Anti-hyperlipidemic and antioxidant potential of different fractions of *Terminalia arjuna* Roxb. bark against PX- 407 induced hyperlipidemia

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The three fractions diethyl ether, ethyl acetate and ethanol of *T. arjuna* exerted hypolipidemic and antioxidative effects at two different doses levels of 175 and 350 mg/kg body weight in Poloxamer (PX)-407 induced hyperlipidemic albino Wistar rats. The hypolipidemic and antioxidant effects of *T. arjuna* fractions were noticed as EtOH>diethyl ether>ethyl acetate. The results suggest that ethanolic fraction of *T. arjuna* possesses the potent properties of being antioxidant and hypolipidemic than other fractions. In turn, it has therapeutic potential for the prevention of coronary arterial disease.

**Keywords:** Antioxidant, Coronary heart disease, Hyperlipidemia, Poloxamer 407, *Terminalia arjuna*

Coronary heart disease caused by atherosclerosis continues to be a leading cause of mortality in developed and developing nations of the world. Hyperlipidemia: the disorders of lipid metabolism have been ranked as one of the greatest risk factors contributing to the prevalence and severity of atherosclerosis, stroke and coronary heart diseases. Hyperlipidemia is characterized by elevated serum total cholesterol, low density lipoprotein, very low-density lipoprotein (LDL, VLDL) cholesterol and decreased high-density lipoprotein (HDL) levels. Therefore, the treatment of hyperlipidemia may reduce the risk of developing ischemic heart disease or the occurrence of further cardiovascular or cerebrovascular disease in patients. Presently existing hypolipidemic drugs have been associated with a number of side effects. The consumption of synthetic drugs leads to hyperuricemia, diarrhoea, nausea, myositis, gastric irritation, flushing, dry skin and abnormal liver function. The herbal treatment for hypercholesterolemia has no side effects, relatively cheap and locally available. They are effective in reducing the lipid levels in the system. Although a variety of *in vitro* screening test systems for hypolipidemic agents have been developed, the results of these experiments do not always match with *in vivo* experiments. Poloxamer (PX)-407 induced hyperlipidemia in rats is one of the most commonly used animal models for the screening of hypolipidemic property of the drugs.

*Terminalia arjuna* Roxb. (Family: Combretaceae) is used in traditional medicine for the treatment of heart disease. It has wound healing, antibacterial, antmutagenic/anticarcinogenic, antioxidant and hypocholesterolemic properties. The active constituents of *T. arjuna* include tannins, triterpenoid saponins (arjunolic acid, arjunic acid, arjunenin, arjunglycosides), flavonoids (arjunone, arjunolone, luteolin), gallic acid, oligomeric proanthocyanidins (OPCs), polyphenols, calcium, magnesium, zinc and copper.

Anti-dyslipidemic and antioxidant activities of different fractions of *T. arjuna* stem bark in Triton WR-1339 induced hyperlipidemia in rats have been reported. In the present study, PX-407 induced hyperlipidemia has been studied in rats. In PX-407

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model, the stable lipid profile is maintained up to 30 h that reaches to normal lipid levels at the end of the 48 h. Whereas, in WR-1339 model, the altered lipid profiles is maintained up to 16 h and it reaches to normal lipid levels at the end of 24 h. Although the mechanism of induction of hyperlipidemia was identical in both the models, PX-407 model has been designed to investigate and document the longer duration of lipid lowering action and free radical scavenging property of the different fractions of T. arjuna up to 24 h.

Materials and Methods

Plant materials—T. arjuna supercritical powder was procured from M/s Elles Pvt Ltd, Chennai and authenticated by the Department of Botany, Centre for Advanced Research in Indian System of Medicine (CARISM), SASTRA University, Thanjavur. A voucher specimen (No.0066) has been deposited in the department.

Fractionation of T. arjuna—Sequential solvent fractionation method was adapted to fractionating the T. arjuna bark powder. Supercritical bark powder (1500 g) was soaked in petroleum ether for 7 days for defatting purpose. Similarly, in subsequence of every 7 days the fractionation was done with different solvents from diethyl ether (TA-01), ethyl acetate (TA-02) and ethanol (TA-03) in the same extract powder as described by Row et al. All the fractions were dried under reduced pressure and the brown colour precipitate was separated out.

Phytochemical screening—Phytochemical screening of fractions TA-01, TA-02 and TA-03 was performed to test the presence of phenolic compounds, tannins, glycosides, saponins, alkaloids and flavonoids in the bark of T. arjuna using appropriate tests.

Animals—Female Swiss albino mice (25-30 g) and male Wistar rats (150–250 g) obtained from National Animal Facility Centre, SASTRA University, Thanjavur, were housed individually in poly propylene cages and kept in a room maintained at an average temperature (22°C ± 3°C) and 55.6% RH, with 12:12 h L:D cycle (lights on, 06:30 – 18:30 h) and fed with standard laboratory diet (Tetragon Pvt. Ltd, Bangalore,) and water ad libitum. They were acclimatized for one week before the start of experiment. The animals were kept in cages with raised floors of wide wire mesh to prevent coprophagy. The study was conducted after obtaining institutional ethical committee clearance (27/SASTRA/IAEC/RPP).

Drugs and chemicals—Poloxamer-407 was obtained from M/s BSFA Pvt. LTD, USA. The biochemical kits for measuring the lipid profiles were procured from Biosys, Bangalore and Ketamine from Neo Pharmaceuticals, Bangalore., All other chemicals and solvents used in this study were obtained from Merck, India and were of analytical grade.

Acute oral toxicity study—In an acute toxicity study, using the up- and down-procedure, all fractions were suspended in peanut oil (OECD 425; accepted vehicle) and administered orally to female Swiss Albino mice. The mice were approximately 6 weeks old and weighed 25-30 g. The general procedure was as follows: one mice was administered with 175 mg/kg body weight and if no mortality or overt toxicity occurred within 48 h, another mice was administered with 550 mg/kg body weight. In the absence of toxicity, a third mice was administered with 2000 mg/kg body weight and, if again no evidence of toxicity was observed, two additional mice were administrated with 2000 mg/kg body weight level. In all cases the volume of test drug was fixed at 10 ml/kg body weight. The mice were observed for clinical signs of toxicity at 0-0.5, 0.5-1, 1-2, 2-4, and 48 h post dosing (with special attention during the first 4 h). The body weight of each mice was recorded prior to administration of test drugs administration and at 7- and 14-days post dosing. Once daily the mice were observed for changes in their skin fur, eyes and mucous membrane (nasal) and also respiratory rate, circulatory (heart rate and blood pressure), autonomic (salivation, lacrimation, perspiration, piloerection, urinary incontinence and defecation) and central nervous system (ptosis, drowsiness, gait, tremors and convulsion) changes. The blood plasma was collected through retro orbital sinus (1ml) for the hematological assessments. The mice were sacrificed by high intravenous dose (22 mg/kg body weight) of ketamine on 14 days post dosing. The time of death, if any, was recorded. Necropsy included a gross examination of all the major organs. Same protocol was adopted for all three fractions. The study was conducted in compliance with OECD Test Guideline 425 (Revised: 17 December 2001) and followed OECD GLP principles reports are interpreted with AOT425StatPgm software.

Selection of dose of the fractions—The lethal dose (LD₅₀) of T. arjuna fractions was calculated as per OECD guidelines for fixing the dose for biological
evaluation. The LD$_{50}$ of all three fractions of TA (TA-01, TA-02 and TA-03) falls under class four values with no signs of acute toxicity at 2000 mg/kg in mice. For biological evaluation in rats, the mice LD$_{50}$ dose was converted into rat dose based on the body surface area and metabolic rate constant. (FDA Guidelines, 2002). Hence, it was found that 1750 mg/kg and from which the biological evaluation was carried out at doses of 175 and 350 mg/kg in rat.

Poloxamer 407 induced hyperlipidemia—Rats were divided into following 10 groups of 6 animals each group. Group 1: control animals, Group 2: normal animals received vehicle (vehicle control), Group 3: PX-407 induced (diseased control) hyperlipidemic animals, Groups 4 and 5: hyperlipidemic animals received TA-01 (175 and 350 mg/kg), Groups 6 and 7: hyperlipidemic animals received TA-02 (175 and 350 mg/kg), Groups 8 and 9: hyperlipidemic animals received TA-03 (175 and 350 mg/kg), Groups 10: Hyperlipidemic animals received atorvastatin (0.4 mg/kg). All three fractions and atorvastatin were suspended in peanut oil and administered orally to the respective groups. Groups 2 and 3 were received peanut oil orally. After 18 h of treatment the animals were anaesthetized with ketamine (20 mg/kg) and midazolam (5 mg/kg) through intraperitoneal injection. Blood (1 ml) sample was withdrawn from retro-orbital sinus puncture and centrifuged at 2500 g for 10 min at 4°C and separated serum was stored in −20°C until the completion of biochemical analysis.

Biochemical analysis—All the samples were used for following biochemical investigations. The blood serum under this model has been analyzed for the marker parameters such as total cholesterol (TC), TC in (high density lipoprotein) HDL, (low density lipoprotein) LDL and (very low density lipoprotein) VLDL as well as TG. The HDL-TC was done by precipitation method$^{22}$. All the parameters were analyzed by semi auto analyzer (GSK-Qualisys, AK601) with biochemical kit$^{23,24}$.

Free radical scavenging activity—Lipid peroxide (LPO)$^{25}$. To 300 µl of the serum sample 1.5 ml of 10% (w/w) triple distilled water was added and was allowed to stand for 15 min at room temperature. The tube was centrifuged and to the supernatant 1.5 ml of TBA solution was added and was heated in a boiling water bath for 15 min. After cooling to room temperature, 3 ml of chloroform was added and the mixture was shaken vigorously for 3 min and centrifuged for 10 min at 1500 rpm. The absorbance of the chromophore was measured at 532 nm. A standard curve was constructed used TEP hydrolysed MDA containing 5-30 µg. The level of lipid peroxide: the thiobarbituric acid reactive substance (TBARS) was expressed as nano mole of malonaldehyde per mg of protein.

Reduced glutathione (GSH)$^{26}$: To 200 µl of the serum 0.4 ml of buffer, 0.2 ml sodium azide, 0.2 ml EDTA, 0.2 ml of hydrogen peroxide and 0.2 ml of reduced glutathione was added and made up to a volume of 2 ml with water. The tubes were incubated at 37°C for 10 min. TAA (1 ml) was added to terminate the reaction. The reaction mixture was centrifuged and with the supernatant, 8 ml of disodium hydrogen phosphate was added. DTNB (1 ml) was added just prior to the analysis. The absorbance was read at 412 nm against a blank which contained only 8 ml phosphate solution and 1 ml DTNB reagent. A standard graph was constructed using 20 to 100 µg of reduced glutathione. The activity was expressed as nmol GSH/mg of protein.

Statistical analysis—All the experimental results were expressed as mean ± SE. Data were analyzed by analysis of variance (ANOVA) followed Dunnett’s test with the level of significance set at $P<0.01$.

Results

Fractionation and phytochemical investigation—The yields (g %) of the fractions TA-01, TA-02 and TA-03 were found to be of 0.42, 0.978 and 24.16%, respectively. The yield of the ethanol fraction was much higher (i.e. 24.16%) than the other fractions. The preliminary phytochemical analysis revealed that above fractions contained phenolics, tannins, triterpenoid, saponins, anthraquinone glycosides, alkaloids and flavonoids.

Acute toxicity studies—The acute oral toxicological study didn’t show any deviation from the normal behaviour of the mice during the entire study period. So, there were no acute toxicological changes for the TA-01, TA-02 and TA-03 fractions of TA up to 2000 mg/kg. Hence, the biological evaluation was carried out at the doses of 175 and 350 mg/kg body weight.

Poloxamer 407 induced hyperlipidemia in rats

Diethyl ether fraction (TA-01)—Though decrease in body weight was observed, it was not significant. No reference had shown that T. arjuna fractions cause decrease in the body weight. Plant control group was required to prove this hypothesis. The drug was able
to decrease the TC and Tg level significantly ($P<0.01$). After 12 h analysis, the fraction decreased the TC and Tg significantly ($P<0.01$) but less significant after 24 h of TC level ($P<0.05$) and Tg were shown non significant ($P>0.05$) with disease control at low dose of 175 mg/kg, whereas in high dose i.e. 350 mg/kg, both 12 h and 24 h had shown a significant effect ($P<0.01$). After 24 h analysis, the fraction was able to decrease the LDL level by 18.71% and Tg level by 5.08% and 17.75% at 175 and 350 mg/kg body weight dose, respectively. These results show that the fraction is hypolipidemic in nature (Table 1). In both the dose levels, HDL has shown the non-significant change when compared to the disease control. The hypolipidemic effect of diethyl ether fraction has already been studied by WX-1339 and the results indicate that diethyl ether fraction is able to decrease cholesterol and triacylglycerol level.

**Ethyl acetate fraction (TA-02)**—No abnormal increase or decrease in body weight was observed during treatment with TA-02 when compared to the control groups. The drug was able to decrease the cholesterol and Tg level significantly ($P<0.01$). After 12 h analysis, the fraction has decreased TC and Tg significantly ($P<0.01$). But less significant after 24 h of cholesterol level ($P<0.05$) and triglycerides had shown non significant ($P>0.05$) with disease control at low dose of 175 mg/kg, whereas in high dose i.e. 350 mg/kg, both 12 h and 24 h analysis had shown a significant effect ($P<0.01$). After 24 hr analysis, the drug was able to decrease the level of cholesterol, LDL, Tg, VLDL and increase the level of HDL at 175 and 350 mg/kg body weight respectively but the lipid lowering activity was not significant ($P>0.05$) (Table 1). The hypolipidemic effect of ethyl acetate fractions was not yet studied.

**Ethanol fraction (TA-03)**—No abnormal increase or decrease in body weight was observed during treatment with ethanol fraction when compared to the control groups. The EtOH fraction was able to decrease TC and Tg level significantly ($P<0.01$). After 12 h analysis, the fraction decreased the total cholesterol ($P<0.01$), triglycerides ($P<0.01$) and HDL ($P<0.05$) significantly at low dose of 175 mg/kg, whereas in high dose i.e. 350 mg/kg, both 12 h and 24 h analysis has shown a significant effect ($P<0.01$) in cholesterol, triglycerides and HDL at the doses of 175 and 350 mg/kg body weight respectively. After 24 h analysis, the drug was able to decrease the level
of cholesterol by 17.52 and 28.3 %, LDL level by 26.16 % and 41.12 % and TGL by 17.07 and 30.01 % at the doses of 175 and 350 mg/kg body weight, respectively (Table 1).

**Free radical scavenging activity**

*Lipid peroxide (LPO)*—Changes in the lipid peroxide in serum was studied for three fractions at high dose level. The inhibition of lipid peroxidation was significant with standard, DE and EtOH fractions ($P<0.01$) treated groups, when compared to the hyperlipidemic control. Non significant effect was shown by the EA fraction ($P>0.05$) (Fig. 1a).

*Reduced glutathione (GSH)*—Changes in the reduced glutathione formation in serum was studied for three fractions at high dose level. The inhibition of reduced glutathione was significant in standard ($P<0.01$) and EtOH fraction ($P<0.05$) treated group when compared to hyperlipidemic control group, whereas diethyl ether and ethyl acetate groups have shown the non significant ($P>0.05$) effect when compared to the disease control (Fig. 1b).

**Discussion**

PX-407 causes these effects by activating HMG-CoA reductase activity and by inhibiting lipoprotein lipase activity. PX-407 induced hyperlipidemia is one of the animal model used for evaluation of hypolipidemic activity of drugs. The PX-407 inducing hyperlipemia model has shown relatively low species differences between experimental animals. Treatment of hyperlipidemic rats with *T. arjuna* fractions TA-01, TA-02 and TA-03 at the doses of 175 and 350 mg/kg po reversed the serum levels of lipid with varying extents. The order of lipid lowering activity by these fractions in above model is: EtOH>diethyl ether>ethyl acetate. The hypocholesterolemic effect of fractions of TA may be due to interference with the absorption of dietary cholesterol as well as bile acids from the intestine, increased elimination of faecal sterols, increased stimulation of bile acid synthesis may lead to an increased utilization of cellular free cholesterol.

Atorvastatin and test fractions of TA are able to decrease the level of LDL-TC. Thus, the present study suggests that the herbal product is a potential agent for reducing or controlling atherogenesis and cholesterol deposition in body tissues including blood vessels which are further strengthened by reduction in the levels of VLDL-TC a precursor of LDL. Low LDL-cholesterol could be due to decrease of VLDL-cholesterol synthesis and secretion from the liver leading to a long term decrease in LDL concentrations. Lowering elevated
levels of LDL cholesterol can retard progression of atherosclerosis.\(^{36,37}\)

Hypertriglyceridemia is a possible risk factor for the development of ischaemic heart disease. The atherogenic property of Tg is related to its lipoprotein transport and metabolism. In hypertriglyceridemia, one expects a marked reduction in clearance of VLDL and LDL which are highly atherogenic. Hypertriglyceridemia is also associated with hypercoagulability due to decreased fibrinolytic activity.\(^{38}\) The results of the present study match well with reported value of Ghatak et al.\(^{39}\) Here the hypcholesteolemic effect of TA results from the increased elimination of cholesterol feces. TA is an important component of increase in the fecal excretion of cholesterol and enhances the serum/plasma lecithin cholesterol acyl transferase (LCAT) activity in addition to accumulation of receptor mediated catabolism of LDL.\(^{40}\)

Hypolipidemic action is mainly due to its anion exchange property. The hypolipidemic action of gugulipid is due to its chloride retention and bile acid sequestration power.\(^{41}\) Such distinctive exchange property is attached here by increase LDL, VLDL, Tg and increases HDL Level. HDL-cholesterol levels reported in human studies have been inconsistent with reports of increases, no effects and decreases in the treated animals.

The data of the present study have shown that ethyl acetate, diethyl ether and ethanol fractions of TA have exerted the lipid lowering activity in vivo. This suggests that arjunic acid as well as its derivatives when undergo biotransformation through hepatic drug metabolizing cascade, produce common active molecules which may be responsible for lipid lowering activity in vivo. The quantity of arjunic acid/ arjunoglycoside (I, II, III and IV) in solvent ether fraction and ethyl acetate is comparatively very less than those of its derivative in ethanolic fraction and due to this, at the same doses ethanolic fraction are more effective than solvent ether and ethyl acetate fraction.

**Conclusion**

In conclusion, the results suggest the effectiveness of different fractions of *T. arjuna* as hypolipidemic and antiatherogenic agent based on its ability to inhibit LDL atherogenic modifications and lipid peroxides formation in hyperlipidemic rats. The flavonoids, tannins and/or phenolic rich fractions intake in the form of diethyl ether, ethyl acetate and ethanolic fraction to PX-407 rats, resulted in beneficial effects on serum lipids, lipoprotein concentrations and antioxidant activities, and thereby delayed the onset of atherosclerosis. Moreover, the ethanolic fraction shows the better effect than the remaining two fractions. The multi-targeted action of ethanolic fraction is due to the presence of beta sitosterol as well as flavones acting on the intestinal absorption of cholesterol and inhibiting the HMG CoA reductase enzyme respectively. However, studies are required in human subjects to prove its clinical efficacy as a hypolipidemic agent.

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