# Herbal medicine AnoSpray suppresses proinflammatory cytokines COX-2 and RANTES in the management of hemorrhoids, acute anal fissures and perineal wounds

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**Abstract.** Hemorrhoids, anal fistula and fissure are common anorectal complications. Anorectal diseases are associated with severe pain, inflammation, swelling, itching and bleeding. These diseases may be managed with different medical treatments or surgical procedures, depending on their severity. Surgical procedures, however, are highly invasive and are associated with higher costs and the possibility of recurrence. In addition, surgical removal of fistula-in-ano leads to the formation of perineal wounds. Therefore, developing therapeutic interventions that are effective in alleviating inflammation and pain are desirable for the effective management of anorectal diseases. Herbal compounds have previously been indicated to suppress inflammation and pain in different pathological conditions. The aim of the present study was to examine the effects elicited by a polyherbal formulation, AnoSpray®, on the migration of inflammatory cells and on the expression of inflammatory cytokines in anorectal diseases. The effect of AnoSpray on cell viability and migration was studied using MTT and wound-migration assays, respectively. Furthermore, the effects of AnoSpray on the expression of the inflammatory cytokines regulated upon activation, normal T cell expressed and presumably secreted (RANTES) and VEGF, as well as on cyclooxygenase-2 (COX)-2, were investigated using western blot analysis. The expression of RANTES and COX-2 in human hemorrhoid specimens was also analyzed to corroborate the *in vitro* findings. The results obtained revealed that AnoSpray did not exhibit any cytotoxic effects; however, it did lead to a significant suppression in the migration of RAW 264.7 and BJ cells. Furthermore, the results suggested that AnoSpray suppressed the expression of the inflammatory cytokines RANTES and VEGF, and also the expression of COX-2. In addition, RANTES and COX-2 were significantly downregulated in the clinical specimens of AnoSpray-treated hemorrhoids compared with the controls. Taken together, the results of the present study suggested that AnoSpray may be a potential therapeutic agent in the treatment of bleeding hemorrhoids, anal fissures and perineal wounds.

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## Introduction

Anorectal conditions are among the most common problems encountered in clinical practice. Anorectal diseases such as hemorrhoids, anal fissure, anorectal abscesses, anal fistula, proctalgia fugax and pruritus ani exhibit overlapping symptoms, which may be distinguished based on consideration of the detailed history of the patient and anorectal examination (1).

Hemorrhoids represent the most common anorectal disorder and arise due to engorgement of vascular cushions in the lower rectum/anus (2,3). Hemorrhoids may be located inside the anal canal (termed 'internal hemorrhoids') or they present at the anal opening ('external hemorrhoids'). If external hemorrhoids become filled with blood clots, this leads to the formation of thrombosed hemorrhoids. Hemorrhoids are traditionally classified into grades I-IV, where grade I hemorrhoids are purely internal, grade II hemorrhoids prolapse on staining but reduce spontaneously, grade III hemorrhoids are characterized by their prolapsing requiring manual reduction and grade IV hemorrhoids are prolapsed and non-reducible (3). According to current estimates, 75% of the world population will suffer from bleeding hemorrhoids at a certain point in their lives (4), indicating that hemorrhoids are a major socioeconomic and medical problem. Hemorrhoids occur commonly in both genders, with a peak incidence arising between the fourth and sixth decades of life (5,6). Even though the precise underlying cause of hemorrhoids has not been fully elucidated, risk

factors for hemorrhoids include straining during defecation as a consequence of constipation, obesity, pregnancy, old age, chronic diarrhea, anal intercourse, cirrhosis with ascites, pelvic floor dysfunction and having a low-fiber diet (7,8). Although 40-55% of cases exhibit no symptoms (9), those patients who are symptomatic frequently exhibit pain, bleeding, prolapse, soiling, grape-like tissue prolapse, itching or a combination of these symptoms (3). Various physiological changes, including abnormal distension of veins, destruction of collagen fibers and fibroelastic tissues, and damage of the anal subepithelial muscle occur during hemorrhoidal progression (10). Inflammatory reactions in the affected area have been indicated to be associated with mucosal ulceration, ischemia and thrombosis (11). Several enzymes, including matrix metalloproteinases, thrombin and plasmin, as well as an array of signaling factors, are involved in the degradation of the supporting tissues in the anal cushions, which consist of collagen, fibronectin and elastin fibers. These events gradually lead to the promotion of angiogenic and proliferative activity mediated by transforming growth factor β as part of the healing process (12,13). Various surgical options, including stapled hemorrhoidopexy and hemorrhoidectomy, are available as treatment procedures; however, these methods are highly invasive and associated with higher costs (2,14). Furthermore, these surgical options are largely unsuccessful, as recurrence, pain and bleeding are major concerns after the procedure (15,16).

An anal fissure is a tear of the anoderm in the anal canal that is caused by mechanical trauma, sphincter spasm or ischemia (17). Anal fissures exhibit overlapping symptoms with hemorrhoids (18) and are always associated with twinges of pain (19). Chronic fissures typically require medical treatment or surgical therapy. Surgical procedures have superior healing rates compared with local medical therapies, although they may result in persistent incontinence (20).

An anal fistula is an epithelialized tract or a connection between the anal canal and the perianal skin. Classical anal fistulas result from a perineal infection and abscess formation (21). Fistulas are also associated with inflammatory bowel disease, radiation, malignancy, chronic diarrhea or pre-existing incontinence (22). Perineal wounds usually result from low pelvic tumors, ablation of the tumor, trauma, perineal infections and electrical or thermal burns (23). Furthermore, various surgical procedures for fistula-in-ano may also lead to poorly healing perineal wounds and impaired continence (24). Patient- and surgery-associated factors, including obesity, being overweight, hypoalbuminemia, extralevator abdominal resection, intraoperative perforation and supine position during the second phase of labor, are associated with delayed perineal wound healing (25-29). Delayed perineal wounds are associated with morbidity, prolonged hospital stays, higher costs, home nursing care needs and lower rates of survival (23,30). Therefore, developing minimally invasive therapeutic interventions that help to alleviate inflammation and pain in anal fissures, bleeding hemorrhoids and perineal wounds would be beneficial for patients.

Herbal products are an important source of medicinal compounds (31). Traditional medicinal compounds are known to suppress pain and inflammation under different pathological conditions (32). Herbal medicines suppress inflammation by downregulating the recruitment of inflammatory cells, as well as through suppressing inflammatory cytokine expression (33). Cytokines such as regulated upon activation, normal T cell expressed and presumably secreted (RANTES), IL-1β and VEGF are proinflammatory cytokines that contribute to inflammation and pain in different pathological settings (33,34). Cyclooxygenase-2 (COX-2) is an enzyme that is involved in the formation of prostaglandins, which are crucially involved in promoting inflammation (35). The expression of COX-2 is regulated by growth factors and different inflammatory cytokines, including IL-1β, IL-6 and tumor necrosis factor-α (TNF-α), and therefore, its expression is upregulated during inflammation (35). The use of natural products for the treatment of these ailments is indeed cost-effective and minimally invasive (32,36). A previous study by our group assessed the safety and efficacy of a polyherbal formulation, AnoSpray® for the treatment of perineal wounds using a single-center, open-label, randomized parallel-group trial. The results indicated that the use of AnoSpray provided a marked improvement in treating perineal wounds as compared to betadine solution (37). However, at present, the underlying mechanism through which AnoSpray exerts its effects on anorectal diseases has remained elusive. The aim of the present study was to investigate the cytotoxicity and molecular action of AnoSpray in anorectal diseases. The data obtained on fibroblasts and macrophages revealed that it was safe to use. Furthermore, it was demonstrated that AnoSpray suppressed the migration of these cells and ameliorated the expression of inflammatory factors in both an *in vitro* model and in clinical specimens of anorectal disease. Collectively, the results confirmed the benefit of AnoSpray treatment in the clinical management of hemorrhoids, anal fissures and perineal wounds.

## Materials and methods

*Cell culture*. The mouse monocyte/macrophage-like cell line RAW 264.7 and human foreskin fibroblasts (BJ cell line) were obtained from the American Type Culture Collection. The RAW 264.7 and BJ cells were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) and Minimum Essential Medium (Eagle) [MEM(E)] (HiMedia Laboratories, LLC) media, respectively. The culture media were supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 100 units of penicillin/100 µg/ml streptomycin (HiMedia Laboratories, LLC), and the cells were grown in a humidified incubator in an atmosphere with 5% $CO_2$ at 37˚C.

*Drug preparation*. The formulation of AnoSpray/PiloSpray® (Healing Hands & Herbs Pvt. Ltd.; https://healinghandsandherbs.in/) was mentioned in a previously published study (37). Similar to AnoSpray/PiloSpray (see https://pilospray.com/AnoSpray-advanced-piles-spray/) is an over-the-counter brand name of the formulation; essentially, AnoSpray/PiloSpray is an Ayurvedic polyherbal formulation in the form of a spray. AnoSpray/PiloSpray consists of lodhara (*Symplocos racemosa*), daruharidra (*Berberis aristata*), mocharas (*Bombax ceiba*), kapur (*Cinnamomum camphora*), pudinah (*Mentha piperita*), til oil (*Sesamum indicum*) and kokam oil (*Garcinia indica*) in aerosol form. 'AnoSpray' or 'PiloSpray' is used as the brand

name to refer to the polyherbal spray formulation in the present study.

*Cell viability assay*. Cell viability was assessed using an MTT assay, following the instructions provided in a previously described protocol (38). In brief, RAW 264.7 and BJ cells (density, $2x10^4$ cells/well) were seeded into 96-well microplates (with flat bottoms) and treated with AnoSpray at concentrations of 0-7.5 $\mu$l/ml for 24 h. MTT (0.5 mg/ml) solution was added to each well and the plates were incubated for 4 h at 37˚C. Subsequently, the MTT solution was carefully aspirated and isopropanol was added to dissolve the formazan crystals. The optical density of the formazan solution was subsequently recorded at 570 nm using an automated microplate reader (EPOCH2; Bio Tek Instruments, Inc.). All experiments were performed in triplicate.

*Wound-closure assay*. Cell migration was studied using a conventional wound-closure/migration assay, as per the standard protocol described previously (39). In brief, RAW 264.7 and BJ cells (density, $2x10^5$ cells) were seeded into 12-well plates and allowed to attain a confluent monolayer. Upon reaching 100% confluence, the monolayers were scratched using a sterile 200-$\mu$l pipette tip and the old medium was removed to remove detached cells. Fresh RPMI-1640 complete medium (1 ml) was added to the cells prior to the treatments. Cells were subsequently incubated at 37˚C with AnoSpray at concentrations of 0-7.5 $\mu$l/ml. Photographs were acquired at 0 and 12/16 h using a using a phase-contrast microscope (magnification, x100; Nikon Corporation). The area of wound closure was measured using Image-Pro Plus 6.0 software (National Institutes of Health).

*Western blot analysis*. Western blot analysis was performed as per a standard procedure described previously (40). Specifically, RAW 264.7 and BJ cells ($5x10^5$ cells) were seeded in 60-mm dishes. On the next day, the cells were treated with different concentrations of AnoSpray (0-7.5 $\mu$l/ml). Cells were harvested by centrifuging the cells at 1,000 x g at room temperature for 10 min and lysed using RIPA buffer. The protein concentration was estimated in cell lysates using Bradford reagent and equal amounts of total protein (30 $\mu$g/lane) were resolved by 10 or 12.5% SDS-PAGE. The separated proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc.) and processed for further analysis Non-nspecific binding sites were blocked by incubating membranes in 5% skimmed milk at room temperature for 1 h. The membranes were subsequently incubated with primary antibodies (obtained from Santa Cruz Biotechnology, Inc.) against COX-2 (cat. no. sc-1746; 1:1,000), RANTES (cat. no. sc-1410; 1:1,000), VEGF (cat. no. sc-7269, 1:1,000 dilution) and β-actin (Santa Cruz Biotechnology, Inc., cat. no. sc-1615, 1:2,000) overnight at 4˚C, followed by incubation with anti-goat HRP (Santa Cruz Biotechnology, Inc.; cat. no. sc-2020; 1:2,000) or anti-mouse HRP antibodies (Santa Cruz Biotechnology, Inc.; cat. no. sc-2005; 1:2,000) for 1 h at room temperature. All blots were visualized using the Clarity Western ECL reagent (Bio-Rad Laboratories, Inc.). Densitometry analysis was performed using ImageJ2 software (National Institutes of Health) and fold-changes were calculated following normalization to β-actin.

*Analysis of clinical specimens*. The present study was approved by the Institutional Ethics Committee of Healing Hands Clinic (Pune, India). Patients with hemorrhoids were treated thrice a day for at least 15 days with AnoSpray. Human hemorrhoid specimens derived from the surgical removal of hemorrhoids (n=10) were collected between March 2020 and February 2021 with the help of a histopathologist from Healing Hands Clinic (Pune, India) and written informed consent was obtained from each of the patients. Paraffin-embedded tissue blocks were prepared and 5-$\mu$m sections were cut and deposited on poly-L-lysine-coated slides. Immunohistochemical analysis was performed using the SuperSensitive™ Polymer-HRP IHC Detection System (BioGenex Laboratories), as per the manufacturer's protocol. In brief, the sections were deparaffinized in xylene and rehydrated in an alcohol gradient. Subsequently, the sections were subjected to antigen retrieval in citrate buffer at 90˚C for 15 min. Sections were covered with peroxide for 10 min to block endogenous peroxidase activity, followed by power block (provided as part of the SuperSensitive™ Polymer-HRP IHC Detection System) to block non-specific binding sites. Sections were then incubated with primary antibodies against COX-2 (1:100) and RANTES (1:100) overnight at 4˚C, and subsequently with specific secondary antibodies for 1 h at room temperature. Liquid DAB chromogen was added at room temperature for 10 min and images of the tissue sections were captured using a Nikon Eclipse microscope (magnification, x200; Nikon Corporation).

*Statistical analysis*. Each experiment was performed in triplicate and the results were expressed as the mean ± SEM. Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Software, Inc.). An unpaired Student's t-test was utilized to assess statistical difference between two groups, whereas the Kruskal-Wallis test was used to measure statistical significance in the case of multiple doses of drug treatments with the Dunn's post-hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Effect of AnoSpray on the viability of fibroblasts*. A previous study by our group (37) reported on the safety of AnoSpray treatment in humans for the management of perineal wounds. The present study aimed to investigate the *in vitro* cytotoxic effects of AnoSpray on macrophage and fibroblast cell lines to assess its safety in therapeutic use. RAW 264.7 (macrophage) and BJ (fibroblast) cells were used for assessing the effect of AnoSpray on cell viability. The RAW 264.7 and BJ cell lines in culture were treated with AnoSpray in a concentration-dependent manner (0-7.5 $\mu$l/ml) to study its effect on cell viability using an MTT assay. The percentage cell viability was measured and the results obtained were statistically analyzed using the Kruskal-Wallis test. The results indicated that AnoSpray did not have any significant effects on the viability of the mouse macrophage cell line RAW264.7 (Fig. 1A). Subsequently, the effect of AnoSpray on the viability of the human normal fibroblast cell line BJ was

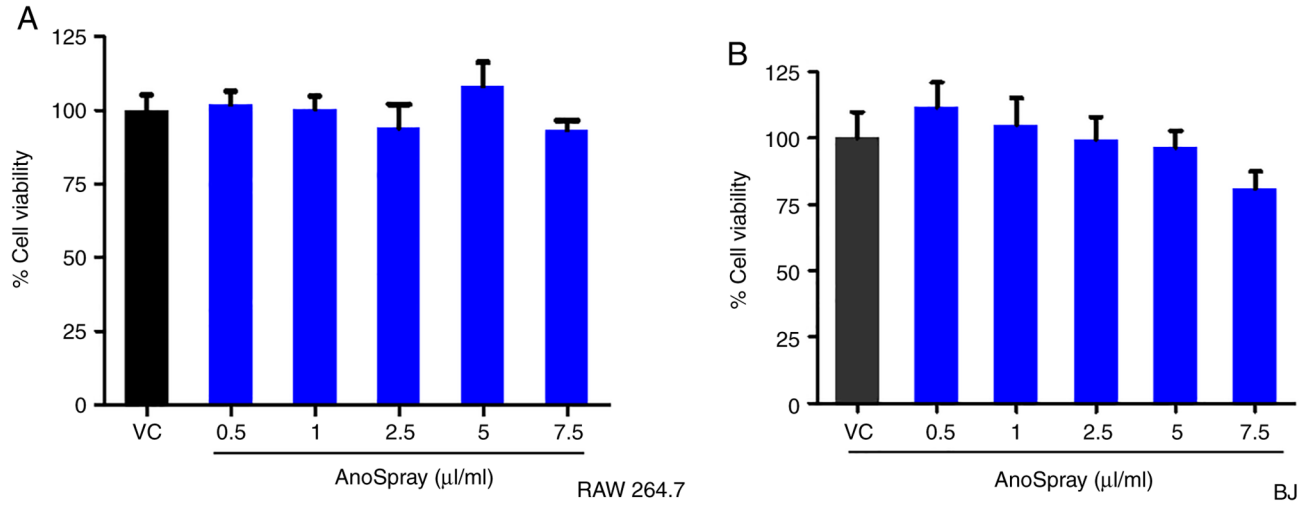


Figure 1. Effect of AnoSpray® on cell viability. (A) RAW 264.7 cells were incubated with VC/AnoSpray® at specified doses (0-7.5 $\mu$l/ml) for 24 h and an MTT assay was performed to examine cell viability. (B) BJ cells were treated with VC/AnoSpray® at specified doses (0-7.5 $\mu$g/ml) and an MTT assay was performed to study the effect of AnoSpray® on cell viability. Bar graphs represent the effect of AnoSpray® on the viability of the above cells. Values are expressed as the mean ± standard error of the mean (n=3). A Kruskal-Wallis test was performed to determine statistically significant differences between different treatment group means vs. VC. VC, vehicle control.

also studied using an MTT assay. Similar to the results for the RAW 264.7 cell line, AnoSpray did not appear to significantly affect the viability of the BJ cells (Fig. 1B). Based on these findings, it was possible to infer that AnoSpray did not affect the viability of macrophages and fibroblasts *in vitro*, thereby demonstrating its safety for therapeutic applications.

*Effect of AnoSpray on the migration of fibroblasts*. Wound healing is a complex and dynamic process that is involved in the recovery of the structure and functions of injured tissues (32). Prolonged inflammation at the site of injury delays the wound-healing process, inducing pathological pain (41,42). In anorectal diseases, inflammation has a fundamental role in the aetiology of hemorrhoids (10), fissures (43) and perineal wounds (44). Macrophages and fibroblasts are recruited at the site of wounds and are involved in the synthesis of extracellular matrix and secretion of proinflammatory cytokines (41,45). Therefore, the effects of AnoSpray treatment on the migration of macrophages and fibroblasts were studied using a conventional wound closure assay. Monolayers of macrophages and fibroblasts in culture were wounded and subsequently treated with different concentrations of AnoSpray (0-7.5 $\mu$l/ml). The percentage migration values were then analyzed and significant differences between the groups were assessed using Student's t-test. The results revealed that the migration of RAW 264.7 cells was significantly reduced upon AnoSpray treatment in a concentration-dependent manner (Fig. 2A and B). Similar results were obtained with the BJ cells. AnoSpray treatment led to a decrease in the migration rates of these cells (Fig. 2C and D), suggesting that AnoSpray has a significant role in impeding the migration of fibroblasts and macrophages.

*Effect of AnoSpray on the expression of inflammatory cytokines*. Inflammatory cytokines are highly expressed in anorectal diseases and are involved in pain, inflammation and itching (44,46-48). Inflammatory mediators, including COX-2, RANTES, VEGF, TNF-α and IL1-β, are known to be highly expressed under different pathological conditions (49,50). Since fibroblasts and macrophages have been reported to express proinflammatory mediators (51,52), macrophages and fibroblasts were used in the present study to examine changes in the expression levels of these cytokines upon treatment with AnoSpray (Fig. 3). Even though the endogenous expression levels of RANTES and VEGF are low in RAW 264.7 cells, the protein expression levels of these cytokines in RAW 264.7 cells were significantly suppressed upon treatment with AnoSpray compared with those in the control cells, as determined using western blot analysis (Fig. 3A). In addition, the endogenous expression of COX-2 was reduced in RAW 264.7 cells upon incubation with AnoSpray (Fig. 3A). The western blots for RANTES, VEGF and COX-2 were subsequently quantified and statistically analyzed using one-way ANOVA. The results revealed that the expression of these cytokines and COX-2 were significantly downregulated in RAW 264.7 cells (Fig. 3B). The expression levels of RANTES and VEGF were also examined in BJ fibroblasts using western blot analysis. The results revealed that the expression levels of these markers were markedly reduced in the AnoSpray-treated cells (Fig. 3C). Densitometric analysis was also performed for the western blot data for RANTES and VEGF, followed by statistical analysis using one-way ANOVA. This analysis revealed that the expression levels of these cytokines were significantly decreased in BJ cells (Fig. 3D). Collectively, these results indicated that AnoSpray treatment downregulated the expression of the two proinflammatory cytokines and COX-2 in macrophages and fibroblasts.

*AnoSpray suppresses the expression of COX-2 and RANTES*. To further corroborate the *in vitro* results in clinical specimens, the expression levels of the proinflammatory cytokines RANTES and COX-2 were examined in hemorrhoidal tissues, where they are known to induce pathological pain. Hemorrhoidal patients were treated with AnoSpray for at least 15 days. The expression levels of RANTES as well as COX-2

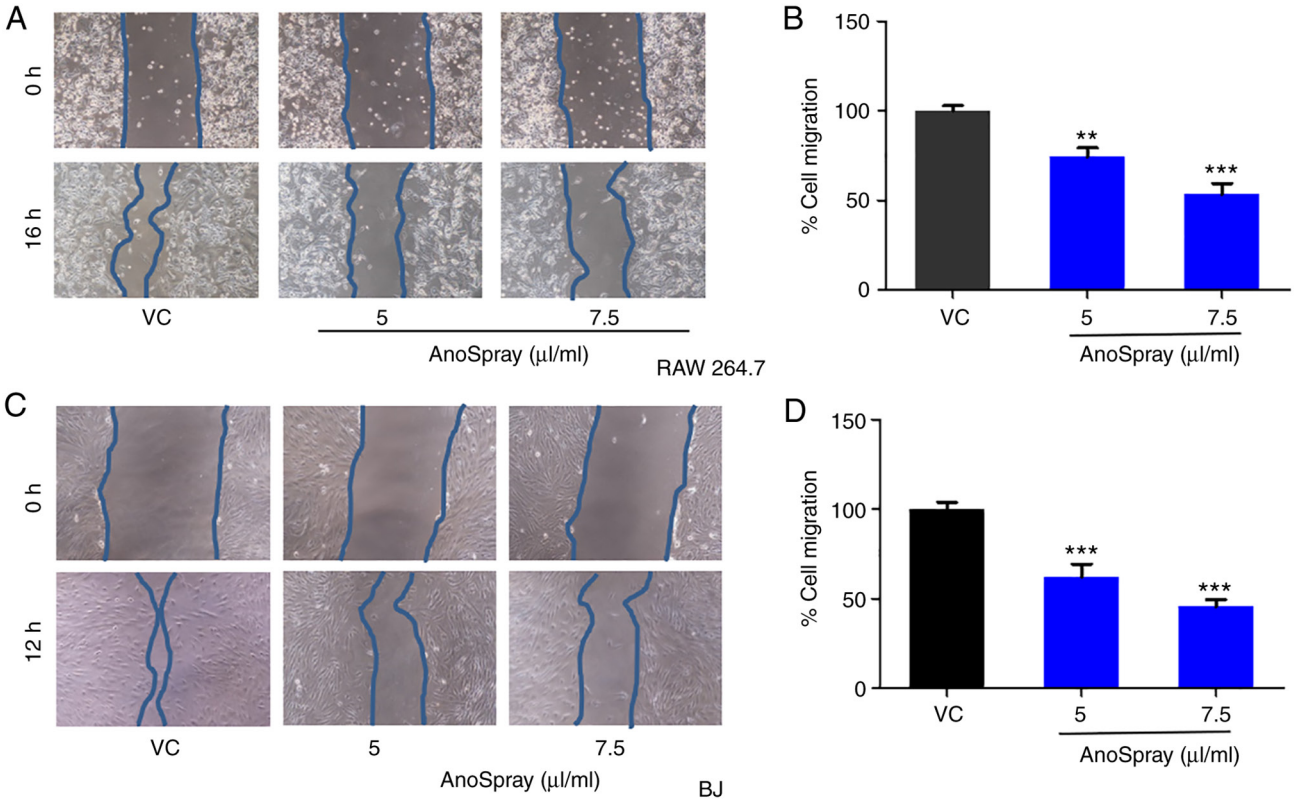


Figure 2. Effect of AnoSpray® on cell migration. RAW 264.7 and BJ cells were treated with AnoSpray® (0-7.5 $\mu$l/ml) and a wound migration assay was performed to study the effect of AnoSpray® on cell migration. Images were captured at 0 and 12/16 h. (A) Images representing the effect of AnoSpray® on the migration of RAW 264.7 cells. (B) Bar graph depicting quantitative wound migration assay results. (C) Images indicating the effect of AnoSpray® on the migration of BJ cells (magnification, x100). (D) Bar graph presenting quantitative wound migration assay results. Values are expressed as the mean ± standard error of the mean (n=3). $^{**}$P<0.01, $^{***}$P<0.001. VC, vehicle control.

were examined in both control (n=5) and AnoSpray-treated (n=5) human hemorrhoid specimens using immunohistochemical analysis. The results of these experiments indicated that RANTES and COX-2 were highly expressed in hemorrhoidal tissues (Fig. 4A and B). Furthermore, it was noted that the expression of RANTES and COX-2 was decreased in AnoSpray-treated (n=5) clinical hemorrhoid specimens (Fig. 4C and D). These results clearly indicated that AnoSpray treatment suppressed the expression of proinflammatory cytokines in hemorrhoids.

## Discussion

Hemorrhoids, anal fissures and fistulas are common benign anorectal diseases (22). These pathological ailments significantly impact the lifestyles of patients afflicted with these diseases, and primary or secondary medical care is usually required, depending on the severity of the disease. Grade I and, in certain cases, grade II hemorrhoids may be treated with dietary and lifestyle modifications or medical treatment options such as sclerotherapy. However, high-grade hemorrhoids require highly invasive surgical options (10,53), which are usually associated with postoperative pain, bleeding and fecal urgency (15,16,54). Furthermore, owing to the high recurrence rates that ensue after performing these procedures, these surgical methods only have partial success (15,16). Anal fissures have several symptoms that are similar to those of hemorrhoids, including severe pain. Anal fissures may be divided into acute and chronic categories. Acute fissures may be effectively treated with conservative therapies (55), whereas chronic fissures typically require medical management or surgical therapy (20). Invasive interventions have greater healing rates compared with the administration of local medical therapies; however, they are associated with a risk of persistent incontinence (20). Anal fistulas may be treated with operative procedures; however, recurrence and the formation of perineal wounds limit the success of operational procedures (24). In addition, the surgical management of anorectal conditions is both invasive and associated with higher costs (2,14). It was observed that delayed wound healing due to inflammation is responsible for disease-associated morbidities, including pain. Therefore, developinging novel minimally invasive and economical therapeutic interventions that exhibit inflammation and pain-suppressive activities may be beneficial for the betterment of the lives of patients with anorectal diseases.

Natural products are an important source of bioactive compounds and have been used since ancient times for the treatment of various diseases (56). Natural products with medicinal properties are also able to facilitate the wound-healing process (32). Several studies on the wound-healing activities of natural products have been performed. Herbal products with anti-inflammatory, antioxidant, antibacterial and pro-collagen synthesis properties have been indicated to elicit positive effects on wound healing. The medicinal properties of herbal products may be attributable to the presence of various bioactive phytochemical constituents, including alkaloids, oils, flavonoids,

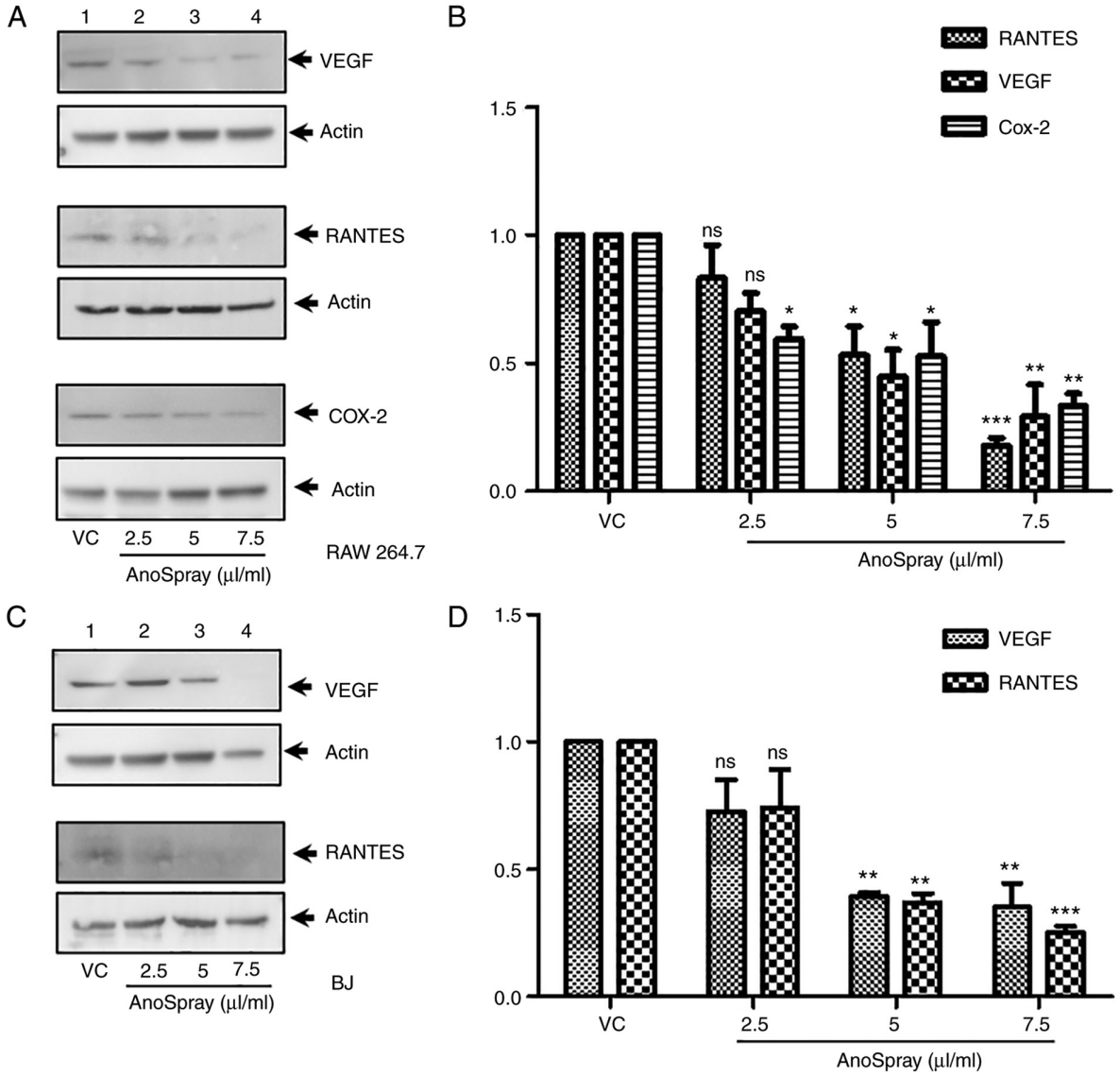


Figure 3. Effect of AnoSpray® on the expression of proangiogenic and proinflammatory factors. RAW 264.7 and BJ cells were stimulated with VC/AnoSpray® (0-7.5 $\mu$g/ml) and immunoblotting was performed to determine the expression of RANTES, VEGF and COX-2. (A) RAW 264.7 cells were treated with AnoSpray® and the expression of VEGF, RANTES and COX-2 was determined by western blot. (B) Densitometry analysis was performed to quantify western blot results for VEGF, RANTES and COX-2 expression, presented in a bar graph. (C) BJ cells were treated with AnoSpray® and the expression of VEGF and RANTES was analyzed by western blot. (D) Densitometry analysis was performed to quantify western blot data for the expression of VEGF and RANTES, presented in a bar graph. Values are expressed as the mean ± standard error of the mean (n=3). Statistical significance was determined by one-way ANOVA. $^{*}$P<0.05, $^{**}$P<0.01, $^{***}$P<0.001. ns, no significance; VC, vehicle control; RANTES, regulated upon activation, normal T cell expressed and presumably secreted; COX-2, cyclooxygenase-2.

tannins, terpenoids, saponins and phenolic compounds (57). Each bioactive agent may have a specific function in relation to the different aspects of the wound-healing process. For instance, saponins are able to augment the synthesis of pro-collagen from fibroblasts, whereas tannins and flavonoids have antiseptic and antibacterial properties, respectively (32,58,59). Hence, these phytochemicals may regulate one or more aspect(s) of the wound-healing process and these components may be easily absorbed by the outer layers of the skin (60). Owing to their anti-inflammatory, wound healing and analgesic properties, herbal products have emerged as an important therapeutic option for the treatment of numerous diseases of different severity. In addition to their biological activity, they also potentially provide important leads for the design of novel synthetic compounds (31,46). A previous study by our group reported that AnoSpray exhibits wound-healing effects on perineal wounds without causing any side effects (37). However, the mechanism underlying this healing activity on perineal wounds was not identified in that study. The present study demonstrated that AnoSpray suppresses the migration of fibroblasts and macrophages, as well as by reducing the expression levels of the proinflammatory cytokines RANTES and VEGF. COX-2 fulfills a crucial role in mitigating acute pain by regulating prostaglandin production and COX-2 inhibitors have been widely used for treating pathological pain associated with numerous diseases (61,62). In the present study, it was determined that AnoSpray reduced the expression of COX-2 in fibroblasts and macrophages. Of note, the present clinical data also indicated that the expression levels of COX-2 and RANTES were downregulated in AnoSpray-treated hemorrhoids. In addition, AnoSpray

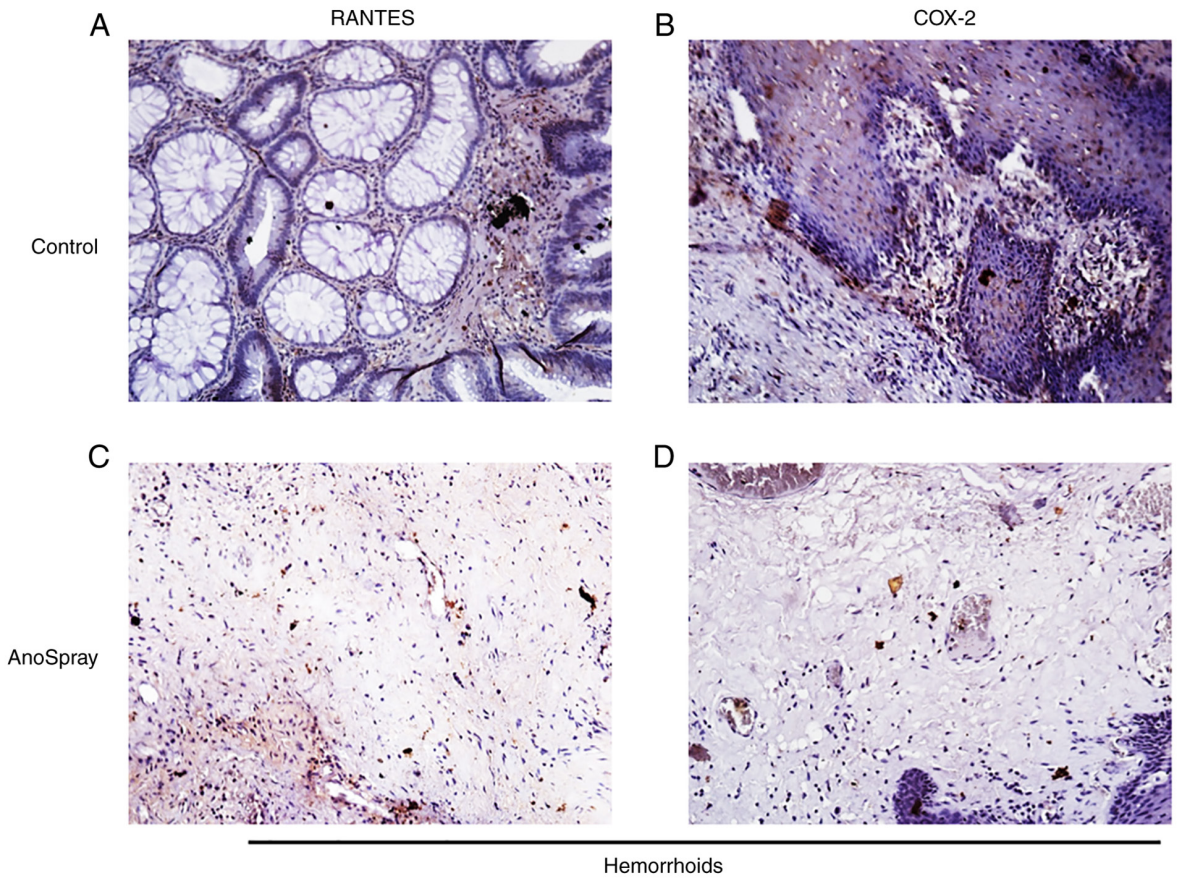


Figure 4. Expression of RANTES and COX-2 in clinical specimens of hemorrhoids. Expression of RANTES and COX-2 in hemorrhoidal disease tissues was analyzed using immunohistochemistry (n=10). Among the 10 hemorrhoidal specimens, 5 patients were untreated, whereas 5 more patients were treated with AnoSpray®. (A and B) Expression of (A) RANTES and (B) Cox-2 in hemorrhoids. (C and D) Expression of (C) RANTES and (D) COX-2 in AnoSpray®-treated hemorrhoids (magnification, x200). RANTES, regulated upon activation, normal T cell expressed and presumably secreted; COX-2, cyclooxygenase-2.

did not have any apparent effects on the viability of macrophages and fibroblasts. The major limitation of the present study was that the expression of RANTES and COX-2 in anorectal disease tissues was not compared with that in control anorectal tissues. AnoSpray is a polyherbal formulation comprising *B. aristata*, *S. racemosa*, *B. ceiba*, *S. indicum*, *G. indica*, *C. camphora* and *M. piperita* extracts. *B. aristata* and *S. racemosa* that was previously reported to exhibit antioxidant, anti-inflammatory, antiangiogenic and wound-healing properties (63,64). Another study also reported that normal and delayed wound healing was enhanced by sesamol derived from *S. indicum* in albino rats (65). As a traditional medicine, *B. ceiba* has been used in the healing of wounds, exhibiting anti-inflammatory, antioxidant and antidiabetic activities (66). An earlier study indicated that Kokum butter, which is derived from *G. indica* and has traditionally been employed for the treatment of wounds and fissures in hands, restores the elasticity of the skin, acting as a moisturizer (67). The leaves of *C. camphora* have been employed as a therapeutic option for the treatment of various skin disorders, anti-inflammatory disorders and antimicrobial diseases, in view of its antioxidant activities (68). Furthermore, an extract of *C. camphora* leaves promoted wound-healing activity in rats (68). Modarresi *et al* (69) demonstrated that the topical application of essential oil derived from *M. piperita* augmented wound healing in an infected mouse model. It is hypothetically possible that the polyherbal formulation of AnoSpray acts at different phases of inflammation, wherein pain is induced or where healing has been initiated, thereby leading to an improvement in patients' lives. The present study highlighted that AnoSpray is both safe to use and therapeutically effective in treating anal fissures, bleeding hemorrhoids and perineal wounds.

In conclusion, the present study suggested that AnoSpray does not exhibit any cytotoxic effects on macrophages or fibroblasts, thereby demonstrating that it is safe to use. Furthermore, its administration leads to a significant attenuation of the migration of these cells and also suppresses the expression of proinflammatory mediators and COX-2, both *in vitro* and in clinical specimens. The results of the present study highlighted the potential implications of AnoSpray as a means of therapy for clinically controlling bleeding hemorrhoids and in the treatment of anal fissures and perineal wounds.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

The study was conceived and designed by AP, GCK, RB and GB. Herbal materials were prepared and supplied by AP, GB and RB. Experiments were performed by RB. The data were analysed and the manuscript was written and edited by RB, AP, GB and GCK. RB, AP, GB and GCK confirmed the authenticity of the data. All authors have read and approved the final manuscript.

## Ethics approval and consent to participate

This study was approved by the Institutional Ethics Committee of Healing Hands Clinic (Pune, India). Human hemorrhoid specimens were collected from Healing Hands Clinic (Pune, India) with informed consent.

## Patient consent for publication

Not applicable.

## Competing interests

GB and RB are employees of Healing Hands & Herbs Pvt. Ltd., who provided the pharamceutical product used in this study.

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