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The pharmacological screening of *Pentanisia prunelloides* and the isolation of the antibacterial compound palmitic acid

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Abstract

The uses of *Pentanisia prunelloides* in Zulu traditional medicine indicate that the plant is believed to be effective in relieving inflammation, bacterial and viral infections and also stimulating uterine contraction. Aqueous, ethanolic and ethyl acetate extracts of leaves and roots were screened for prostaglandin-synthesis inhibitors and antibacterial and antiviral activity. In the results of the anti-inflammatory assay all the extracts showed cyclooxygenase-1 inhibition. The ethanolic and ethyl acetate extracts showed greater antibacterial activity than the aqueous extracts against Gram-positive (*Bacillus subtilis*, *Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*). Both root and leaf extracts were found to inhibit viral replication of the Influenza A virus. The ethyl acetate extract was fractionated by silica vacuum liquid chromatography and anti-inflammatory activity was found to be most pronounced in the more polar fractions. The presence of antibacterial activity was confirmed by running the fractions on a thin layer chromatography (TLC) plate and performing a bioautographic assay. The active fraction was further purified by TLC and the major antibacterial compound in the ethyl acetate root extract was identified by GC/MS as palmitic acid.

Keywords: Antibacterial; Cyclooxygenase inhibition; Influenza A virus; Palmitic acid; *Pentanisia prunelloides*; Traditional medicine

1. Introduction

Medicinal plants are an important part of the South African cultural heritage and Africa is a continent endowed with an enormous wealth of plant resources (Iwu, 1993). Plants produce a diverse range of bio-active molecules, making them a rich source of different types of medicines. In South Africa, a large part of the day to day medicine is still derived from plants and large volumes of plant material, or their extracts, are sold in the informal and commercial sectors of the economy (van Wyk et al., 1997).

The imposing anthropogenic activities that come with economic growth, such as agricultural, industrial and housing developments, are forming an increasing threat to the natural habitats of these medicinal plants. Increased commercialization of medicinal plants in South Africa has resulted in over-harvesting and in some cases near extinction of some valued indigenous plant species (Williams et al., 2000). Documentation of the uses of these plants is therefore of great importance before they vanish. Through training and scientific evaluation of effective remedies it may be possible to bring traditional medicine to a level of efficiency and safety where it can be regarded as an acceptable alternative to the western health system.

Pentanisia prunelloides is a perennial herb with a large, thick tuberous root and leafy branches holding dense groups of small purple flowers. In preparation of traditional medicines mainly the root is used, but sometimes also the leaves (van Wyk et al., 1997). The plant has many uses in Zulu medicine, but most commonly root decoctions are used for swellings, sore joints and

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rheumatism. It has also been reported to be used to relieve pain in the chest and to treat virus infections such as influenza. In the 1918 influenza epidemic a root decoction was used by the Zulu people with marked success (Watt and Breyer-Brandwijk, 1962). A leaf poultice is said to be used for a retained placenta (Watt and Breyer-Brandwijk, 1962; Hutchings, 1996). Previously it has been shown that *P. prunelloides* exhibited smooth muscle activity on ileum and uterus preparations (Kaido et al., 1997; Lindsey et al., 1999). Nothing appears to be known about the chemical compounds of the *Pentanisia* genus. Looking at the uses in Zulu medicine, the plant seems to be effective in relieving inflammation, bacterial and viral infections and also stimulating uterine contraction. The aims of this study were to screen extracts of *P. prunelloides* for anti-inflammatory, antibacterial and antiviral activity and then to try and isolate the active antimicrobiological compounds which would be responsible.

2. Methodology

2.1. Plant material

Leaf and root material of *P. prunelloides* Walp. (Rubiaceae) was collected on 16 October 2000 from the slopes at World's View, Pietermaritzburg by Yff, Jäger and Gardner. The plant was botanically verified by Gardner. A voucher specimen was prepared (Yff1NU) and deposited in the Natal University herbarium. The material was dried in an oven at 50 °C. Once dry, the material was ground.

2.2. Extractions

Two methods were used for the extraction of plant material: sonication and Soxhlet.

Sonication: one gram of the plant material and 10 ml of solvent (water, ethanol or ethyl acetate) was placed in an ultrasound bath for 30 min where after the solvent was filtered through a Whatmann No. 1 filter paper and taken to dryness in front of a fan.

Soxhlet: one gram of root material was extracted with 150 ml of solvent (ethanol or ethyl acetate) for 2 h. The extracts were taken to dryness under vacuum.

2.3. The cyclooxygenase-1 assay for anti-inflammatory activity

The bioassay was performed according to Jäger et al. (1996). The six extracts from the sonication method (redissolved to 10 mg/ml) were tested. The aqueous extracts were redissolved in water and the others in ethanol. The solvent test solutions were made up: 20 μl of aqueous samples; 2.5 μl of ethanolic samples + 17.5 μl water; 2.5 μl of a 8×10^{-4} ethanolic indomethacin (Sigma Chemical Co., MO) solution as standard.

The enzyme stock solution (10 ml of sheep seminal vesical microsomes containing 0.3 mg protein) was added to co-factor solution (L-adrenaline and reduced glutathione, 3 mg of each suspended in 10 ml 0.1 M Tris buffer, pH 8.2) in the ratio 1:5 and pre-incubated on ice for 15 min.

After adding 10 μl 2N HCl to the background samples to inactivate the enzyme, 60 μl of enzyme/co-factor solution were added to every sample. A 5 min incubation was allowed at room temperature and then 20 μl of [^{14}C] arachidonic acid (16 Ci/mole, 30 mM) were added to each sample, followed by incubation in a water bath at 37 °C for 8 min. The reaction was then terminated by adding 10 μl 2N HCl (not to the backgrounds) and the samples were kept in an ice bath.

Column chromatography was then used to separate the [^{14}C] labelled prostaglandins synthesized during the assay from the unmetabolized arachidonic acid.

Pasteur pipettes blocked with glass wool were packed with silica gel in eluent 1 (hexane:dioxane:acetic acid 350:150:1) to a height of 3 cm. One millilitre of eluent 1 and 4 μl of 0.2 mg/ml prostaglandin carrier solution (PGE_2:PGF_2 1:1) were added to each sample and they were loaded onto the column. Four millilitres of eluent 1 further eluted the arachidonic acid and this was discarded. Three millilitres of eluent 2 (ethyl acetate:methanol 425:75) was applied to each column to elute the labelled prostaglandins and this was collected in scintillation vials. Four millilitres of scintillation cocktail (Beckman Ready Solve) were added to the eluent and after 30 min the radioactivity of the samples was counted using a Beckman LS6000LL scintillation counter. Analysing the amount of radioactivity present relative to that in the solvent blanks indicated the percentage of cyclooxygenase inhibition of the test samples. All experiments were carried out in duplicate.

2.4. The microplate antibacterial method

This assay was used to determine the minimum lethal concentration (MLC) using a serial dilution technique (Eloff, 1998). The bacterial cultures used were *Bacillus subtilis* (ATCC 6051), *Escherichia coli* (ATCC 11775), *Klebsiella pneumoniae* (ATCC 13883) and *Staphylococcus aureus* (ATCC 12600). These were maintained on Mueller-Hinton (MH) agar at 4 °C and overnight cultures were prepared in MH broth the day prior to the assay. The assay was performed under sterile conditions on a laminar flow bench.

The plant extracts were dissolved to 50 mg/ml, the aqueous extracts in water and the ethanolic and ethyl acetate extracts in ethanol. The overnight bacterial

cultures were diluted 1:100 in MH broth. Ninety-six-well microplates were used and 100 μl sterile H_2O was added to each well.For each extract a two-fold serial dilution was made down the microplate, starting with a concentration of 25 mg/ml in the first well. Each extract was tested against all the bacterial cultures and standards and controls were made: wells containing MH broth only; each type of bacteria but with no extract; and also a serial dilution of neomycin (Sigma Chemical Co., MO) with each type of bacteria at recommended inhibitory concentrations. The microplates were covered with parafilm and incubated at 37 °C overnight to stimulate the bacterial growth. The following day 40 μl of 0.2 mg/ml INT was added to every well and another incubation period of 10–30 min at 37 °C was allowed.

INT is reduced to a red coloured product by biologically active organisms, so bacterial growth in the well was indicated by a pink/red colour. A clear well indicated inhibition of growth by the plant extract. The MLC is the lowest concentration at which bacterial growth is eliminated and this could be seen in the last clear well in the dilution series.

2.5. Bioautographic assay

An overnight culture of *S. aureus* was grown in MH broth in a water bath at 35 °C. This was then centrifuged at 3000*g* for 10 min and the supernatant decanted. The pellet was resuspended in 10 ml of MH broth and this was sprayed onto the thin layer chromatography (TLC) plate. The plate was then placed on damp tissue in a metal tray covered with plastic to allow 100% humidity. The tray was left in the oven at 35 °C overnight to allow the bacteria to grow.

The following day a 2 mg/ml INT solution was sprayed onto the bacteria covered plate which was then placed back in the oven in 100% humidity for about half an hour. The INT stained the bacteria dark red so that it was clearly visible where bacterial growth had been inhibited. The bands that remained in a clear zone possessed compounds with antibacterial activity.

2.6. Cell cultures

Standard cell culture techniques (Grist et al., 1979), were used for all procedures utilizing cell cultures. Monolayers of secondary vervet monkey kidney (VK) cells (National Institute for Virology, Sandringham, South Africa), between passages four to six, were used. Eagle's minimum essential medium (MEM)(National Institute for Virology) supplemented with 8% heat inactivated foetal calf serum (FCS) (Delta Bioproducts, Kempton Park, South Africa) and containing 100 U/ml penicillin and 100 μg/ml streptomycin was used for the propagation of the cells. Cell cultures were incubated at 37 °C in a humidified CO_2 atmosphere. Maintenance medium was essentially the same as the propagation medium except that it contained only 2% FCS.

2.7. Cytotoxicity assay

Plant extracts were tested for cytotoxicity by exposing monolayers of secondary (VK cells to dilutions of the filter-sterilised plant extracts. Doubling dilutions of the plant extracts, from a concentration of 3.9 to 1000 μg/ml, in serum-free MEM were used for testing on 24-h old monolayers of VK cells. The cells were monitored visually by light microscopy over a period of 7 days and on the seventh day tested for cytotoxicity using a tetrazolium salt reduction (MTT) assay (van Rensburg et al., 1994) based on the method of Hussain et al. (1993). Monolayers of cells exposed to serum-free MEM alone were used as a control.

2.8. Virus stock

Influenza A virus (Inf A): Freshly harvested allantoic fluid containing Inf A (strain Panama) was kindly supplied by the National Institute for Virology. A stock suspension, with a titre of 3.16×10^5 $TCID_{50}$/ml, was prepared by diluting the allantoic fluid in sterile phosphate buffered saline (PBS)(Sigma Chemical Company, St. Louis, MO) containing penicillin (50 μg/ml), streptomycin (50 μg/ml) and neomycin (100 μg/ml) (PSN Antibiotic Mixture [100X], GibcoBRL Life Technologies, Paisley, Scotland). This stock suspension is reportedly stable for 1 week at 4 °C (Barrett and Inglis, 1985). For experimental purposes fresh dilutions of the virus, in serum-free MEM, were prepared immediately before use. The 50% tissue culture infectious dose ($TCID_{50}$) of the virus was calculated according to the Kärber formula as outlined in Grist et al. (1979). The virus was used at a final concentration of 1000 $TCID_{50}$/assay well.

2.9. Antiviral assays

Antiviral assays were only carried out at concentrations of the leaf and root extracts that were not cytotoxic for the cell cultures.

2.9.1. Effect on viral replication

Monolayers of 24-h old VK cells in 16-well Lab-tek7 glass tissue culture chamber slides (Nalge Nunc Int., Naperville, IL) were rinsed with serum-free MEM. Thereafter the serum-free MEM was withdrawn and

1000 $TCID_{50}$ of Inf A was added to each well to adsorb to the cell cultures for 1 h at 37 °C in a humidified CO_2 atmosphere. Cells were rinsed to remove free/unadsorbed virus and 200 μl of dilutions of the plant extract in serum-free MEM were added to the wells which were further incubated at 37 °C in a humidified CO_2 atmosphere for 36 h. Viral infection was monitored by direct immunofluorescence (IF) for the detection of viral antigen. Direct IF was carried out using standard techniques with a mouse anti-influenza A fluorescein isothiocyanate (FITC)-labelled monoclonal antibody (Chemicon International Inc., Temecula, CA) as the detector. The reduction in the number of fluorescent foci indicated antiviral activity in the plant extract. The percentage reduction in fluorescent foci was calculated in relation to controls.

2.9.2. Effect on viral adsorption and subsequent replication

The cell cultures were prepared as before and the appropriate dilution of the plant extract and 1000 $TCID_{50}$ of the virus were added simultaneously to the wells. Incubation at 37 °C in a humidified CO_2 atmosphere and viral infection monitored by direct IF as described previously.

2.9.3. In vitro viral inactivation

Of each dilution of the plant extracts 500 μl were mixed with 500 μl of Influenza A suspension and incubated for 1 h at 37 °C, after which the virus was recovered from the mixture by ultrafiltration using a Centricon®-100 concentrator (Amicon Inc., Beverly, MA). The volume of the retentate was brought up to 500 μl and the cell cultures were inoculated with 100 ml of the retentate. Viral infection was monitored 36 h post infection by direct IF.

2.10. Isolation of antibacterial compound

For the bulk sonication extract, 500 g of root material was placed into a conical flask with enough ethyl acetate to completely cover it. This was sonicated for 30 min and then left to stand for half a day. Once all the root material had settled at the bottom of the flask, the top layer of EtOAc was filtered through a Whatmann No. 1 filter. This process was repeated eight times after which the collected extract was taken to dryness under a vacuum.

2.11. Vacuum liquid chromatography

The extract was fractionated on a large silica vacuum liquid chromatography (VLC) column and eluted with a hexane: ethyl acetate gradient, starting at 100% hexane, decreasing to 85% and then further decreasing in steps of 5% per fraction to 60%. Thereafter, the percentage hexane was decreased by 10% in each fraction. Four hundred millilitre of solvent mixture was used. Each fraction was taken to dryness under vacuum and the yield determined.

Aliquots of all the fractions were loaded onto TLC plates. The plates were developed in hexane:ethyl acetate 3:1. One plate was then stained with anisaldehyde (465 ml ethanol, 5 ml glacial acetic acid, 13 ml concentrated sulphuric acid, 13 ml *para*-anisaldehyde) and heated in an oven at 110 °C for about 15 min, and the other was used in a bioautographic assay.

The fraction which showed the highest antibacterial activity was loaded onto a glass TLC plate (0.25 mm thick) which was run in the same solvent system as before. The band which was responsible for the antibacterial activity was scraped off and eluted with EtOAc, after which the sample was then filtered through a syringe filter (0.45 mm PP). Chemical structures were determined from GC-MS data.

Table 1
Minimum lethal concentration, in mg/ml, of extracts of *P. prunelloides*

	Extract	Strain of bacteria			
		Bs	Sa	Ec	Kp
Leaf	H_2O	12.5	6.25	6.25	3.13
	EtOH	0.78	0.2	1.56	1.56
	EtOAc	1.56	3.13	0.78	0.78
Root	H_2O	12.5	12.5	12.5	12.5
	EtOH (Sonication)	0.78	1.56	1.56	1.56
	EtOH (Soxhlet)	0.78	0.39	3.13	1.56
	EtOAc (Sonication)	1.56	1.56	0.78	1.56
	EtOAc (Soxhlet)	0.78	0.78	0.78	0.39
Neomycin standards (μg/ml)		0.39	3.13	1.56	1.56

Sa: *Staphylococcus aureus*; Ec: *Escherichia coli*; Kp: *Klebsiella pneumoniae*; Bs: *Bacillus subtilis*.

Table 2
Percentage inhibition of cyclooxygenase by extracts of *P. prunelloides*

Extract	Leaf	Roots
H_2O	74	74
EtOH	88	65
EtOAc (Sonication)	72	87
EtOAc (Soxhlet)	–	81

Indomethacin standard (20 μM) 83%.

3. Results and discussion

Table 1 shows the results of the antibacterial screening. Aqueous extracts appear to have less antibacterial activity than ethanolic and ethyl acetate extracts. This is interesting in that the traditional method of treating a bacterial infection would be by giving a root decoction prepared by boiling the root in water, where according to these results an ethanolic solvent would probably be more effective. Soxhlet extracts with EtOH and EtOAc showed comparable activity to sonication extracts with the same solvents. This implies that the compound(s) responsible for antibacterial activity is heat resistant.

In order to isolate the antibacterial compound a bulk extraction of roots was carried out. The total dry mass of the bulk extracts was 2.985 g. When this was put through a VLC column thirteen fractions were collected (labelled A-M as the hexane percentage in the solvent mixture decreased from 100 to 0).

The antibacterial activity in fractions E to H appeared to be caused by the same compound, which seemed to be the major compound in the extract having an Rf value of 0.55. This compound stained green with anisaldehyde. The most pronounced inhibition of bacterial growth was seen in fraction G which was eluted with 60:40 hexane:ethyl acetate. Separation of fraction G (29 mg) on a glass TLC plate enabled the band responsible for the activity to be scraped off, yielding 7 mg, and the compound to be identified by GC-MS. The main component was found to be palmitic acid MS m/z (rel.int.): 256(24)$[M]^+$, 213(22), 185(16), 157(20), 129(52), 97(26), 73(100), 56(14). Palmitic acid was reported to be the major compound in a mixture of fatty acids from *Diplotaxis harra* and *Ericaria microcarpa* (Hashem and Saleh, 1999). These authors reported antibacterial activity against gram-positive and gram-negative bacteria comparable to ampicillin and in the case of *Streptococci*, greater than that of ampicillin (Hashem and Saleh, 1999).

The results of the screening of extracts for prostaglandin synthesis inhibitors are given in Table 2. All the extracts showed a high percentage of inhibition and there was no marked difference between the percentage inhibition caused by sonication extracts and that by the Soxhlet extract. This implies that the compound(s) responsible for the activity is heat resistant. Additionally there was no great difference between the anti-inflammatory activity caused by the root and leaf material. These results show that all the extracts of *P. prunelloides* were effective in reducing prostaglandin synthesis during inflammation by inhibiting the enzyme cyclooxygenase-1 (COX-1).

A COX-1 assay was carried out with the fractions from the VLC at a concentration of 0.1 mg/ml. The highest activity was found in the more polar fractions. Fractions L and M were combined and run on a glass

Table 3
Results of the three antiviral assays showing Influenza A inhibition as a percentage reduction in the number of fluorescent foci on the infected VK cell cultures in relation to controls

Concentration of extract (μg/ml)	Percentage reduction of fluorescent foci on VK cell culture (%)					
	Assay 1		Assay 2		Assay 3	
	Root	Leaf	Root	Leaf	Root	Leaf
Virus control	0	0	0	0	0	0
125	45.4		24.2		96.2	
62.5	29.5		46.5		88.7	
31.25	32.9		44.9		80.3	
15.62	23.6	40.5	39.9	27.0	70.4	83.3
7.81	34.3	28.4	26.8	39.4	64.4	79.5
3.9	16.2	35.7	39.9	39.0	46.0	29.5
1.95		21.8		37.7		21.1
0.98		19.4		18.0		0

Assay 1: Inhibition of replication of 1000 $TCID_{50}$ Influenza A after viral adsorption and incubation of the infected culture in different concentrations of aqueous extract. *Assay 2*: Inhibition of adsorption and replication of 1000 $TCID_{50}$ Influenza A after viral adsorption and incubation of the infected culture in different concentrations of aqueous extract. *Assay 3*: In vitro inactivation of Influenza A after incubation of the virus at 37 °C for 1 h in different concentrations of aqueous extract.

TLC plate and all the bands were separately tested for anti-inflammatory activity. One band was found to be active but further HPLC separation was unsuccessful.

The *P. prunelloides* leaf extract produced gross morphological changes in the VK cell monolayers at concentrations of 62.5 up to 1000 μg/ml in the cytotoxicity assay. The integrity of the monolayer remained at extract concentrations 3.9 up to 31.25 μg/ml. When treated with the root extract of concentrations up to 125 μg/ml the integrity of the monolayers was maintained, however, cytotoxic effects were observed at concentrations of 250 μg/ml or higher.

The results of the three different antiviral assays are shown in Table 3. The antiviral activity against the Inf A is evident by the percentage reduction in the number of fluorescent foci on the infected VK cell cultures. The activity varies at different concentrations of the aqueous extract of *P. prunelloides*. A difference in activity has also been observed between root and leaf extract in the three assays:

3.1. Viral replication

Aqueous leaf extracts at concentrations 0.98–15.62 μg/ml affected the replication of Inf A in cell culture. A direct relationship was noted between the concentration of the leaf extract and the reduction in the number of fluorescent foci. The root extract also exhibited antiviral activity against influenza A viral replication but at higher extract concentrations, i.e. from 3.9 to 125 μg/ml.

3.2. Viral adsorption

Leaf extract concentrations of 1.95–15.62 μg/ml showed similar antiviral activity against the adsorption of influenza A, but activity was much less at 0.98 μg/ml. The root extract showed antiviral activity at concentrations from 3.9 to 125 μg/ml, but there was no clear relationship between concentration of extract and reduction in the number of fluorescent foci.

3.3. In vitro inactivation

Aqueous leaf extracts of concentrations 7.81–15.60 μg/ml showed a marked inactivation of Influenza A. Aqueous root extracts also showed this inactivation, but at higher extract concentrations, i.e. from 3.9 to 125 μg/ml.

The results of the antiviral tests suggest the use of root decoctions for the treatment of influenza-like infections. In 1918 root decoctions of *P. prunelloides* were used during the influenza epidemic, with apparently a marked improvement (Watt and Breyer-Brandwijk, 1962). The combination of antibacterial and anti-inflammatory activity together with antiviral activity against influenza A makes the plant a potentially effective remedy for 'flu relief' and further phytotherapeutic research is warranted.

This study suggests that the use of *P. prunelloides* in traditional medicine may be beneficial for the treatment of selected bacterial and viral infections as well as having anti-inflammatory properties.

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