Research Article

Evaluation of the Analgesic, Anti-Inflammatory and Antimicrobial Activities of Leaf Extracts of *Breynia nivosa*

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Abstract

Breynia nivosa is used in treating headaches, toothaches and tooth infections. This study evaluated some ethnomedicinal uses of leaves of the plant. *B. nivosa* leaves were extracted with ethanol, and then fractionated using methanol, ethyl acetate and dichloromethane. The phytochemical screening of the plant was evaluated and the analgesic, anti-inflammatory, and antimicrobial activities of the extracts/fractions were determined using standard methods. The phytochemical screening of the plant leaves revealed the abundance of alkaloids and flavonoids. Glycosides, tannins, and starch were present in moderate amounts, while saponins were detected in lower quantities. Analgesic activity was recorded by all extracts/fractions of the plant, but best activity was recorded by the dichloromethane fraction whose percentage analgesia was higher than that recorded for the standard drug Diclofenac at all concentrations analyzed (50-200 mg/mL). Anti-inflammatory activity was recorded for all the extracts of *B. nivosa* at 50, 100, and 200mg/mL. But compared to the standard drug Diclofenac (50mg/mL), a higher anti-inflammatory activity was recorded only for the crude ethanol extract and methanol fraction at concentrations of 100 and 200 mg/mL. In the antimicrobial evaluation of the crude ethanol extract of the *B. nivosa*, the plant recorded antimicrobial activity against *S. aureus*, *S. typhi* and *B. subtilis*, with best activity against *S. typhi*. It had no activity against *E. coli*, *A. fumigatus* and *C. albicans*. The extracts of *B. nivosa* leaves are natural analgesic, anti-inflammatory and antimicrobial agent and could have therapeutic potential in the management of various chronic diseases.

Keywords: *Breynia nivosa*, analgesic activity, anti-inflammatory activity, antimicrobial activity.

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Introduction

Plants and plant products have long been used in traditional medicine to treat infectious diseases. The use of medicinal plants remains significant as therapeutic remedies and still plays an important role in primary health care in developing countries such as Nigeria.

For many years, people depended exclusively on leaves, flowers and barks of plants for medicine. Although synthetic drugs have also come into use, in many instances, they are replicas or modifications of chemicals identified in plants [1].

There is growing interest in correlating phytochemical constituents of plants with their pharmacological activity [2]. Flavonoids have been reported to possess antibacterial activity, in which it has the ability to form complex with extracellular soluble proteins and bacterial cell walls [3]. In the same manner, purified alkaloids, as well as their synthetic derivatives, are used for their various biological effects such as analgesic, antispasmodic and bactericidal as remedies for diseases [4]. Investigations into the chemical and biological activities of plants during the past two centuries have yielded compounds for the development of modern synthetic organic chemistry and the emergence of medicinal chemistry as a major route for the discovery of novel and more effective therapeutic agents [5].

In Nigeria, *B. nivosa* is used in folkloric treatment of headaches, toothaches and tooth infections. The stem of *B. nivosa* is commonly used as chewing sticks in South-Eastern Nigeria [6]. Amadi *et al*. [7] reported the antibacterial activity of ethanol and hot water extracts of fresh leaves of *B. nivosa* against *Streptococcus mutans* isolated from dental caries patients. The results of their study confirms the fact that herbal preparations of *B. nivosa* can be used for cleansing of the oral cavity, maintenance of oral hygiene and prevention of dental caries [7].

This study seeks to determine the phytochemical properties/constituents of the plant and to evaluate the analgesic, anti-inflammatory, and antimicrobial properties of leaf extracts of *B. nivosa.*

Experimental

Materials

Figure 1 Leaves of *Breynianivosa*

Chemicals and reagents

Methanol, Ethanol, Ethyl acetate, Dichloromethane, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Ascorbic acid, Distilled water, Dimethyl sulfoxide (DMSO), MacFarland turbidity Standard (prepared from barium chloride, sulfuric acid and distilled water), etc.

Culture media

Nutrient agar, Nutrient broth, Mueller Hinton agar, Sabouraud Dextrose agar, Sabouraud Dextrose broth (Oxoid Limited, England),

Animals

Eighty four (84) Swiss albino mice of both sexes were used in this study. The mice were housed in a neat and well ventilated cage and were allowed free access to food and water.

Test Organisms

Bacterial isolates (*Staphylococcus aureus, Escherichia coli, Bacillus subtilis* and *Salmonella typhi*) and fungal isolates (*Aspergillus fumigatus* and *Candida albicans*) were used in this study.

Methods

Plant Collection, Identification and Preparation

The leaves of *B. nivosa* harvested from a garden in Agulu, Anambra State-Nigeria in the month of March, 2014. The plant was identified by a plant taxonomist in the Department of Pharmacognosy and Traditional Medicine, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka-Nigeria. The plant leaves were then rinsed in clean water and dried under room temperature. The dried leaves were pulverized into a fine powder using a mechanical grinding machine.

The powdered plant material was stored in a plastic container and maintained at room temperature prior to extraction.

Extraction and Fractionation

Ethanol Extract

200g of the pulverized plant sample was macerated in 1 liter of ethanol for 48hrs. The mixture was sieved using porcelain cloth. It was further filtered using No. 1 Whatman filter paper and the filtrate was concentrated using rotary evaporator. The crude concentrate was then stored in the refrigerator.

Ethyl acetate Fraction

The crude ethanol extract was dried to 60% of the total volume. Then 50 mL of the sample was mixed with 150 mL of ethyl acetate in a separating funnel and was shook vigorously. The mixture was allowed to stand for 1hr for proper separation according to their polarity. Then, the ethyl acetate fraction of the extract was collected and concentrated in water bath at the temperature of 50°C.

Methanol Fraction

The ethanol extract was dried to 60% of the total volume. Then 50 mL of the sample was mixed with 150 mL of methanol in a separating funnel and was shook vigorously. The mix was allowed to stand for 1hr for proper separation according to their polarity. Then, the methanol fraction of the extract was collected and concentrated in water bath at temperature of 50°C.

Dichloromethane Fraction

The ethanol extract was dried to 60% of the total volume. Then 50 mL of the sample was mixed with 150 mL of dichloromethane in a separating funnel and was shook vigorously. The mix was allowed to stand for 1hr for proper separation according to their polarity. Then, the dichloromethane fraction of the extract was collected and concentrated in water bath at the temperature of 50°C.

Phytochemical Analysis

In the phytochemical analysis of the powdered *B. nivosa* leaves, tests for alkaloids, flavonoids, glycosides, tannins, saponins, proteins, starch were carried out using standard methods reported by Trease and Evans [8].

Assay for Analgesic Activity

Assay for analgesic activity was carried out using the hot plate analgesic model described by Ezeja *et al*. [9].In this study, a total of 42 mice were used. The animals were grouped into 14 groups of 3 animals per group. Group 1 received 0.5 mL of distilled water, Group 2 received 50mg/kg of Diclofenac; Groups 3, 4, 5 received 50, 100, and 200mg/kg of the crude ethanol extract respectively; Groups 6, 7, 8 received 50, 100, and 200mg/kg of methanol fraction respectively; Groups 9, 10, 11 received 50, 100, and 200mg/kg of dichloromethane fraction respectively; while Groups 12, 13, 14 received 50, 100, and 200mg/kg of ethyl acetate fraction respectively. Each animal in the various groups was placed in a hot plate at a temperature of 50°C before and after treatment with the extract at 30, 60, 90, 120, and 150mins.

The analgesic activity of the plant was measured based on the duration the animal can comfortably stay on the hot-plate without licking the paws or showing any signs of discomfort.

$$\text{Percentage Analgesia (\%)} = 100 - (a/b \times 100)$$

Where;

a = mean time at basal value.

b = mean time at 4 hours.

Assay for Anti-Inflammatory Activity

The anti-inflammatory assay was done using the paw-fluid displacement method described by Winter *et al.* [10]. A using a total of 42 mice was used. The animals were grouped into 14 groups of 3 animals per group. Group 1 received 0.5 mL of distilled water, Group 2 received 50mg/kg of Diclofenac, Group 3, 4, 5 received 50, 100, and 200mg/kg of the crude ethanol extract respectively; Group 6, 7, 8 received 50, 100, and 200mg/kg of methanol fraction respectively; Groups 9, 10, 11 received 50, 100, and 200mg/kg of dichloromethane fraction respectively; while Groups 12, 13, 14 received 50, 100, and 200mg/kg of ethyl acetate fraction respectively. All the treatments were through oral route. One hour post-treatment inflammation was induced in all the animals by single sub-plantar injection of 0.05 mL of egg albumen at the right hind leg. The paw size was checked before and after inducing inflammation at 0.5, 1, 2, 3, and 4hrs. The volume of liquid displaced by the inflamed paw was used as a measure of the edema.

$$\text{Percentage Inhibition (\%)} = (a/b \times 100) - 100$$

Where;

a = mean paw volume of mice at 30 minutes

b = mean paw volume of mice at 4 hours

Antimicrobial Assay

Preparation of Stock Solutions

For the primary antimicrobial screening of the crude ethanol extract of *B. nivosa*, stock solution of the plant extract was prepared by dissolving 1000mg of the extract in 2mL of DMSO to obtain a final concentration of 500mg/mL. For determining the MICs, stock solution of the plant extract was prepared by dissolving 4000mg of the extract in 2mL of DMSO to attain a final concentration of 2000mg/mL. These were transferred to a screw capped bottle and stored at 4°C.

Primary Screening of Plant Extract for Antibacterial and Antifungal Activity

The agar well diffusion assay method described by Perez *et al*. [11] was used to evaluate the antibacterial and antifungal activities of the ethanol extract of *B. nivosa* against the test microorganisms. Dilutions of 250, 125, 62.5, 31.25, and 15.625mg/mL were prepared from the 500mg/mL stock solution of the plant extract in a 2-fold dilution process. Twenty (20) mL of molten Mueller Hinton Agar (MHA) and Sabouraud Dextrose Agar (SDA) (for bacterial and fungal isolates respectively) were poured into sterile Petri dishes (90 mm) and allowed to set. Standardized concentrations (McFarland 0.5) of overnight cultures of test isolates were swabbed aseptically on the agar plates and holes (6mm) were made in the agar plates using a sterile metal cork-borer. Twenty (20µl) of the various dilutions of the plant extract and control were put in each hole under aseptic condition, kept at room temperature for 1 hour to allow the agents to diffuse into the agar medium and incubated accordingly. Gentamicin (10µg/mL) and fluconazole (50µg/mL) were used as positive controls in the antibacterial and antifungal evaluations respectively; while DMSO was used as the negative control. The MHA plates were then incubated at 37^{o}C for 24hrs, and the SDA plates were incubated at room temperature (25-27^{o}C) for 2-3days. The inhibition zones diameters (IZDs) were measured and recorded. The size of the cork borer (6mm) was deducted from the values recorded for the IZDs to get the actual diameter. This procedure was conducted in duplicate and the mean IZDs calculated and recorded.

Determination of Minimum Inhibitory Concentration (MIC) of the Crude Extracts on Test Isolates

Minimum inhibitory concentration (MIC) is defined as the lowest concentration of the antimicrobial agent that inhibits the bacterial growth. The MICs of the plant extract on the test isolates was determined by the agar dilution method as described by Russell and Furr [12]. The stock solution (2000mg/mL) was further diluted in a 2-fold serial dilution to obtain the following concentrations: 1000, 500, 250, 125, 62.5, and 31.25 mg/mL. Agar plates were prepared by pouring 9mL of molten double strength MHA and SDA (for bacterial and fungal isolates respectively) into sterile Petri plates containing 1mL of the various dilutions of the extract making the final plate concentrations to become 200, 100, 50, 25, 12.5, 6.25, and 3.125 mg/mL.

The test isolates which were grown overnight in broth were adjusted to McFarland 0.5 standard and streaked onto the surface of the agar plates containing dilutions of the extract. The MHA plates were then incubated at 37^{o}C for 24hrs and the SDA plates were incubated at room temperature (25-27^{o}C) for 2-3days, after which all plates were observed for growth. The minimum dilution (concentration) of the extracts completely inhibiting the growth of each organism was taken as the MIC. This procedure was conducted in duplicate.

Results and Discussions

Results

The result of the phytochemical analysis of the plant is shown in Table 1. Figures 2, 3, and 4 show the results of the analgesic, anti-inflammatory, and antimicrobial activities of *B. nivosa* respectively. The MICs produced by the crude ethanol extract of the plant are shown in Table 2.

Table 1 Result of Phytochemical Analysis of *Breynia nivosa*

Analysis	Result
Alkaloids	+++
Flavonoids	+++
Glycosides	++
Tannins	++
Saponins	+
Starch	++

+++ = Highly abundant; ++ = Moderately abundant; + = Low

The results of phytochemical analysis of the plant leaves are seen in Table 1 above. The analysis revealed the abundance of alkaloids and flavonoids. Glycosides, tannins, and starch were present in moderate amounts, while saponins were detected in lower quantities.

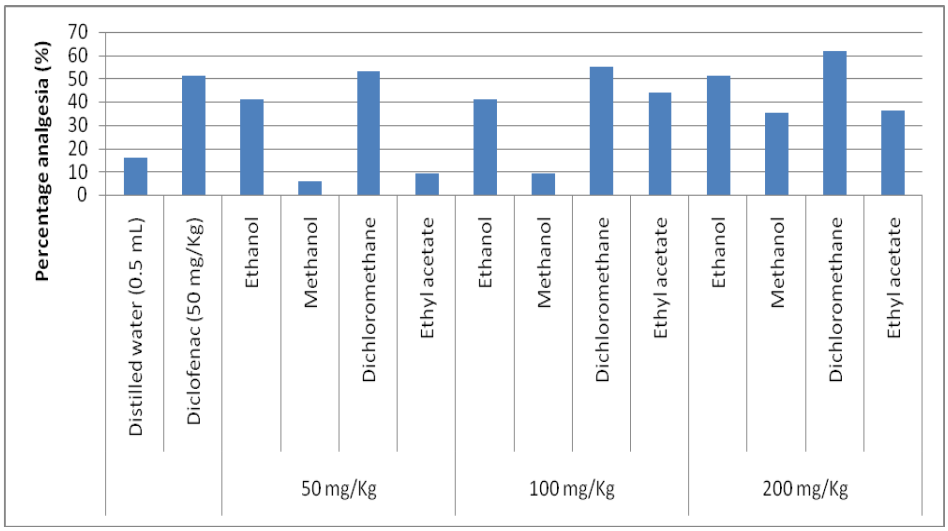

Figure 2 Analgesic activity of *B. nivosa* showing the Percentage analgesia

From Figure 2 above, it can be seen that analgesic activity was recorded for the crude ethanol extract and dichloromethane fraction of the plant at 50mg/Kg. At this concentration, the percentage analgesia recorded by the dichloromethane fraction (53%) was higher than that recorded for the standard drug Diclofenac (51%). At 100 and 200 mg/Kg all extracts of *B. nivosa* recorded analgesic activity (except the methanol extract at 100mg/Kg).

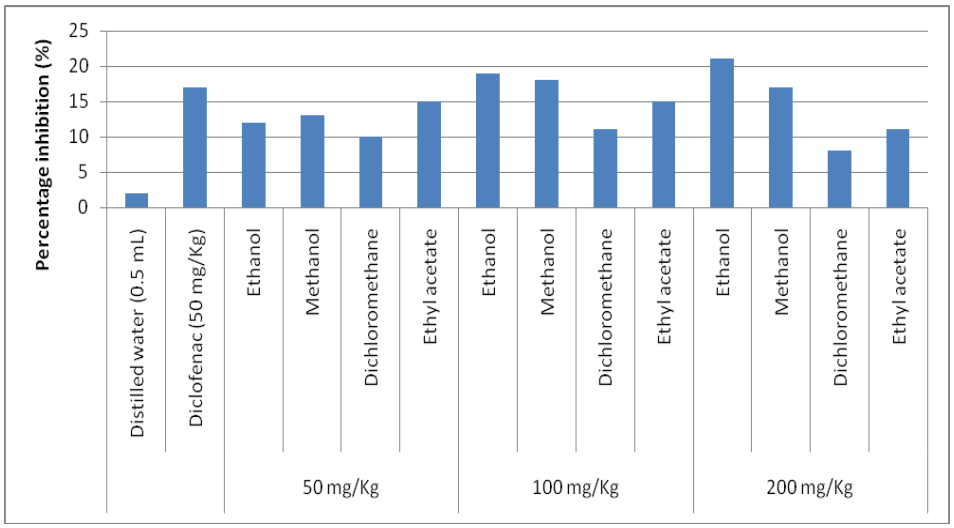

Figure 3 Anti-inflammatory activity of *B. nivosa* showing the Percentage inhibition

Anti-inflammatory activity was recorded for all the extracts of *B. nivosa* at 50, 100, and 200 mg/Kg as seen in Figure 3 above. But compared to the standard drug Diclofenac (50 mg/Kg), a higher anti-inflammatory activity was recorded only for the crude ethanol extract and methanol fraction of the plant at concentrations of 100 and 200 mg/Kg.

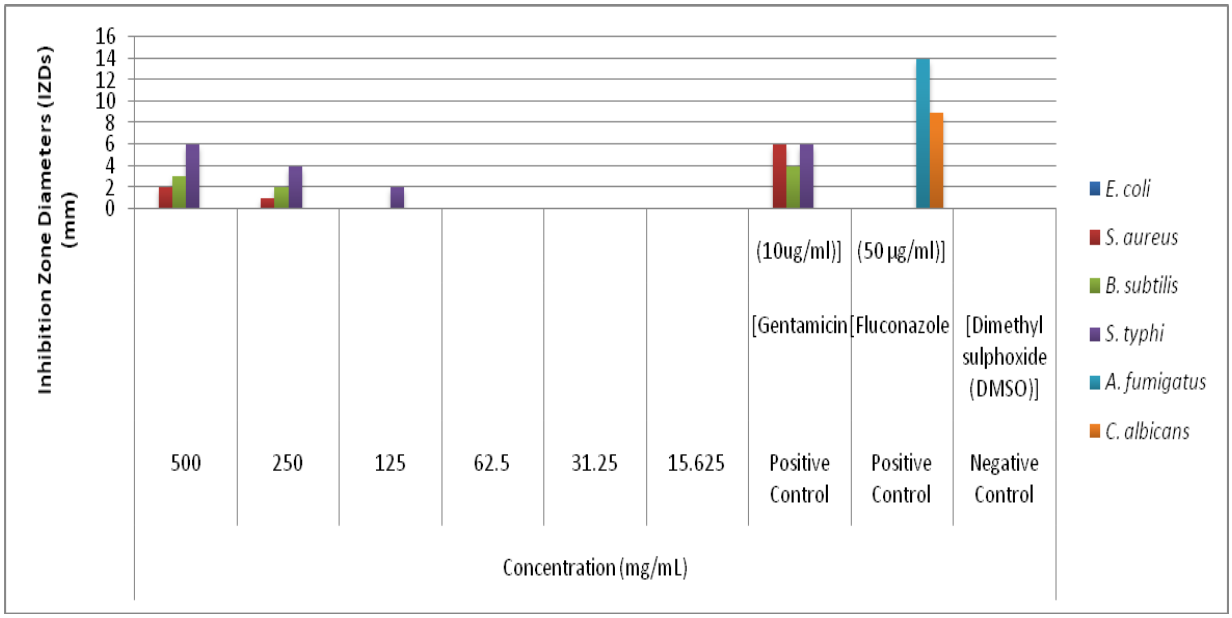

Figure 4 Mean Inhibition Zone Diameters (mm) Produced by the Crude Ethanol Leaf Extract of *B. nivosa* on Test Isolates

The antimicrobial activity of the crude ethanol extract of the plant was observed at concentrations of 500-125mg/mL. At 500mg/mL, inhibition zone diameters**(**IZDs) of 6, 3 and 2mm were recorded for *S. typhi, B. subtilis* and *S. aureus* respectively, at 250 mg/mL IZDs of 4, 2 and 1mm were recorded for *S. typhi*, *B. subtilis* and *S. aureus* respectively and at 125 mg/mL, an IZD of 2mm was recorded only for *S. typhi.*

Table 2 Minimum Inhibitory Concentrations (MICs) of the Crude Ethanol Leaf Extracts of *B. nivosa* on Test Organisms

Test Isolates	MICs (mg/mL)
E. coli	**-**
S. aureus	**200**
B. subtilis	**200**
S. typhi	**100**
A. fumigatus	**-**
C. albicans	**-**

Discussion

The preliminary phytochemical analysis of *B. nivosa* shows the presence of alkaloids, flavonoids, glycosides, tannins, saponins and starch. These phytocompounds have been shown to possess some pharmacological activities [8].

In Figure 1, the results of the assay of the plant extracts for analgesic activity can be observed. At a dose of 50 mg/kg, the plant extracts recorded percentage analgesia that ranged from 6-53%, with the dichloromethane fraction of the plant recording the highest activity (53%). The activity recorded by the dichloromethane fraction of the plant was higher than that recorded by the standard drug Diclofenac (51%) at the same concentration of 50 mg.The hot plate method is one of the most common tests of nociception that is based on a phasic stimulus of high intensity. Pain induced by thermal stimulus of the hot plate is specific for centrally mediated nociception [13]. The ability of the

extract to significantly prolong the reaction latency to thermally-induced pain in mice by the hot plate at 30 and 60min suggests that the extract may have a mild central analgesic activity [14].

Many medicinal plant products are used in Africa for the management of pain and inflammation and their efficacy and potency are traditionally acclaimed [14]. The analgesic activity exhibited by the plant suggests an added advantage in the use of the extract as an anti-inflammatory agent, since most inflammatory conditions are usually associated with pain as a secondary process resulting from the release of algesic mediators [15].

Figure 2 shows the results of the anti-inflammatory study. It can be observed that at 50 mg/kg, the plant extracts/fractions recorded percentage inhibition that ranged from 10-15%, with the ethyl acetate fraction of the plant recording the highest activity (15%). The standard drug Diclofenac, at same concentration (50mg/mL), recorded a percentage inhibition of 17%.This result reveals that the plants possess anti-inflammatory effects which were quite comparable to that of the standard drug Diclofenac.The observed effectiveness of the agent in the acute exudative phase of inflammation suggests the inhibition of histamine and/or serotonin as a likely mechanism of action [16].

B. nivosa has been reported to possess some antimicrobial properties [6]. From the results the antimicrobial screening of the ethanol fraction of *B. nivosa* on the test organisms, it can be observed that the plant has moderate antimicrobial activity against *S. aureus*, *S. typhi* and *B. subtilis.* It recorded no antimicrobial activity against *E. coli, A. fumigatus* and *C. albicans.* The minimum inhibitory concentrations (MICs) of the ethanol extract on the test isolates were determined using the agar dilution method and the results are as shown in Table 2. At the concentrations analyzed, the MICs ranged from 100-200 mg/mL. From Figure 4 and Table 2, it could be observed that the ethanol extract of *B. nivosa* recorded best activity recorded against *S. typhi* compared to other isolates tested.

Conclusion

The findings of this study reveal that *Breynia nivosa* possess anti-inflammatory, analgesic, and antimicrobial properties. This provides a scientific basis for the ethnomedicinal utilization of this plant. Further tests are needed to explore the exact mechanism of action at the molecular level and to know the actual constituents responsible for these activities.

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