Expression of The Fab Enzymes (Fab I And Fab Z) From *Plasmodium Falciparum* After Exposure To *Artemisia Afra* Plant Extracts And Drugs Screening

Ndeye Fatou Kane (nfatoukane86@live.fr)
Pan African University Institute for Basic siences, Technology and Inovation/ JKUAT (PAUISTI-JKUAT)
https://orcid.org/0000-0002-3846-9109
Mutinda Cleophas Kyama
Jomo Kenyatta University of Agriculture and Technology
Joseph Kangethe Nganga
Jomo Kenyatta University of Agriculture and Technology
Ahmed Hassanali
Kenyatta University
Mouhamadou Diallo
Université Cheikh Anta Diop de Dakar: Universite Cheikh Anta Diop de Dakar
Francis Kimani
KEMRI: Kenya Medical Research Institute

Research

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Abstract

Background:

The emergence and spread of drug resistance of the malaria parasite to the main treatment emphasis the need to develop new antimalarial drugs. In this context, the fatty acid biosynthesis (FAS_II) pathway of the malaria parasite is one of the ideal target due to its crucial role in parasite survival.

Method:

We report in this study the expression and the affinity binding of two Fab enzymes (FabI and FabZ) after exposure of the parasite using different extracts of the *Artemisia afra* and after a virtual screening with the different plant compounds. Two differents strains of *Plasmodium falciparum* was used: W2 (CQ_resistant) and D6 (CQ_sensitive) with a parasiteamia of 4%. The parasites were exposed during 2 days to different *Artemisia afra* extracts . Gene expression was done to determine the level of expression of the fab enzymes after treatments. A GCMS was run to determine the different compounds of the plant extracts following by a virtual screening between the fab enzymes and the active compounds using Pyrex.

Result:

The results showed different expression patterns of the Fab enzymes. FabI was downregulated in the W2 and D6 strains by the ethanolic extract in a higher rate for W2. Hexane and DCM extracts increased the production of FabI respectively in W2 and D6 strains. A different expression pattern was observed for Fab Z. The expression of FabZ in the W2 strain were all upregulated. The enzyme was downregulated only in the D6 strain when exposed to the ethanolic and hexane extracts of the plant. After virtual screening, a lot of compounds were found to interact with the two fab enzymes. Hits compounds for FabI and FabZ with high biding energy were detected. 11alpha-Hydroxyprogesterone and Aspidospermidin-17-ol were found to have high binding energy with FabI respectively (-10.7 kcal/mol; -10.2kcal/mol). Fab Z was having also a high affinity with the two following hits compounds: 11alpha-Hydroxyprogesterone (-10 kcal/mol) and Thiourea (-8.4 Kcal/mol).

Conclusion:

The study showed that *Artemisia afra* is a big source of antimalarial drugs, and could act not only as a curative but also as prophylactic due to its effects on the Fab enzymes.

Introduction

Malaria is still a big concern into the Africa health system due to its high number of death yearly. Africa is the most affected where more than 90% of the death occur in those sub-Saharan countries. Today we are facing a big challenge concerning the spread of the resistance to main drugs wildly spread in South East Asia a treat that can drive into Africa. New indigenous gene mutations of the *Plasmodium falciparum*

parasite were discovered in Rwanda in 2020. The mutation is about a new indigenous lineage concerning the *pf*kelch13 R561H that can drive Artemisinin resistance (1). This raises need for developing new drugs that can overcome that resistance. Researchers are getting more and more focused on the use of medicinal plants as a polytherapy that works better into overcoming parasite resistance. *Artemisia afra* is one of those promising medicinal plant with a high antimalarial effect and not toxic in high dosage. This plant is growing wildly from cape to the Eastern part of Africa, and where used for decades by traditional healers it is one of the best known medicinal plant. The list of uses covers a wide range of ailments from coughs, colds, fever, loss of appetite, colic, headache, earache, intestinal worms to malaria (2, 3, 4). In this project different strains of malaria parasite W2 (CQ resistant) and D6 (CQ sensitive) were exposed to different extracts of *Artemisia afra* collected from Burundi, to monitor the expression level of different genes Fab I and Fab Z that belong to the Fab enzymes, type II pathway implyed in the fatty acid biosynthesis of the malaria's parasite. Those Fab enzymes are crucial for the parasites survival and spreading mostly during the liver stage, and are targets for the development of new drugs candidate. Studying their expression when exposing the parasites to the plant extracts can bring a lot of informations about using those genes as target for new drugs.

Materials And Methods

1. Artemisia afra extraction:

1.1 Plant preparation:

Burundi at Rumonge South Burundi. The leaves were harvested before blooming and dried under shade before being sent to Nairobi in paper bags, then kept at room temperature until extraction.

1.2 Extraction Process

Samples of dried and powdered aerial part of *A. afra* weighing each 125 gm were extracted with 600 mL of Ethanol (100%), Hexane (85%), DCM (99.9%) and in water (800mL) in a flat bottomed flask and mixed on an orbital shaker. After gentle maceration for 48 hours, the extracts were filtered through Whatman filter paper n°1. The filtrate was concentrated under reduced pressure using rotary evaporator at 20 rpm and 40°C bath temperature. Finally, concentrated extracts were collected in vials and placed on a water bath at 40°C to evaporate the remaining solvents and stored at room temperature for complete dryness.

2. Gene expression study for Fab_Z and Fab_I from P. falciparum after exposition of the parasites to the crude extracts.

2.1 Culture preparation and incubation

Cultures of *Plasmodium falciparum* (W2 and D6) with a parasitaemia of 4% were incubated with *Artemisia afra* extracts collected from Burundi, e.g.: Burundi ethanolic, hexane and dichloromethane extract to run an inhibition test.

Artemisia afra solution was made for all the extracts. 100 mg of extracts were dissolved in 200ul of DMS; double distilled water was used to dilute the extracts. 20ml of double distilled water was added to the final concentration of 5ug/ul; then the solution was sterilized by filtering it through a microfilter of 0.45um pore size.

- 6ml of W2 cultures were incubated with 666ul of *A. afra* extracts (DCM,ETOH.and Hexane plant extracts) at a final concentration of 0.5µg/µl
- 6ml of W2 culture as negative control incubated with 666ul CMS with DCMS (final DMSO concentration was 0.1%)
- 6ml of D6 cultures were incubated with 666ul of *A. afra* extracts (DCM, EtoH,and Hexane plant extracts) at a final concentration of 0.5µg/µl

  6ml of D6 culture as negative control were incubated with 666ul CMS and DMSO (final concentration of DMSO was 0.1%).

The parasites were then kept for 2 days in the incubation room. After the inhibition test was done, the parasite were kept at -80°C. The samples were used later for RNA extraction to run gene expression of FAB_Z and FAB_I in each sample.

2.2 RNA extraction:

After the inhibition test was done, the total RNA for all samples (W2 and D6) was extracted by following the method of trizol reagent Invitrogen Company (http://tools.thermofisher.com/content/sfs/manuals/trizol_reagent.pdf).

The parasites were thawed in ice, the whole blood was centrifuged, and the supernatant was discarded. The pellet was lysed using 1ml Trizol, then Incubated at 37°C for 5 min to ensure the complete deproteinization of nucleic acids. 200ul of Chloroform was added, the tube was shaken vigorously by hand followed or using a vortex then centrifuged during 15 min at 12,000g at 4^{o}C. The upper aqueous phase was carefully removed and placed in a new tube. Isopropanol was added (to precipitate the RNA) then mixed and centrifuged at 12,000g at 4^{o}C during 15mn. The supernatant was discarded and the pellet suspended with 1ml of 75% ethanol then vortex briefly and centrifuge for 10mn at 7,500 g 4°C. The supernatant was discarded and the pellet air dried for 10mn. The RNA was resuspended with 40 ul of RNAs-free water and incubated in a heat block for 10 minutes at 60°C. The quantity and the quality of the RNA was determined respectively using Nano_Drop. The purity of the RNA sample was defined by the A260/A280 ratio. A ratio between 1.8 and 2.1 was indicative of highly purified RNA. The concentration of the extracted RNA was determined using the following equation:

RNA concentration (µg/µl) = (A260 * 40 * D)/1,000 where D = dilution factor

2.3 cDNA synthesis

The RNA samples were normalized by adding RNA free water to get a concentration of 50ug/ul of RNA for all samples; then the RNA was converted into cDNA by reverse transcription (RT). The oligo (DTs) primers were used for the reverse transcription. cDNA synthesis kit from Solis BioDyne was used for the reaction. The samples were incubated in PCR machine at 50°C during 45 mn to allow the reverse transcription reaction to take place and to be complete, then in 85°C during 5min to inactivate the enzyme and stop the reaction. The cDNA samples were then kept at -20°C awaiting further analysis.

2.4 FAB_Z FAB_I primers

Primers for FAB_Z and Fab_I were created using Prime 3 (http://bioinfo.ut.ee/primer3-0.4.0/) and ordered online. The primers were designed to avoid hairpins and self-annealing with a GC content around 40% – 50% and an annealing temperature of 60°C.

A stock solution of 100uM was prepared for each primers using PCR water buffer. 10ul from the stock solution was added to 90 ul of PCR water to prepare a working solution of 10uM.

- FAB_Z primer

OLIGO start len tm gc% any_th 3'_th hairpin seq

LEFT PRIMER 511 20 58.93 50.00 0.00 0.00 0.00 TTTGCTGGAGTGGATGGAGT

RIGHT PRIMER 687 24 59.91 41.67 0.00 0.00 0.00 CGATAAGGCAAACGTCATTTCTGA

Product size: 177 bp

- FAB_I primer

OLIGO start len tm gc% any_th 3'_th hairpin seq

LEFT PRIMER 333 22 60.36 50.00 0.00 0.00 0.00 CGGGTGGGGTATTGCTAAAGAA

RIGHT PRIMER 510 20 59.31 50.00 1.61 0.00 0.00 AGAAGCGTCAAAGGGTAGCA

PRODUCT SIZE: 178 bp

2.5 Conventional PCR for FAB_I and FAB_Z

The designed primers were tested during the conventional PCR, and their parameters set up. 5x FIREpol Master Mix ready to load with syber green dye was used for the conventional PCR. 4ul of a master mix containing DNA polymerase, 5x reaction buffer, 12.5 mM $Mgcl_2$, 1mM dNTPs, blue and yellow dye added into labelled PCR tubes 1ul of primers solutions were added cDNA samples were thawed and 2ul were added in each PCR tubes and topped up with water till 20 ul of the final volume. The sample was placed into the PCR thermocycler, and man run was set with different parameters until we find the right one that works better. The following amplification program was finally used for the two primers (FAB_Z and

FAB_I): Initial denaturation at 95°C for 5min and then 40 cycles of denaturation at 95°C for 1 min, annealing 60°C for 1min, extension 72°C for 1min, followed by the final extension at 72°C for 10min, and then held at 4°C. After gel electrophoresis was made to make sure that the primers got correctly amplified with those parameters set up.

2.6 Housekeeping gene for *P. falciparum*

To run gene expression of Fab enzymes: Fab_Z (Beta-hydroxyacyl-Acyl-carrier Protein Dehydratase) and Fab _I (Enoyl Acyl-Carrier-Protein Reductase) we needed a housekeeping gene as a reference which is necessary to calculate the expression level of the others genes during real-time PCR. In our study, Actin was chosen as the housekeeping gene for *Plasmodium falciparum*. Primers were made to amplify the actin gene during real-time PCR. Prime 3 was used to make the primers and parameters were set to fit the same parameters than for Fab_z and Fab_I.

- Actin Primers:

OLIGO start len tm gc% any_th 3'_th hairpin seq

FORWARD PRIMER 813 20 59.89 50.00 0.61 0.00 0.00 AGCAGCAGGAATCCACACAA

REVERSE PRIMER 976 20 60.11 50.00 0.00 0.00 0.00 TGGTTGATGGTGCAAGGGTT

SEQUENCE SIZE: 1131

PRODUCT SIZE: 164

A conventional PCR was run first to test the primers chosen for actin as amplified. The following parameters were used: Initial denaturation at 95°C for 5min and then 40 cycles of denaturation at 95°C for 1 min, annealing 60°C for 1min, extension 72°C for 1min, followed by the final extension at 72°C for 10min, and then held at 4°C. After gel electrophoresis was made to make sure that the primers got correctly amplify

2.7 Gel electrophoresis for PCR products

After running the conventional PCR for the genes and the housekeeping gene, a gel was prepared for the PCR product. Agarose gel (2%) in 2x TBE buffer was prepared. Ethidium bromide was included in the gel. Eight µl of each amplification reaction was loaded onto the gel. A molecular weight ladder was included and run at for 40 min.The gel visualized on UV trans-illuminator and photograph gel. The remaining of the 20 µl of the PCR product stored at -20°C.

2.8 Real-time PCR

After a success amplification for all the genes (Fab_I and Fab_Z) and the housekeeping gene (Actin) a Real-time PCR was run to study the expression of the genes Fab_I and Fab_Z when exposed to our active

extracts *Artemisia afra*. 5x HOT FIREPol EvaGreen qPCR mix plus (No Rox) from Solis BioDyne was used for the quantitative real-time PCR. The qPCR master mix composition was: Hot FIREpol DNA polymerase, 5x Evagreen qPCR buffer, 12.5mM $Mgcl_2$, dNTPs, Evagreen dye, No ROX dye. 96 wells plate for real-time PCR were used to set the reaction (Fig. 3.1). In each well 10 µl of total volume solution were prepared each containing 2µl of master mix for qPCR, 0.5 µl for the reverse primers, 0.5µl for the forward primers, 2ul for cDNA and 5µl of water. Each sample was tested in triplicate. LightCycler 96 software was used to set the parameters for Real-time qPCR and to visualize the results

3. Virtual screening with *Artemisia afra* compounds found during GCMS and the Fab enzymes

3.1 Gas Chromatography Mass Spectrometry (GCMS) Analysis of *Artemisia afra* extracts

Artemisia afra from Burundi was collected and extracted with three solvents: Dichloromethane, Ethanol, and Hexane. The extracts were dried using rotary evaporator and kept in the oven at 37C until complete evaporation of the solvents. Pure artemisinin crystal was used as a standard to test any presence of the compound in *Artemisia afra* extracts.

3.1.2 Sample Preparation

Samples were dissolved and diluted in suitable organic solvents i.e. dichloromethane and ethanol extracts were dissolved in methanol solvent, while hexane extract was dissolved in hexane solvent) and passed through carbon black to remove waxes and chlorophylls. The samples were then filtered through 0.45 µm PTFE filters then transferred to sample vials for GCMS analysis.

3.1.3 GCMS Method

A Shimadzu QP 2010-SE GCMS coupled to an autosampler was used for the analysis. Ultrapure He (99.999 %) was used as the carrier gas at a flow rate of 1ml / minute. A BPX5 non-polar capillary column, 30m; 0.25 mm ID; 0.25 µm film thickness, was used for separation. The GC was programmed as follows: 60 ˚C; 10 ˚C /min to 250 ˚C (10 minutes). Total run-time was 30 minutes. Only 1 µL of the sample was injected. The injection was done in split mode, 10:1. Injection was done at 200 ^{0}C. The interface temperature was set at 250 ^{0}C. The EI ion source was set at 200 ^{0}C. Mass analysis was done in full scan mode, 50–550 m/z. A solvent delay time of 2 minutes was used.

3.2 Drug screening with PyRx

After GCMS a library of compounds was prepared based on the GCMS results of *Artemisia afra* extracts from Burundi. The library was screened against two macromolecules (FAB_I FAB_Z) using Autodock Vina in PyRx 0.8 version [5]. PyRx is a Virtual Screening software for Computational Drug Discovery that can be used to screen libraries of compounds against potential drug targets. The software is open access and is available online at http://pyrx.sourceforge.net

3.2.1 Preparation of the library of small molecules

The 3D structures of all the compounds from GCMS were searched from three chemical structure database and downloaded. Three databases were used: ChemSpider (http://www.chemspider.com/), PubChem from NCBI (https://pubchem.ncbi.nlm.nih.gov/search/), and ChEMBL (https://www.ebi.ac.uk/chembl/) from the European Bioinformatics Institute (EBI). The small molecules (Compounds) were downloaded and visualize with Pymol then save in the same file in PDB format.

3.2.2 3D structure of *Plasmodium falciparum* Fab enzymes: Fab Z and Fab I

The 3D structure of the Fab enzymes: Fab_Z and Fab_I were downloaded from the RCSB protein database (PDB), (https://www.rcsb.org/). Fab_Z (PDB entry: 3AZA), [6]; Fab_I (PDB entry: 3LT0), [7]. The macromolecules were crystallized with their ligands. The macromolecules were open in txt format with notepad then all the ligands were removed to free the interaction sites.

3.2.3 Virtual screening with PyRx: Protocol

PyRx (Fig. 1) have the two virtual screening software Autodock 4.0 and Autodock vina. Autodock Vina was used during the drug screening because AutoDock Vina significantly improves the average accuracy of the binding mode predictions compared to AutoDock 4 and is faster (5).

Results

1. Gene expression results

1.1 Gel electrophoresis for PCR product

After parasite RNA extraction with trizol from the 8 samples, we got different concentration of RNA for each sample after measuring with a nanodrop. The concentration of RNA was normalized to get equal concentration for the 8 samples (experimentals and controls) before making the cDNA. A conventional PCR was then run to parameter the primers for the Fab enzyme genes and the housekeeping gene (Actine). A gel was run for the PCR product (Fig. 2).

The results showed that the primers had well amplified the Fab enzymes genes and also the housekeeping gene. Many parameters were set to get the best amplification. The amplification was optimum with these parameters: Initial denaturation at 95°C for 5min and then 40 cycles of denaturation at 95°C for 1 min, annealing 60°C for 1min, extension 72°C for 1min, followed by the final extension at 72°C for 10min, and then held at 4°C.

1.2 Real-time amplification

The same parameter for the conventional PCR was reported to do the real-time PCR for the gene expression study. The target genes were well amplified as shown in Fig. 3

In blue colour, we have the amplification for the housekeeping gene (Actin), in yellow the amplification for FabI and in red colour the amplification for FabZ, In grey the amplification of the negative control and for

the empty wells (that are drawn down of the picture). Actin has started first to be amplify following by FabI then by FabZ. Actin was well amplified and has the best fluorescent curve till 5.7 then followed by Fab I that was also well amplified with a fluorescent curve till 5.7 then for FabZ was also well amplified with a fluorescent curve of 4.8, but the amplification was less compared to the other genes.

1.3 Gene expression for Fab I after exposition of the parasites to A. afra extracts

The fold change expression of FabI and FabZ was calculated from the Ct (cycle threshold is defined as the number of cycles required for the fluorescent signal to cross the threshold) values generated during the real-time PCR, and the result is showed in Table 1 and 2 The fold change for the untreated sample is 1, for the treated samples when the fold change is greater than 1 it means that the targeted gene is upregulated and when it is lower than 1 it means that the targeted gene is downregulated. The Fab I gene from W2 strain of *P. falciparum* was upregulated with the hexane extract and downregulated by the ethanolic and dichloromethane extracts of the plant. In the second train for D6, we have a different path, FabI is upregulated by DCM extract and downregulated by hexane and ethanolic extracts. In the two strains, FabI appeared to be downregulated by the ethanolic extract of *Artemisia afra* (see Table 1).

Table 1
Fold change expression for FabI after treatment with *A. afra* extracts

EXTRACTS (*A. afra*)	**Fold difference W2**	**Fold difference D6**
Untreated	1	1
Hexane	**1.46**	0.47
EToH	0.34	0.37
DCM	0.74	**3.27**

The logarithm 10 of the fold change for FabI in each treatment was calculated to make the histogram (Fig. 4). The histogram is centered in 0 which is the log 10(fold change) of the untreated sample for D6 and W2. The up columns showed upregulation, and the down columns showed downregulation. Depending on the length of the column we can see the level of expression in both side when it is downregulated or when it is upregulated. In this figure Fab I is upregulated the hexane and DCM extract respectively in the W2 and D6 strain. However the upregulation appear to be 3 times higher in the D6 strain compared to W2 strain. Fab I downregulation is higher when exposed to the ethanolic extract in both strains.

1.4 Gene expression for Fab Z after exposition to A. afra extracts

For Fab Z in the W2 strain, the gene is upregulation when exposed to the three different extracts. For the D6 strain, the gene is also upregulated when exposed to the DCM extract but downregulated when

exposed to the others extracts. Fab Z is upregulated by the DCM extract in the two strains of *P. falciparum*.

Table 2
Fold change expression for FabZ after treatment with *A. afra* extracts

EXTRACTS (*A. afra*)	**Fold difference W2**	**Fold difference D6**
Untreated	1	1
Hexane	**1.7**	0.3
EToH	**2.4**	0.3
DCM	**2,9**	**4.7**

The log10 of the fold difference was calculated (Fig. 5) and the histogram made to show the expression of FabZ ; this allowed a better view of the scheme of expression for FabZ depending on the extracts where they are exposed. The histogram is centered in 0 the up columns showed upregulation, and the down columns showed downregulation.

In this figure (Fig. 5) the downregulation of the enzyme Fab Z by the ethanolic and the hexane extract in the D6 strain appear to be almost at the same level.

2. GCMS results

Tableau 3: Main compounds detected by GCMS from dichloromethane extract, hexane and ethanolic extract of *A. afra* and result for GCMS for Artemisinin standard

Standard/Extracts	Name of the compounds	Formula	Retention time (s)	Area %
	Cyclodeca[b]furan-2(3H)-one, 3a,4,5,6,7,8,9,11a-octahydro-3,6,10-trimethyl-		19.718	11.91
Artemisinin	Deoxyartemisinin		20.687	25.71
	1,8-Diazabicyclo[5.4.0]undec-7-en-11-one		20.909	13.3
	Bicyclo[4.1.0]heptane, 1-(3-oxo-4-phenylthiobutyl)-2,2,6-trimethyl-		22.897	49.08
A afra DCM	3,3,6-Trimethyl-1,4-heptadien-6-ol		5.964	1.11
extract	Eucalyptol		6.751	19.77
	1,5-Heptadien-4-one, 3,3,6-trimethyl-		7.054	6.87
	Cyclohexanol, 1-methyl-4-(1-methylethenyl)-, cis-		7.377	0.68
	Linalool		7.76	1.06
	5-Isopropyl-2-methylbicyclo[3.1.0]hexan-2-ol #		7.909	1.79
	Camphor		8.794	1.29
	Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl-, (1S-endo)-		9.176	31.87
	Hexane, 1,6-dimethoxy-		9.235	0.46
	Cyclohexanol, 2-methyl-3-(1-methylethenyl)-, (1.alpha.,2.alpha.,3.alpha.)-		10.013	0.81
	1-Acetoxy-p-menth-3-one		10.472	0.65
	1,7-Octadiene-3,6-diol, 2,6-dimethyl-		10.578	0.56
	Bornyl acetate		10.796	2.63
	(1S,5S,6R)-6-Methyl-2-methylene-6-(4-methylpent-3-en-1-yl)bicyclo[3.1.1]heptane		13.048	1.01
	Methyl (1R,2R,8aS)-2-(methoxycarbonyl)-2-hydroxy-5,5,8a-trimethyl-trans-decalin-1-acetate		14.151	0.52
	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-		14.602	0.52
	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate		14.854	0.71
	Caryophyllene oxide		15.165	1.08

Tableau 3: Main compounds detected by GCMS from dichloromethane extract, hexane and ethanolic extract of *A. afra* and result for GCMS for Artemisinin standard

	.alpha.-Cadinol	15.867	5.27
	(1S,4aS,7R,8aS)-1,4a-Dimethyl-7-(prop-1-en-2-yl)decahydronaphthalen-1-ol	16.258	2.86
	Pulegone	17.514	0.96
	2-Pentadecanone, 6,10,14-trimethyl-	17.837	1.64
	6-C14H26	20.551	0.42
	Ethyl 9,12,15-octadecatrienoate	20.636	0.52
	Phytol	20.704	5.28
	Geranyl ethyl ether 2	21.142	1.99
	11alpha-Hydroxyprogesterone	21.325	2.31
	11alpha-Hydroxyprogesterone	22.075	3.77
	1-Aminocyclopentanecarboxylic acid, N-(but-3-yn-1-yloxycarbonyl)-, but-3-yn-1-yl ester	22.97	1.59
A. afra Hexane	Bicyclo[2.2.1]heptane, 2,2-dimethyl-3-methylene-, (1S)-	5.4	2.68
extract	Eucalyptol	6.745	55.52
	1,5-Heptadien-4-one, 3,3,6-trimethyl-	7.048	24.19
	Undecane	7.621	2.45
	Dodecane	9.231	3.62
	Acetic acid, 1,7,7-trimethyl-bicyclo[2.2.1]hept-2-yl ester	10.783	3.72
	.alfa.-Copaene	12.167	2.95
	Tetradecane	12.221	1.24
	Caryophyllene	12.87	2.42
	4a,8-Dimethyl-2-(prop-1-en-2-yl)-1,2,3,4,4a,5,6,7-octahydronaphthalene	13.764	1.21
A. afra Ethanolic	3,3,6-Trimethyl-1,4-heptadien-6-ol	5.967	0.45
extract	Eucalyptol	6.75	5.58
	1,5-Heptadien-4-one, 3,3,6-trimethyl-	7.054	3.23
	2,7-Dimethyl-2,6-octadien-4-ol	7.417	1.01

Tableau 3: Main compounds detected by GCMS from dichloromethane extract, hexane and ethanolic extract of *A. afra* and result for GCMS for Artemisinin standard

	Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-, (1S)-	8.796	1.02
	Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl-, (1S-endo)-	9.173	30.12
	3,7-Octadiene-2,6-diol, 2,6-dimethyl-	9.227	0.69
	1,7-Octadiene-3,6-diol, 2,6-dimethyl-	10.573	1.11
	Bornyl acetate	10.794	0.96
	3-Cyclohexene-1-methanol, 2-hydroxy-.alpha.,.alpha.,4-trimethyl-	11.932	1.5
	(1S,2S,4S)-Trihydroxy-p-menthane	13.964	1.99
	4-O-Methylmannose	14.289	6.76
	.alpha.-Cadinol	15.863	1.73
	.alpha.-Methyl mannofuranoside	16.132	18.51
	(1S,4aS,7R,8aS)-1,4a-Dimethyl-7-(prop-1-en-2-yl)decahydronaphthalen-1-ol	16.253	2.06
	Longifolenaldehyde	17.907	3.05
	Pentadecanoic acid	19.154	0.75
	Longifolenaldehyde	19.276	1.56
	Phytol	20.703	1.67
	Corymbolone	21.316	3.57
	5.beta.-Androstan-3.alpha.,11.alpha.,17.beta.-triol	22.07	1.58
	2-((2R,4aR,8aS)-4a-Methyl-8-methylenedecahydronaphthalen-2-yl)prop-2-en-1-ol	24.025	3.35
	1H-1,3a-Ethanopentalen-5(4H)-one, 2,3-dihydro-	24.124	3.18
	Thiourea, 1-(adamantane-1-carbonyl)-3-cyclohexyl-	26.574	4.57

After the GCMS of extracts of *Artemisia afra* from Burundi, the results in the table 3 showed that the ethanolic and dichloromethane extract had a high content of Bicyclo [2.2.1]heptan-2-ol, 1,7,7-trimethyl-, (1S-endo)-. The hexane extract had the same compound but at low amount compared to ethanolic and DCM extracts. The major compound in the hexane extract is Eucalyptol (Area 55.52) this compound is

also found in DCM extract though at a lower amount (area 19.77) and also in ethanolic extract of *A. afra* (5.59). Hexane extract has fewer compounds compared to the others. Most of the compounds present in the ethanolic extract are also present in methanolic extract.

3. Drug screening with PyRx:

After GCMS of the ethanolic, hexane and dichloromethane extracts in total, 51 molecules were detected. The affinity of the molecules was tested against FabI and FAB Z with vina. After drug screening, the binding energy for each molecule was determined and expressed in Kcal/mol see Table 4. The results showed that many compounds have a high affinity with the Fab enzymes.

Table 4
Artemisia afra compounds from GCMS and Docking energy with FabI and FabZ

Ligands	Binding Energy (Kcal/mol) (FabI)	Binding Energy (Kcal/mol) (FabZ)
(1S, 2S,4S)-Trihydroxy-p-menthane	-6.4	-6.1
.alfa.-Copaene	-7.9	-7.1
.alpha.-Cadinol	-8.1	-7.4
.alpha.-Methyl_mannofuranoside	-5.7	-5.3
1_6-Dimethoxyhexane	-4.7	-4.9
1_7-Octadiene-3_6-diol__2_6-dimethyl-	-5.7	-6.6
1-Acetoxy-p-menth-3-one	-6.9	-6.9
1-Aminocyclopentanecarboxylic_acid_N-but-3-yn-1-yloxycarbonyl-but-3-yn-1-yl_ester	-7.1	-4.9
1H-1,3a-Ethanopentalen-5(4H)-one_23-dihydro-	-6.7	-6.5
2-((2R,4aR,8aS)-4a-Methyl-8-methylenedecahydronaphthalen-2-yl)prop-2-en-1-ol	-7.9	-6.1
2_2_4-Trimethyl-1_3-pentanediol_diisobutyrate	-6.8	-6.6
2_4H_-Benzofuranone__5_6_7_7a-tetrahydro-4_4_7a-trimethyl-	-7.2	-6.2
2_7-Dimethyl-2_6-octadien-4-ol	-6.3	-6.6
2-Pentadecanone__6_10_14-trimethyl-	-6.2	-5.5
3_7-Octadiene-2_6-diol__2_6-dimethyl-	-6.1	-5.2
3-Cyclohexene-1-methanol__2-hydroxy-.alpha._.alpha._4-trimethyl-	-7.0	-5.8
3-Isopropenyl-2-methylcyclohexanol	-6.7	-6.9
4a_8-Dimethyl-2-_prop-1-en-2-yl_-1,2,3,4,4a,5,6,7-octahydronaphthalene	-8.0	-5.6
4-O-Methylmannose	-6.2	-4.8
5.beta.-Androstan-3.alpha.11.alpha.17.beta.-triol	-9.7	-7.6
6-C14H26_tetradecyne	-5.8	-6.3
Acetic_acid_1_7_7-trimethyl-bicyclo_2.2.1_hept-2-yl_ester	-6.9	-5.9
Aspidospermidin-17-ol, 1-acetyl-19, 21-epoxy-15, 16-dimethoxy	-10.2	-7.6

Ligands	Binding Energy (Kcal/mol) (FabI)	Binding Energy (Kcal/mol) (FabZ)
Bergamotene	-7.5	-8.0
Bicyclo_2.2.1_heptan-2-one__1_7_7-trimethyl-___1S_-	-6.3	-6.7
Bicyclo_221_3d	-6.2	-6.6
Borneol	-6.5	-6.7
Bornyl_acetate	-6.9	-5.9
Camphor	-6.3	-6.7
Caryophyllene	-8.0	-7.3
Caryophyllene_oxide	-8.3	-7.0
Corymbolone	-8.6	-7.3
Dodecane	-5.0	-5.8
Ethyl_9_12_15-octadecatrienoate	-6.6	-5.7
Eucalyptol	-6.4	-5.9
Geranyl_ethyl_ether_2	-5.6	-5.9
Intermedeol_	-7.8	-7.0
Isoartemisia_ketone	-6.0	-6.0
Linalool	-5.8	-6.2
Longifolenaldehyde	-7.9	-7.1
Methyl__1R_2R_8aS_-2-_methoxycarbonyl_-2-hydroxy-5_5_8a-trimethyl-trans-decalin-1-acetate	-8.3	-6.2
Pentadecanoic_acid	-5.6	-4.8
Phytol	-6.4	-7.2
11alpha-Hydroxyprogesterone	-10.7	-10.0
Pulegone	-7.8	-5.7
Tetradecane	-5.2	-5.5
Thiourea, 1-(adamantane-1-carbonyl)-3-cyclohexyl	-9.0	-8.4
Thujanol	-6.3	-6.3
trans–terpineol	-6.8	-6.2
Undecane	-4.7	-5.8

The results showed some hit compounds for FabI and FabZ with high biding energy. 11alpha-Hydroxyprogesterone have high binding energy with FabI (-10.7 kcal/mol) and Fab Z (-10 kcal/mol). The second hit is Aspidospermidin, which also have a high binding energy with FabI (10.2kcal/mol). The third is Thiourea,- which have a binding energy of -9 kcal/mol for FabI and – 8.4 kcal/mol for FabZ.

The binding site for Fab I with 11 alpha-hydroxyprogesterone (hit 1) and with Aspidospermidin (hit 2) were shown respectively in Fig. 4.14 and 4.15. The 3D structure of FabI was download from PDB, and the active sites were determined before docking. After the virtual screening, the ligands were found to bind inside the active site and were in interaction with many residues in the active site (Fig. 6, 7).

Residue with hydrophobic interaction: Ile 369, Phe 368, Tyr 267, Tyr 277, Ala 312, Gly 313, Ser 215, Pro 314, Tyr 111, Leu 265, Ala 320, Ala 319 and hydrogen bound with Ser 317.

Residue with hydrophobic interaction: Tyr 277, Tyr 267, Ala 320, Gly 313, Leu 315, Tyr 111, Ser 317, Gly 110, Met 281, Ala217, Ala 319, Ile 323, Lys 285, and Thr 266.

The binding site for FabZ with 11 alpha-hydroxyprogesterone (hit 1) and Thiourea,-(hit2) were shown respectively in Figs. 8 and 9. There is mostly hydrophobic interactions with the ligands, and also one hydrogen bind between Thiourea and the residue Tyr 100(G).

Residue with hydrophobic interaction: Tyr 100(I), Pro 101(K) 267, Tyr 100(H), Asn 131(K), Asn 131(I), Tyr 100 (L), Pro101(L), Pro 128 (K), Tyr 100(K), Asn 131(L) and Pro 128(L).

Residue with hydrophobic interaction: Phe 129(J), Pro 101(J), Pro 101(I), Tyr 100(I), Pro 128(I), Tyr 100 (I), Tyr 100(K), Asn 131 (L), Phe 129(J), Pro 128(J) and one hydrogen bond with Tyr 100(G).

Discussion

Inhibition test was run by incubating during 48hours the parasite cultures with *A. afra* extracts to study the expression level of the *P. falciparum* Fab enzymes (FabI and Fab Z). The fab Enzymes play an important role in fatty acid synthesis pathway (FAS II). The prodigious proliferative capacity of malarial parasites necessitates access to an abundant source of fatty acids (FAs). These carboxylic acid-linked acyl chains are required for the production of lipid species that are essential for parasite membrane and lipid body biogenesis [8]. It has been shown that FAS II can be exploited for antimalarial drug discovery [9, 10]. In our results, FabZ (D6 strain) was found to be downregulated by the ethanolic and hexane extract of *A. afra*. Fab Z is the primary dehydratase that participates in the elongation cycle of saturated as well as unsaturated fatty acid biosynthesis [11]. Downregulation of FabZ can lead to parasite death due to his crucial role in the fatty acid de novo biosynthesis. Fab I also play a crucial role during liver-stage of malaria infection [12]. A downregulation of Fab I can lead to a failure of the parasite to complete the liver stage by an inability to form intrahepatic merozoites that normally initiate blood-stage infection. The results showed a downregulation of Fab I by the ethanolic and the DCM extracts of *Artemisia afra* for the W2 strain. In the D6 strains the gene is also downregulated by the hexane and ethanolic extracts; this

indicated that *Artemisia afra* could act as a drug with a prophylactic effect that can be able to stop the parasite in the earlier liver stage before the spread into the bloodstream.

GCMS of Artemisia afra extracts

GCMS was ran in all the three extracts: ethanolic; hexane and dichloromethane of *Artemisia afra* leaves from Burundi. 10 compounds were found in hexane extract; the major compound was Eucalyptol (55.5%). Cineole or Eucalyptol has mucolytic, bronchodilating and anti-inflammatory properties and reduces the exacerbation rate in patients suffering from COPD (chronic obstructive pulmonary disease), as well as ameliorates symptoms in patients suffering from asthma and rhinosinusitis [13]. In the dichloromethane extract, 30 compounds were detected by GCMS, and the major compounds were Borneol (33.5%) and Eucalyptol (21.9%). Borneol is a common ingredient in many traditional Chinese herbal formula and has a wide range of uses, It aids the digestive system by stimulating the production of gastric juices; tones the heart and improves circulation; treats bronchitis, coughs, and colds; can relieve pain caused by rheumatic diseases and sprains; reduces swelling; relieves stress; and can be used as a tonic to promote relaxation and reduce exhaustion (http://acupuncturetoday.com/herbcentral/borneol.php). In the methanolic extract, the major compound was also Borneol (Bicyclo [2.2.1] heptan-2-ol, 1, 7, 7-trimethyl-, (1S-endo) (40.6%). Majority of the compounds found in the ethanolic were also found in the dichloromethane and some also in the hexane extract. The same major compound was found by Josphat *et al.* when analysing the essential oil from *Artemisia afra* by GCMS. The major constituents were 1, 8-cineole (67.4%), terpinen-4-ol (6.5%) and borneol (5.1%) [14]. The medicinal effect attributed to that plant can be because of the presence of all those active compounds. The first line against malaria is artemisinin and derivatives, which are extracted from *Artemisia annua*. Artemisinin was not detected in all three extracts of *Artemisia afra*, so this means that the antimalarial activity laid on the other actives compounds of that plant.

Affinity binding with FabI and FabZ

A library was created based on the compounds found during GCMS and was screened against the two fab enzyme (Fab I and FabZ) to test virtualy their potential interactions using Autodock Vina with PyRx.

Autodock Vina is a docking software that aims to predict the ligand-protein complex structure interaction by exploring the conformational space of the ligands within the binding site of the protein. A scoring function is then utilized to approximate the free energy of binding between the protein and the ligand in each docking pose [15].

After the virtual screening all compounds were found to display a binding affinity that varies from high to low binding energies with the Fab enzymes, this may indicate that the all active compounds of *Artemisia afra* may play a significative rule by interacting with the *P. falciparum* Fab enzymes, which has a significant role in parasite survival during malaria infection. Comparison of the different binding energies for all molecules have shown up some dock hits. It was found that 11 alpha-Hydroxyprogesterone, Aspidospermidin-17-ol, 1-acetyl-19, 21-epoxy-15, 16-dimethoxy, and Thiourea, 1-(adamantane-1-

carbonyl)-3-cyclohexyl were the efficient binders, with high binding energy. There is no literature about the pharmacological effects of Aspidospermidin- and Thiourea, 1-(adamantane-1-carbonyl)-3-cyclohexyl. 11alpha-Hydroxyprogesterone has recently been patented for the treatment of skin diseases, particularly psoriasis, it is an important pharmaceutical compound with anti-androgenic and blood-pressure-regulating activity [16]. Those active compounds were also found to interact with most of the residue of the active side of Fab I. However, an *in vitro* test is necessary to confirm their antimalarial activity.

Conclusion

This study shows the high potential of the use of *Artemisia afra* as a source in the search of new antimalarial drugs. The FAS II pathway constitute a prime target for the development of prophylactic and curative drugs.

Abbreviations

GCMS: Gas Chromatography Mass Spectrometry

Ct: Cycle Threshold

PTFE: Polytetrafluoroethylene

KEMRI: Kenya Medical Research Institute

A: Actin

FZ: FabZ

FI: FabI

N: Negative Control

L: Ladder

Declarations

- Trial registration:

No applicable

- Ethical approval:

Ethical approval was provided by KEMRI (Kenya medical research institute).

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- Consent for publication:

No applicable

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- Competing interest

The authors declare no competing interest.

- Availability of data and materials

Not applicable

- Authors contribution

Dr. Ndeye Fatou Kane: Corresponding author, Study Design, performs experimentations, Data analysis, and Article writing.

Dr. Mutinda Cleophas Kyama: Study Design, Data analysis,Writings

Dr. Joseph Kangethe Nganga : Study Design, Writings

Pr. Ahmed Hassanali: Study Design, writtings

Dr. Mouhamadou Diallo : Study Design, writing

Dr. Francis Kimani: Bring facilities to perform experimentations.

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Figures

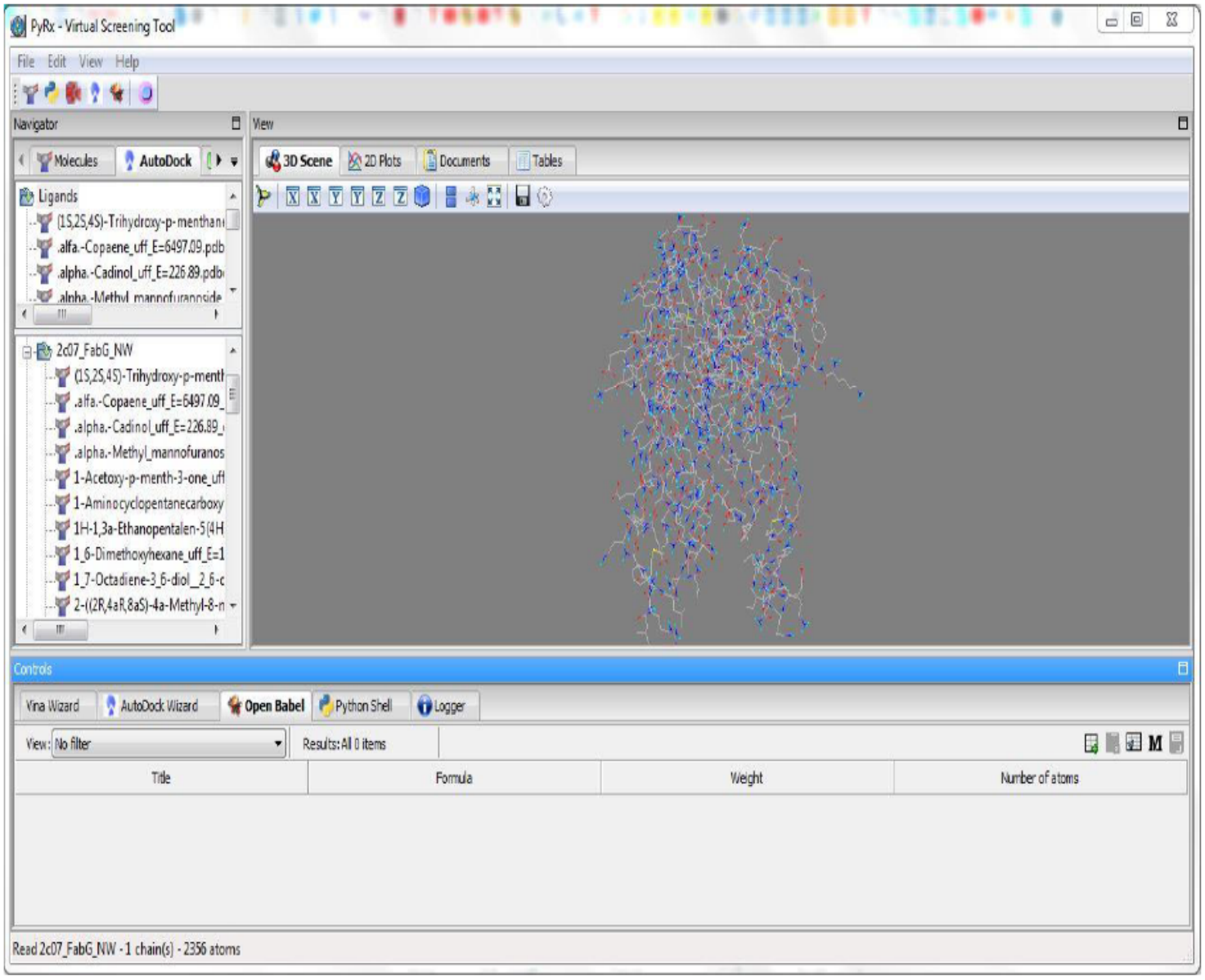


Figure 1

PyRx virtual screening interface.

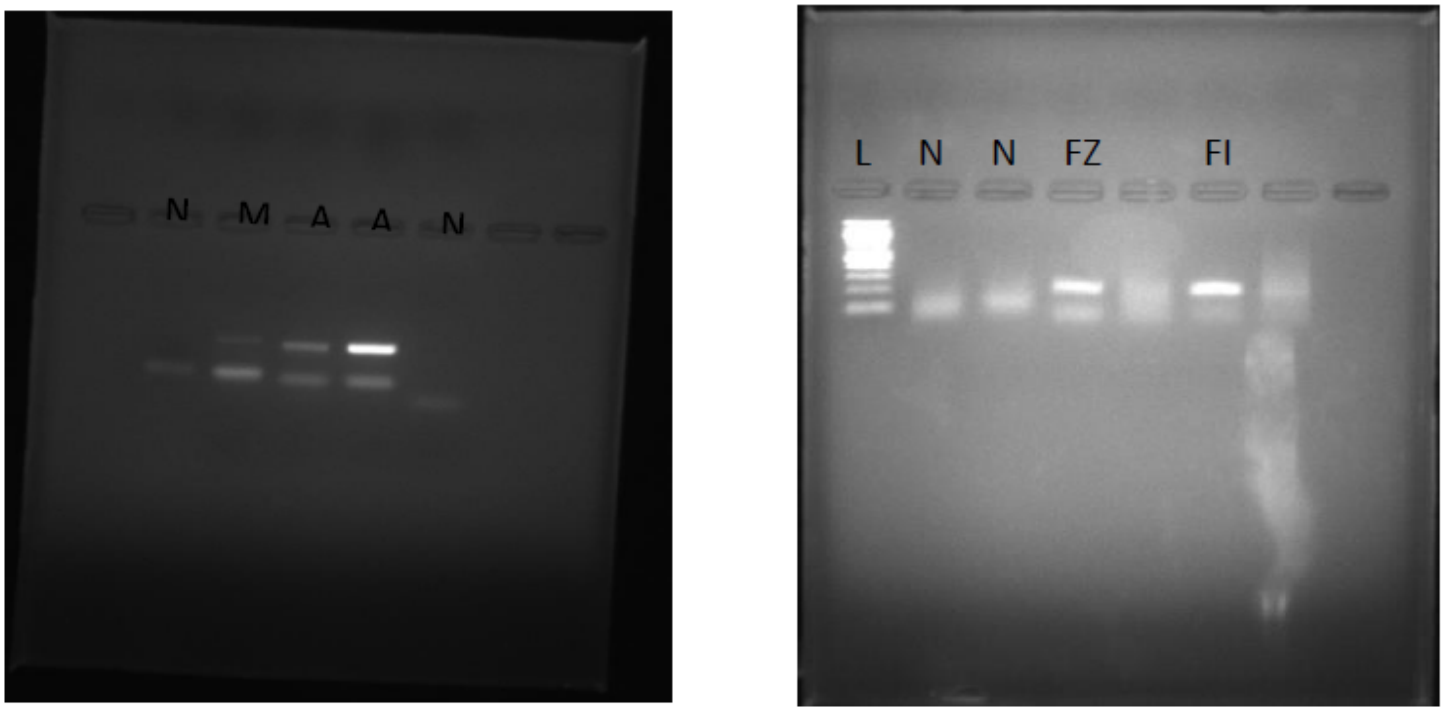


Figure 2

Gel electrophoresis of Actine FabI and FabZ A: Actine FZ: FabZ FI: FabI N: Negative control L: Ladder

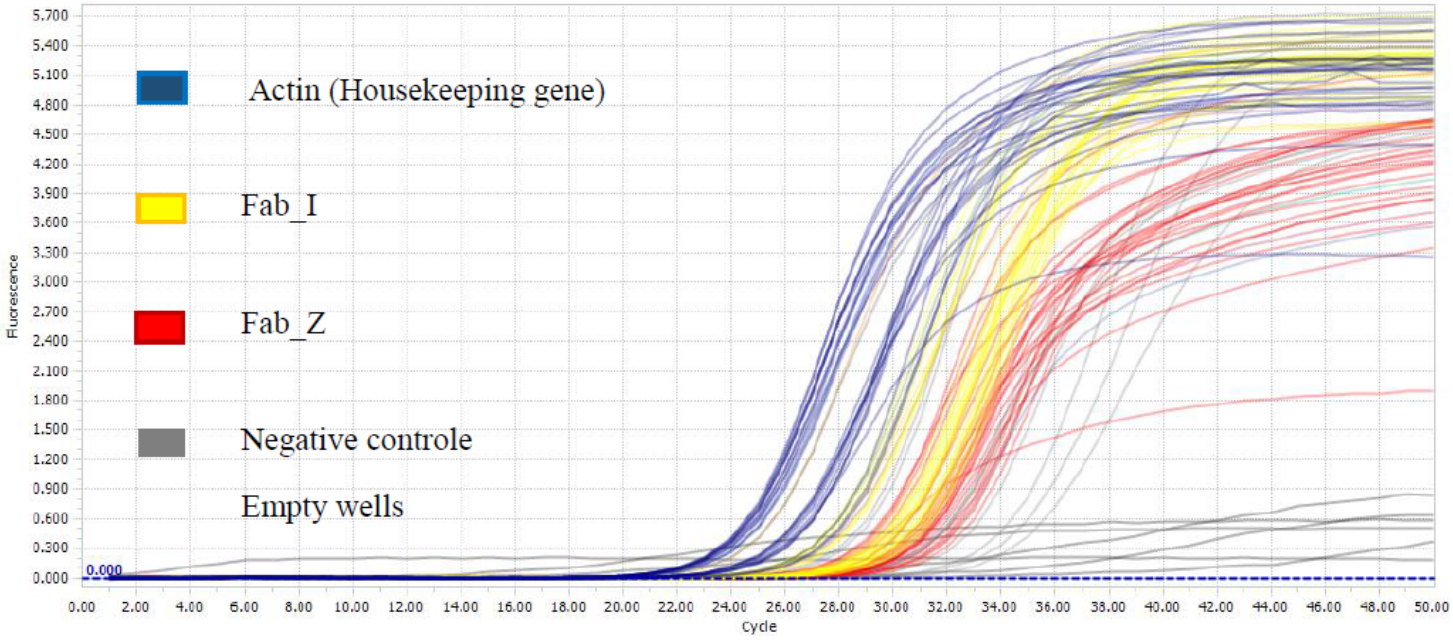


Figure 3

Amplification curves for FabI, FabZ and Actin

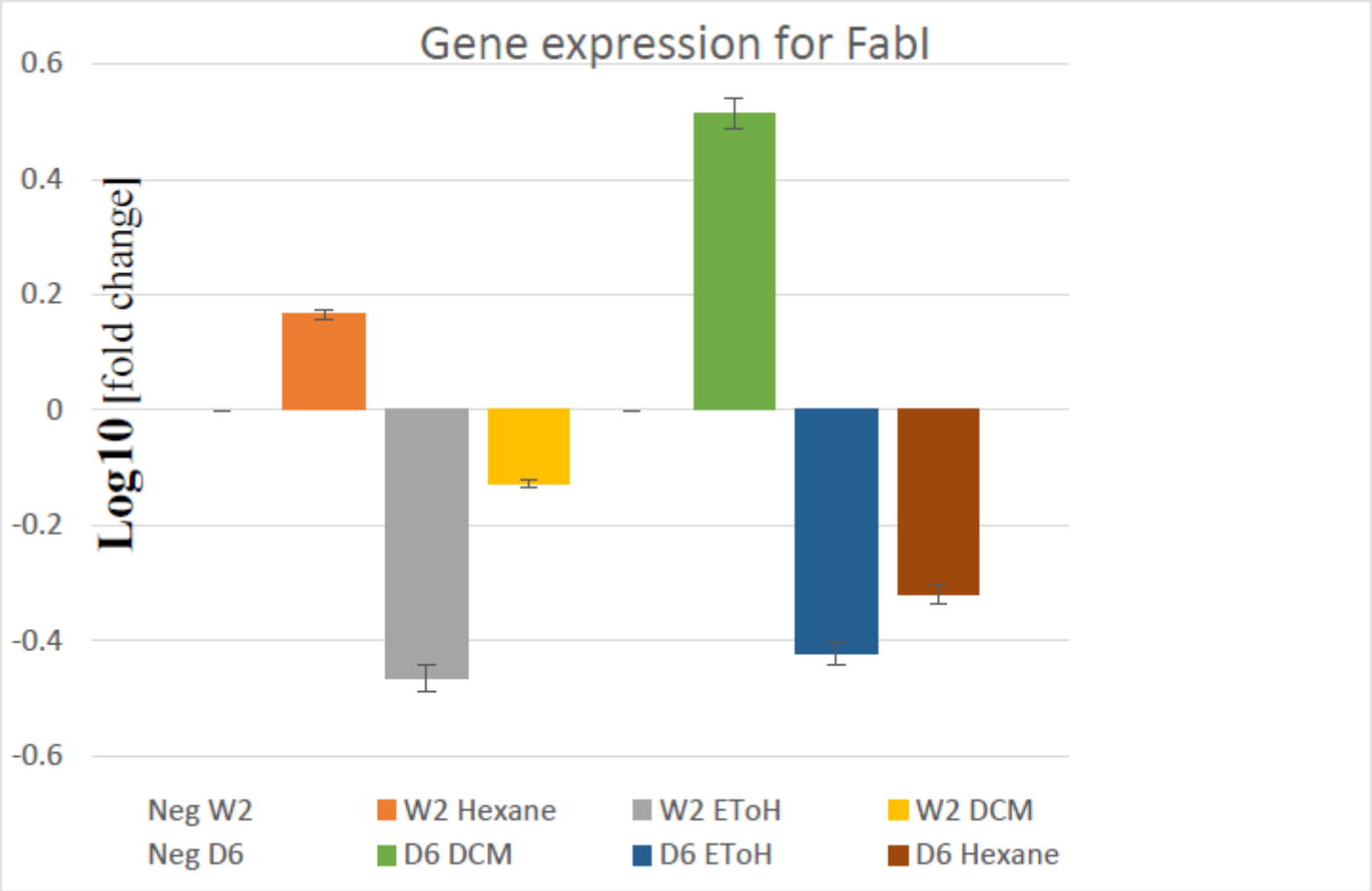


Figure 4

Gene expression for Fab I

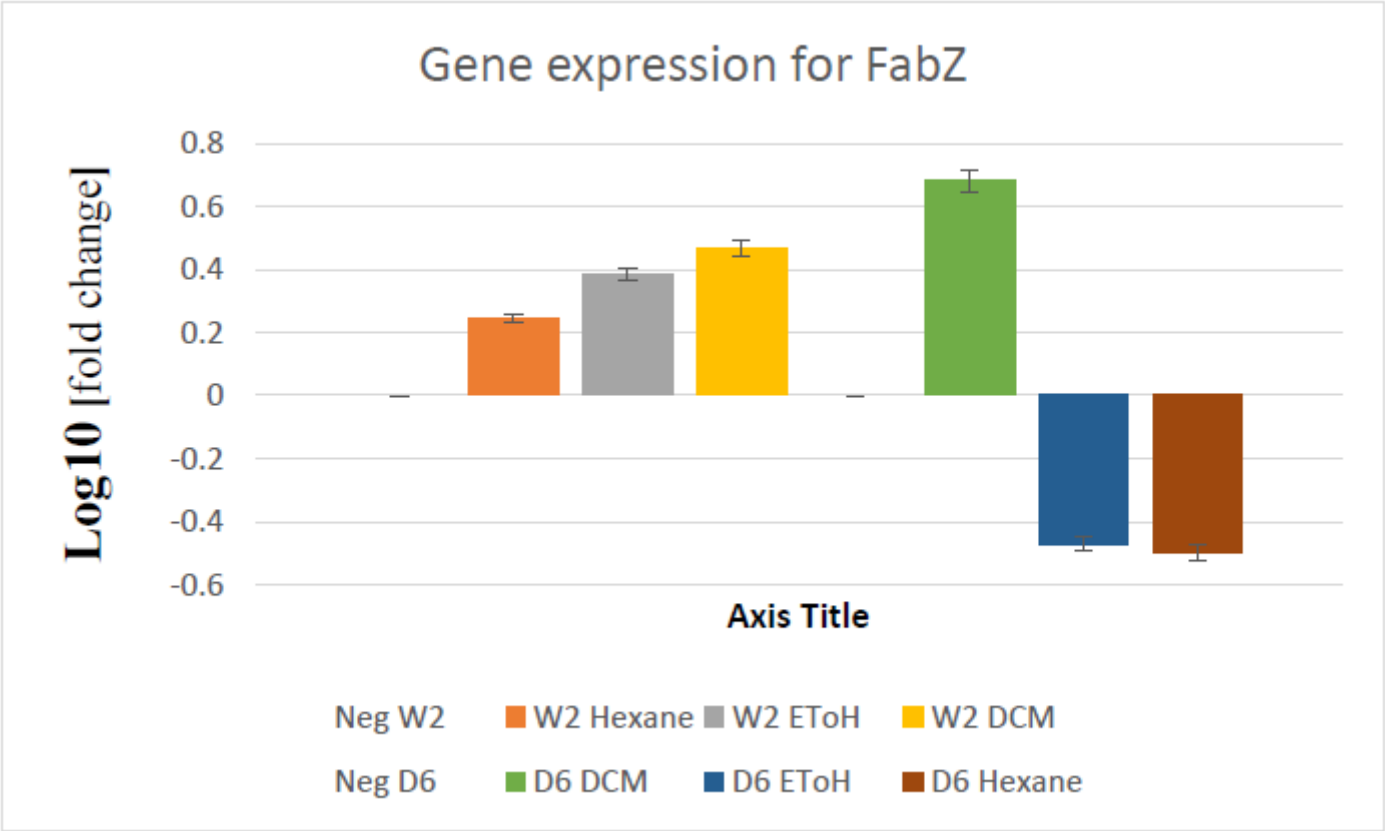
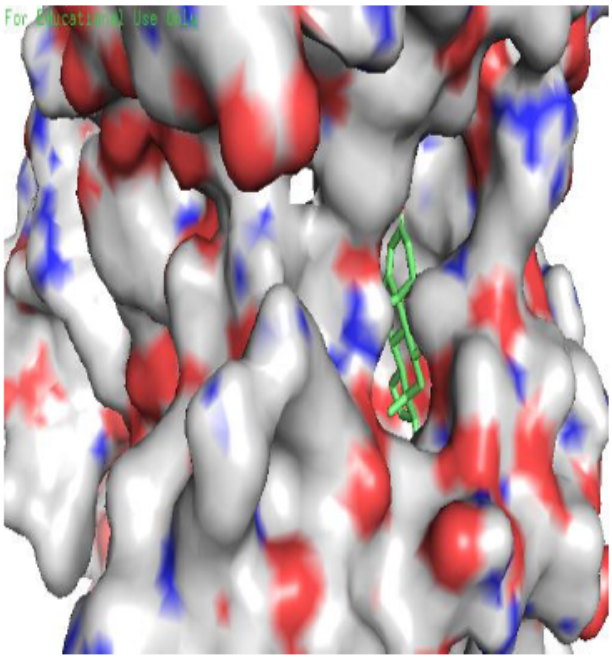


Figure 5

Gene expression for Fab Z



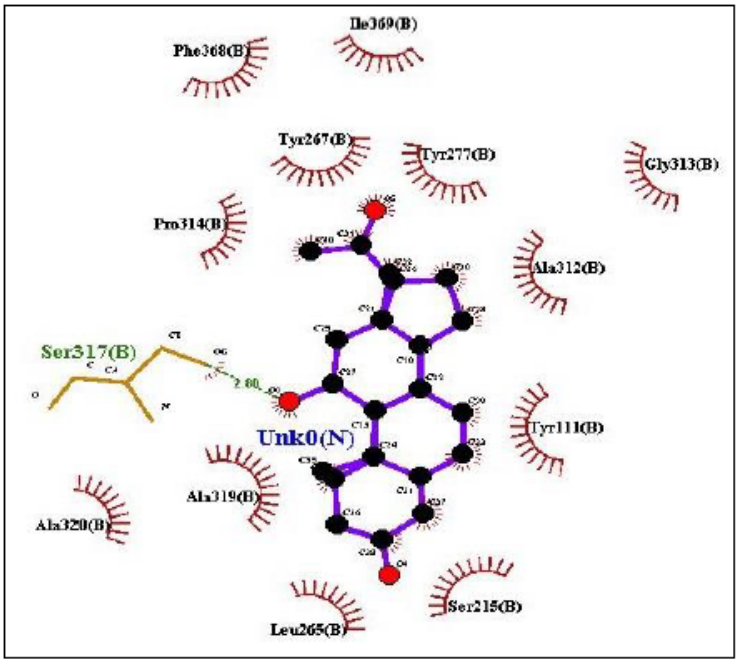


Figure 6

Interaction site for FabI (chain B) and hit N°1 (Hydroxyprogesterone)

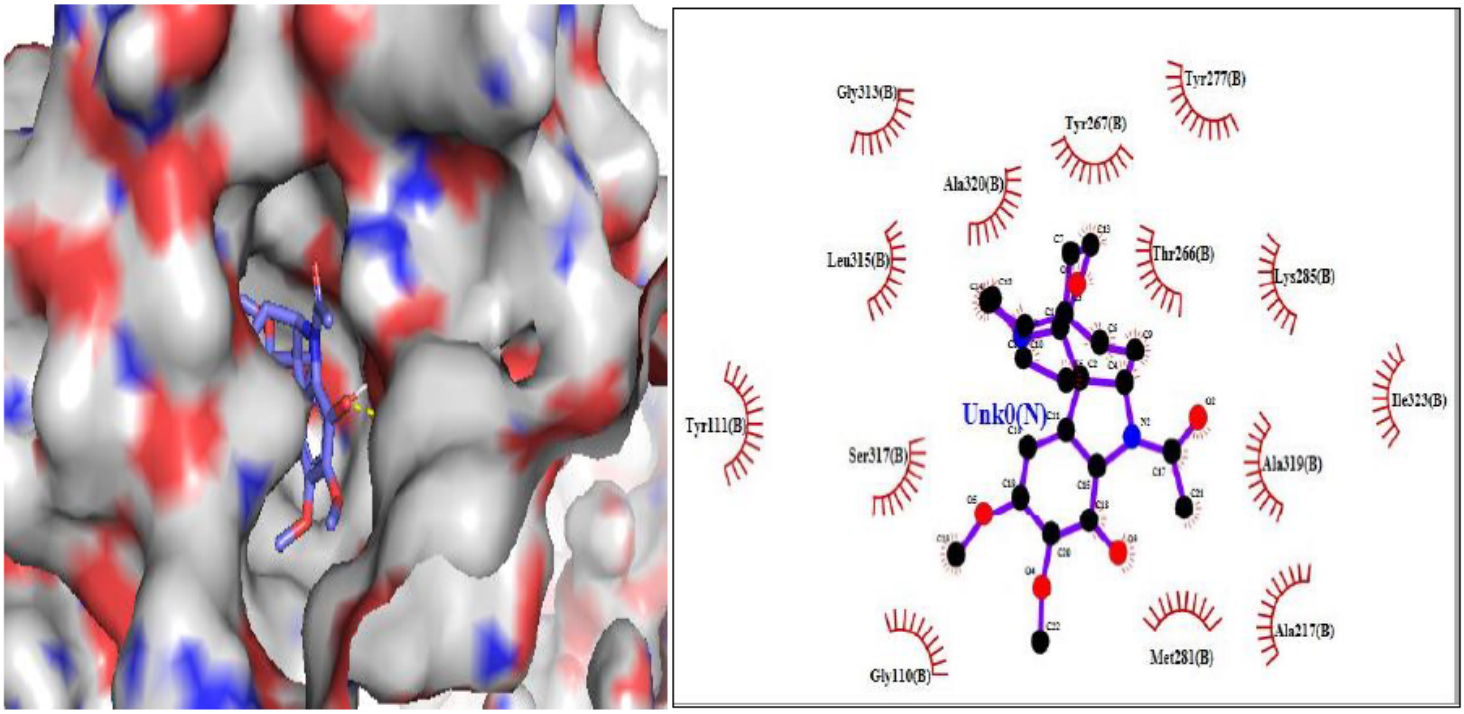


Figure 7

Interaction site for FabI (chain B) and hit N°2 (Aspidospermidin).

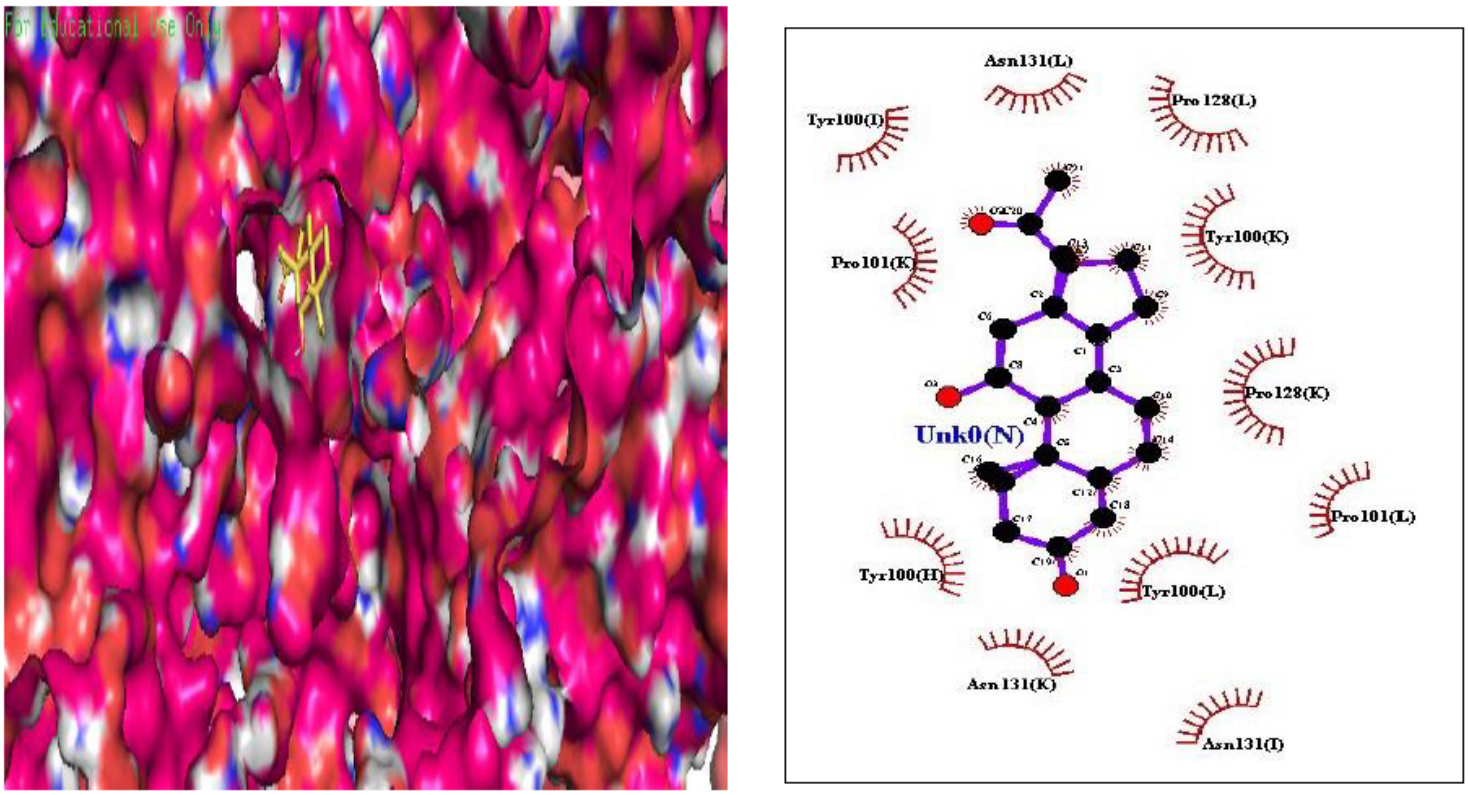


Figure 8

Interaction site for FabZ (chain K, L, I, H) and hit N°1 (Hydroxyprogesterone).

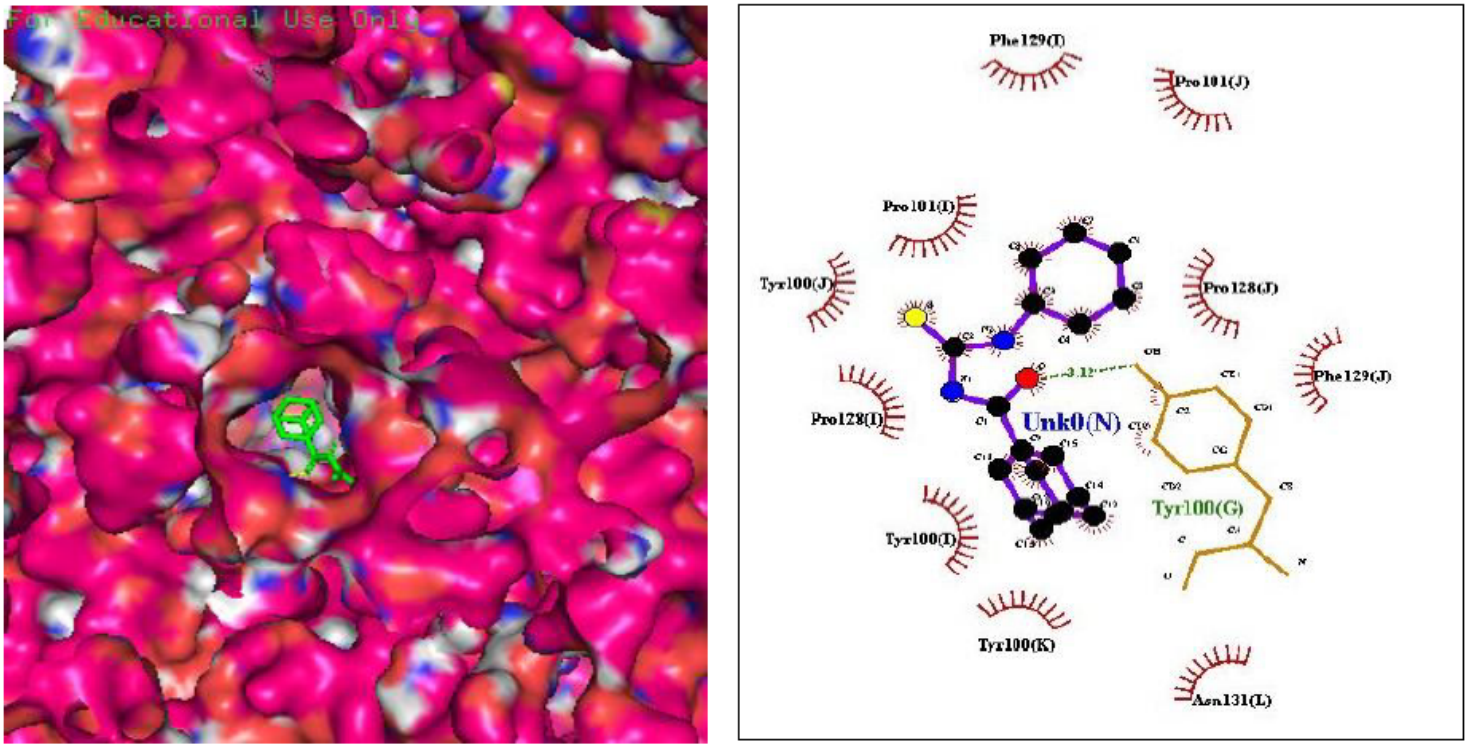


Figure 9

Interaction site for FabZ (chain K, L, I, J, G) and hit N°2 (Thiourea).