Quim. Nova, Vol. 33, No. 4, 867-870, 2010

Artigo

IN VITRO STUDY OF ANTIOXIDANT AND SCAVENGER PROPERTIES OF PHENOLIC COMPOUNDS FROM *Lychnophora* SPECIES

Cristiane F. F. Grael, Alexandre Kanashiro, Luciana M. Kabeya, Christiane O. Jordão, Renata Takeara, Leonardo Gobbo-Neto, Ana Cristina M. Polizello, Yara M. Lucisano-Valim, Norberto P. Lopes e João Luis C. Lopes*
Departamento de Física e Química, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Av. do Café s/nº, 14040-903 Ribeirão Preto - SP, Brasil

Recebido em 9/6/09; aceito em 3/11/09; publicado na web em 11/3/10

This paper describes the antioxidant effects of thirteen phenolic compounds isolated from plants of the genus *Lychnophora*. Two assays were performed to evaluate these effects: a cellular test that measured the luminol-enhanced chemiluminescence produced by neutrophils stimulated with opsonized zymosan and a cell-free test involving horseradish peroxidase-H_2O_2-luminol. In both assays, the antioxidant activity of the phenolic compounds was dependent on their concentration and chemical structure. Our results suggest that the ability of phenolic compounds from *Lychnophora* species to scavenge and inhibit the generation of ROS may be a mechanism underlying the anti-inflammatory activity of extracts from *Lychnophora* spp.

Keywords: antioxidant activity; *Lychnophora*; flavonoids.

INTRODUCTION

Neutrophils are often involved in tissue damage found in many inflammatory disorders, including rheumatoid arthritis, systemic lupus erythematosus, respiratory distress syndromes, blistering skin disorders and ulcerative colitis.[1] Inflammation-induced tissue damage seems to depend on the ability of neutrophils to generate and release great amounts of reactive oxygen species (ROS), such as $O_2^{\bullet-}$ and $HO^{\bullet}$, and the highly reactive HOCl produced by myeloperoxidase.[2] Excessive levels of ROS might overcome the endogenous antioxidant defense system, leading to tissue injury that can amplify the inflammatory process.

Compounds with antioxidant activity have been used to prevent or alleviate tissue damage in inflammatory disorders.[3,4] Phenolic compounds, especially flavonoids, have been studied as promising non-toxic antioxidants in various biological systems.[5,6] Besides their antioxidant properties, such compounds have shown significant anti-inflammatory effects.[3]

Phenolic compounds, such as flavonoids and cinnamic acid derivatives, are commonly found in the *Lychnophora* genus (Vernoniae, Asteraceae). Other constituents that were already reported to some species from this genus are triterpenoids, steroids, sesquiterpene lactones and lignans.[7,8] Species of the *Lychnophora* genus are endemic in the Brazilian "campo rupestre," a special kind of "cerrado" that exists at high altitudes. *Lychnophora* species are popularly known in Brazil as "arnica," "falsa arnica," or "arnica da serra", and their hydroalcoholic preparations, obtained from the leaves, roots and inflorescences, are often used in folk medicine to treat inflammatory disorders.[9] Crude extracts or isolated compounds from these species have been reported to exhibit other pharmacological effects, such as antitumoral, molluscicidal, trypanocidal, anticonvulsant and analgesic activities.[10,11] These different activities may be correlated with environmental conditions. Thus, variations in the total content and in the relative proportions of secondary metabolites in plants can modify the biological analysis and justifies the studies of series of natural products to define different contributions.[12]

The aim of the present study was to evaluate the inhibitory effect of thirteen phenolic compounds extracted from plants of the genus *Lychnophora* on ROS generation by stimulated neutrophils as well as their ROS scavenging properties in a cell-free system. The relationship between chemical structure and both biological effects is discussed.

*e-mail: joaoluis@usp.br

EXPERIMENTAL

Plant material and isolated compounds

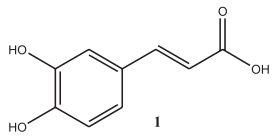
The tested compounds [caffeic acid (**1**), vicenin-2 (**2**), galangin 3-O-methyl (**3**), isorhamnetin 3-O-glucose (**4**), isorhamnetin 3-O-(6''-*p*-coumaroyl)-glucose (**5**), luteolin (**6**), pilloin (**7**), quercetin-3-O-methyl (**8**), tectochrysin (**9**), tilliroside (**10**), pinobanksin (**11**), pinobanksin 3-acetate (**12**) and pinocembrin (**13**)] were previously isolated from crude extracts of leaves plus inflorescences of *L. gardneri* Schultz-Bip (compound **10**),[13] *L. staavioides* Mart (compounds **2**, **3**, **8**, **9**, **11**-**13**),[10] *L. pohlii* Schultz-Bip (compounds **1**-**9**, **11**, **12**)[14] and *L. ericoides* Mart (compound **2**).[15] *L. ericoides* was collected in Ibiraci (Minas Gerais State, Brazil) and the other plant material was collected in Diamantina (Minas Gerais State, Brazil), in July 1998. Voucher specimens of spice plant materials are deposited in the UEC Herbarium (Campinas, SP, Brazil): *L. gardneri* (Lopes456), *L. staavioides* (NPL212), *L. pohlii* (WV451) and *L. ericoides* (NPL122). The chemical structures of the isolated compounds are shown in Figure 1. The tests were performed at the time of isolation of phenolic compounds (2000-2002).

Chemicals

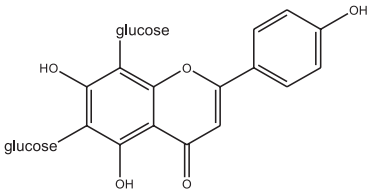
Luminol (5-amino-2, 3-dihydro-1, 4-phtalazinedione), Zymosan A, and Horseradish Peroxidase type VI-A (HRP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethylsulfoxide (DMSO) and H_2O_2 were obtained respectively from Merck-Schuchardt (Hohenbrunn, Germany) and Labsynth (Diadema, SP, Brazil). All other chemicals were of the highest quality available.

Isolation of rabbit neutrophils

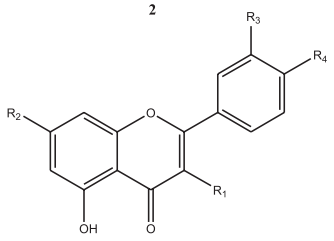
Blood from adult New Zealand White female rabbits (weight about 3 kg) was collected from the central ear artery into Alsever solution (v/v) as anticoagulant. Neutrophils were isolated as des-



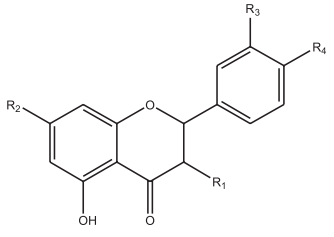
1



2



	R_1	R_2	R_3	R_4
3	OCH_3	OH	H	H
4	O-glucose	OH	OCH_3	OH
5	O-glucose-6´´--coumaroyl	OH	OCH_3	OH
6	H	OH	OH	OH
7	H	OCH_3	OH	OCH_3
8	OCH_3	OH	OH	OH
9	H	OCH_3	H	H
10	O-glucose-6´´--coumaroyl	OH	H	OH



	R_1	R_2	R_3	R_4
11	OH	OH	H	H
12	$OCOCH_3$	OH	H	H
13	H	OH	H	H

Figure 1. *Chemical structures of the phenolic compounds (**1-13**) isolated from Lychnophora species*

cribed[16] and suspended in Hanks Balanced Saline Solution (HBSS) containing gelatin 0.1% (w/v) (HBSS-gel) for use. The resulting cell preparations contained 80-90% of neutrophils, and more than 95% of these cells were viable, as determined by Trypan Blue exclusion.

ROS generation by stimulated neutrophils

Antioxidant activity in the cellular system was evaluated according to Lucisano-Valim *et al*..[17] The concentration of each component in the reaction medium (1 mL) is indicated in parentheses below. Luminol (0.28 mmol/L) and aliquots of each test-compound (2.5 -125 µmol/L) or DMSO (negative control) was added to neutrophil suspension (1 x 10^6 cells/mL), and the reaction mixture was incubated for 3 min at 37 °C. Afterwards, opsonized zymosan (OZ, 1 mg/mL) was added to the reaction tubes, which were carefully homogenized by inversion and transferred to the luminometer measuring chamber (Autolumat LB953, EG&G Bethold, Germany). Luminol and the tested compounds were dissolved in DMSO. Zymosan was prepared and opsonized with normal rabbit serum by the method of Cheung *et al*.,[18] with slight modifications.[6]

The luminol-enhanced chemiluminescence (CL-lum) produced by the stimulated neutrophils was measured for 10 min at 37 ºC. The area under each CL-lum time course curve (AUC), also known as integrated area of CL-lum, was determined. These areas represent the total amount of ROS produced by the cells in 10 min. The method is more sensitive to ROS produced by the myeloperoxidase-H_2O_2-halide pathway. The inhibition of CL-lum promoted by each test-compound was calculated as follows: [1 - (AUC of studied compound / AUC of negative control)] x 100 (%).

ROS generation by cell-free system

ROS-scavenging activity in cell-free system was evaluated according to Krol *et al*.,[19] with slight modifications. The concentration of each component in the reaction medium (1 mL) is indicated in parentheses below. Samples of each test-compound (0.01-1.0 µmol/L) or DMSO (control) were mixed with H_2O_2 (50 µmol/L) and luminol (0.28 mmol/L). The reaction was initiated by adding HRP (0.2 IU/mL). CL-lum was measured for 10 min at 27 ºC. CL-lum inhibition was expressed as percentage, as described in the neutrophil test. Luminol and the tested compounds were dissolved in DMSO; H_2O_2 and HRP were prepared in phosphate buffer (0.1 mol/L, pH 7.4).

Statistical analysis

The data were analysed by one-way analysis of variance (ANOVA) followed by the Tukey's post-hoc test. The compared values were considered significantly different when $P < 0.05$. The analysis was performed using the GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

RESULTS AND DISCUSSION

ROS generation by stimulated neutrophils

Immunomodulatory properties of the phenolic compounds isolated from plants of the genus *Lychnophora* were assessed by inhibition of OZ-stimulated rabbit neutrophils oxidative metabolism, measured by the luminol-enhanced chemiluminescence assay (Figure 2). Under our experimental conditions, compounds **6** and **8** were significantly more active than the other compounds tested, and compound **2** had no significant inhibitory activity, even at the highest concentration tested (125.0 µmol/L).

ROS generation by cell-free system

Free radical scavenging activity of the phenolic compounds isolated from plants of the genus *Lychnophora* was evaluated by inhibition of chemiluminescence produced by HRP-catalyzed oxidation of luminol in the presence of H_2O_2 (Figure 3). In this assay, compounds **6** and **8** were the most actives among the set of compounds tested, while compound **2** had no significant inhibitory activity, even at the

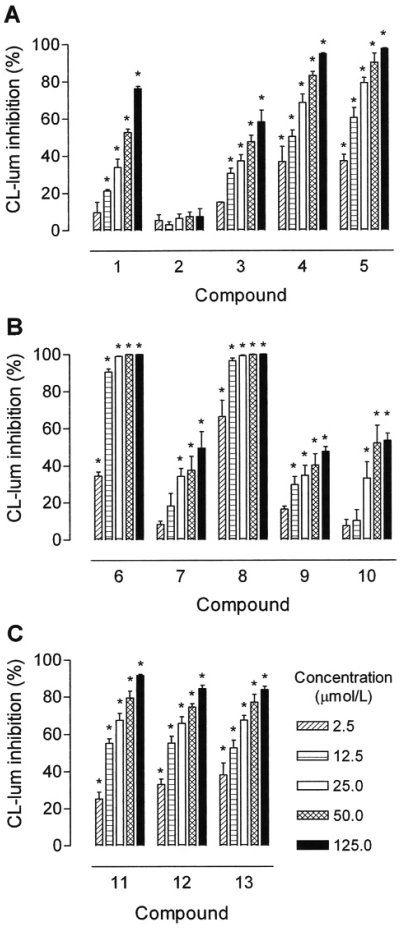


*Figure 2. Effects of phenolic compounds (2.5-125.0 μmol/L) isolated from Lychnophora species on ROS generation by opsonized zymosan-stimulated rabbit neutrophils. Compounds 1-5 (A), 6-10 (B) and 11-13 (C) were shown separately for a better visualization of their concentration-dependent inhibitory effects. Values are shown as mean ± SD of the inhibition (%) of CL-lum of three independent experiments. *Significant difference ($p < 0.05$) from DMSO-treated controls (negative control)*

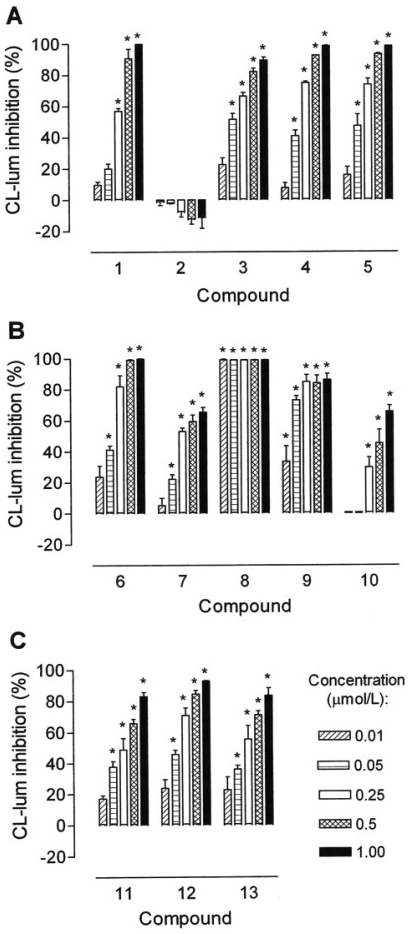


*Figure 3. Effects of phenolic compounds (0.01-1.0 μmol/L) isolated from Lychnophora species on ROS generation by HRP-H_2O_2-luminol chemiluminescence system. Compounds 1-5 (A), 6-10 (B) and 11-13 (C) were shown separately for a better visualization of their concentration-dependent inhibitory effects. Values are shown as mean ± SD of the inhibition (%) of CL-lum of three independent experiments. *Significant difference ($p < 0.05$) from DMSO-treated controls*

highest concentration tested (1.0 μmol/L). The course of CL-lum inhibition and its extent were similar in both tests, except for the phenolic acid **1**, whose inhibitory activity in the cell-free system was higher than in the cellular test.

Due to their sensitivity and reproducibility, chemiluminescence (CL)-based assays have been widely used for monitoring ROS generation by enzymes, cells, and tissues.[20] In the present study, a cellular and a cell-free luminol-enhanced chemiluminescence assays were used to evaluate the antioxidant and ROS scavenger effects, respectively, of thirteen phenolic compounds (Figure 1) isolated from *Lychnophora* species. The former involved rabbit neutrophils stimulated with particles of serum-opsonized zymosan and the later, a system composed of HRP-H_2O_2-luminol.[18] The extent of inhibition was also similar in both tests, except for the phenolic acid **1**, which had a higher inhibitory activity in the cell-free than in the cellular test. This result is particularly important, because **1** is one of the main metabolites of flavonoids produced by digestive enzymes and by microorganisms of the intestinal flora.[21] The reported antioxidant effects were dependent on the concentration and chemical structure of the tested compounds. A structure-activity analysis is presented below.

Being 5,7-dihydroxylated at ring A and having a catechol group at ring B, compounds **6** and **8** were the most active. Compound **3**, which is structurally similar to **8** but lacks the catechol group, was less active than **8**. This finding reinforces the hypothesis that the catechol group is important for antioxidant activity.[5,22] Our results also support the idea that methoxy groups may enhance the antioxidant activity of phenolic compounds, since they increase the electronic density of the aromatic ring.[23] Specifically, we observed that an additional methoxy group in R1 or R3 augmented the antioxidant activity, as can be observed by comparing **8** to **6** and **10** to **5**, although significant differences of effect could be observed only at the lowest concentrations. On the other hand, chemical characteristics of ring B substituents had no influence on the activity of 7-methoxylated compounds **7** and **9**. It thus seems that the position of the methoxy group is a major determinant of the antioxidant activity of flavonoids.

We observed that for compounds without C2-C3 double bond (**11**, **12** and **13**), the change of R2 from H (**13**) to OH (**11**) or ester (**12**) did not influence their ability to inhibit both the cellular and cell-free CL-lum. Similarly, in the case of the *O*-glycosylated flavonoids **4**, **5**, and **10**, an increase of molecular volume of the group attached to R1 (**4** *vs* **5**) did not influence the inhibitory activity.

Information about structure-activity relationship comes also from **2**, which had no effect on ROS generation by the cellular and cell-free systems under the assessed conditions. This compound has

C-glycosidic groups attached to positions 6 or 8 of the A ring, which could promote steric hindrance and impair ROS interaction with the hydroxy group of this ring. This reinforces that, as previously discussed, these interactions determine the antioxidant activity of flavonoids. It may thus seem that antioxidant properties are largely reduced in glycosylated flavonoids, the predominant natural form of flavonoids. In general, glycosylation increases hydrophilicity of a molecule, which in its turn impairs its interaction with cellular membranes and decreases its rate of absorption.[5] However, one must consider that in more complex organisms, gastrointestinal or bacterial enzymes removes the sugar moiety from flavonoid glycosides, making their aglycone forms available to be absorbed by the gut.[24]

The flavonoids **10** and **13** showed marked anti-inflammatory activity *in vivo* in previous studies.[25] Considering the involvement of ROS in the inflammatory process, we suggest that the antioxidant activity of these compounds found in the present work (Figures 2 and 3) may be a mechanism underlying their anti-inflammatory effect reported previously.

In summary, the inhibitory effect of the studied polyphenolic compounds on ROS generation by different systems was dependent on their concentration and chemical structures. The ROS-scavenging activity of these compounds, as evaluated by the cell-free system HRP-H_2O_2-luminol, could be one of the mechanisms responsible for inhibition of CL-lum produced by opsonized zymosan-stimulated neutrophils. Other mechanisms could also be involved, such as inhibition of enzymes that participate in ROS generation in neutrophils, such as NADPH oxidase, phosphoinositide 3-kinase γ, and myeloperoxidase.[26] Therefore, further studies are necessary to elucidate the mechanisms of action of these compounds and the CL-lum studies as reported here thus serve as stepping stone for more in-depth investigations of the drug-neutrophil interactions.

CONCLUSION

The antioxidant activity of phenolic compounds is highly dependent on their chemical structures. The presence of a catechol group and the extended conjugation in the caffeic acid molecule may contribute to its antioxidant activity. Regarding the flavonoids, some requirements may determine or increase the antioxidant activity: ortho-dihydroxy group in ring B; hydroxyl at C-4'; unsaturation between C-2 and C-3 in conjunction with the carbonyl at C-4 (ring C); hydroxyl at C-3 and C-5. Furthermore, the ability of phenolic compounds from *Lychnophora* species to scavenge and inhibit the generation of ROS may be a mechanism underlying the anti-inflammatory activity of hydroalcoholic extracts of many species from *Lychnophora* genus, which are used in Brazilian folk medicine.

ACKNOWLEDGMENT

This study was supported by the Brazilian agencies Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). The authors thank Dr. A. A. Steiner for his critical reading of the manuscript.

REFERENCES

1. Babior, B. M.; *Am. J. Med.* **2000**, *109*, 33.
2. Nathan, C.; *Nat. Rev. Immunol.* **2006**, *6*, 173.
3. Middleton Junior, E.; Kandaswami, C.; Theoharides, T. C.; *Pharmacol. Rev.* **2000**, *52*, 673.
4. Ostrakhovitch, E. A.; Afanas'ev, I. B.; *Biochem. Pharmacol.* **2001**, *62*, 743.
5. Rice-Evans, C. A.; Miller, N. J.; Paganga, G.; *Free Radic. Biol. Med.* **1996**, *20*, 933.
6. Kanashiro, A.; Kabeya, L. M.; Polizello, A. C. M.; Lopes, N. P.; Lopes, J. L. C.; Lucisano-Valim, Y. M.; *Phytother. Res.* **2004**, *18*, 61.
7. Robinson, H.; *Generic and subtribal classification of American Vernonieae – Smithsonian Contributions to Botany*, Smithsonian Institution Press: Washington, 1999.
8. Borsato, M. L.; Grael, C. F. F.; Souza, G. E. P.; Lopes, N. P.; *Phytochemistry* **2000**, *55*, 809.
9. Cerqueira, M. B. S.; Souza, J. T.; Amado Júnior, R.; Peixoto, A. B. F.; *Ciênc. Cult.* **1987**, *39*, 551.
10. Takeara, R.; Albuquerque, S.; Lopes, N. P.; Lopes, J. L. C.; *Phytomedicine* **2003**, *10*, 490.
11. Le Quesne, P. W.; Menachery, M. D.; Pastore, M. P.; Kelley, C. J.; Brennan, T. F.; Onan, K. D.; Raffauf, R. F.; *J. Org. Chem.* **1982**, *47*, 1519; Bazon, J. N.; Lopes, J. L. C.; Vichnewski, W.; Lopes, J. N. C.; Cunha, W. R.; *Fitoterapia* **1997**, *LXVIII,* 92; Grael, C. F. F.; Vichnewski, W.; Souza, G. E. P.; Lopes, J. L. C.; Albuquerque, S.; Cunha, W. R.; *Phytother. Res.* **2000**, *14*, 203; Taleb-Contini, S. H.; Santos, W. F.; Mortari, M. R.; Lopes, N. P.; Lopes, J. L. C.; *Basic Clin. Pharmacol. Toxicol.* **2008**, *102*, 281.
12. Gobbo-Neto, L.; Lopes, N. P.; *Quim. Nova* **2007**, *30*, 374.
13. Jordão, C. O.; Lopes, N. P.; Lopes, J. L. C.; *Resumos da 25ª Reunião Anual da Sociedade Brasileira de Química*, Poços de Caldas, Brasil, 2002.
14. Grael, C. F. F.; Albuquerque, S.; Lopes, J. L. C.; *Fitoterapia* **2005**, *76*, 73.
15. Gobbo-Neto, L.; Santos, M. D.; Kanashiro, A.; Almeida, M. C.; Lucisano-Valim, Y. M.; Lopes, J. L. C.; Souza, G. E. P.; Lopes, N. P.; *Planta. Med.* **2005**, *71*, 3.
16. Lucisano, Y. M.; Mantovani, B.; *J. Immunol.* **1984**, *1322*, 15.
17. Lucisano-Valim, Y. M.; Kabeya, L. M.; Kanashiro, A.; Russo-Carbolante, E. M. S.; Polizello, A. C. M.; Azzolini, A. E. C. S.; Silva, S. C.; Lopes, J. L. C.; de Oliveira, C. A.; Mantovani, B.; *J. Pharmacol. Toxicol. Methods* **2002**, *47*, 53.
18. Cheung, K.; Archibald, A. C.; Robinson, M. F.; *J. Immunol.* **1983**, *130*, 2324.
19. Krol, W.; Czuba, Z.; Scheller, Z.; Shani, J.; *J. Ethnopharmacol.* **1994**, *41*, 121.
20. Dahlgren, C.; Karlsson, A.; *J. Immunol. Methods* **1999**, *232*, 3.
21. Dragsted, L. O.; *Int. J. Nutr. Res.* **2003**, *73*, 112.
22. Kabeya, L. M.; Marchi, A. A.; Kanashiro, A.; Lopes, N. P.; da Silva, C. H. T. P.; Pupo, M. T.; Lucisano-Valim, Y. M.; *Bioorg. Med. Chem.* **2007**, *15*, 1516; Pietta, P. G.; *J. Nat. Prod.* **2000**, *63,* 1035.
23. van den Worm, E.; Beukelman, C. J.; van den Berg, A. J.; Kroes, B. H.; Labadie, R. P.; van Dijk, H.; *Eur. J. Pharmacol.* **2001**, *433*, 225.
24. Blaut, M.; Schoefer, L.; Braune, A.; *Int. J. Vitamin. Nutr. Res.* **2003**, *73*, 79.
25. Sala, A.; Recio, M. C.; Schinella, G. R.; Manez, S.; Giner, R. M.; Cerda-Nicolas, M.; Rios, J. L.; *Eur. J. Pharmacol.* **2003**, *461*, 53.
26. Tauber, A. I.; Fay, J. R.; Marletta, M. A.; *Biochem. Pharmacol.* **1984**, *33*, 1367; Selloum, L.; Reichl, S.; Muller, M.; Sebihi, L.; Arnhold, J.; *Arch. Biochem. Biophys.* **2001**, *395*, 49; 'T Hart, B. A.; Ip Via Ching, T. R.; van Dijk, H.; Labadie, R. P.; *Chem. Biol. Interact* **1990**, *73*, 323.