

Rhamnocitrin ameliorates ovarian fibrosis via PPAR γ /TGF- β 1/Smad2/3 pathway to repair ovarian function in polycystic ovary syndrome rats

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Research

Keywords: polycystic ovary syndrome, Rhamnocitrin, ovarian fibrosis, PPAR γ , TGF- β 1, Smad2/3

Posted Date: March 29th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-312602/v1>

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Abstract

Background:

Polycystic ovary syndrome (PCOS) is one of the major endocrine disorders in women, characterized by androgen excess, chronic anovulation and ovarian fibrosis. Rhamnocitrin is an herbal bioactive flavonoid that has anti-inflammation and antioxidant effects. We intended to investigate the impacts of Rhamnocitrin on PCOS-induced ovarian fibrosis and its underlying mechanisms.

Methods:

Dehydroepiandrosterone (DHEA) induced-PCOS rats were treated with Rhamnocitrin. HE staining was performed to detect ovarian histological features. Ovarian fibrosis was evaluated by Sirius Red and Masson staining. Vaginal smear was examined to exhibit estrus cycle stage and vaginal cornification. The serum hormone levels of FSH, LH, E2 and T were measured with ELISA. The related mRNAs and proteins of fibrosis factors and PPAR γ /TGF- β 1/Smad2/3 signaling were detected by RT-qPCR and western blot. The weights of rat bodies and ovaries were recorded. PPAR γ inhibitor T0070907 and its agonist GW1929 were employed for the mechanistic investigation.

Results:

The corpus luteum and follicles were increased and irregular estrous cycle was restored after Rhamnocitrin treatment in PCOS rats. Rhamnocitrin inhibited ovarian fibrosis and down-regulated the expressions of fibrotic factors. Rhamnocitrin reduced the increased levels of LH, E2, and T, and elevated the decreased FSH level in PCOS rats. Besides, Rhamnocitrin elevated the down-regulated PPAR γ , and suppressed the up-regulated TGF- β 1 and p-Smad2/3 expressions induced by PCOS. These effects of Rhamnocitrin on PCOS rats could be antagonized by T0070907, whereas GW1929 markedly mimics the functions of Rhamnocitrin.

Conclusions:

Rhamnocitrin ameliorates ovarian fibrosis in PCOS rats through regulation PPAR γ /TGF- β 1/Smad2/3 pathway, suggesting it can be a potentially effective therapeutic candidate for PCOS treatment.

Introduction

Polycystic ovary syndrome (PCOS), a common disorder of anovulatory infertility, is one of the major endocrine-metabolic disorder in reproductive age women [1]. Pathophysiology of PCOS is a complex interplay among numerous factors containing androgen excess, disordered gonadotropin secretion, insulin resistance, follicular arrest and ovarian dysfunction [2]. PCOS is characterized by hormonal imbalance, including the leutinizing/follicle-stimulating hormone (LH/FSH) ratio, gonadotrophin-releasing hormone (GnRH), insulin, parathyroid hormone (PTH), estrogens, androgens and cortisol [3]. Significantly,

no general treatment is available for PCOS, and therapeutic approaches for PCOS treatment mainly target hyperandrogenism, ovarian dysfunction and metabolic disorders [4].

Flavonoids are well known to be bioactive polyphenols that associated with anti-inflammation and antioxidant effects due to their highly reactive oxygen radicals [5]. Flavonoids are traditionally utilized to prevent or treat various diseases containing PCOS and reproductive system dysfunction [6]. For instance, supplementation of quercetin down-regulates the level of resistin in plasma and the concentrations of LH and testosterone in overweight PCOS patients[7]. Troxerutin, a natural flavonoid, protects against dihydrotestosterone (DHT)-induced PCOS in rats via modulating neurotransmitter release [8]. Treatment of rutin apparently ameliorates ovarian malfunction and systemic insulin resistance in PCOS rats [9]. Literatures have reported that Rhamnocitrin has the efficacy of anti-inflammation and antioxidant activity [10, 11]. However, limited data are available assessing the effects of Rhamnocitrin on ovarian dysfunction in PCOS.

Polycystic ovarian morphology in PCOS is featured with a thickening of ovarian capsule and stroma induced by up-regulation of collagen deposition and fibrous tissue [12]. As nuclear receptors, peroxisome proliferator-activated receptors (PPARs) are involved in modulating fibrosis [13]. Enormous researches have confirmed that PPAR γ exerts a protective function in tissue from fibrosis and have explored natural products that have latent PPAR γ -activation efficiency [14, 15]. As one of the multifunctional cytokine families, transforming growth factor- β (TGF- β) functions in numerous physiological and pathological processes, such as tissue fibrosis and wound healing [16]. Studies present that TGF- β 1 plays a critical function in extracellular matrix remodeling in ovary [17]. TGF- β 1 acts as a vital regulator in tissue fibrosis largely via activating its downstream small mother against decapentaplegic (Smad) signaling, including Smad2 and Smad3 [18]. Activation of TGF- β 1/Smad3 signaling pathway suppresses development of ovarian follicle through facilitating granulosa cell apoptosis in PCOS [19].

PCOS rodent model induced by dehydroepiandrosterone (DHEA) displays critical characteristics similar to PCOS women, including a high androgen state, abnormal secretion of serum hormones, blocked follicular development, deficient corpora lutea and presence of cystic follicles and so on [20]. Furthermore, DHEA-induced PCOS rats are validated to present ovarian collagen deposition and hyper fibrosis, which compromises ovarian functions [12, 21, 22]. Here, DHEA induced-PCOS rats were applied to explore the role of Rhamnocitrin in PCOS, including ovarian histological features, estrus cycle stage, serum hormone levels, ovarian fibrosis and fibrosis factors expressions. Besides, we then validated whether PPAR γ /TGF- β 1/Smad2/3 pathway was implicated in effects of Rhamnocitrin on ovarian fibrosis to modulate ovarian function. This study aims to provide a potential effective drug for patients with PCOS.

Materials And Methods

Animals

Sprague–Dawley (SD) rats (female, 21 days old, 50–60 g) were obtained from Experimental Animal Center of Guangxi Medical University. Rats were housed in SPF environment at 24 °C with 12:12 h light–

dark cycle. They were provided with free access to water and food. This research was performed according with the guidelines for the use of laboratory animals and approved by the institutional research animal committee of Guilin Medical College. Approval Number: GLMC201803009.

Experiment design

The rats were randomly divided into 7 groups (n=6). For PCOS induction, injectable sesame oil was used to dissolve DHEA. Then rats received a daily hypodermic injection of DHEA solution with a dose of 0.2 ml (6 mg/100 g) at nape of the neck for 21 consecutive days, fed with high fat diet. Naïve rats were adopted as controls. Additionally, some rats also orally treated with three levels of Rhamnocitrin (5mg/kg/day;10mg/kg/day;20mg/kg/day,p.o;Carbosynth,UK) daily during treatment period. For PPAR γ inhibitor treatment, rats were injected intraperitoneally with T0070907 (10 mg/kg, Sigma-Aldrich, St Louis, MO, USA) once daily. For PPAR γ agonist treatment, rats were treated with GW1929 (15 mg/kg, Sigma-Aldrich) by oral gavage once daily. Estrous cycle and body weight of rats were recorded, during process and treatment. At day 21, rats were killed, and the blood and ovaries of rats were collected. After that, fat around the ovaries was removed and the ovaries were weighed.

Serum hormone measurement

The rat orbital veins blood was received administration. After separated from blood samples, serum was stored under -80°C . The Follicle-Stimulating Hormone (FSH), luteinizing hormone (LH), estradiol (E2) and total testosterone (T) in serum were evaluated with ELISA following the guideline. Testing kits for FSH, LH, E2 and T were obtained from Elabscience (Elabscience, USA). Experiments were carried out in triplicate and repeated three times.

Hematoxylin and eosin (H&E) staining

After fixed with 10% neutral-buffered formalin, ovaries were embedded in paraffin. After that, they were sectioned into 3 μm thick slices. Hematoxylin and eosin were utilized to stain paraffin slices and optical microscope (Leica Microsystems, Wetzlar, Germany) was applied to confirm pathological structural variations of rat ovaries.

Sirius Red and Masson staining

After fixed with 10% neutral-buffered formalin, ovaries were embedded in paraffin. Subsequently, they were sectioned into slices (3 μm). They were then stained with Sirius red or Masson staining to reveal ovarian fibrosis and confirm inhibitory impact of Rhamnocitrin on fibrosis. For Masson staining, tissue slides were then incubated at 37°C for 2 h with Bouin solution, which contained acetic acid (5 mL), 10% formalin solution (25 mL) and saturated picric acid (75 mL). After that, according to the manufacturer's direction, slides were stained using Masson Trichrome Staining kit (Sigma-Aldrich) and collagen fibers were stained in blue. For Sirius red staining, tissue sections were administered for 20 min with Picro-Sirius red stain solution kit (Abcam, Cambridge, MA, USA) and washed for 1 min with tap water in accordance with the manufacturer's guideline. The muscle, blood vessels and epithelium appeared yellowish while

collagen fibers were stained in red. These stained specimens were visualized under light microscope (Leica Microsystems).

Determination of the Estrus Cycle Stage

Toluidine blue stain was utilized to determine variations of vaginal exfoliative cytology in rats. After was soaked in physiological saline, aseptic cotton swab was put on vaginal wall of rats and smeared clockwise. Cotton swab was taken out and smeared on slide in same direction. Afterwards, cells on slide were fixed for 15 min with 4% paraformaldehyde. Toluidine blue (Servicebio, MA) was applied to stain vaginal smear following instruction. Microscope imaging system (Nikon, Japan) was used to visualize the images.

Western blot

Ovarian tissues were milled separately with RIPA lysis buffer. After protein collection from each specimen, protein concentration detection was carried out with BCA protein assay kit (Biovision, Milpitas, CA, USA) according with the instruction. The electrophoretic separation is run using SDS-PAGE gels (TakaRa Biotechnology Co., Ltd., Dalian, China). Protein (50 µg) were separated with 10% SDS-PAGE prior to transfer onto PVDF membranes. After that, they were blocked at room temperature in 5% bovine serum albumin for 2 h. Subsequently, they were incubated with primary (12h at 4°C) before treatment with secondary antibodies at room temperature for 2 h. Primary antibodies: anti-PPAR γ (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), anti-TGF- β 1 (1:250; Santa Cruz), anti-Smad2/3 (1:200; Santa Cruz), anti-p-Smad2/3 (1:500, Santa Cruz), anti- α -SMA (1:300; Sigma, St Louis, MO, USA), anti-Collagen1 (1:500, Santa Cruz), anti-MMP-2 (1:500; Abcam, Cambridge, MA, USA), anti-MMP-9 (1:1000; Abcam), anti-TIMP1(1:500, Cell Signaling Technology, Danvers, MA, USA), anti-GAPDH (1:1000, Cell Signaling). Immobilon Western Chemiluminescent HRP Substrate (Abcam) was used to develop the blots. GAPDH was adopted as internal control. Protein expression quantitative analysis was performed with ImageJ software 1.8.0.

Quantitative real-time PCR

RNA was collected with Trizol Reagent (Takara) according with the guideline. cDNA was generated with RNA (1 µg) with Prime Script TM RT Reagent kit (Takara) followed by amplified by RT-PCR with the following cycling conditions: 15 minutes for reverse transcription at 37°C, 5 seconds for inactivating reverse transcriptase at 85°C, followed by cold-storage at 4°C. cDNA was amplified by Brilliant II Fast SYBR green QPCR master mix (Sigma-Aldrich, MO, USA) with ABI 7500 Fast Real-Time PCR System (Applied Biosystems) using the following cycle: 30 seconds for pre-denaturation at 95°C, 5 seconds for PCR amplification at 95°C (45 cycles), and 30 seconds for primer annealing at 60°C. Primer was produced from Macrogen Inc. (Seoul, Korea). GAPDH expression was applied as control. The relative level analysis was achieved by the comparative CT method ($2^{-\Delta\Delta CT}$). The primers were showed in Table 1.

Statistical analysis.

Statistical analyses were evaluated with SPSS 21.0 software. All data are shown as means \pm SEM. Difference between two groups was compared using Student's t test. Results for multiple comparisons was implemented by ANOVA followed by Tukey's post hoc test. The criterion of statistical significance was set as P value less than 0.05.

Results

3.1 Rhamnocitrin recovered ovarian function in PCOS rats

Morphological analysis was performed on ovarian tissues of rats. In model group, the disordered ovary structure was observed, corpus luteum and follicles were noticeably decreased, whereas antral follicles were remarkably increased in comparison by control group (Fig. 1A). Additionally, compared to control group, granular cells were arranged loosely and layer of cells developed thinner (Fig. 1A). Compared to PCOS group, corpus luteum was increased after Rhamnocitrin administration (Fig. 1A). Furthermore, antral follicles were evidently reduced and granular cell layer thickness was increased after Rhamnocitrin administration (Fig. 1A). Results of vaginal smears presented that estrous cycle was regular, with cornified squamous epithelial cells in estrus of control group (Fig. 1B), however, PCOS rats exhibited the irregular estrous cycle, with numerous leucocytes and in continuous diestrus phase (Fig. 1B). After Rhamnocitrin treatment, estrous cycle returned to regular and normal gradually (Fig. 1B). These data revealed that Rhamnocitrin recovered the abnormal ovarian morphology and estrous cycle in PCOS rats.

Among these groups, no obvious difference was exhibited in body weight prior to any intervention, (Fig. 1C). After treated orally by DHEA for 21 days with high-fat diet, we observed significantly increased body weight of rats in comparison with control group (Fig. 1C). Rat weight in rhamnocitr group was apparently decreased compared to PCOS group, especially in high dose Rhamnocitrin rats (Fig. 1C). Ovary weight were also recorded, and the result revealed that in comparison to control group, ovary weight in PCOS rats was effectively increased (Fig. 1D). Whereas the ovary weight was reduced in Rhamnocitrin treated rats, in comparison with that of PCOS rats (Fig. 1D). Besides, the serum hormone levels of FSH, LH, E2 and T were analyzed. The serum levels of LH, E2 and T were obviously up-regulated in PCOS group compared to control group, and Rhamnocitrin administration apparently down-regulated serum LH, E2 and T level when compared to PCOS group (Fig. 1E, F, G). Additionally, Rhamnocitrin treatment significantly elevated the decreased FSH level in PCOS group (Fig. 1H).

3.2 Antifibrotic effects and regulating PPAR γ / TGF- β 1/Smad2/3 signaling of Rhamnocitrin on ovaries of PCOS rats

Sirius Red and Masson staining were used to measure the role of Rhamnocitrin in ovarian fibrosis. Our data demonstrated that in comparison to control group, ovarian interstitial fibrosis was elevated in PCOS rats (Fig. 2A), whereas ovarian interstitial fibrosis was suppressed after Rhamnocitrin treatment (Fig. 2A),

unveiled by Matson staining. In addition, Sirius Red also showed the similar results (Fig. 2B). These data suggested that ovarian fibrosis was inhibited by treatment with Rhamnocitrin.

In rat ovarian tissues, mRNA expressions of fibrosis factors, including α -SMA, Collagen1, MMP-2, MMP-9, TIMP1 were determined by qRT-PCR (Fig. 2C). Data presented that fibrosis factors mRNA expressions in PCOS group were remarkably elevated in control group (Fig. 2C). In ovarian tissues, mRNA expressions of fibrosis factors were decreased considerably in presence of Rhamnocitrin as comparing to PCOS group (Fig. 2C). The protein expression of fibrosis factors exhibited the similar results with qRT-PCR (Fig. 2D). Besides, it was presented that in ovarian tissues from PCOS group, PPAR γ was obviously decreased, while TGF- β 1 and p-Smad2/3 levels were evidently increased in comparison to control group (Fig. 2D). There was a significant increase in expression of PPAR γ , while decrease in expression of TGF- β 1 and p-Smad2/3 after Rhamnocitrin treatment (Fig. 2D). In addition, no significant difference was detected in protein expression of Smad2/3 among these groups (Fig. 2D), suggesting that Rhamnocitrin exerted a role of inhibiting Smad2/3 phosphorylation.

3.3 Rhamnocitrin restored ovarian function by activating PPAR γ in PCOS rats

PPAR γ agonist GW1929 and PPAR γ inhibitor T0070907 were administrated to explore whether PPAR γ signaling pathway was involved in ovarian function recovery of Rhamnocitrin in PCOS rats. We found that in comparison with the PCOS group, High-Rhamnocitrin supplementation showed elevated thickness of granular cell layer, reduced antral follicles and increased corpus luteum (Fig. 3A). Whereas T0070907 reversed the alteration of ovarian tissues induced by Rhamnocitrin in PCOS rats (Fig. 3A). Additionally, GW1929 treatment in PCOS rats exhibited the similar histological characteristics of ovaries with that in High-Rhamnocitrin treated model rats (Fig. 3A). In addition, we found that Rhamnocitrin rescued the irregular estrous cycle induced by PCOS (Fig. 3B). In PCOS rats treated by GW1929, the estrous cycle also returned to regular and became normal, which was similar with that in model rats treated by Rhamnocitrin (Fig. 3B). However, the effect of high-Rhamnocitrin on estrous cycle was antagonized by T0070907 in PCOS rats (Fig. 3B).

In comparison with PCOS group, Rhamnocitrin treatment reduced the weight of PCOS rats, which was similar with GW1929 addition in PCOS rats (Fig. 3C). The reduction of body weight induced by Rhamnocitrin could be reversed after T0070907 treatment (Fig. 3C). Ovary weight were also recorded, and the result was in agreement with body weight (Fig. 3D). We then measured the serum hormone levels of FSH, LH, E2 and T. The data showed that the serum LH, E2 and T levels were obviously increased in PCOS group, compared to that in control group (Fig. 3E, F, G), and both Rhamnocitrin and GW1929 treatment could reduce the elevated LH, E2 and T level of PCOS rats (Fig. 3E, F, G). Compared to control group, FSH level in PCOS group was decreased (Fig. 3H), which could be significantly elevated after Rhamnocitrin or GW1929 treatment (Fig. 3H). On the contrary, Rhamnocitrin induced-alterations of serum hormone levels were antagonized by T0070907 supplementation (Fig. 3E, F, G, H).

3.4 Rhamnocitrin exerted antifibrotic effects on ovaries through PPAR γ /TGF- β 1/Smad2/3 signaling in PCOS rats

Next, we investigated whether PPAR γ signaling pathway was implicated in antifibrotic effect of Rhamnocitrin on ovaries of PCOS rats. Sirius Red and Masson staining were used to evaluate ovarian fibrosis. The data from Masson staining presented that compared with the control group, ovarian interstitial fibrosis was elevated in PCOS rats (Fig. 4A). However, ovarian fibrosis of PCOS rats could be suppressed by administrated with Rhamnocitrin (Fig. 4A), whereas this alteration was reversed by T0070907. GW1929 mimicked the function of Rhamnocitrin on ovarian fibrosis in PCOS rats (Fig. 4A). In addition, the similar results were showed by Sirius Red staining (Fig. 4B). These data suggested that GW1929 or Rhamnocitrin treatment inhibited ovarian fibrosis of PCOS rats, while T0070907 could antagonize antifibrotic effects of Rhamnocitrin on ovaries in PCOS rats.

The qRT-PCR data illustrated that mRNA expressions of fibrosis factors including α -SMA, Collagen1, MMP-2, MMP-9, TIMP1 in PCOS group were remarkably up-regulated, in comparison by control group (Fig. 4C). After Rhamnocitrin or GW1929 treatment, mRNA levels of these fibrosis factors in ovarian tissues were apparently down-regulated (Fig. 4C). Effect of Rhamnocitrin on mRNA levels of fibrosis factors were reversed by T0070907 application (Fig. 4C). The effects of GW1929 and T0070907 on protein expressions of α -SMA, Collagen1, MMP-2, MMP-9, TIMP1 in ovaries detected by western blot showed the similar results with mRNA expressions measured with qRT-PCR (Fig. 4D). Besides, protein expressions of PPAR γ , TGF- β 1 and p-Smad2/3 were also determined. Treatment with Rhamnocitrin or GW1929 in PCOS rats could reverse the down-regulated PPAR γ and the up-regulated TGF- β 1 and p-Smad2/3 in the ovarian tissues, compared to PCOS group (Fig. 4D). Conversely, T0070907 antagonized the effects of Rhamnocitrin on the expressions of PPAR γ , TGF- β 1 and p-Smad2/3 in PCOS rats (Fig. 4D). In addition, there was no significant difference in Smad2/3 protein expression among these groups (Fig. 4D).

Discussion

The judicious application of appropriate therapeutic approaches for treatment of PCOS are required, mainly addressing hyperandrogenism, ovarian dysfunction and associated metabolic disorders [4]. Ovarian fibrosis in PCOS is characterized by increased interstitial fibrosis and collagen deposition, leading to up-regulated ovarian capsule and stroma [12]. Currently, the usage of natural products such as flavonoids in the treatment of different diseases has attracted great attention [6]. In the present work, which is the first of its kind to the best of our knowledge, we evaluated that Rhamnocitrin supplementation had beneficial effects on ovarian morphology and estrous cycle disorders, body and ovary weights, hormonal status, ovarian fibrosis in rats with PCOS. Besides, Rhamnocitrin ameliorates ovarian fibrosis through PPAR γ /TGF- β 1/Smad2/3 pathway in PCOS rats. The present study provides a potentially effective therapeutic candidate of Rhamnocitrin for treating PCOS, the hallmark of anovulatory infertility and endocrine-metabolic disorders in reproductive age women.

Studies have shown that Rhamnocitrin, an herbal bioactive flavonoid, has been reported to exert various pharmacological effects. Rhamnocitrin extracted from *Nervilia fordii* inhibits vascular endothelial activation [10]. Rhamnocitrin isolated from *Prunus padus* var. *seoulensis* is validated to be a reversible human monoamine oxidase (hMAO) inhibitor [23]. Rhamnocitrin 3-O-b-isorhamninoside induces human lymphoblastoid cell apoptosis via the extrinsic apoptosis pathway [24]. Obtained from *Rhamnus alaternus* L. (Rhamnaceae), Rhamnocitrin 3-O-b-isorhamninoside is evaluated to have antioxidant and antigenotoxic activities [11]. However, as far as we know, whether Rhamnocitrin can restore the ovarian damage in PCOS remains unclear. Due to ethical issues, studies on humans have limitations, hence animal models of PCOS aids in studying various aspects beginning from etiology to the treatment [20]. In current study, we first investigate impacts of Rhamnocitrin on DHEA-induced PCOS rats. Our study demonstrated that a number of indicators of ovarian dysfunction, including corpus luteum, antral follicles, the thickness of the granular cell layer and estrous cycle, were restored by Rhamnocitrin supplementation in DHEA treated rats. In addition, the observations that Rhamnocitrin inhibited DHEA-induced up-regulated LH, E2, and T serum levels while promoted the down-regulated FSH serum level, suggest its beneficial role in hormonal secretion and release. Previous study reveals that total flavonoids of dodder can decrease ovary index, affect serum hormone secretion and improve endometrial hyperplasia, achieving a protective effect on PCOS rats [25]. Rutin, a flavonoid, ameliorates hyperandrogenism, acyclicity and infertility to improve PCOS phenotypes [9]. Flavonoids from *Nervilia Fordii* can down-regulate FSH serum level, up-regulate of LH and T serum levels and recover estrous cycle, exerting a therapeutic efficiency for PCOS treatment [26]. Our results are in line with these previous findings that herbal bioactive flavonoids can exert a protective effect on PCOS. Taken together, these observations support the conclusions that Rhamnocitrin has restored ovarian function successfully in PCOS rats induced by DHEA, might probably be considered as one candidate components for PCOS treatment.

Study demonstrates that PCOS rats exhibit ovarian fibrosis which results in function disorder of ovary [22]. As far as we know, in recent studies, the effect of Rhamnocitrin on tissue fibrosis is still unclear. In this study, we discovered Rhamnocitrin was able to repress ovarian interstitial fibrosis, indicated by Matson and Sirius Red staining. The inhibited mRNA and protein levels of fibrotic factors, including α -SMA, Collagen1, MMP-2, MMP-9 and TIMP1, in Rhamnocitrin administration rats may contribute to explain inhibitory mechanism on ovarian fibrosis. Recent researches present that PPAR γ /TGF- β 1/Smad signaling pathway participated in tissue fibrosis development. For example, capsaicin inhibits hepatic fibrosis via activating PPAR γ to suppress TGF- β 1/Smad Pathway [27]. Dual PPAR- α / γ agonist saroglitazar attenuates renal fibrosis via inhibiting TGF- β /Smad signaling pathway [28]. PPAR- γ agonist triterpenoid alleviates fibrogenesis via TGF- β /Smad and Akt pathway [29]. TGF- β 1 from endometriomas modulates TGF- β 1/Smad signaling pathway to promote fibrosis and adhesion to ovary [30]. TGF- β 1/Smad3 pathway participates in ovarian fibrosis inhibition and results in ovarian function restoration in primary ovarian insufficiency (POI) rats after human umbilical cord mesenchymal stem cell (hUMSC) transplantation [31]. Whereas, whether PPAR γ /TGF- β 1/Smad signaling is implicated in ovarian fibrosis and dysfunction of PCOS is still unclear. Here, we demonstrated that PPAR γ was decreased while TGF- β 1

and p-Smad2/3 was elevated in ovarian tissues of PCOS rats, Rhamnocitrin could restore abnormal expressions of PPAR γ , TGF- β 1 and p-Smad2/3. In subsequent study, we demonstrated that PPAR γ inhibitor T0070907 antagonized those beneficial effects of Rhamnocitrin on PCOS rats, including ovarian morphology and estrous cycle disorders, body and ovary weights, hormonal status, ovarian fibrosis and the related fibrosis factors expressions, whereas PPAR γ agonist GW1929 markedly mimicked the functions of Rhamnocitrin. These data further prove that Rhamnocitrin plays its therapeutic efficiency via regulating PPAR γ /TGF- β 1/Smad2/3 pathway.

In addition to the implication into the development of tissue fibrosis, PPAR γ , TGF- β 1 and Smad signaling also participates in other important pathological processes of ovarian dysfunction. Previous research shows that upregulation of PPAR- γ and aromatase Cyp19a1 in ovarian steroidogenic pathway can be a potential cure for PCOS [32]. The decreased bioavailability of TGF- β 1 is correlated with an improvement in some abnormal clinical parameters of PCOS [33]. Enhanced Smad2 level by plasmid can reduce apoptosis and improve cell viability of rat ovarian granulosa cells [34]. TGF- β 1/Smad3 pathway regulates apoptosis of granulosa cells, through inhibiting ovarian follicle development of PCOS [35]. Therefore, what needs to be emphasized is that other potential mechanisms may also exist in the function of Rhamnocitrin on PCOS, which need to be further investigated in the future study.

In conclusion, we demonstrate here that Rhamnocitrin ameliorates ovarian fibrosis in PCOS rats through regulation PPAR γ /TGF- β 1/Smad2/3 pathway, which significantly improves PCOS induced dysfunctions. To our best knowledge, this is the first report to identify Rhamnocitrin, an herbal bioactive flavonoid, as potential novel therapeutic option for PCOS pharmaceutical treatment.

Declarations

Data Availability

The data obtained in this research are available from the corresponding author on reasonable request.

Conflicts of interest

All authors declare having no conflicts of interest relevant to this research.

Author Contributions

YYZ conceived and designed the experiments, YYZ, HL, ZWD and YQW performed the experiments, CJC analyzed the data, YYZ wrote the first draft, and JLS made the amendments and provide financial support.

Consent for publication

Written informed consent for publication was obtained from all participants.

Funding

National Natural Science Foundation of China. [81860767]

Guangxi Natural Science Foundation under Grant. No. 2018GXNSFAA281104

Innovation Project of Guangxi Graduate Education .YCSW2020224

Acknowledgments

None

Ethical Statement

All animal experiments were followed the Universal Declaration on Animal Welfare, and approved by the institutional research animal committee of Guilin Medical College. Approval Number: GLMC2018 03009.

References

1. Azziz R. Polycystic Ovary Syndrome. *Obstet Gynecol.* 2018;132(2):321–36. doi:10.1097/AOG.0000000000002698.
2. Rosenfield RL, Ehrmann DA. The Pathogenesis of Polycystic Ovary Syndrome (PCOS): The Hypothesis of PCOS as Functional Ovarian Hyperandrogenism Revisited. *Endocr Rev.* 2016;37(5):467–520. doi:10.1210/er.2015-1104.
3. Krishnan A, Muthusami S. Hormonal alterations in PCOS and its influence on bone metabolism. *J Endocrinol.* 2017;232(2):R99–113. doi:10.1530/JOE-16-0405.
4. Escobar-Morreale HF. Polycystic ovary syndrome: definition, aetiology, diagnosis and treatment. *Nat Rev Endocrinol.* 2018;14(5):270–84. doi:10.1038/nrendo.2018.24.
5. Chen L, Teng H, Xie Z, Cao H, Cheang WS, Skalicka-Woniak K, Georgiev MI, Xiao J. Modifications of dietary flavonoids towards improved bioactivity: An update on structure-activity relationship. *Crit Rev Food Sci Nutr.* 2018;58(4):513–27. doi:10.1080/10408398.2016.1196334.
6. Rashidi Z, Khosravizadeh Z, Talebi A, Khodamoradi K, Ebrahimi R, Amidi F. Overview of biological effects of Quercetin on ovary. *Phytother Res.* 2020. doi:10.1002/ptr.6750.
7. Khorshidi M, Moini A, Alipoor E, Rezvan N, Gorgani-Firuzjaee S, Yaseri M, Hosseinzadeh-Attar MJ. The effects of quercetin supplementation on metabolic and hormonal parameters as well as plasma concentration and gene expression of resistin in overweight or obese women with polycystic ovary syndrome. *Phytother Res.* 2018;32(11):2282–9. doi:10.1002/ptr.6166.
8. Gao Z, Ma X, Liu J, Ge Y, Wang L, Fu P, Liu Z, Yao R, Yan X. Troxerutin protects against DHT-induced polycystic ovary syndrome in rats. *J Ovarian Res.* 2020;13(1):106. doi:10.1186/s13048-020-00701-z.
9. Hu T, Yuan X, Ye R, Zhou H, Lin J, Zhang C, Zhang H, Wei G, Dong M, Huang Y, et al. Brown adipose tissue activation by rutin ameliorates polycystic ovary syndrome in rat. *J Nutr Biochem.* 2017;47:21–8. doi:10.1016/j.jnutbio.2017.04.012.

10. Lin T, Luo W, Li Z, Zhang L, Zheng X, Mai L, Yang W, Guan G, Su Z, Liu P, et al. Rhamnocitrin extracted from *Nervilia fordii* inhibited vascular endothelial activation via miR-185/STIM-1/SOCE/NFATc3. *Phytomedicine*. 2020;79:153350. doi:10.1016/j.phymed.2020.153350.
11. Bhouri W, Sghaier MB, Kilani S, Bouhlel I, Dijoux-Franca MG, Ghedira K, Ghedira LC. Evaluation of antioxidant and antigenotoxic activity of two flavonoids from *Rhamnus alaternus* L. (Rhamnaceae): kaempferol 3-O-beta-isorhamninol and rhamnocitrin 3-O-beta-isorhamninol. *Food Chem Toxicol*. 2011;49(5):1167–73. doi:10.1016/j.fct.2011.02.011.
12. Takahashi N, Harada M, Hirota Y, Nose E, Azhary JM, Koike H, Kunitomi C, Yoshino O, Izumi G, Hirata T, et al. Activation of Endoplasmic Reticulum Stress in Granulosa Cells from Patients with Polycystic Ovary Syndrome Contributes to Ovarian Fibrosis. *Sci Rep*. 2017;7(1):10824. doi:10.1038/s41598-017-11252-7.
13. Lu Q, Guo P, Guo J, Ares I, Lopez-Torres B, Martinez-Larranaga MR, Wang X, Anadon A, Martinez MA. Targeting peroxisome proliferator-activated receptors: A new strategy for the treatment of cardiac fibrosis. *Pharmacol Ther* 2020:107702.doi:10.1016/j.pharmthera.2020.107702.
14. Wei S, Xu C, Zhang Y, Shi Z, Wu M, Yang B. Ultrasound Assisted a Peroxisome Proliferator-Activated Receptor (PPAR) γ Agonist-Loaded Nanoparticle-Microbubble Complex to Attenuate Renal Interstitial Fibrosis. *Int J Nanomedicine*. 2020;15:7315–27. doi:10.2147/IJN.S262052.
15. Wu L, Guo C, Wu J. Therapeutic potential of PPAR γ natural agonists in liver diseases. *J Cell Mol Med*. 2020;24(5):2736–48. doi:10.1111/jcmm.15028.
16. Morikawa M, Derynck R, Miyazono K. TGF- β and the TGF- β Family: Context-Dependent Roles in Cell and Tissue Physiology. *Cold Spring Harb Perspect Biol* 2016, 8(5).doi:10.1101/cshperspect.a021873.
17. Fang Y, Chang HM, Cheng JC, Klausen C, Leung PC, Yang X. Transforming growth factor- β 1 increases lysyl oxidase expression by downregulating MIR29A in human granulosa lutein cells. *Reproduction*. 2016;152(3):205–13. doi:10.1530/REP-16-0144.
18. Hu HH, Chen DQ, Wang YN, Feng YL, Cao G, Vaziri ND, Zhao YY. New insights into TGF- β /Smad signaling in tissue fibrosis. *Chem Biol Interact*. 2018;292:76–83. doi:10.1016/j.cbi.2018.07.008.
19. Tal R, Seifer DB, Shohat-Tal A, Grazi RV, Malter HE. Transforming growth factor- β 1 and its receptor soluble endoglin are altered in polycystic ovary syndrome during controlled ovarian stimulation. *Fertil Steril*. 2013;100(2):538–43. doi:10.1016/j.fertnstert.2013.04.022.
20. Divyashree S, Janhavi P, Ravindra PV, Muthukumar SP. Experimental models of polycystic ovary syndrome: An update. *Life Sci*. 2019;237:116911. doi:10.1016/j.lfs.2019.116911.
21. Wang D, Wang T, Wang R, Zhang X, Wang L, Xiang Z, Zhuang L, Shen S, Wang H, Gao Q, Wang Y. Suppression of p66Shc prevents hyperandrogenism-induced ovarian oxidative stress and fibrosis. *J Transl Med*. 2020;18(1):84. doi:10.1186/s12967-020-02249-4.
22. Wang D, Wang W, Liang Q, He X, Xia Y, Shen S, Wang H, Gao Q, Wang Y. DHEA-induced ovarian hyperfibrosis is mediated by TGF- β signaling pathway. *J Ovarian Res*. 2018;11(1):6. doi:10.1186/s13048-017-0375-7.

23. Baek SC, Park MH, Ryu HW, Lee JP, Kang MG, Park D, Park CM, Oh SR, Kim H. Rhamnocitrin isolated from *Prunus padus* var. *seoulensis*: A potent and selective reversible inhibitor of human monoamine oxidase A. *Bioorg Chem.* 2019;83:317–25. doi:10.1016/j.bioorg.2018.10.051.
24. Bhouri W, Bouhrel I, Boubaker J, Kilani S, Ghedira K, Ghedira LC. Induction of apoptosis in human lymphoblastoid cells by kaempferol 3-O-beta-isorhamninoside and rhamnocitrin 3-O-beta-isorhamninoside from *Rhamnus alaternus* L. (Rhamnaceae). *Cell Prolif.* 2011;44(3):283–90. doi:10.1111/j.1365-2184.2011.00749.x.
25. Miao M, Peng M, Zhu Z, Yan X, Wei Z, Li M. Effects of dodder total flavone on polycystic ovary syndrome rat models induced by DHEA combined HCG. *Saudi J Biol Sci.* 2019;26(4):821–7. doi:10.1016/j.sjbs.2019.02.007.
26. Zhou Y, Lv L, Liu Q, Song J. Total flavonoids extracted from *Nervilia Fordii* function in polycystic ovary syndrome through IL-6 mediated JAK2/STAT3 signaling pathway. *Biosci Rep* 2019, 39(1).doi:10.1042/BSR20181380.
27. Choi JH, Jin SW, Choi CY, Kim HG, Lee GH, Kim YA, Chung YC, Jeong HG. Capsaicin Inhibits Dimethylnitrosamine-Induced Hepatic Fibrosis by Inhibiting the TGF-beta1/Smad Pathway via Peroxisome Proliferator-Activated Receptor Gamma Activation. *J Agric Food Chem.* 2017;65(2):317–26. doi:10.1021/acs.jafc.6b04805.
28. Makled MN, El-Kashef DH. Saroglitazar attenuates renal fibrosis induced by unilateral ureteral obstruction via inhibiting TGF-beta/Smad signaling pathway. *Life Sci.* 2020;253:117729. doi:10.1016/j.lfs.2020.117729.
29. Wei J, Zhu H, Komura K, Lord G, Tomcik M, Wang W, Doniparthi S, Tamaki Z, Hinchcliff M, Distler JH, Varga J. A synthetic PPAR-gamma agonist triterpenoid ameliorates experimental fibrosis: PPAR-gamma-independent suppression of fibrotic responses. *Ann Rheum Dis.* 2014;73(2):446–54. doi:10.1136/annrheumdis-2012-202716.
30. Shi LB, Zhou F, Zhu HY, Huang D, Jin XY, Li C, Dai Y, Pan YB, Zhang SY. Transforming growth factor beta1 from endometriomas promotes fibrosis in surrounding ovarian tissues via Smad2/3 signaling. *Biol Reprod.* 2017;97(6):873–82. doi:10.1093/biolre/iox140.
31. Cui L, Bao H, Liu Z, Man X, Liu H, Hou Y, Luo Q, Wang S, Fu Q, Zhang H. hUMSCs regulate the differentiation of ovarian stromal cells via TGF-beta1/Smad3 signaling pathway to inhibit ovarian fibrosis to repair ovarian function in POI rats. *Stem Cell Res Ther.* 2020;11(1):386. doi:10.1186/s13287-020-01904-3.
32. Suriyakalaa U, Ramachandran R, Doualathunnisa JA, Aseervatham SB, Sankarganesh D, Kamalakkannan S, Kadalmani B, Angayarkanni J, Akbarsha MA, Achiraman S. Upregulation of Cyp19a1 and PPAR-gamma in ovarian steroidogenic pathway by *Ficus religiosa*: A potential cure for polycystic ovary syndrome. *J Ethnopharmacol* 2020:113540.doi:10.1016/j.jep.2020.113540.
33. Irani M, Seifer DB, Grazi RV, Julka N, Bhatt D, Kalgi B, Irani S, Tal O, Lambert-Messerlian G, Tal R. Vitamin D Supplementation Decreases TGF-beta1 Bioavailability in PCOS: A Randomized Placebo-Controlled Trial. *J Clin Endocrinol Metab.* 2015;100(11):4307–14. doi:10.1210/jc.2015-2580.

34. Yu M, Liu J. MicroRNA-30d-5p promotes ovarian granulosa cell apoptosis by targeting Smad2. *Exp Ther Med.* 2020;19(1):53–60. doi:10.3892/etm.2019.8184.
35. Shen H, Wang Y. Activation of TGF-beta1/Smad3 signaling pathway inhibits the development of ovarian follicle in polycystic ovary syndrome by promoting apoptosis of granulosa cells. *J Cell Physiol.* 2019;234(7):11976–85. doi:10.1002/jcp.27854.

Table

Table 1 Sequences of primers used in qRT-PCR

ID	Sequence (5'- 3')	Product Length(bp)
GAPDH.F	CCTCGTCTCATAGACAAGATGGT	169
GAPDH.R	GGGTAGAGTCATACTGGAACATG	
MMP2.F	ATGGCATTGCTCAGATCCGT	170
MMP2.R	AGCCTTCTCTCCTGTGGGG	
MMP9.F	CTGCGTATTTCCATTCATCTTCG	166
MMP9.R	CGAGTTGCCCCAGTTACAG	
TIMP1.F	TGGGGTGTGCACAGTGTTTC	113
TIMP1.R	CGCTCTGGTAGCCCTTCTCA	
α -SMA.F	GCATCCACGAAACCACCTAT	188
α -SMA.R	GCGTTCTGGAGGAGCAATAA	
Collagen1.F	ACTGGATCGACCCTAACCAA	191
Collagen1.R	CTCGAACTGGAATCCATCGG	

Figures

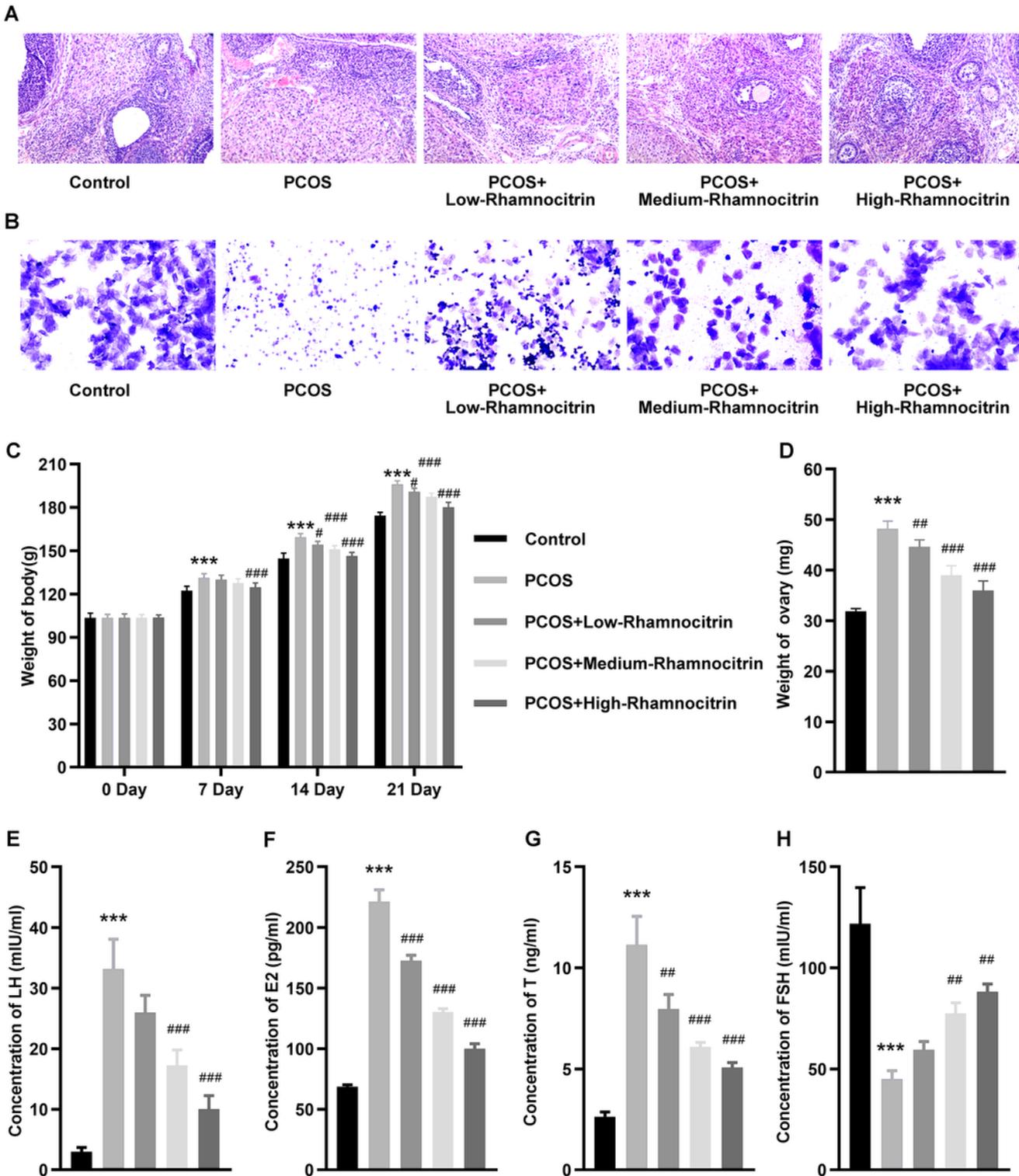


Figure 1

Effect of Rhamnocitrin on ovarian function in PCOS rats. (A) Ovarian tissues from rats were measured by H&E staining. (B) Estrous cycle assessed by Vaginal smears in rats. (C) Body weight was detected before or after different administration. (D) Ovary weight was evaluated after different treatments. (E, F, G, H) LH (E), E2 (F), T (G) and FSH (H) levels in serum were evaluated with ELISA. n = 6. Data are shown as the

mean \pm SEM. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. Control group; # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ vs. PCOS group.

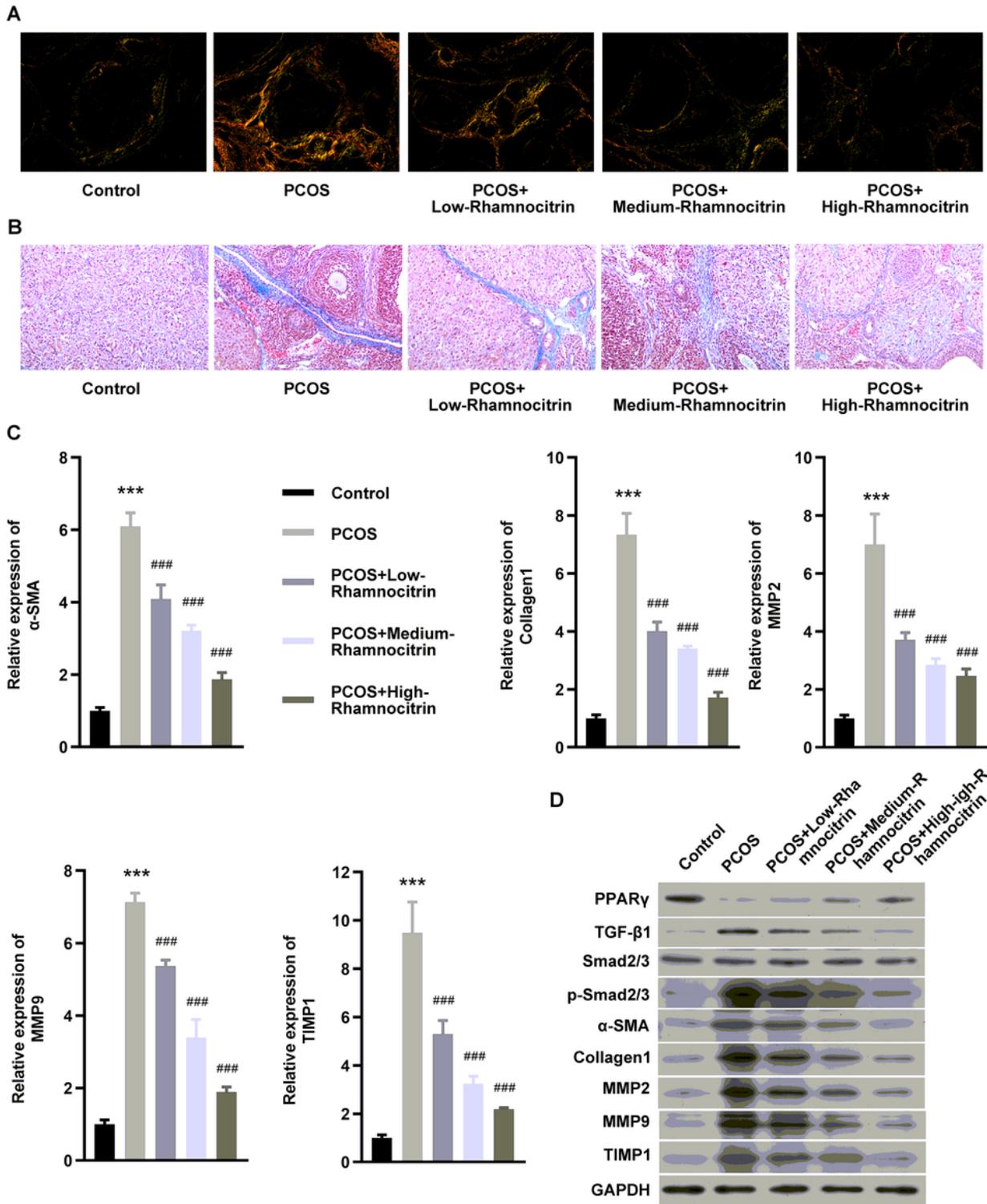


Figure 2

Rhamnocitrin suppresses ovarian fibrosis and regulates fibrosis factors, PPAR γ , TGF- β 1 and Smad2/3 expressions in ovaries of PCOS rats. (A, B) Sirius Red (A) and Masson staining (B) used for Collagen measurement in ovarian slices. Pictures were representative from three independent experiments. (C) qRT-

PCR applied to determine mRNA expression of α -SMA, Collagen1, MMP-2, MMP-9, TIMP1 in the ovaries from rats. (D) The protein expressions of α -SMA, Collagen1, MMP-2, MMP-9, TIMP1, PPAR γ , TGF- β 1, Smad2/3 and p-Smad2/3 in the ovaries from rats detected by western blot. n = 6. Data are shown as the mean \pm SEM. * P < 0.05, ** P < 0.01 and *** P < 0.001 vs. Control group; # P < 0.05, ## P < 0.01 and ### P < 0.001 vs. PCOS group.

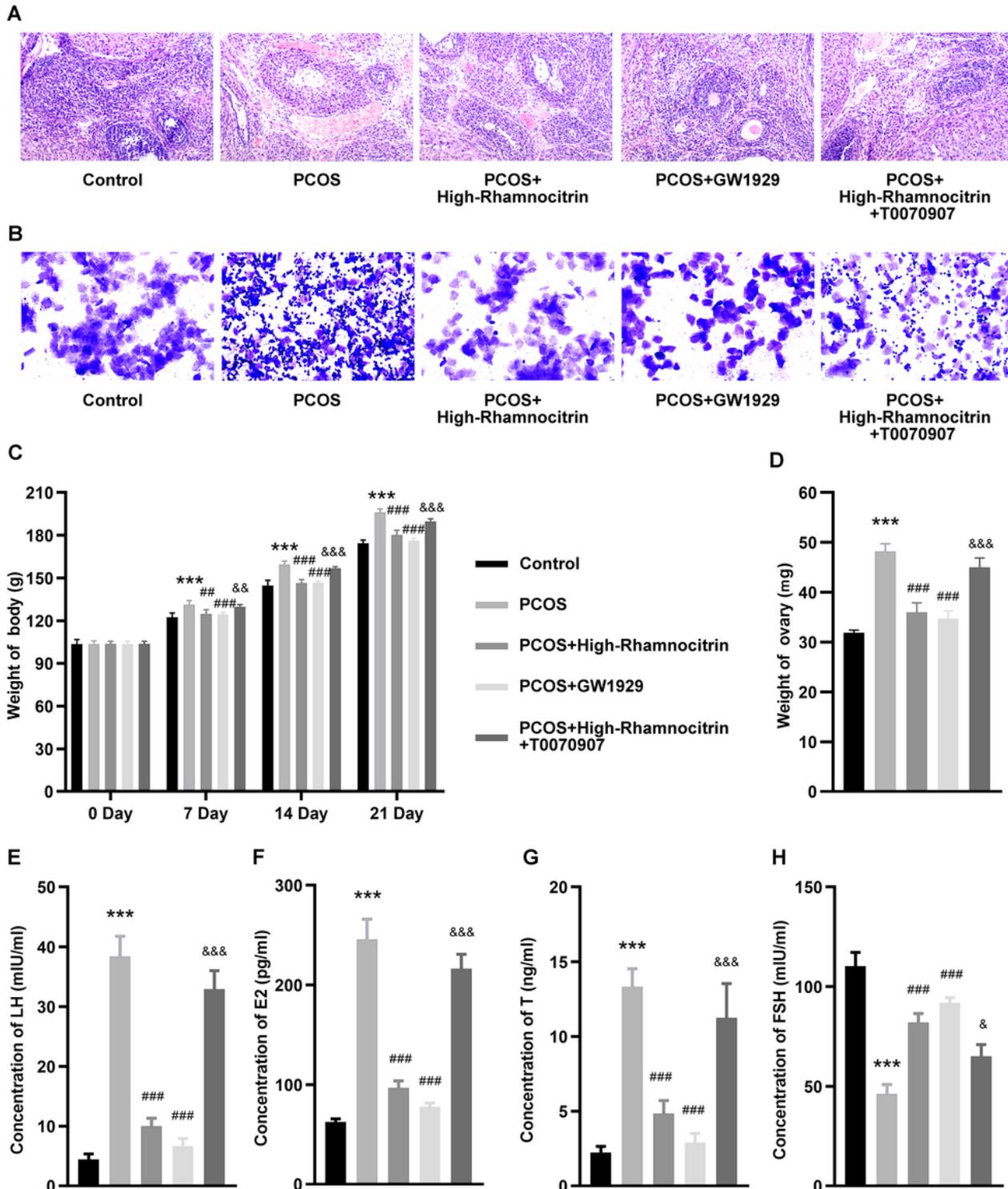
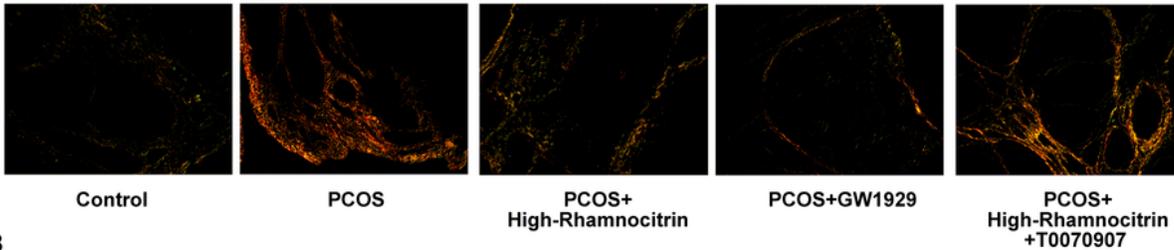


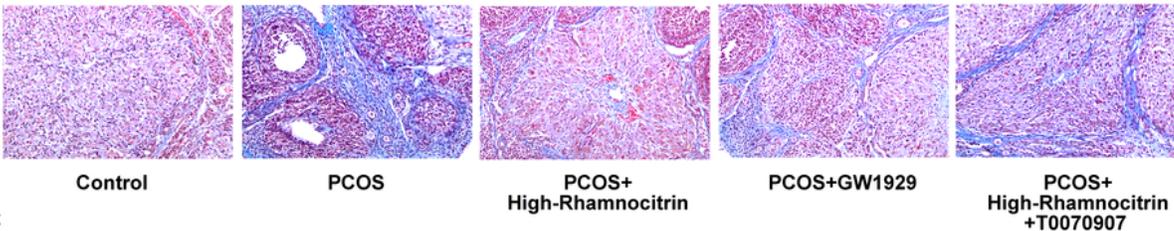
Figure 3

Effect of GW1929 and T0070907 on ovarian function in PCOS rats. (A-H) Rats were divided into five groups. (A) Ovarian tissues measured by H&E staining after treatment. (B) Vaginal smears were carried out to assess the estrous cycle. (C) Body weight recorded before and after different treatments. (D) The ovary weight from rats determined after different administration. (E, F, G, H) LH (E), E2 (F), T (G) and FSH (H) levels in serum of rats after different treatments determined by ELISA. n = 6. Data are shown as the mean \pm SEM. * P < 0.05, ** P < 0.01 and *** P < 0.001 vs. Control group; # P < 0.05, ## P < 0.01 and ### P < 0.001 vs. PCOS group; & P < 0.05, && P < 0.01 and &&& P < 0.001 vs PCOS + High-Rhamnocitrin group.

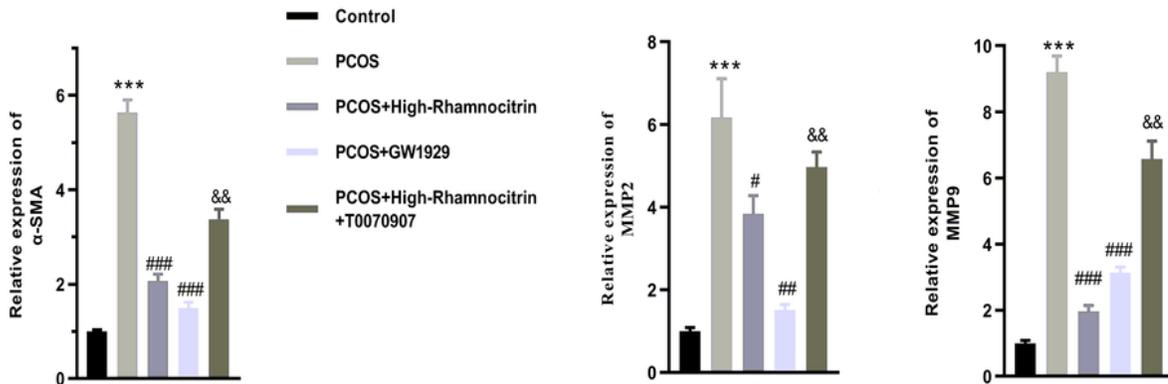
A



B



C



D

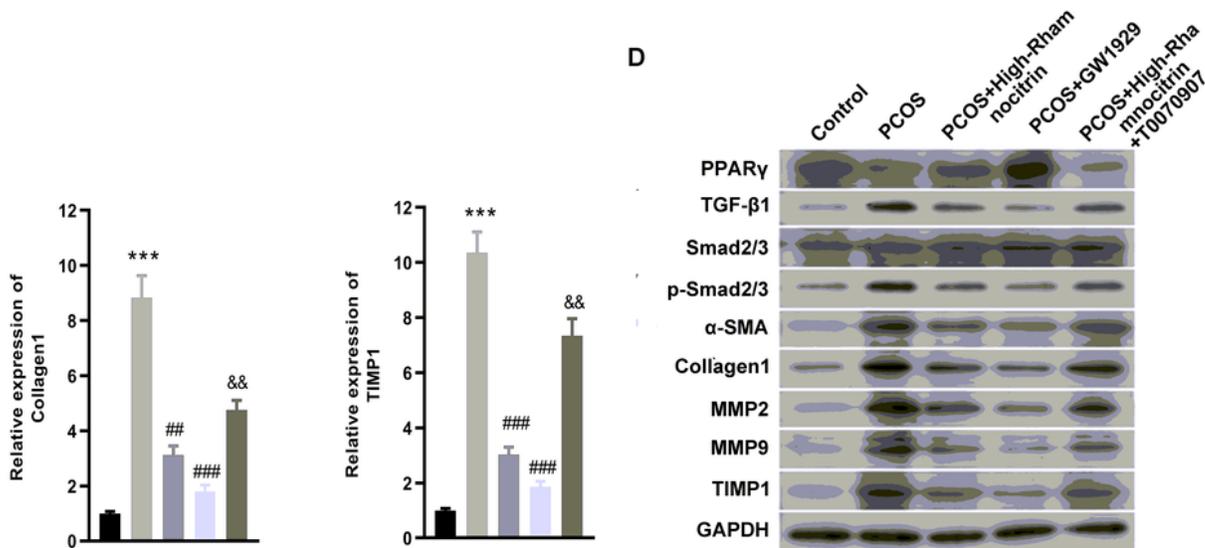


Figure 4

Effects of GW1929 and T0070907 on ovarian fibrosis and the expressions of fibrosis factors, PPAR γ , TGF- β 1 and Smad2/3 in ovaries of PCOS rats. (A, B) Ovarian tissues were obtained from rats. Sirius Red (A) and Masson staining (B) revealed collagen in ovarian slices. Images were representative of three independent experiments with similar results. (C) The mRNA expressions of fibrosis factors of α -SMA, Collagen1, MMP-2, MMP-9, TIMP1 in the ovaries measured with qRT-PCR. (D) Protein expression of fibrosis factors in the ovaries detected with western blot and the protein expression of PPAR γ , TGF- β 1, Smad2/3 and p-Smad2/3 in ovaries also evaluated with western blot. n = 6. Data are shown as the mean \pm SEM. * P < 0.05, ** P < 0.01 and *** P < 0.001 vs. Control group; # P < 0.05, ## P < 0.01 and ### P < 0.001 vs. PCOS group; & P < 0.05, && P < 0.01 and &&& P < 0.001 vs PCOS + High-Rhamnocitrin group.