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Semecarpus anacardium L, nuts inhibit lipopolysaccharide induced NO production in rat macrophages along with its hypolipidemic property

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Traditionally *S. anacardium* is used for rejuvenation, rheumatoid arthritis, fever and neurological disorders. In the present study it was observed that a fraction of *S. anacardium* at dose of 1 mg/100g body wt, significantly reduced serum cholesterol from 378.87 mg/dl in the rats fed with atherogenic diet (AD) to 197.99 mg/dl (45-52%) in the rats fed with AD diet and increased serum HDL-cholesterol (33-37%). The same fraction also inhibited LPS induced NO production in the culture activated rat peritoneal macrophages in the dose dependent manner with IC_{50} value at 50 ng/ml of the culture medium. The drug in the above doses was completely safe and non-toxic, (no change in the enzymes), to liver and kidney functions.

Keywords: Anti-atherosclerotic, Anti-inflammatory, Herbal medicine, Hypolipidemic, Macrophage function, Nitric oxide, *Semecarpus anacardium*.

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Coronary heart disease has now been identified as the manifestation of inflammation and hyperlipidemia[1]. High level of oxidized LDL and low HDL is the basic cause of endothelial dysfunction including wall thickening and fat infiltration[2]. Activated macrophages play a crucial role in the transport of lipid to the vessel wall, along with the production of several cytokines, inflammatory molecules and growth factors[3]. Thus it appears logical to look for a safe medicine, which has both the properties in one formulation. In this paper, the water extract of the whole nut of *Semecarpus anacardium* (purified as per Ayurvedic method) has been investigated under *in vivo* and *in vitro* models in albino rats. For *in vivo* study the standardized plant extract of *S. anacardium* nuts was orally given to rats, kept on normal diet and atherogenic diet for 90 days. At the end of the experiment, peritoneal macrophages were isolated and their response to lipopolysaccharide (LPS) induced NO production was investigated. Further, blood was collected from jugular vein to study lipid profile, kidney and liver function by using standard commercially available kits for different biochemical parameters. Under *in vitro* study thioglycolate-induced macrophages were isolated from normal control rats and cultured. These cells were pre-incubated with different concentrations of sterilized extract of *S. anacardium* and then LPS induced NO production was assessed. Since *Semecarpus anacardium* is treated as the toxic drug in the Ayurvedic literature thus its general effect on liver and kidney function was also observed in rats of different groups.

In the Ayurvedic system of medicine, different preparations of purified nuts of *Semecarpus anacardium* Linn f. (Anacardiaceae) are recommend for insanity, fever, dysentery, loss of appetite, neurological disorders, rasayan drug (rejuvenating drug)[4]. Chemically it contains a number of flavanones like jeediflavanone[5], carpuflavanone, semecarpuflavanone[6] and galluflavanone etc.[7]. Several experimental findings have shown its anticancer property[8-9] and for the management of rheumatoid arthritis[10]. Recently it has been reported that the alcoholic extract of this nut, arrests the cell cycle in the DU-145 prostate cancer cell lines[11] and also on other cells[12]. Although previous workers have shown the hypo-lipidemic effect of de-fatted nuts of *S. anacardium* in rabbits[13], but no such report is available with its oil fraction, on lipid profile and on macrophage functions. For the first time, we have investigated its effect on the above parameters to develop a new anti-inflammatory phytomolecule and also to understand its mechanism of action for its traditional use to prevent inflammation.

Chemicals—Diagnostic kits for total cholesterol was purchased from Diagnostic system International, Cadila Healthcare Ltd., HDL Cholesterol kit was purchased from Zydus Pathline, India. Serum Urea and Serum Glutamate-Pyruvate Transaminase (GPT) kits were obtained from Hind Diagnostic, Varanasi. RPMI-1640 culture media was purchased from Himedia Laboratories Ltd. Mumbai. Rat chow was procured from Pashu Ahar Kendra, Varanasi. Inbred animals of HM strain were procured from the central animal facility of the Institute. The Institute Ethical Committee approved all the protocols.

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Preparation of extract and atherogenic diet—Nuts of *Semecarpus anacardium* were purchased from the Ayurvedic Pharmacy, Banaras Hindu University (BHU), Varanasi, India. Its authenticity was verified by compairing it with the authentic sample, preserved in the herbarium of the Department of Dravyaguna, Faculty of Ayurveda (BHU). The specimen voucher of the studied material was also preserved in the Department herbarium as voucher number MC/YBT/31. Only those nuts were selected for processing, which were drowned into water. They were filtered and dried. The caps of these nuts were removed and then they were broken in to small pieces. It was mixed with the coarse brick powder for 2 days. Then, it was properly washed with the cold water and boiled for 4 hr with distilled water. The upper layer of water, rich in oil fraction (froth-emulsion), was saved and concentrated in low-pressure distillation system. The concentrated fraction was standardized in terms of weight of nuts taken and final volume of the concentrate of oil rich water emulsion (w/v). This *S. anacardium* extract (SA extract) was divided into small aliquots and frozen until experimental use. SA extract was used as stock solution and diluted as per dose requirement.

The atherogenic diet (AD) consists of rat chow-76.2%, milk powder-3.5%, salt- 1.0%, multivitamin-0.1%, cholesterol-2.5%, hydrogenated fat-15.7%, cholic acid-1.0%. It was prepared in small batches, enough for 4 days and kept refrigerated.

Experimental design

In vivo study—Normal albino rats (100-150 g body wt) were randomly divided into four groups (6 animals in each). Animals of Group 1 (Sham control) received the drug vehicle only (1 ml gum acacia, 3% w/v) for three months with normal diet. Group 2 animals received AD, (20 g/day), along with drug vehicle. Group 3 animals received AD diet, (20 g/day) and SA extract, (1 mg/100 g body wt). Group 4 animals received SA extract in the above dose with normal diet. The experiment was continued for 90 days and finally, animals were anaesthetized. Peritoneal fluid was collected in Hank's balance salt solution (HBSS) under sterile condition to isolate the macrophages. The macrophages were isolated from the peritoneal fluid by centrifugation at 250 *g* for 10 min. at 4°C. The isolated macrophages were resuspended in HBSS and counted by haemocytometer. Equal number of macrophages were taken into plate and allowed to attach for 2 hr. The unattached cells were washed out and the attached cells were supplemented with 10% serum rich culture medium. They were exposed to 25 ng/ml of LPS and after 24 hr, medium was isolated to estimate NO by Griess Reagent[14]. From the same animals, venous blood was also collected and other parameters like lipid profile, liver and kidney functions were studied using standard commercial kits. Cholesterol level in plasma was determined by CHOD-PAP: enzymatic photometric test (Artiss *et al*[15].) and HDL-cholesterol by (Allen *et al*[21].) SGPT by 2,4-DNPH method (Rej *et al*[22].) and urea by Berthelot method (Fawlett *et al*[23].).

In vitro study—In another set of experiment, effect of SA extract was studied on activated rat peritoneal macrophages, isolated from the normal healthy rats. Thioglycolate 4% (1 ml) was injected (ip) to these animals and after 4 days, macrophages were isolated. These activated cells were purified and cultured on glass plates (50 mm), as described above. The plates were divided into three different groups. Group A was kept as normal, Group B was treated with LPS (25 ng/ml) and Group C was further divided into 5 subgroups and treated with different doses of SA extract along with LPS (25 ng/ml). After 24 hr of incubation, the culture medium was isolated to determine NO level. The attached cells were carefully subjected to methylene viability test to check number of viable cells and its correlation to NO production.

Culture of macrophages—Equal number of cells were plated in glass plates (50 mm) and kept for 2 hr under humidified incubator maintained with 5%CO_2 at 37°C to attach the cells. Attached cells were finally washed three times with normal saline, and then cultured in RPMI-1640 medium, supplemented with $NaHCO_3$ (2 g/l), penicillin (100 IU/ml), streptomycin (100 µg/ml), gentamicin (20 µg/ml) and fetal calf serum (10%)[16].

Peritoneal macrophages, isolated from the animals, kept on atherogenic diet (AD), showed more production of NO (9-11 $\mu M/3X10^6$ macrophage cells) as compared to the cells isolated from the rats eating normal diet- (5-7 $\mu M/3X10^6$ macrophage cells) in response to same LPS concentration. This showed that the cells in AD feeding rats were more sensitive to LPS response. However, the animals feeding on AD along with SA extract did not show such hyper response to LPS (Table 1).

Lipid profile and serum enzymes in these animals indicated that SA extract significantly increased the serum HDL-cholesterol ratio in the rats fed with the

atherogenic diet. There was significant prevention in the rise of serum cholesterol due to atherogenic diet intake. Surprisingly, the animals kept on atherogenic diet showed significant rise in SGPT and serum urea, which could be due to presence of cholic acid in the diet. However, the animals kept on AD and SA extract did not show such adverse effect of AD. This showed that SA extract was somehow protecting these organs probably by safe and quick metabolism of added cholic acid and cholesterol. (Table 1).

In vitro results indicated that thioglycolate activated macrophages were hypersensitive to LPS and produced NO in the range of 33-36 $\mu M/3\times10^6$ cells, whereas normal animals produced NO in the range of 9-11 $\mu M/3\times10^6$ cells under similar conditions. However, NO production was significantly inhibited by simultaneous and pre-incubation with SA extract in the dose dependent manner (Table 2). This indicated a strong anti-inflammatory property of this fraction with IC_{50} value at 50 ng/ml.

The present results of lipid profile indicated that SA extract had an effect on lipoprotein metabolism. The hypocholesterolemic property of SA extract could be because of—(1) lower intestinal absorption of the dietary cholesterol; or (2) enhanced excretion of ingested cholesterol through the liver. Inhibition of HMG CoA reductase, the key enzyme of cholesterol biosynthesis, which is yet another possibility of cholesterol lowering may not be the case in this study, because SA extract lowered the serum cholesterol even in the presence of the dietary cholesterol feeding. This supported the first two possibilities. Wong *et*

Table 1—Effect of *Semecarpus anacardium* extract on different parameters of rats *in vivo* study.
[Values are mean ± SD of six animals]

Group ↓	LPS response on macrophage	Serum cholesterol (mg/dl)	HDL-cholesterol (mg/dl)	HDL -ratio	SGPT (IU/L)	Urea (mg/dl)
CD sham	6.95 ± 0.33[a]	44.22 ± 2.74	15.28 ± 1.23	53.87 ± 10.12	56.58 ± 11.44	31.98 ± 3.65
AD sham	9.87 ± 0.45[a]	378.87 ± 24.18[a]	19.37 ± 1.59[a]	5.90 ± 1.00[a]	227.55 ± 23.38[a]	189.84 ± 17.57[a]
AD+ SA extract*	7.19 ± 0.34[a]	197.99 ± 10.43[a]	26.25 ± 3.32[a]	15.24 ± 2.84[a]	43.66 ± 3.92[c]	41.62 ± 3.56[a]
CD+ SA extract*	7.31 ± 0.16[a]	69.10 ± 9.60[a]	29.53 ± 1.76[a]	77.06 ± 14.83[b]	54.54 ± 7.00[a]	34.11 ± 2.27[a]

[HDL Ratio =HDL cholesterol/Total cholesterol-HDL cholesterol X 100]
NO was estimated as - NO_2^- $\mu M/3\times10^6$ macrophage cells by Griess Reagent. Statistical significance of LPS response on macrophages is in reference to normal cells i.e. without LPS (4.48 ± 0.28)
Cell viability was measured as methylene blue uptake in form of absorbency at 660 nm, which was constant in all the groups (0.587-0.693) showing no cell death due to extract or LPS in the tested concentrations.
* SA extract given to rats, at the dose of 1 mg/100 g body wt.
P value [a] <0.001, [b]<0.01, [c]<.05 compared to control
Statistical significance of LPS response on macrophages is in terms of no production. It is with reference to the normal cell response to LPS that is 4.48 ± 0.28 $\mu M/3\times10^6$ *cells*.

Table 2—Effect of different concentration of SA extract on LPS induced NO production and cell viability on thioglycolate induced rat macrophages
[Values are mean ± SD of 8 experiments of CD, 12 of AD and 6 of drug treated]

Group →	Normal	LPS (25ng/ml)	LPS (25 ng/ml)+ *S. anacardium* (ng/ml)				
Parameter↓			15	45	90	120	150
NO	10.24 ± 2.31	35.94 ± 3.24[a]	26.55 ± 2.68[a]	19.38 ± 2.48[a]	12.48 ± 0.183[c]	12.69 ± 0.11[b]	8.60 ± 0.74
M B	0.693 ± 0.02	0.774 ±0.11[c]	0.745 ± 0.01[a]	0.730 ± 0.01[a]	0.727 ± 0.009[a]	0.0.713 ± 0.005[b]	0.689 ± 0.09

NO estimated as NO_2^- $\mu M/3X10^6$ macrophage cells.
IC_{50} concentration is 50ng/ml
MB methylene blue uptake in form of absorbency at 660 nm.
P value [a] <0.001, [b]<0.01, [c]<.05 as compared to control

al[17] have shown that several plant sterols inhibit intestinal absorption of the dietary cholesterol. Therefore, SA extract might be acting at the absorption level through its similar phytochemicals (sterols). Secondly the efficacy of cholesterol lowering property of this fraction is higher in comparison to alcoholic extract of de-fatted nuts. Sharma *et al.*[13] have shown reduction (73.3%) in serum cholesterol at the dose of 500 mg/kg body wt. in rabbits, which is 50 times higher in comparison to our results where there is 59% of reduction of serum cholesterol at the dose of 10 mg/kg body wt.

Prevention in the rise of serum urea and SGPT in AD fed animals supplemented with SA extract could be due to safe and rapid excretion of cholic acid and cholesterol. There is a report on slight abnormality in the liver and kidney functions of the animals kept on diet rich in cholic acid [18].

It has been calculated that increase in HDL by 1 mg/dl reduces 2-3% risk of CHD [2]. The present results indicated that even at this low dose, SA extract enhanced the level of HDL by 1.4 times in the animals fed with AD and normal diet. Thus, it appeared that low dose of *S. anacardium* as diet supplement, could protect an individual from the cardiovascular pathology. Although, *Semecarpus anacardium* is treated as a toxic drug in the Ayurvedic literature and should be taken with caution but no adverse response of this fraction was observed on liver and kidney function at the doses used in the present study.

Prevention of LPS induced NO production is an established model to study the anti-inflammatory property of an agent[19]. LPS acts through activation of NFkB transcription factor and produces NO, which is involved in the production of cytokines and other inflammatory factors[20]. Inhibition of NO production by SA extract in a dose-dependent manner indicated its inhibitory role on this transcription factor followed by inhibition of other inflammatory cytokines. SA extract possesses a series of substituted phenolic compounds and variety of flavones, which have known antioxidant property. Besides, S/A extract also possesses anacardic acid, having variety of actions such as inhibition of phoshodiesterase-5 activity[24]. Thus, the traditional use of different preparations of *Semecarpus anacardium* with reference to its anti-inflammatory property and anti-tumour progression property may be attributed to these phytochemicals. Purification step of nuts of *S. anacardium* has not been well studied on the scientific parameters, but probably mixing of crushed nuts with the brick powder might be helping in the removal of the juice and oily part (polar fraction) of nut through absorption[4]. Lower dose of this drug is of great concern, because at higher doses, these phytochemicals may induce apoptosis followed by cell death, which is also evident by the trypon blue exclusion test (data not shown). Anti-proliferation response of *S. anacardium* fraction on DU-145 transformed prostate cancer cell lines[11] and effect of some of its pure constituents on induced programmed cell death in other cell lines[12] support this finding.

Thus, it could be suggested that the oil fraction of *S. anacardium* had a potential to provide a new molecule, which could have a holistic approach for the management of atherosclerosis, through mechanism of anti-inflammation, hypolipidemic and HDL enhancing property.

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