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The Antibiotic Principle of Seeds of Moringa oleifera and Moringa stenopetala[1]

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Key Word Index: Moringa oleifera; Moringa stenopetala; Moringaceae; Antimicrobial Activity; Mustard Oil; 4 (α-L-rhamnosyloxy)benzyl isothiocyanate.

Abstract

4(α-L-Rhamnosyloxy)benzyl isothiocyanate was identified as an active antimicrobial agent form seeds of *Moringa oleifera* and *M. stenopetala*. Roots of *M. oleifera* only contain this compound and benzyl isothiocyanate, but not pterygospermin as previously suggested. Defatted and shell free seeds of both species contain about 8–10 % of 4(α-L-rhamnosyloxy)benzyl isothiocyanate, but this amount is produced from *M. oleifera* only when asorbic acid is added during water extraction. The compound acts on several bacteria and fungi. The minimal bactericidal concentration in vitro is 40 µmol/l for *Mycobacterium phlei* and 56 µmol/l for *Bacillus subtilis*.

Introduction

Moringa oleifera Lam. and *M. stenopetala* (Bak. f.) Cufod. are native to tropical Africa and the former is also found in India, Ceylon and Madagascar. Leaves, roots and seeds are often used in folk medicine [1]. In rural areas of the Sudan in particular, the powdered seeds of *M. oleifera* are traditionally utilized for water purification because of their strong coagulating properties for sedimentation of suspended mud and turbidity [1, 2]. During some coagulation experiments a slight decrease of total bacterial count of the purified water was observed [2] indicating that the seeds might contain substances with antimicrobial activity. Indeed, from roots of *M. oleifera* (syn. *M. pterygosperma* Gaertn.) an antibiotic substance has been isolated and a surprising structure (1) was proposed [3, 4]. The present paper describes purification, elucidation and antimicrobial properties of the antibiotic principle of seeds of *M. oleifera* and *M. stenopetala* and reinvesti-

[1] Part of the projected dissertation of U. Eilert, also presented as a poster during the Intern. Res. Congress on Natural Products as Medicinal Agents, Strasbourg, July 1980 [Planta Medica, *39*, 235 (1980)]

gates the antibiotic substance of roots of *M. oleifera* using its antimicrobial activity for detection [5, 6]. This substance is an isothiocyanate, already known from these plants, but which has never been tested for its antimicrobial activity.

Results and Discussion

Preliminary investigations of aqueous 1:10 extracts of the seeds from *M. oleifera* and *M. stenopetala* indicated distinct antimicrobial effects which are listed in

Table I

Antibiotic activity of an aqueous extract (1:10) from seeds of *Moringa oleifera* tested in the petri plate diffusion assay using the streak-plate method, the cup-test, and the disk-test

(+) = inhibition zone 1– 2 mm
\+ = inhibition zone 2– 5 mm
++ = inhibition zone 5–10 mm
+++ = inhibition zone 10–15 mm

s = saprophytic
h = humanpathogen
p = phytopathogen
s_1 = wood decaying fungus

Organism	Host Substrate	Inhibition
Bacteria		
Bacillus cereus var. *mycoides*	S	+++
Bacillus megaterium	S	+++
Bacillus subtilis 1527	S	+++
Bacillus subtilis Nä	S	+++
Escherichia coli (gram neg.)	S, H	+
Klebsiella aerogenes (gram neg.)	S, (H)	–
Micrococcus aureus (Staphylococcus)	H	+++
Micrococcus luteus (Sarcina)	S	+++
Proteus mirabilis (gram neg.)	S, H	++
Salmonella edinburg (gram neg.)	H	+
Serratia marcescens 1534 (gram neg.)	S, (H)	(+)
Serratia marcescens Nä (gram neg.)	S, (H)	(+)
Streptococcus faecalis	S, H	++
Mycobacterium phlei Nä	P	+++
Mycobacterium phlei Wo	P	+++
Fungi		
Aspergillus oryzae	S	–
Botrytis allii	P	+++
Candida pseudotropicalis	S, H	++
Candida reukaufii	S	+++
Coniophora cerebella	S_1	+++
Fusarium oxysporum, f. *lycopersici*	P, (H)	+
Penicillium expansum	S	+++
Phytophthora cactorum	P	+++
Piricularia oryzae	P	+++
Polystictus versicolor (Polyporus)	S_1	+++
Saccharomyces carlsbergensis	S	(+)
Zygorrhynchus sp.	S	(+)

table I. The active component was not extractable with MeOH or EtOH and seeds boiled for a short time in water showed greatly decreased activity. Water extracts from germinated seeds exhibited a minor activity, whereas extracts from seed shells were completely inactive.

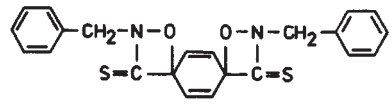
The active principle was extracted from defatted seeds by incubation of the seed powder in water and purified by extraction of the water phase with EtOAc, column chromatography on silica gel and HPLC on RP-18. During all steps the presence of the antimicrobial was monitored by TLC using its antimicrobial activity as an indicator [5, 6]. These tests showed one active substance to be present in seeds of both species with an identical R_f value. This substance was isolated from the HPLC eluate as a white crystalline product, m.p. 74° C (substance A). In the roots of *M. oleifera* in addition a second minor active zone was detected with a greater R_f value (substance B). There was no indication of further antibiotic substances in the roots.

Substance A showed a M^+ of m/e 311 in the MS and contained sulphur as well as nitrogen. λ_{max} in the UV spectrum was 221 (H_2O) and no bathochromic shift was observed upon addition of alkali. The IR spectrum showed a strong and broad absorption at 2160 cm^{-1} and 2080 cm^{-1} indicating a N=C=S grouping. Acidic hydrolysis yielded rhamnose as the only sugar component. The ^{1}H-NMR in acetonitrile-d_3 exhibited a multiplet characteristic of a para disubstituted benzene (7.0–7.5 ppm, 4H), a singlet of an isolated CH_2 group (4.7 ppm, 2H), multiplets in the sugar region between 3.2 and 4 ppm, a doublet of the anomeric rhamnose proton (5.5 ppm, 1H, J = 2 Hz) whose shift indicates the α-configuration at C_1 [7] and a doublet of the CH_3 of the rhamnose (1.2 ppm, 3H, J = 6 Hz). The spectrum is consistent with 4(α-L-rhamnosyloxy)benzyl isothiocyanate (2) which has already been isolated by BADGETT [8] from seeds of *M. oleifera* and by KJAER et al. from myrosinase treated seed extracts of *M. peregrina* [9]. A proton noise-decoupled ^{13}C-NMR was completely compatible with structure 2 and the α-configuration of the sugar was indicated by the $^1J_{C1\text{-}H1}$ value of 165 ± 2 Hz [7] and the chemical shifts of C-3′ (71.37 ppm) and C-5′ (70.01 ppm), which are shifted to higher field when compared to β-rhamnosides [7]. Thus, the antimicrobial substance of water incubated seeds of *M. oleifera* and *M. stenopetala*, as well as roots of *M. oleifera* has structure 2.

Substance B was isolated by preparative TLC from water incubated root powder of *M. oleifera*. The substance had an identical R_f value with benzyl isothiocyanate (TLC, GLC) and an identical ^{1}H-NMR in $CDCl_3$. Thus, the second compound with antimicrobial activity from roots of *M. oleifera* has structure 3.

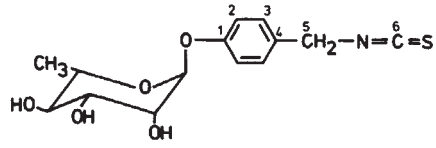
Table II shows the results of the quantitative estimation of 2 and 3 by GLC. When incubated in water *M. stenopetala* seeds released more than twofold the amount of 2 compared to *M. oleifera* seeds, but when incubated with buffer at pH 6.8, together with a small amount of ascorbic acid [10] the content of 2 from *M. oleifera* increased to give a similar amount to that of *M. stenopetala*.

The antimicrobial activity of pure 2 was tested against three species of bacteria and compared to 3: *Bacillus subtilis* (gram +), *Serratia marcescens* (gram −),

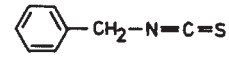


or

1 [3, 4]



2



3

Table II

Contents of *2* and *3* in the defatted seeds without shells of *M. oleifera* and *M. stenopetala* and roots of *M. oleifera* as obtained by GLC using water, as well as phosphate buffer pH 6.8 with 0.1 % ascorbic acid, for incubation.

Seed shells are about 20–25 % of the whole seed; whole seeds contain 26–28 % of extractable lipids.

Incubated with	*M. oleifera*				*M. stenopetala*	
	seeds		roots		seeds	
	2	*3*	*2*	*3*	*2*	*3*
H_2O	3.6 %	–	0.08 %	0.04 %	9.2 %	–
buffer pH 6.8, 0.1 % vit. C	8.9 %	–	–	–	8.5 %	–

and *Mycobacterium phlei*. The results are presented in table III. In the range of concentration used, *B. subtilis* was completely inhibited by 56 µmol/l of 2 and 90 µmol/l of 3 and *M. phlei* by 40 µmol/l of 2 and 42 µmol/l of 3. These values are the minimal bactericidal concentrations as proved by plating.

Both substances only partially inhibited *S. marcescens* in the range of concentrations used. These results indicate that the antimicrobial properties of 4 (α-L-rhamnosyloxy) benzyl isothiocyanate, which at present is the only known glycosidic mustard oil, are similar to or even better than the medicinally utilized benzyl isothiocyanate [11], which is the most active isothiocyanate of about 250 tested by KRISTIÁN et al. [13]. As 2 is a solid that is readily soluble in water (~1.3 mmol/l) and is non volatile, it appears to be a good substitute for 3 which is a volatile

Table III

Results from a dilution tube test with 4 (α-L-rhamnosyloxy) benzyl isothiocyanate (*2*) and benzyl isothiocyanate (*3*).

	Bacillus subtilis				*Mycobacterium phlei*				*Serratia marcescens*			
	2		*3*		*2*		*3*		*2*		*3*	
	µmol/l	µg/ml	µmol/l	µg/ml	µmol/l	µg/ml	µmol/l	µg/ml	µmol/l	µg/ml	µmol/l	µg/ml
minimum bactericidal concentration	56	17.5	90	13.5	40	12.5	42	6.5	–*	–	–△	–
minimum bacterio-static concentration	40	12.5	67	10	40	12.5	42	6.5	–*	–	–△	–
50 % inhibition	30	9.5	35	5	25	7.8	16	2.5	80	25	42	6.5
10 % inhibition	4	1.5	< 3	0.5	3	1.5	10	1.5	30	9.3	–	–

* = final concentration 537 µmol/1

△ = maximal solubility of *3*: 90 µmol/l.

liquid of poor water solubility and acts as a skin irritant. Table II shows that seeds of both *M. oleifera* and *M. stenopetala* are good sources of 2, containing up to about 8–10 % in the defatted shell free seeds. However, in our seed material the myrosinase activity in seeds of *M. oleifera* is not sufficient for liberation of all of 2 from the parent compound 4 (α-L-rhamnosyloxy) benzyl glucosinolate which was obtained as the heptaacetate after acetylation of *M. oleifera* seed extracts by BADGETT [8]. It is known that vitamin C acts as a coenzyme for special glucosidases which hydrolyze glucosinolates [10]. Obviously a glucosidase of this type completes the cleavage to 2 in *M. oleifera* seeds after ascorbic acid has been added.

For water purification normally about 0.2 g/l of the powder made from entire seeds is utilized and ascorbic acid obviously is not present in these systems. From these data a concentration of about 15–30 μmol/l of 2 is calculated, which is on the borderline for a clear antimicrobial effect [2]. Applying the same amount of seeds of *M. stenopetala* (which also are very active as a coagulant, S. A. A. JAHN, pers. comm.), these will be of more value during water purification as they will also have an effect against any microorganisms in the water.

The results from roots of *M. oleifera* indicate 2 and 3 as the only antimicrobial substances in this part of the plant. No evidence for pterygospermine (1) was obtained, at least in the material originating from the Sudan. In view of the surprising structure 1 and the data from this paper the present authors assume 1 to be an artifact of isolation or structural elucidation.

Experimental

Plant material

The air dried plant material was kindly provided by Dr. S. A. A. JAHN (Khartoum, Sudan). The seeds were defatted by extraction with petrol. Incubation of the defatted or undefatted seeds was carried out with 10 parts of water normally for two hours and the filtrate was taken for antimicrobial tests.

Isolation

For isolation the water phase was extracted with EtOAc and the concentrated EtOAc solution was chromatographed on silica gel (3 × 60 cm) with EtOAc/MeOH/H_2O 95:4:1. The active principle was detected by TLC methods [5, 6] in the range 520–900 ml. This fraction was further purified by HPLC on Lichrosorb RP-18 (0.8 × 25 cm) using CH_3CN/H_2O/MeOH 40:53:7 as the eluent (1.3 ml/min, detection at 240 nm, t_R = 12 min). During concentration of the trapped fractions substance A was obtained as white crystals, m.p. 74° C recorded by differential thermo analysis. Another isolation procedure was carried out by chromatography of the EtOAc extract on preparative TLC with MeOH/HCO_2Et 10:90. The same solvent was used for monitoring the antibiotic activity by TLC [5, 6]. In this system substance A shows an R_f of 0.4; extracts of roots additionally show an active zone at R_f 0.7 (substance B) which was collected by elution of this zone with CH_2Cl_2.

NMR-spectra

NMR-spectra were recorded on a Varian T 60 (^1H) and a Varian XL-100 (^{13}C). The proton spectrum is discussed in Results. ^{13}C-NMR: (δ-range, ppm): aromatic carbons at 157.14 (s, C-1), 117.51 (d, C-2), 129.27 (d, C-3), 129.01 (s, C-4); $-CH_2-$: 48.53 (t); -N = C = S: 129.01 or possibly not observed, broad; rhamnosyl residue: 99.11 (d, C-1′), 72.06 (d, C-2′), 71.37 (d, C-3′), 73.26 (d, C-4′), 70.01 (d, C-5′), 18.00 (q, C-6′). The $^1J_{C1\text{-}H1}$ value was determined from the residual coupling in the SFORD ^{13}C-spectrum and H-1 proton chemical shift by the use of known procedures [14]. Although this method is not as accurate as direct determination from a ^1H coupled ^{13}C-spectrum the value of 165 + 2 Hz is characteristic of the α sugar configuration [7].

EI-MS

The EI-MS showed signals at m/e: 311 (8 %), 165 (39 %), 147 (90 %), 146 (100 %), 129 (32 %), 107 (94 %), 85 (83 %), 71 (73 %) as published for 2 in [9].

Quantitative determination

For quantitative determination the defatted seed powder or the pulverized root material (~1 g) was incubated with 5 ml of water (in some experiments with 2.5 ml of water, 2.5 ml of phosphate buffer pH 6.8 containing 0.1 % ascorbic acid) for 12 hours and then kieselgur (Celite 545) and 10 ml CH_2Cl_2 were added. This wet powder was filled into a small glass tube and eluted with 100 ml CH_2Cl_2. The CH_2Cl_2 was removed carefully under vacuum, dissolved in 0.2–0.5 ml pyridine and silylated [12]. 100–200 µl of a standard solution (100 mg 1, 3, 5-triphenylbenzene in 10 ml CCl_4) was added and the supernatant was used for GLC.

GLC-system: OV-101, 2 % on Chromosorb AW-DMCS, 80–100 mesh, 3 m × 1/8´´, glass; temperature program: 17 min isothermally at 90° C, then 10° C/min up to 235° C, 235° isothermally. Detector: FID, 245° C; injector: 225° C; 25 ml/min nitrogen as carrier gas. t_R of 3: 13 min, t_R of 2: 47 min, t_R of standard: 52 min.

Both isothiocyanates were estimated against the internal standard and the correction factor was measured at 1.64 ± 0.11 for 2 and 1.43 ± 0.12 for 3.

Nutrient media: for bacteria: beef extract 0.5 %, meat peptone 0.6 %, glucose 0.4 %, doubly distilled water; pH 7.0 solid medium with 2 % agar. For fungi: Löflunds malt extract 4 %, casein peptone 0.5 %, doubly distilled water, agar 2 %; pH 5.4.

Growth conditions: bacteria 37° C; fungi 25° C.

Methods of antimicrobial assays: preliminary tests: diffusion plate assays; dilution tube test: turbidimetric method (instrument: Trübungsmesser Dr. B. Lange, Berlin). Incubation time: 48 h. TLC-stripe-test as described by WOLTERS [5, 6]; test organisms: *Polystictus versicolor, Bacillus subtilis.*

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