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Screening of natural compounds for ligands to *Pf*TrxR by ultrafiltration and LC–MS based binding assay

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ABSTRACT

In our study, we have screened 133 structurally diverse natural compounds from the MEGx® collection of AnalytiCon Discovery and three synthetic hispolone analogs for binding affinity to *Plasmodium falciparum* thioredoxin reductase (*Pf*TrxR) using an ultrafiltration (UF) and liquid chromatography (LC/MS) based ligand-binding assay newly developed in our laboratory. *Pf*TrxR catalyzes the reduction of thioredoxin (*Pf*Trx) protein. In reduced form, *Pf*Trx is essentially involved in the antioxidative defense and redox regulation of *P. falciparum*. Nine compounds (yohimbine (**1**), catharanthine (**2**), vobasine (**3**), gnetifolin E (**4**), quinidine N-oxide (**5**), 11-hydroxycoronaridine (**6**), hispolone (**7**), hispolone methyl ether (**8**), and hernagine (**9**)) displayed binding affinity for *Pf*TrxR at 1 μM. The ranking order of compound's binding affinities for *Pf*TrxR is **7** > **6** > **2** > **4** > **5** > **8** > **1** > **9** > **3**. On the other hand, compounds **6**, **7**, **2** and **8** demonstrated specific binding to the active site of *Pf*TrxR, when ligands were tested in an equimolar mixture of 1 μM.

1. Introduction

Of malarial parasites, *Plasmodium falciparum* is the most lethal species due to its increased drug resistance towards the current antimalarial drugs. Therefore, there is an urgent need to know more about the sites in the parasite which can be potential targets for plasmodial activity [1]. It has been found that *P. falciparum* is susceptible to oxidative stress [2]. Two antioxidant systems, thioredoxin reductase and glutathione reductase counteract the oxidative stress in *P. falciparum* [2]. *P. falciparum* (*Pf*) thioredoxin reductase (TrxR), a 55 kDa flavoprotein which belongs to a class of pyridine dinucleotide oxidoreductases, catalyzes the NADPH dependent reduction of *P. falciparum* thioredoxin (*Pf*Trx) protein [2]. Reduced thioredoxin regulates the activity of the enzyme by reducing the cellular environment. Reduced *Pf*Trx acts as hydrogen donor to ribonucleotide reductase and methionine sulfoxide reductase, involved in DNA synthesis and protein repair. They can directly reduce hydrogen peroxide and can function as both single oxygen quencher and hydroxyl radical scavenger. These functions of Trx are in response to oxidative stress of the parasite [3]. Because of the involvement of *Pf*TrxR in redox regulation of the parasite, *Pf*TrxR is considered a novel target in the parasite metabolism for plasmodial intervention.

Other different combination therapies available to treat malaria are found to be more expensive, not readily available to people in developing countries, and accompanied with adverse effects. As our contribution to address these problems, we are emphasizing the search for bioactive natural products, since these can be viewed as an inexhaustible reservoir of molecules that can be optimized to be efficient, well tolerated, and safe to use as antimalarial drugs [4,5]. Natural products provide leads for the development of novel therapies for the treatment of malaria. For any compound to intervene with the activity of the enzyme, it has to interact with the target enzyme. In the present work, an ultrafiltration and LC–MS based approach has been used to screen 133 structurally diverse natural compounds from a pure natural product library (MEGx) and three synthetic compounds such as hispolone and their analogs for their binding affinity to *Pf*TrxR.

2. Materials and methods

2.1. Chemicals and enzymes

Solvents used for LC/MS analysis were purchased from Fischer Scientific International (Atlanta, GA). One hundred and thirty-three compounds from AnalytiCon Discovery GmbH (Germany) natural products library (MEGx) (Table 1) were purchased based on compound classes that have been reported for either antimalarial activity or inhibition of TrxR [6,7]. Out of 133 natural products, 26 have been identified as novel plant-derived compounds by AnalytiCon Discovery (Table 1). Three hispolone analogues were

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Table 1
List of one hundred and thirty-three natural products from Analyticon Discovery library tested for binding to *Pf*TrxR.

CAS number	Common/chemical name[a]	Novelty[b]
94-62-2	Piperin	N
3155-53-1	7,8-Dihydro-5,6-dehydromethysticin	N
66648-43-9	Moupinamide	N
64363-86-6	1H-indole-3-ethanaminium	N
89946-11-2	3,3′,5,5′-Tetrahydroxy-4-methoxystilbene	N
1083200-79-6	NA	Y
960509-38-0	2,5-Piperazinedione, 3-[[2-(1,1-dimethyl-2-propen-1-yl)-1H-indol-3-yl]methylene]-6-methyl-, (3Z,6R)-	N
158569-73-4	(−)-Talaumidin methyl ether	N
1212329-33-3	NA	N
152543-09-4	Dihydroconiferyl dihydro-p-coumarate	N
182056-19-5	4(1H)-Quinolinone, 1-methyl-2-(7-tridecenyl)-	N
188113-99-7	4(1H)-Quinolinone, 1-methyl-2-(6,9-pentadecadien-1-yl)-	N
36417-86-4	Paprazine	N
38759-91-0	(±)-Eudesmin	N
220862-05-5	Thunalbene	N
41137-87-5	Hirsutenone	N
886226-15-9	Muricarpone B	N
1083197-74-3	NA	Y
130008-79-6	Phenol, 4-[4-(1,3-benzodioxol-5-yl)-2,3-dimethylbutyl]-2-methoxy-, (R*,R*)-(9CI)	N
1158019-66-9	2-Propen-1-one, 1-(2,4-dihydroxyphenyl)-3-[4-[(3-methyl-2-buten-1-yl)oxy]phenyl]-, (2E)-	Y
76496-57-6	6H-[1,3]Dioxolo[5,6]benzofuro[3,2-c][1]benzopyran-3-ol, 6a,12a-dihydro-	N
112501-42-5	Piperolactam A	N
116086-93-6	Piperolactam D	N
118245-30-0	1,3-Benzodioxole, 5-[(2R,3R)-4-(3,4-dimethoxyphenyl)-2,3-dimethylbutyl]-, rel-	N
886060-78-2	1-(3,4-Dihydroxyphenyl)-7-(4-hydroxyphenyl)-4-hepten-3-one	N
444930-12-5	1,3-Benzodioxole, 5,5′-[(2S,3S)-2,3-dimethyl-1,4-butanediyl]bis-(9CI)	N
78510-20-0	N-trans-feruloyl-4-O-methyldopamine	N
122-69-0	Styracin	N
65026-58-6	Norboldine	N
120693-53-0	4(1H)-Quinoline, 1-methyl-2-(4Z, 7Z)-4,7-tridecadienyl	N
485-61-0	Graveoline	N
36062-05-2	3-Heptanone, 5-hydroxy-1,7-bis(4-hydroxy-3-methoxyphenyl)-hexahydrocurcumin	N
2134-83-0	Vobasine	N
109428-81-1	Cafestol	N
1136618-10-4	Phenol, 3-[(1E)-2-(3-hydroxy-4-methoxyphenyl)ethenyl]-5-methoxy-2-(3-methyl-2-buten-1-yl)-	Y
125564-68-3	2-Propenoic acid, 3-(3,4-dihydroxyphenyl)-, 2-hydroxy-3-[[(2E)-3-(4-hydroxyphenyl)-1-oxo-2-propen-1-yl]oxy]propyl ester, (2E)-	Y
156788-67-9	3-Hydroxy-4′,5-dimethoxy-2-prenylstilbene	N
22313-89-9	1H,3H-Furo[3,4-c]furan, tetrahydro-1,4-bis(3,4,5-trimethoxyphenyl)-	N
212515-38-3	1,3-Benzodioxole, 5,5′-(tetrahydro-1H,3H-furo[3,4-c]furan-1,4-diyl)bis-, (3aR,6aR)-rel-(9CI)	N
71364-33-5	(±)-Tetrahydropalmatine-9-(methoxy-14-C)	N
23095-44-5	Girinimbine	N
56021-85-3	Lyaloside	N
15990-55-6	Bruceolline F	N
139219-98-0	Citracridone III	N
81525-61-3	Citracridone I	N
4339-71-3	Piceatannol	N
146-48-5	Yohimbine	N
100432-87-9	Glucoside, 3-hydroxy-5-(p-hydroxyphenethyl)phenyl (7CI)	Y
859747-99-2	1,3-Benzodioxole, 5-[tetrahydro-4-(3,4,5-trimethoxyphenyl)-1H,3H-furo[3,4-c]furan-1-yl]-	N
2468-21-5	Catharanthine	N
633-65-8	Berberinchlorid	N
1217689-07-0	1H,3H-Furo[3,4-c]furan-1-one, 3-(1,3-benzodioxol-5-yl)tetrahydro-6-(4-hydroxy-3-methoxyphenyl)-	Y
98681-44-8	4H-Dibenzo[de,g]quinolin-2-ol, 5,6,6a,7-tetrahydro-1-methoxy-	N
139101-68-1	β-D-Glucopyranoside, 3-hydroxy-5-[2-(4-hydroxy-3-methoxyphenyl)ethyl]phenyl	Y
140671-07-4	Gnetifolin E	N
56973-65-0	1,7-Bis(4-hydroxyphenyl)-4-hepten-3-one	N
288141-04-8	Platyphyllonol 5-xylpyranoside	N
6871-44-9	Echitamine	N
456-12-2	Aegeline	N
58762-96-2	Pinostilbenoside	N
38963-95-0	Resveratroloside	N
876659-81-3	Ajmalicine	N
79559-61-8	3-Heptanone, 5-hydroxy-7-(4-hydroxy-3-methoxyphenyl)-1-phenyl-	N
79559-59-4	1,7-Diphenyl-4-hepten-3-one	N
845655-40-5	1(2H)-Isoquinoline, 3,4-dihydro-6-hydroxy-7-methoxy	Y
884495-94-7	1,2-Benzenediol, 4-[3,5-dihydroxy-7-(4-hydroxy-3-methoxyphenyl)heptyl]-	Y
132216-13-8	Amaniensine	Y
25924-78-1	Trichostachine	N
34371-47-6	5β-Carboxystrictosidine	N
798569-00-3	D-Tyrosine, N-[3-(3,4-dihydroxyphenyl)-1-oxo-2-propen-1-yl]-3-hydroxy-	N
104821-27-4	Lyalosidic acid	N
91000-14-5	2-Propenamide, N-[4-(acetylamino)butyl]-3-(4-hydroxy-3-methoxyphenyl)-, (E)-(9CI)	Y
21451-50-3	Deoxycordifoline	Y
479-43-6	Canthin-6-one	N
908094-05-3	3-Heptanone, 5-hydroxy-7-(4-hydroxy-3-methoxyphenyl)-1-(4-hydroxyphenyl)-	N
1212137-68-2	[1,3]Dioxolo[4,5-h]-1,3-dioxolo[7,8][2]benzopyrano[3,4-a][3]benzazepine, 5b,6,7,8,13b,15-hexahydro-15-methoxy-6-methyl-	N

Table 1 (*Continued*)

CAS number	Common/chemical name[a]	Novelty[b]
79559-60-7	7-(4-Hydroxy-3-methoxyphenyl)-1-phenyl-4-hepten-3-one	N
33626-08-3	3,5-Dihydroxy-4′-methoxystilbene	N
74133-19-0	Hernagine	N
32507-66-7	Isorhapontigenin	N
NA	Centrolobol-3-rutenoside	Y
1083195-05-4	Benzenepropanol, 4-hydroxy-α-[4-(4-hydroxyphenyl)-3-buten-1-yl]-	Y
79120-40-4	Hannokinol	N
29700-22-9	Oxyresveratrol	N
103188-48-3	N-trans-caffeoyltyramine	N
35387-16-7	Vasicinone	N
19902-91-1	Dihydromethysticin	N
60263-07-2	Jacaranone	N
NA	Indol-3-(6-O-b-D-apiofuranosyl-b-D-glucopyranoside)	Y
1212171-97-5	1,3-Benzodioxole-5-butanol, α-(4-hydroxy-3-methoxyphenyl)-β,γ-dimethyl-	N
7154-01-0	3-(3,4-Methylenedioxyphenyl)-1,2-propanediol	N
1083192-75-9	2-Buten-1-ol, 4-[4-[2-(7-methoxy-1,3-benzodioxol-5-yl)ethyl]phenoxy]-2-methyl-	Y
48216-02-0	Homochelidonine, β-(3Cl)	N
23141-27-7	Vincosamide	N
23512-46-1	Piperanine	N
537-42-8	Pterostilbene	N
517-73-7	Melicopicine	N
130233-83-9	Acerogenin G	N
1083192-76-0	Benzene, 1,2,3-trimethoxy-5-[2-[4-[(3-methyl-2-buten-1-yl)oxy]phenyl]ethyl]-	Y
83-95-4	Skimmianine	N
133-03-9	(+)-Episesamin	N
93559-25-2	(±)-7-(3″,4″-Dihydroxyphenyl)-5-hydroxy-1-(4′-hydroxyphenyl)-3-heptanone	Y
1174388-79-4	NA	Y
1212227-01-4	NA	N
190271-90-0	β-D-Glucopyranoside, 5-(4-hydroxyphenyl)-1-[2-(4-hydroxyphenyl)ethyl]pentyl	N
126722-26-7	Pumiloside	N
104420-85-1	(+)-N-methyllaurotetanine N-oxide	N
58-08-2	Caffeine	N
24192-01-6	Dihydroyashabushiketol	N
4829-55-4	Hetisinone	N
607-91-0	Myristicin	N
105317-66-6	Moskachan D	N
76202-23-8	11-Hydroxycoronaridine	N
70116-00-6	Quinidine N-oxide	Y
115587-58-1	1-Hepten-3-one, 5-hydroxy-1,7-diphenyl-, (1E)-	Y
2051-07-2	Dianisalacetone	N
75821-37-3	5-Hydroxyarborinine	N
149732-51-4	1-(3,4-Dihydroxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione	N
30278-29-6	1,7-Diphenylheptane-3,5-diol	N
83161-95-9	3-Heptanone, 7-(4-hydroxy-3-methoxyphenyl)-5-methoxy-1-phenyl-	N
88660-10-0	Pipernonaline	N
66280-26-0	Pregomisin	N
52617-31-9	Haplamine	N
312608-32-5	Furo[2,3-b]quinolin-7-ol, 5,6,7,8-tetrahydro-4,8-dimethoxy-8-(3-methyl-2-buten-1-yl)-	N
1174387-98-4	NA	Y
1174387-99-5	NA	Y
1174389-03-7	NA	Y
58436-29-6	3-Hydroxy-4′,5-dimethoxystilbene	N
284463-38-3	3,5-Heptanediol, 1-(4-hydroxy-3-methoxyphenyl)-7-phenyl-	Y
10091-84-6	Triacanthin	N
913692-05-4	NA	N
123648-56-6	Germanaism B	N
467-77-6	Coronaridine	N

[a] NA: common/chemical name not assigned in the literature.
[b] Novelty: Y, novel compound; N, known compound.

kindly provided by Dr. G.V. Subbaraju, Aptuit Lauras, Hyderabad, India. Buffer salts and bis-2,4-dinitrophenyl sulfide were purchased from Sigma–Aldrich (Allentown, PA). Deionized water generated by a Milli-Q water system (Millipore, MA) was used in the experiments. *Pf*TrxR (M_r, 55 kDa) enzyme was provided as a gift by Prof. Katja Becker, Justus-Liebig University, Giessen, Germany. The recombinant *Pf*TrxR was prepared and purified using silver-stained SDS page according to the procedure published by Kanzok et al. [8]. The specific activity of *Pf*TrxR (1.9 U/mg) was determined by DTNB [5,5′-dithiobis(2-nitrobenzoic acid)]. Protein concentration of enzymes was determined by Bradford method [9].

2.2. PfTrxR enzyme binding assay using ultrafiltration and liquid chromatography–mass spectrometry

All the compounds were tested for their binding affinity at 1 μM. The binding assay was performed according to the published method [10]. The ligands were reconstituted in 100 μl of MeOH–H_2O (90:10 (v/v)) and were analyzed by LC–MS. Denatured enzyme was used for controlled experiment and assays were carried out in duplicates. Bis-2,4-dinitrophenyl sulfide was used as a reference compound. Binding assay for equimolar mixture (**1**–**9**) was performed in a similar way.

Table 2
LC–MS conditions used for detection of the one hundred and thirty-three natural products.

Batch	Compound class	Test conc. / inj. vol.	Mobile phase type	Mobile phase A	Mobile phase B	Mobile phase proportion		Positive/negative electrospray	Run time
1st, 2nd, 6th, 7th, 10th, 11th	Amides, alkaloids, curcuminoids, coumarins, lignans, quinines, stilbenes	5 μM/5 μl	Gradient	0.1% formic acid in H_2O	95% ACN, 5% H_2O, 0.1% formic acid	**min** 0 2 4 7 8	**%B** 20 50 90 90 20	ESI-positive	10 min
3rd	Amides	10 μM/2 μl	Isocratic	0.1% formic acid in H_2O	0.1% formic acid in MeOH	90:10 (A:B)		ESI-negative	8 min
4th	Alkaloids, amides, coumarins, lignans	1 μM/1 μl	Isocratic	0.1% formic acid in H_2O	95% ACN, 5% H_2O, 0.1% formic acid	5:95 (A:B)		ESI-positive	10 min
5th	Alkaloids	5 μM/5 μl	Gradient	0.1% formic acid in H_2O	95% ACN, 5% H_2O, 0.1% formic acid	**min** 0 2 4 5	**% B** 20 90 90 20	ESI-positive	7 min
8th	Stilbenes	5 μM/5 μl	Gradient	0.1% formic acid in H_2O	95% ACN, 5% H_2O, 0.1% formic acid	**min** 0 2 4 7 8	**% B** 20 40 90 90 20	ESI-negative	10 min
9th	Curcuminoids, alkaloids, diterpenes	1 μM/5 μl	Gradient	0.1% formic acid in H_2O	95% ACN, 5% H_2O, 0.1% formic acid	**min** 0 2 4 7 8	**% B** 20 40 90 90 20	ESI-positive	10 min

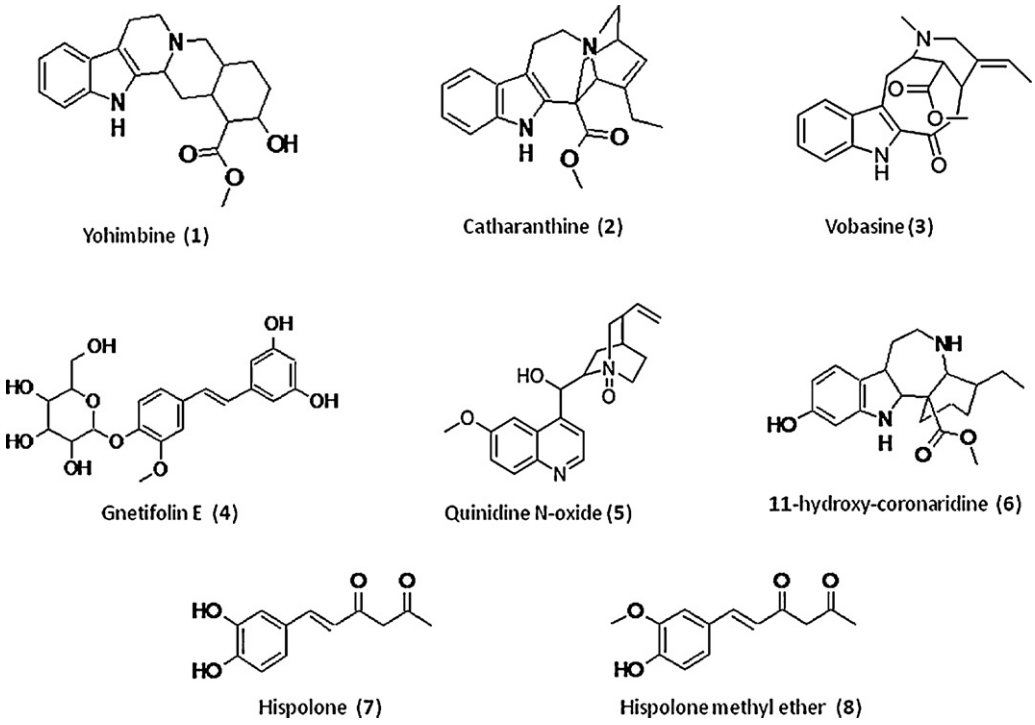

Fig. 1. Structures of active compounds screened for *Pf*TrxR.

2.3. LC/MS analysis

All the test compounds for LC–MS analysis were prepared using MeOH–H_2O (90:10 (v/v)). Prior to screening, the compounds were divided into batches based on their physiochemical properties that determine ionization efficiency and electrospray ionization mode. Liquid chromatography analysis was performed using a ZORBAX Eclipse plus C18 column (2.1 mm × 100 mm, 1.8 μm) at a flow rate of 0.2 ml/min. An Agilent (Little Falls, DE) 6520 accurate-mass quadrupole time of flight (Q-TOF) mass spectrometer equipped with a 1220 rapid resolution liquid chromatography (RRLC) system was used for separation of batch compounds. Detailed information on LC conditions for separation of batch compounds is provided in Table 2.

2.3.1. LC–MS analysis of ligands ***1***, ***2***, ***3***, ***5***, ***6***, ***7***, ***8*** and ***9*** in equimolar mixture of 1 μM

Compounds were analyzed at 1 μM (5 μl inj. vol.) using gradient mobile phase (0 min 5% B to 95% B in 20 min) consisting of (A) 0.1% formic acid in H_2O (v/v) and (B) 0.1% formic acid in 50:50 MeOH–ACN (v/v/v) as solvent system for run time of 20 min with post run 3 min at a flow rate of 0.2 ml/min. The eight compounds (**1**, **2**, **3**, **5**, **6**, **7**, **8** and **9**) were detected in positive ion electrospray.

All the test compounds were detected by MS at capillary voltage of 3200 V for ESI negative mode and 3500 V for ESI positive mode. Nitrogen was supplied as nebulizing and drying gas at flow rates of 25 and 600 l/h, respectively. The drying gas temperature was 350 °C. The fragmentor voltage was optimized to 175–180 eV. Data were acquired and analyzed using Agilent MassHunter Workstation Qualitative Analysis software, version B.02.00. 2.4.

3. Results and discussions

*Pf*TrxR plays an important role in the pathophysiology of malaria disease, regulating the oxidative stress in the enzyme. Considering the significance of *Pf*TrxR as a novel target in the parasite metabolism for therapeutic intervention, UF and LC–MS binding assay [10] was used to screen 133 various classes of natural compounds (alkaloids, amides, coumarins, curcuminoids, diterpenes, lignans, stilbenes) and 3 synthetic compounds which can bind to *Pf*TrxR. Out of the 136 compounds screened, nine compounds showed binding to the enzyme. These compounds were found to be yohimbine (**1**), catharanthine (**2**), vobasine (**3**), gnetifolin E (**4**), quinidine N-oxide (**5**), 11-hydroxycoronaridine (**6**), hispolone (**7**), hispolone methyl ether (**8**), and hernagine (**9**) (Fig. 1).

To screen the ligands for the enzyme, the binding assay was performed by incubating the test compounds with 1 μM *Pf*TrxR at 25 °C for 1 h [10] in a total volume of 200 μl of assay buffer. The bound ligands in the enzyme–ligand complex trapped on the membrane filter were dissociated using MeOH and the dissociated ligands were dried, reconstituted and then analyzed using both positive and negative ESI-MS experiments. All ligands were detected by LC/MS within an error of <1.5 ppm. The relative binding affinities of ligands are shown in Table 3. The relative binding affinity was ascertained by calculating the ratio between the average peak area of compound incubated with active enzyme and the average peak area of compound incubated with denatured enzyme ± standard deviation. According to the binding affinity data obtained in the screening of the 136 compounds against *Pf*TrxR and relative binding affinity of our reference compound (bis-2,4-dinitrophenyl sulfide) [10], compounds that specifically bind the enzyme and displayed relative binding affinity of more than 1.5 were considered as ligands. The relative binding affinity was ranked in the following fashion: non-ligand less than 1.5, weak in the range of 1.5–2.0, moderate between 2.0 and 3.0 and strong when more than 3.0.

The ranking order of compound's binding affinities for *Pf*TrxR is **7** > **6** > **2** > **4** > **5** > **8** > **1** > **9** > **3**. Yohimbine (**1**) has moderate relative binding affinity of 2.0-fold when incubated with 1 μM *Pf*TrxR. Compound **1**, an indole alkaloid, was first isolated from the seeds and bark of a large Venezuelan tree *Aspidosperma excelsum* Benth [11]. Catharanthine (**2**) and gnetifolin E (**4**) both displayed moderate relative binding affinity of 2.5-fold to *Pf*TrxR. Compound **2**, an indole alkaloid, was isolated from the dried leaves of *Catharanthus roseus* (L.) G. Don [12]. Compound **4**, a stilbene glucoside was first isolated

from lianas of *Gnetum parvifolium* (Warb.) W.C. Cheng [13].

Vobasine (**3**) displayed relatively low binding affinity of 1.6-fold to *Pf*TrxR. Quinidine N-oxide (**5**) showed moderate relative binding affinity of 2.6-fold to *Pf*TrxR. Compound **5** was identified as novel structure from plant sources by AnalytiCon Discovery. The 11-hydroxy coronaridine (**6**) has relatively high binding affinity of 3.8-fold compared to yohimbine (**1**). Compound **6**, an indole alkaloid, was isolated from seeds and root-bark of *Pterotabema inconspicua* Stapf [14]. Hernagine showed 1.8-fold relative binding affinity to *Pf*TrxR. Hernagine (**9**), an aporphinoid alkaloid, was first isolated from stem bark of *Hernandia nymphaefolia* Presl [15] and later isolated from other *Hernandia* species like *Hernandia cordigera* Vieill [16]. The extracts of *H. cordigera* displayed antiparasitic activity against other than *Plasmodium* species such as *Leishmania donovani*, *Trypanosoma brucei*, *Trichomonas vaginalis* and *Caenorhabditis elegans* [17]. Compounds **1**, **2** and **6** belong to indole alkaloids and displayed moderate relative binding affinity to *Pf*TrxR. Indole alkaloids isolated from natural sources showed high selectivity to *Plasmodium* species and is reported to have high antiplasmodial activity both *in vitro* and *in vivo* [18].

In addition to the 133 natural products, we also screened 3 synthetic compounds: hispolone (**7**), hispolone methyl ether (**8**), and dehydroxyhispolone (**10**). These compounds are given much attention due to their structural resemblance to curcuminoids (bisdemethoxycurcumin, demethoxycurcumin and curcumin) which are already reported to have high relative binding affinity for *Pf*TrxR [10]. Moreover these compounds have significant anti-inflammatory and anti-proliferative activities when tested on human myeloid leukemia cell line KBM-5 and other human cell lines [19]. For these reasons these compounds were tested for binding affinity to *Pf*TrxR. Hispolone (**7**) showed the highest relative binding affinity of 7.5-fold among all the compounds screened, whereas hispolone methyl ether (**8**) showed moderate relative binding affinity of 2.5-fold and dehydroxyhispolone (**10**) displayed no affinity (less than 1-fold) for *Pf*TrxR. The high binding affinity of hispolone (**7**) can be attributed to the presence of the hydroxyl groups on the phenyl ring. When one of the hydroxyl groups was replaced by a methoxy group in hispolone methyl ether (**8**), there was a significant 3-fold reduction in the binding affinity for *Pf*TrxR. When one of the hydroxyl groups was absent from the ring, as in case of dehydroxyhispolone (**10**) (Fig. 1), there was no binding to *Pf*TrxR. The active site of *Pf*TrxR has two cysteine residues at C-terminal glycine which are essential for enzymatic activity [20]. Therefore it can be hypothesized that hydroxyl groups in hispolone analogues may form a hydrogen bond with enzyme active site. The role of methoxy group as in the case of hispolone methyl ether (**8**) appears optional.

When compounds ionizable by positive mode ESI were assayed with 1 μM of *Pf*TrxR as equimolar solution of 1 μM, compounds **1**,

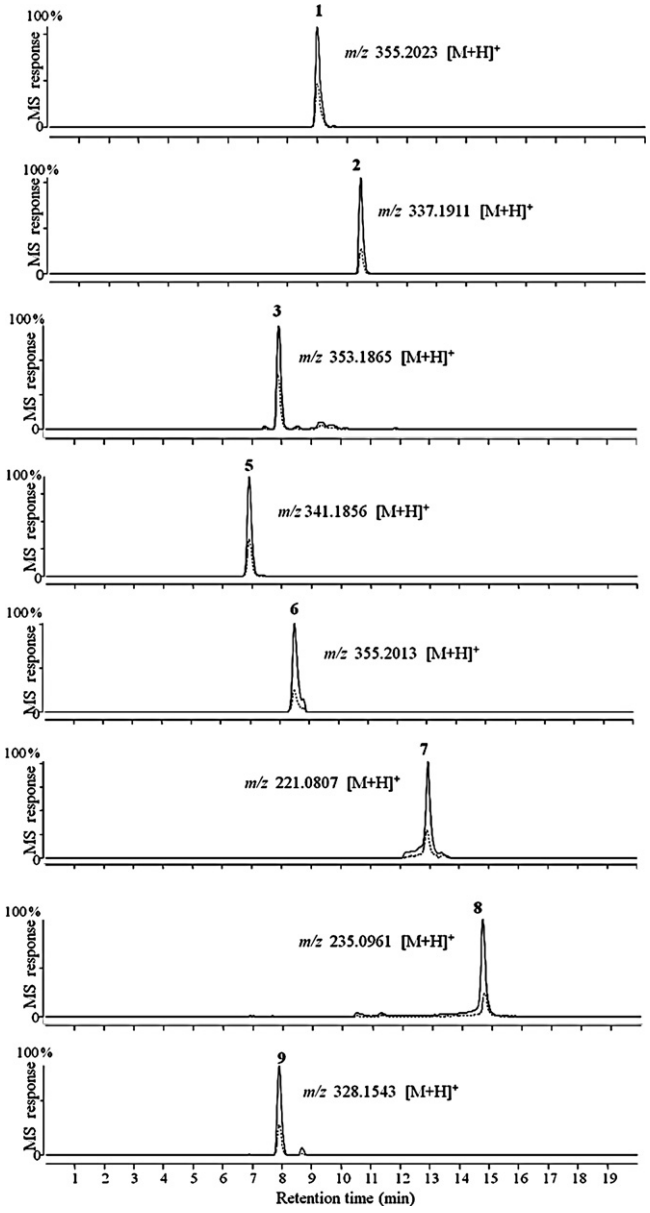

Fig. 2. UF and LC–MS screening of equimolar mixture of compounds **1**, **2**, **3**, **5**, **6**, **7**, **8** and **9** incubated with 1 μM *Pf*TrxR.

Table 3
Relative binding affinities for compounds **1**–**10** when tested against *Pf*TrxR using UF/LC–MS screening method.

Compound	MS detected parent ion[a]	Relative binding affinity of each ligand 1 μM	Relative binding affinity of equimolar mixture of ligands, 1 μM each
Yohimbine (**1**)	355.2013 $[M+H]^+$	2.0 ± 0.13	2.2 ± 0.78
Catharanthine (**2**)	337.1910 $[M+H]^+$	2.5 ± 0.05	3.6 ± 0.59
Vobasine (**3**)	353.1865 $[M+H]^+$	1.6 ± 0.87	1.9 ± 0.56
Gnetifolin E (**4**)	419.1351 $[M-H]^-$	2.45 ± 0.16	ND
Quinidine N-Oxide (**5**)	341.1856 $[M+H]^+$	2.6 ± 0.23	2.6 ± 0.64
11-Hydroxy-coronaridine (**6**)	355.2019 $[M+H]^+$	3.8 ± 0.38	3.7 ± 0.94
Hispolone (**7**)	221.0803 $[M+H]^+$	7.51 ± 0.52	3.6 ± 0.17
Hispolone methyl ether (**8**)	235.0961 $[M+H]^+$	2.05 ± 0.24	3.4 ± 0.42
Hernagine (**9**)	328.1534 $[M+H]^+$	1.8 ± 0.61	2.9 ± 0.70
Dehydroxyhispolone (**10**)	205.0855 $[M+H]^+$	0.95 ± 0.43	ND
2,4-Dinitrophenylsulfide (reference compound) [10]	366.9992 $[M-H]^+$	3.2 ± 0.27	ND

ND: not determined, compounds **4** and **10** were not tested in the equimolar mixture because **4** should be detected in ESI negative mode and **10** was not considered a ligand.
[a] Parent ions detected for ligands (**1**–**10**) in the UF/LC–MS screening when incubated with 1 μM of active and denatured *Pf*TrxR.

2, **3**, **5**, **6**, **7**, **8**, **9** were identified as ligands (Table 3 and Fig. 2). The ranking order of compound's binding affinities in equimolar mixture for *Pf*TrxR is **6** > **7** > **2** > **8** > **9** > **5** > **1** > **3**. Because the amount of *Pf*TrxR available for binding was the same as assayed individually, the ligands **6**, **7**, **2** and **8** competed more effectively for the binding site than compounds **9**, **5**, **1** and **3**. These results suggest that the binding of ligands **6**, **7**, **2** and **8** to *Pf*TrxR was specific to the active site. No previous work related to ligand-binding affinity to *Pf*TrxR or any other in vitro antiplasmodial activity was found to be reported on these compounds **1**–**10**, except for compound **1**, where antiplasmodial activity was reported by Staerk et al. [21].

4. Conclusions

Screening of natural compounds for ligand binding affinity to *Pf*TrxR by UF and LC–MS based approach can be used to address the challenges in the discovery of innovative and cost effective broad spectrum antimalarial agents from plants. LC–MS conditions were developed for all the test compounds prior to the binding assay. Among compounds screened, we found nine compounds (**1**, **2**, **3**, **4**, **5**, **6**, **7**, **8** and **9**) bound to the enzyme. Hispolone (**7**) showed highest relative binding affinity when tested individually. When the selected eight ligands (**1**, **2**, **3**, **5**, **6**, **7**, **8** and **9**) were tested in equimolar mixture, compounds **6**, **7**, **2** and **8** displayed strongly competitive binding for *Pf*TrxR in comparison to the rest of the ligands. Compounds with strong binding affinity to the enzyme will be subjected to further functional assay studies to test their inhibitory activity.

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