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PHYTOCHEMICAL ANALYSIS AND ANTIMICROBIAL ACTIVITY OF NEOCARYAMACROPHYLLA LEAVES EXTRACT

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Abstract

The frequency of life-threatening infections caused by pathogenic microorganisms has increased worldwide and is becoming an important cause of morbidity and mortality especially in developing countries with lower health status indices. The increasing prevalence of multi-drug

resistant strains of bacteria raised the specter of untreatable bacterial infections and adds urgency to the search for new therapeutic strategies.

Along this line, this study investigated phytochemical screening of Neocaryamacrophylla extract using GC-MS and Antibacterial screening of the extract using agar well diffusion method. Three microorganisms were used namely; Escherichia coli, Klebsiellapneumoniae and Staphylococcus aureus. The susceptibility tests result showed inhibition range of 24 and 13mm against K. pneumoniae at 20 and 10 mg/ml illustrating it to be the most sensitive organism. The least was E. coli, (16 and 7mm at 20 and 10 mg/ml). Similarly, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extract indicated K. pneumoniae to be more susceptible followed by S. aureus and E. coli as the least susceptible species.

Generally, in all the model organisms tested, the susceptibility was observed to be concentration dependent. The test results indicated that leaves extract of N. macrophylla has antimicrobial potency and could be used as an alternative antimicrobial therapy.

Keywords

Alkaloid, Antimicrobial; Antioxidant, Gingerbread, Alternative Medicine, Phytotherapy, Extract

1. Introduction

The frequency of life-threatening infections caused by pathogenic microorganisms has increased worldwide and is becoming an important cause of morbidity and mortality especially in developing countries with low health status indices (Rahman *et al.* 2009). The increasing prevalence of multi-drug resistant strains of pathogens has raised the specter of untreatable bacterial infections and adds urgency to the search for new infection-fighting strategies (Shrutika *et al.* 2015). Recent study has highlighted the menace socio-economic burden of multi drug resistant bacteria in cosmopolitan cities (Shashikant *et al.* 2015).

Although several chemosynthetic antibiotics are potent against different pathogenic bacteria, unfortunately were known to have setbacks (Zerazion *et al.* 2016). These include resistance of the microbes towards the antibiotics, resulting in multidrug resistant strains, the recurring side effects as well as the adverse reactions caused by these multispectral antibiotics (Gowsiya S. *et al.* 2014). At times, it has been indicated that drug abuse and mismanagement are among the major cause of pathogenic resistivity (Njood *et al.* 2016). Therefore warranting the need for research and development of new effective antimicrobial drugs.

For ages, plants metabolites have been used across several societies as alternative medicines for different ailments (Rahman *et al.* 2009, Kannan *et al.* 2016). Plants are the cheapest and safer alternative sources of bioactive compounds (Rahman *et al.* 2009), and the antimicrobial properties of such bioactive compounds have been investigated by a number of studies worldwide (Ghaedi *et al.* 2016). The practice of complementary and alternative medicine is now on the increase in developing countries in response to World Health Organization (WHO) directives culminating in several pre-clinical and clinical studies that have provided the scientific basis for the efficacy of many plants metabolites.

Along this line, we reported herein the phytochemical analysis and antimicrobial activity of *Neocaryamacrophylla* (gingerbread plum). This plant belongs to family *Chrysobalanaceae* and is native to Sub-Saharan Africa. It has widely been used in Ethno medicine to cure several ailments including: asthma, skin infections, and treatment of wounds, dysentery, inflammations, as well as eye and ear infections.

2. Materials and Methods

2.1 Sample Collection

Neocaryamacrophylla leaves were collected from a farmland at Daura Local Government Area of Katsina State, Nigeria. The leaves were washed with running tap water and dried under shade for 3 weeks. Dried leaves were ground into fine powder and stored at room temperature (25°C) in a capped jar prior to usage.

2.2 Extraction Procedure

Phytochemical extraction was carried out using maceration technique as reported in literature with slight modification (Joshi *et al.* 2011), by soaking 50g of the dried powdered leaves in 500ml of absolute ethanol in 1000ml volumetric flask, this was left to stay for seven days under shaking at regular intervals. The mixture was then filtered and concentrated using rotary evaporator. The percentage extract yield was calculated using the Eq. 1.0

$$Percentage\ Extract\ yield = \frac{Weight\ of\ Extract}{Weight\ of\ sample\ powder} \times 100 \qquad \text{Eq. (1.0)}$$

2.3Phytochemical Screening using GC-MS Analysis

Phytochemical screening using gas chromatography tandem triple quad mass spectrometry (GC-MS) have been reported in several studies (Hussein *et al.* 2016). In this study, the GCMS analysis was recorded on Agilent triple quadrupole 7000B (Agilent, USA), equipped

with GC-MS triple axis detector carrying Agilent HP-5ms column (30 m length x 0.25 mm internal diameter x 0.25 µm film). A sample of dried crude extract of *Neocaryamacrophylla* leaves (2 mg) was dissolved in 10 ml of chloroform, the mixture was filtered using a 25 ml borosilicate glass syringe equipped with disposable PTFE filter (Ø = 0.22µm). Filtered sample in the form were used for the GC-MS analysis according reported literature (Gumel *et al.* 2014) with slight modification. Briefly, dissolved sample (1 µl) was automatically injected into the GC-MS machine at a split ratio of 10:1. The injection temperature was set at 200^{o}C while the column ramping temperatures were programmed as 40^{o}C for 1 min then increase to 150^{o}C at 10^{o}C min^{-1}, hold for 2 min, and increase to 250^{o}C at 15^{o}C min^{-1} hold for 15 min. Helium was used as carrier gas at 48.3 ml min^{-1} and 0.41 bar pressure. Mass spectra were acquired at 1250 scan speed using electron impact energy of 70eV at 250^{o}C ion-source and 280^{o}C interface temperatures, respectively.

2.4 Bacterial Strains Used

The microbes (i.e. *Escherichia coli*, *Klebsiellapneumoniae* and *Staphylococcus aureus*) were obtained from the Department of Medical Microbiology, Rasheed Shakoni Specialist Hospital Dutse, Jigawa State, Nigeria. The isolates were cultured individually on nutrient agar slants using a sterile wire loop and incubated for 24 hours at 37°C and this served as the stock culture. Growth of microorganisms in the broth was assayed by turbidity (Audu *et al.* 2005). The broth cultures were diluted in normal saline (NaCl). A concentration of about 1.5x10^{8} values based on Mc-Faddin's turbidity standard scale was used (Audu *et al.* 2005).

2.4.1 Biochemical Characterizations

Escherichia coli and *Klebsiellapneumoniae* were characterized biochemically following Indole test using Kovac's reagent, citrate utilization test using Cimon citrate agar, methyl red test using methyl red-Voges Proskauerbroth medium were conducted to confirm each species.

On the other hand, *S.aureus* was typed biochemically using catalase test in presence of 6% H_2O_2. The *S.aureus* was confirmed using coagulase test in presence of human blood plasma.

2.5 Antibacterial Screening of the Extract

The antibacterial activity of ethanolic extract was evaluated usingwell diffusion method. The antibacterial assay was evaluated using different concentrations (20mg/ml and 10 mg/ml) of the extract that were made by dissolving the corresponding weight of the powder in distilled water.

Nutrient agar plates were seeded with 0.1 ml of the standard inoculums of the test microbes (either *E. coli*, *K.pneumoniae* or *S.aureus*) separately; the inoculums were spread evenly by the use of sterile swab stick over the surface of the agar medium. A standard sterile cork borer was used to cut a well at the centre of each inoculated plates and the extract was then introduced into each well on the medium. The inoculated plates were then incubated in non-inverted position at 37°C for 24 hours after which the medium were observed for zones of growth inhibition. The growth inhibition zones were measured with a transparent ruler as per reported somewhere else (Mohamed 2013).

2.5.1 Minimum Inhibitory Concentration of the extract (MIC)

Minimum Inhibitory Concentration is an analysis carried out in order to ascertain the potency of therapeutics on a particular pathogen (Shashikant *et al.* 2015). The MIC of the extract was carried out on the test microbes (i.e. *Escherichia coli, Klebsiellapneumoniae* and *Staphylococcus aureus*) and was done using broth dilution method. A fresh nutrient broth was dispensed into a series of five labeled test tubes, out of which one of the test tube contains 10ml of the broth whereas the other four contained 5ml of it, respectively (Audu *et al.* 2005). These were sterilized at 121°C for 15 minutes; the broth was allowed to cool.

Two-fold serial dilutions of the extract in the broth were made to obtain the concentrations of 20.0mg/ml, 10.0mg/ml, 5.0mg/ml, 2.5mg/ml and 1.2mg/ml respectively. The highest concentration was obtained by dissolving 200 mg of the extract in 10ml of the nutrient broth (Braca *et al.* 2005). Having obtained the different concentrations of the extract in the broth, 0.1 ml of the standard inoculums of the test microbes in the normal saline were inoculated into the test tubes containing different concentration of the extracts. Thereafter, the test tubes were incubated at 37°C for 24 hours. The lowest concentration of the extract in the broth, which inhibited the growth of the microbes, was recorded as the Minimum Inhibitory Concentration (MIC).

2.5.4 Minimum Bactericidal Concentration of the Extract (MBC)

Minimum Bactericidal Concentration (MBC) of the extracts was also carried out to determine whether the tested microbes were killed or only their growth was inhibited. Nutrient agar was prepared according to the manufacturer's instructions (28g in 1litre), sterilized at 121^0C for 15 minutes cooled at 40^0C and poured into sterile petridishes, the plates were allowed to cool and solidify. The contents of the MIC in the serial dilution that show no growth (turbidity) were

sub cultured onto the solidifiedmedium, the plates of the medium were incubated at 37^0C for 24 hours after which the plates were observed for bacterial growth. The MBC was the plate with lowest concentration without colony growth (Audu *et al.* 2005).

3. Results

3.1 Result of Extraction

The weight of the extract with the percentage yield obtained from the powdered leaves of *N. macrophylla* using absolute ethanol is presented in Table 1. The percentage of the extract was calculated according to equation 1.

Table 1: *Result of Phyto-Contents Extraction*

Part of plant	Weight of sample (g)	Weight of extract (g)	Percentage yield (%)
Leaves	50	6.7	13.4

3.2 GC-MS Analysis

The acquired GC-MS chromatogram is presented in Figure 1. The obtained data was parsed against the spectra database of National Institute of Standards and Testing (NIST, USA) 2005 version for similarity checking.

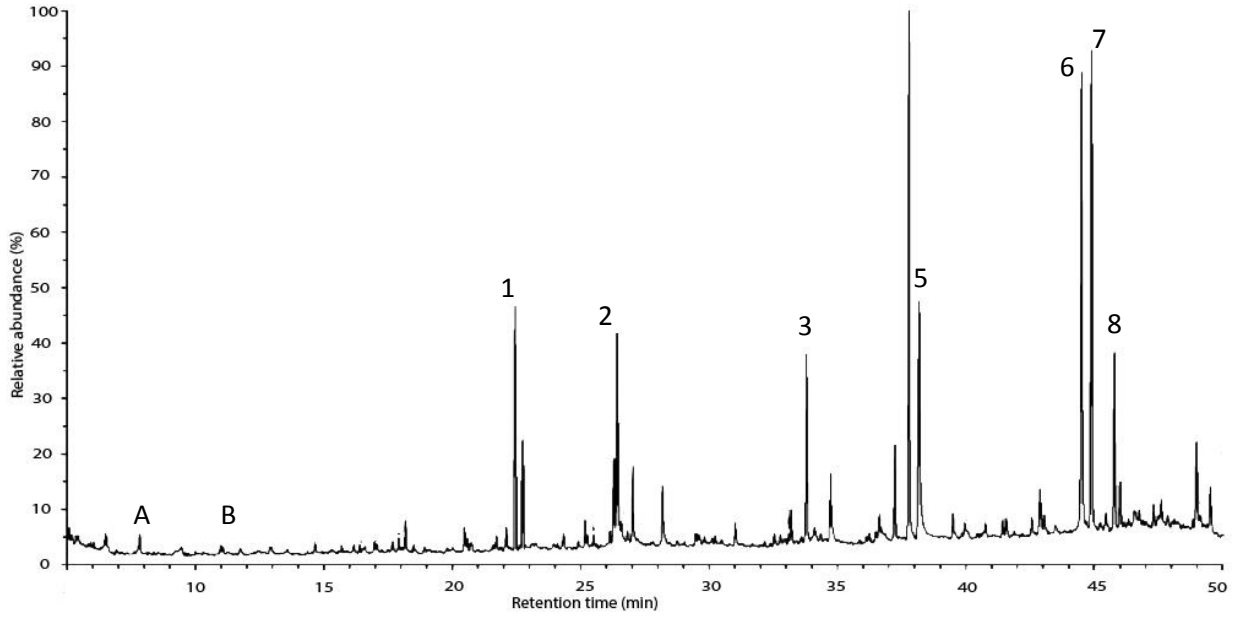

Figure 1: *GC-MS Chromatogram of Neocarya macrophylla Leaf Extract*

KEY:-*A. Alpha tocopherol, B. Beta tocopherol, 1. Palmitoleic acid, 2. Tannins, 3. and 4. Flavonoids, 5.Glycosides.6, 7 and 8.Steroids.*

From the GCMS analysis, several bioactive compounds were detected, and these are illustrated in Table 2 and Figure 2, respectively.

Table 2: *Bioactive Compounds Detected in N. Macrophylla Leaf Extract using GC-MS*

Compounds	**Relative Abundance**	
	Low	**High**
Flavonoids		√
Steroids		√
Palmitoleic acid		√
Alpha tocopherol	√	
Beta tocopherol	√	
Tannins	√	
Glycosides		√

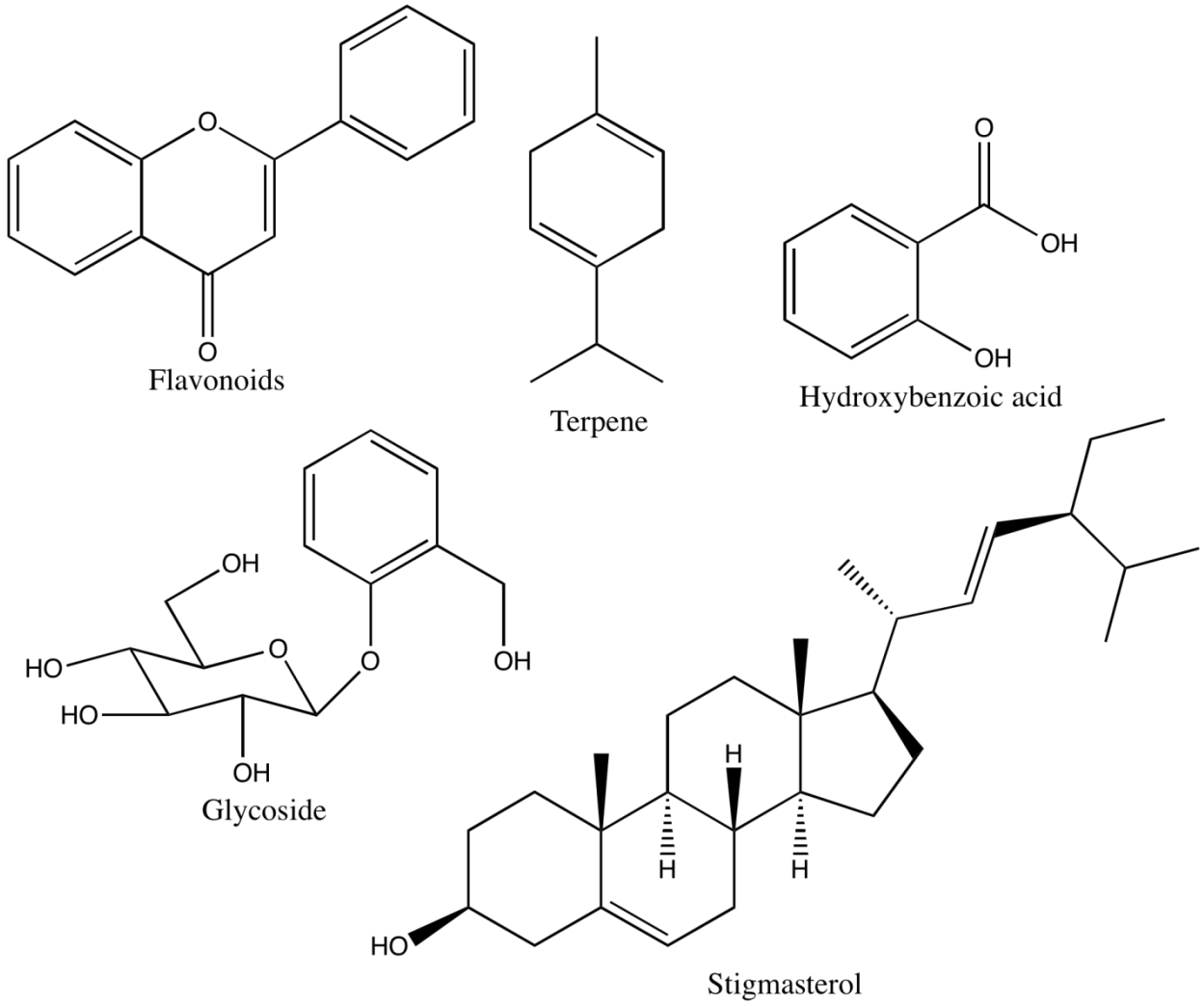

Figure 2: *Chemical Structures of Some of the Detected Compounds*

3.3 Biochemical Confirmatory Test of the Bacteria

Table 3 summarized confirmatory tests observed. These include macroscopic examination, Gram's staining, microscopy and biochemical characterizations.

Table 3: *Confirmatory Biochemical Tests observed*

Test organism	Appropriate media	Macroscopic examination on media	G.R	Microscopic morphology	Biochemical characterization				
					I	Cat	Coa	Cit	MR
E. coli	Eosin Methylene Blue Agar (EMB)	Green metallic sheen, small, spread and slightly raised colonies on EMB	-	Rod-like, reddish, appeared in pairs, single, cluster and chain	+			-	+
K. pneumoniae	MacConkey Agar	Purple, mucoid, small colonies and slightly raised on the media	-	Reddish rod-like appeared and in cluster and single	-			+	-
S. aureus	Mannitol Salt Agar (MSA)	Small, yellow and mucoid colonies on MSA	+	Cocci, appeared in cluster		+	+		

Key:- G.R= Gram's Reaction, I= Indole, Cat.= Catalase, Coa.= Coagulase
Cit. = Citrate utilization, MR= Methyl red, - = Negative and += Positive.

3.4 Results of Antibacterial Assay of *N. macrophylla* Leaf Extract

The well diffusion photo macrograph are shown in Figures 3 and 4. The antibacterial activity of the extract can be seen by the presence of growth inhibitory area on the agar.

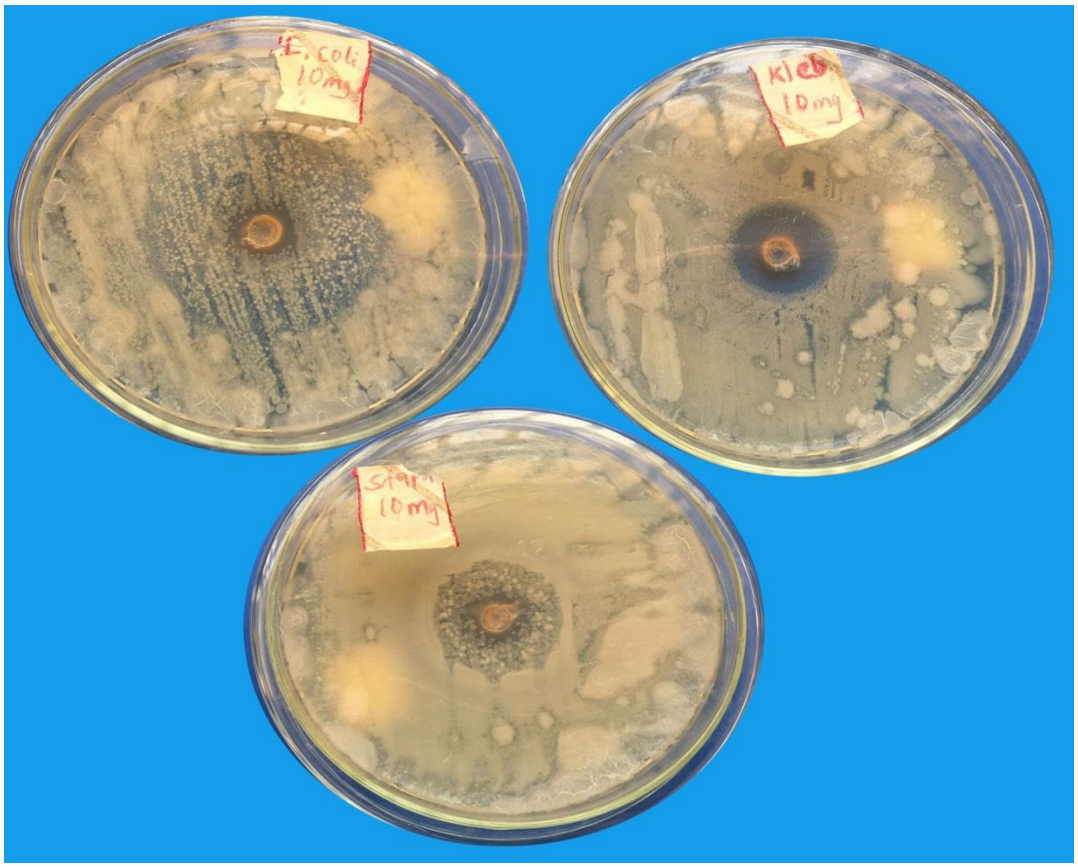

Figure 3: *Photo macrograph of the culture plates showing bacteria growth inhibition zones using disk-well diffusion method at 10 mg extract concentration.*

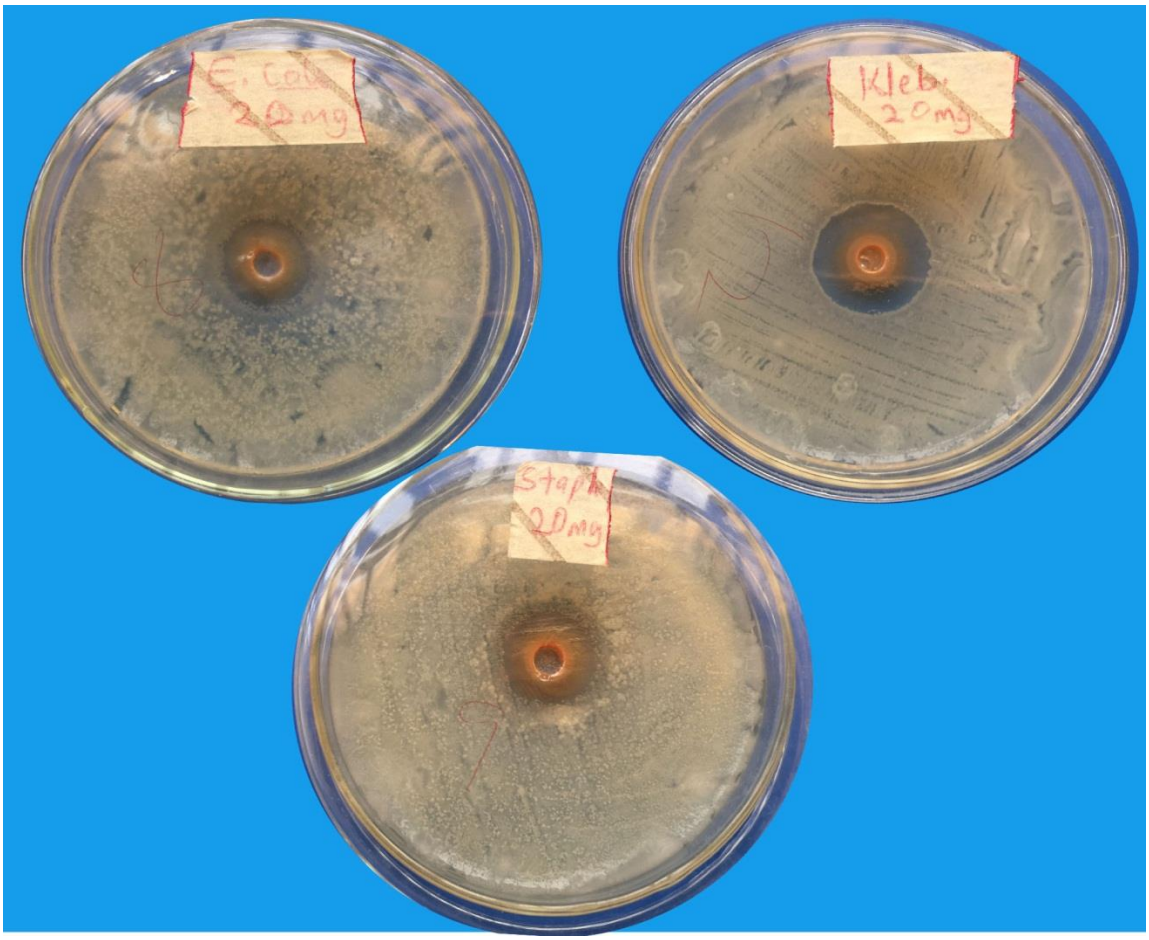

Figure 4: *Photo macrograph of the culture plates showing bacteria growth inhibition zones using disk-well diffusion method at 10 mg extract concentration.*

The antibacterial activity of ethanolic extract of the leaf of *N. macrophylla* is summarized in Tables 4 and 5 as illustrated below:

Table 4: *Result of Antibacterial Screening using Agar Well Diffusion Method*

Test organisms (Bacteria)	**Ethanolic Leave Extract**		**TTC 5 (mg/ml)**	
	20 (mg /ml)	**10 (mg/ml)**	**Positive control**	**Negative control**
Escherichia coli	+	+	+	-
Klebsiella pneumoniae	+	+	+	-
Staphylococcus aureus	+	+	+	-

Key:- (+) Sensitive, (-) Resistance, TTC = Tetracycline

Table 5: *Result of Antibacterial Screening showing Mean Zone of Inhibition*

Test Organisms (Bacteria)	Mean Zone of Inhibition (mm)			
	Concentration of the extracts 20 (mg/ml)	10 (mg/ml)	Pos. Cont. TTC 5 (mg/ml)	Negative Control
Escherichia coli	16	7	22	0
Klebsiella pneumoniae	24	13	19	0
Staphylococcus aureus	18	8	18	0

Key:- TTC = Tetracycline

3.5 Result of Minimum Inhibitory Concentration (MIC) of the Extract

Table 6 below summarized the Minimum Inhibitory Concentration of leaf extract of*Neocaryamacrophylla.*

Table 6: *Result of Minimum Inhibitory Concentration (MIC) of the Extract*

Test Organisms (Bacteria)	Ethanolic Leaves extract Concentration of the extract (mg/ml)					Concentration of TTC (mg/ml) positive control	Negative Control
	20	10	5.0	2.5	1.2	5.0	
Escherichia coli	-	-	MIC	++	+++	-	+++
Klebsiella pneumoniae	-	-	-	MIC	++	-	+++
Staphylococcus aureus	-	-	MIC	+	++	-	+++

Key:- MIC = Minimum Inhibitory Concentration, (-)No turbidity, (+) slightly turbid, (++) Moderate turbidity, (+++) Highly turbid, TTC = Tetracycline.

3.6 Result of Minimum Bactericidal Concentration (MBC) of the Extract

Table 7 summarized the bactericidal activity of leaf extract of *Neocarya macrophylla.*

Table 7: *Result of Minimum Bactericidal Concentration (MBC) of the Extract*

Test Organisms (Bacteria)	Ethanolic Leaves extract			
	Concentration of the extract (mg/ml)			
	20	10	5.0	2.5

Escherichia coli	MBC	+	++	+++
Klebsiella pneumoniae	-	-	MBC	+
Staphylococcus aureus	-	MBC	++	++

Key:- MBC = Minimum Bactericidal Concentration, (-) No colonies growth, (+) Scanty colonies growth, (++) Moderate colonies growth, (+++) Large colonies growth.

4. Discussion

The increase in life-threatening infections caused by pathogenic microorganisms has resulted in the recent surge in the morbidity and mortality especially in developing countries with low health status indices. It has been reported that bacteria such as*E. coli,B.cereus* can cause diarrhea and dysentery (Yusuf *et al.* 2015). In fact, *S.aureus* is known to be associated with skin infections.

The isolated model microorganisms were biochemically typed, and the confirmatory test illustrated *E. coli* to be indole positive which is indicated with red ring appeared at top of inoculated peptone water and it is citrate utilization negative which is indicated on Cimon citrate agar as blue hence, maintaining the colour of the medium. However, it is methyl red positive as indicated by colour change from yellow to red after 48 hours incubation at 37°C on methyl red-Voges Proskauer broth medium. For *K. pneumoniae*, it is found to be indole negative and it is citrate utilization positive which is indicated by colour change from blue to green on Cimon citrate agar. However, it is methyl red negative which is indicated by maintaining the colour of the broth (yellow) after 48 hours incubation at 37°C on methyl red-Voges-Proskauer broth medium. *S. aureus* on the other hand, is found to be catalase and coagulase positive. Similar observations where reported somewhere else (Mac Faddin 1976, Lanyi 1988, Cowan *et al.* 2004).

The increasing prevalence of multi-drug resistant strains of bacteria raised the specter of untreatable bacterial infections and adds urgency to the search for new therapeutic strategies. *Neocarya macrophylla*(gingerbread plum) has become one of those plants that are thought to have antimicrobial potency. Previous literatures have demonstrated the occurrence of flavonoids and triterpenes, glycosides, hydrozybenzoic acid, Isocarthamidin and stigmasterol in *N. macrophylla* and its related species(Castilho *et al.*, 2005; Garo*et al.*, 1997; Braca*et al.*, 2005). In agreement with these literatures, in this study, the presence of these compounds was also

observed. Flavonoids were known to possess antimicrobial potency (Cushnie and Lamb 2005). Similarly, phenolic and benzoic acid derivatives were shown to exhibit antimicrobial efficacy (Park *et al.* 2001).

Antibacterial activity of the extract exhibited varying degree of antimicrobial effect against the test organisms in a concentration dependent manner. The susceptibility test results showed inhibition range of 13mm and 24mm against *K. pneumoniae* at 10 and 20 mg/ml extract loading, respectively. This indicates *K. pneumoniae* to be the mostsensitive organism among those tested. The least susceptibility was observed in E.coli, (7 and 16mm at 10 and 20 mg/ml, respectively). The Minimum Inhibitory Concentration (MIC) of leaf extract of *Neocarya macrophylla* was found to be effective against *Staphylococcus aureus* at the lowest concentration of 5mg/ml. Similarly, the minimum bactericidal activity (MBC) of leaves extract is more effective on *Klebsiella pneumonia* where 5.0mg/ml is the lowest concentration that killed the bacterium, and the lowest concentration that killed *Staphylococcus aureus* was 10mg/ml and the leaves extract is less effective on *E. coli* where the MBC was observed to be 20mg/ml as the lowest concentration that killed it.

Generally, all the microbes model tested were found to be susceptible to extract with variable zone of inhibition. The observed antibacterial activity of the extract against the test organisms could beattributed to the presence of different phytochemical secondary metabolites detected in the plant. In accord with these observations, Audu *et al.* (2005) have reported the antimicrobial activity of the fruits of gingerbread plum against *Escherichia coli*, *Salmonella typhi*, *Candida albican* and *Pseudomonas aeruginosa*. Similarly, Yusuf *et al.* (2015) have reported the susceptibility of *Escherichia coli* and *Staphylococcus aureus* tostem bark extract of *N. macrophylla*. As expected, the researchers ascribed the antimicrobial property to the presence of some of these bioactive compounds.

5. Conclusion

Phytochemical screening conducted on the *N. macrophylla* extract using GC-MS revealed the presence of bioactive compounds that were known to possess antimicrobial potency. Antibacterial screening of the extract based on susceptibility tests revealed *K. pneumoniae* to be the most sensitive organism. Moderate susceptibility was observed in *S. aureus*, whereas *E. coli* was found to be the least susceptible. Generally, in all the model organisms tested, the

susceptibility was observed to be concentration dependent. The test results indicated that leaves extract of *N. macrophylla* has antimicrobial potency and could be used as an alternative antimicrobial therapy.

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