Natural Product Sciences
20(2) : 130-134 (2014)

Cytotoxic Components in an Extract from the Leaves and Stems of *Stauntonia hexaphylla*

Jing Zhao[1,†], Soon-Ho Yim[3,†], Jung-In Um[2], Si-Hwan Park[2], Eun-Sang Oh[2], Da-Woon Jung[2], Darren R. Williams[2,*], and Ik-Soo Lee[1,*]

[1]College of Pharmacy and Research Institute of Drug Development, Chonnam National University, Gwangju 500-757, Republic of Korea
[2] New Drug Targets Laboratory, School of Life Science, Gwangju Institute of Science and Technology, 1 Oryong-Dong, Buk-Gu, Gwangju 500-712, Republic of Korea
[3]College of Public Health and Welfare, Dongshin University, 185 Gunjaero, Naju, Jeonnam 520-714, Republic of Korea

Abstract – An investigation was carried out to identify novel anti-cancer compounds from Korean indigenous plant extracts. Bioassay-guided fractionation and chemical investigation of the EtOAc extract from the leaves and stems of *Stauntonia hexaphylla* resulted in the isolation of two active compounds, hederagenin 3-*O*-α-L-arabinoside (**1**) and quercetin (**2**). The structures of these compounds were elucidated by spectroscopic methods, including UV, IR, MS, NMR techniques and compared with previous spectroscopic data. The cytotoxic effects of fractions and compounds on HCT116 human colon cancer cells were evaluated using the MTT assay. Quercetin showed a stronger anti-cancer effect when compared to hederagenin 3-*O*-α-L-arabinoside.
Keywords – *Stauntonia hexaphylla,* Cytotoxicity, Hederagenin 3-*O*-α-L-arabinoside, Quercetin.

Introduction

The Japanese staunton vine [*Stauntonia hexaphylla* (Thunb.) Decne. (Lardizabalaceae)] is a fast-growing, tender evergreen climbing shrub with compound leaves comprising ovate leaflets with wavy margins. In spring, it bears small, pale-pink, bell-shaped flowers that are fragrant. Flowering may be followed by edible purple fruits in autumn. *S. hexaphylla* is widely distributed in thickets in lowlands and foothills in warmer regions of Korea, Japan, and China. The whole plants of *S. hexaphylla* have been used in Chinese folk medicine as an analgesic, sedative and diuretic.[1] A decoction of the stem and the root of the plant or the pericarp of the fruit has been used as a diuretic. The fruits also activate the circulation and "brighten the eyesight".[2] From the seeds, three acidic triterpene glycosides, mubenins A, B, and C, have been reported.[3] Previous studies of these plants led to the isolation of different compounds, such as triterpenoids and their glycosides,[4] bisepoxylignan glycosides,[5] and phenolic glycosides.[6] Triterpenoids and their glycosides are the major constituents from this plant, and series of triterpene aglycones with oleanane-type, desmethyl oleanane-type, lupine alkane-type mother cores and their glycosides have also been isolated.

As part of search for bioactive compounds, MeOH extract of the aerial part of *S. hexaphylla* was found to exhibit significant cytotoxic activity. Bioassay-directed fractionation of this extract using the cytotoxicity assay resulted in the isolation of two compounds. Detailed isolation and structure elucidation of the two compounds along with their cytotoxic activity are described in this study.

Experimental

General experimental procedures – ^{1}H- and ^{13}C-NMR spectra, with tetramethylsilane (TMS) as the internal standard were recorded using a Varian Unity INOVA 500 spectrometer (Varian, Inc., U.S.A.) for NMR experiments

†These authors contributed equally to this work
*Author for correspondence
Darren R. Williams, New Drug Targets Laboratory, School of Life Sciences, Gwangju Institute of Science and Technology, 1 Oryong-Dong, Buk-Gu, Gwangju 500-712, Republic of Korea.
Tel: +82-62-715-2484; E-mail: darren@gist.ac.kr
Ik-Soo Lee, College of Pharmacy and Research Institute of Drug Development, Chonnam National University, Gwangju 500-757, Republic of Korea.
Tel: +82-62-530-2932; E-mail: islee@chonnam.ac.kr

in pyridine-d_5 and DMSO-d_6. The chemical shifts (δ) were expressed in parts per million (ppm) and coupling constants (*J*) were in Hz. Mass spectra (LC-MS) were measured on a LCMS-2010 (Shimadzu Corp., Japan). IR spectra were obtained on a JASCO FT/IR-300E spectrometer (Jasco Corp., Japan) and UV spectra were recorded on a JASCO V-530 UV/Vis spectrophotometer (Jasco Corp., Japan).

TLC chromatographic analyses were carried out on precoated silica gel 60 F_{254} plates and RP-18 F_{254} plates (Merck). Visualization of the silica gel TLC was performed using anisaldehyde spray reagent which was prepared by mixing 135.0 mL ethanol, 5.0 mL sulfuric acid, 3.7 mL anisaldehyde and 1.5 mL acetic acid. The adsorbent used for column chromatography was silica gel 70 - 230 mesh and RP-18 230 - 400 mesh. The centrifugal chromatography was carried on a CycloGraph chromatography system with an 8 mm silica gel sorbent layer.

Plant material – The leaves and stems of *Stauntonia hexaphylla* were collected in Jeju Island, Republic of Korea, and pulverized using a grinder.

Isolation of active compounds – The dried powder (3 kg) of the leaves and stems from *Stauntonia hexaphylla* was extracted with 100% MeOH, and concentrated *in vacuo* to yield MeOH extract (212.46 g). The MeOH extract was suspended with water and partitioned with HD (hexane-dichloromethane = 9 : 1), DE (dichloromethane-ethyl acetate = 20 : 1), E (100% ethyl acetate), EM (ethyl acetate-methanol = 5 : 1), and A (100% water) in sequence. The EtOAc extract showing strongest activity was loaded onto CycloGraph Chromatography System by using 8 mm silica gel sorbent layer with a gradient of $CHCl_3$-MeOH (95 : 5 → 85 : 15 → 50 : 50 → 0 : 100) to afford 8 fractions. Since it showed a significant cytotoxic activity, the fifth fraction of EtOAc extract from *S. hexaphylla* was subjected onto an RP-18 column with a gradient of MeOH-H_2O (80 : 20 → 78 : 12 → 100 : 0) to give 7 fractions including compound **1** (32.0 mg) and compound **2** (7.2 mg).

Hederagenin 3-*O*-α-L-arabinopyranoside (1) – UV λ_{max} (MeOH) 204 nm; IR (KBr) ν_{max} 3448 (OH), 1686 (acid) and 1655 cm^{-1} (C=C); ^{1}H NMR (in pyridine-d_5, 300 MHz) δ 1.20-1.40 (2H, m, H-1), 1.50-1.70 (2H, m, H-2), 3.30 (1H, m, H-3), 1.30-1.50 (1H, m, H-5), 1.30-1.50 (2H, m, H-6), 1.30-1.50 (2H, m, H-7), 1.70-1.90 (1H, m, H-9), 1.90-2.10 (2H, m, H-11), 5.48 (1H, brs, H-12), 1.20-1.30 (2H, m, H-15), 1.40-1.60 (2H, m, H-16), 2.00-2.20 (1H, m, H-18), 1.30-1.50 (2H, m, H-19), 1.30-1.50 (2H, m, H-21), 1.50-1.70 (2H, m, H-22), 4.45, 4.30 (2H, d, *J* = 8.4 Hz, H-23), 0.94 (3H, s, H-24), 0.95 (3H, s, H-25), 0.97 (3H, s, H-26), 1.01 (3H, s, H-27), 1.03 (3H, s, H-29), 1.26 (3H, s, H-30), 5.01 (1H, d, *J* = 6.9 Hz, H-1'), 4.10 (1H, d, *J* = 8.4 Hz, H-2'), 4.20-4.30 (3H, m, H-3'), 4.20-4.30 (3H, m, H-4'), 4.20-4.30 (3H, m, H-5'); ^{13}C NMR (in pyridine-d_5, 75 MHz) δ 39.30 (C-1), 28.91 (C-2), 82.48 (C-3), 44.02 (C-4), 48.09 (C-5), 18.70 (C-6), 33.87 (C-7), 40.28 (C-8), 48.71 (C-9), 37.49 (C-10), 26.68 (C-11), 122.78 (C-12), 145.73 (C-13), 42.70 (C-14), 31.52 (C-15), 24.41 (C-16), 47.20 (C-17), 42.70 (C-18), 44.02 (C-19), 31.52 (C-20), 34.89 (C-21), 33.41 (C-22), 64.95 (C-23), 14.16 (C-24), 16.65 (C-25), 18.11 (C-26), 26.68 (C-27), 33.87 (C-29), 24.41 (C-30), 107.21 (C-1'), 75.24 (C-2'), 73.61 (C-3'), 70.17 (C-4'), 67.52 (C-5'); ESI-MS *m/z* 603 $[M - H]^-$ ($C_{35}H_{56}O_8$).

Quercetin (2) – UV λ_{max} (MeOH) 374, 256 and 206 nm; IR (KBr) ν_{max} 3410 (OH) 1718 (C=O) 1611, 1523 and 1459 cm^{-1} (aromatic C=C); ^{1}H NMR (in DMSO-d_6, 300 MHz) δ 6.19 (1H, d, *J* = 2.1 Hz, H-6), 6.41 (1H, d, *J* = 2.1 Hz, H-8), 7.54 (1H, dd, *J* = 8.4 Hz, 2.1 Hz, H-2'), 6.89 (1H, d, *J* = 8.4 Hz, H-3'), 7.68 (1H, d, *J* = 2.1 Hz, H-6'), 9.35 (1H, brs, 3-OH), 12.49 (1H, s, 5-OH), 10.79 (1H, brs, 7-OH), 9.59 (1H, brs, 4'-OH), 9.35 (1H, brs, 5'-OH); ^{13}C NMR (in DMSO-d_6, 75 MHz) δ 145.13 (C-2), 135.80 (C-3), 175.91 (C-4), 160.79 (C-5), 98.26 (C-6), 163.95 (C-7), 93.44 (C-8), 156.22 (C-9), 103.08 (C-10), 122.04 (C-1'), 115.13 (C-2'), 146.88 (C-3'), 147.77 (C-4'), 115.67 (C-5'), 120.07 (C-6'); ESI-MS *m/z* 301 $[M - H]^-$ ($C_{15}H_{10}O_7$).

Cell culture – The human colon carcinoma cell line HCT116 was obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM; purchased from Gibco BRL) supplemented with 10% fetal bovine serum (FBS), 50 units mL^{-1} penicillin and 50 μg mL^{-1} streptomycin (PenStrep; purchased from Gibco BRL).

Human fibroblast primary culture was derived from the surgical specimen of an OSCC patient. Informed consent for this study was given by the patient to Yonsei University College of Dentistry, Seoul, Republic of Korea. Isolation procedure of the fibroblasts was described in Jung DW *et al*., 2011.[7] The fibroblasts were cultured and maintained in a 3:1 ratio of DMEM and nutrient mixture F-12 Ham (HAM's-F12; Gibco BRL), supplemented with 10% FBS and 1% PenStrep.

Measurement of cell proliferation – Cell proliferation was measured using the MTT assay. HCT116 colon cancer cells (4×10^3 cells/well) were seeded in a 96 well culture plate overnight. Prior to drug treatment, culture media was changed to serum-free media. Each drug was treated to the cells for a period of 48 h. To measure cell viability, 200 μL of 3-(4,5-dimethylthiazol-

2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole (MTT) reagent was added to the cells for 2-3h. The supernatant was discarded, the precipitate was dissolved in DMSO and optical density was measured with a microplate reader at 570 nm (VersaMax, Molecular Devices, U.S.A.). DMSO was used as a negative control.

Results and Discussion

MeOH extract of *Stauntonia hexaphylla* was selected and screened from a library of 1,021 Korean indigenous plant extracts due to its strong and selective anti-cancer effects (Fig. 2a). The MeOH extract was suspended with water and partitioned with HD (hexane-dichloromethane = 9 : 1), DE (dichloromethane-ethyl acetate = 20 : 1), E (100% ethyl acetate), EM (ethyl acetate-methanol = 5 : 1), and A (100% water) in sequence. Each fraction was subjected to the MTT assays using HCT116 colon cancer cells. Among them, the EtOAc fraction showed strongest cytotoxicity towards the cancer cells. Activity-guided fractionation of the EtOAc extract from *S. hexaphylla* was repeated by column chromatography and led to the isolation of two compounds (Fig. 1). Their structures were elucidated by interpretation of ^{1}H- and ^{13}C-NMR, IR, UV, MS spectra in comparison with the published literature data.

Compound **1** displayed signals for an olefinic group [δ 5.48 (1H, brs, H-12)], six tertiary-CH_3 groups [δ 1.26 (3H, s, H-30), 1.03 (3H, s, H-29), 1.01 (3H, s, H-27), 0.97 (3H, s, H-26), 0.95 (3H, s, H-25), and 0.94 (3H, s, H-24)], besides signals for a set of geminal protons at δ 4.45 and 4.30 (1H each, d, J = 8.4 Hz, H-23) for the triterpenoid skeleton. An anomeric proton signal at δ 5.01 (1H, d, J = 6.9 Hz, H-1') was used as the starting point for the sequential assignment of the monosaccharide resonances. It also exhibited a doublet signal at δ 4.10 (1H, d, J = 8.4 Hz, H-2') and multiplet signals at δ 4.20-4.30 (3H, m, H-3', 4', 5') which was related to the sugar moiety. The 75 MHz ^{13}C-NMR spectrum exhibited thirty five signals, consisting of a pair of olefinic carbon signals at δ 145.76 (C-13) and 122.78 (C-12), a hydroxyl carbon signal at δ 64.95 (C-23) and five arabinosyl signals at δ 107.21 (C-1'), 75.24 (C-2'), 73.61 (C-3'), 70.17 (C-4') and 67.52 (C-5'). On the basis of this evidence and a comparison of the published data,[8-10] compound **1** was determined to be hederagenin 3-*O*-α-L-arabinopyranoside (H3A).

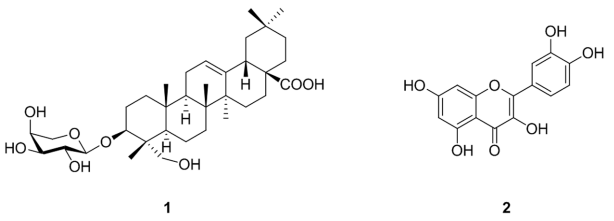

Fig. 1. Chemical structures of compounds **1** and **2**.

Compound **2** was obtained as yellow powder. The ESI mass spectrum of **2** showed a molecular ion peak [M – H]$^{-}$ at *m/z* 301, which was compatible with the molecular formula $C_{15}H_{10}O_7$. The UV spectrum of **2** showed absorption bands at λ_{max} (MeOH) 374, 256 and 206 nm. Its IR spectrum (KBr) exhibited absorption at ν_{max} 3410 (OH) 1718 (C=O) and 1611, 1523, 1459 cm^{-1} (aromatic C=C). The ^{1}H-NMR spectrum of compound **2** displayed characteristic signals for a flavonoid moiety. Thus signals at δ 6.41 (1H, d, J = 2.1 Hz, H-8) and 6.19 (1H, d, J = 2.1 Hz, H-6) suggested 5,7-dihydroxy-substituted A ring, and signals at δ 7.68 (1H, d, J = 2.1 Hz, H-6'), δ 7.54 (1H, dd, J = 8.4 Hz, 2.1 Hz, H-2') and 6.89 (1H, d, J = 8.4 Hz, H-3') suggested a 1,2,4-trisubstituted B ring. It also exhibited a hydrogen-bonded hydroxyl signal at δ 12.49 (1H, s, 5-OH) and three phenolic hydroxyl signals at δ 10.79, 9.59, 9.35 (1H each, br s, 7-OH, 4'-OH and 5'-OH). The ^{13}C-

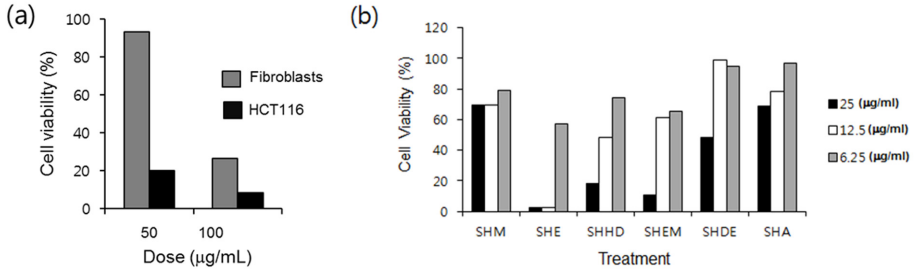

Fig. 2. Anti-cancer effects of the fractions from the MeOH extract of *Stauntonia hexaphylla.* (a) HCT116 colon cancer cells and human stromal fibroblasts were treated with the MeOH extract (50 and 100 μg/mL) for 48h. The cell viability was assessed using the MTT assay. The extract was less toxic to the human stromal fibroblast primary culture compared to the cancer cells, suggesting selective toxicity of the extract. (b) Cytotoxicity of each fractions (6.25, 12.5, and 25 μg/mL) against HCT116 colon cancer cells. SHHD (hexane-dichloromethane = 9 : 1), SHDE (dichloromethane-ethyl acetate = 20 : 1), SHE (100% ethyl acetate), SHEM (ethyl acetate-methanol = 5 : 1), SHA (100% water).

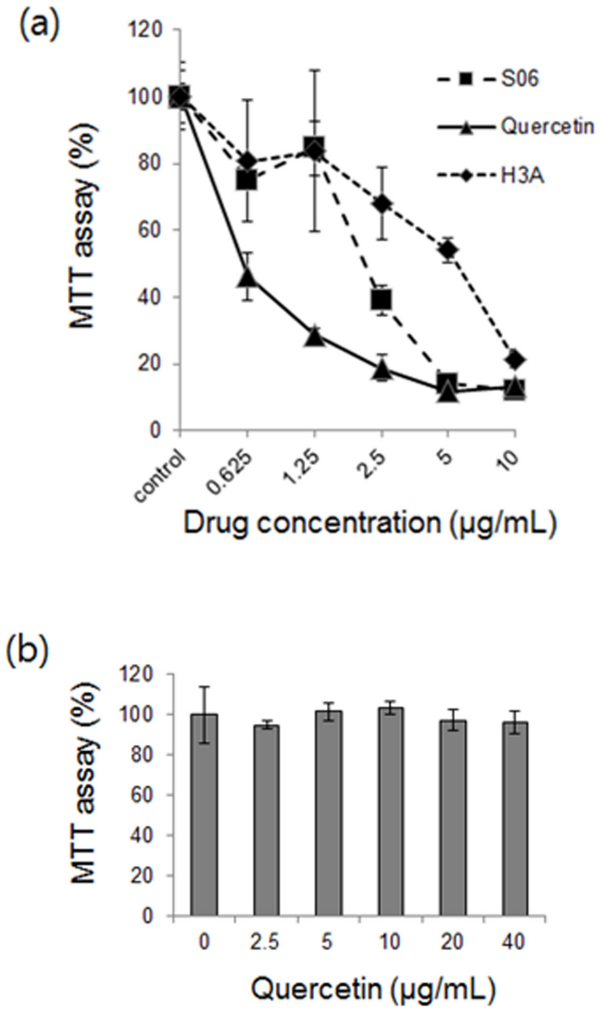

Fig. 3. Effects of compounds **1** and **2** on HCT116 colon cancer cell viability. (a) The cell viability was assessed using the MTT assay. Triazine-based anti-cancer agent S06 served as a positive control. H3A = hederagenin 3-*O*-α-L-arabinopyranoside. Data are average ± SD of triplicate assays. (b) Effect of quercetin on stromal fibroblasts. Quercetin treatment (2.5 - 40 μg/mL) did not show any toxicity against human stromal fibroblasts. Stromal fibroblasts were seeded at a density of 2×10^4 cells/mL. Data are average ± SD of triplicate assays.

NMR spectrum exhibited fifteen signals, consisting of a carbonyl carbon at δ 175.91 (C-4), four phenolic hydroxyl carbons at δ 163.95 (C-7), 160.79 (C-5), 147.77 (C-4') and 115.67 (C-5'), seven aromatic carbons with two fully substituted sp^2 carbons at δ 146.88 (C-3'), 122.04 (C-1'), 120.70 (C-6'), 115.13 (C-2'), 103.08 (C-10), 98.26 (C-6) and 93.44 (C-8), an oxygenated aromatic carbon at δ 156.22 (C-9), and a pair of olefinic hydroxyl carbons at δ 145.13 (C-2) and 135.80 (C-3). On the basis of this evidence and a comparison of the published data,[11,12] compound **2** was determined to be quercetin. The chemical structures of **1** and **2** are shown in Fig. 1.

Two compounds, **1** (Hederagenin 3-*O*-α-L-arabinopyranoside) and **2** (quercetin), showed cytotoxic effects on HCT116 colon cancer cells in a dose-dependent manner (Fig. 3). A previously reported triazine-based anti-cancer compound that targets heat shock protein 90, termed S06,[7] served as a positive control. Quercetin showed the strongest cytotoxic effect against cancer cells when compared to hederagenin 3-*O*-α-L-arabinoside and a synthetic compound S06, previously developed as an anti-cancer agent in our laboratory. In addition, quercetin was relatively non-toxic to human stromal fibroblast primary culture compared to the cancer cells, suggesting selective toxicity of the compound. This selective activity against cancer cells demonstrated by quercetin is a strong point of this compound, because it suggests that quercetin can be developed as an anti-cancer agent that is more effective than compound S06, whilst still retaining selectivity against non-cancer cells. Moreover, the anti-cancer activity demonstrated by hederagenin 3-*O*-α-L-arabinoside suggests that this compound should also be further assessed as a selective anti-cancer agent.

Taken together, we report that the activity-guided fractionation of a *Stauntonia hexaphylla* extract identified quercetin and hederagenin 3-*O*-α-L-arabinoside as anti-cancer components.

Acknowledgments

The authors thank the Gwangju Center, Korea Basic Science Institute for running NMR and MS experiments. This work was supported by a Korea Research Foundation Grant (KRF-2008-220-E00042) from Korean government to Ik-Soo Lee. This research was also supported by a grant (A120326) of the Korean Health Technology R&D Project from Ministry of Health & Welfare, Republic of Korea, to Darren R. Williams. This work was supported by the BioImaging Research Center at GIST, Republic of Korea. We thank Prof. Jin Kim, Yonsei University College of Dentistry, Republic of Korea for offering the stromal fibroblasts.

References

(1) Wang, H. B.; Yu, D. Q.; Liang, X. T.; Watanabe, N.; Tamai, M.; Omura, S. *J. Nat. Prod.* **1990**, *53*, 313-318.
(2) Ikuta, A.; Itokawa, H. *J. Nat. Prod.* **1989**, *52*, 623-628.
(3) Takemoto, T.; Kametani, K. *Justus Liebigs Ann. Chem.* **1965**, *685*, 237-246.
(4) Wang, H. B.; Mayer, R.; Rücker, G. *Phytochemistry* **1993**, *34*, 1389-1394.
(5) Wang, H. B.; Mayer, R.; Rücker, G.; Neugebauer, M. *Phytochemistry* **1993**, *34*, 1621-1624.

(6) Wang, H. B.; Mayer, R.; Rücker, G.; Yang, J. J.; Matteson, D. S. *Phytochemistry* **1998**, *47*, 467-470.
(7) Jung, D. W.; Kim, J.; Che, Z. M.; Oh, E. S.; Kim, G.; Eom, S. H.; Im, S. H.; Ha, H. H.; Chang, Y. T.; Williams, D. R.; Kim, J. *Chem. Biol.* **2011**, *18*, 1581-1590.
(8) Tava, A.; Mella, M.; Avato, P.; Biazzi, E.; Pecetti, L.; Bialy, Z.; Jurzysta, M. *J. Agric. Food Chem.* **2009**, *57*, 2826-2835.
(9) Ding, Q.; Yang, L. X.; Yang, H. W.; Jiang, C.; Wang, Y. F.; Wang, S. *J. Ethnopharmacol.* **2009**, *126*, 382-385.
(10) Hu, H. B.; Zheng, X. D.; Jian, Y. F.; Liu, J. X.; Zhu, J. H. *Arch. Pharm. Res.* **2011**, *34*, 1097-1105.
(11) Ibrahim, L. F.; El-Senousy, W. M.; Hawas, U. W. *Chem. Nat. Compd.* **2007**, *43*, 659-662.
(12) Li, C. Y.; Lin, C. H.; Wu, C. C.; Lee, K. H.; Wu, T. S. *J. Agric. Food Chem.* **2004**, *52*, 3721-3725.

Received November 8, 2013
Revised December 21, 2013
Accepted December 26, 2013