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Investigation of the biological activities of *Siphonochilus aethiopicus* and the effect of seasonal senescence

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Siphonochilus aethiopicus **(Schweinf.) B.L. Burtt (Zingiberaceae), commonly known as wild ginger, is one of the most important and threatened medicinal plants in South Africa. A study of the pharmacological properties of *S. aethiopicus* and the effect of seasonal senescence on antibacterial and anti-inflammatory properties was undertaken. Water, ethanol and ethyl acetate extracts were prepared from the leaves, rhizomes and roots of *S. aethiopicus* plants. The extracts were tested in a variety of pharmacological assays. Results for the general screening showed antibacterial and anti-inflammatory activity. Some cytotoxicity was observed with the aqueous extracts of the rhizome. However, no significant activity against the herpes simplex virus types 1 and 2, the influenza A virus, and in the anthelmintic, antischistosomal and biochemical induction assays were observed. In the microdilution antibacterial assay, no inhibitory activity against the test bacteria was detected with the aqueous extracts. The ethanol and ethyl acetate extracts tested showed greater antibacterial activity at minimal inhibitory concentrations ranging from 0.78 to 3.13mg ml^{-1} against the Gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*) than the Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*). Little difference was observed between the ethanol and ethyl acetate extracts, or between the different plant parts. Ethanol extracts were prepared from the different plant parts before and after seasonal senescence, and tested for antibacterial and anti-inflammatory activity. There appeared to be a loss of antibacterial activity in the leaves with senescence, concomitant with an increase of activity in the alpha-roots. In the cyclooxygenase-1 assay, the aqueous extracts showed no significant prostaglandin synthesis inhibition. For the ethanol and ethyl acetate extracts, the leaves showed the highest levels of activity at a concentration of 250µg ml^{-1} per test solution, in both the cyclooxygenase-1 and -2 assays. Some differences in the levels of anti-inflammatory activity in the roots following senescence were also observed. There was a slight loss of activity as a result of drying the rhizome material prior to extraction. This suggests that fresh rhizome material may be more effective for medicinal use, although it should be noted that the aqueous rhizome extracts displayed moderately high levels of cytotoxicity, and may require further investigation.**

Introduction

Siphonochilus aethiopicus (Schweinf.) B.L. Burtt (Zingiberaceae), commonly known as wild ginger, is a highly sought after plant for use in traditional medicine in South Africa, although it is regarded as being regionally extinct in the wild in KwaZulu-Natal (Scott-Shaw 1999). The rhizome is the part which is traditionally used, although sometimes the roots are also harvested. It is used for colds, coughs, pain relief, asthma, dysmenorrhoea, influenza and is chewed fresh for hysteria (Van Wyk *et al.* 1997). The use of *S. aethiopicus* for pain relief and inflammation has been supported through investigations into the inhibitory action against prostaglandin synthesis (McGaw *et al*. 1997, Lindsey *et al.* 1999, Zschocke *et al.* 2000). However, little is known about other pharmacological properties, and whether there is any seasonal influence on the levels of activity in *S. aethiopicus.* It is possible that plant material of different ages, or harvested at different times, may have varying levels of pharmacological activity. This is an important factor to consider in the use of *S. aethiopicus* by traditional healers. The propagation of medicinal plants by traditional healers and small scale farmers is receiving increasing attention (Jäger and Van Staden 2000). It would be useful to gain a greater understanding of the medicinal properties and seasonal differences in activity of *S. aethiopicus*.

There is very little published information on the chemical constituents of *S. aethiopicus.* The volatile oil contains α-terpineol and various other monoterpenoids, with the main compound being a characteristic sesquiterpenoid (Van Wyk

et al. 1997). None of the terpenoids of the oil of true ginger are present in the essential oil of *S. aethiopicus* (Van Wyk *et al.* 1997).

Extracts of the leaves, rhizomes and roots were screened in the microdilution antibacterial assay, cyclooxygenase-1 and -2 anti-inflammatory assays, vervet monkey kidney cell cytotoxicity assay, herpes simplex virus type 1 and 2 and influenza A antiviral assays, anthelmintic assay, antischistosomal assay and the biochemical induction assay.

Materials and Methods

Extracts for general screening

S. aethiopicus plants were obtained from stock plants at the University of Natal Botanical Garden, Pietermaritzburg. A voucher specimen was deposited in the University of Natal Herbarium (*Light 17* NU). Mature plants were harvested in summer, divided into leaves, rhizomes and roots and dried at 50°C for 2 days. The dried, ground material (10g) was extracted in 100ml solvent (water, ethanol or ethyl acetate), in an ultrasound bath (Branson 5210) for 60min. The extracts were vacuum filtered through Whatman No. 1 filter paper, and dried under vacuum, using a rotary evaporator.

Extracts for comparison of seasonal activity

Plants of *S. aethiopicus* were harvested prior to senescence (green leaves), and after the plants had fully senesced. For each set of extracts, plants were divided into leaves, rhizomes and roots, differentiating between α- and β-rhizomes and roots. The mature rhizomes, and associated roots, from the previous year's growth were classified as α-rhizomes and roots, and the younger rhizomes, and roots, which develop from the α-rhizome at the start of the growing season were classified as β-rhizomes and roots. Part of the freshly harvested material was extracted in 100ml ethanol using a Wareing blender, and then placed in an ultrasound bath (Branson 5210) for 60min. The remaining material was dried at 50°C for 2 days prior to extraction in the same manner. The extracts were vacuum filtered through Whatman No. 1 filter paper, and dried under vacuum, using a rotary evaporator.

Microdilution antibacterial assay

Antibacterial activity was assessed using the microdilution bioassay, as described by Eloff (1998). The residues from the plant extracts were redissolved to a concentration of 50mg ml^{-1} in water (for aqueous extracts) or ethanol (for ethyl acetate or ethanol extracts). Each extract was bioassayed against bacterial strains obtained from the bacterial collection of the Microbiology Department, University of Natal, Pietermaritzburg and maintained on Mueller-Hinton nutrient agar (Biolab) at 4°C. The bacteria used were *Bacillus subtilis, Staphylococcus aureus* (Gram-positive) and *Escherichia coli, Klebsiella pneumoniae* (Gram-negative).

For each test bacterium, 100µl of redissolved extract was serially diluted 2-fold with 100µl sterile distilled water in a sterile 96-well microtitre plate. A similar 2-fold serial dilution of neomycin (100µg ml^{-1}) was used as a positive control for each bacterium. Extract and bacteria-free negative controls were also included. Suspension cultures of the bacteria were inoculated in Mueller-Hinton (MH) broth (Oxoid) from stock cultures and incubated overnight at 37°C in a water-bath on an orbital shaker. Prior to use in the bioassay, the saturated suspension cultures were diluted 1:100 with sterile MH broth. To each of the wells containing the test and control solutions, 100µl of the bacterial cultures were added. The plates were covered and incubated overnight at 37°C. To indicate bacterial growth, 40µl of 0.2mg ml^{-1} *p*-iodonitrotetrazolium chloride (Sigma), were added to each well, and the plates were incubated for a further 30min. The wells which displayed no change in colour represented antibacterial activity. The minimum inhibitory concentration (MIC) was taken as the lowest concentration of plant extract to elicit a bacteristatic or bactericidal effect against the tested bacterium.

Cyclooxygenase-1 assay

The cyclooxygenase-1 (COX-1) assay was performed as described by White and Glassmann (1974), with slight modifications (Jäger *et al.* 1996). The COX-1 enzyme (10µl microsome suspension from sheep seminal vesicles, 0.3µg protein) was activated with 50µl of co-factor solution (0.3mg ml^{-1} adrenaline and 0.3mg ml^{-1} reduced glutathione in 0.1M Tris buffer, pH 8.2) on ice for 15min. The enzyme solution (60µl) was added to the sample solution (2.5µl ethanolic or aqueous plant extract and 17.5µl water) and incubated at room temperature for 5min. The plant extracts were tested at a final concentration of 250µg ml^{-1} per test solution. Indomethacin was tested as a positive control. The reaction was started by adding 20µl [^{14}C]-arachidonic acid (30µM, 16Ci mol^{-1}). Samples were incubated for 8min at 37°C and the reaction terminated by adding 10µl 2M HCl. Prostaglandins and unmetabolised arachidonic acid were separated by column chromatography over silica gel, after addition of 4µl unlabeled prostaglandins (PGE_2:PGF_2 1:1) as a carrier solution. Arachidonic acid was eluted first with *n*-hexane:1,4-dioxan:glacial acetic acid (70:30:0.2). The prostaglandin products were then eluted with ethyl acetate:methanol (85:15) and collected. After mixing with scintillation solution, the samples were counted in a Beckman LS 6000LL scintillation counter. Inhibition refers to reduction of PGE_2 formation in comparison to an untreated sample (2.5µl ethanol in 17.5µl water).

Cyclooxygenase-2 assay

The cyclooxygenase-2 (COX-2) assay was performed as described by Noreen *et al.* (1998), with slight modifications (Zschocke and Van Staden 2000). The COX-2 assay follows a very similar protocol as the COX-1 assay. Purified COX-2 enzyme from sheep placental cotyledons was purchased from Cayman chemical. The enzyme (10µl containing 3 units) was activated with 50µl co-factor solution (0.6mg ml^{-1} adrenaline, 0.3mg ml^{-1} reduced glutathione and 1µM hematin in 0.1m Tris buffer, pH 8.0) on ice for 5min. The enzyme solution (60µl) was added to the sample solution

(2.5µl ethanolic or aqueous plant extract and 17.5µl water) and incubated at room temperature for 5min. The plant extracts were tested at a final concentration of 250µg ml^{-1} per test solution. Positive control measurements were carried out with indomethacin and nimesulide at concentrations of 200µM. The reaction was started by adding 20µl [^{14}C]-arachidonic acid (30µM, 16Ci mol^{-1}). Samples were incubated for 10min at 37°C and the reaction terminated by adding 10µl 2M HCl. Prostaglandins and unmetabolised arachidonic acid were separated and COX-2 inhibition was determined as described for the COX-1 assay.

Preparation of extracts for antiviral testing

A 1 000µg ml^{-1} (w/v) sterile stock suspension of the extract was prepared by diluting an aqueous suspension of plant extract in serum free Eagle's minimum essential medium (MEM) (National Institute for Virology) followed by filter sterilisation through a 0.45µm membrane (Ministart® filter unit, Sartorius). Dilutions of the aqueous plant extracts, in serum-free MEM, were tested for cytotoxicity at concentrations ranging from 3.9µg ml^{-1} up to 1 000µg ml^{-1}, and from 3.9µg ml^{-1} up to 500µg ml^{-1} for the antiviral assays.

Cell culture

Standard cell culture techniques (Grist *et al.* 1979), were used for all procedures utilising cell cultures. Monolayers of secondary vervet monkey kidney (VK) cells (National Institute for Virology) were prepared by seeding 96-well microtitre plates with 200µl of 10^5 cells/ml cell suspension. MEM supplemented with 8% heat inactivated foetal calf serum (FCS) (Delta Bioproducts) and containing 100U ml^{-1} penicillin and 100µg ml^{-1} streptomycin was used for the propagation of the cells. Cell cultures were incubated in a humidified CO_2 atmosphere at 37°C. Maintenance medium was essentially the same as the propagation medium except that it contained only 2% FCS.

Virus stock

The 50 percent tissue culture infectious dose ($TCID_{50}$) of each virus was calculated according to the Kärber formula as outlined in Grist *et al.* (1979).

Stock suspensions of herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2), with titres of 1 x 10^7 and 1 x 10^6 $TCID_{50}$/ml respectively, were prepared from clinical isolates of HSV-1 and HSV-2 (Department of Medical Virology, University of Pretoria). The viruses were diluted in serum-free MEM and used at a final concentration of 100 $TCID_{50}$/microplate well.

Freshly harvested allantoic fluid containing influenza A virus (Inf A) (strain Panama) was kindly supplied by the National Institute for Virology. A stock suspension, with a titre of 3.16 x 10^5 $TCID_{50}$/ml, was prepared by diluting the allantoic fluid in sterile phosphate buffered saline (PBS) (Sigma) containing penicillin (50µg ml^{-1}), streptomycin (50µg ml^{-1}) and neomycin (100µg ml^{-1}) (PSN Antibiotic Mixture [100X], GibcoBRL). This stock suspension is reportedly stable for one 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 virus was used at a final concentration of 1 000$TCID_{50}$/microplate well.

As Inf A does not exhibit a cytopathic effect (CPE) in cell culture, viral infection was monitored by direct immunofluorescence (IF) for the detection of viral antigen. Titrations were done in 16-well Lab-Tek® glass tissue culture chamber slides (Nalge Nunc) instead of 96-well microtitre plates. Forty-eight hours after infection the growth medium was recovered and the slides were washed in PBS and fixed in 100% acetone at -20°C. The direct IF was carried out using standard techniques with a mouse anti-influenza A fluorescein isothiocyanate (FITC)-labeled monoclonal antibody (Chemicon) as the detector. Stained slides were examined for fluorescence using a halogen lamp at 10x magnification. Wells where fluorescent foci were detected were considered to be positive for Inf A infection.

Cytotoxicity assay

Aqueous extracts of the leaves and rhizomes of *S. aethiopicus* were tested for cytotoxicity by exposing monolayers of VK cells to dilutions of the filter sterilised plant extracts. Serial 2-fold dilutions of the extracts, in serum-free MEM, from a concentration of 3.9µg ml^{-1} to 500µg ml^{-1}, were used for testing on 24-hour-old monolayers of VK cells. The cells were monitored visually, by light microscopy, over a period of seven 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.

Antiviral assays

Two separate assays were performed for each virus:

a) To investigate the effect of the aqueous extract of *S. aethiopicus* leaves on the replication of HSV-1 and HSV-2, 24-hour-old monolayers of VK cells in 96-well microtitre plates were starved in serum-free MEM for 1h at 37°C in a humidified CO_2 atmosphere. After starvation the serum-free MEM was withdrawn and 100µl (100$TCID_{50}$) of virus was added to the wells and allowed to adsorb to the cell cultures for 1h at 37°C in a humidified CO_2 atmosphere. After adsorption for 1h the unbound virus was withdrawn and the cells rinsed once with serum-free MEM after which 200µl of the appropriate dilution of plant extract suspension in serum-free MEM was added to each of 6 wells and the cell cultures incubated at 37°C in a humidified CO_2 atmosphere. As a positive control, cells infected with virus were maintained in serum-free MEM, and cells mock-infected with 100µl serum-free MEM and maintained in serum-free MEM served as negative controls. After infection the cell monolayers were examined daily for 7 days, by light microscopy, for the appearance of a typical herpes simplex-like cytopathic effect (CPE) characterised by large refractile cells (Wiedbrauk and Johnston 1993). As the herpes simplex CPE spreads rapidly through the cell monolayer complete destruction of the monolayer was readily discernible by days 4 to 5, post-infection. An obser-

vation period of up to 7 days ensured that no further CPE manifested itself and that unaffected monolayers remained intact. The absence of CPE at a specific concentration of the plant extract was considered to be indicative of antiviral activity.

To investigate the effect of the aqueous extract of the rhizomes of *S. aethiopicus* on the replication of Inf A, the procedure was effectively the same as described above except that the VK cells were grown in 16-well Lab-Tek® glass tissue culture chamber slides and 1 000$TCID_{50}$ Inf A was used per well. Only plant extract concentrations showing no severe cytotoxicity were tested for antiviral activity. Viral infection was monitored by direct IF, in 4 wells per concentration of plant extract, as described previously. Fluorescent foci were counted in three separate fields on each of the 4 wells and the reduction in fluorescent foci in relation to the control wells was calculated.

b) To investigate the effect of the aqueous extract of *S. aethiopicus* leaves on viral adsorption and subsequent replication of HSV-1 and HSV-2 in cell culture, 96-well microtitre plates were prepared and starved as described previously. Equal volumes (100µl) of the appropriate dilution of the plant extract and virus suspension (100$TCID_{50}$) were added simultaneously to each of 6 wells of the 96-well microtitre plate and the cell cultures incubated at 37°C in a humidified CO_2 atmosphere. As a positive control, cells were infected with 100µl (100$TCID_{50}$) virus in serum-free MEM, and as a negative control, serum-free MEM was added to the cells. Cells were examined daily for 7 days, by light microscopy, for the appearance of a CPE. The absence of CPE at a specific concentration of the plant extract was considered to be indicative of antiviral activity.

To investigate the effect of the aqueous extract of the rhizomes of *S. aethiopicus* on the adsorption and replication of Inf A, the procedure was effectively the same as described above except that the VK cells were grown in 16-well Lab-Tek® glass tissue culture chamber slides and 1 000$TCID_{50}$ Inf A was used per well. Viral infection was monitored by direct IF, in 4 wells per concentration of plant extract, as described previously. Fluorescent foci were counted in three separate fields on each of the 4 wells and the reduction in number of fluorescent foci in relation to the control wells was calculated.

Anthelmintic bioassay

A simple anthelmintic bioassay, using *Caenorhabditis elegans* free-living nematodes as test organisms, was carried out as described by McGaw *et al.* (2000). The water, ethanol and ethyl acetate extracts of the leaves, rhizome and roots of *S. aethiopicus* were tested at concentrations of 0.5 and 1mg ml^{-1}. Water and ethanol extracts were redissolved in their extracting solvents, and the ethyl acetate extracts were redissolved in ethanol for use in the assay. A standard concentration of 5µg ml^{-1} levamisole was used as a control. *C. elegans* var. Bristol (N2) nematodes were cultured on nematode growth agar seeded with *E. coli* according to the method of Brenner (1974). For this assay, 500–1000 nematodes (7–10 day-old cultures) in M9 buffer (Brenner 1974) were incubated with the plant extracts for 2h at 25°C in the dark. Nematodes with no plant extracts or levamisole added were included as a control. The percentage of living nematodes, and their movement, was assessed using a dissecting microscope.

Antischistosomal bioassay

The antischistosomal assay was performed according to Sparg *et al.* (2000). Infected *Bulinus africanus* snails were placed into test tubes under a 60W electric light to promote the shedding of cercariae. The cercariae were collected and transformed into schistosomula worms by subjection to a shearing stress, using a syringe with an 0.8ml needle. The bioassay was run in 96-well microtitre plates. The aqueous extracts of the leaves, rhizomes and roots (100µl) were serially diluted 2-fold, giving an initial concentration of 25mg ml^{-1} per test solution. A similar 2-fold serial dilution of praziquantel (Sigma) was used as a positive control. A culture medium blank was included as a negative control. Three schistosomula, in 100µl culture medium, were added to each well. After incubation at 25°C for 1h, the survival of the schistosomula were assessed.

Biochemical induction assay (BIA)

The BIA was performed according to the method described by White *et al.* (1986). Water, ethanol and ethyl acetate extracts of the leaves, rhizomes and roots were resuspended to 100mg ml^{-1} and applied (10µl) to filter paper discs which were placed onto agar assay plates. Plates were incubated for 5 hours at 37°C, after which the chromogenic substrate was added and zones of induction recorded. The strong mutagen 4-nitroquinoline 1-oxide was used at concentrations of 1, 5, 50 and 100µg per spot as positive controls. Appropriate solvent controls were included as negative controls.

Results and Discussion

Results for the general screening of extracts from the different plant parts for antibacterial activity are shown in Table 1. No inhibitory activity against the test bacteria was detected with the aqueous extracts. The ethanol and ethyl acetate extracts tested showed greater antibacterial activity against the Gram-positive bacteria than the Gram-negative bacteria. No distinct differences were observed between the activity obtained with the ethanol and ethyl acetate extracts in the general screening of different plant parts, or between the activity of the different plant parts.

Antibacterial activity has been shown for a number of other species of the Zingiberaceae. In a study by Ahmad and coworkers (1998), wherein 82 Indian medicinal plants were screened for antimicrobial properties, alcoholic extracts of the rhizomes of *Curcuma longa* showed activity against *B. subtilis* and *S. aureus*. Hexane extracts of the seeds of *Elettaria cardamomum* showed activity against *B. subtilis*, *E. coli* and *S. aureus*, and alcoholic extracts showed activity against *B. subtilis*, *E. coli*, *Proteus vulgaris*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, and *S. aureus*.

In the same study hexane and alcoholic extracts of

Table 1: Minimum Inhibitory Concentrations (mg ml^{-1}) of *Siphonochilus aethiopicus* extracts against test bacteria

		Bacterium[a]			
Plant part	Extract	*B.s.*	*S.a.*	*E.c.*	*K.p.*
Leaves	Water	>12.5	>12.5	>12.5	>12.5
	Ethanol	3.13	1.56	3.13	3.13
	Ethyl acetate	1.56	1.56	3.13	6.25
Rhizome	Water	>12.5	>12.5	>12.5	>12.5
	Ethanol	1.56	1.56	3.13	6.25
	Ethyl acetate	1.56	1.56	6.25	6.25
Roots	Water	>12.5	>12.5	>12.5	>12.5
	Ethanol	1.56	0.78	6.25	6.25
	Ethyl acetate	0.78	1.56	6.25	12.5
Neomycin standard (µg ml^{-1})		0.1	0.1	1.56	0.39

Abbreviations: [a] *B.s. B. subtilis*; *S.a. S. aureus*; *E.c. E. coli*; *K.p. K. pneumoniae*

Zingiber officinale, real ginger, showed antibacterial activity against *B. subtilis* and *S. aureus*. The essential oils of *Kaempheria galanga* root and rhizome showed activity against *E. coli* and *S. aureus* (Arambewela *et al.* 1999). In screening 13 species of *Alpinia, Costus* and *Zingiber*, most of the dichloromethane and methanol extracts showed some antibacterial activity against *B. subtilis*, methicillin-resistant *S. aureus* (MRSA) and *P. aeruginosa*. The strongest inhibitory activity of a dichloromethane extract was shown by *Alpinia mutica* with the minimum inhibitory dose of 125µg per disc against both *B. subtilis* and MRSA (Habsah *et al.* 2000).

Results for the study of seasonal effects on antibacterial activity are shown in Table 2. Little difference was observed between the extracts prepared from fresh and dry material for activity against *S. aureus*, as well as for the other test bacteria (results not shown). Extracts prepared from the leaves gave a MIC value of 0.2 (fresh) and 0.1(dry)mg ml^{-1} before senescence and 3.13mg ml^{-1} after senescence, indicating a loss in activity. Extracts prepared from α-roots gave MIC values of 3.13 and 1.56mg ml^{-1} before senescence and 0.1 and 0.2mg ml^{-1} after senescence, indicating an increase in activity.

The results for the COX-1 inhibition assay are shown in Figure 1A. Aqueous extracts showed no significant activity. Ethanol and ethyl acetate extracts of the leaves showed high levels of anti-inflammatory activity. These results confirm the findings of Zschocke *et al.* (2000b) that the leaves of *S. aethiopicus* showed the highest levels of anti-inflammatory activity. The rhizome and root extracts showed much lower levels of activity, with the ethyl acetate extracts having slightly higher activity than the ethanol extracts. Furthermore, the level of activity in the rhizomes was lower than in the roots.

Inhibition of the various extracts in the COX-2 assay are shown in Figure 1B. As was seen in the COX-1 assay, the ethanol and ethyl acetate extracts of the leaves showed the highest levels of activity. Similarly, the rhizome and root extracts showed lower levels of activity, with the ethyl acetate extracts having slightly higher activity than the ethanolic extracts. The aqueous extracts of the roots showed no noteworthy activity, although those of the rhizomes and leaves were slightly higher.

A variety of compounds which demonstrate anti-inflammatory activity have been previously isolated from other species of the Zingiberaceae. Claeson and co-workers (1993) isolated three non-phenolic diarylheptanoids from hexane extracts of the rhizomes of *Curcuma xanthorrhiza*. These compounds showed significant anti-inflammatory activity in the assay of carrageenin-induced hind paw oedema in rats. Bioassay-guided fractionation of extracts of the rhizomes of *Zingiber cassumunar* led to the isolation of three cassumunins with anti-inflammatory activity (Masuda and Jitoe 1994). In further studies on *Z. cassumunar* by Pongprayoon and co-workers (1996), 5 compounds with topical anti-inflammatory activity were isolated from hexane extracts of the rhizome. These compounds gave ID_{50} values ranging from 2 to 62µg/ear in the model of 12-O-tetradecanoylphorbol-13-acetate-induced ear oedema in rats. Two pimarane diterpenes have been isolated from *Kaempferia pulchra*, and were found to have ID_{50} values estimated at 330 and 50µg/ear in the above-mentioned rat ear oedema bioassay (Sematong *et al.* 1996).

Results for the effect of senescence on COX-1 inhibition are shown in Figure 2. As observed with the general screening, the leaves showed the highest levels of activity, with the exception of senesced leaf material that was dried prior to extraction. No differences were observed between activity of the α- and β-rhizomes, before and after senescence. However, there did appear to be a slight loss of activity as a

Table 2: Minimum Inhibitory Concentrations (mg ml$^{-1)}$ of *Siphonochilus aethiopicus* ethanol extracts against *S. aureus*

Extract	Plant part	Before senescence	After senescence
Fresh material	Leaves	0.2	3.13
	Rhizome - α	1.56	1.56
	Rhizome - β	6.25	3.13
	Roots - α	3.13	0.1
	Roots - β	3.13	1.56
Dry material	Leaves	0.1	3.13
	Rhizome - α	3.13	3.13
	Rhizome - β	6.25	6.25
	Roots - α	1.56	0.2
	Roots - β	0.78	0.78
Neomycin standard (µg ml^{-1})		0.39	0.39

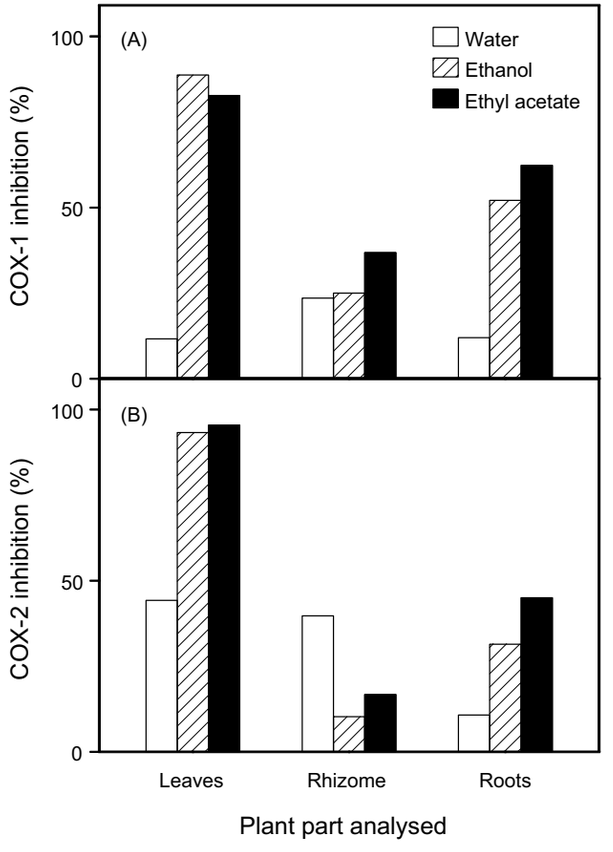

Figure 1: COX-1 (A) and COX-2 (B) inhibition of water, ethanol and ethyl acetate extracts of various plant parts of *S. aethiopicus*. Extracts tested at a final concentration of 250µg ml-1 per test solution. Values represent the mean of double determinations. Indomethacin gave 71% inhibition in the COX-1 assay. In the COX-2 assay, indomethacin and nimesulide standards inhibited the synthesis of prostaglandins by 54% and 33% respectively

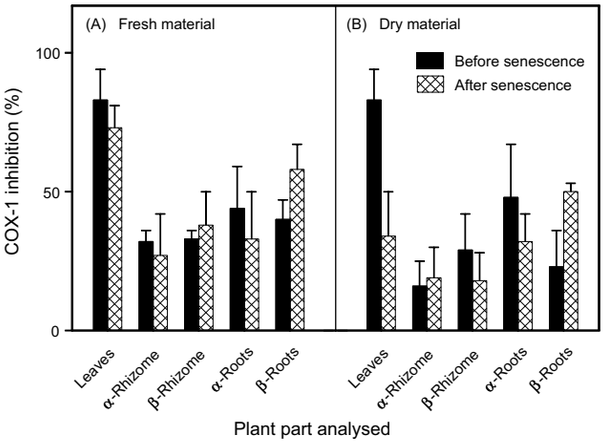

Figure 2: Cyclooxygenase-1 inhibitory activity (± S.D.) of ethanol extracts of various plant parts of *S. aethiopicus* before and after senescence. Extracts tested at a final concentration of 250µg ml^{-1} per test solution. Values represent the mean of double determinations from two experiments. The indomethacin standard inhibited the synthesis of prostaglandins by 54 ± 5.7%

Table 3: Survival of secondary vervet monkey kidney cells in the presence of varying concentrations of aqueous extracts of *S. aethiopicus* leaves and rhizomes

	Survival of VK cells (%)	
Extract concentration (µg ml^{-1})	Leaf extract	Rhizome extract
1 000	53	7
500	58	28
250	68	40
125	75	45
62.5	82	70
31.3	86	62
15.6	89	72
7.8	92	76
3.9	95	85

result of drying the rhizome material before extraction. This does suggest that fresh rhizome material may be more effective for use as a traditional medicine. For the α-roots, there was a slight decrease in activity following senescence. This was seen in both the fresh and dried material. The β-roots showed a trend opposite to this, in that there was an increase in activity following senescence.

In the cytotoxicity assay the integrity of the VK cell monolayers, treated with concentrations of the *S. aethiopicus* aqueous leaf extract, from 3.9µg ml^{-1} up to 1 000µg ml^{-1}, was maintained. Only minimal morphological changes, indicative of cytotoxic effects, were observed during the 7 day observation period at extract concentrations greater than 250µg ml^{-1}. This low level of toxicity was confirmed in the MTT assay (Table 3). Furthermore, the aqueous leaf extracts which were tested against HSV-1 and HSV-2, at concentrations ranging from 3.90µg ml^{-1} to 500µg ml^{-1}, exhibited no antiviral activity when the virus was inoculated onto cell cultures simultaneously with the plant extract (results not shown). The same extracts also showed no activity against the replication of either HSV-1 or HSV-2 after viral adsorption had taken place (results not shown).

Cytotoxicity testing of the aqueous extracts of the rhizome revealed high levels of cytotoxicity (Table 3), as evidenced by a percentage survival of the VK cells of less than 50% at concentrations of 125µg ml^{-1} and higher. In performing the assay, toxicity of the extract was evident by day 3 after inoculation. This extract was also tested in the influenza assay, but did not indicate any noteworthy activity against Inf A (results not shown).

No activity was observed in the anthelmintic, antischistosomal and BIA assays (results not shown). For the BIA test, the *S. aethiopicus* leaf extracts prepared with ethanol and ethyl acetate produced a zone of bacterial growth inhibition around the site of sample application in the BIA assay. However, no red ring of β-galactosidase induction was observed, so it is likely the extracts did not cause any DNA damage, but rather simply exhibited antibacterial activity.

Although no activity in the antiviral, anthelmintic, antischistosomal or biochemical induction assays was detected, anti-inflammatory activity was confirmed, and antibacterial activity observed against both Gram-positive and Gram-negative bacteria. From the experiments on seasonal senescence, it is clear that the time of harvest, and state of the material for extraction may only have a minimal influence on the degree of antibacterial and anti-inflammatory activity. Generally, little differences were observed between the levels of activity of the rhizomes before and after senescence, which is the plant part most used by traditional healers. This would suggest that the rhizomes could be harvested some time just before senescence or after the leaves have fully died back. Results from this study also support the use of the roots, which are sometimes used in traditional medicine. The results from the cytotoxicity assay indicate that the rhizomes are potentially harmful. This is an aspect which would require further investigation in terms of the medicinal use of this plant. These are important factors in the use of *S. aethiopicus* by traditional healers, and should be taken into consideration with regards to cultivation, commercialisation and product quality.

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