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Comparative phytochemical and in vitro antimicrobial activities of the leaf extracts of two medicinal plants growing in North-East, Nigeria

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ABSTRACT

Introduction: The use of plants as medicine is as old as chemistry and common to all societies including the African, notably some parts of Northern Nigeria. Infectious diseases are among the causes of mortality and morbidity in rural areas endemic with hygienic problems in most developing countries including Nigeria. Two plant species with similar ethnomedical reports from different families were used in this study against some microorganisms. Their phytochemicals were also evaluated.

Methods: The leaf samples of *Punica granatum* and *Waltheria indica* were prepared and independently extracted with 80% methanol using maceration technique. The extracts were concentrated to dryness at reduced pressure and then subjected to phytochemical evaluation. Antimicrobial activities were evaluated using hole-in-plate disc diffusion technique.

Results: The phytochemical results of both extracts revealed the absence of anthraquinones. However, both extracts showed the presence of cardenolides, cardiac glycosides, flavonoids, saponins, tannins and terpenoids; while alkaloids were found in *W. indica*. The antimicrobial susceptibility study showed dose-dependent pattern with the highest dose (80 mg/hole) showing inhibition zone of 23.67 ± 0.47 and 23.33 ± 0.47 mm, respectively by *P. granatum* and *W. indica* against *Streptococcus pyogenes* while at 20 mg/hole inhibition was noted as 8.67 ± 0.47 and 7.00 ± 0.00 mm against *Escherichia coli* for *P. granatum* and *W. indica,* respectively.

Conclusion: The findings of this study scientifically support the use of *P. granatum* and *W. indica* in folklore medicine for the cure of infections by microbes.

Implication for health policy/practice/research/medical education:
Waltheria indica showed phytochemicals like cardenolides, cardiac glycosides, flavonoids, saponins, tannins, and terpenoids. However, alkaloids were found in only *W. indica*. The extracts showed dose-dependent broad spectrum antimicrobial activities against the test microorganisms which could be attributed to the secondary metabolites present. Hence both plants might be used as a natural source for preparation of new drugs.

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Introduction

Traditional medical practices use the knowledge and belief of involvement of natural products, spiritual therapies and some exercises applied singly or in combination with the cure of man (1). Historically, man has been using various plant parts in prevention and treatment of several diseases (2). In the early 20th century, traditional medicine was regarded as a practice employed by poor society. However, synthetic or biomedical drugs have been doubted by some developed countries; thus giving interest in natural medicines or therapies (3). A plant becomes medicinal only when its biological activity has been ethnomedically reported or scientifically established (4). Depending on the usage, folk medicine may be regarded as traditional medicine, alternative medicine, indigenous medicine, or natural medicine (5). Traditionally, plants have been used as a source for treatment of diseases through secondary metabolites which play a significant role as medicines,

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flavouring and recreational drug in different parts of the world whose use contributes immensely to the enhancement of primary health care delivery (6,7).

The plant *Punica granatum* Linn. (Lythracea) commonly called pomegranate in English, *"Anar, Anar-ke-per"* in Hindi, *"Anar"* in Urdu, *"Punjabi* and *"Rooman"* in Arabic originated from Iran to the Himalayas in Northern India and found widely distributed the Mediterranean region of Asia, Africa and Europe (8). The rind of the fruit and the bark of the tree are used in the treatment of diarrheoa, dysentery and intestinal parasites, for nose and gum bleeds, toning skin and treating hemorrhoids in ancient Ayurvedic traditional medicine. Its juice is employed as an eye drop to stop the development of cataracts; the seeds and rind are also used as contraceptive and abortifacient. The most abundant phytochemicals in *P. granatum* juice are polyphenols, including the hydrolysable tannins or ellagitannins (9), condensed tannins (10), catechins, gallocatechins and prodelphinidins. The antioxidant property of *Punica granatum* has been reported, previously (11).

Waltheria indica Linn. (Malvaceae) is one of the species of flowering plants in the mallow family which have a tropical distribution. *W. indica* (sleepy morning) commonly called velvet leaf, marsh-mallow, monkey bush, boater bush, leather coat, among other names (12); is abundantly found in West Africa (13). In Nigeria, the plant is locally known as "*hankufa*" or "*hankubah*" in Hausa, "*kori kodi*" in Yoruba, *kafafi*" in Fulani", and "*efu-abe*" in Nupe languages (14). *W. indica* is a plant that has attracted medicinal interest because of its applications in traditional medicine to treat ailments employed as infusion or decoction where febrifugal, purgative, emollient, tonic, analgesic and astringent action is sought (12). The plant is used by Hausas of Northern Nigeria for the treatment of skin diseases, impotency and infertility. It is also used as an aphrodisiac and children's medicine at birth and during teething period (15).

It has been established that infectious diseases especially microbial type constitute a large number of health problems, especially in the developing countries. There is high proliferation of resistance to antimicrobial agents which not only does it result from poor quality drugs manufactured, patient non-compliance and irrational use of antimicrobial agents, but also could be due to mutations within the microbial flora (16). Therefore, the aim of this research was to ascertain the ethnomedical claim by the local populace and to compare the efficacy between the two plant extracts which are locally used here in the treatment of infectious diseases.

Materials and Methods

Sample collection and identification

Fresh leaf sample of *P. granatum* was collected from Gabanni-Babaji Village, Fika Local Government area, Yobe State while *W. indica* was collected from Wadiya Village, Maiduguri Metropolitan Council, Borno State. The plant samples were identified by a taxonomist at Botany section, Department of Biological Sciences, University of Maiduguri, Nigeria. Both plant samples were air-dried at room temperature and freed from twigs and dirt and then ground into coarse-powdered form with wooden mortar and pestle.

Extraction procedure

Two hundred and fifty grams of the powdered plant materials of each sample were independently extracted for 120 hours using 80% methanol in extraction bottle employing maceration technique. The extractives were filtered differently using Whatman No.1. The filtrate was poured into evaporating dish to dryness at reduced temperature and pressure and then kept in a desiccator until use.

Phytochemical screening

Phytochemical screening for the presence or absence of the secondary metabolites such as alkaloids, anthraquinones, cardenolides, cardiac glycosides, flavonoids and saponins were carried out according to the standard procedures as described previously (17-21).

Test for alkaloids

Half gram of the extract was stirred with 5.0 mL of 2 M aqueous hydrochloric acid in a steam bath. 1.0 mL each of the filtrate was separately treated with few drops of Mayer's reagent, Drangendorffs' reagent and Wagner's reagent; appearance of buff-coloured precipitate, orange-red precipitate and a dark-brown precipitate indicated the presence of alkaloid (21).

Test for tannins

Half g of the plant extract was dissolved in 10ml of distilled water and filtered. To 2 ml of the filtrate, few drops of 1% ferric chloride solution was then added. The occurrence of blue-black, green or blue-green precipitate colour indicates the presence of tannins (17).

Test for cardiac glycosides

Liebermann-Burchard's test

To 0.5 g of the extract, 2 mL of acetic anhydride was added. The mixture was cooled in an ice, and then conc. H_2SO_4 was added carefully. Colour development from violet to blue or bluish-green indicates the presence of steroidal ring (20).

Salkowski's test

To 0.5 g of the extract, 2 mL of chloroform was added; followed by conc. H_2SO_4 by the side of the test tube to form a lower layer. The appearance of reddish-brown or yellow colour at the interphase indicates the presence of a steroidal ring (20).

Test for saponins

A small portion of the plant extract was added to distilled water (20 mL) in a 100 mL beaker, boiled and filtered. About 5 mL of the filtrate was shaken vigorously for about 5 minutes. Frothing which persists on warming indicated the presence of saponins (18).

Test for free anthraquinones

About 0.5 g of the plant extract was shaken with 10 mL of benzene and then filtered. Five milliliters of 10% ammonia solution was added to the filtrate. The resultant mixture was shaken. The appearance of pink, red or violent colour at the lower phase indicates the presence of anthraquinones (21).

Test for combined anthraquinones

To 0.5 g of the extract, 10 mL of aqueous H_2SO_4 was added and shaken and transferred while was hot. The filtrate was shaken with 5 mL of benzene. The benzene layer was separated and was added half its own volume with 10% ammonia solution. The presence of pink, red or violet colouration in the ammoniacal phase indicates the presence of combined anthraquinones (21).

Test for flavonoids

Ferric chloride test

To 0.2 g of the extract, 5 mL of water was added, boiled and filtered. To 2 mL of the filtrate, few drops of 10% ferric chloride were added. A green-blue or violet colouration indicates the presence of phenolic hydroxyl group (21).

Shinoda's test

About 0.2 g of the extract was dissolved in 5 mL of ethanol, warmed and then filtered. Few pieces of magnesium chips were added to the filtrate followed by few drops of conc. HCl, a pink, orange or red to purple colouration indicates the presence of flavonoids (19).

Lead acetate test

Exactly 0.5 g of the extract was dissolved in 5 mL of distilled water. Tree milliliters of 10% lead acetate solution were added. The appearance of buff-coloured precipitate indicates the presence of Flavonoids (21).

Test for terpenoids

About 0.2 g of the extract was dissolved in 5 mL of ethanol. One milliliter of acetic anhydride was added, followed by addition of conc. H_2SO_4. The presence of terpenoids in the sample was detected as the colour changes from pink to violet (20).

Test for cardenolides

Keller-Killiani's test

Exactly 0.5 g of the extract was dissolved in 2 mL glacial acetic acid containing a drop of ferric chloride solution. One milliliter of conc. H_2SO_4 was added. The appearance of a brown ring at the interphase shows the presence of digitoxose sugar characteristic of cardenolides (21).

Test for carbohydrates

Molisch's test

Few drops of Molisch's reagent were added to 2 mL of the extract obtained by dissolving in distilled water. To this, 1 mL of conc. H_2SO_4 was added to the side of the test tube. The mixture was allowed to stand for 2 minutes and then diluted with 5 mL distilled water. Formation of red to dull-violet colour at the interphase of the two layers was taken as the positive test (21).

Test for free reducing sugars

Exactly 0.2 g of the extract was dissolved in 5 mL of distilled water and filtered. The filtrate was heated with 5 mL of equal volumes of Fehling's solution A and B formation of a red precipitate of cuprous oxide indicates the presence of reducing sugar's (21).

Test for combined reducing sugars

Exactly 0.2 g of the extract was hydrolyzed by boiling with 5 mL of dilute hydrochloric acid and filtered; the resulting filtrate was neutralized with sodium hydroxide solution. Few drops of Fehling's solution were added and then heated in a water bath for 2 minutes. Formation of reddish-brown precipitate of cuprous oxide indicates the presence of combined reducing sugars (21).

Test organisms

Three strains of gram-positive bacteria used include *Bacillus subtilis, Staphylococcus aureus* and *Streptococcus pyogenes,* Gram-negative were *Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa;* while *Candida albicans* was the only fungal species used in this study. These organisms were clinical isolates obtained from Department of Veterinary Medicine, University of Maiduguri, Maiduguri, Nigeria.

Antimicrobial susceptibility test

The antimicrobial susceptibility test was carried out using the agar plate disc diffusion technique as described by National Committee for Clinical Laboratory Standards (22) and modified by Usman et al (23). The tests were carried out using a stock concentration of 400 mg/mL prepared by dissolving 4 g, 200 mg/mL prepared by dissolving 2 g and 100 mg/mL prepared by dissolving 1 g of the crude extract differently into 10 mL of sterile distilled water. Working volumes was 0.2 mL each of the concentrations prepared and then dispensed into each of the 6 mm bored holes to afford respectively 80, 40 and 20 mg/hole. After incubation, the average diameter of three readings of the clear zone around the hole was recorded as the measure of inhibitory level of the extract against the test bacteria and reported as mean ± SEM. The dilution ratio for gram-positive bacteria and gram-negative

bacteria was 1:1000 and 1:5000 respectively using peptone water (23).

Determination of minimum inhibitory concentration

A serial dilution ranging from 100 to 6.25 mg/mL were made. The bacterial strain was cultured in nutrient broth as suspension; 5 ml peptone water was then added. To the suspension, 5 ml of each extract concentration was added to the nutrient broth cultures containing 1.0×10^7 CFU/mL which were seeded into each test tube and then incubated at 37°C for 18-24 hours. Test tube with no turbidity was taken as the MIC value (24).

Determination of minimum bactericidal concentration

Aliquots from the MIC studies were used for the MBC determination. To a solid nutrient agar, a bacterial streak of the suspension from the MIC test tube was made and the procedure repeated all through the required numbers of corresponding isolates. The isolated organism on the nutrient agar was incubated at 37°C for 18-24 hours. After incubation, the plates were observed for bacterial growth; the lowest concentration of the extract required to kill microorganism was considered as the MBC value (24).

Results

The phytochemical screening and antimicrobial activities were conducted on the methanolic leaf extracts of *P. granatum* and *W. indica*. The results of phytochemical screening are presented in Table 1 while the results of antimicrobial susceptibility are shown in Table 2. The minimum inhibitory concentrations (MICs) of the extracts are presented in Table 3; the results for the minimum bactericidal concentrations (MBCs) of the extracts are expressed in Table 4.

Phytochemical screening

The percentage extractive of *W. indica* was 11.43% and of *P. granatum* was 13.83% w/w. Phytoconstituents found in these extracts were cardenolides, cardiac glycosides, flavonoids, saponins, tannins and terpenoids. However, only *W. indica* indicated the presence of alkaloids. More so, both extracts had not any anthraquinones (Table 1).

Antimicrobial susceptibility

The susceptibility pattern of the extract revealed that the highest activity for both extracts was found at the highest concentration of 80 mg/hole with the DIZ values of 23.67 ± 0.47 and 23.33 ± 0.47 mm, respectively for *T. avicceniodes and W. indica against S. pyogenes*. However, the least activities were also recorded as 8.67 ± 0.47 and 7.00 ± 0.00 mm inhibition zone against *E. coli* at 20 mg/hole (Table 2).

Minimum inhibitory concentration

The minimum inhibitory concentration exhibited by *T. avicceniodes* was indicated as 12.5 mg/ml against *K. pneumonia, P. aeruginosa* and *Candida albicans;* while *W. indica* showed inhibition at the same concentration of 12.5 mg/mL against *S. pyogenes* (Table 3).

Table 1. Phytochemical analysis of crude leaf extracts of *Punica granatum* and *Waltheria indica*

S/No.	Phytochemical tests	Plant species	
		P. granatum	*W. indica*
1	Test of alkaloids		
	Preliminary test for alkaloid	-	+
	Confirmatory test for alkaloid	-	+
2	Test of free anthraquinones	-	-
	Test for combined anthraquinones	-	-
3	Test of carbohydrates		
	Molisch's test	+	+
	Test for combined reducing sugars	+	+
	Test of free reducing sugars	-	-
4	Test for Cardiac glycosides		
	Liebermann-Burchard's test	+	-
	Salkowski's test	-	-
5	Test for cardenolides		
	Keller-Killiani's test	+	+
6	Test of saponins glycosides	+	+
7	Test of tannins	+	+
8	Test for flavonoids		
	Ferric chloride test	-	+
	Shinoda's test	+	+
	Lead ethanoate test	+	+
	Sodium hydroxide test	-	+
9	Test of terpenoids	+	+

Key: + = Present, - = Absent

Minimum bactericidal concentration

The results for the minimum bactericidal concentration revealed that the MBC value of 25.0 mg/ml was exhibited by both extracts while *W. indica* was comparatively effective showing MBC value of 12.5 mg/mL against *P. aeruginosa* and *C. albicans* (Table 4).

Discussion

The results of phytochemical screening of leaf extracts of *P. granatum* and *Waltheria indica* indicate the absence of anthraquinones in *P. granatum* as well as in *W. indica* which corroborated to the earlier report by Mohammed et al (15) that no parts of the plants showed the presence of anthraquinones. However, both extracts revealed the presence of cardenolides, cardiac glycosides, flavonoids, saponins, tannins, and terpenoids. Alkaloids were only present in *W. indica* as reported previously (15). These classes of compounds have been reported to be a remedy against many pathogenic consequences and hence, explain their use traditionally for the treatment of different kinds of diseases (25). Secondary metabolites like tannins, terpenoids, alkaloids, and flavonoids have been reported to have *in vitro* antimicrobial properties (26,27).

Table 2 shows the results obtained from the antimicrobial evaluations of the leaf extracts of *P. granatum* and *W. indica*. The susceptibility pattern at concentrations of

Table 2. Antimicrobial susceptibility of the leaf extracts of *Punica granatum* and *Waltheria indica* at different concentrations

Microorganisms	Extract	Concentration of the extract/diameters inhibition zone (mm), mean±SEM		
		80 mg/hole	40 mg/hole	20 mg/hole
Bacillus subtilis	*P. granatum*	18.67±0.47	14.67±0.47	11.00±0.47
	W. indica	17.00±0.00	13.00±0.00	9.33±0.47
Staphylococcus aureus	*P. granatum*	13.67±0.47	10.00±0.00	11.33±4.71
	W. indica	17.67±0.47	13.67±0.47	10.00±0.00
Streptococcus pyogenes	*P. granatum*	23.67±0.47	18.33±0.47	14.00±0.00
	W. indica	23.33±0.47	18.67±0.47	14.33±0.47
Escherichia coli	*P. granatum*	16.67±0.47	11.67±0.47	8.67±0.47
	W. indica	12.00±0.00	9.00±0.00	7.00±0.00
Klebsiella pneumoniae	*P. granatum*	17.00±0.00	12.67±0.47	9.33±0.00
	W. indica	14.66±0.47	10.66±0.47	7.33±0.47
Pseudomonas aeruginosa	*P. granatum*	21.67±0.47	17.67±0.47	14.00±0.00
	W. indica	18.00±0.00	14.00±0.00	10.33±0.47
Candida albicans	*P. granatum*	19.67±0.47	16.33±0.47	12.33±0.47
	W. indica	18.00±0.00	14.33±0.47	10.67±0.47

Table 3. Minimum inhibitory concentrations (MICs) of the crude leaf extract of *Punica granatum* and *Waltheria indica*

Microorganisms	Extract	Concentration (mg/mL)				
		100.0	50.0	25.0	12.5	6.25
Bacillus subtilis	*P. granatum*	-	-*	+	+	+
	W. indica	-	-*	+	+	+
Staphylococcus aureus	*P. granatum*	-*	+	+	+	+
	W. indica	-	-	-*	+	+
Streptococcus pyogenes	*P. granatum*	-	-*	+	+	+
	W. indica	-	-	-	-*	+
Escherichia coli	*P. granatum*	-	-	-*	+	+
Klebsiella pneumonia	*P. granatum*	-	-	-	-*	+
Pseudomonas aeruginosa	*P. granatum*	-	-	-	-*	+
	W. indica	-	-	-*	+	+
Candida albicans	*P. granatum*	-	-	-	-*	+
	W. indica	-	-	-*	+	+

Key: -*= MIC values.

Table 4. Minimum bactericidal concentrations (MBCs) of the crude leaf extract of *Punica granatum* and *Waltheria indica*

Microorganisms	Extract	Concentrations (mg/mL)				
		100.0	50.0	25.0	12.5	6.25
Bacillus subtilis	*P. granatum*	-	-**	+	+	+
	W. indica	-	-**	+	+	+
Staphylococcus aureus	*P. granatum*	-**	+	+	+	+
	W. indica	-	-**	+	+	+
Streptococcus pyogenes	*P. granatum*	-	-	-**	+	+
	W. indica	-	-	-**	+	+
Escherichia coli	*P. granatum*	-**	+	+	+	+
Klebsiella pneumonia	*P. granatum*	-	-**	+	+	+
Pseudomonas aeruginosa	*P. granatum*	-	-**	+	+	+
	W. indica	-	-	-**	+	+
Candida albicans	*P. granatum*	-	-**	+	+	+
	W. indica	-	-	-**	+	+

Key: -** = MBC values.

80 mg/hole of the extract of *P. granatum* and *W. indica* respectively had 23.67 ± 660.47 and 23.33 ± 0.47 mm. The least activity was recorded at the lowest dosage of 20 mg/hole with 8.67 ± 0.47 and 7.00 ± 0.00 mm against *E. coli* for *P. granatum* and *W. indica* respectively. Generally, the activities due by *P. granatum* were considerably higher than that exhibited by *W. indica.* These observations could not be unrelated to the components as well as compositions of the secondary metabolites, even though they almost contained similar phytoconstituents. Moreover, researches have demonstrated the antimicrobial activities of tannins, flavonoids and saponins (28). It was noticed that the only point where *W. indica* exhibited higher activity than *P. granatum* was against *S. aureus.* The activities against gram-negative, gram-positive as well as fungal species corroborated to the earlier findings which revealed that extractives from the plant were capable of inhibiting these groups of microorganisms. These antimicrobial activities against the test microorganisms may be indicative of the presence of broad spectrum antibiotics in both extracts (29).

The zone of inhibition produced by most antibiotic discs against some of the organisms was found to be greater in relation to those activities produced by most organisms under study though not statistically compared to those produced by the extracts. However, the diameters of antibacterial activity zones of inhibition ≥10 mm around the 21 petri dish were considered active (28). The higher activities presented by *P. granatum* could not be unrelated possibly to the concentrations of the phytoconstituents since both had similar compounds.

Punica granatum showed high MICs of 12.5 mg/ml against three organisms (*Klebsiella pneumoniae, Pseudomonas aeruginosa,* and *C. albicans*). *W. indica* on the other hand exhibited good activity against *Streptococcus pyogenes.* Higher MBCs were observed for both extracts against *S. pyogenes* at 25.0 mg/ml which coincide with the work of Deshi et al (30). More so, *W. indica* had the MBC of 25.0 mg/mL on *C. albicans* and *Pseudomonas aeruginosa.*

Conclusion

In conclusion, these comparative studies have revealed the potency of each of the plant towards the test microorganisms studied. Although the overall susceptibility was shown by *P. granatum* but MBC value further expressed that *W. indica* had a very good bactericidal value on *C. albicans, P. aeruginosa* and also on *S. pyogenes.* Therefore, concurring to the earlier findings, it can be surmised that the extracts from *W. indica* can be said with little reservation that it can stand as a choice for the treatment of disease condition afflicted by *S. pyogenes.*

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Authors' contributions

All authors contributed to the study. AH and ZBA acquired data. HU prepared the drafting. HU and MAT revised it critically for important intellectual content and HU submitted it. All read and confirmed the article ready for publication.

Conflict of interests

The authors declared no competing interests exist.

Ethical considerations

Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission, redundancy) have been completely observed by the authors.

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