Anti-inflammatory, immunomodulatory and antinociceptive activity of *Terminalia arjuna* Roxb bark powder in mice and rats

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*Terminalia arjuna* bark powder (400 mg/kg, po) significantly reduced formalin-induced paw oedema at 24 h but not carrageenan-induced paw oedema. It significantly increased the anti-SRBC antibody titre in the secondary phase of immune response. The same dose significantly reduced the duration of licks and bites in both phases of formalin-induced pain response and showed significant increase in tail flick latency at higher dose (800 mg/kg, po). These effects of *T.arjuna* were antagonised by pretreatment with naloxone (1 mg/kg, ip). In another series of experiments, mice pretreated with morphine for three days in increasing doses (10, 15, 20 mg/kg, ip; twice daily) showed a decreased response in antinociceptive activity of morphine (5 mg/kg, ip). Further, cross tolerance was observed with *T.arjuna* (800 mg/kg, po) in morphine tolerant animals. These findings support the hypothesis that *T.arjuna* has anti-inflammatory potential against some phlogistic agents along with some immunomodulatory activity and also has antinociceptive action probably mediated via central opioid receptors.

**Keywords:** Anti-inflammatory, Antinociception, Formalin-induced pain response, Humoral immune response, Tail flick latency test, *Terminalia arjuna*

Cardiovascular diseases have witnessed a surge in incidence and prevalence in the last few decades. Atherosclerosis is one of the most common disorders involving the cardiovascular system. Atherosclerosis is now recognised as an inflammatory disorder rather than due to hyperlipidemia alone. The inflammatory process has been indicated to be T-cell and macrophage mediated with possible protective role of B-cells in the pathogenesis of an atheroma. At the same time, inflammation and certain humoral mediators have been implicated in the destabilisation of an already existent plaque. Recent studies have also suggested the role of immune response in the pathogenesis of atherosclerosis as evidenced by the immunomodulatory effect of statins in the treatment of atherosclerosis. With the limitation of the currently available molecules, indigenous treatment options are being currently explored for cardiac disorders like atherosclerosis. In Ayurveda also, there is reference of a few drugs available for treatment. One such agent is the bark of *Terminalia arjuna* Roxb Wight & Arn. (Combretaceae).

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The bark of *Terminalia arjuna* (*T.arjuna*), a deciduous tree, has been known in Indian System of Medicine to be beneficial for cardiac ailments. It is an essential ingredient of many Ayurvedic preparations sold as cardiotonics. Animal experiments and clinical studies, have also reported the beneficial effects of dried bark powder of *T.arjuna* in ischaemic heart disease. Various studies have demonstrated the anticoagulant property of petroleum ether extract; cardiotimulatory property of aqueous extract; hypotensive, negative inotropetic and hypocholesterolemic effect of alchoholic extracts of *T.arjuna*. Gauthaman et al. have shown the bark powder of *T.arjuna* to augment endogenous antioxidants and prevent oxidative stress in rabbits against ischemic-reperfusion injury. This bark extract was also reported to reverse endothelial dysfunction in chronic smokers. Some polyherbal preparations, Cap HT2 and BHU X containing *T. arjuna* among other constituents, have been reported to possess antiatherogenic, hypolipidemic and anti-inflammatory activity.

Pain is frequently associated with inflammation and is a feature of numerous cardiovascular diseases like angina and myocardial infarction. Drugs with anti-inflammatory effect very often possess analgesic property as well. The experience of pain is the final
product of a complex information processing network involving the central and peripheral pathways. Many clinical trials, have also demonstrated the beneficial effects of T. arjuna in ischaemic cardiomyopathy and in patients with stable angina. However, the effect of T. arjuna bark powder per se, has not been studied on inflammation, immunomodulation and nociception which may provide a better understanding of its role in atherosclerosis. The present study has therefore been undertaken to investigate the anti-inflammatory, immunomodulatory and antinociceptive, potential of the powdered bark of T. arjuna on rodent models of inflammation, immune response, and nociception.

Materials and Methods

Plant material—T. arjuna bark capsules (Trade name: Arjuna, The Himalaya Drug Company, Bangalore, India) were purchased. Each capsule contained 250 mg of T. arjuna bark powder. Literature provided by the manufacturer indicates that the capsules were standardized to contain not less than 75 mg (30%) of tannins and 1.25 mg (0.5%) arjunolic acid per capsule. The standardization process employed spectrophotometric and high performance liquid chromatography (HPLC) methods. Each batch underwent HPTLC finger printing technology under stringent good manufacturing (GMP) conditions (Batch No. F027009G dated July 2007).

Drugs and treatment schedule—T. arjuna bark capsules (The Himalaya Drug Company India), morphine sulphate (Govt. of India Lab), naloxone hydrochloride (Sigma USA), carrageenan (Sigma USA) and formalin (Merck, India) were used in the present study. The capsules were opened and the bark powder was mixed in distilled water with few drops of Tween 80 to form a suspension of required dose. Morphine sulphate, naloxone and formalin were prepared in normal saline. T. arjuna, morphine and naloxone were administered 60, 30 and 15 min before the test, respectively. T. arjuna was administered orally (po) whereas morphine and naloxone were given intraperitoneally (ip). All drugs were administered in a volume of 10 ml/kg.

Animals—Male Swiss albino mice (30-40 g) and male Wistar rats (150-200 g) were obtained from the Central Animal House, University College of Medical Sciences, Delhi, India. Animals were housed in polypropylene cages (28×22×14 cm), 4 animals per cage, maintained in a room with controlled temperature (22±2°C) under standard condition of light/dark cycle with pellet diet and water available ad libitum, except during the period of experimental observations. Twelve hours before each experiment animals received only water in order to avoid food interference with absorption. Care of animals was taken as per the guidelines of INSA, Animal Welfare Division of the Ministry of Environment & Forests, Council of International Organisation of Medical Sciences (WHO/UNESCO), NIH and PHS and also CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), India. The research protocol was approved by the Institutional Animal Ethics Committee, University College of Medical Sciences, Delhi.

Anti-inflammatory studies—Carrageenan and formalin-induced paw oedema models were used to evaluate the anti-inflammatory potential of T. arjuna. For each model, animals were divided into two groups of 6 each. One group was given vehicle only (control group). In the other group, animals were given T. arjuna (400 mg/kg) (test group). A vernier caliper was used for measurement of paw thickness (mm) of the animals. Oedema was expressed as percentage increase in paw thickness (ΔT) and was calculated from the formula: ΔT = (Tt – To)/To *100; where T0 = right hind paw thickness (mm) before subplantar injection; Tt= right hind paw thickness (mm) at time ‘t’.

Carrageenan-induced oedema in rats—In this method, inflammation was produced by the subplantar administration of 0.1 ml of 1% (w/v) carrageenan in the right hind paw of rats. The thickness (mm) of the paw was measured before injection and at 30, 60, 90, 120 min and at 24 h after injection, with a vernier caliper.

Formalin-induced oedema—In this method, oedema was induced by injecting 50 μl of 1% formalin into subplantar region of right hind paw of mice. Paw oedema was measured before injection and at 24 h.

Haemagglutination titre test—Haemagglutination titre test was performed as described by Mediratta et al. The humoral immune response was measured as haemagglutination titre to sheep red blood cells (SRBC). Rats were immunised with SRBC (0.5×10^9 cells/ml/100 g, ip) on day 0. The animals were then divided into 2 groups of 8 each. Animals in one group were administered normal saline while the second group received T. arjuna (400 mg/kg) per day from day 1 to 6. For primary immune response on day 7, the animals were lightly anaesthetised with ether
and blood was collected from retroorbital plexus. The serum was separated and haemagglutination titre was estimated using microtitre plates. Two fold dilution (0.025ml) of sera were made in microtitre plates with saline. To each well 0.025 ml of 1% (v/v) SRBC was added. The plates were incubated for 1 h at 37 C and then observed for haemagglutination. The highest dilution giving haemagglutination was taken as the antibody titre which was expressed in a graded manner, the minimum dilution (1/2) being ranked as 1.

For secondary immune response, rats were immunised with SRBC on day 0, as described above. Animals received the same dose of antigen, i.e. booster dose on day 7. The animals were then divided into two groups: one group received only vehicle and the other received T.arjuna (400 mg/kg) daily. On the 9th day, the animals were mildly anaesthetised with ether and blood was collected from the retroorbital plexus. Haemagglutination titre was estimated as described above. The mean ranks of different groups were compared for statistical analysis.

Antinociceptive studies—Tail flick test: The tail flick test was used to measure the latency of the response as described by D’amour and Smith24, with modifications. Mice were placed on the tail flick unit so that a constant heat intensity was applied to the lower third of the animal’s tail. When the animal flicked its tail in response to the noxious stimulus, both the heat source and the timer was stopped. A cut-off time of 10s was set to avoid tail damage. Animals were divided into six groups. Control animals (Group I) received similar volume of vehicle as test groups. Mice in Group II and III were pretreated with T.arjuna (400 and 800 mg/kg) respectively, 60 min before the test. To group IV, T.arjuna (800mg/kg) and naloxone (1mg/kg) were given 60 min and 15 min before the test respectively. Group V animals were given morphine (5mg/kg), 30 min before the test and the fifth group was administered morphine (5mg/kg) and naloxone (1mg/kg), 30 min and 15 min before the test, respectively. One group received vehicle only and acted as control. Mice were injected 50μl of 1% formalin into the sub-plantar surface of right hind paw. The nociceptive response was quantified as the duration of licks and bites of the injected paw. The duration of pain response for the early phase (0-5 min) and the late phase (30-35 min) were recorded.

Statistical analysis—Data are expressed as mean ± SE. The data for each test were analysed using standard statistical tests; One way Analysis of Variance (ANOVA) or Student’s t test or Mann Whitney’s U test.

Results

Effect on carrageenan-induced paw oedema—The paw oedema measured at 30, 60, 90, 120 min and 24 h after injection of carrageenan did not show significant reduction in paw thickness when treated with T.arjuna (400 mg/kg) (data not shown).

Effect on formalin-induced paw oedema—The paw oedema measured at 24 h after injection of formalin showed a significant reduction (from 75.32±7.74 to 52.64±5.94 ) in the percentage increase in paw thickness, when treated with T.arjuna (400 mg/kg) compared to control group.

Effect on haemagglutination titre—In rats sensitised with SRBC on day 0, administration of T.arjuna (400mg/kg) daily from day 1 to 6, did not produce a significant change in anti-SRBC antibody titre. On the other hand, 400mg/kg administration from day 7 to 9 after a booster dose of antigen on day 7, significantly raised the anti-SRBC antibody titre (from 5.0±0.36 to 6.6±0.49) compared to control.

Effect on tail flick test—T.arjuna bark powder was evaluated in 2 doses in the tail flick model of pain in
mice. Pretreatment of the animals with doses, 400 and 800mg/kg increased the mean tail flick latency compared to control group. However, the difference was significant only with 800mg/kg dose of T.arjuna. This antinociceptive activity of T.arjuna (800mg/kg) was antagonised by pretreatment with naloxone (1mg/kg). Mice treated with morphine (5 mg/kg) also showed increase in tail flick latency and the antinociceptive effect of morphine was antagonised by pretreatment with naloxone (1mg/kg) (Table 1).

Effect on antinociceptive response in morphine tolerant mice—Mice were pretreated with increasing doses of morphine (10, 15, 20 mg/kg) twice daily for three days, respectively. Antinociceptive response to 5mg/kg morphine was tested on the 4th day, i.e. 24 h after cessation of treatment. Effect of morphine (5mg/kg) was significantly reduced compared to response in morphine-naive animals indicating that the animals have become tolerant to the antinociceptive effect of morphine. Morphine tolerant animals also showed decreased antinociceptive response to T.arjuna (800mg/kg), thus suggesting the development of cross tolerance between the two drugs (Table 2).

Effect on formalin-induced pain response—Injection of 0.05ml, 1% formalin into the subplantar surface of right hind paw of mice, induced pain response which was quantified as the duration of licks and bites. The duration of pain response in the early phase (0-5sec) and late phase (30-35sec) were significantly reduced when treated with T.arjuna (400mg/kg) or morphine (5mg/kg) compared to control. Effect of both these drugs were antagonised by naloxone (1mg/kg) (Table 3).

Discussion

Over the last few decades atherosclerosis has emerged as an inflammatory disorder with underlying cellular and molecular mechanisms. New insights into inflammation in atherosclerosis may help to identify innovative therapeutic strategies to improve outcomes of individuals at risk for or affected by this scourge of growing worldwide importance. Atheroma contains abundant macrophages capable of degrading the collagen that lends strength to the plaque’s protective cap, rendering the cap susceptible to rupture. Interferon arising from the activated T lymphocytes in the plaque can halt collagen synthesis, limiting its capacity to renew the collagen that reinforces the plaque26,27. Macrophages also produce tissue factor, the major procoagulant and trigger to thrombosis found in plaques. Inflammatory mediators regulate tissue factor expression by plaque macrophages, demonstrating an essential link between arterial inflammation and thrombosis28.

A number of natural products are used in the traditional system of medicine in many countries including India. T. arjuna is widely known for its use in various diseases. The present study was conducted to evaluate the antinociceptive activity of T.arjuna (800mg/kg) and its effect on morphine tolerant mice and formalin-induced pain response in mice.

Table 1—Effect of T.arjuna on tail flick latency in morphine-naive mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tail flick Latency (s)</th>
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<tbody>
<tr>
<td>Control (10ml/kg)</td>
<td>2.95± 0.42</td>
</tr>
<tr>
<td>T.arjuna (400mg/kg, po)</td>
<td>4.54±0.63</td>
</tr>
<tr>
<td>T.arjuna (800mg/kg, po)</td>
<td>6.30±0.70*</td>
</tr>
<tr>
<td>T.arjuna (800mg/kg, po) + Naloxone (1mg/kg, ip)</td>
<td>3.34±0.58*</td>
</tr>
<tr>
<td>Morphine (5mg/kg, ip)</td>
<td>7.64±0.32*</td>
</tr>
<tr>
<td>Morphine (5mg/kg, ip) + Naloxone (1mg/kg, ip)</td>
<td>3.53±0.43*</td>
</tr>
</tbody>
</table>

P values: *<0.01 as compared to control; <0.05 as compared to T.arjuna (800mg/kg) morphine-naive group; †morphine (5mg/kg) morphine-naive group.

Table 2—Effect of T.arjuna on tail flick latency in morphine tolerant mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Baseline TFL (s)</th>
<th>TFL after drug administration (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine (5mg/kg, ip)</td>
<td>3.61±1.01</td>
<td>4.60±0.57*</td>
</tr>
<tr>
<td>T.arjuna (800mg/kg, po)</td>
<td>3.1±1.01</td>
<td>2.96±030*</td>
</tr>
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*P<0.05 as compared to morphine(morphine-naive) group, †P<0.01 as compared to T.arjuna (800mg/kg, p.o) morphine-naive group

Table 3—Effect of T.arjuna on formalin-induced pain response in mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Licking response early phase (s)</th>
<th>Licking response late phase (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (10ml/kg)</td>
<td>46.14±4.58</td>
<td>40.14±6.83</td>
</tr>
<tr>
<td>T.arjuna (400mg/kg, po)</td>
<td>20.16±2.97†</td>
<td>6.33±2.48†</td>
</tr>
<tr>
<td>T.arjuna (400mg/kg, po) + Naloxone (1mg/kg, ip)</td>
<td>51.2±1.80b</td>
<td>39.8±4.94b</td>
</tr>
<tr>
<td>Morphine (5mg/kg, ip)</td>
<td>15.75±0.63*</td>
<td>6.50±0.64*</td>
</tr>
<tr>
<td>Morphine (5mg/kg, ip) + Naloxone (1mg/kg, ip)</td>
<td>59.0±6.54c</td>
<td>37.25±6.92c</td>
</tr>
</tbody>
</table>

P<0.05 †as compared to control, ‡<0.001 as compared to T.arjuna (400mg/kg,p.o.) group, <0.01 as compared to morphine (5mg/kg,i.p.) group, ‡<0.001 as compared to control
in cardiovascular diseases like angina and heart failure. It has been reported that polyherbal preparations containing \textit{T. arjuna} among other constituents, possess antiatherogenic, hypolipidemic and anti-inflammatory activity\textsuperscript{31,32}. However, the role of \textit{T.arjuna per se}, in inflammation, immunomodulation and nociception has not been studied so far.

Inflammation is a complex process very often associated with pain. The anti-inflammatory property of \textit{T.arjuna} was screened in formalin and carrageenan models of paw oedema. \textit{T.arjuna} (400mg/kg) significantly decreased formalin-induced paw oedema measured at 24 h. However, it did not have significant effect in reducing carrageenan-induced paw oedema. Anti-inflammatory compounds can act on various levels of the pathophysiological process viz., (a) by blocking the biosynthesis of proinflammatory mediators, by decreasing the enzyme expression or by reducing substrate levels; (b) by inhibiting the release of preformed stored mediators; (c) by blocking mediator–receptor interaction on target cells and (d) immunostimulation which results in less aggressive response to allergen challenge\textsuperscript{30}.

Inflammation has been proposed as the common response of endothelial cells to different factors that attack the arterial intima including dyslipidemia, diabetes, hypertension, immunity, infections and smoking causing atherosclerosis\textsuperscript{31}. The mechanism by which \textit{T.arjuna} exerts its beneficial effects is still unclear. Prominent among the several putative mechanisms suggested, is its antioxidant effect. It has been demonstrated that the antioxidant activity is responsible for its benefits in hepatocellular carcinoma. Besides animal models, trials have demonstrated its antioxidant activity comparable to vitamin E\textsuperscript{7,8,32}. \textit{T.arjuna} is known to contain antioxidant constituents such as flavones (arjunolone), tannins and oligomeric proanthrocyanidins (OPCs). Nair \textit{et al}\textsuperscript{33} have also reported that \textit{T.arjuna} tree bark contains high amount of flavonoids. Arjunolic acid, a new triterpene isolated from the bark of \textit{T.arjuna} has been shown to have antioxidant and cardioprotective activity in rats\textsuperscript{8}. Clinical studies have shown the \textit{T.arjuna} bark powder to reduce LDL cholesterol and also antioxidant potential similar to Vitamin E\textsuperscript{7}. \textit{T.arjuna} bark powder has been shown to increase endogenous antioxidants and prevent oxidative stress in rabbits against ischemic-reperfusion injury. In this model it has also been reported to increase antioxidant enzymes such as super-oxide dismutase and catalase and also reduced glutathione\textsuperscript{14}. This antioxidant property of \textit{T. arjuna} could possibly contribute to its anti-inflammatory activity.

Another mechanism contributing to the efficacy of \textit{T.arjuna} in cardiovascular ailments, especially ischemic heart disease is the antiatherosclerotic and hypolipidemic activity of the plant. The hypolipidemic property has been confirmed in various clinical trials\textsuperscript{7,9,34}. CapHT2, a polyherbal formulation containing among other constituents, \textit{T.arjuna} has been shown to have antiatherogenic, hypolipemic, anti-inflammatory and platelet anti-aggregatory activity. Another polyherbal formulation BHUX containing \textit{T.arjuna} was shown to have anti-inflammatory and antioxidant activity. These two features have been correlated as the possible mechanism for atherosclerosis\textsuperscript{31,32}.

Pain is a feature of numerous cardiovascular diseases like angina and myocardial infarction. In the present study, antinociceptive response was assessed by tail flick model and formalin-induced pain response model, which employ thermal and chemical noxious stimuli respectively. The results clearly demonstrate the antinociceptive activity of \textit{T.arjuna} in doses 400mg/kg in formalin test and 800mg/kg in tail flick test. It was observed that naloxone, an opioid antagonist abolished \textit{T.arjuna}- induced antinociception in tail flick model. These results suggest that \textit{T.arjuna} exhibits antinociceptive activity by central effect probably mediated via central opioid receptors or by promoting the release of endogenous opiopeptides.

In the central regions involving the supra-spinal and spinal control of nociception, such as thalamus and spinal cord, the colocalisation of \mu opioid receptors and nAChRs have been reported\textsuperscript{35}. A close relationship between opioid and cholinergic mechanism has been observed in relation to the augmentation of the release and biosynthesis of endogenous opioid peptides\textsuperscript{36}. \textit{T.arjuna} might be acting on any of these receptors and augmenting the release of endogenous opioid peptides.

Chronic morphine administration, resulting in tolerance can also induce desensitisation (but not down regulation) of \mu opioid receptors\textsuperscript{37}. This effect may diminish the acute antinociceptive property of drugs in morphine tolerant mice. Further, the present results also showed that antinociception induced by \textit{T.arjuna} (800mg/kg) was reduced in morphine tolerant mice indicating the development of cross-
tolerance with *T. arjuna*. Taken together, these findings may suggest a common pathway involved in antinociceptive effects of morphine and *T. arjuna* as well as its development of cross-tolerance.

In contrast to the tail flick test, formalin-induced pain response utilises the long lasting stimulus which facilitates observation of feedback modulation and role of endogenous pain-regulatory systems such as opioid and monoaminergic systems. In rodents, the two distinct phases of the formalin test response may be used to address different aspects of nociception, since the first phase seems to be due to direct chemical stimulation of nociceptors and the second phase is dependant on peripheral inflammation and changes in central processing. This test, thus, has greater relevance for clinical situations. In addition, the time course of pain elicited by formalin injection in humans and the behavioural pattern in animals suggest that the responses are closely related. In the present study, significant reduction in the duration of pain response by *T. arjuna* (400mg/kg) in both early and late phases was observed. The ability of *T. arjuna* bark powder to inhibit both the phases of formalin-induced pain response, in addition to prolong the tail flick latency further indicates the involvement of central opioidergic mechanisms as the antinociceptive activity of *T. arjuna* in both the tests is significantly antagonised by an opioid antagonist, naloxone.

The haemagglutination titre test was performed to assess the effect of *T. arjuna* on humoral immune response. *T. arjuna* significantly increased the secondary immune response, as evidenced by an increase in the anti-SRBC antibody titre, but failed to modulate the primary immune response. Thus, *T. arjuna* bark powder can also be said to possess some immunomodulatory activity. Naloxone, an opioid receptor antagonist, increased IL-2 and interferon gamma levels but decreased the production of IL-4 in BALB/cJ mice. The effect of naloxone could be ascribed to the removal of regulatory effects of endogenous opioid peptides on cytokine production. There have been reports, indicating that an opioid receptor agonist, morphine, has downregulated the phagocytic cell function particularly human peripheral blood mononuclear cells (PBMCs) and neutrophils. Further, phagocytosis, chemotaxis, IL production, generation of active oxygen intermediates and arachidonic acid products have also been reduced by morphine. Mediratta *et al* also reported the role of endogenous opioids in modulation of humoral immune response and antigen-induced histamine release from mixed peritoneal cells of rats. Thus, it can be suggested that *T. arjuna* which probably has effect on opioidergic system, may have some role in inflammation and immunomodulation as well.

### Conclusion

In conclusion, the present findings support the hypothesis that *T. arjuna* has anti-inflammatory potential against some phlogistic agents, immunomodulatory effect, and also has antinociceptive action probably mediated via opioid receptors. Thus *T. arjuna* bark powder may prove to be an important indigenous drug in future for the treatment of atherosclerosis. The new appreciation of the role of inflammation in atherosclerosis provides a mechanistic framework for understanding the clinical benefits of drugs like statins and *T. arjuna* which also have additional immunomodulatory potential. Further identification of the triggers of inflammation and unravelling the details of inflammatory pathways may eventually furnish new therapeutic targets for the treatment of atherosclerosis and other related disorders.

### References


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