Original Article

Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.apjtb.org

doi: 10.4103/2221-1691.319568

Impact Factor: 1.90

Valencene-rich fraction from *Vetiveria zizanioides* exerts immunostimulatory effects *in vitro* and in mice

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ABSTRACT

Objective: To decipher the responsible compound present in the aqueous root extract of *Vetiveria zizanioides* which has tremendous immunomodulatory activity.

Methods: Different fractions of the water extract were collected and analyzed for immunomodulatory activity by analyzing *in vitro* phagocytic activity and nitric oxide production. One fraction VF3 was selected and further analyzed for possible compounds by high performance liquid chromatography and gas chromatography coupled with a mass spectrometer. The *in vitro* immunomodulatory parameters such as phagocytic index, nitrite content, and tumor necrosis factor-α production in murine macrophages were analyzed. *In vivo* studies, sheep red blood cell induced haemagglutination titer, the number of antibody-producing cells, and sheep red blood cell induced delayed-type hypersensitivity were analyzed. Cytotoxic studies in L929 normal fibroblasts were also performed.

Results: One of the fractions, VF3, was selected and confirmed the presence of an active compound valencene. The *in vitro* immunomodulatory parameters were significantly (P<0.05) increased by valencene treatment. *In vivo* studies in Swiss albino mice showed that valencene could significantly (P<0.05) increase haemagglutination titer, the number of antibody-producing cells, and delayed-type hypersensitivity. Cytotoxic studies also showed that valencene did not cause any morphological changes and DNA damage in normal fibroblasts.

Conclusions: Valencene possesses immunomodulatory activities and can be commercially exploited for its immunostimulatory potentials.

KEYWORDS: GC-MS; Immunomodulation; Valencene; *Vetiveria zizanioides*; Thirst quencher

1. Introduction

People around the world are used to depend on thirst quenchers in their daily life. It has become a normal process to include herbs in their drinks. There are various types of thirst quenchers that include synthetic and natural origin. Synthetic thirst quenchers comprise soft drinks. Studies have shown that the consumption of soft drinks has been increasing substantially over the last few decades[1]. The daily use of soft drinks may result in weight gain, high-sugar intake, dehydration, calcium depletion, and other adverse health effects[2]. In their place, traditional thirst quenchers that possess relatively fewer side effects can be effectively used as an alternative for soft drinks.

In most countries, drinking water is boiled with sections of plant roots, leaves, or barks commonly called thirst quenchers. *Acacia catechu*, *Vetiveria zizanioides* (*V. zizanioides*), *Caesalpinia sappan*, *etc* are some of the plants included in this category. People generally believe that water decoctions can clean the blood and can positively influence the general health status of an individual. It is also supposed that regular use of this type of plant decoctions can promote the defense system of the body. These thirst quenchers are also reported to have many pharmacological effects[3,4] and immunomodulatory activity is its major attraction.

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How to cite this article: Sunitha VS, Midhun SJ, Sunil MA, Radhakrishnan EK, Mathew J. Valencene-rich fraction from *Vetiveria zizanioides* exerts immunostimulatory effects *in vitro* and in mice. Asian Pac J Trop Biomed 2021; 11(8): 335-343.

Article history: Received 7 July 2020; Revision 4 August 2020; Accepted 12 January 2021; Available online 9 July 2021

Modulation or rejuvenation of the immune system signifies the change in the immune response that involves the induction or repression of any part or stage of the immune response. A vast variety of natural, synthetic, and/or recombinant molecules are available as immunomodulators. Levamisole, isoprinosine, pentoxifylline, and thalidomide are some of the examples of significant synthetic immunomodulators. Albeit these synthetic immune-modulatory drugs have numerous benefits, their adverse side effect profile and broad-spectrum effects throughout the immune system cause a major constraint to the general conscious use of these drugs. This necessitates the search for effective and safer agents as immunomodulators[5].

The scientific community now focuses on developing more effective and safe naturally occurring immunomodulatory agents. In this scenario, phytochemicals have got much attention because of their natural origin with versatile bioactive potentials. *V. zizanioides*, a perennial grass of the family Poaceae is native to tropical Asia and is now seen in the tropic of both hemispheres. Its thick fragrant roots contain oil, which is used for ulcers, vomiting, nausea, dyspepsia, cough, fever, and low back pain[6]. Studies are available on the chemical composition, antioxidant, and antimicrobial activities of *V. zizanioides* (L.) essential oil derived from its root[7]. But the immunomodulatory activity of this plant along with the identification of responsible compounds from the root extract is scarce. The immunomodulatory activity of the crude extract of *V. zizanioides* was previously reported from our lab[8]. The present study focused on the identification of bioactive compounds present in the aqueous root extract, its *in vitro* and *in vivo* immunomodulatory potentials, and its effect on the viability of normal cells.

2. Materials and methods

2.1. Collection and processing of plant material

Roots of *V. zizanioides* were collected from Alappuzha District, Kerala. The specimens were validated by the plant taxonomist in Post Graduate and Research Department of Botany, St. Berchmans College, Changanacherry, Kerala, and a voucher specimen (No. RHK 6408) was maintained in Regional Herbarium of Kerala (RHK). The samples were washed with distilled water, shade dried, powdered, and stored in airtight containers.

2.2. Reagents

RPMI 1640 medium (Himedia, India), dimethylsulfoxide in phosphate buffer saline (PBS) (Himedia, India), Giemsa's dye (Sigma, India), Griess reagent (Sigma, India), ELISA kit (Biolegend, USA), sheep red blood cell (SRBC) in PBS (Himedia, India), Dulbecco's Modified Eagles Medium (DMEM) (Himedia, India), and MTT (Himedia, India, 5 mg/mL) were used in this study.

2.3. Extraction and fractionation of V. zizanioides root

The fine dried powdered root was subjected to Soxhlet extraction by water. The water after extraction was removed and stored at 4 ℃ until use. The crude water extract of *V. zizanioides* was fractionated in a glass column (450 mm × 30 mm) containing silica gel (60-120 mesh, Merck). Two grams of extract was dissolved in 3 mL of methanol and adsorbed on silica gel and layered on the top of hexane equilibrated silica gel column and subjected to chromatography. The flow rate was set as 2 mL/min and the column was eluted with chloroform: methanol in various proportions (10:0, 8:2, 6:4, 4:6, 2:8, 0:10). Eluted fractions (VF1, VF2, VF3, VF4, VF5, and VF6) were collected and concentrated to dryness and lyophilized. The fractions were further screened for their *in vitro* immunomodulatory activity.

2.4. Determination of in vitro immunomodulatory activity using different fractions of V. zizanioides

2.4.1. In vitro phagocytosis assay

Phagocytosis assay was done based on the method of Frank and Hay[9]. The macrophages were isolated from Swiss albino mice weighing 25-30 g. The macrophages were isolated from the peritoneal fluid based on the method described by Zhang *et al*[10]. Macrophages (2×10^6 cells/mL) seeded in the 24-well plate consisting of a sterilized coverslip were incubated in a CO_2 (5%) humidified incubator for 2 h. The detached cells were washed out in RPMI 1640 medium. The remaining cells present in the wells were grown in RPMI 1640 medium. The plates were then incubated for 24 h. Lipopolysaccharide (LPS) at 5 μg/mL was used as a mitogen and 0.1% dimethylsulfoxide in PBS was used as a control. After incubation and washing, one mL RPMI 1640 medium with 100 μg/mL yeast cells (10^8 particles/mL) was added to each well. The plates were then incubated for 1 h at 37 ℃ in a CO_2 incubator. After incubation, the wells were washed twice with culture medium and fixed with methanol then stained with Giemsa's dye. Based on the following equation the phagocytic index was calculated:

Phagocyte index = number of yeast cells phagocytosed by macrophages/number of macrophages.

2.4.2. Nitric oxide (NO) assay

The NO content was measured by Griess method described by Green *et al*[11]. The peritoneal macrophages were seeded in 24 well plates at a concentration of 2×10^6 cells/well and incubated for 2 h in a CO_2 (5%) humidified incubator, then non-adherent cells were removed by washing in RPMI-1640 medium containing 10%

fetal calf serum and incubated for 24 h with 50 μg/mL of different fractions (dissolved in 0.1% dimethylsulfoxide in PBS) and LPS (1 μg/mL). A total of 100 μL Griess reagent was added to 100 μL culture supernatant and incubated at room temperature for 10 min in a 96-well microplate. The absorbance was recorded at 540 nm in a microplate reader (Thermo Fisher Scientific, USA). The diluted sodium nitrite was used as a standard.

2.5. Identification of bioactive compound in the active fraction

The VF3 fraction which showed potent immunomodulatory activity was further purified by high performance liquid chromatography (HPLC) (Shimadzu Prominence LC 20AP System with pump LC 20AP, UV-VIS detector SPD 20A/20AV, and LC solution software). Column used was Enable RP C18 G250 (250 mm × 4.6 mm × 5 μm, 120A0). HPLC analysis of the fraction was done by a binary gradient of water containing 0.1% trifluoroacetic acid and methanol (50:50). The active compound present in the fraction was further characterized by gas chromatography coupled with a mass spectrometer (GC-MS) analysis along with the standard compound valencene (Sigma Aldrich, USA). The GC-MS analysis was done by Shimadzu GC-MS system (Model NoQP2010s), containing an autosampler and GC-MS with the following conditions: Column Rxi-5Sil MS column (30 mm × 0.25 mm I.D, 0.25 μm thickness) functioning in electron impact mode at 70 eV; helium (99.999%) was used as carrier gas at a constant flow of 1 mL/min and an injection volume of 1 μL, injector temperature 260 ℃; ion-source temperature at 280 ℃. The oven temperature was programmed from 80 ℃ (isothermal for 2 min), with an increase of 10 ℃/min, to 260 ℃, then 5 ℃/min to 280 ℃, ending with a 6 min isothermal at 280 ℃. Mass spectra were taken at 70 eV; a scan speed of 1 000, a gap of 0.5 s, and *m/z* of wreckages starting from 50 to 500 Da. Total GC running time was 35 min. The compounds were identified by comparing the mass spectrum of the unknown with the known compounds having similar spectral data in the library of National Institute Standards and Technology (NIST)-Ⅱ with WILEY 8 instrument.

2.6. Determination of in vitro immunomodulatory effect of active compound (valencene) present in the VF3 fraction

In vitro immunomodulatory activity was analyzed by *in vitro* phagocytosis assay, NO assay, and measurement of proinflammatory cytokine tumor necrosis factor (TNF)-α. The protocols for *in vitro* phagocytosis assay and NO assay were explained in sections 2.3.1 and 2.3.2 correspondingly. The proinflammatory cytokine TNF-α was measured in the cell culture supernatants of murine peritoneal macrophages treated with valencene by ELISA kit (Biolegend, USA) according to the manufacturer's recommendation.

2.7. In vivo immunomodulatory activity of valencene

2.7.1. Experimental animals and selection of doses

Swiss albino male mice (4 month old) weighing 20-30 g were considered in the study. The animals were maintained in the animal maintenance facility of School of Biosciences, Mahatma Gandhi University, Kottayam. The temperature was maintained at (25 ± 2) ℃ and fed *ad libitum* with a standard pellet diet and water. The dosages for the *in vivo* studies were calculated by Irwin test[12]. The test samples were analyzed in PBS at pH 7 and 1/10th – 1/20th of the dose. This dose range was considered as the safe dosage since no physiological alternations were observed.

2.7.2. Treatment protocol

For testing the samples, 24 Swiss albino mice were divided into 4 groups containing six test animals in each experimental group. Group Ⅰ: normal control (PBS); Group Ⅱ: levamisole [25 mg/kg body weight (b.w.), positive control]; Group Ⅲ: valencene 50 mg/kg b.w. (0.2 mL/day for 30 d); Group Ⅳ: valencene 100 mg/kg b.w. orally (0.2 mL/day for 30 d).

2.7.3. Delayed type of hypersensitivity (DTH) response

The DTH response was induced by the protocol described by Raisuddin *et al*.[13]. Animals were injected with 1×10^9 SRBC, subcutaneously on the termination of treatment. On the 5th day after the initial injection, the animals were again challenged with 1×10^8 cells in the left hind footpad. The same volume of physiological saline was injected in the right footpad and served as trauma control for non-specific swelling. Thickening in the footpad was measured after 24 h using a digimatic micrometer (Mitutoyo South Asia).

2.7.4. Haemagglutination antibody (HA) titer

Haemagglutinin titer assay was done by the method of Bin- Hafeez *et al*[14]. At the end of treatment time, animals were injected with 0.2 mL of 10% SRBC intraperitoneally. After immunization (5th day), blood was collected from orbital plexus and used for serum separation. Serum was incubated for 15 min at 56 ℃ for inactivating complements in it. Two-fold dilution of serum was done in 50 μL of PBS (pH 7.2) in microtitre plates (96-well) and mixed with 1% SRBC (50 μL) suspension in PBS. Proper mixing was given and the plates were incubated at 30 ℃ for 2 h.

2.7.5. Plaque forming cell (PFC) assay

The experiment was done by the method of Davis and Kuttan[15]. Animals were intraperitoneally injected with 2.5×10^8 SRBC on the day of treatment termination. The animals were sacrificed on the 5th day after intraperitoneal injection. The spleen suspensions were made in RPMI-1640 medium at a concentration of 1×10^6 cells/mL. A total of 50 μL spleen suspension and 50 μL of SRBC suspension (7%) were mixed with 0.5 mL of 0.5% molten agarose and kept at 45 ℃. The mixture was spread on clean slides. The number of PFC

was measure by Jerne's plaque assay[16].

2.8. Toxicity assessment of valencene

2.8.1. Cytotoxicity assessment of valencene in L929 (fibroblasts) cell line

L929 (fibroblasts) cell line was obtained from National Centre for Cell Science, Pune, India and maintained in DMEM (Himedia). Cultured cell lines were kept at 37 ℃ in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany). Two days old monolayers were trypsinized and suspended in DMEM. The survival of the cells treated with valencene was analyzed by the MTT method[17]. Different concentrations of fractions (6.25-100 μg/mL) in DMEM were added and incubated further for 24 h. After incubation the wells were observed for morphological changes of cells if any using an inverted microscope (Leica, DMi1, India), then 20 μL of MTT (Himedia, India, 5 mg/mL) in PBS. The proliferation percentage was calculated by following equation:

% viability = [(OD of sample – OD of control)×100]/ OD of control

2.8.2. Assessment of genotoxicity of valencene

The L929 (fibroblasts) cells were cultured in 96 well plates treated with the compound (100 μg/mL) and incubated for 24 h at 37 ℃ in a 5% CO_2 incubator (NBS Eppendorf, Germany). After the treatment, trypsinized cells were washed with fresh media and used for comet assay[18]. The slides were photographed using an inverted epifluorescent microscope Olympus CKX41, with Opitka Pro5 CCD camera and the length of DNA migration and tail formation were observed.

2.9. Statistical analysis

The values of all the data were expressed as mean ± SEM (n=6). One-way ANOVA followed by Tukey's HSD was used for statistical analysis (SPSS, version 20, IBM, USA). P<0.05 was considered significant.

2.10. Ethical statement

The study was approved (Register number-MGUSBA/IAEC/2014 B21032014-09) by the Institutional Animal Ethics Committee, School of Biosciences, Mahatma Gandhi University, Kottayam, and followed the guidelines of Government of India, principles of laboratory use and care of animals.

3. Results

3.1. Determination of in vitro phagocytosis and NO content in peritoneal macrophages by fractions of V. zizanioides

The effect of fractions of aqueous extract of *V. zizanioides* on peritoneal macrophages is shown in Figure 1A. Fraction 3 (VF3) showed maximum phagocytic index and it was significantly different from the control. The fraction VF3 showed maximum NO scavenging activity (Figure 1B) compared with other fractions and it was significantly (P<0.05) increased compared with the control and the other groups. Based on this result, the VF3 fraction was selected and further analyzed for the responsible immunomodulatory compound present in it.

3.2. Identification of bioactive compounds from V. zizanioides

The HPLC chromatogram of the VF3 showed a major peak with a retention time at 8.466 (Supplementary Figure 1A) and it was further considered for the GC-MS analysis. The GC-MS analysis of VF3 fraction was depicted in Supplementary Figure 2A. The chromatogram showed a major peak at a retention time of 30.458

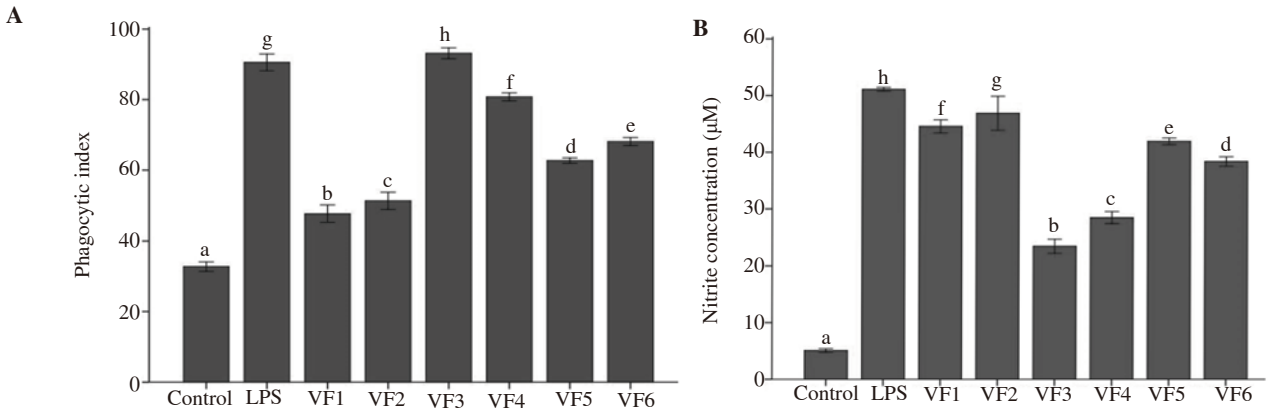

Figure 1. *In vitro* immunomodulatory activity of fractions derived from *Vetiveria zizanioides* L. (A) Effect of fractions on *in vitro* phagocytosis assay; (B) Nitric oxide scavenging activity of fractions. Data stand for the mean ± SEM and values with the same superscript letters are not significantly different (Tukey's HSD; P<0.05). LPS – lipopolysaccharide.

with an *m/z* value of 161.10. The mass spectrum of the peak (Supplementary Figure 2C) showed the following fragments with *m/z* values: 204, 189, 175, 161, 147, 133, 119, 105, 93, 79, 67, 55. By comparing with MS spectra of NIST library, the compound was identified as valencene. In order to confirm the presence of valencene, the GC-MS and HPLC analysis of standard compound valencene was also performed. The retention time of valencene in HPLC analysis was 8.543 (Supplementary Figure 1B) and the GC-MS chromatogram of the standard (Supplementary Figure 2B) showed a peak with a retention time of 30.201 with a similar pattern (Supplementary Figure 2D) as obtained in MS spectra of VF3 fraction (Supplementary Figure 2C).

3.3. Determination of in vitro immunomodulatory activity of the standard compound valencene

Valencene showed a significant (P<0.05) increase in phagocytic index (Figure 2A). Valencene showed maximum phagocytic index at 100 µg/mL concentration. Valencene effectively reduced the generation of NO that leads to an increase in the concentration of nitrite. Valencene showed maximum NO scavenging activity at 100 µg/mL (Figure 2B). The TNF-α production in the macrophages treated with LPS was drastically enhanced compared with the control and valencene treated groups (Figure 2C). In contrast, the administration of valencene significantly (P<0.05) reduced the level of LPS induced TNF-α production in macrophages (Figure 2C).

3.4. Determination of in vivo immunomodulatory activity of valencene

3.4.1. HA titer

A dose-dependent increase in the HA titer was observed in valencene treated groups compared with the control. A maximum increase in antibody titer was observed in animals treated with valencene at a dose of 100 mg/kg b.w. (Figure 3A).

3.4.2. PFC assay

Valencene significantly improved the number of antibody-producing cells in the spleen (Figure 3B). The maximum number of PFC (545 PFCs/10^6 spleen cells) was observed in valencene treated (100 mg/kg b.w.) animals. However, on the 5th day, 361 PFCs/10^6 cells were observed in the control.

3.4.3. DTH

The study on T-cell mediated DTH showed that valencene at 50 and 100 mg/kg b.w. significantly inhibited edema in the foot paw

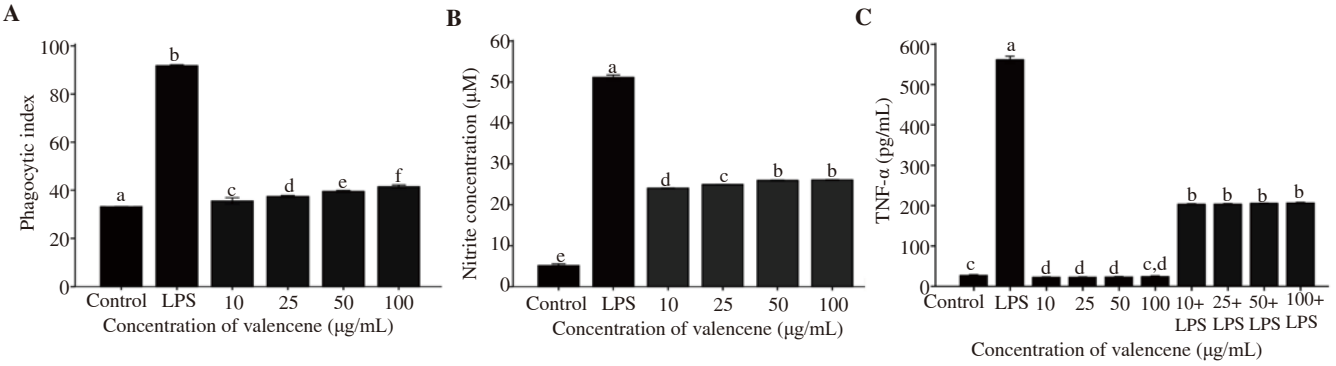

Figure 2. Determination of *in vitro* immunomodulatory activity of valencene in murine peritoneal macrophages. (A) Effect of valencene on *in vitro* phagocytosis; (B) Nitric oxide scavenging activity of valencene; (C) Effect of valencene on tumor necrosis factor (TNF)-α production. Data denote the mean ± SEM and values with the same superscript letters are not significantly different (Tukey's HSD; P<0.05). LPS – lipopolysaccharide.

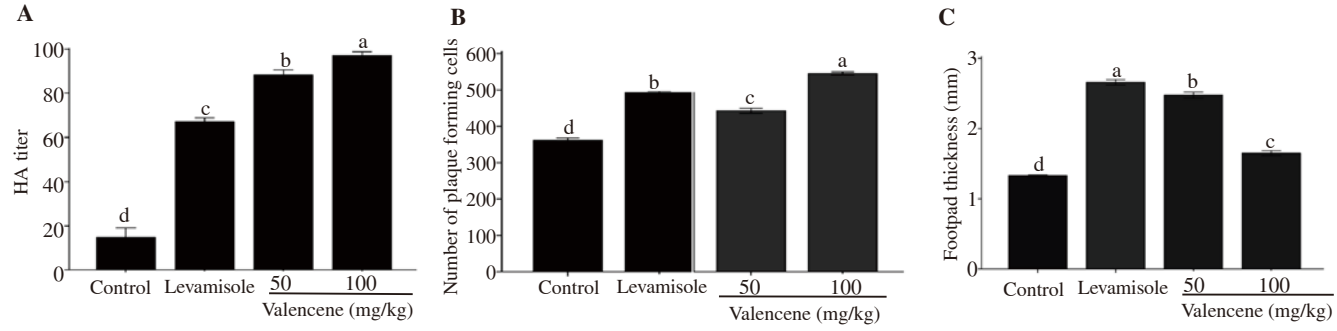

Figure 3. Effect of valencene on haemagglutination antibody (HA) titer (A), antibody-producing cells (B) and and delayed-type hypersensitivity (C). Data denote mean ± SEM (n=6); values with the same superscript letter do not vary significantly (Tukey's HSD; P<0.05).

compared with the control group (Figure 3C).

3.5. Cytotoxicity of valencene on L929 cell line

The administration of valencene did not show any significant reduction in the viability of cells. The percent viability of cells at a concentration of 100 μg/mL was greater than 75%. This indicates the non-cytotoxic effect of valencene on L929 fibroblasts (Figure 4A).

3.6. Genotoxic effect of valencene

The length of the tail is directly proportional to the DNA fragmentation and thus indicates genotoxicity. Valencene at a concentration of 100 μg/mL did not show any significant tail formation compared with the control, which indicated the non-genotoxic effect of the compound (Figure 4B and C).

4. Discussion

Plants are important sources for immunomodulatory compounds and can be functioned as nonspecific immunity modulators[19]. Macrophage activation is an effective way of eradicating pathogens because of their better phagocytic activity, destruction of ingested microbes, augmented cytokine secretions, and remarkable capability to activate T cells[20].

Immune response regulation is quite significant in immune response regulation. They used to be considered as antigen-presenting cells/scavengers because of their ability to pick up and engulf foreign materials and present them to other immune system cells *viz*. T cells and B cells[21]. In the present study, the fractions of *V. zizanoides* showed significant *in vitro* immunomodulatory activity and the fraction VF3 showed a remarkable difference. One of the studies by Piyaviriyakul *et al*.[22] reported that the aqueous root extract of *Acanthus ebracteatus* Vahl. increased the innate immune response in mice macrophages by inducing phagocytosis. In the present study, the VF3 fraction from the aqueous extract of *V. zizanoides* increased the macrophage phagocytic index, which indicated that the fraction could initiate an immune response.

NO is essential for many physiological functions so it is considered as an important inflammatory mediator. It is synthesized by NO synthase and intervenes with varied functions such as vasodilatation, inflammation, and neurotransmission[23]. NO, produced by macrophages, is one of the cytotoxic effector molecules which can be considered as a quantitative indicator of macrophage activation[24]. High reactivity with O_2 and free radical nature provide NO as a possible pro-oxidant molecule, which stimulates oxidative damage and harms cellular targets[25]. The transient and volatile nature of NO makes it unsuitable for the most convenient detection methods. However, nitrite concentration can be taken as an indirect measure of NO production[26]. In this study, the VF3 fraction of the water extract of *V. zizanioides* significantly inhibited NO in mouse peritoneal macrophages. One of the studies reported that *Moringa peregrina* could suppress the production of NO and interleukin-1 beta in macrophages *in vitro*[27]. Another study reported that the essential oil from *V. zizanioides* could reduce NO production in LPS-stimulated RAW 264.7 macrophages. So it can act as an immunomodulant[28]. These studies confirm the present study.

Most of the studies conducted in the field of plant-based immunomodulation are superficial thus detailed studies are necessary to decipher the compounds involved in the immune modulation. The chromatographic technique and mass spectroscopy have been effectively used for plant metabolite profiling[29]. In the present study, HPLC analysis of the fraction VF3 showed a characteristic major peak with a retention time of 8.466, which revealed a bioactive compound present in the fraction. The GC-MS analysis was used for the structural identification of compounds present in the bioactive fraction. GC-MS chromatogram analysis of the HPLC fraction of VF3 was elucidated by the reference mass spectra available in the NIST library. The retention time of the compound, *m/z* value, and

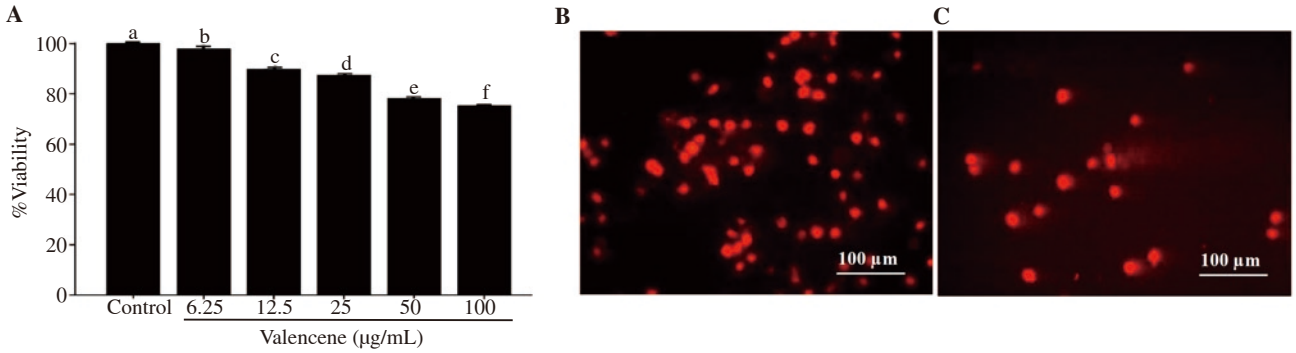

Figure 4. Toxicity assessment of valencene. (A) Cytotoxic test of valencene on L929 (fibroblasts) cells by MTT assay; (B) Genotoxic effect of the control group by comet assay; (C) Genotoxic effect of the valencene treated group (100 μg/mL) by comet assay. Values in the bar graph denote the mean ± SEM and values with alphabet do not vary significantly (Tukey's HSD; $P<0.05$).

molecular formula were compared with the existing database found in the NIST library. The *m/z* value and molecular formula were found to be the same as that of valencene (CAS: 4630-07-3) reported previously. The results obtained from the analysis confirmed that the HPLC fraction of VF3 was rich with the compound valencene. Recently, interesting therapeutic activities of valencene have been reported such as the antiplatelet aggregation effect in rats and anti-proliferative activity towards cancer cell lines[30]. Valencene exhibits antioxidant and antimicrobial activity[31]. Antioxidants reduce oxidative stress in food, cosmetics products, and pharmaceuticals. Antioxidants help to reduce the diseases related to the oxidative-stress human body. Solubility of valencene is 4.581 mg/L at 25℃. The boiling point is 173 to 176 at 760 mm Hg. Several studies are available on the antioxidant and antiproliferative effects of valencene but the studies on immunomodulatory activity *in vitro* or *in vivo* are scarce. So the study has been designed to evaluate the immunomodulatory activity of valencene in experimental rat models. The *in vitro* immunomodulation was analyzed by phagocytic index measurement, NO production, and TNF-α production. Valencene significantly (P<0.05) increased the phagocytic index in murine peritoneal macrophages. It showed a maximum phagocytic index at 100 μg/mL. The bioactive compound valencene effectively reduced the generation of NO which leads to an increase in the concentration of nitrite. A dose-reliant increase in the nitrite concentration had evidenced in the valencene administered groups. One of the studies conducted by Özek *et al*.[32] showed that *Ferula iliensis* essential oils could exhibit phagocyte immunomodulatory activity. This study also characterized many bioactive compounds and collectively reported that these compounds might have immunomodulatory activity. Valencene was one of the compounds reported by them. The present study revealed that valencene could modulate the first line of immune response mediated by macrophages.

The administration of valencene significantly (P<0.05) decreased TNF-α production in macrophages (LPS mediated), which indicated the effectiveness of the compound in fighting with proinflammatory cytokines *i.e.* TNF-α. Increased release of TNF-α is linked with a range of diseases such as autoimmune disorders, infection-related diseases, and cancer. Inhibition of TNF-α production and scavenging of free radicals are the primary mechanism involved in plant extracts or phytocompounds[33]. LPS considerably improved the macrophage TNF-α production and the administration of valencene drastically reduced the LPS induced macrophage TNF-α production.

In vivo immunomodulatory activity of valencene is quite important to analyze because the *in vitro* effect of the compound may not be descended to *in vivo* studies. In the present study, the *in vivo* immunomodulatory assays such as DTH, HA titer, and PFC assays were conducted. All of these studies have revealed that the compound could influence the mice immune system. A study reported by Sunil *et al*.[3] indicated that the heartwood extracts of *Acacia catechu* administered to Swiss albino mice significantly increased the spleen antibody-producing cells as well as antibody titer. The DTH reaction was significantly increased in the heartwood extracts of *Acacia catechu* treated groups. The present study also revealed similar effects of valencene on the *in vivo* immunomodulatory activity in Swiss albino mice.

SRBC mediated DTH was used to evaluate cell-mediated immunity. DTH is an antigen-specific hypersensitivity that causes erythema and an indurated inflammatory response in immunized animals[34]. DTH is characterized by an entry of immune cells such as macrophages and basophils to the location of injection. T cells are necessary to begin the response. The effect of valencene on T cell-mediated DTH showed significant inhibition on footpad edema compared with the control group in the present study. This result indicated the effect of the compound on cell-mediated immunity. Antibodies produced by B-lymphocytes and plasma cells are vital for humoral immunity. IgG and IgM are implicated in the complement activation, opsonization, and neutralization of toxins[35]. The antibody production ability reflects the effectiveness of the immune system in the body and can be analyzed through a haemagglutinating antibody titer against SRBC. The compound valencene produced a major enhancement in the antibody titer rate compared with the control group.

The spleen has a significant function in the progress of immune responses in the bloodstream[36]. Therefore, its function in generating antibody-producing cells is quite important to study. The significant increase in the number of antibody-producing cells in the spleen of the valencene treated animals compared with the untreated control indicated the activated humoral immune response.

Plants and their compounds are extensively applied in health endorsement, disease deterrence, and managing different circumstances[37]. Conversely, in the majority of cases, there is no inclusive technical information regarding their safety. Toxicological evaluations of all medicinal plant-derived compounds are important to ascertain their safety. The compound valencene could be used as a food additive in various preparations. So a toxicity study of the compound is needed to be conducted. The exposure of cells (L929 mammalian fibroblasts) to the compound did not exhibit any remarkable morphological changes. The viability of cells treated with the higher concentration of the compound was above 75%. The single-cell electrophoresis (comet) assay revealed that the compound did not have much DNA damage at its higher concentration.

Extensive immunomodulatory and toxicity studies are necessary for establishing the compound as an immunomodulator. The present study gives basic experimental evidence of its immunomodulatory activity. Molecular mechanisms behind the actions are needed to be addressed. Further studies are necessary for commercialization as well as the marketing of the compound as an immunostimulant.

Conflict of interest statement

We declare that there is no conflict of interest.

Acknowledgments

Authors are extremely thankful to School of Biosciences, MG University for providing necessary facilities.

Authors' contributions

VVSS and MAS were involved in the formulation of hypothesis, executed methodology, analyzed the data using software, and were in charge of investigations; SJM was involved in the writing, reviewing, and editing of the manuscript; EKR and JM supervised the entire work.

References

[1] Yang L, Bovet P, Liu Y, Zhao M, Ma C, Liang Y, et al. Consumption of carbonated soft drinks among young adolescents aged 12 to 15 years in 53 low-and middle-income countries. *Am J Public Health* 2017; **107**(7): 1095-1100.

[2] Tahmassebi JF, BaniHani A. Impact of soft drinks to health and economy: A critical review. *Eur Arch Paediatr Dent* 2019; **1**: 3.

[3] Sunil MA, Sunitha VS, Ashitha A, Neethu S, Midhun SJ, Radhakrishnan EK, et al. Catechin rich butanol fraction extracted from *Acacia catechu* L. (a thirst quencher) exhibits immunostimulatory potential. *J Food Drug Anal* 2019; **27**(1): 195-207.

[4] Sunil MA, Sunitha VS, Radhakrishnan EK, Jyothis M. Immunomodulatory activities of *Acacia catechu*, a traditional thirst quencher of South India. *J Ayurveda Integr Med* 2019; **10**(3): 185-191.

[5] Cohen DJ, Loertscher R, Rubin MF, Tilney NL, Carpenter CB, Strom TB. Cyclosporine: A new immunosuppressive agent for organ transplantation. *Ann Int Med* 1984; **101**(5): 667-682.

[6] Shabbir A, Khan MMA, Ahmad B, Sadiq Y, Jaleel H, Uddin M. *Vetiveria zizanioides* (L.) Nash: A magic bullet to attenuate the prevailing health hazards. In: Ozturk M, Hakeem KR, editors. *Plant and human health*. Switzerland: Springer International Publishing; 2019, p. 99-120.

[7] David A, Wang F, Sun X, Li H, Lin J, Li P, et al. Chemical composition, antioxidant, and antimicrobial activities of *Vetiveria zizanioides* (L.) Nash essential oil extracted by carbon dioxide expanded ethanol. *Molecules* 2019; **24**(10): 1897.

[8] Sunitha VS, Sunil MA, Radhakrishnan EK, Mathew J. Immunomodulatory activity of *Caesalpinia sappan* L. extracts on peritoneal macrophage of albino mice. *Int J Sci Res* 2015; **4**(12): 449-452.

[9] Frank C, Hay OMRW. *Practical immunology*. 4th ed. UK, Oxford: Blackwell Science Ltd; 2002. Doi: 10.1136/jcp.34.7.816-b.

[10] Zhang X, Goncalves R, Mosser DM. The isolation and characterization of murine macrophages. *Curr Protoc Immunol* 2008; **83**(1): 14.1.1-14.1.14. Doi: 10.1002/0471142735.im1401s83.

[11] Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Anal Biochem* 1982; **126**(1): 131-138.

[12] Pandikumar P, Babu NP, Ignacimuthu S. Hypoglycemic and antihyperglycemic effect of *Begonia malabarica* Lam. in normal and streptozotocin induced diabetic rats. *J Ethnopharmacol* 2009; **124**(1): 111-115.

[13] Raisuddin S, Zaidi SIA, Singh KP, Ray PK. Effect of subchronic aflatoxin exposure on growth and progression of ehrlich's ascites tumor in mice. *Drug Chem Toxicol* 1991; **14**(1-2): 185-206.

[14] Bin-Hafeez B, Haque R, Parvez S, Pandey S, Sayeed I, Raisuddin S. Immunomodulatory effects of fenugreek (*Trigonella foenum graecum* L.) extract in mice. *Int Immunopharmacol* 2003; **3**(2): 257-265.

[15] Davis L, Kuttan G. Immunomodulatory activity of *Withania somnifera*. *J Ethnopharmacol* 2000; **71**(1-2): 193-200.

[16] Jerne NK, Nordin AA. Plaque formation in agar by single antibody-producing cells. *Science* 1963; **140**(3565): 405.

[17] Berridge MV, Tan AS. Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): Subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. *Arch Biochem Biophys* 1993; **303**(2): 474-482.

[18] Ostling O, Johanson KJ. Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. *Biochem Biophys Res Commun* 1984; **123**(1): 291-298.

[19] Puri A, Saxena R, Saxena RP, Saxena KC, Srivastava V, Tandon JS. Immunostimulant agents from *Andrographis paniculata*. *J Nat Prod* 1993; **56**(7): 995-999.

[20] Batista O, Duarte A, Nascimento J, Simoes MF, de la Torre MC, Rodríguez B. Structure and antimicrobial activity of diterpenes from the roots of *Plectranthus hereroensis*. *J Nat Prod* 1994; **57**(6): 858-861.

[21] Alberts B, Johnson A, Lewis J. Helper T cells and lymphocyte activation. In: Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD. *Molecular biology of the cell*. New York, United States: Garland Science; 2002, p. 1-8.

[22] Piyaviriyakul S, Yahuafai J, Limpanasithikul W, Jaraswisarutporn Y, Rassamee K, Onsrisawat P, et al. Stimulatory effects of *Acanthus ebracteatus* Vahl. aqueous root extract on innate immune response in mice. *Thai J Pharmacol* 2019; **41**(1): 13-30.

[23] Lowenstein CJ, Snyder SH. Nitric oxide, a novel biologic messenger. *Cell* 1992; **70**(5): 705-707.

[24]Chao WW, Kuo YH, Li WC, Lin BF. The production of nitric oxide and prostaglandin E_2 in peritoneal macrophages is inhibited by *Andrographis paniculata*, *Angelica sinensis* and *Morus alba* ethyl acetate fractions. *J Ethnopharmacol* 2009; **122**(1): 68-75.

[25]Luperchio S, Tamir S, Tannenbaum SR. NO-induced oxidative stress and glutathione metabolism in rodent and human cells. *Free Radic Biol Med* 1996; **21**(4): 513-519.

[26]Ratajczak-Wrona W, Jablonska E, Antonowicz B, Dziemianczyk D, Grabowska SZ. Levels of biological markers of nitric oxide in serum of patients with squamous cell carcinoma of the oral cavity. *Int J Oral Sci* 2013; **5**(3): 141-145.

[27]Albaayit SFA, Al-Khafaji ASK, Alnaimy HS. *In vitro* macrophage nitric oxide and interleukin-1 beta suppression by *Moringa peregrina* seed. *Turkish J Pharm Sci* 2019; **16**(3): 362-365.

[28]Chou ST, Lai CP, Lin CC, Shih Y. Study of the chemical composition, antioxidant activity and anti-inflammatory activity of essential oil from *Vetiveria zizanioides*. *Food Chem* 2012; **134**(1): 262-268.

[29]Jorge TF, Mata AT, António C. Mass spectrometry as a quantitative tool in plant metabolomics. *Philos Trans A Math Phys Eng Sci* 2016; **374**(2079): 20150370. Doi: 10.1098/rsta.2015.0370.

[30]Ambrož M, Boušová I, Skarka A, Hanušová V, Králová V, Matoušková P, et al. The influence of sesquiterpenes from *Myrica rubra* on the antiproliferative and pro-oxidative effects of doxorubicin and its accumulation in cancer cells. *Molecules* 2015; **20**(8): 15343-15358.

[31]Mitropoulou G, Fitsiou E, Stavropoulou E, Papavassilopoulou E, Vamvakias M, Pappa A, et al. Composition, antimicrobial, antioxidant, and antiproliferative activity of *Origanum dictamnus* (dittany) essential oil. *Microb Ecol Health Dis* 2015; **26**: 26543. Doi: 10.3402/mehd.v26.26543.

[32]Özek G, Schepetkin IA, Utegenova GA, Kirpotina LN, Andrei SR, Özek T, et al. Chemical composition and phagocyte immunomodulatory activity of *Ferula iliensis* essential oils. *J Leukoc Biol* 2017; **101**(6): 1361-1371.

[33]Sandoval M, Charbonnet RM, Okuhama NN, Roberts J, Krenova Z, Trentacosti AM, et al. Cat's claw inhibits TNF production and scavenges free radicals: Role in cytoprotection. *Free Radic Biol Med* 2000; **29**(1): 71-78.

[34]Black CA. Delayed type hypersensitivity: Current theories with an historic perspective. *Dermatol Online J* 1999; **5**(1): 7.

[35]Miller RJ, Pickel VM. The distribution and functions of the enkephalins. *J Histochem Cytochem* 1980; **28**(8): 903-917.

[36]Lewis SM, Williams A, Eisenbarth SC. Structure and function of the immune system in the spleen. *Sci Immunol* 2019; **4**(33): eaau6085. Doi: 10.1126/sciimmunol.aau6085.

[37]Van Andel T, De Boer HJ, Barnes J, Vandebroek I. Medicinal plants used for menstrual disorders in Latin America, the Caribbean, sub-Saharan Africa, South and Southeast Asia and their uterine properties: A review. *J Ethnopharmacol* 2014; **155**(2): 992-1000.