Reviewer’s report

Title: Modulation of T cell proliferation and cytokine response by Plumbagin extracted from Plumbago zeylanica in collagen induced arthritis

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Reviewer: T Balakrishna Poduval

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The manuscript (MS) demonstrates the immunomodulatory effects of plant derived napthoquinone plumbagin, in a murine arthritis model. The following concerns need to be addressed.

Major Specific Comments:

1. Selection of Plumbagin and its Dose: What is the need to isolate Plumbagin, when pure plumbagin is available from Sigma chemicals? The purity of the fraction PZE-6(supposed to be Plumbagin) fraction is not shown. The dose of Plumbagin used (mg/Kg body weight) is not stated. The literature survey suggests that LD50 dose of Plumbagin for various strains of mice is in the range of 8-16 mg/Kg. Assuming weight of mice to be between 20-25 grams, the dose used by the authors could be between 16-20 mg/Kg of mice at the higher dose, which is more than the LD50 dose of Plumbagin, as stated above. Authors need to explain why they chose the dose stated in the MS.

2. Hypothesis: The authors are not clear in building up the hypothesis based on the published work. The work cited by the authors (Ref 1), did not show that Plumbagin effectively decreased the production of pro-inflammatory cytokines. It is incorrect. Plumbagin inhibited T cell proliferation in response to polyclonal mitogen Concanavalin A (Con A) by blocking cell cycle progression. It also suppressed expression of early and late activation markers CD69 andCD25 respectively, in activated T cells. At these immunosuppressive doses (up to 5 µM), plumbagin did not reduce the viability of lymphocytes. Further, the work also indicated that the inhibition of T cell proliferation by plumbagin was accompanied by a decrease in the levels of Con A induced IL-2, IL-4, IL-6 and IFN-# cytokines. The introduction is very huge with unnecessary textbook information. It can be shortened.

3. Materials and Methods: The Immunization and Treatment protocols are difficult to understand. It states that treatment was given for four weeks starting from day 0 to day 42, which is actually equivalent to 6 weeks. The experimental design should strict to days (not weeks), starting from day 0. The details for the ELISA method are incomplete.

4. Results: Each results and figures can be stated in details to avoid confusion.

5. The studies did not demonstrate that Plumbagin alleviated the arthritis in CIA
mice, as Plumbagin was given along with the induction of arthritis, for a period not very clear as stated above. It could be possibly preventing the development of the disease. The sentence “From this study we can understand that the Plumbagin has a very significant role in alleviating the synovial arthritis at various stages of the ailment probably by suppressing the IgG response, by reducing the T cells or by decreasing the levels of pro-inflammatory cytokines and hiking the anti-inflammatory cytokines” needs to be rewritten.

6. Discussion: It is not focused to the results obtained. The statement in the discussion “In this study, the effect of Plumbagin purified from the roots of Plumbago zeylanica in alleviating arthritis in the CIA mice was demonstrated” Where is the demonstration. There are no results in this MS to show that Plumbagin reduces the severity and delays the onset of collagen induced arthritis? Clinical assessment of arthritis induction is not performed like evaluation of Arthritic index or incidence. How can the authors be sure that the mice have developed arthritis? The earlier work of the authors (ref NO. 19) indicates that they have done these studies. Please elaborate and discuss the role of IgG subclasses. The conclusion drawn from these studies does not reflect the results obtained by the authors.

7. Arthritis involves various cytokines with IL-1 #, TNF-# and IFN-# being the major cytokines involved. Authors have only studied IFN and not IL-1 # and TNF-#. Moreover, the role of IFN in the development of CIA is controversial as it has been shown to be involved in both promotion as well as prevention of CIA (Mauritz NJ 1988 Arthritis Rheum; Boissier MC 1995, Eur J Immunolo; Manoury-Schwarts BG 1997, J Immunol).

8. Earlier work of the authors and other work have demonstrated the ability of Plumbagin to suppress stimulated T cell proliferation. In this work Plumbagin could reverse the Arthritis induced suppression of Con A proliferation of T lymphocytes. They should explain these results. It becomes important to delineate the Mechanism of action of the anti-arthritis action of Plumbagin. They can look at the inhibition of the nuclear P65 (NF-kB0 in the nuclear extract of paw tissue from collagen immunized mice.

9. The authors claim to observe enhanced T cell proliferation and IL-2 secretion with plumbagin treatment in arthritic mice. It is well known that T cells play an important role in CIA via secretion of cytokines and activation of B cells to secrete arthritogenic anti-c II antibodies. T cells are required for CIA, so if Plumbagin is activating T cell proliferation of IL-2 secretion, how will it suppress CIA. Will an increase in T cell activation not further aggravate the disease? This needs to be looked at.

10. Figures and Legends: The Legends in general are highly confusing. Instead of weeks, days after immunization are more accurate. Day 0 has to be defined clearly.

Fig1: No statistical analysis is indicated. The two sentences “Control groups were treated with olive oil alone and” Control group receive CFA alone, are confusing. The statement “Test group was treated with Plumbagin” does not match with Materials and methods (treatment).
Fig 2: No statistical analysis is indicated. The data is given for only one time point and not 3, 5, 7 weeks after primary immunization as stated in the legend. The results also seem to indicate that IgG2b response is increased in Plumbagin group.

Fig 3: No statistical analysis is indicated. Control group had increased levels of antibody at 5 and 7 week after immunization. No statistical analysis is indicated. Why suddenly use higher doses of Plumbagin, when lower doses which are less toxic were sufficient?

Fig 4: No statistical analysis is indicated. How normal groups are different from control group? How splenic T lymphocytes were isolated. What is the concentration of Con A used per ml of culture? Have you used T cells or splenocytes? There are no details in the materials and methods section.

Fig 5: The data indicate that normal mice are treated with Plumbagin. It is possible that arthritic mice were given Plumbagin and there were no Plumbagin control (i.e. Plumbagin given to normal mice). Do you mean to say that Plumbagin (0.4mg) does not inhibit IL-2 production? On the contrary there is a dose dependent decrease in the cytokine production in normal mice given plumbagin (Ref 1). No statistical analysis is indicated. How splenic T lymphocytes were isolated? What is the concentration of Con A used per ml of culture? There are no details in the materials and methods section.

Other Comments:
1. Weight and sex of the mice are not stated.
2. Abbreviations need to be explained, for eg; what are CIA mice?
3. In M&M: Bhabha Atomic Research Centre does not produce and supply Tritiated thymidine. Baba should be replaced by Bhabha. Further,
4. What are Indometh, emulsion A and B? No details are given.
5. Treatment schedule is very confusing and needs to be rewritten.
6. Antibody and cytokine ELISA protocol are incomplete and incorrect.
7. The concentration of Con A is not given. Why 72 h for IFN-gamma detection?
8. Results section: The details for each figure can be given separately to avoid confusion.
9. What is the rationale of using Indomethacin and what is the concentration used.
10. No discussion about other IgG sub classes in fig 2.