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RESEARCH ARTICLE

Turmocin Plus Suppresses Vascular Endothelial Growth Factor (VEGF) and Macrophage Infiltration in the Management of Perineal Wounds, Anal Fistula, Acute Anal Fissures and Haemorrhoids

Ashwin Porwal[1*], Gopal Kundu[2,3,4], Gajanan Bhagwat[5], Ramakrishna Nimma[2,5] and Jayeeta Chowdhury[3,5]

[1]Healing Hands Clinic, Ground Floor, Millennium Star Extension, Pune – 411001, Maharashtra, India; drashwinporwal@gmail.com
[2]Laboratory of Tumor Biology, Angiogenesis and Nanomedicine Research, National Centre for Cell Science, Pune – 411007, Maharashtra, India
[3]School of Biotechnology, KIIT Deemed to be University, Institute of Eminence, Bhubaneswar – 751024, Odisha, India
[4]Kalinga Institute of Medical Sciences, KIIT Deemed to be University, Institute of Eminence, Bhubaneswar – 751024, Odisha, India
[5]R and D Center, Healing Hands and Herbs Private Limited, Pune – 411002, Maharashtra, India

Abstract

Anorectal problems such as anal fistula, Crohn's disease, haemorrhoids, and fissures are prevalent across the general population. Severe discomfort, inflammation, swelling, itching, and bleeding during defecation are common symptoms of anorectal disorders. Depending on the severity of the condition, several medical therapies or surgical procedures may be used to treat these diseases. Surgical treatments like fistulectomy and sphincterotomy or haemorrhoidectomy are highly intrusive and have a risk of recurrence. Furthermore, surgical procedures cause pain, inflammation, and perineal sores. These will lead to severe socio-economic ramifications in the patient's life. Therefore, treatment options that aid in the reduction of inflammation, pain, and perineal wounds are critical for anorectal disease management. Herbal formulations that comprise turmeric (*Curcuma longa*) extract have anti-inflammatory, pain-relieving, and wound-healing properties. The purpose of the current study was to elucidate the effect of Turmocin Plus on the infiltration of inflammatory cells and the expression of pro-angiogenic factors in anorectal and lower gastrointestinal disorders. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) and wound migration assays were performed to determine the results of Turmocin Plus on the viability and migration of inflammatory cells. The effect of Turmocin Plus on pro-angiogenic factors was determined using Western blot analysis and immunofluorescence. Further, we validate our *in vitro* findings in human fistula specimens using IHC. The investigation showed that Turmocin Plus inhibits immunological (RAW 264.7) cell migration while maintaining their viability. Inflammation and increased levels of Vascular Endothelial Growth Factor (VEGF) were observed in Inflammatory Bowel Disease (IBD), fistula, fissures, and higher-grade haemorrhoids. However, Turmocin Plus suppresses the VEGF expression in macrophages (RAW 264.7) cells. Furthermore, compared to untreated human fistula tissues, decreased expression of VEGF was observed in Turmocin Plus treated patient samples, validating the *in vitro* findings. Our study suggests that Turmocin Plus is a potent therapeutic formulation in treating fistula, perineal wounds, and Crohn's disease.

Keywords: Anorectal Diseases, Immune Cell Infiltration, Inflammation, Turmocin Plus, Turmopain Plus Cytotoxicity

1. Introduction

Haemorrhoids, fissures, abscesses, and fistulas are frequent anorectal diseases that disrupt the normal functioning of the anus and rectum[1]. Haemorrhoids are a frequent anorectal illness characterized by symptomatic vascular cushion expansion and/or distal displacement in the lower rectum and anal canal[2,3].

*Author for correspondence

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Internal and external haemorrhoids are distinguished by their position with respect to the dentate line. Haemorrhoids that arise above the dentate line are internal and those that originate distal to the dentate line are termed external haemorrhoids. Goligher's categorization is used to further classify internal haemorrhoids depending on their appearance and extent of prolapse. Grade I haemorrhoids can cause bleeding but not prolapse. Grade II haemorrhoids prolapse on straining or bowel moment but show a spontaneous reduction. But prolapsing in grade III haemorrhoids needed manual reduction to achieve the original state. Grade IV haemorrhoids are irreducible and permanently prolapse beyond the dentate line; these include acutely thrombosed, incarcerated haemorrhoids[4]. The symptoms of haemorrhoids involve rectal bleeding, irritability, irregular bowel movement, fecal soiling, itching, and swelling around the lower area of the anus or mucus discharge[5]. More than 50% of people on the planet will experience haemorrhoids at some point in their lives[6]. People from the 40-65 years age group, obese and pregnant women come under the risk category for incidence of haemorrhoids[7,8]. Approximately in 40% of instances, patients with haemorrhoids don't manifest any symptoms.[7] The pathophysiology of haemorrhoids is completely unclear, but a widely recognized theory is the sliding anal canal theory. It postulates the disintegration of anal cushion supportive tissue, which leads to distal displacement of the anal cushion and dilation of the venous plexus[9]. Untreated haemorrhoids lead to inflammation; the damaged tissues at the hemorrhoidal sites actuate inflammatory response along with infiltration of neutrophils, macrophages, monocytes, dendritic cells, and mast cells. Anti-inflammatory cytokines are secreted initially, followed by the secretion of proinflammatory cytokines to maintain the inflammation at the haemorrhoid surface[10-12]. Increased expression of Endoglin (CD105); an accessory receptor for TGF- β, was reported in the hemorrhoidal tissue specimens. Moreover, increased microvascular density (neovascularization) was observed in the haemorrhoidal tissues in the disease condition, especially in the presence of stromal VEGF and thrombosis[13]. Also, in another study, enhanced expression of VEGF, a key regulator protein of angiogenesis, was reported in haemorrhoids[14].

Fissures are tears in the anoderm distal to dentate lines. Fissures are arisen due to anal canal trauma[15]. Symptoms of fissures are overlapped with haemorrhoids[16]. Fissures are more common in men compared to women. Surgical intervention is required if fissures are chronic[17].

An Anal fistula is an inflammatory passage arising from the anal gland communicating between the anal canal and the perianal skin[18]. The classical anal fistula is the consequence of perianal infection and abscesses. It is a common colorectal issue occurring in both males and females but more often in males. Although it can happen at any age, the 20 to 40 age range has the highest incidence[19,20]. Fistula is classified into five types transsphincteric, intersphincteric, submucosal, supraspincteric, and extrasphincteric[19,21]. Low transphinteric and intersphincteric fistula are considered simple[21]. Submucosal fistula is the simplest fistula to treat via fistulotomy without any excision of the tract as it does not involve sphincter muscles. Inflammatory bowel disease, Crohn's disease, cancer, radiation, trauma, chronic diarrhoea, or prior incontinence are also connected to them[21,22]. Surgical interventions are a more effective treatment option for anorectal problems like higher-grade haemorrhoids, chronic fissures, and fistula. Although these procedures provide a better cure, they might result in poorly healing perineal lesions and anal incontinence[17,23]. Delayed wound healing, wound infection, wound dehiscence and bleeding, and pelvic abscesses are the most common complications associated with perineal wounds[24]. Natural therapies for perineal wounds, fistula, and other anorectal disorders, including analgesics, antimicrobial, and anti-inflammatory agents, have resurfaced in the spotlight and drew the attention of researchers owing to their efficacy and low invasive nature[25,26]. In this study, we examine the mechanical efficacy of Turmocin Plus, a phytomedicine formulation, in treating pre- and post-perineal wounds brought on by fistula, Crohn's disease, fissures, and haemorrhoids.

2. Materials and Methods

2.1 Cell Culture

The entire experiment was conducted using the mouse macrophage cell line RAW 264.7. The RAW 264.7 cells were procured from the American Type

Culture Collection. RAW 264.7 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum, 100 µg/ml streptomycin,100 units/ml penicillin and incubated in a humidified incubator with 5% CO_2 at 37°C. Overall treatments were done in the complete media.

2.2 Drug Preparation

Turmocin Plus/Turmopain Plus (Healing Hands and Herbs Pvt. Ltd. is a high potency 95% curcumin extract-based herbal formulation in the form of a tablet. The Ethyl Acetate and IPA were used as solvents for the extraction of Curcumin fractions from Turmeric Rhizome. Each Turmocin Plus/Turmopain tablet contains Turmeric Ext (*Curcuma longa*) Curcumin 95% (500 mg); Black pepper (*Piper nigrum*) (250 mg); permitted preservatives and excipients such as Methyl Paraben IP, Propyl Paraben IP, Sodium Benzoate IP, Gum Acacia IP, Talc IP, Magnesium Stearate IP and Starch IP (quantity sufficient). The Same formulation was described in a previous study[27]. The tablet was powdered and dissolved in DMSO (Dimethyl sulfoxide) to treat the cells. "Turmocin Plus"/"Turmopain Plus" are the brand names of the current herbal formulations.

2.3 Cell Viability Assay

Cell viability was assessed using the MTT assay. RAW 264.7 cells ($1x10^4$ cells/well) were seeded into 96-well microplates and treated with Turmocin Plus at concentrations of 0-25 µg/ml for 24 hours. Each well received an addition of MTT (0.5 mg/ml) solution, which was then incubated for 4 hours at 37°C. The absorbance at 570 nm was measured using a microplate reader after the formazan crystals were dissolved in isopropanol. (EPOCH2; Agilent Technologies, Inc.).

2.4 Western Blot Analysis

RAW264.7 cells ($5x10^5$ cells) were seeded in 60-mm dishes for the western blot analysis. Different concentrations of Turmocin Plus (0–25 µg/ml) were used for the treatment of cells. RIPA lysis buffer was used to collect and lysate the cells. The Bradford assay was used to determine the protein concentration of cell lysates. Total protein (30 µg/well) was resolved by 10-12.5% Sodium dodecyl-sulfate polyacrylamide gel electrophoresis. The resolved proteins were added to the PVDF (Bio-Rad Laboratories, Inc.). Then, PVDF membrane and VEGF antibody (Santa Cruz Biotechnology, Inc., cat. no. sc-7269, 1:1000 dilution) were incubated together overnight at 4°C, subsequently by HRP-conjugated secondary antibodies incubated for one hour at room temperature. 5% skimmed milk was used for blocking the PVDF membrane. The actin acts as a loading control.

2.5 Wound Migration Assay

As described, a migration assay was performed[28]. The RAW 264.7 cells ($2x10^5$) were seeded in a 12-well plate. RAW 264.7 cells were allowed to grow until they achieved cobblestone morphology. The sterile tip was used to make uniform-sized wounds. After that, cells were given treatments of either Turmocin Plus or vehicle control at a concentration of 12.5–25 µg/ml. A phase-contrast microscope (Nikon) was used to take images of the wounded area at times of t = 0 hours and t = 12 hours. Using NIS Elements BR software, the migrated area was quantified.

2.6 Immunofluorescence Studies

RAW 264.7 cells were seeded onto sterile coverslips. The RAW 264.7 cells were treated with different concentrations of Turmocin Plus (0-25µg/ml) for 24 hours. The cells were fixed in ice-cold 2% paraformaldehyde for 20 min, quenched with 0.1% glycine, and permeabilized using 0.1% Triton-X-100 for 10 min at room temperature. The cells were then blocked with 10% FBS for 1 hour at room temperature after being rinsed twice for 3 minutes each with 1X PBS. Further, cells were incubated with VEGF antibody (Santa Cruz Biotechnology, Inc., cat. no. sc-7269, 1:100 dilution) overnight at 4°C, followed by fluorescent conjugated secondary antibodies incubation (1:100 dilution) for 1 hour at room temperature. Images were captured by a confocal microscope (Leica SP5) after the coverslips were put onto slides with DAPI and a mounting medium.

2.7 Immunohistochemical Analysis of Anorectal Specimens

A histopathologist from Healing Hands Clinic (Pune, India) assisted in the collection of human anal-fistula specimens; with informed consent and approval from the Healing Hands Clinic Institutional Ethics Committee. Paraffin-embedded tissues were sectioned

onto poly-L-lysine-coated slides. The IHC was done using the Ultra Streptavidin HRP Kit (BioLegend, Cat no: 929501) as per the manufacturer's instructions. Briefly, sections were deparaffinized with xylene, followed by rehydration using gradients of ethanol. Antigen retrieval was achieved using a sodium citrate buffer (pH 6.0) at 90°C for 20 min. Endogenous peroxidase activity was depleted by treating sections with blocking reagent 1 (3% peroxide) from Ultra Streptavidin HRP Kit, followed by blocking reagent 2 to prevent non-specific binding. Sections were incubated with VEGF antibody (Santa Cruz Biotechnology, Inc., cat. no. sc-7269, 1:100 dilution). In the following step, sections were incubated with certain secondary antibodies for 1 hour at room temperature. Images were captured using a Nikon Eclipse microscope after tissue sections were immersed in a DAB substrate for 10 min.

3. Results

3.1 Effect of Turmocin Plus on Cell Viability

The effect of the Turmocin Plus on inflammatory cells' cell viability was elucidated using an MTT assay. To study this, we treated RAW264.7 cells (macrophage) with vehicle control and different concentrations of Turmocin Plus (5-25 µg/ml) for 24 hours, as described in the earlier protocol[29]. The Kruskal-Wallis test was used to statistically analyze cell viability. The findings showed that Turmocin Plus has no significant effect on the viability of RAW264.7 cells (Figure 1). Overall, our findings indicated that Turmocin Plus did not have an impact on macrophage viability.

RAW 264.7 cells were treated with vehicle control or different concentrations of Turmocin Plus (0–25 µg/ml) for 24 hours, and the effect of Turmocin Plus on cell viability was investigated using an MTT assay. The graph represents the percentage of cell viability. Values are represented as the mean ± SEM (n = 3). The statistical significance of differences between the means of the various treatment groups and the vehicle control (VC) was assessed using the Kruskal-Wallis test.

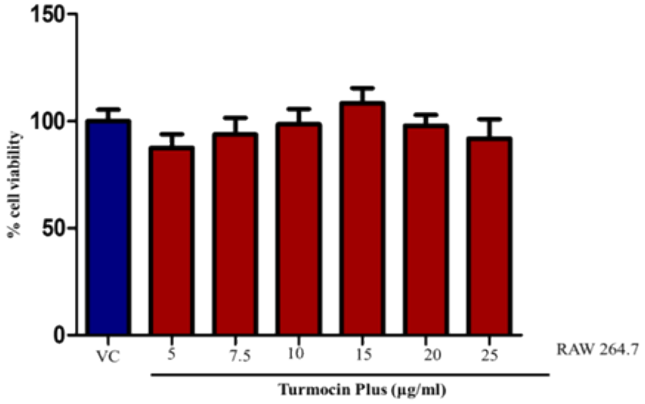

Figure 1. Effect of Turmocin Plus on cell viability.

3.2 Effect of Turmocin Plus on the Migration of Immune Cells

Fistula, perineal sores, fissures, and other anorectal conditions all have an important function for inflammation in their pathogenesis[30-32]. Different types of cells, mainly immune cells like macrophages, endothelial, and other stromal cells, infiltrate the affected site and activate inflammatory reactions and pathological vasculature[33-37]. Thus, in our study, a wound migration assay was used to examine the effect of Turmocin Plus on macrophage migration. Macrophage cells were allowed to form a monolayer, after that, wounds were created with a sterilized tip and

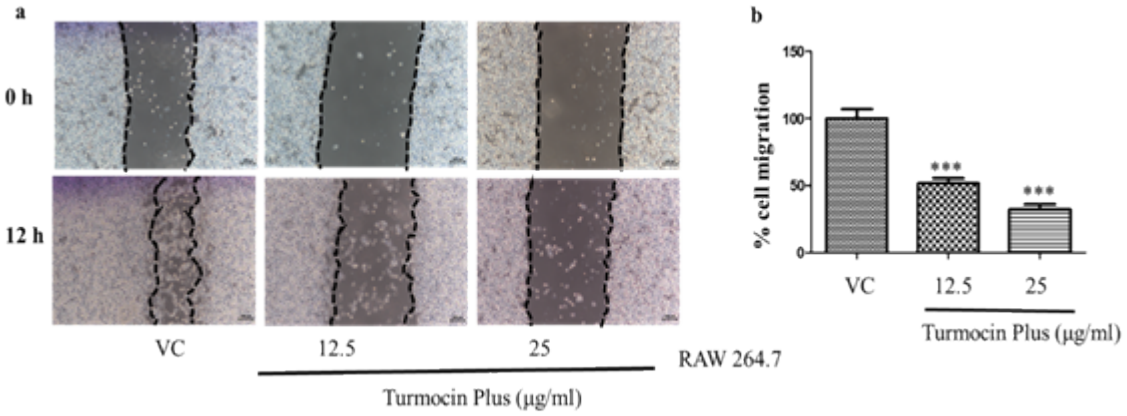

Figure 2. Effect of Turmocin Plus on the migration of macrophages.

then treated by a vehicle and different concentrations of Turmocin Plus (12.5-25 ug/ml). The RAW 264.7 cell migration was downregulated in Turmocin Plus treated condition compared to vehicle control (Figures 2a and b). The results revealed the inhibitory effect of Turmocin Plus on the migration of macrophages.

Migration of the RAW 264.7 cells treated with vehicle control or Turmocin Plus (0–25 g/ml) was determined by the wound-healing assay. Images were captured initially (at 0 hour) and after 12 hours. (a). Images represent the effect of Turmocin Plus on the migration of RAW 264.7 cells (10X magnification). (b) Bar graph depicting the quantification of wound migration assay results. Values are expressed in terms of the mean ± standard error of the mean. ***P<0.001 vs. Vehicle Control (VC).

3.3 Effect of Turmocin Plus on the Expression of Pro-angiogenic Factor VEGF

A significant part of many inflammatory diseases is angiogenesis[37]. Macrophages and other immune cells infiltrate the site of inflammation in the fistula, Crohn's disease, and haemorrhoid conditions[10,22,33,34]. Further, these cells secrete various proinflammatory cytokines and angiogenic factors, which are crucial in developing the inflammatory milieu[38-40]. The impact of Turmocin Plus on the expression of pro-angiogenic factor VEGF was assessed using western blot analysis. In contrast to cells treated with vehicle control, the results showed that Turmocin Plus-treated RAW 264.7 cells had lower levels of VEGF expression (Figure 3a). These findings were further confirmed by immunofluorescence studies (Figure 3b). These results reveal the inhibitory effect of Turmocin Plus on the expression of pro-angiogenic fraction VEGF.

(a) The RAW 264.7 cells were treated with vehicle control or Turmocin Plus (0–25 µg/ml), and the expression of VEGF was examined using Western blot analysis. (b) VEGF expression was further analyzed using immunofluorescence studies by treating RAW 264.7 cells with vehicle control or Turmocin Plus (0–25 µg/ml).

3.4 Turmocin Plus Reduces the Expression of VEGF in Patients with Fistula

To strengthen the validity of our *in vitro* studies in human clinical specimens, we evaluate the expression of pro-angiogenic factor VEGF in human fistula samples. Fistula samples from patients treated with Turmocin Plus along with untreated patient samples. We assessed VEGF expression in both Turmocin Plus treated (n = 3) and control (n = 3) specimens by immunohistochemistry analysis. The findings showed the enhanced expression of VEGF in Fistula specimens (Figure 4a). Furthermore, VEGF expression was significantly reduced in Turmocin Plus treated (n = 3) clinical fistula tissue (Figure 4b).

(a) Expression of VEGF in fistula patient specimens analyzed using immunohistochemistry (n = 3). (b) Expression of VEGF in Turmocin Plus-treated fistula

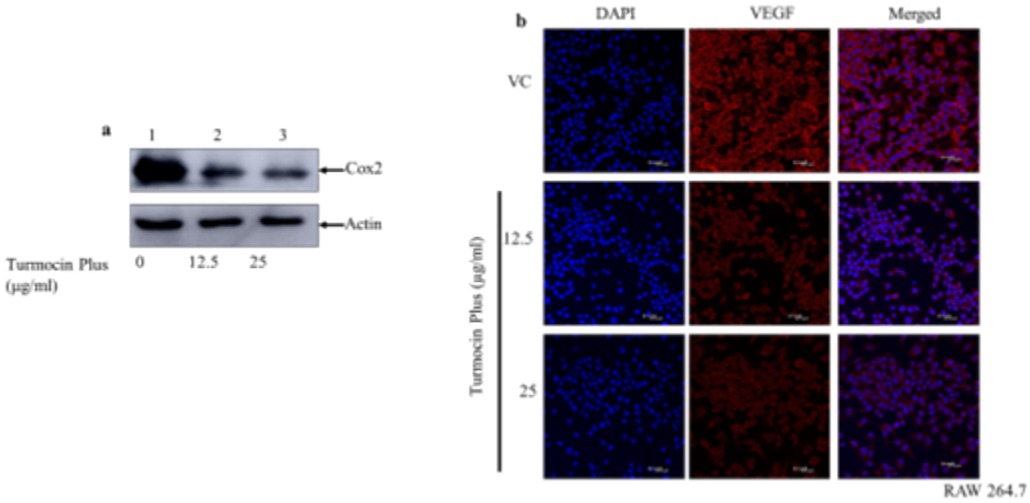

Figure 3. Effect of Turmocin Plus on the expression of the proinflammatory and angiogenic factor VEGF.

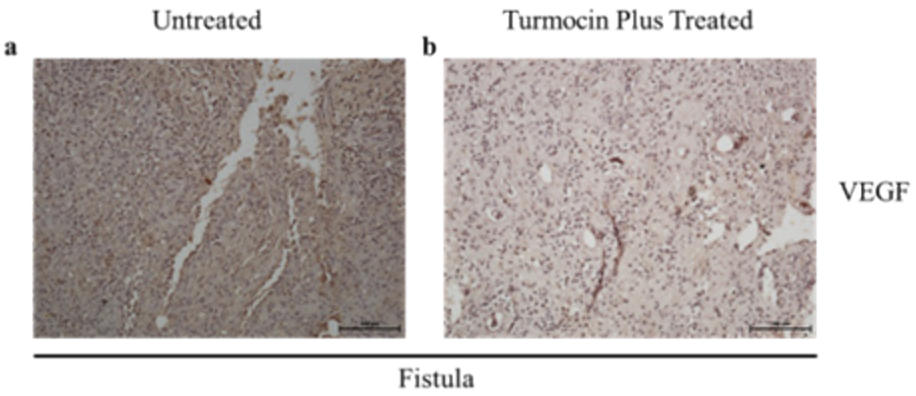

Figure 4. Impact of Turmocin Plus on VEGF expression in patients with fistula.

patient's specimen analyzed using IHC (n = 3). 20x magnification was used to take all of the pictures.

These findings validate our *in vitro* results and demonstrate that Turmocin Plus therapy decreased the pro-angiogenic factor VEGF expression in the fistula. Thus, Turmocin might inhibit the neo-vasculature to ameliorate the condition.

4. Discussion

Crohn's disease, fistula, fissures, abscesses, and haemorrhoids are common gastrointestinal and anorectal problems that cause perineal wounds[1,2,27]. Symptoms of all these diseases include pain, irritation, inflammation, and anorectal bleeding. The quality of life of patients is greatly impacted by these morbidities. Depending on the degree and severity of the condition, there are many treatment options for haemorrhoids and fistula. Conventional treatments like topical application of nitroglycerine and botulinum toxin injection are used to manage acute fissures[41-43]. Surgical interventions such as hemorrhoidectomy and lateral internal sphincterotomy were required in case of higher-grade haemorrhoids and chronic fissures[1,44,45]. An anal fistula can be treated through different surgical procedures, including regular lay-open fistulotomy. However, Post-surgical complications like perineal lesions and reoccurrence are the main limitations of these methods[1,46,47]. Prolonged inflammation hinders the wound healing process, is accountable for disease-related morbidities, and also increases the probability of wound infection[35,48,49]. Therapeutic agents that show anti-inflammatory, analgesic, and wound-healing properties are effective in treating anorectal disorders, including post-operative wound healing[50,51]. Several herbal medications have been shown to be beneficial for treating anorectal disorders and post-operational perineal wounds owing to their anti-inflammatory activity[51-53].

An earlier clinical study conducted by our group on patients who had undergone perianal surgery established the wound-healing property of a polyherbal formulation, Turmocin Plus[54]. In the current study, we showed the possible mode of action of Turmocin Plus on perineal wounds, fistula, and other anorectal and autoimmune diseases. Our results indicated that Turmocin Plus inhibits the migration of macrophages. VEGF is essential for the pathological angiogenesis-induced inflammation and infiltration of immune cells. It acts as both proinflammatory and angiogenic factors[55-57]. In many inflammatory diseases, angiogenesis and inflammation are interdependent and lead to chronic conditions[58-61]. Agents that target VEGF are frequently used to treat various inflammatory conditions like haemorrhoids and fistula[51,52]. In our current study, we established that Turmocin Plus inhibits the expression of VEGF in macrophages. Furthermore, we also observed reduced levels of VEGF in Turmocin Plus-treated patient's fistula samples. Turmocin Plus is an herbal formulation consisting of *Curcuma longa* Ext. (Curcumin 95%) and *Piper nigrum*[27]. These are conventional phytomedicines and are used to treat different disease conditions. The role of curcumin in the management of various ailments has been extensively reported in several studies. Curcumin exhibits antioxidant, anti-inflammatory, anti-cancer, anti-diabetic, wound healing, and antimicrobial activity. All these properties make it a potential

therapeutic agent for treating various diseases[62]. Earlier studies also revealed the anti-inflammatory, antioxidant, analgesic, and antimicrobial properties of *Piper nigrum*[63]. It also enhances the bioavailability of curcumin[27]. Our current findings are also persistent with the anti-inflammatory properties of these compounds. Altogether, our study demonstrated that Turmocin Plus inhibits the migration of immune cells and reduces VEGF expression, thereby aiding in managing perineal wounds, fistula, and fissures. However, a comprehensive molecular mechanism underlying the therapeutic effect of Turmocin Plus has to be further validated in a bigger size of anorectal sample sets.

5. Conclusion

Our study revealed that Turmocin Plus has no significant effect on the viability of macrophages. But it significantly slows down macrophage migration. Moreover, it inhibited the expression of the pro-angiogenic factor VEGF. Overall, our findings demonstrated that Turmocin Plus exhibits its effect on perineal wounds and fistula by attenuating the infiltration of macrophages and inhibiting pro-angiogenic and inflammatory factors, VEGF at the site of inflammation. The current findings highlighted the therapeutic role of Turmocin Plus in managing symptoms of anal fistula, fissures, higher-grade haemorrhoids, and perineal wounds both before and after surgery.

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7. References

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