Immunomodulatory Potential of *Leptadenia arborea* in Immune-Challenged Rats

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**ABSTRACT**

**Background:** Plant-based remedies play a crucial role as powerful health aid worldwide. In recent years, the usage of complementary and alternative medicines has grown rapidly. The objective of the present study was to evaluate the immunomodulatory effects of methanolic extract of *Leptadenia arborea* leaves in BCG-immunized rats.

**Materials and Methods:** *L. arborea* extract suspended in 1% carboxymethyl cellulose (CMC) was administered in three doses viz 50, 100 and 200 mg/kg, p.o. All animals were challenged with 0.05 ml BCG, sc on day 0. Levamisole (18 mg/kg body weight; p.o.) was used as a positive control group. **Results:** *L. arborea* extract exhibited a significant dose- and time-dependent increase in the plasma levels of IFN-γ and TGF-β. On day 14, plant’s extract significantly increased TNF-α level at dose 200 mg/kg. Moreover, *L. arborea* extract elicited an inhibitory effect on IL-10 levels that was significantly observed at dose 200 mg/kg of the plant extract. LD₅₀ value was calculated to be 15.5 g/kg body weight and the extract didn’t display any behavioral changes or clinical signs after 12 hr of treatment. **Conclusion:** *L. arborea* methanol extract showed immunostimulatory effects through elevation of IFN-γ and thereby activation of cell-mediated immunity.

**Key words:** *Leptadenia arborea*, Cell-mediated immunity, TH1 cytokines, TH2 cytokines, Immunomodulator.

**INTRODUCTION**

The use of herbal medicines over the past decade has increased tremendously across the world both in developing and developed countries including the UK and the rest of Europe, as well as in North America and Australia.¹ It is estimated that approximately 80% of the world’s populations rely on herbal medicinal products as a primary source of health care,² and herbal medicines constitute a central part of the medical system in countries like India and China as a traditional system of medicine.³ The importance of plant and plant-derived compounds cannot be denied due to the fact that the modern pharmacopeias contain at least 25% drugs derived from plants and many other synthetic analogs that are isolated from them⁶,⁷ such as paclitaxel, camptothecin, etoposide, mevastatin and artemisinin.⁸,⁹ *Leptadenia arborea* (Asclepiadaceae) is a valuable medicinal plant, widely distributed in tropical Africa and the Arabian Peninsula. In Sudan, it is called “sha’aloub or sho’bait” and is widely spread along the White Nile province and Gash Delta in Eastern Sudan.¹⁰,¹¹ *Leptadenia* species have a rich phytoconstituent profile containing beta-amyрин, alpha-amyрин, β-sitosterol, stigmasterol, campesterol, triterpene alcohol and several flavonoids such as apigenin, kaempferol, luteolin, diosmetin, astragalin and isoquercitrin.¹²,¹³ *Leptadenia arboresa* is used traditionally in Sudan to treat...
rheumatoid arthritis, gonorrhea, constipation, colic and snake bites.\textsuperscript{11} Plant-derived natural products such as polysaccharides, flavonoids, alkaloids, sesquiterpene lactones and triterpenes have received considerable attention in recent years due to their diverse pharmacological properties such as immunomodulatory, anti-inflammatory, cytotoxic, cancer chemopreventive and anti-HIV.\textsuperscript{15-19} The use of plant products as immunomodulator has a traditional history from the ancient times and the modern characterization techniques have isolated the bioactive constituents of plants that are found to stimulate the immune system.\textsuperscript{20,21} The therapeutic potential of immunomodulatory agents from plant sources and their curative properties have been reported in previous studies. However, the present work was carried out to evaluate the immunomodulatory potential of methanolic extract of \textit{L. arborea} leaves in Bacillus Calmette-Guérin (BCG)-immunized rats.

**MATERIALS AND METHODS**

**Animals and maintenance**

Albino rats (average weight 180 g) of either sex were obtained from the animal house of Faculty of Pharmacy, University of Khartoum, Sudan. They were housed in standard plastic cages and maintained under standard laboratory conditions of temperature (25°C±1°C), light (12 hr light/dark cycle) and controlled humidity. They had free access to standard diet and clean water \textit{ad libitum}. The experimental protocol was approved by the local Ethics Committee (Ethics Committee for Animal Experimentation, Faculty of Pharmacy - University of Khartoum, Sudan) and the experiments were performed in accordance with Institutional Animal Welfare Guidelines.

**Plant collection and extraction**

\textit{L. arborea} plant was collected from Khartoum state in central Sudan. It was identified and authenticated by the Department of Medicinal and Aromatic Plants at the National Centre for Research, Khartoum, Sudan. Leaves of the plant were air-dried in the shade and then they were pounded into coarse powder. The extraction procedures were carried out at laboratory of medicinal and aromatic plants at National Centre for Research, following the established extraction procedure of plant materials. 100 g of the powdered plant was macerated in one liter of 80% methanol at room temperature for 24 hr with occasional shaking. It was then concentrated under reduced pressure, using the rotary vacuum evaporator and dried to a solid mass under air at room temperature. The dry extract was stored at 4°C until analysis was carried out.

**Experimental protocol**

Thirty rats (average body weight 180 g) were randomly allocated into five groups (\(n=6\)). The extract was suspended in 1% carboxymethyl cellulose (CMC) (Chemcolloids Ltd. UK). On day 0, all groups were sensitized subcutaneously with 0.05 ml BCG. Group 1 (vehicle control group) received 1% CMC (50 mg/kg body weight; p.o) whereas group 2 (positive control group) was dosed orally with 18 mg/kg levamisole (Sigma-Aldrich Inc. USA). Group 3, 4 and 5 (plant treated groups) received three doses viz 50, 100 and 200 mg/kg body weight; p.o. of the extract, respectively. The dosing was continued for a duration of 14 days. On the 1\textsuperscript{st} day (before challenge), 7\textsuperscript{th} and 14\textsuperscript{th} day, blood was withdrawn from retro-orbital plexus of each animal under mild ether anesthesia (Figure 1). Plasma was separated after centrifugation and stored at -80°C until biochemical analysis.

**Measurement of T Helper cell (TH) 1 and T Helper cell (TH) 2 cytokines in plasma**

Plasma levels of tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) and interferon \(\gamma\) (IFN-\(\gamma\)) (TH1 cytokines) in plasma were quantified using rat antibody specific enzyme linked immunosorbent assay (ELISA) kits (Peprotech Company, USA), according to the manufacturer’s instructions. Tumor growth factor \(\beta 1\) (TGF-\(\beta 1\)) and interleukin-10 (IL-10) (TH2 cytokines) were analysed using rat antibody specific ELISA kits (Bender Medsystem Company/eBioscience, USA), following the manufacturer’s protocol.

**Acute oral toxicity study**

Methanolic extract of \textit{L. arborea} leaves was tested for acute toxicity studies as per the procedure given in Organization for Economic Cooperation and Development (OECD) guidelines 425 and limit test method was followed.\textsuperscript{22} Albino rats weighing between 120 to 210 g of either sex were randomly distributed into six groups (\(n=8\)). The animals were deprived of food but not water (16-18 h) prior to administration of the plant extract. Five groups of animals were fed with single oral doses of the plant extract (2, 4, 8, 11 and 13 g/kg/bodyweight), suspended in 1% CMC. The 6\textsuperscript{th} group (control group) received 1% CMC (50 mg/kg body weight; p.o.). Following oral administration of extracts, the animals were closely observed for general and behavioral changes that were recorded systematically at 2, 4, 6 and 12 h after the dosing. The visual observations included skin and fur, eyes,
nose, motor activity, respiration, lacrimation and feces consistency. Further, the animals were observed at 24 and 48 h after dosing for mortality rate. The number of animals died from each group were recorded and the toxicological effects were assessed based on mortality, which was expressed as LD$_{50}$ value (the median lethal dose that kills 50% of animal population). LD$_{50}$ value was determined by using a computer program SPSS-probit analysis. The higher the LD$_{50}$ value, the lower will be the toxicity of the plant extract.

**Statistical analysis**

Data were expressed as Mean ± S.E.M. Levels of difference among all groups were determined by one-way analysis of variance (ANOVA) followed by Dunnett’s $t$-test. $P$-values ≤ 0.05 were considered as statistically significant.

**RESULTS**

**L. arborea extract enhanced IFN-γ production in immune challenged rats**

$L. arborea$ extract exhibited dose-dependent increase in IFN-γ levels on day 7 ($p$ ≤ 0.05) and day 14 ($p$ ≤ 0.05) with a highly significant increase seen at dose 200 mg/kg ($p$ ≤ 0.01). Moreover, the effect of $L. arborea$ extract on the time course of IFN-γ levels revealed a significant time-dependent increase in IFN-γ levels at a dose of 200mg/kg of the plant extract ($p$ ≤ 0.01). Interestingly, the increased IFN-γ levels at 200 mg/kg dose was somewhat similar to that of the positive control group, i.e. levamisole (Figure 2 and Figure 3).

**L. arborea extract induced TGF-β production in immune-challenged rats**

With regard to TGF-β, $L. arborea$ extract exerted a significant dose and time-dependent increase in the plasma levels of TGF-β on 7th and 14th day ($p$ ≤ 0.05), as compared with the control group. Further, TGF-β level at the dose of 200 mg/kg was significantly higher than that of positive control group (levamisole) on 7th and 14th day ($p$ ≤ 0.05) (Figure 2 and Figure 3).

**Acute oral toxicity study**

The lethality was measured 24 hr after treatment with $L. arborea$ extract; and then LD$_{50}$ value was calculated to be 15.5 g/kg body weight, using probit analysis (Table 1). Furthermore, the behavioral patterns of rats including changes in skin and fur, eyes, nose, motor activity, respiration, lacrimation and feces consistency, were observed upto 12 hr after administration of the treatment. $L. arborea$ extract did not display any behavioral changes or general signs in treated and untreated rats after plant administration (Table 2).

**Table 1: Acute oral toxicity for Leptadenia arborea methanolic extract.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose (g/kg)</th>
<th>Log dose</th>
<th>Total N</th>
<th>%Dead</th>
<th>Probit</th>
<th>LD$_{50}$ (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group2</td>
<td>2</td>
<td>0.30103</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Group3</td>
<td>4</td>
<td>0.60206</td>
<td>8</td>
<td>12.5</td>
<td>3.82</td>
<td></td>
</tr>
<tr>
<td>Group4</td>
<td>8</td>
<td>0.90309</td>
<td>8</td>
<td>12.5</td>
<td>3.82</td>
<td></td>
</tr>
<tr>
<td>Group5</td>
<td>11</td>
<td>1.041393</td>
<td>8</td>
<td>25</td>
<td>4.33</td>
<td></td>
</tr>
<tr>
<td>Group6</td>
<td>13</td>
<td>1.13943</td>
<td>8</td>
<td>25</td>
<td>4.33</td>
<td></td>
</tr>
<tr>
<td>Group1 (control)</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>15.5</td>
</tr>
</tbody>
</table>

N- Number of rats.

Figure 1: Experimental protocol.
Table 2: General appearance and behavioral observations for rats after *Leptadenia arborea* administration.

<table>
<thead>
<tr>
<th>Observations</th>
<th>Control group</th>
<th>Treated groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 hrs</td>
<td>4 hrs</td>
</tr>
<tr>
<td>Skin/fur</td>
<td>normal</td>
<td>normal</td>
</tr>
<tr>
<td>Eyes</td>
<td>normal</td>
<td>normal</td>
</tr>
<tr>
<td>Nose</td>
<td>normal</td>
<td>normal</td>
</tr>
<tr>
<td>Respiration</td>
<td>normal</td>
<td>normal</td>
</tr>
<tr>
<td>Lacrimation</td>
<td>normal</td>
<td>normal</td>
</tr>
<tr>
<td>Motor activity</td>
<td>normal</td>
<td>normal</td>
</tr>
<tr>
<td>Feces consistency</td>
<td>normal</td>
<td>normal</td>
</tr>
</tbody>
</table>

Discussion

The history of drug discovery revealed that plants are an important source of novel pharmacologically active compounds and the plant-based or derived remedies play a crucial role in the medical system worldwide. A burgeoning interest in medicinal plants encourages large scale pharmacological screening of herbs and search for new leads derived from plant origin for development of potent and safe agents. In the last few years, considerable attention has been paid to plant-derived natural products as potential immunomodulatory agents. As immunomodulators, medicinal plants may act by stimulating both specific and Non-specific immunity. Phytochemicals such as flavonoids, polysaccharides, lactones, alkaloids, diterpenoids and glycosides have been reported to be responsible for the immunomodulating properties of the plants. Plant-based immunomodulators can provide an effective and safe alternative to conventional chemotherapy for a variety of immune disorders including cancer, immunodeficiency, autoimmune diseases and various infectious and inflammatory conditions.

This study aimed to determine the immunomodulatory effects of *L. arborea* that has been used traditionally to treat various infectious diseases in Sudan. Four cytokines (IFN-γ, TNF-α, IL-10 and TGF-β) were quantified by ELISA. Cytokines have been demonstrated to play a crucial role in differentiation of TH cell into Th1 and...
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Figure 3: Time-dependent effects of Leptadenia arborea extract on IFN-γ, TNF-α, IL-10 and TGF-β levels. *Rats were treated with L. arborea extract (50, 100 and 200 mg/kg), levamisole (18 mg/kg) or vehicle for 14 days. Data were presented as mean ± SEM. *, p ≤ 0.05 versus day 0

Figure 3: Time-dependent effects of Leptadenia arborea extract on IFN-γ, TNF-α, IL-10 and TGF-β levels. *Rats were treated with L. arborea extract (50, 100 and 200 mg/kg), levamisole (18 mg/kg) or vehicle for 14 days. Data were presented as mean ± SEM. *, p ≤ 0.05 versus day 0

Th2. These cytokines were chosen due to their critical role in innate and adaptive immunity that could be either cell-mediated or antibody-mediated immunity. Indeed, the reciprocal control mechanisms of Th1 and Th2 differentiation, using Th1 cytokines (IL-2, IL-12, IFN-γ and TNF-α) and Th2 cytokines (IL-4, IL-5, IL-10 and TGF-β), is critical for directing the immune response toward cell-mediated or humoral-mediated responses. Thus, any factors that can interfere with Th1/Th2 axis might affect the outcome of the immune response.29,30 This study demonstrated that the L. arborea methanolic extract exhibited a significant dose- and time-dependent increase in TGF-β level which is an indicative marker for the anti-inflammatory activity of the plant extract. These findings are in line with a previous study where ethanolic extract of L. arborea showed anti-inflammatory activity against carrageenan-induced paw edema in rats.31 Anti-inflammatory activity of this plant is attributed to its enriched phytochemical profile including flavonoids, beta-amyrin and alpha-amyrin, as also reported in previous studies.32,33 Furthermore, L. arborea extract elicited a dose- and time-dependent increase on IFN-γ and TNF-α levels while exhibited an inhibitory effect on IL-10 production. Accordingly, the plant extract provoked stimulatory effect on cell-mediated immunity through induction of TNF-α and IFN-γ production; and in contrary inhibition of IL-10 level. On the basis of these findings, L. arborea could be recommended as a good candidate for treatment of various infectious diseases and support its traditional claim as anti-rheumatic agent.11 Several previous reports have found immunopotentiating activity in Leptadenia species which are not clearly distinguished from L. arborea.34 A previous study demonstrated that L. reticulata produces a stimulatory effect on the immune system,13 and potentiates antibody titer values and percentage neutrophil adhesion to nylon fibers as well as phagocytosis in carbon clearance assay. Therefore, L. reticulata may play an important role in reducing the risk of developing immunodeficiency disorders.35 Moreover, another in vitro study has confirmed that the aqueous extract of L. reticulata alleviates the immunosuppressive conditions induced by the immunotoxicant, chromate.36 The immunomodulatory activity of L. heterophylla may be attributed to the presence of a mixture of sterols (campesterol, stigmasterol and ß-sitosterol), triterpene alcohol and several flavonoids that are previously documented as plant-derived immunomodulatory products.26,37 In the present study, the lethal dose (LD₅₀ = 15.5 g/kg) of methanolic extract of L. arborea strongly suggests the low oral toxicity of the extract, as LD₅₀ value greater than 5000 mg/kg by the oral route is regarded as being safe or of low toxicity.38 Additionally, according to OECD guidelines for acute oral toxicity,39 the substances with LD₅₀ dose of 2000 mg/kg and above are categorized as non-toxic and safe compounds. Moreover, absence of any behavioral changes after administration of different oral doses of L. arborea supported the safety of this plant extract.

CONCLUSION

To conclude, we show that treatment with methanolic extract of L. arborea in immune challenged rats for a duration of 14 days exhibits immunostimulatory effects as demonstrated by higher plasma levels of IFN-γ and TGF-β and lower levels of IL-10. Further, our results suggests low oral toxicity of the extract as evident by LD₅₀ value greater than 500 mg/kg. The absence of any sign of behavioral change after administration of L. arborea extract at different oral doses further supported the safety of this plant extract. Therefore, these safety findings may explain and support the wide traditional use of L. arborea in Sudan for therapeutic management of several ailments.
Data availability

The data used to support the findings of this study are included within the methodology section of the article.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

ANOVA: Analysis of Variance; BCG: Bacillus Calmette-Guérin; CMC: Carboxy Methyl Cellulose; ELISA: Enzyme linked immunosorbent assay; HIV: Human Immunodeficiency Virus; IFN-γ: Interferon γ; IL-10: Interleukin 10; LD₅₀: Lethal dose 50; OECD: Organization for Economic Cooperation and Development; TGF β: Tumor growth factor β; TH1: T Helper cell 1; TH2: T Helper cell 2; TNF-α: Tumor necrosis factor-α.

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**Leptadenia arborea** is widely distributed in Sudan and is traditionally used to treat rheumatoid arthritis, gonorrhea, constipation, colic and snake bites.

- Immunomodulatory potential of methanolic extract of *L. arborea* leaves in Bacillus Calmette-Guérin (BCG)-immunized rats was investigated.
- *L. arborea* extract exhibited a significant dose- and time-dependent increase in the plasma levels of IFN-γ and TGF-β and decrease in IL-10 levels.
- LD₅₀ value was calculated to be 15.5 g/kg body weight and the extract didn't display any behavioral changes or clinical signs after 12 hr of treatment.

**Summary**

- *Leptadenia arborea* is widely distributed in Sudan and is traditionally used to treat rheumatoid arthritis, gonorrhea, constipation, colic and snake bites.
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