Original Article

Turmeric based oral rinse "HTOR-091516" ameliorates experimental oral mucositis

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Abstract

Background: Prevalence and incidence of oral mucositis (OM) are rigorously increasing and there is no effective treatment. The herbal formulation "HTOR-091516" containing *Curcuma longa*, *Triphala* and honey were evaluated for the treatment of OM. **Aim:** The aim of this study was to evaluate the safety and efficacy of HTOR-091516, employing cellular model, human gingival fibroblasts-1 (HGF-1), and 5-fluorouracil (5-FU)-induced mucositis model in rats. **Materials and Methods:** The cell viability was assessed using 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay and the inhibitory effect of HTOR-091516 on tumor necrosis factor-alpha (TNF-α) was evaluated using TNF-α bioassay in lipopolysaccharides-induced HGF-1. 5-FU and glacial acetic acid were used to induce OM in rats. Animals were divided into two groups, group 1 served as mucositis control and group 2 was treated with HTOR-091516 at the dose of 200 µl and TNF-α was estimated in plasma samples. **Results:** The *in vitro* safety of HTOR-091516 was evaluated in reconstructed human oral epidermis and was found to be nontoxic and exhibited concentration-dependent TNF-α inhibition in HGF-1. The treatment with HTOR-091516 reduced mucositis scores and mortality rate and also decreased the plasma TNF-α level. **Conclusion:** The present data indicate that HTOR-091516 is effective in the treatment of OM.

Keywords: 5-fluorouracil, anti-inflammatory, Ayurvedic formulation, oral mucositis, tumor necrosis factor-alpha

Introduction

Oral mucositis (OM) is a common complication in the patients receiving chemotherapy, radiotherapy and stem cell transplantation. Major characterizations of OM are atrophy and ulceration of stratified squamous epithelium, vascular tissue damage and infiltration of inflammatory lymphocytes to the basement regions.[1] The prevalence and rigorousness of mucositis varies from patient to patient, which also varies with the different treatment regimen. The incidence of mucositis with head and neck radiotherapy is 85%–100% and with patients receiving aggressive myeoloablative chemotherapy can approach 90%–100%.[2]

The pathogenesis of mucositis includes epithelial damage caused by the initial injury, followed by local cytokine production, which leads to inflammation followed by ulceration. Multiple inflammatory components are involved in mucositis such as nuclear factor-kappa B (NF-kB), cyclooxygenase-2 (COX-2) and pro-inflammatory cytokines such as interleukin 1 beta (IL-1 β), IL-6 and tumor necrosis factor-alpha (TNF-α) are linked to the pathogenesis of mucositis.[3] The use of 5-flurouracil (5-FU) is one of the most common causes of OM. It is an anti-metabolite and commonly used for the treatment of malignant tumors, particularly of the breast, colon or rectum, uterine, ovarian and bladder carcinomas.[4]

Even though there is no effective treatment for OM, certain clinically used treatments are local anesthetics, palifermin, glutamine, caphosol mouth rinse, amifostine and antimicrobial agents.[1] Many preclinical and clinical studies support the use of medicinal herbs with good anti-inflammatory, antimicrobial, and antiseptic properties for the prevention and treatment of OM. Different herbal preparations/formulations are used in

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various dosage forms to prevent or treat OM,[5-8] but these existing formulations were not found to be promising in the treatment of OM.

There are many herbs with useful pharmacotherapeutic actions used for the treatment of OM.[9,10] Hence, a herbal formulation HTOR-091516 which contains *Curcuma longa* L.(turmeric), *Triphala*, (the combination of *Phyllanthus emblica* Linn. *Terminalia chebula* Retz. and *Terminalia bellerica* (Gaertn.) Roxb) and honey has been formulated based on the Ayurvedic wisdom and the evidence available in the modern literature on the individual herbs [Table 1]. *Curcuma longa* which has been used extensively in Ayurvedic medicine for centuries, as it is nontoxic and has a variety of therapeutic properties such as anti-oxidant, analgesic, anti-inflammatory, antiseptic, anti-carcinogenic, antibacterial, properties, etc., Recently, many studies have reported curcumin's role in the prevention and reduction of fibrosis caused by harmful factors.[11-13] *Triphala* is rich in anti-oxidants, possess antibacterial, anti-viral anti-cancer and radioprotection properties.[14] Anti-inflammatory effect of *Triphala* shows significant inhibition in levels of lysosomal enzymes, lipid peroxidation (LPO) and inflammatory mediator TNF-α.[15] Honey has good anti-inflammatory, antibacterial activity; on application of honey on the wound, it visibly reduced inflammation and edema surrounding wounds.[16,17] Thus, the present study was designed to evaluate the efficacy and safety of HTOR-091516 in experimental models of OM.

Materials and Methods

In vitro studies

Chemicals

Dulbecco's Modified Eagle Medium (DMEM) (high glucose), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), neutral red dye, dimethyl sulfoxide were obtained from Sigma Chemicals. Fetal bovine serum (FBS) was purchased from Invitrogen, USA. Glacial acetic acid, absolute ethanol, ethylene diamine tetraacetic acid (EDTA) was procured from Merck, India. 5FU was purchased from Biochem Pharmaceutical industries Ltd. Enzyme-linked immunosorbent assay (ELISA) kits for TNF-α were purchased from Krishgen Biosystems, India.

Cell lines and its maintenance

Human gingival fibroblasts-1 (HGF-1) and L929 (Mouse connective tissue) were obtained from the National Centre for Cell Sciences, Pune, India. HGF-1 and L929 cells were grown in DMEM (high glucose) and DMEM (low glucose) media, respectively. All media were supplemented with 10% heat-inactivated FBS, penicillin (100 U/mL) and streptomycin (100 µg/mL) and cultured under a humidified atmosphere (95% air and 5% CO_2) at 37°C and the monolayer cultures were routinely subcultured by using trypsin-EDTA. The reconstructed human oral epidermis was obtained from Skin Ethic, France.

Table 1: Composition of HTOR-091516

Ingredients	Quantity (each 100 ml contains)
Haridra dry extract *(Curcuma longa)*	10.5 mg
Triphala dry extract	400 mg
Madhu (Honey)	10 g

Cell viability

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used to determine cell viability, which reflects initial cell death. HGF-1 cells were cultured in 96-well plates (1×10^4 cells/mL) and treated with various concentrations (15.625–1000 µg/mL) of HTOR-091516. After 24 h incubation, cytotoxicity was tested by MTT (10 µL/well containing 100 µL of cell suspension; 5 mg/mL of stock in PBS) solution and the absorbance were read at 540 nm using Synergy HT multi-detection microplate reader (Bio-Tek, Winooski, VT). The nontoxic concentration of HTOR-091516 was taken for further experiments.

Tumor necrosis factor-alpha inhibitory studies using bioassay

The study was carried out in HGF-1 cells. The cells with different concentrations of drug (250 µg/ml and 500 µg/ml) were treated with 1 µg/ml of lipopolysaccharide (LPS) and incubated at 37°C with 5% CO_2 for 24 h. After incubation, the cell supernatant was separated by centrifugation. TNF-α bioassay was carried out using L929 cells which are sensitive toward TNF-α (Varma *et al*., 2011). The L929 cells were grown in 96 well plate using DMEM-LG with 2%FBS and treated with the collected cell supernatant and incubated for 24 h. The cell viability is a direct indication of inhibitory properties of HTOR-091516 against LPS-induced TNF production in HGF-1 cells which was determined by MTT assay. Dexamethasone (DXM) 100 µM was used reference standard.

In vitro safety study on the reconstructed human oral epithelium

The experiment was conducted as per the *INVITTOX* SKINETHIC™ skin irritation test protocol. A volume of 16 µl of HTOR-091516 was transferred on the top of epithelial tissue and incubated for 42 min at 37°C with 5% CO2. After incubation, the treatment was washed with PBS and traces of PBS were drained with filter paper and further incubated in growth medium for 42 h at 37°C with 5% CO_2. After incubation, the treated tissues were transferred in the prefilled MTT solution and incubated for 3 h at 37°C. The formazan was extracted by isopropanol and the absorbance was measured at 570 nm. The percentage viability was calculated from absorbance values at 540 nm of treated and control groups.

In vivo studies

Animals

Twenty male Wistar rats of 12–16 weeks old weighing between 200 300 g were received from the in-house animal breeding facility with Animal Ethics Committee Approval (Protocol No. 127/13) for the experiment. The animals were

housed in polycarbonate cages with free access to standard rat feed and Aquaguard RO water (Eureka Forbes Limited, Bombay, India.), and acclimatized to a constant temperature of 22 ± 3°C. They were maintained with equal light and dark cycle.

Experimental design

Animals were kept for acclimatization for 7 days. After the acclimatization period, animals were randomized into two groups of ten each based on the body weight. Group-1 served as mucositis control and group-2 was mucositis treated with HTOR-091516-(200 µl).

Induction of mucositis

The animal model for chemotherapy-induced OM was based on the modified method described by Sonis *et al*.[18] Animals were injected with 100 mg/kg on day-1 and 80 mg/kg on day-3 with intra-peritoneal injection of 5-FU.[19] On day-2 mucositis was induced with acetic acid swab (phlogistic agent). A cotton swab dipped in glacial acetic acid and extra acid was removed by dabbing on tissue paper. The swab was rotated with light pressure on the right cheek pouch mucosa. The treatment was started from day-4.

Animals were treated with HTOR-091516 by slowly pouring the solution (200 µl) drop by drop on the induced aphthae for every animal in the treatment period. The oral mucositis score (OMS) was evaluated in grading format [Table 2]. This format was prepared corresponding to the WHO grading system which is based on clinical background.[20]

The scoring was observed twice in a week in the treatment period with the agreement of two independent observers and the survival rate was calculated. All animals were sacrificed on day-14 and blood was collected in heparinized tubes, plasma was separated and processed for TNF-α estimation.

Statistical analysis

All values are expressed as the mean ± standard error of the mean. The results were statistically analyzed using paired/unpaired Student's *t*-test method using Graphpad Prism software version 6.07, CA, USA. GraphPad Prism version 6.07, La Jolla CA, USA. $P < 0.05$ was considered statistically significant.

Results

In vitro studies

Cell viability assay by 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide

HTOR-091516 was found to be nontoxic to HGF-1 cells. The IC_{50} value of HTOR-091516 was >1000 µg/ml in HGF-1 cells [Figure 1]. Hence, nontoxic concentrations (250 and 500 µg/ml) were taken for further studies.

Effect of HTOR-091516 on tumor necrosis factor-alpha

Studies have reported that TNF-α plays a key role in mucositis as a pro-inflammatory cytokine and being the main target for treatment for mucositis,[21] the inhibitory effect of HTOR-091516 on TNF-α was measured using

Table 2: Oral mucositis scoring system

Grade	Description
0 (none)	None
I (mild)	Oral soreness, erythema
II (moderate)	Oral erythema, ulcers, solid diet tolerated
III (severe)	Oral ulcers, liquid diet only
IV (life-threatening)	Oral alimentation impossible

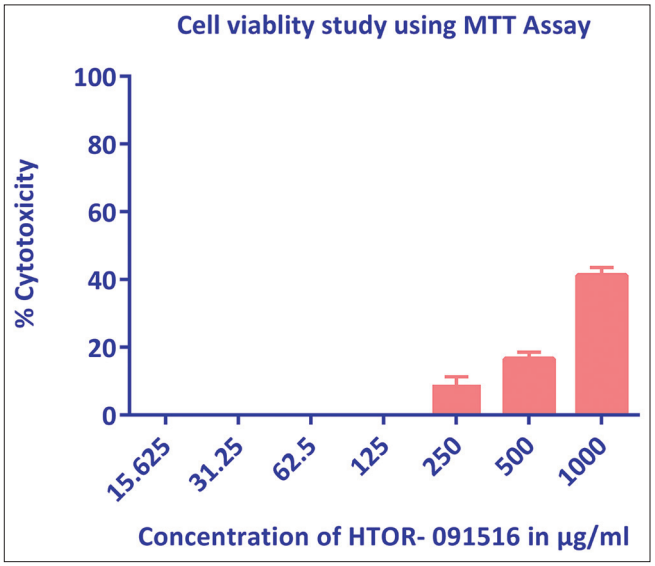

Figure 1: Effect of HTOR-091516 on cell viability by 3-(4,5-dimethy lthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay that reflects initial cell death

TNF-α inhibitory bioassay. HTOR-091516 showed a concentration-dependent TNF-α inhibition in LPS-induced HGF-1 cells. HTOR-091516 showed 34.1% and 24.9% TNF-α inhibition at 500 µg/ml and 250 µg/ml, respectively [Figure 2]. Dexamethasone (100 µM) positive control used in the present study showed 34.3% TNF-α inhibition.

In vitro safety study using reconstructed human oral epidermis for irritation

The relative percentage viability of HTOR-091516 was found to 100% over cell control [Figure 3]. Hence, it can be concluded that HTOR-091516 is nontoxic and nonirritant.

In vivo studies

Effect of HTOR-091516 on body weight

All animals were weighed weekly twice from day 0 to day 14. Over the experimental period, there was a decrease in body weight in both groups. However, the decrease in the treatment group was not to an extent of mucositis control group. The mean bodyweight of the treatment group was significantly high as compared to mucositis control group [Figure 4a and b].

Effect of HTOR-091516 oral mucositis score

The intraperitoneal administration of 5-FU followed by phlogistic agent (acetic acid) trauma in cheek mucosa of the rats, caused clear ulceration up to day 14 in all rats were with

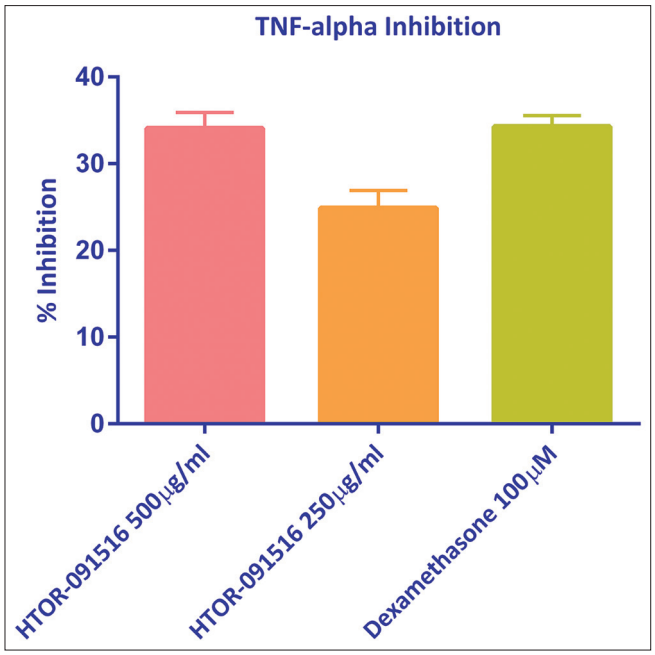

Figure 2: Inhibitory effect of HTOR-091516 on tumor necrosis factor-alpha using HGF-1 cells

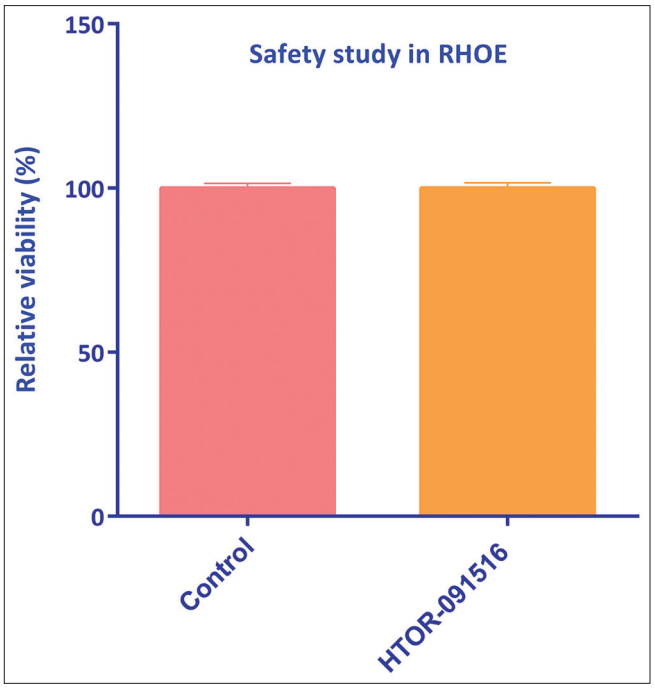

Figure 3: Safety of HTOR-091516 on reconstructed human oral epithelium skin model and was conducted as per the Invittox Skinethic™ Skin Irritation test protocol

the maximum score of 3.0. The scoring was done as shown in Table 2. Treatment with HTOR-091516 showed a significant reduction in OMS compared to mucositis control [Figure 5].

Mortality rate

The mortality rate was recorded during 14 days of the study period in each group. In mucositis control group, one rat died on the 12th day and 4 rats died on the 14th day of the study period, and hence, the percentage of mortality was found to be 50% at the end of the study period. In HTOR-091516 treated group, none of the rats died till 12th day, but 2 died on the 14th day of the experiment period, the percentage of mortality was 20% at the end of the experiment. Treatment with HTOR-091516 showed a protective effect against the toxicity of 5-FU by decreasing mortality proportion and increasing survival proportion during 14 days of experiment period compared to mucositis control [Figure 6].

Effect of HTOR-091516 in plasma tumor necrosis factor-alpha level

The plasma concentration of cytokine TNF-α was quantified using the ELISA kit by Krishgen Biosystems. Treatment with HTOR-091516 suppressed the elevation of TNF-α level when compared to the mucositis control group [Figure 7].

Discussion

Mucositis induced by antineoplastic drugs is an important dose-limiting side effect of anticancer treatment, bone marrow transplantation, and local irradiation for tumors in the head-and-neck area.[22] Oral mucosa comprises membranes of rapid epithelial turnover and maturation rates with a high mitotic index. This renders the mucosa vulnerable to the adverse effects of chemotherapy and radiotherapy.[23] It is well accepted that pathophysiology of OM results from the direct inhibitory effects of chemoradiotherapy on DNA replication and mucosal cell proliferation, resulting in the reduction and renewal capabilities of the basal epithelial cells.[3]

The major treatment for OM in the clinical scenario is to relieve pain with local anesthetics, or coating the oral mucosa and to locally administer bactericidal or anti-inflammatory agents.[24] In this perspective, HTOR-091516 a combination of *Curcuma longa, Triphala* and honey, were evaluated in experimental models of OM. Human gingival fibroblast (HGF-1) was taken as the cellular model for evaluating the safety and efficacy of HTOR-091516. It was found that HTOR-091516 was nontoxic with a CTC_{50} value >1000 µg/ml. Tumor necrosis factor-α plays an important role in the development of OM[21] and also it has been reported that pro-inflammatory cytokines are increased in saliva samples of cancer treatment patients.[25-27] It was observed that HTOR-091516 significantly inhibited TNF-α secretion. With the positive results obtained from cellular and reconstructed skin model experiments, further studies were carried out in the animal model of OM. An animal model reported by SonisSonis ST *et al*., was modified for the evaluation of HTOR-091516 in OM, which includes the usage of acetic acid along with 5-FU to induce OM.[18] Treatment with HTOR-091516 had showed the protective action against ulcerated lesions and a significant reduction in mucositis score. Mortality is one of the important parameters in the chemotherapy-induced mucositis model. HTOR-091516 showed a protective effect against the toxicity of 5FU by decreasing mortality proportion and increasing survival proportion in the experiment period compared to mucositis

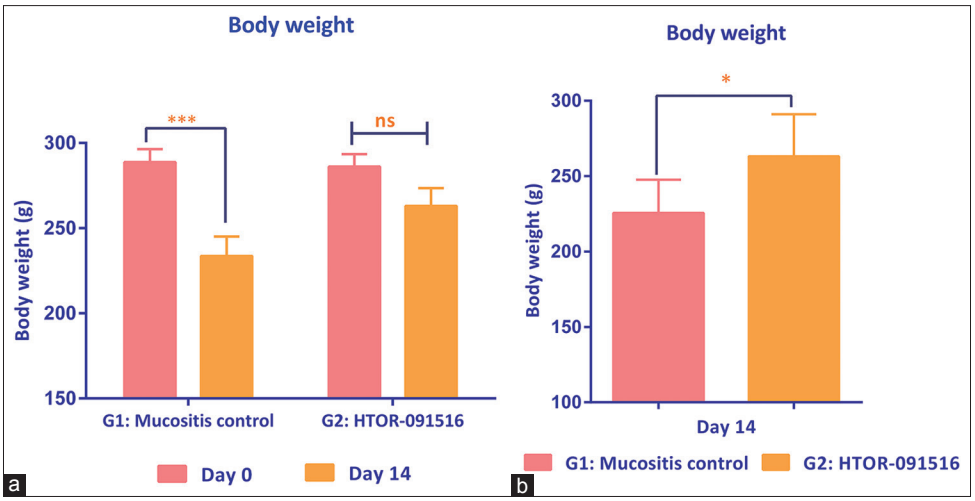

Figure 4: Effect of HTOR-091516 on mean body weight of 5-Fluorouracil induced oral mucositis rats. (a) ***$P < 0.001$ compared to day 0. (b) *$P < 0.05$ compared to mucositis control

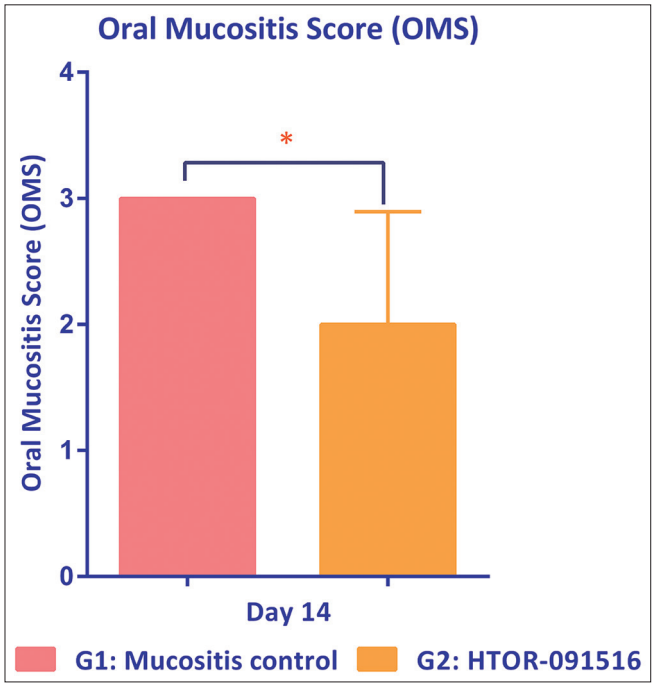

Figure 5: Effect of HTOR-091516 on oral mucositis score of the rats at the end of study period. *$P < 0.05$ compared to mucositis control

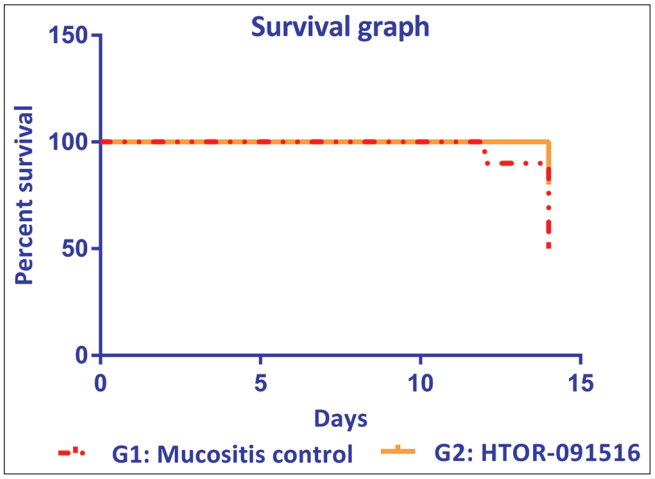

Figure 6: Longevity of oral mucositis rats treated with HTOR-091516 is expressed as percentage survival

control. There is a link between low immune competence and mucositis severity with weight loss.[18,24,28] At the end of the study period, a significant improvement in body weight was observed in the treatment group compared to the mucositis control group. This may be explained on the basis that healing of the OM was faster in the treated groups, which increased the feed intake and body weight.

The beneficial effect of HTOR-091516 on OM may be due to various mechanisms reported for individual active ingredients of the formulation. Like curcumin which is one of the ingredients, is reported to suppress the acute and chronic inflammation, it exerts anti-inflammatory activity by inhibiting a number of different molecules that participate in the process of inflammation.[29] The expression of several genes that are regulated by NF-κB has shown to be suppressed by curcumin.[11] These include cell surface adhesion molecules, chemokines, TNF, MMP9, COX2, and NOS. It also has a fibrinolytic property due to its ability to inhibit LPO and check cellular proliferation, thereby reducing the rate of collagen synthesis which can help in mucositis.[12] *Triphala*, which is the major ingredient of the formulation shows significant inhibition in levels of lysosomal enzymes and inflammatory mediators TNF-α.[21] It also protected whole-body irradiated mice through the inhibition of oxidative damage in cells and organs, which may help in reducing the inflammation associated with mucositis.[30] Honey is commonly used as an antibacterial and anti-inflammatory agent. The antibacterial effects of honey are based on high osmotic properties and the presence of glucose oxidase enzyme which produces hydrogen peroxide.[16] Due to its acidic nature and its high sugar content, it can prevent infection

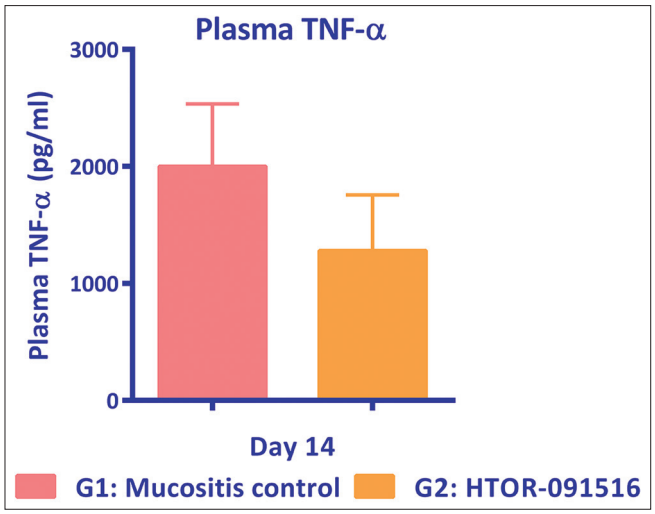

Figure 7: Effect of HTOR-091516 on plasma tumor necrosis factor-alpha levels of the oral mucositis rats

by forming a physical protective barrier. The topical application of honey significantly reduced chemotherapy-induced mucositis and facilitates wound healing process.[31] The individual herbs present in HTOR-091516 may be acting in synergism on various pathophysiological pathways of OM to exert its beneficial effect.

Conclusion

The polyherbal formulation 'HTOR-091516' showed beneficial effect on oral mucositis in cellular, animal and reconstructed skin models. Thus it can be concluded that 'HTOR-091516' is safe and effective in the treatment of oral mucositis and may be recommended for the prevention of chemotherapy-induced oral mucositis.However, further clinical studies are in progress to substantiate the same.

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Conflicts of interest

There are no conflicts of interest.

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