Original Article

Antioxidant effect of isoflavones: A randomized, double-blind, placebo controlled study in oophorectomized women

Rakesh Mittal, Niti Mittal, Debasish Hota[1], Vanita Suri[2], Neelam Aggarwal[2], Amitava Chakrabarti[1]

Department of Pharmacology, Pt B D Sharma Postgraduate Institute of Medical Sciences, Rohtak, Haryana, [1]Departments of Pharmacology, and [2]Obstetrics and Gynaecology, Postgraduate Institute of Medical Education and Research, Chandigarh, India

Abstract

Background: One of the postulated mechanism for cardioprotective potential of isoflavones is their ability to exert antioxidant action. However, various reports give conflicting results in this area. **Aim:** The present study was conducted with an objective to probe into the cardioprotective mechanism of isoflavones by evaluating their antioxidant potential in oophorectomized women. **Materials and Methods:** This was a randomized, double-blind, parallel, placebo controlled study. A total of 43 women were randomized to receive 75 mg/day isoflavones tablet or placebo for 12 weeks. Red blood cell antioxidant parameters including lipid peroxidation, superoxide dismutase (SOD), catalase and glutathione peroxidase (GSH-Px) were determined at baseline and at the end of the study. **Results:** After 12 weeks of administration of isoflavones, there was no statistically significant difference in lipid peroxidation (*P* value for isoflavones: 0.37; for placebo: 0.37), catalase (*P* value for isoflavones: 0.35; for placebo: 0.84), SOD (*P* value for isoflavones: 0.41; for placebo: 0.28) and GSH-Px (*P* value for isoflavones: 0.92; for placebo: 0.29). There was no statistically significant difference in the proportion of patients experiencing adverse events in the two groups (*P* -1.00). **Conclusion:** The study strengthens the concept that the cardioprotective mechanism of isoflavones might be due to some other reason apart from the antioxidant pathway.

Key words: Antioxidant, cardioprotection, isoflavones, oophorectomized women

Introduction

Phytoestrogens are a group of naturally occurring plant compounds with demonstrated estrogenic and/or anti-estrogenic activity and hold great promise for development as botanical dietary supplements for postmenopausal women. There are three main classes of phytoestrogens: isoflavones, coumestans and lignans. The major dietary sources of these include: Legumes and soybean products (isoflavones); whole grain cereals, fruits, seeds and alcoholic sources (lignans); bean sprouts and fodder crops (coumestans). Isoflavones make up the most common form of phytoestrogens.[1]

Phytoestrogens can act as idealized selective estrogen response modifiers with the desirable properties of being anti-estrogenic in breast and uterine tissue, but being pro-estrogenic in bone and brain and in lipid metabolism.[2] A number of cardioprotective benefits have been attributed to dietary isoflavones including a beneficial effect on lipid profile; inhibition of platelet aggregation and an improvement in vascular reactivity; antioxidant action and inhibition of pro-inflammatory cytokines, cell adhesion proteins and inducible nitric oxide production.[3]

In an *in vitro* study, Rüfer and Kulling demonstrated the antioxidant efficacy of isoflavones at concentrations within the range found in the plasma of subjects consuming soy products.[4] Genistein has been shown to inhibit hydrogen peroxide production and increase the activity of antioxidant enzymes, such as catalase, superoxide dismutase (SOD),

Address for correspondence: Dr. Rakesh Mittal,
Department of Pharmacology,
Pt B D Sharma Postgradaute Institute of Medical Sciences,
Rohtak, Haryana, India.
E-mail: rakesh7878@yahoo.co.in

Access this article online

Quick Response Code:

Website: www.ijabmr.org

DOI: 10.4103/2229-516X.125686

glutathione peroxidase (GSH-Px) and glutathione reductase. Furthermore, genistein and to a lesser degree daidzein, can inhibit superoxide anion generation by xanthine/xanthine oxidase.[5] low-density lipoprotein (LDL) exposed to flavonoids demonstrated reduced oxidizability and flavonoids suppressed the formation of lipid peroxides and thiobarbituric acid reactive substances (TBARS).[6]

In an *in vivo* study conducted in male Sprague-Dawley rats, Anderson *et al*. studied the effects of a low isoflavone soy protein isolate and a genistein-rich soy protein isolate. High-genistein diet profoundly decreased all parameters of lipoprotein oxidation.[7] In a study conducted in healthy humans, high flavonoid (HF) diet did not significantly inhibited lipid peroxidation as compared to low flavonoid (LF) diet.[8] Hsu *et al.*, assessed the effect of soy isoflavones on erythrocyte antioxidant enzyme activities in postmenopausal women and concluded that isoflavone supplementation did not appear to have antioxidant activities.[9]

A review of the literature revealed the above-mentioned conflicting reports about the antioxidant potential of isoflavones. Moreover, there is a lack of such data in postmenopausal women, the group which is most commonly prescribed with isoflavones for the relief of menopausal symptoms. Hence, the present study was planned to evaluate the antioxidant effect of isoflavones in oophorectomized menopausal women.

Materials and Methods

Subjects

All the women who reported to the obstetrics and gynecology out-patient department of Post Graduate Institute of Medical Education and Research, Chandigarh, India were screened for inclusion in the study. They were included in the study if they had undergone bilateral oophorectomy, were less than 55 years of age and were willing to comply with the protocol. They were excluded if any of the following criteria were present: (i) Already on isoflavones (ii) taken hormone replacement therapy/estrogen replacement therapy within previous 8 weeks (iii) presence of renal and/or hepatic disease (iv) active major psychiatric disorders (v) history of thrombophlebitis or thromboembolism or cerebrovascular disorders (vi) uncontrolled hypertension with blood pressure > 180/100 mmHg (vii) uncontrolled diabetes (viii) presence of active infection or malignancy (ix) present or past history of soya or nut related food allergies.

The study protocol was approved by the Institutional Ethics Committee and all the women participating in the study signed a written informed consent prior to enrolment. The study was conducted in accordance with ICMR ethical guidelines for biomedical research on human subjects. Eligible women were randomized and hence that they had an equal probability of assignment to either of the two groups. The randomization code was developed using a random number table to select random permuted blocks of length four. The identity of the drugs was hidden by packing in numbered opaque envelopes to ensure concealment of the sequence until assigned. Randomization, allocation sequence generation and packing of envelopes was done by investigators not directly involved in dispensing and evaluating the treatments so that it was double-blind both the patient and evaluating clinician not aware of the assigned treatment. The participants remained on the same allocation throughout the study period if they continued. The randomization code was revealed to the investigators once recruitment, data collection and laboratory analyses were complete. The participants attended the clinic at the time of randomization (baseline) and at the interval of every 3 week for 12 weeks.

Study interventions

The participants were randomized to either placebo or study drug to be administered once a day orally for 12 weeks. The study drug and matching placebo tablet was obtained as a free gift sample from Dr. Reddy's Laboratory, Hyderabad (there was no conflict of interest), which contained 75 mg of soy isoflavones extract standardized to provide isoflavones 40% genistein and genistin 25%; daidzein and daidzin 15%. Placebo tablet matching in color, size, shape and the taste was used as control treatment. Patients were advised to take one tablet daily at bedtime with 150 ml of plain water for a total 12-week period. All the patients were instructed to stop the dietary isoflavones consumption by avoiding soy, seeds and sprouts, beans and legumes during the study period. However, concomitant treatments for other illnesses were allowed. Patients were asked to bring the drug envelopes during subsequent visits and compliance was assessed by pill counting. More than 80% of compliance was considered as an adequate.

Sample collection

Fasting blood sample was taken for antioxidant parameters before the start of study medications and at the end of 3-month study period. A total volume of 4 mL blood was collected in ethylenediaminetetraacetic acid (EDTA) vacutainer and the sample kept in icebox and was later processed for separating out red blood cells (RBCs) and plasma.

RBC preparation

Freshly drawn venous blood was centrifuged at 2000 g for 10 min at 4°C to separate plasma and erythrocytes. Plasma

was immediately stored at −80°C. Erythrocytes were washed thrice with 0.9% NaCl solution containing 0.5% of pyrogallol as an antioxidant agent. The final hematocrit suspension was made up to 50% (approximately 5 × 10^6 erythrocytes/ml) with the addition of distilled water containing 2 μmol/L of butylated hydroxytoluene and these aliquots of RBC suspensions were coded and stored at −80°C until used for analysis of antioxidant enzyme activities and lipid peroxidation.

On the day of analysis, the sample was thawed and diluted 5:1 (by volume) with distilled water and further 2:1 by a phosphate buffer containing dithiothreitol (DTT) (100 mmol/L KH_2PO_4, 1 mmol/L ethylenediaminetetraacetic acid (EDTA) and 2 mmol/L DTT, pH 7.4) and the final dilution of 1:20 was made. These hemolysates were used for all analyses. All the analyses were performed in a period of 2 days; TBARS and SOD were done on 1st day while GSH-Px and catalase were done on 2nd day using spectrophotometer. All the chemicals and reagents used in the study (manufacturer: Sigma-Aldrich) and were of analytical reagent grade.

Measurement of RBC antioxidant parameters

Lipid peroxidation

Lipid peroxidation was estimated by a previously described method with modification.[10] Briefly, the reaction mixture contained 200 μL of sample and 800 μL of phosphate buffer saline (pH = 7.4). After incubating the samples at 37°C for 90 min, 2 ml of 8.1% sodium dodecyl sulfate was added followed with 1.5 ml of 20% acetic acid (pH 3.5). The amount of malondialdehyde (MDA) formed during incubation was estimated by adding 1.5 ml of 0.8% thiobarbituric acid and heating the samples at 95°C for 45 min. After cooling, the samples were centrifuged and the TBARS were measured in supernatants at 532 nm by recording optical density (OD).

The formula used was

$$\text{Lactoperoxidas eactivity} = \frac{\text{OD} \times \text{reaction volume}}{\text{E} \times \text{Hb concentration}}$$

where E is extinction coefficient = 1.53 × 10^5/M/cm.

SOD

SOD was estimated by the method of Kono.[11] This method is based on the principle of the inhibitory effect of SOD on reduction of nitroblue tetrazolium (NBT) dye by superoxide anions, which are generated by photooxidation of hydroxylamine hydrochloride ($NH_2OH.HCl$).

Reagents

- Solution A: 50 mM sodium carbonate solution, pH 10.0, containing 0.1 mM EDTA
- Solution B: 96 μM NBT in solution A
- Solution C: 0.6% triton-X-100 in solution A
- Solution D: 20 mM hydroxylamine hydrochloride in double distilled water, pH adjusted to 6.0 by 0.1 N NaOH.

Assay

To the test cuvette, 1 ml of solution A, 0.5 ml of solution B and 0.1 ml of solution C were added. The contents were incubated at 37°C for 10 min. Reaction was initiated by the addition of 0.1 ml of solution D to the reaction mixture in test cuvette and the rate of NBT reduction in the absence of enzyme source was recorded. Following this, 10 μl of the sample was added to the test cuvette. Percentage inhibition in the rate of NBT reduction was noted at 560 nm. One unit of enzyme was expressed as inverse of the amount of hemoglobin (g) required to inhibit the reduction rate of NBT by 50%. Sample volume was adjusted accordingly.

SOD = 1/hemoglobin (g) causing 50% reduction.

Catalase

The activity of catalase was measured by a previous described method.[12] Briefly, the reaction mixture contained tris (50 mM) EDTA (5 mM) buffer, pH 7.0, 30 mM H2O2 (in 0.1 M KH_2PO_4 buffer, pH 7.4) in test cuvette. The reference cuvette contained tris-EDTA solution and distilled water only. The contents of both the cuvettes were incubated at 37°C for 10 min. the reaction was initiated by the addition of 10 μl sample (diluted) to the test cuvette. The rate of elimination of hydrogen peroxide by catalase was measured by recording the rate of change of absorbance/min at 240 nm for 3 min. The formula used was

$$\text{Catalase activity} = \frac{\Delta\,\text{OD/min} \times \text{total reaction volume} \times \text{dilution factor}}{\text{E} \times \text{volume of sample/assay} \times \text{Hb concentration}}$$

where E is extinction coefficient = 43.6/M/cm.

GSH-Px

GSH-Px was estimated by the method of Paglia and Valentine.[13] Briefly, the reaction mixture in the test cuvette contained 50 mM phosphate buffer, pH 7.4, 0.1 mM EDTA, 0.37 M sodium azide, 0.1 M GSH, 100 μl per assay glutathione reductase and 2 mM nicotinamide adenine dinucleotide phosphate (NADPH) in 0.1% sodium bicarbonate. The contents were incubated at 37°C for 10 min. the reaction was started by adding 35 μl of 2.2 mM H2O2 in the cuvette. The enzyme activity was expressed as amount of NADPH oxidized to NADP + by using the extinction coefficient of 6.22/mM/cm at 340 nm.

The formula used was:

$$\text{GSH-Px activity} = \frac{\Delta\ \text{OD/min} \times \text{total reaction volume} \times \text{dilution factor}}{E \times \text{volume of sample/assay} \times \text{Hb concentration}}$$

where E is extinction coefficient = 6.22/M/cm.

Statistical analysis

Data was expressed as mean ± standard deviation unless specified and numbers (percentages). Proportions were compared using Chi-square tests with continuity correction or Fisher's exact test when appropriate. The within groups and between group comparisons were carried out using paired *t* test and unpaired Student's *t*-test, respectively. Two sided significance tests were used throughout. $P < 0.05$ was considered to be significant. All the statistical analyses were performed using SPSS statistical software version 16.

Results

A total of 43 oophorectomized women underwent randomization, with 21 and 22 assigned to the isoflavone and placebo groups respectively [Figure 1]. The two groups were comparable with respect to the demographic profile [Table 1]. At baseline, there was no difference in RBC antioxidant parameters between two study groups [Table 2].

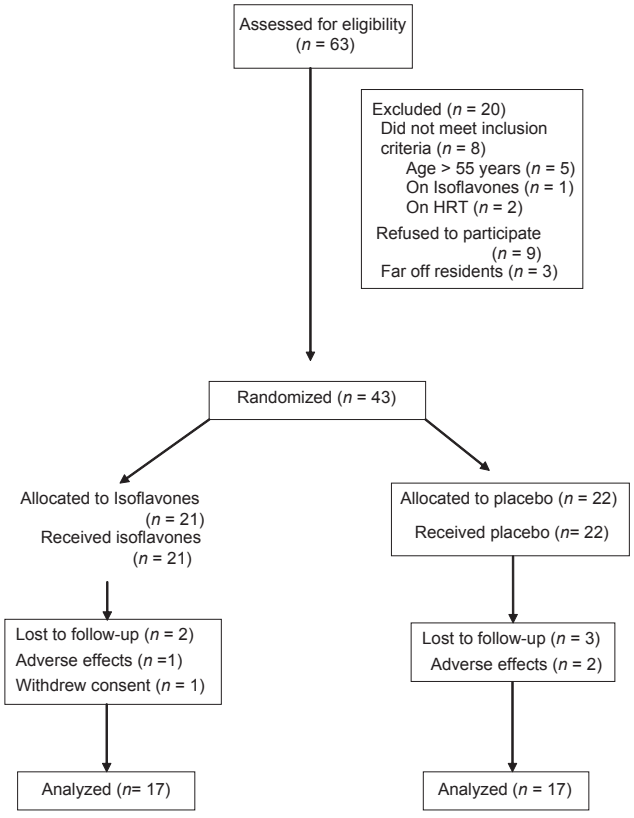

Figure 1: Consort flowchart

The alteration in antioxidant parameters at 12 weeks compared with baseline was not significant in either group [Figure 2].

One patient out of 21 enrolled (2%) in the isoflavone group discontinued treatment after 4 weeks because of

Table 1: Demographic profile and clinical characteristics of patients in isoflavone and placebo groups at baseline

Characteristic	Isoflavones (*n*=21)	Placebo (*n*=22)	*P* value
Age in years (mean±SD)	49.14±6.83	46.28±6.14	0.15
BMI in kg/m^2 (mean±SD)	24.99±4.58	24.94±4.03	0.97
Time since oophorectomy, in months; median (IQR)	6 (1.5-33)	4.5 (1-21.75)	0.44
Past HRT use, *n* (%)	3 (14.3)	1 (4.5)	0.34
Coexisting illness, *n* (%)			
Hypertension	9 (42.8)	9 (40.9)	0.90
Type 2 diabetes	3 (14.3)	2 (9.1)	0.99
Paraumbilical hernia	0	1 (4.5)	0.99
Joint disease	0	1 (4.5)	0.99
Others	0	2 (9.1)	0.49
Past history, *n* (%)			
Gallstones	1 (4.7)	1 (4.5)	0.99
Renal stones	1 (4.7)	0	0.49

SD: Standard deviation; IQR: Interquartile range; BMI: Body mass index; HRT: Hormone replacement therapy

Table 2: Baseline comparison for RBC antioxidant enzyme (mean±SD)

Parameter	Isoflavones (*n*=21)	Placebo (*n*=22)	*P* value
Lipid peroxidation#	330.46±63.84	347.13±95.91	0.71
Catalase*	48.86±23.56	58.61±18.91	0.41
Superoxide dismutase*	336.61±90.9	337.79±68.02	0.98
Glutathione peroxidase*	345.67±80.56	368.73±103.56	0.65

#LPO measured as activity in TBARS units/g Hb; *Catalase, SOD, GSH-Px measured as units/g Hb (units described as M/cm/min); RBC: Red Blood cell; SD: Standard deviation; LPO: Lipid peroxides; TBARS: Thiobarbituric acid-reactive substances; Hb: Hemoglobin; SOD: Superoxide dismutase; GSH-Px: Glutathione peroxidase

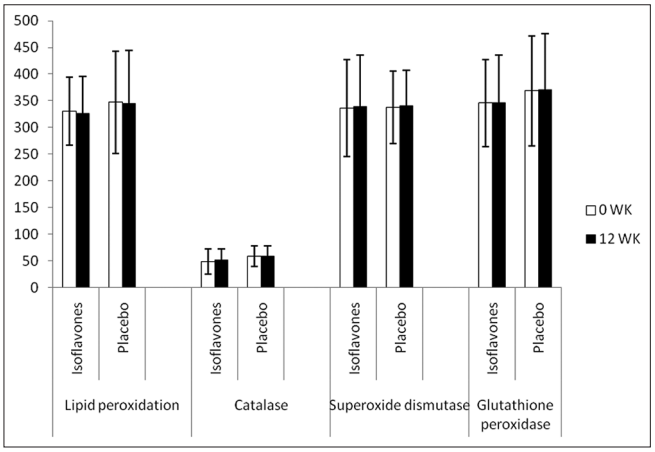

Figure 2: Red blood cells antioxidant parameters at 12 weeks compared to baseline. #Lipid peroxidation measured as activity in thiobarbituric acid reactive substances units/g hemoglobin. *Catalase, superoxide dismutase, glutathione peroxidase measured as units/g Hb (units described as M/cm/min)

disturbed sleep, anxiety and restlessness. In the placebo group, two patients (5%) experienced adverse effects in the form of generalized body aches and increased fatigability and they discontinued treatment at 2 weeks and 2 days respectively (P = 1.00).

Discussion

Isoflavones have been implicated to be cardioprotective by virtue of several direct and indirect vascular effects. One of the postulated mechanisms is its antioxidant effect. In this study, isoflavones (75 mg/day) were evaluated for the presence of antioxidant activity by evaluating erythrocyte antioxidant enzyme activities including catalase, SOD, GSH-Px and lipid peroxidation in surgical menopausal women. However, none of the enzyme activities was altered to a significant level with isoflavones compared with placebo group, demonstrating a lack of antioxidant potential of isoflavones under the study conditions.

Similar findings have been observed in the study by Hsu *et al*.,[9] in which the effect of soy isoflavones on erythrocyte antioxidant enzyme activities was assessed in postmenopausal women. A total of 37 postmenopausal women were included in this 6 month trial and were given 150 mg/day of isoflavone supplements twice daily. At 3 and 6 month measures of erythrocyte antioxidant enzyme activities did not differ significantly from baseline, thereby concluding that isoflavone supplementation did not appear to have antioxidant activities. Reilly *et al*.,[8] investigated the effects of consuming a HF diet enriched with onions and black tea on indexes of oxidative damage *in vivo* compared with a LF diet in 32 healthy humans in a randomized crossover design. Indexes of oxidative damage used were plasma F2-isoprostanes (a biomarker of lipid peroxidation *in vivo*) and the titer of antibodies to MDA-modified LDL. There were no significant differences in the intake of macronutrients or assessed micronutrients, plasma F2-isoprostane concentrations and MDA-LDL autoantibody titer between the HF and LF dietary treatments. It was concluded that flavonoid consumption in onions and tea had no significant effect on plasma F2-isoprostane concentrations and MDA-LDL autoantibody titer in this study and thus does not seem to inhibit lipid peroxidation in humans.

The results of the present study contradict the finding of antioxidant effect in rats.[7] However, this can be attributed to species variation. The above study strengthens the concept that the cardioprotective mechanism of isoflavones is due to some other reason apart from antioxidant pathway.

However, a number of *in vitro*[4,5,14] and rodent studies[7] have demonstrated the potential of isoflavones to exert antioxidant action. The postulated mechanisms for such action include inhibition of hydrogen peroxide production and increase the activity of antioxidant enzymes, such as catalase, SOD, GSH-Px and glutathione reductase.[5]

Few limitations in the present study can be the lack of pharmacokinetic data for isoflavones, which would have provided an opportunity to ascertain the compliance and bioavailability and understand the kinetic–dynamic correlation for the agents. However, the concentration of their metabolites differ widely among individuals and species even after administration of controlled quantity of food supplements due to gut microflora, antibiotic use, bowel disease, gender difference and the concomitant dietary intake.[1,15]

Secondly, it was a fixed dose study for a limited duration, whereas the postmenopausal women consume soy products over prolonged periods of time and the use of active comparator in the form of vitamin E, a reference antioxidant used widely would have been more justifiable.

Conclusion

In this study conducted on surgically induced menopausal women, the effect of 75 mg/day isoflavones on RBC antioxidant parameters was assessed and no significant antioxidant activity was found at the end of 3-month study period when compared with the placebo group. However, future studies for longer duration can be planned with different doses of isoflavones, using active comparator group and including objective pharmacokinetic measurements to provide a conclusive proof regarding the use of isoflavones in clinical practice.

References

1. Murkies AL, Wilcox G, Davis SR. Clinical review 92: Phytoestrogens. J Clin Endocrinol Metab 1998;83:297-303.
2. Tham DM, Gardner CD, Haskell WL. Clinical review 97: Potential health benefits of dietary phytoestrogens: A review of the clinical, epidemiological, and mechanistic evidence. J Clin Endocrinol Metab 1998;83:2223-35.
3. Rimbach G, Boesch-Saadatmandi C, Frank J, Fuchs D, Wenzel U, Daniel H, *et al*. Dietary isoflavones in the prevention of cardiovascular disease: A molecular perspective. Food Chem Toxicol 2008;46:1308-19.
4. Rüfer CE, Kulling SE. Antioxidant activity of isoflavones and their major metabolites using different *in vitro* assays. J Agric Food Chem 2006;54:2926-31.
5. Wei H, Bowen R, Cai Q, Barnes S, Wang Y. Antioxidant and antipromotional effects of the soybean isoflavone genistein. Proc Soc Exp Biol Med 1995;208:124-30.
6. Safari MR, Sheikh N. Effects of flavonoids on the susceptibility of low-density lipoprotein to oxidative modification. Prostaglandins Leukot Essent Fatty Acids 2003;69:73-7.
7. Anderson JW, Diwadkar VA, Bridges SR. Selective effects of different antioxidants on oxidation of lipoproteins from rats. Proc Soc Exp Biol Med 1998;218:376-81.

8. O'Reilly JD, Mallet AI, McAnlis GT, Young IS, Halliwell B, Sanders TA, *et al*. Consumption of flavonoids in onions and black tea: Lack of effect on F2-isoprostanes and autoantibodies to oxidized LDL in healthy humans. Am J Clin Nutr 2001;73:1040-4.
9. Hsu CS, Shen WW, Hsueh YM, Yeh SL. Soy isoflavone supplementation in postmenopausal women. Effects on plasma lipids, antioxidant enzyme activities and bone density. J Reprod Med 2001;46:221-6.
10. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979;95:351-8.
11. Kono Y. Generation of superoxide radical during auto-oxidation of hydroxylamine and assay for superoxide dismutase. Arch Biochem Biophys 1978;186:189-95.
12. Luck H. Catalase. In: Bergmeyer HW, editor. Sec. 3. Methods of Enzymatic Analysis. New York: Academic Press; 1963. p. 885-94.
13. Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med 1967;70:158-69.
14. Ruiz-Larrea MB, Mohan AR, Paganga G, Miller NJ, Bolwell GP, Rice-Evans CA. Antioxidant activity of phytoestrogenic isoflavones. Free Radic Res 1997;26:63-70.
15. Adlercreutz H, Höckerstedt K, Bannwart C, Bloigu S, Hämäläinen E, Fotsis T, *et al*. Effect of dietary components, including lignans and phytoestrogens, on enterohepatic circulation and liver metabolism of estrogens and on sex hormone binding globulin (SHBG). J Steroid Biochem 1987;27:1135-44.

How to cite this article: Mittal R, Mittal N, Hota D, Suri V, Aggarwal N, Chakrabarti A. Antioxidant effect of isoflavones: A randomized, double-blind, placebo controlled study in oophorectomized women. Int J App Basic Med Res 2014;4:28-33.

Source of Support: Nill. **Conflict of Interest:** None declared.