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ORIGINAL PAPER

Capparis spinosa protects against oxidative stress in systemic sclerosis dermal fibroblasts

Yue-lan Cao · Xin Li · Min Zheng

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Abstract High reactive oxygen species (ROS), Ha-Ras, and active ERK1/2 in fibroblasts play an essential role in the pathogenesis of systemic sclerosis (SSc). The present study was carried out to evaluate the effects of the ethanol extract from fruits of *Capparis Spinosa* L. (ECS) on oxidative stress and ROS-ERK1/2-Ha-Ras signal loop in SSc dermal fibroblasts in vitro. Cultured dermal fibroblasts from three SSc patients and three normal controls were treated with ECS by different concentration (10, 50, 100 μg/ml). ECS significantly reduced the production of O_2^-, H_2O_2, and ROS in SSc fibroblasts in a dose-dependent manner. ECS effectively minimized the loss of cell viability and apoptosis induced by H_2O_2 in normal and SSc fibroblasts. Furthermore, the protective effect of ECS on SSc fibroblasts was more significant than on normal ones. ECS decreased the expression of P-ERK1/2 and Ha-Ras in a dose-dependent manner. In conclusion, ECS exhibits a notable activity in protecting against oxidative stress and interrupting of ROS-ERK1/2-Ha-Ras signal loop in SSc, suggesting its potential protective effects against skin sclerosis.

Keywords Systemic sclerosis · Reactive oxygen species · Oxidative stress · Ha-Ras · *Capparis spinosa* L.

Y. Cao (✉) · M. Zheng
Department of Dermatology,
Second Affiliated Hospital, School of Medicine,
Zhejiang University, 88 Jiefang Road,
310009 Hangzhou, China
e-mail: CYL123321@sohu.com

X. Li
Department of STD and AIDS Prevention,
Hangzhou Center for Disease Control and Prevention,
310006 Hangzhou, China

Abbreviations

SSc	Systemic sclerosis
ECS	The ethanol extract from fruits of *Capparis Spinosa* L.
ROS	Reactive oxygen species
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
ERK	Extracellular signal-regulated kinase
DCFH-DA	Fluorescent probe 2′-7′-Dichlorodihydrofluorescin diacetate

Introduction

Capparis spinosa L. belongs to the Capparidaceae Family. This green spiny shrub distributes throughout the Mediterranean basin and grows wildly in the north-west region of China. Apart from its use as flavoring, *C. spinosa* has been known as a traditional herbal medicine for its diuretic, antihypertensive and tonic properties for centuries [7]. Previous chemical studies have reported that alkaloids, lipids, polyphenols, flavonoids, indole and glucosinolates were isolated from *C. spinosa* [19, 22]. Various extracts of *C. spinosa* have been showed antifungal [1], anti-inflammatory [18], antiallergic [26], antihepatotoxic [12], anti-diabetic [10], and hypolipidemic effects [11]. The methanol extract of *C. spinosa* showed a noteworthy antioxidant/free radical scavenging effectiveness in various in vitro models [6, 13], and this extract has been suggested to treat oxidative stress-based pathological diseases.

Systemic sclerosis (SSc) is a connective tissue disorder that typically results in fibrosis of the skin and internal organs [5]. Although the pathogenesis of SSc is unknown, the pathologic increase in tissue collagen deposition in affected organs is mainly the result of its overproduction by

dermal fibroblasts [14, 15]. Recently, it is proposed that excessive oxidative stress might be involved in the pathogenesis of SSc [20, 21, 25]. Fibroblasts derived from SSc patients contain high ROS and Ha-Ras levels and exhibit constitutive activation of ERK1/2. The activation of ROS-Ha-Ras-ERK1/2 loop in SSc fibroblasts lead to collagen over-synthesis. Inhibition of either ROS, or ERK1/2, or Ha-Ras converted SSc fibroblasts to normal ones [20, 25].

On the basis of the properties of the active compounds contained in *C. spinosa*, we presume that *C. spinosa* may have important clinical applications in scleroderma. We observed for the first time that the ethanol extract of *C. spinosa* (ECS) could significantly reduce the expression of α_2(I) collagen mRNA and type I collagen protein in SSc fibroblast in a dose- and time-dependent manner [8]. In the present work we investigated the effects of the pool of active compounds contained in ECS on oxidative stress and the ROS-Ha-Ras-ERK1/2 loop in SSc fibroblasts.

Materials and methods

Reagents

Collagenase, Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), L-glutamine, pancreatic trypsin were obtained from Gibco (Milan, Italy). Dimethylsulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide(MTT), *N*-acetyl-L-cysteine(NAC), ferricytochrome C, Superoxide dismutase, homovanilic acid, horseradish peroxidase were obtained from Sigma-Aldrich Co (St. Louis, MO, USA). Fluorescent probe 2′-7′-dichlorodihydrofluorescin diacetate (DCFH-DA) purchased from Molecular Probes (Eugene, OR, USA). Annexin V/PI assay kit purchased from Bender (Vienna, Austria). The bicinchoninic acid protein assay kit was from Pierce (Lockford, IL, USA). Anti-p-ERK1/2, anti-ERK1/2, anti-Ha-ras, anti-Ki-ras and antirabbit IgG horseradish peroxidase antibodies were purchased from Chemicon (CA, USA). Polyvinylidene difluoride membranes were from Millipore (Milford, MA). The chemiluminescence detection kit was acquired from Amersham Pharmacia (Arlington Heights, IL, USA).

Preparation of the extract of *C. spinosa*

Fruits of *C. spinosa* were collected from Xinjiang province in August 2006 and air-dried at 37°C. They were authenticated by Associate Professor Liurong Chen (College of Pharmaceutical Sciences, Zhejiang University, China). Voucher specimens (XJ0608) were deposited at the herbarium of Department of Dermatology, Second Affiliated Hospital, School of Medicine, Zhejiang University, China, for further reference. The fruits (100 g) were chopped into small pieces and then extracted twice with 95% ethanol (800 ml) by reflux at 80°C for 1 h and the solution pooled. The solution was filtered and evaporated to dryness under reduced pressure to obtain the extract 10.26 g. For in vitro tests, ECS was reconstituted in DMSO, sterilized with 0.22 μm filter and further diluted with serum-free medium to defined concentrations prior to use. The amount of total flavonoids in the ethanol extract was $5.439 \pm 0.736\%$ (w/w) (as rutin equivalents) determined by ultraviolet spectrophotometric analysis as described previously [9]. In addition, the level of rutin was calculated as $0.353 \pm 0.007\%$ (w/w) by HPLC analysis [13].

Primary fibroblasts

Human dermal fibroblasts were obtained from punch biopsies taken from the leading edge of affected areas (abdomen or dorsal forearm) of three early stage (<2 years) patients with diffuse cutaneous SSc, who fulfilled the American Rheumatism Association preliminary criteria for the diagnosis of SSc [24]. Control fibroblasts were obtained by skin biopsies from three age- and sex-matched healthy donors. SSc and control samples were processed at equal pace. Primary fibroblasts were maintained in 25 cm^2 culture flasks in DMEM containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. Monolayer cultures were incubated at 37°C in 5% CO_2. Fibroblasts between the fourth and the sixth subpassage were used in all experiments. Confluent quiescent fibroblasts were treated with different concentrations of ECS (10, 50, 100 μg/ml) for 48 h.

ROS assay

The superoxide anion release from fibroblasts into the overlying medium was estimated using the superoxide dismutase-inhibitable cytochrome c reduction [21]. The hydrogen peroxide release was measured by a modified method described by Valletta and Berton [28]. The levels of reactive oxygen species (ROS) in living fibroblasts, loaded with fluorescent probe 2′-7-dichlorodihydrofluorescin diacetate (DCFH-DA) [17, 20], were observed under Olympus IX70 Inverted Microscope (Olympus, Middlesex, UK), and further measured by flow cytometric analysis using a FACS Calibur (BD Biosciences, San Jose, CA, USA). General ROS scavenger NAC (10 mM) was used as a reference compound.

Cell viability assay

The protective effect of ECS against the cytotoxic effect on normal and SSc fibroblasts induced by H_2O_2 was evaluated by MTT tests [16].The cells were incubated for 2 h with

6 mM H_2O_2 alone or pretreated for 48 h with different concentration of ECS, washed with PBS, and treated with 10 µl MTT dye. After 4 h incubation, the growth medium was removed and the formazan crystals were dissolved with 100 µl DMSO. The absorbance was quantified at 570 nm using an ELX800 Microplate Reader (Bio-Tek Instruments, Vermont, USA).

Apoptosis assay

The protective effect of ECS on the apoptosis of normal and SSc fibroblasts induced by H_2O_2 was evaluated by flow cytometric analysis. When grown to 80–90% confluence, fibroblasts were preincubated with 100 µg/ml ECS for 48 h followed by treatment with 2 mM H_2O_2 for 2 h. After the removal of the stimulus, apoptosis was detected by flow cytometric analysis using annexin V/PI assay kit.

Immunoblot analysis

After treatment, cytoplasmic extracts were lysed with lysis buffer containing 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mMNa_2EDTA, 1 mM EDTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mMNaF, 1 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The concentration of protein extracts was determined using the bicinchoninic acid protein assay kit. Twenty micrograms of protein were separated in a 12% polyacrylamide gel and transferred onto Polyvinylidene difluoride membranes. After blocked for 2 h, the membranes were incubated with the following antibodies overnight at 4°C: anti-Ha-Ras (1:800), anti-Ki-Ras (1:800), anti-P-ERK1/2 (1:1000), anti-ERK1/2 (1:1000), and incubated with secondary antibodies (1:5,000) for 1 h at room temperature, then washed, visualized by the chemiluminescent assay, and quantitated using Gel-Pro 3.1 imaging software (Media Cybernetics, Silver Spring, MD).

Statistical analysis

Results are expressed as the mean ± SD. Statistical analysis using SPSS 11.0 software was carried out with one-way ANOVA followed by the Student-Newman–Keuls test for comparison between groups. P values less than 0.05 were considered to be significant.

Results

ECS reduces the release of O_2^- and H_2O_2 in SSc fibroblasts

As shown in Fig. 1, The amounts of superoxide anion and H_2O_2 released by unstimulated SSc fibroblasts were

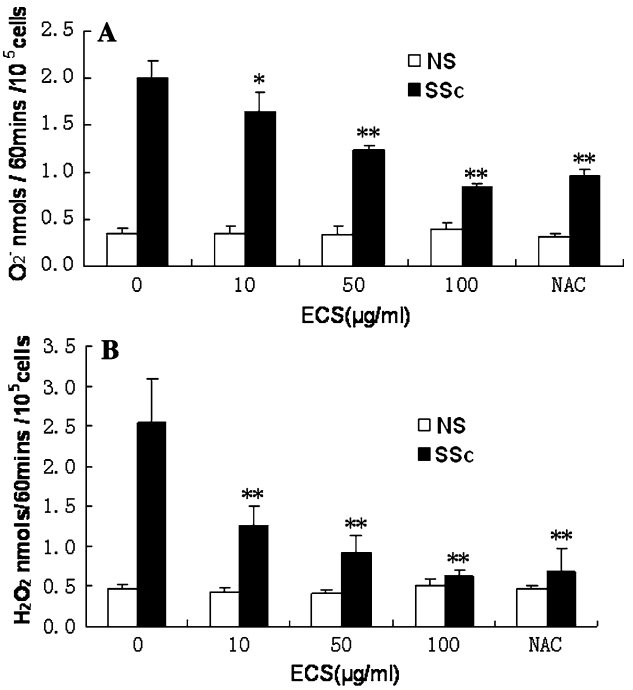

Fig. 1 ECS reduced the release of O_2^- and H_2O_2 in SSc fibroblasts. After incubated with different concentrations of ECS (10, 50, 100 µg/ml) and 10 mM NAC for 48 h, the superoxide anion levels generated by normal (*open bars*) and SSc (*closed bars*) fibroblast were measured using the superoxide dismutase-inhibitable cytochrome c reduction (**a**). H_2O_2 release was assayed using a modification of the method of Valletta and Berton (**b**). Values are the mean ± SD of three independent experiments performed in triplicate. * $P < 0.05$ versus control, ** $P < 0.01$ versus control

significantly greater than those generated by normal ones. The levels of O_2^- and H_2O_2 generated by normal fibroblasts did not change when the cells were treated with ECS and NAC. The treatment of SSc fibroblasts with ECS caused a dose-dependent inhibition on O_2^- (Fig. 1a) and H_2O_2 (Fig. 1b) production. 10 mM NAC decreased $O_2^-$and H_2O_2 production and these effects were similar to 100 µg/ml ECS.

ECS reduces the levels of ROS in SSc fibroblasts

The fluorescent probe DCFH-DA distributed within the different cell organelles, where it emitted according to the levels of intracellular ROS. The nuclear signal in normal fibroblasts was enhanced, whereas the cytoplasmic signal was reduced. The distribution pattern of the probe in quiescent SSc fibroblasts was as the same as in normal cells, but the signal was significantly higher, approaching saturation in both nuclear and perinuclear regions. After treatment with 100 µg/ml ECS, the fluorescent signal in SSc fibroblasts was markedly reduced to approximately the same as that in normal cells. This is also shown by treatment with 10 mM NAC (Fig. 2a). Trypan blue stain was used to demonstrate that 100 µg/ml ECS and 10 mM NAC did not

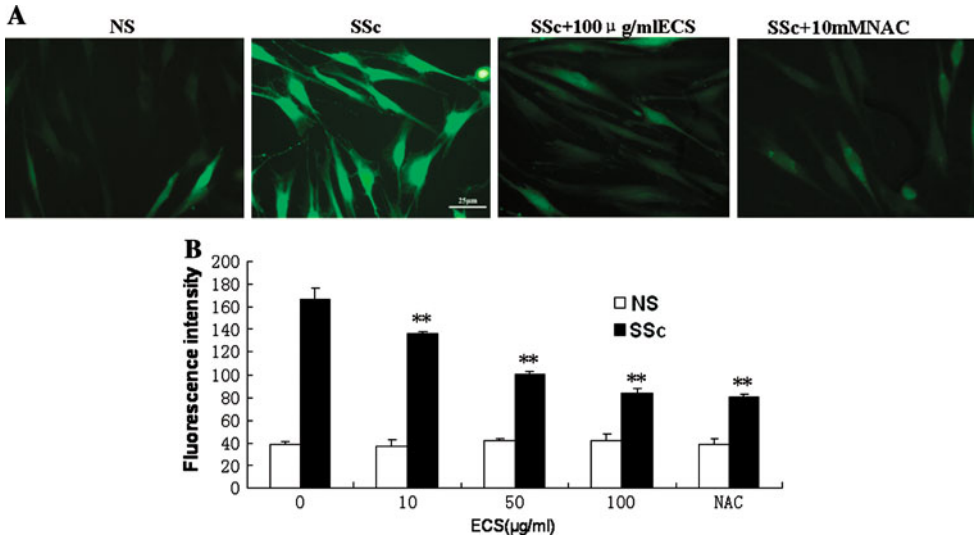

Fig. 2 ECS reduced the generation of ROS in SSc fibroblasts. **a** Imaging of ROS generation in fibroblast with the fluorescent probe DCFH-DA. Signal intensity is proportional to oxidative activity. Fluorescence microscopy fields of living, quiescent fibroblasts from healthy subject, SSc patient treated with 100 μg/ml ECS and 10 mM NAC for 48 h are shown. The result is representative of three separate experiments. *Bar* = 25 μm. **b** Levels of ROS evaluated as DCFH-DA fluorescence intensity by FACS analysis in normal (*open bars*) and SSc (*closed bars*) fibroblast lines after incubation with different concentrations of ECS (10, 50, 100 μg/ml) and 10 mM NAC for 48 h before ROS assay. Values are the mean ± SD of three independent experiments performed in triplicate. * $P < 0.05$ versus control, ** $P < 0.01$ versus control

cause cell death. The effect of different concentrations of ECS on the levels of ROS in living fibroblasts was further investigated as DCFH-DA fluorescence intensity by flow cytometric analysis. The levels of ROS in SSc fibroblasts were fourfold higher than that in normal controls. The treatment of SSc fibroblasts with ECS caused a dose-dependent inhibition in the ROS production. ECS at the dose of 100 μg/ml showed 49.30% inhibition, an antioxidant activity approximately equal to that of 10 mM NAC (51.24%) (Fig. 2b).

ECS protects against apoptosis and cell death induced by H_2O_2 in normal and SSc fibroblasts

MTT assay were performed to study the protective effect of ECS against the apoptotic death on normal and SSc fibroblasts induced by H_2O_2. Treatment with 6 mM H_2O_2 for 2 h, a high fraction of normal and SSc fibroblasts showed a decreased viability as compared to untreated controls. However, pretreatment with ECS at concentration of 10–100 μg/ml for 48 h before exposure to 6 mM H_2O_2 significantly prevented the loss of viability in a dose-dependent manner (Fig. 3a). Flow cytometric analysis was used to examine whether ECS protected against H_2O_2-induced apoptosis. A significant increase of apoptosis was observed in normal and SSc fibroblasts treated with 2 mM H_2O_2 as compared with control cells. SSc fibroblasts were more sensitive to oxidative stress-induced apoptosis than normal fibroblasts. However, ECS-pretreated fibroblasts showed significant resistance to H_2O_2-induced apoptosis. The protective effect of ECS on SSc fibroblasts was more significant than on normal fibroblasts (Fig. 3b, c).

ECS inhibits Ha-Ras expression and ERK/2 activation in SSc fibroblasts

After we confirmed that ECS can reduce the generation of ROS in SSc fibroblasts and protect them from H_2O_2-induced apoptosis, we further investigated the effects of this herbal extract on ROS-Ha-Ras-ERK1/2 loop. A significant increase of Ha-Ras protein and phosphorylated forms of ERK/2 was detected in SSc fibroblasts as compared with normal controls. After 48 h treatment, ECS significantly suppressed the protein levels of Ha-Ras and P-ERK1/2 in SSc fibroblasts in a dose-dependent manner (Fig. 4a, b).

Discussion

We have shown that ECS possessed a notable anti-fibrotic effects in SSc [8]. In the present work, we further investigated into mechanisms which might be implicated in the protective effects of ECS in vitro. It has been reported that low concentration of H_2O_2 could greatly stimulate the transcription of collagen genes in normal fibroblasts, suggesting ROS may be involved in the pathogenesis of SSc [25], Furthermore, the addition of ROS scavengers induced a dose-dependent down-regulation of a1(I) and a2(I) collagen genes in SSc [20]. Several reports have provided indirect [23] and direct [20, 21, 25] evidence of abnormal ROS

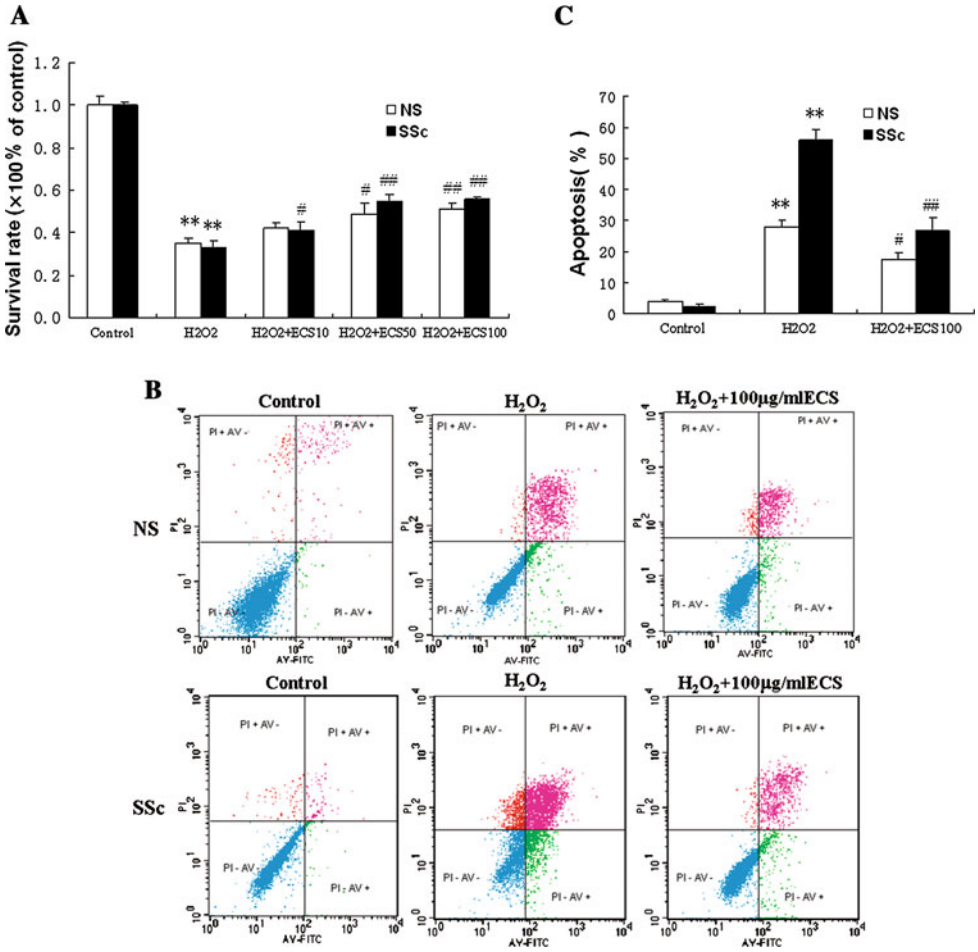

Fig. 3 ECS protected against cell apoptosis and death induced by H_2O_2 in normal and SSc fibroblasts. **a** The viability measured by MTT assay in normal and SSc fibroblast lines that were pretreated with different concentrations of ECS (10, 50, 100 μg/ml) for 48 h before exposure to 6 mM H_2O_2 for 2 h. Values are the mean ± SD of three independent experiments performed in triplicate. **b** The apoptosis of normal and SSc fibroblast was determined by FACS analysis of annexin V-PI stained cells pretreated with 100 μg/ml ECS for 48 h before exposure to 2 mM H_2O_2 for 2 h. Results are representative of three separate experiments. **c** *Bar graph* representing the percentage of apoptosis in the same samples. The data shown are the mean ± SD of three independent experiments performed in triplicate.* $P < 0.05$ versus control, ** $P < 0.01$ versus control; $^{\#}$ $P < 0.05$ versus H_2O_2-treated group, $^{\#\#}$ $P < 0.01$ versus H_2O_2-treated group

generation in this disease. Our findings are consistent with previous reports that SSc fibroblasts release increased amounts of H_2O_2 and O_2^- in vitro in the absence of stimulation. The generation of ROS in fibroblasts from SSc patients was significantly higher than those in normals. Although the antioxidant activity of extracts from *C. spinosa* has been reported in different in vitro tests, no report had been demonstrated before this study in terms of its effect on SSc fibroblasts. This study was undertaken to examine the effect of ethanol extract of *C. spinosa* on the production of ROS in SSc. To the best of our knowledge, the present study for the first time demonstrates that addition of this extract of natural compounds to SSc fibroblasts cultures could reduce the release of H_2O_2 and O_2^-, and suppress production of ROS in a dose-dependent manner.

It has been recently found that platelet-derived growth factor (PDGF) and ROS induce Ha-Ras expression through ERK1/2 in primary fibroblasts. This has revealed a novel pathway, the ROS-ERK1/2-Ha-Ras signaling loop. This signaling loop is relatively amplified in fibroblasts in patients with SSc [25]. In SSc patients, an increased level of PDGF receptor resulting from abnormal TGF-β signaling [27] and stimulatory autoantibodies against PDGF receptor [4] triggers increased production of ROS by activation of NADPH oxidase [20]. ROS, in turn, activates the extracellular signal-regulated kinases 1 and 2(ERK1/2). Activation of ERK1/2 and high ROS levels stabilize Ha-Ras protein by inhibiting proteasomal degradation rather than change mRNA levels of Ha-Ras. The long-term persistence of Ras-ROS-ERK1/2 loop ultimately results in accumulating DNA damage, stimulating collagen-gene expression and becoming prone to stress-induced apoptosis. Inhibition of any of the components of this loop (ROS, ERK1/2, or Ras) down-regulates the system and restores the normal phenotype in SSc fibroblasts [25].

Consistent with previous reports, our experiments showed that SSc fibroblasts were more sensitive to oxidative stress-induced apoptosis than normal fibroblasts. Effects of ECS in vitro suggest it could be therapeutic by blocking oxidative stress in SSc. In this study, ECS in a range of 10–100 μg/ml remarkably inhibited the cytotoxicity of normal and SSc fibroblasts caused by H_2O_2. Moreover, Flow cytometry analysis using annexin V showed that ECS significantly reduced the apoptosis in H_2O_2 treated cells. This indicates that ECS at certain concentrations exerts a significantly protective effect by inhibiting H_2O_2-induced cell apoptosis and death. The protective effect of ECS on SSc fibroblasts was more significant than on

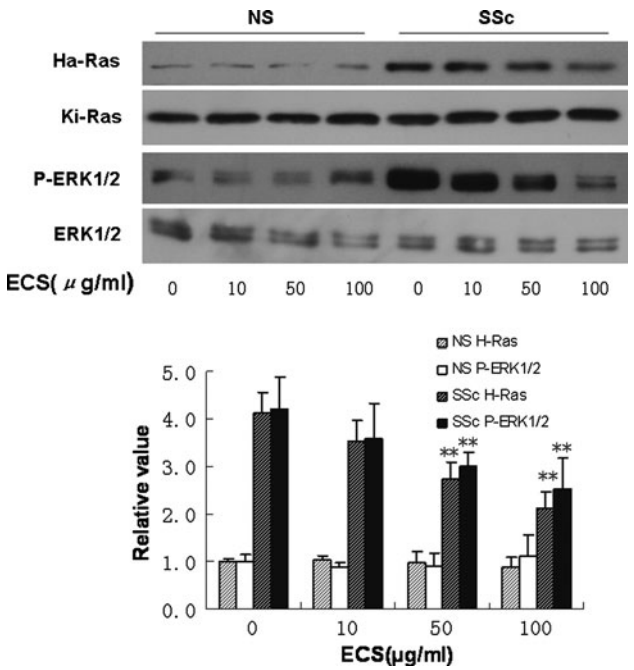

Fig. 4 ECS inhibited the expression of Ha-Ras and the activation of ERK1/2 in SSc fibroblasts. **a** After treatment for 48 h with 10, 50 and 100 μg/ml ECS, the protein expression of Ha-Ras and P-ERK1/2 in normal and SSc fibroblasts were determined by western blotting analysis. **b** Densitometric analyses of western blot are presented as the mean ± SD for three independent experiments performed in triplicate. * $P < 0.05$ versus control, ** $P < 0.01$ versus control

normals. The present study demonstrated that fibroblasts derived from SSc patients exhibited high levels of Ha-Ras and constitutive activation of ERK1/2. Furthermore, the herbal extract, ECS, could significantly inhibit activation of ERK1/2 and expression of Ha-Ras protein in a dose-dependent manner. Since the association of Ras-ROS-ERK1/2 loop and collagen-gene expression has been well documented, our findings point out that ECS suppresses the expression of α_2(I) collagen mRNA and type I collagen protein in SSc fibroblast, at least in part, through its antioxidant action and inhibition of the ROS- ERK1/2-Ras loop.

The pharmacological activities of ECS under our experimental conditions may be partially related to the high level of phenolic active constituents, especially flavonoids. Previous studies have reported that the methanol extract of *C. spinosa* is rich in flavonoid compounds such as quercetin derivatives (2.52% as rutin equivalents), kaempferol derivatives (3.28% as kaempferol-3-Orutinoside equivalents), and small amounts of hydroxycinnamic acids (caffeic acid, ferulic acid, p-cumaric acid, and cinnamic acid) [6]. The content of total flavonoids of ECS in our experiment was determined as 5.439 ± 0.736% (as rutin equivalents). The polyphenolic structures of flavonoids endow them with the ability of free radicals scavenge and transition metals chelation, a basis for their potent antioxidant abilities [3]. Flavonoids can interact with both lipid and protein components of biological membranes and alter their properties. They stabilize membranes by decreasing membrane fluidity [2]. The anti-apoptotic or cytoprotective action of flavonoids in ECS which prevent cell death caused by ROS is mainly associated to their protective effects on membranes. But active components, which are exactly responsible for the anti-fibrotic and antioxidant activity of ECS remain unknown, and await further research.

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