



Impact of Soybean Phytoestrogen-Rich Extract on Markers of Inflammation Markers in 4-Vinyl Cyclohexane Diepoxide-Induced Menopause in Albino Rats

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Abstract: The vasomotor symptoms of menopause, including hot flashes, sweating, physical and psychological discomfort, and emotional changes, are accurate and experienced by many of the menopausal and postmenopausal female population. In addition, it causes osteoporosis and slowed metabolism, raising the chance of developing many different ailments. Given that hormone replacement therapy (HRT) has been linked to an increased cancer risk, this investigation was undertaken to identify viable alternatives. The study aimed to investigate the impact of Soybean phytoestrogen-rich extract on some markers of inflammation of 4-vinyl cyclohexene diepoxide-induced menopause in albino rats. Sixty-five (65) female albino Wistar rats were employed in the investigation. Each one was induced with 80mg/kg of 4-vinyl cyclohexene diepoxide before being treated with either normal estradiol therapy (14ug/kg) or varying concentrations of the soybean phytoestrogen-rich extract (200 mg/kg, 400 mg/kg, and 600 mg/kg). Inflammatory markers (C-reactive protein (CRP), interleukin-6 (IL-6), and tumour necrosis factor-alpha (TNF- α)) were measured by ELISA methods. Statistical software SPSS (IBM) version 23.0 was used to analyze the data. Compared to the positive control group, the soybean phytoestrogen-rich extract therapy group saw a dose-dependent reduction in CRP and IL-6 levels ($p < 0.05$) but not in TNF- α ($P > 0.05$). Data from this research demonstrate the anti-inflammatory effects of soybean phytoestrogen-rich extract therapy in menopause-induced female Wistar rats. Soybean phytoestrogen-rich extract therapy in a high dose appears to show no significant difference from hormone replacement therapy as an alternate estrogen source in managing inflammation as a chronic disease.

Keywords: 4-vinyl cyclohexane diepoxide; inflammation; menopause; phytoestrogen

INTRODUCTION

Postmenopausal women are at an increased risk for developing chronic diseases, highlighting the need for preventative care. Regular physical exercise training and/or the consumption of phytoestrogens may be essential strategies for minimizing or preventing the low-grade chronic inflammation and oxidative stress that are common in postmenopausal women (Alvehus et al., 2012). Puberty,

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menstruation, pregnancy, and menopause are just a few of the life stages that women go through (Suri & Suri, 2014).

After 12 months of amenorrhea, women are considered to have reached menopause, officially marking the end of their reproductive years (Butler & Santoro, 2011). When a woman enters her 40s or 50s, her reproductive hormone levels naturally drop. Natural menopause, premature or early menopause, and surgical or forced menopause are the three forms of menopause recognized by the World Health Organisation (Nelson, 2008).

Menopause, like adolescence, is a natural occurrence in a woman's life. It often begins between the ages of 42 and 56, though symptoms can appear much earlier and persist for much longer. Due to growing globalization, urbanization, awareness, and greater lifespan, menopause is becoming an issue among middle-aged Nigerian women living in metropolitan areas. About 467 million women aged 50 and more globally 1990 (World Bank, 1993). By 2020, this figure is predicted to have risen to 564 million (WHO, 1993; Hill, 1996). Out of Nigeria's total female population 59.5 million, 5%-8% are currently experiencing menopause. Symptoms of menopause affect 46.9% of women between the ages of 45 and 59. Menopausal symptoms include hot flashes for 5.7% of women and nocturnal sweats for 5.2%. Dizziness affects 22.2% of women after menopause; heart palpitations affect 15%; irritability affects 17.3%; headaches affect 18%; insomnia affects 16.4%; and depression affects 2.2%. (WHO, 2007).

Seventy percent of women have menopausal symptoms, which can endure during menopause and beyond. Later symptoms include cardiovascular issues, oxidative stress, osteoporosis, weight gain, inflammation of the vaginal wall, hair loss, changes in libido (sex drive), joint and muscle aches and pains. Because estrogen levels drop dramatically, these symptoms become more noticeable throughout menopause. The negative consequences of low estrogen levels may be mitigated by phytoestrogens (Somekawa et al., 2001)

Hormonal and non-hormonal therapy is used to address menopausal symptoms. Hormone therapy is widely utilized, specifically progesterone, estrogen, and combination progesterone. Soybean and green leafy vegetables, as well as calcium and vitamin supplements, are suggested examples of dietary changes that can be used instead of hormone therapy (Dutta, 2004). Due to the Women's Health Initiative study results, vasomotor symptoms, osteoporosis, and cardiovascular illnesses can no longer be prevented by estrogen therapy. There is a clear need for estrogen therapy in women with distressing symptoms, particularly in the non-systemic form. However, the treatment is still contentious because the hazards may outweigh the benefits in women with symptomatic menopause. A third of women have itching, irritation, dryness, and dyspareunia without it, which can significantly lower their quality of life. Improvements in sexual function, urinary tract infection frequency, and incontinence are just some of the quality-of-life indicators that can be dramatically enhanced with local estrogen treatment in the form of soybeans (Krause, 2009). When weighing the advantages and disadvantages of estrogen, the distribution route is crucial. Compared to oral administration, transdermal estrogen has been associated with a reduced incidence of thromboembolic events like deep vein thrombosis (DVT), cholecystitis, osteoporosis, and stroke. Long-term usage of estrogen medication has also been shown to benefit patients at high risk for osteoporotic fractures (Valdes & Bajaj, 2023).

Many diseases, including obesity, atherosclerosis, rheumatoid arthritis, and cancer, have been linked to inflammation, the body's quick biological response to

damaging stimuli. Leukocyte migration into wounded tissues is facilitated by the increased vascular permeability caused by inflammation. Tumor necrosis factor- α , interferon α , interleukins (IL), and chemokines are all considered inflammatory mediators (Sadeghalvad et al., 2019). Inflammation is typically treated with steroidal or non-steroidal anti-inflammatory drugs (NSAIDs). However, these medications might have unwanted side effects and are not a viable therapeutic choice for treating chronic inflammatory illnesses (Barnes, 2010). Among the many conditions that crude plant extracts treat in alternative medicine are acute and chronic inflammation. Recent studies have shown that the active components of these extracts display anti-inflammatory action both in vitro and in vivo in addition to anticancer, antibacterial, and antiviral activities (Tidke et al., 2018).

Isoflavones have been studied for their potential as anti-inflammatory drugs due to their ability to inhibit cytokine-induced signal transduction. Over the past several years, isoflavones have been shown to have anti-inflammatory effects in a growing body of research. The research showed that various isoflavones had varying impacts on inflammatory responses. Human epithelial colorectal adenocarcinoma cells treated with genistein showed decreased IFN-induced signal transducer and activator of transcription 1 (STAT1) phosphorylation (Paradkar et al., 2004). Jantaratnotai et al. (2013) came to a similar conclusion, stating that genistein and daidzein inhibited microglia activation by LPS. Reducing expression of monocyte chemoattractant protein-1 (MCP-1) and interleukin-6 (IL-6) was observed, and interferon regulatory factor-1 and phosphorylated STAT1 were implicated as mediators of these effects (Jantaratnotai et al., 2013). Several immune cells can produce inflammatory cytokines (IL-6, IL-8, TNF-, and IL-12) suppressed by equol and other isoflavone metabolites.

Against D-galactosamine-induced inflammation and hepatotoxicity, isoflavones have been tested in animal studies for their therapeutic potential (Ganai et al., 2015). After exposure to D-galactosamine, Isoflavones inhibited the production of proinflammatory cytokines like TNF- and IL-1 in male Wistar rats (Ganai et al., 2015). Thirty-two (32) healthy, non-obese postmenopausal women who were not on hormone replacement therapy were split into two groups: those who exercised and took a placebo and those who exercised and took 100 milligrams of isoflavone supplements (Giolo et al., 2018).

Lipid profile, IL-6, IL-8, SOD, CAT, TBARS levels, and other antioxidant and inflammatory markers were measured in blood samples. Isoflavones did not promote additive or independent effects on the lipid profile and inflammatory and oxidative stress markers in non-obese postmenopausal women. However, the study's participants consumed too few of the supplements. The study was too brief to draw any firm conclusions about the effects of isoflavone supplementation in conjunction with combined exercise (Giolo et al., 2018). Lebon et al. (2014) found more encouraging results when they tested the effects of exercise and isoflavones on obese and overweight women. Mixed-exercise training improved body composition and C-reactive protein in overweight or obese postmenopausal women, while TNF levels increased (Lebon et al., 2014).

The inflammatory markers C-reactive protein, IL-6, and TNF- were lowered in the soy group when given isoflavone-containing soy-based dietary supplements (soy group) or isoflavone-free milk protein (control group) for eight weeks. This suggests that isoflavones have anti-inflammatory properties in both in vitro and in vivo forms of inflammation. The primary way isoflavones exert anti-inflammatory activities is by reducing the production of inflammatory cytokines and chemokines (Blay et al.,

2010). Isoflavones inhibit proinflammatory cytokines and chemokines in vivo and in vitro (Lesinski et al., 2015) and in various animal models and people. Research evaluating the impact of soybean phytoestrogen-rich extracts on inflammatory markers requires more extensive data. At the same time, some studies did not find a significant effect of soybean phytoestrogen-rich extracts on inflammatory markers; this study aims to investigate the impact of phytoestrogen-rich soybean extracts against several markers of 4-vinyl cyclohexene diepoxide-induced menopausal inflammation in albino rats.

MATERIALS AND METHODS

This experimental study was conducted at the Faculty of Pharmacy laboratory and animal house, University of Benin, Benin City. It involved 65 female matured (aged 6-8 weeks) Wistar rats (*Rattus norvegicus*) weighing between 120 and 240g. Toxicity (n=20), preliminary (n=15), and animal model (n=30) studies of chemically induced menopause were conducted on the animals. Sexually matured female Wistar rats were used in the study, while those less than six weeks old and male Wistar rats were excluded. The animals were kept at the animal house at the University of Benin, Benin City, for two weeks to stabilize before the experiments began. The Wistar rats were given a conventional rodent cube diet from Ewu feeds and flour mills limited in Ewu, Edo state, Nigeria, and have free access to water ad libitum. Before each experiment, all animals were fasted overnight. Because of their ability to mimic the symptoms of perimenopause and postmenopause in humans—such as estrous acyclicity and fluctuating, then undetectable, estrogen levels—Wistar rats were chosen for this study to enable the separation of the impact of hormone levels from that of aging. The study aimed to investigate the impact of Soybean phytoestrogen-rich extract on some markers of inflammation of 4-vinyl cyclohexene diepoxide-induced menopause in albino rats.

The approval for the study was sought from the Animal Studies Ethics Review Committee of the Faculty of Pharmacy, Department of Pharmacology and Toxicology, University of Benin, Benin City. The approval was given after experimental protocols (where animals were housed and cared for under natural lighting conditions). Because some of the Laboratory investigations were done at the Federal University of Technology, Akura, a second Ethical approval and clearance was obtained from the ethics and research committee of the Federal University of Technology Akure, Ondo (protocol number FUTA/ETH/21/14). The sample size used in the study was sixty-five (65) adult female rats.

The soybeans were purchased from the Oba market in Benin and were authenticated by a plant taxonomist at the Department of Plant Biology and Biotechnology (PBB) laboratory, University of Benin, Benin City, and was given a voucher number (UBH-G628).

In preparing the flour, the soybean seeds were carefully picked, separated from debris, and rinsed in water. After washing, the grains were transferred to a large, clean bowl and left to soak overnight. The soybean chaff was washed off, drained to eliminate as much water as possible, and dried in the sun until completely dried. The grains were heated in a frying pan over medium heat and stirred until they turned brown, but careful not to allow them to burn. Once the beans were browned, the grains were removed immediately. The roasted soybeans were immediately ground into a fine powder in a Kitchen blender. A quick transfer from a hot frying pan into the blender ensures the seeds grind smoothly to powder. The powder was stored in an airtight container. The method of Cvejic et al. (2009) was used with

modification. Here, a known quantity of the Soybean flour was loaded into a thimble and placed in the Soxhlet extractor chamber (SER-148, Italy) until it was defatted using hexane in the Soxhlet extractor. After defatting, the powder was dried and re-extracted with methanol, using the Soxhlet extractor to obtain the methanol extract (phytoestrogen-rich extract). The extract was concentrated using a rotary evaporator (N-1300(8) ENYELA, NY, USA) and then dried thoroughly using a thermostatically controlled hot air oven (BST/HAO-1122 Bionics Scientific, India).

Animals were divided into six (6) groups of five (5) animals in each group and induced intraperitoneally with 80mg/kg of VCD obtained during the preliminary study. Group 1: 80 mg/kg of 4-vinylcyclohexene diepoxide + 200mg/kg phytoestrogen - rich extract. Group 2: 80 mg/kg of 4-vinylcyclohexene diepoxide + 400 mg/kg of phytoestrogen - rich extract. Group 3: 80 mg/kg of 4-vinylcyclohexene diepoxide + 600 mg/kg of phytoestrogen - rich extract. Group 4: 80 mg/kg of 4-vinylcyclohexene diepoxide + 14µg/100g estrogen of body weight. Group 5: 80 mg/kg of 4-vinylcyclohexene diepoxide (positive control). Group 6: Normal rats (negative control). The Soybean was administered daily at a single dose for 28 days- using oral gavage. The animals were observed closely for signs of toxic manifestation and toxicity, and none of the Wistar rats died after 28 days of treatment.

At the end of the 28-day treatment period, the animals were sacrificed under chloroform anesthesia; blood sample was collected directly from the abdominal aorta and the heart chamber with a needle mounted on a 10 mL syringe (Agary Pharmaceutical LTD, Nigeria) into lithium heparin anticoagulant and plane sample bottles. Biochemical analyses were performed on the serum sample obtained after centrifugation of whole blood at 2500 rpm for 10 min. The serum was kept frozen at -200 degrees Celsius until biochemical analysis was performed.

C-reactive protein (CRP), Interleukin 6 (IL-6), and Tumor Necrosis Factor Alpha (TNF-α) were determined by immunoenzymatic assay using ELISA technique (ELK biotechnology Monobind Inc. CA ELK8383, China) described by Eckersall et al., 1991, Santer *et al.*, 2010, Kaneko et al., 2008 respectively.

The data were statistically analyzed using SPSS Software (IBM) version 23.0. The various results obtained from this study were expressed as Mean ± Standard deviation (SD). The differences between the groups were determined by one-way ANOVA. The Tukey-Kramer Multiple Comparisons Test was used as the post hoc test to determine significant differences between Means. A P-value (≤ 0.05) was considered to be statistically significant, and a P-value (>0.05) was considered not statistically significant.

RESULTS AND DISCUSSION

The impact of soybean phytoestrogen-rich extract on markers of inflammation in 4-vinyl cyclohexane diepoxide-induced menopause in albino rats is displayed in Table 1. The inflammatory indicators were reduced after treatment with soybean phytoestrogen-rich extract (200mg/kg, 400mg/kg, and 600mg/kg) and estradiol (14ug/100g) in female rats given 80mg/kg 4-vinyl cyclohexane diepoxide (VCD). One-way analysis of variance followed by Tukey's post hoc test revealed that C-reactive protein ($F=17.96$, $p=0.001$), interleukin-6 ($F=7.45$, $p=0.001$), and tumour necrosis factor (TNF) all differed significantly from one another. However, TNF exhibited no significant difference ($F=1.93$, $p=0.126$). C-reactive protein levels varied significantly ($p<0.05$) across groups, while differences in actual CRP levels were consistent across groups 1, 2, 3, 4, and 6. The differences seen in the IL-6 group

were the same as those shown in groups 2, 3, 4, and 6, but there was no such difference in the TNF group.

Table 1. Levels of Inflammatory Markers in Female Wistar Rats Induced With 4-Vinylcyclohexane Diepoxide (VCD)

	Parameters		
	CRP (ug/ml)	TNF (pg/ml)	IL-6 (pg/ml)
Group 1	1116.64±73.00 ^a	10.86±3.30 ^a	82.42±3.88 ^b
Group 2	911.22±89.31 ^a	5.39±1.56 ^a	68.38±4.89 ^a
Group 3	640.74±145.06 ^a	2.64±0.55 ^a	45.71±6.96 ^a
Group 4	527.32±56.46 ^a	3.56±2.34 ^a	27.59±8.53 ^a
Group 5	2617.60±431.49 ^b	23.19±13.83 ^a	95.03±21.13 ^c
Group 6	373.04±43.80 ^a	1.27±0.37 ^a	30.96±4.29 ^b
F	17.96	1.93	7.45
ANOVA (P-value)	0.001	0.126	0.001

Values are expressed in mean ± SD. The value with different superscript showed difference from each other (p<0.05) while value with same superscript are not statistically difference from each other (p>0.05). Group 1: 80mg/kg VCD+200mg/kg Soybean, Group 2: 80mg/kg VCD+400mg/kg Soybean, Group 3: 80mg/kg VCD+600mg/kg Soybean, Group 4: 14ug/100g Estradol, Group 5: 80mg/kg VCD, Group 6: Control, CRP: C-reactive protein, TNF: Tumor necrosis factor, IL-6: Interleukin-6.

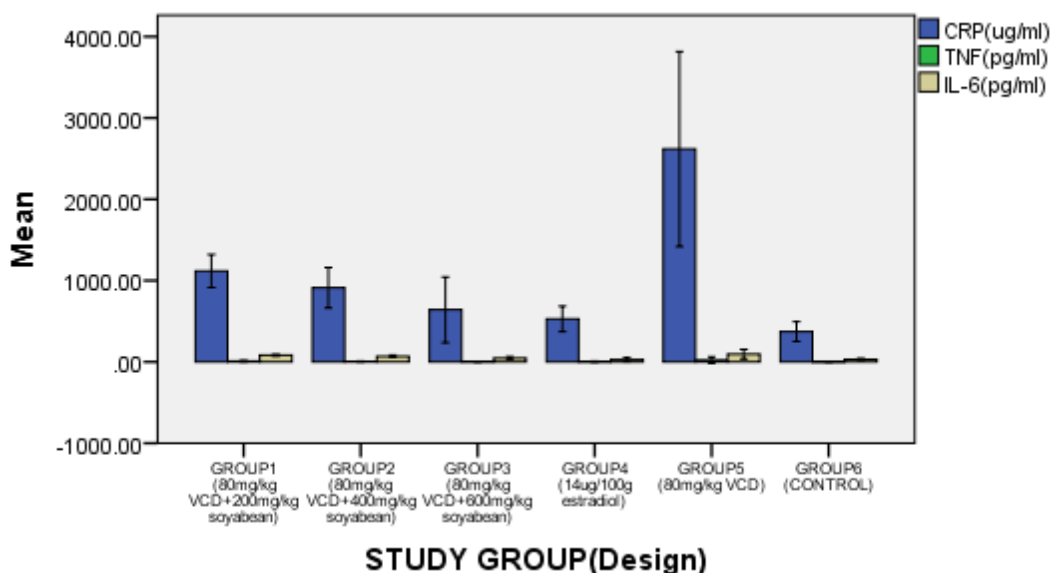


Figure 1. Showing the Impact of Soybean Phytoestrogen-Rich Extract on Markers of Inflammation Markers In 4-Vinyl Cyclohexane Diepoxide-Induced Menopause in Albino Rats

Postmenopausal women are at an increased risk for developing chronic diseases, highlighting the need for preventative care. Regular physical exercise training and/or the consumption of phytoestrogens may be essential strategies for minimizing or preventing the low-grade chronic inflammation and oxidative stress

that are common in postmenopausal women (Alvehus et al., 2012). This study aimed to determine the impact of soybean phytoestrogen-rich extract on markers of inflammation in 4-vinyl cyclohexane diepoxide-induced menopause in albino rats. Soybean phytoestrogen extract contains genistein, a compound structurally similar to 17 estradiol that can attach to estrogen receptors and compete with endogenous estrogens. The affinity of genistein for estrogen receptor (ER) is 87%, which is roughly 20-30 times higher than the affinity of ER- for 17 -estradiol (4%). Genistein's interactions with estrogen receptors activate a genetic mechanism called the estrogen receptor element (ERE) (Pilsakova et al., 2010; Park et al., 2012). Because of their structural similarity to estrogen, anthocyanins can bind to estrogen receptors and exert some estrogenic action, albeit at a much weaker level than endogenous estrogens (Chalopin et al., 2010).

In light of the preceding, it is essential to note that the soybean (*Glycine max*) compounds genistein and anthocyanins interact with estrogen receptors, which can then trigger the activation of the estrogen receptor element (ERE) for gene transcription. The aortic membrane expresses both ER- α and ER- β in male and female vascular tissues (Mendelsohn & Karas, 2005). According to research by Villablanca et al. (2013), the primary estrogen receptors ER- α and ER- β regulate estradiol production in the male aortic endothelial cell by converting testosterone to estradiol. Soybean extract (*Glycine max*) administration has been shown to increase estradiol levels in the blood of rats. Since the ovaries are the primary source of estradiol and their function has been suppressed by 4-vinyl cyclohexane diepoxide.

Many women experience distress due to menopause's associated symptoms, which might include hot flashes and mood swings (Krebs et al., 2004). In addition, it causes osteoporosis and a slowed metabolism, both raising the chance of developing different ailments. Postmenopausal women often have distressing vasomotor symptoms, such as hot flashes and perspiration (Guo et al., 2019). Estrogen is still recognized as the most effective treatment for these menopausal symptoms by the US Food and Drug Administration (FDA) (Hill et al., 2016). The usage of HRT has dropped since a 2002 research by the Women's Health Initiative (WHI) indicated that it raised the risk for breast cancer, stroke, and coronary heart disease in otherwise healthy postmenopausal women (Barber et al., 2004). Given the abovementioned risks, HRT is often given at the minimum effective dose for the shortest possible duration (De Franciscis et al., 2019). When treating menopausal symptoms, the Food and Drug Administration advises women first to try HRT's non-estrogen alternatives (De Franciscis et al., 2019). Since then, postmenopausal females and their medical care providers have sought out HRT substitutes that are both effective and safe (Guo et al., 2019). Nitric oxide (NO) is produced through the fermentation of isoflavone extract, although its use in relieving menopausal symptoms has only been the subject of a few clinical investigations. In addition to antioxidant and anti-inflammatory effects, the contribution of NO was anticipated to optimize the effectiveness of isoflavones.

Table 1 results showed that when compared to the menopause-induced rat (positive control), the levels of CRP and pro-inflammatory cytokines IL-6 were significantly lower ($P < 0.05$) except for TNF- α ($p > 0.05$). Significant reductions in CRP levels were seen after administration of soybean milk at either of three doses (200mg/kg, 400mg/kg, or 600mg/kg) in combination with estradiol at a dose of 14ug/kg. The present study found a decrease in CRP and interleukin, consistent with prior research (Tousoulis et al., 2012; Kao et al., 2007). Vascular dilation and an anti-inflammatory effect were achieved by a decrease in pro-inflammatory cytokines

(TNF-) IL-6 and a down-regulation of cytokine-induced signal transduction events in immune cell membranes (Verdrergh, 2003). The primary way isoflavones exert anti-inflammatory activities is by reducing the production of inflammatory cytokines and chemokines. Macrophages release the majority of pro-inflammatory cytokines. Transcriptomic profiling has indicated that pretreatment with isoflavones reduces the overproduction of nitric oxide (NO) and prostaglandin E2 (PGE2) in RAW 264.7 macrophages in response to LPS plus IFN- (Blay et al., 2010). Isoflavone has significantly inhibited LPS-induced IL-6 and TNF- overproduction in RAW 264.7 macrophages (Ji et al., 2012). Soybean was studied for its impact on inflammation and vascular endothelial function in a randomized crossover clinical trial involving women with metabolic syndrome. The effects of the DASH, a soy protein, and a soy nut diet on metabolic syndrome in 42 postmenopausal women were studied over eight weeks. C-reactive protein (CRP) expression, a marker of inflammation, was also analyzed. C-reactive protein levels were lower in the soy nut and soy protein groups compared to the control diet group by 8.9% ($p < 0.01$) and 1.6% ($p < 0.01$), respectively (Azadbakht et al., 2007). Previous research has demonstrated that nitric oxide (NO) can boost vascular health by preventing atherosclerosis, clot formation and increasing blood flow (Tousoulis et al., 2012).

This study shows that when compared to the treatment group with phytoestrogen-rich extract, menopause-induced female Wistar rats had significantly dose-dependent lower levels in C-reactive protein (CRP), interleukin-6 (IL-6), except tumour necrosis factor-alpha (Figure 1) his demonstrated that pituitary and gonadal hormone levels could be altered due to 4-vinyl cyclohexane diepoxide (4-VCD) acting as an endocrine disruptor and inflammatory agent. In animal models, administration of phytoestrogen-rich extract at varying concentrations exhibited impressive anti-inflammatory properties, including decreased pro-inflammatory enzyme activities and cytokine levels. This study can reawaken interest in natural diets and refresh some of the public's understanding of the role of isoflavones in the inflammatory process. The biochemical and metabolic panel of 4-vinyl cyclohexane dioxide (4-VCD)-induced rats improved after four weeks of treatment. Treatment with phytoestrogen isoflavones in menopause-induced female Wistar rats resulted in beneficial effects on inflammation. Compared to the gold standard of estrogen therapy, it appears that high-dose phytoestrogen-rich extract therapy is more effective than hormone replacement therapy. The limitations of this study are its small sample size and research lack of funding from any source; hence, it could not cover all interleukins.

CONCLUSION

This study shows that when compared to the treatment group with phytoestrogen-rich extract, menopause-induced female Wistar rats had significantly higher levels of liver enzymes, C-reactive protein (CRP), interleukin-6 (IL-6), and tumour necrosis factor-alpha. This demonstrated that pituitary and gonadal hormone levels could be altered due to 4-vinyl cyclohexane diepoxide (4-VCD) acting as an endocrine disruptor and inflammatory agent. In animal models, administration of phytoestrogen-rich extract at varying concentrations exhibited impressive anti-inflammatory properties and decreased pro-inflammatory enzyme activities and cytokine levels. Compared to the gold standard of estrogen therapy, it appears that high-dose phytoestrogen-rich extract therapy is more effective than hormone replacement therapy.

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CONFLICT OF INTEREST

There are no stated conflicts of interest by the authors.

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