general must not violate more than one of the following criteria: The hydrogen bond donors must not be more than 5 (the summation of nitrogen–hydrogen and oxygen–hydrogen bonds), hydrogen bond acceptors must not exceed 10 (all nitrogen or oxygen atoms), the molecular mass of the compound must be less than 500 Da, an octanol-water partition coefficient (log *P*) must not exceed 5 [27]. The results obtained from Table 1 as regarding the lipinski's rule shows that cannabidiol and its C_2H_5 analogue violated one of the lipinski's rule, but this singular violation does not affect their drug likeness characteristics since the lipinski's rule allows the violation of not more than one of the rule of five.

The P-glycoprotein (P-gp) is involved physiologically in the reduction of the harmful effects of toxic compounds, xenobiotics and drugs which the body is exposed to by constantly pumping them out of cells. The need for the role played by the P-glycoprotein has led to the recognition of the modulation it confers on many important and clinical therapeutic agents and this pharmacokinetic importance has led to the incorporation of its screening in any process involving drug discovery [28]. Drug pharmacokinetic parameters can also be affected through various drug induced induction or inhibition directed at modulating drug transporters and this can lead to a significant drug-drug interaction [29]. Cannabidiol, its

C_2H_5analogue and cycloguanil were no substrates to the P-glycoprotein hence their oral bioavailability remains intact.

Cytochrome P450 constitute the most important family of biotransformation enzymes involved indrug metabolism, playing an important role in the disposition of drugs and their pharmacological and toxicological effects. It comprises a superfamily of isoformsthat are vital in the metabolism of drugs. The most common used isoforms are CYP3A4, CYP2C9, CYP2D6, CYP1A2, and CYP2C19. To maximize oral absorption, a successful CNS drug must have no significantCYP2C9 metabolism and be a nonpotent CYP3A4 inducer. CYP3A4 and CYP2C9 are of great interest because oftheir high probability to be involved in the metabolism of many coadministered drugs [30]. The oral bioavailability of cannabidiol and its C_2H_5 analogue might be higher than cycloguanil as shown in their CYP2C9 inhibitory effect in table 1.

The boiled-egg is a robust and intuitive graph prediction of passive intestinal absorption and brain penetration, as a function of lipophilicity and apparent polarity (described by WLOGP and TPSA, respectively). If plotted molecule falls inside the white ellipse, the probability of a good intestinal absorption is high. If plotted molecule falls inside the yellow ellipse (i.e. the yolk), the probability of a good BBB crossing is high. The white and yolk of the BOILED-Egg are not mutually exclusive and molecules predicted as not absorbed by the GI and BBB non-permeant are located in the grey area or even further outside the range of the plot [31]. The experimental compounds cycloguanil, cannabidiol and its C_2H_5 analogue were plotted in the boiled-egg plot (Figure 5). Perceptions demonstrate that every one of the compounds shows high GI assimilation. This perception legitimizes the position of cycloguanil and the C_2H_5analogue of cannabidiol (molecule 1 and 3 respectively) which falls in the white district of the boiled-egg plot as against the position of cannabidiol (molecule 2) which were spotted in the yolk (yellow) region of the boiled-egg plot.. None of the compounds fall in the dark locale of the plot, which affirms that every one of these mixes shows high GI ingestion with the exemption of cannabidiol which is largely BBB penetrable (Figure 5).

The molecular docking examination of this experiment was concentrated to investigate the firm binding of cycloguanil, cannabidiol and its modified analogue against the *P. falciparum* dihydrofolate reductase utilizing atomic docking approach [32]. Results from the docking experiment has shown that cannabidiol and its C_2H_5 analogue might be the best competitive inhibitor for the *P. falciparum* dihydrofolate reductase haven exhibited the lowest binding scores (Table 1) compared to the binding energy of cycloguanil which served as the control drug. This means that the C_2H_5analogues might be an alternative antimalarial agent against the *P. falciparum* dihydrofolate reductase for the treatment of malaria.

CONCLUSION:

In the bead to discover alternative therapeutic agents for the treatment of *P. falciparum*-linked malaria, the C_2H_5 cannabidiol analog was designed as a potentially effective oral dihydrofolate reductase inhibitor using series of computer-aided drug design processes, such as the in-silico ADMET and molecular docking protocols. The C_2H_5 analogue of cannabidiol haven been modified to lose its BBB permeation ability and retain its therapeutic properties has been shown to be the most ideal inhibitor for the *P. falciparum* DHPR in malaria treatment. This poor interaction with the CNS might make it highly effective in the treatment of the pre-erythrocytic intrahepatic forms of *P. falciparum*-linked malaria infections. In addition, in-silico ADMET study showed good properties for the C_2H_5 analogue of cannabidiol alongside its binding energy. We therefore recommend the laboratory synthesis of this compound for further preclinical studies with the focus on the discovery of alternative potent therapeutic agents for malaria treatment.

The overall results indicate that the molecular docking protocols and ADMET properties can be used to predict novel *P. falciparum* DHPR inhibitors and guide the further discovery of new potential therapeutic analogs.

Conflict of Interest

The authors declare there is no conflict of interest.

REFERENCES:

1. Crabb BS and Cowman AF (1996) Characterization of promoters and stable transfection by homologous and nonhomologous recombination in Plasmodium falciparum. ProcNatlAcadSci USA 93:7289–7294.
2. Crabb BS, Triglia T, Waterkeyn JG, and Cowman AF (1997) Stable transgene expression in Plasmodium falciparum. MolBiochemParasitol 90:131–144.
3. WHO. *World Malaria Report 2017*. Geneva: World Health Organization (2017).
4. WHO. *World Malaria Report. 2015*. Geneva: World Health Organization (2015).

5. Wooden JM, Hartwell LH, Vasquez B, and Sibley CH (1997) Analysis in yeast of antimalaria drugs that target the dihydrofolate reductase of Plasmodium falciparum. MolBiochemParasitol 85:25–40.
6. Wu Y, Kirkman LA, and Wellems TE (1996) Transformation of Plasmodium falciparum malaria parasites by homologous integration of plasmids that confer resistance to pyrimethamine. ProcNatlAcadSci USA 93:1130–1134.
7. Foote SJ, Galatis D, and Cowman AF (1990) Amino acids in the dihydrofolate reductase-thymidylate synthase gene of Plasmodium falciparum involved in cycloguanil resistance differ from those involved in pyrimethamine resistance. ProcNatlAcadSci USA 87:3014–3017.
8. Peterson DS, Milhous WK, and Wellems TE (1990) Molecular basis of differential resistance to cycloguanil and pyrimethamine in Plasmodium falciparum malaria. ProcNatlAcadSci USA 87:3018–3022.
9. Sirawaraporn W, Sathitkul T, Sirawaraporn R, Yuthavong Y, and Santi DV (1997) Antifolate-resistant mutants of Plasmodium falciparum dihydrofolate reductase. ProcNatlAcadSci USA 94:1124–1129.
10. R. Mechoulam. The pharmacohistory of cannabis sativa, in Cannabinoids as Therapeutic Agents, (Ed: R. Mechoulam). CRC Press, Roca Raton, 1986, pp. 1–19.
11. L. Grinspoon, J.B. Bakalar, Cannabis as a medicine, in Marijuana: The Forbidden Medicine. Yale University Press: New Haven, 1993, pp. 67–81.
12. Berman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., Shindyalov, I. N. and Bourne, P. E. (2000). The Protein Data Bank. *Nucleic Acids Research*, **28**(1): 235–242.
13. Laskowski, R. A., Hutchinson, E. G., Michie, A. D., Wallace, A. C., Jones M. L. and Thornton, J. M. (1997). "PDBsum: a Web-based database of summaries and analyses of all PDB structures". *Trends in Biochemical Science*, **22**(12): 488–490.
14. Anon, H. (2014). Hard data: It has been no small feat for the Protein Data Bank to stay relevant for 100,000 structures. *Nature*, **509**(7500): 260-268.
15. Chen, J., Swamidass, S. J., Dou, Y., Bruand, J., and Baldi, P. (2005). ChemDB: a public database of small molecules and related chemoinformatics resources. *Bioinformatics*. **21**(22): 4133–4139.
16. Gasteiger E., Hoogland C., Gattiker A., Duvaud S., Wilkins M.R., Appel R.D., Bairoch A. (2003). *Protein Identification and Analysis Tools on the ExPASy Server.[software]*.http://us.expasy.org/tools/protparam.
17. Noel, M. O., Michael, B., Craig, A. J., Chris, M., Tim, V. and Geoffrey, R. (2011). Hutchison Open Babel: An open chemical toolbox. *Journal of Cheminformatics*,**3:** 33
18. Antoine Daina, Olivier Michielin, Vincent Zoete (2017) SwissADME: a free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules. *Sci Rep.* 7: 42717. Published online 2017 Mar 3. doi: 10.1038/srep42717
PMCID: PMC5335600
19. Sousa, S. F., Fernandes, P. A. and Ramos, M. J. (2006). Protein-ligand docking: Current status and future challenges". *Proteins: Structure, Function, and Bioinformatics*. **65**(1): 15–26.
20. Chou PY, Fasman GD (1974). "Prediction of protein conformation". *Biochemistry*. **13** (2): 222–
245. doi:10.1021/bi00699a002. PMID 4358940
21. Gasteiger E., Hoogland C., Gattiker A., Duvaud S., Wilkins M.R., Appel R.D., Bairoch A.; Protein Identification and Analysis Tools on the ExPASy Server; (In) John M. Walker (ed): The Proteomics Protocols Handbook, Humana Press (2005). pp. 571-607
22. Kyte, J. and Doolittle, R.F. (1982) A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. 157, 105-132. [PubMed: 7108955]
23. Ciechanover, A. and Schwartz, A.L. (1989) How are substrates recognized by the ubiquitin-mediated proteolytic system? Trends Biochem. Sci. 14, 483-488. [PubMed: 2696178]
24. Bachmair, A., Finley, D. and Varshavsky, A. (1986) In vivo half-life of a protein is a function of its amino-terminal residue. Science 234, 179-186. [PubMed: 3018930]
25. Gill, S.C. and von Hippel, P.H. (1989) Calculation of protein extinction coefficients from
amino acid sequence data. Anal. Biochem. 182:319-326(1989). [PubMed: 2610349]
26. Lipinski, C. A., Lombardo, F., Dominy, B. W. and Feeney, P. J. (2001). Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Advanced Drug Delivery Reviews*, **4**(1–3): 3–26.
27. Leo A, Hansch, C. and Elkins, D. (1971). Partition coefficients and their uses. *Chemical Reviews*, **71**(6): 525–616.
28. Wolak, D. J.and Thorne, R. G. (2013). Diffusion of macromolecules in the brain: implications for

drug delivery. Molecular Pharmaceutics, **10**:1492–1504.
29. Williams, W. C. and Sinko, P. J. (1999). Oral absorption of the HIV protease inhibitors: A current update. *Advanced Drug Delivery Reviews*,**39**: 211–238.
30. GraafdeC, Vermeulen EPN, Feenstra NK. Cytochrome P450 in silico: an integrative modeling approach. *J Med Chem* 48:2726– 2755, 2005.
31. M. J. Waring, J. Arrowsmith, A. R. Leach, P. D. Leeson, S. Mandrell, R. M. Owen, G. Pairaudeau, W. D. Pennie, S. D. Pickett, J. Wang, O. Wallace, A. Weir, Nat. Rev. Drug Discov. 2015, 14, 475–486.
32. Seeliger, D. and De Groot, L. B. (2010) Ligand Docking and Binding Site Analysis with PyMOL and Autodock/Vina. *Journal of Computer Aided Molecular Design*, **24**: 417-422.
33. Schellman JA, Schellman CG (1997). "Kaj Ulrik Linderstrøm-Lang (1896–1959)". *Protein Sci*. **6** (5):1092 100. doi:10.1002/pro.5560060516. PMC 2143695. PMID 9144781.
34. Abrusan G, Marsh JA (2016). "Alpha helices are more robust to mutations than beta strands". *PLoS Computational Biology*. **12** (12): 1–16. doi:10.1371/journal.pcbi.1005242. PMC 5147804. PMID 27935949
35. Rocklin GJ, et al. (2017). "Global analysis of protein folding using massively parallel design, synthesis, and testing". *Science*. **357** (6347): 168–175. doi:10.1126/science.aan0693. PMC 5568797. PMID 28706065