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Phytochemical and *In Vitro* Antibacterial Properties of Leaves of Some Selected Plant Species in Maiduguri, Nigeria

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Abstract The phytochemical analyses of the leaves of some selected plants species (*Amaranthus caudatum, Brasica oleracea, Ipomea batata, Manihot esculenta* and *Telfera occidentalis*) and *in vitro* antimicrobial activities on some bacteria species were determined using aqueous and ethanol solvents in Maiduguri, Nigeria. Analysis revealed the presence of tannin, cardiac glycoside, saponin, glycoside, flavonoid and tapenoid in all the plants, while anthraquinine, phylobatanin were not detected. Alkaloid was only present in *Manihot esculenta* leaves. The antibacterial assay of the leaf extracts on the tested bacteria showed that the extracts are effective against *Salmonella typhi, Escherichia coli, Pseudomonas auriginosa, Staphylococcus aureus* and *Streptococcus feacalis*, this was determined using crude antibiotic disc to investigate its potential use as antibacterial agent and standard antibiotic drugs as positive control for comparison. The extracts showed higher antibacterial activities against *E. coli* and *S. aureus.*

Keywords Phytochemical, *in vitro*, secondary metabolites, antimicrobial, antibiotic

Introduction

The plant kingdom is a good source of drugs used for the treatment of various human and animal diseases, some of which are malaria, diabetes, cancer, high blood pressure, malnutrition, obesity, cardiovascular and cerebrospinal diseases [1]. The usefulness of plants in the treatment of diseases has been demonstrated to be as a result of the presence of certain chemicals in plants, some of which include glycosides, flavonoids, alkaloids, saponins, tannin and steroids [2]. These compounds are found to be chemo preventive and possess antioxidant effects [3].

Phytochemical Compounds are non –specific in their action and can exhibit several functions, viz antibacterial [4], anti–fungal [5], anti–viral and anti–spasmodic [6]. Again, phytochemical compounds have antioxidant effects, and are now used as ingredients in dietary supplements to maintain good health, as prophylactic agents in cancer and coronary heart diseases [7], as food preservatives and in cosmetics [8-9]. Medicinal herbs, ignored during some ages and even dismissed in others, have been waiting quietly and patiently for several thousand years for man to turn his eyes to them in order to know, study and use them [10].

Microorganism has been a major problem to man from time being, though microbes have been utilized in many ways to man's advantage, their negative roles or adverse effects cannot be over looked. Many measures have been implemented to tackle these microorganisms, among which is the production of antibiotics and antimicrobials from various plant extracts. These antibiotics and antimicrobials of plant extracts have been effective against microorganism over the years. But the emergence of human pathogenic and other food spoilage microorganism that

are resistant to major classes of antibiotics and antimicrobials have increased in recent years, due to the indiscriminate use of these substances and this has become a major problem to man [11].

Bacteria constitute a large domain of prokaryotic microorganisms typically ranging from 0.15 to 4 micrometers in length. Bacteria have a number of shapes, ranging from spheres to rods and spirals. They were among the first life forms to appear on earth and are present in most habitats. Bacteria inhabit soil, water, acidic springs and radioactive waste and deep portions of earth crust [12]. Bacteria also live in symbiotic and parasitic relationship with plants and animals. There are typically million bacterial cells in a gram of soil and a million bacterial cells in a milliliter of fresh water. There are approximately 5150 bacteria on earth [13] forming a biomass which exceeds that of all plants and animals [14]. According to reports, they are extremely adaptable to conditions and survive wherever they are, [15]. The most common fatal bacterial diseases are respiratory infections with tuberculosis alone killing about 2 million people a year, mostly in sub-Saharan Africa [11, 16-18]. There is very little research on the phytochemicals and antimicrobial properties of the selected plant species, especially in the Northern Eastern part of Nigeria. These phytochemicals and minerals are available in plants which are yet to be identified. Many plants have been tested for their antimicrobial properties but only few have such property.

Therefore, research for development of new antimicrobials agents from plant extracts containing certain phytochemicals is an urgent need in order to ensure consumers safety and reduce food loses due to spoilage [1]. In view of the above problem, this research was conducted to analyze the phytochemical and antimicrobial properties of the selected plant species leaves commonly consumed in Maiduguri the Borno state capital.

Materials and Methods

Study area

The experiment was conducted in Maiduguri the state capital of Borno. The state has a land area of about 73,273km^3 and lies between latitude 11^o 09^1 and 11^o 45^1 N and longitude 09^o and 14^o 20^1 E [19]; [20]. Borno shares international border with Niger Republic in the North, Chad Republic in the North-East and Cameroon Republic in the East. Agriculture is the main stay of economy in the state. Crops grown reflect the nature agro-ecological zone. The major crops cultivated include: millet, sorghum, groundnut, maize, cowpea and vegetables (onion, pepper, tomatoes, Amaranthus etc). The vegetation of state is mostly mesophyte and xerophytes plants due to the semi-arid nature of the Sahel and Northern Sudan Savannah [20].

Samples Collection and Processing

Fresh leaves of the selected plants were collected from University of Maiduguri campus and local markets (Kasuwan Tashan Bama) along Bama road, in Maiduguri, Borno State in July 2014. The leaves were transported to Department of Biological Sciences University of Maiduguri in a standard container for identification and authentication. Prior to the analysis the leaves were washed with tap water and then rinsed with distilled water. The residual moisture was evaporated at room temperature and thereafter the leaves were oven dried at 60^{o}C until properly dried. The dried leaves were then ground in porcelain mortar, sieved through 2mm mesh sieve and stored in polythene bags. The powdered samples were used for proximate, mineral, phytochemical and *in-vitro* antimicrobial properties.

Sources of the Bacteria

E. coli was isolated from sewage water collected from the University of Maiduguri sewage dam, *Salmonella typhi* was obtained from contaminated tiger nut juice extract. The *Streptococcus feacalis***,** was found in fecal sample collected from abattoir, cattle market in Maiduguri, while the *Staphylococcus aureus* was obtained in processed dried beef commonly called *Kilishi* Northern Nigeria, and the *Pseudomonas aeruginosa* was found in a fresh dairy cow milk locally called *Kindrmu* in Hausa language.

Bacterial isolation

For the bacteria extraction quadrant streaking was done to obtain pure colonies. The inoculation of the culture was made on the agar surface by back and forth streaking with the inoculation loop over the solid agar surface, making dilution gradient across the agar plate. Upon incubation, individual colonies arose from the media. In order to obtain

the pure culture of organism, the isolated colonies were aseptically transferred on to different nutrient agar slant tubes and incubated overnight at 37 ºC.

The following characteristics features of the colonies were observed on solid agar media and were shown in Table 1.

Table 1: Characteristic features of the bacteria colonies

Shape	Size (mm)	Elevation	Surface	Edges	Colour	Nature
Circular	Small	Elevated	Smooth	Entire	Yellow	Discrete
Irregular	Medium	Convex	Wavy	Undulated	Green	Confluent
	Large	Concave	Rough	Crenated	Golden	Coliform
		Unbonate	Granular	Fimbriate	Blue	Spreading
		Umbitricate	Papilate	Curied		

Source: Glistening

Gram staining

A smear of the test organisms were made on different clean slides and were allowed to air dried for few seconds. The smears were flooded with crystal violet and were allowed to stand for 30 seconds. The stains were washed off with distilled water and were allowed to drain off. The stains were covered with logoes iodine and allowed to stand for 30 seconds before washing off with distilled water. The stains were flooded with acetone and washed off immediately. The smears were later counterstained with safranin and let to stand for 1 minute. The stains were washed off with distilled water and allowed to air –dry. They were examined under oil immersion (100 xs).

Biochemical reaction

The Biochemical test was done according to the method described by MacFadin (1980), based on the method Indole test was carried out to differentiate gram- negative rods especially *E-coli* that breakdown the amino acid tryptophan with the release of indole. The organisms were cultured in a test tubes containing 3ml of sterile peptone water and was incubated at 37^0C for 48 hours. After the incubation 0.5ml of Kovac's indole reagent was added to the tubes, stirred and was allowed to stand for 10 mins. A red colour in the surface layer was observed. Catalase test was carried out in which a small amount of the organisms were smeared in different slides, a drop of hydrogen peroxide were added on each slide and were observed for vigorous bubbling. This test was done to identify organisms that produce the enzyme, catalase. This enzyme detoxifies hydrogen peroxide by breaking it down into water and oxygen gas. The bubbles resulting from production of oxygen gas clearly indicate a catalase positive result. Oxidase test was used to identify microorganisms containing the enzyme cytochrome oxidase. It is commonly used to distinguish between oxidase negative *Enterobactericeae* and *Pseudomonadaceae*.

Reagent preparation

Oxidase reagent is specially prepared as 10g/l or 1% solution of tetra methyl-p-phenylenediaminedihyhydrochloride. Filter paper method; a piece of filter paper was placed in petri dish and three drops of freshly prepared oxidase reagent were added. Using a sterile glass rod, a colony of test organisms were removed from a culture plate and smeared on on the filter paper. Oxidase positive organisms give blue colour within 5-10 seconds, and in oxidase negative organisms, colour does not change. The coagulase test was done to differentiate strains of staphylococcus species.Test tubes containing 0-1ml of plasma were labelled. The organisms were inoculated differently in the plasma tubes, capped and stored in a water bath at 37^0C for several hours. The bottles were checked for clumping solidification or semisolid.

Preliminary phytochemical screening

Photochemical screening of the extracts was carried using standard methods. The components analyzed were as follows; glycosides, flavonoids, saponins, tannins, tapenoids, phylobataninins, alkaloids and anthraquinine. 25g of air dried plant material were pulverized using wooden mortar and pestle. The samples were transferred into 1 liter round bottom flask and sufficient amount of 95% ethanol were added until it covered the sample. The mixture were refluxed for about 1(one) hour, the solution was removed and decanted. This was repeated twice using new solvent. After the sample had been extracted, the solution was filtered and concentrated on water bath 40-50 ^{o}C. After

evaporating, a black colour was obtained. The weight was taken and transferred to air dried container for further analysis as done by [22]**.**

Test for Tannins

The extract (0.5g) was stirred with about 10ml of distilled water and then filtered. The filtrate was boiled with 3 drops of 10% HCl and 1 drop of methanol, a red precipitate was taken as evidence for the presence of tannins, as described by [23] and [24].

Test for Phylobatanin

Small amount of extract was boiled with distilled water and then filtered. The filtrate was further boiled with 1% aqueous HCl. The appearance of a red precipitate shows the presence of a Phylobatanin as done by [25].

Test for Glycosides

For the plant extract (0.5g) to be tested, 10ml of benzene was added and then filtered. 5ml of 10% ammonia solution was added to the filtrate. The mixture was then shaken and the appearance of a pink color in the ammoniacal (lower) phase was taken as the presence of free Anthraquinine [25].

Test for Tapenoid

A little portion of the extract was dissolved in ethanol to it 1ml of acetic anhydride was then added followed by the addition of con H_2SO_4; a color change from pink to violet indicates the presence of terpenoid [22].

Test for Saponin

Was done according to the method describe by [23] and Vishnoi (1979) based on the methods, 1g of the plant extract was boiled with 5ml of distilled water, filtered and the filtrate divided into two portions, the first portion, about 3ml of distilled water was added and then shaken for about 5minutes. Frothing which persist on warming is an evidence for the presence of saponnins [23]. The second portion 2.5ml of a mixture of equal volume of Fehling's solution A and B was added, appearance of a brick red precipitate was taken as the presence of saponins glycosides [26].

Test for Flavonoid

The flavonoids test was carried out using the following methods:

The Shinoda's Test was done using the method described by Markham [27], 0.5g of the extract was dissolved in ethanol warmed and then filtered. Three pieces of magnesium chips was added to the filtrate followed by a few drops of con HCl. Purple coloration, indicate the presence of flavonoids [27], the Ferric Chloride Test was done by boiling the leaf extract with distilled water and then filtered into 2ml of the filtrate, few drops of 10% ferric chloride solution was added, a green blue indicate the presence of a phenolic hydroxyl group as done by [25]. While the Sodium Hydroxide test done also using the method of [25], small quantity of the extract was dissolved in water and filtered, to this 2ml of the 10% aqueous sodium hydroxide was added to produce a yellow coloration, a change in color from yellow to colorless on addition of dilute hydrochloric acid indicates the presence of flavonoids [25].

Test for Alkaloid

For the alkaloid test, extract (0.5g) 5ml of the 1% aqueous HCl stirred on water bath and then filtered. Of the filtrate, 3ml was taken and divided equally into 3 portions in a test tube. To the first portion few drops of dragendorff's reagent was added; the occurrence of orange red precipitate was taken as positive. To the second portion 1ml of Mayer's reagent was added and appearance of buffer-colored precipitate indicate the presence of alkaloids and to the last portion, 1ml few drops of Waquer's reagent was added and a dark-brown precipitate indicates the presence of alkaloids as done by [28].

Results and Discussion

Results

The qualitative phytochemical analysis of the selected plant species reveals that the plants had glycoside, tannin, Saponin, terpenoid and flavonoids while alkaloids was only found in *M.esculenta*.

Table 2: Qualitative phytochemical components of leaves of the selected plant species

Plant	Glycoside		Terpenoid		Saponin		Flavonoid		Tannin		Phylobatanin		Anthraquinine		Alkaloid	
	EE	**AE**	**EE**	**AE**	**EE**	**AE**	**EE**	**AE**	**EE**	**AE**	**EE**	**AE**	**EE**	**AE**	**EE**	**AE**
Amaranthus caudatum	+	+	+	+	-	-	+	+	-	-	-	-	-	-	-	-
Brassica oleraceae	+	+	+	+	+	+	+	+	+	+	_	_	_	_	_	_
Ipomoea batatas	+	+	+	+	_	+	+	+	+	+	_	_	_	_	_	_
Manihot esculenta	-	-	+	+	-	-	-	-	+	+	_	_	_	_	+	_
Telfairia occidentalis	+	+	+	-	+	+	-	-	+	+	_	_	_	_	_	_

EE=Ethanol extract, AE= Aqueous extract, + = presence, - = absence

Confirmatory test

Staphylococcus aureus was light cream in colour, cocci in shape. It was positive to gram reaction, positive on nutrient agar and Mackonkey agar, positive to catalase and coagulase test and negative on indole. *E- Coli* were pink in colour and rod in shape, negative on gram reaction, indole positive. *Streptococcus feacalis* was green in colour and spherical in shape, positive in catalase, indole and coagulase test. *Pseudomonas aeruginosa* was gray and straight in shape it is positive in catalase test and negative in indole and coagulase test while *Salmonella typhi* were blue in colour and rod in shape it was positive on gram and catalase but negative on indole and coagulase.

Table 3: Confirmatory test for the organisms

Characteristic	**Species**				
	S. aureus	***E. coli***	***S. feacalis***	***P. aeruginosa***	***S. typhi***
Colours of colony	Light Green on MacConkey agar	PinkMacConky agar	Green on MacConkey agar	Gray on nutrient agar	Blue on nutrient agar
Shapes	Cocci	Rod	Spherical	Straight	Rod
Gram reaction	+	_	+	_	+
Nutrient agar	+	+	+	+	+
MacConkey agar	+	+	+	+	+
Catalase test	+	N/A	_	+	+
Indole test	_	+	+	_	_
Coagulase test	+	N/A	+	_	+

+=Positive; - =Negative; N/A=Not applicable

Effect of ethanol extracts against bacterial species

P. auriginosa had the highest zones of inhibition (4.3mm) using *T. occidentalis* using ethanol extracts while *S. aureus* had the least (2.4mm).There is significance difference in the zones of inhibition of *A. caudatum* , *P. auriginosa* shows highest zones of inhibition (10.8mm) but *S. aureus* and *S. typhi* had the lowest (4.6mm). *B. oleraceae* had the highest sensitivity on *S.typhi* (5.3mm) and *S. aureus (*1.1mm). The leaves extracts of *M. esculenta* inhibited the *E. coli* giving the zone of inhibition of (12.2mm) but it had least inhibition on *S.feacalis*having (1.6mm). *I. batatas* leaves extract shows highest sensitivity on *E. coli* (6.1mm) while it had least sensitivity on *S. aureus* (1.1mm).

Table 4 shows zones of inhibition (mm) of leaves of the selected plants extracts against five different bacterial species using ethanol as the solvents.

Table 4: Zone of inhibition (mm) of ethanol extract selected plant leaves against some bacteria

Bacteria	**Plant**				
	A. Caudatum	***B. oleraceae***	***M. esculenta***	***I. batatas***	***T. occidentalis***
Escherichia coli	6.7^{c}	5.1^{b}	12.2^{a}	6.1^{a}	6.1^{a}
Staphylococcus aureus	4.6^{d}	2.2^{e}	7.3^{c}	1.1^{d}	1.1^{d}
Streptococcus feacalis	6.3^{c}	2.4^{d}	1.6^{d}	3.4^{b}	3.4^{b}
Pseudomonas auriginosa	10.8^{b}	2.5^{c}	7.4^{c}	4.5^{b}	4.5^{b}
Salmonella typhi	4.6^{d}	5.3^{a}	10.3^{b}	3.1^{c}	3.1^{c}
SE±	0.15	0.02	7.8	0.03	0.03

Values present mean (n=3); SE±= standard error of means. Values within a column with different superscript are significantly different (P<0.05)**.**

Effect of aqueous extracts the bacterial species

The leaves extracts of *T. occidentalis* shows highest zone of inhibition on *E. coli* ,*S. feacalis* and *P. auriginosa*,(3.3mm),(2.6) and (3.0mm) respectively at P<0.05 while *S. aureus* had the least (2.3mm). *A. caudatum* leaves extract had highest sensitivity on *S. aureus* (17.5mm) at P<0.05 significace but least in *S. feacalis* (1.5mm). *B. oleraceae* inhibited *E-coli* with zone of (2.0mm) giving the highest zone of inhibition at P<0.05 significance while the least inhibition was (0.1mm) on *S. typhi*. *M. esculenta* leaves was sensitive against *E-coli* (14.0mm) which was the highest zone of inhibition at P<0.05, and lowest in *S. feacalis* (2.3mm). *I. batatas* leaves extract was sensitive against all the tested bacteria giving the highest inhibition on *E-coli* (7.5mm) at P<0.05 significance but least sensitivity was observed in *S. feacalis* and *S.typhi* (1.0mm).

Table 4: Zone of inhibition (mm) of the aqueous leaves extracts on some bacteria species

Test organism	**Plant**				
	A. caudatum	***B. oleraceae***	***I. batatas***	***M. esculenta***	***T. occidentalis***
E. coli	1.9^{c}	2.0^{b}	7.5^{a}	14.0^{a}	3.3^{b}
S. aureus	2.5^{a}	2.5^{a}	3.0^{c}	8.3^{b}	2.3^{b}
S. feacalis	0.1^{e}	1.0^{c}	1.0^{d}	2.3^{e}	2.6^{a}
P. auriginosa	0.9^{b}	0.1^{d}	4.5^{a}	7.0^{c}	3.0^{a}
S. typhi	1.0^{d}	1.0^{c}	1.0^{d}	5.0^{d}	3.1^{a}
SE±	0.3	1.3	3.4	7.3	2.9

Values present mean (n=3); SE±= standard error of means. Values within a column with different superscript are significantly different (P<0.05)**.**

Table 5 shows the combined antibacterial properties of crude aqueous of selected plant species *E. coli* showed high sensitivity 5.7mm to the extracts while while *S. aureus* showed low sensitivity 0f 2.2 mm . Combined antibacterial properties of ethanol extracts of selected plant species where *E.coli* had the highest sensitivity 7.5mm while *P.auriginosa* had little sensitivity of 3.0mm.

Table 5: Combined antibacterial properties of different extracts of selected plant species

Test organism	**Zone of inhibition (mm)**	
	Aqueous	**Ethanol**
E. coli	5.74	7.5
P. auriginosa	3.72	3.0
S. typhi	3.0	3.9
S. aureus	2.2	5.8
S. feacalis	3.08	5.18

Table 6 shows the effects of standard antimicrobial disc on the test organisms. Where cpx10 µg shows high sensitivity against all the test organisms, followed by l5µg while l30µg showed least sensitivity in the test organisms.

Table 6: Zone of inhibition (mm) using standard antibiotic drugs on bacterial species

Antibiotics	***E-coli***	***S. aureus***	***S. feacalis***	***P. auriginosa***	***S. typhi***
CPX 10µg	15.0	10.0	24	14	24
AMX25µg	1.0	-	1.0	4.0	-
OFL5µg	14.0	9	24	14	24
ERY5µg	14	9	4	9	-
CHL30µg	1	1	4	1	4
STR10µg	1	1	1	9	1
COT25µg	16	1	-	0.2	0.2
GEN10µg	0.5	0.5	0.4	4	14
PEF5µg	15	4.0	16	14	9

Source: Fondoz Dics Diagnostic Laboratories

Discussion

The phytochemical screening of some selected plant species leaves in table 4, indicates that they contain glycosides, tapenoid, saponin, flavonoid, tannin and alkaloid .The findings in the study agreed with earlier studies which also found that, not all phytochemicals are present in all plants part and that those present differ according to the type of the extracting solvent used [29]; [30].These compounds have been found to inhibit bacterial growth and are capable of protecting certain plants against bacterial infections [31;[32]. Flavonoids are widely distributed in plants fulfilling many functions. They have been shown to have antifungal activity *in-vitro* [33].The potent antioxidant activity of flavonoids reveals their ability to scavenge hydroxyl radicals superoxide and lipid peroxy radicals, this may be the most important function of flavonoids[33]. Saponin was also detected in the leaves of five plant species, saponins have been shown to possess both beneficial (Cholesterol lowering) and deleterious (Cytotoxic; permeabilization) of the intestine) properties [34]. Although some saponins have been shown to be highly toxic under experimental conditions, acute poisoning is relatively rare in animals [35]. Due to its ability to form froth, soap is being produced locally from it for bathing. The presence of tannins in these selected plant leaves agreed with the works of [36], which also discovered 235 traces of tannins in the leaves grown in Uganda. Tannins are polyphenols that are obtained from various parts of different plants belonging to multiple species. Tannins can also be effective in curbing haemorarhages as well as restrict bare swellings. Herbs possessing tannins are widely used as mouthwashes, eyewashes, snuff and even as vaginal douches and also treat rectal disorders [37].

The cultural and morphological characteristics of test organisms studied reveals that all the five bacterial species have different colours including, light green, pink, Gray and blue-black colouration. *S.aureus* was cocci in shape, negative on indole, *E-coli* was rod in shape and indole positive, *S.feacalis* was green in colour,spherical in shape and indole positive, *P. auriginosa* was gray in colour and straight in shape negative on indole. All the five bacteria studied grows well on nutrient agar and McConkey agar, this study closely agrees to the findings reported by [38]

The plant extracts differ in their inhibitory behavior against the microorganisms tested. Most of the extracts showed antimicrobial activities against *E. coli* (60%) *S. aureus* (30%). The inhibitory activity of *T. occidentalis* against *E. coli* agrees with the finding of [38] who reported that the ethanol extract of *T. occidentalis* inhibited the growth of *E. coli.* The action of ethanol extracts on five bacterial species, the leaves of *T. occidentalis* inhibited *P. auriginosa* with 3.1mm zone this is similar to zone of inhibition obtained for $Gentamycin_{10\mu g}$ used on the same bacteria. The result agrees with earlier research carried on the same plant by other researchers like [38] and [39]. Most of their result showed a higher antimicrobial activity against the following organism namely *P. auriginosa*, *E-coli,* and *S.aureus*14.0mm, 9.0mm and 7.5mm respectively. *Staphylococcus aureus a* common food poisoning bacterium, the antimicrobial activity of *M. esculenta* leaves could inhibit the growth of this bacterium in both ethanol and Aqueous

extracts [40] .Aqueous extracts of *M. esculenta* had inhibited *E-coli* with 14mm zone this is similar to standard antibiotics drug disc $ERY_{5\mu g}$ and $OFL_{5\mu g}$ which also gave 14mm zone against the same organism.

The relatively low antibacterial activities of the combined extracts of five plant species observed in this study are surprising. It contradicts and confounds the belief of many people that a combination of these plants in food promotes better health with particular reference to microbial infection than taking each of them in isolation. A similar reduction in antibacterial property was observed by [41]. This may be attributed to possible interference among the constituents of the various extracts in the combined form or interactions that produce products with lesser or no antibacterial properties [42]. Further investigation on the phytochemical composition of these plants and their interactions in the combined form is necessary to further elucidate the mechanisms behind the lowered antibacterial properties of the plants in combined forms.

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