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Ibiba Reuben Jack
Department of Chemistry, Rivers State University, Nkpolu-Oroworukwo, Port Harcourt, Rivers, Nigeria

Rachael Elijah Ekong
Department of Chemistry, Rivers State University, Nkpolu-Oroworukwo, Port Harcourt, Rivers, Nigeria

Gloria Ihuoma Ndukwe
Department of Chemistry, Rivers State University, Nkpolu-Oroworukwo, Port Harcourt, Rivers, Nigeria

Corresponding Author:
Gloria Ihuoma Ndukwe
Department of Chemistry, Rivers State University, Nkpolu-Oroworukwo, Port Harcourt, Rivers, Nigeria

Phytochemical constituents of dichloromethane fraction and essential oil of *Napoleonaea imperialis* rind

Ibiba Reuben Jack, Rachael Elijah Ekong and Gloria Ihuoma Ndukwe

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Abstract
Napoleonaea imperialis P. Beauv. (Family Lecythidaceae) is a medicinal plant predominantly found in South-Eastern Nigeria. Essential oil (1.92 g, 0.4%) was extracted from the rind of *N. imperialis* through hydro distillation using a Clevenger-apparatus. Thirteen compounds constituting 99.96% of the overall components were identified using gas chromatography-mass spectrometry (GC-MS). Monoterpenoids (geranial, neral and citronellol) made up 53.46% of the total constituents. (Z,Z)-3-Hexenyl-3-hexenoate (10.56%) and (Z)-9, 17-octadecadienal (7.75%) were also identified as major constituents of the essential oil. Methanol crude extract of the rind was partitioned with n-hexane, dichloromethane, and methanol to yield n-hexane fraction (7.4%), dichloromethane fraction (23.7%) and methanol fraction (68.7%). GC-MS analysis of the dichloromethane fraction showed the presence of thirty-six compounds constituting 98% of the total constituents with oleic acid having the highest percentage (28.04%). n-Hexadecanoic acid was identified in both essential oil and dichloromethane fraction of the rind. Fatty acid methyl esters, fatty acids and glycosides were also present in the dichloromethane fraction. Phytochemical screening of compounds in the three fractions were carried out using thin layer chromatography with different spray reagents. Anisaldehyde-sulphuric acid spray reagent produced purple, violet and grey spots confirming the presence of terpenoids, saponins and steroids respectively in the fractions. Ferric chloride spray reagent showed green spot indicating the presence of tannins in n-hexane fraction while sulphuric acid-methanol spray reagent revealed five spots in n-hexane fraction, four spots in dichloromethane fraction and three spots in methanol fraction.

Keywords: Phytochemical, rind, *Napoleonaea imperialis,* essential oil, thin layer chromatography

Introduction
Plant usage for medicinal purpose depends on phytochemicals for its biological activities [2]. These phytochemicals generally present in plants include flavonoids, alkaloids, saponins, coumarins, terpenes, tannins, and glycosides [1, 2]. Thin layer chromatography (TLC) is one of the methods used in separating and quantifying phytochemicals from plant extracts [3, 4]. It gives a quick answer to the number of components present in an extract and treating developed TLC plate with phytochemical screening reagents causes change in colour based on the phytochemicals present in the plant extract [5].

Essential oil is the volatile oil obtained from different plant parts mainly through hydro distillation. It is a promising group of compounds in the development of novel products in pharmaceuticals, agriculture, food packaging, food preservation, perfumery and cosmetic, traditional, and modern medicine, and natural therapies [6-8]. Terpenes and their oxygenated derivatives are the most distributed natural products and primary constituents of essential oil of medicinal plants [9]. Tropical fruit wastes comprise of seed, flower, leaf, rind, and part of the fruit often discarded after consumption. The outer covering of a fruit is known as the peel or rind [10]. Studies show that rind of fruits contain several phytochemicals which acts as immunostimulant, anticancer, antioxidant, anti-inflammatory, antimicrobial, and anti-tumor agents which can be useful for pharmaceutical purposes [11, 12]. Waste materials from fruits and vegetables are regarded as important source of flavor and aroma and utilized as dietary fiber, animal feed and biofuel [13, 14]. The conversion of waste into useful products also helps in environmental pollution reduction [15]. Flavonoids, coumarins, polyphenols, limonoids, alkaloids, carotenoid, sugar, ascorbic acid, dietary fiber, saponins, natural enzymes and essential oils from the rind of fruits have been investigated [13, 10, 14, 16, 17]. Some monoterpenoids present in fruit rind essential oils include geranial, neral, D-limonene, geranyl acetate, citronellol and thymol [18-25].

The medicinal plant, *Napoleonaea imperialis*, has been reported to show antihypertensive, wound healing, anti-inflammatory, anti-oxidative, anti-diarrhoeal, anti-plasmodial, hepatoprotective effects and used during postnatal recovery [26-31]. *N. imperialis* rind is derived from the fruit of *N. imperialis,* known as 'mkpodu' in South-Eastern Nigeria and predominantly found in bush fallows and secondary bushes [26]. Studies carried out by Ndukwe *et al.* showed *N. imperialis* rind and seed as potent antibacterial agent when tested against both Gram-negative and Gram-positive bacteria [32]. Phytochemicals of *N. imperialis* have been investigated and reported [33, 34]. The bark and fruit rinds are used in treatment of respiratory tract infections while the twigs are used as traditional chew stick for oral hygiene [32]. Chronic leg ulcer is treated with herbal ointments of the leaves extract, and the juice from the fruits is consumed by humans while the seeds have good performance when fed on finisher broilers [35, 36]. Volatile phytochemicals such as the two isomers of citral, estragole and linalool have been reported as the major constituents of the essential oils obtained from the twig, leaves and stem bark of *N. imperialis* respectively [37]. The seed extract has also been found to inhibit corrosion of mild steel and aluminium [38, 39].

This work reports the extraction, and characterization of phytochemicals from *Napoleonaea imperialis* rind.

Materials and methods

Reagent preparation

Sulphuric acid-methanol: Reagent was prepared by adding 10 ml concentrated sulphuric acid to 90 ml methanol. This spray reagent was used to detect organic components present in the extracts [40].

Anisaldehyde-sulphuric acid: Glacial acetic acid (15 ml) and anisaldehyde (0.5 ml) were added to 85 ml methanol. 5 ml of conc. sulphuric acid was carefully poured into the beaker from the sidewall. This reagent was used to detect the presence of saponins, terpenoids and steroids [41].

Drangendorff's reagent: Bismuth nitrate (0.4 g) was dissolved in 5 ml glacial acetic acid. The mixture was diluted to 25 ml by slowly adding distilled water. 10 g of potassium iodide was accurately weighed and dissolved in 25 ml of distilled water into a 50 ml volumetric flask, 5 ml of solution A and B was pipette and brought to volume with 10% (v/v) aqueous sulphuric acid solution. 0.2 ml of 30% H_2O_2 was added to the mixture. This was used to detect the presence of alkaloids [42].

Vanillin-sulphuric acid: Vanillin (1 g) was weighed and added to 100 ml of concentrated sulphuric acid. This spray reagent was used to detect the presence of flavonoids [43].

Ferric chloride reagent: Ferric chloride (5 g) was weighed and dissolved in 100 ml of distilled water, 10 ml of the solution was added to 90 ml of ethanol. The presence of tannins was confirmed using this spray reagent [44].

Essential oil extraction

Fruits of *N. imperialis* were harvested from Etche Local Government Area, Rivers State, Nigeria. The rinds were separated from the seeds, chopped into small pieces, and weighed. The chopped rind (463.04 g) was loaded (in batches) in a round bottomed flask seated on a heating mantle and distilled water poured into the flask. A Clevenger-apparatus was tightly fitted to the flask. The plant material was subjected to hydro distillation and the volatile oil collected after 50 mins [45]. The volatile oil, 1.92 g (0.4%), was stored in an airtight glass vial at 4 °C until required for spectroscopic analysis.

Crude extraction and partitioning

Rinds of *N. imperialis* were air-dried and pulverized. The dried pulverized rind (617.2 g) was extracted using methanol through the process of maceration [40], and subsequently, the extract was concentrated using a rotary evaporator to give a brown-sticky crude extract (148.12 g). Crude methanol extract (148.12 g) was partitioned successively with n-hexane, dichloromethane (DCM) and methanol (MeOH) in a separatory funnel [46]. Each of the partitioned fraction was concentrated to dryness using a rotary evaporator at 40 °C to yield n-hexane fraction (11.01 g), DCM fraction (35.16 g) and MeOH fraction (101.05 g).

GC-MS analysis

Agilent Technologies GC system of GC-7890A/MS-5975C model (Agilent Technologies, Santa Clara, CA, USA) with HP-5MS column (length 30 m in length; diameter 250 µm and 25 µm film thickness) was used for the analysis. GC-MS with high energy electrons (70 eV) using pure helium (99.995%) as the gas carrier with flow rate of 1mL/min was utilized. The temperature was initially set at 50-150 ºC and increase at 3 °C/min while holding for about 10 min. 1 µl of the prepared 1% sample was injected in splitless mode for the analysis. A chromatogram showing the constituents and their peak area (%) was obtained.

Constituents of the essential oil and DCM fraction of *N. imperialis* rind were verified based on GC retention time in HP-5MS column and comparing the spectra with computer software data of standards. It was further verified by comparing their fragmentation pattern with those reported in the mass spectra library database (Replib and Mainlab MS HP, USA) [47].

Thin layer chromatography

Thin layer chromatography (TLC) was used to identify phytochemical groups and the number of constituents in the three fractions of *N. imperialis* rind. 0.5 g each of the n-hexane, DCM, and MeOH fractions were dissolved in 2 ml n-hexane, DCM and MeOH, respectively. The prepared solutions were spotted manually using a capillary tube on an analytical TLC plate (5x5 cm with 0.25 mm thickness). The spotted plates were air-dried and placed into separate chromatographic tanks containing n-hexane:ethyl acetate (8:2) and acetone:water (9.5:0.5) solvent systems. The mobile phase then migrates up the plate through the adsorbent by capillary action. TLC run time was about 5 mins each and the developed plates were removed from the chromatographic tanks and air dried. Sulphuric acid-methanol spray reagent was used to detect the number of spots on the developed TLC plates, while anisaldehyde-sulphuric acid, Dragendorff's, vanillin-sulphuric acid, and ferric chloride spray reagents (table 1) were used to identify phytochemical groups present in the three fractions. The R_f values of the separated constituents were calculated and recorded.

Table 1: Spray Reagents Used in Identifying Phytochemicals

Phytochemical group	Spray reagent	Colour
Saponins	Anisaldehyde-sulphuric acid	Violet
Terpenoids	Anisaldehyde-sulphuric acid	Purple
Alkaloids	Dragendorff's reagent	Orange
Steroids	Anisaldehyde-sulphuric acid	Grey
Flavonoids	Vanillin-Sulphuric acid	Yellow/Orange
Tannins	Ferric chloride	Green

Results

Constituents of *N. imperialis* rind essential oil

The gas chromatogram of *N. imperialis* rind essential oil (figure 1) shows thirteen peaks. The thirteen compounds detected from GC-MS analysis represented 99.96% of the total constituents. The major compounds which are monoterpenes (geranial, neral, citronellol, D-limonene and geranyl acetate) added up to 61.87% of the total constituents. trans-Nerolidol (a sesquiterpenoid, 2.33%) and 6,11-dimethyl-2,6,10-dodecatrien-1-ol (2.77%) were also identified (table 2).

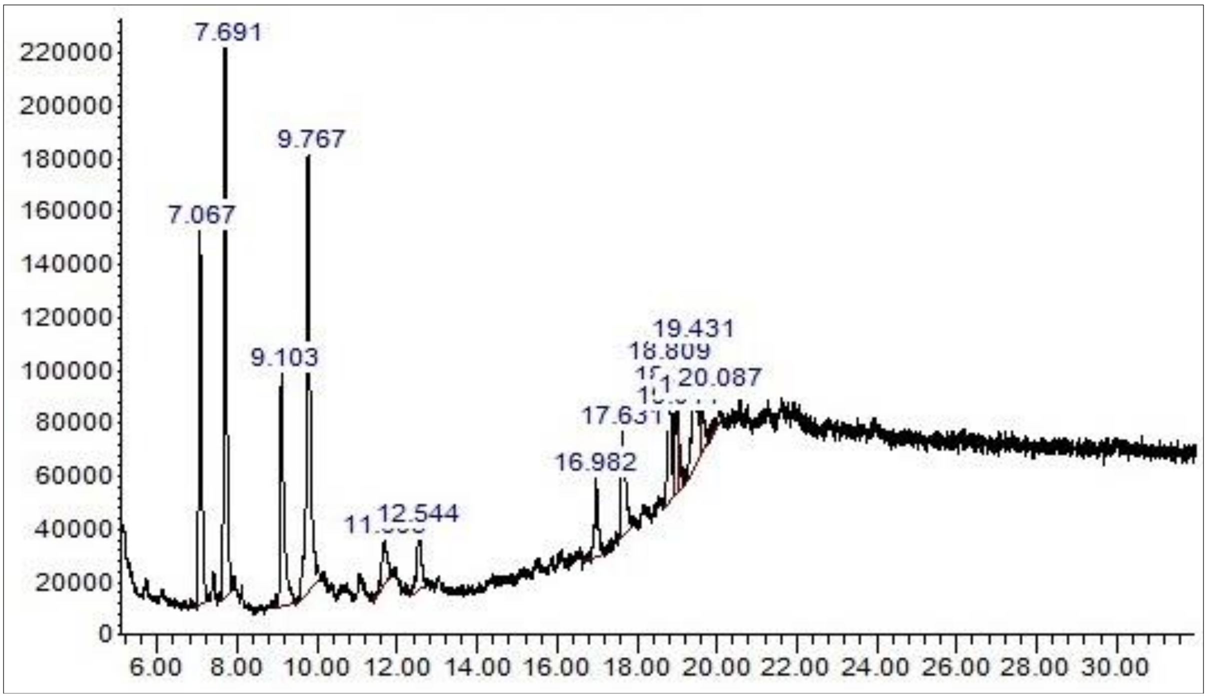

Fig 1: Gas chromatogram of essential oil of *N. imperialis*

Table 2: Compounds in the Essential Oil of *N. imperialis* Rind

S. No.	Retention Time	Compound	Molecular Weight (gmol^{-1})	Concentration (%)
1	7.067	Neral	152	12.86
2	7.691	Geranial	152	39.51
3	9.103	(Z, Z)-3-Hexenyl-3-hexenoate	196	10.56
4	11.693	(E)- 6-Hydroxy- 4-methy- methyl-4-hexenoate	158	3.08
5	12.544	D-limonene	136	3.48
6	16.982	Methyl hexadecanoate	270	3.75
7	17.631	n-Hexadecanoic acid	256	5.39
8	18.809	Geranyl acetate	284	4.93
9	18.972	6,11-Dimethyl-2,6,10-dodecatrien-1 -ol	208	2.77
10	19.041	Methyl 16-methylheptadecanoate	298	2.49
11	19.431	(Z)-9,17-Octadecadienal	264	7.75
12	19.605	trans-Nerolidol	222	2.33
13	20.087	Citronellol	156	1.09

Composition of the DCM fraction of *N. imperialis* rind

Thirty-six compounds (figure 2 and table 3) constituting 98% of the total composition were identified. The major compounds were oleic acid (28.04%), (Z)-6-octadecenoic acid (7.41%), n-hexadecanoic acid (6.13%), 1-cyclohexylnonene (4.07%), 2-Octyl- cyclopropane octanal (3.49%), 3-Hydroxy-ethyl butanoate (3.34%), cyclononene (3.29%) and dodecyl propyl ether (3.18%).

Table 3: Compounds in DCM Fraction of *N. imperialis* Rind

S. No.	Retention Time	Compound	Molecular Weight (gmol^{-1})	Concentration (%)
1	6.755	4-Ethyl cyclohexanone	126	3.05
2	7.115	4,4-Dimethyl-oct-5-enal	154	2.29
3	7.942	1-Nitro-2-acetamido-1,2- dideoxy-d-glucitol	252	2.95
4	8.586	3-Hydroxy-ethyl butanoate	132	3.34
5	8.675	Methyl-6-O-[1-methylpropyl]-beta-d-galactopyranoside	250	2.31
6	9.908	6-Acetyl-β-d-mannose	222	2.05
7	10.206	Undecyl-undec-10-ynoate	336	1.28
8	10.466	trans-Cyclononene	124	3.29
9	10.705	Dodecyl propyl ether	228	3.18
10	10.819	2,3-Dihydroxypropyl-decanoate	246	2.12
11	11.068	Undecylenic acid	184	0.31
12	11.377	n-Hexadecanoic acid	256	6.13

13	11.460	Methyl-4,6-ethylidene-alpha-d-galactopyranoside	206	1.22
14	12.076	Tetradecyl-undec-10-ynoate	378	3.09
15	12.381	2-Hydroxy-cyclopentadecanone	240	2.04
16	12.550	Myristoleic acid	226	2.10
17	13.156	Methyl palmitate	270	1.38
18	14.292	(Z,Z)-4,16-Octadecadien-1-ol acetate	308	3.05
19	14.477	3-Dodecen-1-ol	184	2.06
20	14.829	Falcarinol	244	1.93
21	15.184	Olealdehyde	266	0.01
22	15.528	Methyl-11-octadecenoate	296	1.05
23	15.886	Methyl stearate	298	0.31
24	16.113	(Z)-9,10-Epoxyoctadecan-1-ol	284	0.12
25	16.406	(Z)-13-Octadecenoic acid	282	2.42
26	16.676	Stearic acid	284	0.95
27	17.714	11-Dodecen-1-ol-trifluoroacetate	280	0.16
28	17.768	2-Octyl- cyclopropane octanal	280	3.49
29	17.910	1,3,12-Nonadecatriene	262	0.06
30	18.431	(E)-11-Hexadecenal	238	0.16
31	18.610	(Z)-9,17-Octadecadienal	264	0.01
32	18.902	Oleic acid	282	28.04
33	19.840	(E)-9-Octadecenoic acid	282	0.11
34	20.871	Petroselinic acid	282	7.41
35	21.134	1-Cyclohexylnonene	208	4.07
36	22.821	(Z)-2-Hydroxy-1-(hydroxymethyl) ethyl 9-octadecenoate	354	0.46

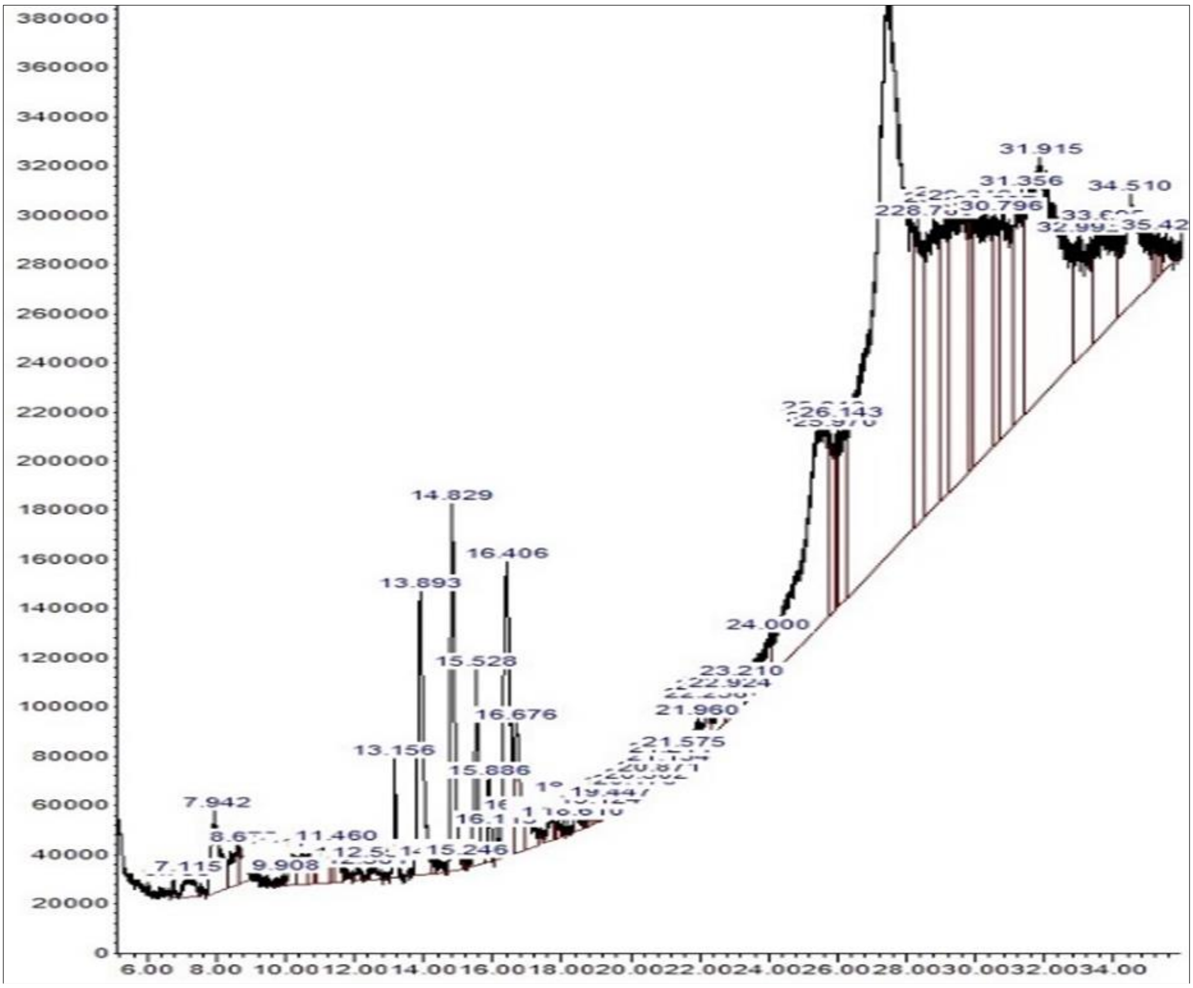

Fig 2: Gas-chromatogram of DCM fraction of *N. imperialis* rind

Phytochemical groups in the three fractions of *N. imperialis* rind

Partitioning of the crude extract produced three fractions (table 4). MeOH fraction had the highest yield (62.3%) followed by DCM fraction (23.7%) and n-hexane fraction (7.4%). Five, four and three spots were detected in n-hexane, DCM and MeOH fractions respectively when developed TLC plates were treated with sulphuric acid-methanol spray reagent and heated for 5 mins at 105 °C (table 5). R_f values range from 1 to 0.2 for spots detected in n-hexane fraction, 1 to 0.4 for spots in DCM fraction and 1 to 0.4 for MeOH fraction. Alkaloids and flavonoids were absent in all fractions. Saponins were detected as violet spots in the DCM and MeOH fractions while terpenoids appeared as purple spots in the three fractions. Steroids were confirmed in n-hexane and DCM fractions as grey spots while tannins was detected as a green spot in n-hexane fraction (table 6).

Table 4: Profile of Fractions

Fraction	Colour	Nature	Weight (g)	Yield (%)
n-hexane	Green	Oily semi solid	11.01	7.4
DCM	Light brown	Solid	35.16	23.7
MeOH	Dark brown	Solid	101.75	68.7

Table 5: Components in the Fractions of *N. imperialis* Rind

Fraction	TLC solvent system	No. of spots	R_f Values
n-Hexane	n-hexane: ethyl acetate (8:2)	5	1.0, 0.9, 0.7, 0.5, 0.2
DCM	Acetone: water (9.5:0.5)	4	1.0, 0.7, 0.6, 0.4
MeOH	Acetone: water (9.5:0.5)	3	1.0, 0.7, 0.4

Table 6: Phytochemical Groups Detected in *N. imperialis* Rind

Phytochemical group	Spray reagent	Colour after treatment with spray reagent	n-Hexane fraction	DCM fraction	MeOH fraction
Saponins	Anisaldehyde-sulphuric acid	Violet	-	+	+
Terpenoids	Anisaldehyde-sulphuric acid	Purple	+	+	+
Alkaloids	Dragendorff's reagent	-	-	-	-
Steroids	Anisaldehyde-sulphuric acid	Grey	+	+	-
Flavonoids	Vanillin-Sulphuric acid	-	-	-	-
Tannins	Ferric chloride	Green	+	-	-

Discussion

The essential oil of *N. imperialis* rind had a characteristic aroma and was composed mainly of geranial and neral (52.37%) which are the two isomers of citral (Table 2). This result is similar to the high percentage of citral in *Cymbopogon citratus* essential oil with a characteristic aroma [21, 48]. Studies show that the antimicrobial activities observed in essential oils of *Citrus medica* and *Backhousia citriodora* were ascribed to high content of citral, while Ndukwe and Ekong posited that the high percentage of citral in the essential oil of *N. imperialis* twig could justify the twig's effectiveness for oral hygiene when used as a chew stick [49, 37]. An unsaturated long chain aldehyde, (Z)-9,17-octadecadienal, identified as one of the major compounds in the essential oil of *N. imperialis* rind has been reported as a constituent of the essential oil of *Citrus sinensis* rind and has anti-inflammatory and antioxidant properties [24, 50]. Citronellol though present in small concentration (1.09%) in *N. imperialis* rind essential oil, is a natural acyclic monoterpene alcohol reported as a mosquito repellent and primary constituent of *Java citronella* grass and *Citrus maxima* essential oils [51, 22, 20]. trans-Nerolidol is the major constituent of *Psidium guajava L.* (white fruit) essential oil, while (Z,Z)-3-hexenyl-3-hexenoate is a volatile compound present in organic and conventional fruit pulp of *Passiflora edulis* used as a flavouring agent [52-54]. n-Hexadecanoic acid identified in the essential oil and DCM fraction of *N. imperialis* rind (tables 2 and 3) has been reported to occur with petroselinic acid in the crude methanolic extract of *N. imperialis* fruit pulp [33]. n-Hexadecanoic acid is used as flavouring and immunostimulant agent and has been found to be present in the essential oil rinds of *Citrus medica*, *Citrus sinensis*, *Citrus aurantium*, *Citrus reticulata* and in the methanol extract of *Limonia acidissima* rind [50, 18, 19, 55, 23, 56]. Oleic acid having the highest concentration (20.02%) in the DCM fraction of *N. imperialis* rind (table 3) is the most widely occurring fatty acid in nature and its high content in olive oil is accountable for the hypotensive effect observed in olive oil [57-59]. The reported antihypertensive effect of *N. imperialis* could be attributed to the high concentration of oleic acid in the plant [26]. Among the phytochemicals identified in the DCM fraction of *N. imperialis* rind (table 3) was falcarinol, a natural pesticide isolated from carrots and red ginseng and reported to show some bioactivities [60, 61]. Saponins, terpenoids, tannins, and steroids are phytochemicals present in *N. imperialis* rind (table 6). Saponins have been reported as a major phytochemical of the fruit rind of *Couroupita guianensi* (cannon ball tree) belonging to Lecythidaceae family [4]. Saponins have cardioprotective activity and hypocholesterolemia effect, antibacterial and antifungal, anticancer and adjuvant activities [62-64]. The foaming property of saponins is useful in food industry as food additive and flavor modifier, foaming agents in carbonated beverages and cosmetics, and as emulsifiers [65]. The application of saponins in food processing as antimicrobial, anti-yeast agents and in food preservation have been reported [66]. More recently, Paul *et al.* suggested the possible use of saponin as a substitute for sodium lauryl sulphate in toothpaste [67]. Terpenoids were present in the three extracts of *N. imperialis* rind as shown in table 6. Terpenoids possess antitumor, antimalarial, anti-inflammatory, antiviral, antibacterial, hypoglycemic effect and are used in prevention and treatment of cardiovascular disease [68, 69]. The use of terpenoids as fragrances in perfumes, natural food flavor additives, and other biological activities has been reported [70, 71]. Pharmacological effects of tannins include antioxidant and free radical scavenging activity, antimicrobial, anti-cancer, anti-nutritional and cardio-protective properties, treatment of neurodegenerative diseases and neuropsychiatric disorders [72, 73]. Tannins are responsible for the astringent taste of many fruits and vegetables and are applied in food preservation, tanning of leather and used as wood adhesives [72, 74, 75]. Steroids are used as antimicrobial agent and reported to have potential clinical efficacy as anticonvulsants, anesthetics, hypnotics, and anxiolytics [76, 77]. Pintiaux *et al.* have reported the gynaecological uses of steroids [78].

Conclusion

The fruit rind of *Napoleonaea imperialis* which is regarded as waste contains interesting phytochemicals, monosaturated and monounsaturated fatty acids. Gas chromatography-mass spectrometry analysis of the essential oil and DCM fraction of *N. imperialis* rind indicated thirteen and thirty-six compounds, respectively. The essential oil and DCM fraction of *N. imperialis* rind are rich sources of monoterpenoids and oleic acid, respectively. Thin layer chromatography phytochemical screening of *N. imperialis* rind revealed the presence of saponins, terpenoids, tannins and steroids.

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