บทความวิชาการ

สารยับยั้งกลไกการดื้อยาที่เกิดจากการทำงานของ NorA Efflux Pump ในเชื้อแบคทีเรีย *Staphylococcus aureus*

Inhibitors of NorA Multidrug Resistance Efflux Pump in *Staphylococcus aureus*

สิริธร สโมสร*

Siritron Samosorn

Introduction

Superbugs, antibiotic resistance microorganisms, are a problem worldwide and need to be controlled by effective strategies. One of the major resistance mechanisms is conferred by an efflux pump, membrane transporter protein, which extrudes antibiotic out of bacterial cell, resulting in a sub-lethal concentration of the antibiotic within the cell. To date, five families of bacterial drug efflux pumps have been identified based on the energy source used for substrate extrusion from the cells and on sequence similarity. The Major Facilitator Superfamily (MF), Resistance-Nodulation Division Family (RND), Small Multidrug Resistance Family (SMR), and Multidrug and Toxic Compound Extrusion Family (MATE) use a proton motive force (PMF), pH gradient and electrochemical formation to efflux antibacterial agents in exchange for protons. In contrast, the ATP-Binding Cassette Superfamily (ABC) of transporter proteins derives its energy from ATP hydrolysis [1].

NorA efflux pump is a major multidrug transport protein in the human pathogenic bacterium *S. aureus,* an organism which possesses several multidrug efflux pump proteins, and is located in the cytoplasmic membrane [2]. The NorA pump is a member of the MF Superfamily and is encoded by the naturally occurring chromosomal *norA* gene, conferring an intrinsic resistance to a variety of structurally unrelated antibiotics [3]. Both naturally occurring and synthetic compounds can be pump substrates, including hydrophilic fluoroquinolones and monocationic

ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ

* ผู้นิพนธ์ประสานงาน, e-mail: siritron@swu.ac.th

organic compounds such as berberine, ethidium bromide, tetraphenylphosphonium, benzalkonium, chlorhexidine, pentamidine, norfloxacin and others (Figure 1) [2, 4]. The precise mechanism of multidrug recognition of the NorA pump is unclear due to the difficulty of performing high-resolution X-ray structural analysis on integral membrane proteins. Unfortunately none of the efflux pump proteins belonging to the MF superfamily have been crystallized with bound substrates, which would provide a better understanding of how the efflux pump proteins can bind and transport a wide range of structurally dissimilar substrates.

NorA Efflux Pump Inhibitors

Recently, efflux pump inhibitors (EPIs) of the NorA efflux pump have been investigated in order to potentiate the activity of antibiotics which are substrates of the efflux pump. A variety of natural product and synthetic inhibitors of the NorA efflux pump have been reported.

Reserpine (Figure 2), an indole alkaloid obtained from root extracts of *Rauwolfia serpentina*, was the first compound identified [4] as a potential inhibitor of NorA, which, when combined with a hydrophilic fluoroquinolone antibiotic, can potentiate the antibiotic potency. Reserpine enhanced the activity of ciprofloxacin (MIC = 8 μg/mL) against a fluoroquinolone-resistant strain of *S. aureus* SA-1199B, a strain that overexpresses NorA, by eight-fold at a concentration of 20 μg/mL [5]. Reserpine also prevented emergence of fluoroquinolone resistance in *S. aureus* [6] and *Streptococcus pneumoniae* [7]. Since reserpine is neurotoxic to humans at the concentration required for NorA inhibition, it cannot be used clinically as a NorA inhibitor [8]. This leads to a search for other compounds with more selective activity.

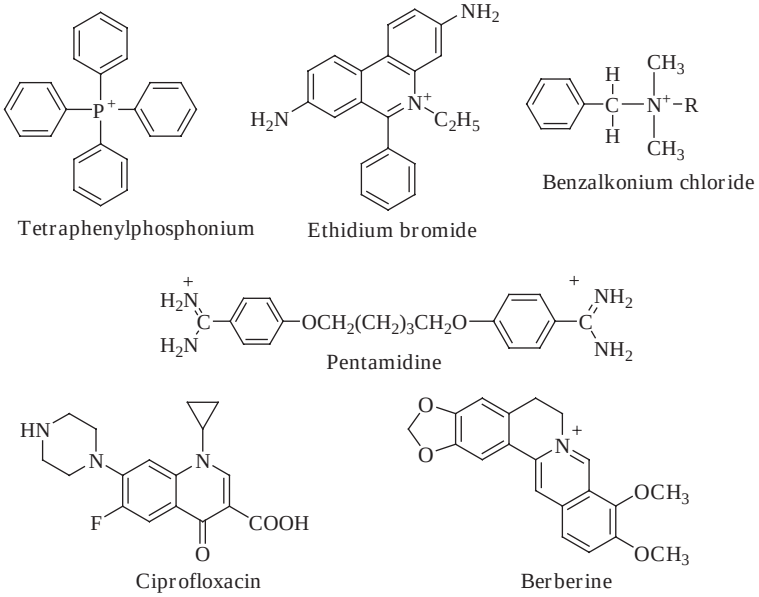


Figure 1 Substrates of NorA MDR pump.

A number of structurally different inhibitors of NorA, that possess chemical substructures inherent to the core nucleus of reserpine including the synthetic INF analogues, have been reported by Influx Inc. (Chicago, IL) (Figure 2). Screening of a chemical library containing 9,600 compounds for NorA inhibitor activity resulted in 399 compounds (4%) which were structurally unrelated, and which had inhibitory activity at least equipotent to that of reserpine. Five of the most potent INF analogue inhibitors including INF55, INF240, INF271, INF277, and INF392 (Figure 2) were tested in combination with ciprofloxacin against *S. aureus* resistant strain, SA-1199B, and were found to act synergistically and also reversed ciprofloxacin resistance four-fold (MIC of ciprofloxacin 2 versus 8 μg/mL) at a concentration of 0.2, 0.4, 0.8, 1.5 and 1.5 μg/mL for INF392, INF240, INF277, INF271, and INF55, respectively [8]. The most potent inhibitor of all the INF analogues was INF392, which was more potent than reserpine by fifty-fold. Also, INF392 reduced the MIC values of ethidium bromide (EtBr) and ciprofloxacin by eight-fold against SA-1199B at a concentration of 0.4 μg/mL [9].

Further development of a small molecule of the potent inhibitor INF55 to improve pump inhibitory activity was achieved by Samosorn *et al.* [10]. A variety of functionalisation in the aryl substituent group of INF55 derivatives were synthesized in order to assess their NorA pump inhibitory activity compared to that of the parent INF55. The compound [4-benzyloxy-2-(5-nitro-1*H*-2-yl)-phenyl]-methanol (Figure 2) was the most potent pump inhibitor in this series at a

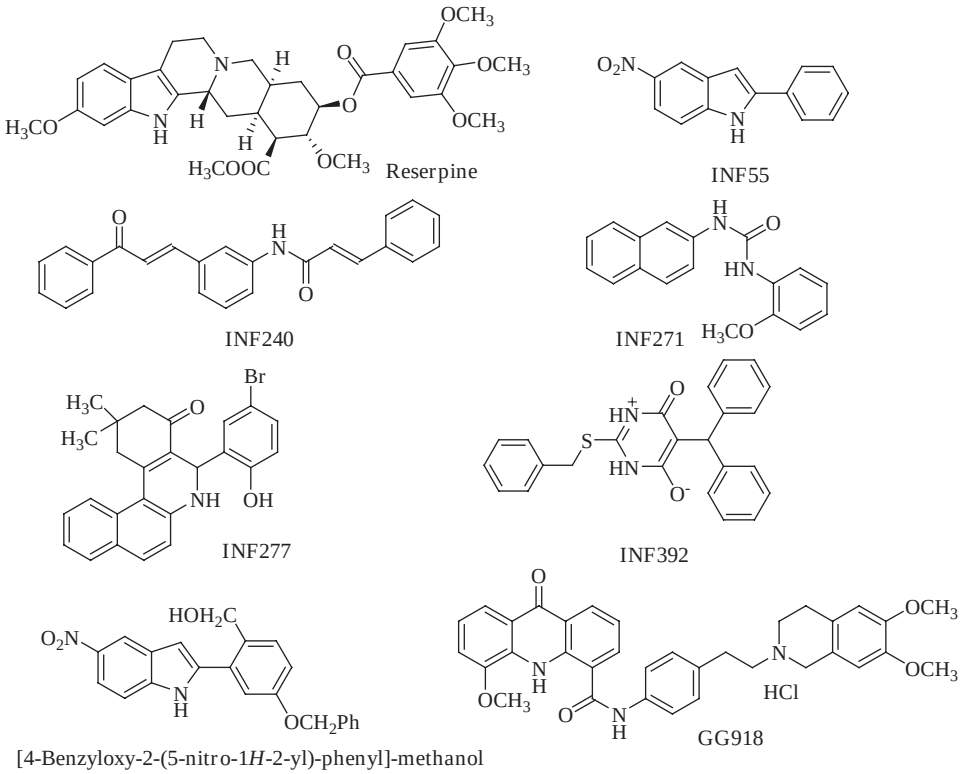
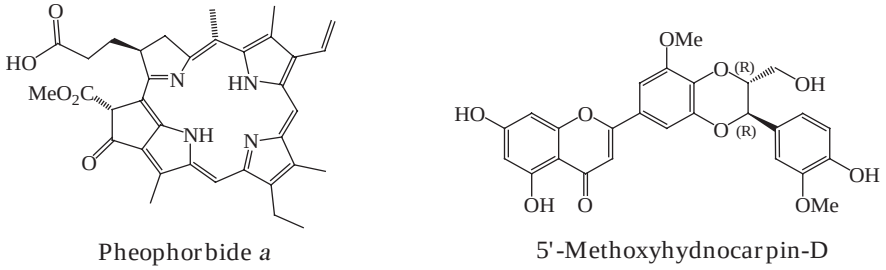


Figure 2 Inhibitors of NorA MDR pump.

concentration of 0.8 μg/mL, which was four-fold more potent than INF55 in increasing the potency of berberine against the *S. aureus* resistant strain, K2361.

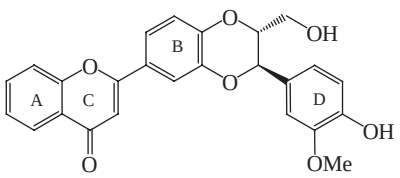
A synthetic inhibitor of P-glycoprotein-mediated mammalian tumour multidrug resistance, GG918 (Figure 2), was discovered to be a NorA inhibitor and was equipotent to reserpine in potentiating the activity of norfloxacin and ciprofloxacin against some strains of *S. aureus.* In combination with ciprofloxacin, GG918 reduced the MIC of ciprofloxacin against SA-1199B eight-fold at a concentration of 10 μg/mL, but did not show synergistic activity at the same concentration against the RN4220 strain of *S. aureus*, which carries a gene encoding the MsrA macrolide efflux protein. Moreover, GG918 enhanced the activity of norfloxacin eight-fold against SA-1199B and by four-fold against RN4220 at a concentration of 10 μg/mL [5].

Berberine-producing plants from *Berberis* species have been found to produce potent NorA inhibitors, including porphyrin pheophorbide *a* and the flavonolignan 5′-methoxyhydnocarpin (5′-MHC-D) (Figure 3) [11, 12, 13], that act synergistically with the antibacterial berberine against *S. aureus.* Berberine contains a planar aromatic cationic centre which is recognized by efflux proteins in bacterial cells, resulting in its extrusion from the bacterial cells. Pheophorbide *a*, and 5′-MHC-D, have no antibacterial activity alone but have been shown to boost the activity of an ineffective antibiotic such as berberine against a NorA-producing strain of *S. aureus.* These results may help to explain why *Berberis* plants are relatively free of bacterial plant infections. The MIC of pheophorbide *a* and 5′-MHC-D were 0.5 and 1.2 μg/mL, respectively, in the presence of 30 μg/mL of berberine (which is one-eighth of its MIC against wild-type *S. aureus* RN4222) [13]. Many synthetic flavonolignans and simple flavones were synthesized in order to determine structure-activity relationships (SARs) for synergistic activity with berberine against *S. aureus* RN4222. Many of those compounds showed a synergistic action with a sub-inhibitory concentration of berberine (30 μg/mL) in the MIC range of 0.08-163 μg/mL. The most potent flavonolignan-based NorA inhibitor was 5,7-deoxyhydnocarpin-D and the most potent flavone-based inhibitor was 4′-*n*-propoxyflavone (Figure 4) with MIC values of 0.08 and 0.4 μg/mL, respectively, in combination with a sub-inhibitory concentration of berberine in *S. aureus.* The results revealed that the free hydroxy groups in ring A were not necessary for NorA inhibitory activity [14]. The effect of stereochemistry on activity was not reported for the 5,7-deoxyhydnocarpin-D flavonolignan.

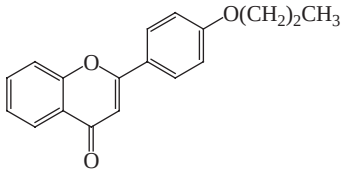


Pheophorbide *a* 5'-Methoxyhydnocarpin-D

Figure 3 Naturally occurring NorA inhibitors from *Berberis* species

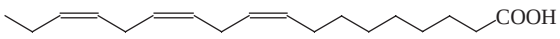


5,7-Deoxyhydnocarpin-D

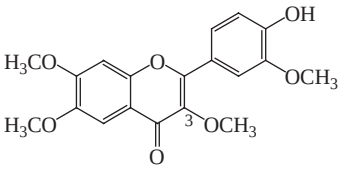


4'-*n*-Propoxyflavone

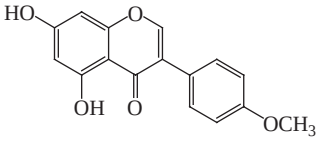
Figure 4 The most potent synthetic flavonolignan (relative configuration shown) and flavone of NorA inhibitors



Linolenic acid



Chrysoplenetin



Biochanin A

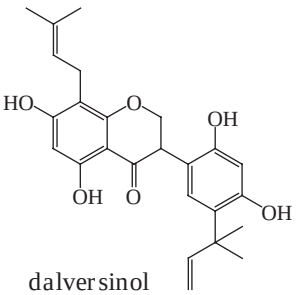
Figure 5 Natural flavonol and isoflavone NorA inhibitors, and the antibacterial, α-linolenic acid

Recently, some naturally occurring flavonol and isoflavone NorA inhibitors (Figure 5) have been identified. A weak antibacterial, α-linolenic acid (MIC, 62.5 μg/mL against *S. aureus* wild-type 8325-4), was isolated from the leaf and stem extracts of *Lupinus argenteus*, along with the flavonoid biochanin A which potentiated the antibacterial activity of that acid. Biochanin A was shown to completely inhibit *S. aureus* and *Bacillus megaterium* (11561, M. Cannon) growth at a concentration of 6.25 μg/mL in combination with a sub-inhibitory concentration of berberine (30 μg/mL) or a sub-inhibitory concentration of α-linolenic acid (30 μg/mL) [14,15]. Chrysoplenetin, a natural flavonol extracted from *Artemisa annua*, at a concentration of 6.25 μg/mL with a sub-inhibitory dose of berberine, completely inhibited the growth of *S. aureus* 8325-4 [16].

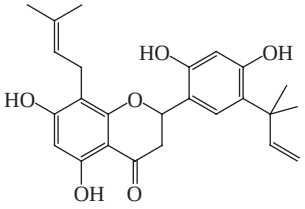
More recently, a new antibacterial flavonoid and known phenolic compounds (Figure 6) were isolated [17] from the plant, *Dalea versicolor*, and they showed direct or synergistic activities against *S. aureus* and *B. cereus*. Both the methoxychalcone derivative and stilbene derivative were equally the most potent inhibitors in this plant at a concentration of 3.3 μg/mL in combination with a sub-inhibitory concentration of berberine against *S. aureus* 8325-4, whereas dalversinol and the tetrahydroxyflavanone derivative had direct activity with MICs of 31.3 and 7.8 μg/mL, respectively. Typically, medicinal plants produce efflux pump inhibitors to potentiate their own weak antibiotics. However, *Dalea versicolor* was the first plant reported in the literature that produced both strong antibacterials and efflux pump inhibitors. It is likely that dalversinol and the tetrahydroxyflavanone derivative, which have a flavanone nucleus, might not be NorA pump substrates.

Polyacylated neohesperidosides (Figure 7) isolated from *Geranium caespitosum* were found to potentiate the antibacterial activity of berberine, rhein (an anthraquinone), ciprofloxacin and norfloxacin against *S. aureus* 8325-4. Neither the monoester nor diester derivatives showed potentiation activity with berberine, whereas the tetraester and pentaester derivatives did with MIC values of 3.12-6.25 μg/mL in the presence of a sub-inhibitory concentration of berberine [18].

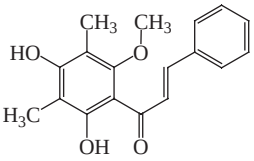
The latest natural NorA inhibitor, 2,6-dimethyl-4-phenyl-pyridine-3,5-dicarboxylic acid diethyl ester (Figure 7), was extracted from the rhizome of *Jatropha elliptica* (Pohl) Muell Arg.. This compound at a concentration of 50 μg/mL potentiated the activities of ciprofloxacin and EtBr (three- and four-fold, respectively) against the NorA resistant strain, SA-1199B, which were similar to that of the reference inhibitor reserpine at a concentration of 20 μg/mL [19].



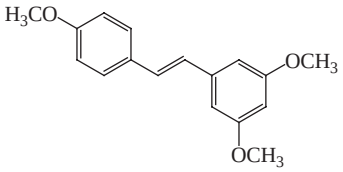
dalversinol



2(*S*)-5'-(1''', 1'''-dimethylallyl)-8-(3'', 3''-dimethylallyl)-2', 4', 5, 7-tetrahydroxyflavanone

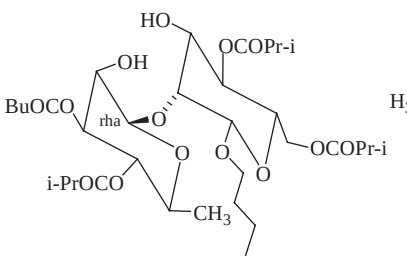


4', 6'-dihydroxy-3', 5'-dimethyl-2'-methoxychalcone

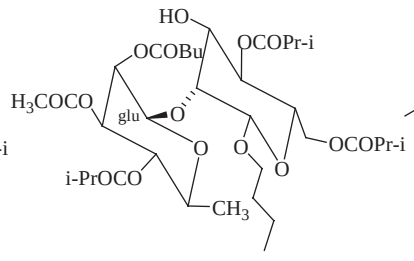


3, 5, 4'-trimethoxy-trans-stilbene

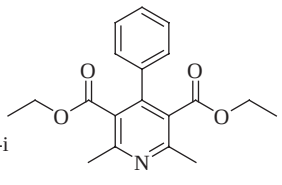
Figure 6 Natural phenolic compounds from *Dalea versicolor*



Tetraester of neohesperidoside



Pentaester of neohesperidoside



2,6-Dimethyl-4-phenyl-pyridine-3,5-dicarboxylic acid diethyl ester

Figure 7 Natural polyacylated neohesperidoside and penta-substituted pyridinedicarboxylate ester efflux inhibitors

Conclusions and Future Prospects

A number of NorA pump inhibitors have been discovered. The synthetic NorA inhibitor 5,7-deoxyhydnocarpin-D [14], with an MIC value of 0.08 μg/mL, is the most potent inhibitor discovered so far. In comparison to reserpine having efflux pump inhibitory activity at a concentration of 20 μg/mL in combination with a sub-inhibitory dose of berberine [16], it was found that 5,7-deoxyhydnocarpin-D was more active than reserpine by 250-fold against *S. aureus* 8325-4. In the future prospects for clinical utility of such EPIs, one strategy could be achieved by a combination of EPI as an antibiotic-potentiating agent with a pump substrate antibiotic compound to restore the antibacterial activity against bacteria expressing efflux pumps, and may delay the development of bacterial resistance problem.

Acknowledgements

I wish to thank the University of Wollongong, Australia and Srinakharinwirot University, Thailand for supporting this work.

References

1. Lomovskaya, O. and Watkins, W. J. 2001. Efflux Pumps: Their Role in Antibacterial Drug Discovery. 8: 1699-1711.
2. Hsieh, P. C., Siegel, S. A., Rogers, B., Davis, D. and Lewis, K. 1998. Bacteria Lacking a Multi-Drug Pump: A Sensitive Tool for Drug Discovery. *Proc. Natl. Acad. Sci. U.S.A.* 95: 6602-6606.
3. Kaatz, G. W. and Seo, S. 1995. Inducible NorA-Mediated Multidrig Resistance in *S. aureus. Antimicrob. Agents Chemother.* 39: 2650-2655.
4. Neyfakh, A. A., Borsch, C. M. and Kaatz, G. W. 1993. Fluoroquinolone Resistance Protein NorA of *S. aureus* is a Multidrug Efflux Transporter. *Antimicrob. Agents Chemother.* 37: 128-129.
5. Gibbons, S., Oluwatuyi, M. and Kaatz, G. W. 2003. A Novel Inhibitor of Multidrug Efflux Pumps in *Staphylococcus aureus. Antimicrob. Agents Chemother.* 51: 13-17.
6. Markham, P. N. and Neyfakh, A. A. 1996. Inhibition of the Multidrug Transporter NorA Prevents Emergence of Norfloxacin Resistance in *S. aureus. Antimicrob. Agents Chemother*. 40: 2673-2674.
7. Markham, P. N. 1999. Inhibition of the Emergence of Ciprofloxacin Resistance in *Streptococcus pneumoniae* by the Multidrug Efflux Inhibitor Reserpine. *Antimicrob. Agents Chemother.* 43: 988-989.
8. Markham, P. N., Westhaus, E., Klyachko, K., Johnson, M. E. and Neyfakh, A. A. 1999. Multiple Novel Inhibitors of the NorA Multidrug Transporter of *S. aureus. Antimicrob. Agents Chemother.* 43: 2404-2408.
9. Kaatz, G. W. 2002. Inhibition of Bacterial Efflux Pumps: a New Strategy to Combat Increasing Antimicrobial Agent Resistance. *Expert Opin. Emerging Drugs* 7: 223-233.
10. Samosorn, S., Bremner, J. B., Ball, A. and Lewis, K. 2006. Synthesis of Functionalised 2-Aryl-5-Nitro-1*H*-Indoles and Their Activity as Bacterial NorA Efflux Pump Inhibitors. *Bioorg. Med. Chem.* 14: 857-865.
11. Guz, N. R. and Stermitz, F. R. 2000. Synthesis and Structures of Regioisomer Hydnocarpin-Type Flavonolignans. *J. Nat. Prod.* 63: 1140-1145.

12. Stermitz, F. R., Lorenz, P., Tawara, J. N., Zenewicz, L. A. and Lewis, K. 2000. Synergy in a Medicinal Plant: Antimicrobial Action of Berberine Potentiated by 5'-Methoxyhydnocarpin, a Multidrug Pump Inhibitor. *Proc. Natl. Acad. Sci. U.S.A.* 97: 1433-1437.
13. Stermitz, F. R., Tawara-Matsuda, J., Lorenz, P., Mueller, P., Zenewicz, L. and Lewis, K. 2000. 5'-Methoxyhydnocarpin-D and Pheophorbide A: *Berberis* Species Components that Potentiate Berberine Growth Inhibition of Resistant *S. aureus. J. Nat. Prod.* 63: 1146-1149.
14. Guz, N. R., Stermitz, F. R., Johnson, J. B., Beeson, T. D., Willen, S., Hsiang, J. F. and Lewis, K. 2001. Flavonolignan and Flavone Inhibitors of a *Staphylococcus aureus* Multidrug Resistance Pump: Structure-Activity Relationships. *J. Med. Chem.* 44: 261-268.
15. Morel, C., Stermitz, F. R., Tegos, G. and Lewis, K. 2003. Isoflavones as Potentiators of Antibacterial Activity. *J. Agric. Food Chem.* 51: 5677-5679.
16. Stermitz, F. R., Scriven, L. N., Tegos, G. and Lewis, K. 2002. Two Flavonols from *Artemisia annua* which Potentiate the Activity of Berberine and Norfloxacin against a Resistant Strain of *Staphylococcus aureus. Planta Med.* 68: 1140-1141.
17. Belofsky, G., Percivill, D., Lewis, K., Tegos, G. P. and Ekart, J. 2004. Phenolic Metabolites of *Delea versicolor* that Enhance Antibiotic Activity against Model Pathogenic Bacteria. *J. Nat. Prod.* 67: 481-484.
18. Stermitz, F. R., Cashman, K. K., Halligan, K. M., Morel, C., Tegos, G. P. and Lewis, K. 2003. Polyacylated Neohesperidosides from *Geranium caespitosum:* Bacterial Multidrug Resistance Pump Inhibitors. *Bioorg. Med. Chem. Lett.* 13: 1915-1918.
19. Marquez, B., Neuville, L., Moreau, N. J., Genet, J. P., Feitosa Dos Santos, A., Cano De Andrade, M. C. and Sant'ana, A. E. G. 2005. Multidrug Resistance Reversal Agent from *Jatropha elliptica. Phytochemistry* 66: 1804-1811.

ได้รับบทความวันที่ 3 เมษายน 2549
ยอมรับตีพิมพ์วันที่ 20 เมษายน 2549