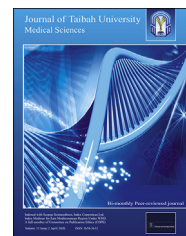




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Original Article

Effect of *Prunus dulcis* and *Salvia hispanica* in the management of polycystic ovary syndrome in Wistar rats

Somasundaram Ramachandran, PhD^{a,*}, Jasthi Nikitha, M.Pharm^a,
Chandravadivelu Gopi, PhD^b, Masa Amala, M.Pharm^a and
Magharla D. Dhanaraju, PhD^c

^a Department of Pharmacology, GIET School of Pharmacy, Rajahmundry, India

^b Department of Pharmaceutical Chemistry, GIET School of Pharmacy, Rajahmundry, India

^c Research Lab, GIET School of Pharmacy, Rajahmundry, India

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المخلص

أهداف البحث: أظهرت الأبحاث أن معظم السيدات يعانين من العقم بسبب متلازمة المبيض المتعدد الأكياس. الأدوية المستخدمة لعلاج متلازمة المبيض المتعدد الأكياس تميل أساساً لعلاج الأعراض بدلاً من علاج المرض. إضافة إلى ذلك، تؤدي الأدوية إلى آثار جانبية حادة. هذه الآثار الضارة لها تأثير على جودة الحياة للمريضات أيضاً. مما يستلزم الحاجة إلى دواء طبيعي يعالج بفاعلية متلازمة المبيض المتعدد الأكياس من دون آثار جانبية.

طرق البحث: تم تحريض فئران ويستر البالغة بمتلازمة المبيض المتعدد الأكياس عن طريق إعطائهم ليتروزول 1 مجم/كجم لمدة 21 يوماً عن طريق الفم. كما تم إعطاء برفوق دولسيسز (الجوز) والقصعين الإسباني (بنور الشيا)، بشكل فردي ومعاً، لفئران التجارب لمدة 15 يوماً. أي من يوم 22 إلى 36 بعد إحداث متلازمة المبيض المتعدد الأكياس. بعد ذلك، تم فحص الفئران للخصائص الشكلية، والبيوكيميائية والمرضية.

النتائج: كان هناك انخفاض كبير في وزن الجسم ووزن المبيضين. وتم الحفاظ على الهرمونات على مستويات مطلوبة بعد استهلاك برفوق دولسيسز والقصعين الإسباني بشكل فردي ومع بعضهم. حثت هذه المواد على الإباضة لدى حيوانات التجارب بواسطة منع ضعف خلايا المبيض بسبب وجود مركبات البوليفينول. تمت مقارنة هذه النتائج بمجموعات التحكم والمجموعات القياسية.

الاستنتاجات: تشير هذه الدراسة إلى أن الحيوانات التي تم تغذيتها برفوق دولسيسز والقصعين الإسباني بشكل فردي ومعاً منعاً للعقم. كما تمت استعادة مستويات الهرمون واضطرابات الأيض عند حيوانات التجارب. لذلك، يمكن

استخدام برفوق دولسيسز والقصعين الإسباني كعوامل علاجية لعلاج مرضى العقم بسبب ضعف البويضة والإباضة.

الكلمات المفتاحية: متلازمة المبيض المتعدد الأكياس؛ تشكل المبيض المتعدد الأكياس؛ ليتروزول؛ سترات الكلوميدين؛ مركبات البوليفينول

Abstract

Objective: Research has shown that polycystic ovary syndrome (PCOS) is a common cause of infertility in women. The drugs used to treat PCOS tend to manage the symptoms rather than cure the disease. Furthermore, these drugs have severe side-effects and influence the quality of life for the patients. There is therefore a need for natural medicine that can effectively treat PCOS without side-effects.

Method: PCOS was induced in adult female Wistar rats by daily oral administration of letrozole (1 mg/kg) for 21 days. From day 22 until the end of the experiment (day 36), these rats were given a daily oral dose of either *Prunus dulcis* (walnut) or *Salvia hispanica* (chia seed) alone, or in combination. Animals were subsequently examined for morphological, biochemical, and histopathological changes.

Result: When compared with the control and standard groups, rats who had consumed *P. dulcis* and *S. hispanica*, either as individual agents or in combination, had significantly lower body and ovarian weights, and hormone concentrations were maintained at healthy levels. The presence of polyphenolic compounds in these substances induced ovulation in the PCOS model animals.

* Corresponding address: Department of Pharmacology, GIET School of Pharmacy, NH-16, Rajahmundry, Andhra Pradesh, 533 296, India.

E-mail: ramsnetin@yahoo.com (S. Ramachandran)

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Conclusion: This study demonstrated that animals fed with *P. dulcis* and *S. hispanica* either individually or in combination were able to overcome infertility. Hormone levels and metabolism were restored in these animals. Therefore, *P. dulcis* and *S. hispanica* can be used as therapeutic agents to treat patients who are infertile due to suboptimal oocyte competence and anovulation.

Keywords: Clomiphene citrate; Letrozole; Polyphenolic compounds; Polycystic ovary morphology; Polycystic ovary syndrome

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Introduction

Polycystic ovary syndrome (PCOS) is a leading cause of infertility in women worldwide and is an endocrine disorder that affects 15–20% of the female population at reproductive age. It is caused by an imbalance in the female reproductive system, which manifests as an excess of androgen, irregular menstrual cycles, ovulatory dysfunction, hirsutism, subfertility, anovulatory infertility, polycystic ovary morphology, and weight gain.^{1–3} First described by Stein and Leventhal in 1935,⁴ there is no solid evidence to indicate the cause of PCOS, but scientist believes that a high level of androgen and endogenous hormones could be the reason behind this disease.⁵ In PCOS, oestrogen dominance can arise from hyperandrogenism.^{6–10} Research studies have revealed that women with PCOS have metabolic abnormalities such as insulin resistance, dyslipidemia, obesity, type II diabetes mellitus, cardiovascular dysfunction, oxidative stress, and genetics problems.^{11–13} Ovarian function is affected by elevated luteinising hormone levels and hyperandrogenism, which leads to premature granulosa cell luteinisation and meiosis. Consequentially, the growth of antral follicles is arrested, and oocyte quality degrades.^{14,15} Mild PCOS is associated with normal ovulation and hyperandrogenism. This can progress to full PCOS over time when progesterone biosynthesis is affected and follicular oestrogen is produced.¹⁶

Current treatments for PCOS have substantial side-effects such as joint and muscle pain, and arthritis, which decreases the quality of life for patients, and there is no definite cure for this disease.^{17,18} Furthermore, most of these treatments focused on managing the symptoms instead of curing the disease.¹⁹ Therefore, there is a need to find effective treatment in natural products, which can have fewer side-effects.^{20,21} In the last two decades, armed with extensive knowledge of the pathogenesis of this disease, researchers worldwide have focused on identifying PCOS treatments from natural medicines.

Prunus dulcis is a dry fruit that is used by a diverse number of ethnic groups for various ailments.²² People from 7000 BC have often used this as one of the key ingredients in traditional and alternative medicine.²³ It contains secondary metabolites, including polyphenolic compounds such as terpenes, tannins, and essential oils,²⁴ which have a variety of biological activities against inflammation,²⁵ atherosclerosis,²⁶ and

oxidative damage.²⁷ *Salvia hispanica* is an oilseed plant that belongs to the Lamiaceae family.²⁸ The plant is native to Bolivia, Guatemala, Mexico, Ecuador, Argentina, and Australia,²⁹ and contains many health-related substances such as phenolic compounds, dietary fibre, alpha-linolenic acid, vitamins, and proteins.³⁰ As *S. hispanica* has a number of different secondary metabolites, it is known to have significant antimicrobial,³¹ antioxidant,³² anti-cancer,³³ and pro-neurological properties.³⁴ To the best of our knowledge, this is the first study to demonstrate that *P. dulcis* and *S. hispanica*, as either single agents or in combination, have an effect against the PCOS induced by letrozole in a rat model. This report suggests that these compounds are effective treatments for PCOS with fewer side-effects and is an important milestone in the development of naturally-derived PCOS treatments.

Materials and Methods

Collection of materials

Good quality *P. dulcis* and *S. hispanica* were procured from a local Ayurvedic supplier in Rajahmundry, Andhra Pradesh in February 2018.

Drugs and chemicals

Letrozole (Novartis Pharmaceuticals, India) and clomiphene citrate (Aventis, India) tablets were purchased from a local pharmacy in Rajahmundry.

Experimental animals

Virgin adult female Wistar albino rats (160–200 g) were procured from the Sainath Agencies, Hyderabad, Andhra Pradesh, India. The animals were housed and maintained under standard laboratory conditions: in controlled temperature ($22 \pm 3^\circ\text{C}$), humidity ($55 \pm 5\%$), and a 12 h light/dark cycle. The animals had ad libitum access to a standard rat pellet diet and water.^{35,36} The experiment protocol was approved by the Institutional Animal Ethics Committee at GIET School of Pharmacy in Rajahmundry, Andhra Pradesh, India (GSP/IAEC/2017/03/03). All experimental animals were sacrificed using diethyl ether, and their ovaries were harvested.

Experimental method

Experimental animals that were induced to undergo PCOS by letrozole were fed *P. dulcis* and *S. hispanica*. Animals were initially divided into six groups ($n = 6$): a negative control, a positive control (placebo), a standard, and three treatment groups. Group 1 (negative control) was given daily oral doses of 1 mg/kg in 0.5% carboxymethyl cellulose (CMC) for 21 days. The remaining animals were administered with 1 mg/kg letrozole daily for 21 days to induce PCOS,³⁷ and were further divided into five groups. Group 2 rats were the positive control group and were administered with 40 g of placebo. Groups 3–5 were the treatment groups, with group 3 receiving an oral dose of *S. hispanica* (40 g/day); group 4, an oral dose of *P. dulcis* (40 g/day); and group 5, an oral combination of *P. dulcis* and *S. hispanica* (20 g each). A sixth group was also administered with an oral dose of clomiphene citrate at 1 mg/kg in 0.5% CMC (standard group). Other than group 1, all groups were

treated with their respective treatments for 15 days (from day 22–36). The animals were anaesthetised and sacrificed with diethyl ether at the end of the experiment, and the organs were extracted to assess for various morphological, biochemical, histopathological investigations.³⁸

Morphological parameters

Body weight measurements

Animal body weight was measured by placing the animal on a weighing scale. The body weight of individual rats was recorded on day 1 and 37 as a difference in weight between these two days.³⁹

Ovarian weight measurements

The ovaries were removed after the rats were euthanised and dissected, and were washed thoroughly with 0.9% saline solution to remove the uterine tissue. The ovaries were then weighed with a weighing balance.⁴⁰

Biochemical analysis

Blood samples were collected from the experimental rats by retro-orbital puncture 24 h after the last dose of treatment and stored in Eppendorf tubes containing heparin sodium for further processing. The plasma samples for hormone assays were separated by centrifugation at 3000 rpm/min for 15 min and stored in a freezer at 20 °C. The oestradiol and testosterone levels were determined by a chemiluminescent immunoassay method using a commercially available kit (Siemens, New York, NY, USA, mean inter- and intra-assay coefficient of variation (CV) of 4.4% and 6.2%, respectively). To measure the plasma progesterone and cholesterol levels, we used a microparticle enzyme immunoassay and a method published by Azeez et al.,⁴¹ using a commercially available kit (Court-Acount progesterone; Los Angeles, California, USA, mean inter- and intra-assay coefficient of variation (CV) of 14.4% and 4.9%, respectively). All blood sample measurements were recorded twice: 21 days after letrozole induction (that is, on the 22nd day of the study) and 15 days following treatment with *P. dulcis* and *S. hispenica* (that is, on the 37th day of study).⁴²

Histopathological examinations

All rats were sacrificed at the end of the experiment according to the Committee for the Purpose of Control and Supervision of Experiments on Animals as per Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines, and the ovaries were extracted, cleansed to remove fat, and weighed. Histopathological examination of these ovaries was carried out as per the standard

procedure. Ovary sections were prepared from paraffin-embedded blocks and were stained with haematoxylin-eosin. Light microscopy was used to examine these sections.⁴³

Statistical analysis

Results were statistically assessed by paired t-test and one-way ANOVA. The results of the treatment groups were compared to control and standard groups, respectively.^{44,45}

Results

Body weight

Animals were weighed at the beginning of, and upon completion of the experiment (on the 37th day of the experiment), and expressed as a difference in body weight between these two days. The body weights of experimental animals decreased significantly after single or combination treatment with *P. dulcis* and/or *S. hispenica*. The body weight results are summarized in Table 1.

Measuring ovarian weight

The weight of both ovaries extracted from the treatment groups (rats treated with *P. dulcis* and/or *S. hispenica* as single agents or in combination) was compared to those extracted from the control groups. These results are summarised in Table 2.

Hormone profile

The experimental rats treated with *P. dulcis* and/or *S. hispenica*, and these rats produced testosterone and oestradiol within the normal ranges. Whereas, progesterone levels were lower in the letrozole-treated PCOS groups (groups 2–6) than in the negative control group. These natural products also affected the cholesterol level in these animals. The hormone levels of all animals tested are summarised in Table 3.

Histopathological studies

Transverse sections of the left and right ovaries were prepared and were histopathologically studied. The ovaries extracted from the negative control group had healthy follicles at various stages of maturation and were surrounded by a thick ovarian stroma and corpus luteum (Fig 1A). Letrozole causes tissue damage, inducing the formation of free radicals, which lead to the development of polycystic

Table 1: The body weight of experimental animals before and after administration of *Prunus dulcis* and *Salvia hispenica*.

S.No	Group	Initial body weight Mean ± SEM (gms) n = 6	Final body weight Mean ± SEM (gms) n = 6	Difference in body weight Mean ± SEM (gms) n = 6
1	Negative control	198.50 ± 2.06	207.17 ± 2.44	8.67 ± 0.38
2	Positive control	193.17 ± 3.85**	231.0 ± 3.01*	37.83 ± 0.84*
4	<i>Prunus Dulcis</i>	211.95 ± 3.86**	227.33 ± 2.80**	15.38 ± 1.06**
5	<i>Salvia Hispenica</i>	198.94 ± 3.92*	213.00 ± 5.89**	14.06 ± 1.97*
6	<i>Prunus Dulcis</i> and <i>Salvia Hispenica</i>	199.83 ± 4.55*	212.83 ± 4.80**	13.00 ± 0.25**
3	Standard (Clomiphene citrate)	199.33 ± 2.99**	211.50 ± 2.83*	12.17 ± 0.16**

**P < 0.005, *P < 0.001 as compared to negative control. Statistical analysis-One way ANOVA.

Table 2: The effects of *Prunus dulcis* and *Salvia hispanica* on the weight of ovaries extracted from Wistar albino rats induced to undergo PCOS.

S.No	Group	Left ovary (n = 6) gms	Right ovary (n = 6) gms
1	Negative control	54.8950 ± 1.4316	55.7933 ± 1.3547
2	Positive control	61.8417 ± 1.3490**	62.8267 ± 1.3754*
3	<i>Prunus Dulcis</i>	60.0033 ± 1.2965**	60.1827 ± 1.0246*
4	<i>Salvia Hispanica</i>	58.7350 ± 1.3951*	58.8217 ± 1.2856**
5	<i>Prunus Dulcis</i> and <i>Salvia Hispanica</i>	57.5067 ± 1.1324**	57.4583 ± 1.0989**
6	Standard (Clomiphene citrate)	55.8583 ± 1.51300**	56.9600 ± 1.0246*

**P < 0.005, *P < 0.001 as compared to blank and standard respectively. Statistical analysis-One way ANOVA.

Table 3: The effects of *Prunus dulcis* and *Salvia hispanica* on hormone levels in Wistar albino rats induced to undergo PCOS.

S.No	Group	Testosterone (ng/dl) n = 6	Progesterone (ng/dl) n = 6	Estradiol (pg/dl) n = 6
1	Negative control	54.8500 ± 0.8164	32.4867 ± 0.5192	22.7867 ± 0.4189
2	Positive control	73.6167 ± 0.5944**	21.2583 ± 0.6855*	85.2833 ± 0.3952**
4	<i>Prunus Dulcis</i>	63.1983 ± 0.8079*	22.4900 ± 0.5210**	35.1700 ± 0.4473*
5	<i>Salvia Hispanica</i>	59.0417 ± 0.8618**	24.6600 ± 0.3916*	29.1150 ± 0.3968**
6	<i>Prunus Dulcis</i> and <i>Salvia Hispanica</i> and Chia	57.4800 ± 0.6058**	25.4500 ± 0.2837**	26.4767 ± 0.3856**
3	Standard (Clomiphene citrate)	56.2733 ± 0.7069*	28.2667 ± 0.5136*	25.7417 ± 0.4434**

**P < 0.005, *P < 0.001 as compared to blank and standard respectively. Statistical analysis-One way ANOVA.

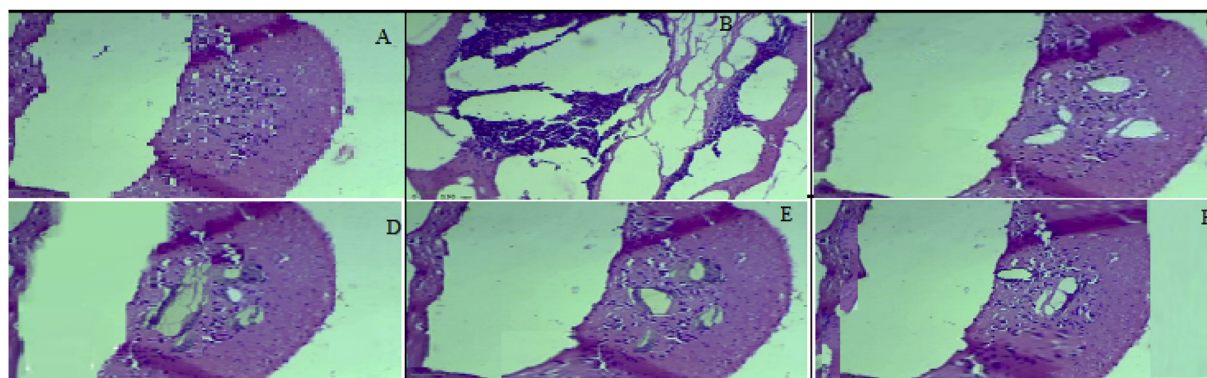


Figure 1: Histopathological sections of ovaries extracted from rats in all experimental groups. The images were captured using light microscopy after the sections were stained with haematoxylin-eosin. A) Negative Control group; B) Positive control (Placebo) group C) Standard clomiphene citrate-treated group D) *Prunus dulcis*-treated group E) *Salvia hispanica*-treated group F) *Prunus dulcis* and *Salvia hispanica* combined treatment group.

ovaries in the experimental animals. In the positive control group, this is demonstrated by the appearance of multiple cystic follicles lined by a thin granulosa layer, follicular atresia, stromal hyperplasia, and vacuolated stroma (Fig 1B). *Prunus dulcis* treatment reversed some of these follicular changes, with smaller cysts and fewer pyknotic granulosa cells (Fig 1C). In rats treated with *Salvia hispanica*, the effect of letrozole was completely reversed, with ovaries that had healthy-looking follicles and stroma (Fig 1D). Interestingly, this morphological reversal correlated with that of progesterone and oestrogen levels, which also returned to normal ranges. These effects were comparable to those observed in the rats treated with the

standard drug (clomiphene citrate), which is known to mask letrozole's effect (Fig 1E).

Discussion

This study was designed to identify the effect of *P. dulcis* and *S. hispanica* in the management of PCOS in rats. In this study, letrozole was used to induce PCOS in the experimental animals. Clomiphene citrate, which reverses letrozole's effect, was used as a marker to assess the effectiveness of the test compounds. Both letrozole and clomiphene citrate were prepared in 0.5% CMC and administered orally at a dose of 1 mg/kg as per published protocol. To induce PCOS, letrozole was administered to all rats for 21 days

except those belonging to the negative control group (group 1). Group 2 animals were the positive controls for this experiment. Groups 3, 4, and 5 were given a daily dose of *P. dulcis* (40 g/kg), *S. hispenica* (40 g/kg), or a combination of both (20 g/kg each) respectively, for 15 days. Group 6 animals were treated with clomiphene citrate. After drug treatment, the body and ovarian weights, hormone levels, and the histopathological changes were measured from animals sacrificed on the 37th day of the experiment.

The results revealed that feeding rats with these natural compounds, either individually or in combination, significantly reduced the body weight of the experimental animals. The results revealed that feeding rats with these natural compounds reversed the effect of letrozole when PCOS was induced. This include a reduction of the body and ovarian weight gained from 199.83 ± 4.55 , 57.5067 ± 1.1324 and 57.4583 ± 1.0989 . The rats that were treated with a combination of both *S. hispenica* and *P. dulcis* exhibited a reduction of 13 ± 0.25 g in body weight, which was comparable to the clomiphene citrate-treated group. Furthermore, *S. hispenica* (Group 3) or *P. dulcis* (Group 4) treatments alone considerably increased the body weight of these rats by 14.06 ± 1.97 g and 15.38 ± 1.06 g, respectively. These results are summarised in Table 1.

Experimental animals were sacrificed on the 37th day of the experiment, and the left and right ovaries were extracted and weighed. The result showed that ovaries extracted from the combined-treatment animals did not form cysts, therefore the mean ovarian weight was 57.5067 ± 1.1324 (left) and 57.4583 ± 1.0989 (right). These values were similar to those of the negative control group. In animals that were treated with either compound, an ovarian mean weight of 58.7350 ± 1.3951 , 58.8217 ± 1.2856 (*S. hispenica*) and 60.0033 ± 1.2965 , 60.1827 ± 1.0246 (*P. dulcis*) was observed. The weights of both ovaries are summarised in Table 2.

At the end of the study, hormone levels and histopathological changes were measured when the animals were sacrificed. In the letrozole-treated rats, both testosterone and oestradiol concentrations increased, whereas progesterone levels were reduced. Combination treatment with *P. dulcis* and *S. hispenica* restored hormone levels to normal in these animals because these products contained polyphenolic compounds. These plant products protect the ovaries from the harmful effects of letrozole, resulting in healthy ovarian stroma. Both hormone levels and histopathology of experimental animals are summarised in Table 3 and Figure 1. Similar results were also obtained in studies conducted by other scientists, who found that *P. dulcis* and *S. hispenica* were responsible for alleviating ovarian dysfunction by improving metabolic and endocrine parameters in tested animals.^{46–49} Because of these benefits, natural compounds should be considered as viable treatment options for PCOS and other emerging diseases. These natural compounds should be further researched to investigate their role in the management of PCOS and other hormone-related diseases caused by the modern lifestyle.

Conclusion

Our study demonstrated that individual or combined treatment with *P. dulcis* and *S. hispenica* improves ovarian functions in letrozole-induced PCOS rats. Letrozole-induction is an accurate method to induce PCOS in experimental animals. These natural products contain polyphenolic compounds, which are the active ingredients to treat metabolic and reproductive dysfunctions. Therefore, hormone levels are restored in these experimental animals. Thus, *P. dulcis* and *S. hispenica* are promising agents for the management of PCOS and other metabolic disorders in women.

Recommendation

We recommend that further studies should be carried out on the therapeutic activities of *P. dulcis* and *S. hispenica* as we have shown that they have significant effects against metabolic and reproductive dysfunctions by restoring normal hormone levels without producing side-effects.

Source of funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interest

There are no conflicts of interest.

Ethical approval

The experimental protocol was approved by the Institutional Animal Ethics Committee at the GIET School of Pharmacy, Rajahmundry, Andhra Pradesh, India (GSP/IEAC/2017/03/03).

Authors contributions

SR designed the study and proposed the hypothesis that *P. dulcis* and *S. hispenica* can manage letrozole-induced PCOS in Wistar rats. CG and JN carried out the experiment and recorded all data from the animals. MDD contributed to the writing of the manuscript, in particular the structure of the manuscript and its pharmacological content. All authors have read and approved the final manuscript. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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