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Effectiveness of *Tagetes patula* against chronic nonbacterial prostatitis in rat model

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Abstract

The effectiveness of water decoction of *Tagetes patula* was studied against the chronic nonbacterial prostatitis in a rat model induced by estradiol subcutaneously in a castrated male rat. The low-dose decoction of *T. patula* significantly ameliorated the prostatitis. The low-dose of polysaccharide and supernatant constituent of *T. patula* decreased the levels of IL-1β, TNF-α, PSA, and EGF, and improved the levels of testosterone and dihydrotestosterone *in vivo*. Further, the chemical study indicated that flavonoids were predominant in the supernatant constituent of *T. patula*. The flavonoids and polysaccharides were the effective constituents against prostatitis and this effect may be mediated by keeping hormone balance and a down-regulation of the inflammatory mediator level of the prostate.

Introduction

Prostatitis, an inflammatory response, is caused by the microbials and other pathogens or certain non-infectious factors (Krieger and Mcgonagle, 1989). Its clinical manifestations usually exhibit discomfort or pain of prostate region, abnormal urethral discharge and other symptoms (Liu et al., 2016). Until now, etiology and natural history of chronic nonbacterial prostatitis are poorly understood and thus effective and appropriate treatment is still need to be explored.

Here, for the first time, we attempted to evaluate the anti-prostatitis effect of the water extract of *Tagetes patula* stem and leaf using a chronic nonbacterial prostatitis rat model and provided insight into the mechanism involving the inflammatory mediators and hormone levels.

Materials and Methods

Materials and reagents

The stem and leaf of *T. patula* was provided by the Dalian Wuzhou Holy Herb Scientific and Technological Co. Ltd. (China). It was identified by Prof. Wang Bing in the Liaoning University of Traditional Chinese Medicine and the voucher specimens (No. 20160911) were deposited in the Herbarium of College of Pharmacy, Liaoning University of Traditional Chinese Medicine. Estradiol benzoate was purchased from the Ningbo Second Hormone Factory (China). Levofloxacin hydrochloride was obtained from the Cisen Pharmaceutical Co., Ltd. (China). Dihydrotestosterone and prostate-specific antigen ELISA kit were from Shanghai Qiaodu Biotechnology Co. Ltd. (China). T, TNF-α, IL-1β and EGF ELISA kit were all purchased from the Nanjing Jiancheng Biotechnology Co. Ltd. (China). All the other chemicals used in the experiments were commercial products of analytical grade.

Preparation of water extract

The stem and leaf of *T. patula* (150 g) was pulverized and extracted with 10 times distilled water with 4 hours soaking and 1 hour boiling. The resulting decoction was placed steadily to room temperature and filtered with the filter cloth (200 mesh). Then, the residual was

Table I

Preparation of sample solution administrated to rat

	m_1 (g)	yield (%)	m_2 (g)	V_{CMC-Na} (mL)	C (mg/mL)
Water decoction	36.9	24.1	7.8	180.0	43.4
Essential oil	3.0	0.3	0.1	200.0	0.5
Polysaccharide	141.3	14.1	5.1	200.0	25.4
Supernatant	115.3	11.5	4.2	200.0	20.8
Pule'an tablets	-	-	12.5	231.0	54.0
Levofloxacin hydrochloride	-	-	1.0	222.0	4.5

Notes: m_1 represents total quantity of freeze-dried powder; m_2 represents weighted quantity of freeze-dried powder for intragastric administration; V_{CMC-Na} represents volume of solvent CMC-Na; C represents solution concentration of intragastric administrated

extracted with 8 times distilled water for another 1 hour again, and then, filtered. The combined extracts were centrifuged at 3,000 rpm for 10 min. The resulting supernatant was evaporated in vacuo to recover the water and the concentrated extract was lyophilized to give the water extracts powder. Before administration to rat, the powder was dissolved in 0.5% CMC-Na water solution. Yield and preparation methods of solution administrated to rat is shown in Table I.

Preparation of the separated constituents

The stem and leaf of *T. patula (*1 kg) was pulverized and extracted with 10 times distilled water for 4 hours soaking and 1 hour boiling with essential oil extractor to give the essential oil components. The resulting decoction was placed steady to room temperature and filtered with the filter cloth (200 mesh). The residual was continued to extract with 8 times distilled water for another 1 hour again, and then, filtered. The filtrates and essential oil were collected respectively. The collected filtrates were concentrated to 1.5 L after centrifugation. Then, adding 95% alcohol to the concentrated solution to meet 70% alcohol for two times to provide the precipitate and supernatant constituents, respectively. Further, the supernatant was evaporated in vacuo to recover the solvent and the concentrated extracts were lyophilized to give the samples of supernatant constituent and the precipitate was dried in vacuo to provide the polysaccharide constituent. Before administration to rats, the powder was dissolved in 0.5% CMC-Na water solution respectively. Yields and preparation methods of solution administrated to rat is shown in Table I.

Animals

Adult male SPF level Sprague-Dawley rats weighing approximately 180–200 g were obtained from the Changsheng Biotechnology Limited in Liaoning of China (License Key: SCXK (Liao) 20150001). The rats were housed in an animal room maintained at a constant temperature (20 ± 2°C) and 40 ± 10% relative humidity with a 12 hours light/dark cycle. Food and water were taken freely. Experiments were carried out by the Guide for Care and Use of Laboratory Animals of Liaoning University of Traditional Chinese Medicine (131/2010).

Pharmacological effects of the water extract of T. patula

In this study, we selected the human dosage of 20 g/day (equivalent dosage of 285 mg/kg/day extracts or 1.8 g/kg/day for rats equivalent dosage) as the low-dose reference and 5.4 g/kg/day for the high-dose reference to investigate the anti-prostatitis effect of different doses of decoction of *T. patula* on chronic non-bacterial prostatitis rat model. The rats were randomly divided into four groups with 10 animals for each, i.e. the sham-operated group (SO), the untreated chronic nonbacterial prostatitis model group (MO), low-dose treatment group (LD) and high-dose treatment group (HD). Chronic nonbacterial prostatitis was induced in the model group according to the method was established as described previous (Robinette, 1988). Except SO group, all the other groups began to be injected with estradiol benzoate (0.3 mg/kg/day in sunflower oil) subcutaneously in the back of the rats and SO group was injected with sunflower oil subcutaneously for 30 days. On the 30th day, the rats were intragastrically administrated with different drugs for one week. The low-dose treatment group and high-dose treatment group were intragastrically administrated with final doses of 1.8 g/kg/day and 5.4 g/kg/day in 0.5% CMC-Na solution, and the sham-operated group and the untreated chronic nonbacterial prostatitis model group were intragastrically administrated with 0.5% CMC-Na. The treatment was intragastric administered at 9 a.m. each day. All groups were anesthetized by intraperitoneal injection 3% pentobarbital sodium (1 mL/kg) at 24 hours after the last administration. Blood was taken from the abdominal aorta until blood loss and death. The prostate, kidney, spleen and thymus tissues were collected. The serum was collected by centrifugation at 2,500 rpm for 20 min and frozen in -80°C. After weighing left lateral lobe part

of the prostate, kidney, spleen and thymus tissues were fixed in 4% paraformaldehyde solution over 24 hours, embedded in paraffin and sectioned at a thickness of 5 µm for histological evaluation. The sections were stained with hematoxylin-eosin and examined microscopically. The remaining right lateral lobe part of the prostate tissues were quickly frozen at -80°C.

Pharmacological effects of different constituents of T. patula

The rats were randomly divided into eight groups of 8 animals for each: The sham-operated group (SOG), the untreated chronic nonbacterial prostatitis model group (MOG), water decoction treatment group (WDG), essential oil treatment group (EOG), polysaccharide treatment group (POG), supernatant treatment group (SUG), Pule'an tablet treatment group (PAT) and levofloxacin treatment group (LHG). Pule'an is a clinical Traditional Chinese Medicine and levofloxacin is a drug commonly used to treat prostatitis, both are used as positive control. The model was established in the same way. At the end of the month, the rats were intragastrically administrated with different drugs for one week. The water decoction treatment group (1.8 g/kg/day), essential oil treatment group (5.4 mg/kg/day), polysaccharide treatment group (254.3 mg/kg/day), supernatant treatment group (207.5 mg/kg/day), Pule'an tablets treatment group (540 mg/kg/day) and levofloxacin treatment group (45 mg/kg/day) were intragastrically administrated with the corresponding solution. The sham-operated group and the untreated chronic nonbacterial prostatitis model group were intragastrically administrated with 0.5% CMC-Na. The treatment was intragastrically administrated at 9 AM each day. All groups were anesthetized. Blood and prostate tissues were collected. The serum was finally collected and stored at -80°C before estimation.

Physiological indexes

The concentrations of testosterone, dihydrotestoterone, and prostate-specific antigen in serum were determined by ELISA. Homogenate was obtained according to the ratio of tissue of the right lateral lobe of prostate and saline 1:9. The supernatant was collected by centrifugation for 10 min at 3,000 rpm. The level of TNF-α, IL-1β, and EGF in the supernatant of prostate homogenate were measured by using a commercially available ELISA kit according to the manufacturer's protocol.

HPLC analysis

The Agilent 1260 HPLC system (Agilent Technologies, USA) comprised G1329B quat pump, G1329B autosampler, G1315D column thermostat, G1315D diode-array detection (DAD) was used. The chromatographic conditions are as follows: All samples were performed on an Agilent 1260 HPLC equipped with DAD detector over Agilent eclipse plus C_{18} (4.6 × 150 mm, 5 µm). The mobile phase was composed of 0.1% phosphoric acid-water (A) and acetonitrile (B) with gradient elution (16% B in 0-18 min, 16-52% B in 0-30 min, 52% B in 30-35 min, 52-80% B in 35-50 min, 80% B in 50-60 min). The flow rate of the mobile phase was 1 mL/min. The wavelength of the detector was 254 nm. The temperature was maintained at 28°C and the sample injection volume was 10 µL.

The preparation of sample solutions is as follows: The water extracts powder, supernatant powder and essential oil were diluted with 60% methanol in a volumetric flask of 10 mL and added to the constant volume, then filtered with 0.5 µm millipore membranes before HPLC analysis.

The preparation of the standards are as follows: The standard compounds, separated and identified by our laboratory, patuletin, quercetin-7-O-α-L-rhamnosepyranoside, quercetin-3-O-α-L-arabinopyranoside, kaempferol-3-O-β-D-glucopyranoside, kaempferol-3-O-β-D-xylopyranoside, kaempferol-3-O-α-L-rhamnosepyranoside, kaempferol, kaempferol-3-O-α-L-arabonipyranoside, were diluted in methanol and dissolved under a ultrasonic wave, then filtered with 0.5 µm millipore membranes before HPLC analysis.

Statistical analysis

Data were analyzed statistically and expressed as the mean ± standard deviation. IBM SPSS Statistics ver. 22.0 (IBM Co., USA) was used to evaluate the data. One-way ANOVA was used for statistical analysis of the differences between the groups. A value of $p<0.05$ was considered statistically significant.

Results

Morphological observation and histological analysis of prostate

The prostates of rats in SO showed soft texture. The color was pink. The surface was smooth surface and shiny. There was no adhesion with the surrounding tissues. However, the prostates in MO exhibited significantly increased in volume, slightly hard quality, or significant induration, and showed dark red, dull glands. There was adhesion with the surrounding tissues (Figure 1).

Histological analysis of the prostates of the rat from the model group revealed hallmarks of inflammation, confirming the presence of prostatitis. The forms of glandular cavity of prostate were different and there was a small amount of normal secretions in it. There was no obvious inflammatory cell infiltration in the prostatic stromal. The structure of the tissue was complete and there was no hyperplasia of fibrous tissue. The pathological finding in MO showed

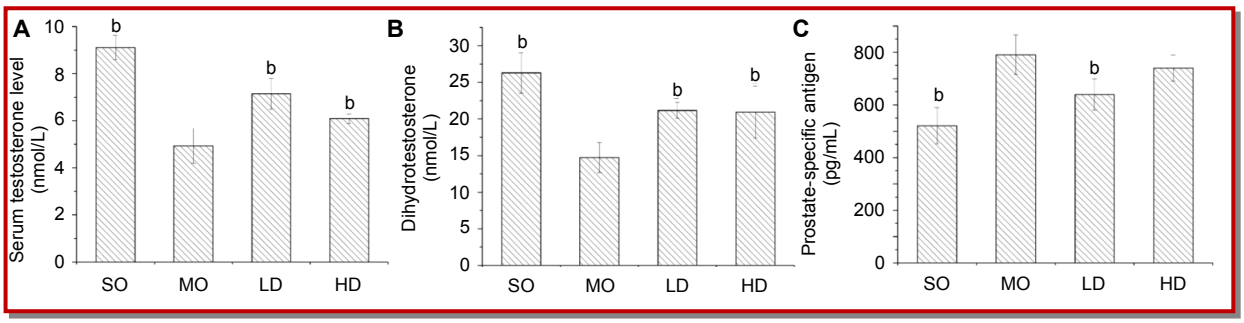

Figure 1: Effect of *T. patula* water extract on the levels of serum testosterone, dihydrotestosterone and prostate-specific antigen levels. Values are in mean ± SD; (n = 10), [a] = p<0.05, [b] = p<0.01 Vs the model group (MO)

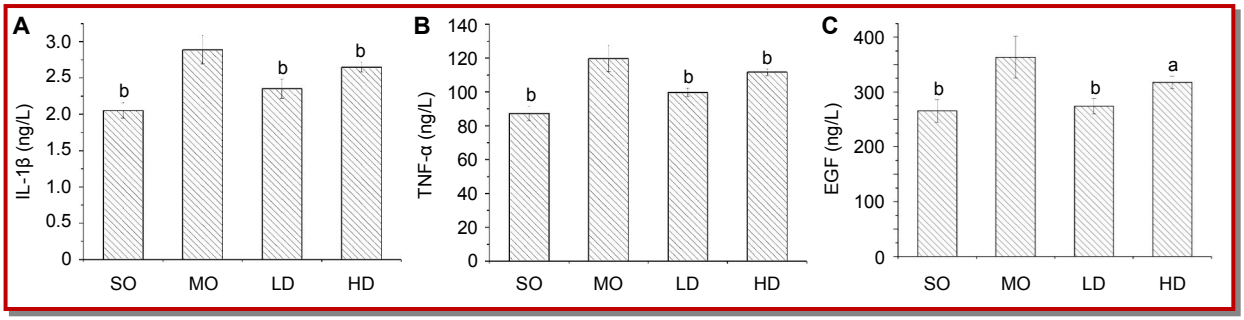

Figure 2: Effect of *T. patula* water extract on the contents of IL-1β, TNF-α and EGF in prostate tissue. Values are in mean ± SD; n = 10; [a] = p<0.05, [b] = p<0.01 Vs the model group (MO)

papillary proliferation of acinar cells and there were some secretions in the glandular cavity. The prostatic stromal was loose and edematous. There was inflammatory cell infiltrate in it (Figure 2).

To demonstrate the specificity of prostatitis model, we conducted the same histological analysis in other tissues. There was no pathological change similar to that of the prostate in hematoxylin-eosin staining of kidney, spleen and thymus (Figure 3, Figure 4).

Effects on prostate wet weight

The prostate wet weight was significantly decreased in LD and HD compared with MO (p<0.01). In order to eliminate individual differences, we took the ratio of prostate wet weight and body mass to express the anti-chronic nonbacterial prostatitis effect. The ratio of prostate wet weight and body mass in LD and HD were significantly lower than in MO (p<0.01), indicating that whether low-dose or high-dose SLTP water extract both could alleviate the prostate wet weight changes caused by chronic nonbacterial prostatitis (Table II).

Effects on prostate wet weight of different constituents of extract

The prostate wet weight and the ratio of prostate wet weight and body mass were significantly less in PAT, WDG and SUG (p<0.01, p<0.05) compared with MOG, indicating that supernatant could alleviate prostatitis

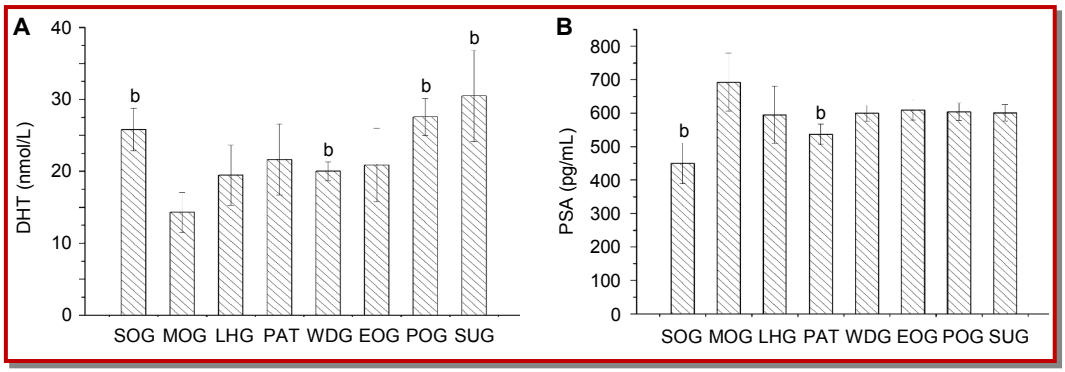

Figure 3: Effect of different constituents of *T. patula* on the levels of serum DHT and PSA. Values are in mean ± SD; n = 8; [a] = p<0.05, [b] = p<0.01 Vs the model group (MOG)

Table II

Effects of water extract on the prostate weight

Group	Prostate wet weight (mg)	Prostate wet weight / body mass ratio (mg/g)
SO	193.1 ± 10.7[b]	0.8 ± 0.1[b]
MO	284.9 ± 20.0	1.6 ± 0.0
LD	182.6 ± 13.9[b]	1.0 ± 0.1[b]
HD	242.1 ± 15.7[b]	1.4 ± 0.1[b]

Values are in mean ± SD; n = 10; [a] = p<0.05, [b] = p<0.01 Vs the model group (MO)

symptom (Table III).

The levels of physiological indexes

Effects of water extract on the levels of indexes

The contents of testosterone and dihydrotestosterone in LD and HD were significantly increased (p<0.01). Prostate-specific antigen, IL-1β, TNF-α and EGF were significantly decreased in LD (p<0.01) and the levels of IL-1β and EGF in HD were decreased compared with MO. The results indicated that water extract has the ability to alleviate the prostatitis, especially low-dose. In view of LD in the dose and the advantages of its superior efficacy, we selected the low-dose as the standard to explore the anti-prostatitis active constituents of the extract (Figure 1; Figure 2).

Table III

Effect of different constituents of extract on the prostate weight

Group	Prostate wet weight (mg)	Prostate wet weight / body mass ratio (mg/g)
SOG	192.3 ± 65.3[b]	0.8 ± 0.2[b]
MOG	392.9 ± 81.1	2.1 ± 0.5
LHG	252.0 ± 77.3	1.3 ± 0.3
PAT	202.5 ± 82.4[b]	1.0 ± 0.4[b]
WDG	210.5 ± 71.9[b]	1.1 ± 0.3[b]
EOG	228.5 ± 79.0[a]	1.3 ± 0.4
POG	239.3 ± 105.6	1.2 ± 0.3[a]
SUG	237.5 ± 52.6[a]	1.2 ± 0.2[a]

Values are in mean ± SD; n = 8; [a] = p<0.05, [b] = p<0.01 Vs the model group (MOG)

The levels of indexes in different constituents of the extract: Compared with MOG, the concentrations of testosterone and dihydrotestosterone in POG and SUG were increased statistically (p<0.01). There was a certain trend to subside the contents of prostate-specific antigen in other treatment groups. The levels of TNF-α were less in POG and SUG, the contents of IL-1β in prostatic tissue in SUG also had a downward trend. The concentrations of EGF were statistically decreased in SUG (p<0.05). Comprehensive of the above results, polysaccharide and supernatant constituents were

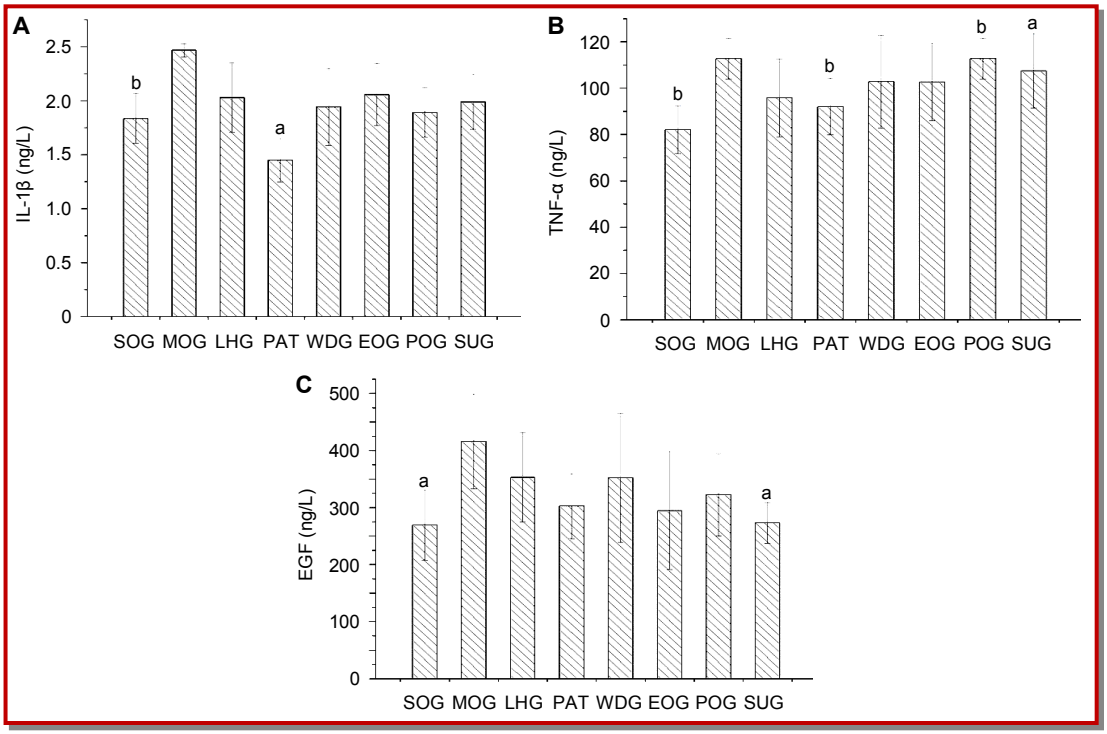

Figure 4: Effect of different constituents of *T. patula* on prostate IL-1β, TNF-α and EGF levels. Values are in mean ± SD; n = 8; [a] = p<0.05, [b] = p<0.01 Vs the model group (MOG)

effective against chronic nonbacterial prostatitis (Figure 3; Figure 4).

HPLC analysis

The main components of water decoction and supernatant are flavonoids and flavonoid derivatives. The HPLC fingerprints of water decoction and supernatant constituents of the herb were collected through the HPLC/DAD analysis and all standard compounds could be identified in the HPLC fingerprints (Figure 4).

Discussion

The principle of chronic nonbacterial prostatitis treatment mainly practiced by heat-clearing, detoxifying and treatment according to different symptoms in TCM (Bao, 2001). *T. patula*, name *Kong Que Cao* in Chinese, is a traditional drug in Chinese Yi ethnomedicine firstly recorded in Guizhou Herbs and has the function of clearing heat removing dampness. It is annual herb belonging to the family Asteraceae and commonly known as French marigold (Bano et al., 2002). The dried and ground flower petals constitute a popular spice in the Republic of Georgia in the Caucasus and is also a well-nigh essential ingredient in the spice mixture Khmeli-Suneli, which is popular in Georgian cookery.

The essential oil of *T. patula* was discovered to have antifungal activity, including treatment of candidiasis and fungal infections in plants (Romagnoli et al., 2005; Mares et al., 2004). The dried leaves or florets of *T. patula* have been used internally for indigestion and anti -inflammation, and externally for the treatment of sore eyes and rheumatism (Soule, 1993). In Argentina, the plant extract is taken internally as a diuretic which is associated with the prostatitis treatment (Kasahara et al., 2002). The etiology and pathogenesis of prostatitis is complicated, Chinese medicine found that the main pathogenesis of chronic prostatitis is damp-heat (Bao, 2001). Poorly physical quality is an important intrinsic factor that causes chronic prostatitis (Zeng, 2005). In this study, the method of castration combine with estrogen induction was selected to establish the rat model of chronic nonbacterial prostatitis by change hormone and endocrine balance to induce prostatitis. Here we investigated the anti-prostatitis activity of *T. patula*, we measured the testosterone, dihydrotestosternoe and prostate-specific antigen levels in serum and concentrations of TNF-α, IL-1β and EGF in the prostate.

90% of testosterone comes from the testicle, and testosterone is the main component of androgen. After the testicular castration in rats, the levels of androgen in the body were decreased, estradiol was injected subcutaneously as a class of estrogens, and leading that the estrogen and androgen levels in the ovariectomized rats were out of balance. The formation of benign prostatic hyperplasia is closely related to hormone imbalance which is usually a complication of prostatitis. Studies have shown that estrogen has a synergistic effect on androgen, which is achieved by increasing the level of the androgen receptor (Lan, 2007). Testosterone in blood is often used clinically as a biochemical indicator for the diagnosis of prostatitis. When the inflammation occurs in the prostate, testis, and epididymis, the blood testosterone levels will have varying degrees of reduction. Blood testosterone levels are associated with the treatment of the prostate and reproductive system. If the blood testosterone levels increase, blood circulation will accelerate, the immune system will be enhanced, tissue repaired will be faster, the symptoms will be improved the more obvious. Testosterone is converted to dihydrotestosterone by 5-alpha reductase and play biological effects. DHT is more bioactive than testosterone and has more affinity with androgen receptor (Lan, 2007). Dihydrotestosterone androgen receptor complexes are more stable. Dihydrotestosterone content was negatively correlated with the degree of prostatitis inflammation.

Prostate-specific antigen is an important adjuvant diagnostic indicator for prostate cancer. It has high specificity and sensitivity to prostate tissue. The hyperplasia, infection, and inflammation of prostatic and other benign diseases will also increase the level of prostate-specific antigen in serum (Guo and Li, 2007). The concentration of prostate-specific antigen is positively proportional to the severity of inflammation, however, the mechanism of elevated prostate-specific antigen levels in serum caused by prostatitis remains unclear. At present, the most likely mechanism is the so -called "prostate-specific antigen leakage theory", that prostatitis will destroy the integrality of the original physiological barrier of the prostate gland duct and acinar, prostate-specific antigen in the prostate gland duct and acinar are leaked out into the blood circulation, leading to increasing the level of prostate-specific antigen in serum (Kuznar, 1995). Cytokines as an immune mediator involved in inflammatory response, it can reflect the strength of inflammation. As important pro-inflammatory cytokines, TNF-α can induce the production of chemokines and the expression of adhesion molecules in epithelial cells and lymphocytes, mediating inflammatory cells to inflammatory local aggregation, activation and releasing of inflammatory mediators (Yang, 2007). IL-1β plays a key role in the local inflammatory response of prostatitis, which is through the induction of adhesion molecules, activating of neutrophils to adhesion and degranulation, releasing the oxygen free radicals and proteolytic enzymes, mediating the expression of prostate endothelial cell, leading to leakage of leukocytes from the blood vessels and invading into prostate tissue, and also can expand the inflammatory response by inducing inflammatory cells and endothelial cells, fibroblasts and

other secreting cytokines (Zhao and An, 2009). It can be used as a new method of differential diagnosis of prostatitis that in foreign studies the levels of TNF-α and IL-1β in prostate secretions are significantly higher in prostatitis patients compared with normal (Nadler et al., 2000). Peptide growth factor is widely present in the microenvironment of the prostate, and is a kind of biologically active peptides, which regulates the cell cycle and apoptosis of the prostate by sending signal (Wang, 2005). But if the peptide growth factor or its receptor abnormal expression, which will directly lead to uncontrolled growth of prostate cells. EGF is an important member of the prostate peptide growth factor superfamily. EGF regulates the growth of prostate epithelial cells by binding to its receptor and sending a signal. Not only binds to the receptor to produce proliferation but also can induce apoptosis of prostate cells by inhibiting TGF-β. The against between two factors keep the balance in prostate cells. The growth internal balance of prostate depends on the internal balance of the interaction of different growth factors, overactive or weakened of growth factors will both interfere with the internal balance of prostate cells, resulting in benign prostatic hyperplasia (Cao and Zhou, 2004). Therefore, we chose the common indicators of inflammation IL-1β, TNF-α as an indicator of our chronic prostatitis, at the same time they also serve as an indicator of the strength of inflammation of prostatitis, and testosterone, dihydrotestosterone, prostate-specific antigen as a reflection of the impact of prostate inflammation *in vivo* hormone indicators, EGF as an adjunct to determine the relationship between prostatitis and benign prostatic hyperplasia.

The main ingredients of the essential oil of *T. patula* are 2-carene and β-cortisene, (Hu, 1992) which is used for the compounding of high-grade perfumes and it is also a fly-repellent (Bano et al., 2002). Polysaccharide constituent of the extract is mostly macromolecular compounds, including polysaccharides and protein, which usually can improve immunity and enhance physical fitness (Zhou and Xu, 1994). From the HPLC analysis results, it can be seen that the polysaccharide of *T. patula* has good effects on chronic nonbacterial prostatitis, which may be achieved by improving the immunity of the body. Studies have shown that flavonoids and their derivatives have a wide range of pharmacological effects, they can inhibit leukocyte adhesion, improve microcirculation and reduce local inflammation (Cui, 2014). Supernatant constituent of the extract contains flavonoids and small molecules of sugar. Its anti-prostatitis effect is likely to be achieved through the above mechanism, but the specific mechanism remains to be further explored.

In this paper, we investigated pharmacological effects of water extract firstly. It is found that the low-dose water decoction of *T. patula* has the best effect on the treatment of chronic nonbacterial prostatitis. Therefore, we further investigated the effects of different constituents of *T. patula* on chronic nonbacterial prostatitis rats. These findings confirm that polysaccharide and supernatant constituents which main components are flavonoids and their derivatives were effective against chronic nonbacterial prostatitis. It is suggested that this effect may be mediated by keeping hormone balance in the body and a down-regulation of the inflammatory mediator TNF-α and IL-1β at the level of the prostate. These studies offer a new strategy to treat chronic nonbacterial prostatitis and further research will be needed to fully explain which specific pathway to produces this therapeutic effect.

Conclusion

The water decoction of *T. patula* has the best effect on the treatment of chronic nonbacterial prostatitis. Polysaccharide and flavonoids present within the *T. patula* are effective. This effect may be mediated by keeping hormone balance in the body and a down-regulation of the inflammatory mediator TNF-α and IL-1β at the level of the prostate.

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Conflict of Interest

The authors declare no conflict of interest.

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