



Research article

Chungtaejeon (CTJ) inhibits adhesion and migration of VSMC through cytoskeletal remodeling pathway

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ABSTRACT

Introduction: Vascular remodeling is crucial for the progression of vascular disease such as atherosclerosis. We utilize the *in vitro* experimental model of atherosclerosis to elucidate the activity of Chungtaejeon (CTJ), a Korean fermented tea on adhesion and migration of human aortic vascular smooth muscle cells (HASMC).

Materials and methods: Various *in vitro* assays such as cell viability, cell adhesion, Western blot, immunofluorescence, were carried out on HASMC to explore pathway associated with cytoskeletal remodeling during the progression of atherosclerosis.

Results: In result, CTJ significantly inhibited adhesion of HASMC as revealed by collagen assay. Similarly, CTJ inhibited the β 1-integrin protein expression as well as FAK phosphorylation. Treatment of CTJ also inhibited stress fiber formation. Likewise, adherence of cells on collagen optimally increased the expression of both RhoA and Cdc42, however, treatment of CTJ dose dependently decreased their expression. The lysophosphatidic acid stimulation of HASMC rapidly increased the level of phosphorylated forms of MLC20 within 15 min, followed by an extended level of MLC20 phosphorylation. The treatment of CTJ at a dose of 50, 100 and 250 μ g/ml remarkably reduced the diphosphorylated form while decreased the level of monophosphorylated form of MLC20.

Conclusions: Our results suggests that, with further validation CTJ could be a promising herbal resource for prevention of atherosclerosis.

1. Introduction

The process of vascular remodeling leading to progression of atherosclerosis follows complex mechanism, incorporating participation of macrophages for vascular inflammation, degradation of extracellular matrix (ECM), role of growth factor such as platelet derived growth factor (PDGF), cytokines such as tumor necrosis factor that facilitate proliferation and migration of vascular smooth muscle cells (VSMC), and reaction oxygen species that results in endothelial dysfunction [1–4]. Apart from cellular and molecular composition, vascular remodeling also involves alteration in vasculature structure, including its size, shape. These alterations are not only observed in atherosclerosis but also in diverse cardiovascular disorders such as heart failure and hypertension. Dynamic changes

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in the endothelial cells, fibroblasts, VSMC, pericytes or other blood vessel cells underlie remodeling. Furthermore, immune cells such as macrophages and lymphocytes, may infiltrate vessels and initiate inflammatory signaling that facilitates progression of vascular inflammation. Apart from inflammation, these immune cells also contribute to VSMC cell proliferation, apoptosis, migration, and extracellular matrix reorganization, which as key for vascular remodeling [5,6]. ECM provides the milieu for VSMC to be involved in the adhesive, proliferative, and migratory events that collectively results in development of atherosclerotic lesion [7,8]. Various transcriptomics are involved in vascular remodeling or different stage of atherosclerosis development. Integrins are transmembrane receptors in various cells including VSMC and they consist of α and β subunits. Upon ligation and/or clustering of integrin, focal adhesion kinase (FAK) becomes activated through phosphorylation of tyrosine moiety followed by interaction with multiple targets. These interactions provide cellular signals that have been implicated in cell migration, proliferation, growth factor signaling, and apoptosis [9]. Cell adhesion to ECM components, such as collagen, laminin, and fibronectin, is predominantly mediated by integrin receptors [10]. Rho-family GTPases [example RhoA (Ras homolog family member A (RhoA) and cell division kinase 42 (cdc42)] are considered as cellular molecular switches that govern the assembly and disassembly of actin cytoskeletal structures (stress fibers; lamellipodia and filopodia) and also plays role as a molecular framework to support cell motility [11]. It is established that the myosin light chain (MLC20) phosphorylation activates the actin-stimulated ATPase of myosin-II and facilitate binding of myosin to actin followed by contraction of the actin-cytoskeleton to provide necessary force for migration [12]. Chungtaejeon (CTJ) is a commonly consumed Korean tea prepared by processing that involved fermentation of *Camellia sinensis* [13]. *Camellia sinensis* is very popular plant as tea prepared from its leaves is consumed globally and it is also well-known for its high polyphenol content [14] that possess different health promoting effects like anti-mutagenic, anti-diabetic, anti-bacterial and anti-inflammatory activities [15]. Consumption of tea has been practicing since antiquity for the management of diabetes [16], obesity [17], and hypertension [18], which are the key risk factors of atherosclerosis. We have already demonstrated that the inhibitory effect of CTJ on PDGF induced chemotaxis of human aortic smooth muscle cells (HASMC) in our previous study [19] and preventive effect of CTJ on high fat atherogenic diet fed rat model [13]. As CTJ is produce by fermentation of *Camellia sinensis*, it is important to know the chemical constituent *Camellia sinensis* as well as pre-dominant microorganism involved in the fermentation process of CTJ that may exert potential biological activities. In this regard DG Moon 2020 has identified that primary species involved in fermentation process was *Pantoea* sp. and *Klebsiella oxytoca*. Similarly, the phylogenetic analysis showed that dominant species in CTJ was γ -proteobacteria. The main chemical constituent of *Camellia sinensis* are catechin, caffeine, gallic acid, epicatechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate [20]. The chemicals are well known to exert various biological activity such as anti-cancer, anti-angiogenic, hepatoprotective, and renoprotective, [21,22]. The objective of our study was to investigate the effect of CTJ on the collagen induced adhesion of HASMC and various receptor, protein or signaling pathway involved on it.

2. Materials and methods

2.1. Materials

Cell culture media (DMEM), phosphate buffer saline, fetal bovine serum (FBS), penicillin and streptomycin mix, trypsin, trypan blue, bovine serum albumin, formalin, Triton X-100, fluorescein-5-isothiocyanate (FITC)-conjugated phalloidin, PDGF, lysophosphatidic acid (LPA), trichloroacetic acid (TCA), dithiothreitol (DTT), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxy-fluorescein acetoxymethyl ester (BCECF/AM), Collagen type I, hematoxylin and eosin (H/E) staining solution, and antibody to myosin light chain (MLC20) were obtained from Sigma. Antibodies of focal adhesion kinase (FAK) and phospho-FAK (pFAK) were obtained from cell signaling; β 1-integrin was obtained from Millipore; RhoA, Cdc42 and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were obtained from Santa Cruz. All other chemical reagents were purchased from Sigma Aldrich and were of analytical grade.

2.2. Processing and extraction of Chungtaejeon

The processing of CTJ was performed as described in our previous study [13]. 112 g of the lyophilized tea leaves was extracted with 3300 ml of distilled water for the period of 3 h followed by evaporation of extract and freeze-drying to collect final powder form of CTJ. We collected 12 % of the extract as final product and stored it in 4 °C until further use for experiments. For each set of experiments, various concentration of CTJ was freshly prepared by diluting the CTJ extract.

2.3. Cell culture

HASMC were obtained from ATCC, USA (PCS-100-102) and tested for the presence for mycoplasma (ThermoFisher, A55124). After confirmation of mycoplasma negative, cells were cultured in DMEM with 10 % fetal bovine serum (FBS) and 1 % antibiotic mix in a standard cell culture incubator. For each set of assays, early passages (p1-p10) of HASMC were grown up to 80 % confluence and in a typical experiment, the cells were quiescent in 0.4 % FBS for 24 h to synchronize their cell cycle phase.

2.4. Cell viability assay

HASMC viability was assessed using an MTT colorimetric assay as described in our previous study [23]. Cells were plated in 96 well plates at a density of 1×10^4 cells/well and after quiescent treated with various concentrations of CTJ for 24 h. MTT stock of 5 mg/ml

was added 10 μl to each well of 96 well plate and incubated. Four hours later, the formazan crystals formed by enzymatic activity of live cells were solubilized in 100 μl of DMSO and the chromophore was measured at 540 nm.

2.5. Adhesion assay

Adhesion assay was carried as describe previously [24]. Ninety-six well plates were coated overnight with type I collagen and then blocked with 10 % bovine serum albumin (BSA) for 1 h. Suspended HASMC, labeled with 10 $\mu\text{g}/\text{ml}$ of BCECF/AM were washed and then resuspended in media at 1×10^5 cells/ml. Then, cells were incubated with indicated concentrations of CTJ for an hour followed by seeding into the 96-well plates and additional 4-h incubation for cell adhesion. After PBS washing, fluorescence was measured at excitation 485 and emission 535 nm. For morphological analysis, the cells were stained with H/E and photographs were taken.

2.6. Western blot

Western blot was carried out as mentioned previously [25]. Briefly, the cells, pretreated with indicated concentrations of CTJ for 1 h, were plated on collagen coated plates for 4 h. Then, protein was extracted, and equal amount of protein were loaded for electrophoresis followed by development of blot for pFAK, FAK, $\beta 1$ -integrin, RhoA, Cdc42 and GAPDH. Finally, the band of these proteins were detected using picotect™ Western blot chemiluminescent substrate.

2.7. Immunofluorescence

Immunofluorescence microscopy was performed as described previously [24]. Briefly, HASMC, pretreated with various concentration of CTJ for 1 h were plated on a coverslip pre-coated with collagen and incubated for 4 h. Cells were washed with chilled PBS, fixed with 5 % formalin, permeabilized with 0.2 % Triton X-100, and blocked with BSA followed by incubation with FITC-conjugated phalloidin (1:200 dilution) for an hour at room temperature. Coverslips were mounted with a mounting media and the fluorescence images were taken with a fluorescence microscope at 400 \times magnification.

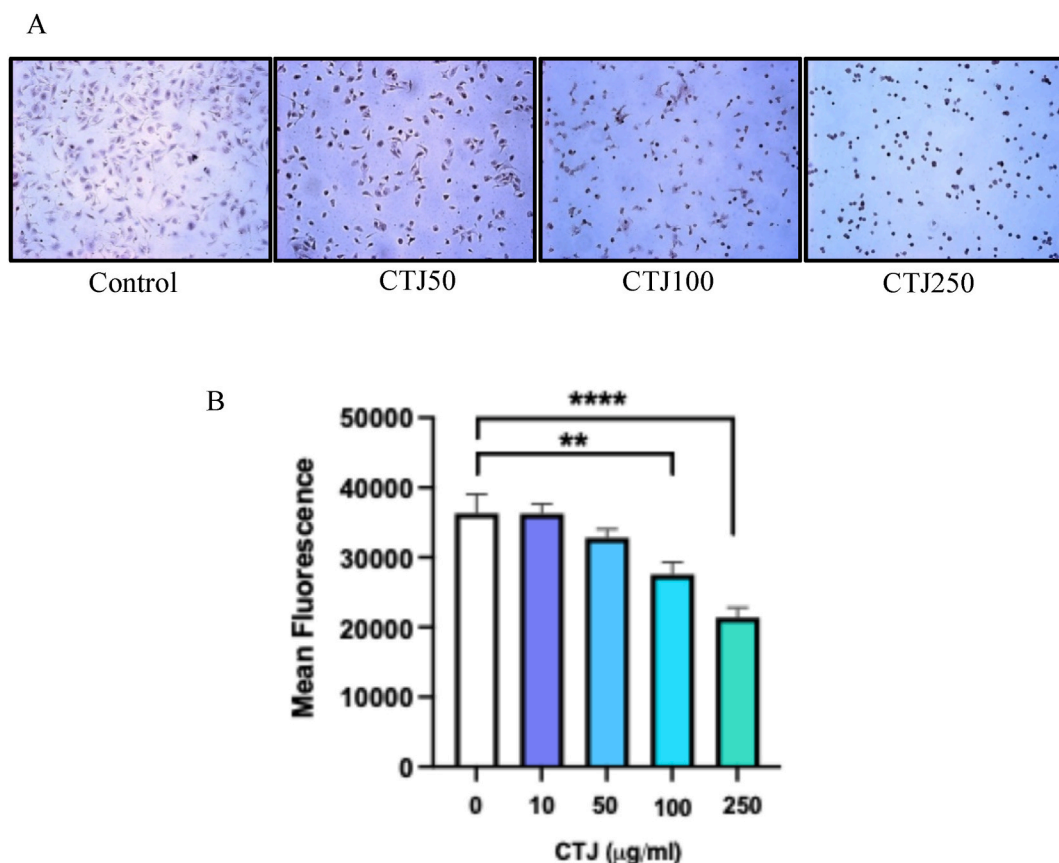


Fig. 1. Effect of CTJ on adhesion of HASMC. a) Morphological analysis of HASMC stained with hematoxylin and eosin (H/E) after allowing HASMC to adhere on collagen for 4 h, b) Fluorescence intensity quantification of HASMC labeled with BCECF/AM and pretreated with indicated concentrations of CTJ. $**p < 0.01$ and $****p < 0.0001$ vs control (sample untreated).

2.8. Urea-glycerol polyacrylamide gel electrophoresis (urea-glycerol PAGE)

HASMC, starved for 24 h, were pre-treated with indicated dose of CTJ for 1 h, followed by stimulation with PDGF (20 ng/ml) or LPA (30 μ M) for 0–4 h. Afterwards, ice-cold 10 % TCA containing 2 mM DTT was added to the cells and scrapped off the dishes followed by washing with chilled acetone containing DTT (10 mM). The cell extract was suspended in urea sample buffer. Unphosphorylated, monophosphorylated and di-phosphorylated forms of MLC20 were separated using urea/glycerol gel electrophoresis as mentioned previously [24].

2.9. Statistical analyses

The data are represented as the mean \pm standard error mean (SEM) of three biological replicates. Statistical significance between the groups was determined by a one-way ANOVA followed by Tukey multi-comparison test. Values of * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ were considered significant.

3. Results

3.1. Effect of CTJ on viability of HASMC

We performed the cell viability assay to confirm the safe dose range of CTJ on HASMC using MTT colorimetric assay. The treatment of CTJ up to the concentration of 250 μ g/ml for 24 h did not affect the viability of HASMC. However, CTJ at the concentration of 500 μ g/ml possessed cytotoxicity (data not shown). Therefore, the subsequent experiment was carried with CTJ treatment not exceeding 250 μ g/ml.

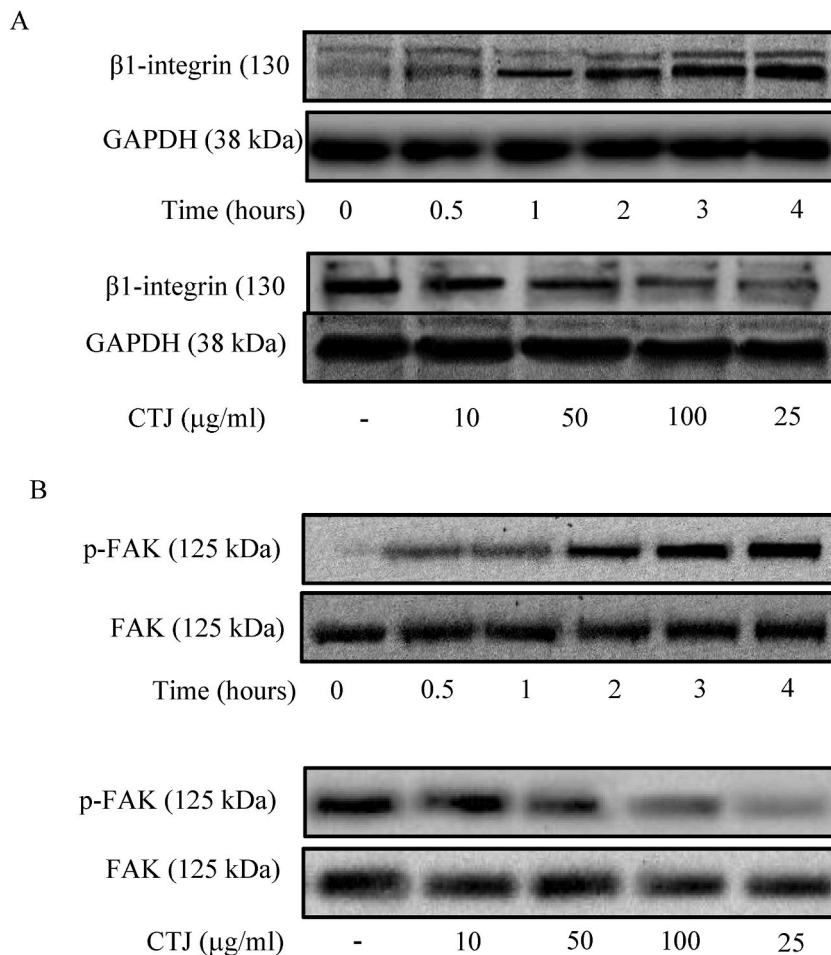


Fig. 2. Effect of CTJ on expression of β 1-integrin and phosphorylation of focal adhesion kinase (FAK) HASMC, pre-treated with CTJ for 1 h, were seeded on previously collagen coated plates and allowed to adhere for indicated period. Then, the cells were lysed, and Western blot was performed. A) β 1-integrin expression, and B) Phosphorylation of FAK.

3.2. Effect of CTJ on adhesion of HASMC

The effect of CTJ on adhesion of HASMC on collagen was evaluated by using a fluorimeter and H/E staining as shown in Fig. 1. The pretreatment of CTJ at concentrations of 50, 100 and 250 $\mu\text{g/ml}$ dose dependently inhibited the adhesion of HASMC on collagen. Under a light microscope, it was observed that HASMC fully spread and adhere to collagen on the plate. However, treatment of CTJ changes the morphology of HASMC to round shape (Fig. 1A). Similarly, the fluorescence quantification of 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxy-fluorescein acetoxymethyl ester (BCECF/AM) labeled HASMC treated with CTJ also showed dose dependent decrease in mean fluorescence intensity suggesting inhibition of HASMC adherence on collagen (Fig. 1B)

3.3. Effect of CTJ on $\beta 1$ -integrin expression and FAK activation

Fig. 2 shows the effect of CTJ on $\beta 1$ -integrin expression (Fig. 2A) and FAK activation (Fig. 2B). Both $\beta 1$ -integrin expression and FAK phosphorylation were not obviously observed in suspended HASMC at time 0 h, but their expression was increased when the cells adhered to collagen in a time-dependent manner. The adherence of cells on collagen for 4 h caused the optimum expression of $\beta 1$ -integrin and FAK phosphorylation. The treatment of CTJ inhibited the $\beta 1$ -integrin expression and FAK phosphorylation; however, GAPDH and total FAK remained unchanged.

3.4. Effect of CTJ on stress fiber formation

Fluorescence microscopy was carried to determine the effect of collagen on actin cytoskeleton reorganization. As shown in Fig. 3, collagen caused well spreading of the cells and typical long stress fibers running across the HASMC body were observed. However, treatment of CTJ significantly inhibited the stress fiber formation. Morphologically, the cells in CTJ treatment group were round compared to media control group.

Immunofluorescence images of HASMC treated with various dose of CTJ. Cells were stained with fluorescein-5-isothiocyanate (FITC)-conjugated phalloidin the images were taken with fluorescence microscope at 400 \times magnification.

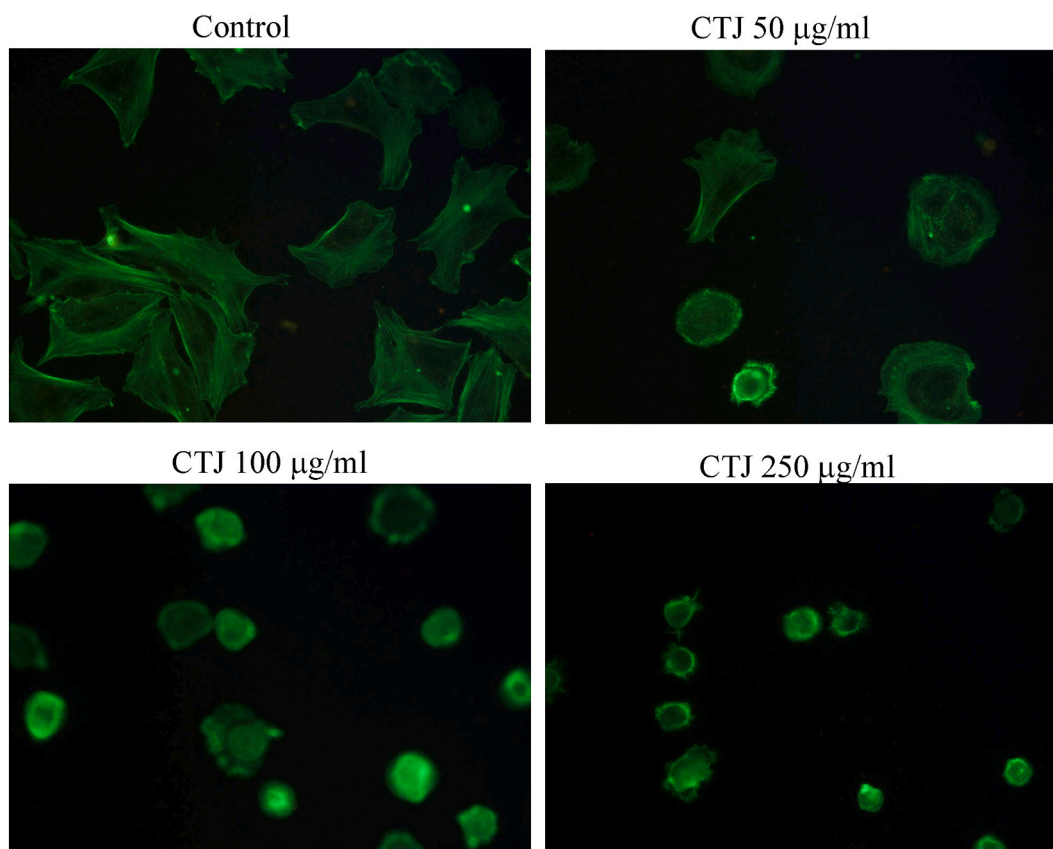


Fig. 3. Effect of CTJ on actin cytoskeleton organization.

3.5. Effect of CTJ on Rho family proteins

To investigate whether Rho family proteins are associated with reduction in the cell migration by CTJ, the expression of RhoA and Cdc42 protein were analyzed by immunoblot. The adherence of cells on collagen for 4 h optimally increased the expression of both RhoA and Cdc42. However, treatment of CTJ dose dependently decreased their protein expression (Fig. 4).

HASMC pre-treated with CTJ for 1 h, were seeded on previously collagen coated plates and allowed to adhere for indicated period. Then, the cells were lysed, and Western blot was performed for RhoA and Cdc42 protein expression. GAPDH was used as housekeeping protein.

3.6. Effect of CTJ on MLC20 phosphorylation

Stimulation of HASMC with 30 μ M of LPA results in notable raise in the protein expression of both monophosphorylated and diphosphorylated forms of MLC20 within 15 min, followed by a prolonged increase in MLC20 phosphorylation for up to 4 h. Comparatively, prolonged increase of monophosphorylated form was observed (up to 240 min) than diphosphorylated form (up to 30 min). Interestingly, stimulation of HASMC with 20 ng/ml of PDGF did not results in MLC20 phosphorylation (second lane of Fig. 5A). The treatment of CTJ at 50, 100 and 250 μ g/ml significantly inhibited the diphosphorylated form while decreased the level of monophosphophorylated form of MLC20 (Fig. 5B).

4. Discussion

The removal of vascular endothelium after percutaneous transluminal coronary angioplasty (PTCA) initiates process of VSMC migration to the tunica intima which is closely associated with high collagen production [26,27]. Previous studies have revealed that in restenotic tissue composition, maximum portion is ECM while the cellular portion is very few highlighting that the ECM could be more crucial for the formation of restenosis [28]. We have already studied the effect of CTJ on the growth factors induced proliferation, expression of MMPs and their roles in migration [13,19]. In this study, we attempted to demonstrate the effect of CTJ on adhesion induced by collagen, a major protein in ECM. HASMC is seeded on type I collagen coated plates formed firm adhesion within 4 h, suggesting the involvement of integrin receptors in binding. Moreover, the suspended cells did not express β 1-integrin while its expression was increased with time. The treatment of CTJ inhibited the adhesion of HASMC on collagen because of decrease in β 1-integrin protein expression as revealed by immunoblot. Integrin β 1, predominantly found in both VSMC *in vivo* and in cultured VSMC, is associated with in increased proliferative and migratory events of VSMCs [29]. Integrins are thought to recruit intracellular signaling molecules such as FAK to their cytoplasmic domains. FAK, a cytosolic tyrosine kinase, is phosphorylated in response to β 1-integrin as well as growth factors and mitogenic neuropeptides that allows cell adhesion with ECM [29]. Interestingly, in our study, there was no phosphorylation of FAK in suspended cells while the phosphorylation of FAK was increased with time. The phosphorylation of FAK was correlated with the β 1-integrin expression which suggest that the clustering of β 1-integrin during cell adhesion causes phosphorylation of FAK. Upon FAK activation, RhoA and Cdc42 can be transiently stimulated followed by formation of contraction-dependent stress fibers and filopodia respectively [30]. In our study, staining of F-actin with phalloidin showed characteristics long stress fibers running across the HASMC, observed after 4 h of adhesion on collagen. In contrast, CTJ treatment to HASMC inhibited the formation of stress fibers resulting in the cells morphology to rounded possibly because of inhibition of RhoA and Cdc42 expression. After the cell is widespread over the ECM, the contractile forces needed to cell motility are transmitted through the integrin-cytoskeleton interaction. The rigidity of the substrate directly influences how strongly the integrin is coupled to the cytoskeleton. The robust this coupling, the more coherently force can be transduced through the migrating cell, demonstrating the importance of the ECM-integrin-cytoskeleton linkage [31]. During cell motility, phosphorylation of MLC20 is noticed at the rear of cells and within lamellipodia, proposing that MLC20 phosphorylation is crucial for cell motility. LPA is a bioactive phospholipid produce by activated platelet (such as those stimulated by thrombin), and it induces the proliferation of VSMC and stimulates the migration/invasion of various cells [32]. We have demonstrated in our study that LPA, but not PDGF, increases the MLC20 phosphorylation indicating that LPA induced HASMC migration in our previous study [24]. The treatment of CTJ inhibited both monophosphorylated and diphosphorylated levels of MLC20. Chemical analysis of *C. sinensis* demonstrated the presence of tannins, flavonoids, glycosides, and volatile oils [14]. Previous study has reported that polyphenols from tea can inhibit the adhesion and migration of rat VSMC induced by ECM protein collagen and laminin by interfering the cell-ECM interaction [33]. In our study, we

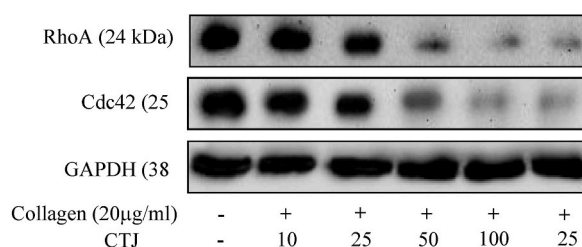


Fig. 4. Effect of CTJ on the expression of RhoA and Cdc42.

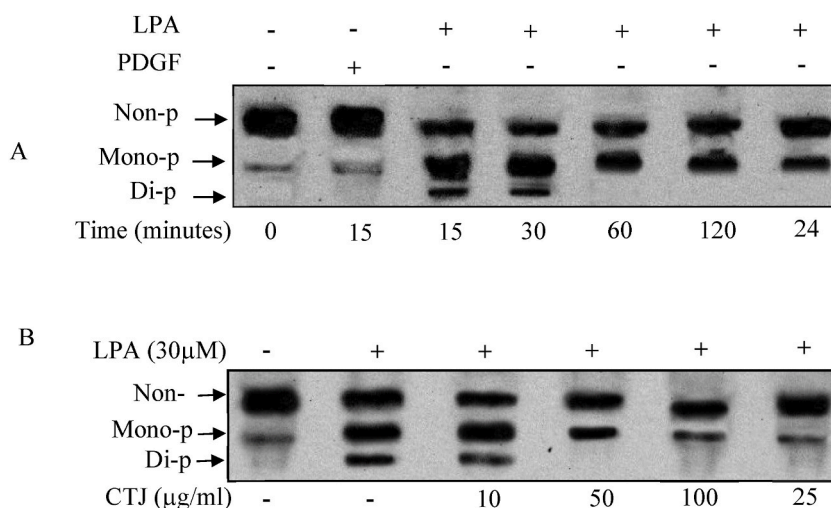


Fig. 5. Effect of CTJ on myosin light chain (MLC20) phosphorylation

A) HASMC were stimulated with LPA or platelet derived growth factor (PDGF) for indicated period and lysate were subjected to urea-glycerol electrophoresis. B) HASMC pretreated with CTJ, were stimulated with LPA for 15 min and then the effect of CTJ on MLC20 phosphorylation was determined.

observed the similar findings, and we also revealed the mechanism based on β 1-integrin, FAK, Cdc42, MLC20 protein expression. In summary, we provided here the evidence showing that CTJ inhibited HASMC adhesion to collagen by inhibiting immobilized collagen-induced β 1-integrin expression and FAK phosphorylation. In addition, CTJ inhibited cytoskeletal reorganization by inhibiting RhoA and Cdc42 expression. Thus, we speculate that CTJ could be a beneficial in the prevention of blood vessel disorder related with vascular remodeling such as atherosclerosis and restenosis after angioplasty. While our study showed promising benefit of CTJ against vascular remodeling *in vitro* there are certain limitation in the study. These limitations serve research platform for us and other research to further expand the study. First, our study model is entirely *in vitro* therefore our data should be validated using *in vivo* animal models of atherosclerosis. Second, we did Western blot to see the protein expression of various protein involved in vascular remodeling. There is scope of studying the gene expression for the same protein to validate the activity by both proteomics and transcriptomics. For specific mechanism, it would be interesting to choose top gene involved in vascular remodeling and created a mouse knockout model to study the potency of CTJ.

5. Conclusions

Taken together, our *in vitro* results suggests that CTJ could be a promising functional food with potential to inhibit HASMC adhesion to collagen by targeting β 1-integrin expression and FAK phosphorylation. In addition, the inhibiting of cytoskeletal reorganization by targeting RhoA and Cdc42 expression could be beneficial to control the vascular remodeling VSMCs adhesion and migration are critical events in disease such as atherosclerosis and restenosis after angioplasty. The pharmacological potential of CTJ must be further validated using pre-clinical animal models and advance techniques such as CRISPR/cas9 specific gene (related to vascular remodeling) knockout cells or mice, followed by human clinical trials to translate it from bench side to bed side.

Data and code availability

Data will be made available on request.

CRedit authorship contribution statement

Keshav Raj Paudel: Writing – review & editing, Writing – original draft, Validation, Software, Methodology, Investigation, Formal analysis. **Nisha Panth:** Writing – review & editing, Software, Formal analysis. **Dong Wook Kim:** Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Rajendra Karki:** Writing – review & editing, Writing – original draft, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Co-author Dr. Keshav Raj Paudel is currently serving as associate editor of Heliyon journal.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.heliyon.2024.e38508>.

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