Trop J Nat Prod Res, July 2018; 2(7):358-361 **ISSN 2616-0684 (Print)**
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Inhibitors of Sporangia Formation of *Phytophthora capsici* from *Polygonum capitatum*

Umer Farooq[1], LiHong Cheng[1], Hui Zhang[1], Qian Wang[1], YuanChao Wang[2], Hirokazu Kawagishi[3], JianHua Qi[1]*

[1] *College of Pharmaceutical Sciences, Zhejiang University, Yu Hang Tang Road 866, Hangzhou 310058, China.*
[2] *College of Plant Protection, Nanjing Agricultural University, Nanjing 210095, China.*
[3] *Research Institute of Green Science and Technology, Shizuoka University, 836 Ohya, Surugaku, Shizuoka 422-8529, Japan.*

ARTICLE INFO

Article history:
Received 21 June 2018
Revised 29 June 2018
Accepted 05 July 2018
Published online 08 July 2018

ABSTRACT

The genus *Phytophthora* comprises of nearly 120 pathogenic species which causes both economic and enviormental damage. This study aims to isolate active compounds from natural products for inhibition of sporangia formation of *Phytophthora capsici.* The ethyl acetate (EtOAc) layer of the methanol (MeOH) extract of *Polygonum capitatum* exhibited significant activity. The ethyl acetate (EtOAc) fraction was then subjected to bioassay-guided separation and purification to afford five active flavonoids which significantly inhibited sporangia formation on *Phytophthora capsici*. The structures were elucidated by spectroscopic analysis and comparison of spectroscopic data with those reported. The active compounds were identified as myricetin 7-*O*-*α*-L-rhamnopyranoside (**1**), quercetin 3-*O*-*β*-D-glucopyranoside (**2**), quercetin 4′-*O*-*α*-L-rhamnopyranoside (**3**), quercetin 3-*O*-(2″-galloyl)-*α*-L-rhamnopyranoside (**4**), and kaempferol 3-*O*-*α*-L-rhamnopyranoside (**5**). Compounds **1**, **2,** and **3** were isolated from *P. capitatum* for the first time. Moreover, the stereo structure of sugar moiety of **3** was corrected by chemical derivatization means. Compounds (**1**-**5**) isolated from *P. capitatum* significantly inhibited sporangia formation of *Phytophthora capsici.*

Keywords: Flavonoids, Inhibitors, *Phytophthora capsici*, *Polygonum capitatum*, Sporangia formation

Introduction

The genus *Phytophthora*, known as "plant destroyer," consists of approximately 120 pathogenic species.[1-2] These species could bring serious effects on agriculture, environment, and related industries. Potato late blight caused by *Phytophthora infestans* is economically the most important and most destructive potato and tomato disease worldwide. The disease causes annual losses of several billion dollars and globally threatens the potato market.[3] In the middle of the 19th century, *P. infestans* destroyed a significant part of potato crop plantations in the USA and Europe; this pathogen is widely known as the cause of the Irish potato famine in 1845, resulting in the death of more than a million people.[4]

Various preventive control strategies are used to prevent *Phytophthora* infection. Metalaxyl, is the most effective and commonly used fungicide against *Phytophthora*. However, the long-term use of this fungicide led to serious resistance and environmental issues.[5] Therefore, development of new methods to control *Phytophthora* is a very urgent task for researchers.

The life cycle *of Phytophthora* can be separated into asexual and sexual cycles. To control the sexual reproduction of *Phytophthora*, we previously determined the chemical structures of two signaling molecules, namely, hormones *α*1 and *α*2, which stimulate sexual reproduction in heterothallic species.[6-8] The asexual cycle is the driving force of rapid polycyclic epidemics in crops and forest trees during the growing season. The infected plants produce and release numerous sporangia into the atmosphere. These sporangia germinate directly or indirectly to produce zoospores, which not only directly infect the plants but also grow to hypha and complete the asexual cycle. Hence, screening of inhibitors of sporangia formation of *Phytophthora* species will be a promising way to control the disease.[9]

Materials and Methods

General

Optical rotations of isolated compounds were obtained on a JASCO P-1030 digital polarimeter. NMR spectra were recorded on Bruker AV III-500 spectrometer. Chemical shifts in □□(ppm) were referenced to the solvent peaks of □H 3.30 ppm for CD_3OD. Mass spectra were obtained on 6224A accurate mass TOF LC/MS system. Preparative HPLC analysis was conducted using ELITE P-230 pumps. Column chromatography was performed using silica gel (Qingdao Haiyang Chemical Co. Ltd., Qingdao, China) and ODS (Cosmosil 75 C18-OPN, Nacalai Tesque, Japan). TLC analysis was conducted using pre-coated silica gel (0.25 mm) and RP-18 plates (0.25 mm).

Plant Material

The whole plant of P. capitatum was purchased from Bozhou City, Anhui Province, China in January 2016. The plant was authenticated by Professor Jianhua Qi from the College of Pharmaceutical Sciences, Zhejiang University. A voucher specimen (20160103) was deposited at the Institute of Materia Medica, Zhejiang University.

Extraction and Isolation

The dried plant of P. capiatum (250 g) was powdered, soaked in methanol (MeOH), and continuously stirred at room temperature (25 °C) for 2 days. The supernatant was separated by filtration and concentrated under reduced pressure to obtain crude methanol (MeOH) extract. The crude methanol (MeOH) extract was partitioned between ethyl acetate (EtOAc) and water to obtain ethyl acetate (EtOAc) and

*Corresponding author. E mail: qijianhua@zju.edu.cn
Tel: +86-571-88208627

Citation: Farooq U, Cheng L, Zhang H, Wang Q, Wang Y, Kawagishi H, Qi J. Inhibitors of Sporangia Formation of *Phytophthora capsici* from *Polygonum capitatum*. Trop J Nat Prod Res. 2018; 2(7):358-361. doi.org/10.26538/tjnpr/v2i7.10

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

water layers. The active ethyl acetate (EtOAc) fraction (Figure 1) was loaded on silica gel column and successively eluted with stepwise gradients of n-hexane/CH_2Cl_2 (10:0, 9:1, 7:3, 5:5, 3:7, 1:9, and 0:10), CH_2Cl_2/EtOAc (9:1, 7:3, 5:5, 3:7, and 0:10), and MeOH (100%) to obtain eight fractions. The active fraction eluted with 100% MeOH was separated through an ODS open column and eluted with MeOH/H_2O step gradient (30:70, 35:65, 40:60, 50:50, 60:40, 70:30, 90:10, and 100:0) to yield eight fractions. Fractions 3 and 4 were active. The active fraction 3 was separated by ODS open column and eluted with MeOH/H_2O (30:70, 50:50, and 100:0) to obtain four fractions. The active fraction eluted with MeOH/H_2O (50:50) was further purified by HPLC using solvent MeOH/H_2O (38:62) to yield active compounds **1**, **2**, and **3**. Similarly, the active fraction 4 was separated through ODS open column and eluted with MeOH/H_2O (30:70, 50:50, and 100:0) to afford 3 fractions. The active fraction eluted with MeOH/H_2O (50:50) was further purified by HPLC using solvent MeOH/H_2O (48:52) to obtain active compounds **4** and **5**.

Acid Hydrolysis of 3 and Sugar Analysis

The absolute configuration of sugar moiety of compound **3** was determined according to the reported methods.[10-11] Firstly, the aldose thiocarbamate standards were synthesized: L-rhamnose was derivatized with L-cysteine methyl ester, D-cysteine methyl ester, and o-tolylisothiocyanate, according to the published papers.[10] Then, compound **3** (0.5 mg) was hydrolyzed at 80 °C, for 4 h in 2.0 M HCl (CH_3OH, 1 mL). The product was concentrated and dried in vacuo, and re-dissolved in pyridine (0.2 mL) containing 2.5 mg/ml L-cysteine methyl ester. The reaction was heated at 60 °C for 1 h. Approximately 0.1 mL of o-tolylisothiocyanate solution (2.5 mg/mL) in pyridine was added to the mixtures, which were then further heated at 60 °C for 1 h. The reaction products (2 µL) were diluted by methanol (98 µL) and directly analyzed by Agilent technologies 6224A accurate mass LC-TOF-MS under the following conditions: Agilent Extend C18 column (3.5 µm, 3.0 mm × 100 mm); detected at 210 nm; t = 0 min MeOH/H_2O/formic acid (30:70:0.1), t = 15 min MeOH/H_2O/formic acid (95:5:0.1); and flow rate: 0.45 mL/min.

The L-rhamnose was identified as the sugar moiety of **3** by comparing the retention time of its aldose thiocarbamate derivative (t_R = 7.42 min) with those of the aldose thiocarbamate standards: D-cysteine- L-rhamnose (t_R = 6.11 min), L-cysteine- L-rhamnose (t_R= 7.36 min).

Bioassay Method

More than 100 kinds of methanol (MeOH) extracts of plants were screened for inhibition of the sporangia formation of P. capsici. For the bioassay, the A2 mating type of P. capsici was inoculated on a solid medium containing 10% V8 juice, 0.02% $CaCO_3$, and 2% agar. The inoculated solid medium was incubated at 25 °C for 3–4 days. A piece of hypha was cut from the edge of the Phytophthora colony and placed in the center of a Petri dish containing liquid medium, that is, 10 mL of 3% V8 juice. After 48 h, the liquid medium was replaced with 10 mL of distilled water containing either the sample at various concentrations or the solvent control. After 24 h, the number of sporangia was counted under a microscope to evaluate inhibitory activity. Inhibition ratio was calculated according to the following equation:

$I_R=(N_C-N_S)/N_C\times100\%$

where I_R is the inhibition rate, N_C is the number of sporangia after treatment with the solvent control, and N_S is the number of sporangia after treatment with the samples. Metalaxyl, a well-known fungicide against Phytophthora, was used as positive control to evaluate the reliability of the bioassay system.

Statistical Analysis

The data was presented as means ± SE values of three replicates. Data was analyzed with paired Student t-test using GraphPad Prism software (GraphPad Software). $P < 0.05$ was considered statistically significant.

Results and Discussion

During our screening study, the ethyl acetate (EtOAc) layer, obtained from the partitioning of the methanol (MeOH) extract of *P. capitatum*, exhibited a potential inhibitory activity against the sporangia formation of *P. capsici* (Figure 1). *P. capitatum* is used as a traditional Chinese medicine for treatment of urinary tract infection, hematuria, eczema, diarrhea, and dysentery.[12] The major constituents isolated from *P. capitatum* include volatile oils, glycosides, lignans, chromone glycosides, and flavonoids.[12-13]

This research focused on bioassay guided separation and purification of inhibitors of sporangia formation of *Phytophthora capsici* from the ethyl acetate (EtOAc) layer of *P. capiatum.* As a result, five active compounds (**1-5**) were obtained.

Structure Elucidation

Structures of the isolated compounds (**1-5**) (Figure 2) were determined by spectroscopic analyses, particularly MS and ^{1}H NMR spectroscopy, and comparison of spectroscopic data with published literature data.[14-19] The isolated compounds were identified as myricetin 7-*O*-*α*-L-rhamnopyranoside (**1**),[14] quercetin 3-*O*-β-D-glucopyranoside (**2**),[15] quercetin 4′-*O*-*α*-L-rhamnopyranoside (**3**),[16] quercetin 3-*O*-(2″-galloyl)-*α*-L-rhamnopyranoside (**4**),[17] and kaempferol 3-*O*-*α*-L-rhamnopyranoside (**5**),[18-19] respectively (Figure 2). Although ^{1}H NMR data reported by literature for compound **3** was the same as we obtained,[16] the configuration of the sugar moiety was incorrect. Therefore, to confirm the absolute configuration of sugar moiety of compound **3**, chemical derivatization was conducted according to the reported method.[10-11] Finally, the configuration of sugar moiety was identified as L-rhamnose and the structure of compound **3** was revised (Figure 2). Compounds **1-3** were isolated for the first time from *P. capiatum*.

Spectroscopic data of compounds 1-5

Myricetin 7-*O*-*α*-L-rhamnopyranoside (**1**): yellow solid, $[\alpha]_D^{21}$ -120.0° (*c* 0.14; MeOH), ^{1}H NMR (500 MHz, CD_3OD) □: 0.96 (3H, d, *J* = 6.0 Hz, H-6’’), 3.35-3.79 (3H, m, H-3’’-5’’), 4.22 (1H, br s, H-2’’), 5.31 (1H, s, H-1’’), 6.20 (1H, d, *J* = 2.0 Hz, H-6), 6.36 (1H, d, *J* = 2.0 Hz, H-8), 6.95 (2H, s, H-2’ and 6’), ESI-TOF-MS *m/z* 465 $[M+H]^+$.

Quercetin 3-*O*-*β*-D-glucopyranoside (**2**): yellow solid, $[\alpha]_D^{21}$ -15.5° (*c* 0.08; MeOH), ^{1}H NMR (500 MHz, CD_3OD)□□: 3.20-3.47 (4H, m, H-2’’- 5’’), 3.57 (1H, dd, *J* = 12.0, 6.2 Hz, H-6b’’), 3.70 (1H, dd, *J* = 12.0, 2.2 Hz, H-6a’’), 5.26 (1H, d, *J* = 8.0 Hz, H-1’’), 6.20 (1H, d, *J* = 2.0 Hz, H-6), 6.39 (1H, d, *J* = 2.0 Hz, H-8), 6.86 (1H, d, *J* = 8.5 Hz, H-5’), 7.58 (1H, dd, *J* = 8.5, 2.0 Hz, H-6’), 7.70 (1H, d, *J* = 2.0 Hz, H-2’), ESI-TOF-MS *m/z* 465 $[M+H]^+$.

Quercetin 4'-*O*-*α*-L-rhamnopyranoside (**3**): yellow solid, $[\alpha]_D^{21}$ -179.2° (*c* 0.3; MeOH), ^{1}H NMR (500 MHz, CD_3OD) □: 0.94 (3H, d, *J* = 6.0 Hz, H-6’’), 3.39-3.51 (2H, m, H-4’’ and H-5’’), 3.75 (1H, dd, *J* = 9.5, 3.0 Hz, H-3’’), 4.22 (1H, br s, H-2’’), 5.35 (1H, s, H-1’’), 6.20 (1H, d, *J* = 1.25 Hz, H-6), 6.37 (1H, d, *J* = 1.25 Hz, H-8), 6.91 (1H, d, *J* = 8.0 Hz, H-5’), 7.31 (1H, dd, *J* = 8.0, 2.0 Hz, H-6’), 7.34 (1H, d, *J* = 2.0 Hz, H-2’), ESI-TOF-MS *m/z* 449 $[M+H]^+$.

Quercetin 3-*O*-(2′′-galloyl)-*α*-L-rhamnopyranoside (**4**): yellow solid, $[\alpha]_D^{21}$ -9.5° (*c* 0.32; MeOH), ^{1}H NMR (500 MHz, CD_3OD) □: 1.02 (3H, d, *J* = 6.0 Hz, H-6’’), 3.46 (2H, m, H-4’’ and 5’’), 4.00 (1H, dd, *J* = 9.0, 3.4 Hz, H-3’’), 5.50 (1H, d, *J* = 1.5 Hz, H-1’’), 5.62 (1H, dd, *J* = 3.4, 1.5 Hz, H-2’’), 6.19 (1H, d, *J* = 2.0 Hz, H-6), 6.36 (1H, d, *J* = 2.0 Hz, H-8), 6.93 (1H, d, *J* = 8.5 Hz, H-5’), 7.06 (2H, s, H-2’’’ and 6’’’) 7.34 (1H, dd, *J* = 8.5, 2.0 Hz, H-6’), 7.36 (1H, d, *J* = 2.0 Hz, H-2’), ESI-TOF-MS *m/z* 601 $[M+H]^+$.

Kaempferol 3-*O*-*α*-L-rhamnopyranoside (**5**): yellow solid, $[\alpha]_D^{21}$ -149.6° (*c* 0.21; MeOH), ^{1}H NMR (500 MHz, CD_3OD)□□: 0.92 (3H, d, *J* = 5.5 Hz, H-6’’), 3.33-3.35 (2H, m, H-4’’-5’’), 3.71 (1H, dd, *J* = 9.0, 3.3 Hz, H-3’’), 4.22 (1H, dd, *J* = 3.3, 1.5 Hz, H-2’’), 5.37 (1H, d, *J* = 1.5 Hz, H-1’’), 6.18 (1H, d, *J* = 2.0 Hz, H-6), 6.35 (1H, d, *J* = 2.0 Hz, H-8), 6.93 (2H, d, *J* = 9.0 Hz, H-3’ and 5’), 7.77 (2H, d, *J* = 9.0 Hz, H-2’ and 6’), ESI-TOF-MS *m/z* 433 $[M+H]^+$.

*Bioassay results of compounds **1-5***

Compounds **1-5** significantly inhibited the sporangia formation of *Phytophthora* in comparison with the positive (metalaxyl) and negative controls (0.2% DMSO) (Figure 3). Compounds **1** and **2** (30 µM) showed 74% and 65% inhibition rates, respectively. Similarly, Compounds **3** and **4** (3 µM) showed inhibition rates of 86% and 78%, respectively. Compound **5** showed 90% inhibition rate at 10 µM. All of these compounds exhibited inhibitory activity 24 h after the treatment.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

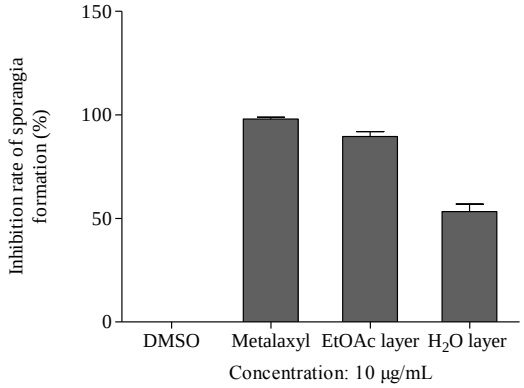

Figure 1: Inhibition rate of the EtOAc and H_2O layers of MeOH extract of *P. capiatum* in comparison with metalaxyl (positive control) and DMSO (negative control).

Figure 4 shows photomicrographs of sporangia formed on *P. capsici* mycelia 24 h after treatment of negative control, positive control and compounds **1**-**5** at the optimal dose, respectively. Few sporangia were observed on mycelia after treatment of positive control, compounds **3** and **5** at indicated doses, respectively.

Conclusion

These results indicated that methanol (MeOH) extract of *P. capitatum* contains active components possessing inhibition activity on sporangia formation of *Phytophthora. P. capitatum* is a traditional Chinese medicinal plant and with wide distribution in China. Therefore, methanol (MeOH) extract of *P. capitatum* and its active components could be potential agents for development of fungicides to control *Phytophthora* infection which is a big threat to forests, crops and ecosystem in the world. The mechanism of action of the most active compound (**3**) will be investigated in future study.

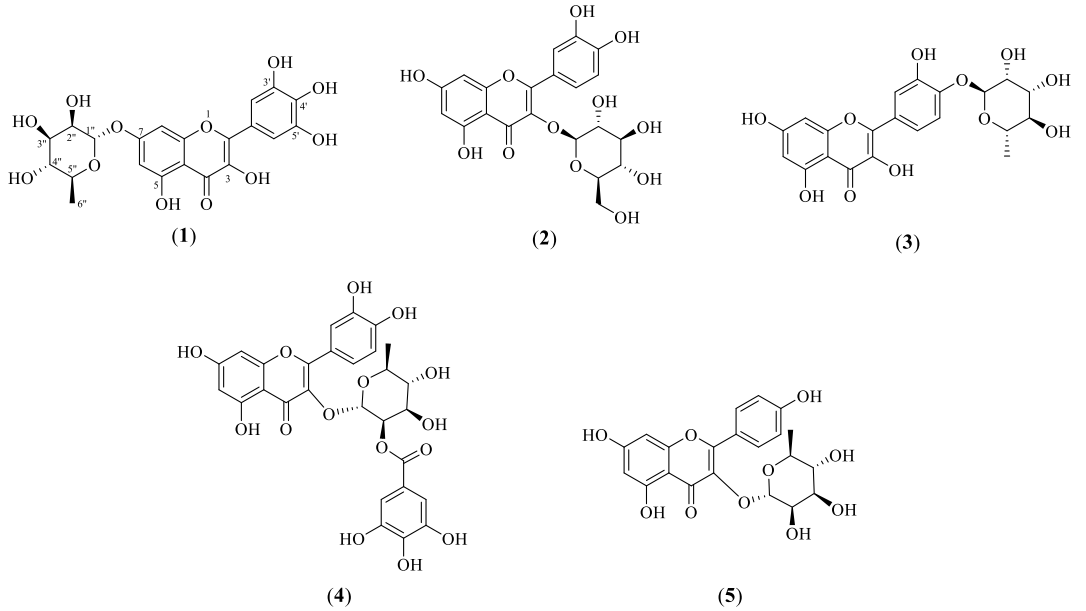

Figure 2: Chemical structures of myricetin 7-O-α-L-rhamnopyranoside (**1**), quercetin 3-O- β -D-glucopyranoside (**2**), quercetin 4′-O-α-L-rhamnopyranoside (**3**), quercetin 3-O-(2″-galloyl)-α-L-rhamnopyranoside, (**4**) and kaempferol 3-O-α-L-rhamnopyranoside (**5**).

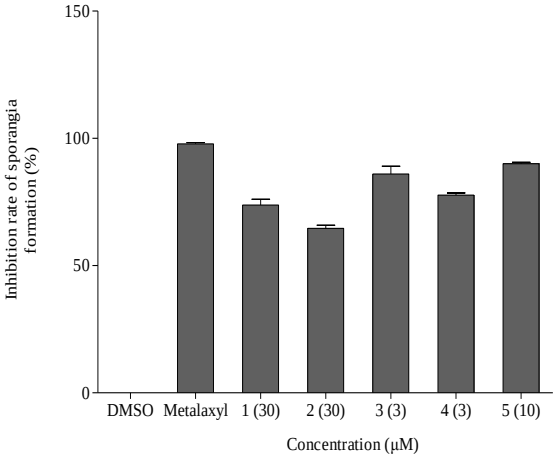

Figure 3: Inhibition rate of compounds 1-5 in comparison with P (positive control) and C (negative control).

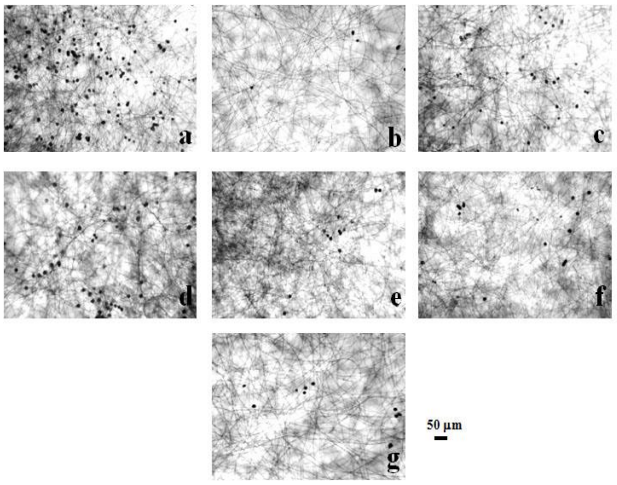

Figure 4: Photomicrographs of *P. capsici* mycelia under phase-contrast microscope 24 h after treatment (a)-(g). Scale bar, 50 □m. (a) Negative control, (b) positive control (30 □g/mL), (c) **1** (30 □M), (d) **2** (30 □M), (e) **3** (3 □M), (f) **4** (3 □M), and (g) **5** (10 □M).

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgments

This work was financially supported by National Natural Science Foundation of China (Grant No. 21572204 and 21661140001) and Special Fund for Agro-scientific Research in the Public Interest (Grant No. 201303018).

References

1. Hyun IH, Choi W. *Phytophthora* species, new threats to the plant health in Korea. Plant Pathol J. 2014; 30:331-342.
2. Kroon LP, Brouwer H, de Cock AW, Govers F. The genus *Phytophthora* anno 2012. Phytopathol. 2012; 102:348-364.
3. Cooke DEL, Lees AK. Markers, old and new, for examining *Phytophthora infestans* diversity. Plant Pathol. 2004; 53:692-704.
4. Smart CD, Fry WE. Invasions by the late blight pathogen: renewed sex and enhanced fitness. Biol Invasions 2001; 3:235-243.
5. Jaimasit P, Prakob W. Characterization of *Phytophthora infestans* population in potato crops from Chiang Mai and Tak Provinces. J Agric Technol. 2010; 6:117-125.
6. Ojika M, Molli SD, Kanazawa H, Yajima A, Toda K, Nukada T, Mao H, Murata R, Asano R, Qi J, Sakagami Y. The second *Phytophthora* mating hormone defines interspecies biosynthetic crosstalk. Nat Chem Biol. 2011; 7:591-593.
7. Qi J, Asano T, Jinno M, Matsui K, Atsumi K, Sakagami Y, Ojika M. Characterization of a *Phytophthora* mating hormone. Sci. 2005; 309:1828-1828.
8. Yajima A, Qin Y, Zhou X, Kawanishi N, Xiao X, Wang J, Zhang D, Wu Y, Nukada T, Yabuta G, Qi J, Asano T, Sakagami Y. Synthesis and absolute configuration of hormone α1. Nat Chem Biol. 2008; 4:235-237.
9. Glendinning D, MacDonald JA, Grainger J. Factors affecting the germination of sporangia in *Phytophthora infestans*. Trans Br Mycol Soc. 1963; 46:595-603.
10. Tanaka T, Nakashima T, Ueda T, Tomii K, Kouno I. Facile discrimination of aldose enantiomers by reversed-phase HPLC. Chem Pharm Bull. 2007; 55:899-901.
11. Berrué F, McCulloch MWB, Boland P, Hart S, Harper MK, Johnston J, Kerr R. Isolation of steroidal glycosides from the Caribbean sponge *Pandaros acanthifolium*. J Nat Prod. 2012; 5:2094-2100.
12. Yang Y, Wu ZJ, Chen WS. Chemical constituents of *Polygonum capitatum*. Chem Nat Compd. 2015; 51:332-335.
13. Li X, Yu M, Meng D, Li Z, Zhang L. A new chromone glycoside from *Polygonum capitatum*. Fitoterapia 2007; 78:506-509.
14. Korul'kina LM, Shul'ts EE, Zhusupova GE, Abilov ZA, Erzhanov KB, Chaudri MI. Biologically active compounds from *Limonium Gmelinii* and *L. Popovii* I. Chem Nat Compd. 2004; 40:465-471.
15. Han JT, Bang MH, Chun OK, Kim DO, Lee CY, Baek NI. Flavonol glycosides from the aerial parts of *Aceriphyllum rossii* and their antioxidant activities. Arch Pharm Res. 2004; 27:390-395.
16. Raziq N, Saeed M, Ali MS, Zafar S, Ali MI. In vitro anti-oxidant potential of new metabolites from *Hypericum oblongifolium* (Guttiferae). Nat Prod Res. 2015; 29:2265-2270.
17. Lee CS, Jeong EB, Kim YJ, Lee MS, Seo SJ, Park KH, Lee MW. Quercetin-3-O-(2″-galloyl)-α-L-rhamnopyranoside inhibits TNF-α-activated NF-κB-induced inflammatory mediator production by suppressing ERK activation. Int Immunopharmacol. 2013; 16:481-487.
18. Braca A, Politi M, Sanogo R, Sanou H, Morelli I, Pizza C, De Tommasi N. Chemical composition and antioxidant activity of phenolic compounds from wild and cultivated *Sclerocarya birrea* (Anacardiaceae) leaves. J Agric Food Chem. 2003; 51:6689-6695.
19. De Almeida AP, Miranda M, Simoni IC, Wigg MD, Lagrota M, Costa SS. Flavonol monoglycosides isolated from the antiviral fractions of *Persea americana* (Lauraceae) leaf infusion. Phytother Res. 1998; 12:562-567.