

EVALUATION OF ANALGESIC AND ANTI-INFLAMMATORY ACTIVITIES OF COMPOUND HERBS
PUXING YINYANG SAN

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Abstract

Background: Bovine mastitis is one of the most relevant and problematic diseases to treat and control in practice. Puxing Yinyang San (PYS) is a compound of herbs to treat bovine mastitis in China. This study was performed to evaluate the analgesic and anti-inflammatory activities of PYS in mice and rats.

Materials and Methods: The analgesic and anti-inflammatory activities of PYS were determined using acetic acid-induced writhing response, hot plate test, xylene-induced ear swelling test, carrageenan-induced paw edema test, and acetic acid-induced capillary permeability and leukocyte infiltration test with oral doses of 155, 310 and 620 mg/kg-bw in mice or rats.

Results: The acetic acid-induced writhing response was dose-dependently inhibited by oral administration of PYS and the latency time to thermal stimuli was increased in the hot plate test, especially 90 minutes after treatment. In the xylene-induced ear swelling, PYS significantly decreased swelling degree in a dose-dependent manner. Additionally, PYS significantly suppressed the peritoneal capillary permeability and leukocyte infiltration in mice induced by intraperitoneal injection of acetic acid. PYS also significantly reduced the carrageenan-induced rat paw edema at 2, 3, and 4 h after the carrageenan injection. The results suggested that PYS possessed significant analgesic and anti-inflammatory activities.

Conclusion: This study was the first to demonstrate that oral administration of PYS might play an important role in the process of analgesia and anti-inflammation, supporting its treatment for mastitis. Future investigations will focus on the broader involvement of the ingredients and mechanisms responsible for pharmacological activities of PYS.

Keywords: Puxing Yinyang San, analgesic activity, anti-inflammatory activity, herb.

Introduction

Mastitis is the most common disease in dairy cows and the principal cause of economic losses in the dairy industry (Demon et al., 2013). It undermines udder health, reduces milk quality, loses milk production and entails prohibitive costs (Piepers et al., 2007). The incidence of subclinical mastitis in dairy cows was 22%~65% in the world (Biiiragohin and Dutta, 1999; Gitau et al., 2014; Lam et al., 2013; Mukesh et al., 2014; Ramírez et al., 2014), and it was 27%~55% in China (Xiao, 2011). Although it has been studied for more than one hundred years, bovine mastitis is still one of the most relevant and problematic diseases to treat and control in practice because of the complexity and mutability of the condition (Green and Bradley, 2013). Recently, it is common to cure mastitis in dairy cows with intra-mammary antimicrobials in practice (Hill et al., 2009; Pol and Ruegg, 2007). However, antimicrobial treatment increases the risk of antibiotic residues in milk and antimicrobial resistance,

which may endanger public security. Therefore, it is still important to search for more effective and safer medicine to treat or prevent dairy cows with mastitis. Many Chinese herbs possess bacteriostatic or anti-inflammatory ability, and provide holistic therapy for interrelated diseases.

Puxing Yinyang San (PYS), pharmaceutical product named "Ru Ning San", consists of three Chinese herbal medicines: *Taraxacum mongolicum* Hand.-Mazz., *Vaccaria segetalis* (Neck.) Garcke, and *Epimedium brevicornu* Maxim (Table 1). *Taraxacum mongolicum* is a broad-spectrum herb (Li et al., 2008; Song et al., 2006). The polysaccharides and total flavonoids are main antibacterial components of *Taraxacum mongolicum* (Gu and Wang, 2007; Song et al., 2010); taraxasterol and phenolic acids show significant anti-inflammatory ability (Liu et al., 2012; Park et al., 2011). *Vaccaria segetalis* possesses abilities of activating blood circulation, regulating menstrual disturbances, dispelling edema, and promoting lactation for centuries in China (Li and Liang, 2007). Polysaccharide isolated from *Epimedium* has inhibitory effects on *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus aureus* (Su et al., 2011). Total Flavonoids of *Epimedium* has anti-inflammatory activity (Zhang et al., 1999), and icariin is a safe and effective natural anti-inflammatory drug (Wu et al., 2009). PYS has been shown effective to treat subclinical mastitis in our previous studies and the effective rate was 88.89% (Wang, 2013). It has also been demonstrated that PYS is not toxic if orally administered in acute toxicity tests in mice (maximum daily dose=40 g/kg-bw) and subchronic toxicity test at doses of 750, 1500, and 3000 g/kg-bw (Wang et al., 2013). However, its mechanism to treat subclinical mastitis has not been studied.

The aim of the present study was to evaluate the analgesic and anti-inflammatory effect of PYS through five animal models, including acetic acid-induced mouse writhing, hot plate test, xylene-induced mouse ear swelling, acetic acid-induced mouse capillary permeability and leukocyte infiltration and carrageenan-induced rat hind paw edema.

Materials and Methods

Reagents and Drugs

Carrageenan, indometacin, Evans blue and Crystal violet were purchased from Sigma–Aldrich Chemical Co. (St. Louis, USA). Acetic acid was obtained from Tianjin Damao Chemical Reagent Factory (Tianjin, China). Xylene was purchased from Tianjin Fuchen Chemical Reagent Factory (Tianjin, China). The control medicinal materials of *Taraxacum mongolicum*, *Vaccaria segetalis*, and *Epimedium* were purchased from National Institutes for Food and Drug Control (Beijing, China). The reference substances of vaccarin (95.1%), icariin (100%), and caffeic acid ($\geq 98\%$) were also purchased from National Institutes for Food and Drug Control (Beijing, China).

Preparation of PYS

PYS was composed of three herbs - *Taraxacum mongolicum*, 110 g; *Vaccaria segetalis*, 60 g; *Epimedium*, 30 g. Plant parts and origin used in the formula had been shown in Table 1. The herbs were pre-treated by washing and drying. Following that, herbs were mixed as previously described and crushed through 120 mesh sieve.

Table 1: Component herbs of PYS

Herb name	Plant part	Origin	Grams, g	Content (%)
Pu gong ying (<i>Taraxacum mongolicum</i> Hand.-Mazz.)	whole plant	Gansu, China	110	55
Wang bu liu xing (<i>Vaccaria segetalis</i> (Neck.) Garcke)	Seed	Hebei, China	60	30
Yin yang huo (<i>Epimedium brevicornu</i> Maxim)	Leaf	Sichuan, China	30	15

Thin Layer Chromatography (TLC) Analysis of PYS

The TLC was carried out according to the protocol described by Chinese pharmacopoeia (2010). Briefly, 2.50 g PYS was dissolved in 40 mL 5% formic acid, extracted for 20 minutes with ultrasonic, filtered and the filtrate was volatilized until dry. Following that, the residue was dissolved in 10 mL distilled water and filtered. The filtrate was extracted with 10 mL ethyl acetate for two times. Then the ethyl acetate extraction was volatilized until dry and the residue was dissolved in 1 mL methanol and spotted on a silica G thin layer plate (Taizhou Luqiao Sijia Biochemical Plastics Factory, China) with 2 μ L, and developed at room temperature with butyl acetate-formic acid-water (7:2.5:2.5). The solution of *Taraxacum mongolicum* and caffeic acid were used as the reference substance. The TLC spots were visualized by spraying with alchlor reagent and the color stripes were displayed at 366 nm (CabUVIS, DESAGA GmbH, Germany). 3.00 g PYS was dissolved in 40 mL 70% methanol, extracted for 30 minutes with ultrasonic, filtered and spotted on a polyamide film plate (Taizhou Luqiao Sijia Biochemical Plastics Factory, China) with 2 μ L, and water-methanol (6:4) was used as the developing reagent. The solution of *Vaccaria segetalis* and vaccarin were used as the reference substance. The TLC spots were visualized by spraying with alchlor reagent (2%) dissolved in ethanol solvent and the color stripes were displayed at 366 nm. 2.30 g PYS was macerated in 20 mL ethanol for 30 minutes, then filtered and the filtrate was volatilized until dry. Following that, the residue was dissolved in 10 mL distilled water and the filtrate was extracted with 10 mL ethyl acetate for two times. Then the ethyl acetate extraction was volatilized until dry. The residue was dissolved in 1 mL ethanol and spotted on a silica H plate (Taizhou Luqiao Sijia Biochemical Plastics Factory, China) with 2 μ L, and developed with ethyl acetate-butanone-formic acid-water (10:1:1). The solution of *Epimedium* and icariin were used as the reference substance. The TLC spots were visualized by spraying with alchlor reagent and the color stripes were displayed at 366 nm.

Animal Care and Handling

Male or female Balb/C mice (18~22 g) and SD rats (160~200 g) were obtained from the experimental animal center of Lanzhou University. They were housed in plastic cages at a temperature of 22 \pm 1 $^{\circ}$ C, relative humidity 50 \pm 10% with free access to food and water. This study was approved by the Institutional Animal Care and Use Committee of Lanzhou Institute of Husbandry and Pharmaceutical Sciences of the Chinese Academy of Agricultural Sciences (SCXK20008-0003). The animal protocols were in compliance with the ethical guidelines for the treatment of animals of the International Association for the Study of Pain (Zimmermann, 1983).

Analgesic Tests

Acetic Acid-induced Writhing Response

The writhing response in mice was carried out by using the method of previous study (Young et al., 2005). PYS was administrated orally to mice at doses of 155, 310, and 620 mg/kg-bw, respectively. Mice received indometacin (3 mg/kg-bw) in reference group and normal saline (10 mL/kg-bw) in control group. After 60 minutes treatment, each mouse was intraperitoneally administered 0.7% acetic acid (10 mL/kg-bw). The number of writhing movements was recorded in a period of 0-30 minutes after the injection of acetic acid. The inhibition ratio was calculated using the following equation:

$$\text{Inhibition ratio} = (N_c - N_t) / N_c \times 100\%$$

N_c represents the number of writhing in the control group, and N_t represents the number of writhing in the drug treated group.

Hot Plate Test

This test was carried out according to previously described method (Shinde et al., 1999). Mice whose forepaw licking or jumping on the heated plate (55 \pm 1 $^{\circ}$ C) within 30 s was used in this experiment. Mice were treated orally with PYS (155, 310, and 620 mg/kg-bw) or indometacin (3 mg/kg-bw) as reference. Normal saline was used in control animals (10 mL/kg-bw). Before and 30, 60, and 90 min after treatment, mice were individually put on the heated plate and the time for forepaw licking or jumping was registered as the latency time to thermal stimuli with a cut-off time of 60 s to avoid paw lesions.

Anti-inflammatory Tests**Xylene-induced Ear Swelling**

Experiments were carried out according to previously described method (Kou et al., 2005). Mice were pre-treated with PYS (155, 310, and 620 mg/kg·bw) 60 min prior to the induction of ear swelling. Ear swelling was induced by smearing 30 μ L of xylene on the anterior and posterior surfaces of the right ear. The positive control received indometacin (3 mg/kg·bw) and the control group received the same volume of normal saline. One hour later, the mice were sacrificed by cervical dislocation and circular sections were taken from both ears at the same place with a diameter of 8 mm punch, and weighed. The swelling degree and inhibition ratio were calculated using the following equations:

$$\text{Swelling degree (SD)} = (W_r - W_l) / W_l \times 100\%$$

$$\text{Inhibition ratio} = (SD_c - SD_t) / SD_c \times 100\%$$

W_r represents weight of the right ear, and W_l represents weight of the left ear of the same mouse; SD_c represents the swelling degree in the control group, and SD_t represents the swelling degree in the drug treated group.

Leukocyte Infiltration and Capillary Permeability Caused by Intraperitoneal Injection of 0.7% Acetic Acid

Experiments were carried out according to previously described method (Lucena et al., 2007). PYS (155, 310, and 620 mg/kg·bw), indometacin (3 mg/kg·bw) and normal saline (10 mL/kg·bw) was administered orally to mice, respectively. After 60 min, 0.5% Evans blue solution (10 mL/kg·bw) was intravenously injected to the tail veins of mice; 10 min later, mice were injected with 0.7% acetic acid solution in normal saline by intraperitoneal route. 20 min after the intraperitoneal injection, mice were sacrificed by cervical dislocation and the peritoneal cavity was opened and washed with 5 mL sterile saline. The peritoneal washes were collected. Finally, 50 μ L of peritoneal fluid was taken and diluted in 450 μ L of Türk's solution for total leukocyte counts under microscope (Olympus Company, Japan). The remainder of the peritoneal fluid was centrifuged at 3000 rpm for 15 min. The absorbance of the supernatant was read at 606 nm using an Evolution 300 UV-VIS spectrophotometer (Thermo Scientific, USA). The concentration of Evans blue leaked into the peritoneal cavity was calculated according to the standard curve of Evans blue, which indicated the peritoneal capillary permeability induced by acetic acid (Lucena et al., 2007). The inhibition ratios of leukocyte infiltration (IRLI) and capillary permeability (IRCP) in drug treated groups were calculated using the following equations:

$$\text{IRLI} = (L_c - L_t) / L_c \times 100\%$$

$$\text{IRCP} = (E_c - E_t) / E_c \times 100\%$$

L_c represents the number of leukocytes in the control group, and L_t represents the number of leukocytes in the drug treated group; E_c represents the concentration of Evans blue in the control group, and E_t represents the concentration of Evans blue in the drug treated group.

Carrageenan-induced Paw Edema

This test was based on the method of the previous study (Kou et al., 2005). PYS (155, 310, and 620 mg/kg·bw), indometacin (3 mg/kg·bw) and normal saline (10 mL/kg·bw) was administered orally to rats, respectively. One hour later, the edema of the right hind paw was induced by hypodermic injection with 100 μ L of 1% carrageenan suspension in normal saline. Paw thickness was determined with a vernier caliper before and 1, 2, 3 and 4 h after the injection of carrageenan. The swelling degree and inhibition ratio were calculated using the following equations:

$$\text{Swelling degree (SD)} = (PT_t - PT_0) / PT_0 \times 100\%$$

$$\text{Inhibition ratio} = (SD_c - SD_t) / SD_c \times 100\%$$

PT_0 represents the right hind paw thickness before carrageenan injection, and PT_t represents the right hind paw thickness 1, 2, 3, or 4 h after carrageenan injection; SD_c represents the swelling degree in the control group, and SD_t represents the swelling degree in the drug tested group.

Statistical Analysis

The data were expressed as mean \pm SE. Data using SPSS software program version 17.0 by a one-way ANOVA followed by LSD as the

post hoc test. The results were considered statistically significant at $p < 0.05$.

Results and Discussion

Owing to the complexity and mutability of the condition, bovine mastitis is still one of the most relevant and problematic diseases to treat and control in practice (Green and Bradley, 2013). PYS has been shown to have obvious effect on preventing and treating subclinical mastitis of dairy cows in our previous studies (Wang, 2013). In this present study, it demonstrated that PYS had analgesic and anti-inflammatory activities according to five animal models.

TLC Chromatograms of PYS

PYS is a mixture of *Taraxacum mongolicum*, *Vaccaria segetalis*, and *Epimedium*. The results of TLC analyses were shown in Figure 1, Figure 2, and Figure 3, which were in accord with the requirement of the detection of traditional Chinese herbal medicine in Chinese Pharmacopoeia. PYS contains several kinds of compounds and only the three marked components, caffeic acid, vaccarin, and icariin, were detected using TLC, because the low concentration of other compounds or lack of single component standards. In addition, the preparation of PYS was only through physical process, smash and mixture and the chemical components would not be changed between PYS and the three herbs.

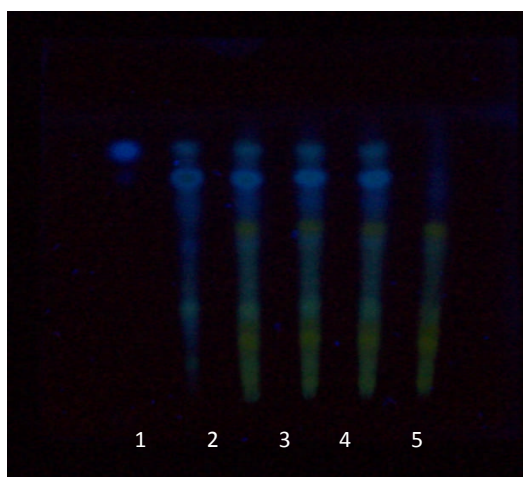


Figure 1: The TLC Chromatograms of Caffeic Acid (1), *Taraxacum mongolicum* Hand.-Mazz (2), PYS (3, 4, and 5), and negative control (6).

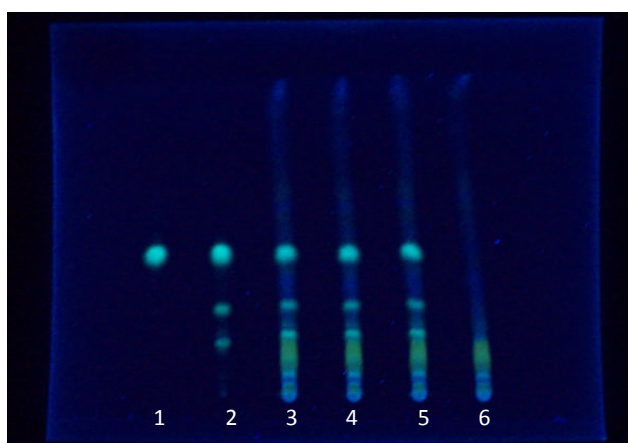


Figure 2: The TLC Chromatograms of Vaccarin (1), *Vaccaria segetalis* (Neck.) Garcke (2), PYS (3, 4, and 5), and negative control (6).

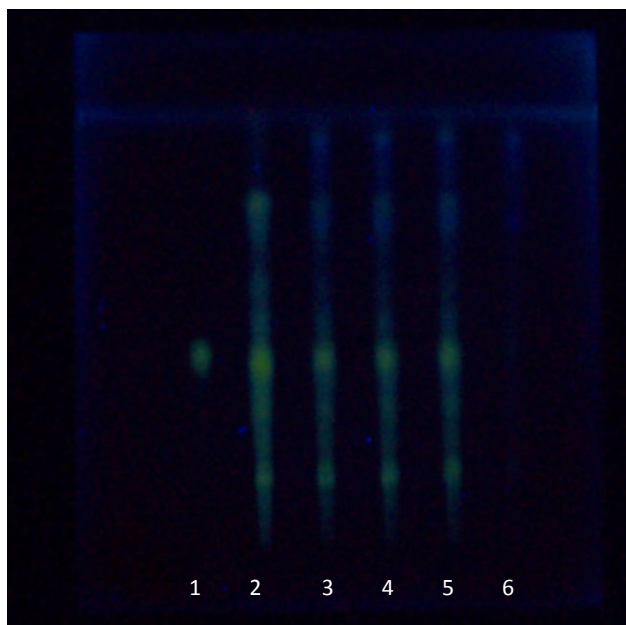


Figure 3: The TLC chromatograms of icariin (1), *Epimedium brevicornu Maxim* (2), PYS (3, 4, and 5), and negative control (6).

Analgesic Activity of PYS

Two animal models, the acetic acid-induced writhing response and the hot plate test, were employed to investigate the analgesic activity of PYS in this study. Acetic acid-induced writhing test is believed to indicate the involvement of peripheral mechanisms (Deraedt et al., 1980), whereas the hot plate test is believed to investigate the central mechanisms (Ferreira et al., 2004). The acetic acid-induced writhing model is a visceral pain model. In the process, arachidonic acid releases by cyclooxygenase and prostaglandins biosynthesis increases such as prostaglandins, serotonin, and histamine, which play an important effect on the nociceptive mechanism (Duarte et al., 1988). Table 2 showed the analgesic effect of PYS on the acetic acid-induced writhing test in mice. PYS (155, 310, and 620 mg/kg·bw) and indometacin significantly restrained the number of abdominal writhing compared with the control group. It indicated the good peripheral analgesia of PYS and the mechanism of the analgesic effect may be associated with blockage of arachidonic acid metabolite synthesis.

Table 2: Effect of PYS on the acetic acid-induced writhing test in mice.

Groups	Dose (mg/kg·bw)	Number of writhing	Inhibition (%)
Control	-	41.10±5.69	-
Indometacin	3	16.20±3.94**	60.58
	155	29.00±2.55**	29.44
PYS	310	26.33±6.60**	35.94
	620	21.11±3.66**	48.64

Each value represents mean ± SE. (n=10).

** $P < 0.01$ significantly different from the control group.

To check for the possible central analgesic activity of PYS, the hot-plate test was carried out, as it was sensitive to strong analgesics and

had limited tissue damage (Ferreira et al., 2004). In the hot-plate test (Table 3), although no significant effect was observed after 60 min of drug administration at doses of 155, and 310 mg/kg-bw, significant increase in the latency to the thermal stimuli was observed at 620 mg/kg-bw after 60 min of drug administration. Interestingly, in both cases, the effect was long lasting, and PYS increased the latency time could be observed at 90 min after drug administration, at doses of 155, 310, and 620 mg/kg-bw, respectively. In this study, it was possible that PYS exerted an analgesic effect at least in part through central mechanisms, because of the specific central nociception of hot plate test. Given both results of the two tests, we suggested that PYS had strong analgesic activity.

Table 3: Effect of PYS on the hot plate test in mice.

Groups	Dose (mg/kg-bw)	Latency period (s)			
		0 min	30 min	60 min	90 min
Control	-	22.23±4.53	18.59±3.87	18.16±4.98	17.14±5.85
Indometacin	3	21.89±3.44	23.32±8.47	26.84±6.14**	33.41±7.77**
	155	22.16±4.06	18.43±4.56	20.81±5.66	27.83±6.14**
PYS	310	22.54±4.24	19.11±5.04	23.38±5.91	27.57±6.37**
	620	22.37±6.05	24.23±8.30	24.79±5.97*	29.71±5.52**

Each value represents mean ± SE. (n=10). * $P < 0.05$, ** $P < 0.01$ significantly different from the control group at each corresponding time.

Anti-inflammatory Activity of PYS

The process of acute inflammatory resulted from vasodilation, capillary permeability accentuation, and edema formation at the inflammatory site. The xylene-induced ear swelling was a common inflammatory model to evaluate vasodilation and substance P partially participated in the process (Luber-Narod et al., 1997). Administration of xylene on ears brought about a significant increase of ear weight in the control group (Table 4). Compared with the control group, PYS significantly suppressed xylene-induced ear swelling in mice and it was dose-dependent. The results indicated that PYS might reduce the release of substance P or antagonize ear swelling induced by xylene.

Peritoneal capillary permeability and leukocyte infiltration in the abdominal cavity could be aggravated by intraperitoneal injection of acetic acid (Li et al., 2010). Injection of acetic acid led to a significant increase of total leukocytes and Evans blue content extruded into mice peritoneal cavity in the control group (Table 5 and Table 6). Compared with the control group, PYS as well as indometacin significantly reduced total leukocytes and Evans blue content induced by acetic acid in mice. These results indicated that peritoneal leukocyte infiltration and capillary permeability induced by acetic acid were suppressed by oral administration of PYS in a dose-dependent manner.

Table 4: Effect of PYS on the xylene-induced ear swelling test in mice.

Groups	Dose (mg/kg-bw)	Swelling (%)	Inhibition (%)
Control	-	64.16±5.20	-
Indometacin	3	30.76±4.29**	52.06
	155	55.48±5.59**	13.53
PYS	310	43.60±4.70**	32.04
	620	38.92±5.83**	39.34

Each value represents mean ± SE. (n=10). ** $P < 0.01$ significantly different from the control group.

The carrageenan-induced paw edema, including edema and hyperalgesia, is a reliable and repeatable model to evaluate the anti-inflammatory activity of natural products (Huang et al., 2011). The hind paw edema is a biphasic phase of various mediators release in sequence to generate the inflammatory response (Vinegar et al., 1969). The first phase is correlated with the release of histamine, serotonin and bradykinin (Di Rosa et al., 1971). The late phase, a complement-dependent reaction, contributes to elevated production of prostaglandins in tissues (Di et al., 1972; García et al., 2004; Posadas et al., 2004). Injection of carrageenan brought about a significant increase of paw thickness

in the control group and the inflammation approximately reached its maximum at 3 h after the carrageenan injection (Table 7). Compared with the control group, the edema was significantly decreased by administering PYS at 2, 3, and 4 h after the carrageenan injection. The results demonstrated that the anti-inflammatory effect of PYS mainly played in the late phase in the carrageenan-induced rat paw edema test. The anti-inflammatory mechanism of PYS might inhibit the synthesis of prostaglandins. According to all the anti-inflammatory tests, we suggested that PYS displayed a significant anti-inflammatory effect.

Table 5: Effect of PYS on the acetic acid-induced leukocyte infiltration test in mice.

Groups	Dose (mg/kg·bw)	Total leukocytes (×10 ⁶)	Inhibition (%)
Control	-	5.13±0.72	-
Indometacin	3	3.27±0.36**	36.26
	155	4.52±0.55**	11.89
PYS	310	3.76±0.24**	26.71
	620	3.66±0.31**	28.65

Each value represents mean ± SE. (n=10). **P<0.01 significantly different from the control group.

Table 6: Effect of PYS on the acetic acid-induced Evans blue leakage test in mice.

Groups	Dose (mg/kg·bw)	Evans blue (ug/mL)	Inhibition (%)
Control	-	7.13±0.99	-
Indometacin	3	4.68±0.20**	34.36
	155	5.86±0.72**	17.81
PYS	310	5.33±0.37**	25.25
	620	5.10±0.24**	28.47

Each value represents mean ± SE. (n=10).; **P<0.01 significantly different from the control group.

Table 7: Effect of PYS on the carrageenan-induced paw edema test in rats

Group	Dose (mg/kg·bw)	1 h		2 h		3 h		4 h	
		Swelling (%)	Inhibition (%)	Swelling (%)	Inhibition (%)	Swelling (%)	Inhibition (%)	Swelling (%)	Inhibition (%)
Control	-	32.94±5.7	-	58.59±3.75	-	71.61±9.92	-	62.61±4.66	-
Indometacin	3	30.94±3.5	6.07	44.13±4.80*	24.68	45.12±3.43*	36.99	26.95±7.29*	56.96
	155	32.29±6.1	1.97	58.42±4.67	0.29	70.89±6.50	1.01	49.82±4.98*	20.43
PYS	310	31.29±5.1	5.01	47.14±7.85*	19.54	52.90±2.97*	26.13	31.56±3.49*	49.59
	620	31.19±2.9	5.31	47.73±4.04*	18.54	49.76±5.68*	30.51	31.62±5.21*	49.50
	2			*		*		*	

Each value represents mean ± SE. (n=10).

**P<0.01 significantly different from the control group at each corresponding time.

Conclusion

This study suggested that PYS possessed significant analgesic and anti-inflammatory activities, supporting its use to treat mastitis in dairy

cows; and more investigations for ingredients and mechanisms responsible for the pharmacological activities of PYS should be studied further.

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