

In vitro cysteine protease inhibitory activity of selected Indian antimalarial plants

Jigna Vadalia*, Shruti Vekariya, Mihir Raval & Navin Sheth

*Department of Pharmaceutical Sciences, Saurashtra University, Rajkot-360 005, Gujarat, India
E-mails: jigna.vadalia@gmail.com, s.a.vekariya@gmail.com, rmihir@yahoo.com, navin_sheth@yahoo.com.

Received 27 June 2016, revised 22 December 2016

In this study few plants having antimalarial activity (leaves of *Nyctanthes arbor-tristis* L., *Caesalpinia crista* L., *Ailanthus excelsa* Roxb., *Bauhinia variegata* L., seed of *Balanites aegyptiaca* Delile, entire plant of *Enicostema littorale* Blume, fruits of *Momordica charantia* L.) were screened for cysteine protease inhibitory activity. Cysteine protease inhibitory activity was done by papain inhibition assay. Water and methanol extracts of all selected plants were screened for *in vitro* enzyme inhibition assay. Percentage inhibition of papain was measured and IC₅₀ for all the extracts were calculated. Comparative study of above selected medicinal plants methanolic and water extract showed the maximum inhibition in leaves of *N. arbor-tristis*, 87.8051 %, IC₅₀ – 13.03 µg/ml and 85.6189 %, IC₅₀ - 16.54 µg/ml, respectively. The present study has provided scientific validity to leaves of *N. arbor-tristis* against cysteine protease inhibition activity and it is concluded that protease inhibitor of *N. arbor-tristis* leaves are an indicator of wide range of pharmacological activities such as anticancer, antimalarial, osteoarthritis, osteoporosis, etc., isolation of cysteine protease inhibitors may provide a lead compound for development of novel therapeutic agents in the above areas.

Keywords: *Nyctanthes arbor-tristis*, Falcipain, Cysteine protease inhibitor, Papain inhibition, Malaria.

IPC Int. Cl.⁸: A61K 36/00, A61K, C12P, A61K 38/43, C12N 9/00, C12N 11/00, A01D 20/46

Cysteine proteases are widely distributed throughout nature, having been found in viruses, bacteria, protozoa, plants, mammals and fungi¹. Papain like cysteine proteases (henceforth referred to as the papain super family) belongs to clan CA and includes cathepsin B and calpains. Cysteine proteases of the papain super family have been implicated in numerous diseases and cellular processes and are thus attractive targets for therapeutic drugs². Papain is the most extensively studied protease, and the first one for which a crystal structure was determined. Papain is isolated from the tropical papaya (*Carica papaya*) fruit. Papain is considered to have a broad specificity relative to other cysteine proteases. *Plasmodium* cysteine proteases (falcipains: *P. falciparum*, *P. vivapains*, *P. vivax*, *P. berghepains*, *P. berghei*, *P. yoeli-pains*, *P. yoelii*) play important role during activation of pro-enzymes, hemoglobin degradation and egress of parasites from red blood cells, erythrocytes rupture^{3,4}. Malaria, the most prevalent protozoal infection worldwide, remains a major public health problem globally. A number of drugs are currently available to treat malaria, however, treatment is becoming complicated

by frequent drug resistance strain of *Plasmoium* to existing antimalarial drug, toxicity, and high cost⁵. Recently, drug resistance against the new effective drug artemisinin, is also emerging and we need new effective drugs to treat malaria. Therefore, the development of other classes of effective antimalarials, especially compounds that act against novel biochemical targets, is required. Among potential new targets for antimalarial chemotherapy are *Plasmodium* proteases. Proteases are drugable targets, and at present protease inhibitors are now licensed as well as in clinical development to treat different diseases for example osteoporosis, diabetes, cancer, hypertension, and infectious diseases⁶.

Cysteine protease inhibitors blocked the invasion of hepatocytes by *P. falciparum* as well as blocked invasion of red blood cells⁷ and the disruption of cysteine protease gene of *Plasmodium berghei* which prevented sporozoite egress from oocyst⁸ indicating that cysteine protease plays an important role in both erythrocytic stage and non-erythrocytic stage parasites. Since malarial cysteine proteases have broad specificity, it is important to regulate their activity for the survival of the parasite and host. The aim of the present work was to evaluate cysteine

*Corresponding author

protease inhibitory activity of leaves of *N. arbor-tristis*^{9,10}, *C. crista*¹¹, *A. excelsa*¹², *B. variegata*¹³, seed of *B. aegyptiaca*¹⁴, entire plant of *E. littorale*¹⁵ and fruit of *M. charantia*¹⁶ which are official in *Ayurveda* and *Charaksamhita* to treat malaria and are also proved scientifically for their antimalarial activity.

Materials and methods

Collection and identification of materials

Leaves of *N. arbor-tristis* and seeds of *B. aegyptiaca* were collected from Kunkavav, Amreli (Gujarat). Leaves of *A. excelsa* and *B. variegata* were collected from Saurashtra University campus, Rajkot. Leaves of *C. crista* were collected from Dhoraji, Rajkot. Fruits of *M. charantia* were purchased from market of Rajkot, entire plants of *E. littorale* were collected from Dharala, Rajkot. The plants were identified and authenticated by Botanist Prof Vrunda S Thakkar, Department of Bioscience, Saurashtra University, Rajkot and the plant specimen number SU/DPS/HERBS/63 were deposited in the Department of Pharmaceutical Sciences, Saurashtra University, Rajkot. Papain enzyme obtained from Meteoric Life Sciences Private Limited, Ahmedabad.

Preparation of powdered material

Leaves *N. arbor-tristis*, *C. crista*, *A. excelsa*, *B. variegata*, seeds of *B. aegyptiaca*, entire plants of *E. littorale*, fruits of *M. charantia* were collected and properly cleaned and dried in oven at 45 °C to remove excess of moisture. The dried plant material was subjected to size reduction to coarse powder, stored in air tight container and then used for further investigation.

Hot extraction of plant materials

Place about 5 gm of coarsely powdered air dried materials of leaves *N. arbor-tristis*, *C. crista*, *A. excelsa*, *B. variegata*, seeds of *B. aegyptiaca*, entire plants of *E. littorale* and fruits of *M. charantia* inaccurately weighed two set of glass-stoppered conical flask. Add 100ml of methanol in one flask and add 100 ml water in other flask to prepare methanolic and water extract of plants materials, weight contain the total weight including the flask. Shake well and to allow stand for 6 hrs. Attach reflux condenser to the flask and boil gently for 1 hr; cool and weigh. Shake well and filter rapidly through a dry filter. Transfer whole of the filtrate to a porcelain dish and evaporate to dryness on a water-bath at 60 °C, cool in a

desiccators for 30 min and dry extract stored in air tight container at -40 °C temperature.

Optimization of assay conditions

Optimization of physiochemical parameters are like temperature, pH, incubation time, buffer and enzyme concentration. A temperature range of 37-40 °C and pH range 6-10 were screened for optimization. Also incubation period of papain with inhibitor (15min, 2 hrs, and over night) and papain concentration (0.1, 0.5, and 1 %) were optimized. Optimization of casein preparation (water, 0.05M Na₂PO₄, 0.01 M Na₂PO₄, 0.1 M and 0.0068 M KH₂PO₄ buffer) was done. The assay was performed described as above. The absorbance was measured at 280 nm and a graph of papain concentration vs. % inhibition was plotted in order to study the effect of different concentrations of papain on proteolytic activity of papain.

Papain inhibition assay¹⁷⁻¹⁹

Pipette 2 ml of 1 % papain (prepared in phosphate-cysteine-EDTA buffer solution) and 2 ml Inhibitor (plant extracts of concentration of 5, 10, 30, 50, 80, 100, 500, 1000 µg/ml prepared in 0.5 % DMSO) into each of a series of tube (size 15 x 100 mm) is incubated for 15 min at 40 ± 0.1 °C. Pipette 5 ml of 1 % casein substrate (prepared in 0.068 M potassium phosphate monobasic pH 8) into other each series of tubes and equilibrate the tubes for 15 min in an incubator maintained at 40 ± 0.1 °C. Then add enzyme and inhibitor solution into each of a series of tube into the equilibrated substrate, starting the stopwatch at zero time. Mix each by swirling, stopper and place the tubes back in incubator. After 60.0 min, add 3 ml of 30 % TCA Solution to each tube (caution: Do not use mouth suction). Mix each tube immediately by swirling. The reaction mixture is centrifuged, and absorbance of the supernatant is measured at 280 nm. The reading is corrected for a blank in which the enzyme is added after addition of TCA (Fig. 1).

Screening of plant extracts by papain inhibition assay

Methanolic and water extract of leaves of *N. arbor-tristis*, *C. crista*, *A. excelsa*, *B. variegata*, seed of *B. aegyptiaca*, entire plant of *E. littorale*, fruit of *M. charantia* are screened by papain inhibition assay to study cysteine protease inhibitor activity of plant extracts as per above mentioned protocol.

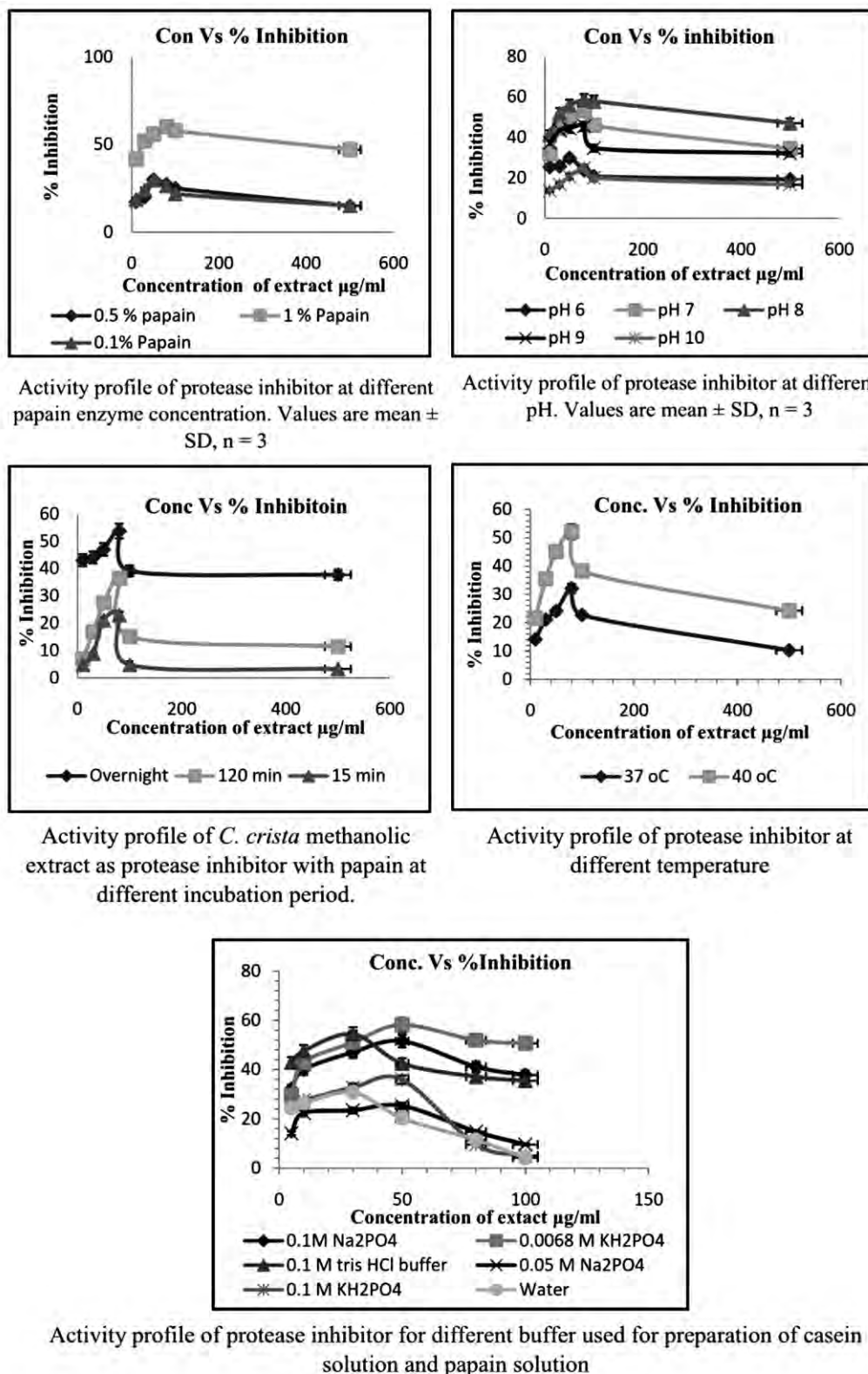


Fig.1 — Optimization of different parameter for papain inhibition assay

Statistical analysis

All data were expressed as the mean $\pm$ SEM. Data was subjected to one way ANOVA followed by Dunnett test. The statistical analysis was conducted with Graph pad InStat Software (Version 3, USA). The values of $P < 0.05$ were considered as statistically significant.

Results

Optimization of assay condition

Assay conditions are optimized by using methanolic extract of *C. crista* leaves as protease inhibitor (Fig. 1). Optimized parameter are 1 % papain (60.11 % inhibition), pH 8 (58.50 % inhibition), Overnight incubation (53.75 % inhibition), 40 °C (52.12 % inhibition) and 0.0068M KH₂PO₄ for casein preparation (58.13 % inhibition).

Screening of methanolic extracts of plants materials by papain inhibition assay

Most of the natural protease inhibitor are proteinaceous in nature and are located mainly in seed, leaves and tubers which act as specific defense and regulatory proteins. Many reports are available on the isolation, purification and characterization of protease inhibitor from seeds of legume plants²⁰. Hence, few plants belonging to different families of Leguminosae, Oleaceae, Zygophyllaceae, Cucurbitaceae were screened for protease inhibitor activity. The results are presented in Fig. 2 shows percentage papain inhibition activity by different methanolic extracts of above plants. Maximum activity is shown by methanolic extract of leaves of *N. arbor-tristis* (87.8051 %, IC₅₀ -13.03 µg/ml) followed by *A. excelsa* (71.3414 %, IC₅₀ -5.01 µg/ml), *B. variegata*

(63.9691 %, IC₅₀ -18.29 µg/ml), *C. crista* (60.1156 %, IC₅₀ -28.19 µg/ml), *E. littorale* (59.7916 %, IC₅₀ -14.91 µg/ml), *M. charantia* (57.4380 %, IC₅₀ -11.74 µg/ml), *B. aegyptica* (54.5454 %, IC₅₀ -44.61 µg/ml).

Screening of water extracts of plants materials by papain inhibition assay

The results presented in Fig. 3 shows that maximum percentage papain inhibition activity is shown with their IC₅₀ value by water extracts of *N. arbor-tristis* leaves (85.6189 %, IC₅₀ -16.54 µg/ml) followed by *A. excelsa* (69.7832 %, IC₅₀ - 6.31µg/ml), *B. variegata* (63.8407 %, IC₅₀ -21.55 µg/ml), *C. crista* (56.7758 %, IC₅₀ -13.28 µg/ml), *E. littorale* (58.6805 %, IC₅₀ -14.91 µg/ml), *M. charantia* (54.8898 %, IC₅₀ -21.55 µg/ml), *B. aegyptiaca* (54.6143 %, IC₅₀ - 44.98 µg/ml).

Discussion

Comparative studies of methanolic and water extracts on cysteine protease (papain) inhibition profile is described and it indicate that, the methanolic and water extract of leaves of *N. arbor-tristis* is promising candidate for cysteine protease inhibition activity. There is no significant difference between the methanolic and water extracts. So, it is concluded that both the solvent extracts show almost equivalent cysteine protease inhibition activity. A major reason for the continued severity of the worldwide malarial problem is the increasing resistance of malarial parasites to available drugs²¹. Proteases appear to be required for the rupture and subsequent reinvasion of erythrocytes by merozoite-stage parasites and for the degradation of hemoglobin by intra-erythrocytic trophozoites²². A malarial cysteine proteinase participates in the dissociation of the hemoglobin

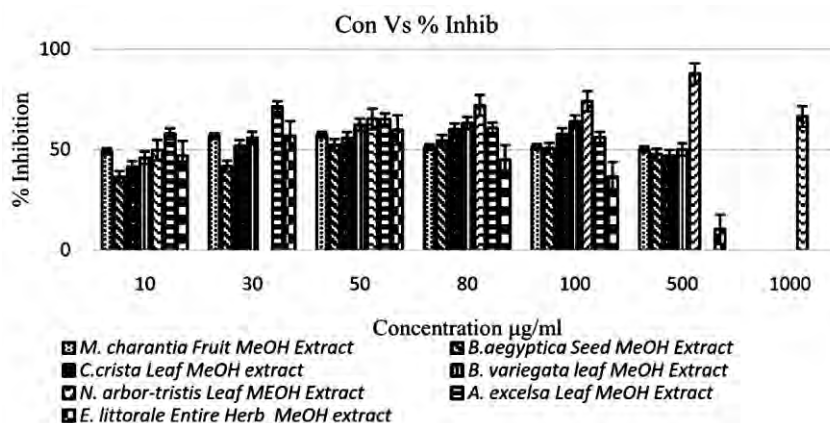


Fig. 2 — Screening of methanolic extracts of selected medicinal plants for papain inhibitory activity

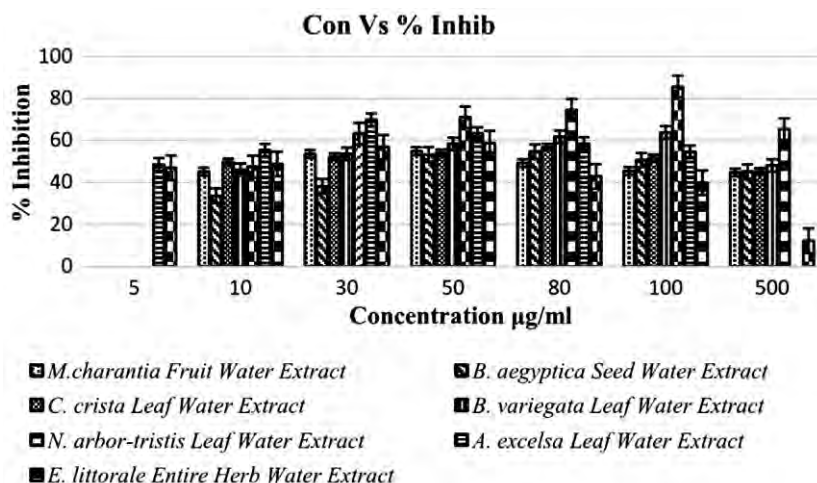


Fig. 3 — Screening of water extracts of selected medicinal plants for papain inhibitory activity

tetramer and the release of heme/haem from globin. Thus, the malarial cysteine proteinase is a promising target for antimalarial chemotherapy²³. It is necessary to evaluate new modes of therapy directed against cysteine protease like target.

Cysteine protease inhibitors are an indicator of wide range of pharmacological activities such as anticancer, antimalarial, osteoarthritis, osteoporosis, autoimmune diseases, etc. Hence, the results of this project may provide a lead compound for development of novel therapeutic agents in the above areas.

In the past, the antimalarial plants of traditional system took a long time to enter the global mainstream use. This time lag was costly in terms of morbidity and mortality in malaria. Hence, it is vital to pursue this experimental hit by an effective and trans-disciplinary research path. Biological evaluation of plants used traditionally for primary healthcare are one way in which searching for new leading compounds should concentrate. *In vitro* biological evaluation for the detection of antimalarial activity in plant extracts are currently rapid way for new lead. Efficacy and safety of traditional medicines can be validated and their mechanism can be identified by this study.

Conflict of interest statement

The authors declare no conflicts of interest related to this work.

References

- Mehdi S, Cell-penetrating inhibitors of calpain, *Trends Biochem Sci*, 16 (1991)150-153.
- Mehrtens JM, *The design, synthesis and biological assay of cysteine protease specific inhibitors*, (University of Canterbury, 2007).
- Pandey KC, Macromolecular inhibitors of malarial cysteine proteases—an invited review, *J Biomed Sci Eng*, 6 (2013)885-895.
- Rosenthal PJ, Cysteine proteases of malaria parasites, *Int J Parasitol*, 4 (2004) 1489-1499.
- Solomon A, Tilahun T, Ariaya H, Berhanu E & Mirutse G, Antimalarial Medicinal Plants used by Gumuz people of Mandura Woreda Benishangol- Gumuz regional state, Ethiopia, *Indian J Tradit Knowle*, 15 (4) (2016) 546-552.
- Pandey KC & Dixit R, Structure-Function of Falcipains: Malarial Cysteine Proteases, *J Trop Med*, (2012) 2201, Article ID 345195.
- Pandey KC, Singh N, Arastu Kapur S, Bogyo M & Rosenthal PJ, Falstatin: A cysteine protease inhibitor of *Plasmodium falciparum* facilitates erythrocyte invasion, *PLoS Pathogens*, 2 (2006)11 article e117.
- Annika R, Christine L, Anna H, Tina W, Guido H, *et al.*, Exoerythrocytic *Plasmodium* parasites secrete a cysteine protease inhibitor involved in sporozoite invasion and capable of blocking cell death of host hepatocytes, *PLoS Pathogens*, (2010) 6,e1000825.
- Pinky Kumari, Dinkar S, Jain SK & Virander Ch, Bioactivity guided fractionation of leaves extract of *Nyctanthus arbor tristis* (Harshringar) against *P. falciparum*, *PLoS One*, 7 (12) (2012) e51714.
- Agrawal J, Shanker K, Chanda D & Pal A, *Nyctanthus arbor-tristis* positively affects immunopathology of malaria-infected mice prolonging its survival, *Parasitol Res*, 112 (2013) 2601-2609.
- Suryawanshi HP & Patel MR, Traditional uses, medicinal and Phytopharmacological properties of *Caesalpinia crista* linn - an overview, *Inter J Res Pharm Chem*, 1 (4) (2011) 179-84.
- Kumar D, Bhat Z, Singh P, Shah M & Bhujbal S, *Ailanthus excelsa* Roxb. is really a plant of heaven?, *Inter J Pharmacol*, 6 (2010) 535-550.
- Ali Esmail & Al-Snafi, The pharmacological importance of *Bauhinia variegates*—a review, *Int J Pharm Sci Res*, 4 (2013) 160-164.
- Peter K, Susanne D, Sabine S, Rudeka M, Stefanie H, *et al.*, *In vitro* and *in vivo* Antimalarial activity Assays of seeds from *Balanites aegyptiaca* compounds of the extract

- show growth inhibition and activity against Plasmodial Aminopeptidase, *J Parasit Res*, (2011) Article ID 368692.
- 15 Rajamani S, Thirunavukkarasu T, Munisami H, Ranganathan B & Ernest D, Pharmacognosy of *Enicostemma littorale*: A review, *Asian Pacific J Trop Biomed*, 3 (1) (2013) 79-84.
 - 16 Ananya P & Sarmistha S, Medicinal uses and molecular identification of two *Momordica charantia* varieties – a review, *Electronic J Biol*, 6 (2) (2010) 43-51.
 - 17 Food chemical codex, Institute of Medicine (US), Committee on Food Chemicals Code ed, plant proteolytic cativity, *Nat Acad Press*, 5 (2003) 198-199.
 - 18 Trivedi V, Rathore RPS, Kamble PR, Goyal M & Singh N, Pepsin, Papain and Hyaluronidase Enzyme Analysis: A Review, *Int J Res Pharm Sci*, 3 (1) (2013) 01-18.
 - 19 Bijina B, *Isolation, purification and characterization of protease inhibitor from Moringa oleifera*, Ph D thesis, University of Science and Technology, Cochin, 2006.
 - 20 Bijina B, Sreeja Ch, Soorej MB, Elyas KK, Ali H & Chandrasekaran M, Protease inhibitor from *Moringa oleifera* leaves, *Saudi J Bio Sci*, 18 (3) (2011) 273–281.
 - 21 Andrey S, Jed E. Olson & Rosenthal PJ, Antimalarial Synergy of Cysteine and Aspartic Protease Inhibitors, *Antimicrobiol Agents Chem*, 42 (9) (1998) 2254–2258.
 - 22 Rosenthal PJ, Proteases of Malaria Parasites: New Targets for Chemotherapy, *Emerg Infect Dis*, 4 (1) (1998) 49-57.
 - 23 Gamboa de Dominguez ND & Rosenthal PJ, Cysteine proteinase inhibitors block early steps in hemoglobin degradation by cultured malaria parasites, *Blood*, 87 (10) (1996) 4448-54.