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Antimicrobial and Anti-inflammatory Effects of *Piliostigma reticulatum* Leaf Extract

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Abstract: This study aimed to evaluate the effectiveness of the extraction method employed in traditional herbal medicine by comparing the antimicrobial and anti-inflammatory activities of the leaf extracts of *Piliostigma reticulatum* and *Piliostigma thonningii*. The suitability of the two species as substitute was also determined. The agar dilution method was employed to compare the activity of crude leaf extracts of the two species against sixty bacterial and fungal isolates selected from diverse genera. The antimicrobial spectra of the extracts from both species were very similar. In 44 (73.3%) of the test strains, MIC values for the two extracts were identical and in 6 (10.0%) the MIC values differed by only one dilution factor. Chlorocresol was used as standard. The crude extract and solvent fractions obtained from the leaf of *P. reticulatum* were evaluated for anti-inflammatory activity using the carrageenan-induced rat paw oedema model. The aqueous fraction at 100 mg kg^{-1} produced the highest inhibition of oedema (61.2%), while the crude extract at 100 mg kg^{-1} exerted a low anti-inflammatory effect (13.2%). Indomethacin at 5 mg kg^{-1} inhibited oedema by 86.2%. This study has established that *P. reticulatum* and *P. thonningii* have similar antimicrobial spectra and anti-inflammatory activity. The traditional extraction method is not effective in extracting the antimicrobial agents from the plants.

Key words: *Piliostigma reticulatum*, *Piliostigma thonningii*, Caesalpinaceae, antimicrobial effect, anti-inflammatory activity

INTRODUCTION

Piliostigma thonningii (Schum) Milne Redhead (family: Caesalpinaceae) is used in ethnomedicine in Africa for the treatment of wounds, chronic ulcer, toothache and gingivitis, indications that are all related to infections and inflammation[1]. Bioactivity directed fractionation of the leaf extract of *P. thonningii* has led to isolation of some C-methylflavonols that are responsible for both antibacterial and anti-inflammatory activities[2,3]. *P. reticulatum* (DC.) Hochst, is the only locally available co-generic species of *P. thonningii,* which is often confused with the latter due to morphological similarity. Both piliostigma species are undististinctly used in ethnomedicine[4] and a number of similar C-methylflavonols have been reported from *P. reticulatum*[5]. In this study, the effectiveness of the extraction method employed in traditional herbal medicine has been investigated by comparing the antimicrobial spectrum and anti-inflammatory activity of the extracts from both species. Whether *P. reticulatum* could be used instead of *P. thonningii* for treating infections and/or inflammations will also be determined. This will assist in herbal drug formulation.

MATERIALS AND METHODS

Plant material: Leaves of *P. reticulatum* and *P. thonningii* were collected in May 1998 at Ikire and the Teaching and Research Farm, Obafemi Awolowo University, Ile-Ife, respectively. Mr. G. Adesakin of the Herbarium section, Faculty of Pharmacy, OAU Ile-Ife, authenticated the plants where voucher specimens were deposited.

Preparation of the extracts: The collected plants were air dried for two weeks and powdered. The powdered leaf from *P. reticulatum* and *P. thonningii* were extracted separately with 50% aqueous ethanol at room temperature for 24 h and filtered. Extracts were concentrated under reduced pressure at 40°C. The crude extract from *P. reticulatum* and *P. thonningii* were stored in the refrigerator until required.

The crude extract of *P. reticulatum* was re-suspended in distilled water and extracted with EtOAc and BuOH, successively.The combined organic layers were evaporated to dryness *in vacuo* to afford the EtOAc fraction as a dark brown solid and a butanol fraction.

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Microorganisms: Bacteria were maintained as frozen stocks in laboratory[6]. The strains used included type cultures, archetypal strains and recent clinical isolates. Fungal strains were obtained from the Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Nigeria. Table 1 lists the strains, their sources and their voucher specimen numbers.

Animals: Male Wistar rats (weighing 180-200 g) were allotted into groups of five animals each. The animals were acclimatized in the laboratory area for a week, prior to experiments. The animals were bred and housed in the Animal Holding, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Nigeria, which was properly ventilated. They were fed on standard diet (Guinea Feeds, Benin City) and water *ad libitum*. The animals were kept in standard animal facility environment, at a temperature between 25 and 29°C (12 h light/12 h dark light cycle).

Microbiological studies: Minimum Inhibitory Concentrations (MIC) were determined by the agar dilution method[7]. Plate concentrations containing 10,000,

Table 1: Antimicrobial spectra of the crude extract of *P. thonningii* and *P. reticulatum*

Strain	Reference or Source	*P. thonningii* (μg mL^{-1})	*P. reticulatum* (μg mL^{-1})	Chloro-cresol (μg mL^{-1})	No. of isolates
Gram positive bacteria					
Bacillus subtilis	NCTC 8236	2,500	2,500	≤12.5	1
Bacillus sp.	Environmental	≤1,250	2,500	≤12.5	3
		2,500	2,500	50	1
		≤1,250	5,000	25	1
Micrococccus luteus	Environmental	≤1,250	5,000	25	1
Staphylococcus aureus	NCTC 6571	2,500	2,500	≤12.5	1
Staphylococcus aureus	Clinical	10,000	10,000	100	1
		2,500	5,000	50	1
		≤1,250	2,500	50	1
		≤1,250	2,500	≤12.5	1
		≤1,250	≤1,250	100	1
Staphylococcus sp. (coagulase negative)	Clinical	≤1,250	≤1,250	50	1
Gram negative bacteria					
Acinetobacter baumanii	[9]	10,000	10,000	100	2
Chrysogenum violaceum	ATCC	2,500	5,000	≤12.5	1
Escherichia coli	NCTC 10418	10,000	10,000	50	1
E. coli K-12	DH5α	10,000	5,000	50	1
Enteroaggregative *E. coli*	[9,10]	10,000	10,000	50	6
Enterohaemorrhagic *E. coli* O157:H7	[11]	10,000	10,000	50	1
Enteropathogenic *E. coli*	[9]	10,000	10,000	50	
Enterotoxigenic *E. coli*	[9]	10,000	10,000	100	2
Faecal *E. coli*	[12,13]	10,000	10,000	50	10
Klebsiella pneumoniae ssp. Pneumoniae	Clinical	10,000	10,000	50	2
Proteus vulgaris	Clinical	10,000	5,000	50	1
Pseudomonas aeruginosa	ATCC 10145	10,000	10,000	>100	1
Pseudomonas putida	[9]	10,000	10,000	50	1
Pseudomonas sp.	Environmental	10,000	10,000	50	1
		5,000	5,000	50	2
Salmonella Cholerasius	Clinical	10,000	10,000	100	1
Salmonella Typhi	Clinical	10,000	10,000	100	1
Salmonella Paratyphi A	[9]	10,000	10,000	>100	1
Shigella sp.	Clinical [13]	10,000	10,000	50	1
Yersinia enterocolitica	Clinical	10,000	10,000	100	1
Vibrio cholerae O1	[14]	5,000	5,000	>100	1
Vibrio cholerae	Clinical [13]	2,500	10,000	50	1
Vibrio sp. (non cholerae)	Clinical [13]	10,000	5,000	100	1
E. coli (lactose negative)	Clinical	10,000	5,000	50	1
		10,000	10,000	100	1
Fungi					
Candida-pseudotropicalis	NCYC 6	5,000	≤1,250	>100	1
Cochlibolus lunatus	Micro, OAU	≤1,250	5,000	≤12.5	1
Trichoderma sp.	Micro, OAU	≤1,250	≤1,250	>100	1

5,000, 2,500 and 1,250 μg mL^{-1} of *P. thonningii* and *P. reticulatum* crude extracts in Iso-sensitest agar (Oxoid, England) were prepared. Plates containing chlorocresol 100.0, 50.0, 25.0 and 12.5 μg mL^{-1} were similarly prepared as a positive control and Isosensitest Agar containing no antimicrobials was used as negative control. Duplicate plates were prepared for each assay.

Bacteria were grown overnight in nutrient broth. The cultures were diluted to a final density of $2x10^5$ cfu mL^{-1} in normal saline and applied to the surface of nutrient agar plates containing dilutions of *P. thonningii, P. reticulatum,* chlorocresol or solvent (50% methanol) alone employing a multi-point inoculator. Plates were incubated at 37°C for 48 h Inoculum density was verified by viable counts on nutrient agar.

Yeasts were grown on Sabouraud-Dextrose Agar (SDA) slants for 3 days. The slants were washed with 2 mL normal saline and diluted to an inoculum density of $0.5\text{-}2.5x10^3$ cfu mL^{-1}. Filamentous fungi were grown on SDA for seven days after which the growth was washed with 5 mL normal saline and allowed to settle for 5 min. The upper part of the washings (consisting mainly of conidia) was diluted to a concentration of about $1x10^4$ cfu mL^{-1}, which was employed as inoculum. Inoculum counts were verified by viable counts on SDA. When the counts differed by more than 10 fold of the expected count, the assays were repeated. Test plates for fungal determinations were prepared with SDA, the organisms were surface inoculated with a multi-point inoculator and the plates were incubated for 5 days at 25°C.

All plates were observed for growth and the least dilution completely inhibiting the growth of each organism, was taken as the MIC. Statistical analysis employing a two-tailed Students t-test was used to compare the MICs obtained with *P. thonningii* and *P. reticulatum* extracts.

Anti-inflammatory activity: The method used for evaluating anti-inflammatory activity of the crude extract and solvent fractions was based on that described by Winter *et al.*[8]. The method measures the rate of the development of oedema following challenge by carrageenan.

The crude extract, ethyl acetate fraction, butanol fraction and aqueous fraction of *P. reticulatum* were suspended in 2.5% Tween 80. The extract and fractions (100 mg kg^{-1}) were administered intraperitoneally to the groups of rats. Indomethacin (5 mg kg^{-1}, I. p.) was administered to rats in the reference group, while control animals received Tween 80 at a dose of 10 mL Kg^{-1}. Thirty minutes after treatment, all the rats were injected 0.1 mL of 1% (w/v) carrageenan into the subplantar tissue of the right hind paw under the subplantar tissue. Measurement of paw volume was carried out using a plethysmometer (Ugo Basile, Italy, Model 7140). Measurements of paw volume of each rat (which was taken as a measure of oedema) were taken just before and at 1, 2, 3, 4 and 5 h after carrageenan injection. Oedema inhibitory activity in each group was determined at the 3rd h following carrageenan injection and was calculated using the formula:

$$\text{Percentage Inhibition} = \frac{(C_3 - C_0)\ \text{Control} - (C_3 - C_0)\ \text{Treated}}{(C_3 - C_0)\ \text{Control}}$$

Where, C_3 = Mean Paw Volume at the 3rd h after carrageenan injection.

C_0 = Mean Paw Volume before carrageenan injection.

RESULTS AND DISCUSSION

Determination of MICs of the extracts: The crude *P. reticulatum* and *P. thonningii* extracts showed a broad spectrum of activity at the test concentrations. Sixty strains belonging to diverse genera were employed, all of which grew on the blank plates. The reference standard was chlorocresol. The test strains included Gram positive and Gram negative bacteria as well as yeasts and moulds. The antimicrobial spectra of extracts from the two species were very similar (Table 1).

Anti-inflammatory activity: Table 2 shows the effects of crude extract, EtOAc fraction, butanol fraction and aqueous fraction of *P. reticulatum* on carrageenan-induced paw oedema in rats. The paw volumes in all the groups increased from the 1st h and peaked at the 3rd h following carrageenan injection.

In 44 (73.3%) of test strains, the MIC values for the two extracts were identical and in 6 (10.0%) the MIC values differed by only one dilution factor ($p>0.05$, Students t-test). Differences were most commonly found with Gram positive bacteria and fungi. *P. thonningii* appeared slightly more active against Gram positive organisms and moulds; where differences were seen with Gram negative organism *P. reticulatum* was slightly more active. These data are sufficient to provide a rational for the indistinctly use of both species in ethnomedicine although the MICs against most of the Gram negative organisms and fungi are too high to support the development of the extracts using the traditional extraction method as clinical antimicrobial therapies.

At the 3rd h following carrageenan injection, the mean paw volume of the control rats was 4.05±0.20,

Table 2: The effects of the crude extract and some solvent fractions of *Piliostigma reticulatum* on carrageenan-induced rat paw oedema over a 5 h period. Percentage inhibition is given in parenthesis at the 3rd h

	Time (h)					
Treatment (s)	0	1	2	3	4	5
Control (Tween 80)	1.09±0.30	2.46±0.19	3.29±0.34	4.05±0.20	3.72±0.24	3.18±0.24
Crude (100 mg kg^{-1})	1.03±0.24	2.04±0.27	2.71±0.30	3.61±0.12 (13.2%)	3.30±0.17	3.22±0.37
EtOAc (100 mg kg^{-1})	1.29±0.25	2.16±0.21	2.40±0.22	2.83±0.25 (48.0%)	2.57±0.29	2.56±0.37
BuOH (100 mg kg^{-1})	1.30±0.26	2.49±0.27	3.16±0.37	3.57±0.45 (23.2%)	3.41±0.38	3.37±0.51
Aqueous (100 mg kg^{-1} g)	1.27±0.23	2.11±0.21	2.33±0.18	2.42±0.26 (61.2%)	2.34±0.26	2.40±0.46
Indomethacin (5 mg mL^{-1})	0.93±0.14	1.15±0.12	1.25±0.13	1.35±0.15 (86.8%)	1.23±0.55	1.07±0.16

Values are presented as mean±SEM for five rats in each group

whereas it was 3.61±0.12 in the crude extract treated group. Remarkable reductions in mean paw volumes were found with the EtOAc and aqueous fractions treated rats. Indomethacin (5 mg kg^{-1}) significantly decreased the paw volumes over the 5 h period. Table 2 at the 3rd h shows the percentage inhibition by the various treatments at the 3rd h (peak oedema period) following carrageenan challenge in the rat paws. The standard drug used (Indomethacin, 5 mg kg^{-1}) produced the highest inhibition of oedema formation (86.8%). It was also observed that the aqueous fraction gave the highest inhibition of oedema formation at the 3rd h (61.2%). An appreciable (48%) inhibition was also exhibited by the EtOAc fraction.

In a related study[15], to determine the best extractant for the screening and isolation of antimicrobial components from plants. Of the eight different extractants employed, ethanol and water were scored the lowest on the overall extraction parameters investigated. This is probable because most antimicrobial agents isolated are hardly soluble in water.

This comparative study showed that *P. reticulatum* and *P. thonningii* have similar antimicrobial spectra and anti-inflammatory activity. *P. reticulatum* can substitute for *P. thonningii*. However, the traditional extraction method is not effective in extracting the antimicrobial agents from the plants. It is important to screen the non-polar extracts of the leaf of these plants.

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