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***trans*-Cinnamic acid, but not *p*-coumaric acid or methyl cinnamate, induces fibroblast migration through PKA- and p38-MAPK signalling pathways**

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**Declarations of interest:** none

***trans*-Cinnamic acid, but not *p*-coumaric acid or methyl cinnamate, induces fibroblast migration through PKA- and p38-MAPK signalling pathways**

**Abstract**

*Aim:* Hydroxycinnamic acids their derivatives have various pharmacological properties. The hydroxycinnamic acid derivatives, methyl cinnamate, *trans*-cinnamic, and *p*-coumaric acids have been the object of study in the treatment of skin wounds. However, it is unclear whether these derivatives exert a direct beneficial effect on fibroblast function. In this study, we evaluated the effects of methyl cinnamate, *trans*-cinnamic, and *p*-coumaric acids on fibroblast migration *in vitro*.

*Materials and methods:* NIH 3T3 and L929 fibroblast cell lines were exposed to each drug at several concentrations and the effect on cell viability, cell cycle, and extracellular matrix production were assessed by MTT assay, flow cytometry, and immunofluorescence staining, respectively. The effect on cell migration was examined using scratch assay.

*Results:* The results showed that hydroxycinnamic acid derivatives not affect cell viability, but increase fibroblast migration in the *in vitro* scratch-wound healing assay. They also induced an increase in S and G2/M phases accompanied by a decrease in the G0/G1 phase of the cell cycle. The cell proliferation inhibitor mitomycin C abolished the effect induced by *p*-coumaric acid and methyl cinnamate, indicating that only the *trans*-cinnamic acid stimulated migration. A transwell migration assay confirmed that *trans*-cinnamic acid-treated fibroblasts exhibited increased migration compared with untreated cells. *trans*-Cinnamic acid-induced fibroblast migration was decreased by PKA inhibitor and p38-MAPK inhibitor but not by JNK inhibitor. Additionally, *trans*-

cinnamic acid-treated fibroblasts showed an increase in the production of laminin and collagen type I.

*Conclusion:* Our study showed that *trans*-cinnamic acid improves fibroblast migration and modulates extracellular matrix synthesis, indicating its potential for accelerating the healing process.

**Keywords:** fibroblast; *trans*-cinnamic acid; methyl cinnamate; *p*-coumaric acid; wound healing

## 1. Introduction

Wound healing is a dynamic process involving complex biological and molecular events, which requires the participation of many types of cells, including macrophages, keratinocytes, endothelial cells, and fibroblasts [1]. During this process, fibroblasts play an important role by depositing extracellular matrix (ECM) [2], which guides angiogenesis [3] and supports the migration and proliferation of other cells that eventually form the scar [4]. Thus, drugs targeting fibroblast function may contribute to skin wound healing.

The increasing popularity of natural therapies has stimulated research into the use of plant-derived compounds for wound management [5, 6]. It is worth pointing out that medicinal plants containing phenolic compounds, such as the species *Achyrocline alata*, *Strychnos pseudoquina*, and *Grammatophyllum speciosum* have been used in traditional medicinal practices worldwide to treat skin wounds [7-9]. Indeed, phytochemicals have been used to treat wounds for years. In particular, hydroxycinnamic acid derivatives are an important group of compounds present in fruits and foods, including apples, pears, peas, beans, and tomatoes, that exhibit a wide spectrum of biological activities [10]. Recent studies have reported that hydroxycinnamic acids exert health-promoting effects such as reduction of blood glucose levels [11] and possess anti-microbial, anti-tumour, anti-inflammatory, and wound healing properties [12, 13].

Hydroxycinnamic acids derivatives such as *trans*-cinnamic acid, *p*-coumaric acid, and methyl cinnamate have been reported to possess antioxidant effects, an important characteristic for wound healing [6]. Moreover, it has been reported that *trans*-cinnamic acid ameliorates the UVA-induced dermatotoxic effects on human dermal fibroblasts and mouse skin [14]. In addition, *trans*-cinnamic acid also increases

the capability of phagocytic innate immune cells, a phenomenon important for the healing process [15]. Studies have also found that *p*-coumaric acid has free radical scavenging ability, anti-ulcer activity [16], and stimulates bone formation [17]. Previous studies have demonstrated the effect of methyl cinnamate on inducing nitric oxide, an important inducer of collagen deposition and stimulator of wound healing [18]. In addition, hydroxycinnamic acid derivatives also stimulates distinct intracellular signaling pathways such as protein kinase A (PKA), c-Jun N-terminal kinase (JNK) and mitogen-activated protein kinases (MAPK) [19], which are associated with multiple biological functions, including changes in the actin cytoskeleton, migration and extracellular matrix (ECM) production [20]. All properties listed above indicate the potential of hydroxycinnamic acid derivatives in promoting wound healing. However, the effects of *trans*-cinnamic acid, methyl cinnamate, and *p*-coumaric acid on fibroblast functions related to healing, such as migration, have not been demonstrated previously.

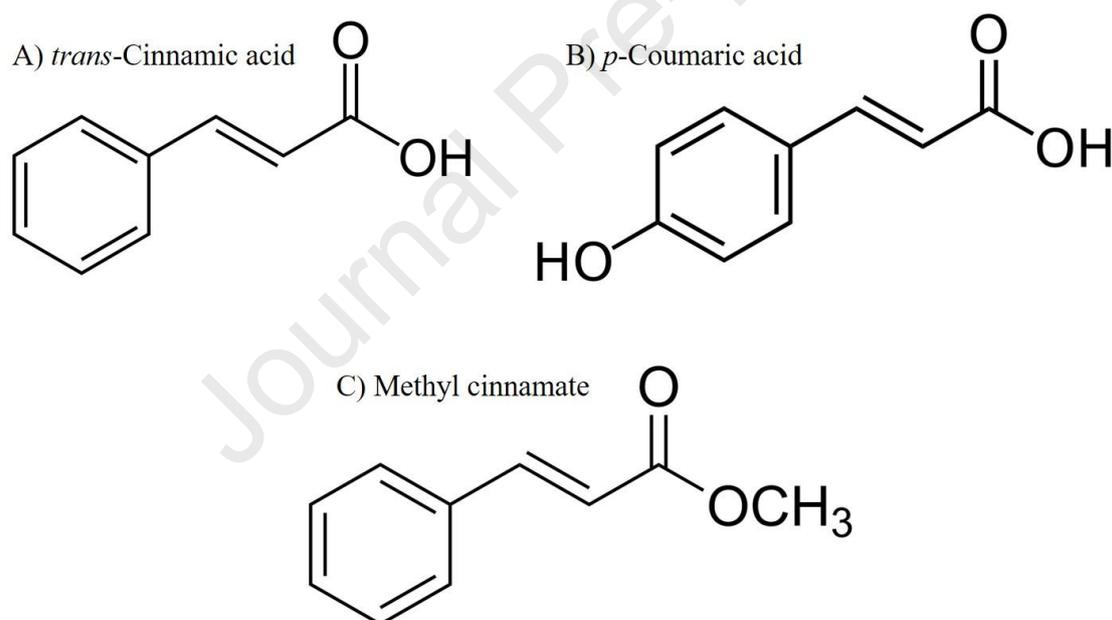
Thus, the aim of this study was to evaluate the effect of the three hydroxycinnamic acid derivatives, *trans*-cinnamic acid, methyl cinnamate, and *p*-coumaric acid, on fibroblast migration and suggest the underlying mechanism. Moreover, their effect on the synthesis of ECM and the cell cycle was also investigated.

## **2. Methods**

### ***2.1. Chemical and reagents***

The following reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA): *trans*-cinnamic acid (PubChem CID: 444539, Figure 1A), *p*-coumaric acid (PubChem CID: 637542, Figure 1B), methyl cinnamate (PubChem CID: 637520, Figure

1C), Tween-20, dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS), Dulbecco's modified Eagle medium (DMEM), mitomycin C, propidium iodide (PI), methylthiazolyldiphenyl-tetrazolium bromide (MTT), 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI), SB203580 (p38-MAPK inhibitor), JNK-IN-8 (inhibitor of JNK1/2/3), and PKI-(6-22)-amide (inhibitor of cAMP-dependent protein kinase, CREB). Foetal bovine serum (FBS), trypsin, penicillin, streptomycin, and L-glutamine were purchased from Invitrogen (Carlsbad, CA, USA). Antibodies against laminin, fibronectin, and collagen type I were purchased from Novotec (St. Martin-LaGarenne, France), while anti-goat IgG-fluorescein isothiocyanate (FITC) was from Santa Cruz Biotechnology (CA, USA).



**Figure 1.** The chemical structure of the *trans*-cinnamic acid (A), *p*-coumaric acid (B), and methyl cinnamate (C) used in the present study.

## 2.2. Cell culture

NIH 3T3 mouse embryonic fibroblasts or L929 fibroblast cell line were cultured in DMEM, supplemented with 10% FBS, 2 mM L-glutamine, and 0.02% penicillin/streptomycin, at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. The

assay was performed using cells between 3 and 5 passages. Hydroxycinnamic acid derivatives were dissolved in DMSO and then diluted with DMEM. The final concentration of DMSO during treatments did not exceed 0.1% (v/v) in cell cultures. DMEM containing of dimethyl sulfoxide (0.1% DMSO) was used as the vehicle.

### **2.3. Cell viability assay**

The effect of *trans*-cinnamic acid, *p*-coumaric acid, and methyl cinnamate on cell viability was evaluated by the MTT assay [21]. NIH 3T3 fibroblasts were plated in 96-well plates ( $7 \times 10^3$ /well) and treated with vehicle (negative control) or hydroxycinnamic acid derivatives at concentrations of 3, 30, and 300  $\mu$ M for 24 h. Cells lysed by 1% Triton-X-100 were used as the positive control. Next, the medium was replaced with fresh DMEM containing 5 mg/mL MTT and the plates were incubated for a further for 4 h in a humidified CO<sub>2</sub> incubator at 37 °C and 5% CO<sub>2</sub>. The resulting culture supernatant was removed and DMSO (150  $\mu$ L/well) was added to each culture plate. After incubation at room temperature for 15 min, the absorbance of the solubilized MTT formazan product was spectrophotometrically measured at 540 nm. Three individual wells were assayed for each treatment, and the percentage viability relative to the control sample was determined as (absorbance of treated cells/absorbance of untreated cells) $\times$ 100%.

### **2.4. Scratch wound healing assay**

The scratch wound-healing assay was applied as previously described [22]. NIH 3T3 fibroblast cells ( $2 \times 10^5$ ) were seeded into each well of a 24-well plate and maintained for 24 h until they reached 90% confluency. Next, a 200  $\mu$ l sterile pipette tip was used to create a straight cell-free scratch. The wells were washed with PBS to

remove dead cells and debris, and then subjected to treatment with 30  $\mu\text{M}$  of *trans*-cinnamic acid, *p*-coumaric acid, or methyl cinnamate in serum-free medium. As a control, the cells were treated with vehicle. After 8, 12 or 24 h of treatment with hydroxycinnamic acid derivatives, the cells that migrated onto the scratched region were photographed using an inverted microscope with a digital camera (Nikon Eclipse TS100, Nikon Corporation, Tokyo, Japan). In another set of experiments, the scratched monolayer was treated with 10  $\mu\text{M}$  SB203580, 10  $\mu\text{M}$  JNK-IN-8, or 10  $\mu\text{M}$  PKI-(6-22)-amide, followed by the addition of 30  $\mu\text{M}$  *trans*-cinnamic acid for 24 h.

The same procedure described above was used to evaluate the effect of *trans*-cinnamic acid, *p*-coumaric acid, or methyl cinnamate on L929 dermal fibroblast cell migration. After reaching confluence, the monolayer of L929 fibroblasts was scratched. Cells were then treated with 30  $\mu\text{M}$  of *trans*-cinnamic acid, *p*-coumaric acid, or methyl cinnamate. As a control, the cells were treated with vehicle (DMSO 0.1% in DMEM). After 24h of treatment, cells were photographed using an inverted microscope with a digital camera to measure wound closure. To prevent cell proliferation, mitomycin C (4  $\mu\text{g/ml}$ ) was added to each well, including vehicle and hydroxycinnamic acid derivatives-treated cells; this ensured that only migration contributed to wound closure. Cell migration was analysed using ImageJ software, and was expressed as the percentage of closure of the initial wound (scratch width at time zero) using the control group (vehicle) as 100% and the experimental group compared with the control group to ascertain the difference between the groups. Hydroxycinnamic acid derivatives-exposed and vehicle control cultures were compared. All the experiments were done in triplicate and repeated 3 times.

## **2.5. Transwell migration assay**

Migration assays were performed using a method described by Suvarna et al. (2018) [22] using 24-well culture chambers and transwell inserts with polycarbonate membranes (diameter, 10 mm; pore size, 8.0  $\mu\text{m}$ ; Corning Costar, Cambridge, USA). NIH 3T3 fibroblasts ( $5 \times 10^4$ ) were seeded into the upper chamber in 200  $\mu\text{L}$  DMEM medium devoid of serum and containing 30  $\mu\text{M}$  *trans*-cinnamic acid. As a control, the cells were treated with vehicle (DMSO 0.1% in DMEM). Cells were allowed to migrate for 8 hours at 37°C in a 5%  $\text{CO}_2$  incubator. At the end of the incubation period, the membrane was fixed with 3.7% formaldehyde, and stained with 1% crystal violet and subjected to inverted microscope image under 10 $\times$  objective. Cells that penetrated the filter were counted in three predetermined fields. Three independent experiments were performed, each performed in triplicate, and a representative image is shown.

## 2.6. Cell-cycle analysis

The cell cycle analysis using flow cytometry was carried out as described previously by Pozarowski and Darzynkiewicz (2004) [23]. In brief, cells were seeded on 24-well plates ( $6 \times 10^4$  cells/well), and 24 h later, these cells were treated with vehicle (DMSO 0.1% in DMEM) or hydroxycinnamic acid derivatives (30  $\mu\text{M}$ ). Subsequently, cells were trypsinized, washed with PBS, and then fixed in 70% ice-cold ethanol for 1 h. Later, the ethanol was removed by centrifugation (460  $\times g$  for 10 min), and the cell pellets were resuspended in 500  $\mu\text{L}$  of DNA-staining buffer containing 5  $\mu\text{g}/\text{mL}$  PI, 1% Triton X-100, and 0.05  $\text{mg}/\text{mL}$  RNase. After 30 min of incubation at room temperature in the dark, cells were examined using the FACSCanto II flow cytometer system (BD Biosciences, San Jose, CA, USA).

## 2.7. Immunofluorescence staining

Fibroblasts ( $6 \times 10^4$ ) were cultured in 8-well Lab-tek chamber glass slides (Nunc, NY, USA) with complete medium (DMEM) for 24 h. The medium was then replaced and the cells were treated with 30  $\mu\text{M}$  of *trans*-cinnamic acid for 24 h. Cells maintained in vehicle (DMSO 0.1% in DMEM) under the same conditions were used as controls. After treatment, the cultures were washed with PBS, fixed with 100% methanol for 10 min, and subjected to an indirect immunofluorescence assay, as previously described Smaniotto et al. (2005) [24]. Briefly, cells were rehydrated in PBS and incubated for 1 h with PBS containing 1% BSA to block non-specific binding. Next, samples were incubated with primary specific anti-laminin, anti-fibronectin, or anti-collagen type I antibodies (1:50) for 1 h in a humidified chamber, washed with PBS, and incubated with appropriate FITC-conjugated secondary antibody (1:200) for 45 min at room temperature. DAPI staining was used to visualize the nuclei. Immunostained samples were analysed by fluorescence microscopy. Controls comprised specimens in which primary antibodies were replaced by unrelated Igs and generated no significant labelling. The staining of each section was analysed to obtain a mean optical density value (MOD), which represented the strength of the staining signal per positive pixel/ $\mu\text{m}^2$  [25].

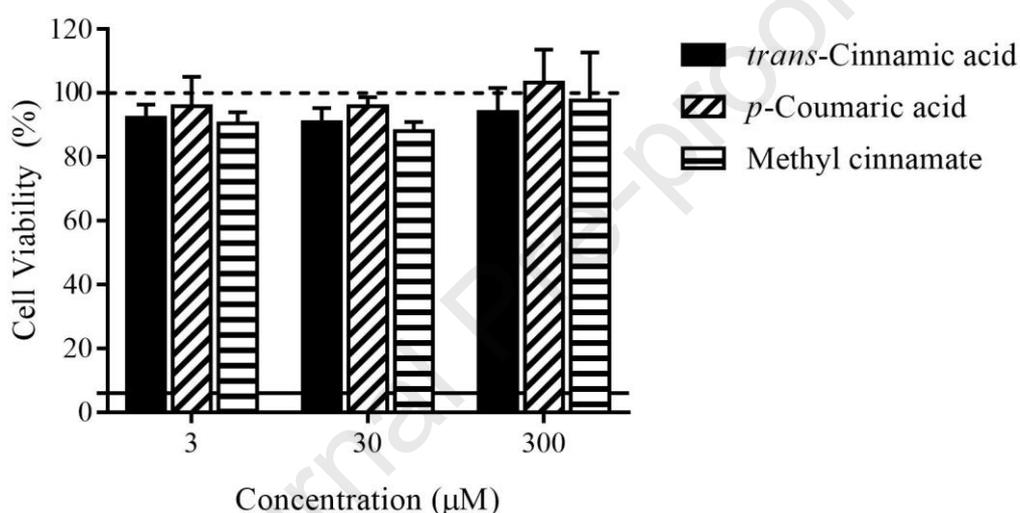
## **2.8. Statistical analysis**

Data are reported as mean  $\pm$  standard deviation (SD) and were analysed using GraphPad Prism software, version 5.0 (San Diego, CA, USA). Comparisons between the experimental groups were performed either by one-way ANOVA followed by Tukey's test, or by two-way ANOVA followed by the Bonferroni post hoc test. P values less than 0.05 were considered statistically significant.

### 3. RESULTS

#### 3.1. Effect of *trans*-cinnamic acid, *p*-coumaric acid, or methyl cinnamate on cell viability

The MTT assay was used to test the potential cytotoxic effect of the hydroxycinnamic acid derivatives on fibroblasts. As presented in Figure 2, we identified that the fibroblast viability was not significantly affected by treatment for 24 h with *trans*-cinnamic acid, *p*-coumaric acid, or methyl cinnamate at doses 3, 30, or 300  $\mu$ M.

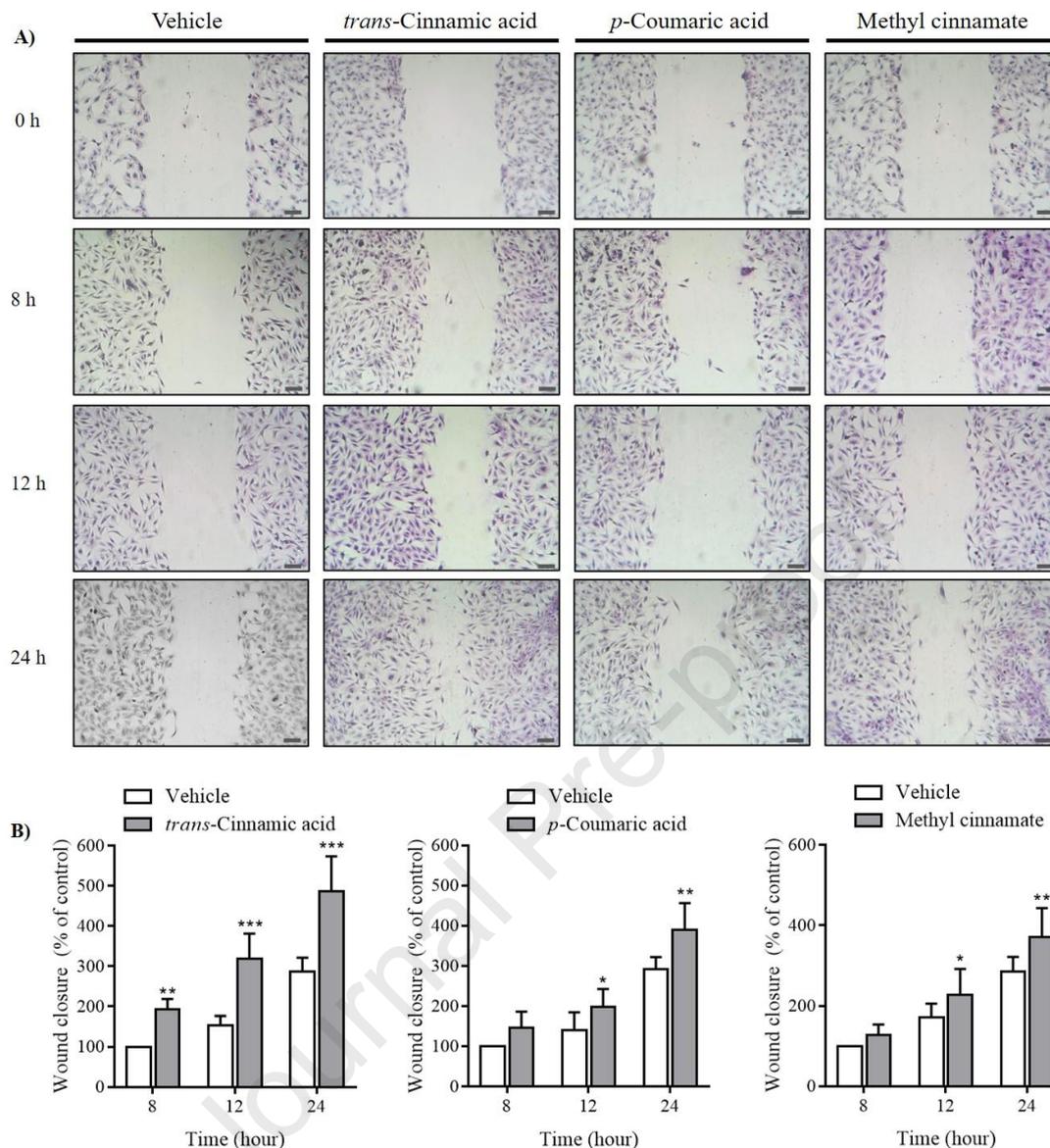


**Figure 2.** Effect of *trans*-cinnamic acid, *p*-coumaric acid, or methyl cinnamate on the viability of NIH 3T3 fibroblasts. Cells were plated at  $7 \times 10^3$  cells/well and treated with hydroxycinnamic acid derivatives (3–300  $\mu$ M) for 24 h. Cell viability was measured by MTT assay. The dashed line represents cells maintained only with vehicle (DMSO 0.1% in DMEM), while the solid line represents the cells lysed by 1% Triton-X-100 (positive control). The bars represent mean  $\pm$  SD of three independent experiments, each performed in triplicate.

#### 3.2. Effects of hydroxycinnamic acid derivatives treatment on fibroblast motility

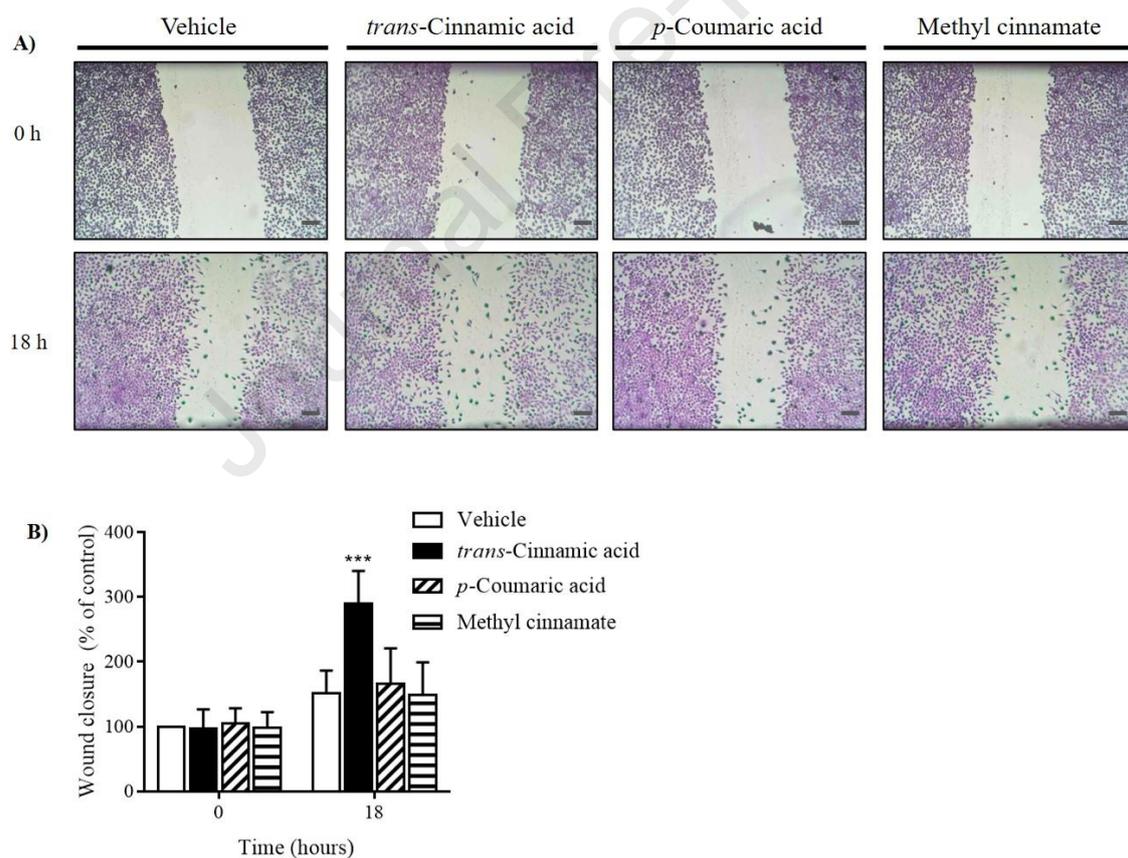
The effect of *trans*-cinnamic acid, *p*-coumaric acid, or methyl cinnamate on fibroblast migratory activity was examined using a scratch assay. Scratches were made on the monolayer of confluent cells and then treated with vehicle or hydroxycinnamic

acid derivatives at concentration of 30  $\mu\text{M}$ . Cell migration into the scratch area was evaluated at 8, 12 and 24 h. As shown in Figure 3A, fibroblasts treated with *trans*-cinnamic acid, *p*-coumaric acid, or methyl cinnamate exhibited a faster scratch closure rate compared to vehicle (control). In Figure 3B, after 8, 12, and 24 h of culture, basal levels of scratch closure in vehicle-treated cells were 10%, 16% and 29% respectively, while fibroblasts cultured in the presence of 30  $\mu\text{M}$  of *trans*-cinnamic acid exhibited respectively, an increase in migration to 19% at 8 hours, 26% at 12 hours and 52% at 24 hours. A similar profile of migration occurred in cells cultured with 30  $\mu\text{M}$  *p*-coumaric acid (Figure 3B), where cells had their migratory capacity increased to 15% at 8 hours, 20% at 12 hours and 40% at 24 hours (Figure 3B). As observed in the two preceding treatments, exposure of the cells to 30  $\mu\text{M}$  of methyl cinnamate increased the migration rate to 13% at 8 hours, 23% at 12 hours and 37% at 24 hours (Figure 3B).



**Figure 3.** The effect of *trans*-cinnamic acid, *p*-coumaric acid, and methyl cinnamate on the cell migration of NHI 3T3 fibroblasts assayed by the scratch assay. Fibroblasts were treated with 30  $\mu$ M of each hydroxycinnamic acid derivative, and images were captured to calculate the scratch closure. A) Representative photomicrography images showing the scratch area of cells treated with vehicle (DMSO 0.1% in DMEM), *trans*-cinnamic acid, *p*-coumaric acid, or methyl cinnamate and the cell migration towards the cell-free area after 8, 12 and 24 h. Scale bars, 50  $\mu$ m. B) The percentage of scratch covered was measured by quantifying the total distance the cells moved from the edge of the scratch towards the center of the scratch, using ImageJ software, followed by conversion to a percentage of the wound covered. The bars represent mean  $\pm$  SD of three independent experiments, each performed in triplicate. (\*)  $P < 0.05$ , (\*\* )  $P < 0.01$  and (\*\*\*)  $P < 0.001$  compared with vehicle-treated cells at the respective time points.

We also used the established L929 fibroblast cell line in the scratch assay to assess the effects of hydroxycinnamic acid derivative on fibroblast migration. Figure 4A shows representative images captured under the inverted microscope of cells treated with vehicle (DMSO 0.1% in DMEM), *trans*-cinnamic acid, *p*-coumaric acid, or methyl cinnamate. As indicated in Figure 4B, quantitative analysis of the migratory response of L929 fibroblasts treated with mitomycin C (4  $\mu$ g/ml) and exposed to *trans*-cinnamic acid (30  $\mu$ M) showed a significant increase in total migration at 24 hours post-scratch. However, the migratory response of L929 fibroblasts was only slightly increased by treatment with *p*-coumaric acid or methyl cinnamate, and these increases, compared with vehicle-treated cells, were not significant.

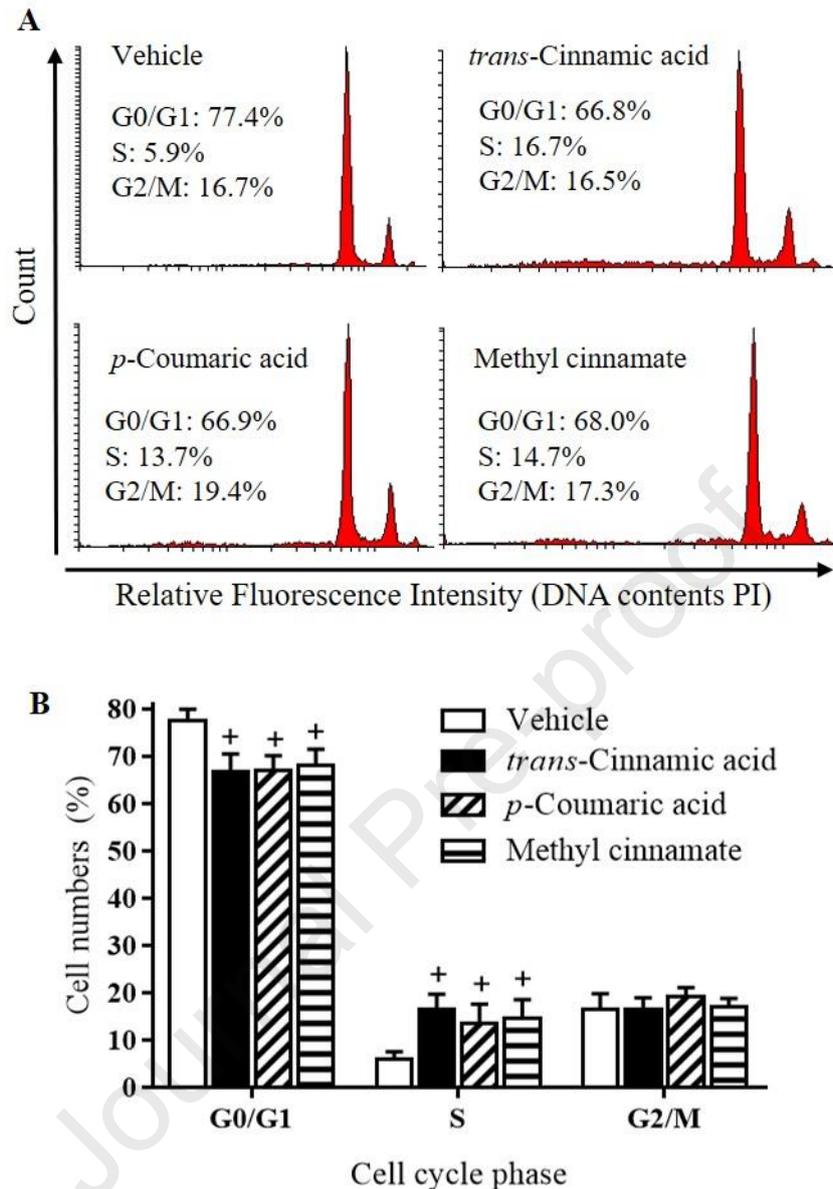


**Figure 4.** The effect of *trans*-cinnamic acid, *p*-coumaric acid, and methyl cinnamate on cell migration by L929 fibroblasts, assayed by the scratch assay. Fibroblasts were treated with 30  $\mu$ M of each hydroxycinnamic acid derivative or vehicle (DMSO 0.1% in DMEM), and photomicrographs were captured to calculate scratch closure. A) Representative photomicrographic images showing the scratch area of cells treated with

vehicle, *trans*-cinnamic acid, *p*-coumaric acid, or methyl cinnamate, and cell migration into the cell-free area after 24 hours. Scale bars, 50  $\mu\text{m}$ . B) The percentage of scratch covered was measured by quantifying the total distance the cells moved from the edges of each scratch towards the center, using ImageJ software, followed by conversion to a percentage of the wound covered. The bars represent mean  $\pm$  SD of three independent experiments, each performed in triplicate. (\*\*\*)  $P < 0.001$  compared with respective vehicle-treated groups at time 0.

### ***3.3. The effect of hydroxycinnamic acid derivatives on cell cycle progression and migration***

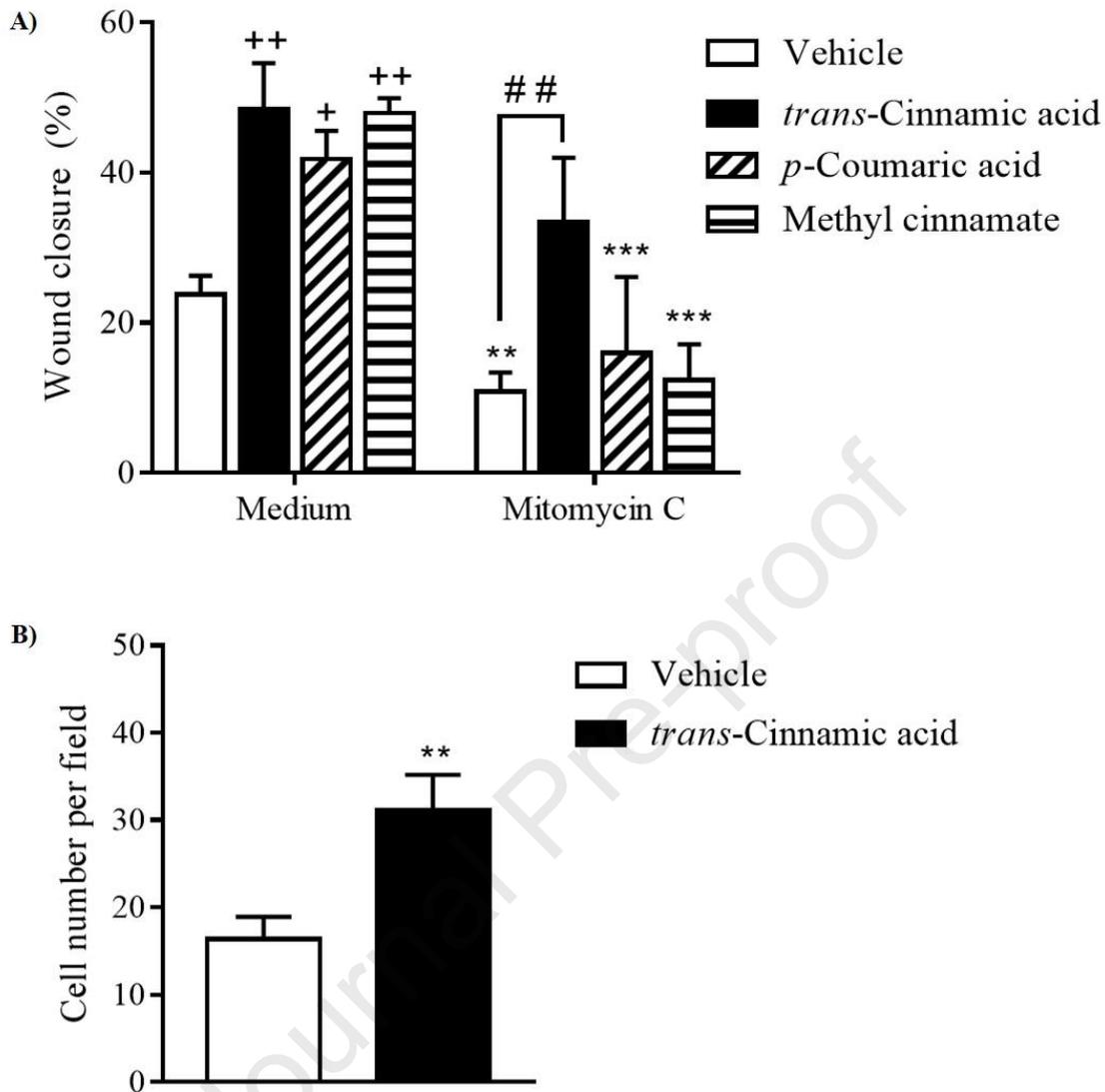
To determine the effect of each hydroxycinnamic acid derivative on cell cycle progression, flow cytometry was performed. As shown in Figure 5, cells cultured in vehicle for 24 h (control group) exhibited a cell cycle distribution at G0/G1, S, and G2/M phases of 77.4%, 5.9%, and 16.7%, respectively. Compared with the control group, the group treated with 30  $\mu\text{M}$  of *trans*-cinnamic acid showed a reduction in the G0/G1 phase population to 66.8% and a concomitant 16.7% increase in the S phase population without any change in the G2/M phase population (16.5%) (Figure 5). Similarly, the treatment with *p*-coumaric acid or methyl cinnamate decreased, respectively, the cells in G0/G1 phase to 66.9% and 68% and, simultaneously, increased the cell population in S phase to 13.7% and 14.7%, while maintaining the numbers of cells in G2/M phase at 19.4% and 17.3%, respectively (Figure 5). These results indicate that a proliferative state of the fibroblasts was induced by treatment with the hydroxycinnamic acid derivatives.



**Figure 5.** Effect of *trans*-cinnamic acid, *p*-coumaric acid, and methyl cinnamate on cell cycle progression in fibroblasts. Cells were exposed to the treatment for 24 h. Cell cycle progression in fibroblasts was assessed by flow cytometry by analysing propidium iodide fluorescence in the slow acquisition and with doublet discrimination; 10,000 events were recorded. A) Histogram showing cell cycle of cells treated with 30  $\mu$ M hydroxycinnamic acid derivatives. B) Quantification of G0/G1-, S-, and G2/M-stage in fibroblasts. The bars represent mean  $\pm$  SD of three independent experiments, each performed in triplicate. Statistical significance between groups was determined by ANOVA followed by Bonferroni's test. (+)  $P < 0.05$  compared with vehicle-treated cell in the respective phase.

To eliminate the possibility that the scratch wound closure is dependent on proliferation, we treated the fibroblasts with mitomycin C, a potent inhibitor of cell proliferation. As shown in Figure 6A, cells were pre-treated with 4  $\mu\text{g/ml}$  of mitomycin C for 1 h and then exposed to 30  $\mu\text{M}$  hydroxycinnamic acid derivatives for 24 h. It was observed that the cell proliferation was halted by mitomycin C, while the migratory response was maintained at approximately 54% when compared to vehicle-treated cells (white bars). However, the migratory response of fibroblasts after treatment with *p*-coumaric acid or methyl cinnamate was reduced to the basal levels after exposure to mitomycin C (Figure 6A). This situation was different from that where fibroblasts were pre-treated with *trans*-cinnamic acid, wherein mitomycin C treatment did not cause a significant reduction in migratory response of fibroblasts (Figure 6A).

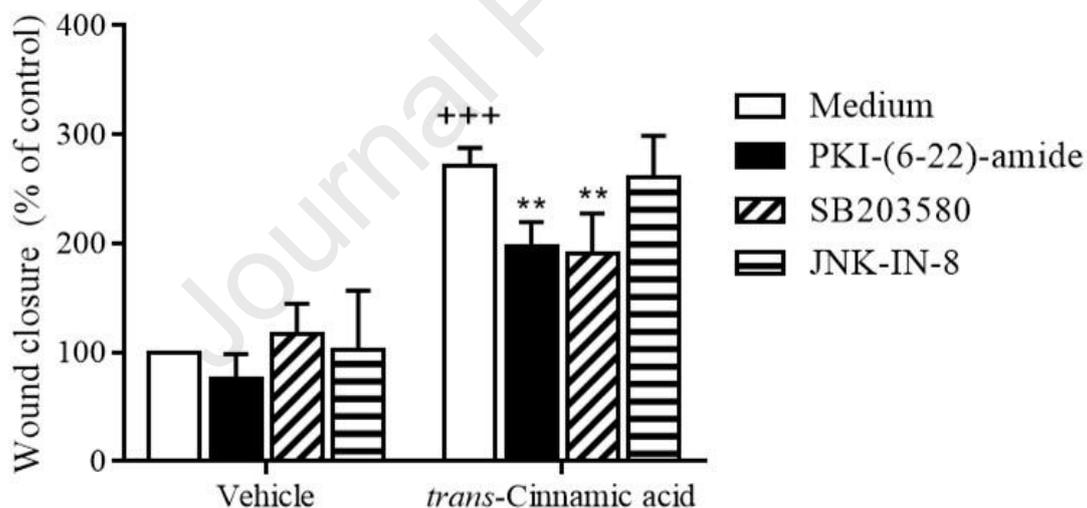
To confirm the effect of *trans*-cinnamic acid on migration, we performed transwell migration assays (Figure 6B). NIH 3T3 fibroblasts were seeded into the upper chamber with the filters, and were allowed to migrate for 8 hours. *trans*-Cinnamic acid-treatment resulted in a significant (close to 50%) increase in fibroblast migration (Figure 6B), thereby reinforcing the suggestion that *trans*-cinnamic acid plays a direct role in fibroblast migration *in vitro*.



**Figure 6.** *trans*-Cinnamic acid promotes fibroblast migration. A) NIH 3T3 fibroblasts were treated with mitomycin C before adding 30  $\mu$ M of each hydroxycinnamic acid derivative. Cell migration in the scratch assay was determined by photomicrography at 0- and 24-hours post scratch with the aid of an inverted microscope. B) NIH 3T3 Fibroblast migration analyses via transwell cell migration assay. Cells were seeded into transwell chambers in serum-free medium containing 30  $\mu$ M *trans*-cinnamic acid or vehicle (DMSO 0.1% in DMEM), and allowed to migrate across the membranes for 8 hours. Membranes were stained with crystal violet, and images were captured with an inverted microscope. Bars represent mean  $\pm$  SD of three independent experiments, each performed in triplicate. Statistical significance between groups was determined by ANOVA followed by Bonferroni's test. (+)  $P < 0.05$  and (++)  $P < 0.01$  compared with medium-treated cells, (\*\*)  $P < 0.01$  and (\*\*\*)  $P < 0.001$  compared with vehicle-treated cells. (##  $P < 0.01$ ).

### 3.4. The role of the p38-MAPK, JNK, and PKA signalling pathways in *trans*-cinnamic acid-induced fibroblast migration

To identify the possible molecular mechanisms of *trans*-cinnamic acid-induced migration, specific inhibitors of intracellular signalling were used. As shown in Figure 7, the PKA inhibitor (PKI-(6-22)-amide, 10  $\mu$ M) and p38-MAPK inhibitor (SB203580, 10  $\mu$ M) inhibited the *trans*-cinnamic acid-induced migration of fibroblasts by 27% and 29%, respectively. Treatment with the JNK inhibitor (JNK-IN-8, 10  $\mu$ M) did not cause a significant alteration in *trans*-cinnamic acid-induced fibroblast migration (Figure 7). These results indicate that *trans*-cinnamic acid exerted a positive effect on fibroblast migration via specific mechanisms.

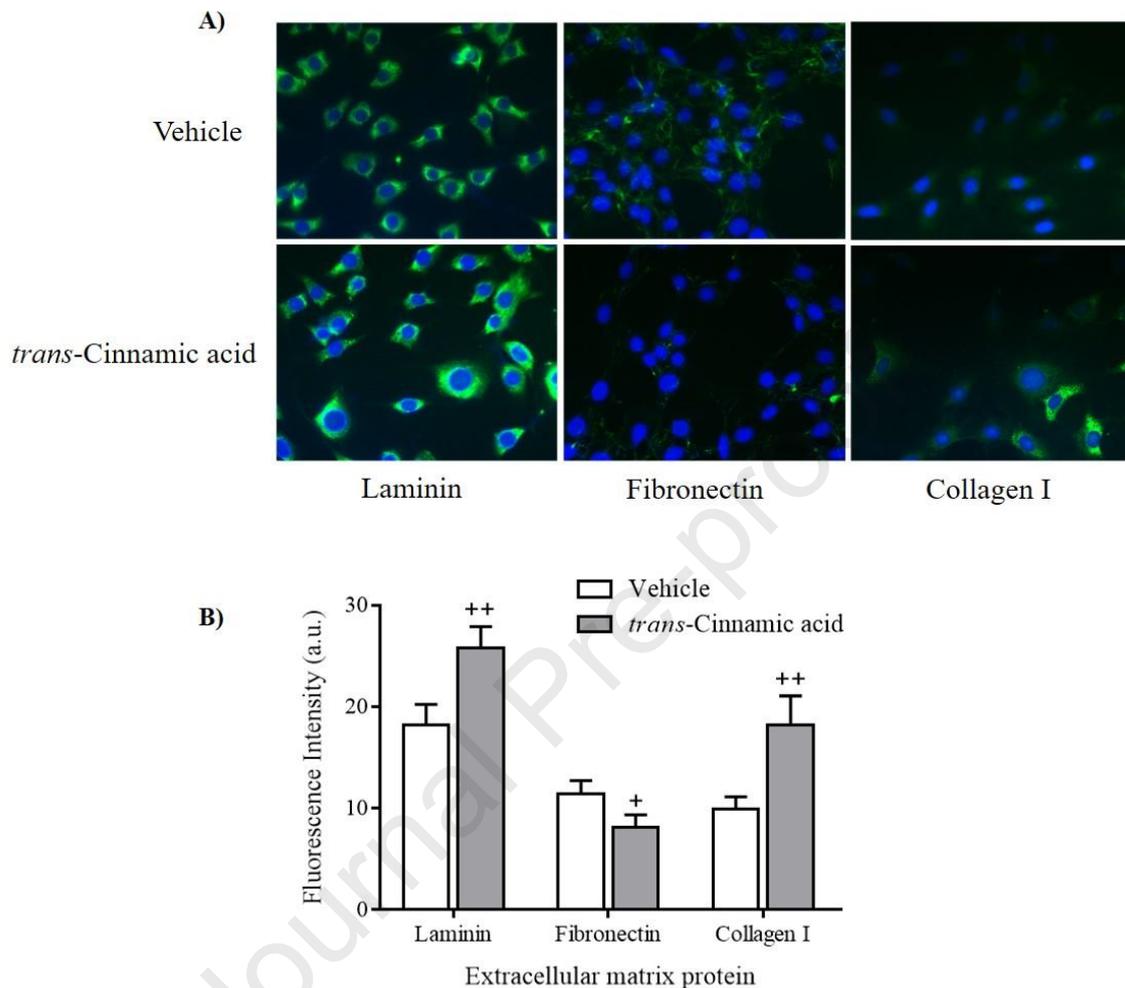


**Figure 7.** Inhibition of PKA and p38-MAPK but not JNK signalling pathways attenuates fibroblast migration induced by *trans*-cinnamic acid. Scratch wounds were created in cell monolayers of fibroblasts using a sterile pipette tip. Then, cells were cultured only with vehicle (DMSO 0.1% in DMEM) or PKI-(6-22)-amide (a PKA inhibitor, 10  $\mu$ M), SB203580 (a p38-MAPK inhibitor, 10  $\mu$ M), or JNK-IN-8 (a JNK inhibitor, 10  $\mu$ M) in the presence of *trans*-cinnamic acid (30  $\mu$ M) for 24 h. The bars represent mean  $\pm$  SD of three independent experiments, each performed in triplicate. Statistical differences were detected with two-way ANOVA followed by the Bonferroni test. (++++) P < 0.001 compared to vehicle-treated cells, (\*\*) P < 0.01 compared to *trans*-cinnamic acid-treated cells.

### 3.5. Effect of *trans*-cinnamic acid on ECM deposition by fibroblasts

Considering the importance of the ECM for the various functions of fibroblasts, including migration, we evaluated whether *trans*-cinnamic acid treatment could affect ECM protein synthesis. To this end, fibroblasts were exposed for 30 h to *trans*-cinnamic acid and the laminin, fibronectin, and collagen type I production was assessed by immunofluorescence analysis. As shown in Figure 8A, cells treated with vehicle showed a basal level of laminin protein production organized around the cell nucleus. The treatment with *trans*-cinnamic acid (30  $\mu$ M) intensified this immunofluorescence staining of cytoplasmic laminin. Image analysis showed a 30% increase in the intracytoplasmic fluorescence reflecting laminin levels after treatment (Figure 8B). The intracytoplasmic level of fibronectin in fibroblasts treated with vehicle was relatively low and appeared to be organized in small clusters around the cell nucleus (Figure 8A). The treatment with *trans*-cinnamic acid caused a significant reduction in the amount of fibronectin produced by fibroblasts. This reduction was confirmed after image analysis showed a 27% decrease in the cytoplasmic fluorescence for fibronectin after treatment with *trans*-cinnamic acid (Figure 8B). When the collagen type I expression was evaluated, fibroblasts treated with vehicle exhibited a weak immunofluorescence staining that was diffused in the cytoplasm (Figure 8A). Treatment with *trans*-cinnamic acid (30  $\mu$ M) increased the immunofluorescence staining of collagen type I in cells. When the image was analysed, it showed an increase in collagen type I content by 45% in the intracytoplasmic fluorescence (Figure 8B). This immuno-labelling of collagen type I in the fibroblasts was increased after *trans*-cinnamic acid treatment (30  $\mu$ M).

Analysis of this image showed an increase by 45% in the intracytoplasmic fluorescence for collagen type I content (Figure 8B).



**Figure 8.** Effect of *trans*-cinnamic acid on the levels of laminin, fibronectin, and collagen type I in fibroblasts using immunofluorescence analysis. A) Fibroblasts were cultured with or without 30  $\mu$ M *trans*-cinnamic acid, and after 24 h, the cells were fixed and extracellular matrix was immunostained using antibodies against laminin, fibronectin, and collagen type I. Nuclei were stained with DAPI. Each panel shows an image of one representative field from three independent experiments. B) Graph showing the results of the quantification of extracellular matrix synthesis of images from panel A. Bars represent mean  $\pm$  SD of three independent experiments, each performed in triplicate. Statistical significance between groups was determined by ANOVA followed by Bonferroni's test. (+)  $P < 0.05$  and (++)  $P < 0.01$  compared with respective vehicle-treated group.

#### 4. DISCUSSION

Hydroxycinnamic acid derivatives constitute a class of compounds originating from plants with a wide range of pharmacological effects that may be useful for treating various pathological conditions, including cutaneous wounds [26, 27]. In the healing process, the migration of fibroblast towards the injury area is a vital mechanism behind natural wound healing [28]. Thus, identification of compounds that can improve fibroblast migration can lead to the discovery of new and cost-effective drugs for treating wounds that do not heal. In the present study, we demonstrated that *trans*-cinnamic acid, but not its derivatives *p*-coumaric acid and methyl cinnamate, enhanced the migration of fibroblasts *in vitro* by a signalling mechanism involving PKA and p38-MAPK pathways. Additionally, we also identified a modulatory effect of *trans*-cinnamic acid on the production of ECM, which resulted in an increase of synthesis of laminin and collagen type I and reduction in fibronectin synthesis by fibroblasts.

Fibroblast functions during the wound healing process are essential for restoring tissue structural integrity and maintaining tissue functionality. The initial responses of fibroblasts adjacent to the wound site include proliferation, migration, and ECM deposition [28]. It is important to mention that the lack of these functions can compromise healing, and fibroblast dysfunction is a key factor that leads to the non-healing of chronic venous leg ulcers [29], as well as the impairment of wound healing observed in diabetic patients [30]. Thus, these observations clearly demonstrate that compounds with the ability to improve fibroblast functions can have a positive role in wound healing.

Previous studies have shown that hydroxycinnamic acids have preferential cytotoxicity for cancer cell lines [31, 32] and that changes in the structure of these molecules can change their biological activity or even impart new activities [26]. Here,

we used MTT assay to estimate the cytotoxic potential of the hydroxycinnamic acid derivatives. The MTT assay has been widely used in experimental research to assess cell viability [33]. In addition, the advantages of the MTT assay include ease and rapidity of performance, reproducibility of the results, and observed clinical correlation between *in vitro* and *in vivo* testing [34]. In the present study, we evaluated the *in vitro* cytotoxicity of *trans*-cinnamic acid, *p*-coumaric acid, and methyl cinnamate, and we noted no toxic effects on fibroblasts at dose ranges from 3–300  $\mu$ M and 24 h exposure. This result confirms previous reports that demonstrated the absence of cytotoxicity of these hydroxycinnamic acid derivatives in fibroblasts [14, 35, 36].

Whereas that mouse fibroblastic lines are comparable to human cell lines in responsiveness to migration pattern [37], we decided to evaluate the effect of hydroxycinnamic acid derivatives on mouse fibroblast migration via the scratch assay. This assay is a standard *in vitro* methodology, which enables screening of substances that are able to regulate cellular migration [38]. For this, a cell-free area is created in a confluent monolayer through mechanical damage, and the cells that remain move towards the artificial gap, which allows for measuring the rate of gap closure (wound closure) [39].

In this assay, the mouse fibroblast cell lines are commonly used in screening drugs that can improve wound healing [40]. In addition, the importance of NIH/3T3 and L929 cells as model for translational study have been recognized by many biomedical and pharmaceutical companies [7]. In our study, *trans*-cinnamic acid, *p*-coumaric acid, or methyl cinnamate were used as a treatment during the scratch assay, and all these compounds were found to accelerate the closure of the scratched area in a time-dependent manner, suggesting that these compounds have a direct effect on fibroblasts.

These results are consistent with a previous study in which an hydroxycinnamic acid, ferulic acid, significantly improved fibroblast motility [41].

It is well-known that during a scratch assay, cells not only migrate into the gap, but also proliferate. Thus, we decided to characterize the effect of these hydroxycinnamic acid derivatives on cell cycle phases. Our results revealed that *trans*-cinnamic acid, *p*-coumaric acid, or methyl cinnamate promoted proliferation of fibroblasts, accelerating the entry of cells into the S phase of the cell cycle. It is well-established that serum starving is the most common non-pharmaceutical method for minimizing proliferation in wound healing assays [38]. Even so, we decided to carry out the scratch assay with hydroxycinnamic acid derivatives using mitomycin C, a drug used to arrest mitosis at various stages. To our surprise, we found that the use of mitomycin C prevented the migration of fibroblasts treated with *p*-coumaric acid or methyl cinnamate, but not *trans*-cinnamic acid, in the scratch assay. Such an effect of *trans*-cinnamic acid in accelerating closure of the scratched area was also observed when L929 fibroblasts were used in the scratch assay. To further confirm this effect on fibroblast motility, *trans*-cinnamic acid-treated cells showed markedly increased migratory activity compared with untreated cells when evaluated using transwell migration assays. These observation supports the notion that *trans*-cinnamic acid directly affect fibroblast migration. This selective influence on cell migration is not unique to *trans*-cinnamic acid, the gallic acid, a hydroxybenzoic acid occurring mostly in red fruits, also exerts migration-stimulating effects on human dermal fibroblasts without inducing proliferation [39]. Furthermore, this finding clearly shows that the chemical modifications in the structure of these compounds have a direct impact on the profile of their biological activities, indicating the need for further studies on the structure-activity relationship of these hydroxycinnamic acid derivatives. A probable,

albeit speculative, explanation may be that the chemical structure of *p*-coumaric acid has one -OH moiety in its aromatic ring, while methyl cinnamate has one -CH<sub>3</sub> moiety in its carboxyl group; these modifications may affect electronic charge distribution across the whole structure, bringing changes in these molecules' polarity, and causing loss of activity. Prior chemical studies have demonstrated that simple chemical modifications may result in unexpected effects in hydroxycinnamic acids, including new modes of action [42, 43]. Further studies are required to investigate this concept.

Fibroblast migration is an event mediated by multiple intracellular signalling pathways, including protein kinase A (PKA), CREB, and p38 and JNK MAPK pathways [44]. However, despite scientific advancements in recent years, knowledge about the fibroblast signalling pathways that are activated by *trans*-cinnamic acid remains scarce. Thus, to evaluate whether the pro-migratory effect of *trans*-cinnamic acid is exerted via any intracellular signalling pathways, we evaluated fibroblast migration using the scratch assay in the presence of inhibitors of distinct signalling pathways. Our results demonstrated that inhibitors PKI-(6-22)-amide and SB203580, but not the inhibitor of JNK-IN-8, attenuated the *trans*-cinnamic acid-induced fibroblast migration, indicating the involvement of both signalling pathway PKA and p38-MAPK in fibroblast migration. These data are in agreement with previous studies showing that *trans*-cinnamic acid induces an increase in PKA and p38-MAPK phosphorylation in other cell types, such as adipocytes [45] and epithelial cells [46]. Therefore, the involvement of these intracellular signalling pathways in the positive regulation of fibroblast migration induced by *trans*-cinnamic acid supports the proposition of this compound as a new tool for improving fibroblast migration. However, further studies are required to determine how *trans*-cinnamic acid leads to both PKA and p38-MAPK activation.

It is also worth highlighting that previous studies demonstrated that activation of PKA and p38-MAPK intracellular signalling pathways induces the upregulation of ECM production by fibroblasts [47, 48]. Considering that the deposition of laminin, collagen type I and fibronectin plays a crucial role in fibroblast migration, adhesion, and wound healing, we evaluated whether *trans*-cinnamic acid also affected the production of these ECM proteins. Indeed, we verified that *trans*-cinnamic acid increased the synthesis of laminin and collagen type I and inhibited the synthesis of fibronectin. In line with our study, previous findings have also shown this dual effect of cinnamic acid in stimulating laminin and collagen synthesis while simultaneously inhibiting fibronectin production in epidermal cells [26, 49]. Considering that ECM can induce intracellular signals or interact with transduction pathways triggered by specific receptors, thereby promoting cell migration, we can infer that *trans*-cinnamic acid might represent a new chemical that can induce fibroblast migration.

## CONCLUSION

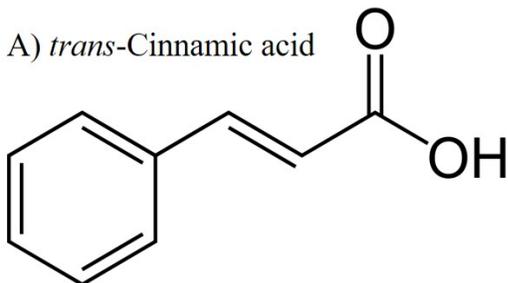
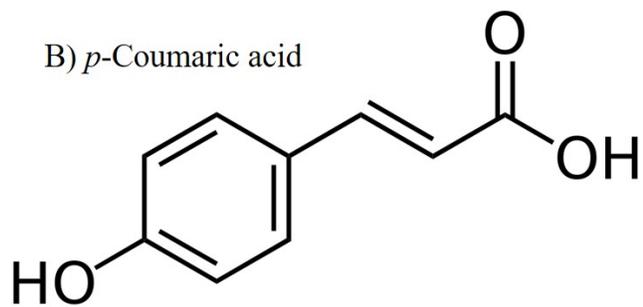
In conclusion, we have presented for the first time that in addition to stimulating laminin and collagen type I synthesis, *trans*-cinnamic acid significantly stimulates fibroblast function to promote their migration via a mechanism that seems to involve both the PKA and p38-MAPK signalling pathways. These effects might have an important role in the wound healing process and are relevant for wound therapies.

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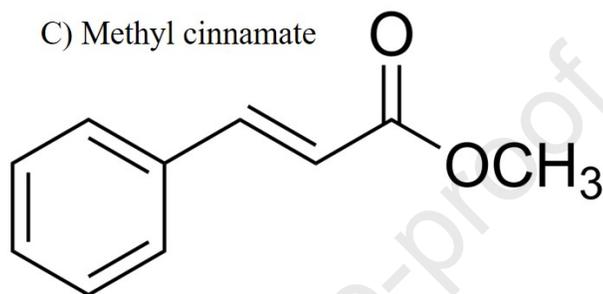
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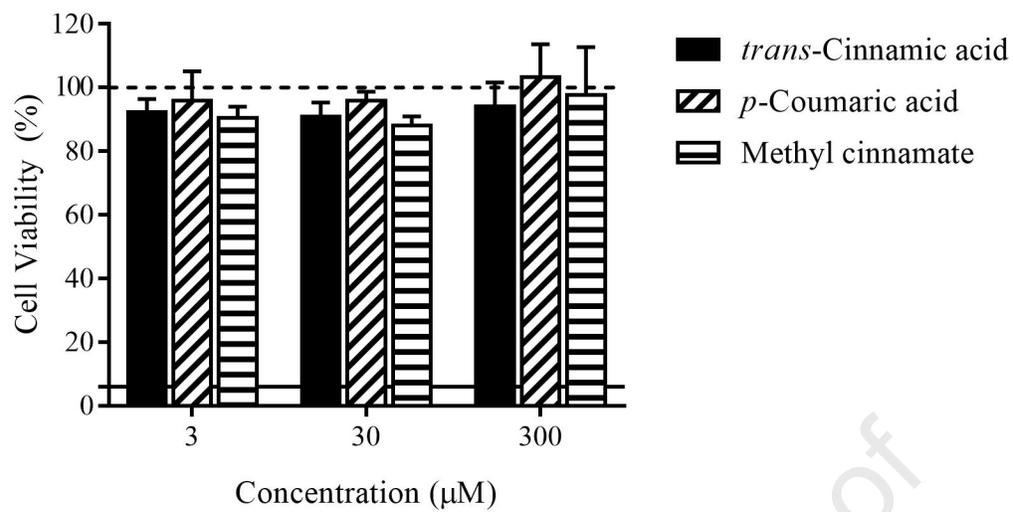
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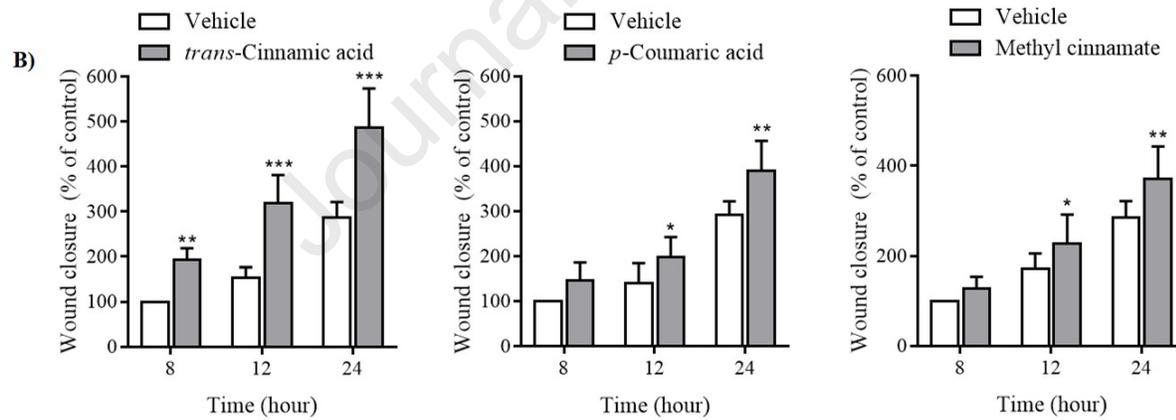
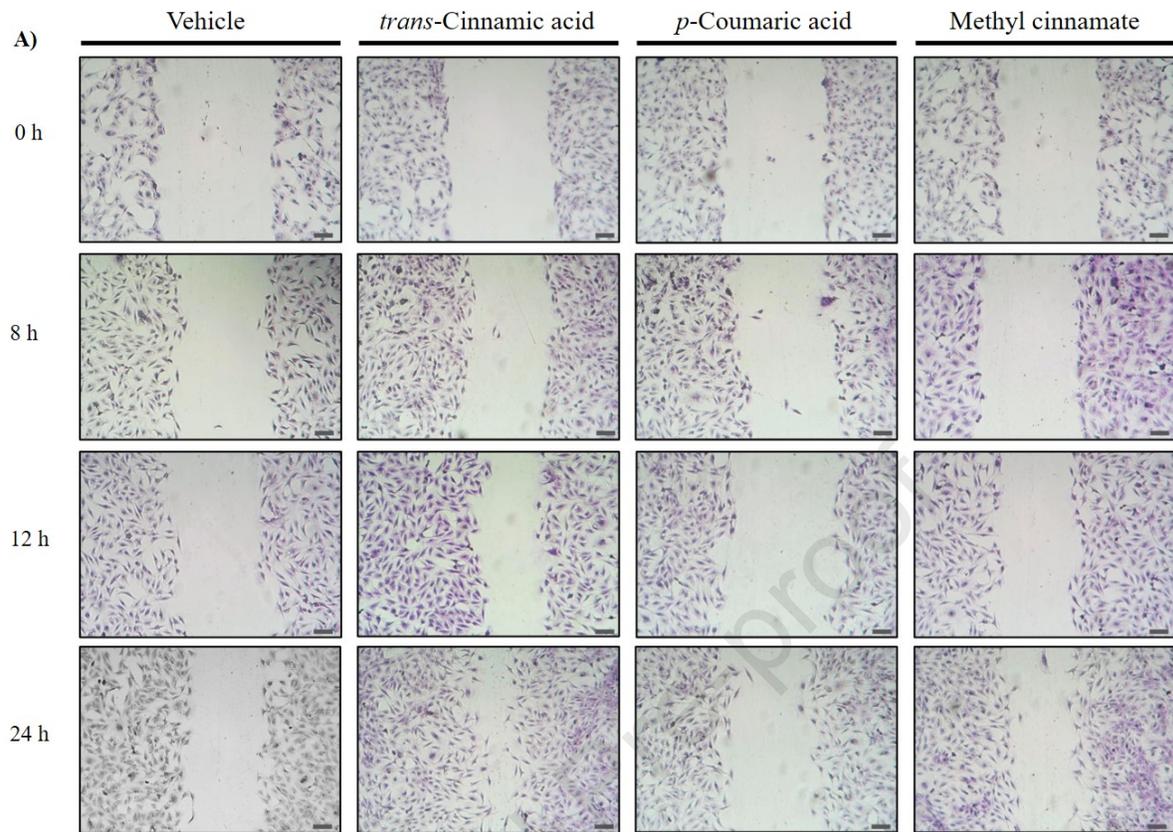
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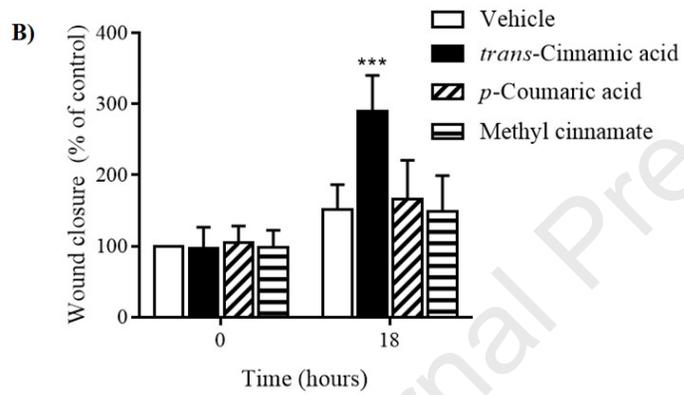
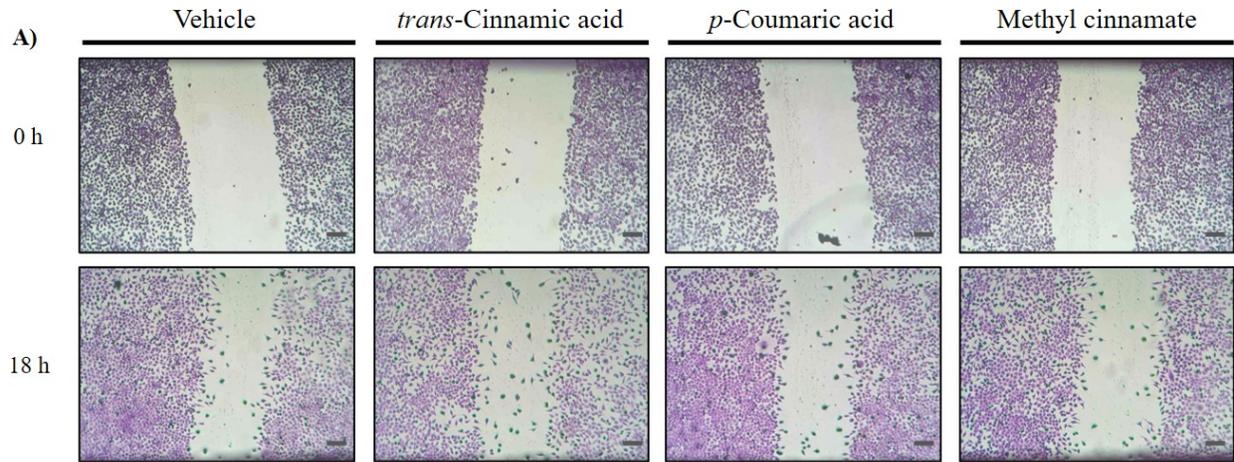
A) *trans*-Cinnamic acidB) *p*-Coumaric acid

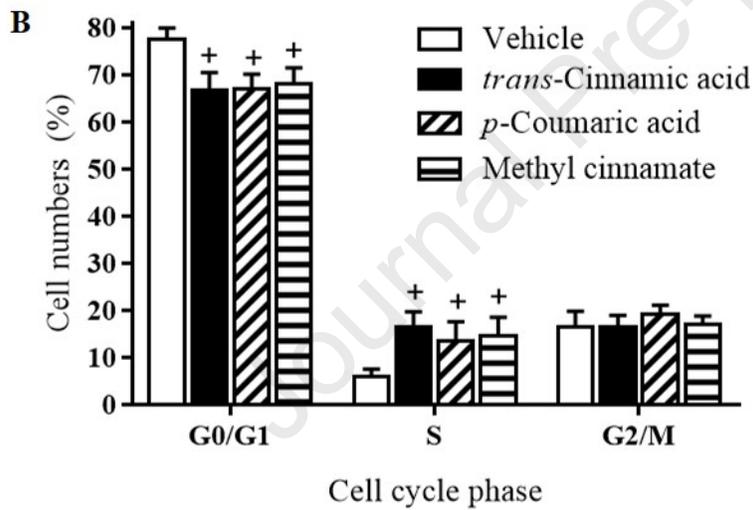
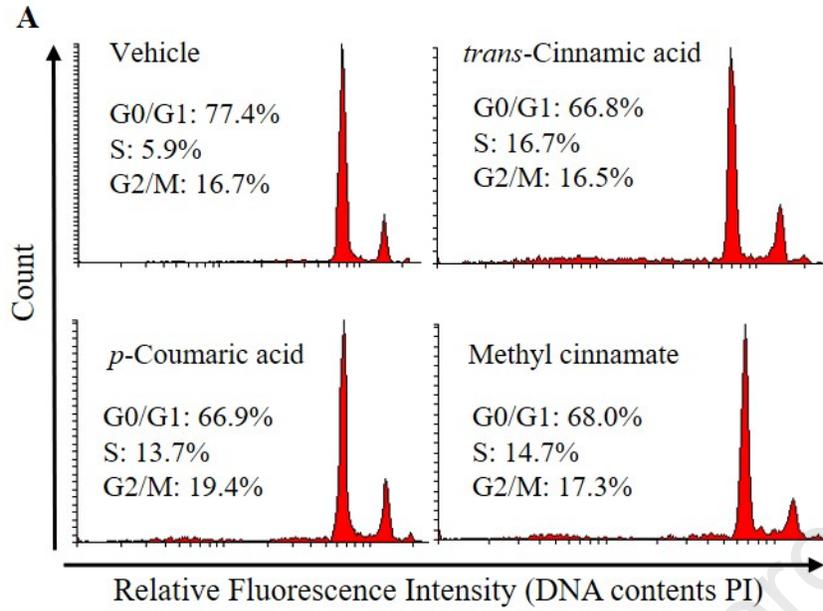
C) Methyl cinnamate

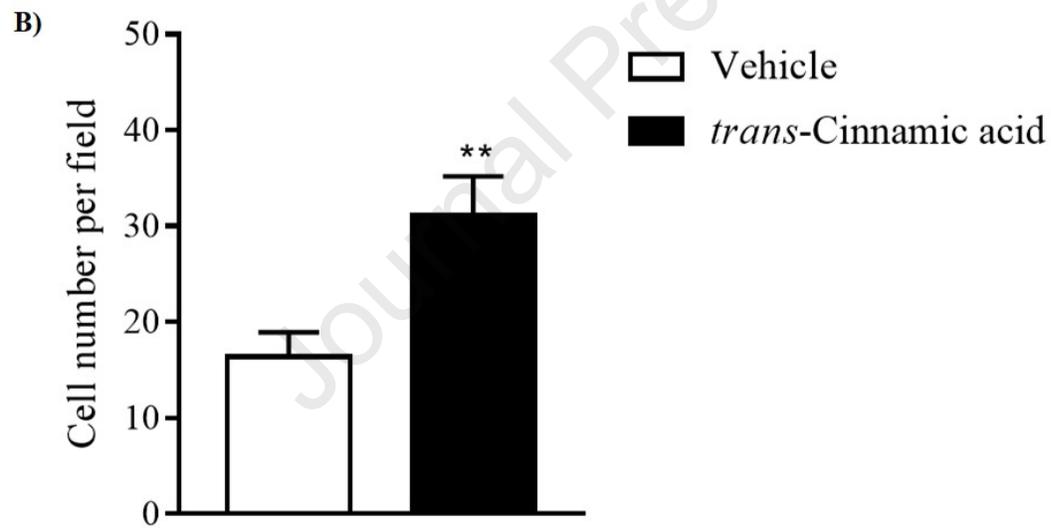
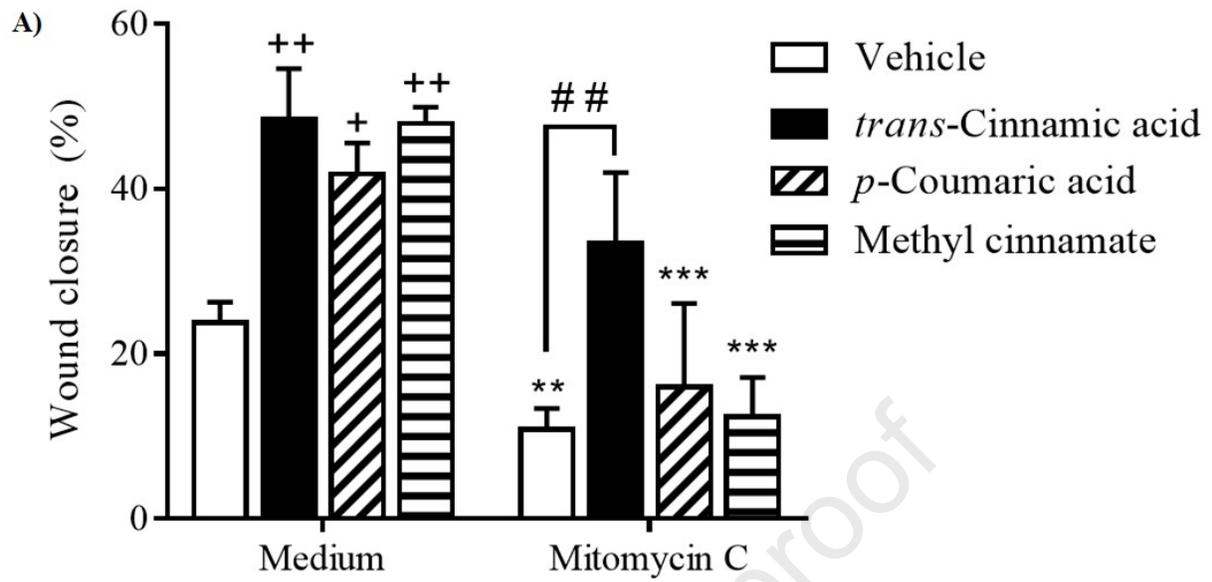


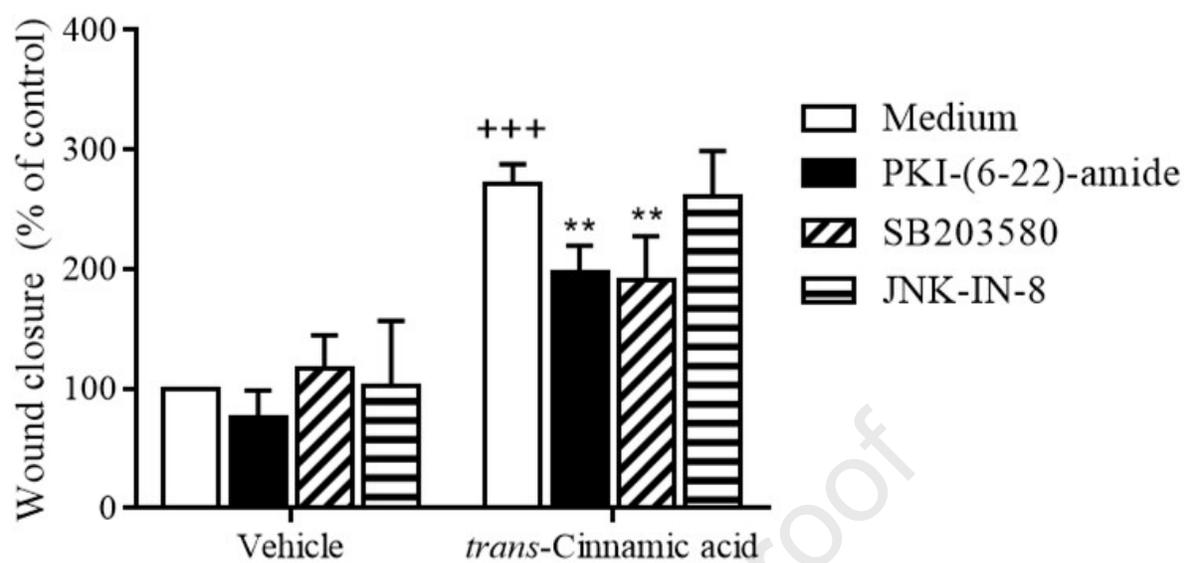


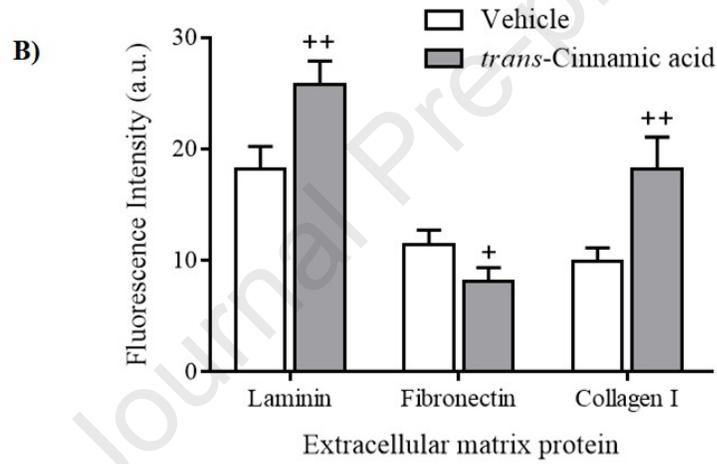
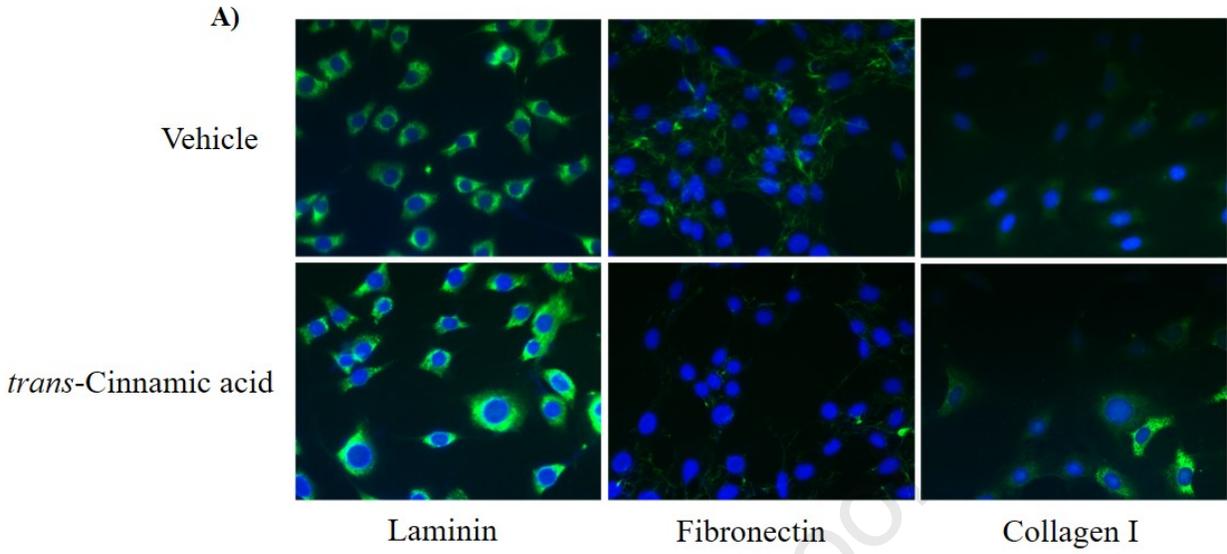












## Highlights

- Treatment with *trans*-cinnamic acid, but not methyl cinnamate or *p*-coumaric acid, caused an increase in fibroblasts migration.
- *trans*-Cinnamic acid-induced fibroblast migration was dependent of PKA and p38-MAPK signaling pathways.
- *trans*-Cinnamic acid provoked in fibroblasts the synthesis of laminin and collagen type 1, but not of fibronectin.

### **Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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