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Original article

In vitro and *in vivo* immunomodulatory potential of *Pedicularis longiflora* and *Allium carolinianum* in alloxan-induced diabetes in rats

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A R T I C L E I N F O

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A B S T R A C T

Pedicularis longiflora Rudolph (Orobanchaceae) and *Allium carolinianum* Linn (Alliaceae) are two important medicinal plants found in *trans*-Himalayan Changthang. The immunomodulatory potential of these plants has not been explored. In the present study, we evaluated the *in vitro* and *in vivo* immunomodulatory potential of *P. longiflora* and *A. carolinianum* in alloxan-induced diabetes in Wistar rats. The ethanol extracts of the aerial parts of *P. longiflora* and whole plant parts of *A. carolinianum* were used for studying the *in vitro* immunomodulatory activity using lymphocyte stimulation and cytokine release assays. For the *in vivo* study, 5 groups of 6 rats per group, including alloxan-induced diabetic and plant extract-treated rats, were evaluated for cell-mediated immune (CMI) and humoral immune (HMI) responses in a 42-day experimental trial using doses of 500 mg/kg b.wt. for *P. longiflora* and 250 mg/kg b wt. for *A. carolinianum*. For *P. longiflora*, the median effective dose was found to be 500 mg/kg. The *in vitro* lymphocyte stimulation index for *P. longiflora* was significantly higher (1.73 ± 0.04, $p < 0.05$) than that for *A. carolinianum* (1.27 ± 0.06). However, the release of transforming growth factor-β1 (TGF-β1, 15.63 ± 1.00, $p < 0.05$) by *P. longiflora* was significantly lower than that by *A. carolinianum* (21.61 ± 1.19), suggesting a better immune response by *P. longiflora* than by *A. carolinianum*. *P. longiflora* significantly increased the ear thickness (53.12%), inflammatory cellular infiltration (200.00 ± 11.42), and total leukocyte count (7.44 ± 0.02) compared to *A. carolinianum* (47.57%, 165.83 ± 3.96, and 7.01 ± 0.01, respectively). *P. longiflora* significantly reduced the percentage of leukocytes with depolarized mitochondria (3.24 ± 0.16%) and apoptosis (1.81 ± 0.07%), and induced a better CMI response than *A. carolinianum*. Significantly ($p < 0.05$) higher hemagglutination titer (28.37 ± 0.80) and IgG production (6.43 ± 0.34 mg/mL) were observed in the *P. longiflora*-treated group than in the *A. carolinianum*-treated group (23.93 ± 0.58 and 6.23 ± 0.37 mg/mL). Plasma tumor necrosis factor-α (TNF-α) and TGF-β1 levels, and nuclear factor-κB (NF-κB) expression were significantly ($p < 0.05$) lower in the *P. longiflora*-treated group than in the *A. carolinianum*-treated group. This may be due to better HMI responses produced by *P. longiflora* than by *A. carolinianum.* This is the first study to show that *P. longiflora* ethanol extract has more potent *in vitro* and *in vivo* immunomodulatory activities than *A. carolinianum*, especially in alloxan-induced diabetic rats. However, further research is needed to identify the different molecular mechanisms involved in mediating this immunomodulatory response.

1. Introduction

Diabetes mellitus (DM) has become the most common metabolic disorder worldwide, resulting from a defect in insulin secretion, insulin action, or both [1,2]. At present, more than 285 million people globally have diabetes. By the year 2030, this number is expected to increase to 438 million (2–5-fold increase), especially in developing countries, with 70% of the deaths being due to diabetes and its associated complications [3,4]. Diabetes is rampant in India, with more than 62 million diabetes patients currently diagnosed every year [5]. It is predicted that

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by the year 2030 it may increase to 79.4 million, posing a serious challenge [6,7]. Hence development of prophylactics and therapeutics for prevention and treatment of diabetes seemed imperative from time to time [8–10]. These entities have evolved from the use of traditional medicines and practices in the ancient times to the application of synthetic chemical drugs and hormones in medieval and now a days a recent trend of utilizing novel target oriented therapeutics and potential antidiabetic medicinal plants [8,10]. Each class of these antidiabetes medicines has shown effectiveness in the management of diabetes to some extent for a certain period of time but has resulted in limitations, chemical or synthetic ones being on the top of having serious and life threatening consequences and side effects [8,9,11,12]. They have shown many adverse effects on health, are costly and rarely available at all places, while on the other hand medicinal plants are proving to be quite effective, cheaper and easily available [11,12]. Conventional antidiabetic medicines include injectable insulins, sulfonylureas, biguanides, glucosidase inhibitors and glinides [11,12]. Recent approaches in drug discovery have contributed to the development of new class of therapeutics like inhalable insulins, incretin mimetics, amylin analogues, gastric inhibitory polypeptide analogues, peroxisome proliferator activated receptors and dipeptidyl peptidase-4 inhibitors [12,13]. The costs of treating diabetes and its related complications are staggering with conventional therapeutics that are expensive, prone to side effects and rarely being available in most of the developing or under developed countries [11,12]. Novel technologies and therapies such as nanotechnology, stem cells, gene therapy, were explored but these have again proven to be a costly affair, not affordable by all and not available everywhere [11,12,14]. Hence novel therapies and targets are being explored for curing diabetes [12,15,16,17]. Subsequently, the identification and clinical investigation of bioactive substances from plants and herbs have revolutionized the research on drug discovery and lead towards the identification of effective drug candidates for diabetes management and cure [11,12]. Several medicinal plants contain phytoactive constituents that have shown potent antidiabetic potential [10,18,19,20,21] including garlic (*Allium sativum*) [22], dog wood (*Corni fructus*) [23], soursop (*Annona muricata*) [24], HemoHIM [25], cowplant (*Gymnema sylvestre*) [26], cocoyam (*Colocasia esculenta*), unripe plantain (*Musa paradisiaca*) [3], jamun (*Syzygium cumini*) [27], eyeball plant (*Spilanthes Africana*), purslane (*Portulaca oleracea*) and arrowleaf sida (*Sida rhombifolia*) [28]. Recently we explored such potential in louse wort (*Pedicularis longiflora* Rudolph) [10].

DM affects multiple body systems, including the immune system [7,29]. It causes dysfunction of the innate immunity due to impaired phagocytosis and loss of chemotaxis [29,30], along with defects in adaptive immunity due to abnormal delayed-type hypersensitivity (DTH) and decreased serum antibody levels [31,32]. Consequently, the production and functioning of immune cells, antibodies, and cytokines are impaired, thereby reducing the resistance to pathogenic infections. This results in high morbidity and mortality in diabetes patients [29].

Immunomodulators have been reported to be beneficial to overcome immune dysfunction in DM [3,33–36]. Natural immunomodulators from medicinal plants are preferred to synthetic ones because of their fewer side effects, cost effectiveness, and easy availability [34,37,38]. *Pedicularis longiflora* Rudolph (Orobanchaceae), commonly known as lousewort or Luguruk serpo, and *Allium carolinianum* Linn (Alliaceae), commonly known as wild *Allium* or wild garlic, are important medicinal plants found in *trans*-Himalayan Changthang (a high-altitude, cold, and arid region in Ladakh); they are used in traditional medicine for curing hepatic, pancreatic, kidney, and urinary diseases [10,39]. *Pedicularis* sp. are rich in flavonoids, phenylethanoids, phenylpropanoids, iridoid glycosides, and polyphenols [40–43]. These phytochemicals have antioxidant, antimicrobial, antitumor, anti-fatigue, and DNA repairing properties [40,44,45]. They increase the activities of glutathione reductase, catalase, and superoxide dismutase, and decrease the levels of lipid peroxides [10]. Their antimicrobial potential may be due to their phenolic content [44]. They have been shown to inhibit exercise-induced synthesis of 5-hydroxytryptamine (5-HT) and tryptophan hydroxylase, and to increase 5-HT1B expression, resulting in anti-fatigue effects [46]. Extracts from different species of *Pedicularis* have been found to be beneficial in diabetes treatment, as they increase the level of insulin and decrease the levels of glucose and glycated products [10,43,47]. Diallyl sulfide, S-allyl cysteine sulfoxide, and S-methyl cysteine sulfoxide, isolated from *Allium* sp. have been found to possess antibacterial, anticancer, antioxidant, antidiabetic, and immunomodulatory properties [36,48–50]. However, the immunomodulatory potential of *Pedicularis* sp. and *A. carolinianum*, especially in diabetes, has not been evaluated and compared with that of standard immunomodulators like vitamin E.

Vitamin E is a potent antioxidant and modulates host immune functions [51,52]. It helps to maintain B cell functions and improves macrophage functions [51]. Vitamin E supplementation results in the suppression of pro-inflammatory cytokines and chemokines [52]. Therefore, vitamin E can be utilized as a standard immunomodulator for evaluating the immunomodulatory potential of the extracts of *P. longiflora* and *A. carolinianum*.

The present study was designed to evaluate the *in vitro* and *in vivo* immunomodulatory potential of *P. longiflora* and *A. carolinianum* in alloxan-induced diabetic rats.

2. Materials and methods

2.1. Plant materials

Aerial parts of *P. longiflora* and whole plant parts of *A. carolinianum* were collected in August 2013 (summer season) from the *trans*-Himalayan Changthang region of Ladakh, India. The plants were authenticated by a scientist (Dr. Kunzes Angmo) at High Mountain Arid Agricultural Research Institute, Ladakh, India (Voucher specimen No. PL-3/2014 for *P. longiflora* and AC-5/2014 for *A. carolinianum*). The plants were shade-dried and then powdered using an electric blender.

2.2. Ethanol extraction

The powdered plant material was extracted three times at room temperature (RT) with 60% ethanol in a Soxhlet extraction apparatus. The extract was filtered using Whatman filter paper no. 1 and dried in a rotary evaporator at RT. The extract yielded a dark brown, sticky, semisolid mass with a yield of 24.62% (w/w) for *P. longiflora,* and a brown black, semisolid mass with a yield of 23.98% (w/w) for *A. carolinianum.*

2.3. Qualitative phytochemical analyses

Qualitative phytochemical analyses of both the ethanol extracts were performed to screen for various phytoconstituents, such as steroids, alkaloids, terpenoids, anthraquinones, glycosides, saponins, sulfur-containing compounds, phenolics, flavonoid compounds, and carbohydrates, using the procedures described by Trease & Evans [53], Mikail [54], Parekh & Chanda [55], and Saminathan [56].

2.4. Quantitative analyses of phytochemical constituents

2.4.1. Total phenolic content estimation

Total phenolic contents were measured with the Folin–Ciocalteu (FC) reagent. A stock solution (5 mg/mL) of gallic acid was prepared and used as a standard. From the stock solution, different concentrations (*i.e.,* 0.25, 0.5, 0.75, and 1.0 mg/mL) were prepared. The diluted ethanol extract (20 μL) was mixed with 1.58 mL of distilled water (DW); 100 μL of FC reagent and 300 μL of saturated Na_2CO_3 (20%) were then added. The solution was incubated at 40 °C for 30 min, and the absorbance was measured at 765 nm with a spectrophotometer. Linearity was observed in the range of 0.25–1.0 mg/mL. Total phenols in both