Inhibition of Vascular Smooth Muscle Cell Proliferation by *Gentiana lutea* Root Extracts

Rushendhiran Kesavan¹, Uma Rani Potunuru¹, Branislav Nastasijević², Avaneesh T¹, Gordana Joksić², Madhulika Dixit¹*

¹Laboratory of Vascular Biology, Department of Biotechnology, Indian Institute of Technology Madras, Chennai, India, ²Department of Physical Chemistry, Vinca Institute of Nuclear Sciences, Belgrade, Serbia

**Abstract**

*Gentiana lutea* belonging to the *Gentianaceae* family of flowering plants are routinely used in traditional Serbian medicine for their beneficial gastro-intestinal and anti-inflammatory properties. The aim of the study was to determine whether aqueous root extracts of *Gentiana lutea* consisting of gentiopicroside, gentisin, bellidifolin-8-O-glucoside, demethylbellidifolin-8-O-glucoside, isovitexin, swertiamarin and amarogentin prevents proliferation of aortic smooth muscle cells in response to PDGF-BB. Cell proliferation and cell cycle analysis were performed based on alamar blue assay and propidium iodide labeling respectively. In primary cultures of rat aortic smooth muscle cells (RASMCs), PDGF-BB (20 ng/ml) induced a two-fold increase in cell proliferation which was significantly blocked by the root extract (1 mg/ml). The root extract also prevented the S-phase entry of synchronized cells in response to PDGF. Furthermore, PDGF-BB induced ERK1/2 activation and consequent increase in cellular nitric oxide (NO) levels were also blocked by the extract. These effects of extract were due to blockade of PDGF-BB induced expression of iNOS, cyclin D1 and proliferating cell nuclear antigen (PCNA). Docking analysis of the extract components on MEK1, the upstream ERK1/2 activating kinase using AutoDock4, indicated a likely binding of isovitexin to the inhibitor binding site of MEK1. Experiments performed with purified isovitexin demonstrated that it successfully blocks PDGF-induced ERK1/2 activation and proliferation of RASMCs in cell culture. Thus, *Gentiana lutea* can provide novel candidates for prevention and treatment of atherosclerosis.

**Introduction**

Pathogenesis of atherosclerosis and neo-intimal thickening post angioplasty involves excessive migration and proliferation of smooth muscle cells (SMCs) from media into the lumen of blood vessels. Increased expression of several growth factors such as basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF)-BB contribute to atheroma formation [1]. These agonists due to blockade of PDGF-BB induced expression of iNOS, cyclin D1 and proliferating cell nuclear antigen (PCNA). Docking analysis of the extract components on MEK1, the upstream ERK1/2 activating kinase using AutoDock4, indicated a likely binding of isovitexin to the inhibitor binding site of MEK1. Experiments performed with purified isovitexin demonstrated that it successfully blocks PDGF-induced ERK1/2 activation and proliferation of RASMCs in cell culture. Thus, *Gentiana lutea* can provide novel candidates for prevention and treatment of atherosclerosis.

**Materials and Methods**

2.1 Materials Used

DMEM-F12 medium and antibiotic solution consisting of penicillin and streptomycin were from HiMedia Labs, Mumbai,
India. Fetal bovine serum of South American origin was from GIBCO, Invitrogen, NY, USA. Antibodies against phospho- and total forms of eNOS, ERK1/2, PDGFR-β, IKKα, iNOS, cyclinD1 and PCNA were from Cell Signaling Technology Inc., Beverly MA, USA. EKR1/2 activation inhibitor (328000) was from Calbiochem, Inc. La Jolla, USA. PDGF-BB, EGF, bFGF, Insulin, Elastase, Collagenase, Trypsin inhibitor, iNOS PCR primers, L-arginine, Diaminofluorescein –2 Diacetate (DAF2-DA), Alamaran blue reagent, propidium iodide, NOS inhibitor L-NAME and all the other dry lab chemicals were from Sigma Aldrich, St. Louis, MO, USA. HPLC grade acetonitrile and methanol were from J.T.Baker (Deventer, Netherlands).

2.2 Plant Material and Extract Preparation

Gentiana lutea plant roots were purchased from the Institute of Medicinal Plant Research “Dr. Josif Pancic”, Belgrade, Serbia. Aqueous root extracts were prepared by boiling the gentian roots in water (in ratio 1:20, m/V) for 10 minutes, followed by filtration through 0.45 μm filters (Millipore Co. Ltd) as described previously [14,15]. Frozen extracts were lyophilized and stored in sample tubes until further analysis at a dry place. At the time of experimentation the extracts were reconstituted in sterile distilled water (at a concentration of 10 mg/ml) and filtered through 0.22 μm filter for further use.

2.3 Isolation and Culture of Smooth Muscle Cells

Male Wistar rats weighing 100–150 g were procured from Kings Institute, Chennai. The protocol employed for isolation and culture of aortic smooth muscle cells was approved by the Institutional Animal Ethics Committee at the Indian Institute of Technology Madras in accordance with Indian Council of Medical Research, Government of India guidelines. These guidelines are formulated in accordance with U.S. National Institutes of Health guidelines for care and use of laboratory animals. Thoracic aorta were obtained and subjected to collagenase based enzymatic digestion for isolation of smooth muscle cells as previously published [16]. Cells were seeded and cultured on to collagen coated 6-well tissue culture dishes in DMEM-F12 medium supplemented with 10% (v/v) FBS, penicillin, streptomycin, fibroblast growth factor and epidermal growth factor in a humidified atmosphere of 5%CO₂-95% air as reported previously [16]. Prior to experimental treatments, cells were serum-starved over-night. All experiments were done with quiescent cells up to passage two.

2.4 Immunoblotting

Cells were washed with ice-cold phosphate buffer saline followed by lysis in Triton X lysis buffer (20 mmole/LTris, 100 mmole/L NaCl, 10% triton X-100, 1 mmole/L EDTA, 1 mmole/L sodium orthovanadate, 2.5 mmole/L sodium pyrophosphate, 0.5% sodium deoxycholate, 1X protease inhibitor from Sigma). Following separation on polyacrylamide gels, proteins were electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes from Amersham. Transferred proteins were incubated with respective primary and secondary antibodies as per manufacturer’s instructions and equal loading of blots was confirmed by re-probing the blots with β-actin. Enhanced chemiluminescence based ECL plus detection kits were obtained from Cell Signaling Technology Inc. Beverly, MA, USA.
2.5 Nitric Oxide (NO) Measurements via DAF2-DA Imaging

Cultured SMCs were serum starved prior to treatment with PDGF-BB (20 ng/ml) in presence or absence of Gentiana lutea extract for given time duration. For measurement of intracellular NO, cells were co-incubated with 10 µmole/L DAF2-DA and 1 mmole/L L-arginine as nitric oxide synthase substrate. Release of NO was scored as appearance of green fluorescence due to binding of cellular NO to DAF2 dye. DAF2-DA is a cell permeable dye, which loses its diacetate moiety upon entering the cell due to action of cellular esterases and fluoresces upon binding to NO [17]. Images were captured using Olympus immunofluorescence microscope equipped with ProgResCapture-Pro 2.7 camera followed by analysis with ImageJ software (NIH).

2.6 Alamar Blue Assay

Cell viability was measured by Alamar Blue assay [18]. Briefly, Alamar blue (Resazurin sodium salt from Sigma) was dissolved in phosphate buffered saline pH 7.4 to make a stock of 1 mg/mL and a final working concentration of 0.1 mg/mL in cell culture medium. Resazurin is a redox indicator, which measures the reducing environment of the cell by reducing to a pink colored resorufin. Following experimental treatments with PDGF-BB (20 ng/ml, 24 hours) in presence or absence of Gentiana extract (1 mg/ml), the cells were treated with alamar blue dye. Colour change was monitored colorimetrically at 590 nm and 570 nm to evaluate oxidized versus reduced forms respectively of the reagent by using multi-mode plate reader from Spectramax.

2.7 Cell Cycle Analysis

RASMCs were seeded on collagen coated 6-well dishes and were maintained in growth medium until they reached 70% confluence. They were then serum starved over-night for synchronization followed by treatment with PDGF-BB (20 ng/ml) with or without G. lutea extract for up to 24 hours. Cells were trypsinized, centrifuged at 12000 rpm for 10 minutes and the pellets were re-suspended in 0.3 ml PBS. Cell fixation was done with ice-cold 70% ethanol at 4°C for 16 hours. Fixed cells were vortexed and briefly centrifuged at 12000 rpm for 5 minutes, followed by re-suspension of pellet in PBS containing 10 mg/ml RNAaseA and propidium iodide (50 µg/ml) for staining. Following incubation at 37°C for 1 hour, the PI-DNA complex in the nucleus of each cell was measured using FACS Diva (Becton and Dickinson Co., Franklin Lakes, NJ, USA). Subsequent analysis to determine percentage of cells in various stages of cell cycle was done using FlowJo software.

2.8 Docking Analysis

Docking analysis of Gentiana lutea constituents onto human MEK1 were performed using the Lamarckian genetic algorithm of AutoDock4 [19]. Putative docking sites were first identified using the entire macromolecules as search space followed by rigid docking at the sites of high binding energy. The PDB ids of MEK1 used for the analysis were 3EQF and 3EQH [20]. These are crystal structures of ternary complexes of MEK1 with Mg2+ATP and inhibitors K252a and U1026 respectively. The chemical ids of the ligands used for the study were: CID 9912413 for K252A, CID 3006531 for U1026, CID 88708 for gentipicroside, CID 115149 for amarogentin, CID 162350 for isovitexin, CID 442435 for gentipicroside, CID 115149 for amarogentin, CID 162350 for isovitexin, CID 442435 for...
The search parameters for docking analysis were as follows: Grid parameters: number of points, 626262, spacing: 0.375 Å (Rigid docking), 1.0 Å (Blind docking), number of runs: 100, initial population size: 150, maximum number of evaluations: 2500000, maximum number of generations: 27000 and default values were used for all the other parameters.

2.9 Statistical Analysis
Results are expressed as mean±SEM for a minimum of four independent experiments and statistics were performed using Student’s t-test using GraphPad Prism software. P values <0.05 were considered to be statistically significant.

Results
3.1 Determination of Cytotoxic Concentrations of the Extract
UPLC and MALDI-TOF analyses previously carried out on the extract [14], reported presence of following compounds: gentisin, bellidifolin-8-O-glucoside, demethylbellidifolin-8-O-glucoside, isovitexin, swertiamarin, amarogentin and getiopicroside. Among these, the major constituent of the extract was gentiopicroside as already reported [14]. We first determined the concentration of the extract at which it will inhibit proliferation by 50% (IC50) for primary cultures RASMCs, rat and human specific aortic smooth muscle cell lines A7r5 and ATCC CRL-1999 respectively. These were found to be 2.22 mg/ml for primary cultures of RASMCs, 2.7 mg/ml for A7r5 and 3.43 mg/ml for human cell line as shown in figure 1.

3.2 Effect of Extract on PDGF-BB Induced Cell Proliferation
We then sought to determine whether co-incubation of smooth muscle cells with *G. lutea* extract (1 mg/ml) for 24 hours blocks PDGF-BB (20 ng/ml) induced proliferation. As seen in figure 2, the extract significantly blocked PDGF-BB induced conversion of resazurin (alamar blue reagent) to its reduced form resorufin both in primary cultures of rat aortic smooth muscle cells (fig. 2A) and A7r5 cell line (fig. 2B). This effect was however not due to induction of apoptosis which was scored as Annexin V labeling (fig. 2C), neither was it due to interference of resazurin indicator with extract alone (data not shown). We then analyzed the effect of extract on cell cycle progression. Over-night serum starvation was done to achieve 83±2% synchronization of cells in G0/G1 phase. Cells were then treated with 20 ng/ml PDGF-BB and were analyzed at 12 and 18 hours post treatment for entry into S and G2/M phase using propidium iodide labeling (fig. 3A). The percentage of cells in S phase increased from 5.5±2% in control cells to 16.25±1.1% and 24.25±1.1% at 12 and 18 hours respectively in response to PDGF-BB (fig. 3B). While number of cells in G2/M phase increased from 9.25±0.75% at control to 16.00±1.3% at 12 hours and 21.00±1.6% at 18 hours. Treatment with *G. lutea* extract significantly inhibited entry of synchronized cells in to S and G2/M phase in response to PDGF-BB as seen in figure 3A and B.

3.3 PDGF-BB Activates ERK1/2-nitric Oxide Axis
PDGF-BB treatment time dependently increased the activation of ERK1/2 which was measured as increase in Threonine 202 and Tyrosine 204 phosphorylation of ERK1 and dual phosphorylation of ERK2 at Threonine 185 and Tyrosine 187 (fig. 4A). ERK1/2 activation was seen from one minute onwards and was observed till 30 minutes. PDGF treatment also increased levels of intracellular nitric oxide (NO) as early as 30 seconds (fig. 4B&C). Early increase in nitric oxide coincided with Akt mediated Ser 1177 phosphorylation of endothelial isoform of nitric oxide synthase (eNOS) (fig. 4D). This residue in eNOS activates the NO synthase activity [21]. An increase in the expression of iNOS was also seen in response to PDGF from 30 minute onwards (fig. 4E). Inhibition of both ERK1/2 and nitric oxide synthases via 328000 and L-

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**Figure 3. Cell cycle analysis of RASMCs.** A) Representative flow cytogram depicting cells in various stages of cell cycle upon treatment with PDGF-BB (20 ng/ml) in presence and absence of *G. lutea* extract, and B) Bar graph summarizing data for four independent experiments. ***P<0.001 versus control, †††<0.001 versus PDGF treatment.

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svertiamarin, and CID 5281636 for gentisin. The search parameters for docking analysis were as follows: Grid parameters: number of points, 62 × 62 × 62, spacing: 0.375 Å (Rigid docking), 1.0 Å (Blind docking), number of runs: 100, initial population size: 150, maximum number of evaluations: 2500000, maximum number of generations: 27000 and default values were used for all the other parameters.
Figure 4. Effect of PDGF-BB (20 ng/ml) on ERK1/2 and NO signaling. A) Time course of ERK1/2 activation in response to PDGF, B&C) Time course of generation of intracellular nitric oxide in response to PDGF, D) Activation of eNOS through phosphorylation of Ser1177 residue in response to PDGF and E) PDGF-induced expression of iNOS. Bar graphs summarize data for a minimum of four independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001 versus control.
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NAME respectively blocked PDGF-BB induced proliferation (fig. 5A&B). We then determined the effect of root extract on PDGF-BB induced cellular NO. The *G. lutea* root extract, completely blocked cellular NO induced by PDGF-BB (fig. 5C).

### 3.4 *G. lutea* Extract Blocks PDGF-BB Induced Cell Signaling

We then sought to determine the intracellular signaling affected by the root extract. The aqueous extract failed to block PDGF induced PDGF receptor tyrosine auto-phosphorylation at Tyr 751 (fig. 6A) suggesting that its mode of action is down-stream of the receptor. However, it blocked PDGF-BB induced ERK1/2 activation (fig. 6B). Consequently the phosphorylation of IKK-α a downstream target of ERK1/2 was also blocked by the extract (fig. 6C). Since IKK-NFκB axis is directly involved in activating transcription of iNOS [22], we determined the effect of the extract on PDGF-BB induced expression of iNOS. Indeed the extract blocked PDGF induced expression of iNOS (fig. 6D&E). Among the cell cycle regulators, the *G. lutea* extract decreased PDGF-BB induced expression of PCNA and cyclin D1 (fig. 6D&E).

### 3.5 Docking Analysis with Extract Components

Since MEK1 is the known dual specificity kinase which phosphorylates the threonine and tyrosine residues in the activation loopes of ERK1 and ERK2 in order to activate them [23,24], we set out to determine if any of the extract components interact with MEK1 through docking analysis. Due to lack of crystal structure for MEK2 and ERK1 we could not perform the analysis on these molecules. Using the Lamarckian algorithm of the AutoDock4 program blind and rigid docking of the extract constituents were done with x-ray structure of human mitogen-activated protein kinase kinase1 (MEK1) as described in methods. It should be noted that human MEK1 shares considerable homology with rat MEK1. Figure 7A depicts complexes of known inhibitors K252a and U1026 as well as isovitexin with MEK1. Among the components tested the best binding energies were predicted for isovitexin as seen in table 1. Isovitexin was found to bind to similar sites as K252a (for PDB id: 3EQF) and U1026 (PDB id: 3EQH) in MEK1 (fig. 7A). Residues which were found to be in close contact with isovitexin in 3EQF were Lys97, Leu115, Leu118, Val127, Met143, Glu144, His145, Met146, Leu197 Cys207, Asp208, Phe209, Gly210 and Val211. Among these, isovitexin exhibits propensity of hydrogen bonding with Lys97, Met146, Asp208 and Val211. Residues which were in close contact with isovitexin in 3EQH were Lys97, Leu118, Ile141, Glu144, His145, Met146, Leu197 Cys207, Asp208, Phe209, Gly210 and Val211. Among these, isovitexin exhibits possibility of hydrogen bonding with Val211. We then set out to determine experimentally whether purified isovitexin indeed blocks PDGF-BB induced signaling and proliferation in primary cultures of RASMCs. As seen in figure 7B, isovitexin at varying concentrations successfullly blocked PDGF-induced ERK1/2 activation and entry of cells in to S-Phase. Effect of isovitexin on PDGF-induced proliferation was also confirmed via alamar blue assay (fig. 7C).

### Discussion

Epidemiological studies demonstrate an inverse correlation between intake of dietary polyphenols and progression of chronic
Figure 6. Effect of *G. lutea* extract on PDGF-induced cell signaling in RASMCs. A) PDGFR-β phosphorylation, B) ERK1/2 activation, C) Phosphorylation of IKKα and D&E) Representative blot and bar graph indicating expression of cyclin D1, PCNA and iNOS for a minimum of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 versus control and {**P<0.01 and {***P<0.001 versus PDGF treatment.

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Proliferation and migration of VSMCs in response to PDGF triggers intimal thickening post-angioplasty [2]. Use of PDGF receptor antagonists blocks both in vitro proliferation of VSMCs and atherosclerosis in animal models [2,26]. In the present study, we demonstrate that aqueous extract of G lutea roots effectively blocks PDGF-BB induced cell cycle progression by interfering with ERK1/2-iNOS signaling (as summarized in fig. 8). These effects of the extract were neither due to cytotoxicity nor due to apoptosis.

Entry and progression of cells through different stages of cell cycle is a tightly orchestrated process. It involves sequential activation and expression of regulatory proteins such as cyclins, cyclin dependent kinases (CDKs), CDK inhibitors, p53 and pRb [27,28]. Activation of CDK2 and CDK1 in complex with cyclin D1 mediates progression of cells from G0/G1 to S phase. CDK2 in turn is also involved in phosphorylation of pRb and accumulation of proliferating cell nuclear antigen (PCNA) [29]. Phosphorylation of pRb is also mediated by ERK1/2, wherein activated ERK1/2 directly phosphorylates pRb at Ser780 and Ser795 residues [30]. This event precedes cyclin D1 expression and is necessary for the release of transcription factors in order to promote DNA synthesis. We observed an increase in ERK1/2 phosphorylation upon PDGF-BB treatment, which was blocked by the G lutea extract. Consequently, the extract also blocked PDGF-BB induced expression of cyclin D1 and PCNA. However, the extract failed to block PDGF-BB induced PDGF-receptor tyrosine phosphorylation, thereby indicating that the site of action for the extract and its constituents is downstream of the receptor. Effects of ERK1/2 activation on cell cycle vary depending upon the type of cells involved. For instance, in fibroblasts ERK1/2 activation leads to G0 to S phase entry via cyclin D1 expression, while in epithelial cells it is required for G0 to M phase entry through cyclinB1 and c-fos [31]. Although we did not look for effect of G lutea extract on cyclinB1 and c-fos expression, given that the extract effectively blocks ERK1/2 activation we believe, it utilizes the same mechanism for blocking G2 to M phase entry of cells in response to PDGF-BB.

In the present study we also observed that PDGF-BB induced production of intracellular NO was significantly blocked by the G lutea extract. The exact effect of nitric oxide on cell cycle depends upon its local concentration. At low concentrations it promotes cell proliferation while at higher doses it induces cytostatic and/or apoptotic effects [32,33]. Since in this study NOS inhibitor L-NAME blocked PDGF-BB induced cell proliferation we believe that intracellular NO in response to PDGF-BB promotes smooth muscle cell proliferation. Although the molecular mechanisms by which NO promotes cell proliferation are not well understood, recent studies have highlighted its ability to inhibit apoptosis by preventing the DNA binding activity of p53 [34] and through S-nitrosylation of caspases [35,36].

The major sources of NO in cells are the various isoforms of NO synthases (NOS) such as iNOS (inducible isoform), nNOS (neuronal isoform) and eNOS (endothelial isoform) [37,38]. Among these eNOS and nNOS are constitutively expressed while iNOS expression is transcriptionally induced in a MAPK-dependent manner upon agonist activation [37]. ERK1/2 activates the IKK-NFκB axis in order to activate transcription of iNOS [22]. We observed that PDGF-BB induced activation of ERK1/2 in vascular smooth muscle cells was responsible for increased expression of iNOS. Intriguingly, abundant expression and activation of ERK1/2 is seen in atheroma samples derived from hyperlipidemic mice [39]. Similarly, increased iNOS expression and activity is reported in VSMCs obtained from diabetic rats [40]. Involvement of iNOS in pathology of atherosclerosis is also supported by the fact that its deficiency reduces atherosclerotic plaques [41] and neo-intimal thickening [42] in rodent models. These reductions are either due to decreased LDL oxidation [41] or due to G0/G1 arrest of iNOS deficient VSMCs [43]. We also observed G0/G1 arrest of VSMCs in presence of G lutea extract. Additionally the extract blocked PDGF-BB induced iNOS expression. Given these observations, it is tempting to speculate that the active principles of Gentiana lutea root extract will prevent neo-intimal thickening by blocking ERK1/2-iNOS activation.

MEK1/2 is the only known upstream kinase which can activate ERK1/2 [23]. We hence carried out docking analysis of identified constituents of the G lutea extract with the available crystal structures of human MEK1. Two crystal structures of MEK1 as ternary complexes with known competitive inhibitor K252a (PDBID: 3EQF) and allosteric inhibitor U1026 (PDBID: 3EQH) were used for the analysis [20]. It was observed that among the individual components, isovitexin was seen to dock to the same binding pocket in MEK1 as K252a and U1026. It should be noted that inhibitor binding site in MEK1 is adjacent to the Mg2+/ATP binding site and these two sites are physically separated by the side

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### Table 1. Predicted binding energies for G lutea constituents and known MEK1. inhibitors.

| Ligand          | Best predicted binding energy in Kcal/mol for 3EQF | Best predicted binding energy in Kcal/mol for 3EQH |
|-----------------|--------------------------------------------------|--------------------------------------------------|
| Amarogentin     | −6.39                                            | −1.83                                            |
| Gentiotricoside | −4.18                                            | −4.02                                            |
| Gentisin        | −5.76                                            | −5.89                                            |
| Isovitexin      | −8.16                                            | −7.38                                            |
| Swertiamarin    | −3.8                                             | −2.94                                            |
| K252a           | −10.41                                           |                                                  |
| U1026           | −8.85                                            | −10.79                                           |

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chains of Lys^{97} and Met^{146}, while the other neighboring amino acids Leu^{118}, Ileu^{141}, and Phe^{209} form a deep hydrophobic pocket within the core kinase domain extending from amino acid 53 to 369^{[20,44]}. Binding of K252a induces a conformational change due to hydrogen bonding with Glu^{144} and Met^{146} and thus affects catalytic activity of MEK1^{[20]}. On the other hand, although the exact molecular mechanism of allosteric inhibition of MEK1 is still unclear, crystal structure studies have predicted that binding of allosteric inhibitors adjacent to active site induces and stabilizes a basal and naturally occurring inactive form of MEK1^{[44]}. Conformational change induced by these allosteric inhibitors breaks a conserved ion pair formation between Glu^{144} and Lys^{97}. 

Figure 8. Summary figure depicting the pathway blocked by *G. lutea* extract to prevent PDGF-induced RASMC proliferation.
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in the ATP binding site of the enzyme. Among the G lutea components analyzed, isovitexin was found to dock to the same site as K232a and U1026 and demonstrated a propensity of hydrogen bonding with catalytic residues Lys97, Met46, Aap208, or Val251. Furthermore in cell culture experiment, isovitexin successfully blocked PDGF induced proliferation of RASMCs. 

Plant derived polyphenols and carotenoids delay the onset and progression of atherosclerosis by modulating serum lipid levels and by reducing oxidative stress and lipid peroxidation. Some of these also block VSMC proliferation such as ellagic acid [45], Naringenin [46], lutelone [47] and luettein [48]. Binding of PDGF-BB to its cognate receptor followed by auto-phosphorylation, triggers generation of intracellular reactive oxygen species (ROS) [49]. ROS then reversibly inactivate protein tyrosine phosphatases (PTPase), the known negative regulators of PDGF receptor signaling [50]. Alternatively these free radicals activate ERK1/2 pathway by activating ERK1/2 phosphorylating kinases such as Src, Pyk2 and Syk [51]. Since polyphenolic content of an extract imparts greater antioxidant capacity and given that both water and ethanol extracts of Gentiana lutea roots have high polyphenol content as already reported by us [15], it is likely that the extract through its radical scavenging activity is directly modulating PDGF induced ERK1/2 activation. Additionally others have shown that polyphenols obtained from numerous other Gentiana species act as antioxidants and effectively block lipid peroxidation [52–55]. As the receptor tyrosine phosphorylation was unaltered by the extract (Fig. 5A), we believe that the anti-oxidant effect of the extract would lie downstream of the receptor but upstream of ERK1/2 activation. Thus antioxidants such as isovitexin [56] and gentisin present in the water extract may inactivate these kinases thereby blocking ERK1/2 activation. This however needs to be tested in our future studies.

Diabetes increases the risk of atherosclerosis. Hyperglycemia observed in these patients favors glucose metabolism through polyol pathway and induces formation of advanced glycation end products (AGEs) [57]. These pathways along with pro-inflammatory cytokines and growth factors promote diabetic micro- and macro-vascular complications. Aldose reductase (AR) the rate limiting enzyme in polyol pathway, upon over-expression, accelerates atherosclerotic lesions in apoE−/− mice [58]. Furthermore, its inhibition successfully blocks bFGF, angiotsentin II, AGEs, hyperglycemia and PDGF-AB induced proliferation of vascular smooth muscle cells [59–61]. These effects of AR inhibitors are through their ability to attenuate ERK1/2-NFκB signaling leading to cell cycle arrest at G1 phase [61]. Intriguingly, the methanol and ether extracts of Gentiana lutea roots were recently shown by our co-authors to inhibit rat and human isoforms of AR, thereby preventing sorbitol accumulation under high glucose conditions [14]. Molecular docking studies identified amarogentin as a potential inhibitor of AR [14]. The present study identifies isovitexin as yet another anti-atherosclerotic agent in aqueous extract of Gentiana lutea roots. Similar studies on cancer cell lines have demonstrated the anti-tumor activity of root extracts of Gentiana triflora [62], but to the best of our knowledge this is the first study demonstrating anti-proliferative effects of Gentiana lutea extract on vascular smooth muscle cells. Although nothing is reported regarding the anti-diabetic effects of Gentiana lutea, isoorientin obtained from another Gentianaceae member, Gentiana oliveti, exhibits hypoglycemic and anti-hyperlipidemic effects in streptozotocin induced diabetic rats [63]. Thus it becomes imperative to study even the hypoglycemic effects of Gentiana lutea extracts in future. Recent work by Nastasjevic et.al., has shown that gentiopicroside, isovitexin and amarogentin present in the root extracts of Gentiana lutea are effective inhibitors of myeloperoxidase [64]. It should be noted that myeloperoxidase is an early marker of vascular dysfunction and plays a crucial role in oxidative modification of LDL and hence in pathogenesis of atherosclerosis [65]. Given these observations and our current study we propose that Gentiana extracts and their constituents can provide drug leads to come up with effective anti-atherosclerosis therapy in future.

Conclusion

In conclusion, aqueous root extract of Gentiana lutea and its constituent isovitexin effectively blocks PDGF-BB induced proliferation of rat aortic smooth muscle cells by blocking ERK1/2 activation and consequent iNOS expression.

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Author Contributions

Conceived and designed the experiments: MD GJ. Performed the experiments: RR URP BN AT. Analyzed the data: RK MD GJ AT. Contributed reagents/materials/analysis tools: BN GJ. Wrote the paper: MD.

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