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Isolation and pesticide activity of daphnanes of *Lasiosiphon kraussianus*

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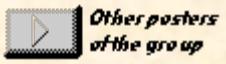

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Introduction:

The significance of rodent damage to health, agricultural production or structures has come to be widely recognised as economically very important (1). Plants offer an excellent source of biologically active natural products. The most important botanical rodenticides are scilliroside (2), reserpine (3) and strychnine (4). The natural products used as rodenticides per se are all acute poisons until the discovery of the anticoagulant rodenticides in the early 1950s (4). The spread of rat resistance to anticoagulant rodenticides limited the effectiveness of rat control (5). Therefore, and beside resistance management (6), the development of new natural products, to be directly used or as lead for synthesis, will be the most practicable method to solve most rat problems.

The family Thymelaeaceae is distributed throughout the tropical and temperate parts of the world. The toxicity of plants in the Thymelaeaceae, and the compounds isolated from plants in this family were summarized (7). On the other hand, some pharmacological investigations have been carried out in this plant roots (8-10). A detailed description of Lasiosiphon kraussianus (Mesin) had been reported (11). The leaves of this plant were reported to be poisonous when fed to livestock (12). Literature survey showed no reference to natural products that have been isolated from the leaves of this plant and thus far, no pesticidal investigation has been reported. Depending on that, the present work deals with the isolation and structure elucidation of a diterpenoid having daphnane skeleton, Kraussianin, exhibiting potent acute activity against mice Mus musculus. It is worth noting that, Kraussianin is also known as a potent antileukemic compound (13).

Materials and methods

- Preparation of the plant leaves

The local name of L. kraussianus in Darfour (west of Sudan) is "Komma" or "Mahjiria". The leaves of this species were collected in November 1995 at Jebel Marra (Wadi Mertegello, 1160 meter above sea level). This plant was identified by K. Uhlig and A.A. Adam, Jebel Forest Circle, Golol, GTZ Project, Sudan. A voucher specimen is available in their herbarium. The leaves were shade-dried for two weeks and then miled and used in a powder form.

- Extraction and fractionation
    - Extraction

Powdered leaves (300 g) were macerated with methanol (2 L) for one week under room temperature. After filtration under suction through hyflosupercel (Prolabo, France), the dried debris was percolated with another two litres of methanol. The filtrate and percolate were combined and evaporated upto dryness using a rotary evaporator (water-bath temperature, 40° C). The extract was finally dissolved in 30 ml of methanol (50%) and subjected to flash chromatography.

    - Fractionation

The flash chromatography was carried out in microboron glass filter (5 cm i.d. and, 5 cm high) with silinised silica gel. The elution started with methanol 50% and, then methanol and dichloromethane (75%., 25%; v/v). The last fraction (where the activity was found) was evaporated upto dryness and dissolved in dichloromethane (30 ml).
This fraction was loaded on to silica gel (kieselgel) column (90 cm high and 2.5 cm i.d.), packed with 80 grams of silica gel (particle size 0.063-0.125 mm; 120-230 mesh ASTM), slurry packed in dichloromethane. For elution, the fraction was subjected to gradient chromatography started with dichloromethane (100%) and ended with ethyl acetate and methanol (60%/ 40%, v/v) in an increasing polarity, at a constant flow rate of 3 ml/min, using HPLC (Waters 600, multisolvent delivery system), a programmable photodiode array detector (Model 994) and equiped with a fraction collector (Seive type 192-N). One hundred and five tubes (18X180 mm) of 17 ml each were collected. All tubes were subjected to a TLC single-spot analysis (Polygram sil G/UV 254, layer thickness 0.25 mm (Macherey-Nagel) and developed first with dichloromethane (15 ml) and hexan (35 ml) and ended with dichloromethane (25 ml), ethyl acetate (25 ml) and methanol (5 ml) in increasing polarity, Followed by visulization under UV light at 254 nm and, then collected in 22 fractions.
The active fraction was then chromatographed over sephadex LH-20. The conditions were: column: 90 cm x 2.5 cm; sephadex LH-20 (Pharmacia) particle size 25-100 µm, the pump used was Gilson 301; 2138 UVICORD S detector (LKB), wavelength 254 nm; 2-channel recordor LKB; Fraction collector used was Foxy, isco. Eluted with methanol and dichloromethane (80% / 20%, v/v) with a flow rate of 2 ml/min. Sixty-one tubes (13 x 100 mm) of 7 ml were collected. After TLC analysis all tubes were combined in nine subfractions. The active subfraction was then rechromatographed on a short-column of silica gel (30 cm high and 1 cm i.d.). Elution started with hexane and ethyl acetate (95% / 5%, v/v), the percentage of the ethyl acetate was then increased to 10% and 15% and then ended with Hexan, ethyl acetate and methanol (70% / 20% / 10%, v/v). The collection was programmed to be 10 min/tube, 0.5 mm/min and, hence 5 mm/tube. Sixty tubes were collected. After TLC analysis (developed with hexane and ethyl acetate, 70% /30%, v/v), all tubes were combined in eight sub-subfractions. The active sub-subfraction was further separated by a preparative TLC, which developed with dichloromethane, ethyl acetate and methanol (85% /13% / 2%, v/v). The 14 bands which were detected under UV-light, were marked. Each band was taken in a small conical flask and dissolved with ethyl acetate, evaporated up to dryness, and redissolved in methanol. Band 4 was subjected to preparative reversed phase HPLC (Waters 600, multisolvent delivery system). The conditions were: column: 220 mm x 4.5 mm, packing material: C18, 5 µm, pressure: 2150 PSI, mobile phase: methanol and water, solvent gradient, flow rate: 1.5 ml/min). Each peak was collected manually and, the purified active peak was then subjected to identification and bioassay.

- Spectroscopic analysis
  - Mass spectrometry (MS)

The MS and MS-MS spectra were obtained with a Nermag R 30-10 (Quad Service, Poissy, France) triple quadrupole instrument. Source conditions were set as follows: temperature, 130 °C; filament current, 50 µA; electron energy, 95 eV; reagent gas (NH3 or ND3) pressure was set at 10-4 Torr in the source housing. For MS-MS experiments, the collisional activated dissociation (CAD) spectra were obtained at 20 eV collision energy and with argon (7 x 10-2 Torr) as collision gas in the second quadrupole. Sample introduction was done by desorption chemical ionization (DCI) in both positive (PICI) and negative (NICI) modes.

  - 1H NMR

The active compound was subjected also to 1H NMR analysis (Varian Gemini-300, 300 Mz) in solution in CDCl3, using CHCl3 as internal reference (7.27 ppm) and supported by the 13C NMR (75.5 MHz) recorded using CDCl3 (77.14 ppm) as reference. Confirmation of the attribution of the 13C resonances was achieved through analysis of a 2D 1H- 13C correlation optimized for a C-H coupling constant of 140 Hz.

- Testing procedure

Swiss white mice Mus musculus, 45 days-old, were purchased and acclimatised to laboratory conditions before testing. Standard laboratory diet and water were available. Ten mice (five females and five males) were taken for each concentration. Distilled water was used for the dilution of the stock solution. The concentrations used were 0.25, 0.5, 1 and 2 mg/kg. Methanol in water (20% / 80%, v/v), was used as control. A volume of 500 *l of each concentration was administered orally for each mouse. The sex, weight, dosing schedule and mortality are given in Table1. The LD50 was calculated after 24 hr and the live mice were observed for one week for the acute signs of toxicity and mortality. Percentage kill were corrected for control mortality by Abbott's formula and, the LD50 values were calculated using the probit analysis (14), with a special microcomputer programme (15).

Results :

- Identification of the active constituent

The chemical structure was determined by NH3 -DCI-MS (and MS-MS) and NMR spectroscopy. The molecular weight of this compound was established by using both positive and negative NH3-DCI conditions. PICI (NH3 as reagent gas) gave ions at m/z 672, 655 and 637 which might be interpreted as [M + NH4]+, MH+ and [MH - H2O]+, whereas NICI (using NH3) yielded an ion at m/z 654 (M-°). These data were consistent with MW 654.
Some of the functionalities were determined as follows :
- ND3-PICI showed 4 exchangeable hydrogens (possibly alcohol groups)
-The CAD spectrum of the MH+ ion obtained under PICI gave several daughter-ions especially in the high mass region indicating 4 consecutive losses of H2O eventually combined with one elimination of CO. The pattern in the intermediate region was quite different from that of daphnanes excoecaria toxin and wikstrotoxin D (isolated from the roots of this plant, data submitted for publication) spectra.
-The CAD of the M-° ion was mostly characterized by an abundant ion at m/z 532 corresponding to the elimination of a benzoic acid neutral (122 u).
These data associated with those from NMR were in agreement with the structure of kraussianin (Fig 1) already reported (13).

- Activity against white mice

The LD50 within 24 hours of oral administration, was found to be 1.39 mg/kg (Table 1). The symptoms of kraussianin toxicity were found to be : hyperthermy, hyperventilation, collapse, atony leading to death.

Discussion

This work was started depending on the traditionally known toxicity of this plant (12). Literature survey revealed that no chemical investigations are reported in the leaves of L. kraussianus. Kraussianin was reported for the first time in 1984 in the roots of this plant as a potent antileukemic product (13). Thus far, it is the first time to report kraussianin in the leaves of this plant and, its potent fast-activity to mice.
From activity point of view, it can be said that this natural product possesses an acute toxicity comparable to that of the already commercially used rodenticides of plant origin. Reserpine is an alkaloid extracted from the roots of various species of Rauwolfia notably R. serpentina Benth and R.. vomitoria Afz. It is much more effective in controlling mice than rats, with an acute oral LD50's of about 200 and 420 mg/kg to mice and rats, respectively (3). Strychnine is an alkaloid which occurs in the seeds of the species of genus Strychnos, particularly S. nux-vomica and S. wallichiana. The LD50 values of 4.8 and 6-8 mg/kg, for R. norvegicus, have been reported (4). The LD50's of scilliroside (isolated from Scilla maritima L) through ingestion to rats, from 0.43 (female) to 2.15 (male) mg/kg and to mice from 0.43 (female) to 0.35 (male) mg/kg (5).
It can be concluded that, this product may be used directly and/or as lead for synthesis. In addition, it is worth noting that, the symptoms of the nervous system toxicity (e.g. convulsions) that prohibited the use of the previously mentioned three rodenticides was not demonstrated by this product. These symptoms may indicate a new mode of action and, hence, can be used in the development of a new class of rodenticides. On the other hand, field and laboratory investigations were organised to be carried out, aiming at the possible use of this crude-extract directly and against wild rodents.

References : follow this link
