Introduction

The structural and functional changes of the immune system caused by drugs and plants are responsible for the immunosuppression or immunostimulation, which, in turn, may cause adaptation of the host defense mechanism against cancer and infection. These changes may also stimulate the abnormal immune response causing autoimmunity and allergy.[1,2] Mefenamic acid belongs to nonsteroidal anti-inflammatory class of drugs having analgesic, anti-inflammatory, and antipyretic activities. It is used to relieve moderate to severe pain such as muscular aches, menstrual cramps, headache, and dental pain.
Effects of Mefenamic Acid on Cell-mediated Immunity

Delayed type hypersensitivity test

Intraperitoneal treatment with mefenamic acid and immunomodulator was started at day 1 and continued for 8 days. Mice groups were carefully shaved for DTH assay. All the mice were sensitized with 0.1 ml of 2% dinitrochlorobenzene (DNCB) at day 2, except negative control group which was sham-sensitized with 0.1 ml acetone only. Before applying DNCB, skin thickness of mice was measured with the help of vernier caliper. Previously, sensitized mice were again challenged with 0.2 ml of 2% DNCB at day 8, except negative control group which was false-challenged with 0.2 ml acetone. The skin thickness was measured at 24 h, 48 h, and 72 h after DNCB challenge.

Cyclophosphamide-induced myelosuppression

Mice of the experimental groups were treated with mefenamic acid and immunomodulator for consecutive 10 days. About 200 mg/kg cyclophosphamide was administered subcutaneously at the 10th day. Blood samples were collected from rat tail before and 72 h after administration of cyclophosphamide. Total white blood cell (WBC) count, RBC count, hemoglobin (Hb) content, lymphocyte levels, and neutrophil levels were measured using automated hemocytometer.

Effects of Mefenamic Acid on Humoral Immunity

Hemagglutination test

Preparation of sheep red blood cells

Sheep RBCs were separated from freshly collected blood of sheep. For this purpose, 5 ml blood was taken in a centrifuge tube, and volume was made up to 15 ml with phosphate buffer saline. Blood was centrifuged at 5000 rpm for 10 min. The RBC pellet was collected after removal of supernatant fluid and procedure was repeated twice. SRBCs were counted under the microscope using the neubauer chamber and number of cells was adjusted to 0.5 × 10^6 cells/0.1 ml. This concentration of cells is known to induce immunological response in mice.

Procedure

Mice were weighed and divided into five groups. All the groups were immunized with 0.5 × 10^6 cells/0.1 ml of SRBCs at day 0. All the groups were intraperitoneally treated for seven consecutive days. On the 7th day, blood sample was collected from the tail of each mouse and centrifuged at 5000 rpm for 15 min to separate the serum. Antibody titer was determined using HA titer method. 96-well plates were used for performing HA test, and 25 µl of PBS was added in all the wells, except the last column which was denoted as control. Serum (25 µl) was then placed in the first row of microtiter plate and was 2-fold serially diluted up to the 8th row of the microtiter plate. Twenty-five microtiter of 10 v/v SRBCs were then dispensed in microtiter plate and the plate was incubated at 37°C for 1 h.

Mice lethality test

Preparation of Pasteurella multocida culture

Pasteurella multocida was reconstituted in normal saline. LD50 (10^2 cells/0.5 ml) dose of P. multocida was injected subcutaneously into one rabbit. After death of the rabbit, blood samples were collected, and postmortem was performed.
Specific organs (liver, heart, spleen, and kidney) were separated, cut into small pieces, and preserved. A small piece of any organ was then placed into blood agar media (pyrogen free) using a Petri dish and incubated for 24 h.

Procedure

All the mice groups were treated with experimental drugs for 21 days starting from day 1. On 7th and 17th day of experiment, all the groups were immunized through intraperitoneal route with hemorrhagic septicemia vaccine, except negative control. On the 21st day of experiment, all the mice were challenged with lethal dose of *P. multocida* subcutaneously and were examined for about 72 h.

Statistical Analysis

The data obtained from above mention experiments were statistically analyzed using GraphPad Prism version 6 software. All the data were expressed as mean ± standard error of the mean and analyzed using one-way ANOVA followed by Tukey’s test or Student’s t-test where applicable. *P < 0.05* was considered statistically significant.

Results

**Treatment with Mefenamic Acid Significantly Reduced Delayed Type Hypersensitivity Test After 24 h, 48 h, and 72 h**

We found that treatment with DNCB caused increase in skin thickness in all groups after 24 h. The data showed a significant (1.5 ± 0.1; *P < 0.001*) increase in skin thickness in positive control group as compared with negative control group (0.01 ± 0.005). Treatment with low (0.9 ± 0.1; *P < 0.01*), medium (0.8 ± 0.08; *P < 0.001*), and high (0.7 ± 0.07; *P < 0.001*) doses of mefenamic acid significantly inhibited the increase in skin thickness as compared with positive control group. Immunomodulator also showed a significant (1.5 ± 0.1; *P < 0.001*) increased skin thickness [Figure 1a].

Analysis after 48 h showed a significant increase in skin thickness in positive control group (1.3 ± 0.1; *P < 0.001*) as compared with negative control group (0.020 ± 0.001). Treatment with immunomodulator (1.8 ± 0.13; *P < 0.001*) also showed a significant increase as compared with negative control group. Treatment with low dose (0.7 ± 0.07; *P < 0.05*), medium dose (0.46 ± 0.07; *P < 0.01*), and high dose (0.36 ± 0.04; *P < 0.01*) caused significant decrease in skin thickness as compared with positive control group [Figure 1b].

Analysis after 72 h showed that treatment with low dose, medium dose, and high dose showed significantly (0.32 ± 0.06; *P < 0.001*, 0.16 ± 0.05; *P < 0.001*, and 0.07 ± 0.01; *P < 0.001*, respectively) decreased skin thickness when compared with positive control group. We determined a significant elevation in positive control group (0.7 ± 0.06; *P < 0.001*) as compared with negative control group (0.04 ± 0.01). Treatment with immunomodulator (1.18 ± 0.1; *P < 0.001*) also showed a significant increase in skin thickness as compared to negative control [Figure 1c].

**Mefenamic Acid Significantly Reduced White Blood Cell Counts, Lymphocyte Levels, Neutrophil Levels, Red Blood Cell Counts, and Hemoglobin Content in Healthy Mice**

We compared control group (10.7 ± 0.1) with experimental groups and results showed nonsignificant difference of immunomodulator (11.5 ± 0.2) as compared with control group. The data revealed that there were significant reduction in WBC counts in low dose (9.3 ± 0.3; *P < 0.01*), medium dose (7.1 ± 0.2; *P < 0.001*), and high dose (5.6 ± 0.2; *P < 0.001*) treated groups [Figure 2a].

Treatment with low dose (71.5 ± 3; *P < 0.05*), medium dose (65 ± 1.4; *P < 0.01*), and high dose (51.0 ± 2.7; *P < 0.001*) showed a significant decrease in lymphocytes levels as compared with control group. However, nonsignificant difference was found when the result of immunomodulator (74.8 ± 1.6) group was compared with control group (79.5 ± 1.6) [Figure 2b].

We found nonsignificant difference in neutrophil levels when immunomodulator group (80.8 ± 0.9) was compared with control group. Treatment with low dose (69.8 ± 2.8; *P < 0.05*), medium (65.8 ± 1.8; *P < 0.001*), and high doses (52.2 ± 3.6; *P < 0.001*) showed a significant alleviation as compared with control group (81.3 ± 1.6) [Figure 2c].

We did not find statistical significant difference when low dose (6.5 ± 0.1) and immunomodulator (7.0 ± 0.2) groups were compared with control group (6.8 ± 0.2). The data showed a significant reduction in RBC counts in medium (6.2 ± 0.1; *P < 0.05*) and high dose treated groups (6.0 ± 0.1; *P < 0.01*) as compared with control group [Figure 2d].

Treatment with low dose (10.5 ± 0.4) of mefenamic acid and immunomodulator (11.2 ± 0.37) showed nonsignificant difference in Hb levels as compared to control group (11.2 ± 0.3). Whereas medium (9.0 ± 0.5; *P < 0.01*) and high dose groups (8.2 ± 0.4; *P < 0.001*) demonstrated significant decrease in Hb levels when compared with control group [Figure 2e].

**Mefenamic Acid Significantly Reduced White Blood Cell Counts, Lymphocyte Levels, Neutrophil Levels, Red Blood Cell Counts, and Hemoglobin Content in Cyclophosphamide-induced Myelosuppressive Mice**

We found significant (*P < 0.001*) alleviation in WBC counts in positive control group (8.4 ± 0.1; *P < 0.001*) as compared with negative control group (10.7 ± 0.1). Treatment with low dose (6.4 ± 0.1), medium dose (5.2 ± 0.1), and high dose (4.3 ± 0.2) of mefenamic acid significantly (*P < 0.001*) attenuated WBC counts as compared with positive control. Whereas immunomodulator (10.4 ± 0.15; *P < 0.01*) showed a significant prevention in WBC reduction caused by cyclophosphamide administration [Figure 3a].

Lymphocytes levels were found significantly (*P < 0.001*) alleviated in positive control (79.5 ± 1.6) as compared with negative control group (61.4 ± 1.6). Treatment with low dose (51.7 ± 0.7), medium dose (48.8 ± 1.6), and high dose (40.8 ± 1.4) of mefenamic acid significantly (*P < 0.001*) attenuated the lymphocyte levels as compared with positive control group. Treatment with immunomodulator (75.6 ± 2; *P < 0.001*) caused significant elevation as compared to positive control [Figure 3b].

We found significant suppression of neutrophil levels in positive control group (59.3 ± 3.6; *P < 0.001*) as compared with negative control group (81.3 ± 1.6). Treatment with immunomodulator (73.83 ± 2.1; *P < 0.05*) nearly normalized the neutrophils level. Therapy with low dose (43.3 ± 3.8; *P < 0.01*), medium dose (29.5 ± 2.8; *P < 0.001*), and high dose (19.3 ± 2.1; *P < 0.001*) displayed a significant reduction in neutrophil levels as compared with positive control group [Figure 3c].
Treatment with low dose (4.6 ± 0.2) and medium dose (4.1 ± 0.3) did not show significant difference in RBC counts as compared with positive control group. However, high dose treated group showed (3.4 ± 0.1; P < 0.001) significant reduction as compared with positive control group. We also found significant (P < 0.001) alleviation in RBC counts in positive control group (4.7 ± 0.1; P < 0.001), as compared with negative control group (6.8 ± 0.06). Treatment with immunomodulator (7.3 ± 0.2; P < 0.001) normalized the RBC counts [Figure 3d].

We found significantly decreased levels of Hb in the positive control (8.4 ± 0.17; P < 0.001) group as compared with negative control group (11.3 ± 0.3). Treatment with low dose (6.7 ± 0.09), medium dose (5.46 ± 0.1), and high dose (4.6 ± 0.2) of mefenamic acid showed a significant (P < 0.001) attenuation of Hb content as compared with positive control group. Treatment with immunomodulator significantly (10.4 ± 0.15; P < 0.001) prevented the cyclophosphamide induce decrease in Hb content [Figure 3e].

**Mefenamic Acid Significantly Suppressed Antibody Titer in Hemagglutination Assay**

We found that treatment with low (85.8 ± 2.3), medium (79.8 ± 0.4), and high (65.03 ± 2.15) doses of mefenamic acid significantly (P < 0.001) decreased the antibody titer as compared with the control group (136 ± 11). Similarly, treatment with cyclophosphamid (reference drug) also significantly (52.7 ± 1.7; P < 0.001) attenuated the antibody titer [Figure 1d].

**Mefenamic Acid Increased the Mortality Rate in Mice Lethality Test**

Administration of *P. multocida* caused 100% mortality in 24 h in the negative control group, whereas 33% mortality was observed in positive control group within 72 h. Treatment with low dose and medium dose increased the mortality rate to 50% and 66.6%, respectively as compared with positive control group. We observed the death of one mouse after 48 h and two mice later after 72 h in low dose treated group. In medium dose treated group, we found one mouse dead within 12–24 h. Subsequently, two mice died after 48 h, and one mouse was found dead after 72 h. Treatment with high dose of mefenamic acid caused 100% mortality within 24 h, whereas cyclophosphamide used as a reference drug caused death of all five mice within 12 h [Table 1].

**Discussion**

We evaluated the immunosuppressive effect of mefenamic acid through DTH assay. DNCB was used as an allergen to cause hypersensitivity reaction. When DNCB is applied on the skin, it causes accumulation of macrophages on reaction site which release IL-12 and IL-18. These cytokines, in turn, are responsible for differentiation of Th1 cell. These factors cause
increase in skin thickness at the site of reaction which is widely considered as a measure of DTH. In our study, inflammatory response toward allergen was greater in positive control group as compared with mefenamic acid treated groups. In mefenamic acid treated groups, we found dose-dependent reduction in skin thickness as compared with positive control group. DTH is a T-cell-mediated reaction, and T-cell-mediated immunity is regulated by different prostaglandins that are product of cyclooxygenase (COX) pathway, for example, PGE2, PGI2, and PGD2, and by T-cell specific cytokines. NSAIDs inhibit COX pathway which eventually leads to T-cell suppression. Ketoprofen is another NSAID, which is known to suppress the T-cell-mediated DTH reaction.

Besides, DTH assay, we also evaluated the immunomodulatory effects of mefenamic acid on cell-mediated immunity using cyclophosphamide-induced myelosuppression assay. Cyclophosphamide causes bone marrow suppression by alkylation of DNA. Effects of mefenamic acid were evaluated in cyclophosphamide-induced neutropenic mice, as well as, in healthy mice. Mefenamic acid showed immunosuppressive effect by dose-dependently decreasing the total leukocyte counts (TLCs) and differential leukocyte counts (DLCs). WBCs are fundamental constituent of immune system. Elevation or attenuation in WBCs directly affects the immune system as they are known to identify the pathogen and support the immune response. Neutropenia is associated with the treatment of different disease conditions such as cancer chemotherapy, rheumatoid arthritis, and allergic

**Table 1:**

| Groups          | Within 12 h | 12-24 h | After 48 h | After 72 h | Total mortality | Percentage mortality |
|-----------------|-------------|---------|------------|------------|-----------------|---------------------|
| Negative control| 3           | 3       | -          | -          | 6/6             | 100                 |
| Positive control| -           | -       | 1          | 1          | 2/6             | 33                  |
| Low dose        | -           | -       | 1          | 2          | 3/6             | 50                  |
| Medium dose     | -           | 1       | 2          | 1          | 4/6             | 66.6                |
| High dose       | 4           | 2       | -          | -          | 6/6             | 100                 |
| Cyclophosphamide| 5           | 1       | -          | -          | 6/6             | 100                 |

Figure 2: Mefenamic acid showed immunosuppressive effect by significantly reducing white blood cell counts (a), lymphocyte levels (b), neutrophil levels (c), red blood cell counts (d), and hemoglobin content (e) in healthy mice. Immunomodulator prevented the suppression in hematological parameters. Mean ± standard error of the mean is given to represent the data, where *P < 0.05, **P < 0.01, and ***P < 0.001 represent comparison of experimental groups with positive control.
Lymphocytes are considered as an essential parameter of immune system which plays a key role in modulating immune system through the differentiation and proliferation of T-cells and B-cells. Previous studies showed that other NSAIDs such as ketoprofen demonstrated immunosuppressive effects by significantly decreasing the TLC and DLC, before and after administration of cyclophosphamide. It was suggested that NSAIDs enhance cell apoptosis and reduce granulocyte colony stimulating factor which plays an important role in the development of new neutrophils.

Antigen-specific antibody production is the main component of humoral immunity. In this study, we evaluated the effects of mefenamic acid on humoral immunity through HA assay and mice lethality test. For experimental purpose, the most frequently used method for evaluating humoral immunity is testing of antibody titer against sheep RBCs. Antigens are neutralized either directly by antibody or antibodies convert antigen in such form that is easily phagocytized. Antibody titer is the direct way to evaluate humoral immunity. In the current study, all the doses of mefenamic acid were found to decrease antibody titer in a dose-dependent manner as compared with control group, similar to the effect of cyclophosphamide used as a reference drug. Hamdani et al. suggested that ketoprofen treated mice decreased the production of IgG and IgM antibody against sheep RBCs. The decreased antibody titer found in our study might also be due to the decrease production of IgG and IgM antibodies in the serum of mefenamic acid-treated mice. However, further studies are required to confirm evaluate the effects of mefenamic acid on IgG and IgM levels.

Mice lethality test is used to evaluate the survival rate after injecting challenging dose of P. multocida in hemorrhagic septicemia vaccine immunized mice. P. multocida is pathogenic to experimental animals, so mice are first immunized with vaccine to produce antibodies and then P. multocida is administered to evaluate the effect of drugs or plant extracts on lethality ratio. If the drug is immunostimulant and increases the antibody production, it would cause survival of mice against P. multocida culture, and if the drug is immunosuppressant, it would cause the lethality in experimental animal. In the current study, mefenamic acid treated group showed increase in mice lethality ratio as compared with positive control group. Immunization with hemorrhagic septicemia vaccine produces IgG and IgM antibodies in mice blood which causes mice to survive against P. multocida antigen. It may be assumed that mefenamic acid treated groups might have decreased antibodies (IgG and IgM) production which led to increase in the mortality rate as compared to positive control.

Conclusion

The data suggest that mefenamic acid possesses immunosuppressive activity which is evident by reduction in TLCs, lymphocytes levels, neutrophil levels, RBC counts, and Hb content in healthy mice. Mefenamic acid showed

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**Figure 3:** Treatment with mefenamic acid significantly reducing white blood cell counts (a), lymphocyte levels (b), neutrophil levels (c), red blood cell counts (d), and hemoglobin content (e) in cyclophosphamide-induced myelosuppressive mice. Immunomodulator significantly prevented the suppression in hematological parameters. Mean ± standard error of the mean is given to represent the data, where n = 6. *P < 0.05, ***P < 0.001 represent comparison of experimental groups with positive control. #**P < 0.001 represents comparison with negative control group.
immunosuppressive effect on cell-mediated immunity by attenuation of DTH and all hematological parameters in a mouse model of cyclophosphamide-induced myelosuppression. Immunosuppressive activity of mefenamic acid on humoral immunity was also evident in our study as different doses significantly reduced antibody titer in HA assay and increased mice lethality ratio.

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Nil.

Conflicts of Interest
There are no conflicts of interest.

References
1. Hule AK, Juvekar AR. Evaluation of immunomodulatory effects of methanol extract of Elaeocarpus ganitrus seeds. J Nat Remed 2010;10:1-10.
2. Heroor S, Beknal AK, Mahurkar N. Immunomodulatory activity of methanolic extracts of fruits and bark of Ficus glomerata Roxb. in mice and on human neutrophils. Indian J Pharmaco 2013;45:130-5.
3. Somchit N, Sanat F, Gan EH, Shahrin IA, Zuraini A. Liver injury induced by the non-steroidal anti-inflammatory drug mefenamic acid. Singapore Med J 2004;45:530-2.
4. Kushima K, Oda K, Sakuma S, Furusawa S, Fujitama M. Effect of prenatal administration of NSAIDs on the immune response in juvenile and adult rats. Toxicology 2007;232:257-67.
5. Barasoain I, Rojo JM, Portolés A. “In vivo” effects of acetylsalicylic acid and two other derived compounds on primary immune response and lymphoblastic transformation. Immunopharmacology 1980;2:293-300.
6. Shabbir A, Shahzad M, Ali A, Zia-ur-Rehman M. Anti-arthritic activity of N0-[(2,4-dihydroxyphenyl) methylidene]-2-(3,4-dimethyl-5,5-dioxidopyrazolo[4,3-c] [1,2]benzothiazin-1 (4H)-yl) acetohydrazide. Eur J Pharmacol 2014;738:263-72.
7. Aleksandrova GM, Rozhkova VN, Borisova LN. Comparative characteristics of the immunotropic activity of sodium diclofenac, indomethacin and sodium salicylate. Farmakol Toksikol 1981;44:450-3.
8. Moed H, Stoff TJ, Boorisma DM, von Blomberg BM, Gibbs S, Bruynzeel DP, et al. Identification of anti-inflammatory drugs according to their capacity to suppress type-1 and type-2 T cell profiles. Clin Exp Allergy 2004;34:1868-75.
9. Colin V, Turcu D, Petrut T. Immunological determination in rabbits after immune response potentiation by using immunomodulators. J Vet Med 2011;57:25-32.
10. Omer MO, Ashraf A, Javeed A, Maqbool A. Immunostimulatory effect of ivermectin on macrophage. J Anim Plant Sci 2012;22:250-5.
11. Lagrange PH, Mackaness GB, Miller TE. Influence of dose and route of antigen injection on the immunological induction of T cells. J Exp Med 1974;139:528-42.
12. Heden CG. On the estimation of fifty percent end-points in serological titrmetry. J Pathol Bacteriol 1946;56:477-81.
13. Fulzele SV, Satvir PH, Joshi SB, Dorle AK. Study of the immunomodulatory activity of Haridradi ghrita in rats. Indian J Pharmaco 2003;35:51-4.
14. Dannenberg AM Jr. Roles of cytotoxic delayed-type hypersensitivity and macrophage-activating cell-mediated immunity in the pathogenesis of tuberculosis. Immunobiology 1994;191:461-73.
15. Kobayashi K, Kaneda K, Kasama T. Immunopathogenesis of delayed-type hypersensitivity. Microsc Res Tech 2001;53:241-5.
16. Daiji S, Chengcan Y, Shuh N. Emerging role of prostanooids in T cell-mediated immunity. Life 2010;8:591-6.
17. Paccagni SR, Bonorriste M, Ullieriez C, D’Ellos MM, Del Prete G, Baldari CT. Nonsteroidal anti-inflammatory drugs suppress T-cell activation by inhibiting p38 MAPK induction. J Biol Chem 2002;277:1509-13.
18. Hamdani DA, Javeed A, Ashraf M, Nazir J, Ghaffoor A, Yousaf MS. Effects of ketoprofen on cellular immune responses in mice. Pak J Zool 2015;47:551-7.
19. Kondo N, Takahashi A, Ono K, Ohnishi T. DNA damage induced by alkylation agents and repair pathways. J Nucleic Acids 2010;2010:543531.
20. Sultana R, Khanam S, Devi K. Immunomodulatory effect of methanol extract of Solanum xanthocarpum fruits. Int J Pharm Sci Res 2011;2:93-7.
21. Parham P. The Immune System. 3rd ed. New York: Garland Science; 2009. p. 9-10.
22. Vivier E, Ugelini S, Blaise D, Chabannon C, Brossay L. Targeting natural killer cells and natural killer T cells in cancer. Nat Rev Immunol 2012;12:239-52.
23. Rastogi B, Tiwari U, Dubey A, Babara B, Chauhan NS, Saraf DK. Immunomodulatory and cytotoxic activity of Cocculus hirsutus on immunosuppressed rat. Pharmacologyonline 2008;3:38-57.
24. Gokhale AB, Damre AS, Saraf MN. Investigations into the immunomodulatory activity of Argyreia speciosa. J Ethnopharmacol 2003;84:109-14.
25. Rishi P, Batra N, Sood S, Tiwari RP. Modulatory effects of Salmonella LAP-LPS on murine macrophages. Indian J Med Microbiol 2002;20:167-93.