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Isolation and structural elucidation of compounds from the non-alkaloidal extract of *Nicandra physaloides* and the antimicrobial activity of withanicandrin.

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The aerial parts of *Nicandra physaloides* plant collected from Kenyatta National Hospital grounds were dried and subjected to acid-base extraction and partitioned to obtain alkaloidal and non-alkaloidal extracts. The non-alkaloidal extract yielded three compounds; withanicandrin, β-sitosterol and stigmasterol after column chromatography. Withanicandrin exhibited antifungal activity against *Saccharomyces cerevisiae* and *Candida albicans* but lacked antibacterial activity.

Key words: *Nicandra physaloides*, withanicandrin, β-sitosterol, stigmasterol, antifungal activity.

INTRODUCTION

Nicandra physaloides is a monotypic genus of flowering plants in the Solanaceae family. The plant is commonly known as Apple of Peru or shoo-fly and is commonly used for ornamental purposes. Native to Peru, it is recognized elsewhere as an exotic species. It is a coarse, erect annual plant reaching 3-8 feet in height and about half as much in width although the size of the plant varies depending on soil fertility and moisture availability [1]. The preference is full or partial sun, moist conditions and a loamy fertile well drained soil. Most vegetative growth occurs during the late spring and summer. In Kenya, the plant tends to grow well during the rainy seasons. It has large alternate leaves, ovate-cordate in shape, measuring up to 10 cm in length that resemble *Datura* leaves. All parts of the plant are mildly poisonous due to the presence of alkaloids [2].

The plant is used for the treatment of diuresis, mydriasis, analgesia, antibacterial and inflammation in Tibetan medicine [3]. Several compounds, nicaphysalins, have been isolated from the plant [4]. It has also been confirmed to have insecticidal properties [5]. This study sought to isolate compounds from the non-alkaloidal extract of aerial parts of the plant and to test some of the isolated compounds for antimicrobial activity.

MATERIALS AND METHODS

Plant material

Aerial parts *of Nicandra physaloides* plant were collected from Kenyatta National Hospital grounds in Nairobi County, Kenya in August 2009. The plant was identified and authenticated by a taxonomist before collection. A voucher specimen (SOP/NAM/2009/01) was deposited at the School of Pharmacy, University of Nairobi. The material was air-dried and ground before use.

Solvents, materials and reagents

General purpose reagent grade ethyl acetate, chloroform and methanol (Kobian Kenya Ltd, Nairobi, Kenya) were distilled and used for extraction, fractionation an isolation of compounds. Analytical grade methanol and acetone (Sigma-Aldrich GmbH, Seelze, Germany) were used for recrystallization of compounds. Sulphuric acid (Loba Chemie PVT Ltd, Mumbai, India), and ammonia solution (Pharmacos Ltd, Essex, England) were used in extraction of alkaloids. Dimethyl sulfoxide (Fischer Scientific, Loughborough, United Kingdom) was used in preparation of suspensions for antimicrobial activity. Filtration to remove particulate matter from crude extracts and solutions of isolated compounds were done

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using Whatmann filter paper No.1 (Whatmann International Ltd, Maidstone, England). Column chromatography on normal phase silica gel of porosity 60 Å and particle size 63-200 µm (Sigma–Aldrich GmbH, Seelze, Germany) was used for fractionation of the crude extracts. Thin Layer Chromatography (TLC) was carried out on pre-coated aluminium plates with a 0.2 mm thick layer of normal phase silica gel 60 GF_{254} (Sigma-Aldrich GmbH & Co., Seelze, Germany). Vanillin (1% w/v), prepared using vanillin powder (BDH Chemicals Ltd, Poole, England) in concentrated sulphuric acid and iodine resublimed general reagent (Merck, Darmstadt, Germany) were used as visualizing agents on TLC.

Equipment

A Heidolph VV2000® rotary vacuum evaporator (Heidolph Electro GmbH & Co. KG, Kelheim, Germany) connected to a Laborota4000 cooler (Polyscience, Niles, USA), a WB2000® water bath (Heidolph Electro GmbH & Co. KG, Kelheim, Germany) and a diaphragm vacuum pump (KNF Neuberger GmbH, Freiburg, Germany) was used to reduce to dryness extracted material for column chromatography and antimicrobial activity testing. The extracts were fractionated using a glass column whose dimensions were 2 cm internal diameter and 50 cm long equipped with a glass wool filter. The chromatographic fractions were collected using a SuperFrac™ automatic fraction collector (Pharmacia LKB Biotechnology, Uppsala, Sweden). Thin layer chromatograms were visualized using Mini UV/Vis® ultraviolet light lamp (Desaga GmbH, Heidelberg, Germany). Extracts and isolated compounds for recrystallization were refrigerated at 4-6 °C. The nuclear magnetic resonance spectroscopic data was obtained using a Varian-Mercury 200 MHz spectrometer (Varian Inc. Palo Alto, Carlifornia, USA) with a magnet from Oxford Instruments (Oxford, UK). The data was acquired using an on-line computer (Sun Micro-Systems, California, USA) and analysed using Varian software. Mass spectrometric data was obtained using an Agilent 6230 Accurate-Mass Time-of-Flight (TOF) LC/MS (Agilent Technologies, Carlifonia, USA) operating at 175eV. A Fourier transform infrared spectrophotometer (IRPrestige-21, Shimadzu Corporation, Kyoto, Japan) was used for infrared spectroscopy. A hydraulic press machine (Perkin-Elmer GmbH, Germany) was used to prepare the sample IR discs and IR solution software was used in analysis and recording.

Extraction

The extraction was carried out by wetting 500 g of dried and ground aerial parts of *N. physaloides* with 450 ml ammonium hydroxide (NH_4OH) (50 % v/v) followed by maceration using 2 litres of ethyl acetate overnight at room temperature with continuous stirring. The material was washed twice with 2 litres of ethyl acetate. The filtered extract was partitioned between equal volumes of 2 % aqueous sulphuric acid and ethyl acetate in a separating funnel. The organic layer was reduced to dryness using a rotary vacuum and subjected to column chromatography.

Fractionation and Isolation

For this purpose, 10 g of the non-alkaloidal extract was loaded onto a chromatographic column packed with 120 g of silica gel. The column was eluted with a chloroform-methanol mixture by gradient elution, starting with 100 % chloroform and reducing polarity by 10 % to 50 % chloroform in methanol. The eluents were collected in 5 ml fractions using a fraction collector. Analysis of the fractions was done by use of thin-layer chromatography. Fractions showing similar profiles were pooled to yield seven fractions coded F1 to F7. All the fractions were evaporated *in vacuo* to a volume of about 10 ml and dried in open air on the laboratory bench. Fraction F2 formed a precipitate at ambient temperature after four days which was washed with methanol and dissolved in chloroform. The chloroform solution was left overnight to crystallise. Recystallization from methanol-chloroform (10:90) under slow evaporation at ambient temperature produced colourless crystals. Fraction F3 formed clear crystals on the test tube wall after three days at ambient temperature. The crystals were scraped off, redissolved in ethyl acetate and allowed to

recystallize. Further recrystallization was done using acetone under slow evaporation producing clear crystals.

Antimicrobial activity testing

Antibacterial testing was carried out using *Staphylococcus aureus* (NC 07447*), Escherichia coli* (ATCC 25922) and *Bacillus pumillus* (NC 08241). The fungal micro-organisms tested were *Candida albicans* (NCPF 3179) and *Saccharomyces cerevisiae* (ATCC 9763).
The plate agar diffusion method described by Kavanagh was adopted [6]. Nutrient agar was prepared according to the manufacturer's instructions. Gentamicin and nystatin were employed as standards. The standards and test compound were each diluted to concentrations of 30 μg/ml in DMSO.

RESULTS AND DISCUSSION

Structure elucidation

Using spectroscopic methods, three compounds were identified; two compounds (β-sitosterol and stigmasterol) were isolated from fraction F2 whereas one compound was isolated from fraction F3 and identified as withanicandrin. The structures of withanicandrin, β-sitosterol and stigmasterol are shown in Figure 1. The data obtained was in agreement with those reported in literature [4, 7].

Withanicandrin: IR ν_{max} KBr (cm^{-1}): 3477 (H-bonded, OH str), 1700 (α,β-unsaturated δ-lactone), 1650 (C=O str); MS (m/z): 507.235 $[M+K]^+$, 975 $[2M+K]^+$, 976 $[2M+H+K]^+$, 977 $[2M+2H+K]^+$; ^{1}H-NMR ($CDCl_3$, 200 MHz) δ: 0.98 (3H, d, *J* = 7.0 Hz, H-21), 1.12 (3H, s, H-18), 1.25 (3H, s, H-19) 1.88 (1H, m, H-14), 3.55 (1H, dd, *J* = 16.6 Hz, 4.0 Hz, H-7), 1.57 (2H, m, H-15), 1.89 (3H, s, H-27), 1.49 (2H, m, H-16), 1.95 (3H, s, H-28), 2.00 (1H, m, H-9), 2.22 (1H, m, H-8), 1.68 (1H, m, H-17), 2.48 (2H, m, H-23), 2.17 (1H, m, H-20), 2.68 (2H, m, H-11), 2.41 (2H, m, H-4), 3.09 (1H, d, *J* =4.0 Hz, H-6), 4.40 (1H, m, H-22), 6.59 (1H, d, *J* = 8.8Hz, H-2), 5.83 (1H, m, H-3); ^{13}C-NMR ($CDCl_3$, 50 MHz) δ: 201.7 (C-1), 129.0 (C-2), 140.2 (C-3), 36.8 (C-4), 73.4 (C-5), 56.4 (C-6), 57.1 (C-7), 35.7 (C-8), 37.8 (C-9), 51.6 (C-10), 38.5 (C-11), 212.5 (C-12), 57.8 (C-13), 53.3 (C-14), 23.8 (C-15), 27.3 (C-16), 42.8 (C-17), 11.6 (C-18), 14.9 (C-19), 39.9 (C-20), 13.7 (C-21), 76.6 (C-22), 30.1 (C-23), 149.8 (C-24), 122.0 (C-25), 167.3 (C-26), 12.7 (C-27) and 20.8 (C-28).

The ^{1}H-NMR spectrum of withanicandrin was characteristic of the steroidal structure for the withanolide class of compounds [8]. It was similar to those observed for other steroidal lactones having a 17α-oriented side chain [9]. There was a distinct doublet at 6.59 (H-2) due to the coupling by the single proton at H-3. Another doublet observed at 3.07 is due to the proton at H-6 which is coupled to a single proton at C-7. The H-7 proton is coupled to 2 protons (H-6 and H-8) producing a doublet of doublets at 3.55. There was only one methyl proton at 0.98 (H-21) that is coupled to H-20 producing a doublet. The other 4 methyl protons (H-18, H-19, H-27 and H-28) were attached to quaternary carbons thus appearing as singlets.

The ^{13}C-NMR values of isolated withanicandrin were in good agreement with reported values [4]. Further confirmation using the distortionless enhancement by polarization transfer (DEPT) spectrum indicated that there were signals for five methyl, five methylene and ten methine carbon atoms, hence eight quaternary carbons. This confirmed that there were 28 carbons in the molecule.

The infrared (IR) spectrum of withanicandrin indicates the presence of hydroxyl groups (3477 cm^1) [8]. There was a broad band at 1700 which indicates the presence of an α, β-unsaturated δ-lactone, an ester and an α,β-unsaturated ketone. A peak at 1650 cm^{-1} indicates the presence of C-C double bonds [10].

Further support for the structure of the compound was obtained from mass spectroscopy based on ESI TIC scan. A pseudomolecular ion peak at *m/z* 507.235 $[M+K]^+$ and a dimer at 975 $[2M+K]^+$ were indicative of withanicandrin. This indicates that the molecular ion has mass value of 468. Together with the NMR data, the molecular formula was proposed to be $C_{28}H_{36}O_6$.

β-sitosterol; IR ν_{max} KBr (cm^{-1}): 3469 (H-bonded, OH str), 2940 (CH_3 C-H str), 2869 (CH_2 C-H str), 1367 (=CH C-H str); MS (m/z): 414: ^{13}C-NMR ($CDCl_3$, 50 MHz) δ: 37.6 (C-1), 31.8 (C-2), 71.0 (C-3), 42.4 (C-4), 141.7 (C-5), 120.9 (C-6), 31.9 (C-7), 32.0 (C-8), 50.5 (C-9), 36.6 (C-10), 21.1 (C-11), 39.9 (C-12), 42.3 (C-13), 56.9 (C-14), 24.3 (C-15), 28.4 (C-16), 56.3 (C-17), 11.7 (C-18), 19.8 (C-19), 36.2 (C-20), 18.7 (C-21), 34.0 (C-22), 26.1 (C-23), 46.0 (C-24), 29.2 (C-25), 19.1 (C-26), 19.4 (C-27), 23.1 (C-28), 11.6 (C-29).

Stigmasterol: IR ν_{max} KBr (cm^{-1}): 3456 (H-bonded, OH str), 2943 (CH_3 C-H str), 2871 (CH_2 C-H str), 1358 (=CH C-H str); MS (m/z): 412; ^{13}C-NMR ($CDCl_3$, 50 MHz) δ: 37.6 (C-1), 31.8 (C-2), 71.0 (C-3), 42.3 (C-4), 141.7 (C-5), 120.9 (C-6), 31.9 (C-7), 32.0 (C-8), 50.5 (C-9), 36.6 (C-10), 21.1 (C-11), 39.9 (C-12), 42.3 (C-13), 56.9 (C-14), 24.3 (C-15), 28.4 (C-16), 56.1 (C-17), 11.9 (C-18), 19.8 (C-19), 40.7 (C-20), 21.2 (C-21), 138.7 (C-22), 129.4 (C-23), 51.5 (C-24), 31.9 (C-25), 19.1 (C-26), 21.0 (C-27), 25.4 (C-28), 11.6 (C-29).

The ^{13}C-NMR data were generally in agreement with those of β-sitosterol and stigmasterol. The ^{13}C-NMR chemical shifts at 71, 120, 129, 138 and 141 were characteristic of stigmasterol whereas 71, 120 and 141 were characteristic of β-sitosterol with only one double bond. From the LC/MS data, which indicates a mixture of compounds, the m/z values of 414 and 412 further confirm the presence of β-sitosterol and stigmasterol, respectively.

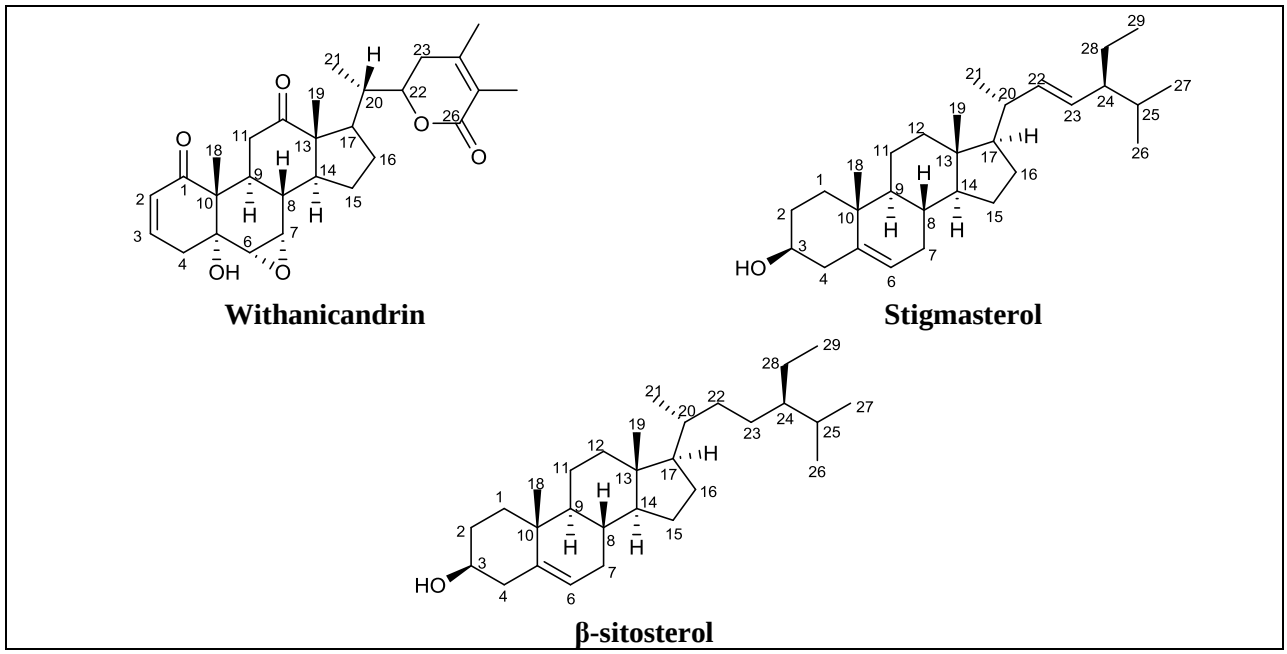

Figure 1: Chemical structures of withanicandrin, stigmasterol and β-sitosterol

Withanicandrin has previously been reported from *N. physaloides* [4]. However, to the best of our knowledge, this is the first report of the isolation of β-sitosterol and stigmasterol from this plant.

Antimicrobial activity of withanicandrin

The antimicrobial activity was expressed as activity index calculated as the ratio of the zone of inhibition of each test solution to that of the standard. Activity indexes less than 1 indicate that the compound is more active than the standard. Although withanicandrin exhibited no appreciable antibacterial activity against all the three bacterial species screened, it was more active than nystatin against *Saccharomyces cerevisiae* and *Candida albicans* with activity indexes of 0.56 and 0.72, respectively. This finding indicated that withanicandrin possesses

greater antifungal activity against *C. albicans* compared to *S. cerevisiae* and may lend scientific credence to the traditional use of *Nicandra physaloides* in wound management [2].

CONCLUSION

Three compounds namely stigmasterol, β-sitosterol and withanicandrin, were isolated from the aerial parts of *Nicandra physaloides*. This is the first report of the isolation of stigmasterol and β-sitosterol from this plant. Antimicrobial testing showed that one of the compounds isolated, withanicandrin, was active against the two fungal species tested but lacked appreciable activity against the three bacterial species screened. This finding may justify the traditional use of the plant to treat wounds.

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