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Modulation of Gene Expression by *Polyalthia longifolia* in Postmenopausal Women with Coronary Artery Disease: An In Vitro Study

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Abstract Chronic underlying inflammation is involved in the pathophysiology of coronary artery disease (CAD). *Polyalthia longifolia* var. *pendula* bark extract (PLE) is known to exhibit anti-inflammatory activity and has high content of phytosteroids. Since phytosteroids mimic estrogen structurally, we postulated that PLE may provide protection in postmenopausal women against CAD. Thus the effect of PLE has been explored on expression of estrogen receptors (ERα and ERβ) and inflammatory inducible nitric oxide synthase (iNOS) genes in vitro in peripheral blood mononuclear cells (PBMCs) obtained from postmenopausal women. A total of 20 postmenopausal women were included in the present study. Group I (N=10) included women with angiographically proven CAD, and group II (N=10) is composed of equal number of age-matched healthy postmenopausal females as controls. Significantly low levels of serum 17-β estradiol were observed in subjects of group I as compared to group II ($p<0.01$). A marked increase in L-citrulline levels ($p>0.05$) and significantly augmented levels of reactive nitrogen intermediates ($p<0.05$) were observed in group I subjects. PLE significantly attenuated PMA-induced expression of both ERα and ERβ receptors and inflammatory iNOS gene in vitro in a dose- and time-dependent manner and had an additive effect on these genes when compared with tamoxifen. Ours is the first report to demonstrate that PLE contains certain bioactive principles, which possess anti-inflammatory and estrogenic properties, and thereby hold the promise to be screened for their anti-atherogenic potential in experimental animals to favorably alter several other markers of cardiovascular risk.

Keywords Coronary Artery Disease · *Polyalthia longifolia* var. *pendula* · SERMs · Estrogen Receptors · iNOS

Introduction

Coronary artery disease (CAD) is the main contributor of morbidity and mortality among postmenopausal women, and atherosclerosis is the main biological process determining CAD. Menopause-associated estrogen deficiency has both metabolic and vascular consequences that increase the risk for CAD [1, 2]. Clinical data support the notion that CHD is sensitive to estrogens, but debate exists concerning the effects of the hormone on atherosclerosis and its complications. Whereas hormone replacement therapy in postmenopausal women increases the coronary artery risk, epidemiological studies and experimental studies demonstrate a major atheroprotective action of estradiol (E2) [3]. Inducible nitric oxide synthase (iNOS) elaborated by

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macrophages propagates inflammation leading to acceleration and vulnerability of atherosclerotic plaques [4, 5], and estrogen has been reported to have a beneficial effect on metabolic and vascular factors influencing the incidence of CAD. It has a direct effect on the vascular endothelium, since it potentiates endothelial nitric oxide (NO) and prostacyclin production, thus promoting beneficial effects such as vasorelaxation and inhibition of platelet aggregation [6]. Depre et al. [3] and Dubey et al. [7] reported that estrogen can also increase bioactive NO levels via inhibition of superoxide production and also accelerate endothelial regrowth, thus favoring vascular healing. Most of these effects of E2 are mediated by estrogen receptor alpha (ERα) and are independent of estrogen receptor beta (ERβ) [3, 7]. Thus a better understanding of the deleterious and beneficial effects of estrogens is required not only on the normal and atheromatous arteries but also on innate and adaptive immune responses, which should help to optimize the prevention of cardiovascular disease after menopause. Estrogen receptor (ERα/β) is a member of the nuclear hormone family of intracellular receptors, which is activated by the hormone 17β-estradiol [8]. Selective estrogen receptor modulators (SERMs) are compounds capable of binding the estrogen receptor to induce a functional profile distinct from estrogens. The possibility that SERMs may shift the estrogenic balance on cardiovascular risk toward a more beneficial profile has generated interest in recent years. There is considerable information on the effects of SERMs on distinct areas that are crucial in atherogenesis [9].

In India, the use of medicinal herbs is as old as 1500 BC. In Ayurveda, various herbal plants like *Terminalia arjuna* (arjuna), *Allium sativum* (garlic), and *Aegle marmelos* (Bael) have been well documented for their cardio-protective potentials [10]. The most untouched plant in this category is *Polyalthia longifolia*, which has been shown to posses antimicrobial and anti-fungal, cytotoxic, and apoptosis-inducing properties [11–13]. Besides other 23 known compounds and phytosteroids, different clerodane diterpenoids isolated from the bark of *P. longifolia* var. *pendula* showed significant cytotoxicity toward Hep G2 and Hep 3B hepatoma cell lines and exhibited potent anti-inflammatory activity toward formyl-L-methionyl-L-leucyl-L-phenylalanine/cytochalasin B (fMLP/CB)-induced superoxide generation by neutrophils with IC50=0.60±0.09 μg/ml [11, 13, 14]. As the bark extract of*P. longifolia* has high content of phytosteroids, these may mimic estrogen structurally.

Therefore, against this background, we have carried out a pilot study to determine the effect of *P. longifolia* bark extract on the expression of iNOS and estrogen receptors (ERα/β) in peripheral blood mononuclear cells (PBMCs) obtained from postmenopausal women with coronary artery disease in vitro. The purpose of this study was to examine whether the bark extract of *P. longifolia* possesses anti-inflammatory and estrogenic activity.

Materials and Methods

Selection of the Subjects

Inclusion Criteria Postmenopausal females with CAD (N=10; group I) who were of >50 years of age had a history of myocardial infarction (chest pain associated with electrocardiography (ECG) evidence of myocardial infarction or raised cardiac enzymes or both), unstable angina (cardiac pain associated with dynamic ECG abnormalities), and angiographically proven coronary artery disease (>50% stenosis in one or more major epicardial vessel in multiple projections). Equal number of unrelated matched postmenopausal women with angiographically proven normal coronary arteries served as controls (N=10; group II).

Exclusion Criteria Subjects were excluded if they had a history of cardiomyopathy, a past or present history of hypertension (BP>120/80 mmHg), diabetes or any inflammatory or infectious disease, serious organ disease, systemic illness, chronic alcohol abuse, smoking, serious psychiatric illness, and/or anticonvulsant therapy.

Routine laboratory investigations, including a complete hemogram, urine analysis, blood biochemistry, and lipid and lipoproteins profile, were carried out in all the study subjects. A 12-lead ECG was recorded, and a chest X-ray was taken. Blood pressure was recorded according to JNC VI Criteria [15]. The study was approved by The Medical Ethics Committee of PGIMER, Chandigarh. Informed written consent was taken from all the study subjects prior to their participation in this study.

Sampling

Venous blood was collected from the overnight fasted individuals in the morning from antecubital vein into vacutainers for serum and in EDTA for plasma. Serum/plasma was separated and stored at −80°C for further analysis.

Measurement of Lipid and Lipoprotein Profile

Total cholesterol (TC), triglycerides (TG), and high-density lipoprotein cholesterol levels (HDL-C) levels were measured with commercially purchased standard enzymatic kits (Accurex Biomedical Pvt. Ltd.). Low-density lipoprotein cholesterol (LDL-C) and very low-

density lipoprotein (VLDL-C) values were calculated using Friedwald's formula [16].

Determination of Nitric Oxide

Determination of Plasma Nitrite

NO is a very unstable molecule and has a half-life of few seconds. Therefore, we measured plasma nitrite (NO_2^-) as a stable end product of NO production by the method of Green et al. [17].

Determination of L-Citrulline

L-Citrulline is a by-product of nitric oxide synthesis and used for NO estimation. L-Citrulline levels were measured by the method of Boyde and Rahmattulah [18].

Determination of Serum 17β-Estradiol

Circulating levels of 17β-estradiol were determined in serum samples of all the study subjects using Enzyme Immunoassay Kit (Cat no. DKO 003; Diametra) following manufacturer's instructions. The immunoassay inter- or intra-assay coefficient of variation was 7.25% and 6.81%, respectively.

Plant Material and Preparation of the Extract

The bark of *P. longifolia* var. *pendula* was obtained from a single tree from Northern part of India (PGIMER Campus, Chandigarh) and identified by a certified Botanist (Vide Letter No. BAES/08). Fifteen grams of bark was crushed to a coarse powder and then repeatedly extracted with 70% *v/v* alcohol at boiling temperature till complete exhaustion using a Soxhlet apparatus for 70–80 h. The alcoholic extract was concentrated and dried at 60°C under vacuum using Rotavapor [19].

The dried material was powdered and weighed around 1.92 g. The extract was then reconstituted in serum-free RPMI 1640 cell culture media for use in in vitro experiments. The extract was further purified using high-performance liquid chromatography (HPLC) and stored in amber-colored bottles under atmosphere of nitrogen at −20°C (HPLC data not shown).

In Vitro Studies

Isolation of Peripheral Blood Mononuclear Cells

PBMCs were isolated from whole blood of the study subjects by the method of Boyum [20] by density gradient centrifugation, using Histopaque-1077 (Cat no.10771; Sigma). These cells were cultured in RPMI-1640 media supplemented with 10% fetal calf serum, 2 mM L-glutamine, 20 mM sodium bicarbonate, 20 mM HEPES, and antibiotics at 37°C in a humidified 5% carbon dioxide (CO_2) atmosphere.

Cell Culture

The human leukemic monocytic cell line (THP-1) was obtained from the National Center for Cell Sciences (NCCS), Pune, India. The cells were maintained in RPMI-1640 media supplemented with 10% heat-inactivated calf serum (Sigma) as described previously [21, 22]. THP-1 monocytic cell line was used as a model for human monocytes to avoid the limited availability and individual variations of monocytes observed, when isolated from the peripheral human blood. THP-1 cells are often used as fair substitutes for simulating human monocytes-macrophages in analyzing the production of inflammatory mediators/cytokines, including MMPs, in response to various inflammatory stimuli [21, 23].

Effect of *P. longifolia* Bark Extract on Cell Viability and Cell Proliferation

The effect of ethanolic extract of *P. longifolia* (PLE) on cell viability and cell proliferation was assessed by trypan blue exclusion assay and anti-BrdU cell proliferation assay (Cat no. 11647229001, Roche Diagnostics, Germany) in the presence or absence of various concentrations (10, 50, 100, 200, 250, 300, 350, and 400 μg) of the extract up to 72 h.

Expression of iNOS

Semi-quantitative RT-PCR was used to determine the expression of iNOS gene. Total RNA was isolated from the cultured cells by the method of Chomczynski et al. [24]. RT-PCR was performed using a two-step RT-PCR kit (MBI Fermentas). The cDNA was then amplified by PCR using human iNOS and β-actin specific primer pair (Table 1). The amplicons were resolved in 2% agarose gels containing ethidium bromide. The ethidium bromide-stained products were photographed, and the intensities of bands were analyzed using Scion Image analysis software (USA). The levels of mRNA expression of each gene were given by normalizing the RT-PCR band intensities of that gene to β-actin band intensity, which was used as an invariant control. The intensity ratio of the target mRNA to β-actin mRNA was expressed as percentage value.

Expression of Estrogen Receptors

Expression of estrogen receptors was determined by Western Blot. For this, total cell lysates were prepared in

Table 1 Table showing different primer sequences and program for RT- PCR

Gene	Primer pair and PCR program			No. of cycles
β-actin (257 bp)	Forward: 5′-CAT GTA CGT TGC TAT CCA GGC-3′			30
	Reverse:5′-CTC CTT AAT GTC ACG CAC GAT-3′			
	i.	94°C	5 min (initial denaturation)	
	ii.	94°C	45 s	
		58°C	30 s	
		72°C	45 s	
	iii.	72°C	10 min (final extension)	
iNOS (395 bp)	Forward: 5′-TATGCTGATGCGCAAGACAATGGC-3′			
	Reverse: 5′-ATGTCATGAGCAAAGGCGCAGAAC-3′			
	i.	94°C	4 min (initial denaturation)	40
	ii.	94°C	45 s	
		56°C	45 s	
		72°C	1 min	
	iii.	72°C	5 min (final extension)	

the cell lysis buffer (50 mM Tris–HCl pH7.4, 300 mM NaCl, 5 mM EDTA, 1% Triton X-100) supplemented with a protease inhibitor cocktail (Cat no. 11697498001, Roche). Cells were then sonicated three times for 10 s, and cell lysates were then centrifuged at 10,000 rpm for 30 min at 4°C. The protein levels were quantified using Bradford's reagent. Equal amounts of proteins (40 μg/lane) were resolved by SDS-PAGE and transferred onto PVDF membrane for at 100 V for 4 h/4°C. Membranes were blocked in 5% nonfat dry milk in PBS for overnight at 4°C. Primary antibodies (ERα (1:700; Cat no. sc8002, Santacruz Inc.) and ERβ (1:500; Cat no. GR39, Calbiochem)) were incubated with the membrane in the blocking buffer for 2 h at 37°C under agitation on a rotary shaker. Proteins immune-reactive with primary antibodies were detected with HRP-conjugated secondary antibodies (1:1,000; Cat no. A9044, Sigma) for 1 h at 37°C, and the bands were visualized by DAB–H_2O_2 system.

Time- and Dose-Dependent Studies for Gene Expression

PLE was added in different concentrations, i.e., 50, 100, and 200 μg/10^6 cells/ml for different time intervals, i.e., 3, 6, 12, 24, and 48 h after stimulation with PMA for 48 h. Cells stimulated by PMA alone served as controls. The cells were harvested at each interval and were then processed to determine the transcriptional expression of iNOS by RT-PCR and translational expression of ER-α and ER-β by Western Blot analysis. β-Actin was used as an invariant control for both the transcriptional and translational expression.

Effect of Tamoxifen on Expression of Genes

Further, to know whether PLE acts as an agonist or antagonist for the expression of these genes and behaves like SERMS, THP-1 cells were cultured in the presence of 10^{-7} to 10^{-6}M tamoxifen alone and in the presence of PLE (200 μg/ml) along with tamoxifen (10^{-7} to 10^{-6}M) up to 72 h, and expression of ER-α, ER-β, and iNOS genes were determined.

Statistical Analysis

Data were analyzed using statistical software SPSS 14.0. The data obtained from study subjects in different groups were compared using unpaired Student's*t* test. Results of in vitro experiments with PBMCs were compared by paired Student's *t* test, whereas Mann–Whitney test was used for in vitro experiments with THP-1 cells.

Results

Baseline Characteristics

Baseline characteristics of both the study groups are depicted in Table 2. As far as the age, body weight, and body mass index (BMI) are concerned, we did not observe any statistically significant difference between the two groups, though BMI was moderately higher in the patient group ($p>0.05$) as determined by using unpaired Student's *t* test.

Biochemical Parameters

The two study groups were not matched for nitrite, estradiol, or lipid parameters as determined by using unpaired Student's *t* test. On inter-group comparisons, mean levels of TC, TG, LDL-C, and VLDL-C were observed to be significantly higher in group I when compared with group II subjects ($p<0.05$; Table 2). Although, the values of HDL-C were

Table 2 Physical and biochemical characteristics of the study subjects

Parameters	Group I (n=10)	Group II (n=10)
Age range (years)	50–60	52–60
Body mass index (kg/m^2)	25.9±5.2	30.6±5.14
Smoking	None	None
Alcohol intake	None	None
TC (mg/dl)	168.68±21.75	225.68±51.77*
TG (mg/dl)	119.4±20.1	137.5±32.7**
HDL-C (mg/dl)	39.3±12.6	37.7±9.85
LDL-C(mg/dl)	105.5±19.27	162.8±50*
VLDL-C (mg/dl)	23.9±4.0	27.48±6.55**
Plasma nitrite (μmol/ml)	26.28±9.66	46.17±23.36*
L-Citrulline (μmol/ml)	7.75±2.0	11.72±1.9
17β- Estradiol (pg/ml)	37.16±19.6	14.1±4.56***

Results are expressed as mean±SD

n number of individuals in each group

*p<0.05 group I vs group II; ***p<0.001 group I vs group II

slightly lower in group I subjects, the values were statistically not different between the two groups (p>0.05). Nitrite levels were found to be significantly higher in the plasma of CAD patients as compared to their control counterparts (46.17± 23.36 vs 26.28±9.66 μM/ml; p<0.05). L-Citrulline levels were found to be higher in the patient group as compared to the control group but the difference between the two groups was statistically not significant (p>0.05). Serum 17β-estradiol levels were determined in all the study subjects to verify whether the postmenopausal study subjects with CAD had low estradiol levels. As expected, we observed significantly higher levels of 17β-estradiol in the controls as compared to those of postmenopausal women (p<0.01; Table 2).

Effect of *P. longifolia* Bark Extract on Cell Viability and Cell Proliferation

Trypan blue exclusion assay and anti-BrdU assay revealed that there was no significant decrease in the viability and proliferation of PBMCs and THP-1 cells in the presence or absence of 250 μg of PLE up to 48 h (viability>95%; p>0.05), whereas a dose of ≥300 μg PLE for 24 h caused a significant decrease in the cell viability and cell proliferation (p<0.05). However, even a minimal dose of PLE, i.e., 100 μg/ml, was able to affect cells adversely at 72 h both in terms of viability and proliferation (Fig. 1).

Effect of *P. longifolia* Bark Extract on iNOS

mRNA expression of iNOS was observed to be moderately higher in PBMCs of CAD subjects as compared to their control counterparts. However, the difference between the two study groups remained statistically insignificant (p>0.05). The PBMCs of these subjects were activated with 100 ng/ml PMA for different time periods, i.e., 24, 48, and 72 h. PMA acts as an analogue of diacylglycerol and induces stimulation of lymphocytes and monocytes by activating PKC pathway. PMA-stimulated basal expression of iNOS was found to be maximum at 48 h, and the difference between 24 and 48 h was not statistically significant. Therefore, in further experiments with PLE, we induced PBMCs with 100 ng/ml PMA for 48 h.

We observed that PMA-stimulated mRNA expression of iNOS was decreased in the presence of different doses of PLE both at 24 and 48 h. PLE at a dose of 100 μg/ml had no significant effect on the expression of iNOS at 24 h (p>0.05) when compared with the cells stimulated with PMA alone. However, a statistically significant decrease in iNOS expression was observed at 48 h with the same dose of PLE (Fig. 2). Further, at a higher dose of PLE, i.e., 200 μg/ml, the expression of iNOS was found to be significantly inhibited both at 24 and 48 h, respectively, (p<0.01), and the observed decrease was more pronounced at 48 h (p<0.01). Inter-group comparison between both the study groups showed non-significant difference as far as the expression of iNOS is concerned (p>0.05; Fig. 2).

Effect of *P. longifolia* Bark Extract on Estrogen Receptors

We observed that the constitutive expression of estrogen receptors, i.e., ERα and ERβ, was significantly low at translational levels in postmenopausal women with CAD as compared to the females of the control group (p<0.05). Therefore, the PBMCs of these subjects were activated with 100 ng/ml PMA for different time periods, i.e., 24, 48, and 72 h. After PMA stimulation, the basal expression of ERβ was found to be maximum at 48 h, whereas ERα expression was elevated maximally at 24 h in both the study groups. Also, the difference between 24 and 48 h was not significant for ERα expression. Therefore, in further experiments with PLE, we induced PBMCs with 100 ng/ml PMA for 48 h (Figs. 3 and 4).

Similar to the findings as observed with iNOS expression, expression of both the ERs under consideration was found to be inhibited in the presence of different doses of PLE both at 24 and 48 h when compared to the cells stimulated with PMA alone, whereas PLE at a dose of 100 μg/ml showed no significant inhibitory effect on the expression of either ERα or ERβ at 24 h (p>0.05), and the same dose was found to inhibit expression of both the genes significantly at 48 h, respectively (p<0.01).

Further, as observed with iNOS, an increment in the dose of PLE to 200 μg/ml significantly inhibited the expression of ERα and ERβ both at 24 and 48 h,

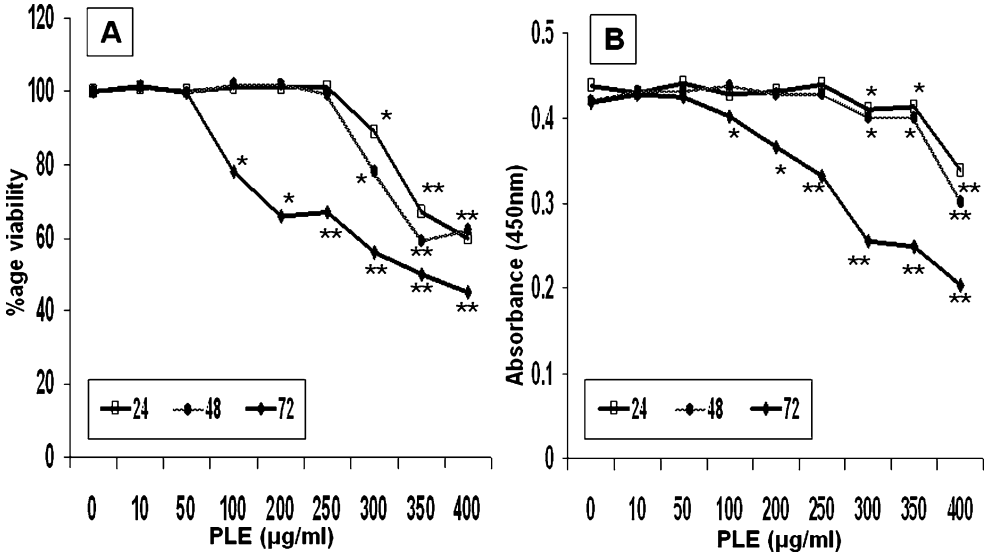

Fig. 1 Time- and dose-dependent effects of PLE (μg/ml) on cell viability (**a**; trypan blue exclusion assay) and cell proliferation (**b**; cell proliferation ELISA BrdU (colorimetric assay, Roche Diagnostics Cat no. 11647229001)). *$p<0.05$ vs basal; **$p<0.01$ vs basal. Data presented are means of three experiments with similar results

respectively ($p<0.01$). On comparison, both genes were found to be inhibited more at 48 h as compared to 24 h ($p<0.01$). However, inter-group analysis showed that ERα and ERβ expression did not differ significantly between both the groups ($p>0.05$) (Figs. 3 and 4).

Effect of Tamoxifen on Gene Expression

We observed that tamoxifen significantly decreased PMA-induced expression of ERα/β and iNOS in THP-1 cells in a dose-dependent manner ($p<0.05$). Further, when PLE (200 μg/ml) and tamoxifen (10^{-7} and 10^{-6}M) were added simultaneously, a significant additive effect of PLE was observed in attenuating the expression of ERα/β and iNOS genes ($p<0.05$; Fig. 5).

Discussion

There are several risk factors for development of CAD but menopause is a condition which stands out for the development of CAD in postmenopausal women [1]. For this particular reason, postmenopausal women with CAD having undetectable or low endogenous estradiol represent a good model where effect of natural compounds with anti-inflammatory and estradiol-like properties can be studied simultaneously.

Our observations revealed that BMI and age of both the study groups were statistically not different, which signifies that both the study groups under consideration were homogenous. We observed significantly higher levels of lipids and lipoproteins in the postmenopausal women with

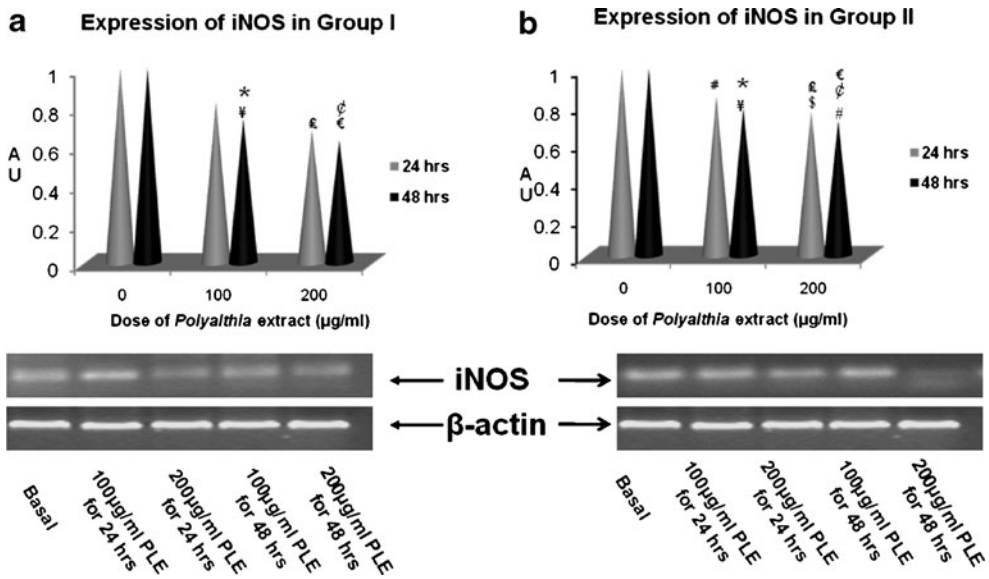

Fig. 2 Depicts mRNA expression of iNOS by PBMCs (*A*) in group I subjects ($N=10$) and (*B*) in group II subjects ($N=10$) in the presence or absence of *P. longifolia* bark extract (*PLE*) at 24 and 48 h, as determined by semi-quantitative RT-PCR. *$p<0.05$ basal vs 100 μg/ml at 48 h; €$p<0.05$ basal vs 200 μg/ml at 48 h; $p<0.05$ basal vs 200 μg/ml at 24 h; $p<0.05$ 100 μg/ml vs 200 μg/ml at 24 h; #$p<0.05$ 100 μg/ml vs 200 μg/ml at 48 h; ¥$p<0.001$ 100 μg/ml 24 h vs 48 h; ¢$p<0.001$ 200 μg/ml 24 h vs 48 h

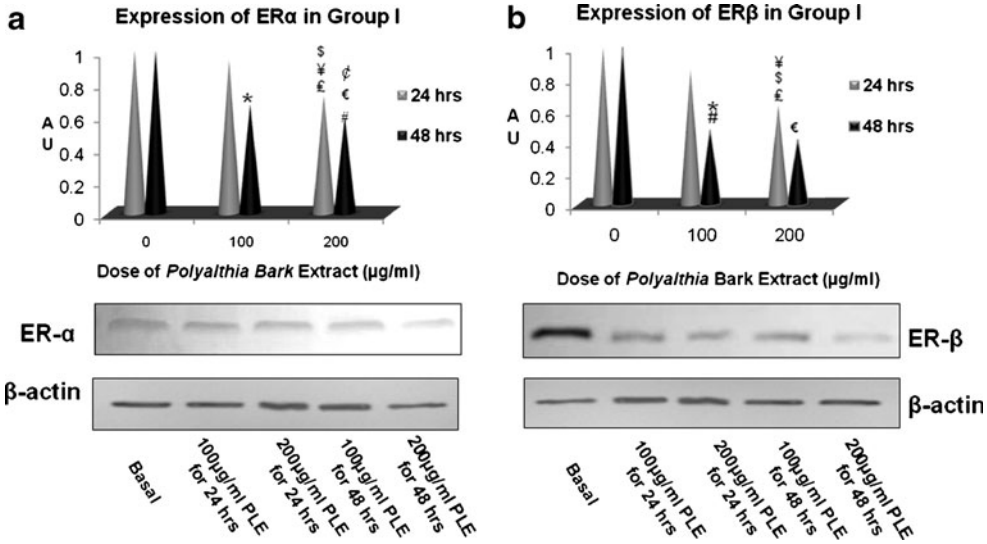

Fig. 3 Depicts protein expression of *A* ERα and *B* ERβ in PBMCs of group II (*N*=10) subjects cultured in the presence or absence of *P. longifolia* bark extract (*PLE*) at 24 and 48 h, as determined by Western Blot analysis. **p*<0.05 basal vs 100 µg/ml at 48 h; ¥*p*<0.001 100 µg/ml 24 h vs 48 h; €*p*<0.05 basal vs 200 µg/ml at 48 h; *p*<0.05 basal vs 200 µg/ml at 24 h; $*p*<0.001 100 µg/ml vs 200 µg/ml at 24 h; #*p*<0.001 100 µg/ml vs 200 µg/ml at 48 h; ¢*p*<0.001 200 µg/ml 24 h vs 48 h

CAD as compared to the women without CAD. Our findings are in tune with the reports of Castelli et al. [25] and Gordon et al. [26] who demonstrated similar findings. This implies the fact that menopause due to deprivation of estrogen acts as a risk factor by increasing the harmful lipoproteins and reducing the beneficial ones.

Yoon et al. [27] reported high nitrite (NO_2^-) levels in the plasma of postmenopausal women with CAD, and our data reconfirm that there is an increased nitric oxide generation in these women. Our results are also in accordance with similar findings observed earlier in CAD, hypertension, and other inflammatory diseases [27–29]. However, there is one contradictory report from Soysal et al. [30] who reported low nitrite levels in CAD patients. Our results suggest that increased generation of $NO^{\cdot}$ may occur as a compensatory and adaptive response.

The incidence of cardiovascular disease (CVD) is low in premenopausal women but increases substantially after menopause due to depletion of estradiol suggesting that estrogen protects the females from CVD [1, 2]. Decreased levels of circulating 17-β estradiol as observed in the present study further corroborate these findings. Estrogen provides protection against atherosclerosis through its genomic and nongenomic effects and ERα and ERβ both mediate its action.

Our data show that in stimulated cells, ERα is expressed earlier than ERβ. Similar findings have been reported by Cutolo et al. [31], where they reported that treatment of THP-1 cells with phorbol diester 12-*O*-tetradecanoylphorbol 13-acetate caused early expression of ERα, whereas ERβ was induced later. The possible reason for an early expression of ERα could be that ERα acts as a main

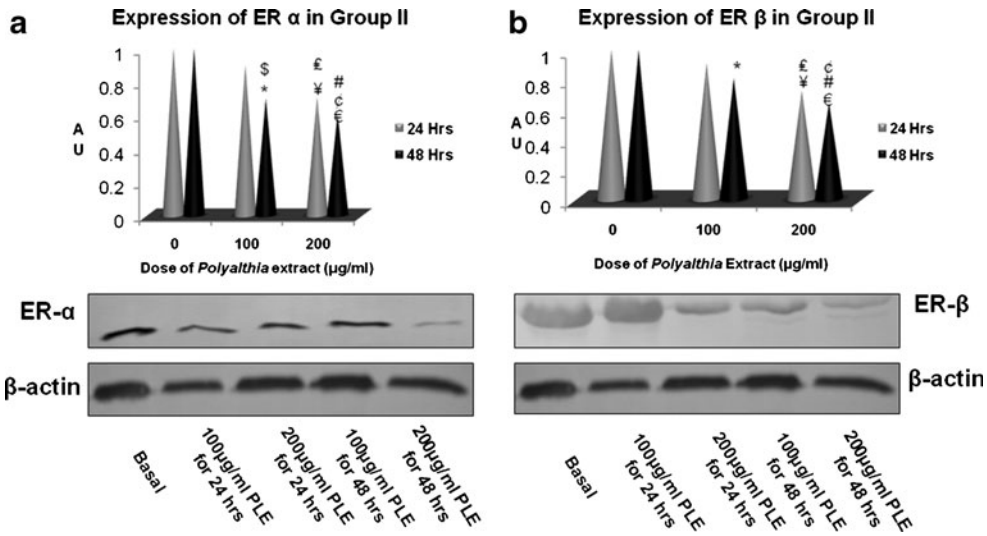

Fig. 4 Depicts protein expression of *A* ERα and *B* ERβ in PBMCs of group I (*N*=10) subjects cultured in the presence or absence of *P. longifolia* bark extract (*PLE*) at 24 and 48 h, as determined by Western Blot analysis. **p*<0.05 basal vs 100 µg/ml at 48 h; €*p*<0.05 basal vs 200 µg/ml at 48 h; *p*<0.05 basal vs 200 µg/ml at 24 h; ¥ *p*<0.001 100 µg/ml vs 200 µg/ml at 24 h; #*p*<0.05 100 µg/ml vs 200 µg/ml at 48 h; $ *p*<0.001 100 µg/ml 24 h vs 48 h; ¢*p*<0.001 200 µg/ml 24 h vs 48 h

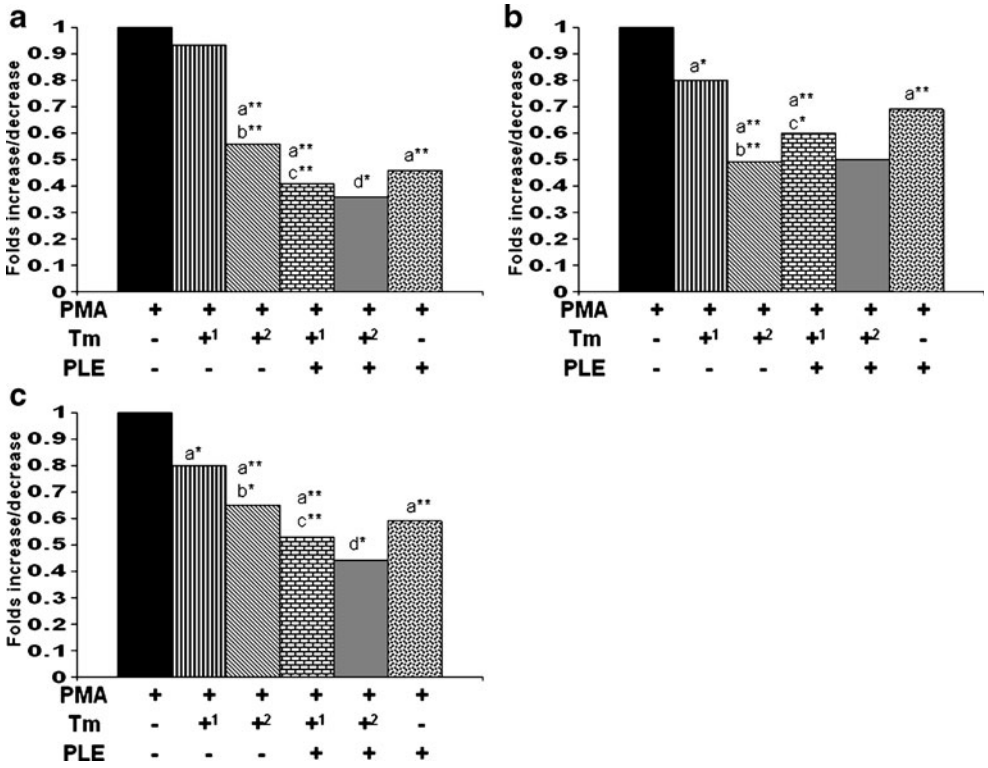

Fig. 5 Effect of PLE (200 μg/ml) on PMA-induced expression of ERα (**a**), ERβ (**b**), and iNOS (**c**) in the absence or presence of tamoxifen (*Tm*) ($^{1}10^{-7}$ and $^{2}10^{-6}$M) in THP-1 monocytic cell line. *$p<0.05$; **$p<0.01$; *a* vs PMA; *b* 10^{-7}M Tm vs 10^{-6} M Tm; *c* 10^{-7}M Tm vs 10^{-7}M Tm+PLE; *d* 10^{-6}M Tm vs 10^{-6} M Tm+PLE

receptor for estradiol-mediated signaling and is expressed earlier, and when defective ERα is present, ERβ mediates the effect of estradiol and SERMs.

Our observations demonstrated decreased expression of both ERα and ERβ receptors in PBMCs of postmenopausal women with CAD as compared to those women without CAD. Decreased expression of ERα has been correlated with the incidence of development of CAD in females [32]. Pollak et al. [33] reported that ERα polymorphisms are responsible for decreased ERα expression in these women. However, in this study, it was interesting to observe that ERβ expression was also reduced in patient group as compared to their controls. Survey of literature shows that ours is the first study to report that ERβ expression is low in postmenopausal women with CAD.

Besides these clinical and biochemical parameters, we also conducted in vitro experiments in PBMCs obtained from these subjects using PLE. The main aim of this study was to examine whether the bark extract of *P. longifolia* could modulate expression of genes associated with inflammation and CAD and possess anti-inflammatory property and estrogenic activity.

To date, there is no evidence and scientific validation of this plant that supports its role as a cardio-protective agent, and therefore, it was not ethical to administer the plant extract to the study subjects for in vivo experiments. It was for this reason that we carried out in vitro experiments using PBMCs from the study subjects. Though, in true sense, a significant SERM-like activity can only be assessed by receptor binding studies with pure isolated compounds, our data clearly demonstrate that the bioactive components present in bark extract of PLE are estrogenic, modulate estrogen receptors, and thus may have SERM-like action. These observations are further strengthened by results of our in vitro experiments in a human leukemic monocytic cell line with tamoxifen and PLE where an additive effect of PLE in attenuation of both ERα, ERβ, and inflammatory iNOS genes was observed. Tamoxifen is a nonsteroidal agent which has been demonstrated to exhibit anti-estrogenic properties, and also competes with estradiol for estrogen receptor proteins and thus behaves like SERMs. Further, the presence of phytosteroids in the PLE can have structural similarity with estrogen, and their SERM-like action may have an effect on the downstream signaling cascade driven through estrogen receptors.

iNOS isoform is a known marker for inflammation, is upregulated by cytokines and endotoxins in many types of cells, and is also expressed by activated macrophages in atherosclerotic plaques [3]. Our findings demonstrated that the expression of iNOS was moderately higher in PBMCs of postmenopausal women with CAD as compared to their control counterparts without CAD. Ravalli et al. [34] reported that iNOS produces large amount of $\dot{N}O$ in response to inflammatory cytokines and further suggested that the high output NO pathway and possibly the oxidant peroxynitrite might be involved in the process leading to the development of CAD. In their study, Ferreiro et al. [35] reported that iNOS levels were elevated in subjects with

heart failure in comparison to subjects without heart failure. Thus, finding high induction of iNOS in our subjects is not surprising and supports prevalent underlying inflammation in these patients.

On one hand, the increased $NO^{\cdot}$ production can be protective by its vasodilatory, antiaggregatory, and anti-proliferative effects, whereas on the other hand, the formation of peroxynitrite from $NO^{\cdot}$ may favor vasospasm and thrombogenesis. Reduced bioavailability and increased production of $NO^{\cdot}$ during CAD as found in our study may, therefore, lead to the production of excess reactive nitrogen intermediates which instead of providing the protection may worsen the situation, considering that oxidative stress is prevalent in such conditions.

Recently, in a couple of studies, Chang et al. [11, 36] have reported that a clerodane diterpenoid from Formosan *P. longifolia* var. *pendula* significantly inhibited the generation of superoxide anion and the release of elastase in activated human neutrophils in a concentration-dependent fashion, thereby demonstrating that the stem bark of *P. longifolia* possesses anti-inflammatory activity. Also, the bark extract has been shown to have high content of phytosteroids, and these may mimic estrogen structurally. However, the present study is not without certain limitations. It is a pilot study, and though we have used HPLC-purified bark extract, the bioactive principles need further characterization and receptor binding studies with pure isolated principles, and further their signaling pathways need to be elucidated. Another limitation in our study is the small sample size for analysis of the variables in the plasma/serum, and these observations need to be reproduced in a larger study cohort.

Based on current promising observations of our study, we hypothesize that the bark extract of *P. longifolia* var. *pendula* indeed possesses some bioactive components with strong anti-inflammatory and estrogen receptor modulating activity. However, there is no specific evidence that the bark extract acts as a SERM. As far as the survey of literature goes, such a study has not been conducted so far. However, future investigations are warranted for this medicinal plant to be exploited and screened for its potential as a true SERM in experimental animals and to favorably alter several other established markers of cardiovascular risk, which might play a significant role in treatment of menopause in near future.

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