Modulatory Effect of Paste of Dried seeds of *Prunus amygdalus* on Ovotoxicity Induced Postmenopausal Complications in Rat

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ABSTRACT-Menopause is a natural process in women's reproductive life at the age of 40 to 50 years and leads to deficiency of estrogen causing various complications in women's health. *Prunus amygdalus* nuts are abundant source of various phytoestrogens and produces effect similar to that of estrogen and can be used in menopausal women in place of HRT.

In the present study ovotoxicity and menopause was induced in female rats by 4-vinyl cyclohexane diepoxide (165 mg/kg, i.p. five times a week for 15 days). After induction menopause the animals were treated with the paste of dried seeds of *Prunus amygdalus* at the doses of 100, 200 and 400 mg/kg, p.o. for 45 days. Blood estrogen level was checked before and after administration of VCD to confirm induction of menopause. After the treatment period of 45 days blood was collected from the animals, centrifuged and serum was separated and used for the estimation of estrogen, cholesterol, triglyceride, HDL, LDL and VLDL. Ovaries were isolated from the treated animals, kept in ice cold tris buffer, homogenized and centrifuged. The supernatant was used for the estimation of malondialdehyde (MDA) and antioxidant enzyme (superoxide dismutase and glutathione peroxidase). Results indicate that administration of seeds of *Prunus amygdalus* at different dose levels do not have any effect on serum estrogen level but significantly improved lipid profile and decreased atherogenic index in postmenopausal rats. A significant decrease in the MDA and increase in antioxidant enzymes level was observed in postmenopausal rats after treatment with paste of dried seeds of *Prunus amygdalus*. Results conclude that administration of seeds of *Prunus amygdalus* for a long period of time may improve the cardiovascular complications of women's health after menopause.

Keywords: Estrogen, Menopause, Phytoestrogen, *Prunus amygdalus*, 4-vinyl cyclohexane diepoxide.

Introduction

Menopause is defined as 1 year without menses and characterised by the failure of ovaries to produce estrogen. This failure often begins in the late 30s, and most women experience near-complete loss of production of estrogen by their mid-50s. The transition from normal ovarian function to ovarian failure is described as the menopausal transition. Menopause can occur at different times (Mirghafourvand M.*et al*.,2014). Normally most women receive menopause status at age 45 and 55. At the age of 51, permanent amenorrhea lead to decline level of estrogen in women(Ghazanfarpour M.*et al*.,2015).The decreased level of estrogen in women shows symptomatic changes, such as sleep disorders, night sweats, hot flashes, mood changes, sexual complaints, urogenital symptoms and dementia (Henderson, V.W 2008).The dropping level of estrogen and progesterone also affects the brain and skin tissue which causes psychological disturbances(Elavsky E and Mc Auley, 2009).

During the end of reproductive age, the majority of women generally uses estrogen based therapy to

keep down the physiological changes in body. Hormone Replacement Therapy is used as primary treatment for relieving the symptoms of menopausal related disorders, such as osteoporosis and coronary heart disease (Holloway D, 2011). The regular intake of estrogen and progesterone lead to increase risk of breast cancer, ovarian disease, venous thromboembolism, pulmonary embolism, stroke, heart disease, endometrium cancer and vascular dementia (Cauley JA.*et al*.,2001). Studies from literature indicates the use of plant based oestrogens also known as phytoestrogens are useful to treat the menopausal complications without adverse effects. Phytoestrogens are non-steroidal, polyphenolic plant derived metabolite that mimic or modulate actions of estrogen, having structure similar to 17β-estradiol (Whitten PL.*et al*.,1997). Phytoestrogen act through binding to estrogenic receptors (ER)-alpha and beta.They also show some antioxidant property.Phytoestrogens are considered natural therapeutic alternative to synthetic hormonal therapies, which are not associated with chronic indications and adverse effects. According to literature survey it is known that the fruit of*Prunus amygdalus*(almond) family *Rosaceae*. Almonds contains high number of phytoestrogens which are beneficial for human health.It contains large amount of phytoestrogens such as flavonols, flavanones, phenolic acids, anthocyanins(Spiller GA. e*tal*., 2012). It also contains large amount of vitamins such as vitamin E, folic acid, vitamin B6, proteins, etc which are responsible for its beneficial effects on health (Frison-Norrie S. *et al*., 2002). Traditionally almonds have a consistent LDL cholesterol lowering effect in healthy individuals and in individuals with high cholesterol and diabetes. It also possess a memory enhancing activity.Vitamin E present in almonds is known to be potent antioxidant properties and free radicals may contribute to the pathological processes of cognitive impairment. Literature survey showed that almonds improved the immune response due to production high level of cytokines such as INF- α, INF- γ, IL-12 and TNF- α etc. (Adriana Arena, *et al,*2010). One of the other almonds health benefits is that it helps in maintaining a proper blood pressure and indirectly helps in prevention of diseases caused due to high blood pressure **(**Phillips KM. *et.al*., 2012).

Materials and Methods

Reagents

Estradiol and 4-vinyl cyclohexene diepoxide were purchased from Sigma Aldrich. Other chemicals were obtained from local sources and were of analytical grade. Diagnostic kits were used for the estimation of all biochemical parameters.

Animals

Female Wistar rats weighing between 150-200g and 6-7 weeks of age were used for study. The rats were housed in standard cages and were allowed to have free access to food and water ad libitum under controlled room temperature (24±2°C) in a 12-h light-dark cycle. The guidelines of the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA) of the Government of India were followed and prior permission was taken from the Institutional Animal Ethical Committee(273/PO/Re/S/2000/CPCSEA/002/2015-2017) for conducting the animal experimental studies.

Induction of ovotoxicity and Menopause

Ovotoxicity was induced in mice by the Accelerated Ovarian Failure (OVF) method. An ovotoxic chemical, 4-vinylcyclohexene diepoxide (VCD) was injected at a dose of 160 mg/kg, i.p. five times per week for 15 days in female mice. The blood estrogen level is checked in normal female mice before injecting 4-vinylcyclohexene diepoxide (VCD) and after 15 days of injecting the chemical in order to confirm the induction of menopause (Hoyer PB., *et.al.*2001).

Preparation of test sample and dose selection

The nuts of *Prunus amygdalus*were purchased from the local market and authenticated at the Botany Department of forest Research Institute, Dehradun, India. The fine paste of the PA nuts was prepared in distilled water and sonicated for 20 min to obtain a fine suspension.

Then, the paste was administered orally to the mice at three doses of 100, 200 and 400 mg/kg/day. The above dose levels were selected by the conversion of conventional human dose into animal dose. The human dose of PA was five to six nuts daily (approximately 6 g).

Treatment protocol

The ovotoxic animals were divided into six groups each group having six animals and one group of normal animal was taken into the study. The first group of ovotoxic animals received normal saline (1 ml/kg,p.o.). The ovotoxic animals received the following treatments for a period of 45 days.

Group 1: Normal animals received normal saline (1mg/kg p.o.)

Group 2: Ovotoxic animals received normal saline 1ml/kg, p.o.

Group 3: Ovotoxic animals received the paste of seeds of *Prunus amygdalus* (100 mg/kg, p.o.) for 45 days.

Group 4: Ovotoxic animals received the paste of seeds of *Prunus amygdalus* (200 mg/kg, p.o.) for 45 days.

Group 5: Ovotoxic animals received the paste of seeds of *Prunus amygdalus* (400 mg/kg, p.o.) for 45 days.

Group 6: Ovotoxic animals received estrogen therapy (60/mg/kg) for 45 days.

At 0 day and after the treatment period of 15 and 60 days, hormonal and biochemical parameters and oxidative stress parameters were studies. The blood was collected from the animal by retro orbital puncture under mild ether anaesthesia, it was centrifuged at 2500 rpm in cooling centrifuge and serum was separated to evaluate the biochemical parameters using autoanalyser and spectrophotometer. Ovaries were was quickly removed and place in ice cold 10% formaldehyde solution. The ovarian tissue was weighed and homogenized in 0.1M phosphate buffer (pH 7.2). The homogenate was centrifuged at 300 in the homogenizer (Remi motors, Mumbai) for 10 min and the resultant cloudy supernatant liquid was used for estimation of oxidative stress.

Estimation of Estrogen

The estimation of estrogen was carried out from serum using an ELISA kit(MACDONALD et al., 1979).

Estimation of lipid profile for evaluation of cardiovascular risk factors

Serum cholesterol, triglyceride, high density cholesterol (HDL-cholesterol) level were estimated by CHOD-PAP method with LCF(Castelli et al., 1977), Enzymatic Colorimetric Method or GPO (Rust et al., 2006), and Phosphotungstic Acid Method(Miller et al., 1977), respectively using ERBA diagnostic kits. The concentration of VLDL, LDL and atheraogenic index was obtained by using the formula.

LDL= Triglyceride/5

VLDL= TC-HDL

Atherogenic Index= TC-HDL/HDL

Estimation of lipid peroxidation and superoxide dismutase and reduced glutathione from ovaries

Estimation of lipid peroxidation was done from the tissue supernatant by the method of Slater and Sawer, 1971 (Slater and Sawyer, 1971). Superoxide dismutase and reduced glutathione were estimated by the method of Mishra et. al 1972 and Moron et.al, 1979 respectively using supernatant from ovaries.

Statistical analysis

The statistical analysis was carried out using Graph Pad Prism 4.0 software. All values were expressed as Mean ± SEM. Multiple comparison between different groups was evaluated statistically using analysis of variance (ANOVA) followed by Dunetts Multiple comparison test. Difference level at $P< 0.05$ was considered as statistically significant condition.

Results

Effect of paste of dried seeds of *Prunus amygdalus* on serum level of estrogen in ovotoxicity induced postmenopausal complications in rats

Result summarized in table no.3.1 indicates the effect of VCD on the estrogen level in mice. Result shows that administration of VCD at a dose of 160mg/kg for 15 days (5 days a week) caused significant (P<0.01) decrease in estrogen level as compared to normal rats. The result obtained from the study confirmed induction of menopause and ovarian failure in rats. Result presented in table-1 reveals that the seeds of *Prunus amygdalus* does not have any significant effect on serum estrogen level in menopausal mice. Treatment of ovotoxic post menopausal rats by the seeds of *Prunus amygdalus* at a dose of 400mg/kg and 200mg/kg for 45 days caused slight increase(84.6 ±0.75&79.6 ± 0.64 respectively) in estrogen level in ovotoxic animals. At the dose level of 100mg/kg however the seed powder does not have significant effect on estrogen level (65.56 ± 0.4) in ovotoxic animal. However in estrogen treated animals, the level of estrogen was significantly improved.

Effect of seeds of *Prunus amygdalus* on cardiovascular parameters (Cholesterol, triglycerides, HDL, LDL, VLDL and atherogenic index) in ovotoxicity induced postmenopausal complications in rats

Results shown in table no. 2 reveals that after VCD administration, the level of cholesterol, triglycerides, LDL and VLDL were significantly (P<0.001) increased in postmenopausal women indicating impairment of lipid profile. Treatment of the ovotoxic animals by the seeds of *Prunus amygdalus* at a dose of 400mg/kg and 200mg/kg for 45 days caused significant (P<0.001 and P<0.01 respectively) decrease in blood cholesterol, triglyceride, LDL and VLDL levels. Treatment with 100 mg/kg caused moderate decrease ((P<0.05) in the level of above mentioned parameters. HRT by estrogen at a dose of 60mg/kg for 45th days caused most significant (P<0.001) decrease in lipid profile in postmenopausal rats. HRT maintain the estrogen level in the menopausal animalsand restored the lipid profile to the normal value.

Results indicate that VCD administration severely decreased the level of HDL in rats. Administration of seeds of *Prunus amygdalus* to the postmenopausal ratsat a dose of 200mg/kg and 400 mg/kg for 45 days significantly (P<0.001) increased (32.125±0.18 and 37.29± 0.6 respectively)the level of HDL ac compared to positive control animals. Estrogen therapy also normalized the level of HDL from 20.61±0.15 to 38.65±1.25. Atherogenic index was also increased significantly (P<0.001) in female rats after induction of menopause. Table 3 shows a highly significant (P<0.001) decrease in the level of AI in the group administered with the paste of *Prunus amygdalus* at a dose of 200 mg/kg and 400 mg/kg. Estrogen therapy also normalized the AI level.

Effect of paste of dried seeds of *Prunus amygdalus* on the level of lipid peroxidation, superoxide dismutase and reduced glutathione in ovotoxicity induced postmenopausal rats

Result summarized in table-4 shows that administration of VCD significantly (P<0.001) increased the level of malondialdehyde (MDA), an indicator of lipid peroxidation and decreased the levels of superoxide dismutase (SOD) and reduced glutathione (GSH)in the ovaries of female rats indicating increased oxidative stress. Treatment with the paste of dried seeds of *Purus amygdalus*at a dose of 400mg/kg, p.o. for 45 days caused a remarkable decrease (P<0.001) in the level of MDA in postmenopausal rats as compared to ovotoxic

control animals. At a dose of 200mg/kg the paste of seeds also caused significant (P<0.01) decrease (54.15±1.28) in MDA level in postmenopausal rats. At a dose level of 100 mg/kg seeds of *Prunus amygdalus* does not exhibit any effect on MDA. Estrogen therapy given for 45 days at a dose of 60 mg/kg also caused significant ((P<0.001) decrease (48.05±1.92) in the level of TBARS which indicates decrease in free radicals formation in postmenopausal rats. Treatment of the ovotoxic rats for 45 days by the paste of seeds of *Prunus amygdalus* (400mg/kg and 200mg/kg) significantly (P<0.001) increased (213.09±2.1 and 208.54±0.64respectively) the level of reduced glutathione in ovotoxic animals as compared to the ovotoxic control group (99.6±1.52). However treatment of ovotoxic animal by the seeds of *Prunus amygdalus* at 100 mg/kg do not have any significant effect on ovarian reduced glutathione level in ovotoxic animals. The paste of seeds of *Prunus amygdalus at a dose of* 400mg/kg and 200mg/kg also caused significant (P<0.001 and P<0.01) increase in level of SOD in ovotoxic animal as compared to positive control group. Estrogen therapy for 45 days caused significant increase ((P<0.001) in the level of reduced glutathione and SOD in ovotoxic postmenopausalrats.

Discussion

The present study was aimed to evaluate the effect of paste of dried seeds of *Prunus amygdalus* on ovotoxicity induced postmenopausal complications in rats. Study was mainly designed to evaluate the effect of *Prunus amygdalus*seeds at different dose level on cardiovascular parameters, estrogen level and oxidative stress in ovotoxicity induced postmenopausal complications in female rat.VCD is an ovotoxic chemical, used for induction of ovotoxicity and menopause in experimental animals. It acts by gradual degeneration of primary follicles and thereby induces menopause similar to the natural menopause in humans (Hoyer PB., *et.al.*2001). Results of the study reveals that VCD caused a destruction and gradual decline in the no. of oocytes as indicated by changes in vaginal cytology and decreased estrogen level. The process of decline in the serum estrogen level in rats was gradual and was similar to natural menopause in humans.

In the present study the paste of dried seeds of *Prunus amygdalus* does not have any significant effect on serum estrogen level. So it can be assumed that the phytoconstituents present in *Prunus amygdalus* does not have any effects on estrogen synthesis. The phytoestrogens of *Prunus amygdalus* only binds with estrogen receptor and produces estrogen-like effects, thereby decreases menopausal complications.

In women estrogen has direct metabolic actions on many non reproductive tissues such as bone, vascular endothelium, liver etc. The level of oestrogen has its one of the major effects on serum lipid proteins and triglycerides. Oestrogen reduces the level of total serum cholesterol. It increases the level of good cholesterol,.i.e. HDL (high density lipoprotein) and reduces the levels of lipoprotein A and low density lipoproteins(LDL) which is mediated by ERα**.** Oestrogen also enhances lipolysis and reduces lipogenesis which is mediated by increase in the hepatic expression of apoprotein genes and LDL receptors while decreases the transcription of lipoprotein lipase (LPA) gene which is mediated by ERα. After menopause the level of oestrogen decreases that causes increase in the LPL activity that further enhances the accumulation of free fatty acids on abdomen. There is a greater chance of development of central obesity if expression of ERα and ERβ is reduced on proliferating adipocytes which is associated with an increase atherogenic profile that results in increased risk of heart disease.

In postmenopausal women due to low level of estrogen, the level of TG, cholesterol, LDL and VLDLgets elevated and HDL level is reduced and is responsible for increasing the chances of cardiovascular diseases in postmenopausal women. In the present study, in ovotoxic postmenopausal animals the serum level of TG, cholesterol. LDL and VLDL were increased significantly whereas significant decrease was observed in the levelof HDL indicating abnormalities in serum lipoprotein level.

Treatment of ovotoxic animals with paste of dried seeds of *Prunus amygdalus*for a long period caused significant improvement in lipid profile and decrease in atherogenic index, thereby decreasing the chances of cardiovascular complications. Studies indicates that phytoestrogens exerts their cardioprotective action by regulating the vascular tone and altering the lipid metabolism. The vascular tone is regulated by mechanisms involving endothelial (dependent or independent) vasodilator systems (NO) and it also mediates vasodilatory action by inhibiting the vasoconstriction mechanisms (Doshi sejal B.*et.al.,*2013). We can correlate the findings of our studies with such studies on phytoestrogens and assume that the improvement in lipid metabolism by Prunus amygdalus is due to phytoestrogen present in it (WILMINK et al., 2000).

In menopause declining level of estrogen, loss of protective effects of estrogen and deficient levels of endogenous antioxidants leads to increased oxidative stress in the body that is responsible for various complications related to menopause such as atherosclerosis, cardiovascular diseases, etc. In menopause the disturbances between the formation and elimination of free radicals leads to various changes in the body like dryness, reduced collagen content, elasticity and fragility etc (Hall G and Phillips TJ, 2005).

In the present study, high level of malondialdehyde, which is a product of lipid peroxidation and decreased levels of endogenous antioxidant enzymes in postmenopausal rats indicated increased oxidative stress in the body.The paste of dried seeds of *Prunus amygdalus* caused significant decrease in lipid peroxidation and increase in the level SOD and GSH in postmenopausal rats. It may be assumed that the phytoestrogens present in the seeds of *Prunus amygdalus* are responsible for decreasing oxidative stress. The phytoestrogens by binding to estrogen receptors produces antioxidant effect and decreases oxidative stress in the body.

Conclusion

From the results it can be concluded that consumption of the seeds of *Prunus amygdalus* for long period of time and on regular basis can protect the females from menopause induced health problem including cardiovascular diseases. However future study are required to establish the effect of seeds of *Prunus amygdalus* on various other complications of menopause such as depression, hot flushes, anxiety, dementia, osteoporosis etc. in postmenopause rats.

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Table -1 Effect of paste of dried seeds of *Prunus amygdalus* on serum level of estrogen in ovotoxicity induced postmenopausal complications in rats

Treatment (i.p/ p.o)	Oestrogen Level (pg/ml)		
	0th Day	15th Day	60th Day
Normal control (normal saline 1ml/kg p.o)	165.85±0.9	162.4±0.6	168±0.42
Positive control (VCD 160 mg/kg i.p, 5 times a week for 15 days)	155.06±0.4	27.80±0.15 [a]	30.4±0.6
VCD+ Paste of dried seeds of *Prunus amygdalus* (100 mg/kg, p.o)	154±0.8	30.4±0.44 [a]	65.56 ± 0.4
VCD+ Paste of dried seeds of *Prunus amygdalus* (200mg/kg,p.o)	160.9±0.36	29.85±0.64 [a]	79.6 ± 0.64*
VCD+ Paste of dried seeds of *Prunus amygdalus* (400mg/kg, p.o)	169.95±0.32	38.6±0.75 [a]	84.6 ±0.75*
VCD+ estradiol (60mg/kg, p.o)	163±0.94	29.02±0.95 [a]	158.27 ± 0.95***

The statistical significance of difference between means was calculated by ANNOVA followed by t-test for unpaired comparison. N= 6
Values are expressed as Mean ± SEM, *P< 0.05, **P< 0.01, ***P< 0.001 when Groups Compared: II:III, II:IV, II:V, II:VI on 60th day of the treatment.
[a]P<0.001 when results of day 15 were compared with day one of the study.

Table -2 Effect of paste of dried seeds of *Prunus amygdalus* on serum level of Cholesterol, HDL, LDL and VLDL in ovotoxicity induced postmenopausal complications in rats.

Treatment (i.p/ p.o)	Cholesterol (mg/dl)	HDL (mg/dl)	Triglyceride (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)
Normal control (normal saline, 1ml/kg p.o)	60.505 ± 2.6	40.7 ± 0.98	69.7± 1.25	13.94±0.25	24.505±1.62
Positive control (VCD 160mg/kg i.p, 5 times a week for 15 days)	96.54 ± 1.2[a]	20.61±0.15 [a]	182.5± 0.32 [a]	36.5±0.064 [a]	75.54±1.05 [a]
VCD +Paste of seeds of PA (100mg/kg, p.o)	80.26±1.35*	23.62±0.15	153.3±0.12*	30.6± 0.024*	118.7±0.096*
VCD+ Paste of seeds of PA (200mg/kg,p.o)	66.25± 2.0***	32.125±0.18**	136.5± 1.56**	27.3±0.312**	109.2±1.28**
VCD+ Paste of seeds of PA (400mg/kg, p.o)	64.05±1.59***	37.29± 0.6***	85.5± 0.25***	17.1±0.05***	68.4±0.02***
VCD+ Estradiol (60mg/kg, p.o)	63.19 ± 2.5***	38.65±1.25***	76.15±0.16***	15.23±0.032***	60.92±0.128***

The statistical significance of difference between means was calculated by ANNOVA followed by t-test for unpaired comparison. N= 6

Values are expressed as Mean ± SEM, *P< 0.05, **P< 0.01, ***P< 0.001 when Groups were compared with the positive control group.

[a]P<0.001 when results of positive control group were compared with the normal animals.

Table - 3 Effect of paste of dried seeds of *Prunus amygdalus* on atherogenic index in ovotoxicity induced postmenopausal complications in rats.

Treatment (i.p/ p.o)	Atherogenic Index
Normal control (normal saline, 1ml/kg p.o)	56.84 ± 0.43
Positive control (VCD 160mg/kg i.p, 5 times a week for 15 days)	94.44 ± 0.12[a]
VCD +Paste of seeds of PA (100mg/kg, p.o)	83.76 ± 0.51
VCD+ Paste of seeds ofPA (200mg/kg,p.o)	63.26 ± 0.61**
VCD+ Paste of seeds of PA (400mg/kg, p.o)	61.50 ± 0.11**
VCD+ Estradiol (60mg/kg, p.o)	59.78 ± 0.8 ***

The statistical significance of difference between means was calculated by ANNOVA followed by t-test for unpaired comparison. N= 6

Values are expressed as Mean ± SEM, *P< 0.05, **P< 0.01, ***P< 0.001 when Groups were compared with the positive control group.

[a]P<0.001 when results of positive control group were compared with the normal animals.

Table 3.4. Effect of paste of dried seeds of *Prunus amygdalus* on serum level of MDA, SOD and GSH in ovotoxicity induced postmenopausal complications in rats.

Treatment (i.p/ p.o)	MDA (mol/l)	SOD (IU/l)	GSH (µg of Tissue/ml)
Normal control (normal saline, 1ml/kg p.o)	45.06±2.3	114.14±1.96	230±0.09
Positive control (VCD 160mg/kg i.p, 5 times a week for 15 days)	93.75±1.46[a]	60.19±2.01[a]	99.6±1.52[a]
VCD +Paste of seeds of PA (100mg/kg, p.o)	78.16±0.52*	74.34±0.98	102.81±2.2
VCD+ Paste of seeds of PA (200mg/kg,p.o)	54.15±1.28***	98.84±0.39**	208.54±0.64***
VCD+ Paste of seeds of PA (400mg/kg, p.o)	51.625±0.49***	111.13±0.28***	213.09±2.1***
VCD+ Estradiol (60mg/kg, p.o)	48.05±1.92***	112.55±1.56***	222.98±2.96**

The statistical significance of difference between means was calculated by ANNOVA followed by t-test for unpaired comparison. N= 6

Values are expressed as Mean ± SEM, *P< 0.05, **P< 0.01, ***P< 0.001 when Groups were compared with the positive control group.

[a]P<0.001 when results of positive control group were compared with the normal animals.