# Regulation of IL-2 and IL-4 cytokines by fractionated *Lawsonia inermis* Linn. leaves extract

**Goel Anjana*, Sharma Ritesh and Pathak Juhi**
Department of Department of Biotechnology, GLA University, Mathura, U.P, INDIA
*anjana.goel@gla.ac.in; anjanagoel2000@yahoo.com

***Abstract***

*The medicinal potential of Lawsonia inermis Linn, different fractions of its leaves extract was investigated for its immunomodulating potential by studying its effect on splenocyte proliferation and altering the expression of IL-2 and IL-4 cytokines in in vitro system. Hexane (HLE), ethyl acetate (ELE) and methanolic (MLE) fractions of leaves extract were made by successive extraction method using Soxhlet apparatus. Effect of these extract fractions on splenocytes proliferation was measured by MTT assay. 200µl of the spleen cell suspension containing $2\times10^6$ cells/ml was added in each well of a cell culture plate and incubated for 72 hrs in the presence of different concentration of all the three extract fractions. Absorbance was taken by ELISA reader after the addition of MTT dye for 4 hrs. For the quantitation of IL-2 and IL-4 cytokines, culture supernatants of splenocytes were tested after 48 hrs of incubation. IL-2 and IL-4 cytokines were quantitatively determined in spleen cell culture supernatant according to the kit protocol provided by R. D. system (USA) and B.D. Bioscience (USA) respectively.*

*Among all fractions, hexane fraction showed highest stimulation of 40.93% with 500 µg/ml extract concentration of a maximum. 9.89% and 39.9% stimulations were observed with 50 µg/ml concentrations of ethyl acetate and methanolic extract. IL-2 expression was stimulated by all the fractions but maximum stimulation was observed by hexane fraction. IL-4 expression was inhibited by all the fractions as compared to control. Results obtained during this study clearly indicate that leaves of L. inermis have wide spectrum medicinal potential based on in vitro bioassay experiments employing Wister albino rat model. All fractions of L. inermis leaves under influence of IL-2 induce Th1 lymphocytes which can improve cell mediated responses against numerous infections.*

**Keywords**: Cytokines, Immune-modulation, Medicinal plants, Splenocytes proliferation.

## Introduction

Sensing the medicinal values of plants, human beings around the world have developed their traditions to make the utmost use of these plants in treating the different ailments of mankind and animals. Over centuries in doing so, several important traditional systems have emerged and have been established.[16] Among them Ayurveda, an Indian system of medicine is one of the most eminent and ancient systems to cure human beings and animals. In Ayurveda, about 2000 plants have been prized for their medicinal potential.[11]

A novel research effort to describe the merits of traditional systems of medicine concerning for their safety and efficacy in terms of antimicrobial activities and immunomodulation could result in better use of the complementary system of medicines in regulating the defence mechanisms involving innate and adaptive immune systems.[22] Scientific understanding encompasses many experimental pieces of evidences suggesting their genuine effectiveness required to treat the illness of humans and animals or to promote defence mechanism against pathogens.

*Lawsonia inermis* is native of South-West Asia and North Africa and used for the dye and decorative purposes. This plant is grown widely in various countries including India.[6] It was initially used in various cosmetics from the ancient time but due to the presence of various phytochemicals, this plant also has good medicinal value.[4,19]

However, there is very less information regarding immunomodulation and cytokine regulation of *L. inermis* which is a shred of important evidence to evaluate the therapeutic value of a medicinal plant. Therefore, the present study has been conducted to find out the medicinal potential of *L. inermis* by regulating the expression of cytokines with scientific evidence.

## Material and Methods

**Collection of Plant leaves:** *Lawsonia inermis Linn.* plant leaves were collected from GLA University campus, Mathura and were authenticated by Dr. (Mrs.) A. S. Upadhye (Voucher no. L-081), Botany group, Plant Science Division, Agharkar Research Institute, Pune. Leaves were shade dried, coarsely powered and were packed in airtight bottles for the preparation of different extracts.

**Experimental animals:** Wistar albino rats of both sexes (90-130 gm. of body wt.) were procured from IVRI Izatnagar Bareilly. These animals were used for splenocytes proliferation assay as well as for IL-2 and IL-4 cytokine induction. The animals were housed in animal cages at animal house of Institute of Pharmaceuticals Research GLA University, Mathura. Rats were fed with commercial pellet food and water *ad libitum*. All the rats were accustomed for

10 days before starting the study and were exposed to 12:12 hr light: dark photoperiod (lights on at 06:00) until they were needed for the experiment.

The individual animal was marked using 1% picric acid. All experimental protocols and animal handling procedures were as per the guidelines of the animal ethical committee of the institute with CPCSEA (GLAIPR/CPCSEAC/ IAEC/2016/Biotech/02).

**Splenocytes preparation:** Splenocytes preparation was done as per the method of Goel et al.[9] Briefly, the spleen was collected aseptically from a rat in PBS and then suspended into RPMI-1640 medium. The cells were disaggregated by the mechanical method using sterile syringe and forceps. RPMI-1640 with 10% FBS and 1% antibiotics were used for suspending cells followed by centrifugation at 2700rpm for 10mins at 25°C. Centrifuged cells were then incubated with chilled 0.15M $NH_4Cl$ solution for 10 minutes at RT to lyse erythrocytes. Cells were washed thrice with RPMI-1640 medium to remove the traces of lysis buffer.

Cells viability was determined by dye exclusion with 0.1% solution of trypan blue and counted by haematocytometer. Cells concentration was adjusted to $2x10^6$ cells/ml. These cells were used for splenocytes proliferation/inhibition and IL-2/IL-4 cytokine analysis.

**Splenocytes proliferation assay:** After adjusting the cell concentration to 2 X $10^6$ spleen cell/ml in RPMI-1640 medium, 200µl of cell suspension was added in each well into the cell culture plate. The optimized concentration of Con A i.e. 5µg/ml was added in each well. Different concentrations i.e. 20µg/ml, 50µg/ml, 100µg/ml, 250µg/ml and 500µg/ml of *Lawsonia inermis* leaves were added in spleen cells of respective rows.

This culture plate was incubated at $37^0$C and 5% $CO_2$ in $CO_2$ incubator and analysed by MTT assay. Results were calculated in mean value and compared with control.

The % stimulation index was determined for different concentrations of plant fractions:

$$\% \text{ Stimulation index} = \frac{\text{Absorbance of plant fraction} - \text{Absorbance of control}}{\text{Absorbance of control}} \times 100$$

**Determination of different extracts of plant leaves on cytokine (IL2 and IL4) production:** For *in vitro* effect of different extracts of plant leaves, normal spleen cells were isolated and cultured in the presence of ConA (5µg/ml).

Spleen cells were cultured in wells of respective rows along with different concentrations (20µg/ml, 50µg/ml, 100µg/ml, 250µg/ml and 500µg/ml) of different plant leaves fractions. Spleen cell culture was incubated for 48 hrs. and then supernatants were collected for the determination of IL-2/IL-4 cytokine.

**Determination of Cytokines (IL-2 and IL-4) using sandwich ELISA:** Determination of IL-2 and IL-4 cytokine induction in spleen cell culture supernatant was done according to the kit protocol provided by R. D. system (USA) and B.D. Bioscience (USA) respectively. Cytokine detection from spleen cell culture was done by using a Sandwich ELISA method. Different standard concentrations of IL-2 (Std.1- 4000pg/ml, Std.2- 2000pg/ml, Std.3- 1000pg/ml, Std.4- 500pg/ml, Std.5- 250pg/ml, Std.6- 125pg/ml, Std.7- 62.5pg/ml) and IL-4 (Std.1- 100pg/ml, Std.2- 50pg/ml, Std.3- 25pg/ml, Std.4- 12.5pg/ml, Std.5- 6.3pg/ml, Std.6- 3.1pg/ml, Std.7- 1.3pg/ml) were used respectively. These standard concentrations were provided in the kit of R. D. System and B. D. Bioscience. Concentrations of IL-2 and IL-4 cytokines were quantitated in spleen cell culture supernatants by sandwich ELISA as per the protocol. Optical density was evaluated at 450nm.

$$\% \text{ Stimulation/inhibition index} = \frac{\text{Conc. of Cytokine in the test well - Conc. of cytokine in control well}}{\text{Conc. of cytokine in control well}} \times 100$$

**Statistical analysis:** Data were calculated as Mean ± S.E. Effect of different fractions between groups was compared using student's t- test. The level of significance was recorded at the 1% and 5% level of confidence.

## Results

**Effect of different fractions of L. inermis leaves on splenocytes proliferation:** 5 different concentrations (20µg/ml, 50µg/ml, 100µg/ml, 250µg/ml and 500µg/ml) of hexane fraction of *L. inermis* leaves were used for splenocytes modulation. Hexane fraction has stimulated the splenocytes in a dose-dependent manner and the highest % stimulation index was found at 500 µg/ml concentration (40.93%). At 50 and 100µg/ml concentrations of *L. inermis*, significant ($p<0.05$) increase were found whereas highly significant ($p<0.01$) results were found at 250 and 500 µg/ml concentrations (Fig. 1A).

9.89 % stimulation index was estimated with 50 µg/ml concentration of ethyl acetate fraction and at this concentration, significant ($p<0.05$) increase was found. At high concentrations, 250 µg/ml and 500 µg/ml fraction showed toxicity (Fig. 1B). In methanol fraction, highest % stimulation index value (39.9%) was recorded with 50µg/ml while 100µg/ml also showed 26.5 % stimulation index. At both concentrations results showed highly significant ($p< 0.01$) data. Higher concentrations were found toxic for the cells (Fig. 1C).

***In vitro* effect of different fractions of *L. inermis* leaves on IL-2 production:** In hexane fraction, all the concentrations stimulated the IL-2 production (Fig. 2A).

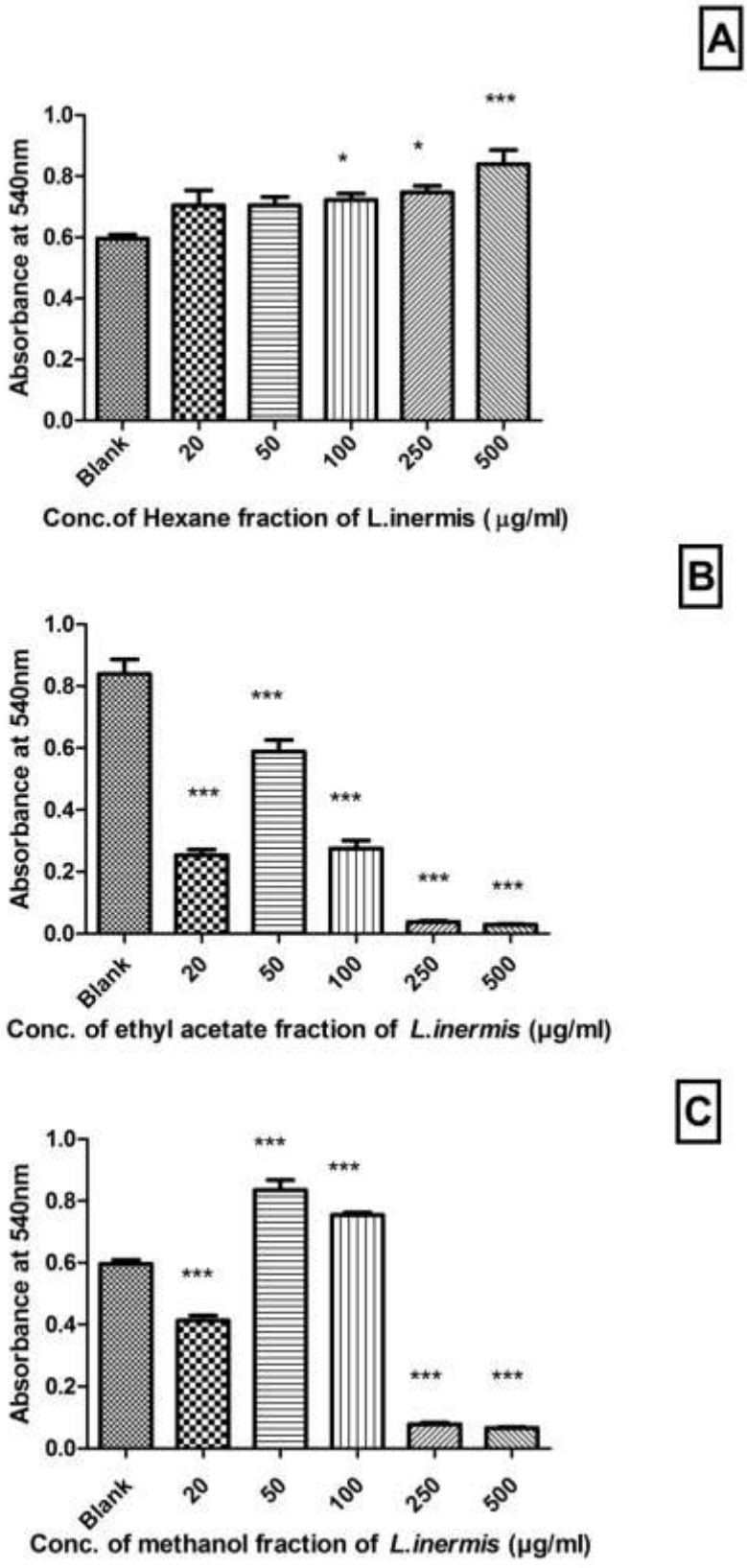


**Figure 1: Effect of *L.inermis* extracts on splenocyte proliferation. Spleens were taken out from Wistar albino rats. Single cell suspension was made and 0.2 ml of 2X10$^6$ cells /ml concentration were cultured in 96 well culture plate in the presence of A: Hexane extract of *L.inermis* leaves; B: Ethyl acetate extract of *L.inermis* leaves; C:Methanolic extract of *L.inermis* leaves. Proliferation was measured by MTT assay. The results are expressed as the mean ± SEM; The results are analysed using Graph Pad Prism 5.1 software and expressed as the mean ± SEM. *p < 0.05, **p < 0.01 and ***p < 0.001 compared with the untreated control (one-way analysis of variance/ Dunnett's test).**

However, the maximum IL-2 production was recorded at 500µg/ml. 20µg/ml concentrations also stimulated the IL-2 production but 500µg/ml stimulated maximally i.e. 35.5%. Significant ($p<0.05$) increase was recorded at 20 and 50µg/ml conc. Highly significant ($p<0.01$) results were obtained at higher concentrations i.e. 100, 250 and 500 µg/ml in the comparison of control.

In the ethyl acetate fraction at 20 µg/ml and 50 µg/ml, IL-2 production was 89.57 ± 1.17 and 100.32 ± 2.56 pg/ml. Above this concentration, IL-2 production was inhibited. Highest stimulation index (10.81%) was found at 50 µg/ml. Significant ($p<0.01$) increase was recorded at 50 µg/ml (Fig. 2B).

IL-2 production was found maximum at 50 and 100 µg/ml of methanol fraction of *L. inermis* with the value of 116.0 ±4.62 and 108.96±2.99 pg/ml. Both concentrations showed highly significant ($p < 0.01$) data with the comparison of control. Toxicity was observed at 250 and 500 µg/ml concentrations of methanol fraction. Highest stimulation index (33.04%) was at 50µg/ml (Fig. 2C).

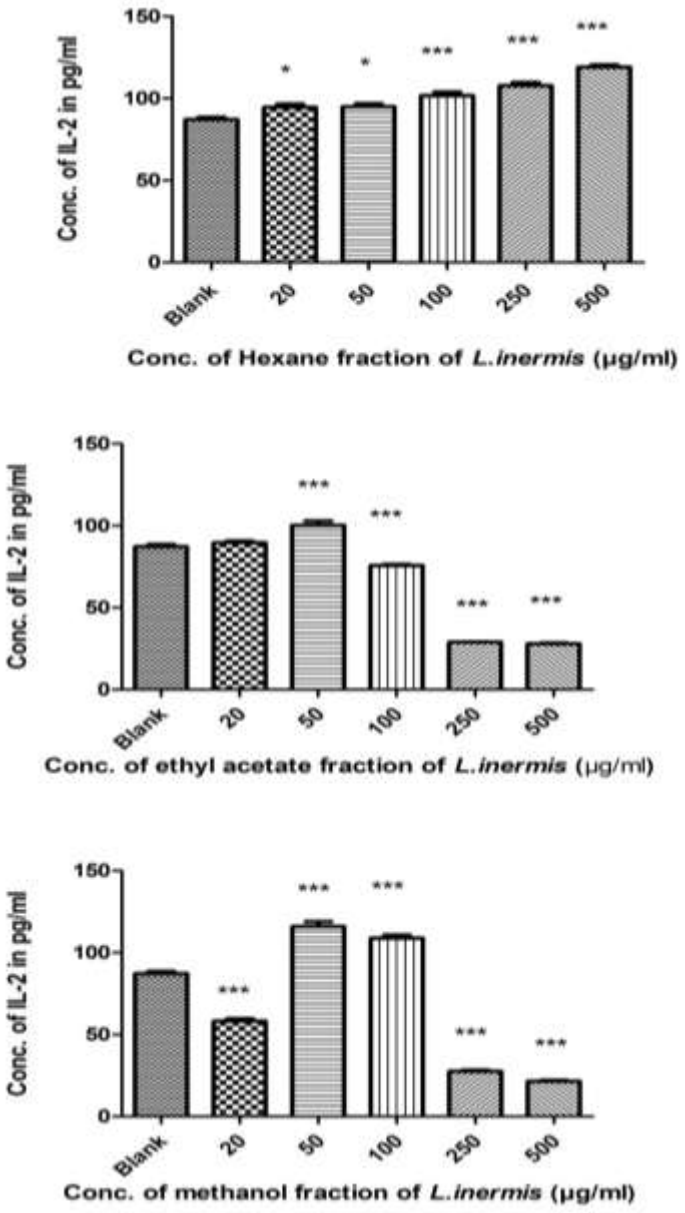


**Figure 2: Effect of *L.inermis* extracts on IL-2 cytokine modulation. Spleens were taken out from Wistar albino rats. Single cell suspension was made and 0.2 ml of $2X10^6$ cells /ml concentration were cultured in 96 well culture plate in the presence of different concentrations (25, 50, 100, 250 and 500µg/ml) of A: Hexane extract of *L.inermis* leaves; B: Ethyl acetate extract of *L.inermis* leaves; C:Methanolic extract of *L.inermis* leaves. IL-2 cytokine was quantitated, in the culture supernatant of 48 hrs splenocyte culture in the presence of different concentrations of all the fractions of *L.inermis* extract, by sandwich ELISA. The results are analysed using Graph Pad Prism 5.1 software and expressed as the mean ± SEM. *p < 0.05, **p < 0.01 and ***p < 0.001 compared with the untreated control wells (one-way analysis of variance/ Dunnett's test).**

***In vitro* effect of different fractions of *L. inermis* leaves on IL-4 production:** In hexane fraction, all the concentrations down-regulate the IL-4 production (Fig. 3A). However, the maximum IL-4 inhibition was recorded at 500µg/ml i.e. 1.70±0.05. The 20 µg/ml concentrations also inhibit the IL-4 production but it was non-significant. Significant ($p<0.05$) decrease was recorded at 50, 100 and 250 µg/ml. 500 µg/ml conc. showed maximum inhibition i.e. 30.04% and highly significant ($p<0.01$) result was obtained with the comparison of control.

In the ethyl acetate fraction, highest IL-4 inhibition was found at 100 µg/ml i.e. 1.81± 0.04pg/ml and above this concentration, toxicity occurred and IL-4 concentration was very less. Highest inhibition 25.5% was found at 100 µg/ml. 20 and 50 µg/ml also showed inhibition but significant ($p<0.01$) decreases were recorded at 100 µg/ml (Fig.3B).

IL-4 inhibition was found maximum at 100 µg/ml of methanol fraction of *L. inermis* with the value of 1.79 ± 0.04pg/ml and this concentration showed highly significant ($p < 0.01$) data with the comparison of control. Toxicity was observed at 250 and 500 µg/ml concentrations of methanol fraction. % inhibition was also observed at 20 and 50 µg/ml. The highest inhibition index (26.34%) came at 100µg/ml (Fig. 3C).

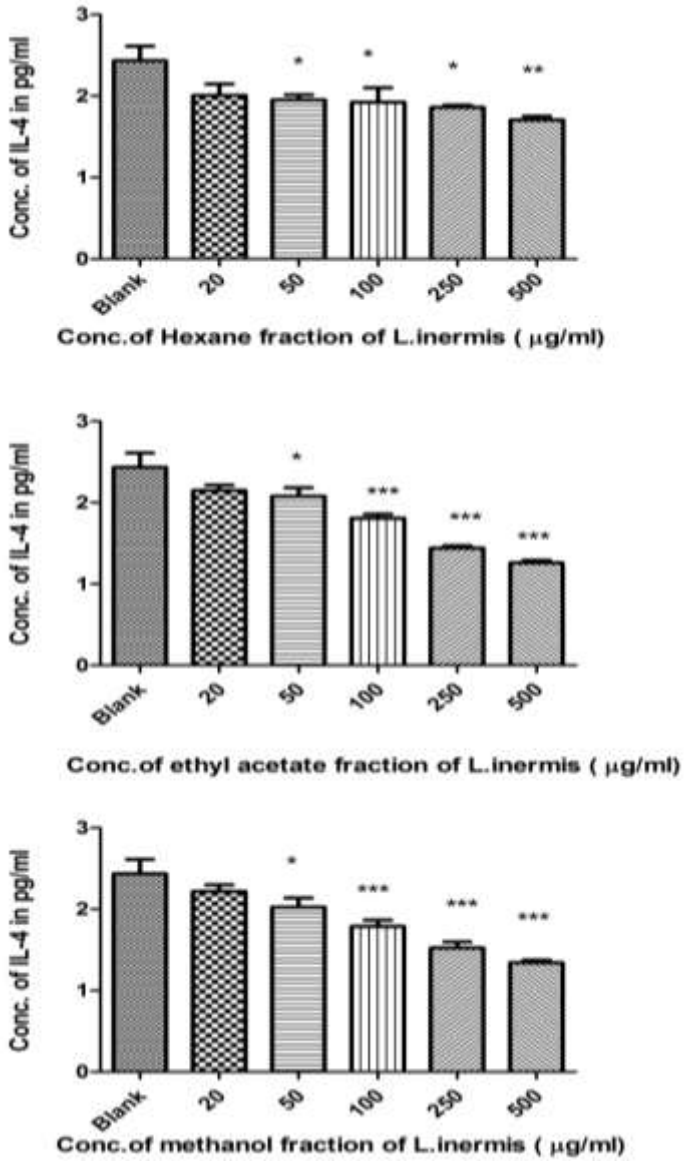


**Figure 3: Effect of *L.inermis* extracts on IL-4 cytokine modulation. Spleens were taken out from Wistar albino rats. Single cell suspension was made and 0.2 ml of $2X10^6$ cells /ml concentration were cultured in 96 well culture plate in the presence of different concentrations (25, 50, 100, 250 and 500µg/ml) of A: Hexane extract of *L.inermis* leaves; B: Ethyl acetate extract of *L.inermis* leaves; C:Methanolic extract of *L.inermis* leaves. IL-4 cytokine was quantitated, in the culture supernatant of 48 hrs splenocyte culture in the presence of different concentrations of all the fractions of *L.inermis* extract, by sandwich ELISA. The results are analysed using Graph Pad Prism 5.1 software and expressed as the mean ± SEM. *p < 0.05, **p < 0.01 and ***p < 0.001 compared with the untreated control wells (one-way analysis of variance/ Dunnett's test).**

## Discussion

The spleen is a major secondary lymphoid organ that responds to blood-borne infection and traps blood-borne antigen and contributes to humoral as well as cell-mediated immune response. Therefore, for determination of the effect of all fractions on splenocytes, *in vitro* experiment was designed in Wistar albino rat model.

Among all fractions, hexane showed the highest stimulation and it exhibited dose dependent induction of Con-A treated splenocyte proliferation. 20µg/ml, 50µg/ml, 100µg/ml, 250µg/ml and 500µg/ml of hexane fraction enhanced 18.12%, 18.28%, 22.48%, 25.5% and 40.93% proliferation respectively in respect to control. In ethyl acetate and methanol fraction, highest stimulation index was obtained at 50µg/ml i.e. 9.89% and 39.9% respectively. All the results showed significant increase (Fig. 1). In the previous study, Mikhaeil et al[12] also reported the immunostimulatory activity of methanolic fractions of *L. inermis* in ConA stimulated lymphocyte transformation assay. Methanolic extract of *L.inermis* was also found to enhance the nonspecific cellular and humoral response in fishes against *A. hydrophila*.[20]

Naphtoquinone, present in large quantities in the extract of *L.inermis*, has been shown to have immunomodulatory activity.[7] Squalene has been reported to have immune stimulatory activity.[18] In our previous studies[17], GC-MS studies clearly indicate the presence of squalene in hexane and methanol fraction which might be responsible for immune stimulation recorded with these fractions. Many other compounds present in the extract might have a synergistic effect on the proliferation of lymphocytes.

Thymus is the primary immune organ in which T cells mature. These cells mature based on the glycoproteins i.e. CD8 glycoprotein (cytotoxic) and CD4 glycoprotein (helper T cells). Th cells differentiate into main three subsets Th1, Th2 and Th17 which are characterized by different cytokine profiles and these subsets of Th cells have different functions in the immune system of an organism.[14] Th1 releases IFN-γ and TNF; Th2 releases IL-4 (an important survival factor for B-type lymphocytes), IL-5 and IL-13 and Th17 produces IL-17 (a cytokine playing an important role in host defence against bacteria and fungi[14], It is now well documented that cytokines, low molecular weight glycoproteins play major roles in inducting and regulating the development of effective immune responses involving cells of immune, inflammatory and haematopoietic systems.[13]

Cytokine IL-2 and IL-15 have been given as therapeutic agent in cancer treatment.[21] Looking at the clinical significance of cytokines, *in vitro* modulation of IL-2 and IL-4 cytokines from *L. inermis* treated splenocyte cell culture was determined. Outcomes of this study revealed that different doses of hexane fractions of *L. inermis* significantly stimulated the production of IL-2 as compared to control wells. Dose-dependent increase was also observed and highest % stimulation index was found at 500 µg/ml concentration (40.93%) (Fig. 2A). In ethyl acetate fraction, (10.81%) stimulation index was estimated with 50 µg/ml concentration and at this concentration, significant ($p<0.05$) increase was found (Fig. 2B). In methanol fraction, highest % stimulation index value (33.04%) was recorded at 50µg/ml while 100µg/ml also showed 26.5 % stimulation index (Fig. 2C). At both concentrations, results showed highly significant ($p< 0.01$) data. *L.inermis* was also reported to have cytotoxicity against several cancer cell lines.[8] A study conducted on albino mice showed the reduction of diethyl nitrosamine induced hepatocellular carcinoma by methanolic extract of *L.inermis*.[1]

Moreover, some of the workers[3,10] described clinical use of IL-2 as a therapy to improve the functional efficacy of the immune system of the AIDS patient by promoting IFN-γ (Th1 cytokine) secretion. FDA (Food and Drug Administration, US) agrees to use IL-2 as an immunotherapeutic agent for treating renal cancer, metastasis and malignant melanoma.[2,15]

Different doses of all fractions of *L. inermis* leaves as compared to control also down regulate the level of IL-4. Percentage inhibition of IL-4 was found to be 30.04% in splenocytes culture of albino rats treated with 500 µg/ml hexane fraction of plant leaves in relation to control whereas in ethyl acetate and methanol fraction, down regulation 25.51% and 26.34% were also observed at 100 µg/ml. IL-4 and IL-10 are the key cytokines of Th-2 subset of T helper cells and anti inflammatory in nature. Chaibi et al[5] investigated the anti inflammatory activity of methanol extract and found it superior than that of hexane and chloroform extract.

In conclusion, results indicate the presence of immunomodulatory metabolite in leaves of *L. inermis* which provide scientific evidence to the ethnomedical use of this plant. Cytokine modulation study indicates that leaves of *L. inermis* have wide spectrum medicinal potential based on *in vitro* bioassay experiments employing Wistar albino rat model. Furthermore, well designed clinical studies using well précised and standardized methodology are needed in immune compromised individuals to assess and analyzes whether *L. inermis* could be used for its medicinal potential.

## Acknowledgement

The authors thank Director and Head, IAH, GLA University for providing the facilities needed.

## References

1. Abdel-Hamid N.M., Mohafez O.M., Nazmy M.H., Farhan A. and Thabet K., The effect of co-administration of *Lawsonia inermis* extract and octreotide on experimental hepatocellular carcinoma, *Environ Health Prev Med*., **20**, 195–203 **(2015)**

2. Atkins M.B., Lotze M.T., Dutcher J.P., Fisher R.I., Weiss G., Margolin K., Abrams J., Sznol M., Parkinson D., Hawkins M., Paradise C., Kunkel L. and Rosenberg S.A., High dose

recombinant interleukin-2 therapy for patients with metastatic melanoma: analysis of 270 patients between 1985 and 1993, *J Clin Oncol*, **17(7)**, 2105-63 **(1999)**

3. Bernstein Z.P., Porter M.M., Gould M., Lipman B., Bluman E.M., Stewart C.C., Hewitt R.G., Fyfe G., Poiesz B. and Caligiuri M.A., Prolonged administration of low dose interleukin-2 in human immunodeficiency virus associated malignancy results in selective expansion of innate immune effectors without significant clinical toxicity, *Blood*, **86**, 3287-94 **(1995)**

4. Benaissa K. and Belhamra M., An optimization of the extraction and the physicochemical proprieties of the essential oil of *Lawsonia inermis* L. cultivated in Biskra (Department of Algeria), *Indian J. Pharm. Educ.*, **51(3)**, 286-89 **(2017)**

5. Chaibi R., Drine S. and Ferchichi A., Chemical study and biological activities of various extracts from *Lawsonia inermis* (Henna) seeds, *Acta Medica Mediterranea*, **33**, 981-6 **(2017)**

6. Chaudhary G.D., Poonia P., Kamboj P. and Kalia A.N., Hepatoprotective potential of *Lawsonia inermis* (seeds), *Int. J. Phytopharmacol*, **3**, 66–73 **(2012)**

7. Dikshit V., Dikshit J., Saraf M., Thakur V. and Sainis K., Immunomodulatory activity of naphthoquinone fraction of *Lawsonia inermis* Linn, *Phytomedicine*, **7**, 102-03 **(2000)**

8. Endrini S., Rahmat A., Ismail P. and Taufiq-Yap Y.H., Comparing of the cytotoxicity properties and mechanism of *Lawsonia inermis* and *Strobilanthes crispus* extract against several cancer cell lines, *J Med Sci*., **7(7),** 1098-1102 **(2007)**

9. Goel A, Singh D.K. and Bhatia A.K., Effect of *Ocimum sanctum* extract on the induction of IFN-γ and IL-10 cytokines and their mRNA expression, *Journal of Immunology and Immunopathology*, **12(1),** 29-41 **(2010)**

10. Jacobson E.L., Pilaro F. and Smith K.A., Rational interleukin2 therapy for HIV positive individuals: daily low doses enhance immune function without toxicity, Proc. Natl. Acad. Sci. U.S.A., **93**, 10405-10 **(1996)**

11. Kumar H.C., Ramesh A., Kumar J.N.S. and Ishaq B.M., A Review on Hepatoprotective Activity of Medicinal Plants, *International Journal of Pharmaceutical sciences and Research*, **2(3)**, 501-15 **(2011)**

12. Mikhaeil B.R., Badria F.A., Maatooq G.T. and Amer M.M.A., Antioxidant and immunomodulatory constituents of henna leaves, *Zeitschrift fuer Naturforschung C*, **59**, 468-76 **(2014)**

13. Owen J., Punt J., Stranford S.A. and Jones P.P., Kuby Immunology, 7th ed., 1-691 **(2013)**

14. Raphael I., Nalawade S., Eagar T.N. and Forsthuber T.G., T cell subsets and their signature cytokines in autoimmune and inflammatory diseases, *Cytokine*, **74**, 5-17 **(2015)**

15. Rosenberg S.A., Treatment of 283 consecutive patients with metastatic melanoma or renal cancer using high-dose bolus interleukin-2, *Journal of the American Medical Association*, **271(12),** 907-13 **(1994)**

16. Shankar K. and Liao L.P., Traditional Systems of Medicine, *Physical Medicine and Rehabilitation Clinics of North America*, **15**, 725-47 **(2004)**

17. Sharma R.K. and Goel A., Identification of phytoconstituents in *Lawsonia inermis* Linn. leaves extract by GC-MS and their antibacterial potential, *Pharmacogn J*, **10**, 1101-8 **(2018)**

18. Sivasubramanian R. and Brindha P., *In vitro* cytotoxic, antioxidant and GC-MS studies on *Centratherum punctatum* cass, *International Journal of Pharmacy and Pharmceutical Science*, **5(3)**, 364-67 **(2013)**

19. Snafi A.E.A., A review on *Lawsonia inermis*: A potential medicinal plant, *International Journal of Current Pharmaceutical Research*, **11(5)**, DOI: 10.22159/ijcpr.2019v11i5.35695 **(2019)**

20. Soltanian S. and Fereidouni M.S., Effect of Henna (*Lawsonia inermis*) extract on the immunity and survival of common carp, *Cyprinus carpio* infected with *Aeromonas hydrophila*, *Int Aquat Res*, DOI 10.1007/s40071-016-0141-2 **(2016)**

21. Waldmann T.A., The biology of interleukin-2 and interleukin-15: implications for cancer therapy and vaccine design, *Nature Review Immunology*, **6(8)**, 595-60 **(2006)**

22. WHO, Traditional Medicine, WHO, Geneva **(2003)**.

(Received 11th May 2020, accepted 18th July 2020)