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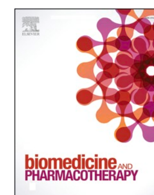

Anti-oxidant and anti-inflammatory effects of ellagic and punicic acid in an in vitro model of cardiac fibrosis

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ABSTRACT

Cardiac fibrosis is a pathological process characterized by an excessive deposition of extracellular matrix (ECM) and an increased production of fibrillar collagen in the cardiac interstitium, mainly caused by the activation of cardiac fibroblasts and their transition into myofibroblasts. Oxidative stress is deeply implicated in the pathogenesis of cardiac fibrosis both directly and via its involvement in the tumor growth factor β1 (TGF-β1) signaling. Ellagic acid (EA) and punicic acid (PA) are the main components of the Punica granatum L (pomegranate) fruit and seed oil respectively, whose antioxidant, anti-inflammatory and anti-fibrotic effects have been previously described. Therefore, the aim of this study was to investigate the effects of EA or PA or EA+PA in an in vitro model of cardiac fibrosis. Immortalized Human Cardiac Fibroblasts (IM-HCF) were stimulated with 10 ng/ml of TGF-β1 for 24 h to induce a fibrotic damage. Cells were then treated with EA (1 μM), PA (1 μM) or EA+PA for additional 24 h. Both EA and PA reduced the pro-fibrotic proteins expressions and the intracellular reactive oxygen species (ROS) accumulation. The anti-oxidant activity was also observed by Nrf2 activation with the consequent TGF-β1-Smad2/3-MMP2/9 and Wnt/β-catenin signaling inhibition, thus reducing collagen production. EA and PA significantly inhibit NF-κB pathway and, consequently, TNF-α, IL-1β and IL-6 levels: the greater effect was observed when EA and PA were used in combination. These results suggest that EA, PA and in particular EA+PA might be effective in reducing fibrosis through their antioxidant and anti-inflammatory properties by the modulation of different molecular pathways.

1. Introduction

Cardiac fibrosis is a pathological process characterized by an excessive deposition of extracellular matrix (ECM) and an increased production of fibrillar collagen in the cardiac interstitium, mainly caused by the activation of cardiac fibroblasts and their transition into myofibroblasts [1]. This pathological condition may be observed following myocardial ischemia and hypoxia consequent to myocardial infarction which is considered one of the main causes of fibrosis induction; indeed, during the early onset of cardiac dysfunction, fibroblasts are activated into myofibroblasts to preserve myocardium structural integrity; however, the lasting proliferation of myofibroblasts causes an excessive deposition of ECM which may lead to heart failure [1]. Oxidative stress is deeply implicated in the pathogenesis of cardiac fibrosis both directly and stimulating the tumor growth factor β1 (TGF-β1) signaling. Thus, TGF-β1 signaling triggering mediated by reactive oxygen species (ROS) may lead to fibroblast activation, thus contributing to an increase of ECM deposition in the cardiac interstitium [2,3]. TGF-β1 signaling activation engages Smad-2/3 that stimulate the transcription of different pro-fibrotic genes, such as COL1A1, COL3A1, MMP-2 and MMP-9 [4,5]. Furthermore, the TGF-β1-Smad-2/3 axis induces the production of canonical WNTs proteins, such as Wnt1 and Wnt3 [6]; these proteins boost the nuclear translocation and accumulation of β-catenin that has been identified as a crucial step for the induction of a

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pro-fibrotic state through myofibroblast activation [7–9]. Both Wnt/β-catenin pathway and oxidative stress may induce an inflammatory state thus promoting the activation of different pro-inflammatory pathways and the production of pro-inflammatory cytokines such as interleukin 1β (IL-1β), interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α), which negatively affects heart function [10]. In this scenario, the nuclear factor erythroid 2–related factor 2 (Nrf2) plays a pivotal role in the prevention of oxidative and inflammatory damage in cardiomyocytes [11]. In fact, Nrf2 usually activates the expression of cytoprotective genes in response to ROS [12] and it is also able to exert anti-inflammatory effects through the down-regulation of the Nuclear Factor-κB (NF-κB) which is one of the master regulators of the inflammatory response [13,14]; Nrf2 not only balances oxidative stress and reduces inflammation, but also negatively affects TGF-β1-mediated pro-fibrotic signaling, suggesting that Nrf2 targeting could have anti-fibrotic cardiac effects [15,16]. Treatment of cardiac fibrosis still has limited efficacy and currently there is no drug approved for the treatment of this condition [17]. For this reason, finding new alternative approaches for the management of cardiac fibrosis is of great interest. Therefore, the use bioactive compounds with antioxidant and anti-inflammatory effects could be a new strategy for the treatment of ROS-related conditions, including cardiac fibrosis [12,15]. *Punica granatum* L (pomegranate) is a fruit whose extracts showed significant therapeutic effects; in particular, ellagic acid (EA) represents the main component of the pomegranate fruit with well recognized antioxidant and anti-inflammatory activities [18,19]. EA anti-fibrotic effects were also observed in lungs, pancreas and heart in in vivo models through the inhibition of fibroblasts activation and Wnt/β-catenin signaling suppression [20–22]. Also, the use of Punicic acid (PA), which constitutes around 70–76 % of the pomegranate seed oil, was effective in reducing oxidative stress and inflammation with no toxic effects as well as EA [23–25]. Therefore, the aim of this study was to investigate the effects of ellagic or punicic acid or a combined treatment of ellagic and punicic acid in an in vitro model of cardiac fibrosis.

2. Material and methods

2.1. Cell culture

Immortalized Human Cardiac Fibroblasts (IM-HCF) were purchased by Innoprot (Derio, Spain). IM-HCF cells were cultured in Fibroblast basal Medium-2 (FM-2) (Innoprot, Derio, Spain) supplemented with 5 % of fetal bovine serum (FBS) (Innoprot, Derio, Spain), 1 % of penicillin/streptomycin (Innoprot, Derio, Spain) and 1 % of Fibroblast Growth supplement-2 (Innoprot, Derio, Spain) in a humidified incubator at 37 °C with a percentage of 5 % CO_2. The culture medium was replaced every 2–3 days.

2.2. Cell treatments

IM-HCF cells were seeded in six well plates at a density of 1.5×10^6 cells/well; upon reaching confluence, cells were stimulated with TGF-β1 10 ng/ml (Sigma Aldrich, Milan, Italy) for 24 h to reproduce a fibrotic damage. The day after, cells were treated with Ellagic acid 1 μM (Sigma Aldrich, Milan, Italy), Punicic acid 1 μM (Cayman Chemical, Ann Arbor, MI, USA) or the combination of EA and PA for additional 24 h.

EA and PA doses were titrated on the basis of their effects on Nrf2 expression (Supplemental Fig. 1) while TGF-β1 dose was chosen in accordance with previous published paper [26].

2.3. MTT assay

Cell viability assay was used to evaluate EA and PA cytotoxic effects. In detail, IM-HCF cells were cultured in a 96-well plate at a density of 1×10^5 cells/well and were incubated with TGF-β1 (10 ng/ml), TGF-β1 + EA (1 μM), TGF-β1 + PA (1 μM), TGF-β1 + EA and PA (1 μM) for 24 h. Twenty μl of tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Alfa Aesar, Heysham, UK) was dissolved in sterile PBS and was added into each well 5 h before the end of the incubation; 200 μl/well of dimethyl sulfoxide (DMSO) (Sigma Aldrich, Milan, Italy) were then added to dissolve the insoluble formazan crystals and to measure cell viability by using VICTOR Multilabel Plate Reader (Perkin Elmer; Waltham, Ma, USA) at λ 540 and 620 nm. Results are expressed as percentage of cell viability compared to untreated cells.

2.4. Intracellular ROS levels measurement

5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) probe was used to evaluate the accumulation of intracellular ROS in IM-HCF cells stimulated with TGF-β1 and treated with EA and PA alone or EA+PA. CM-H2DCFDA probe 5 μM (Thermo Fisher, Carlsbad, CA, USA) was added into each well for 1 h at 37 °C; cells were washed 2–3 times with sterile PBS and observed with a fluorescent microscope. Fluorescence quantification was performed by using ImageJ software for Windows (Softonic, Barcelona, Spain).

2.5. Real-Time quantitative PCR (RTqPCR)

Total RNA was isolated from IM-HCF cells with Trizol LS Reagent Kit (Life Technologies, Monza, Italy) and 1 μg was reverse transcribed by using the Superscript IV RT Master Mix (Invitrogen, Carlsbad, CA, US) following quantification with a spectrophotometer (NanoDrop Lite, Thermo Fisher, Carlsbad, CA, USA). qPCR reaction was carried out with 1 μl of cDNA, BrightGreen qPCR Master Mix (ABM, Richmond, Canada) and specific primers at the concentration of 10 μM in a total volume of 20 μl/well in order to evaluate Collagen 1a1, metalloproteinase 2 (MMP-2), metalloproteinase 9 (MMP-9), Wnt1 and β-Catenin mRNA expression. QuantStudio 6 Flex (Thermo Fisher, Carlsbad, CA, USA) was used to monitor qPCR reaction and the data were quantified using the $2^{-\Delta\Delta CT}$ method using GAPDH as housekeeping gene [27,28].

The primers for targets and reference genes are listed in Table 1.

2.6. Western Blot

Total protein content was extracted from IM-HCF cells using RIPA buffer with proteinase inhibitors and cell supernatants were used for quantification with Bradford method following centrifugation. Proteins (30 μg) were run by electrophoresis on a 10 % SDS polyacrylamide gel and transferred to PVDF membranes (Amersham, Little Chalfont, UK) at 200 mA for 1 h. The obtained membranes were incubated with non-fat dry milk (5 %), washed 3 times with TBS/0.1 % Tween buffer, and then incubated with primary antibodies for COl1a1, β-Catenin, pNF-κB (Cell Signaling, Danvers, MA, USA), Nrf2 (Abcam, Cambridge, UK) and Wnt1 (Thermo Fisher, Carlsbad, CA, USA) diluted in TBS-0.1 % Tween, overnight at 4 °C. The day after, a secondary peroxidase-conjugated goat anti-rabbit antibody (KPL, Gaithersburg, MD, USA) was used for 1 h at

Table 1
Primer list.

Gene Name	Sequence
GADPH	Fw:5'GAGTCAACGGATTTGGTCGT3' Rw:5'TTGATTTTGGAGGGATCTCG3'
β-catenin	Fw:5'GCCGGCTATTGTAGAAGCTG3' Rw:5'GAGTCCCAAGGAGACCTTCC3'
Col1a1	Fw:5'GTGCTAAAGGTGCCAATG3' Rw:5'CTCCTCGCTTTCCTTCCTCT3'
Wnt-1	Fw:5'TTCTCCGGGTCCTCCTAAGT3' Rw:5'ATGGCTCCACGACAGAGACT3'
MMP2	Fw:5'ATGACAGCTGCACCACTGAG3' Rw:5'ATTTGTTGCCCAGGAAAGTG3'
MMP9	Fw:5'TTGACAGCGACAAGAAGTGG3' Rw:5'GCCATTCACGTCGTCCTTAT3'

room temperature. Images were obtained and quantified by a scanning densitometry using a bio-image analysis system (C-DiGit, Li-cor, Lincoln, NE, USA). Data were expressed as integrated intensity using β-actin (Cell Signaling, Danvers, MA, USA) as control for equal loading samples [29,30].

2.7. Enzyme-linked immunosorbent assay (ELISA)

MMP-2, MMP-9, IL-1β, IL-6 and TNF-α protein levels were measured in the cell culture supernatants, using Enzyme-Linked Immunosorbent Assay (ELISA) kits (Abcam, Cambridge, UK), in agreement with the instructions given by the manufacturer.

2.8. Statistical analysis

All the results are expressed as mean ± standard deviation (SD). The reported values are the results of at least three experiments. All assays were performed in duplicate to ensure reproducibility. The differences between the groups were evaluated by one-way ANOVA with Tukey's post-test. A p value less than 0.05 was considered significant. Graphs were prepared using GraphPad Prism Version 8.0 for macOS (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

3.1. Ellagic and Punicic acid concentrations do not affect cell viability

Cell viability was evaluated in IM-HCF cells stimulated with TGF-β1 and treated with EA and PA, alone or in combination. One hundred percent of viability was observed in control cells (unstimulated and untreated) following 24 h; neither TGF-β1 incubation at the concentration of 10 ng/ml nor EA or PA or EA+PA in combination did not affect cardiac fibroblast viability. These results demonstrated that the natural compounds used in this study were safe and did not cause cell death (Fig. 1).

3.2. Ellagic and Punicic acid show anti-oxidant effects

Intracellular ROS levels was markedly increased in IM-HCF challenged with TGF-β1 for 24 h compared to unstimulated cells (Fig. 2B). EA and PA significantly reduced ROS accumulation compared to untreated IM-HCF cells stimulated with TGF-β1 (Fig. 2C-D); the co-incubation of EA and PA showed a greater effect than that observed when EA or PA were used alone, as demonstrated by the reduction of fluorescence signal showed in Fig. 2E. These results were quantified and summarized in Fig. 2F. The anti-oxidant effects of the EA and PA were also confirmed by evaluating Nrf2 levels, which finely regulates oxidative stress in favor of the antioxidant response. A significant decrease of Nrf2 expression was observed in TGF-β1-challenged cells compared to controls (Fig. 2G), as a consequence of the oxidative stress induced by TGF-β1. EA and PA significantly increased Nrf2 levels (Fig. 2G) and also the co-incubation with EA and PA markedly improved Nrf2 protein expression with a greater effect than that detected when EA or PA were used alone (Fig. 2G).

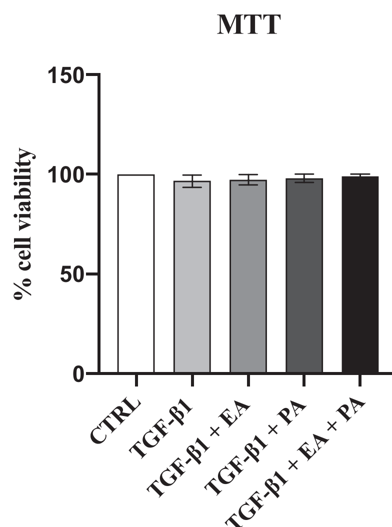

Fig. 1. The graph shows the cytotoxicity assay performed following 24 h of incubation with both TGF-β1(10 ng/ml) and ellagic acid (EA; 1 μM), punicic acid (PA; 1 μM) and EA+PA in IM-HCF cells. Values are expressed as the means and SD.

3.3. Ellagic and Punicic acid reduce the inflammatory panel

The protein expression of the transcription factor NF-κB and of the pro-inflammatory cytokines' TNF-α, IL-1β and IL-6 was investigated to study whether TGF-β1 might induce inflammation and EA and PA might modulate the inflammatory panel. TGF-β1 stimulus induced a significant upregulation of pNF-κB protein expression compared to controls (Fig. 3A). EA and PA significantly reduced pNF-κB protein expression compared to untreated cells (Fig. 3A), with a greater effect when the natural compounds were used together (Fig. 3A). TGF-β1 stimulus and the consequent NF-κB activation also caused a significant increase of TNF-α, IL-1β and IL-6 protein levels in TGF-β1-challenged cells compared to controls (Fig. 3 B-D). These pro-inflammatory cytokines were significantly reduced following EA, PA and especially EA+PA treatment, with a greater effect than that of EA or PA alone in inhibiting the NF-κB pathway (Fig. 3B–D).

3.4. Ellagic and Punicic acid modulates fibrosis and pro-fibrotic markers

mRNA and protein levels of Collagen1a1, MMP2 and MMP9 were studied to investigate the anti-fibrotic effects of EA, PA or their combination. A marked increase of mRNA expression of all tested pro-fibrotic markers was detected in IM-HCF cells stimulated with TGF-β1 (Fig. 4A–C); EA and PA, alone or in combination, significantly reduced Collagen1a1 and MMPs 2/9 mRNA expression compared to TGF-β1-challenged cells (Fig. 4A–C), thus demonstrating their ability in modulating these pro-fibrotic markers. These anti-fibrotic effects were also confirmed in the mature protein of MMPs 2/9 thus a significant reduction of the TGF-β1-induced protein levels was observed following EA, PA and EA+PA treatment, whereas Collagen1a1 protein expression was significantly decreased just following PA and EA+PA treatment (Fig. 4D–F).

3.5. Ellagic and Punicic acid inhibit Wnt/β-Catenin pathway

Both Wnt1 and β-Catenin mRNA and protein expression were investigated to evaluate the anti-fibrotic effect of EA and PA in cardiac fibroblasts. In fact, TGF-β stimulus caused a marked increase of Wnt1 and β-Catenin mRNA and protein expression (Fig. 5A–D); EA and PA alone or in combination significantly reduced mRNA expression and protein levels of both these pro-fibrotic molecules compared to TGF-β1 untreated group (Fig. 5A–D), thus demonstrating that these polyphenolic compounds might have an anti-fibrotic potential through Wnt/β-Catenin pathway inhibition (Fig. 5A–D).

4. Discussion

The activation of cardiac fibroblasts and their transition into myofibroblasts is one of the main events that characterize myocardial fibrosis [31]. Oxidative stress activation as well as inflammation with the consequent dysregulation of cytokine balance are related to tissue changes and may be considered as triggers of

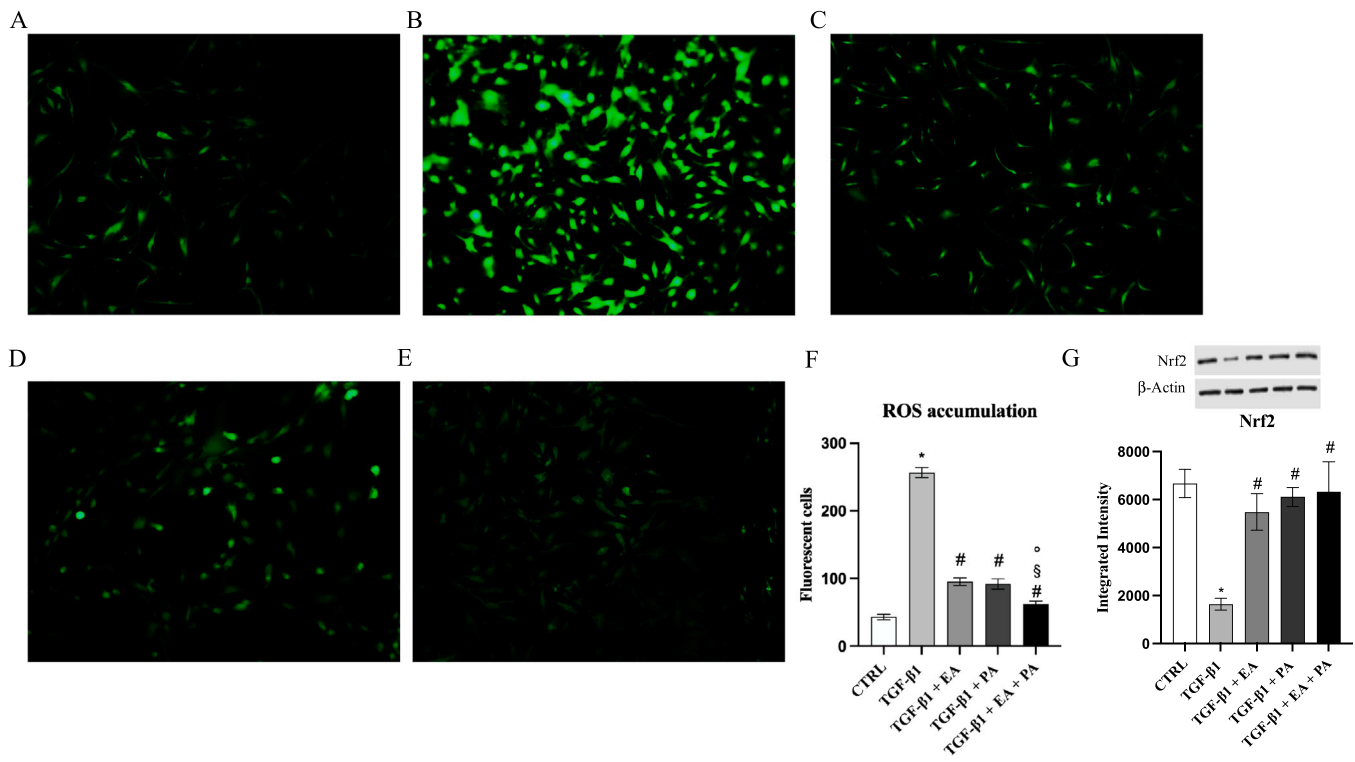

Fig. 2. Intracellular ROS accumulation evaluated by CM-H2DCFDA fluorescent probe from CTRL (A), TGF-β1 10 ng/ml (B), TGF-β1 + EA 1 μM (C), TGF-β1 + PA 1 μM (D), TGF-β1 + EA + PA (E) groups. All images were captured at 10X of magnification. Panel (F) Shows the number of FluoreScent Cells. Panel (G) shows EA and PA effects on Nrf2 protein levels. The data are expressed as means ± SD. *p < 0.05 vs. CTRL; #p < 0.05 vs. TGF-β1. §p < 0.05 vs TGF-β1 + EA; ∘p < 0.05 vs TGF-β1 + PA.

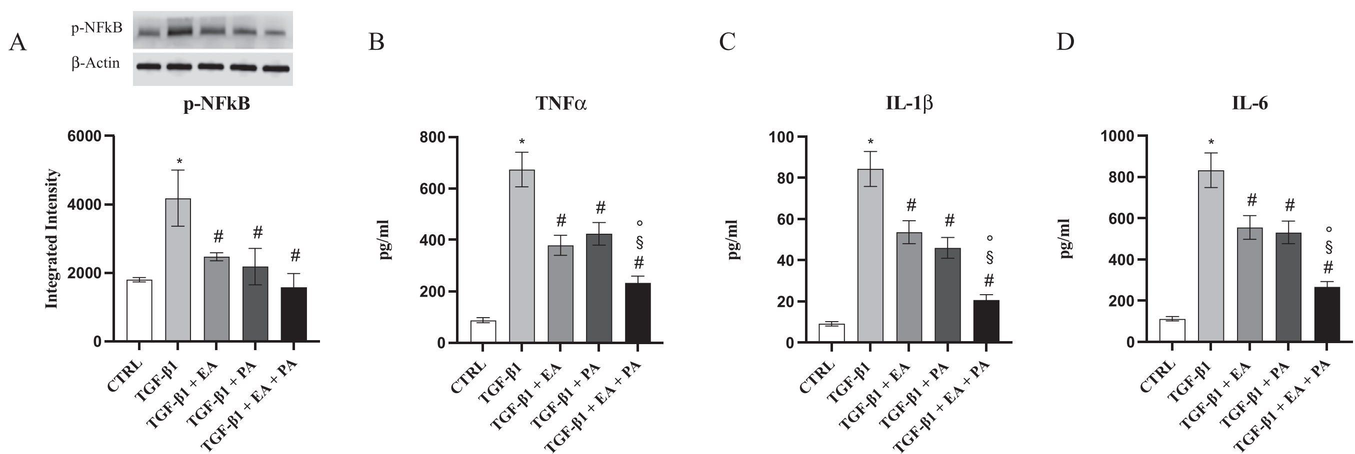

Fig. 3. The graphs represent p-NF-κB (A) protein expression (Western blot analysis) and TNF-α (B), IL-1β (C) and IL-6 (D) protein levels (ELISA assay) in IM-HCF cells stimulated with TGF-β1 (10 ng/ml) and treated with EA (1 μM), PA (1 μM) and EA+PA. Values are expressed as the means and SD. *p < 0.001 vs CTRL; #p < 0.05 vs TGF-β1; §p < 0.05 vs TGF-β1 + EA; ∘p < 0.05 vs TGF-β1 + PA.

fibroblast-to-myofibroblast differentiation. Oxidative stress may directly affect different molecular pathways such as TGF-β1 pathway with the involvement of Smad2/3 and MMP2/9 and the Wnt/β-catenin signaling that play important roles in cardiomyocyte regulation both physiologically and pathologically [32]. Previous studies reported the anti-oxidant and anti-inflammatory effect of ellagic and punicic acid in particular for the treatment of chronic disorders and ROS-related diseases, such as lung and pancreatic fibrosis [20,21,24]. Therefore, in the present study an in vitro model of cardiac fibrosis induced by TGF-β1 challenge was used to evaluate the anti-fibrotic effects of ellagic or punicic acid or a combined treatment of EA+PA. In fact, TGF-β1 may be considered a pro-fibrotic stimulus thanks to its ability in inducing a fibrotic damage, thus promoting structural and functional alterations: TGF-β1 significantly increases ROS levels, stimulating TGF-β1-associated fibroblast activation and myofibroblast differentiation and ROS themselves in turn stimulate TGF-β1 signaling [33]. In this experimental setting, IM-HCF cells challenged with TGF-β1 showed a significative increase of intracellular ROS levels together with a decreased Nrf2 expression compared to unstimulated cells. Both ellagic and punic acid significantly reduced ROS levels and increased the depressed expression of Nrf2 observed in TGF-β1 challenged cells, these positive effects were most evident when ellagic and punic acid were used in combination. These results were in accordance with previous papers that showed that Nrf2 stimulation is tightly related to the functional integrity of myocardial tissue during

Fig. 4. The graphs represent Col1a1 (A), MMP2 (B), MMP9 (C) mRNA expression (RTqPCR analysis) and Col1a1 (D) (Western blot analysis), MMP2 (E), MMP9 (F) protein levels (ELISA assay) in IM-HCF cells stimulated with TGF-β1 (10 ng/ml) and treated with EA (1 μM), PA (1 μM) and EA+PA. The data are expressed as the means and SD. *p < 0.05 vs CTRL; #p < 0.05 vs TGF-β1.

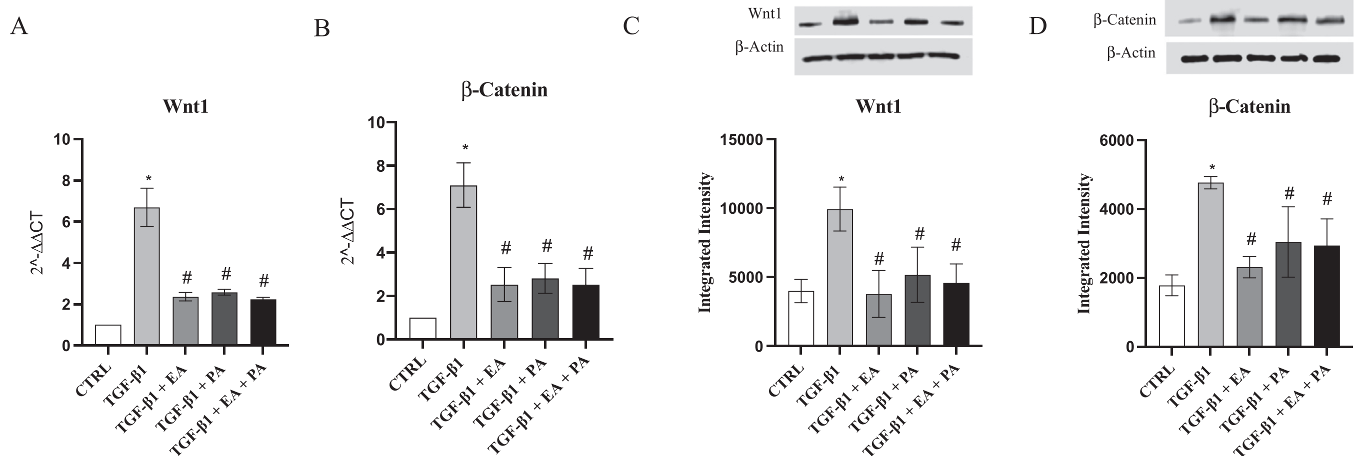

Fig. 5. The graphs represent Wnt1 (A), β-Catenin (B) mRNA expression (RTqPCR analysis) and Wnt1 (C), β-Catenin (D) protein levels (Western blot analysis) in IM-HCF cells stimulated with TGF-β1 (10 ng/ml) and treated with EA (1 μM), PA (1 μM) and EA+PA. The data are expressed as the means and SD. *p < 0.05 vs CTRL; #p < 0.05 vs TGF-β1.

cardiac remodeling, blocking also TGF-β1-induced fibrotic genes [16, 34]. Nrf2 has also been revealed to have anti-inflammatory properties thanks to its ability in modulating transcription factors, such as NF-κB and consequently pro-inflammatory cytokines (TNF-α, Il-1β and IL6) production [35,36]. NF-κB is surely one of the main transcription factors involved in the inflammatory response but also plays a critical role in

fibrosis, and in particular in the progression of inflammation associated to cardiac fibrosis [37,38]. The results obtained in this experimental condition showed an enhanced expression of NF-κB and increased levels of TNF-α, Il-1β and IL-6; both ellagic and punic acid and even more their combination significantly reduced NF-κB expression and its down-stream signal in accordance with previous papers that reported the anti-inflammatory effects of these natural compounds [39–41]. Cardiac fibrosis is also influenced by Smad-2/3 and MMPs 2/9 as result of the TGF-β1 signaling [42,43], thus further stimulating cardiac fibroblasts conversion into myofibroblasts, contributing to interstitial fibrosis, cardiomyocyte death and increased heart stiffness [44]. In our study, TGF-β1 challenge was able to induce the up-regulation of mRNA expression and protein levels of MMP2, MMP9 and Col1a1 in IM-HCF cells, probably as a consequence of the TGF-β1 pathway, with the involvement of Smad-2/3 which participate in metalloproteinases and collagen secretion. Ellagic and punic acid also inhibited Smad-2/3 signaling, reducing the protein levels of MMP2, MMP9 and Col1a1, in accordance with previous papers that demonstrated the efficacy of these natural compounds in the management of lung fibrosis [20,45]. Moreover, TGF-β1 signaling also activates the Wnt/β-catenin pathway likewise implicated in the pathogenesis of lung, dermal and liver fibrosis as well as in scarring after myocardial fibrosis [46–48]. In the present study TGF-β1 challenged cells showed a significant increase of both Wnt and β-catenin levels and ellagic and punic acid treatments significantly reduced the mRNA expression and protein levels of these pro-fibrotic mediators, thus confirming the results of previous papers that showed the ability of these natural products in the downregulation of the Wnt/β-catenin signaling with consequent inhibition of fibroblast activation and ECM production [20,49]. In conclusion, our results suggest that ellagic and punic acid may inhibit TGF-β1-Smad-2/3-MMP2/9 and Wnt/β-catenin signaling through Nrf2 activation, which also contributes to the modulation of the transcription factor NF-κB and its down-stream signal and highlight the beneficial effects of these natural compounds against the inflammatory process associated to cardiac fibrosis. Nowadays a specific therapeutic approach has not been approved for the treatment of cardiac fibrosis; however, experimental studies showed that novel target might be used for myocardial fibrosis management, such as epigenetic enzymes, genes, and signaling molecules [50]. Among the approved and used drugs, the renin-angiotensin system (RAS) inhibitors represent the standard therapy for cardiac fibrosis thanks to their effect on fibroblast differentiation inhibition. However, fibrosis may also persist in MI patients treated with RAS inhibitors, therefore these therapeutic approaches could be considered not enough, thus indicating the need to develop novel and adjuvant antifibrotic therapies. Therefore, these preliminary findings are intriguing and collectively suggest that ellagic and punicic acid and even more their association could be considered in the future as adjuvant approaches for the management of cardiac fibrosis. However, additional studies will be needed to confirm their efficacy in the field of cardiac fibrosis and their possible use in the clinical practice.

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CRediT authorship contribution statement

Federica Mannino: Writing – original draft. **Chiara Imbesi:** Investigation, Formal analysis. **Alessandra Bitto:** Validation. **Letteria Minutoli:** Validation. **Francesco Squadrito:** Funding acquisition. **Giovanni Pallio:** Writing – review & editing, Supervision. **Natasha Irrera:** Conceptualization, Project administration.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

Data Availability

Data will be made available on request.

Acknowledgments

None.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.114666.

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