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Original Article

Modulation of IL-17/IL-23 axis by chloroform fraction of *Boerhavia erecta* L.: a potential route for treating autoimmune disorders

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ABSTRACT

In view of remarkable similarities between two autoimmune disorders, rheumatoid arthritis and type 1 diabetes, and associated long-term adverse effects of pharmaceutical drugs in the treatment of such chronic metabolic disorders, herbal drugs are being targeted. This study evaluated immuno-modulatory activity of chloroform fraction of standardized hydro-alcoholic extract of root and stem parts of *Boerhavia erecta* L. (BE) containing 1-(4-hydroxytridecyloxy) pentadecan-4-ol by gene expression, *in vitro* and *in vivo* methods. A new bioactive compound, 1-(4-hydroxytridecyloxy) pentadecan-4-ol, hitherto unreported, was isolated from BE. The isolated compound was characterized by NMR and FTIR and the chloroform fraction containing the bioactive compound was subjected to *in vivo* anti-inflammatory and antidiabetic studies. Carrageanan-induced paw edema and cotton pellet granuloma in Wistar rats were considered as acute and chronic inflammatory models, respectively, with 5 groups (6 animals per group) in each study. After oral glucose tolerance test (OGTT), streptozotocin (STZ)-induced diabetic rat model with 6 groups of 6 animals each was used for antidiabetic study. In *in vitro* studies on RAW cell lines, the cell lines were stimulated with Lipopolysaccharide (LPS) and then treated with interleukin (IL) IL-23, IL-1β and IL-6 that were co-cultured both in the presence and absence of different doses (10 mg, 20 mg and 40 mg) of chloroform fraction of BE extract and finally the inhibition of IL-17 expression was recorded. The chloroform fraction having 1-(4-hydroxytridecyloxy) pentadecan-4-ol, at 40 mg/kg body weight, was effective as an antidiabetic and anti-inflammatory agent and exhibited statistical significance with that of control animal groups ($p < 0.01$). The study showed the inhibition of IL-17 significantly ($p < 0.0001$) in the presence of IL-23 and other cytokines. These immuno-modulatory activities are probably through IL-17/ IL-23 axis which needs to be to be further confirmed by human clinical trials targeting at least two autoimmune diseases.

KEYWORDS: auto-immune, PGE2, COX-2, interleukin, 1-(4-hydroxytridecyloxy) pentadecan-4-ol.

INTRODUCTION

It is now well established scientifically that in vertebrates, besides innate immune response as first line of defense, the adaptive responses are highly specific to a particular pathogen, providing long-lasting protection. Earlier, it was thought that different sub-populations of T helper (Th) cells, the common lymphoid progenitor cells that develop from bone marrow and migrate to thymus and develop as Th cells, could shape immune responses by differential cytokine production. The two subsets of Th cells were initially identified

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and were termed as “Th1 and Th2” [1]. As per this model Th1 cells mediate “cellular immunity,” wherein naïve Th cells are not predetermined to be either Th1 or Th2, but rather that the environment in which they encounter antigen dictates their subsequent role. In accordance with this idea, it was shown that IL-12, usually derived from antigen-presenting cells (APCs) such as dendritic cells, directs differentiation of naïve CD4+ T cells into the Th1 lineage. This is characterized by macrophage activation and opsonizing antibodies through the actions of the cytokine interferon-γ (IFNγ). Conversely, Th2 cells mediate “humoral immunity” wherein the cytokine IL-4 promotes development to the Th2 lineage. This is characterized by activation of B cells and effector antibodies mediated by interleukin (IL)-4, IL-5 and IL-13. The Th1–Th2 hypothesis does not adequately explain the regulation of CD4+ T cells during autoimmunity and infection. IL-17 (often referred to as IL-17A), although primarily derived from T cells, was not obviously a Th1 or Th2 cytokine. Therefore, a new CD4+ T cell lineage could possibly be involved in the production of another cytokine [2]. It has become clear that T helper (Th17) cells, a distinct lineage of CD4+ T helper cells, produce interleukin IL-17. These cells play an important role in the host defensive against extra cellular bacterial pathogens and participate in the pathogenesis of multiple inflammatory and autoimmune disorders. The identification of two distinct Th17 populations, one with pathogenic properties (Teff 17) and another with regulatory and protective properties (Treg 17), may explain the conventional nature of Th17 cells in autoimmunity, particularly, Type 1 Diabetes (T1D).

Recent studies on rheumatoid arthritis and the role of Th17 cells also suggests that IL-17A plays an imperative role in the pathogenesis of rheumatoid arthritis (RA). IL-17 is the signature cytokine of the newly-described “Th17” T helper cell population and has been implicated in the pathogenesis of numerous autoimmune diseases including rheumatoid arthritis [3]. Rheumatoid synovial tissue produces IL-17A, which causes cartilage and bone degradation in synovial and bone explants. Over expression of IL-17A induces synovial inflammation and joint destruction in animal RA models. Levels of IL-17A were found to be higher in RA synovial fluid when compared with osteoarthritis (OA) synovial fluid [4]. Controlling the release of pathogenic IL-17A cytokine could be a potential therapeutic approach for patients with RA and T1D. Indeed, IL-23 was shown to stimulate production of IL-17 in murine CD4+ T cells and it was found that IL-23 is indispensable for promoting autoimmunity; this led to a re-evaluation of the established paradigm of Th1-Th2 cytokine imbalance in autoimmunity [5-8]. In 2008, it was found that IL-23 is required for the *in vivo* development of Th17 cells and for the induction of EAE-experimental autoimmune encephalomyelitis [9]. However, in 2013, IL-17-specific and IL-23p19-specific antibodies (also known as IL-23A), have shown beneficial effects in trials for psoriasis, AS-ankylosing spondylitis, RA-rheumatoid arthritis and MS-multiple sclerosis.

Our recent studies on standardized *Boerhavia erecta* L. (BE) crude extract, a native to the United States, Mexico, Central America and Western South America, West and South Africa regions, have shown immuno-modulatory properties when compared with other herbs at preliminary level [unpublished data]. In the current study, we have also focused on *in vitro* and *in vivo* studies of chloroform fraction of the BE extract containing the isolated compound and reported probable mode of action through which it exhibits immuno-modulation activity.

2. MATERIALS AND METHODS

2.1. Drugs & chemicals

Fetal bovine serum (FBS), phosphate buffered saline (PBS), Dulbecco’s modified eagle’s medium (DMEM) and trypsin were obtained from Sigma Aldrich Co, St Louis, USA; EDTA, glucose and antibiotics from Hi-Media Laboratories Ltd., Mumbai; Dimethyl sulfoxide (DMSO) and propanol were purchased from E.Merck Ltd., Mumbai, India.

2.2. Plant material

Boerhavia erecta L. (BE) was identified and authenticated by Dr. K. Madhava Chetty, Professor of Botany, Sri Venkateswara University, Tirupathi, India. *B. erecta* (erect spiderling) is available at

cosmopolitan tropical and sub-tropical regions; for the study purpose it was collected from Sheshalam forests, India and deposited in the herbarium with voucher specimen numbers # T1404. For macroscopic & organoleptic characters, visible distinct characters were documented prior to initiation of the actual studies.

2.3. Gene expression studies

2.3.1. Cell lines and culture medium (for COX-2 and PGE2)

RAW (macrophage) cell line was procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in DMEM supplemented with 10% inactivated FBS, penicillin (100 IU/ml), streptomycin (100 μg/ml) and amphotericin B (5 μg/ml) in an humidified atmosphere of 5% CO_2 at 37 °C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm^2 culture flasks and all experiments were carried out in 96 micro-titre plates (Tarsons India Pvt. Ltd., Kolkata, India).

The mRNA expression levels of COX-2 and PGE2 were carried out using semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Briefly, the RAW cells were cultured in 60 mm petri-dish and maintained in DMEM medium for 24 h. The DMEM medium was supplemented with FBS and Amphotericin. To the dish, the required concentration of test sample was added along with LPS and incubated for 24 h. Total cellular RNA was isolated from the untreated (control) and treated cells using Tri Reagent as per standard protocol. cDNA was synthesized from total isolated RNA by reverse transcriptase kit according to manufacturer's instructions (Thermo scientific). 20 μl of the reaction mixture was subjected to PCR for amplification of COX-2/PGE2cDNAs. It is done separately using specifically designed primers procured from Eurofins India as an internal control, and the house keeping gene GAPDH was co-amplified with each reaction at 94 °C for 2 min. This is followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min. This was followed by final extension at 72 °C for 10 min, oligod T primer was used as I^{st} strand synthesis for PGE2 and COX-2 gene. For II^{nd} strand synthesis of PGE2, forward primer used was 5' GGC AGT GGG CGG ATG A 3' and reverse primer was 5' TCG GCA GGT GTT CGG TAC A 3' and the product size was 307 bp. For II^{nd} strand synthesis of COX-2, forward primer used was 5' TTC AAA AGA AGT GCT GGA AAA GGT 3' and reverse primer was 5' GAT CAT CTC TAC CTG AGT GTC TTT T 3' and the product size was 305 bp.

2.4. Isolation procedure

For column chromatography, hydro-alcoholic BE extract was adsorbed to 3 g of silica gel using SG-60/120 mesh and the mixture was dried until the silica gel was free flowing. Petroleum ether fraction: The order of solvent system in CC was Pet Ether : Chloroform (Pure Pet Ether; 8:2; 6:4; 4:6; 2:8); Chloroform : Ethyl Acetate ($CHCl_3$; 8:2; 6:4; 4:6; 2:8); Ethyl Acetate : Methanol (Ethyl acetate; 8:2; 6:4; 4:6; 2:8; Methanol). Only chloroform fraction was considered for further *in vitro* and *in vivo* studies because of its greater efficacy than petroleum ether fraction as per the results of preliminary gene expression studies.

2.4.1. Spectral analysis

The isolated compound from chloroform fraction was characterized by FTIR using Nicolet Avatar 330 FTIR spectrometer, ^{1}H and ^{13}C-NMR (Mercury Plus 300 MHz, Varian) and MS (LCMS-2010A, Shimadzu).

2.5. *In vitro* studies

The murine macrophage cell line RAW264.7 (RAW cells) was obtained from Microbial Type Culture Collection (MTCC, India) and maintained in a Complete medium, having lymphoid RPMI1640 medium supplemented with 2 mM glutamine, 100 units/ml of penicillin and streptomycin and 10% FBS (Sigma, St. Louis, MO, endotoxin NMT 10.0 EU/ml). Mouse bone marrow-derived macrophages (BMDMs) were generated from bone marrow cells and cultured with complete RPMI1640 medium containing 10 ng/ml of macrophage colony-stimulating factor (M-CSF) for one week. After 7-days culture, the fully differentiated BMDMs were used for experiments, 3×10^6 bone marrow cells were

cultured with RPMI1640 complete medium containing 40 ng/ml mouse IL-23 and 10 ng/ml IL-1β and IL-6 each and co-cultured with different doses of (10 mg, 20 mg and 40 mg) test sample administered as 1 μg/mL. Supernatants from BMDM cultures were harvested at 24 h after IL-23 and LPS stimulation and stored at −70 °C. IL-17 expression was recorded using mouse IL-17 Quantikine ELISA Kit (USA) following the standard protocol.

2.6. *In vivo* studies

Animal study was initiated post ethics committee approval, vide reference number RR/IAEC/03-2015. The research was conducted in accordance with internationally accepted principles for laboratory animal use and care, and committee for the purpose of control and supervision of experiments on animals (CPCSEA) guidelines. Male Wistar rats were used in our experimental work and were housed separately in the standard polypropylene cages and provided with food and water *ad libitum*. The animals were housed under standard environmental conditions with controlled conditions of temperature (23 ± 2 ºC), humidity (50 ± 5%) and 12 h light-dark cycles. The animals were acclimatized for a period of 2 weeks prior to the experimental work performed in accordance to the current guidelines for the care of the laboratory animals [10].

2.6.1. Acute toxicity study

The oral acute toxicity of chloroform fraction of *Boerhavia erecta* was determined in Wistar rats maintained under standard conditions [11]. The animals were maintained in fasting condition overnight prior to the experiment. Fixed dose (OCED Guideline no. 420) method of CPCSEA was adopted for toxicity studies [12]. The test article was administrated orally and no mortality was observed at a dose of 4000 mg/kg body weight.

2.6.2. Anti-inflammatory activity

Anti-inflammatory activity was evaluated by two models, namely carrageenan induced paw edema and cotton pellet granuloma.

2.6.2.1. Carrageenan-induced paw edema (acute model)

Animals were divided into five groups of rats, six in each group. Inflammation was induced by injecting 0.1 ml of 1% w/v carrageenan sodium salt subcutaneously in the sub-plantar region of the rat right hind paw. The test article (10, 20, 40 mg/kg) or diclofenac sodium (5 mg/kg) was administered orally [13] 1 h before carrageenan injection while control group received only saline at the doses of 10 ml/kg body weight. The hind paw volume was measured plethysmometrically before and after the carrageenan injection at hourly intervals for 3 h.

% inhibition of edema = (Vc-Vt)/Vc ×100

where, Vt = mean paw volume of test group; Vc = mean paw volume of control group.

2.6.2.2. Cotton pellet granuloma (chronic model)

Five groups of rats, six in each group, were included in this study. For cotton pellet granuloma, a 50 mg sterilized cotton pellet was implanted subcutaneously on the back of neck in rats under ether anesthesia. Animals in the control group received only the vehicle at the dose of 10 ml/kg body weight. Animals in treated group received the test article at the doses of 10, 20 and 40 mg/kg body weight once daily for 14 consecutive days. Diclofenac sodium (5 mg/kg b. wt.) was given as reference drug. On the 14th day the animals were sacrificed with ether, the pellets granulomas were removed, fixed from extraction tissue, dried overnight at 55 ± 0.5 ºC and weighed [14].

2.6.3. Anti-diabetic activity

2.6.3.1. Oral glucose tolerance test (OGTT) – acute model

OGTT was carried out in the diabetic-induced wistar rats divided into 5 groups with 6 animals in each group. Group I - Normal control received 0.9% saline, Group II - Diabetic rats treated with 2 mg/kg b. wt. of standard glibenclamide, Group III - Diabetic rats treated with test sample/article at 10 mg/kg b. wt., Group IV - Diabetic rats treated with test sample at 20 mg/kg b. wt., Group V - Diabetic rats treated with test sample at 40 mg/kg b. wt. After 60 min. of drug administration, the rats of normal and diabetic groups were orally treated with 2 g/kg of glucose and blood samples were collected through femoral vein at 0 min, 30 min, 1 h, and 2 h for estimation of glucose levels

using GOD-POD (glucose oxidase –peroxidase) method.

2.6.3.2. Streptozotocin-induced diabetes – chronic model

Experimental diabetes was induced by single intraperitoneal injection of 60 mg/kg of streptozotocin (STZ), freshly dissolved in cold citrate buffer, pH 7.4 [15, 16]. Control animals received only citrate buffer. Six groups of 6 rats each were used in this experiment. Group 1, normal control (the animals were given normal saline only). Group 2, diabetic group induced by streptozotocin (60 mg/kg b. wt.). Group 3, Positive control (the diabetic rats treated with glibenclamide at 2 mg/kg b. wt. Group 4, treatment group diabetic animals treated with test sample at 10 mg/kg b. wt. Group 5, treatment group diabetic animals treated with test sample at 20 mg/kg b. wt. Group 6, treatment group diabetic animals treated with test sample at 40 mg/kg b. wt. The animals were weighed and dose was given through oral intra-gastric tube every day. The test sample and reference standard drugs were given orally and the experiment was terminated in overnight fasted rats at the end of 15 days. After the experimental regimen, the animals were sacrificed by cervical dislocation after giving mild anesthesia using chloroform. Blood was collected using EDTA as the anticoagulant and serum was separated by centrifugation at 2500 rpm.

3. RESULTS

3.1. Isolation and spectral analysis

Bioactivity-guided isolation using PGE2 and a COX2 marker was followed in the present study. Relative mRNA inhibitory levels of PGE2 and COX2 by different fractions of hydro-alcoholic extracts of root and stem parts of *Boerhavia erecta* L. were considered for relative comparison of their efficacies. Chloroform fractions exhibited highest inhibition of these two pro-inflammatory gene expressions when compared to that of LPS-induced gene expressions. From the thin layer chromatography (TLC) study, the R_f value was found to be 0.75. In the IR spectra the absorption bands at 2951, 2849 and 1473 shows the presence of C-H bonds in the molecule. ^{1}H-NMR showed signals at δ 0.89 for a methyl group, δ 1.27 for a long chain of methylene protons and at δ 1.59 for methyl groups attached to unsaturated systems. It also exhibited signals at δ 3.64 showing the presence of a proton under oxygen function. In the ^{13}C-NMR signals were observed at δ 58.42 and 60.14 for carbon atoms attached to oxygen. The signals at δ 79.96 and 81.58 are due to carbon atoms attached to the functional oxygen (to hydroxyl groups), The signals at δ 17.45 and 18.95 are due to two terminal methyl carbon atoms, at 28.19, 30.69 and 31.03 are due to long chain methylene carbon. The signals at δ 155.96, 157.29 and 176.19 may be due to some impurities or contaminants. ESI-MS (Positive mode) spectra showed a pseudo molecular ions at m/z 467 for $[M+H+Na]^{++}$ ion. The compound may have a structure with IUPAC name as 1-(4-hydroxytridecyloxy) pentadecan-4-ol. The yield of the compound was 5.7% from chloroform fraction of the hydro-alcoholic extract.

3.2. Anti-inflammatory activity (Paw edema & Cotton pellet)

Results of the present study suggest that the test sample predominantly inhibits the release of prostaglandin-like substances or phospholipase as such inhibitors are able to decrease inflammatory response to carrageenan in the rats [17]. The results of anti-inflammatory effect of different doses of test sample on carrageenan-induced paw edema are shown in Table 1.

Test sample against the cotton pellet granuloma technique (Table 2) established the anti-inflammatory activity of the root extract at different doses. The extract showed significant ($p < 0.05$) anti-inflammatory effect in a dose dependent manner. The repairing phase of inflammation is initiated as a proliferation of fibroblasts and a multiplication of small blood vessels. Proliferating cells penetrate the exudates producing a highly vascularized reddened mass known as granulation tissue [18]. Significant reduction of the cotton pellet-induced granuloma in rats suggests that the test sample may involve in the proliferative phase of inflammatory process.

3.3. Anti-diabetic activity (OGTT & STZ induced diabetes)

Glucose-lowering effects were found after oral administration of test samples in Wistar rats.

Table 1. Effect of chloroform fraction of *B. erecta* extract on carrageenan-induced paw edema in Wistar rats (acute model).

Treatment N = 6	Dose (mg/kg)	Paw volume mL (Mean ± SEM)			
		0 hrs	1 hrs	2 hrs	3 hrs
Control	-	0.70 ± 0.04	1.14 ± 0.04	1.19 ± 0.02	1.25 ± 0.03
Diclofenac – Positive control	5	0.71 ± 0.04	0.94 ± 0.03*	0.85 ± 0.05***	0.78 ± 0.04***
Test - Dose 1	10	0.69 ± 0.02	0.96 ± 0.04*	0.92 ± 0.04**	0.90 ± 0.04**
Test - Dose 2	20	0.68 ± 0.01	0.93 ± 0.03*	0.90 ± 0.03**	0.86 ± 0.02***
Test - Dose 3	40	0.70 ± 0.03	0.89 ± 0.02**	0.84 ± 0.03***	0.80 ± 0.02***

*p < 0.05; **p < 0.01; ***p < 0.001 when compared to control group.

Table 2. Effect of chloroform fraction of *B. erecta* extract on cotton pellet-induced granuloma in Wistar rats (chronic model).

Treatment N = 6	Dose (mg/kg)	Mean weight of granuloma (mg) on Day 14 (mean ± SD)
Control	-	101.17 ± 1.35
Diclofenac – Positive control	5	62.75 ± 1.48**
Test - Dose 1	10	92.21 ± 1.74*
Test - Dose 2	20	80.13 ± 1.39*
Test - Dose 3	40	71.52 ± 1.11**

*p < 0.05; ** p < 0.01 when compared to control group.

Administration of test sample significantly lowered ($p < 0.05$) the blood glucose level as compared to normal control rats. This may be due to the presence of hypoglycemic phenols, flavonoids, terpenes or saponins or fatty acids that required further investigation. In STZ-induced diabetic rats, the blood glucose level was significantly ($p < 0.05$) higher in diabetic rats as compared to normal rats. Administration of test compound lowered the blood glucose level as compared to diabetic and normal rats. The highest dose of the test sample (40 mg/kg b. wt.) reduced the elevated blood sugar levels similar to that of the standard glibenclamide with no statistical significance between the two groups.

3.4. *In vitro* studies

IL-17 proteins were negligibly expressed from LPS-stimulated RAW cells that were cultured with BMDM. However, when complete medium (RAW cells, RPMI1640, glutamine, penicillin, streptomycin, FBS) was stimulated with LPS and cultured with 3 different interleukins IL-23, IL-1β and IL-6, it resulted in the triggering of IL-17 protein expression. Different doses of (10 mg, 20 mg and 40 mg/kg b. wt.) test sample admixtured at a concentration of 1 μg/mL, and when co-cultured with a similar set up, demonstrated inhibition of IL-17 cytokine expression. Interestingly, the results obtained show statistically significant difference ($p < 0.0001$) at all three doses of test sample. The obtained results are the mean values of two independent experiments.

4. DISCUSSION

The identification of two distinct Th17 populations, one with pathogenic properties (Teff 17) and another with regulatory and protective properties

(Treg 17), may explain the conventional nature of Th17 cells in autoimmunity, particularly Type 1 Diabetes (T1D). Before the discovery of the Th17 subset as a distinct $CD4^+$ effector population, it was considered that Th1, Th2 and B cells were the main mediators of pathology in autoimmunity. IL-17 neutralization prevented the development of diabetes when given post initiation of insulitis but not earlier. This suggests IL-17's interference with the effector phase of the disease [19] was perceived to have a role in the autoimmune disease caused by T cell-mediated destruction of the insulin producing pancreatic β cells. More recently, Th17 immunity has been demonstrated in the development of autoimmune diabetes in animal models [20]. IL-23 appears to be the key player in the terminal differentiation of Th17 cells and generation of pathogenic Th17 cells. Treg cells can be converted to Teff cells by culturing with IL-23 *in vitro* [7, 21]. Th17 cells are characterized by their ability to secrete high levels of IL-17 promoting an inflammatory profile. The differentiation of Th17 cells is dependent upon IL-6 and transforming-growth factor-β (TGF-β) stimulation, and the presence of this subpopulation of CD4+ cells has been correlated with the onset and progression of autoimmune diseases, such as T1D [22]. IL-23 is an inflammatory cytokine involved in the expansion and commitment of Th17 cell populations and one of its main sources is classically activated macrophages (CaMφs). In diabetic mice induced by streptozotocin (STZ), it has been shown that the administration of IL-23 increases IL-17, TNF-α, and IFN-γ secretion, which is associated with the onset of extremely severe T1D, implicating CaMφs in the recruitment, differentiation, and expansion of pathogenic Th17 lymphocytes contributing to β cell death and T1D induction [23]. Therefore, CaMφs and Th17 cells, together with CD8+ cytotoxic T cells, are considered to be the main cell populations favoring the development of T1D.

In the present study, chloroform fraction which was initially found to inhibit PGE2 (Figure 1A) and COX2 (Figure 1B), the pro-inflammatory markers, was tested to check if it would have effects on the IL-17 cytokines. The compound obtained from chloroform fraction was subjected to spectral analysis (Figures 2a-2e). Active fraction of chloroform was further explored for preliminary anti-inflammatory (Tables 1 and 2) and anti-diabetic activities (Tables 3 and 4). It has been well established that inhibition of pro-inflammatory markers like PGE2 and COX2 have a significant influence on IL-23 [24].Therefore, IL-23 was cultured with IL-6, IL-1β and we found that our results are comparable to that of reported literature on expression of IL-17 under the influence of the said cytokines. So, the effects of different doses/strengths of the isolated compound revealed interesting results. The test sample at highest dose tested did inhibit the expression of IL-17 from LPS-stimulated BMDM cells (Figure 3) when cultured with IL-23, IL-6 and IL-1β cytokines.

GMCSF is important for the pathogenicity of Th17 cells and TGF-β suppresses its production. Th17 cells that lack the ability to produce GMCSF do not transfer autoimmune disorder. Initially gene expression studies on inflammatory markers like PGE2 and COX2 were carried with BE extract. Subsequently, effects of different doses (10 mg, 20 mg and 40 mg/kg b. wt.) of chloroform fraction on the expression of IL-17A were explored by co-culturing with IL-23, IL-1β and IL-6. All three doses of active fraction under test inhibited the expression of IL-17A. The spectral analysis suggests a fatty acid 1-(4-hydroxytridecyloxy) pentadecan-4-ol as the active constituent. It can therefore be regarded as a potential biomarker to develop promising therapeutic agents against diabetes and inflammations with higher affinity and better selectivity.

The *in vitro* and *in vivo* results of the present study suggest that IL-17 inhibition could play a potential role in the immuno-modulatory activity by the compound isolated, i.e. 1-(4-hydroxytridecyloxy) pentadecan-4-ol. The dosage for efficacy studies in animals were based on the dose derived from the acute oral toxicity testing. This being an isolated compound from a herbal extract, the lowest possible dose of 10 mg/kg b.wt. and highest dose of 40 mg/kg b. wt. were considered for the acute toxicity test. PGE2 induces resident immature DCs to generate IL-23 which in turn induces IL-17, a potent pro-inflammatory cytokine secreted by activated

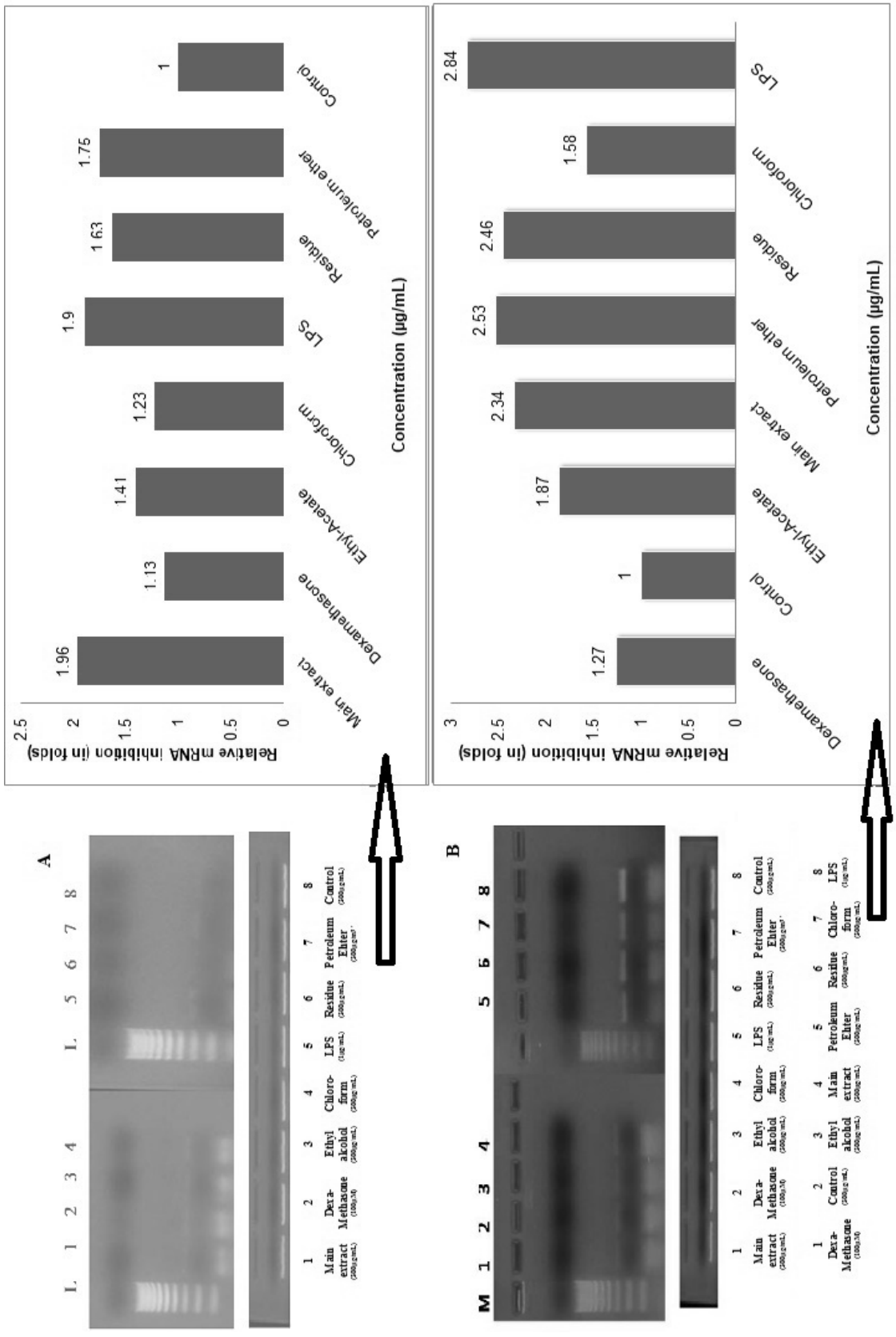

Figure 1. Effect of hydro-alcoholic extracts of *Boerhavia erecta* L on relative inhibition of PGE2 gene expression (A) and COX-2 gene expression (B). The relative level of gene expression was normalized to GAPDH and expressed as arbitrary values.

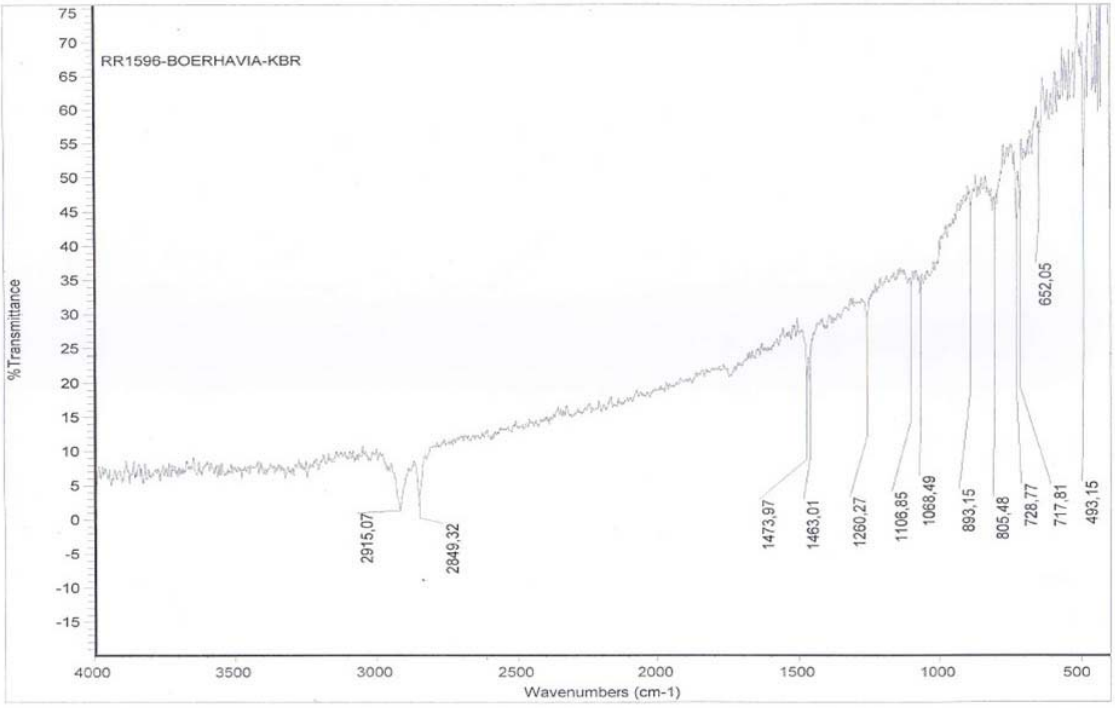

Figure 2a. FTIR analysis of chloroform fraction of hydro-alcoholic extract of root and stem parts of *Boerhavia erecta* L.

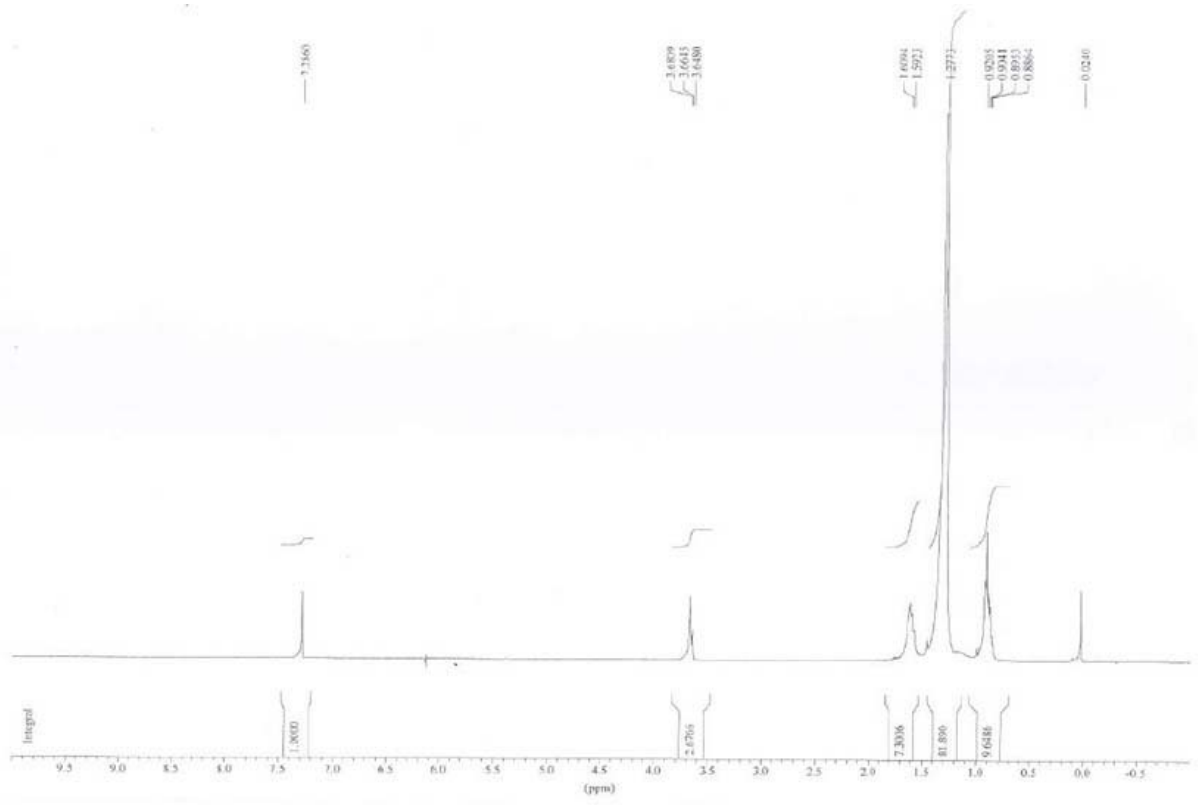

Figure 2b. ^{1}H-NMR of chloroform fraction of hydro-alcoholic extract of root and stem parts of *Boerhavia erecta* L.

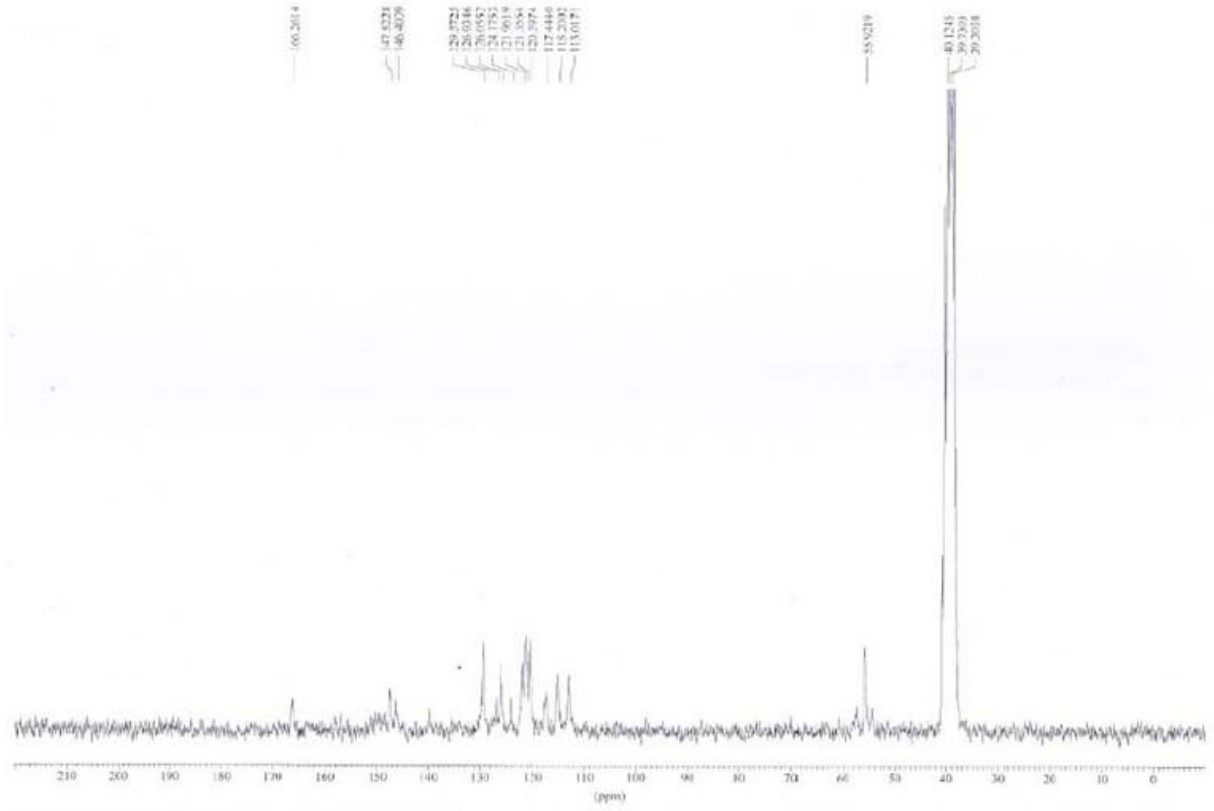

Figure 2c. ^{13}C-NMR of chloroform fraction of hydro-alcoholic extract of root and stem parts of *Boerhavia erecta* L.

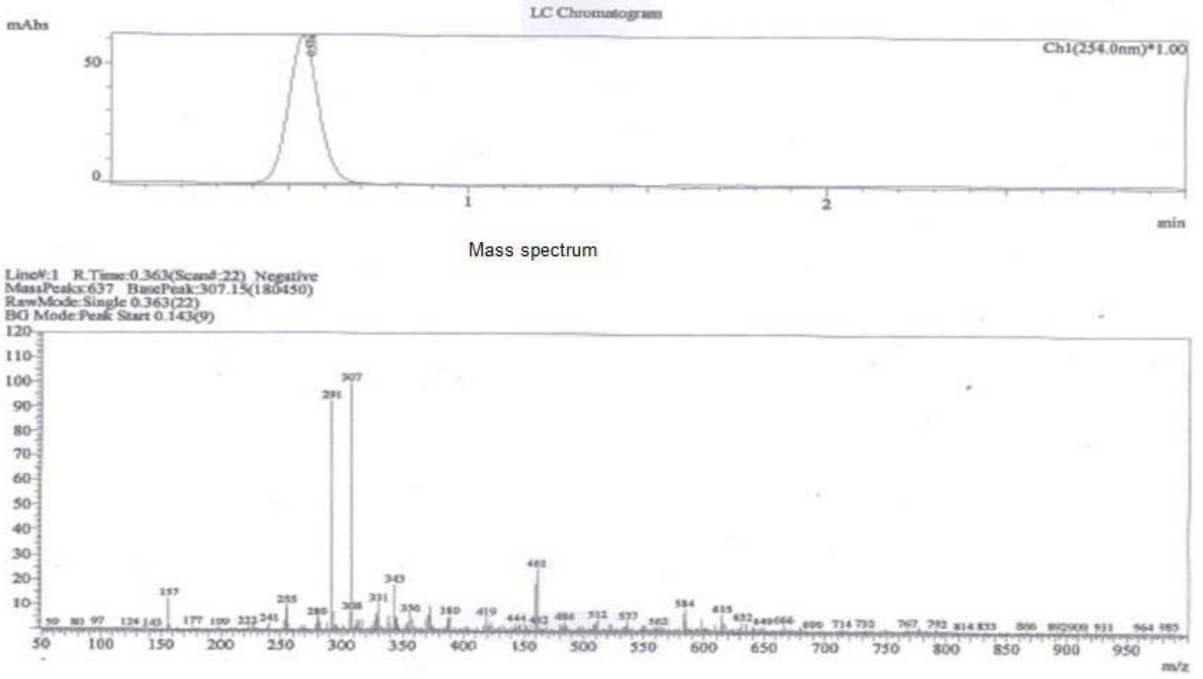

Figure 2d. Mass spectrum of the chloroform fraction of hydro-alcoholic extract of root and stem parts of *Boerhavia erecta* L.

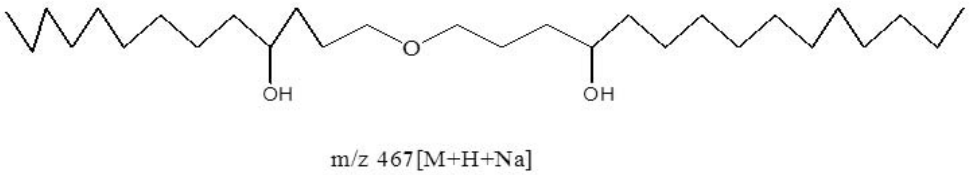

Figure 2e. Structure of the isolated compound.

Table 3. Effect of chloroform fraction of *B. erecta* extract on Oral Glucose Tolerance Test in Wistar rats (acute model).

Treatment N = 6	Dose (mg/kg)	Blood glucose values in mg/dL (mean ± SEM)				
		0 min	30 min	60 min	90 min	120 min
Control	-	91.85 ± 0.04	134.72 ± 0.04	152.31 ± 0.02	121.42 ± 0.03	82.53 ± 0.03
Glibenclamide – Positive control	2	73.72 ± 0.04	109.48 ± 0.03**	128.37 ± 0.05*	102.98 ± 0.04*	72.64 ± 0.04*
Test - Dose 1	10	84.62 ± 0.02	118.28 ± 0.04	137.56 ± 0.04*	118.72 ± 0.04*	78.41 ± 0.04*
Test - Dose 2	20	76.29 ± 0.01	113.75 ± 0.03	132.32 ± 0.03	117.45 ± 0.02**	75.93 ± 0.02**
Test - Dose 3	40	74.11 ± 0.03	103.14± 0.02**	129.22 ± 0.03**	110.17 ± 0.02**	70.69 ± 0.02**

*p < 0.05; ** p < 0.01 when compared to control group.

Table 4. Effect of chloroform fraction of *B. erecta* extract on blood glucose levels – against STZ-induced diabetic rats (chronic model).

Group	Dose (mg/kg)	Blood glucose levels in mg/dL (Mean ± SD)				
		Initial	Day 1	Day 5	Day 10	Day 15
Normal Control	-	70.78 ± 7.03	65.05 ± 9.33	66.70 ± 9.85	67.00 ± 7.41	65.48 ± 5.88
Negative Control	-	249.76 ± 8.85	262.28 ± 14.75	285.85 ± 4.78	309.20 ± 8.09	313.28 ± 4.73
Glibenclamide – Positive control	2	249.62 ± 8.53	247.51 ± 8.11*	190.07 ± 11.04**	167.84 ± 9.37**	123.93 ± 5.89**
Test - Dose 1	10	250.85 ± 8.40	256.57 ± 5.57*	249.23 ± 8.42**	204.38 ± 5.84**	192.03 ± 5.80**
Test - Dose 2	20	250.85 ± 8.40	252.49 ± 5.57*	237.85 ± 8.40**	199.85 ± 8.40**	182.85 ± 7.66**
Test - Dose 3	40	251.84 ± 4.90	249.85 ± 8.40*	213.45 ± 6.30**	192.77 ± 4.89**	154.85 ± 9.42**

*p < 0.05; ** p < 0.01 when compared to control group.

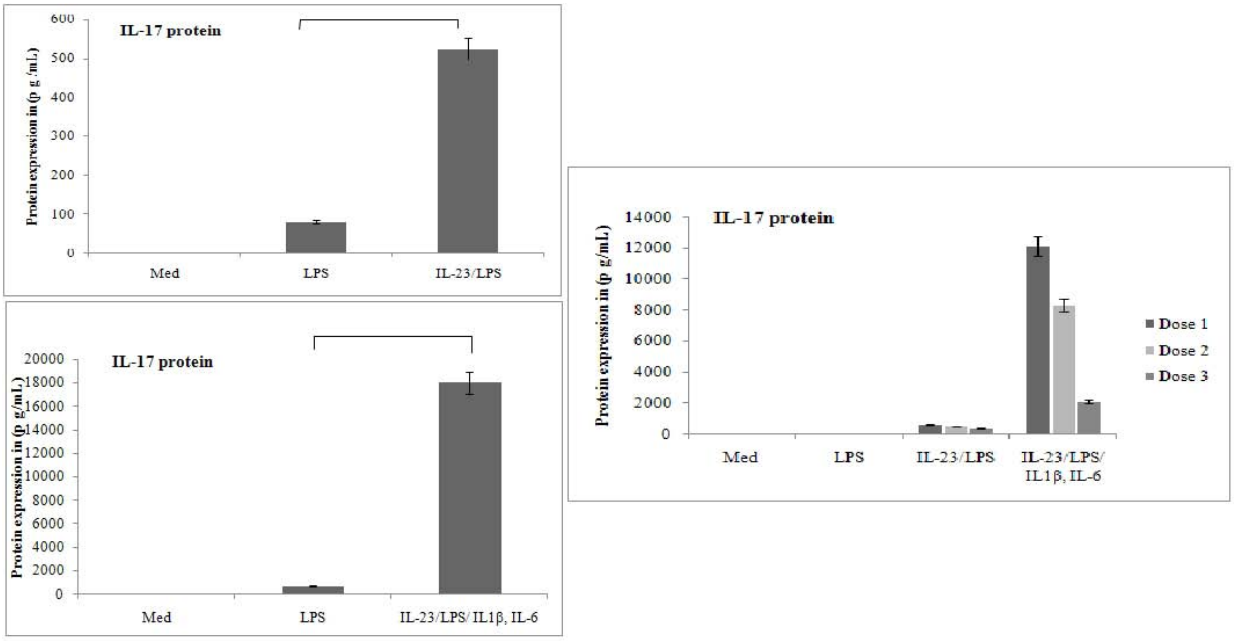

Figure 3. Influence of different doses of chloroform fraction of *Boerhavia erecta* L. on IL-17 expression when co-cultured with other cytokines in LPS stimulated BMDM cells.

T cells and synovial cells and found at high levels in the synovial fluid of RA patients [25]. Amir *et al.* reported a novel mechanism through which the COX-1/2 inhibitors play a beneficial role of in rheumatoid arthritis, i.e., the inhibition of local IL-17/ IL-23 production mediated through the reduction in endogenous PGE2 release [26]. The identification of other factors/cytokines acting in conjunction with PGE2 in the induction of IL-23 could lead to the development of new combined therapies for autoimmune diseases such as rheumatoid arthritis and experimental autoimmune encephalomyelitis, where IL-23 has been shown to play an essential role [6, 27]. However, given the fact that pathological or harmful effects of IL-17 are more under the influence of GM-CSF and such an effect could not be tested by us, we consider this as the limitation for the present study.

5. CONCLUSION

The chloroform fraction of BE extracts containing 1-(4-hydroxytridecyloxy) pentadecan-4-ol elicited immuno-modulatory activity probably through IL-17/IL-23 axis. Based on the study, rheumatoid arthritis (RA) and type 1 diabetes (T1D), that were considered as auto-immune prototype disorders, have been reduced remarkably by this extract. However, this probable mode of action has to be confirmed in an animal model targeting at least two autoimmune diseases together on a single animal model.

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CONFLICT OF INTEREST STATEMENT

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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ABBREVIATIONS

COX, Cyclooxygenase; BMDM, Mouse bone marrow derived macrophages; DMEM, Dulbecco's modified eagle's medium; DMSO, Dimethyl sulfoxide; EDTA, Ethylene-diamine-tetra-acetic acid; FBS, Fetal bovine serum; IL, Interleukin; PBS, phosphate buffered saline; PGE, prostaglandin; RAW 264.7, mouse leukaemic monocyte macrophage cell line; RT-PCR, Reverse Transcription Polymerase Chain Reaction; TPVG, Trypsin Phosphate Versene Glucose; IFNγ, interferon-γ; Th, T helper; CPCSEA, Committee for the purpose of control and supervision of experiments on animals.

REFERENCES

1. Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A. and Coffman, R. L. 1986, J. Immunol., 136, 2348.
2. Aarvak, M., Chabaud, P., Miossec, P. and Natvig, J. 1999, J. Immunol., 16, 1246.
3. Gaffen, L. S. 2009, Curr. Rheumatol. Rep., 11, 365.
4. Stamp, L. K., Easson, A., Pettersson, L., Highton, J. and Hessian, P. A. 2009, J. Rheumatol., 36, 2403.
5. Parham, C., Chirica, M., Timans, J., Vaisberg, E., Travis, M., Cheung, J., Pflanz, S., Zhang, R., Singh, K. P., Vega, F., To, W., Wagner, J., O'Farrell, A. M., McChanahan, T., Zurawski, S., Hannum, C., Gorman, D., Rennick, C., Kastelein, R. A., Malefyt, R. and Moore, K. M. 2002, J. Immunol., 168, 5699.
6. Cua, D. J., Sherlock, J., Chen, Yi., Craig, A., Murphy, B. J., Brian, S., Lucian, L., To, W., Kwan, S., Churkova, T., Zurawski, S., Wiekoswski, M., Sergio, A., Daniel, G., Robert, A. K. and Sedwick, J. D. 2003, Nature, 421, 744.
7. Langrish, C. L., Chen, Yi., Blumenschein, W. M., Mattson, J., Basham, B., Sedgwick, J. D., McClannahan, T., Kastelein, R. A. and Cua, D. J. 2005, J. Exp. Med., 201, 233.
8. Weaver, C. T., Hatton, R. D., Mangan, R. R. and Harrington, L. E. 2007, Ann. Rev. Immnol., 25, 821.
9. McGeachy, M. J., Chen, Yi., Tato, C. M., Laurence, A., Shaikh, B. J., Blumenschein, W., McClanahan, W., Oshea, J. J. and Cua, D. J. 2009, Nat. Immunol., 10, 314.
10. Zimmerman, M. 1983, Pain Headache, 16, 109.
11. Ecobichon, D. J. 1999, The Basis of Toxicology Testing, CRC Press, New York.
12. Prema, V. 2003, Expert Consultant CPCSEA, OECD Guideline No. 420.
13. Adeyemi, O. O., Okpo, S. O. and Ogunti, O. O. 2002, Fitoterapia, 73, 375.
14. Cuman, R. K. N., Bersani-Amadio, C. A. and Fortes, Z. B. 2002, Inflam. Res., 50, 460.
15. Erejuwa, O. O., Sulaiman, S. A., Wahab, M. S., Salam, S. K., Salleh, M. S. and Gurtu, S. 2010, Int. J. Mol. Sci., 11, 2056.
16. Pandit, R., Phadke, A. and Jagtap. 2010, J. Ethnopharmacol., 128, 462.
17. Ferrandiz, M. L. and Alcaraz, M. L. 1991, Agents Action, 32, 283.
18. Miyake, A., Yamamoto, H., Kubota, E., Hamaguchi, K., Kouda, A., Honda, K. and Kawashima, H. 1993, Br. J. Pharmacol., 110, 447.
19. Honkanen, J., Janne, K. and Nieminen, Ru. 2010, J. Immunol., 185, 1959.
20. Annunziato, F., Cosmi, L. and Santarlasci, V. 2007, J. Exp. Med., 204, 1849.
21. McGeachy, M. J., Bak-Jensen, K. S. and Chen, Y. 2007, J. Nat. Immunol., 8, 1390.
22. Zhu, S. and Qian, Y. 2012, Clinical Science, 122, 487.
23. Mensah, E. P. K., Shahin, A. and Al-Shamisi, M. 2006, Eur. Journal. Immunol., 36, 216.
24. Katia, B., Kirstian, S. and Bak, J. 2010, J. Exp. Med., 206, 535.
25. Zhang, G., Gran, B. and Yu, S. 2016, J. Immunol., 170, 2153.
26. Amir, T., Iman, T. and Jing, H. 2004, The FASEB, 122, 2122.
27. Zhang, G., Yu, S. and Gran, B. 2003, J. Immunol., 171, 4485.