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Piceatannol bolsteres fetal haemoglobin formation in K562 cells via p38 map kinase activation and ERK inactivation

ABSTRACT

Elevation of the level of fetal haemoglobin (HbF) by pharmacological agents is a safe and a promising approach for treating beta thalassemia. In this study, the effect of piceatannol was studied in human erythroleukemic K562 cells for their role in gamma-globin mRNA and HbF induction. The role of p38 mitogen activated protein kinase (MAPK) and extracellular regulated protein kinase (ERK) signaling pathways were also examined. It was found that piceatannol significantly increased gamma-globin mRNA and HbF levels in dose and time dependent manner in K562 cells. This was determined by enzyme linked immunosorbent assay (ELISA) and western blot analysis. Pretreatment with p38 MAPK inhibitor (SB203580) obstructed the stimulatory effect of piceatannol in total and HbF activation. In contrast, no change in HbF level was observed in K562 cells when treated with ERK inhibitor (PD98059). Moreover, piceatannol activated p38 MAPK and inhibited ERK signaling pathways in K562 cells as shown by western blot analysis. Besides, the inhibitor SB203580 inhibited p38 MAPK activation when cells were pre-treated with piceatannol. In summary, piceatannol was found to be a strong inducer of HbF production in K562 cells. The results mark the role of p38 MAPK and ERK signaling as molecular targets for stimulation of HbF synthesis upon treatment with piceatannol.

Key words: piceatannol, pharmacological agent, fetal haemoglobin inducer

Introduction

Beta-thalassemia syndromes describe a group of genetic blood disorders caused by decreased or absent synthesis of the beta-globin chain, resulting in reduced amount of haemoglobin in red blood cells (RBC), low RBC production and anaemia (Galanello & Origa, 2010; Kukreja et al., 2013a). It occurs mainly due to mutation in the beta globin gene present on chromosome 11p15.5. It is a cause of morbidity and mortality, including hepatosplenomegaly (Rund & Rachmilewitz, 2005). Increased level of HbF can counter balance the low level of adult haemoglobin (HbA) in patients suffering from beta thalassemia (Olivieri & Weatherall, 1998). Some chemical agents, such as 5-azacytidine and hydroxyurea have been used to augment HbF production in patients of beta thalassemia(Heller & Desimone, 1984; Watanapokasin et al., 2005), but associated side effects such as myelotoxicity, carcinogenesis and modest responses to treatment have limited the clinical utility of these chemical agents (Randi et al., 2005; Strouse et al., 2008).

Piceatannol possesses various health benefits. It has anti-adipogenic property, due to which it modulates the development of adipose tissue. It is helpful in stimulating the osteoblastic activity and leads to bone formation. It is also known to possess antimelanogenic activity. It also prevents type II diabetes (Kukreja et al., 2013b). But, scientific evidences on its biological activities are very limited. One study showed that piceatannol inhibited the proliferation and induced apoptosis *in vitro* (Piotrowska et al., 2012). K562 (human erythroleukemia) cell line is found to be Philadelphia-chromosome positive. In this cell line, the expression level of both HbF and embryonic Hb (HbPortland and HbGower) is markedly increased after exposure to hemin (Smith et al., 2000). This cell line is committed to the embryo fetal erythroid differentiation pathway and produces

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haemoglobin independently of terminal differentiation (Gambari & Fibach, 2007). Therefore, it is used as a model system for the study of human globin gene expression and HbF inducer screening (Atweh & Schechter, 2001).

To determine the HbF inducing effect of piceatannol, the present study was designed to study the effect of piceatannol on K562 erythroid differentiation. It was observed that piceatannol promoted gamma globin mRNA expression and HbF synthesis in K562 cell line. The inducing effect of piceatannol was observed due to the activation of p38 MAPK and inactivation of ERK signaling pathway.

Materials and Methods

K562 cell culture protocol

Human erythroleukemic K562 cells were purchased from National Centre for Cell Science, Pune. K562 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (BSA) and 1% penicillin-streptomycin in a CO_2 incubator. The atmosphere inside the CO_2 incubator was humidified with 5% CO_2 and temperature was maintained at 37°C. When cells reached semi-confluence state, they were then sub-cultured at 1×10^4 cells/mL.

K562 cell treatment protocol with standard and reference compound

Individual The solution of piceatannol was prepared in dimethyl sulfoxide (DMSO) for cell culture. The final concentration of DMSO was kept <0.1% of final concentration of cell culture medium so that it remains non cytotoxic to the cells. K562 cells were treated with piceatannol reconstituted in DMSO. Hydroxyurea at different concentrations was used as a known positive control. To study the cytotoxic effect, K562 cells were seeded at 4×10^3 cells/well in RPMI in 96 well plates and specified amount of drug was applied for 48 h at 37°C. For estimating the level of HbF and total haemoglobin (THb), the cells were seeded in 96 well plates at 4×10^3 cells/well and readings were observed daily for 6 consecutive days. To confirm the underlying signaling pathways, inhibitors of ERK (PD98059, 25 µM) and p38 MAPK (SB203580, 10 µM) pathways were added into the medium containing K562 cells prior to application of piceatannol. This was followed by the quantification HbF and THb levels as well as western blot analysis. The concentration of piceatannol was optimized at 20 µg/mL for mechanistic studies in our experiments.

Cytotoxicity assay

The cytotoxic effect of piceatannol was estimated by using 1-(4,5-dimethylthiazol-2-yl)-(3,5)-diphenyl-formazan (MTT) colorimetric assay. According to this assay, after 48 h of treatment with piceatannol, MTT solution (prepared by dissolving 5 mg of MTT per ml of phosphate buffer saline) was added to each well of the plate and the plate containing MTT solution was incubated at 37°C for 3 h. The blue formazan crystals that formed as a result of reduction of MTT by living cells were dissolved in DMSO and the absorbance was observed at 540 nm. Cells survival rate was calculated as a percentage of viable cells in cells containing known amount of drug versus non drug containing cells i.e., control cells.

THb assay

The relative amount of THb produced in K562 cells was determined by TMB (3,3,5,5-tetramethylbenzidine) assay. This assay is based on peroxidase like activity of heme portion of haemoglobin. In brief, after each time interval, the cells of each treatment group were allowed to react with 5% solution of TMB for 10 minutes at 37°C in dark atmosphere followed by addition of H_2SO_4 solution (2M) to terminate the reaction. The number of blue (stained) and pale yellow (unstained) cells were counted by using inverted microscope. The relative total haemoglobin production was expressed as (number of blue cells/total number of cells) × 100. The quantitative amount of haemoglobin was determined with the help of plasma Hb kit (Sigma).

HbF assay

HbF level of K562 cells was determined quantitatively with the help of ELISA kit (Sigma-Aldrich). At each time interval, cell lysate diluted in 50 mM Tris-Cl/1% BSA solution was transferred to 96-well plate pre-coated with anti-HbF followed by incubation for 1 h at room temperature. The secondary antibody (horse radish peroxidase) was added to 96-well plate containing primary antibody and the plate was incubated for 1 h at 37°C. This was followed by the addition of 10% TMB solution. Finally, the reaction was stopped by adding 2M H_2SO_4 solution and absorbance was measured at 450 nm. The concentration of HbF was calculated with the help of standard curve and expressed as HbF/total proteins.

Western Blot analysis

K562 cells treated with piceatannol were lysed in 200 µL of ice-cold lysis buffer (150 mM NaCl, 50 mM Tris, 50 mM

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NaF, 2 mM EDTA, 1% SDS, 0.2 mM sodium vanadate, 1 mM phenylmethylsulphonyl fluoride, 1 µg/mL leupeptin and 1 µg/mL aprotinin). The protein concentration in the samples was calculated by Bicinchoninic acid assay. The cytosolic extracts (20 µg) were run on 10% of sodium dodecyl sulphate-polyacrylamide gel electrophoresis and were transferred to polyvinylidene uoride membranes (BioRad). Membranes were blocked with Tris-buffer saline containing 10% dry milk and 0.1% Tween-20 for 1 h. Then, membranes were incubated overnight at room temperature with diluted primary antibodies (anti-ERK1/2 total, anti-ERK1/2 phosphorylated, anti-p38 phosphorylated and anti-p38 total antibodies; Calbiochem). Following incubation, these membranes were washed with TBS-T and were incubated with secondary antibodies conjugated with horseradish peroxide diluted at 1:3000 for 1 h. The proteins of interest were observed by the chemiluminent protein detection system (BioRad).

Results

Cytotoxic effect of piceatannol on K562 cells

The cytotoxic effect of piceatannol on K562 cells is shown in Figure 1. It can be observed that as the concentration of piceatannol increases the percentage of viable cells decreases. This indicates a dose-dependent inhibitory effect of piceatannol. Significant cytotoxic effect was observed in cells when piceatannol was used at a concentration 80 µg/ml or above it. The following mentioned figures (Figure 1 and Figure 2) represent the effect of piceatannol on cell viability and THb induction in K562 cells.

THb assay

Our results confirmed that piceatannol induced THb formation in the specified range without any cytotoxic effects. At 40 µg/ml, piceatannol showed its maximal effect on THb production by 79.0±4.2% (Figure 2). Beyond the concentration of 40 µg/ml, the amount of haemoglobin induction was decreased. This effect was due to the increasing cytotoxic effect of piceatannol as found in MTT assay.

Figures 3, 4 and 5 depict the time dependent response of piceatannol on THb induction. It was seen that there was a significant increase in the percentage of TMB-positive cells at all test concentrations of piceatannol from day 3 to day 6 when compared with their respective basal values of day 1.

Our data represented that the optimal dose of piceatannol was 40 µg/ml, which showed similar result as compared with the positive control HU at 25 µg/ml. There was a significant difference in viability of cells ($P<0.05$) when compared with non-treated cells. Also, a significant difference ($P<0.05$) was found in the number of cells stained with TMB when compared with non-treated cells. In control experiment, the percentage of TMB-positive cells was increased to lesser extent (<15%). It represented that auto-differentiation of K562 cells was observed in prolong culture.

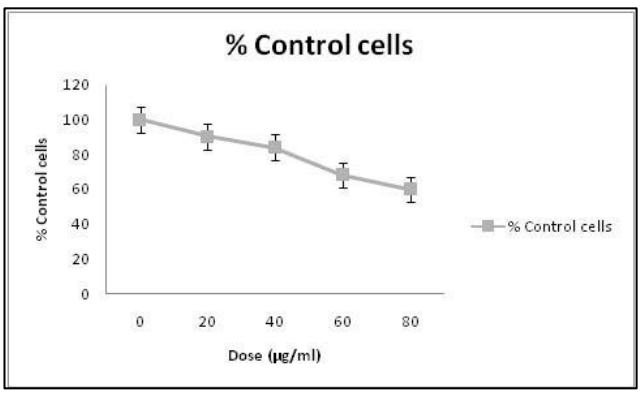

Figure 1. *The cytotoxic effect of piceatannol on K562 cells. The cells were treated with different concentrations of piceatannol for 2 days. Later, the cell viability % (filled rectangle) was calculated by MTT assay. This result is expressed as mean ± SD of three cultures.*

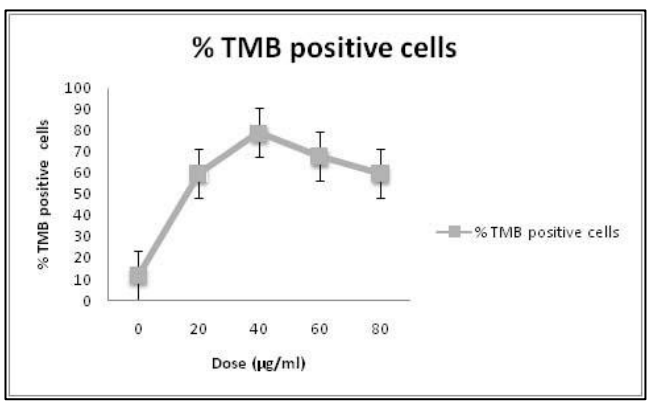

Figure 2. *THb induction in K562 cells. The cells were treated with different concentrations of piceatannol for 6 days. The haemoglobin positive cells were stained by TMB assay. The percentage of stained cells (filled rectangle) was estimated. The data is representative of three separate experiments.*

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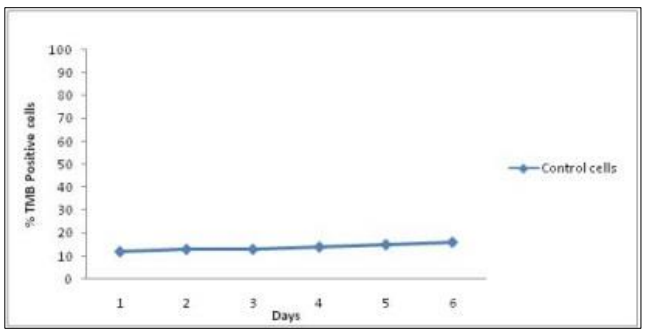

Figure 3. *Levels of THb in non-induced K562 cells at different time intervals. In this experiment, Hb positive cells were stained by TMB assay. The result is expressed as mean ± SD of three cultures.*

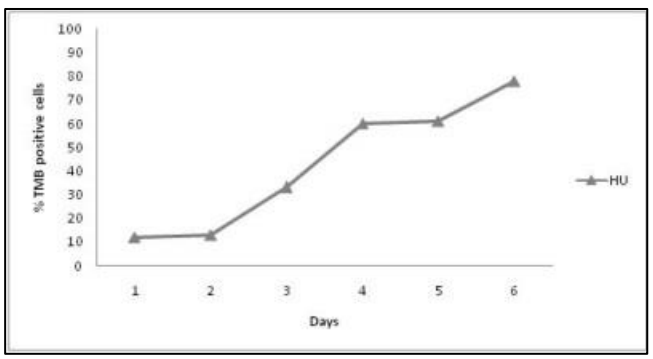

Figure 4. *The effect of HU (25 µg/ml) on induction of THb in K562 cells at different time intervals. K562 cells were treated with HU for 6 days. In this experiment, Hb positive cells were stained by TMB assay. The result is expressed as mean ± SD of three cultures.*

HbF percentage analysis

Significant increase in HbF level was observed in K562 cells when treated with different concentration (20-80 µg/mL) of piceatannol as compared with untreated control cells (Figure 3 and Figure 5). Dose dependent response of piceatannol was detected in the range of 20-40 µg/mL. Maximal response was seen at 40 µg/ml. Beyond the concentration of 40 µg/mL, the HbF inducing effect of piceatannol was reduced, possibly due to cytotoxic effect of piceatannol at higher concentration. Time dependent effect of piceatannol was observed in K562 cells during 6 days of incubation. Similar increase in HbF level was observed in K562 cells treated with HU (25 µg/ml).

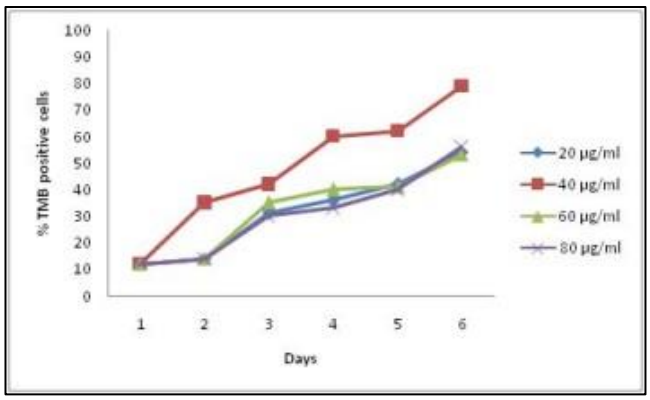

Figure 5. *The effect of piceatannol on induction of THb in K562 cells at different time intervals. K562 cells were treated with different concentrations of piceatannol for 6 days. In this experiment, Hb positive cells were stained by TMB assay. The results are shown as mean ± SD of three cultures.*

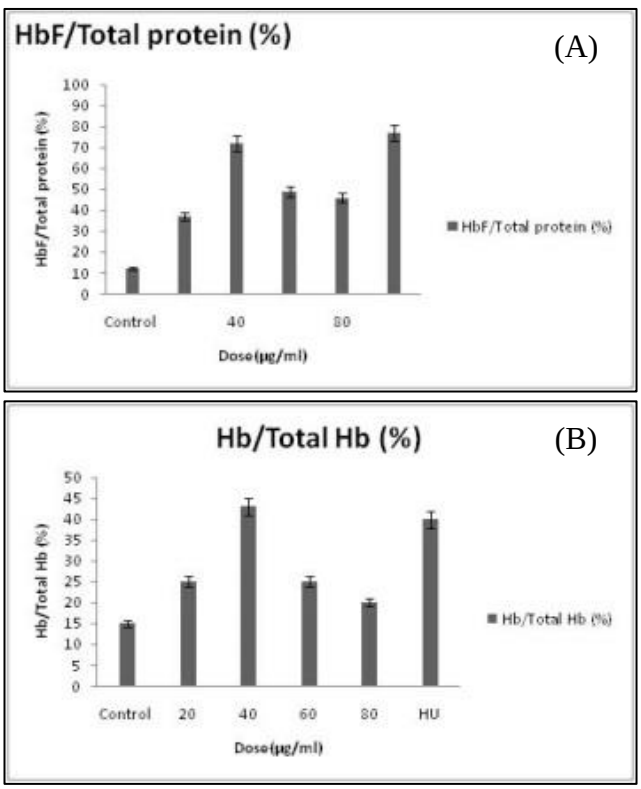

Figure 6. *The effect of piceatannol and HU (25 µg/ml) on HbF production of K562 cells. Dose-dependent effect of piceatannol in HbF-inducing on K562 cells after 6 days of treatment, depicted in (A) % of HbF per total proteins and (B) % of HbF per total Hb. The HbF concentration was quantified by ELISA kit. The data are representative of three distinct experiments. P<0.05, significantly different from non-treated control cells.*

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It increased the concentration of HbF over 6 days of culture period. While doing the experiment, auto-differentiation of K562 cells was observed in untreated cells (control cells) starting from 4 to 6 days. But, the degree of increase in HbF level was smaller than both positive control and treated cells.

To examine the specificity of piceatannol in HbF induction, we measured HbF to THb ratio (Figure 6). Piceatannol was able to increase the level of HbF in comparison to total K562 cells. At concentration of 40 µg/mL, piceatannol caused a 2.8 fold increase in HbF ratio compared with untreated cells (control group). HbF inducing effects of piceatannol were found similar to positive control cells (HU group).

Inhibitory effects on piceatannol treated cultures

To find whether MAPK signaling pathways are responsible in piceatannol driven HbF stimulation, the actions of SB203580 (p38 inhibitor) and PD98059 (ERK inhibitor) in piceatannol treated K562 cells were examined. As shown in Figure 7A, p38 inhibitor significantly inhibited piceatannol driven Hb production by 48%. However, ERK inhibitor did not produce any effect on piceatannol treated K562 cells. According to Figure 7B, p38 inhibitor reduced piceatannol induced HbF production and ERK inhibitor had no effect in K562 cells. On day 6, percentage of HbF/Total proteins of piceatannol plus SB group was 19.2±0.7, which was lower than piceatannol group by 50%. On the other hand, ERK inhibitor did not change the production of HbF synthesis (P >0.05) when compared with piceatannol treatment cells.

Western blot analysis on signaling pathways

The p38 MAPK and ERK pathways are supposed to be involved in HbF activation. We tested if piceatannol affected these pathways in K562 cells. As shown in Figure 8A, significant change in basal levels of p-ERK were observed, as both the proteins were dynamically involved in cell survival and proliferation at undifferentiated state. After 10 minutes of addition of piceatannol, p38 was substantially phosphorylated. This phosphorylation remained constant for entire observation period. However, ERK was de-phosphorylated on addition of piceatannol in time dependent manner. Moreover, addition of p38 inhibitor (SB203580) down-regulated the piceatannol induced phosphorylation of p38 MAPK pathway as shown in figure (Figure 8B). The results indicated that activation of p38 MAPK pathway is necessary for piceatannol induced HbF production.

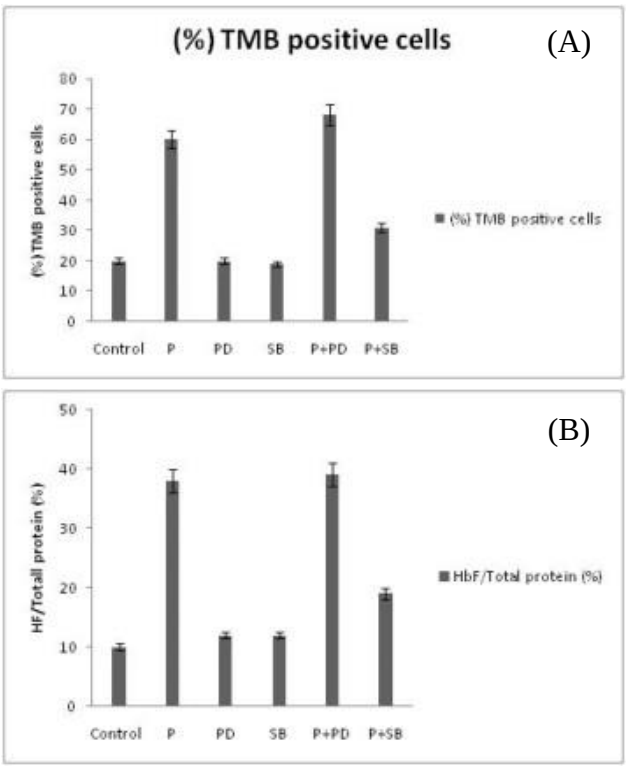

Figure 7. *Number of TMB stained and unstained cells. Relative amount of THb production was expressed as a percentage of number of stained versus total number of cells (A) or percentage of HbF per total proteins in cells (B).*

Discussion

In this study, we showed that piceatannol is a potent inducer HbF. Effectiveness of piceatannol was compared with HU. It has been observed that HbF inducing activity of piceatannol was more than HU in K562 cells, as shown by the result of quantitative ELISA using anti-HbF antibody and western blot analysis. It was found that the activation of HbF production involved the activation of p38 MAPK and inhibition of ERK pathways as depicted in western blotting. Inhibition of p38 MAPK signaling pathway by SB203580 inhibited piceatannol mediated activation of Hb synthesis.

Till date, several therapeutic agents are being used in treatment of beta-thalassaemia – HU, 5-azacytidine and some natural haemoglobin inducers such as rapamycin (*Streptomyces hygroscopicus*), resveratrol (grapes), angelicin and linear psoralens (*Aeglemarmelos* and *Angelica areangelica*), ethanol extract of *Fructus trichosanthis*, mithramycine, cucurbitacin D (Fontecave et al., 1998; Frémont, 2000; Smith et al., 2000; Atweh & Schechter, 2001; Rodrigue et al., 2001; Fibach et al., 2003; Iyamu et al., 2003;

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Nepote et al., 2004; Woessmann et al., 2004; Gambari & Fibach 2007; Bianchi et al., 2009). Among all of them, HU is only FDA approved for the treatment of beta-thalassemia. But in comparison to HU, these therapeutic agents are more responsive and have fewer side effects.

Piceatannol is a natural compound having functional properties. And we found that it is a potent inducer of HbF. But still, studies on large scale are needed to be done to check its HbF inducing property and its cytotoxic effect, if any.

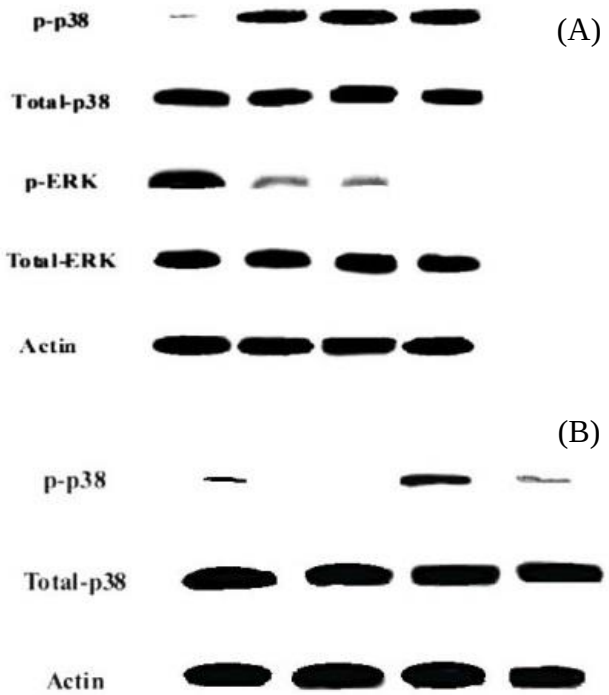

Figure 8. *Effect of piceatannol on p38 MAPK mediated signalling pathways. (A) Piceatannol was applied on K562 cells for 10 min. Cells were broken open and analyzed by western blot with antibodies probing phosphor and total forms of p38 and ERK. (B) The K562 cells were pre-treated with p38-inhibitor (SB203580) for 10 min in presence of piceatannol. Cells were broken open and examined by western blot analysis.*

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