



Pharmacological Study

Anti-inflammatory effect of *Pueraria tuberosa* extracts through improvement in activity of red blood cell anti-oxidant enzymes

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Abstract

Changing life style and over-nutrition causes low-grade inflammation (LGI), with obesity and hyper-lipidemia as basic factors. The physiological state polarizes macrophages to classical type (M1), which is pro-inflammatory and promotes ectopic fat deposition in the body. Both factors induce inflammatory cascade, where free radicals (FRs) play an important role. Thus, pharmacological and non-pharmacological interventions would be effective in the management of LGI and plant products would be used as food supplement or as a drug. Previously, a study has reported the anti-oxidant potential of methanolic extract of tubers of *Pueraria tuberosa* (PTME) and inhibitory role of tuberousin on lipopolysaccharides-induced expression of inducible nitric oxide synthase in macrophages in an *in vitro* study model. Here, the effect of PTME has been explored on carrageenan-induced inflammatory changes in rats. The activity of antioxidant enzymes in red blood cell hemolysate has been assessed. PTME was orally given to rats for 9 days and periodical changes (every 3rd day) in the activity/concentration of superoxide dismutase (SOD), catalase, reduced glutathione (GSH), lipid peroxides (LPO), and C-reactive proteins (CRP) were monitored. The PTME significantly prevented carrageenan-induced decline in GSH content, lowering of catalase and SOD activity, and rise in LPO and CRP in rats in a time-dependent, sequential manner. Thus, it could be suggested that the anti-inflammatory role of PTME is primarily mediated through its FR scavenging potential.

Key words: Anti-inflammatory, anti-oxidant, *Pueraria tuberosa*

Introduction

The changing life style and improper dietary habits are the causes of several chronic metabolic diseases including metabolic syndrome.^[1] The expanded adipose tissue and high immunological reactions in the body are some of the important factors.^[2,3] The low-grade inflammation (LGI) is also induced by hormonal imbalances, neurological hyper-stimulation, recurrent infections, autoimmune disorders, aging process, nutrition, life style, and environmental factors. A person with high caloric diet and western life styles are more prone to develop atherosclerosis, if pre-conditioned with LGI, involving various pathways e.g., oxidative stress and aberrant immune activity.^[4-6]

The excess accumulation of reactive oxygen species (ROS) has been reported to damage cellular macromolecules, leading to accumulation of lipid peroxides (LPO) or protein peroxides,^[7] resulting to systemic LGI. Thus, pharmacological and non-pharmacological approaches, with multi-targeted action would be helpful in the management of atherosclerosis and diabetes and other associated complications. Here exploring the medicinal plants, to be used as food supplement would be beneficial.

The tubers of *Pueraria tuberosa* Linn (*Fabaceae*), (PT) are already used as medicine by Ayurvedic physicians for the management of fertility disorders, general weakness, and also as anti-ageing.^[8] It is known as *Bidarikand* in Hindi and Indian Kudzu in English. Its various formulations are prescribed as nutritive, diuretic, expectorants, and for the management of rheumatism, fever, and bronchitis. Various *in vitro* experimental models earlier have established its anti-oxidant and anti-inflammatory property.^[9,10] Some of its other documented biological properties are anti-hyperglycemic, anti-hyperlipidemic, anti-fertility in male rats, and hepatoprotective.^[11-14] PT tubers

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are rich in isoflavonoids and terpenes with daidzein, puerarin, puerubersoanol, and tuberosin^[15,16] as bioactive phytochemicals.

However, its effect on *in vivo* model, especially against LGI has not been studied so far. Here, it is proposed to explore the effect of polar fraction of the tubers of *Pueraria tuberosa* Linn (*Fabaceae*), (PT) toward its anti-oxidant and anti-inflammatory potential, in an *in vivo* rat-model, where, carrageenan has been used to establish systemic LGI.^[17] A correlation of anti-inflammatory properties with activity of anti-oxidant enzymes in red blood cell (RBC) (erythrocytes) has been explored.

Materials and Methods

Materials

Carrageenan, trichloroacetic acid (TCA), nitroblue tetrazolium (NBT), sodium dodecyl sulfate (SDS), Riboflavin, were purchased from Hi Media, Mumbai, India. Other chemicals were of analytical grade. The experimental protocol was approved by the Institutional Animal Ethics committee of the Institute of Medical Sciences. (Dean/2011-12/208, dated 28-6-11).

Experimental design

The inbred albino rats of Charles Foster strain were purchased from the central facility of our Institute. They were acclimatized in the institutional laboratory condition for a week and then randomly divided into three groups, having six animals in each. The 1st Group was normal-control, where animals were kept on normal diet with drug vehicle only. The Group 2 was experimental control. Here, the animals received one dose of carrageenan through injection in the air-pouch (made on the back of each animal) and treated with drug vehicle. The extract-treated group was further divided into three sub-groups, where animals received different doses of *Pueraria tuberosa* (PTME) as 10 mg (3rd Group), 20 mg (4th Group), and 40 mg (5th Group) per 100 g body weight. The treatment was continued for 9 consecutive days. From each animal, blood was collected on 3rd, 6th, and 9th day of carrageenan administration and subjected to various biochemical tests as described below.

Estimation of lipid peroxides and C-reactive protein

The blood was collected in a plain tube and serum was separated. The degree of LPO was measured as thiobarbituric acid reactive substances (TBARS)/mg protein.^[18] Here, 100 μ l of serum was added to the reaction mixture (0.2 ml 10% SDS, 1.5 ml 0.8% TBA in water, 1.5 ml 20% acetic acid). It was mixed properly and heated in boiling water bath for 1 h. Finally, it was cooled and absorbance was read on 532 nm. Standard solution of tetra ethoxy propane (TEP) was used as a standard.

The C-reactive protein (CRP) was estimated by a commercially available kit (Hind diagnostic, Karaundi, Varanasi India). The 10 μ l of serum (with different dilutions) was mixed with a drop of the given reagent, on a ceramic plate. That dilution point was recorded, which did not show any agglutination. It was calculated by multiplying dilution factor (D) with 0.6 and expressed as mg/ml.^[19]

Assay of anti-oxidant enzymes

The blood was collected in a heparinized tube (0.5 ml) and RBC

was separated after centrifugation. It was washed two times with phosphate buffer saline (PBS) and finally re-suspended in 2 ml of distilled water to prepare the hemolysate, which was used as the enzyme source. The hemoglobin (Hb) content was measured in this by Drabkin's, reagent.^[20,21]

The superoxide dismutase (SOD) activity was measured by the method of Beauchamp and Fridovich, as described earlier.^[22,23] Here, the instant superoxide-ions were generated by photoreaction using NBT (0.75 mM) in the presence of riboflavin. The reaction mixture was incubated with varying concentrations of hemolysate, (the source of SOD). The change in absorbance was read at 560 nm with reference to time. Thus, the kinetics of formation of blue-formazone, in presence of SOD indicated its activity, which was expressed as units/mg Hb). The 1 unit was defined as the amount of enzyme capable to inhibit formazone formation by 50%.

The catalase activity was assessed by method of Ashru and Sinha.^[24] The reaction was initiated by adding H₂O₂ (20 mM) to 500 μ l of RBC hemolysate and after 1 min, 1 ml of 5% potassium dichromate-in acetic acid (3:1 ratio) was added. The absorbance was read at 620 nm and the activity was expressed as unit/mg Hb.

For measurement of reduced glutathione (GSH) content, 25 μ l of fresh heparinized blood was immediately transferred to a tube containing 50 μ l of metaphosphoric acid. It was mixed with 1 ml of precipitating reagent (0.5% m-phosphoric acid + 20 mM NaCl) and incubated for 20 min at room temperature. Finally, the reaction mixture was centrifuged at 2000 rpm. 800 μ l of its supernatant was mixed with 200 ml of freshly prepared 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) solution (in 0.03% sodium citrate, pH 6.8). Absorbance was taken at 412 nm and GSH content was reported as μ g/mg of Hb.

All data were expressed as mean \pm SD and Pearson's correlation analysis (SPSS 7.5 for Windows, SPSS Inc. IBM) was used to test the level of significance of correlation (Pandey *et al.*, 2007).

Observations and Results

Characterization of *Pueraria tuberosa*

The % yield of PTME was found to be 16.04%, which was calculated on the basis of weight of total raw material taken for extraction. The PTME showed the absence of proteins, proteinases and carbohydrates. It also failed to show any response on macrophages in relation to NO production, which indicated the absence of endotoxin in PTME. The content of tuberosin in PTME was found to be 11.5 mg/g of extract.

In vivo study of *Pueraria tuberosa* against carrageenan-induced changes in inflammatory markers in serum

In the experimental control group, the carrageenan injection did not raise the LPO and CRP content up to 6th day. However on 9th day, there was a significant increase in these parameters. In the PTME-treated group, significant inhibition was observed in the rise of LPO and CRP. The response was concentration-dependent [Table 1].

Effect of *Pueraria tuberosa* against carrageenan-induced changes in anti-oxidant enzymes in RBC

In the experimental control groups, carrageenan administration

induced significant decline in GSH content on the 3rd day, without any significant change in activity of catalase and SOD. On the 6th day, the activities of both of these enzymes were significantly dropped. This low activity was also observed on the 9th day. In contrast, PTME treatment significantly prevented these changes in a concentration-dependent manner [Table 2]. The fold change (calculated to normal value) in all the parameters on 9th day, has also been presented in Figure 1.

Discussion

Carrageenan is a high-molecular-weight sulfated polysaccharide. It produces an inflammation primarily by generating (1) excess free radicals (FRs), (2) release of histamine, serotonin, and bradykinin and also by (3) activation of toll-like receptor-4 receptors.^[25] FRs further produce LPO, resulting in initiation of inflammation, which raises CRP in the blood and other disturbance in the homeostasis of the cell function. It also depletes glutathione content of the cell and inactivates various anti-oxidant enzymes.^[26] Therefore, interruption at one of

these steps, by using herbal products, could be an effective method for controlling these pathogenic processes. These results clearly indicate that PT extract reduces the CRP in the last days of treatment, showing its anti-inflammatory property. Interestingly, at this time point, TBARS was also raised, (9th day), suggesting the role of FRs in the process, as lipid peroxidation is the end product oxidative stress. The FRs activate the expression of several inflammatory enzymes such as inducible nitric oxide synthase (iNOS), COX-2 etc., and cytokines, via activation of transcription factors like nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) and activator protein-1 (AP-1).^[27] These inflammatory markers affect the liver to induce expression of acute phase proteins, e.g. CRP. This indicated that inflammation by carrageenan is a delayed process and there must be several steps involved upstream with these changing parameters.

It was earlier reported the inhibitory potential of PT extracts against lipopolysaccharides (LPS)-induced nitric oxide release in macrophages and inhibition of iNOS expression.^[28] The tuberosin has been identified as the active principle, so the PTME has been standardized in terms of presence of tuberosin

Table 1: Effect of methanolic extract of *Pueraria tuberosa* tubers on carrageenan induced thiobarbituric acid and C-reactive proteins active substances

Days	Normal control	Experimental control	PT (mg/100 g BW)		
			PT (10 mg)	PT (20 mg)	PT (40 mg)
TBARS (mmol/mg protein)					
3	0.652±0.093	0.672±0.103	0.669±0.090	0.643±0.078	0.654±0.089
6	0.658±0.105	*0.780±0.095	0.753±0.097	0.756±0.085	0.683±0.113*
9	0.654±0.086	0.976*±0.105	0.964±0.103	0.878±0.098	0.656±0.092*
CRP (mg/ml)					
3	<0.6 mg/ml	1.2 mg/ml	0.6 mg/ml**	0.6 mg/ml**	<0.6 mg/ml
6	<0.6 mg/ml	2.4 mg/ml	1.2 mg/ml**	0.6 mg/ml**	<0.6 mg/ml
9	<0.6 mg/ml	3.6 mg/ml	2.4 mg/ml**	1.2 mg/ml**	<0.6 mg/ml

Significance level (P**<0.001) when compared with respective control values. PT: *Pueraria tuberosa*, TBARS: Thiobarbituric acid reactive substances, CRP: C-reactive proteins

Table 2: Effect of methanolic extract of *Pueraria tuberosa* tubers on changes in carrageenan induced glutathione, catalase, and superoxide dismutase

Days	Normal	Exptl. control	PTME (per 100 g/bw)		
			10 mg	20 mg	40 mg
GSH (µM/mgHb)					
3	177±2.09	98±1.98*	98±1.67	122±1.01	161±2.67*
6	165±3.08	91±1.54*	91±1.85	119±2.00	169±2.11*
9	169±2.67	89±1.87*	88±1.54	117±2.01*	170±2.07*
Catalase activity (U/mgHb)					
3	19.98±0.98	39.66±2.08*	41.98±1.98	42.98±1.78	18.06±1.34*
6	22.28±1.54	40.05±2.02*	34.28±1.74	37.28±1.51	17.56±1.62*
9	17.69±0.63	42.23±2.03*	41.90±2.06	37.67±1.32	18.63±1.28*
SOD activity (U/mgHb)					
3	82.43±4.06	86.33±3.71	95.78±2.86	85.78±3.06	95.78±2.86
6	75.20±3.70	126.60±3.90*	121.20±2.70	114.34±2.08	85.20±2.70*
9	84.66±3.91	158.67±3.60*	147.66±2.91	120.43±2.91*	87.66±2.91*

All the observations in different groups showed significant (P* < 0.05) relationship when compared with control. SOD: Superoxide dismutase, GSH: Glutathione, PTME: Methanolic extract of *Pueraria tuberosa*

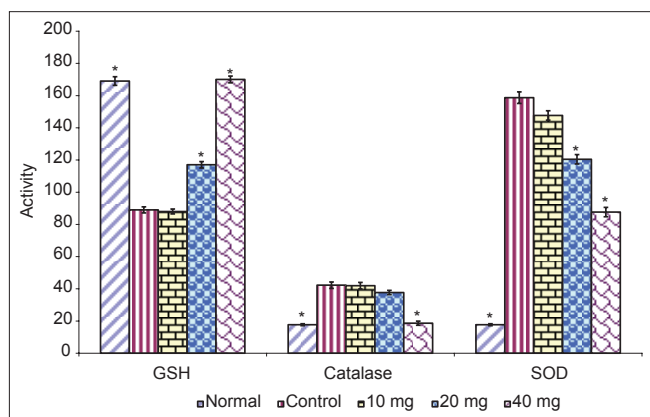


Figure 1: The fold change (calculated to normal value) in all the anti-oxidant enzymes on 9th day

in PTME. It is a polyphenolic compound of flavones group. Besides its FR scavenging property, PTME also possesses metal chelation property.^[10] These earlier reports support its observed anti-oxidant and anti-inflammatory potential.

Although there are several other metabolites in the blood, which act as FR trapper/scavenger, GSH plays a primary role. In the body, GSH is the 1st line of defense against oxidative stress. It directly neutralizes the FRs and works in association with other anti-oxidants such as vitamin E/C. They help in recycling the oxidized glutathione disulfide (GSSG) to reduced GSH.^[29] Thus, its reduction on 3rd day is logical because of its continuous utilization in trapping the carrageenan-induced FRs. This is also supported by no rise in CRP and TBARS on 3rd day.

However, the second line of protecting molecules is the anti-oxidant enzymes,^[30] which are located in the RBC. The SOD is found to be very high in mitochondria, because it is the primary site for super oxide generation during electron transport chain for adenosine tri phosphate production. When superoxides (SO) are dismutated, they produce hydrogen peroxide, which may produce high level of hydroxyl radical (OH), if not decomposed. The OH is another highly active FR. Thus, catalase comes to work for hydrolyzing H₂O₂ to water. Accordingly, the data show the decline in SOD and catalase activity on the 6th day. There might be compensatory increase in these enzymes in early days, but persistent over-load of FRs might reduce their level on 6th day. When all the protective tools in the system get exhausted, then lipid peroxidation is initiated by FRs. This has been observed by high TBARS and CRP on the 9th day. Its late rise suggests the net effect of FR-mediated lipid peroxidation and related inflammation.

The treatment with PT extract prevented the rise in CRP on the 9th day, which follows the rise in activity of SOD and catalase on the 6th day. The GSH level was also raised on the 3rd day. This reversal of anti-oxidant enzymes could be due to low FR stress in the blood. It could be because PTME directly traps the carrageenan-induced FRs and spares the use of GSH and other anti-oxidant enzymes from use. This logic is supported by our earlier report,^[10] where it has been shown that PT is capable to trap all species of FRs along with metal chelation property [Figure 2]. However, the rise in catalase and SOD activity could be also due to their high expression at the

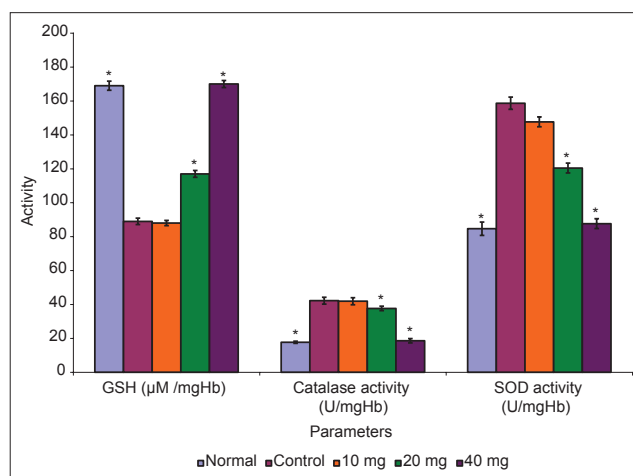


Figure 2: Activities of antioxidant enzymes - glutathione, superoxide dismutase, catalase and effect of *Pueraria tuberosa* on these enzymes

transcriptional/translational level, but our existing data are not enough to answer this question.

Conclusion

It can be concluded that the polar fraction of PT tuber (PTME) is rich in polyphenolic compounds. It traps the FRs and spares the use of GSH, catalase, and SOD in reducing FR stress. Its anti-inflammatory property (reported by low CRP and low TBARS) is primarily mediated through its FR scavenging potential, which is involved in the early steps of signal transduction in the process of inflammation.

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हिन्दी सारांश

विदारी कंद सत्व का शोथहर प्रभाव- आरबीसी एंटीऑक्सीडेंट एंजाइम कार्य वृद्धि के संदर्भ में

निधी पंड्या, दूर्गावती यादव, विवेक पंड्या, यामिनी बी. त्रिपाठी

जीवन शैली के परिवर्तन और अधिक भोजन के कारण जो शोथ होता है उसमें लो ग्रेड इनफ्लेमेशन एक महत्वपूर्ण लक्षण है। इसकी जानकारी हेतु रक्त में बढ़े हुए सी-रियेक्टिव प्रोटीन का बढ़ना बताया गया है। यह स्थिति मोटापा एवं हाइपरलिपिडिमिया एवं मधुमेह (मेटाबोलिक सिंड्रोम) में अधिक पायी जाती है। इसके बढ़े रहने से हृदयरोग एवं अन्य मेटाबोलिक रोग होने की संभावना बढ़ जाती है। इस प्रक्रिया में बढ़े हुए फ्री-रेडिकल की मुख्य भूमिका होती है। अतः इसकी चिकित्सा हेतु औषध द्रव्य, भोज्य पदार्थ एवं रहन-सहन में बदलाव के साथ-साथ औषधियों का सेवन लाभकर है। इस प्रक्रिया में विदारी कंद की जड़ का प्रयोग लाभकारी पाया गया। इसके मथनालिक सत्व में एन्टीआक्सीडेंट, एन्टीइनफ्लेमेटरी गुण मिला और इसमें ट्यूब्रोसीन नामक मुख्य अंश पाया गया। एक प्रयोग में देखा गया कि कैरजेनीन द्वारा उत्पन्न शोथ से संबंधित चूहों के रक्त में कैटिलेज, एस.ओ.डी. एवं ग्लुटाथयोन की कमी तथा लिपिडपराक्सिडेशन एवं सी. आर. पी. में वृद्धि होती है। तथा विदारी कंद के सत्व के उपयोग से इसे रोका जा सकता है। अतः इस आयुर्वेदिक औषधि का प्रयोग उपरोक्त रोगों की चिकित्सा में सफलतापूर्वक किया जा सकता है। यह एन्टीआक्सीडेंट इनजाइम्स को बढ़ाने एवं फ्री-रेडिकल्स को निष्क्रिय करने के माध्यम से कार्य सम्पन्न करता है।