Anticancer action of garcinol in vitro and in vivo is in part mediated through inhibition of STAT-3 signaling

Aamir Ahmad, Sanila H. Sarkar, Amro Aboukameel, Shadan Ali, Bernhard Biersack, Sebastian Seibt, Yiwei Li, Bin Bao, Dejuan Kong, Sanjeev Banerjee, Rainer Schobert, Subhash B. Padhye, and Fazlul H. Sarkar

Introduction

Phytochemicals, particularly in their ability as cancer chemopreventive and chemotherapeutic agents, have generated considerable interest in recent years. These compounds are considered safe for human use and have been shown to modulate critical cellular signaling pathways leading to their anticancer effects (1). Garcinol, a polyisoprenylated benzophenone derivative extracted from Garcinia indica (2), has shown some promising anticancer activity (3). Earlier, we have reported anticancer action of garcinol against the breast cancer estrogen receptor (ER)-positive MCF-7 cells, as well as the triple negative MDA-MB-231 cells (4). We have also shown that garcinol effectively inhibit the cell growth of prostate cancer (AR-positive LNCaP, AR-positive but androgen non-responsive C4-2B cells and AR-negative PC3 cells), as well as pancreatic cancer BxPC-3 cells (5). The activity of garcinol was found to be cancer-cell specific because the normal breast epithelial MCF10A cells were not affected (4). These investigations also suggested a role for nuclear factor-kap-paB (NF-kB) signaling in eliciting the anticancer activity of garcinol; however, the exact mechanism of action of garcinol is still not clear.

The signal transducer and activator of transcription (STAT-3) is known to cross talk with NF-kB in various cancer models (6). There is ample evidence to suggest a cooperation between these signaling hubs leading to enhanced tumor progression and metastasis (7). STAT-3 is also known to play an important role in the tumor survival and progression through regulation of prosurvival factors (8). It is constitutively phosphorylated in cell lines, as well as human tumors, and regulates the expression of several genes that are involved in metastases (9). Targeted inhibition of STAT-3 has been demonstrated to exert antitumor effects and therefore STAT-3 offers an attractive target for therapy for aggressive cancers. Several factors such as Janus-activated kinases, interleukin-6 (IL-6), epidermal growth factor receptor and Src family kinases are known activators of STAT-3. Once activated via phosphorylation of key tyrosine and serine residues, STAT-3 dimerizes, translocates to nucleus and induces the expression of its downstream target genes by binding to DNA response elements in the promoter region of the target genes. There is evidence to suggest the role of STAT-3 in chemoprevention (10) but the effect, if any, of garcinol on STAT-3 signaling leading to its anticancer action has never been investigated, which prompted us to undertake the current study. We investigated the mechanism of anticancer action of garcinol, involving the regulation of STAT-3 signaling pathway, in cell models representing breast, prostate and pancreatic cancers. In addition to our in vitro studies in the cell-line models, we corroborated our results in vivo in a mouse xenograft model using human breast cancer cells.

Materials and methods

Cell lines and reagents

Human breast cancer cell line MDA-MB-231 and pancreatic cancer cell line BxPC-3 were cultured in Dulbecco’s modified Eagle’s medium, whereas prostate cancer cell line DU145 was cultured in RPMI media, with 10% fetal bovine serum and penicillin/streptomycin. All cells were cultured in 5% CO2-humidified atmosphere at 37°C. The cell lines have been tested and authenticated by the core facility (Applied Genomics Technology Center at Wayne State University) through short tandem repeat profiling using the PowerPlex 16 System from Promega. Antibodies were purchased from following sources—STAT-3 (Cell Signaling, Danvers, MA), pSTAT-3 (Tyr 705/Ser 727), vascular endothelial growth factor (VEGF), urokinase-type plasminogen activator (uPA) (Santa Cruz Biotechnology, Santa Cruz, CA), matrix metalloproteinase (MMP)-9 (R&D Systems) and β-actin (Sigma-Aldrich, St Louis, MO). Garcinol was isolated and purified using published methods (11) and the results validated using commercially available garcinol from Biomol/Enzo Life Sciences International, Inc. (Plymouth Meeting, PA).

Cell invasion assay

Cell invasion assay was performed using 24-well transwell permeable supports with 8 µm pores (Corning, Lowell, MA) as described previously (12). Cells were either treated with vehicle control or garcinol for indicated time period. At the end of the treatment, cells were harvested by trypsinization, viable cells were counted under microscope and equal number of viable cells from each experimental condition (control versus garcinol-treated) were suspended in serum-free medium and seeded into the transwell inserts, coated with growth factor-reduced matrigel (BD Biosciences, Bedford, MA). Bottom wells were filled with media containing complete media. Cells were not exposed to vehicle/garcinol during this part of the experiment. After 24 h, cells were stained with 4 µg/ml calcien AM (Invitrogen) in phosphate-buffered saline at 37°C for 1 h and photographed under a fluorescence microscope. The cells were detached...
from inserts by trypsinization and fluorescence of the invaded cells was read in ultra-multifunctional microplate reader (TECAN, San Jose, CA).

Luciferase assays for STAT-3 transcriptional activity

Transcriptional activity of STAT-3 was studied using CIGNAL Reporter Assay kit (SA Biosciences, Frederick, MD) that uses dual-luciferase assays consisting of a STAT-3-specific firefly reporter and a Renilla luciferase normalization reporter. The assay was performed in 96-well plate, as per vendor’s recommended protocol. After the initial overnight seeding of cells, CIGNAL reporters were transfected using Fugene6 transfection reagent (Roche, Indianapolis, IN). Cells were treated with indicated concentrations of garcinol 24h post-transfection. In the assays involving IL-6 stimulation, cells were exposed to 5 ng/ml IL-6 for 1 h before garcinol treatment. Luciferase assay was developed by using Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Briefly, cells were lysed within the 96-well plates with 20 µl passive lysis buffer provided in the kit. About 100 µl luciferase assay reagent II was then added to the wells to measure luciferase activity followed by addition of 100 µl stop and glo reagent, which quenched the luciferase luminescence with simultaneous initiation of Renilla control luciferase. Luminescence was measured in ultra-multifunctional microplate reader.

Real-time reverse transcription–PCR

Total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. Real-time PCR was used to quantify messenger RNA expression. Sequences of primers for VEGF, MMP-9 and glyceraldehyde 3-phosphate dehydrogenase were the same as reported earlier (13) and the amount of RNA was normalized to glyceraldehyde 3-phosphate dehydrogenase expression (14).

Western blot analysis

For western blot analysis, cells were lysed in RIPA buffer containing complete mini ethylenediaminetetraacetic acid-free protease inhibitor cocktail (Roche, Indianapolis, IN) and phosphatase inhibitor cocktails 1 and 2 (Sigma-Aldrich, St. Louis, MO) as described earlier (12). After resolution through 12% polyacrylamide gels under denaturing conditions, proteins were transferred to nitrocellulose membranes, incubated with appropriate primary and horseradish peroxidase-conjugated secondary antibodies and further visualized using chemiluminescence detection system (Pierce, Rockford, IL).

ELISA assay for VEGF/MMP-9

To investigate the effect of garcinol on IL-6-stimulated STAT-3 signaling, MDA-MB-231 cells were stimulated with IL-6 and treated with garcinol, followed by VEGF/MMP-9 quantitation using VEGF/MMP-9 ELISA kit (R&D Systems). At the end of treatment, the culture media were collected and centrifuged to remove cellular debris. The cells in the plate were trypsinized and the total number of cells was determined by cell counting. The levels of VEGF and MMP-9 secreted in the conditioned medium were determined following instructions supplied by the manufacturer and the results were normalized to the cell number.

In vivo studies

Female homozygous ICR SCID mice, ages 4 weeks, were used for our study. Animal experimental protocol was approved by the Committee on the Ethics of Animal Experiments, the Wayne State University Institutional Users of Animal Care Committee. To initiate the xenografts, 5 × 10⁶ MDA-MB-231 cells (in serum-free medium) were injected s.c. bilaterally in the flank areas of SCID mice. Animals were examined thrice per week until they developed palpable tumors. Then the animals were randomly divided into two groups of six animals each (12 tumors per group because of bilateral tumors in each animal). Group I was assigned as control and received only sesame seed oil as a representative model cell line for these studies. Treatment of MDA-MB-231 cells, with IL-6 (5 ng/ml) for an hour, resulted in the activation of STAT-3, as evidenced by increased pSTAT-3 (Tyr 705) and Ser 727) (Figure 4A). These results indicate that in addition to the inhibition of STAT-3 expression, garcinol also inhibits the activation of STAT-3 (transcriptional activity) in a dose-dependent manner. In cancer cells, activated STAT-3 induces the activity of several downstream targets involved in cell growth, metastasis and angiogenesis through regulation of their transcription. Therefore, next assessed whether the inhibition of STAT-3 transcriptional activity could also correlate with transcriptional inhibition of its target genes, and we chose VEGF and MMP-9 as the representative genes (18). We observed a dose-dependent inhibition of transcription of both the genes by garcinol in all the cell lines tested (Figure 3B), thus confirming an inhibitory action of garcinol on transcription of STAT-3.

Effect of garcinol on IL-6 induced STAT-3

STAT-3 is known to be activated by cytokine IL-6, and IL-6-induced STAT-3 is an accepted model for studying the inhibition of STAT-3 signaling by novel compounds (10). Since we observed very similar effects of garcinol on MDA-MB-231, DU145 and BxPC-3 cells in all the assays described above, we chose MDA-MB-231 cells as a representative model cell line for these studies. Treatment of MDA-MB-231 cells, with IL-6 (5 ng/ml) for an hour, resulted in the activation of STAT-3, as evidenced by increased pSTAT-3 (Tyr 705 and Ser 727) (Figure 4A)—second lane in pSTAT-3 immunoblots. Incubation of these IL-6 stimulated cells with garcinol for 48h resulted in a dose-dependent inhibition of pSTAT-3 levels detected by western blotting (Figure 4A). Further, IL-6-induced MDA-MB-231 cells had higher basal STAT-3-directed transcription (Figure 4B—second bar in the bar-graph), as measured by luciferase assay, but the treatment of cells with 10 µM garcinol significantly (P < 0.05) inhibited this IL-6-induced STAT-3 activity, whereas treatment with 25 µM garcinol brought down the STAT-3 activity to the levels below those in unstimulated cells (Figure 4B). In order to further verify the
Attenuation of IL-6-stimulated STAT-3 activation in garcinol-treated cells in a reciprocal setup, we first treated MDA-MB-231 cells with garcinol for 48 h and then exposed the control (vehicle-treated) and 10 µM garcinol-treated cells to IL-6 (5 ng/ml) for 20 min. As seen in Figure 4C, whereas garcinol inhibited the phosphorylation as expected, exposure to IL-6 increased the levels of pSTAT-3 (both Tyr 705 and Ser 727) only in control cells. Garcinol-treated cells exhibited a significantly less activation of STAT-3, as evidenced by significantly lower levels of phosphorylated STAT-3 (lane 4 versus lane 3 in pSTAT-3 immunoblots).

**Effect of garcinol on STAT-3 targets**

Having established an inhibitory action of garcinol on constitutive, as well as IL-6 stimulated, STAT-3 signaling, we next assessed the effect of garcinol on STAT-3 targets in MDA-MB-231 cells. Activated STAT-3 leads to elevated uPA, VEGF and MMP-9 levels, all of which are also known to be stimulated by IL-6 (18,19). Accordingly, we found significantly elevated levels of uPA, VEGF and MMP-9 in IL-6-stimulated MDA-MB-231 cells (Figure 5A—second lanes in uPA, VEGF and MMP-9 immunoblots). However, treatment with garcinol completely attenuated the IL-6-induced activation of uPA, VEGF and MMP-9 expression. We also quantitated the secreted VEGF and MMP-9 levels by ELISA to further validate our results and found that, whereas IL-6 stimulation resulted in increased VEGF (Figure 5B) and MMP-9 (Figure 5C) production, treatment with garcinol not only attenuated the IL-6 stimulated effects but further decreased the levels below those in control cells (P < 0.05).

**In vivo effects of garcinol**

We employed a xenograft mouse model to study the anticancer activity of garcinol in vivo. We first assessed the effect of garcinol administration on tumor burden in SCID mice. Administration of garcinol for 4 weeks was found to significantly reduce the tumor growth in mice (P < 0.05) (Figure 6A). Tumor remnants were processed for further studies, and analyses of proteins, by western blotting, showed down-regulation of total as well as pSTAT-3 (Tyr 705) in tumors of garcinol-administered mice (Figure 6B). These results fully support our in vitro observations described above, suggesting an effective inhibition of STAT-3 signaling by garcinol leading to its observed biological effects in cancer cells.

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**Fig. 1.** (A) Chemical structure of garcinol. Garcinol inhibited the invasion of (B) MDA-MB-231 breast cancer, (C) DU145 prostate cancer and (D) BxPC-3 pancreatic cancer cells. Bar graphs represent quantitation of the degree of invasiveness under each experimental condition. C: control, G: 25 µM garcinol. **P < 0.01 versus control.

**Fig. 2.** Effect of increasing doses of garcinol on total STAT-3 and phosphorylated STAT-3 in breast, prostate and pancreatic cancer cells. Cells were treated with vehicle control or indicated doses of garcinol for 48 h. β-actin was used as the internal loading control.

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assay, which measures the invasiveness of cancer cells in vitro. We started our investigation by performing a functional assay, that is the invasion assay, which measures the invasiveness of cancer cells in vitro. The results clearly demonstrated an inhibitory effect of garcinol on the invasiveness of breast, prostate and pancreatic cancer cells, which prompted us to investigate further the signaling pathways that might influence the invasive potential of cancer cells. A pathway that stood out was STAT-3 signaling pathway. This pathway is known to cross talk with NF-κB signaling (6,7) and a recent report by Zhang et al. (16) has suggested that a small-molecule inhibitor of STAT-3 signaling blocks STAT-3-NF-κB cross-talk leading to reduced migration and invasion of breast tumor cells. Our results are in agreement with this report showing that inhibition of STAT-3 signaling by garcinol results in the inhibition of invasion not only in breast cancer cells but also in prostate and pancreatic cancer cells. Further, the report by Zhang et al. (16) also demonstrated an inhibitory effect of inhibition of STAT-3 on tumor growth in a human breast xenograft model. We observed an in vivo inhibitory action of garcinol in a breast xenograft model consistent with lowered STAT-3 levels (total and phosphorylated forms) in the tumor remnants. This seems to suggest that inhibition of STAT-3 signaling by garcinol is one of the mechanisms important for the in vivo efficacy of this agent.

Recently, it has been suggested that STAT-3-selective inhibitor, CPA-7, has a potent antitumor activity in only immunocompetent mice with little to no activity in immunodeficient SCID mice (36). This makes sense given the established role of STAT-3 as a negative regulator of inflammatory responses in immune cells. However, this study conducted in lymphoma model contradicted an earlier study (37), which was also carried out in a lymphoma model but used a different STAT-3 inhibitor, atiprimod. The earlier study (37) noted a significant reduction in tumor weight when SCID mice were administered atiprimod. A close examination of the experimental setup revealed some subtle differences between the two studies that might help explain this discrepancy (36). First, CPA-7 is a highly selective inhibitor of STAT-3 unlike atiprimod, which modulates NF-κB, as well as upstream JNK. Second, the study with atiprimod used human cancer cells, whereas the study with CPA-7 used murine cells. Garcinol, the anticancer agent used in the current study, is a known inhibitor of NF-κB (4,5,14,38,39), which might explain its significant antitumor

Discussion
Cancer is the second leading cause of deaths worldwide. Aside from non-melanoma skin cancer, breast cancer is the most common cancer among women and prostate cancer is the most common cancer among men (20). Although pancreatic cancer ranks at number 10 in the list of estimated cancer diagnosis for the year 2012, it ranks at number 4 in men, as well as in women, for the estimated deaths in 2012 (20,21), which highlights the aggressiveness of pancreatic cancer. The use of naturally occurring compounds for the prevention and/or therapeutic intervention of cancer has long been advocated but seems to be gaining more ground in recent years (1,3). A major advantage offered by these phytochemicals as anticancer agents is their relative non-toxic nature. Garcinol is one such phytochemical, which has demonstrated remarkable biological activities (2,3). Early studies focused on the antioxidant ability of garcinol (2,22,23) but its anticancer activity was later recognized as well (3,23–33). However, in spite of the increasing interest in garcinol’s anticancer activity, its exact molecular mechanism of action is not yet known. A number of different mechanisms have been proposed, including inhibition of histone deacetylases (34) and induction of reactive oxygen species (33).

Our earlier work has demonstrated the regulation of NF-κB signaling pathway by garcinol as one of the mechanisms by which it inhibits cell proliferation and induces apoptosis. We were the first to report such an activity of garcinol in breast (4), as well as in prostate and pancreatic, cancer cells (5). Such NF-κB modulating activity of garcinol was shown to occur through a direct inhibition of constitutive NF-κB activity, as well as downregulation of NF-κB-regulated genes. Our results were particularly significant because we found the activity of garcinol to be cancer-cell specific without any effect on the normal breast epithelial, MCF10A cells.

We designed the current study to further elucidate the mechanism of garcinol action particularly in view of the observations that NF-κB signaling cross-talks with multiple pathways (35). We started our investigation by performing a functional assay, that is the invasion assay, which measures the invasiveness of cancer cells in vitro. The

Fig. 3. (A) Effect of increasing doses of garcinol on transcriptional activity of STAT-3. Cells were treated with vehicle control or indicated doses of garcinol for 48 h and assayed for STAT-3-directed transcriptional activity by STAT-3 reporter assay kit. Cells were transfected with plasmid-encoding firefly luciferase and renilla luciferase was used to normalize the values. (B) Effect of increasing garcinol doses on transcription of STAT-3 target genes VEGF and MMP-9, as determined by real-time RT–PCR. Glyceraldehyde 3-phosphate dehydrogenase messenger RNA expression was used as the internal control for normalization of our data. *P < 0.05 and **P < 0.01 versus control.
action even in SCID mice \((P < 0.039)\). Our recent study with garcinol has further highlighted its pleiotropic nature where we have shown its action against Wnt signaling and epithelial-to-mesenchymal transition \((14)\). Such pleiotropic activity of garcinol, especially its inhibition of NF-\(\kappa B\), is similar to the STAT-3 inhibitor atiprimod and could explain its \(in vivo\) action in a SCID mouse xenograft model. A number of studies, including some recent ones, have demonstrated a correlation between STAT-3 inhibition \((40–42)\) or STAT-3 ectopic expression \((43)\) with tumor growth in SCID mice. Because of the inhibition of STAT-3, garcinol might also exhibit antitumor activity against immunocompetent mice, an idea that needs to be tested in future studies in relation to its role \(if any\) in the modulation of immune response.

Although in inflammatory cells, STAT-3 is known for its tumor-suppressive action, in tumorigenic cells, both STAT-3 and NF-\(\kappa B\) have been implicated for their oncogenic activity \((6,7)\). STAT-3 enhances the proliferation of cancer cells \((7)\). We observed an inhibition of cell proliferation by garcinol in breast, prostate and pancreatic cells in our earlier studies \((4,5)\), and our present results showed the effect of garcinol on STAT-3 signaling, which is consistent with our previous findings. Both NF-\(\kappa B\) and STAT-3 signaling pathways are known to

![Fig. 4](image)

**Fig. 4.** (A) MDA-MB-231 breast cancer cells were stimulated with 5 ng/ml IL-6 for 1 h, exposed to indicated doses of garcinol for 48 h and then probed for activated STAT-3 as assessed by western blotting. (B) For transcriptional assays, cells were transfected with firefly luciferase/enhila luciferase for 24 h before stimulation with IL-6 and garcinol treatment for 48 h. (C) MDA-MB-231 breast cancer cells were treated with 10 \(\mu M\) garcinol for 48 h, stimulated with 5 ng/ml IL-6 for 20 min and then probed for activated STAT-3 as assessed by western blotting. \(\beta\)-actin was used as the internal loading control in the western blots for normalization of our data. G10: 10 \(\mu M\) garcinol, G25: 25 \(\mu M\) garcinol. *\(P < 0.05\) and **\(P < 0.01\) versus control.

![Fig. 5](image)

**Fig. 5.** (A) Treatment with garcinol inhibited IL-6-stimulated downstream targets of STAT-3 signaling—uPA, VEGF and MMP-9 in MDA-MB-231 cells, as determined by western blot analysis. \(\beta\)-actin was used as the internal loading control. Secreted VEGF (B) and MMP-9 (C) were quantitated by collecting conditioned media and assayed using ELISA kits. Results were normalized to calculated cell numbers. G10: 10 \(\mu M\) garcinol, G25: 25 \(\mu M\) garcinol. *\(P < 0.05\) versus control.

![Fig. 6](image)

**Fig. 6.** Garcinol inhibits STAT-3 signaling \(in vivo\). MDA-MB-231 cells were injected in SCID mice subcutaneously (bilaterally) and mice were randomized into two groups \(six mice each\)—control and garcinol-treated. Garcinol \((5\text{ mg/mice/day})\) was administered orally for 28 days. (A) Garcinol-administered mice exhibited decreased tumor growth rate. Tumor remnants were used to isolate proteins. (B) Expression of total STAT-3 and phosphorylated STAT-3 in representative three tumors each from control and garcinol-administered mice. C: control, G: garcinol.
control apoptosis (7), which explains our earlier published results on the induction of apoptosis by garcinol (4,5). Not only do NF-κB and STAT-3 physically interact, many genes downstream of NF-κB and STAT3 signaling pathways are intertwined and require transcriptional cooperation between the two factors (6). It is conceivable that inhibition of just one pathway might be compensated by routing the prosurvival mechanisms in cancer cells through the alternate pathway. Our earlier results characterizing the NF-κB inhibitory activity coupled with the present observations on STAT-3 inhibitory activity underlines a very potent anticancer action of garcinol, which is important because garcinol will inactivate both of these signaling pathway simultaneously. Further, combining these results with our recent report (14) on the inhibition of Wnt signaling pathway clearly defines a pleiotropic nature of garcinol that involves modulation of multiple signaling pathways leading to a more efficient antitumor action. Although we observed an inhibitory action of garcinol against STAT-3 phosphorylation and activation, the exact mechanisms involved in such action of garcinol need to be further elucidated. It is possible that such action of garcinol may include inhibition of upstream kinases. Janus kinase 2 phosphorylates STAT-3 when stimulated by IL-6, whereas receptor tyrosine kinases (such as EGFR and c-met), as well as non-receptor tyrosine kinase src, are also known to phosphorylate STAT-3.

In our study, we evaluated the expression levels of uPA, VEGF and MMP-9, as well as secreted VEGF/MMP-9, as a read-out of STAT-3 activity. Stimulation with IL-6 increases the levels of active (phosphorylated) STAT-3. The increased expression levels of uPA, VEGF and MMP-9, and the secreted VEGF and MMP-9 levels, upon stimulation by IL-6 confirmed the connection between STAT-3 activation and increased uPA, VEGF and MMP-9 levels. More importantly, the dose-dependent inhibition of uPA, VEGF and MMP-9 by garcinol demonstrated an inhibitory action of garcinol on STAT-3 signaling. There is evidence for such action of another phytochemical, benzyl isothiocyanate (BITC), on STAT-3 signaling (44). In this study, BITC was found to block the invasion of BxPC-3 cells, reduce the secretion of VEGF, reduce in vivo tumor growth and reduce the expression of phosphorylated STAT-3 in vivo. Our results closely resemble these findings, suggesting inhibition of STAT-3 by phytochemicals as one of the mechanisms of their anticancer action.

In summary, we have identified STAT-3 signaling as a novel target for the anticancer action of garcinol. The major conclusions of our study are (i) garcinol inhibits the invasive capacity of breast cancer MDA-MB-231, prostate cancer DU145 and pancreatic cancer BxPC-3 cells, (ii) it effectively downregulates both the constitutive total and phosphorylated STAT-3 as well as IL-6-induced STAT-3 signaling in a dose-dependent manner, (iii) downregulation of STAT-3 signaling and its transcriptional activity correlates with downregulation of its downstream targets uPA, VEGF and MMP-9 that are known to play a role in metastasis and angiogenesis, (iv) garcinol inhibits tumor progression in vivo in a xenograft model and (v) garcinol-treated animals showed marked reduction in the levels of total, as well as phosphorylated, STAT-3 in tumor remnants. Based on these promising results, we suggest that further pre-clinical and clinical studies are warranted in order to firmly establish the anticancer action of garcinol.

Conflict of Interest Statement: None declared.

References


Received June 4, 2012; revised August 29, 2012; accepted September 5, 2012