

Mechanistic Evaluation of Anti-hemorrhoidal Activity of *Newbouldia laevis* (P. Beauv.) Seem. ex-Bureau Extract via Modulation of NF- κ B/Nrf2 Signaling in Experimentally Induced Hemorrhoids in Wistar Rats

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ABSTRACT

Background and Objectives: It is currently unknown which molecular mechanisms underlie the extensive use of *Newbouldia laevis* stem bark in African traditional medicine to treat hemorrhoids. This study investigated the anti-hemorrhoidal impact of *N. laevis* stem bark extract (NLSBE) via altering the nuclear factor-kappa B (NF- κ B) and nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathways in experimentally produced hemorrhoids in rats.

Materials and Methods: Wistar rats were treated with croton oil to induce hemorrhoids. The animals received NLSBE (100, 200, and 400 mg/kg, p.o.) or a standard anti-hemorrhoidal drug for 14 consecutive days. Hemorrhoidal severity, histology, oxidative stress indices, inflammatory cytokines, and the expression of genes and proteins associated with the NF- κ B/Nrf2 pathway were evaluated using biochemical assays, RT-qPCR, immunohistochemistry, and Western blotting.

Results: The 400 mg/kg group's hemorrhoid index dropped by 71.8% ($p < 0.001$) in comparison to untreated rats, suggesting that NLSBE significantly and dose-dependently decreased hemorrhoidal severity. Histological study revealed a considerable restoration of anorectal architecture with reduced edema, inflammatory cell infiltration, and vascular congestion. Reduced glutathione (4.28 ± 0.37 to $8.51 \mu\text{mol/g}$ tissue), glutathione peroxidase (27.3 ± 2.0 to 49.4 ± 3.1 U/mg protein), catalase (21.5 ± 1.8 to 43.6 ± 2.7 U/mg protein), and superoxide dismutase (18.7 ± 1.4 to 3.1 U/mg protein). NLSBE dramatically reduced the expression of NF- κ B p65, TNF- α , IL-1 β , IL-6, COX-2, and iNOS by 41–68% while increasing Nrf2, HO-1, and NQO1 by 2.1-, 2.8-, and 2.4-fold, respectively, as compared to the disease control.

Interpretation & Conclusion: NLSBE improved experimental hemorrhoids by reducing oxidative damage and promoting tissue healing by blocking NF- κ B-mediated inflammation and activating the Nrf2 antioxidant defense mechanism.

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INTRODUCTION

Hemorrhoids, one of the most common benign anorectal diseases worldwide, affect 4-5% of the general population and have a lifetime prevalence of more than 50% in people over 50 [1]. The syndrome is characterized by symptomatic expansion and distal displacement of the normal anal cushions due to deterioration of the supporting connective tissue and rising venous pressure within the hemorrhoidal plexus. Clinical signs of hemorrhoids that considerably impair patients' quality of life include rectal bleeding, pain, pruritus, prolapse, mucus discharge, and discomfort during feces [1-3]. Hemorrhoids are rarely fatal, but their high recurrence rate and chronic nature put a heavy financial burden on healthcare systems worldwide.

Anorectal microcirculation dysregulation, oxidative stress, vascular hyperplasia, chronic inflammation, and connective tissue degradation are all part of the complicated aetiology of hemorrhoids. Risk factors that increase intra-abdominal pressure include chronic constipation, prolonged straining during defecation, obesity, pregnancy, sedentary lifestyle, and insufficient dietary fiber intake. This leads to venous congestion and the progressive degradation of hemorrhoidal cushions [4,5]. Recent studies have shown that persistent inflammation and oxidative stress are important contributors to hemorrhoids. Overproduction of reactive oxygen species (ROS) disrupts the cellular redox equilibrium, leading to tissue damage in the anorectal mucosa, lipid peroxidation, endothelial dysfunction, and inflammatory cell recruitment [6]. The edema, vascular dilatation, and mucosal deterioration that are characteristic of severe hemorrhoidal disease are ultimately encouraged by these pathological processes.

The nuclear factor-kappa B (NF- κ B) signaling pathway is activated to cause and sustain hemorrhoidal inflammation at the molecular level. NF- κ B translocates pro-inflammatory genes into the nucleus in response to oxidative and inflammatory stimuli, including cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), interleukin-1 beta (IL-1 β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF- α) [6]. Long-term activation of these mediators results in tissue edema and pain, increases vascular permeability, and enhances inflammatory responses [6-8]. These inflammatory markers have been demonstrated to be expressed more frequently in hemorrhoidal tissues, highlighting NF- κ B as an

important therapeutic target for disease management.

Oxidative stress is prevented by nuclear factor erythroid 2-related factor 2 (Nrf2), a master regulator of endogenous antioxidant defense [9-12]. Nrf2 is biologically linked to Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm. The cytoprotective enzymes that are expressed when Nrf2 separates from Keap1 and translocates into the nucleus, where it binds to antioxidant response elements (AREs), include heme oxygenase-1 (HO-1), NAD(P)H quinone oxidoreductase-1 (NQO1), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and enzymes involved in glutathione synthesis [11,12]. There is mounting evidence that by reducing oxidative tissue damage and indirectly blocking NF- κ B-mediated inflammatory signaling, pharmacological activation of the Nrf2 pathway enhances tissue repair and restores vascular integrity. Because of this, therapeutic medications that can both boost Nrf2 signaling and reduce NF- κ B activation are becoming recognized as viable treatments for hemorrhoids.

Current hemorrhoid therapy methods include dietary modifications, topical corticosteroids, flavonoids, analgesics, venotonic medications, minimally invasive therapies, and surgical hemorrhoidectomy, depending on the severity of the problem [1-3]. Even though these procedures are frequently helpful, many of them are associated with adverse effects, recurrence, postoperative pain, or high treatment costs. Additionally, prolonged corticosteroid use may result in mucosal atrophy, delayed wound healing, and a higher risk of infection. Because of these limitations, medicinal plants are becoming more popular as safer and less costly ways to treat anorectal issues.

Known as the "tree of life" in many African communities, *Newbouldia laevis* (family Bignoniaceae) is a widely distributed medicinal plant that is used in traditional medicine to treat a variety of gastrointestinal disorders, ulcers, diabetes, wounds, and inflammation [6,13,14]. Its stem bark preparations have also been used in ethnomedical records to treat hemorrhoids, indicating strong anti-inflammatory and wound-healing qualities. Flavonoids, phenolic acids, tannins, alkaloids, saponins, iridoids, and naphthoquinone derivatives are among the many bioactive components found in stem bark, according to phytochemical studies. Many of these compounds have antioxidant, anti-inflammatory, analgesic, antimicrobial, and vasoprotective properties [6,15]. *Newbouldia laevis* may target several pathogenic pathways implicated in hemorrhoidal illness, according to these pharmacological characteristics.

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Although *N. laevis* extracts have been shown to have anti-inflammatory and antioxidant properties in a variety of disease types, little is known about the molecular mechanisms behind their anti-hemorrhoidal actions. Specifically, whether *N. laevis* stembark extract influences the NF- κ B/Nrf2 signaling axis during hemorrhoidal inflammation has not been thoroughly studied. Its traditional therapeutic use would be validated by clarifying these mechanisms, which would also shed light on its possible development as a phytopharmaceutical agent for anorectal illnesses.

With a focus on the regulation of NF- κ B-mediated inflammatory signaling and Nrf2-dependent antioxidant defense pathways, the current study sought to examine the anti-hemorrhoidal activity of *N. laevis* stembark extract in croton oil-induced hemorrhoids in Wistar rats. In order to clarify the mechanistic underpinnings of the extract's therapeutic actions, the study also assessed histopathological alterations, oxidative stress biomarkers, inflammatory cytokines, and the expression of important molecular mediators implicated in hemorrhoidal pathogenesis.

MATERIALS AND METHODS

Chemicals and Reagents

Croton oil, phenol, hematoxylin and eosin (H&E), TRIzol® reagent, reverse transcription and quantitative PCR kits, radioimmunoprecipitation assay (RIPA) buffer, bicinchoninic acid (BCA) protein assay kit, enhanced chemiluminescence (ECL) detection kit, and primary antibodies against NF- κ B p65, I κ B α , Nrf2, HO-1, NQO1, TNF- α , IL-1 β , IL-6, COX-2, iNOS, and β -actin were obtained from standard commercial suppliers. Assay kits for malondialdehyde (MDA), reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) were purchased from recognized manufacturers of biochemical reagents. All chemicals and solvents used were of analytical grade.

Collection and Authentication of Plant Material

Fresh stembark of *Newbouldia laevis* was collected from a natural population in Nsukka, Nigeria, in the evening hours during the rainy season. The plant was authenticated by a taxonomist, Dr. C.A. Ukwubile, in the Department of Pharmacognosy, University of Maiduguri, where a voucher specimen (Voucher number UMM/FPH/BIN/001) was deposited in the departmental herbarium for future reference.

Preparation of *Newbouldia laevis* Stembark Extract

After the gathered stembark was properly cleaned with distilled water to get rid of any debris, it was allowed to air dry for three weeks at room temperature (25–28°C) in the shade. A mechanical grinder was used to grind the dried material into a coarse powder. For 72 hours, 2.0 kg of powdered stembark was macerated in 80% ethanol (1:10 w/v) while being shaken periodically. To maximize phytochemical extraction, the marc was extracted twice under comparable conditions after the extract was filtered through Whatman No. 1 filter paper. To create a dark-brown crude extract, the mixed filtrates were concentrated under reduced pressure using a rotary evaporator at 40°C and then dried in a vacuum oven. The extract was kept at 4°C until it was needed, and the % yield was computed. Before being taken orally, fresh extract suspensions were prepared daily in 1% carboxymethyl cellulose (CMC).

Experimental Animals

Thirty adult male Wistar rats weighing 180–220 g were obtained from the Animal House Facility of the Department of Pharmacology and Toxicology, University of Maiduguri. The animals were housed in polypropylene cages under standard laboratory conditions (22 $\pm$ 2°C; relative humidity 55 $\pm$ 10%; 12 h light/12 h dark cycle) and allowed free access to standard laboratory pellets and clean drinking water. The animals were acclimatized for one week before the commencement of the experiment. All experimental procedures complied with the guidelines for the care and use of laboratory animals and were approved by the Departmental Animal Ethics Committee.

Acute Oral Toxicity Study

The acute oral toxicity of the *Newbouldia laevis* stembark extract was evaluated according to the Organization for Economic Co-operation and Development (OECD) Guideline 425. Female Wistar rats received a single oral dose of 2,000 mg/kg body weight of the extract and were observed continuously during the first 4 h and daily for 14 days for signs of toxicity, behavioral changes, morbidity, and mortality. The median lethal dose (LD₅₀) was estimated based on OECD recommendations.

Induction of Experimental Hemorrhoids

Experimental hemorrhoids were induced according to a modified croton oil method. Briefly, the rats were lightly anesthetized with ketamine (50 mg/kg) and xylazine (10 mg/kg). A cotton swab impregnated with 100 μ L of croton

oil solution (6% croton oil in a mixture of pyridine, diethyl ether, and distilled water; 4:1:5, v/v/v) was carefully inserted approximately 20 mm into the rectoanal region for 10 s. This procedure produced localized inflammation, edema, vascular congestion, and mucosal injury resembling human hemorrhoidal pathology [1,2,7].

Experimental Design

The animals were randomly divided into six groups (n = 5 per group):

- i) Group I: Normal control (vehicle only)
- ii) Group II: Hemorrhoid control (croton oil + vehicle)
- iii) Group III: Standard drug (croton oil + Daflon® 100 mg/kg/day)

iv) Group IV: Croton oil + *Newbouldia laevis* stem bark extract (100 mg/kg/day)

v) Group V: Croton oil + *Newbouldia laevis* stem bark extract (200 mg/kg/day)

vi) Group VI: Croton oil + *Newbouldia laevis* stem bark extract (400 mg/kg/day)

Treatments were administered orally once daily for 14 consecutive days.

Evaluation of Hemorrhoidal Severity

At the end of treatment, animals were euthanized, and the distal rectoanal tissues were excised. The severity of hemorrhoids was assessed by determining the rectoanal coefficient:

$$\text{Rectoanal coefficient} = \frac{\text{Weight of rectoanal tissue (mg)}}{\text{Body weight (mg)}} \quad (1)$$

Macroscopic observations, including edema, erythema, congestion, ulceration, and bleeding, were recorded [3,4,7].

Histopathological examination

Rectoanal tissues were fixed in 10% neutral-buffered formalin for 24 h, dehydrated through graded ethanol, cleared in xylene, embedded in paraffin wax, sectioned at 5 µm thickness, and stained with hematoxylin and eosin (H&E). Tissue sections were examined under a light microscope for epithelial integrity, inflammatory cell infiltration, edema, vascular congestion, and mucosal architecture [7].

Determination of Oxidative Stress Biomarkers

Fresh rectal tissues were homogenized in ice-cold phosphate-buffered saline (PBS, pH 7.4) and centrifuged at 10,000 × g for 15 min at 4°C. The supernatants were used for biochemical analyses. The following parameters were determined according to the manufacturers' instructions [16,17].

- Malondialdehyde (MDA)
- Reduced glutathione (GSH)
- Superoxide dismutase (SOD)
- Catalase (CAT)
- Glutathione peroxidase (GPx)

Protein concentrations were determined using the BCA assay [18,19].

RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted from rectoanal tissues using TRIzol® reagent according to the manufacturer's protocol. RNA purity and concentration were assessed spectrophotometrically. Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using a reverse transcription kit. Quantitative PCR was performed using SYBR Green Master Mix on a real-time PCR system. Gene expression levels of NF-κB p65, TNF-α, IL-1β, IL-6, COX-2, iNOS, Nrf2, HO-1, and NQO1 were determined. β-Actin served as the housekeeping gene. Relative gene expression was calculated using the 2^{−ΔΔCt} method [20,21].

Western Blot Analysis

Rectoanal tissues were homogenized in RIPA lysis buffer containing protease and phosphatase inhibitors. Protein concentrations were quantified using the BCA assay. Equal amounts of protein (30–40 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% non-fat milk and incubated overnight at 4°C with primary antibodies against NF-κB p65, IκBα, Nrf2, HO-1, NQO1, COX-2, iNOS, and β-actin. Following incubation with horseradish peroxidase-conjugated secondary antibodies, protein bands were visualized using enhanced chemiluminescence and quantified by densitometry [22–24].

Immunohistochemistry

Paraffin-embedded tissue sections were deparaffinized, rehydrated, and subjected to antigen retrieval. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide before blocking with normal serum. The sections were incubated overnight with primary antibodies against NF- κ B p65, Nrf2, and COX-2, followed by incubation with biotinylated secondary antibodies and streptavidin-horseradish peroxidase. Immunoreactivity was visualized using 3,3'-diaminobenzidine (DAB), counterstained with hematoxylin, and examined under a light microscope [25-27]

Ethical Approval

All experimental procedures involving animals were conducted in accordance with the principles outlined in the Guide for the Care and Use of Laboratory Animals published by the National Research Council (8th edition, 2011) and complied with the guidelines of the National Institutes of Health for the care and use of laboratory animals. The study protocol was reviewed and approved by the Institutional Animal Ethics Committee (AREC) of the Department of Pharmacology and Toxicology, University of Maiduguri, Nigeria (Approval No.: FP/03/26/20/11/01/063), before the commencement of the experiment. All efforts were made to minimize animal suffering and reduce the number of animals used. Animals were monitored daily throughout the study for signs of pain or distress, and all invasive procedures were performed under ketamine-xylazine anesthesia. At the end of the experiment, the animals were humanely euthanized under deep anesthesia before tissue collection.

Statistical Analysis

Data were expressed as mean $\pm$ SD ($n = 3$ replicates). Statistical analyses were performed using GraphPad Prism version 10.0 (GraphPad Software, San Diego, CA, USA). Differences among groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc test. Values of $p < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Acute Oral Toxicity of *Newbouldia laevis* Stembark Extract

Over the course of the 14-day observation period, a single oral dosage of *Newbouldia laevis* stembark extract at a limit dose of 2,000 mg/kg body weight did not result in any deaths or treatment-related symptoms of toxicity. The treated rats

continued to be active and showed typical respiration, feed and water consumption, grooming behavior, exploratory activity, and locomotor performance. During the first four hours following the dose or the ensuing observation period, no unusual clinical symptoms, such as tremors, convulsions, salivation, piloerection, diarrhea, lethargy, respiratory distress, or coma, were noted. Additionally, there was no discernible difference in body weight gain between day 0 (184.6 ± 5.8 g) and day 14 (205.8 ± 6.3 g) ($p > 0.05$), suggesting normal growth and no systemic toxicity (Table 1). Major organs such as the liver, kidneys, heart, lungs, and spleen showed no obvious abnormal changes during the experiment's gross necropsy. The oral median lethal dose (LD_{50}) of *Newbouldia laevis* stembark extract was calculated to be greater than 2,000 mg/kg body weight based on the lack of mortality at the limit dose, indicating that the extract has a low acute oral toxicity profile in accordance with the OECD Guideline 425 classification. The findings indicate that *N. laevis* stembark extract is relatively safe following acute oral administration and supports its traditional use as a medicinal plant. According to the Globally Harmonized System (GHS) for the classification of chemicals, substances with an oral LD_{50} exceeding 2,000 mg/kg are generally considered to have low acute toxicity. The absence of behavioral abnormalities and mortality further suggests that the phytochemical constituents present in the stembark extract do not produce immediate neurotoxic or systemic adverse effects at the tested dose.

The observed safety profile may be attributed to naturally occurring antioxidant phytochemicals, including flavonoids, phenolic compounds, and iridoids, previously reported in *N. laevis*. These metabolites have been associated with favorable toxicological profiles and have demonstrated minimal acute toxicity in experimental animal models. Similar findings have been reported for extracts of *N. laevis* leaves and stembark, where oral administration at doses up to 2,000–5,000 mg/kg produced no mortality or significant toxic manifestations, supporting the broad margin of safety of the plant [6,15]. Determining the extract's acute safety is a necessary precondition for assessing its pharmacological effectiveness in experimental hemorrhoids and supports the choice of the therapeutic dosages of 100, 200, and 400 mg/kg employed in this investigation. Overall, these findings show that the stembark extract from *N. laevis* has a good acute safety profile, which supports its continued development as a plant-derived medicinal agent for the treatment of inflammatory anorectal diseases.

Table 1. Acute oral toxicity assessment of *Newbouldia laevis* stembark extract (2,000 mg/kg, OECD Guideline 425)

Parameter	Observation
Experimental animals	Female Wistar rats (n = 5)
Dose administered	2,000 mg/kg body weight (single oral dose)
Observation period	14 days
Mortality	0/5
Estimated oral LD ₅₀	>2,000 mg/kg body weight
General appearance	Normal
Skin and fur	No abnormalities observed
Eyes and mucous membranes	Normal
Respiration	Normal
Salivation	Absent
Tremors	Absent
Convulsions	Absent
Piloerection	Absent
Diarrhea	Absent
Lethargy	Absent
Grooming behavior	Normal
Locomotor activity	Normal
Food intake	Normal
Water intake	Normal
Initial body weight (g)	184.6 ± 5.8
Final body weight (g)	205.8 ± 6.3
Body weight gain (g)	21.2 ± 3.7
Relative body weight gain (%)	11.5 ± 1.9
Gross pathological findings	No visible abnormalities in the liver, kidneys, heart, lungs, or spleen

Values are presented as mean ± SE (n = 5). No mortality or treatment-related signs of toxicity were observed during the 14-day observation period. The oral LD₅₀ of *Newbouldia laevis* stembark extract was estimated to be >2,000 mg/kg body weight in accordance with OECD Guideline 425.

Effect of *N. laevis* Stembark Extract on Hemorrhoidal Severity

In the hemorrhoid control group (Group II), the production of hemorrhoids with croton oil resulted in significant anorectal inflammation, indicated by edema, erythema, vascular congestion, ulceration, and sporadic bleeding. Rats given *Newbouldia laevis* stembark extract, on the other hand, showed dose-dependent reduction of these clinical symptoms. Similar to animals treated with the reference medication, Daflon® (100 mg/kg), animals receiving the maximum dose (400 mg/kg) displayed almost normal anorectal appearance with little swelling and congestion. The successful induction of hemorrhoids was confirmed by the rectoanal coefficient, a commonly used measure of hemorrhoidal edema and inflammatory tissue enlargement, which increased significantly in the hemorrhoid control group (2.83 ± 0.14 mg/g) compared to the

normal control group (1.31 ± 0.08 mg/g, $p < 0.001$) (Table 2). In comparison to the hemorrhoid control, oral administration of *Newbouldia laevis* stembark extract significantly decreased the rectoanal coefficient in a dose-dependent manner to 2.28 ± 0.11, 1.86 ± 0.09, and 1.55 ± 0.07 mg/g at doses of 100, 200, and 400 mg/kg, representing reductions of 19.4%, 34.3%, and 45.2%, respectively (all $p < 0.05$). The rectoanal coefficient decreased to 1.47 ± 0.06 mg/g, or 48.1%, after treatment with Daflon®.

These quantitative results were corroborated by macroscopic analysis. Severe circumferential edema, strong erythema, prominent vascular engorgement, mucosal ulceration, and recent rectal bleeding were all present in the hemorrhoid control animals. When *Newbouldia laevis* stembark extract was administered, these severe pathological alterations gradually

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subsided. While the 200 mg/kg dose significantly improved mucosal integrity and decreased vascular congestion, the 100 mg/kg dose resulted in minor decreases in edema and erythema. A few animals showed only slight residual congestion after the maximum dose (400 mg/kg) had entirely restored normal anorectal shape. The total gross improvement was similar to what Daflon® produced, indicating significant anti-hemorrhoidal effectiveness.

After receiving therapy with *Newbouldia laevis* stembark extract, the rectoanal coefficient decreased, indicating a reduction in the vascular swelling and inflammatory edema linked to hemorrhoidal illness. By activating the phospholipase A₂ and protein kinase C pathways, croton oil causes acute anorectal inflammation [1,4,7,28]. This leads to an overabundance of prostaglandins, leukotrienes, reactive oxygen species (ROS), and pro-inflammatory cytokines that increase tissue edema and vascular permeability [4]. As a result, in experimental hemorrhoids, the rectoanal coefficient is regarded as a trustworthy measure of the severity of the condition and the effectiveness of treatment. The current results are in line with earlier research showing that medicinal plants with antioxidant and anti-inflammatory qualities greatly lessen the severity of hemorrhoids in animal models. For instance, flavonoid-rich formulations like Daflon® have been demonstrated to improve venous tone, decrease capillary hyperpermeability, and inhibit leukocyte infiltration into inflamed hemorrhoidal tissue, while oral administration of *Aesculus hippocastanum* seed extract reduced anorectal edema and vascular congestion through venotonic and anti-inflammatory mechanisms [29-31]. Similarly, through antioxidant and vascular protective properties, polyphenol-rich plant extracts from *Myrtus communis* have

been shown to reduce rectoanal edema and speed mucosal healing [32].

The various phytochemical components of *Newbouldia laevis* stembark extract, such as flavonoids, tannins, phenolic acids, iridoids, and naphthoquinones, are probably responsible for the anti-hemorrhoidal activity seen [6,13,14]. While tannins have local astringent actions that lessen edema and encourage the contraction of inflammatory tissues, flavonoids maintain vascular endothelial cells by decreasing capillary permeability and preventing leukocyte adherence [33-35]. Additionally, phenolic substances inhibit lipid peroxidation and protect against endothelial damage by scavenging ROS produced during inflammation caused by croton oil. Together, these complementary pharmaceutical effects help to restore normal anorectal architecture, lessen vascular congestion, and diminish inflammatory exudation. The 400 mg/kg dose produced effects that were strikingly similar to those of Daflon®, and the therapeutic response showed a distinct dose-dependent pattern. This result implies that the inflammatory cascade and vascular dysfunction underlying hemorrhoidal illness may be more effectively modulated at larger concentrations of the bioactive ingredients. The potential of *Newbouldia laevis* stembark extract to aid tissue repair is further supported by the observed improvement in gross morphology; this effect is further investigated by histological and molecular investigations in the sections that follow.

All of these findings confirm the traditional use of *Newbouldia laevis* stembark extract in the treatment of anorectal diseases and offer compelling evidence that it successfully reduces the clinical signs of experimental hemorrhoids.

Table 2. Effect of *N. laevis* stembark extract on rectoanal coefficient and macroscopic hemorrhoidal severity in croton oil-induced hemorrhoids

Group	Treatment	Rectoanal coefficient (mg/g)	Reduction vs. hemorrhoid control (%)	Gross pathological findings
I	Normal control	1.31 ± 0.08	—	Normal anorectal mucosa
II	Hemorrhoid control	2.83 ± 0.14 ^a	—	Severe edema, erythema, vascular congestion, ulceration, and bleeding
III	Daflon® (100 mg/kg)	1.47 ± 0.06 ^b	48.1	Minimal edema and congestion; normal mucosal appearance
IV	<i>N. laevis</i> stembark extract (100 mg/kg)	2.28 ± 0.11 ^b	19.4	Moderate edema and erythema; mild congestion
V	<i>N. laevis</i> stembark extract (200 mg/kg)	1.86 ± 0.09 ^c	34.3	Mild edema; reduced vascular congestion; improved mucosa
VI	<i>N. laevis</i> stembark extract (400 mg/kg)	1.55 ± 0.07 ^c	45.2	Near-normal anorectal morphology with minimal residual congestion

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Values are presented as mean $\pm$ SE (n = 5). ^ap < 0.001 versus normal control. ^bp < 0.05 versus hemorrhoid control. ^cp < 0.001 versus hemorrhoid control (one-way ANOVA followed by Tukey's multiple comparison test).

Effect of *N. laevis* Stembark Extract on Histopathological Alterations in Croton Oil-Induced Hemorrhoids

Figure 1 shows photomicrographs of rectoanal tissues stained with hematoxylin and eosin (H&E). The normal control group's histopathological analysis (Figure 1A) showed intact anorectal mucosal architecture with a continuous epithelial lining, well-organized mucosal glands, normal connective tissue, and no edema, vascular congestion, or inflammatory cell infiltration. These results verified that the rectoanal tissues in healthy rats had normal histological integrity. The hemorrhoid control group, on the other hand, showed significant pathological changes that were consistent with anorectal inflammation brought on by croton oil (Figure 1B). Extensive epithelial erosion and ulceration, significant submucosal edema, dense infiltration of inflammatory cells, primarily neutrophils and mononuclear leukocytes, severe vascular congestion, and distortion of the mucosal architecture were all observed in the rectoanal tissues. These histological lesions validate the successful formation of the experimental hemorrhoid model and support the gross pathological findings.

These pathological alterations were significantly reduced by treatment with Daflon® (100 mg/kg) (Figure 1C). With little inflammatory cell infiltration, decreased edema, and the restoration of normal vascular architecture, the rectoanal mucosa showed almost total epithelial regeneration. These results align with the well-established venotonic and anti-inflammatory characteristics of micronized pure flavonoid fractions that are employed in clinical settings to treat hemorrhoidal illness. Rectoanal histopathology improved in a dose-dependent manner when *Newbouldia laevis* stembark extract was administered. When compared to the untreated hemorrhoid group, rats given 100 mg/kg (Figure 1D) showed minor submucosal edema, decreased inflammatory infiltration, and moderate preservation of epithelial integrity. Significant mucosal architectural repair was seen at 200 mg/kg (Figure 1E), as evidenced by significantly less vascular congestion, fewer inflammatory cells, and better glandular organization. With intact epithelial lining, well-preserved mucosal glands, few inflammatory infiltrates, and no appreciable edema or vascular congestion, the 400 mg/kg therapy group (Figure 1F) showed nearly total normalization of rectoanal histology. This group's histological appearance was similar to that of the animals treated with Daflon®.

The significant histological improvement brought about by *Newbouldia laevis* stembark extract implies that inflammatory tissue damage is effectively suppressed and mucosal healing is improved. By activating phospholipase A₂ and releasing arachidonic acid metabolites, croton oil causes anorectal inflammation. This leads to an overabundance of prostaglandins, leukotrienes, reactive oxygen species, and inflammatory cytokines [34,36,37]. Epithelial ulceration and edema result from these mediators' promotion of endothelial damage, leukocyte recruitment, vascular leakage, and tissue degradation [4]. Thus, a decrease of inflammatory infiltrates and maintenance of epithelial integrity are regarded as trustworthy markers of anti-hemorrhoidal effectiveness. The current results are consistent with other studies showing that medicinal herbs high in flavonoids considerably improve hemorrhoidal histology by lowering vascular congestion and inflammatory cell infiltration. Anorectal mucosal architecture has been restored after oxidative stress and inflammatory responses have been suppressed, according to experimental research on *Aesculus hippocastanum* [29-31]. Similarly, it has been observed that Daflon®, the standard comparator in this investigation, reduces capillary permeability, enhances venous tone, and prevents leukocyte adhesion, all of which speed up tissue recovery in hemorrhoidal lesions.

The many flavonoids, phenolic chemicals, tannins, iridoids, and naphthoquinone derivatives found in *Newbouldia laevis* stembark extract are probably responsible for its defensive properties. While tannins have local astringent actions that lessen tissue edema and capillary permeability [29], flavonoids have strong anti-inflammatory and antioxidant properties that strengthen vascular endothelium and prevent neutrophil migration [38,39]. Furthermore, phenolic compounds efficiently scavenge reactive oxygen species, preventing lipid peroxidation and maintaining the integrity of cellular membranes [40-42]. The extraordinary histological healing seen after therapy with the extract may be explained by these complementary pharmacological activities taken together. Additionally, the molecular findings presented in the next sections are strongly supported by the dose-dependent restoration of normal anorectal architecture, especially the activation of the Nrf2 antioxidant defense pathway and the suppression of NF- κ B-mediated inflammatory signaling. The decrease in inflammatory damage, improvement in tissue regeneration,

and general improvement in hemorrhoidal disease shown in the extract-treated groups are probably due to these mechanisms taken together.

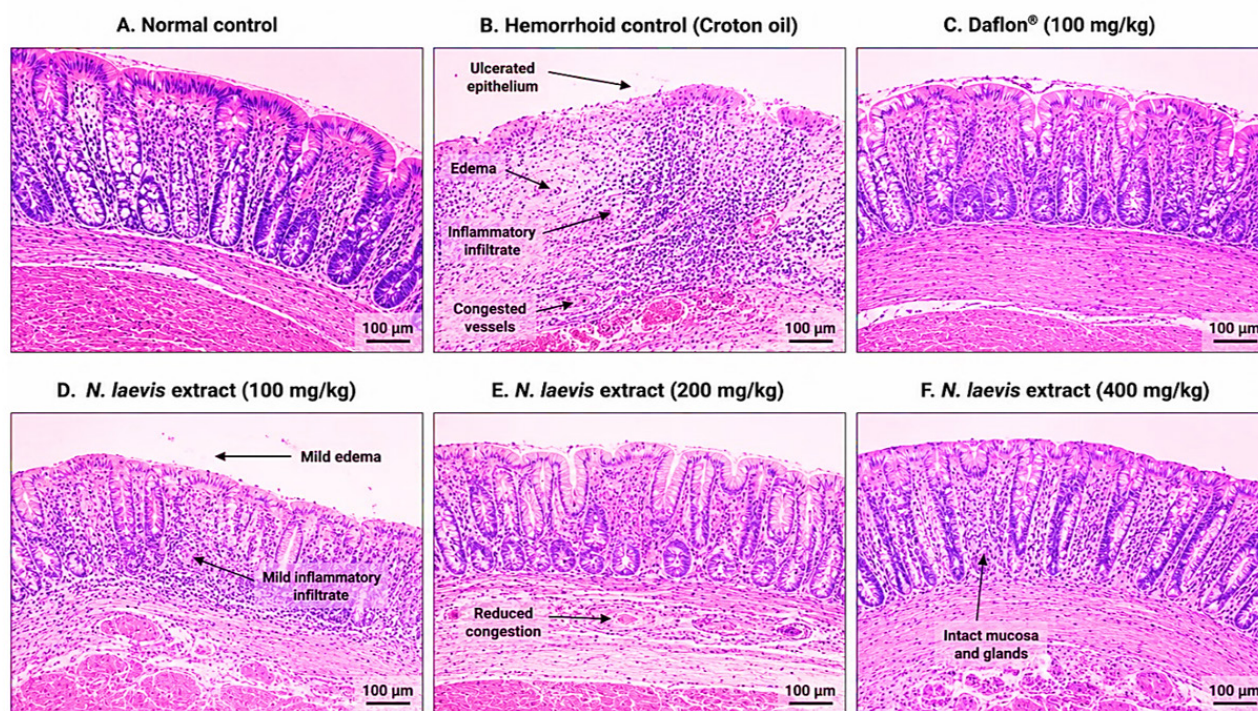


Figure 1. Histopathological effects of *Newbouldia laevis* stem bark extract on rectoanal tissues in croton oil-induced hemorrhoids. Hematoxylin and eosin (H&E)-stained sections (5 µm; original magnification ×200; scale bar = 100 µm) of rectoanal tissues from experimental rats. **(A)** Normal control showing intact epithelial lining, well-organized mucosal glands, and normal tissue architecture. **(B)** Hemorrhoid control demonstrating epithelial ulceration, severe inflammatory cell infiltration, submucosal edema, and vascular congestion. **(C)** Daflon® (100 mg/kg) shows marked restoration of mucosal architecture with minimal inflammatory infiltration. **(D)** *Newbouldia laevis* stem bark extract (100 mg/kg) showed a moderate reduction in edema and inflammatory cell infiltration. **(E)** *Newbouldia laevis* stem bark extract (200 mg/kg) showing improved epithelial integrity and reduced vascular congestion. **(F)** *Newbouldia laevis* stem bark extract (400 mg/kg) shows near-complete restoration of normal rectoanal histoarchitecture with minimal inflammatory changes.

Effect of *N. laevis* stem bark Extract on Oxidative Stress Biomarkers in Croton Oil-Induced Hemorrhoids

Through the overproduction of reactive oxygen species (ROS), which encourage lipid peroxidation, endothelial dysfunction, inflammatory cell recruitment, and tissue damage, oxidative stress plays a key role in the development and progression of hemorrhoidal illness. After 14 days of therapy, oxidative stress indicators were measured in rectoanal tissues to determine if the anti-hemorrhoidal efficacy of *Newbouldia laevis* stem bark extract is linked to the reduction of oxidative damage. Tissue redox homeostasis was severely disrupted when hemorrhoids were induced with croton oil, as Table 3 illustrates. Malondialdehyde (MDA), an indication of lipid peroxidation, increased significantly in the hemorrhoid control group from 2.41 ± 0.18 to 8.41 ± 0.52 nmol/mg protein ($p < 0.001$). On the other hand,

endogenous antioxidant enzyme activity was significantly decreased. In comparison to normal animals, the levels of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and reduced glutathione (GSH) dropped by 48.3%, 45.6%, 42.7%, and 52.6%, respectively, indicating significant oxidative stress inside the inflamed anorectal tissues.

Antioxidant defense was considerably and dose-dependently restored by oral administration of *Newbouldia laevis* stem bark extract. MDA levels decreased to 6.72 ± 0.43 , 4.81 ± 0.34 , and 3.18 ± 0.29 nmol/mg protein at dosages of 100, 200, and 400 mg/kg, corresponding to decreases of 20.1%, 42.8%, and 62.2%, respectively, when compared to the normal control ($p < 0.05$ – 0.001). The maximum dose resulted in an MDA concentration (2.94 ± 0.24 nmol/mg protein) that was statistically similar to the Daflon® group. Similarly, SOD, CAT, GPx, and GSH

activities were markedly elevated by treatment with *Newbouldia laevis* stem bark extract. At 400 mg/kg, SOD rose from 18.7 ± 1.4 to 35.9 ± 2.1 U/mg protein, CAT from 21.5 ± 1.8 to 43.6 ± 2.7 U/mg protein, GPx from 27.3 ± 2.0 to 49.4 ± 3.1 U/mg protein, and GSH from 4.28 ± 0.37 to 8.92 ± 0.51 mol/g tissue ($p < 0.001$ vs hemorrhoid control). These data showed similar antioxidant efficacy to that seen after Daflon® therapy.

The current results unequivocally show that *Newbouldia laevis* stem bark extract successfully restores redox equilibrium and that oxidative stress is a significant pathogenic consequence of hemorrhoids caused by croton oil. Phospholipase A₂ and protein kinase C are known to be activated by croton oil, which results in an excessive production of reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide, and hydroxyl radicals [5,7,28]. This reactive species cause membrane lipid peroxidation, which raises MDA levels and weakens intracellular antioxidant defenses. Therefore, severe oxidative damage and reduced antioxidant capacity are indicated by the observed rise in MDA and concurrent decrease in SOD, CAT, GPx, and GSH in untreated hemorrhoidal tissues. After *Newbouldia laevis* stem bark extract is administered, antioxidant enzyme activities are restored, indicating that the extract effectively neutralizes ROS and strengthens endogenous antioxidant defenses. Superoxide radicals are dismutated into hydrogen peroxide by SOD, and hydrogen peroxide is then detoxified into water by CAT and GPx to stop the production of hydroxyl radicals [17,43-45]. GSH is a key intracellular antioxidant and a necessary substrate for detoxification processes mediated by GPx [46]. The concurrent increase in these antioxidant indicators suggests that cellular antioxidant capability and defense against oxidative tissue damage are fully reinforced.

These results are in line with earlier research showing *Newbouldia laevis* extracts' strong antioxidant action in experimen-

tal inflammatory diseases. In models of hepatic damage, gastric ulcers, and inflammatory disorders, extracts of the stem bark and leaves have been shown to considerably lower lipid peroxidation while raising SOD, CAT, GPx, and GSH activities. Similar antioxidant effects have also been reported for flavonoid-rich medicinal plants used in hemorrhoidal therapy, such as *Aesculus hippocastanum*, where improved vascular integrity and faster mucosal healing were linked to the restoration of endogenous antioxidant defenses [29,31]. The phytochemical makeup of *Newbouldia laevis* stem bark is probably responsible for the antioxidant activity seen in this investigation. The stem bark contains a large number of flavonoids, phenolic acids, tannins, iridoids, and naphthoquinone derivatives, according to earlier phytochemical studies [6,14]. These substances chelate transition metals, prevent lipid peroxidation, maintain endogenous antioxidant enzymes, and have potent free radical-scavenging abilities [47,48]. It has been demonstrated that flavonoids in particular activate the Nrf2 signaling pathway, leading to the transcriptional overexpression of antioxidant enzymes such as HO-1, NQO1, SOD, CAT, and GPx [49]. As a result, the current study's observation of tissue antioxidant status restoration offers biochemical proof that the Nrf2-dependent antioxidant defense system has been activated.

Furthermore, one of the main ways that *Newbouldia laevis* stem bark extract reduces hemorrhoidal disease may be through the attenuation of oxidative stress. Reduced oxidative damage promotes mucosal repair and anorectal architecture preservation by limiting endothelial dysfunction, vascular leakage, inflammatory cell recruitment, and tissue edema. These biochemical results confirm the following molecular data showing activation of Nrf2 signaling and reduction of NF-κB-mediated inflammatory responses, and they are consistent with the histopathological improvements shown in the extract-treated groups.

Table 3: Effect of *Newbouldia laevis* stem bark extract on oxidative stress biomarkers in rectoanal tissues of croton oil-induced hemorrhoidal rats

Group	MDA (nmol/mg protein)	GSH (μmol/g tissue)	SOD (U/mg protein)	CAT (U/mg protein)	GPx (U/mg protein)
Normal control	2.41 ± 0.18	9.04 ± 0.48	36.2 ± 2.0	45.1 ± 2.6	50.8 ± 3.2
Hemorrhoid control	8.41 ± 0.52^a	4.28 ± 0.37^a	18.7 ± 1.4^a	21.5 ± 1.8^a	27.3 ± 2.0^a
Daflon® (100 mg/kg)	2.94 ± 0.24^c	8.71 ± 0.46^c	37.8 ± 2.3^c	44.8 ± 2.5^c	51.2 ± 3.0^c
<i>N. laevis</i> stem bark extract (100 mg/kg)	6.72 ± 0.43^b	5.82 ± 0.41^b	24.9 ± 1.6^b	28.6 ± 2.1^b	33.8 ± 2.4^b
<i>N. laevis</i> stem bark extract (200 mg/kg)	4.81 ± 0.34^c	7.31 ± 0.44^c	30.7 ± 1.8^c	36.5 ± 2.3^c	42.6 ± 2.8^c
<i>N. laevis</i> stem bark extract (400 mg/kg)	3.18 ± 0.29^c	8.92 ± 0.51^c	35.9 ± 2.1^c	43.6 ± 2.7^c	49.4 ± 3.1^c

Citation: Ukwubile CA, Makinde AO. (2026). Mechanistic Evaluation of Anti-hemorrhoidal Activity of *Newbouldia laevis* (P. Beauv.) Seem. ex-Bureau Extract via Modulation of NF-κB/Nrf2 Signaling in Experimentally Induced Hemorrhoids in Wistar Rats. *Pharmacogn.* 4(1):7.

Values are expressed as mean $\pm$ SE (n = 5). ^ap < 0.001 versus normal control. ^bp < 0.05 versus hemorrhoid control. ^cp < 0.001 versus hemorrhoid control (one-way ANOVA followed by Tukey's multiple comparison test).

Effect of *N. laevis* Stembark Extract on The mRNA Expression of NF-Kb/Nrf2 Signaling-Related Genes

Quantitative real-time PCR was used to measure the relative mRNA expression of important inflammatory and antioxidant genes in rectoanal tissues to clarify the molecular mechanisms behind the anti-hemorrhoidal effect of *Newbouldia laevis* stembark extract. Figure 2 displays the expression profiles of NF- κ B p65, TNF- α , IL-1 β , IL-6, COX-2, iNOS, Nrf2, HO-1, and NQO1. Hemorrhoids caused by croton oil significantly increased the expression of inflammatory genes while reducing the expression of antioxidant defense genes. The relative mRNA expression of NF- κ B p65 (2.38-fold), TNF- α (2.66-fold), IL-1 β (2.49-fold), IL-6 (2.63-fold), COX-2 (2.51-fold), and iNOS (2.62-fold) was significantly higher in the abnormal control group compared to the normal control (p < 0.001). On the other hand, compared to the normal control, the expression of Nrf2, HO-1, and NQO1 dropped dramatically to 0.42-, 0.44-, and 0.49-fold, respectively (all p < 0.001), suggesting a severe disruption of endogenous antioxidant signaling.

These transcriptional changes were markedly and dose-dependently reversed by treatment with *Newbouldia laevis* stembark extract. The extract decreased the relative expression of NF- κ B p65, TNF- α , IL-1 β , IL-6, COX-2, and iNOS to roughly 1.05-, 1.02-, 0.98-, 1.06-, 0.95-, and 1.00-fold, respectively, at the maximum dose (400 mg/kg). These reductions were 56–62% when compared to the hemorrhoid control (p < 0.001). Antioxidant gene expression was significantly elevated concurrently by the extract, with Nrf2, HO-1, and NQO1 increasing to 2.52-, 2.68-, and 2.54-fold, respectively, indicating around six-fold activation in comparison to untreated hemorrhoidal tissues. These results were quite similar to those seen in the group treated with Daflon®. The current results show that *Newbouldia laevis* stembark extract suppresses the NF- κ B signaling pathway to produce strong anti-inflammatory effects. The expression of many inflammatory mediators involved in vascular inflammation and hemorrhoidal development is regulated by the master transcription factor NF- κ B [50,51]. After being activated by oxidative stress, NF- κ B translocates to the nucleus and triggers the transcription of TNF- α , IL-1 β , IL-6, COX-2, and iNOS, which increases tissue edema, leukocyte recruitment, vascular permeability, and nitric oxide overproduction [51–53]. Molecular proof that the extract stops the inflammatory cascade causing anorectal tissue damage is thus provided by

the significant decrease of these inflammatory genes shown in the extract-treated groups.

Since both COX-2 and iNOS are important downstream targets of NF- κ B activation, the decrease in their expression is very significant. While iNOS produces excess nitric oxide that combines with superoxide to form peroxynitrite, a highly reactive oxidant that can cause endothelial dysfunction and epithelial injury [51], COX-2 catalyzes prostaglandin production, which promotes vasodilation, pain, and inflammatory edema. Therefore, the decreased rectoanal edema, vascular congestion, and inflammatory cell infiltration seen in the macroscopic and histological examinations can be explained by their downregulation. Importantly, the stembark extract from *Newbouldia laevis* significantly increased the expression of Nrf2, HO-1, and NQO1, suggesting that the endogenous antioxidant defense system was activated. By triggering the transcription of antioxidant and cytoprotective enzymes, Nrf2, which is acknowledged as the primary regulator of cellular redox homeostasis, shields tissues from oxidative damage. By boosting HO-1 and NQO1 expression, Nrf2 activation improves free radical detoxification, prevents lipid peroxidation, and maintains cellular integrity. The considerable restoration of SOD, CAT, GPx, and GSH activities, along with the decreased MDA concentrations mentioned in the previous section, is consistent with the transcriptional activation of these genes seen in our work.

There is growing evidence that the NF- κ B and Nrf2 signaling pathways interact extensively. While chronic NF- κ B activation inhibits Nrf2-dependent antioxidant responses, Nrf2 activation reduces intracellular ROS and inhibits nuclear translocation of NF- κ B, hence suppressing NF- κ B-mediated inflammation. Thus, it is highly likely that *Newbouldia laevis* stembark extract restores redox equilibrium through coordinated modulation of the NF- κ B/Nrf2 signaling axis based on the simultaneous downregulation of inflammatory genes and overexpression of antioxidant genes found in this study. These results are consistent with other studies that found that *Newbouldia laevis*'s high concentration of flavonoids, phenolic acids, iridoids, and naphthoquinones gives it strong anti-inflammatory and antioxidant qualities. In models of inflammatory bowel disease, gastric ulceration, vascular inflammation, and oxidative tissue damage, many flavonoids extracted from medicinal plants have been demonstrated to concurrently activate Nrf2-mediated antioxidant signaling and inhibit NF- κ B

activation. *Aesculus hippocastanum*, another flavonoid-rich medicinal herbs utilized in hemorrhoidal therapy, has also been shown to have similar molecular processes [29,31].

The current study's dose-dependent regulation of these genes offers strong mechanistic support for the conventional usage of *Newbouldia laevis* stem bark in the treatment of hemorrhoids. The extract's capacity to simultaneously increase anti-

oxidant defense and reduce pro-inflammatory gene transcription indicates that one of the main molecular mechanisms underlying its anti-hemorrhoidal action is the control of the NF- κ B/Nrf2 signaling pathway. These transcriptional results offer a molecular foundation for the therapeutic potential of *Newbouldia laevis* in inflammatory anorectal diseases and further support the biochemical and histological improvements shown after treatment.

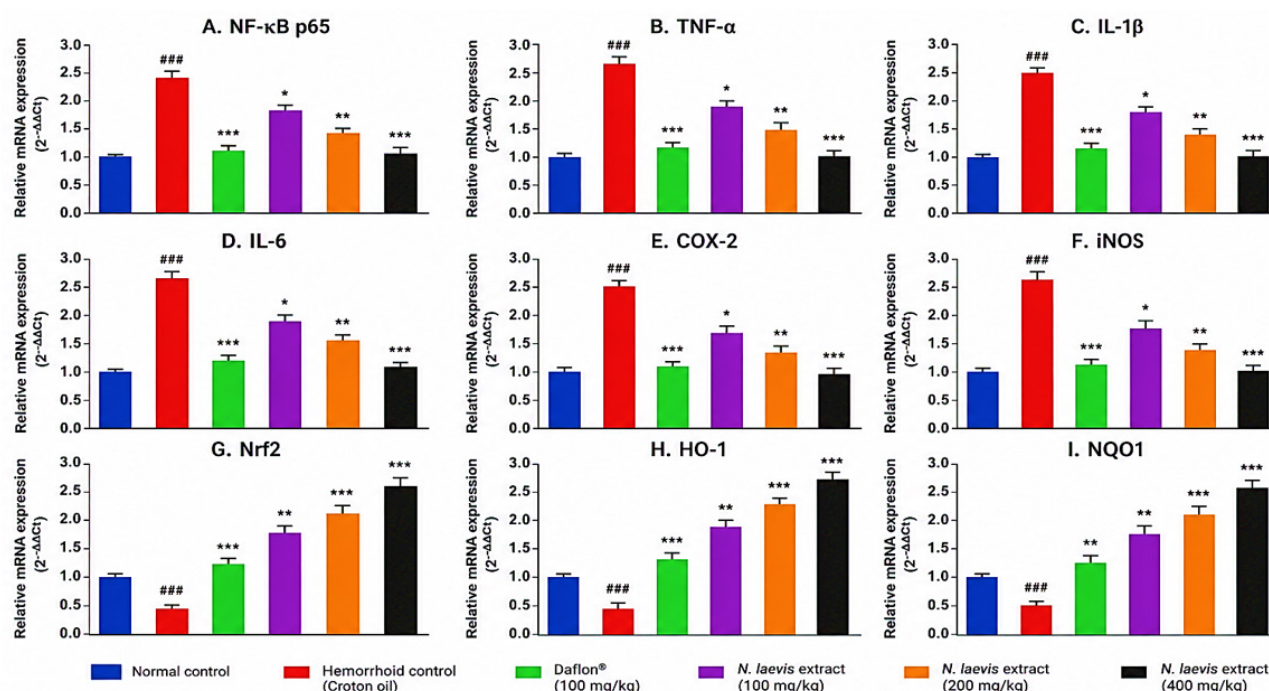


Figure 2: Effect of *Newbouldia laevis* stem bark extract on the relative mRNA expression of inflammatory and antioxidant genes in rectoanal tissues of croton oil-induced hemorrhoidal rats. Relative gene expression was determined by quantitative real-time PCR using the $2^{-\Delta\Delta C_t}$ method and normalized to β -actin. Panels show the relative expression of (A) NF- κ B p65, (B) TNF- α , (C) IL-1 β , (D) IL-6, (E) COX-2, (F) iNOS, (G) Nrf2, (H) HO-1, and (I) NQO1. Data are expressed as mean $\pm$ SE (n = 5). $^{\wedge}###^{\wedge}$ p < 0.001 versus normal control; p < 0.05, *p < 0.01, and $^{**}p$ < 0.001 versus hemorrhoid control (one-way ANOVA followed by Tukey's multiple comparison test). Treatment with *Newbouldia laevis* stem bark extract dose-dependently suppressed inflammatory gene expression while enhancing antioxidant gene expression, indicating modulation of the NF- κ B/Nrf2 signaling pathway.

Effect of *Newbouldia laevis* Stem bark Extract on NF- κ B/Nrf2 Signaling Proteins in Rectoanal Tissues

Western blot analysis was used to measure the protein expression of important mediators in the NF- κ B/Nrf2 signaling pathway to confirm the transcriptional findings. Figure 3 displays representative immunoblots together with the related densitometric analysis. Figure 3A–B illustrates how croton oil-induced hemorrhoids led to a considerable activation of inflammatory signaling, as seen by the significant overexpression of NF- κ B p65, COX-2, and iNOS and the downregulation of I κ B α , Nrf2, HO-1, and NQO1 in comparison to the normal control

(p < 0.001). Densitometric analysis showed that whereas COX-2 and iNOS increased by 2.5 and 2.6 times, respectively, NF- κ B p65 protein expression increased by about 2.7 times. On the other hand, in hemorrhoidal tissues, expression of I κ B α , Nrf2, HO-1, and NQO1 decreased to 42%, 39%, 36%, and 41% of normal control levels, indicating significant inflammatory activation and impaired antioxidant defense. These protein changes were considerably and dose-dependently altered by treatment with *Newbouldia laevis* stem bark extract. NF- κ B p65, COX-2, and iNOS expression were moderately reduced in rats given 100 mg/kg, while I κ B α , Nrf2, HO-1, and NQO1 were partially restored. At 200 mg/kg, more noticeable effects were

seen, and at 400 mg/kg, protein expression was nearly entirely restored. In comparison to the hemorrhoid control, NF- κ B p65, COX-2, and iNOS protein levels dropped by roughly 58%, 55%, and 60% at this dose ($p < 0.001$), while I κ B α , Nrf2, HO-1, and NQO1 increased by 2.3-, 2.6-, 2.9-, and 2.5-fold, respectively. These reactions were statistically similar to those seen in the group that received Daflon[®] treatment.

The current study's protein expression profile reveals that *Newbouldia laevis* stembark extract controls the NF- κ B/Nrf2 signaling axis at both transcriptional and translational levels, which is strongly consistent with the qRT-PCR results. Under normal circumstances, NF- κ B is kept in the cytoplasm by binding to its inhibitory protein, I κ B α . I κ B α is phosphorylated and degraded by proteases after inflammatory stimulation, which enables NF- κ B to go into the nucleus and trigger the production of many inflammatory genes, such as COX-2 and iNOS. Thus, prolonged NF- κ B activation and ongoing inflammatory signaling are responsible for the significant decrease in I κ B α expression seen in untreated hemorrhoidal tissues. Inhibition of NF- κ B nuclear activation is suggested by the restoration of I κ B α expression after treatment with *Newbouldia laevis* stembark extract. Therefore, prostaglandin synthesis, nitric oxide overproduction, vascular permeability, and inflammatory edema would all be decreased by suppressing downstream proteins like COX-2 and iNOS [54]. The reported decreases in the rectoanal coefficient, vascular congestion, inflammatory infiltration, and epithelial damage discussed in the preceding sections are compatible with these molecular events. The significant activation of the Nrf2 antioxidant pathway is an equally significant discovery. The primary transcription factor controlling cellular defense against oxidative stress is well known to be Nrf2. When Nrf2 is activated, it moves into the nucleus and promotes the development of cytoprotective enzymes, including HO-1 and NQO1, which are essential for reducing oxidative tissue damage and neutralizing reactive oxygen species. Following treatment with *Newbouldia laevis* stembark extract, there was a significant overexpression of Nrf2, HO-1,

and NQO1 proteins. This is direct proof that the extract enhances cellular resistance to oxidative damage by activating natural antioxidant signaling.

The idea of reciprocal regulation of both pathways is supported by the simultaneous suppression of NF- κ B and activation of Nrf2 seen in this work. Excessive ROS produced during hemorrhoidal inflammation prolongs inflammatory damage by activating NF- κ B and inhibiting Nrf2 signaling [29,49]. On the other hand, Nrf2 activation decreases NF- κ B-dependent inflammatory responses, stabilizes I κ B α , and lowers intracellular oxidative stress. Thus, *Newbouldia laevis* stembark extract's coordinated control of both pathways constitutes a highly effective technique for breaking the hemorrhoidal disease's self-amplifying cycle of oxidative stress and inflammation. These results are consistent with earlier studies showing that in inflammatory illness models, flavonoids and phenolic compounds extracted from *Newbouldia laevis* and other medicinal plants stimulate Nrf2-mediated antioxidant defense while inhibiting NF- κ B activation. Polyphenol-rich extracts of *Aesculus hippocastanum*, which have demonstrated effectiveness in lowering vascular inflammation and fostering anorectal tissue repair, have been shown to exhibit similar dual regulation of NF- κ B/Nrf2 signaling [29]. Therefore, the observed molecular effects are probably caused by the phytochemical components of *Newbouldia laevis* stembark, specifically flavonoids, phenolic acids, iridoids, and naphthoquinones.

All things considered, the Western blot results offer strong mechanistic proof that the anti-hemorrhoidal action of *Newbouldia laevis* stembark extract is mediated by activation of the Nrf2 antioxidant defense system and regulation of the NF- κ B inflammatory pathway. These molecular changes are in line with the biochemical, histopathological, and gene expression findings, which together show that one of the main mechanisms underlying the extract's therapeutic effects against experimental hemorrhoids is modulation of the NF- κ B/Nrf2 signaling axis.

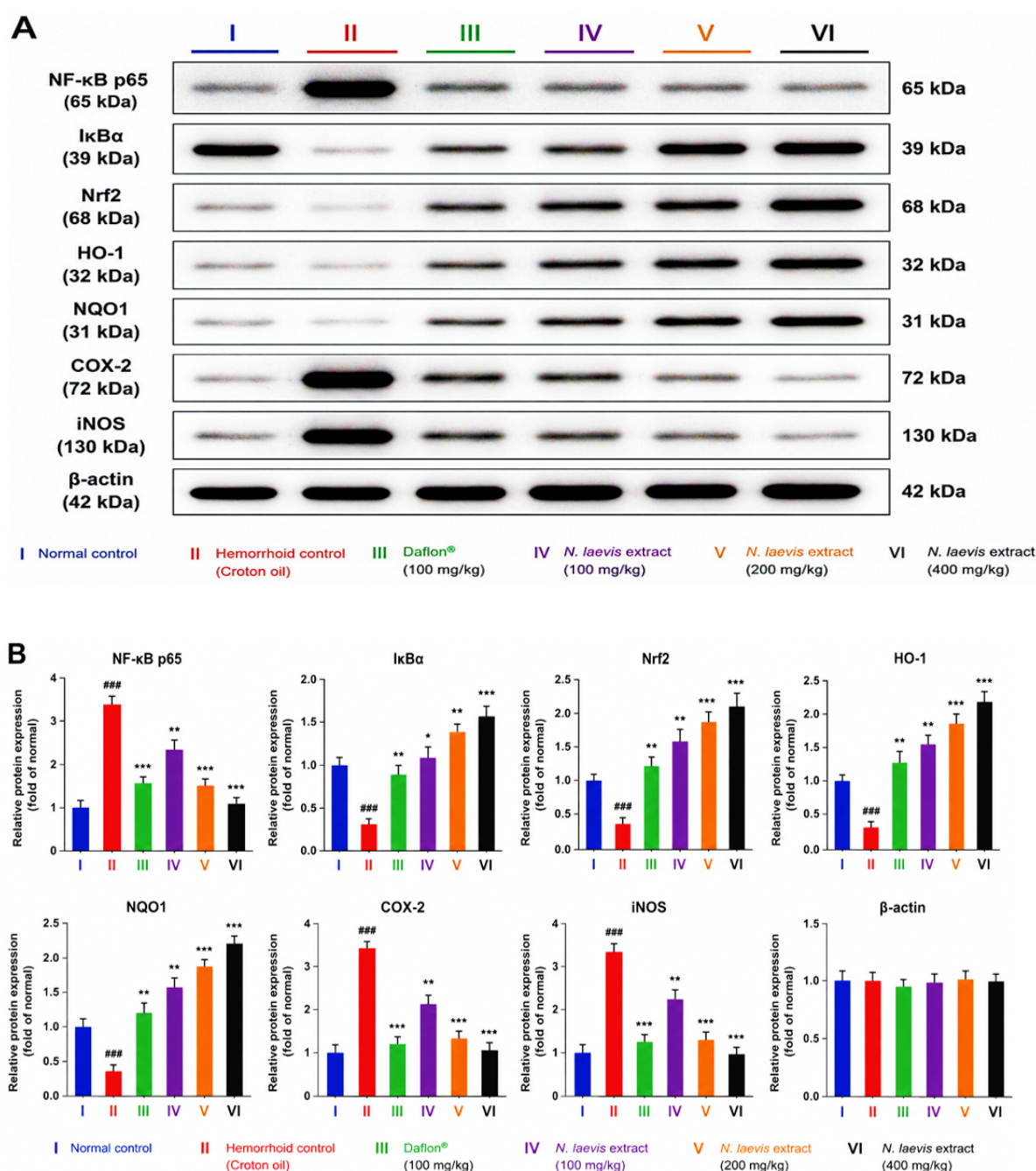


Figure 3: Effect of *Newbouldia laevis* stem bark extract on the protein expression of NF-κB/Nrf2 signaling molecules in rectoanal tissues of croton oil-induced hemorrhoidal rats. **(A)** Western blot bands showing protein expression of NF-κB p65, IκBα, Nrf2, HO-1, NQO1, COX-2, iNOS, and β-actin in the six experimental groups: Normal Control (NC), Hemorrhoid Control (HC), Daflon[®] (100 mg/kg), *Newbouldia laevis* stem bark extract (100, 200, and 400 mg/kg). **(B)** Corresponding densitometric analysis of protein expression normalized to β-actin and expressed relative to the normal control. Data are presented as mean ± SE (n = 5). ### p < 0.001 versus normal control; * p < 0.05, ** p < 0.01, and *** p < 0.001 versus hemorrhoid control (one-way ANOVA followed by Tukey's multiple comparison test). Treatment with *Newbouldia laevis* stem bark extract dose-dependently suppressed NF-κB p65, COX-2, and iNOS while restoring IκBα, Nrf2, HO-1, and NQO1, indicating inhibition of inflammatory signaling and activation of antioxidant defense pathways.

Effect of *Newbouldia laevis* Stembark Extract on Immunohistochemical Expression of NF- κ B p65, Nrf2, And COX-2 in Rectoanal Tissues

Immunohistochemical localization of NF- κ B p65, Nrf2, and COX-2 proteins was carried out in rectoanal tissues to further validate the molecular mechanisms underlying the anti-hemorrhoidal activity of *Newbouldia laevis* stembark extract. Figure 4 displays photomicrographs and semi-quantitative immunohistochemistry (IHC) scores. While robust nuclear Nrf2 staining was seen throughout the epithelium and submucosal layers, indicating normal antioxidant activity, the normal control group showed poor cytoplasmic and nuclear immunoreactivity for NF- κ B p65 and COX-2 (Figure 4A). The hemorrhoid control group, on the other hand, showed strong NF- κ B p65 and COX-2 immunostaining, primarily in injured epithelial cells, vascular endothelial cells, and infiltrating inflammatory cells. Concurrently, Nrf2 immunoreactivity was significantly reduced, indicating a compromised endogenous antioxidant defense after oxidative damage caused by croton oil. Daflon® treatment significantly reduced COX-2 and NF- κ B p65 immunostaining while boosting Nrf2 expression. Similarly, these indicators were modulated in a dose-dependent manner when *Newbouldia laevis* stembark extract was administered. NF- κ B p65 and COX-2 staining were somewhat decreased by the 100 mg/kg dose, but Nrf2-positive nuclei were partially restored. Rats given 200 mg/kg showed enhanced Nrf2 immunoreactivity and significant decreases in inflammatory protein expression. Only modest COX-2 and NF- κ B p65 staining persisted at 400 mg/kg, whereas Nrf2 staining intensified and spread widely throughout the rectoanal mucosa, closely mimicking the normal and Daflon®-treated groups.

These findings were corroborated by semi-quantitative analysis (Figure 4B–D). NF- κ B p65 and COX-2 immunohistochemistry scores were significantly higher in the hemorrhoid control group (3.12 ± 0.24 and 3.21 ± 0.22 , respectively) than in the normal control group (0.71 ± 0.11 and 0.69 ± 0.10) ($p < 0.001$). On the other hand, in hemorrhoidal tissues, the Nrf2 immunohistochemistry score drastically decreased from 3.02 ± 0.23 to 0.63 ± 0.09 ($p < 0.001$). NF- κ B p65 and COX-2 values were considerably lowered to 0.62 ± 0.08 and 0.58 ± 0.07 , respectively, after treatment with 400 mg/kg of *Newbouldia laevis* stembark extract, while the Nrf2 score increased to 2.94 ± 0.19 ($p < 0.001$ against hemorrhoid control). The Daflon® group's values and these values did not differ appreciably. *Newbouldia laevis* stembark extract inhibits inflammatory signaling while

boosting endogenous antioxidant responses, as demonstrated directly at the tissue level by the immunohistochemistry results. In untreated hemorrhoidal tissues, increased nuclear localization of NF- κ B p65 indicates that this transcription factor is activated in response to inflammatory and oxidative stress. Activated NF- κ B promotes leukocyte infiltration, vascular permeability, edema, and tissue damage by stimulating the transcription of many pro-inflammatory genes, including TNF- α , IL-1 β , IL-6, COX-2, and iNOS. Consequently, the significant decrease in NF- κ B p65 immunostaining after extract administration suggests that inflammatory signal transduction has been effectively inhibited.

Similarly, NF- κ B activation was highly correlated with COX-2 expression. In addition to directly causing vasodilation, pain sensitivity, and inflammatory edema linked to hemorrhoidal illness, COX-2 is an inducible enzyme that is in charge of prostaglandin synthesis during inflammation. Therefore, the decreased rectoanal edema, vascular congestion, and inflammatory cell infiltration shown by macroscopic and histological investigations are compatible with the significant decrease in COX-2 immunoreactivity seen in the treated groups. On the other hand, the recovery of Nrf2 immunostaining clearly implies that antioxidant defense systems have been activated. Nrf2 is generally kept at low intracellular levels by Keap1-mediated degradation, but when antioxidant phytochemicals activate it during oxidative stress, it quickly builds up within the nucleus. Nuclear Nrf2 then triggers the transcription of cytoprotective genes, such as HO-1 and NQO1, which lower reactive oxygen species, maintain mitochondrial function, and shield tissues from oxidative injury. The higher expression of Nrf2, HO-1, and NQO1 proteins found by Western blotting and the previously reported restoration of antioxidant enzyme activity are compatible with the increased nuclear localization of Nrf2 seen in this work.

These results are in line with earlier research showing that the NF- κ B and Nrf2 signaling pathways are mutually regulated. While excessive NF- κ B activation reduces the transcription of antioxidant genes, which prolongs chronic inflammation, Nrf2 activation suppresses oxidative stress by inhibiting NF- κ B nuclear translocation. Therefore, the hypothesis that the extract disrupts the oxidative stress–inflammation cycle responsible for hemorrhoidal progression is strongly supported by the coordinated decrease in NF- κ B p65 and COX-2 expression along with increased Nrf2 immunoreactivity observed after administration of *Newbouldia laevis* stembark extract.

The high flavonoids, phenolic chemicals, tannins, and iridoids found in *Newbouldia laevis* stem bark are probably responsible for the reported immunohistochemistry effects. In experimental models of inflammatory bowel illness, gastric ulceration, vascular inflammation, and oxidative tissue injury, these phytochemicals have been shown to promote Nrf2 nuclear translocation while inhibiting NF- κ B activation. Other medicinal herbs that have historically been utilized in hemorrhoid-

al therapy, such as *Aesculus hippocastanum* [31] and *Centella asiatica*, have also been shown to have similar molecular processes [55]. Overall, the immunohistochemical results support the biochemical, histopathological, qRT-PCR, and Western blot data, offering strong in situ proof that *Newbouldia laevis* stem bark extract has strong anti-hemorrhoidal effects by simultaneously activating the Nrf2 antioxidant pathway and inhibiting NF- κ B/COX-2-mediated inflammatory signaling.

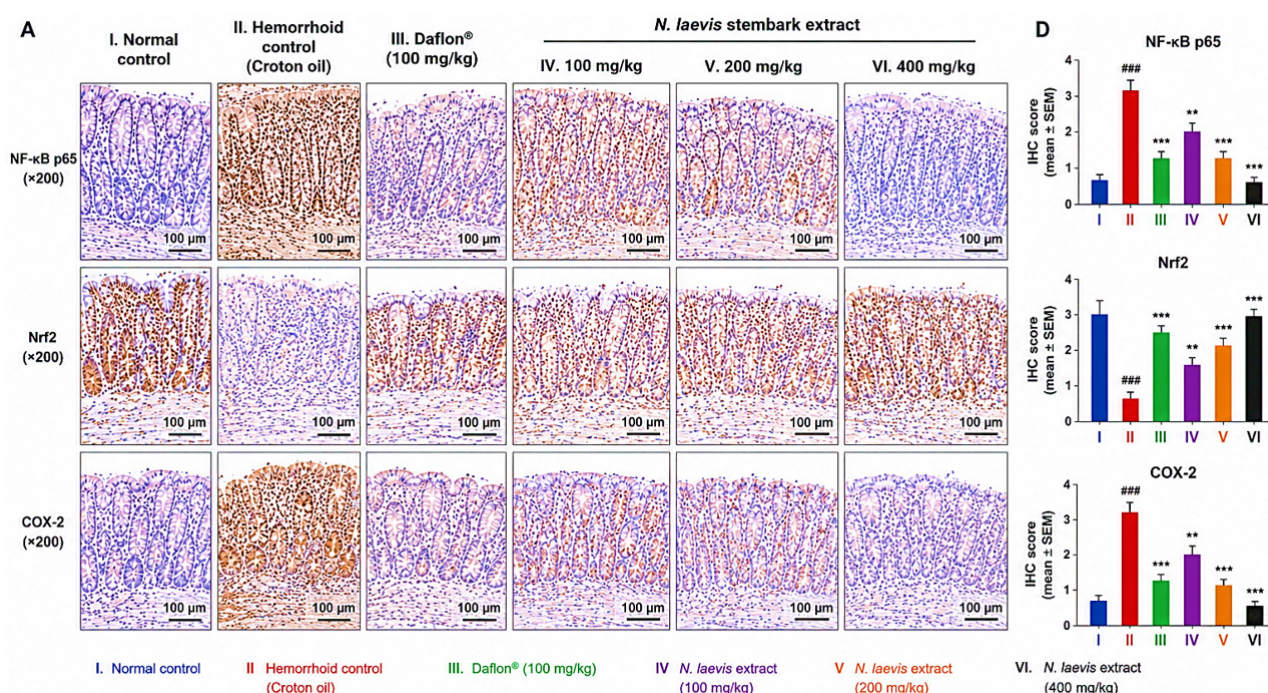


Figure 4: Immunohistochemical localization of NF- κ B p65, Nrf2, and COX-2 in rectoanal tissues following treatment with *N. laevis* stem bark extract in croton oil-induced hemorrhoidal rats. **(A)** Immunohistochemical photomicrographs (DAB chromogen, brown; hematoxylin counterstain, blue; original magnification $\times 200$; scale bar = 100 μ m) showing expression of NF- κ B p65, Nrf2, and COX-2 in rectoanal tissues from the six experimental groups. **(B–D)** Semi-quantitative immunohistochemical scores for NF- κ B p65, Nrf2, and COX-2, respectively. Data are presented as mean $\pm$ SE (n = 5). ### p < 0.001 versus normal control; p < 0.01 and p < 0.001 versus hemorrhoid control (one-way ANOVA followed by Tukey's multiple comparison test). *Newbouldia laevis* stem bark extract dose-dependently reduced NF- κ B p65 and COX-2 immunoreactivity while enhancing nuclear Nrf2 expression, indicating suppression of inflammatory signaling and activation of antioxidant defense in rectoanal tissues.

CONCLUSION

Through coordinated suppression of inflammation and an increase in endogenous antioxidant defenses, the current study shows that *Newbouldia laevis* stem bark extract has considerable anti-hemorrhoidal efficacy in a rat model produced by croton oil. With an estimated oral LD₅₀ more than 2,000 mg/kg body weight, the extract demonstrated a favorable acute oral safety profile and low acute toxicity. By reducing the rectoanal coefficient, attenuating edema, vascular congestion, erythe-

ma, and ulceration, as well as significantly restoring normal rectoanal histoarchitecture, treatment with *Newbouldia laevis* stem bark extract reduced hemorrhoidal severity in a dose-dependent manner. Significant decreases in lipid peroxidation and the restoration of antioxidant status through elevated activities of superoxide dismutase, catalase, glutathione peroxidase, and reduced glutathione followed these therapeutic effects. Mechanistically, the extract upregulated the antioxidant signaling molecules Nrf2, HO-1, and NQO1 at the gene and protein levels while downregulating the expression of import-

ant inflammatory mediators such as NF- κ B p65, TNF- α , IL-1 β , IL-6, COX-2, and iNOS. Immunohistochemical results further verified decreased tissue expression of COX-2 and NF- κ B p65 together with increased nuclear localization of Nrf2, offering compelling *in situ* proof that the extract's protective effects are due to modification of the NF- κ B/Nrf2 signaling axis. The reported pharmacological activity's mechanistic foundation is strengthened by the agreement between biochemical, histological, molecular, and immunohistochemical data.

When taken as a whole, these results show that regulation of the NF- κ B/Nrf2 signaling pathway is a key mechanism of action for the traditional usage of *Newbouldia laevis* stem bark in the treatment of hemorrhoids. The study also emphasizes *Newbouldia laevis*'s medicinal potential as a source of bioactive phytochemicals for the creation of innovative anti-hemorrhoidal drugs. However, since the current study was restricted to an experimental animal model, more research is necessary to identify and describe the active ingredients, examine their pharmacokinetic characteristics, assess long-term safety, and verify efficacy in carefully planned preclinical and clinical trials before therapeutic use in humans.

STATEMENTS

Conflict of Interest

The authors declare there is no conflict of interest.

Authors' Contributions

Cletus Anes Ukwubile: Conceptualization, methodology, investigation, formal analysis, data interpretation, supervision, writing original draft, manuscript review; Abdulmuizz Olawale Makinde: Formal analysis, investigation, data curation, software, visualization, manuscript review. All authors read and approved the final version of the manuscript for submission.

Human Ethics and Consent to Participate

Not applicable. This study did not involve human participants, human tissues, or human data. All experimental procedures were performed using laboratory animals and were approved by the Institutional Animal Ethics Committee (AREC), Department of Pharmacology and Toxicology, University of Maiduguri, Nigeria (Approval No.: FP/03/26/20/11/01/063), in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 8th edition, 2011) and the National Institutes of Health guidelines for the care and use of laboratory animals.

Consent for Publication

Both authors agreed to publish this research.

Data Availability

The data generated and analyzed during the current study are available from the corresponding author upon reasonable request.

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