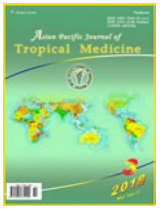

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Steroidal alkaloids ameliorate cell proliferation, oxidative stress, inflammation and histology outcome *in vitro* and *in vivo*

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ABSTRACT

Objective: To investigate the effects of steroidal alkaloids and sarcovagine D isolated from *Sarcococca hookeriana* var. *digyna* on cell-proliferation and secretion of inflammatory cytokines in TNF-α-induced human RA-FLS MH7A *in vitro*, and against complete Freund's adjuvant (CFA)-induced arthritis rats *in vivo*. **Methods:** CCK-8 assay was utilized to evaluate the anti-proliferation activity *in vitro*. In *in-vivo* study, rats were randomly divided into control group, model group (CFA), steroidal alkaloids (STA) groups (5.0, 2.5 and 1.25 g/kg BW), and sarcovagine D (SD) groups (50, 100 and 200 mg/kg BW), 10 rats for each group. To evaluate the anti-inflammation effect, the histology, biochemical parameters and expression of inflammatory cytokines were detected. **Results:** Steroidal alkaloids and sarcovagine D showed strong anti-proliferative activity during MH7A cell culture proliferation and downregulated NO levels, and inflammatory cytokines (IL-1β, IL-6 and PGE_2) *in vitro*. The rats treated with CFA induced ankle inflammation and lymphocyte infiltration, upregulated IL-1β, TNF-α, IL-6, PGE_2, MDA and NO levels, and downregulated IL-10 and SOD. On the contrary, steroidal alkaloids ameliorated all the changes induced by CFA in rats, suggesting the anti-inflammatory effect of steroidal alkaloids and sarcovagine D. **Conclusions:** The results suggest that *Sarcococca hookeriana* var. *digyna* has anti-inflammatory effect. Steroidal alkaloids and sarcovagine D has the potential to cure RA.

1. Introduction

The systemic, autoimmune, chronic inflammatory disorder-rheumatoid arthritis (RA) is characterized by persistent synovial proliferation[1,2]. Recent researchers have found that fibroblast-like synoviocytes (FLS) played an important role in the establishment and maintenance of RA[2]. Excessive inflammatory cytokines such as IL-6, IL-8, IL-10 and PGE_2, and proteolytic enzymes responded to oxidative stresses, such as superoxide dismutase (SOD) are released from RA-FLS in hyperplastic synovial tissue, resulting in degradation and destruction of cartilage and subchondral bone[3-5]. *Sarcococca hookeriana* var. *digyna* (*S. hookeriana* var. *digyna*) has long been used in folk medicine as an anti-rheumatic remedy for the treatment of trauma and bruises[6]. Crude extracts and its purified steroidal alkaloids (STA) are potential cholinesterase inhibitors (ChEIs)[7]. The cholinergic anti-inflammatory pathway protect the body during infection, and CHEIs show potential anti-inflammatory properties[8,9].

Sarcovagine D (SD), a steroidal alkaloid isolated from *Sarcococca*

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vagans, *Sarcococca ruscifolia* and *S. hookeriana* var. *digyna*, showed cytotoxic activities in many cancer cell lines[10,11], and showed significant immunosuppressive effect on IL-2 production and T-cell proliferation in a dose dependent manner[10].

To the best of our known, few studies have described the effects of STA and pure compound of *S. hookeriana* var. *digyna* on RA *in vitro* or *in vivo*; therefore, we aimed to explore its potential anti-proliferation of MH7A *in vivo* and the potential effect to cure complete Freund's adjuvant (CFA)-induced arthritic rat model *in vivo*.

2. Materials and methods

2.1. Reagents

Human RA-FLS MH7A cell line was obtained from Enzyme Research Technology (GuangZhou Jennio Biotech Co., Ltd, China). CCK-8 kit, human cytokine ELISA assay kits were purchased from Beyotime Biotechnology, China. Different aldehydes were purchased from Tianjin Fuchen Reagent Co., Ltd.

2.2. Processing and extraction of plant material

Whole plant of *S. hookeriana* var. *digyna* was gathered from the forest area of Banqiao, Enshi, Hubei of China, and authenticated by the author. The voucher specimen was deposited in the Herbarium of Hubei University for Nationalities, Hubei, China.

After authentication, the plant was dried and subjected to size reduction to get coarse powder (1.5 kg), defatted with petroleum ether (5 L), then infiltrated with ammonia and extracted with chloroform (5 L) to obtain the STA (17 g). The yield was about 1.13%.

To yield SD, a pH-zone-high speed countercurrent chromatography (HSCCC) method was utilized. Briefly, a quaternary solvent system-n-hexane: ethyl acetate: ethanol: water (6:1:7.2:1), with 10 mM trimethylamine in the upper phase and 5 mM HCl in lower phase was applied to isolation. Approximately 140 mg SD were obtained from 2 g STA for each isolation.

2.3. Cell lines and cell culture

MH7A cells[12] were cultured in DMEM/HIGH Glucose medium containing 10% fetal bovine serum (FBS; Hyclone Life Technologies, Australia) and 100 mg/L streptomycin (Solarbio, China) and 1×10 μ/L penicillin (Solarbio, China) and cultured at 37 ℃ in a 5% CO_2 incubator saturated humidity environment.

2.4. Cell viability assays

A CCK-8 assay was utilized to measure cytotoxicity of STA and SD. Approximately, 100 μL/well of MH7A (2×10^5 cell/mL) were added in a 96-well tissue culture plates, incubated for 24 h in 5% CO_2 at 37 ℃. Then, 10 μL/well of the test solution (6.25-500 μg/mL) was added and incubated for an additional 12 h. Afterwards 10 μL of CCK-8 was added and incubated for another 4 h. OD_{450} absorbance was determined using a full-wavelength automatic microplate reader (Thermo Scientific, USA). Results represented as cell viability. All reactions were run in triplicates.

Cell viability * (%) = [administration OD_{450}-blank OD_{450}] / [normal OD_{450}-blank OD_{450}] × 100.

2.5. NO inhibition

The secretion of NO was measured on MH7A. The experiment was divided into control group (without TNF-α and test solution), TNF-α treated group (50 ng/mL TNF-α), and test group (with 50 ng/mL TNF-α and appropriate concentration test solution). Briefly, 100 μL/well of MH7A (2×10^5 cell/mL) were plated in a 96-well tissue culture plates, and incubated for 24 h in 5% CO_2 at 37 ℃. Then 10 μL /well of the test solution was added and incubated for another 24 h. Afterwards, 50 μL of the supernatants was respectively incubated into a microtiter plate, then 50 μL of Griess Ⅰ and Griess Ⅱ was added, respectively. After mixing, OD_{540} absorbance was determined using a full-wavelength automatic microplate reader (Thermo Scientific, USA). The NO was analyzed by using NO kit according to manufacturer's instructions.

2.6. PGE_2, IL-1β and IL-6 inhibition

Pro-inflammatory cytokine IL-1β, IL-6 and PGE_2 was secreted from MH7A cell line. The experiment was divided into control group (without TNF-α and test solution), TNF-α treated group (50 ng/mL TNF-α), and test group (50 ng/mL TNF-α and appropriate concentration test solution). Each group of cells were mixed with 2×10^5 cell/mL in a 96-well tissue culture plates and incubated in 5% CO_2 at 37 ℃ for 24 h. Supernatants were analyzed for PGE_2, IL-1β and IL-6 production by using commercially available human cytokine ELISA assay kits according to manufacturer's instructions.

2.7. Animals and drug administration

Eighty male SD rats weighing (200 ± 20) g were feed for 7 d for acclimation. All the rats were randomly divided into control group, model group, STA groups (5.0, 2.5 and 1.25 g/kg BW), and SD groups (50, 100 and 200 mg/kg BW) with 10 rats for each group. All the rats except the control group were injected with 100 μL of CFA (Shanghai Yuanye biological Co., Ltd, China) into the left hind limb to induce arthritis model[5]. After the inflammation, the rats in the STA groups and SD groups were given gavage once daily for 28 d. Finally all animals were sacrificed and joint tissues and serum samples were prepared for histology, ELISA, and biochemical parameter measurements[5].

2.8. Histology analysis

Rats were sacrificed after removal of blood from the femoral artery. Foot and ankle joints of inflamed rats were fixed, dehydrated, embedded, sectioned and stained with hematoxylin-eosin (HE), and inflammation degree was assessed as previously described[5].

2.9. Biochemical parameter measurements

Serum suspensions by homogenizing in a solution containing potassium phosphate[5] were subjected to the preliminary phytochemical investigation by employing standard procedures and tests according previous methods described by Umar *et al*.[13]. The measurement included SOD, MDA and NO level.

2.10. Cytokines measurements

After 28 d of administration, the femoral arteries of the rats in each group were sacrificed after blood was taken out and centrifuged at 4 000 r/min for 10 min. The supernatant was stored at 4 ℃ for further testing. The levels of IL-1β, IL-6, IL-10, TNF-α, PGE_2 in serum were determined by ELISA using ELISA kits (Beyotime Biotechnology, China) according to the manufactures' instruction.

2.11. Statistical analysis

All values were presented as the mean ± standard deviation. For multiple variables comparison, data were analyzed by ANOVA followed by Dunnett's multiple comparison and Tukey's test when necessary. Differences were considered statistically significant at $P<0.05$.

3. Results

3.1. MH7A cell viability 24 h after STA and SD administration

The impact of STA and SD on MH7A cell viability was examined. CCK-8 assay data indicated that MH7A cell proliferation declined in a dose dependent manner with increasing doses of STA and SD. When the concentration of SD and STA was 25 μg/mL, the proliferation inhibition rate of MH7A cells reached 59.95 % and 32.63 %, respectively. When the concentration up to 50 μg/mL, the inhibition rate up to 87.94 % and 86.37 %, respectively. IC_{50} of SD and STA were 26.85 and 34.22 μg/mL, respectively. This suggested that STA and SD treatment could inhibit MH7A cell proliferation.

3.2. NO secretion

The secretion of NO was measured on MH7A. The data indicated that the content of NO was significantly increased when incubated with STA and SD compared with blank group, but significantly decreased compared with the TNF-α model group (Table 1).

Table 1
Effects of STA and SD on production of inflammatory cytokines.

Group	NO (μmol/L)	IL-6 (pg/mL)	IL-1β (pg/mL)	PGE_2 (ng/L)
Control	3.97 ± 0.57	48.08 ± 1.23	85.02 ± 0.58	3.97 ± 0.21
TNF-α treated	5.21 ± 0.37**	62.01 ± 0.87**	172.12 ± 0.48**	5.21 ± 0.96**
MTX positive	3.84 ± 0.59**	51.02 ± 0.35**	135.03 ± 0.74**	3.84 ± 0.85**
STA50	4.24 ± 0.23*	56.04 ± 0.45*	162.14 ± 0.58*	4.24 ± 0.19*
STA100	4.19 ± 0.84**	43.03 ± 0.37**	142.23 ± 0.89**	4.19 ± 0.84**
STA200	3.84 ± 0.73**	18.02 ± 0.47**	95.13 ± 1.32**	3.84 ± 0.29**
SD25	4.37 ± 0.37*	15.03 ± 0.28*	140.15 ± 2.25*	3.92 ± 0.25*
SD50	3.76 ± 0.92**	5.05 ± 0.18**	127.17 ± 1.25**	3.85 ± 0.12**
SD100	3.14 ± 0.14**	3.02 ± 0.55**	98.15 ± 0.47**	3.12 ± 0.47**

STA50, STA 100, STA 200, SD25, SD50 and SD100 represents that the cells were treated with 50, 100, and 200 μg/mL STA, and 25, 50 and 100 μg/mL SD, respectively. Data are means±standard deviation of n=3 independent experiments. *$P<0.05$, **$P<0.01$, compared to control group.

3.3. Pro-inflammatory cytokines production in TNF-α-activated MH7A

To evaluate the anti-inflammatory effect of STA and SD in TNF-α-activated MH7A, IL-1β, IL-6 and PGE_2 were detected. The results showed that those cytokines were significantly upregulated when activated cells were incubated with STA and SD compared with the control group, but significantly decreased compared with the TNF-α model group (Table 1).

3.4. STA and SD decreases CFA-induced ankle inflammation

The histology analysis showed CFA-induced significant ankle inflammation. Large number of proliferation of synovial cells, and large number of lymphocyte infiltration into the joints, or the formation of blood vessels, cartilage surface damage was observed in CFA model groups, as compared to the controls. On the contrary, STA and SD decreased ankle inflammation, the synovial cells thickening, fibrous tissue exudation had improved, and lymphocyte infiltration into the joints was reduced in a dose-dependent manner by STA and SD. The lymphocyte infiltration into the joints was significantly reduced in 5.0 g/kg and 200 mg/kg, as compared with model, respectively. The result demonstrated the anti-inflammatory effect of STA and SD to CFA-induced arthritis.

Table 2

Effects of STA and SD on CFA-induced rats.

Group	SOD (unit/mg protein)	NO (μM/mg protein)	MDA (nM/mg protein)	IL-6 (pg/mL)	IL-1β (pg/mL)	TNF-α (pg/mL)	PGE_2 (ng/L)	IL-10 (pg/mL)
Control	12.57 ± 1.34	4.13 ± 0.47	6.12 ± 0.76	0.93 ± 0.16	1.03 ± 0.28	0.99 ± 0.12	143.00 ± 3.74	124.00 ± 2.74
Model	6.37 ± 0.97**	8.97 ± 0.77***	12.67 ± 0.95***	2.17 ± 0.23**	4.25 ± 0.67***	1.72 ± 0.23**	178.00 ± 3.57**	81.75 ± 1.25*
STA1.25	7.32 ± 0.45$^{\#}$	8.25 ± 0.68	9.26 ± 0.43**	1.78 ± 0.25$^{\#}$	3.87 ± 0.55$^{\#}$	1.58 ± 0.37$^{\#}$	165.00 ± 2.15$^{\#}$	114.88 ± 3.89**
STA2.5	8.58 ± 0.58$^{\#\#}$	7.83 ± 0.85$^{\#}$	8.97 ± 0.28$^{\#}$	1.59 ± 0.58$^{\#\#}$	3.36 ± 0.21$^{\#\#}$	1.45 ± 0.37$^{\#\#}$	162.00 ± 1.87$^{\#}$	115.14 ± 4.87$^{\#}$
STA5.0	10.13 ± 0.37$^{\#\#}$	6.25 ± 0.37$^{\#\#}$	7.45 ± 0.47$^{\#\#}$	1.36 ± 0.75$^{\#\#}$	1.84 ± 0.74$^{\#\#\#}$	1.37 ± 0.42$^{\#\#}$	154.00 ± 2.52$^{\#\#}$	117.89 ± 5.25$^{\#\#}$
SD50	7.87 ± 0.54$^{\#}$	8.34 ± 0.88	9.32 ± 0.32**	1.75 ± 0.35$^{\#}$	3.76 ± 0.32$^{\#}$	1.62 ± 0.28$^{\#}$	168.00 ± 3.47$^{\#}$	112.54 ± 1.87**
SD100	8.92 ± 0.49$^{\#\#}$	7.76 ± 0.92$^{\#}$	8.48 ± 0.28$^{\#}$	1.62 ± 0.39$^{\#\#}$	3.13 ± 0.20$^{\#\#}$	1.47 ± 0.13$^{\#\#}$	164.00 ± 1.95$^{\#}$	113.48 ± 3.85$^{\#}$
SD200	10.34 ± 0.84$^{\#\#}$	6.17 ± 0.74$^{\#\#}$	7.31 ± 0.47$^{\#\#}$	1.47 ± 0.41$^{\#\#}$	1.92 ± 0.43$^{\#\#\#}$	1.40 ± 0.28$^{\#\#}$	152.00 ± 1.74$^{\#\#}$	116.42 ± 2.87$^{\#\#}$

STA5.0, STA 2.5, and STA 1.25 represents that arthritic rats received 5.0, 2.5 and 1.25 g/kg wt STA; SD50, SD100 and SD200 represents that arthritic rats received 50, 100 and 200 mg/kg wt SD, respectively. $^{**}P<0.01$ versus control, $^{***}P<0.001$ versus control, $^{\#}P<0.05$ versus model. $^{\#\#}P<0.01$ versus model, $^{\#\#\#}P<0.001$ versus model.

3.5. Evaluation of biochemical parameters

SOD, MDA and NO levels were measured to evaluate the influence of STA and SD on biochemical parameters. The results indicated MDA and NO levels were upregulated by CFA as compared to the controls ($P<0.05$, Table 2), but SOD was significantly reduced ($P<0.05$). As expected, STA and SD administration attenuated all the CFA-induced changes in biochemical parameters in a dose-dependent manner. The group of STA5.0 and SA200 showed the lowest MDA and NO levels, and the highest SOD levels in comparison with the model rats ($P<0.05$, Table 2). The results demonstrated that STA and SD could reduce the generation of MDA and NO, and increase the content of SOD.

3.6. Pro-inflammatory cytokines production in CFA-induced arthritic rats

To evaluate the anti-inflammatory effect of STA and SD in arthritis rats, IL-1β, IL-6, TNF-α, IL-10 and PGE_2 in serum were detected. The results showed that IL-1β, IL-6 and TNF-α were significantly upregulated, and IL-10 were downregulated in serum of CFA-induced arthritic rats as compared with the control ($P<0.05$, Table 3). The levels of IL-1β, IL-6 and TNF-α in serum of rats were reduced, while IL-10 were significantly increased ($P<0.01$) in STA and SD administration. The expression of PGE_2 was also increased in arthritis rats ($P<0.01$, Table 3), and reduced by STA and SD administration ($P<0.05$). The results indicated STA and SD showed anti-inflammatory effect on CFA induced arthritis.

4. Discussion

This study was to explore the chemical bases and possible mechanism of *S. hookeriana* var. *digyna* to treat trauma and bruises. Moreover, STA and SD show anti-proliferation activity on MH7A cell lines, and possess therapeutic potential for arthritis.

RA synovial dysplasia resembles the nature of the tumor, causing irreversible damage to the cartilage and bone, resulting in stiffening of the joint as well as loss of function or complete loss. MH7A cells belong to a mature cell line of RA-FLSs, which has the characteristics of rapid proliferation, multiple passages and close to the *in vivo* state of patients[14]. Therefore, MH7A cells were selected as the cell model for RA study in this study. Pro-inflammatory cytokines, such as IL-6, IL-8, IL-10 and PGE_2 are involved in the process of RA[13,15,16]. TNF-α is one of the most important cytokines in RA, which can cause tumor-like proliferation of cells and produce a large number of inflammatory factors, resulting in destruction of cartilage and bone[17]. IL-1β is the major cytokine that causes the RA immune response to amplify and switch to a destructive reaction. IL-6 is an important inflammatory cytokine, and plays an important role in the development of RA. NO, an effective factor of inflammatory response and immune regulation, plays an important role in the inflammatory cascade, and can also promote the release of inflammatory factors such as IL-1β, PGE_2 as well as TNF-α[18,19]. When excessive inflammatory factors produced can cause a series of inflammatory damage, PGE_2 is an important mediator of inflammation. The occurrence and development of inflammation is closely related to the content of local PGE_2. Therefore, to cure RA, IL-1β and TNF-α must be double-blocked to prevent joint destruction. The present results indicated that 50 μg/L of TNF-α can significantly induce the proliferation of MH7A cells and promote the secretion of cytokines NO, PGE_2, IL-1β, IL-6. However, 50-500 μg/mL of STA, 25-500 μg /mL of SD could significantly inhibited the cell proliferation and inflammatory cytokines secretion in a concentration-dependent manner.

As confirmed in the previously study[5], pro-inflammatory cytokines, such as IL-6, IL-1β, TNF-α, PGE_2, etc. are increased significantly in CFA-induced arthritis. The similar results were found in our study. On the other hand, arthritic rats treated with STA and SD showed relative remission of arthritis, declined CFA-induced IL-6, IL-1β, TNF-α and PGE_2 production, and increased IL-10 production. These indicated STA and SD have the anti-inflammatory

effect against CFA-induced rats model *in vivo*.

In CFA-induced arthritic rats, MDA and NO level were elevated, and H2O2-removing enzymes SOD were decreased, demonstrated the rise of oxidative stress[13,20,21]. The results of this present study demonstrate the antioxidant effects of STA and SD. Moreover, the antioxidant effect might be one of the potential mechanisms of the anti-RA effect.

From this study, we can reach the conclusion that STA and SD can inhibit the proliferation of MH7A cells and the secretion of NO, PGE_2, IL-1β and IL-6 in a concentration-dependent manner *in vitro*, which may be one of the mechanisms of its anti-RA effect.

STA and SD downregulate the production of IL-6, IL-1β, TNF-α, PGE_2 and NO, or hinder the effect, alleviate the synovial tissue inflammatory response and tissue damage, hinder or delay pathological development of RA. STA and SD can protect rats against oxidative stress, neutrophil infiltration, and inflammation by ameliorating CFA-dysregulated oxidative related enzymes and pro-inflammatory cytokines. This study has shown the chemical bases and possible mechanism of *S. hookeriana* var. *digyna* used in folk medicine as an anti-rheumatic remedy for the treatment of trauma and bruises.

Conflict of interest statement

We declare that we have no conflict of interest.

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