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ANTI-HEMORRHOIDAL ACTIVITY OF HYDROETHANOLIC EXTRACT OF NEPHROLEPIS BISERRATA WITH PHYTOCHEMICAL CHARACTERIZATION, ANTIOXIDANT POTENTIAL AND IN VIVO EVALUATION IN A RAT MODEL

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ABSTRACT

Hemorrhoids are a common disorder characterized by inflammation and alteration of anorectal tissues, leading to pain and discomfort. Natural plant-based treatments are being explored for their potential safety and efficacy. In this context, the present study aimed to evaluate the anti-hemorrhoidal potential of the hydroethanolic extract of *Nephrolepis biserrata* by correlating its phytochemical composition, antioxidant activity, and biological effects in rats. The methodology involved preparing aqueous-ethanolic extracts of *N. biserrata*, performing phytochemical screening, and administering two doses (250 and 500 mg/kg) to rats following experimental induction of hemorrhoids. Studied parameters included plasma protein levels, liver enzymes (ALT, AST), recto-anal coefficient, and antioxidant activity (DPPH assay). Results showed that *N. biserrata* is rich in flavonoids (38.5 mg QE/g), tannins, alkaloids, and total polyphenols (75 mg GAE/g), with

high antioxidant activity (IC_{50} = 9.13 × 10^{-3} mg/mL). Treatment with 250 mg/kg normalized plasma protein levels (65.4 mg/dL), significantly reduced the recto-anal coefficient (0.00156), and decreased ALT and AST to 88.7 and 172.19 U/L, respectively, sometimes surpassing the efficacy of Daflon. The 500 mg/kg extract was also effective but slightly less so for certain parameters, suggesting an optimal dose at 250 mg/kg. These effects are likely related to the synergistic action of the chemical compound families present in the plant. In conclusion, *Nephrolepis biserrata* appears to be a promising candidate for hemorrhoid treatment, combining anti-inflammatory, antioxidant, and hepatoprotective activities, with an identified optimal dose of 250 mg/kg.

KEYWORDS: Anti-hemorrhoidal potential, hydroethanolic extract, *Nephrolepis biserrata*, phytochemical screening, antioxidant activity.

I- INTRODUCTION

Hemorrhoids are a common anorectal disorder characterized by pathological dilation of the venous plexuses in the anal canal. These vascular structures, normally involved in continence, become symptomatic when inflamed or congested, causing pain, pruritus, bleeding, and swelling. Hemorrhoids are classified as internal or external according to their location. Their occurrence is promoted by various factors such as chronic constipation, sedentary lifestyle, pregnancy, and repeated straining during defecation.[1,2]

Globally, hemorrhoids represent a major public health issue, affecting up to 50–85% of individuals during their lifetime, with a high prevalence among middle-aged adults.[3,4] In Africa, prevalence ranges from 7% to over 50% depending on the context.[5–8] In Benin, although precise data are limited, the widespread use of traditional medicine suggests a high incidence.[9,10]

Conventional treatments have limitations related to cost, side effects, and frequent recurrences.[11,12] Therefore, medicinal plants appear as promising alternatives due to their anti-inflammatory, antimicrobial, and wound-healing properties.[13–15]

Among these, *Nephrolepis biserrata* has attracted growing interest for its antimicrobial, anti-inflammatory, and antioxidant activities, attributed to its richness in bioactive compounds.[16–20]

The objective of this study is to evaluate the anti-hemorrhoidal potential of *Nephrolepis biserrata*, an activity that remains poorly documented scientifically. This work aims to fill this gap and highlight its use in traditional therapeutic practices.

II- MATERIALS AND METHODS

2.1. Materials

2.1.1. Plant Materials

The leaves of *Nephrolepis biserrata* were collected in the morning from palm trees in a palm plantation located in the commune of Pobè, Republic of Benin.

2.1.2. Chemical materials

The chemical materials specifically included methanol, ethanol, hydrochloric acid, gallic acid, and quercetin, supplied by Sigma-Aldrich and Acros Organics. Various types of laboratory glassware were also used throughout the experiment.

2.1.3. Animal material

The animal material consisted of twenty-five (25) Wistar albino rats with an average body weight of 130 g. These rats were randomly divided into five (5) groups of five rats each and housed in standard cages for a two-week acclimatization period prior to experimentation. They were maintained at a constant temperature of 22 ± 2°C and subjected to a 12 h light/12 h dark cycle in the animal facility of the Research Unit in Applied Microbiology and Pharmacology of Natural Substances, University of Abomey-Calavi.

2.2. Methods

2.2.1. Preparation and chemical analysis of extracts

2.2.1.1. Powder preparation

The leaves of *Nephrolepis biserrata* were carefully washed and then air-dried at room temperature for 7 days.[21] The dried leaves were then finely ground using an electric grinder. The resulting powder was sieved and stored in glass containers protected from moisture until further use.

2.2.1.2. Preparation of extracts

Sixty grams (60 g) of *Nephrolepis biserrata* powder were mixed with 600 mL of a water/ethanol (96%) solution in a 30:70 ratios, with the water preheated to 100°C. This

method has previously demonstrated efficiency in optimizing the extraction of bioactive compounds.[22]

After agitation and homogenization, the mixture was filtered using Whatman filter paper. The filtrate was concentrated using a rotary evaporator at 45–50°C under reduced pressure with the aid of a vacuum pump. Final drying was carried out in an oven at 35°C for 72 hours. The dry extract obtained was stored at 4°C in a refrigerator.

2.2.1.2. Qualitative phytochemical screening of hydroethanolic extract of *N. biserrata*

Qualitative phytochemical screening was performed based on colorimetric and precipitation reactions. The tests were carried out directly on the hydroethanolic extract of *N. biserrata* seed powder according to the method described by Houghton and Raman (1998).[23]

2.2.1.3. Quantitative phytochemical screening of hydroethanolic extract of *N. biserrata*

Quantitative phytochemical analyses were performed according to the method of Harborne (1984), adapted to our laboratory conditions.[24,25]

2.2.1.3.1. Determination of total polyphenols

Total polyphenol content was determined using the Folin–Ciocalteu colorimetric method. This reagent is reduced during the oxidation of phenolic compounds into a mixture of blue oxides of tungsten and molybdenum. The resulting blue coloration shows maximum absorbance around 750 nm. The absorbance, compared to a standard calibration curve obtained with gallic acid, allowed the quantification of total phenolic content expressed as mg gallic acid equivalents per gram of extract (mg GAE/g).[25]

2.2.1.3.2. Determination of total flavonoids

Total flavonoid content was estimated using the aluminum chloride ($AlCl_3$) method. Quercetin was used as the reference compound for establishing the calibration curve.[25]

Polyphenol and flavonoid contents were calculated using the following formula:

$$T = (C \times Vr) / (Vp \times Cp)$$

Where: T = content of compounds; C = concentration obtained from the calibration curve; Vr = reaction volume; Vp = volume of extract used; Cp = concentration of the extract solution.

2.2.1.4. *In vitro* evaluation of antiradical activity

The antiradical activity of the extracts was evaluated using the DPPH• (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method, following a procedure similar to that described by Fagbohoun (2014).[25] This method is based on the reduction of the stable free radical DPPH• in the presence of a hydrogen-donating compound at a wavelength of 517 nm. The percentage of DPPH radical scavenging was calculated using the following formula:

% DPPH = 100 × (Abs blank – Abs sample) / Abs blank

Where: Abs blank = absorbance of the control (DPPH solution); Abs sample = absorbance of the test sample;

The IC_{50} value, defined as the concentration of extract required to neutralize 50% of DPPH radicals, was determined from the graph plotting percentage inhibition against extract concentration. Each test was performed in duplicate.[26]

2.2.2. Evaluation of biological parameters

2.2.2.1. Grouping of animals

Table 1 presents the grouping of rats, the substances administered, and the corresponding doses used. Each group consisted of five (5) rats.

Table 1: Composition of groups.

Groups	Codes	Substances to be administered
Group 1 Normal Control	**GN1**	No induction and treatment with distilled water
Group 2 Negative Control	**GN2**	Induction without treatment
Group 3 Reference Control	**RCD 10 mg/kg**	Induction and treatment with Daflon at 10 mg/kg
Group 4	**NB 250 mg/kg**	Induction and treatment with NB at 250 mg/kg
Group 5	**NB 500 mg/kg**	Induction and treatment with NB at 500 mg/kg

NB = hydro-ethanolique extract of *Nephrolepis biserrata*

2.2.2.2. Induction of hemorrhoids

Hemorrhoids were induced in rats using a 6% croton oil solution prepared by mixing distilled water, pyridine, acetone, and croton oil in a ratio of 1:4:5:10. Prior to induction, the rats were subjected to overnight fasting.

Induction was performed in all groups except the normal control by inserting sterile cotton swabs (4 mm in diameter), soaked with 100 µL of the 6% croton oil solution, into the anus

(recto-anal region, approximately 20 mm from the anal opening). This procedure was carried out once daily for three consecutive days.[27]

On day 4, blood samples were collected to measure erythrocyte sedimentation rate and total protein levels. Photographs of the animals were also taken.

2.2.2.3. Anti-hemorrhoidal test and measurement of biological parameters

On the fourth day, each rat in the test groups received the corresponding treatment dose (Table 1) orally for 5 days. The animals were weighed and anesthetized using ether.

A 1 cm of the rectum was surgically excised from each rat. The rectal tissues were weighed and analyzed to evaluate weight variations between control and treated groups.[28]

Photographs of rats from the different groups were also taken.

2.2.2.4. Recto-anal coefficient (RAC)

The recto-anal coefficient (RAC) was calculated using the following equation:

RAC = Recto-anal tissue weight (mg) / Body weight (g)

2.2.2.5. Inflammatory index

The inflammatory index was assessed on day 9. To evaluate the severity of inflammation in the recto-anal region, a scoring system based on clinical hemorrhoidal presentation and severity index was used. Hemorrhoids were graded on a scale from I to IV: **Grade I:** Anal cushions bleed but do not prolapse; **Grade II:** Anal cushions prolapse during straining but reduce spontaneously; **Grade III:** Anal cushions prolapse during straining and require manual reduction; **Grade IV:** Prolapsed tissue remains permanently outside and is irreducible.[28]

2.2.2.6. Biochemical parameters

On day 9, blood samples were collected from all animals to measure erythrocyte sedimentation rate, total protein levels, aspartate aminotransferase (ASAT), and alanine aminotransferase (ALAT), in accordance with OECD guidelines (2025).[29]

2.2.3. Data processing and analysis

The collected data were entered into Microsoft Excel 2013 and subjected to appropriate statistical analyses. All results were expressed as mean ± standard deviation. Analysis of

variance (ANOVA), followed by Tukey's multiple comparison test, was used to compare percentages and variation rates. The level of statistical significance was set at 5% ($p < 0.05$).

III- RESULTS AND DISCUSSION

3.1. Yield and Physical Appearance of the Extract

The table 2 presents the *Nephrolepis biserrata* extract obtained by maceration using a water/ethanol mixture (30/70). The extract appears as a paste, with an extraction yield of 16.36 ± 1.83%, coded as "**NB**"

Table 2: Physical Appearance and Codes.

Extracts	Physical appearance	Extraction yield (%)	Codes
Nephrolepis biserrata water/ethanol (30/70)	Paste	16,36 ± 1,83	**NB**

This yield reflects the efficiency of the hydroalcoholic solvent in extracting bioactive compounds such as flavonoids, phenols, and tannins.[24,30] The pasty consistency suggests a high concentration of secondary metabolites, which is valuable for pharmacological and antioxidant applications. The paste-like appearance may also indicate the presence of lipophilic compounds. Compared to other ferns, this yield is moderate, indicating an interesting potential but requiring process optimization.[31]

3.2. Phytochemical Composition of *Nephrolepis biserrata* (NB)

The table highlights the significant phytochemical richness of *Nephrolepis biserrata*, characterized by the presence of several classes of secondary metabolites such as reducing compounds, saponins, alkaloids, flavonoids (38.5 ± 0.01 mg QE/g), terpenoids, catechin tannins, gallic tannins, free anthracenes, leuco-anthocyanins, quinone compounds, and total polyphenols (75 ± 0.03 mg GAE/g).

Table 3: Phytochemical Composition of *Nephrolepis biserrata* (NB).

Compounds	NB	Compounds	NB
Reducing compound	+	Saponin	+
Alkaloids	+	Coumarin	nd
Flavonoids (mgEQ/g)	38,5 ± 0,01	Terpenoids	+
Tanins catechic	+	Mucilages	-
Tanins gallic	+	Cartenoids	-
Anthocyanins	-	Free Anthracenics	+
Leuco-anthocyanins	+	O-heterosides	-
Quinonics compound	+	Polyphenols totaux (mgEAG/g)	75 ± 0,03

+: Presence; - : Absence

The presence of total polyphenols (75 ± 0.03 mg GAE/g) and flavonoids (38.5 ± 0.01 mg QE/g) indicates a strong antioxidant potential, in agreement with the work of Prior et al. (2005), who associated these compounds with free radical scavenging. The simultaneous presence of catechin and gallic tannins, alkaloids, saponins, and terpenoids also suggests anti-inflammatory and pharmacological properties.[31,32] These variations are consistent with the observations of Harborne (1998).[24] These results confirm that this fern represents a promising source of bioactive compounds, although further biological studies are required to validate its therapeutic applications.

3.3. Antioxidant Activity by the DPPH Assay

Figure 1 illustrates the change in absorbance as a function of different concentrations of *NB*. Solving the corresponding equation allowed the determination of a very low IC_{50} (9.13×10^{-3} mg/mL), reflecting a high antioxidant capacity.

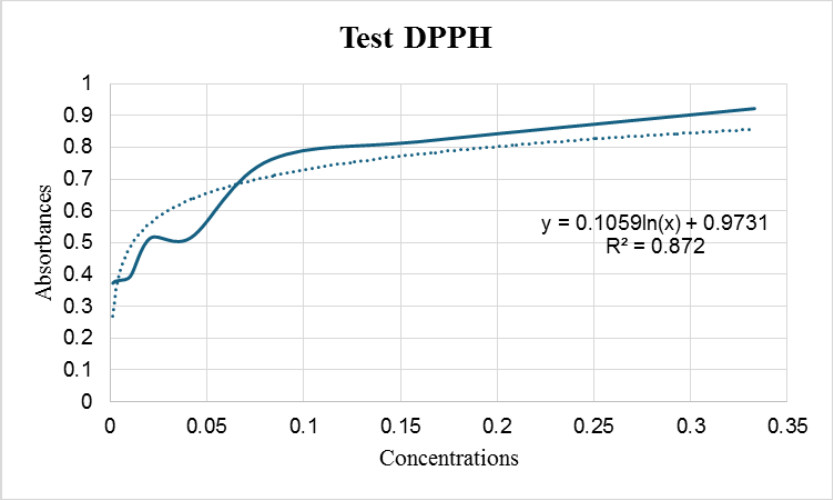


Figure 1: Antioxidant Activity by the DPPH Assay.

The initial DPPH value was 0.960, corresponding to y = 0.480. Solving the equation yielded an IC_{50} of 9.13×10^{-3} mg/mL. The observed antioxidant activity could significantly contribute to the reduction of inflammation and tissue protection. The effectiveness at low concentrations indicates a richness in secondary metabolites, known to neutralize free radicals and limit oxidative stress.[33] *Nephrolepis biserrata* therefore appears to be a promising source of natural antioxidant compounds.

3.4. Evaluation of Anti-Hemorrhoidal Activity

3.4.1. Distribution of Experimental Groups

The table in the Materials and Methods section summarizes the distribution of experimental groups and the treatments administered to the rats. Group 1 (GN1) served as the normal control, without induction or treatment, receiving only distilled water. Group 2 (GN2) served as the negative control, subjected to induction without treatment. Group 3 (RCD 10 mg/kg) received Daflon following induction. Groups 4 (NB 250 mg/kg) and 5 (NB 500 mg/kg) were treated with *Nephrolepis biserrata* extract at doses of 250 mg/kg and 500 mg/kg, respectively, after induction.

This setup allows for a comparative evaluation of treatment efficacy. The experimental design is appropriate, as it enables clear comparisons between the absence of treatment, the standard treatment, and the tested extract, thereby facilitating interpretation of potential therapeutic effects.

3.4.2. Effect of Treatments on Erythrocyte Sedimentation Rate in Rats After Hemorrhoid Induction

Figure 2 highlights variations in erythrocyte sedimentation rate (ESR) in rats following hemorrhoid induction, reflecting a more pronounced initial inflammatory state in the treated groups, particularly NB 250 mg/kg (4.66) and NB 500 mg/kg (3.33).

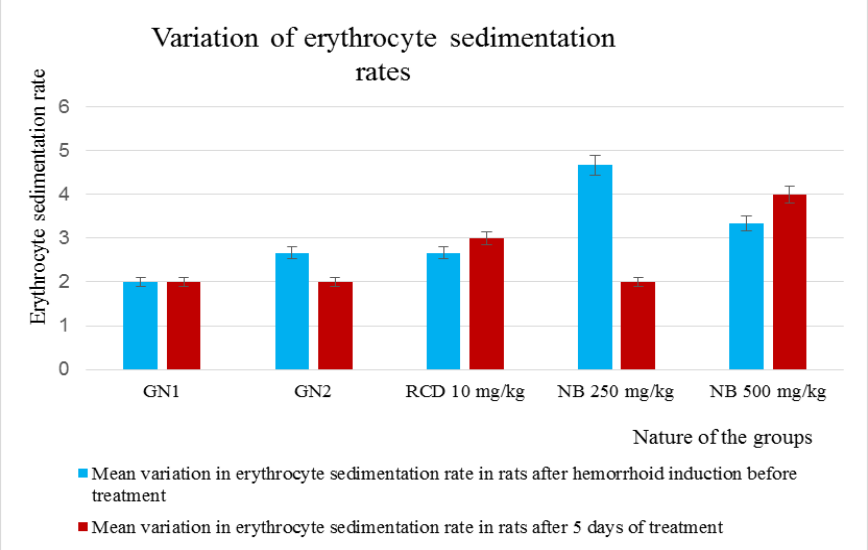


Figure 2: Erythrocyte Sedimentation Rate in Rats After Hemorrhoid Induction.

After 5 days of treatment, a marked decrease in ESR was observed for NB 250 mg/kg (from 4.66 to 2), suggesting a significant anti-inflammatory effect ($p < 0.05$), whereas the increase

observed at 500 mg/kg may indicate a non-linear dose-dependent effect or biological saturation. This reduction in ESR is generally associated with a decrease in acute-phase proteins and, consequently, inflammation.[34,35]

The observed activity may be related to the richness of secondary metabolites in *Nephrolepis biserrata*, notably alkaloids and tannins, which are known for their anti-inflammatory and vasoprotective properties. Alkaloids can modulate inflammatory mediators, while tannins exert astringent and antioxidant effects contributing to tissue protection. [33] However, since ESR is a non-specific marker, these results require confirmation through targeted biochemical assays and further studies.

3.4.3. Effect of the Extract on Total Protein Levels in Rats After Hemorrhoid Induction and Treatment

Figure 3 illustrates the changes in mean plasma protein levels in rats after hemorrhoid induction and 5 days of treatment. Prior to treatment, protein levels were homogeneous across groups (57.5–63 mg/dL), providing a comparable physiological baseline.

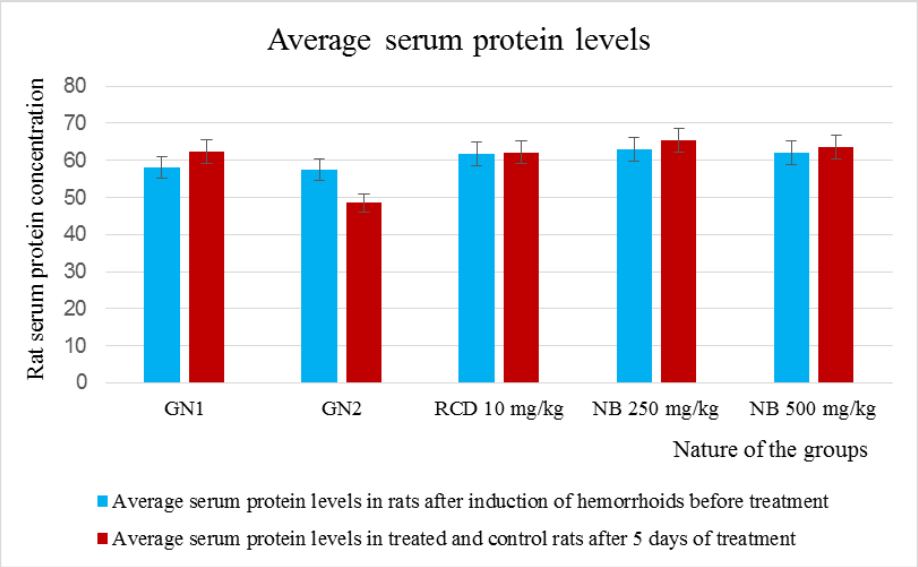


Figure 3: Total Protein Levels in Rats After Hemorrhoid Induction and Treatment.

After 5 days, the normal control (GN1) and the reference control treated with Daflon showed an increase or stabilization of serum protein levels (62.4 and 62.15 mg/dL), reflecting inflammation resolution, as observed in experimental models of acute inflammation where protein levels tend to normalize following effective treatment (Chen et al., 2022). In contrast,

the negative control (GN2) exhibited a significant decrease (48.5 mg/dL), indicating protein degradation associated with uncontrolled inflammation.

The groups treated with *Nephrolepis biserrata* extract (NB 250 and NB 500 mg/kg) displayed a greater increase in plasma protein levels (65.4 and 63.5 mg/dL), suggesting a potentially superior therapeutic effect. This effect is likely related to the activity of secondary metabolites, known for their anti-inflammatory properties and promotion of protein synthesis.[36] These metabolites may limit inflammation-induced protein degradation and support regulation of protein metabolism. However, more specific biochemical analyses are required to confirm these mechanisms.

3.4.4. Effect of the Extracts on the Variation of the Recto-Anal Coefficient in Rats After Hemorrhoid Induction

The table presents the mean variation of the recto-anal coefficient (RAC) after treatment of rats with different extracts and controls. The normal control (GN1) displayed a very low value (0.00335), serving as a physiological reference.

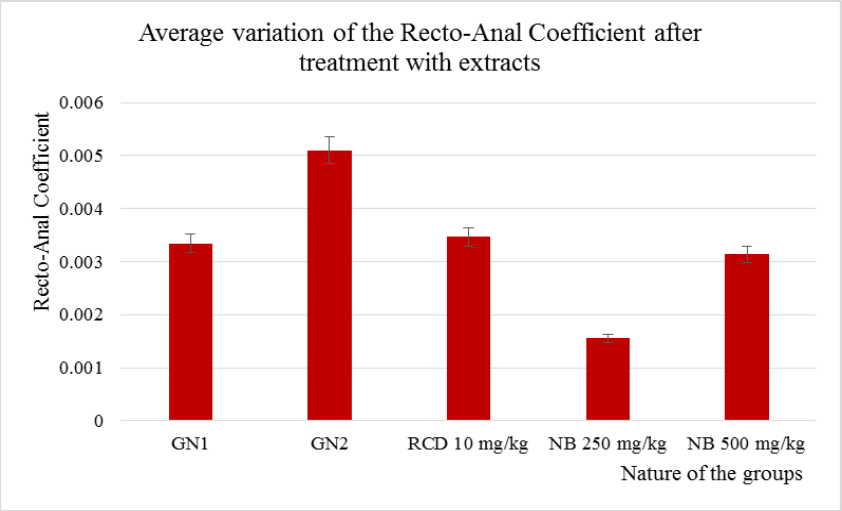


Figure 4: Variation of the Recto-Anal Coefficient in Rats After Hemorrhoid Induction.

The negative control (GN2) showed a notable increase (0.00510), indicating recto-anal coefficient (RAC) impairment due to untreated inflammation. This corroborates previous findings of increased ESR and decreased plasma protein levels in the same group, reflecting a persistent inflammatory state.

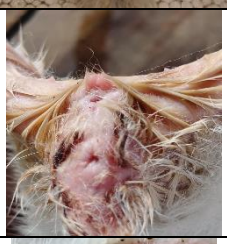
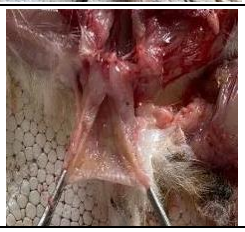
The reference control (Daflon 10 mg/kg) exhibited a RAC close to the normal control (0.00346), confirming the efficacy of this standard treatment. The *Nephrolepis biserrata*

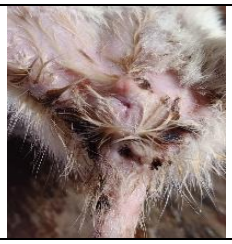
extracts demonstrated a dose-dependent effect: NB 250 mg/kg produced the lowest variation (0.00156), suggesting a protective effect superior to Daflon, whereas NB 500 mg/kg (0.00314) remained slightly above the normal control, possibly reflecting saturation or a non-linear effect at higher doses.

These observations are consistent with the improvements in plasma protein levels and the reduction of inflammation reported previously, highlighting the combined effect of polyphenols, alkaloids, and tannins in the extract, known for their anti-inflammatory and vasoprotective properties.[35] Recent studies confirm that modulation of the RAC is a reliable indicator of anorectal inflammation resolution.[34] Therefore, these results suggest that NB 250 mg/kg may be the optimal dose to restore recto-anal function following experimental hemorrhoids.

3.4.5. Inflammatory Index

Table 2: Clinical Assessment of Hemorrhoids After 5 Days of Treatment Across. Experimental Groups

Groups	Before traitment	After traitment	Appreciation after treatment
GN1		 	
GN2		 	Grade II: The anal cushions prolapse through the anus during straining but reduce spontaneously
RCD 10 mg/kg		 	Grade I: The anal cushions bleed but do not prolapse
NB 250 mg/kg		 	Grade I: The anal cushions bleed but do not prolapse

NB 500 mg/kg		 	Grade I: The anal cushions bleed but do not prolapse

The results show that the treated groups (RCD 10 mg/kg and *Nephrolepis biserrata* at 250 and 500 mg/kg) exhibited a notable improvement in symptoms, progressing from grade II to grade I, characterized by the absence of prolapse. This regression indicates a potential therapeutic effect, likely related to the anti-inflammatory and vasoprotective properties of the plant extracts. The lack of improvement in the control groups confirms the treatment effect. Furthermore, the comparable efficacy between doses suggests activity even at low concentrations. These observations are consistent with studies demonstrating the role of plant compounds in reducing inflammation and vascular disorders.[36]

3.4.6. Effect of Extracts on Serum ASAT Levels in Rats After Hemorrhoid Induction and Treatment

Figure 5 presents the mean serum aspartate aminotransferase (ASAT) levels in rats after five days of treatment. The normal control (GN1) exhibited a value of 325.22 U/L, representing the reference level. The negative control (GN2) showed a marked increase to 438.98 U/L, indicating liver injury. The group treated with Daflon at 10 mg/kg showed a decrease to 369.98 U/L, indicating a moderate effect. The groups treated with *Nephrolepis biserrata* displayed a more pronounced improvement: 88.7 U/L at 250 mg/kg and a further decrease to 81.25 U/L at 500 mg/kg.

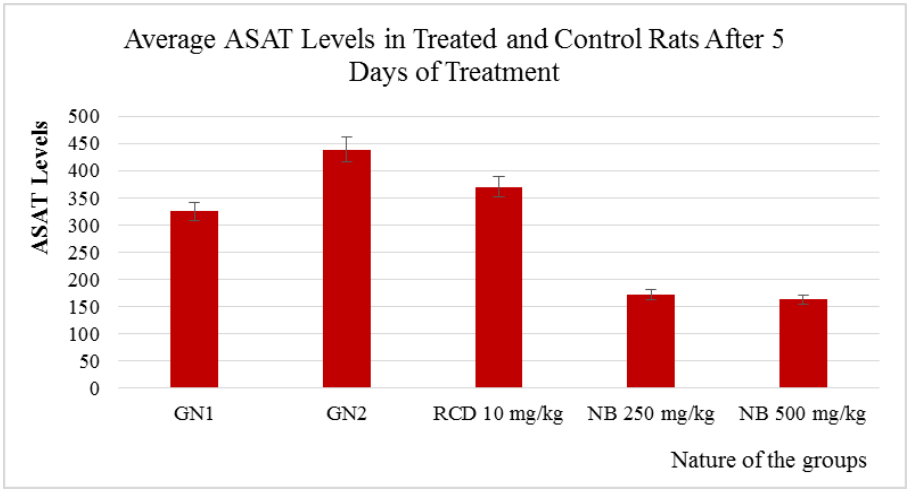


Figure 5: ASAT Levels in Rats After Hemorrhoid Induction and Treatment.

The normal control group (GN1) exhibited a baseline level of 325.22 U/L, reflecting physiological liver activity. The negative control (GN2) showed a notable increase to 438.98 U/L, indicating hepatocellular stress likely associated with hemorrhoid-induced inflammation, consistent with previous findings of increased ESR and decreased plasma protein levels. The reference control (Daflon 10 mg/kg) presented an intermediate level (369.98 U/L), suggesting a partial hepatoprotective effect.

Rats treated with *Nephrolepis biserrata* extract demonstrated a pronounced reduction in ASAT: NB 250 mg/kg at 172.19 U/L and NB 500 mg/kg at 163.25 U/L, indicating a strong hepatoprotective effect. This effect may be attributed to the richness in polyphenols, flavonoids, and alkaloids, known for their antioxidant properties and ability to mitigate hepatic oxidative stress.[36,37] These results corroborate previous observations, where the extract improved plasma protein levels and reduced the recto-anal coefficient, suggesting that *Nephrolepis biserrata* exerts both anti-inflammatory and liver-protective effects.[37,38]

3.4.7. Effect of Extracts on Serum ALAT Levels in Rats After Hemorrhoid Induction and Treatment

Figure 6 illustrates the mean serum alanine aminotransferase (ALAT) levels in rats after five days of treatment. The normal control (GN1) showed a low value of 50.22 U/L. The negative control (GN2) exhibited a marked increase to 118.47 U/L, indicating liver injury. The group treated with Daflon at 10 mg/kg presented an even higher level (180.36 U/L), suggesting limited efficacy in this case. Rats treated with *Nephrolepis biserrata* extract showed relative improvement: 88.7 U/L at 250 mg/kg and 81.25 U/L at 500 mg/kg.

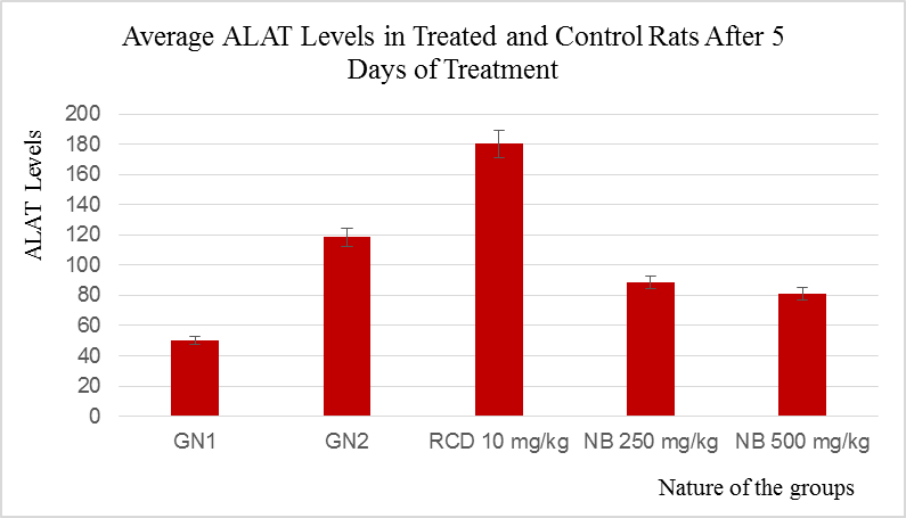


Figure 6: ALAT Levels in Rats After Hemorrhoid Induction and Treatment.

The normal control (GN1) exhibited a low level of 50.22 U/L, representing normal physiological liver activity. The negative control (GN2) showed a marked increase to 118.47 U/L, reflecting liver injury induced by hemorrhoid-related inflammation, consistent with previous observations for ESR and plasma protein levels, where persistent inflammation was evident. The reference control (Daflon 10 mg/kg) displayed an even higher value (180.36 U/L), which may reflect variability or secondary hepatic stress due to pharmacological treatment, a phenomenon occasionally reported in recent animal studies.[38]

Nephrolepis biserrata extracts showed a significant reduction in ALAT, with NB 250 mg/kg at 88.7 U/L and NB 500 mg/kg at 81.25 U/L, indicating a dose-dependent hepatoprotective effect. This improvement is consistent with earlier observations on plasma protein levels and the recto-anal coefficient, where the extract reduced inflammation and improved physiological markers. The protective effects may be attributed to the presence of polyphenols, flavonoids, and alkaloids, known for their antioxidant and anti-inflammatory properties.[39] These results confirm the therapeutic potential of *Nephrolepis biserrata* in restoring liver function and limiting cellular damage associated with anorectal inflammation.[38,39]

CONCLUSION

In summary, this study demonstrates that *Nephrolepis biserrata* extract exhibits significant therapeutic potential against experimental hemorrhoids. Its richness in flavonoids, tannins, alkaloids, and polyphenols contributes to its antioxidant, anti-inflammatory, and hepatoprotective effects. Administration at 250 mg/kg showed optimal efficacy, normalizing plasma protein levels, reducing the recto-anal coefficient, and modulating liver enzymes, in some cases surpassing the reference treatment. These findings suggest that *N. biserrata* could be a promising natural alternative for hemorrhoid management, warranting further pharmacological investigations.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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