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The anti-climacterium effects of red clover dry extracts combined with pomegranate concentration powder in ovariectomized rats

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Abstract

Objective : In this study, the addition of dried pomegranate concentrate powder (PCP) was affected the anti-climacterium activity of red clover dry extracts (RC) in ovariectomized (OVX) rats.

Materials and methods : After bilateral OVX surgery, RC 40 mg/kg, PCP 20 mg/kg and RC:PCP 2:1 mixture (g/g) 120, 60 and 30 mg/kg (of body weight) were orally administered, once a day for 84 days, and then the changes on the serum estradiol levels, abdominal fat pad and uterus weights were observed for estrogenic effects. In addition, liver weights, serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were also evaluated for hepatoprotective effects, and serum total cholesterol (TC), low density lipoprotein (LDL), high density lipoprotein (HDL) and triglyceride (TG) levels were monitored for hypolipidemic effects.

Results : As a result of OVX, the estrogen-deficient climacterium symptoms, increments of abdominal fat pad weights, serum AST, ALT, TC, LDL and TG levels with decrease of uterus and liver weights, serum estradiol levels, were demonstrated. However, these estrogen-deficient climacterium symptoms induced by bilateral OVX in rats were significantly inhibited by continuous oral treatment of RC 40 mg/kg, PCP 20 mg/kg and RC:PCP 2:1 mixture (g/g) 120, 60 and 30 mg/kg, respectively.

Conclusion : The results suggested that RC:PCP 2:1 mixtures synergistically increased the anti-climacterium effects of RC in OVX rats. It, therefore, is expected that RC:PCP 2:1 mixture will be promising as a new potent protective agents for relieving the climacterium symptoms.

Key words : dried pomegranate concentrate powder; red clover dry extracts; 2:1 mixtures; anti-climacterium effects; ovariectomy; rats

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I. Introduction

Climacteric suffering corresponds to the period of life during which women gradually lose their reproductive capacity as a result of aging[1]. It is closely related to loss of activity of ovarian follicles, with consequent estrogen deficiency[2]. Approximately 70% of women report some type of symptom during the climacteric period. In general, these symptoms are resulted from the estrogen deprivation. The most common complaints are vasomotor symptoms and night sweats, also, vaginal dryness, dyspareunia and urinary urgency, which are associated with urogenital atrophy. It may interfere with the sex life and quality of life of postmenopausal women[2]. In addition, the postmenopausal state is associated with an increased risk of metabolic diseases such as obesity, heart disease, diabetes, and hypertension[3].

To minimize the impact of ovarian failure on women's health, hormone therapy is often recommended. However, it has been raised serious concerns with regard to the use and safety of hormone replacement therapy in relation to cardiovascular events and breast cancer in long treatment[4]. For this reason, many researchers have sought alternative therapies, such as the use of phytoestrogens to relieve menopausal symptoms[1]. Phytohormones comprise several compounds that can be extracted from plants and, when purified, can enhance their activity in the body as well as improve their bioavailability[5]. Phytoestrogens are substances with chemical structure and function similar to that of estrogens and have been shown to bind to estrogen receptors due to the presence of a phenolic ring[6]. Isoflavones, especially those derived from plants, have various biological activities and can improve the metabolic symptoms of menopause[7]. Phytoestrogen acts as an estrogen mimic[8], and these estrogenic effects in biological systems believed to relate its structural similarity to estrogen. The isoflavonoids exert a weak estrogenic-like effect by binding to both estrogen receptors [ER]-α and ER-β in various tissues[9].

Pomegranate (*Punica granatum* L) fruit consumption is suitable for people of all ages[10]. Pomegranates are popularly consumed as fresh fruit, beverages, other food products, dietary supplements, and herbal medicine ingredients[11]. The main active substances in pomegranate are polyphenols, which have anti-oxidant, anti-mutagen, anti-inflammatory, and antimicrobial abilities[10]. Polyphenols are present in different parts of pomegranate fruits[12]. It has recently been reported that pomegranate contains some species of flavonoids and anthocyanidins in their seed oil and juice, and shows an antioxidant activity three times more potently than red wine and green tea extract [13]. Red clover (RC, *Trifolium pratense* L) botanical dietary supplements have received much attention recently for their potential use in the treatment of menopause symptoms, maintenance/improvement cardiovascular health and for the reported benign effects on the breast, endometrium and neural structure besides for its safety[14]. The estrogenic activity of RC is mainly due to isoflavones and to a smaller part to coumestans[15]. Therefore, it can be confirmed that beneficial effects of each RC[15] and dried pomeanate concentrate powder (PCP)[16] on postmepausal symptoms have been well documented as summarized by other investigators, mainly related to their phytoestrogenic effects of isoflavonoids and related antioxidant and anti-inflammatory pathways[17].

It, therefore, considered that appropriated mixres consisted of RC and PCP will be showed more favorable synergic anti-climacterium effects as increases the diversity of bioactive isoflavonoids. In the present study, addition of PCP whether increases the anti-climacterium activity of RC were observed on ovariectomized (OVX) rats, a

well-documented rodent models resembles with women postmenopausal climacterium symptoms[18]. In this study, anti-climacteric effects of RC comned with PCP were evaluated on ovariectomized (OVX) rats separated into estrogenic effects, anti-bese, hypolipidemic effects and hepatoprotective effects.

II. Materials and methods

1. Animals and husbandry

Total 77 virgin VAF Outbred-Rats, Crl:CD [Sprague-Dawley, SD] specific pathogen-free rats (6-week old old upon receipt; OrientBio, Seungnam, Korea) were used after acclimatization for 7 days. Animals were allocated four per polycarbonate cage in a temperature (20-25℃) and humidity (45-55%) controlled room. Light : dark cycle was 12 hr : 12 hr and feed (Samyang, Korea) and water were supplied free to access. After 28 days after OVX operation, eight rats per group were selected based on the body weight deviations. All laboratory animals were treated according to the national regulations of the usage and welfare of laboratory animals, and approved by the Institutional Animal Care and Use Committee in Daegu Haany University (Gyeongsan, Gyeongbuk, Korea) prior to animal experiment. In addition, experiments on osteoporosis were conducted based on US FDA Guideline "Guidelines for Preclinical Evaluation of Agents Used in The Prevention or Treatment Postmenopausal Osteoporosis" April, 1994, Division of Metabolic and Endocrine Drug Products, USA.

2. Preparations and administration of test substances

Standardized RC, PCP and their 2:1 (g/g) mixtures were supplied by sponsor (HEALTH LOVE. Ltd., Anyang, Korea). RC contains 8% total isoflavones; 0.62% Genistein, 5.43% Biochanin A, 3.66% Formononetin and 0.47% Daidzein, and well suspended in distilled water. PCP contains 0.90 mg/g Ellagic acid, and well dissolved in distilled water. RC:PCP 2:1 mixture (g/g) prepared by sponsor were also well suspended in distilled water. From 28 days after OVX, RC 40 mg/kg, PCP 20 mg/kg and RC:PCP 2:1 mixture (g/g) 120, 60 and 30 mg/kg (of body weight) were orally administered, once a day for 84 days. In OVX and sham control rats, only distilled water as vehicle, were orally administered as equal volumes and periods, instead of herbal formulas in this experiment.

3. Menopause inducement - bilateral OVX

Rats were anesthetized with 25 mg/kg intraperitoneal injection of Zoletile mixture (Zoletile 50™; Virbac Lab., Carros cedex, France) and maintained with 1 to 1.5% isoflurane (Hana Pharm. Co., Hwasung, Korea) in the mixture of 70% N_2O and 28.5% O_2. The surgical protocol was carried out according to our established methods[19]. The OVX treatment group underwent open surgery involving bilateral OVX via a midline incision of linea alba. Following surgery, the incision was closed in two layers. The muscular layers were sutured independently from peripheral tissues using dissolvable 3-0 vicryl sutures, and the skin closed by continuous sutures using silk (3-0). The second group of rats underwent a sham operation, in which a similar incision in the *linea alba* was made but bilateral OVX were not performed.

4. Organ weight measurements

At Sacrifice, the left sides of abdominal fat pad deposited into dorsal abdominal wall, total liver and uterus including vagina located in abdominal cavity were collected after eliminations of the

surrounding connective tissues, muscles and any debris. The weights of organs were measured at g levels regarding absolute wet-weights.

5. Serum biochemistry

For serum biochemistry, 10 ml of whole blood was collected from vena cava at sacrifice, and separated the serum by centrifugation at 15,000 rpm for 10 min under 4℃, using clotting activated serum tube. All serum samples were frozen at −150℃ until they were assayed. Serum AST, ALT, TC, LDL and TG levels were detected using an automated blood analyzer (Hemagen Analyst; Hemagen Diagnostic, Columbia, MD, USA), and HDL levels were also measured by other typed automated blood analyzer (AU400; Olympus, Tokyo, Japan). In addition, serum estradiol contents were also measured using the chemiluminescent immunoassay technique (ECLIA, Modular E 170C, Estradiol II 03000079 122, Roche, Germany) from the separated serum at sacrifice in all individual rats.

6. Statistical analyses

All values are expressed as mean ± S.D. of eight rats in this experiment. Multiple comparison tests for different dose groups were conducted. Variance homogeneity was examined using the Levene test. If the Levene test indicated no significant deviations from variance homogeneity, the obtain data were analyzed by one way ANOVA test followed by least-significant differences (LSD) multi-comparison test to determine which pairs of group comparison were significantly different. In case of significant deviations from variance homogeneity were observed at Levene test, a non-parametric comparison test, Kruskal-Wallis H test was conducted. When a significant difference is observed in the Kruskal-Wallis H test, the Mann-Whitney U (MW) test was conducted to determine the specific pairs of group comparison, which are significantly different. Statistical analyses were conducted using SPSS for Windows (Release 14K, SPSS Inc., USA). A p-values < 0.05 were considered significantly different.

III. Results

1. Effects on the abdominal fat pad weights

Significant ($p<0.01$) increases of abdominal fat pad deposited into left dorsal abdominal muscles, absolute and relative, weights were noticed in OVX control rats as compared with sham control rats, respectively. However, significant ($p<0.01$) decreases of abdominal fat pad weights were observed in RC 40 mg/kg, PCP 20 mg/kg, RC:PCP 2:1 mixture (g/g) 120, 60 and 30 mg/kg treated rats as compared with OVX control rats, respectively. Especially, RC:PCP 2:1 mixture (g/g) 120 and 60 mg/kg treated rats also showed significant ($p<0.01$) decreases of abdominal fat pad absolute and relative weights as compared with those of single formula of RC 40 mg/kg and PCP 20 mg/kg treated rats(Table 1, Fig 1).

2. Effects on the uterus weights

Significant ($p<0.01$) decreases of the uterus absolute and relative wet-weights were observed in OVX control rats as compared with sham control rats, respectively. However, significant ($p<0.01$) increases of the uterus weights were noticed in all test substance treated rats including RC:PCP 2:1 mixture (g/g) 120, 60 and 30 mg/kg as compared with OVX control rats, respectively. Especially, RC:PCP 2:1 mixture (g/g) 120 and 60 mg/kg treated rats also showed significant ($p<0.01$) increases of the uterus absolute and relative weights as compared with those of single formula of RC 40 mg/kg and PCP 20 mg/kg treated rats (Table 1, Fig 2).

Table 1. Abdominal Fat Pad, Uterus and Liver Weights in OVX Rats

Groups	Absolute wet-weight (g)			Relative wet-weight (% of body weight)		
	Abodminal fat pad	Uterus	Liver	Abodminal fat pad	Uterus	Liver
Controls						
Sham	3.6081.146	0.6480.148	7.3740.837	1.1400.351	0.2090.055	2.3490.245
OVX	14.7032.682[a]	0.0820.007[a]	7.3720.351	3.2350.604[a]	0.0180.002[a]	1.6230.118[a]
RC 40mg/kg	8.6831.261[ac]	0.1040.014[ac]	7.6800.606	2.0870.270[ac]	0.0250.004[ac]	1.8490.147[ad]
PCP 20mg/kg	9.3551.279[ac]	0.1010.014[ac]	7.6630.351	2.2380.329[ac]	0.0240.004[ac]	1.8300.066[ac]
RC:PCP 2:1 mixture (g/g)						
120mg/kg	5.1731.220[bceg]	0.1360.023[aceg]	8.4770.444[acfg]	1.3070.296[ceg]	0.0340.006[aceg]	2.1460.096[ceg]
60mg/kg	6.5040.979[aceg]	0.1250.011[aceg]	8.5140.736[aceg]	1.5900.181[aceg]	0.0310.003[aceg]	2.0910.188[gcfg]
30mg/kg	8.3861.613[ac]	0.1050.014[ac]	7.7370.638	2.0120.391[ac]	0.0250.004[ac]	1.8530.122[ac]

Values are expressed mean S.D. of eight rats. [a] $p<0.01$ and [b] $p<0.05$ as compared with sham control, [c] $p<0.01$ and [d] $p<0.05$ as compared with OVX control, [e] $p<0.01$ and [f] $p<0.05$ as compared with RC 40 mg/kg treated rats, [g] $p<0.01$ as compared with PCP 20 mg/kg treated rats. OVX = Bilateral ovariectomy, PCP = Pomegranate Concentrate Powder, RC = Red clover dry extracts.

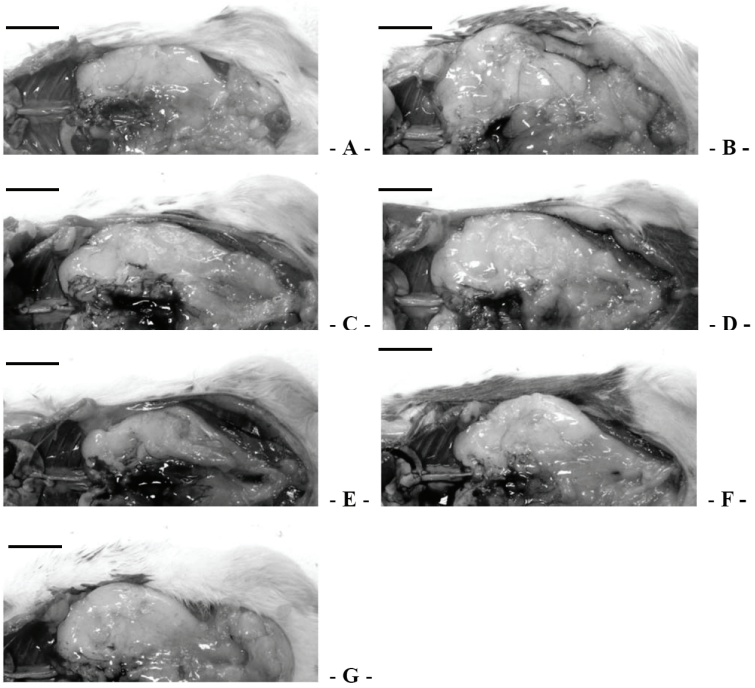

Figure 1. Representative Gross Images of Abdominal Fat Pads, Taken from Intact or OVX Rats Deposited into Left Abdominal Muscles. Note that noticeable increases of abdominal fat pad deposited into left dorsal abdominal muscles were demonstrated in OVX control rats as compared with sham control rats, but they were dramatically normalized by treatment of RC 40 mg/kg, PCP 20 mg/kg, RC:PCP 2:1 mixture (g/g) 120, 60 and 30 mg/kg treated rats as compared with OVX control rats, respectively. Especially, RC:PCP 2:1 mixture (g/g) 120 and 60 mg/kg treated rats also showed more decreases of abdominal fat pad depositions as compared with those of single formula of RC 40 mg/kg and PCP 20 mg/kg treated rats, respectively. RC:PCP 2:1 mixture (g/g) 30 mg/kg treated rats did not showed any significant changes on the abdominal fat pad depositions as compared with those of single formula of RC 40 mg/kg and PCP 20 mg/kg treated rats, in this experiment. A = Intact control rat B = OVX control rat C = RC 40 mg/kg treated rat D = PCP 20 mg/kg treated rat E = RC:PCP 2:1 mixture (g/g) 120 mg/kg treated rat F = RC:PCP 2:1 mixture (g/g) 60 mg/kg treated rat G = RC:PCP 2:1 mixture (g/g) 30 mg/kg treated rat. OVX = Bilateral ovariectomy; PCP = Pomegranate Concentrate Powder; RC = Red clover dry extracts. Scale bar = 24mm

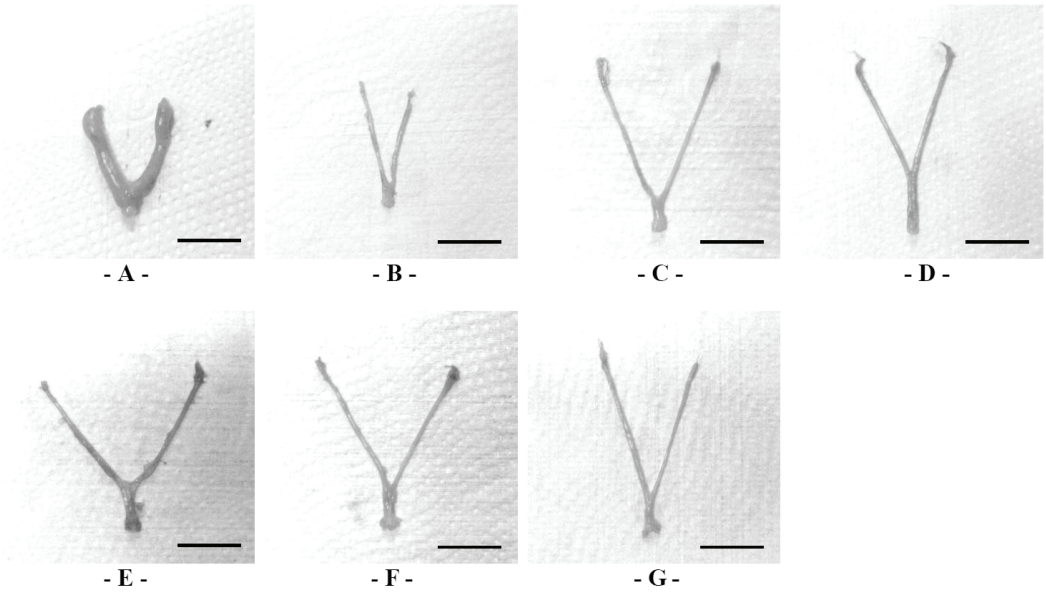

Figure 2. Representative Gross Images of Uterus, Taken from Intact or OVX Rats. Note that marked atrophic changes of uterus were observed in OVX control rats as compared with sham control rats, but noticeable inhibitory activities against uterine atrophy were observed in all test substance treated rats as compared with OVX control rats, respectively. Especially, RC:PCP 2:1 mixture (g/g) 120 and 60 mg/kg treated rats also showed obvious increases of the uterus volumes as compared with those of single formula of RC 40 mg/kg and PCP 20 mg/kg treated rats, respectively. RC:PCP 2:1 mixture (g/g) 30 mg/kg treated rats did not showed any significant changes on the uterus as compared with those of single formula of RC 40 mg/kg and PCP 20 mg/kg treated rats at gross inspections, in this experiment. A = Intact control rat B = OVX control rat C = RC 40mg/kg treated rat D = PCP 20mg/kg treated rat E = RC:PCP 2:1 mixture (g/g) 120mg/kg treated rat F = RC:PCP 2:1 mixture (g/g) 60mg/kg treated rat G = RC:PCP 2:1 mixture (g/g) 30mg/kg treated rat. OVX = Bilateral ovariectomy PCP = Pomegranate Concentrate Powder RC = Red clover dry extracts. Scale bar = 18mm.

3. Effects on the liver weights

Significant ($p<0.01$) decreases of the liver relative wet-weights were detected in OVX control rats as compared with sham control rats, but significant ($p<0.01$ or $p<0.05$) increases of the liver relative weights were demonstrated in all test substance treated rats including single formula of RC 40 mg/kg as compared with OVX control rats, respectively. Especially, RC:PCP 2:1 mixture (g/g) 120 and 60 mg/kg treated rats also showed significant ($p<0.01$) increases of the liver absolute weights as compared with OVX control rats, and they also showed significant ($p<0.01$ or $p<0.05$) increases of the liver absolute and relative wet-weights as compared with those of single formula of RC 40 mg/kg and PCP 20 mg/kg treated rats (Table 1).

4. Effects on the serum biochemistry

Significant ($p<0.01$) increases of the serum AST, ALT, TC, LDL and TG levels, and significant ($p<0.05$) decreases of serum HDL levels were noticed in OVX control rats as compared with sham control rats, respectively. However, significant ($p<0.01$ or $p<0.05$) decreases of the serum AST, ALT, TC, LDL and TG levels and non-significant increased trends in the serum HDL levels were demonstrated in all test material treated rats as compared with OVX control rats, respectively. Especially, RC:PCP 2:1 mixture (g/g) 120 and 60 mg/kg treated rats also showed noticeable significant ($p<0.01$) increases of the serum HDL contents as compared with OVX control, and they also showed significant ($p<0.01$ or $p<0.05$) normalized serum AST, ALT, TC, LDL,

Table 2. Serum Biochemistry: AST, ALT, TC, LDL, HDL and TG Levels in OVX Rats

Groups	Serum biochemistrical values					
	AST (U/L)	ALT (U/L)	TC (mg/dl)	LDL (mg/dl)	HDL (mg/dl)	TG (mg/dl)
Controls						
Sham	96.8612.54	68.2514.62	62.5013.48	32.5010.94	23.504.81	53.8813.15
OVX	171.5021.79[a]	117.7519.56[a]	128.1319.70[a]	85.5015.92[a]	19.133.23[b]	119.5029.09[a]
RC 40mg/kg	146.636.80[ac]	93.387.93[ac]	97.3810.01[ac]	66.3811.61[ac]	20.252.19	86.6310.84[ac]
PCP 20mg/kg	150.757.81[ac]	98.007.58[ad]	101.2514.36[ac]	64.5011.07[ac]	21.753.96	90.0012.06[ad]
RC:PCP 2:1 mixture (g/g)						
120mg/kg	117.2513.33[aceg]	76.639.16[ceg]	76.389.13[ceg]	47.639.83[bceg]	29.134.09[bceg]	68.5010.69[bceg]
60mg/kg	127.2515.98[acfg]	83.257.59[bcfg]	81.0014.01[bcfg]	51.637.67[acfh]	27.134.05[ceh]	75.387.61[acfh]
30mg/kg	141.2519.38[ac]	90.7518.22[bd]	91.7517.43[ac]	63.7512.40[ac]	22.136.56	83.2520.87[ad]

Values are expressed mean S.D. of eight rats. [a] $p<0.01$ and [b] $p<0.05$ as compared with sham control, [c] $p< 0.01$ and [d] $p<0.05$ as compared with OVX control, [e] $p<0.01$ and [f] $p<0.05$ as compared with RC 40 mg/kg treated rats, [g] $p<0.01$ and [h] $p<0.05$ as compared with PCP 20 mg/kg treated rats. OVX = Bilateral ovariectomy, PCP = Pomegranate Concentrate Powder, RC = Red clover dry extracts, ALT = Alanine aminotransferase, AST = Aspartate aminotransferase, LDL = Low density lipoprotein, TC = Total cholesterol, TG = Triglyceride, HDL = High density lipoprotein.

HDL and TG levels as compared with those of single formula of RC 40 mg/kg and PCP 20 mg/kg treated rats(Table 2).

Significant ($p<0.01$) decreases of the serum estradiol levels in OVX control rats as compared with sham control rats, respectively. However, significant ($p<0.01$ or $p<0.05$) increases of the serum estradiol levels were demonstrated in RC 40 mg/kg, PCP 20 mg/kg, RC:PCP 2:1 mixture (g/g) 120, 60 and 30 mg/kg treated rats as compared with OVX control rats, respectively. Especially, RC:PCP 2:1 mixture (g/g) 120 mg/kg treated rats also showed noticeable significant ($p<0.01$) increases of the serum estradiol contents as compared with those of single formula of RC 40 mg/kg and PCP 20 mg/kg treated rats(Fig 3).

IV. Discussion

In this study, anti-climacteric effects were evaluated separated into several categories; 1) estrogenic effects, 2) anti-obese, 3) hypolipidemic effects and 4) hepatoprotective effects. Twenty-eight days after bilateral OVX surgery, RC 40 mg/kg, PCP 20 mg/kg and RC:PCP 2:1 mixture (g/g) 120, 60 and 30 mg/kg (of body weight) were orally administered, once a day for 84 days, and then the changes on the serum estradiol levels, abdominal fat pad and uterus weights were evaluated for estrogenic effects. In addition, liver weights, serum AST and ALT levels were also evaluated for hepatoprotective effects, and serum TC, LDL, HDL and TG levels were monitored for hypolipidemic effects. The results of RC:PCP 2:1 mixture (g/g) were compared with those of each single RC and PCP formula treated OVX rats, in the present study.

As a result of OVX, noticeable increases of weights of abdominal fat pad deposited in dorsal abdominal cavity, serum AST, ALT, TC, LDL and TG levels were demonstrated in this experiment with decrease of uterus and liver weights, serum estradiol levels. It suggested that the estrogen-deficient climacterium symptoms were induced by OVX. However, these estrogen-deficient climacterium symptoms induced by bilateral OVX in

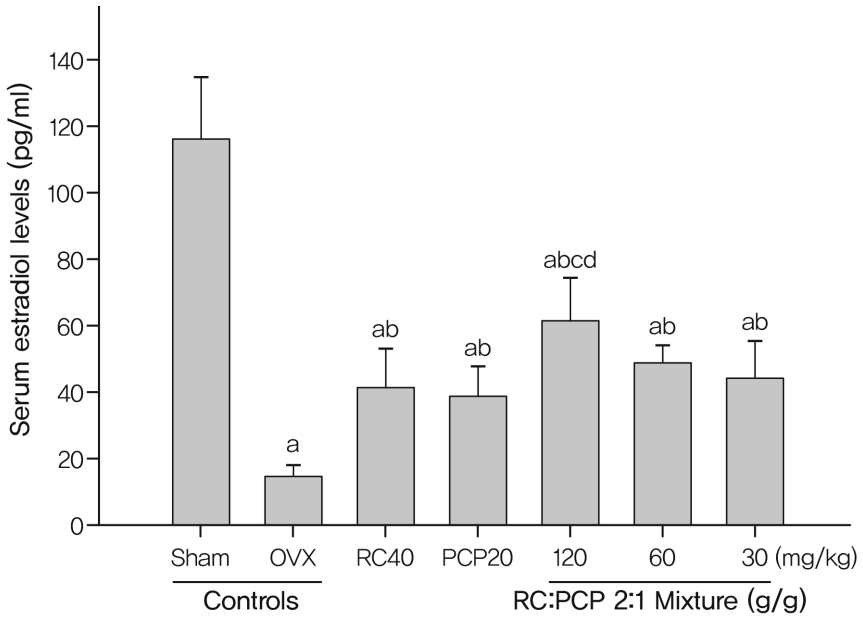

Figure 3. Serum Estradiol Levels in OVX Rats. Note that significant decreases of the serum estradiol levels in OVX control rats as compared with sham control rats, respectively. However, significant increases of the serum estradiol levels were demonstrated in RC 40 mg/kg, PCP 20 mg/kg, RC:PCP 2:1 mixture (g/g) 120, 60 and 30 mg/kg treated rats as compared with OVX control rats, respectively. Especially, RC:PCP 2:1 mixture (g/g) 120 mg/kg treated rats also showed noticeable significant increases of the serum estradiol contents as compared with those of single formula of RC 40 mg/kg and PCP 20 mg/kg treated rats. Values are expressed mean S.D. of eight mice. [a] $p<0.01$ as compared with sham control, [b] $p<0.01$ as compared with OVX control, [c] $p<0.01$ as compared with RC 40 mg/kg treated rats, [d] $p<0.01$ as compared with PCP 20 mg/kg treated rats. OVX = Bilateral ovariectomy, PCP = Pomegranate Concentrate Powder, RC = Red clover dry extracts.

rats were significantly inhibited by 84 days of continuous oral treatment of RC 40 mg/kg, PCP 20 mg/kg and RC:PCP 2:1 mixture (g/g) 120, 60 and 30 mg/kg. Especially, RC:PCP 2:1 mixture (g/g) 120 and 60 mg/kg treated OVX rats showed significant more favorable inhibitory activities against estrogen-deficient climacterium symptoms induced by OVX as compared with those of single formula of RC 40 mg/kg and PCP 20 mg/kg treated rats. Although RC:PCP 2:1 mixture (g/g) 30 mg/kg treated rats also showed more inhibitory effects against estrogen-deficient climacterium symptoms as compared to those of RC 40 mg/kg and PCP 20 mg/kg single formula treated rats in some indices, but it did not showed any statistical significant changes. These results are considered as direct evidences that addition of appropriated amounts of PCP, RC:PCP 2:1 mixtures (g/g) synergistically increased the anti-climacterium effects of RC in OVX rats, may be increased the diversity of isoflavonoids in mixtures.

Estradiol is produced within the follicles of female ovaries, but also in other tissues (including fat, liver, adrenal and breast tissues). Estradiol has been shown to control body weight by modulating the potency of feedback signals that control intake[20]. The interaction between cholecystokinin (CCK) and estradiol is the best result, the data indicate that estradiol increases the satiating potency of exogenous and endogenous CCK[21]. Similar mechanisms may be operational for glucagon because the effects of glucagon and of glucagon antibodies to decrease or increase meal size, respectively, were both amplified by estradiol in OVX rats[22]. It is a well-established pheno-

menon that the absence of estradiol leads to a temporary increase the intake and a sustained gain the body weight[23]. This phenomenon is of clinical relevance because estradiol levels decrease in postmenopausal women; importantly, postmenopausal women occupy a high frequency of the obese population[24]. Estrogen depletion by OVX in rats induced severe increases of food and water intakes but did not in urine volume and fecal excretions, as summarized by other investigators facilitated the body fat depositions, especially on abdominal cavity[25]. The accumulation or increase of fat deposition in the body is a major characteristic of obesity, and cellular hypertrophy appears to be the major mode of expansion of the intra-abdominal adipose tissue in rodents[26]. In the present study, OVX also induced marked and noticeable increases of abdominal fat depositions. However, these estrogen-deficiencies related obese was dramatically inhibited by treatment of RC, PCP and all three different dosages of RC:PCP 2:1 mixture (g/g). Especially, RC:PCP 2:1 mixture (g/g) 120 and 60 mg/kg treated OVX rats showed significant more favorable inhibitory activities against obese induced by OVX as compared with those of single formula of RC and PCP treated rats, suggesting RC:PCP 2:1 mixtures (g/g) synergistically increased the anti-obese of RC in OVX rats.

Estrogens play a vital role in the regulation and function in numerous female target organs such as uterus, vagina, and skeletal and cardiovascular systems[27]. Therefore, estrogen deficiency results from ovarian production cessation is associated with many complaints experienced by menopausal women, climacterium symptoms[28]. For decades, the hormone replacement therapy has been used as an alternative for managing menopause-induced complaints[29]. Although hormone replacement therapy is presumably a safe treatment for short-term therapy, it is implicated in adverse outcomes after long-term usase such as increased risk of endometrial and breast cancer, stroke and pulmonary thromboembolism[4]. For these reasons, women turned to natural health remedies and then the interest in phytoestrogens, from plant derived, raised significantly in the last decades[25]. Phytoestrogen behaves as an estrogen mimic[8], and these estrogenic effects in biological systems believed to relate its structural similarity to estrogen, isoflavonoids exert a weak estrogenic-like effect by binding to both estrogen receptors [ER]-α and ER-β in various tissues[9]. Estrogen deficiency is accompanied with a marked atrophy of organs such as uterus and vagina[25]. In addition, marked atrophic changes of uterus were induced by OVX-induced estrogen deficiency[25]. Our results show that OVX induced a significant decrease in uterine weights with marked decreases of serum estradiol levels. However, these estrogen-deficient uterine atrophy induced by bilateral OVX in rats were significantly inhibited by 84 days of continuous oral treatment of RC, PCP and RC:PCP 2:1 mixture (g/g) 120, 60 and 30 mg/kg, respectively. Especially, RC:PCP 2:1 mixture (g/g) 120 and 60 mg/kg treated OVX rats showed significant more favorable inhibitory activities against estradiol depletion and related uterine atrophic changes as compared with those of single formula of RC and PCP treated rats, respectively. These results are considered that RC:PCP 2:1 mixtures (g/g) synergistically increased the estrogenic effects of RC in OVX rats. The estrogenic activity of RC is mainly due to isoflavones and to a smaller part of coumestans[15]. The isoflavones, formononetin, biochanin A, genistein and daidzein, are present in red clover as glycosides and malonates[30]. In addition, it has recently been reported that PCP also contains some species of flavonoids and anthocyanidins in their seed oil and juice[10]. The increase of uterine mass is mainly attributed to the uterine water imbibition and/or a cell proliferation[31] and mediated through ERα[9], and they also believed to

involve the mechanism of the protective effects of RC and PCP, and their combinations against OVX-induced uterine atrophy. However, more detail mechanism should be studied in future.

Estrogen deficiency is associated with atherogenic lipid profile, the principal cause of increased risk of developing cardiovascular disease[32]. In this study, OVX induced a significant increase of TC, LDL and TG while decreasing HDL. This result is in accordance with the literature, which reports a significant increase of TC, LDL, TG, and low HDL levels in postmenopausal women[33] and similar changes on serum lipids were also induced by OVX[25]. In the previous reports, estradiol significantly decreases the TC and LDL levels[25] as compared with OVX controls. This effect of estradiol on serum lipid profiles is thought to be mediated by inhibiting the activity of the rate-limiting enzyme involved in cholesterol synthesis, the 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA)[34], and also in the present study. However, these OVX-induced hyperlipidemia in rats were significantly inhibited by 84 days of continuous oral treatment of RC, PCP and RC:PCP 2:1 mixture (g/g) 120, 60 and 30 mg/kg. Especially, RC:PCP 2:1 mixture (g/g) 120 and 60 mg/kg treated OVX rats showed significant more favorable hypolipidemic activities as compared with those of single formula of RC and PCP treated rats, respectively. These results are considered that RC:PCP 2:1 mixtures (g/g) synergistically increased the hypolipidemic effects of RC in OVX rats, may be through enhancement of HMG-CoA activities, which were involved in cholesterol synthesis[34].

Because of main target organ of HMG-CoA reductase is liver[35], hypertrophy and fatty change of hepatocytes are accompanied by increased AST and ALT activities, serum biochemistrical indicators of hepatic damages[36], in OVX rats, related to afore-mentioned estrogen-deficiency mediated obese and hyperlipidemia[35], and these liver steatosis were also induced by OVX in this experiment. However, these OVX-induced hepatic steatosis in rats were significantly inhibited by oral treatment of RC, PCP and RC:PCP 2:1 mixture (g/g) 120, 60 and 30 mg/kg, and especially, RC:PCP 2:1 mixture (g/g) 120 and 60 mg/kg treated OVX rats showed significant more favorable hepatoprotective activities as compared with those of single formula of RC and PCP treated rats, respectively. These results are considered that RC:PCP 2:1 mixtures (g/g) synergistically increased the hepatoprotective effects of RC in OVX rats, may be through enhancement of estrogenic properties.

V. Conclusion

The results obtained in this study as follows.

1. The addition of appropriated amounts of PCP (RC:PCP 2:1 mixtures, g/g) synergistically increased the anti-climacterium effects of RC - estrogenic, anti-obese, hypolipidemic and hepato protective effects in OVX rats, through synergic anti-inflammatory and anti-oxidative activities.

2. RC:PCP 2:1 mixture (g/g) 120 and 60 mg/kg treated OVX rats showed significant more favorable inhibitory activities against estrogen-deficient climacterium symptoms induced by OVX as compared with those of single formula of RC 40 mg/kg and PCP 20 mg/kg treated rats, respectively.

3. Therefore, it is expected that RC:PCP 2:1 mixture will be promising as a new potent protective agents for relieving the climacterium symptoms, especially on estrogen depletion, obese, hyperlipidemia and hepatic steatosis in menopausal women.

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