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ANTIBACTERIAL ACTIVITY OF THE AQUEOUS AND METHANOLIC SEED EXTRACTS OF *NIGELLA SATIVA* LINN. (RUNUNCULACEAE) IN MAIDUGURI

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ABSTRACT

The present study examined the antibacterial activity of the aqueous and methanolic seed extracts of *Nigella sativa* Linn. against some Gram negative (*Escherichia coli, Salmonella typhi, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) and Gram positive (*Staphylococcus aureus, Streptococcus pyogenes, Bacilus subtilis* and *Corynbacterium* spp.) bacteria. The dried seed powder was subjected to reflux method of extraction using distilled water and methanol for the aqueous and methanolic extracts, respectively. The seed extracts were further subjected to phytochemical screening using a standard method and *in vitro* antibacterial sensitivity tests using the disc diffusion method. Zones of inhibition, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined. The percentage yields were 18.24 and 16.03% ($^{w}/_{w}$) for aqueous and methanolic extracts, respectively. The phytochemical screening revealed presence of carbohydrates, terpenoids, cardiac glycosides, saponin glycosides, tannins, alkaloids and flavonoids in both the aqueous and methaolic seed extracts. The results of the sensitivity test showed that the aqueous extract was active against only *S. pyogenes* at concentrations of 200 and 300 mg/ml while methanolic extract inhibited the growth of *S. aureus, B. subtilis, Corynbacterium spp., S. typhi* and *P. aeruginosa*. In addition, the methanolic extract was most active at the highest concentration of 300 mg/ml against *S. aureus*. The results of the MIC showed that *P. aeruginosa* had the MIC of 50 mg/ml, *S. aureus* and *C. specie* had the MIC value of 25 mg/ml while *B. subtilis* and *S. typhi* had the MIC value of 12.5 mg/ml. The results of the MBC for *P. aeruginosa*, *S. aureus*, *C. specie*, *S. typhi* and *B. subtilis* were 100, 50, 50, 25 and 12.5 mg/ml, respectively. In conclusion, the seed extracts are rich in phytochemicals and the methanolic extract demonstrated more antibacterial activity than the aqueous extract. These findings may provide justification for the traditional uses of the plant.

KEYWORDS: Antibacterial activity, *Nigella sativa*, phytochemical constituents, zone of inhibition, MIC, MBC.

INTRODUCTION

Plants produce many chemical compounds as defence mechanism against insects, fungi and herbivorous mammals. Chemical compounds produced by these plants mediate their effects in human body through processes identical to those used by conventional drugs; thus herbal medicines do not differ greatly from conventional drugs interms of their mechanism of action. This enables herbal medicines to have beneficial pharmacological properties such as antibacterial activity, but also gives them the same potential as conventional pharmaceutical drugs to cause harmful side effects.[1]

Nigella sativa Linn. (family Ranunculaceae) commonly known as black seed, black caraway, nutmeg flower, "habbatus sauda" in Hausa and "Asofeye je" in Yoruba, is one of the medicinal plants with reported pharmacological activities. It has been used as spice, food preservative and as protective and curative agent of various diseases. Traditionally, such diseases include ascites, cough, jaundice, hydrophobia, fever, paralysis, conjuctivitis, piles, skin diseases, anorexia, dyspepsia, flatulence, abdominal disorder, diarrhoea, dysentery, hemorrhage and amenorrhea.[2,3] Previous studies have shown that the extract of the plant has pharmacological activities such as hypoglycemic,[2,4] anticancer,[2,5] analgesic,[2,6] diuretic, antihypertensive, bronchodilating, gastroprotective, hepatoprotective, immunomodulatory, antimicrobial, anti-inflammatory, spasmolytic, renal protective and antioxidant effects.[2]

Infectious diseases are still one of the major health challenges leading to death of millions of people especially in developing countries.[7] This may be partly due to widespread resistance to commonly used antibacterials and this has accentuated the need for alternative medicine.[7,8]

Several researchers have studied the antimicrobial activity of *N. sativa* Linn. crude extracts against different microorganisms.[9,10,11] However, extreme range of altitude, climate and soil across geographical areas may influence phytoconstituents in plant and in turn affect their pharmacological activities. Thus, this study investigated the phytochemical constituents and antibacterial activity of *N. sativa* Linn. in Maiduguri, Nigeria.

METHODS

Collection and Identification of Plant Seeds

The seeds of *N. sativa* Linn. used in this research procedure which was obtained from the market in Maiduguri Metropolitan Council, Borno state, Nigeria. The seed was identified and authenticated by a plant taxonomist, Prof. S. S. Sanusi of Department of Biological Sciences, Faculty of Science, University of Maiduguri. Portion of *N. sativa* Linn. identified was kept in the Herbarium, Faculty of Pharmacy for further reference.

Preparation of Seed Extracts

The dried seeds of *N. sativa* Linn. was pulverized into powder using wooden mortar and pestle. The extraction was by reflux method using distilled water and methanol. Briefly, 150 g and 130 g of the powdered material were transferred into conical flasks containing 2 L each of distilled water and methanol, respectively and allowed to run for 2 hours. The extracted sample was filtered, dried on a water bath and then stored in a desiccator. Percentage yield was determined.[12]

Bacterial Isolates

A total of eight bacterial isolates were used in this study; four of which are Gram positive (*Staphylococcus aureus, Streptococcus pyogenes, Bacillus subtilis* and *Corynbaterium spp.*) and the other four are Gram negative (*Escherichia coli, Salmonella typhi, Klebsiella pneumonia* and *Pseudomonas aeruginosa)*. These organisms were obtained from the Department of Veterinary Microbiology and Parasitology, Faculty of Veterinary Medicine, University of Maiduguri, Nigeria.

Phytochemical Screening

A small quantity of each of the extracts (aqueous and methanol) was subjected to phytochemical screening to detect the presence of alkaloids, carbohydrates, flavonoids, saponins, tannins, glycosides (cardiac, steroidal), terpenes/terpenoids, fatty acids and resins using previously described methods (Table 1).

Assessment of Antibacterial Activity of the Seed Extracts

The antibacterial activity of the seed extracts was assessed using the methods described by Usman and Osuji,[20] using 100 mg/ml, 200 mg/ml and 300 mg/ml of the aqueous and methanolic extracts. The dilution ratios for Gram positive bacteria and Gram negative bacteria were 1:1000 and 1:5000, respectively using peptone water.[21] About 0.5 ml of the dilute cultures was aseptically inoculated on the surface of the sterile petri dishes containing sterile solid nutrient agar. Discs were impregnated with the extract by dipping in various concentrations of the extract. The extract-impregnated discs were asceptically mounted on the inoculated agar and incubated at 37^{o}C for 24 hours. The inhibition zone was observed and then recorded in millimetres using a transparent meter rule. The tests were conducted in triplicate for each of the isolates. The standard antibacterial discs used were Ciprofloxacin (5 µg) and Tetracycline (30 µg).[20]

Table 1: Phytochemical Screening techniques/methods.

Phytochemical	Test	Reference
Carbohydrates	Molisch's Test	[18]
Reducing Sugar	Fehling's Test	[18]
Combined Reducing Sugars	Fehling's Test	[19]
Ketones	Salivanoff's Test	[14]
Monosaccharides	Barfoed's Test	[13]
Soluble starch	5% potassium hydroxide and tetraoxosulphate (VI) acid Test	[13]
Anthraquinones	Bontrager's Test	[18]
Cardiac Glycosides	Salkowiski's Test	[16]
	Liebermann-Burchard's test	[16]
Terpenoids	Acetic anhydride and tetraoxosulphate (VI) acid Test	[16]
Flavonoids	Ferric Chloride Test	[18]
	Shinoda's Test	[15]
	Lead Ethanoate Test	[13]
	Sodium Hydroxide Test	[18]
Saponin Glycoside	Frothing Test	[17]
	Fehling's solution A and B Test	[14]
Phlobatannins	1 % aqueous hydrochloric acid Test	[18]
Tannins	1 % ferric chloride Test	[17, 18]
	10 % lead ethanoate Test	[17, 18]
	10 % hydrochloric acid and methanol Test	[17, 18]
Alkaloids	Dragendoff's reagent	[13]
	Mayer's reagent	[13]

Determination of Minimum Inhibitory Concentration (MIC) of the Seed Extracts

The modified method used by Usman and Osuji[20] was employed to determine the minimum inhibitory concentration (MIC). Double dilutions of of the seed extracts were prepared in distilled water to yield 6.125-100 mg/ml. Then, 0.2 ml of fresh bacteria culture prepared in nutrient broth was exposed to each concentration of the extract and incubated at 37^{o}C for 18 hours. The cultures were observed for turbidity. The least concentration where no turbidity was observed was determined and recorded as MIC.

Determination of Minimum Bactericidal Concentration (MBC) of the Seed Extracts

The Minimum Bactericidal Concentration (MBC) was determined from the broth dilution test resulting from the MIC test as described by Usman and Osiju.[16] The broth was inoculated on nutrient agar plates. The plates were then incubated at 37^{o}C for 24 hours. The lowest concentration of extract with no bacteria growth after subculturing was taken as MBC.[22]

Statistical Analysis

The statistical tool used was GraphPad Software package, Version 5.01.[23] The zones of inhibition of the extracts were expressed as means and standard error of mean and were compared with that of the standard drugs. Significance was inferred at $p<0.05$.

RESULTS

The Seed Extracts

The colour of both the aqueous and methanolic extracts was black with pungent smell. The methanolic extract was oily in texture while the aqueous extract was powdery. The percentage yields of the aqueous and methanolic extracts were 18.24 % ($^{w}/_{w}$) and 16.03 % ($^{w}/_{w}$), respectively.

The Phytochemical Constituents

The results of the phytochemical screening of both the aqueous and methanolic extracts are shown in Table 2. The result shows the presence of similar phytochemicals in both extracts including cardiac glycosides, terpenoid, cardenolides, saponin glucosides, tannins, alkaloids, flavonoids and carbohydrates.

Antibacterial Activities of the Seed Extracts

The results of the *in vitro* antibacterial susceptibility test of the extracts are shown in Tables 3 and 4. The aqueous extract produced no activity against tested organisms except at 200 and 300 mg/ml against *S. pyogenes*. This inhibition was significantly lower than that of standard drugs ($p<0.05$) [Table]. In addition, the methanolic extract produced a dose-dependent antibacterial activities against *S. aureus*, *B. subtilis*, *Corynbaterium species*, *S. typhi* and *P. aeruginosa*. The activity of the methanolic extract against *B. subtilis* and *Corynbaterium species* is similar to that of tetracycline ($p>0.05$). The highest zone of inhibition of 18.0 mm was produced at 300 mg/ml against *S. typhi* while the lowest zone of inhibition of 8.0 mm was produced at concentration of 100 mg/ml against *P. aeruginosa* [Table 4].

MIC of the Seed Extracts

The results of the MIC are presented in Table 5. The least MIC of 12.5 mg/ml was recorded against *B. subtilis* and *S. typhi* while the highest of 50 mg/ml was recorded against *P. aeruginosa*.

MBC of the Seed Extracts

The results of the MBC are presented in Table 6. The least MBC of 12.5 mg/ml was recorded against *B. subtilis* while the highest of 100 mg/ml was recorded against *P. aeruginosa*.

Table 2: Phytochemicals of the Aqueous and Methanolic Extracts of *Nigella sativa* Linn

Phytochemical	Test	Aqueous	Methanolic
Cabohydrates	Molisch's Test	+	+
	Barfoed's Test	-	-
	Fehling's Test	+	+
	Test for combined reducing sugar	+	+
	Test for ketoses	+	+
Soluble starch	Test for soluble starch	-	-
Anthraquinones	Test for free anthraquinone	-	-
	Test for combined anthraquinone	-	-
Cardiac glycosides	Salkowski's test	+	+
	Lieberman-Burchard's test	+	+
Terpenoids	Test for Terpenoids	+	+
Flavonoids	Shinoda's Test	+	+
	Ferric chloride's Test	+	+
	Lead acetate' Test	-	-
	Sodium hydroxide's Test	-	-
Saponins	Frothing's Test	+	+
Phlobatannins	Test for phlobatannins	-	-
Tannins	Ferric chloride's Test	+	+
	Lead acetate's Test	-	-
Alkaloids	Dragendoff's reagent	+	+
	Mayer's reagent	+	+
Cardenolides	Keller-Killiani's Test	+	+

- = Absent
+= Present

Table 3: The zone of inhibition produced by the Aqueous Extracts of *Nigella sativa* Linn

Bacterial Isolates	Zones of Inhibition (mm)				
	100 mg/ml	200 mg/ml	300 mg/ml	Ciprofloxacin	Tetracycline
S. aureus	R	R	R	26±0.00	R
S. pyogene	R	7.67±0.33	9.67±0.33	29.33±0.37	16.67±0.48
B. subtilis	R	R	R	26±0.00	16±0.00
Corynbaterium spp	R	R	R	34.67±0.33	12.67±0.32
E. coli	R	R	R	25.33±0.28	R
S. typhi	R	R	R	32.33±0.43	27±0.00
K. pneumonia	R	R	R	15±0.00	12.33±0.42
P. aeruginosa	R	R	R	33.67±0.52	R

Results are expressed as Mean±Standard Error of Mean
n = 3 per group
R = Resistance

Table 4: The zone of inhibition produced by the Methanolic Extracts of *Nigella sativa* Linn

Bacterial Isolates	Zones of Inhibition (mm)				
	100 mg/ml	200 mg/ml	300 mg/ml	Ciprofloxacin	Tetracycline
S. aureus	9.0±0.00	11.0±0.00	13.67±0.33	26±0.00	R
S. pyogene	R	R	R	29.33±0.37	16.67±0.48
B. subtilis	12.0±0.00	14.67±0.33	17.67±0.33	26±0.00	16±0.00
Corynbaterium spp.	9.33±0.33	11.67±0.33	15.0±0.00	34.67±0.33	12.67±0.32
E. coli	R	R	R	25.33±0.28	R
S. typhi	12.0±0.00	15.33±0.33	18.0±0.00	32.33±0.43	27±0.00
K. pneumonia	R	R	R	15±0.00	12.33±0.42
P. aeruginosa	8.0±0.00	10.67±0.33	14.0±0.00	33.67±0.52	R

Results are expressed as Mean±Standard Error of Mean
n = 3 per group
R = Resistance

Table 5: Minimum Inhibitory Concentration of Methanolic Seed Extract of *Nigella sativa* Linn

Bacterial Isolate	Concentrations (mg/ml)				
	6.125	12.5	25	50	100
S. aureus	+	+	β	-	-
B. subtilis	+	β	-	-	-
Corynbaterium spp.	+	+	β	-	-
S. typhi	+	β	-	-	-
P. aeruginosa	+	+	+	β	-

+ = Growth
- = No growth
β = MIC

Table 6: Minimum Bactericidal Concentration against Methanolic Seed Extract of *Nigella sativa* Linn

Bacterial Isolates	Concentrations in mg/ml				
	6.125	12.5	25	50	100
S. aureus	+	+	+	α	-
B. subtilis	+	α	-	-	-
Corynbaterium spp.	+	+	+	α	-
S. typhi	+	+	α	-	-
P. aeruginosa	+	+	+	+	α

+ = Growth
- = No growth
α = MBC

DISCUSSION

In the present study, the phytochemicals and antibacterial activity of aqueous and methanolic seed extracts of *N. sativa* Linn.were determined in other to justify some of the traditional uses of the seeds.

The findings from the phytochemical screening revealed presence of several bioactive constituents in both the aqueous and methanolic which include: carbohydrates, terpenoids, saponins, tannins, flavonoids, alkaloids and cardiac glycosides. This result is in accordance with previous work done by Abdulrohman,[24] Khan *et al.*,[25] Eloff[26] and Hajhashemi *et al*.[27] This may justify the traditional uses of the seeds in the management of cardiovascular conditions as glycosides are known with cardiac effect.[28] Terpenoids are essentially lipids and are known for their aromatic properties. Different functions have been attributed to terpenoids including growth regulating and antimicrobial activity.[24] Saponins present in the extract are glycosidic in nature and have physical characteristic of producing soapy foam. Saponins have expectorant activity which is very useful in the management of inflammation of the upper respiratory tract. In addition, saponins present in many plants are known to be cardiotonic in nature.[19] Anthraquinones, phlobatannins and soluble starch were not detected in this study. This corroborated a previous study carried out on the plant in which, anthraquinones, phlobatannins and soluble starch were absent in the extracts of *N. sativa* Linn.[29]

The results of the *in vitro* disc diffusion antibacterial activity showed variability in the inhibitory nature of the extracts. The aqueous extract produced no antibacterial activity against most of the isolates at the concentrations tested despite the presence of phytochemicals with known antibacterial activity. This could be an indication that using water as a solvent did not yield sufficient amount to produce therapeutic effect. This does not agree with the work of Nor' Aisha *et al*.[30] which stated that aqueous extract demonstrated antibacterial activity even at lowest concentration of 20 mg/ml. The discordance in the two studies could be attributed to the strains of the bacteria tested.

However, five (5) of the isolates tested (*S. aureus*, *B. subtilis*, *C. specie*, *S. typhi* and *P. aeruginosa*) demonstrated dose-dependent sensitivity to the methanolic extract of the seeds. Previous studies have shown superiority of methanol as solvent over water.[30] In addition, Parekh *et al*.[31] reported that most of the antimicrobial active compounds were soluble in polar solvents such as methanol instead of water. Thus, the methanol used in this case might have yielded sufficient amount of the phytochemicals resulting in the antibacterial activity observed. This activity may be attributed to the presence of tannins, saponins, glycosides and flavonoids. Flavonoids are known to have antibacterial activity which inhibit the growth of bacterial cell and tannins, a group of polymeric phenolic substances capable of precipitating gelatin thereby toxic to microbes such as bacteria, filamentous fungi and yeast.[32] The antibacterial activity demonstrated by the methanolic extract is in accordance with findings by Nor' Aisha *et al*.[30] who reported antibacterial activity by methanolic extract. In contrast, *S. pyogenes*, *E. coli* and *K. pneumoniae* were not sensitive to the methanolic extract as against Ferdous *et al*.[33] that reported that *E. coli* was sensitive to the seed extract. This variation may be as a result of different strains of bacteria used i.e. the strains used in present study might have developed resistance overtime.

Interestingly, *E. coli* isolate used in the present study demonstrated insensitivity to both aqueous and methanolic extract as well as tetracycline used as positive control. Similarly, *S. aureus* and *P. aeruginosa* were resistant to tetracycline. These provides possible evidence of resistance development among the bacterial isolates. This in turn might have affected their sensitivity to the extract. The methanolic seed extract were further subjected to MIC and MBC determination. The result indicated that the methanolic extract is bacteriostatic at lower concentration than the bactericidal effect except for *B. subtilis* where same concentration produced both inhibitory and lethal effect.

In conclusion, the results obtained from the phytochemical analysis revealed the presence of carbohydrates, terpenoids, flavonoids, saponins, tannins, cardiac glycosides and alkaloids in both the aqueous and methanolic extracts of *N. sativa* Linn. The methanolic extract was found to be more active against the tested organisms than the aqueous extract. The result of the susceptibility study implies that the plant could be used locally for the management of bacterial related infections, especially those caused by *S. aureus*, *B. subtilis*, *C. species*, *S. typhi* and *P. aeruginosa*. However, caution must be ensured to avoid potential harmful effect(s).

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