

Naringenin and morin reduces insulin resistance and endometrial hyperplasia in the rat model of polycystic ovarian syndrome through enhancement of inflammation and autophagic apoptosis

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Polycystic Ovary Syndrome (PCOS) is a gynecologic disorder with unsatisfactory treatment options. Hyperandrogenism and insulin resistance (IR) are two symptoms of PCOS. The majority of PCOS patients (approximately 50% to 70%) have IR and moderate diffuse inflammation of varying degrees. We investigated *in-vitro* and *in-vivo* effects of naringenin, morin and their combination on PCOS induced endometrial hyperplasia by interfering with the mTORC1 and mTORC2 signaling pathways. The vaginal smear test ensured the regular oestrous cycles in female rats. Serum cytokines (TNF- α & IL-6) were assessed using the ELISA test, followed by *in-vivo* and *in-vitro* determination of prominent gene expressions (mTORC1 & C2, p62, LC3-II, and Caspase-3 involved in the inflammatory signaling mechanisms through RT-PCR, western blotting, or immunohistochemical analysis. In addition, the viability of naringenin or morin treated cells was determined using flow cytometry analysis. The abnormal oestrous cycle and vaginal keratosis indicated that PCOS was induced successfully. The recovery rate of the oestrous cycle with treatments was increased significantly ($P < 0.01$) when compared to the PCOS model. Naringenin, morin, or a combination of the two drugs substantially decreased serum insulin, TNF- α , IL-6 levels with improved total anti-oxidant capacity and SOD levels ($P < 0.01$). Treatments showed suppression of HEC-1-A cells proliferation with increased apoptosis ($P < 0.01$) by the upregulation of Caspase-3 expression, followed by downregulation of mTORC, mTORC1, and p62 ($P < 0.01$) expressions with improved LC3-II expressions ($P < 0.05$) respectively. The histological findings showed a substantial increase in the thickness of granulosa layers with improved corpora lutea and declined the number of cysts. Our findings noticed improved inflammatory and oxidative microenvironment of ovarian tissues in PCOS treated rats involving the autophagic and apoptotic mechanisms demonstrating synergistic *in-vitro* and *in-vivo* therapeutic effects of treatments on PCOS-induced endometrial hyperplasia.

Keywords: PCOS, Morin, Naringenin, hyperplasia

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Abbreviations: COX-2, Cyclooxygenase-2; DAB3, 3'-diaminobenzidine; FBG, Fasting blood glucose; HEC-1-A, Human endometrial adenocarcinoma cells; hCG, Human chorionic gonadotropin; IL-6, Interleukin-6; MAPK, mitogen-activated protein kinase; mTORC1, mammalian target of rapamycin complex 1; LC3-II, Microtubule-

associated protein light chain 3-2; NO, Nitric oxide; PCOS, polycystic ovarian syndrome; SOD, Superoxide dismutase; TNF- α , Tissue necrosis factor

INTRODUCTION

Insulin resistance and hyperandrogenism are the main causes of polycystic ovary syndrome (PCOS), which affects about 15% of women of reproductive age (Moran *et al.*, 2011). Premalignant lesions are called endometrial hyperplasia (EH). It was estimated, however, that approximately 3–12% of endometrial hyperplasia cases lead to infiltrating cancer. The incidence of EH in women with PCOS without treatment of contraceptive steroids was over 30% (Kurman *et al.*, 1985). As per the previous study, 25% and 2% of Polycystic Ovarian Syndrome patients developed simple EH and EC respectively (Cheung, 2001). Unlike endometrial cancer (EC), simple endometrial hyperplasia was precluded as a pre-malignant condition and infrequently lead to endometrial cancer (Kurman *et al.*, 1985). The levels of many endometrial markers experience major changes during the implantation of the embryos, suggesting that simple endometrial hyperplasia can be initiated before polycystic ovary syndrome induced infertility (Park *et al.*, 2011). The EH decreased the rates of embryos implantation (Goncharenko *et al.*, 2013; Ip *et al.*, 2013). The simple endometrial hyperplasia treatment could enhance the pregnancy rate and decrease the abortion rate in PCOS patients. There was evidence that cardio-metabolic risk, insulin resistance, and weight gain are associated with PCOS (Ardawi & Rouzi, 2005; Bulun *et al.*, 2005; Rakha *et al.*, 2012). Obesity also induced insulin resistance through the release of tumor necrosis factor (TNF)- α and PPAR-dependent pathways (Barber *et al.*, 2016).

Phytoestrogens are present in different plant types and include flavonoids, coumestan (Neisy *et al.*, 2019) are capable of binding to the oestrogen receptor (ER) (Miksicek, 1994; Ververidis *et al.*, 2007). Phytoestrogens display several actions through reduced pro-inflammatory COX-2 and NO synthase (Kuiper *et al.*, 1998); boosting the expression of GLUT-4 transporter triggering sensitivity of insulin; antioxidant genes activation and adipogenesis inhibition by interfering the AMPK signaling cascade (Medjakovic *et al.*, 2010).

The beneficial actions of foods containing naringenin and morin on oestrogen deficiency diseases had been identified in the past. Breast cancer (linked to oestrogen deficiency) was found to be substantially less common in

areas where flavonoid-rich foods are consumed (Harris *et al.*, 2004; Palma-Duran *et al.*, 2015).

According to the research, autophagy de-regulation is said to be an important part of the development of EH in PCOS patients (Sarkaki *et al.*, 2015; Zhang *et al.*, 2017), and both naringenin and morin have been shown to have a synergistic therapeutic effect on autophagy (Lin *et al.*, 2018; Singh *et al.*, 2019). The naringenin had effectively deactivated mTORC2 signaling in HCC-94 cells and further improved its effects on MAPK signaling and mTORC1 suppression (Raha *et al.*, 2015; Zhang *et al.*, 2018). However, the effects of morin on inhibition of the MAPK and mTORC1 and signaling pathways were not studied. The naringenin and morin were shown to improve the auto-phagocytosis and programmed cell death of HCC-94 cells (Lin *et al.*, 2018; Raha *et al.*, 2015; Singh *et al.*, 2019; Zhang *et al.*, 2018). Therefore, the pro-autophagy treatment offered by naringenin and morin can give an ideal route for treating HEC-1-A-cells. Hence, our study involved the investigation of *in-vitro* and *in-vivo* beneficial effects of naringenin, morin, and their combined treatment on PCOS induced endometrial hyperplasia through the inhibition of the proliferation of HEC-1-A-cells by interfering with the mTORC1 and mTORC2 signaling pathways.

MATERIALS AND METHODS

Experimental design

Female rats of two months old were used for the study. All rats (60) were received clean water in separate pathogen-free animal facilities and food was provided. The rats were maintained at a temperature of 30°C. All rats were tested before the start of the analysis to ensure that they had regular oestrous cycles. A standard procedure for vaginal smear test confirmed the existence of regular oestrous cycles. The rats were subsequently assigned to control, polycystic ovary syndrome (PCOS), PCOS+naringenin, PCOS+morin, and PCOS+naringenin+morin groups. Naringenin (purity>8%, 67604-48-2) and Morin (purity>97%, 480-16-0), both were procured from Merck, USA. A homogenous suspension of naringenin and morin was prepared separately using sodium carboxymethylcellulose (CMS, 1% w/v) suitable for oral administration.) Each group was comprised of 12 rats. The control rats were given saline only. The insulin+hCG (Human chorionic gonadotropin) was given to rats in the PCOS group to stimulate both symptoms of excess androgen and higher insulin levels in the blood. The insulin+hCG treatment was given as per standard procedures and doses followed a method previously described (Li *et al.*, 2013; Tamadon *et al.*, 2018). Insulin was first administered at a dosage of 0.5 IU per day, then steadily augmented to 5 IU/day to induce hyperinsulinemia and insulin resistance. PCOS induction was deemed successful in rats with keratosis in their vaginal epithelial cells for 10 days (Yu *et al.*, 2014). PCOS rats' orbital venous blood was obtained and tested after 15 hours for fasting insulin and blood glucose concentrations. The evaluation of the homeostatic model (Keskin *et al.*, 2005) $HOMA-IR = \text{Fasting Blood Glucose (mmol/L)} \times \text{Fasting Insulin concentration (mU/L)} / 22.5$ was evaluated in conjunction with the HOMA-IR. The Insulin Resistance in PCOS rats with HOMA-IR was greater than 3.0 (Coniglio *et al.*, 2012). The hCG was administered to trigger hyperandrogenism with 3.0 IU per day after insulin resistance and hyperinsulinemia

were confirmed. The subcutaneous injections of hCG twice a day were given to all rats until the experiment was completed.

In the PCOS+naringenin group, the rats received a dose of 200 mg per kg of naringenin dissolved in sodium carboxymethylcellulose (CMS, 1 percent w/v) orally on the 23rd day of the test using gavage. Similarly, in the PCOS + Morin group, the rats received oral 200 mg per kg of morin dissolved in sodium carboxymethylcellulose (CMS, 1 percent w/v). The PCOS+naringenin+morin group received orally 200 mg per kg of naringenin dissolved in sodium carboxymethylcellulose (CMS, 1 percent w/v), plus 200 mg per kg of morin dissolved in sodium carboxymethylcellulose (CMS, 1 percent w/v) respectively.

The blood was collected from each rat following anesthesia after the study. The uterus of the rats was also taken for a real-time polymerase reaction and a Western blot examination. Ovaries of all treated rats were collected surgically, set in formalin, and then placed in alkanes (paraffin). The 5 µm thick sections were stained with eosin and hematoxylin until the morphological features of the ovaries were blindly evaluated by two pathologists. The research protocol was carried out with the permission of the Institute's Animal Ethics Committee under the national and international guidelines for the care and use of laboratory animals.

Fasting Plasma Glucose

The blood was collected after anesthesia following fasting for 15 hours from the orbital vein. The blood was measured for fasting plasma glucose with a glucometer.

Insulin Release Test

The insulin production was evaluated in rats that had been fasted for 15 hours. The rats were treated with glucose by gavage to the normal dosage of 4 g/kg body weight. Blood was collected immediately after oral administration from the orbital vein at 30 min, 60 min, and 120 min after gavage. For 20 minutes, the blood was centrifuged at 4500 rpm. The serum was then placed in a freezer at -80°C for further studies.

At each stage, the blood insulin was measured using an immunosorbent assay kit connected to the insulin enzyme-linked. A microplate reader at 480 nm was used to measure the optical density values. The blood insulin release curve (IUC) area was calculated as follows: $IAUC = \text{FINS} \times 0.25 + 60\text{-minute insulin} \times 0.75 + 120\text{-minute insulin} \times 0.5$ (Bahceci *et al.*, 2004).

Enzyme-Linked Immunosorbent Assay (ELISA)

Superoxide dismutase function, total antioxidant potential, and total oxidative stress levels in rat ovarian tissues were determined utilizing ELISA kits as per the manufacturer's instructions to ascertain the antioxidant effects of naringenin or morin or their combinations. The activity of SOD was expressed as mol per µg protein and total antioxidant potential and total oxidative stress levels were expressed as nmol per mg protein after determining the relative protein content in each sample.

Culture of cells

The Human endometrial cancer-1-A cells were collected and seeded on a 96-well plate 50 hours after surgery. The cells were cultivated in a Dulbecco modified Eagle medium (SigmaAldrich, USA) with 12 percent fe-

tal calf serum, 80 and 120 µg per ml of penicillin and streptomycin respectively at 40°C with the supply of 5 percent CO₂. The cells were sub-cultured once or twice a week and harvested for subsequent experiments after a specified exponential growth period. The HEC-1-A cells were divided into four classes to understand the effect of morin and naringenin on multiple factors associated with cell proliferation and apoptosis and treated accordingly: (a) control group; (b) naringenin group (500 µM of naringenin); (c) Morin group (500 µM of morin), and (d) combined treatment group (500 µM of naringenin and 500 µM of morin) to investigate the outcome of morin and naringenin on cellular proliferation and cell death-related factors. Cells were extracted from treated and untreated groups after 2 days of therapy to test the levels of the corresponding cellular markers. The experiments were replicated three times.

Cell proliferation assay

The Human endometrial cancer-1-A cells were collected and seeded on a 96-well plate 50 hours after surgery (In 100 µl of cell culture medium, with a density of 10⁵ cells per well). To 90 percent confluence of the cells, various naringenin or morin or combined concentrations at 0 µmol/L, 10 µmol/L, 20 µmol/L, 40 µmol/L, 60 µmol/L, and 80 µmol/L were applied to the cell culture medium. The cells were then incubated for 4.5 hours in the dark with a reagent after 48 hours of incubation. To evaluate the viability of the cells, their optical density was measured at 480 nm. There were three repetitions of each experiment.

Immunohistochemistry

The levels of inflammatory cytokines such as IL-6 and TNF-α in the rat ovaries were determined using immunohistochemistry on tissue slices. The samples were fixed in 3 percent formaldehyde, engrained in paraffin, and sectioned into 5 µm thick parts for the assay. The sections were again incubated for one hour at 70°C before being dewaxed in xylene and dehydrated in alcohol. Following that, the sections were incubated for 45 minutes at room temperature in 2 percent hydrogen peroxide to inhibit endogenous peroxidase activity. The samples were then blocked with 4 percent goat serum for 45 minutes at room temperature before being incubated with primary antibodies (1:2000 dilutions) overnight at 4°C. The sections were incubated with the secondary antibodies of goat-antirabbit conjugated with biotin (1:2000 dilutions) for one hour at room temperature on the next day. Additionally, the sections were stained for 15 minutes with DAB, then counterstained with hematoxylin for 5 minutes at room temperature, and briefly dehydrated in ethanol, and mounted in neutral gum for examination under the microscope.

Western blot analysis

The supernatant was obtained after the cells specimens were lysed in a lysis buffer and separated by centrifugation at 14000 rpm for 15 minutes. A bicinchoninic acid assay kit was used to assess the protein content in the lysate. Following that, isolated protein samples (30 µg each) were inactivated by boiling for 10 minutes at 80°C before being electrophoresed on a 10% SDS. The protein components were then transferred onto a nitrocellulose membrane followed by blocking for 2 hours with 3% skim milk, then incubated overnight with primary antibodies against mTORC1 (catalogue number:

ab120224; 1:2000; Abcam, USA) & mTORC2 (catalogue number sc-1549; 1:2000; Santa Cruz Biotechnology), p62 (catalogue number – ab91526; 1:2000; Abcam, USA), Caspase-3 (catalogue number – ab145046; 1:2000; Abcam, USA), LC3-II (catalogue – number-ab192890; 1:2000; Abcam, USA) and GAPDH (catalogue number – ab8245; 1:4000; Abcam, USA). The membrane was incubated with secondary antibodies for one hour at room temperature on the second day (1:2000; Abcam, USA). The membrane was fully dipped in a chemiluminescence reagent after three additional PBS washes, and the gel colour produced in the dark was visualized. The target and the GAPDH protein bands ratio were used to measure the mTORC1 and mTORC2, LC3-II, p62, and Caspase-3 relative protein expressions. Each experiment was carried out three times in total.

Real time PCR assessment

The HEC-1-A cells (1106 /ml) were cultivated in six well plates. The cells were harvested 4 hours after treatment with naringenin, morin, or their combination, and were used to conduct the experiments. TRIzol reagent (ThermoFisher Scientific, CA, USA) was used to extract total RNA, which was then reversed transcribed into cDNA using RNA assay kit. The cDNA samples were combined with SYBR-Green PCR-Mastermix (Sigmaaldrich, USA) and TNF-α, IL-6, and β-actin primers. A Bio-rad thermal Cycler was used to perform quantitative real-time PCR assessment (Liu *et al.*, 2016).

Apoptosis analysis

The HEC-1-A cells were washed with PBS three times after 2 days of treatments and flow cytometry was used to detect cell apoptotic profiles with annexin V-fluorescein detection kit following the manufacturer's instructions.

Statistical analysis

Using SPSS 21.0 software, variance analysis was carried out. The mean ± SEM was expressed for all datasets. All experiments were replicated three times. The student's *t*-test was applied to compare the inter-group statistical significance. For time-course studies, two-way ANOVA was used to analyze the data. If the 2-way ANOVA showed significant variation then Bonferroni's *post-hoc* test was applied to assess the intergroup variation. The *P*<0.05 was considered statistically significant.

RESULTS

Insulin tolerance in a rat model of PCOS

The rats' vaginal cytological changes were examined using the methylene blue staining technique after 24 days of hCG injections. There was an abnormal oestrous cycle and vaginal keratosis in a group of 75 rats, 48 indicated that PCOS was successfully induced following the assessment of FBS and fasting insulin. All the PCOS rats showed Homeostatic Model Assessment of Insulin resistance > 2.91. In PCOS rats, the incidence rate of IR was 66.1 percent

Oestrous cycles improved, weight increased and ovary co-efficient increased with treatments

The rats in the naringenin, morin, and naringenin+morin groups showed restored oestrous cy-

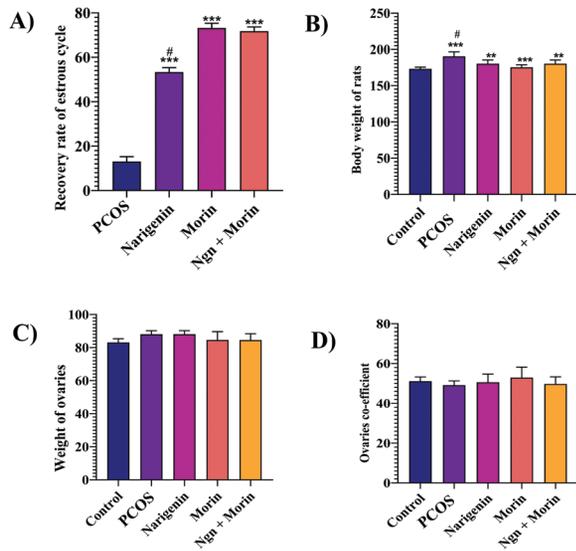


Figure 1. The effects of naringenin (Ngn), morin and their combination on polycystic ovary syndrome (PCOS) rats.

A) Recovery rate of oestrous cycles. **B)** Rat body weights. **C)** Weight of ovaries. **D)** ovary coefficient. Data was statistically analysed using Student's *t*-test. * $P < 0.05$ vs PCOS group; ** $P < 0.01$ and *** $P < 0.001$ vs control & PCOS group respectively. $N = 12$ rats.

cles after treatment. The recovery rates were 8.33 percent, 58.33 percent, 66.67 percent, and 76.54 percent respectively in the model, naringenin, morin, naringenin+morin groups (Fig. 1A). After treatment, the naringenin, morin, and the combined groups had lower body weights (Fig. 1B). The ovarian ratio was evaluated as follows: ovarian coefficient = ovarian weight per body weight ($\text{mg} \times 100$ per gram) (Coniglio *et al.*, 2012). Ovarian weight and ovarian coefficients did not vary statistically significant from the model coefficients in the naringenin, morin, and naringenin+morin groups (Fig. 1C and D).

Rat Ovarian Morphology

The therapeutic effect of naringenin or morin or both on rat ovaries in PCOS was linked to the morphology of rat ovaries in various groups. The control groups'

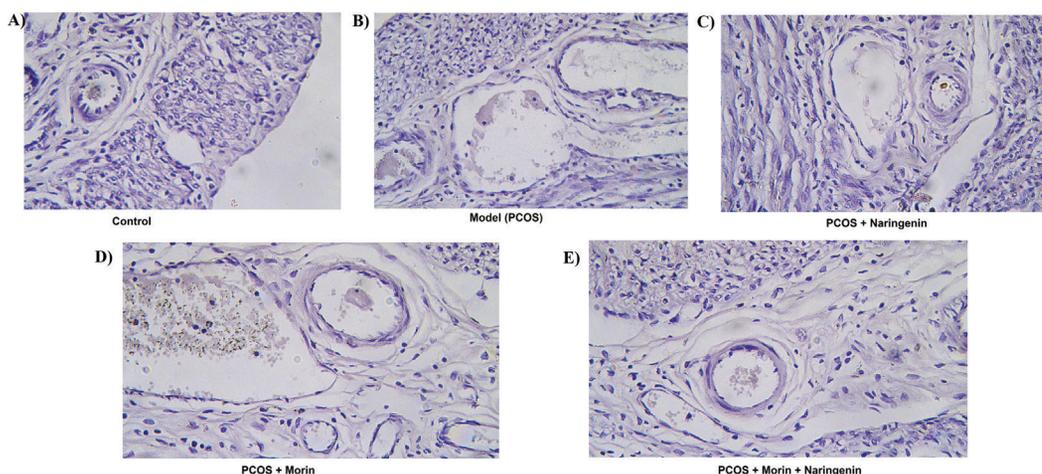


Figure 2. The effects of naringenin (Ngn), morin and their combination showed improved the morphology of the ovarian follicles and increased the granulosa cells of the PCOS rats.

A) Control, **B)** PCOS model **C)** PCOS model + naringenin treatment, **D)** PCOS model + morin treatment and **E)** PCOS model + combined treatment (naringenin + morin). (Magnification $\times 100$).

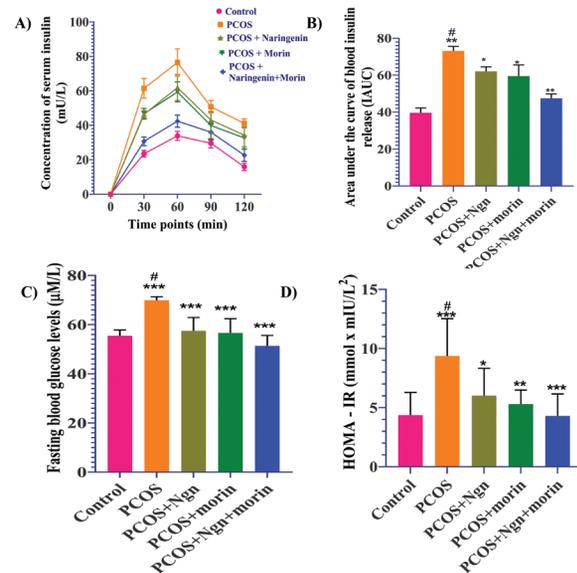


Figure 3. The effects of naringenin (Ngn), morin and their combination on serum insulin levels in PCOS rats

A) Serum insulin levels in rats. The release of insulin in rats at 30 min ($P < 0.01$; Naringenin or morin or Naringenin+morin groups vs PCOS group); 60 min ($P < 0.01$; Naringenin or morin or Naringenin + morin groups vs PCOS group); 90 min ($P < 0.05$; $P < 0.01$; Naringenin or morin or Naringenin+morin groups vs PCOS group); 120 min ($P < 0.01$; $P < 0.01$; Naringenin or morin or Naringenin+morin groups vs PCOS group). **B)** Area under the curve of blood insulin release (IAUC). **C)** Fasting blood glucose levels. **D)** Homeostasis model of assessment of insulin resistance (HOMA-IR). Data was statistically analysed using Student's *t*-test. Data from time course studies was analysed using two-way ANOVA and Bonferroni's post-hoc test. * $P < 0.05$ vs PCOS group; ** $P < 0.01$ and *** $P < 0.001$ vs control & PCOS group respectively. $N = 12$ rats.

ovaries had multiple lutea follicles at different locations, with up to nine granulosa layers in the prevailing follicle. The PCOS group ovaries showed cystic dilatation without oocyte and corona radiation of the follicles. There was a decrease in the number of granulosa cells within the follicle by 3 layers and a decrease in the number of luteal cells. At different points, the naringenin, morin, or combined group ovaries showed follicles and more lutea

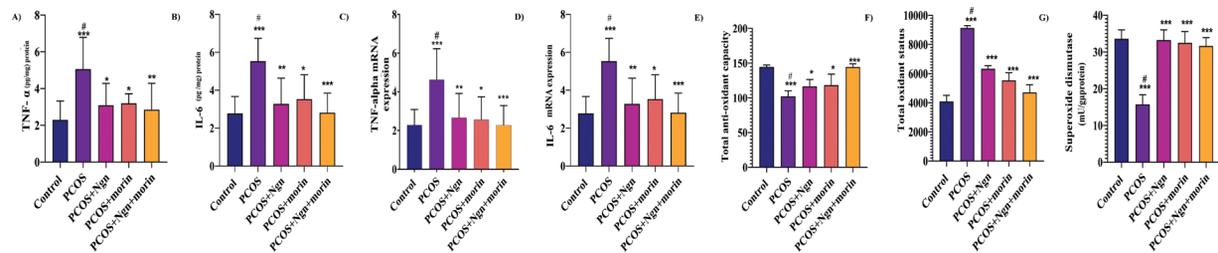


Figure 4. The effect of Naringenin (Ngn), morin and their combination on the concentration of (A) tumour necrosis factor- α (TNF- α) (pg/mg protein), (B) interleukin-6 (IL-6) (pg/mg protein), (C, D) relative expression of TNF- α and IL-6 mRNA in the rat ovary, (E) the total antioxidant capacity (TAC) (F) the total oxidant status (TOS) and (G) the activity of the superoxide dismutase (SOD) in the ovary tissue of the PCOS control group compared to the treated groups and control group.

Data are represented as mean \pm S.E.M. (n=12 in each group). Data was statistically analysed using Student's *t*-test. *Significantly different between treated groups and PCOS control group. Significantly different from the control group (* P <0.05, ** P <0.01, *** P <0.001)

with the granulosa cells increasing in layers (3–7) compared to PCOS rats (Fig. 2).

Release of serum insulin assessment

An insulin release was established at various time points. The PCOS group had higher insulin levels at 30 min, 60 min, and 120 minutes than the control group. The levels of insulin, as well as IAUC and FBG at different times, were all lower than in the PCOS group as compared to naringenin, morin, and combined groups (Fig. 3A–C; P <0.05). The Homeostatic model assessment-IR value was significantly greater than the control value (P <0.01) in the PCOS group. The HOMA-IR values of naringenin, morin, and the combined groups, on the other hand, were considerably lesser when compared to the PCOS group (Fig. 3D; P <0.01).

Interleukin-6 and TNF- α protein levels

To observe the therapeutic effects of naringenin, morin, and their combination on inflammatory cytokines, immunohistochemistry was performed on tissues to compare Interleukin (IL)-6 and TNF- α levels among the different groups. Compared to the control group, the levels of Interleukin-6, TNF- α were high in the PCOS group. Naringenin, morin, and combined therapy have substantially inhibited the levels of these inflammatory cytokines (Fig. 4). Moreover, naringenin, morin and their combination significantly suppressed the mRNA expression of TNF- α and IL-6 in PCOS group as compared to control rat (Liu *et al.*, 2016). In the results of the PCOS group, the accumulation of oxidants was substantially greater with a significant decrease in the total antioxidant capacity (P <0.05) than in the control group (Fig. 4). When compared to the PCOS group, the total antioxidant potential in the naringenin or morin or their combined groups was significantly higher (P <0.01). In contrast, when compared to the PCOS group, different doses of naringenin or morin, or their combinations, drastically (P <0.001) decreased total oxidant stress in ovarian tissues of treated groups. Additionally, the activity of the SOD in the ovarian tissue of the naringenin or morin or their combined groups was considerably higher (P <0.001) than in the control.

Effects of naringenin and morin on endometrial and myometrial regions

The endometrial (Fig. 5a), myometrial (Fig. 5b) areas of the PCOS group were elevated relative to those

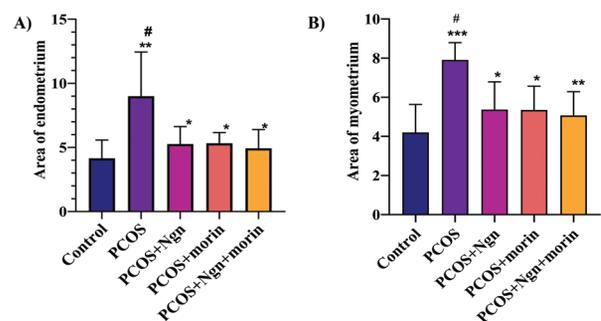


Figure 5. Effect of naringenin (Ngn), morin and their combination on the areas of endometrium and myometrium.

The naringenin and morin significantly reduced the areas of the endometrium (A) and myometrium (B) in PCOS, and the inhibitory effect of naringenin+morin was highly significant when compared to the control and PCOS groups. Data was statistically analysed using Student's *t*-test. * P <0.05, ** P <0.01, *** P <0.001

of the control group, as shown in Fig. 5. Although the endometrial and myometrial areas of naringenin, morin, and the combined groups were all decreased, the greatest inhibitory effect on endometrial areas was shown that in PCOS rats than on myometrium. The combined therapy was found to be more effective than naringenin or morin alone (Fig. 5).

Impact on mTORC-1, mTORC-2, LC3-II, p62 protein, and Caspase-3 levels of naringenin and morin *in-vivo*

To learn more about the mechanisms that underpin the role of naringenin and morin in the polycystic ovary syndrome, the mTORC-1, mTORC-2, Light Chain 3-II, and Caspase-3 levels were compared between the groups. The mTORC1 protein level (Fig. 6a) was much higher in the PCOS group than in the control group, as indicated in Fig. 6, whereas the abnormal mTORC1 protein levels were restored by treatment with naringenin or morin or their concomitant treatment. The abnormal levels of mTORC-1 in the PCOS+naringenin group were restored to a level similar to that of the PCOS+morin group. Similarly, the abnormal mTORC2 protein levels were restored after treatment with naringenin or morin or their combined treatment.

The PCOS group had slightly lower LC3-II (Fig. 6b) and Caspase-3 (Fig. 6c) protein levels than the control group, but naringenin or morin therapy partially restored

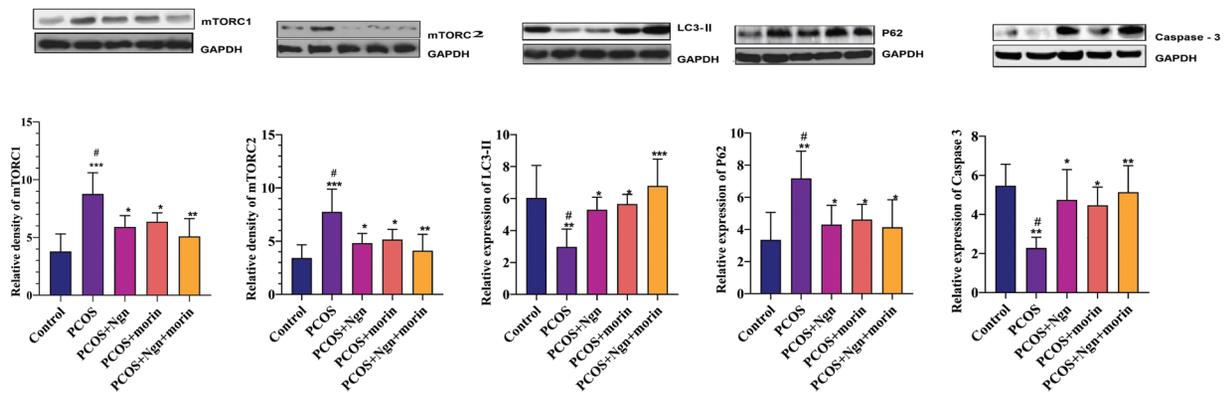


Figure 6. Effect of naringenin (Ngn), morin and their combination on the protein expression of mTORC1, mTORC2 the levels of LC3-II, p62 AND Caspase-3 were detected by western blot analysis.

Data was statistically analysed using Student's *t*-test. *indicates significantly different between treated groups And PCOS control group (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) respectively.

the reduced LC3-II and Caspase-3 expressions in PCOS rats. However, there was a significant impact noticed with the combined treatment (naringenin+morin) in the LC3-II and Caspase protein levels. The up-regulated p62 (sequestosome 1/p62 is a multifunctional protein and a signaling hub that shuttles ubiquitinated proteins to the lysosome during autophagy) expressions in PCOS rats were inhibited by naringenin or morin therapy compared to the control group, and the greatest inhibitory effect on p62 expressions was noticed with the combined treatment of naringenin and morin therapy, which displayed a degree of p62 expression close to that in the control group.

The effects of naringenin and morin therapy on the proliferation of cells and apoptosis

A flow cytometry analysis was used to evaluate the apoptotic and viability of HEC-1-A cells treated with naringenin, morin, or naringenin+morin. The HEC-1-A cell viability was slightly lower in the naringenin and morin group than in the PCOS group, while cell viability for the combined treatment (naringenin+morin) was the lowest (Fig. 7a). The naringenin or morin or their combined treatment also substantially improved the ability to cell apoptosis of both treated and untreated apoptotic HEC-1-A cells (Fig. 7b). There was a comparable impact of naringenin and morin on cell apoptosis and the combined effect (naringenin+morin therapy) showed

a synergistic effect. The active caspase-3 levels of the HEC-1-A cells treated with naringenin and morin were significantly higher than the control group. The combined treatment showed the highest caspase-3 levels compared to the control group. Naringenin and morin reduced mTORC1 and mTORC2 expressions that triggered autophagy by downregulation causing apoptosis of cells, which increased Caspase-3 and LC3-II levels. The combined treatment of naringenin and morin can be used as a unique strategy in treating EH induced by polycystic ovary syndrome since cell apoptosis showed a significant role in EH production during polycystic ovary syndrome.

DISCUSSION

Asians have a lower incidence of oestrogen-related disease than Westerners, which has prompted researchers to investigate the therapeutic agents in relevant plants. The therapeutic effects of naringenin and morin in oestrogen related disorders such as PCOS were scarce (Rehman *et al.*, 2018; Roy *et al.*, 2016). The most significant characteristic of naringenin or morin was its estradiol-like structure and phytoestrogen production (Rehman *et al.*, 2018; Roy *et al.*, 2016). The previous research had focused primarily on the anti-inflammatory and antioxidant actions of naringenin in PCOS. The anti-inflammatory and antioxidant effects may be linked to KEAP up-regulation and iNOS and COX-2 down-regulation, which are part of the effects of phytoestrogens (Kandemir *et al.*, 2020). Keap1 (Keech-like ECH-associated protein 1) is required for host defense towards oxidative and electrophilic damage. Keap1 acts as an adapter for ubiquitin E3 ligase and facilitates proteasomal degradation in the absence of stress (Taguchi *et al.*, 2012). Several studies were shown that naringenin or morin can inhibit the cascade of the NF- κ B signal inflammation and decrease the levels of inflammatory cytokines (including tumor necrosis factor- α and Interleukins) (Arisha *et al.*, 2019; Wali *et al.*, 2020). Previous diabetes and its complications research have shown that by treating both hyperglycemia and oxidative stress using animal models, naringenin or morin had a protective role as an antidiabetic agent (Arisha *et al.*, 2019; Wali *et al.*, 2020).

In the current analysis, we successfully developed a rat PCOS model. Typically, the metabolic and reproductive characteristics resembling to human PCOS, such as

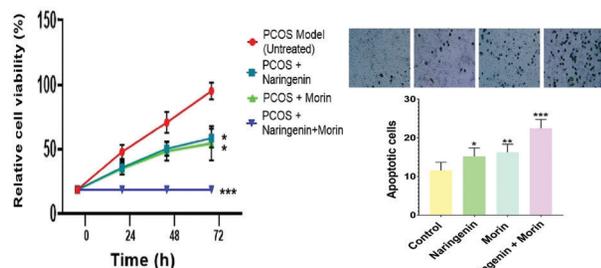


Figure 7. Proliferation and apoptosis of HEC-1-A cells were affected by the treatment with naringenin (Ngn), morin and their combination.

Data was statistically analysed using Student's *t*-test. Data from time course studies was analysed using two-way ANOVA and Bonferroni's post-hoc test. *Indicates significantly different between treated groups and PCOS control group (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) respectively.

irregular reproductive cycles, increase in weight of the body, ovarian co-efficient, and elevated FBG and exogenous insulin levels were reproduced in the rat model. The injected rats showed insulin resistance in 66.7 percent of cases. In rats with PCOS, the oestrous cycle was restored by treatment with morin or naringenin. The body weights of the PCOS rats, ovarian morphology, and serum insulin levels were increased. These findings were shown that naringenin or morin induces a strong therapeutic effect on insulin-resistant PCOS in rats.

The etiology and pathology of PCOS were further discussed by recent studies, wherein long-term inflammation (besides IR and hyperandrogenism), especially low and persistent inflammation, is a hallmark of polycystic ovary syndrome (Escobar-Morreale *et al.*, 2005). The immune response was associated with moderate chronic inflammation but without the presence of acute inflammatory symptoms including swelling, redness, pain, or heat (Lee & Lee, 2014). Inflammation and IR's cause and effect were not well understood, IR and the coexistence of inflammation were widely recognized (Escobar-Morreale *et al.*, 2005; Lee & Lee, 2014). This result indicates that oxidative stress has been correlated with IR in PCOS. The study showed that the PCOS group's IL-1 β , IL-6, and TNF- α expression levels were higher than those of the controls. Insulin is both a reproductive and a metabolic hormone, as evidenced by the link between the insulin resistance and PCOS. It modulates ovarian steroidogenesis by acting as a co-gonadotropin *via* its associated receptor. This effect is maintained despite resistance to insulin's metabolic activities in the peripheral and ovary. Ovulation appears to be dependent on insulin signalling in the central nervous system (CNS). Hyperinsulinemia boosts androgen production in PCOS, according to human research. Because reducing insulin levels has no effect on circulating androgen concentrations in healthy women, it appears that intrinsic defects in steroidogenesis are required for this insulin activity to occur. Insulin has an essential role in the synthesis of SHBG. Insulin resistance can be reduced, and ovulatory menstrual periods can be restored (Dunaif, 1997).

The morin effectively deactivated naringenin-induced mTORC2 signal activation in HEC-1-A cells as a result, combined treatment with morin and naringenin decreased HEC-1-A cell proliferation. The concomitant naringenin and morin therapy further decreases the *in-vitro* and *in-vivo* tumor cell proliferation by interrupting the mTORC1/mTORC2 signal response circuit. (Kumar *et al.*, 2017; Rajput *et al.*, 2021). The synergistic effect of naringenin and morin leads to the promotion of autophagy and apoptosis of HEC-1-A cells. An anti-autophagic strategy could also be employed when naringenin and morin were used together to treat HEC-1-A. In the present research, the effect of naringenin and morin in the levels of mTORC-1, mTORC-2, LC3-II, and Caspase-3 were investigated in western blot and immunohistochemistry studies. The results indicated that polycystic ovary syndrome decreased the concentrations of, LC3-II, and Caspase-3, while the treatment with naringenin increased them. In contrast, naringenin reduced the mTORC1, mTORC2, p62 levels regulated by PCOS. The effect of morin increased the levels of Caspase-3 and LC3-II with a reduction in the p62, mTORC1, and mTORC2 levels respectively.

A process called autophagy involves autophagosomes ingesting organelles and proteins from the cytoplasm. LC3-phosphatidylethanolamine conjugate (LC3-II) is formed when a cytosolic form of LC3 (LC3-I) is coupled to phosphatidylethanolamine and driven to autophago-

somal membranes. During autophagosome fusion with lysosome formation, intra-autophagosomal elements are destroyed by lysosomal hydrolases, which are found in the lysosomes. Simultaneously, LC3-II is destroyed in the autolysosomal lumen. The autophagosomal marker LC3-II undergoes rapid turnover in response to starvation, and the detection of LC3 by immunofluorescence or immunoblotting has proven to be a consistent method for assessing autophagy-related mechanisms, such as autophagic cell death, in animal models of starvation (Tanida *et al.*, 2008).

In response to cytotoxic stress, autophagy and apoptosis govern cancer cell survival; nevertheless, their causal link is unknown. p62/sequestosome 1 is a protein complex that also serves as a signalling hub during autophagy, transporting misfolded proteins components to the lysosome for degradation. Inhibiting autophagy increases p62 expression, and prior studies imply that p62 may induce apoptosis. The study findings showed that the protein p62 can modulate a caspase-8-dependent apoptosis against the BH3 mimic ABT-263 in a cell line model. It was demonstrated that up-regulation of p62 enhanced ABT-263-induced caspase-8 stimulation, which was Bax-dependent and occurred via mitochondrial amplification. Caspases-8 dependence was demonstrated utilising caspase-8-deficient cells and caspase-8 siRNA. Ectopic wild-type p62 was found to co-localize with caspase-8 and accelerate its self-aggregation in ABT-263-treated cells, but not in p62 mutants with reduced ability to trigger apoptosis, as demonstrated by a bimolecular fluorescence test. In the presence of ABT-263 and an autophagy inhibitor, endogenous p62 was found to be co-localized with caspase-8. The autophagosome marker LC3II interacts and co-localizes with caspase-8, according to a study. The p62 gene was knocked out, which reduced the binding of caspase-8 to LC3II, whereas the p62 gene was overexpressed, which increased the co-localization of caspase-8 aggregation with LC3. In contrast to the connection between caspase-8 and p62, the LC3 knockdown had no effect on this interaction, suggesting that p62 may assist caspase-8 translocation to the autophagosomal membrane. When TRAIL, a direct activator of caspase-8, was used alone or in combination with ABT-263, it caused caspase-8 aggregation and co-localization with p62, which was associated with a synergistic pharmacological interaction, as reported previously. These findings suggest that up-regulation of p62 can cause apoptosis by activating caspase-8 in the presence of autophagy inhibitory factors. LC3, the mammalian homolog of the yeast Atg8 protein, undergoes conversion from LC3-I to LC3-II during autophagy and acts as a valid marker of autophagosomes during lysosome degradation. Caspase-8-dependent and mitochondria-mediated apoptotic pathways can be enhanced by autophagy inhibition through unknown mechanisms (Huang *et al.*, 2013)

Naringenin, a traditional hypoglycemic agent used to treat type 2 diabetes mellitus, may enhance hyperglycemic symptoms without causing endogenous secretion of insulin (Sarkar *et al.*, 2012; You *et al.*, 2016) The effect of naringenin on ischemic brain injury, Huntington disease, and Parkinson's disease, among other central nervous system disorders (Chtourou *et al.*, 2015; Sarkar *et al.*, 2012; You *et al.*, 2016) were shown to be therapeutic that could minimize the intensity of EH caused by tamoxifen and estrogen, by inhibiting the activation of S6K1 signaling by mTOR (Dirimanov & Högger, 2019). The study (Xu *et al.*, 2011) also showed that naringenin can increase progesterone receptor expression by suppressing mTOR

activation in EC cells. Naringenin has been shown to control cell differentiation and proliferation by acting as an anti-mitogen. It has been shown that mTOR signaling suppression effectively postpones the development of endometrial, breast, and ovarian cancers (Martínez-Rodríguez *et al.*, 2020; Nowak *et al.*, 2020; Trecek *et al.*, 2006). Morin also improved ACR-induced hepatotoxicity and nephrotoxicity by modulating the protein levels of mTOR signalling pathways (Rajput *et al.*, 2021).

The remarkable suppression of adhesion and migration of SKOV-3 and A2780 cell lines together with the distinct regulation of autophagy and significant apoptotic induction was noticed after treatment with morin, although the exact autophagic mechanism of morin is still unknown (Nowak *et al.*, 2020; Pal Singh *et al.*, 2020). Autophagy activation has been implicated in cisplatin resistance, while autophagy suppression improves the effectiveness of morin in SKOV-3 cells (Bieg *et al.*, 2018; Pal Singh *et al.*, 2020). A study found that morin can induce melanoma cell apoptosis with ROS regulating pro and anti-apoptotic proteins (Lee *et al.*, 2019).

The flow cytometry and western blot analyses were used to evaluate the apoptotic and viability profiles of HEC-1-A cells treated with naringenin, morin, and their combination therapy. The findings discovered that naringenin and morin therapies inhibited HEC-1-A cell proliferation while increasing Caspase-3 expression, which facilitated apoptosis. The mTOR pathway can efficiently control autophagy action by activating chemotherapeutic effects and apoptotic mechanisms (Jung *et al.*, 2010; Nicoletti *et al.*, 2011). Autophagy can either facilitate or impede tumor cell formation, depending on the circumstances. In HEC-1-A cells, concomitant treatment of naringenin and morin will increase the level of autophagy. As a result, autophagy caused by naringenin and morin is anti-apoptotic rather than apoptotic. The mTOR can keep a balance of growth factors, uptake of glucose, amino acids, environmental stress, and hormones as a key change in many biological processes. The mTOR kinases were also strongly preserved in many organisms and participate in the regulation of stress autophagy. The two different signaling pathways, mTORC1 and mTORC2, mTOR kinases serve as catalytic components. The mTORC1 can stimulate p70S6K, which phosphorylates the ribosomal proteins (4E-BP1) and regulates protein translation. The mTORC2 complex, which consists of mTORRictor, mSIN1, and Protor-1 in reverse, directly stimulates Akt signaling by phosphorylating Serine 473. The results showed that naringenin or morin or their combined treatments while inhibiting p62 expression, increased mTORC1, mTORC2, and LC3-II expression (Kumar *et al.*, 2017; Shahid Ali Rajput *et al.*, 2021). It can be inferred that naringenin and morin can reduce the insulin resistance and endometrial hyperplasia in the rat model of PCOS *via* regulation of inflammation and autophagic apoptosis. Earlier studies also showed attenuating effects of naringenin and morin on mTOR signaling and caspase-3 activity in different animal models and in vitro studies (Kucukler, 2020; Park, 2008). Furthermore, parallel to the findings of previous studies, naringenin and morin reduced expression of inflammatory cytokines and enhanced the level of endogenous antioxidants that might have decreased insulin resistance, improved glucose levels, and attenuated endometrial hyperplasia in PCOS model (Moran *et al.*, 2011; Neisy *et al.*, 2019).

CONCLUSION

Our findings demonstrated that the concomitant naringenin and morin therapy decreases the *in-vitro* and *in-vivo* tumor cell proliferation by interrupting the mTORC1/mTORC2 signal response circuit. The synergistic effect of naringenin and morin leads to the promotion of autophagy and apoptosis of HEC-1-A cells. In the present research, the effect of naringenin and morin in the levels of mTORC-1, mTORC-2, LC3-II, and Caspase-3 were investigated. The results indicated that polycystic ovary syndrome decreased the concentrations of, LC3-II, and Caspase-3, while the treatment with naringenin increased them. In contrast, naringenin reduced the mTORC1, mTORC2, p62 levels regulated by PCOS. The effect of morin increased the levels of Caspase-3 and LC3-II with a reduction in the p62, mTORC1, and mTORC2 levels respectively. Consequently, we found the *in-vitro* and *in-vivo* synergistic therapeutic effects of naringenin and morin on PCOS-induced endometrial hyperplasia thus regulating the autophagy process.

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