PRELIMINARY ANTIMICROBIAL AND PHYTOCHEMICAL STUDY OF THE AQUEOUS, ETHANOL, METHANOL AND CHLOROFORM EXTRACTS OF THE LEAVES OF *NAPOLEONAEA IMPERIALIS* P. BEAUV. (LECYTHIDIACEAE)

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Abstract: The antimicrobial activity and phytochemical analysis of *Napoleonaea imperialis* P. Beauv. (Lecythidiaceae) was done using aqueous, ethanol, methanol and chloroform leaf extracts to determine its antimicrobial and phytochemical constituents. The antimicrobial activities of the extracts were tested against bacteria and fungi isolates using the agar well diffusion method. Commercial antibiotics were used as positive reference standards to determine the sensitivity of the isolates. The leaf extract was subjected to phytochemical analysis using standard experimental procedures. The extracts showed significant inhibitory activity against the bacterial and fungal isolate (Bacterial isolates- *Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Klebsiella pneumonia, Proteus mirabilis, Pseudomonas aeruginosa;* fungal isolates- *Penicillum notatum, Aspergillus niger, Fusarium oxysporum, Saccharomyces cerevisae,* and *Candida albicans*). The MIC values obtained using the Agar-dilution test ranged from 0.5-10mg/ml. The results showed that the extract of *N. imperialis* plant leaves have broad spectrum of antimicrobial activity. These results suggest that it will be useful in the treatment of microbial infections.

Keywords: Aqueous extract, antimicrobial activity, chloroform extract, ethanol extract, methanol extract, *Napoleonaea imperialis*, phytochemical analysis

INTRODUCTION

Medicinal plants are the oldest sources of pharmacologically active compounds. They provide virtually the only source of medicinally useful compounds for centuries (Cordell, 1981). Benefits derived from using medicine obtained from plants are that they are relatively safer than synthetic alternatives by offering profound therapeutic benefits and more affordable treatment (Iwu *et al*., 1999). Antimicrobial agents are among the most commonly used and misused of all drugs. The inevitable consequences of the wide spread use of antimicrobial agents has been the emergence of antibiotic resistant pathogens, fuelling an ever increasing need for drugs (Chambers, 2006).

Herbal remedies used in traditional medicine provide an interesting and still largely unexplored chemotherapy which might help to overcome the growing problem of drug resistance and also the toxicity of currently available commercial antibiotics (Al-Wadh-Ali *et al*., 2001). The plant *Napoleonaea imperialis* P. Beauv. belongs to the family lecythidiaceae. It is a tree that grows often more than 6m high. It is the most widespread *Napoleonaea* in Nigeria. The leaves are large, sometimes reaching nearly 30cm length and 8cm breadth. The fruit of *Napoleonaea imperialis* is brownish to reddish, spotted with a depressed centre often slightly warted. Ethnobotanically, *Napoleonaea imperialis* is given as a treatment of asthma and cough using the bark decoction in low doses. The twigs are chewed as chewing sticks for cleaning teeth.

This study aims at determining the phytochemical and antimicrobial properties of the aqueous, ethanol, methanol and chloroform extracts of the leaves of *Napoleonaea imperialis*.

MATERIAL AND METHOD

Collection, Identification and Preparation of Plant materials

The leaves of *Napoleonaea imperialis were* collected from farmlands around the University of Benin and environs. It was identified by Professor Macdonald Idu of the Department of Plant Biology and Biotechnology, University of Benin, Benin City. The leaves were dried in an open air under shade to prevent ultra violet rays from inactivating the chemical constituents. The leaves were grounded to fine particles. 50g of the leaves were soaked in 300ml of sterile water; 50g of grounded leaves were also soaked in 250ml ethanol, methanol and chloroform, all in their respective bottles. They were left for 24hrs at room temperature with occasional thorough stirring. The extracts were filtered and concentrated to dryness by evaporation and was stored in refrigerator until required for use.

Test organisms

The microorganisms used for the study include clinical isolates of *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Proteus mirabilis, Aspergillus niger, Penicillum notatum, Saccharomyces cerevisae, Fusarium oxysporum* and *Candida albicans* for the bacterial and fungal isolates respectively. The microorganisms were obtained from pure cultures of the laboratory stock of the Department of Microbiology, University of Benin Teaching

Hospital and Edo State Environmental Laboratory, Benin City, Nigeria.

Test for Antimicrobial activity

The diluted aqueous, ethanol, methanol and chloroform extracts of leaves of the plant was tested for its antimicrobial properties using agar well diffusion method (Pelezar *et al.*, 1993; Barry and Thornsberry, 1995). Nutrient agar was used as the medium for testing the bacteria. Potato dextrose agar was also used as the medium for testing the fungi. Plates of the agar were prepared. Each plate was seeded with a test organism. After which, wells were bored on the surface of inoculated agar plates using 6mm cork borer. The wells were filled separately with the different concentrations of each extracts and were sufficiently spaced to prevent the resulting zones of inhibition from overlapping. The plates were then incubated at 37℃ for 24 hours for bacteria, and also incubated at 25℃ for 72 hours for fungi cultures. The resulting zones were measured and recorded. Standard antibiotic disc was placed on the agar plates as positive control after which the plates were incubated overnight at 37℃ and 25℃ for bacteria and fungi cultures respectively. At the end of the incubation period, the diameter of inhibition zone(s) were measured and recorded. Standard commercial antibiotic disc was placed on the agar plates as positive control (susceptibility testing) after which the plates were incubated overnight at 37°C and 25°C for bacteria and fungi cultures respectively. At the end of the incubation period, the diameter of inhibition zone(s) were measured and recorded.

Determination of Minimum Inhibitory Concentration (MIC)

The standard agar dilution protocol with doubling dilution was used. The extract was incorporated into nutrient agar at concentrations of 10mg/ml, 5mg/ml and 0.5mg/ml. A control without the extract was also set up. Each of the test organisms previously diluted was used to inoculate the plates. These were incubated at 37°C for 24hours for bacteria and 25°C for 48hours for fungi after which the results were recorded after observing for growth. The minimum inhibitory concentration (MIC) of the extract for each test organism was regarded as the agar plate with the lowest concentration without growth.

Minimum Bactericidal Concentration (MBC) Determination

The minimum bactericidal concentration (MBC) of the plant extracts was determined by a modification of the method of Spencer and Spencer (2004). Samples were taken from plates with no visible growth in the MIC assay and sub-cultured on freshly prepared nutrient agar plates and later incubated at 37℃ for 24hours for bacteria. The MBC was taken as the concentration of the extract that did not show any growth on a new set of agar plates.

Phytochemical Screening of the Extracts of the Leaf

The aqueous, ethanol, methanol and chloroform leaf extracts were tested for the presence of saponins, flavonoids, tannins, phlobatannins, steroids, terpenoids, cardiac glycosides, alkaloids, anthracene and reducing sugar using standard procedures to identify the constituents as described by Sofowora (1993), Trease and Evans (1989) and Harborne (1973). Quantitative determination was also carried out.

Statistical analysis

Results were expressed as means ± standard error of mean (S.E.M) and the level of significance between means were computed by student's t- test using SPSS 14.0 computer software package. The level of significance was determined at 0.05.

RESULTS

The results of the antimicrobial activity of the the aqueous, ethanol, methanol and chloroform leaf extracts of *N. imperialis are* shown in Tables 1 to 4. The inhibitory activity of the commercial antibiotics (standard sensitivity disc and ketoconanzone) on the test bacteria and fungi respectfully are presented in Table 5.Table 6 and 7 represents the results of the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) for the leaf extracts of the plant. The results of the phytochemical screening of all the leaf extracts of *N. imperialis* showed the presence of tannins, saponins, flavonoids, steroids, terpenoids, cardiac glycosides and reducing sugars (Table 8) . Alkaloid was found present only in the aqueous extract of *N. imperialis* while steroids, flavonoids and terpenoids were observed present only in the chloroform leaf extract..

Table 1. The effect (zone of inhibition in mm) of aqueous leaf extract of *Napoleonaea imperialis* of various concentrations on test organisms.

Test Organisms	Zone of Inhibition (mm)			Sterile distilled water
	10mg/ml	5mg/ml	0.5mg/ml	
Bacillus subtilis	$17.0^{a} \pm 0.0$	$12.5^{b} \pm 0.5$	$9.0^{c} \pm 0.0$	-

Escherichia coli	$19.0^{a} \pm 1.0$	$11.0^{ab} \pm 3.0$	$6.5^{b} \pm 3.5$	-
Proteus mirabilis	$16.5^{a} \pm 2.5$	$11.0^{b} \pm 3.5$	$4.0^{c} \pm 3.52$	-
Pseudomonas aeruginosa	$14.5^{a} \pm 2.5$	$9.5^{a} \pm 3.5$	$6.5^{a} \pm 3.5$	-
Staphylococcus aureus	$16.5^{a} \pm 3.5$	$12.5^{a} \pm 4.5$	$4.0^{a} \pm 4.0$	-
Klebsiella pneumonia	$19.0^{a} \pm 5.0$	$11.0^{ab} \pm 1.0$	$5.0^{b} \pm 1.0$	-
Penicillum notatum	$11.5^{a} \pm 0.5$	$7.5^{ab} \pm 1.5$	$2.0^{b} \pm 2.0$	-
Aspergillus niger	$17.0^{a} \pm 1.0$	$4.0^{b} \pm 0.0$	$1.5^{b} \pm 1.5$	-
Fusarium oxysporum	$19.5^{a} \pm 0.5$	$6.5^{b} \pm 3.5$	$1.5^{b} \pm 1.5$	-
Saccharomyces cerevisae	$16.5^{a} \pm 0.5$	$6.0^{b} \pm 1.0$	$1.0^{c} \pm 1.0$	-
Candida albicans	$4.5^{a} \pm 0.5$	$3.0^{a} \pm 1.0$	$1.0^{a} \pm 1.0$	-

Note: Values are means ± S.E.M of two measurements across each zone of inhibition. Means ± S.E.M with different superscript within a row are significantly different, $P < 0.05$.

Table 2. The effect (zone of inhibition in mm) of ethanol leaf extract of *Napoleonaea imperialis* of various concentrations on test organisms.

Test Organisms	**Zone of Inhibition (mm)**		
	10mg/ml	**5mg/ml**	**0.5mg/ml**
Bacillus subtilis	$9.0^{a} \pm 3.0$	$5.0^{a} \pm 1.0$	$4.0^{a} \pm 2.0$
Escherichia coli	$7.0^{a} \pm 7.0$	$10.5^{a} \pm 0.5$	$3.0^{a} \pm 1.0$
Proteus mirabilis	$11.0^{a} \pm 3.0$	$7.0^{a} \pm 0.0$	$2.5^{a} \pm 1.0$
Pseudomonas aeruginosa	$12.0^{a} \pm 2.0$	$7.0^{ab} \pm 1.0$	$2.5^{b} \pm 2.5$
Staphylococcus aureus	$11.0^{a} \pm 3.0$	$7.0^{a} \pm 1.0$	$2.5^{a} \pm 2.5$
Klebsiella pneumonia	$10.5^{a} \pm 1.5$	$6.0^{ab} \pm 2.0$	$2.0^{b} \pm 2.0$
Penicillum notatum	$26.5^{a} \pm 0.5$	$19.0^{b} \pm 2.0$	$5.0^{c} \pm 1.0$
Aspergillus niger	$17.0^{a} \pm 5.0$	$3.5^{b} \pm 0.5$	$1.0^{b} \pm 1.0$
Fusarium oxysporum	$20.5^{a} \pm 2.5$	$6.5^{b} \pm 0.5$	$4.5^{b} \pm 0.5$
Saccharomyces cerevisae	$10.5^{a} \pm 2.5$	$4.0^{b} \pm 0.0$	$2.0^{b} \pm 0.0$
Candida albicans	$14.5^{a} \pm 1.5$	$7.5^{b} \pm 0.5$	$3.5^{b} \pm 0.5$

Note: Values are means ± S.E.M of two measurements across each zone of inhibition. Means ± S.E.M with different superscript within a row are significantly different, $P < 0.05$.

Table 3. The effect (zone of inhibition in mm) of methanol leaf extract of *Napoleonaea imperialis* of various concentrations on test organisms.

Test Organisms	**Zone of Inhibition (mm)**		
	10mg/ml	**5mg/ml**	**0.5mg/ml**
Bacillus subtilis	$14.5^{a} \pm 2.5$	$9.5^{a} \pm 4.5$	$5.5^{a} \pm 3.5$
Escherichia coli	$13.0^{a} \pm 1.0$	$4.5^{b} \pm 0.5$	$4.0^{b} \pm 1.0$
Proteus mirabilis	$13.0^{a} \pm 3.0$	$8.0^{b} \pm 0.0$	$4.0^{c} \pm 0.0$
Pseudomonas aeruginosa	$17.5^{a} \pm 2.5$	$12.5^{a} \pm 0.5$	$6.0^{a} \pm 6.0$
Staphylococcus aureus	$17.5^{a} \pm 0.5$	$11.5^{b} \pm 1.5$	$10.5^{b} \pm 1.5$
Klebsiella pneumonia	$13.0^{a} \pm 2.0$	$7.0^{ab} \pm 1.0$	$4.5^{b} \pm 1.5$
Penicillum notatum	$13.5^{a} \pm 0.5$	$4.5^{b} \pm 0.5$	$0.0^{c} \pm 0.0$
Aspergillus niger	$12.0^{a} \pm 2.0$	$5.0^{b} \pm 0.0$	$1.0^{b} \pm 1.0$
Fusarium oxysporum	$9.5^{a} \pm 1.5$	$4.5^{b} \pm 0.5$	$1.0^{b} \pm 0.0$
Saccharomyces cerevisae	$13.5^{a} \pm 2.5$	$4.5^{b} \pm 0.5$	$0.0^{b} \pm 0.0$
Candida albicans	$17.0^{a} \pm 1.0$	$10.5^{ab} \pm 0.5$	$5.0^{b} \pm 3.0$

Note: Values are means ± S.E.M of two measurements across each zone of inhibition. Means ± S.E.M with different superscript within a row are significantly different, $P < 0.05$.

Table 4. The effect (zone of inhibition in mm) of chloroform leaf extract of *Napoleonaea imperialis* of various concentrations on test organisms.

Test Organisms	Zone of Inhibition (mm)		
	10mg/ml	5mg/ml	0.5mg/ml
Bacillus subtilis	6.0 ± 1.0^{a}	$2.0^{ab} \pm 2.0$	$0.0^{b} \pm 0.0$
Escherichia coli	$6.5^{a} \pm 1.5^{a}$	$3.0^{a} \pm 1.0$	$1.5^{a} \pm 0.0^{a}$
Proteus mirabilis	$7.5^{a} \pm 0.5^{a}$	$4.0^{b} \pm 0.0$	$0.0^{c} \pm 0.0$
Pseudomonas aeruginosa	$8.0^{a} \pm 1.0^{a}$	$5.5^{ab} \pm 1.5$	$0.0^{b} \pm 0.0$
Staphylococcus aureus	$3.5^{a} \pm 1.5^{a}$	$1.0^{a} \pm 1.0$	$0.0^{a} \pm 0.0$
Klebsiella pneumonia	$4.5^{a} \pm 0.5^{a}$	$0.0^{b} \pm 0.0$	$0.0^{b} \pm 0.0$
Penicillum notatum	0.0± 0.0	0.0± 0.0	0.0± 0.0
Aspergillus niger	$2.5^{a} \pm 2.5^{a}$	$0.0^{a} \pm 0.0^{a}$	$0.0^{a} \pm 0.0^{a}$
Fusarium oxysporum	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
Saccharomyces cerevisae	$3.5^{a} \pm 0.5^{a}$	$0.0^{b} \pm 0.0$	$0.0^{b} \pm 0.0$
Candida albicans	$6.5^{a} \pm 1.5^{a}$	$0.0^{b} \pm 0.0$	$0.0^{b} \pm 0.0$

Note: Values are means ± S.E.M of two measurements across each zone of inhibition. Means ± S.E.M with different superscript within a row are significantly different, $P < 0.05$.
0 = No inhibition

Table 5. Susceptibility testing (sensitivity test) on the test organisms.

Test Organisms	Zone of Inhibition (mm)		Ketoconazole (200 mg/ml)
	Gentamycin (10µg)	Ciprofloxacin (10µg)	
Proteus mirabilis	24		
Pseudomonas aeruginosa	20		
Klebsiella pneumonia		20	
Staphylococcus aureus		18	
Escherichia coli		20	
Bacillus subtilis		15	
Penicillum notatum			20
Aspergillus niger			20
Fusarium oxysporum			18
Saccharomyces cerevisae			16
Candida albicans			22

Table 6. Minimum inhibitory concentration of aqueous, ethanol, methanol and chloroform leaf extracts of *napoleonaea imperialis* on test organisms.

Test Organisms	Concnetration of Extracts (mg/ml)			
	Aqueous	Ethanol	Methanol	Chloroform
Bacillus subtilis	5	10	10	10
Escherichia coli	0.5	0.5	5	0
Proteus mirabilis	10	10	10	0
Pseudomonas aeruginosa	0.5	0.5	10	0
Staphylococcus aureus	0.5	0.5	0.5	0
Klebsiella pneumonia	10	0.5	0.5	0
Penicillum notatum	10	10	0	0
Aspergillus niger	0	0	0.5	0

Fusarium oxysporum	5	10	0.5	0
Saccharomyces cerevisae	10	0.5	0	0
Candida albicans	0	10	10	0

Key: 0= Growth at All Concentrations (i.e. MIC not determined).

Table 7. Minimum Bactericidal Concentration Of Aqueous, Ethanol, Methanol And Chloform Leaf Extracts of *Napoleonaea imperialis* on the test bacteria.

Test Bacteria	Concentration of Extracts(mg/ml)			
	Aqueous	Ethanol	Methanol	Chloroform
Bacillus subtilis	5	10	10	0
Escherichia coli	10	0.5	5	0
Proteus mirabilis	0	0	10	0
Pseudomonas aeruginosa	0.5	0	0	0
Staphylococcus aureus	0	0.5	0.5	0
Klebsiella pneumonia	0	0.5	10	0

Key: 0 = Not bactericidal

Table 8. Qualitative determination of Leaf extracts of *Napoleonaea imperialis*

Phytochemical constituents	Aqueous extract	Ethanol extract	Chloroform extract	Methanol extract
Saponins	+	+	-	+
Tannins	+	+	-	+
Phlobatannins	-	-	-	-
Steroids	+	+	+	+
Flavonoids	+	+	+	+
Terpenoids	+	+	+	+
Cardiac glycosides	+	+	-	+
Alkaloids	+	-	-	-
Reducing sugars	+	+	-	+
Anthracene	-	-	-	-

Key: - = Absent, + = Present

Table 9. Quantitative determination of the phytochemical constituents of the leaf of *Napoleonaea imperialis*.

Plant	Tannin (%)	Saponin (%)	Terpenoid (%)	Flavonoid (%)	Reducing sugar (%)
Napoleonaea imperialis	0.17	7.77	0.10	4.53	17.21

DISCUSSION

Plants are important source of potentially useful substancess for the development of new chemotherapeutic agents. Scientific reports abounds on the antiviral, antibacterial, antifungal, antihelmintic, antimolluscal and anti-inflammatory properties of plants (Samy and Ignacimuthu, 2000, Palombo and Semple, 2001).

The aqueous, ethanol, and methanol extracts of *N. imperialis* revealed strong antimicrobial activities against all the test organisms than the chloroform extract. It was observed that with increasing concentration of the extracts sensitivity increased. *N. imperialis* showed inhibitory activities against both the Gram positive and Gram negative bacteria as well as the tested fungi isolates. The activity of the aqueous leaf extract of *Napoleonaea imperialis* at different concentrations (10mg/ml, 5mg/ml, and 0.5mg/ml) was significantly different ($p < 0.05$) on the test organisms (*Bacillus subtilis, Escherichia coli, Proteus mirabilis,* and *Klebsiella pneumonia*). The effect of the ethanol leaf extract of *Napoleonaea imperialis* was significantly different ($p < 0.05$) from one concentration to another on *Pseudomonas aeruginosa* and *Klebsiella pneumonia.* There was no significant difference in all the concentrations on *Bacillus subtilis, Escherichia coli, Proteus mirabilis,*

and *Staphylococcus aureus* (Table 2). The minimum inhibitory concentration (MIC) values of all the extracts (aqueous, ethanol, methanol and chloroform) of the plant, is a confirmation of its ethnomedical use for the treatment of stomach disturbances, urinary tract infections, skin infections and fungal infections. These results agree with the findings of Kurosaki and Nishi (1983) that higher concentrations of antimicrobial substances of the same extracts could show appreciably more growth inhibitions in being both bacteriostatic and bactericidal. Chah *et al.,* (2006) demonstrated antibacterial and wound healing properties of *N. imperialis* in rats. In another experiment, Esimone *et al.,* (2005) prepared an herbal ointment of the methanolic extract of *N. imperialis* and evaluated its wound healing effect by excision wound model on guinea pigs.

Identifying the phytochemical constituents of a plant can help one speculate on its medicinal value. Tannins, saponins, flavonoids, steroids, terpenoids, cardiac glycosides and reducing sugars were all observed present in *N. imperialis* (Table 8). Tannins have antimicrobial (Ya *et al.,* 1988) and antioxidant properties. Parekh and Chanda (2007) reported that tannins are known to react with proteins to provide the typical tanning effect which is important for the treatment of inflamed or ulcerated tissues. These observations therefore support the use of *N. imperialis* in herbal remedies. The presence of tannins also showed that the leaves of the plant could be used as purgative, for cough, asthma and hay fever according to Gill (1992). Saponin was found to be present in *N. imperialis* and supported the usefulness of the plant in managing inflammation. Saponins has been shown to have immense significance as antihypercholesterol, hypotensive and cardiac depressant properties (Trease and Evans, 1985; Price, 1987). Steroidal compounds present in *N. imperialis* extracts are of importance and interest due to their relationship with various anabolic hormones including sex hormones (Okwu, 2001). Cardiac glycosides have been used for over two centuries as stimulants in cases of cardiac failure (Trease and Evans, 1985; Olayinka *et al.,* 1992). This perhaps justifies the functions of the plant in treatment and management of hypertension.

CONCLUSION

In conclusion, this plant is confirmed as useful antimicrobial agents. Bioactive substances from this plant can therefore be employed in formulation of antimicrobial agents for the treatment of various bacterial and fungal infections and can be used for treatment of chronic and degenerative diseases such as cardiovascular diseases.

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