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Research Article

Evaluation of Antimicrobial Activity of a Sudanese Herbal Plant (*Piliostigma reticulatum*)

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Abstract

Background: *Piliostigma reticulatum* is a plant that is found in a wide area of Sahelo-Sudanian region of Africa. It is widely used in Africa as a traditional medicine for the treatment of a wide range of diseases including epilepsy, anxiety, and agitation. The leaf extract was found to have antimicrobial activity. In Sudan (Nuba mountains in particular), it is widely used to dress new wounds and as well puerperal sepsis. Moreover it's fruit is eaten and used to prepare juice. Reported studies concerning antimicrobial activity of the plant in Sudan could not be found. This study therefore aimed to evaluate the antimicrobial action of Ethanolic and Aqueous extract of leaves and barks of the plant.

Methods: Barks and leaves of *P. reticulatum* were obtained from North Kordofan State. They were then air dried in the shade and milled into powder using Mortar. Methanolic and water extract of each part of the plant was prepared using a Soxhlet apparatus. The following concentrations of extracts of each part (bark and leaves) of the plant were prepared using Distilled water (50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, 3.125 mg/ml, and 1.56 mg/ml). Antimicrobial action of the different concentrations of the extracts of the two parts of the plant on selected bacterial and fungal species was performed using well diffusion technique. Antimicrobial susceptibility of the tested organisms to serial concentrations (40 µg, 20 µg, 10 µg, and 5 µg) of three antibacterial (Gentamicin, Ampicillin, and Tetracycline) and 2 anti-fungal (Nystatin and clotrimazole) was evaluated using well diffusion method.

Results: The methanolic extract of *P. reticulatum* leaves showed high antibacterial activity against *Bacillus subtilis* (inhibition zone 22 mm), *S. aureus* (25 mm), *P. aeruginosa* (23 mm), and *E.coli* (20 mm). The extract also showed antifungal activity against *A. niger* (23 mm) and *C. albicans* (23 mm). The aqueous extract revealed low activity against *P. aeruginosa* (10 mm) and no action on the rest of the microorganisms.

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Conclusion: In this study, the methanolic extract of the leaves inhibited the growth of all the tested bacteria and fungi but with varied activity (inhibition zones between 8 mm and 20 mm). The highest activity was against *B. subtilis* (inhibition zone 20 mm), followed by *A. niger* and *C. albicans* (19 mm each), then *P. aeruginosa* (18 mm). Methanolic extract of the leaves revealed moderate activity against *E. coli*(13 mm) and low activity against *S. aureus* (8 mm).

1. Introduction

Piliostigma reticulatum is a plant that occurs in the Sahelo-Sudanian region of Africa from Senegal, Mauritania to Sudan and has been introduced to Mozambique (Fibres, 1846). The plant is a dioeciously shrub or small tree up to 10–15 meters tall, bole short, rarely straight, up to 30 cm in diameter. The outer bark is deeply fissured, cracked grey to brown, and the inner bark pink to red crown rounded and dense. The branches are grey, waxy, and glabrous. The leaves alternate conspicuously bilobed, petiole 1–3.5 cm long, swollen at both ends, blade 5–12 cm X 4–18 cm, chordate or rounded at base, lobes rounded more or less cuneate (Figure 1).

The flowers are unisexual, 2.5 cm in diameter, calyx 5 toothed, 15–20 cm long, petals 5 obovate, white with pink stripes, male flowers with 10 stamens, anthers brown (Figure 2). Fruit an oblong pod 15–30 cm X 2.5–5 cm, straight undulate woody, hard, glabrous, or sparsely pubescent, brown flat, pruinose, sometimes twisted and cracked, indehiscent and persisting, many seeded [1].

The plant is used widely in Africa as a traditional medicine for the treatment of many diseases, such as malaria, tuberculosis, and diarrhea [2]. *Piliostigma reticulatum* is used in traditional medicine in Cameron to treat epilepsy, anxiety, and agitation. In fact the results of a study done in Cameron suggested that it possesses anxiolytic and antipyretic properties in mice and could really be helpful in the treatment of anxiety [3].

The leaf extract from the plant was found to exhibit anti-microbial activity against some bacteria and fungi such as *Staphylococcus aureus* (NCTC 6571, *Escherichia coli* (NCTC 10418), *Bacillus subtilis* (NCTC 8236), *Proteus vulgaris* (NCTC 4175), *Aspergillus niger* (ATCC 10578), and *Candida albicans* (NCTC 10231) [4]. Vibriocidal action of *P. reticulatum*

among other medicinal plants was studied and proved to be effective in killing *Vibrio spp*. [5]. In Nuba mountains in Sudan, the plant is widely used. The young leaves with their acidic taste are eaten. The fruits after maturation and drying is also eaten for its sweet taste and nowadays used for preparation of juice. The fresh bark of the plant is used for fresh wound dressing as it coagulates blood and is believed to enhance healing of the wound by keeping it clean. In the old days before the introduction of primary healthcare and midwifery facilities, the inner soft bark of *P. reticulatum* was widely used after delivery to cover the episiotomy wound (based on personal observation, one of the authors being part of the Nuba community).

This study was conducted to evaluate the antimicrobial activity of *P. reticulatum* on selected bacterial and fungal spp.

Figure 1: Fruits and leaves of *P. reticulatum.*

Figure 2: Leaves and flowers of *P. reticulatum.*

2. Materials and Methods

2.1. Preparation of the different plant's parts

Barks and leaves of *P. reticulatum* were obtained from North Kordofan State. The bark and leaves of the plant were dried in the shade and then milled using Mortar and Pestle to prepare a powder.

2.2. Preparation of the extracts

Eighty five grams of the air-dried and coarsely powdered material of each plant part was exhaustively extracted for 20 hours with methanol (40–60°C) in a Soxhlet apparatus. The methanolic extract was filtered and evaporated under reduced pressure again using Rota-vapor. Each residue was weighed and the yielded percentage was determined. The methanol residue was redissolved or suspended in methanol. The final volume was adjusted to give the specific concentration (100 mg/ml) and kept in a refrigerator until used.

Aqueous extract of each dried, ground plant part (10 g) was prepared by infusion using boiled distilled water. It was allowed to soak in a beaker on water bath with occasional shaking for four hours. The residue was then dried and weighed and the yield percent was obtained. The final volume of the residue was adjusted to 10 ml sterile distilled water, and used immediately.

2.3. Preparation of the standard test organisms

The standard organisms were obtained from the department of Microbiology and Parasitology, Medicinal and Aromatic Plant Research Institute, Khartoum and inoculated into broth that was then incubated at 37°C aerobically for 24 hours.

2.4. Preparation of the standard bacterial suspensions

One ml aliquots of a 24-hours broth culture of the test organisms were aseptically distributed onto nutrient agar slopes and incubated at 37°C for 24 hours. The bacterial growth was harvested and washed off with 100 ml sterile normal saline, to produce a suspension containing about 10^8–10^9 colony forming units per ml. The suspension was stored in the refrigerator at 4°C till used. The average number of viable organisms per ml of the stock suspension was determined by the means of the surface viable counting technique. Serial dilutions of the stock suspensions were made in sterile normal saline solution and 0.02 ml volumes of the appropriate dilution were transferred by micropipette to the surface of dried nutrient agar plates. The plates were allowed to stand for 2 hours at room temperature for the drops to dry, and then incubated at 37°C for 24 hours. After incubation, the number of developed colonies in each drop was counted. The average number of the colonies per drop (0.02ml) was multiplied by 50 and by the dilution factor to give the viable count of stock suspensions, expressed as the number of colony forming unit per ml of suspension. Each time fresh stock suspension was prepared, all the aforementioned experimental conditions were maintained constant so that suspensions with very close viable count would be obtained [6, 7].

2.5. Preparation of standard fungal organisms

The standard fungi were obtained from the department of Microbiology and Parasitology, Medicinal and Aromatic Plant Research Institute, Khartoum and were maintained on Sabouraud's dextrose agar, incubated at 25°C for 4 days. The fungal growth were harvested and washed with sterile normal saline and finally suspended in 100 ml of sterile normal saline and the suspension was stored in refrigerator till used.

2.6. Testing of antibacterial susceptibility

The paper disc diffusion method was used to screen the antibacterial activity of plant extracts and performed by using Mueller Hinton agar (MHA). The experiment was carried out according to the National Committee for Clinical Laboratory Standards Guidelines [8]. Bacterial suspension was diluted with sterile physiological solution to 10^8cfu/ml (turbidity = McFarland standard 0.5). One hundred microliters of bacterial suspension were swabbed uniformly on surface of MHA and the inoculum was allowed to dry for 5 minutes. Sterilized filter paper discs (Whatman No.1, 6 mm in diameter) were placed on the surface of the MHA and soaked with 20 µl of a solution of each plant extracts. The inoculated plates were incubated at 37 ºC for 24 h in the inverted position. The diameters (mm) of the inhibition zones were measured.

2.7. Testing for antifungal activity

The same method as for antibacterial activity was used. Sabouraud dextrose agar was used instead of nutrient agar. The inoculated medium was incubated at 25ºC for three days for the *A. niger* and two days for *C. albicans*.

2.8. Determination of minimum inhibitory concentration (MIC) by agar plate dilution method

The principle of the agar plate dilution is the inhibition of growth on the surface of the agar by the plant extracts incorporated into the medium. Plates were prepared in the series of increasing concentrations of the plant extract. The bottom of each plate was marked off into six segments. The organisms tested were inoculated into broth media over night to obtain 10^9 CFU/ml. A loop-full of diluted culture was spot with a standard loop that delivers 0.001 ml on the surface of each segment and then incubated at 37ºC for 18 hour (9). *Piliostigma reticulatum* extract was prepared in the series of decreasing concentrations in the following order 50, 25, 12.5, 12.5, 6.25, 3.125, and 1.56 mg/ml. The end point (MIC) is the least concentration of antimicrobial agent that completely inhibits the growth. MIC for each microbe was reported in term of mg/ml.

2.9. Antibacterial activity of reference drugs

In the present work, three antibiotics (Ampicillin, Gentamicin, and tetracycline) were used as reference drugs. Antibacterial drugs were tested at different concentrations obtained by dissolving 0.1 g of each powdered drug in 100 ml sterile distilled water to give a concentration of 1000 µg/ml followed by serial dilutions to give concentrations of 40, 20, 10, and 5 µg/ml. These drugs were tested against reference bacteria, that is, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*.

2.10. Antifungal activity of reference drugs

In the present work, two antifungal agents (Nystatin and Clotrimazole) were used as reference drugs. Antifungal drugs were tested at different concentrations obtained by dissolving 0.1 g of each powdered drug in 100 ml sterile distilled water to give a concentration of 1000 µg/ml followed by serial dilutions to give concentrations of 40, 20, 10, and 5 µg/ml. These drugs were tested against reference fungi. *A. niger* and *C. albicans*.

3. Results

3.1. Screening for antimicrobial activity of methanolic and aqueous extracts of Bark and Leaves of P. reticulatum (kharoub)

The methanolic leaves extract of *P. reticulatum* showed high antimicrobial activity against Gram positive and negative bacteria with inhibition zone of 20 mm against *B. subtilis*, 18 mm against *P. aeruginosa*. It also showed high activity against *C. albicans* and *A. niger* (inhibition zone 19 mm against each one). The methanolic leaves extract revealed moderate activity against *E. coli* and low activity against *S. aureus* (inhibition zones of 13 mm and 8 mm, respectively) (plates 2–3).

The aqueous extract showed moderate activity against *A. niger* (inhibition zone 12 mm) and no activity against the rest of the tested organisms (Table 1).

The methanol Bark extract of *Piliostigma reticulatun* showed pronounced activity against *S. aureus* (inhibition zone 25 mm), and high activity against *P. aeruginosa*, *B. subtilis,* and *E.coli* (inhibition zones of 23 mm, 22 mm, 20 mm, respectively). The methanolic bark extract also showed pronounced activity against *A. niger* and high

activity against *C. albican* (inhibition zones of 24 mm and 23 mm, respectively) (Table 1 and plate-1).

The aqueous extract showed low activity (10 mm) against *P. aeruginosa* and inactive against the rest of the organisms (Table 1).

3.2. Comparison between the antimicrobial activity of the most active Bark and Leaves methanolic extracts with standard reference drugs

E. coli was found to be resistant to all the three drugs (ampicillin, gentamicin, and tetracycline) at all concentrations while sensitive to the leaves and bark ethanolic extract of the plant. It was also found to be resistant to tetracycline and ampicillin at all concentrations but sensitive to leaves and bark ethanolic extracts of *P. reticulatum* (Table 2). *Bacillus subtilis* revealed sensitivity to all the three antibiotics used and the ethanolic leaves and bark extracts of the plant but resistant to the aqueous extracts (Tables 1 and 2).

Candida albicans was found to be sensitive to both antifungal used in all concentrations and as well methanolic leaves and bark extract of the plant but resistant to aqueous extracts. *Aspergillus niger* showed sensitivity to all extracts except the aqueous bark extract. It also revealed sensitivity to nystatin and clotrimazole except in low concentration (Tables 1 and 3).

The leaves methanolic extract of *P. reticulatum* activity against *B. subtilis* was equivalent to the activity 10 µg/ml gentamicin.

The Bark methanol extract of *P. reticulatum* activity against *B. subtilis* was similar to the activity of 20 µg/ml gentamicin. The methanol bark extract activity against *S. aureus* was similar to that of 40 µg/ml tetracycline. The diameter of the inhibition of methanolic bark extract against *C. albicans* was similar to that caused by 12.5 µg/ml nystatin (Tables 2 and 3).

3.3. Determination of the minimum inhibitory concentrations

The minimum inhibitory concentrations of the bark methanolic extracts of *P. reticulatum* was found to be 3.125 mg/ml for *S. aureus* and *P. aeruginosa* and 1.56 mg/ml for *B. subtilis*, *E. coli*, *A. niger*, and *C. albicans*. Regarding the methanolic leaves extract, MIC for *S. aureus* was 50 mg/ml, 6.25 mg/mg for *C. albicans*, and 3.125 mg/ml for *B. subtilis*, *P. aeruginosa*, and *A. niger* (Table 4).

Table 1: The diameter of the inhibition zone in mm of the methanolic and water extracts of *P. Reticulatum* against standard tested organisms.

Type of extract	The size of inhibition zone (mm) against the different standard organisms					
	E. coli	P. aeruginosa	B. subtilis	S. aureus	A. niger	C. albicans
Leaves methanolic extract	13	18	20	8	19	19
Leaves water extract	Resistant	Resistant	Resistant	Resistant	12	Resistant
Bark methanolic extract	20	23	22	25	24	23
Bark water extract	Resistant	10	Resistant	Resistant	Resistant	Resistant
Interpretation of results: Mean diameter of inhibition zone in mm (MDIZ): (> 8 mm: Sensitive, 12–15 mm: Intermediate : < 8 mm: Resistant.						

Table 2: Antibacterial activity of reference drugs against standard bacteria.

Type of the antibiotic	Concentration of the drug in the antibiotic disc	The diameter of the inhibition zone in mm			
		E. coli	P. aerugi-nosa	B. subtilis	S. aureus
Ampicillin	40	Resistant	*Resistant*	*15*	*25*
	20	*Resistant*	*Resistant*	*14*	*20*
	10	*Resistant*	*Resistant*	*13*	*18*
	5	*Resistant*	*Resistant*	*12*	*15*
Gentamicin	40	*Resistant*	29	9	35
	20	*Resistant*	21	22	33
	10	*Resistant*	20	20	30
	5	*Resistant*	19	17	28
Tetracycline	40	*Resistant*	*Resistant*	25	25
	20	*Resistant*	*Resistant*	23	*Resistant*
	10	*Resistant*	*Resistant*	19	*Resistant*
	5	*Resistant*	*Resistant*	18	*Resistant*

4. Discussion

The methanol and aqueous extracts of the Leaves and Bark of *P. reticulatum* were screened for their anti-microbial activity against six microorganisms, two standard Gram-positive bacteria (*S. aureus* and *B. subtilis*), two Gram-negative bacteria (*E. coli* and *P. aeruginosa*) and two standard fungi (*A. niger and C. albicans)* using disc diffusion method.

In this study, the methanolic extract of *P. reticulatum* bark and leaves extracts were found to have broad spectrum antimicrobial activity against the gram-positive and negative bacterial species tested as well as the two fungal species. The bark ethanolic

Table 3: Antifungal activity of reference drugs against standard fungi.

The antifungal Drugs	Conc. Used µg\ml	The diameter of the inhibition zone in mm	
		A. niger	C. albicans
Nystatin	500	27	32
	50	17	28
	25	14	26
	12.5	Resistant	23
Clotrimazole	40	30	42
	20	22	40
	10	20	31
	5	Resistant	28

Table 4: Minimum inhibitory concentrations (MIC) of *P. reticulatum* methanolic extracts against the standard microorganisms.

Part Used	Solvent	Conc. Used mg/ml	The diameter of the inhibition zone in mm					
			B. subtilis	S. aureus	E. coli	P. aerugi-nosa	A. niger	C. albicans
Bark	Methanol	50	22	20	16	13	14	16
		25	14	19	15	12	14	14
		12.5	14	11	14	11	14	13
		6.25	14	11	13	11	13	13
		3.125	11	0	10	0	10	11
		1.56	0	0	0	0	0	0
leaves	Methanol	50	18	8	13	17	17	18
		25	17	0	12	15	13	16
		12.5	14	0	11	15	13	14
		6.25	13	0	11	14	13	12
		3.125	11	0	0	11	10	0
		1.56	0	0	0	0	0	0

extract showed higher activity against all tested bacteria and fungi compared to leave extract. Based on microbial species, the bark ethanolic extract showed higher effect on *S. aureus* inhibition zone of 25 mm followed by *A. niger* (24 mm), then *P. aeruginosa* and *C. albicans* (23 mm each).

Contrary to our results, Daniel and Malomo reported that *P. reticulatum* was not active against *P. aruginosa* but showed high activity (with inhibition zone 22 mm against *B. subtilis* and 20 mm against *E. coli* [11]. The antimicrobial effect of the methanolic bark and leave extract were even better than the drugs used (ampicillin, gentamicin, and tetracycline).

Plate 1: *Invitro* antimicrobial activity of *P. reticulatum* Bark extract (m) methanol extract (w) water extract against *Staphylococcus aureus.*

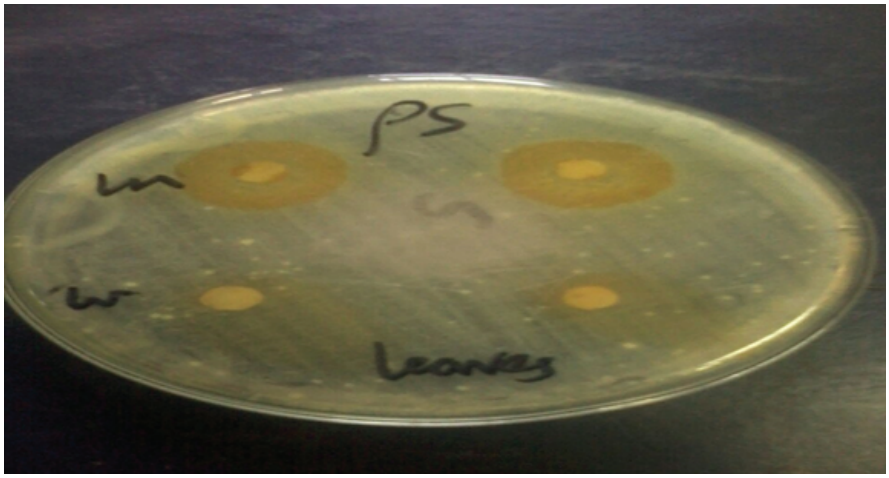

Plate 2: *Invitro* antimicrobial activity of *P. reticulatum* Bark extract (m) methanol extract (w) water extract against *Pseudomonas aeruginosa.*

The aqueous extract of the plant showed low antibacterial activity against *P. aeruginosa* (inhibition zone10 mm), while inactive against rest bacterial and fungal organisms tested. Similar results were reported by a study done in 2009 in which the hot and cold aqueous extracts of *P. reticulatum* exhibited low antimicrobial activity against *E. coli, Shigella dysenteriae*, *Salmonella typhimrium, Staphylococcus aureus,* and *Pseudomonas aruginosa* with zones of inhibition ranging between 8–10 mm and 4–7 mm for bacterial and fungal species, respectively [12].

However, one of the previous studies found no difference between the methanolic and aqueous extract of the plant [10]. The active ingredient is possibly alcohol soluble and water insoluble resulting in the variation in the antimicrobial effect of the two

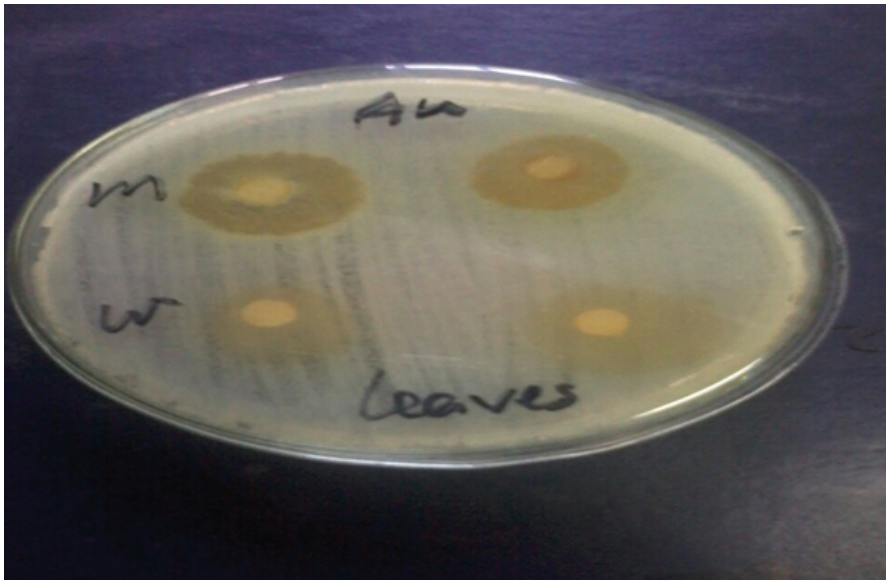

Plate 3: *Invitro* antimicrobial activity of *P. reticulatum* Leaves extract (m) methanol extract (w) water extract against *A. niger.*

types of extract, moreover the concentration of the active ingredient is probably higher in the bark than the leaves. This can explain why bark extract was more effective than the leave extract.

In conclusion, the methanolic extract of bark showed more anti-microbial activity than leaves extract.

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