Biologically active substances from *Zanthoxylum capense* (thunb.) Harv.

A chemical investigation into the composition of Zanthoxylum capense *yielded several biologically active compounds, including pellitorine. A convenient HPLC method was developed to determine the presence of pellitorine in crude extracts from plants rapidly and qualitatively.*

In our continuing search[1] for biologically active substances of natural origin, the stems, twigs and leaves of *Zanthoxylum capense* (thunb.) Harv. were collected on three occasions in the Potchefstroom and Parys areas of South Africa. Four metabolites of interest, viz. pellitorine, xanthoxylum-γ,γ-dimethylallyl ether, β-sitosterol and sitosterol-β-D-glucoside, were isolated and characterised by physico-chemical techniques. The reported biological activity of *Z. capense* is evidently related to those of pellitorine and sitosterol-β-D-glucoside.

The tree, *Z. capense*, is indigenous to South Africa. Parts of the tree were used by early white pioneers and are still being widely used as a medicine by traditional healers.[2] The latter use the plant for a number of ailments such as colic, flatulent colic, gastric intestinal disorder and as a cure against intestinal parasites. It is also used to cure palsy and as a stomachic. It was reported to have been employed as a snakebite remedy, and is administered by rubbing the powdered root into snakebite wounds after the wounds have been lanced; also, the bark is swallowed repeatedly at fifteen-minute intervals until the snake venom-induced swelling subsides. The bark is also used to cure cattle of gall-sickness.

Zanthoxylum capense was a popular treatment in South Africa during the influenza epidemic of 1918, and was subsequently also used against colds and flu. The root acts against violent chronic coughing and the bark makes a tonic and a cure for sores and blood impurities. The Zulus use the powdered bark to relieve toothache, and against tuberculosis and paralysis. It was employed by whites as an epilepsy remedy and to 'disinfect' anthrax-infected meat, either by boiling the meat with some leaves, or by drinking a leaf infusion after eating the roasted meat.[2] The Mpondo people use the powdered root to cure pimples and 'blood poisoning' in general. The leaves serve as a purgative and parasiticide. The Thonga use the root against bronchitis and as a mouthwash for aphthae in children; a lotion made from the root is applied against acne.[2]

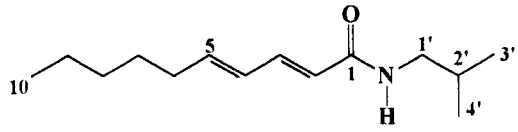
Juritz[3] reported in 1914 the only chemical investigation of *Z. capense* and related that 'A fairly large proportion of a resinous body was extracted, together with tannins and traces of a yellow colouring matter, for which no characteristic tests were ascertained'.

Our collection of plant material took place on three occasions, namely, 1) January 1995 (Parys district, farm Koedoesfontein); 2) July 1995 (Machavie district, Potchefstroom Agricultural College); 3) January 1996 (farm Koedoesfontein). During the exploratory collection in January 1995, only pellitorine was isolated. Large amounts of plant material were collected on the two subsequent occasions and were fractionated as described in the experimental section.

Only β-sitosterol (**2**) and sitosterol-β-D-glucoside (**3**) were isolated during the July collection (winter season), and pellitorine and xanthoxylol-γ,γ-dimethylallyl ether were extracted in addition to β-sitosterol in January (summer).

The most salient spectroscopic data, in particular IR, ^{1}H and ^{13}C NMR characteristics, of pellitorine (**1**) and xanthoxylol-γ,γ-dimethylallyl ether (**4**) are reported in the experimental section. The NMR assignments are based on the interpretation of DEPT, COSY and HETCOR experiments, which were conducted on all the substances.

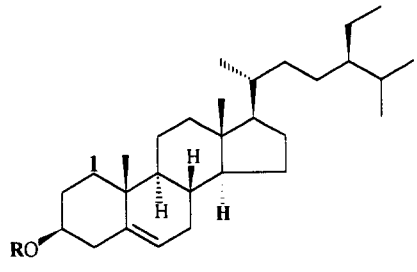
Pellitorine, isolated from *Z. capense*, occurs in several other *Zanthoxylum* species, e.g. *Z. macrophylla*,[4] *Z. zanthoxyloides*,[5] *Z. gillettii*,[6,7] *Z. acutifolium*,[8] *Z. petiolare*[9] and *Z. zanthoxyloides*.[10]



1: Pellitorine

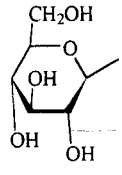
The bioactivity of pellitorine has been established; it was found to exhibit potent ovicidal action against the potato beetle, *Leptinotarsa decemlineata*, even at very low concentrations.[10] Kubo *et al.*[4] isolated several insect growth inhibitors from *Z. macrophylla*, such as pellitorine. Pellitorine has been incorporated into artificial diets, optimised against several economically important agricultural pests, e.g. pink bollworm (*Pectinophora gossypiella*), tobacco budworm (*Heliothis virescens*), corn earworm (*H. zea*) and fall armyworm (*Spodoptera frugiperda*). In these studies, the LD_{90} for pellitorine against *P. gossypiella* was determined as 25 ppm. Pellitorine was used in tests against the house mosquito (*Culex pipiens*) and the freshwater snail (*Biomphalaria glabratus*), both having medical significance. Pellitorine was the most potent compound tested against *C. pipiens*.

Sitosterol-β-D-glucoside (**3**) is a biologically active compound which exhibits visible antigastro-ulcerative properties against acetic acid-induced ulcers, as well as a visible effect against cold stress-induced ulcers.[11] Sitosterol-β-D-glucoside is the major constituent of a phytomedicine which is sold in Germany for the regeneration of the prostate gland, it has a stimulatory effect on the immune system, and a prophylactic effect on a variety of diseases of civilisation.[12]



2: Sitosterol; R = H

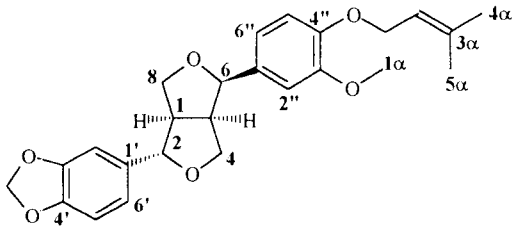
3: Sitosterol-β-D-glucoside; R =



Several lignans are known to be biologically active, however, the activity of xanthoxylol-γ,γ-dimethylallyl ether, isolated in this study, has not been investigated.

Some of the reported beneficial properties of *Z. capense*[2] may therefore be satisfactorily explained by the secondary metabolites (pellitorine and sitosterol-β-D-glucoside) characterised in this study.

A fast and convenient HPLC method was developed to



4: Xanthoxylol-γ,γ-dimethylallyl ether

determine rapidly and qualitatively the presence of pellitorine in crude extracts from plants. It is important to note that pure pellitorine, upon standing, forms a mixture of at least three amides, containing the fully conjugated *trans-trans*-diene (pellitorine) as the major component.[13] This mixture of isomers will be referred to as the 'pellitory mixture' in subsequent explanations. Maximum absorption of the pellitory mixture occurred at 254 nm. Using MeOH/H_2O (85:15) as eluent at a flow rate of 1 cm^3 min^{-1}, a good chromatographic separation of the three isomers, eluting at retention times of 2.4, 3.1 and 3.9 min, was obtained. A diode array detector was used to measure absorption at 189–440 nm.

This HPLC method permits a seasonal study of *Z. capense* to determine if the occurrence of pellitorine in the plant is influenced by seasonal changes, since no pellitorine was isolated from plant material collected during the winter months.

Experimental

Infrared spectra were recorded on a Nicolet 550 series II spectrometer using KBr pellets. UV spectra were obtained on a Shimadzu UV-240 spectrophotometer. Mass spectra were recorded on a VG Micromass 7070-E double focusing mass spectrometer. ^{13}C and ^{1}H nuclear magnetic resonance spectra were recorded on a Varian Gemini-3 spectrometer at 75 MHz and 300 MHz for ^{13}C and ^{1}H NMR nuclei, respectively.

HPLC separations were conducted using a Hewlett Packard series 1050 HPLC system equipped with a quaternary pump, diode array detector and auto sample injector. An HP ODS Hypersil column (100 mm × 4.6 mm) packed with material of 5 μm particle size was used.

Extraction of plant material collected during the winter season

Twigs, stems and leaves of *Z. capense* were collected in the Machavie district on the property of the Potchefstroom Agricultural College during July 1995. This material was dried at 50°C over a period of 24 h and subsequently milled to a coarse powder (7500 g). This powder was extracted by stirring at room temperature for 12 h with $CHCl_3$/MeOH (50 l, 1:1). The filtrate was concentrated under reduced pressure at 60°C to yield a crude extract (265 g) which was purified according to Fig. 1 to yield β-sitosterol (120 mg) and sitosterol-β-D-glucoside (180 mg).

The crude extract was partitioned between $CHCl_3$:water (10 l, 1:1) and the $CHCl_3$ layer was concentrated under reduced pressure at 60°C to yield a sticky mass of material (135 g), which was the subject of further investigation. The $CHCl_3$ extract (135 g) was chromatographed on a silica gel column (8 × 40 cm, 1000 g silica gel) using $CHCl_3$ as eluant. Appropriate fractions (20 cm^3) were collected, and those showing similar TLC analyses were combined and evaporated under reduced pressure at 60°C yielding six samples.

Fractions 2–4 were combined (55.6 g) and chromatographed on a silica gel column (8 × 50 cm, 1300 g silica gel) using acetone:*n*-hexane (1:10). Appropriate fractions (25 cm^3) were collected and those showing similar TLC analyses were combined and concentrated under reduced pressure at 60°C to yield six samples. The β-sitosterol (0.12 g, 0.0016%, based on dry material), crystallised from acetone to yield pure β-sitosterol mp 143°C.

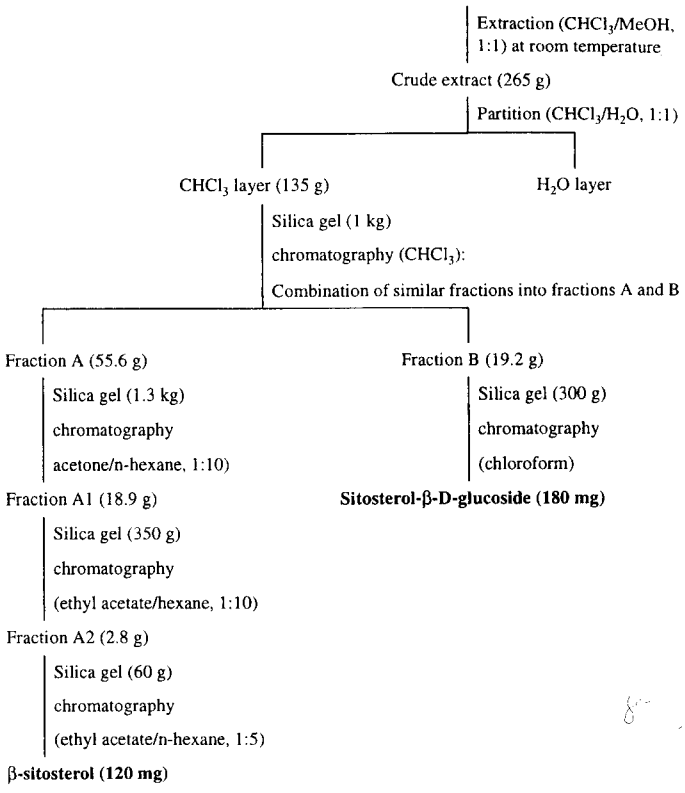


Fig. 1. Extraction of dried plant material (7.5 kg) in July 1995.

Fraction 6 (19.2 g) was purified by silica gel column chromatography to yield pure sitosterol-β-D-glucoside (0.18 g, 0.0024%, based on dry material), mp 296°C.

Extraction of plant material collected during the summer season

Fresh twigs and leaves of *Z. capense* were collected in the Parys district on the farm Koedoesfontein during January 1996. The material was dried at 50°C over a period of 24 h and subsequently milled to a coarse powder (7500 g). This powder was extracted at room temperature for 12 h with $CHCl_3$/MeOH (50 l, 1:1). The filtrate was concentrated under reduced pressure at 60°C to yield a crude extract (255 g), which was partitioned between $CHCl_3$ and water, and the organic layer between hexane and 90% MeOH. The lipid layer contained some β-sitosterol. The 90% MeOH layer was concentrated and its residue partitioned between water and $CHCl_3$. The latter layer (86 g) was purified by extensive chromatography on silica gel to yield xanthoxylol-γ,γ-dimethylallyl ether and pellitorine. The xanthoxylol-γ,γ-dimethylallyl ether crystallised from *n*-pentane (0.07 g, 0.0024%, based on dry material), mp 58°C, R_f 0.28 (ethyl acetate/*n*-hexane, 1:4), *m/z* 424, λ_{max} (MeOH)/233 nm (ε/dm^3 mol^{-1} cm^{-1} 18100), 280 (8925). IR: ν_{max}/cm^{-1} 3000–2800, 1600, 1510, 1450, 1300–1200, 1150, 1050, 1000, 800 and 700. $[\alpha]_D$ – 86.3°. The ^{13}C and ^{1}H NMR spectral data of xanthoxylol-γ,γ-dimethylallyl ether are summarised below.

δ_C 149.40 (S) C-4″, 148.02 (S) C-4′, 147.33 (S) C-3″, 147.26 (S) C-3′, 137.62 (S) C-3α, 135.22 (S) C-1′, 130.98 (D) C-1″, 120.03 (D) C-2α, 119.58 (D) C-6″, 117.62 (D) C-6′, 112.96 (D) C-5″, 109.12 (D) C-5′, 108.15 (D) C-2″, 106.54 (D) C-2′,

101.03 (T) -OCH_2O, 87.63 (D) C-2, 82.02 (D) C-6, 70.91 (T) C-4, 69.70 (T) C-8, 65.72 (T) C-1α, 55.84 (Q) -OCH_3, 54.52 (D) C-1, 50.03 (D) C-5, 25.70 (Q) C-5α, 18.07 (Q) C-4α.

δ_H 6.90 (m), 6.85 (m), 6.82 (m), 6.76 (m), 6.38 (m), 6.74 (m), 5.92 (s, 2H), 5.50 (t, 1H, J 6.7 Hz), 4.82 (d, 1H, J 5.6 Hz), 4.54 (d, 2H, J 6.7 Hz), 4.38 (d, 1H, J 7.1 Hz), 4.07 (d, 1H, J 9.3 Hz), 3.86 (s, 3H), 3.81 (m, 2H, J 9.0 Hz), 3.29 (m, 2H, J 9.0 Hz), 2.85 (q, 1H, J 7.3 Hz), 1.75 (s, 3H), 1.70 (s, 3H).

Pure pellitorine (2.2 g, 0.029%, based on dry material) was obtained, mp 71°C (ethyl acetate), R_f 0.28 (ethyl acetate/*n*-hexane, 1:4), *m/z* 223, λ_{max} (MeOH)/256 nm (ε/dm^3 mol^{-1} cm^{-1} 1390), IR ν_{max} (KBr)/cm^{-1} 3300, 3100, 3000–2800, 1650, 1610, 1550, 1450, 1300, 1250, 1150 and 1000. The ^{13}C and ^1H NMR spectral data for pellitorine are summarised below.

δ_C 166.60 (S) C-1, 143.25 (D) C-3, 141.33 (D) C-5, 128.27 (D) C-4, 121.82 (D) C-2, 46.85 (T) C-1′, 32.97 (T) C-6, 31.24 (T) C-8, 28.50 (D) C-2′, 28.35 (T) C-7, 22.23 (T) C-9, 19.99 (Q) C-3′ and C-4′, 13.85 (Q) C-10.

δ_H 7.15 (m, J 9.7, 9.8 and 13.8 Hz), 6.01 (m, J 9.8 and 13.9 Hz), 5.74 (d, J 11.1 Hz), 5.35 (m), 3.10 (t, J 6.5 Hz), 2.08 (m), 1.74 (m), 1.34 (m), 1.24 (m), 1.21 (m), 0.85 (d, J 6.7 Hz), 0.81 (t, J 7.0 Hz).

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Nutrient limitation affects growth and attachment of two food spoilage bacteria, *Bacillus subtilis* and *Pseudomonas fluorescens*

The attachment was studied of the food spoilage bacteria Pseudomonas (P.) fluorescens *and* Bacillus (B.) subtilis *to stainless steel test surfaces suspended in Minimal Salts Medium (MSM) or Standard One Nutrient Broth (SONB). Attached cells were enumerated on Standard One Nutrient Agar after being dislodged from test surfaces by being shaken with beads. Counts of planktonic cells grown in SONB and MSM were evaluated in parallel with counts of attached cells. Higher counts of attached* P. fluorescens *and* B. subtilis *cells (c. 1 log cfu cm^{-2}) were recovered from surfaces suspended in SONB compared to those in MSM. Counts of planktonic* B. subtilis *cells grown in SONB exceeded those in MSM by c. 1 log cfu ml^{-1}. By contrast, counts of planktonic* P. fluorescens *in SONB and MSM were not significantly different (P > 0.05). Scanning electron microscopy showed attached cells of* B. subtilis *grown in SONB to be rod-shaped, while coccoid shapes were observed for corresponding cells grown in MSM. Attached cells of* P. fluorescens *were rod-shaped in both SONB and MSM. Evidence of extracellular polymeric substance formation was observed for attached cells of both bacteria in SONB and MSM. These findings have implications for the contamination of foodstuffs by these bacteria.*

Biofilms of a wide variety of bacteria can develop *in situ* on food processing equipment.[1–3] The initial stages of adhesion of bacterial cells to surfaces are thought to be, in part, influenced by the nutrient status of the surrounding liquid medium.[4] The types and availability of nutrients on moist food contact surfaces *in situ* are variable.[5] Often, nutrient-rich conditions are encountered in food processing plants during the production cycle, due to residues of proteins and fats being deposited on equipment surfaces.[6] However, low nutrient conditions can also be encountered when cleaning programmes aimed at removal of food residues from food contact surfaces have been completed after production.[5]

Different opinions prevail on bacterial adhesion under nutrient-rich and nutrition-limited conditions. It has been postulated that bacterial cells attach in high numbers to surfaces as a survival strategy when nutrients in the surrounding medium are limited. This is believed to be the result of a higher concentration of nutrients at a surface compared to the corresponding bulk fluid or surrounding liquid medium.[4] Nutrient limitation in the environment adjacent to solid surfaces has also been shown to condition bacteria for adherance,[4,7,8] because they can benefit from an enhanced nutrient status.[7] Although nutrient-limitation tends to favour bacterial attachment to surfaces, certain low-nutrient conditions have been shown to induce detachment.[9] Conversely, other workers have proposed that bacterial adhesion to surfaces also occurs when nutrient concentrations are high in the surrounding fluid medium.[10]

The objective of this *in vitro* study was to simulate conditions for bacterial growth which might occur in food processing environments and hence determine the effect of nutrient limitation on the growth and attachment of *Pseudomonas (P.) fluorescens* and *Bacillus (B.) subtilis* as examples of typical food spoilage bacteria.[11,12]

Materials and methods

A rope-inducing *Bacillus (B.) subtilis* strain[11] and a *Pseudomonas (P.) fluorescens* strain, isolated from poultry,[12]