

SUPPLEMENTARY MATERIAL

Silymarin, a polyphenolic flavonoid impede *Plasmodium falciparum* growth through interaction with heme

Pooja Rani Mina¹, Yogesh Kumar², Ajeet Kumar Verma¹, Feroz Khan², Sudeep Tandon³, Anirban Pal¹ and Mahendra Pandurang Darokar^{1**}

¹*Molecular Bioprospection Department, CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow-226015, U.P., India*

²*Metabolic & Structural Biology Department, CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow-226015, U.P., India*

³*Chemical Processing Department, CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow 226 015, India*

****Corresponding author:**

Dr. M.P Darokar

Molecular Bioprospection Department,

CSIR-Central Institute of Medicinal and Aromatic Plants,

P.O. CIMAP, Lucknow - 226015, India

mpdarokar@yahoo.com, mp.darokar@cimap.res.in

Running Head: Silymarin-heme interaction causing malaria parasite death

ABSTRACT

A polyphenolic flavonoid, Silymarin isolated from *Silybum marianum* is widely known for its hepatoprotective action. In the present study anti-plasmodial activity of Silymarin has been demonstrated for the first time having IC₅₀ of 14±0.33 µM against the NF-54 strain of *P. falciparum* with high selectivity index (>100). The parasitostatic action is exerted through inhibition of β-hematin/hemozoin formation which is due to the interaction (*K_d*=3.63±0.9µM) of silymarin with free heme in a Stoichiometry of 1:1 silymarin: heme complex resulting into heme-induced membrane damage in the parasite. Silymarin could hinder the glutathione and hydrogen peroxide-induced heme detoxification. Silymarin also

induces apoptosis in the parasite through the elevation of caspase-3 level in a dose-dependent manner. Results from the docking studies suggest that silymarin interacts with heme.

Keyword: Silymarin, β -haematin, Hemozoin, *P. falciparum*, Heme, Chloroquine

Experimental

P. falciparum culture

Chloroquine sensitive strain NF-54 of *P. falciparum* was cultured in RPMI-1640 medium supplemented with human O+ red blood cells, 25 mM HEPES, 0.2% sodium bicarbonate, 100 μ M hypoxanthine, 10 μ g/ml gentamycin, and 0.5% albumax II at 37°C (using the method described previously by Trager and Jensen 1976). Culture was maintained in a standard gas mixture consisting of 5% CO₂. The medium was changed after every 24 h and monitored through Geimsa staining. The culture was synchronized using 5% D-sorbitol treatment to obtain ring-stage parasites (Lambros and Vanderberg 1979).

In vitro antiplasmodial assay

Silymarin was dissolved in DMSO and further diluted with culture medium to achieve the required concentrations. Precautions was taken that the final concentration of DMSO does not exceed 1%. Parasite growth was determined spectrophotometrically in control and treated cultures using a parasite lactate dehydrogenase assay (pLDH) as described previously with minor modifications (Makler et al. 1993). A synchronous ring stage culture with 1% parasitemia and 1% hematocrit was incubated in 96-well tissue culture plate with different concentrations of silymarin (1, 10, 50 and 100 μ g/ml) at 37°C for 72 h. Chloroquine and artemisinin were used as positive control. After incubation, plates were subjected to three 20 minute freeze–thaw cycles to release cell content. Parasite culture was carefully mixed and aliquots of 20 μ l were added to another 96-well plate containing 25 μ l of NBT–PES (1.9 μ M NBT and 0.24 μ M PES) and 100 μ l of Malstat reagent (0.125% Triton X-100, 130 mM L-lactic acid, 30 mM Tris buffer and 0.62 μ M APAD) solution per well. The plate was incubated in dark for 30 minute and absorbance was recorded at 650 nm using a microplate reader (FLUOStar Omega, BMG Labtech). The antiplasmodial activity of silymarin was expressed as IC₅₀ (mean \pm SEM), calculated from dose–response curve data by nonlinear regression analysis.

The percent growth inhibition at different concentration was calculated using the following formula:

$$\text{Growth inhibition (\%)} = \frac{[\text{absorbance of control} - \text{absorbance of test}]}{[\text{absorbance of control}]} \times 100.$$

Drug-haematin-induced red blood cell lysis

Experiments that evaluated the lysis of human red blood cells by haematin and drug-haematin complexes were conducted by incubating 0.03% (vol/vol) cell suspensions in phosphate-buffered saline (pH 7.4) at 37°C for 1 h with various concentrations of haematin in the absence or presence of silymarin and measuring the decrease in absorbance at 700 nm.

Determination of selectivity ratio

Selectivity index (SI) is used as a parameter of clinical significance. Generally, selectivity index > 2.0 is considered as safe for natural products. SI of silymarin was calculated from the following expression as described previously (Omoregie and Sisodia 2012):

$$\text{SI} = \text{IC}_{50} \text{ of against Vero cells} / \text{IC}_{50} \text{ against } P. \textit{falciparum}$$

In vitro β-haematin formation inhibition assay and Measurement of hemozoin content in parasite lysate

In order to determine the mode of action of silymarin, its ability to inhibit β-hematin (or hemozoin) formation by the ferriprotoporphyrin IX (FP) biomineralization was studied (Ncokazi and Egan 2005). Briefly, the assay was performed in a 96-well plate, each well containing a mixture of 50 µl silymarin solution of different concentrations, 50 µl of 0.5 mg/ml hemin chloride freshly dissolved in DMSO, and 100 µl of 0.5 M sodium acetate buffer (pH 4.4). After incubation at 37 °C for 12 h, the plate was centrifuged at 550 X g for 8 minute . The supernatant was discarded by vigorously flipping the plate upside down and pellet was washed twice with 200 µl DMSO. Then the pellet was dissolved in 200 µl of 0.1 M NaOH solution, and absorbance was measured at 405 nm using a micro plate reader (FLUOStar Omega, BMG Labtech). The results were expressed as percent of inhibition compared to the negative control (DMSO). The positive control was chloroquine (in water). All the experiments were performed in triplicate.

P. falciparum culture of 10 % parasitemia was treated with different concentrations of silymarin, after incubation at 37°C for 36 h parasite was pellet down by centrifugation at

2,000 rpm and lysed by using 0.08% saponin (1X PBS). After centrifugation at 10,000 rpm, supernatant was discarded and pellet was washed thrice with an equal volume of 2.5% SDS buffered with 25 mM Tris-HCl (pH 7.4). The pellet was hydrolyzed to monomeric heme in 1N NaOH and measured spectrophotometrically using methods described earlier (Huy et al. 2002). β -haematin /hemozoin formation inhibition calculated as :

$$\frac{\text{Heme content in control} - \text{heme content in sample}}{\text{Heme content in control}} \times 100$$

Silymarin-haematin interaction assay

The silymarin-haematin interaction was evaluated by differential optical spectroscopy as mentioned earlier (Sarkar et al. 2016). Briefly, 100 μ M hemin solution and silymarin was added at different concentrations and soret spectrum was recorded at 300-700 nm using (FLUOStar Omega, BMG Labtech). The interaction was evaluated in term of equilibrium dissociation constant (Kd) by the equation used by Sarkar et al. 2016.

$$1/\Delta A = (K_D/\Delta A_{\infty}) 1/S + 1/\Delta A_{\infty}$$

Where S is the concentration of Silymarin, ΔA is the absorption changes at a particular wavelength and at saturating concentration of the ligand (Silymarin), absorption changes are expressed as ΔA_{∞} .

Stoichiometry between silymarin and heme was determined at pH 7.4 and pH 5.6 through continuation variation jobs plot (Auparakkitanon *et al.* 2003) in aqueous DMSO (40% v/v) solution 0.2 M HEPES and 0.2M MES respectively. 10 μ M haematin and 10 μ M silymarin mixed in in different molar ratios were prepared as follows: 0:10, 1:9, 1:4, 3:7, 2:3, 1:1, 3:2, 7:3, 4:1, 9:1, and 10:0. Spectra between 240 and 700 nm were recorded using FLUOStar Omega, BMG Labteck.

Inhibition of hydrogen peroxide-mediated hemin degradation

The monitoring of peroxidative decomposition of hemin was adapted from (Loria et al. 1999). Briefly, a freshly prepared hemin stock solution (300 μ M in 0.1 M NaOH) was added to the 96-well plate and incubated for 20 minute at room temperature. Finally 180 μ l bovine serum albumin ((1 mg/ml) prepared in 0.2 M sodium acetate (pH 5.2) along with 100 μ M

silymarin was added to the plate. To start peroxidative degradation of hemin, 10 μ l of 200 mM H_2O_2 was added and reduction in absorption was recorded at 405 nm at 0, 30 and 60 minute time point using (FLUOStar Omega, BMG Labtech). H_2O was used as a negative control instead of H_2O_2 included in each experiment. Results were expressed as the percentage of unchanged hemin in the solution at various time points.

Inhibition of glutathione-mediated hemin degradation

Inhibition of glutathione-mediated hemin degradation was performed according to method described by Garavito et al. 2007 where GSH is detected as an OPA-GSH adduct. Fresh hemin stock solution prepared in 0.1 M NaOH and distributed in 96-well plate for final concentration of 300 μ M, after that 1 mM DTPA was added to chelate free ferric which may oxidize GSH (Atamna and Ginsburg 1995), then GSH was added at 250 μ M concentration and silymarin at 50 μ M concentration. Reaction was started by adding 2.5 mM OPA (o-phthalaldehyde) to obtained OPA-GSH adduct which was measured using microplate reader (FLUOStar Omega, BMG Labtech) at 37°C at 0, 30 and 60 minute using $\text{ex} = 355$ nm and $\text{em} = 460$ nm.

Positive control contains hemin without the drug and negative control consist of NaOH (0.1 M) instead of hemin. Results were expressed as the percentage of remaining GSH after 30 or 60 minute incubation.

Silymarin-haematin induced red blood cell lysis

The effect of silymarin-haematin induced hemolysis was evaluated by following the method described earlier (Huy et al. 2007). Briefly, 100 μ l of heme (0 to 20 μ M) was incubated with or without silymarin (10 μ M) for 10 Then 50 μ l of erythrocyte suspension in PBS was added and incubated 2 h at 37°C and centrifuged at 2000 rpm for 2 minute, 150 μ l of supernatant transferred to 96-well plate and absorbance was measured at 630 nm. The absorbance of the control well, which contained only erythrocytes, was considered to be 0% hemolysis and was used to convert the remaining absorbance values to the percentage hemolysis.

Cytotoxicity and Hemocompatibility

Cytotoxicity was performed using Vero cell line (VERO C1008; ATCC CRL-1586) which was cultured in RPMI-1640 medium supplemented with 0.2% NaHCO_3 , 10% FBS, and 1 \times antibiotic-antimycotic solution at 37°C and 5% CO_2 . The cells were seeded in flat bottom 96-well cell culture plate and allowed to grow for 24 h. Cells were treated with different

concentrations (1 to 100 µg/ml) of silymarin and incubated for 48 h. Cytotoxicity was assessed in terms of cell viability using MTT assay as described earlier (Woerdenbag et al. 1993). Experimentally After incubation 10 µl of MTT (5 mg/ml in PBS) solution was added to each well, gently mixed and incubated for another 4 h at 37°C. After incubation culture medium was removed and 100 µl of DMSO was added to each well and mixed gently. The absorbance of control and treated wells was recorded using a microplate reader (FLUOStar Omega, BMG Labtech) at 570 nm. Cytotoxicity of test compounds was expressed as IC_{50s} (mean ± SEM), calculated from dose-response curve data by nonlinear regression analysis. Hemolysis was performed as described (Huy et al. 2007). Briefly Fresh blood from healthy donors was collected in heparinized tube and 250 µl of 10% (v/v) RBCs suspension was incubated under agitation at room temperature for 2 h containing 1 to 25 µg/ml concentration of Silymarin . Triton X-100 1% (v/v) was used as a positive control (100% lysis) and PBS (pH 7.4) as a negative control (0% lysis). The mixtures were then centrifuged at room temperature for 5 min at 8,000 × g and the absorbance of the supernatants was measured at 550 nm with a microplate reader (FLUOStar Omega, BMG LabTech). The red blood cell lysis percentage was determined as follows:

$$\text{Percent RBCs lysis} = (\text{OD}_{550\text{nm sample}} - \text{OD}_{550\text{nm PBS}}) / (\text{OD}_{550\text{nm Triton X-100 1\%}} - \text{OD}_{550\text{nm PBS}}).$$

Molecular docking study

Molecular docking study of silymarin and chloroquine were performed with designed hemozoin 3D structure. The docking study was performed by AutoDockVina (<http://www.vina.scripps.edu/>), (Molecular Graphics Lab at the Scripps Research Institute, USA). 21 PyRx v0.8 (<http://pyrx.sourceforge.net>) (Vanichtanankul et al., 2011).

Statistical analysis

One-way analysis of variance (ANOVA) was used. Dunnett's test was used to compare the treatment and control. $P < 0.05$ is considered statistically significant.

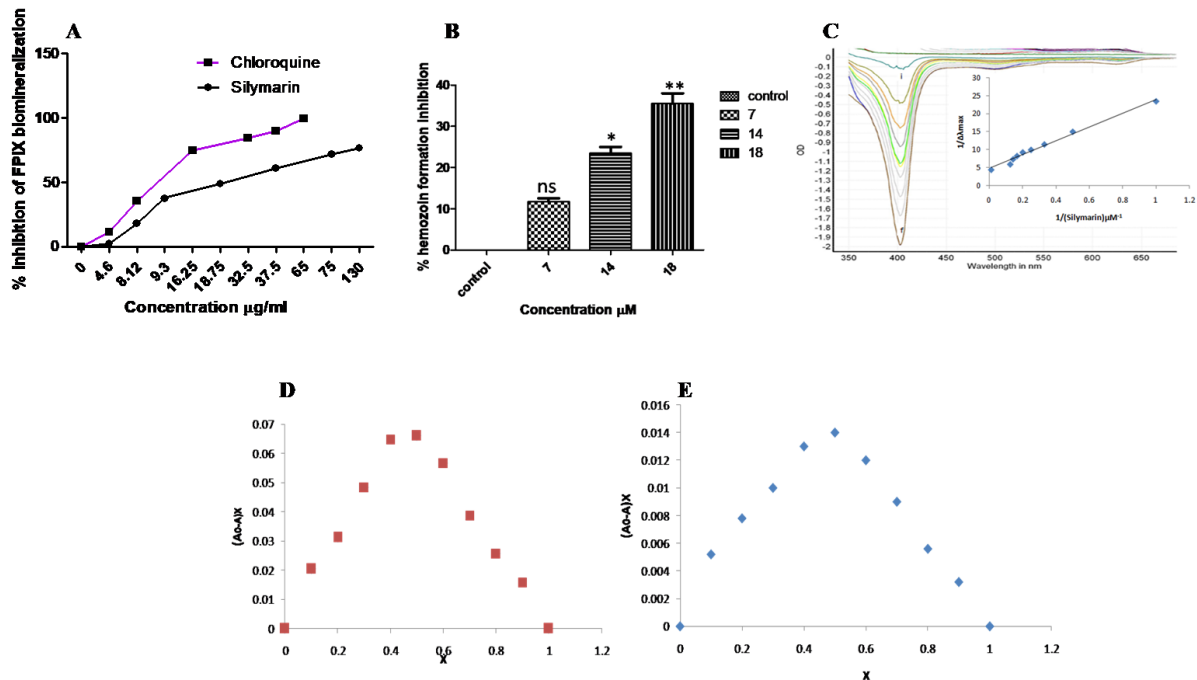


Figure S1. Interaction of silymarin with heme (A) *in vitro* concentration-dependent inhibition of β -haematin formation in presence of silymarin and chloroquine (B) concentration-dependent inhibition of hemozoin formation in *P. falciparum* culture (C) differential Soret spectroscopy spectra using various concentrations of silymarin-heme complex, (inset) plot of $1/\lambda_{\text{max}}(\text{nm})$ versus $1/[\text{silymarin}]$ used to calculate K_d values of silymarin-heme interaction; Job's plot of monomeric heme complex formation with silymarin at (D) pH 7.4 and (E) pH 5.6. Mole fraction of silymarin (X) = $[\text{Silymarin}]/([\text{Silymarin}] + [\text{heme}])$; A_0 is the absorbance, when $x = 1$ and A is the absorbance at respective values of X . data expressed as mean values \pm SEM. * $P < 0.05$, ** $P < 0.01$ vs. control: Dunnet test

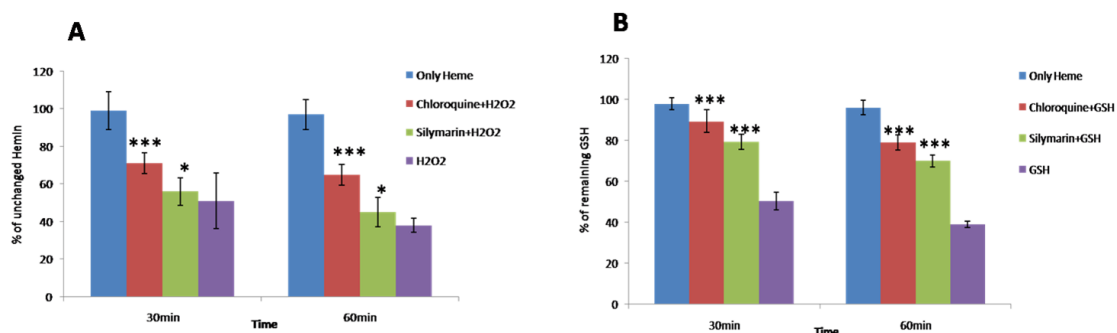


Figure S2. Inhibition of heme detoxification in presence of silymarin (A) peroxidative degradation of hemin at 20 μM of H_2O_2 and 100 μM of silymarin and chloroquine each; (B) GSH-mediated degradation at 50 μM of GSH and 100 μM of silymarin and chloroquine each. Data expressed as mean values \pm SEM. * $P < 0.05$ *** $P < 0.001$ vs control.

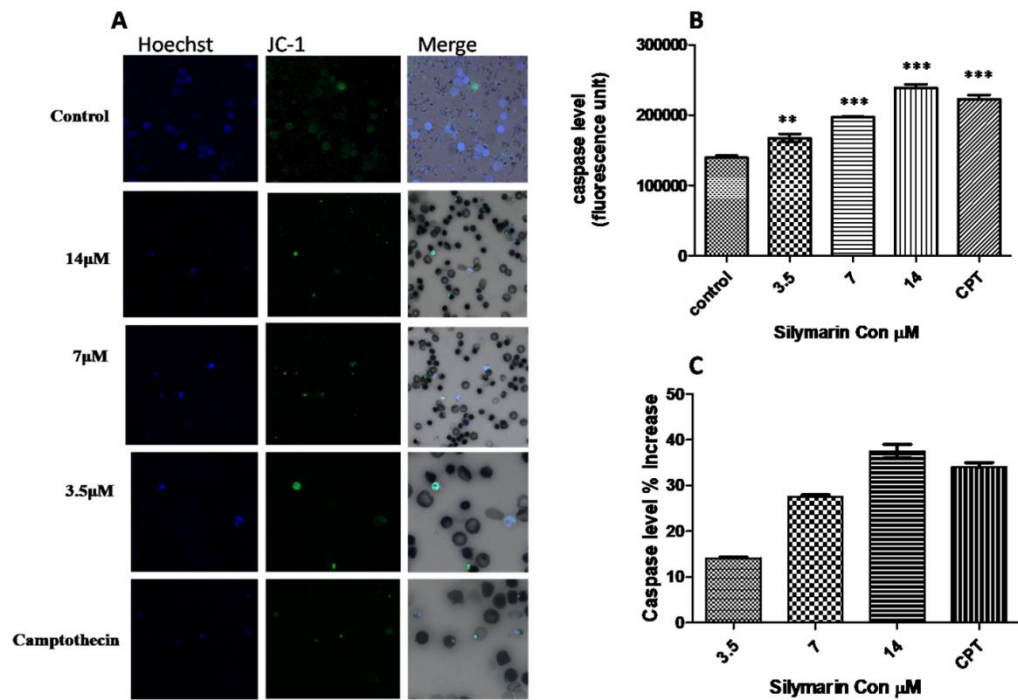


Figure S3. Pro-apoptotic effect of silymarin (A) concentration-dependent effect of silymarin on chromatin (Hoechst 33342) and mitochondria (JC-1) of *P. falciparum* (B) increase in caspase-3 level in terms of fluorescence units (C) percent increase in caspase-3 level over the control. Data expressed as mean values \pm SEM. , ** $P < 0.01$, *** $P < 0.001$ vs control Dunnet test.

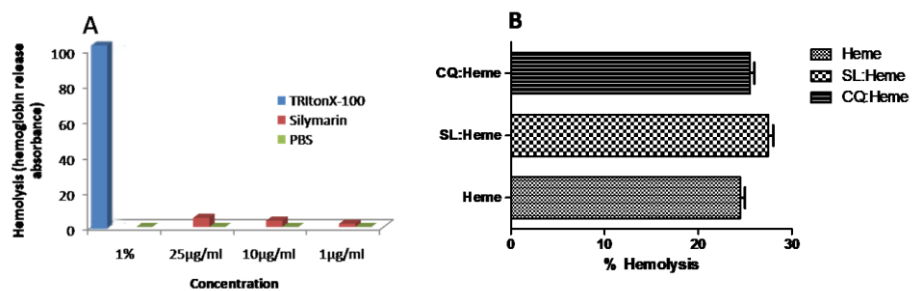


Figure S4. Hemolytic effect using RBC lysis measuring hemoglobin in presence of (A) silymarin and (B) silymarin-heme complex

References

- Auparakkitanon S, Noonpakdee W, Ralph RK, Denny WA, Wilairat P. 2003. Antimalarial 9-anilinoacridine compounds directed at hemozoin. *Antimicrobial agents and chemotherapy*.47(12):3708-3712.
- Huy NT, Kamei K, Yamamoto T, Kondo Y, Kanaori K, Takano R, Tajima K, Hara S. 2002. Clotrimazole Binds to Heme and Enhances Heme-dependent Hemolysis proposed antimalarial mechanism of clotrimazole. *Journal of Biological Chemistry*.277(6):4152-4158.
- Huy NT, Mizunuma K, Kaur K, Nhien NTT, Jain M, Uyen DT, Harada S, Jain R, Kamei K. 2007. 2-tert-butyl-8-quinolinamines exhibit potent blood schizontocidal antimalarial activity via inhibition of hemozoin crystallization. *Antimicrobial agents and chemotherapy*.51(8):2842-2847.
- Lambros C, Vanderberg JP. 1979. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. *The Journal of parasitology*65(3):.418-420.
- Loria P, Miller S, Foley M, Tilley L. 1999. Inhibition of the peroxidative degradation of haem as the basis of action of chloroquine and other quinoline antimalarials. *Biochemical Journal*.339(2):363-370.
- Makler M, Ries J, Williams J, Bancroft J, Piper R, Gibbins B, Hinrichs D. 1993. Parasite lactate dehydrogenase as an assay for *Plasmodium falciparum* drug sensitivity. *The American Journal of Tropical Medicine and Hygiene*.48(6):739-741.
- Ncokazi KK, Egan TJ. 2005. A colorimetric high-throughput β -hemozoin inhibition screening assay for use in the search for antimalarial compounds. *Analytical biochemistry*.338(2):306-319.
- Omeregbe E, Sisodia B. 2012. In vitro antiplasmodial activity and cytotoxicity of leaf extracts of *Jatropha tanjorensis* JL Ellis and Soroja. *Bayero Journal of Pure and Applied Sciences*.5(1):90-97.

Sarkar S, Siddiqui AA, Saha SJ, De R, Mazumder S, Banerjee C, Iqbal MS, Nag S, Adhikari S, Bandyopadhyay U. 2016. Antimalarial activity of small-molecule benzothiazole hydrazones. *Antimicrobial agents and chemotherapy*.60(7):4217-4228.

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

Vanichtanankul, J., Taweechai, S., Yuvaniyama, J., Vilaivan, T., Chitnumsub, P., Kamchonwongpaisan, S., & Yuthavong, Y. (2011). Trypanosomal Dihydrofolate Reductase Reveals Natural Antifolate Resistance. *ACS Chemical Biology*, 6(9), 905–911.

Woerdenbag HJ, Moskal TA, Pras N, Malingré TM, El-Feraly FS, Kampinga HH, Konings AW. 1993. Cytotoxicity of artemisinin-related endoperoxides to Ehrlich ascites tumor cells. *Journal of Natural Products*.56(6):849-856.