Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.apjtb.com

Document heading doi:10.12980/APJTB.4.2014APJTB-2014-0043

Proteomics analysis of antimalarial targets of *Garcinia mangostana* Linn.

Wanna Chaijaroenkul[1], Artitiya Thiengsusuk[1], Kanchana Rungsihirunrat[2], Stephen Andrew Ward[3], Kesara Na-Bangchang[1*]

[1]*Chulabhorn International College of Medicine, Thammasat University (Rangsit Campus), Pathumtani 12121, Thailand*

[2]*College of Public Health Sciences, Chulalongkorn University, Bangkok 10330, Thailand*

[3]*Molecular and Biochemical Parasitology, Liverpool School of Tropical Medicine, University of Liverpool, Liverpool L3 5QA, United Kingdom*

PEER REVIEW

Peer reviewer

Parnpen Viriyavejakul, Mahidol University, Faculty of Tropical Pathology. E-mail: parnpen.vir@mahidol.ac.th

Co-reviewer: Raewadee Wisedpanichkij, Bangkok, Thailand.

Comments

The paper is a basic research which looked at new antimalarial target-mangosteen extract and concluded its effect through glycolysis pathway.

Details on Page 519

ABSTRACT

Objective: To investigate possible protein targets for antimalarial activity of *Garcinia mangostana* Linn. (*G. mangostana*) (pericarp) in 3D7 *Plasmodium falciparum* clone using 2-dimensional electrophoresis and liquid chromatography mass-spectrometry (LC/MS/MS).

Methods: 3D7 *Plasmodium falciparum* was exposed to the crude ethanolic extract of *G. mangostana* Linn. (pericarp) at the concentrations of 12μg/mL (IC_{50} level: concentration that inhibits parasite growth by 50%) and 30 μg/mL (IC_{90} level: concentration that inhibits parasite growth by 90%) for 12 h. Parasite proteins were separated by 2-dimensional electrophoresis and identified by LC/MS/MS.

Results: At the IC_{50} concentration, about 82% of the expressed parasite proteins were matched with the control (non-exposed), while at the IC_{90} concentration, only 15% matched proteins were found. The selected protein spots from parasite exposed to the plant extract at the concentration of 12 μg/mL were identified as enzymes that play role in glycolysis pathway, *i.e.*, phosphoglycerate mutase putative, L-lactate dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase, and fructose-bisphosphate aldolase/phosphoglycerate kinase. The proteosome was found in parasite exposed to 30 μg/mL of the extract.

Conclusions: Results suggest that proteins involved in the glycolysis pathway may be the targets for antimalarial activity of *G. mangostana* Linn. (pericarp).

KEYWORDS

Malaria, Proteomics, *Garcinia mangostana* Linn.

1. Introduction

The success in elucidation of genome sequences of several micro-organisms including *Plasmodium falciparum (P. falciparum)* in the pre-genomic era has challenged research for identification of potential proteomic markers correlated disease pathology, as well as protein targets of candidate drugs and vaccines in the post genomic era. Proteomics analysis is the large-scale study of proteins, particularly their structures and functions. It determines global protein expression including sub-cellular localization and post-translational modification. The approach is much more complicated than the genomics approach due to the fact that, while the genome of an organism is relatively stable, its proteome differs from cell to cell and from time to time. This is because distinct genes are expressed in distinct cell

*Corresponding author: Prof. Dr. Kesara Na- Bangchang, Chulabhorn International College of Medicine, Thammasat University (Rangsit Campus), Pathumtani 12121, Thailand.

Tel: +662-564-4440-79 Ext.1803

Fax: +662-564-4398

E-mail: kesaratmu@yahoo.com

Foundation Project: supported by The National Research Council of Thailand, (Grant No.034/2556) and Thailand Research Fund, (Grant No. MRG5380192).

Article history:

Received 28 Apr 2014

Received in revised form 5 May, 2nd revised form 13 May, 3rd revised form 20 May 2014

Accepted 15 Jun 2014

Available online 28 Jul 2014

types. Detection of a marked change in the expression of proteins that are vital for the survival of a micro-organism represents one of the most important strategies in drug discovery research. Two-dimensional gel electrophoresis (2-DE) is the common technique applied for investigation of the change in these expressed proteins. The proteins are firstly electrophoresed in one direction, followed by another direction which allows for the visualization of a small change in protein expression which is finally identified by mass spectrometry[1,2].

A large number of medicinal plants have been identified as potential antimalarial agents. The antimalarial activity of *Garcinia mangostana* (*G. mangostana*) Linn. or mangosteen has previously been reported with IC_{50} (concentration which inhibits parasite growth by 50%) ranging from 4.5 to 12.6 μg/mL[3,4]. Variety of biological activities of xanthones isolated from this fruit pericarp has also been reported[5-7]. The aim of the present study was to identify protein targets for antimalarial activity of *G. mangostana* Linn. (pericarp) in 3D7 *P. falciparum* clone using 2-DE and liquid chromatography mass-spectrometry (LC/MS/MS).

2. Materials and methods

2.1. Crude extract of G. mangostana Linn. (pericarp), chemicals and reagents

The *G. mangostana* fruit pericarps were washed, cut into small pieces, air-dried, weighed and ground into powder. The powder was then soaked in ethanol until exhaustion. The ethanol extract was filtrated and evaporated under reduced pressure by rotary evaporation. The extract yield was weighed and stored at -20 °C until used.

2.2. Assessment of antimalarial activity of crude ethanolic extract of G. mangostana Linn. in vitro

3D7 *P. falciparum* clone was used in this study. The parasite was cultured according to the method of Trager and Jensen with some modifications[8]. Antimalarial activity of the crude ethanolic extract of *G. mangostana* Linn. (pericarp) was investigated using SYBR Green I assay[9,10]. Highly synchronous ring stage parasite was used in each assay. An aliquot of parasite inoculum (50 μL) with 2% parasitaemia and 1% haematocrit was added into each well of a 96-well microtiter plate. The 96-well drug plates were dosed with the extract at eight final concentrations as follows: 0.78125, 1.5625, 3.125, 6.25, 12.5, 25, 50 and 100 μg/mL. The experiment was done in triplicate. The IC_{50} and IC_{90} values used as indicators of antimalarial activity were determined from a log-dose-response curve plotted using the Calcusyn™ version 1.1 (BioSoft, Cambridge, UK).

2.3. Extraction of P. falciparum proteins following exposure to the ethanolic extract of G. mangostana Linn.

Synchronized 3D7 *P. falciparum* was exposed to the ethanolic extract of *G. mangostana* Linn. at the IC_{50} and IC_{90} for 12 h. Parasite culture was harvested and cell pellet was re-suspended in 0.15% saponin and incubated on ice for 1 h in order to lyse red blood cells. The lysate was collected through centrifugation at 8 500 r/min for 5 min (4 °C) and washed three times with 1 mL of 50 mmol/L Tris (pH 7.5) until the supernatant was clear. The parasite pellet was re-suspended in 500 μL of rehydration buffer (8 mol/L urea, 2 mol/L thiourea, 2% CHAPS, 65 mmol/L DL-Dithiothreitol, 1% ampholyte (pH 3-10), and 1× of protease inhibitor). Sample was vortexed and sonicated on ice for four times, six seconds each (21% amplitude, 6 seconds, interspersed with 9 seconds), followed by centrifugation at 8 500 r/min for 1 h (4 °C). Protein concentration was measured using Bradford reagent (BioRad Co. Ltd., California, USA) and the supernatant was subjected to 2-DE analysis[11,12].

2.4. 2-DE

The extract of parasite protein (100 μg) was mixed with rehydration buffer (8 mol/L urea, 1% CHAP, 15 mmol/L dithiothretol, and 0.001% bromophenol blue) to prepare 125 μL of protein mixture and then applied onto 7 cm immobilized pH gradient strips (non-linear) with a pH range of 3-10 in an isoelectric focusing (IEF) system (PROTEAN® i12™ IEF Cell, BioRad Co. Ltd., California, USA). The IEF was initially performed at 250 V for 15 min, followed by 4 000 V for 1 h and 4 000-20 000 volt-hour. The focused strips were equilibrated in equilibration solution I [10 mL of 50 mmol/L Tris-HCl pH 8.8, 6 mol/L urea, 30% glycerol, and 2% sodium dodecyl sulfonate (SDS)] containing the reducing agent DL-Dithiothreitol (100 mg) for 10 min, followed by equilibration solution II (5 mL) containing iodoacetamide (450 mg) for additional 10 min. Finally, the strips were equilibrated with 1× electrode buffer (pH 8.3) for 10 min. The strips were then loaded onto 12% SDS-polyacrylamide gel electrophoresis for second dimension separation. The gels were run on 1× electrode buffer (pH 8.3). After electrophoresis, gels were fixed and stained with silver stain (BioRad Co. Ltd., California, USA) according the manufacturer's recommendation. The 2-DE gel images were scanned and analyzed using PDQuest™ software (BioRad Co.

Ltd., California, USA). At least four independent gels were analyzed for each sample group.

2.5. Identification of proteins

Selection of protein spots separated by 2-dimensional gels was guided through images on quadruple gels. Briefly, the excised gel spot was put into a 1.5 mL centrifuge tube and acetonitrile (400 μL) was added. The mixture was incubated at room temperature for 10 min. The gel slices were then incubated overnight at 37 °C in 10-20 μL of sequence grade trypsin (Sigma, Dorset, UK) (10 μg/mL of 25 mmol/L ammonium bicarbonate with 10% acetonitrile). The supernatant was collected through centrifuge at 6 560 r/min (4 °C), and the gel was incubated with 20 μL of the mixture of acetonitrile and 100 mmol/L ammonium bicarbonate (2:1, v/v) at room temperature for 15 min. The supernatant was removed and pooled together with the first aliquot. The combined supernatant was dried in a rotational vacuum concentrator (at 37 °C for 3 h), and the dried samples were kept at -80 °C until used.

The sample was re-suspended in 20 μL of 0.1% formic acid and centrifuge at 8 500 r/min (4 °C) for 20 min. Proteins were separated using nano-flow high-performance liquid chromatography on a C18 reverse phase Dionex Ultimate 3000 column (Thermo Scientific, California, USA) and the mobile phase consisting of 50% acetonitrile for 30 min. The elute proteins were detected using LTQ ion-trap mass spectrometer (Thermo Scientific, California, USA). The resulting MS/MS spectra were submitted to TurboSequest Bioworks version 3.1 (Thermo Electron, California, USA) and the individual spectrum was merged into an MGF file before submitting to Mascot (v2.2).

3. Results

The median (range) IC_{50} and IC_{90} values of the ethanolic extract of *G. mangostana* Linn. (pericarp) for the 3D7 *P. falciparum* clone were 12.6 (10.5-13.2) and 27.3 (24.7-48.4) μg/mL, respectively. The patterns of protein spots separated from the parasite extract following exposure to 12 (IC_{50}) and 30 (IC_{90}) μg/mL of the plant extract including the non-exposed parasite are shown in Figure 1. The number of protein spots from the parasite extract following exposure to 12 and 30 μg/mL and the control were 102, 73 and 95, respectively. The number of matched protein spots with the control for the parasite exposed to the plant extract at the concentrations of 12 and 30 μg/mL were 84 (82%) and 11 (15%), respectively. Among these matched spots, the number of clear up-regulated protein spots (at least 3 out of 4 spots) were 22 and 11 for the parasite extract following exposure to 12 and 30 μg/mL extract, respectively (Table 1 and 2). The six protein spots at least two-fold up-regulated were identified by LC/MS/MS as phosphoglycerate mutase putative (PGM1), L-lactate dehydrogenase (LDH)/glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and fructose-bisphosphate aldolase/phosphoglycerate kinase (PGK) (Table 3). All these proteins were enzymes that play role in glycolysis pathway. The proteosome subunit putative protein was found only in parasite extract following exposure to 30 μg/mL of the plant extract.

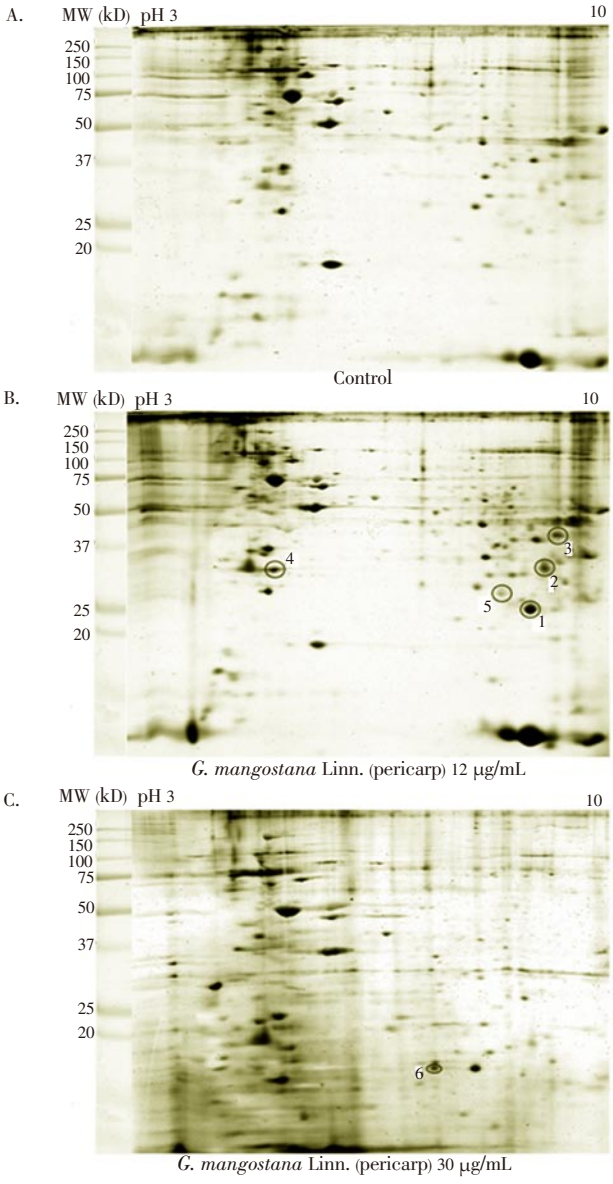

Figure 1. Differential expression of protein spots from the extract of 3D7 *P. falciparum* separated by 2-DE: (A) control (non-exposed), (B) following exposure to the extract of *G. mangostana* Linn. (pericarp) at the IC_{50} level (12 μg/mL), and (C) following exposure to the extract of *G. mangostana* Linn. (pericarp) at the IC_{90} level (30 μg/mL).

Table 1
Comparison of 2-DE protein spots from 3D7 *P. falciparum* exposure to *G. mangostana* Linn. (pericarp) at 12 μg/mL with at least 2-fold increase in density compared with control.

Spot ID	Protein spot density		Ratio of spot density (exposed/control)
	Control	Exposed to 12 μg/mL	
1102	327228.30	–	0.00
2006	70497.30	–	0.00
2108	308001.80	–	0.00
2211	511576.90	–	0.00
2507	962410.40	–	0.00
5205	72382.30	–	0.00
5309	141748.70	–	0.00
5414	493481.40	–	0.00
7120	6336.00	–	0.00
8109	349470.80	–	0.00
8208	224686.70	–	0.00
8013	118752.20	367943.30	3.10
6005	56925.70	177043.60	3.11
6007	75021.20	236373.40	3.15
8012	69251.20	218654.80	3.16
6112	292829.80	1055952.10	3.61
6205	158713.30	691401.70	4.36
2210	198064.60	897992.90	4.53
5104	85343.10	406396.40	4.76
8106	146820.80	718545.10	4.89
3108	193890.50	955672.50	4.93
7115	175944.10	914203.40	5.20
6307	54313.00	315164.60	5.80
2408	33665.60	203455.10	6.04
7114	126117.20	795175.10	6.31
6204	118583.80	780371.60	6.58
4408	107065.50	876504.40	8.19
7113	74850.90	706481.40	9.44
5412	7313.60	80676.10	11.03
6113	66245.50	749835.30	11.32
7004	86445.00	1323992.80	15.32
4211	32429.70	551161.00	17.00
7204	25699.70	715906.10	27.86

Table 2
Comparison of 2-DE protein spots from 3D7 *P. falciparum* exposure to *G. mangostana* Linn. (pericarp) at 30 μg/mL with at least 2-fold increase in density compared with control.

Spot ID	Protein spot density		Ratio of spot density (exposed/control)
	Control	Exposed to 30 μg/mL	
1005	937120.0	1097044.0	1.17
1006	642015.9	1031071.0	1.61
1102	3241389.0	957180.4	0.30
2006	1225975.1	858408.8	0.70
2007	32429.7	638622.9	19.69
2008	902078.9	513838.9	0.57
2009	511576.9	340800.0	0.67
2106	728049.3	306116.8	0.42
2107	1072162.8	258182.8	0.24
2108	308001.8	198814.9	0.65
2109	170356.2	122522.1	0.72

Table 3
Identification of protein spots (by LC/MS/MS) separated from the extract of 3D7 *P. falciparum* following exposure to the extract of *G. mangostana* Linn. (pericarp) at either concentration of IC_{50} (12 μg/mL) or IC_{90} (30 μg/mL).

No	Spot ID	Protein name	Accession Number	MW (kDa)	pI	Sequence coverage	Score	PlasmoDB
1	7004	PGM1	XP_001347879.1	28.8	8.28	12.40	19.03	PF3D7_1120100
2	7114	LDH	XP_001349989	34.1	7.55	27.22	50.84	PF3D7_1324900
		GAPDH	XP_001348772.1	36.6	7.69	47.48	36.80	PF3D7_1462800
3	7115	Fructose-bisphosphate aldolase	XP_001348599.1	40.1	8.12	20.05	26.76	PF3D7_1444800
		PGK	XP_001352096.1	45.4	7.83	11.06	26.32	PF3D7_0922500
4	3108	Unknown	–	–	–	–	–	–
5	6005	Unknown	–	–	–	–	–	–
6	6225	Proteasome subunit, putative	XP_001350251.1	27.2	6.55	26.97	36.54	PF3D7_1353900

4. Discussion

The proteomics approach is a potential tool for target identification in the discovery of medicines and vaccines. For malaria research in the post-genomics era, this tool has facilitated the search for new promising targets that are selective and biologically significant for parasite's growth and survival. Nevertheless, the application of 2-DE in the investigation of malaria parasite proteomes has been limited due to the problem relating to the extraction and solubilization of parasite proteins. In our previous study, the ethanolic extract of *G. mangostana* Linn. (pericarp) was shown to exhibit good to moderate antimalarial activity with IC_{50} of 4.5-12.6 μg/mL[4]. In the present study, the proteomics approach was applied to analyze possible malarial parasite's protein targets of *G. mangostana* Linn. (pericarp). Difference in protein patterns of 3D7 *P. falciparum* extract following exposure to the plant extract at the concentrations of 12 (IC_{50}) and 30 (IC_{90}) μg/mL was observed. At the IC_{50} concentration, about 82% of the expressed parasite proteins were found to be matched with the control, while at the IC_{90} concentration, only 15% matched proteins were found. The possible explanation for this phenomenon is that the concentration at IC_{90} level was too high to detect an initial change in protein expression. This supposition was supported by the detection of proteasome subunit protein expressed following parasite's exposure to the extract at the IC_{90} level. Although malaria parasites critically rely on lysosomes and the ubiquitin proteasome system for their growth and survival[13,14], this proteasome subunit protein plays a major role in the degradation of cellular contents of the damaged parasites[13,14]. Following parasite's exposure to the extract at the IC_{50} level on the other hand, an initial change in protein expression was observed, but with absence of this protein. The expression of proteins that were markedly changed (at least three-fold up-regulated) are proteins which are involved in the glycolysis pathway, *i.e.*, PGM1, LDH/GAPDH, and fructose-bisphosphate aldolase/PGK[15,16]. The malaria parasite utilizes glucose for energy production, which is converted to lactate[15]. Several glycolytic intermediates may be diverted into other products that serve as precursors for the synthesis of other final products, particularly those involved in the redox system[16]. In the footprinting metabolites, pyruvates were expressed at the level of about

six times of the control (Chaijaroenkul *et al*, unpublished data). The metabolism of pyruvate may provide intermediates in several biosynthetic pathways. Furthermore, direct evidence showed that *G. mangostana* Linn. (pericarp) might affect glucose or glycolysis pathway of malaria parasite.

The proteomics approach is a potential tool for target identification in the drug discovery. Investigation of possible protein targets for antimalarial activity of ethanolic extract of *G. mangostana* Linn. suggested that the glycolysis pathway may be the major protein target from this extract.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

The study was supported by The National Research Council of Thailand, (Grant No.034/2556) and the Commission on Higher Education, Ministry of Education of Thailand (NRU Project). Wanna Chaijaroenkul receives financial support from Thailand Research Fund, (Grant No. MRG5380192). We would like to thank Dr. Gavin Laing for protein analysis (LC/MS/MS).

Comments

Background

Though, its clinical application is far from present, the present paper has generated a significant contribution of the proof of concept, and deeper understanding of using mangosteen extract as antimalarial drug target.

Research frontiers

The paper addresses the effect of mangosteen extract on malaria glycolysis pathway. Methodology applied is straight forward and has yielded appreciable results. The work generated scientific idea to bridge between "concept" and future "clinical application".

Innovations and breakthroughs

The paper concluded that mangosteen affects parasite glycolysis pathway. The paper initiates further research interest in looking for new antimarial drug target using medicinal plants/fruits parts.

Applications

The proteomics approach is a potential tool for target identification in the drug discovery. Investigation of possible protein targets for antimalarial activity of ethanolic extract of *G. mangostana* Linn. suggested that the glycolysis pathway may be the major protein target from this extracts.

Peer review

The paper is a basic research which looked at new antimalarial target-mangosteen extract and concluded its effect through glycolysis pathway.

References

[1] Brewis IA, Brennan P. Proteomics technologies for the global identification and quantification of proteins. *Adv Protein Chem Struct Biol* 2010; **80**: 1-44.

[2] Stoyanov A. IEF-based multidimensional applications in proteomics: toward higher resolution. *Electrophoresis* 2012; **33**(22): 3281-3290.

[3] Mahabusarakam W, Kuaha K, Wilairat P, Taylor WC. Prenylated xanthones as potential antiplasmodial substances. *Planta Med* 2006; **72**(10): 912-916.

[4] Thiengsusuk A, Chaijaroenkul W, Na-Bangchang K. Antimalarial activities of medicinal plants and herbal formulations used in Thai traditional medicine. *Parasitol Res* 2013; **112**(4): 1475-1481.

[5] Shan T, Ma Q, Guo K, Liu J, Li W, Wang F, et al. Xanthones from mangosteen extracts as natural chemopreventive agents: potential anticancer drugs. *Curr Mol Med* 2011; **11**(8): 666-677.

[6] Obolskiy D, Pischel I, Siriwatanametanon N, Heinrich M. *Garcinia mangostana* L.: a phytochemical and pharmacological review. *Phytother Res* 2009; **23**(8): 1047-1065.

[7] Pedraza-Chaverri J, Cardenas-Rodriguez N, Orozco-Ibarra M, Perez-Rojas JM. Medicinal properties of mangosteen (*Garcinia mangostana*). *Food Chem Toxicol* 2008; **46**(10): 3227-3239.

[8] Trager W, Jensen JB. Human malaria parasites in continuous culture. *Science* 1976; **193**(4254): 673-675.

[9] Bennett TN, Paguio M, Gligorijevic B, Seudieu C, Kosar AD, Davidson E, et al. Novel, rapid, and inexpensive cell-based quantification of antimalarial drug efficacy. *Antimicrob Agents Chemother* 2004; **48**(5): 1807-1810.

[10] Smilkstein M, Sriwilaijaroen N, Kelly JX, Wilairat P, Riscoe M. Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening. *Antimicrob Agents Chemother* 2004; **48**(5): 1803-1806.

[11] Makanga M, Bray PG, Horrocks P, Ward SA. Towards a proteomic definition of CoArtem action in *Plasmodium falciparum* malaria. *Proteomics* 2005; **5**(7): 1849-1858.

[12] Rungsihirunrat K, Chaijaroenkul W, Siripoon N, Seugorn A, Thaithong S, Na-Bangchang K. Comparison of protein patterns between *Plasmodium falciparum* mutant clone T9/94-M1-1(b3) induced by pyrimethamine and the original parent clone T9/94. *Asian Pac J Trop Biomed* 2012; **2**(1): 66-69.

[13] Chung DW, Ponts N, Prudhomme J, Rodrigues EM, Le Roch KG. Characterization of the ubiquitylating components of the human malaria parasite's protein degradation pathway. *PLoS One* 2012; doi: 10.1371/journal.pone.0043477.

[14] Rosenthal PJ. Falcipains and other cysteine proteases of malaria parasites. *Adv Exp Med Biol* 2011; **712**: 30-48.

[15] Sander BJ, Lowery MS, Kruckeberg WC. Glycolytic metabolism in malaria infected red cells. *Prog Clin Biol Res* 1981; **55**: 469-490.

[16] MacRae JI, Dixon MW, Dearnley MK, Chua HH, Chambers JM, Kenny S, et al. Mitochondrial metabolism of sexual and asexual blood stages of the malaria parasite *Plasmodium falciparum*. *BMC Biol* 2013; **11**: 67.