Extract of gum resins of *Boswellia serrata* L. inhibits lipopolysaccharide induced nitric oxide production in rat macrophages along with hypolipidemic property

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Received 20 April 2004; revised 15 January 2005

*Boswellia serrata*, Linn F (Burseraceae) is commonly used in Indian system of medicine (Ayurvedic) as an anti-inflammatory, analgesic, anti-arthritic and anti-proliferative agent. This study was planned to investigate the water-soluble fraction of the oleoresin gum of *Boswellia serrata* (BS extract) on lipopolysaccharide (LPS) induced nitric oxide (NO) production by macrophages under *in vivo* and *in vitro* conditions. In the previous condition, rats were fed on atherogenic diet (2.5% cholesterol, 1% cholic acid, 15.7% saturated fat) along with the BS extract for 90 days. Blood was collected for lipid profile and toxicological safety parameters. Peritoneal macrophages were isolated and cultured to see the LPS induced NO production. Under *in vivo* experiment, BS extract significantly reduced serum total cholesterol (38-48%), increased serum high-density lipoprotein-cholesterol (HDL-cholesterol, 22-30%). Under *in vitro* experiments with thioglycolate activated macrophages, it inhibited LPS induced (NO) production with IC$_{50}$ value at 662 ng/ml. Further, this fraction, in the dose of 15 mg/100 g body wt for 90 days, did not show any increase in serum glutamate-pyruvate transaminase (SGPT) and blood urea in normal control animals. However, it significantly reversed the raised SGPT and blood urea in the atherogenic diet-fed animals. Transverse section of liver and kidney also supported its protective effect. Thus it may be concluded that water extract of *Boswellia serrata* possesses strong hypocholesteremic property along with increase in serum HDL. It inhibits the LPS induced NO production by the activated rat peritoneal macrophages and show hepato-protective and reno-protective property.

**Keywords:** Anti-atherosclerotic, Anti-inflammatory, Ayurveda, *Boswellia serrata*, Herbal medicine, Hypolipidemic, Macrophage function, Nitric oxide

Hyperlipidemia and inflammation are considered as major risk factors for coronary heart disease (CHD). Blood cholesterol and lipid are distributed in different types of lipoproteins namely chylomicrons, very low-density lipoproteins (VLDL or pre-β-lipoproteins), low-density lipoproteins (LDL or β-lipoproteins), and high-density lipoprotein (HDL or α-lipoproteins). They are synthesized in liver and intestine. Among lipoproteins, HDL-cholesterol is considered as good cholesterol as it is responsible for reverse transport of the cholesterol from tissue store to liver for its metabolism and excretion. It also enhances endothelial repair, stabilizes prostacyclin and protects against lipid peroxidation. Other lipoproteins are considered as atherogenic. Under the influence of free radical attack, LDL is oxidized (Ox-LDL) and taken up by the monocytes derived macrophages through the scavenger receptors to be converted to foam cells, responsible for plaque formation. Thus, high level of oxidized LDL and low-level of HDL is considered as one of the parameters leading towards endothelial dysfunction including wall thickening and fat infiltration.

Recent evidences indicate inflammation as another important factor for atherosclerosis. Release of different cytokines such as Interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), activation of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) are the factors, which have been associated with the progression of inflammation and atherosclerosis. Thus, it appears logical to look for a safe medicine, which has both the properties in one i.e. hypolipidemic as well as anti-inflammatory.

In Ayurvedic system of medicine, *Boswellia serrata* L. (Burseraceae) has been used as a traditional remedy for inflammation, pain, arthritis, chronic colitis, ulcerative colitis, Crohn’s disease, bronchial...
asthma and brain oedema. B. serrata, also known as Sallai Guggal, grows in hilly areas of Western, Central India and Coromandel Coast. Boswellia tree is a green/ash/yellow-colored exfoliating bark and its gum is used as medicine. The oleo gum resin exudates of B. serrata consists of 23.5% Boswelli gum and 55% resin, D-galactose (46.22%), D-arabinose (11.6%) and their uronic acid hydride (30.9%) with small amounts of D-mannose and D-xylose. It also contains methyl chavicol, α- and β-amyrin and a diterpene alcohol (Serratol) (C_{22}H_{34}O). Besides, there are eight tetracyclic triterpene acids in the resin, whose structure have been identified as β-boswellic acid, acetyl-β-boswellic acid, acetyl-11 keto-beta-boswellic acid (AKBA), keto-beta-boswellic acid (KBA), 3-α-acetoxytirucall-8, 24-diene-21-oic acid, 3-α-hydroxytirucall-8, 3-β-hydroxytirucall. Although this plant product is in extensive use by the Ayurvedic physicians, the role of water-soluble fraction on macrophage function and its detailed toxicological study with atherogenic diet has not been reported.

In this communication, the water extract of B. serrata has been investigated under in vivo and in vitro models in albino rats to answer the above questions. For in vivo study, the standardized water extract of B. serrata was given orally to rats (15 mg/100 g body weight), with and without atherogenic diet for 90 days and serum HDL, total cholesterol, urea, creatinine and SGPT were determined. Further peritoneal macrophages were isolated to assess its response against lipopolysaccharide (LPS) induced NO production. Under in vitro study, thioglycolate activated peritoneal macrophages were used to study the protective response against LPS pretreatment (25 ng/ml).

Materials and Methods

Chemicals—Diagnostic kit for lipid profile was purchased from Diagnostic System International, Cadila Healthcare Ltd and Serum Urea and Serum Glutamate-PyruvateTransaminase (GPT) kits from Hind Diagnostics, Varanasi. RPMI-1640 culture media, antibiotics and fetal calf serum were purchased from Himedia Laboratories Ltd. Mumbai. Rat chow was procured from Pashu Ahar Kendra, Varanasi. Animals of inbreed nature of HM strain were procured from the central animal facility of the Institute. The Institute Ethical Committee approved all the protocols.

Preparation of atherogenic diet—The atherogenic diet (AD) was prepared by mixing 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.

Preparation of the extract of B. serrata oleoresin and its standardization—The authentic raw material (oleo-gum resin of B. serrata), was purchased from the Ayurvedic pharmacy, Banaras Hindu University and preserved in the departmental herbarium as voucher number MC/YBT/20. For standardization purpose, the alcoholic extract of B. serrata was prepared by soxhlet extraction method. The solvent free extract was again dissolved in toluene and subjected to thin layer chromatography (TLC) on silica G in different solvent systems. Major spots were identified in different batches of raw material (Table 1). However, for biological experiments, raw oleo-gum resin of B. serrata was purified as per classical Ayurvedic method by boiling with double distilled water for 4 hr. Water was added to maintain its volume in the range of 1 : 16, during the boiling period. After 4 hr, it was cooled and smashed properly and then filtered by cheesecloth. The filtrate was dried on slow heating device and this powder was used for experiments.

Experimental design

In vivo study—Normal albino rats (140-150 g body wt.) were randomly divided into 4 groups of 6 animals each. Animals of Group 1 (normal control) received normal diet with the drug vehicle only (1 ml gum acacia, 3% w/v). Group 2 animals were kept on atherogenic diet (AD), 20 g/day and drug vehicle (sham control). Group 3 animals received AD diet, as in group 2 and B. serrata extract, 15 mg/100 g body wt.
wt. Group 4 animals received only B. serrata extract in the above dose along with normal diet. After 90 days of treatment, animals were anaesthetized. Peritoneal fluid was collected in Hank’s balance salt solution (HBSS) under sterile condition to isolate the macrophages. It was centrifuged at 250 g for 10 min at 4°C. The pellet rich in macrophages were re-suspended in HBSS and counted by haemocytometer. Macrophage cells (3x10^6) were taken to different 50 mm culture plates, and allowed to attach for 2 hr in incubator, maintained at 37°C with 5% carbon dioxide. After 2 hr, unattached cells were washed off and the attached macrophages were 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 inactivated FCS (10%) in incubator as maintained above. The attached cells were exposed to 25 ng/ml of LPS (final concentration) and after 24 hr; culture supernatant was isolated to estimate NO by Griess reagent. From the same animals, blood was also collected by cardiac puncture and parameters related to lipid profile, liver and kidney functions were assessed. In brief, cholesterol by CHOD-PAP: enzymatic photometric test, HDL-cholesterol by the method of Allen et al., SGPT by 2,4-DNPH method and urea by Berthelot method. Further liver and kidney were dissected out and fixed in 10% formaldehyde solution and processed for routine histological examination. Serial sections (5µm thick) were cut by manual microtome and were stained by Hematoxylin and Eosin. Degrees of necrosis was observed in ten different fields in each group.

In vitro study

Cultures of macrophages—In another set of experiment, effect of B. serrata extract was studied on activated rat peritoneal macrophages. Here, 1 ml thioglycolate (4%) was injected intraperitoneally (ip). After four days, peritoneal fluid was collected; macrophages were isolated and cultured in 50 mm glass petridishes, as described above. The plates were divided into three different groups. Group A was kept as normal, Group B was treated with lipopolysaccharide (LPS; 25 ng/ml) and Group C was further divided into 5 sub-groups and treated with different doses of B. serrata extract along with LPS (25 ng/ml). After 24 hr of incubation, the culture supernatant was collected to determine NO level. The attached cells were carefully subjected to methylene blue viability test to check the number of viable cells and its correlation to NO production.

Estimation of nitric oxide—Nitric oxide (NO) in the culture media was estimated by using Griess reagent, in terms of nitrite concentration as described earlier. Griess reagent consists of (1:2 of 1% sulphanilamide in 2.5% H_3PO_4 and 0.1% N-(1-naphthyl) ethylene diamine dihydrochloride). Briefly, a small amount of aliquot was removed from conditioned medium and spin down for any cell debris. Culture media (100 µl) was incubated with an equal volume of freshly prepared Griess reagent. The tubes were mixed and incubated in dark, at room temperature for 10 min. The absorbance at 540 nm, determined the concentration of nitrite by using NaNO_2 as a standard.

Methylene blue viability test—The attached cells were carefully subjected to methylene blue viability test. In short, the culture media was removed and cell mono-layer was washed with PBS and incubated with methylene blue solution (final concentration of 0.25 µM.) in HBSS for one hour in humidified 5% CO_2 at 37°C to allow macrophage uptake of methylene blue. The HBSS medium was decanted and cells were washed 3-4 times up to clearances of background methylene blue. The cells uptake methylene blue, which was extracted by incubating cell monolayer in 1.0 ml of 0.1% HCl for 10 min. The amount of viable cells was counted by reading absorbency at 660.00 nm.

Statistical analysis—The data were analysed statistically using Student’s t-test. Significance values of each experimental group were compared in respect to their sham control. i.e. AD Sham is compared to CD sham, AD + B. serrata is compared to AD Sham and CD+ B. serrata is compared to CD sham.

Results

In vivo study—Peritoneal macrophages, isolated from the animals, fed on atherogenic diet, showed more production of NO (42.01 % higher) in response to same concentration of LPS exposure, as compared to the cells, isolated from the rats eating normal diet. This showed that the cells from the AD fed rats were more sensitive to LPS response. However, the animals kept on atherogenic diet along with B. serrata extract, did not show such hyper response to LPS (Table 2).

The result of lipid profile (Table 2) indicated that in atherogenic diet treated animals, serum cholesterol level was significantly elevated (P<0.001) as...
Table 2—Effect of *B. serrata* extract on different parameters of in vivo study in albino rats

<table>
<thead>
<tr>
<th>LPS Response on NO production by macrophage (µM/3×10⁶ cells)</th>
<th>Serum cholesterol (mg/dl)</th>
<th>HDL-cholesterol (mg/dl)</th>
<th>Tc: HDL-cholesterol ratio</th>
<th>SGPT (IU/L)</th>
<th>Urea (mg/dl)</th>
<th>Body wt. (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.95 ± 0.33</td>
<td>44.22 ± 2.74</td>
<td>15.28 ± 1.23</td>
<td>2.91 ± 0.41</td>
<td>56.58 ± 2.74</td>
<td>31.98 ± 12.50</td>
</tr>
<tr>
<td>AD sham</td>
<td>9.87 ± 0.05</td>
<td>378.87 ± 19.37</td>
<td>19.70 ± 1.59</td>
<td>5.65 ± 0.76</td>
<td>227.55 ± 19.84</td>
<td>31.98 ± 47.50</td>
</tr>
<tr>
<td>AD+ <em>B. serrata</em></td>
<td>4.68 ± 0.37</td>
<td>212.42 ± 25.06</td>
<td>25.60 ± 3.04</td>
<td>8.55 ± 0.81</td>
<td>41.41 ± 4.68</td>
<td>112.22 ± 32.50</td>
</tr>
<tr>
<td>CD+ <em>B. serrata</em></td>
<td>4.60 ± 0.16</td>
<td>54.33 ± 26.18</td>
<td>26.18 ± 2.10</td>
<td>3.23 ± 0.43</td>
<td>33.25 ± 3.88</td>
<td>40.77 ± 6.66</td>
</tr>
</tbody>
</table>

Tc: HDL-Ratio is a calculated value, obtained by dividing Total cholesterol by HDL cholesterol.

NO was estimated as—NO₂⁻: µM/3×10⁶ macrophage cells by Griess reagent.

Statistical significance values of each experimental group were compared in respect to their sham control. i.e. AD Sham is compared to CD sham, AD + *B. serrata* is compared to AD Sham and CD+ *B. serrata* is compared to CD sham. *P* value: *a* < 0.001, *b* < 0.05

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.

Compared to the animals kept on normal diet, which was prevented in the *B. serrata* extract treated animals in the range of 38-48%. Further, there was significant increase in HDL cholesterol (*P* < 0.01) in the BS extract treated animals, in both the groups of animals that were kept on normal diet and atherogenic diet. Total cholesterol to HDL-cholesterol ratio (Tc: HDL-ratio), a potent predictor of cardiovascular disease, was also found to be low as compared to atherogenic sham control animals. The atherogenic diet treated animals also showed an increased level of serum SGPT and urea (*P* < 0.001) in comparison to normal control. The animal treated with *B. serrata* showed the normal level of SGPT and significant prevention in serum urea (*P* < 0.001). The normal control animals showed a decreased change in body weight (BW), but there was significant increase in body weight in *B. serrata* treated control animals. However, atherogenic diet eating animals showed significant increase in body weight, which was significantly inhibited in AD+ *B. serrata*, although it was higher than the CD and CD+ *B. serrata* group.

**In vitro study**—Thioglycolate activated macrophages produced more NO (250% more than the normal) in response to exposure of 25 ng/ml LPS. However, *B. serrata* inhibited this NO production. The changes were significant in the concentration dependent manner with the IC₅₀ value at 662 ng/ml (Table 3).

**Histological study**—The control animals treated with *B. serrata* did not show any structural change, as liver and kidney were similar to normal structure (Fig. 1A and 2A). The histological picture of liver and kidney from atherogenic diet treated animals revealed several changes such as congestion, change in cellular architecture, fatty infiltration near central vein, inflammation, hepatic cell necrosis and degeneration (Fig. 1B and 2B) which were prevented in the *B. serrata* treated animals (Fig. 1C and 2C).

**Discussion**

*Boswellia* extract showed significant hypocholesterolemic response, which may be at the level of the intestinal absorption of ingested atherogenic components or at the level of high excretion of ingested cholesterol through the liver. Several plant sterols inhibit intestinal absorption of the dietary cholesterol. The polyphenols of other several plants have shown rise in serum HDL, finally leading to removal of body lipid and decrease in the body weight. Thus the above effect of *B. serrata* extract may be attributed to its similar constituents. However
Figs 1 and 2—Histological section of (1) liver and (2) kidney of different groups of *J. serrata* [A: control sham; B: atherogenic sham; C: AD+ *J. serrata* (H&E staining, × 40)]
Table 3—Effect of different concentrations of \textit{BS extract} on LPS induced NO production and cell viability on thioglycolate induced rat macrophages (in vitro study).

<table>
<thead>
<tr>
<th>Normal cells (25 ng/ml)</th>
<th>LPS (25 ng/ml)+ \textit{B. serrata} extract (ng/ml)</th>
<th>5</th>
<th>50</th>
<th>500</th>
<th>25,000</th>
<th>50,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO</td>
<td>10.24±2.31</td>
<td>33.62±2.89</td>
<td>21.92±2.51</td>
<td>13.58±1.70</td>
<td>12.34±0.99</td>
<td>9.29±1.61</td>
</tr>
<tr>
<td>MB</td>
<td>0.69±0.02</td>
<td>0.74±0.01</td>
<td>0.73±0.01</td>
<td>0.72±0.009</td>
<td>0.71±0.009</td>
<td>0.68±0.09</td>
</tr>
</tbody>
</table>

NO estimated as NO$_2$ μM /3X10$^6$ macrophase cells.
IC$_{50}$ concentration is 662ng/ml.
MB : Methylene blue uptake in form of absorbency at 660 nm.
Statistical significance of LPS group is compared with normal and drug treated with LPS.

\( P \) value *<0.001, *<0.01, *<0.05

previous workers have also shown its hypolipidemic response, but the proposed mechanism was through the inhibition of cholesterol synthesis$^{29}$. Increase in HDL by \textit{B S} extract was seen in both the conditions, i.e., with normal diet and with AD diet. This increase shows the cardio protective effect of \textit{BS extract}. Although there are not much compounds, which have shown significant change in HDL, some medicinal plants, polyphenols, fibrates and butylated hydroxy toluene (BHT), etc. have shown significant increase in HDL$^{28,30,31}$. Therefore, lowering of total cholesterol and increase in HDL cholesterol, defined as its ratio (Tc: HDL cholesterol ratio) shows its cardio-protective property$^{26}$.

Further, it was found that atherogenic diet produced significant rise in SGPT and serum urea. This adverse effect could be due to presence of high fat and cholic acid in the high fat-cholesterol diet, because the lithogenic effect of cholic acid$^{32}$. It promotes biliary cholesterol hyper secretion and cholelithogenesis by enhancing intestinal absorption, hepatic bioavailability, and phase separation of cholesterol in bile. Further, hyperlipidemia caused by atherogenic diet may help in increasing Ox-LDL and cholesterol, which may be activating inflammatory cascade as shown by Dichtl \textit{et al.}$^{33}$ and also observed in histological pictures of the present study. Significant protection was seen in the atherogenic diet induced histological changes especially fatty infiltration, inflammation and necrosis. \textit{BS extract} treatment lowered the raised SGPT level to normal range. Serum urea level was also significantly lowered than atherogenic sham control but it was still higher than the normal animals. This indicates the protective role of \textit{BS extract} on the liver damage. The significant decrease in body weight in \textit{B. serrata} treated atherogenic animals also conform the hypolipidemic role of this drug.

Experiments with macrophages showed the prevention of LPS induced NO production with \textit{B. serrata} treatment. LPS induced changes in the macrophage function are suggested to be an appropriate model to assess the potential of any anti-inflammatory agent$^{34}$. It induces the synthesis of inflammatory molecules including NO via activation of NF-kB$^{35}$. Inhibition of LPS induced NO production by \textit{B. serrata} extract (Table 2) was thought to be its interaction with the inflammatory cascade, as has been shown earlier by Syrovates \textit{et al.} who have reported that one of pentacyclic triterpene from \textit{B. serrata}, known as acetyl-α-boswellic acid is a potent inhibitor of NF-kB and p38 MAP kinase pathway in activated macrophage$^{36}$. Further, it has also been reported that boswellic acid blocks the synthesis of pro-inflammatory 5-lipoxygenase products, including 5-hydroxyeicosatetraenoic acids (5-HETE) and leukotriene B4 (LTB4) and affects the activity of iNOS$^{37}$. Thus the protective effect of \textit{BS extract} may be associated with these phytochemicals.

\textbf{Acknowledgement}

The authors are thankful to the Department of Biotechnology, Govt. of India for financial support and to Mr O.P. Tiwari for help in TLC experiments.

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