

Antidiarrheal property of *Napoleona imperialis* may be due to Procyanidins and Ellagic acid derivatives

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ABSTRACT

Diarrhea is a global epidemic. Majority of the populace in developing Countries including Nigeria depend on *Napoleona imperialis* as a safer, more effective and affordable alternatives for treatment of diarrhea. This study evaluated the antidiarrheal potential of ethanol (leaf) extract and fractions of *N. imperialis* in Swiss albino mice. Acute toxicity test was performed to determine safe dose range before *in vivo* experiments. Castor-oil induced diarrhea and charcoal meal gastrointestinal motility test models were used. Antimicrobial activity on bacteria-implicated diarrhea, and HPLC analysis of the aqueous fraction (AF) were also evaluated. The result of the acute toxicity tests show that no death occurred at the test doses. Preliminary antimicrobial screening shows that the inhibitory zone diameter (IZD) of the extract has a weak antibacterial activity against sample organisms. The presence of procyanidin, 9- α -OH-pinoresinol, isoprunein and ellagic acid derivatives in the aqueous fraction were highlighted by the HPLC analysis. The AF produced more significant ($P < 0.05$) decrease in diarrhea than the other groups. This study shows that *N. imperialis* possesses antidiarrheal activity and hence justifies its folkloric use in the treatment of diarrhea.

INTRODUCTION

Diarrhea has been the second leading cause of death among children under the age of five globally (Victoria *et al.*, 2000). Nearly one in five children dies (about 1.5 million each year) from diarrhea. It kills more young children than AIDS, malaria or measles. Africa and South Asia are home to more than 80 per cent of child deaths due to diarrhea. Complications of diarrhea include malnutrition, diminished growth, and impaired cognitive development in resource-limited countries (UNICEF/WHO, 2009). There is need for effective control of diarrhea globally. Majority of the world's population living in developing countries depend on herbal remedies for treatment of diarrhea (Ezekwesili *et al.*, 2010). Current control measures for diarrhea

include oral rehydration therapy, zinc therapy, probiotics, antibiotics and anti-motility agents. Problems associated with these control measures include lack of accessibility, high cost and adverse effects of these medicines (Etuk *et al.*, 2009). Ways to achieve better control of this disease is to verify claims of antidiarrheal property of herbal remedies, as well as promotion of effective herbal remedies as alternative therapies. This idea makes sense since a large proportion of diarrhea patients use indigenous plants as medicine (Bako *et al.*, 2010). Furthermore, plant screening and isolation of active compounds can generate lead compounds for the drug market. Plants used in ethnomedicine such as; *Alchornea cordifolia* (Emudainohwo *et al.*, 2015), *Phoenix dactylifera* (Agbon *et al.*, 2013), and *Rubia Cordifolia* (Sayeed *et al.*, 2011) has been proven scientifically to elicit antidiarrheal properties.

Napoleona imperialis, (family *lecythidaceae*) is an indigenous plant used in some Nigerian communities for the treatment of diarrhea, and bacterial infections. The bark and fruit pulp are chewed to alleviate pulmonary problems.

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Its wound healing (Esimone *et al.*, 2005), anti-hypertensive (Omale *et al.*, 2011), anti-plasmodial (Ogbuehi *et al.*, 2014), and antimicrobial properties (Onyegbule *et al.*, 2011) has been demonstrated. Its leaf extract contains glycosides, tannins, proteins and saponins (Omale *et al.*, 2011). In this study, we sought for justification for the folkloric use of the leaves of *N. imperialis* as an antidiarrheal agent. This was done by use of castor-oil induced diarrhea and charcoal meal gastrointestinal motility test models. Since bacteria can cause diarrhea, and elimination of such infecting organism resolves the problem, we also assessed the antimicrobial activity of the test samples on common bacteria that cause diarrhea. Finally, HPLC analysis of the most active fraction was done to identify possible compounds responsible for the activity. The outcome of the study will either encourage or discourage the use of *Napoleona imperialis* in ethnomedicine. Positive outcome may result in development of affordable herbal formula for treatment of diarrhea. Very promising results would encourage further research towards identification of pure compounds responsible for any antidiarrheal property observed.

MATERIALS AND METHOD

Materials

Plant material

The leaves of *Napoleona imperialis* were collected in the morning from Agulu, Anambra State, Nigeria in the month of March, 2014, and were identified and authenticated by Mr. P.O Ugwuozo of the Department of Botany, Nnamdi Azikiwe University Awka, Anambra State, Nigeria. A voucher specimen number of 'PCG 474/A/040' was assigned to the plant sample.

Animals

Albino mice (20-28 g) of both sexes from the animal house of the Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria were housed under standard laboratory conditions for one week before the experiments began. They were properly fed with Vital Feeds (Nigeria) and had unrestricted access to water. The animals were handled in compliance with the National Institute of Health Guidelines for care and use of laboratory animals (Pub No. 85-23, revised 1985), as approved by the University's ethical committee on the use of Laboratory animals.

Test organisms

Pure cultures of *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, and *Klebsiella pneumonia*, obtained from the Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka-Nigeria were used in the study.

Culture Media, Reagents and other materials

Culture media used were nutrient agar, nutrient broth and Mueller-Hinton agar (Oxoid Limited, England). Reagents and

other materials used include: McFarland 0.5 turbidity standard (prepared from barium chloride, sulphuric acid and water), ethanol (Sigma-Aldrich Inc., Germany), sodium chloride (BDH Chemicals, England), distilled water, tween 80, dimethyl sulphoxide (DMSO), loperamide, castor oil, atropine, deactivated charcoal, oral cannula, needles and syringes.

Methods

Preparation of plant extract and fractions

The leaves of *N. imperialis* were air-dried at room temperature and pulverized. Then, 1 kg of powdered leaf was cold macerated with 70 % aqueous ethanol for 48 h, and filtered. Portions of the crude extract were used to obtain fractions (n-hexane, ethyl acetate, butanol and water) by solvent partitioning. The extract and fractions were dried at 40 °C using rotary evaporator and stored at -4 °C before use.

Acute toxicity test

The acute toxicity test was carried out as described by Lorke (1983). The doses (100, 500 and 1000 mg/kg) of the ethanol extract were administered to the mice after an overnight fast as phase 1 experiment. The animals were monitored for 24 h for mortality and signs of toxicity. Subsequently, other animals were given 2000, 3000, 4000 and 5000 mg/kg of the ethanol extract as phase 2 experiments, and monitored for 24 h.

Screening of extracts for antibacterial activity

The antibacterial activity of the extract was determined by the agar well diffusion method (Boyan *et al.*, 2008). Gentamicin (10 µg/ml), and DMSO were used as positive and negative controls respectively. Sample plates (n=2) were incubated at 37 °C for 24 h and the inhibition zone diameters (IZDs) measured.

Determination of minimum inhibitory concentration (MIC) of extracts on test isolates

The MIC of the plant extract was determined by the agar dilution method. The test isolates were grown for 18 h in Nutrient broth. Culture suspensions (adjusted to McFarland 0.5) were streaked onto the surface of the agar plates (n = 2) containing dilutions (25, 12.5, 6.25, 3.13, 1.56 and 0.78 mg/ml) of the extract. The plates were incubated at 37 °C for 24 h, and afterwards observed for growth.

Castor oil induced diarrhea

The animals were divided into 12 groups (n = 5). Castor oil induced diarrhea was carried out following the method described by Shah *et al.* (2011), with slight modification. The mice were fasted for 18 h followed by oral administration of test samples (250 and 500 mg/kg), which were dissolved in tween 80. Loperamide (2 mg/kg) and tween 80 (10 ml/kg) were used as positive and negative controls respectively. Thirty minutes later, diarrhea was induced by single oral administration of castor oil (0.5 ml). Then, the frequency and weights of feces (wet and solid)

released were monitored for 4 h. Percentage protection against the castor oil-induced diarrhea was calculated based on the number of dry feces in each cage in comparison to the wet feces.

Gastrointestinal motility test

The animals were divided into 12 groups ($n = 5$). The gastrointestinal motility test was carried out as described by Creuz *et al.*, (2009). The animals orally received test samples (250 and 500 mg/kg), which was followed 30 min later by oral administration of 0.5 ml of 5 % deactivated charcoal in mucilage of tragacanth. Atropine (10 mg/kg) and tween 80 (10 ml/kg) were used as positive and negative controls respectively. Animals were allowed further 30 min before being sacrificed by cervical dislocation. The distance traveled by the charcoal plug from the pylorus to the caecum was measured.

HPLC analysis

A quantity (2 mg) of the aqueous fraction was reconstituted with 2 ml of HPLC grade methanol. The mixture was sonicated for 10 min and thereafter centrifuged at 3000 rpm for 5 min. A quantity (100 μ g) of the sample was dissolved with 500 μ l of HPLC grade methanol. HPLC analysis was done using Dionex P580 HPLC system coupled to photodiode array detector (UVD340S, Dionex Softtron GmbH, Germany). Detection was at 235, 254 and 340 nm. The separation column (125 X 4 mm; length X internal diameter) was prefilled with Eurospher-10 C18 (Knauer, Germany), and a linear gradient of nano pure water (adjusted to pH 2 by addition of formic acid). Methanol was used as eluent. Compounds were detected using diode array and identified based on similarity with data in the inbuilt library

Data analysis

Results were expressed as mean \pm SEM (Standard error of mean). Student's *t*-tests were done using statistical package for social science (SPSS) version 16. Statistical significance was established when $P < 0.05$. Graphical illustrations were carried out using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

RESULTS

Acute toxicity studies

No sign of toxicity or death was observed in both phases.

Screening of extracts for antibacterial activity

Table 1 shows the crude extract exhibited antibacterial activity against all the test isolates. However, inhibitory zone

diameter of gentamicin (positive control) was almost twice that of the highest concentration of extract tested.

Determination of minimum inhibitory concentration (MIC) of extracts on test isolates

Result of the MIC presented in Table 2 shows that *E. coli* and *K. pneumonia* shared the same value and have a wider inhibition zone. Similarly, *S. aureus* and *S. typhi* shared the same value and have a lower inhibition zone.

Table 2: Minimum inhibitory concentrations (MICs) of ethanol extract of *N. imperialis*.

Test organisms	MICs (mg/ml)
<i>E. coli</i>	3.13 \pm 0.01
<i>S. aureus</i>	1.56 \pm 0.01
<i>S. typhi</i>	1.56 \pm 0.01
<i>K. pneumonia</i>	3.13 \pm 0.01

Values were presented as mean \pm Standard error of mean (SEM), $n = 2$.

Antidiarrheal activity

The order of inhibition on number of dry feces at 250 mg/kg dose is ethyl acetate fraction > crude extract > butanol fraction (Fig.1). At 500 mg/kg dose, the order is ethyl acetate fraction > crude extract > n-hexane fraction. The values for n-hexane fraction (250 mg/kg), butanol fraction (500 mg/kg), and aqueous fraction (at both doses) were similar.

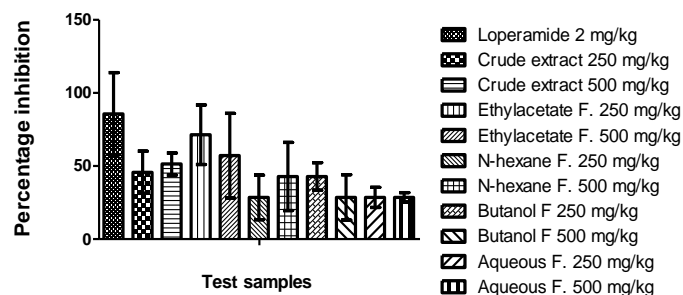


Fig. 1: percentage inhibition of ethanol extract and fractions of *N. imperialis* on number of dry feces in castor oil induced diarrhea.

The order of inhibition on number of wet feces at 500 mg/kg dose is aqueous fraction > ethyl acetate fraction > crude extract (Fig. 2). The values at 250 mg/kg for the crude extract and all the fractions were similar.

At 250 mg/kg dose, the order of inhibition of the weight of dry feces was; crude extract > aqueous fraction > butanol fraction > n-hexane fraction > ethyl acetate fraction (Fig. 3). At 500 mg/kg dose, the order of inhibition of the weight of dry feces was; aqueous fraction > butanol fraction = n-hexane fraction > crude extract > ethyl acetate fraction (Fig. 3).

Table 1: Preliminary antimicrobial screening of effect of ethanol extract of *N. imperialis* by agar well diffusion showing mean inhibitory zone diameter (mm).

Test Organism	Concentrations (mg/ml)							Controls	
	500	250	125	62.5	31.25	15.63	7.81	Positive	Negative
<i>E. coli</i>	11 \pm 0.01	10 \pm 0.01	8 \pm 0.01	5 \pm 0.00	4 \pm 0.00	4 \pm 0.00	4 \pm 0.00	24 \pm 0.00	0 \pm 00
<i>S. aureus</i>	8 \pm 0.00	7 \pm 0.00	6 \pm 0.00	4 \pm 0.00	3 \pm 0.00	2 \pm 0.00	2 \pm 0.00	19 \pm 0.01	0 \pm 00
<i>S. typhi</i>	9 \pm 0.01	8 \pm 0.01	7 \pm 0.01	6 \pm 0.00	5 \pm 0.00	5 \pm 0.00	3 \pm 0.00	21 \pm 0.00	0 \pm 00
<i>K. pneumonia</i>	10 \pm 0.00	8 \pm 0.00	6 \pm 0.00	5 \pm 0.00	3 \pm 0.00	3 \pm 0.00	2 \pm 0.01	19 \pm 0.01	0 \pm 00

Values were presented as mean \pm Standard error of mean (SEM). **Positive control:** Gentamicin, **Negative control:** Dimethyl sulphoxide.

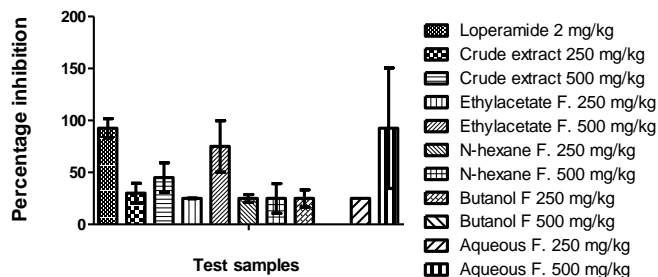


Fig. 2: percentage in hibition of ethanol extract and fractions of *N. imperialis* on number of wet feces in castor oil induced diarrhea.

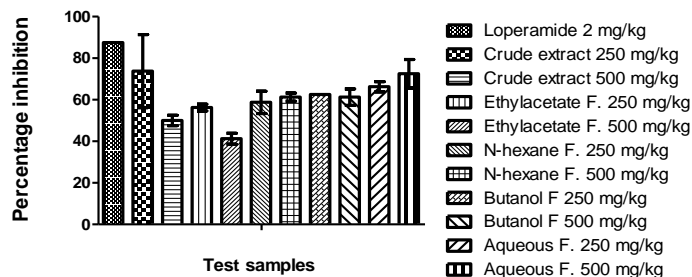


Fig. 3: percentage in hibition of ethanol extract and fractions of *N. imperialis* on weight of dry feces in castor oil induced diarrhea.

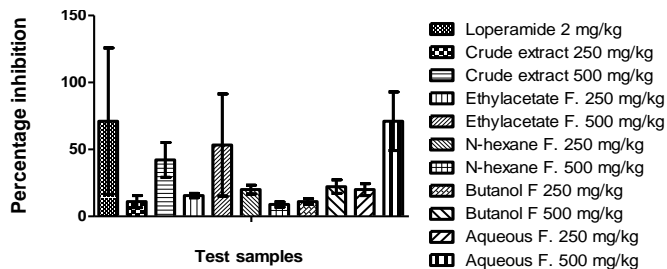


Fig.4: percentage in hibition of ethanol extract and fractions of *N. imperialis* on weight of wet feces in castor oil induced diarrhea.

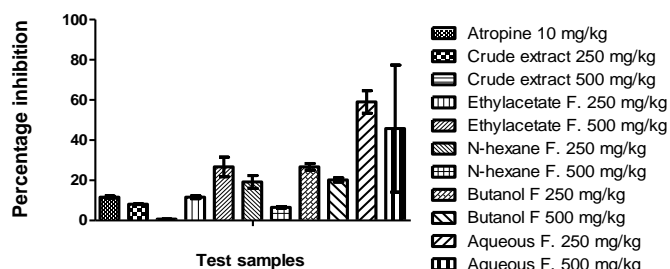


Fig.4: percentage inhibition of ethanol extract and fractions of *N. imperialis* on hyperkinetic gastro-intestinal tract.

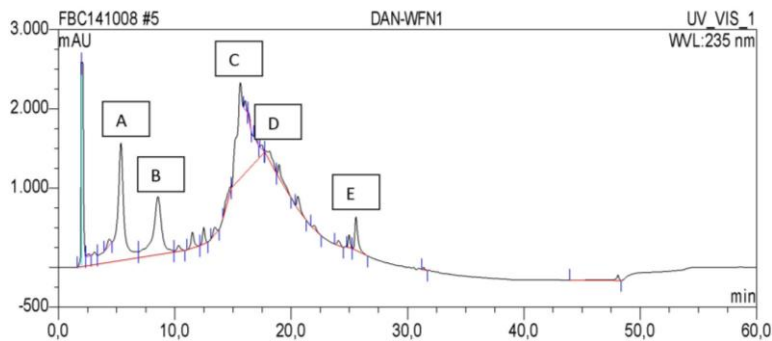


Fig. 6: HPLC analysis of the components in AF of *N. imperialis*. Where 'A' and 'B' represent procyanidin derivatives. 'C' represents 9, alpha-OH-pinoresinol derivative. 'D' represents Isoprunetin derivative and 'E' represents ellagic acid derivative.

At 500 mg/kg dose, the order of inhibition in weight of wet feces was; aqueous fraction > ethyl acetate fraction > crude extract (Fig. 4). At 250 mg/kg dose, the order of inhibition of gastrointestinal motility was aqueous fraction > butanol fraction > n-hexane fraction > ethyl acetate fraction > crude extract (Fig. 5).

At 500 mg/kg dose, the order of inhibition of gastrointestinal motility was aqueous fraction > butanol fraction > ethyl acetate fraction > n-hexane fraction > crude extract (Fig. 5).

HPLC analysis in figure 6 shows that the aqueous fraction contains larger quantities of 9, alpha-OH-pinoresinol and isoprunetin derivatives, than other components (ellagic acid and procyanidin derivatives).

DISCUSSION

The morbidity and mortality associated with diarrhea underscores the need for its effective control (Victoria *et al.*, 2000). Due to problems enumerated earlier, there is need for

alternative measures that will drive the achievement of the Millenium Development Goals. One of the alternatives may lie within the highly patronized plant and plant products used as medicines in many developing countries (Ezekwesili *et al.*, 2010). In addition, plants hold promise as sources of new drugs. In the light of this, we investigated the antidiarrheal potential of ethanol leaf extract and fractions of *Napoleona imperialis*.

The absence of death and toxic signs in both phases of acute toxicity tests suggests that the crude extract has a high safety margin.

The observed antidiarrheal property may not result from the anti-bacteria property of the plant. Bacteria known to cause diarrhea include *E. coli*, *S. aureus*, *Sal. typhi* and *K. pneumonia* (Serangi *et al.*, 2010). Diarrhea resulting from infections is quickly resolved when the infecting organism is dislodged. The Kirby-Bauer and MIC tests suggest that the extract has low activity against the isolates. This is understood considering that the IZDs of gentamicin (positive control) were almost twice that of the

highest tested extract concentrations. The values of the IZD show that the extract has a comparatively weak antibacterial activity against the organisms. Similarly, the MIC values (which are in mg/ml) are generally higher than values used in clinics for treatment. Taken together, it is obvious that the extract lacks strong anti-bacterial property.

Castor oil induces diarrhea by releasing ricinoleic acid, thereby causing a change in the integrity of the fluid and electrolyte balance in the mucosa of the gastrointestinal tract (Rajeev *et al.*, 2010). In this study, castor oil increased the number of wet and dry feces as well as weight of solid and wet feces. Agents with antidiarrheal properties are evaluated by their ability to delay diarrhea latency and decrease weight and number of diarrhea feces (Atiqur *et al.*, 2011).

It appears most of the antidiarrheal compounds lie in the aqueous fraction. Although the weights of dry feces for crude extract (250 mg/kg) and aqueous fraction (500 mg/kg) were comparable in the castor oil induced diarrhea (Fig. 1-4), the aqueous fraction (500 mg/kg) had better control in number and weight of wet feces than the crude extract. Similarly, it is noteworthy that the performance of the aqueous fraction (500 mg/kg) is close to that of the positive control in weight of dry feces, and number and weight of wet feces.

In this study, the charcoal meal test demonstrates that the aqueous fraction has a superior inhibition of the gastrointestinal movement than the reference drug (atropine) at the test doses (Fig. 5). Antidiarrheal agents decrease distance traveled by charcoal meal, or increase re-absorption of water (Rajabhai *et al.*, 2011). The aqueous fraction exhibited more activity than the other test samples. Although, ethylacetate is next to aqueous fraction in ranking of strength of inhibition, it is noteworthy that some of the compounds in the aqueous fraction may also be present in the aqueous fraction due to the polar status of both solvents.

Procyanidins and ellagic acid may be responsible for the observed antidiarrheal property since previous studies in other plants have identified them to be responsible for such activities (Hai-Tao Xiaoa, *et al.*, 2013). Procyanidins are condensed tannins while ellagic acid is the dilactone of hexahydroxydiphenic acid. Both compounds are natural polyphenolic compounds. Plants produce ellagic acid from hydrolysis of tannins such as ellagitannin and geraniin. Raji *et al.*, (2001) reported that methanol extract of the stem bark of *Irvingia gabonensis* which possesses 2,3,8-Tri-O-methyl ellagic acid has antidiarrheal and antiulcer properties in rats. Furthermore, Derivatives of pinoresinol and isoprunetin are not known for possessing antidiarrheal property. Consequently, it is reasonable to suspect that the procyanidins and ellagic acid derivatives present in *N.imperialis* (Fig. 6) may be responsible, either partly or wholly for the antidiarrheal property observed.

CONCLUSION

This study has demonstrated that the ethanol extract and fractions of *N. imperialis* exhibit antidiarrheal properties, which

may be due to derivatives of procyanidin and ellagic acid. This conclusion justifies its ethno-medicinal use in the treatment of diarrheal. Further studies on the isolated compounds are necessary to ascertain the possible mechanism(s) of action.

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DECLARATION OF INTEREST

The authors declare that there are no conflicts of interest.

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